Association of fibrotic–related extracellular vesicle microRNAs with lung involvement in systemic sclerosis

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GRAPHICAL ABSTRACT Overview of the study. SSc: systemic sclerosis; ILD: interstitial lung disease; HS: healthy subjects; sEV: small extracellular vesicle; RT–qPCR: quantitative reverse transcriptase PCR; FC: fold change; ECM: extracellular matrix.

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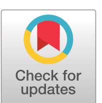

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An sEV–based biomarker approach enabled identification of a promising four–miRNA signature characteristic of ILD in SSc patients, with a strong correlation with disease severity. SSc–ILD sEVs present a profibrotic property which may affect SSc progression. https://bit.ly/4fARNNo

Cite this article as: Guiot J, André B, Potjewijd J, *et al*. Association of fibrotic–related extracellular vesicle microRNAs with lung involvement in systemic sclerosis. *Eur Respir J* 2024; 0: 2400276 [DOI: 10.1183/13993003.00276-2024].

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Received: 8 Feb 2024
Accepted: 17 Nov 2024

Abstract

Background There is a pressing need to identify early biomarkers of lung involvement in systemic sclerosis to start antifibrotic therapy as soon as possible. We aimed to identify extracellular vesicle–derived microRNAs (miRNAs) that are differentially expressed between systemic sclerosis patients with and without interstitial lung disease, and to explore their diagnostic value and functional properties. Q2

Methods Small extracellular vesicles derived from plasma were isolated from 91 well–characterised patients with systemic sclerosis with (n=45) and without (n=46) interstitial lung disease and 43 matched healthy subjects. Small RNA-sequencing followed by quantitative reverse transcriptase PCR were used to identify and validate small extracellular vesicle miRNAs associated with systemic sclerosis–associated interstitial lung disease. Correlations between systemic sclerosis–associated interstitial lung disease miRNAs and clinical parameters were assessed, as well as the effect of related miRNAs/small extracellular vesicles on fibrosis.

Results We identified a four–miRNA signature associated with interstitial lung disease in the context of systemic sclerosis (miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p) (area under the receiver operating characteristic curve=0.85, 95% CI 0.76–0.94, p<0.0001). Deeper analysis revealed a correlation of these candidates with pulmonary function tests (diffusing capacity of the lung for carbon monoxide and forced vital capacity), highlighting their use in monitoring lung fibrosis progression in systemic sclerosis patients. Furthermore, small extracellular vesicle miRNAs associated with systemic sclerosis–associated interstitial lung disease are positively correlated with and enriched in circulating lymphocytes, suggesting that these immune cells are their cellular source. Finally, functional studies highlighted altered functional properties of small extracellular vesicles in the context of systemic sclerosis–associated interstitial disease, mainly due to the transfer of profibrotic miR–584–5p in lung fibroblasts.

Conclusions Our small extracellular vesicle–based biomarker approach identified a promising four–miRNA signature characteristic of interstitial lung disease in systemic sclerosis patients. Furthermore, the profibrotic properties of small extracellular vesicles associated with systemic sclerosis–associated interstitial lung disease suggest a prominent role of these vesicles in systemic sclerosis severity.

Introduction

Systemic sclerosis (SSc) is a heterogeneous autoimmune disease of unknown origin characterised by systemic inflammation and vasculopathy, leading to skin and internal organ fibrosis [1, 2]. The main complication of SSc responsible for one third of its mortality is lung fibrosis, also known as interstitial lung disease (ILD) [3, 4]. SSc-associated ILD (SSc–ILD) shows great diversity and high heterogeneity regarding the extent and types of lung parenchymal abnormalities, as well as the clinical progression rate and outcome [4, 5]. The course of SSc–ILD progression has a wide spectrum, ranging from gradual progressive respiratory failure to rapid deterioration of respiratory function through acute exacerbations [6, 7]. There are no valid biomarkers to predict the occurrence of SSc–ILD. Chest auscultation and pulmonary function tests are important diagnostic tools but lack sensitivity to detect the early phase of the disease. Thus, there is an urgent need to identify specific biomarkers associated with ILD for early SSc–ILD diagnosis and therapeutic intervention.

Recent advances in technologies for extracellular vesicle (EV) analysis have provided new tools to identify EV–related biomarkers for clinical applications [8]. In this regard, microRNAs (miRNAs) derived from small EVs (sEVs) and exosomes are of particular interest. sEVs are nanovesicles (30–200 nm) that are constitutively secreted into biofluids (*e.g.* blood, urine, saliva and sputum) [9] and play a key role in intercellular communication *via* the delivery of their cargo to target cells [10]. These vesicles contain numerous bioactive molecules (*e.g.* RNAs, miRNAs, proteins and lipids) that confer their biological activities. miRNAs are small noncoding RNA molecules (20–22 nucleotides) that modulate gene expression post–transcriptionally by regulating the stability and/or translation of target mRNAs by binding to the 3′ untranslated region of mRNAs [11, 12]. In a pathological context, sEVs can transport dysregulated miRNAs between cells, thereby contributing to miRNA–based signalling alterations, which may play a role in several diseases, including ILD–related diseases [13–15]. Given their deregulation associated with disease progression, sEV–related miRNAs have been investigated as diagnostic and prognostic biomarkers for lung diseases [16–18].

In this study, we aimed to identify specific biomarkers to sort SSc patients at high risk of developing ILD based on their profibrotic profile. For this, we investigated sEV miRNAs that are differentially expressed between well–characterised SSc patients with (n=45) and without (n=46) ILD. Correlations between SSc–ILD–related miRNAs and clinical parameters were assessed, as well as the effect of related miRNAs/sEVs on the fibrotic process.

Materials and methods

Detailed methods on the demographic and clinical characteristics of cohorts, pulmonary function tests, small RNA library (preparation, sequencing and analysis), quantitative reverse–transcriptase PCR (RT–qPCR), nanoparticle tracking analysis, Western blotting, high–density lipoprotein/low–density lipoprotein cholesterol assays, *in silico* analysis, flow cytometry, purification of circulating neutrophils and lymphocytes, cell culture and statistical analysis can be found in the supplementary material.

Demographic and clinical characteristics of cohorts

Patients were required to fulfil 2013 American College of Rheumatology/European Alliance of Associations for Rheumatology classification criteria for SSc [19]. Patients with SSc (with and without ILD) (n=91) were enrolled at University Hospital of Liège (CHU Liège) (Liège, Belgium) and Maastricht University Medical Center (MUMC+) (Maastricht, Netherlands). Patients were divided into two groups according to their high–resolution computed tomography scan to determine the presence of ILD: 45 SSc patients with ILD (SSc–ILD) and 46 SSc patients without ILD (SSc–no ILD). The protocols were approved by the ethics committee of CHU Liège (B707201422832; reference 2014/302), and MUMC+ (NL57351.068.17, reference 172021). All subjects gave written consent before their enrolment.

The first cohort of SSc patients enrolled at CHU Liège (SSc–ILD, n=10; SSc–no ILD, n=10; healthy control subjects (HS), n=10), matched in age and sex, was used to study the effect of lung involvement on the composition of SSc–derived sEVs and to identify new candidate biomarkers for SSc–ILD (*via* small RNA–sequencing (RNA–seq)) (discovery cohort). The second cohort enrolled at CHU Liège and MUMC+ (SSc–ILD, n=35; SSc–no ILD, n=36; HS, n=33) was used to validate the newly identified SSc–ILD biomarkers (*via* RT–qPCR) (validation cohort) (figure 1a). Table 1 provides an overview of the demographic and clinical characteristics of the discovery and validation cohorts.

Plasma processing and sEV isolation

Blood was collected and transferred to EDTA–containing tubes. Plasma was isolated from the blood by centrifugation at 1500 g for 10 min at 4°C to remove blood cells, followed by centrifugation at 2000 g for

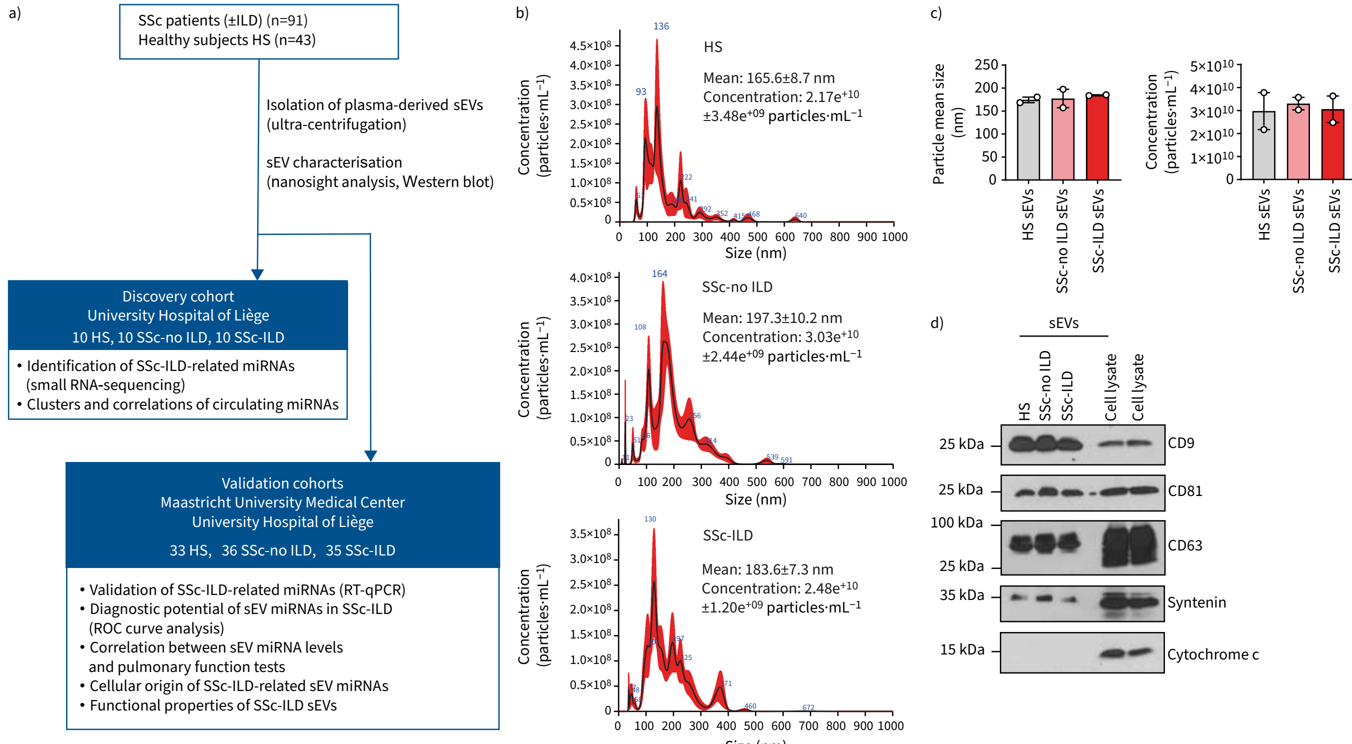

FIGURE 1 Workflow of study design and characterisation of plasma small extracellular vesicle (sEVs). a) Schematic representation of the approach used to identify a microRNA (miRNA) signature for systemic sclerosis (SSc) with interstitial lung disease (ILD). b) Particle size distribution analysis of sEVs isolated from plasma of SSc patients (with/without ILD) and healthy control subjects (HS). c) Quantification of the mean particle size and the particle concentration (particles·mL^{-1}) of indicated EVs (n=2 biological replicates). d) Western blot analysis of exosomal markers (CD9, CD81, CD63, syntenin) and mitochondrial cytochrome c in protein extracts from plasma sEVs of SSc patients (with/without ILD) and HS and from cell lysates. 5 µg of sEV or cell protein were loaded in the Western blot for every sample. RT–qPCR: quantitative reverse transcriptase PCR; ROC: receiver operating characteristic.

15 min at 4°C to remove platelets and cell debris. Then, plasma (2 mL) was resuspended in PBS (25 mL) and precleared by centrifugation at 12 000 g for 45 min at 4°C. The supernatants were passed through a 0.22–µm filter (Millipore). To isolate sEVs, the precleared supernatants of plasma were ultracentrifuged at 110 000 g for 120 min at 4°C, followed by washing of the sEV pellet with PBS at 110 000 g for 120 min at 4°C (Optima XPN–80 Ultracentrifuge + SW32 rotor; Beckman Coulter). The supernatant was discarded, and the sEV pellet was resuspended in PBS or lysed with QIAzol Lysis Reagent (QIAGEN) and stored at −80°C. The protein levels of the sEV preparations were measured using the BCA Protein Assay kit (Pierce) following the manufacturer's instructions. sEVs were characterised by nanoparticle tracking analysis and Western blotting.

Results

Lung involvement in SSc patients affects miRNA expression profile of plasma sEVs

To study the effect of lung involvement on the composition of circulating sEVs in the context of SSc, libraries of small RNAs were generated from plasma sEVs of SSc-ILD patients (n=10), SSc–no ILD patients (n=10) and HS (n=10) matched in age and sex (admitted to the University Hospital of Liège, Belgium) (figure 1a, table 1 and supplementary tables S1–S3). Isolated sEVs were characterised by nanoparticle tracking analysis and Western blotting. Particle analysis showed a predominance of vesicles within 90 and 200 nm, consistent with the size of sEVs (figure 1b, c). No difference was observed in sEV concentration between SSc patients with and without ILD and HS (figure 1c). Standardised clinical chemistry profiling was also performed to assess the presence of lipoprotein particles (*e.g.* high–density lipoprotein/low–density lipoprotein cholesterol) in isolated sEVs (supplementary figure S1). This analysis revealed that sEVs isolated from plasma did not contain detectable levels of lipoprotein particles, which could confound EV functional experiments. Western blotting demonstrated that isolated vesicles presented an enrichment of several exosomal markers, CD9, CD81, CD63 and synthenin, and an absence of mitochondrial cytochrome c, which confirms the purity of our plasma–derived sEV preparations (figure 1d).

TABLE 1 Clinical characteristics of SSc patients (±ILD) and healthy volunteers for whom plasma–derived sEVs were used for small RNA–seq (identification cohort) and for RT–qPCR (validation cohort)

	Identification cohort				Validation cohort			
	HS	SSc-no ILD	SSc-ILD	p-value	HS	SSc-no ILD	SSc-ILD	p-value
Subjects (n)	10	10	10		33	36	35	
Demographic characteristics								
Female	7 (70)	7 (70)	6 (60)	0.86	16 (48.5)	24 (66.6)	20 (57.1)	0.40
Age (years)	52.7 (45.3–57.5)	60.5 (49.5–68.7)	55.5 (48.7–60.5)	0.47	60.0 (53.7–65.0)	60.0 (51–65)	64 (55–72)	0.11
BMI (kg·m^{-2})		22.0 (21–29.5)	26.5 (22–28)	0.82		24.4 (22.0–30.0)	24.0 (21.6–27.0)	0.55
Skin involvement								
Limited cutaneous		8 (80)	6 (60)	0.33		26 (72.2)	22 (62.8)	0.40
Diffuse cutaneous		0	3 (30)	0.06		3 (8.3)	7 (20)	0.15
mRSS		3.5 (0.5–6.7)	6 (2.0–25.0)	0.26		2.0 (2.0–5.5)	5.0 (3.0–17.0)	0.005
Auto-antibodies								
Anti-Scl70		0	5 (50)	0.009		4 (11.1)	10 (28.6)	0.06
Anti-centromeres		4 (40)	0	0.02		18 (50)	1 (2.8)	<0.0001
PFTs								
D_{LCO} (%)	–	58.5 (41.5–72.2)	49.5 (35.2–62.7)	0.25	–	73 (62–87)	51 (43–59)	<0.0001
FVC (% pred)	–	88.5 (67.2–95.0)	80.0 (64.7–94.2)	0.54	–	93.5 (86.2–103.8)	81.5 (67.0–95.2)	0.01
FEV_1 (% pred)	–	83.0 (67.7–94.5)	80.5 (71.0–96.7)	0.79	–	74 (54–102)	69 (59–93)	0.82
FEV_1/FVC (% pred)	–	80.0 (68.0–84.2)	83.5 (80.7–87.0)	0.06	–	74 (68–80)	80 (71–83.5)	0.07
K_{CO} (%)	–	69.0 (59.5–87.5)	68.0 (61.0–78.7)	0.60	–	79.0 (64–86)	67 (60–77.5)	0.02
PAH		2 (20)	1 (10)	0.53		3 (8.3)	4 (11.4)	0.66
Blood cells (%)								
Neutrophils	–	57.9 (55.1–70.4)	72.4 (68.5–77.5)	0.01	–	63.4 (57.5–69)	72.4 (67.1–86.9)	0.0013
Lymphocytes	–	24.3 (20.0–34.4)	19.5 (16.9–22.4)	0.05	–	22.2 (20–28.9)	15.5 (7.7–21.2)	0.0013
Monocytes	–	8.3 (8.1–10.5)	5.2 (4.8–6.2)	0.002	–	8.3 (6.7–9.8)	6.8 (4.9–9.1)	0.23
Eosinophils	–	1.55 (0.9–3.6)	0.9 (0.7–2.9)	0.50	–	3.1 (1.9–3.7)	1.6 (0.2–2.4)	0.01

Data are presented as median (interquartile range) or n (%), unless otherwise indicated. Comparisons between two groups of continuous variables were performed using parametric unpaired two–tailed t–test (for normally distributed data) or non–parametric two–tailed Mann–Whitney test (for not normally distributed data). Significance between more than two groups of continuous variables was assessed using ordinary one–way ANOVA with Tukey's correction (for normally distributed data). Differences in categorical data were compared using chi-squared test. HS: healthy control subject; SSc: systemic sclerosis; ILD: interstitial lung disease; BMI: body mass index; mRSS: modified Rodnan skin score; PFT: pulmonary function test; D_{LCO}: diffusing coefficient of the lung for carbon monoxide; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; K_{CO}: transfer coefficient of the lung for carbon monoxide; PAH: pulmonary arterial hypertension.

A miRNome analysis of plasma sEVs from SSc–ILD, SSc–no ILD and HS revealed that a total of 123 and 43 miRNAs were differentially expressed between SSc–ILD patients and HS (figure 2a–c, supplementary figure S2a and table S4) and between SSc–no ILD patients and HS (figure 2b, supplementary figure S2b and table S5), respectively (both fold–change >1.5, false discovery rate (FDR) <0.1). Interestingly, 28 miRNAs were similarly altered in SSc–ILD and SSc–no ILD patients compared to HS (figure 2b). We then focused our investigation on miRNAs differentially expressed in SSc patients according to lung involvement. A total of 10 miRNAs were differentially expressed between SSc–ILD and SSc–no ILD patients, among which seven were upregulated and three downregulated in the context of ILD (figure 2d and supplementary table S6).

Furthermore, we performed a hierarchical clustering analysis of the most altered miRNAs in SSc–ILD patients compared to HS (fold–change >2, FDR<0.02). As expected, seven out of 10 miRNAs altered in SSc patients according to ILD clustered and correlated together, and with well–known inflammation– and fibrotic–related miRNAs (figure 2e). For example, miR–744–5p clustered and correlated with SSc–ILD–related miRNAs miR–1307–3p (r=0.59, p<0.0001) and miR–146–5p (r=0.39, p<0.0001), with fibrosis–related miRNAs miR–382–5p (r=0.59, p<0.0001) and miR–199a–3p (r=0.45, p<0.0001), and with inflammatory–related miRNAs miR–134–5p (r=0.66, p<0.0001) and miR–432–3p (r=0.56, p<0.0001). These results suggest that SSc–ILD–related miRNAs are strongly associated with inflammation and fibrosis pathways, which are major processes involved in the initiation and progression of lung fibrosis.

Q3

Q4

RT–qPCR validation of sEV miRNAs related to lung involvement in SSc patients

Having identified SSc–ILD–related miRNAs from plasma sEVs, we aimed to validate these candidates in a multicentre cohort study of SSc patients recruited at CHU Liège (SSc–ILD, n=22; SSc–no ILD, n=23) and

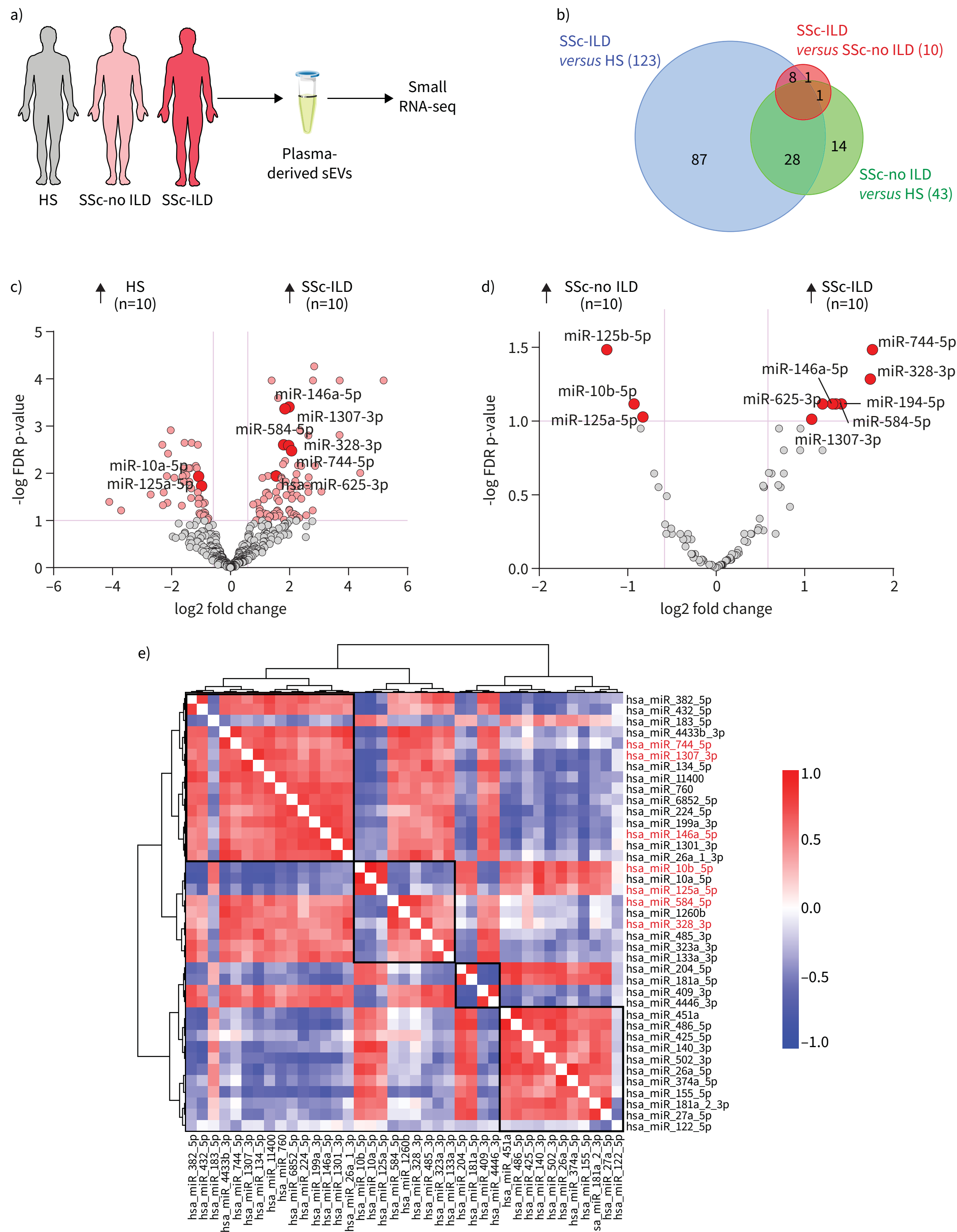

FIGURE 2 Identification of small extracellular vesicle (sEV)–related microRNAs (miRNAs) specific to lung involvement in systemic sclerosis (SSc) patients *via* small RNA-sequencing (RNA–seq). a) Overview of the procedure to identify sEV miRNAs associated with SSc–associated interstitial lung disease (ILD). Small RNA–seq of plasma sEVs from SSc patients with ILD (SSc–ILD, n=10), without ILD (SSc–no ILD, n=10) and healthy control subjects (HS, n=10). b) Venn diagram illustrating the number of shared and distinct miRNAs across the differential expression analysis: SSc–ILD

versus HS, SSc–no ILD *versus* HS and SSc–ILD *versus* SSc–no ILD (fold change >1.5 and false discovery rate (FDR) <0.1). c) Volcano plot showing plasma sEV miRNAs that were differentially expressed between HS (n=10) and SSc–ILD patients (n=10). There were 123 miRNAs differentially expressed in SSc–ILD patients compared to HS (fold change >1.5 and FDR<0.1): 80 upregulated and 43 downregulated. Highlighted are miRNAs that were associated with ILD in SSc patients. d) Volcano plot showing plasma sEV miRNAs that were differentially expressed between SSc–ILD patients (n=10) and SSc-no ILD patients (n=10). There were 10 miRNAs differentially expressed in SSc–ILD patients compared to SSc–no ILD patients (fold change >1.5 and FDR<0.1): seven upregulated and three downregulated. e) Clusters and correlations of sEV miRNAs measured by small RNA–seq in SSc–ILD patients. The heatmap represents a hierarchical cluster analysis conducted upon a Spearman correlation network of miRNA levels in SSc patients that were found to be differentially expressed between SSc–ILD patients and HS (fold change >1.5, FDR<0.02) and for which a critical role in lung fibrosis and inflammation has been shown previously.

at MUMC+ (SSc–ILD, n=13; SSc–no ILD, n=13) (table 1). SSc patients with and without ILD did not differ in age and sex. RT–qPCR was used to measure the relative expression levels of seven miRNA candidates. Having determined that levels of endogenous controls (identified by RNA–seq data) did not vary between SSc patients (with/without ILD) and HS (supplementary figure S3), we used miR–16–5p, miR–191–5p and miR–222–3p to normalise miRNA expression levels.

In agreement with small RNA–seq results, there were higher levels of miR–584–5p, miR–744–5p and miR–1307–3p in plasma sEVs from SSc–ILD patients than from SSc–no ILD patients (FDR<0.01, FDR<0.05, FDR<0.05, respectively), while there was a lower level of miR–10b–5p (FDR<0.01) (figure 3a). miR–625–3p showed pronounced changes in SSc patients in comparison to HS (FDR<0.001), but was not significantly affected by lung involvement in SSc patients (supplementary figure S4).

sEV–related miRNA signature of lung involvement in SSc

To evaluate the diagnostic performance of sEV–related miRNAs for lung involvement in SSc, we performed receiver operating characteristic curve analysis of different combinations of miR–744–5p, miR–584–5p, miR–1307–3p and miR–10b–5p. Binary and triplet miRNA combinations could serve as potential markers to discriminate SSc patients with and without ILD, with miR–10b–5p+miR–584–5p being the best binary signature (area under the curve (AUC)=0.81, 95% CI 0.72–0.91, p<0.0001) and miR–584–5p+miR–10b–5p+miR–744–5p being the best triplet signature (AUC=0.84, 95% CI 0.75–0.93, p<0.0001) (supplementary table S7). The quadruplet miRNA signature (miR–10b–5p+miR–584–5p+miR–744–5p+miR–1307–3p) showed the best performance to distinguish SSc patients with ILD from those without ILD (AUC=0.85, 95% CI 0.76–0.94, p<0.0001) (figure 3b and supplementary table S7). Interestingly, this four–miRNA signature has a similar diagnostic value for detecting lung involvement in SSc patients as diffusing coefficient of the lung for carbon monoxide (D_{LCO}) (AUC=0.86, p<0.0001) (figure 3b), a clinical practice currently used for SSc–ILD diagnosis.

In order to investigate biological processes associated with SSc–ILD–related miRNAs, we submitted miR–744–5p, miR–584–5p, miR–1307–3p and miR–10b–5p to the DIANA miRPath v4.0 tool (http://diana.imis.athena–innovation.gr/DianaTools). The biological processes highlighted in this analysis are in agreement with SSc–ILD pathophysiology, such as apoptosis (FDR=1.4e^{-3}), transforming growth factor β (TGF–β) signalling (FDR=1.2e^{-2}) and epithelial–mesenchymal transition (FDR=1.3e^{-2}) (figure 3c and supplementary table S8).

Correlation between sEV miRNA levels and lung function in SSc patients

Given the alteration of sEV miRNA levels in SSc patients with and without ILD, we assessed the correlation of these miRNAs (miR–744–5p, miR–584–5p, miR–1307–3p and miR–10b–5p) with D_{LCO} and forced vital capacity (FVC), two key pulmonary function tests commonly used to predict the progression of lung involvement in ILD patients. Correlation studies revealed a negative correlation between FVC and miR–584–5p (r= −0.29, p<0.05) and miR–1307–3p (r= −0.28, p<0.05) levels, and a positive correlation between D_{LCO} and miR–10b–5p levels (r=0.33, p<0.01) in SSc patients (figure 4a–d). Interestingly, these correlations were observed in the SSc–no ILD subgroup (figure 4e–h). Indeed, there was a negative correlation between miR–1307–3p levels and FVC (r= −0.42, p<0.05) and a positive correlation between miR–10b–5p levels and D_{LCO} (r=0.40, p<0.05) in the SSc–no ILD subgroup (figure 4e–h). These results show that candidate miRNAs are associated with SSc–ILD progression.

Cellular origin of sEV miRNAs associated with lung involvement in SSc

To investigate cellular sources of deregulated sEV miRNAs in the blood of SSc–ILD patients, we performed correlation analysis of miRNA expression levels with peripheral blood cells, including

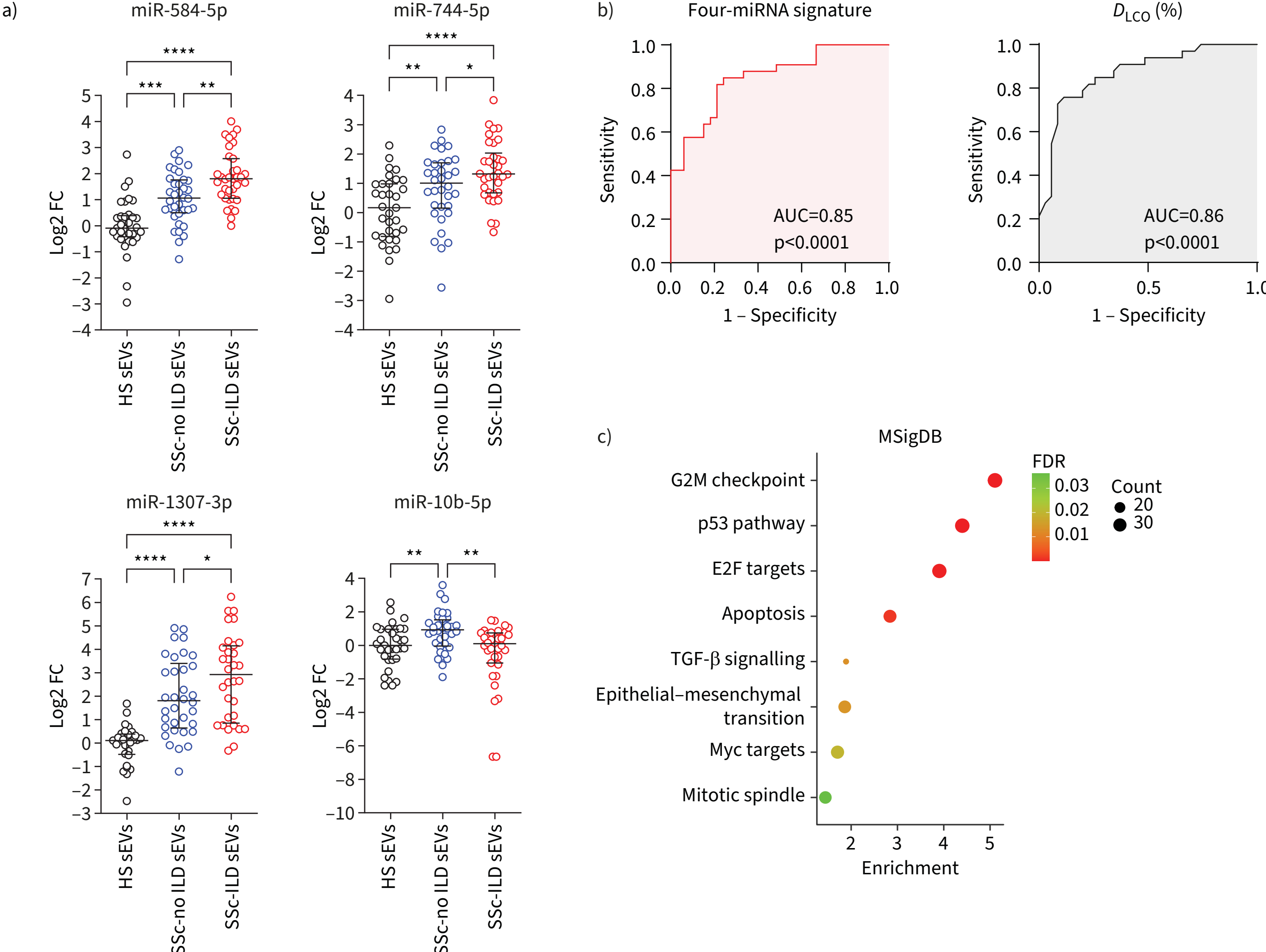

FIGURE 3 Quantitative reverse transcriptase PCR (RT-qPCR) validation of microRNAs (miRNAs) specific to lung involvement in systemic sclerosis (SSc) patients and assessment of their diagnostic value. a) RT–qPCR of specific miRNAs miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p in plasma small extracellular vesicles (sEVs) of SSc patients with interstitial lung disease (ILD) (SSc-ILD, n=35), without ILD (SSc-no ILD, n=36) and healthy control subjects (HS, n=33). Data were non–normally distributed and analysed using a non–parametric Kruskal–Wallis test, followed by Benjamini, Krieger and Yekutieli's false discovery rate (FDR) correction for multiple comparisons. FC: fold change. *: FDR<0.05; **: FDR<0.01; ***: FDR<0.001; ****: FDR<0.0001. b) Receiver operator characteristic (ROC) curves with corresponding area under the curves (AUCs) for comparing the ability of the four–miRNA signature (miR–584–5p+miR–744–5p+miR–1307–3p+miR–10b–5p) and diffusing coefficient of the lungs for carbon monoxide (D_{LCO}) (%) to discriminate SSc-ILD patients (n=35) from SSc–no ILD patients (n=36). c) Molecular Signatures Database (MSigDB) hallmark targeted by SSc–ILD–related miRNAs (miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p). *In silico* analysis was performed with the DIANA–miRPath v4.0 tool using Tarbase v.8. TGF–β: transforming growth factor β.

neutrophils and lymphocytes, which are associated with pulmonary function impairment and fibrotic manifestations of SSc [20, 21]. This analysis revealed that miR–744–5p levels were positively correlated with circulating lymphocytes (r=0.55, p=0.023) and monocytes (r=0.54, p=0.026), and negatively correlated with circulating neutrophils (r= −0.58, p=0.014) in the SSc–ILD subgroup (figure 5a–e), suggesting that lymphocytes and monocytes are responsible of the elevation of miR–744–5p levels in plasma sEVs of SSc–ILD patients.

To confirm this observation, circulating neutrophils and lymphocytes were isolated from the blood of HS, and the levels of SSc–ILD–related miRNAs were assessed (figure 5f, g). As expected, circulating lymphocytes were enriched with miR–584–5p and miR–744–5p compared with neutrophils (figure 5h). Furthermore, an analysis of tissue–specific miRNA expression with the miRNA Tissue Atlas tool suggested that the main source of miR–10b–5p is the kidney (supplementary figure S5). **Q5**

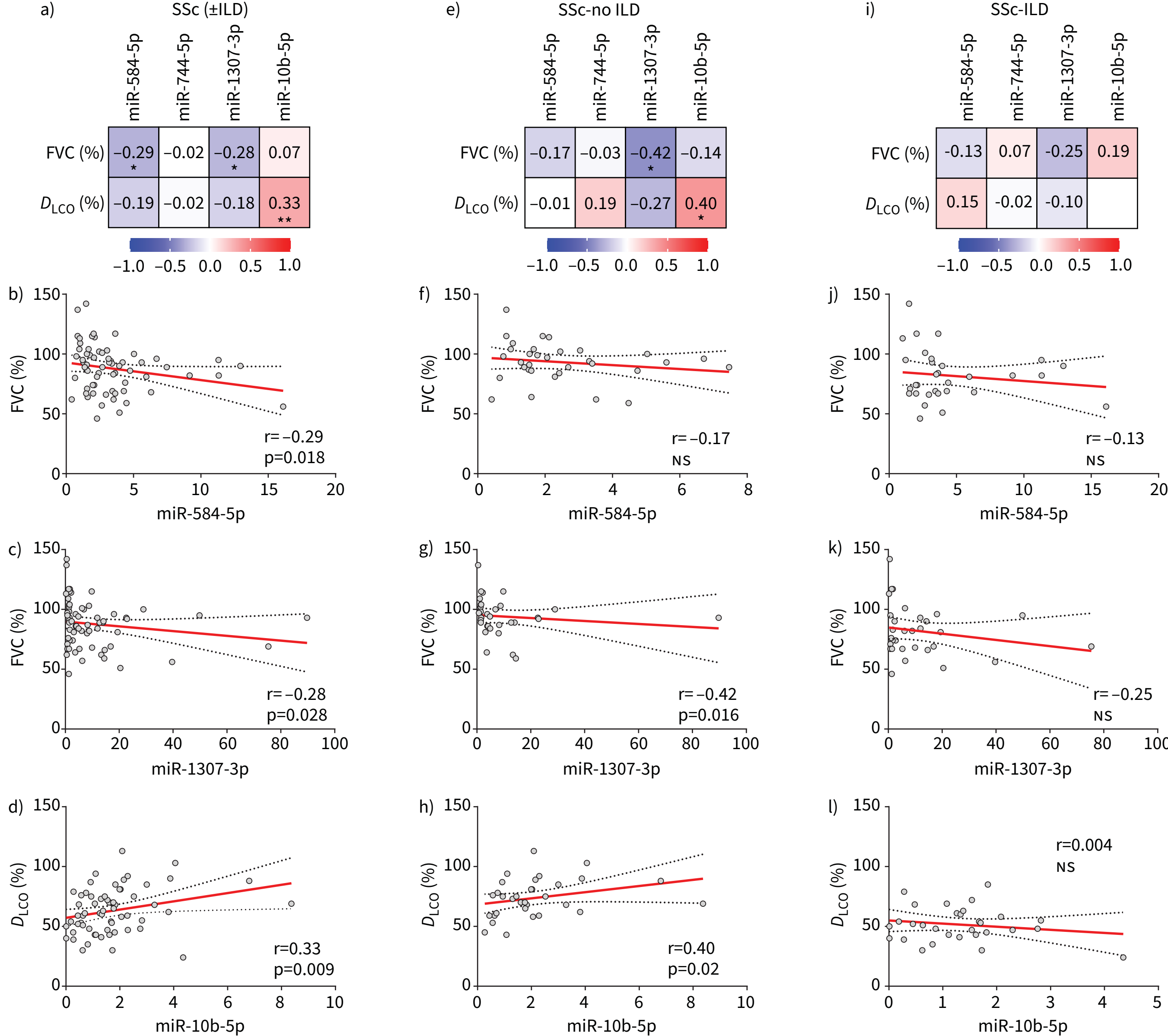

FIGURE 4 Correlation analysis of small extracellular vesicle (sEV)–related microRNAs (miRNAs) and lung function in systemic sclerosis (SSc) patients. a, e, i) Heatmaps showing correlations between lung function (assessed by forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (D_{LCO})) and the levels of SSc-associated interstitial lung disease (ILD)–related miRNAs (miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p) in a) all SSc patients, e) SSc patients without ILD (SSc–no ILD) and i) SSc patients with ILD (SSc–ILD). b, f, j) Correlation between FVC and the levels of sEV miR–584–5p in b) all SSc patients, f) SSc–no ILD patients and j) SSc–ILD patients. c, g, k) Correlation between FVC and the levels of sEV miR–1307–3p in c) all SSc patients, g) SSc–no ILD patients and k) SSc–ILD patients. d, h, l) Correlation between D_{LCO} and the levels of sEV miR–10b–5p in d) all SSc patients, h) SSc–no ILD patients and l) SSc–ILD patients. Data were non–normally distributed and analysed using Spearman correlation (two–tailed). *: p<0.05; **: p<0.01.

Taken together, these results suggest that circulating lymphocytes are a cellular source of high levels of sEV miR–744–5p and miR–584–5p in the context SSc–ILD, and the kidney is responsible for the deregulation of sEV miR–10b–5p levels.

SSc–ILD–related sEVs transfer miR–584–5p and miR–744–5p to recipient lung fibroblasts

Based on our observations so far, we postulated that the packaging of specific miRNAs in SSc–ILD–related sEVs might affect their biological properties, particularly on fibrosis. First, we tested whether SSc–ILD sEVs were able to transfer miRNA cargo in target cells. For this, MRC–5 lung fibroblasts were

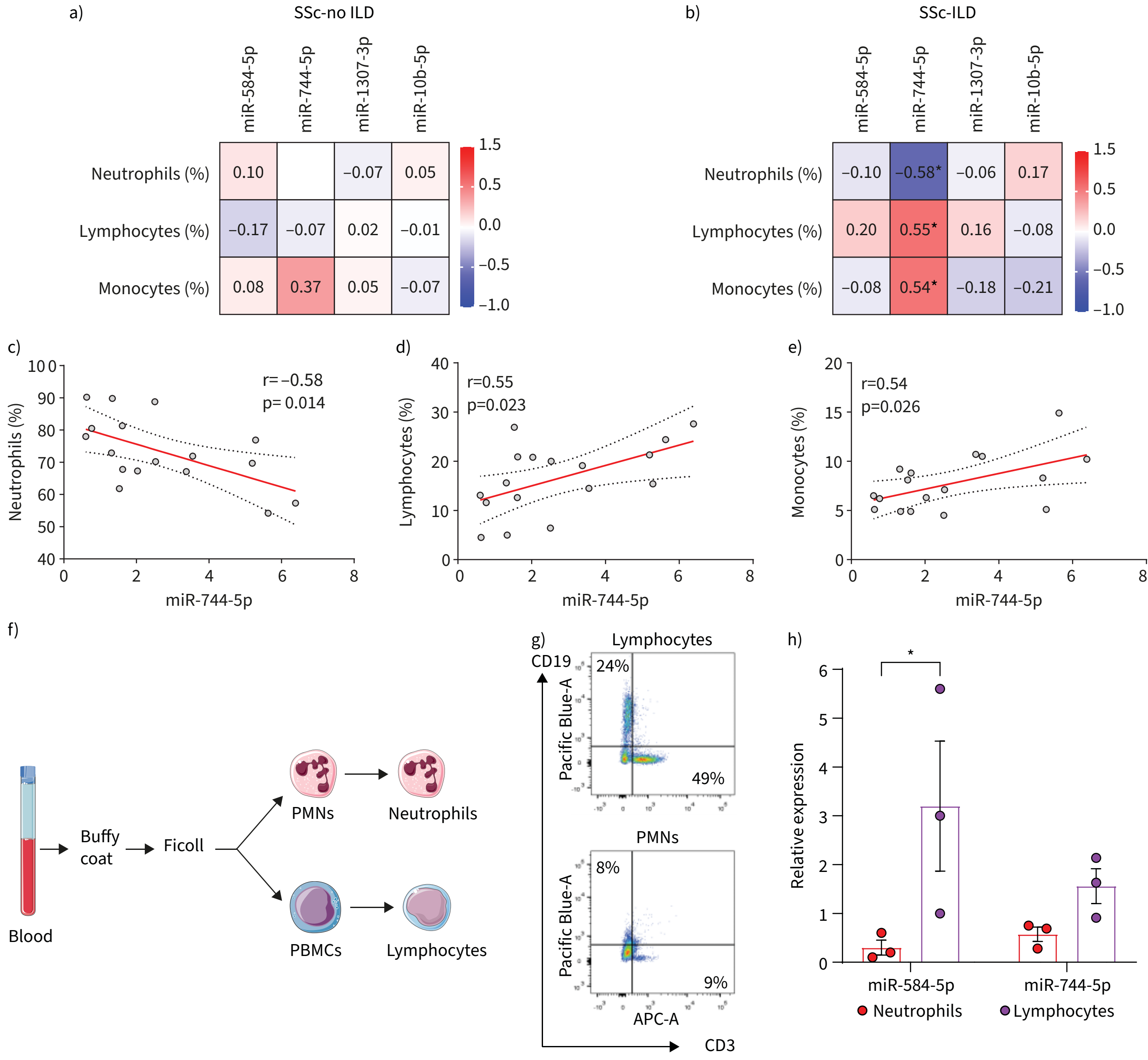

FIGURE 5 Cellular origin of small extracellular vesicle (sEV) microRNAs (miRNAs) associated with lung involvement in systemic sclerosis (SSc) patients. a,b) Heatmaps showing correlations of SSc-associated interstitial lung disease (ILD)–related miRNAs (miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p) with plasma–derived cells (neutrophils, lymphocytes and monocytes) in a) SSc patients without ILD (SSc–no ILD) and b) SSc–ILD patients. Data were normally distributed and analysed using Pearson correlation (two–tailed). c–e) Correlation between plasma levels of sEV miR–744–5p and c) circulating neutrophils, d) lymphocytes and e) monocytes in SSc–ILD patients. f) Schematic representation of the purification of circulating neutrophils and lymphocytes from buffy coats of healthy control subjects (HS). g) Purity was evaluated by flow cytometry after staining of lymphocytic fraction and polymorphonuclear leukocyte (PMN) fraction with anti–CD3 allophycocyanin (APC) and anti–CD19 Brilliant Violet 421 (BV421). h) Expression level of miR–584–5p and miR–744–5p in circulating neutrophils and lymphocytes purified from buffy coats of HS. Data were analysed using ordinary two–way ANOVA with Sidak's correction. PBMCs: peripheral blood mononuclear cells. *: p<0.05.

Q7
Q8

incubated with plasma sEVs derived from SSc–ILD (n=8) or SSc–no ILD (n=9) patients, and from HS (n=12) for 24 h (figure 6a). Incubation with SSc–ILD sEVs significantly increased the levels of miR–584–5p and miR–744–5p in MRC–5 cells (p<0.05 for both compared to SSc–no ILD sEVs) (figure 6b). Importantly, the levels of these miRNAs rapidly increased in MRC–5 cells treated with SSc–ILD sEVs, but the precursors pre–miR–584 and pre–miR–744 transcripts were not induced, suggesting a lack of

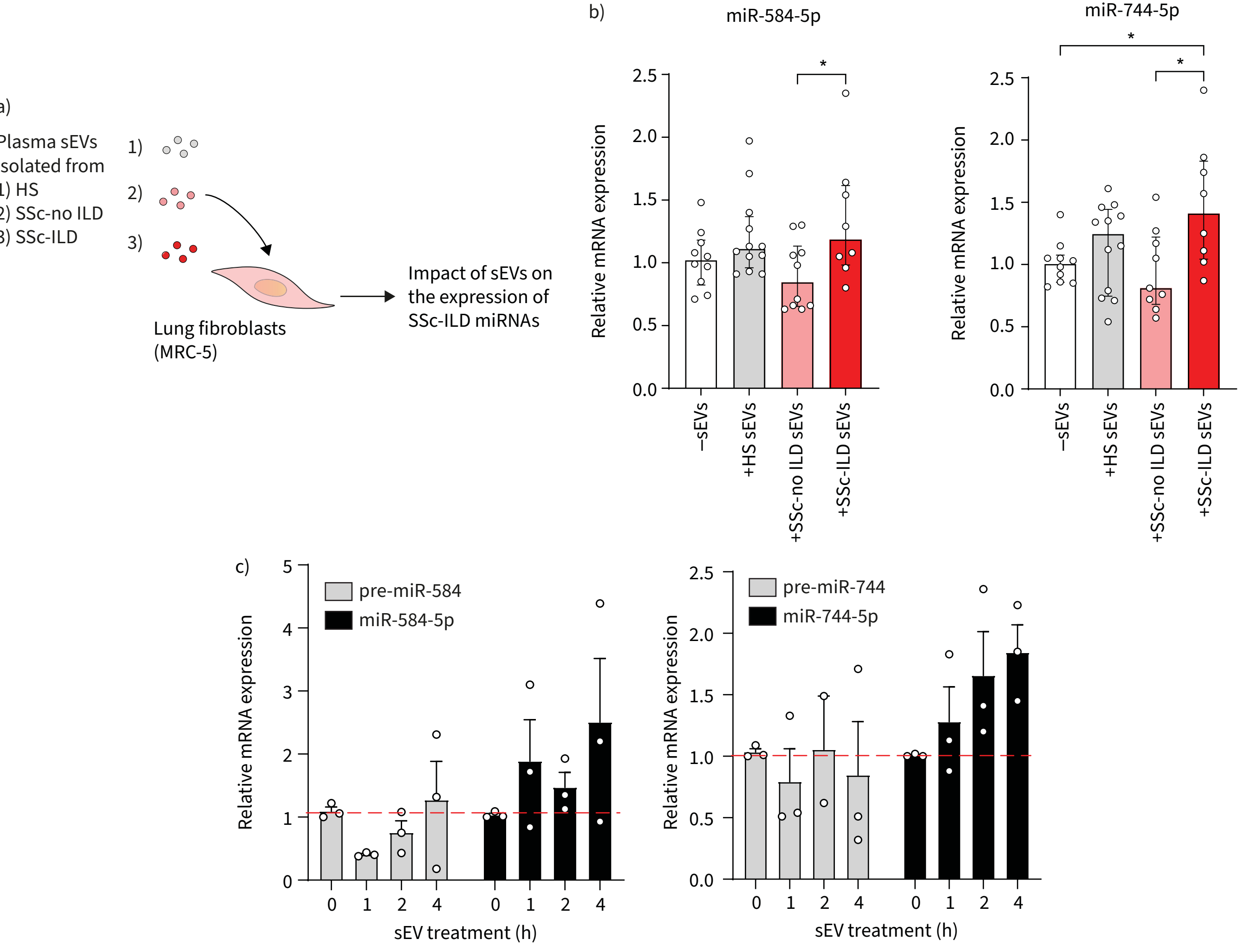

FIGURE 6 Plasma small extracellular vesicle (sEVs) from patients with systemic sclerosis (SSc) with interstitial lung disease (ILD) are able to transfer miR–584–5p and miR–744–5p to recipient lung fibroblasts. a) Schematic representation of the treatment of lung fibroblast cell line MRC–5 with plasma sEVs (5 µg·mL^{-1}) derived from 1) healthy control subjects (HS), 2) SSc patients without ILD (SSc–no ILD) or 3) SSc–ILD patients for 24 h. b) Transcript levels of SSc–ILD–related microRNAs (miRNAs) (miR–584–5p and miR–744–5p) in lung fibroblast cell line MRC–5 treated with corresponding sEVs (5 µg·mL^{-1}) for 24 h assessed by quantitative reverse transcriptase PCR (HS, n=12; SSc–no ILD, n=10; SSc–ILD, n=8). Data are expressed as median±IQR and were analysed using ordinary one–way ANOVA with Tukey's correction (normally distributed data). *: p<0.05. c) Kinetics of the expression of mature miR–584–5p and miR–744–5p and their precursors (pre–miR–584 and pre–miR–744, respectively) after treatment of MRC–5 cells with SSc–ILD sEVs (5 µg·mL^{-1}) (n=3).

Q9

transcriptional induction (figure 6c). Taken together, these data provide evidence that miR–584–5p and miR–744–5p are directly transferred from SSc–ILD sEVs to target cells.

miR–584–5p induces a profibrotic phenotype in lung fibroblasts

We next sought to investigate the biological effect of SSc–ILD–related miRNAs on fibrotic response. For this, miR–584–5p and miR–744–5p were overexpressed by mimic transfection in MRC–5 lung fibroblasts and their effect on the expression of profibrotic genes was assessed. The overexpression of miR–584–5p increased the induction of profibrotic genes by TGF–β stimulation in MRC–5 cells (fibronectin (*FN*), p<0.01; actin α2 smooth muscle (*ACTA2*), p=0.055; collagen type I α1 (*COL1A1*), p<0.01; and collagen type III α1 (*COL3A1*), p<0.01) (figure 7a). The upregulation of FN by miR–584–5p was confirmed at the protein level by fluorescence-activated cell sorting (figure 7e). Furthermore, the reduction of miR–584–5p in MRC–5 cells was able to repress the expression of profibrotic genes in the presence of TGF–β (*FN*, p<0.05; *ACTA2*, p<0.05) (figure 7b). MiR–744–5p did not affect the expression of profibrotic genes in MRC–5 fibroblasts (supplementary figure S6). These data highlight the profibrotic property of miR–584–5p in lung fibroblasts.

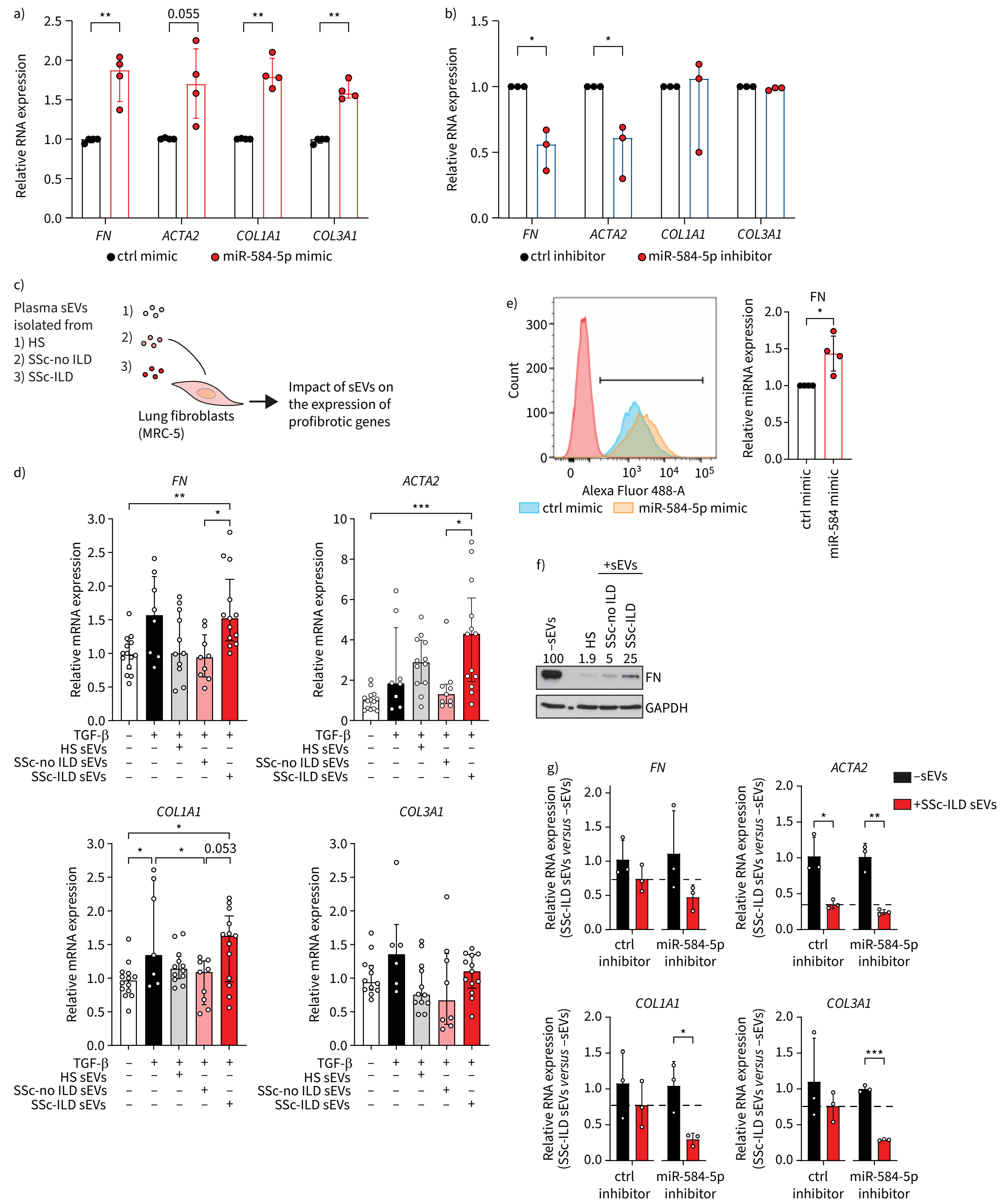

FIGURE 7 Plasma small extracellular vesicle (sEVs) from patients with systemic sclerosis (SSc) with interstitial lung disease (ILD) have miR–584–5p–dependent profibrotic properties. a) miR–584–5p overexpression increases the expression of profibrotic genes (*FN*, *ACTA2*, *COL1A1* and *COL3A1*) in transforming growth factor β1 (TGF–β1)–treated MRC–5 cells (n=4), as assessed by quantitative reverse transcriptase PCR (qRT–PCR). b) miR–584–5p inhibition decreases the expression of profibrotic genes (*FN* and *ACTA2*) in TGF–β1–treated MRC–5 cells (n=3), as assessed by qRT–PCR. Data are expressed as median±IQR and were analysed using multiple paired t–test (a and b). c) Schematic representation of the treatment of lung

fibroblasts (MRC–5) with plasma sEVs (5 $\mu g \cdot mL^{-1}$) derived from 1) healthy control subjects (HS), 2) SSc patients without ILD (SSc–no ILD) or 3) SSc–ILD patients for 24 h, followed by TGF–β stimulation for 4 h. d) Plasma sEVs from SSc–ILD increased the expression of profibrotic genes (*FN*, *ACTA2*, *COL1A1* and *COL3A1*) in MRC–5 cells compared to the ones from SSc–no ILD, as assessed by qRT–PCR (HS, n=12; SSc–no ILD, n=9; SSc–ILD, n=13). Data are expressed as median±IQR and were analysed using ordinary one–way ANOVA with Tukey's correction (for normally distributed data) or Kruskal–Wallis test with Dunn's correction (for non–normally distributed data). e) miR–584–5p overexpression increases the protein expression of fibronectin (FN) in TGF–β1–treated MRC–5 cells (n=3), as assessed by flow cytometry. f) Plasma sEVs from SSc–ILD increase the protein expression of FN in MRC–5 cells compared to those from SSc–no ILD, as assessed by Western blot. g) Inhibiting miR–584–5p in MRC–5 cells restores the sEV-dependent regulation of profibrotic genes in treated cells. Data are expressed relative to cells without sEV treatment (n=3). Data are expressed as mean±SD and were analysed using multiple unpaired t–test. All statistical analyses were two–tailed. ctrl: control. *: p<0.05; **: p<0.01; ***: p<0.001.

Q10

Plasma sEVs have altered functional properties in SSc–ILD

Because SSc–ILD–derived sEVs are able to transfer profibrotic miR–584–5p to target cells, we anticipated that these sEVs would induce a profibrotic phenotype in lung fibroblasts. To test this, MRC–5 cells were incubated with plasma sEVs from SSc patients with (n=13) or without (n=9) ILD and from HS (n=12) for 24 h, followed by TGF–β stimulation for 4 h (figure 7c). While sEVs from HS and SSc–no ILD patients were able to decrease the expression of profibrotic genes (*FN*, *COL1A1* and *COL3A1*) in the presence of TGF–β, SSc–ILD sEVs lost this modulatory effect (figure 7d). Indeed, expression levels of profibrotic markers were higher in SSc–ILD sEV–treated fibroblasts than in ones treated with sEVs from HS and SSc–no ILD (*FN*, p<0.05; *ACTA2*, p<0.05; *COL1A1*, p=0.053; compared to SSc–no ILD sEVs) (figure 7d). This observation was confirmed at the protein level, with a partial loss of the modulatory effect of sEVs on FN expression in the context of SSc–ILD (figure 7f). These results clearly show that plasma sEVs lose their modulatory effect against fibrosis in the context of SSc–ILD.

To further assess the role of miR–584–5p in the fibrotic property of SSc–ILD sEVs, we inhibited miR–584–5p in MRC–5 cells prior to treating them with corresponding sEVs. While SSc–ILD sEVs had a moderate effect on the expression of profibrotic genes in control inhibitor–treated MRC–5 cells, these sEVs were able to strongly repress the expression of these genes in miR–584–5p–inhibited MRC–5 cells (figure 7g). This result demonstrates that profibrotic miR–584–5p contributes to the fibrotic property of SSc–ILD sEVs.

Discussion

There is a pressing need to identify early biomarkers of lung involvement in SSc patients to propose the best treatment at the earliest stage and to slow down disease progression. In our present study, among three independent cohorts of SSc patients we found that the expression of four sEV miRNAs (miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p) constitutes a molecular signature significantly associated with ILD. Using small RNA–seq, we identified sEV miRNAs that differed in SSc patients with lung involvement. Several of these miRNAs have previously been associated with other autoimmune connective tissue diseases (CTDs) and lung fibrosis. Using RT–qPCR, we determined the miRNA signature showing the best performance to distinguish SSc patients with ILD from SSc patients without ILD. Interestingly, this four–miRNA signature had a similar diagnostic value for detecting lung involvement in SSc patients as D_{LCO}, a clinical practice currently used for SSc–ILD diagnosis.

The hallmarks of SSc are autoimmunity and inflammation, widespread vasculopathy and progressive fibrosis of the skin and multiple internal organs [22]. In line with this, our hierarchical clustering analysis of the expression of altered miRNAs in SSc–ILD identified several miRNA patterns related to inflammatory and fibrosis processes. One of these miRNA patterns presented a strong correlation of miR–744–5p or miR–1307–3p with inflammatory–related miRNAs, such as miR–146–5p, miR–224–5p and miR–155–5p, which likely reflects the proinflammatory state in SSc. A study by Vreca *et al*. [23] showed that miR–146a–5p may be a predisposing factor for the development of lung fibrosis and a more progressive form of SSc. Another study reported that elevated expression of miR–146–5p in skin samples may be correlated with the occurrence of vascular abnormalities in SSc [24]. miR–155–5p has been identified as a critical regulator of immune responses and it is involved in various CTDs, including SSc, rheumatoid arthritis and systemic lupus erythematosus [25, 26]. Expression of miR–155 is higher in peripheral blood mononuclear cells and skin lesions of SSc patients than in those of HS [27], and its serum level is highly associated with SSc–ILD progression [28, 29]. In addition, the strong correlation of SSc–ILD miRNAs with several actors of lung fibrosis (such as miR–122–5p [30, 31], miR–26a–5p [32] and miR–155–5p [33]) suggests a fundamental role of sEV miRNAs in the initiation and progression of ILD in SSc patients. We have previously identified an miRNA signature associated with idiopathic

pulmonary fibrosis [16]. Several idiopathic pulmonary fibrosis–related miRNAs are deregulated in SSc–ILD. Four fibrotic–related miRNAs, miR–33a–5p, miR–29b–3p, miR–30a–5p and miR–142–3p, are dysregulated in both pathological contexts.

We validated the alteration of the levels of four sEV miRNAs in SSc–ILD: miR–584–5p, miR–744–5p, miR–1307–3p and miR–10b–5p. Combining the miRNAs of interest into biomarker signatures enabled us to identify a four–miRNA signature presenting the best diagnostic performance for lung involvement in SSc. These candidates correlated with two risk factors for the progression and mortality of SSc–ILD, D_{LCO} and FVC [5, 7], highlighting their capacity to monitor the progression of lung fibrosis in SSc patients.

One of the major questions in this study is the cellular sources of sEV miRNAs associated with SSc–ILD. Our data suggest the cellular source of these sEV miRNAs is circulating lymphocytes, which play a critical role in systemic autoimmunity and SSc progression through various functions, such as cytokine production, lymphoid organogenesis and the induction of other immune cell activation in addition to autoantibody production [34, 35]. Indeed, our data showed that miR–744–5p is positively correlated with and enriched in circulating lymphocytes. In accordance with this, it has been found that miR–155–5p and miR–146a–5p, two SSc–ILD–associated miRNAs, are upregulated in lymphocytes from patients with rheumatoid arthritis [36, 37]. In addition, several studies have reported an alteration of miR–10b–5p levels in T lymphocytes in autoimmune CTDs [38, 39]. There is evidence that the kidneys may be another source of miR–10b–5p in the circulation [40, 41]. miR–10b–5p has also been implicated in the development of various fibrotic diseases, including renal fibrosis [40, 42]. Given that the main source of miR–10b–5p is the kidneys, the positive association between sEV miR–10b–5p and D_{LCO} is likely a consequence of the same pathological mechanism that leads to their decrease in blood (inflammation/fibrosis of the kidneys) rather than a functional relationship with the lung.

In silico analysis revealed that these miRNAs target cellular processes associated with SSc–ILD pathophysiology, such as apoptosis and cellular senescence [43–45]. In accordance with this, recent studies have demonstrated that miR–584–5p and miR–744–5p promote apoptosis [46–48] and senescence [49]. Furthermore, several studies have reported an alteration of these miRNAs in inflammatory diseases and their impact on the regulation of inflammatory pathways [50]. Indeed, miR–584–5p can regulate the expression of *RAB23*, thereby influencing the development of the pulmonary inflammatory response in lung adenocarcinoma [51]. A study by Pilson *et al.* [52] revealed a key role for miR–744–5p in mediating ocular inflammation in primary Sjogren's syndrome by targeting Pellino3. In another study, Zhang *et al.* **Q6** showed that miR–744–5p regulates the type I interferon signalling pathway in renal inflammation. Alteration of miR–10b–5p levels has been associated with autoimmune CTDs. A study by Tu *et al.* [38] showed that elevated levels of miR–10b–5p in rheumatoid arthritis patients promotes disease progression by disrupting the balance between subsets of $CD4^{+}$ T lymphocytes. In addition, miR–10b–5p is elevated in T lymphocytes from patients with systemic lupus erythematosus and promotes T–cell hyperactivity by targeting serine/arginine-rich splicing factor 1 [39]. Given that SSc–ILD–related miRNAs are associated with inflammatory diseases, this suggests a prominent role of related sEVs on SSc progression.

Finally, we investigated the impact of sEVs on the progression of lung fibrosis in SSc patients. We observed differential fibrotic properties of plasma sEVs from SSc patients according to disease severity, with a loss of the modulatory effect against fibrosis in SSc–ILD. To our knowledge, this is the first study highlighting an alteration of the fibrotic property of sEVs associated with lung involvement in SSc disease. This observation is in line with the work of Wermuth *et al.* [53] who showed that exosomes purified from SSc patients induced a profibrotic phenotype in cultured normal dermal fibroblasts, with a greater induction of profibrotic gene expression with exosomes from patients with diffuse SSc compared to those from patients with limited SSc. Interestingly, they showed that SSc–derived exosomes have an altered profile of fibrotic miRNAs, and several of them have been identified in our study, such as miR–125b–5p, miR–146a–5p, miR–155–5p and miR–133a–3p. A similar study by Nakamura *et al.* [54] investigated the effect of SSc serum exosomes on gene expression patterns of normal dermal fibroblasts, and showed that these vesicles were able to increase the expression of mRNA levels of *COL1A1* and *COL1A2*. A study by Leleu *et al.* [55] showed that another type of EV, microparticles, present greater profibrotic potential associated with the clinical parameters of SSc patients, in particular vasculopathy and lung involvement. A recent study by Corallo *et al.* [56] demonstrated that SSc–derived exosomes contain specific profibrotic miRNA signatures that can induce myofibroblast differentiation *in vitro*. In our study, we found that SSc–ILD sEVs have profibrotic properties due in part to the delivery of miR–584–5p to target cells. Previously, this miRNA has been associated with the pulmonary inflammatory response [51], but this is the first study demonstrating its profibrotic property. Further investigations need to be performed to assess the effect of sEV miR–584–5p on lung fibrosis (*e.g.* bleomycin model).

A limitation of our study is the use of a lung fibroblast cell line (MRC–5) instead of primary lung fibroblasts to perform functional experiments. However, previous studies have shown that the MRC–5 cell is a good model to study the impact of miRNA/EVs on lung fibrosis process *in vitro* [10]. Another limitation is the use of a constant amount of sEV proteins instead of a set sEV number as vesicle normalisation for functional experiments.

In summary, our sEV–based biomarker approach identified a promising four–miRNA signature characteristic of ILD in SSc patients, which correlated with clinical parameters (D_{LCO}, FVC, circulating monocytes/lymphocytes). In addition, our results suggest that SSc–ILD–derived sEVs might play a crucial role in disease progression *via* the delivery of profibrotic miR–584–5p to target cells. The biomarker status of this four–miRNA signature needs to be validated in large independent cohorts. Further studies are needed to determine whether this four–miRNA signature has the capacity to aid in prognosis assessment by monitoring lung involvement in SSc, which could help therapeutic decision–making.

Acknowledgements: We are grateful for the support of the technology platforms at the GIGA Research Center (University of Liège). We would like to thank Géraldine Piel from the Laboratory of Pharmaceutical Technology and Biopharmacy (University of Liege) for the use of the NanoSight. Figure 5f and the graphical abstract were drawn in part using images from Servier Medical Art. Thanks are due to all co–authors for their contribution.

Data availability: The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found in the ArrayExpress database under accession number E–MTAB–14290.

Ethics statement: All subjects gave written consent before their enrolment. The protocols were approved by the ethics committee of CHU of Liège (B707201422832, ref: 2014/302) and MUMC+ (NL57351.068.17, reference 172021).

Author contributions: B. André, J. Potjewijd, P. Jacquerie, R. Tobal and F. Gester contributed to participant recruitment, sample collection and clinical data collection. M. Henket and L. Giltay contributed to sample processing and clinical data collection. L. Idoufkir, S. Cremers, C. Remacle, M. Hamaïdia and B. Polese contributed to the laboratory data generation and analysis. C Moermans, I. Struman, E. Louis, M. Malaise, D. de Seny, P. van Paassen, R. Louis and C. Ribbens critically commented on the study and revised the manuscript. J. Guiot and M–S. Njock designed and supervised the study, analysed data and wrote the manuscript. All authors critically reviewed the manuscript and approved the final draft for submission.

Conflict of interest: R. Louis reports grants from Chiesi, AstraZeneca and GSK; consultancy fees from AstraZeneca and GSK; payment or honoraria for lectures, presentations, manuscript writing or educational events from AstraZeneca and GSK; and participation on a data safety monitoring board or advisory board with AstraZeneca. J. Guiot reports payment or honoraria for lectures, presentations, manuscript writing or educational events from Boehringer Ingelheim, Janssens, GSK, Roche and Chiesi; support for attending meetings from Chiesi, Roche, Janssens, Boehringer Ingelheim and AstraZeneca; patents planned, issued or pending with Radiomics (Oncoradiomics SA); and participation on a data safety monitoring board or advisory board with GSK, Janssens, Chiesi, AstraZeneca and MSD. The rest of the authors declare that they have no conflicts of interest.

Support statement: This study was supported by the "Fonds d'Investissement de Recherche Scientifique" (FIRS) and "Fonds Léon Fredericq" of University Hospital of Liège. Funding information for this article has been deposited with the Crossref Funder Registry.

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