Comprehensive analysis of inflammatory markers in chronic thromboembolic pulmonary hypertension patients

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ABSTRACT   Chronic thromboembolic pulmonary hypertension (CTEPH) is associated with chronic inflammation but the pathological mechanisms are largely unknown. Our study aimed to simultaneously profile a broad range of cytokines in the supernatant of pulmonary endarterectomy (PEA) surgical material, as well as prospectively in patients with CTEPH to investigate whether circulating cytokines are associated with haemodynamic and physical characteristics of CTEPH patients.

Herein, we show that PEA specimens revealed a significant upregulation of interleukin (IL)-6, monocyte chemoattractant protein-1, interferon-γ-induced protein-10 (IP)-10, macrophage inflammatory protein (MIP)1α and RANTES compared to lung tissue from healthy controls.

In prospectively collected serum, levels of IL-6, IL-8, IP-10, monokine induced by interferon-γ (MIG) and MIP1α were significantly elevated in CTEPH patients compared to age- and sex-matched healthy controls. In serum of idiopathic pulmonary arterial hypertension (IPAH) patients, only IP-10 and MIG were significantly increased. In CTEPH but not in IPAH, IP-10 was negatively correlated with cardiac index, 6-min walking distance and carbon monoxide diffusion capacity. *In vitro*, IP-10 significantly increased migration of freshly isolated adventitial fibroblasts.

Our study is the first to show that IP-10 secretion is associated with poor pulmonary haemodynamics and physical capacity in CTEPH and might be involved in the pathological mechanism of PEA tissue formation.

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Increased circulating IP-10 associated with poor pulmonary haemodynamics and physical capacity in CTEPH patients http://ow.ly/yDX7U

Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is a disease associated with high morbidity and mortality [1]. CTEPH is caused by obstruction of proximal pulmonary arteries as a consequence of acute and/ or recurrent pulmonary emboli, *in situ* thrombosis of larger proximal and segmental pulmonary arteries and/ or small vessel pulmonary arteriopathy [2]. The resulting pulmonary hypertension leads to right ventricular dilatation, elevation of end-systolic and end-diastolic pressures, and right heart failure. Although typical risk factors involved in the development of thrombosis, such as mutations in antithrombin, protein S, protein C, factor II or factor V Leiden, were not significantly associated with CTEPH, elevated levels of antiphospholipid antibodies associated with lupus anticoagulant were detected [3]. In addition, the fibrin β-chain N-terminus from those patients seems to be more resistant to thrombolysis, suggesting an important role for the development of CTEPH but possibly also for pulmonary arterial hypertension (PAH) [4]. Furthermore, a recent study suggests that the differentiation of mesenchymal progenitor cells present in the neointima of the occluded vessels of CTEPH patients might be triggered by factors present in the microenvironment of the included vessel, leading to progressive occlusion [5].

The mechanisms resulting in the persistence and progression of the obstructing fibrothrombotic material in CTEPH requires further elucidation. There is a growing interest in the role of inflammation, and even infection, for the progression of the disease. BONDERMAN *et al.* [6] have described *Staphylococcus* infection in CTEPH thrombi. Additionally, they detected that *Staphylococcus* infection delayed the thrombus resolution in a murine model [6]. In CTEPH, inflammatory cells (*e.g.* CD45$^+$) and collagen-secreting cells have been found to be abundant in the vascular wall [7, 8]. Furthermore, circulating levels of inflammatory factors such as tumour necrosis factor-α, monocyte chemoattractant protein (MCP)-1 and C-reactive protein were shown to be elevated in CTEPH, and MCP-1 showed a positive correlation with pulmonary vascular resistance [9–12].

We have recently reported that CXCL10, also known as interferon-γ-induced protein-10 (IP-10), causes endothelial dysfunction in primary endothelial cells from human pulmonary arteries [13]. Our working hypothesis was that cytokines like IP-10 mediate the obstructive remodelling of pulmonary arteries in CTEPH. Apart from IP-10, we investigated a broad range of inflammatory factors (interleukin (IL)-8, IL-6, MCP-1, monokine induced by interferon-γ (MIG), RANTES, CX3CL1, macrophage inflammatory protein (MIP)1α and CXCL12) that may be involved in the inflammatory response and may attract cell migration. These factors were investigated in pulmonary endarterectomy (PEA) tissue, PEA supernatant and the serum of newly diagnosed CTEPH and idiopathic pulmonary arterial hypertension (IPAH) patients. We found that in CTEPH patients, IP-10 was negatively correlated with cardiac index, 6-min walking distance (6MWD) and diffusing capacity of the lung for carbon monoxide (D_{LCO}). In addition, IP-10 increased migration of freshly isolated human adventitial fibroblasts *in vitro*. This suggests an important role of IP-10 in the pulmonary vascular remodelling in CTEPH.

Materials and methods

Surgical PEA tissue

PEA tissue was obtained from eight CTEPH patients undergoing PEA. The patient characteristics are presented in table 1. The PEA tissue was transported directly from the operating room to the cell laboratory in endothelial culture media (VascuLife VEGF Cell Culture Medium; LifeLine Technology, Walkersville, MD, USA) and was processed immediately thereafter. For this study, proximal and distal PEA tissue was evaluated. The study protocol for tissue donation was approved by the Institutional Review Board of the Medical University of Vienna (Vienna, Austria) in accordance with national law. Written informed consent was obtained from each individual patient.

In addition, samples from donor lungs, not used for lung transplantation, were used for gene expression studies. This study was also approved by the Institutional Review Board of the Medical University of Vienna in accordance with national law.

Serum samples from CTEPH and IPAH patients and healthy controls

Blood samples were prospectively taken from a separate cohort of patients who were treated at the Medical University of Graz (Graz, Austria) when undergoing diagnostic or follow-up right heart catheterisation. 16 CTEPH and 11 IPAH patients were enrolled, as well as 16 healthy controls sex- and age-matched with the CTEPH patients and 11 healthy controls sex- and age-matched with the IPAH patients. The samples from the healthy controls (n=98) were prospectively collected by the Biobank of the Medical University of Graz between 2011 and 2013.

All samples underwent the same procedure. Blood samples were left for 30 min at room temperature and then centrifuged at 1500 × g for 10 min. Serum was stored in separator tubes (Vacuette premium tubes;

TABLE 1 Clinical features and haemodynamic parameters

	PEA tissue collection	Serum sample collection			
	CTEPH	CTEPH	Control	IPAH	Control
Subjects	8	16	16	11	11
Female/male	2/6	8/8	8/8	8/3	8/3
Age years	61 (44–69)	64 (34–86)	63 (34–86)	65 (30–83)	63 (34–86)
Heart rate bpm	95 (92–98)	77.5 (65.5–87.5)	ND	78 (67–80)	ND
PAWP mmHg	14.5 (13.8–17)	8.5 (6.8–10)	ND	12 (6–14.5)	ND
P_{CO_2} mmHg	42.5 (39.5–46.3)	30.8 (27.5–34.3)	ND	37.1 (33.8–41.4)	ND
PVR dyn·s·m^{-5}	851 (733–1194)	788 (499–993)	ND	550 (462–722)	ND
mPAP mmHg	54 (48–57)	45.5 (40–59)	ND	40 (35.5–46)	ND
Cardiac output L·min^{-1}	4.5 (3.6–4.7)	3.8 (3.5–5.1)	ND	5.3 (4.6–5.7)	ND
Cardiac index L·min^{-1}·m^{-2}	2.2 (2–2.2)	2.1 (1.9–2.5)	ND	2.8 (2.1–3.1)	ND
Right atrial pressure mmHg	ND	10 (5.8– 13)	ND	7 (5–10)	ND
6MWD m	208 (185.5–241.5)	350 (248–428)	ND	408 (327.8–441)	ND
CRP mg·L^{-1}	0.64 (0.34–1.04)	4.9 (1.9–6)	ND	6.3 (3.5–8.4)	ND
Bilirubin mg·dL^{-1}	ND	1.0 (0.6–1.4)	ND	0.6 (0.5–0.9)	ND
Uric acid mg·dL^{-1}	ND	7.1 (6.1–8.5)	ND	7 (5.2–9.9)	ND
NT-proBNP pg·mL^{-1}	ND	2747 (1217–4104)	ND	535 (251–1496)	ND
NYHA					
I	0	1	ND	0	ND
II	0	6	ND	4	ND
III	8	9	ND	7	ND
IV	0	0	ND	0	ND

Data are presented as n or median (interquartile range), except for age which is presented as median (range). PEA: pulmonary endarterectomy; CTEPH: chronic thromboembolic pulmonary hypertension; IPAH: idiopathic pulmonary arterial hypertension; PAWP: pulmonary artery wedge pressure; P_{CO_2}: partial pressure of carbon dioxide; PVR: pulmonary vascular resistance; mPAP: mean pulmonary artery pressure; 6MWD: 6-min walking distance; CRP: C-reactive protein; NT-proBNP: N-terminal pro-brain natriuretic peptide; NYHA: New York Heart Association; ND: not defined.

Greiner Bio-One GmbH, Kremsmünster, Austria). All samples were aliquoted and stored at -80°C in the Biobank of the Medical University of Graz. Written informed consent was obtained from all subjects. The study was approved by the Institutional Review Board of the Medical University of Graz in accordance with national law.

Isolation of adventitial fibroblasts from donor lungs
Adventitial fibroblasts were isolated from pulmonary arteries from non-transplantable donor lungs by removing the adventitial layer. The adventitial fibroblast tissue was cut and the fragments were resuspended in adventitial fibroblast medium (FibroLife Medium, LS-1038 supplements; LifeLine) and cultured in T75 culture flasks.

Immunohistochemistry
Fresh surgical PEA material and human donor lungs were fixed in 4% formaldehyde for 24 h and embedded in paraffin blocks. The sliced paraffin tissue (2-µm thick) was placed on Capillary Gap Microscope Slides (Dako REAL; Dako, Ely, UK) and kept for further use at room temperature. The immunohistochemical stainings for IP-10 (1:250, ab9807; Abcam, Cambridge, UK), IL-8 (1:100; ab7747; Abcam), MCP-1 (1:100, ab73680; Abcam) and IL-6 (1:100, ab6672; Abcam) were performed with the 3,3′-diaminobenzidine peroxidase substrate kit from R&D Systems (Minneapolis, MN, USA). For the haematoxylin-eosin staining, the protocol of Gill was used. The staining was analysed by an experienced pathologist who was blinded to the patient characteristics. For the collagen fibre staining, the Masson's Trichrome staining protocol was used according to the manufacturer's instructions. The stainings for vimentin (1:100, ab11256; Abcam), α-actin (1:500; Everest Biotech Ltd, Upper Heyford, UK), fibronectin (1:250, ab23750; Abcam), CD45 (1:200, ab10558; Abcam), CD68 (1:250, sc-20060; Santa-Cruz Biotechnology Inc., Dallas, TX, USA), MIP1α (1:50, PA5-32496; Thermo Scientific, Rockford, IL, USA) and CXCL12 (1:100, sc-28876; Santa-Cruz Biotechnology Inc.) were performed with the Vektor ImmPRESS reagent kit (Vector Laboratories Ltd, Peterborough, UK).

Determination of the supernatant of PEA tissue

After removal, the PEA samples from the eight CTEPH patients were immediately put into endothelial cell basal medium supplemented with 5% fetal calf serum (FCS) and antibiotics (VascuLife VEGF Cell Culture Medium; LifeLine Technology) [14]. The tissue was cut into small pieces and incubated for 18 h at 37°C in DMEM-F12 medium (Gibco) with 0.3% FCS, glutamine, penicillin and streptomycin (1% each).

A similar procedure was performed for supernatant collection from primary human cells from donor lungs (pulmonary arterial endothelial cells, smooth muscle cells and fibroblasts). The supernatant was collected and stored at -80°C.

Measurement of cytokines

IP-10, IL-8, RANTES, MIG and MCP-1 were measured with the Human Chemokine Kit Cytometric Bead Array (Becton Dickinson, Franklin Lakes, NJ, USA), whereas IL-6, fractalkine and MIP1α contents in the supernatant and serum were determined using the IL-6, fractalkine and MIP1α Flex sets (558276; 560265; 558325; Becton Dickinson). The measurements were performed on a FACS Canto II flow cytometer (Becton Dickinson). CXCL12 was measured in the supernatant and serum *via* ELISA (human CXCL12/SDF1α Quantikine ELISA kit; R&D Systems).

RNA isolation, c-DNA synthesis and real-time PCR

The trizol protocol was applied for total RNA isolation from PEA material of eight patients and samples from control healthy donor lungs not used for lung transplantation. A Nanodrop 2000c spectrophotometer (PeQlab, Sarisbury Green, UK) was used to quantify the concentration and the purity of the isolated total RNA. The total RNA was converted to cDNA using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas; Thermo Fischer Scientific, Schwerte, Germany). Real-time PCR analysis was performed by a Lightcycler 480 (Roche Diagnostic International AG, Rotkreuz, Switzerland). As a housekeeping gene human B2M was used. Primers used are listed in table S6.

Migration assay

Adventitial fibroblasts were serum-starved over-night in basal medium (FibroLife Medium; LifeLine Cell Technology, Frederick, MD, USA). Sub-confluent cells were harvested with trypsin-EDTA and 8.0×10^5 cells were placed in cell culture inserts (PIEP12R48; Merck Millipore, Darmstadt, Germany). The bottom of the wells were filled with 2 µg·mL^{-1} isotype control or isotype control with IP-10 (10 ng·mL^{-1}). Additional wells were filled with IP-10 (10 ng·mL^{-1}) with CXCR3 blocking antibody (2 µg·mL^{-1}) or IP-10 neutralising antibody (2 µg·mL^{-1}). After 6 h the cells were fixed with methanol and stained with haematoxylin. 20 pictures of five random regions for each filter were made with a light microscope (20X LCPlanFl objective; Olympus Optical Company LTD, Tokyo, Japan). The number of cells that migrated to the bottom of the filters was counted.

Proliferation assay

To investigate the effect of IP-10 on human adventitial fibroblast proliferation the following protocol was applied: 10 000 cells (from three different donors) were seeded in 96-well plates. Prior to stimulation adventitial fibroblasts were kept in quiescent medium (VascuLife basal medium with 1% penicillin/streptomycin and 0.1% FCS; LifeLine Technology) for 24 h. Cells were then treated for 24 h with 1, 10 and 100 ng·mL^{-1} IP-10 or were kept under control conditions. The proliferation of adventitial fibroblasts was determined by [^{3}H]-thymidine (Biotrend Chemikalien GmbH, Cologne, Germany) incorporation as an index of DNA synthesis and measured as radioactivity by a scintillation counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter and Luminometer; PerkinElmer Life and Analytical Sciences, Turku, Finland). Experiments were performed in quintuplicate and repeated four to seven times.

Statistical analysis

Data are presented as median (interquartile range (IQR)), unless otherwise stated. Intergroup differences were assessed by factorial analysis of variance with *post hoc* Bonferroni test or Mann–Whitney U-test as appropriate. The Spearman test was performed for intergroup correlations. A p-value <0.05 was considered significant.

Results

Inflammatory factors secreted by PEA tissue

The clinical features of the patients with CTEPH are reported in table 1. Cytokines released by the resident cells of proximal and distal PEA tissue from eight patients were measured in the supernatant. IL-8, IL-6, MCP-1, IP-10, CXCL12, MIP1α, RANTES and MIG were present at high concentrations as compared to

CX3CL1/fractalkine (fig. 1). Low levels of cytokines were detected in the supernatant of primary human pulmonary arterial endothelial, smooth muscle cells and fibroblast (table S1). In the PEA material itself, mRNA levels were determined. IP-10, IL-6, MCP-1, MIP1α and RANTES mRNA were significantly upregulated compared to healthy human lung tissue (fig. 2a–i). Immunohistochemical staining of the PEA tissue showed expression of IP-10, IL-8, IL-6, MCP-1, CXCL12, and MIP1α (fig. 2j–o). Stainings for the same factors were also performed in donor lungs (fig. S1). The receptors for all these factors were also present in the PEA tissue (fig. S2).

Circulating inflammatory cytokines in CTEPH and IPAH patients

We prospectively enrolled CTEPH and IPAH patients and sex- and age-matched healthy controls to investigate the circulating inflammatory cytokines. Blood sampling was performed during right heart catheterisation. The clinical features and haemodynamic parameters of the patients and the controls are shown in table 1. Flow cytometry and ELISA assay performed on the serum samples showed a significantly elevated level of IL-8, IL-6, IP-10, MIG and MIP1α in the serum of CTEPH patients as compared to healthy controls (fig. 3 and table S2), while serum levels of RANTES were significantly decreased. To elucidate if the observed cytokine profile is specific for CTEPH, we assessed the same inflammatory cytokines in the serum of IPAH patients and their age- and sex matched controls. The demographics and pulmonary haemodynamics of the IPAH patients are listed in table 1. In IPAH, IP-10 and MIG but not IL-6, IL-8 and MIP1α were significantly elevated (fig. 4).

IP-10 and IL-6 are significantly correlated with haemodynamics in CTEPH

Next we performed Spearman analysis between the significantly elevated cytokine levels and haemodynamics. The detailed analysis is shown in table S3. In CTEPH, there was a significant negative correlation between IP-10 and cardiac output ($\rho = -0.71$) and cardiac index ($\rho = -0.54$) (fig. 5). In addition, there was a significant positive correlation between IL-6 and pulmonary vascular resistance ($\rho = 0.52$), right atrial pressure ($\rho = 0.62$) and N-terminal pro-brain natriuretic peptide (NT-proBNP; $\rho = 0.51$) (fig. 5). None of the other inflammatory cytokines demonstrated any significant correlations with haemodynamic parameters. In addition, IP-10 serum levels were negatively correlated with D_{LCO} ($\rho = -0.57$ for single breath D_{LCO} and $\rho = -0.62$ for D_{LCO}/alveolar volume).

In the IPAH patients, IL-6 but not IP-10 was significantly correlated with haemodynamic parameters (tables S4 and S5). IL-6 was significantly negatively correlated with cardiac output ($\rho = -0.63$) and cardiac index ($\rho = -0.86$). There was a significant positive correlation with New York Heart Association functional class ($\rho = 0.66$), C-reactive protein ($\rho = 0.67$), uric acid ($\rho = 0.71$) and NT-proBNP ($\rho = 0.85$). Taken together, these results indicate that IL-6 is strongly associated with prognostic markers in IPAH, but that IP-10 is only associated with haemodynamics in CTEPH.

Correlations of cytokine levels with 6MWD

In CTEPH patients, only IP-10 and IL-6 were significantly correlated with exercise capacity, as assessed by 6MWD ($\rho = -0.66$ and $\rho = -0.64$, respectively) (fig. 5 and table S3).
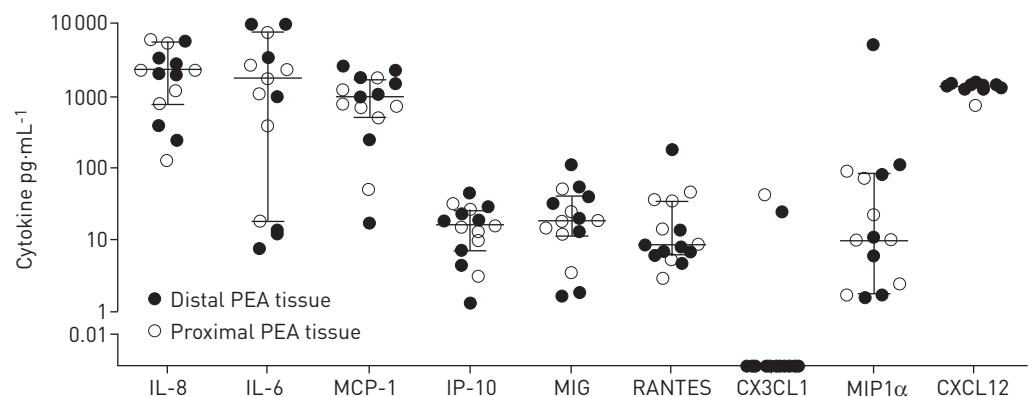

FIGURE 1 Cytokines in the supernatant of pulmonary endarterectomy (PEA) tissue. Results of flow cytometry and ELISA measurements in the supernatant of PEA tissue obtained from eight different patients. Data are presented as median (interquartile range). Each data point is a mean of ELISA/fluorescence-activated cell sorter measurements obtained from three to 12 independent PEA materials. IL: interleukin; MCP: monocyte chemoattractant protein; IP: interferon-γ-induced protein; MIG: monokine induced by interferon-γ; MIP: macrophage inflammatory protein.
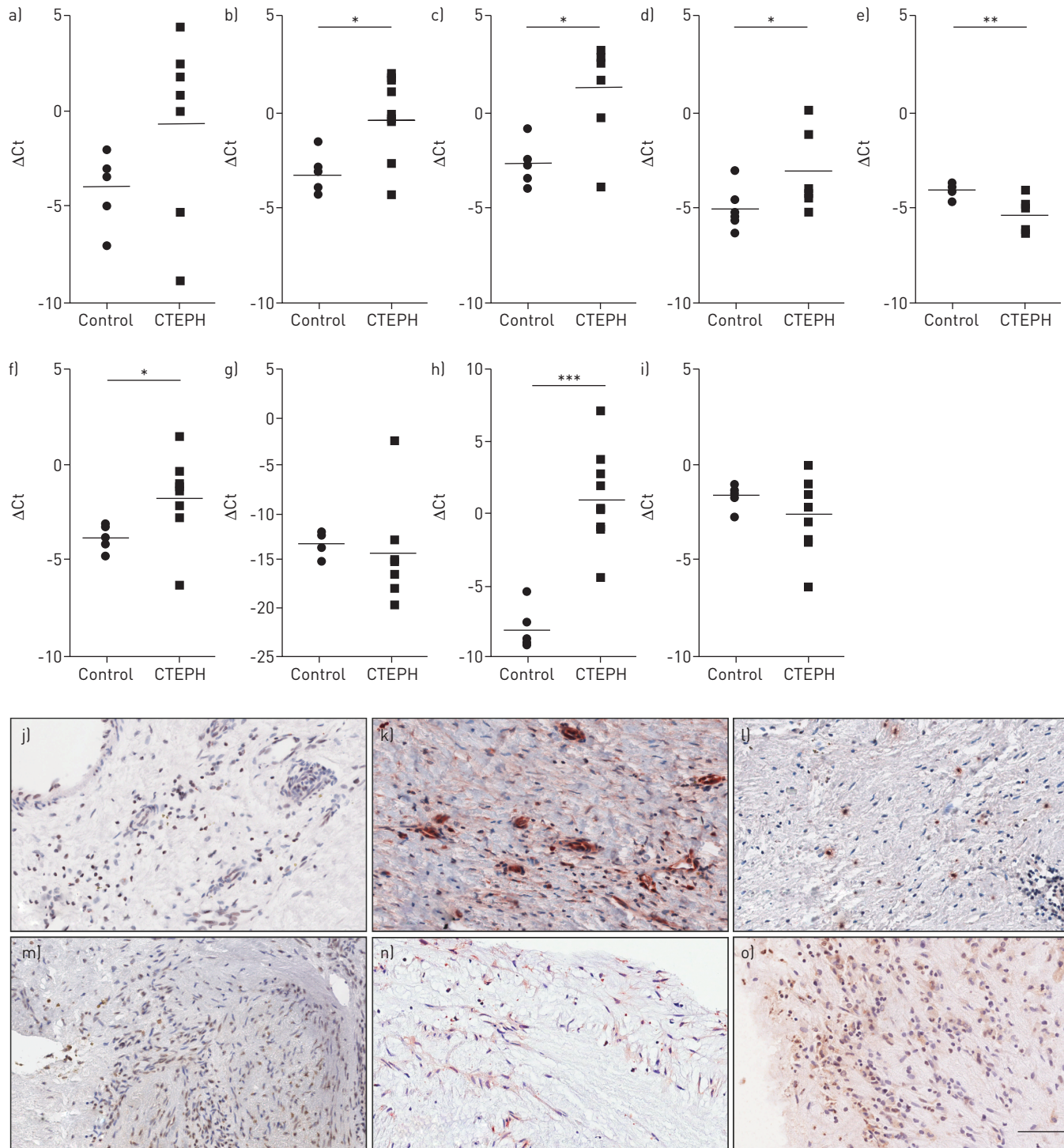
FIGURE 2 Cytokines in pulmonary endarterectomy (PEA) tissue. Gene upregulation of a) interleukin (IL)-8, b) IL-6, c) monocyte chemoattractant protein (MCP)-1, d) interferon-γ-induced protein (IP)-10, e) monokine induced by interferon-γ, f) RANTES, g) CX3CL1, h) macrophage inflammatory protein (MIP)1α and i) CXCL12 in PEA tissue compared to control lung. *: p<0.05; **: p<0.01; ***: p<0.001. Immunohistochemical staining of j) IL-8, k) IL-6, l) MCP-1, m) IP-10, n) MIP1α and o) CXCL12. CTEPH: chronic thromboembolic pulmonary hypertension. Scale bar=100 μm.

Infiltration of the PEA material by fibroblasts and inflammatory cells

Fixed PEA tissue in serial sectional analysis from Trichrome staining showed an accumulation of collagen (blue) and cell infiltration (red) (fig. 6). Immunohistochemistry showed that most of the infiltrating cells were positively stained for α-actin, fibronectin and vimentin in the PEA tissue, especially in the distal part of the removed tissue. In addition, inflammatory cells (CD45⁺) were also detected, predominantly mononuclear cells
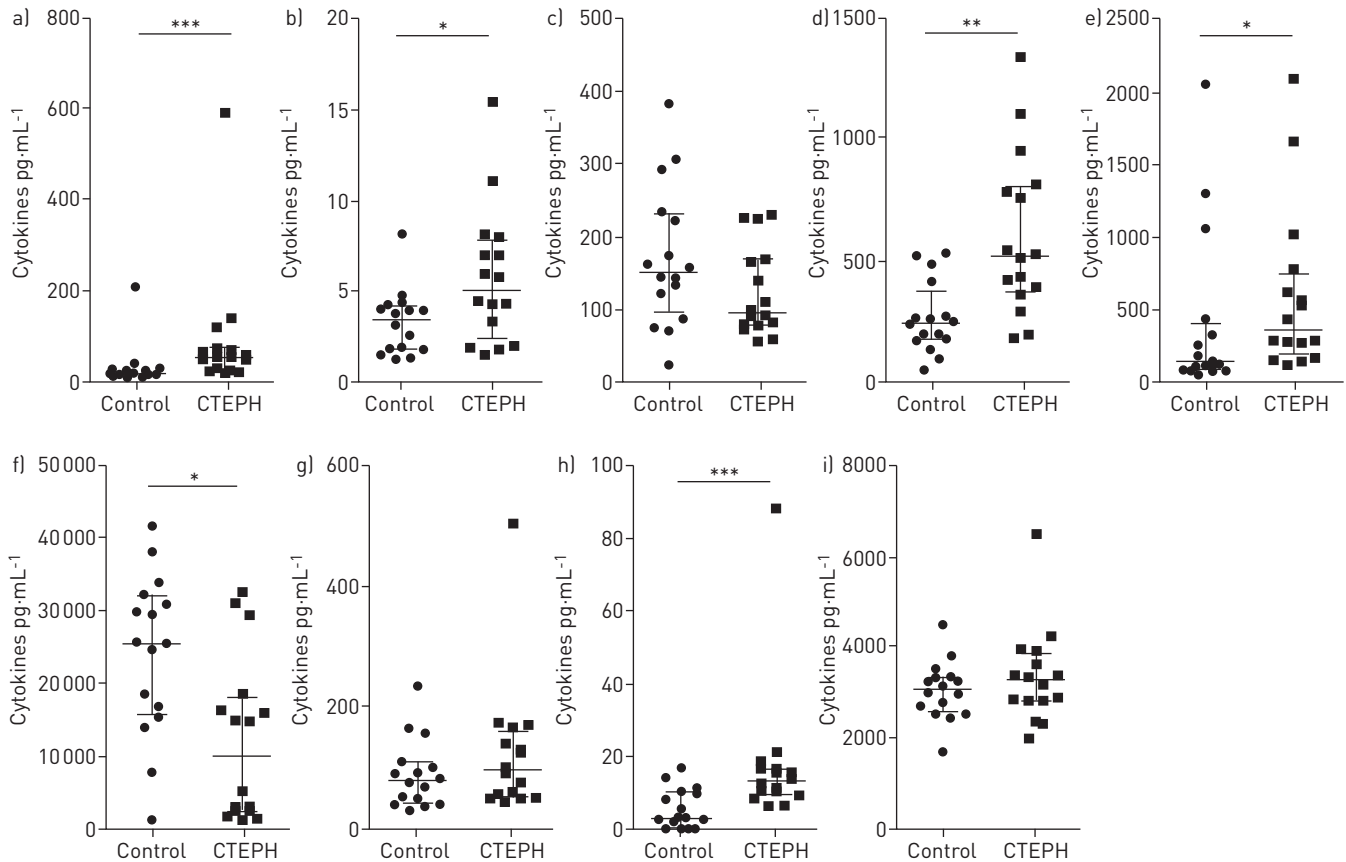
FIGURE 3 Cytokines in the serum of chronic thromboembolic pulmonary hypertension (CTEPH) patients *versus* matched controls. a) Interleukin (IL)-8, b) IL-6, c) monocyte chemoattractant protein-1, d) interferon-γ-induced protein-10, e) monokine induced by interferon-γ, f) RANTES, g) CX3CL1, h) macrophage inflammatory protein-1α and i) CXCL12. Serum samples were taken from venous blood during right heart catheterisation in CTEPH patients and compared to matched controls. *: p<0.05; **: p<0.01; ***: p<0.001.

consistent with lymphocytes, with a minority of macrophages and neutrophils (fig. 6 and fig. S3). Collagen α-actin, fibronectin, vimentin and CD45 were also shown in human donor lungs (fig. S4).

IP-10 induces adventitial fibroblast migration

In transwell migration assays, we found that IP-10 significantly attracted human pulmonary arterial adventitial fibroblasts. The effect of IP-10 was diminished by co-treatment with an CXCR3 blocking antibody or an IP-10 neutralising antibody (fig. 7a). In contrast, IP-10 did not alter either the proliferative behaviour of the adventitial fibroblast or lead to increased activation of F-actin even with the highest used dosage (fig. 7b and c).

Discussion

This study is the first to simultaneously profile a broad range of cytokines in PEA surgical material, its supernatant and in an independent cohort of patients with CTEPH. In addition, we compared the circulating cytokine levels obtained from CTEPH patients with IPAH patients and control subjects. Our results indicate a significant dysregulation of circulating inflammatory cytokines in both CTEPH and IPAH patients. Among IPAH patients, factors indicating decompensation of the right ventricle, such as 6MWD, uric acid, NT-proBNP, *etc.* were strongly associated with IL-6 levels. This agrees with previous findings from Soon *et al.* [15] and Humbert *et al.* [16]. We found that in CTEPH patients such associations were also present with IP-10 levels. IP-10 may be of particular importance because it induces fibroblast migration, a factor that may contribute to the high cellularity of the distal PEA material with collagen producing cells, as observed by Ogawa *et al.* [17]. We demonstrated that the dysregulation of serum cytokines in CTEPH does not only include IP-10, IL-6 and IL-8, but also MIG and MIP1α. Correspondingly, in PEA tissue we detected significantly increased levels of IL-6, MCP-1, IP-10, RANTES and MIP1α and we showed that the proteins are present in the PEA tissue.
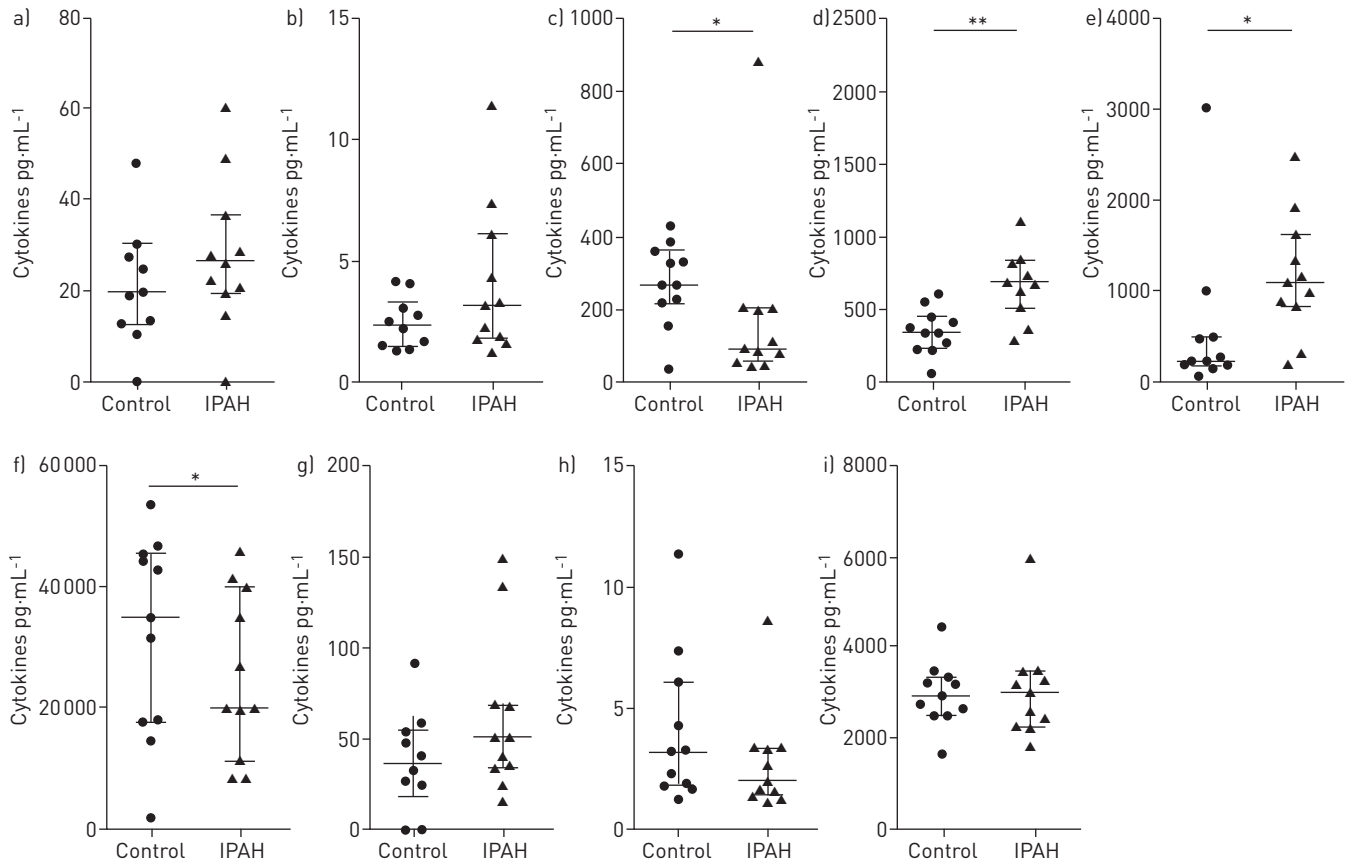
FIGURE 4 Cytokines in serum of idiopathic pulmonary arterial hypertension (IPAH) patients compared to matched controls. a) Interleukin (IL)-8, b) IL-6, c) monocyte chemoattractant protein-1, d) interferon-γ-induced protein-10, e) monokine induced by interferon-γ, f) RANTES, g) CX3CL1, h) macrophage inflammatory protein-1α and i) CXCL12. Samples were taken from venous blood during right heart catheterisation. *: p<0.05; **: p<0.01.

CTEPH is a rare and late consequence of venous thromboembolism and it is associated with prominent obstructions in larger pulmonary arteries [18]. Despite some evidence of dysregulated thrombolysis, the pathological mechanisms of CTEPH remain poorly understood and may involve inflammation, *in situ* thrombosis and endothelial dysfunction. A deleterious effect of chronic inflammation in CTEPH patients is suggested by studies showing that inflammation not only contributes to the pathogenesis of CTEPH, but also to the development of the disease [19]. An increased prevalence of chronic inflammatory diseases was found in CTEPH patients [20] and the presence of *Staphylococcal* antigen in most patients with ventriculoatrial shunt or pacemaker-related CTEPH [6]. Another retrospective study reported that the inflammatory marker C-reactive protein was significantly elevated in patients with CTEPH [12]. In a follow-up study, based on their *in vitro* data, WYNANTS *et al.* [21] postulated that C-reactive protein may contribute to the persistence of obstructing material in pulmonary arteries in CTEPH.

Pro-inflammatory cytokine activation, including IL-6, has been described in patients with IPAH [15, 16], and recently in patients with isolated right ventricular dysfunction due to CTEPH [22]. In addition, haemodynamic instability after PEA has been associated with higher post-operative plasma concentrations of IL-6 and IL-8 [15], suggesting that these cytokines may act as biomarkers to identify patients at risk for developing residual pulmonary hypertension after PEA surgery. Our study confirms the finding of increased levels of IL-6 in the CTEPH serum and extends it by a significant correlation with markers of right ventricular function. In addition, it shows that IL-8 is not only elevated in post-operative but also in preoperative patients. In both the IPAH and CTEPH cohorts, IL-6 was significantly correlated with haemodynamics, although the average level was not significantly increased in IPAH compared to matched controls.

Previous studies investigating the role of chemokines in pulmonary hypertension have primarily focused on PAH. In lung homogenate of IPAH patients (formerly precapillary pulmonary hypertension), increased mRNA levels of the CC chemokine MIP1α [23], and subsequently RANTES, was reported [24]. We detected
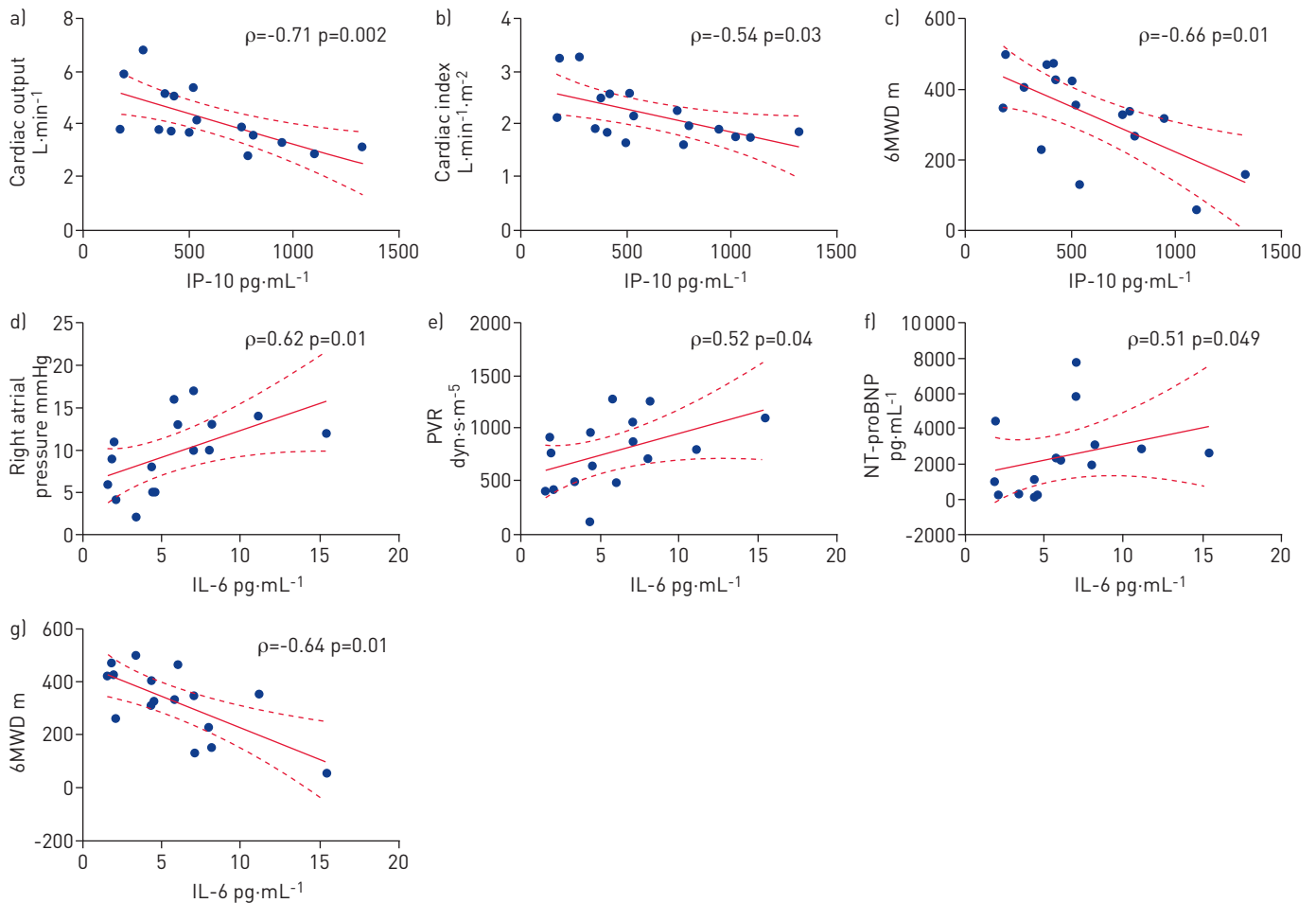
FIGURE 5 Correlation of the serum levels of interferon-γ-induced protein (IP)-10 and interleukin (IL)-6 with haemodynamic and physical parameters in chronic thromboembolic pulmonary hypertension (CTEPH) patients. IP-10 correlation with a) cardiac output, b) cardiac index and c) 6-min walking distance (6MWD). IL-6 correlation with d) right atrial pressure, e) pulmonary vascular resistance (PVR), f) N-terminal pro-brain natriuretic peptide (NT-proBNP) and g) 6MWD. ——: Spearman correlation coefficient (ρ); - - - -: 95% CI of the fit.

significantly increased MlP1α and RANTES in the PEA tissue; however, in the serum of CTEPH patients, only MlP1α was elevated and RANTES was decreased. KIMURA *et al.* [11] reported a correlation of elevated pulmonary vascular resistance with expression of the CCL2 chemokine MCP-1 in the plasma and large pulmonary arteries of CTEPH patients. However, they did not compare their findings in the CTEPH population with healthy controls. Our study included sex- and age matched healthy controls for both the CTEPH and IPAH patients. This showed no significant change of MCP-1 serum level. In our CTEPH population, MIG was significantly elevated as compared with healthy controls. Finally, in line with previous observations in PAH [25], we found that CXCL10/IP-10 was significantly elevated in CTEPH patients. This chemokine is secreted by the cells in the PEA tissue and may attract fibroblasts into the thromboembolic material. Therefore, it could be a link between the persistence of the obstructing fibrothrombotic material and the haemodynamic deterioration.

A major strength of our study is the comprehensive correlation analysis of circulating cytokines with haemodynamics, gas exchange and physical parameters of CTEPH patients. IP-10 was increased both on the mRNA and protein level in PEA tissue, as well as in the serum of CTEPH patients. We observed a significant negative correlation of IP-10 with classic indexes of right heart function (cardiac output and cardiac index), as well as 6MWD. The consistent upregulation of IP-10 in the PEA tissue together with its effect as an inducer of fibroblasts migration and the correlation with haemodynamics points to an important pathological function in the development of CTEPH.

This study was exploratory and included a relatively low amount of PEA material. We employed multiple testing on the circulating levels of cytokines and patients haemodynamics with numerous correlation analyses and comparisons between small numbers of patients and their matched controls. However, we
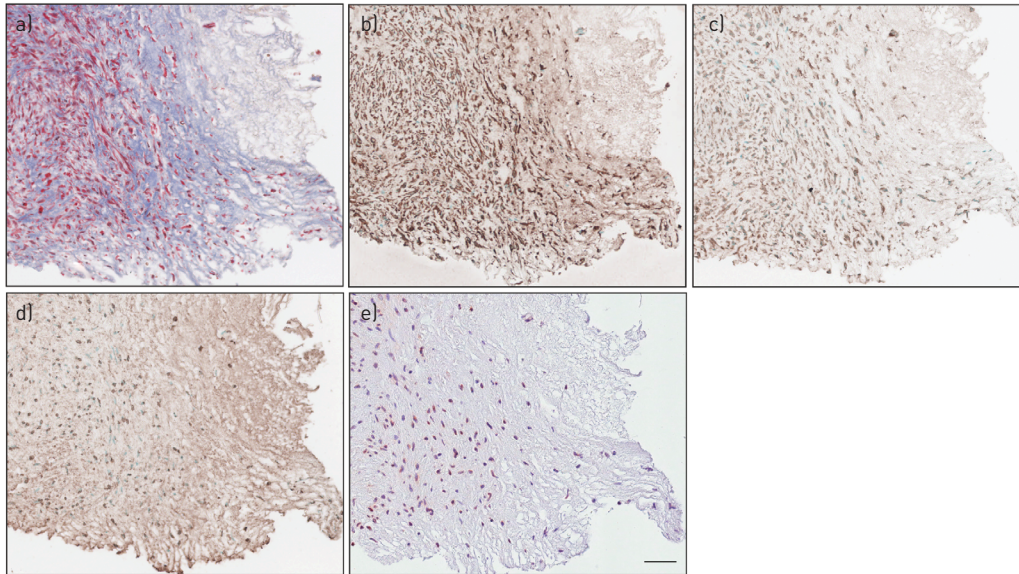
FIGURE 6 Representative photomicrographs of serial sections of pulmonary endarterectomy tissue. Sections were stained with a) trichrome to show collagen, or immunostained for b) vimentin, c) α-smooth muscle actin, d) fibronectin and e) CD45. All images are representative of eight patients. Scale bar=50 μm.

included clinically well-characterised CTEPH and IPAH patients and sex- and age matched healthy controls that were independently and prospectively enrolled in the Biobank of the Medical University of Graz. To confirm the diagnostic value of the inflammatory markers, such investigations should be extended to a larger number of patients in a prospective study.
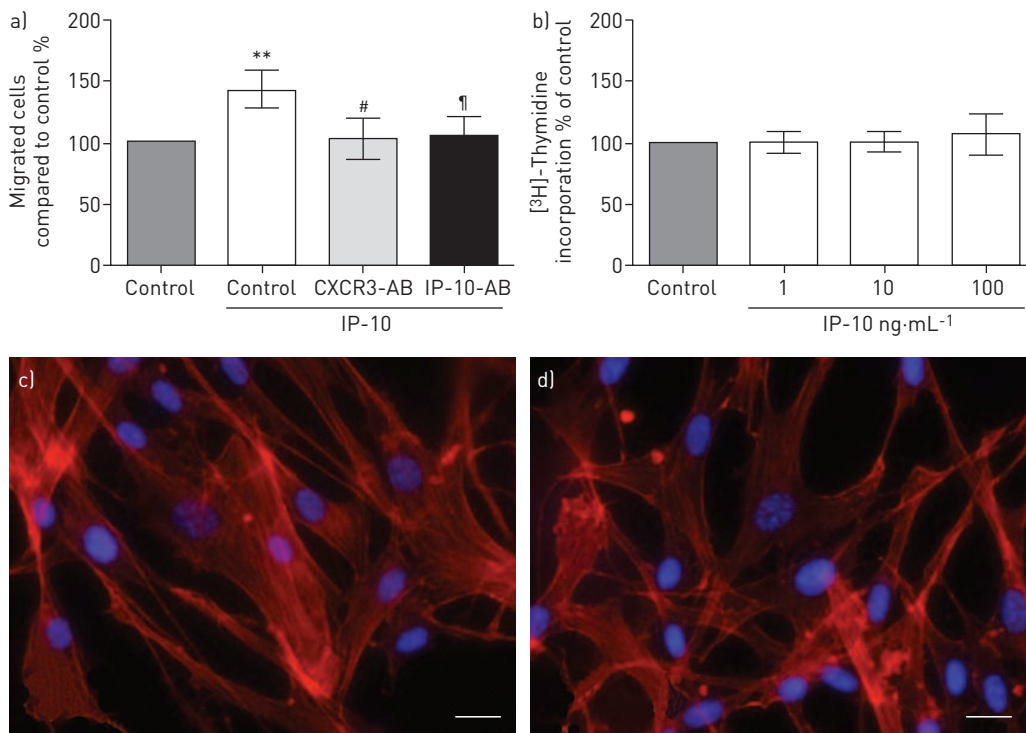

FIGURE 7 Interferon-γ-induced protein (IP)-10 causes adventitial fibroblast migration. a) Migrated adventitial fibroblasts towards IP-10 stimuli compared to untreated control, or treatment with CXCR3-antibody (AB) or with IP-10 neutralising AB. **: $p<0.01$; #: $p<0.01$, ¶: $p<0.05$ compared to IP-10 treated control. b) Proliferating adventitial fibroblasts on 1, 10 and 100 ng·mL^{-1} IP-10 stimuli compared to unstimulated control. Adventitial fibroblasts stimulated for 24 h c) without or d) with IP-10 were stained for F-actin. Scale bar=20 μm.

We can only speculate on the mechanisms underlying progressive obstruction of the pulmonary arteries in CTEPH. However, the importance of IP-10 for endothelial dysfunction in the pulmonary circulation has been recently shown [13]. In the current study we showed that IP-10 specifically boosts adventitial fibroblast migration. Thus, local IP-10 may attract fibroblasts from the adventitia or perhaps circulating fibrocytes into the thromboembolic material leading to a persistent accumulation of those cells that secrete collagen and may cause growth and stiffening of this endovascular material. Also MCP-1, MIP1α and IL-6 were shown to promote migration of monocytes, macrophages and fibroblasts [26, 27]. Furthermore, MCP-1-induced inflammation can lead to neointimal hyperplasia [28, 29] and also stimulates fibroblast proliferation and collagen secretion [30]. Finally, in line with our observations, inflammatory cell infiltration was observed in the distal vascular end of the PEA material [8]. These findings constitute further evidence that cellular infiltration and proliferation as well as collagen production are among the factors that lead to progressive occlusion of the pulmonary arteries in CTEPH and that IP-10 may play a central role.

In conclusion, we demonstrate that IP-10 is elevated on the mRNA and protein level of PEA tissue, as well as in the serum of CTEPH patients. The pathophysiological relevance of this upregulation is supported by the fact that circulating IP-10 levels are correlated with poor haemodynamic and physical parameters. In addition, IP-10 facilitates fibroblast migration, which might contribute to the progression of the disease. Further studies to define the temporal changes of IP-10 during disease progression and treatment are warranted.

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