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Pathogenesis of Systemic Sclerosis: pro-inflammatory role of ET-1 receptors

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1. SYSTEMIC SCLEROSIS

1.1. DEFINITION AND GENERAL ASPECTS

Systemic Sclerosis (SSc), or scleroderma, is a rare autoimmune disease that involves the connective tissue of skin and internal organs, with a remarkable heterogeneity in the clinical features and course of the disease, resulting in high morbidity and mortality. The disease is characterized by severe and diffuse endothelial cell damage and by overproduction and accumulation of collagen and other extracellular matrix proteins, resulting in thickening of the skin and fibrosis of the affected organs. The pathogenic mechanisms involve three main components: a) vascular dysfunction and injury, b) activation of the immune system and c) increased secretion of collagen by fibroblasts.

Currently the standard classification criteria for SSc are the 1980 preliminary criteria for the classification of SSc, suggested by the American College of Rheumatology. These criteria allow the identification of three different forms of the disease: localized cutaneous scleroderma, SSc and overlap syndromes [1].

Localized scleroderma is characterized only by skin involvement. The main subtypes are plaque morphea, linear scleroderma, generalized morphea, and pansclerotic morphea. With few exceptions, the disorder does not have systemic involvement, but it can cause considerable morbidity. These forms are more common in childhood and are rarely associated with systemic involvement, unlike SSc [2,3].

SSc is characterized by affecting the skin and various organs and apparatuses. Considering the cutaneous extension of fibrosis, two subsets of illness can be identified: limited SSc (lSSc) and diffuse SSc (dSSc) [4-8]. The limited form is characterized by thickening of the

skin of the face and of the distal part of the limbs. Usually, cutaneous sclerosis arises after a long history of Raynaud's phenomenon, which sometimes precedes the systemic disease for decades. Visceral involvement is usually present although delayed: pulmonary hypertension can complicate the course of the disease in 10-15% of cases only after 3 years after the onset of the disease. In 70-80% of the cases lSSc is associated with the presence of anti-centromeric antibodies. The diffuse form of SSc is characterized by a cutaneous thickening that involves also the proximal portions of the limbs and often also the trunk and abdomen. Usually cutaneous sclerosis appears simultaneously with Raynaud's phenomenon or a short while after its onset. Initial visceral involvement is frequent. dSSc is associated in 30% of cases with the presence of autoantibodies against DNA topoisomerase-1 (Scl-70).

There are also cases of Systemic Lupus Erythematosus, Rheumatoid Arthritis, Sjogren's Syndrome and other types of connectivitis which develop cutaneous and/or visceral sclerosis. In these cases, a diagnosis of Overlap Syndrome can be performed when the criteria for diagnosis of SSc and of another autoimmune disease are met.

Rarely, in 1% of cases, SSc can manifest without cutaneous involvement; this is then defined as SSc without scleroderma [6].

Recently, because of the low sensitivity of the 1980 criteria, and as a result of advances in the knowledge of SSc, the ACR and the European League Against Rheumatism (EULAR) have established a committee to suggest a joint proposal for new classification criteria for SSc. The aims are to develop criteria that 1) encompass a broader spectrum of SSc including patients whose disease is in the early stage as well as those in the late stage; 2) include vascular, immunological

and fibrotic manifestations; 3) are simple enough to be used in daily clinical practice; and 4) are in accordance with the criteria used for diagnosis of SSc in clinical practice [9]. These criteria may be endorsed as inclusion criteria for SSc studies. However, such criteria need to be validated in different populations before accepted in routine practice.

1.2. EPIDEMIOLOGY

The epidemiology of SSc is difficult to estimate. Varying prevalence and incidence rates of SSc have been reported due to the differences in the geographical area surveyed, the definition of the disease and the method of case ascertainment [10]. It is estimated that SSc has a prevalence between 50 and 300 cases per million inhabitants and an incidence ranging from 2.3 to 22.8 cases per million inhabitants per year in the general population [11].

The disease shows a marked predilection for the female sex, with a female/male ratio varying from 3:1 to 14:1 according to different studies. The onset of the disease can occur at any age; however subjects between the third and fifth decade of life are preferentially hit. Higher incidence and a younger age of onset are reported among African-Americans [12]. The latter have also a greater probability of developing diffuse cutaneous disease and pulmonary fibrosis; generally they have a worse prognosis than Caucasians [7].

1.3. AETIOPATHOGENESIS

SSc is a disease with sporadic onset and an aetiology which is not yet fully known. Besides genetic factors, various environmental factors may play an important role in the aetiopathogenesis of the disease. We

still lack an overall view of the process which eventually leads to sclerodermic damage. In the last few decades research has made considerable progress, trying to have a deeper inside into the three fundamental aspects of the disease: the vasculopathy; the alterations of cellular and humoral immune response; the excessive deposition of collagen and other macromolecules in the skin and in the target organs. One of the objectives of the present study is to try to identify initial events that eventually lead to the onset of the disease. The comprehension of these pathogenetic aspects will enable us to identify new potential therapeutic targets.

1.4. GENETIC FACTORS

The role of genetic factors is supported by the fact that different cases have been observed in the same family, by the frequency of SSc is higher in families already suffering from other autoimmune pathologies, and by the greater risk of developing the disease in certain ethnic groups than in others. Twin studies have highlighted a greater probability of developing the disease among monozygote twins compared with dizygote twins, although with low concordance percentages (lower than 5%) [7]. Various studies have shown that SSc is associated with particular gene polymorphisms which code for cytokines and their receptors, chemokines and proteins of the extracellular matrix [13]. Furthermore, there is evidence of association between certain class II histocompatibility antigens, presentation of the disease and autoantibody structure [14].

1.5. ENVIRONMENTAL FACTOR

Environmental factors may play a role in triggering the disease on predisposing genetic background. Recent studies underline the importance of epigenetic modifications (methylation, acetylation, phosphorylation, sumoylation, microRNA) [15] that may link genes and environment in the pathogenesis of SSc.

The principal environmental factors involved are: viruses, drugs, radiation and chemicals.

The hypothesis that infectious agents may be involved in the pathogenesis of the disease is based on different mechanisms; the most studied mechanism is the presence of a molecular mimicry between autoantigens and proteins of viral origin. It is interesting to note that there is a certain structural similarity between retroviral proteins and topoisomerase I, an antigen recognized by anti-Sc1-70 autoantibodies. It has emerged that the human Cytomegalovirus (HCMV) may contribute to the onset of SSc through its ability to infect endothelial cells and to induce cell activation and apoptosis. Patients affected by SSc present antibodies directed against the HCMV derived protein UL94; such antibodies are able to induce apoptosis of human endothelial cells and to stimulate fibroblast proliferation: two typical aspects of SSc [16-19].

Occupational chemicals which could play a role in the onset of SSc are: silica dust, polyvinyl chloride, trichloroethylene, organic solvents. Indications have also been reported for pesticides, hair tinctures and industrial fumes [20]. Cigarette smoke seem not associated with SSc; however, exposure to cigarette smoke is capable of aggravating and accelerating the course of sclerodermic vasculopathy and lung involvement.

Drugs correlated with the development of SSc-like syndromes are bleomycin (a fibrosing agent), pentazocine, cocaine and fenfluramine, which is associated with the onset of pulmonary hypertension. Even silicone prosthetic mammary implants had initially been associated with the onset of the disease, but successive large-scale epidemiological studies have not confirmed this initial observation.

Radiant treatment for neoplasia has been associated both with the exacerbation of pulmonary fibrosis in sclerodermic patients, and with the onset *de novo* of the disease [7].

1.6. SCLERODERMIC VASCULAR DAMAGE

Vascular damage is considered the *primary event* in the pathogenesis of SSc [21]. It is a systemic process which involves the small blood vessels, in particular the arterioles. It precedes fibrosis [22,23] and can potentially affect any organ [24,25].

Endothelial cells are the first cells to be involved in sclerodermic damage. Alterations of endothelial cells lead to: increased expression of adhesion molecules, recruitment and activation of leucocytes, thickening of the basal lamina, proliferation of pericytes, fibroblasts and smooth muscle cells, platelet adhesion and activation, and formation of perivascular infiltrate. Perivascular infiltrate is represented in the first phases by myeloid dendritic cells, by CD4+ T lymphocytes and by mastocytes, and in successive phases by polymorphonucleates, B and T lymphocytes [26-31]. Growth factors, oxidative stress and cytokines stimulate transdifferentiation of the fibroblasts into myofibroblasts within the vessel wall, with consequent thickening of the vessel wall itself and further reduction of the regional blood flow by narrowing the vessel lumen. This is

exacerbated by activation of platelets and of the coagulation system, with the formation of microthrombi. Unrespective of the progressive loss of capillaries and of the high plasma levels of endothelial growth factor (VEGF) in response to tissue hypoxia, a deficit in angiogenesis is observed [32]. Many consequences of the endothelial damage are related to imbalance of vasoactive factors, with hyperproduction of endothelin-1 and hypoproduction of nitric oxide and prostacyclin. Imbalance of these mediators has an effect not only on vascular tone, but also on inflammation, on platelet activation, on myofibroblastic induction and on fibrosis, and therefore favouring the perpetuation of vasculopathy.

Chronic perivascular inflammation, in advanced sclerodermic lesion, is replaced by fibrosis, which contributes to the permanent alteration of the vessel architecture, with thickening of the vessel wall and progressive narrowing of the lumen. At cutaneous level, fibrosis begins at the deep derma and extends to involve the entire derma and the more superficial layers, and a similar course is encountered in the other organs affected by the disease. The composition of the extracellular matrix varies according to the stage of the disease: proteoglycans and elastic fibres such as fibrillin are typical of the first phases of the fibrotic process, while type I collagen is found in the advanced phases.

Although the initial manifestations of disease are often limited to Raynaud's phenomenon, sclerodermic vascular damage is not limited to the skin and at the extremities; indeed the clinical consequences can be widespread and associated with organ malfunction and significant morbidity and mortality [33].

1.7. IMMUNOLOGICAL DYSFUNCTION

Immunological alterations are a typical feature of SSc and it still unclear whether they are precede or follow vascular damage; it is, however, clear that activation of the immune response is an early event in the development of the illness. Alterations both of the "first line" defences, such as innate immunity, and of the adaptive immune system, may induce vascular damage, precipitate autoimmune responses, favour the production of autoaggression, and eventually lead to tissue fibrosis [34].

1.7.1. INNATE IMMUNITY

Toll-like Receptors

In the initial phases of the sclerodermic lesion the perivascular infiltrate is mainly represented by myeloid dendritic cells and by CD4+ T lymphocytes [26,27]. In the early stages of the disease the myeloid dendritic cells of patients with SSc show an increased response mediated by Toll Like Receptors (TLR) [35,36]. The TLRs are a family of highly conserved receptors, which recognize stereotyped non-self molecular patterns, common to numerous pathogenic micro-organisms. Among the ligands there are components of the bacterial surface, such as lipopolysaccharides (LPS) or endotoxin, and RNA and DNA ligands; various ligands can also be generated by molecules of the extracellular matrix during tissue damage processes [37,38]. TLRs characterize innate immunity cells and can be located on the cell surface, like TLR4, or at endosomal level, like TLR3. Activation of the TLRs on dendritic cells and on monocytes/macrophages stimulates the production of cytokines, antigen presentation and trigger the adaptive immune response.

In SSc patients the detection of high circulating levels of pro-inflammatory mediators, such as TNF-α and IL-6, often secreted by myeloid dendritic cells after stimulation via TLR [35,39], combined with observation of the presence of endogenous agonists for TLR4 in the serum [40], supports a role for dendritic cells and TLRs in the early stages of the disease. There is also the interesting observation that polyinosinic/polycytidylic acid, a TLR3 ligand, is capable *in vitro* to stimulate the production of type I interferon and TGF-β by dermic fibroblasts of patients with SSc; also, when injected subcutaneously in mice, it is able to induce cutaneous fibrosis [39].

Further studies on TLR7, show that its activation can induce maturation of autoreactive B lymphocytes [41]. Activation of TLRs may also lead to fibrosis, probably via TGF-α [42]. Recent observations suggest that activation of TLRs may directly stimulate myofibroblastic transdifferentiation through TLR3 [43] and/or TLR9 [44].

Interferons

Patients with SSc show an increased expression of genes responsive to interferon (IRGs) [45,46]. Interferons (IFNs) are distinguished into Type I IFNs, such as IFN-α and IFN-β; Type II, such as IFN-γ, and Type III. Dendritic cells, in particular plasmacytoids (pDCs), are the principal source of IFN-α; they produce large amounts of this cytokine following activation of TLR7 or TLR9. The serum of patients suffering from SSc can stimulate the production of IFN-α by peripheral blood mononuclear cells (PBMC) [47]. Fibroblasts are the principal producers of IFN-β. Th1 lymphocytes and NK cells are the principal producers of IFN-γ.

Monocytes/macrophages, IL-4 and IL-13

Activation of the monocytes/macrophages can occur by means of IFN-γ, a cytokine typically produced by the Th1 lymphocytes, or by means of IL-4 and IL-13, typically produced by the Th2 lymphocytes. IL-4 is capable of favouring the production of collagen by the fibroblasts, and increases the production of TGF-β. IL-13 can stimulate the monocytes/macrophages to develop a profibrotic phenotype, probably by stimulating the production of TGF-β, and this activation may play an important role in fibrosis in patients affected by SSc. In line with these findings it has been observed that the alveolar macrophages of patients with pulmonary fibrosis produce high quantities of IL-13; serum levels of IL-13 are high in patients suffering from SSc [48-50].

1.7.2. ADAPTIVE IMMUNITY

T Lymphocytes

SSc is typically considered a predominant Th2 disease [51]. Indeed in the serum of SSc patients a preferential production of cytokines derived from Th2 lymphocytes, such as IL-4, IL-13, IL-6 and IL-10 has been observed. IL-4 and IL-13 are among the principal fibrogenic cytokines in SSc; they are also able to promote the production of antibodies. High levels of IL-17, produced by Th17 lymphocytes have also been observed in the serum and the derma of patients with SSc. This cytokine favours the proliferation of fibroblasts and stimulates the macrophages to produce TNF-α and IL-1, which in turn stimulate the fibroblasts to produce collagen. IL-17 also stimulates endothelial

cells to produce IL-1 and IL-6, and to express the adhesion molecules ICAM-1 and VCAM-1.

Although Th2 cells play a crucial role in fibrogenesis and in inducing the humoral response, it seems that Th1 cells producing IFN-γ play an important role in SSc. Indeed, an increased percentage of IFN-γ positive cells among the circulating T cells is observed in the peripheral blood of patients. It is therefore possible that Th1 cytokines such as TNF-α and IL-1 are involved in the pathogenesis of SSc, particularly in the very early stages of the disease. The Th2 cells are principally activated by the B lymphocytes producing IL-6 and IL-10, while the Th1 cells are principally activated by the dendritic cells producing IL-12. CD4+ cells represent the majority of the T lymphocytes infiltrating the sclerodermic skin, whereas CD8+ cells represent the majority of the T cells infiltrating the pulmonary interstitium and are responsible for alveolitis. Both CD4+ T cells in the skin and CD8+ T cells in the lung are responsible for the fibrotic process. The expansion of T cells in infiltrated tissues seems to be oligoclonal, suggesting an antigen driven T cell response, although the putative antigens are in large part unknown [52-54].

sclerodermic lesions, while they are not reduced in peripheral blood [55]; indeed they are often increased in the serum of patients and their levels correlate with the activity and severity of the disease [56], probably because defective activity of these cells in SSc [57].

B lymphocytes

B cells are activated in SSc. This is suggested by the frequent presence of hypergammaglobulinaemia, by the production of autoantibodies and by the overexpression of CD19 in the peripheral

blood cells. Moreover expansion of naïve B cells and activation of B memory cells have been reported in SSc patients; however the number of the latter is reduced by apoptosis. It is possible that the continous loss of memory B cells leads to increased medullary production of naïve B cells, so that B cell homoeostasis is maintained.

Polymorphisms have been demonstrated in the genes of CD19 and CD22 in sclerodermic patients. These are associated with hyperexpression of CD19, a molecule implicated in positive selection of B cells in their process of development in the bone marrow and the lymphoid organs, and with hyperexpression of CD22, a molecule involved in negative selection.

For activation, production of autoantibodies and accumulation in sclerodermic lesions, B lymphocytes require interaction with T lymphocytes. This cross-talk, crucial in the pathogenesis of SSc, is carried out by B cells presenting the antigen to CD4+ lymphocytes through MHC class II molecules and also by B lymphocytes secreting cytokines such as IL-6, IL-10 and TGF-β.

The activated B lymphocytes contribute to the fibrosis: indeed IL-6 and IL-10 promote a predominant Th2 immune response, which favours collagen synthesis. In addition, IL-6 as well as TGF-β production by activated B cells, directly induces fibrosis [53,54,58].

Autoantibodies

SSc is characterized by the production of various autoantibodies, some of which are important diagnostic markers.

Usually autoantibodies directed against intracellular antigens are correlated with particular clinical subsets: typically, the diffuse form is associated with the presence of anti-Scl-70 antibodies, while the

limited form is associated with the presence of anti-centromere antibodies. The association is also known between particular autoantibodies and certain clinical manifestations: the presence of anti topoisomerase-1 autoantibodies correlates with an increased risk of interstitial lung disease; anti-RNA polymerase-3 autoantibodies correlate with a greater probability of developing sclerodermic renal crisis, while they are rarely present in patients with severe pulmonary involvement [59,60]. Anticentromere and anti-U3 RNP autoantibodies are frequently found in the serum of patients with pulmonary hypertension [61]. Anti-U3 RNP autoantibodies are also associated with sclerodermic cardiopathy. Anti U11/U12 RNP autoantibodies are present in sclerodermic patients with pulmonary hypertension and they seem to be correlated with the severity of the prognosis [62].

Although these autoantibodies correlate with the severity of the disease and with the risk of developing complications, they are commonly believed to have no pathogenetic role. Autoantibodies against surface antigens, such as autoantibodies against endothelial cells [63], the PGDF receptor [64], the receptor for angiotensin-1 and the receptor ET_A for ET-1 [65], are thought to have a pathogenetic role in triggering the endothelial cell damage and the fibrotic process.

Among the anti-endothelial cell autoantibodies, anti-NAG2 antibodies are present in large numbers of SSc patients, without differences between the limited and diffuse clinical forms. NAG2 is a tetraspanin expressed on the surface of the epithelial cell associated with integrins. It has a high sequence homology with the HCMV derived protein UL94; patients present cross-reactive antibodies which can recognize the viral epitope of HCMV and NAG2 through a molecular mimicry mechanism. In vitro, it has been demonstrated that anti-

NAG2 antibodies are able to induce apoptosis of the epithelial cells and to promote the proliferation of fibroblasts. These antibodies could thus induce the disease by directly favouring two of the basic pathogenetic mechanisms of SSc: endothelial damage and fibrosis [18] (Figure 1).

2. ENDOTHELIN-1

Endothelin-1 (ET-1) is a key mediator known for its vasoconstrictor effect. Various other effects of ET-1 have recently been identified: inflammation, cell adhesion, fibrosis and angiogenesis. All these aspects are important in the pathogenesis of SSc. For these reasons, besides the anti-vasoconstrictor effect, ET-1 receptor antagonists (ERAs) represent one of the most promising therapeutic options in sclerodermic patients.

It has been shown that ET-1 and its receptors play an important role in the pathogenesis of various diseases, among them: atherosclerosis and cardiovascular pathologies, pre-eclampsia, renal pathologies, pulmonary pathologies including pulmonary hypertension, carcinogenesis and other fibrosing pathologies besides SSc.

2.1. ISOFORMS AND EXPRESSION

There are four isoforms of endothelin: ET-1, ET-2, ET-3 and ET-4.

ET-1 was first identified in endothelial cells. It is expressed by a large number of cells, among them smooth muscle cells in the vessel wall, fibroblasts and myofibroblasts, mastocytes, monocytes/macrophages, polymorphonucleates, dendritic cells and many others [66,67]. ET-2 is mainly expressed by intestinal and renal cells. ET-3 is found at high concentrations in CNS. Little is known about ET-4 (Table I).

2.2. SYNTHESIS

The gene coding preproET-1 is located on the short arm of chromosome 6, distally to locus HLA. Activation of this gene is regulated by transcription factors among which c-fos, c-jun, NFkB-1, AP-1 and GATA-2. Early transcription codes for preproET-1 which,

following post-transcriptional modifications, is first converted to pre-ET-1 and then to big-ET-1. The latter, which is also present in the extracellular compartment and in the serum, is then converted to ET-1 by dedicated enzymes (Figure 2).

Production of ET-1 is stimulated by hypoxia, by exposure to cold, by low wall tension, by angiotensin II, by growth factors and by cytokine including TGF-β [66,67]. Its production is inhibited by nitric oxide (NO), by prostacyclin, by natriuretic peptide, by increase in blood flow and by increase in wall tension [68].

2.3. ENDOTHELIN CONVERTING ENZYME AND MATRIX METALLOPROTEASE-1

Endothelin converting enzyme (ECE) and matrix metalloprotease-1 (MMP-1) have opposite effects on the metabolism of endothelin: the first is the principal activator, the latter is its principal inactivator.

ECE is a protease, bound to the plasma membrane or the Golgi network membrane, which cleaves BigET-1 into the biologically active form: ET-1 [66,69]. Three isoforms of ECE are known. ECE-1, comprising the isoforms ECE-1a, ECE-1b, ECE-1c, ECE-1d, performs its function optimally if the pH is around 7 and is expressed preferentially by the smooth muscle cells. Its function is not only to hydrolize Big-ET, but also to hydrolize other molecules such as bradykinin, substance P and insulin. It has a greater specificity for ET-1.

ECE-2 is expressed in the membranes of the Golgi network. It is found in large quantities in the cells of nervous system and in the endothelial cells, and works optimally at a pH of 5. It has greater specificity for ET-1. Little is known about the ECE-3.

MMP-1 cleaves mature ET-1, making the peptide biologically inactive [70,71].

2.4. SECRETION

Secretion of ET-1 occurs by means of granules after stimulus and constitutively as a basal secretion [65]. In the first case the mechanism is regulated and secretion occurs only through specific stimuli: hypothermia, mechanical stress, or through the action of histamine and thrombin. Basal secretion on the other hand is carried out principally by the basolateral portions of the endothelial cell as a continuous secretion; however, it can be affected by vasodilatation and by the release of NO.

3. RECETTORI DELL'ET-1ET-1 RECEPTORS

3.1. ISOFORMS

Three receptor isoforms are known: ET_A, ET_B and ET_C.

ET_A binds both ET-1 and ET-2 with high avidity and ET-3 with low avidity; it is more specific for ET-1. ET_B binds ET-1, ET-2 and ET-3 with the same specificity. Little is known about the role of ET_C.

Endothelin receptors are expressed on various cell types. The effects depend, besides the type of receptor bound and the cell type stimulated, also on the tissue conditions. Indeed both the number and the avidity of the receptors can be different in pathological conditions, such as during an inflammatory process [65,71].

3.2. EARLY AND LATE RESPONSES TO ET-1.

Early responses mediated by the endothelin receptors occur within a few minutes and are transient. They principally promote cellular contraction.

Late responses are slower and take hours or days to be evident. They consist of transcriptional activation of genes important for regulating the cell matrix, strengthening the contractile function of the smooth muscle cells, and altering the tissue composition [72].

3.3. EXPRESSION AND ROLE OF ET_A AND ET_B

There is evidence that the majority of cells potentially involved in the pathogenesis of SSc, among them endothelial cells, smooth muscle cells, fibroblasts and myofibroblasts, monocytes/macrophages,

polymorphonucleates, mastocytes, platelets and dendritic cells express the receptors for ET-1 [65,66,69,71]. These have also been observed on myocardiocytes. ET-1 also plays an important role in cardiac remodelling (Table II).

3.4. ET-1 RECEPTORS IN ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS OF THE VESSEL WALL

Endothelial cells express exclusively ET_B, while smooth muscle cells express both ET_A and ET_B.

ET-1 binds ET_B on endothelial cells and stimulates the production of NO, a potent vasodilator, by eNOS activation. In physiological conditions the vascular smooth muscle cells receive stimuli both from NO and from ET-1, in such a way that there is a balance between relaxation and contraction of the myofibrils (Figure 3).

ET-1, binding ET_A on smooth muscle cells, induces both signal transduction mechanisms capable of promoting contraction and pathways which induce hypertrophy and hyperplasia [73,74] (Figure 4).

3.5. ET-1 RECEPTORS ON FIBROBLASTS

Fibroblasts are cells capable of producing large quantities of collagen. Various profibrotic stimuli converge on them; among these an important role is played by ET-1 and TGF-β, besides the Th2 cytokines and IL-17.

The fibroblasts express both ET_A and ET_B and are capable of producing ET-1. Some studies suggest that ET-1 can activate the production of collagen and induce the expression of ICAM-1 on the surface of fibroblasts. It may also stimulate the fibroblasts themselves

to transdifferentiate into myofibroblasts [75,76] and to reprogram the functional phenotype of the smooth muscle cells and of the microcirculation pericytes into a pro-fibrogenic cell population with myofibroblastic properties [77]. Furthermore, it has been observed that the production of collagen by fibroblasts induced by ET-1 is not reduced by the selective antagonism of ET_A or ET_B, while it is reduced by combined ET_A/ET_B antagonism. This would suggest the existence of cross-talk between the two receptors inducing signal transduction pathways that lead to the production of collagen. Moreover, the combined ET_A/ET_B antagonism reverts the phenotype of the fibrotic fibroblasts to that of normal fibroblasts [76,78]. Conversely, the selective ET_A blockage does not reduce the production of collagen whereas it reduces the expression of β-SMA and the contraction of the extracellular matrix [77].

Various mechanisms have been proposed for the signal transduction pathways involved in the induction of tissue fibrosis stimulated by ET-1 [76,79]. These would imply cross-talk between the ET_A and ET_B receptors, which are associated with protein Gq/12/13, with the activation of signal transduction pathways which would stimulate the expression of α-SMA, cellular contraction and the production of collagen [77].

The importance of ET-1 in the genesis of fibrosis is strengthened by evidence of the existence of autocrine loops producing ET-1 and of induction of fibrosis by the fibroblasts. The latter in fact can both express the receptors ET_A and ET_B and produce ET-1. TGF-β may function as a trigger for establishing these loops which could potentially be self-maintaining. TGF-β and ET-1 would therefore work in concert in the pathogenesis of fibrosis in SSc. In fibroblasts,

TGF-β stimulates the production of collagen through a classic transduction pathway depending on ALK5/Smad; induction of the production of ET-1 may occur through a pathway dependent on ALK5/JNK/Ap-1 [80,81].

It is interesting to note that fibrosis in SSc is accompanied by imbalance in the ratio between ET_A and ET_B in favour of ET_B in the fibroblasts [75,82] and alteration of the equilibrium between metalloproteases (MMPs) and tissue inhibitors of metalloprotease (TIMP), with an increase in many MMPs, in particular MMP-2, which can convert the inactive precursors of ET-1 into active ET-1 [83].

3.6. ET-1 RECEPTORS IN THE MONOCYTES/MACROPHAGES

ET-1 is among the first mediators to be produced in the inflammatory cascade [84].

The monocytes/macrophages can both produce ET-1 and express receptors ET_A [85] and ET_B [86]; this can trigger an autocrine loop and hyperstimulate the production of pro-inflammatory cytokines.

Some observations suggest that ET-1 is responsible for the amplification of the inflammatory cascade in monocytes/macrophages [87-89], activating the transcription of specific mRNAs, through a signal transduction pathway triggered by ET_B.

In particular, in the monocytes/macrophages, the stimulation of ET_B by ET-1 induces an increase in the production of pro-inflammatory molecules, such as TNF, PGE2, IL-1β; a larger production of free radicals of oxygen through activation of iNOS; and the regulation of the mechanisms of cell adhesion and recruitment. *In vitro* it has been observed that the receptor antagonist ET_A/ET_B (Bosentan) may

attenuate the response of the human monocytes/macrophages to ET-1 [86].

3.7. ET-1 RECEPTORS IN THE POLYMORPHONUCLEATES

Polymorphonucleates (PMNs) express both ET_A [85] and ET_B [90]. A recent study has shown that the platelets and neutrophils can synthesize 20-hydroxyeicosatetraenoic acid (20-HETE), a metabolite of arachidonic acid important in the regulation of vascular tone and in the control of renal function and arterial pressure, in response to angiotensin II and ET-1. In both cell types the synthesis of 20-HETE may be induced mainly through the binding of the ligands to the respective receptors AT2 and ET_B [90].

3.8. ET-1 RECEPTORS IN THE DENDRITIC CELLS

The dendritic cells (DCs) produce large quantities of ET-1 and significantly increase the expression of ET_A and ET_B after maturation. ET_A is important for the maturation of the DCs, the production of IL-12, the stimulation of the T lymphocytes and for inhibiting the apoptosis of the DCs. ET_B, on the other hand, is however, important for inhibiting maturation and inducing apoptosis of the DCs [91].

3.9. ET-1 RECEPTORS AND AUTOANTIBODIES

Riemekasten *et al.* have recently demonstrated the presence of autoantibodies against ET_A in the majority of patients with SSc. Their presence may be associated with more serious clinical manifestations, with higher mortality and an increased expression of TGF-β by the endothelial cells. According to these authors, anti-ET_A autoantibodies bind the receptor expressed on the endothelial cells [92]. It is not clear

why these antibodies have been found only in endothelial cells since these cells exclusively express the receptor ET_B.

4. ENDOTHELIN-1 AND TREATMENT

Although a considerable progress has been made in the last few decades, at the moment there is no treatment able to significantly modify the natural history of the disease. The choice of each therapeutic regimen is a challenge both because of the heterogeneity of the clinical manifestations, because of their progression and their severity, and because the pathogenesis of SSc is not yet altogether understood.

Corticosteroids at low dosages are useful for controlling the inflammatory symptoms, such as oedema in the early phases of cutaneous involvement, myositis, serositis, arthritis and tenosynovitis. Long-term treatment with these drugs should be avoided, also because they can precipitate a sclerodermic renal crisis.

The therapeutic choices must be individually tailored, and for this reason it is necessary to carry out an accurate staging of the disease, before starting any “DMARDs” treatment, and follow its course over time. The available “DMARDs” drugs act principally on the vascular damage and on the alterations to the immune system with no or little effects on the fibrotic process [7].

4.1. ANTAGONIST DRUGS TO ET-1 RECEPTORS

The introduction of ET-1 receptor antagonist drugs has improved the management and prognosis of patients with recurrent digital ulcers and with pulmonary hypertension associated with SSc. Clinical studies have provided encouraging results even on cutaneous fibrosis and on Raynaud's phenomenon. Furthermore recent observations

about the anti-inflammatory effects of ERAs make these drugs promising for the future as disease modifying drugs.

Since ET-1 is a mediator implicated in various pathogenetic aspects of SSc, including vasoconstriction, mitogenesis and hypertrophy of the vascular smooth muscle cells, collagen deposition and myofibroblastic induction, its use in affected patients is reasonable. ET-1 levels are also increased in plasma, derma and internal organs of patients with SSc. In particular ET-1 levels are high in the broncoalveolar washing liquid of patients with SSc and pulmonary involvement, including those with pulmonary arterial hypertension, in which ET-1 levels correlate with the severity and the prognosis of this complication [93-95].

Among the disadvantages of ERAs are the slow clinical response, generally 3-6 months from the beginning of treatment, the cost and the adverse effects. The principal one of these, common to all of them, is an increase in hepatic transaminases, which may or may not be associated with gastrointestinal symptoms. For Bosentan this effect is usually moderate and transitory, occurring in about 10% of cases. It is therefore advisable to monitor the hepatic cytolysis enzymes before starting treatment, every 15 days for a month and then monthly after the beginning of treatment. If an increase in transaminases occurs it is necessary to reduce dosage of the drug or suspend it. Full blood count must be checked as well as renal function. Moreover the possible interaction with other drugs must be considered: Bosentan is both a substrate and an inducer of the cytochromes CYP2C9 and CYP3A4 and can therefore reinforce their activity, thus altering the metabolism of numerous other drugs commonly used; among them oral anticoagulants, statins, oral hypoglycaemiants, tricyclic

antidepressants, benzodiazepines and some antibiotics. Oedema at the extremities appears frequently [96]. Ambrisentan, a selective ET_A inhibitor, has a toxicity profile similar to that of Bosentan [97].

Bosentan.

Bosentan is a sulfonamide derivative for oral administration with affinity for the receptors ET_A and ET_B. The usual dosage is 65 mg twice a day for the first four weeks and, if well tolerated, 125 mg twice a day.

It was the first ERA approved in Europe and the USA (from 2002) for the treatment of pulmonary hypertension associated with SSc, in patients with WHO functional class grade III, i.e. those patients with marked limitation of their physical activities. Bosentan has demonstrated significant efficacy in reducing the mean pressure in pulmonary artery (mPAP), in improving the tolerance to exercise and in delaying the progression of the pulmonary arterial hypertension (PAH) not only in patients in WHO grade III but also in asymptomatic patients. It is also capable of reversing the process of cardiac remodelling and improving right ventricular function and cardiac output [98-100].

Bosentan is also approved for treating recurring ischaemic digital ulcers in patients with SSc. It reduces the incidence of new ulcers, pain and disability but has no effect on recovery from ulcers already present [101,102].

Clinical studies have shown that treatment with Bosentan in patients with SSc and severe Raynaud's phenomenon significantly improves the frequency, duration and severity of painful attacks, especially in patients with previous ischaemic digital ulcers. In patients without this

complication, however, it improves functional disability [103-105]. It has been observed that the degree of cutaneous fibrosis improves significantly in treated patients especially those with dSSc [106].

The observations on the anti-inflammatory effects of Bosentan used *in vitro* and *in vivo* are also interesting. *In vitro* Bosentan amplifies the inflammatory cascade, stimulating the production of TNF and other mediators by monocytes/macrophages [86]. *In vivo*, after 12 months of treatment we observe the normalization of the concentration of soluble adhesion proteins in patients' serum [107]. Furthermore, in patients with PAH and SSc, there is a significant reduction in the serum levels of IL-6, ICAM-1 and BNP, accompanying haemodynamic and clinical improvement [108].

In the vascular smooth muscle cells, the predominant effect is the blocking of ET_A, responsible for the pathological modifications of the vessel wall. It is therefore hypothezised that Bosentan opposes the vasoconstrictive and proliferative effect induced by ET-1 by blocking the ET_A and it induces an anti-inflammatory and anti-fibrotic effect by blocking the ET_B. In the fibroblasts, moreover, the production of collagen induced by ET-1 is not reduced by the selective antagonism of ET_A or ET_B, while it is reduced by combined ET_A/ET_B antagonism.

Ambrisentan

Ambrisentan is a non-sulfonamide ERA, selective for ET_A (260:1 ET_A *vs* ET_B), approved in the USA in 2007 and subsequently in Europe for the treatment of PAH associated with SSc. Like Bosentan, Ambrisentan too has demonstrated beneficent effects in patients in the early stage of the disease, and is indicated in patients with PAH of WHO I, II and III. The usual dosage is 5-10 mg once a day. The drug improves haemodynamic parameters, tolerance to exercise and WHO grade and retards the clinical worsening of the patients treated [109].

5. AIM OF THE WORK

ET-1 plays a pivotal role in vasoconstriction, fibrosis and inflammation, the three major aspects in the pathogenesis of SSc. The ET-1 receptors are ET_A and ET_B. The receptors are expressed on the majority of cells involved in SSc, such as endothelial cells, smooth muscle cells and fibroblasts. Little is known about the expression of ET-1 receptors on leukocytes, except for macrophages and monocytes; there is no information about the expression of ET_A and ET_B on lymphocytes, neutrophils or other cells involved in the innate and acquired immune response. Endothelin receptor antagonists are used in the treatment of scleroderma patients with recurrent ischemic digital ulcers and/or pulmonary arterial hypertension. They have beneficial effects on vasoconstriction and fibrosis, but less is known about their anti-inflammatory role.

We aimed at studying the link between ET-1 and inflammation in SSc. Since T and B cells, monocytes and neutrophils are among the most important cells in inflammatory responses in SSc, we studied ET_A and ET_B expression on these cells with flow cytometry, and also ET_A- and ET_B-coding mRNA expression in CD4+ T cells and neutrophils using RT-PCR. In studying T and B cells, we observed the differences in receptor expression between patients and controls, and we made correlations with the cutaneous form of the disease, pulmonary arterial hypertension, interstitial lung disease, Bosentan therapy, and ischemic digital ulcers in SSc patients. We also studied the modulation of receptors on activated T cells with flow cytometry, and how ET-1 influences the production of some cytokines such as IFN-γ, IL-4, IL-6, IL-10 and IL-17 in CD4+ T cells, by ELISA and RT-PCR in different

condition (with and without blocking ET_A and ET_B) and time points, in order to understand the timing of CD4+ T cells response to stimulation with ET-1, its hypothesized proinflammatory effects and the anti-inflammatory effects of Bosentan. Finally we studied the response of neutrophils to ET-1 stimulus by evaluating, with ELISA, the production of IL-8, MMP9, TNF-α, VEGF, IFN-γ and IL-17.

6. MATERIALS AND METHOD

Patients and controls

We enrolled 41 SSc patients (mean age 56.4±13.5 years; 5 male and 36 female) attending the Unit of Autoimmune Diseases of this University Hospital, and 22 healthy controls, sex and age matched.

All patients fulfilled the ACR criteria for the diagnosis of SSc [35]. The distinction between limited and diffuse cutaneous SSc was made according to the criteria of Le Roy at al. [36]. Of these patients, 32 was affected by lSSc and 9 by dSSc.

Ischaemic DUs were defined as necrotic areas located at distal digits with loss of both epidermis and dermis; whereas scleroderma contracture ulcers were defined as ulcers overlying bony prominence at the site of joint contractions. Skin fissures and areas of calcium extrusion were not included.

All the patients underwent examination and laboratory evaluation comprehensive of detection of antibodies to antinuclear proteins and anti-extractable nuclear antigens, respectively by indirect immunofluorescence on Hep-2 cells and the enzyme-linked immunosorbent assay method.

All patients underwent the following instrumental investigations: chest radiograph, pulmonary function test with diffusing capacity for carbon monoxide adjusted to haemoglobin, Doppler echocardiogram to estimate pulmonary artery systolic pressure (sPAP). If no tricuspid regurgitation could be detected, sPAP was presumed normal, and if estimated sPAP was >35 mmHg it was considered abnormal. Pulmonary fibrosis was detected by X-Ray and High Resolution Computed Tomography (HRCT) of the chest, and it was considered

pathological only if there were ground glass or honeycombing areas in the lung.

We also collected information about patients' treatment, particularly with Bosentan.

Blood samples (20 ml) were collected from patients and controls in heparinized Falcon tubes (Becton Dickinson, NJ, USA).

Isolation of peripheral blood mononuclear cells and flow-cytometry

Peripheral blood mononuclear cells (PBMC) were isolated through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, Norway), and separated in tubes, each containing 1 million cells.

Monocyte-dedicated tubes were pre-incubated for 10 minutes at room temperature with mouse serum (DAKO, Glostrup, Denmark). We then used the following antibodies: rabbit polyclonal anti-ETA (Acris Antibodies GmbH, Herford, Germany), sheep polyclonal anti-ETB (Lifespan Biosciences, Seattle, WA, USA), phycoeritrin (PE)-conjugated goat anti-rabbit IgG monoclonal secondary for ET_A (R&D Systems, Minneapolis, MN, USA), PE-conjugated donkey anti-sheep IgG monoclonal secondary for ET_B (R&D Systems). We incubated PBMC for 1 hour at 4°C with anti-ETA (0.25 mg/ml) and anti-ETB (0.2 mg/ml) antibodies, and for 30 minutes at 4°C with PE-conjugated secondary antibodies. Samples were also stained for 20 minutes at room temperature in a dark room with allophycocyanin (APC)-conjugated anti-CD3 and anti-CD14 antibodies (BD Biosciences, San Jose, CA, USA).

Resuspended samples were analysed on a FACSCalibur cytometer (BD Biosciences): 20,000 CD3+ or CD14+ cells per sample were

acquired in live gating. CellQuest software (BD Biosciences) was used to analyse data. Median florescence intensity (MFI) was determined for each sample. MFI was calculated for samples incubated with both primary anti-receptor and PE-conjugated secondary antibodies (receptor MFI) and for samples incubated only with PE-conjugated secondary antibody (IgG MFI). ET_A and ET_B expression on T cells and monocytes was then assessed by the following equation: ΔMFI= receptor MFI - IgG MFI. We also determined the ETA/ETB ratio (ΔMFI of ETA/ΔMFI of ETB).

T cell stimulation

In order to assess receptor expression on activated CD4+ and CD8+ T cells, PBMCs were obtained from 4 patients and 4 sex- and age-matched healthy controls and stimulated for 24 hours with anti-CD3/CD28 antibody coated microbeads: Dynabeads Human T-Activator (Dynal, Oslo, Norway). Cells were stimulated according to the manufacturer's recommendations. In order to distinguish T cells from other cells and CD4+ from CD8+ cells, we used the following antibodies: peridinin chlorophyll protein (PerCp)-conjugated anti-CD3, APC-H7-conjugated anti-CD4, APC-conjugated anti-CD8. Activated cells were detected by incubating cells with fluorescein (FITC)-conjugated anti-CD25 antibodies. All reagents were purchased from BD Biosciences. Cells were also stained with anti-ETA, anti-ETB and secondary antibodies. Incubations were performed as described above.

Samples were analysed on a FACSCanto cytometer (BD Biosciences). FlowJo software (Treestar, Ashland, OR, USA) was used to analyse data. The receptor expression variation was expressed as difference

between the MFI of activated cells and the MFI of unactivated cells MFI (ΔΔMFI).

Isolation of CD4+ T cells

We isolated CD4+ T cells from healthy donor buffy coat. Isolation of CD4+ T cells was a preliminary to studying the expression of ETA- and ETB-coding mRNA (with RT-PCR). Isolation was obtained through negative selection using CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Cologne, Germany) and MidiMACS Starting Kit, including MACS LD column and MACS Separator (Miltenyi Biotec), following manufacturer's instructions.

Isolation of RNA and RT-PCR from CD4+ T cells

Total RNA was extracted from CD4+ T cells using TRIzol Reagent (Gibco BRL, Billings, MT, USA) according to the manufacturer's protocol.

In order to verify the production of ETA- and ETB-coding mRNA by CD4+ T cells, we generated first-strand cDNA using the Super Script III System (Invitrogen, Carlsbad, CA, USA), with random hexamers, according to the manufacturer's recommendations. RNA was previously treated with DNAse I (Invitrogen). Fibroblast cDNA was used as a positive control for the expression of ETA- and ETB-coding mRNA. CD4+ T and fibroblasts cDNA coding to ETA- and ETB- was amplified with sequence-specific primers: ET_A forward 5'-ATGCACAACTATTGCCCACA-3', ET_A reverse 5'-GGACAGGATCCAGATGGAGA-3', ET_B forward 5'-GCACATCGTCATTGACATCC-3', ET_B reverse 5'-CAGAGGGCAAAGACAAGGAC-3' (Sigma-Aldrich, Saint Louis,

MO, USA). Sequence-specific primers were used to amplify cDNA coding vimentin (housekeeping gene), as a PCR reaction control. Amplification was performed using the AmpliTaq Gold PCR MasterMix system (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Reaction components were mixed and PCR tubes dedicated to the production of ET_A-, ET_B- and vimentin-coding amplicons were prepared. Thermic cycles with the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) were performed as follows: 10 minutes at 95°C to start reaction, 60 cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 53°C for ETA, at 55°C for ETB, at 53°C or 55°C for vimentin) and extension (1 minute at 72°C), and 7 minutes at 72°C to stop reaction. Amplicons (length: 447 bp for ET_A, 558 bp for ET_B, 266 bp for vimentin) were separated on agarose gel (1.5%). VersaDoc video documentation system (Bio Rad, Hercules, CA, USA) was used to document the electrophoretic separation.

Study of cytokine response to ETA- and ETB-stimulation by ET-1 in CD4+ T cells

In order to study cytokine response to ET_A- and ET_B-stimulation by ET-1 in CD4+ T cells, we distributed the CD4+ isolated cells in microplates and we incubated the cells at first with selective antagonists for ET_A and ET_B; we then added the stimulus with ET-1. We studied the cytokine response with ELISA at different times (3, 6 and 24 hours) in order to quantify IFN-γ and IL-4 production in microplate supernatants and the timing of the response of the CD4+ T cells to stimulation with ET-1.

Receptor blockage/stimulation test

CD4+ T cells were distributed in 5 plates. We incubated cells with neither ET_A (BQ123)- nor ET_B (BQ788)-antagonists nor stimulus (ET-1) in one plate (basal cells). We made the following incubations in the other plates: ET-1; BQ123 and ET-1; BQ788 and ET-1; BQ123, BQ788 and ET-1. BQ123 and BQ788 at the concentration of 10-6 M were incubated for 45 minutes, ET-1 at the concentration of 10-7 M was incubated for 24 hours. In plates in which we incubated both the antagonists and ET-1, the antagonists were incubated first. All reagents were purchased from Sigma Aldrich.

RT-PCR and evaluation of IFN-γ-, IL-4-, IL-6-, IL-10- and IL-17-coding mRNA in response to ET-1 stimulus

Total RNA was extracted from CD4+ T cells using TRIzol Reagent (Gibco BRL, Billings, MT, USA) according to the manufacturer's protocol.

In order to verify the production of IFNγ-, IL-4-, IL-6-, IL-10- and IL-17-coding mRNA by CD4+ T cells, we used sequence-specific TaqMan probes marked with the fluorophore 6-carboxy-fluorescein (FAM) and that have as quencher dihydrochloro-pyrrole (MGB).

The PCR reactions were prepared in 96-well plates by mixing 12.5 µl of TaqMan MasterMix (Applied Biosystems) containing Taq Gold polymerase, uracilDNA glycosylase, deoxyribonucleotides (dATP, dGTP, dCTP, dTTP and dUTP) and buffer, 1.25 µl of TaqMan assay, 2 µl of cDNA and 9.25 µl of water (total volume 25 µl). In thermocycler Sequence Detection System 7300 (Applied Biosystems), the samples were subjected to a thermal cycle which consisted of 2 minutes at 50° C, to allow the activation of uracilDNA glycosylase, 10

minutes at 95° C to allow the activation of Taq Gold polymerase and a subsequent 40 cycles of denaturation (15 seconds at 95°C) and annealing/extension (1 minute at 60°C). The amplification of the cDNA was quantified by evaluating the increase of fluorescence during the exponential phase of the PCR reaction, using the software Sequence Detector System (Applied Biosystems). We obtained the ΔCT for each condition by subtracting the C_T (threshold cycle) of the endogenous control 18s (a constitutively expressed housekeeping gene) from the C_T of the transcript that we considered. We then calculated ΔΔCT by subtracting the ΔCT of the basal condition from the ΔCT of non-basal conditions. We thus obtained a fold change (FC) for each condition using the equation: $FC = 2^{-\Delta\Delta Ct.}$

Evaluation of cytokine response by ELISA

The supernatant concentrations of IFN-γ and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA). IFN-γ kit and IL-4 kit (Quantikine Human IFN-γ Immunoassay and Quantikine Human IL-4 Immunoassay respectively) were purchased from R&D Systems. Samples containing supernatants at appropriate dilutions were prepared and the assay was performed following the manufacturer's recommendations. Sunrise absorbance reader for microplates (Tecan, Salzburg, Austria) was used to determine optical density for each sample.

Isolation of neutrophils and RT-PCR

We isolated neutrophils from healthy donor buffy coat in order to study ETA and ETB expression on the cell surface by flow cytometry and the expression of ETA- and ETB-coding mRNA by RT-PCR.

Highly purified granulocytes (neutrophils > 96.5%) were isolated and prepared under endotoxin-free conditions using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, Norway). Ficoll-Paque–isolated neutrophils were further enriched by positively removing all contaminating cells with mAb against CD3, CD56, CD19, CD36, CD49d, and Gly-A using a custom-made EasySep® kit (StemCell Technologies, Vancouver, BC, Canada), to reach > 99.7% purity (high purity neutrophils).

Total RNA was extracted from neutrophils using TRIzol Reagent (Gibco BRL, Billings, MT, USA) according to the manufacturer's protocol.

In order to verify the production of ETA- and ETB-coding mRNA by neutrophils, we generated first-strand cDNA using the Super Script III System (Invitrogen, Carlsbad, CA, USA), with random hexamers, according to the manufacturer's recommendations. RNA was previously treated with DNAse I (Invitrogen). Fibroblast cDNA was used as a positive control for the expression of ETA- and ETB-coding mRNA. Neutrophils and fibroblasts cDNA coding to ET_A- and ET_B- was amplified with sequence-specific primers: ETA forward 5'-ATGCACAACTATTGCCCACA-3', ET_A reverse 5'-GGACAGGATCCAGATGGAGA-3', ET_B forward 5'-GCACATCGTCATTGACATCC-3', ET_B reverse 5'-CAGAGGGCAAAGACAAGGAC-3' (Sigma-Aldrich, Saint Louis, MO, USA). Sequence-specific primers were used to amplify cDNA coding vimentin (housekeeping gene), as a PCR reaction control. Amplification was performed using the AmpliTaq Gold PCR MasterMix system (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Reaction components were

mixed and PCR tubes dedicated to the production of ET_A-, ET_B- and vimentin-coding amplicons were prepared. Thermic cycles with the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) were performed as follows: 10 minutes at 95°C to start reaction, 45 cycles of denaturation (1 minute at 95°C), annealing (1 minute at 50°C for ET_A, at 58°C for ET_B, at 72°C for vimentin) and extension (1 minute at 72°C), and 10 minutes at 72°C to stop reaction. Amplicons (length: 447 bp for ET_A, 558 bp for ET_B, 266 bp for vimentin) were separated on agarose gel (1.5%). VersaDoc video documentation system (Bio Rad, Hercules, CA, USA) was used to document the electrophoretic separation.

Study of the cytokine response after stimulation by ET-1 in neutrophils

In order to study the cytokine response after stimulation by ET-1, we distributed the isolated neutrophils in microplates and incubated with or without 100 ng/mL Ultrapure *E. coli* LPS (Invivogen, San Diego, CA) and with or without ET-1. In this way we considered 4 conditions: neutrophils without stimulus as negative control, neutrophils with ET-1 or LPS alone and with both ET-1 and LPS. We studied the cytokine response with ELISA at different times (3 and 10 hours) in order to quantify IL-8, MMP9, TNF-α, VEGF, IFN-γ and IL-17 production in microplate supernatants and the timing of neutrophils response to ET-1 stimulus.

Evaluation of cytokine response by ELISA

The supernatant concentrations of IL-8, MMP9, TNF-α, VEGF, IFN-γ and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA). Quantikine Human Immunoassay for these specific

cytokines was purchased from R&D Systems. Samples containing supernatants at appropriate dilutions were prepared and the assay was performed following the manufacturer's recommendations. Sunrise absorbance reader for microplates (Tecan, Salzburg, Austria) was used to determine optical density for each sample.

Statistical analysis

All the calculations were performed with SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). All results are expressed as mean ΔMFI ± standard deviation, except when otherwise indicated. Quantitative data were assessed using the Student's t-test. A value of $P<0.05$ was considered statistically significant.

7. RESULTS

ET_A and ET_B expression on T and B cells and on monocytes and neutrophils.

For the first time we show ET_A and ET_B expression on the surface of T and B cells and of monocytes. We have observed receptors expression on T cells by flow-cytometry (Figure 5) and we have also found the expression of ET_A and ET_B-coding mRNA in CD4+ T cells by reverse transcription-PCR (Figure 6).

Flow cytometry reveal the presence of surface ET_A and ET_B on B cells and monocytes (Figure 7).

Neutrophils express both ET-1 receptors as shown by flow cytometry (Figura 8) and by reverse transcription-PCR (Figura 9).

Quantification of ET_A and ET_B expression on T cells and monocytes.

T and B cells and monocytes of both patients and controls expressed ET_A and ET_B on their surface. We assessed receptor expression on these cells referring to ΔMFI, and then we compared numerical data using mean (ΔMFI or ET_A/ET_B ratio) ± standard deviation.

In both patients and controls, T cells and monocytes express ET_A (patients: ΔMFI=100,61±45,21 and 212,24 ± 64,27 respectively; controls: ΔMFI= 110,45 ± 35,89 and 188,4 ± 35,61 respectively) at higher levels than ETB (patients: 46,85 ± 29,78 and 91,14 ± 27,44 respectively; controls: ΔMFI= 49,23 ± 29,16 and 98,74 ± 54,66 respectively) ($p<0,001$) (Table III).

Patients affected by diffuse SSc showed lower levels of ET_B on T cells than patients affected by limited SSc (28,6±17,9 vs 51,9±31,1) ($p<0,01$); a similar pattern was found on monocytes (74,4±29,6 vs 97,2±24,5) ($p<0,05$).

There were no significant differences in ET_A expression on T cells and monocytes in patients with diffuse or limited form (94,8±48,2 vs 99,1±42,1 and 251,2±116,3 vs 199,3±34,5 respectively) (Table IV).

No significant correlation regarding ET_A or ET_B expression was found in patients treated or not treated with Bosentan (T cell: ETA: 97,9±52 vs 102,6±44,3; ET_B: 47,9±17,7 vs 47,6±32,3, respectively; Monocytes ET_A: 240,7±130,6 vs 205,1±34,9; ET_B: 89,6±22,9 vs 93,1±27,7) (Table V).

ET_A and ET_B expression on T cells or monocytes showed no correlation with the presence or absence of ischaemic digital ulcers (T cell: ET_A 121,4±69 vs 98,8±41,6 and ET_B 40,8±20,1 vs 48,6±31 respectively; Monocytes: ET_A: 221±4,3 vs 211,3±69,6; ET_B: 80,4±25,3 vs 93,6±27,5) (Table VI).

Patients with pulmonary arterial hypertension (PAH) showed a lower ET_B expression on monocytes compared with patients without PAH (77,2±23,4 vs 96,9±27,3), especially when we considered the limited form of the disease (77,6±17,6 vs 102,3±24,4) ($p<0,05$) (Table VII).

Furthermore, limited SSc patients with interstitial lung disease (ILD) showed a lower ET_A expression on T cells compared with patients without ILD (77,8±34,2 vs 111,6±43,9) ($p<0,05$) (Table VIII).

ET_A is expressed at higher levels than ET_B on the surface of B cells of both patients treated with Bosentan (ET_A: 281.33±43.47 vs ET_B: 161.33±43.97) and untreated patients (ET_A: 270.00±28.16 vs ET_B: 171.33±35.47). However, there are significant differences in ET_A and ET_B expression in patients with or without therapy. ET_A and ET_B show the same pattern of expression also in B lymphocytes from healthy donors.

Quantification of ET_A and ET_B expression on activated T CD4+ and CD8+ cells.

According to flow cytometry results for T cells, both CD4+ and CD8+ T lymphocytes of patients and controls express ET_A and ET_B on their surface. As observed in T cells, we found a higher ET_A expression in unstimulated CD4+ and CD8+ cells. When activated, by contrast, both CD4+ and CD8+ cells reduce ET_A and raise ET_B expression (Figure 10).

Cytokine response to ET_A- and ET_B-stimulation by ET-1 in T CD4+ cells.

Evaluation of the IFN-γ-coding mRNA was performed at 3, 6, 24 and 48 hours with or without receptor blockers, in T lymphocytes of a control and a patient. In CD4+ T cells from the control, incubation with ET-1 for 3 hours without receptor blockade showed a decrease in IFN-γ-coding mRNA (F.C: -2,14). We observed the same after 6 and 24 hours' incubation. Cells incubated with ET-1 in the presence of ET_A blocker alone, or both ET_A and ET_B receptor blockers result in the same decrease in IFN-γ-coding mRNA (Figure 11). After 48 hours IFN-γ-coding mRNA increased; IFN-γ-coding mRNA increase is greatest in presence of ET-1 and ET_B blockade (F.C: -1,17). By contrast, using CD4+ T cells obtained from one patient, incubation with ET-1, both alone (F.C: -20,72) or in the presence of ET_A and ET_B inhibitors (F.C: -17,62 and -28,46 respectively), lead to a significant decrease in IFN-γ-coding mRNA after 3 hours, with a return to the baseline value of the IFN-γ-coding mRNA after 6 hours. We also investigated transcripts for IL-4 and IL-17 after 3 and 6 hours cells culture. After 6 hours incubation with ET-1 alone (F.C: 8,02) or together with ET_A blockade (F.C: 5,54) or ET_A and ET_B inhibitors

(F.C: 5,21) lead to an increase of IL-4-coding mRNA. Conversely, ET-1 together with ET_B inhibitor induces a decrease in IL-4-coding mRNA (F.C: -1,22) (Figure 12).

Using CD4+ T cells isolated from a healthy control, we observed that after incubation with ET-1 for 3 and 6 hours, IL-17-coding mRNA decreases (F.C: -5,22 and -1,07 respectively). The same result was obtained when cells were incubated with ET-1 in presence of ET_B inhibitor or both ET_A and ET_B blockade (F.C: -4,26 and -19,17 respectively). In contrast using cells from a patient in the same conditions, IL-17-coding mRNA showed a similar decrease after 3 hours, while after 6 hours using ET-1 alone we observed an increase of the transcript (F.C 3,08) (Figure 13).

IL-6- and IL-10-coding mRNA behaved as IL-17-coding mRNA when we considered cells obtained from healthy control.

We used supernatants of culture of the same cells to evaluate cytokine production. In particular after 24 hours of incubation with ET-1, IFN-γ was 9.5 times higher than in cell culture without ET-1 (7,6 pg/ml vs 0,8 pg/ml. IFN-γ levels after selective ET_A- or ET_B-blockade in presence of ET-1 were 1,2 pg/ml and 1,6 pg/ml respectively. By contrast, the simultaneous blocking of ET_A and ET_B, which mimics a Bosentan condition in vitro, caused a marked reduction of IFN-γ in supernatants and we could not detect the cytokine (Figure 14). We also studied IL-4 production by CD4+ T cells. After 24 hours of ET-1 incubation IL-4 was not detectable in the medium culture. Conversely, the production of IL-4 increased in the presence of ET-1 and selective inhibition of ET_A (711,42 pg/ml) in a more significant manner than in the presence of ET_B receptor blockade (694,47 pg/ml). In the presence of both ET_A and ET_B receptor blockade, the production of IL-4, after 24 hour of incubation with ET-1, decreased (682 pg/ml).

Cytokine response to ET_A- and ET_B-stimulation by ET-1 in neutrophils

The IL-8, TNF-α, MMP-9, VEGF, INF-γ and IL-17 was performed at 1, 3, and 10 hours with ET-1 or LPS or both ET-1 and LPS on isolated neutrophils from a healthy donor buffy coat.

In neutrophils from the healthy donor buffy coat, the incubation with ET-1 only for 1 hour showed a marked increase of MMP-9 (165,11 ηg/mL). On the contrary, after 1 hour incubation with ET-1 we did not observe significant changes in the production of the other cytokines studied, compared with the control.

After 3 hours of incubation with LPS and ET-1 the neutrophils production of IL-8 is higher (67,69 pg/mL) than with ET-1 alone (7,69 pg/mL) or with LPS alone (59,23 pg/mL). As far as MMP-9 concerns, no difference was observed after stimulus with both ET-1 and LPS (205,59 ηg/mL), or with LPS alone (203,78 ηg/mL).

The production of TNF-α increased much more by cells incubated for 10 hours with only ET-1 (23,41 pg/mL) than with both ET-1 and LPS (2,05 pg/mL) or with LPS alone (-0,23 pg/mL). After 10 hours the incubation with ET-1 alone also increases the production of IL-17 (3,50 pg/mL) much more than with both ET-1 and LPS (-2,50 pg/mL) or with LPS alone (-0,50 pg/mL). After 10 hours of incubation, the production of IL-8 and MMP-9 also increased, if the neutrophils were stimulated with both ET-1 and LPS (134,62 pg/mL and 220,69 ηg/mL respectively) rather than with ET-1 alone (16,62 pg/ml and 63,29 pg/mL respectively) or with only LPS (93,85 pg/mL and 205,29 ηg/mL respectively).

8. DISCUSSION

ET-1 is a mediator known for its vasoconstrictor effect. In the last few years other different effects of ET-1 have been identified: it can play a role in inflammation, in cell adhesion, in fibrosis and in angiogenesis. All these aspects are important in the pathogenesis of SSc, which involves three pivotal components: vascular dysfunction and injury, activation of the immune system and increased secretion of collagen by fibroblasts. Moreover, in patients with SSc, increased levels of ET-1 are also found in the plasma, derma and internal organs [93-95].

Considering these novel properties of ET-1, ERAs may not only have a vasodilator effect, but may also have a role in inflammation, although less is known about their potential anti-inflammatory effects.

Little is known about the expression of ET-1 receptors (ET_A and ET_B) on leukocytes. Some studies report that polymorphonuclear cells and dendritic cells express both ET_A and ET_B [65,66,69,71]. Conflicting data are reported in the literature about the expression of ET-1 receptors in monocytes. Mencarelli *et al.* observed only ET_A-coding mRNA expression, in contrast Juergens *et al.* noticed only ET_B-coding mRNA expression in monocytes [85,86]. Juergens *et al.* also suggested a proinflammatory role for ET_B on monocytes/macrophages: its stimulation with ET-1 was correlated with an increase of proinflammatory cytokines, able to activate Th1 response. Consistently with this, blocking ET_B with Bosentan reduced proinflammatory cytokine production [86].

In order to better clarify the role of ET-1 in the immune system, we first studied the expression of ET_A and ET_B on lymphocytes, monocytes and neutrophils. We observed that T cells and monocytes express both ET-1 receptors. We validated our data in a large cohort

of patients and controls, with the aim to better understanding the possible correlation of ET-1 receptor surface expression and particular clinical features. We confirmed not only that T cells and monocytes but also B lymphocytes constitutively express ET_A and ET_B on their surface. Moreover, the expression of ET_A was greater than the expression of ET_B both in patients and controls in T cells and monocytes, while for B cells there was no difference in the expression of the two receptors between patients and controls.

Considering that a high ET_A/ET_B ratio correlates with the extension of cutaneous fibrosis in SSc patients and in particular that ET_B expression was lower in dSSc- rather than lSSc-patients, ET_A signalling seems to be important in the cutaneous profibrotic effects of ET-1. Since a lower ET_B expression on monocytes correlated with PAH and a lower ET_A expression on T cells correlates with ILD, we can hypothesize that a different pattern of receptor expression is associated with a different response of T cells or monocytes in the preferential induction of PAH or ILD. ET_A signalling and ET_B signalling may therefore be characteristic of paricular clinical features. Moreover, the higher expression of ET_B in patients than in healthy controls and its increase in activated T cells, lead to the consideration that ET_B signalling plays a major role in inflammation.

In order to evaluate potential receptor modulation in a proinflammatory microenvironment, we stimulated T cells with anti-CD3/CD28 antibody-coated microbeads, which mimic physiological stimulation and costimulation of T cells by antigen-presenting cells. Since we found a reduction in ET_A and an increase in ET_B on CD4+ and CD8+ T cells, we suppose that once CD4+ and CD8+ T cells are in a proinflammatory microenvironment, such as in scleroderma active lesion, they may modulate receptor expression in favour of

ET_B. This observation supports the hypothesis that ET_B signalling plays a major role in inflammation. These data are also in favour of a benefit of a double ET-1 receptors blockage.

Another point of interest is that ET_A and ET_B are not up- or down-regulated with ER_A therapy. This supports the clinical observation that ERAs induce neither pharmacodynamic nor inverse tolerance, although we observed this feature only on T cells and monocytes. It is likely, however, that the same happens in vascular cells.

Furthermore, we investigated how T CD4+ cells respond to ET-1 stimulus in the presence or absence of the selective blocking of one or both receptors.

We focused our attention on INF-γ and IL-6, a proinflammatory cytokine, and IL-4, an antinflammatory cytokine.

The results, obtained by RT-PCR, on the expression of IFN-γ-coding mRNA, show an increase of 2.83 times compared with the basal condition, if ET_B is stimulated and ET_A blocked. ET-1 therefore seems to promote Th1 responses through ET_B. By blocking both receptors, the production of transcripts is reduced by 4 times compared with only ET_A blockage. In contrast, ET-1 in T cells does not stimulate the production of IL-4- and IL-6-coding mRNA. However, in the same cells, ET-1 induces a slight increase in transcripts encoding for IL-6, while it inhibits the production of IL-4.

Most of these effects can be attributed to ET_B, because this receptor is mainly expressed on activated T CD4+ cells.

By ELISA assay we quantified the levels of IFN-γ released in the supernatants by CD4+ T cells. We found that ET-1 causes a proinflammatory response by T CD4+ cells. The engagement of both ETA and ETB triggered production of IFN-γ in the supernatants 9.5 times higher than in the basal condition. When both receptors were

blocked, which simulates a Bosentan condition in vitro, we found a profound reduction in IFN-γ production. Interestingly neither selective ET_A- nor ET_B-stimulation caused a IFN-γ production as high as the stimulation of both the receptors; and neither selective ET_A- nor ET_B-blockage induced IFN-γ production as low as the blockage of both the receptors. These observations support the hypothesis of ET_A-ET_B cross-talk in transductional pathways leading to a pro-inflammatory response in CD4+ T cells. Stimulating or blocking both ET_A and ET_B therefore has synergistic effects. Recently, similar observations have been noted in scleroderma fibroblasts: blocking both ET_A and ET_B suppressed collagen production more effectively than ET_A- or ET_B-selective blockage, with synergistic effects [59]. Considering these observations and our results, the blockage of both the receptors seems to inhibit inflammation and the consequent fibrosis more than selective blockage.

Finally, we focus our attention on innate immunity. Neutrophils participate in the early stages of SSc and contribute to endothelial damage, by production of reactive oxygen species, fibroblast activation and recruitment of T and B cells. We showed that neutrophils express ETA and ETB on their surface. After ET-1 stimulus we observed an increased production of IL-8, TNF-α, MMP-9 and IL-17. IL-8, TNF-α and IL-17 are proinflammatory cytokines while MMP-9 is a metalloproteinase involved in tissue remodelling. So ET-1, through ET_A and ET_B, can contribute to triggering neutrophil activation, which leads to vascular damage.

If this is the case, Bosentan-therapy would be effective also in early-stage SSc, when inflammation is prominent and facilitates vascular damage progression and evolution into fibrosis.

9. CONCLUSION

In conclusion our result suggest that the ET-1 system has an important role in inflammation and in fibrosis in SSc patients, especially through a combined ET_A-ET_B signalling. In particular ET-1 can promote the activation of neutrophils, which are involved in the early stage of sclerodermic lesion. By stimulating ET-1, neutrophils are able to produce IL-8, which induces chemotaxis of proinflammatory cells at the site of inflammation, and promotes angiogenesis. Neutrophils also produce TNF-α and IL-17, two proinflammatory cytokines that promote the recruiting of monocyte/macrophages and lymphocytes at the site of inflammation. Neutrophils, therefore, in the presence of high plasma levels of ET-1, may lead to vascular injury and immune system activation, two important aspects in the first phase of SSc vascular damage. After neutrophil activation, T cells migrate to the site of inflammation, and their response can be modulated by ET-1. Initially T cells when activated express high levels of ET_B, which leads to the production of pro-inflammatory cytokines, such as INF-γ. These contribute to the perpetuation of inflammation. In the second stage T lymphocytes down-modulate the expression of ET_B and increase expression of ET_A, as we observed in patients with a long history of SSc. In this way T cells can promote a profibrotic effect, that lead to an increase in collagen and matrix protein production by fibroblasts.

These results pave the way for a more exhaustive study on the role of ET-1 in the pathogenetic changes in the immune system in SSc. Bosentan may therefore also exert anti-inflammatory effects.

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10. TABLE AND FIGURES

TABLE I. ET-1, ET-2, ET-3 producing cells [Modified from Khimji AK, Rockey DC.: Endothelin, Biology and disease. Cellular Signalling 2010; 22: 1615–1625].

Endothelin-1	*Endothelin-2*	*Endothelin-3*
Endothelial cells	Epithelial cells of the kidney	Neuronal stromal cells
Fibroblasts and myofibroblasts	Stromal cells of the gastrointestinal tract	Glial cells
Smooth muscle cells on the vasal wall	Cardiac myocytes	Cells of the adrenal cortex
Leukocytes (mast cells, macrophages, polymorphonuclear, Dendritic cells)	Trophoblast cells	Lung epithelial cells
Cardiac myocytes	Cellule epiteliali dell'epitelio ghiandolare dell'utero	Cellule epiteliali della mucosa intestinale
Epithelial cells of the kidney		Epithelial cells of the kidney
Mesangial cells of the kidney		Epithelial cells of the intestinal mucosa
Astrocytes		
Neuronal cells stromal		
Epithelial cells of the breast		
Endometrial cells		
Sertoli cells		
Epithelial cells of the bile ducts		

TABLE II. Cells expressing ETA and/or ETB, and their main functions [Modified from Khimji AK, Rockey DC.: Endothelin, Biology and disease. Cellular Signalling 2010; 22: 1615–1625].

Cells	*Receptors*	*Role play*
Endothelial cells	ET_B	Regulation of vascular tone
Smooth muscle cells	ET_A, ET_B	Contraction of smooth muscle of the vassal wall, cell proliferation
Fibroblasts and myofibroblasts	ET_A, ET_B	Contractility, synthesis of extracellular matrix proteins
Cardiac myocytes	ET_A, ET_B	Cell proliferation, contraction, hypoxia-induced apoptosis
Hepatocytes	ET_A, ET_B	Regulation of metabolism (glycogenolysis and gluconeogenesis)
Epithelial cells of the renal collecting ducts	ET_B	Tubular reabsorption
Neurons	ET_A, ET_B	Lengthening of neurites; cellular response to stress and apoptosis
Osteoblasts	ET_A, ET_B	Proliferation, differentiation, synthesis of extracellular matrix proteins
Keratinocytes	ET_A, ET_B	Proliferation and melanogenesis
Adipocytes	ET_A, ET_B	Regulation of lipid and glucose metabolism
Monocytes / macrophages	ET_A [110], ET_B [123]	Inflammations
PMNs	ET_A [110], ET_B [111]	Inflammations
Mast cells	ET_A ? [151,152]	???
Platelets	ET_A [111], ET_B [153]	Regulation of mechanisms of adhesion and platelet aggregation
Dendritic cells	ET_A [127], ET_B [127]	Regulation of maturation, functions and apoptosis

Table III. SSc patients vs healthy controls.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
SSc PATIENTS	T cells	A	100.6±45.2
		B	46.8±29.8
	monocytes	A	212.2±64.3
		B	91.1±27.4
HEALTHY CONTROLS	T cells	A	110.4±35.9
		B	49.2±29.2
	monocytes	A	188.4±35.6
		B	98.7±54.7

Table shows differences among SSc patients and healthy controls about ET_A and ET_B expression on T cells and monocytes. ΔMFI (Δ median fluorescence intensity) represents the quantification of receptors expression, according to the following equation: ΔMFI= receptor MFI - IgG MFI. Receptor MFI represents the MFI of samples incubated with both primary anti-receptor and PE-conjugated secondary antibodies. IgG MFI represents the negative control, i.e. the MFI of samples incubated only with PE-conjugated secondary antibody. ET_A/ET_B ratio represents the ratio between ET_A ΔMFI and ET_B ΔMFI. ΔMFI (for ET_A and ET_B) and ET_A/ET_B ratio on T cells and monocytes are here reported as mean ± standard deviation among samples of patients or controls.

Table IV. dSSc vs lSSc patients.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
dSSc PATIENTS	T cells	A	94.8±48.2
		B	28.6±17.9
	monocytes	A	251.2±116.3
		B	74.4±29.6
lSSc PATIENTS	T cells	A	99.1±42.1
		B	51.9±31.1
	monocytes	A	199.3±34.5
		B	97.2±24.5

Table shows differences among dSSc and lSSc patients about ET_A and ET_B expression on T cells and monocytes.

Table V. Bosentan-treated vs Bosentan-free SSc patients.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
BOSENTAN TREATED PATIENTS	T cells	A	97.9±52
		B	47.9±17.7
	monocytes	A	240.7±130.6
		B	89.6±22.9
BOSENTAN FREE PATIENTS	T cells	A	102.6±44.3
		B	47.6±32.3
	monocytes	A	205.1±34.9
		B	93.1±27.7

Table shows differences among Bosentan-treated and Bosentan-free SSc patients about ET_A and ET_B expression on T cells and monocytes.

Table VI. SSc patients with vs those without ischemic digital ulcers.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
SSc PATIENTS WITH DUs	T cells	A	121.4±68
		B	40.8±20.1
	monocytes	A	221±4.3
		B	80.4±25.3
SSc PATIENTS WITHOUT DUs	T cells	A	98.8±41.6
		B	48.6±31
	monocytes	A	211.3±69.6
		B	93.6±27.5

Table shows differences among SSc patients with or without ischemic digital ulcers about ET_A and ET_B expression on T cells and monocytes.

Table VII. SSc patients with vs those without pulmonary arterial hypertension.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
SSc PATIENTS WITH PAH	T cells	A	97.3±43.3
		B	44.4±21
	monocytes	A	229.7±111
		B	77.2±23.4
SSc PATIENTS WITHOUT PAH	T cells	A	100.4±44.5
		B	49.1±33.7
	monocytes	A	206.1±34.7
		B	96.9±27.3

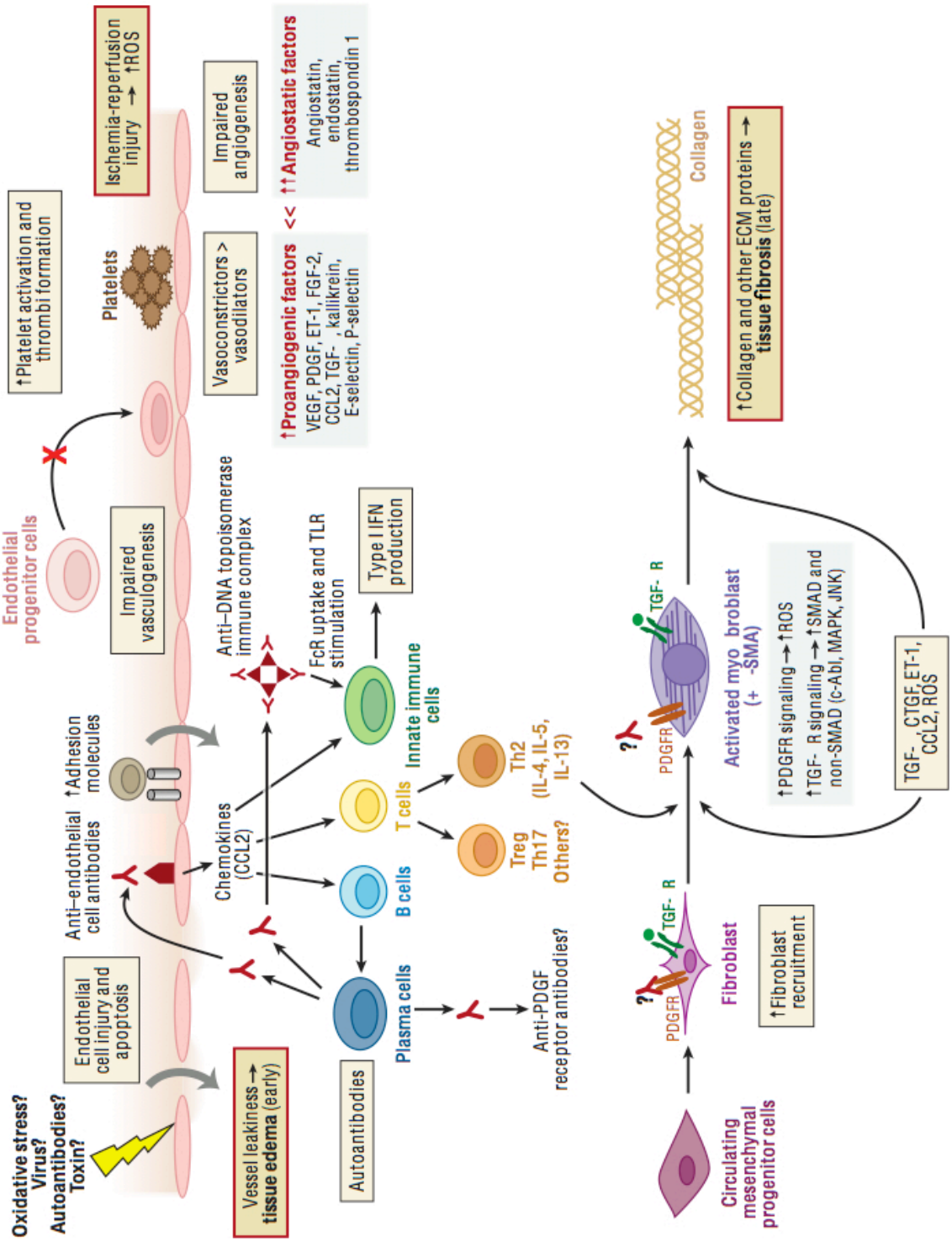
Table shows differences among SSc patients with or without pulmonary arterial hypertension about ET_A and ET_B expression on T cells and monocytes.

Table VIII. SSc patients with vs those without interstitial lung disease.

SAMPLE	CELLS	RECEPTOR	ΔMFI MEAN ± STANDARD DEVIATION
SSc PATIENTS WITH ILD	T cells	A	88.3±43.6
		B	41.6±38.7
	monocytes	A	224.3±89.5
		B	83.1±30.5
SSc PATIENTS WITHOUT ILD	T cells	A	112.4±46.6
		B	52.1±21
	monocytes	A	202±34.3
		B	97.2±24.5

Table shows differences among SSc patients with or without interstitial lung disease about ET_A and ET_B expression on T cells and monocytes.

FIGURE 1 [Modified from Katsumoto TR, Whitfield ML, Connolly MK. The pathogenesis of systemic sclerosis. Annu Rev Pathol. 2011;6:509-37]. Pathogenesis of systemic sclerosis.



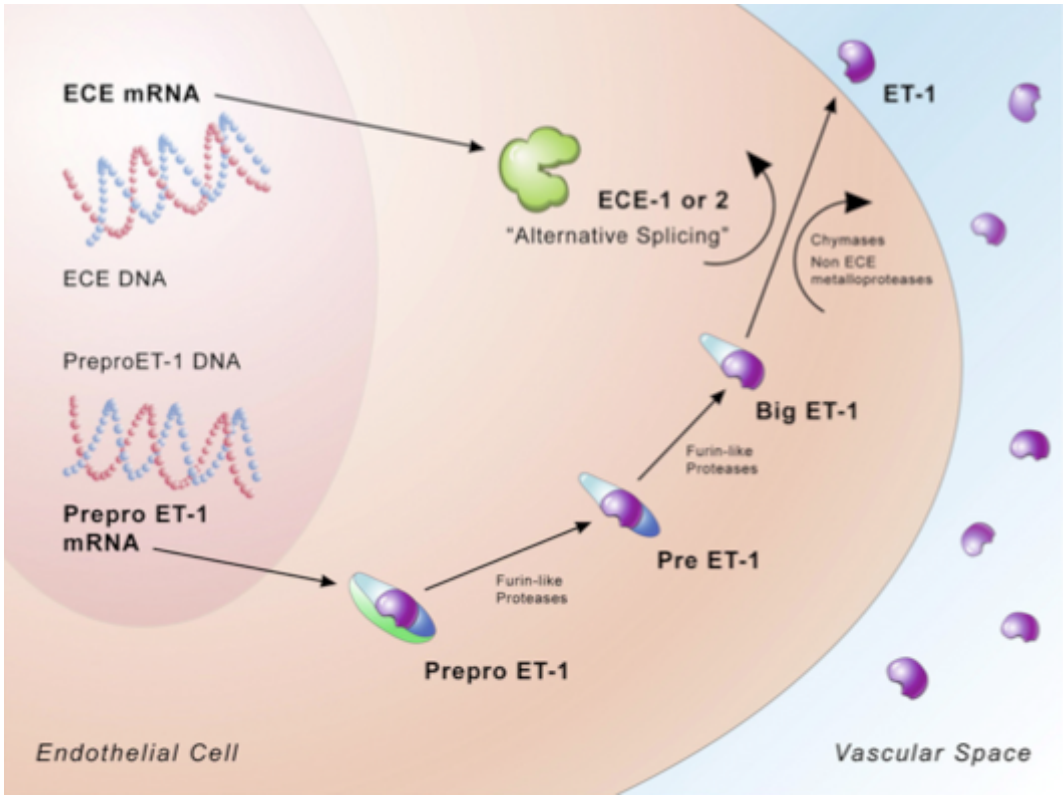


FIGURE 2 [Modified from Al-karim Khimji, Don C. Rockey.: Endothelin, Biology and disease. Cellular Signalling 2010; 22: 1615–1625]. The ET-1 is formed from the gene encoding the pre-proET-1, which is converted first into preET-1 and then in BigET-1 due to cleavage of the protease. The latter is cleaved by enzymes, especially ECE-1 or 2, to form the biologically active form of the peptide, namely the ET-1

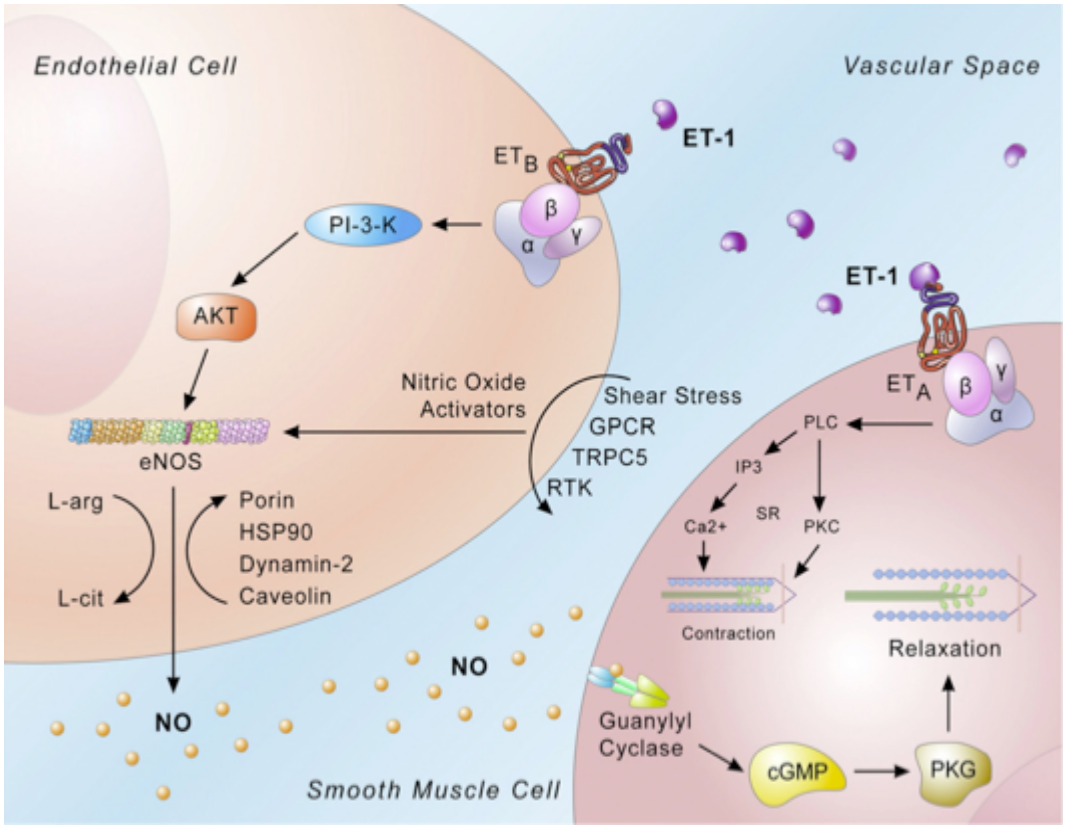


FIGURE 3 [Modified from Al-karim Khimji, Don C. Rockey.: Endothelin, Biology and disease. Cellular Signalling 2010; 22: 1615–1625]. On the left is shown an endothelial cell, in which the ET-1 triggers, via the ETB receptor binding and signal transduction, the production of NO, which induces in the vascular smooth muscle cell (shown on the right) relaxation myofibrils. In vascular smooth muscle cell, the ET-1, however, through binding to the ETA receptor, has a direct effect of contraction of myofibrils, so that under normal conditions the effects of vasoconstriction and vasodilatation are balanced.

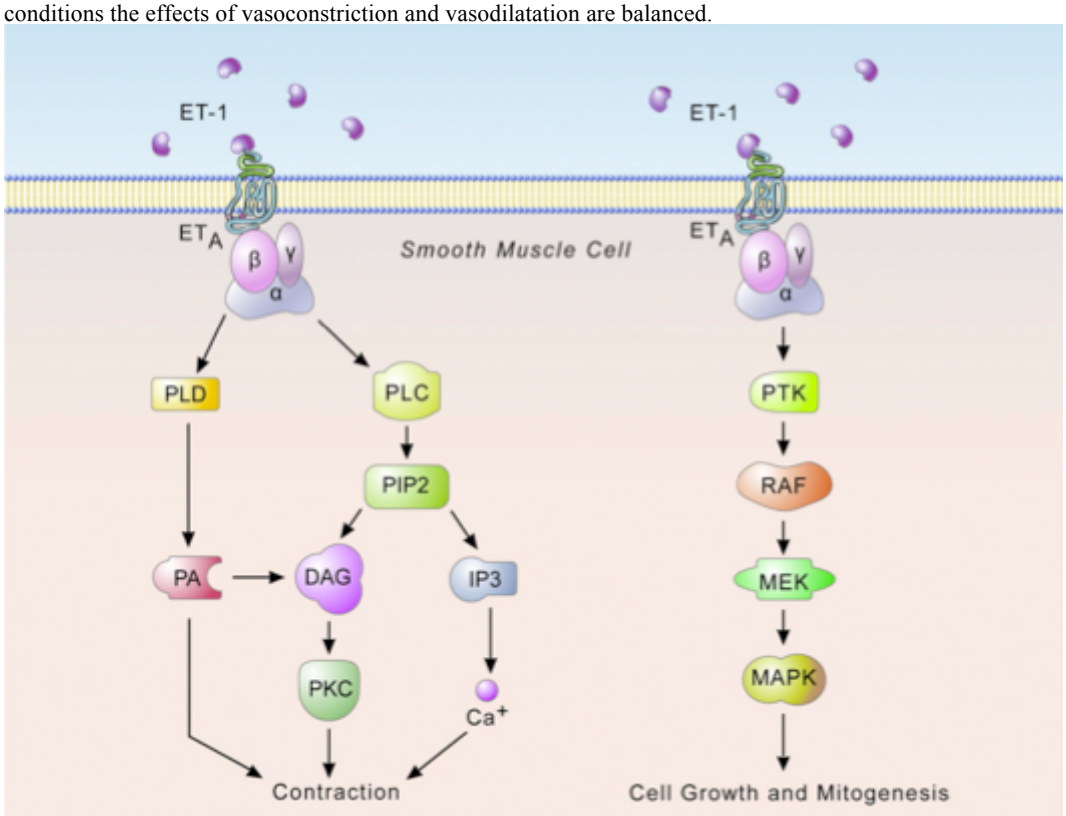


FIGURE 4 [Modified from Al-karim Khimji, Don C. Rockey.: Endothelin, Biology and disease. Cellular Signalling 2010; 22: 1615–1625]. In the smooth muscle cell stimulation of ET-1, through binding to the ETA receptor, is able to induce two signal transduction pathways which lead respectively to contraction of myofibrils and mitogenesis and cell growth. These aspects are particularly important in vascular perturbation in SSc. The ETA receptor antagonist ambrisentan specifically interfere with these molecular mechanisms.

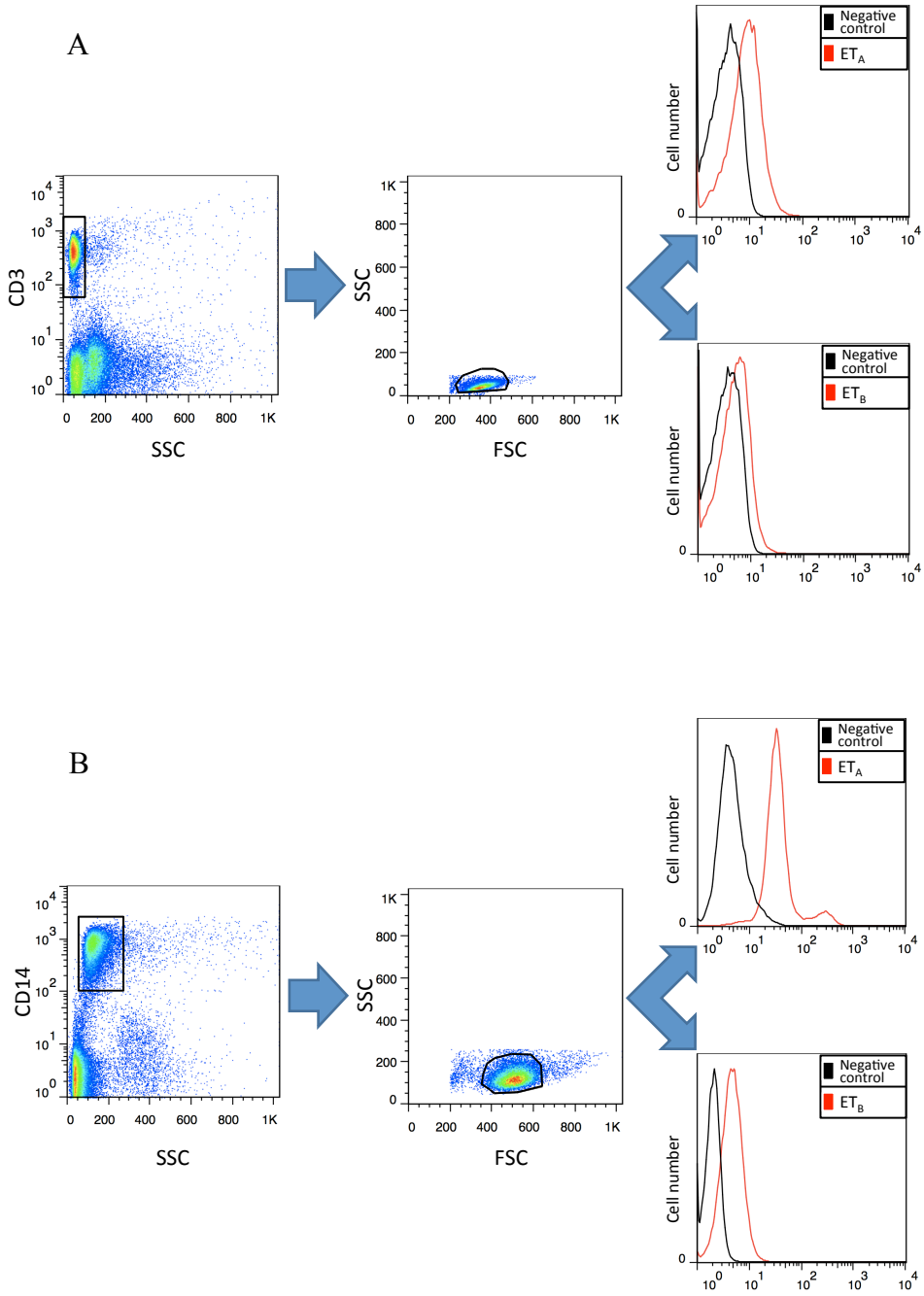


Figure 5. ET_A and ET_B expression in T cells and monocytes detected by flow cytometry. Here is shown only one paradigmatic case. T cells were selected by locating a gate on CD3+ cells and another on scattering features (A). Monocytes were picked out by locating a gate on CD14+ cells and another on scattering features (B). The quantification of receptors expression on these cells is represented by the difference of fluorescence intensity between sample (red line) and its negative control (black line).

FIGURE 6. Agarose gel electrophoretic separation of amplicons ETA and ETB. The amplification reaction occurred starting from the cDNA obtained from CD4+ lymphocytes, at basal and after stimulation with CD3/CD28, and fibroblasts. The amplification of the cDNA encoding vimentin (housekeeping gene) is the control of the method of RT-PCR. Fibroblasts are a positive control for the expression of the ETA and ETB. The CD4+ T cell express ETA and ETB.

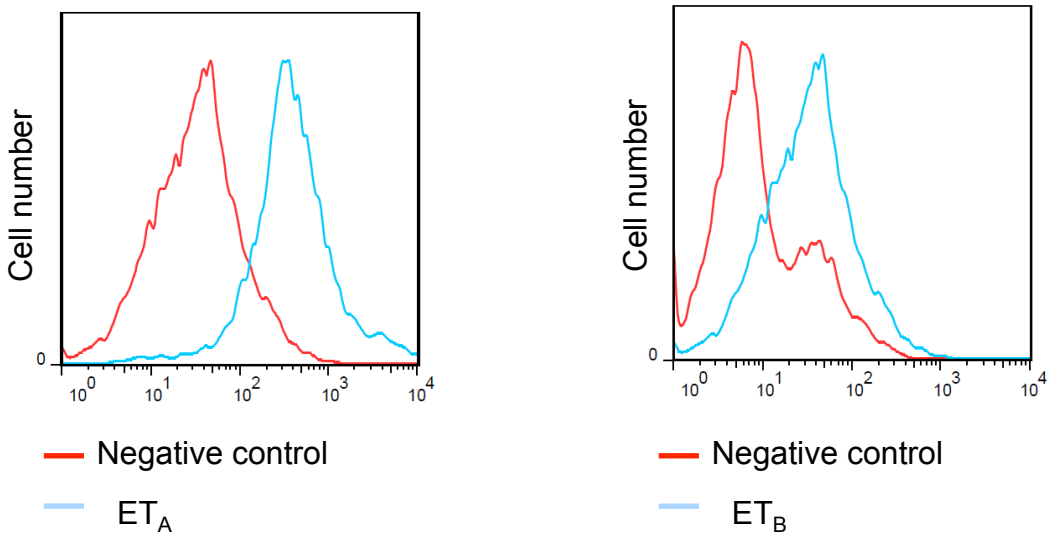


Figure 7. ET_A and ET_B expression in B cells by flow cytometry.

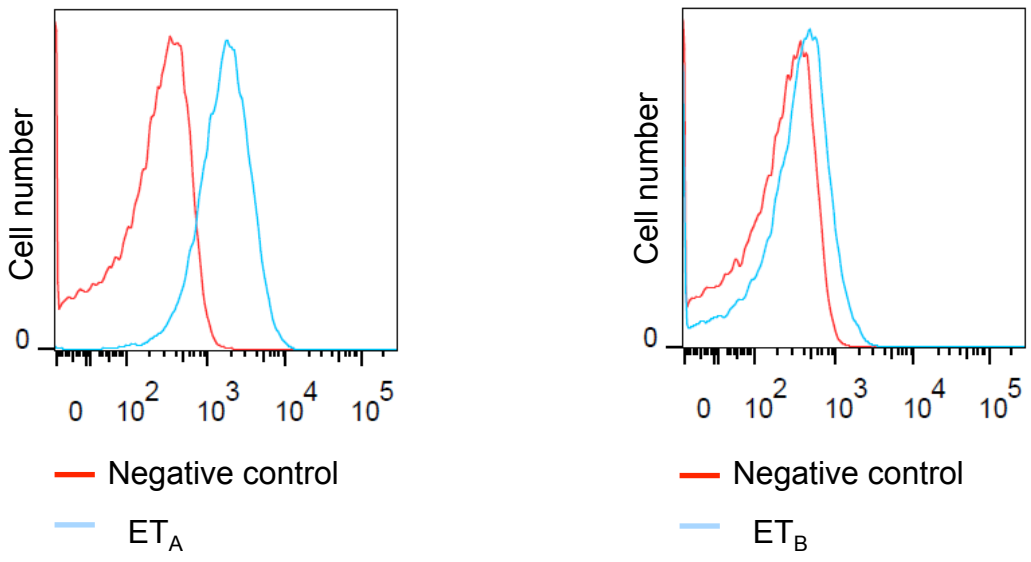


Figure 8. ET_A and ET_B expression in neutrophil by flow cytometry.

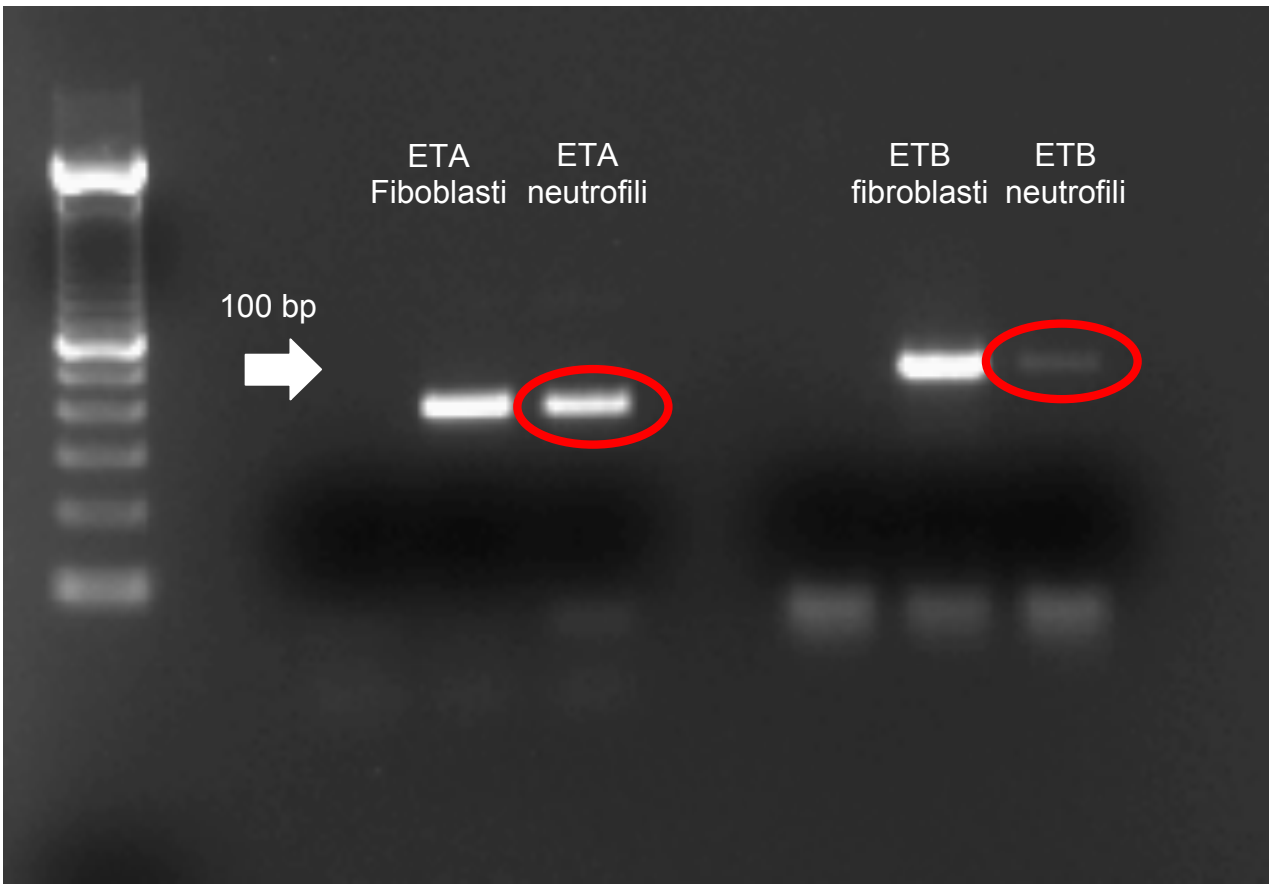


FIGURE 9. Agarose gel electrophoretic separation of amplicons ETA and ETB. The amplification reaction occurred starting from the cDNA obtained from neutrophils. Fibroblasts are a positive control for the expression of the ETA and ETB. Neutrophil express ETA and ETB.

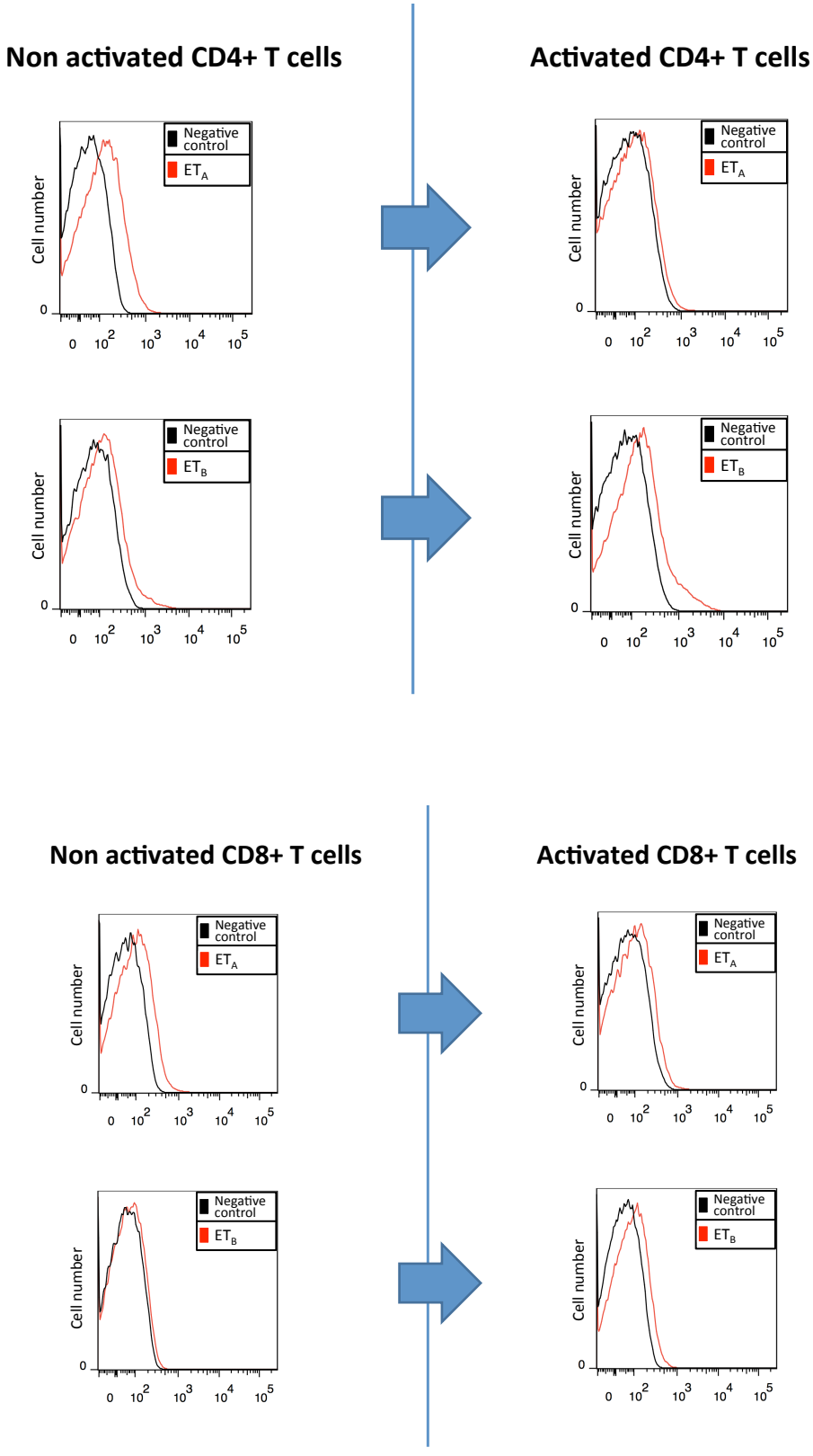


Figure 10. Change in ET_A and ET_B expression on activated T CD4+ and CD8+ cells. The stimulation of cells, performed with microbeads coated by anti-CD3/CD28 antibodies, leads to a reduction of ET_A and a raise of ET_B expression, both in CD4+ (A) and CD8 cells (B). Here is shown only one of four similar experiments.

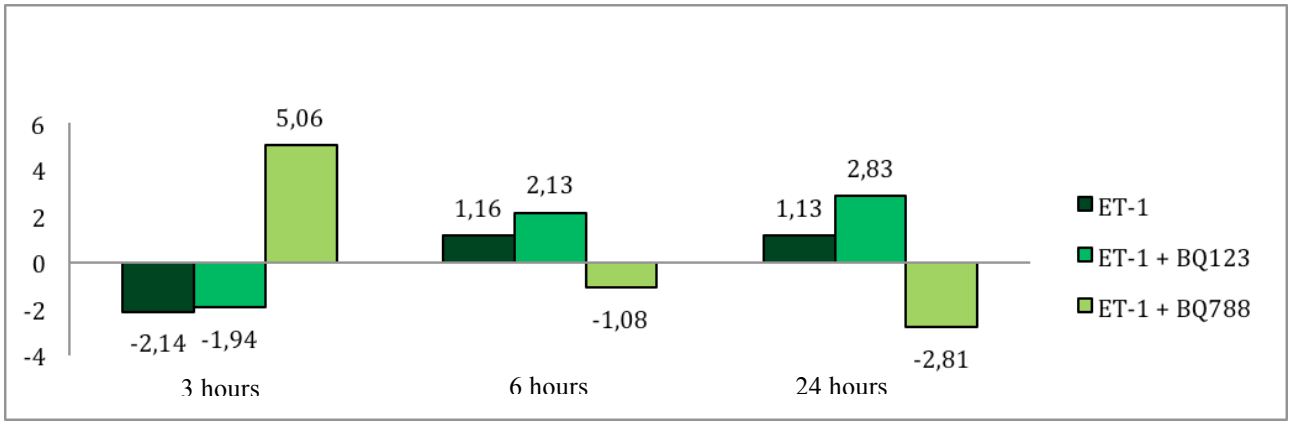


Figure 11. INF-γ, RT-PCR. The IFN-γ-coding mRNA was performed at 3, 6, 24 and 48 hours with or without receptor blockers, on T lymphocytes of a control.

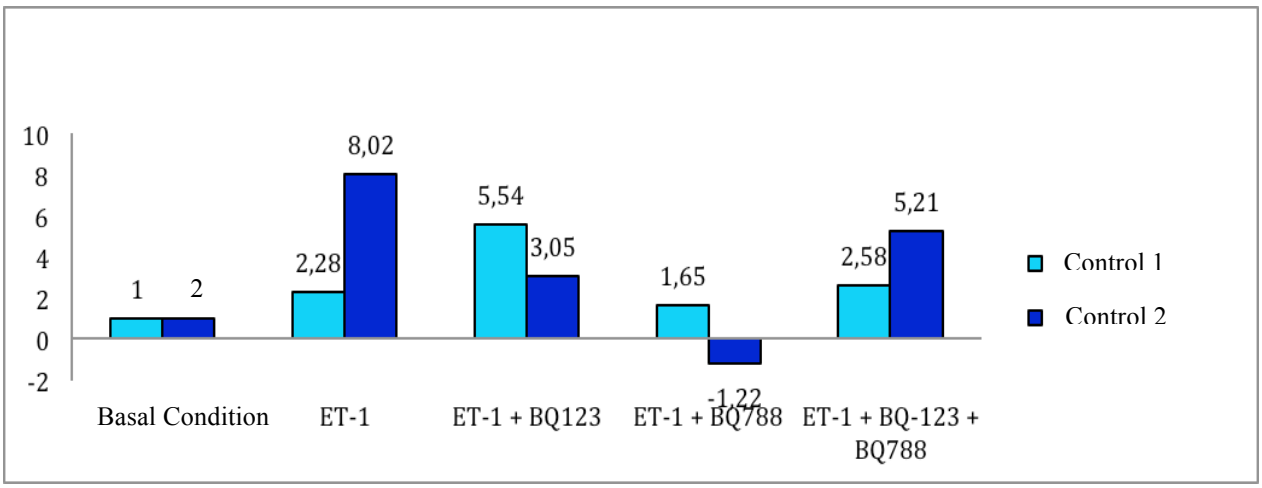
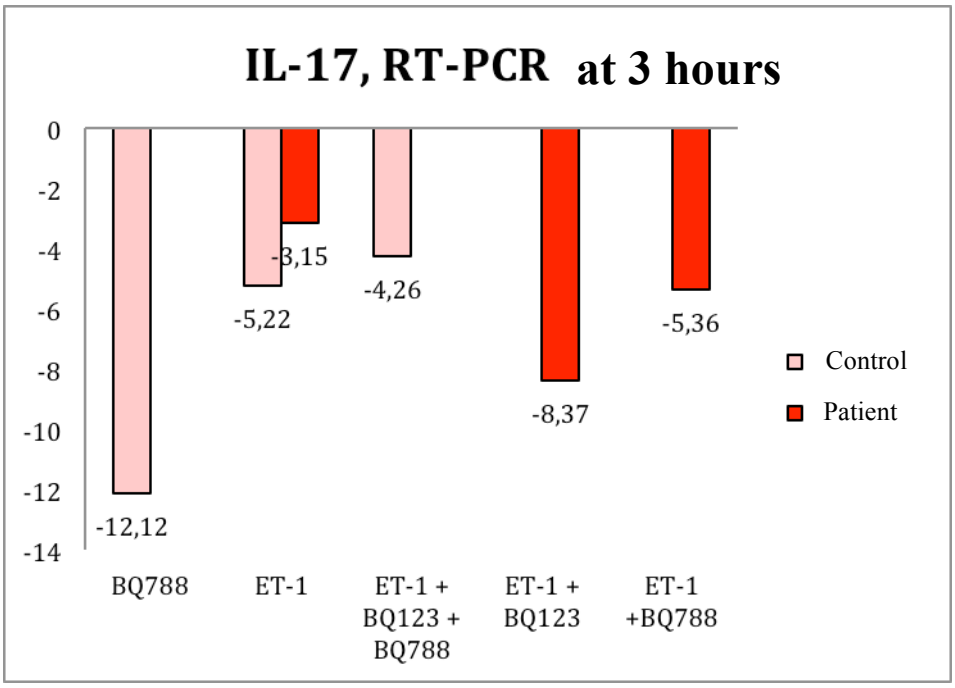


Figure 12. IL-4, RT-PCR at 6 hours. After 6 hours incubation with ET-1 alone (F.C: 8,02) or together with ET_A blockade (F.C: 5,54) or ET_A and ET_B inhibitors (F.C: 5,21) lead to an increase of IL-4-coding mRNA. Conversely ET-1 together with ET_B inhibitor induces a decrease of IL-4-coding mRNA (F.C: -1,22).



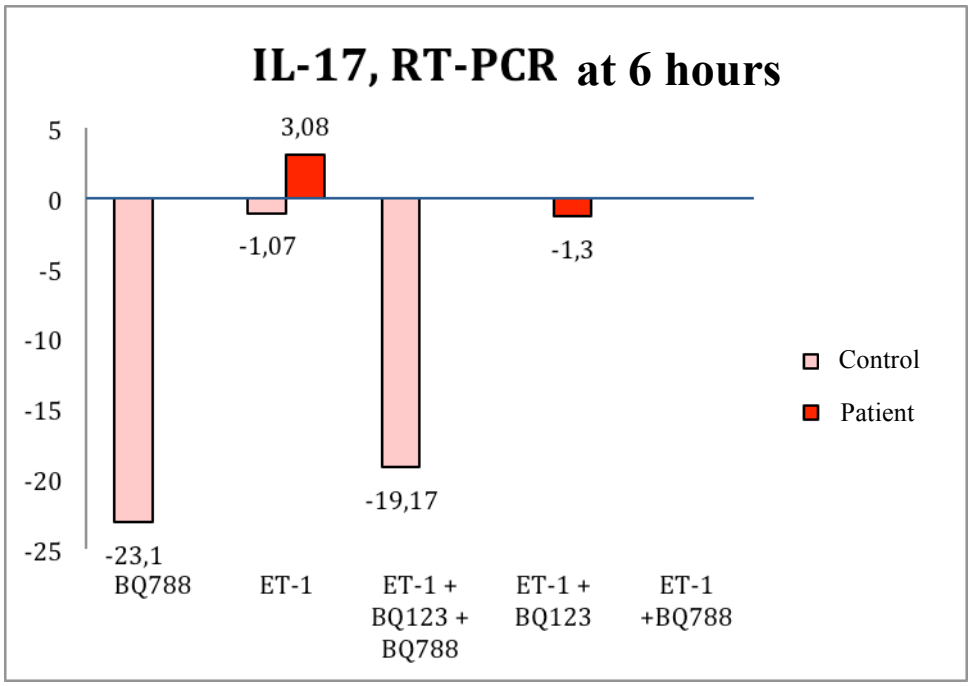


Figure 13. Using CD4+ T cell isolated from healthy control, we observed that after incubation with ET-1 for 3 and 6 hours IL-17-coding mRNA decrease. The same was obtained when cells were incubated with ET-1 in presence of ET_B inhibitor or both ET_A and ET_B blockade. In contrast using cells from patient in the same conditions, IL-17-coding mRNA showed similar decrease after 3 hours while after 6 hours using ET-1 alone we observed an increase of the transcript (F.C 3,08).

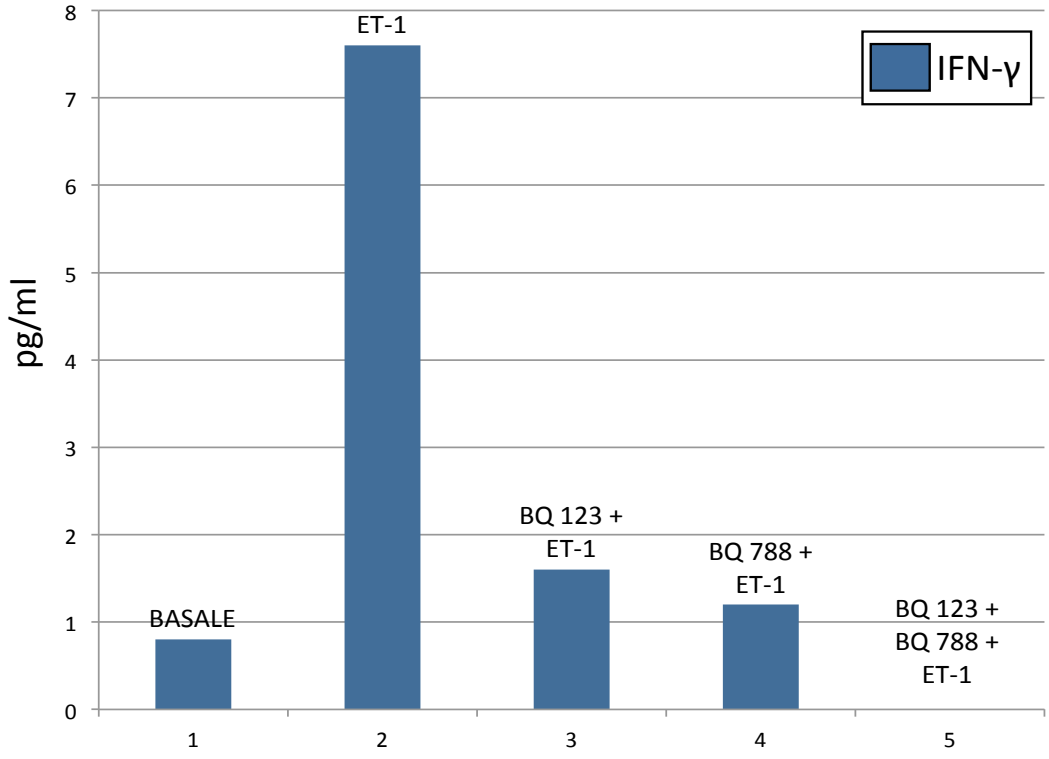


Figure 14. Proinflammatory response to ET_A and ET_B stimulation with ET-1 in T CD4+ cells. Figure shows IFN-γ production by T CD4+ cells in 5 different conditions: basal condition (1), ET_A and ET_B stimulation with ET-1 (2), ET_A blockage with BQ123 and ET_B stimulation with ET-1 (3), ET_B blockage with BQ788 and ET_A stimulation with ET-1 (4), blockage of both ET_A and ET_B and stimulation with ET-1. IFN-γ production in the supernatants of plates was measured in pg/ml and assessed on the basis of a standard curve of IFN-γ concentrations, from 0 to 1000 pg/ml. IFN-γ productions resulted: 0,8 pg/ml in condition 1; 7,6 pg/ml in condition 2; 1,6 pg/ml in condition 3; 1,2 pg/ml in condition 4; less than measurable in this system in condition 5. In condition 2, when both ET_A and ET_B were stimulated with ET-1, IFN-γ production was 9,5 times higher than basal condition. Synergic effects were observed both for the stimulation and the blockage of ET_A and ET_B.