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Venous lower limb microenvironment: an open window on the Chronic Venous Disease

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INDEX

Preface

The title of this thesis might mislead by inducing to think that it will be only a careful examination of the numerous factors involved in the onset of the Chronic Venous Disease (CVeD) and in its progression, but the words "*open window*"are far from being trivial. Different points of view can justify their use, starting from that historical which enables us to dredge up that the Greek physicians Hippocrates (460-377 before Christ) and Galeno (130-210 before Christ), with their first observations and theories about the CVeD and the physiology of venous circulation, have marked the begin, or rather *the opening* of a research season which nowadays goes on[1]. Figures as Vassaseus (1544), who described venous valves and their function, Harvey (1628), a pioneer of modern medicine, who revolutionized the Galenic theory by clarifying the venous valve role in blood flow maintenance, Brodie, (1846) who described symptoms and signs of Chronic Venous Insufficiency (CVI)and later Linton, who founded the pathophysiological theory for CVeD on the concept of ambulatory venous hypertension[1,2], have begun to give voice to the still current need for improving the knowledge about the most deep mechanisms of the widespread venous disorders, although this goal is still long way off. In effect, questions as, are there further CVeD risk factors besides those well-known? What are the deep cellular and molecular mechanisms triggering the switch among the CVeD stages? What is their timing? What are the real moderators of the biochemical interactions characterizing the venous disorders? What criteria should be used to consider a therapeutic treatment as successful? These might represent some of the

partially *opened* questions which inspire the efforts to find suitable answers to lean out of that *open window* consciously.

The favourable implication of this positive disposition consists in the contribution to a knowledge shift from the only CVeD pathophysiology framework to the details which could make the difference in developing of targeted therapeutic approaches.

Chapter 1

Introduction

1.1 Epidemiological, diagnostic and therapeutic background of the Chronic Venous Disease

In the centuries, numerous clinical studies have endeavoured to elucidate the multifaceted magnitude of the macro- and microcirculatory impairments and the potential molecular mechanisms responsible for the multiple symptoms and signs of CVeD. Although these efforts are certainly praiseworthy also due to their implications in the unbated research of the suitable therapeutic options, they represent only one side of the medal. In fact, it should not be neglected the socio-economic and psychological impact that the different manifestations of venous disorders and their consequent management have on the patient quality of life. The costs of diagnosis, therapeutic treatments and the loss of working days represent only some aspects of the heavy burden bore by the worldwide Western country population affected by telangiectasies (80%), varicose veins (from 20% to 64%), the more advanced stages of CVeD edema, hyperpigmentation, lipodermatosclerosis (5%) and active or healed ulcers (from 1% to 2%)[1,3]. This prevalence rates should be certainly considered with relation to some of well-known risk factors, as age, gender, ethnicity, obesity, familiar history, in order to understand their real significance[1,4]. In this regard, the correlation between the increased appearance of varicose veins and chronic venous insufficiency (CVI) and the older age and female gender remains one of the most confirmed epidemiological evidence of the progression from the CVeD asymptomatic signs to the most sever clinical

manifestations[5,6,7]. In particular, the second of the above-mentioned factors is strictly correlated with the pregnancy, which seems to have its weight in venous disorder appearance[8]. Furthermore, the obesity (BMI >30 Kg/m^2) and a CVeD familiar positive history might also contribute to some hemodynamic disfunctions, despite the genetics role remains unclear in several respects nowadays[3,9,10]. This risk factor summary might be enriched by considering other factors, as oral contraceptive assumption, low fibre intake or constipation, smoking, hypertension, prolonged orthostatism (> 4 hours daily) and physical activity, whose potential relationship with CVeD should be clarified in some respects[6]. However, the population ethnicity might be considered a persistent background in both the worldwide spread of the progressive CVeD manifestations and in the risk factor contribution to these[11]. In this regard, several recent studies have interestingly noticed that the CVeD is one of the diseases with the major prevalence in the western world population compared to that Asian (Tab. 1.1.1)[3,12,13].

CEAP	Asia	Eastern Europe	Latin America	Western Europe	Total
0	n = 5907 (48.07%)	n = 8980 (31.89%)	n = 3195 (31.89%)	n = 18 000 (38.35%)	n = 36 082
1	n = 2257 (18.37%)	n = 5712 (18.97%)	n = 2238 (25.16%)	n = 10 057 (21.42%)	n = 20 264
2	n = 1686 (13.72%)	n = 6414 (21.30%)	n = 1920 (19.16%)	n = 7207 (15.35%)	n = 17 227
3	n = 1665 (13.55%)	n = 5276 (17.52%)	n = 1340 (13.37%)	n = 7437 (15.84%)	n = 15 718
4	n = 616 (5.01%)	n = 2864 (9.51%)	n = 930 (9.28%)	n = 3457 (7.36%)	n = 7867
5	n = 73 (0.59%)	n = 628 (2.09%)	n = 250 (2.50%)	n = 578 (1.23%)	n = 1529
6	n = 84 (0.68%)	n = 236 (0.78%)	n = 147 (1.47%)	n = 205 (0.44%)	n = 672
Total	n = 12 288	n = 30 110	n = 10 020	n = 46 941	n = 99 359

Region	CVD (C1-C6)	CVI (C3-C6)
Asia	51.93%	19.84%
Eastern Europe	70.18%	29.90%
Latin America	68.11%	26.62%
Western Europe	61.65%	24.88%
Overall	63.69%	25.95%

Tab. 1.1.1 Clinical and general prevalence of CVeD in the main world geographical areas[3]. The tables show data collected during the period 2009-2013 and relating to the different CVeD manifestations in worldwide scale

Although it is fairly quick, this epidemiologic review enables to set the CVeD in a framework of implications highlighting that it certainly represents a serious clinical problem. Nevertheless, the increased pain, the reduced physical function and mobility, which often accompanied the most advanced CVeD stages, contribute to convert the venous disorders in a real psycho-social burden often characterized by depression and social isolation feelings[9]. These uneasiness sensations might be defined only as the tip of an iceberg represented by a series of health dimensions, as physical functionalities and limitations, emotional problems, pain, vitality, mental and general health perception, which are evaluated through different Quality-of-Life (QoL) assessments. They consist in questionnaires which are often specific for well-defined CVeD manifestations (e.g. AAVQ for varicose veins, CIVIQ for the CVI early manifestations) and so prone to poorly flexibility, but their features enable them to represent a mirror of the CVeD whole spectrum severity useful to elaborate an accurate evaluation of patient clinical condition and its therapeutic outcomes[14,15,16]. Sure enough, the QoL measurements are affected by the patient sociocultural context besides their aging, the disease perception and the presence of comorbidities, thus they might be considered a promising instrument halfway through the population epidemiologic characterization and the effective diagnosis of CVeD symptoms and signs. In this regard, the chances of mastering the management of venous disorders is also related to the opportunity of recognizing their hallmarks from the first appearance, by a thorough disease classification. The Clinical-Etiology-Anatomy-Pathophysiology classification (CEAP) lives up to this expectation, though it provides only a descriptive approach consistent with the original aim of refining the CVeD description and ensuring a straightforward

scientific and clinical communication. This remark enables to get to the heart of the CEAP classification, which defines the CVeD as a series of morphologic and functional venous disorders, rather than as disease, through its four above-mentioned points of view (Tab. 1.1.2)[1,17]. In particular, the first provides a clinical focus on the CVeD manifestations by arranging them in seven worldwide adopted sequential classes (C0-C6) in order to explain the potential venous disorder progression from the absolute absence of disease signs (C0) to the appearance of the first structural and functional abnormalities, as telangiectasies and varicose veins (C1-C2), followed by the arising of chronic venous insufficiency corresponding to the development of edema, skin changes, eczema, lipodermatosclerosis and healed or active venous ulcers (C3-C6)[17].

*C*linical Classification	*E*tiologic Classification	*A*natomical Classification	*P*athophysiologic Classification
C_0: No visible or palpable indicators of venous disease	E_C: Congenital	A_S: Superficial veins	P_R: Reflux
C_1: Telangiectases	E_P: Primary	A_D: Deep system	P_O: Obstruction
C_2: Varicosities	E_S: Secondary (post-thrombosis)	A_P: Perforator system	P_{RO}: Combination of reflux and obstruction
C_3: Edema	E_N: No venous cause identified	A_N: No venous identified	
C_4: Venous skin changes (hemosiderosis, dermatitis, lipodermatosclerosis)			
C_5: Venous skin changes *plus* healed ulceration			
C_6: Venous skin changes *plus* active ulceration			
	S: Symptomatic (aches, pain tightness, skin irritation)		
	A: Asymptomatic		

Tab. 1.1.2 The complete clinical, etiologic, anatomical and pathophysiologic (CEAP) classification of the CVeD. The table shows the clinical, etiological, anatomical and pathophysiological features of the CVeD manifestations

However, it should be borne in mind that this progression through these worldwide adopted CEAP clinical classes representing the whole spectrum of CVeD is prone to different influencing factors, which might lead to arrest it in one of them. As above-anticipated, the etiological, anatomical and pathophysiological CEAP classification might be considered from three further points of view which enable to pin the CVeD down by specifying its primary or secondary aetiology, the exact affected venous system

(superficial, deep or perforator veins) and the pathologic events at the origin of the disorder (obstruction, reflux or other pathophysiologic processes) (Tab. 2)[17].

Overall, these information doubtless contribute to build a reliable characterization of the clinical CVeD manifestations, but they are lacking in considering the appearance of some key symptoms and in pointing out the disorder severity[1]. This deficiency is often filled by resorting to the venous severity scores (VSS), which are suitable to report also faint changes in disease symptoms and severity. However, their limitations, as the poor reliability of the patient-reported symptoms, the existence of comorbidities and surveys of CVeD not specific symptoms, make them complementary instruments with CEAP classification and QoL assessments in evaluating both the clinical picture and outcomes[14]. Although all these aforementioned diagnostic instruments are affected by clinicians or patient disorder perceptions, they still provide the basis for a more thorough investigation of the venous system general state and potential functional disorders by more or less invasive diagnostic tools. The cornerstone in the CVeD symptom detection consists in the Duplex Ultrasonography (DUS) which offers the considerable advantage of observing the vein anatomy and the presence of valvular incompetence or venous obstructions by combining ultrasound imaging and pulsed wave Doppler. Its use has supplanted the outdated handheld Continuous Wave Doppler (CW Doppler) mainly due to its weak reliability in venous anatomical and hemodynamic impairment detection[18,19]. Furthermore, the DUS consists in a non-invasive diagnostic approach and its high accuracy is increased by the frequent concurrent performance with colour flow imaging which quickens the visualization of also deep venous system incompetence[20]. These assets have led to overtake invasive diagnostic tools, as

phethysmography and air-plethysmography (APG) based on venous volume and pressure measurements, or the further imaging technique phlebography[21,22]. Moreover, the DUS diagnostic results might be enhanced by the acquisition of three-dimensional vein structure processed by the alternative methods Computed Tomography (CT) and Magnetic Resonance Venography (MRV) in order to determine a careful CVeD diagnosis and choose the most suitable treatments[23]. This latter aspect, which represents the desirable result of the whole diagnostic effort, might consist in different therapeutic options. In this regard, the compression therapy constitutes the undisputed gold-standard among the numerous conservative therapeutic strategies[24]. Its non-invasive approach, consisting in the exercise of a controlled pressure on both lower limb superficial and deep venous systems, and its versatility are the effective advantages that allow it to work on the calf pump functionality to restore the physiological ambulatory pressure[25,26,27]. The main consequence of this mechanical action is the prevention or the management of venous hypertension, which represents one of the first pathophysiological processes at the origin of the different CVeD manifestations. Numerous garments, as elastic stockings, tights, and elastic or non- bandages commercially available have physical properties able to provide a proper graduated or an intermittent pneumatic compression (Tab. 3)[28,29]. In particular, the stiffness, which represents a direct correlation between the increase of pressure applied and the leg circumference increase (mmHg/cm), and the number of layers constituting the compression devices determine specific resting and walking pressure values suitable for treating the different CVeD manifestations[26]. Multilayer elastic compression stockings with high stiffness and a graduated compression degree around 20-40mmHg are

recommended in the active treatment of varicose veins, lipodermatosclerosis and edema due to their ability to apply a pressure which decreases from the ankle up to the thigh and results higher during the walking than the resting position (Tab. 1.1.3)[28,30,31].

Compression Class	USA	UK (BS 6612)	France	Germany
I	15–20 (moderate)	14–17 (light)	10–15	18–21 (light)
II	20–30 (firm)	18–24 (medium)	15–20	23–32 (medium)
III	30–40 (extra firm)	25–35 (strong)	20–36	34–46 (strong)
IV	40+		>36	>49 (very strong)

Tab. 1.1.3 Pressure values (mmHg) and respective compression classes of stockings used in different world countries[28]. The table shows the pressure values applied by compression garments to a hypothetical cylindrical ankle; (1 mmHg = 1,333 hPa)

In this regard, the wanted effect is comparable with a strong massage on the calf during the patient deambulation able to reduce pain and swelling. These functional characteristics, based on the La Place's law stating that the pressure applied around a cylinder is directly proportional to the radius of the cylinder one, make them useful also in maintenance treatment of lower limb healed ulcers and lymphoedema, even if elastic bandages are preferred in these cases[25,32,33].

As previously mentioned, non-elastic bandages, as the Unna's boot impregnated with zinc oxide paste or adjustable velcro straps, and intermittent pneumatic compression devices, obtained through inelastic cuff intermittently pumped up by a pump which produces different pressure degree in established time intervals, complete the range of the available compression approaches[1,25]. Furthermore, other non-invasive CVeD treatments consist in leg physiotherapy, which seems to improve the calf muscle functionality by selected exercises, leg elevation, which is recommended especially in C2-C6 treatment by enhancing the microcirculation and reducing edema, and the leg massage, which is mainly aimed to ameliorate the tissue edema by making deep

massages around the ulcer area before wearing the elastic stockings or bandages[34,35]. Nevertheless, a noteworthy common feature of these numerous CVeD non-invasive therapeutic approaches often consists in their concurrent use with those invasive. In this regard, a real cornerstone in the CVeD treatment is the well-known surgery. Different surgical strategies are developed over the time from the varicose vein ablation through the High Ligation with Stripping (HL/S) often associated with the ambulatory phlebectomy, the Ambulatory Selective Varices Ablation under Local anaesthesia (ASVAL), a less invasive phlebectomy technique aimed to preserve the undamaged segments of saphenous trunk, or the Cure conservatrice et Hémodynamique de l'Insuffisance Veineuse en Ambulatoire (CHIVA), improving the superficial venous system hemodynamics, up to the innovative Transilluminated Powered Phlebectomy (TIPP) consisting in large varicose vein cluster ablation with a considerably reduced number of incisions[36,1]. All these treatments are effectively followed by the recommended wearing of compression devices with proper pressure levels in order to ameliorate the post-operative outcomes by giving relief from pain and other potential complications. Moreover, this represents the same clinical procedure followed after performing the superficial venous system treatment by the sclerotherapy[37,38]. This consists in a therapeutic strategy for the chemical ablation of varicosities, venules or telangiectasias by the injection of liquid or foam sclerosant agents which damage the venous endothelial lining due to their close contact with it and induce the collagen and smooth muscle basal layer exposition accompanied by vasospasms and the treated vessel transformation in a fibrous cord[39]. However, it should not be neglected that this sclerosant effect is distance and time-dependent due to the direct contact between the

chemical agents and the blood circulating cells which take away them and determine their dilution, also defined consumption[40,41,42]. This interaction is delayed by the more efficient blood displacement induced by foam than liquid sclerosant by extending the treatment effects[43]. In fact, the chemical nature of the sclerosants and their physical state (liquid or foam) result decisive for the treatment outcome[44,45]. The best known sclerosant polidocanol and sodium tetradecyl sulphate have respectively non-ionic and anionic nature which determines their membrane solubilization and protein denaturing properties on circulating and non- components following their injection in the damaged vessel[46,47]. Furthermore, several studies have interestingly highlighted time-dependent pro-coagulant effects at low concentrations of both the sclerosant agents unlike those anti-coagulant revealed at increased concentrations[48,49,50]. The sclerotherapy is only one of a series of alternative and less invasive approaches developed for the saphenous vein incompetence treatment also including the Endovenous Laser Ablation (EVLA), the Endovenous Thermal Ablation (EVTA), the Radiofrequency Laser Ablation (RFA) and the more recent Mechanochemical Ablation (MOCA) and cyanoacrylate glue injection[1,51,36]. These strategies are equally aimed to induce endothelial damages through different percutaneous procedures by causing the treated vessel occlusion. Thus, all these treatment features might enable to set them halfway between the aforementioned invasive clinical approaches and the controversial pharmacological strategies. In this regard, drugs as alpha-benzopyrones (Coumarin), gamma-benzopyrones (flavonoids and their micronized purified fraction or MPFF), saponines (escin, ruscus extract), other plant extracts (anthocyans, proantocyanidins) and calcium dobesilate, benzarone,

naftazon are overall defined as natural or synthetic venoactive drugs (VAD), which differ from the nonvenoactive drugs (pentoxifylline, acetylsalicylic acid) (Tab. 1.1.4)[52,53,13].

Group	Substance	Origin
Alpha-benzopyrones	Coumarin	Melilot (*Melilotus officinalis*)
		Woodruff (*Asperula odorata*)
Gamma-benzopyrones (flavonoids)	Diosmin	Citrus spp. (*Sophora japonica*)
	Micronized purified flavonoid fraction	*Rutaceae aurantiae*
	Rutin and rutosides	*Sophora japonica*
	0-(β-hydroxyethyl)-rutosides (troxerutin, HR)	*Eucalyptus* spp.
		Fagopyrum esculentum
Saponins	Escin	Horse chestnut seed extracts (*Aesculus hippocastanum* L)
	Ruscus extract	Butcher's broom (*Ruscus aculeatus*)
Other plant extracts	Anthocyans	Bilberry (*Vaccinium myrtillus*)
	Proanthocyanidins (oligomers)	Red wine leaves extracts, Maritime pine (*Pinus maritimus*)
	Extracts of Ginkgo, heptaminol and troxerutin	Ginkgo biloba
	Total triterpene fraction	Centella asiatica
Synthetic products	Calcium dobesilate	Synthetic
	Benzaron	Synthetic
	Naftazon	Synthetic

Tab. 1.1.4 Classification of the main venoactive drugs (VAD)[52]

The firsts are able to increase the venous tone also by exploiting the noradrenaline pathway, to reduce the venous permeability and the inflammation by counteracting the leucocyte-endothelium interactions, the second act by limiting the white cell and platelet activation with consequent anti-inflammatory effects. Furthermore, this action spectrum encompasses also the therapeutic effects of some glycosaminoglycans (e.g. Sulodexide) which contribute to restore the endothelial physiological functionality and to reduce inflammatory processes[54,55,13]. Clearly, all these effects make the medical approaches fit for each CVeD stage treatment often in association with the compression therapy.

1.2 The structural and hemodynamic players of the Chronic Venous Disease

The occurrence of visible dilated blue or red/purple dermal veins, corresponding to reticular veins or telangiectasies (C1), with a smaller diameter (<4 mm) than the palpable and tortuous varicose veins (>4 mm) (C2) up to the unsightly skin changes and ulcerations represent some of the signs of the primary and secondary CVeD[56,17,57]. The choice of drawing the attention to these CVeD clinical signs is consistent with the perspective of considering them mirror of break points in the structural and mechanical integrity of the intricate lower extremity vascular system. Three different venous components, the superficial, deep and perforating veins, provide the correct blood distribution in the supplied districts through their specific localization[56,58]. However, their role runs down far from being simple and passive conduits since they have structural features actively related to the main hemodynamic events responsible for the physiologic drainage of lower limbs, besides for the blood return to the heart. In detail, the set of reticular veins lining the dermis, which accompany the Great Saphenous Vein (GSV) and the Small Saphenous Vein (SSV) with their numerous tributaries and a series of associated nerves, represent the abovementioned superficial venous system implicated in the skin microcirculation[56,58,59]. This function is also consistent with their spatial organization in a superficial sub-compartment, known as saphenous compartment, in which the GSV and the SSV are confined between the saphenous and the muscular fascia[56]. The ascending GSV course starts from the dorsal pedal venous arch up to the joining with the common femoral vein in the thigh through the saphenofemoral junction (SFJ) after crossing the medial malleolus and the tibia. Tributary veins, principally organized in the Leonardo's arch, enable the GSV to drain

numerous ankle, tibial and anterior or posterior calf veins. Moreover, the GSV is also connected with the SSV and collects blood from the superficial external pudendal, epigastric and circumflex iliac veins by spilling it into the femoral vein. The small or short saphenous vein equally rises up from the dorsal pedal arch by passing behind the malleolus and proceeds posteriorly up to the calf, where it crosses the gastrocnemius muscles and joins the popliteal vein through the saphenopopliteal junction (SPJ)[60,56].

The functionality of this intricate system of lower limb superficial veins is sustained by the equally complex arrangement of the deep vein system. They might be described according with their anatomical localization in foot, calf and thigh deep veins. In particular, the two plantar veins give origin to both the saphenous veins and are organized in a calcaneus plexus involved in the ambulatory pressure control besides in ejecting blood in the paired posterior tibial veins. These last with the corresponding anterior tibial veins, the peroneal, soleal and gastrocnemius veins consist in the deep calf venous system. The soleal and gastrocnemius veins, which are connected with the popliteal vein, give rise to muscular venous plexuses essential for the calf pump function. The tibial and soleal veins are usually connected with the peroneal veins to form the popliteal vein, which goes up the thigh and becomes the femoral vein after running into the thigh adductor magnus muscle. The popliteal and femoral veins represent a fundamental deep check-point in the preservation of the calf pump functionality through their valvular competence[59]. Furthermore, the femoral vein runs through most of the thigh up to the inguinal region where it joins the deep femoral vein and the GSV to form the common femoral vein besides to accompany the femoral arteries and receive numerous muscle tributary veins. The common iliac vein, which takes origin

from the connection between the external (derivative of the common femoral vein) and the internal iliac vein, and the pelvic vein, which is organized in a series of venous plexuses interconnecting deep and superficial veins with pelvis, visceral and parietal districts, enrich the deep thigh venous system[59,61].

The perforating veins exactly located in four anatomical districts, consisting in the foot, the medial and lateral calf and the thigh, represent "the glue" of the whole lower limb drain system by just favouring blood exchange between deep and superficial veins cited until now[56,59]. However, the collecting station of the whole blood circulation through this venous network lies in the Inferior Vena Cava (IVC) which takes origin from the deep common iliac vein and gathers blood from lumbar, renal, inferior phrenic, right gonadal and hepatic veins before heading for the heart through the diaphragm and pericardium[59].

Structural and mechanical features of these intricate vascular systems represent the basis for the correct physiologic blood drainage in lower limbs and their competence ensures the awaited results of this transport process. In this regard, the valvular systems, variously distributed in the lower vascular architecture, have a noteworthy role in association with some essential pressure phenomena and compressive mechanisms mainly induced by muscles variously distributed in lower extremities. They consist in bicuspid and unidirectional venous valves showing half-moon-shaped cusps which are characterized by a thin collagen and endothelial layer consistent with the vein wall structural composition[2,62]. In particular, each cusp has the margin attached to the vein wall which appears thicker in the point of contact than the opposite free (Fig. 1.2.1b). These valve components, whose length is strictly related to the respective venous

calibre, present two different sides named luminalis and parietalis. The first, consisting in the cusp side directly exposed to the lumen of the vessel and, thus, to the blood flow, is made up of an internal and fairly thick elastic layer covered by medial connective tissue and an external endothelial lining disposed along the vessel major axis, whereas the second, representing the cusp side facing the vein wall of the valve sinus, is characterized by a depleted connective and muscular layer covered by endothelial cells transversally stretched. The mechanical implication of this variable endothelial cell orientation accompanied by the presence of plentiful smooth muscle cells and elastic fibres in specific valve portions (e.g. the join between the valve cusps and the vein wall) consists in the acquisition of properties useful in tackling blood flow fluctuations[62]. In this regard, the very existence of the venous valves and their open-and-closure mechanism passively regulated by a transvalvular pressure gradient determine the fragmentation of the blood column in multiple segments with controlled pressure[1,57]. The reversal of this gradient triggers the valve closure after a physiological quick reflux (< 0.5 sec)[59,63,64]. These events occur continuously in each valvular system to generally favour the blood drainage from the superficial to the deep venous system despite their variable distribution progressively less abundant by raising along the leg[56]. Nonetheless, the competent valvular functionality is further sustained by the structural arrangement of the vein wall three layers which spread to the bicuspid valves (Fig. 1.2.1a). In particular, the most internal tunica intima is mainly characterized by the presence of endothelial cells which rest on their basement membrane followed by an intimal elastic lamina whose fibres stretch out in the valvular cusps accompanied by some muscular bundles. This thin monolayer, endowed with anti-thrombogenic or pro-coagulant

properties dependent on endothelium stimulations, is sequentially followed by the tunica media organized into three superimposed smooth muscle layers accompanied with scattered collagen and elastin fibres in addition to an adrenergic innervation[65]. These consistent venous muscular and elastic components take part of the valvular portions subjected to the major mechanical stress in order to develop an appropriate resistance besides to prevent an excessive venous dilatation[62]. The last and thickest venous wall tunica consists in the adventitia which is mainly composed of collagen fibres which sustain the just mentioned functional aim[56].

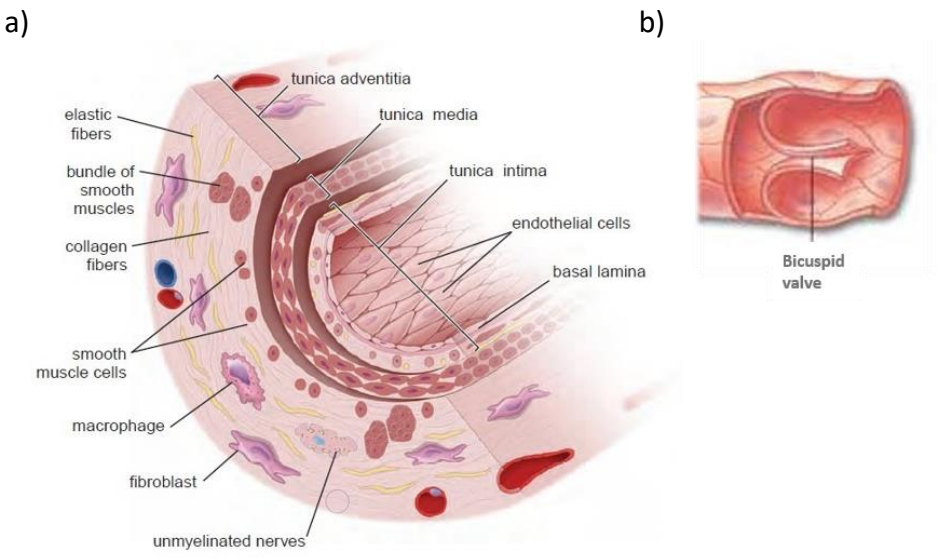


Fig. 1.2.1 Representation of the intimate venous wall and bicuspid valve structure. The figure in *a)* shows the three overlapped layers (tunica intima, tunica media, tunica adventitia) and their cellular components; the figure in *b)* shows the structural organization of the venous bicuspid valve

Thus, the evidenced structural features should be counted as the mechanical requirements for providing both the unidirectional valve role of preventing the blood reflux occurrence and the physiologic vascular tone and compliance[1,66]. These last

aspects, closely related to the contractile ability of the muscular component and the adaptation of vascular capacitance to the blood flow entity, in addition to the valvular competence, the hydrostatic, ambulatory and dynamic pressures and the respiration contribution represent the main physiological forces influencing the venous blood return to the right atrium[18,67]. Although the respiratory airflow might seem the least pertinent factor among those affecting the lower limb circulation, this involuntary act makes its indirect contribution to the physiologic process. In fact, the inspiration determines a consistent reduction of the thoracic cavity pressure accompanied by a consequent increased blood flow in the upper portion of trunk and the opposite effect on the abdominal pressure with a correspondent decrease in lower extremities blood outflow. The expiration reverses these anatomical and pressure adjustments leading to a heavy blood return from the lower body districts[59,68]. In line with the essential need to provide the correct occurring of this last physiologic process, noteworthy assets might be also credited to the dynamic pressure. It directly depends on the heartbeat propagation firstly in the arteries followed by the other vascular systems with a progressive decrease related to different factors, as the arterial precapillary vasoconstriction (12-18mmHg in capillary venous side). However, this pressure is affected by the influence of the lower still atrial pressure (4-7mmHg), which induces the presence of an effective gradient favouring the blood return to the heart[1]. This flow modulation, mainly predominant in supine position, is heavily influenced by the other two mentioned pressure systems. The ambulatory pressure, as its name implies, is closely related to the well-organized contractile activity of the principal foot, calf and thigh muscles during the daily ambulation. In this regard, it should be noted that the

functional competence of these real pumping systems also depends on the elliptical geometry typical of the calf and foot veins which enhances the venous capacitance by minimizing the effect of blood volume and pressure changes. Their flaccid appearance at low pressure value alternates with more circular sectional geometry consistent with an increased blood volume are a representative confirm of this their property[59]. Therefore, the high capacitance and low resistance of vessels and the prevalent activity of the muscular component make the calf pump the main driving force of the blood return to the heart (Fig. 1.2.2)[69]. Its ejection fraction (EF) of about 65% is tightly associated with the ambulatory pressure, which still consists in a gradient involving the thigh and the distal leg districts and exactly generated by the calf muscle activity[70]. In particular, the contraction determines the increase of venous pressure and the consequent centripetal blood expulsion from the deep popliteal and femoral veins to the superficial GSV (pressure of about 140 mmHg), whereas induces a pressure fall around 25-30 mmHg in the lower leg and foot. The calf pump relaxation reverses this effect with a consequent centrifugal blood flow directed from the GSV, characterized by high pressure, to deep venous system[56,69].

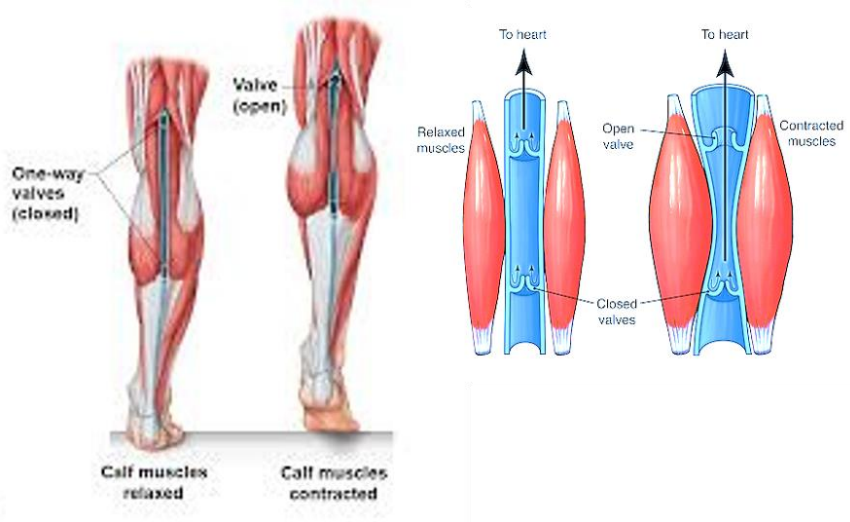


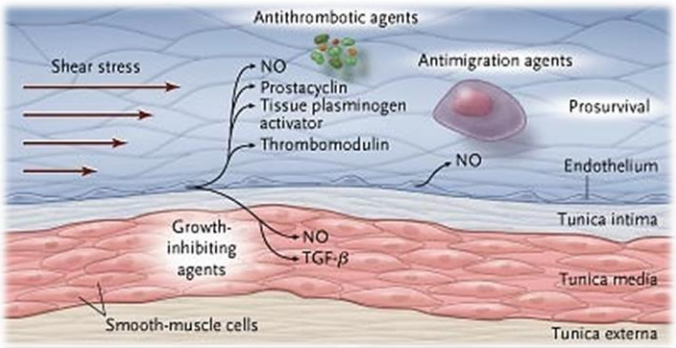
Fig. 1.2.2 Representation of the venous calf pump. The figure schematically shows the structural organization of the calf pump muscles and the mechanical effects induced on the retrograde blood return

The very existence of this horizontal flow depends on the venous perforating system, which spreads the pressure changes between the other two venous systems due to its medial position. Furthermore, the physiologic muscular activity of the calf pump is also able to indirectly affect the retrograde blood return to the right atrium by influencing the hydrostatic pressure[71]. This consists in an additional blood pressure component arisen from the gravitation force effects on the lower limb veins during the quiet standing position. Interestingly, it can be defined as the vertical distance between the heart, which corresponds to the pressure zero line, and a specific body anatomical district by depending on the subject height. The hydrostatic pressure generally increases of about 0.8mmHg/cm as proceeding from the right atrium to the ankle, where it amounts to 80-100mmHg[67]. Although it is not subjected to variations both in deep and superficial venous systems at the resting position, however the ambulation induces the calf pump activation, which determines the drop of the high hydrostatic pressure to

about 25-30mmHg in the calf and foot during its muscular contraction. The pump relaxation causes a slow pressure increase to the initial value instead[67].

On the basis of this general focus on the roles of each dynamic variable involved in the regulation of the physiological blood circulation in distal leg and the vital retrograde blood flow, there needs to point out the essentiality of the valvular competence as common denominator[18]. In this regard, the previously hinted open-and-closure mechanism of the venous valve leaflets (about 20 times/min in standing position) determines a pulsatile blood flow which is characterized by two dynamic components consisting in the directed and the vortical flows. The first is mainly sustained by the ambulation effects on foot and calf muscle functionality, which accelerate the flow by reducing the pressure applied on the luminal side of the valve cusps, while the second is consequent to the previous by preventing the blood stasis in the valvular parietalis side and favouring its exposition to the shear stress effects (Fig. 1.2.3a)[72]. Furthermore, the low pressure of the blood directed jet compared to that of the vortical flow is essential to determine the valve leaflets closure, despite the appearance of the previously mentioned quick reflux[57].

a)



b)

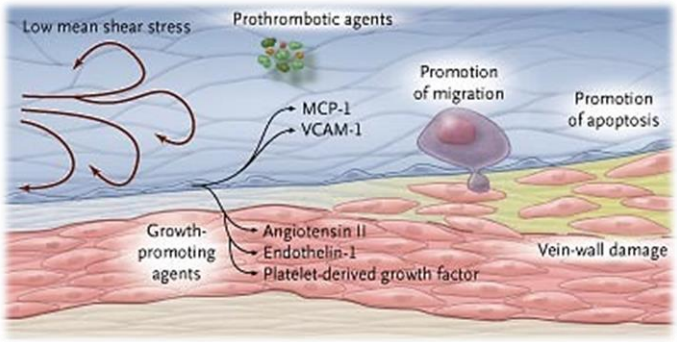


Fig. 1.2.3 Schematic representation of the shear stress change effects[57]. The figure in *a)* shows the laminar and steady shear stress effects ensuring the endothelium and venous wall integrity; the figure in *b)* shows the venous wall damages with specific secretive responses to the low and irregular shear stress

The shear stress is a further physiological consequence of the fluid dynamics in the blood vessels[73]. In effects, its relevance might be attributed both to the direct contact with the endothelial glycocalyx, which transduces its laminar or low and irregular turn by triggering different cellular response pathways, and to the high leucocyte responsiveness to its variations (Fig. 1.2.3a,b)[74,75]. These additional details enable to understand the real significance of venous valve functional impairment often associated to leaflet structural damages or vein wall alterations (e.g. hypertrophic region with dysregulated collagen synthesis, smooth muscle and elastin fibres scatter)[57,76]. Consequently, incompetent venous valves become unable to fragment the blood column and to tackle its high pressure induced by muscle pump activity in deep venous system by abolishing the two opposite poles of the previously said pressure gradients. The immediate result of the knock-on effect sparked off by these last functional and structural disturbances, which are reflected in hemodynamic perturbation, consists in hypertension development characterizing the different CVeD manifestations[76,77].

1.3 The cellular and biochemical players of the Chronic Venous Disease

Despite controversial opinions about the right sequence between the venous valve and wall impairment occurrence have followed one another over the years, it might be intuitive thinking that a series of preliminary alterations of the vein wall structure induce vessel dilation and, thus, valve closure failure. The extracellular matrix (ECM) depletion in laminin and elastin concentration accompanied by compromised deposition of collagen by the vascular smooth muscle cells and fibroblasts are effectively the structural premises of venous hypertension[52,57,78]. In this regard, the pointed-out imbalance between the collagen I and III ratio in favour of the first seems to be coherent with the venous loss of elasticity and distensibility characterizing this pathological condition[79]. Hence, the vein walls weak and stiff become unable to handle the whole spectrum of physiological pressure and hemodynamic fluctuations with the mechanical breakdown of the valvular unidirectional checkpoints. The consequent abolition of the previously discussed pressure gradients and the general increase of pressure in all districts of lower extremities force to shift the focus on their effects on the venous endothelial layer. This acts as a sort of condenser capable of gathering the physiological mechanical stress induced by the very blood flow with all its dynamic components and transducing them in proliferative, secretive and transcriptional stimuli in the nearby cells[79,80]. However, the real transducer is the glycocalyx consisting in some glycosaminoglycans (e.g. heparan and chondroitin sulphate, hyaluronan), proteoglycans and glycoproteins which line the luminal side of the vascular endothelial layer[73]. It represents an effective interface between the shear stress effects and the overhanging cellular lining, besides a selective permeability barrier able to provide the positive

regulation of coagulation and fibrinolysis processes and the necessary hindrances to the preliminary inflammatory events[81]. For all these reasons, the venous wall homeostasis can be guaranteed until the glycocalyx integrity is preserved. In fact, the decrease of shear stress, induced by the hemodynamic alterations characterizing the venous hypertension, firstly results in a dropped synthesis of glycocalyx components by the vascular endothelial cells[73,82]. Thus, the depletion of this functional interface consists in the starting point of a vicious circle of maladaptive events favouring the hypertension perpetuation in the different CVeD manifestations. The endothelial cells, directly exposed to the widespread hemodynamic dysregulation, strengthen the current pronounced venous dilatation by the increased synthesis of nitric oxide (NO) through the stimulation of the inducible nitric oxide synthase (iNOS) rather than of the constitutive and Ca^{2+}-dependent endothelial enzyme isoform (eNOS). In effect, the increased release of NO, prostacyclin (PGI2), endothelin-1 (ET-1) and endothelium-derived hyperpolarizing factor (EDHF) is coherent with the overbalanced vasodilation compared to the vasoconstriction mainly revealed in warped varicose vein segments (C2)[57,79]. The correlated venous distension is also sustained by the smooth muscle cell relaxation induced by the enhanced release of acetylcholine as further consequence of shear stress alteration[79]. Furthermore, the NO overexpression appears increasingly related to the interesting vascular distension mechanism sustained by the up-regulated matrix metalloproteinases (MMP)[83,84]. In fact, these enzymes are able to favour the hyperpolarization of the varicose vein vascular smooth muscle cells by involving some protease-activated receptors upregulated in endothelial and muscle cells and platelets, besides an hyperpolarizing factor and the hypoxia-induced transcription factor-1A and -

2A (HIF-1A and HIF-2A), which inhibit the cellular Ca^{2+}-intake to reduce the contraction[85,86].

Of note, the high responsiveness of MMPs to the hemodynamic impairments and their extensive involvement in the numerous vascular remodelling processes mirror their variable expression by the different cellular components of the venous wall. They consist in a superfamily of 26 zinc-dependent endopeptidases (only 23 expressed in human) which are synthesized as pre-pro-MMPs, although the signal peptide is lost during the translational process (Tab. 1.3.1)[87,88]. They share three constant structural features consisting in the sequence homology with the collagenase 1 or MMP-1, the pro-domain cysteine switch motif PRCGXPD essential to maintaining the enzyme inactive by the interaction with the Zn^{2+} ion of the catalytic domain, and the Zn^{2+}-binding motif characterized by three histidine residues in the conserved sequence HEXGHXXGXXH, a conserved glutamate and a methionine residue placed in a downstream sequence (XBMX) from the catalytic domain and essential as support of its structure[89]. In addition, the repeated type II fibronectin domain, the linker-domain located between the catalytic site and the hemopexin domain and this last are differently distributed among the family members (Fig, 1.3.1).

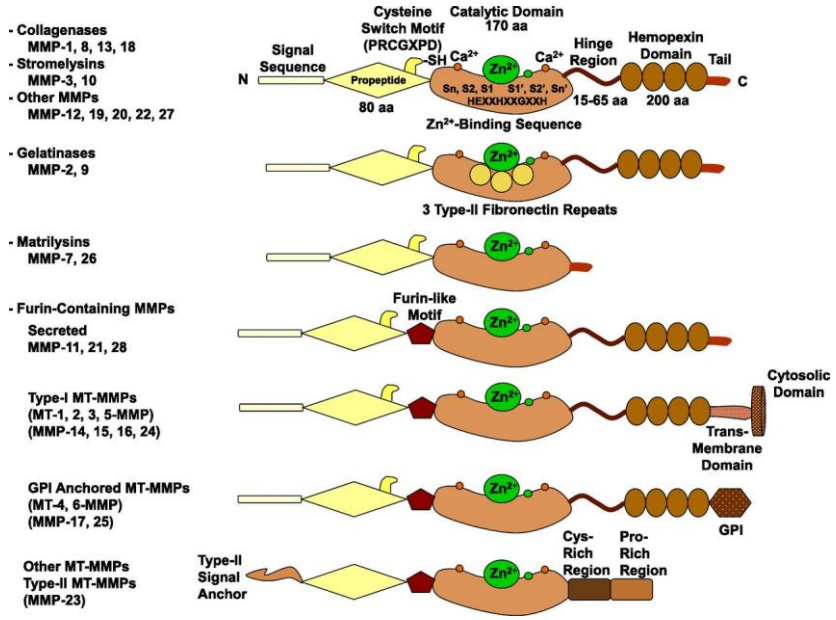


Fig. 1.3.1 Schematic representation of MMP structure[89]**.** The figure represents the main MMP domains consisting in the pro-peptide domain characterized by the cysteine switch motif PRCGXPD and associated with the signal sequence, the catalytic domain containing the Zn^{2+}-binding sequence HEXGHXXGXXH and two or three Ca^{2+} ions with stabilisation function, the linker domain and the hemopexin domain; three type II fibronectin repeats in the catalytic site are showed in the gelatinase structure. Further alternative structures are associated to other MMP family members

These are usually set in six different groups on the basis of their substrates, including the collagenases MMP-1, -8 and -13 which, as their name suggests, mainly interact with the collagen I, II and III by releasing fragments of variable length; the gelatinase-A or MMP-2 and -B or MMP-9 cleaving the gelatine (consisting in denatured collagen), collagen and laminin through their repeated fibronectin II domain in the catalytic site; the stromelysin-1 or MMP-3 characterized by more efficient proteolytic activity on different collagen types, other ECM structural components (elastin, fibronectin, laminin) and numerous proteins than the stromelysin-2 or MMP-10; the matrilysin-1 or MMP-7 and -2 or MMP-26, which lack hemopexin domain and interact with limited collagen types (collagen IV and X) and further non-ECM substrates; the membrane-type MMPs

(MT-MMPs) consisting in the type-I transmembrane proteins MT1-MMP (MMP-14), which mainly digests collagen I, II and III and is involved in the proMMP-2 activation, and the MT2-, MT3- and MT4-MMP (MMP-15, -16, -24) accompanied by the glycosylphospatidylinositol (GPI)-anchored proteins MT5- and MT6-MMP (MMP-17, MMP-25); and the remaining stromelysin-3 (MMP-11) presenting different sequence and substrate specificity compared to the MMP-3, the metalloelastase (MMP-12) cleaving the elastin and other ECM structural components, and the MMP-19, -20 , -22, -23, -28, some of which characterized by different domain organization or recently identified, are just defined as the other MMPs (Tab. 1.3.1; Fig. 1.3.1)[86,90]. Additional metalloproteinase families consisting in ADAMs (A Disintegrin And Metalloproteinases) and ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs), which are similarly known to exercise their Zn^{2+}-dependent catalytic activity on collagen and other different ECM fibrillar components[91–93].

MMPs	Other Names	Mol. Wt. KDa		Tissue Distribution/Disease Condition	Collagen Substrate	Other Substrates
		Proform	Active			
Vascular						
Collagenases						
MMP-1	Collagenase-1	55	45	Fibroblast, Interstitial, Tissue collagenase	I, II, III, VII, VIII, X	Aggrecan, gelatin, MMP-2, MMP-9
MMP-8	Collagenase-2	75	58	Neutrophil, or PMNL Collagenase	I, II, III, V, VII, VIII, X	Aggrecan, elastin, fibronectin, gelatin, laminin
MMP-13	Collagenase-3	60	48	SMC, varicose veins, pre-eclampsia, breast cancer	I, II, III, IV	Aggrecan, gelatin
Gelatinases						Aggrecan, elastin, fibronectin, gelatin, laminin, proteo-glycan, MMP-9, MMP-13
MMP-2	Gelatinase-A	72	66	Aortic aneurysm, varicose veins	I, II, III, IV, V, VII, X, XI	
MMP-9	Gelatinase-B	92	86	Aortic aneurysm, varicose veins	IV, V, VII, X, XIV	Aggrecan, elastin, fibronectin, gelatin
Stromelysins				SMC, synovial fibroblasts, CAD, HTN, tumor invasion	II, III, IV, IX, X, XI	Aggrecan, elastin, fibronectin, gelatin, laminin, proteoglycan, MMP-7, MMP-8, MMP-13
MMP-3	Stromelysin-1	57	45			
MMP-10	Stromelysin-2	57	44	Uterine, preeclampsia, arthritis, atherosclerosis, carcinoma cells	III, IV, V	Aggrecan, elastin, fibronectin, gelatin, laminin, MMP-1, -8
Matrilysins						Aggrecan, elastin, fibronectin, gelatin, laminin, proteoglycan, MMP-1, MMP-2, MMP-9
MMP-7	Matrilysin-1	28	19	Uterine	IV, X	
Membrane-Type MMPs	MT1-MMP	66	56	Human fibroblasts, SMC, VSMC, uterine, angiogenesis	I, II, III	Aggrecan, elastin, fibronectin, gelatin, laminin, MMP-2, MMP-13
MMP-14						
MMP-15	MT2-MMP	72	50	Fibroblasts, leukocytes, preeclampsia, cancer (breast, prostate, colon)	I	Fibronectin, gelatin, laminin, MMP-2
MMP-16	MT3-MMP	64	52	Leukocytes, angiogenesis human cancer	I	MMP-2
MMP-24	MT5-MMP	57	53	Leukocytes, brain tumor, astrocytoma/glioblastoma,	None identified	Fibrin, gelatin
Other MMPs						
MMP-11	Stromelysin-3	51	44	Uterine, Angiogenesis, hepatocellular carcinoma	Does not cleave	Aggrecan, fibronectin, laminin
MMP-12	metallo-elastase	54	45 and 22	Macrophage	IV	Elastin, fibronectin, gelatin, laminin
MMP-21	XMMP	62	49	Human placenta		α1-anti-trypsin
Non-Vascular						
MMP-18	Xenopus Collagenase-4	70	53	Xenopus (amphibian)	I	Gelatin
MMP-26	matrilysin-2, endometase	28	19	Human endometrial tumor	IV	Gelatin, fibronectin, gelatin
MMP-17	MT4-MMP	57	53	Brain specific cerebellum, breast cancer,	None identified	Fibrin, gelatin
MMP-25	MT6-MMP, Leukolysin	34	28	Leukocytes, anaplastic astrocytomas, glioblastomas	IV	Gelatin, fibronectin, laminin, fibrin
MMP-19	RASI-1	54	45	Liver	IV	Fibronectin, aggrecan, COMP, laminin, gelatin
MMP-20	Enamelysin	54	22	Tooth enamel	V	Aggrecan, amelogenin, COMP
MMP-22	CMMP	51		Chicken fibroblasts	Unknown	Gelatin
MMP-23	cysteine array MMP	28	19	Reproductive tissues	Unknown	Unknown
MMP-28	Epilysin	56	45	Skin keratinocytes	Unknown	Unknown

Tab. 1.3.1 The MMP family members[86]. The table summarizes the MMP family members associated with their tissue expression and their different substrates.

The passage from the pro- to the active MMP is marked by the interruption of Cys-Zn^{2+} coordination and the removal of the hemopexin domain representing the starting point of the MMP proteolytic activition[94]. The enzyme is then ready to interact with its substrate through the Zn^{2+} ion which gives rise to a nucleophilic attack mediated by a carbonyl oxygen atom of the substrate by releasing a water molecule. This process is primarily due to the presence of a water molecule accompanying the conserved glutamate residue, which determines the penta-coordination of the catalytic Zn^{2+} ion with the three histidine, methionine and substrate oxygen atoms. Furthermore, the MMP-11, -21 and -28 as well as the MT-MMPs contain a furin domain comprised between the pro-peptide and the catalytic site, which is intracellularly cleaved by the endopeptidase furin following the recognition of a specific sequence in C-terminus of their pro-peptide[89,95]. These pro-MMP activation mechanisms can involve either enzymatic family members, as demonstrated by the MMP-3 and all the MT-MMPs (with the only exclusion of the MT4-MMP), or different treatments including heat, pH decrease and chemical agents (4-aminophenylmercuric acetate, mercury chloride, sodium dodecyl sulphate, reactive oxygen species) by determining structural interferences[86,96]. Interestingly, the proMMP-2 activation requires the concurrent involvement of the MT1-MMP (MMP-14) and the tissue inhibitor of matrix metalloproteinases 2 (TIMP-2) on the cell membrane. The TIMP-2-proMMP-2 complex involving the C-terminal domains can interact through the TIMP-2 N-terminal domain with the MT1-MMP anchored on the cell surface. Thus, the pro-MMP2 bound to the

membrane might become substrate for another MT1-MMP molecule, which determines its activation. However, it might also occur an early interaction between TIMP-2 and MT1-MMP on the cell surface, which inhibited binds the hemopexin domain of proMMP2 to expose it to a free MT1-MMP which performs the activation[84,86,95]. The mention of this TIMP-2 function allows to make remarks on the other three tissue MMP inhibitors TIMP-1, -3 and -4. Each one is characterized by a C-terminal domain and a N-terminal domain responsible for the MMP inhibition through the direct interaction with the protease catalytic site[97,98]. TIMPs are specifically distributed among the different venous wall layers, where exercise their activity on all MMPs, despite some peculiarities consisting in the TIMP-1 lack of MT1-MMP interaction, the just mentioned TIMP-2 role in the MMP-2 activation, the TIMP-3 ADAM inhibition and the TIMP-4 activity mainly localized in cardiovascular systems. Moreover, they are endowed with additional functions, as the pro-angiogenic TIMP-1 properties, the endothelial cell proliferation inhibition by TIMP-2 and TIMP-3, which may prevent cellular migration and angiogenesis by interacting with VEGF receptor[86,99]. Further endogenous factors able to interfere with the MMP and other different endopeptidase catalytic activity are the α_2-macroglobulins provided of four identical subunits with Zn^{2+}-binding domain fundamental to their function. In particular, they determine MMP-complex formation which is degraded by endocytosis mechanisms[89].

This overall picture enables to understand both the modulation of MMP targeted activity on the main ECM components (e.g. collagens, elastin, fibronectin, vitronectin, tenascin, laminin), which explains the intensive involvement in the physiologic and pathologic venous wall remodelling, and the protease further interactions with factors,

which differentially determine the CVeD functional impairments[100,101,102]. In these functions as well as in the cell proliferation, migration and differentiation control, the MMP/TIMP balance is an essential requirement to provide the venous physiological homeostasis. In fact, the loss of this mutual counterbalance and the significant venous pressure increase effects on MMP transcriptional and post-translational processes might be considered determinant in the appearance of both hypertrophic and atrophic segments of varicose veins (C2)[103,104]. Interestingly, this evidence is confirmed by the pressure stress on vascular endothelial and smooth muscle cells, which induces them to synthesize large amount of MMP-2 following the transcription factor activator protein-1 (AP-1) activation by the reactive oxygen species (ROS) release[96]. This event is exacerbated by the endothelial mechanical and inflammatory activation, which determines an hyperexpression of the NADP(H) oxidases[79]. A similar MMP-2 concentration increase is also associated to other CVeD manifestations including hyperpigmentation($C4_a$), lipodermatosclerosis or atrophie blanche ($C4_b$). They share a common hypoxic microenvironment accompanied by a huge oxidative stress and hemosiderin deposition, which enhance the MMP and the vascular endothelial growth factor (VEGF) expression by determining the appearance of typical clinical signs [57,105]. In addition to the MMP-2 widely present in all venous wall layers, the increase of the MMP-1, mainly synthesized by fibroblasts, endothelial cells and smooth muscle cells, and the MMP-9, highly expressed by endothelial and muscle cells, influences the proliferation and migration of venous smooth muscle cells in varicose veins (Tab. 1.3.1)[106]. In this regard, the proteolytic degradation of the ECM components is one of the immediate structural assets offered to the varicose vein dedifferentiated muscle cells, which are

then induced to migrate. The removal of some adhesion sites and the exposition of binding sites for different factors enhanced by the MMP increased secretion potentiate the migration process[79]. This mechanical implication is accompanied by an effective smooth muscle cell phenotype switch from contractile to proliferative and secretive which materializes through increased ECM-muscle cell interactions and the release of growth factors in the hypertrophic regions of varicose veins[65]. Furthermore, MMP-3, -7 and -13, equally up-regulated in this pathological condition, might contribute to the venous wall weakening by determining the ECM component degradation. Of note, the decreased TIMP release and the consequent uncontrolled MMP-3 proteolytic activity are responsible of some collagen III post-translational modifications inducing its deposition imbalance with the collagen I in thinned and tortuous atrophic regions of varicose veins[79,84,107]. Bearing in mind all these MMP proteolytic activity implications and the wide cellular expression of these enzymes in the vascular system, it might result expectable to associate their involvement also with the other CVeD stages likewise characterized by ECM alterations and diffused inflammation. In effects, this condition is further sustained by the MMP effects on the activated endothelial cells interacting with similarly activated leucocytes and the modulation of some inflammatory mediators, as cytokines, chemokines and growth factors[108]. In this regard, one of the most representative process might consist in the sequence of the four wound healing time-dependent phases (hemostasis, inflammation, granulation and re-epithelialization). The initial inflammatory events and the ECM turnover, facilitating keratinocyte and fibroblast migration, pave the way to the progressive granulation tissue formation and the angiogenesis stimulation by leading to the endothelial basement membrane and

skin barrier restoration[109,110]. The matrilysin MMP-7, expressed by vascular endothelial and muscle cells, takes part to this process through both the activation of the latent tumor necrosis factor-α (TNF-α) stimulating chemoattractant mechanisms and macrophage infiltration, and the alterations of some cell-cell and ECM-cell interactions to promote cellular migration[107]. Similarly, the MMP-12, mainly secreted by macrophages (Tab. 1.3.1), might be involved in the inflammatory processes by triggering the TNF-α activation, besides in the reparative phases due to its ECM elastin, fibronectin, collagen VI and laminin degradation[111,112,113]. The restoration of the tissue integrity in the final phases of the wound closure is also favoured by the up-regulation of the collagenases MMP-1, MMP-8 and MMP-13 which are expressed in endothelial cells, smooth muscle cells and fibroblasts and are determinant mainly in promoting the proliferation and migration of these last during the ECM remodelling (Tab. 1.3.1)[114,115]. An undebated role in the prolonged inflammatory state is attributed to increased concentrations of the MMP-2 and MMP-9 during the healing process, although their proteolytic activity are strictly moderated by their inhibitors TIMP-1 and TIMP-2[99,116,117]. Thus, their loss might induce the reduction of the angiogenesis and the impairment in the correct ECM and tissue deposition characterizing the wound chronicity.

Furthermore, as reported on several occasions during the chapter, the switch from a laminar and pulsatile to an irregular and low shear stress is comparable to squeezing a trigger which sparks off self-reinforcing inflammatory events, besides structural impairments (Fig. 1.2.3b). In effects, this hemodynamic force acts as a physiological modulator of the circulating leucocytes, which undergo their superficial integrin CD11b/CD18 (Mac-I) proteolytic shedding and cytoskeleton F-actin depolymerization

with consequent pseudopod retraction, if exposed to a laminar blood flow. The shear stress drop is then a key event in determining the leucocyte recruitment on the vascular endothelial layer through the integrin increased expression, the pseudopod sprouting and the loss of the cellular spherical shape to give rise to contact regions[118,119,120]. Although the integrin CD11b/CD18 up-regulation is an effective requirement to the leucocyte migration and subsequent penetration in the vascular endothelial layer, however further adhesion molecules, as the endothelial E-selectin, the intercellular adhesion molecule-1 (ICAM-1), the vascular adhesion molecule-1 (VCAM-1), and the leucocyte L-selectin, the lymphocyte function-associated antigen-1 (LFA-1) and the very late activation antigen-4 (VLA-4), are determinant in this respect[57,110,121]. Interestingly, the reduced expression of the CD11b/CD18 on leucocyte cellular surface and the correspondent increase of soluble L-selectin plasmatic levels might be representative of cellular migration into the endothelium[110,79]. The generalized activation state of the endothelium is also prompted by the shear stress-induced glycocalyx damages, which unmask the adhesion molecules promoting leucocyte docking and inflammation advance[57]. These events are further exacerbated by the previously mentioned sharp reduction of endothelial NO synthesis by the eNOS, which physiologically plays an essential anti-inflammatory role, besides inhibiting smooth muscle cell proliferation and stimulating their relaxation. The nitric oxide depletion is counterbalanced by the endothelial increased levels of the pro-inflammatory agent angiotensin II (AngII), which stimulates the ROS production and the adhesion molecule and inflammatory cytokine release (Fig. 1.2.3b)[57,122]. Furthermore, the compromised and activated endothelium gives rise to a prothrombotic environment sustained by the presence of plasminogen

activator inhibitor-1 (PAI-1), factor VIII (FVIII) and Von Willebrand factor (Vwf), which might be considered endothelial disfunction markers. The high concentration of these last along with D-dimer, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and C reactive protein (CRP) revealed in the lower limb circulating blood might be associated with a fibrinolytic profile characterizing the CVeD[79]. The presence of these just mentioned cytokines mirrors the heavy secretion of inflammatory mediators which act as constant background of the whole spectrum of the hemodynamic and structural impairments approached so far. In this regard, the cytokines consist in a multifaceted family of glycoproteins and peptides able to induce their effect on the basis of the timing and the environment of their release, the potential presence of inhibitor or synergistic factors, the cellular distribution of their receptors and the balance between inflammatory and anti-inflammatory family members (Tab. 1.3.2). Cytokines as, interleukin (IL) -4, -10, -13, interferon-α (IFN-α) and variously the transforming growth factor-β (TGF-β) are known to counteract inflammatory reactions induced by tumour necrosis factor-α (TNF-α), other interleukins as IL-1, -6, -8, -11, -12, -19, IFN-β and IFN-γ, TGF-β, macrophage inflammatory protein-1α and -1β (MIP-1α, MIP-1β), the regulated on activation, normal T-expressed and secreted (RANTES), the platelet factor-4 (PF-4) and the monocyte chemoattractant protein-1, -2, -3 (MCP-1, -2, -3)[122]. This initial distinction, manly based on the general functional roles of cytokines, enables to refer to their specific organization in subfamilies including the numerous interleukins, the tumour necrosis factors (TNF-α and -β), the interferons (IFN-α, -β, -γ), the colony stimulating factors (CSF) distinct in granulocyte (G-CSF), monocyte (M-CSF) and granulocyte-monocyte (GM-CSF), the transforming growth factors (TGF-β1, - β2, -β3)

accompanied by the bone morphogenetic proteins (BMPs), the activins and the inhibins, the chemokines (IL-8/CXCL8, PF-4/CXCL4, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MCP-1/CCL2, MIP-2/CCL8, MIP-3, MIP-4), and other members(Tab. 1.3.2).

Cytokine	Producing cell/ serum level	Receptor	Target	Function
GM-CSF	Th cells, macrophages, Ecs, lymphocytes <8pg/mL	GM-CSFRα GM-CSFRβ	Progenitor cells, hematopoietic stem cells, neutrophils, macrophages	Growth and differentiation of monocytes, dendritic cells, granulocytes, macrophages pro-atherogenic
IFN-α	Leucocytes	IFNαR1 IFNβR2	Various	Viral replication, major histocompatibility complex (MHC i) expression
IFN-γ	Macrophages, Th1 cells, Tc cells, B cells, Natural Killer (NK) cells, VSMCs	IFN-Γr1 IFN-γR2	Various, macrophages, lymphocytes, NK cells, Ecs, SMC, Activated B cells, Th2 CELLS, VSMCs, ECM	Viral replication proinflammatory, promotes Th1 immune responses/secretion ofTh1-associated cytokines, inhibits ECM synthesis by SMC, MHC I expression, pathogen elimination, Ig class switch to IgG_{2a}, proliferation, migration, remodeling pro-atherogenic
MIP-1α MIP-1β	Macrophages	IL-10R2	Monocytes, T cells	Chemotaxixs
TGF-β1 TGF-β2 TGF-β3	T cells, monocytes	TGF-βR1 TGF-βR2	Monocytes, macrophages, activated B cells, various	Chemotaxis, IL-1synthesis, IgA synthesis, proliferation anti-atherogenic
TNF-α	Macrophages, mast cells, NK cells, VSMCs, T cells, B cells <6pg/mL	TNFR1 (p55) TNFR2 (p75)	Macrophages, various, tumouor cells, ECs	Cytokine expression proinflammatory, feevr, neutrophil activation, bone resorption, anticoagulant, cell death, tumor necrosis, increase permeability, adhesion molecules stimulation, pro-atherogenic
TNF-β	Th1 cells, Tc cells, platelets, macrophages, Th3, Treg cells, B cells, SMC	Type I and type II Ser/Thr kinase receptors	Phagocytes, Ecs, VSMCs, various	Phagocytosis, NO production, pro-atherogenic, angiogenesis, anti-inflammatory, profibrotic, promotes wound healing, suppresses Th1 and Th2 immune responses
IL-1α (30,6 kDa) IL-1β (30,7 kDa) IL-1δ IL-1ε	Monocytes, macrophages, T cells, B cells, DC Ecs, VSMCs < 8pg/mL	IL-1R type 1 IL-1R type 2	Th cells, B cells, NK cells, verious, Ecs, VSMCs	Co-stimulation, proinflammatory, maturation, proliferation, activation, inflammation, acute phase response, fever, EC and SMC activation, migration, proliferation, pro-atherogenic
IL-2 (17,6 kDa)	Activated T cells, Th1 cells <50 pg/mL	IL-2Rβ IL-2Rγ	Macrophages, T cells, B cells, NK cells	Growth, prolifera.tion, activation, pro-atherogenic
IL-3 (17 kDa)	T cells, NK cells, monocytes, B cells, mast cells	IL-3Rα Il-3Rβ	Stem cells, hematopoietic progenitor cells, mast cells	Growth, proliferation, differentiation, histamine release, pro-atherogenic
IL-4 (17,4 kDa)	Th2 cells, mast cells <15 pg/mL	IL-4Rα IL-4Rγ	Activated B cells, macrophages, T cells, B cells, mast cells, macrophages, hematopoietic progenitor cells	Proliferation, differentiation, IgG and IgE synthesis, pro-atherogenic, MHC II expression, proliferation and differentiation B cells (Ig switching to IgG1 and IgE) and Th2 cells (anti-inflammatory by inhibiting Th1 immune responses), VCAM-1 stimulation
IL-5	Th2 cells, T cells, mast cells, ECs	IL-5Rα IL-5Rβ IL-3Rβ	B cells	Growth, proliferation and differeatiation, IgA switching

Cytokine	Producing cell/ serum level	Receptor	Target	Function
IL-6 (23,7 kDa)	Monocytes, macrophages, Th2 cells, Ecs, VSMCs, fibroblasts, stromal cells 0.16-7.25 pg/mL)	IL-6Rα IL-6Rβ	Activated B cells, plasma cells, stem cells, various, VSMCs, EC, T cells	Porliferation, differentiation ito plasma cells, pro-atherogenic, growth, IgA switching, antibody secretion, acute phase response
IL-7	Marrow stroma, thymus stroma, platelets	IL-7Rα IL-7Rγ	Stem cells, monocytes, T cells, B cells	Differentiation into progenitor B and T cells, proinflammatory
IL-8	Macrophages, Ecs, monocytes, T cells< 15pg/mL	IL-8R	Neutrophils, T cells, monocytes	Chemotaxis, proinflammatory, promotes leucocytes arrest, pro-atherogenic
IL-9 (15.9 kDa)	Th2 cells	IL-9Rα	T cells, B cells, mast cells, eosinophils, neutrophils, epithelial cells	Anti-atherogenic, promotes proliferation and ifferentiation of mast cells, stimulates IgE production, inhibits monocyte activation, stimulates TGF-β in monocytes
IL-10 (20,5 kDa)	Macrophages, Th2 cells, Treg cells, B cells, monocytes, mast cells <8 pg/mL	IL-10R-1 IL-10R-2	Macrophages, T cells, B cells	Inhibit proinflammatory cytokine production, anti-atherogenic, anti-inflammatory, Anti-inflammatory, inhibits Th1 responses, promotes proliferation and differentiation of regulatory T Cells, B cells activation
IL-11 (21,4 kDa)	Ecs, macrophages	IL-11Rα	Hematopoietic progenitor cells	Hematopoiesis, anti-atherogenic
IL-12α (24,8 kDa) IL-12β (37kDa)	Macrophages, B cells, Th1 cells	IL-12Rβ2	Activated Tc cells, T cells, macrophages, NK cells	Differentiation into cytolytic T cell (CTL), proinflammatory, promotes NK and cytotoxic lymphocyes activity, induces IFN-, pro-atherogenic
IL-13	Th2 cells	IL-4Rα	B cells,	Activation of Ig trascription
IL-14	ECs, macrophages, monocytes		B cells	B cell growth factor
IL-15 (18kDa)	ECs, macrophages, monocytes	IL-2Rβ	T cells, B cells, NK cells, monocytes	Enhances neutrophil chemokine production, cytoskeletal rearrangement, phagocytosis, delays apoptosis, pro-atherogenic
IL-16	Mast cells, $CD4^+$ and $CD8^+$ cells		$CD4^+$	$CD4^+$ T-cell growth factor, proinflammatory, enhances lymphocyte chemotaxis, adhesion molecule, and IL-2 receptor and HLA-DR expresssion
IL-17	Human T cells, mouse thymocytes		Fibroblasts, keratinocytes, epithelial cells, ECs	Secretion of IL-6, IL-8, PGE2, MCP-1 and G-CSF, induces ICAM-1 expression, T- cell proliferation, pro-atherogenic
IL-18 (22,3 kDa)	Macrophages, ECs, VSMCs	IL-1BRα IL-18Rβ	ECM, T cells, NK cells, myeloid, monocytic erythroid and megakaryocytic cells	Remodeling, proinflammatory, induces IFN-γ and other Th1 cytokines, promotes Th1 development and NK activity, pro-atherogenic

Tab 1.3.2 Cytokine, chemokine and growth factor family members[122]. The table summarizes some of the most representative cytokines, chemokines and growth factors and their corresponding cellular sources and targets, receptors and functions

Interestingly, the chemokines, so named in reference to their chemotactic properties, are distinct in further 4 groups characterized by the presence or not of a conserved cysteine (C) residue in the N-terminus accompanied by a variable region (X). These consist in XC group, whose members (XCL1, XCL2) are involved in lymphocyte chemoattraction, CC group acting on monocytes, CXC group including ELR^+-CXC members with a N-terminus sequence glutamic acid-leucine-arginine (ELR motif), which interact with neutrophils, and ELR^--CXC members lacking in the ELR sequence and mainly acting on lymphocytes, and CX_3C group including the fractalkine[122,123]. The pleiotropic ability of cytokines to trigger different physiological responses in equally different

cellular types is consistent with their variable release by macrophages and T cells, which synthesize different inflammatory and anti-inflammatory interleukins (IL-1, -6, -10, -12) besides TGF-β, TNF-α, IFN-γ and some chemokines (MCP-1/CCL2, MCP-4/CCL13, IL-8/CXCL8), platelets, which store in their secretion granules numerous cytokines and chemokines (IL-1β, PF4/CXCL4, MIP-1/CCL3, RANTES/CCL5), and other non-inflammatory cells as the vascular endothelial and smooth muscle cells secreting IL-1α, IL-1β and TNF-α (Tab. 1.3.2). However, the efficacy of their biological functions is strongly tied to the interaction with specific receptors which often represent the starting point of an activation cascade culminating in the synthesis of new products or in mechanisms of transcriptional modulation. Four receptor structures are located on the target cellular surface to mediate cytokine effects by binding them through their extracellular domains. In particular, the hematopoietin receptor, which is characterized by a dimeric or trimeric structure with some conserved cysteine residues in the extracellular domain, interacts with different interleukins, as IL-2 and IL-7, and with the GM-CSF, the IFN receptors equally presenting some conserved cysteine residues, the TNF receptors, and the chemokine receptors which are G-protein coupled and typically characterized by seven transmembrane domains[122]. Numerous ILs, IFNs and CSFs, binding their aforementioned receptors, give rise to their dimerization activating the JAK-STAT signalling pathway, which involves the Janus kinases (JAK) and the signal transducer and activator of transcription proteins (STAT) recruitment. Alternatively, the inflammatory IL-1 and IL-18 with the TNFs exploit the NF-kB signalling to influence the expression of some adhesion molecules (E-selectin, VCAM-1, ICAM-1), the iNOS and some MMPs besides other cytokines and growth factors determinant in the

inflammatory process. Instead, the TGF-βs induce numerous endothelial alterations and inflammation mediator expression through the Smad-signalling pathway (Tab. 1.3.2). The existence of strict regulation systems, as specific inhibitors or phosphatases, is required to ensure the appropriate balancing of these transductional mechanisms. In this regard, the increased concentrations of the basic fibroblast growth factor (bFGF) in the activated endothelium as well as of the TNF-α, IFN-γ, CSFs, MCPs, different interleukins and growth factors are evidence of these mediator negative feedback system disturbances favouring the uncontrolled inflammatory state which characterizes numerous CVeD manifestation. In effects, the association of TGF-β1 enhanced expression with the varicose veins (C2) is consistent with its ability to stimulate elastin and collagen synthesis and to down-regulate the TIMP expression to determine the stiffening of the vein walls[57,124]. The role of TGF-β1 in promoting the ECM remodelling and cellular migration is also compatible with the early phases of the wound healing process, in which the reorganization of vascular basement membrane and some angiogenic stimulator release occur[109]. Furthermore, the pro-inflammatory cytokine induces the production of bFGF exercising its mitogenic function principally on fibroblasts and vascular smooth muscle cells in varicose veins. For their part, muscle cells are stimulated to produce IL-6 and the chemokine MCP-1 in presence of thrombin by promoting monocyte recruitment[122]. Furthermore, TGF-β1, as well as TNF-α and IFN-γ, seems to be involved in the up-regulation of the iNOS especially in the varicose vein tortuous segments[122,125]. These cytokines are also correlated with the endothelial increased permeability, which characterizes the whole CVeD. In particular, TNF-α determines a loosening of the endothelial junctions by inducing the up-regulation of the

vascular endothelial growth factor (VEGF), bFGF, IL-1α and IL-6, which stimulate the MMPs expression, and iNOS which increases the NO levels[79,122,126]. This structural alteration is detectable from the first CVeD sign appearance sustained by a pronounced venous hypertension and progressively worsened by the extravasation of some macromolecules, as fibrinogen, red blood cell degradation fragments and proteins stimulating leucocyte migration and trapping in the fibrin cuff meanwhile accumulated in the interstitial space[57,109]. The consequent edema and the hemosiderosis occurrence generate a microenvironment heavily hypoxic which represents an undebated stimulator factor for MMP, pro-inflammatory cytokine and growth factor synthesis characterizing the different CVI manifestations[105,106,127]. The same TNF-α induced by the urokinase-type plasminogen activator (u-PA) and accompanied by IL-17 and -19 seems to enhance the MMP-9 expression in varicose veins [79,122]. However, a similar effect is produced by TNF-α and IL-1β on MMP-1, -2, -3, -9, -13 and MT1-MMP to maintain the inflammatory state in chronic wound healing. The up-regulation of MMPs is in line with the general chemotactic role of the IL-8, which is also able to stimulate angiogenesis, besides the proliferation and migration of keratinocytes during the wound closure[128]. Differently, the inflammatory events are moderated by IL-10, which prevents the endothelial cell-leucocyte interactions and inhibits the pro-inflammatory cytokine release. High expression of this interleukin is consistent with the containing of the inflammatory phase to determine the tissue restoration during the healing process[122,112,126]. Overall, an interesting implication of the activation of this complex biochemical machine regulated by effective chain reactions involving different cellular types and numerous mediators consists in its stimulation of nociceptors located

between the endothelial and smooth muscle cells of the venous wall by determining the onset of pain[52].

Certainly, this last detail as well as the different aspects approached until now is consistent with the aim of this introductive focus on CVeD to provide instruments suitable to properly contextualising the implications of the evidences which are presenting.

Chapter 2

Thesis aim

The mechanical and cellular players related to the physiological compartmentalization of the micro- and macrocirculation in the lower limbs could be considered them as the main agents for ensuring the modulation of the blood dynamics and the venous homeostasis[58,59]. Their mutual influence in pressure gradient and hemodynamical force determination are often mirrored by a pleiotropic biochemical environment which similarly contributes to the essential blood return to the heart. However, any impairment at every level of this complex biologic system characterized by cellular and biochemical balances strictly regulated and sensitive may trigger a vicious circle of pathological events corresponding to the different CVeD manifestations[57,79]. The multifaceted pathological profile of the CVeD in many respects unexplored contributes to exacerbate its worldwide diffusion with profound social, political and economic effects[3,12].

In this regard, the study of the different expression and activity profile modulation of some of the main mediators involved in the CVeD occurrence represents the general aim of this three-year research project to trace the disease progression starting from its last to the early clinical manifestations. In particular, four intermediate purposes are established in order to reach what just mentioned and they consist in (Fig. 2.1): I) biochemical characterization of C6 microenvironment through the determination of the three TGF-β isoforms and sEng expression in wound fluid (WF) samples from inflammatory (Infl) and granulating (Gran) non-healing venous leg ulcers (VLU), the

evaluation of the expression profile of these mediators and MMPs in serum-free supernatants from THP-1 monocyte cells stimulated with Infl and Gran WF in presence or absence of sulodexide (SDX) and finally, the MMP-2 and MMP-9 proteolytic activity observation in the same serum-free supernatants; II) biochemical characterization of $C4_a/C4_b$ microenvironment through the MMP expression determination in plasma samples from $C4_a/C4_b$ patients underwent three different gravitational conditions including wearing under-knee graduated compression stockings (23-32 mmHg, class II); III) *in vitro* characterization of detergent sclerosant biological and cellular effects through the study of the MMP proteolytic and expression profile modulation by the addition of different concentration of polidocanol and sodium tetradecyl sulphate to normal plasma samples, and the observation of human HECV endothelial cell morphological changes induced by the same detergents concentrations prepared in saline solution and in plasma; IV) biochemical characterization of C1/C2 microenvironment through the cytokine, chemokine and growth factor expression determination in serum samples from C1/C2 patients treated with the sclerosant polidocanol.

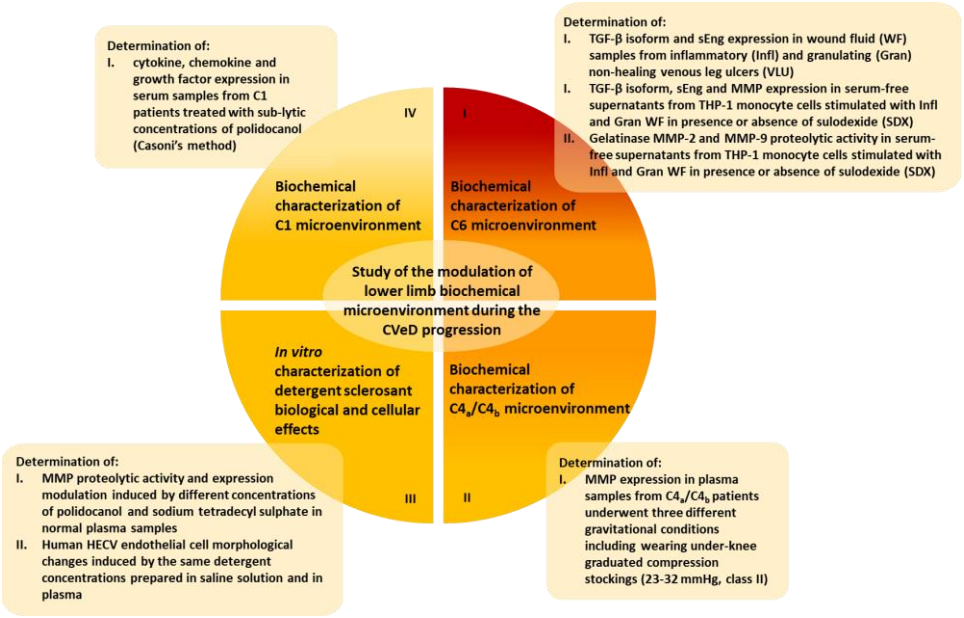


Fig. 2.1 Graphic representation of the study aims. The graph shows the four intermediate study aims organized in clockwise direction around the general purpose in the midpoint. The boxes at the side of each aim contain its schematic description

Chapter 3

Materials and methods

3.1 Biochemical characterization of C6 microenvironment: TGF-β isoform, sEng and MMP expression profile

3.1.1 Patient recruitment and sample collection

The investigation of the TGF-β isoform and its co-receptor soluble Endoglin (sEng) expression in inflammatory and granulating wound fluid (WF) has entailed the recruitment of thirty patients affected by non-healing venous leg ulcer (VLU) classified as C6 stage according with the CEAP classification[17].

Prearranged exclusion criteria, as age less than 18 years, pregnancy or breast-feeding, arterial disease, renal insufficiency, insulin-dependent diabetes mellitus, vasculitis, autoimmune diseases, cortisone, immunosuppressant or hormonal therapies, previous venous surgery or sclerotherapy, are observed to start the study in accordance with the ethical standards of the Helsinki Declaration of 1975 (revised in 2000) and after the approval by the local ethics committee (Barbantini Clinics of Lucca and University "Carlo Bo" of Urbino).

Patients provided their signed written informed consent and underwent Duplex ultrasound scanning (DUS) in standing position with the weight on the contralateral leg. Moreover, the calf compression-release manoeuvre and the compression ultrasounds respectively allowed to detect venous reflux (> 0.5 s in superficial venous system and > 1 s in deep venous system) and thrombosis occurrence[1,59]. On the basis of the clinical examination evidences, the WFs collected during the patient admission to the hospital

are divided in inflammatory (n= 20, Infl) and granulating (n= 10, Gran). Bacterial analysis of the patient ulcer bed is performed through biopsies and the visual analogue scale (VAS), based on a numeric scale from 0 = no pain, 1-3 = mild pain, 4-6 = moderate pain up to 7-10 = severe pain, is used to determine the intensity of patient pain perception. Furthermore, all patients are treated with inelastic multilayer compression (≥ 60mmHg; class VI)[28] in supine position and differently underwent skin grafting or sclerotherapy after WF sample collection.

This is performed through the application of proper cotton gauzes on the ulcer bed. The saturated gauzes were collected in centrifuge tubes without additives or antiproteases and centrifuged at 10,000xg for 15 minutes at 4°C. The obtained supernatants are stored at -80°C until the TGF-β isoform, sEng and MMP determinations through the multiplex suspension immunomagnetic assays (Bio-Plex®, Bio-Rad, Hercules, CA, USA).

3.1.2 Cell culture and treatments

The human monocytic THP-1 cell line (ATCC® TIB-202™) is used for stimulation with inflammatory and granulating WF in presence or absence of Sulodexide (SDX) provided by Alfa Wasserman (Milan, Italy). The THP-1 cells, obtained from American Type Culture Collection (Manassas, VA, USA), grown in standard 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, are seeded at 1,500,000/mL in serum-free culture media for performing experiments, in order to avoid potential interferences due to TGF-b, sEng, or MMPs expressed in fetal bovine serum.

The inflammatory and granulating WF pools used in the THP-1 cell treatment are obtained from aliquots with the same volume of 10 samples randomly selected from each group. The pools, after their filtration (0.45 µm tissue culture filter unit) to remove eventual bacteria and cellular debris, are added to the serum free culture media of THP-1 cells to a final concentration of 5% v/v, in presence or absence of SDX co-treatment (0.12 LSU/mL)[1] for 24 hours. Each experiment on serum-free conditioned medium was performed in triplicate in at least two independent experiments. The trypan blue exclusion test is used to evaluate the cell viability. The sterile compounds for cell culture are from JET BIOFIL Bio-filtration Products Co (Guangzhou, China) whereas chemicals and reagents for cell culture are from Carlo Erba Reagents S.r.l. (Milan, Italy).

3.1.3 Multiplex suspension immunomagnetic assay

The assay allows to quickly detect up to a hundred analytes in the same sample by exploiting signal emitted by fluorescently dyed polystyrene magnetic beads (diameter 6,5 µm) conjugated with monoclonal antibodies. In particular, the detection of the investigated analytes is determined by the specific interaction between the primary monoclonal antibody-protein complex, generated on the beads during the assay, and a biotinylated secondary antibody which in turn is recognised by streptavidin-phycoerythrin molecules. The fluorescence produced is dependent on the markers loaded in the beads (specific for each analyte) and its intensity is directly proportional to the phycoerythrin bound to the analyte. In fact, the Bio-Plex® 200 array reader is

[1] The lipasemic units (LSU) are related to the SDX stimulating effects on the lipoprotein lipase activity; 1 mg = 10 LSU[185]

composed of two lasers, the first determines the fluorescence emission by the marker loaded in order to identify the beads involved in the assay, whereas the second is related to the phycoerythrin stimulation to obtain a reporter signal detected by a photomultiplier integrated in the instrument. The signal is then processed by a Bio-Plex® Manager software (6.1 version) which converts it in the concentration value of the sample analytes expressed in pg/mL, besides further parameters.

The kits employed to determine the TGF-β isoform and sEng concentration in WF and in serum-free supernatants from cell culture assays respectively consist in the Pro™Human TGF-β 3-plex Assay (including the three isoform TGF-β1, TGF-β2 and TGF-β3) and in the single-plex Endoglin, which is part of the Pro™Human Cancer-panel 2 Assay. In the same samples the MMP expression is evaluated through the Pro™Human MMP 9-plex Assay.

The effective quantification is based on a standard curve resulting from nine serial dilutions (1:3 or 1:4 depending on the panel of analytes considered) of the assay standard in a specific diluent both provided by the manufacturer.

The WF and culture media samples are maintained at room temperature before to be centrifuged at 10,000 rpm for 10 minutes (4°C).

The WF and supernatant samples need to be activated in order to determine the immunoreactive TGF-β isoforms (this step is required only for the measurement of TGF-β isoforms, all the other quantification assays did not require this step). According with the manufacturer instructions, the activation step is performed through the addition of 10 µl HCl to 50 µl WF samples which are mixed and incubated for 10 minutes at room temperature. An immediate neutralization is required by adding 10 µl NaOH to the previous solution. The samples are then loaded (50 µl/well) on a 96-well plate containing

the beads conjugated with the primary monoclonal antibody (50 µl/well) previously resuspended in the assay buffer and washed two times through a magnetic Wash Station (Bio-Rad) to remove primary antibodies uncoupled by preventing false negative occurrence. The plate incubation in the dark for 1 hour (sEng and MMPs) or 2 hours (TGF-β isoforms) on a shaker at 850±50 rpm is followed by three washing steps through the magnetic Wash Station to remove the unbound proteins, according to the manufacturer instructions. Then, the addition of the biotinylated secondary antibody properly diluted in the antibody diluent to each well (25 µl/well) is performed to induce the interaction with the analyte-primary antibody complex generated on the bead surface in the previous step. In the last step the plate incubated in the dark for 30 minutes (sEng and MMPs) or 1 hour (TGF-β isoforms) on the shaker at 850±50 rpm is washed three times to remove the unbound antibodies and then, loaded with the fluorescent marker streptavidin-phycoerythrin (50 µl/well) with a further incubation in the dark of 30 minutes (TGF-β isoforms) or 10 minutes (sEng and MMPs) on the shaker (850±50 rpm). The next washing step through the magnetic Wash Station is followed by the addition of the assay buffer (125µl/well) in each well and a rapid shake for 30 seconds to resuspend fluorescently-dyed immunocomplexes before the plate reading and the data collection.

3.1.4 Zymography assay

The serum-free supernatants of THP-1 cells are also used to perform zymography assays in order to evaluate the proteolytic activity of the gelatinases MMP-2 and MMP-9 after the treatments. The assay, as derivation of the sodium dodecyl sulphate polyacrylamide

gel electrophoresis (SDS-PAGE) performed in denaturing and nonreducing conditions, consists in inducing the migration of the samples containing MMPs through a SDS-PAGE gel copolymerized with a specific substrate (e.g. gelatine) after their treatment with the anionic detergent sodium dodecyl sulphate (SDS). This provides them a general negative charge directly proportional to their molecular mass. In fact, the SDS binding every two amino acids causes the enzyme linearization due to the electronegative repulsion between the numerous negative charges. Furthermore, the denaturing detergent is also responsible for the cysteine residue dissociation from the active site Zn^{2+} ion by inducing the activation of latent MMPs during the assay.

The denatured enzymes begin then to move from the electric field cathode (negative) to the anode (positive) through the pores of a discontinuous polyacrylamide gel composed of a stacking and a separating gel. These resulted from the head-to-tail radical polymerization reaction between the acrylamide monomers and the cross-linker bis-acrylamide or piperazine diacrylamide (PDA) monomers. The reaction is catalysed by the catalyser ammonium persulphate (APS) and the initializer N-tetramethylendiamine (TEMED). This last induces the APS homolytic decomposition in free sulfuric radical ($\bullet SO_4^-$), which allows to obtain gel with variable porosity on the basis of the cross-linker monomer (bis-acrylamide or PDA) percentage compared to the total monomers (acrylamide and bis-acrylamide). The larger pores of the stacking gel than those of the separating gel below and their discontinuous pH buffer (lower in stacking than in separating) heavily influence the migration of the MMPs from the first, in which are induced to concentrate in a very tight zone at the interface with the second, where they give rise to bands localized at different heights corresponding to their molecular weight.

The presence of these bands is due to the acrylamide co-polymerization with the MMP-2 and MMP-9 substrate gelatine in the separating gel solution by favouring the proteolytic digestion during the overnight gel incubation in enzyme incubation buffer (50 mM Tris-HCl, pH 7.5; 5 mM $CaCl_2$; 100 mM NaCl; 1 mM $ZnCl_2$; 0.3 mM NaN_3, 0.2 g/l of Brij®-35; and 2.5 mL/l of Triton X-100) at 37°C. However, this last phase is following to the enzyme renaturation process consisting in two passages in the non-ionic detergent Triton X-100 2,5% v/v solution able to remove the SDS and allow the enzyme partial renaturation and activity recovery.

The MMP substrate used in these assays was the 0,3% w/v gelatine 90 bloom (PANREAC®) useful to evaluate the gelatinolytic MMP-2 and MMP-9 activity. Generally, a separating gel (7.5% acrylamide w/v) and a stacking gel (4% acrylamide w/v) containing bis-acrylamide as cross-linker in a 37.5:1 acrylamide:bis-acrylamide ratio are required to perform the zymography assay. In the present set of experiments a homemade 100:1 acrylamide:PDA ratio is used (unless otherwise specified) both for separating and stacking gels. The final composition of the separating gel (10 mL) is 0.375 M Tris HCl, pH 8.8; 7.5% acrylamide:PDA; 0.3% gelatin; 0.1% SDS, 0.1% APS, TEMED whereas stacking gel (2.5 mL) is 0.125 M Tris HCl, pH 6.8; 4% acrylamide:PDA; 0.1% SDS, 0.1% APS, TEMED. The separating gel solution is loaded between two juxtaposed glass plates previously assembled in their proper support to polymerize at room temperature. Afterwards, a polyethylene comb is pushed in the stacking gel solution to create the sample wells during the gel polymerization on the top of the separating gel at room temperature. The discontinuous gel obtained is then located in an electrophoretic chamber filled with a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), which ensures the continuous passage of

charges generated by the chamber connection with an electric power supply (120 Volt) for about 2 hours. This passage is crucial for the migration of MMPs contained in the serum-free THP-1 cell supernatant samples (20µl) centrifuged at 10,000 rpm for 10 minutes (4°C) and loaded in the gel wells after the addition of about 6µl of the marker dye Zymogram sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue). A peripheral blood sample prepared as 1:15 dilution in zymogram sample buffer is loaded (20µl) as molecular weight standard. The final visualization of clear bands on dark blue background is realised through a first passage in the 0,2% w/v staining solution of the Remazol Brilliant blue R250 dye (30% distilled water, 20% glacial acetic acid, 50% methanol, 0,2% w/v Remazol Brilliant blue R250) (VWR international srl; Sigma-Merck) for 15 minutes and a second in the Coomassie Brilliant Blue R250 dye (30% distilled water, 20% glacial acetic acid, 50% methanol, 0,2% w/v Coomassie Brilliant Blue R250) (VWR international srl; Sigma-Merck) for about 10 minutes. A destaining solution (70% distilled water, 10% glacial acetic acid, 20% methanol) (Sigma-Merck, VWR international srl) might be necessary to remove the dye excess.

The digital gel analysis by the specific Lab Image 1D software (Kapelan Bio-Imaging) provides both the correspondence between the band localization and its molecular weight through the comparison with the standard loaded (peripheral blood sample) and the volume band quantification. All chemicals and reagents for the zymography assays are obtained from Bio-Rad, unless otherwise specified.

3.1.5 Statistical analysis

The variables showed in the graphs are expressed as the mean ± standard error (SEM) of the mean. All the data are statistically analysed through Fisher Exact test, Mann-Whitney, one-way analysis of variance ANOVA with repeated measures followed by Dunn's post-hoc test according with the variable characteristics. The tests performed are two-tailed and the significance is set at $p < 0.05$. The analyses and the graphs are carried out through the Prism software for Windows 7 (version 3.1; Graph-Pad, San Diego, CA, USA).

3.2 Biochemical characterization of $C4_a/C4_b$ microenvironment: MMP expression profile

3.2.1 Patient recruitment and sample collection

The study of the MMP expression profile modulation by different gravitational positioning and compression treatment is approved by the London Queen Square Research Ethics Committee and is designed in accordance with the STROBE statement checklist on the reporting observational studies.

In particular, it involved fourteen healthy volunteers, members of the Ealing hospital (London, UK) staff without clinical evidence of venous disorders and classified as C0 and C1 stages in accordance with the CEAP classification, and fourteen $C4_a/C4_b$ patients enrolled in the waiting list to undergo venous laser ablation for primary saphenous reflux (>5s) detected through the ultrasound standing method after the calf compression-release manoeuvre execution[1,17]. Varicose veins, skin changes, corona phlebectatica paraplantaris and edema occurrence are the exclusion criteria followed in

the first group recruitment, whereas leg ulceration, thrombophlebitis and previous intervention are those applied in the second. General exclusion criteria are past history of deep venous thrombosis, post-thrombotic syndrome, chronic systemic inflammatory disease, cancer, pregnancy, anti-inflammatory or anticoagulant pharmacological treatments and tissue loss evidences. All participants signed their written informed consent and each one represented the own control.

The collection of their blood samples from the ankle region is immediately performed at the end of each of the three gravitational conditions imposed by the study plan. These consist in: a) stationary standing position on a paper square (40cm X 40cm) by leaning on an orthopaedic aluminium support frame; b) lying down in a supine position with the legs elevated at 20° with the knee joint supported by a stuffed platform to preventing hypertension; c) stationary standing by wearing a below knee and graduated elastic compression stocking (CONFORT medical compression stockings, 23-32 mmHg, class II; SIGVARIS, Andover, UK). Each state is maintained for 1 hour in three different attendances to favour the correct sample collection without inflammatory events related to the previous blood sampling.

The local blood is preferred because more representative of the functional and hemodynamic alterations affecting the lower limb drained tissue than the systemic. The samples are centrifuged in citrated collection tubes at 3,500 rpm for 10 minutes to obtain the plasma supernatant separated by red cells and stored at -20°C. These study phases are took place in London (Ealing Hospital, Ealing; Department of Surgery and Cancer, Imperial College, London; West London Vascular and Interventional Centre, London North West University Healthcare NHS Trust, Harrow)[129]. The samples received

under ice conditions are stored at -80°C until performing the MMP expression evaluation.

3.2.2 Multiplex suspension immunomagnetic assay

The assay is performed through the Pro™Human MMP 9-plex Assay based on the multiplex suspension immunomagnetic assay (Bio-Plex®, Bio-Rad, Hercules, CA, USA), as previously described.

Unlike the previously described sample treatments, plasma samples were diluted 1:4 in sample diluent for the execution of the assay, as required by the manufacturer. All the analytical steps of the multiplex suspension immunomagnetic assay followed the guidelines described in the previous subchapter.

3.2.3 Statistical analysis

The MMP expression differences between volunteers and patients in the same gravitational condition is statistically evaluated through the Mann-Whitney test. The Wilcoxon signed-rank test is applied to determine the biomarker differences within the same participant between each gravitational condition compared with the stationary standing position. The tests performed are two-tailed and the variables are expressed as mean ± standard error of the mean (SEM). The significance is set at $p < 0.05$. The analysis and the graphs are obtained through the Prism software for Windows 7 (version 3.1; Graph-Pad, San Diego, CA, USA).

3.3 *In vitro* characterization of detergent sclerosant biological and cellular effects

3.3.1 Sample collection and treatment

The blood samples of two healthy subjects are collected in citrated collection tubes (0,109 M sodium citrate) and centrifuged at 3,200xg for 15 minutes at 24°C to obtain plasma samples (Hospital Santa Maria della Misericordia, Urbino). The samples are then treated with the two detergent polidocanol (Atossisclerol, Gloria Med Pharma S.r.l.) and sodium tetradecyl sulphate (Fibro-Vein, MAC PHARMA S.r.l) as follows: a) 500µL of 3-0.6% v/v polidocanol previously diluted in distilled water are added to the plasma samples (500µL) to reach the final concentrations of 0.3%, 0.6%, 0.9%, 1.2% and 1.5%, as showed in table 1 (Tab. 3.3.1);

Detergent	dH_2O volume in 500 µL	3% detergent volume (µL) in 500 µL	Detergent concentration (%) in 500 µL	Detergent volume (µL)	Detergent final concentration (%)	Plasma volume (µL)
Pol	500 µL	0 µL	0%	0 µL	0%	500 µL
Pol	400 µL	100 µL	0.6%	500 µL	0.3%	500 µL
Pol	300 µL	200 µL	1.2%	500 µL	0.6%	500 µL
Pol	200 µL	300 µL	1.8%	500 µL	0.9%	500 µL
Pol	100 µL	400 µL	2.4%	500 µL	1.2%	500 µL
Pol	0	500 µL	3%	500 µL	1.5%	500 µL

Tab. 3.3.1 Schematic description of polidocanol concentrations. The table shows the detergent dilution set both in dH_2O and in plasma to reach the final concentrations

b) 500µL of 3-0.3% v/v sodium tetradecyl sulphate similarly diluted in distilled water and added to the plasma samples (500µL) to reach the final concentrations of 0.15%, 0.3%, 0.4%, 0.45%; 0.5%, 0.6%; 0.9%; 1.2% and 1.5%, as showed in the following table (Tab. 3.3.2).

Detergent	dH_2O volume in 500 µL	3% detergent volume (µL) in 500 µL	Detergent concentration (%) in 500 µL	Detergent volume (µL)	Detergent final concentration (%)	Plasma volume (µL)
STS	500 µL	0 µL	0%	0 µL	0%	500 µL
STS	450 µL	50 µL	0.3%	500 µL	0.15%	500 µL
STS	400 µL	100 µL	0.6%	500 µL	0.3%	500 µL
STS	366,7 µL	133,3 µL	0.8%	500 µL	0.4%	500 µL
STS	350 µL	150 µL	0.9%	500 µL	0.45%	500 µL
STS	333 µL	167 µL	1%	500 µL	0.5%	500 µL
STS	300 µL	200 µL	1.2%	500 µL	0.6%	500 µL
STS	200 µL	300 µL	1.8%	500 µL	0.9%	500 µL
STS	100 µL	400 µL	2.4%	500 µL	1.2%	500 µL
STS	0	500 µL	3%	500 µL	1.5%	500 µL

Tab. 3.3.2 Schematic description of sodium tetradecyl sulphate concentrations. The table shows the detergent dilution set both in dH_2O and in plasma to reach the final concentrations

Aliquots from the treated plasma samples incubated for 30 minutes at room temperature are further diluted in distilled water (1:10) to be used for the gelatinases MMP-2 and MMP-9 activity determination through the zymography assay and for the SDS-PAGE protein analyses.

3.3.2 Zymography assay

The potential effects of the sclerosant detergents polidocanol and sodium tetradecyl sulphate on MMP-2 and MMP-9 gelatinolytic activity in plasma samples are determined through the zymographic assay. According with the classical zymography protocol the separating gel (7.5% w/v acrylamide, copolymerized with gelatin 0.3%) and the stacking gel (4% w/v acrylamide) are characterized by the cross-linker bis-acrylamide in a 37.5:1 acrylamide:bis-acrylamide ratio and the assay is performed as previously stated by

loading 1:10 diluted aliquots (25 µl) of plasma samples treated with each detergent concentration.

3.3.3 SDS-PAGE assay

The same samples are also employed to investigate the potential modulation of protein expression after the treatment with the different concentrations of the two sclerosant detergents. The electrophoretic assay is performed in reducing and denaturing conditions generated by the beta mercaptoethanol (βME) and the heat treatment, besides by the SDS. The βME (Bio-Rad) determines the removal of the disulphide bonds between the protein cysteine residues by their reduction. It was diluted (1:20) in the marker dye Laemmli sample buffer (65,8mM Tris-HCl, pH 6.8, 26,3% glycerol, 2,1% SDS, 0.01% bromophenol blue; Bio-Rad). The subsequent sample boiling (100°C; 5 minutes) in presence of SDS, which is contained in the marker dye Laemmli sample buffer added (7µL), speeds up the complete denaturation of the proteins. These sample treatments are in accordance with the assay principle to induce the migration of proteins in a gel subjected to an electric field on the basis of their molecular weight, as previously elucidated. In this regard, the stacking and the separating gel are obtained in accordance with the protocol mentioned for the zymography in absence of the gelatine.

The samples loaded (15µL of 1:10 dilution) give rise to dark blue protein bands on clear background after the electrophoretic run and staining. Their visualization is performed through the Remazol Brilliant Blue R250 and Coomassie Brilliant Blue R250 staining solutions (Bio-Rad) followed by a destaining step, as afore mentioned.

The molecular weight standard consists in the standard Plus Protein Unstained (Bio-Rad) loaded in the gel according with the manufacturer instructions. The digital analysis is performed through the Lab Image 1D software (Kapelan Bio-Imaging).

3.3.4 Cell culture and treatment

The polidocanol and sodium tetradecyl sulphate effects on cellular membrane are evaluated through the treatment of human endothelial vascular cell line HECV cell line obtained from Interlab Cell Line Collection (ICLC). The HECV cells are cultured in complete high glucose DMEM/F-12 (supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine and 1% antibiotics) at 37°C in humidified air with 5% CO_2. For the assays, they are seeded at 15,000 cells/cm^2 in each well of four 24-well plates up to reaching the confluence and undergoing the treatments with the previously specified sclerosant dilutions in a final volume of 100µL.

The sclerosant dilutions are obtained as follows: a) 50 µL of 3-0.6% v/v polidocanol previously diluted in distilled water are added to plasma samples (50 µL) and saline solution (PBS and glucose 0,9g/L) (50 µL) to reach the final concentrations of 0.3%, 0.6%, 0.9%, 1.2% and 1.5% in a final volume of 100 µL; b) 50 µL of 3-0.15% v/v sodium tetradecyl sulphate similarly diluted in distilled water and added to the plasma samples (50 µL) and saline solution (50 µL) to reach the final concentrations of 0.075%, 0.15%, 0.3%, 0.4%, 0.45%; 0.5%, 0.6%; 0.9%; 1.2% and 1.5% in a final volume of 100 µL.

The polidocanol and sodium tetradecyl sulphate solutions both in saline and in plasma are added in each well of the corresponding plate placed under the microscope in order

to provide an immediate observation with video captures (OPTIKAM HDMI Pro OPTIKA Microscopes).

The sterile compounds for cell culture are from JET BIOFIL Bio-filtration Products Co (Guangzhou, China) whereas chemicals and reagents are from Carlo Erba Reagents S.r.l. (Milan, Italy).

3.4 Biochemical characterization of C1/C2 microenvironment: cytokine, chemokine and growth factor expression profile

3.4.1 Patient recruitment and sample collection

The study of the sclerotherapy treatment effects on the expression of some inflammatory mediators is performed through the serum sample collection from twelve patients affected by the early CVeD manifestations classified as C1/C2 stages of the CEAP classification[17]. The patients come from the Ippocrate clinics of Parma (Italy) where they undergo the sclerotherapy treatment based on the Casoni's method after giving their signed written informed consent. This clinical strategy consists in intravenous injection of sub-lytic concentrations of polidocanol, which vary on the basis of the treated vessel dimensions (0.10% in 15mL; 0.10% in 20mL; 0.05% in 20mL).

The blood sample collection is performed before the first treatment (T0), after 24 hours (T24h), 7 days (T7d) and 30 days (T30d) from the first treatment. In some patients the blood collection is also carried out at 30 minutes (T30'), 45 minutes (T45') and 3 hours (T3h) from the first treatment. A second treatment is executed after the blood sampling at T7d. All samples are stored at -20°C until the inflammatory mediator determination

through the multiplex suspension immunomagnetic assays (Bio-Plex®, Bio-Rad, Hercules, CA, USA).

3.4.2 Multiplex suspension immunomagnetic assay

The assay is performed through the Pro™Human Cytokine 27-plex, group I Assay (Bio-Plex®, Bio-Rad, Hercules, CA, USA), which provides the simultaneous quantification of IL-1β, IL-1ra, IL-2, -4, -5, -6, -7, -8, -9, -10, -12, -13, -15, -17, bFGF, Eotaxin, G-CSF, GM-CSF, INF-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF.

The required standard curve is obtained through nine serial dilutions (1:4) of the assay standard in a specific diluent both provided by the manufacturer. The serum samples are maintained at room temperature before to be centrifuged at 10,000 rpm for 10 minutes (4°C). Sample diluted 1:4 in sample diluent are loaded (50 µl/well) on the 96-well plate containing the beads conjugated with the primary monoclonal antibody (50 µl/well) previously resuspended in the assay buffer and washed two times through a magnetic Wash Station (Bio-Rad), as previously explained. The plate incubation in the dark for 30 minutes on a shaker at 850±50 rpm is followed by three washing steps through the magnetic Wash Station to remove the unbound proteins. Then, the addition of the biotinylated secondary antibody properly diluted in the antibody diluent to each well (25 µl/well) is performed to induce the interaction with the analyte-primary antibody complex generated on the bead surface. In the last step the plate incubated in the dark for 30 minutes on the shaker at 850±50 rpm is washed three times to remove the unbound antibodies and then, loaded with the fluorescent marker streptavidin-phycoerythrin (50 µl/well) with a further incubation in the dark of 10 minutes on the

shaker (850±50 rpm). The next washing step through the magnetic Wash Station is followed by the addition of the assay buffer (125µl/well) in each well and a rapid shake for 30 seconds to resuspend fluorescently-dyed immunocomplexes before the plate reading and the data collection.

3.4.3 Statistical analysis

The statistical analysis is performed through the Friedman test followed by Dunn's multiple comparison post-hoc test according with the variable characteristics. The significance is set at $p < 0.05$. Each variable is expressed as mean ± standard error of the mean (SEM). The analyses and the graphs are carried out through the Prism software for Windows 7 (version 3.1; Graph-Pad, San Diego, CA, USA).

Chapter 4

Results

4.1 Biochemical characterization of C6 microenvironment

4.1.1 Demographic data

According with the inclusion criteria, the population of thirty patients involved in the study of TGF-β isoform, sEng and MMP expression in WF of venous leg ulcers was characterized by adult individuals ranged from 43 to 91 years (mean age 73.7 years) of both sexes (10 males and 20 females) among which 11 showed ulcers at the first occurrence and 19 are affected by relapse. The ulcers had a mean duration of 41.6-54.5 moths and an average size of 10.7 cm^2 (range 0.2-60 cm^2). The collected WF samples were respectively divided in inflammatory (n=10) and granulating (n=20) groups on the basis of the wound healing process phase (inflammatory or granulating phases) and the clinical examinations of the tissues involved.

4.1.2 TGF-β isoform and sEng expression profile in wound fluid

The results obtained from multiplex suspension immunomagnetic assay allowed us to observe the only significant increase of TGF-β3 concentration in Infl WF compared to Gran WF samples (p= 0.033). This evidence might be consistent with the antagonist role of the TGF-β isoform against the TGF-β1. On the contrary, the TGF-β1 and TGF-β2 quantification resulted not statistically different between the two groups of WF samples (p= 0.947 and p= 0.301, respectively) (Fig. 4.1.1).

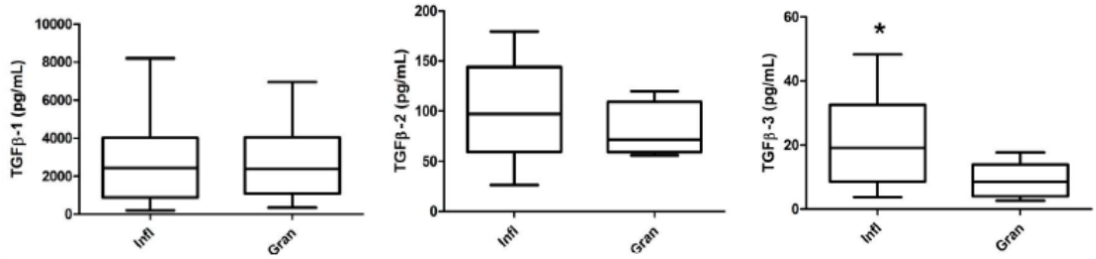


Fig. 4.1.1 Quantitative determination of TGFβ isoform concentration. The graphs show the three TGFβ isoform concentrations in inflammatory (n= 10) and granulating (n= 20) WF samples; (*= $p < 0.05$)

With regard of the sEng quantitative levels detected in Gran and Infl WF samples, they appeared statistically relevant in the former compared to the latter (p= 0.002) (Fig. 4.1.2). This implication might be correlated with the proteolytic environment which varies during the healing process by influencing the sEng shedding and function.

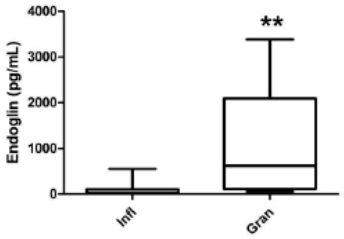


Fig. 4.1.2 Quantitative determination of sEng concentration. The graph reveals the sEng concentration in inflammatory (n=10) and granulating (n=20) WF samples; (**= $p < 0.01$)

4.1.3 TGF-β isoform, sEng and gelatinase expression profile in WF-stimulated THP-1 co-treated with sulodexide

The Infl and Gran WF pools were also employed as inflammatory stimulation of the human monocyte THP-1 cells in order to evaluate the responses produced by monocyte during the healing process.

The THP-1 cell viability after the treatment with 5% of both WF pools was obtained through the previously mentioned trypan blue exclusion test, which showed a cellular viability >85% and >95% in presence of Infl and Gran WF, respectively.

The addition of the 5% v/v WF pools was carried out in presence or absence of the glycosaminoglycan mixture sulodexide (SDX) (0.12 LSU/mL) composed of heparin and dermatan sulphate and used in the VLU treatment[13,54]. All the treatments lasted 24 h. However, the TGF-β isoform as well as the sEng release by the monocyte THP-1 cells was not significantly modified neither by the Infl and Gran WF stimulation nor by the presence or absence of SDX co-stimulation (Fig. 4.1.3; Fig. 4.1.4).

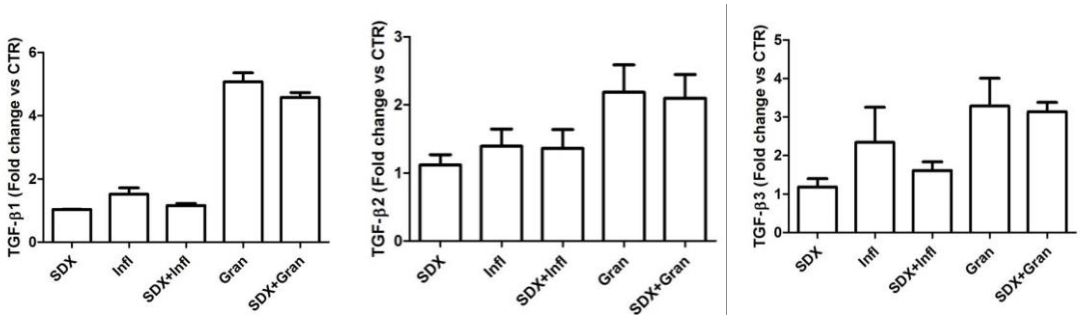


Fig. 4.1.3 Quantitative determination of TGFβ isoform concentration in WF-stimulated THP-1 monocyte cells in presence or absence of SDX co-treatment. The TGF-β isoform levels in serum-free culture media supernatants from THP-1 cells treated with 5% v/v Infl and Gran WF in presence or absence of SDX (0.12 LSU/mL) for 24h

An opposite effect was detectable about the sEng concentration, which showed positive variations after the co-treatment with both WF pools and SDX, although statistically relevant evidences were only pointed out after the association of Gran WF with SDX ($p < 0.001$) (Fig. 4.1.4). This might be consistent with a potential glycosaminoglycan ability to enhance the proteolytic sEng shedding, which appeared high in Gran WF, as showed in Fig. 4.1.2.

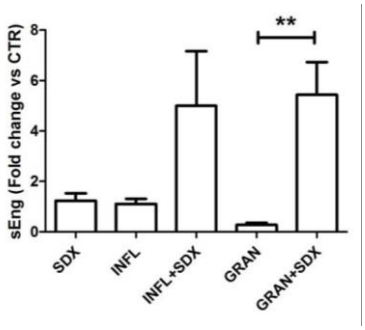


Fig. 4.1.4 Quantitative determination of sEng concentration in WF-stimulated THP-1 monocyte cells in presence or absence of SDX co-treatment. The sEng concentration in serum-free culture media supernatants from THP-1 cells treated with 5% v/v Infl and Gran WF in presence or absence of SDX (0.12 LSU/mL) for 24h; (** = $p < 0.01$)

This speculation seems to be sustained by the statistically raised MMP-2 expression from THP-1 cells stimulated with the same Gran WF and SDX co-treatment compared with the other treatment conditions ($p < 0.005$). The MMP-9 concentration appeared statistically pronounced in presence of Infl WF compared to the Gran WF ($p= 0.0001$), despite a general increase results also evident after the co-treatment with Infl WF and SDX (Fig. 4.1.5).

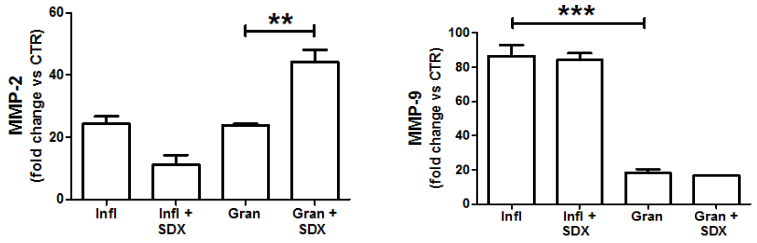


Fig. 4.1.5 Quantitative determination of MMP-2 and MMP-9 in WF-stimulated THP-1 monocyte cells in presence or absence of SDX co-treatment. The MMP-2 and MMP-9 concentrations in serum-free supernatants from THP-1 cells treated with 5% v/v Infl and Gran WF in presence or absence of SDX (0.12 LSU/mL) for 24h; (**= $p < 0.005$; ***= $p < 0.0005$)

4.1.4 Proteolytic activity profile of MMP-2 and MMP-9 in WF-stimulated THP-1 co-treated with sulodexide

The results of zymography assays of THP-1 cell serum-free culture media supernatants enriched the just stated evidences by showing a diffused increase of the MMP-9 (92 kDa and 225 kDa isoforms) gelatinolytic activity after the treatment with Gran WF compared with the corresponding Infl WF effects. Additionally, the co-treatment with the glycosaminoglycan caused a reduction of the gelatinase isoform activity in combination with both Infl WF and Gran WF compared with the pools alone (Gran WF/SDX vs Gran WF: -53% and -61% for the 92 and 225 kDa isoforms, respectively; Infl WF/SDX vs Infl WF: -72% and -96% for the 92kDa and 225 kDa isoforms, respectively) (Fig. 4.1.6).

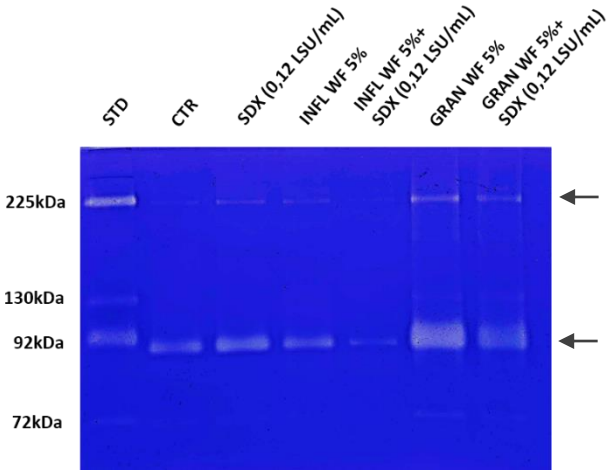


Fig. 4.1.6 Zymography assay of serum-free culture media supernatants from WF-stimulated THP-1 monocyte cells in presence or absence of SDX co-treatment. The gel image shows the proteolytic profile of MMP-2 (72 kDa) and MMP-9 isoforms (see the arrows) in serum-free culture media supernatants from THP-1 cells after the different treatments consistent with the gel lanes read starting from left. The first lane (STD) consists in the gelatinolytic activities of the MMP-2 monomer (72kDa) and MMP-9 multimeric isoform (225 kDa), NGAL/MMP-9 complex (130 kDa) and MMP-9 monomer (92 kDa) detectable in peripheral blood sample; the second lane (CTR) corresponds to a serum-free culture media supernatant sample from THP-1 cells without any treatment

However, the MMP-2 gelatinolytic profile seemed to be not affected by the four treatments (Fig. 4.1.6)[130,131]

These evidences are consistent with the involvement of these enzymes in the different healing process phases and with the SDX anti-inflammatory properties.

4.2 Biochemical characterization of $C4_a/C4_b$ microenvironment

4.2.1 Demographic data

The study of the biochemical mediators characterizing the CVeD microenvironment during its progressive manifestation and their modulation was expanded through the investigation of the potential effects induced by some recommended clinical treatments, including the gold-standard compression therapy. In this regard, C0 and C1 adult healthy volunteers (n=14), ranged from 33 to 56 years (mean age 48), and $C4_a/C4_b$ adult patients (n=14), ranged from 38 to 61 (mean age 51) are enrolled, according to the specified inclusion and exclusion criteria. Although the patient group was characterized by higher height and weight than the volunteer group, there were no differences in the body mass index (BMI) value. Patients showed ankle and calf circumference increased compared with the volunteers. Additionally, their venous clinical severity score (VCSS) is significantly higher than volunteers.

Each group undergoes three different laboratory conditions, among which wearing medical compression stockings providing pressure values at the ankle comprised in the manufacturer's specified range of 23-32 mmHg during the standing position in accordance with the study plan.

4.2.2 MMP expression profile in plasma samples from healthy volunteers and $C4_a/C4_b$ patients

The Pro™Human MMP 9-plex assay allowed to detect a significant statistical decrease of the MMP-2 and MMP-13 expression in plasma samples from patients after the compression treatment compared to the stationary standing position ($p = 0.0245$ and $p = 0.0171$, respectively) (Fig. 4.2.1).

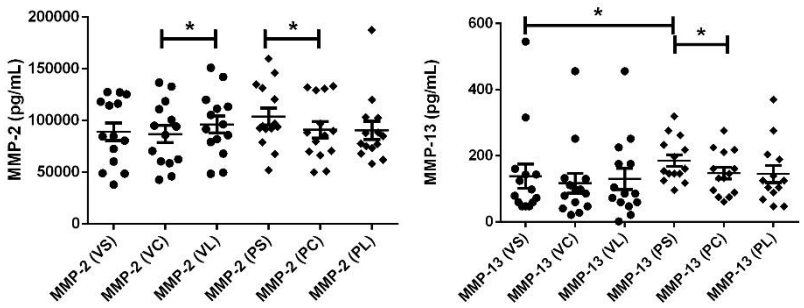


Fig. 4.2.1 Quantitative determination of the gelatinase MMP-2 and the collagenase MMP-13 in plasma samples from healthy volunteers (V) and $C4_a/C4_b$ patients (P). The expression profile of MMP-2 and MMP-13 in plasma samples from healthy volunteers (n=14) and $C4_a/C4_b$ patients (n=14) in stationary standing position (VS, PS), standing position by wearing compression stockings (VC, PC) and lying down position (VL, PL), respectively; (*= $p < 0.05$)

The result might be consistent with the gelatinase and collagenase proteolytic involvement in the extracellular matrix alterations (collagen degradation) and the skin changes associated with the $C4_a/C4_b$ CVeD stages. A statistical increase is observed in MMP-2 expression during the lying down position versus the compression treatment in volunteers ($p = 0.0245$). This might be associated with possible hemodynamic variations occurring in the passage from one position to another.

Additionally, the assay disclosed a general statistically relevant decrease of concentration of the other investigated MMPs in plasma samples from both patients and volunteers undergoing lying down position compared to stationary standing

position and the compression treatment, as shown in figure 4.2.2. In particular, this was observed for MMP-3 (VC vs VL: p = 0.0419; VS vs VL: p = 0.0398) and MMP-7 (VS vs VL: p = 0.0419) in volunteers, and for MMP-3 (PC vs PL: p = 0.0295; PS vs PL: p = 0.0017), MMP-7 (PC vs PL: p = 0.0203; PS vs PL: p = 0.0006), MMP-8 (PC vs PL: p = 0.0327; PS vs PL: p = 0.0012), MMP-9 (PC vs PL: p = 0.0494; PS vs PL: p = 0.0295), MMP-10 (PS vs PL: p = 0.0269) and MMP-12 (PC vs PL: p = 0.0785) in patients.

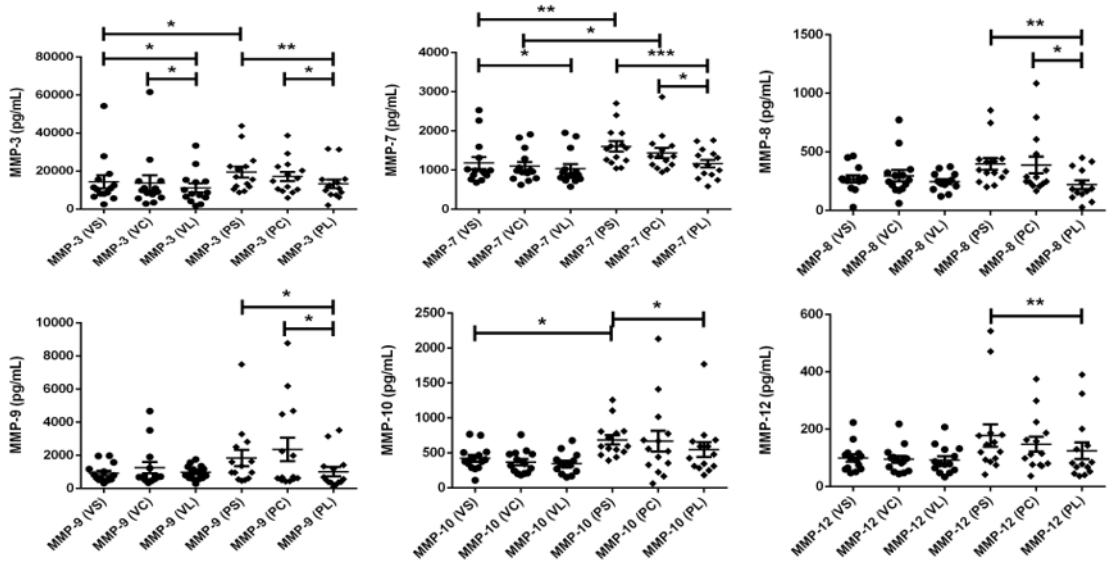


Fig. 4.2.2 Quantitative determination of MMPs in plasma samples from healthy volunteers and $C4_a/C4_b$ patients. The expression profile of the MMP-3, -7, -8, -9, -10 and -12 in plasma samples from healthy volunteers (n=14) and $C4_a/C4_b$ patients (n=14) in stationary standing position (VS, PS), standing position by wearing compression stockings (VC, PC) and lying down position (VL, PL), respectively; (*= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$)

Additionally, by comparing the expression profiles of MMPs observed in patients vs. volunteers, within the same group defined by the leg position, some further statistically significant differences emerged. In this respect, $C4_a/C4_b$ patients subjected to standing position showed significantly increased levels of MMP-3 (p = 0.0409), MMP-7 (p = 0.0063), MMP-10 (p = 0.0014) and MMP-13 (p = 0.013) compared to healthy volunteers

maintaining the same position (Fig. 4.2.1 and Fig. 4.2.2). Furthermore, MMP-7 levels were found significantly increased in PC vs. VC ($p = 0.0203$) (Fig. 4.2.2).

The MMP-1 expression data are not available by considering the instrument detection limit based on the standard curve.

In regard of the results showed, the remarkable responsiveness of the MMP expression to the physiological hemodynamic fluctuations characterizing the blood supply to the lower extremities and their impairment during the CVeD might represent the conceptual basis of these evidences.

4.3 *In vitro* characterization of detergent sclerosant biological and cellular effects

4.3.1 Proteolytic activity profile of MMP-2 and MMP-9 in normal plasma samples treated with polidocanol and sodium tetradecyl sulphate

The zymography assays of plasma samples from two healthy subjects after the treatment with the different dilutions of the non-ionic sclerosant detergent polidocanol (POL) did not reveal evident differences about the gelatinolytic activity of the two gelatinases after the treatment with increasing concentrations of POL, as shown in the figure 4.3.1.

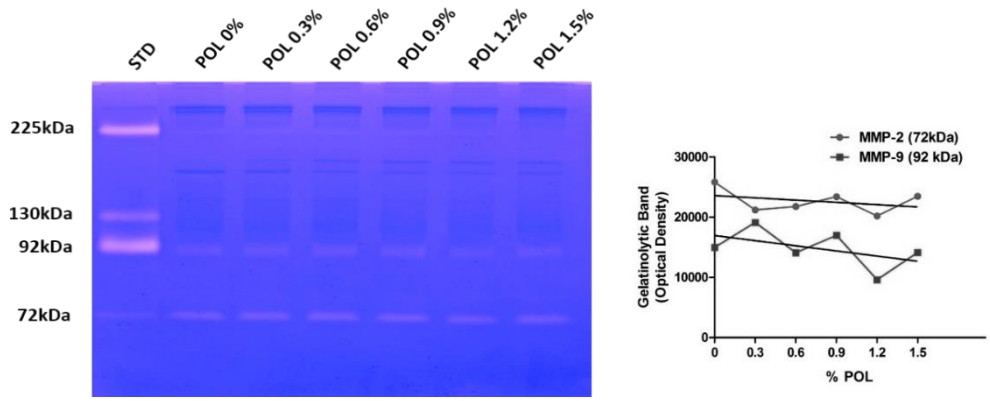


Fig. 4.3.1 Zymography assay of plasma samples from healthy subjects treated with polidocanol. The proteolytic profile of MMP-2 and MMP-9 without significant variations after the addition of polidocanol dilutions corresponding to the gel lanes; the standard lane (STD) consists in the gelatinolytic activities of the MMP-2 monomer (72kDa) and MMP-9 multimeric isoform (225 kDa), NGAL/MMP-9 complex (130 kDa) and MMP-9 monomer (92 kDa) detectable in peripheral blood sample. The graph on the right shows the optical density data of the MMP bands obtained through the Lab Image 1D software analysis

However, the same plasma samples treated with the anionic sclerosant detergent sodium tetradecyl sulphate highlighted the appearance of a gelatinolytic band around 160 kDa and comprised within the 0.3% and 0.6% STS concentrations. No significant differences were visible about the MMP-9 and MMP-2 proteolytic activity after the treatments (Fig. 4.3.2).

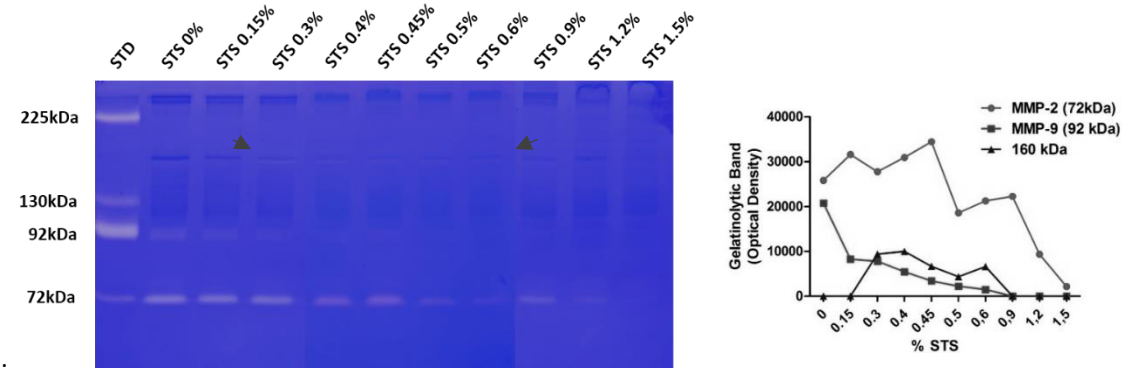


Fig. 4.3.2 Zymography assay of plasma samples from healthy subjects treated with sodium tetradecyl sulphate. The MMP-2 and MMP-9 activity determination and the appearance of the gelatinolytic band of about 160 kDa between the STS concentrations indicated by the arrows; the standard lane (STD) consists in the gelatinolytic activities of the MMP-2 monomer (72kDa) and MMP-9 multimeric isoform (225 kDa), NGAL/MMP-9 complex (130 kDa) and MMP-9 monomer (92 kDa) detectable in peripheral blood sample. The MMP optical density graph (Lab Image 1D software data) similarly evidences the 160 kDa band presence between the 0.3% and 0.6% STS concentrations

These evidences might be correlated with potential interactions between the sclerosant agents and the protein contents of the plasma samples by explaining the additional band

appearance (possible sclerosant-enzyme complex around 160 kDa) and a potential modulation of MMP-2 and MMP-9 activity especially by the STS during the treatment of the CVeD manifestations.

4.3.2 Electrophoretic profile of plasma protein content after the treatment with polidocanol and sodium tetradecyl sulphate

The property of the detergents to generate potential protein complexes in normal plasma samples was deepened through the investigation of the protein profile induced by the treatment with different sclerosant concentrations. In this respect, the most interesting result consisted in a protein band visible around 100 kDa, which began to increase between 0.4% and 0.6% STS concentrations to gradually reducing up to 1.5% STS concentration (Fig. 4.3.3).

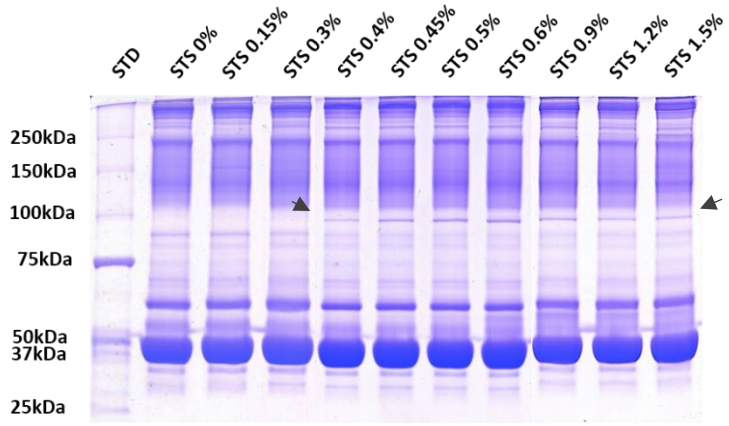


Fig. 4.3.3 SDS-PAGE assay of plasma samples from healthy subjects treated with sodium tetradecyl sulphate. The electrophoretic determination of protein content of normal plasma samples treated with different STS concentrations; visualisation of the 100 kDa protein band between 0.4% and 1.5% as indicated by the arrows

However, the results from the SDS-PAGE assays of the normal plasma samples treated with the increasing polidocanol concentrations did not show any significant difference in the protein profile (Fig. 4.3.4).

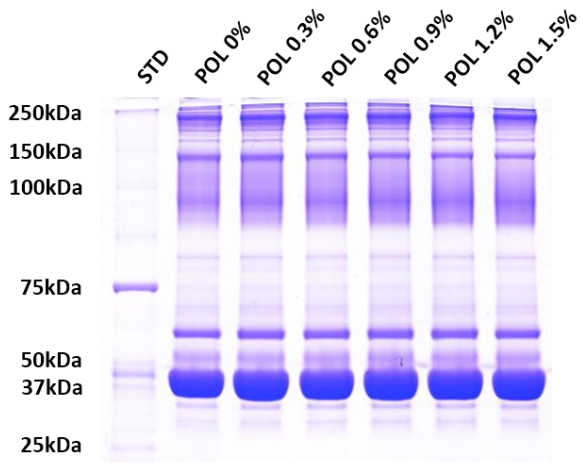


Fig. 4.3.4 SDS-PAGE assay of plasma samples from healthy subjects treated with polidocanol. The electrophoretic determination of protein content of normal plasma samples treated with different increasing concentrations of POL

4.3.3 Cellular morphological changes in HECV endothelial cells induced by polidocanol and sodium tetradecyl sulphate treatment

According with the literature[49], the same POL and STS dilutions prepared both in normal plasma samples and in saline solution were employed to observe the potential structural effects induced on the human endothelial HECV cells. The addition of the lowest polidocanol concentration 0.3% determined cellular size reduction and cytoplasmatic condensation more evident in saline preparation than in plasma during the videotaping time. Intense cellular blebbing characterized the HECV cells treated with the sclerosant preparation in plasma (Fig. 4.3.5c).

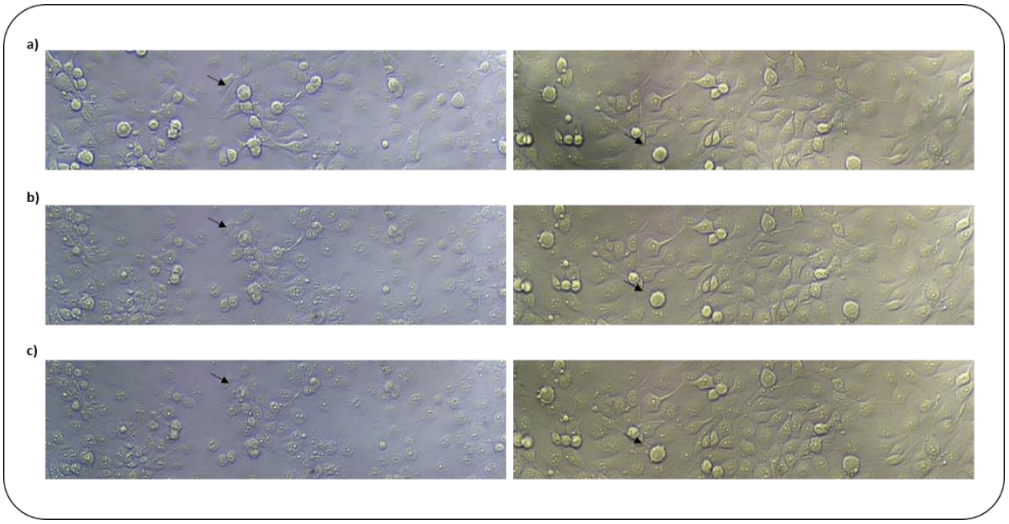


Fig. 4.3.5 Morphological changes in HECV endothelial cells after the treatment with the lowest POL concentration. The video frames relating to 0 (a), 3 (b) and 8 seconds (c) from the addition of the 0.3% POL solution prepared in saline (on the lefts) and in plasma (on the right); the arrows allow to focus the attention on a single cell to observe the morphological changes described

These morphological alterations were also detected in the following treatments with the other polidocanol concentrations (video frames not shown). The modifications culminate in a rapid cellular swelling, cytoplasmatic destruction and intense cellular degranulation and lysis observed after the addition of both saline and plasma polidocanol solutions at the highest concentration of 1.5% (Fig. 4.3.6).

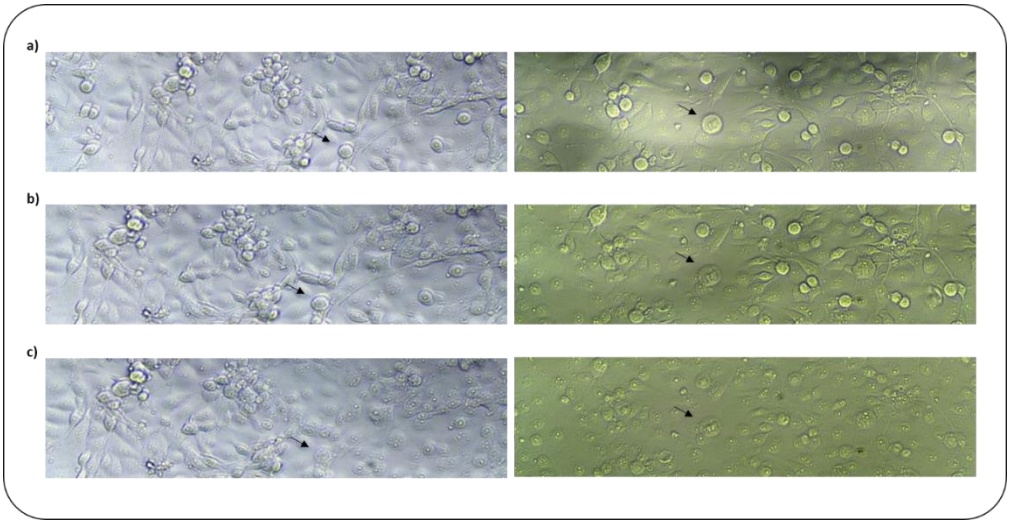


Fig. 4.3.6 Morphological changes in HECV endothelial cells after the treatment with the highest POL concentration. The video frames relating to 0 (a), 3 (b) and 8 seconds (c) from the addition of the 1.5% POL solution prepared in saline (on the lefts) and in plasma (on the right); the arrows allow to focus the attention on a single cell to observe the morphological changes

The video frames related to the lowest concentration of the STS corresponding to 0.075% are representative of the HECV pronounced structural changes due to the almost immediate cellular lysis and shrinkage, which appeared delayed when the detergent solution was prepared in plasma compared to saline (Fig. 4.3.7b, c). This evidence might be related with the plasma modulation of STS lytic function[40].

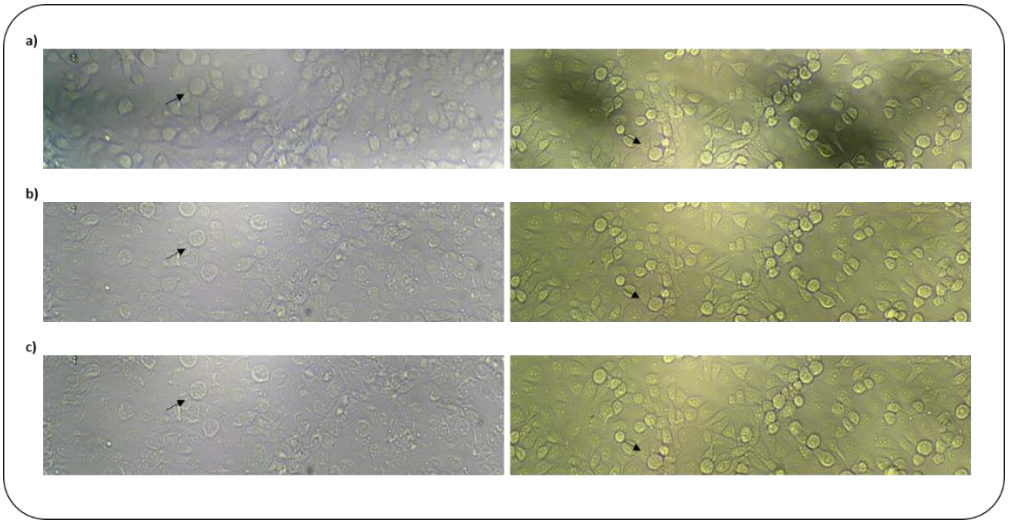


Fig. 4.3.7 Morphological changes in HECV endothelial cells after the treatment with the lowest STS concentration. The video frames relating to 0 (a), 3 (b) and 8 seconds (c) from the addition of the lowest STS concentration (0.075%) in saline (on the lefts) and in plasma (on the right) solutions; the arrows allow to focus the attention on a single cell to observe the different timing of the effects induced by the two sclerosant preparations

Quick fragmentation, shrinkage and cluster formation of HECV cells at the side margins of the plate wells were the structural alterations derived from the treatment with 1.5% STS concentration and obtained both in plasma and in saline solutions (Fig. 4.3.8c, d). These represented the greater expression of the morphological changes which occurred and became increasingly rapid during the treatment with the intermediate STS concentrations up to the highest.

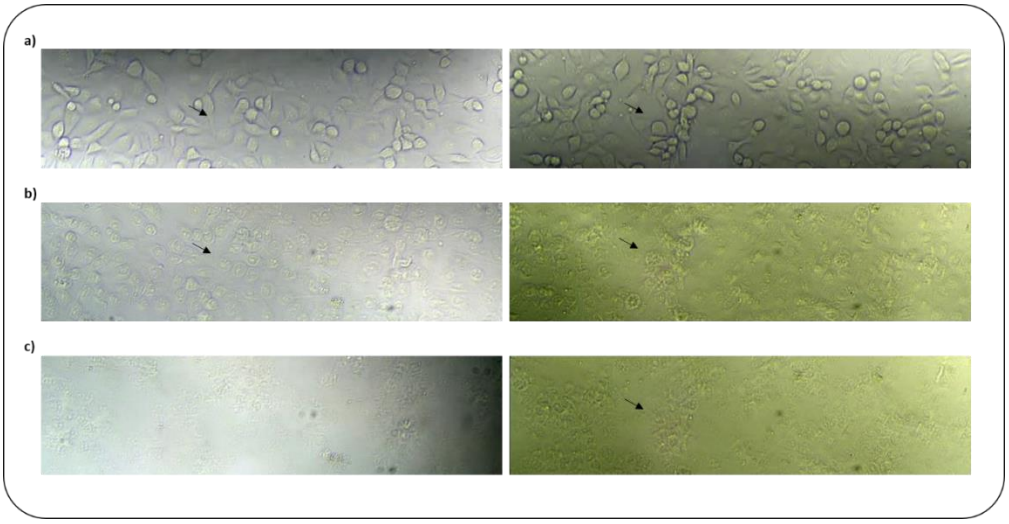


Fig. 4.3.8 Morphological changes in HECV endothelial cells after the treatment with the highest STS concentration The video frames relating to 0 (a), 3 (b) and 8 seconds (c) from the addition of the 1.5% STS concentration in saline (on the lefts) and in plasma (on the right) solutions; the arrows allow to observe the fast degrading effects induced by the treatment on the HECV cells indicated

All the mentioned alterations observed *in vitro* on the HECV cells might be correlated with the chemical nature of the two sclerosant agents which justifies probable membrane-sclerosant interactions at the origin of the bleb formation, increased membrane permeability and further biological processes as cellular activation or death[49].

4.4 Biochemical characterization of C1/C2 microenvironment

4.4.1 Demographic data

The quantitative analysis of the inflammatory mediators in serum samples from twelve C1/C2 patients (11 female and 1 male) treated with sub-lytic doses of the sclerosant

polidocanol was performed to enrich the biochemical CVeD microenvironment characterization. However, a partial group of 11 patients was considered for the statistical elaboration of the data obtained through the Pro™Human Cytokine 27-plex Assay due to the lack of T30d experimental time in one patient. Furthermore, the T30', T45' and T3h times available only for three patients were exploited to evaluate a potential inflammatory short-term response induced by polidocanol.

4.4.2 Inflammatory mediator expression profile in serum samples from C1/C2 patients

A general upward trend without statistical significance was detected for inflammatory cytokines as IL-1β, IL-7, IL-12, IL-17, IFN-γ, TNF-α around the T30', but it was followed by a rapid decrease. Interestingly, the increment of IL-7 at T30'/T45' resulted then in a gradual and statistically relevant reduction up to T30d ($p < 0.05$) (Fig. 4.4.1).

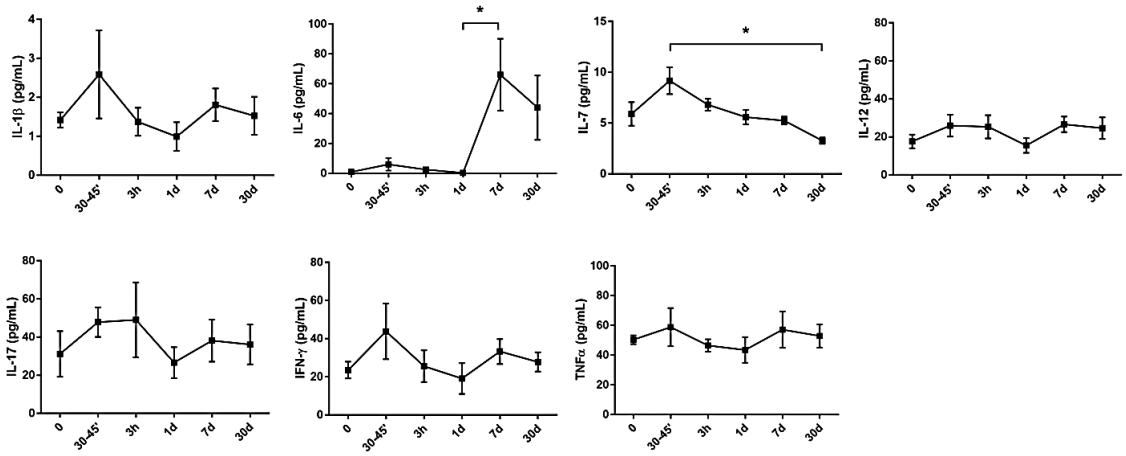


Fig. 4.4.1 Quantitative determination of pro-inflammatory cytokines in patient serum samples. The expression profile of the pro-inflammatory cytokine IL-1β, -6, -7, -12, -17, IFN-γ and TNF-α in patient serum samples (n=3) in all experimental times; (* = $p < 0.05$)

The inflammatory cytokine IL-6, the anti-inflammatory IL-1ra, IL-4, -5, -9 and -13 showed a constant expression profile at short-term, except for the IL-1ra peak at T30' correlated

with the IL-1β trend and the slight increase of IL-9 and IL-13 at T3h and T30'/T45', respectively (Fig. 4.4.1; Fig. 4.4.2).

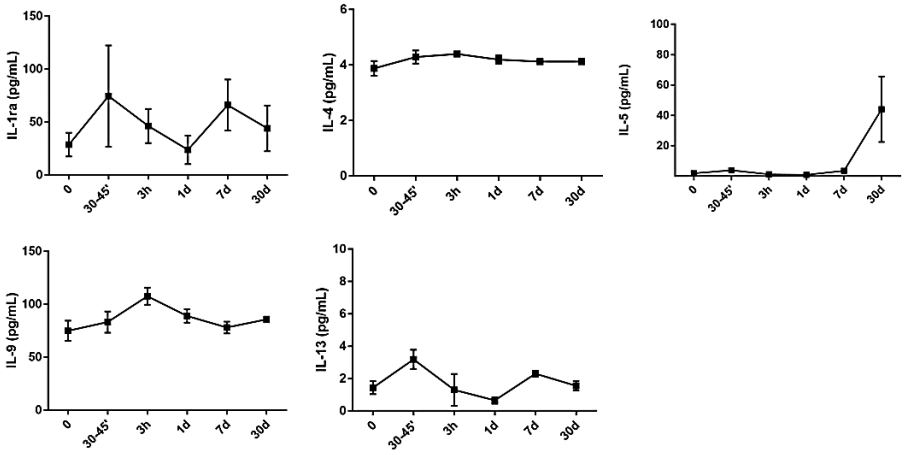


Fig. 4.4.2 Quantitative determination of anti-inflammatory cytokines in patient serum samples. The expression profile of the anti-inflammatory cytokine IL-1ra, -4, -5, -9 and -13 in patient serum samples (n=3) in all experimental times

The maintenance of a nearly unchanged concentration was also observed for G-CSF, the chemokine IL-8/CXCL8, IP-10/CXCL10, MIP-1α/CCL3, RANTES/CCL5, Eotaxin/CCL11, MIP-1β/CCL4 and the growth factor bFGF, PDGFbb and VEGF immediately after the sclerosant treatment, although IL-8 and VEGF showed a sharp increase at T30'/T45' (Fig. 4.4.3).

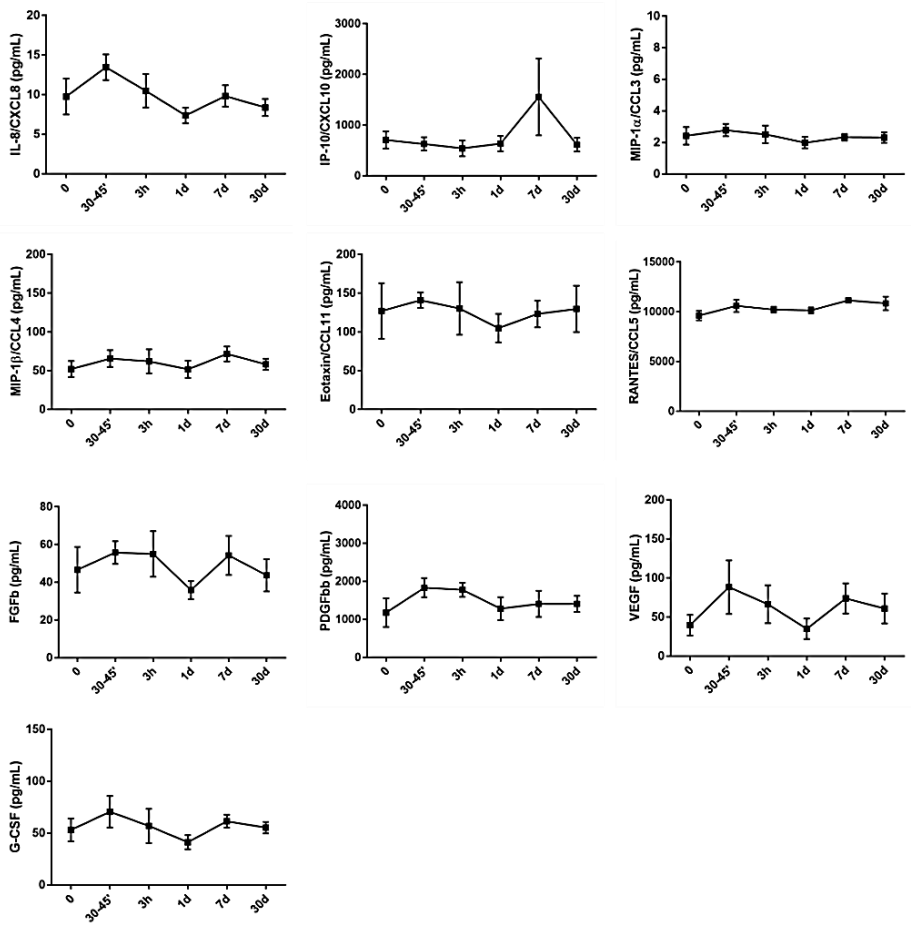


Fig. 4.4.3 Quantitative determination of chemokines and growth factors in patient serum samples. The expression profile of the chemokines IL-8, IP-10, MIP-1α, MIP-1β, Eotaxin, RANTES, the growth factors FGFb, PDGFbb, VEGF and the cytokine G-CSF in patient serum samples (n=3) in all experimental times

The data relative to the first experimental times are used to evaluate the occurrence of an early potential inflammatory response induced by the sub-lytic concentrations of polidocanol. Although a slight increase is evident for most of the mediators, however the small number of samples available for these times might explain the absence of statistical significance.

Results statistically significant began to appear at long-term, as showed by the pro-inflammatory cytokine IL-6, which drastically raises at T7d by considering its expression profile within all the experimental times ($p < 0.05$) (Fig. 4.4.1). The pro-inflammatory

mediators including IL-1β, -12, -17, IFN-γ, and TNF-α were characterized by an upward trend, which became more evident by considering the only medium- and long-term experimental times (Fig. 4.4.4). In fact, IL-12 and IL-17 progressively increased starting from T7d up to T30d after the treatment, although a statistical significance can be assigned only to IL-12 at T7d and T30d compared to T0 ($p < 0.05$). This evidence is also valid for the IL-1β at T7d ($p < 0.05$) as well as for IFN-γ and TNF-α, whereas an opposite tendency was statistically detectable for IL-7 from T7d up to T30d, as previously mentioned ($p< 0.01$) (Fig. 4.4.4). However, these data represent only preliminary and descriptive observations about the potential modulation of the inflammatory reaction induced by sub-lytic concentrations of polidocanol.

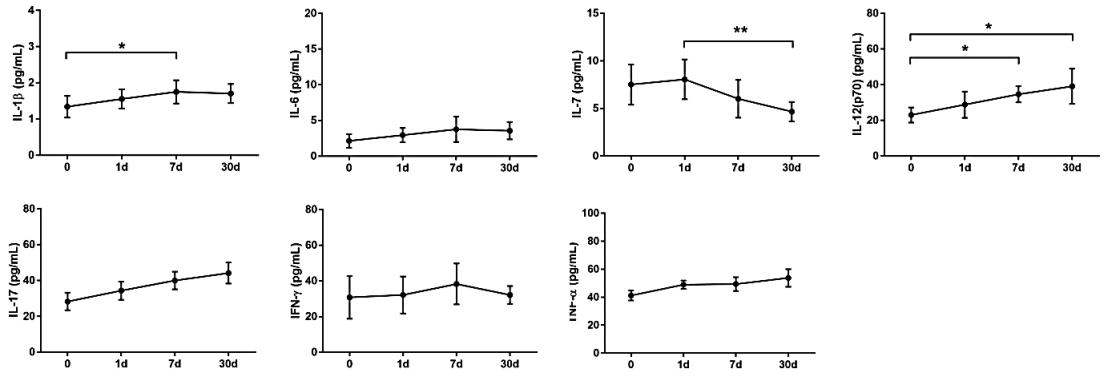


Fig. 4.4.4 Quantitative determination of pro-inflammatory mediators in patient serum samples. The expression profile of the pro-inflammatory cytokines IL-1β, -4, -7, -12, -17, IFN-γ and TNF-α in patient serum samples (n=11) at medium- and long-term experimental times; (* = $p < 0.05$; ** = $p < 0.01$)

The analysis focused on the main medium- and long-term expression profile of the considered mediators also highlighted a diffused increase of the anti-inflammatory cytokines (Fig. 4.4.5), although it flattened some evidences especially showed about IL-5 and IL-13 by considering all the experimental intervals (Fig. 4.4.2). Interestingly, IL-1ra represented the only anti-inflammatory cytokine up-regulated among each

experimental interval by resulting statistically significant at T7d ($p < 0.05$) (Fig. 4.4.5). This observation might be correlated with its antagonist role against IL-1β.

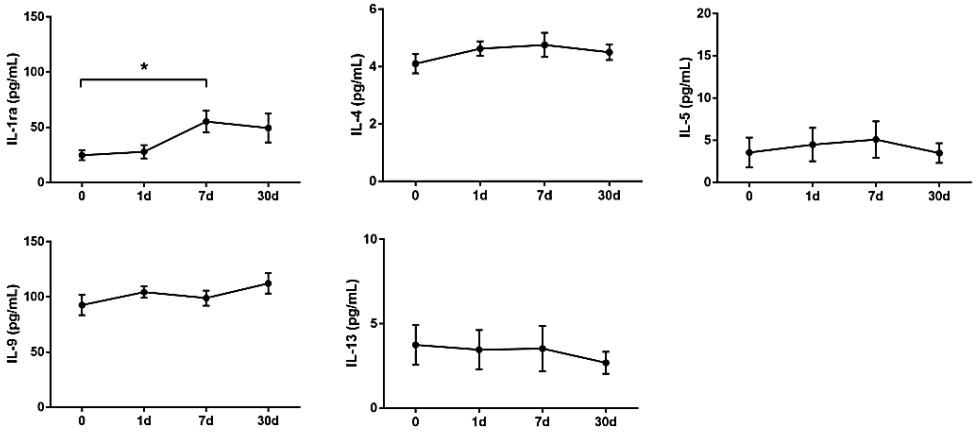


Fig. 4.4.5 Quantitative determination of anti-inflammatory mediators in patient serum samples. The expression profile of the anti-inflammatory cytokine IL-1ra, -4, -5, -9 and -13 in patient serum samples (n=11) at medium- and long-term experimental time; (* = $p < 0.05$)

The growth factor bFGF, PDGFbb, VEGF and the chemokine MIP-1β/CCL4 appeared gradually raising at long-term, although only VEGF and MIP-1β/CCL4 concentrations were statistically relevant at T7d compared to T0 ($p<0.01$; $p< 0.05$, respectively) (Fig. 4.4.6).

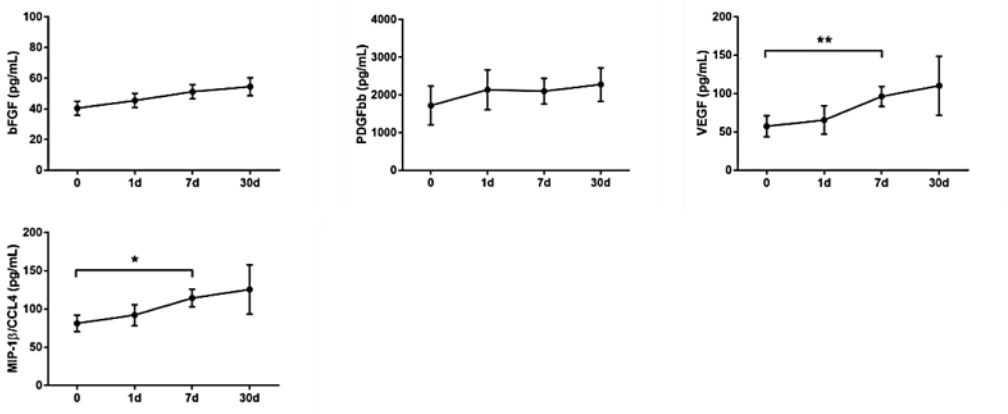


Fig. 4.4.6 Quantitative determination of chemokines and growth factors in patient serum samples. The expression profile of the chemokine MIP-1β and the growth factor bFGF, PDGF and VEGF in patient serum samples (n=11) at medium- and long-term experimental times; (* = $p < 0.05$; ** = $p<0.01$)

Other mediators as G-CSF and the chemokine Eotaxin/CCL11, IP-10/CXCL-10, RANTES/CCL5, IL-8/CXCL8 and MIP-1α/CCL3 continued to show a constant expression profile, excepted for the last two which were characterized by an increment at T30d as well as IP-10/CXCL-10 at T7d (Fig. 4.4.7; Fig. 4.4.3).

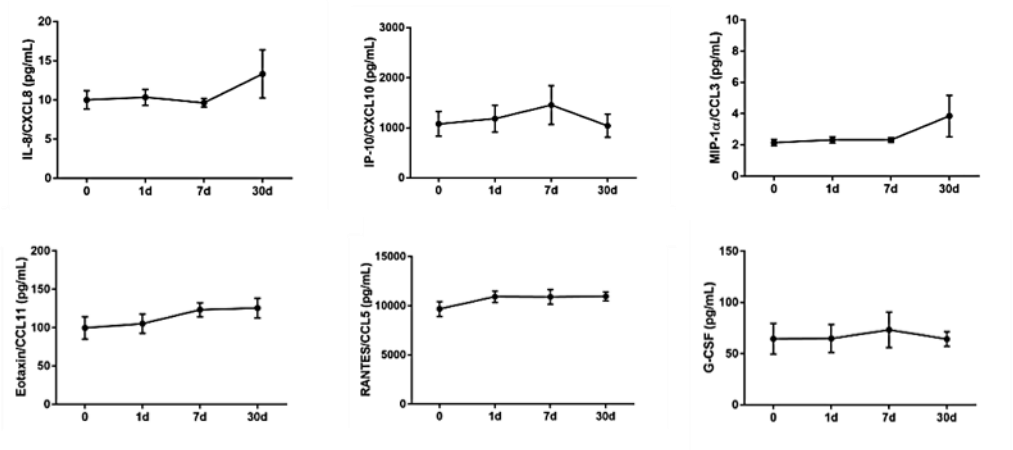


Fig. 4.4.7 Quantitative determination of cytokine and chemokines in patient serum samples. The expression profile of G-CSF and the chemokine IL-8, IP-10, MIP-1α, Eotaxin and RANTES in patient serum samples (n=11) at medium- and long-term experimental times

The IL-2, -10, -15, GM-CSF and MCP1/CCL2 expression data are not been showed due to their concentration lower than the instrument detection limit on the basis of the standard curve employed during the assay.

Additionally, zymography assays of the patient serum samples at the different experimental times are performed, though did not reveal significative differences in the modulation of the gelatinolytic profile of MMP-2 and MMP-9 after the treatment with sub-lytic doses of polidocanol (data not shown).

Chapter 5

Discussion

The whole sequence of anabolic and catabolic events that follow one another by fostering the CVeD progression from the early functional disorders (C1-C2) up to the CVI occurrence (C3-C6) represents the pathological framework variably modulated but characterized by some constant cellular and biochemical key points[17]. The venous wall intimate structure and its related mechanical dynamics, the blood hemodynamic and the involvement of specific lower limb anatomical districts are the driving forces vouching their functional balances essential to maintain the physiological blood return to the heart. Their reciprocal influence is the main reason of the harmful cascade of CVeD pathological events often triggered by the propagation of a single wrong process part of this complicated biological machine. In effects, the impairment in collagen deposition and in smooth muscle cell organization inducing the venous wall weakening, the consequent valvular incompetence accompanied by blood reflux occurrence and flow changes give rise to microcirculation disturbances and localized hypertension in the lower limbs. Overall, these events result in endothelial dysfunction and activation with a heavy increased venous wall permeability which allows leucocyte infiltration and the onset of a self-reinforcing inflammatory reaction. The chemoattractant role exercised by macromolecules, interstitial proteins and red blood cell degradation debris extravasated in the dermal interstitium is crucial for the leucocyte recruitment in this district[52]. The presence of white blood cells and their release of ROS and different

inflammatory mediators leads to skin changes and ulceration which represent the extreme CVeD manifestations[80,104].

Focusing the attention on the just hinted inflammatory state becomes interesting due to its crossroads function during the venous leg ulcer (VLU) progression in the healing process. In fact, the impairment of the closely modulated balances among the different biochemical mediators involved in the VLU inflammatory phase as well as in the progression to the next ones can determine the stop of the healing process in a persistent inflammatory state causing the VLU chronicity.

The absence of wound closure at least within six months represents a common factor between the inflammatory chronic venous leg ulcer (CVLU) characterized by the just mentioned condition and the granulating CVLU which, as its definition suggests, achieves the healing process without accomplishing it[112,110,117].

Bearing in mind these keys to the reading, the study results about the expression of TGF-β isoforms and their co-receptor sEng contribute to deepen their functions in the whole CVLU microenvironment, whose soluble mediators are differently modulated during the healing process. The TGF-β is member of a superfamily including its three isoform TGF-β1, -β2 and -β3, the bone morphogenetic proteins (BMP), the activin A, B and C, the inhibins and the anti-Müllerian hormone variously involved in development, homeostasis, disease and repair processes[126,128]. In this regard, the three TGF-β isoforms are secreted as latent precursors bound to the latent TGF-β-binding protein (LTBP) by fibroblasts, keratinocytes, platelets, macrophages and in low amount by bone cells and placental tissue[126,132]. The LTBP extracellular proteolytic cleavage makes them available to interact with their specific receptor complexes consisting in the heteromeric

organization of type I (five type I TGF-β receptors known) and type II TGF-β serine-threonine kinase receptors (seven type I TGF-β receptors known). However, the binding of TGF-β to the type II receptor occurs after its exposition by a type III receptor (betaglycan) and this event may determine either the mediator translocation on the flanked type I receptor becoming phosphorylated and inducing the downstream activation of Smad-signaling pathway or the inhibition of TGF-β functions through its interaction with transmembrane glycoprotein endoglin[132,133]. This represents a non-signaling homodimeric co-receptor of type II TGF-β receptors and is similarly characterized by cytosolic, transmembrane and extracellular domains among which this last is directly involved in the TGF-β binding. Furthermore, the RGD motif (Arg-Gly-Asp tripeptide) located in the zona pellucida of the extracellular domain is the structural feature which enables the auxiliary receptor to interact with the leucocyte $\alpha_v\beta_1$-integrin favoring the white blood cell transmigration[134,135,136]. The endoglin extracellular domain is amenable to a proteolytic shedding by different proteases, also including MMP-14, which allows to distinguish between a membrane-anchored long endoglin (L-endoglin) and a soluble short endoglin (S-endoglin) characterized by a cytoplasmic domain of only 14 residues[135]. The sEng seems to exercise anti-angiogenic effects contrary to the pro-angiogenic membrane isoform of the TGF-β co-receptor, which also contributes to the physiological vascular homeostasis through the eNOS structural stabilization[136–138]. According with the general sEng function to antagonize its membrane-anchored opposite, its increase detected in Gran VLU WF samples seems to be consistent with a potential inhibition of TGF-β1 by binding and sequestering it away from the receptor complex (Fig. 5.1). Additionally, this implication might be strictly correlated with both

the necessary TGF-β1 and -β2 down-regulation and the sEng property to interact with the leucocyte $\alpha_v\beta_1$-integrin adhesion molecules to limit the proliferative stimuli and the leucocyte endothelial trans-migration promoting the wound repair. In fact, these TGF-β isoforms are up-regulated and widely released in the early phases of the wound healing process, immediately after the clotting cascade activation, the platelet-induced vasoconstriction and the subsequent vasodilation with release of chemotactic factors involved in the leucocyte recruitment (hemostasis phase). Firstly, the neutrophils, which are active in the local bacteria killing and in the necrotic tissue proteolytic debridement, and then the macrophages, which sustain the phagocytic activity and secrete growth factors, chemokines and cytokines, pave the way to the next proliferative and reparative phases. These phases (hemostasis and inflammation) of the healing process effectively provide a storage of cytokines, as TNF-α, IL-1α, IL-1β, IL-6, the chemokine IL-8/CXCL8, and growth factors, as bFGF, VEGF, PDGF, EGF and TGF-β, ensuring the migration of leucocytes, fibroblasts, keratinocytes and endothelial cells on the provisional matrix and the wound resolution[128,139,140]. These events are also sustained by an intense expression of MMPs which are involved in the activation of some cytokines released in latent form (e.g TNF-α) and in the structural orchestration of the wound healing progression[113,141] (Fig. 5.1). However, a corresponding TIMP down-regulation is necessary to ensure these processes[142,99]. Although the study results have not shown significative differences in the TGF-β1 and -β2 expression between Infl and Gran WF samples, both the mediator isoforms take part in these overlapped healing process phases as well as in the next re-epithelialization. They stimulate fibroblast proliferation and a renewed matrix deposition (collagen II and III, fibronectin, vitronectin, tenascin and proteoglycans)

characterized by a balanced relation between collagen synthesis and fibroblast presence in order to provide a structural support to the next keratinocytes organization (granulation and re-epithelialization phase)[109,139,143]. These last similarly induced to proliferate by both mediator isoforms are able to migrate on the collagen and fibronectin fibers moving from the wound edges to the center through the proteolytic destruction of their intercellular desmosomes also involving MMPs, and the actin fiber synthesis[144,145]. This keratinocyte shuffling process regulated by the release of some inflammatory mediators, as the cytokine IL-1, culminates with the conjunction of the opposite wound edges accompanied by new adhesion molecules appearance and the ECM reorganization[139]. Furthermore, the TGF-β1 and -β2 are also involved in the vascularization and final remodeling of the new tissue. They stimulate the release of specific growth factors as VEGF, PDGF and bFGF which interact with their receptors on the endothelial cell membrane of pre-existent vessels by inducing the secretion of proteases destroying their basal membrane. The activated endothelial cells migrate through their membrane adhesion molecules to organize themselves in tubular structures in the wound progressively reinforced by smooth muscle cells and pericytes and interconnected with each other. However, the angiogenic process as well as the cellular proliferation progressively slow down and give way to the ECM strengthening through collagen III replacing with collagen I and the wound contraction through the myofibroblast activity[109,139]. These events further involve TGF-β1 and -β2[128,145].

The physiological sequence of these overlapped phases modulating each other through a time-dependent regulation of their specific cellular and biochemical microenvironment appears impaired in chronic venous ulcers in which prevails a cellular

hyperproliferative and a biochemical inflammatory profile[109]. In this regard, the Infl WF sample analysis allowed to observe the increased expression of TGF-β3 which might be correlated with the previously stated inflammatory CVLU features (Fig. 5.1). This TGF-β isoform is well-known for its ability to counteract the TGF-β1 effects for determining a fibroblast and keratinocyte proliferation slowdown accompanied by a senescent phenotype development and the new ECM deposition delay[109,128,132]. These effects are coherent with the VLU chronicity.

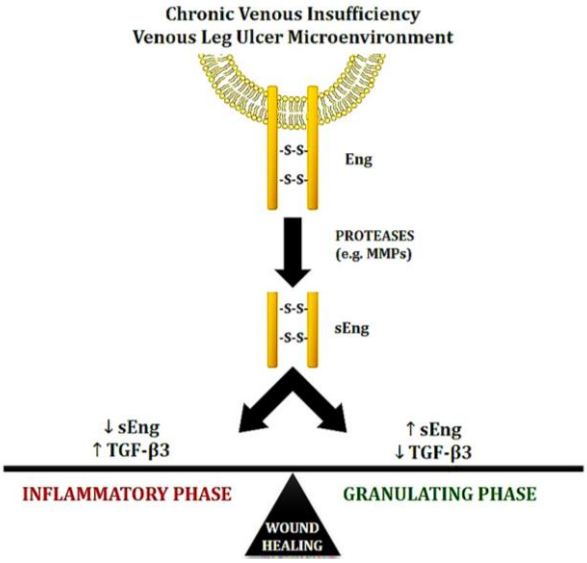


Fig. 5.1 Graphical summary of the evidences about the characterization of the C6 biochemical microenvironment[134]. The graph shows the different TGF-β3 isoform, sEng and MMP expression profile modulation during the inflammatory and granulating phase of the wound healing process

Additionally, the MMP-2 and -9 high expression by monocyte THP-1 cells stimulated with Infl WF might be considered as further factors contributing to this pathological condition. The statistically significant increase of MMP-9 seems to be in line with the TGF-β3 up-regulation due to the mediator positive effect on the gelatinase expression[146,147]. This TGF-β isoform promotes the ECM proteolytic degradation and the

chronic inflammatory state through the release of TNF-α, IL-1, IL-6 and the chemokine IL-8/CXCL8 and MCP-1/CCL2, which may be activated by TNF-α and regulate the neutrophil and macrophage recruitment, besides their involvement in the angiogenic processes[139,148]. However, the reduced levels of the MMP-9 observed after the THP-1 monocyte cells co-treatment with the Infl WF and SDX counterbalance these evidences. The glycosaminoglycan mixture is known to have anti-inflammatory properties which justify the MMP-9 reduced release mainly by platelets and leucocytes widely involved in the early phases of the wound healing process as well as in the iterative CVLU inflammatory state[130,131,146]. This result is consolidated by the gelatinolytic profile determined through the zymographic assay which shows the decreased activity of MMP-9 in supernatants from THP-1 monocyte cells treated with both Infl and Gran WF in presence of SDX. Although MMP-2 is strongly involved in the ECM collagen component degradation during the healing process, however it is known to be mainly released by fibroblasts, which have a prominent role in the matrix reorganization during more advanced phases than the early ones[146,110,106]. This might explain its similarly expression in THP-1 cells stimulated with Infl and Gran WF. In addition, the afore-mentioned SDX anti-inflammatory properties in association with Gran WF potentially causes a negative modulation of some mediators, also including TGF-β1, able to promote MMP-2 expression, as highlighted by the study results[131,149]. This last evidence might be also correlated with the sEng increased expression in THP-1 monocyte cells supernatants in presence of SDX and Gran WF. The glycosaminoglycan mixture may enhance the TGF-β co-receptor shedding by stimulating the proteolytic environment and influencing the TGF signaling[134]. This is also sustained by the SDX role of inducing

the expression of mediators, as the growth factor bFGF, which is able to interact with endothelial receptors and induce a cellular activation resulting in protease secretion[131,139,150]. Overall, these evidences highlight some of the potential imbalances of mediators which might contribute to the impaired wound healing process.

According with the declared aim of this work to deepen the biochemical players involved in the pathophysiologic progression of CVeD, the results related to the modulation of MMP expression in $C4_a/C4_b$ patients allow to add a further piece to the investigated pathological framework. The pronounced fibrosis associated with an irregular fibrinolytic activity and a heavy hypoxic environment characterizing the lipodermatosclerosis (C4) show an effective correlation with the leucocyte-induced inflammatory reactions and MMP involvement[110,151]. However, the hemodynamic impairments and the diffused hypertension may be considered as the common denominator of these events which contribute to the pathologic state occurrence. As the denomination lipodermatosclerosis suggests, the typical features of this condition consist in broad changes in subcutaneous adipocytes and reticular dermis ECM organization. In fact, the leucocyte infiltration triggering intense inflammatory reactions through the release of various mediators induces adipocyte necrosis in the fat lobules localized in this dermis district followed by vacuole appearance and consistent lipomembranous alterations[152]. These events are exacerbated by the influence of pro-fibrinolytic factors which degrade the fibrinogen extravasated in the dermal interstitium afterwards the generalized increasing of venous wall permeability to promote the anomalous deposition of a perivascular fibrin cuff[52,152]. The limited oxygen availability and the ROS release by leucocytes chemoattracted in this dermis district result in the

hypoxic environment which promotes microthrombotic and necrotic events characterizing this pathological condition. Furthermore, the dermal interstitium revealed the presence of abundant hemosiderin accumulation strictly correlated with the erythrocyte diapedesis followed by an intense red blood cell degradation and release of Fe^{3+} ions[153]. Different studies speculated on the primary involvement of the hemosiderin in the C4 skin pigmentation occurrence. However, its role seems to be only marginal compared to the hypermelanisation process determining a dysregulated melanin accumulation in the dermal keratinocytes. The alteration of melanogenesis occurring in melanocytes may be induced by the plentiful release of inflammatory cytokines and growth factors exactly sustained by the hemosiderin[154,155,156]. This evidence is also consistent with the enhanced MMPs expression in the subcutaneous district which is liable to fibrotic changes due to the abnormal deposition of collagen I and III and ECM components counterbalanced by the lack of elastic fibers[127,152]. Moreover, the edematous state may contribute to the MMP proteolytic activity by providing them a mechanical stimulus which worsens the already considerable tension associated with the dermal structural impairments. In this regard the high concentration of the collagenase MMP-13 and the gelatinase MMP-2 in plasma samples of C4 patients in stationary standing position is consistent with their heavy involvement in the pathological condition (Fig. 5.2). In particular, the first is often associated with the proteolytic activation of inflammatory cytokines released in latent form, as the TNF-α, besides with the degradation of ECM component mainly consisting in collagen I, II, III and IV[111]. Most of these are abundantly deposited in the venous wall by the proliferating fibroblasts stimulated by the presence of inflammatory mediators also including TNF-α

and TGF-β. The same substrates are fragmentated by the gelatinase which appears also active in the cleavage of some endothelial intercellular junction components, as the occludine[111]. All these evidences highlight the MMP-2 role both in the vascular permeability increase and in the skin weakening by contributing to the clinical signs of lipodermatosclerosis[89,106]. Furthermore, the hypoxic stress, ROS and ferric ion release specifically characterizing the sclerotic dermis represent known biochemical mechanisms influencing the gelatinase expression profile[127,154,157]. The same effect may be also reported about the MMP-9 which results similarly modulated in the C4 plasma samples related to the standing position. The pronounced inflammatory environment and the ECM collagen abundance represent the favourable conditions which might explain this evidence. Furthermore, the MMP-9 cooperates with the other gelatinase in promoting the vascular permeability and the cellular transmigration through the depletion of the intercellular adhesion systems[89,111,127]. These functions are also ascribable to both the matrilysin MMP-7 and the macrophage metalloelastase MMP-12. The first, normally involved in the tissue remodeling processes, may contribute to the widespread ECM proteoglycan and fibrous component degradation coherent with the structural impairment of the C4 stage. However, this matrilysin might also promote the proteolytic release of syndecan-1 and E-cadherin influencing cellular interaction[111,158]. This function allows to consider its increased expression correlated with the similar effects induced by some of the just mentioned MMPs. In this regard, the MMP-12 role in ECM elastin cleavage and in modulation of macrophage transmigration might justify the results revealed. The collagenase MMP-8 and the stromelysin MMP-3 and MMP-10, responsible of the activation of the MMP-1, -7, -8, -9, are similarly encompassed in the

speculated functional associations between the results showed about the other MMPs and their potential involvement in the C4 sequence of pathological events. These results might be effectively consistent with the occurrence of the typical C4 clinical signs in lower extremities which show the characteristic aspect of an inverted champagne bottle. In fact, the affected limb initially exhibits an intense reddish aspect accompanied by diffused pain and high sensitiveness which evolves in a real fibrotic induration of the skin appearing hyperpigmentated due to the melanin accumulation in the keratinocytes and very stretched. This clinical evolution strictly mirroring the biochemical and structural dysregulation is consistent with the transition from a lipodermatosclerosis acute to chronic phase[152,78]. Furthermore, the different manifestation of the cutaneous alterations identifying the C4 stage justifies the distinction between the pigmentation ($C4_a$) and the more deep tissue damages as dermal sclerosis and skin atrophy ($C4_b$)[17,155]. As previously mentioned these aspects are accompanied by the persistent occurrence of the interstitial edema induced by the hypertension. The physiological hemodynamic force restoration determining the correct modulation of the blood flow and the different pressure gradients are some of the effects induced by the compression therapy[28,159]. This clinical strategy is effectively recommended in lipodermatosclerosis edema treatment, although the existence of numerous therapeutic alternatives including devices aimed to produce specific leg movements and pumping systems applying a defined compressive force[159]. The edema prevention or resorption appears more effective during the application of low-stretch compression wraps (30-40 mmHg) than the graduated compression stockings in the treatment of acute lipodermatosclerosis. This evidence is consistent with a prolonged mechanical effect induced on the

subcutaneous tissue resulting in the modulation of the interstitial fluid filtration[152,159]. However, the intense pain characterizing the pathological condition is often incompatible with a lasting and strong compression by explaining the alternative garment (class I and II) use[160,29].

The decrease of the subcutaneous pressure following the venous dimension reduction, the restoration of the regulated venous wall permeability and the blood hemodynamic induced by the compression treatment might be also associated to the modulation of the biochemical environment of lipodermatosclerosis. Although this observation is statistically confirmed only by the MMP-2 and MMP-13 expression results, the decreased levels of the other MMPs observed in plasma samples of C4 patients in supine lying down position suggest that the hemodynamic fluctuations are a preponderant influencing factor (Fig. 5.2). The same consideration might be also extended to the results about the volunteer plasma samples, though they show only MMP-2, -3 and -7 expression modulation after the compression treatment and the lying down position correlated to the standing position. Additionally, this last evidence allows to confirm that the MMPs expressed under physiological conditions are strongly sensitive to the pressure gradient alterations (Fig. 5.2).

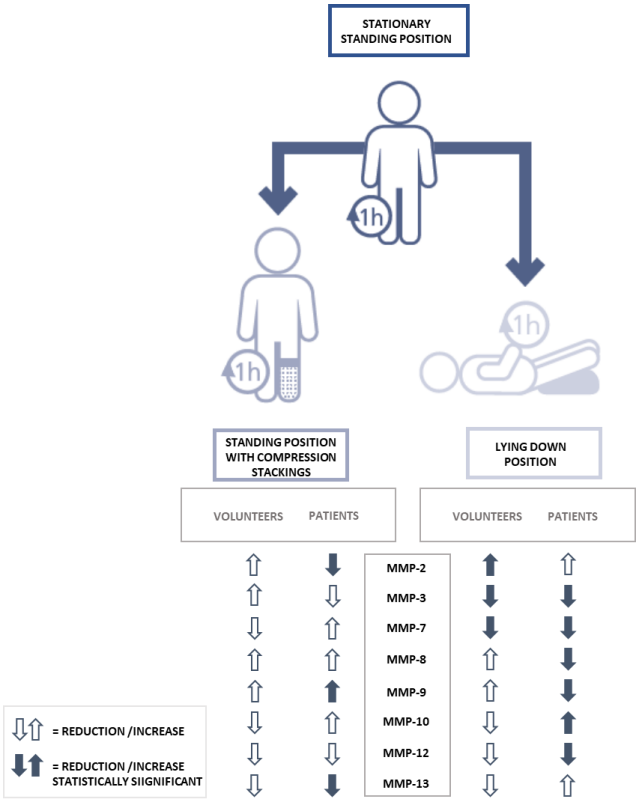


Fig. 5.2 Graphical summary of the evidences about the characterization of the $C4_a/C4_b$ biochemical microenvironment[129]**.** The graph shows the MMP expression profile modulation by the three different gravitational conditions

As afore mentioned, the MMPs, which are physiologically involved in tissue remodeling and repair, may head up different inflammatory events by degrading the ECM components and generating cytokine, chemokine and growth factor gradients associated with the CVeD manifestations[111,89]. Although the ECM is often considered as a passive structural scaffold for the vascular tissues and cells, its composition makes it a real operations room for cellular migration, differentiation and signaling pathways triggered by MMPs during the pathophysiologic evolution of CVeD. In effects, collagen I, II, III, IV, V, VI, VII, VIII, IX, X, and XIV as well as laminin, fibronectin, vitronectin, entactin, tenascin, aggrecan and elastin are well-known MMP substrates[86,161]. Considering their wide distribution in the vein walls, the concomitant MMP proteolytic

activity may be strictly influenced by systemic factors as pressure and hemodynamic fluctuations with their mechanical implications and inflammatory states often associated with oxidative stress and hypoxia[89]. However, the speculation about the potential effects induced by some clinical treatments on the MMP expression and activity might be useful to better understand the biochemical dynamics characterizing the CVeD microenvironment. This observation certainly refers to the results showed about the modulation of the MMP-2 and -9 gelatinolytic profile by different POL and STS detergent concentrations.

The clinical use of these sclerosant agents recommended for the treatment of intradermal, subcutaneous and transfascial reticular veins or telangiectasies (C1) and varicose veins (C2) is aimed to destroy the vessel endothelial lining to determine the ECM fibrillar component exposition and the smooth muscle cells contraction[17,39]. The resultant vasospasm promotes the treated vessel isolation from the lower extremities blood circulation by reducing it in a fibrous cord. Although side effects with different degree of medical significance are evidenced, including tissue necrosis, stroke, transient ischemic attack (TIA), deep venous thrombosis (DVT), pulmonary embolism, headache and migraine, chest tightness, matting, pigmentation, the sclerotherapy is a moderately invasive and an alternative strategy to the surgery able to ensure the restoration of the venous functional integrity and the prevention of the CVeD progression with a remarkable impact on the patient quality of life[39]. Nevertheless, the sclerotherapy treatment is often followed by wearing suitable graduated compression stockings of class I (20-30mmHg) for a variable period from one week to one month[39,38,162]. This allows to avoid haematoma occurrence, pain and adverse outcomes, besides to provide

a better sclerosant displacement long the vessel and a prolonged contact with the endothelial lining[38,].

Most of the two detergent effects depend on their chemical nature and their physical form at the moment of the intravascular injection. According with the European guideline, liquid sclerotherapy is strongly recommended as C1 reticular varicose vein treatment, whereas the foam sclerotherapy represents its potential alternative mainly indicated for the C2 varicose veins[39]. The diameter and the general dimensions of the treated vessel are the structural features determining this distinction and the POL and STS different concentrations which are included in 0.1-1% and 1-3% ranges for the C1 and C2 treatment by POL and in 0.05-1% and 1-3% ranges for the same treatments by STS[39,46]. On the basis of these technical observations the foam sclerotherapy is suitable in the treatment of medium and large vessels to effectively displace the blood content and favor the direct interaction of the surfactants with the vascular endothelial cells[40,41].

Several strategies have been described to obtain foams different both in air-liquid ratio and in sclerosant concentrations, although one of the most employed is the so-called Tessari's method nowadays. This consists in one part of the STS sclerosant dispersed in four parts of room air through a dual syringe system characterized by a three-way stopcock[162]. The resultant foam is defined wet due to its lower air content (1 + 4) compared to the opposite composition of the dry foams (1 + 8). It shows bubbles with variable dimensions (foam coarseness) and stability based on the sclerosant concentration and the dilution solvent nature[43,47].

Furthermore, POL and STS are defined surfactants due to their ability to counteract the surface tension characterizing the structural organization of various substances and

their interactions with the surrounding microenvironment. Interestingly, the non-ionic and anionic nature of polidocanol and sodium tetradecyl sulphate, respectively determined by the absence of charges and the presence of a negative charge on their hydrophilic head, allow them to differently exercise their surfactant role[46]. This is additionally influenced by their organization in micellar structures when are dispersed in an aqueous or non-aqueous solution correspondent to their critical micellar concentration (CMC) above which detergent micelles and monomers coexist in a differently regulated balance. Reaching the CMC is the essential step for the POL and STS membrane solubilization activity[46]. The first sclerosant, consisting in an alcohol ethoxylate and commonly known as anesthetic agent, is characterized by a lower CMC (0.002% both in saline and in water) than the CMC of the sodium tetradecyl sulphate (0.075% in saline; 0.2% in water) often employed to enhance the antiseptic properties of some pharmaceutical preparations[46,47].

On the basis of this observation, the polidocanol is able to solubilize more efficiently the cellular membrane components giving rise to toroidal structures without inducing any protein structural modifications compared to the STS. In fact, its negative charge and small dimensions make its membrane crossing hard and delayed with protein denaturation and phospholipid insertion in its micelles[46].

According with these literature evidences, the zymography assay of STS-treated normal plasma samples, showing the appearance of a gelatinolytic band (160 kDa) between 0.3% and 0.6% STS concentrations in normal plasma samples, might be consistent with a detergent-enzyme interaction to generate a high molecular weight product with gelatinolytic activity. The band might be considered as the result of the just mentioned

STS denaturation properties on the plasma protein content. The same speculation might be advanced about the protein band (100 kDa) revealed by the SDS-PAGE assay and corresponding to the STS concentrations from 0.4% up to 1.5% in normal plasma samples. In particular, the gel analysis allows to pinpoint that the band gradually increases between 0.4% and 0.6% and then begins to decrease up to 1.5%. This might reflect the reaching of a potential equilibrium-zone between the STS molecules and the proteins present in plasma samples only in that concentration range (0.4% - 0.6%) by promoting the association in a transient complex. However, no significant evidences appear after the plasma sample treatment with different POL concentrations in the same experimental conditions. It might be supposed that the plasma components also including the albumin are responsible for a consumption effect on the sclerosant by sequester it[40,41].

Noteworthy, different target cells are exposed to the sclerosant agents after their injection in the vessel. In this regard, several literature evidences exist about the POL and STS pro-coagulant properties at low concentrations mainly due to their interaction with the membrane of platelets by inducing their cytosolic Ca^{2+} increase from the intracellular store and exposition of the phospholipid phosphatidylserine on cellular surface inducing platelet activation and fragmentation in platelet-derived microparticles (PMP) provided of pro-coagulant functions[41,46,48]. This sequence of events is commonly observed both at long distance from the injection point and after the early phases of the treatment by justifying its association with the sclerosant low concentrations[50,48]. An opposite effect is accompanied by the denaturation of some clotting factors and the destruction of PMP induced by STS besides a generalized cellular lysis affecting platelets,

red blood cells, leucocytes and endothelial cells at the higher detergent concentrations[41,49]. The results obtained from the HECV endothelial cell treatment with POL and STS high doses (1.5%) confirm these lytic effects respectively consisting in a rapid cellular swelling with consequent cytoplasmic degranulation and membrane fragmentation with cluster formation. These evidences might be coherent with the afore mentioned sclerosant chemical properties which induce the potential membrane protein denaturation and phospholipid disorganization resulting in cellular destruction after the STS addiction. The same observation might be useful to explain the effects caused by the POL high concentrations potentially associated with the sclerosant ability to cooperatively interact with the membrane component culminating in a cellular volume increase. Additionally, the cytoplasmic condensation and the cellular emptying observed respectively after the treatment with the lowest POL and STS concentrations (0.3% and 0.075%) continue to be in line with the just stated evidences, though these effects seem to be quite delayed by eventual interactions occurring between detergents and plasma components[40,41,163].

Moreover, as fully discussed on several occasions, the vicious circle of hemodynamic, structural and functional events traced in the progression among the different CVeD manifestations is strictly associated with the leucocyte recruitment and the concurrent inflammatory microenvironment. This is triggered by the general impairment of blood pressure gradient in lower limbs resulting in a low and irregular shear stress able to affect the endothelial cell homeostasis as well as the leucocyte inflammatory responses. The increased expression of leucocyte integrin CD11b/CD18 and L-selectin as well as the endothelial adhesion molecule ICAM-1, VCAM-1 and E-selectin accompanied by the

glycocalyx depletion are key events to promote the white blood cell infiltration in venous walls[119,164]. The concomitant eNOS activity decrease and iNOS induction worsen this event, besides contributing to the venous wall relaxation/contraction imbalance and the smooth muscle cell switch from contractile to proliferative phenotype[165,166]. Also, the fibroblast stimulation and the irregular ECM remodeling by the up-regulated MMP proteolytic activity are responsible for the increased collagen I/collagen III ratio and elastin depletion inducing venous wall weakening[165,167]. The resultant appearance of both vascular atrophic and hypertrophic tortuous regions accompanied by an abnormal dilation represent the structural distinctive signs of varicose veins[79,165,13]. These morphological features sustained by the venous hypertension resultant from hemodynamic alterations and valvular incompetence with their cellular and biochemical implications appear from the early CVeD manifestation consisting in reticular veins or telangiectasies (C1). This last aspect supports the intention to evaluate the inflammatory microenvironment characterizing this CVeD stage and its potential modulation by the recommended sclerotherapy treatment[39]. In particular, the results achieved concern the use of the sclerosant polidocanol at sub-lytic doses compared to those indicated by the European guideline in treatment of reticular veins (0.25-1%)[39]. Literature evidences associate these detergent concentrations with procoagulant effects through the platelet activation. In fact, the POL monomers are able to interact with the platelet membrane without compromising its structure but promoting the cellular activation through the phosphatidylserine exposition and the subsequent release of PMP[48,168,169]. This might be consistent with the increased expression of the pro-inflammatory IL-6 and IL-1β characterized by procoagulant properties and strictly correlated with the platelet

reactivity[122,170]. In the same way the chemokine IL-8 which shows similar function and appears increased both at short- and long-term[170]. These evidences might be associated with the time- and distance-dependent pro-coagulant function of the low doses of detergent, besides with a potential inflammatory reaction induced by the release of platelet-derived microparticles[48,171,172].

Furthermore, the POL sub-lytic doses seems to be also responsible for the leucocyte oncosis consisting in a cellular death mechanism correlated with the membrane ionic transport impairment culminating in cellular destruction and release of the inflammatory mediators[41,173,49]. This might be consistent with the enhancement of the variegate inflammatory environment revealed.

Although, the evaluation of the inflammatory profile among all experimental intervals is only related to three C1/C2 patients, however this allows to observe its potential modulation at short-time. Interestingly, the pro-inflammatory IL-1β and the chemokine IL-8 result increased immediately after the sclerosant injection. This seems to be correlated with the involvement of these mediators in the early inflammatory response associated with the neutrophil recruitment[174,123]. Additionally, they are also involved in the up-regulation of some MMPs, as MMP-1, -3, -7 and -9, in the vascular smooth muscle cells and fibroblasts to promote the ECM remodeling[79,122,175].

The pro-inflammatory cytokine IL-6 shows a spike at one week from the first treatment appearing coherent with its release during an initial acute inflammation, besides the stimulation of other inflammatory factors, as IFN-γ, similarly involved in the neutrophil activation, and VEGF with angiogenic and ECM remodeling properties [122,174,176]. Also, the increase of IL-17 and TNF-α observed in the early experimental intervals might concern

the release of IL-6, IL-8/CXCL8, G-CSF, besides the up-regulation of MMPs and some cellular adhesion molecule, as ICAM-1[122,175,126]. The progressive decrease of the cytokine IL-7 among the different experimental intervals from T30'/45' might be correlated with its role in lymphocyte homeostasis and maturation and in modulation of the innate immunological response[177–179]. The release of this cytokine often associated with the vascular endothelial tissue and involved in various inflammatory skin, cardiovascular and autoimmune diseases appears strongly influenced by the POL treatment allowing to speculate a potential immune-modulator role of the sclerosant detergent[177,179,180].

Although these mediators show an overall up-ward trend immediately after the treatment, however responses statistically significative are observed from T7d. In this regard, the pro-inflammatory cytokine IL-12 reveals an evident up-regulation at long-term consistent with its role to stimulate both the immune response induced by IFN-γ responsible for macrophage activation[176,181]. It contributes to enhance the inflammatory reaction similarly to IL-6[172]. Interestingly, the anti-inflammatory cytokine IL-1ra exerting a protective role towards the vascular tissue increases in line with its opposite pro-inflammatory IL-1β[182]. The rising levels of the chemokines IL-8/CXCL8, IP-10/CXCL10, MIP-1β/CCL4 and RANTES/CCL5 involved in the monocyte/macrophage recruitment as well as in the stimulation of fibrotic processes and angiogenesis might be consistent with the hypothesis of a potential delayed inflammatory response to the treatment[172,175]. The growth factor PDGFbb, bFGF and VEGF result increased and strictly correlated with the smooth muscle cell and fibroblast proliferation besides with the angiogenesis and ECM remodeling by the MMP modulation[122,176,183,184].

Overall, these evidences seem to reveal the absence of a consistent endothelial damage induced by the sclerosant polidocanol, which triggers a delayed inflammatory reaction also correlated with its procoagulant properties at low concentration.

The study represents a preliminary attempt to characterize the biochemical microenvironment of the CVeD and its modulation by tracing its progression from the last to the initial manifestations. In this regard the future perspective lies in outdoing the weak point consisting in the limited patient cohort by expanding it in order to reproduce the evidences obtained and correlate them with potential clinical implications.

Chapter 6

Conclusions

The main aim of this three-year research project to investigate the biochemical microenvironment modulation of CVeD allowed to correlate the different mediators examined with their potential roles in the disorder progression along its pathological manifestations.

In particular, the statistically significant increased expression of the TGF-β3 in inflammatory wound fluid is representative of its well-known role to counteract the TGF-β1 effects enhancing the release of inflammatory mediators and MMPs, including the gelatinase MMP-9, by contributing to the healing process impairment and the ulcer chronicity. Also, the raised concentration of the sEng in granulating wound fluid confirms its anti-inflammatory functions by interfering with its membrane-bound form and TGF-β signaling to promote the wound resolution. The TGF-β co-receptor seems to be sustained in this role by the SDX anti-inflammatory effects, which modulate the proteolytic environment, as shown by the *in vitro* qualitative and quantitative results. All these evidences lead to conclude that the different expression of the TGF-β isoforms, its accessory receptor endoglin and gelatinases are probably a faithful mirror of their distinct roles in the healing process. Furthermore, the quantitative determination of the MMP profile during the gravitational study, also including the wearing of below-knee compression stockings (class II), confirms the mediator susceptibility to the hemodynamic fluctuations, which result more evident in lying down position compared to the stationary standing position both in volunteer and in patients. This evidence might

be representative of the MMP modulation induced by the venous hypertension characterizing the pathological condition, besides by the physiological blood dynamics, as demonstrated by the volunteer group.

Interestingly, the *in vitro* assays of normal plasma samples treated with increasing concentrations of polidocanol and sodium tetradecyl sulphate highlighted differences both in the MMP proteolytic profile and in the plasma protein expression consistent with interactions based on the different chemical nature of the sclerosant agents. This last aspect is also implicated in the morphological changes induced in HECV endothelial cells and associated with increased cellular permeability probably accompanied by cellular activation and different cellular death mechanisms. The final step of this observational investigation leads to observe the absence of an early and acute inflammatory response to the sub-lytic concentration of the polidocanol. This evidence is in accordance with the time- and concentration-dependence of the pro-coagulant, pro-angiogenic and pro-inflammatory effects of these detergent doses able to induce only mild endothelial damages and a delayed inflammatory reaction. This might be also associated with the detergent interaction with circulating cells, including platelets and leucocytes, by stimulating them to secrete inflammatory mediators.

This study produced evidences useful to set all the investigated mediators in the biochemical framework characterizing the CVeD progression, in which take place mutual interactions responsible for the impairment of physiological balances essential for the venous homeostasis. In this regard, it might be favourable to deepen the mechanisms which are triggered by both the involved mediators and some of the recommended

clinical treatments to prevent irreversible impairments and select the most suitable approach.

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