Supplementary Material

Supplementary Methods

Studies involving humans

Patients with chronic thromboembolic pulmonary hypertension

The study protocol was conducted in accordance with the amended Declaration of Helsinki and was approved by the local Ethics Committee (AZ 44/14). All patients gave written informed consent prior to participation in the study.

The study design has been described in detail before [1]. Diagnosis of chronic thromboembolic pulmonary hypertension (CTEPH) was confirmed according to current guideline recommendations [2]. Patients who declined participation in the study, patients with a diagnosis of pulmonary artery sarcoma and patients with missing data were excluded from the analysis. Baseline clinical data including comorbidities, risk factors for CTEPH, symptoms and functional status, medication and results from clinical tests, such as right heart catheterisation, transthoracic echocardiography, 6-minute walk test and laboratory measurements, or surgical procedures and in-hospital outcomes were collected using a standardised case report form. Pulmonary endarterectomy (PEA) was performed, as previously reported [3, 4]. In a subgroup of patients, PEA tissue material was kept in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (Gibco) and rapidly processed for subsequent (immuno-)histological analyses. All treatment decisions were in the responsibility of the caring physicians and not influenced by the study protocol at any time. Follow-up data were collected using a standardized case report form by reviewing the medical records.

Patients with pulmonary embolism

The study protocol was conducted in accordance with the amended Declaration of Helsinki and approved by the local Ethics Committee (AZ 14/6/10). All patients gave written informed consent prior to participation in the study.

The study design of the Pulmonary Embolism Registry Goettingen (PERGO) has been described in detail elsewhere [5-7]. Management strategies complied with existing guideline recommendations [8] throughout the study period, and treatment decisions were made by the responsible physicians and not influenced by the study protocol at any time. Patients were excluded from the analysis if pulmonary embolism (PE) was asymptomatic and/or an accidental finding during the diagnostic workup for another suspected disease, if patients died during the in-hospital stay or if consent for participation in the study was denied or withdrawn.

Long-term follow-up data were collected at least six months after the initial PE event during a routine clinical follow-up visit as part of the standard clinical care at the Clinic for Cardiology and Pneumology, University Medical Centre Goettingen, Germany [7] or by contacting the treating general practitioner. Diagnosis of CTEPH was confirmed according to current guideline recommendations [2]. All outcomes, including cause of death, were independently adjudicated by two of the authors (L.H. and C.N.) by reviewing medical and autopsy records, if available, or by contacting the last treating physician; disagreement was resolved by a third author (M.L.).

Measurement of human and murine circulating biomarkers

Venous blood samples for subsequent biomarker measurements were collected from patients with acute PE, CTEPH before and after PEA, IPAH and healthy volunteers. Blood samples

were processed using standard operating procedures, immediately stored at -80°C and analysed in batches after a single thaw. Routine laboratory parameters were measured as part of the clinical routine at the Department of Clinical Chemistry of the University Medical Centre Goettingen, Germany, in study patients with PE and at the Department of Haemostaseology and Transfusion Medicine, Kerckhoff Clinic, Bad Nauheim, Germany, in study patients with CTEPH.

Immunohistological analyses

Antibodies directed against human and mouse ANGPT2 (NBP2-15385, Novus Biological; Littelton; USA), ANGPT1 (ab133425; abcam; Cambridge; UK) and phosphorylated TIE2 (pTIE2; MBS8502462; Mybiosource; Vancouver; Canada) were used. Endothelial cells were detected using antibodies against cluster of differentiation (CD) 31 (in humans: clone JC70A; Dako; Glostrup; Denmark; in mice: DIA-310; Dianova; Hamburg; Germany). All morphometric analyses were performed using image analysis software (Image ProPlus, version 7.0). The immunosignal was manually marked as a red-coded area and subsequently measured automatically using the "count-size" function for each region of interest (i.e. complete murine thrombi or a 300 x 150 µm area in histologic regions of human tissue material removed from PEA) and expressed in (%).

Immunofluorescence confocal microscopy

Human tissue material removed during PEA was cryoembedded in Tissue-Tek O. C. T. compound (Sakura, Finetek, Torrance, CA, USA). Five µm-thick tissue sections were post-fixed with acetone followed by permeabilisation using 0.05 % Triton X-100. Primary antibodies against ANGPT2 and CD31 (as listed above) and secondary antibodies (MFP488- or MFP555-conjugated [MoBiTec, Berkheim, Germany]) were used. Cell nuclei were

detected using DRAQ5 (Life Technologies). Confocal fluorescence microscopy images were evaluated using a Zeiss Observer Z1 confocal microscope (Zeiss, Jena, Germany).

Studies involving mice

Administration of ANGPT2 via osmotic pumps

Mice were treated with recombinant murine ANGPT2 (7186-AN; R&D Systems; Minneapolis; USA) via osmotic pumps (ALZET osmotic minipumps, DURECT Corporation; Cupertino; USA) implanted subcutaneously between the shoulder blades one day after surgical IVC ligation. According to the scheduled treatment time, ALZET pumps with different delivery rates were selected (1.0 µl per hour for 3 days, 0.5 µl per hour for 7 days, 0.25 µl per hour for 14 days and 0.11 µl per hour for 21 days) and filled with ANGPT2 dissolved in 0.9% sodium chloride according to the intended treatment dose of 75 ng per hour [9].

Mice with endothelial cell-specific overexpression of ANGPT2

ANGPT2 was overexpressed specifically in endothelial cells in mice (CD1 background) using tetracycline-controlled transcriptional activation system (TetOFF). Mice were fed with doxycycline (Dox)-containing food pellets (100 mg/kg) from ssniff Spezialdiaeten GmbH, Soest, Germany. To allow transgene expression, Dox was removed. Transgenic and control wild-type (WT; CD1 background) male mice were used at 14 to 16 weeks of age. ANGPT2:Tie1 double transgenic (DT) animals were identified by PCR from tail biopsies.

Mouse model of stagnant flow venous thrombosis

The study protocol was in accordance with the guideline of the European Union 2010/63/EU and the protection of animal act (TierSchG, §8) in Germany. The study protocol and animal experiments were approved by the local authorities (Landesuntersuchungsamt Rheinland-

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Pfalz/ 23 177-07/G 14-1-044). Thrombi were induced using an established murine model of stagnant flow venous thrombosis by surgical subtotal ligation of the infrarenal inferior vena cava (IVC) [10]. After midline laparotomy incision and exploration of the IVC below the left renal artery, the IVC was surrounded with a 4-0 silk suture and a 5-0 prolene suture was placed alongside the IVC. Stagnant flow in the vein was achieved by tying the silk suture around the IVC; to allow minimal blood flow through the stenosis, the prolene suture was pulled out. Anesthesia during surgical IVC ligation and implantation of osmotic pumps was induced by intraperitoneal injection of midazolam (5.0 mg/kg body weight [BW]), medetomidine (0.5 mg/kg BW) and fentanyl (0.05 mg/kg BW). At the end of each procedure, anaesthesia was antagonized with atipamezole (0.05 mg/kg BW) and flumazenile (0.01 mg/kg BW) and analgesia was achieved with subcutaneous buprenorphine hydrochloride (0.075 mg/kg BW).

Non-invasive analysis of venous thrombus resolution

Abdominal sonography was performed in mice under anaesthesia with 2.5% isoflurane inhalation using B-mode, pulse wave (pw) Doppler and power Doppler imaging (PDI) with high frequency ultrasound (Vevo770 system; Toronto; Canada) to assess thrombus size (area). The change of thrombus size over time is expressed as percentage (%) change of the thrombus size from each time point, which was assessed after IVC ligation by abdominal sonography at different time points (day 3, 7, 14 and 21) [11].

Blood and thrombus sampling

At the indicated time points, venous thrombi were harvested by midline laparotomy incision after anaesthesia and lethal blood sampling by cardiac puncture. After dehydration, using a standardised descending alcohol series, thrombi were embedded in paraffin wax (Leica). For

histological analyses, 5 µm-thick transverse sections were cut using a microtome (Leica RM2250; Germany).

Additionally, to assess the influence of surgical procedures on Angpt-2 plasma concentrations and thrombus size, mice underwent lethal blood sampling by cardiac puncture only (control group A), midline laparotomy without IVC ligation and without osmotic pump implantation followed by lethal blood sampling by cardiac puncture (control group B) and implantation of osmotic pumps filled with 0.9% sodium chloride after IVC ligation followed by lethal blood sampling by cardiac puncture (control group C).

Studies involving cells

Culture of human endothelial cells

Human pulmonary arterial endothelial cells (HPAECs) isolated from healthy individuals (PromoCell) and CTEPH endothelial cells (CTEPH-ECs) outgrown from the PEA tissue were grown on 0.2% gelatin-coated cell culture plates in Endothelial Cell Growth Medium MV2 Kit (PromoCell). Cell were analysed between passage 3 and 5. Unstarved HPAECs were treated with recombinant human TGF-β1 (10 ng/ml in endothelial cell medium) for 7 days, and TGF-β1 was replaced along with the fresh medium on day 4. To induce chemical hypoxia, HPAECs were treated with $CoCl_2$ (150 µM in endothelial cell medium) for 16 hours. Cells treated with an equal volume using endothelial cell medium were used as control. Changes in cell viability and toxicity were examined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega) and the lactate dehydrogenase activity (Sigma-Aldrich) assay, respectively, following the manufacturers' instructions.

RNA isolation and microarray analysis of CTEPH endothelial cells

Cells were lyzed in Trizol® reagent (Ambion) to obtain total RNA. RNA quality was checked using Bioanalyzer (Agilent Technologies) and biotinylated cRNA was prepared. Afterwards,

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200 ng of cRNA was used to prepare one-stranded cDNA. Fragmented and biotinylated cDNA was used for hybridisation on MOGENE 2.0 ST Arrays (Affymetrix GeneChip WT PLUS Reagent Kit). To analyse signal intensity of gene expression, Affymetrix Transcriptom Analysis Expression Console Software (version 1.4) was used.

Quantitative real time polymerase reaction

Total RNA was isolated using Trizol® reagent, treated with DNase I (Promega) and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BioRad). Primer sequences and quantitative real-time polymerase chain reaction conditions have been described before [12].

Statistical analysis

Comparison of the prognostic performance of ANGPT2 was performed by calculation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The prognostic relevance of biomarker cut-off values as well as single predictors with regard to study outcomes was tested using univariable logistic regression analysis and presented as Odds ratios (OR) with the corresponding 95 % confidence intervals (CI). For microarray analysis volcano plot showing statistical significance of ANGPT2 and TEK expression (p-value; y-axis) versus magnitude of change (-fold change; x-axis). P values are shown within the graph and were determined using Affymetrix Transcriptome Analysis Console (TAC 4.0) for mRNA expression levels.

Supplementary Results

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

On admission for PEA, 16 (23.5 %) patients received specific drugs. Of those, 11 (68.8 %) a combination therapy. In more detail, 11 patients were treated with riociguat, six with an endothelin-receptor antagonist, six with a phosphodiesterase-5 inhibitor and one patient with an inhaled prostacyclin analogue.

No association between circulating levels of ANGPT2 and persistent PH during follow-up

At postoperative follow-up conducted a median time of 388 (IQR, 342-437) days after surgery, 56 (65.1%) of the patients underwent right heart catheterization; this showed a median mPAP of 26 (IQR 20-33) mmHg and median PVR of 231 (IQR 170-374) dyn*sec*cm-5. Overall, 15 (26.8%) patients were found to have PH. However, preoperatively measured ANGPT2 was not associated with persistent PH during follow-up (OR for ANGPT2 $\geq$5.5 ng/ml: 4.80, 95% CI 0.40-58.01, p=0.217).

Supplemental Tables

Table S1. Baseline characteristics, medical history and initial presentation of 84 PE patients (stratified according diagnosis of CTEPH at follow-up)

Parameter	All study patients (n=84)	No CTEPH (n=78)	CTEPH (n=6)	p-value
Age (years)	67 (51-76)	67 (51-76)	69 (54-72)	0.900
Sex (male)	41 (48.8 %)	41 (52.6 %)	2 (33.3 %)	0.427
Comorbidities				
Previous PE	13 (15.5 %)	13 (16.7 %)	0 (0 %)	0.584
Cancer*	15 (17.9 %)	15 (19.2 %)	0 (0 %)	0.585
Chronic heart failure	11 (13.1 %)	8 (10.3 %)	3 (50.0 %)	**0.027**
Coronary artery disease	14 (16.7 %)	14 (17.9 %)	0 (0 %)	0.583
Chronic pulmonary disease	11 (13.1 %)	8 (10.3 %)	3 (50.0 %)	**0.027**
Diabetes mellitus	12 (14.3 %)	12 (15.4 %)	0 (0 %)	0.587
Renal insufficiency†	28 (33.3 %)	25 (32.1 %)	3 (50.0 %)	0.395
Symptoms and clinical findings on admission				
Dyspnoea	78 (92.9 %)	72 (92.3 %)	6 (100.0 %)	1.000
Haemoptysis	2 (2.5 %) n=81	2 (2.7 %) n=75	0	1.000
Chest pain	46 (55.4 %) n=83	44 (57.1 %) n=77	2 (33.3 %)	0.400
Syncope	15 (17.9 %)	13 (16.7 %)	2 (33.3 %)	0.290

Tachycardia‡	30 (35.7 %)	29 (37.2 %)	1 (16.7 %)	0.414
Hypoxia§	20 (27.8 %) n=72	19 (27.5 %) n=69	1 (33.3 %) n=3	1.000
RV dysfunction (on TTE)	27 (47.4 %) n=57	24 (46.2 %) n=52	3 (60.0 %) n=5	0.660
NT-proBNP ≥600 pg/ml	44 (53.0 %) n=83	38 (49.4 %) n=77	6 (100.0 %)	0.027
hsTnT ≥14 pg/ml	53 (65.4 %) n=81	49 (64.5 %) n=76	4 (80.0 %) n=5	0.654
Angpt2 ≥5.5 ng/ml	9 (10.7 %)	4 (5.1 %)	5 (83.3 %)	**<0.001**

* defined as active or anti-tumour therapy within the last 6-months, or metastatic state

† defined as glomerular filtration rate (GFR) <60 ml/min/1.73m²

‡ defined as heart rate ≥100 beats per minute

§ defined as arterial oxygen saturation <90 % or arterial oxygen partial pressure (PaO_2) <60 mmHg

Abbreviations: *hsTnT*, high-sensitivity troponin T; *NT-proBNP*, N-terminal pro-brain natriuretic peptide; *PE*, pulmonary embolism; *RV*, right ventricular; *TTE*, transthoracic echocardiography.

Table S2. Baseline characteristics, medical history and initial presentation of 68 patients with CTEPH and 38 patients with PAH

Parameter	CTEPH patients (n=68)	PAH patients (n=38)	p-value
Age (years)	63 (55-72)	68 (62-72)	0.070
Sex (male)	33 (48.5 %)	17 (44.7 %)	0.840
Comorbidities			
Cancer*	2 (2.9 %)	0	0.536
Chronic heart failure	3 (4.4 %)	0	0.426
Coronary artery disease	15 (22.1 %)	21 (55.3 %)	**0.001**
Chronic pulmonary disease	22 (32.4 %)	25 (65.8 %)	**0.001**
Diabetes mellitus	8 (11.8 %)	11 (28.9 %)	**0.036**
Renal insufficiency†	16 (23.5 %)	31 (81.6 %)	**0.001**
Thrombophilia‡	9 (13.2 %)	1 (5.0 %)	0.444
Splenectomy	1 (1.5 %)	0	1.000
Thyroid disease	20 (29.4 %)	3 (8.1 %)	0.103
Symptoms and clinical findings on admission			
Dyspnoea (NYHA class III-IV)	52 (77.6 %)	8 (21.1 %)	**0.001**
Haemoptysis	5 (7.7 %) n=65	0	0.154

Distance (m) in 6-minute walk test	364 (279-433) n=33	360 (255-480) n=37	0.733
Laboratory values			
NT-proBNP (pg/ml)	792 (173-2271) n=65	-	-
BNP (pg/ml)	-	152 (53-327)	-
C-reactive protein (mg/dl)	3.0 (1.3-8.0)	3.5 (1.9-6.4)	0.506
Right heart catheterisation			
Mean pulmonary artery pressure (mmHg)	43 (34-51) n=67	44 (36-51)	0.722
Pulmonary vascular resistance (WU)	6.4 (4.4-10.6) n=66	8.3 (5.0-14.3)	0.188
Cardiac index (l/min/m²)	2.4 (1.9-2.9) n=63	2.4 (1.9-2.8)	0.921

* defined as active or anti-tumour therapy within the last 6-months or metastatic state

† defined as GFR <60 ml/min/1.73 m²

‡ defined as antiphospholipid syndrome, heterozygous or homozygous factor V Leiden mutation, heterozygous or homozygous prothrombin mutation or protein S or C deficiency

§ defined as inflammatory bowel disease (e.g., ulcerative colitis or Crohn disease) or rheumatic disorder (e.g., systemic lupus erythematosus, connective tissue disease, or vasculitis)

Abbreviations: *CTEPH*, chronic thromboembolic pulmonary hypertension; *NT-proBNP*, N terminal pro-brain natriuretic peptide; *NYHA*, New York Heart Association; PAH, pulmonary arterial hypertension.

Figure S1. Western blot analysis HIF1α protein expression in response to CoCl₂
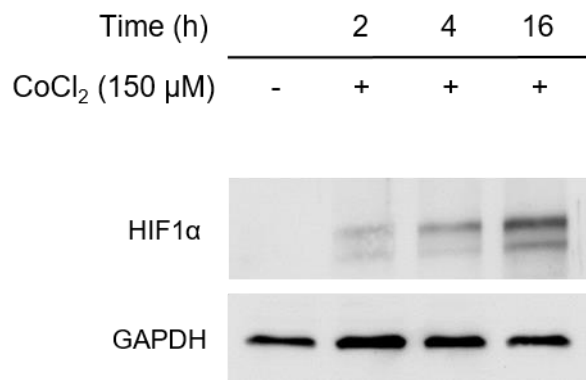
Abbreviations: *CoCl₂,* cobalt(II) chloride; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *h*, hours; *HIF1α*, hypoxia-inducible factor 1-alpha; *μM*, micromolar.

Supplementary References

1.   Lankeit M, Krieg V, Hobohm L, Kolmel S, Liebetrau C, Konstantinides S, Hamm CW, Mayer E, Wiedenroth CB, Guth S. Pulmonary endarterectomy in chronic thromboembolic pulmonary hypertension. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2017.

2.   Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M, Hoeper M. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Respir J* 2015: 46(4): 903-975.

3.   Guth S, Wiedenroth CB, Kramm T, Mayer E. Pulmonary endarterectomy for the treatment of chronic thromboembolic pulmonary hypertension. *Expert Rev Respir Med* 2016: 10(6): 673-684.

4.   Jenkins D, Madani M, Fadel E, D'Armini AM, Mayer E. Pulmonary endarterectomy in the management of chronic thromboembolic pulmonary hypertension. *Eur Respir Rev* 2017: 26(143).

5.   Lankeit M, Friesen D, Aschoff J, Dellas C, Hasenfuss G, Katus H, Konstantinides S, Giannitsis E. Highly sensitive troponin T assay in normotensive patients with acute pulmonary embolism. *Eur Heart J* 2010: 31(15): 1836-1844.

6.   Hellenkamp K, Schwung J, Rossmann H, Kaeberich A, Wachter R, Hasenfuss G, Konstantinides S, Lankeit M. Risk stratification of normotensive pulmonary embolism: prognostic impact of copeptin. *Eur Respir J* 2015: 46(6): 1701-1710.

7.   Klok FA, Tesche C, Rappold L, Dellas C, Hasenfuss G, Huisman MV, Konstantinides S, Lankeit M. External validation of a simple non-invasive algorithm to rule out chronic thromboembolic pulmonary hypertension after acute pulmonary embolism. *Thromb Res* 2015: 135(5): 796-801.

8.   Konstantinides SV, Torbicki A, Agnelli G, Danchin N, Fitzmaurice D, Galie N, Gibbs JS, Huisman MV, Humbert M, Kucher N, Lang I, Lankeit M, Lekakis J, Maack C, Mayer E, Meneveau N, Perrier A, Pruszczyk P, Rasmussen LH, Schindler TH, Svitil P, Vonk Noordegraaf A, Zamorano JL, Zompatori M, Task Force for the D, Management of Acute Pulmonary Embolism of the European Society of C. 2014 ESC guidelines on the diagnosis and management of acute pulmonary embolism. *Eur Heart J* 2014: 35(43): 3033-3069, 3069a-3069k.

9.   Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med* 2006: 12(2): 235-239.

10.   Alias S, Redwan B, Panzenbock A, Winter MP, Schubert U, Voswinckel R, Frey MK, Jakowitsch J, Alimohammadi A, Hobohm L, Mangold A, Bergmeister H, Sibilia M, Wagner EF, Mayer E, Klepetko W, Holzenbein TJ, Preissner KT, Lang IM. Defective Angiogenesis Delays Thrombus Resolution A Potential Pathogenetic Mechanism Underlying Chronic Thromboembolic Pulmonary Hypertension. *Arteriosclerosis Thrombosis and Vascular Biology* 2014: 34(4): 810-819.

11.   Alias S, Redwan B, Panzenboeck A, Winter MP, Schubert U, Voswinckel R, Frey MK, Jakowitsch J, Alimohammadi A, Hobohm L, Mangold A, Bergmeister H, Sibilia M, Wagner EF, Mayer E, Klepetko W, Hoelzenbein TJ, Preissner KT, Lang IM. Defective angiogenesis delays thrombus resolution: a potential pathogenetic mechanism underlying chronic thromboembolic pulmonary hypertension. *Arteriosclerosis, thrombosis, and vascular biology* 2014: 34(4): 810-819.

12.   Bochenek ML, Leidinger C, Rosinus NS, Gogiraju R, Guth S, Hobohm L, Jurk K, Mayer E, Munzel T, Lankeit M, Bosmann M, Konstantinides S, Schafer K. Activated Endothelial TGFbeta1 Signaling Promotes Venous Thrombus Nonresolution in Mice Via Endothelin-1: Potential Role for Chronic Thromboembolic Pulmonary Hypertension. *Circulation research* 2020: 126(2): 162-181.