

# Intrapulmonary vascular shunt pathways in alveolar capillary dysplasia with misalignment of pulmonary veins

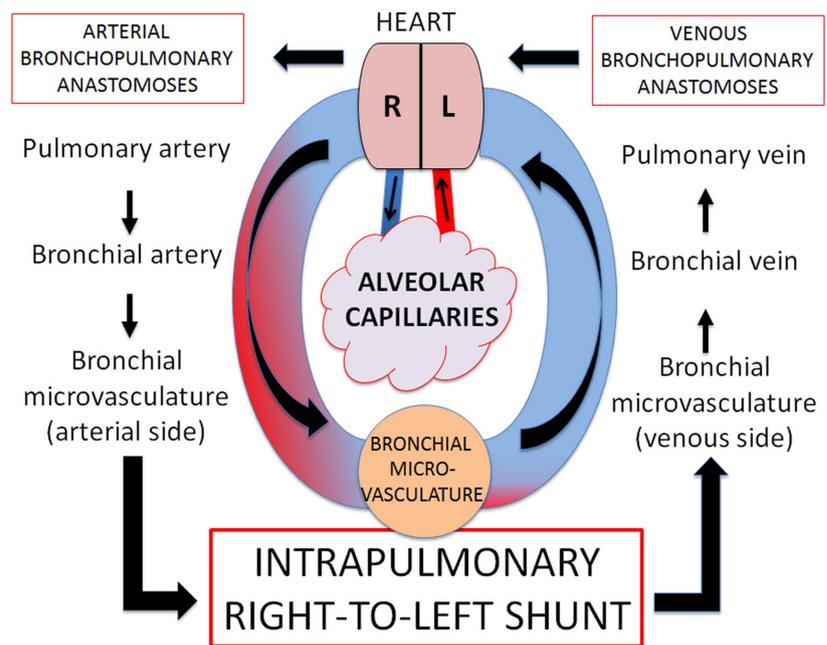
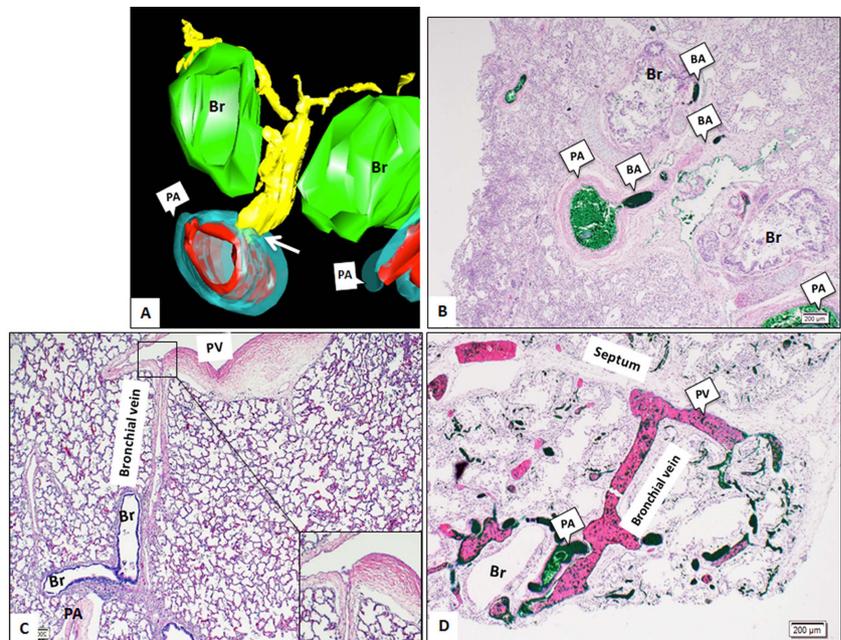
## Abstract

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) is a lethal neonatal lung disease characterised by severe pulmonary hypertension, abnormal vasculature and intractable hypoxaemia. Mechanisms linking abnormal lung vasculature with severe hypoxaemia in ACD/MPV are unknown. We investigated whether bronchopulmonary anastomoses form right-to-left shunt pathways in ACD/MPV. We studied 2 infants who died of ACD/MPV postmortem with direct injections of coloured ink into the pulmonary artery, bronchial artery and pulmonary veins. Extensive histological evaluations included serial sectioning, immunostaining and 3-dimensional reconstruction demonstrated striking intrapulmonary vascular pathways linking the systemic and pulmonary circulations that bypass the alveolar capillary bed. These data support the role of prominent right-to-left intrapulmonary vascular shunt pathways in the pathophysiology of ACD/MPV.

Alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) is a rare and lethal neonatal lung disease associated with severe respiratory failure and pulmonary hypertension.<sup>1</sup> Despite improved respiratory care, infants with ACD/MPV die of severe hypoxaemia after birth.<sup>2</sup> Abnormal vascular lung histology of ACD/MPV includes dysplastic capillaries, hypertensive arterial remodelling, and prominent veins located close to distal airways and arteries ('MPV'). Cardiac catheterisation studies have revealed absent 'capillary blush' phase during angiography in infants with ACD/MPV, suggesting intrapulmonary shunting.<sup>3</sup> However, the contribution of MPV to the pathophysiology of ACD/MPV remains unknown. Precarinal anatomical communications between systemic (bronchial) and pulmonary vascular systems has been extensively described<sup>4</sup>; whether they form an alternate right-to-left vascular pathway bypassing distal lung vasculature in ACD/MPV is unclear. Postmortem injections of pulmonary arteries (PA), bronchial arteries (BA) and pulmonary veins (PV) were performed with coloured inks (green, blue and black, respectively). Histology, immunohistochemistry and three-dimensional reconstructions were done as previously described with slight modification (see supplement). Infants' clinical course and microscopic

pathology was typical of ACD/MPV. Injection of green and blue ink marked the vessels of PA, BA, bronchial vein (BV), PV including the 'MPV' and the bronchial microvessels (mv), while black ink was visible within the PV, BV, mv and 'MPV'

(see online supplements 1–3). No ink filling was seen in alveolar capillaries or lymphatics (see online supplements 4 and 5). MPV bridging mv and PV were frequently noted and contained all three inks (see online supplements 1–3) suggesting that MPV are BV



**Figure 1** Upper panel: Main components of intrapulmonary vascular anastomotic pathways are shown. Three-dimensional image reconstruction (A) and histological sections (B) show direct connections between pulmonary arteries (PA) and bronchial arteries (BA) (white arrow) (BA-yellow, PA-red and blue, Bronchus (Br)-green). In an age-matched control lung, BV and pulmonary veins (PV) connections are present but appear very delicate, almost invisible (C, insert magnifies BV-PV connection). In alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), the bronchial vessels are enlarged, especially the BV (D) appearing as dilated, thin-walled channels within the bronchovascular bundle ('misaligned pulmonary veins'). Lower panel: Schematic of intrapulmonary right-to-left shunt in ACD/MPV lung is shown. The majority of right heart blood is shunted through the arterial bronchopulmonary anastomoses (PA, BA, mv) that drains via venous bronchopulmonary anastomoses (microvessels (mv), BV, PV) leading to significantly decreased flow within the alveolar capillary bed.

and may act as venous 'shunt vessels', as BV normally link mv associated with airways, large vessels, pleura, and lymph nodes with PV (figure 1C).<sup>5</sup> In addition to the expanded venous bronchopulmonary anastomoses we also identified arterial bronchopulmonary anastomoses via PA-BA connections (figure 1, see online supplement 1) as part of intrapulmonary right-to-left to vascular shunt circuit (figure 1). The prominence of these vessels in ACD/MPV is likely due to enhanced flow through blood shunting from PA to systemic vessels that dilate mv and BV, which become visible as extra vessels within bronchoarterial bundles ('MPV'). Thus, we propose that rather than representing 'mal-developed' vessels, MPV are BV that appear markedly dilated due to increased blood flow in PA-BA anastomotic vessels in ACD/MPV. In summary, we report striking intrapulmonary vascular anastomoses in ACD/MPV that could contribute to fatal hypoxaemia due to the shunting of blood away from the alveolar capillary bed.

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**Contributors** CG: designed experiments, performed experiments, analysed the data, drafted and revised the paper. He is guarantor. SS-L: performed, collected and analysed 3D data, reviewed manuscript. NA: collected clinical data, discussed experimental design, reviewed manuscript. JG: collected clinical data, discussed experimental design, reviewed manuscript. MKD: Performed experiments, reviewed manuscript. SHA: designed experiment, reviewed and revised manuscript.

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**Patient consent** Obtained.

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Supplemental Material:

Intrapulmonary Vascular Shunt Pathways in Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins.

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### **Supplement Material- Methods.**

Patient data

Patient demographic data were extracted from the autopsy report and chart.

Ink injections.

After removal of heart-lung block according to routine autopsy protocol, the PA was identified and injected with green ink in Case 1. In Case 2 the BAs were initially isolated after clamping the ductus arteriosus, aortic arch and distal thoracic aorta. The isolated aortic segment containing BA branches was then injected with blue ink. Then the left PA was injected with green ink followed by injection of the right main PV with black ink. This combination of ink injection allowed us to track pathways connected with the PA, and BA separately. Ink (Tissue Marking Dyes, Cancer Diagnostic Inc., Durham, NC, USA) was prepared with 1:1 dilution with tap water to ensure low viscosity, and injection was done with 20 ml syringe equipped with a 22 gauge needle. Once the needle entered the vessel, an additional clamp was used to secure the needle tip to the vessel wall it through which it entered. During BA injection however, some leakage was seen through unclamped intercostal arteries. We also encountered some minor leakage at the site of BV injection likely due to puncturing the more delicate venous wall. Approximately 30 ml ink was injected. The injection lasted approximately 5 minutes and performed with hand applied semi gentle pressure. Ink was never found in the heart excluding extrapulmonary right to left ink shunting.

Histology, immunohistochemistry, and 3D reconstructions were performed as previously described (1) with slight modification.

Histology

Autopsy tissue from 2 patients who died with ACD/MPV was studied. The lungs were inflated with formalin, and all lobes were sampled, including central and peripheral areas. There were 21 HE sections/lung/case 1 and 58 HE sections/lung/case 2; one section/slide. All slides were reviewed and analyzed.

Immunohistochemistry

Immunohistochemistry was performed by routine streptavidin-biotin peroxidase methods using an automated slide stainer (BenchMark; Ventana Medical Systems, Tucson, Arizona). Primary antibodies included CD31 (Clone JC70A, Dako North America, Inc, Carpinteria, CA), SMA (Clone IA4, Dako North America, Inc, Carpinteria, CA) and D2-40 (Dako, Carpinteria, California).

### Serial sectioning

Representative areas with PA-BA connection were serially sectioned at 5-mm intervals and stained with hematoxylin and eosin along with the immunomarkers for CD31, SMA, and D2-40, to identify endothelium, smooth muscle and lymphatics, respectively.

### Three-dimensional reconstruction

A representative area with PA-BA connection was chosen, serially sectioned at 5-mm intervals. A total of 20 sections were obtained and serial stack was prepared with hematoxylin and eosin staining. Sections were placed onto a microscope equipped with software to trace and reconstruct the lung vasculature in 3 dimensions (Stereo Investigator; MBF Bioscience, Williston, Vermont), as described previously (2). Each section was then traced for ink content and distinct vascular and airway elements with selective colors: yellow for bronchial artery, red for pulmonary arterial endothelium, light blue for pulmonary arterial wall muscle, and green for airways. The neighboring section was then aligned and traced. This process continued until the entire stack was completed. The stack of images was then 3D-reconstructed, and images and movies were generated.

### Supplement Material- Figures:

Supplement 1. Injection of ACD/MPV lung with green ink via PA highlights bronchopulmonary anastomotic vascular pathways (Case 1). Green ink is diffusely present in pulmonary arteries (PA) (A, C), bronchial arteries (BA) (A), dilated bronchial microvessels (mv) (B), misaligned pulmonary vein (MPV) (C) and pulmonary vein (PV) (D). Ink does not appear in distal PA vessels. D2-40 immunostaining identifies small lymphatics that do not contain ink (C). Smooth muscle alpha-actin (SMA) immunostaining highlights bronchial and vascular walls (D). Serial sectioning identifies bronchopulmonary arterial channels (BPC) between PA and BA (E,F,G,G'). Ink injected into the PA perfuses smaller channels that neighbor a large airway (C, C', SMA stain). A schematic illustrates the bronchopulmonary anastomotic pathways suggested from the PA ink injection.

Supplement 2. Injection of ACD/MPV lung with green ink via PA and blue ink via BA further highlights bronchopulmonary arterial anastomoses (Case 2, left lung). Both green and blue ink are present in PA (A,B), BA (A,B), bronchial mv (C, D), MPV (E,F) and PV (G,H). Mixing of green and blue ink shows connections between that PA and BA vascular pathways (C, D, E, F, H). A schematic based on findings from separate PA and BA ink injections demonstrate common intrapulmonary vascular pathways linking PA, BA, mv, BV and PV, but not alveolar capillaries.

Supplement 3. Injections of blue and black ink into the BA and PV, respectively, further illustrate anastomotic vascular pathways in ACD/MPV (Case 2, right lung). Blue and black inks are both present in mv (A), while black ink is present in mv (B), MPV (C) and PV (D). Presence of blue and black ink shows that BA and PV vascular pathways intersect (A, insert highlights a mixture of blue and black ink within mv). Schematic of anastomotic pathways based on the findings from BA and PV ink injections is shown.

Supplement 4. Ink injection of ACD/MPV lung via PA and BA reaches the smallest abnormally thickened PA (pa), but not the alveolar capillaries. Green ink injected via PA (A) and blue ink injected via BA (B) is present in pa, but not in the surrounding capillaries indicating no ink flow toward the left heart via alveolar capillaries. Alveolar capillary integrity is not compromised as shown by diffuse CD31 staining in both cases (Case 1—C, Case 2—D). CD-31 immunoreactivity

highlights the pathognomonic feature of dilated and “dysplastic” capillaries within the thick alveolar wall (C,D).

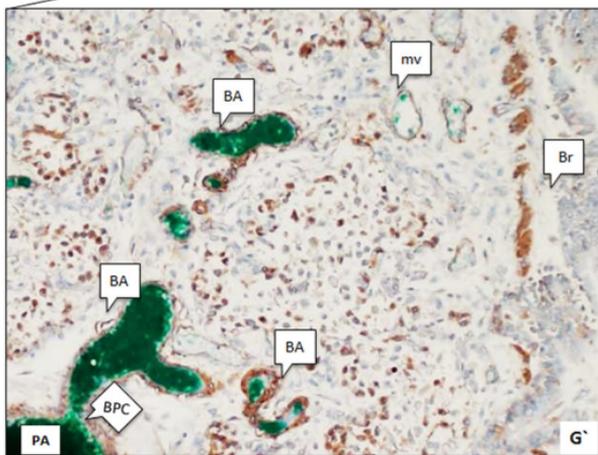
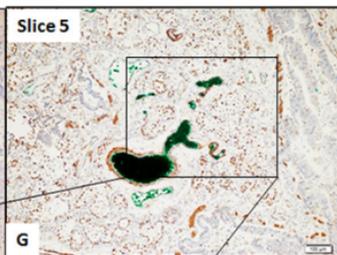
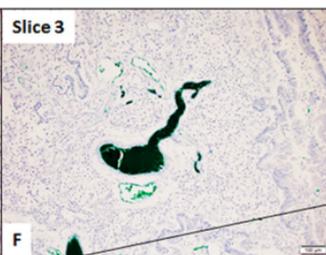
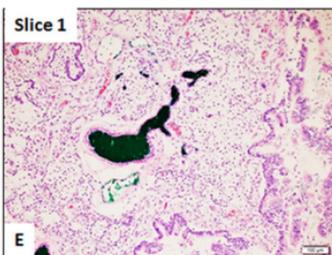
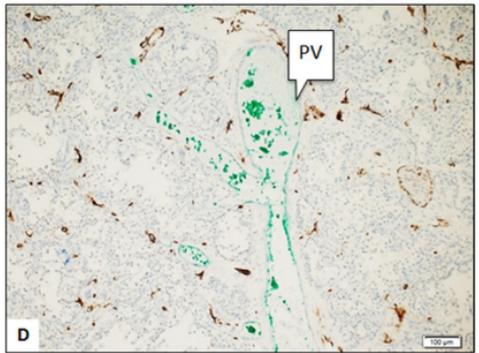
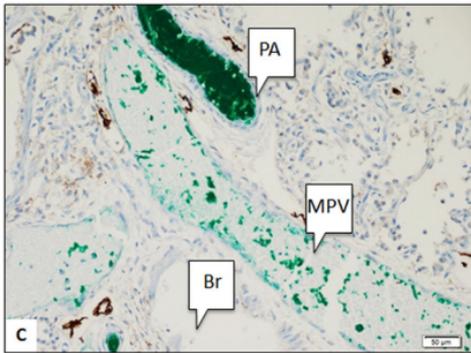
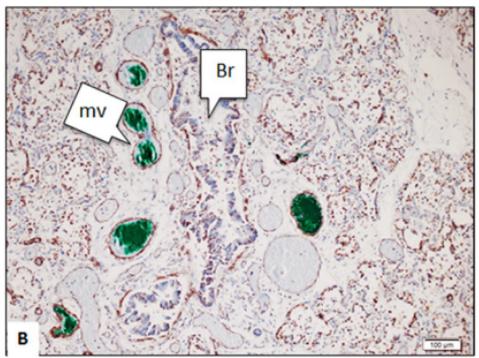
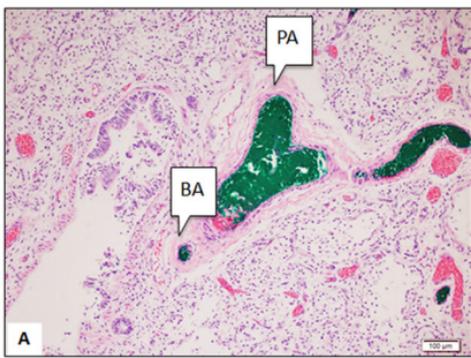
Supplement 5. Lymphatic vessels are not distinct from sites of intrapulmonary anastomoses. Immunoreactivity to D2-40, specific lymphatic immunomarker highlights several channels within the interlobular septum (A, B, D) and around the bronchovascular bundles (C). These findings confirm that ink containing vessels (black ink and green in PV [B,D], blue ink in BV [C]) are not lymphatics.

**Supplement Material - Video.**

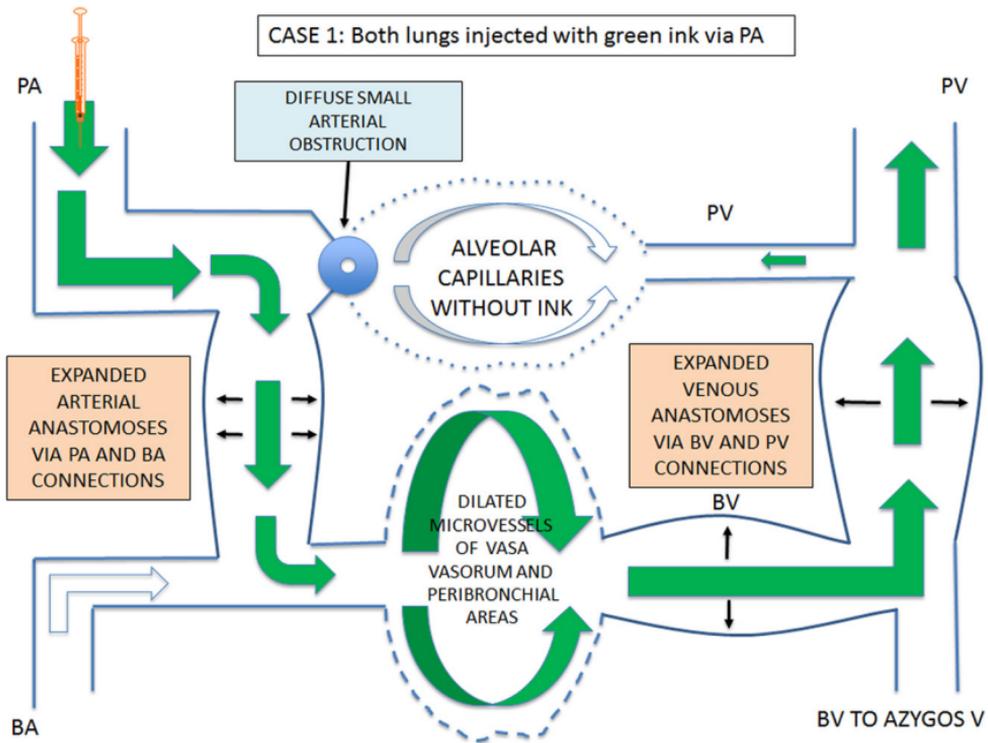
Arterial bronchopulmonary anastomosis is present in ACD/MPV lung between pulmonary and bronchial artery. Video clip generated by 3D reconstruction of stacked microscopic images highlights direct connection between PA and BA (BA-yellow, PA-red and blue, Airway-green).

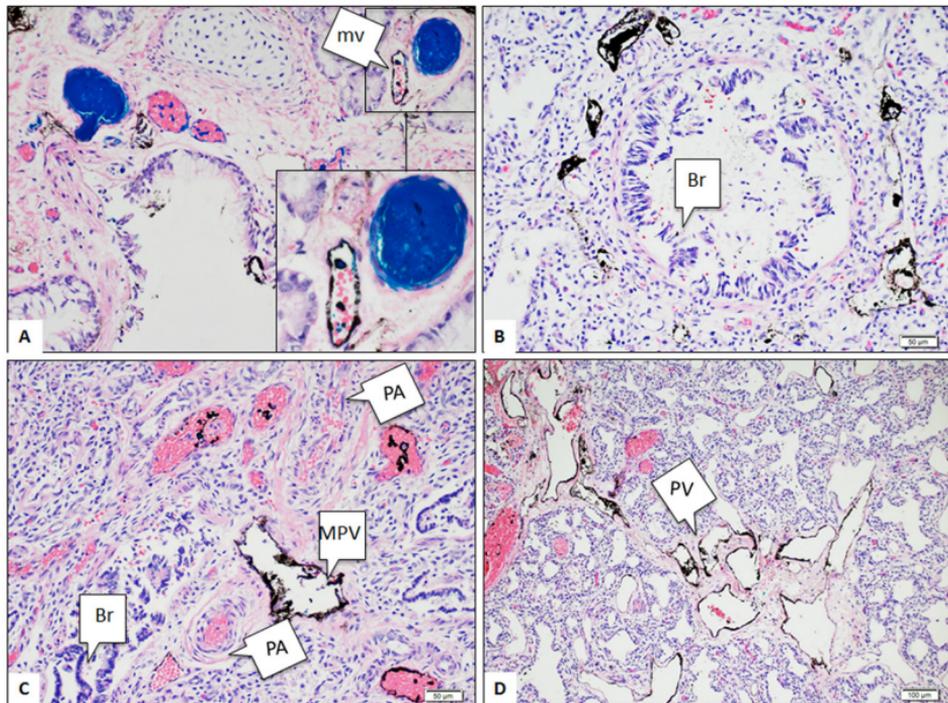
**Reference** for Supplement Data and Method.

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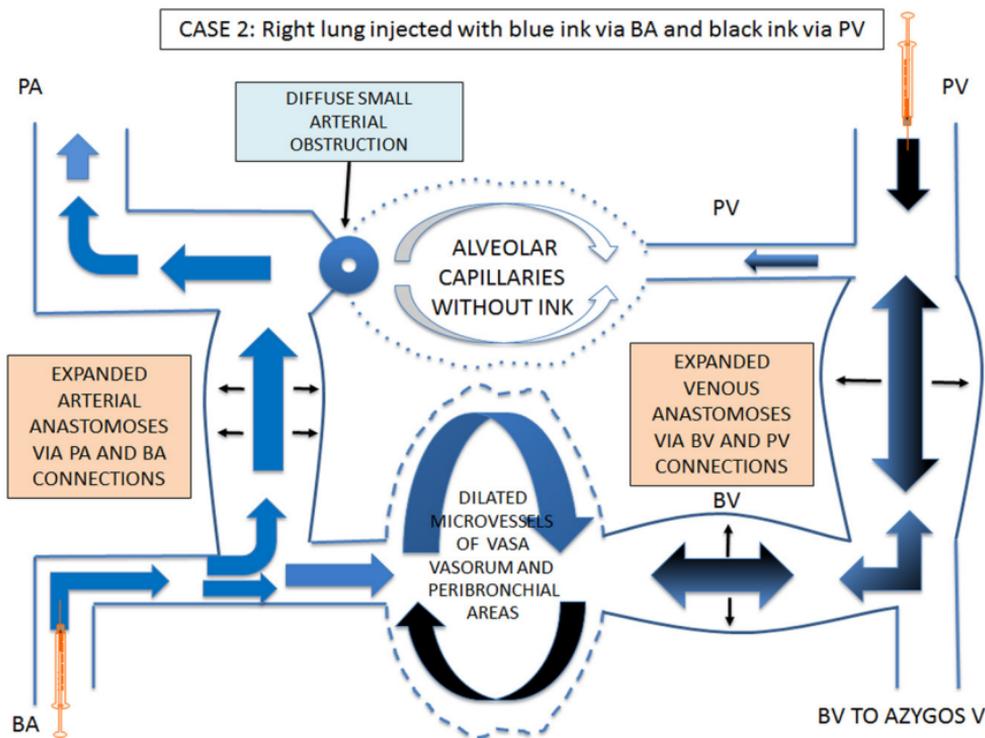


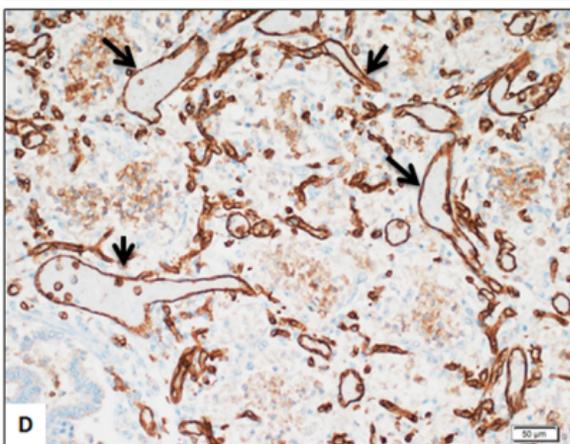
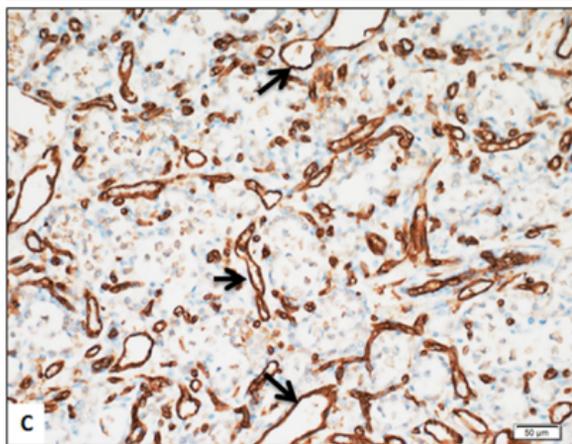
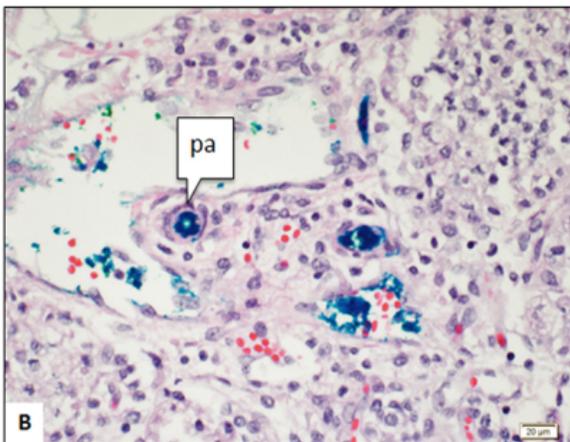
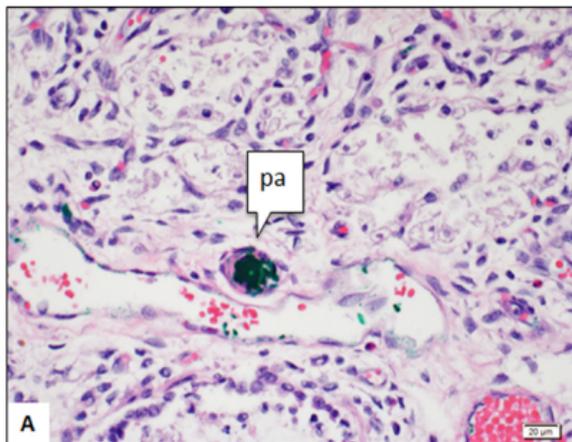
CASE 1: Both lungs injected with green ink via PA

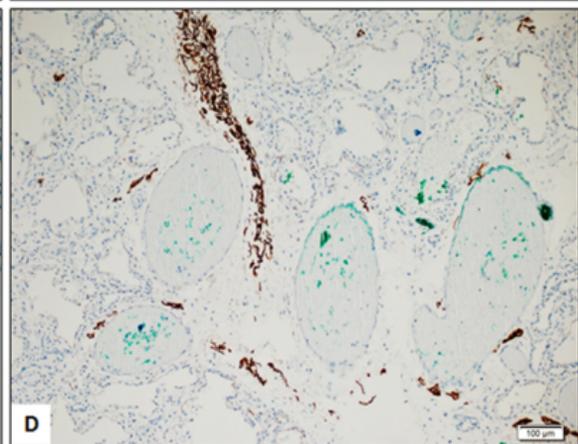
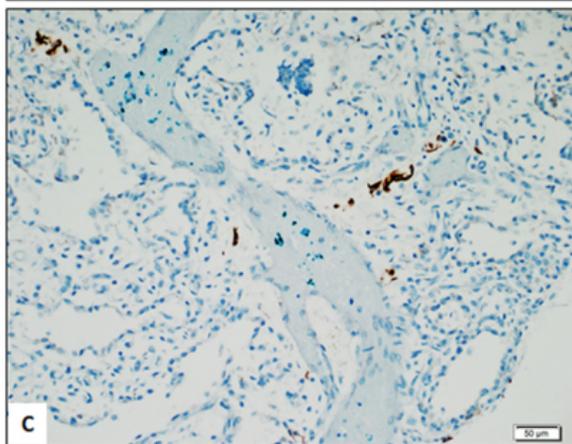
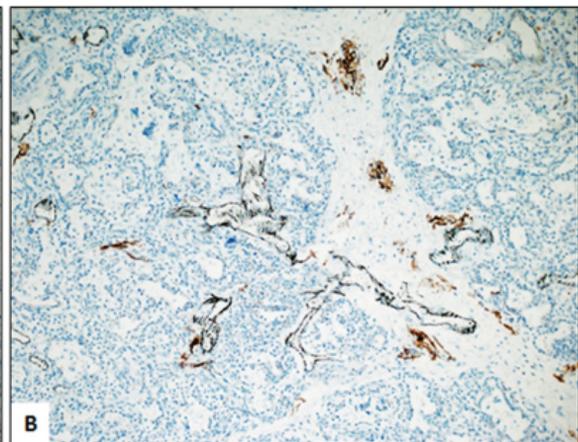
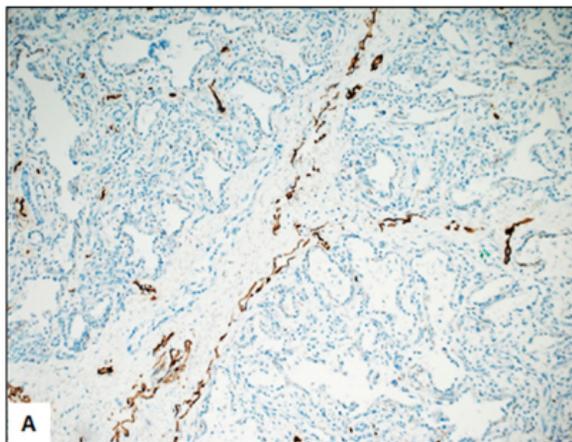


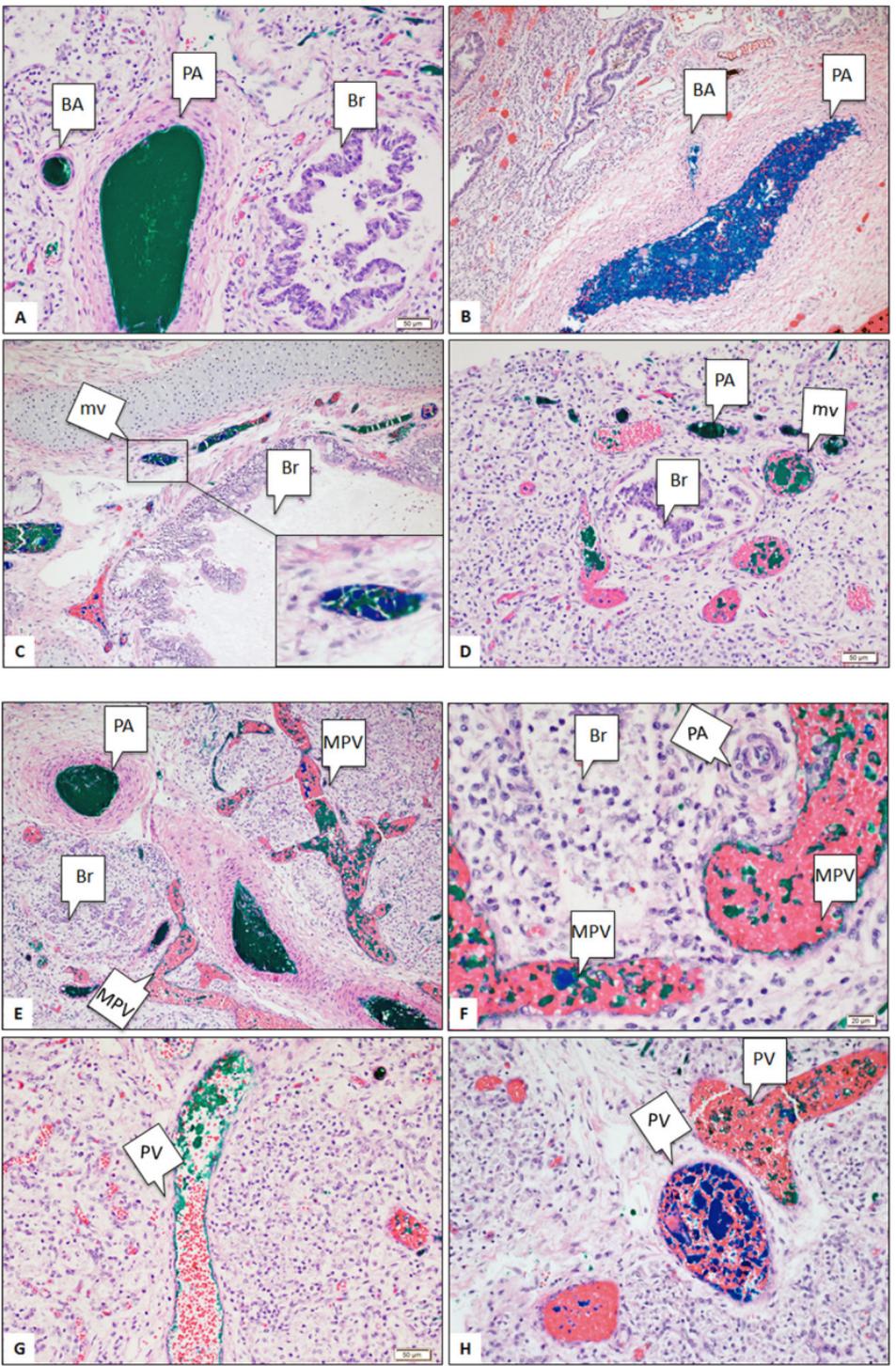


**CASE 2: Right lung injected with blue ink via BA and black ink via PV**









CASE 2: Left lung injected with green ink via PA and blue ink via BA

