



SHORT COMMUNICATION

Relationship of Structural to Functional Impairment during Alveolar-Capillary Membrane Development



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Bronchopulmonary dysplasia is a chronic lung disease of extreme preterm infants and results in impaired gas exchange. Although bronchopulmonary dysplasia is characterized histologically by alveolar-capillary simplification in animal models, it is clinically defined by impaired gas diffusion. With the use of a developmentally relevant model, we correlated alveolar-capillary structural simplification with reduced functional gas exchange as measured by the diffusing factor for carbon monoxide (DF_{CO}). Neonatal mouse pups were exposed to >90% hyperoxia or room air during postnatal days 0 to 7, and then all pups were returned to room air from days 7 to 56. At day 56, DF_{CO} was measured as the ratio of carbon monoxide uptake to neon dilution, and lungs were fixed for histologic assessment of alveolar-capillary development. Neonatal hyperoxia exposure inhibited alveolar-capillary septal development as evidenced by significantly increased mean linear intercept, increased airspace-to-septal ratio, decreased nodal density, and decreased pulmonary microvasculature. Importantly, alveolar-capillary structural deficits in hyperoxia-exposed pups were accompanied by a significant 28% decrease in DF_{CO} (0.555 versus 0.400; $P < 0.0001$). In addition, DF_{CO} was highly and significantly correlated with structural measures of reduced alveolar-capillary growth. Simplification of alveolar-capillary structure is highly correlated with impaired gas exchange function. Current mechanistic and therapeutic animal models of inhibited alveolar development may benefit from application of DF_{CO} as an alternative physiologic indicator of alveolar-capillary development. (*Am J Pathol* 2015, 185: 913–919; <http://dx.doi.org/10.1016/j.ajpath.2014.12.007>)

Bronchopulmonary dysplasia (BPD) is the most common significant morbidity associated with extreme prematurity and results in chronically impaired lung function.^{1–7} Although necessary to sustain life after extreme preterm birth, animal studies have suggested that mechanical ventilation and supplemental oxygen disrupt the intricate molecular networks responsible for normal alveolar-capillary membrane development.^{8–11} Structurally, lungs of infants dying of BPD demonstrate interstitial thickening with minimal fibrosis, inhibited subdivision of distal airspaces, blunted pulmonary microvasculature growth, and impaired growth of the alveolar-capillary surface area for gas exchange.^{12–15} Early in life, BPD is characterized functionally by increased work of breathing and prolonged

dependence on supplemental oxygen.¹⁶ We previously demonstrated that infants with BPD have reduced pulmonary diffusion to carbon monoxide (DL_{CO}) but normal alveolar volume (V_A), which is consistent with the impaired development of fewer but larger alveoli and decreased pulmonary capillary density observed from autopsy studies.^{12,13,17} Similar findings of decreased DL_{CO} in infants with BPD and older children with a history of BPD support

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the hypothesis that BPD results in a persistent impairment of alveolar-capillary development. However, it is not likely that there will ever be a clinical study in human infants to show a correlation between lung parenchymal structure and function under conditions of impaired alveolar development, and we will clinically always depend on *in vivo* functional measurements.

Animal models have demonstrated that brief exposure of the developing neonatal lung to hyperoxia results in a persistent impairment in alveolarization and pulmonary microvessel density, which closely resemble several cardinal pathologic features of human BPD.^{18–22} Preclinical BPD models have primarily focused on the ability of an intervention to restore the alveolar-capillary membrane structure; however, clinical efficacy is determined functionally as the ability to restore alveolar-capillary gas exchange, the surrogate for which is the absence of the need for supplemental oxygen at 36 weeks postmenstrual age.¹⁶ Therefore, preclinical models assess lung structure without a functional assessment of gas exchange, whereas clinical studies assess gas exchange without assessment of lung structure. The purpose of our study was to bridge this gap and to directly evaluate whether there was a correlation between lung structure and lung function in a murine model of BPD.

Fallica et al²³ described a simple method to evaluate pulmonary gas exchange in rodents by measuring the diffusion factor to carbon monoxide (DF_{CO}), which is the relation of carbon monoxide uptake relative to the dilution of neon, a nonabsorbable gas, during a single breathhold maneuver. DF_{CO} is similar to the carbon monoxide transfer factor measured in humans, the ratio of DL_{CO} to V_A (DL_{CO}/V_A).^{24,25} With the use of a murine model of inhibited alveolar-capillary development, we demonstrate that alveolar-capillary membrane simplification correlates with reduced DF_{CO}. Our findings support the notion that alveolar-capillary membrane simplification accounts for the impaired pulmonary diffusing capacity observed clinically in infants with BPD. By providing a functional assessment, DF_{CO} may contribute to a more robust clinically relevant preclinical evaluation of inhibited alveolar-capillary membrane development in studies designed to treat and/or prevent BPD.

Materials and Methods

Murine Neonatal Hyperoxia Exposure Model of BPD

All procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (protocol 10598) and were previously described.¹¹ For each individual experimental exposure, two or more litters of wild-type C57BL/6J mice born within 6 hours of one another were pooled and separated into two equal groups of six to eight mice. By 12 hours of age, half of the pups were placed in a 30" × 20" × 20" polypropylene chamber (BioSpherix, Lacona, NY) in which the

oxygen concentration was maintained at >90% O₂, and the other half were maintained in room air (RA; 21% O₂). Nursing dams were rotated between groups every 48 hours to prevent oxygen toxicity in the dams. Humidity and carbon dioxide levels were maintained within the ambient range of the facility. To use a developmentally appropriate model to correlate the long-term structural and functional consequences of early lung injury, we limited hyperoxia exposure to the first 7 postnatal (P) days (P0 to P7), which includes murine saccular and early alveolar development.²⁶ Pups in the oxygen group were, therefore, exposed to >90% O₂ continuously from P0 to P7. To simulate the period of clinical recovery typically observed in preterm infants through childhood and adolescence, mice were allowed to recover in RA until P56 when rodent alveolar development is essentially complete.²⁷ All structural and functional analyses were performed on P56.

Assessment of the DF_{CO}

DF_{CO} was assessed as initially described by Fallica et al.²³ Mice were anesthetized with 50 mg/kg pentobarbital *i.p.*, a tracheostomy was performed with an 18-g syringe adapter secured with 3-0 silk suture, and the lungs were ventilated with tidal volume 10 mL/kg at 150 breaths/minute and 100% O₂ with the flexiVent (SCIREQ, Montreal, QC, Canada). With the use of a 3-mL syringe, the lung was inflated via the tracheal catheter with 0.8 mL of test gas (0.5% Ne; 0.5% CO; 20% O₂; balance nitrogen), which was held inflated for 6 seconds, and then 0.8 mL was withdrawn back into the syringe. The 0.8 mL of exhaled gas was diluted with RA to a final volume of 2 mL, and concentrations of neon and carbon monoxide were immediately determined with a 3000 Micro GC bench top gas chromatographer (INFICON, East Syracuse, NY). DF_{CO} was calculated as the ratio of carbon monoxide uptake to neon dilution measured in the sample after the 6-second breathhold by using the following equation:

$$DF_{CO} = 1 - \frac{[CO]_{6s}}{[Ne]_{6s}} \quad (1)$$

Because carbon monoxide is highly diffusible and binds to hemoglobin in the pulmonary capillary vessels, [CO]_{6s} represents the uptake of carbon monoxide across the alveolar-capillary membrane during the 6-second breathhold. Conversely, neon does not diffuse across the alveolar-capillary membrane; therefore, [Ne]_{6s} represents the degree of neon dilution by the alveolar gas and estimates lung volume. Values for DF_{CO} range from 0 (no uptake) to 1 (complete uptake). Measurements were obtained in triplicate with each measurement separated by a 150-second period of mechanical ventilation to ensure disappearance of carbon monoxide and neon from the previous measurement. For each mouse, DF_{CO} was expressed as the average of the three measurements.

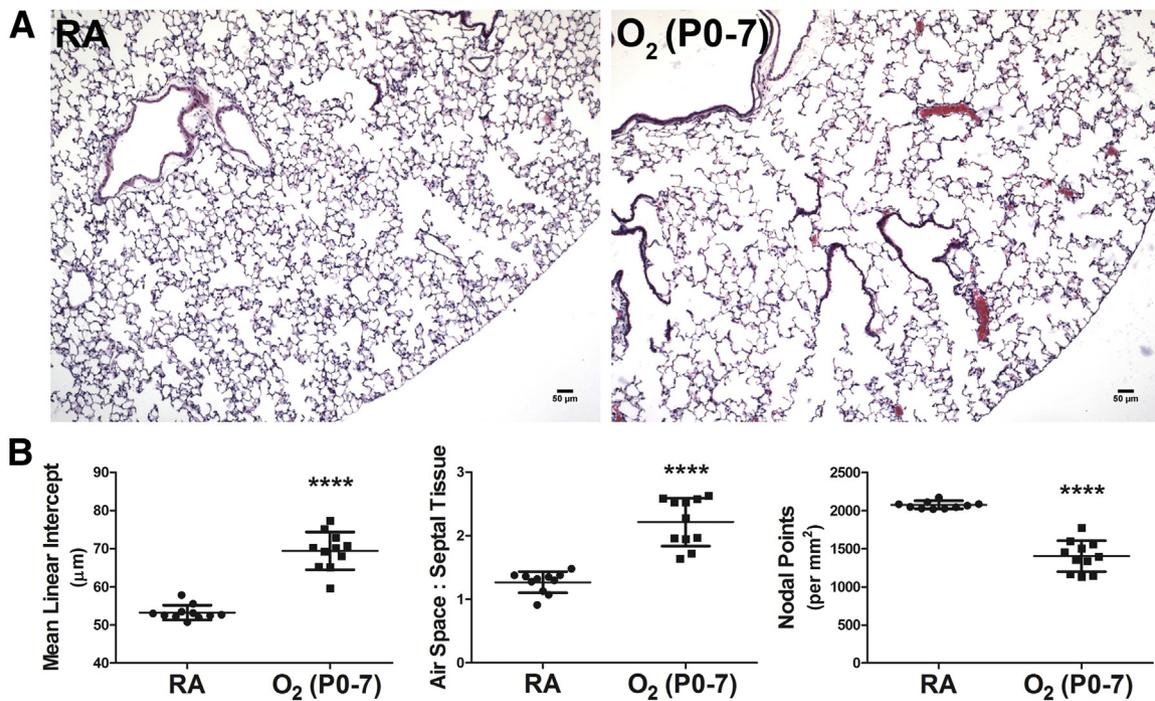


Figure 1 Neonatal hyperoxia results in simplified distal lung structure in the mature lung. **A:** At 2 months of age, compared with mice raised entirely in RA, mice exposed to $\geq 90\%$ O₂ from P0 to P7 display larger, simpler airspaces with a reduction in total septal tissue despite returning to RA for 7 weeks. **B:** Standard morphologic analysis confirms a larger mean linear intercept, increased airspace-to-septal ratio, and a decreased density of nodal points in mice previously exposed to postnatal hyperoxia, indicative of larger, less complex distal airspace structures. Values are expressed as means \pm SEM. $n = 11$ mice per group, composed of three separate litters per group. **** $P < 0.0001$ versus RA control by two-tailed Student's t -test. Representative photomicrographs are stained with H&E. Original magnification, $\times 50$. P, postnatal day; RA, room air.

Lung Histology and Morphometric Analysis

Immediately after measurements of DF_{CO}, the pulmonary vessels were perfused free of blood with cold phosphate-buffered saline and inflation-fixed with 4% paraformaldehyde at a constant pressure of 20 cm H₂O for 45 minutes with the chest wall intact. Then, a suture was

tied around the trachea to maintain fixation pressure, the chest was opened, and the lungs and heart were removed *en bloc* and immersed in 4% paraformaldehyde overnight. To ensure that morphometric analysis was not confounded by differences in lung expansion during tissue fixation, the left lung was dissected free, and left lung volumes were measured by water immersion. The left lung was then

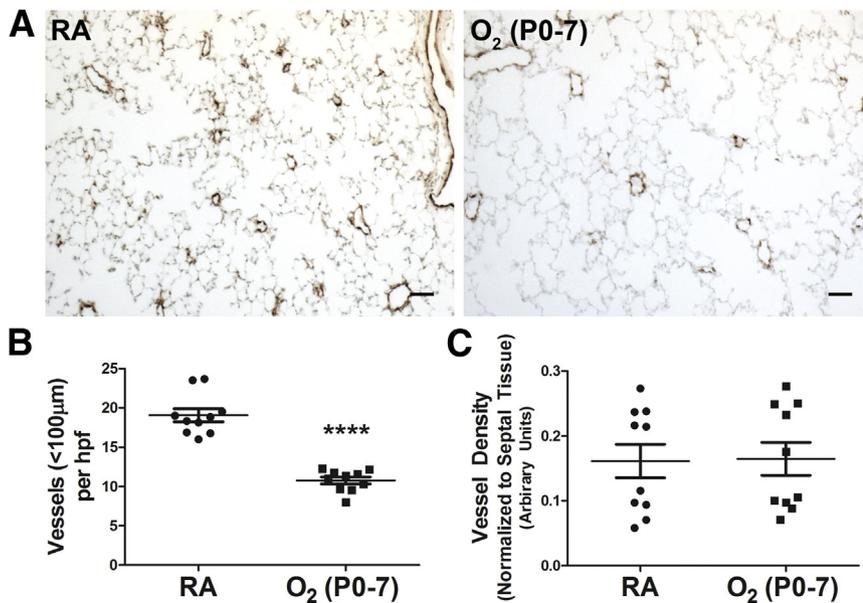


Figure 2 Reduced number but similar density of small vessels in the distal lung of mice formerly exposed to neonatal hyperoxia. **A:** Representative photomicrographs show immunohistochemistry for von Willebrand factor. **B:** At 2 months of age, mice raised entirely in RA have increased numbers of distal vessels compared with adult mice exposed to $\geq 90\%$ O₂ from P0 to P7, quantified as the number of vessels (<50 μm) per $10\times$ field. **C:** However, when corrected for the amount of septal tissue per field, the density of vessels is similar. Values are expressed as means \pm SEM. $n = 10$ mice per group, composed of three separate litters per group. **** $P < 0.0001$ versus RA control by two-tailed Student's t -test. Scale bars = 50 μm . Original magnification, $\times 100$. Hpf, high-powered field; P, postnatal day; RA, room air.

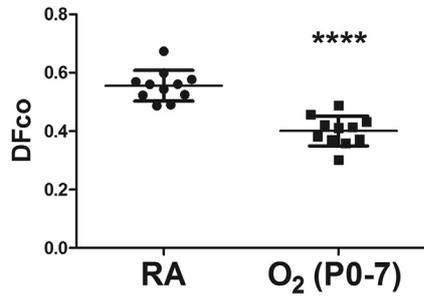


Figure 3 Gas exchange reduces in adult mice exposed to brief postnatal hyperoxia. The DF_{CO} was measured at 2 months of age in mice continuously raised in RA or exposed to $\geq 90\%$ O_2 from P0 to P7. Mice previously exposed to hyperoxia have a significantly reduced capacity for carbon monoxide transfer per alveolar volume. Values are expressed as means \pm SEM. $n = 11$ mice per group, composed of three separate litters per group. **** $P < 0.0001$ versus RA control by two-tailed Student's t -test. DF_{CO} , diffusing factor for carbon monoxide; P, postnatal day; RA, room air.

processed in graduated ethanols, embedded in paraffin, and serially sectioned as previously described.¹¹ Morphometric analysis was performed on 10 to 12 non-overlapping fields from six equidistant 7- μ m thick transverse sections stained with hematoxylin and eosin obtained from the left lung. Random fields that contained distal airspaces were photographed with a 20 \times objective on a Zeiss Axioskop2Plus microscope (Carl Zeiss Inc., Thornwood, NY) at 1292 \times 968 pixels. To assess distal airspace maturation, computer-aided morphometric analysis was performed with ImageJ version 1.44 with a custom plug-in to measure mean linear intercept, airspace-to-septal tissue ratio, and nodal density as previously described.¹¹ Development of distal lung vasculature was assessed by immunohistochemical staining for the endothelial marker, von Willebrand factor [vWF; rabbit polyclonal anti-human vWF; dilution 1:2500; (A0082); Dako, Carpinteria, CA], using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine (Vector Laboratories) as previously described.¹¹ The number of vWF⁺ vessels $< 100 \mu$ m in diameter per 10 \times field were quantified and expressed as vessels per high-powered field. To correct for alveolar septal tissue density, a grid of 500 points was superimposed over the same images; the ratio of points falling on vWF⁺ tissue to points falling on septal tissue was calculated and expressed as vessel density normalized to septal tissue. With the use of three slides obtained from the left lung, at least six non-overlapping fields were analyzed per mouse. For morphometric and vessel density analysis, only fields consisting of alveolar septa were used; fields containing large vessels or conducting airways were avoided.

Statistical Analysis

Results are representative of three litters per group from separate experiments. Statistical analysis was performed

with Prism software version 5.02 (GraphPad Inc., San Diego, CA). Comparisons between experimental groups (RA versus hyperoxia) were made with two-tailed unpaired Student's t -test. Correlation between DF_{CO} and structural measures of alveolar-capillary complexity were assessed by simple linear regression and reported as r^2 values. $P < 0.05$ was considered statistically significant. All data are presented as means \pm SEM.

Results

Representative histologic images of the distal lung at P56 are illustrated in **Figure 1A** for a RA control mouse and a mouse exposed to hyperoxia as a neonate. Compared with the mouse initially raised in RA (**Figure 1A**), the lung of the mouse exposed to $>90\%$ O_2 from P0 to P7 had simplified distal airspaces. When expressed relative to RA controls, mice exposed to hyperoxia as neonates had similar left lung volumes (1.000 ± 0.03 versus 0.947 ± 0.05 ; $P = 0.34$). (Unless otherwise specified, values without units specified represent values with arbitrary units.) Morphometric analysis (**Figure 1B**) revealed 30% increase in airspace size (mean linear intercept: 53.2 ± 0.6 versus $69.5 \pm 1.5 \mu$ m; $P < 0.0001$), 75% increase in the proportion of airspace relative to septal tissue (1.27 ± 0.05 versus 2.22 ± 0.11 ; $P < 0.0001$), and 32% decrease in septal tissue complexity (nodal point density: 2077 ± 16 versus 1402 ± 61 points/ mm^2 ; $P < 0.0001$). Likewise, analysis of distal pulmonary vascular development by vWF staining (**Figure 2**) demonstrated 44% reduction of pulmonary vessels per high-powered field in mice previously exposed to hyperoxia as

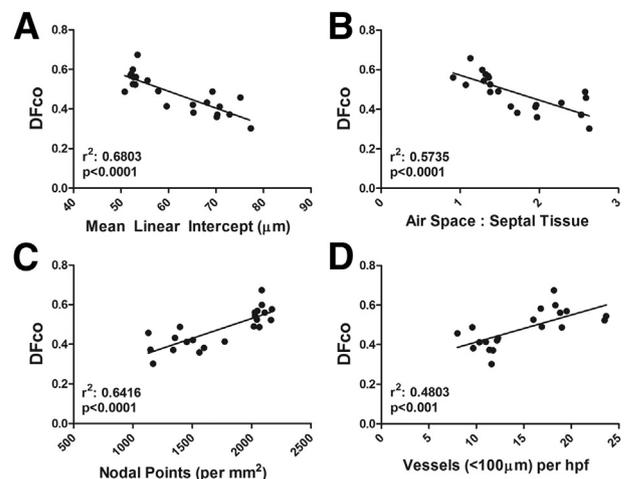


Figure 4 Correlation of DF_{CO} with standard measures of distal airspace morphology. A lower DF_{CO} is significantly correlated with increasing mean linear intercept (**A**), increasing airspace-to-septal tissue ratio (**B**), and decreasing nodal density (**C**), indicating a direct correlation between structural complexity and functional gas exchange. **D**: Similarly, a lower DF_{CO} is significantly correlated with reduced number of pulmonary vessels per field. Analyses were determined by linear regression. $n = 11$ mice per group, composed of three separate litters per group. DF_{CO} , diffusing factor for carbon monoxide; hpf, high-powered field.

neonates (19.1 ± 0.8 versus 10.8 ± 0.4 vessels; $P < 0.0001$) (Figure 2, A and B). However, the density of vessels per alveolar septal tissue was similar between the two groups (0.16 ± 0.03 versus 0.16 ± 0.03 ; $P = 0.93$) (Figure 2C).

In agreement with the observed histologic simplification, mice previously exposed to $>90\%$ O_2 from P0 to P7 had a significant (28%) decrease in DF_{CO} compared with RA controls (0.555 ± 0.016 versus 0.400 ± 0.0215 ; $P < 0.0001$) (Figure 3). When each component of DF_{CO} was analyzed separately, $[CO]_{6s}$ was significantly higher (30% increased) in hyperoxia-exposed mice (0.061 ± 0.002 versus 0.080 ± 0.003 ; $P < 0.0001$), indicating less carbon monoxide uptake and, thus, less pulmonary diffusion of carbon monoxide. However, although statistically significant, the two groups of mice differed minimally ($<3\%$) for $[Ne]_{6s}$ (0.138 ± 0.001 versus 0.134 ± 0.002 ; $P = 0.037$), indicating similar dilution of neon and, thus, similar lung volumes.

With the use of linear regression analysis we evaluated whether significant correlations existed between the structural and functional measurements we obtained (Figure 4). DF_{CO} , our functional assessment of gas exchange, was highly correlated with all of our structural measures of alveolar septal and pulmonary vascular tissue. DF_{CO} decreased as mean linear intercept increased ($r^2 = 0.6803$, $P < 0.0001$) (Figure 4A) and the airspace-to-septal tissue ratio increased ($r^2 = 0.5735$, $P < 0.0001$) (Figure 4B). In addition, DF_{CO} decreased as nodal point density decreased ($r^2 = 0.6416$, $P < 0.0001$) (Figure 4C). Finally, DF_{CO} also decreased as vessel density decreased ($r^2 = 0.4803$, $P < 0.001$) (Figure 4D).

Discussion

The present study is the first to demonstrate *in vivo* functional impairment of gas exchange in a rodent model of BPD. In addition, with the use of a developmentally relevant rodent model of BPD with a persistent impairment of alveolar development, we demonstrated a high correlation between the standard measures of alveolar-capillary structural development and the functional assessment of gas exchange by using DF_{CO} . Our findings support the use of the DF_{CO} as a functional end point in assessing alveolar-capillary development in murine models of BPD, and add support to the interpretation of clinical assessment of gas exchange in infants with BPD.

Extremely preterm infants that develop BPD are typically exposed to hyperoxia during the saccular and early alveolar periods of alveolar development.²⁶ Therefore, we used a model that limited hyperoxia exposure to the first 7 postnatal days, which includes the murine period of saccular and early alveolar development.²⁶ In addition, we simulated the period of clinical recovery through childhood by allowing the mice to recover in RA until day 56 when rodent alveolar development is essentially

complete.²⁷ Our adult mice, which were previously exposed to neonatal hyperoxia, displayed reduced complexity and enlargement of distal airspaces, and a reduction in the pulmonary microvasculature, which recapitulates several of the pathologic findings of human BPD.^{12–15} It is important to note that infants dying of BPD have also demonstrated interstitial thickening, a characteristic that is lacking in the rodent hyperoxia model of BPD. In addition, follow-up studies that examine lung function in infants, children, adolescents, and adults formerly diagnosed with BPD have revealed significant heterogeneity (reviewed in Ahlfeld and Conway¹⁰), most likely a result of population variance. Although our study included mice from three litters derived from separate experiments, additional numbers of litters may have been necessary to reveal significant population variance, as was observed in clinical studies.

Our BPD mice also had lower DF_{CO} , indicating reduced gas exchange, which was primarily related to lower carbon monoxide uptake during the 6 seconds of gas exchange, whereas there was essentially no difference in the final neon concentration, indicating similar lung volumes for gas exchange. This physiologic finding in our murine BPD model is similar to follow-up studies of infants and older children with BPD (reviewed in Ahlfeld and Conway¹⁰).^{5,6,17,28–31} There, the reduced DL_{CO}/V_A is secondary to a reduction in DL_{CO} in the presence of a normal V_A , which is consistent with fewer but larger alveolar-capillary units within the same lung volume, thus less surface area for gas exchange. In our murine model of BPD, we were able to demonstrate a strong correlation between the reduction in DF_{CO} and the increased alveolar size, which produces a decrease in surface area for the same lung volume. Gas exchange across the alveolar-capillary unit depends on the surface area of the alveolar membrane and the pulmonary capillary blood volume, which is indirectly reflected by the capillary vessel density. We found that a decrease in DF_{CO} not only correlated with larger, more simplified alveoli, which reflects a smaller alveolar surface area, but also with a lower microvessel density. However, the correlation was stronger with the overall septal tissue density than the microvessel density. This difference in the strengths of the correlations we observed between these two structural assessments of impaired alveolar development may have resulted from how our measurements were obtained. Our mice were ventilated with 100% O_2 before our measurements of DF_{CO} , which competes with carbon monoxide for hemoglobin binding, thus, decreasing the vascular contribution to diffusion and decreasing the measured DF_{CO} . The technique of measuring pulmonary diffusion under conditions of RA and again breathing high concentrations of oxygen can be used to separate the relative contributions of the alveolar membrane and the pulmonary capillary to the overall resistance to diffusion.²⁴ Therefore, our measurement of DF_{CO} under high oxygen

concentrations probably reflects the alveolar membrane component more than the vascular component, thus accounting for the higher correlation with septal tissue than with microvessel density. This, and a shorter breathhold, may also account for the lower DF_{CO} we found in healthy mice compared with the values reported by Fallica et al.²³ In addition, the sensitivity and specificity of vWF staining we used to assess microvessels may have not adequately limited our measurement to pulmonary capillaries, but may also include pre- and post-capillary vessels, thus not accurately assessing the vascular component of diffusion.

Despite technologic advances in the neonatal intensive care, which has resulted in improved survival of infants born extremely preterm, BPD continues to develop in most of these infants.¹ Animal models of inhibited alveolar septation have improved our understanding of BPD pathogenesis; however, translation into clinically effective therapies has been disappointing. The use of *in vivo* assessment of gas exchange in developmentally relevant models of inhibited alveolar-capillary development may advance our ability to translate novel therapies from pre-clinical to clinical trials by using similar outcomes of alveolar-capillary development. Although the described murine model of inhibited alveolar-capillary development does not recapitulate every aspect of BPD, it provides an effective, refined method to study the effect of alveolar-capillary simplification on gas exchange, which is one cardinal feature of BPD.

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