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KEYWORDS:
pulmonary endarterectomy; microvascular disease; pulmonary hypertension; chronic thromboembolic pulmonary hypertension; endothelin-1; interleukin-6

BACKGROUND: Pulmonary microvascular disease (PMD) develops in both occluded and non-occluded territories in patients with chronic thromboembolic pulmonary hypertension (CTEPH) and may cause persistent pulmonary hypertension after pulmonary endarterectomy. Endothelin-1 (ET-1) and interleukin-6 (IL-6) are potential PMD severity biomarkers, but it remains unknown whether they are related to occluded or non-occluded territories. We assessed PMD and ET-1/IL-6 gene expression profiles in occluded and non-occluded territories with and without chronic lung reperfusion in an animal CTEPH model.

METHODS: Chronic PH was induced in 10 piglets by left pulmonary artery (PA) ligation followed by weekly embolization of right lower lobe arteries with enbucrilate tissue adhesive for 5 weeks. At Week 6, 5 of 10 animals underwent left PA reperfusion. At Week 12, animals with and without reperfusion were compared with sham animals ($n = 5$). Hemodynamics, lung morphometry and ET-1/IL-6 gene expression profiles were assessed in the left lung (LL, occluded territories) and right upper lobe (RUL, non-occluded territories).

RESULTS: At Week 12, mean PA pressure remained elevated without reperfusion (29.0 ± 2.8 vs 27.0 ± 1.1 mm Hg, $p = 0.502$), but decreased after reperfusion (30.0 ± 1.5 vs 20.5 ± 1.7 mm Hg, $p = 0.013$). Distal media thickness in the LL and RUL PAs and systemic vasculature to the LL were significantly lower in the reperfused and sham groups compared with the non-reperfused group. PMD progression was related to ET-1 and IL-6 gene expression in the RUL and to the ET-A/ET-B gene expression ratio in the LL.

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CONCLUSIONS: PMD regressed in occluded and non-occluded territories after lung reperfusion. Changes in ET-1 and IL-6 gene expression were associated with PMD in non-occluded territories.

Chronic thromboembolic pulmonary hypertension (CTEPH) is due to chronic pulmonary artery (PA) obstruction by organized clots persisting after pulmonary embolism.[1,2] However, in addition to proximal obstruction by clots, pulmonary microvascular disease (PMD) develops in non-occluded territories, contributing to elevate the pulmonary pressure.[3] PMD is induced by increases in blood flow[4–6] and pressure, possibly with a contribution of circulating factors,[7] resulting in non-specific pathologic lesions typical of pulmonary hypertension (PH).[5,8] In occluded territories, post-obstructive pulmonary vasculopathy (POPV) develops in response to the chronic PA occlusion. POPV manifests as increases in PA media thickness and in the systemic blood supply to the lung.[9–12] Surgical treatment of CTEPH by pulmonary endarterectomy (PEA) decreases the pulmonary arterial resistance,[13] thereby preventing progression to right heart failure.[14,15] However, in some patients, PEA is followed by persistent PH,[15] which may be due to the previous development of PMD in occluded and non-occluded territories.[5,16]

To date, there is no evidence that PMD in non-occluded territories and/or POPV can regress after PEA. In several animal models, pulmonary vasculopathy induced by high blood flow regressed after high-flow correction[4] and POPV regressed after PA reperfusion.[9,17] However, the relevance of these findings to CTEPH is limited, as none of these studies were done in reliable CTEPH models.

Endothelin-1 (ET-1), a peptide produced mainly by endothelial cells, exerts mitogenic and vasoconstricting effects on adjacent smooth muscle cells, and is involved in distal PA remodeling in patients with PH.[18] Interestingly, endothelial PA cells were found to overexpress ET-1 when exposed to increased shear stress and pulsatility, as seen in patients with PH.[19,20] Herein we hypothesized that the ET-1 gene expression profile may be chiefly increased in non-occluded vascular territories, where blood flow is higher than in occluded territories. In patients with CTEPH, serum ET-1 elevation before PEA was associated with an increased risk of persistent PH.[21]

In addition, inflammation was recently identified as a key contributor to PA remodeling in PH lungs.[22] Interleukin-6 (IL-6) is a pleiotropic cytokine that influences inflammatory reactions and is a main inducer of C-reactive protein (CRP) secretion.[23] In CTEPH patients, serum CRP levels predict the prognosis after PEA.[24] Moreover, serum IL-6 elevation correlates with the hemodynamic severity of primary PH.[25]

These data suggest a role for ET-1 and IL-6 in PMD development in CTEPH. An unresolved issue of considerable interest is whether these cytokines are differentially expressed in occluded and non-occluded territories.

We recently developed a piglet model of CTEPH modeling the hemodynamic changes due to chronic PA occlusion, with occluded and non-occluded vascular territories, in which PMD could be studied separately.[21,26]

The aims of this study were to determine whether PMD in occluded and non-occluded territories regressed after surgical lung reperfusion used to replicate PEA, and to look for differences between occluded and non-occluded territories in expression levels of IL-6, ET-1 and the two endothelin receptors, ET-A and ET-B.

Methods

All procedures were approved by our institutional animal care committee according to institutional guidelines that complied with the "Principles of Laboratory Animal Care," developed by the National Society for Medical Research, and *The Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health.

CTEPH animal model and surgical interventions

We studied 15 male Large White piglets, each weighing between 20 and 25 kg. CTEPH was induced in 10 piglets, as described by Mercier et al,[26,27] by left PA ligation, which was followed by weekly embolization of the right lower lobe arteries with enbucrilate (Histoacryl; B. Braun, Melsungen, Germany) under fluoroscopic control, for 5 weeks. The right upper lobe (RUL) remained non-occluded, whereas all remaining right lung territories were progressively occluded. At Week 6, 5 piglets underwent surgical left PA revascularization (reperfused CTEPH group) to mimic PEA, by interposition through a left thoracotomy of a vascular prosthesis (diameter 8 mm, length 10 mm) between the pulmonary trunk and left PA. The other 5 piglets were followed up for 6 additional weeks (CTEPH group). These two groups were compared with a sham group ($n = 5$) given weekly saline injections into the PAs.

Hemodynamic assessment

Hemodynamic measurements were performed using a Swan–Ganz catheter connected to a monitor (Vigilance; Edwards Lifesciences, Irvine, CA) at baseline, Week 6 and Week 12. The total pulmonary resistance index (TPRI, in WU/m^2) was calculated by dividing mean pulmonary artery pressure (MPAP, in mm Hg) by cardiac index (CI, in liters/min/m^2). Body surface area (m^2) was computed as: weight (kg)$^{0.656}$ / 10.[28]

Lung sampling, light microscopy and vascular morphometry

At Week 12, random biopsy specimens weighing 300 to 500 mg were taken from the RUL and left lung (LL), snap-frozen in liquid nitrogen, and stored at –80°C or fixed in 4% paraformaldehyde solution instilled into the airway. For light microscopy and

morphometry measurements, three fixed RUL and LL sections per piglet were processed using standard histologic techniques and embedded in paraffin.

Morphometric measurements were performed in a blinded manner by our pathologist (P.D.) using imaging software (NIS-Elements BR, version 2.30; Nikon USA, Melville, NY). PA media thickness (MT) of RUL and LL pulmonary arteries, measuring 40 to 100 μm and 101 to 200 μm in diameter, respectively, was calculated as described elsewhere.[4,17] MT has been quantified in a total of 1,254 pulmonary arteries as: [external media diameter (μm) – internal media diameter (μm)] / external media diameter (μm).

Systemic vasculature to the LL was quantified as described elsewhere[17] around bronchioles >220 μm in diameter. Systemic vessels supplying 48 bronchioles were examined. Bronchial arteries were counted per 100 μm of bronchiole perimeter. Percentage of bronchial artery occlusion was assessed as the ratio of media thickness over external radius. The total bronchial artery lumen area (TBALA) per 100 μm of perimeter of associated bronchial mucosa was determined.

Real-time quantitative polymerase chain reaction

RNAs were extracted from whole RUL and LL tissues using the total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA), then retranscribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). ET-1, ET-A, ET-B, IL-6 and β-actin gene expression profiles were quantified using real-time quantitative polymerase chain reaction (RTqPCR) with TaqMan gene expression assays (gene [assay ID number]: β-actin [Ss03376081_u1]; ET-1 [Ss03392453_m1]; ET-A [Ss03394413_m1]; ET-B [Ss03379833_u1]; and IL-6 [Ss03384604_u1]; Applied Biosystems, Foster City, CA), as reported elsewhere.[29] RTqPCR was performed in a StepOnePlus RTqPCR system (Applied Biosystems). Relative gene expression data were obtained using the ΔΔCt method with 1 sham group piglet as the calibrator and β-actin as the reporter gene.

Human plasma ET-1, IL-6 and CRP levels

Our institutional review board approved the collection of samples from patients, and all patients provided written informed consent before sample collection.

Blood samples were collected from 59 CTEPH patients before PEA and 20 control patients before lung resection for localized lung cancer. Pre-operative transthoracic echocardiography confirmed the absence of PH in the controls. Patients and controls were matched for gender, age and smoking. ET-1 and IL-6 levels were measured using an enzyme-linked immunoassay (ELISA; R&D Systems, Lille, France). Plasma ET-1, IL-6 and CRP levels were compared between controls and the overall patient population, patients with TPR >900 dynes/s/cm^5 and patients with TPR ≤900 dynes/s/cm^5.

Statistical analysis

Hemodynamics, morphometry and gene expression profiles are presented as mean ± SEM with 5 animals per group. We performed 3 × 3 comparisons of continuous variables across and within the three groups of piglets at the three time-points (baseline, Week 6, Week 12) using 1-way analysis of variance (ANOVA), followed by Fisher's protected least-significant-difference test, using statistical software (StatView, version 5; Abacus, Berkeley, CA). Student's *t*-test and 1-way ANOVA were used to compare plasma IL-6, ET-1 and CRP levels in patients with CTEPH and controls. $p < 0.05$ was considered significant.

Results

Hemodynamics

MPAP and TPRI showed similar significant increases from baseline to Week 6 in the CTEPH group (MPAP: from 15 ± 0.4 to 29 ± 2.8 mm Hg, $p = 0.0003$; TPRI: from 3.77 ± 0.48 to 8.32 ± 1.33 WU/m^2, $p = 0.0022$) and reperfused CTEPH group (MPAP: from 14 ± 0.6 to 30 ± 1.9 mm Hg, $p < 0.0001$; TPRI: from 3.03 ± 0.19 to 9.31 ± 0.52 WU/m^2, $p < 0.0001$) (Figure 1). At Week 6, MPAP and TPRI were not different between the CTEPH and reperfused CTEPH groups ($p = 0.7331$ and $p = 0.4104$, respectively). In the sham group, no significant differences were found between baseline and Week 6 for MPAP (12 ± 0.9 vs 13 ± 1.3 mm Hg, $p = 0.3234$) or TPRI (3.09 ± 0.36 vs 4.12 ± 0.55 WU/m^2, $p = 0.1840$).

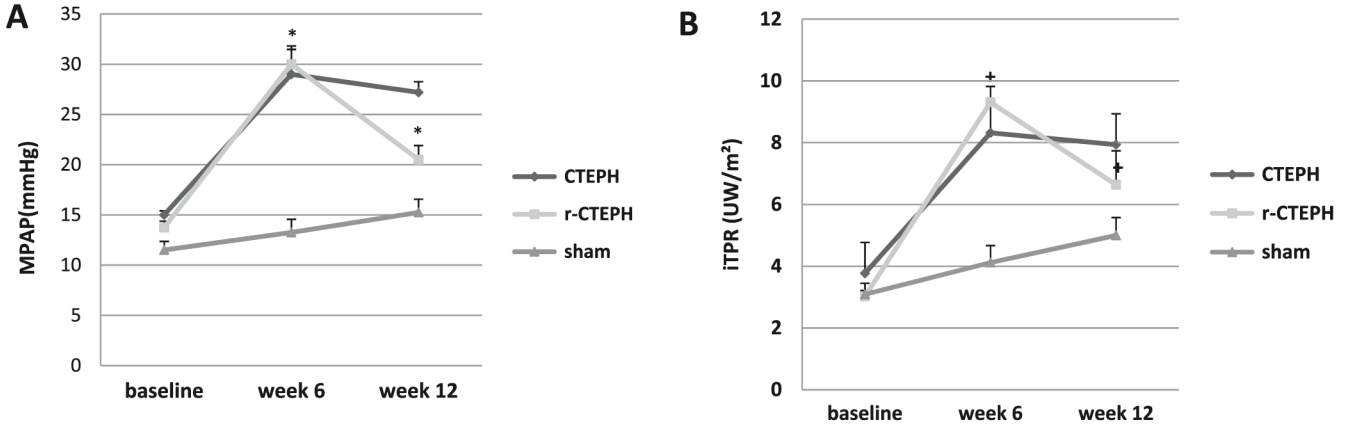

Figure 1 Right heart catheterization data at baseline, 6 weeks and 12 weeks in the three groups. (A) MPAP shown with standard error bars. All animals in the CTEPH and reperfused CTEPH (r-CTEPH) groups had pulmonary hypertension at Week 6. In the r-CTEPH group, MPAP decreased significantly 6 weeks after left PA reperfusion ($^*p < 0.05$), but remained elevated in the CTEPH group. (B) ITPR shown with the standard error bars. The changes mirror those of MPAP, with a significant decrease in the r-CTEPH group ($^+p < 0.05$) at 6 weeks after left PA reperfusion, which is in contrast to the persistent elevation in the CTEPH group.

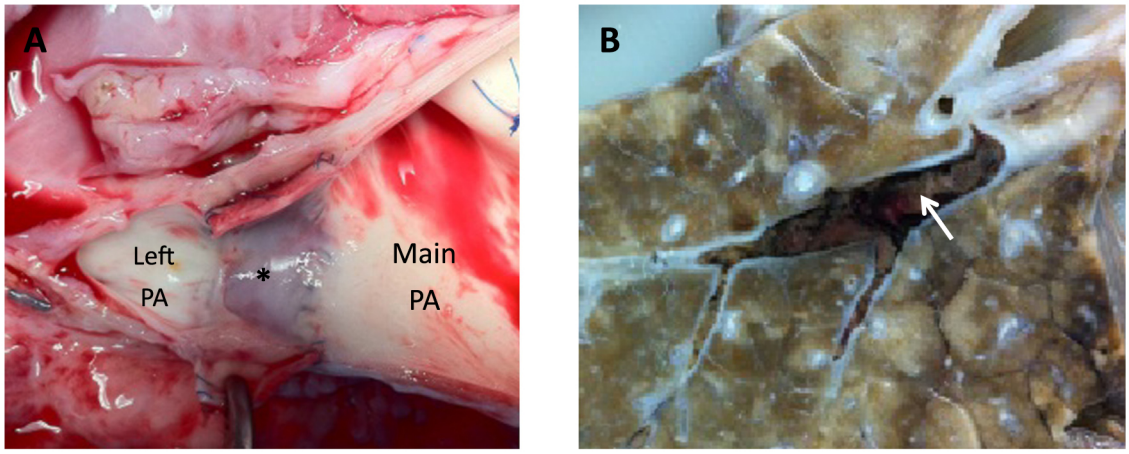

Figure 2 Gross appearance of pulmonary arteries at Week 12. (A) Longitudinal arterial section in a reperfused CTEPH animal showing a patent vascular graft (*) sewn between the main PA and left PA. (B) Intravascular adhesive material (enbucrilate) persisted in the PA of the right lower lobe (white arrow) at 7 weeks after the last embolization.

MPAP and TPRI decreased significantly between Week 6 and Week 12 in the reperfused CTEPH group (MPAP: from 30 ± 1.9 to 20.5 ± 1.7 mm Hg, $p = 0.0129$; TPRI: from 9.31 ± 0.52 to 6.6 ± 1.1 WU/m^2, $p = 0.0192$), but remained elevated compared with baseline (MPAP: 20.5 ± 1.7 vs 14.0 ± 0.6 mm Hg, $p = 0.018$; TPRI: 6.6 ± 1.1 vs 3.0 ± 0.19 WU/m^2, $p = 0.0026$). In the CTEPH group MPAP and TPRI showed no decline between Weeks 6 and 12 (MPAP: 29 ± 2.8 vs 27 ± 1.1 mm Hg, $p = 0.502$; TPRI: 8.32 ± 1.33 vs 7.9 ± 0.6 WU/m^2, $p = 0.730$) and remained stable in the sham group (MPAP: 13 ± 1.3 vs 15 ± 1.3 mm Hg, $p = 0.2630$; TPRI: 4.12 ± 0.55 vs 5.0 ± 0.58 WU/m^2, $p = 0.2520$) (Figure 1).

Gross lung anatomy at Week 12

Qualitatively, the RUL parenchyma, vessels and airways in the CTEPH and reperfused CTEPH groups were not different from those in the sham group. The LLs from the CTEPH group exhibited an extensive network of neovessels at the surface of the visceral pleura and in the walls of the proximal arteries and bronchi, as compared with the sham and reperfused CTEPH groups. In the reperfused CTEPH group, the vascular bypass between the main PA and left PA remained patent in all animals (Figure 2A). In the right lower lobes, proximal PA occlusion persisted in the CTEPH and reperfused CTEPH groups (Figure 2B).

Pathology and morphometry

RULs at Week 12. In the CTEPH group, media thickness of the distal pulmonary arteries was increased (35.5 ± 0.8% for 40- to 100-μm arteries and 34.1 ± 1.4% for 101- to 200-μm arteries; Figure 3B) compared with the reperfused CTEPH group (24.5 ± 0.5%, $p < 0.0001$; and 20.3 ± 1.2%, $p < 0.0001$, respectively) and sham group (24 ± 0.8%, $p < 0.0001$; and 19 ± 1.7%, $p < 0.0001$, respectively) (Figure 3D). In the reperfused CTEPH group, media thickness of RUL distal pulmonary arteries returned to approximately sham-group values (Figure 3A and C).

LLs at Week 12. Media thickness of distal pulmonary arteries was significantly increased in LLs from the CTEPH group (37.4 ± 0.1% for 40- to 100-μm arteries and 35.4 ± 1.6% for 101- to 200-μm arteries) compared with the reperfused CTEPH group (22.2 ± 0.6%, $p < 0.0001$; and 21 ± 1.6%, $p < 0.0001$, respectively) and sham group (23.1 ± 0.5%, $p < 0.0001$; and 18.6 ± 1.1%, $p < 0.0001$) (Figure 4D). Neither of these two values differed significantly between the reperfused CTEPH group and the sham group ($p = 0.35$ and $p = 0.28$, respectively)

The TBALA per 100 μm of bronchial mucosa (Figure 5A) and number of bronchial arteries per 100 μm of bronchial mucosa (Figure 5B) were significantly increased the CTEPH group (395 ± 66 μm^2 per 100 μm and 1.16 ± 0.11 per 100 μm, respectively) compared with the sham group (13 ± 4 μm^2 per 100 μm, $p < 0.0001$; and 0.29 ± 0.098 per 100 μm, $p < 0.0001$, respectively) and to the reperfused CTEPH group (97 ± 32 μm^2 per 100 μm, $p = 0.0003$; and 0.434 per 100 μm, $p < 0.0001$, respectively). Neither of these two values differed significantly between the sham and reperfused CTEPH groups ($p = 0.38$ and $p = 0.37$, respectively).

Outer bronchial artery diameters were not significantly different between the CTEPH and reperfused CTEPH groups (52.7 ± 6.5 μm and 56.6 ± 7.16 μm, respectively; $p = 0.65$), but the values in these two groups were significantly higher than in the sham group (13.4 ± 1.9 μm; $p = 0.0003$ and $p = 0.0006$, respectively) (Figure 5C).

Percentage of bronchial artery occlusion was higher in the reperfused CTEPH group (73 ± 3.8%) than in the sham group (50 ± 3.1%; $p < 0.0001$) and CTEPH group (53 ± 2.5%; $p = 0.0001$). There was no difference between the sham and CTEPH groups ($p = 0.5019$) (Figure 5D).

No thrombus or endothelial changes were found in any of the three groups.

These results were consistent with an increase in distal PA media thickness and functional systemic blood supply to the LLs, indicating POPV in the CTEPH group, as compared with the sham group. These manifestations of POPV regressed 6 weeks after left PA reperfusion in the reperfused CTEPH group (Figure 6).

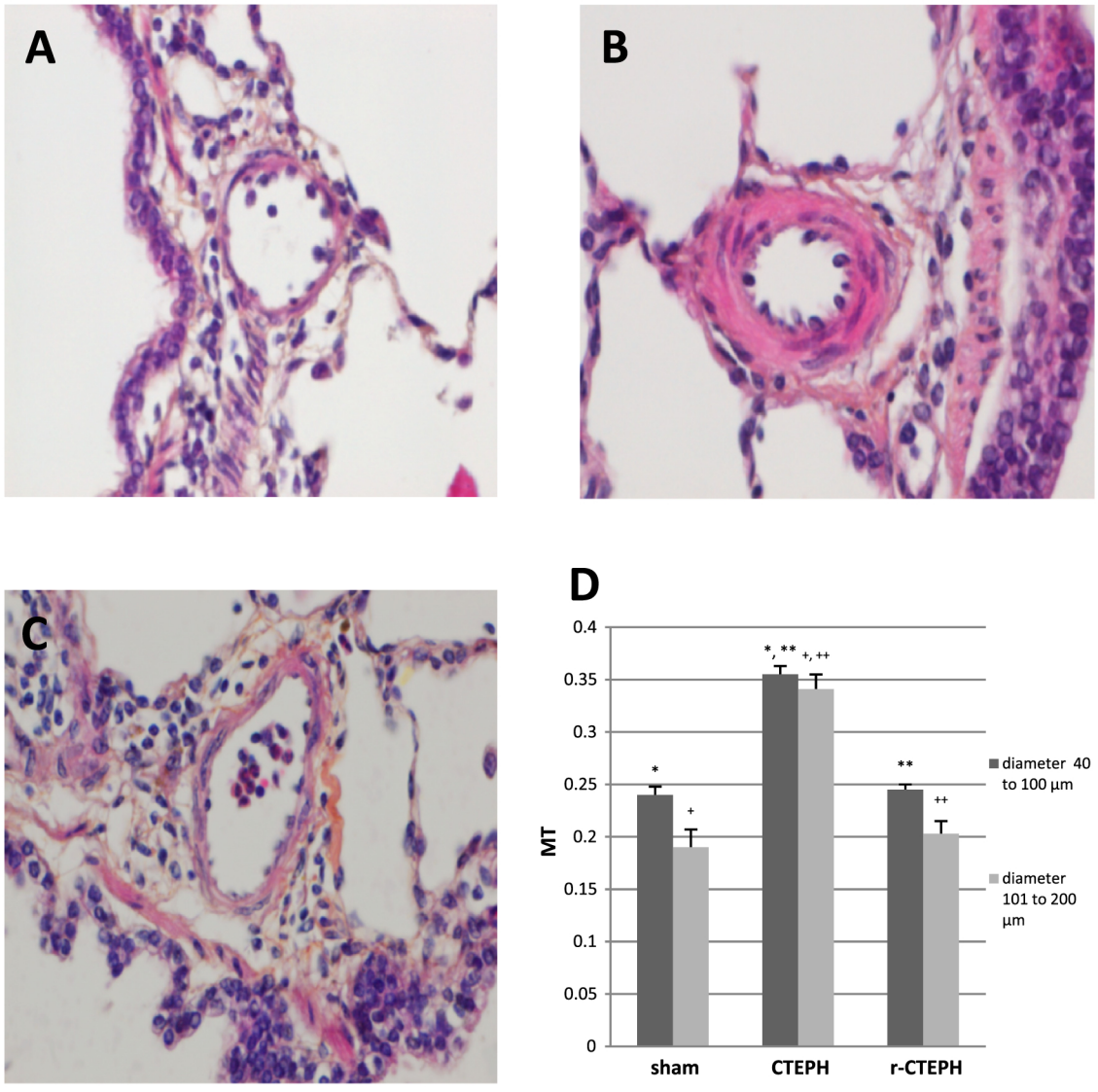

Figure 3 Distal PA morphometry in the right upper lobes. (A–C) Cross-sections (original magnification: ×600; hematoxylin eosin saffron stain) of pulmonary arteries <200 µm in diameter in the distal right upper lobes from the sham group (A), CTEPH group (B) and reperfused CTEPH (r-CTEPH) group (C). (D) Histogram of media thickness (MT), with the standard error bars, of distal pulmonary arteries (40 to 100 µm and 101 to 200 µm in diameter), taken from the right upper lobes. Values were higher in the CTEPH group compared with the sham group ($^{*}p < 0.05$ and $^{**}p < 0.05$ for both vessel diameters) or r-CTEPH group ($^{+}p < 0.05$ and $^{++}p < 0.05$ for both vessel diameters).

Tissue gene expression profiles

RULs at Week 12 (Figure 7A). ET-1 and IL-6 tissue gene expression profiles were increased 2-fold in RULs from the CTEPH group compared with the sham group (ET-1, $p = 0.02$; IL-6, $p = 0.03$). In the reperfused CTEPH group, ET-1 gene expression profiles in RULs at Week 12 tended to decrease and were not significantly different from those of the sham group ($p = 0.34$). IL-6 was significantly overexpressed in RULs from the CTEPH group compared with the reperfused CTEPH group ($p = 0.040$) and returned to sham values in the reperfused CTEPH group ($p = 0.9138$).

ET-A gene expression profile and ET-A/ET-B gene expression profile ratios in RULs were similar in the three groups. However, the ET-B gene expression profile in the RULs was significantly lower in the reperfused CTEPH group compared with the sham group ($p = 0.049$).

LLs at Week 12 (Figure 7B). ET-1, ET-A, ET-B and IL-6 tissue gene expression profiles in the LLs were not significantly different between the reperfused CTEPH and CTEPH groups.

The ET-A/ET-B expression profile ratios in LLs was increased 4-fold in the CTEPH group compared with the sham group ($p = 0.002$). The ET-A/ET-B gene expression ratio increase was due to a significant decrease in ET-B gene expression profile in the CTEPH group compared with the sham group ($p = 0.0003$). The ET-A expression profile in LLs in the CTEPH group was similar to that in the sham group ($p = 0.4509$) (Figure 7B). In the reperfused CTEPH group, ET-A/ET-B tended to be lower compared with the CTEPH group ($p = 0.1682$) and increased 3-fold compared with the sham group ($p = 0.0304$) (Figure 7B).

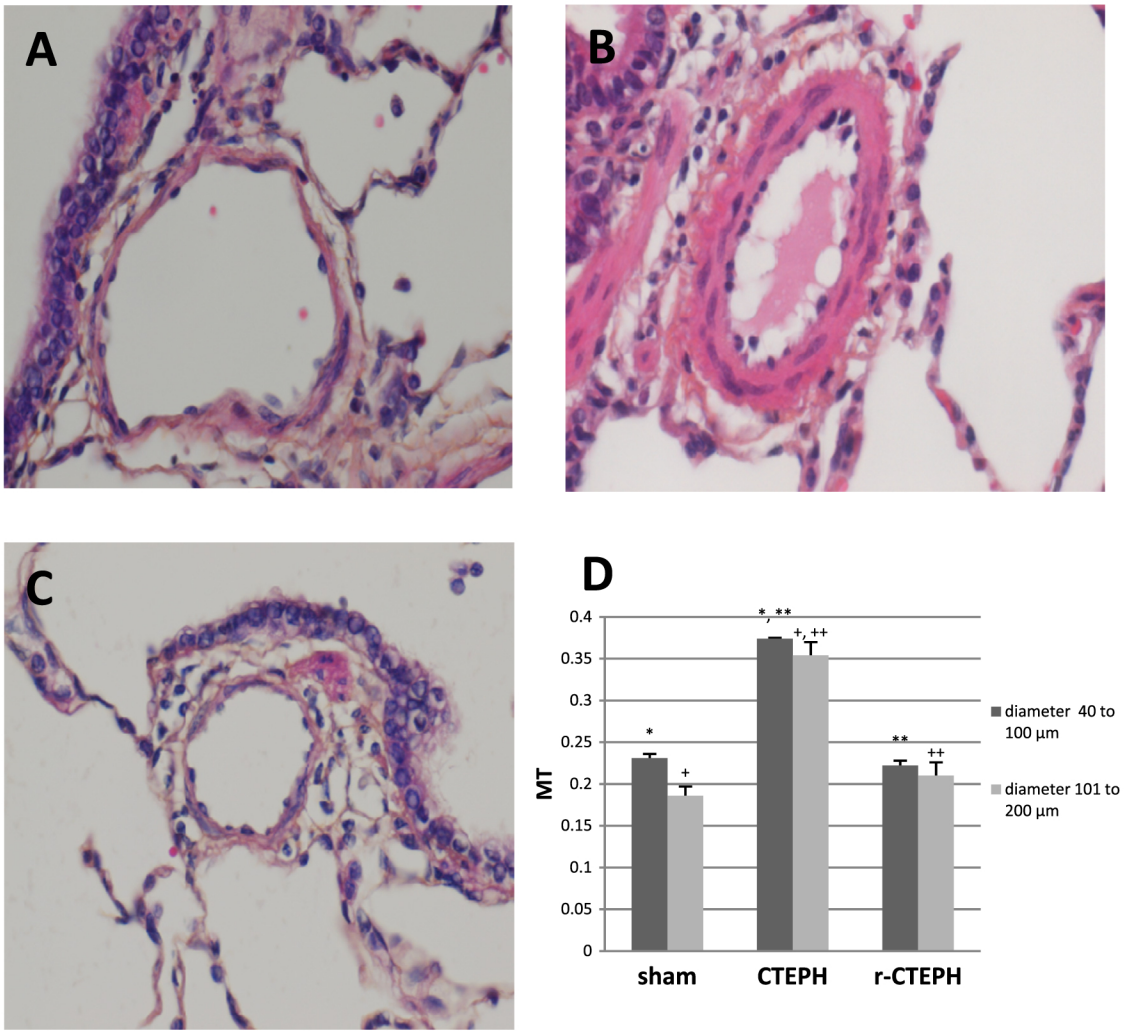

Figure 4 Distal PA morphometry of the left lungs. (A–C) Cross-sections (original magnification: ×600; hematoxylin eosin saffron stain) of distal pulmonary arteries <200 µm in diameter taken from the left lungs of animals in the sham group (A), CTEPH group (B) and reperfused CTEPH (r-CTEPH) group (C). (D) Histogram of mean media thickness (MT), with the standard error bars, of distal pulmonary arteries 40 to 100 µm in diameter and 101 to 200 µm in diameter, taken from the left lungs. Values were higher in the CTEPH group compared with the sham group ($^{*}p < 0.05$ and $^{**}p < 0.05$ for both vessel diameters) or r-CTEPH group ($^{+}p < 0.05$ and $^{++}p < 0.05$ for both vessel diameters), respectively.

Human plasma ET-1, IL-6, and CRP levels

Table 1 lists the main patient characteristics. The only significant between-group difference was the higher systolic PA pressure in the CTEPH group.

Plasma IL-6 level was elevated in the CTEPH group compared with the control group ($p = 0.001$) and was significantly higher ($p = 0.045$) in the CTEPH subgroup with TPR >900 dynes/s/cm^5, a value suggesting PMD (Figure 8A). Similarly, plasma ET-1 level was significantly elevated in CTEPH patients compared with controls ($p = 0.001$), with a trend toward higher levels in the subgroup with TPR >900 dynes/s/cm^5 (Figure 8B). Plasma CRP levels were not different between controls and the overall CTEPH population or either of the CTEPH subgroups based on TPR (Figure 8C).

Discussion

In this study we replicated the features of human CTEPH in a piglet model with both occluded and non-occluded territories. Surgical reperfusion of occluded and non-occluded territories modeled the effects of PEA. We found that: (i) PMD persisted in both territories 7 weeks after the last PA embolization in the absence of surgical reperfusion of occluded territories; (ii) PMD had regressed in both territories 6 weeks after surgical reperfusion of occluded territories; (iii) ET-1 and IL-6 genes were overexpressed in non-occluded territories, and the ET-A/ET-B gene expression ratio was increased in occluded territories; (iv) IL-6 gene overexpression in non-occluded territories had regressed 6 weeks after surgical reperfusion of occluded territories; and (v) plasma IL-6 and ET-1 levels were higher in CTEPH patients than in controls and were highest in the patient subgroup with TPR >900 dynes/s/cm^5.

Persistent PH is an established independent risk factor for in-hospital and 1-year mortality after PEA. Persistent PH was noted after 16.7% of PEAs in a recent international prospective registry analysis,[30] and was ascribed to the pre-operative development of PMD.[5,13] Pulmonary vascular resistance decreases gradually over the months after

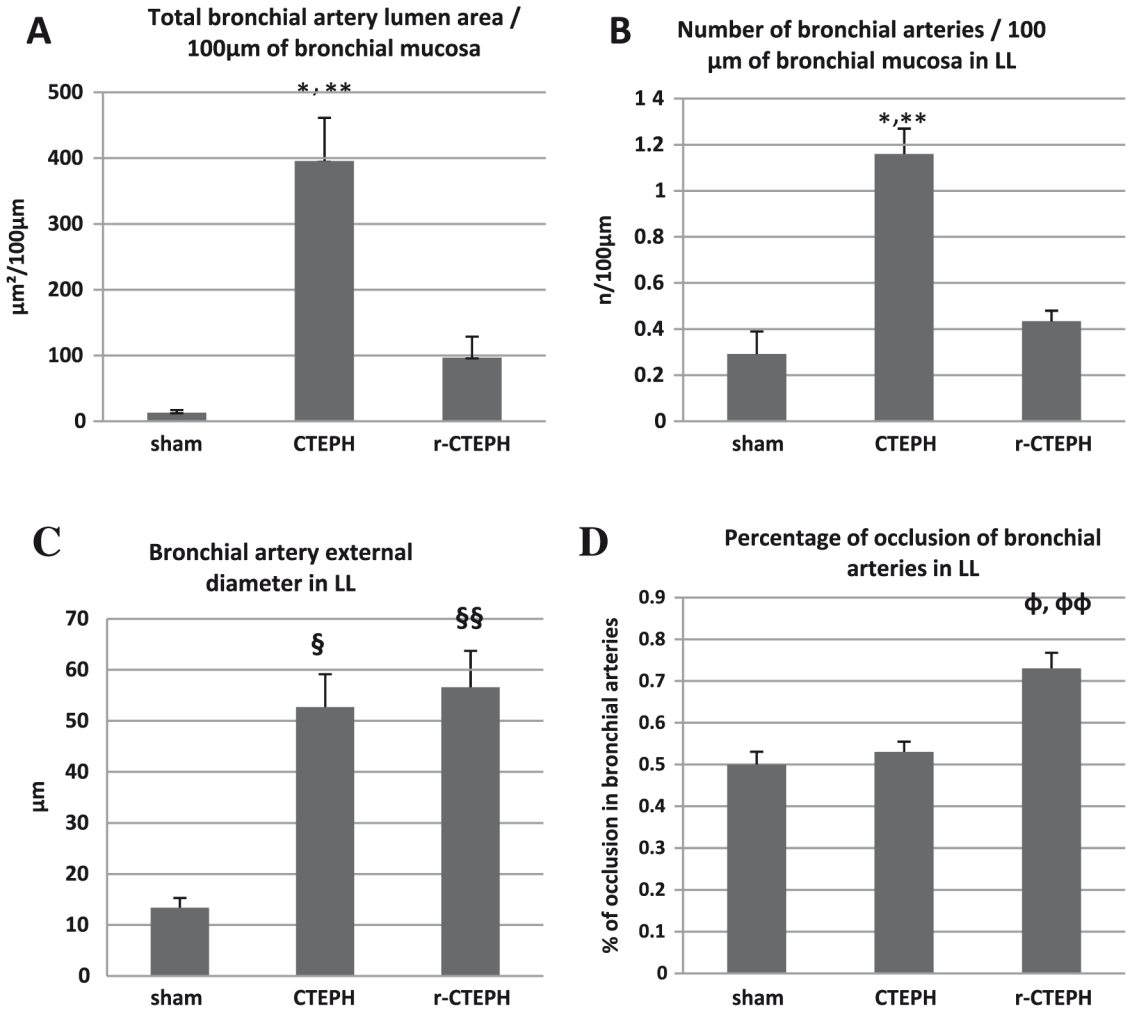

Figure 5 Quantification of the systemic blood supply to the left lungs in each group via morphometry assessment at Week 12 of bronchial arteries to bronchioles >200 µm in diameter. Total bronchial artery lumen area (TBALA) per 100 µm of bronchial mucosa perimeter (A) and number of bronchial arteries per 100 µm of bronchial mucosa perimeter (B) were significantly increased in the CTEPH group compared with the reperfused CTEPH (r-CTEPH) and sham groups ($^{*}p < 0.05$ and $^{**}p < 0.05$). Bronchial artery external diameters (C) were significantly larger in the CTEPH (§) and r-CTEPH (§§) groups compared with the sham group ($^{\S}p < 0.05$ and $^{\S\S}p < 0.005$). However, the percentage of bronchial artery occlusion (D) was significantly greater in the r-CTEPH group than in the CTEPH (ΦΦ) and sham (Φ) groups ($^{\Phi}p < 0.05$ and $^{\Phi\Phi}p < 0.05$).

PEA,[30] but it remains unclear whether this change is ascribable to PMD regression. PMD complicating CTEPH is defined as increased thickness of the arterial media and intima.[5] In our animal model, media thickness decreased after surgery and the intima remained normal. Although we did not perform a histopathologic evaluation of microvascular lesions at Week 6, our results indicate PMD regression after LL reperfusion, and we previously reported that PMD lesions were visible in both occluded and non-occluded territories at Week 6 in our CTEPH model.[26,27] Consequently, the absence of microvascular media remodeling and the occlusion of LL bronchial arteries in the reperfused CTEPH group, in contrast to the persistent microvascular remodeling in the CTEPH group, indicated PMD regression induced by surgical reperfusion of the occluded territories.

The increase in IL-6 tissue gene expression in non-occluded (i.e., RULs), but not in occluded territories (i.e., LLs), is a novel finding from this study. IL-6 overexpression in non-occluded territories resolved concomitantly with PH reversal in the reperfused CTEPH group. A role for IL-6 in the pathogenesis of various forms of PH remains unproven. Serum IL-6 levels were elevated in patients with various types of PH,[25,31–33] including PH associated with Castleman's disease, a lymphoproliferative disorder characterized by IL-6 overexpression.[34] In mice, IL-6 overexpression increased the development of microvascular remodeling and hypoxia-induced PH.[35,36]

In our study the IL-6 gene overexpression profile was found only in the RULs (i.e., non-occluded territories), which were exposed to high PA blood flow and pressure.

A recent study of rat pulmonary smooth muscle cells showed that ET-1 induced the production of IL-6.[37] In addition, high blood flow and pressure in the PAs has been demonstrated to increase ET-1 expression by PA endothelial cells.[19,20] Therefore, our results support the hypothesis that ET-1 and IL-6 gene overexpression profiles are involved in the microvascular lesions that can develop in non-occluded lung territories in patients with CTEPH.

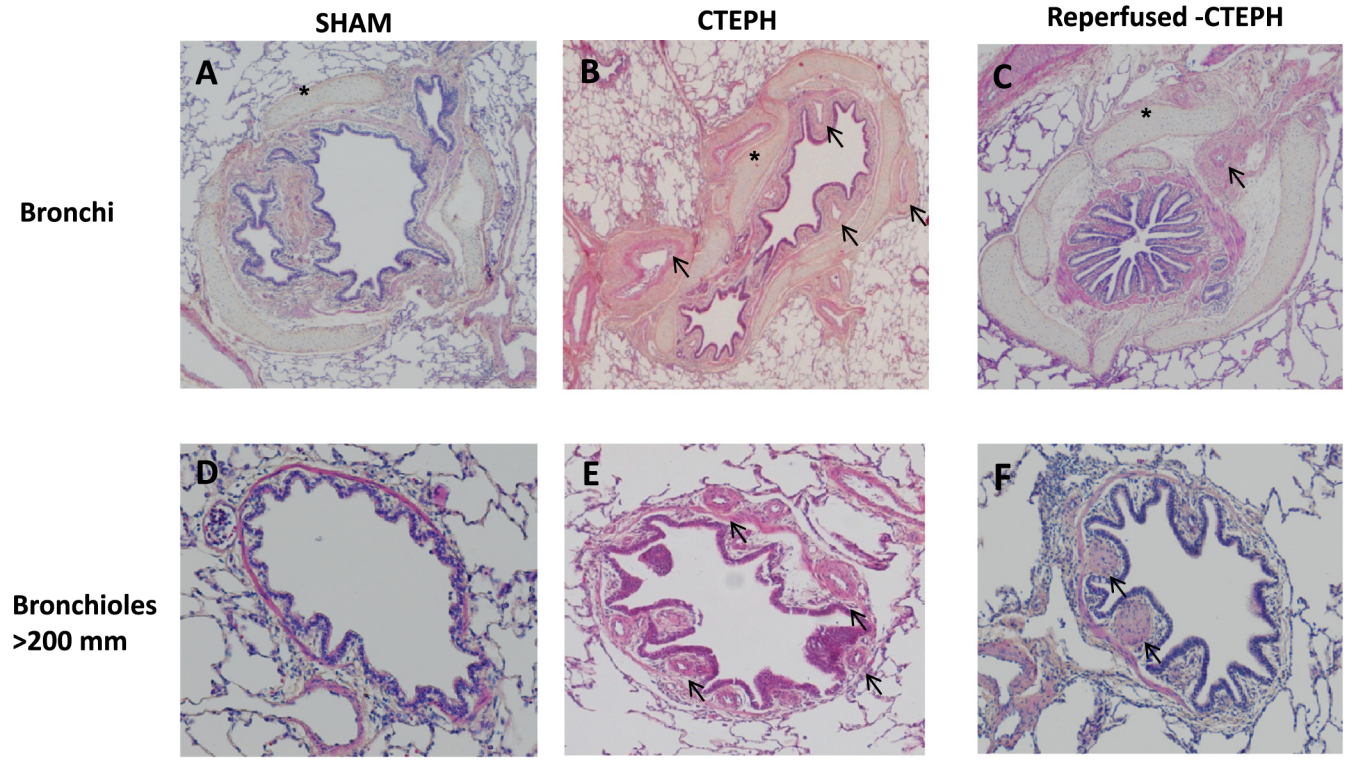

Figure 6 Evaluation of the bronchial arteries supplying the left lungs at 12 weeks. (A–C) Left lung slices [original magnifications: ×40 (A, C) and ×20 (B); hematoxylin eosin saffron stain] at the level of the bronchi with cartilage (*) and bronchial arteries (arrows) larger and more numerous in the CTEPH group (B) than in the sham (A) and reperfused CTEPH (r-CTEPH) (C) groups. (D–F) Left lung slices (original magnification: ×200; HES stain) showing that bronchial arteries of the distal airways (bronchioles >200 μm in diameter) were larger and had visible lumen in the CTEPH group (E) compared with the sham group (D). The r-CTEPH group had similarly sized bronchial arteries (arrows) as the CTEPH group, but with occluded lumen (F).

Conversely, the IL-6 tissue gene expression profile in LLs 12 weeks after left PA ligation was not different from sham values. However, IL-6 upregulation in the lung has been reported early after acute PA ligation.[38,39] Hence, IL-6 overexpression in CTEPH may reflect the acute pathologic process in recently occluded territories and, at a later stage, the chronic PMD process in non-occluded territories.

In humans, ET-1 expression is associated with the severity of PH[40] and CTEPH in particular.[21] In addition, elevated serum levels of the circulating ET-1 precursor, big ET-1, were associated with persistent PH and poor outcomes after PEA,[41] suggesting a predominant role in persistent PH for peripheral PMD compared with proximal obstruction. In another clinical study,[21] higher serum ET-1 levels were associated with fatal and non-fatal persistent PH after PEA and predicted poor outcomes with a high sensitivity (79%) and specificity (85%).

Interestingly, in our CTEPH group, ET-1 expression was increased only in non-occluded territories (i.e., RUL) at Week 12. Thus, ET-1 expression may be a marker for PMD in non-occluded territories, where the distal vessels are subjected to high flows and pressures. These findings may explain why pre-operative serum ET-1 elevation predicts poor hemodynamic results after PEA in humans, as increased ET-1 expression may indicate persistent microvascular remodeling in non-occluded territories where PEA has no immediate benefits.

MPAP and TPRI did not return to baseline values in the reperfused CTEPH group despite LL reperfusion, PMD regression in both territories, and normalization of IL-6 expression in non-occluded territories. The sustained MPAP elevation may be ascribable to persistent right lower lobe artery occlusion by the tissue adhesive and also to persistent ET-A/ET-B elevation in the LLs, as high ET-A/ET-B values correlate with increased reactivity to ET-1 and PA tone.[42] Our results are also consistent with the steady improvement in hemodynamics over the first 6 months after PEA, as media thickness decreased in both occluded and non-occluded territories in our CTEPH model. However, these results were obtained in growing animals and may not be applicable to adults. Further studies using this model are needed to determine whether hemodynamics continue to improve beyond 6 weeks after LL reperfusion, the time-point at which ET-A/ET-B returned to normal.

Despite the gene expression differences between the occluded and non-occluded territories, media thickness of the distal pulmonary arteries was similar in the two territories. Thus, two different pathways may lead to similar distal PA remodeling.

Our results identify IL-6 and ET-1 as potential biomarkers for microvascular remodeling in non-occluded territories. Pre-operative assessment of PMD is crucial to predict the effects of PEA. To date, no objective tools are available for evaluating the extent of PMD in patients with CTEPH. Our results suggest that ET-1 and IL-6 may help to assess PMD in non-occluded territories. However, we

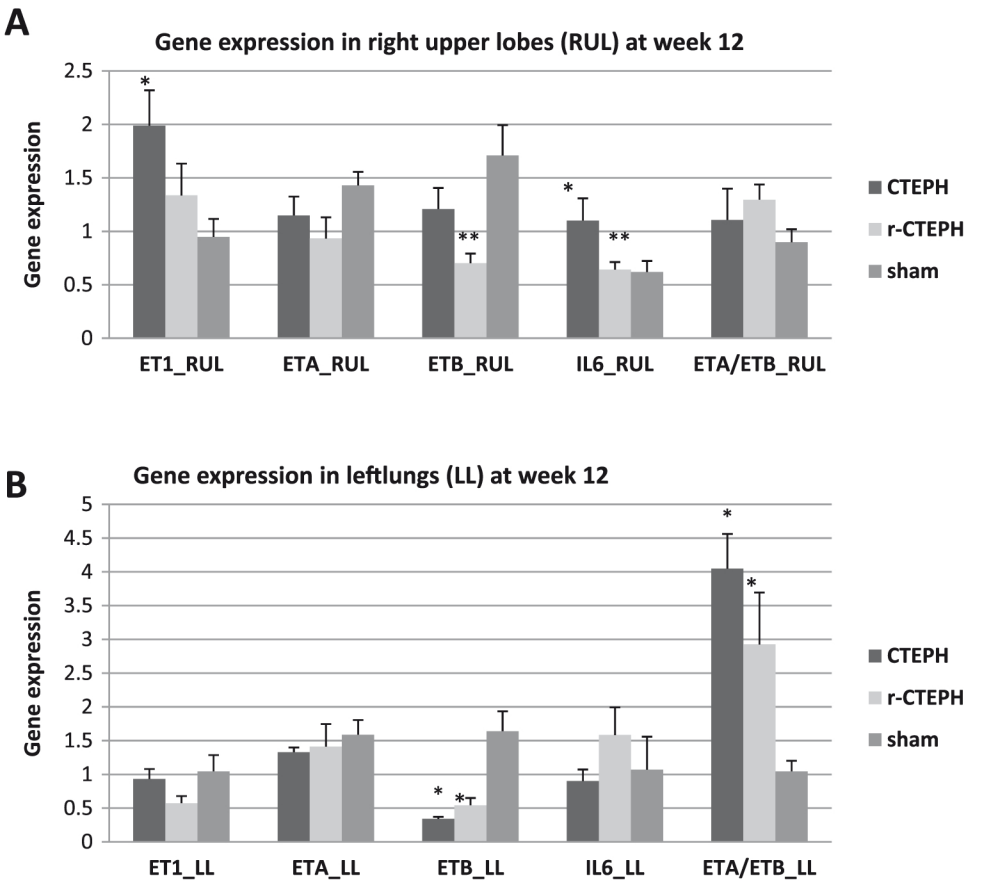

Figure 7 Tissue gene expression profiles in the right upper lobes (A) and left lungs (B) at Week 12. (A) ET-1 and IL-6 were overexpressed in the right upper lobe of CTEPH animals compared with sham group animals ($^{*}p < 0.05$). IL-6 and ET-B expression levels were significantly lower ($^{**}p < 0.05$) in the right upper lobes of reperfused CTEPH (r-CTEPH) animals, which also had non-significantly lower ET-1 expression compared with CTEPH animals. The ET-A/ET-B tissue gene expression profile ratio was not different between the CTEPH and r-CTEPH groups. (B) ET-A/ET-B gene expression profile ratios were elevated in left lungs from CTEPH and r-CTEPH animals compared with sham animals ($^{*}p < 0.05$) due to a significant decrease in ET-B expression, with ET-A expression levels similar to those in the sham group. No significant differences across groups were noted for ET-1 and IL-6 tissue gene expression profiles.

studied 3-week-old growing piglets, whereas most CTEPH patients are adults. Interestingly, plasma IL-6 and ET-1 levels were higher in CTEPH patients compared with controls and were highest in patients with TPR >900 dynes/s/cm^5. The exact correlations between PMD severity and TPR are unknown, but higher TPR is associated with greater PMD severity. Thus, the results in human samples were consistent with those in our animal model. Nevertheless, further prospective studies with larger sample sizes are needed to establish whether serum IL-6 and ET-1 levels correlate with PMD severity in non-occluded territories and with the risk of persistent PH after PEA. Conceivably, ET-1 and IL-6 may hold promise as biomarkers for selecting patients for targeted pharmacotherapy before PEA to

Table 1 Main Characteristics of Control Subjects and Patients With Chronic Thromboembolic Pulmonary Hypertension Scheduled for Pulmonary Endarterectomy

	Control group ($n = 20$)	CTEPH group ($n = 59$)	p-value
Age (years)	58 ± 10	59 ± 14	0.72
Male / female ratio (n)	11 / 9	35 / 24	0.79
Smoker [n (%)]	5 (25%)	21 (35%)	0.42
SPAP (mm Hg)	27 ± 2	79 ± 23	0.0001[a]
MPAP (mm Hg)	—	47 ± 11	—
Cardiac index (liters/min/m^2)	—	2.6 ± 0.7	—
Total pulmonary resistance (dynes/s/cm^5)	—	815 ± 313	—

Data expressed as mean ± standard deviation, unless noted otherwise. CTEPH, chronic thromboembolic pulmonary hypertension; MPAP, mean pulmonary artery pressure; SPAP, systolic pulmonary artery pressure.

[a]$p < 0.05$ (statistically significant).

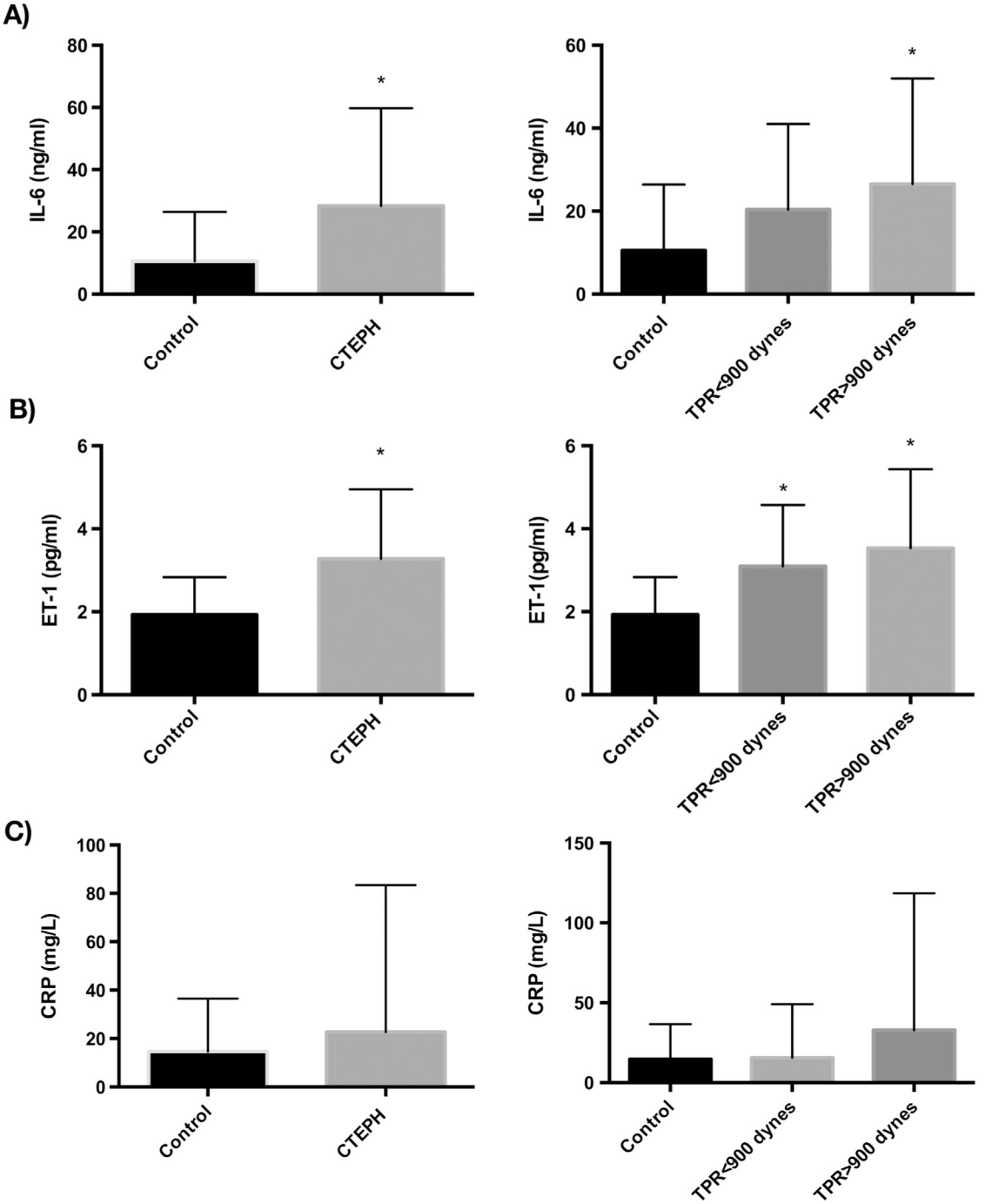

Figure 8 Plasma levels of IL-6, ET-1 and CRP in patients with CTEPH and controls undergoing lung resection for localized cancer and having no evidence of pulmonary hypertension by pre-operative echocardiography. (A) Plasma IL-6 levels were higher in the CTEPH group compared with the control group ($p = 0.001$) and were highest in the CTEPH subgroup with pre-operative TPR >900 dynes/s/cm^5, consistent with microvascular disease ($p = 0.045$). (B) Plasma ET-1 levels were significantly higher in the overall CTEPH group ($p = 0.001$) and in each TPR-based CTEPH subgroup ($p < 0.05$) compared with the control group. Plasma ET-1 levels were non-significantly higher in the CTEPH subgroup with TPR >900 dynes/s/cm^5 than in the CTEPH subgroup with ≤ 900 dynes/s/cm^5. (C) CRP plasma levels were not significantly different between the sham group and the overall CTEPH group or either of the CTEPH subgroups based on TPR. Note the wide range of CRP values in the CTEPH groups. Plasma CRP level may not depend exclusively on plasma IL-6 level and may be a less accurate indicator of microvascular disease when compared with other biomarkers.

combat predominant PMD in non-occluded territories suggesting a high risk of persistent PH after PEA.

Disclosure statement

The authors have no conflicts of interest to disclose.

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