



HAL
open science

Upregulation of Stromal cell derived factor-1 α in Collagen Vascular Diseases - associated interstitial pneumonias (CVDs-IPs)

Giorgos A. Margaritopoulos, Katerina M. Antoniou, Giannoula Soufla, Konstantinos Karagiannis, Athanasia Proklou, Ismini Lasithiotaki, Nikolaos Tzanakis, Demetrios A. Spandidos, Nikolaos M. Siafakas

► To cite this version:

Giorgos A. Margaritopoulos, Katerina M. Antoniou, Giannoula Soufla, Konstantinos Karagiannis, Athanasia Proklou, et al.. Upregulation of Stromal cell derived factor-1 α in Collagen Vascular Diseases - associated interstitial pneumonias (CVDs-IPs). *Pulmonary Pharmacology & Therapeutics*, 2010, 23 (2), pp.115. 10.1016/j.pupt.2009.10.008 . hal-00614331

HAL Id: hal-00614331

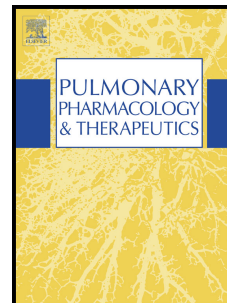
<https://hal.science/hal-00614331>

Submitted on 11 Aug 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript



Title: Upregulation of Stromal cell derived factor-1 α in Collagen Vascular Diseases - associated interstitial pneumonias (CVDs-IPs)

Authors: Giorgos A. Margaritopoulos, Katerina M. Antoniou, Giannoula Soufla, Konstantinos Karagiannis, Athanasia Proklou, Ismini Lasithiotaki, Nikolaos Tzanakis, Demetrios A. Spandidos, Nikolaos M. Siafakas

PII: S1094-5539(09)00122-9

DOI: [10.1016/j.pupt.2009.10.008](https://doi.org/10.1016/j.pupt.2009.10.008)

Reference: YPUPT 963

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 19 May 2009

Revised Date: 8October2009

Accepted Date: 21 October 2009

Please cite this article as: Margaritopoulos GA, Antoniou KM, Soufla G, Karagiannis K, Proklou A, Lasithiotaki I, Tzanakis N, Spandidos DA, Siafakas NM. Upregulation of Stromal cell derived factor-1 α in Collagen Vascular Diseases - associated interstitial pneumonias (CVDs-IPs), *Pulmonary Pharmacology & Therapeutics* (2009), doi: [10.1016/j.pupt.2009.10.008](https://doi.org/10.1016/j.pupt.2009.10.008)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Upregulation of Stromal cell derived factor-1 α in Collagen Vascular Diseases - associated interstitial pneumonias (CVDs-IPs)

¹Giorgos A. Margaritopoulos, ¹Katerina M. Antoniou, ²Giannoula Soufla, ¹Konstantinos Karagiannis, ¹Athanasia Proklou, ¹Ismeni Lasithiotaki, ¹Nikolaos Tzanakis, ²Demetrios A. Spandidos, ¹Nikolaos M. Siafakas

¹ Department of Thoracic Medicine, Interstitial Lung Disease Unit, Medical School, University of Crete, Heraklion, Crete, Greece,

² Department of Clinical Virology, Medical School, University of Crete, Heraklion, Crete, Greece

Subtitle: Different angiogenesis between CVDs-IPs and IPF

Statement: The authors have no conflicts of interest to declare

Corresponding author:

Katerina M. Antoniou, MD, PhD

Lecturer of Thoracic Medicine

Dept of Thoracic Medicine,

University Hospital, Medical School, University of Crete,

Heraklion 71110 Crete, Greece

Tel: +30-2810 392 433,

Fax: +30 2810 542 650

Email: pneumon@med.uoc.gr

Abstract

Objective: We speculated that distinct angiogenic profiles are involved in idiopathic interstitial pneumonias (IIPs) in comparison with interstitial pneumonias associated with collagen vascular disease (CVD-IPs). This hypothesis was investigated by measuring the expression of a cardinal biologic axis, the vascular endothelial growth factor (VEGF) –stromal derived growth factor [SDF-1 α , transcripts 1 and 2 (TR1 and TR2)] and receptor, CXCR4 and the angiogenetic receptors CXCR2 and CXCR3 in bronchoalveolar lavage fluid (BALF) in both conditions.

Methods: We studied prospectively 25 patients with fibrotic IIPs (f -IIPs), [20 with idiopathic pulmonary fibrosis, (IPF) and 5 with idiopathic non-specific interstitial pneumonia, (NSIP)] and 16 patients with CVD-IPs. mRNA expression was measured by Real-Time RT-PCR and protein was evaluated by Western Blotting.

Results: A significant greater value has been detected in SDF-1 α -TR1 mRNA expression levels of CVD-IPs ($p=0.05$) in comparison with IPF group. A similar trend has been also detected in protein expression in favor of CVD-IPs group. In addition, VEGF mRNA levels have been found significantly increased in CVD-IPs in comparison with the NSIP group ($p=0.05$). No significant difference has been found in SDF-1 α -TR2 -CXCR4 mRNA and CXCR2 –CXCR3 between the two groups.

Conclusion: These results showed increased expression of SDF-1 α in CVD-IPs, suggesting different angiogenic procedures. Further studies are needed in order to better explore the angiogenetic pathway in these disorders.

Introduction

Connective tissue disorders (CTDs) represent an heterogeneous group of immunologically mediated inflammatory disorders with a large variety of affected organs. Interstitial pneumonia (IP) is frequent in CTDs with an overall incidence estimated at 15% (1). It has been reported that patients with CTD-IP have a better prognosis than patients with idiopathic interstitial pneumonia (IIP) (2-6), and that was primarily related to a higher frequency of non specific interstitial pneumonia (NSIP) in patients with CTDs. It has been recently observed that even patients with CTD-usual interstitial pneumonia pattern (UIP) have better prognosis than patients with idiopathic pulmonary fibrosis (IPF), despite the same histologic pattern (7).

The underlying pathogenesis of fibrosis in these diseases may be distinctly different, however, poorly investigated in the current literature. It has been previously demonstrated that there is a lower profusion of fibroblastic foci in patients with CTD-UIP than in patients with IPF-UIP, despite a similar amount of fibrosis on HRCT (3). Recent theories implicate recurrent injurious exposure, imbalance that shifts Th1/Th2 equilibrium towards Th2 response and angiogenesis in the pathogenesis of pulmonary fibrosis (8).

The concept of vascular remodeling in IPF was originally identified by Turner-Warwick (9). In more recent morphologic reports, Renzoni et al have also observed vascular remodeling in both IPF and fibrosing alveolitis associated with systemic sclerosis (10). Moreover, Cosgrove and coworkers provided further support of vascular remodeling in IPF demonstrating decreased vascular density within the fibroblastic foci (11). Furthermore, our department has demonstrated a distinct profile of angiogenic and angiostatic chemokines in IPF and sarcoidosis patients that may explain differences between these two diseases in terms of pathogenetic mechanisms (12).

It is of note that vascular endothelial growth factor (VEGF) is a potent angiogenic factor. Stromal derived factor 1 (SDF 1, CXCL12) is a CXC chemokine which despite the lack of ELR motif has an angiogenic activity, and its receptor is CXCR4. It has been demonstrated that there is a positive feed-back loop of VEGF inducing CXCR4 and CXCL12 expression by endothelial cells and conversely CXCL12 interaction with CXCR4 enhances VEGF expression of these cells (13-15). Furthermore, known

ligands for CXCR3 are three angiostatic ELR⁻CXC chemokines (Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11) (9-12). We have shown that sarcoidosis exhibits a distinct angiostatic profile, as shown by an ELR⁻CXC chemokine upregulation in comparison to IPF patients (12). On the other hand, ELR⁺CXC chemokines (IL-8, ENA-78 and GRO- α) sharing the same receptor, CXCR2, were found increased in IPF (12), while a downregulation of their levels was recently shown after treatment (13).

The purpose of our study was to assess levels of these angiogenic factors in the BALF of patients with f-IIPs (IPF and NSIP) and CVD-IPs in order to test the hypothesis that a distinct angiogenic profile between these two diseases could partially explain the differences in terms of pathogenetic mechanisms and different prognosis.

Patients and Methods

1. Patients

From April 2006 to January 2008, forty one (41) consecutive patients from the Interstitial Lung Disease Unit of the Department of Thoracic Medicine, University Hospital of Heraklion were enrolled in the study: 25 patients with idiopathic fibrotic pneumonias (f-IIPs) and 16 patients with fibrosis related to CVDs (CVD-IPs).

First group (f-IIPs): The diagnosis was based on internationally accepted clinical and imaging criteria (16). 20 patients were characterized as IPF and 5 as NSIP. The diagnosis of IPF was made in 7 cases by video-assisted thoracoscopic surgery (VATS) biopsy (in the correct clinical context, detailed below); the histologic diagnosis of Usual Interstitial Pneumonia (UIP) was obtained in six cases, while the pattern of NSIP was found in one patient. In the remaining 18 cases the diagnosis was made on the basis of clinical and high-resolution computed tomography (HRCT) criteria: (1) bilateral basal or widespread crackles; (2) restrictive ventilatory defect or isolated depression of DL_{CO}; (3) computed tomography (CT) appearances indicative of IPF with predominantly basal and subpleural microcystic or macrocystic honeycombing, with variably extensive ground-glass and reticular abnormalities but no consolidation, nodular abnormalities, or other parenchymal abnormalities (apart from centrilobular emphysema); and (4) no environmental exposure to a fibrogenic

agent or connective tissue disease (16). According the aforementioned criteria a known cause of pulmonary fibrosis, such as a connective tissue disorder, has been excluded by both immunologic screening and rheumatological clinical evaluation.

Second group (CVD-IPs): The diagnosis was based on clinical and HRCT criteria in accordance with the international societies (7, 17, 18). In detail, a. 7 patients with Rheumatoid Arthritis (RA): Four with HRCT characteristics of UIP, 1 with NSIP and two with imaging features of Organizing Pneumonia (OP), b. 4 patients with systemic sclerosis (SSc) and HRCT appearance of NSIP, c. 2 patients with systemic lupus erythematosus (SLE) and HRCT features of NSIP, d. one patient with Sjogren syndrome and histologically proven fibrotic NSIP, e. one patient with dermatomyositis-polymyositis and HRCT features of NSIP, f. one patient with mixed connective tissue disease and NSIP imaging on HRCT. Thus, the second group was consisted by 4 UIP, 10 NSIP and two patients with OP.

2. Methods

Pulmonary function tests

All patients were evaluated spirometrically and by measurement of lung volumes, diffusion capacity and arterial blood gases (at rest). Spirometry and lung volumes (helium-dilution technique) and $T_{L,CO}$ (corrected for the haemoglobin) using the single-breath method were performed by a computerised system (Jaeger 2.12; MasterLab, Würzburg, Germany). Predicted values were obtained from the standardised lung function testing of the European Coal and Steel Community, Luxembourg (1993) (19). Arterial blood gases were measured by a arterial blood gas analyser (AVL330; MasterLab system).

BAL fluid processing

BALF was obtained from patients with f- IIPs and CVD- IPs by methods previously described (12, 13). Briefly, a flexible bronchoscope was wedged into a subsegmental bronchus of a predetermined region of interest based on radiographical findings. A

BAL was performed by instilling a total of 240 mL of normal saline in 60-mL aliquots, each retrieved by low suction. The BALF fractions were pooled and split equally into two samples. One sample was sent to the clinical microbiology and cytology laboratory and the other sample was placed on ice and transported to the research laboratory. The research sample was filtered through sterile gauze (Thompson, Ontario, Canada) and centrifuged at 400 g for 15 minutes at 4°C. Total cell counts were determined using an improved Neubauer counting chamber, and expressed as the total number of cells per mL of aspirated fluid. The pellet was washed three times with cold PBS-Dulbecco's and the cells were adjusted to a final concentration of 10⁶ cells/mL with RPMI1640 plus 2% FCS. The slide preparation was performed as previously reported (20). The authorization has been achieved from the Ethical Committee of the University General Hospital of Heraklion.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

RNA extraction and reverse transcription

Total RNA was extracted from each specimen using a power homogenizer and the TRIzol® reagent (Invitrogen, Carlsband, CA) according to the manufacturer's instructions. cDNA was synthesized using the Strascript reverse transcriptase kit (Stratagene, La Jolla, CA) as previously described (21).

Real-time RT-PCR

Peptide growth factors mRNA expression was measured using a real-time RT-PCR assay with SYBR-Green I. Primers were designed to span introns (21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, in order to normalize VEGFA, SDF-1 α or CXCL12, transcripts 1 and 2 CXCR4 and CXCR2, CXCR3 expression levels (Table 1). Specifically, 1 μ l cDNA from pathological or control samples was amplified in a PCR reaction containing 2X Brilliant SYBR-Green I QPCR Master Mix, 300 nM of each primer and 30 μ M ROX passive reference dye, in a final volume of 20 μ l. After an initial denaturation at 95°C for 10 min, the samples were subjected to 40 cycles of amplification, comprised of

denaturation at 95°C for 30 sec, annealing at appropriate temperature for each primer pair for 30 sec and elongation at 72°C for 30 sec, followed by a melt curve analysis, in which the temperature was increased from 55°C to 95°C at a linear rate of 0.2°C/sec. Data collection was performed both during annealing and extension, with two measurements at each step, and at all times during melt curve analysis. In each PCR reaction two non-template controls were included. All PCR experiments were conducted on the Mx3000P real-time PCR thermal cycler using the software version 2.00, (Stratagene, La Jolla, CA). To verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide and photographed on a UV light transilluminator. Primer sequences, annealing temperatures and PCR products length for all the growth factors analyzed, as well as for GAPDH, are described in Table 1.

All reactions were run in triplicates, and peptide growth factor transcript levels were calculated and normalized to each specimen's house keeping gene mRNA (GAPDH) as well as the appropriate calibrators, using the $\Delta\Delta C_t$ method for relative quantification. Specifically, after amplification, standard curves were constructed from samples used in a series of consecutive dilutions, for both the gene of interest (GF) and the internal control (GAPDH). Growth factor and GAPDH amplification efficiencies were the same, reaching 100%. IPF and control data were first normalized against variation in sample quality and quantity. Normalized values to GAPDH, ΔC_t s, were initially calculated using the following equation: $\Delta C_{t_{\text{sample}}} = C_{t_{\text{GF}}} - C_{t_{\text{GAPDH}}}$.

Western blotting

Proteins extracts (20 μ g) were electrophoresed through a 10% polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with an anti-VEGF antibody (Cat. No. MAB293, R&D systems, UK) at a dilution of 1:500, an anti-SDF antibody (Cat. No. MAB350, R&D systems, UK) at a dilution of 1:500 and with an anti-actin antibody (sc-47778, Santa-Cruz biotechnology, USA) at a dilution 1:1000. Antibody binding was revealed by a peroxidase labelled secondary antibody. Bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA), according to the manufacturer's protocol. The analysis was performed twice for each sample. The film was photographed with the Alpha Imager © system (Alpha Innotec Corp., USA). VEGF and SDF protein levels in f- IIPs and CVD-IPs were

quantified using Alpha Innotec image analysis software, with the beta-actin protein levels of each sample used as an internal control.

Statistical analysis

Peptide growth factors mRNA levels were first evaluated by the one-sample Kolmogorov-Smirnov goodness of fit test, in order to determine whether they follow a normal or not distribution. Based on the results, the non-parametric Spearman test was used to examine correlations. Proportions were compared using chi-square test. The Kruskal-Wallis followed by a posthoc analysis for pairwise significance and the Mann-Whitney U test were used as indicated to examine VEGF, SDF-1 α -TR1, SDF-1 α -TR2 and CXCR2 - 4 expression status among f-IIPs and CVD-IPs groups. Statistical analysis was carried out using SPSS 13.0 Chicago IL, USA. Statistical significance was set at the 95% level (P -value < 0.05).

Results

Patients' characteristics are shown in Table 2. Patients with idiopathic disease enrolled in the study had more severe disease than those with CVD-IPs, as it is shown in Table 2. They showed significant decrease in all cardinal parameters of restrictive defect (TLC and DLco) (Table 2).

SDF-1 α -TR1 transcript levels were found to be substantially different between IPF and CVD-IPs patients (Table 3, Figure 1). Specifically, SDF-1 α -TR1 exhibited significantly higher mRNA levels in CVD-IPs subjects compared to IPF ($p=0.05$). SDF-TR2 CXCR4 and CXCR2 and CXCR3 mRNA levels were not significantly different between the two patients' groups (Table 3).

In addition, VEGF mRNA levels were significantly higher in the CVD-IPs group when compared with NSIP patients' group ($p=0.05$) (Table 3).

Moreover, we tried to identify if there is any correlation between molecular (VEGF, SDF-TR1 and SDF-TR2 mRNA expression levels) with lung function parameters (FVC and DLCO). We found a significant correlation between SDF-1 α -TR1 and TL_{CO} in the study population ($r=0.461$, $p=0.04$).

Western Blotting analysis

We evaluated VEGF and SDF-1 α protein expression by western blot in 7 BALFs samples from IPF patients, 5 samples from CVD-IPs patients. VEGF expression was observed only in one (1/7) IPF patient (14%) while VEGF protein was present in three (3/5) CVD-IPs patients (60%). However, due to the limited number of samples, according to the Fisher exact statistical test, the above difference in VEGF expression is not statistically significant ($p=0.22$). SDF-1 α protein expression was observed in 2 out of 7 samples of IPF patients (29%) and in 2 out of 5 samples of CVD-IPs patients (40%) respectively. This difference is not found to be statistically significant between the two patients' groups ($p=0.57$, Fisher's exact test) (Figure 2).

Discussion

We hypothesized that angiogenesis differs in CTD-IPs and f-IIPs. To the best of our knowledge, this is the first study to investigate the expression of different angiogenic factors in patients with CTD-IPs and f-IIPs. The most important finding of our study is the presence of a different angiogenic profile in CTD-IPs compared to IPF patients. In particular, BALF levels of the angiogenic factor SDF-1 α were found significantly elevated in patients with CTD-IPs in comparison to IPF, in both mRNA and protein expression. Furthermore, we detected a post-translational overexpression of VEGF in CVD-IPs in comparison with IPF.

However, there are several limitations of this study that merit consideration. We tried to investigate an expression signal in a more homogenous material from the lung, like the BALF pellet. Thus, the limitation is that the analysis is not in lung tissue. However, as it is obvious from two previous elegant reports (10, 11), lung tissue in IPF presents regional heterogeneity. In detail, Renzoni and coworkers have demonstrated vascular remodeling in both IPF and fibrosing alveolitis associated with

systemic sclerosis (FASSc). They have found that vascular remodelling is particularly active in the areas immediately adjacent to fibroblastic foci and by contrast they observed an absence of vessels within fibroblastic foci (10). Later, Cosgrove et al have also observed a relative absence of vessels in the fibroblastic foci of IPF as well as a significant vascularity in the areas of fibrosis around the fibroblastic foci. These findings are similar to those of Renzoni et al and supports the concept of regional heterogeneity of vascularity in IPF (10, 11). We cannot perform a direct comparison between our and the above studies. However, it is of note that Cosgrove et al found a different VEGF expression between OP and IPF, demonstrating a lack of expression in this angiogenic factor in IPF, in accordance with our finding in BALF.

Moreover, because of the diversity of CVDs themselves, it is very difficult to include enough subjects in order to analyze data separately for individual CVDs and for individual histologic patterns. Our subgroup analysis could not be confident for the aforementioned reason of small patients' groups. Furthermore, the post-transcriptional analysis of this mRNA signal into protein level is needed in a larger population in order to further investigate the signal of our analysis. Furthermore, IPF has not the same profile and prognosis as NSIP, however, BALF findings do not discriminate between usual interstitial pneumonia and NSIP and have no prognostic value (22).

Previously, the issue of different pathogenesis and prognosis between idiopathic and autoimmune disease has been investigated (3), however, not at the angiogenic aspect. Flaherty et al, in a series of patients diagnosed as CTD-UIP and IPF by surgical lung biopsy, have shown that the profusion of fibroblastic foci may correlate with the prognosis of these diseases. In fact, they have found that patients with CTD-UIP had a lower profusion of fibroblastic foci in comparison to patients with IPF and they have also confirmed an improved survival for patients with CTD-UIP (3). However, in this study patients with CVDs were younger than patients with idiopathic disease and this was a considerable limitation (3). In the present study CVD-IPs patients were not younger than patients with idiopathic disease, however, they had milder pulmonary disease at baseline, such as Flaherty's group (3).

Two Japanese reports suggest that different pathogenesis between idiopathic and autoimmune disease is due to impaired Th1/ Th2 balance, investigating two angiogenic receptors like CXCR3 and CCR4 (23, 24). In detail, Shimizu and coworkers have examined the distribution of helper T-cell subsets in open lung biopsy

specimens from patients with CTD (RA, PM/DM, SSc, primary sjogren syndrome SS) and for comparison patients with IPF. They have analyzed immunohistochemically the expression of CXCR3 and CCR4, which are two chemokine receptors associated in vitro with Th1 and Th2 cells respectively. However, they failed to demonstrate a clear cut-off in Th1/Th2 ratio between IPF and systemic diseases (24).

Although, there is lack of studies regarding expression of angiogenic factors in the lung in patients with systemic disease, evidence suggests impaired angiogenesis in autoimmune disease without lung involvement. It is of note that in rheumatoid arthritis there is an imbalance between inducers and inhibitors of angiogenesis that favours angiogenesis. There is evidence suggesting that, in RA, VEGF expression is induced by the hypoxic environment of the inflamed rheumatoid joint. Hypoxia activates the VEGF gene via binding of hypoxia inducible factor and this in turn augments IL-1 or transforming growth factor TGF- β induced sinovial fibroblast VEGF (25). This procedure contributes significantly to angiogenesis in the synovium and progression of the disease. In contrast to RA, systemic sclerosis (SSc) is characterized by insufficient angiogenesis that leads to failure to replace damaged vessels, resulting in a reduction in capillary density (26). Paradoxically, it has been observed an increased expression of proangiogenic factors despite the defective angiogenesis in SSc. VEGF is markedly overexpressed in various cell types in the skin of SSc patients. VEGF receptors are up regulated on endothelial cells in SSc. Serum levels of VEGF are increased in SSc patients through-out different disease stages (27-30). The mechanism of VEGF induction in SSc is unclear. Hypoxia does not seem to play an important role (31), whereas induction by cytokines as platelet-derived growth factor and TGF- β appear to be more important. Unfortunately, we could not perform an analysis of the angiogenic parameters in each distinct diseases, because of the small numbers of patients included.

Emerging data implicate the SDF-1/CXCR4 axis in the pathogenesis of bleomycin-induced pulmonary fibrosis in mice, but whether similar pathogenetic processes are operative in pulmonary fibrosis in humans remains to be shown (32). Although a number of recent data have dealt with CXCL12-CXCR4 pathway in the pathogenesis of pulmonary fibrosis, the mRNA expression pattern and interaction of this biological axis has not been investigated in autoimmune disease with lung involvement. It has

been found that the concentration of CXCL12 is elevated in plasma and synovial fluid specimens from patients with RA (33, 34), and an overexpression of CXCL12 is also observed in RA synovial tissue (35-36). CXCL12 promotes CD4⁺ memory T-cells recruitment and accumulation (37) and monocyte migration into synovium (38) and induces pseudoemperipolesis of both T and B cells (39). It can also promote joint destruction by inducing angiogenesis (36). Little is known about the regulation and induction mechanisms of CXCL12. Recently has been found that IL-17, a T-cell derived proinflammatory cytokine, is able to regulate the expression of CXCL12 in RA, and that the production and action of CXCL12 results from the interplay of T-cells and fibroblast-like synoviocytes (40).

Our most important finding is the increased expression of CXCL12 in the BALF of patients with autoimmune disease in comparison with IPF. Moreover, this overexpression could have prognostic implications, as it has been found significantly correlated with the diffusing capacity of these patients. However, increased protein levels of CXCL12 has been detected in lung tissue from five patients with UIP and fibrotic NSIP, when compared them to levels in normal lung tissue (41). The above findings are not in disagreement with our findings as the authors investigated post-transcriptional expression of SDF-1 in five patients of an heterogeneous group. Moreover, in accordance with the current data in BALF pellet, we detected a significant decrease in both transcripts of SDF-1 in lung tissue of 25 IPF tissues in comparison with controls (42). In agreement with our findings, Cipriani et al have demonstrated a differential expression of CXCL12 and its receptor CXCR4 in skin and endothelial cells of systemic sclerosis patients (43). They have found that CXCL12 and CXCR4 are up-regulated in the skin of patients with early SSc, both in the diffuse and limited cutaneous forms of the disease, and progressively decreased with the lowest expression in the latest stages of both SSc subsets. Our patients with systemic sclerosis had severe pulmonary disease with severe reduction of diffusing capacity.

In conclusion, we have shown that patients with CVD-IPs have a markedly increased expression of the biological axis VEGF-CXCL12 compared with patients with idiopathic fibrotic disease indicating possibly different angiogenetic procedures.

References

1. Antoniou KM, Margaritopoulos G, Economidou F, Siafakas NM. Pivotal clinical dilemmas in collagen vascular diseases associated with interstitial lung involvement. *Eur Respir J*;33:882-96
2. Agustí C, Xaubet A, Roca J, Agustí AG, Rodriguez-Roisin R. Interstitial pulmonary fibrosis with and without associated collagen vascular disease: results of a two year follow up. *Thorax* ;47 :1035-40
3. Flaherty KR, Colby TV, Travis WD, Toews GB, Mumford J, Murray S, et al. Fibroblastic foci in usual interstitial pneumonia: idiopathic versus collagen vascular disease. *Am J Respir Crit Care Med*. 2003 May 15;167 :1410-5.
4. Papiris SA, Vlachoyiannopoulos PG, Maniati MA, Karakostas KX, Constantopoulos SH, Moutsopoulos HH. Idiopathic pulmonary fibrosis and pulmonary fibrosis in diffuse systemic sclerosis: two fibroses with different prognoses. *Respiration*. 1997;64 :81-5
5. Wells AU, Cullinan P, Hansell DM, Rubens MB, Black CM, Newman-Taylor AJ, et al. Fibrosing alveolitis associated with systemic sclerosis has a better prognosis than lone cryptogenic fibrosing alveolitis. *Am J Respir Crit Care Med*. 1994; 149: 1583-90
6. Douglas WW, Tazelaar HD, Hartman TE, Hartman RP, Decker PA, Schroeder DR, Ryu JH. Polymyositis-dermatomyositis-associated interstitial lung disease *Am J Respir Crit Care Med*. 2001; 164: 1182-5
7. Park JH, Kim DS, Park IN, Jang SJ, Kitaichi M, Nicholson AG, Colby TV. Prognosis of Fibrotic Interstitial Pneumonia: Idiopathic Versus Collagen Vascular Disease-Related. *Am J Respir Crit Care Med*. 2007; 175: 705-711
8. Strieter RM: Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel. *Chest* 2005; 128:526S-532S
9. Turner-Warwick M. Precapillary systemic-pulmonary anastomoses. *Thorax* 1963; 18:225-17

10. Renzoni EA, Walsh DA, Salmon M, Wells AU, Sestini P, Nicholson AG, Veeraraghavan S, Bishop AE, Romanska HM, Pantelidis P, Black CM, Du Bois RM. Interstitial vascularity in fibrosing alveolitis. *Am J Respir Crit Care Med.* 2003; 167:438-43
11. Cosgrove GP, Brown KK, Schiemann WP, Serls AE, Parr JE, Geraci MW, Schwarz MI, Cool CD, Worthen GS. Pigment epithelium-derived factor in idiopathic pulmonary fibrosis: a role in aberrant angiogenesis. *Am J Respir Crit Care Med.* 2004;170:242-51
12. Antoniou KM, Tzouveleki A, Alexandrakis MG, Sfiridaki K, Tsiligianni I, Rachiotis G, Tzanakis N, Bouros D, Milic-Emili J, Siafakas NM. Different angiogenic activity in pulmonary sarcoidosis and idiopathic pulmonary fibrosis. *Chest.* 2006;130:982-8
13. Antoniou KM, Tzanakis N, Tzortzaki EG, Malagari K, Koutsopoulos AV, Alexandrakis M, Wells AU, Siafakas NM. Different angiogenic CXC chemokine levels in bronchoalveolar lavage fluid after interferon gamma-1b therapy in idiopathic pulmonary fibrosis patients. *Pulm Pharmacol Ther.* 2008;21:840-4.
14. Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol.* 1999; 154:1125-35
15. Schrader AJ, Lechner O, Templin M, Dittmar KE, Machtens S, Mengel M, Probst-Kepper M, Franzke A, Wollensak T, Gatzlaff P, Atzpodien J, Buer J, Lauber J. CXCR4/CXCL12 expression and signalling in kidney cancer. *Br J Cancer.* 2002 22;86:1250-6
16. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med* 2002; 165: 277-304
17. American Rheumatism Association Diagnostic and Therapeutic Criteria Subcommittee for Scleroderma. Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1988; 23: 581-590
18. Arnett FC, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31:315-324

19. Quanjer PH, Tammeling GJ, Cotes JE, et al. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J, Suppl* 1993; 16:5-40
20. Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. *Eur Respir J.* 1999; 14:245-8
21. Soultzis N, Karyotis I, Delakas D, Spandidos DA. Expression analysis of peptide growth factors VEGF, FGF2, TGFB1, EGF and IGF1 in prostate cancer and benign prostatic hyperplasia. *Int J Oncol* 2006; 29:305-14.
22. Veeraraghavan S, Latsi PI, Wells AU, Pantelidis P, Nicholson AG, Colby TV, Haslam PL, Renzoni EA, du Bois RM. BAL findings in idiopathic nonspecific interstitial pneumonia and usual interstitial pneumonia. *Eur Respir J.* 2003;22:239-44
23. Shimizu S, Yoshinouchi T, Naniwa T, Nakamura M, Sato S, Ohtsuki Y, Fujita J, Yamadori I, Eimoto T, Ueda R. Distribution of CXCR3- or CCR4-positive cells in interstitial pneumonia associated with primary Sjogren's syndrome. *Virchows Arch.* 2004;445:477-84
24. Shimizu S, Yoshinouchi T, Niimi T, Ohtsuki Y, Fujita J, Maeda H, Sato S, Yamadori I, Eimoto T, Ueda R Differing distributions of CXCR3- and CCR4-positive cells among types of interstitial pneumonia associated with collagen vascular diseases. *Virchows Arch.* 2007;450:51-8
25. Cho ML, Cho CS, Min SY, Kim SH, Lee SS, Kim WU, Min DJ, Min JK, Youn J, Hwang SY, Park SH, Kim HY. Cyclosporine inhibition of vascular endothelial growth factor production in rheumatoid synovial fibroblasts. *Arthritis Rheum* 2002; 46:1202-1209
26. Distler JH, Gay S, Distler O: Angiogenesis and vasculogenesis in systemic sclerosis. *Rheumatology (Oxford)* 2006, Suppl 3: iii26-iii27
27. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, Gay RE, Michel BA, Bruhlmann P, Muller-Ladner U, et al.: Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; 4:R11

28. Choi JJ, Min DJ, Cho ML, Min SY, Kim SJ, Lee SS, Park KS, Seo YI, Kim WU, Park SH, et al.: Elevated vascular endothelial growth factor in systemic sclerosis. *J Rheumatol* 2003; 30: 1529-1533
29. Allanore Y, Borderie D, Lemarechal H, Ekindjian OG, Kahan A: Nifedipine decreases sVCAM-1 concentrations and oxidative stress in systemic sclerosis but does not affect the concentrations of vascular endothelial growth factor or its soluble receptor 1. *Arthritis Res Ther* 2004; 6:R309-R314
30. Kuryliszyn-Moskal A, Klimiuk PA, Sierakowski S: Soluble adhesion molecules (sVCAM-1, sE-selectin), vascular endothelial growth factor (VEGF) and endothelin-1 in patients with systemic sclerosis: relationship to organ systemic involvement. *Clin Rheumatol* 2005; 24:111-116
31. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, Michel BA, Gay RE, Muller-Ladner U, Matucci-Cerinic M, et al.: Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ Res* 2004; 95:109-116
32. Strieter RM, Gomperts BN, Keane MP. The role of CXC chemokines in pulmonary fibrosis. *J Clin Invest* 2007; 117: 549- 556
33. Mittal GA, Joshi VR, Deshpande A. Stromal cell-derived factor-1 α in rheumatoid arthritis. *Rheumatology (Oxford)* 2003; 42:915–6
34. Kanbe K, Takagishi K, Chen Q. Stimulation of matrix metalloprotease 3 release from human chondrocytes by the interaction of stromal cell–derived factor 1 and CXC chemokine receptor 4. *Arthritis Rheum* 2002; 46:130–7
35. Grassi F, Cristino S, Toneguzzi S, Piacentini A, Facchini A, Lisignoli G. CXCL12 chemokine up-regulates bone resorption and MMP-9 release by human osteoclasts: CXCL12 levels are increased in synovial and bone tissue of rheumatoid arthritis patients. *J Cell Physiol* 2004; 199: 244–51
36. Pablos JL, Santiago B, Galindo M, Torres C, Brehmer MT, Blanco FJ, et al. Synoviocyte-derived CXCL12 is displayed on endothelium and induces angiogenesis in rheumatoid arthritis. *J Immunol* 2003; 170:2147–52
37. Nanki T, Hayashida K, El-Gabalawy HS, Suson S, Shi K, Girschick HJ, et al. Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium. *J Immunol* 2000; 165:6590–8

38. Blades MC, Ingegnoli F, Wheller SK, Manzo A, Wahid S, Panayi GS, et al. Stromal cell-derived factor 1 (CXCL12) induces monocyte migration into human synovium transplanted onto SCID mice. *Arthritis Rheum* 2002; 46: 824–36
39. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, Kipps TJ. Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *J Clin Invest* 2001; 107:305–15
40. Kim KW, Cho ML, Kim HR, Ju JH, Park MK, Oh HJ, Kim JS, Park SH, Lee SH, Kim HY. Up-regulation of stromal cell-derived factor 1 (CXCL12) production in rheumatoid synovial fibroblasts through interactions with T lymphocytes: role of interleukin-17 and CD40L-CD40 interaction. *Arthritis Rheum.* 2007;56:1076-86
41. Mehrad B, Burdick MD, Zisman DA, Keane MP, Belperio JA, Strieter RM. Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease. *Biochem Biophys Res Commun.* 2007; 353:104-8
42. Antoniou KM, Soufla G, Lymbouridou R, Economidou F, Lasithiotaki I, Manousakis M, Drositis I, Spandidos DA, Siafakas NM. Expression analysis of angiogenic growth factors and biological axis CXCL12/CXCR4 axis in IPF. *Connect Tissue Res.* *in press*
43. Cipriani P, Franca Milia A, Liakouli V, Pacini A, Manetti M, Marrelli A, Toscano A, Pingiotti E, Fulminis A, Guiducci S, Perricone R, Kahaleh B, Matucci-Cerinic M, Ibba-Manneschi L, Giacomelli R. Differential expression of stromal cell-derived factor 1 and its receptor CXCR4 in the skin and endothelial cells of systemic sclerosis patients: Pathogenetic implications. *Arthritis Rheum.* 2006;54:3022-33

Table 1: Primer sequences used for quantitative Real-time RT-PCR

<i>Growth factor or Cytokine</i>	<i>Primer pair Sequence (5'-3')</i>	<i>Annealing temperature</i>	<i>Product size</i>
VEGF	ATGACGAGGGCCTGGAGTGTG CCTATGTGCTGGCCTTGGTGAG	60°C	91
SDF1-TR1	TGAGAGCTCGCTTTGAGTGA CACCAGGACCTTCTGTGGAT	55°C	233
SDF1-TR2	CTAGTCAAGTGCGTCCACGA GGACACACCACAGCACAAAC	55°C	221
CXCR4	GGTGGTCTATGTTGGCGTCT TGGAGTGTGACAGCTTGGAG	55°C	229
GAPDH	GGAAGGTGAAGGTCGGAGTCA GTCATTGATGGCAACAATATCCACT	60°C	101
CXCR2	GGCCACTCCAATAACAGCAGGTC GTAGAAAAGGGGGCAGGGTAGAGC	60°C	197
CXCR3	AAAGCAGAGGGGCAGGCAGCACAC AGGGCGGGGAGGTACAGCACGAGT	65°C	181

Table 2 Demographic and lung function characteristics of patients with f- IIPs and CVD-IPs. Values are expressed as mean \pm SD, and age as median (range).

Characteristics	f-IIPs	CVD-IPs	p value
Number	25	16	
Sex (Male/Female)	19/6	5/11	0.002
Age median (yrs)	66.5	64.60	NS
Smokers/Nonsmokers	19/6	5/11	0.01
FEV ₁ (% pred)	77.15	107.50	NS
FVC (% pred)	74.80	93.10	0.02
TLC (% pred)	73.60	91.00	0.02
DLco (% pred)	58.00	71.700	0.05
PaO ₂ (mmHg)	72.50	75.00	NS
TCCx10 ⁵ /ml*	28.6 \pm 3.2	30.7 \pm 4.6	NS
Macrophages%	82.2 \pm 4.3	76.2 \pm 2.5	NS
Neutrophils %	6.2 \pm 2.2	8.9 \pm 2.8	NS
Lymphocytes%	7.7 \pm 3.0	9.7 \pm 3.5	NS
Eosinophils %	3.7 \pm 2.4	4.1 \pm 1.3	NS

Abbreviations:

FVC: Forced Vital Capacity, TLC: Total Lung Capacity, DL_{CO}: Diffusing Capacity for Carbon Monoxide, PaO₂: Arterial Partial Pressure of Oxygen

* TCC: Total cell counts were available in 23 patients with f- IIPs and 12 patients with CVD- IPs

Table 3: mRNA expression of angiogenic parameters in f-IIPs (IPF and NSIP) and CVD-IPs. Values are expressed as median (mean \pm SD).

mRNA expression	f-IIPs	IPF	NSIP	CVD-IPs	p*
VEGF	0.25 (11.91 \pm 10.47)	0.34 (14.34 \pm 12.66)	0.00 (0.35\pm0.34)	2.00 (867.48 \pm 584.31)	0.05
SDF-1^{α}-TR1	1.83 (473.70 \pm 254.79)	0.93 (168.07\pm132.16)	13.99 (1303.27 \pm 839.93)	162.81 (776.05\pm527.48)	0.05
SDF-1 ^{α} -TR2	3.94 (237.92 \pm 152.82)	3.02 (141.90 \pm 125.94)	37.29 (512.26 \pm 479.48)	166.68 (592.66 \pm 362.03)	NS
CXCR4	2.26 (433.94 \pm 307.10)	3.15 (174.14 \pm 121.46)	0.44 (1176.20 \pm 1148.46)	1.18 (1348.21 \pm 1322.22)	NS
CXCR2	0.0 (0.07 \pm 0.05)	0.0 (0.08 \pm 0.05)	0.0 (0.00 \pm 0.00)	0.0 (0.10 \pm 0.06)	NS
CXCR3	0.0 (0.11 \pm 0.08)	0.0 (0.12 \pm 0.08)	0.00 (0.00 \pm 0.00)	0.01 (0.03 \pm 0.01)	NS

*Kruskal-Wallis followed by a posthoc analysis using the Mann-Whitney U test for pairwise comparisons

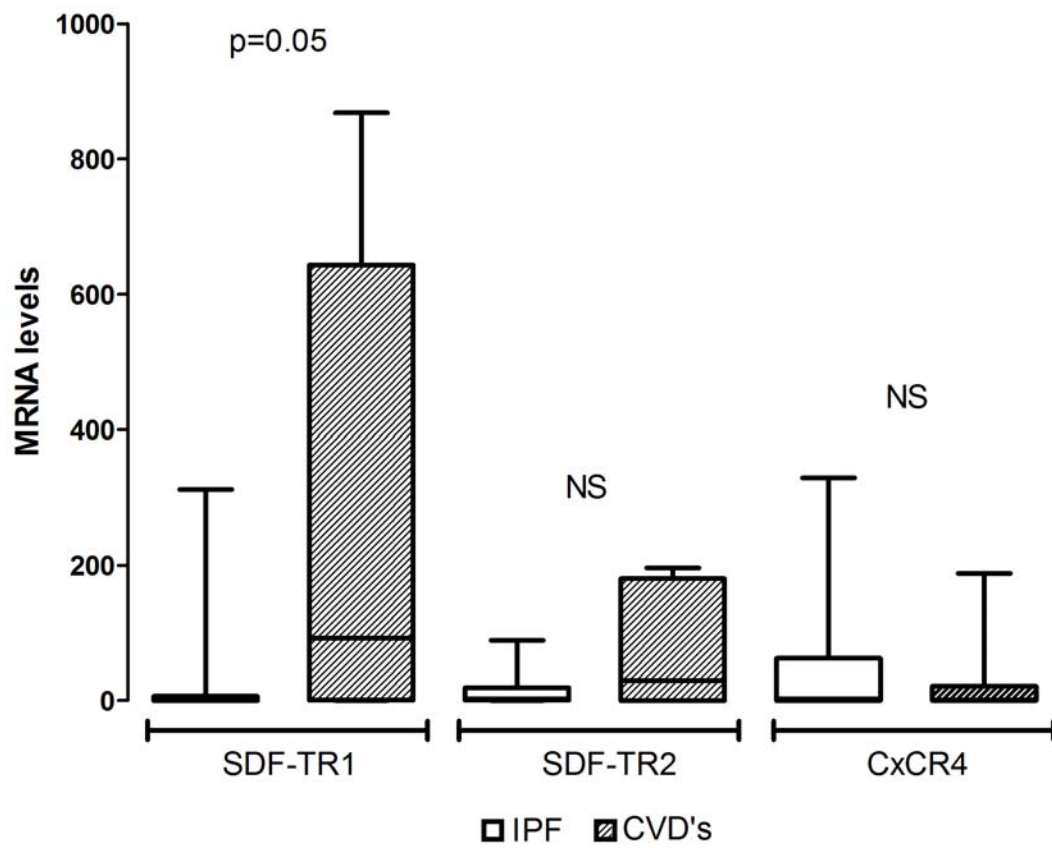
- Bold text indicates angiogenic parameter and pairs with statistical significance

Legends for figures

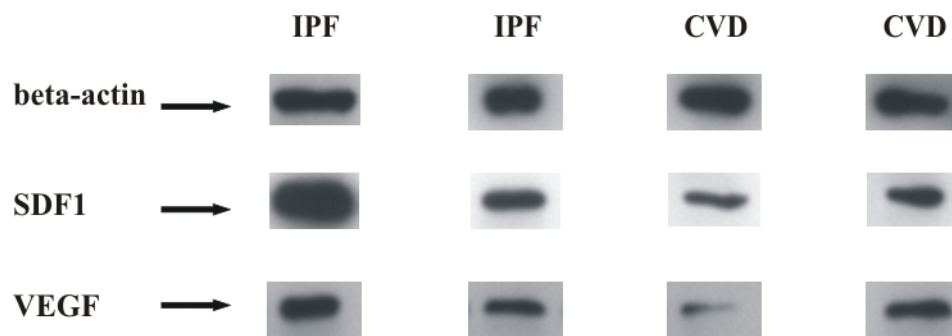
Figure 1: mRNA levels of SDF-1 α and its receptor, CXCR4 in CVD-IPs and IPF

Figure 2: Western blot analysis

ACCEPTED MANUSCRIPT



ACCEPTED



ACCEPTED MANUSCRIPT