Review

One year in review 2017: systemic sclerosis

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ABSTRACT

Systemic sclerosis is a rare acquired systemic disease characterised by heterogeneous evolution and outcome. Each year novel insights into the pathogenesis, diagnosis and treatment of this severe disease have been published. We herewith provide our overview of the most significant literature contributions published over the last year.

Introduction

Systemic sclerosis (SSc) is a chronic multisystem disease characterised by three pathogenetic landmarks, microvascular involvement, activation of the immune system and increase of extracellular matrix deposition in the skin and internal organs (1).

In this manuscript we will provide our annual update of the recent advances in the pathogenesis, diagnosis and treatment of systemic sclerosis. A systematic MedLine search has been performed using the term "systemic sclerosis" (MeSH terms and semantic search), focusing on the most relevant contributions to the medical literature published between January 2016 and January 2017.

Pathogenesis

Despite the still unclear pathogenetic complexity of SSc, various new pathways have been investigated in the last year, leading to hypothesising new possible targets in the therapeutic area. After initial considerations regarding new insights in risk factors and genetic predisposition, although the main pathogenetic branches are connected to each other, we will consider the main areas of inflammation, vasculopathy and fibrosis, separately.

Genetics and other risk factors

Genetic predisposition was investigated in various areas, in particular regarding human leukocyte antigen (HLA) and non-HLA mutation risk for the development of SSc.

Studying the Chinese Han population, Guo *et al*. found 31 copy number variations in 20 SSc Han Chinese patients, some observed in the HLA region. Consequently, in a much larger population of 365 SSc and 369 ethnically-matched patients, the authors showed risk increased of SSc for copy number variations (CNVs) of HLA-DQA1 (as a protective factor, OR 0.09) and of APOBEC3A/3B (involved in the innate immunity, OR 1.6), which were identified as a genetic factor risk for SSc (2). Combining studies on rheumatoid arthritis (RA) and SSc patients, Kanaan *et al*. investigated the presence of both genetic HLA predisposition and X-chromosome inactivation mosaicism in SSc, showing that an increased number of X-chromosome inactivation mosaicism in SSc was not significantly associated with amino acid motifs of DRB1 and DRB5, thus representing two independent predisposing genetic factors to SSc (3).

Moving to specific HLA locus, Lee *et al*. investigated the genetic susceptibility for HLA-DPB1 alleles in anti-topoisomerase I positive (ATA+) SSc, showing that *09:01 and *13:01 variants are associated with increased risk of SSc (OR 2.97 and 3.04, respectively). Investigating HLA-DP β chain and negatively charged amino-acido sequences (NCTs), they showed that the number of negatively charged triplets was higher in ATA + SSc patients, with 82–85 binding groove hypothesised to function as an anchor antigen for the ATA+ charged peptides, further requiring that NCTs are present at 55–57 and/or 67–69 to perpetuate its action and present the antigen more effectively (4).

HLA predisposition to SSc was also confirmed by Stevens *et al*. in the juvenile SSc (jSSc) population, in

particular with HLA-DRB1*01 and DQA1*05, while protection from jSSc in the case of DQBQ*A6. They were also the first to identify a novel significant association between jSSc and HLA-DRB1*10 (OR 7.48, CI 2.79-20, p=0.0002), distinguishing HLA genetic risk for juvenile and adult onset (5). Investigating the role of killer immunoglobulin-like receptors, which interact with HLA and determine interferon (IFN) α and tumour necrosis factor (TNF) α secretion and cytotoxic activity, Tozkir *et al*. found a higher KIR2DS3 gene phenotype in 25 SSc patients *versus* 40 healthy controls (HC) (OR 3.1, CI 1.080–9.362 p=0.032) and it was more frequently combined with HLA-C1 in increasing the risk for SSc. The same ligand also increased the risk for SSc when combined with KIR2DL2 and KIR2DS2, while the coupling of KIR3DL1 with HLA-Bw4 was protective (OR 0.2, 0.098–0.842, p=0.02). These results suggest that variations of some KIR genes may have a role in the pathogenesis of SSc (6).

Regarding non-HLA genetic predisposition to SSc, various papers showed significant results identifying mutations with increased risk for SSc development: for example Pehlivan *et al*. showed some single nuclear polymorphisms (SNPs) in Rho-K1, Rho-K2, Rho-A and Rho-C genes, postulating a role for these polymorphisms in perpetuating an activated pathogenetic cascade leading to some specific SSc features, based on Rho pathway mediated ET-1 effects (7). Five IL1 family gene SNPs were investigated by a Chinese group in a case-control study followed by a meta-analysis, showing increased allele frequency for IL-1A-899C/T (OR 2.345, CI 1.518–3.662) in the SSc Chinese patients group. Unfortunately, this result was not confirmed by a meta-analysis then performed afterwards, which instead found a significant association between IL-1B-511C/T and SSc (OR 1.267, CI 1.016–1.580) (8).

Whole exome sequencing analysis is a tool that can identify gene allelic variants which may determine contribution to SSc development. While Mak et al. mostly focused on SSc-ILD and identified many novel genetic variants but also confirmed previous results for BANK1 in SSc and TERT in interstitial lung disease (ILD) (9), Gao *et al*. used this methodology on a larger study population and were able to identify ATP8B4 rs55687265 single missens variant as associated with SSc in both derivation and replication cohorts and also in the Fisher meta-analysis combining these two results (cumulative OR 2.5, p=1.92x10-7). Although they found no differences between pulmonary arterial hypertension (PAH) complicated and non-complicated patients, they observed a correlation between these allelic variants and peripheral blood mononuclear cell (PBMC) expression of ATP8B4, identifying this gene over-expression as a potential SSc biomarker (10). Recently, an integrated, multicohort analysis of SSc transcriptome data across 515 samples was performed, identifying a 415-gene set that robustly distinguishes SSc patients from healthy control subjects across independent heterogeneous cohorts of skin biopsies, allowing precise objective monitoring of skin disease during treatment. In fact they defined a disease severity measure called the SSc skin severity score (4S) that showed a significant correlation with SSc severity as measured by mRSS across all skin biopsy datasets and could be used as a prognostic tool (11). Finally, Rech *et al*. analysed matrix metalloproteinase (MMP) polymorphisms (MMP1 -1607 1G/2G, MMP3 -1171 5A/6A, and MMP9 -1562 C/T), reporting lack of association with SSc susceptibility, although MMP1 1G/1G genotype was associated with interstitial lung disease and MMP3 5A/5A genotype correlated with the presence of anti-topoisomerase I antibodies and reduced diffusing capacity for carbon monoxide (12).

Other genes were investigated based on the shared epitope theory, two SNPs for the CD2, a T cell surface antigen involved in the adhesion to antigen presenting cells and induces Treg effects, a gene known to increase risk for RA, was also studied in a wide SSc European population by Koumaris *et al*. This showed increased risk for SSc only for the rs624988 CD2 gene SNP, with particular risk for anti-centromere positive SSc (13). Despite previous associations between vitamin D receptor genetic variants and increased risks for some autoimmune diseases, such as RA and systemic lupus erythematosus, Kamai et al. showed no difference in genotype and allele frequencies for vitamin D receptors in a small SSc population, as previously proven for Sjögren's syndrome (14). In a large population meta-analysis, Xu *et al*. established the bases for a pathogenetic role for STAT4 and IRF5 pathways in SSc: STAT4 is a key nuclear signalling transducer for IL12, IL23 and IFNγ, while IRF5 is a transcription factor involved in type I IFN signalling. The authors showed an increased risk for SSc in the case of SNPs STAT4 rs7574865 and IRF5 rs2004640, as previously proven for RA and SLE (15). Still in the JAK-STAT pathway, considering that TYK2 is known to mediate IL12 family cytokine activity, a large multicentre study proved that allele variant V362F (rs2304256), involved in RA development risk, was also significantly associated with SSc risk, reinforcing the hypothesis in previous reports of an IL12 cytokine family role in SSc pathogenesis (16). Finally, in a case-control study including 300 SSc patients and 280 HC, Koca *et al*. investigated IL-33 gene variants in the susceptibility for SSc, as previously shown for RA: their study demonstrated increased SSc risk for rs7044343 SNP, with significant lower prevalence in the anti-Topo I positive population, confirming the hypothesis for IL-33 genetic predisposition to SSc (17).

Concluding this section, CYP2D6 (involved in both drug and xenobiotic metabolism) polymorphisms were investigated in 81 SSc patients *versus* 150 HCs, demonstrating that heterozygote *1/*4 extensive metabolisers had a 2.9 increased risk of developing SSc (1.63–5.02, p=0.0002), while this risk was significantly lower for homozygotic *1/*1 extensive metabolisers (OR 0.4, 0.23–0.71, p=0.001) (18). This is in line with a recent metanalysis that pooled the 14 studies involving

1657 SSc patients and 3838 healthy controls, confirming previous reports for increased risk of SSc for solvent exposed patients (OR 2.72, CI 1.21–6.09), in particular more significant for men *versus* women (OR 5.28, CI 3.46–8.05), identifying aromatic solvents, tricholoethylene, halogenated solvents and ketons as being significantly associated (19). Wei et al. also performed a genome-wide study on the interaction between genetic and environmental factors in a complex SSc fibroblast model. They reported a strong association between a previously identified SSc locus of TNFAIP3 (SNP rs58905141) and silica-induced profibrotic responses of the fibroblasts. Similar associations were observed in two other reported SSc loci of IL2RA and ITGAM. In addition, several other genetic loci and genes unrelated to SSc susceptibility were also strongly associated with expression of the ECM genes of silica-stimulated fibroblasts in a Caucasian-only analysis and/or meta-analysis (20).

Immunity/inflammation

Various cells types have been studied recently in both the B and T lines. Involving primary Raynaud's patients, early SSc and advanced SSc without fibrotic manifestations, Cossu *et al*. showed an increased response of isolated CD56+ cells (NK and NK-like cells) to TLR stimulation, in particular to TLR1/2. This increased response above all involves IL6, TNFα and MIP-1α, able to differentiate the three subgroups, thus hypothesising a role for innate immunity in different pre-fibrotic stages (21). Analysing complement classic pathway activation (with C4d), complement alternative activation (C3B-BbP convertase) and soluble terminal complement complex (sTCC) representing the lytical terminal pathway, Okròj *et al*. showed increased values of all three markers in SSc *versus* healthy controls. In particular, when analysing a subgroup with a history of scleroderma renal crisis, and/or RNApol-III positivity, the authors demonstrated an increased C4d while C3b-BbP and sTCC were decreased, indicating a significant activation of the alternative complement pathway. This was also in line with the increased staining for C3b in both skin biopsies of SRC and skin of ARA positive patients, postulating complement activation as an important feature in the pathogenesis of SRC (22). Investigating the possible role of nucleosomes among all possible toll-like receptor (TLR) ligands in SSc pathogenesis, Yoshizaki *et al*. showed significantly increased levels of nucleosomes in both plasma and serum of SSc patients, in particular, in patients with dcSSc, pulmonary fibrosis and pitting scars/ulcers. Subsequently, they showed that nucleosomes were potent inducers of B and T cell activity, which was significantly increased in SSc patients and led to increased levels of IL4 and IL17 mRNA when stimulating T cells, and increased IgG production when testing B cells. Finally, they demonstrated that nucleosome effects were mostly mediated by TLR9 and CD19, as proven by showing reduced skin thickening and cellular activation when using a bleomycin mouse model treated with TLR9 antagonist and CD19-/- mouse-derived B cells (23).

Investigating phenotype and activation of memory B cells in 28 SSc *versus* 15 HC, Simon *et al*. demonstrated a reduced CD27+ memory cell repertoire in SSc patients, mostly due to a decrease in CD27+IgD+ non-switched memory B cells, particularly true for lcSSc. Conversely, CD27-IgD- double negative memory B cells were significantly elevated when ATA+, diffuse skin involvement and pulmonary fibrosis were present. This subgroup also presented higher numbers of CD27+CD95+ activated memory B cells and CD27-IgD-CD95+ double negative (DN) memory B cells, possibly representing a negative disease course biomarker to be further studied and validated (24).

Mavropoulos *et al*. described the transitional and memory Breg population in early and established SSc patients *versus* HC, showing a decrease in both categories in SSc patients, in particular, in the case of higher fibrotic burden such as dcSSc or pulmonary fibrosis. This was paralleled by a decreased IL-10 production by these cells in SSc patients and a reduced activity of its intracellular effects, as demonstrated by an impaired activation of MAPK and STAT3 pathways. This is in line with autoimmunity development, as IL-10 is a known regulatory cytokine in preventing and suppressing autoimmune responses (25).

Klein *et al*. investigated the role of CD8+ T cells in 26 SScc patients, showing that these cells were strong producers of type II cytokines, in particular, IL-4 and IL-13, known pro-fibrotic cytokines. When SSc lymphocytes were stimulated with non-specific T cell receptors, SSc patients showed a higher prevalence of memory CD8+ T cells, hypothesising that CD8+ T cell proliferation was not altered, though not sufficiently suppressed by T-regs. Regarding chemokines, a reduced circulating number of CXCR3+ and CD161+CCR6+T cells in diffuse SSc was noted, possibly justified by cellular homing into inflamed tissues. Regarding anti-inflammatory IL-10, CD8+ T cells, the authors speculated on the possible existence of a regulatory production by a specific CD8+ T cell subtype, accompanying the remaining inflammatory mechanisms (26).

In contrast with the above mentioned results on mice by Lei *et al*. (27), Almanzar *et al*. did not find increased levels of IL17A or other Th17-associated inflammatory cytokines in SSc human blood, but did find increased serum levels of both IL17-producing CCR6+ Th cells and Foxp3+ Treg cells in patients with dcSSc, with elevated transcriptional level of CCR6 and IL17 and demonstration of hypermethylation of Th17-associated transcription factor RORC. This inflammatory response was not sufficiently counteracted by IL10 serum level and gene expression, also supported by a lower suppression of lymphocyte activity by SSc-derived *versus* HC-derived Tregs. These results support the imbalance of the Th7/Treg ratio particularly during the inflammatory phases of early SSc (28).

It has recently been shown that innate lymphoid cells (ILC) play an important role monitoring helper T cells in their cytokine profile. Among ILCs, it emerged that ILC group 1 played an important

role in coordinating type I inflammatory response. Roan *et al*. investigated ILCs groups in SSc patients, demonstrating an increased frequency of ILC1 subset cells expressing CD4 antigen (ILC1-CD4+), which were strong producers of TNFα, Granulocyte macrophage colony-stimulating factor (GM-CSF) and IL2 but have a low expression of IFNγ. ILC1-CD4+ cells were also the most responsive to IL6 in SSc although with a decreased IL6Rα expression, the latter as a postulated down-regulatory effect of T cell stimulation. Given all these activities and phenotype, the authors propose further studies on ILC1 as a possible future target for biologic treatments in SSc (29). On the same topic, using skin biopsies and blood samples from SSc patients versus healthy controls, Wohlfahrt *et al*. demonstrated a significantly increased level of type 2 innate lymphoid cells in SSc patients and their levels paralleled different fibrotic manifestations, being associated with diffuse skin subset and pulmonary fibrosis but also correlating with mRSS, thus supporting a role for this cell type in fibrosis development (30).

Given previous reports of γδ T cells infiltration in the involved skin of SSc patients and the critical role of CD27 as a costimulatory for survival of this cell type, Henriques et al. showed decreased levels of circulating γδ T cells in SSc patients versus controls, with an increase of the effector Vγ9 positive repertoire in patients with dcSSc and pulmonary fibrosis, which also determined a significantly higher production of IFNγ and TNFα. Moreover, an increase in the CD27+ γδ T cells expressing granzyme B or perforine was seen, in particular in dcSSc. This suggests a dysregulation in both cytotoxic activity and proinflammatory status in SSc (31). Immunological activation, especially of T cells, is considered a key point in the development of the vascular abnormalities and fibrosis. CD8+CD28- T cells could represent a pathogenic T-cell subset in SSc, especially in the early stage of skin disease, being increased in SSc blood and skin and correlating with disease duration and skin fibrosis extent. Most CD8+CD28-T cells were skin-tropic or skin-resident and exhibited IL13 dependent pro-fibrotic and cytotoxic functions and effector-memory phenotype, while circulating SSc CD8+CD28- cells possess a strong cytolytic activity *ex vivo* (32).

Interestingly, Elhai *et al*. showed that OX40L, a costimulatory molecule required for full activation of T cells, is implicated in the development of inflammation-driven skin, lung, and vessel fibrosis in SSc, as it was over expressed in SSc sera and skin, and its blockage could then prevent severe fibrosis in SSc (33).

IFN signature was previously demonstrated in SSc: recently, Brkic *et al*. observed higher IFN scores in SSc *versus* HC, with an IFN score ≥4.12 as a pathologic cut-off. Moreover, they demonstrated that an IFN type I signature is observed in patients with SSc from the earliest phases of the disease, even before overt skin fibrosis, since it is correlated with B cell activating factor (BAFF) mRNA expression and serum type III procollagen N-terminal propeptide (PIIINP) levels, supporting a contribution in the pathogenesis and progression of SSc (34).

Vasculopathy

Vascular dysfunction represents a disease-initiating event in SSc, with recent studies suggesting epigenetic dysregulation impacting on elevated pro-angiogenic but insufficient compensatory angiogenesis, thus not allowing a sufficient vasculature recovery.

Tsou *et al*. analysed the expression of the pro-angiogenic chemokines, namely growth-regulated protein-g (CXCL3), granulocyte chemotactic protein 2 (CXCL6) and their receptor CXCR2 in endothelial cells (ECs) isolated from SSc skin, demonstrating an increased expression in SSc serum although SSc ECs were unable to respond to all three pro-angiogenic chemokines and could not promote their migration (35). In SSc, ECs proliferation and migration can also be controlled by histone deacetylases (HDACs), whose overexpression was demonstrated by Tsou *et al*. They identified novel HDACs-regulated target genes associated with fibrosis and impaired angiogenesis, such as CYR61, PVRL2, and FSTL1 (36). McMahan *et al*. showed that the expression of the anti-interferon-inducible protein 16 (anti-IFI-16), CD31 and CENPs, which are associated with severe vascular complication in SSc, are increased in vascular lineage cells, from progenitors through mature endothelial cells, as well as in SSc-biopsy specimens, but not in circulating endothelial cells (37). This is in line with Patschan *et al*. who demonstrated a comparable regenerative early endothelial progenitor cell (eEPC) capacity of SSc *versus* controls, which was not paralleled by the number of circulating eEPCs, since they were significantly decreased in SSc. Moreover they confirmed that these cells were more prone to mesenchymal transdifferentiation and also showed an impaired migration to wound area when cultured with SSc sera, an effect that was reduced when these cells were co-cultured with Bosentan. This study confirms a central role for eEPCs in both altering vascular regeneration and promoting collagen deposition (38). In a small group of lung tissue biopsies from SSc patients with ILD, Mendoza *et al*. reported that CD31+/CD102+ EC simultaneously expressed mesenchymal cell-specific proteins (collagen I, collagen III and fibronectin), EC-specific genes (collagen IV and VE-cadherin), profibrotic genes (TGF-β and CTGF), and genes encoding endothelial to mesenchymal (EndoMT)-related transcription factors (TWIST1 and SNAI2), indicative of the occurrence of endothelial to EndoMT phenotypic transition (39).

As a second mechanism of vascular damage, vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR) system disturbances and Neuropilin-1 (NRP1), which a receptor for both class-3 semaphorins (Sema3s) and VEGF-A, a molecule required for optimal VEGF-A/VEGFR-2 signalling, were analysed by Romano *et al*. This group demonstrated that serum levels and dermal expression of NRP1 were significantly decreased in SSc and correlated with the severity of NVC abnormalities and the presence of

digital ulcers; this led to the hypothesis that NRP1 suppression is due to Fli1 transcription factor deficiency as an additional factor in the perturbed VEGF-A/VEGFR-2 defective angiogenesis (40).

Fibrosis

As a third clinically significant pathogenetic pathway, vascular damage may lead to the activation of fibroblasts and the abnormal accumulation of extracellular matrix (ECM), mainly collagen, leading to fibrosis. In SSc, fibroblasts in lesional areas are mostly myofibroblasts and constitutively secret ECM components and exert excessive scarring of the skin. The decreased susceptibility of SSc fibroblasts to apoptosis is an important driver of dysregulated fibrosis in SSc. Last year Jafarinejad-Farsangi *et al*. analysed microRNA-21 (miR-21), a pro-fibrotic factor with high expression in fibrotic areas of SSc skin and fibroblasts, describing an overexpression of miR-21 through Bcl-2 driven reduced SSc fibroblast apoptosis. Conversely, the antisense inhibition of miR-21 induced a high rate of apoptosis in SSc fibroblasts probably associated with modification in Bcl-2 and Bax/Bcl-2 ratio expression (41). Wang *et al*. reported that long non-coding RNAs (lncRNAs) levels were over-expressed in SSc dermal fibroblasts; among them, in particular, serum TSIX levels were upregulated in SSc fibroblasts as a possible result of activated endogenous TGF-β signalling (42).

The known role of endothelin-1 (ET-1) in SSc was also further supported in the fibrotic pathway by Akashi *et al*., who demonstrated ET-1 receptor type B (ETB) signalling as an important inductor of skin fibrosis: in fact. ETB receptor-knockout (ETBKO) mice presented a significantly reduction of dermal thickness, subcutaneous fat atrophy, and myofibroblast counts, and lower gene expressions of α-smooth muscle actin and collagen 1α1 compared to wild type mice, after BLM treatment (43).

Consistent with previous studies, using proteomic analysis, some authors demonstrated that fibroblasts from affected and unaffected skin from limited SSc patients act as two distinct entities: in fact, an increase in ECM components (in particular collagen α-1 and α-2), upregulation of Col IA and vimentin as typical markers of activated myofibroblasts and overexpression of peroxiredoxin-1 (PRDX1) as antioxidant defence by fibroblasts, were seen in affected areas' fibroblasts; there results were also confirmed by immunohistochemistry analysis on total skin samples and by qRT-PCR analysis on fibroblasts (44). In a recent study, Wang *et al*. performed a gene expression analysis using induced pluripotent stem cells (iPSC) from cultured SSc dermal fibroblasts (SSc-iPSC), and fibroblast re-differentiated from SSc-iPSC (SSc-iPSC-FB), reporting a significant down-regulation of S100A8, Smad6, and TGF-b2 in SSc-iPSC, although not significantly differently expressed in cultured SSc fibroblasts or SSc-iPSC-FB compared to normal controls. Conversely, the expression of collagen and integrins (a and b) was up-regulated in SSc fibroblasts but normalised in iPSC, suggesting epigenetic gene modifications as a mechanism of collagen accumulation and gene reprogramming as a novel therapeutic approach (45).

The role of platelets in the profibrotic way was analysed by Truchetet *et al*., who described that serum thymic stromal lymphopoietin (TSLP) levels were significantly increased in SSc patients *versus* healthy donors and were associated with a higher frequency of vasculopathy (presence of digital ulcers, scleroderma renal crisis, and/or PAH). Moreover, the proportion of TSLP-positive dermal cells was increased in the SSc skin biopsies and correlated with modified Rodnan skin score. In SSc dermis, TSLP was mainly expressed by CD31-positive endothelial cells and *in vitro* activated platelets induced TSLP production by human dermal microvascular endothelial cells in an interleukin-1b-dependent mechanism (46).

On the pivotal role of transforming grow factor (TGF) β in SSc fibrosis, Palumbo-Zerr *et al*. identified TWIST1 as a crucial regulator upregulated factor in SSc fibroblasts, as it was induced by TGFβ and amplified the pro-stimulatory effects of TGFβ on fibroblasts. On the other hand, inactivation of Twist1 protected from TBRI-induced fibrosis *in vivo* and knockdown of TWIST1 ameliorated mice skin fibrosis induced by bleomycin (47). The nuclear factor erythroid 2-related factor 2 (Nrf2) was is known to mitigate fibrotic responses by blocking canonical TGF-β-Smad signalling. Wei *et al*. first demonstrated that Nrf2 expression and activity is decreased in SSc skin biopsies and then used bleomycin treatment on Nrf2-null mice resulting in exaggerated fibrosis, whereas a novel pharmacologic Nrf2 agonist 2-trifluoromethyl-2'-methoxychalcone prevented this over-response (48). Kanno *et al*. recently demonstrated that α2-antiplasmin (α2AP), a physiological inhibitor of plasmin, has a profibrotic effect probably by inhibiting fibrinolysis and inducing the production of TGF-β. They reported that α2AP expression was elevated in SSc dermal fibroblasts and the administration of α2AP in mice as an inductor of profibrotic changes. Conversely, α2AP neutralisation exerted an antifibrotic effect and also prevented autoantibody production in an SSc bleomycin-induced mouse model (49).

Although TGFβ is recognised as being a key trigger of fibroblast activation in SSc, prominent innate immunity suggests that additional pathways contribute to disease persistence. Tenascin-C is an endogenous TLR activator that drives TLR4-dependent fibroblast activation and organ fibrosis. In a small cohort of SSc patients, Tenascin was elevated in SSc serum, fibroblasts and skin samples. This was also seen in mouse models, as Tenascin-C deficiency determined the attenuation of skin and lung fibrosis (50). Moreover, mtDNA and other damage-associated TLR9 ligands in SSc skin might trigger localised activation of TLR9 signalling, TGF-β production, and consequent fibroblast activation (51). Ciechomska *et al*. demonstrated a novel role of histone demethylation induced by DZNep on Fra-2-mediated tissue inhibitor of metalloproteinase 1

(TIMP-1) production by monocytes in the presence of TLR-8 agonist, leading to fibroblast transdifferentiation (52).

Some regulatory proteins, implicated in several cellular processes such as transcription, apoptosis and cellular metabolism, can be involved in the pathogenesis of SSc. Recently, it has been postulated that norepinephrine (NE) is associated to fibrosis in SSc through IL-6 secretion: some authors demonstrated that NE enhanced IL-6 production and proliferation more significantly in SSc fibroblasts, in particular through ARβ activity (53). Akamata *et al*. revealed a role of the mitochondrial deacetylase sirtuin 3 (SIRT3) in modulating fibrotic responses through blocking intracellular TGF-β signalling and fibrotic responses, reducing reactive oxygen species (ROS) enhanced production and mitigating the activated phenotype of SSc fibroblasts. This suggested the ability of a novel pharmacological SIRT3 agonist to attenuate fibrosis *in vitro* and *in vivo* in SSc patients (54). Nakayama *et al*. showed IL-23 up-regulated type I collagen expression in SSc fibroblasts *versus* normal controls and reported that the paradoxical effects of IL-23 in SSc fibroblasts are also mediated by the balance between miR-4458 and miR-18a expression (55). Takahashi *et al*. reported that LL-37 is upregulated in SSc dermal fibroblasts, perivascular inflammatory cells, keratinocytes and dermal small vessels; LL-37 upregulation potentially contributes to dermal fibrosis and pulmonary fibrosis through its antiapoptotic effect on those cells (56). Similarly, Cathepsin L (CTSL), a lysosomal proteolytic enzyme involved in vascular and extracellular matrix remodelling, was reported by Yamashita *et al*. as over-expressed in dermal small vessels of SSc patients and associated to nailfold bleeding, telangiectasia and digital ulcers. Moreover, CTSL mRNA expression was enhanced in dermal small vessels of endothelial cell-specific Fli1 knockout mice, reminiscent of SSc vasculopathy; simultaneously, CTLS mRNA levels were significantly decreased in the lesional skin of BLM-treated mice, suggesting that its down-regulation is likely to be associated with dermal fibrosis development (57). O'Reilly *et al*. investigated the IL-13 pathway in SSc, showing that this cytokine is responsible for increasing collagen expression, which is independent of TGFβ1. The authors were also able to demonstrate that IL13-induced collagen production was strongly determined by STAT6 signalling; on the other hand, when fibroblasts were transfected with miRNA-135b, collagen production was significantly reduced, in line with the report for reduced miRNA-135b serum levels in SSc patients. These results demonstrate that IL13 effects in SSc patients are mediated by both STAT6 and miRNA136, suggesting interaction with these two mediators as a possible therapeutic target (58). Given previous reports of the involvement of lysophosphatidic acid (LPA) in SSc dermal fibrosis, Castelino *et al*. confirmed these results and demonstrated loop activity between LPA, autotaxin (enxyme converting lysophospholipids to LPA) and IL6 (whose production is induced by LPA). In fact, their elegant study showed that autotaxin inhibition by PAT-048 was able to prevent and treat dermal fibrosis in the bleomycin model, reducing both dermal thickness and hydroxyproline/αSMA/TGFβ levels. Moreover, they were able to demonstrate that autotaxin-LPA-IL6 levels were respectively stimulating each other, both as a positive feedback direct effect and as a negative feedback effect when IL6 gene was silenced or ATX inhibited. These results confirm that autotaxin is required for bleomycin mouse model dermal fibrosis and its effect is amplified by both LPA and IL6, two possible targets for SSc treatment (59). Based on previous reports that a transient receptor potential channel of the vanilloid subfamily TRPV4 is pivotal for TGFβ1-induced lung fibroblast proliferation and development of pulmonary fibrosis in mice, Goswani *et al*. investigated the role of this Ca++ membrane channel in SSc human samples and mouse models. They demonstrated an increased αSMA+ myofibroblast positive staining for TRPV 4 in both the human and bleomycin SSc mouse model and the role of TRPV4 was confirmed by demonstrating reduced skin thickness and collagen deposition when bleomycin was administered to a TRPV4 knock out mice (60).

Animal models and miscellaneous

Further characterising currently available animal models for SSc, Manetti *et al*. demonstrated the presence of typical myocardial histopathological features of scleroderma cardiomyopathy in both right and left ventricules in the urokinase-type plasminogen activator receptor-deficient mice. This was present in 24-week-old mice, but not in younger 12-week-old ones, representing an age-related complication which could be used to further investigate and evaluate potential treatment for SSc-related cardiomyopathy (61). Similarly, Cipriani *et al*. performed further analysis on the University of California at Davis line 200 (UCD-200) chickens, demonstrating progressive vascular loss in the comb of the chicken model; in particular, this was associated with a progressive reduction of chemokine SDF1 and CXCR4 in vascular cells, supporting the initial inflammatory phase. Moreover, the impaired vascularisation was paralleled by an increase of VEGF-A, ET1 and ET1 receptors A and B and platelet-derived growth factor (PDGF), mirroring the human progressive vasculopathy and fibrotic changes, thus supporting its use in translational research on the early SSc disease phases (62).

To further characterise the pathogenetic role of TGFβ in the Fos-related antigen 2 transgenic mice, Tsujino *et al*. treated this mouse model with a pan-TBFβ-blocking antibody and showed a reduction in the occlusive pulmonary vasculopathy, which was unfortunately associated with an increase in the granulocyte inflammatory pulmonary infiltrate. This was supposed to be related with a TGFβ receptor (called bone morphogenic protein type 2 receptor - BMPR2). Similar results were also obtained when TGFβ receptor type 2 was deleted from pulmonary vessels smooth muscle cells, resulting in the amelioration of the same pulmonary vascular features and postulating a role

for this receptor in the remodelling process (63).

Using the bleomycin murine (BLM) model for both skin and lung fibrosis, Lei *et al*. showed that CD4+IL17A+ Th17 cells were significantly increased in bleomycin mice skin and lungs *versus* controls. Simultaneously, serum concentrations of IL17A and other Th-17-related cytokines (TGF-β1, IL6) and levels of their mRNA transcripts were significantly increased in BLM mice skin and lungs, positively correlated with skin and lung hydroxyproline levels. Moreover, rIL17A cultured pulmonary fibroblasts showed enhanced fibrosis with increased type I collagen and TGF-β1 mRNA transcripts secretion, which was lowered when co-cultured with anti-mouse IL17A antibody, establishing the basis for anti-IL17A treatment in SSc (27).

The enzyme Vanin-1 pathway, involved in vitamin B5 generation and cysteamine-dependent reactive oxygen species scavenging activity, has a shown pro-inflammatory effect in many conditions. This was also proven in two SSc mouse models, namely hypochlorous acid induced (HoCl) and bleomycin-induced mouse models, showing reduced mouse skin thickening, hydroxyproline and αSMA content in both models, reduction of endothelial activation markers in the HoCl model. Consequently, increased levels of serum vanin-21 and pantothenic acid were found in 51 SSc patients *versus* 30 healthy controls, in particular with higher values in dcSSc and PAH patients, proposing a role for vanin-1 inhibition in SSc treatment (64). Moreover, heme-oxygenase is an enzyme that produces bilirubin, caron monoxide and ferritine, exerting anti-inflammatory, anti-oxidant and anti-fibrotic activities; van Bon *et al*. showed significantly reduced levels of heme oxygenase 1 in lymphoid and myeloid cells from SSc patients *versus* healthy controls, which were not related to an intrinsic reduced expression but due to the increased CXCL4 levels – dependent down-regulation. Moreover, showing that the induction of heme oxygenase 1 could inhibit TLR4 and TLR7/8 mediated cytokine production, the authors postulated a role for heme oxygenase stimulator in SSc treatment (65). Recently, Ogawa *et al*. used a scleroderma mouse to show that transplantation of MHC compatible, minor antigen mismatched bone marrow stromal/stem cells (BMSCs) played a role in the pathogenesis of fibrosis and suggested an interaction between transplanted and residual host T cells in the induction of the autoimmune phenotype observed in SSc mouse model fibrosis (66). Finally, it was also demonstrated that BLM-induced extracellular hydrogen peroxide (H2O2) was transported into fibroblasts by AQP3 and activated transforming growth factor-β1 (TGF-β1) signalling in a BLM-induced mouse model of SSc. It means that silencing AQP3 decreases TGF-β1, COL1, and COL3 expression by reducing H2O2 levels in fibroblasts and attenuating fibrosis in BLM-treated mice (67).

Biomarkers

Identification of reliable and consistent biomarkers, which can predict the course of the disease, is required for a better stratification and management of patients and it would be of great utility in the setting of a multifaceted disease as SSc. In the last year, several attempts have been made to correlate possible laboratory biomarkers with distinct clinical features of SSc. Dantas and colleagues showed that levels of TGFβ1 were higher in the serum of patients with SSc compared to healthy controls; moreover, levels of TGFβ1 were even higher in patients with lung fibrosis and diffuse SSc (68). Abdel-Magied *et al*. showed that levels of IL-6 were increased in the serum of patients with SSc and significantly correlated with high resolution computerised tomography (HRCT) scores, and also with diffusing capacity of the lung for carbon monoxide (DLCO), six-minute walking distance (6MWD), and echocardiographic abnormalities of the right side of the heart. This contributes to the evidence of a possible role of IL-6 in the development and disease activity of cardiopulmonary manifestations in SSc patients, as tested in ongoing clinical trials (69).

In addition to cytokines, new molecules that reflect cellular activation and cellular functions are emerging as interesting biomarkers. Programmed death 1 (PD-1), a member of the CD28/B7 family of costimulatory molecules, is a protein with a central role in regulating T cell activation and in the maintenance of tolerance. PD-1 is expressed by activated T cell, and binds to PD-L1 and PD-L2. In particular, PD-L2 is expressed on antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells. In the work of Fukasawa *et al*., serum levels of sPD-1 and sPD-L2 were found to be elevated in patients with SSc and to correlate with the extent of fibrosis (given that these molecules are higher in the serum of patients with diffuse SSc, patients with pitting scars/ulcers, and with higher modified Rodnan skin scores - MRSS scores) (70). Interestingly, PD-L2 was expressed at high levels on B cells producing interleukin-10 (IL-10), indicating another possible function of B cells in regulating T cell functions and cytokine production in SSc (70).

The complement system has been implicated in the pathogenesis of SSc. Okrój *et al*. showed that by measuring products of the activation of both the classical and alternative pathways of the complement cascade, these products were increased in SSc patients compared to healthy controls, confirming an ongoing complement activation in SSc (22). Moreover, by classifying the patients into subgroups depending on different clinical features, a different distribution of complement activation markers was detected in patients with scleroderma renal crisis (SRC), who showed significantly higher amounts of C4d (a product of the classical pathway) and significantly lower levels of C3bBbP (a product of the alternative pathway) than patients without kidney involvement (22). This pointed to a specific pattern of complement activation during SRC. Okrój *et al*. also showed by functional assay that a significant decrease in complement haemolytic activity characterised patients with scleroderma renal crisis, thus confirming complement consumption in these patients (22). Furthermore, deposits of C3b were

found in kidney biopsies of patients with scleroderma renal crisis (22). These evidences point out the potential role of interfering with the complement activation cascade as a novel approach to SRC.

Lung disease is a severe complication of SSc. Several works have investigated biomarkers to identify lung involvement early in the disease course, and also as predictive factors of response to treatment. CXCL4 is a chemokine secreted by plasmacytoid dendritic cells (pDC) and it has been shown to be increased in patients with SSc and to correlate with the development of lung fibrosis and pulmonary arterial hypertension (71). Furthermore, CXCL4 was found to be higher also in patients with undifferentiated connective tissue disease with features of SSc, but not yet meeting its classification criteria (72). However, further work conducted on a larger number of cases is needed to confirm a possible prognostic value of this chemokine (72). Volkmann and colleagues showed that baseline serum CXCL4 levels were significantly higher in SSc patients with ILD compared with HCs (73). However, in this study, no significant correlations were identified between CXCL4 levels and the extent of ILD at baseline. In the same study, patients were then assigned to receive either cyclophosphamide (CYC) or mycophenolate mofetil (MMF): plasma CXCL4 levels decreased significantly from baseline to 12 months in all patients regardless of the type of treatment. Furthermore, patients with the largest decline in CXCL4 levels during the first 12 months of treatment showed a significant improvement on pulmonary function test at both 1 and 2 years follow-up (73).

Wu and colleagues endeavoured to study a possible predictive significance of several cytokines for long-term progression of ILD and survival in patients with early SSc. The chemokine CCL2 and IL-10 were found to be significant predictors of ILD progression, with higher levels of CCL2 predicting a faster decline in respiratory function, while higher IL-10 levels predicted a slower decline (74). Higher levels of CCL2 also predicted a poorer survival of SSc patients (74). The group of Rolla *et al*. analysed the levels of cytokines present in exhaled breath condensate and serum of SSc patients and reported higher levels of Th-17-derived cytokines in exhaled breath condensate of patients with diffuse SSc. Moreover, levels of IL-1 beta in exhaled breath condensate and serum levels of IL-23, TNFα and IL-10 were significantly associated with thoracic CT-scan score of ILD. Interestingly, levels of IL1β, IL-17 in exhaled breath condensate and serum levels of IL-10 were negatively related to DLCO (75). These data point to a role of Th17 in the development of ILD in SSc, highlighting the importance of targeting Th17 cells in ILD and exploiting Th17-derived cytokines as biomarkers.

In keeping with an activation of the complement cascade in SSc, Miyagawa *et al*. showed that SSc patients with ILD tended to have decreased serum levels of H-ficolin, an oligomeric lectin involved in the activation of complement (76). Moreover, serum H-ficolin levels showed a tendency to positively correlate with diffusing capacity for carbon monoxide (76). Finally, Yamakawa *et al*. reported that serum levels of Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) correlated with ILD activity in SSc measured as pulmonary function tests and disease extent on HRCT: serum levels of KL-6 were significantly related to changes in forced vital capacity, making it a promising biomarker to monitor ILD activity after treatment, whereas high serum levels of SP-D were a significant predictor of forced vital capacity decline over time (77).

Although all these biomarkers need further research and validation in large multicentre studies, they showed interesting correlations with clinical features of SSc, with disease activity and response to treatment, opening up new horizons in the field of precision medicine and early intervention for SSc. It is interesting to speculate that in the near future a biomarker, or a combination of different ones, may be of use to the clinician to identify, early in the disease course, the risk of developing several features of SSc (such as ILD), possibly helping to design early aggressive treatments in order to reduce, or even prevent, tissue damage, and thus disability. Moreover, biomarkers can point out to determined pathways active in certain clinical manifestations of the disease, therefore identifying new possible therapeutic strategies and patients susceptible to treatment with specific drugs.

Clinical aspects

The severity of SSc and epidemiological data accounting for it have been investigated in numerous studies in the general SSc population and in subgroups divided according to time of diagnosis or for disease-specific organ involvement (PAH representing the most frequent group studied). Recently, a large multinational study of SSc subjects recruited from Australia, Canada and Spain estimated mortality and determined causes of death in those recruited within 4 years of disease onset ('inception' cohort) and a 'prevalent' cohort of patients including all SSc patients followed at the involved centres (n=3218) (78). Standardised mortality rates were higher in the inception cohorts (4.06 (95% CI 3.39–4.85)) that in the corresponding prevalent cohorts (3.39 (95% CI 3.06–3.71)). Mortality was SSc-related in 62% of the patients in the inception cohort while the disease-specific effect lowered in the prevalent cohort (55%), whereas the non-SSc-related causes increased in percentage (24–34%). Causes of death were similar between the 2 cohorts: in most cases they were represented by heart-lung disease (proportionally more in the prevalent cohort): PAH, ILD and PAH combined with ILD, myocardial involvement, followed by gut involvement, renal crisis (proportionally more in the inception cohort, as expected). Other causes of death listed in the inception cohort but not in prevalent cohort were pericardial effusion (1.1%) and sepsis due to ischaemic digit or decubitus ulcer (1.1%). Non-SSc-related causes were in both cases malignancy (38.2%), sepsis (14.7%), cerebrovascular disease (11.8%) and ischaemic heart disease (8.8%). Multivariable hazards

regression analysis showed that male gender, older age at disease onset, diffuses disease subtype, PAH and SRC were independent predictors of risk, and longer disease duration at recruitment was an independent protective factor. Among the risk factors, PAH had the highest hazard ratio (2.35 (1.29–4.29) p=0.06) in the inception cohort. The prevalent patients showed similar figures with slight differences: among significant predictors in multivariate analysis ILD was an independent predictor along with male gender, older age at disease onset, diffuse disease subtype, PAH. Anticentromere antibodies and longer disease duration at recruitment showed a protective effect. The results of this multicentre international study point out that SSc prevalent cohorts underestimate early mortality in SSc, which has a substantial impact on mortality mainly dependent on the negative prognostic value of PAH. ILD seems to be a risk factor for poor long-term survival rather than early death but further studies need to address it. That SSc may be a paraneoplastic manifestation in some patients, especially those diagnosed with breast cancer synchronous to SSc, has been hypothesised from data coming mainly from the antiRNA polymerase (RNAP) 3 autoantibody subset (79-81). Because of this, a web-based Delphi consensus between EUSTAR centres has been set up with a high consensus that screening for cancer is appropriate at diagnosis in SSc and that prospective studies should clarify how tight and for how long the cancer surveillance related to the development of SSc should persist and by which techniques (81).

In light of these results it emerges the need to distinguish the type of populations in the study, not only according to disease subtypes but also whether they are incident or prevalent cases. Many studies lately have included this distinctive feature in their analyses. Morrisroe *et al*. (82) have demonstrated that incident patients with group 1 PAH enrolled in the Australian Scleroderma Cohort Study (ASCS), an important SSc cohort study encompassing annual screening for the pulmonary vascular involvement with the combination of echocardiographic and functional respiratory tests, had a significant impact on mortality from older age at PAH diagnosis (HR 1.1, 95% CI 1.0–1.1, p=0.03), presence of mild ILD (HR 2.8, 95% CI 1.4–5.6, p=0.01), worse WHO functional class (HR 2.0, 95% CI 1.1–3.9, p=0.03), higher mPAP at diagnosis (HR 1.1, 95% CI 1.0–1.1 mmHg, p=0.001) and presence of digital ulcers ever (HR 3.1, 95% CI 1.4–7.2, p=0.01). Of note, despite annual screening the functional class at diagnosis was more frequently represented by New York Heart Association (NYHA) III and this has a great impact itself on mortality. These data point out on a relevant type of patient, the incident, newly diagnosed at times when our knowledge is mostly updated, that screening is still significantly fallacious in the early identification of SSc PAH group 1 patients. The reasons for these faults may lie in the different checkpoints of the timetable. In the cohort study of ASCS, based on the European Society of Cardiology (ESC)/European Respiratory Society (ERS) guidelines (83) and further stratifying patients into low, moderate and high risk for PH, the estimated annual prevalence of PAH was 1.4% (1.2% in lcSSc, 0.9% in dcSSc). Strikingly, most of the patients were in NYHA class III/IV (85%) and showed longer disease duration and higher age than non-PAH patients (84). In this study it was evident that adherence to the screening algorithm was high (84%), but right heart catheterisation (RHC) referral was very scarce due to a number of practical and/or cost-effective reasons. As a result, even when considering "incident" cases, higher functional NYHA classes were reported already at diagnosis with all the consequences involved in terms of prognosis and survival. These evidences highlight the necessity of screening and its implementation in SSc for PAH. In 2014 Coghlan *et al*. (85) reported the first evidence-based algorithm for the screening of PAH in SSc (DETECT), obtaining higher performances and fewer missed diagnoses than the 2009 European Society of Cardiology (ESC)/European Respiratory Society (ERS) guidelines (86). The DETECT algorithm is based on entry criteria (disease duration of at least 3 years and DLCO<60% of predicted) and is a score calculated on 2 successive steps, the first on clinical and laboratory measures and the second on echocardiographic parameters. If positive, the DETECT score indicates the need for performing RHC to diagnose PAH. In 2017 Guillén-Del Castillo *et al*. (87) retrospectively analysed their cohort of patients with indication to RHC due to a different clinical presentation than DETECT-predicted (but all satisfying the DETECT inclusion criteria). They compared both the RHC-confirmed PH and patients with no PH for the *a posteriori* satisfaction of the DETECT 2-step process and also analysed the ability to identify borderline PH (21-24 mPAP at RHC). The DETECT screening algorithm was tested against the ESC/ERS 2009 guidelines. The RHC referral rate is higher for the DETECT algorithm, and also by this method there were no missed diagnosis while with the ESC/ERS guidelines they represented 8.5%. There were only slight differences in sensitivity (100% *vs*. 91%, respectively) while specificity was low (42.9%) for DETECT and higher (85.7%) for the ESC/ERS 2009 guidelines. Positive predictive value was 68.6% using DETECT compared to 88.9% using the ESC/ERS 2009 guidelines. Negative predictive value (NPV) instead reached 100% (75.7–100) using the DETECT algorithm, while using the ESC/ERS 2009 guidelines it was 88.9% (71.9–96.1). With respect to borderline PH, a condition that could represent a negative prognostic factor on mortality (88), the DETECT algorithm showed better performances in terms of sensitivity and NPV than the ESC/ERS 2009 guidelines. The issue with this study, beyond its retrospective analysis applied to a screening method, is that the population is selected and corresponds entirely to the DETECT enrichment criteria. Recently, another study has addressed the performances on *unselected*, consecutive SSc patients of 4 different screening methods in PH: ESC/ERS 2009, DETECT and

ESC/ERS 2015 update including only echocardiographic indication or combined with DETECT for high-risk patients (89). In their analysis, Vandecasteele *et al*. discovered that referral rates for RHC are 3 times higher for DETECT than other screening algorithms (ESC/ERS 2009 and their updated version of 2015 including DETECT for high risk patients, the costs also being as much higher for the DETECT method. RHC instead was performed in a higher percentage of patients for whom it was indicated based on the ESC/ERS 2009 (100% *vs*. 83% and 80% for DETECT and ESC/ERS 2015, respectively). The 1-year incidence of PAH was 1.5%·year and all the PAH patients were recommended for RHC by all screening algorithms. The positive predictive value (PPV) was 23% for the combined approach, 18% for the 2009 guidelines, 6% for the DETECT algorithm, 11% for the 2015 echo screening and 9% for the 2015 combined screening algorithm. These results show that in the whole SSc cohort, which is indeed as a whole at risk of developing PAH, the use of a combined and multiparametric approach remains the mainstay of screening (90).

A study by D'Andrea *et al*. (91) has studied the associations of right atrial lateral strain as evaluated by speckle-tracking two-dimensional strain echocardiography. This parameter is an indirect measure of cardiac function used for both ventricular and atrial chambers. In 90 consecutive SSc patients compared to 55 healthy controls it correlates independently with pulmonary artery pressure systolic (PAPs) during exercise, right atrial area (RAA), left ventricle (LV) stroke volume and inferior vena cava (IVC) diameter. In the subgroup of patients with pulmonary fibrosis measures of IVC diameter, PAPs, PAPm and RAA were significantly higher than in patients without lung fibrosis. The 2D strain analysis was reproducible but this was a monocentric study so it needs to be further validated in larger and multicentre cohorts. The prevalence and clinical associations of elevated PAPs on echocardiography has been further investigated in a large cross-sectional cohort of patients with early SSc included in the European Scleroderma Trials and Research group (EUSTAR) database (92). The study has included 1188 patients with early SSc (less than 3 years of duration from the first non-Raynaud's symptom). Elevated PAPs have been found in 17% of them, with equal frequencies in both limited and diffuse cutaneous SSc. Patients with PAPs >40 mmhg were older at disease onset, had lower DLCO, more frequent digital ulcers, cardiac conduction blocks and proteinuria. In the multivariate analysis filtered for the lc-SSc subset, older age at the first non-Raynaud's symptom, ACA positivity, restrictive lung function test, lower DLCO and joint contractures were independent factors associated with high PAPs. In the dc-SSc subset again older age at disease onset, but also longer time between Raynaud onset and the first non-Raynaud's, digital ulcers, cardiac conduction blocks and proteinuria were independent predictors of elevated PAPs. Because of the cross-sectional nature of the analysis and the lack of confirmation of the systolic PAP by right heart catheterism (RHC) its elevation cannot be attributed entirely to the presence of PH but also of important visceral complications of SSc such as primitive myocardial, peripheral vascular, renal and lung fibrotic involvement, altogether impacting on mortality even in the early stages of the disease. These results encourage the performance of echocardiography as a precious screening tool in SSc. The utility of other screening tools in PAH has been investigated by the Pulmonary Hypertension assessment and Recognition of outcomes in Scleroderma (PHAROS) registry (93), a multicentre prospective cohort of patients with SSc at high risk of developing PAH or definitive PH. The biomarkers BNP or its metabolite NT-proBNP have been studied in an at-risk PAH population (SSc-AR-PAH, n=157) and in definite SSc-PH patients (172 patients). Their predictive value of survival has also been tested in the total of 329 patients enrolled in the study. Median Brain natriuretic peptide (BNP) (135, 9-5534 pg/ml) and N-terminal pro-brain natriuretic peptide (NTproBNP) (503, 1-9999 pg/ml) levels were higher in PH patients with respect to at-risk patients (43.5, 3–2560 pg/ml and 82 (4–1223 pg/ml). Higher levels were seen in group 2 PH. In the SSc-AR-PAH during a mean follow-up time of 3.5 years 26 developed PH and among them 16 have PAH. BNP and NTproBNP levels were moderately but significantly correlated to PAPm and pulmonary vascular resistance (PVR) at RHC. NT-proBNP had a moderate negative correlation with cardiac output. Significant predictors of PH development were PAPs≥40 mmHg and FVC/DLCO≥1.8, whereas for PAH independent predictors were PAPs≥40 mmHg and creatinine levels. BNP or NT-proBNP values were not significantly predictive of PH development or mortality within this group of patients. To improve the accuracy of PH detection beyond echocardiography and laboratory biomarkers, a recent study has also investigated the correlation of cardiopulmonary exercise testing (CPET) parameters with the diagnosis obtained by pulmonary haemodynamics (94). The cohort studied was enriched with patients with a high suspect of PH (right heart enlargement or elevated PAP by echocardiography, reduced DLCO, elevated NT-proBNP serum levels). In this setting, 27.8% of patients were diagnosed with PAH and 5.8% with postcapillary PH by RHC. Differences in peak VCO_2, AT, nadir $VE/VCO_2$and peak $PETCO_2$ statistically distinguished patients with normal haemodynamics *versus* PAH patients and PH with significant reciprocal correlations to resting pulmonary haemodynamics (mPAP, PVR or transpulmonary pressure gradient - TPG) for peak oxygen consumption (VO_2) and peak $PETCO_2$and linear correlation of nadir VE/VCO_2. Cardiac index and cardiac output showed weaker correlations. By ROC analyses, peakVO_2 reached highest values of all analysed gas exchange parameters with a sensitivity of 87.5% and a specificity of 74.8% at a threshold of 13.8 mL/min/kg, specificity (84.6%) was higher for peak $PETCO_2$but with a lower sensitivity (81.3%) with a

threshold of 31.3 mmHg. A peak VO_2 threshold level of >18.7 mL/min/kg was reached in 38/173 patients (22%) with a negative predictive value of 1.0. In addition, the nadir VE/VCO_2 was most predictive of PAH, as all patients with a nadir VE/VCO_2 >45.5 had PAH (positive predictive value 1.0). The performance of these parameters was further improved in patients with a pulmonary capillary wedge pressure (PAWP) <12 mmHg, a cut-off value considered useful to exclude the possible coexistence of a precapillary component of a left heart disease to the pulmonary vascular reactivity. The performance of a cardiopulmonary exercise test in SSc patients with suspect PAH encourages the addition of this test to the diagnostic algorithm to enrich of the population candidate to RHC.

A condition potentially associated to early vasculopathy and subsequently to PAH and its ominous prognosis is exercise precapillary PH (ePH) although, as suggested by the panel of experts who developed the current guidelines (83), it still needs more evidences to be regarded as a standard definition, and in SSc this is no exception and in fact it represents the disease setting in which exercise haemodynamics have been mostly investigated (95). In a recent study from the University of Zurich (96), the authors reviewed exercise RHC performed in SSc patients and the prognostic value of exercise pulmonary haemodynamics in terms of transplant-free survival. Patients were diagnosed with ePH by mPAP at maximal exercise >30 mmHg with a mPAP/cardiac output >3mmHg·min·L-1, PAWP <20mmHg. This condition was associated to impaired survival even correcting for other known prognostic factors with mPAP increase during exercise (hazard ratio 1.097, 95% CI 1.002–1.200), mPAP increase per watt (hazard ratio 8.131, 95% CI 2.209–29.928) and distensibility index α (hazard ratio 0.100, 95% CI 0.012–0.871) predictors of transplant-free survival. Interestingly in this analysis resting mPAP did not predict survival, confirming that other factors linked to vascular resistance are impacting on the right heart and on prognosis. Characterising SSc-PAH patients from idiopathic PAH in terms of physiology is indeed a strong correlation between the ventricular mass index (VMI) and pulmonary vascular resistance (PVR) over the range of PVR as evaluated by RHC and contemporaneous cardiac magnetic resonance (CMR) as demonstrated in a large cohort of precapillary PH patients followed at the University of Sheffield (97). This feature may contribute to the significant difference in survival between SSc-PAH and IPAH besides epidemiological factors including older age at diagnosis in SSc-PAH. The complementarity of CMR analysis is a precious tool in the setting of PAH and in general in the heart involvement of SSc but unfortunately it is still is a research test for which specific indications and protocols are lacking and it deserves active research in the near future (98).

Vasculopathy of the pulmonary micro-circulation itself is not the only factor influencing the development of PH in SSc. Increasing evidences are pointing out that heart failure with preserved ejection fraction causing PH (SSc-PH-HFpEF) and classified in group 2 by guidelines (83) is a rather frequent condition in SSc and for which much attention must be paid to the off-label use of PAH-specific therapy. Recently, however, Hassoun *et al*. (99) have shown that SSc-PH-HFpEF is associated to poor survival without any difference with SSc-PAH but to a higher risk of death after adjusting for haemodynamics. HFpEF-PH patients showed higher pulmonary and right atrial pressures and, furthermore, they had higher effective arterial elastance (Ea), a measure of total RV afterload *versus* PAH patients and interestingly similar transpulmonary gradients (TPG) which instead should be a discriminatory parameter between the two conditions in ideal cases. The results indicate the polymorphic nature of PH in SSc and shed some light on accurately performing the diagnostic flowchart of guidelines but also looking for clinical, instrumental and laboratory features of its different causes in SSc to specifically target therapeutically where it is most needed (the pulmonary vascular bed, the heart, the lungs or hopefully in the future all of them with SSc disease-modifying anti-rheumatic drugs - DMARDs).

In terms of the interstitial counterpart of lung involvement in SSc (SSc-ILD), recently a multiparametric approach similar to that applied in PH has been tested for the prediction of its development and of patients survival combining clinical data, HRCT scans of the thorax and respiratory function (100). A recent publication of the same group (101) demonstrated that pulmonary function trends at one and two years were predictive of intermediate and long-term mortality but only in the extensive lung involvement cohort. These findings have implications not only for the support of pulmonary function monitoring in SSc-ILD as in idiopathic pulmonary fibrosis but also as an indication for cohort enrichment in SSc ILD clinical trials. A further aid in the prognostic stratification of patients, and in line with the discrimination made possible by the method proposed by Goh (100), is the use of quantitative chest CT available through free software and which is operator-independent. This method is able to distinguish groups of SSc-ILD patients with different mortality as data from a multicentre Italian study have recently shown (102).

One of the most urgent and once lethal complications of SSc is scleroderma renal crisis. As already described (78), this manifestation does not have the impact on disease-specific mortality that it used to have in past decades, but there is still controversy on its rare occurrence and optimal management. Our knowledge on it has been hampered by a lack of consensus on its definition. In a recent review of the literature, Hoa *et al*. (103) described 40 original definitions of SRC from 36 original studies, 9 reviews and 2 editorials and pointed out the need for definitive criteria on which future research can be conducted, both from the pathogenetic and from the therapeutic point of view.

SSc is an extremely heterogeneous disease and the consideration of its different clinical aspects accounts for its complexity and difficult

Table I. Revised EUSTAR activity index (107).

Item	Weight
Dskin	1.5
Digital ulcers	1.5
mRSS>18 or for mRSS up to 18	1.5 score*0.084
Tendon friction rubs	2.25
CRP>1 mg/dl	2.25
DLCO<70% of the predicted value	1

clinical management. Because of this heterogeneity and the mixed presence of activity and damage clinical manifestations at the same time validated biological markers are lacking and difficult to validate (104). The activity index (AI) proposed by the European Scleroderma Study group (EScSG) in 2001 (105) is a composite measure of different areas of involvement in SSc: scores above 3 indicate an active disease. Its predictive validity has been recently assessed in a longitudinal analysis of the Canadian Scleroderma Research Group (CSRG) database (106). The study has considered changing the AI with its course during 10 years of follow-up (area under the curve of EScSG-AI over time) or a persistently active disease/flare or an increase in disease activity (from inactive at baseline to active at the follow-up visit). The adjusted mean of the EScSG-AI was the best predictor of severity of internal organ involvement at the 3-year follow-up visit and also predicted the decline in FVC and DLCO or the progression of visceral disease.

In 2017 the disease activity score has been revised to adapt its features to a different SSc patient population than it was originally formulated on (107). The index has also been validated for the first time. SSc patients enrolled in the study fulfilled the 2013 ACR/EULAR criteria. In the derivation study a list of items significantly associated to disease activity were identified and so it was their weight. ROC analyses could define a score of ≥2.5 as the one with the best match of sensitivity (80%, 64.4–90.9) and specificity (91.2%, 80.7–97.1). Table I lists the proposed preliminary EUSTAR index of disease activity.

Validation of these criteria has been performed on an independent cohort confirming the identification of active/very active disease in patients with an index ≥2.5 with 73.9% sensitivity and 78.3% specificity. Over time the score is subject to change: in the study after a median follow-up of 13 months (6-38) changes in disease activity significantly correlated with those in the Medsger Severity Score (108) albeit the correlation was weak (r=0.33, 0.162–0.479, p=0.0002). Despite limitations, the revised EUSTAR activity index should be included in further studies to improve its performance and acquisition in the routine assessment of SSc patients. The mRSS is a fundamental measure in SSc, in both clinical practice and in trials, especially in the subset of dcSSc (109). Contributors to its deterioration in this setting are well clarified: short disease duration, joint synovitis and low scores at baseline (110). To gain better insight into the change of this parameter and to enrich clinical trial population, a recent analysis of the EUSTAR database has focused instead on its regression in dcSSc (111). The most significant independent predictor of improvement within a year was the baseline mRSS itself and the best cut-off value indicating progression *versus* regression of the skin involvement is 18, which predicts the highest proportion of progressors and the lowest of regressors in the EUSTAR cohort. This cut-off is consistent with that identified in the activity index study (107) and should be regarded as an important indicator of a tighter management in SSc patients at their baseline evaluation. To improve the skin-related evaluation in patients also taking their perspective into account, a patient-reported outcome (PRO) was recently developed (112). For the analysis, 4 different constructs were considered: physical limitation (PL) imposed by skin tightness, physical effects, emotional effects and social effects. The analyses performed included test-retest reliability, internal consistency and exploratory factor analysis (EFA) and the final items that significantly affected the score, called SSPRO, are 18 questions directly approaching the patients on the personal feeling about their skin or the practical effects of the skin involvement in their daily and social life. Interestingly, SSPRO had face, content and construct validity in the study but did not correlate well with physician assessed skin scores (mRSS) in both subsets, indicating the complimentarily added by the evaluation of this PRO, which could aid clinical studies assessing the specific efficacy on skin fibrosis of different study drugs, antifibrotic/immunomodulatory agents.

One of the most validated and used PRO in SSc is the health assessment questionnaire (HAQ), adapted with 5 specific VAS to the scleroderma population, each one of them including a specific focus on disease-related features such as Raynaud's phenomenon (RP), digital ulcers (DUs), gastrointestinal symptoms (GI), respiratory symptoms, as well as the overall severity (SHAQ) (113, 114). Recently, the Desscipher initiative within the EUSTAR database (115) has analysed this prospective cohort for predictors of disability and factors contributing to the impairment. The results indicated that the strongest predictors of functional disability were dyspnea (as NYHA class), gastrointestinal symptoms, fibromyalgia, muscle weakness and the presence of DU in contrast to more objective parameters that generally drive the physician judgment about the severity of the disease. Strikingly, HAQ scores reported in the study are >4 times higher than those reported in the general French population, and in line with other systemic rheumatic diseases. The discordance between patients and physician judgment is a key message of the paper, indicating that even in the best clinical setting that EUSTAR has established in adhering centres, patients needs and their quality of life is still not at the centre of the attention and not targeted by the therapeutic management in SSc.

Treatment of SSc

The treatment of SSc, is targeted against the three processes that are mainly involved in the pathogenesis of the dis-

ease, including microvascular abnormalities, immune system activation and tissue fibrosis (116).

The treatment of immune system activation with immunosuppressive drugs is crucial to target the inflammatory processes involved in SSc pathogenesis. Data regarding synthetic immunosuppressant confirmed the role of cyclophosphamide (CYC) and mycophenolate mofetil (MMF) in the treatment of SSc. CYC represents an anchor drug in the treatment of SSc-related ILD and also recent reports confirm the efficacy of CYC pulse therapy in stabilising lung function in patients (117), with a relatively low percentage of side effects (118). Patients who did not improve after the first six months of CYC pulse therapy probably will not improve also after additional CYC doses (119). After CYC therapy, maintenance treatment with azathioprine may be a good option for SSc-ILD patients (120).

Results from the scleroderma lung study (SLS) I and II suggest that MMF may be an additional option in the treatment of SSc-related ILD (121); although the treatment with MMF is not more effective than CYC (122, 123), MMF may be preferred because of its better safety profile (123). MMF safety is also better compared to azathioprine although gastrointestinal adverse events are common (124). Novel data about predictive factor for the response to the treatment have been published. In particular, the decrease in the circulatory levels of a chemokine, CXCL4, seems to be related to a better prognosis in the progression of ILD during the treatment with MMF and CYC (73). Moreover, plasma levels of plasmin-alpha2-plasmin inhibitor complex (PIC), may predict the efficacy of endovenous CYC in the treatment of ILD.

Data derived from the SLS I and II confirmed the efficacy of both MMF and oral CYC also in skin involvement of patients with SSc compared to placebo (125).

B cells are involved in several pathogenic mechanisms that may responsible for SSc (126, 127), and several recent articles investigated the effect of the treatment with rituximab (RTX), a chimeric monoclonal antibody directed against CD20 + B cells, in the treatment of SSc. Novel data have confirmed that the treatment with RTX is associated to a better outcome in lung function (increase of FVC) and in skin fibrosis compared to conventional treatment with immunosuppressants, without an increase of adverse events (128). Other data reported only a stabilisation of lung function in SSc patients (129). RTX seems to be effective both in early and in long-term disease (130). Despite the effects on skin fibrosis, RTX was not recommended for the treatment of calcinosis cutis (131, 132).

Riociguat is a novel drug, stimulator of soluble guanylate cyclase (sGC). Riociguat is approved for the treatment of pulmonary artery hypertension, pulmonary hypertension and chronic thromboembolic pulmonary hypertension. Recently published data on patients with PAH associated with connective tissue disease confirmed the efficacy of riociguat also in this subgroup of patients with an improvement in the 6-minute walking distance, WHO functional class that persisted at 2 years. Moreover, the safety profile is similar in patients with CTD compared the entire group treated with riociguat (133). Based on these data and on the antifibrotic effect demonstrated in animal models by riociguat, a novel study, RISE-SSc, is investigating the effects of this drug in patients with dc-SSc, particularly in reducing skin fibrosis, improving the mRSS from baseline (134).

Also pirfenidone, a drug approved for idiopathic pulmonary fibrosis may have a potential role in the treatment of SSc-ILD (135), and the treatment appears relatively safe in this subgroup of patients (136).

Vascular manifestations, in particular pulmonary arterial hypertension (PAH) and digital ulcers (DUs), represent one of the most severe involvements in SSc patients. Several drugs are now available to treat vasculopathy in SSc. Several vasoactive drugs are now available for the treatment of SSc and the use of vasoactive drugs increased in the last eight years (137).

Endothelin receptors antagonists (ERA) in SSc patients are used in particular in the treatment of pulmonary arterial hypertension (PAH) and digital ulcers.

Novel data are available about the efficacy of treatment with ambrisentan in improving clinical symptoms and RP in SSc patients (138); however, until now, only bosentan has demonstrated efficacy in preventing DUs in this subgroup of patients (139). Moreover, in a recent trial, treatment with macitentan, a novel endothelin-1 blocker, failed to achieve a significant reduction in SSc digital ulcers compared to placebo (140). The treatment with ERA is not free from adverse events and it may be associated to an increased risk of cardiovascular events, oedema and fluid retention, anaemia, and increased liver enzymes (141).

The treatment with phosphodiesterase type 5 (PDE5) inhibitors has been associated to a reduction of time of healing of SSc-DUs (142). Unfortunately, in a recent placebo controlled trial, the treatment with sildenafil failed to demonstrate a reduction of healing time of DUs; how-ever, the authors found a reduction in the number of DUs in patients treated with sildenafil compared to the placebo group (143).

Although data on treprostinil, a synthetic analogue of prostacyclin, in the treatment of SSc-related DUs are conflicting, a recent study report that patients that discontinue the oral treatment may experience an increasing incidence of DUs (144). Also the treatment with digital treprostinil iontophoresis may improve skin blood flow in the hand during local cooling and rewarming phase (145).

Selexipag, an orally available selective, non-prostanoid prostaglandin I2 receptor agonist, has recently been approved for the treatment of PAH. Moreover, this medication delays the progression of PAH and was well-tolerated among PAH-CTD patients, including those with PAH-SSc and PAH-SLE (146).

Combination therapy

Recent data underlines the importance of a combination therapy in the treatment of vascular disorders in SSc patients.

In particular, the post hoc results of the AMBITION trial, demonstrated a better

outcome, defined as reduced risk of clinical worsening, of patients treated with combination therapy (ambrisentan plus tadalafil) compared to monotherapy (ambrisentan or tadalafil) (147).

Analysing data from a registry of patients with SSc-related PAH, the combination therapy with ERA and PDE5 inhibitor is associated to a better time to clinical worsening compared to the treatment with ERA alone (148).

Also in the treatment of SSc peripheral vasculopathy, the treatment with ERA in combination with other vasoactive drugs is associated to a better response. The treatment with bosentan associated with sildenafil allows a better improvement of Raynaud's phenomenon and nailfold videocapillaroscopy (NVC) compared to the single drugs alone (149). Moreover, also the addition of bosentan to iloprost seems to be associated to an improvement in NVC, with an increase in particular in ramified capillaries that is not observed in patients treated with iloprost alone (150).

Cellular therapy

Cellular-based therapy represents an emerging therapeutic option in patients with SSc. In the last years, several data have been published about autologous haematopoietic stem cell transplantation (AHSCT) in patients with severe dc-SSc. These data were confirmed also by recent reports that showed a prolonged survival, reduction of skin involvement and disease activity in patients treated with AHSCT compared to patients treated with CYC (151). In the case of disease relapse after AHSCT, a second transplantation may be a therapeutic option in selected cases (152). To mobilise peripheral blood stem cells, a treatment scheme with low-dose CYC may be equally effective compared to classic higher dose approaches (153).

Autologous adipose tissue injection has demonstrated good efficacy both in the treatment of hand and face disability in SSc patients. In particular, the treatment may improve the skin sclerosis, movement of fingers and mouth, and reduce hand and facial pain (154, 155). The efficacy of the treatment in hand involvement may last up to 1 year (156).

Ulcers

The treatment of DUs, in addition to pharmacological treatment, is based on local wound care. The treatment of DUs requires debridement to remove devitalised tissue and/or foreign material from a wound. Debridement may be painful and may cause discomfort in patients; the application of local anesthetics, such as lidocaine, may reduce pain and allow a safer and better debridement procedures (157). Also an oral administration of opioid may be useful for optimise wound debridement (158). When classical therapy is insufficient in the treatment of SSc-related DUs, the use of innovative approaches may be helpful. For example, extracorporeal shock wave therapy (ESWT) may be used for decrease the number of DUs and their size, improving also the health related quality of life (159).

Although the pathogenesis of calcinosis cutis in SSc patients has not been defined yet, the presence of this difficult to treat manifestations is associated with high morbidity and disability (160). ESWT may be a promising approach allowing a reduction in pain and calcification size (161). Also, local application of sodium metabisulfite 25% may be a valid alternative in the treatment of calcinosis cutis, allowing a reduction in pain and lesion size (162).

Hand involvement is a serious and disabling concern for patients with SSc (163). At the present time, no pharmacological intervention has demonstrated efficacy in improving the range of motion and reducing digital deformity and joint contracture. A combined approach with occupational therapy and self-administered stretching exercises may improve and maintain hand function, consequently improving the quality of life of patients (164).

Ongoing study

The use of tocilizumab, an interleukin-6 inhibitor, has a pathogenic rational in the treatment of SSc (165, 166). Recently, tocilizumab has been studied in SSc patients in a phase II study; the results of this study are promising in skin and lung involvement, although the differences with placebo in skin thickening were not statistically significant (167). A phase III study is needed to clarify the potential efficacy of tocilizumab in SSc.

To date, several clinical trials on SSc are ongoing. In particular, we would like to mention the study of two new molecules: selexipag in the treatment of Raynaud's phenomenon in SSc patients (168) and abituzumab in the treatment of SSc-related ILD (169).

Conclusion

The use of tocilizumab, an interleukin-6 inhibitor, has a pathogenic rational in the treatment of SSc (165, 166). Recently, tocilizumab has been studied in SSc patients in a phase II study; the results of this study are promising in skin and lung involvement, although the differences with placebo in skin thickening were not statistically significant (167). A phase III study is needed to clarify the potential efficacy of tocilizumab in SSc.

To date, several clinical trials on SSc are ongoing. In particular, we would like to mention the study of two new molecules: selexipag in the treatment of Raynaud's phenomenon in SSc patients (168) and abituzumab in the treatment of SSc-related ILD (169).

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