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Microparticles in systemic sclerosis: Potential pro-inflammatory mediators and pulmonary hypertension biomarkers

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Abstract

Background and objective: Endothelial microparticles (EMP) are submicron vesicles released from endothelial cells. We aimed to determine the utility of EMP as bio-markers of pulmonary arterial hypertension (PAH) in systemic sclerosis (SSc) patients and the pathogenic role of microparticles (MP) in vascular inflammation.

Methods: Levels of EMP (CD144+, CD31+, CD62E+ and CD143+) were compared between three groups (10 SSc patients with PAH, 10 SSc patients without pulmonary hypertension (no-PH) and 10 healthy age- and sex-matched controls). Human pulmonary artery endothelial cells (HPAEC) were exposed in vitro to MP obtained from SSc patients or healthy controls, and levels of cytokines and inflammatory adhesion molecules were compared.

Results: CD144+ EMP were significantly higher in the SSc-PAH group compared to either the SSc-no PH or healthy controls (diagnostic accuracy 80%, $P = 0.02$). Compared to controls, SSc patients had higher CD31 +/CD62E+ ratios, indicating larger contributions of apoptosis to EMP

release ($P = 0.04$). Patients with limited SSc had significantly higher levels of CD143+ EMP compared to those with diffuse subtype ($P = 0.008$). When HPAEC were exposed to MP from SSc patients, there was a significant increase in inflammatory cytokines and adhesion molecules. Interestingly, exposure to healthy control MP caused a reduction in inflammatory markers.

Conclusion: EMP (particularly CD144+) are promising biomarkers of PAH in SSc but require further study. MP isolated from SSc patients induced an increase in endothelial cell inflammation and may be an important pathogenic factor in SSc.

Clinical trial registration: NCT02331225 at clinicaltrials.gov

Keywords

biomarkers; cardiovascular; cytokines and inflammatory mediators; respiratory; scleroderma

INTRODUCTION

Pulmonary arterial hypertension (PAH) occurs in up to 12% of patients with systemic sclerosis (SSc)¹ and is the second leading cause of death in this condition.² Screening for PAH in SSc is indicated because it is highly prevalent, a pre-clinical phase where PAH is asymptomatic is common,³ and early identification and treatment hold the hope of preventing or delaying clinical worsening.⁴ However, efforts at early PAH identification have thus far been ineffective, indicating a need for more discriminate biomarkers.

As SSc⁵ and PAH are both intrinsically vascular diseases, identification of biomarkers of endothelial cell activation could be useful in predicting SSc-PAH. Microparticles (MP) are submicron vesicles that are released in healthy and diseased conditions⁶ from various cell types including endothelial cells (endothelial MP, EMP), platelets (platelet MP, PMP) and leucocytes (LMP). EMP appear to play an active role in the pathogenesis of vascular disease by modulating cell–cell signalling. Circulating EMP are increased in pulmonary hypertension (PH) associated with a wide variety of conditions,⁶ correlate with haemodynamic severity⁶ and are predictive of poor outcomes.⁷ Prior measurements of EMP in SSc have demonstrated conflicting results^{8–11} and have not directly addressed the potential of EMP to serve as biomarkers of SSc-PAH. We hypothesized that (i) SSc patients would have higher levels of EMP compared to healthy controls, (ii) SSc-PAH patients would have higher levels of EMP than SSc patients without PH and (iii) MP isolated from SSc patients would induce greater endothelial cell (EC) inflammation in vitro compared to those isolated from healthy controls.

METHODS

Patient selection

In this pilot study, a sample of 20 consecutive adult SSc patients (10 with a current diagnosis of PAH and 10 without PH) fulfilling American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria¹² were recruited from 2015 to 2016. We excluded patients who have other conditions known to increase EMP levels (Appendix S1, Supplementary Information). PAH was defined as a mean pulmonary artery

pressure (mPAP) 25 mm Hg on right heart catheterization (RHC) and fulfilling criteria for group 1 PAH.¹³ As a comparison group, 10 healthy age- and sex-matched, non-smoking controls were recruited. This study was conducted in accordance with the amended Declaration of Helsinki and was approved by the LSUHSC IRB (#8772); written informed consent was obtained from all patients.

Disease characterization and clinical testing

SSc patients were characterized as having limited or diffuse SSc based on Leroy criteria.¹² A detailed history and physical examination was conducted by a scleroderma specialist (L.A.S.) with assessment for digital ulcers/pitting, telangiectases, osteolysis and modified Rodnan skin score. Pulmonary function testing¹⁴ and 6-min walk testing¹⁵ were performed according to the American Thoracic Society (ATS) guidelines. Transthoracic echocardiography was performed by a certified echocardiographer and RHC data were collected historically.

EMP collection

EMP were isolated from venous blood by modification of a published protocol using differential centrifugation¹⁶ (see Appendix S1 for the complete protocol, Supplementary Information). EMP were incubated with anti-CD42b (a marker of PMP), anti-CD31 (PECAM-1, reflecting apoptosis as a cause for EMP release¹⁷), anti-CD62E (*E*-selectin, reflecting inflammation as a cause for EMP release¹⁷), anti-CD143 (ACE, originating mainly from the pulmonary capillary bed¹⁸) and anti-CD144 (VE-Cadherin, reflecting destruction of the junction between endothelial cells¹⁹). Flow cytometry was conducted and EMP are reported as the number of MP/100 μ L of plasma.

In vitro experiments

To study the differential inflammatory effects of MP, human pulmonary artery endothelial cells (HPAEC) were exposed to pooled MP from either healthy controls ($n = 10$) or SSc patients ($n = 10$, 5 with PAH and 5 without PH) for 24 h (see Appendix S1 for complete protocol, Supplementary Information). To simulate conditions where MP were exposed to proinflammatory signalling,^{20,21} HPAEC were pretreated with MP and stimulated with 0.1 ng TNF- α (Fig. S1, Supplementary Information) for 3 or 24 h. Quantitative reverse transcriptase PCR (RT-PCR) using primers for human ICAM-1, VCAM-1, MCP-1 or GAPDH was conducted.^{22,23} Cytokines and chemokines were assayed using the Millipore MILLIPLEX (Burlington, Massachusetts, USA) MAP human cytokine/chemokine system. Biologically relevant cytokines (Granulocyte-macrophage colony-stimulating factor) (IL-2, IL-4, IL-6, MIP-1 β , GM-CSF) with *P*-values of 0.05–0.20 in the multiplex were re-measured using Bio-Rad Bioplex (Hercules, California, USA).

Statistical analysis

Baseline characteristics were compared using an unpaired t-test, Mann–Whitney U-test or chi-square test, as appropriate, and are presented as mean $\pm$ SD, median (interquartile range) or percentages. The a priori primary outcome was CD144+ EMP among the three groups (healthy controls, SSc-PAH and SSc-no PH). EMP were compared using Kruskal–Wallis

with Dunn's post-test and Bonferroni correction and are displayed as median (interquartile range). To compare EMP between SSc subtypes, Mann–Whitney test was used followed by adjustment for PAH status, as PAH rates differ based on disease subtype.²⁴ A receiver operating characteristic (ROC) curve analysis was used to calculate area under the curve (AUC). To internally validate CD144+ EMP as a biomarker of SSc-PAH and calculate an optimism-corrected performance estimate, resampling by bootstrapping was performed for the ROC-AUC with 1000 resamples.²⁵ Pearson correlation analysis was undertaken to correlate EMP with disease severity measures (haemodynamics, log-transformed B-type natriuretic peptide (BNP) and 6-min walk distance (6MWD)). There were no comparable studies to base a power analysis on, but the sample size in this pilot study allowed for detection of a 32% difference in CD144+ EMP levels between SSc groups.⁹ For the in vitro analyses, one-way analysis of variance (ANOVA) was conducted with a Tukey's post-test to compare TNF- α -stimulation alone, TNF- α + healthy MP and TNF- α + SSc-MP. Statistical analysis was conducted with STATA (version 13, StataCorp., College Station, TX, USA) and GraphPad (version 5, GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Clinical characteristics

There were no statistically significant differences in multiple parameters previously described to differentiate SSc-PAH from SSc-no PH patients,^{3,26,27} nor was there a difference in functional class or 6MWD (Tables 1–2). There was a trend towards more limited disease subtype in the SSc-PAH patients (90%) versus the SSc-no PH group (40%, $P = 0.06$, Table 1). BNP levels were higher in the SSc-PAH group (102 (52, 135) vs 37 (20, 44) pg/mL, $P = 0.02$ in the SSc-no PH, ROC-AUC = 0.81).

Historical RHC showed haemodynamic differences (expected based on group definitions), with the SSc-PAH patients having an mPAP of 40 ± 18 mm Hg and pulmonary vascular resistance (PVR) of 6.7 ± 5.4 Wood units (Table 2). Only 20% of SSc-PAH patients had right ventricular dysfunction on echocardiography. Importantly, all SSc-PAH patients were on at least one PAH-approved medication at the time of EMP measurement. Fifty percent of patients were on two PAH-approved medications and 20% were on triple therapy.

EMP biomarker results (SSc-PAH vs SSc-no PH vs healthy controls)

CD144+ EMP (the pre-defined marker of interest) were significantly higher in the SSc-PAH group compared to both the SSc-no PH patients and healthy controls (1310 (800, 1472) vs 563 (284, 1150) vs 501 (439, 605) MP/100 μ L, respectively, $P = 0.02$, Table 3, Fig. 1A). The ROC-AUC was 0.80 (95% CI: 0.60–0.99, $P = 0.02$) for CD144+ EMP to differentiate SSc-PAH patients from those without PH (Fig. 1B). Internal validation by bootstrap resampling generated a similar ROC-AUC of 0.78 (95% CI: 0.55–0.93). Right atrial pressure (RAP) on RHC correlated with log-transformed CD143+ ($r = 0.72$, $P = 0.03$) and CD62E+ EMP ($r = 0.74$, $P = 0.02$). However, no significant correlations existed between EMP and RAP or other disease severity measures after Bonferroni correction for multiple comparisons.

SSc patients had lower levels of CD62E+ EMP compared to healthy controls (74 (63110) vs. 54 (43, 63) vs. 96 (72111) MP/100 μ L for SSc-PAH vs. SSc-no PH vs. healthy controls, respectively, $P = 0.02$). SSc patients had higher CD31+/CD62E+ ratios than healthy controls, suggesting that their EMP were released more from apoptotic than inflammatory mechanisms¹⁶ ($P = 0.04$, Table 3).

CD143+ (111 (59, 147) vs 32 (31, 66) MP/100 μ L, $P = 0.02$) and CD62E+ (68 (63, 110) vs. 44 (41, 61) MP/100 μ L, $P = 0.02$, Fig. 1C) EMP were significantly higher in limited SSc patients compared to diffuse SSc patients; only CD143+ EMP were significantly different after adjusting for the presence of PAH ($P = 0.008$, Table S1 (Supplementary Information); AUC: 0.84, 95% CI: 0.65–1.00, $P = 0.02$, Fig. 1D). CD42b + MP (platelet-derived) were not different between controls and SSc patients, SSc-PAH vs SSc-no PH and limited vs. diffuse SSc subtypes (Tables 3, S1, Supplementary Information).

In vitro experiments (SSc vs healthy controls)

Following stimulation of MP-pretreated HPAEC with low-dose TNF- α , there were two noticeable patterns between MP from healthy controls versus those of SSc subjects (pooled samples from SSc-PAH and SSc-no PH). Granulocyte-colony stimulating factor (G-CSF), interferon-gamma (IFN- γ) and macrophage inflammatory protein 1-alpha (MIP-1 α) levels were significantly reduced when HPAEC were exposed to healthy MP, but were increased when exposed to SSc-MP (Fig. 2, Table S2, Supplementary Information). Levels of IL-2, IL-4, IL-6, IL-12, IL-1 β , MIP-1 β , Vascular endothelial growth factor (VEGF) and IL-1RA significantly increased when HPAEC were exposed to SSc-MP (Fig. 2, Table S2, Fig. S2, Supplementary Information).

After 3 h of TNF- α stimulation, HPAEC exposed to SSc-MP had significantly increased expression of VCAM-1 (Fig. 3). After 3 or 24 h of TNF- α stimulation, HPAEC exposed to healthy MP had significantly lower ICAM-1 expression. Compared to healthy MP, exposure to SSc- MP led to increased ICAM-1 at both time points. Lastly, expression of MCP-1 by HPAEC exposed to SSc-MP was increased after 24 h of TNF- α stimulation compared to either healthy MP exposure or TNF- α stimulation alone.

DISCUSSION

We found that circulating CD144+ EMP were significantly higher in SSc-PAH patients compared to SSc patients without PH (80% diagnostic accuracy for differentiation). Additionally, SSc patients had a distinct EMP population compared to healthy controls. We also demonstrated that when HPAEC were exposed to MP isolated from SSc patients versus healthy controls, SSc- MP increased inflammatory cytokines and adhesion molecules that are intimately linked to the disease, while healthy MP reduced these inflammatory mediators. This is a novel description of EMP as a potential biomarker of PAH in SSc and is the first time that MP obtained from human subjects with SSc have been shown to be pro-inflammatory in vitro.

EMP are microvesicles measuring 100–1000 nm released from EC constitutively and during endothelial activation or stress.⁶ Although others demonstrated that EMP were significantly

increased in patients with various forms of PH compared to healthy controls,⁶ it would be highly significant and clinically beneficial if EMP could differentiate patients with and without PAH within a high-risk group like SSc. We believe that our study provides evidence for the potential utility of EMP as a distinguishing biomarker. Prior investigations of EMP in SSc patients have yielded mixed results. We found that EMP populations were different between SSc patients and healthy controls, with a relative increase in CD31+ and decrease in CD62E+ EMP. One prior study noted that CD31+ and CD62E+ EMP levels were higher in SSc patients compared to controls¹¹ while another study found the opposite.¹⁰ We also found that SSc patients with a limited subtype had higher levels of CD143+ EMP than diffuse subtype patients, even after adjustment for baseline PAH status. This is intriguing as CD143+ EMP are primarily derived from pulmonary capillaries¹⁸ and limited SSc patients are at higher risk for PH.²⁴ It is difficult to make direct comparisons between our study and prior investigations of EMP in SSc as differences in patient populations and EMP processing can influence the results. Our PAH patients were treated aggressively with medications that could have decreased EMP; future studies may help characterize whether greater differences exist in EMP between treated and treatment-naïve PAH patients.

To our knowledge, we are the first to describe differences in EMP based on PH status defined by RHC. We found higher median levels of all measured EMP in SSc-PAH patients compared to SSc patients without PH. Our pre-defined primary marker CD144+ EMP was significantly higher in SSc-PAH patients, with an overall diagnostic accuracy of 80% to detect PAH. CD144 is the major component of endothelial adherens junctions and plays an important role in the control of vascular permeability.¹⁹ Interestingly, loss of EC barrier function may contribute to PAH pathogenesis by allowing inflammatory mediators to cross the barrier.²⁸ All of these factors increase the relevance of CD144+ EMP as a biomarker of SSc-PAH.

In contrast to other studies that enrolled more heterogeneous PAH populations,⁶ we did not find a correlation between EMP and disease severity measurements after adjusting for multiple comparisons. Larger samples are needed to assess whether this is a strength of the bio-marker, being able to differentiate mild PAH from those without PAH. BNP, the only clinically utilized biomarker of PH, was significantly different between SSc patients with and without PAH and had a similar diagnostic accuracy to CD144+ EMP. Interestingly, there was no correlation between BNP and CD144+ EMP, suggesting that each is indicating a different disease feature (e.g. cardiac stretch and endothelial abnormalities, respectively). Combining BNP with CD144+ EMP did lead to an increase in the ROC-AUC (0.83, 95% CI: 0.64–1.00).

Our in vitro data show that SSc-MP upregulate many pro-inflammatory cytokines and chemokines in HPAEC that are known to be directly involved in SSc and PAH pathogenesis. For example, IL-6 production in peripheral blood mononuclear cells²⁹ and fibroblasts³⁰ has been implicated in the pathogenesis of SSc and PAH. Furthermore, IL-6 overexpression leads to PH in mice³¹ while IL-6 knockouts are resistant to hypoxia-induced PH.³² IL-2,^{33,34} IL-4,^{35,36} MIP-1 α C-C ligand (CCL3),³⁷ MIP-1 β (CCL4)³⁸ and monocyte chemoattractant protein (MCP)-1^{37,39} are all elevated in SSc patients (serum, skin and bronchoalveolar

lavage fluid) and participate in the fibrotic pathogenesis of SSc. Additionally, experimentally blocking MCP-1 and MIP-1 β prevents skin fibrosis in an animal model of SSc.⁴⁰

Herein, we provide the first description of a potentially pathogenic role of MP derived from humans with SSc that may have relevance to the development of PAH. Prior studies have demonstrated that MP obtained from rodents with PH have pathogenic effects in vitro and in vivo, leading to right ventricular hyper-trophy and pulmonary vascular remodelling when injected intravenously into healthy mice.⁴¹ We found that exposure of HPAEC to SSc-MP increased the expression of inflammatory adhesion molecules. Similarly, exposure of PAEC to MP isolated from a rat model of PH increased ICAM-1 expression.⁴² We are the first to expose HPAEC in vitro to SSc-MP; however, we acknowledge that in vitro models cannot characterize the full complexity of the pathogenesis of pulmonary vascular disease in humans.⁴³

An additional novel finding from our experiments is that MP obtained from healthy control subjects *decreased* several pro-inflammatory cytokines and adhesion molecules in vitro. The role of MP in health is supported by prior experiments demonstrating EMP from human coronary artery EC reduced ICAM-1 expression and monocyte adhesion, without affecting VCAM levels.²¹ Similarly, we found for the first time that MP obtained from healthy humans significantly decreased ICAM-1 in HPAEC without altering VCAM levels.

In addition to the limitations listed above, a few other caveats must be acknowledged. Although CD144+ EMP could accurately differentiate patients with SSc-PAH from those without PH, our sample size was small and therefore the precision of this observation may be limited. Although we performed internal validation by boot-strapping, external validation in a larger separate SSc cohort is needed prior to implementation of this bio-marker into clinical practice. While we looked at MP derived from endothelial cells and platelets, we did not investigate MP from other cell types (e.g. leucocytes). It is important to mention that the SSc patients without PAH had significant functional limitations and a high rate of interstitial lung disease; whether EMP would perform differently when compared to a SSc cohort without pulmonary disease is unknown. It should be emphasized that the HPAEC were exposed to MP, which included not only EMP but also MP derived from other cells (e.g. platelets and leucocytes). MP used in our in vitro experiments were pooled from an equal number of SSc patients with and without PAH, so we cannot comment on whether there is a differential effect of MP in these SSc subgroups. Future investigations will need to investigate MP content (e.g. miRNA) on the pathogenic mechanisms of pulmonary vascular disease.

In conclusion, EMP (particularly CD144+) are promising biomarkers of SSc-PAH and may be involved its pathogenesis. Future studies are needed to confirm these findings and to determine the utility of EMP to predict future PAH development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

6MWD	6-min walk distance
ANA	anti-nuclear antibody
AUC	area under the curve
BNP	B-type natriuretic peptide
CCL	C-C ligand
DL_{CO}	diffusion capacity for carbon monoxide
EC	Endothelial cell
EMP	endothelial microparticle
FC	functional class
FVC	forced vital capacity
HPAEC	human pulmonary artery endothelial cell
ICAM	intercellular adhesion molecule
IL	interleukin
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MP	microparticle
mPAP	mean pulmonary artery pressure
PAH	pulmonary arterial hypertension
PCR	polymerase chain reaction
PH	pulmonary hypertension
PMP	platelet MP
RAP	right atrial pressure
RHC	right heart catheterization
ROC	receiver operating characteristic
SSc	systemic sclerosis

TNF-α	tumour necrosis factor alpha
VCAM	vascular cell adhesion protein

REFERENCES

1. Mukerjee D, George D St, Coleiro B, Knight C, Denton CP, Davar J, Black CM, Coghlan JG. Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: application of a registry approach. *Ann. Rheum. Dis* 2003; 62: 1088–93. [PubMed: 14583573]
2. Steen VD, Medsger TA. Changes in causes of death in systemic sclerosis, 1972–2002. *Ann. Rheum. Dis* 2007; 66: 940–4. [PubMed: 17329309]
3. Steen VD, Graham G, Conte C, Owens G, Medsger TA Jr. Isolated diffusing capacity reduction in systemic sclerosis. *Arthritis Rheum* 1992; 35: 765–70. [PubMed: 1622414]
4. Humbert M, Yaici A, de Groote P, Montani D, Sitbon O, Launay D, Gressin V, Guillemin L, Clerson P, Simonneau G et al. Screening for pulmonary arterial hypertension in patients with systemic sclerosis: clinical characteristics at diagnosis and long-term survival. *Arthritis Rheum* 2011; 63: 3522–30. [PubMed: 21769843]
5. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J. Clin. Invest* 1996; 98: 785–92. [PubMed: 8698871]
6. Amabile N, Heiss C, Real WM, Minasi P, McGlothlin D, Rame EJ, Grossman W, De Marco T, Yeghiazarians Y. Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension. *Am. J. Respir. Crit. Care Med* 2008; 177: 1268–75. [PubMed: 18310479]
7. Amabile N, Heiss C, Chang V, Angeli FS, Damon L, Rame EJ, McGlothlin D, Grossman W, De Marco T, Yeghiazarians Y. Increased CD62e(+) endothelial microparticle levels predict poor outcome in pulmonary hypertension patients. *J. Heart Lung Transplant* 2009; 28: 1081–6. [PubMed: 19782291]
8. Iversen LV, Ostergaard O, Ullman S, Nielsen CT, Halberg P, Karlsmark T, Heegaard NH, Jacobsen S. Circulating microparticles and plasma levels of soluble E- and P-selectins in patients with systemic sclerosis. *Scand. J. Rheumatol* 2013; 42: 473–82. [PubMed: 24016306]
9. Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, Gay RE, Pisetsky DS, Gay S, Matucci-Cerinic M et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis Rheum* 2008; 58: 2845–53. [PubMed: 18759303]
10. Jung C, Drummer K, Oelzner P, Figulla HR, Boettcher J, Franz M, Betge S, Foerster M, Wolf G, Pfeil A. The association between endothelial microparticles and inflammation in patients with systemic sclerosis and Raynaud's phenomenon as detected by functional imaging. *Clin. Hemorheol. Microcirc* 2015; 61: 549–57. [PubMed: 26410864]
11. Michalska-Jakubus M, Kowal-Bielecka O, Smith V, Cutolo M, Krasowska D. Plasma endothelial microparticles reflect the extent of capillaroscopic alterations and correlate with the severity of skin involvement in systemic sclerosis. *Microvasc. Res* 2017; 110: 24–31. [PubMed: 27889559]
12. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, Matucci-Cerinic M, Naden RP, Medsger TA Jr, Carreira PE et al. 2013 Classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann. Rheum. Dis* 2013; 72: 1747–55. [PubMed: 24092682]
13. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M et al. 2015 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension: the Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur. Heart J* 2016; 37: 67–119. [PubMed: 26320113]
14. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P et al. Standardisation of spirometry. *Eur. Respir. J* 2005; 26: 319–38. [PubMed: 16055882]

15. ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories. ATS statement: guidelines for the six-minute walk test. *Am. J. Respir. Crit. Care Med* 2002; 166: 111–7. [PubMed: 12091180]
16. Gordon C, Gudi K, Krause A, Sackrowitz R, Harvey BG, Strulovici- Barel Y, Mezey JG, Crystal RG. Circulating endothelial microparticles as a measure of early lung destruction in cigarette smokers. *Am. J. Respir. Crit. Care Med* 2011; 184: 224–32. [PubMed: 21471087]
17. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb. Res* 2003; 109: 175–80. [PubMed: 12757771]
18. Danilov SM, Gavriluk VD, Franke FE, Pauls K, Harshaw DW, McDonald TD, Miletich DJ, Muzykantov VR. Lung uptake of anti-bodies to endothelial antigens: key determinants of vascular immunotargeting. *Am. J. Physiol. Lung Cell. Mol. Physiol* 2001; 280: L1335–47. [PubMed: 11350815]
19. Dejana E, Orsenigo F. Endothelial adherens junctions at a glance. *J. Cell Sci* 2013; 126: 2545–9. [PubMed: 23781019]
20. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum tumor necrosis factor- α levels in patients with systemic sclerosis: association with pulmonary fibrosis. *J. Rheumatol* 1997; 24: 663–5. [PubMed: 9101498]
21. Jansen F, Yang X, Baumann K, Przybilla D, Schmitz T, Flender A, Paul K, Alhusseiny A, Nickenig G, Werner N. Endothelial micro-particles reduce ICAM-1 expression in a microRNA-222-dependent mechanism. *J. Cell. Mol. Med* 2015; 19: 2202–14. [PubMed: 26081516]
22. Ghonim MA, Pyakurel K, Ibba SV, Wang J, Rodriguez P, Al-Khami AA, Lammi MR, Kim H, Zea AH, Davis C et al. PARP is activated in human asthma and its inhibition by olaparib blocks house dust mite-induced disease in mice. *Clin. Sci. (Lond.)* 2015; 129: 951–62. [PubMed: 26205779]
23. Ghonim MA, Pyakurel K, Ibba SV, Al-Khami AA, Wang J, Rodriguez P, Rady HF, El-Bahrawy AH, Lammi MR, Mansy MS et al. PARP inhibition by olaparib or gene knockout blocks asthma-like manifestation in mice by modulating CD4(+) T cell function. *J. Transl. Med* 2015; 13: 225. [PubMed: 26169874]
24. Cox SR, Walker JG, Coleman M, Rischmueller M, Proudman S, Smith MD, Ahern MJ, Roberts-Thomson PJ. Isolated pulmonary hypertension in scleroderma. *Intern. Med. J* 2005; 35: 28–33. [PubMed: 15667465]
25. Steyerberg E *Clinical Prediction Models: A Practical Approach to Development, Validation, and Updating*, 1st edn. New York, Springer, 2009.
26. Steen V, Medsger TA Jr. Predictors of isolated pulmonary hypertension in patients with systemic sclerosis and limited cutaneous involvement. *Arthritis Rheum* 2003; 48: 516–22. [PubMed: 12571862]
27. Coghlan JG, Denton CP, Grunig E, Bonderman D, Distler O, Khanna D, Muller-Ladner U, Pope JE, Vonk MC, Doelberg M et al. Evidence-based detection of pulmonary arterial hypertension in systemic sclerosis: the DETECT study. *Ann. Rheum. Dis* 2014; 73: 1340–9. [PubMed: 23687283]
28. Francis M, Xu N, Zhou C, Stevens T. Transient receptor potential channel 4 encodes a vascular permeability defect and high-frequency Ca(2+) transients in severe pulmonary arterial hypertension. *Am. J. Pathol* 2016; 186: 1701–9. [PubMed: 27083517]
29. Giacomelli R, Cipriani P, Danese C, Pizzuto F, Lattanzio R, Parzanese I, Passacantando A, Perego MA, Tonietti G. Peripheral blood mononuclear cells of patients with systemic sclerosis produce increased amounts of interleukin 6, but not transforming growth factor beta 1. *J. Rheumatol* 1996; 23: 291–6. [PubMed: 8882034]
30. Takemura H, Suzuki H, Fujisawa H, Yuhara T, Akama T, Yamane K, Kashiwagi H. Enhanced interleukin 6 production by cultured fibroblasts from patients with systemic sclerosis in response to platelet derived growth factor. *J. Rheumatol* 1998; 25: 1534–9. [PubMed: 9712097]
31. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 overexpression induces pulmonary hypertension. *Circ. Res* 2009; 104: 236–44 28p following 244. [PubMed: 19074475]

32. Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S, Eddahibi S. Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. *Respir. Res* 2009; 10: 6. [PubMed: 19173740]
33. Kahaleh MB, LeRoy EC. Interleukin-2 in scleroderma: correlation of serum level with extent of skin involvement and disease duration. *Ann. Intern. Med* 1989; 110: 446–50. [PubMed: 2784042]
34. Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. *Arthritis Rheum* 1992; 35: 67–72. [PubMed: 1731816]
35. Tsuji-Yamada J, Nakazawa M, Minami M, Sasaki T. Increased frequency of interleukin 4 producing CD4+ and CD8+ cells in peripheral blood from patients with systemic sclerosis. *J. Rheumatol* 2001; 28: 1252–8. [PubMed: 11409116]
36. Salmon-Ehr V, Serpier H, Nawrocki B, Gillery P, Clavel C, Kalis B, Birembaut P, Maquart FX. Expression of interleukin-4 in scleroderma skin specimens and scleroderma fibroblast cultures. Potential role in fibrosis. *Arch. Dermatol* 1996; 132: 802–6. [PubMed: 8678573]
37. Codullo V, Baldwin HM, Singh MD, Fraser AR, Wilson C, Gilmour A, Hueber AJ, Bonino C, McInnes IB, Montecucco C et al. An investigation of the inflammatory cytokine and chemokine network in systemic sclerosis. *Ann. Rheum. Dis* 2011; 70: 1115–21. [PubMed: 21285114]
38. Bolster MB, Ludwicka A, Sutherland SE, Strange C, Silver RM. Cytokine concentrations in bronchoalveolar lavage fluid of patients with systemic sclerosis. *Arthritis Rheum* 1997; 40: 743–51. [PubMed: 9125259]
39. Carulli MT, Handler C, Coghlan JG, Black CM, Denton CP. Can CCL2 serum levels be used in risk stratification or to monitor treatment response in systemic sclerosis? *Ann. Rheum. Dis* 2008; 67: 105–9. [PubMed: 17604287]
40. Kimura M, Kawahito Y, Hamaguchi M, Nakamura T, Okamoto M, Matsumoto Y, Endo H, Yamamoto A, Ishino H, Wada M et al. SKL-2841, a dual antagonist of MCP-1 and MIP-1 beta, prevents bleomycin-induced skin sclerosis in mice. *Biomed. Pharmacother* 2007; 61: 222–8. [PubMed: 17147981]
41. Aliotta JM, Pereira M, Amaral A, Sorokina A, Igbinoba Z, Hasslinger A, El-Bizri R, Rounds SI, Quesenberry PJ, Klinger JR. Induction of pulmonary hypertensive changes by extracellular vesicles from monocrotaline-treated mice. *Cardiovasc. Res* 2013; 100: 354–62. [PubMed: 23867631]
42. Blair LA, Haven AK, Bauer NN. Circulating microparticles in severe pulmonary arterial hypertension increase intercellular adhesion molecule-1 expression selectively in pulmonary artery endothelium. *Respir. Res* 2016; 17: 133. [PubMed: 27765042]
43. Distler JH, Akhmetshina A, Dees C, Jungel A, Sturzl M, Gay S, Pisetsky DS, Schett G, Distler O. Induction of apoptosis in circulating angiogenic cells by microparticles. *Arthritis Rheum* 2011; 63: 2067–77. [PubMed: 21437873]

SUMMARY AT A GLANCE

CD144+ endothelial microparticles (MP) were significantly higher in systemic sclerosis (SSc) patients with pulmonary hypertension (PH) compared to SSc patients without PH, suggesting that they may be a biomarker of SSc-PH. MP from SSc patients also increased endothelial inflammation in vitro, indicating a possible pathogenic role in this condition.

Figure 1.

(A) CD144+ endothelial microparticles (EMP) from healthy controls versus patients with systemic sclerosis (SSc) with pulmonary arterial hypertension (PAH) and without pulmonary hypertension (PH). (B) Receiver operating characteristics (ROC) curve for CD144+ EMP levels to differentiate SSc patients with PAH from SSc patients without PH. A CD144+ EMP cut-off of 479 yielded 90% sensitivity and 50% specificity to detect SSc-PAH, whereas a cut-off of 1278 provided 60% sensitivity and 100% specificity (area under the curve (AUC): 0.80, 95% CI: 0.60–0.99, $P = 0.02$). (C) CD143 + EMP for SSc patients with diffuse versus limited sub-type. (D) ROC curve for CD143 + EMP to differentiate SSc patients with diffuse versus limited subtype. A CD143+ EMP cut-off of 42.5 yielded 92% sensitivity and 71% specificity, whereas a cut-off of 85 yielded 62% sensitivity and 100% specificity for limited versus diffuse SSc (AUC: 0.84, 95% CI: 0.65–1.00, $P = 0.02$).

Figure 2.

Levels of macrophage inflammatory protein (MIP)-1α (A), G-CSF (B), interferon-gamma (IFN-γ) (C), IL-2 (D), IL-4 (E) and MIP-1β (F) under three in vitro conditions: human pulmonary artery endothelial cell (HPAEC) stimulated by TNF-α alone, TNF-α + microparticles (MP) isolated from healthy subjects and TNF-α + MP from systemic sclerosis (SSc) patients. Data are displayed as mean ± SEM. Overall P -value is shown in the upper right hand corner and intergroup comparisons are displayed as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 3.

Reverse transcriptase PCR (RT-PCR) for VCAM-1 (A), ICAM-1 (B) and MCP-1 (C) after human pulmonary artery endothelial cell (HPAEC) were exposed to microparticles (MP) isolated from healthy subjects or systemic sclerosis (SSc) patients and stimulated for 3 h with TNF- α , and (D-F) after 24 h of TNF- α stimulation. Data are displayed as mean $\pm$ SEM. Overall P -value is shown in the upper right hand corner and intergroup comparisons are displayed as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1

Clinical characteristics of the SSc patients with PAH and without PH

Parameter	SSc with PAH (n = 10)	SSc without PH (n = 10)	P-value
Age (years)	57 ± 11	55 ± 11	0.71
Sex (% female)	90%	100%	1.00
Mycophenolate use	60%	80%	0.63
SSc duration (years from first non-Raynaud symptom)	7.0 (5.6, 16.2)	11.4 (4.2, 17.5)	1.00
SSc subtype (% limited)	90%	40%	0.06
Digital ulcers	20%	40%	0.63
Digital pitting	20%	10%	1.00
Telangiectases	80%	50%	0.35
Osteolysis	20%	40%	0.63
Modified Rodnan skin score	6 ± 6	13 ± 8	0.11
Autoantibodies [†]			0.97
ANA positive	70%	100%	
ANA nucleolar	30%	30%	
Anti-centromere	40%	30%	
Anti-Scl70	20%	20%	
Anti-RNA polymerase	20%	20%	
WHO FC (% FC III)	50%	40%	1.00
UCSD SOB questionnaire	47 ± 19	63 ± 35	0.31
BNP (pg/mL)	102 (52, 137)	37 (20, 44)	0.02

Data are presented as median (interquartile range), mean ± SD or percentages.

[†]Individual patients may have more than one antibody and thus percentages add to up greater than 100%.

ANA, anti-nuclear antibody; BNP, B-type natriuretic peptide; FC, functional class; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SOB, shortness of breath; SSc, systemic sclerosis; UCSD, University of California-San Diego; WHO, World Health Organization.

Table 2

Pulmonary function, echocardiography and RHC variables

Parameter	SSc with PAH (<i>n</i> = 10)	SSc without PH (<i>n</i> = 10)	<i>P</i> -value
FVC (% predicted)	66 ± 27	69 ± 20	0.76
DL _{CO} (% predicted)	43 ± 20	50 ± 21	0.46
FVC/DL _{CO} ratio	1.79 ± 0.77	1.62 ± 0.81	0.67
Echo left atrial size (cm)	3.4 ± 0.6	3.3 ± 0.8	0.58
Echo diastolic dysfunction	40%	40%	1.00
Echo pericardial effusion	10%	0%	1.00
Echo right ventricular dysfunction	20%	0%	0.47
RHC right atrial pressure (mm Hg) [†]	6 ± 5	5 ± 4	0.61
RHC mPAP (mm Hg) [†]	40 ± 18	19 ± 6	0.005
RHC PAWP (mm Hg) [†]	11 ± 6	9 ± 5	0.57
RHC cardiac output (L/min) [†]	5.6 ± 1.8	5.3 ± 1.7	0.76
RHC PVR (Woods units) [†]	6.7 ± 5.4	1.9 ± 0.9	0.03
6-min walk distance (m)	379 ± 91	383 ± 60	0.90

Data are presented as mean ± SD or percentages.

[†] All 10 patients with PH and 9 patients without PH had historical RHC.

DL_{CO}, diffusion capacity for carbon monoxide; FVC, forced vital capacity; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PAWP, pulmonary artery wedge pressure; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; RHC, right heart catheterization; SSc, systemic sclerosis.

Table 3

EMP levels in healthy controls, patients with SS_c-PAH, and patients with SS_c-no PH

MP	Healthy controls	SS _c -PAH	SS _c -no PH	P-value	Control vs SS _c -no PH	Control vs SS _c -PAH	SS _c -PAH vs SS _c -no PH
CD144	501 (439, 605)	1310 (800, 1472)	563 (284, 1150)	0.02	>0.05	0.02	0.03
CD31	2200 (1468, 3379)	4000 (2980, 9338)	3682 (1348, 8970)	0.13	—	—	—
CD62E	96 (72, 111)	74 (63, 110)	54 (43, 63)	0.02	0.01	>0.05	>0.05
CD143	110 (69, 163)	85 (50, 139)	49 (32, 111)	0.16	—	—	—
CD31/CD62E ratio	23.7 (13.6, 40.7)	50.6 (31.7, 105)	40.7 (22.8, 128.6)	0.04	0.05	0.03	>0.05
CD42b	2005 (1198, 3879)	3114 (1362, 4719)	1927 (1252, 3300)	0.57	—	—	—

Data are presented as median (interquartile range). Displayed are *P*-values from the Kruskal–Wallis testing between the three groups as well as Dunn’s post-test for intergroup comparisons. MP are displayed as MP/100 µL of plasma. All EMP were CD42b⁺. CD42b⁺ + MP were platelet-derived.

EMP, endothelial MP; MP, microparticle; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SS_c, systemic sclerosis.