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PhD Program in Human Health

Cycle XXXV

INCREASED EXPRESSION OF THE ECTOENZYME CD38 IN PERIPHERAL BLOOD PLASMABLASTS AND PLASMA CELLS OF PATIENTS WITH SYSTEMIC SCLEROSIS

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Years 2019-2022

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ABSTRACT

Objective: CD38 is a type II glycoprotein highly expressed on plasmablasts and on short- and long-lived plasma cells, but weakly expressed by lymphoid, myeloid, and non-hematopoietic cells. CD38 is a target for therapies aimed at depleting antibody-producing plasma cells. Systemic sclerosis (SSc) is an immune-mediated disease with a well-documented pathogenic role of B cells. We therefore analyzed CD38 expression in different subsets of peripheral blood mononuclear cells (PBMCs) from a cohort of SSc patients.

Methods: Cell surface expression of CD38 was evaluated on PBMCs from SSc patients using eight-color flow cytometry analysis performed with a FacsCanto II (BD). Healthy individuals were used as controls (HC).

Results: Forty-six SSc patients (mean age 60, range 23-79 years; 38 females and 8 males), and thirty-two age- and sex-matched HC were studied. Twenty-eight patients had the limited cutaneous form and eighteen the diffuse cutaneous form of SSc. The mean disease duration was 7 years. Fourteen patients were on immunosuppressive therapy (13 MMF, 5 RTX).
The total percentages of T, B and NK cells were not different between SSc and HC. Compared to HC, SSc patients had higher levels of CD3+CD38+ T cells ($p<0.05$), higher percentage ($p<0.001$) of CD3+CD4+CD25+FOXP3+ regulatory T cells, lower percentage ($p<0.05$) of CD3-CD56+ NK cells.
Moreover, SSc patients had higher levels of $CD24^{high}CD19+CD38^{high}$ regulatory B cells than HC ($p<0.01$), while the amount of CD24+CD19+CD38+CD27+ memory B cells was lower ($p<0.001$).
Finally, the percentages of circulating $CD38^{high}CD27+$ plasmablasts and $CD138+CD38^{high}$ plasma cells were both higher in the SSc group than in HC ($p<0.001$).
We did not observe any correlations between these immunophenotypes and disease subsets or duration, and ongoing immunosuppressive treatment.

Conclusions: The increased expression of CD38 in peripheral blood plasmablasts and plasma cells of SSc patients may suggest this ectoenzyme as a candidate therapeutic target, under the hypothesis that depletion of these cells may beneficially downregulate the chronic immune response in SSc patients. Validation of this data in multicenter cohorts shall be obtained prior to clinical trials with existing anti-CD38 drugs.

1. INTRODUCTION and BACKGROUND

1.1. Classification and epidemiology of Systemic Sclerosis

Systemic sclerosis or scleroderma (SSc) is a multisystem connective tissue disease characterized by immune dysregulation, obliterative vasculopathy, and fibrosis of skin and internal organs. It has the highest disease-related mortality and morbidity with an impaired quality of life among the rheumatologic illness [3]. It is believed that its complex pathophysiology involves genetic and environmental factors, vascular and immune system functions, as well as fibroblasts and extracellular matrix alterations. Clinically it is characterized by the massive deposition of collagen fibers in tissues such as the gastrointestinal tract, lungs, heart and kidneys, by alterations of the microvasculature and by disturbances of the immune system at both the cellularand humoral level. Patients with SSc almost universally present numerous autoantibodies, some of which seem to be disease-specific.

The results of studies of the prevalence and incidence of SSc are conflicting because of methodological variations in case ascertainment and geographic differences in these measurements.

The available data indicate a prevalence ranging from 50 to 300 cases per 1 million persons and an incidence ranging from 2.3 to 22.8 cases per 1 million persons per year. Women (age range mainly between 30 and 50 years) are at much higher risk for scleroderma than men, with a ratio ranging from 3:1 to 14:1 [4].

Subsets of SSc can be discerned: limited cutaneous SSc (lcSSc), diffuse cutaneous SSc (dcSSc), and SSc without skin involvement [5].

In limited cutaneous SSc, fibrosis is mainly restricted to the hands, arms, and face. Raynaud's phenomenon is present for several years before fibrosis appears and pulmonary hypertension (PAH) is frequent. The prognosis is significantly more favourable when compared with patients with the diffuse variant. Limited SSc indeed presents a more benign disease course and a lower incidence of renal involvement (Figure 1A).

Diffuse cutaneous SSc is a rapidly progressing disorder that usually begins with symmetric finger and hand swelling that generalizes to involve the forearms, arms, face, trunk, and lower extremities. Over time, the oedema evolves into firm bound-down induration and fibrosis that

eventually cause deformities of the digits, which typically show fixed-flexion contractures of the proximal interphalangeal joints. In the majority of diffuse scleroderma patients, the onset of edema takes place soon after the first episode of Raynaud's phenomenon. In dSSc the internal organ involvement is early and severe and compromises one or more organs. Anti-topoisomerase I antibodies are present in approximately 40% of dSSc patients (Figure 1B).

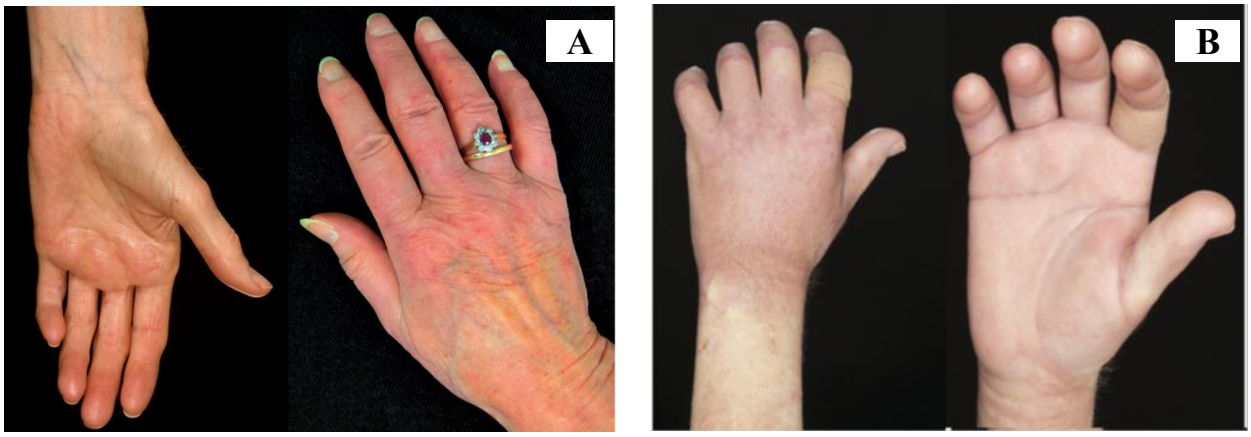

Figure 1. (**A**) **Limited cutaneous systemic sclerosis**. Limited cutaneous SSc is associated with mild skin involvement distal to the elbows and knees, with or without face and neck involvement, and sparing of the chest and abdomen. A representative image of sclerodactyly. (**B**) **Diffuse cutaneous systemic sclerosis**. Hand function is affected in these patients and is often associated with severe digital ulcers and ulceration over areas of pressure or trauma [6].

In the past the term CREST (calcinosis, Raynaud's phenomenon, esophageal motility dysfunction, sclerodactyly, and telangiectasia) syndrome was comprised within the limited scleroderma subset, but it no longer used. A small number (<5%) of patients have clinical features (most commonly Raynaud's phenomenon, digital ulcers, and pulmonary arterial hypertension) and autoantibodies that are specific to systemic sclerosis, but no skin involvement (so-called sine scleroderma). Patients with scleroderma plus evidence of systemic lupus erythematosus, rheumatoid arthritis, polymyositis, or Sjogren's syndrome are considered to have an overlap syndrome. In some patients, organ-based manifestations of the disease are observed, which might include lung fibrosis, pulmonary arterial hypertension, renal failure (usually with accelerate-phase hypertension and a thrombotic microangiopathy clinical picture), or gastrointestinal complications [6].

The involvement of visceral organs is a major factor in determining the prognosis; severely debilitating esophageal dysfunction is the most common visceral complication, and lung involvement is the leading cause of death [7].

Fibrosis is caused by fibroblast activation, proliferation and increased deposition of extracellular matrix (ECM) proteins such as fibronectin and collagen in various organs. Progression of organ fibrosis leads to end-stage organ failure as a result of the loss of normal structure and function. Notably, interstitial lung disease (ILD) is currently one of the most significant complications of SSc. ILD is currently the leading cause of death in patients with SSc. Furthermore, skin fibrosis causes reduction of wrinkles and disappearance of the cutaneous furrows. These features lead to diminished mouth opening and width, concomitance of sicca syndrome, and finger joint contractures with impaired quality of life [6].

In general, the survival rate lies between 34 and 73% [8]. It is, however, lower and associated with a poorer prognosis in men and older patients, while women and younger patients generally present a higher survival score and better prognosis. In SSc patients, it has also been observed an increased susceptibility to the development of cancer, mainly of the lung [9].

1.2 Pathogenesis of Systemic Sclerosis

SSc is a clinically heterogeneous disease; despite extensive investigations, the key pathogenic links between these disease hallmarks remain obscure, as well as the etiology underlying the beginning of this complex disorder.

Familial clustering of the disease, the high incidence of other autoimmune disorders in families of patients with scleroderma [10], and differences in phenotype among racial and ethnic groups, suggest that genetic factors contribute to SSc. The most prominent genetic factor is gender. Epidemiologic studies have shown a significant increase in the risk of SSc in first-degreerelatives of patients with the disease: 1.6% versus a 0.026% risk in the general population, and there is strong evidence of familial clustering of cases [11]. Twin concordance rates for scleroderma are low; in the latest publication to date involving 42 twin pairs, the concordance rate for monozygotic twin pairs was 4.2% and for dizygotic twin pairs 5.6% [12].

Candidate gene association studies and gene linkage studies (GWAS) have clearly indicated both human leukocyte antigen (HLA) and non-HLA genes that are associated with scleroderma, its subphenotypes and its specific autoantibodies.

The HLA gene loci show the strongest association and particularly with specific autoantibodies (e.g. anti-topoisomerase 1 with DPB1*1301, anti-fibrillarin with DRB1*1302). The association

with the non-HLA loci is only modest, but more importantly, these gene associations have implicated a number of immune pathways likely relevant in pathogenesis. These associated genes codes for proteins involved in immune processing, antigen presentation and inflammation, immune signalling, T-cell differentiation and activation, and innate immunity [13] Further, manyof these latter genes have also been associated with other known autoimmune disorders (e.g. systemic lupus erythematosus (SLE), thyroid disease, Type 1 diabetes mellitus) [14].

Viral agents have been traditionally considered as potential etiopathogenic triggers of SSc. The most compelling evidences in favor of a viral etiopathogenesis of SSc are focused on cytomegalovirus (CMV) and Parvovirus B19. Chronic CMV infections in humans may play an important role in the pathogenesis of vascular injury that involves small vessels, particularly the arterioles. Along with smooth muscle cells, epithelial cells are the predominant targets for virus CMV replication which might induce epithelial-mesenchymal transition (EMT), a relevant step in fibrosis [15]. Recent studies are focusing on the involvement of adenovirus.

In the pathogenesis of scleroderma play a central role: endothelial damage, excessive production and deposition of collagen involving fibroblasts, oxidative stress and reactive oxygen species (ROS), oxygenation of the tissues, and finally autoimmunity, which reflects the coordinated activation of innate and adaptive immune responses (Figure 2).

Endothelial dysfunction is considered a pivotal factor contributing to peripheral vessel remodelling in SSc. Endothelial-to-mesenchymal Transition (EndoMT) may represent a crucial link between endothelial cells (ECs) dysfunction and development of fibrosis.

EndoMT is a phenotypical conversion in which ECs downregulate the expression of theirspecific markers, such as CD31/platelet-EC adhesion molecule-1, von Willebrand factor (vWF) and vascular endothelial (VE)-cadherin, and acquire mesenchymal cell products including α- SMA, S100A4/fibroblast-specific protein-1 (FSP1) and type I collagen, together with stabilisation and nuclear translocation of the transcriptional regulator Snail1, a crucial trigger of mesenchymal transition.

Multiple pathways such as transforming growth factor-β (TGFβ), endothelin-1 (ET-1), notch, sonic hedgehog and Wnt pathways may participate in the molecular mechanisms of the EndoMT process.

This evidence was demonstrated by Matucci *et al* in a study involving SSc patients and two mouse models of the disease [16].

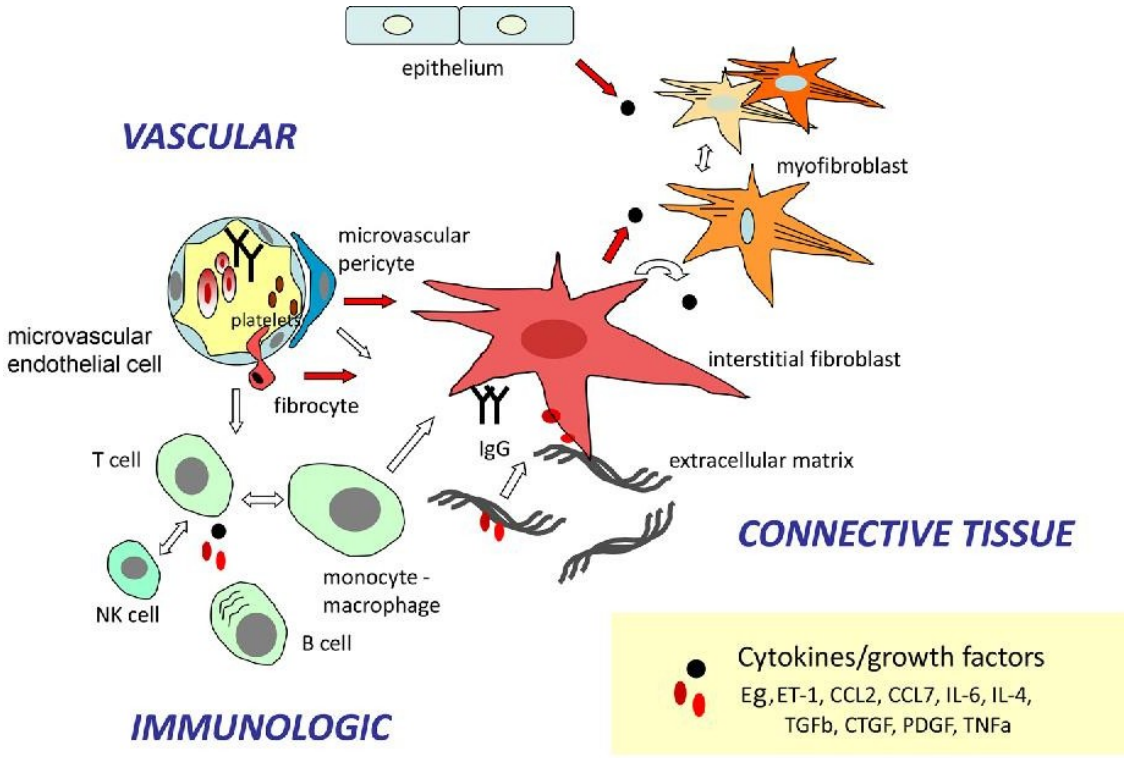

Figure 2. Cellular interplay in the pathogenesis of SSc. CCL2, chemokine ligand 2; CCL7, chemokine ligand 7; CTGF, connective tissue growth factor; ET-1, endothelin-1; IgG, immunoglobulin G; NK, natural killer; PDGF, platelet-derived growth factor; TGFb, transforming growth factor beta; TNFa, tumor necrosis factor alpha [11].

Regarding excessive extracellular-matrix production, Le Roy *et al* provided data demonstrating that *in vitro* the scleroderma fibroblast produces more soluble collagen, synthesizes collagen more rapidly, and fourfold more of its protein synthetic activity is directed to collagen production than in the normal skin fibroblast. Proline hydroxylase levels, an enzyme involved in collagen synthesis, have been variably elevated in skin biopsy material from scleroderma patients [17].

Recent studies have demonstrated that fibroblasts of SSc patients produce, in vitro, high levels of reactive oxygen species (ROS). The resulting oxidative stress induces the activation of fibroblasts, resulting in an increased proliferation rate and augmented transcription of collagen genes. ROS are also able to induce the expression and stabilization of Ras protein, through the activation of extracellular-signal-regulated kinases (ERK1/2) [18]. It is known that the production of ROS can also be triggered by the binding of PDGF to its receptor expressed on the cell surface of fibroblasts, and by the subsequent activation of NADPH oxidase.

Profound alterations characterize the adaptive immune response in systemic sclerosis, and several layers of evidence support a prominent role exerted by immune cellular effectors and humoral mediators in the pathogenesis of this disease. These include (i) the presence of oligoclonal T cells in tissues undergoing fibrosis consistent with (auto)antigen-specific recruitment, (ii) the preferential expansion of polarized CD4+ and CD8+ T cells producing pro- fibrotic cytokines such as IL-4 and IL-13, (iii) the presence of increased number of cells producing mediators belonging to the IL-17 family, including IL-22, which may drive and participate in inflammatory pathways involving epithelial cells as well as fibroblasts, (iv) the deficient or redirected function of T regulatory cells favoring fibrosis, and (v) the enhanced expression of CD19 and CD21 on naïve B cells, and the upregulation of co-stimulatory molecules in mature B cells, which together with the increased levels of B cell activating factor (BAFF) underlie the propensity to an exaggerated humoral response possibly favoring fibrogenesis [19].

T lymphocytes and cytokines

The abundance of CD4+ T lymphocytes in the perivasal infiltrates and in the neo-formed fibrotic tissue, commonly found in scleroderma patients as a result of a generalized state of inflammation, suggests that these cells could hold a pathogenic role in the disease [20]. CD4+ cells constitute the "helper" class of lymphocytes (Th). T lymphocytes of SSc patients show a remarkable increase of the Th2 subset, with consequently increased IL-4, IL-6, IL-10 and IL-13 serum levels. Moreover, the plasmatic level of monocyte chemoattractant protein 1 (MCP-1) is significantly increased during the course of scleroderma, contributing to the unbalance towards the Th2 phenotype. Indeed, MCP-1 is an important pro-fibrotic factor in vitro and it has been shown to regulate the migration of monocytes and Th2 lymphocytes at sites of inflammation [21].

B cells in SSc and fibrosis

Analysis of circulating B cell repertoire in SSc has shown expansion of the (CD27-) naïve B cell subset and the concurrent decline of memory B cells and plasmacellular components.

However, both naive and memory B cells from SSc patients overexpressed a positive response regulators such as CD19; loss of CD19 expression attenuates skin and lung fibrosis in the bleomycin-induced SSc mouse model. Members of the tumor necrosis factor (TNF) superfamily exerting important homeostatic functions on B cells, BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand), are increased in SSc patients and associated with specific

clinical manifestations such as extent of skin involvement and the presence of pulmonary fibrosis. In addition, B cells were shown to induce contact-dependent human dermal fibroblasts activation with upregulation of, among other mediators, type I collagen [19].

1.3 Autoantibodies and Systemic Sclerosis

SSc is also characterized by the presence of circulating autoantibodies to several cellular and extracellular components. Around 95% of patients with SSc have detectable autoantibodies, which in most cases are highly specific for the disease. The target antigens for the commonly seen antibodies are all intranuclear.

Distinct specificities of antinuclear antibodies (ANAs) are selectively detected in SSc patients and are associated with unique disease manifestations, but do not have a proven pathogenic role. A new group of autoantibodies reactive with cell surface receptors have been identified in SSc patients. They have been shown to directly activate pathways that may contribute to tissue and vascular damage [22].

1.3.1. Anti-nuclear antibodies (ANA)

One representative feature of the immunological abnormalities in SSc patients is the presence of antinuclear antibodies (ANA). ANA are present in more than 90% of SSc patients, and these ANA react against various intracellular components.

Anti-centromere antibodies (ACA) and anti-topoisomerase I antibodies (anti-topo I, formerly anti-Scl-70) are the classic ANA found in SSc.

Autoimmune disease detection protocol starts with determination of ANA performed through immunofluorescence staining (IF) using HEp-2 cells and a positive ANA test leads to further investigation of extractible nuclear antigens (ENA) [23].

Except for ACA, it is difficult to identify the specific ANA by IF because epitopes cannot be well defined using this technique.

Therefore, additional techniques such as enzyme-linked immunosorbent assay (ELISA), immunodiffusion and immunoprecipitation are required to confirm ANA in a patient's sera.

a) Anti-centromere antibodies (ACA)

Although the frequency of ACA in patients with SSc has been reported to be 20–30% overall, it varies among different ethnic populations. The presence of ACA are predictive for development of pulmonary hypertension but not pulmonary fibrosis [24, 25]. ACA-positive SSc patients possess a more favourable prognosis (associated with limited skin involvement) than patients with other SSc-related ANA [25].

The class of ACA comprises autoantibodies specific for six different centromeric polypeptides (CENP A-F). For clinical use, an ELISA system based on a cloned fusion protein of the CENP-B antigen has been established.

b) Anti-topoisomerase I antibodies

Anti-topo I antibodies were found in approximately 40% of patients with dSSc, but in less than 10% of patients with lSSc. Anti-topo I are usually not found in healthy individuals, in patients with other connective diseases or primary Raynaud's syndrome [26]. They are associated with a higher risk for severe pulmonary fibrosis early in disease.

In vitro studies have revealed direct pathogenic effects of these autoantibodies found in SSc. Henault *et al* [27] have reported that the autoantigen topoisomerase I was bound specifically to fibroblasts, where it was recognized by anti-topo I from SSc patients.

The binding of anti-topo I subsequently stimulated adhesion and activation of cultured monocytes. Topo I released from apoptotic endothelial cells also bound specifically to fibroblasts. Thus, it is possible that, in vivo, topoisomerase I binds to fibroblast surfaces, recruits anti-topo I and subsequently induces monocyte adhesion and activation, leading to the development of SSc.

c) Other anti-extractable nuclear antigens (ENA) antibodies

<u>Anti-RNA polymerase I, II and III antibodies</u>

The co-presence of anti-RNA polymerase RNAP I and III antibodies is specific to SSc with a prevalence of approximately 20% and is usually associated with diffuse cutaneous involvement and higher risk of renal crisis.

Antibodies to RNAP II alone are rare and not specific for SSc because they are also detectable in SLE and overlap syndrome.

Anti-Th/To antibodies (anti-Th/To, known as anti-7-2RNA antibodies)

Anti-Th/To are present in a small subset of patients with SSc (2–5%) and are associated with limited skin involvement, but a high risk for severe organ involvement and therefore they stand for a worse overall prognosis.

Some antinuclear antibodies can be found in SSc-myositis overlap:

✓ Anti-U1RNP antibodies (anti-U1RNP) – found in 90% of patients with mixed connective tissue disease and in approximately 6% of SSc patients.

✓ Anti-RM-Scl (anti-PM-Scl) – found in 4-11% of SSc-myositis overlap patients, but only in 2% of patients with scleroderma only.

1.3.2 Antibodies other than Anti-nuclear antibodies

In SSc patients there is another group of autoantibodies reactive with cell surface receptors. These include the antibodies anti-endothelial cells (AECA), anti-angiotensin II type 1 receptor (AT1R), anti-endothelin-1 type A receptor (ETaR) and a new class of antagonistic autoantibodies, the anti-muscarinic-3 receptor (M3R) antibodies.

- AECA are indicative of the entity of vascular damage and the extent of visceral involvement; these autoantibodies are able to increase in endothelial cells the expression of inflammatory cytokines (IL-1, IL-6 and MCP-1) and adhesion molecules (ICAM-1), thus may contribute to SSc vascular lesions.
- Angiotensin II type 1 receptor and endothelin-1 type A receptor are widely expressed on cells of the vascular system and on immune cells. Anti-AT1R and anti-ETAR antibodies were found in about 85% of SSc patients. Higher levels of anti-AT1R and anti-ETAR antibodies were associated with severe SSc vascular manifestations such as digital ulcers and PAH. In human dermal microvascular endothelial cells these autoantibodies induce ERK1/2 phosphorylation and increased TGFß gene expression; their role were tested also in vivo.
- Autoantibodies against muscarinic-3 receptor (M3R):

  Gastrointestinal (GI) involvement leading to dysmotility is frequent in SSc as a result of disturbance of cholinergic neurotransmission and smooth muscle atrophy, in which stimulation of the muscarinic-3 receptor (M3R) is the principal excitatory mediator of GI. Anti-M3R autoantibodies may lead to failure of the cholinergic neurotransmission and, in turn, result in GI motility dysfunction. Several studies found a high incidence of anti-myenteric neuronal antibodies in the sera of SSc patients with GI symptoms [22].

1.4. Diagnosis of Systemic Sclerosis

The difficulties inherent in diagnosing, screening and treating SSc are due to the complex pathology of the disease, which involves interplay between the immune system, vasculature and components of connective tissue. Therefore, a cross-disciplinary collaboration in the screening and diagnosis of patients is important. There are multiple tools used in clinical practice, such as clinical history and physical examination (RSS - Rodnan skin score to measure the extent of skinfibrosis), laboratory tests (detection of autoantibodies as markers of disease) and instrumental exams (capillaroscopy and exams for internal organ involvement). In the absence of a diagnostic test proving absence or presence for SSc, several sets of classification criteria have been developed.

Because of the insufficient sensitivity of the 1980 criteria and advances in knowledge about SSc, the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) established a new classification criteria for SSc. The experts concluded that `skin thickening of the fingers of both hands that extends proximal to the metacarpophalangeal joints' was sufficient to classify a subject as having SSc. Further, patients with skin thickening sparing the fingers' are classified as not having SSc. The newly developed classification system includes the four items of the 1980 ARA classification criteria as well as the items of the 2001 proposal for classification of SSc by Leroy and Medsger.

There are 7 domains, some have several possible manifestations and each manifestations have a score: Skin thickening of the fingers (Puffy fingers, Sclerodactyly), Fingertip lesions (Digital Tip Ulcers and Finger Tip Pitting Scars), Telangiectasia, Abnormal nailfold capillaries, Pulmonary arterial hypertension and/or Interstitial lung Disease, Raynaud's phenomenon, Scleroderma related antibodies.

The new classification includes the concept of specific serum autoantibodies such as anti-topoisomerase I, anti-centromere, and anti-RNA polymerase III. There is the possibility that other SSc autoantibodies such as anti-Th/To, anti-U3 RNP and others may become more widely available. The maximum possible score is 19 and patients who have 9 points or more are classified as having SSc.

The sensitivity and specificity of the new SSc criteria were 0.91 and 0.92 in the validation sample; the new system is more inclusive and also perform well in patients with early disease, meaning a time since diagnosis of 0–3 years [5].

1.5. Raynaud's phenomenon and early diagnosis of Systemic Sclerosis

The most peculiar vascular dysfunction in SSc is the Raynaud's phenomenon. This clinical feature is caused by the dysregulation of the vascular tone of fingers and toes. It is an episodic digital ischemic vasospasm triggered by cold- or emotional-stress leading to a pale and cyanotic skin with a postischemic phase of hyperemia [28].

Raynaud's phenomenon is further divided into primary Raynaud's disease and secondary Raynaud's phenomenon, related to a connective tissue disease. Primary Raynaud's disease is common, with a prevalence of 3% to 5% in the general population, and remains uncomplicated without permanent injury, while 95% of scleroderma patients have Raynaud's phenomenon, often as the first symptom of disease. The incidence of progression from isolated Raynaud's phenomenon to definite SSc is 12,6% [29] and the incidence of progression to any connective tissue disease (CTD) is 13,6% [29, 30].

In SSc, in addition to an imbalance in endothelial signals (for example, an increased release of vasoconstrictive endothelin), other factors, including impaired vasodilatory mechanisms (such as lowering of nitric oxide levels or of endothelial-dependent relaxation factor), enhanced platelet aggregation and reduced neuropeptide levels, contribute to the vasospastic propensity. Other harmful factors, such as toxic factors, proteases (e.g., granzyme 1), lipoperoxides, and anti-endothelial autoantibodies may contribute to this process [31, 32].

The diagnosis of SSc and, consequently, an appropriate therapy are delayed until the appearance of skin involvement and/or clinically detectable internal organ involvement when microvascular remodeling, tissue fibrosis, or atrophy are already irreversible.

In a recent Delphi exercise [33], four signs/symptoms have been identified as necessary for the very early diagnosis of SSc: Raynaud's phenomenon (RP), puffy swollen digits turning into sclerodactyly, antinuclear antibodies and specific SSc antibodies (ACA and anti-topo I antibodies), and abnormal capillaroscopy with scleroderma pattern.

1.6. Therapy of Systemic Sclerosis

At present there is no etiopathogenetic therapy that can cure SSc, but only symptomatic drugs that can control the most common ailments of the disease. The drugs generally used in the treatment of SSc are:

Immunosuppressing medications: (nonsteroidal anti-inflammatory, high-dose corticosteroids, cyclophosphamide in combination with steroids, cyclosporine) have poor efficacy and their use is rationally restricted to forms of scleroderma characterized by a strong inflammatory component, to control joint muscle and tendon pain. Cyclophosphamide is the only disease- modifying drug, since it is approved and effective for active SSc with lung involvement [34].

Symptomatic organ-specific individual SSc patients have variable and different severity of organ involvement. The treatment must be individualized, to minimize the symptoms and to preserve as much as possible the functionality of each organ. Some examples of organ-specific therapy are: analogs of prostacyclin, with vasoactive action, for the treatment of ulcers and pulmonary hypertension, the proton pump inhibitors and prokinetic drugs for reflux esophagitis, blood pressure medications (particularly angiotensin converting enzyme (ACE) inhibitors) for high blood pressure or kidney problems.

Thanks to the advances made in research in recent years new experimental therapies have been adopted, greatly helping to improve the quality and life expectancy of SSc patients. The drugs currently used are [35, 36]:

• Inhibitors of inflammatory pathways: anti CD-20 (Rituximab), blockers of IL-6 receptor (Tocilizumab)

• Anti-fibrotic drugs: tyrosine kinase inhibitors (Nintedanib)

• Endothelin receptor antagonists (Bosentan)

Some of these drugs, such as rituximab and nintedanib are now used extensively in clinical practice having shown remarkable efficacy in clinical trials with regard to the pulmonary involvement of the disease. Notwithstanding recent advances, translational and clinical research studies aiming to find disease-modifying therapies in SSc is very much needed.

1.7. Cluster of differentiation 38 (CD38) and its role in human health

CD38 (Cluster of Differentiation 38) is a multifunctional ecto-enzyme that metabolizes NAD+ and mediates nicotinamide dinucleotide (NAD+) and extracellular nucleotide homeostasis as well as intracellular calcium. CD38 is also an emerging therapeutic target under conditions in which metabolism is altered including infection, aging, and tumorigenesis [37].

CD38 is expressed predominately on immune cells in response to stimulation by cytokines, endotoxins, and interferon. Expression of the enzyme is regulated by a promoter region containing

binding sites for NF-kB, RXR, LXR, and STAT suggesting that it plays a key role in the inflammatory response. CD38 expression causes a substantial decline in cellular NAD+ levels, thus altering the availability of substrates for enzymes regulating cellular homeostasis. Thus, infiltration of CD38-expressing immune cells during infection, aging, or tumorigenesis has the potential to: alter NAD+ homeostasis in parenchymal tissues or the tumor microenvironment; disrupt normal metabolic processes; and undermine tissue integrity.

The identification of CD38 as a key modulator of NAD+ metabolism in the context of cell signaling, aging, and tumor biology suggests that the enzyme is a target of promising therapeutic potential. CD38 is paradoxical in its mode of action, diverse in its locale, and functionally pleotropic, and thus presents numerous drug design challenges and opportunities [37].

CD38 is positioned in the cellular membrane with its catalytic site facing toward the extracellular environment in a type II orientation (Figure 3). Functionally, over 90% of CD38 acts as an ecto-NADase that catabolizes b-NAD+. Given the abundance of intracellular NAD+, the presence of an extracellular catalytic site presents a topological paradox. In addition to NAD+, CD38 metabolizes extracellular NAD+ precursors (NMN and NR) prior to their transport into the cell for NAD+ biosynthesis. It has been shown that the ecto-NMNase activity of CD38 plays a critical role in the regulation of nicotinamide nucleotides during the aging process.

A role of CD38 as a second messenger enzyme for the synthesis of cADPR has also been proposed. Interestingly, CD38 is a very inefficient cyclase and must degrade nearly 100 molecules of NAD+ to generate one molecule of cADPR.

Another role for CD38 is the regulation of extracellular adenosine, which requires consumption of NAD+. The synthesis of adenosine from NAD+ is a complementary mechanism similar to the CD39/CD73-mediated catabolism of ATP to adenosine (Figure 3)[38]. Adenosine is important in immune modulation because it has been implicated in immune suppression through purinergic receptor binding and in the immunomodulation of multiple myeloma and lung cancer. Thus, involvement of CD38 in the regulation of NAD+ and adenosine homeostasis has led to some speculation that CD38 may function as an immune check point molecule [37].

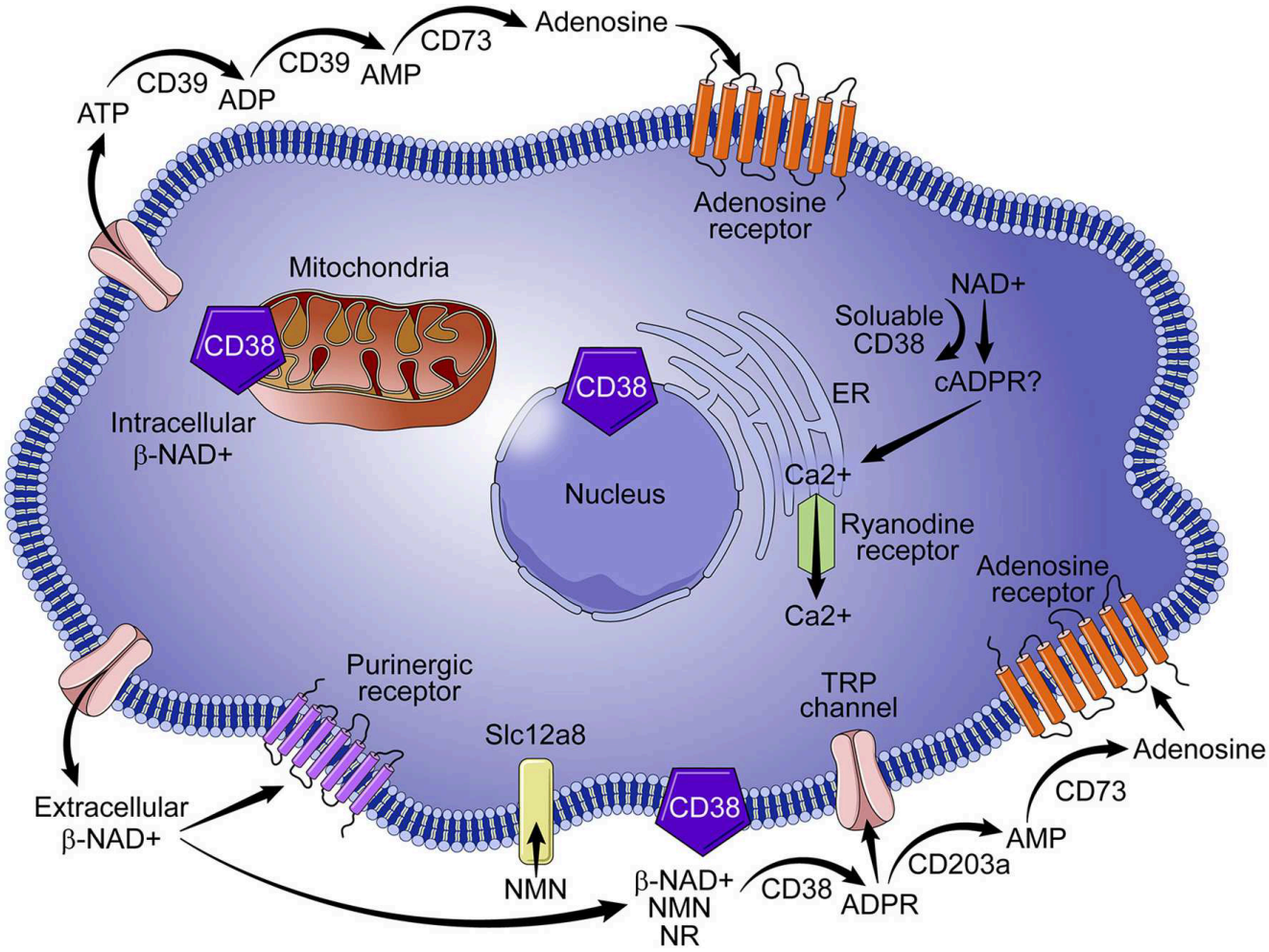

Figure 3. Role of CD38 in NAD+ metabolism. CD38 is predominantly expressed on immune cells and metabolizes nicotinamide nucleotides (NAD+ and NMN) to ADPR and cADPR, which results in the mobilization of calcium. Although intracellular CD38 is present in the cytoplasm and in the membranes of organelles, a vast majority of CD38 activity is extracellular, which results in degradation of NAD+ precursors (e. g., NMN) necessary for NAD+ synthesis. Extracellular activity of CD38 has wide ranging implications for NAD+ homeostasis in the context of infection, metabolic dysfunction, aging, and tumor biology.

2. AIM OF THE STUDY

Despite all efforts, there is currently no curative therapy and SSc is still a severe disease, causing disability with morbidity and mortality directly linked to the extent of fibrosis [39].
Since immune dysregulation is believed to play a major role in the pathogenesis of SSc, therapeutic regimens based on immunosuppressive drugs like mycophenolate mofetil still remain the main therapeutic option, especially in SSc with severe lung disease [40].

Autoantibodies, some of which potentially pathogenic [41], are a hallmark of SSc and, together with B cell infiltrates in skin biopsies of SSc patients, suggest the involvement of adaptive immunity in the pathogenesis of the disease and fostered the therapeutic use of B-cell depleting drugs such as Rituximab (RTX). Rituximab is an anti B-cell antigen CD20 but its mechanism of action in connective tissue diseases is still unclear and probably goes beyond depletion of B cells. Conflicting data have been reported about its efficacy in SSc [42]. In a recent systematic review and meta-analysis, Goswami and colleagues showed that RTX, as a treatment of SSc interstitial lung disease, improved FVC and DLco during the first year of treatment [43]. Two other systematic reviews and meta-analysis [44-45] and an observational study which enrolled 254 SSc patients [46] indicated improvement of skin score and stabilization of organ involvement. Despite these observations on possible beneficial effects, long-lasting results have not been documented.
A possible drawback for RTX efficacy may come from the fact that this drug is certainly efficacious in B cell depletion but plasma cells and hematopoietic stem cells are not targeted.

CD38, a multifunctional ectoenzyme present on plasma cells and other lymphoid and myeloid cell populations, may be pharmacologically attractive for targeting plasma cells as demonstrated by the beneficial effect obtained by two monoclonal antibodies, daratumumab and isatuximab, targeting CD38 expressing plasma cells in patients with multiple myeloma. Furthermore, a possible role of CD38 in the pathogenesis of SSc has been postulated by Shi et al who showed that CD38 is an important promoter of fibrosis via nicotinamide adenine dinucleotide (NAD) depletion [47].

Therefore, in light of the hypothesis that plasma cell depletion may be therapeutically rewarding in SSc patients, the present study aimed at evaluating the levels of circulating $CD38^{high}$ plasma cells and the expression of CD38 in other peripheral blood mononuclear cells in a cohort of patients with SSc.

3. MATERIALS AND METHODS

3.1 Patients

Forty-six consecutive patients with SSc and thirty-two healthy controls (HC) were enrolled. The inclusion criteria were as follows:

1) age > 18 years;
2) fulfilment of ACR/EULAR 2013 classification criteria [48];
3) capability to provide a written informed consent.

There were no exclusion criteria.

HC were enrolled among blood donors and were matched to SSc patient for gender and age.

The following SSc features were recorded: gender, age, duration since the first non-Raynaud's symptom, type and extent of systemic involvement, subsets (limited or diffuse skin disease [49], autoantibody profile, modified Rodnan Skin Score (mRSS), previous and ongoing immunosuppressive therapy.

The study was approved by the local ethics committee (Comitato Etico Regionale delle Marche, n°2020/159) and was conducted in accordance with the Declaration of Helsinki, 5th edition (2000). Written informed consent was obtained from all patients.

3.2 Blood samples - Peripheral blood mononuclear cells

Heparinized peripheral blood was obtained by venipuncture and peripheral blood mononuclear cells (PBMCs) were separated on Ficoll-Hypaque gradient (Amersham) and washed twice with PBS before use.

3.3 Flow cytometric analysis

Cell surface expression of CD38 was evaluated on total PBMCs from HCs and SSc patients using eight-color flow cytometry analysis performed by FacsCanto II (Becton- Dickinson, Franklin Lakes, NJ). Single cells were stained according to standard protocols.

Briefly, for the surface-staining cells were incubated for 15 minutes at room temperature with optimal dilution of following conjugated monoclonal antibodies (Becton-Dickinson): CD138*FITC, CD24*PE, CD19*PE-Chlorophyll-Protein, HLADR*PE-Chlorophyll-Protein, CD25*PE-cyanine 7, CD38*PEcyanine 7, CD56*PE-cyanine 7, CD27*allophycocyanin, CD4*allophycocyanin-cyanine 7, CD19*allophycocyanin-cyanine 7, CD20*allophycocyanin-cyanine 7, CD38*V450 and CD3*V500.

Subsequently, for intracellular staining, cells were fixed with Reagent A and then permeabilized with Reagent B (Becton-Dickinson); cells were then stained with the conjugated monoclonal antibody FOXP3*PE-Chlorophyll-Protein (Becton-Dickinson) for 15 minutes at room temperature.

A minimum of 500,000 cells per tube were acquired; frequencies of the different subpopulations and CD38 mean fluorescence intensity (MFI) were subsequently calculated by FacsDiva software (Becton-Dickinson).

We firstly identified the population of interest gating on CD3-CD56+ NK cells, CD3+CD4+ T helper cells, CD3+CD8+ cytotoxic T cells, CD3+HLA-DR+ or CD3+CD38+ activated T cells, CD3+CD4+CD25+FOXP3+ regulatory T cells, CD19+ B cells; the expression of ectoenzyme was evaluated on $CD24^{high}$+CD19+$CD38^{high}$ immature B cells, CD24+CD19+CD38+CD27+ memory B cells, $CD38^{high}$CD27+ plasmablasts, CD138+ $CD38^{high}$ plasma cells. The gating strategy is shown in Figure 4.

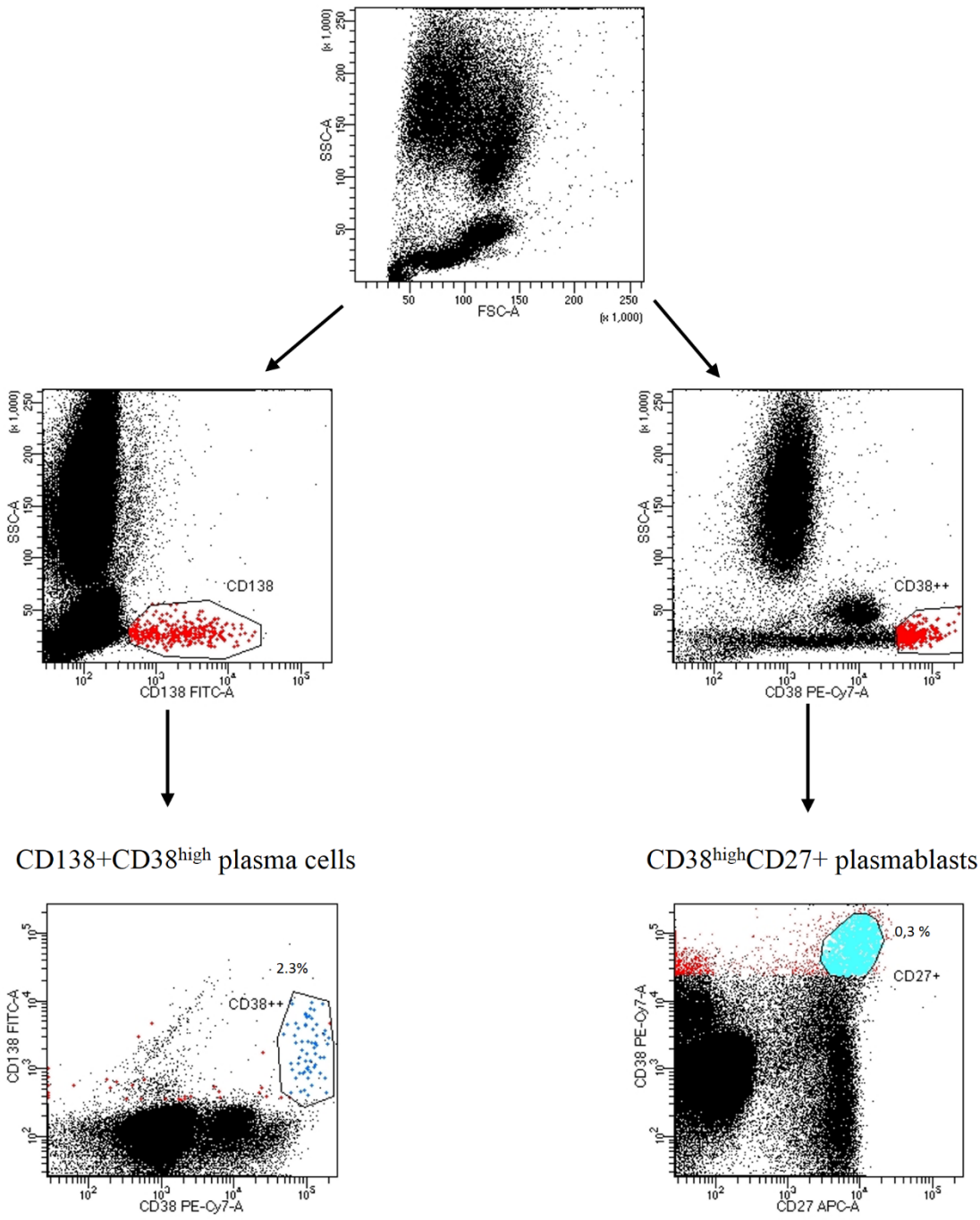

Figure 4. Gating strategy of CD138CD38^{high} plasma cells and CD38^{high}CD27+ plasmablasts. Representative dot plots from a SSc patient. Lymphocytes were identified and gated according to SSC-A (granularity) vs FSC-A (size). Plasma cells were distinguished by CD138+CD38^{high} expression; plasmablasts were identified by CD38^{high}CD27+ expression.

3.4 Statistical analysis

Flow cytometry data were summarized with percentages of positive cells and summarized as median. CD38 MFI levels were summarized as mean.

Stratified analyses were conducted comparing patients with limited or diffuse cutaneous involvement, early (≤ 3 years) or late (< 3 years) disease duration and use or not of immunosuppressive treatment.

The differences between groups were compared using the Mann-Whitney test for independent data or the Student's t test, as appropriate. Datasets were analyzed by Prism 9.0 (GraphPad Software).

Statistical significance was set at a level of $p < 0.05$.

4. RESULTS

4.1 Patients

Thirty-eight females and 8 males (mean age 56, range 23-79 years) composed the SSc group and 32 age- and sex-matched healthy subjects the control group (mean age 49, range 26-71 years; 26 females and 6 males). Clinical and demographic characteristics of the patients are summarized in Table 1. Twenty-eight patients had the limited cutaneous form (lcSSc) and eighteen the diffuse cutaneous form (dcSSc) of SSc. The mean disease duration since the first non-Raynaud's phenomenon symptom was 7 years. Twenty-six patients (56.2%) were anti-topoisomerase I positive, and skin, esophagus and lung were the most affected organs. At the time of sample collection, 14 patients were under active immunosuppressive treatment with MMF (5 of them also received RTX earlier than 6 months before enrolment), while 24 patients (52.1%) had never received immunosuppressive therapy..

Table 1. Demographic characteristics of SSc patients and controls

	SSc (n=46)	Controls (n=32)
Age (years)	56 ± 13.7	49 ± 10.1
Sex (F)	38 (82.6%)	26 (81.2%)
Disease subgroups	lcSSc 28 (61%) dcSSc 18 (39%)	N/A
Mean duration of disease (years)*	7 ± 6.4	N/A
Autoantibodies	ANA† 8 (17.4%) Anti-centromere 9 (19.5%) Anti-topo I§ 26 (56.2%) Other 3 (6.9%)	N/A
Organ involvements	Lung 33 (71.7%) Skin 38 (82.6%) Esophageal 35 (76%) Other 7 (15.2%)	N/A
Mean mRSS	8 ± 10	N/A
Immune-suppressive therapy	*Previous* RTX 4 (8.6%) CYC 4 (8.6%) MMF 4 (8.6%) *Ongoing* RTX° 5 (10.8%) MMF 14 (30.4%)	N/A

Data are presented as mean ± SD or number (%) as appropriate. N/A: not applicable. lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis; †ANA: anti-nuclear antibodies; §anti topo I: anti-topoisomerase I antibodies; mRSS: modified Rodnan Skin Score; RTX: rituximab; CYC: cyclophosphamide; MMF: mycophenolate mofetil.
*from the first non-Raynaud's symptoms.
° Rituximab was considered ongoing if received < 6 months before the study

4.2 B and T cell compartments in patients with SSc compared to HC

Compared to HC, SSc patients showed no substantial differences in the percentage of CD19+ B lymphocytes (9.6% vs 9%), CD3+ lymphocytes (74.6% vs 73.2%), CD3+CD4+ T helper lymphocytes (48.4% vs 45.7%), CD3+CD8+ T cytotoxic lymphocytes (23% vs 23.2%).

However, compared to HC, within the total T lymphocyte population CD38+ cells were more frequent in SSc patients (32.9% vs 24.6%, $p<0.05$; mean fluorescence intensity (MFI) level 419.1 vs 262.8 $p<0.05$). No difference was found within the B cell subset (Figure 5). No correlation was found between

CD3+CD38+ and SSc subsets, disease duration and immunosuppressive treatments (Figure 6).

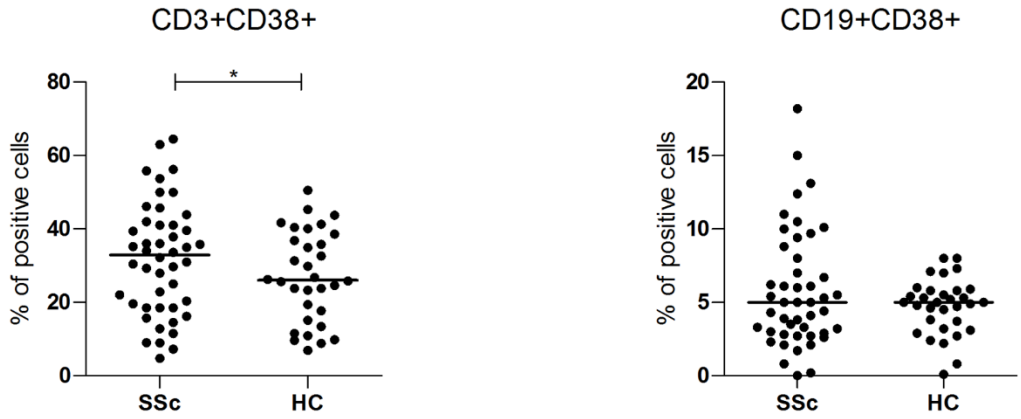

Figure 5. CD3+CD38+ and CD19+CD38+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) were analyzed by flow cytometry. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$.. Mann-Whitney test was used for inter-groups analysis.

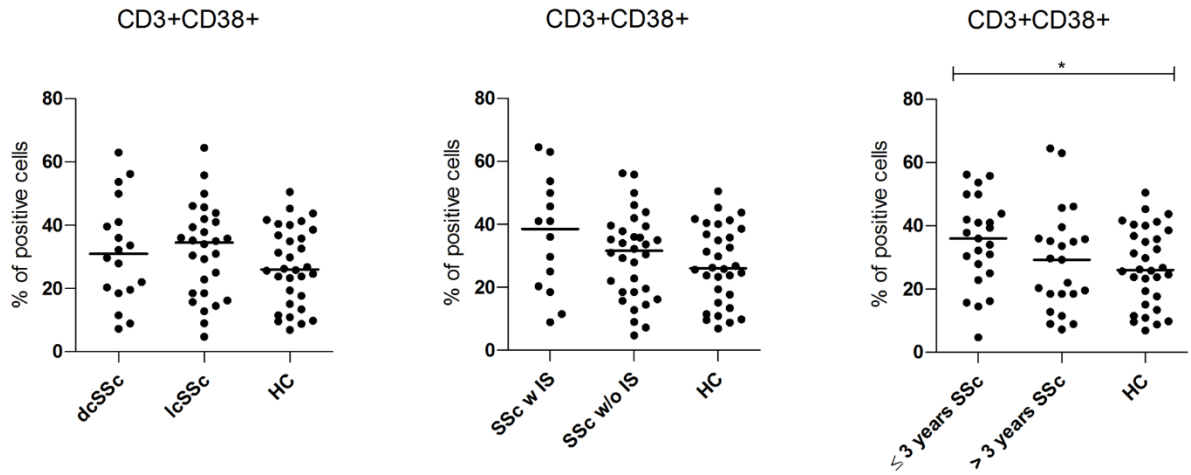

Figure 6. The CD3+CD38+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$.. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years SSc: disease duration.

4.3 Plasmablasts and plasma cells in patients with SSc compared to HC

Flow cytometry analysis showed significantly higher levels of both circulating CD138+ CD38^{high} plasma cells (1.9% vs 0.3%, p<0.001) and CD27+ CD38^{high} plasmablasts (0.3% vs 0.1%, p<0.001) in SSc patients compared to HC (Figure 7).

There was no statistically significant difference in CD38 mean fluorescence intensity (MFI) levels, suggesting an expansion of the CD38^{high} plasma cells and plasmablasts rather than increased expression per cell.

When the levels of CD38+ plasma cells and CD38+ plasmablasts were analyzed in dcSSc and lcSSc, no difference was found in patients with disease duration less or more than 3 years, and in relation to treatment, although HC demonstrated significantly fewer CD38+ cells compared to all subgroups (Figure 8). Moreover, we observed a non-significant higher trend in the percentage of CD38+ cells in patients with lcSSc compared to dcSSc and in patients not immunosuppressed compared to those immunosuppressed (Figure 8).

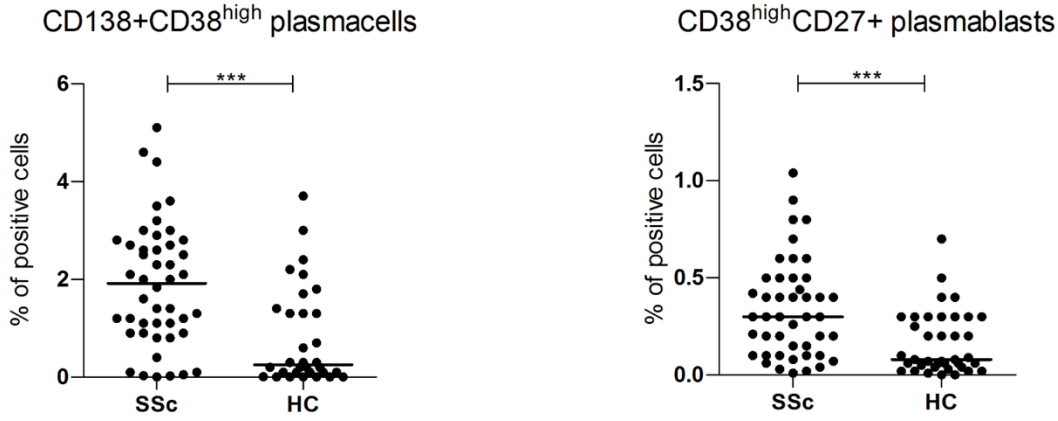

Figure 7. CD138+CD38^{high} and CD38^{high}CD27+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) were analyzed by flow cytometry. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *** = p < 0.001. Mann-Whitney test was used for inter-groups analysis.

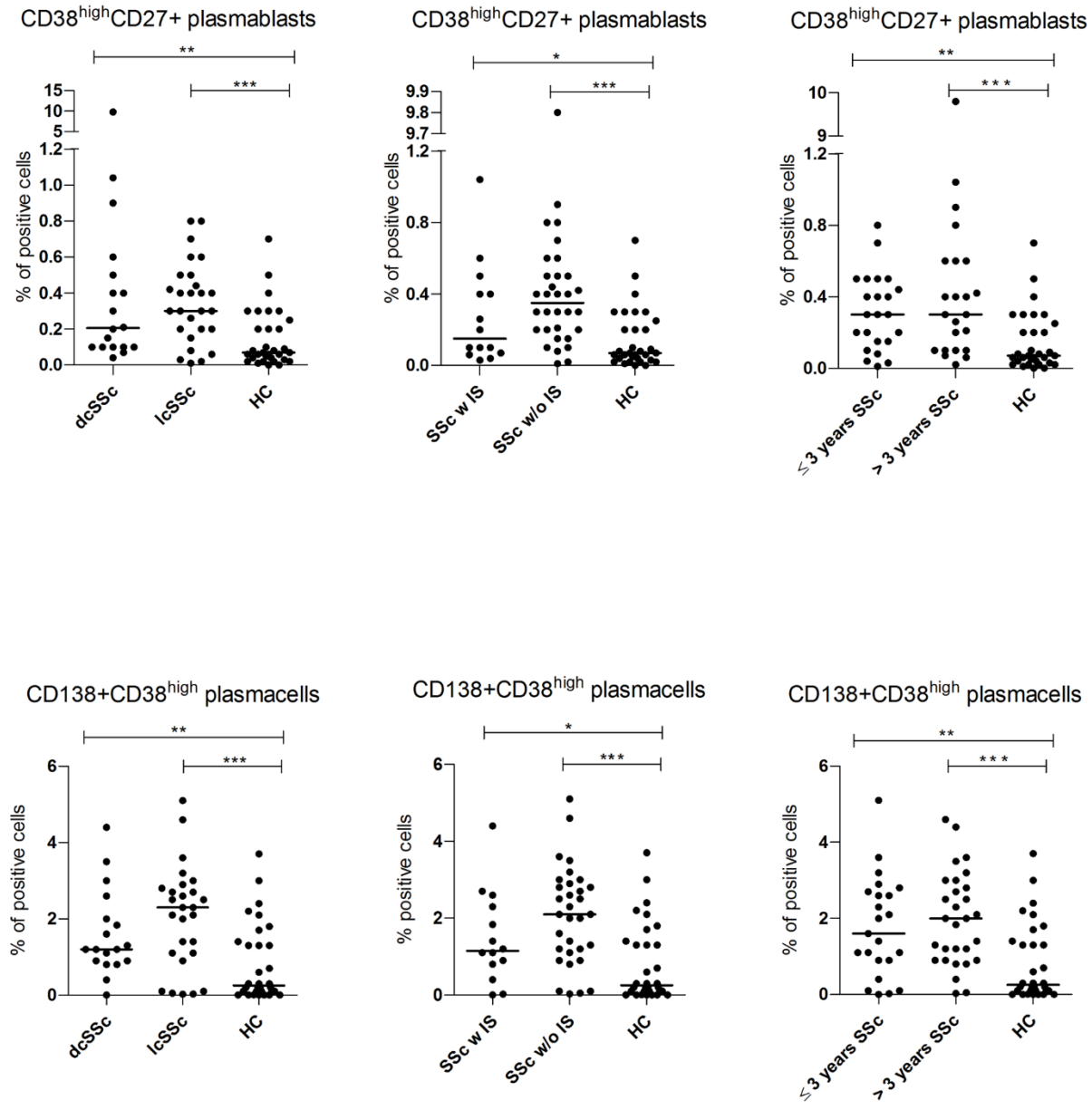

Figure 8. CD138+CD38^{high} plasma cells surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years SSc: disease duration.

CD138^{high}CD27+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years: disease duration.

4.4 NK and NKT cells in patients with SSc compared to HC

Since it has been suggested that SSc can be triggered by viruses and natural killer (NK) and NKT cells are important effector cells of the innate immune system during infections we investigated the frequency of these cell populations in peripheral blood of patients and controls.

Interestingly, SSc patients showed similar levels of CD3-CD56+ NK cells (11.6% vs 11%) compared to HC, but they had an overall reduced frequency of CD3+CD56+ NKT cells (4.1% vs 6%, $p<0.05$) with a higher percentage of CD38+ cells within this population (10% vs 3.7%, $p<0.001$; MFI 434.4 vs 158.6 $p<0.0001$) (Figure 9).

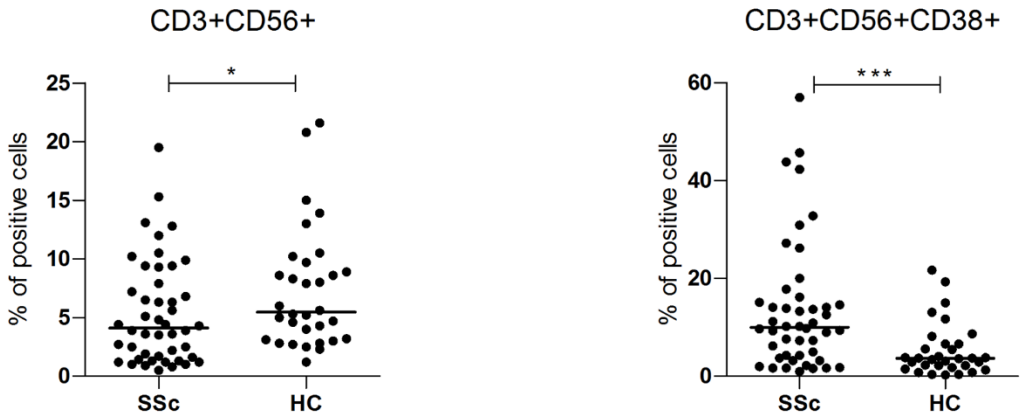

Figure 9. CD3+CD56+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) and CD3+CD56+CD38+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) were analyzed by flow cytometry. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$;*** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis.

4.5 Regulatory T and B cells in patients with SSc compared to HC

SSc patients showed increased frequency of CD3+CD4+CD25+Foxp3+ regulatory T lymphocytes (3.1% vs 0.6%, p<0.001), but, in this population, there was no difference in the percentage of cells expressing CD38 (40.2% vs 40%) (Figure 10). Regulatory T cells were higher in lcSSc and dcSSc compared to HC, independently of treatment and disease duration (Figure 11).

Finally, the subpopulations of regulatory B cells were also investigated. As shown in Figure 12, compared to HC, SSc patients showed a higher frequency of $CD24^{high}CD19+CD38^{high}$ immature B cells (15.3% vs 9%, p<0.01; MFI 4119 vs 1344, p<0.05) and a lower occurrence of CD24+ CD19+CD38+CD27+ memory B lymphocytes (1.7% vs 3.5%, p <0.001) (Figure 12). There was no statistically significant difference in CD38 mean fluorescence intensity (MFI) levels of memory B lymphocytes, suggesting a reduction of the CD38 population rather than decreased expression per cell.

Moreover, immature B cells were higher in lcSSc and dcSSc compared to HC, independently of treatment and disease duration. Interestingly, memory B cells were lower than HC in all the subsets analyzed except for SSc patients without immunosuppressive therapy, where the number of these cells were higher than HC (Figures 13 and 14).

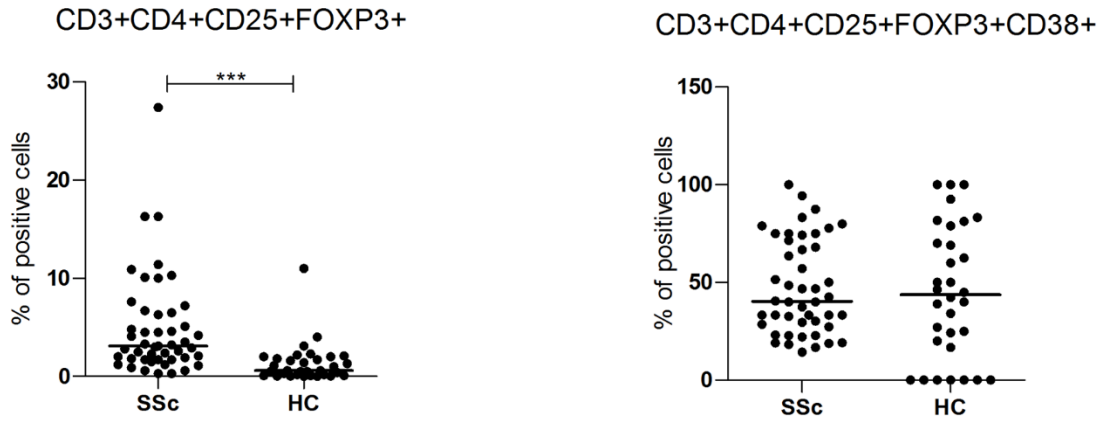

Figure 10. CD3+CD4+CD25+FOXP3+ and CD3+CD4+CD25+FOXP3+CD38+ intracellular (FOXP3) and surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) were analyzed by flow cytometry. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *** = p < 0.001. Mann-Whitney test was used for inter-groups analysis.

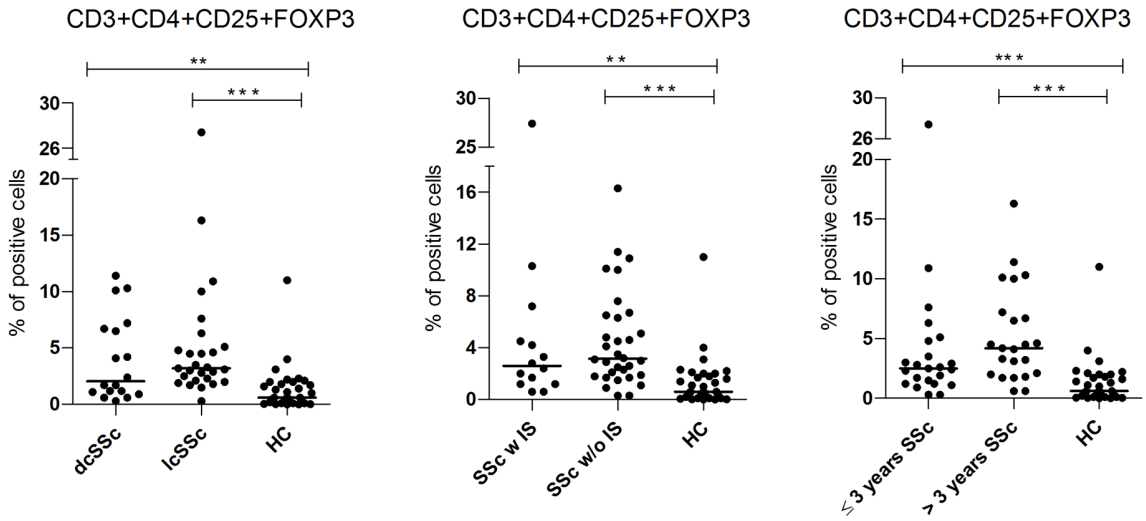

Figure 11. CD3+CD4+CD25+FOXP3+ intracellular (FOXP3) and surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values;** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years SSc: disease duration.

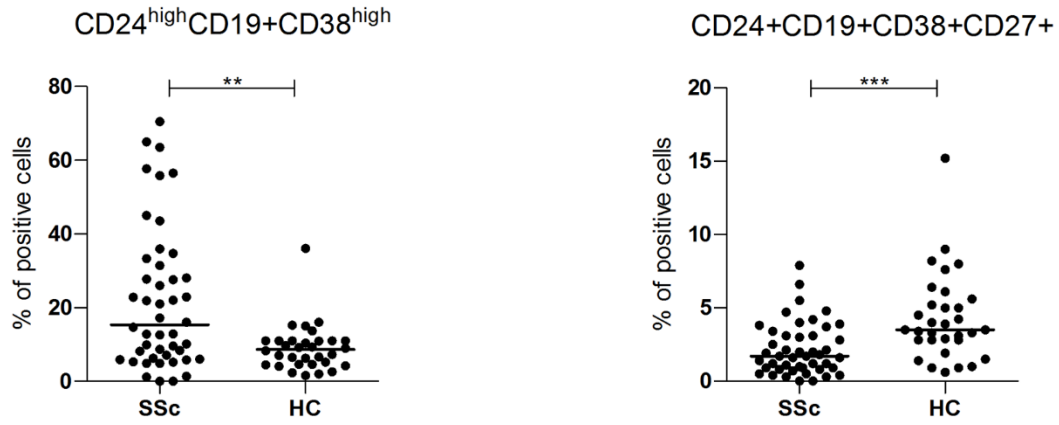

Figure 12. $CD24^{high}CD19+CD38^{high}$ and CD24+CD19+CD38+CD27+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) were analyzed by flow cytometry. Results are expressed as percentage of positive cells. Horizontal bars represent median values; ** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis.

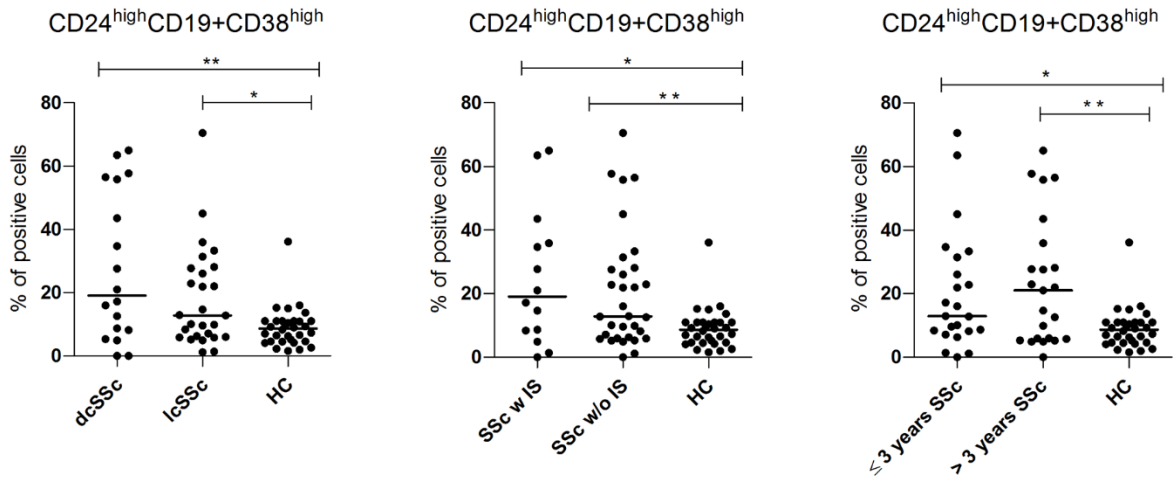

Figure 13. CD24^{high}CD19+CD38^{high} surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years SSc: disease duration.

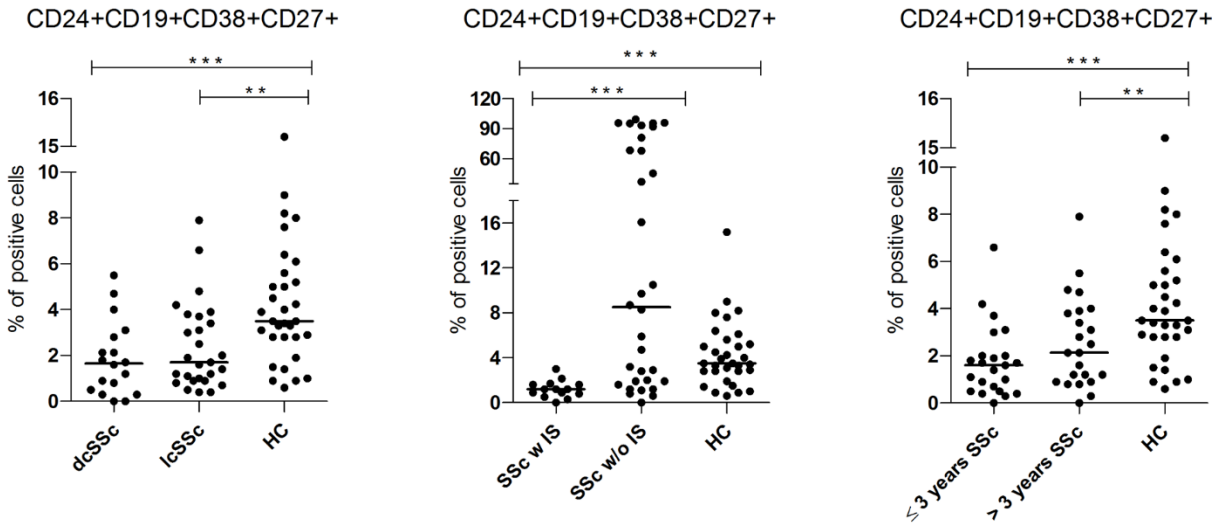

Figure 14. CD24+CD19+CD38+CD27+ surface antigen expression of PBMC from SSc patients (SSc; n = 46) and healthy controls (HC; n = 32) analyzed by flow cytometry. SSc patients were stratified for disease subgroups, immunosuppressive therapy at the time of blood draw and disease duration. Results are expressed as percentage of positive cells. Horizontal bars represent median values; *= $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$. Mann-Whitney test was used for inter-groups analysis. dcSSC: diffuse SSc, lcSSc: limited SSc; SSc w IS: immunosuppressed SSc, SSc w/o IS: not immunosuppressed SSc; ≤ or > of 3 years SSc: disease duration.

5. DISCUSSION

CD38 is a ubiquitous protein mainly localized on the cell surface and highly expressed in hematopoietic tissues, with an important functional role showing both ADP-ribosyl cyclase and cADPR hydrolase enzymatic activities [38]. The significant expression of CD38 in immune cells suggests also a relevant contribution to immune cell homeostasis, as already observed in several inflammatory, neoplastic and immune-mediated diseases [48].

In this study, we have characterized the expression pattern of CD38 on circulating PBMCs of patients with SSc, demonstrating a higher number of CD38+ cells within total T lymphocytes, higher levels of circulating CD38+ plasma cells and plasmablasts, an increased frequency of CD38+ cells within immature B cells and NKT cells in SSc patients compared to HC. Although no difference in the expression of CD38 within total B lymphocytes was observed between SSc and HC, the finding of lower levels in the subgroups treated with immunosuppressive drugs may indicate some effect of these therapeutics on humoral adaptive immunity [49].

Thus, we found that CD38 expression is increased in many peripheral blood cell populations of SSc patients, although its functional role has not been completely defined. Albeit CD38 is usually considered a marker of T cell activation [48], previous studies indicate that its role may be rather multifaceted, ranging from differentiation to effector function and senescence [50].

The data reported herein expand the results by Gumkowska-Sroka et al, who recently reported that SSc patients show a higher frequency of regulatory B lymphocytes, lower levels of memory B cells, NK and NKT cells, and an increase of circulating plasmablasts in patients with SSc compared to HC, that may correlate with disease activity [49]. The present study strengthens these findings by demonstrating a higher expression and/or number of CD38+ cells within these populations, paving the way to larger studies supporting the use of CD38 as a target for SSc treatment.

The most significant result of this study is the demonstration of increased levels of circulating $CD38^{high}$ plasma cells and plasmablasts in patients with SSc compared to HC. Several lines of evidence indicate an altered homeostasis of the B cell compartment in PBMCs of patients with SSc [51], although the precise role of B cells in the pathogenesis of SSc is still controversial [52-53].
In general, B cells of SSc patients are characterized by enhanced pro-inflammatory and pro-fibrotic properties, as well as impaired immunosuppressive activity. Activated B cells may contribute to the

pathogenesis of SSc by promoting the differentiation of Th2 cells, thereby shifting cytokine production towards cytokines such as IL-6, IL-4, and IL-13, which in turn promote antibody production and tissue fibrosis [53]. Furthermore, the discovery of pathogenetic autoantibodies strongly implicate the humoral compartment of adaptive immunity in the development of SSc [54].

Therefore, it is conceivable that B cells may play an important role in the pathogenesis of SSc and other immune-mediated diseases, at least at early stages [55]. The partial clinical benefit obtained in SSc patients by depleting B cells with anti-CD20 drug RTX might be explained by lack of expression of CD20 in long-lived plasma cells [56]. Having reached the highest degree of differentiation, these plasma cells are able to produce antibodies in the protective niche of the bone marrow, relatively spared from RTX depletion. Thus, an effective immunosuppressive treatment would require elimination of these antibody-producing long-lived plasma cells.

The high expression of CD38 in antibody-producing cells suggests its therapeutic use in targeted therapies against immune-mediated diseases like SSc [56]. Additionally, CD38 has several important enzymatic activities strongly implicated in the homeostasis of the immune system [37]. In fact, over 90% of CD38 acts as an ecto-NADase that catabolizes NAD+. Another role of CD38 is the regulation of extracellular adenosine, which requires consumption of NAD+. The synthesis of adenosine from NAD+ is a complementary mechanism similar to the CD39/CD73-mediated catabolism of ATP to adenosine. Adenosine has an important role in immune modulation because it has been implicated in immune suppression. Mechanistically, Shi et al. demonstrated that in SSc the inhibition of CD38 may boosts NAD+ levels, which in turn increases sirtuin (SIRT) activation and, at last, induces the downregulation of TGFb-SMAD pathway, which is strongly implicated in SSc pathogenesis [47].

Thus, our study and the above mentioned reports prompt the use of anti-CD38 therapy in SSc; however, it is unclear whether the best approach would be the use of a cytotoxic monoclonal antibody or a reversible enzymatic inhibitor. Of note, in a recent proof-of-concept study, two patients with systemic lupus erythematosus (SLE) refractory to multiple lines of therapy were successfully treated with anti-CD38 daratumumab, with long-term efficacy that was maintained with belimumab co-administration [57]. More recently, Yalcin Mutlu et al. successfully treated a patient with SLE and refractory cerebral vasculitis with daratumumab [58].

Given its peculiar expression pattern on PBMCs, the importance of targeting CD38 in SSc may go beyond the B cell compartment of adaptive immunity. For example, in rheumatoid arthritis CD38 is involved in the regulation of the cellular Treg/Th17 ratio through its expression in NK and NKT cells.

Therefore, CD38 may also be an important regulator of NK, NKT and T cell lineages and their function, and our finding that CD38 is highly expressed in these populations in SSc suggests an additional potential effect of its drug targeting.

Although we found an increase of CD38+ regulatory B and T cells in this SSc cohort, suggesting a potential limitation of the use of anti-CD38 therapy in patients with SSc, it is thought that these populations are functionally impaired in SSc, thus their role is highly controversial [59-60].
Tregs function is thought to be impaired in SSc through mechanisms including reduced production of IL-10 and TGF-b, and reduced plasticity of Treg cells which can differentiate into Th2- or Th17-like cells when exposed to a pro-inflammatory environment [59-60].
Thus, it is not predictable that anti-CD38 treatment would lead to an impairment of the regulatory compartment of adaptive immunity.

Finally, another important issue potentially hampering the safety of CD38 inhibition in SSc is the higher risk of infection and sepsis associated with this treatment as shown by studies in multiple myeloma. The mechanisms through which the inhibition of CD38 increases the infectious risk include not only the depletion of natural and adaptive immune cells (including memory B cells) but also the impairment of several regulatory functions of immune system [61]. This suggests the opportunity of further investigating the safety of anti-CD38 cytotoxic antibodies versus reversible CD38 inhibitors.

This study has several limitations. First of all, the sample size is limited and patients are highly heterogeneous with respect to disease subgroups, duration and treatment, which can hinder the interpretation of study results. Importantly, to overcome this potential issue we have conducted stratified analyses taking into account disease characteristics and treatment, although the study was not adequately powered to show differences between groups, if any. The second important limitation is that we did not evaluate the functional role of CD38 in the different compartments of the immune cells, that may be important with regard to the enzymatic activity of this molecule. Therefore, we were unable to provide mechanistic insights into the role of CD38 inhibition in the interaction between PBMCs.

Further studies are therefore needed to validate the pathophysiologic mechanisms of CD38 and to investigate the role of targeted therapies against CD38 in SSc and other immune-mediated diseases.

6. BIBLIOGRAPHY

1. Moroncini G, Grieco A, Nacci G, Paolini C, Tonnini C, Pozniak KN, et al. Epitope Specificity Determines Pathogenicity and Detectability of Anti-Platelet-Derived Growth Factor Receptor alpha Autoantibodies in Systemic Sclerosis. Arthritis Rheumatol. 2015;67(7):1891-903. PubMed PMID: 25808833.

2. Luchetti MM, Moroncini G, Jose Escamez M, Svegliati Baroni S, Spadoni T, Grieco A, et al. Induction of Scleroderma Fibrosis in Skin-Humanized Mice by Administration of Anti-Platelet-Derived Growth Factor Receptor Agonistic Autoantibodies. Arthritis Rheumatol. 2016;68(9):2263-73. PubMed PMID: 27111463.

3. Watanabe T, Nishimoto T, Mlakar L, Heywood J, Malaab M, Hoffman S, et al. Optimization of a murine and human tissue model to recapitulate dermal and pulmonary features of systemic sclerosis. PLoS One. 2017;12(6):e0179917. PubMed PMID: 28651005.

4. Chifflot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and prevalence of systemic sclerosis: a systematic literature review. Semin Arthritis Rheum. 2008;37(4):223-35. PubMed PMID: 17692364.

5. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47. PubMed PMID: 24122180.

6. Denton CP, Khanna D. Systemic sclerosis. Lancet. 2017;390(10103):1685-99. PubMed PMID: 28413064.

7. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360(19):1989- 2003. PubMed PMID: 19420368.

8. Silman AJ. Epidemiology of scleroderma. Ann Rheum Dis. 1991;50 Suppl 4:846-53. PubMed PMID: 1750796.

9. Silman AJ. Scleroderma demographics and survival. J Rheumatol Suppl. 1997;48:58-61. PubMed PMID: 9150120.

10. Frech T, Khanna D, Markewitz B, Mineau G, Pimentel R, Sawitzke A. Heritability of vasculopathy, autoimmune disease, and fibrosis in systemic sclerosis: a population-based study. Arthritis Rheum. 2010;62(7):2109-16. PubMed PMID: 20506251.

11. Stern EP, Denton CP. The Pathogenesis of Systemic Sclerosis. Rheum Dis Clin North Am. 2015;41(3):367-82. PubMed PMID: 26210124.

12. Feghali-Bostwick C, Medsger TA, Jr., Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. Arthritis Rheum. 2003;48(7):1956-63. PubMed PMID: 12847690.

13. Arnett FC, Gourh P, Shete S, Ahn CW, Honey RE, Agarwal SK, et al. Major histocompatibility complex (MHC) class II alleles, haplotypes and epitopes which confer susceptibility or protection in systemic sclerosis: analyses in 1300 Caucasian, African-American and Hispanic cases and 1000 controls. Ann Rheum Dis. 2010;69(5):822-7. PubMed PMID: 19596691.

14. Hewagama A, Richardson B. The genetics and epigenetics of autoimmune diseases. J Autoimmun. 2009;33(1):3-11. PubMed PMID: 19349147.

15. Moroncini G, Mori S, Tonnini C, Gabrielli A. Role of viral infections in the etiopathogenesis of systemic sclerosis. Clin Exp Rheumatol. 2013;31(2 Suppl 76):3-7. PubMed PMID: 23910606.

16. Manetti M, Romano E, Rosa I, Guiducci S, Bellando-Randone S, De Paulis A, et al. Endothelial-to-mesenchymal transition contributes to endothelial dysfunction and dermal fibrosis in systemic sclerosis. Ann Rheum Dis. 2017;76(5):924-34. PubMed PMID: 28062404.

17. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest. 1974;54(4):880-9. PubMed PMID: 4430718.

18. Sambo P, Baroni SS, Luchetti M, Paroncini P, Dusi S, Orlandini G, et al. Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. Arthritis Rheum. 2001;44(11):2653-64. PubMed PMID: 11710721.

19. Chizzolini C, Boin F. The role of the acquired immune response in systemic sclerosis. Semin Immunopathol. 2015;37(5):519-28. PubMed PMID: 26152639.

20. Prescott RJ, Freemont AJ, Jones CJ, Hoyland J, Fielding P. Sequential dermal microvascular and perivascular changes in the development of scleroderma. J Pathol. 1992;166(3):255-63. PubMed PMID: 1517881.

21. Distler JH, Akhmetshina A, Schett G, Distler O. Monocyte chemoattractant proteins in the pathogenesis of systemic sclerosis. Rheumatology (Oxford). 2009;48(2):98-103. PubMed PMID: 18984611.

22. Moroncini G, Svegliati Baroni S, Gabrielli A. Agonistic antibodies in systemic sclerosis. Immunol Lett. 2018;195:83-7. PubMed PMID: 29032187.

23. Orton SM, Peace-Brewer A, Schmitz JL, Freeman K, Miller WC, Folds JD. Practical evaluation of methods for detection and specificity of autoantibodies to extractable nuclear antigens. Clin Diagn Lab Immunol. 2004;11(2):297-301. PubMed PMID: 15013979.

24. Ho KT, Reveille JD. The clinical relevance of autoantibodies in scleroderma. Arthritis Res Ther. 2003;5(2):80-93. PubMed PMID: 12718748.

25. Hesselstrand R, Scheja A, Shen GQ, Wiik A, Akesson A. The association of antinuclear antibodies with organ involvement and survival in systemic sclerosis. Rheumatology (Oxford). 2003;42(4):534-40. PubMed PMID: 12649400.

26. Reveille JD, Solomon DH. Evidence-based guidelines for the use of immunologic tests: anticentromere, Scl-70, and nucleolar antibodies. Arthritis Rheum. 2003;49(3):399-412. PubMed PMID: 12794797.

27. Henault J, Robitaille G, Senecal JL, Raymond Y. DNA topoisomerase I binding to fibroblasts induces monocyte adhesion and activation in the presence of anti-topoisomerase I autoantibodies from systemic sclerosis patients. Arthritis Rheum. 2006;54(3):963-73. PubMed PMID: 16508979.

28. Kahaleh B, Matucci-Cerinic M. Raynaud's phenomenon and scleroderma. Dysregulated neuroendothelial control of vascular tone. Arthritis Rheum. 1995;38(1):1-4. PubMed PMID: 7818557.

29. Koenig M, Joyal F, Fritzler MJ, Roussin A, Abrahamowicz M, Boire G, et al. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud's phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum. 2008;58(12):3902-12. PubMed PMID: 19035499.

30. Spencer-Green G. Outcomes in primary Raynaud phenomenon: a meta-analysis of the frequency, rates, and predictors of transition to secondary diseases. Arch Intern Med. 1998;158(6):595-600. PubMed PMID: 9521223.

31. Carvalho D, Savage CO, Black CM, Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. Induction of adhesion molecule expression and involvement of endothelium-derived cytokines. J Clin Invest. 1996;97(1):111-9. PubMed PMID: 8550821.

32. Salojin KV, Le Tonqueze M, Saraux A, Nassonov EL, Dueymes M, Piette JC, et al. Antiendothelial cell antibodies: useful markers of systemic sclerosis. Am J Med. 1997;102(2):178-85. PubMed PMID: 9217568.

33. Avouac J, Fransen J, Walker UA, Riccieri V, Smith V, Muller C, et al. Preliminary criteria for the very early diagnosis of systemic sclerosis: results of a Delphi Consensus Study from EULAR Scleroderma Trials and Research Group. Ann Rheum Dis. 2011;70(3):476-81. PubMed PMID: 21081523.

34. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. N Engl J Med. 2006;354(25):2655-66. PubMed PMID: 16790698.

35. Beyer C, Distler O, Distler JH. Innovative antifibrotic therapies in systemic sclerosis. Curr Opin Rheumatol. 2012;24(3):274-80. PubMed PMID: 22450392.

36. Ramos-Casals M, Fonollosa-Pla V, Brito-Zeron P, Siso-Almirall A. Targeted therapy for systemic sclerosis: how close are we? Nat Rev Rheumatol. 2010;6(5):269-78. PubMed PMID: 20386562.

37. Hogan KA, Chini CCS and Chini EN (2019) The Multi-faceted Ecto-enzyme CD38: Roles in Immunomodulation, Cancer, Aging, and Metabolic Diseases. Front. Immunol. 10:1187. doi: 10.3389/fimmu.2019.01187

38. Horenstein AL, Bracci C, Morandi F and Malavasi F (2019) CD38 in Adenosinergic Pathways

and Metabolic Re-programming in Human Multiple Myeloma Cells: In-tandem Insights From Basic Science to Therapy. Front. Immunol. 10:760. doi: 10.3389/fimmu.2019.00760

39. Denton CP, Khanna D. Systemic sclerosis. Lancet. 2017 Oct 7;390(10103):1685-99.

40. Roofeh D, Distler O, Allanore Y, Denton CP, Khanna D. Treatment of systemic sclerosis-associated interstitial lung disease: Lessons from clinical trials. J Scleroderma Relat Disord. 2020 Mar;5(2 Suppl):61-71.

41. Moroncini G, Svegliati Baroni S, Gabrielli A. Agonistic antibodies in systemic sclerosis. Immunol Lett. 2018 Mar;195:83-7.

42. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Ann Rheum Dis. 2014 Jun;74(6):1188-94.

43. Goswami RP, Ray A, Chatterjee M, Mukherjee A, Sircar G, Ghosh P. Rituximab in the treatment of systemic sclerosis-related interstitial lung disease: a systematic review and meta-analysis. Rheumatology (Oxford). 2021 Feb 1;60(2):557-67.

44. Tang R, Yu J, Shi Y, Zou P, Zeng Z, Tang B, et al. Safety and efficacy of Rituximab in systemic sclerosis: A systematic review and meta-analysis. Int Immunopharmacol. 2020 Jun;83:106389.

45. Moradzadeh M, Aghaei M, Mehrbakhsh Z, Arab-Bafrani Z, Abdollahi N. Efficacy and safety of rituximab therapy in patients with systemic sclerosis disease (SSc): systematic review and meta-analysis. Clin Rheumatol. 2021 Oct;40(10):3897-918.

46. Elhai M, Boubaya M, Distler O, Smith V, Matucci-Cerinic M, Alegre Sancho JJ, et al. Outcomes of patients with systemic sclerosis treated with rituximab in contemporary practice: a prospective cohort study. Ann Rheum Dis. 2019 Jul;78(7):979-87.

47. Shi B, Wang W, Korman B, Kai L, Wang Q, Wei J, et al. Targeting CD38-dependent NAD(+) metabolism to mitigate multiple organ fibrosis. iScience. 2020 Jan 22;24(1):101902.

48. Piedra-Quintero ZL, Wilson Z, Nava P, Guerau-de-Arellano M. CD38: An Immunomodulatory Molecule in Inflammation and Autoimmunity. Front Immunol. 2020;11:597959.

49. Gumkowska-Sroka O, Jagoda K, Owczarek A, Helbig G, Giemza-Stoklosa J, Kotyla PJ. Cytometric Characterization of Main Immunocompetent Cells in Patients with Systemic Sclerosis: Relationship with Disease Activity and Type of Immunosuppressive Treatment. J Clin Med. 2019 May 8;8(5).

50. Kar A, Mehrotra S, Chatterjee S. CD38: T Cell Immuno-Metabolic Modulator. Cells. 2020 Jul 17;9(7).

51. Thoreau B, Chaigne B and Mouthon L (2022) Role of B-Cell in the Pathogenesis of Systemic Sclerosis. Front. Immunol. 13:933468. doi: 10.3389/fimmu.2022.93346

52. Forestier A, Guerrier T, Jouvray M, Giovannelli J, Lefevre G, Sobanski V, et al. Altered B lymphocyte homeostasis and functions in systemic sclerosis. Autoimmun Rev. 2018

Mar;17(3):244-55.

53. Yoshizaki A, Fukasawa T, Ebata S, Yoshizaki-Ogawa A, Sato S. Involvement of B cells in the development of systemic sclerosis. Front Immunol. 2022;13:938785.

54. Benfaremo D, Svegliati Baroni S, Manfredi L, Moroncini G, Gabrielli A. Putative functional pathogenic autoantibodies in systemic sclerosis. Eur J Rheumatol. 2020 Oct;7(Suppl 3):S181-S6.

55. Werner A, Schafer S, Zaytseva O, Albert H, Lux A, Kristic J, et al. Targeting B cells in the pre-phase of systemic autoimmunity globally interferes with autoimmune pathology. iScience. 2021 Sep 24;24(9):103076.

56. Peclat TR, Shi B, Varga J, Chini EN. The NADase enzyme CD38: an emerging pharmacological target for systemic sclerosis, systemic lupus erythematosus and rheumatoid arthritis. Curr Opin Rheumatol. 2020 Nov;32(6):488-96.

57. Ostendorf L, Burns M, Durek P, Heinz GA, Heinrich F, Garantziotis P, et al. Targeting CD38 with Daratumumab in Refractory Systemic Lupus Erythematosus. N Engl J Med. 2020 Sep 17;383(12):1149-55.

58. Yalcin Mutlu M, Wacker J, Tascilar K, Taubmann J, Manger B, Kronke G, et al. Effective and safe treatment of anti-CD38 therapy in systemic lupus erythematosus associated refractory cerebral vasculitis induces immune tolerance. Rheumatology (Oxford). 2022 Jul 8.

59. Frantz C, Auffray C, Avouac J, Allanore Y. Regulatory T Cells in Systemic Sclerosis. Front Immunol. 2018;9:2356.

60. Kobayashi S, Nagafuchi Y, Shoda H, Fujio K. The Pathophysiological Roles of Regulatory T Cells in the Early Phase of Systemic Sclerosis. Front Immunol. 2022;13:900638.

61. Glaria E, Valledor AF. Roles of CD38 in the immune response to infection. Cells (2020) 9(1):228. doi: 10.3390/cells9010228.