Supplement 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 immunreactivity
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.**
CASE 1: Both lungs injected with green ink via PA

DIFFUSE SMALL ARTERIAL OBSTRUCTION

EXPANDED ARTERIAL ANASTOMOSES VIA PA AND BA CONNECTIONS

ALVEOLAR CAPILLARIES WITHOUT INK

EXPANDED VENOUS ANASTOMOSES VIA BV AND PV CONNECTIONS

DILATED MICROVESSELS OF VASA VASorum AND PERIBRONCHIAL AREAS

BV TO AZYGOS V
CASE 2: Right lung injected with blue ink via BA and black ink via PV

DIFFUSE SMALL ARTERIAL OBSTRUCTION

EXPANDED ARTERIAL ANASTOMOSES VIA PA AND BA CONNECTIONS

DILATED MICRONECRESSES OF VASA VASORUM AND PERIBRONCHIAL AREAS

ALVEOLAR CAPILLARIES WITHOUT INK

EXPANDED VENOUS ANASTOMOSES VIA BV AND PV CONNECTIONS
CASE 2: Left lung injected with green ink via PA and blue ink via BA