Role of angiopoietin-2 in venous thrombus resolution and chronic thromboembolic disease

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These findings in patients and mouse models reveal a new role for angiopoietin-2 in the pathophysiology of CTEPH, suggesting that its overexpression in pulmonary endothelium may contribute to defective angiogenesis and persistent vascular occlusion https://bit.ly/3gotczC

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Abstract

Background Defective angiogenesis, incomplete thrombus revascularisation and fibrosis are considered critical pathomechanisms of chronic thromboembolic pulmonary hypertension (CTEPH) after pulmonary embolism. Angiopoietin-2 (ANGPT2) has been shown to regulate angiogenesis, but its importance for thrombus resolution and remodelling is unknown.

Methods ANGPT2 plasma concentrations were measured in patients with CTEPH (n=68) and acute pulmonary embolism (n=84). Tissue removed during pulmonary endarterectomy (PEA) for CTEPH was analysed (immuno)histologically. A mouse model of inferior vena cava ligation was used to study the kinetics of venous thrombus resolution in wild-type mice receiving recombinant ANGPT2 *via* osmotic pumps, and in transgenic mice overexpressing ANGPT2 in endothelial cells.

Results Circulating ANGPT2 levels were higher in CTEPH patients compared to patients with idiopathic pulmonary arterial hypertension and healthy controls, and decreased after PEA. Plasma ANGPT2 levels were elevated in patients with pulmonary embolism and diagnosis of CTEPH during follow-up. Histological analysis of PEA specimens confirmed increased ANGPT2 expression, and low levels of phosphorylated TIE2 were observed in regions with early-organised pulmonary thrombi, myofibroblasts and fibrosis. Microarray and high-resolution microscopy analysis could localise ANGPT2 overexpression to endothelial cells, and hypoxia and transforming growth factor-β1 were identified as potential stimuli. Gain-of-function experiments in mice demonstrated that exogenous ANGPT2 administration and transgenic endothelial ANGPT2 overexpression resulted in delayed venous thrombus resolution, and thrombi were characterised by lower TIE2 phosphorylation and fewer microvessels.

Conclusion Our findings suggest that ANGPT2 delays venous thrombus resolution and that overexpression of ANGPT2 contributes to thrombofibrosis and may thus support the transition from pulmonary embolism to CTEPH.

Introduction

In patients surviving an episode of pulmonary embolism, the long-term course may be complicated by persisting and progressive haemodynamic and functional impairment [1]. At the far end of the severity spectrum lies chronic thromboembolic pulmonary hypertension (CTEPH), which is associated with poor prognosis if left untreated [1, 2]. Incomplete thrombus resolution followed by vascular remodelling is considered a critical pathomechanism for the development of CTEPH after pulmonary embolism [3]. Thrombi resolve through complex processes of degradation and organisation, which involves leukocyte recruitment and the formation of microvascular channels within the thrombus [4]. Angiogenesis, and an intact angiogenic response after acute thrombosis or thromboembolism, are key mediators of normal thrombus resolution [5, 6].

Angiopoietin-2 (ANGPT2), an antagonist ligand of the endothelial-specific TIE2 receptor, inhibits the protective and stabilising influence of ANGPT1 [7–10]. ANGPT2 is stored in endothelial Weibel–Palade bodies and released from the endothelium upon activation by diverse stimuli, including thrombin and hypoxia [11]. Elevated ANGPT2 plasma concentrations have been associated with impaired haemodynamics and poor prognosis in patients with idiopathic pulmonary arterial hypertension (IPAH) and chronic right ventricular (RV) failure [12]. The present study, involving measurements in humans with CTEPH in comparison to other types of acute and chronic pulmonary hypertension, and experiments in primary endothelial cells and gain-of-function mouse models, aimed to examine the hypothesis that ANGPT2 overexpression contributes to incomplete thrombus resolution and may thus support the development of CTEPH after one or multiple episodes of pulmonary embolism.

Methods

Studies involving patients and controls

Consecutive patients aged ⩾18 years with objectively confirmed operable CTEPH referred for pulmonary endarterectomy (PEA) to the Department of Thoracic Surgery, Kerckhoff Clinic, Bad Nauheim, Germany, a national referral centre for PEA surgery, were included in the CTEPH Registry Bad Nauheim (CEPRA). Venous blood samples for biomarker measurements were collected from patients with CTEPH, before and after PEA (not earlier than 48 h after surgery). Details on the study design are provided in the supplementary material.

Consecutive patients aged ⩾18 years with objectively confirmed acute pulmonary embolism were prospectively included in the single-centre Pulmonary Embolism Registry of Göttingen (PERGO) at the University Medical Centre Göttingen, Germany. Diagnosis of CTEPH during long-term follow-up was a prospectively defined outcome of the registry. Details on the study design are provided in the supplementary material.

Consecutive patients aged ⩾18 years with objectively confirmed IPAH were prospectively included in the single-centre Pulmonary Hypertension Registry Mainz (PHYREM), Germany. The IPAH group consisted of 38 patients. The diagnosis of IPAH was based on standard criteria as recommended by current guidelines [13].

The control group consisted of 36 apparently healthy volunteers and has been described elsewhere [12].

Model of stagnant flow venous thrombosis in gain-of-function mouse models and controls

Venous thrombosis was induced using an established murine model of stagnant flow venous thrombosis [5]. To study thrombus resolution over time, mice underwent abdominal vascular ultrasound on days 1, 3, 7, 14 and 21 after inferior vena cava (IVC) ligation. At each time point, a subset of mice underwent lethal blood sampling by cardiac puncture to obtain plasma, and thrombi were harvested for histological analysis. Osmotic pumps (Alzet) filled with recombinant murine ANGPT2 (R&D Systems) were implanted into C57BL/6 wild-type mice. C57BL/6 mice receiving osmotic pumps filled with sterile 0.9% sodium chloride (Gibco) or without pump implantation were used as controls. The generation and characterisation of mice with targeted ANGPT2 expression in endothelial cells (Angpt2:Tie1 double transgenic (DT) mice) has been described [7]. CD1 wild-type mice were used as controls. Only male mice were examined throughout the study.

Measurement of plasma ANGPT2 levels

ANGPT2 levels in plasma of patients and controls, or in mice, were measured using specific ELISA, detecting both the endogenous and the recombinant protein, according to the manufacturer's instructions (R&D Systems). Soluble TIE2 plasma concentrations were determined only in humans using specific ELISA (R&D Systems).

Histological and immunohistological analyses

Human tissue material removed during PEA and murine thrombi were examined by light microscopy after Masson's trichrome (MTC) staining [14] and stratified into six distinct histological regions of interest, as described previously [15]. Immunohistochemical studies were performed as described in the supplementary material. The immunosignal was manually marked as a red-coded area and automatically measured using the "count-size" function for each region of interest (*i.e.* complete murine thrombi or a 300×150 µm area in histological regions of human tissue material removed from PEA) and expressed as a percentage.

Studies involving cells

Human pulmonary arterial endothelial cells (HPAECs) isolated from healthy controls (PromoCell) and endothelial cells outgrown from PEA tissue (CTEPH-ECs) were cultivated, as suggested by the manufacturer or as published [15]. Endothelial cell outgrowth was observed primarily from Jamieson type II lesions (organised thrombus and intimal thickening proximal to segmental arteries) [16], and cells outgrown from those samples were used for gene expression analysis. Cells were treated with cobalt chloride ($CoCl_2$; 150 µM in endothelial cell medium) for 16 h.

Statistical analysis

Fisher's exact test or Chi-squared test was used to compare categorical variables, which are expressed as absolute numbers or percentage. Continuous variables did not follow a normal distribution when tested with the modified Kolmogorov–Smirnov test (Lilliefors test); therefore, these variables are expressed as medians with the corresponding interquartile range (IQR), and were compared using the unpaired Mann–Whitney U-test. For comparison of two groups and normal distribution, a t-test was performed. If more than two groups and different time points were compared, two-way ANOVA followed by Bonferroni multiple comparison testing was performed. Receiver operating characteristics (ROC) curve analysis was performed to determine the area under the curve (AUC). Youden index quantification was used to identify the optimal ANGPT2 cut-off values for the prediction of study outcomes. A two-sided significance level of $\alpha<0.05$ was defined appropriate to indicate statistical significance. All statistical analyses were performed using SPSS software (version 21.0; SPSS, Chicago, IL, USA).

Results

Elevated circulating levels and pulmonary endothelial expression of ANGPT2 in patients with CTEPH

Between June 2014 and September 2015, 68 patients (51.5% female; median (IQR) age 63 (55–72) years) with confirmed CTEPH referred for PEA were included in the study. The baseline characteristics of the study patients are shown in table 1. ANGPT2 plasma concentrations ranged from 1.2 to 14.7 $ng \cdot mL^{-1}$ (median (IQR) 8.8 (4.1–13.4) $ng \cdot mL^{-1}$) and correlated with haemodynamic parameters (mean pulmonary artery pressure r=0.430, p<0.001; pulmonary vascular resistance r=0.588, p=0.001) and biomarkers indicating RV dysfunction (N-terminal pro-brain natriuretic peptide r=0.763, p<0.001). ANGPT2 plasma levels were higher in CTEPH patients compared to patients with IPAH (median (IQR) 4.7 (2.8–6.7) $ng \cdot mL^{-1}$; p=0.0393) and those in healthy controls (median (IQR) 2.0 (1.4–2.5) $ng \cdot mL^{-1}$; p<0.0001) (figure 1a). Patients with IPAH were older and had a significantly higher number of comorbidities, but their haemodynamic profile did not differ from that of patients with CTEPH. Circulating ANGPT2 levels decreased significantly following PEA, *i.e.* surgical removal of the thrombofibrotic tissue (n=26 patients; figure 1b). Of note, pre- and post-operatively measured ANGPT2 levels were associated with post-operative persistent pulmonary hypertension (ANGPT2 pre-operatively AUC 0.69, 95% CI 0.54–0.85, p=0.025; ANGPT2 post-operatively AUC 0.74, 95% CI 0.55–0.94, p=0.045).

ANGPT2 is abundantly expressed in areas of thrombus (non)resolution in pulmonary endarterectomy tissue from CTEPH patients

Local ANGPT2 expression patterns and their association with thrombus organisation and angiogenesis were examined in tissue specimens removed during PEA from six patients with CTEPH. MTC-stained cross-sections were used to distinguish six regions of interest corresponding to chronological stages of (defective) thrombus organisation [15]; immunostaining of CD31 was used to visualise angiogenesis. Representative images are shown in figure 2a. ANGPT2-positive immunosignals were predominantly detected in early-stage organised thrombi (7.6%, 4.6–10.2%), late-stage organised thrombi (7.1, 4.2–9.8%) and vessel-rich regions (7.5, 3.5–11.4%) (figure 2b). In comparison, immunosignals for ANGPT1 were less frequently observed; they were detected predominantly in late-stage organised thrombi (0.3, 0.2–0.4%) (figure 2c). In line with an inhibitory effect of ANGPT2 on angiogenic signalling, immunosignals for its phosphorylated receptor pTIE2 (figure 2d) and for CD31 (figure 2e) were found to be low in early-stage organised thrombi and regions with myofibroblasts or fibrosis compared to regions with fresh and late-organised thrombi and vessel-rich regions.

TABLE 1 Baseline characteristics, medical history and initial presentation of 68 patients with chronic thromboembolic pulmonary hypertension (CTEPH) referred for pulmonary endarterectomy (PEA) (stratified according to their median angiopoietin-2 (ANGPT2) plasma concentrations)

	All study patients	ANGPT2 <3.4 ng·mL^{-1}	ANGPT2 ⩾3.4 ng·mL^{-1}	p-value
Subjects	68	33	35	
Age (years)	63 (55–72)	62 (49.0–70.5)	64 (59.0–76.0)	0.210
Sex (male)	33 (48.5)	20 (60.6)	15 (42.9)	0.156
Comorbidities and risk factors for CTEPH				
Previous pulmonary embolism	65 (95.6)	32 (97.0)	33 (94.3)	1.000
Cancer[#]	2 (2.9)	2 (6.1)	1 (2.9)	0.608
Chronic heart failure	3 (4.4)	0	3 (8.6)	0.239
Coronary artery disease	15 (22.1)	6 (18.2)	9 (25.7)	0.563
Chronic pulmonary disease	22 (32.4)	9 (27.3)	13 (37.1)	0.444
Diabetes mellitus	8 (11.8)	2 (6.1)	0	0.262
Renal insufficiency[¶]	16 (23.5)	5 (15.2)	11 (31.4)	0.155
Thrombophilia[+]	9 (13.2)	7 (21.2)	5 (14.3)	0.534
Splenectomy	1 (1.5)	0	1 (2.9)	1.000
Systemic inflammatory disease[§]	9 (13.2)	3 (9.1)	6 (17.1)	0.478
Thyroid disease	20 (29.4)	8 (24.4)	12 (34.4)	0.431
Clinical symptoms and functional capacity				
Dyspnoea (NYHA class III–IV)	52 (77.6)	20 (60.6)	32 (91.4)	**0.007**
Haemoptysis	5 (7.7) n=65	3 (9.1) n=34	2 (6.1) n=33	0.708
6-min walk distance (m)	364 (279–433) n=33	401 (319–443) n=16	325 (235–423) n=17	0.102
Laboratory values				
NT-proBNP (pg·mL^{-1})	792 (173–2271) n=65	173 (61–573) n=32	2239 (1165–4508) n=33	**<0.001**
C-reactive protein (mg·dL^{-1})	3 (1.3–8.0) n=68	2 (1.0–4.0) n=33	5 (2.0–9.0) n=35	**0.003**
Right heart catheterisation				
Mean pulmonary artery pressure (mmHg)	43 (34–51) n=67	39 (30–48) n=33	48 (41–52) n=34	**0.004**
Pulmonary vascular resistance (Wood units)	6.4 (4.4–10.6) n=66	5.0 (3.6–6.6) n=33	9.4 (6.0–13.1) n=33	**<0.001**
Cardiac index (L·min^{-1}·m^{-2})	2.4 (1.9–2.9) n=63	2.8 (2.4–3.1) n=32	1.91 (1.6–2.4) n=31	**<0.001**

Data are presented as n, median (interquartile range) or n (%), unless otherwise stated. Bold type represents statistical significance. NYHA: New York Heart Association; NT-proBNP: N-terminal pro-brain natriuretic peptide. [#]: active or anti-tumour therapy within the past 6 months or metastatic state; [¶]: glomerular filtration rate <60 mL·min^{-1}·1.73 m^{-2}; [+]: antiphospholipid syndrome, heterozygous or homozygous factor V Leiden mutation, heterozygous or homozygous prothrombin mutation or protein S or C deficiency; [§]: inflammatory bowel disease (*e.g.* ulcerative colitis or Crohn's disease) or rheumatic disorder (*e.g.* systemic lupus erythematosus, connective tissue disease or vasculitis).

ANGPT2 overexpression is localised in pulmonary endothelium and increases in response to hypoxia or transforming growth factor-β1

Microarray analysis of endothelial cells outgrown from PEA tissue (CTEPH-ECs) identified higher *ANGPT2* mRNA transcript levels compared to human pulmonary arterial endothelial cells (HPAECs) (p=0.008), with a parallel reduction in mRNA expression of endothelial tyrosine kinase receptor *TEK* (also known as *TIE2*; p=0.040; figure 3a and b). In contrast, gene transcripts of *ANGPT1*, vascular endothelial growth factor A (*VEGFA*) and its main receptor (*KDR*), or of the endothelial marker platelet endothelial cell adhesion molecule (*PECAM1* or *CD31*) did not significantly differ between CTEPH-ECs and HPAECs (figure 3b or not shown). Confocal fluorescence microscopy analysis of vessel-rich regions within PEA specimens confirmed co-localisation of ANGPT2 and the endothelial cell marker CD31 (figure 3c). *ANGPT2* mRNA levels significantly increased in HPAECs following cultivation in the presence of $CoCl_2$ in order to prevent hypoxia-inducible factor (HIF)1α degradation (supplementary figure S1) and thus to mimic hypoxia (figure 4a); they also increased in response to recombinant human transforming growth factor (TGF)-β1 (figure 4b). Both stimuli, HIF1α and TGF-β1, are present at elevated levels in human CTEPH and murine thrombus nonresolution [15, 17]. Of note, $CoCl_2$ treatment did not alter cell viability, as determined using MTC staining (p=0.1113; n=3 biological replicates) and the lactate dehydrogenase activity assay (p=0.1636; n=3 biological replicates).

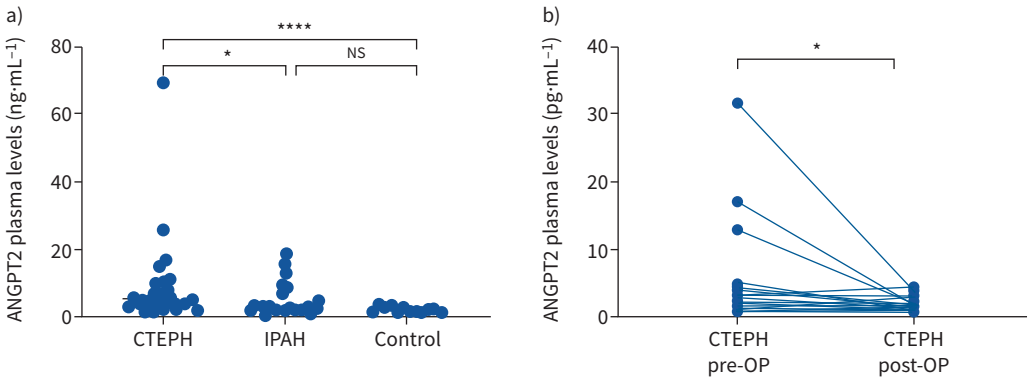

FIGURE 1 Plasma angiopoietin-2 (ANGPT2) levels in patients with chronic thromboembolic pulmonary hypertension (CTEPH) and controls. a) ANGPT2 levels were determined in plasma of patients with CTEPH (n=68) and idiopathic pulmonary arterial hypertension (IPAH) (n=38) and compared to healthy controls (n=36). *: p<0.05, ****: p<0.0001, determined using one-way ANOVA. b) ANPGT2 plasma levels were measured in 26 patients with CTEPH immediately before (CTEPH pre-OP) and after (CTEPH post-OP) pulmonary endarterectomy. *: p<0.05, determined using Wilcoxon matched-pairs signed rank test. NS: nonsignificant.

Circulating ANGPT2 levels in patients with acute pulmonary embolism are associated with diagnosis of CTEPH at follow-up

Circulating ANGPT2 levels were examined in 84 patients with acute pulmonary embolism and diagnosis of CTEPH during clinical follow-up (n=6, 7.1%; median (IQR) time to diagnosis 260 (44–900) days). The baseline characteristics of the study patients are shown in supplementary table S1. Patients who were later diagnosed with CTEPH had higher plasma ANGPT2 levels on admission for the acute event compared to patients not diagnosed with CTEPH at follow-up (median (IQR) 9.0 (4.7–19.6) *versus* 2.0 (1.4–3.3) $ng \cdot mL^{-1}$; p<0.0001) (figure 5a). Plasma levels of soluble TIE2 also were higher in patients with pulmonary embolism developing CTEPH during follow-up (p=0.0095; figure 5b). ROC analysis yielded an AUC of 0.92 (95% CI 0.78–1.00; figure 5c) for ANGPT2 with regard to the diagnosis of CTEPH at follow-up. The calculated optimal ANGPT2 cut-off value of 5.5 $ng \cdot mL^{-1}$ was associated with a sensitivity of 83%, a specificity of 95%, a PPV of 56% and a NPV of 99%. Patients with ANGPT2 $\geqslant$5.5 $ng \cdot mL^{-1}$ on admission had a >90-fold increased risk of a diagnosis of CTEPH at follow-up (OR 92.5, 95% CI 8.6–999.6; p<0.001).

Exogenous administration and endothelial cell-specific overexpression of ANGPT2 in mice attenuate resolution of venous thrombi

To investigate the possible role of ANGPT2 during venous thrombus resolution, a murine model of subtotal IVC ligation and experimental venous thrombosis was employed. In total, 31 male wild-type mice were treated with recombinant ANGPT2 *via* osmotic minipumps (treatment group) and compared to 57 male untreated wild-type animals. ANGPT2 administration was initiated on day 1 after surgery to exclude any effects on thrombus formation, and no differences in thrombus size were observed on day 1 prior to osmotic pump implantation (figure 6a; arrow). Sonographic analyses of thrombus size revealed significantly larger venous thrombi in mice receiving recombinant ANGPT2 compared to untreated mice at day 3, day 7 and day 14 after IVC ligation (figure 6a). Analysis of changes of thrombus size over time confirmed delayed thrombus resolution in mice treated with ANGPT2 compared to control mice (figure 6b). ANGPT2 plasma concentrations were confirmed to be higher in mice treated with recombinant ANGPT2 compared to control mice (p<0.003; figure 6c) and found to correlate with thrombus size (r^2=0.314; p=0.023; figure 6d). Of note, thrombus size did not differ between wild-type controls and wild-type mice treated with buffer alone (0.9% sodium chloride *via* osmotic pumps; n=6; p=0.129). In addition, unchanged ANGPT2 plasma levels were observed in mice with surgical laparotomy only (median (IQR) 14.3 (11.4–15.1) $ng \cdot mL^{-1}$; p=1.000) and those with IVC ligation (13.2 (11.1–14.8) $ng \cdot mL^{-1}$; p=0.516) compared to those without any surgical intervention (13.4 (12.2–15.8) $ng \cdot mL^{-1}$).

Immunohistochemical analysis of murine venous thrombi harvested at different time points after IVC ligation revealed more pronounced ANGPT2 immunosignals in thrombi of ANGPT2-treated mice compared to control mice at day 3 (p=0.0461), day 7 (p=0.0016) and day 14 (p=0.0253) (figure 7b). Representative images after IVC ligation are shown in figure 7a. Similar to human PEA material,

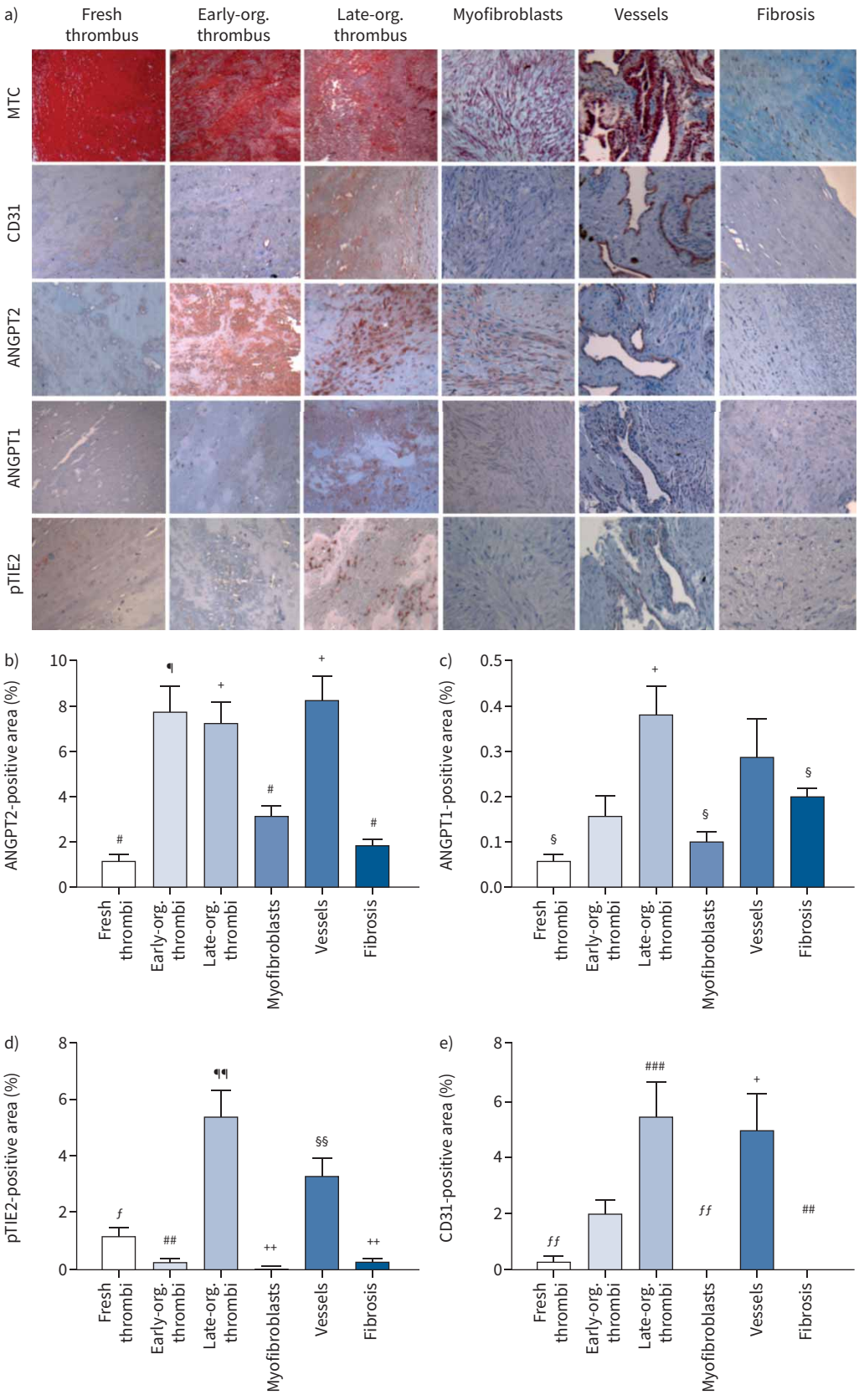

FIGURE 2 (Immuno)histological analysis of human pulmonary endarterectomy (PEA) tissue material. a) Representative images of findings in six distinct histological regions of interest after staining of paraffin-embedded cross-sections of PEA with Masson's trichrome (MTC) stain or following

immunohistochemistry with domain-specific antibodies against human CD31, ANGPT2, ANGPT1 and pTIE2 (phosphorylated TIE2) are shown. Boxplots show the results of the quantitative morphometric analyses after immunohistochemical staining for b) ANGPT2; c) ANGPT1; d) pTIE2; and e) CD31 per 300×150 µm areas in six distinct histological regions of interest. Comparisons were made using one-way ANOVA. #: *versus* early-organised (org.) thrombi, late-org. thrombi, vessels; ¶: *versus* myofibroblasts, fibrosis; +: *versus* fresh thrombi, myofibroblasts, fibrosis; §: *versus* late-org. thrombi; ƒ: *versus* late-org. thrombi, myofibroblasts, vessels, fibrosis; ##: *versus* late-org. thrombi, vessels; ¶¶: *versus* fresh thrombi, early-org. thrombi, myofibroblasts, vessels, fibrosis; ++: *versus* fresh thrombi, late-org. thrombi, vessels; §§: *versus* fresh thrombi, early-org. thrombi, late-org. thrombi, myofibroblasts, fibrosis; ƒƒ: *versus* vessels; ###: *versus* fibrosis.

immunosignals for ANGPT1 were found to be less prominent and did not differ significantly between thrombi of ANGPT2-treated mice and controls (figure 7c). Importantly, pTIE2 immunosignal was almost undetectable in thrombi of mice having received ANGPT2, whereas a nonsignificant trend towards increase over time was observed in controls (figure 7d). Newly formed microvessels, detected by CD31-immunopositive cells, were also less frequently present in thrombi of ANGPT2-treated mice compared to control mice (figure 7e).

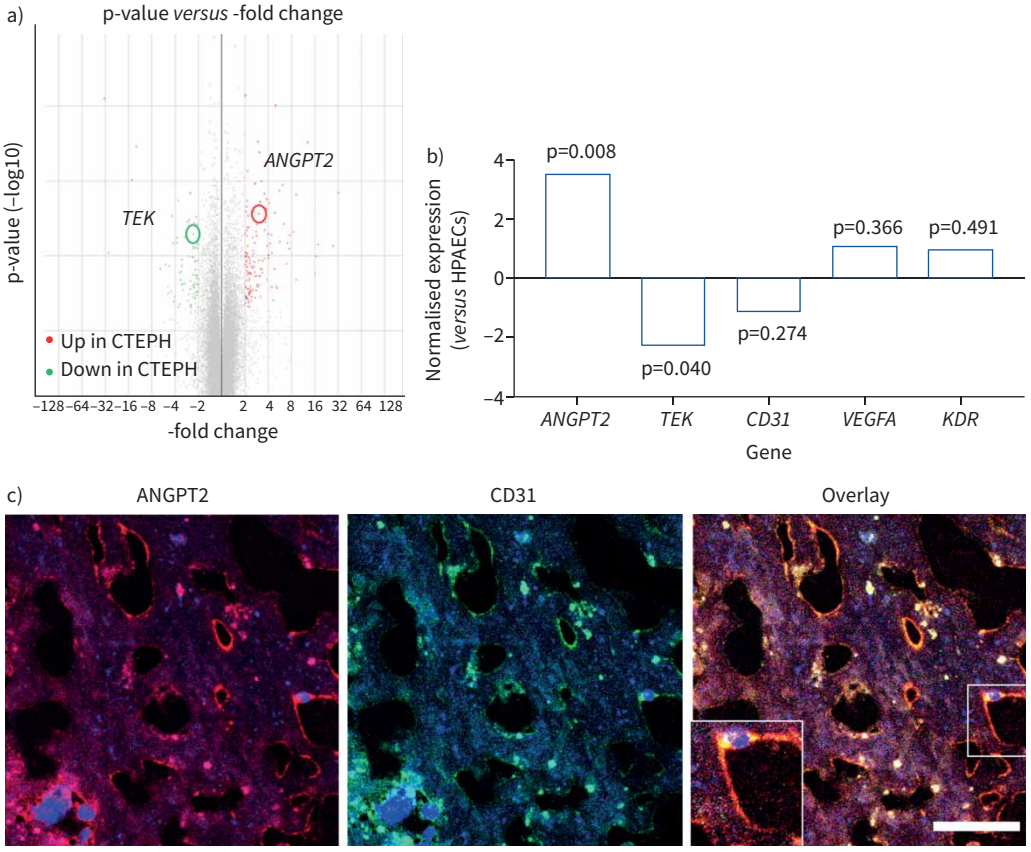

FIGURE 3 Microarray analysis of endothelial cells outgrown from human pulmonary endarterectomy (PEA) tissue. Endothelial cells outgrown from PEA tissue (chronic thromboembolic pulmonary hypertension (CTEPH)-ECs) and human pulmonary arterial endothelial cells (HPAECs) were subjected to microarray gene expression analysis. a) Volcano plot showing statistical significance of angiopoietin-2 (*ANGPT2*) and *TEK* expression (p-value; y-axis) *versus* magnitude of change (fold change; x-axis). b) Changes in mRNA expression levels in CTEPH-ECs *versus* HPAECs. p-values are shown within the graph and were determined using Affymetrix Transcriptome Analysis Console (TAC 4.0). c) Immunofluorescence confocal microscopy analysis of ANGPT2 (red signal) and platelet endothelial cell adhesion molecule (PECAM1; green signal) in endothelial cells within a vessel-rich region of human PEA tissue material. Scale bar=100 µm.

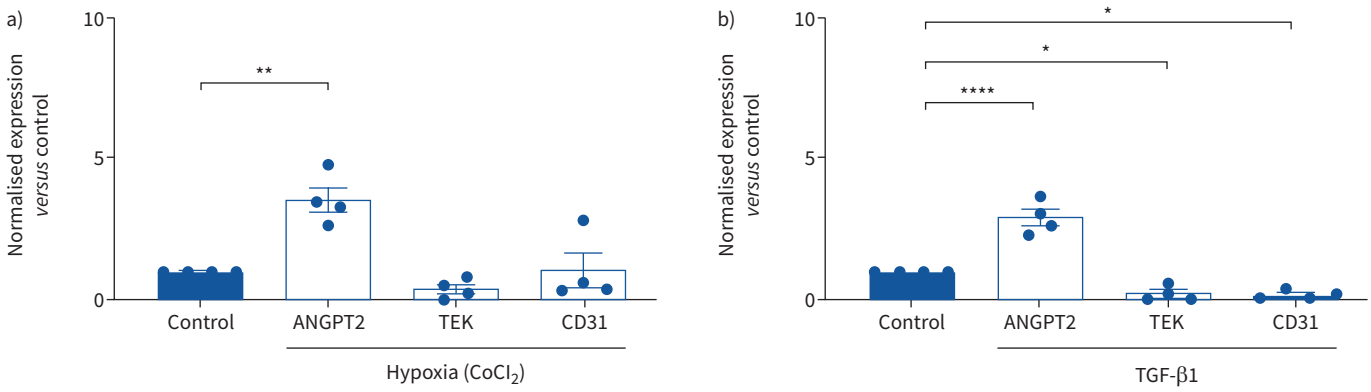

FIGURE 4 Effect of hypoxia and transforming growth factor (TGF)-β1 stimulation on angiopoietin-2 (ANGPT2) mRNA expression in human pulmonary arterial endothelial cells (HPAECs). Quantitative real-time PCR analysis was employed in HPAECs to determine the effects of a) cobalt chloride ($CoCl_2$)-induced chemical hypoxia or b) stimulation with recombinant human TGF-β1 on the mRNA expression of ANGPT2 or its receptor (TIE2) and the endothelial marker platelet endothelial cell adhesion molecule (PECAM1). *: p<0.05, **: p<0.001, ****: p<0.0001 *versus* control-treated cells, as determined by one-way ANOVA.

The importance of ANGPT for thrombus remodelling was validated in a second model using mice with endogenous, endothelial cell-specific ANGPT2 overexpression (ANGPT2 DT). IVC ligation followed by repeated ultrasound analyses of thrombus size revealed larger venous thrombi in ANGPT2 DT mice compared to wild-type (CD1) controls beginning from day 3 until day 14 after surgery (figure 8a). Histological analysis of changes of thrombus size over time demonstrated less pronounced alterations in ANGPT2 DT mice indicating delayed thrombus resolution (figure 8b and c). Moreover, immunohistological analysis confirmed a marked reduction of thrombus angiogenesis by showing low and unaltered CD31 immunosignals in thrombi of ANGPT2 DT mice (figure 8c and d). Of note, ANGPT2 level in plasma of those mice were highly elevated (median (IQR) 41.9 (24.2–67.9) *versus* 0 (0–0) $ng \cdot mL^{-1}$; p<0.0001) compared to wild-type (CD1) controls (figure 8e).

Discussion

The angiopoietin–TIE2 ligand–receptor system has been identified as a major signalling pathway controlling angiogenesis and vascular remodelling. However, the importance of ANGPT2 for misguided thrombus resolution and persistent or progressive vascular occlusion after pulmonary embolism, and consequently its potential contribution to the development of CTEPH, has not yet been systematically studied. In the present study, we investigated the potential role of ANGPT2 in patients with pulmonary embolism and CTEPH, and employed experimental venous thrombosis in two mouse models of elevated circulating ANGPT2 levels. Our findings in humans, mice and primary endothelial cells suggest that ANGPT2 delays venous thrombus resolution, and that overexpression of ANGPT2 contributes to thrombofibrosis and may thus support the transition from acute pulmonary embolism to CTEPH. Circulating ANGPT2 levels were higher in patients with CTEPH compared to patients with IPAH and healthy controls, and they identified pulmonary embolism patients at higher risk for being diagnosed with CTEPH during follow-up. ANGPT2 could be localised to the pulmonary vessel endothelium, and high numbers of ANGPT2-positive cells, paralleled by reduced TIE2 phosphorylation, were present in early-stage organised thrombi and myofibroblast-rich regions in PEA tissue; findings were similar in experimental murine thrombi. Exogenous ANGPT2 administration or, alternatively, endothelial cell-specific overexpression of ANGPT2 prevented or misguided murine venous thrombus resolution.

Pathophysiological importance of ANGPT2 for the development of CTEPH after pulmonary embolism

The angiopoietin–TIE-2 ligand–receptor system is essential during embryonic vessel assembly and maturation, and functions as a key regulator of adult vascular homeostasis [18]. Transgenic overexpression of ANGPT2 leads to disruption of blood vessel formation and thus embryonic lethality [8, 19]. TIE2 loss-of-function experiments demonstrated its importance for angiogenesis, particularly for vascular network formation in endothelial cells [20]. ANGPT2 acts as an antagonist ligand of the endothelial tyrosine kinase receptor TIE2 inhibiting the protective and stabilising influence of ANGPT1 [8–10]. Increased expression of ANGPT2 in human and murine venous thrombi has been described earlier and suggested to contribute to thrombofibrosis [5, 21]. Studies also support the angiostatic effect of ANGPT2

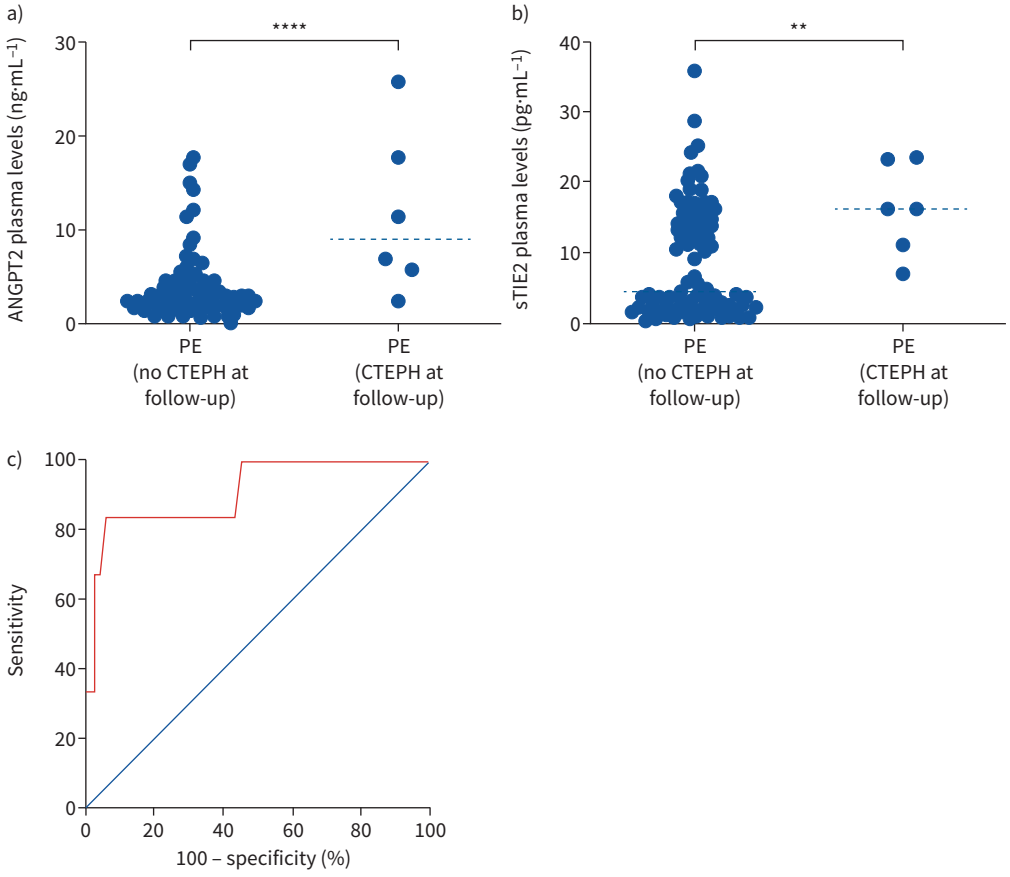

FIGURE 5 Plasma angiopoietin-2 (ANGPT2) and soluble (s)TIE2 levels in patients with acute pulmonary embolism. Plasma concentrations of a) ANGPT2 and b) sTIE2 were determined in patients with pulmonary embolism (n=26), with and without diagnosis of chronic thromboembolic pulmonary hypertension (CTEPH) during follow-up. **: p<0.01, ****: p<0.0001 as determined using unpaired t-tests. c) Prognostic performance of ANGPT2 plasma levels regarding the diagnosis of CTEPH in 84 patients with pulmonary embolism who underwent clinical follow-up.

and its role in vessel rarefaction in other disease contexts, for example in the myocardium of diabetic mice [22] or during cardiac hypoxia and inflammation after myocardial ischaemia [23].

Angiogenesis is crucial for the degradation and organisation of thrombi and restoration of vascular patency by forming microchannels to allow for blood flow, as shown by our group [5] and others [6, 24, 25]. Conversely, the incomplete resolution of thrombus material followed by fibrotic remodelling is considered a critical mechanism underlying the development of CTEPH after pulmonary embolism [3, 26]. To explore the importance of ANGPT2, we established a murine model of continuous exogenous administration of recombinant ANGPT2 using subcutaneously implanted osmotic pumps. Our results show that mice treated with recombinant murine ANGPT2 following IVC ligation developed larger thrombi and also exhibited a delayed decrease in thrombus size over time. The inhibiting effect of ANGPT2 on TIE2 receptor signalling was confirmed by the almost complete absence of detectable immunosignals of phosphorylated TIE2 in venous thrombi of ANGPT2-treated mice, whereas their numbers showed a trend towards increase over time in control mice. Additionally, newly formed microvessels, detected by means of CD31-positive cells, were less frequently present in thrombi of ANGPT2-treated mice compared to controls, in line with an inhibitory effect of ANGPT2 on (neo)angiogenesis. In line with this, larger venous thrombi and fewer newly formed microvessels were observed in mice with endogenous ANGPT2 overexpression in endothelial cells. Comparable findings were observed in tissue material removed from CTEPH patients during PEA, revealing elevated ANGPT2 expression paralleled by the absence of relevant pTIE2 and CD31immunosignals in early organised thrombi and regions rich in myofibroblasts and fibrosis. These findings suggest that ANGPT2 mediated inhibition of ANGPT1/TIE2 signalling contributes to venous thrombus nonresolution and persistent vascular obstruction. Other authors have also reported low relative

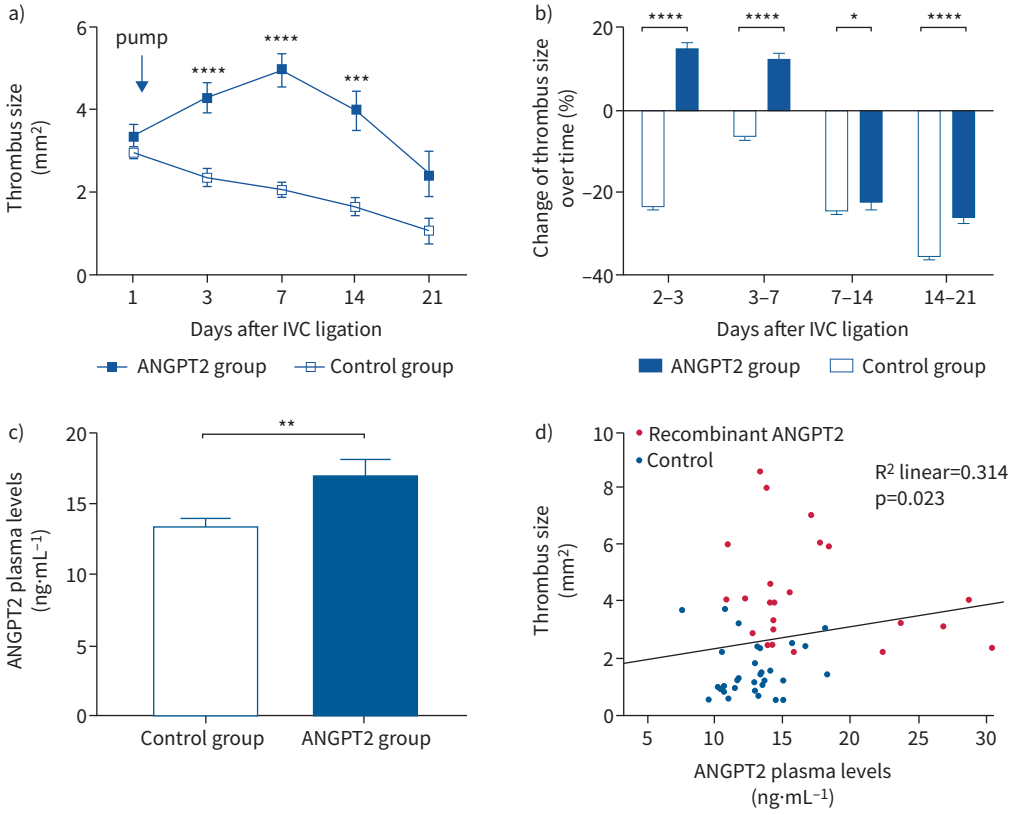

FIGURE 6 Effect of recombinant angiopoietin-2 (ANGPT2) on murine venous thrombus resolution. Wild-type mice were subjected to inferior vena cava (IVC) ligation followed by osmotic pump implantation on day 1 after surgery (arrow in panel a)). The a) thrombus size (mm^2) and b) change of thrombus size over time (%) was assessed by abdominal sonography at different time points (days 3, 7, 14 and 21) after IVC ligation in ANGPT2-treated mice and control mice. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001 *versus* the control group, as determined by two-way ANOVA. c) Plasma ANGPT2 levels in mice treated with recombinant ANGPT2 compared to mice without (p<0.003). d) Correlation of ANGPT2 levels with thrombus size (r^2=0.314; p=0.023) in mice treated with recombinant ANGPT2 compared to mice without.

expression of CD31 mRNA [24] and an abundant presence of angiostatic factors such as platelet factor 4 [21] in human CTEPH tissue material removed during PEA.

ANGPT2 upregulation induced by hypoxia and profibrotic stimuli

ANGPT2 is rapidly released into the circulation from activated endothelium and promotes capillary leakage and impaired endothelial integrity [11, 20]. Elevated ANGPT2 plasma concentrations were reported to be associated with poor prognosis in patients with cardiogenic shock [27] and in patients with IPAH [12]. In the present study, we observed higher levels of ANGPT2 in plasma of patients with CTEPH compared to IPAH and healthy controls. Importantly, circulating ANGPT2 levels in patients with confirmed CTEPH were also higher compared to those in patients with IPAH, which haemodynamically did not differ from patients with CTEPH, suggesting a specific (or stronger) activation stimulus and/or cellular source. In this regard, microarray analysis demonstrated increased *ANGPT2* mRNA expression levels in endothelial cells outgrown from PEA specimens compared to HPAECs, whereas those of *PECAM1*, *ANGPT1* or *VEGF* were not altered. Mechanistically, endothelial ANGPT2 expression may have occurred following activation of the endothelium associated with hypoxia [28, 29], thrombin [30] or inflammation [23]. Hypoxia is considered a main stimulus of venous thrombosis initiation [31], and the importance of hypoxia and HIF1A induced signalling for venous thrombus angiogenesis and resolution has been demonstrated before [6, 32]; we have documented the presence of hypoxia in CTEPH using a systematic histological analysis of tissue microarrays [15]. Moreover, we found that circulating ANGPT2 levels decreased significantly following PEA, suggesting that surgical removal of thrombus (a source of ANGPT2 expression) and the post-operative reduction in hypoxia may both have contributed to the reduction of ANGPT2 levels. Besides the decrease of ANGPT2 after surgical treatment in CTEPH,

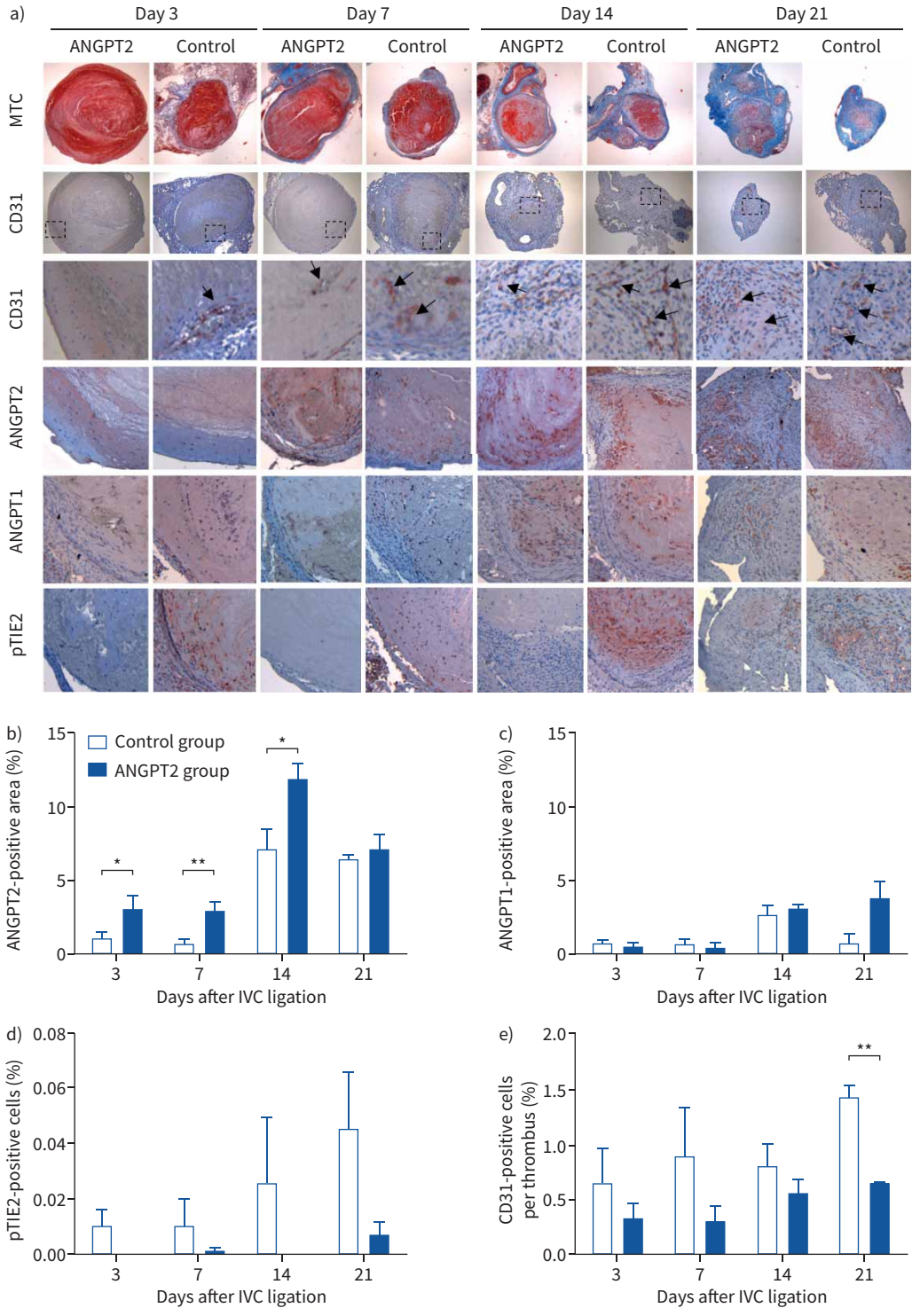

FIGURE 7 Histochemical analysis of venous thrombi resolution in mice with and without angiopoietin-2 (ANGPT2) treatment. Serial cross-sections of paraffin-embedded venous thrombi harvested at different time points (day 3, 7, 14 and 21 after inferior vena cava (IVC) ligation) were analysed using Masson's trichrome (MTC) staining or antibodies directed against mouse ANGPT2, ANGPT1, pTIE2 and CD31. a) Representative images are shown. Magnification ×200. Results of the quantitative morphometric analysis of the b) ANGPT2, c) ANGPT1-immunopositive area or the relative number of cells immunopositive for d) pTIE2 or e) CD31 are shown in thrombi of ANGPT2-treated mice and control mice. *: $p<0.05$, **: $p<0.01$, as determined by two-way ANOVA.

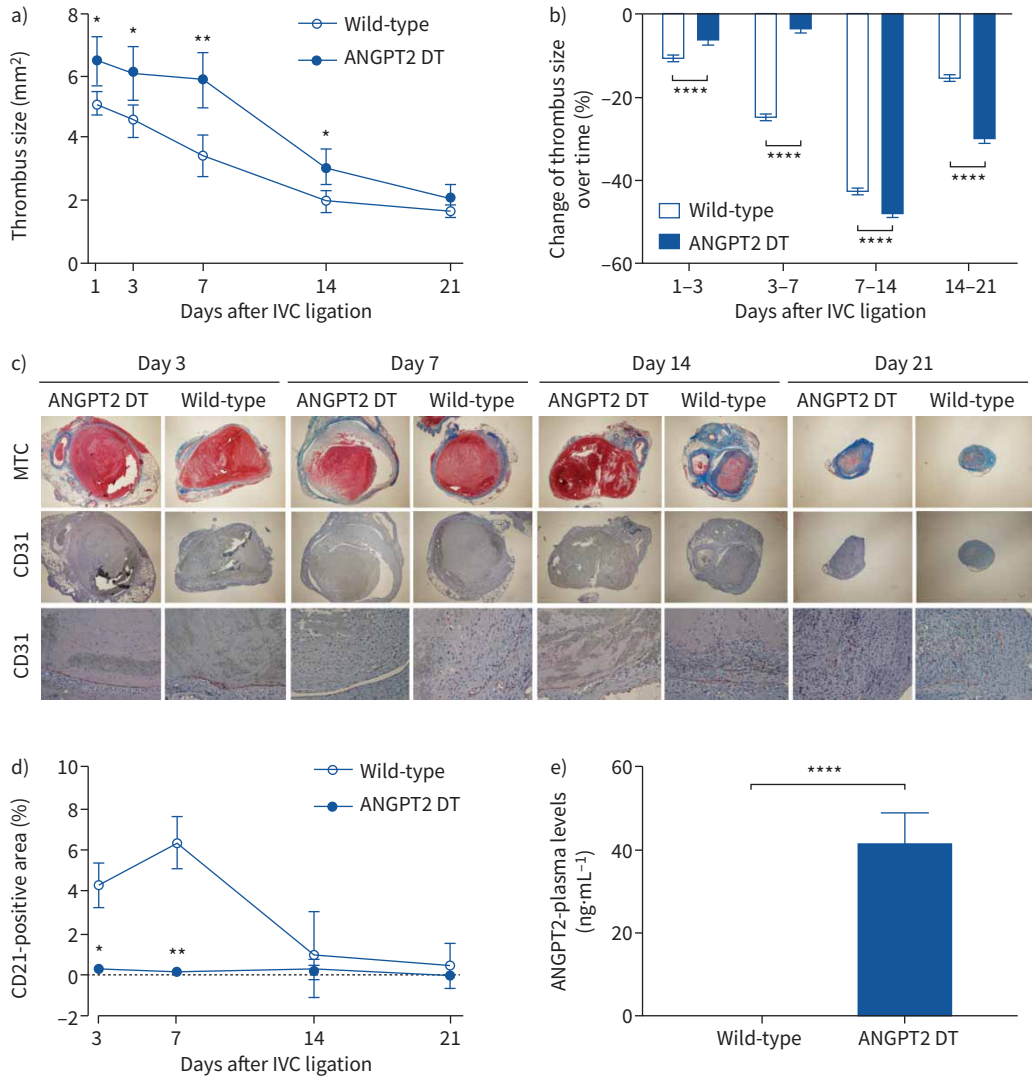

FIGURE 8 Effect of endothelial-specific angiopoietin-2 (ANGPT2) overexpression on venous thrombus resolution. Mice genetically engineered to overexpress ANGPT2 in the endothelium (ANGPT2 DT) and wild-type control mice were subjected to inferior vena cava (IVC) and thrombus formation and resolution followed by ultrasound as well as histologically over 3 weeks. Graphs show the results of the quantitative analysis of ultrasound of a) thrombus size (mm^2) and b) change of thrombus size over time (%) in wild-type mice and ANGPT2 DT mice at days 1, 3, 7, 14 and 21 after IVC ligation. *: p<0.05, **: p<0.01, ****: p<0.0001 *versus* wild-type mice as determined by two-way ANOVA. c) Serial cross-sections of paraffin-embedded venous thrombi harvested at different time points (days 3, 7, 14 and 21 after IVC ligation) were analysed using Masson's trichrome (MTC) staining or rat monoclonal antibodies directed against mouse CD31. d) Quantitative analysis of CD31-immunopositive area in venous thrombi at different time points. *: p<0.05, **: p<0.01 *versus* wild-type mice as determined by two-way ANOVA. e) Plasma ANGPT2 levels in wild-type and ANGPT2 DT mice before IVC ligation. ****: p<0.0001.

pro-inflammatory markers such as IL-6, TNF-α or CRP were found to decrease post-operatively [33, 34]. As shown previously, loss-of-function mutations for the transcriptional mediator Smad4, one of four TGF-β pathway members, causes the formation of inappropriate, fragile connections between arteries and veins, while ANGPT2 transcription in endothelial cells is increased. Interestingly, the endothelial size and shape defects could be rescued by ANGPT2 inhibition, which highlights the importance of ANGPT2 as mediator of inappropriate neovascularisation [35]. In the present study, we found that *ANGPT2* mRNA transcripts increased in response to TGF-β1; a finding in line with our recent studies demonstrating a role for activated endothelial TGF-β1 signalling in venous thrombus nonresolution in mice and the development of thrombofibrosis in CTEPH [17]. Our findings now suggest that ANGPT2 may be a

downstream mediator of hypoxia and profibrotic TGF-β1 signalling in CTEPH. The link between ANGPT2 and the degree of vascular obstruction and hypoxia also appears to be supported by the fact that, in the present study, ANGPT2 plasma levels correlated with indicators of haemodynamic impairment in patients with CTEPH as well as with thrombus size in mice.

Circulating ANGPT2 may identify pulmonary embolism patients at higher risk for CTEPH

In the present study, 7% of survivors of acute pulmonary embolism were diagnosed with CTEPH during follow-up. These patients had higher ANGPT2 levels at the time of pulmonary embolism diagnosis compared to pulmonary embolism patients who did not develop CTEPH, and ANGPT2 plasma concentrations above the calculated optimal cut-off value of 5.5 $ng \cdot mL^{-1}$ were associated with an impressively increased risk (93-fold) for CTEPH diagnosis at follow-up. Our study cannot answer the question whether these patients subsequently developed CTEPH "because of" ANGPT2 overexpression in their endothelium, or if this process was already ongoing and ANGPT2 was a biomarker of pre-existing CTEPH. In either case, if confirmed in independent cohorts, our results may contribute to establishing ANGPT2 levels as an additional, relatively simple prognostic laboratory marker in acute pulmonary embolism. Its elevation could increase awareness of possibly developing CTEPH and thus reinforce the current guideline recommendations on post pulmonary embolism care and early detection of late sequelae [1].

Limitations

The present study has limitations that need consideration: first, although we investigated ANGPT2 in a murine model of venous thrombus resolution, it must be mentioned that a mouse model mimicking CTEPH does not exist so far. Second, although we were able to demonstrate an increased risk for subsequent diagnosis of CTEPH patients with pulmonary embolism and high ANGPT2 plasma levels, further research is needed to determine whether ANGPT2 may help in clinical decision making and prognostic assessment of individual patients. Finally, external validation is essential to address the accuracy of ANGPT2 as a potential marker in pathogenesis and early prediction for patients with acute pulmonary embolism and CTEPH.

Summary and conclusion

Taken together, our findings in patients and mouse models reveal a new role for ANGPT2 in the pathophysiology of CTEPH, suggesting that ANGPT2 overexpression in pulmonary endothelium may contribute to defective angiogenesis and persistent vascular occlusion. Patients with acute pulmonary embolism and elevated ANGPT2 levels demonstrated an increased risk of CTEPH at follow-up. Depending on external validation of our findings, ANGPT2 might play an important role in the pathogenesis of CTEPH and may help to identify patients with acute pulmonary embolism and pre-existing CTEPH or with an increased risk for developing CTEPH.

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Author contributions: L. Hobohm: conception and design of the study, data collection, performance of laboratory experiments, analysis of the data, interpretation of data, drafting of the manuscript and revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; S. Kölmel: acquisition and analysis of the data, performance of laboratory experiments, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; C. Niemann and V.J. Krieg: acquisition and analysis of the data, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; P. Kümpers: acquisition and analysis of the data, interpretation of data, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; M.L. Bochenek and A.H. Lukasz: performance of laboratory experiments, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; Y. Reiss and K-H. Plate: performance of laboratory experiments, interpretation of data, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; C. Liebetrau, C.B. Wiedenroth, S. Guth, T. Münzel, G. Hasenfuß, P. Wenzel and E. Mayer: revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; S.V. Konstantinides and K. Schäfer: conception and design of the study, interpretation of data, revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted; M. Lankeit: conception and design of the study, data collection, analysis of the data, interpretation of

data, drafting of the manuscript and revising of the manuscript critically for important intellectual content, final approval of the manuscript submitted.

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