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Adipokine expression in systemic sclerosis lung and gastrointestinal organ involvement

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ABSTRACT

Objectives: The immunomodulatory properties of adipokines have previously been reported in autoimmune disorders. Less is known about the role of adipokines in systemic sclerosis (SSc). Lung and gastrointestinal tract are frequently involved in SSc; therefore, these organs were analyzed for adipokine expression as well as pulmonary samples of patients suffering from idiopathic pulmonary fibrosis (IPF) as comparison.

Methods: Gastric samples (antrum, corpus) of SSc were analyzed immunohistochemically for adiponectin, resistin and visfatin compared with non-SSc related gastritis. Inflammatory cells were quantified in gastric samples and correlated with adipokine expression. Lung samples of SSc, IPF and healthy controls were also analyzed. Protein levels of lung tissue lysates and bronchoalveolar lavages (BAL) in minor fibrotic stages were measured by ELISA.

Results: Lung sections of donor parenchyma showed significantly stronger adiponectin signals as IPF and SSc (donor vs. IPF: $p < 0.0001$). In SSc and IPF, resistin and visfatin were increased within immune cell infiltrates, but overall no difference in expression for resistin or visfatin compared to controls was observed. In BAL and lung protein lysates of early stages of fibrosis, adiponectin and visfatin were not reduced in IPF and SSc compared to controls. In gastric samples collected by standard endoscopic gastric biopsy, adiponectin was also significantly reduced in SSc- compared to non-SSc gastritis ($p = 0.049$) while resistin and visfatin were comparable although deeper fibrotic layers were not included in the respective samples. Adiponectin-positive tissues showed higher amounts of $CD4^+$ but not $CD8^+$ T cells. Controls showed no correlation between $CD4^+$ T cells and resistin, whereas SSc showed significantly more $CD4^+$ T cells in resistin-negative tissues.

Conclusion: Adipokines are expressed in gastric and lung samples of patients with SSc and in lung samples affected by IPF. Prominently, adiponectin levels were reduced in fibrotic SSc gastritic tissue as well as in IPF and SSc lung tissue. Consequently, adiponectin expression seems to be associated with fibrotic progression in the context of SSc and IPF.

1. Introduction

Systemic sclerosis (SSc) is a rare autoimmune connective tissue disease. Its multifaceted pathophysiology involves an overactive immune system with increased matrix deposition in skin and internal organs alongside disturbed angiogenesis and general vasculopathy. SSc can affect the majority of internal organs. SSc patients are classified as diffuse cutaneous SSc (dcSSc) with early, more severe internal organ involvement or as limited cutaneous SSc (lcSSc) with less severe and a later onset of organ involvement [1,2]. The severity of organ involvement is the main reason for SSc-related morbidity and mortality. Besides the skin as primarily involved organ, gastrointestinal tract (GI), lung and heart are most commonly involved. At least 90% of SSc patients report GI symptoms caused by increasing stiffness and reduced

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motility. Cardio-pulmonary complications include pulmonary arterial hypertension and interstitial lung disease which are associated with reduced life expectancy.

SSc pathogenesis combines vasculopathy, autoimmunity and fibrosis. Activation and subsequent apoptosis of endothelial cells (EC) occur early [2] leading to increased vessel permeability allowing lymphocytes and macrophages to transmigrate thus forming perivascular infiltrates. Macrophage and $CD4^+$ T cell numbers in SSc skin infiltrates are increased. Via lymphocyte activation, local fibroblasts are activated and attracted, leading to increased matrix synthesis. The initial inflammatory phase leads to fibroblast transformation into myofibroblasts, being the major source of pro-fibrotic TGF-β. The resulting fibrosis is mostly responsible for organ dysfunction or even failure [3,4]. Autoimmunity is represented by an increased B cell response leading to production of SSc-associated antibodies like anti-centromere, anti-topoisomerase or anti-RNA polymerase III antibodies.

Adipokines are bioactive substances with metabolic and immunomodulatory functions mainly produced by adipose tissue. In rheumatoid arthritis (RA) for example, a chronic inflammatory joint disease, synovial fibroblasts respond to adiponectin with secretion of inflammatory and proangiogenic factors and matrix degrading enzymes [5]. Other cells in RA (e.g. EC, chondrocytes, lymphocytes) respond in a similar way [6]. For other autoimmune diseases, including systemic lupus erythematosus (SLE), an association with altered systemic adiponectin levels could be shown [7]. Nevertheless, for an association between SSc and adipokine levels in serum the studies were inconsistent [8–12], but interestingly a negative correlation to skin thickness of affected regions was described [13]. In liver fibrosis, adiponectin reversed the activation of stellate cells [14] and in angiotensin-II-induced cardiac fibrosis, adiponectin showed protective anti-fibrotic effects by inhibiting TGF-β signaling. In pulmonary hypertension, affecting 20–30% of SSc patients, adiponectin levels were increased [15] and induced activation of EC. Adiponectin suppressed vascular inflammation [16–18], an important feature due to the vascular alterations in SSc. Resistin, a predominantly pro-inflammatory adipokine, increases production of inflammatory factors by macrophages [19] and is involved in EC activation during inflammation [20,21]. Resistin is involved in the pathogenesis of arteriosclerosis [22]. Serum concentrations are increased in rheumatic diseases such as RA, SLE and Sjögren's syndrome [23–25] and in chronic inflammatory gastrointestinal diseases such as ulcerative colitis and Crohn's disease [26]. Visfatin has insulin-mimetic but also immunomodulatory effects. It activates leukocytes and induces inflammatory factors and expression of costimulatory surface molecules and EC activation [27,28]. In arteriosclerosis, visfatin is detectable in arteriosclerotic plaques and induces matrix degrading enzymes in EC [29,30]. In RA, visfatin serum and synovial fluid levels are increased [31,32] as well as in Crohn's disease and ulcerative colitis [26].

The number of available SSc lung tissue was very limited. Therefore, tissues of patients with idiopathic pulmonary fibrosis (IPF) were included for comparison with another fibrotic lung disease. IPF represents the most common fibrotic lung disease with an estimated prevalence ranging from two to 29 cases per 100,000 [33]. Histopathological and radiographic findings are in line with a pattern of an interstitial pneumonia (UIP). Before the diagnosis of IPF can be made, other causes of interstitial lung disease have to be excluded [34]. The pathophysiology is not fully understood, but the concept of IPF as a form of abnormal wound healing due to myofibroblast proliferation has been accepted [35]. Genetic and environmental risk factors in combination with local micro-injuries lead to activation of alveolar epithelial cells and secretion of fibrogenic factors [36]. Similar to SSc fibrosis, the transforming growth factor β (TGF β) plays a major role in the IPF pathogenesis [37].

Due to the known involvement of adipokines in inflammation and potentially also in fibrosis, the aim of the study was to investigate expression patterns of adipokines in SSc and IPF lung tissue as well as in GI samples of patients with SSc by analyzing different areas of the affected tissues and correlating the results with the presence of key inflammatory cells.

Table 1
Characteristics of patients included in the adipokine analysis of gastric samples.

Characteristics of the participants		
	Gastritis (N = 11)	SSc (N = 9)
Age (years)		
Median (range)	39.0 (21–72)	58.0 (33–51)
Sex (male, %)	45.5	33.0
Clinical severity of gastritis (0–3)	0.9	1.3
Histologic severity of gastritis (0–3)*	1.3	0.7
Other upper GI pathology		
Hernia (%)	36.4	33.3
Reflux esophagitis (%)	36.4	77.8
PPI (%)**	22.2	0.0

* no information available for two patients of the SSc-group.
** no information available for one patient of SSc-group.

2. Material and methods

2.1. Tissues

Lung samples were formalin-fixed and paraffin-embedded. Small tissue samples from all groups were snap frozen for protein isolation and ground into powder using liquid nitrogen, mortar and pestle. Pulverized samples were mixed with lysis buffer (0.05 M Tris, 1% Nonidet-P40, Roche miniComplete® protease-inhibitor cocktail) and sonicated 30sec on ice. Undissolved fractions were removed by centrifugation (5 min 800 × g, 15 min 10,000 × g). Protein content was determined with the BCA protein assay kit (Thermo Fisher Scientific).

Standard forceps biopsies of antrum and corpus including mucosa and submucosa were collected during gastroscopy (adipokine analysis: nine SSc, twelve non-SSc related gastritis controls without rheumatic diseases, table 1). Samples were embedded in OCT-TissueTek and snap frozen. Lung tissues from eight IPF and two SSc patients were obtained during lung transplantation and were compared to eight lung tissue preparations from organ donors obtained during atypical resection due to size incompatibility (referred to as donor lungs). In addition, bronchoalveolar lavage (BAL) liquids were collected from 26 IPF, 3 EAA (extrinsic allergic alveolitis), 2 SSc and 10 healthy subjects, centrifuged and stored at −80 °C. Small lung tissue biopsies for protein lysates were taken during bronchoscopy in appropriate patients. All human samples were obtained in the frame of existing ethical votes of the ethics committees of the Universities Regensburg and Giessen (eurIPFreg AZ 111/08, AZ 94/95, AZ 84/05) and all patients gave written informed consent.

2.2. Immunohistochemistry

5 µm frozen acetone-fixed sections were blocked for endogenous peroxidase activity and unspecific bindings (5% milk powder or 10% horse serum). Primary antibody (suppl. 1) was incubated overnight at 4 °C. Sections were incubated for 40 min with the Histofine detection system (Medac). For visualization, the AEC kit was used (Vector-Laboratories). 5 µm paraffin-embedded tissue sections were deparaffinized followed by rehydration. Slides were digested with proteinase K and stained as described above. Nuclei were stained with hematoxylin. Isotype controls and controls without primary antibody were used. For evaluation, "PhotoScape"™ software was used. Large lung tissues were scanned (Carl Zeiss) and evaluated using "MiraxViewer"™ software allowing high resolution evaluation.

All samples were encoded and randomized before evaluation which was performed at once. For each patient, two different gastric samples

were evaluated and the mean calculated. Evaluation of CD4/CD8/CD68 was performed quantitatively for three equally sized areas/sample (1 × submucosa, 2× mucosa). Mean of all areas was calculated (size: 300 × 200 dpi = 200 × 135 µm). Numbers and location of inflammatory infiltrates were quantified separately. Adipokine protein expression was analyzed qualitatively: Presence (1) or absence (2) of staining was scored and anatomic-morphological areas evaluated: glandular epithelium, lamina propria and basal lamina.

For lung samples, adipokine evaluation was performed semi-quantitatively for the whole section followed by anatomic-morphological area evaluation (score 0: no staining, score 1: few stained areas or single cells, 2: intermediate staining with a minimum of half the section stained, score 3: most of the section stained with intermediate to strong intensity). Surfactant protein C (SPC) staining served as control for anatomic-morphological area assignments: vessel wall of large vessels, bronchial epithelium with kinocilia, alveolar septate and fibrosis areas (suppl. 2). Presence and number of inflammatory infiltrates were evaluated.

2.3. Immunoassays

Adipokine levels in liquids from BAL (concentrated using Microcon® devices, Millipore) and protein lysates were measured by ELISAs (BioVendor & AdiopGen).

2.4. Fibrosis scoring of lung tissue

Formalin-fixed and paraffin-embedded lung samples were deparaffinized followed by rehydration and then stained with a trichrome staining kit (Abcam). For every patient two sections were analyzed. SSc and IPF samples were from the same patients as used in previous analyses, whereas the healthy controls were equivalent samples. For each slide, images of four microscopic fields in three different magnifications (25×/50×/100×) were taken. The samples were encoded, randomized and each 100× magnification scored by a blinded investigator. For scoring a modified Ashcroft scale as described by Hübner et al. [38] was used. For each patient, the mean of the eight scorings was calculated and used for statistical analysis.

2.5. Statistics

Using GraphPad Prism version 6.0, mean, standard deviation, median and variation were calculated. For non-normally distributed data, non-parametric tests were performed. For evaluation of quantitative and semiquantitative data (CD4/CD8/CD68/adipokines/fibrosis score), the Mann-Whitney U test was used. For evaluation of qualitative data (adipokines in stomach tissues), Fisher's exact test was used and a contingency table generated. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Severity of pulmonary fibrosis

The lung parenchyma of patients suffering from IPF and SSc were damaged severely (Fig. 1). This was confirmed by the fibrosis scores ranging between 3.6 and 7.3, whereas healthy controls showed mostly normal lung tissues with fibrosis scores lower than 1.6. The mean score of IPF tissues was 6.1 and higher in comparison to healthy controls ($p = 0.002$; Fig. 1). SSc samples showed a comparable strong fibrosis to IPF lung tissue confirmed by a score of 5.9.

3.2. Adiponectin in lung samples

In lung parenchyma, strong cell-specific adiponectin signals were detectable in controls whereas fibrotic lungs from SSc and IPF showed very low or absent adiponectin expression (Fig. 2A/D, Table 2). Adiponectin was specifically expressed in larger vessels with fibro-muscular walls (Fig. 2E, suppl. 3) and in thin vessels mainly consisting of the endothelium with the strongest signals detectable in donors (mean 2.38), followed by IPF (1.75) and SSc (1.5) (Fig. 2B, Table 2). However, statistical significance was not reached ($p = 0.059$). In contrast, bronchial epithelium showed no or very weak adiponectin signals in all groups (suppl. 3) mainly located within kinocilia. Of note, parenchyma of healthy donors showed adiponectin signals especially in thin septa. Lung septa in fibrotic lungs were thickened and in part single septa no longer distinguishable. Tissue lysates prepared from tissues corresponding to immunohistochemical staining confirmed the reduced adiponectin expression in IPF (vs. control $p < 0.0001$) and SSc (Fig. 2C).

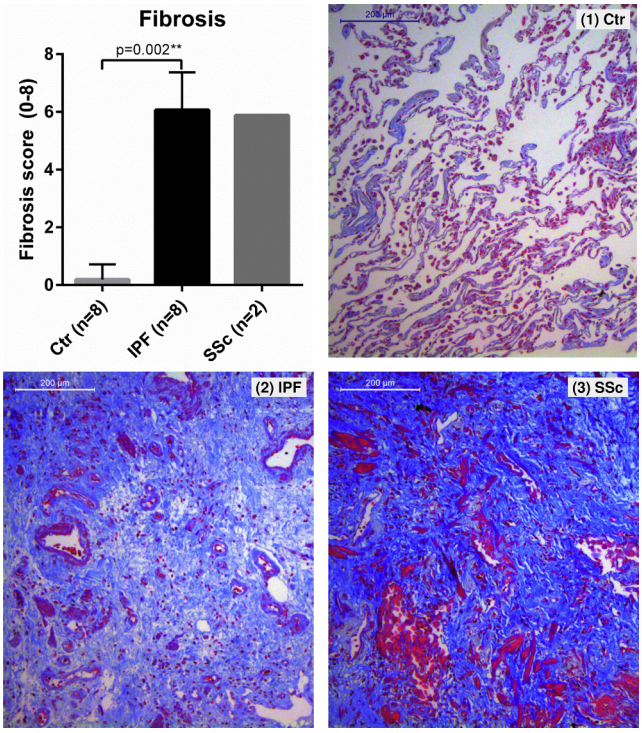

Fig. 1. Severity of pulmonary fibrosis in the included tissues. The fibrosis score was higher in lung tissues of IPF and SSc compared to healthy donors (Ctr). By trichrome staining with collagen stained blue, healthy controls showed a normal lung structure, whereas in IPF (2) and SSc (3) the alveolar septa were mostly not existent and the alveoli were obliterated with fibrous masses.

BAL liquid and additional protein samples of lung tissue biopsies representing earlier, inflammatory stages of SSc lung involvement, confirmed the presence of adiponectin in early inflammatory SSc lung disease (Fig. 2F/G). In BAL liquid and in protein lysates of lung tissue biopsies, adiponectin was higher in patients with inflammatory lung involvement including SSc vs. controls.

3.3. Resistin and visfatin in lung samples

Resistin staining in lung was predominantly cell-associated. Resistin was present in all tissue areas but slightly decreased in IPF (Fig. 3A – C). However, there was no relevant difference in overall resistin expression between fibrotic vs. control samples. All tissues showed intermediate resistin signals (suppl. 4). Fibrotic parenchyma contained low numbers of resistin-positive cells, whereas in IPF and SSc resistin-positive cells were increased within immune cell infiltrates, which were usually absent in controls (suppl. 4). Epithelial cells of the bronchial system showed intermediate to strong resistin signals without significant difference between IPF and donors (Fig. 3B). In walls of larger vessels,

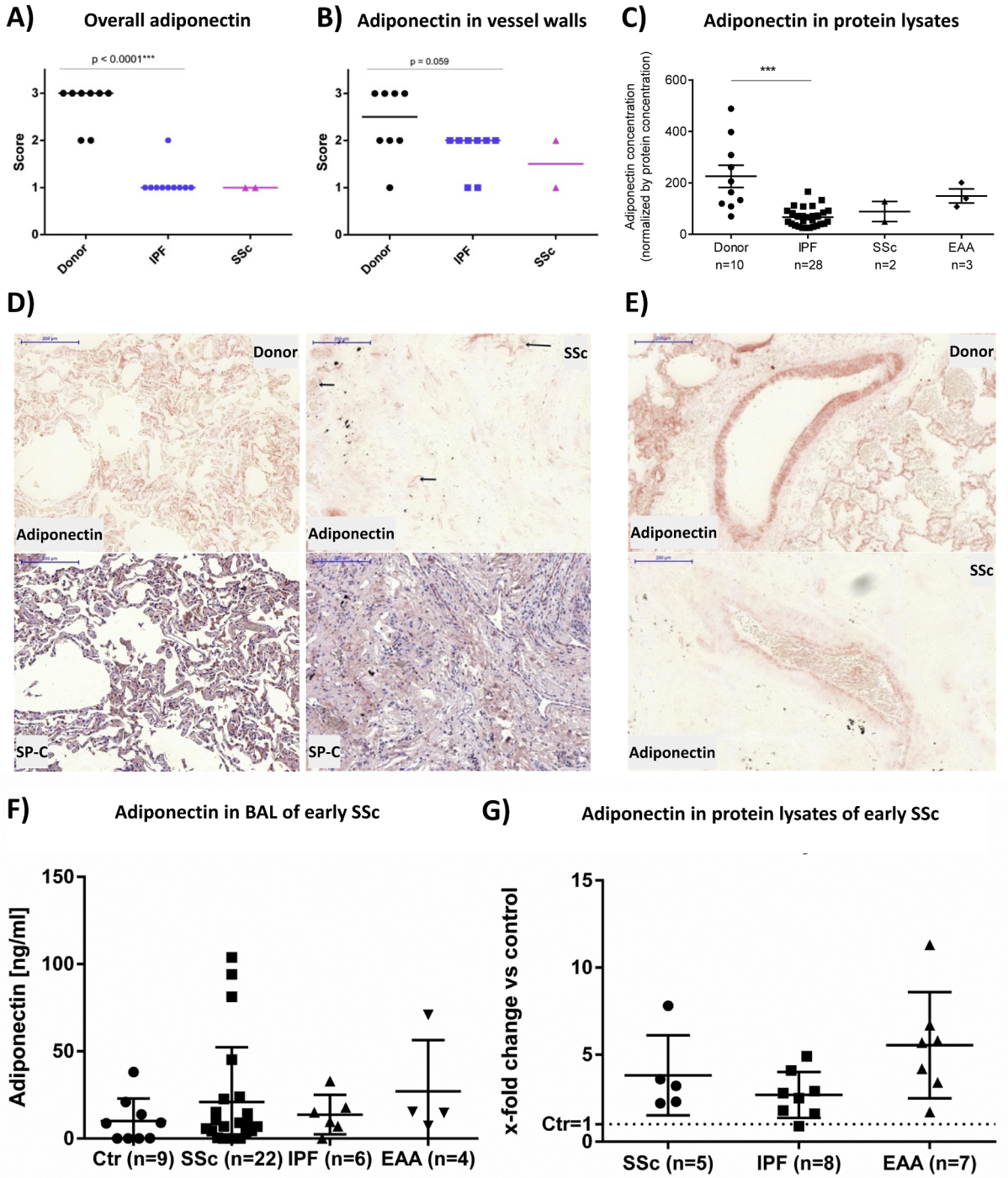

Fig. 2. Adiponectin in lung tissues and bronchoalveolar lavage (BAL) of healthy donors, EAA, IPF and SSc. (A) Overall adiponectin staining was strongly reduced in IPF and SSc. (B) Adiponectin intensity was lowest in SSc vessels. (C) Protein lysates (histology matched) confirmed the reduced adiponectin expression in lung tissue with IPF compared to healthy controls. (D) Adiponectin in lung parenchyma was strongly reduced in SSc (arrows: vessels) compared to donors. In the lower panels epithelial cells can be identified by SP-C staining (red). Nuclei were stained blue (hematoxylin). (E) Donor vessels expressed more adiponectin than SSc. In early stages of SSc, adiponectin was higher in BAL liquid (F) and in protein lysates of lung tissue biopsies (G).

resistin-positive cells were detectable in all groups (Fig. 3C).

All tissues showed intermediate or strong visfatin staining (Fig. 4A/E, suppl. 4). In contrast to immunohistological staining, corresponding lung tissue lysates showed that visfatin was significantly reduced in IPF ($p < 0.0001$) but not SSc compared to control (Fig. 4B). Within parenchyma, all tissues expressed visfatin with a regular distribution of positive cells in donors. In contrast, visfatin expression especially in IPF but also in SSc showed few, solitary visfatin-positive cells within fibrotic areas (Fig. 4E). However, visfatin staining was increased in inflammatory infiltrates with strong intensity (suppl. 3, suppl. 4). The strongest visfatin expression was localized within the bronchial epithelium including kinocilia (Fig. 4E, suppl. 3, suppl. 4).

BAL liquid and additional small protein samples of lung tissue biopsies representing earlier, inflammatory stages of SSc lung involvement confirmed the presence of visfatin in early inflammatory SSc lung disease (Fig. 4C/D). In BAL liquid, visfatin was higher in patients with inflammatory lung involvement including SSc vs. controls, but the variability was high in the collected liquid (SSc vs. control: visfatin

Table 2
Adipokine expression in anatomic areas of donor, IPF and SSc lung tissues. Median, mean and standard deviation of the semi-quantitative scoring (0 = low to 3 = strong). Due to the low number of available SSc lung samples, statistical evaluation refers to donor vs. IPF group.

Adiponectin:		Donor (n = 8)	IPF (n = 8)	SSc (n = 2)	p value
Overall expression in tissue	median	3	1	–	< 0.0001***
	mean	2.75 +/− 0.16	1.10 +/− 0.1	1.0 +/− 0.0	
vessel wall	median	2.5	2.0	–	0.059
	mean	2.38 +/- 0.26	1.75 +/- 0.16	1.5 +/- 0.0	
parenchyma	median	3.0	1.0	–	< 0.0001***
	mean	2.75 +/- 0.16	1.0 +/- 0.19	1.0 +/- 0.0	

p = 0.06).

3.4. Adipokines and inflammatory cell distribution in gastric samples

Standard endoscopic gastric biopsies were evaluated which do not include the deeper fibrotic areas located in the muscularis mucosase and deep regions of the lamina propria which are not included in the more superficial endoscopic samples [39]. To evaluate whether there is a difference in the endoscopic biopsies regarding adipokines and inflammatory cells, adipokine and cell marker staining were performed. SSc tissues contained significantly more $CD68^+$ cells in mucosa and submuosa compared to non-SSc related gastritis (suppl. 5). However, the numbers of $CD4^+$ or $CD8^+$ cells in SSc gastritis did not differ from non-SSc related gastritis (suppl. 5) and could, therefore, not distinguish between both etiologies. The decrease of adiponectin in SSc and IPF lung tissue indicated a possible role in pathogenesis of SSc. Indeed, a significant decrease in adiponectin expression was observed between SSc-gastritis and non-SSc related gastritis (p = 0.049, Fig. 5A) although

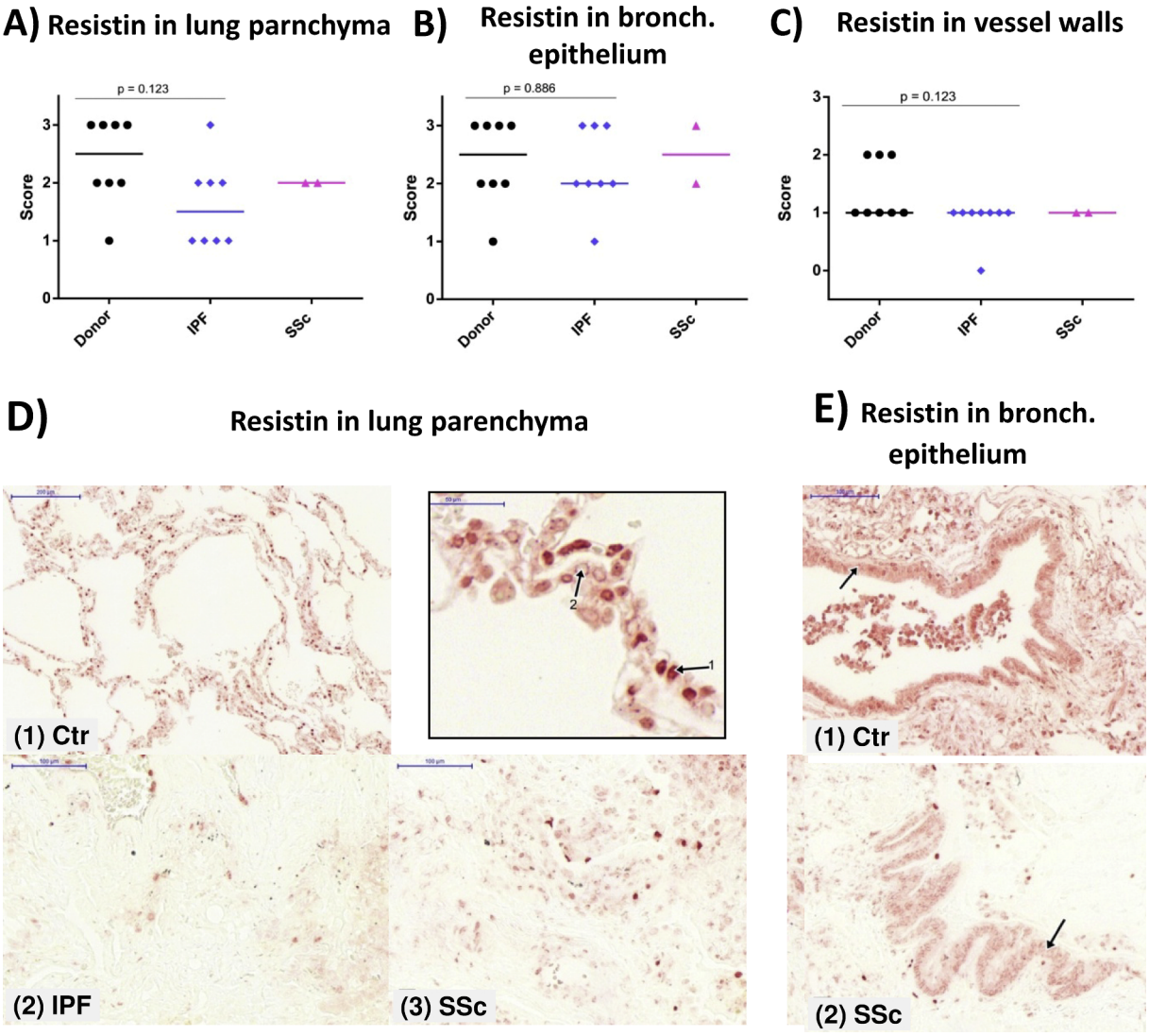

Fig. 3. Resistin in lung tissues of healthy donors, IPF and SSc. (A) Resistin expression was in general decreased in both, IPF and SSC lung parenchyma with no difference in bronchial epithelium (B) or vessel walls (C). (D) Donor parenchyma showed regular, cell associated resistin-signals ((1) Ctr, arrow1: strong, arrow2: intermediate); IPF ((2) IPF) and SSc ((3) SSc) showed reduced resistin-signals. (E) In bronchial epithelium, donor ((1) Ctr), IPF and SSc ((2) SSc) showed regular resistin-signals.

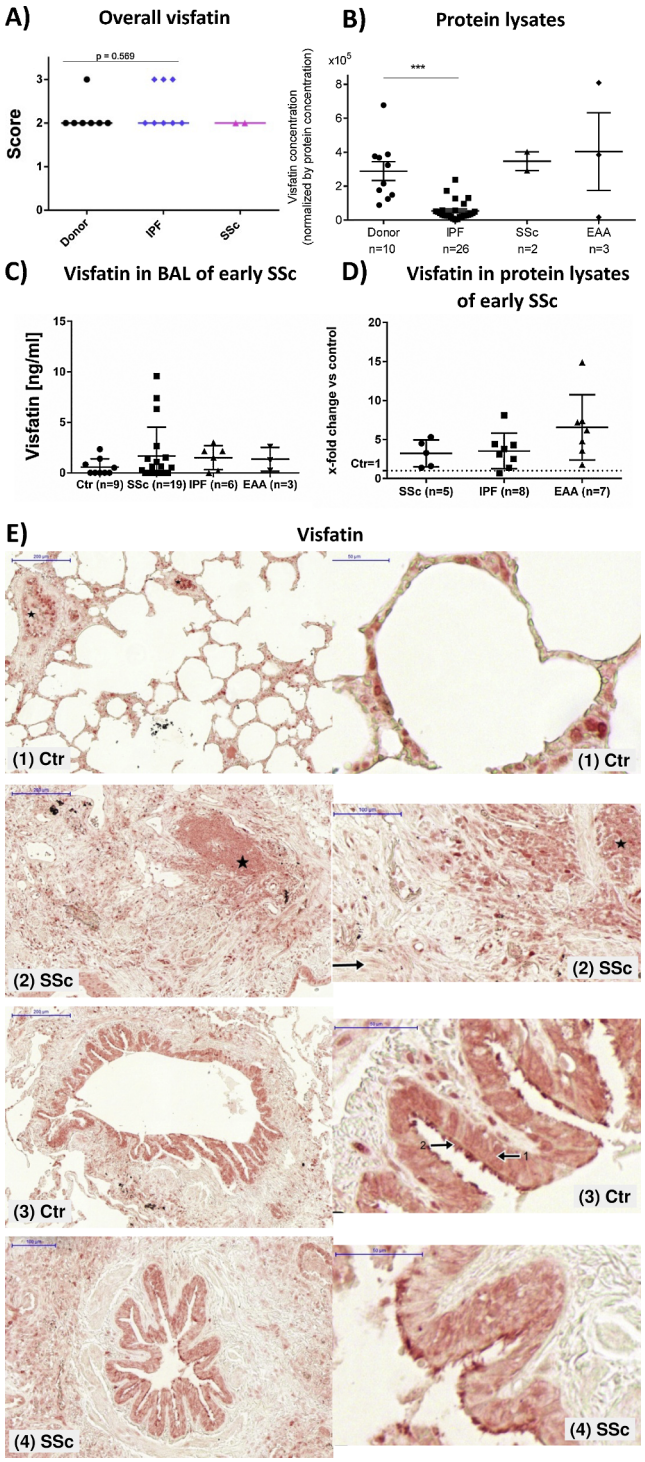

Fig. 4. Visfatin in lung tissues and bronchoalveolar lavage (BAL) of healthy donors, EAA, IPF and SSc. (A) Overall visfatin staining was comparable in donors, IPF and SSc. (B) In contrast, protein lysates of IPF lung tissue showed decreased visfatin concentrations compared to donors. (C,D) In early stages of SSc, in IPF and in EAA visfatin was higher in BAL liquid and in protein lysates of lung tissue biopsies compared to controls. (E) Donor parenchyma showed regular visfatin-signals ((1) Ctr, star: immune cells) but irregular staining in IPF and SSc ((2) SSc star: infiltrate). All bronchial epithelia (arrow1) expressed visfatin ((3) Ctr: arrow2: kinocilia, (4) SSc).

the areas of fibrosis are not included in the respective samples. Interestingly, adiponectin-positive tissues showed higher amounts of $CD4^+$ cells (Fig. 5B, Table 3), whereas $CD8^+$ T cell numbers were comparable (suppl. 6). $CD68^+$ cell number was higher in adiponectin-negative tissues in both groups (Fig. 5B, Table 3). Adiponectin was expressed in the walls of small vessels, submucosa and single cells in the lamina propria of gastritis controls (Fig. 5C). In controls, 91% of tissues were adiponectin-positive in vessels and/or cells of the lamina propria. In 44% of SSc patients, adiponectin-positive staining was only detectable in vessels (suppl. 6).

Most SSc and gastritis tissues expressed resistin without significant difference (Fig. 6A). In some patients, resistin staining was limited to the lamina propria and basal lamina, whereas in other tissues the mucosal glandular epithelium was resistin-positive (Fig. 6C). In SSc significantly more $CD4^+$ T cells were present in resistin-negative tissues (Fig. 6B, suppl. 7), whereas in controls no correlation between $CD4^+$ T cell numbers with resistin expression was seen.

All SSc patients as well as 91% of controls showed visfatin expression (Fig. 6B). When evaluating anatomical compartments, similar areas expressed visfatin comparable to resistin (suppl. 6). In SSc as well as controls, visfatin was expressed in the lamina propria, glandular epithelium and basal lamina (suppl. 6). Visfatin was expressed in more than one anatomical region in most tissues.

The presence of resistin and visfatin corresponded more strongly with each other than with the presence of adiponectin (suppl. 7). Interestingly, all visfatin-negative tissues were adiponectin-positive in controls, whereas in SSc visfatin-negative tissues were also adiponectin-negative.

4. Discussion

This is the first study to investigate adipokine distribution in two dominantly affected tissues of patients suffering from SSc and in lung tissues of patients suffering from IPF. Expression differed between the adipokines and also between the compartmental and cellular distribution. Although systemic adipokine levels are altered in chronic inflammatory rheumatic diseases, e.g. RA and SSc (e.g. [8,40,41]), knowledge is limited on adipokine tissue localization.

Decreased adiponectin levels in fibrotic skin areas and in sera of diffuse cutaneous SSc (dc-SSc) [42] and the inverse correlation with skin scores in dc-SSc [43] were previously shown. Data on organs other than skin are limited due to tissue availability. For the adipokine leptin a correlation between elevated plasma levels and an acute exacerbation of IPF is known [44]. However, there are no data regarding the expression of adiponectin or other adipokines in the affected lung tissue. We could show that adiponectin expression in lung tissue correlated inversely with lung fibrosis of patients suffering from IPF, while healthy lungs showed strong adiponectin expression. The same correlation between lung fibrosis and adiponectin expression was also observed in SSc tissues, however, the value of this statement is limited due to the small number of included patients.

Interestingly, adiponectin-deficient mice developed a spontaneous lung phenotype with activated lung endothelium, perivascular immune cell infiltration and increased pulmonary arterial pressures [45] and they showed increased eosinophilic inflammatory vascular responses to allergic lung challenge [46]. It is also known, that adiponectin is a regulator of tissue remodeling and cell proliferation in lung [40] and adiponectin receptor 1 is present on lung epithelium [47].

In rheumatoid arthritis adiponectin displays local pro-inflammatory effects [6,48]. The anti-fibrotic properties of adiponectin to attenuate liver fibrosis, to prevent myocardial fibrosis associated with pressure overload and ischemia [11,49], to suppress type I collagen expression in normal and SSc skin fibroblasts and to attenuate stimulation of profibrotic effects by TGF-β [50] were previously shown. Additionally, adiponectin was significantly decreased in SSc patients with active interstitial lung disease vs. healthy controls, however, significantly increased after intravenous pulse cyclophosphamide treatment [10]. Therefore, not only adiponectin levels *per se* but also response of adiponectin to treatment may be relevant.

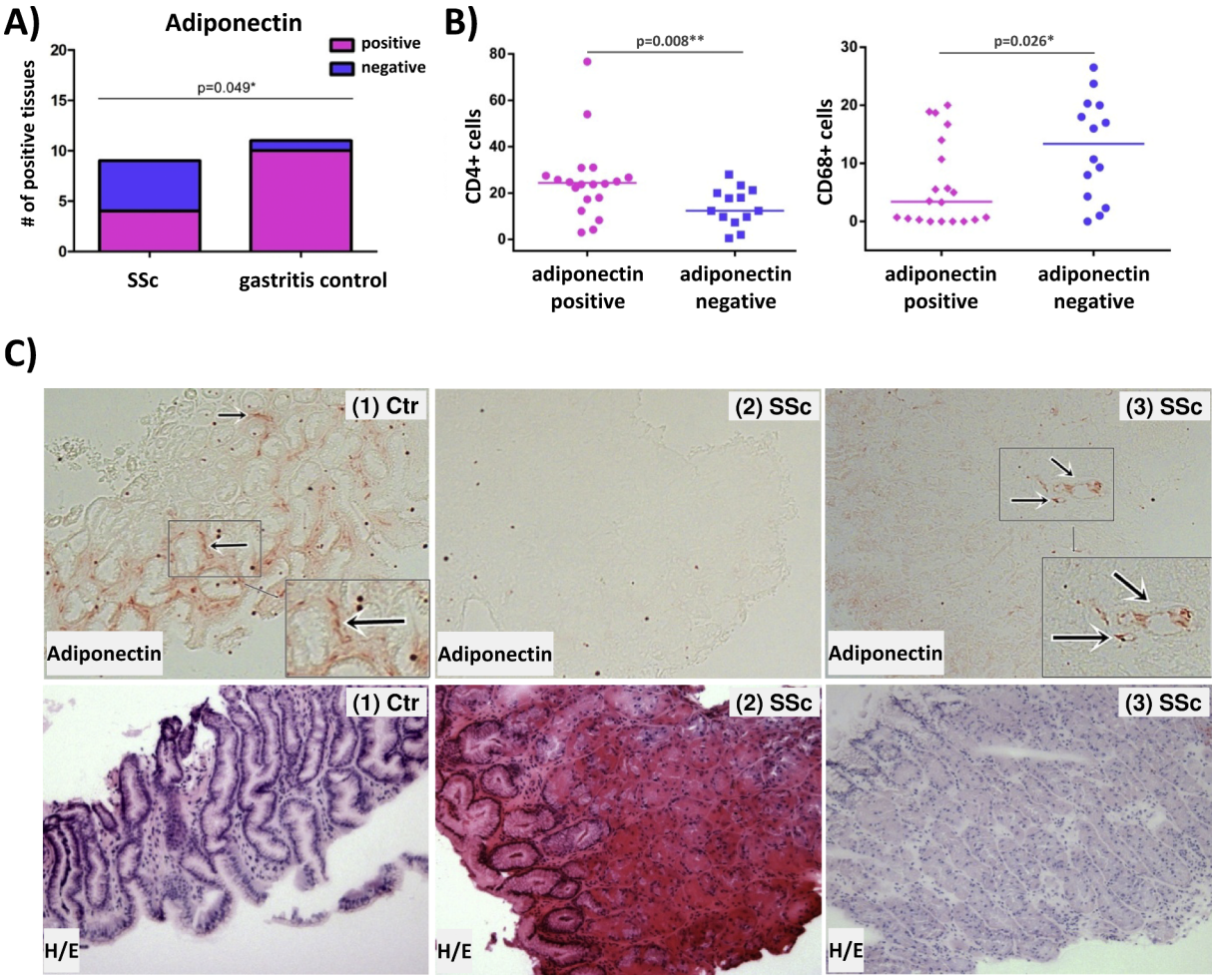

Fig. 5. Adiponectin in SSc and gastritis controls. (A) 44% of SSc and 91% controls were adiponectin-positive. B) Adiponectin-positive tissues contained more $CD4^+$ cells. Adiponectin-negative tissues showed more $CD68^+$ cells. C) Adiponectin was expressed in gastritis controls ((1) Ctr), especially in the lamina propria ((1) Ctr, arrow/box), whereas SSc were mostly adiponectin-negative ((2) SSc) except of small vessels ((3) SSc arrow/box).

Table 3
Correlation of adipokines with immune cells and between stomach SSc and gastritis control samples. Median and p values are shown.

	adiponectin + tissues	adiponectin - tissues	p value
Number of $CD4^+$ cells	24.4	12.3	0.008**
SSc	22.0	15.0	0.543
Gastritis control	24.4	9.7	0.019*
Number of $CD68^+$ cells	3.4	14.4	0.026*
SSc	5.0	17.0	0.251
Gastritis control	3.3	9.3	0.189
	resistin + tissues	resistin- tissues	p value
Number of $CD4^+$ cells	12.3	24.0	0.024*
SSc	12.3	27.4	0.004**
Gastritis control	21.7	23.7	0.846

The SSc tissues in our study were collected during lung transplantation representing very advanced fibrosis. Access to early inflammatory stages of SSc lung tissues is limited. However, protein expression in BAL and small lung samples confirmed the expression of all adipokines in earlier SSc lung disease and IPF and the strongly reduced expression could only be observed in the end-stage disease tissues. Therefore, the vital difference observed regarding down-regulation of adiponectin seems dependent on fibrotic progression.

Knowledge on adipokine alterations in gastric SSc involvement is even more limited. The inflammation due to SSc is characterized by T and mature B cell infiltration as well as by an increased $CD4^+/CD8^+$ - ratio as previously shown [51]. To evaluate whether the SSc-specific changes can be identified in standard gastroscopic biopsy samples not containing the deeper areas of fibrosis [39], non-SSc related gastritis samples were used to avoid comparison of non-inflammatory healthy conditions with inflammation and fibrosis processes in SSc. Indeed, the number of $CD4^+$ and $CD8^+$ T cells was comparable in SSc and non-SSc related gastritis and could not distinguish between both etiologies. However, we found a higher number of patients with reduced adiponectin expression in gastric tissues of SSc patients compared to controls although the deeper fibrotic tissue areas were not collected due to the depth of the standard gastroscopy biopsy collection procedure. This finding is in line with the results detected in the lung tissues of two SSc patients. Few samples displayed adiponectin in the lamina propria and submucosa although fibrosis takes place in deeper regions as previously shown [39,51]. Interestingly, adiponectin-positive tissues showed significantly higher $CD4^+$ T cell numbers in controls and SSc and comparable numbers of $CD8^+$ T cells. $CD68^+$ cell numbers were higher in adiponectin-negative tissues of both groups. Therefore, SSc gastric samples are not only characterized by increased $CD4^+/CD8^+$ T cell ratio in comparison to normal controls as previously shown [51] but also by decreased adiponectin expression specifically in comparison to non-SSc related gastritis.

Taken together, the known anti-fibrotic properties of adiponectin and the decreased expression in IPF and SSc tissue indicates a possible role of adiponectin in fibrosis in these two diseases. It is unclear whether adiponectin is really included in the pathogenesis or whether the

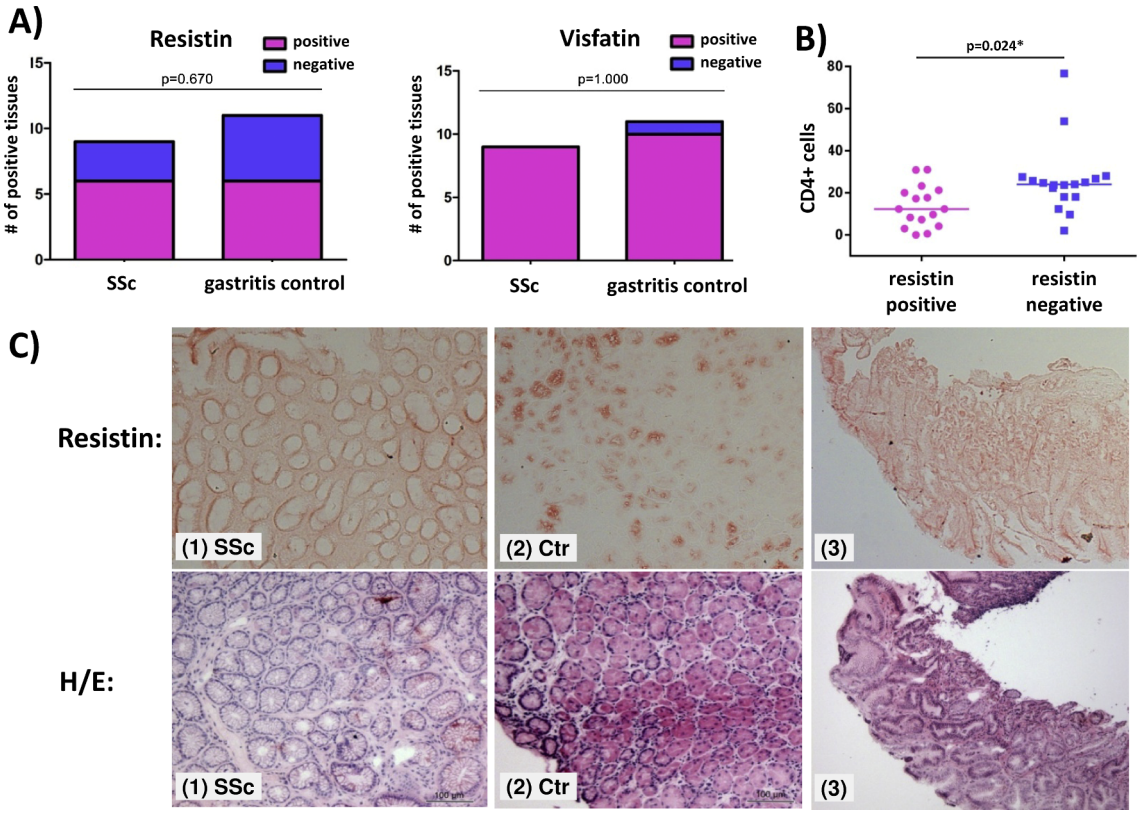

Fig. 6. Resistin and Visfatin in SSc and gastritis controls. A) 67% of SSc-patients and 55% of controls expressed resistin. B) Resistin-negative tissues had more $CD4^+$ cells. C) Most SSc tissues ((1) SSc) expressed resistin in lamina propria and basal lamina whereas glandular epithelium was stained in controls ((2) Ctr). Some samples showed general staining (3).

reduction of adiponectin is a result of the fibrosis. Further studies are required to first confirm the hypothesis of reduced adiponectin in fibrotic tissues and then to clarify the role in the pathogenesis.

For resistin, *in vitro* knowledge is limited in the context of SSc or IPF. We found no significant difference in resistin expression between fibrotic lungs and controls and also no difference in the examined gastric SSc samples. However, fibrotic lung parenchyma areas in IPF and SSc showed increased numbers of resistin-positive cells within immune cell infiltrates (usually absent in healthy controls). This suggests resistin to be associated with inflammation as shown for other inflammatory diseases [19–21], but not yet for fibrosis.

Interestingly, visfatin, also displaying mainly pro-inflammatory properties, was not differentially expressed systemically in SSc and control, dc- or lc-SSc [52]. We also detected visfatin expression in nearly all lung tissues without differences between controls and IPF or SSc. However, only few single visfatin-positive cells were found in fibrotic areas (IPF and SSc) but increased numbers in inflammatory infiltrates. This is in line with findings that visfatin is induced by pro-inflammatory stimuli e.g. in RA [53].

Comparing adipokine patterns, overlaps between resistin and visfatin were obvious, while adiponectin differed. In donors, intermediate expression of all adipokines was detectable. Fibrotic tissues (IPF and SSc) showed intermediate to strong resistin and to a lesser extent visfatin expression whereas adiponectin was strongly reduced. In fibrotic tissues, inflammatory infiltrates were resistin- and visfatin-positive.

In summary, decreased expression of adiponectin is linked to fibrotic changes in idiopathic pulmonary fibrosis and probably in SSc organ involvement, whereas resistin and visfatin were found to be associated with inflammation.

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Competing Interests

There are no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.11.013.

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