Serum IGFBP-2 in Systemic Sclerosis as a Protective Factor of Lung Dysfunction

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Abstract

Background Systemic sclerosis (SSc) is a rare connective tissue disease associated with rapid evolving interstitial lung disease (SSc-ILD), driving its mortality. Specific biomarkers associated with the progression of this lung disease are highly needed. We aimed to identify specific biomarkers of SSc-ILD to predict the evolution of the disease.

Methods We compared prospectively serum levels of several biomarkers associated with lung fibrosis in SSc patients (n = 102) compared to healthy subjects (HS) (n = 39). We also performed a longitudinal study in a subgroup of 28 patients analyzing biomarkers variations and pulmonary function tests over a period of 2 years. Furthermore, we performed in vitro analysis to study the impact of Insulin like Growth Factor Binding Protein (IGFBP)-2 on fibrotic activity of human lung fibroblasts.

Results Serum levels of IGFBP-1, IGFBP-2, interleukin-8 and matrix metallopeptidase-9 were significantly increased in SSc patients compared to HS while IGF-1 and IGFBP-3 were reduced. The variation of IGFBP-2 between baseline and 2-year follow-up was positively correlated with pulmonary function (assessed by carbon monoxide transfer coefficient (KCO)) at 2-year follow-up (r = 0.6, p < 0.001). Receiver operating characteristic curve analysis enabled us to identify that baseline IGFBP-2 < 105 ng/ml was associated with a better outcome (low risk to display KCO < 70% predicted) at 2-year follow-up (area under the curve = 0.75 at 75% sensibility and 68% specificity, p < 0.05). In vitro functional study showed that IGFBP-2 significantly reduced fibroblast proliferation and pro-fibrotic activity.

Conclusions We showed for the first time that serum levels of IGFBP-2 might predict the evolution of SSc-ILD. Baseline IGFBP-2 above 105 ng/ml might be a prognostic factor of alveolo-capillary dysfunction.

Background

Systemic sclerosis (SSc) is a complex systemic disease of unknown origin associated with a multi-organic affection involving a complex interplay of microvasculopathy, disturbances in fibroblastic function and abnormalities of the immune system [1, 2]. One of the major complications of that pathology driving the mortality of the patients is the interstitial lung disease (ILD) [3, 4] with an estimated incidence ranging from 40−75% [5, 6]. The main pathological pattern associates vascular inflammation leading to a progressive fibrosing process characterized by excessive collagen production. This collagen deposition is leading to tissue fibrosis of the skin and the internal organs reducing its functionality [7]. The main organ involved in the SSc is the skin. Besides the skin, kidneys and lungs are frequently attacked. In lungs, SSc is expressed as a chronic interstitial lung disease (SSc-ILD) having many common clinical and pathological characteristics with some other major ILDs, mainly idiopathic pulmonary fibrosis (IPF) [8−11].

As it is described in many other ILDs, SSc-ILD is associated with multiple clinical phenotypes. It can progress with a wide spectrum of course ranging from a rapid progressive lung disease as well as a slow evolving ILD but also a non-progressive ILD. Contrary to what is seen in IPF [12], treatment is mainly

based on an aggressive immunosuppressive therapy specifically proposed in the progressive forms of SSc-ILD. One of the major problem clinicians have to deal with, as in other ILD, is to identify patients at increased risk of progression for early intervention [5, 13–16]. In this context, biomarkers are highly needed in order to help clinicians for diagnosis, prognosis and the evaluation of the therapeutic response. The ideal biomarker would be a good predictors in SSc-ILD [14, 17]. Although antinuclear antibodies (ANA) were the first biomarkers available, those biomarkers do not allow for disease activity monitoring [18, 19].

The transforming growth factor beta (TGF-β) is known to be involved in the pathophysiology of many lung fibrotic diseases by stimulating the deposition of collagen and increasing lung remodeling [20, 21]. TGF-β is also widely known as an inducer of fibroblast activity. Besides TGF-β, previous studies identified that insulin-like growth factor-binding proteins (IGFBPs) were also clearly associated with IPF [22] and of interest as new potential biomarkers [23].

Insulin-like growth factors (IGFs) are peptides growth factors playing a crucial role in normal growth and development in many tissues as well as in maintaining cellular homeostasis by regulating several cellular processes (such as cell proliferation, differentiation, metabolism) [24]. IGFs are found in serum and other biofluids associated with IGFBPs. Through the sequestration of IGFs, IGFBPs regulate IGF stability, transport and tissue distribution, thus determining the bioavailability and biological activity of IGFs [25]. It has been shown that IGFBPs can inhibit or potentiate IGF activities in vitro depending on cell type and experimental conditions [26]. The main IGFBPs described in ILD context are IGFBP-1 and IGFBP-2. It has been shown that IGFBP-1 and – 2 were able to improve tissue repair in inflammatory conditions by increasing fibroblast proliferation and migration [27]. In SSc context, IGF-1 and IGFBP-3 are involved in abnormal repair process [28, 29]. Further, elevated levels of IGFBP-2 were found in broncho-alveolar lavage fluid of ILD patients [30], but also in serum and sputum of IPF patients [15, 31].

We focused our study on the quantification of several serum growth factors including IGFs and IGFBPs in order to identify a potential new biomarker for SSc and sought to determine whether these biomarkers might influence the evolution of the disease.

Methods

Subject characteristics

In this study, we prospectively recruited patients with SSc (n = 102) from our ambulatory clinic at CHU Liege from 2010 to 2018 and compared them to healthy subjects (HS) (n = 39) (Fig. 1). The diagnosis of SSc was made according to the international recommendations of ACR/Eular [7]. SSc-ILD was defined by a combination of specific HRCT images of at least 10% of all parenchyma (reticulations, honey combing and/or ground glass opacities) with clinical signs (velcros or crackles) or symptoms (cough, shortness of breath) and alteration of pulmonary function tests. We excluded all other causes of interstitial lung disease (such asbestosis, idiopathic pulmonary fibrosis, idiopathic non specific interstitial pneumonia,

hypersensitivity pneumonitis or toxic pneumonitis). All cases were validated after a multidisciplinary discussion in order to confirm the presence or absence of SSc-ILD. Then, we performed a longitudinal study, resampling blood 2 years after the first analysis (n = 28) (Fig. 1). HS were recruited by advertisement in our policlinic waiting room. They all denied any respiratory disease and had normal spirometric values with FEV1 > 80% predicted and FEV1/FVC ratio > 70%. The protocol was approved by the ethics committee of CHU of Liège, and all subjects gave written consent be for their enrollment (Belgian number: B707201422832 ; ref : 2014/302).

Pulmonary function tests

All tests were performed according to the recommendations of the European Respiratory Society (ERS). The results were expressed in percent predicted. The total lung capacity (TLC) was measured by body plethysmography and expressed in percent predicted. The diffusion capacity of CO (DLCO) and the report DLCO/AV (alveolar volume) were measured by the single-breath carbon monoxide gas transfer method and expressed in percent predicted (SensorMedics2400He /CO Analyzer System, Bilthoven, Netherlands).

Biomarkers measurements in serum

Levels of Interleukin (IL)-8, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)-7, Chitinase-3-like protein 1 (YKL-40). IGFBP-1 and IGFBP-3 were assessed by ELISA multiplex using Fluorokine-1. Multianalyte Profiling Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The detection limit for this assays were 3-3-200-230-170-705 pg/ml respectively. The concentration of the other proteins were measured separately by ELISA: TGF-β, MMP-9, IGF-1, IGFBP-2. (DuoSet kit, R&D systems); The detection limits for these kits were 7-25-25-32 pg/ml respectively.

Fibroblast isolation

Primary control human lung fibroblast (HLF) were obtained from around 500 mg specimen of healthy lung from surgery indicated for lung tumor resection in University Hospital from Liège, Belgium. The specimen were cut in part of 3 to 5 mm size and transferred in a gentle MACS™ C-tube (Myltenyi biotech™, Benelux) containing a mixture of HBSS with red phenol (Lonza™, Verviers, Belgium), inactivated FCS 5% (Invitrogen™), collagenase A 1 mg/ml (Roche™) and Dnase I 0.05 mg/ml (Roche™). The tissue was dissociated with a gentle MACS™ Dissociator (Myltenyi biotech, Benelux) according to manufacturer's recommendations and digested in the enzymatic mixture during one hour at 37 °C under agitation. After a supplementary step of dissociation, the cell suspension was filtered on a 70 μm cells strainer (Corning Falcon®) and washed in 50 ml PBS (Lonza™) + 10 mM EDTA. Then cells were plated in a 75 cm flask and cultured in DMEM (Gibco®) containing 2mMGlutamine (Gibco®), 10% inactivated Foetal Calf Serum (FCS) (Invitrogen®), 0.1% fungizone (Gibco®) 100 U/ml penicillin and100mg/ml steptomycin (Gibco®) in a humidified incubator at 37 °Cand 5% CO2. The medium was changed twice a week and after 14 days, cells reached confluence. Cells were harvested by treatment with trypsin /EDTA (Lonza™). All experiment were performed with cell between passage 3 and 5

Protein measurement

Cell culture

60000 cells were plated into12 well plate and cultured in 800 µl DMEM supplemented with 2mMGlutamine, 0.1% fungizone (Gibco®) 100 U/ml penicillin and100mg/ml steptomycin (Gibco®) and 10% inactivated FCS. When cells reached 80% of confluence, the medium was removed and replaced by medium with 2% inactivated serum or without serum alone or in presence of TGF-β 5 ng/ml (R&D Systems Europe, Abingdon, UK). After 48 h while culture mediums were recovered and conserved at -20 °C for protein measurement, cells were used after trypsinisation for FACS analysis and an immunocytochemistry to alpha-Smooth Muscle Actin (α-SMA) were performed on adherent cells

IGFBP-2 and Pro-col-1 ELISA

IGFBP-2 and Pro-col-1 were measured in culture supernatant by commercial Kits ELISA (DUOSET kit from R&D Systems Europe, Abingdon, UK) according to instructions provided by the manufacturer. The detection limits was 32 and 62 pg/ml respectively. The culture medium was diluted 5x for IGFBP-2 and 1000x for Pro-Col-1 measurement.

α-SMA immunocytochemistry

After permeabilization with cold methanol and blocking 30 minute with SAB 2% in DPBS, cells were washed and stained with Human α-SMA mouse monoclonal antibody (DAKO M0851) diluted 1/1000. The detection was performed with Envision + HRP system anti mouse (DAKO K4000) and the DAB + substrate chromogen (DAKO K3468) according to instructions provided by the manufacturer. Cells were counterstained 5 secondes with Hemacolor® solution fixation 3 (Merck)

α-SMA FACS analysis

Cells from each well were washed with DPBS fixed in 4% paraformaldehyde for 10 min washed twice with FACS buffer: DPBS, Serum Albumin Bovine 0.2%. Saponin 0.5%, Sodium Azide 0.1% (Sigma-Aldrich, Belgium). And were stained with a human α-SMA-APC-conjugated Antibody (R&D systems) or with the corresponding isotype control: Mouse IgG2A APC-conjugated Antibody (R&D systems). After 20 min. of incubation at 4 °C, cells were washed with DPBS and analysed on a FACS Aria II (BD) flow cytometer .

Viability and Proliferation measurement

8000 cells were plated into 96 well plate and cultured in 200 µl DMEM supplemented with 2 mM Glutamine, antibiotics and 10% inactivated FCS. When cells reached 80% of confluence, cells were deprived of serum during 24 h then cultured in medium free serum. After 48 h, HLF viability and proliferation were evaluated. BrdU (5-bromo-2-deoxyuridine) cell Proliferation Assay Kit were performed according to instructions provided by the manufacturer Cell signaling Technology®,Europe : At the end of the culture, 20 µl BrDU were added into wells. After 4 h of incubation (37°c -5% CO2) cells were fixed and labeled with an BrdU antibody. The revelations were performed with the system Biotin/HRP. The BrdU incorporation was detected by measuring the absorbance at 450 nm

Statistical analysis

Demographic and functional data were expressed as mean ± standard deviation (SD). The biomarkers levels were expressed as median (IQR). Comparisons between groups were performed by Mann-Witney and unpaired "t" test according to the distribution of the variable. Correlations between variables were performed using Spearman's rank correlation test. A $p < 0.05$ was considered as significant. Statistical analysis and graph were performed with Prism Graph Pad software® v6. San Diego.

Results

Study population, patient characteristics, and clinical data

We prospectively recruited patients with SSc (n = 102) from our ambulatory care policlinic at CHU Liege and compared them to healthy subjects (HS) (n = 39) (Fig. 1). Demographic, functional and treatment characteristics of the subjects are given in Table 1. The average age of patients compared to healthy subjects (HS) was similar. FEV1 and FVC were moderately lowered in the SSc group ($p < 0.01$ and $p < 0.001$, respectively). SSc patients displayed a reduced DLCO (66 ± 20% predicted). Of note 38% of our SSc cohort were suffering from SSc-ILD based on chest CT. The blood cell count analysis showed that patient with SSc exhibited higher levels of neutrophils ($p < 0.001$) and reduced levels of lymphocytes ($p < 0.001$) compared to HS. By contrast, circulating monocytes, eosinophils and basophils were similar in the two groups (Table 1). Of note 30% of patients were receiving maintenance treatment with immunosuppressive drugs and 27% were receiving oral corticosteroids.

Table 1
Demographic and clinical characteristics of HS and SSc patients.

	HS (n = 39)	SSc (n = 102)
Age, yrs	59 ± 10	58 ± 12
Gender (M/F)	13/26	27/75
BMI, Kg/m²	26 ± 4	25 ± 5
Smokers (NS/FS/S)	17/16/6	54/28/20
Paq-year	12 ± 18	9 ± 13
leuco x10e3/µl	7 ± 1.5	7.5 ± 2.2
Neutrophil %	55 ± 7	65 ± 9***
Cell/mm³	3842 ± 1156	4999 ± 1985**
Lymphocyte %	35 ± 7	24 ± 9***
Cell/mm³	2389 ± 570	1739 ± 684***
Monocyte %	7.7 ± 2.3	7.9 ± 2.4
Cell/mm³	531 ± 168	586 ± 221
Eosinophil %	2.3 ± 1.8	2.1 ± 1.3
Cell/mm³	155 ± 109	151 ± 96
Basophil %	0.5 ± 0.3	0.5 ± 0.3
Cell/mm³	36 ± 15	37 ± 21
FEV1 post-BD, %pred.	106 ± 18	94 ± 23**
FVC post-BD, %pred.	112 ± 18	98 ± 22***
TLC, %pred.	-	95 ± 18
DLCO, %pred.	-	66 ± 20
KCO, %pred.	-	77 ± 18
Immunosupressor (yes/no)	0/39	31/71
OCS (yes/no)	0/39	28/74
ILD (yes/no)	-	39/63
Disease duration (y)	-	6,9 ± 7,9
Rodnan skin score	-	3,9 ± 5,8

	HS (n = 39)	SSc (n = 102)
ACR/Eular score	-	11 ± 5,5
Limited SSc/lcSSc/dSSc/ SS	-	31/51/10/3
Musculoskeletal involvement (%)	-	11
Renal crisis (%)	-	3
Cardiac involvement (%)	-	3

Data are expressed as mean ± SD. *p < 0.05 **p < 0.001 ***p < 0.0001 compared to HS.

Abbreviations: dSSc = diffuse cutaneous SSc; DLCO = Diffusion lung capacity for CO; FEV1 = Forced expired volume in one second; FS = Former smoker; FVC = Forced Vital Capacity; HS = healthy subjects; ILD = Interstitial lung disease; IT = Immunosuppressive therapy (Mycophenolate Mofetil, methotrexate, cyclophosphamide); KCO = The carbon monoxide transfer coefficient; lcSSc = limited cutaneous SSc; NS = Non smoker; OCS = Oral corticosteroid; S = smoker; SS = Sine scleroderma; SSc = Systemic sclerosis; TLC = Total lung capacity.

Serum biomarkers at baseline

First, we compared the levels of different serum biomarkers associated with lung fibrosis (IGF-1, IGFBP-1, IGFBP-2, IGFBP-3, TGF-β, IL-8, TNF-α, YKL-40, MMP-7, MMP-9 and CRP) between HS and SSc groups (concentrations are listed in Table 2). There is a significant increase in IGFBP-1 (8 to 12.9 ng/ml, p < 0.05), IGFBP-2 (83 to 117 ng/ml, p < 0.001), IL-8 (3.6 to 9.3 pg/ml, p < 0.001), MMP-9 (412 to 967 ng/ml, p < 0.001) and CRP (0.7 to 2.1 mg/l, p < 0.001) levels in SSc patients compared to HS (Fig. 2b, c, e, f) (Table 2). Of note, IGF-1 and IGFBP-3 were significantly reduced SSc patients compared to HS (13 to 8.9 ng/ml, p < 0.05; and 806 to 694 ng/ml, p < 0.05, respectively) (Fig. 2a, d). We did not find any significant correlations between biomarkers and therapies at baseline (immunosuppressive agent or systemic corticosteroids).

Table 2
Concentrations of serum biomarkers in HS and SSc patients.

	HS (n = 39)	SSc (n = 102)
IGF-1 (ng/ml)	13 (8−17)	8.9 (5.1−15.3)*
IGFBP-1 (ng/ml)	8 (3−16)	12.9 (5.4−24)*
IGFBP-2 (ng/ml)	83 (51−109)	117 (73.8-177.6)***
IGFBP-3 (ng/ml)	806 (675−926)	694(571−861)*
ratio IGF-1/IGFBP-1	5 (3−15)	2.8(0.9−10.2)*
ratio IGF-1/IGFBP-2	0.7 (0.4−1.4)	0.3(0.2−0.9)***
ratio IGF-1/IGFBP-3	0.1 (0 -0.1)	0.1(0-0.1)
TGF-β (ng/ml)	26 (24−31)	29 (24−35)
IL-8 (pg/ml)	3.6 (1.5 -7)	9.3 (3.8−17.4)***
TNF-α (pg/ml)	ND	ND
YKL-40 (ng/ml)	33 (24−49)	42 (22−63)
MMP-7 (ng/ml)	1.7 (1.4 -2)	2 (1.3−3.5)
MMP-9 (ng/ml)	412 (221−818)	967 (444−1422)***
CRP (mg/l)	0.7 (0.3−1.2)	2.1 (0.8−5.4)***

Data are expressed as median (interquartile range). * $p < 0.05$ **$p < 0.01$ ***$p < 0.001$ compared to healthy subjects. Abbreviations: ND = not detectable. CRP = C-Reactive Protein; HS = healthy subjects; IGF-1 = Insuline like growth factor-1; IGFBP-1,-2,-3 = Insuline-like growth factor − 1,-2,-3; IL-8 = Interleukin-8; MMP-7,-9 = Metalloproteinase − 7 and − 9; SSc = Systemic sclerosis; TGF-β = Transforming growth factor β; TNF-α = Tumor necrosing factor α; YKL-40 = Chitinase-3-like protein 1.

Correlation between serum biomarkers and pulmonary function tests at baseline

We performed correlation analysis to assess whether biomarkers were associated with pulmonary function tests at baseline. The correlation matrix is shown in Table 3. IGFBP-2 was negatively correlated with alveolo-capillar function assessed by carbon monoxide transfer coefficient (KCO) (r=-0.29, $p < 0.01$) (Fig. 3). In addition, YKL-40 was also negatively correlated with Forced Vital Capacity (FVC) as well as the Diffusion Lung capacity for CO (DLCO) (r=-0.31, $p < 0.01$ and r=-0.24, $p < 0.05$ respectively) (Table 3).

Table 3

Spearman correlation evaluating serum biomarkers at baseline in comparison with pulmonary function tests.

	FEV1%pred.	FVC %pred.	TLC, %pred.	DLCO, %pred.	KCO, %pred.
IGF-1	-0.08	-0.15	-0.23*	-0.09	0.02
IGFBP-1	0.05	0.1	0.28**	0.05	-0.2
IGFBP-2	-0.13	-0.12	0.16	-0.16	-0.29**
IGFBP-3	0.08	0.06	-0.01	0.07	0.12
TGF-β	0.04	-0.03	-0.02	0.15	0.03
IL-8	-0.12	-0.06	-0.03	-0.12	-0.17
TNF-α	-0.12	-0.07	0.14	-0.08	-0.15
YKL-40	-0.3**	-0.31**	-0.14	-0.24*	-0.12
MMP-7	-0.16	-0.18	-0.2*	-0.16	-0.05
MMP9	-0.11	-0.14	-0.18	-0.04	0.07
CRP	-0.31**	-0.32**	-0.26*	-0.13	0.15

Numbers represent the correlation coefficient (r), $p < 0.05$, **$p < 0.01$.

Abbreviations: CRP = C-Reactive Protein; DLCO = Diffusion lung capacity for CO; FEV1 = Forced expired volume in one second; FVC = Forced Vital Capacity; IGF-1 = Insuline like growth factor-1; IGFBP-1,-2,-3 = Insuline-like growth factor − 1,-2,-3; IL-8 = Interleukin-8; KCO = The carbon monoxide transfer coefficient; MMP-7,-9 = Metalloproteinase − 7 and − 9; TGF-β = Transforming growth factor β; TLC = Total lung capacity; TNF-α = Tumor necrosing factor α; YKL-40 = Chitinase-3-like protein 1.

Longitudinal analysis on serum biomarker variations and pulmonary function tests

To assess whether the variation over the time of the levels of serum biomarkers was associated with pulmonary function declines, we performed a longitudinal study in a subgroup of 28 patients analyzing biomarkers variations and pulmonary function tests over a period of 2 years (Fig. 1). Demographic and biological characteristics of SSc patients at baseline and after 2 years are given in Table 4. The 2-year longitudinal analysis of pulmonary function revealed that only KCO was significantly reduced (Baseline: 81 (± 14) % and 2-years 73 (± 12) %, $p < 0.001$) (Fig. 4a). There was no significant variation of the levels of serum biomarkers between baseline and 2-year follow-up (supplementary Table S1). Next, we performed analysis to determine if pulmonary function decline was associated to the variation of serum biomarkers (Table 5). Interestingly, we found a positive correlation between the variation of IGFBP-2 and KCO at 2-year follow-up ($r = 0.6$, $p < 0.001$) (Fig. 4b). ROC curve analysis enabled us to identify that baseline IGFBP-

2 < 105 ng/ml was associated with a better outcome assessed by KCO at 2 years follow-up in the overall SSc cohort (AUC 0.75 at 75% sensibility and 68% specificity) (Fig. 4c). Of note, we did not find any correlations between biomarkers evolution and immunosuppressive therapies. These results suggest a protective effect of IGFBP-2 on the progression of SSc disease.

Table 4
Demographic and biological characteristics of SSc patients at baseline and 2-year follow-up.

	Baseline (n = 28)	2 years follow-up (n = 28)
Age, yrs	57 ± 12	59 ± 12
Gender (M/F)	22/6	22/6
BMI, Kg/m²	24 ± 4	24 ± 4
Smokers (NS/ES/S)	14/10/5	14/10/5
Paq-year	0 (0−26)	1 (0−26)
leuco x10e3/µl	7 ± 1.7	7.8 ± 2.8
Neutrophil %	66 ± 10	64 ± 12
Cell/mm³	4676 ± 1696	5257 ± 2885
Lymphocyte %	23 ± 9	24 ± 10
Cell/mm³	1542 ± 674	1694 ± 624
Monocyte %	8 ± 2.3	8.1 ± 2.7
Cell/mm³	555 ± 199	595 ± 269
Eosinophil %	2.6 ± 1.3	2.6 ± 1.6
Cell/mm³	172 ± 92	185 ± 126
Basophil %	0.5 ± 0.3	0.5 ± 0.3
Cell/mm³	35 ± 16	40 ± 22
FEV1 post-BD, %pred.	100 ± 20	98 ± 19
FVC post-BD, %pred.	103 ± 20	101 ± 18
TLC %pred.	95 ± 16	94 ± 13
DLCO %pred.	66 ± 17	65 ± 13
KCO %pred.	81 ± 14	73 ± 12***
ILD yes/no	8/20	11/17

Data are expressed as mean (SD). * $p < 0.05$ **$p < 0.01$ ***$p < 0.001$ compared to Baseline.

Abbreviations: DLCO = Diffusion lung capacity for CO; FEV1 = Forced expired volume in one second; FS = Former smoker; FVC = Forced Vital Capacity; ILD = Interstitial lung disease; IT = Immunosuppressive therapy (Mycophenolate Mofetil, methotrexate, cyclophosphamide); KCO = The carbon monoxide transfer coefficient; NS = Non smoker; OCS = Oral corticosteroid; S = smoker; SSc: Systemic sclerosis; TLC = Total lung capacity.

	Baseline (n = 28)	2 years follow-up (n = 28)
IT yes/no	10/18	10/18
OCS yes/no	5/23	5/23
Data are expressed as mean (SD). * p < 0.05 **p < 0.01 ***p < 0.001 compared to Baseline.		
Abbreviations: DLCO = Diffusion lung capacity for CO; FEV1 = Forced expired volume in one second; FS = Former smoker; FVC = Forced Vital Capacity; ILD = Interstitial lung disease; IT = Immunosuppressive therapy (Mycophenolate Mofetil, methotrexate, cyclophosphamide); KCO = The carbon monoxide transfer coefficient; NS = Non smoker; OCS = Oral corticosteroid; S = smoker; SSc: Systemic sclerosis; TLC = Total lung capacity.		

Table 5
Spearman correlation evaluating pulmonary function tests at 2 years follow-up in comparison with biomarker variation (Δ) between baseline and 2-year follow-up.

	FEV1%pred.	FVC %pred.	TLC %pred.	DLCO %pred.	KCO %pred
Δ IGF-1	0.02	0.00	0.11	0.34	0.26
Δ IGFBP-1	-0.09	-0.12	-0.03	-0.08	0.03
Δ IGFBP-2	-0.02	-0.08	-0.28	0.29	0.60***
Δ IGFBP-3	0.16	0.09	0.20	0.42*	0.11
Δ TGF-β	0.03	0.14	0.09	0.33	0.31
Δ IL-8	-0.01	-0.10	-0.29	-0.08	0.09
Δ YKL40	0.07	-0.04	-0.07	-0.21	-0.02
Δ MMP7	0.05	-0.12	-0.25	-0.08	0.12
Δ MMP9	0.03	0.00	0.14	-0.09	-0.22
Numbers represent the correlation coefficient (r), *p < 0.05, **p < 0.01, *** p < 0.001.					
Abbreviations: DLCO = Diffusion lung capacity for CO; FEV1 = Forced expired volume in one second; FVC = Forced Vital Capacity; IGF-1 = Insuline like growth factor-1; IGFBP-1,-2,-3 = Insuline-like growth factor – 1,-2,-3; IL-8 = Interleukin-8; KCO = The carbon monoxide transfer coefficient; MMP-7,-9 = Metalloproteinase – 7 and – 9; SSc = Systemic sclerosis; TGF-β = Transforming growth factor β; TLC = Total lung capacity; YKL-40 = Chitinase-3-like protein 1.					

Impact of IGFBP-2 on fibrotic and proliferative processes

In order to elucidate the impact of IGFBP-2 on the progression of SSc disease, we investigated its effect on SSc-related processes *in vitro*.

IGFBP-2 secretion is reduced in pro-fibrotic context

First, we investigated the secretion of IGFBP-2 by human lung fibroblasts (HLFs) at basal state (FCS free) and in pro-proliferative condition (FCS, 2%). Both quiescent and proliferating HLFs actively secrete IGFBP-2. Proliferating HLFs produce more IGFBP-2 (4062 ± 141 pg/ml) than quiescent HLFs (958 ± 445 pg/ml) (Fig. 5a). Then, we investigated the impact of pro-fibrotic factor TGF-β on the secretion of IGFBP-2. Under TGF-β stimulation, we observed a reduction of IGFBP-2 secretion in proliferating HLFs (2945 ± 1074 pg/ml) ($p < 0.05$). TGF-β stimulation has no effect on the secretion of IGFBP-2 on quiescent HLFs.

IGFBP-2 reduces the proliferation of HLF fibroblasts

Then, we studied the effect of IGFBP-2 on HLF proliferation by BrdU assay. In presence of IGFBP-2 (100 ng/ml), we observed a significant decrease in BrdU incorporation at 48 h, which reveals a reduction of HLF proliferation ($p < 0.05$) (Fig. 5b). As expected, the positive control (TGF-β) increases the proliferation of HLF fibroblasts.

2.5.3. IGFBP-2 reduces the fibrotic activity of HLF fibroblasts

The impact of IGFBP-2 on fibrotic activity of HLF fibroblasts was first assessed by measuring the level of α-SMA *via* immunocytochemistry (Fig. 5c) and FACS analysis (Fig. 5d), then completed by the quantification of pro-collagen I secretion (Fig. 5e). In presence of IGFBP-2, we observed by FACS a decrease of α-SMA levels in HLF fibroblasts (Fig. 5d). Furthermore, the secretion of pro-collagen I was also significantly reduced by 35% in presence of IGFBP-2 ($p < 0.05$) (Fig. 5e). The immunocytochemistry analysis did not show any difference without or with IGFBP-2.

Taken together, these results highlight the anti-proliferative and anti-fibrotic properties of IGFBP-2.

Discussion

Our study highlights the involvement of the IGF/IGFBP pathway in SSc disease. SSc patients featured a marked increase in serum levels of IGFBP-1, IGFBP-2, IL-8 and MMP-9 whereas IGF-1 and IGFBP-3 were significantly reduced compared to HS. Of interest, IGFBP-2 was correlated to KCO at baseline. Two-year longitudinal analysis determined that IGFBP-2 variation was positively correlated with the KCO measurement. Of great interest, initial levels of IGFBP-2 at baseline under 105 ng/ml were associated with better KCO at 2-years, suggesting the protective effect of IGFBP-2 on lung disease. *In vitro* functional analysis confirmed the anti-fibrotic property of IGFBP-2. Indeed, we identified that IGFBP-2 was able to reduce fibroblastic proliferation as well as global fibrogenic process (α-SMA and pro-col-I).

In previous studies, we have identified that IGFBP-2 was positively associated with lung fibrosis in serum and induced sputum of IPF patients [22, 31]. Moreover, IGFBP-2 was reduced in IPF patients receiving anti-fibrotic therapy, although serum levels remained higher in IPF patients than in HS [22]. Other studies focusing on IGFBP-2 in lung fibrosis identified a significant increase in broncho-alveolar lavage fluid and in lung tissue of interstitial lung diseases without focusing on systemic sclerosis [30]. In this study, we

showed that patients suffering from SSc exhibited higher levels than HS but to a lesser extend than patients suffering from IPF as previously shown in one of our study [22, 31]. Of interest, we identified that level variation of IGFBP-2 was associated with the severity of lung evolution. Indeed, baseline serum level of IGFBP-2 under 105 ng/ml allows identifying patients with a good prognosis based on a better KCO at 2-year follow-up (above 70% pred) (DLCO/VA). This interesting observation suggests the potential prognostic value of baseline IGFBP-2 to identify SSc patients with risk of rapid evolution. Integrating new biomarkers in the follow up of SSc-ILD is challenging taking into account the variability of other clinical markers like symptoms, CRP, DLCO or FVC. Moreover, it is suitable to avoid repeated chest imaging in the follow-up of the patients to limit as much as possible irradiation. The use of serum biomarker IGFBP-2 could be a good candidate to predict the progression of SSc-ILD and need to be explored.

IGFBP-2 was able to reduce significantly fibroblast proliferation and the expression of two pro-fibrotic factors, pro-col-I and α-SMA, revealing its capacity to fight against fibrosis. The upregulation of anti-fibrotic IGFBP-2 in SSc patients is probably a process to fight against fibrogenesis and to slow the progression of the disease. Similarly, other studies focusing on human dermal fibroblasts identified that IGFBP-2 inhibited IGF-induced fibroblastic proliferation [25, 32, 33]. Of note, the inhibitory effect of IGFBP-2 was reduced when it was cleaved by metalloproteases like MMP-9 [34], which was also increased in our present study in SSc group. Accordingly, increased MMP-9 levels due to neutrophilic activation could reduce simultaneously the regulatory effect of IGFBP-2.

We also identified that IGFBP-3 was reduced in SSc without any specific correlation with regards to the severity of lung involvement. Conversely to our observation, many studies have identified that alveolar IGFBP-3 is associated to fibrotic lung process in SSc without any specifications of its circulating levels. Of interest our previous study focusing on IPF patients identified that untreated IPF patients were exhibiting lower serum levels of IGFBP-3 than HS and IPF patients treated with specific anti-fibrotic therapies. We also underlie that patients with ARDS as well as those suffering from cystic fibrosis or COPD presented similar variation of IGFBP-3 than those shown in our study [35].

In our study, serum levels of TGF-β were similar for all groups even though TGF-β is widely known to be associated with the pathophysiology of fibrosing lung disease [36]. Similarly, our previous study focusing on IPF did not find any difference in TGF-β levels between HS and patients suffering from IPF leading to the conclusion that serum TGF-β is not a good biomarker of lung fibrosis [15].

YKL-40 was negatively correlated with pulmonary function tests in our study (FEV1, FVC, DLCO) even if there were no significant differences of the serum level between the control group and the SSC group. In the same line, there was no significant difference with regard to the follow-up level at 2 years. Of great interest, the basal level of YKL-40 is still negatively correlated with the FVC at 2-year foolw-up. YKL-40 is a chitinase 3-like protein 1, regulates cell proliferation and survival and is produced by activated macrophages. It acts as a growth factor for connective tissue. Confirmatory to previous studies, we identified that YKL-40 is associated with the lung function impairment of patients suffering from SSc

[37–39]. Therefore, these observations need further explorations to see whether YKL-40 could act as a predictor of lung degradation for patients with SSc.

IL-8 was also increased in our study in SSc patients. IL-8 is known to be a strong chemotactic agent for neutrophils and can take part to the pathophysiological process of SSc [40] by recruiting neutrophils in lungs [41]. Of interest, it should be noted that blood neutrophils were increased in SSc compared to HS. In the same line, MMP-9 was also increased in SSc context. MMP-9 is known to be actively secreted by neutrophils [42, 43], which are increased in SSc patients.

The impact of maintenance of immunosuppressive drugs on cell count and biomarker levels was not relevant in our study.

Conclusions

We conclude that IGFBP-2 is increased in patients with SSc in comparison to HS and may play a protective role against the progression of the disease by blocking fibrogenesis process. We certainly need to validate those results in a larger longitudinal trial to confirm the clinical value of these observations.

Abbreviations

ANA: Antinuclear Antibodies

BRDU: Bromodeoxyuridine

CRP: C-Reactive Protein

DLCO: Diffusion lung capacity for CO

FEV1: Forced expired volume in one second

FVC: Forced vital capacity

IGF-1: Insuline like growth factor-1

IGFBP-1,-2,-3: Insulin like Growth Factor Binding Protein – 1,-2,-3

IL-8: Interleukin-8

ILD: Interstitial lung disease

IPF: Idiopathic pulmonary fibrosis

IQR: Interquartile range

KCO: The carbon monoxide transfer coefficient

MMP-7,-9: Matrix metalloproteinase – 7 and – 9

Pro-Col I: Pro-collagen type I

SD: Standard deviation

SSc: Systemic sclerosis

SSc-ILD: Systemic sclerosis associated interstitial lung disease

TGF-β: Transforming growth factor β

TLC: Total lung capacity

TNF-α: Tumor necrosing factor α

YKL-40: Chitinase-3-like protein 1

Declarations

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of Hospitalo-Facultaire Universitaire de Liège (CHU Hospital of Liège, Belgian number: B707201422832 ; ref: 2014/302). All subjects gave written consent be for their enrollment.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. MH is employee of Belgian Volition SPRL.

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Author contribution

JG, MSN, RL, MGM designed the study and coordinated the research. JG, FG and MH contributed to collect samples, and carried out the clinical evaluation of patients. MSN, MH, BA, CM and DDS performed

experiments and analysed the datas. JG and MSN drew figures and wrote the manuscript. All authors reviewed the final version of the manuscript. All authors read and approved the final version of the manuscript. JG, MSN, RL, MGM guaranty the integrity of the work as a whole, from inception to published article.

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Figures
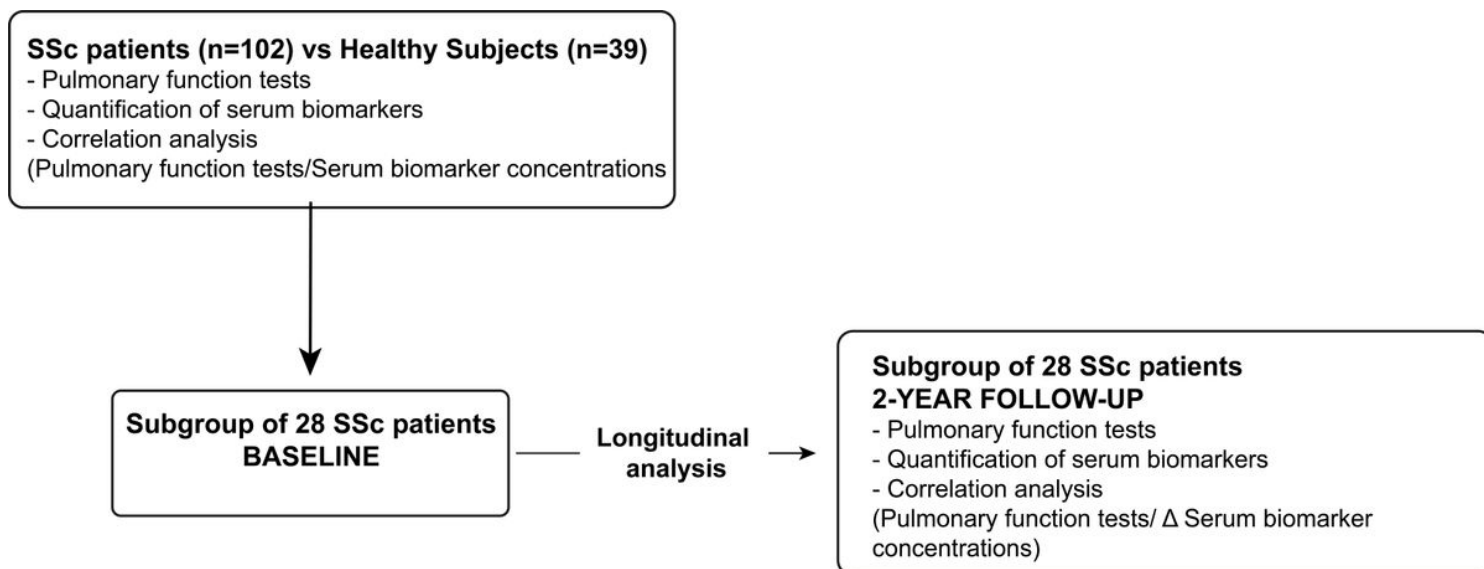
Figure 1

Schematic illustration of the study design
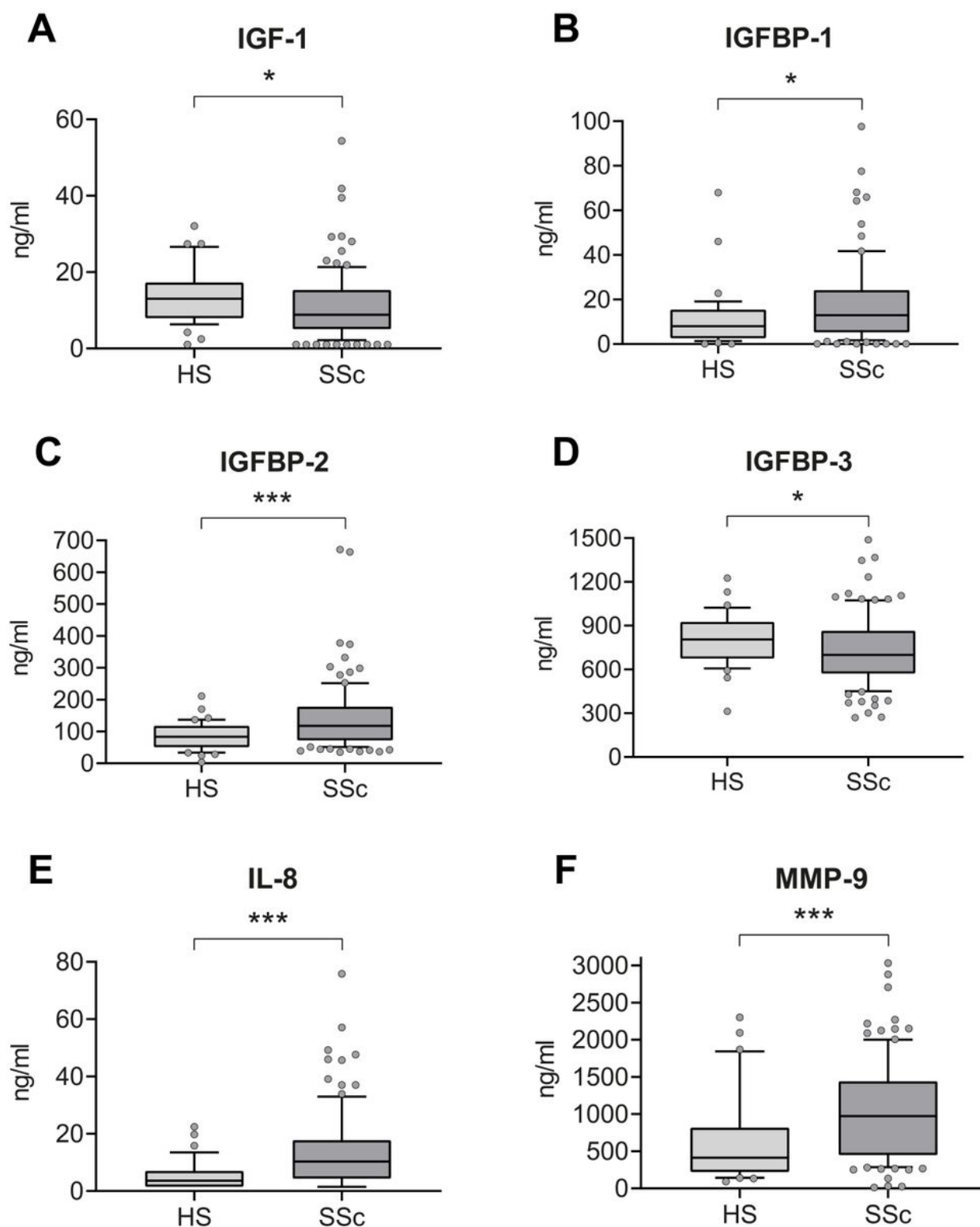
Figure 2

Serum biomarkers in SSc patients compared to HS. Comparison of the concentration of (a) IGF-1, (b) IGFBP-1, (c) IGFBP-2, (d) IGFBP-3, (e) IL-8 and (f) MMP-9 in SSc patients and HS. Data are expressed as median (IQR − CI 90%). * p<0.05 **p<0.01 ***p<0.001 compared to HS. Abbreviations: HS = healthy subjects; IGF-1 = Insuline like growth factor-1; IGFBP-1 , -2, -3 = Insulin like Growth Factor Binding Protein -1,-2,-3; IL-8 = Interleukin-8; MMP-9 = Matrix metalloproteinase-9; SSc = Systemic sclerosis.
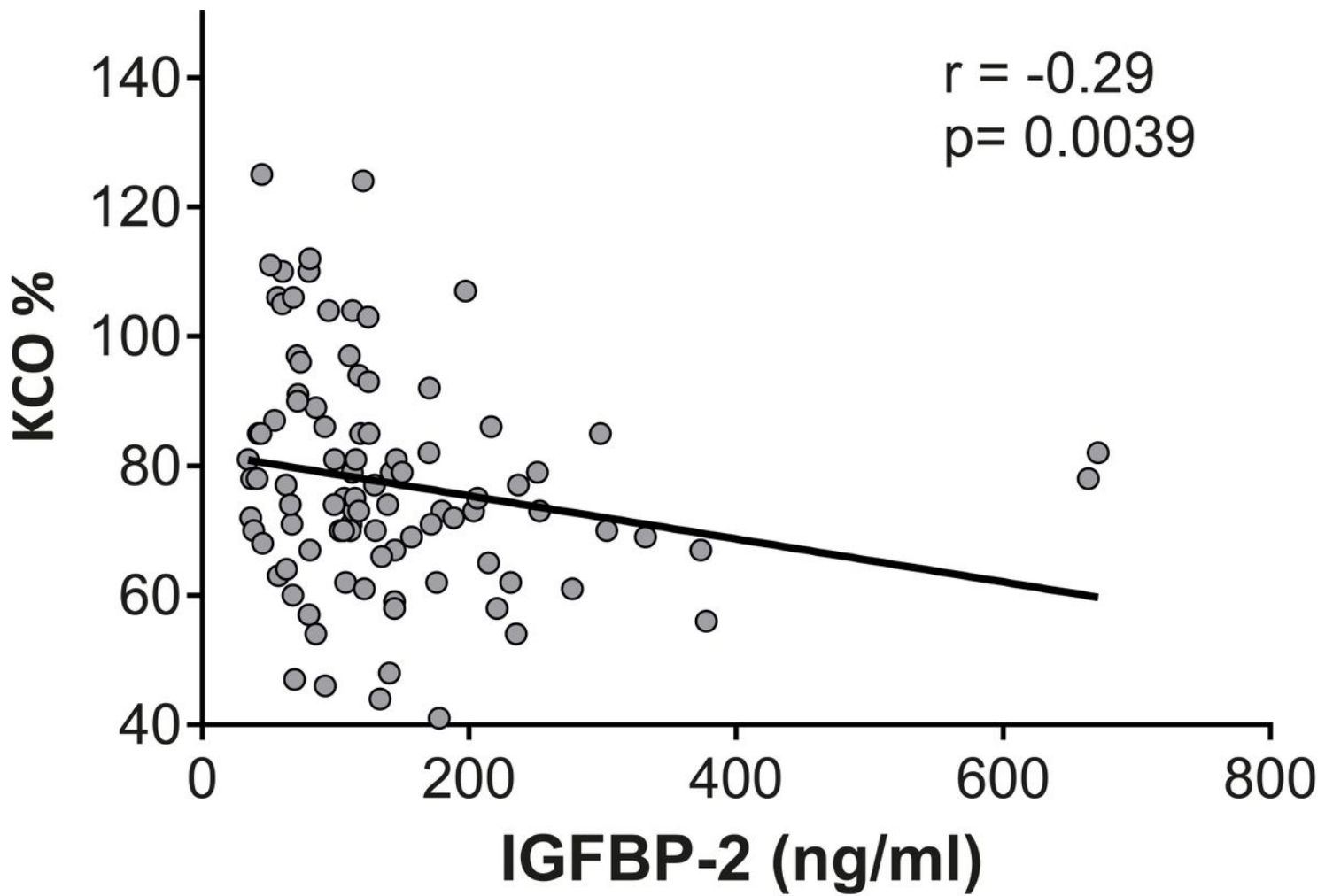
Figure 3

Correlation at baseline between KCO (% pred) and IGFBP-2 levels in SSc cohort. Abbreviations: IGFBP-2 = Insuline-like growth factor-2; KCO = The carbon monoxide transfer coefficient.
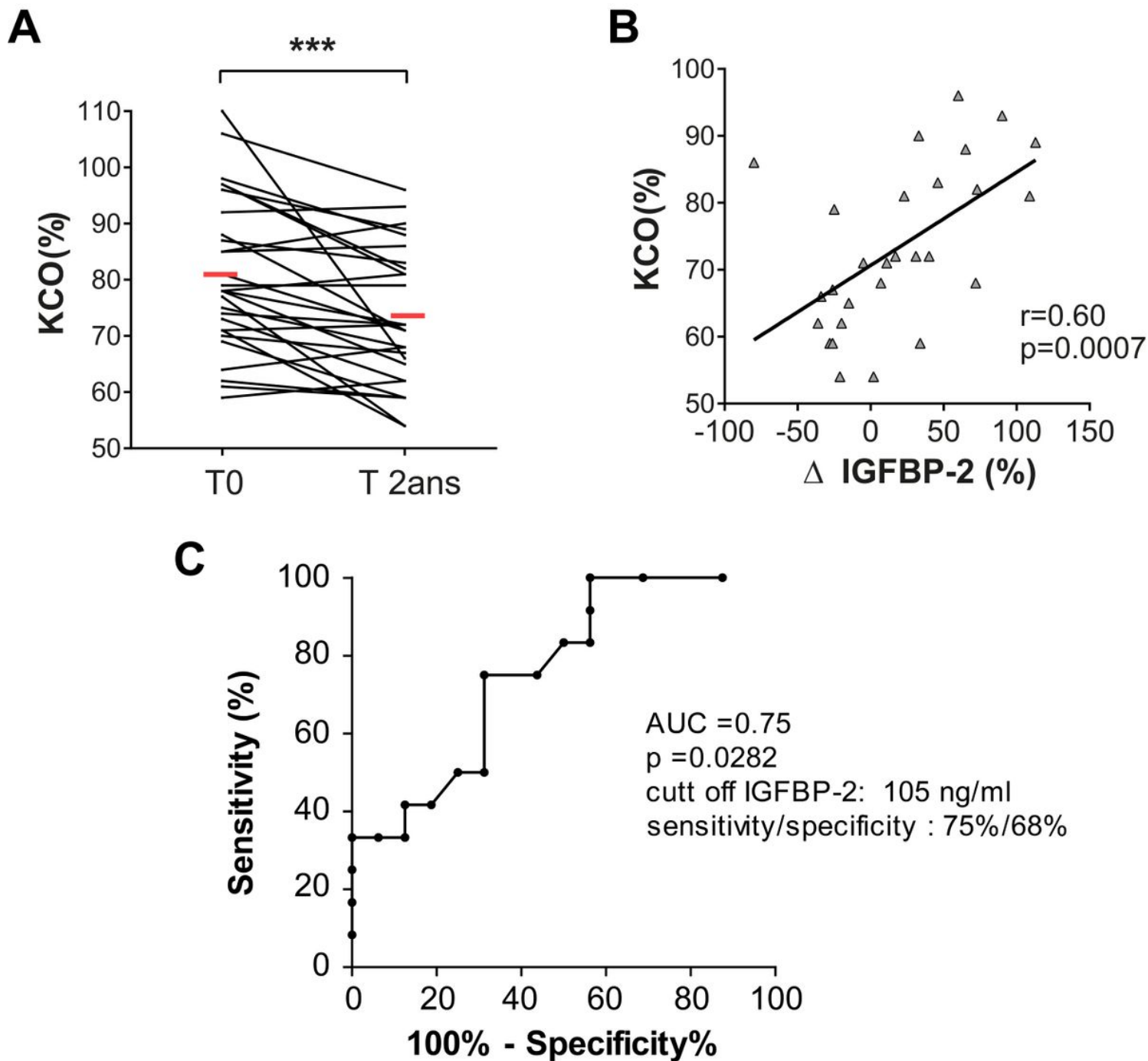
Figure 4

Longitudinal analysis. (a) KCO variation in the 2-year longitudinal analysis. *** p<0.001. (b) Correlation study between KCO at 2-yars and IGFBP-2 variation between baseline and 2-year follow-up. (c) ROC curve analysis showing that baseline levels of IGFBP-2 < 105ng/ml predict preserved KCO (>70% pred) at 2 years. Abbreviations: AUC = area under the curve; IGFBP-2 = Insuline-like growth factor-2; KCO = The carbon monoxide transfer coefficient.
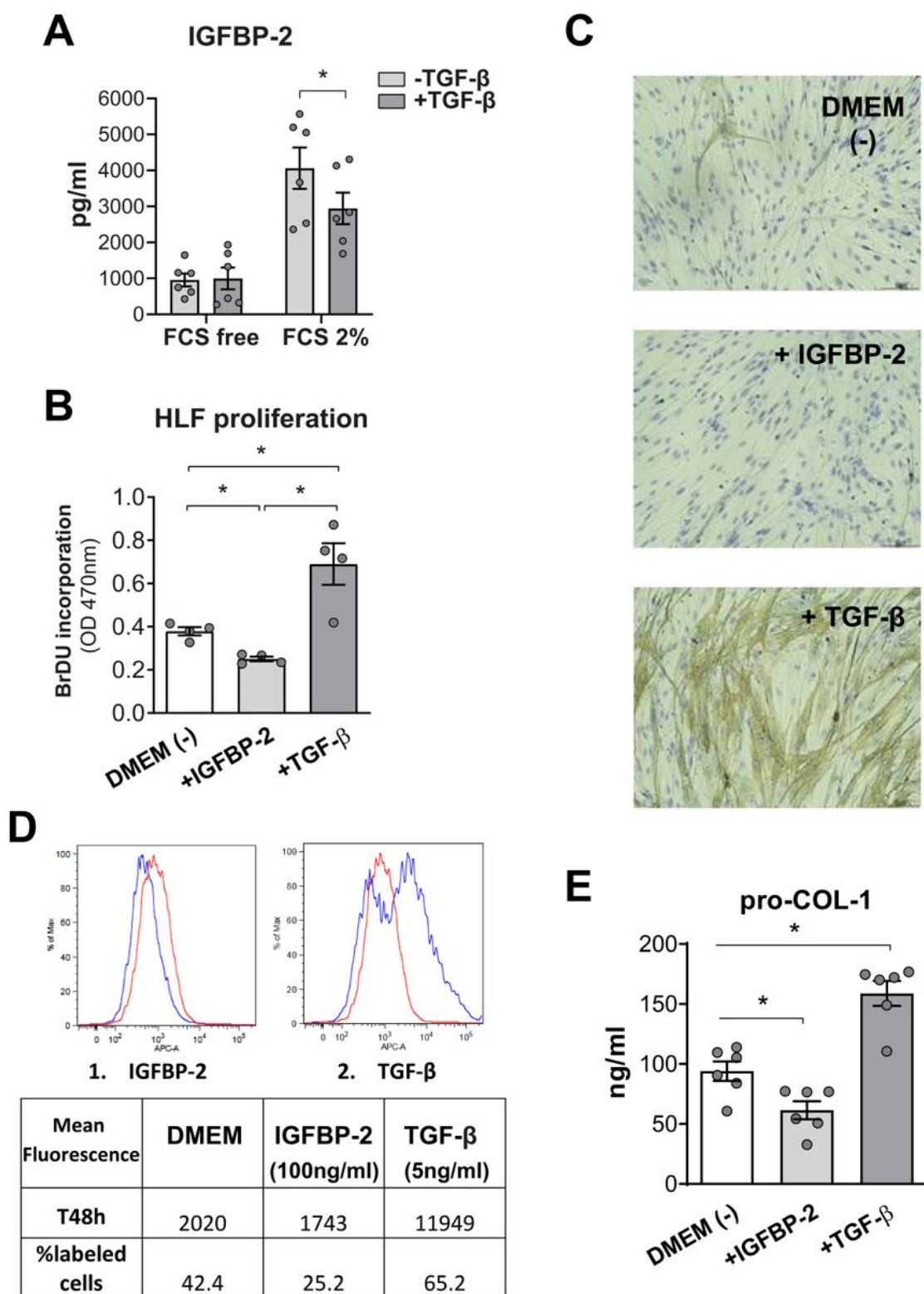
Figure 5

Evaluation of IGFBP-2 properties on human lung fibroblasts. (a) Secretion of IGFBP-2 by HLF fibroblasts at basal state (FCS free) and in pro-proliferative condition (FCS, 2%) without or with TGF-β (n=6). (b) Effect of IGFBP-2 on HLF proliferation assessed by BrdU assay. (c-d) Effect of IGFBP-2 on fibrotic activity of HLF fibroblasts assessed by measuring α-SMA by (c) immunocytochemistry (α-SMA, brown) and (d) FACS analysis. FACS analysis of α-SMA comparing HLF fibroblasts under DMEM condition (red) and

studied medium (blue) (in presence of IGFBP-2 or TGF-β) (n=2). (e) Effect of IGFBP-2 on the secretion of pro-collagen I of HLF fibroblasts.* p<0.05, **p<0.001, ***p<0.0001. Abbreviations: BrdU = Bromodeoxyuridine; DMEM = Dulbecco's Modified Eagle Medium; FCS = Fetal Calf Serum; IGFBP-2 = Insuline-like growth factor-2; Pro-Col I = Pro-collagen type I; TGF-β = Transforming growth factor β.