Ph.D Program

Translational and Molecular Medicine

Characterization of soluble biomarkers and regulatory B cell subset useful to improve systemic scleroderma clinical diagnosis and management

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INTRODUCTION

SYSTEMIC SCLERODERMA

The Scleroderma disorders comprise a heterogeneous group of autoimmune conditions linked by the presence of sclerotic, thickened skin lesions. The classification of scleroderma includes different subtypes, ranging from localized to systemic forms of the disease, which are more rapidly advancing types accompanied by a frequent visceral involvement. Systemic scleroderma or systemic sclerosis (SSC) is a multisystem disease characterized by an unpredictable course, high mortality and resistance to therapy[1] . SSC is a rare disease observed in all ethnic groups throughout the world, affecting females more frequently than males with a ratio of 4-5:1 and with a peak of incidence between ages 30 and 50. It is rare in children with an incidence rate per million children per year of 0.27[2]. The principle pathognomonic disease manifestation is the thickening of the skin, that can vary from normal skin (called systemic sclerosis sine scleroderma) to mild involvement with puffy fingers, to a more severe and diffuse cutaneous involvement. Other sclerotic features may include thickening of the skin of the fingers, that become spindle-shaped (sclerodactyly) from resorption of the fingertips, flexion contractures, tendon friction rubs, pulmonary and gastrointestinal fibrosis[3] (figure 1).

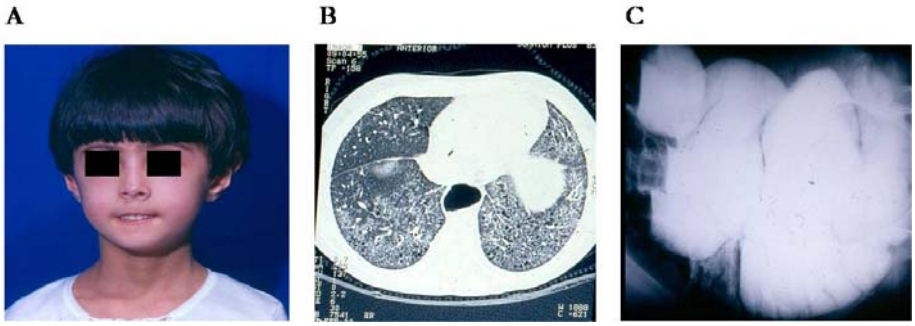

Figure 1. A) Amimic face due to the diffuse cutaneous thickening **B)** bilateral basilar pulmonary fibrosis **C)** fibrotic involvement of the gastrointestinal tube.

1.1 CLASSIFICATION OF SYSTEMIC SCLERODERMA

Due to the lack of a single diagnostic test, classification criteria are crucial to discriminate systemic sclerosis from other similar conditions and to identify subgroups of patients for inclusion into research studies. Preliminary Criteria for the Classification of Systemic Sclerosis were published in 1980. According to this classification, the presence of either a major criterion (proximal scleroderma defined as thickening, induration proximal to the metacarpophalangeal or metatarsophalangeal joints) or two or more minor criteria: (1) sclerodactyly, (2) digital pitting scars of the fingertips or loss of the substance of the distal pad, and (3) bilateral basilar pulmonary fibrosis, were enough to diagnose a condition of systemic sclerosis. This classification was widely adopted but, over the time, in order to ameliorate the diagnosis of early and mild systemic sclerosis, alternative classification systems including vascular and serologic features were proposed (Table 1).

Reference	SSC classification criteria
ARA criteria 1980 [6]	1 major criterion: proximal scleroderma defined as tightening, thickening, and non-pitting induration proximal to the metacarpophalangeal or metatarsophalangeal joints or 2 or more minor criteria: (1) sclerodactyly, (2) digital pitting scars of the fingertips or loss of the substance of the distal pad, (3) bilateral basilar pulmonary fibrosis
Nadashkevich et al. 2004 [7,8]	Any 3 of ABCDCREST: (1) autoantibodies to: centromere proteins, ScL-70 (topoisomerase-1, fibrillin), (2) bibasilar pulmonary fibrosis, (3) contractures of the digital joints or prayer sign, (4) dermal thickening proximal to the wrists, (5) calcinosis cutis, (6) Raynaud's phenomenon, (7) esophageal distal hypomotility or reflux esophagitis, (8) sclerodactyly or non-pitting digital edema, (9) telangiectasia
LeRoy and Medsger 2001 [9]	Limited SSC (lSSC) (1) Raynaud's phenomenon and (2) abnormal wide-field nailfold capillaroscopy or (3) SSC selective autoantibodies. Limited cutaneous (lcSSC): criteria for lSSC and cutaneous changes distal to the elbow, knees, and clavicles. Diffuse cutaneous (dcSSC): criteria for lSSC and cutaneous involvement of the arms, chest, abdomen, back, or thighs
Avouac et al. 2011, VEDOSS criteria [10]	(1) Raynaud's phenomenon, (2) puffy fingers, (3) antinuclear antibodies, and (4) capillaroscopy or (5) SSc-specific antibodies

Table 1. Adopted from Sindhu R. *et al. Curr Rheumatol Rep* 2015; 17(5):506 (VEDOSS Very Early Diagnosis of Systemic Sclerosis)

In particular in 2001 Le Roy and Medsger introduced for the first time the differentiation between limited and diffuse SSC: patients with acral skin involvement are classified as limited SSC (lSSC), whereas patients with thickening of the skin of the torso and acral regions are defined diffuse SSC (dSSC)[9]. Finally, the American College of Rheumatology(ACR) and the European League Against Rheumatism (EULAR) presented in 2013 a new classification system including previous criteria (proximal scleroderma, sclerodactyly, digital pits, pulmonary fibrosis, Raynaud's phenomenon, and scleroderma specific autoantibodies) but underlying the importance of the typical vasculopathic manifestations of the disease[11] (Table 2).

Items	Sub-items	Weight
SUFFICIENT CRITERION		9
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints		
Skin thickening of the fingers *(only count the highest score)*	Puffy fingers Whole Finger, distal to MCP	2 4
Finger tip lesions *(only count the highest score)*	Digital Tip Ulcers Pitting Scars	2 3
Telangiectasia		2
Abnormal nailfold capillaries		2
Pulmonary arterial hypertension and/or Interstitial lung Disease		2
Raynaud's phenomenon		3
Scleroderma related antibodies	(any of anti-centromere, anti-topoisomerasel [anti-ScL 70], anti-RNA polymerase III)	3
TOTAL SCORE:		
Patients having a total score of 9 or more are classified as having definite systemic sclerosis.		

Table 2. Adopted from Sindhu R. *Current Rheumatology Reports* April 2015, 17:32

1.2 PHYSIOPATHOLOGY OF SISTEMIC SCLERODERMA

The pathogenesis of SSC is still partially unknown, but a genetic predisposition concomitant with environmental stimuli results in immune activation, vascular injury and excessive collagen production and accumulation (Figure 2). Environmental factors may play a role in triggering the disease. These may include viruses (such as cytomegalovirus), chemicals (such as vinyl chloride, some pesticides, benzene derivatives and silica), and drugs (such as cocaine, appetite suppressants, penicillamine and vitamin K).

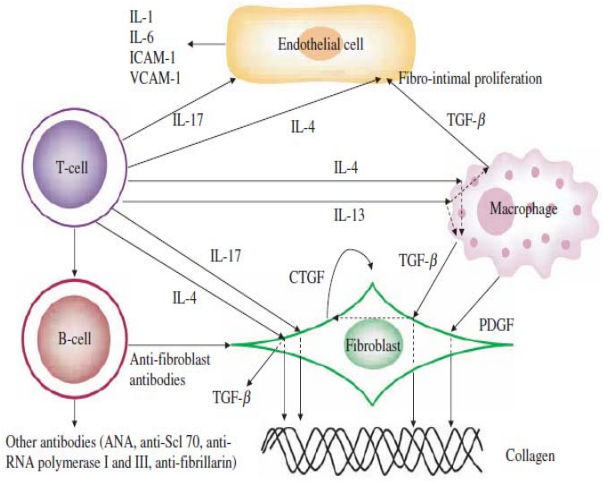

Figure 2. Pathogenesis of systemic scleroderma.

1.2.1 THE ROLE OF IMMUNOLOGICAL MEDIATORS IN SSC

The immune system plays a central role in the physiopathology of SSC. Highly specific autoantibodies and activated B lymphocytes are detectable in SSC patients.

1.2.1.1 Role of autoantibodies

Autoantibodies directed against several nuclear, cytoplasmic and extracellular autoantigens represent a serological hallmark of systemic sclerosis. Some of these autoantibodies are associated with specific disease subtypes and with distinct clinical features, while others have no apparent role in the pathogenesis[12] . The autoantibodies with a direct pathogenic function in scleroderma are anti-endothelial cell, antifibroblast, anti–matrix metalloproteinase (MMP) and antifibrillin antibodies. Anti-endothelial cell antibodies (AECA) have been identified in SSC patients as important players in the progression of the endothelial damage; their presence and high titer is considered as an adjunctive risk factor for a more severe disease course[13] . The exposure of endothelial cells to anti-endothelial cell antibodies (AECA) results in up-regulation of the adhesion molecules ICAM-1, VCAM-1 and E-selectin, leading to enhanced leukocyte adhesion[14,15] while anti-fibrillin-1 antibodies, detectable in more than 50% of SSC patients, can stimulate fibroblasts and the release of TGF-β. Antifibroblast antibodies induce fibroblast activation *in vitro*, resulting in an increased expression of ICAM-1 and production of the proinflammatory cytokine IL-6[16.] Another class of potentially pathogenic autoantibodies are the anti-PDGF receptor

antibodies, that can stimulate the deposition of collagen I and convert resting fibroblasts into activated myofibroblasts. Currently, these autoantibodies are not routinely analyzed in the clinical setting. Antinuclear antibodies (ANA) are present in the sera of more than 95% of scleroderma patients, with certain clinical phenotypes associated with specific ANAs. Anticentromere (ACA) and antitopoisomerase I antibodies (ATA) are important diagnostic markers of SSC and are associated with different clinical phenotypes. ACA autoantibodies occur in about 50–90% of patients with CREST syndrome or limited cutaneous SSC[17], although they are not specific for this conditions and have been described in patients with other diseases, like primary biliary cirrhosis and systemic lupus erythematosus[18,19]. On the other hand, diffuse SSC is classically associated with antitopoisomerase-I antibodies (anti-topo-I or anti-Scl-70) and the absence of anticentromere antibodies (ACA)[20]. Overlap syndromes, such as mixed connective tissue disease (MCTD), with features of systemic lupus erythematosus, rheumatoid arthritis and scleroderma, are associated with the presence of anti-U1-small nuclear ribonucleoprotein particle (U1-snRNP) antibodies[21]. These autoantibodies are currently used in the clinical settings for the diagnosis of SSC different subgroups of patients. However, the low sensitivity of anti-Scl-70 and ACA assays limits their usefulness in the pathology prediction before clinical signs develop. Moreover, since about 40% of SSC patients are likely to be negative for autoantibodies, a negative result is not conclusive to exclude SSC diagnosis.

1.2.1.2 Role of the immune system

Few conflicting reports are available on blood lymphocyte homeostasis in SSC patients. A prominent role of CD4+T cell activation has clearly been demonstrated in the development and maintainance of SSC. Activated T lymphocytes are detectable in the blood as well as in affected organs (skin and lung) of SSC patients, presenting, beyond their activated phenotype, numerical alterations. An immune imbalance between regulatory T (Treg) and Th17 cells is also typical of SSC: in particular an increased frequency of CD4(+)CD25(+)FoxP3(+) Treg has been described in SSC patients, together with an impairment in the immunosuppressive function of these cells [22].

The current view is that abnormalities in the B cell compartment could play a role in SSC pathogenesis. B cells present different immunological functions, but are generally considered positive regulators of immune responses by their ability to produce antigen-specific antibody and to induce CD4+ T cell activation. Recently, it has been demonstrated that B cells perform additional functions, including the production of several cytokines, the presentation of antigens and also the capacity to suppress immune responses in a variety of mouse models of inflammation and autoimmunity [23,24,25,26]. The identification of inhibitory functions mediated by B cells, such as the suppression of Th1, Th2 and Th17-mediated responses, the stimulation of Foxp3 regulatory T cells (Tregs) and the conversation of effector T cells into Treg1 cells (Foxp3-IL10+CD4+) rose the idea of the existence of specific subsets of B cells with regulatory functions [27,28]. A rare

CD1dhiCD5+CD19hi subset of regulatory B cells has been characterized, in the spleens of wild type mice and in mouse models of autoimmune diseases, by their ability to exclusively produce IL-10. The cells displaying regulatory phenotype have been named B10-cells and represents 1% to 3% of spleen B cells in mice. Their competence to down regulate immune responses and inflammatory conditions is fully attributable to IL-10[29,30.] Additional B cells within the CD1dhiCD5+ B-cell subpopulation achieve the capacity to function like B10-cells following 48 hours of *in vitro* stimulation with TLR or agonistic CD40 monoclonal antibody[29.] Among TLR agonists, LPS and CpG represented the most potent stimuli for inducing the maturation of human blood B10 progenitor (B10pro cells) into IL-10-competent B cells. These B10pro cells become so far able to secrete cytoplasmic IL-10 after 5 hours of stimulation with PMA, ionomycin, and monensin[31]. Recently, an equivalent IL-10 competent B cell subset has been characterized in the human blood[32]. Although IL10 production remains the best phenotypic marker for defining human B10-cells, the majority of the CD19+CD5+CD1dhiB cells (71%), previously reported to be regulatory in experimental models of inflammation[33], are contained within the CD4+CD24hiCD38hi B cell subset. Moreover, it has recently been confirmed that, after CD40 stimulation, human CD19+CD24hiCD38hi cells are able to suppress the function of CD4+Th1 cells, via the secretion of IL-10, representing the human B10-cell subset: these CD19+CD24hiCD38hi B cells exhibit the ability to restrain pro-inflammatory cytokine production (TNFα and IFNγ) by CD4+ T Cells[34]. Up

to date, the possible role of these B regulatory cells in the pathogenesis of SSC has not been investigated yet.

1.2.2 THE ROLE OF FIBROBLASTS IN SSC

Fibroblasts play a central role in the maintenance of the structural integrity of the connective tissue, through the secretion of fibrillar procollagens and fibronectin and the regulation of the turnover and composition of the extra cellular matrix (ECM) via highly specific proteases such as collagenase. Fibroblasts can be divided in different subtypes distinguishable by their gene expression profiles and their different functional activities[35,36]. Following tissue damage, during the inflammation phase and the wound healing, quiescent fibroblasts are activated to produce granulation tissue and a provisional matrix. In SSC patients, activated fibroblasts are responsible for the development of fibrosis and accumulation of ECM molecules, through an overproduction of collagen and the induction of collagen-modifying enzymes. Activated fibroblasts in SSC may derive from several origins[37]. Mesenchymal progenitors may be recruited from the bone marrow through the circulation and resident tissue-specific precursors may be mobilized from the surrounding tissue. Different mechanisms may activate these quiescent fibroblasts including the stimulation by soluble pro-fibrotic mediators including TGF-β, connective tissue growth factor (CTGF), and endothelin-1 or by cell-to-cell interaction[38,39,40]. These findings suggest a major role for fibroblasts in the pathogenesis of SSC (Figure 4). Finally, the principle sites of skin fibrosis follow the same typical distribution patterns of sites involved in thermoregulation (fingers, feet, face, and lower arms),

suggesting a causative relationship between the vascular injury and the skin fibrosis[41].

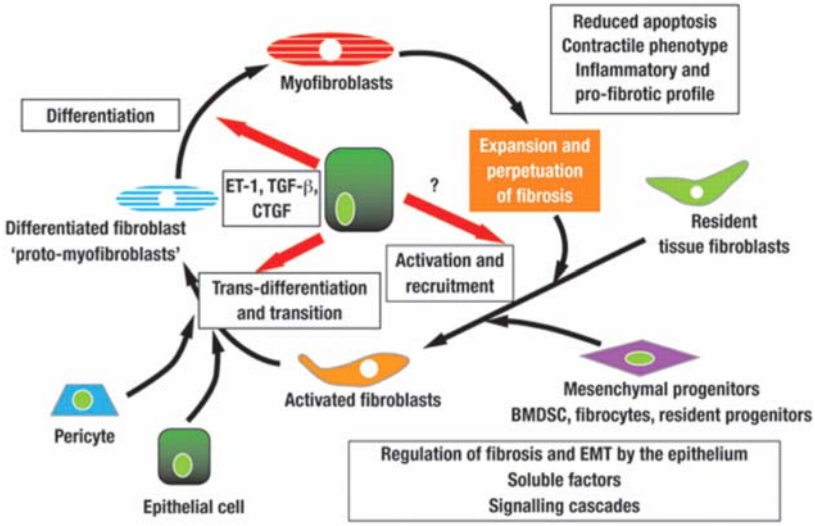

Figura 4. The role of the epithelium in the pathogenesis of SSC. Adopted from D. J. Abraham *et al. Rheumatology* 2009 Jun;48 Suppl 3:3-7.

1.2.3 THE ROLE OF ENDOTHELIUM IN SSC

The vascular involvement is of fundamental importance in the pathogenesis of SSC, since it is present in every patient and appears to be responsible for the earliest clinical manifestations as well as for the major life-threatening complications of the disease, contributing to an important morbidity and mortality. SSC is so far considered a primarily vasculopathic disease[41]. The vascular injury can also be due to the activation of cellular and humoral immunity, through the autoantibodies secretion and the release of products of activated T cells able to alter the endothelium functions[42]. Characteristic of SSC are the endothelial activation with enhanced expression of adhesion molecules, intimal proliferation till capillary occlusion, leading to a vicious cycle of ischemia-reperfusion injury, further exacerbated by extracellular matrix accumulation due to fibrosis[43,44]. Persistent digital hypoperfusion can lead to digital pitting, digital ulceration and gangrene.

Raynaud phenomenon (RP), that frequently represents the first sign of the vascular involvement of the disease, is characterized by the tricolor change featuring pallor (ischemic phase), cyanosis (deoxygenation phase), and erythema (reperfusion phase) induced by cold or stress and triggered by endothelial injury[45]. The underlying physiopathological mechanisms are still not completely understood, however the dysfunction of the endothelium results in an imbalance of vasoactive factors, including an increased production of the vasoconstrictor endothelin1 (ET-1) and a reduced secretion of the vasodilator prostacyclin and nitric oxide, that lead to a dysregulation of vascular tone control, clinically evident as Raynaud's

phenomenon [41,42,46] (Figure 4). Abnormal nailfold capillaries, gastric antral vascular ectasia, scleroderma renal crisis and pulmonary arterial hypertension represent other vasculopathic manifestations characteristic of SSC. Microvascular damage in SSC patients can be detected in structural changes of the capillaries of the skin and can be easily assessed by nailfold videocapillaroscopy (NVC). The characteristic alterations include a progressive reduction of capillary density, enlargement of the capillaries and microhaemorrhagies: these scleroderma patterns may be distinguished in an "early", an "active" and a "late" pattern of SSC capillaroscopy based on the different degree of vascular disturbance and are frequently used as an important diagnostic tool in order to distinguish SSC patients from uncomplicated primary Raynaud phenomenon. The nailfold capillary irregularities are considered an important predictor of disease severity and of the development of peripheral vascular complications and are accompanied by abnormal levels of different vascular mediators[47]. The utility of recognized serum biomarkers able to predict the evolution of the vascular disease as well as the outcome and the response to therapy is well established[48]. Nonetheless, up to date no definitive candidate serum biomarkers is available to really evaluate the disease activity and progression in SSC patients.

2. PLASMA BIOMARKERS OF DISEASE ACTIVITY IN SYSTEMIC SCLERODERMA

In the majority of SSC cases, severe visceral involvement occurs within the first three years of the disease and skin sclerosis rarely progresses after five years[49]. Therefore, the possibility to predict the disease course may be of particular importance for SSC patients at their first visits but, as we have previously reported, no definitive candidate serum biomarkers are available for SSC patients. In SSC, a pro-inflammatory status and an enhanced production of pro-angiogenic factors are followed by the prevalent activity of several angiostatic factors, resulting in a loss of capillary density and extensive avascular areas. So far, a variety of circulating markers mirroring the degree of vascular injury have been described as potential biomarkers of SSC vasculopathy[50].

Endothelial cell adhesion molecules mediate the cell-cell and cell-extra cellular matrix interaction, playing an important role in the pathogenesis of SSC[51]. Increased plasma concentrations of adhesion molecules released by endothelial cells, such as ICAM-1, VCAM- and E-selectin, have been described. ICAM-1 (CD54) is a member of the Ig superfamily that is constitutively expressed not only on endothelial cells, but also on epithelial cells and fibroblasts[52]. ICAM-1 mediates the transmigration of leukocytes across vascular endothelia[53]. Several studies have demonstrated that serum levels of ICAM-1 may be considered a prognostic biomarker of respiratory dysfunction in SSC patients. Unlike VCAM-1, serum levels of E-selectin were found to correlate with the presence of a "late" scleroderma pattern with avascular areas at the NVC, suggesting its potential role as a biomarker of disease activity[54,55,56,57]. Finally,

the role in SSC pathogenesis of junctional adhesion molecules (JAMs), involved in the ischemia–reperfusion injury and in the recruitment of leukocytes during inflammation, was assessed. Plasmatic levels of soluble JAM-A and soluble JAM-C were found significantly raised in SSC patients with "early" or "active" NVC patterns and in SSC patients with active digital ulcers, thus suggesting a role of JAMs in early endothelial cell activation and in the inflammatory and vascular disease[58].

Chemokines represent a family of molecules classified according to the position of the NH(2)-terminal cysteine motif. Chemokines are critical mediators of leukocytes migration through endothelia. In SSC patients they play a pivotal role in the process of fibrosis, mediating leukocyte and mononuclear cells chemotaxis and the release of pro-fibrotic growth factors[59]. The role of different chemokines in SSC pathogenesis and their correlation with the degree of vascular involvement has already been investigated in previous works[60]. In a recent study, greater plasmatic amounts of CCL2, CXCL8 and other chemokines were observed in SSC patients compared to healthy controls; however no significant correlation between NVC findings and the plasmatic levels of such biomarkers was demonstrated[61].

Vascular endothelial growth factor (VEGF) was found to be highly overexpressed in the circulation and in the skin of SSC patients. If increased levels of VEGF may stimulate angiogenesis, a chronic prolonged overexpression, as seen in SSC patients, may lead to a vascular injury rather than to new vessel formation[62,63,64]. Significantly higher level of VEGF were detected in peripheral blood of SSC patients compared to healthy controls[65],

but no significant correlation was observed in the expression of VEGF between SSC patients with more or less NVC damage. Endothelin-1 represents a vasoconstrictor agent, involved also in vascular wall cell inflammation and fibrosis[66]. Higher amounts of endothelin-1 have been recognized in SSC patients. Moreover, endothelin-1 plasma levels demonstrated a strict correlation to capillary dimension and reduction, resulting higher in SSC patients with "active" NVC compared to those with "early" and "late" patterns[67] and suggesting a possible role of endothelin-1 in the microvascular and fibrotic injury in SSC patients. Recent papers investigated the role of other pro-angiogenic factors, such as angiopoietin-like protein 3, galectin-3, tissue kallikrein and interleukin-33 in the physiopathology of SSC, but no correlation with the different NCV findings and the severity of vascular involvement was found[68,69,70,71].

Conflicting results have been reported on the role of the main inhibitor of angiogenesis, endostatin, in the pathogenesis of SSC, but no significant correlation was found between serum levels of endostatin and the disease activity[72]. Anti-angiogenic VEGF isoform levels were found to be significantly increased in SSC patients either with "active" or "late" NVC patterns in comparison to controls, suggesting the role of this variant in the loss of microvessels[73]. Matrix metalloproteinases (MMP-12) have been correlated to impaired angiogenesis. Augmented plasmatic levels of MMP-12 were detected in patients with SSC compared to controls and increased amounts of MMP-12 were associated with the severity of NVC abnormalities, suggesting their part in the disturbance of microvessels in SSC patients[74]. Although all these inflammatory cytokines and vascular

mediators have been proposed as candidate biomarkers for SSC, none has been accepted for clinical use yet. So far, more specific and sensible biomarkers are needed for a better management of SSC patients.

3. TWO NEW POTENTIAL BIOMARKERS FOR SYSTEMIC INFLAMMATORY DISEASES: ELAFIN and TNFRI

3.1 ELAFIN

A newly discovered promising biomarker for inflammatory immune-mediated conditions is elafin (also known as SKALP), a serine elastase inhibitor[75], first isolated from psoriatic skin and human bronchial secretions[76,77]. It has recently been demonstrated that plasma levels of elafin well correlate with the disease activity in patients with severe psoriasis, a chronic inflammatory skin condition[78]. Moreover, the decrease in serum elafin levels during therapy correlates with the clinical course of psoriasis, providing an important tool for monitoring not only the disease activity, but also the response to treatment[78]. Elafin is present in normal human skin only at low levels in the granular cell layer, but is strongly induced during inflammatory conditions such as psoriasis and acute Graft-versus-Host Disease (aGVHD). The induction of elafin/SKALP gene expression in psoriasis is closely related to the infiltration of neutrophils into the epidermis, showing an important role in protecting the skin components against the tissue damage caused by the infiltrated leukocytes[79].

Another immune-mediated condition in which elafin has demonstrated an important role as biomarker of cutaneous involvement is the aGVHD, a serious complication of allogeneic hematopoietic stem cell transplantation, that presents clinical features similar to those observed in SSC patients. Although secreted locally in response to pro-inflammatory cytokines, in aGVHD patients elafin is promptly identified in the systemic circulation, showing a value not only as a non-invasive diagnostic test in

GVHD, but also as a prognostic biomarker, providing important information independent of the extent of skin GVHD at presentation. Moreover, elafin was also found to be overexpressed in skin biopsies of patients with acute (aGVHD) and chronic lichenoid (clGVHD), but not chronic sclerotic GVHD (csGVHD)[80]. Elafin-high aGVHD/clGVHD lesions are associated with poor prognosis and decreased overall survival in aGVHD and corticosteroid resistance in clGVHD[80].

Elafin is also able to restrain the endogenous vascular elastase (EVE), a serine protease produced after vascular injury[81,82], indicating its potential role as biomarker of vascular involvement in inflammatory systemic diseases. In some vascular disorders, such as pulmonary arterial hypertension (PAH) and arterial injury, endogenous vascular elastase (EVE) plays a pivotal role in matrix accumulation and vascular smooth cell proliferation, causing intimal thickening. Recent studies showed the ability of elafin to prevent the elastase-induced keratinocyte hyperproliferation and the vascular remodeling.

3.1.2 BIOLOGICAL CHARACTERSITICS OF ELAFIN

Elafin is an endogenous human protein composed of an N-terminal transglutaminase region and a C-terminal-domain with anti-proteolytic activity[83,84]. It is constitutively expressed in epithelial tissues where specifically inhibits the neutrophil-derived serine proteases elastase (HNE) and the proteinase-3 by a competitive tight-binding mechanism[76,85]. Elafin is secreted by the squamous epithelium of the skin, with an increased expression during inflammatory skin conditions[86]. It has also been detected

in other epithelia, such as tongue, gengiva, esophageal lining, vagina, mammary[87], stomach and intestine[86]. Recent immunohistochemical studies on autopsy samples of human coronary arteries revealed the expression of elafin also in the endothelium, medial vascular smooth muscle cells and in the extraxcellular matrix of coronary artheries[88]. Elafin presents several biological functions, suggesting its role in inflammation and cellular proliferation. The inhibition of elastase and proteinase-3[89] (figure 5) leads to a reduction of intensity of several key processes in the inflammatory cascade. Elafin is able to prevent CD14 cleavage from macrophages membrane, leading to apoptotic cell recognition and clearance[90] and inhibits extracellular matrix degradation and endothelial disruption mediated by human neutrophil elastase and proteinase 3[91]. In addition, elafin shows a role in the antimicrobial defense screen on epithelial and mucosal surfaces. Moreover, elafin seems to encourage the development of Th1-type immune responses: this finding is consistent with the high concentrations of elafin found in the psoriasic skin, a condition characterized by a prevalent type-1 immune response[92] (figure 5).

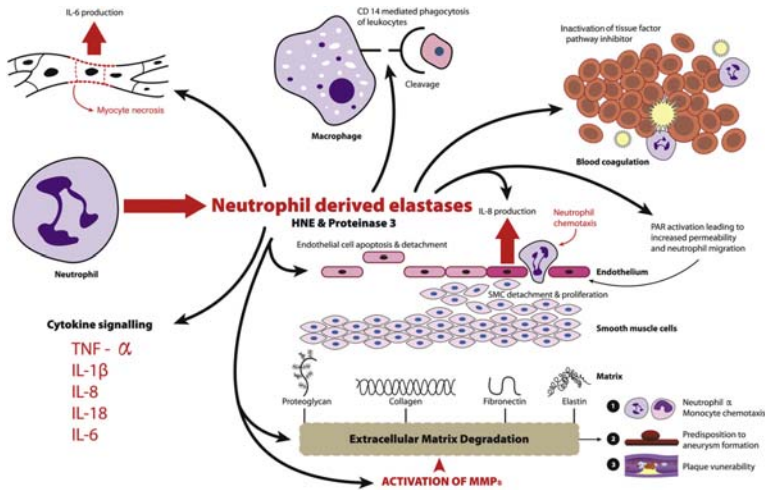

Figure 5. Functions of neutrophil-derived elastases. Adopted from Shaw L, *et al. Biochem Soc Trans.* 2011 Oct;(5):1450-4.

3.1.3 POTENTIAL THERAPEUTIC APPLICATIONS OF ELAFIN

Elafin presents not only a value as potential diagnostic biomarker, as previously described, but it has also been demonstrated to play a role as a potential therapeutic tool for the treatment of inflammatory vascular, systemic and pulmonary diseases. Therapeutic inhibition of neutrophil-derived elastases holds promise with powerful treatment effects observed in various preclinical vascular injury models (Figure 6) In these models, elafin administration or gene overexpression reduced elastase activity, tissue damage and inflammatory cell infiltration[93]. Pre-clinical studies have

suggested therapeutic benefit of elafin administration in cardiovascular disease. In several models of ischemia-reperfusion injury, for example, the administration or the over-expression of elafin resulted in reduced infarct expansion and neutrophil infiltration; the increased elastase and matrix metalloproteases activity after the infarction are significantly suppressed in elafin overexpressing transgenic mice in comparison to wild type[94,95]. Moreover, elafin showed the ability to reduce the disease progression in atherosclerosis and pulmonary hypertension models[96], providing also beneficial effects in a murine model of viral myocarditis[97].

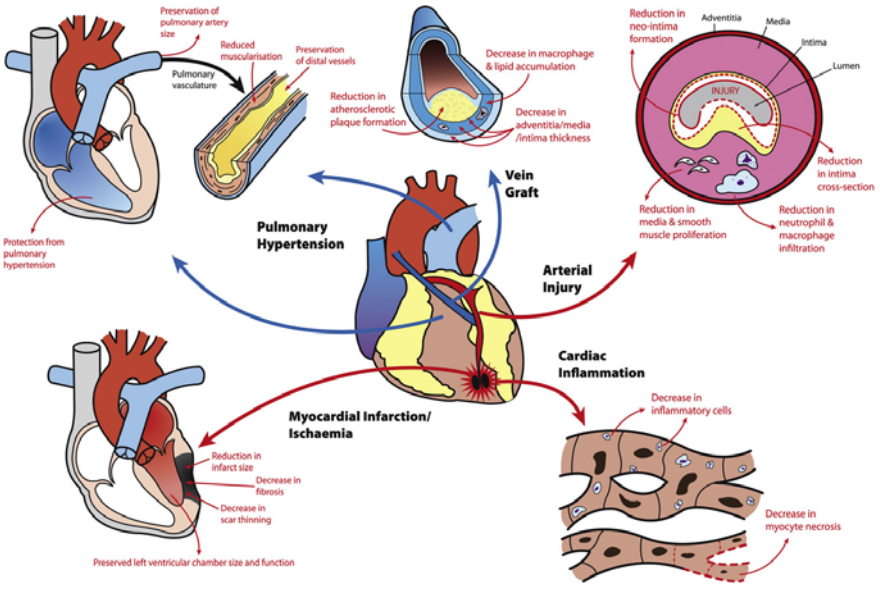

Figure 6. Potential therapeutic application of elafin in preclinical vascular injury models. Adopted from Alam SR *et al. Biochem Pharmacol.* 2012; 83(6):695-704.

In conclusion, pre-clinical studies of inflammatory vascular damage have demonstrated that the administration of elafin is able to limit tissue injury and to preserve organ function. Using yeast-derived recombinant human elafin, administered intravenously, a Phase I clinical trial in healthy subjects raised favorable results in terms of toxicity. Phase II clinical trials evaluating the protective role of elafin in myocardial ischemia- reperfusion injury in patients after coronary bypass surgery, are still ongoing[98].
Up to date, the potential role of elafin in SSC remains to be elucidated.

3.2. TNFRI

Another promising plasma biomarker, recently identified in different immune-mediated and systemic inflammatory conditions, is the soluble tumor necrosis factor receptor-I (s-p55TNFR or sTNFRI). Endogenous soluble TNFRI concentrations appear to reflect the activation state of the TNFα/TNF receptor system. Soluble TNFRI is constitutively released in the circulation[99] and its levels increase during different diseases[100] and after TNF stimulation[101,102]. Thus, soluble TNFRI and TNFRII levels show high accuracy in the follow-up and prognosis of various diseases, such as HIV infection and sepsis. Moreover, determination of soluble TNFRI also gives useful data for monitoring cancer and autoimmune diseases. The information provided is often even superior to that obtained with classical disease markers, probably due to the direct involvement of the TNF system in the physiopathology of these conditions.

It has been demonstrated that knock-in mice expressing a mutated non-sheddable TNFRI develop Toll-like receptor-dependent innate immune

hyperreactivity, with a more efficient control of intracellular bacterial infections. Notably, gain of function for antibacterial host defenses is achieved at the cost of misbalanced inflammatory responses leading to pathology. Mutant mice develop spontaneous hepatitis, TNF-dependent arthritis and experimental autoimmune encephalomyelitis[103]. In humans, mutations on the TNFRI gene, causing reduced levels of shedding, were proposed to be responsible for a newly defined class of autoinflammatory diseases, named TNFRI-associated periodic syndromes (TRAPS), characterized by recurrent fever attacks and inflammation[104]. The assessment of soluble TNFRI may so far presents a prognostic value in infectious, inflammatory and also autoimmune diseases. Circulating levels of TNFRI revealed to be differently associated with inflammation depending on the pathology. Recently it has been demonstrated that plasma levels of TNFRI could provide useful information about disease activity in systemic lupus erythematosus (SLE) patients, representing a promising biological marker for the follow up of these patients, where acute phase protein response is generally low during disease flares[105,106]. Recent evidences has showed also the importance of the soluble TNFRI as a good biomarker of renal disease activity in murine models of lupus as well as in human lupus nephritis[107].

The detection of TNFRI plasma levels is actually used in the clinical setting to better diagnose aGVHD. In fact it has recently been described that a biomarker panel consisting of IL-2 receptor-α and TNFR1 correlate with clinical diagnosis as well as survival of aGVHD[108].

Finally, it is known that TNF elicits distinct signaling pathways in vascular endothelial cells (ECs) via the interaction with TNFRI and

TNFRII[109]. The vascular endothelium is a major regulator of inflammation. Different inflammatory conditions result from inflammation in microvascular beds, in which endothelial activation causes transcriptional expression of leukocyte adhesion receptors such as E-selectin[110]. Endothelia of both large and small blood vessels express TNFRI[111], which is critical to this process, as confirmed by the evidence that inflammation is abolished in mice lacking TNFRI[112]. TNFRI and TNFRII play differential roles in ischemia-mediated arteriogenesis and angiogenesis. TNFRI signaling inhibits, whereas TNFRII signaling promotes, this adaptive response, probably due to their opposite effects on EC survival and migration[109]. So far, the specific inhibition of TNFRI signaling in ECs appears to be a promising novel target for the treatment of vascular diseases. Since systemic sclerosis is considered a primarily vasculopathic disease and considering the potential diagnostic and prognostic value of TNFRI in different inflammatory and autoimmune conditions, it could represent a potential interesting biomarker for SSC.

3.2.2 BIOLOGICAL CHARACTERSITICS OF TNFRI

The TNF receptor superfamily is characterized by the ability to bind the tumor necrosis factors (TNF) via an extracellular cysteine-rich domain[113]. TNFRI is expressed ubiquitously, whereas TNFRII expression is tightly regulated and found predominantly on ECs and hematopoietic cells. These two TNFRs display structurally similar extracellular domains, but signal through distinct intracellular regions. TNFRI is initially synthesized as membrane-anchored proteins, which can subsequently be released from the

cell surface by proteolysis, forming soluble molecules able to bind the TNF ligand (sTNFRI)[114,115](figure 7).

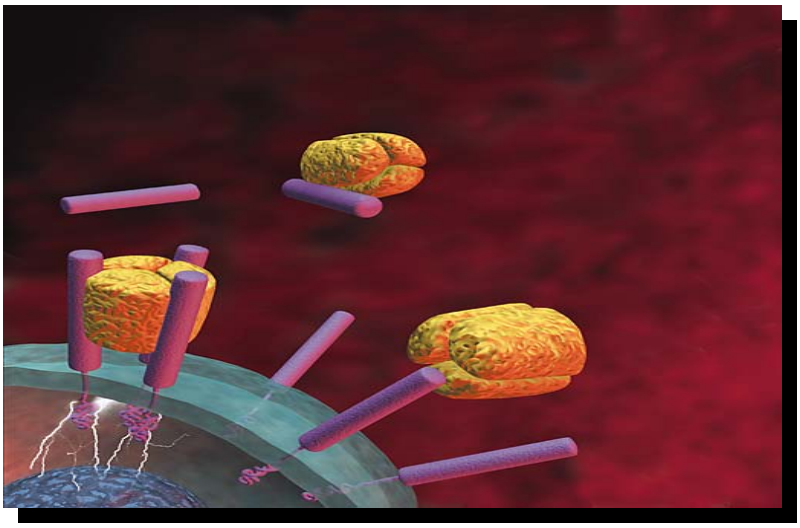

Figure 7. Shedding of TNFRI from cell surface.

In different cell culture systems, soluble receptors are rapidly secreted in response to several different stimuli such as TNF [116], LPS[117], PMA, IL-10 or after T cell[118] and neutrophil activation[119]. The pool of soluble receptors secreted could function as physiological attenuators of the TNF activity, competing for the ligand with the cell surface receptors. Moreover, it has been proposed that soluble receptors can stabilize circulating soluble TNF functioning as TNF agonists[120], dampening inflammatory responses and playing a major role in establishing the balance that regulate progression of the inflammatory processes from defense to injury. The role of TNFRI as potential biomarker of SSC has not been investigated yet.

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SCOPE OF THE THESIS

Systemic scleroderma or systemic sclerosis (SSC) is a multisystem disease characterized by an unpredictable course, high mortality and resistance to therapy. The immune pathogenesis of SSC is not completely understood as it is the result of a complex interaction between genetic, environmental and immunological factors. Moreover, there are no specific biomarkers able to accurately predict disease activity, progression and therapeutic response.

The best hope for continued progress lies in the development of innovative treatments, thanks to a better understanding of SSC pathogenesis and in the identification of new easily measurable disease markers able to ameliorate SSC diagnosis and management. Along these hypotheses, the project comprises two lines of research.

1) The first one is focused on the role of elafin and TNFRI as new potential biomarkers of disease activity in SSC patients
2) The second line of research is focused on the characterization of a specific subset of regulatory B10-cells in the pathogenesis of SSC

CHARACTERIZATION OF SOLUBLE BIOMARKERS AND REGULATORY B CELL SUBSET USEFUL TO IMPROVE SYSTEMIC SCLERODERMA CLINICAL DIAGNOSIS AND MANAGEMENT

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ABSTRACT

Systemic scleroderma or systemic sclerosis (SSC) is a multisystem disease characterized by an unpredictable course, high morbidity and mortality. Actually there are no specific biomarkers able to accurately predict disease activity, progression and response to treatment. The aims of our study consist in the identification of easily measurable disease candidate biomarkers and in the characterization of a specific subset of regulatory B cells in order to ameliorate SSC patient management. Elafin, a serine elastase inhibitor, has been recently identified as a potential biomarker of disease activity and as a potential therapeutic tool in several inflammatory immune-mediated conditions. We evaluated in 69 SSC patients plasma levels of elafin and we observed a significant higher concentration of the protein compared to healthy controls (mean ± SEM 17440 pg/ml ± 750 and 11980 pg/ml ± 724.5 respectively with $p \leq 0.001$). A linear regression analysis showed a significant negative correlation between elafin concentrations and the disease activity scores, highlighting the potential role of elafin as a biomarker of disease activity. Moreover, the immunohistochemical analysis of skin, heart and lung biopsies from SSC patients showed the lack of elafin in the analyzed tissues. In accordance with the pathogenic role of T-cells, which have been documented to mediate damage in different SSC involved organs, we further observed increased plasma concentration of the TNFRI in SSC compared to healthy donors, independent from the disease activity and internal organ involvement. Few conflicting reports are available on blood B lymphocyte homeostasis in SSC

patients. In accordance to recent findings in other autoimmune conditions, we observed higher B10-cell frequencies in SSC patients compared to healthy controls (mean ± SEM = 10.56 % ± 0.854 and 4.9 % ± 0.497; $p \leq 0.0001$). Interestingly, unless higher frequencies of B10-cells in the peripheral blood, the mean percentages of CD4+ IFN-γ producing T cells resulted comparable and even slightly increased in SSC patients compared to HD (mean CD4+ IFN-γ+= 26.6% ± 6.4 and 14.1%±3.38 respectively). In conclusion, the identified soluble markers, potentially mirroring the disease activity and the molecular inflammatory status in SSC patients, could be harnessed to better design patient-shaped therapy. Moreover, the investigation of the pathogenic role of B cells, focusing in particular on regulatory B10-cells, may shed new light on the pathogenesis of systemic scleroderma, leading to new possible therapeutic targets.

INTRODUCTION

Systemic scleroderma or systemic sclerosis (SSC) is a multisystem disease characterized by an unpredictable course, high mortality and resistance to therapy[1]. Due to the lack of a single diagnostic test, classification criteria are crucial to discriminate systemic sclerosis from other similar conditions and to identify different subgroups of patients[2,3]. The classification of SSC includes different subtypes, ranging from limited to diffuse forms, linked by the presence of sclerotic skin lesions, characterized by a frequent gastrointestinal and pulmonary involvement. Actually there are no specific biomarkers able to accurately predict disease activity, progression and therapeutic response. Systemic sclerosis is considered a primarily vasculopathic disease[4,5]. Nonetheless, the immune pathogenesis of SSC is not completely understood as it is the result of a complex interaction between genetic, environmental and immunological factors. The best hope for continued progress lies in the development of innovative treatments, thanks to a better understanding of SSC pathogenesis and in the identification of new easily measurable disease markers able to ameliorate SSC diagnosis and management. Several inflammatory cytokines, that are increased in plasma of SSC patients, have been identified as candidate biomarkers for SSC, but, up to date, none has been developed for clinical use. Elafin is a serine elastase inhibitor expressed predominantly in epithelial tissue and able to suppress several key processes in the inflammatory cascade[6,7,8], with particular regard to the inflammation triggered by reperfusion injury. This raises the possibility that elafin might be an effective

treatment in inflammatory vascular, systemic and pulmonary conditions[9,10,11]. Moreover, Elafin has been recently recognized as a biomarker of inflammatory immune-mediated pathologies, such as psoriasis[12,13] and Graft Versus Host Disease, a severe complication of allogeneic hematopoietic stem cell transplantation with clinical features similar to those observed in SSC patients[14]. Another promising plasma disease biomarker is the Tumor Necrosis Factor Receptor I (TNFRI), synthesized as a membrane-anchored protein, subsequently released from the cell surface by proteolysis, forming soluble molecules able to bind the TNF ligand (sTNFRI)[15,16]. Soluble TNFRI was recently considered as a new tool to gain information about immune processes and able to provide valuable insight into a variety of autoinflammatory and autoimmune conditions, such as systemic lupus eritematosus[17,18,19].

Dysregulation in B cell number and functions are known to play a major role in the pathogenesis of different autoimmune diseases. It has been recently demonstrated that a particular subset of regulatory B cells, characterized by a CD19+CD24^{hi}CD38^{hi} phenotype[20], showed an increased frequency and presented a functional impairment in some autoimmune conditions, lacking their capacity to suppress the production of IFNγ by CD4+ T Cells[21].

In our paper we analyzed elafin and TNFRI as new potential biomarkers in SSC pathology. In addition, we studied the potential pathogenic alterations of the B cell compartment, focusing in particular on a specific subset of regulatory B10-cells.

We demonstrated for the first time, in an homogeneous cohort of patients, the potential role of elafin and TNFR1 as two new potential disease biomarkers and the alteration in frequency of regulatory B10-competent cells in SSC patients. Further studies are needed to better comprehend the mechanisms by which these proteins and this subset of regulatory B cell can be involved in SSC pathogenesis.

METHODS

Patients

Sixty-nine SSC patients, referred to the Rheumatologic Centre of Gaetano Pini Hospital of Milan from 2012 to 2015, classified according to 2013 criteria[22] and 18 healthy donors (HD) were prospectively enrolled in the study. Concerning SSC patients, the concomitant presence of other inflammatory, infectious or malignant diseases represented exclusion criteria. Patients' characteristics such as age, sex, disease duration (defined as time from disease diagnosis to the last follow-up), the presence anticentromere (ACA) and anti-topoisomerase I (anti-Scl-70) autoantibodies[23,24], the eventual presence of ongoing immunosuppressive therapies were summarized in Table 1.

SSC patients were classified in two subgroups according to the extent of skin involvement. In particular patients with limited cutaneous disease were characterized by sclerosis of distal extremities, not above the elbow and knees, with or without sclerosis of neck and face, while patients with diffuse cutaneous disease were characterized by sclerosis of both distal and proximal extremities, with or without involvement of the trunk[25,26]. To further stratify patients, the following clinical information were collected for all patients:

- Organ system involvement: defined as previously described[24], with some modifications[27]: PI = pulmonary involvement investigated with the diffusion capacity of the lung (DLCO), considering pathological a reduction of diffusion lung of carbon monoxide < 75% of predicted[28]; pulmonary arterial hypertension (PAH) = clinical evidence of pulmonary hypertension and

elevated pulmonary artery pressure (>45 mmHg) documented by echocardiography in the absence of severe pulmonary interstitial fibrosis; VI= microvascular involvement investigated by the presence or absence of digital ulcers at time of sample collection.

- Disease Activity Scores: i) the modified Rodnan skin score (mRSS), which uses clinical palpation to estimate skin thickness, is currently considered the most appropriate technique for measuring skin involvement in SSC. The grading of the modified Rodnan skin thickness score, evaluating 17 different cutaneous areas[29,30], is as follows: 0= normal, 1= thickened skin, 2= thickened and unable to pinch, 3= thickened and unable to move. Several studies demonstrated that assessment of mRSS is easily applicable and is reliable and sensitive to change in the context of clinical trials. More extensive skin involvement coincides with more severe internal organ manifestations, poor prognosis and increased disability[31,32,33,34]; ii) the European Scleroderma Study Group (EScSG) score is a simple and feasible instrument that evaluates both clinical and laboratory items, including the modified Rodnan skin score, carbon monoxide diffusing capacity (DLCO), presence of scleredema, digital ulcers, arthritis, erythrocyte sedimentation rate, hypocomplementaemia and patient-reported worsening of skin, vascular and cardiopulmonary symptoms[35,36]. It has been recently validated as a new reliable index to assess disease activity in SSC.

Samples collection and ELISA assay

Ten to 20 mL of whole blood was obtained from patients and healthy donors in heparin-containing tubes to prevent clotting. Plasma was separated from cell fraction by centrifugation and cryopreserved before the use. Two plasma biomarkers were studied: elafin and TNFRI. Plasma elafin and TNFRI levels were measured by ELISA using commercially available kits (R&D, Minneapolis, MN). The limit of detection was 15.62 pg/ml for Elafin and 12.5 pg/ml for TNFRI. All assays were performed according to the manufacturer's instructions.

Analysis of frequency and functionality of B10 cells

Blood mononuclear cells (PBMCs) were isolated from fresh blood by centrifugation over a Ficoll gradient. Cells were resuspended (2 × 106 cells/mL) in medium (RPMI 1640 media containing 10% fetal bovine serum, 200 μg/mL penicillin, 200 U/mL streptomycin, and 4 mM L-glutamine [all from Invitrogen]). The frequencies of IL-10+ B-cells were determined after *in vitro* PBMC stimulation with CD40L (1 μg/mL; R&D Systems) and CpG (ODN 2006, 10 μg/mL; Invivogen) for 48 hours. Phorbol-myristyl-acetate (PMA, 50 ng/mL; Sigma-Aldrich), ionomycin (1 μg/mL; Sigma-Aldrich) and brefeldin A (BFA, 1 × solution/mL; BioLegend) were added during the last 5 hours of culture. At the end of the culture PBMCs were firstly stained with an anti-CD19 antibody (PC7, Beckman Coulter). Then PBMCs were permeabilized using Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer's instructions. After Fc receptor blocking (FcγR-binding

inhibitor, eBioscience), to prevent unspecific binding, PBMCs were further stained with anti–IL-10 antibody (PE, Biolegend).

To assess the functionality of B10-cells, we evaluated their ability to suppress CD4 mediated-cytokine production. So far, PBMCs, isolated from fresh peripheral blood of patients with SSC and HD, were cultured for 72 hours with 0.5 mg/ml soluble anti-CD3 antibody (purified, BD Pharmingen). PMA and ionomycin were added during the last 6 hours of culture. Cultured PBMCs were then surface stained with anti-CD4 antibody (PerCP-Cy5.5, BD Pharmingen), permeabilized, and stained with anti-IFN-γ antibody (FITC, BD Pharmingen).

Immunohistochemical evaluation of skin biopsies

Formalin fixed skin biopsies from 7 SSC patients were collected, together with acute GVHD and normal control skin biopsies respectively used as positive and negative controls. Pulmonary and human coronary artery biopsies collected at autopsy from a diffuse SSC patient dead for hypertrophic cardiomyopathy were also analysed. In this case a coronary artery biopsy obtained from a patients dead for heart infarction was used as positive control. All the samples were immunohistochemically stained using a polyclonal antibody directed against elafin (FL-117, dilution 1:250; Santa Cruz Biotechnology). Positive expression was defined as significant staining of at 50% of the depth of the epidermis, excluding the granular cell layer and the acrosyringium. Original magnification 400X.

Statistics

Statistical analysis were performed by Prism software (GraphPad, La Jolla, USA) using two-tailed T test.

RESULTS

Elafin levels were elevated in plasma, but not in skin, lung and coronary artery biopsies of SSC patients

Plasma samples were prospectively collected from healthy donors (n=18) and SSC patients (n=69), divided in limited (n= 27) and diffuse (n=42) subgroups. Main clinical, serological and demographic features of SSC patients enrolled in the study were reported in Table 1. Digital skin ulcers and interstitial lung disease were more frequent in the diffuse SSC in comparison to limited SSC subgroup (43% vs 15% and 80% vs 63% respectively). As expected, ACA autoantibodies occur in 63% of patients with limited cutaneous SSC, whereas anti-Scl-70 were detected in 91 % of diffuse SSC. Most patients were undergoing treatment with immunomodulatory agents including methotrexate and/or low doses of corticosteroids (Table1).

Table 1. Patients' characteristics		
	LIMITED SSC	DIFFUSE SSC
Number	27	42
Mean age at disease onset, years (range)	50.8 (25-80)	49.9 (26-79)
Disease duration, years (range)	9 (2-30)	10.8 (1-30)
Sex, n. (%)		
Female	25 (93%)	36 (86%)
Male	2 (7%)	6 (14%)
Autoantibodies, n. (%)		
Anticentromere antibodies (ACA)	17 (63%)	1 (2%)
Antitopoisomerase I antibodies (anti-Scl-70)	1 (4%)	38 (91%)
Immunosuppressive therapy at sample collection n. (%)	11 (41%)	35 (83%)
Mean modified Rodnan skin score, n. (range)	1.8 (0-4)	6.6 (0-18)
Mean European Scleroderma Study Group score n. (range)	1.1 (0-3.5)	2.5 (0.5-6)
Presence of ulcers, n. (%)	4 (15%)	18 (43%)
Diffusion capacity of the lung <75% of predicted n. (%)	17(63%)	34 (80%)
Pulmonary artery pressure>45 mmHg , n. (%)	2 (7%)	3 (7%)

Plasma levels of elafin were significantly increased in SSC patients compared to healthy controls (mean ± SEM 17440 pg/ml ± 750 and 11980 pg/ml ± 724.5 respectively with p ≤0.001) (Figure 1A). Elafin plasma levels were then analyzed according to SSC clinical features, as previously described. With this purpose, SSC patients were firstly divided in two subgroups with limited or diffuse disease.

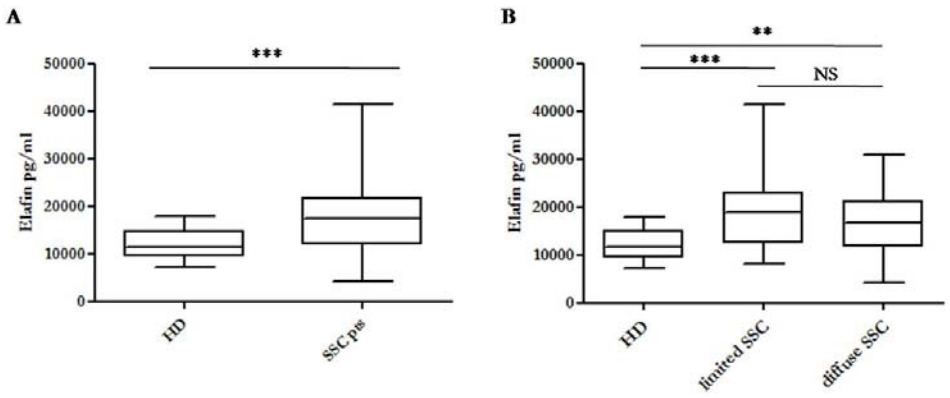


Figure 1. Elafin plasma levels significantly increased in SSC patients. A) Elafin plasma levels were measured by ELISA assay in the peripheral blood of SSC patients and Healthy Donors (HD). Mean values, maximum and minimum were plotted in the graphs. **B)** Mean elafin plasma concentrations were determined in the two subgroups of SSC patients with limited or diffuse forms and compared to HD; *** p≤0,001 **p≤0,01.

Elafin levels resulted significantly higher in both SSC subgroups in comparison to HD (mean ± SEM 18350 pg/ml ± 1243 in limited SSC, 16770 pg/ml ± 924 in diffuse SSC, with a p ≤0.001 and p≤0.01 respectively). However, no differences between the two subgroups of limited and diffuse SSC were observed (Figure 1B). In order to evaluate the role of elafin as potential biomarker of disease activity, protein levels were then correlated with the modified Rodnan skin score and EScSG score. Linear regression analysis showed a significant negative correlation between elafin plasma levels and both disease activity scores (R2=0.1733 with p≤0.001 and R2 0.2079 with p≤0.0001, respectively) (Figure 2A and 2B).

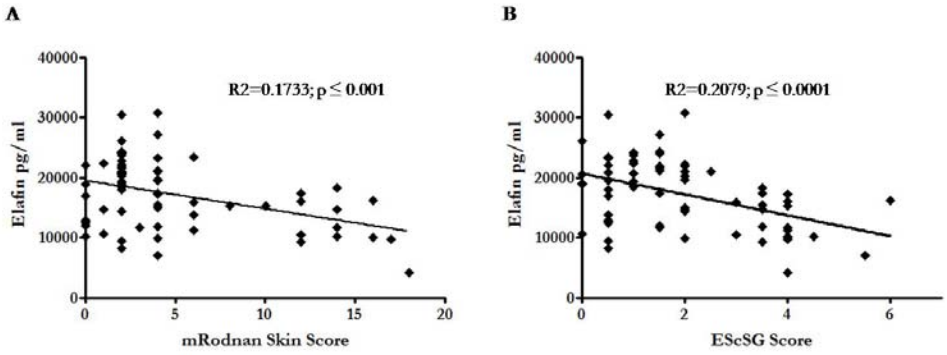
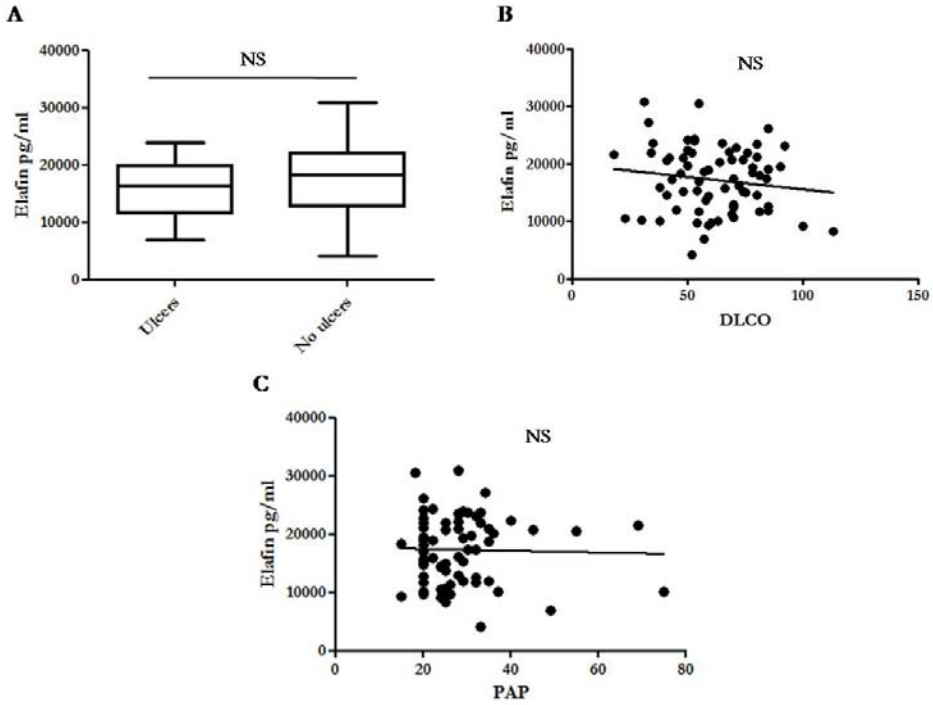


Figure 2. Elafin plasma levels inversely correlated with the modified Rodnan (mRodnan) skin score and the European Scleroderma Study Group Score (EScSG). Elafin plasma levels measured in the peripheral blood of SSC patients were correlated by linear regression analysis with the extension of skin involvement and the disease activity, investigated by the mRodnan Skin Score **(A)** and the EScSG Score **(B)**.

Finally, elafin plasma level were further correlated with the following variables: active digital ulcers, interstitial lung disease (DLCO assessment) and pulmonary arterial hypertension (PAP evaluation). Any significant correlation with these variables could be determined (Figure 1A, 1B, 1C Supplementary).



Supplementary Figure 1. Correlation between elafin plasma levels and the degree of pulmonary and vascular involvement. A) Elafin levels measured in the plasma of SSC patients were analyzed according to the presence or absence of ulcers. Mean values, maximum and minimum were plotted in the graph. **(B)** Elafin levels were further correlated by linear regression analysis with the diffusion capacity of the lung (DLCO) and with the presence of pulmonary arterial hypertension decteted through pulmonary artery pressure (PAP) **(C)**.

We next examined the expression of elafin in skin biopsies of 7 patients with both limited and diffuse SSC. An acute GVHD skin biopsy and a skin biopsy from a normal donor were used as positive and negative controls. In contrast to what observed in GVHD skin biopsies, immunohistochemistry studies highlighted the presence of a basal elafin expression at the level of the granular, but not in the spinous or basal cell layers (Figure 3), similarly to what observed in normal skin biopsies (data not shown). Moreover, we demonstrated the absence of elafin in pulmonary and heart biopsies from autopsy specimens of a diffuse SSC patient dead for hypertrophic cardiomyopathy (Figure 3).

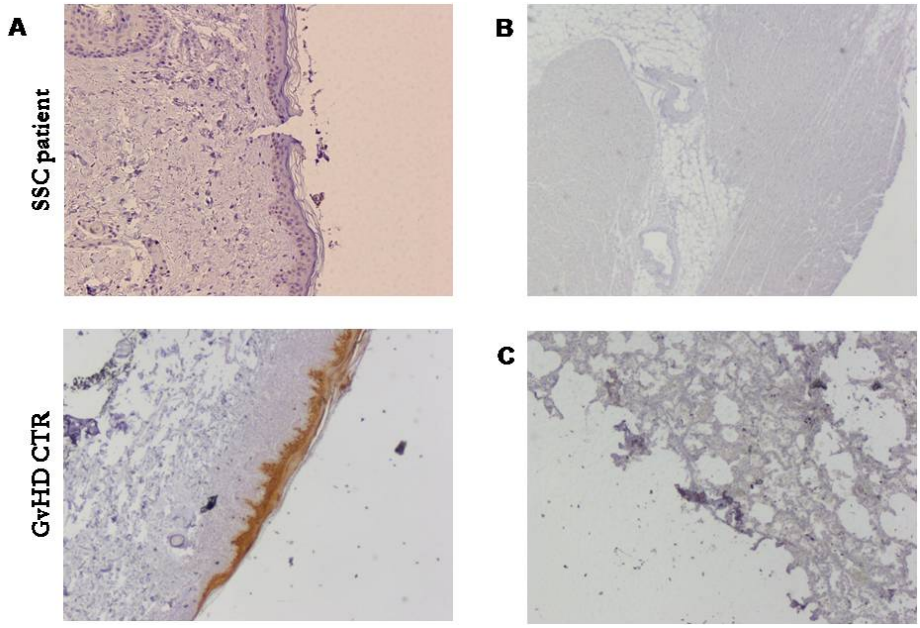


Figure 3. Elafin immunohistochemistry staining of tissues specifically involved in SSC disease. A) Skin biopsies from a SSC patient (upper panel) and from a positive GvHD control (GvHD CTR, lower panel) were immunohistochemically stained to evaluate elafin expression. Elafin staining of a hearth biopsy **(B)** and a pulmonary biopsy **(C)** from autopsy specimens of a diffuse SSC patient, dead for hypertrophic cardiomyopathy.

Plasma levels of TNFRI were elevated in SSC patients

We also analyzed plasma levels of Tumor Necrosis Factor Receptor I (TNFRI) in SSC patients and HD. Plasma levels of TNFRI were significantly increased in SSC patients compared to HD (mean ± SEM 1971 pg/ml ± 127.3 and 1206 pg/ml ± 83.52 respectively; p ≤0.001) (Figure 4A). As already mentioned, we then compared TNFRI plasma levels in limited and diffuse SSC patients. Mean values of TNFRI concentrations resulted comparable in the two subgroups (Figure 4B), but in both cases significantly increased compared to HD (mean ± SEM 1998 pg/ml ± 222.2 in limited SSC, 1951 pg/ml ± 151.0 in diffuse SSC; p≤0.01 and p ≤0.001 respectively).

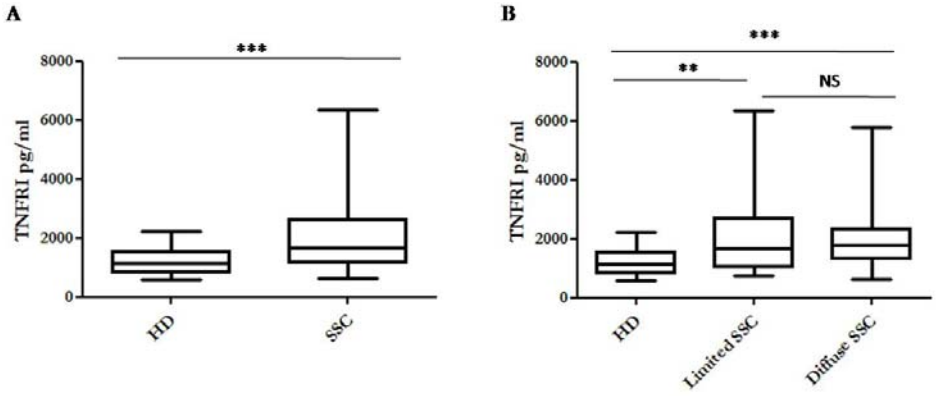
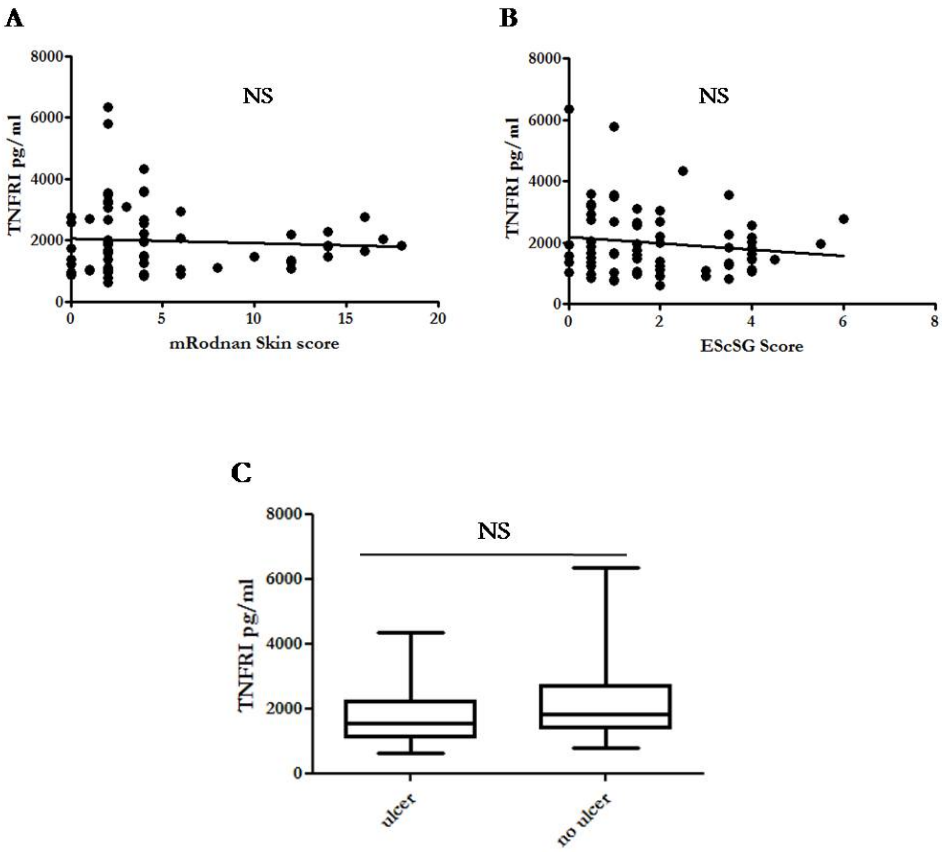
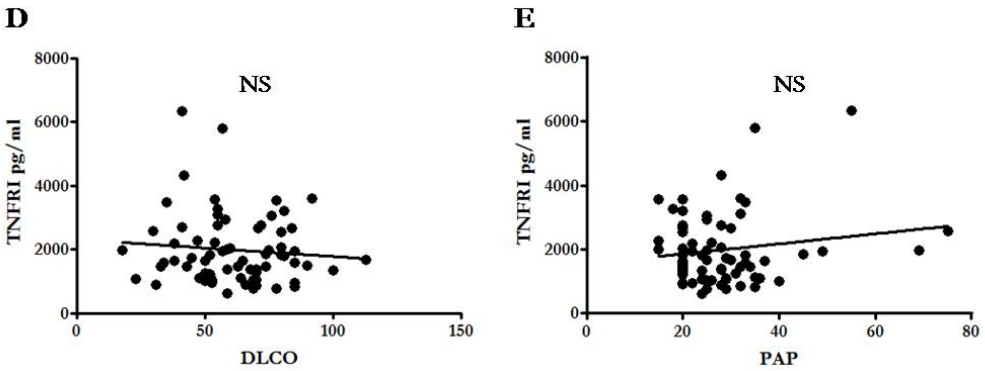


Figure 4. Tumor necrosis factor receptor I (TNFRI) plasma levels significantly increased in SSC patients. A) TNFRI levels were measured by ELISA assay in the plasma of SSC patients and HD. **B)** Mean TNFRI plasma concentrations were determined in two subgroups of SSC patients with limited or diffuse forms and compared to HD. Mean values, maximum and minimum were plotted in the graphs; *** p≤0,001 **p≤0,01.

We next analyzed the possible association of TNFRI plasma concentrations with disease activity, evaluated by the modified Rodnan skin score and the EScSG score, however without finding any significant correlation (Figure 2A and 2B Supplemetary). Moreover, TNFRI plasma levels were correlated with the presence of active digital ulcers, interstitial lung disease and pulmonary arterial hypertension. These analysis did not show any significant variation of TNFRI levels, which resulted independent from all the investigated clinical variables (Figure 2C, 2D, 2E Supplementary).





Supplementary Figure 2. Correlation of TNFRI plasma levels with disease activity, vascular and pulmonary involvement. A) TNFRI plasma levels, measured in the peripheral blood of SSC patients, were correlated by linear regression analysis with the extension of skin involvement and the disease activity, investigated by the mRodnan Skin Score **(A)** and the EScSG Score **(B)**. TNFRI plasma levels were further correlated with the presence or absence of ulcers **(C),** with the diffusion capacity of the lung (DLCO) **(D)** and with the presence of pulmonary arterial hypertension detected through pulmonary artery pressure (PAP) **(E)**.

Alterations of B10-cells frequency in SSC

To determine whether peripheral blood B10-cell numbers were altered in SSC patients, we examined the frequencies of B10-cells in 20 SSC patients (10 limited SSC and 12 diffuse SSC) compared to HD (Figure 5A). B10-cell frequencies, expressed as percentage of CD19+ cells on gated PBMCs, resulted significantly increased in SSC patients compared to HD (mean ± SEM = 10.56 % ± 0.854 and 4.9 % ± 0.497; p ≤0.0001). Considering the different subgroups of SSC patients, B10-cells frequencies resulted comparable in limited and diffuse SSC (mean ± SEM= 10.5 % ± 1.482 in limited SSC, 10.15 % ± 0.964 in diffuse SSC; p ≤0.01 and p ≤0.001 respectively)(Figure 5B).

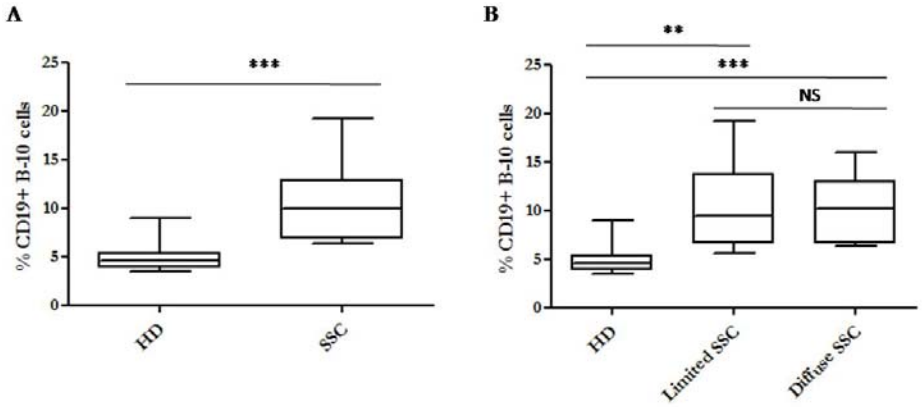
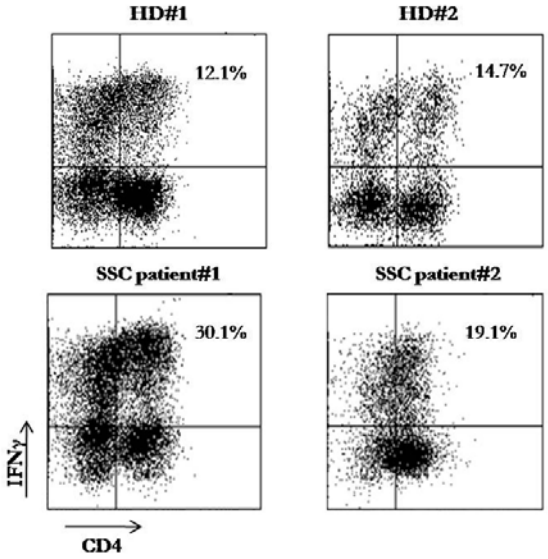


Figure 5. Increased frequency of B10-cells in SSC patients. A) The frequency of B10-cells, evaluated as CD19+ IL10+ cells, was measured in the peripheral blood of SSC patients and in HD after 48 hours of stimulation with CD40L and Cpg. PMA, ionomycin and brefeldin A were added during the last 6 hours of culture. **B)** B10-cell frequencies were analyzed in limited and diffuse SSC patients and compared with HD. Mean frequencies of CD19+ IL10+ B cells, minimum and maximum were plotted in the graphs; *** p≤0,001 **p≤0,01.

We then studied the functionality of B10-cells, by analyzing their ability to suppress T cell-mediated cytokine production. In particular, after stimulating PBMCs from HD with anti-CD3, the mean percentage of CD4+ IFN-γ producing T cells resulted 14.1% ± 3.38 (n=5) (Supplementary Figure 3). Interestingly, unless higher frequencies of B10-cells in the peripheral blood of SSC patients, after stimulating patient PBMCs under the same conditions, the mean percentage of CD4+ IFN-γ producing T cells resulted slightly increased compared to HD (mean CD4+ IFN-γ+= 26.6% ± 6.4, n=5) (Supplementary Figure 3).



Supplementary Figure 3. IFN-γ production by CD4 T cells of SSC patients and HD. The frequency of CD4+ T cells producing IFN-γ was measured in the peripheral blood of HD (upper panels) and SSC patients (lower panels), after culturing PBMCs for 72hours in the presence of soluble anti-CD3. Two representative flow cytometry plots out of five were shown for each group.

DISCUSSION

Systemic scleroderma (SSC) is a multisystem disease characterized by an unpredictable course, high mortality and resistance to therapy[1]. At present, there are no specific biomarkers able to accurately predict disease activity and response to treatment[38]. Therefore, new biomarkers are needed to help clinicians with objective indications to improve SSC patient management and outcomes. Elafin represents a newly discovered promising biomarker for different inflammatory immune-mediated conditions, such as psoriasis[12,13] and Graft-versus-Host Disease[14]. We demonstrated for the first time that the plasma concentrations of elafin are significantly increased in both limited and diffuse SSC patients compared to healthy controls. Even if we could not find any correlation with single clinical parameter, such as the presence of digital ulcers, interstitial lung disease and pulmonary arterial hypertension, we highlighted an inverse correlation of elafin plasma levels with the modified Rodnan skin score and with the EScSG score. In particular, the recently validated EScSG score, a reliable index to assess disease activity in SSC, represents a feasible instrument that evaluates both clinical and laboratory items, including the modified Rodnan skin score (MRSS), vascular and cardiopulmonary symptoms together with the measurement of acute phase reactants[35,36,37]. The significant inverse correlation demonstrated between plasma concentration of elafin and the above mentioned disease activity scores leads us to hypothesize a protective role of elafin in SSC pathology. This theory is in accordance with recent studies which have demontrated the therapeutic value of elafin in restraining

tissue damage and preserving organ function in ischemia-reperfusion injury mice models, thus showing a promising protein role in the treatment of inflammatory vascular, systemic and pulmonary diseases[38,39,40,41,42]. In view of all these findings, further studies are needed to understand if elafin could represent a possible future therapeutic approach for SSC disorder. Differently from previous findings about elafin expression in tissues affected by several inflammatory, immune-mediated pathologies, such as Graft-versus-Host Disease and psoriasis[12,13,14], biopsies of target organs mainly involved in SSC, such as skin, lungs and heart, did not show any elafin expression. However, this observation is in accordance with our data at the plasmatic level describing low elafin levels in patients with more severe disease. In particular, the lack of the protein in biopsies of SSC patients with high disease activity score or bad prognosis, could mirror the absence of a potentially protective pathway. Further analysis correlating elafin expression in SSC involved tissues with the disease severity are needed to corroborate our hypothesis.

Activated T lymphocytes are detectable in the blood as well as in the affected organs of SSC patients, presenting, beyond their activated phenotype, an alteration in number and frequency[43,44,45]. TNFRI is synthesized as a membrane-anchored protein, subsequently released in circulation by activated T cells[15,16]. It has already been demonstrated that TNFRI represents a disease biomarker in autoimmune pathologies, such as systemic lupus erythematosus[17,18,19]. Furthermore, TNFRI, in association with other plasma biomarkers, has a prognostic and predictive value in acute Graft-versus-Host Disease, supporting the stratification of patients and

helping clinicians to timely adopt anti-GVHD treatment[46]. We demonstrated an increased plasma concentration of TNFRI in SSC patients compared to healthy donors, independently from the specific organ involvement, but in accordance with the activation of the T-cell compartment, which has been widely documented to mediate damage in different SSC involved organs.

Considering the pivotal role of activated T lymphocytes in SSC pathogenesis and chronic inflammation, we next analyzed a recently identified subset of B cells with immune-regulatory activity, characterized by the ability to modulate T cell inflammatory responses partially via IL-10 production, named B10-cells[20,21]. According to what described in other autoimmune conditions, a significant increased frequency of regulatory B10-cells was observed in SSC patients compared to healthy donors. Interestingly, unless higher frequencies of B10-cells in the peripheral blood of SSC patients, the stimulation of PBMCs from HD and SSC patients with anti-CD3 revealed a trend of increase in CD4+ T cell producing IFN-γ in SSC patients. This preliminary finding suggests a possible functional impairment of the regulatory B compartment in SSC disease, potentially resulting in a chronic T cell mediated production of cytokines responsible for organ damage. Further studies with a larger cohort of patients will be needed to better clarify the potential alteration of B10-cell frequency and functionality in SSC disease. If our data will be confirmed, the impairment of the axis B10-cells - T lymphocytes, could represent a promising cellular pathway to target for the treatment of SSC patients.

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CONCLUSIONS AND FUTURE PERSPECTIVES

Systemic scleroderma (SSC) is an orphan multisystem autoimmune disorder characterized by significant morbidity and mortality[1]. Although there have been significant progresses over the years in therapeutic options[2,3], the mainstay for ameliorating SSC diagnosis and management lies in the development of innovative treatments, thanks to a better understanding of SSC pathogenesis and in the identification of new easily measurable disease markers. In the last years many groups focused their attention on the identification of potential soluble biomarkers able to accurately predict disease activity[4], but, up to date, no specific molecule has been identified. In our work we evaluated if elafin and TNFRI, two recently described plasma biomarkers of disease activity in other inflammatory immune-mediated conditions[5,6,7], could represent potential biomarkers also in the case of SSC pathology. We observed a significant inverse correlation between plasma concentration of elafin and the modified Rodnan skin score and the EScSG score[8,9], leading us to hypothesize the potential role of elafin as biomarker of disease activity and its protective role in SSC physiopathology. This findings are in line with the absence of the protein observed in skin, lung and heart biopsies of SSC patients with high disease activity score or bad prognosis and with the recently proposed therapeutic role of elafin in the treatment of inflammatory vascular, systemic and pulmonary diseases.

In view of all these observations, to understand if elafin could represent a possible future therapeutic approach for SSC, we will test the effect of its injection in different mouse models of the pathology. In detail,

we will firstly set up a modified model of Graft-versus-Host induced Systemic Sclerosis, already described and characterized by the group of Dr. Garman[10,11], which recapitulates several features of the human pathology. By injecting spleen cells from B10.D2 mice into RAG-2 knock out mice on the Balb/c background, is possible to obtain a SSC model characterized by dermal thickening, progressive fibrosis of internal organs and autoantibody generation. In this SSC model, we will assess if the injection of a single dose or repeated administrations of elafin could impact on the pathology, by evaluating at different time points the disease severity in different organs, such as skin, lung and heart and the presence of autoantibodies in the peripheral blood.

In accordance with the pathogenic role of T-cells, widely documented in SSC[12,13], we further observed increased plasma concentration of the TNFRI, a membrane protein released in circulation by shedding from the activated T cell surface, in SSC compared to healthy donors. It has already been demonstrated the role of TNFRI as a disease biomarker with a prognostic and predictive value in other autoimmune pathologies, such as systemic lupus erythematosus[14,15,16] and acute Graft-versus-Host Disease[17]. So far, we are planning to analyze plasma levels of the molecule in a large cohort of patients presenting nonspecific alteration at naildfold videocapillaroscopy, eventually associated with the presence of autoantibodies, in order to assess if TNFRI levels could be used as predictive factor for risk stratification.

In view of the important role of activated T cells in SSC pathogenesis and development, we analyzed a recently identified subset of B cells, characterized by the ability to suppress T cell inflammatory cytokine production partially via IL-10 production, named B10-cells[18,19]. In line with other autoimmune conditions, a significant increased frequency of regulatory B10-cells was observed in SSC patients compared to HD. Interestingly, unless higher circulating frequencies of B10-cells in SSC patients, we observed percentages of CD4+ IFN-γ producing T cells comparable and even slightly increased in SSC patients compared to HD, suggesting a possible functional impairment of the B regulatory subset. To corroborate these preliminary data, we are planning to culture B10-cells, characterized by $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype and sorted by flow cytometry, 1:1 with autologous magnetic-bead purified CD4+CD25 T cells. Cultures will be then stimulated with an anti-CD3 antibody and intracellular stained to evaluate the frequencies of IFNγ CD4+ T cells. We will next observe whether the depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells from PBMCs of HD and SSC patients might impact the production of pathogenic pro-inflammatory cytokines by T cells. All these studies in a large cohort of patients will elucidate the potential alteration of B10-cell functionality in SSC disease and their role in SSC pathogenesis.

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