Contribution of inflammation and impaired angiogenesis to the pathobiology of chronic thromboembolic pulmonary hypertension

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ABSTRACT Deficient angiogenesis and systemic inflammation could be involved in the pathophysiology of chronic thromboembolic pulmonary hypertension (CTEPH). We aimed to characterise the histopathology of pulmonary vascular lesions from 52 CTEPH patients who underwent a pulmonary endarterectomy (PEA) and investigate a potential link between clinical, biological and morphometric parameters.

Collagen, elastin, fibrin, lipid, endothelial, smooth muscle and inflammatory cell content was investigated using immunohistochemistry. Qualitative changes were evaluated using severity scores. Circulating levels of inflammatory mediators were measured using ELISA.

Neointima, thrombotic, recanalised and atherosclerotic lesions were found. Accumulation of macrophages, T-lymphocytes and neutrophils was found mainly in atherosclerotic and thrombotic lesions. Angiogenesis was observed in all kinds of lesions; low-scored angiogenesis predicted adverse outcome, including persistent pulmonary hypertension post-PEA, start of medical therapy and poor survival. C-reactive protein (CRP), interleukin-10, monocyte chemotactic protein-1, macrophage inflammatory protein-1α and matrix metalloproteinase (MMP)-9 were significantly elevated in CTEPH patients. Plasma CRP and MMP-9 levels correlated with neutrophil and macrophage accumulation, respectively.

Enhanced systemic inflammation parallels local inflammatory cell infiltration in major pulmonary arteries at advanced stages of CTEPH. Impaired neovascularisation is associated with poor survival, start of medical treatment and persistent pulmonary hypertension post-PEA. These findings suggest that inflammation and impaired angiogenesis could contribute to the progression of the disease.

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Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is one of the main causes of precapillary pulmonary hypertension. CTEPH is characterised by the presence of unresolved thromboemboli associated with fibrous stenosis in the major pulmonary arteries, resulting in obstruction of major pulmonary arteries, increased pulmonary vascular resistance (PVR), pulmonary hypertension and progressive right heart failure. It may be caused by massive pulmonary embolism and local formation of thrombi. Whereas precapillary arteriopathy may occur in nonoccluded and occluded lung areas [1], the obliteration of major pulmonary arteries, removable by pulmonary endarterectomy (PEA) up to subsegmental level, is the major feature observed in CTEPH [2, 3].

Several authors have reported on the histomorphometry and cellular content of pulmonary vascular material collected from patients who underwent PEA. THISTLETHWAITE et al. [4] proposed a surgical classification based on a macroscopic observation of the presence of fresh thrombotic material and of fibrotic tissue either at a proximal or more distal level. ARBUSTINI et al. [5] described fibrous plaques, including angioneogenesis, and core-rich atherosclerotic plaques, consisting of immunoreactive material including cholesterol clefts, macrophages, T-lymphocytes and calcification. BERNARD and YI [6] performed a clinicopathological study of 200 consecutive PEA samples which evidenced organised thrombi, inflammation, cholesterol clefts, calcification and increased cellularity. The cellular protagonists of major-vessel pulmonary remodelling in CTEPH patients remain poorly characterised.

In addition, there is evidence supporting the involvement of systemic inflammation in the pathogenesis of CTEPH, including elevated prevalence of inflammatory diseases in CTEPH patients [7], prevention of resolution of thrombi in rodents following bacterial infection [8] or splenectomy [9] and elevated tumour necrosis factor-α plasma levels in CTEPH [10]. Elevated PVR is correlated with increased expression of CC chemokine ligand (CCL)2 in plasma and large pulmonary arteries of CTEPH patients [11]. Circulating levels of various cytokines including interleukin (IL)-1β, IL-4, IL-8 and IL-10 are increased in CTEPH patients [12]. Increased expression of intercellular adhesion molecule-1 and raised serum levels of its soluble form are found in various conditions associated with endothelial activation, such as pulmonary hypertension [13]. Increased expression of CCL3 has been reported in lung biopsies from patients with pulmonary arterial hypertension (PAH) [14]. Elevated circulating biomarkers of tissue remodelling, such as matrix metalloproteinases (MMPs) [15], and disordered angiogenesis, mainly characterised by impaired vascular endothelial growth factor (VEGF) expression [16] have also been observed in pulmonary hypertension. This inflammatory concept is further supported by our recent observations that CTEPH patients display elevated circulating C-reactive protein (CRP) levels, which decrease significantly after PEA [17], and that CRP may increase endothelium dysfunction and the proliferating capacities of endothelial and smooth muscle cells isolated from PEA material [18, 19].

More recently, a concept of deficient angiogenesis in CTEPH has been proposed [20], based on the presence of angiogenic factors such as VEGF or basic fibroblast growth factor (bFGF) in resolving venous thrombi [21], and on the enhancement of venous thrombus recanalisation and organisation caused by VEGF in a venous thrombosis rat model [22]. In addition, a deficiency in angiogenic gene expression has been observed in PEA material [23].

We hypothesised that inflammation and angiogenesis could be involved in the pathogenesis of CTEPH. The purpose of the current study was to evaluate the histomorphometry and cellular content of major-vessel lesions from CTEPH patients and to investigate a potential link with clinical and biological parameters. Therefore, we have analysed major-vessel lesions from CTEPH patients who underwent a PEA, by immunohistochemistry, in parallel with the analysis of the systemic inflammatory mediator and growth factor profile.

Material and methods

Pulmonary vascular material was obtained from 52 patients diagnosed with CTEPH, who underwent a PEA between April 1, 2004 and January 31, 2009 at the University Hospital Leuven (Belgium). The study protocol was approved by the institutional ethics committee of the University Hospital Leuven and all participants gave written informed consent.

Details of the material and methods are available in the online supplementary material.

Results

Characteristics of the study population

The demographics and clinical characteristics at diagnosis of the 52 CTEPH patients from whom pulmonary vascular material was obtained are reported in table 1. 40 CTEPH patients (77%) had a history of acute venous thromboembolism (VTE). Among the 52 patients, 18 patients (34.6%) had at least one of the established thrombotic risk factors including lupus anticoagulant/antiphospholipid antibodies (15.4%),

TABLE 1 Study population characteristics

	CTEPH patients	Control subjects
Subjects n	52	20
Age years	58 (54–62)	54 (49–59)
Female	60	50
BMI kg·m^{-2}	27 (25–28)	27 (25–29)
Ever smoking	65	55
AHT	33	20
DMII	5.8	15
History of pulmonary embolism and/or DVT	77	NA
Coagulopathy	71	NA
6MWD m	363±122	NA
NYHA functional class	2.7 (2.6–2.9)	NA

Data are presented as mean (95% CI), % or mean±SD, unless otherwise stated. CTEPH: chronic thromboembolic pulmonary hypertension; BMI: body mass index; AHT: arterial systemic hypertension; DMII: diabetes mellitus type II; DVT: deep venous thrombosis; 6MWD: 6-min walking distance; NYHA: New York Heart Association; NA: not applicable.

protein S and C deficiency (13.5% and 1.9%, respectively), activated protein C resistance, including factor V Leiden mutation, prothrombin gene mutation and antithrombin deficiency (5.8%). In addition, factor VIII was elevated in 9.5% of the patients. Finally, 16 patients (31%) had hyperhomocysteinaemia (>15 μmol·L^{-1}). After surgery, endarterectomised patients displayed a decrease in mean pulmonary artery pressure (mPAP) (−14±13 mmHg; p<0.0001) and in PVR (−570±388 dyn·s·cm^{-5}; p<0.0001) and an increase in cardiac index (+0.91±0.93 L·min^{-1}·m^{-2}; p<0.0001) (table 2). 12 (23%) patients had persistent pulmonary hypertension after PEA, defined by mPAP ⩾35 mmHg 2–3 days after surgery. 12 patients started medical treatment for PAH after PEA: seven with bosentan; four with epoprostenol and one with iloprost. Moreover, eight (15%) patients had received PAH medical treatment before PEA: five received bosentan and three received epoprostenol; in three of them mPAP was normalised and PAH medical treatment stopped after 1 week (epoprostenol), 4 months (epoprostenol) and 4 months (bosentan).

During the observation period, 12 patients displaying persistent pulmonary hypertension after PEA died. Six in-hospital deaths were observed: right heart failure (n=2), lung reperfusion oedema (n=1), inflammatory reaction (n=1), bleeding (n=1) and undetermined reasons (n=1). The six later deaths were attributed to sudden death (n=2), myocardial infarction (n=1), sepsis (n=1), undetermined reasons 3–6 years after PEA (n=1) and heroin overdose 6 months after surgery (n=1). Among patients with normalised hemodynamics after PEA, no deaths were observed. The median "age of the lesions", calculated as the time lapse between the first acute episode of pulmonary embolism and the PEA was 18 months (range 4 months to 15 years). The median time between first detection of pulmonary hypertension and PEA was 15 months with a range of 1 month to 9 years. The median time between CTEPH diagnosis with right heart catheterisation and PEA was 2.4 months (range 0.7–10.5 months); this time interval also corresponds to the period between blood collection and surgery.

Morphometric analysis of thromboembolic disease
The 103 specimens were classified macroscopically (table 3) according to Jamieson classification [24]. Four different kinds of lesion were observed, including neointima formation, atherosclerotic lesions, thrombotic

TABLE 2 Patient haemodynamic data before and after pulmonary endarterectomy (PEA)

	Pre-PEA	Post-PEA	Change post–pre	p-value
RAP mmHg	6 (5–8)	8 (9–11)	+3±6	0.0002
mPAP mmHg	46 (43–49)	33 (30–36)	−14±13	<0.0001
PVR dyn·s·cm^{-5}	795 (696–907)	299 (258–347)	−570±388	<0.0001
CI L·min^{-1}·m^{-2}	2.0 (1.9–2.1)	3.0 (2.8–3.1)	0.91±0.93	<0.0001

Data are presented as geometric mean (95% CI) or mean±SD, unless otherwise stated. Pre-PEA: at diagnosis; post-PEA: at day 2–3; RAP: right atrium pressure; mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; CI: cardiac index.

	Type I	Type II	Type III
Right specimen	28	15	9
Left specimen	15	30	6

Data are presented as n. Type I: fresh thrombus in the main and/or lobar pulmonary arteries; type II: intimal thickening and fibrosis proximal to the segmental arteries; type III: disease within distal segmental arteries only.

lesions and recanalised regions (fig. 1). Neointima is characterised by the presence of α-smooth muscle actin (SMA)-positive cells (fig. 1a). Atherosclerotic lesions displayed macrophage-derived foam cells containing lipid droplets (fig. 1b). Thrombotic lesions either contained fresh thrombi or were more organised (fig. 1c). Fresh thrombus was characterised by the presence of fibrin, which disappears from organised thrombi (fig. 2a and b). Recanalised lesions were characterised by the presence of neovessels (fig. 1d).

Whereas neointima formation was observed in 94% of the patients, atherosclerotic lesions were more rarely found (31% of the patients); thrombotic and recanalised lesions were present in 75% and 63% of the patients, respectively (fig. 3a). Interestingly, in patients bridged with PAH medical therapy prior to PEA, atherosclerotic lesions were more often observed (63% *versus* 25%, p=0.03) and thrombotic lesions were more rarely found (25% *versus* 70%, p=0.01). The distribution of the different type of lesions according to Jamieson surgical classification (fig. 3b) shows that mural thrombus was dominant in type I material, whereas neointima was dominant in type II material. Recanalised lesions were more frequent in type II compared to type I material and atherosclerotic lesions were marginal in both material types. Regarding the two patients harbouring type III material, thrombotic, neointima, atherosclerotic and recanalised lesions were observed. The endothelium monolayer was absent or disrupted in 50%, 70% and 80% of the
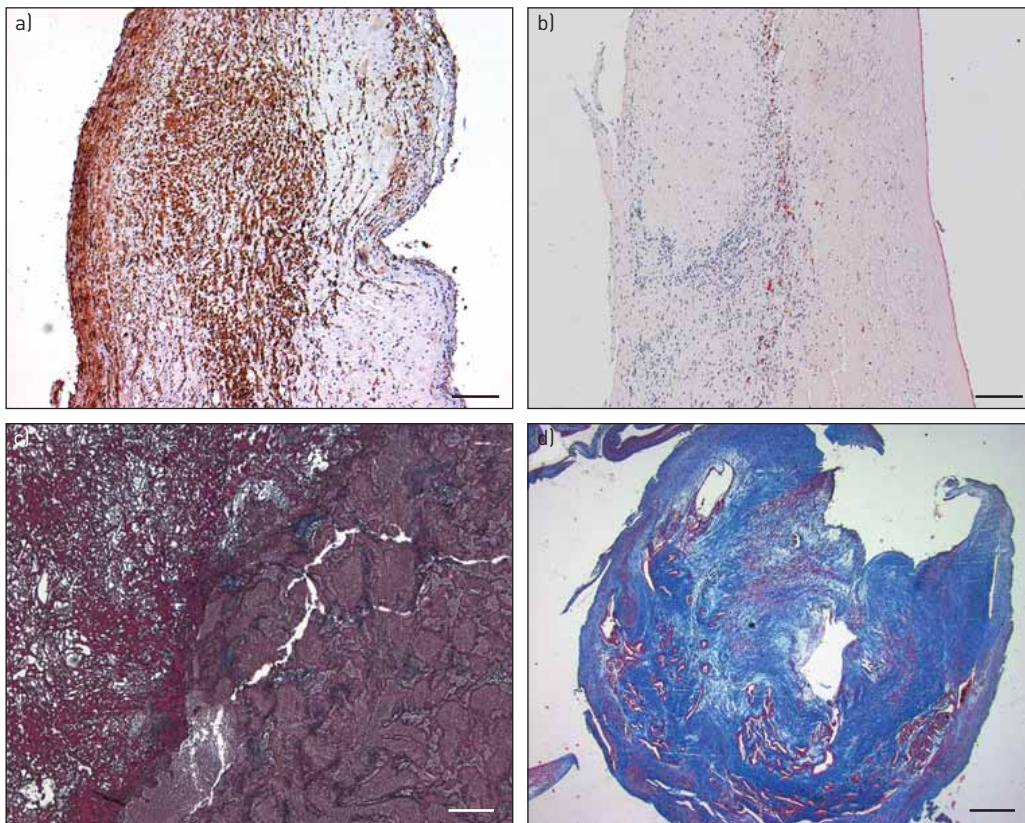

FIGURE 1 Different kinds of lesion observed in large pulmonary vascular material of patients with chronic thromboembolic pulmonary hypertension. a) Neointima immunolabelled by an anti-α-smooth muscle actin antibody; b) atherosclerotic lesion labelled with oil red O; c) thrombotic lesion labelled with phosphotungstic acid; d) recanalised lesion labelled using Masson staining. a–c) Scale bars=400 μm; d) scale bar=2 mm.
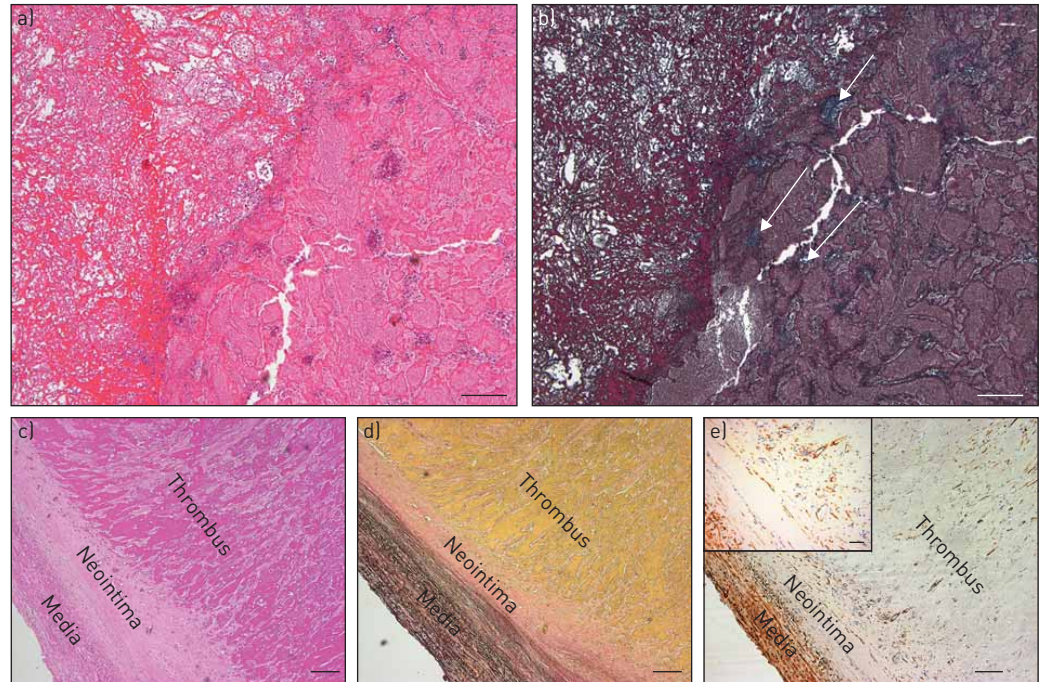
FIGURE 2 Histomorphometry of thrombotic lesions. a) Cell nuclei labelled with haematoxylin and eosin (HE); b) fibrin labelled with phosphotungstic acid (arrows); c) cells labelled with HE; d) elastin labelled using Verhoeff–van Gieson stain; e) smooth muscle cells immunolabelled using an anti-α-smooth muscle actin antibody. a–e) Scale bars=400 μm; e) inset scale bar=100 μm.

neointima, atherosclerotic and thrombotic lesions, respectively. An intracellular lipid deposit was found in 90% of the analysed atherosclerotic lesions. Fresh thrombi were rarely observed in thrombotic lesions, whereas organised thrombi were found in >90% of thrombotic lesions. Interestingly, colonisation of organised thrombotic lesions by intimal α-SMA-positive cells was observed (fig. 2c–e). Among the 41 CTEPH patients with a documented history of VTE, we found the presence of mural thrombi in 26 (63%) patients and no thrombi in 15 (37%) of them. Similarly, among the 10 patients without history of VTE, six (60%) had mural thrombi. Completeness of endarterectomy, evaluated by the number of thrombotic tails [25], was similar in patients with persistent pulmonary hypertension and in patients whose haemodynamics normalised after PEA (9.3±8.1 versus 9.8±5.1).

Angiogenesis

The presence of CD31-positive cells or endothelial cells was observed in all kinds of lesion. Angiogenic process was evidenced by the presence of isolated endothelial cells, sprouting capillaries (early stages) and developing and mature neovessels (late stages) (fig. 4). Only one patient displayed no angiogenesis and
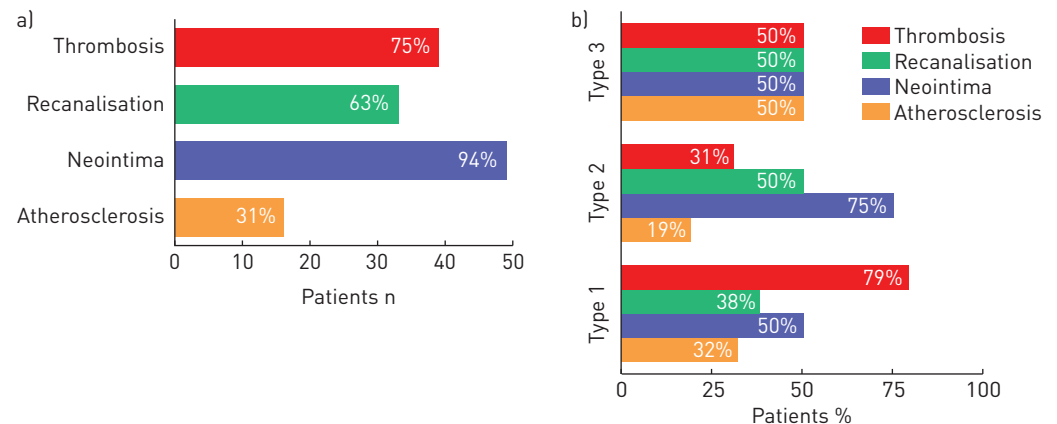

FIGURE 3 Repartition of the different kinds of lesion a) among patients and b) according to Jamieson surgical classification [4].
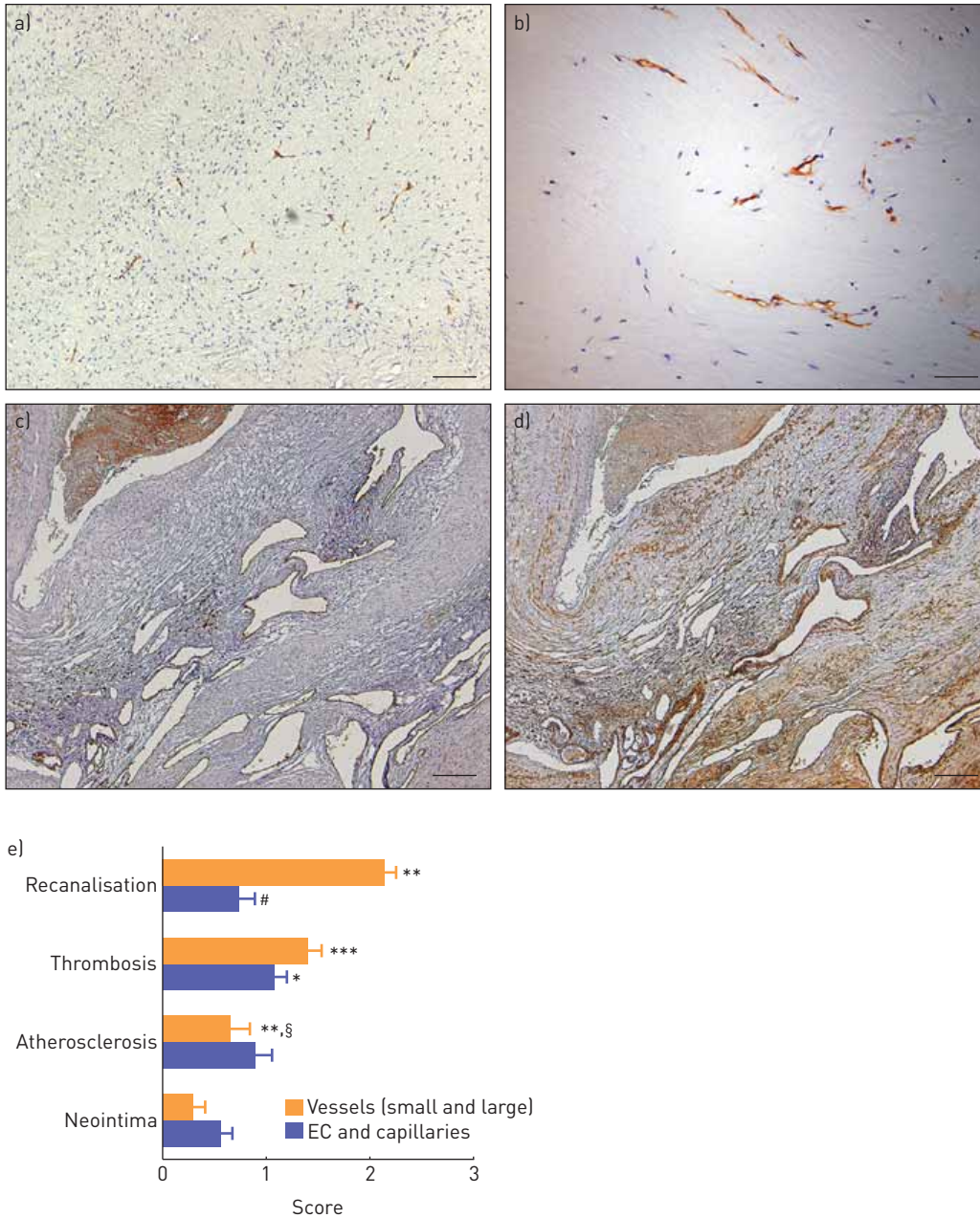
FIGURE 4 Angiogenesis. Different kinds of lesions were immunolabelled with an anti-CD31 antibody and a) cords, b) capillaries, c) small vessels and d) large vessels were identified. a, c, d) Scale bars=400 µm; b) scale bar=200 µm. e) CD31 immunolabelling was scored in the different kinds of lesion and for the different stages of angiogenesis. ANOVA for endothelial cells (EC) and capillaries: p=0.02; *: $p<0.05$ versus neointima; #: $p<0.0001$ versus vessels. ANOVA for vessels: p<0.0001; **: $p<0.01$ versus thrombosis; ***: $p<0.001$ versus neointima; §: $p<0.001$ versus recanalisation.

four did not harbour late angiogenesis. Whereas the amount of isolated endothelial cells and capillaries was comparable in the four kinds of lesions, the number of neovessels progressively increased from neointima and atherosclerotic to thrombotic and recanalised lesions (fig. 4e). Moreover, in neointima and atherosclerotic lesions, cords and capillaries were predominant, whereas in thrombotic and recanalised lesions, neovessels were more represented, with a significant difference in recanalised lesions (fig. 4e). The overall angiogenesis score was significantly lower in nonsurviving CTEPH patients (0.66 ± 0.37 versus 1.04 ± 0.46, p=0.009) (online supplementary fig. S1b), and tended to be lower in CTEPH patients with persistent pulmonary hypertension after PEA (0.76 ± 0.46 versus 1.01 ± 0.46, p=0.10) and in CTEPH patients who started medical treatment (0.80 ± 0.44 versus 1.03 ± 0.46, p=0.08), but did not reach statistical significance. More specifically, late angiogenesis was significantly lower in CTEPH patients with persistent pulmonary hypertension post-PEA, in the nonsurvivors and in those patients who started medical

treatment (0.65±0.64 *versus* 1.20±0.78, p=0.03; 0.49±0.44 *versus* 1.26±0.80, p=0.001; and 0.73±0.67 *versus* 1.11±0.45, p=0.01, respectively) (fig. S1d–f). By contrast, early angiogenesis was similar in all groups. In addition, Kaplan–Meier analysis showed that patients with an overall angiogenesis score >0.85 (fig. 5a) and with a late-scored angiogenesis >0.80 (fig. 5b) displayed a significantly higher rate of survival; patients with a late-scored angiogenesis <0.70 (fig. 5c) harboured a greater risk of starting medical treatment. Finally, overall and late angiogenesis are both predictive for survival, start of medical treatment and persistent pulmonary hypertension post-PEA (fig. 5d and e). Overall angiogenesis was inversely correlated with the age of the lesion (r=−0.57, p=0.0001). Age of lesions was higher in nonsurvivors compared to that of survivors (66±74 *versus* 27±33 months, p=0.04). Although patients who died in hospital harboured a lower angiogenesis score, analysis of odds ratios showed that low angiogenesis did not predict early in-hospital death. Scored angiogenesis was similar in patients bridged with PAH medical therapy prior to PEA and in patients who did not receive any PAH medical therapy prior to PEA.

α-SMA-positive cells were found as isolated smooth muscle cells in the four types of lesion, and as organised neovessels only in recanalised lesions (fig. 6a). Isolated α-SMA-positive cells were evenly distributed in neointima, atherosclerotic and thrombotic lesions, whereas significantly higher proportions were observed in recanalised lesions. Neovessels were mainly found in recanalised lesions and were almost absent from the other kinds of lesion (fig. 6b). Similar to angiogenesis, CTEPH patients with persistent pulmonary hypertension post-PEA displayed a significantly lower score for α-SMA-positive cells (1.06±0.56 *versus* 1.48±0.68, p=0.03). In addition, the amount of mature vessels was significantly lower in these patients (0.23±0.50 *versus* 0.84±0.85, p=0.008), whereas the amount of isolated α-SMA-positive cells was similar in the two subgroups of patients (1.72±0.97 *versus* 2.12±0.69, p=0.095).

Inflammatory cells

B-lymphocytes, T-lymphocytes, macrophages and neutrophils were observed in the four kinds of lesion (fig. 7). Whereas B-lymphocyte accumulation was very limited, macrophages, neutrophils and T-lymphocytes were more abundant (fig. 8a). Atherosclerotic and thrombotic lesions displayed an elevated inflammatory cell accumulation compared to recanalised and neointima lesions (fig. 8b). Whereas B-lymphocytes, when present, were mainly rather deeply localised within the lesion and close to the native media, macrophages and T-lymphocytes were transmurally distributed and neutrophils were mainly found at the surface of the lesion, close to the residual lumen (fig. 8c). CTEPH patients with a history of VTE displayed a significantly higher score for CD68 (macrophages) compared to CTEPH patients without
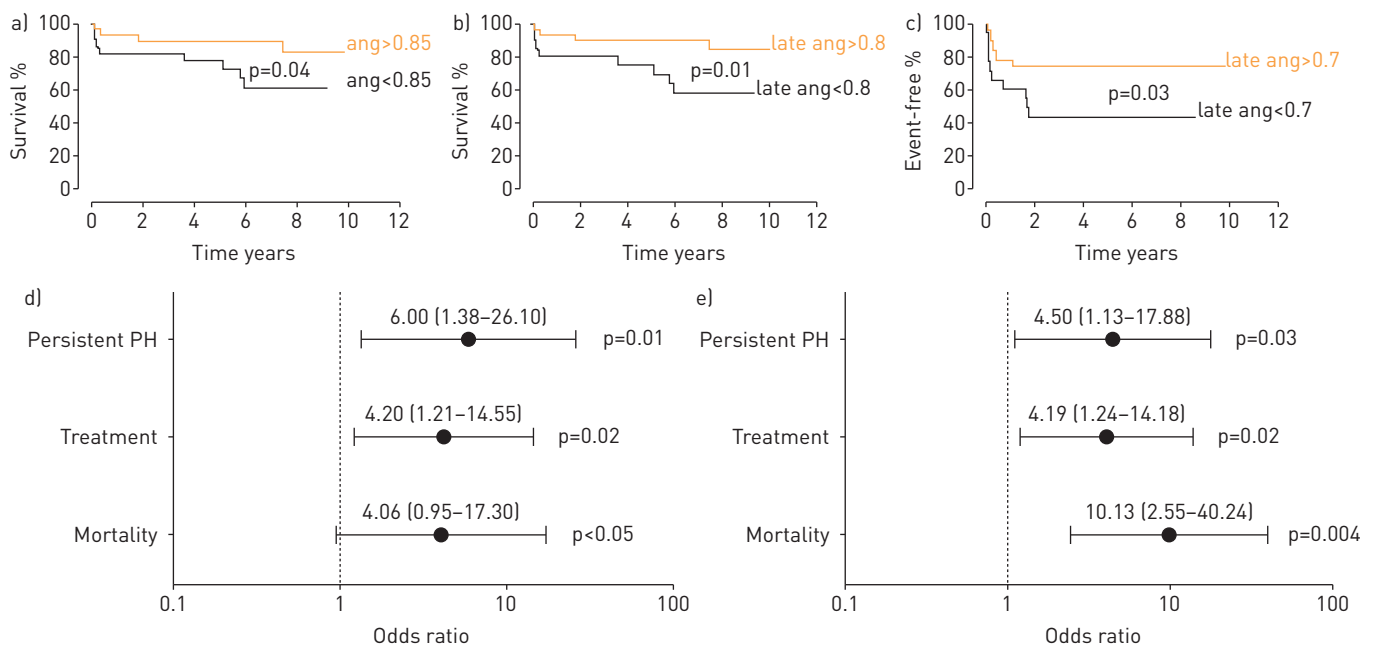

FIGURE 5 Angiogenesis and prediction of adverse outcome in chronic thromboembolic pulmonary hypertension. CD31 immunolabelling was scored in the different kinds of lesion and for the early and late stages of angiogenesis. Lesions were stratified according to persistent pulmonary hypertension (PH) after pulmonary endarterectomy (PEA), survival and start of a medical treatment. Kaplan–Meier curves comparing a) survival with scored overall angiogenesis (ang) <0.85 or >0.85; b) survival with scored late angiogenesis <0.80 or >0.80; and c) start of a medical treatment with scored late angiogenesis <0.70 or >0.70 (cut-off values with best sensitivity and specificity upon receiver operating characteristic analysis). Odds ratios (95% CI) of d) overall scored angiogenesis and e) late scored angiogenesis, evaluating the risk of persistent PH after PEA, start of a medical treatment and mortality.
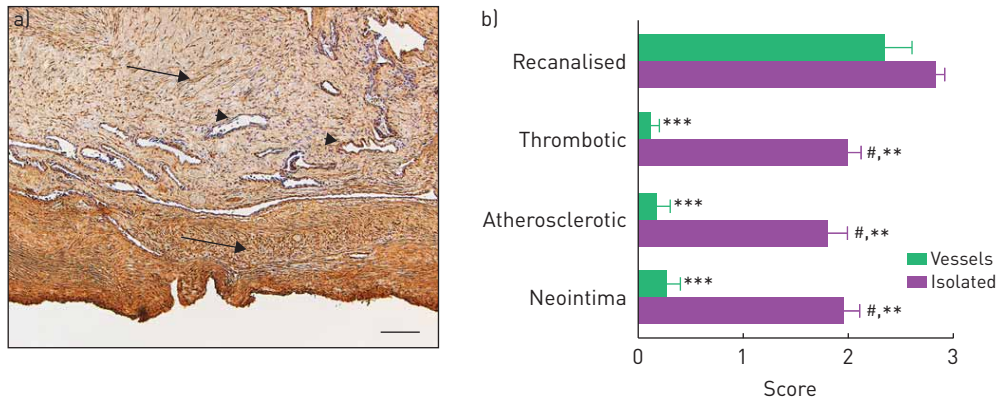
FIGURE 6 α-smooth muscle actin (SMA)-positive cell distribution. a) Lesions were immunolabelled with an anti-α-SMA antibody and isolated smooth muscle cells (arrows) and smooth muscle cells organised in neovessels (arrowheads) were identified; scale bar=400 μm. b) α-SMA immunolabelling was scored into the different types of lesion and divided into isolated α-SMA-positive cells and neovessels. ANOVA for neovessels p=0.003; ***: p<0.001 *versus* recanalisation; ANOVA for isolated smooth muscle cells: p=0.0003; **: p<0.01 *versus* recanalisation; #: p<0.0001 *versus* neovessels.

history of VTE (1.59±0.68 *versus* 1.10±0.61, p=0.04). In patients bridged with PAH medical therapy before PEA, T-lymphocytes scored accumulation was significantly lower (0.88±0.27 *versus* 1.66±0.13, p=0.02).

Circulating inflammatory and angiogenic mediators

Plasma levels of inflammatory mediators measured in CTEPH patients and in healthy control subjects are presented in table 4. CRP, IL-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and MMP-9 are significantly higher in CTEPH patients compared to healthy control
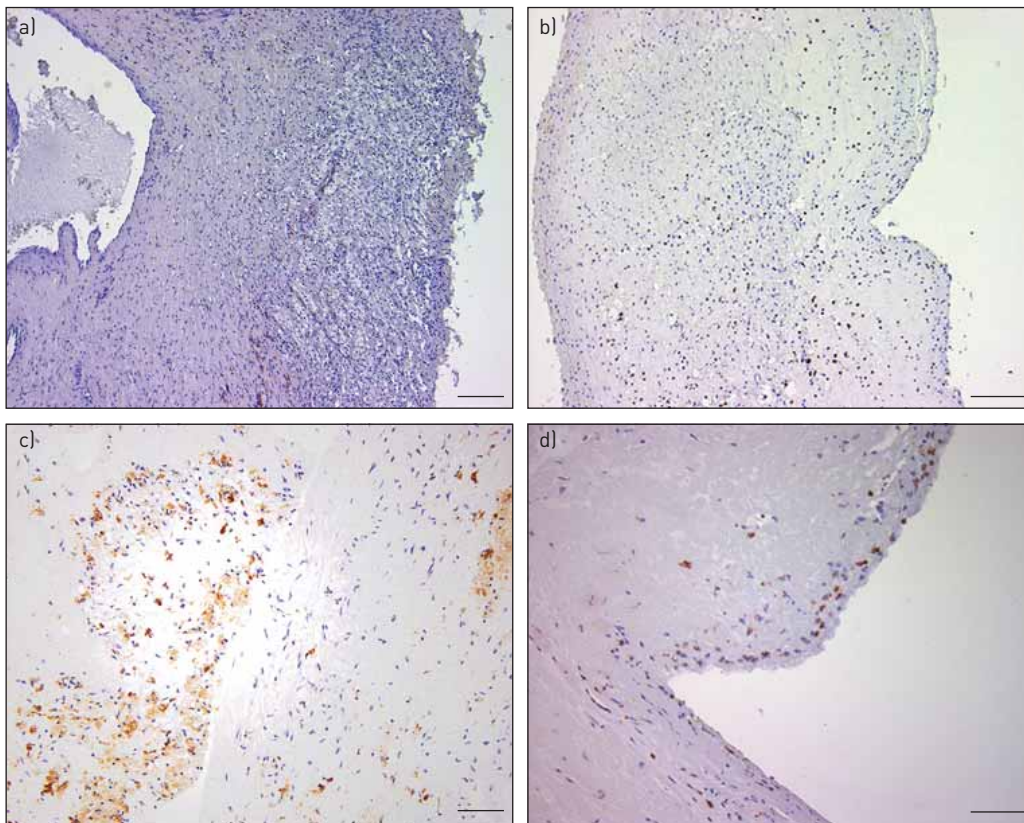

FIGURE 7 Inflammatory cells. Lesions were immunolabelled with a) anti-CD20 (B-lymphocytes); b) anti-CD3 (T-lymphocytes); c) anti-CD68 (macrophages); and d) anti-myeloperoxidase (neutrophils) antibodies. a, b) Scale bars=400 μm; c, d) scale bars=200 μm.
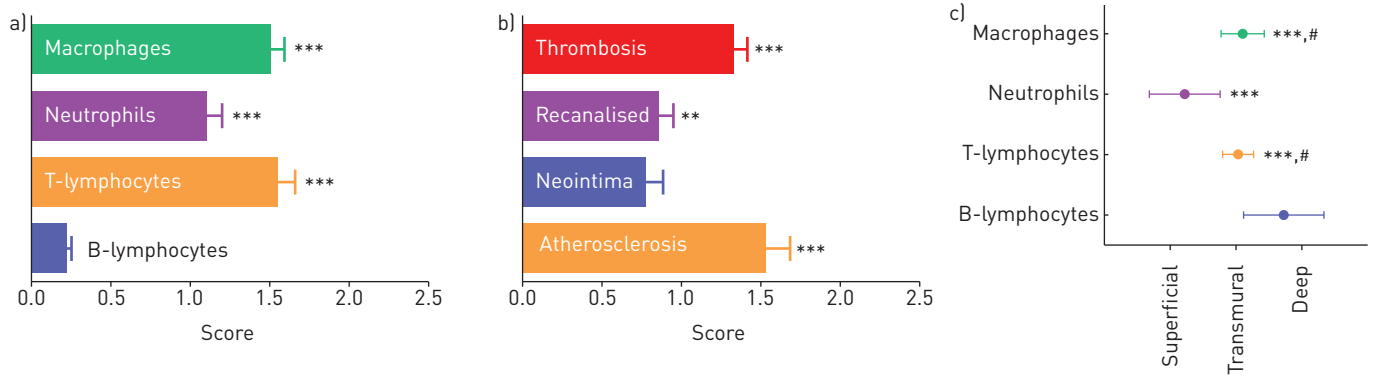
FIGURE 8 Inflammatory cell accumulation and topographic distribution. a) Inflammatory cell accumulation scores in different lesion types according to inflammatory cell type; ANOVA p<0.0001; ***: p<0.001 versus B-lymphocytes. b) Inflammatory cell accumulation scores according to the type of lesion; ANOVA p<0.005; ***: p<0.001 versus neointima; **: p<0.01 versus atherosclerosis. c) Topographic distribution of inflammatory cells type; ANOVA p<0.0001; ***: p<0.001 versus B-lymphocytes; #: p<0.001 versus neutrophils.

subjects, whereas IL-1β and IL-6 levels were similar in the two groups. CRP and MMP-9 levels were positively correlated with inflammatory cell infiltration (r=0.34, p=0.01; and r=0.55, p=0.01, respectively) (online supplementary fig. S5A and C). More precisely, plasma CRP was correlated with neutrophil local accumulation (r=0.36, p=0.01) (fig. S5B) and plasma MMP-9 was correlated with macrophage infiltration (r=0.54, p=0.01) (fig. S5D). VEGF levels tended to be lower in CTEPH patients without reaching statistical significance (17.8, 95% CI 5.5–57.9 versus 32.5, 95% CI 18.4–57.4 pg·μL^{-1}; p=0.09). VEGF was significantly lower in CTEPH patients with persistent pulmonary hypertension post-PEA (26.2±13.6 versus 83.1±17.0 pg·μL^{-1}, p=0.03) as well as MMP-9 (5561±597 versus 8566±1238 pg·μL^{-1}, p=0.04). Circulating VEGF and MMP-9 did not correlate with scored angiogenesis. Interestingly, VEGF was inversely correlated with mPAP measured 3 days after PEA (r=−0.51, p=0.04) and VEGF <48 pg·mL^{-1} predicted persistent pulmonary hypertension post-PEA (OR 24.00, 95% CI 1.74–331.00; p=0.0075).

Discussion

In the present study, we analysed major-vessel lesions of CTEPH patients using immunohistochemistry and found the presence of four types of lesion: neointima, thrombotic, atherosclerotic and recanalised lesions. Accumulation of inflammatory cells, including T-lymphocytes, macrophages and neutrophils was found in all types of lesions. Inflammatory mediators including IL-10, MCP-1, MIP-1α and MMP-9 were elevated in CTEPH patient plasma. An association between local and systemic inflammation was observed. Angiogenesis and α-SMA-positive cells were observed in all types of lesions, with a predominance of neovessels in recanalised lesions. Interestingly, neovessels were less abundant in material from patients with postoperative persistent pulmonary hypertension, and low circulating levels of VEGF predicted

TABLE 4 Plasma levels of inflammatory proteins in chronic thromboembolic pulmonary hypertension (CTEPH) patients and healthy subjects

	Healthy controls	CTEPH	p-value
Subjects n	20	20	
CRP mg·L^{-1}	2.4 (1.6–3.4)	6.4 (4.3–9.4)	**0.0005**
IL-1β pg·mL^{-1}	0.12 (0.09–0.15)	0.12 (0.07–0.20)	0.86
IL-6 pg·mL^{-1}	0.66 (0.43–1.02)	1.17 (0.63–2.20)	0.11
IL-10 pg·mL^{-1}	0.25 (0.17–0.37)	0.71 (0.37–1.32)	**0.006**
MCP-1 pg·mL^{-1}	109 (91–130)	158 (134–186)	**0.002**
MIP-1α pg·mL^{-1}	8.7 (7.3–10.5)	14.2 (11.6–17.2)	**0.0006**
MMP-9 pg·mL^{-1}	1.29 (0.49–3.38)	6.70 (5.50–8.17)	**0.0009**
VEGF-A pg·mL^{-1}	32.5 (18.4–57.4)	17.8 (5.5–57–9)	0.09

Data are presented as geometric mean (95% CI), unless otherwise stated. Bold type represents statistical significance. CRP: C-reactive protein; IL: interleukin; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.

postoperative persistent pulmonary hypertension, suggesting that impaired angiogenesis could predict postoperative persistent pulmonary hypertension.

As previously found [5, 6, 26], we observed the presence of resident organised thrombi, fibroproliferative intima including atheroma and intimal inflammation in the pulmonary vascular material collected during PEA. The mechanisms leading to the remodelling of unresolved thromboemboli into fibrotic tissue, narrowing and obstruction of major pulmonary arteries, increased PVR and right ventricular dysfunction still remain to be unravelled.

Thrombotic material

Thrombotic risk factors are frequently observed in CTEPH patients [27] and Arbustini et al. [5] reported recent thrombotic material in 86% of specimens. By contrast, we rarely observed fresh fibrinous clots, confirming the findings of Blauwet et al. [26] and Bernard and Yi [6], who retrieved fresh thrombi in no patients and in only 0.8% of the analysed specimens, respectively. These discrepancies could be attributed to subjective differences in the interpretation by the pathologist of the degree of organisation of each thrombotic specimen or to therapeutic anticoagulation, which is mandatory for ⩾3 months before PEA in our institution. Blauwet et al. [26] found evidence of the presence of thrombi in each resected specimen, whereas we observed thrombotic lesions in 75% of the patients and mural thrombi in 36% of the patients.

Neointima and atherosclerosis

Although intimal thickening was described as the result of thrombus reorganisation, accompanied by fibrotic tissue incorporation into the intimal layer [26], our findings indicated that neointimal lesions should be distinguished from evolutionary thrombotic lesions regarding angiogenesis and inflammatory cell content. Similarly, neointimal thickening should be distinguished from atherosclerotic lesions, considering their different inflammatory cell content. Atherosclerotic lesions are mainly characterised by accumulation of inflammatory cells, cholesterol clefts and collagen fibres [5, 26], whereas the intimal layer mainly involves collagen and elastin fibre deposition, and α-SMA-positive cells potentially deriving from dedifferentiated smooth muscle cells migrating from the media [28, 29] or from circulating progenitor cells [30, 31]. Whereas a paucity of inflammatory cells was observed in the intimal layer, atherosclerotic lesions were massively infiltrated by macrophages, and to a lesser extent by neutrophils and T-lymphocytes.

Systemic and local inflammation

Infiltration of various inflammatory cells including macrophages, B- and T-lymphocytes and neutrophils was observed, as previously found [5, 6, 26]. By scoring inflammatory cell accumulation, we could quantify the inflammatory process within major-vessel lesions of CTEPH patients. Macrophages, T-lymphocytes and neutrophils were found in the different types of lesion, whereas B-lymphocytes were rarely found, except in thrombotic lesions. Whereas inflammatory cell infiltrates were often observed in atherosclerotic and thrombotic lesions, recanalised and intimal lesions were almost devoid of inflammatory cells, suggesting differential distribution of chemokines and cytokines within the lesions. Moreover, the topographic analysis revealed a superficial localisation, close to the residual lumen, of neutrophils, and a transmural distribution of macrophages and T-lymphocytes, whereas B-lymphocytes were mostly localised deep within the lesion, close to internal elastic lamina and native media. Since material collected during PEA refers to advanced lesions and consequently chronic inflammation, the presence of neutrophils, key players of the innate immune system, rapidly recruited at the injury site following a trauma and mostly considered as a hallmark of acute inflammation, was quite unexpected. The presence of macrophages and T-lymphocytes, also observed in plexiform lesions in PAH patients [32, 33], was predictable and illustrate a chronic inflammatory process. Accordingly, CD4+ T-lymphocytes were observed in CTEPH patients [34]. Interestingly, circulating inflammatory mediators, namely CRP, IL10, MCP-1, MIP-1α and MMP-9 were elevated in CTEPH patients, suggesting mild chronic inflammation, as previously mentioned [11, 17].

In addition, among those enhanced inflammatory mediators, CRP and MMP-9 were found associated with local accumulation of inflammatory cells, namely neutrophils and macrophages, respectively. Bacterial infection, as well as splenectomy, by potentiating the inflammatory insult, could also contribute to progression of the disease [8, 35]. Whereas Kimura et al. [11] observed a correlation between circulating MCP-1 and PVR, we did not find any association between haemodynamic parameters and circulating inflammatory markers, nor with local inflammatory cell accumulation. By contrast, circulating CRP was correlated with local neutrophil accumulation. A correlation between plasma CRP and blood toxic granulation neutrophils has been established in inflammatory diseases [36, 37]. CRP has been shown to elicit white blood cell activation [38]. CRP could prevent neutrophil chemotaxis [39, 40], which could partially explain the neutrophil distribution at the surface of the lesion. Local production of CRP has been observed in vulnerable plaques and in injured coronary arterial walls [41] and in the major large pulmonary arterial tissue from CTEPH patients [18]. CRP-induced production of MMP-9 has been found

in macrophages [42]. MMP-9 is produced by neutrophils, but can also activate neutrophils, and macrophage-derived MMP-9 may contribute to neovascularisation of ischaemic tissue [43].

Angiogenesis and recanalisation

In >60% of the analysed lesions, we observed recanalised lesions characterised by fibrotic material including smooth muscle cells, collagen and elastin, and vascular neoangiogenesis, as previously described [5, 6]. One major feature of recanalised lesions is the presence of neovessels characterised by CD31-positive endothelial cells and α-SMA-positive smooth muscle cells. Recanalisation is a process commonly involved in thrombus resolution and involves recruitment of various cell types including neutrophils, monocytes, endothelial cells and myofibroblasts [44]. For instance, this is illustrated by the colonisation of organised thrombi by migrating α-SMA-positive cells (fig. 2e). It has been suggested that angiogenesis plays a key role in the resolution of thrombi in a rat model of venous thrombosis, highlighting a potential role of angiogenic factors such as VEGF and bFGF [21, 22]. Angiogenesis was observed in all analysed specimens, as previously observed [5], although neovessels were mainly present in thrombotic and recanalised lesions. Interestingly, we observed that patients with adverse outcome displayed fewer neovessels; we also found that low angiogenesis scores could predict adverse outcomes. In addition, plasma VEGF 1) tended to be lower in CTEPH patients compared to healthy subjects; 2) was lower in CTEPH patients with persistent pulmonary hypertension post-PEA; 3) was inversely correlated with mPAP measured 3 days after PEA; and 4) low VEGF predicted persistent pulmonary hypertension post-PEA. With respect to these findings, we may speculate that enhanced pulmonary arterial angiogenesis could be responsible for partial permeability of the obstructive material with maintenance of a preserved postobstructive vascular bed through/by circulating vasoactive factors. Alternatively, in patients displaying less angiogenesis in the removed surgical material, inaccessible distal fibrothrombotic material could be more abundant and less recanalised, resulting in persistent pulmonary hypertension post-PEA. MOSER et al. [1] suggested that endothelial cells or platelets stimulated by the pulmonary hypertensive state could contribute to distal vessel remodelling by producing and releasing proliferative and/or anti-apoptotic mediators. Accordingly, LANG et al. [20] observed a deficiency of vessels in areas distal to occluded vessels and defective angiogenesis was shown to delay thrombus resolution [23]. Additionally, angiostatic factors, which cause changes in calcium homeostasis, consequently affecting pulmonary endothelial cell proliferation and migration, and vessel formation, could be involved in impaired calcium homeostasis and endothelial dysfunction in CTEPH [45]. Finally, impaired neovascularisation was associated with adverse outcome, including poor survival, start of medical treatment and persistent pulmonary hypertension post-PEA in CTEPH patients. Pulmonary hypertension-related mortality, such as right heart failure or reperfusion injury represented only half the inpatient deaths, and none of the later deaths were attributed to right heart failure. However, persistent pulmonary hypertension can complicate the course of intercurrent diseases such as inflammatory reaction, bleeding, myocardial infarction, or sepsis; consequently, all-cause mortality was selected [46].

Limitations

The authors are aware of the limited volume of surgeries, high incidence of mortality (11%) and of postoperative pulmonary hypertension reported in this cohort (23%). However, the current article reports on long-term outcome of patients operated upone 5–10 years ago. Results of surgery, with regard to mortality and persistent pulmonary hypertension, were then less favourable than currently, even in larger centres [47]. Experienced surgeons can currently remove more distal material, which could possibly influence the results of this study. However, whereas CTEPH with a poor outcome had an impaired angiogenesis in the major pulmonary arteries, no difference was observed in the number of thrombotic tails, suggesting that the way in which the surgical specimen was distally removed was not a major concern. Finally, it would have been of great interest to investigate the role of bronchial artery collaterals, since HOEY et al. [48] suggested that collateral vessels from the systemic circulation can grow to reperfuse areas distal to complete obstruction. However, it does not seem to be easy to distinguish bronchial artery collaterals, as recently evoked by DORFMÜLLER et al. [49], who developed an elegant technique using different coloured ink to discriminate systemic arteries, and which also requires the disposal of the complete lung, which is absolutely not the case with material withdrawn during PEA.

Conclusion

Although the present study is basically a descriptive pathological study, the scoring of the different lesions represents an alternative approach compared to previous published investigations. Moreover, elevated inflammatory mediators as well as the association between local and systemic inflammation at advanced stages of the disease highlight the potential role of chronic inflammation in the pathogenesis of CTEPH. Finally, angiogenesis as a potential predictor of postoperative persistent pulmonary hypertension is an interesting finding, which deserves further investigation.

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