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Effect of Perflubron-Induced Lung Growth on Pulmonary Vascular Remodeling in Congenital Diaphragmatic Hernia

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Introduction

Congenital diaphragmatic hernia (CDH) affects approximately 1 in 2500 live births [1]. The morbidity and mortality associated with the disease are largely due to both pulmonary hypoplasia and pulmonary hypertension (PH). Although overall survival has improved to 70%, the most severe CDH patients suffer disproportionate mortality and long term morbidity [2, 3].

Many innovative strategies have been developed to address pulmonary hypoplasia in patients with severe CDH using the principles of mechanical transduction. Prenatally, fetoscopic tracheal occlusion has been shown to enhance lung growth by preventing egress of lung fluid and providing an intratracheal distending force. This approach is promising, and is being studied in a well-designed prospective randomized clinical trial (TOTAL trial) [4]. Postnatal mechanical transduction to stimulate lung growth has been performed using Perflubron-induced lung growth (PILG) [5]. Perflubron is a perfluorocarbon liquid which has been used for gas exchange (partial liquid ventilation and total liquid ventilation). It is used solely as a distending agent in the PILG technique. The PILG technique requires ECMO support for the patient. The Perflubron is instilled in the endotracheal tube until a meniscus is seen, and a constant pressure of 8 cm of H_2O is applied using a CPAP machine. Experimental studies in the neonatal lamb have demonstrated lung growth with alveolar proliferation using Perflubron as a distending agent [5]. Furthermore, experimental studies in piglets have demonstrated increased lung DNA synthesis after perfluorocarbon distention [6]. A pilot clinical study of CDH patients requiring ECMO demonstrated that one week of PILG improved survival [7]. Since evidence from the laboratory indicated that 3 weeks of distention are required to promote maximal lung growth, another prospective randomized clinical study was performed [5]. In this study, although PILG induced impressive lung growth, it did not ameliorate pulmonary hypertension and there was a higher mortality rate than conventional therapy [8].

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It is known that CDH patients have pathologic remodeling of the pulmonary vasculature that occurs during gestation and in the immediate postnatal period [9]. Pathologic remodeling of the pulmonary vascular tree, also known as pulmonary vascular remodeling, is a complex process that involves many cell types within the vascular wall, complex intercellular signaling, and alterations in the extracellular matrix of the pulmonary vasculature [10]. Extensive pulmonary vascular remodeling can lead to severe pulmonary hypertension. One of the key alterations that leads to the hypertensive state within the blood vessels is a change in the composition of the extracellular matrix that leads to an increase in collagen and decrease in elastin, altering the quantitative relationship between the two proteins in the vessel wall [11, 12]. The increase in collagen in the vessel wall alters vessel wall function by increasing the stiffness of the pulmonary arteries, contributing to PH [13]. Furthermore, there is hypertrophy and increased accumulation of smooth muscle cells in the media of vessel walls [14], contributing to increased cell proliferation in the vessel walls. This hypertrophy and accumulation leads to thickened vessel walls and decreased vessel lumens, further contributing to PH. Studies have also shown that there is an increased systemic inflammatory response in patients with CDH [15, 16], however the relationship between this and pulmonary vascular remodeling is still unclear. Unfortunately, therapies for the PH of CDH have not been developed because the mechanisms driving the pulmonary vascular remodeling that leads to the development of PH in CDH remain unknown. Studies have shown that more severe PH is associated with worse survival, but currently CDH patients are managed with ventilator strategies, ECMO, and pharmacologic measures that are all only supportive in nature [17]. Although there are other factors that play an important role in PH, such as vasoconstriction, in this study we focus on the histology of vessel remodeling because the process of vessel wall remodeling is not understood, and currently there are no therapies to reverse pulmonary vascular remodeling.

Although PILG stimulates lung growth in CDH patients, the effect on pulmonary vascular remodeling and the PH component of CDH remains unknown. We aim to determine the effect of PILG on pulmonary vascular remodeling in neonates with CDH requiring extracorporeal membrane oxygenation (ECMO)

Methods

Lung tissue samples were obtained from deceased patients enrolled in a prospective randomized trial of PILG in neonates with CDH at the University of Michigan [8]. There was a total of 16 patients in this original study that were randomized to control (conventional mechanical ventilation + ECMO) or treatment (PILG + ECMO) groups. A total of four patient tissue samples were available and all were analyzed. Three samples are from patients treated with PILG + ECMO, and one was treated with conventional mechanical ventilation + ECMO (control). All four patients eventually succumbed to PH, as documented by their final echocardiogram prior to death. Demographic information was collected on all patients and the mean value was calculated for each item in the PILG group where possible. The probability of survival was determined by using the CDH Study Group equation [18]. Liver position was also noted as a factor related to severity of clinical course in CDH.

The lung tissue was preserved in paraformaldehyde and embedded into paraffin. Lung tissue was cut into 5 micrometer sections and placed on slides for histologic evaluation. Tissue was stained with Masson's trichrome stain in order to assess for collagen distribution in the vascular wall and peri-vascular space. Increased collagen deposition in the vessel wall is a component of pulmonary vascular remodeling. Overall collagen thickness as well as the presence or absence of collagen in the media and adventitia was assessed in order to evaluate for vascular remodeling.

Immunohistochemistry was performed with an antibody against phosphorylated Histone H3 protein (pH3b) to assess cell proliferation in the vascular wall. Rabbit antibody for pH3b was used at a 1:500 dilution (Cell Signaling #9701, Danvers, MA) with a secondary goat anti-rabbit antibody at 1:500 dilution (Jackson #111-065-144, Westgrove, PA). Immunohistochemistry with antibody against macrophage antigen F4/80 was performed to determine macrophage levels in the vascular wall. Rat antibody for F4/80 was used at a 1:100 dilution (AbD Serotec #MCA497, Raleigh, NC) with a secondary goat anti-rat antibody at 1:500 dilution (Jackson #112-065-167, Westgrove, PA).

Immunofluorescence analysis was performed to specifically evaluate the morphometry of the pulmonary vasculature. The slides were double stained with rabbit antibodies for CD31 (1:100 dilution; Abcam AB28364, Cambridge, MA) and mouse antibody for alpha-smooth muscle actin (1:400 dilution; Dako M0851, Carpinteria, CA). Secondary antibodies were used for detection by immunofluorescence (1:500 dilution) with a goat anti-rabbit AF 488 and a goat anti-mouse AF594 respectively (Invitrogen, Waltham, MA). Slides were visualized and images acquired with Olympus BX61 epifluorescence microscope, and the images were analyzed using Image-J software (National Institutes of Health). 20x images were used to perform total vessel counts of each sample. Vessels were counted in 15 high powered fields (HPF) per lung specimen. Higher power images at 40x were focused on vessels 20-75 um in diameter to capture arterioles. Pulmonary vasculature was identified by the presence of both CD31 and alpha-smooth muscle actin. For each patient, the vessel wall diameter (calculated as vessel wall thickness), lumen diameter, total area, and number of smooth muscle cell layers was calculated. Calculations were also performed to determine the ratio of lumen diameter to vessel wall diameter for each vessel.

Once the raw data was calculated, a mean for each sample was determined for the characteristics of analysis. Calculations were performed using STATA13 and Microsoft Excel software. Due to the fact that only one control sample was available, formal statistical analysis was not possible. The mean from each sample was individually compared to control, but significance was not determined. The data derived are descriptive in nature.

Results

Two patients were female, including the control patient, and two male. Average birthweight in the PILG group was 2.92 kgs. (range 2.51 to 3.15) versus 3.29 kgs. in the control. Estimated gestational age was 38, 38, and 39 weeks for the three PILG patients, PILG A, PILG B, and PILG C, respectively, and 40 weeks for the control patient. The mean days of life at death in the PILG group was 35 (range 23-51) vs. 135 in the control. All four patients

succumbed to PH, as documented by final echocardiogram prior to death. Mean number of days on ECMO was 20 (range 11-28) in the PILG group vs. 18 in the control. Probability of survival was lower in the PILG group as all three had the liver in the chest when the control did not; furthermore, the calculated probability of survival based on the CDHSG equation was 9% and 40% in two out of three PILG patients and 67% in the control. The third PILG patient was born by EXIT; thus, probability could not be calculated.

To determine changes in the extracellular matrix of the pulmonary vasculature, we evaluated lung samples with Masson's Trichrome stain to assess collagen distribution. Histologic evaluation demonstrates the presence of collagen in the media and adventitia of the vessel wall in PILG and control samples. However, there is a larger distribution of collagen in the PILG patients versus control, and more specifically, accumulation of collagen in the adventitia of the vessel wall (Fig. 1a). When systematically measured at the level of arterioles (20-75 μm in diameter), collagen thickness of the vessel walls was greater in 2 of 3 of the PILG patients compared to the control (PILG: 14.23, 35.66, and 38.46μm versus control: 22.16μm) (Fig. 1b).

To measure another facet of pulmonary vascular remodeling, cell proliferation was evaluated. PILG patients A, B and C had fewer nuclei stained with pH3b when compared to the control patient, with an average of 2.93, 3.13, and 2.53 nuclei per HPF versus 7.13 nuclei per HPF, respectively (Fig. 2). This indicates that there was lower cell proliferation present in the lungs of the PILG patients.

To evaluate infiltration of inflammatory cells into the pulmonary vasculature, macrophage invasion into the vessel wall was evaluated as a surrogate for the degree of inflammatory cell invasion in the lungs and pulmonary vessels. PILG had fewer macrophages per high powered field when compared to the control patients. (PILG: 1.2, 1.8, and 0.5 mean number of macrophages/HPF vs. control: 4.9 mean number of macrophages/HPF) (Fig. 3).

To better evaluate changes that occur in the pulmonary vasculature during pulmonary hypertension, vessel and vessel wall morphometry were evaluated. Mean vessel wall diameter (calculated as vessel wall thickness) was increased in all PILG patients compared to the control (PILG: 6.86, 10.28, and 10.35μm versus control: 4.35 μm) (Table 2). Vessel lumen diameter was decreased in PILG patients compared to the control patient (PILG: 48.99, 41.75, and 36.2μm versus control: 51.56μm) (Table 2). To make sure the vessel size was appropriately matched across patients, total vessel area in each patient was assessed and they were similar (Data not shown). Lumen to vessel wall diameter ratio for each patient was determined. The ratio was decreased in each PILG treated patient compared to the control (PILG: 7.14, 4.06, and 3.49 versus control: 11.85) (Table 2). This indicates a higher degree of pulmonary vascular remodeling in PILG patients.

To assess for smooth muscle cell hyperplasia typically seen in pulmonary vascular remodeling, smooth muscle cell layers were quantitated. An increased number of smooth muscle cell layers was present in the PILG pulmonary vessels. (PILG: 2.47, 2.8 and 2.8 layers vs. Control: 2.13 layers) (Table 2 and Fig. 4). Finally, the total number of vessels was determined in all four patient samples. There was a slight increase in the total number of

vessels per 20x HPF in the PILG patients when compared to the control (PILG: 4.4, 5.7, and 4.6 vessels per HPF vs. control: 4 vessels per HPF).

Discussion

Despite advances in mechanical ventilation and ECMO, PH in CDH remains difficult to manage as current available therapies are merely supportive in nature. PILG has been shown to be an innovative strategy to promote lung growth in order to address the pulmonary hypoplasia component of CDH. Animal studies show a link between airway growth and blood vessel growth in the lung [19]; thus, it is presumed that airway growth from Perflubron therapy may also increase blood vessel growth and ameliorate pulmonary hypertension in CDH. However, despite promoting lung growth, 50% of patients died in the PILG arm of the clinical study due to suprasystemic pulmonary hypertension [8]. The effect of PILG on pulmonary vascular remodeling has previously not been studied.

In our study, it appears that PILG does not decrease the pulmonary vascular remodeling that leads to PH in CDH patients. The three PILG treated patients in our study had increased collagen thickness, increased vessel wall diameter, decreased vessel lumen, decreased lumen to vessel wall diameter ratio despite similar total vessel area, and increased number of smooth muscle cell layers when compared to the control patient treated with conventional mechanical ventilation + ECMO alone. This suggests a higher degree of pulmonary vascular remodeling in the PILG patients compared to the control. This is likely related to the fact that the PILG patients had a more severe form of CDH, as evidenced by all three having liver present up in the chest, compared to the control, who did not. Presence of "liver up" is known to be associated with lower survival and higher rates of ECMO use [20]. This coincides with the higher degree of pulmonary vascular remodeling in these three patients, despite the use of PILG.

Pulmonary vascular remodeling involves increased accumulation of smooth muscle cells in the media of vessel walls, indicated by the higher number of smooth muscle cell layers in the vessel wall in the PILG patients [10]. Furthermore, this increase in smooth muscle cells in the media results in larger vessel wall diameters and a narrowed lumen diameter, both also seen in the PILG patients compared to the control. The combination of effects thus results in a decreased lumen to vessel wall diameter ratio. Pulmonary vascular remodeling also involves alterations in the extracellular matrix of the pulmonary vasculature, including an increase in collagen [10]. We also saw evidence of this in the PILG patients, with increased collagen thickness in the vessel wall upon staining with Masson's Trichrome. The combination of remodeling in both the matrix (increased collagen) and the media (smooth muscle cell hyperplasia and hypertrophy) of the vessel wall leads to a non-compliant vessel that is fixed in an incompletely dilated state that leads to increased resistance in the pulmonary vasculature [21]. PILG did not ameliorate increased extracellular matrix production nor did it decrease smooth muscle cell proliferation in the treated patients in comparison to control. Despite treatment, PILG patients had a greater degree of pulmonary vascular remodeling and more severe pulmonary hypertension. Based upon the subjects in this study, PILG is not a therapy that improves pulmonary hypertension in patients with CDH.

We also assessed for cell proliferation by performing immunohistochemistry with pH3b. We found a decreased level of cell proliferation in the PILG patients compared to the control. Given the increase in amount of collagen and increased accumulation of smooth muscle cells in the vessel walls, we anticipated a higher degree of cell proliferation in the PILG patients, where pulmonary vascular remodeling seemed more severe. It is unclear why the cell proliferation was decreased. The pH3b staining was not specific to the vasculature, and it is unclear as to the exact cell type that was stained. It is therefore difficult to say whether the cells with increased proliferation in the control patient were inflammatory cells, smooth muscle cells, fibroblasts, pneumocytes, or any other cell type. Furthermore, perfluorocarbons have been shown to decrease inflammation by suppressing the secretion of inflammatory cytokines and by clearing inflammatory cells from the airways; thus, the decrease in cellularity may be related to the anti-inflammatory properties of Perflubron [22-25]

Finally, it has been shown that CDH is associated with a systemic inflammatory response, with increased levels of chemokines and cytokines in the serum of patients with CDH [15, 16]. We therefore used immunohistochemistry with antibody against F4/80 to target macrophages as a sign of inflammation. The PILG patients had a decreased amount of macrophages compared to the control patient. Again, perfluorocarbons have been shown to decrease inflammation, thus, it is plausible that the decreased number of macrophages present in the PILG group is related to the anti-inflammatory properties of Perflubron [22-25]. Future studies must investigate the presence of other inflammatory cell types as they were not assessed in this study.

Our study has several limitations. The sample size is small, included only deceased patients, and we had only one control for comparisons. Although all four patients have severe CDH based on the CDH study group equation, liver position, and need for ECMO, there still remains some degree of heterogeneity amongst these patients. Pulmonary vascular remodeling is influenced by many clinical factors; as such, standardization amongst our 4 patients in these very important regards was not possible. Due to the limited sample size and inadequate number of control patients, we were unable to perform statistical analysis to determine if differences that were discovered are significant. Thus our study is descriptive in nature, but it does demonstrate findings that may stimulate questions for further study. Further cases for analysis are needed in the future to determine the statistical significance of our current findings. Another limitation to the study is that the tissue samples were labelled as to which study group they came from (treatment or control) and therefore the observer conducting the measurements of the tissue samples was not blinded to the nature of the samples. However, effort was made to account for this, and all calculations were made in a standardized fashion in order to minimize the potential for bias.

Although PILG stimulated lung growth in patients with CDH on ECMO in the clinical trial, all of the PILG patients who died had suprasystemic PH [8]. Though this study is only descriptive in nature due to the small sample size, this study demonstrates that PILG does not appear to have a salutary effect on the pulmonary vascular remodeling related to the PH of CDH. Since alveolar growth is linked to angiogenesis, the hope was that vascular remodeling and growth would parallel alveolar growth. We can only speculate that a longer duration of PILG would ameliorate PH using this strategy. Unfortunately, no specific therapy

exists for PH in CDH, only supportive treatment strategies. Further investigation into the mechanisms that lead to pulmonary vascular remodeling is needed in order to develop targeted treatment strategies for PH in CDH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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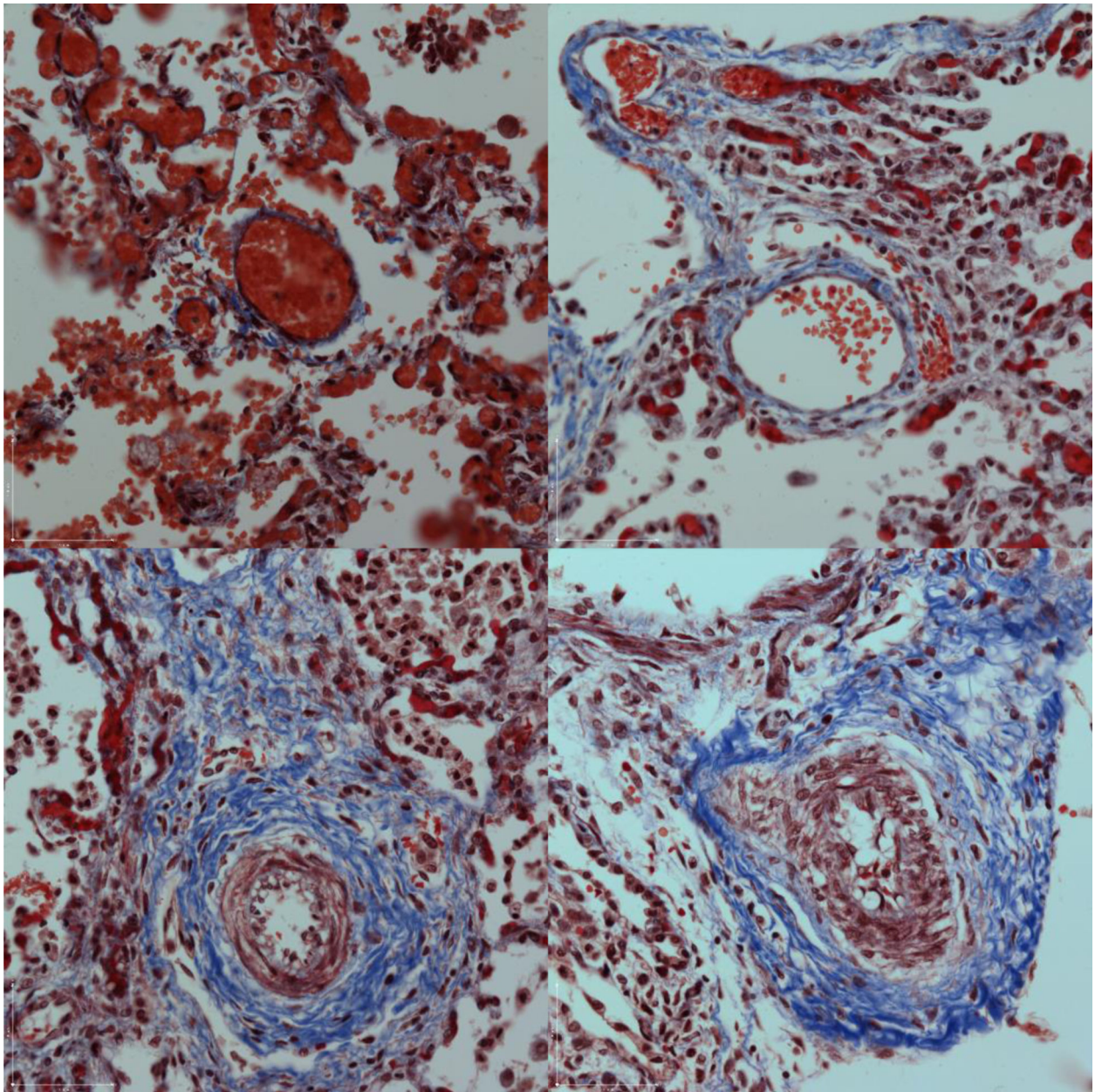
Fig. 1a.
Collagen thickness in arterioles as assessed by Masson's Trichrome staining. Collagen fibers are stained blue. Images acquired at 40x. Clockwise from top left: Control, PILG A, PILG B, PILG C. PILG patients have more collagen in the vessel wall, adventitia, and peri-vascular space
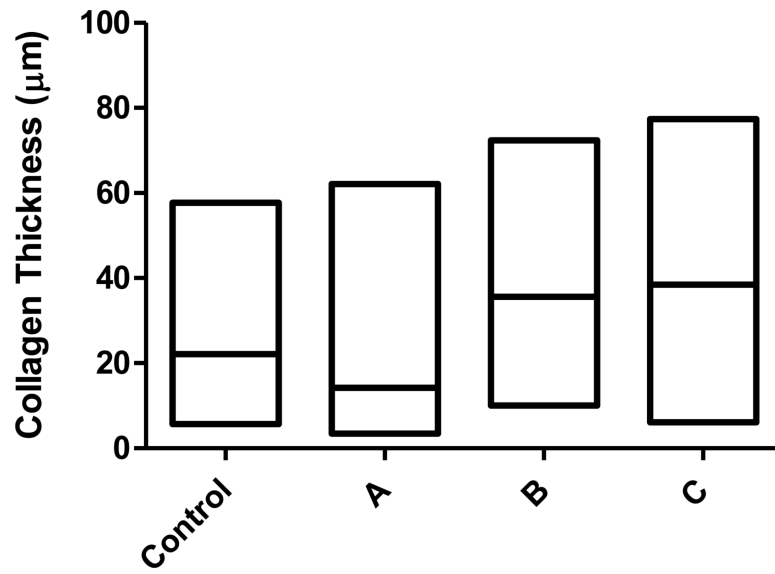
Fig. 1b.

Collagen thickness in the vessel wall measured in micrometers. The mean of sampled vessels is represented by the line in the bar with the upper and lower limits of the bar representing the range of measurements. Vessels in PILG samples B and C have on average greater collagen thickness than the control
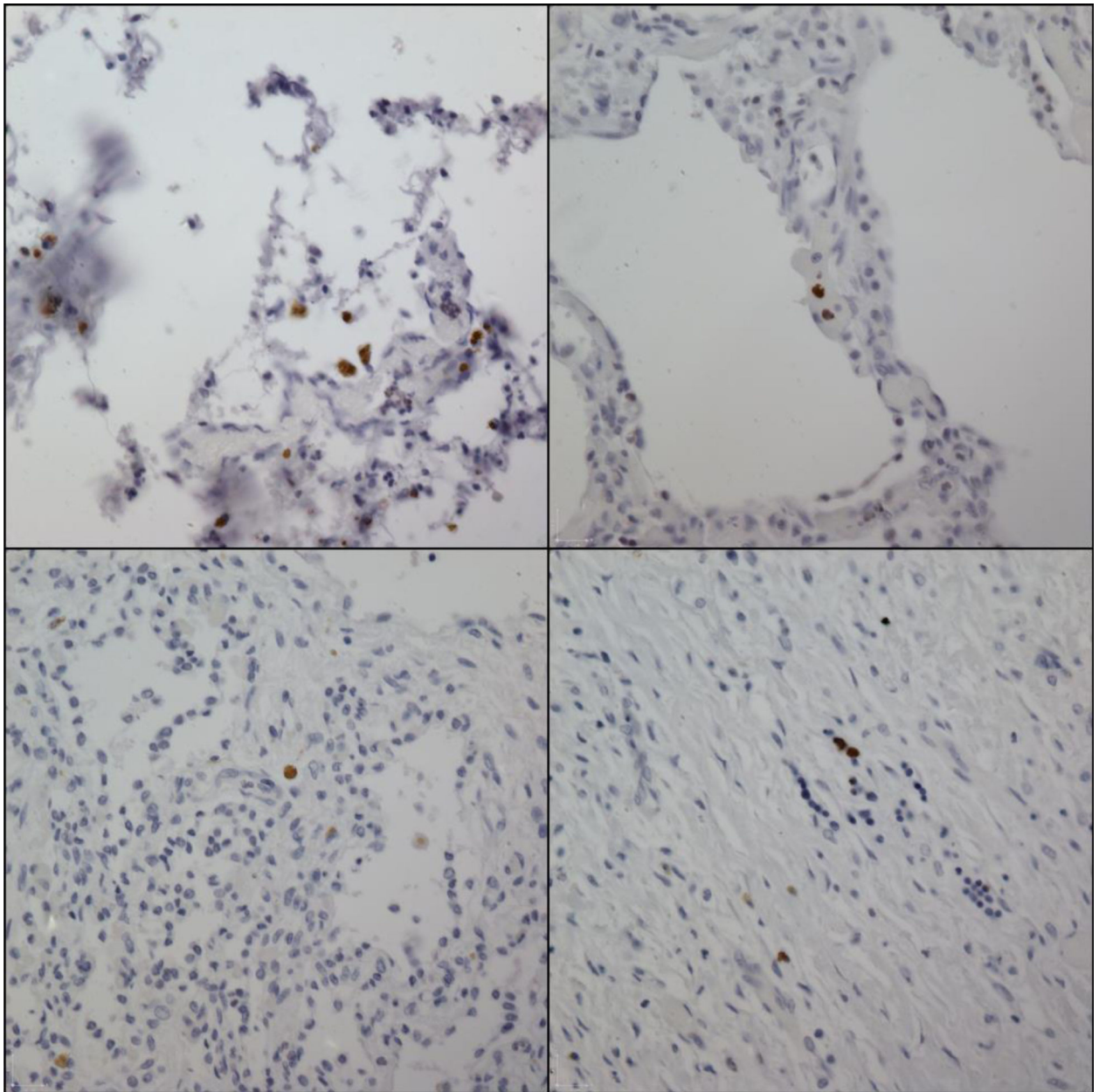
Fig. 2a.
Assessment of cell proliferation. Montage of images at 40x- Clockwise from the top left:
Control, PILG A, PILG B, PILG C. Immunohistochemistry was performed with an antibody
targeting the cell proliferation marker Phosphorylated Histone H3 (pH3b). Positive cells are
brown. Few cells in the PILG lung tissues are pH3b positive, indicating a lack of cell
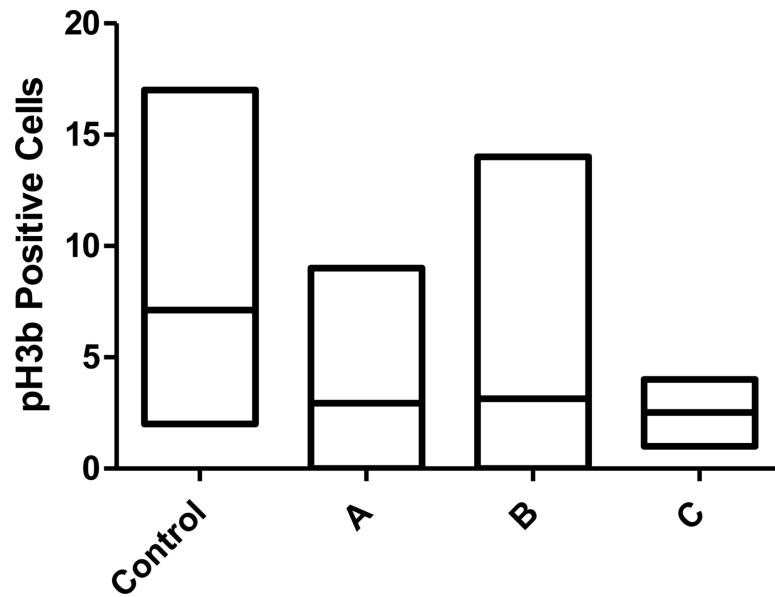proliferation.

Fig. 2b.

Assessment of cell proliferation. The bar graph is a numerical representation of the average number of cells positive for pH3b in each high powered field surveyed (The bar is the range and the mean is represented by the line). All PILG patients had lower mean cell proliferation than the control.
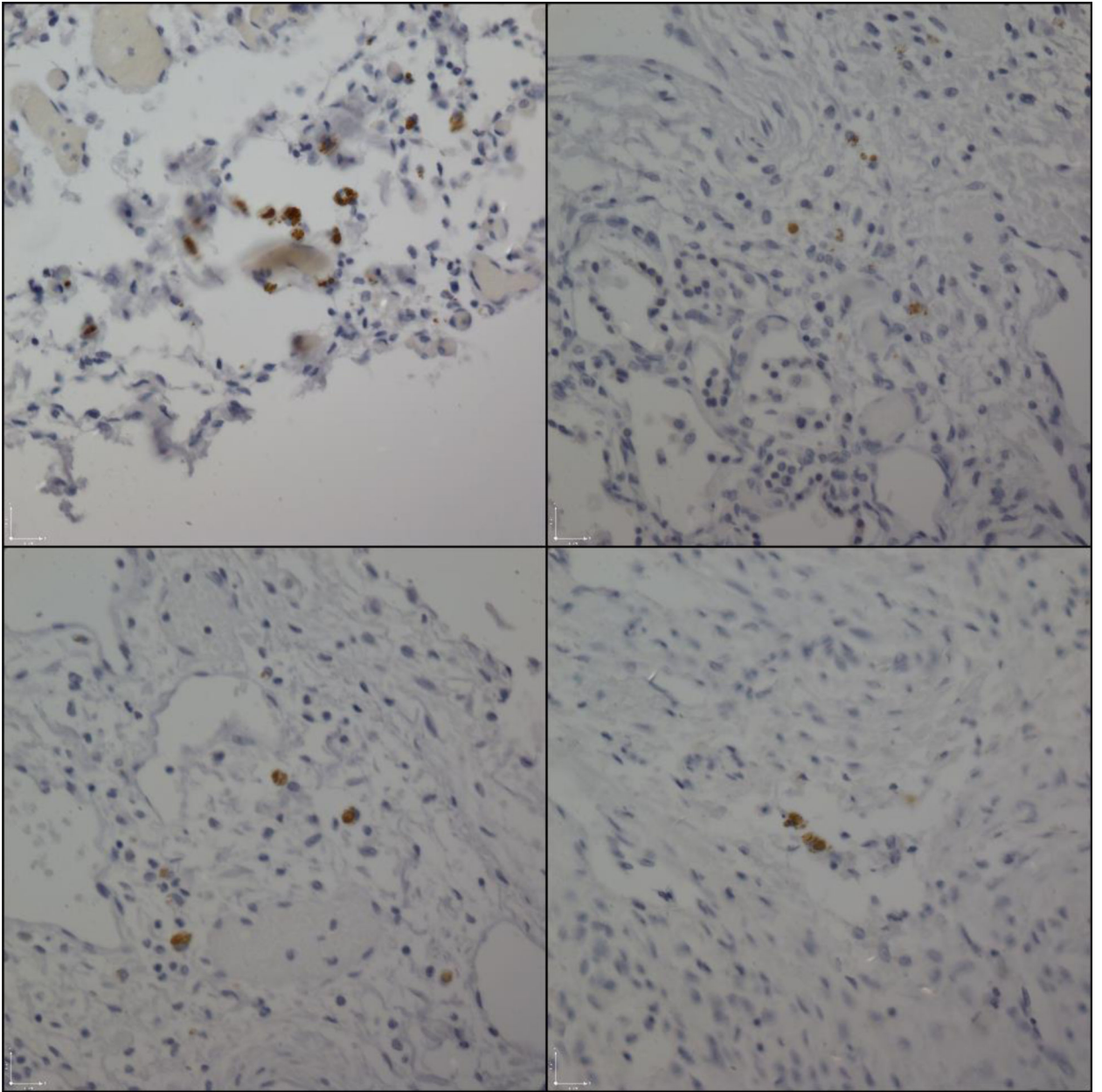
Fig. 3.
Assessment of inflammation. Images acquired at 40x- Clockwise from the top left: Control, PILG A, PILG B, PILG C. Lung tissue was stained with an antibody targeting the macrophage antigen F4/80. PILG patients show decreased macrophage infiltration in the lung compared to control. Macrophages are brown.
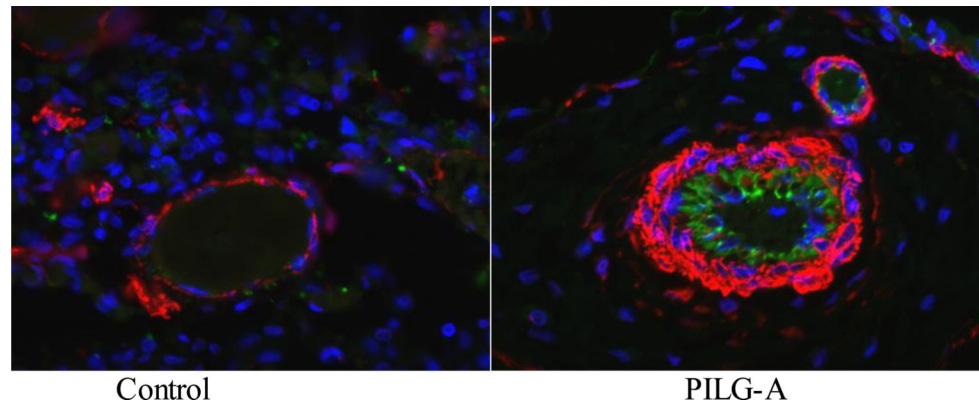
Fig. 4.
Vessel Wall Morphometry. Images acquired at 40x. Immunofluorescence was performed with antibodies targeting smooth muscle cells (α-smooth muscle actin-red), endothelial cells (CD31- green), and DNA stain (DAPI-blue). In comparison to the Control, the PILG-A vessel wall shows an increased number of smooth muscle cells and more prominence of the endothelial cells. These are indicators of a higher degree of pulmonary vascular remodeling in PILG treated patients

Table 1

Characteristics of PILG and Control patients with CDH requiring ECMO

	Control (n=1)	PILG (n=3)
Days of Life at Death	135	35 (23-51)
Female Sex	1	1
Birth weight (kgs.)	3.29	2.92 (2.51-3.15)
Estimated Gestational Age (weeks)	40	38.33 (38-39)
Liver in chest	0	3
Length of ECMO Run (Days)	18	20 (PILG A: 11, PILG B: 21, PILG C: 28)
Day of Life of Repair (Days)	1	7.67 (2-19)

Table 2

Characteristics of the pulmonary vasculature as determined at the level of arterioles within the lung tissue sections

	Control	A	B	C
Mean Wall Diameter (μm)	4.35	6.86	10.28	10.35
Mean Lumen Diameter (μm)	51.56	48.99	41.75	36.2
Ratio of Lumen to Vessel Wall Diameter	11.85	7.14	4.06	3.49
Smooth Muscle Cell Layers	2.13	2.47	2.8	2.8
Mean Number of Vessels per HPF	4.0	4.4	5.7	4.6