Biochimica et Biophysica Acta 1862 (2016) 1900–1908

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Chronic venous disease – Part II: Proteolytic biomarkers in wound healing

Daniela Ligi [a], Giovanni Mosti [b], Lidia Croce [a], Joseph D. Raffetto [c,d], Ferdinando Mannello [a,*]

[a] Department of Biomolecular Sciences, Section of Clinical Biochemistry and Molecular Genetics, University "Carlo Bo", Urbino, Italy
[b] Department of Angiology, Barbantini's Clinic, via del Calcio 2, 55100 Lucca, Italy
[c] Vascular Surgery Division, VA Boston Healthcare System, West Roxbury, MA, USA
[d] Harvard Medical School, Brigham and Women's Hospital, Boston, MA, USA

ARTICLE INFO

Article history:
Received 30 May 2016
Received in revised form 4 July 2016
Accepted 22 July 2016
Available online 25 July 2016

Keywords:
Chronic Venous Insufficiency
Wound healing
Glycosaminoglycan
Venous leg ulcer
Matrix metalloproteinase
Tissue inhibitors of metalloproteinase

ABSTRACT

Venous leg ulcers (VLU) are characterized by sustained proteolytic microenvironment impairing the healing process. Wound fluid (WF) reflect the biomolecular activities occurring within the wound area; however, it is unclear if WF from different healing phases have different proteolytic profiles and how VLU microenvironment affects the wound healing mechanisms. We investigated the proteolytic network of WF from distinct VLU phases, and in WF- and LPS-stimulated THP-1 monocytes treated with glycosaminoglycan sulodexide, a well known therapeutic approach for VLU healing.

WF were collected from patients with VLU during inflammatory (Infl) and granulating (Gran) phases. WF and THP-1 supernatants were analyzed for nine matrix metalloproteinases (MMP) and four tissue inhibitors of metalloproteinases (TIMP) by multiplex immunoassays. Our results demonstrated that: 1) WF from Infl VLU contained significantly increased concentrations of MMP-2, MMP-9, MMP-12, TIMP-1, and TIMP-2 compared to Gran WF; 2) WF from Gran VLU showed significantly increased levels of MMP-1, MMP-7, MMP-13, and TIMP-4 compared to Infl WF; 3) LPS- and WF-stimulation of THP-1 cells significantly increased the expression of several MMP compared to untreated cells; 4) Sulodexide treatment of both LPS- and WF-stimulated THP-1 significantly down-regulated the release of several MMPs. Our study provides evidence-based medicine during treatment of patients with VLU. WF from Infl and Gran VLU have different MMP and TIMP signatures, consistent with their clinical state. The modulation of proteolytic pathways in wound microenvironment by glycosaminoglycan sulodexide, provide insights for translating research into clinical practice during VLU therapy.

1. Introduction

Venous leg ulcer (VLU) is the most severe clinical class of Chronic Venous Insufficiency (CVI), which affects between 1% and 1,5% of the adult population worldwide, and is one of the major clinical problem in term of public healthcare spending for treatment and patient suffering [1,2].

VLU is the result of a complex cascade of events starting with a condition of prolonged venous hypertension in the lower extremities, related to valvular incompetence, superficial and deep vein insufficiency, venous reflux and/or obstruction, which arises from endothelial activation and white blood cells accumulation in the vein microenvironment [3,4].

The subsequent leukocyte activation initiates a series of inflammatory processes involving different cell types of the venous microenvironment (e.g. white blood cells, fibroblasts, keratinocytes and endothelial cells) which produce free radicals, inflammatory mediators and proteolytic enzymes (i.e. matrix metalloproteinase, MMP) [5], involved in the pathological remodeling of the vein structures and skin characteristic of the ulceration, such as hyperpigmentation, dermatitis and lipodermatosclerosis.

In particular, MMPs, and their four endogenous inhibitors tissue inhibitor of matrix metalloproteinases (TIMPs) play a key role in regulating the progression of dermal wounds [3].

The MMPs are a family of calcium- and zinc-dependent endopeptidases normally divided into 6 groups depending on their substrates and consisting in collagenases (MMP-1, −3, −8), gelatinases (MMP-2, −9), stromelysins (MMP-3, −10), matrilysins (MMP-7, −26), membrane-type MMPs (MT-MMP), such as MMP-14, MMP-15, MMP-16 and MMP-24, and other MMPs (MMP-11, −12, −19, −20, −22, −23, −28) [6].

Abbreviation: CEAP, Clinical, Etiological, Anatomical, and Pathophysiological; CVeD, chronic venous disease; CVI, Chronic Venous Insufficiency; VLU, venous leg ulcer; ECM, extracellular matrix; ICAM, intercellular adhesion molecule; LPS, Lipopolysaccharide; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; WF, Wound Fluid.

* Corresponding author at: Department of Biomolecular Sciences, Section of Clinical Biochemistry and Molecular Genetics, University "Carlo Bo", via O. Ubaldini 7, 61029 Urbino (PU), Italy.
E-mail address: ferdinando.mannello@uniurb.it (F. Mannello).

http://dx.doi.org/10.1016/j.bbadis.2016.07.011
0925-4439/

Their involvement has been highlighted in each phase of chronic venous disease (CVeD) evolution, where increasing levels of collagenases, stromelysins and gelatinases contribute to the appearance of typical pathological signs such as thickened venous wall relaxation and dilatation accompanied by a consequent valvular incompetence [6]. Moreover, the tight control of MMP proteolytic activity is also essential to conduct the different events of wound healing process [7]. In this respect, during the inflammatory, proliferative and remodeling phases of wound healing MMPs participate in regulating cytokine/chemokine availability, gradient and activity by cleaving them from cell surface or by processing the pro-enzymatic forms. In addition, MMP actions can be involved in the degradation of proteins involved in cell-cell and cell-extracellular matrix (ECM) interaction participating hence in the processes of cell migration and proliferation [8–10].

Therefore, the expression of the different MMP classes varies during the different cellular events typical of the wound repair phases, reflecting modifications in ulcer microenvironment and ulcer wound fluid (WF) composition [11,12].

Moreover, TIMPs are also well known to possess additional functions, beside the regulation of the MMP activity [13]; nevertheless, little is known about their expression in WF from VLU at different healing stages.

As a consequence, an imbalance between MMPs and TIMPs could affect the progression of VLU within the healing process [14], by amplifying leukocyte infiltration and activation, altering the functions of resident cells, remodeling the ECM, and bringing an aberrant distribution and activity of cytokines, growth factors and proteases [15].

Several in vitro studies evaluated the effect of WF from chronic and acute ulcers added to cell cultures, highlighting that chronic WF showed pro-inflammatory properties [16], decreased cellular mitogenic activities related with growth factor degradation by MMPs [17], and the ability of increasing MMP expression by cultured dermal and human fibroblasts [18]. In particular, it has been observed that chronic WF is often characterized by increased expression of the collagenases, gelatinases and stromelysins [19]. On the contrary, the addition of WF from healing wounds to 3 T3 fibroblasts caused significant increased proliferation [20], suggesting that healing wounds are characterized by an improved extracellular environment, which is favorable for the healing response, and that delayed wound healing depends not on an ineffective inflammatory response, but on an increased synthesis of inflammatory mediators, together with an unbalanced MMP proteolytic activity [13,14]. Therefore, detecting any alteration in MMP and TIMP levels in chronic wounds could potentially lead to the development of novel, accurate and objective tests for predicting chronic wound outcome before the appearance of the clinical signs and to provide additional biochemical therapeutic targets.

In this regard, we previously investigated the effect of sulodexide (Alfa Wassermann, Bologna, Italy) in suppressing the inflammatory response in monocyte exposed to LPS stimulation [2], and in modulating the proteolytic activity of gelatinases MMP-2 and MMP-9 [21]. Sulodexide is a mixture of glycosaminoglycans used in the treatment of a number of vascular disorders (e.g. intermittent claudication, peripheral arterial occlusive disease and post-myocardial infarction), due to its known antithrombotic and profibrinolytic activities, as well as to its anti-inflammatory, endothelial-protective and pleiotropic effects [21–24].

A body of evidences supported the use of sulodexide as adjuvant therapy in combination with local wound care and bandages for improving healing in patients with persistent VLU [25–27], and it has been recently suggested in the UIP guidelines as grade 1B for the treatment of VLU [28].

Moreover leucocytes, in particular monocytes/macrophages, are one of the main sources of MMPs, as well as of cytokines and growth factors usually involved in the healing process, and are able to change their phenotype according to micro-environmental stimuli they receive. The investigation of biochemical and cellular mechanisms leading to monocyte/macrophage responses in the Infl and Gran ulcer microenvironments and the possibility to modulate pharmacologically their activities, could represent important tools to promote the completion of wound repair and to translate biochemical and immunological investigation results in a potential prevention of clinical signs and pathophysiologic development of VLU.

The purpose of the present study was: I) to characterize the MMP profile of WF from Infl and Gran VLU; II) to determine the expression of all the four to date known TIMP (TIMP-1, −2, −3, and −4) in WF from patients with VLU in the Infl and in the Gran phase; (III) to investigate the effects of ulcer WF on THP-1 monocyte MMP expression with respect to LPS-stimulation; (IV) to study the modulatory properties of sulodexide on MMP expression by LPS- and WF-activated monocytes.

2. Materials and methods

2.1. Patient selection and recruitment criteria

Thirty-four patients with non-healing Infl and Gran VLU were admitted to the hospital for surgical debridement and skin grafting, and recruited for studying MMP and TIMP profiles in the WF.

There were 34 patients enrolled, and there are patients that had inflammatory VLU that then went on to develop granulating VLU, so these patients wound fluid was collected at each wound phase. The importance is that the wound fluid is the unit of measure, and therefore accounts for the 34 patients but with a total of 48 wound fluid collections between Infl and Gran VLU.

Inclusion criteria: patients of both sexes, older than 18 years, with chronic, non-healing VLU, presenting with primary and recurrent VLU. A non-healing VLU was defined as an ulcer that did not demonstrate a decrease in size after six months of treatment with dressing and compression therapy.

Exclusion criteria were: age <18 years, pregnant or breast feeding women, presence of arterial disease, renal insufficiency, insulin-dependent diabetes mellitus, vasculitis, autoimmune disease, cortisone or immunosuppressant therapies, previous venous surgery or sclerotherapy.

Insulin-dependent diabetes in patients with wounds, can affect the state of inflammation. It is also unknown if exogenous insulin affects MMPs in wound fluid. Therefore, to decrease confounding variables, we elected to not include insulin-dependent diabetes in this analysis.

Data on the medical history of all patients was recorded, and clinical and duplex ultrasound venous examinations were performed. Venous pathophysiology, was identified according to the clinical presentation and confirmed with Duplex scanning examination. Duplex was performed in the standing position with the weight on the contralateral leg. Venous reflux was elicited by means of calf compression-release maneuver, and diagnosed when venous reflux was >0.5 s in the superficial venous system and >1 s in the deep venous system [29]. The presence of thrombosis was evaluated with compression ultrasound. CVeD was classified according to the CEAP classification [30].

Patients with both primary and secondary venous disease and an active VLU were enrolled.

Wound fluid was collected at patients admission to the hospital when VLU were divided in two groups: I) Inflammatory (Infl) and II) granulating (Gran). When granulation tissue was obtained by means of a sharp debridement of Infl wounds, WF examination was repeated. In these cases we performed two WF examinations both at the beginning in the Infl stage, and after debridement in the Gran phase. Skin grafting and/or foam sclerotherapy was performed after WF sampling.

All patients pain was assessed with a visual analog scale (VAS), which rates pain intensity on a scale from 0 to 10, where 0 = no pain; 1–3 = mild pain; 4–6 = moderate pain, 7–10 = severe pain [31].

Infection was determined quantitatively by having more than 10E + 05 bacteria from a biopsy, defined as when colony forming unit were >100,000 per cm^2.

A written informed consent from patients was received by all the patients. The study was approved from the local ethics committee (both Barbantini Clinics of Lucca and University of Urbino) and was in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000.

2.2. Chronic venous ulcer wound fluid protocol

WF was collected by applying cotton gauze to the ulcer bed until saturated WF was transferred in a collecting tube without additives or antiproteases, and the WF centrifuged at 10,000 × g with the supernatant stored at −80 °C until further analysis. Because WF is the unit of measure in this study, in some cases, patients with an Infl wound that then became Gran, had both WF sampled for both wound types.

2.3. Cell culture and treatments

Human monocytic THP-1 (ATCC® TIB-202™) cell line obtained from American Type Culture Collection, Manassas, VA, USA was grown in standard culture conditions (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% antibiotics) and maintained at 37 °C in humidified air with 5% CO_2. The experiments were performed in serum free conditions to avoid the recovery of endogenous bovine serum cytokines.

THP-1 cells were seeded at 1,500,000/mL and treated for 18 h with sulodexide (0.12 LSU/mL) or LPS 10 μg/mL for 8 h, or a combination of LPS and sulodexide (LPS 10 μg/mL, 8 h, followed by removal of the culture media, and addition of culture media supplemented with sulodexide 0.12 LSU/mL for 18 h) to evaluate the effects of sulodexide on the extracellular release of MMP both in basal conditions and after the pro-inflammatory stimulation.

In addition, THP-1 cell line was stimulated with WF collected from patients with Infl or Gran VLU. For this purpose, aliquots (with the same volume) from ten randomly selected samples of WF from each group ($n = 10$ Infl and $n = 10$ Gran WF) were pooled. After filtration with 0.45 μm tissue culture filter unit to remove large debris, including bacteria, the two pooled Infl and Gran WF were immediately diluted in serum free RPMI 1640 culture media to a final concentration of 5% v/v, where treated cells were grown for 24 h, in the presence or absence of sulodexide co-treatment to a final dose of 0.12 LSU/mL. Cell viability was assessed by trypan blue exclusion test. Each experiment on serum-free conditioned medium was performed in triplicate in at least two independent experiments.

2.4. Magnetic multiplex immunoassay

MMP and TIMP concentrations in WF and serum free culture media samples were determined through the Pro™ Human MMP 9-plex Assay (including: MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13), and the Pro™ Human TIMP 4-plex Assay (including: TIMP-1, TIMP-2, TIMP-3, and TIMP-4), multiplex suspension immunomagnetic assays based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific for the target proteins, according to the manufacturer's instructions (Bioplex, Bio—Rad Labs, Hercules, CA, USA).

Although the commercially available kit of proteinases allow to analyze several biological fluids other than plasma, to exclude in assays the possible WF 'matrix' artifacts caused by possible interference substances, we serially diluted randomly selected WF samples, reanalyzing them for the response linearity. According to the manufacturer's data, the lower detection limit was 1.0 and 1.6 pg/mL(MMP and TIMP, respectively), while the mean intra-assay variability was <10% as specified by manufacturer's instructions.

Levels of all analytes were determined using a Bio-Plex 200 array reader, based on Luminex X-Map Technology (Bio—Rad Labs, Hercules, CA, USA) that detects and quantifies multiple targets in a 96-well plate with a single small fluid volume (50 μL). Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v.6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve.

2.5. Chemicals

Sulodexide was provided by Alfa Wassermann (Bologna, Italy). All chemicals of reagent grade and lipopolysaccharide (LPS from *E. Coli*, code L3129) was obtained from Sigma (Milan, Italy), whereas the sterile compounds for cell culture were from JET BIOFIL Bio-filtration Products Co (Guangzhou, China), and chemicals and reagents for cell culture were from Carlo Erba Reagents S.r.l. (Milan, Italy).

2.6. Statistical analysis

Each variable was expressed as the mean ± standard error of the mean, unless otherwise specified. Statistical analyses were carried out through Fisher Exact test, Mantel-Haenszel $\chi 2$, Mann-Whitney, or Student's *t*-test according to variable characteristics. All statistical tests were two-tailed, and significance was set at $p < 0.05$. Data and graphs were analyzed with Prism software for Windows-7, version 3.1 (Graph-Pad, San Diego, CA, USA). We defined this study as pilot preclinical study, therefore we did not determine a power calculation. Accordingly, the results can only be labeled as an exploratory study.

3. Results

3.1. Demographic data

Thirty-four patients with VLU were enrolled in the study. According to the phase of wound healing and to the clinical observation of the ulcer tissues, WF were subdivided into two groups: Infl ($n = 32$) and Gran ($n = 16$).

The biological samples examined were collected from both sexes (19 males and 29 females), with a mean age 72 years (range: 43–91 years), and presenting ulcers both at first episode ($n = 16$) or recurrent ($n = 32$). The mean ulcer duration was 40 ± 47.9 months. The average ulcer size was 12.9 cm^2 (range 0,2–60 cm^2). Complete demographic variables for the study population are presented in Table 1.

3.2. Pain scale

Patient with Infl WF had significantly increased pain scales compared to patients with Gran WF (5.0 ± 0.24 vs. 3.4 ± 0.29, respectively, $p = 0.0003$)(Table 1).

3.3. MMP profile in venous ulcer wound fluids

The quantitative expression of MMPs in 48 WF samples from VLU, in both the Infl and the Gran ulcer group, revealed that MMP-1, MMP-2, MMP-7, MMP-9, MMP-12, and MMP-13 showed significant statistical differences between the two groups, whereas MMP-3, MMP-8 and MMP-10 showed a similar distribution in the Infl and Gran phases (Fig. 1).

In particular, we observed significantly increased levels of MMP-2 (Infl vs. Gran: 943,900 ± 119,600 vs. 414,700 ± 65,300 pg/mL, respectively; $p = 0.0026$), MMP-9 (Infl vs. Gran: 483,100 ± 68,190 vs. 173,900 ± 47,060 pg/mL, respectively; $p = 0.0025$), and MMP-12 (Infl vs. Gran: 67,550 ± 12,350 vs 22,780 ± 7478 pg/mL, respectively; $p = 0.0367$) in the WF from Infl compared with Gran VLU. However, MMP-1 (Infl vs. Gran: 79,460 ± 26,370 vs. 142,800 ± 26,370 pg/mL, respectively; $p = 0.0016$), MMP-7 (Infl vs. Gran: 623.6 ± 165.8 vs. 3072 ± 1076 pg/mL, respectively; $p = 0.0002$), and MMP-13 (Infl vs. Gran: 3093 ± 930.3 vs. 10,290 ± 3775 pg/mL, respectively; $p = 0.0016$) were significantly over-expressed during the Gran compared

Table 1
Demographic and clinical characteristics.

Characteristic	Inflammatory	Granulating	*p* value
Number, n (%)	32 (66.7)	16 (33.3)	
Age Range, years	43–91	65–85	
Mean Age (± SD), years	69.1 ± 14.8	77.8 ± 6.5	0.03
Sex			0.12
Male, n (%)	10 (31.3)	9 (56.3)	
Female, n (%)	22 (68.7)	7 (43.7)	
Comorbidities			
Diabetes, n (%)	10 (31.3)	2 (12.5)	0.29
Hypertension, n (%)	19 (59.4)	15 (93.8)	0.02
Hyperlipidemia, n (%)	18 (56.3)	2 (12.5)	0.01
Smoking, n (%)	6 (18.8)	0 (0)	0.16
Rheumatic disease, n (%)	2 (6.3)	0 (0)	0.55
Ulcer History			0.52
Primary	12 (37.5)	4 (25.0)	
Recurrent	20 (62.5)	12 (75.0)	
Venous insufficiency			0.52
Superficial , n (%)	24 (70.6)	14 (73.7)	
Deep, n (%)	5 (14.7)	4 (21)	
Mixed, n (%)	5 (15.7)	1 (5.3)	
Infection	22 (68.8)	0 (0)	<0.001
Duration, months, mean ± SD	48.0 ± 56.6	44.4 ± 85.5	0.79
Surface area, cm^2, mean ± SD	146.0 ± 187.4	95.3 ± 80.3	0.85
VAS score, mean ± SD	5.0 ± 0.24	3.4 ± 0.29	0.0003

VAS: visual analog scale.

to the Infl phase. MMP-3, MMP-8 and MMP-10 were found similar in the two groups (Infl vs. Gran: 19,480 ± 4124 vs. 25,700 ± 4508 pg/mL, $p = 0.0646$; 163,700 ± 9914 vs. 169,200 ± 10,270 pg/mL, $p = 0.8870$; 16,490 ± 3483 vs. 15,120 ± 3820 pg/mL, $p = 0.7346$, respectively).

3.4. TIMP profile in venous ulcer wound fluids

The analyses of the quantitative expression of TIMPs in 48 WF samples from VLU, in both the Infl and the Gran ulcer group, demonstrated that the four TIMPs displayed a different behavior with TIMP-1 and TIMP-2 (11,420 ± 673.6 vs. 8332 ± 929.6, $p = 0.0104$ and 16,980 ± 1233 vs. 12,200 ± 1503, $p = 0.0296$, respectively) overexpressed in the Infl VLU WF, and TIMP-4 (173.2 ± 24.9 vs. 331.7 ± 57.1, $p = 0.0130$) over-expressed in Gran VLU WF. TIMP-3 did not show significant differences between the two microenvironments (537.7 ± 42.7 vs. 581.3 ± 73.5, $p = 0.8231$) (Fig. 2).

3.5. Effects of sulodexide on LPS-activated THP-1 monocytes

The evaluation of the MMP secretion profile in THP-1 cells after LPS-induced inflammation, in the absence and presence of sulodexide, revealed that LPS treatment promoted an increased release of MMP-1, MMP-9 (~1.6-fold, $p = 0.0402$ and $p = 0.0086$, respectively), and MMP-12 (~2-fold, $p > 0.05$) compared to untreated control cells, while MMP-2, MMP-7, MMP-10 and MMP-13 levels were not significantly different from controls (Fig. 3).

Treatment with sulodexide 0.12 LSU/mL of the LPS-exposed THP-1 resulted in a different modulation of the MMP classes, suggesting that sulodexide possessed a broad spectrum of targets among the proteolytic enzymes. Sulodexide + LPS-treated THP-1 monocytes down-regulated significantly MMP-1 (~30%; $p = 0.0318$), and insignificantly MMP-9 (~13%; $p > 0.05$), and MMP-12 (~23%; $p > 0.05$) compared to LPS-treated cells, but the levels remained higher with respect to control cells ($p > 0.05$, $p = 0.0139$, and $p = 0.0074$, respectively). Sulodexide treatment of LPS-stimulated THP-1 resulted in an increased release of MMP-7 (~27%; $p = 0.0334$) and MMP-10 (~90%; $p = 0.0161$) (Fig. 3). The secretion of MMP-2 and MMP-13 was not modified by sulodexide treatment, whereas levels of MMP-3 and MMP-8 were undetectable in all the experimental conditions explored.

3.6. MMP profile in WF-stimulated THP-1 cells

The MMP profile in WF-stimulated THP-1 was obtained after subtracting the level measured in WF to consider only the fraction of

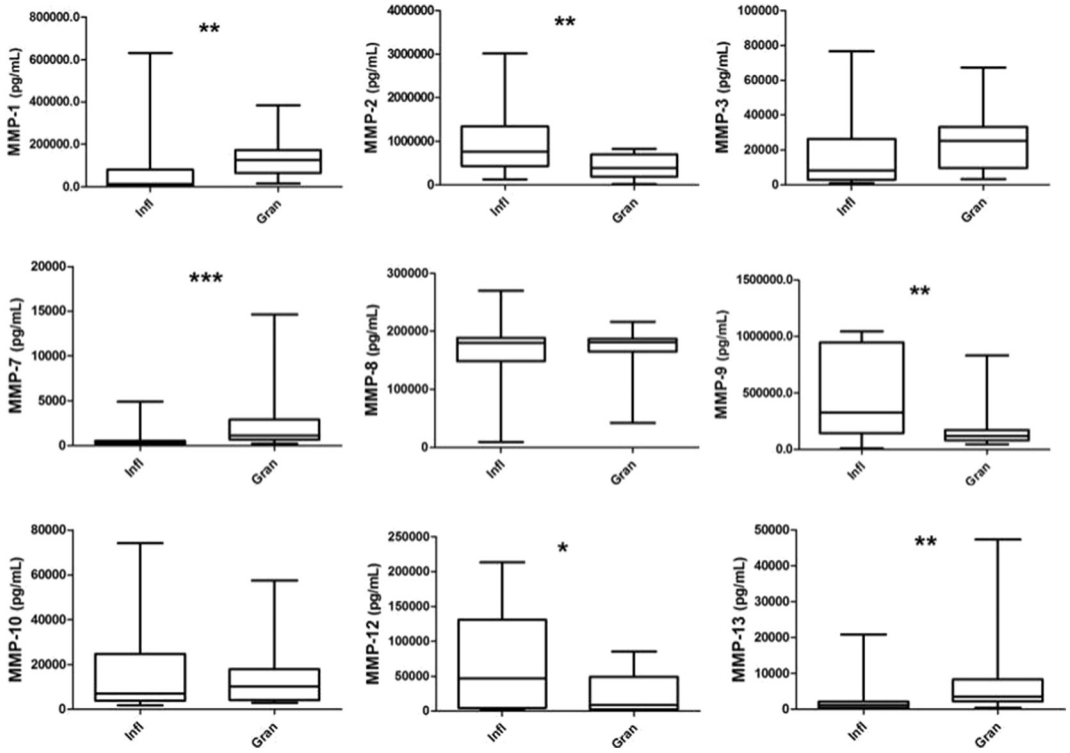

Fig. 1. Levels of MMP in inflammatory (Infl, $n = 32$) and granulating (Gran, $n = 16$) venous ulcer wound fluids. Values are expressed as pg/mL (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

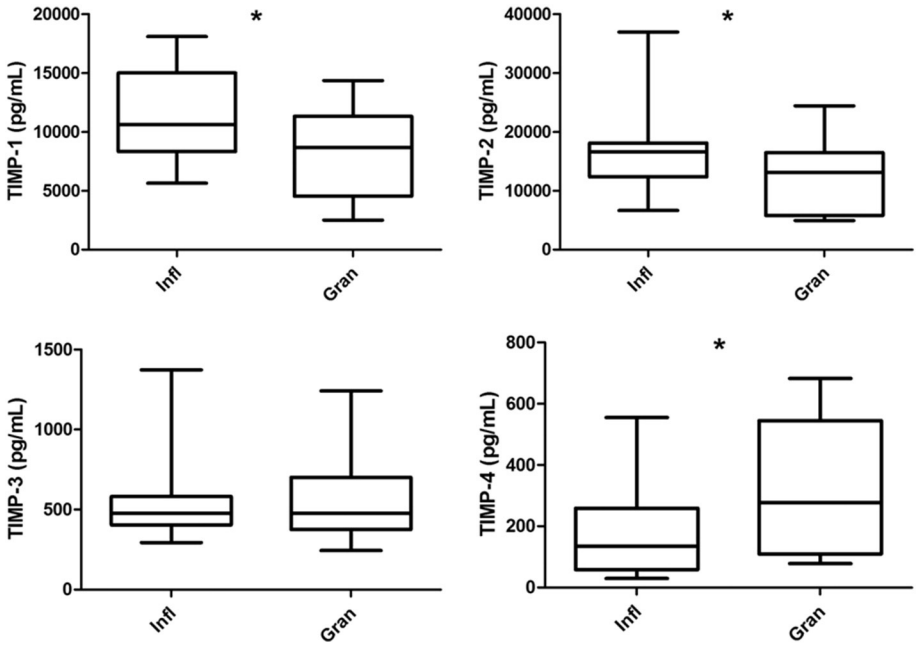

Fig. 2. Levels of TIMP in inflammatory (Infl, $n = 32$) and granulating (Gran, $n = 16$) venous ulcer wound fluids. Values are expressed as pg/mL (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

biomolecules released by THP-1 cells and avoid interferences with those of WF. The treatment of THP-1 cell line with WF from Infl and Gran VLU led to a significantly increased release of almost all the analytes evaluated compared with untreated control cells. Compared to untreated control THP-1 cells, culture media from Infl and Gran WF-exposed THP-1 cells showed increased levels of MMP-2 (24.3-fold, $p = 0.0021$ and 23.9-fold, $p < 0.0001$, respectively), MMP-3 (2.1-fold, $p = 0.0027$ and 16.1-fold, $p < 0.0001$, respectively), MMP-7 (1.6-fold, $p = 0.0008$ and 3.2-fold, $p < 0.0001$, respectively), MMP-8 (496.5-fold, $p = 0.0002$ and 287.9-fold, $p < 0.0001$, respectively), MMP-9 (86.7-fold, $p = 0.0006$ and 18.2-fold, $p < 0.0001$, respectively), MMP-10 (11.7-fold, $p < 0.0001$ and 11.3-fold, $p < 0.0001$, respectively), and MMP-12 (22.5-fold, $p = 0.0273$ and 69.9-fold, $p < 0.0001$, respectively), compared to untreated control cells (Table 2). We observed that the treatment with Infl WF did not significantly alter the release of MMP-1 and MMP-13, whereas MMP-1 (2.48-fold, $p < 0.0001$) was significantly reduced by treatment with granulating WF compared with control cells (Table 2).

3.7. Effects of sulodexide on WF-stimulated monocytes

The exposure of THP-1 cells with either Infl or Gran WF resulted in a different MMP release from monocytic cells. MMP-1, MMP-8, and MMP-9 were significantly higher expressed by Infl- compared to Gran WF-treated THP-1 ($p < 0.0001$, $p = 0.0059$, and $p = 0.0001$, respectively), whereas levels of MMP-3, MMP-7, and MMP-13 resulted significantly increased levels after the treatment with Gran WF compared to Infl WF ($p < 0.0001$, $p = 0.0059$ and $p = 0.0001$, respectively)(Fig. 4).

No differences were found for MMP-2 and MMP-10 levels after the treatment with the two pools. The co-treatment of THP-1 with sulodexide and Infl WF revealed that levels of MMP-8 ($p < 0.05$) were significantly reduced compared with Infl WF stimulation, whereas we found significantly increased levels of MMP-2 ($p < 0.005$) and decreased levels of MMP-7 ($p < 0.05$) in sulodexide + Gran WF treated monocytes compared to Gran WF exposed cells (Fig. 4).

4. Discussion

The increasing incidence of chronic VLU has led research to focus on the wound microenvironment in order to gain information regarding the prolonged inflammatory and proteolytic profiles characterizing the different phases of the disease [2,11,32]. In this regard, the characterization of the ulcer WF, reflecting the ulcer milieu, represents an important and non invasive tool to investigate the inflammatory and proteolytic processes occurring during the different phases of wound

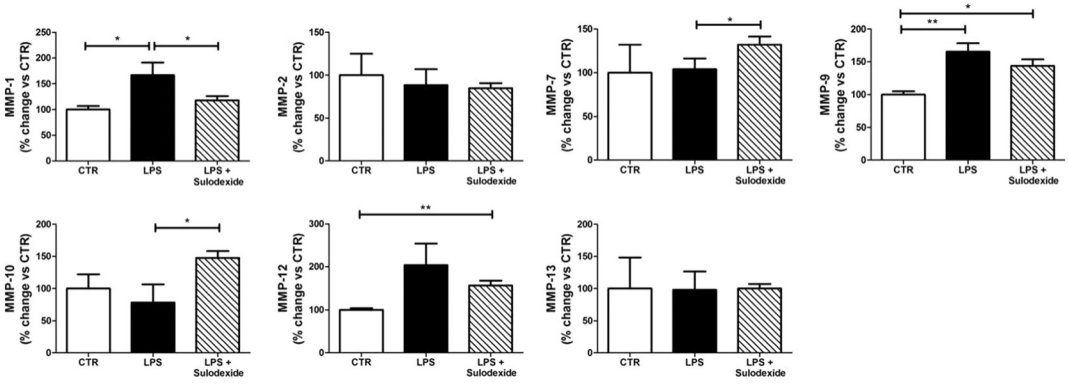

Fig. 3. MMP profile in LPS- and LPS + Sulodexide-stimulated THP-1 monocytes. Values are expressed as fold change versus unstimulated THP-1 control cells, referred as 100% (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

Table 2
Levels of MMP in culture media of THP-1 treated with inflammatory (Infl) and granulating (Gran) WF compared to untreated control cells.

		UWF vs CTR	
		Infl (p)	Gran (p)
Matrix Metalloproteinases	**MMP-1**	↑	↓↓↓ (***)
	MMP-2	↑↑ (**)	↑↑ (***)
	MMP-3	↑ (**)	↑↑ (***)
	MMP-7	↑ (***)	↑ (***)
	MMP-8	↑↑↑ (***)	↑↑↑ (***)
	MMP-9	↑↑↑ (***)	↑↑ (***)
	MMP-10	↑↑ (***)	↑↑ (***)
	MMP-12	↑↑ (*)	↑↑↑ (***)
	MMP-13	↓	↑

↑↑↑ ≥ 60-fold; ↑↑ = 15–25-fold; ↑ ≤ 5-fold; ↓↓↓ ≥ 1-fold; ↓↓ = 1–0.5-fold; ↓ ≤ 0.2-fold.
*** = $p < 0.0005$ ** = $p < 0.005$ * = $p < 0.05$.

healing, possibly providing a proteomic array of molecules regulating the ulcer microenvironment [2]. This study demonstrated that i) There is differential expression of MMPs and TIMPs between the WF of Infl and Gran VLU; ii) LPS treated monocytes express MMPs which are modulated by sulodexide; iii) Monocytes treated with either WF from Infl or Gran VLU have a differential expression of MMPs, and which for certain MMPs sulodexide is able to decrease or increase their expression.

Several studies hypothesized that chronic wounds are blocked in a persistent inflammatory phase, characterized by excessive white blood cell infiltration, inflammatory mediator release and proteolytic extracellular matrix remodeling. Nevertheless, to date there are no clinical or biochemical markers useful to assist clinicians in diagnosis and monitoring the prognosis of these conditions, and chronic wounds still remain a crucial problem [2,33]. Similarly, to date, treatments for VLU are mainly addressed to the correction of the hemodynamic alterations and compression therapy, and no biochemical targets have been determined and tested clinically.

The differences in MMPs and TIMPs are reflected clinically, in that patients with Infl WF had significantly higher pain scores. To our knowledge, this is the first description of such a phenomenon in two different VLU wound types, and likely is a result of the differences of inflammatory and anti-inflammatory biomolecules present in the WF (Ligi et al., manuscript under consideration in Clin Sci, CS-2016-0289). In fact, pain associated with VLU is induced by the inflammatory reaction in response to wounding, by wound complications (e.g. infection) and by damaged peripheral nerves, as previously described [34].

A large body of evidence highlights the loss of physiological and functional balance between MMPs functioning as proteolytic enzymes required to promote the removal of damaged tissue and to support migration and neovascularization during the wound healing process [14], and their respective inhibitors TIMPs. The imbalance between MMPs and TIMPs can lead to an uncontrolled degradation of ECM elements, cytokines, growth factors and their receptors, interfering with the healing process and contributing to the onset of pathological states, such as chronic wounds [35].

A number of studies investigated the expression profile of MMPs during wound healing of both acute and chronic VLU, but to our knowledge none of them examined simultaneously nine MMPs and four TIMP from the WF of VLU healing in different phases (Infl vs. Gran) [36–45].

According to literature data, our results demonstrated that WF from Infl VLU were characterized by significantly increased levels of MMP-2 and MMP-9[32,41,46,47] supporting the hypothesis that the chronic venous ulcer bed is rich in proteases active in destroying the underlying ECM [48]. Similarly, we also observed that Infl VLU showed an increased expression of metalloelastase (MMP-12), a macrophage product, previously studied only in ulcer tissues from patient with malignant and non-malignant chronic ulcers [49], and in ulcer tissue before and after compression therapy [40], but never investigated in Infl and Gran ulcer WF. MMP-12 has been recently assessed in vitro as a protease released by macrophages activated by alternative pathway (i.e., M2 polarized macrophages), able to support healing processes and wound closure [50]. The release of MMP-12 in the VLU inflammatory

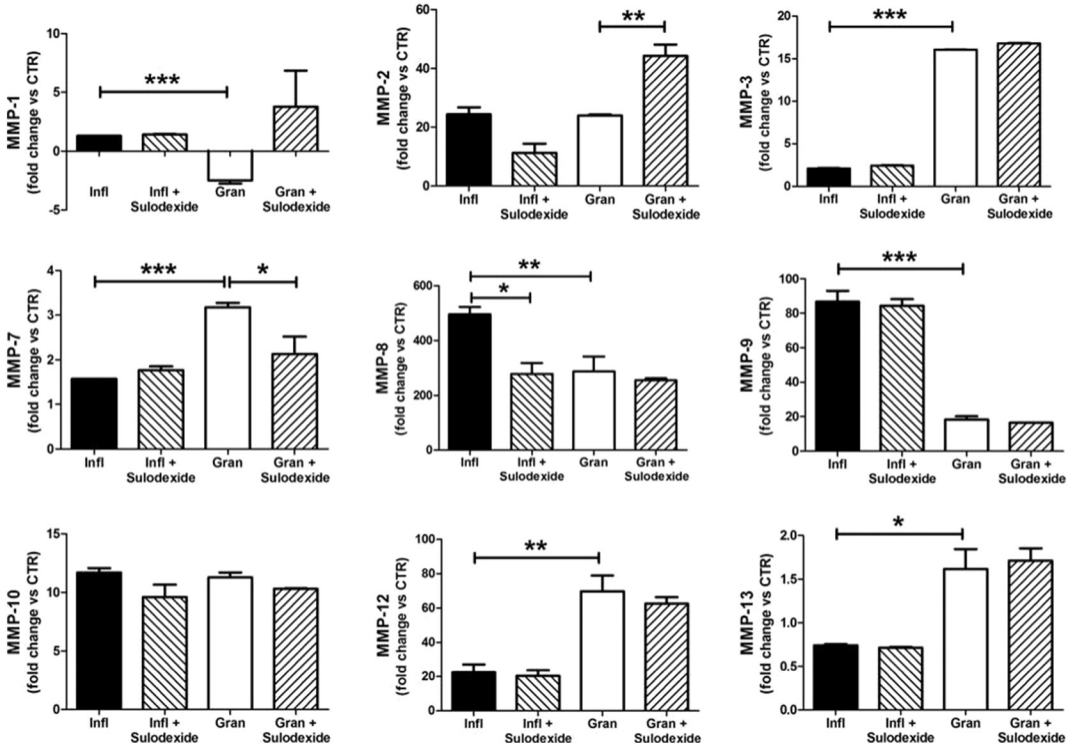

Fig. 4. MMP profile in UWF- and UWF + Sulodexide-stimulated THP-1 monocytes. Values are expressed as fold change versus unstimulated THP-1 control cells, referred as 100% (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

microenvironment from M2 polarized macrophages [51] may shed light on the presence of metallo-elastase MMP-12 as a biomolecular signal promoting the transition toward the proliferative Gran phase.

However, this study revealed that Gran VLU WF showed increased concentrations of MMP-1, MMP-13, and MMP-7, compared with Infl VLU WF, suggesting potential roles for these proteases in skin re-epithelization and wound closure.

Studies investigating venous ulcer tissues found increased expression of MMP-1 protein [40] and mRNA [44] in ulcer tissue compared to normal skin, and in chronic compared to acute wounds [37], with contrasting results in relation with ulcer healing [39,40,43,52]. Other authors reported also that an increased MMP-1 level in WF may depend on ulcer infection [42]. Similarly, MMP-13 expression has been previously investigated only in ulcer tissue, where it was demonstrated to be over-expressed in ulcer compared to normal tissue [40], and in malignant ulcers [49].

However, MMP-1 and MMP-13, are involved in granulation tissue formation and remodeling facilitating fibroblast and keratinocyte migration, and regulating fibroblast survival and contraction during dermal wound healing [37,39,53]. Our findings would be consistent in that Gran WF VLU had increased levels of MMP-1 and MMP-13, which would indicate a favorable healing environment compared to an Infl VLU.

Unlike the other MMPs, MMP-7 was not thoroughly investigated neither in the context of ulcer WF, nor during the physiological evolution of a wound. Our study demonstrated that MMP-7, an epithelial-derived MMP, was significantly higher in Gran compared with Infl VLU, suggesting that MMP-7 may have a role during the formation of granulation tissue and wound closure. Our results are in agreement with several findings obtained in animal models or in other human tissues [54–56] suggesting that MMP-7 is important for epithelial repair in mucosal and airway tissues, regulation of the chemokine gradients for leukocyte adhesion and migration [57], and control of cell apoptosis, thus enhancing epithelial repair. As observed for the other tissues, MMP-7 may represent a pivotal mediator in promoting angiogenesis [58], re-epithelization/wound closure and remodeling by shedding proteoglycans, chemokines and integrins affecting cell-cell and cell-matrix interactions and leukocyte functions [59].

In addition, our data demonstrated that TIMP-1 and TIMP-2, showed an enhanced expression in Infl compared to Gran VLU WF, whereas TIMP-4 was significantly increased in Gran WF compared with Infl WF. The increased levels of TIMP-1 and TIMP-2 might be related to the need to counteract the higher concentrations of MMP-2 and MMP-9 observed during the inflammatory phase [60]. Moreover the increased secretion of MMP-2 by fibroblasts also promotes the expression of TIMP-2 to protect the matrix from excessive degradation [61]. Furthermore, TIMPs possess other MMP-independent functions, such as controlling angiogenesis [62–64] needed for the neovascularization process during the wound repair. On the contrary, little is known on TIMP-4, suggested to be highly expressed in the cardiovascular structures [65,66]. Its increased expression in Gran WF compared to Infl WF in this study, may be related to the ability of TIMP-4 in regulating apoptotic events [67], occurring during the maturation of the granulation tissue in wound repair [68].

Therefore the simultaneous expression of these two classes of molecules is in agreement with the need for their homeostatic balance to remove damaged tissue, bacterial cells or other detrimental materials and to prepare the wound to the next phases of the healing process.

In this regard, monocytes and macrophages are crucial players in all the phases of the dermal wound repair, orchestrating the healing phases through the release of MMPs [8,9] and inflammatory mediators [69]. Thanks to their plasticity, monocytes could have dual roles during wound progression, responding with different signals, as the ulcer microenvironment changes during wound repair [51].

In this regard, our results support the literature data suggesting that LPS-treated monocytes are characterized by an increased release of MMP-1, MMP-9 and MMP-12 [70–73]. Similarly, the treatment of THP-1 monocytic cells with WF induced a significant increased secretion of all the MMP classes, with MMP-1, MMP-8 and MMP-9 mainly induced by Infl WF, and MMP-3, MMP-7, MMP-12 and MMP-13 by Gran WF.

It has been previously described that human monocytes stimulated with WF displayed an inflammatory response similar to that observed in LPS-treated monocytes [16]; however, to date, no study reported how the MMPs release profile was affected by WF exposure of monocytes. Our data suggest the presence of unique mediator(s) in WF, characteristics of the two phases of wound healing, which affects the release of MMP from monocytes and could drive the wound repair process.

In addition, we previously reported that the release of cytokines from LPS-stimulated monocyte could be modulated by glycosaminoglycan sulodexide treatment [74], and that sulodexide also affected the proteolytic activity of gelatinase MMP-9 [21]. In the present study we identified that sulodexide, which has been recently included within the adjuvant treatments for VLU by the evaluation of the International Union of Phlebology (level of evidence 1B) on the guidelines set forth by the Society for Vascular Surgery and American Venous Forum [28], was also able to modulate a large spectrum of MMP, exhibiting a different activity on LPS- or WF-stimulation.

Specifically, we observed that after LPS exposure of monocytes, sulodexide was able to reduce levels of MMP-1, MMP-9, and MMP-12, whereas MMP-7 and MMP-10 were increased, leading us to suppose that the two glycosaminoglycan-based fractions could bind to monocyte receptors, affecting the MMP release. In this respect, a large body of evidence has highlighted the ability of glycosaminoglycans to interact and modulate the activity of MMPs [21,75–79], by directly interacting with MMP [75] or affecting their synthesis [76] or interfering with the signaling cascade activated by bacterial LPS [74,80]. However, a different profile was observed in WF + sulodexide treated monocytes. In fact, this study demonstrated that sulodexide treatment significantly reduced MMP-8 levels during Infl WF stimulation, whereas increased the release of MMP-2 and reduced MMP-7 during Gran WF exposure.

It has been proposed that due to the ability to activate TLR receptors, WF had an inflammatory activity close to that of known inflammatory stimuli [16], and that glycosaminoglycan sulodexide treatment showed a similar modulatory profile in LPS- or WF-stimulated monocytes (Ligi et al., manuscript under consideration). However, this does not appear completely true for MMP, suggesting that other mechanisms are involved in WF-induced MMP expression and glycosaminoglycan modulation.

Collectively, these data highlighted the complex nature of MMP and TIMP modulation within the wound microenvironment, and provide novel biochemical evidences on the involvement of MMPs in venous ulcers, possibly suggesting future panel of biomarkers useful to monitor the progression of the disease.

Further investigations are needed to better understand the intricate signaling regulating MMP roles in venous ulcer wound healing and their modulation by glycosaminoglycans. The limitations of this study are that it was explorative and therefore may be underpowered, but the findings provide proof of concept for future work. In addition, WF was collected from a single time point of two types of clinically classified VLU wounds, Infl and Gran. There was no follow up to determine if the Infl or the Gran WF was correlated to ultimately healing of the VLU. Future work will need to identify MMPs and TIMPs within VLU that are predictive of VLU healing.

Finally, a thorough investigation on the broad-spectrum of MMP-dependent and independent TIMP roles might allow to exploit them as endogenous regulators in wound progress for limiting cellular and molecular alterations occurring in the development of chronic wounds.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] R.T. Eberhardt, J.D. Raffetto, Chronic venous insufficiency, Circulation 130 (2014) 333–346.
[2] F. Mannello, D. Ligi, M. Canale, J.D. Raffetto, Omics profiles in chronic venous ulcer wound fluid: innovative applications for translational medicine, Expert. Rev. Mol. Diagn. 14 (2014) 737–762.
[3] J.D. Raffetto, Dermal pathology, cellular biology, and inflammation in chronic venous disease, Thromb. Res. 123 (Suppl. 4) (2009) S66–S71.
[4] A.N. Nicolaides, Investigation of chronic venous insufficiency: a consensus statement (France, March 5–9, 1997), Circulation 102 (2000) E126–E163.
[5] J.D. Raffetto, Inflammation in chronic venous ulcers, Phlebology 28 (Suppl. 1) (2013) 61–67.
[6] J.D. Raffetto, R.A. Khalil, Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease, Biochem. Pharmacol. 75 (2008) 346–359.
[7] U.K. Saarialho-Kere, Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers, Arch. Dermatol. Res. 290 (Suppl) (1998) S47–S54.
[8] S.E. Gill, W.C. Parks, Metalloproteinases and their inhibitors: regulators of wound healing, Int. J. Biochem. Cell Biol. 40 (2008) 1334–1347.
[9] V.L. Martins, M. Caley, E.A. O'Toole, Matrix metalloproteinases and epidermal wound repair, Cell Tissue Res. 351 (2013) 255–268.
[10] P. Martin, R. Nunan, Cellular and molecular mechanisms of repair in acute and chronic wound healing, Br. J. Dermatol. 173 (2015) 370–378.
[11] K. Moore, E. Huddleston, M.C. Stacey, K.G. Harding, Venous leg ulcers - the search for a prognostic indicator, Int. Wound J. 4 (2007) 163–172.
[12] D.R. Yager, R.A. Kulina, L.A. Gilman, Wound fluids: a window into the wound environment? Int. J. Low Extrem. Wounds 6 (2007) 262–272.
[13] C.S. Moore, S.J. Crocker, An alternate perspective on the roles of TIMPs and MMPs in pathology, Am. J. Pathol. 180 (2012) 12–16.
[14] N.J. Trengove, M.C. Stacey, S. MacAuley, N. Bennett, J. Gibson, F. Burslem, G. Murphy, G. Schultz, Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors, Wound Repair Regen. 7 (1999) 442–452.
[15] L. Nissinen, V.M. Kahari, Matrix metalloproteinases in inflammation, Biochim. Biophys. Acta 1840 (2014) 2571–2580.
[16] B.S. Pukstad, L. Ryan, T.H. Flo, J. Stenvik, R. Moseley, K. Harding, D.W. Thomas, T. Espevik, Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers, J. Dermatol. Sci. 59 (2010) 115–122.
[17] R.W. Tarnuzzer, G.S. Schultz, Biochemical analysis of acute and chronic wound environments, Wound Repair Regen. 4 (1996) 321–325.
[18] K. Subramaniam, C.M. Pech, M.C. Stacey, H.J. Wallace, Induction of MMP-1, MMP-3 and TIMP-1 in normal dermal fibroblasts by chronic venous leg ulcer wound fluid*, Int. Wound J. 5 (2008) 79–86.
[19] D.R. Yager, B.C. Nwomeh, The proteolytic environment of chronic wounds, Wound Repair Regen. 7 (1999) 433–441.
[20] N.J. Trengove, H. Bielefeldt-Ohmann, M.C. Stacey, Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers, Wound Repair Regen. 8 (2000) 13–25.
[21] F. Mannello, V. Medda, D. Ligi, J.D. Raffetto, Glycosaminoglycan sulodexide inhibition of MMP-9 gelatinase secretion and activity: possible pharmacological role against collagen degradation in vascular chronic diseases, Curr. Vasc. Pharmacol. 11 (2013) 354–365.
[22] G.M. Andreozzi, Sulodexide in the treatment of chronic venous disease, Am. J. Cardiovasc. Drugs 12 (2012) 73–81.
[23] S. Coccheri, F. Mannello, Development and use of sulodexide in vascular diseases: implications for treatment, Drug Des. Dev. Ther. 8 (2014) 49–65.
[24] R. Serra, L. Gallelli, A. Conti, G. De Caridi, M. Massara, F. Spinelli, G. Buffone, F.G. Calio, B. Amato, S. Ceglia, G. Spaziano, L. Scaramuzzino, A.G. Ferrarese, R. Grande, S. de Franciscis, The effects of sulodexide on both clinical and molecular parameters in patients with mixed arterial and venous ulcers of lower limbs, Drug Des. Dev. Ther. 8 (2014) 519–527.
[25] M. Saviano, O. Maleti, L. Liguori, Double-blind, double-dummy, randomized, multicentre clinical assessment of the efficacy, tolerability and dose-effect relationship of sulodexide in chronic venous insufficiency, Curr. Med. Res. Opin. 13 (1993) 96–108.
[26] G. Scondotto, D. Aloisi, P. Ferrari, L. Martini, Treatment of venous leg ulcers with sulodexide, Angiology 50 (1999) 883–889.
[27] P. Gloviczki, A.J. Comerota, M.C. Dalsing, B.G. Eklof, D.L. Gillespie, M.L. Gloviczki, J.M. Lohr, R.B. McLafferty, M.H. Meissner, M.H. Murad, F.T. Padberg, P.J. Pappas, M.A. Passman, J.D. Raffetto, M.A. Vasquez, T.W. Wakefield, The care of patients with varicose veins and associated chronic venous diseases: clinical practice guidelines of the Society for Vascular Surgery and the American Venous Forum, J. Vasc. Surg. 53 (2011) 2 s–48 s.
[28] G. Mosti, M. De Maeseneer, A. Cavezzi, K. Parsi, N. Morrison, O. Nelzen, E. Rabe, H. Partsch, A. Caggiati, M. Simka, A. Obermayer, M. Malouf, M. Flour, O. Maleti, M. Perrin, L. Reina, E. Kalodiki, F. Mannello, K. Rerkasem, A. Cornu-Thenard, Y.W. Chi, M. Soloviy, O. Bottini, N. Mendyk, L. Tessari, R. Varghese, R. Etcheverry, F. Pannier, M. Lugli, A.J.C. Lantz, P. Zamboni, M. Zuolo, M.F. Godoy, J.M. Godoy, D.P. Link, M. Junger, A. Scuderi, Society for Vascular Surgery and American Venous Forum Guidelines on the management of venous leg ulcers: the point of view of the International Union of Phlebology, Int. Angiol. 34 (2015) 202–218.
[29] N. Labropoulos, J. Tiongson, L. Pryor, A.K. Tassiopoulos, S.S. Kang, M. Ashraf Mansour, W.H. Baker, Definition of venous reflux in lower-extremity veins, J. Vasc. Surg. 38 (2003) 793–798.
[30] B. Eklof, R.B. Rutherford, J.J. Bergan, P.H. Carpentier, P. Gloviczki, R.L. Kistner, M.H. Meissner, G.L. Moneta, K. Myers, F.T. Padberg, M. Perrin, C.V. Ruckley, P.C. Smith, T.W. Wakefield, Revision of the CEAP classification for chronic venous disorders: consensus statement, J. Vasc. Surg. 40 (2004) 1248–1252.
[31] H. Breivik, P.C. Borchgrevink, S.M. Allen, L.A. Rosseland, L. Romundstad, E.K. Hals, G. Kvarstein, A. Stubhaug, Assessment of pain, Br. J. Anaesth. 101 (2008) 17–24.
[32] A.N. Moor, D.J. Vachon, L.J. Gould, Proteolytic activity in wound fluids and tissues derived from chronic venous leg ulcers, Wound Repair Regen. 17 (2009) 832–839.
[33] J.D. Raffetto, W.A. Marston, Venous ulcer: what is new? Plast. Reconstr. Surg. 127 (Suppl. 1) (2011) 279 s–288 s.
[34] M. Briggs, E.A. Nelson, M. Martyn-St James, Topical agents or dressings for pain in venous leg ulcers, Cochrane Database Syst. Rev. 11 (2012), Cd001177.
[35] K. Brew, H. Nagase, The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity, Biochim. Biophys. Acta 1803 (2010) 55–71.
[36] M. Vaalamo, T. Leivo, U. Saarialho-Kere, Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, −2, −3, and −4) in normal and aberrant wound healing, Hum. Pathol. 30 (1999) 795–802.
[37] M. Vaalamo, L. Mattila, N. Johansson, A.L. Kariniemi, M.L. Karjalainen-Lindsberg, V.M. Kahari, U. Saarialho-Kere, Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds, J. Invest. Dermatol. 109 (1997) 96–101.
[38] J. Norgauer, T. Hildenbrand, M. Idzko, E. Panther, E. Bandemir, M. Hartmann, W. Vanscheidt, Y. Herouy, Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers, Br. J. Dermatol. 147 (2002) 1180–1186.
[39] F.J. Meyer, K.G. Burnand, S. Abisi, J.M. Tekoppele, B. van Els, A. Smith, Effect of collagen turnover and matrix metalloproteinase activity on healing of venous leg ulcers, Br. J. Surg. 95 (2008) 319–325.
[40] S.K. Beidler, C.D. Douillet, D.F. Berndt, B.A. Keagy, P.B. Rich, W.A. Marston, Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy, Wound Repair Regen. 16 (2008) 642–648.
[41] R. Serra, G. Buffone, D. Falcone, V. Molinari, M. Scaramuzzino, L. Gallelli, S. de Franciscis, Chronic venous leg ulcers are associated with high levels of metalloproteinases-9 and neutrophil gelatinase-associated lipocalin, Wound Repair Regen. 21 (2013) 395–401.
[42] R. Serra, R. Grande, G. Buffone, V. Molinari, P. Perri, A. Perri, B. Amato, M. Colosimo, S. de Franciscis, Extracellular matrix assessment of infected chronic venous leg ulcers: role of metalloproteinases and inflammatory cytokines, Int. Wound J. 13 (2016) 53–58.
[43] B. Amato, G. Coretti, R. Compagna, M. Amato, G. Buffone, D. Gigliotti, R. Grande, R. Serra, S. de Franciscis, Role of matrix metalloproteinases in non-healing venous ulcers, Int. Wound J. 12 (2015) 641–645.
[44] S. Saito, M.J. Trovato, R. You, B.K. Lal, F. Fasehun, F.T. Padberg Jr., R.W. Hobson 2nd, W.N. Duran, P.J. Pappas, Role of matrix metalloproteinases 1, 2, and 9 and tissue inhibitor of matrix metalloproteinase-1 in chronic venous insufficiency, J. Vasc. Surg. 34 (2001) 930–938.
[45] B. Mwaura, B. Mahendran, N. Hynes, D. Defreitas, G. Avalos, T. Adegbola, M. Adham, C.E. Connolly, S. Sultan, The impact of differential expression of extracellular matrix metalloproteinase inducer, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-2 and PDGF-AA on the chronicity of venous leg ulcers, Eur. J. Vasc. Endovasc. Surg. 31 (2006) 306–310.
[46] J.F. Tarlton, A.J. Bailey, E. Crawford, D. Jones, K. Moore, K.D. Harding, Prognostic value of markers of collagen remodeling in venous ulcers, Wound Repair Regen. 7 (1999) 347–355.
[47] T. Grzela, J. Niderla-Bielinska, M. Litwiniuk, R. White, The direct inhibition of MMP-2 and MMP-9 by an enzyme alginogel: a possible mechanism of healing support for venous leg ulcers, J. Wound Care 23 (278) (2014) 280-272–284–275.
[48] U. Mirastschijski, U. Impola, T. Jahkola, T. Karlsmark, A.G. MS, U. Saarialho-Kere, Ectopic localization of matrix metalloproteinase-9 in chronic cutaneous wounds, Hum. Pathol. 33 (2002) 355–364.
[49] U. Impola, L. Jeskanen, L. Ravanti, S. Syrjanen, B. Baldursson, V.M. Kahari, U. Saarialho-Kere, Expression of matrix metalloproteinase (MMP)-7 and MMP-13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds, Br. J. Dermatol. 152 (2005) 720–726.
[50] T. Roch, O. Akymenko, A. Kruger, F. Jung, N. Ma, A. Lendlein, Expression pattern analysis and activity determination of matrix metalloproteinase derived from human macrophage subsets, Clin. Hemorheol. Microcirc. 58 (2014) 147–158.
[51] A. Sindrilaru, K. Scharffetter-Kochanek, Disclosure of the culprits: macrophages-versatile regulators of wound healing, Adv. Wound Care (New Rochelle) 2 (2013) 357–368.
[52] B.C. Nwomeh, H.X. Liang, I.K. Cohen, D.R. Yager, MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers, J. Surg. Res. 81 (1999) 189–195.
[53] M.J. Toriseva, R. Ala-aho, J. Karvinen, A.H. Baker, V.S. Marjomaki, J. Heino, V.M. Kahari, Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts, J. Invest. Dermatol. 127 (2007) 49–59.
[54] M. Swee, C.L. Wilson, Y. Wang, J.K. McGuire, W.C. Parks, Matrix metalloproteinase-7 (matrilysin) controls neutrophil egress by generating chemokine gradients, J. Leukoc. Biol. 83 (2008) 1404–1412.

[55] Q. Li, P.W. Park, C.L. Wilson, W.C. Parks, Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury, Cell 111 (2002) 635–646.
[56] W.C. Powell, B. Fingleton, C.L. Wilson, M. Boothby, L.M. Matrisian, The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis, Curr. Biol. 9 (1999) 1441–1447.
[57] A.M. Manicone, I. Huizar, J.K. McGuire, Matrilysin (matrix metalloproteinase-7) regulates anti-inflammatory and antifibrotic pulmonary dendritic cells that express CD103 (alpha(E)beta(7)-integrin), Am. J. Pathol. 175 (2009) 2319–2331.
[58] N. Huo, Y. Ichikawa, M. Kamiyama, T. Ishikawa, Y. Hamaguchi, S. Hasegawa, Y. Nagashima, K. Miyazaki, H. Shimada, MMP-7 (matrilysin) accelerated growth of human umbilical vein endothelial cells, Cancer Lett. 177 (2002) 95–100.
[59] P. Chen, L.E. Abacherli, S.T. Nadler, Y. Wang, Q. Li, W.C. Parks, MMP7 shedding of syndecan-1 facilitates re-epithelialization by affecting alpha(2)beta(1) integrin activation, PLoS ONE 4 (2009), e6565.
[60] Y. Herouy, A.E. May, G. Pornschlegel, C. Stetter, H. Grenz, K.T. Preissner, E. Schopf, J. Norgauer, W. Vanscheidt, Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: implications for venous ulcer formation, J. Invest. Dermatol. 111 (1998) 822–827.
[61] V. Arpino, M. Brock, S.E. Gill, The role of TIMPs in regulation of extracellular matrix proteolysis, Matrix Biol. 44-46 (2015) 247–254.
[62] H. Liu, B. Chen, B. Lilly, Fibroblasts potentiate blood vessel formation partially through secreted factor TIMP-1, Angiogenesis 11 (2008) 223–234.
[63] W.G. Stetler-Stevenson, Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities, Sci. Signal. 1 (2008), re6.
[64] J.H. Qi, Q. Ebrahem, N. Moore, G. Murphy, L. Claesson-Welsh, M. Bond, A. Baker, B. Anand-Apte, A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2, Nat. Med. 9 (2003) 407–415.
[65] I. Koskivirta, O. Rahkonen, M. Mayranpaa, S. Pakkanen, M. Husheem, A. Sainio, H. Hakovirta, J. Laine, E. Jokinen, E. Vuorio, P. Kovanen, H. Jarvelainen, Tissue inhibitor of metalloproteinases 4 (TIMP4) is involved in inflammatory processes of human cardiovascular pathology, Histochem. Cell Biol. 126 (2006) 335–342.
[66] F.G. Spinale, Matrix metalloproteinases: regulation and dysregulation in the failing heart, Circ. Res. 90 (2002) 520–530.
[67] Y.H. Guo, W. Gao, Q. Li, P.F. Li, P.Y. Yao, K. Chen, Tissue inhibitor of metalloproteinases-4 suppresses vascular smooth muscle cell migration and induces cell apoptosis, Life Sci. 75 (2004) 2483–2493.
[68] A. Desmouliere, M. Redard, I. Darby, G. Gabbiani, Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar, Am. J. Pathol. 146 (1995) 56–66.
[69] B. Mahdavian Delavary, W.M. van der Veer, M. van Egmond, F.B. Niessen, R.H. Beelen, Macrophages in skin injury and repair, Immunobiology 216 (2011) 753–762.
[70] U.T. Shankavaram, D.L. DeWitt, L.M. Wahl, Lipopolysaccharide induction of monocyte matrix metalloproteinases is regulated by the tyrosine phosphorylation of cytosolic phospholipase A2, J. Leukoc. Biol. 64 (1998) 221–227.
[71] T. Suzuki, S. Hashimoto, N. Toyoda, S. Nagai, N. Yamazaki, H.Y. Dong, J. Sakai, T. Yamashita, T. Nukiwa, K. Matsushima, Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE, Blood 96 (2000) 2584–2591.
[72] D. Min, A.G. Moore, M.A. Bain, S.N. Breit, J.G. Lyons, Activation of macrophage promatrix metalloproteinase-9 by lipopolysaccharide-associated proteinases, J. Immunol. 168 (2002) 2449–2455.
[73] B. Reel, G.B. Sala-Newby, W.C. Huang, A.C. Newby, Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes, Br. J. Pharmacol. 163 (2011) 1679–1690.
[74] F. Mannello, D. Ligi, M. Canale, J.D. Raffetto, Sulodexide down-regulates the release of cytokines, chemokines, and leukocyte colony stimulating factors from human macrophages: role of glycosaminoglycans in inflammatory pathways of chronic venous disease, Curr. Vasc. Pharmacol. 12 (2014) 173–185.
[75] R.D. Kenagy, S.T. Nikkari, H.G. Welgus, A.W. Clowes, Heparin inhibits the induction of three matrix metalloproteinases (stromelysin, 92-kD gelatinase, and collagenase) in primate arterial smooth muscle cells, J. Clin. Invest. 93 (1994) 1987–1993.
[76] G.M. Campo, A. Avenoso, S. Campo, A. D'Ascola, P. Traina, D. Sama, A. Calatroni, Glycosaminoglycans modulate inflammation and apoptosis in LPS-treated chondrocytes, J. Cell. Biochem. 106 (2009) 83–92.
[77] W. Hornebeck, B. Gogly, G. Godeau, H. Emonard, B. Pellat, Heparin and fragments modulate the expression of collagen-degrading enzymes (matrix metalloproteinases 1 and 2) by human gingival fibroblasts, Ann. N. Y. Acad. Sci. 878 (1999) 625–628.
[78] W.H. Yu, J.F. Woessner Jr., Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7), J. Biol. Chem. 275 (2000) 4183–4191.
[79] Y.G. Fulcher, R.R. Sanganna Gari, N.C. Frey, F. Zhang, R.J. Linhardt, G.M. King, S.R. Van Doren, Heparinoids activate a protease, secreted by mucosa and tumors, via tethering supplemented by allostery, ACS Chem. Biol. 9 (2014) 957–966.
[80] F. Mannello, D. Ligi, J.D. Raffetto, Glycosaminoglycan sulodexide modulates inflammatory pathways in chronic venous disease, Int. Angiol. 33 (2014) 236–242.