

Alveolar epithelial-to-mesenchymal transition in scleroderma interstitial lung disease: Technical challenges, available evidence and therapeutic perspectives

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Abstract

The alveolar epithelial-to-mesenchymal transition is the process of transformation of differentiated epithelial cells into mesenchymal-like cells through functional and morphological changes. A partial epithelial-to-mesenchymal transition process can indirectly contribute to lung fibrosis through a paracrine stimulation of the surrounding cells, while a finalized process could also directly enhance the pool of pulmonary fibroblasts and the extracellular matrix deposition. The direct demonstration of alveolar epithelial-to-mesenchymal transition in scleroderma-related interstitial lung disease is challenging due to technical pitfalls and the limited availability of lung tissue samples. Similarly, any inference on epithelial-to-mesenchymal transition occurrence driven from preclinical models should consider the limitations of cell cultures and animal models. Notwithstanding, while the occurrence or the relevance of this phenomenon in scleroderma-related interstitial lung disease have not been directly and conclusively demonstrated until now, pre-clinical and clinical evidence supports the potential role of epithelial-to-mesenchymal transition in the development and progression of lung fibrosis. Evidence consolidation on scleroderma-related interstitial lung disease epithelial-to-mesenchymal transition would pave the way for new therapeutic opportunities to prevent, slow or even reverse lung fibrosis, drawing lessons from current research lines in neoplastic epithelial-to-mesenchymal transition.

Keywords

Scleroderma-related interstitial lung disease, epithelial-to-mesenchymal transition, systemic sclerosis, transitional research.

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Introduction

Systemic sclerosis (SSc) is the condition associated with higher mortality among rheumatic diseases, mainly as a direct or indirect consequence of lung involvement.¹ Scleroderma-related interstitial lung disease (SSc-ILD) is a severe fibrotic and vascular complication characterized by an excessive deposition of extracellular matrix (ECM) that leads to impaired gas exchanges and increased vascular resistance of the pulmonary circulation.

Two main distinct histologic patterns have been recognized in SSc-ILD, namely nonspecific interstitial pneumonia (NSIP) and usual interstitial pneumonia (UIP). The NSIP pattern is associated with more than two-thirds of SSc-ILD patients and is characterized by a variable extent

of alveolar septa thickening, lymphocytic interstitial infiltrate, fibrosis and neo-vascularization.² The UIP pattern is conversely observed in a smaller proportion of patients

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and replicates the abnormalities of idiopathic pulmonary fibrosis (IPF)³ with fibrotic areas mainly organized into foci of fibroblasts, myofibroblasts and a few inflammatory cells adjacent to normal parenchyma.³

Despite these substantial histologic dissimilarities, it is still substantially unknown whether different triggers or biological pathways could lead to distinct parenchymal structural changes. Nevertheless, crucial differences between NSIP and UIP in SSc have not been conclusively recognised in terms of epidemiology, progression rate, treatment response or related mortality.⁴

Multiple sources have been demonstrated or postulated for the hyperactive fibroblast pool involved in SSc-ILD ECM deposition, including activation of quiescent resident tissue fibroblasts, recruitment of circulating fibrocytes or transformation of pericytes, endothelial cells and alveolar epithelial cells (AECs).⁵

The epithelial-to-mesenchymal transition (EMT) is a process of gradual transformation of fully differentiated epithelial cells towards mesenchymal-like cells through functional and morphological changes.⁶ While physiological EMT events take place during organogenesis (type 1), pathological EMT has been linked to abnormal healing processes in fibrosis (type 2) and carcinoma metastatic invasion (type 3).⁷ Of note, the EMT is a gradual, reversible and sometime incomplete process, so a full transformation of epithelial cells into fibroblast or myofibroblast could be often non-finalized. It is crucial to stress the concept of partial activation of an EMT programme because it could explain some conflictual experimental data, without disproving a significant contribution to fibrogenesis and inflammation through the paracrine stimulation of the surrounding cells.⁸

EMT within alveolar epithelium

Distal airways as a predisposing context for EMT

The alveolar microenvironment plays an important role in the induction of EMT. AECs are considered dynamic cytotypes in a strategic interface location that can adapt to physiological and pathological stimuli and reprogram themselves through a set of transcription factors and microRNAs. The functional unit of the lung – the alveolus – is composed by the alveolar epithelium and the alveolar septa, which contain the pulmonary capillaries participating in gas exchange and some connective tissue. The alveolar epithelium layer is made up of two types of AECs. Type 1 AECs are flat cells that regulate gas transport, fluid balance among the alveoli and surfactant production by type 2 AECs through mechanical stimulation. Conversely, type 2 AECs are progenitor cells that differentiate into type 1 AECs, produce surfactant and can also present antigens to immune cells.⁹

This peculiar anatomical organization is functional to gas exchanges and directly exposes AECs to alveolar, interstitial and intravascular environments. These cells are consequently in a close relationship with mesenchymal interstitial cells, endothelial cells and immune cells dwelling in the alveolar and interstitial spaces, most of which are macrophages in both physiological and pathological conditions¹⁰ (Figure 1).

Although type 2 AECs are postulated to be the main responsible for both EMT processes and type AEC differentiation, it is also known that type 1 can differentiate back into type 2 cells suggesting the participation of all epithelial cells in the mesenchymal transition process.¹¹

Methods and caveats of direct alveolar EMT assessment strategies

The easiest and most used method to demonstrate the occurrence of EMT is to report the cellular co-expression of mesenchymal and epithelial markers through western blotting, immunohistochemistry or flow cytometry. Mesenchymal markers include surface molecules such as N-Cadherin, cytoskeletal components such as vimentin, alpha-Smooth Muscle Actin and fibroblast-specific protein 1 and ECM components such as fibronectin or type 1 collagen. Nevertheless, transcription factors such as Snail, Slug, ZEB-1, ZEB-2, Twist and β -catenin could also be tested as markers reflecting the activation of four main interconnected pathways involved in the EMT process, namely canonical and non-canonical transforming growth factor β 1 (TGF- β 1), Wnt-Catenin and Notch signalling.¹²

This approach based on marker co-expression has shown some limitations the first of which is the intention to record a dynamic EMT process through a static biological picture. Furthermore, all these markers are liable to a variable degree of specificity in different types of cells and a simultaneous evaluation of a group of them is, therefore, necessary.¹³ Finally, in the case of a complete EMT, epithelial markers are not expressed anymore, and the epithelial origin of a differentiated fibroblast must be unequivocal also in the more complex in vivo systems.

Single cell RNA sequencing, genetic fate tracking and intravital microscopy are new techniques of real-time EMT assessment that aim to overcome the limitations described above. Single cell RNA sequencing is a genomic approach for the detection and quantitative analysis of messenger RNA molecules in specific populations of cells, including AECs. Single cell RNA sequencing could evaluate the transcriptome as a proxy of the proteome to explore the co-expression of mesenchymal and epithelial markers but still presents some standardization and interpretation challenges¹⁴ and still provides a static description of the cellular processes. Lineage tracing relies on site-specific recombinase-mediated DNA excision to switch the expression of some reporter molecules and allow therefore the

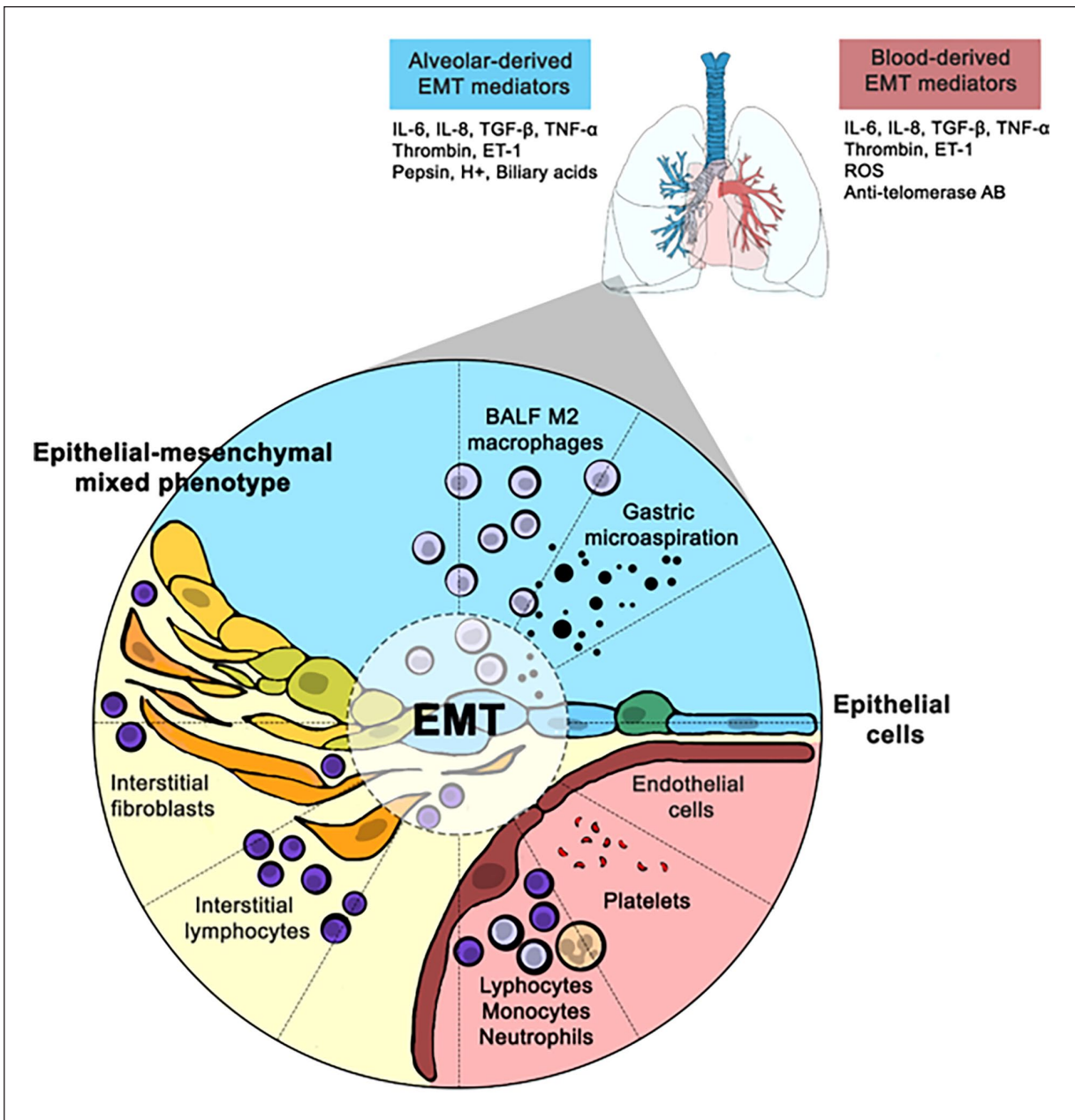


Figure 1. Putative mechanisms of EMT alveolar induction in SSc-ILD patients.

AB: antibody; BALF: bronchoalveolar lavage fluid; EMT: epithelial-to-mesenchymal transition; ET: endothelin; IL: interleukin; ILD: interstitial lung disease; ROS: reactive oxygen species; SSc: systemic sclerosis; TGF- β : transforming growth factor β ; TNF- α : tumour necrosis factor α .

identification of the cellular progeny arising from an individual cell.¹⁵ It remains technically challenging and is prone to criticism about choices, so it sometimes provided conflicting evidence.^{16–18} Intravital microscopy – that is, microscope imaging of live animals – is an increasingly adopted technique that has been mainly applied to EMT evaluation in neoplastic lesions. Of note, this kind of real-time evaluation is thought to be applied to the lungs because of their enclosed position within the body, respiratory movements and physical impact of the heartbeat.¹⁹

Alveolar EMT in SSc-ILD patients and preclinical models

The stronger evidence of EMT occurrence is through the direct analysis of human biopsy lung samples but indirect evidence is commonly derived from cell cultures and animal models of SSc-ILD. Of note, also the bronchoalveolar lavage fluid (BALF) analysis is currently available as a less-invasive tool to directly assess the alveolar environment as a surrogate of lung biopsies.²⁰ Although the BALF

analysis is not able to directly characterize the lung parenchyma, it indeed explores in terms of cellular and acellular components the biological compartment that is in closer contact with AECs.

By the time of this review, EMT has never been assessed though epithelial and mesenchymal marker co-expression in lung samples of patients with SSc as it was previously done in IPF^{16,21} or lung-transplanted patients.²² One of the reasons is that surgical lung biopsy is seldom performed in SSc compared to IPF or transplanted patients since current evidence did not support its routine diagnostic or prognostic use in SSc-ILD. A single RNA-assessment study, including the sequencing of AEC transcriptome, have indicated the simultaneous transcription of mesenchymal and epithelial markers in a specific subpopulation of aberrant basaloid cells in lung biopsies. Although this observation clearly supports an EMT occurrence, it was derived from a small population of patients with UIP pattern and long-stage disease and therefore preclude wider inferences on SSc-ILD pathophysiology.²³

An *in vivo* active pathophysiological contribution of AECs to SSc-ILD is anyway clearly indicated by the increased serum level of AEC-secreted glycoproteins, such as surfactant protein D (SP-D) and Krebs von den Lungen 6 (KL-6),²⁴ and by functional nuclear medicine studies showing that alveolar epithelium permeability is increased in SSc-ILD and predicts a worse prognosis.^{25,26}

Given the mentioned limitations, lung epithelium cell cultures are by far the most diffuse source of biological information on EMT in SSc-ILD.²⁷ As detailed below, inferences on SSc-ILD pathophysiology were driven from experimental conditions that reproduced biological or chemical stimuli that are over-represented in SSc lung²⁸ since direct exposure to patient serum or BALF was never tested until now, and AEC lines are hard to develop from lung biopsies of SSc patients. Of note, many inferences have been driven from immortalized or tumour-derived cell lines because of technical advantages and relatively low costs, although the related results should be cautiously interpreted since EMT is known to be fostered by immortalization and cancer genesis.^{29,30}

In addition to the mentioned *in vitro* studies, bleomycin-induced lung fibrosis is an extensively used and informative animal model of lung fibrosis *in vivo* in which EMT has been proven to occur also using lineage tracking methods.^{17,31,32} Although this model has been applied to both IPF and SSc-ILD evaluation, it is by far closer to the latter. The proximity of bleomycin-induced lung fibrosis to SSc-ILD is related to different reasons. First, the histology pattern shows pulmonary vascular endothelium damage at early stages (in case of intravascular, intraperitoneal or subcutaneous administration), followed by inflammation infiltration and homogeneous secondary fibrotic changes.³³ Second, bleomycin-exposed animals often develop seropositivity to antinuclear antibodies (ANAs),

anti-topoisomerase-I, anti-U1 RNP and anti-histone antibodies.³⁴ Finally, the role of immune response is so striking that bleomycin-induced lung damage can improve after corticosteroid and mycophenolate mofetil administration.^{35,36}

Microvascular dysfunction as EMT-inducer in SSc-ILD

Structural and functional affection of microcirculation are milestones in the pathogenesis of SSc that are known to occur early in the natural history of the disease. These abnormalities are the presumptive consequence of multiple processes such as infections, immune-mediated cytotoxicity, anti-endothelial antibodies,³⁷ or ischaemia-reperfusion³⁸ and are manifested as aberrant vasomotor tone and Nitric Oxide metabolism, intravascular coagulation, leucocyte diapedesis, barrier permeability and possibly a direct contribution to fibrosis via endothelial-to-mesenchymal transition.^{39,40}

A causal relationship between microcirculation abnormalities and pulmonary fibrosis could be driven by the clinical association of SSc-ILD with digital ulcers,⁴¹ capillaroscopy structural changes,⁴² anti-endothelium antibody positivity⁴³ and increased serum markers of endothelial dysfunction such as E-selectin, P-selectin, von Willebrand Factor Antigen and ICAM1.^{44,45} Furthermore, the available BALF characterization data consistently show increased levels of endothelin-1⁴⁶ and thrombin⁴⁷ in SSc-ILD patients. Of note, even if endothelial cells are known to be major players in the synthesis or activation of the above paracrine mediators, these can also derive from epithelial cells, macrophages or mesenchymal cells as a part of a complex cellular crosstalk.^{48,49}

The theory of a mechanistic relationship between microvascular disease and EMT is also supported by the *in vitro* effect of the main mediators of endothelial dysfunction on AECs. Oxidative stress,⁵⁰⁻⁵² NO deficiency,⁵³ endothelin-1⁵⁴ and thrombin⁵⁵ are all demonstrated to be EMT inducers in cellular or animal models of ILD.

Inflammation as EMT-inducer in SSc-ILD

In SSc-ILD, immune activation is a main driver of fibrosis compared to the idiopathic forms as suggested by auto-antibody negativity and the relatively reduced presence of immune cells in IPF lung biopsies.⁵⁶ The role of immune cells is highlighted by the numerous correlations of BALF cellularity with respiratory symptoms, lung imaging, pulmonary function and even mortality in SSc-ILD patients.⁵⁷ The macrophages are the most represented immune cell type in the alveolar space and interstitium, and their circulating precursors are prone to develop an alternative M2 activation profile with pro-fibrotic properties^{58,59} and an expected promotion of EMT.⁶⁰ The specific role of

lymphocytes in the EMT process has been poorly defined despite the known efficacy of mycophenolate mofetil and rituximab in the treatment of this lung complication.⁶¹

In addition to BALF cells, some alveolar cytokines have been reported to be hyper-represented in BALF of SSc-ILD patients and show a correlation of their level with the severity of lung impairment. Of note, alveolar immune cells must be considered only one of the stakeholders involved in cytokine production in response to a plethora of biological, chemical or even mechanical stimuli. Pulmonary mesenchymal, endothelial and epithelial cells are indeed proven to contribute to the definition of the alveolar immune microenvironment in a series of self-sustained paracrine circles.

TGF- β , interleukin (IL)-6, tumour necrosis factor α (TNF- α) and IL-8 are the cytokines more consistently reported to be abnormal and associated with alveolitis in SSc-ILD. TGF- β 1 has been implicated as a pivotal inducer of lung fibrosis with multiple mechanisms, including EMT transition⁶²⁻⁶⁴ and is known to be increased in BALF of SSc-ILD⁶⁵ with immunohistochemical evaluations indicating alveolar macrophages and type 2 AECs as main producers⁶⁶ and to be increased expression of TGF- β target genes in SSc lung.⁶⁷ Similarly, the evidence of IL-6 and TNF- α , and IL-8 hyperexpression in SSc-ILD lung and their ability to induce EMT in pre-clinical models⁶⁸⁻⁷⁰ further support the pathophysiological hypothesis of an immune-induced EMT in SS-ILD.

Micro-aspiration of gastric content as EMT-inducer in SSc-ILD

Gastro-oesophageal disease is an early and highly prevalent organ involvement of impaired distal oesophageal peristalsis, lower oesophageal sphincter pressure, reduced oesophageal acid clearance and gastroesophageal micro-aspiration.⁷¹ Of note, because of the multi-organ nature of SSc, the almost constant association of pulmonary interstitial fibrosis and oesophageal disease is a unicum in human pathology. The severity of ILD has been shown to be associated with the degree of oesophageal involvement so gastric micro-aspiration has been proposed as a key mechanism of the progression of lung fibrosis in SSc.⁷² Both acid and non-acid components of the reflux have been associated with worse pulmonary involvement. These clinical data are interestingly consistent with pre-clinical studies showing that EMT in lung tissue can be determined in animal models where pepsin,⁷³ biliary acid⁷⁴ or acid solution⁷⁵ are delivered to the lungs via aspiration.

Telomere dysfunction as EMT-inducer in SSc-ILD

The telomeres are complexes of DNA repeats and binding proteins that protect eukaryotic chromosomal ends, preserved across cellular divisions by the telomerase

ribonucleoprotein and finally, prevent DNA damage responses and cellular senescence. Telomere dysfunction of AEC2s has been considered a key event in IPF pathogenesis,⁷⁶ but also SSc patients were reported to show shorter age-standardized telomere length compared to healthy controls, especially in the presence of ILD.⁷⁷ Nevertheless, one SSc patient in 10 is known to express autoantibodies that target telomere proteins and are associated with a more severe ILD and shorter telomere length.⁷⁸

In preclinical models, telomere shortening enhances TGF- β -mediated pulmonary fibrosis after bleomycin exposure⁷⁹ but the evidence about a direct pathogenetic connection between EMT and telomeres is still poor and conflicting. The telomerase has been shown to be down-regulated by TGF- β through SNAIL1 in murine mesenchymal staminal cells⁸⁰ but also to promote EMT in lung and oral cancer cells.^{29,81}

Could alveolar EMT be a future therapeutic target in SSc-ILD?

The licenced anti-fibrotic medications, namely nintedanib and pirfenidone, have shown *in vitro* an ability to counteract EMT that could contribute to their therapeutic value together with the anti-proliferative effect towards fibroblast and, possibly, anti-angiogenetic effect. Furthermore, current immunosuppressive medications with an established efficacy in SSc-ILD, – namely cyclophosphamide, mycophenolate mofetil, tocilizumab and rituximab – are certainly able to address the multiple pro-inflammatory signals that have been demonstrated to be potent inducers of EMT. Nevertheless, medications such as azithromycin, atorvastatin and losartan showed anti-EMT effects with a demonstrated or postulated marginal clinical effect on lung fibrosis.⁸²

Current efforts towards the development of drugs that are specifically able to interfere with EMT have been mainly made in cancer research with more than one-hundred drugs under investigation given the known involvement of the process in neoplastic progression. Different strategies have been proposed to counteract the process in different stages, but given the complexity and the interconnections of the involved pathways, it is often hard to distinguish between anti-EMT and anti-proliferative effects. These approaches include blockage of EMT upstream extracellular signals such as TGF- β or Wnt/ β -catenin pathways, targeting EMT-related transcription factors including members of the SNAIL, TWIST and ZEB families, or even targeting the mesenchymal cells themselves by inhibiting the functions of mesenchymal-specific proteins such as vimentin, fibronectin and N-cadherin or their ability to interact with EMC components.⁸³

Accumulating the evidence on the EMT role in SSc-ILD could pave the way for anti-EMT treatment also in this disease with EMT reversing as a specifically attractive

therapeutic option. On the other side, SSc-ILD patients could be exposed to specific risks related to the interference with normal homeostatic processes and in particular negative effects on healing of skin ulcers. Nevertheless, patients and disease stages in which SSc-ILD patients would benefit the most from an anti-EMT therapy should be identified.

Conclusion

While there is an abundance of pre-clinical and clinical evidence to support the role of EMT in SSc-ILD development and progression, two main research priorities should be addressed by the research agenda on the topic.

First, the effective contribution of EMT process to lung fibrosis in SSc should be demonstrated. As reported above, this evidence is quite hard to be derived from animal or cellular models and a direct characterization of SSc-ILD lung tissue is needed. Given the high mortality associated with lung involvement, the use of lung biopsies or BALF collection of selected patients for research purpose could provide definitive evidence on SSc-ILD pathophysiology and should be explored in future research proposals. Of note, both these procedures are already extensively used in IPF and lung-transplanted patients and are now safe and associated with a limited patient discomfort when performed in experienced centres. Nevertheless, considering that routinary resort to invasive procedures is not feasible in clinical practice, the investigation of serum markers of epithelial damage or activation, should be implemented to easily extend the inference driven from lung-derived samples to the largest number of SSc-ILD patients.

The second point of the research agenda should be focused on the efficacy assessment of the available medications against EMT in SSc-ILD patients. The preliminary evaluation of the potentially different role of EMT in different SSc-ILD patients could guide an enriched enrolment in randomized controlled trials based on clinical characteristics and validated EMT markers. Nevertheless, the possibility of SSc-specific adverse events that are less relevant in neoplastic patients should be considered, with a specific reference to digital ulceration and tissue healing.

Data consolidation on SSc-ILD EMT could in conclusion pave the way for new therapeutic opportunities to prevent, slow or even reverse lung fibrosis.

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