

Comprehensive three-dimensional morphology of neoangiogenesis in pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis

Lavinia Neubert^{1,2†*}, Paul Borchert^{1,2†}, Hoen-Oh Shin^{2,3}, Friedemann Linz⁴, Willi L Wagner^{5,6}, Gregor Warnecke^{2,7}, Florian Laenger^{1,2}, Axel Haverich^{2,7}, Helge Stark^{1,2}, Marius M Hoepfer^{2,8}, Mark Kuehnel^{1,2}, Maximilian Ackermann^{4,9‡} and Danny Jonigk^{1,2‡}

¹Institute of Pathology, Hannover Medical School, Hannover, Germany

²Member of the German Center for Lung Research (DZL), Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Hannover, Germany

³Institute for Diagnostic and Interventional Radiology, Hannover Medical School, Hannover, Germany

⁴Institute of Functional and Clinical Anatomy, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

⁵Department of Diagnostic and Interventional Radiology, University of Heidelberg, Heidelberg, Germany

⁶Member of German Center for Lung Research (DZL), Translational Lung Research Center Heidelberg (TLRC), Heidelberg, Germany

⁷Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany

⁸Clinic for Pneumology, Hannover Medical School, Hannover, Germany

⁹Institute of Pathology, Medical Faculty, Heinrich-Heine-University and University Hospital Düsseldorf, Düsseldorf, Germany

*Correspondence: Lavinia Neubert (née Maegel), Institute of Pathology, Hannover Medical School, Carl-Neuberg Straße 1, 30625 Hannover, Germany. E-mail: neubert.lavinia@mh-hannover.de

†These authors contributed equally and share first authorship.

‡These authors contributed equally and share last authorship.

Abstract

Pulmonary veno-occlusive disease (PVOD) is a rare lung disease characterized by fibrotic narrowing of pulmonary veins leading to pulmonary hypertension (PH) and finally to death by right heart failure. PVOD is often accompanied by pulmonary capillary hemangiomatosis (PCH), a marked abnormal proliferation of pulmonary capillaries. Both morphological patterns often occur together and are thought to be distinct manifestations of the same disease process and accordingly are classified together in group 1' of the Nice classification of PH. The underlying mechanisms of these aberrant remodeling processes remain poorly understood. In this study, we investigated the three-dimensional structure of these vascular lesions in the lung explant of a patient diagnosed with PVOD by μ -computed tomography, microvascular corrosion casting, electron microscopy, immunohistochemistry, correlative light microscopy and gene expression analysis. We were able to describe multifocal intussusceptive neoangiogenesis and vascular sprouting as the three-dimensional correlate of progressive PCH, a process dividing pre-existing vessels by intravascular pillar formation previously only known from embryogenesis and tumor neoangiogenesis. Our findings suggest that venous occlusions in PVOD increase shear and stretching forces in the pulmonary capillary bloodstream and thereby induce intussusceptive neoangiogenesis. These findings can serve as a basis for novel approaches to the analysis of PVOD.

Keywords: pulmonary veno-occlusive disease; pulmonary capillary hemangiomatosis; pulmonary hypertension; pulmonary vascular remodeling; intussusceptive neoangiogenesis

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Introduction

Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) are rare and prognostically very unfavorable diseases involving the

pulmonary vasculature, which manifest with pulmonary hypertension (PH) and impaired gas exchange [1]. Even though PVOD and PCH are currently classified together in group 1' of the Nice classification of PH, it is still debated whether PVOD and PCH

represent distinct manifestations of the same underlying disorder [1]. Familial cases of isolated PCH without the manifestation of PVOD indicate differences between a hereditary (primary) and a secondary type of PCH, associated with PVOD [2]. PVOD is characterized by fibrotic narrowing of the pulmonary veins, while PCH shows circumscript proliferation of capillaries within the alveolar septae with concomitant hemorrhage. Despite recent advances including the discovery of genetic mutations associated with the development of PVOD such as *eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4)* mutations, the pathogenesis of PVOD remains poorly understood and even the architecture of the vascular remodeling present has not been fully investigated, so far [3,4]. Here, we undertake for the first time a comprehensive analysis of PVOD by microvascular corrosion casting, three-dimensional (3D) scanning electron microscopy (SEM), μ -computed tomography (μ -CT), correlative (immuno-)histochemical staining and gene expression analysis from the lungs of a 53-year-old female patient with PVOD and PCH.

Materials and methods

Patient characteristics

The patient presented in 2010 with exertional dyspnea and hypoxemia and was diagnosed with PH *via* right heart catheterization showing a mean pulmonary arterial pressure of 30 mmHg, a pulmonary arterial wedge pressure of 7 mmHg and a pulmonary vascular resistance of $287 \text{ dyn s cm}^{-5}$. At that time, treatment with endothelin receptor antagonists and phosphodiesterase-5-inhibitors was initiated. Because of worsening hypoxemia and progressive centrilobular ground glass opacities in CT scans, a thoracoscopic lung biopsy was performed in 2012, which confirmed the clinical diagnosis of PVOD: histopathological examination revealed focal (sub)total obliteration of small pulmonary veins and concomitant sclerosis of pulmonary arteries without the formation of concentric or plexiform lesions. High resolution CT in 2016 showed dilated pulmonary arteries without pruning of peripheral pulmonary vessels. Moreover, centrilobular ground glass opacities and interstitial thickening were found evenly distributed over both lungs (Figure 1). In 2017, the patient underwent bilateral lung transplantation because of end-stage PH. Preparation of fresh human lung explants was carried out as described previously [5].

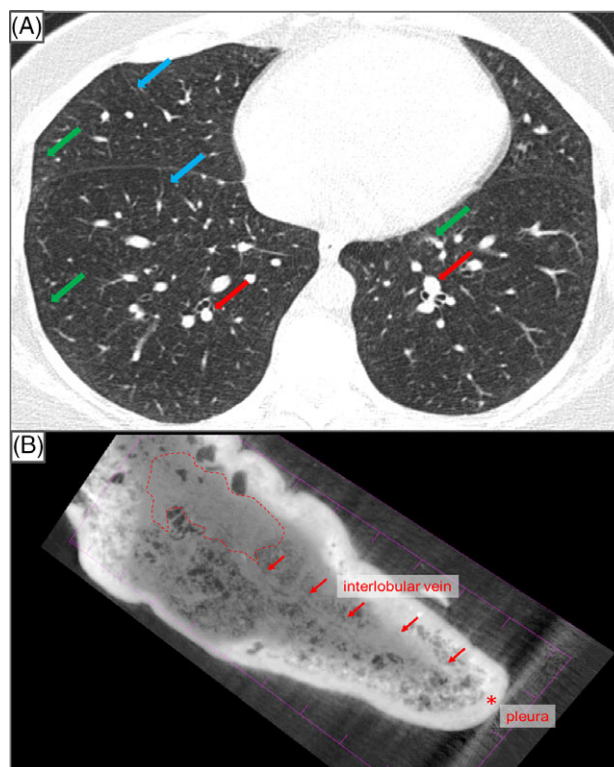


Figure 1. (A) High-resolution computed tomography of the patient's thorax shows dilated main branches of the pulmonary arteries without pruning of peripheral pulmonary vessels (red arrows). Sparse centrilobular ground glass opacities (green arrows) and interstitial thickening (blue arrows) are distributed homogeneously over both lungs. (B) μ -CT of PVOD and PCH showing thickened interlobular septa with subtotally obliterated pulmonary veins next to a dense opacified area which likely represents capillary proliferation. The lung parenchyma shows mild interstitial fibrosis and enlarged alveoli. Arrows indicate an interlobular vein. The dotted red line delineates an area probably consisting of PCH.

The study was designed and performed following the requirements of the local ethics committee at MHH (Ethics vote no. 2702-2015).

Conventional light microscopy

For (immuno)histochemical staining formalin-fixed and paraffin-embedded (FFPE) tissue was cut to $1 \mu\text{m}$ thick slices using a conventional microtome. Staining, including hematoxylin–eosin (H&E), Elastica van Giesson (EvG), CD34, smooth muscle actin, CD31 and podoplanin was performed following our established protocols. Conventional histopathological assessment was then performed at a routine diagnostic light microscope.

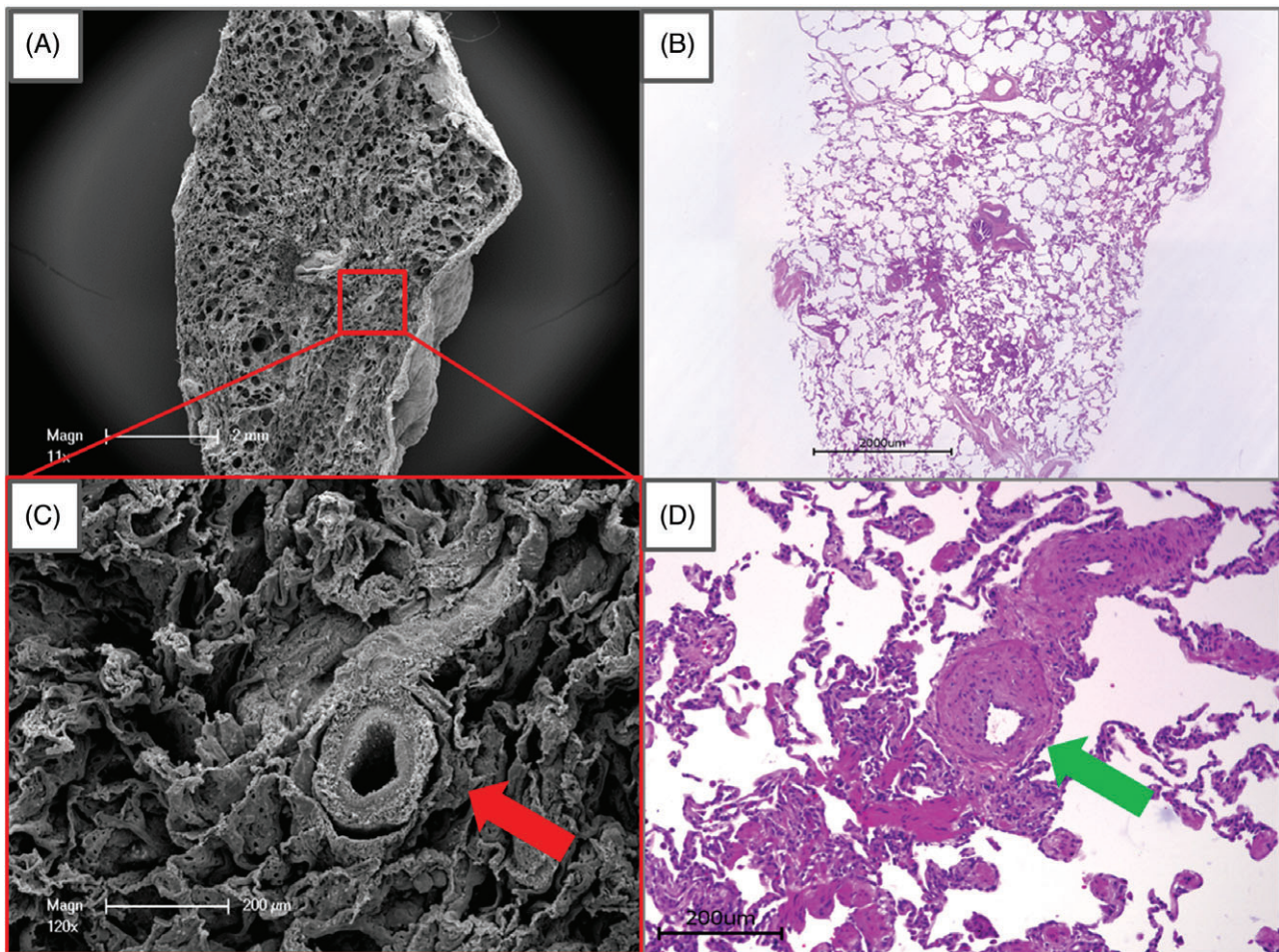


Figure 2. Structure and architecture of PVOD and PCH. Cross-sectional view of the lung parenchyma with mosaic-like, mild interstitial fibrosis and focal rarefaction of alveolar septae in (A) a SEM and (B) complementary H&E staining. Arrows indicate hypertrophic remodeling of the intima and media of a pulmonary vein in a septum by (C) SEM and (D) H&E staining.

Vascular corrosion casting and SEM

In brief, at the time of tissue collection, afferent vessels were cannulated with an olive-tipped cannula. The vasculature was flushed with saline (at body temperature) followed by glutaraldehyde fixation solution (2.5%, pH 7.4, Sigma Aldrich, Munich, Germany). Fixation was followed by injection of prepolymerized PU4ii resin (VasQtec, Zurich, Switzerland) mixed with hardener (40% solvent) and blue dye as casting medium. After curing of the resin, the lung tissue was macerated in 10% KOH (Fluka, Neu-Ulm, Germany) at 40 °C over 2–3 days. Specimens were then rinsed with water and frozen in distilled water. The casts were freeze-dried, cut and mounted on specimen holders, sputtered with gold in an argon atmosphere and examined using a Philips ESEM XL-30 scanning

electron microscope (Philips, Eindhoven, Netherlands) [6].

Microcomputed tomography

High resolution μ -CT analysis was obtained using a Bruker Skyscan 1176 μ -CT scanner. FFPE specimen scanning was performed using the following settings: 50 kV tube potential, 0.5 mm aluminum filtration, 500 μ A tube current, 0.18° rotation step over rotation of 180° and a detector pixel size of 8.6 μ m (spatial resolution), applying a frame averaging of 5. Volume image data (2667 slices of 3396x3396 pixels² each) was reconstructed with NRecon (v1.7.0.4: Bruker Biospin, Rheinstetten, Germany) and analyzed with CTVox: (v. 3.1.1: Bruker Biospin). Volumetric

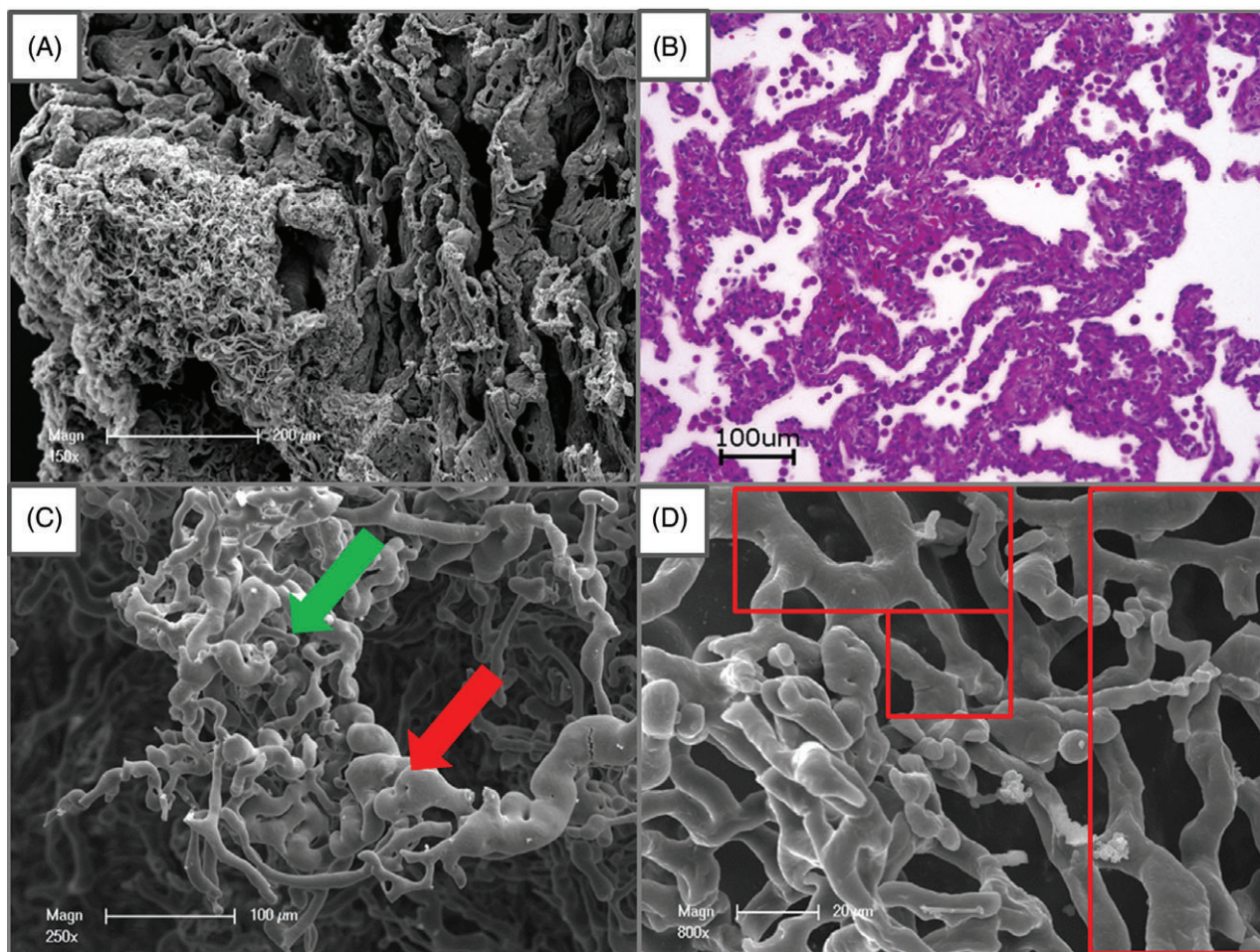


Figure 3. Structure and architecture of PVOD and PCH. Prominent and back-to-back proliferation of capillaries in an area with PCH (A) by SEM and (B) in a complementary H&E stain. (C,D) SEM following microvascular corrosion casting of affected lung tissue reveals capillary neo-formation by sprouting (green arrow, C) and intussusceptive vascular pillars (red arrow, C) next to non-remodeled pulmonary capillaries (red frames, D).

analysis was conducted using CTAn (v.1.15.4: Bruker Biospin).

Results

Histopathological and 3D examination of the patient's lung explants demonstrated, in addition to extensive sclerosis of the pulmonary veins, prominent remodeling of the pulmonary arteries with the formation of concentric lesions and extensive capillary hemangiomatosis (PCH), confirming the diagnosis of PVOD accompanied by PCH. Pulmonary veins and venules showed section by section narrowing by extracellular matrix deposition and smooth muscle proliferation in the intima and media (Figures 2 and 3). Sparse

lymphohistiocytic infiltrates could be found in the perivascular, widened connective tissue (see supplementary material, Figure S1). PCH areas showed a diffuse proliferation of back-to-back capillaries, with concomitant luminal narrowing of the adjacent veins/venules. In the capillary-enriched PCH areas, there was multifocal vascular sprouting and intussusception (Figures 2 and 3 and see supplementary material, Figures S2 and S3), a vascular process defined by (1) formation of intravascular laminae which (2) subdivide pre-existing capillaries or small vessels [7]. Furthermore, CD34/CD31-positive capillary channels within the proliferating PCH lesions were surrounded by sparse podoplanin-positive lymphatic vessels (see supplementary material, Figure S1).

Complementary analysis of gene expression using the nCounter[®] Analysis System (NanoString Technologies,

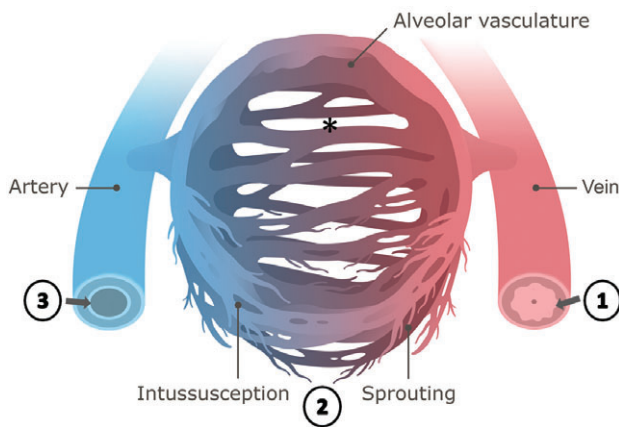


Figure 4. Pathogenesis of vascular remodeling in PVOD. (1) Marked fibrosis of the intima and hypertrophy of the media lead to occlusion of the pulmonary postcapillary vasculature. (2) Venous occlusions cause increasing blood pressure in the pulmonary capillaries (*). Pressure overload induces excessive neoangiogenesis by sprouting and intussusceptive pillar formation likely driven by increased flow and shear stress, which results in the formation of PCH. (3) Congestion of the pulmonary capillary and postcapillary vasculature result in pulmonary hypertension associated with subsequent sclerotic remodeling of pulmonary arteries and arterioles.

Seattle, WA, USA) in further PVOD lung explants, archived in our Institute of Pathology, as compared to controls, revealed increased expression of *glucose-6-phosphate dehydrogenase (G6PD)* (P value = 0.033598), increased expression of *tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1)* (P value = 0.005339), decreased expression of *matrix metalloproteinase 9 (MMP9)* (P value = 3.58E-05), decreased expression of *C-X-C chemokine receptor type 2 (CXCR2)* (P value = 0.000108), decreased expression of *C-X-C chemokine receptor type 1 (CXCR1)* (P value = 8.45E-05), and decreased expression of *angiopoietin-1 receptor (TEK)* (P value = 0.177383) (see supplementary material, Figure S4).

Discussion

PVOD remains as a life-threatening cause of PH with a lack of definite curative therapies, except for lung transplantation [1]. Therefore, the underlying morphological changes and disease mechanisms need to be investigated to improve therapeutic approaches for patients suffering from this rare form of post-capillary vascular remodeling. Among the vascular alterations found in the PVOD spectrum of disease, PCH is likely

to represent mostly a reaction to an injury process induced by postcapillary obstruction, and developing secondary to venous occlusions, a hypothesis supported by the observation of PCH lesions in patients with post-capillary PH [8,9]. Venous occlusions change the vascular dynamics in the capillaries, increase shear and stretching forces and therefore induce pre-obstruction neoangiogenesis at this '*locus minoris resistentiae*' (Figure 4). Correspondingly, we found alterations in shear stress-associated genes in human PVOD lung explants as compared to healthy controls (see supplementary material, Figure S4): here, shear stress appears to shift vascular homeostasis to neoangiogenic and proliferative remodeling, as indicated by increased expression of *G6PD*, *TIE1* and decreased expression of *MMP9*, *CXCR2*, *CXCR1*, and *TEK* in PVOD.

In-vitro cell culture experiments have shown that increased laminar shear stresses can downregulate the gene expression of *CXCR1* and *CXCR2* in human EA-hy926 cells, suggesting that downregulation of *CXCR1* and *CXCR2* influence the abnormal endothelial cell migration present in PVOD [10]. Moreover *MMP9* signaling, which is affected in PVOD, is known to prevent extracellular matrix remodeling in human saphenous veins exposed to high pressure [11]. Also, alterations in *TIE1* and *TEK* signaling are associated with atherosclerosis, hypoxia and neovascularization and may demonstrate a proangiogenic microenvironment in pulmonary vascular remodeling [12]. Furthermore, *G6PD* inhibition leads to vascular smooth muscle cell contraction and seems to change flow dynamics in PVOD in this way [13].

The morphologic correlate of this abnormal gene regulation is probably sprouting and especially intussusceptive neoangiogenesis, as outlined above, which has recently been described as a mechanism capable of changing intraluminal flow dynamics [14]. Furthermore, perivascular and interstitial fibrosis due to chronic inflammation in PVOD appears also to further induce intussusceptive neoangiogenesis [14]. Hereby, extraluminal inflammation is thought to induce proliferation of smooth muscle cells, (myo)fibroblasts and endothelial cells and contribute to narrowing and occlusion of pulmonary veins and venules. Pulmonary capillaries might therefore compensate for the resulting intraluminal increase of shear forces by intussusceptive neoangiogenesis, triggered by mechanosensors. This phenomenon of pulmonary microvascular neoangiogenesis has recently been described in chronic thromboembolic PH (CTEPH), particularly in plexiform lesions, which represent complex glomeruloid (neo) formations of proliferating vascular channels [6].

Herein, CD34-positive endothelial progenitor cells (EPCs) migrate into small pulmonary vessels and are thought to drive intussusceptive vessel formation. This suggests that PCH due to PVOD could be the morphologic analog of the formation of plexiform vasculopathy in the context of PAH with presumably similar underlying disease mechanisms.

Moreover, we observed dilated bronchial vessels in our PVOD patient, suggesting that these *vasa privata* might (incompletely) compensate pressure overload in the pulmonary circulation. In contrast, isolated PCH, which can occur on its own e.g. in rare familial cases, represents another entity altogether that develops independently from PVOD [2].

In summary, we here present intussusceptive angiogenesis in PVOD with secondary PCH for the first time. This form of neoangiogenesis may turn out to be the key process of progressive vascular remodeling as a reaction to injury pattern in this rare and potentially fatal pulmonary vascular disease and consequently may serve as a basis for further investigations.

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Author contributions statement

LN generated the hypotheses, developed the experimental design and concept of the study, histologically evaluated human PAH lung tissue samples, supervised experimental work and data analysis, wrote, edited and revised the manuscript for important intellectual content. PB generated the hypotheses, histologically evaluated human PAH lung tissue samples, performed experiments, performed data analysis, wrote, edited and revised the manuscript for important intellectual content. HS supported clinical data and revised the manuscript. Friedemann Linz performed vascular casting and electron microscopy and revised the manuscript. WLW assisted in the microcomputed imaging and revised the manuscript. GW supported clinical data and revised the manuscript. Florian Laenger

revised the manuscript for important intellectual content. AH supported clinical data and revised the manuscript. HS performed the gene expression analysis of the NanoString® data and revised the manuscript. MMH supported clinical data and revised the manuscript. MK supervised experimental work, supervised data analysis and revised the manuscript for important intellectual content. MA developed the experimental design and concept of the study, supervised experimental work and data analysis, wrote, edited and revised the manuscript for important intellectual content. DJ generated the hypotheses, developed the experimental design and concept of the study, supervised experimental work and data analysis, wrote, edited and revised the manuscript for important intellectual content.

References

1. Galie N, Humbert M, Vachiery J-L, *et al.* ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Heart J* 2016; **37**: 67–119.
2. Langleben D, Heneghan JM, Batten AP, *et al.* Familial pulmonary capillary hemangiomatosis resulting in primary pulmonary hypertension. *Ann Intern Med* 1988; **109**: 106–109.
3. Eyries M, Montani D, Girerd B, *et al.* EIF2AK4 mutations cause pulmonary veno-occlusive disease, a recessive form of pulmonary hypertension. *Nat Genet* 2014; **46**: 65–69.
4. Lantuejoul S, Sheppard MN, Corrin B, *et al.* Pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis a clinicopathologic study of 35 cases. *Am J Surg Pathol* 2006; **30**: 850–857.
5. Länger F, Stark H, Braubach P, *et al.* Injury patterns in interstitial lung diseases. *Pathologe* 2018; **39**: 262–271.
6. Ackermann M, Gaumann A, Mentzer SJ, *et al.* Plexiform vasculopathy in chronic thromboembolic pulmonary hypertension. *Am J Respir Crit Care Med* 2017; **196**: e48–e51.
7. Mentzer SJ, Konerding MA. Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. *Angiogenesis* 2014; **17**: 499–509.
8. Montani D, Girerd B, Jaïs X, *et al.* Clinical phenotypes and outcomes of heritable and sporadic pulmonary veno-occlusive disease: a population-based study. *Lancet Respir Med* 2017; **5**: 125–134.
9. Wangi K, Inenaga T, Yamada S. Failure due capillary to aortic stenosis. *J UOEH* 2009; **31**: 339–344.
10. Zeng Y, Sun HR, Yu C, *et al.* CXCR1 and CXCR2 are novel mechano-sensors mediating laminar shear stress-induced endothelial cell migration. *Cytokine* 2011; **53**: 42–51.
11. Longchamp A, Alonso F, Dubuis C, *et al.* The use of external mesh reinforcement to reduce intimal hyperplasia and preserve the structure of human saphenous veins. *Biomaterials* 2014; **35**: 2588–2599.
12. Woo KV, Baldwin HS. Role of Tie1 in shear stress and atherosclerosis. *Trends Cardiovasc Med* 2011; **21**: 118–123.

13. Chettimada S, Joshi SR, Dhagia V, *et al.* Vascular smooth muscle cell contractile protein expression is increased through protein kinase G-dependent and -independent pathways by glucose-6-phosphate dehydrogenase inhibition and deficiency. *Am J Physiol Hear Circ Physiol* 2016; **311**: H904–H912.
14. Filipovic N, Tsuda A, Lee GS, *et al.* NIH Public Access. *Cell Tissue Res* 2010; **78**: 286–293.

SUPPLEMENTARY MATERIAL ONLINE

Figure S1 (Immuno-)histochemical staining of a broncho-vascular bundle

Figure S2 Scanning electron micrographs (SEM) following microvascular corrosion casting in pulmonary veno-occlusive disease, for use with red-green anaglyph glasses

Figure S3 Scanning electron micrographs following microvascular corrosion casting of human tissue showing physiologic alveolar capillaries

Figure S4 Boxplots showing normalized log₂ counts of mRNA expression