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Intrauterine endotoxin-induced impairs pulmonary vascular function and right ventricular performance in infant rats and improvement with early vitamin D therapy

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Mandell E, Powers KN, Harral JW, Seedorf GJ, Hunter KS, Abman SH, Dodson RB. Intrauterine endotoxin-induced impairs pulmonary vascular function and right ventricular performance in infant rats and improvement with early vitamin D therapy. *Am J Physiol Lung Cell Mol Physiol* 309: L1438–L1446, 2015. First published October 16, 2015; doi:10.1152/ajplung.00302.2015.—High pulmonary vascular resistance (PVR), proximal pulmonary artery (PA) impedance, and right ventricular (RV) afterload due to remodeling contribute to the pathogenesis and severity of pulmonary hypertension (PH). Intra-amniotic exposure to endotoxin (ETX) causes sustained PH and high mortality in rat pups at birth, which are associated with impaired vascular growth and RV hypertrophy in survivors. Treatment of ETX-exposed pups with antenatal vitamin D (vit D) improves survival and lung growth, but the effects of ETX exposure on RV-PA coupling in the neonatal lung are unknown. We hypothesized that intrauterine ETX impairs RV-PA coupling through sustained abnormalities of PA stiffening and RV performance that are attenuated with vit D therapy. Fetal rats were exposed to intra-amniotic injections of ETX, ETX+vit D, or saline at 20 days gestation (term = 22 days). At postnatal day 14, pups had pressure-volume measurements of the RV and isolated proximal PA, respectively. Lung homogenates were assayed for extracellular matrix (ECM) composition by Western blot. We found that ETX lungs contain decreased α-elastin, lysyl oxidase, collagen I, and collagen III proteins ($P < 0.05$) compared control and ETX+vit D lungs. ETX-exposed animals have increased RV mechanical stroke work ($P < 0.05$ vs. control and ETX+vit D) and elastic potential energy ($P < 0.05$ vs. control and ETX+vit D). Mechanical stiffness and ECM remodeling are increased in the PA ($P < 0.05$ vs. control and ETX+vit D). We conclude that intrauterine exposure of fetal rats to ETX during late gestation causes persistent impairment of RV-PA coupling throughout infancy that can be prevented with early vit D treatment.

chorioamnionitis; bronchopulmonary dysplasia; extracellular matrix; vascular remodeling; arterial stiffening

CHANGES IN HEMODYNAMIC COMPONENTS, such as pulmonary vascular resistance (PVR), proximal pulmonary artery (PA) impedance, and right ventricular (RV) afterload, to the pathogenesis of pulmonary hypertension (PH) as bronchopulmonary dysplasia (BPD) in the neonate are not well defined and could offer insight into the mechanisms of the disease and treatment. PVR, a measure of heart workload, is a long established measure of pulmonary hypertension progression (1, 2, 15, 16, 31). PVR measures the resistance to flow and clinically describes the degree of vasoconstriction or angiogenic development of the distal vasculature. More recently, proximal PA stiffness has increasing interest as an important indicator of disease progression (20, 32, 35, 36). Proximal PA stiffness through extracellular matrix (ECM) remodeling increases impedance that increase RV afterload (9, 14). Both PVR and impedance components contribute to the pathogenesis and RV dysfunction in PH (35) but are poorly understood in the setting of BPD. Furthermore, neonatal complications of BPD have been clinically associated with global RV structural and functional differences in adult life (21), motivating the need to understand RV-PA function in the neonate.

Chorioamnionitis, a disease characterized by intra-amniotic inflammation, is associated with increased risk for perinatal morbidities and a high risk for chronic lung disease in preterm infants known as BPD (13, 18, 28, 30). Infants with severe BPD have sequelae that include prolonged need for ventilator or oxygen support, pulmonary hypertension, recurrent respiratory infections, abnormal lung function, exercise intolerance, late neurodevelopmental outcomes, and a high risk for death (5).

There is increasing evidence that vitamin D (vit D) deficiency is an independent risk factor for lung and cardiovascular disease, and vit D treatment can improve endothelial dysfunction (12, 22, 29, 38). Transgenic knockout of the endothelial vit D receptor (VDR) causes a significant impairment of eNOS expression (25) and increases systemic artery stiffness and

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vascular impedance through ECM remodeling, increases in collagen and decreases in elastin protein content (3). Using a rat model of chorioamnionitis caused by intrauterine amniotic exposure to endotoxin (ETX), we previously reported high mortality at birth, PH as assessed by right ventricular hypertrophy (RVH), and decreased distal alveolar and vascular growth (37). Vit D treatment reduces mortality, decreases RVH, and increases distal alveolar and vascular growth in this model (23). But whether antenatal [intra-amniotic (IA)] ETX impairs right ventricular performance, proximal PA stiffness, and ECM development in the lung and the impact of early vit D therapy remains unknown.

RV performance is a critical reflection of the pulmonary vascular structure and function. Clinically, RV performance has been shown as a sensitive marker for pulmonary vascular disease diagnosis and prognosis (6). Simultaneous RV measurement of pressure and volume gives a complete description of the performance of the pulmonary vasculature both at the proximal and distal levels through measures of RV-PA coupling. However, the RV pressure-volume measurement has primarily been used as a clinical marker in older patients with a more progressive pulmonary hypertension and little is known about RV and arterial function in the BPD infant.

Therefore, we hypothesized that intrauterine ETX impairs RV-PA coupling owing to ECM remodeling causing sustained impairments of RV performance. Furthermore, antenatal vit D treatment will preserve normal ECM composition reducing PA stiffness and improving RV performance. To test these hypotheses, we studied the effects of vit D treatment on 2-wk-old rat pup RV function, PA artery stiffness, and whole lung ECM composition after antenatal ETX in vivo exposure.

METHODS

Animal model. The Institutional Animal Care and Use Committee at the University of Colorado Denver Anschutz Medical Campus approved all procedures and protocols. We utilized an animal model of chorioamnionitis as previously described (37) and vit D therapy as also previously described (23). In short, timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver altitude (1,600 m; barometric pressure 630 mmHg; inspired oxygen tension 122 mmHg) for 1 wk prior to full term. A laparotomy was performed at 20 days gestation (term: 22 days) during the late canalicular stage of lung development in the rat to parallel the similar stage of human lung development in 24- to 26-wk premature newborns, which are highest risk for BPD. The pregnant rats were randomly assigned to saline control groups (50 μl of normal saline per amniotic sac; $n = 6$); the ETX group (10 μg of *Escherichia coli* 055:B55 diluted into 50 μl of saline per sac; Sigma Chemical, St. Louis, MO; $n = 6$); or the ETX with the biologically active form of vit D [1,25-$(OH)_2D_3$] treatment [ETX+vit D; 50 pg diluted into 50 μl of normal saline; 1,25-$(OH)_2D_3$; $n = 6$]. At full term, 2 days after IA injections, rat pups were delivered via cesarean section and placed with a foster mother to be raised through 14 days. On day 14 of life, the following procedures were performed.

Pressure-volume measurements. Animals were anesthetized with 4–5% isoflurane in room air at 1,000 ml/min for ~2 min and placed in the supine position, a tracheal cannula was inserted and connected to a Hallowell EMC Micro Vent, and anesthesia was maintained at 1.5–2.5% isoflurane in 100% oxygen. Peak airway pressure was maintained between 16–18 cmH_2O, breaths per minute at 50–150, and the oxygen flow at 0.5–0.8 l/min. The RV, PA, aortic arterial (Ao), and left ventricular (LV) pressures were directly measured with a 1.2-Fr FTX-1212B-4018 Pressure-Volume Catheter (Transonic/Scisense, London, Ontario, Canada) inserted into the heart directly through the wall via a diaphragmatic approach. The pericardium was resected and a small hole was made at the base of the RV with a 30-gauge needle. The pressure-volume catheter was inserted in the hole and advanced along the length of the RV. Bleeding was minimal because of the small size of the hole and the larger catheter plugging it. Steady-state hemodynamics was collected with short pauses in ventilation (up to 10 s) to eliminate ventilator artifact from the pressure-volume recordings. Occlusions of the inferior vena cava were also performed to decrease filling to obtain an accurate end-systolic pressure-volume relationship. The catheter was advanced into the PA and pressure measurements were recorded. One final hole was made in the apex of the LV and the catheter was placed along the length of the LV for measurement of steady-state data in the LV. The catheter was advanced into the Ao to record systemic pressure measurements. Data were recorded continuously with LabScribe 2 (iWorx, Dover, NH) and analyzed offline. At the termination of an experiment, the animal was given an intracardiac overdose of pentobarbital sodium and left pulmonary arteries were collected for pressure myography experiments; the heart was sectioned, weighed, and fixed for histology; and the whole lung samples were flash frozen for Western blot analysis.

Pressure myography measurements. The left PA (LPA) was mounted in a pressure myograph (Living Systems Instrumentation, St. Albans, VT). The artery was immersed in physiological saline solution at 37°C. The artery was pressurized from 0 to 30 mmHg in 5-mmHg increments and from 30 to 120 mmHg in 10-mmHg increments. Transmural pressure and outer diameter were recorded. Tested arteries were paraformaldehyde fixed and imaged by using second harmonic generation to visualize mechanical constituents of collagen and elastin.

Biochemical analysis. The ECM components were assessed both in the proximal vasculature and the whole lung. The right and main pulmonary arteries were lyophilized and weighed for dry mass. The dried tissue was hydrolyzed in 6 M HCl and dried with a SpeedVac. The amount of elastin and collagen in the ECM were quantified with standard measurements of tissue desmosine and hydroxyproline content, respectively (33, 34).

Western blotting. Whole lung protein expression was determined as described previously (11). Blood was drawn from the right heart. Then the right lung was removed, flash frozen in liquid nitrogen, and stored at −80°C. The lung was pulverized and then homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors (Roche) and 0.01% phosphatase inhibitor cocktail (Sigma). Equal volume (35 μg) of protein was loaded on a Bis-Tris gradient gel

Table 1. *Blood pressure measurements*

	CTL	ETX	ETX + VD
Heart rate, beats/min	311 ± 21	264 ± 10	283 ± 4
Systemic systolic BP, mmHg	37.6 ± 5.0	46.2 ± 5.4	51.5 ± 1.8
Systemic diastolic BP, mmHg	17.3 ± 2.9	20.7 ± 1.7	26.7 ± 1.6*
Systemic mean BP, mmHg	27.0 ± 5.1	31.5 ± 3.4	39.2 ± 1.9
Pulmonary systolic BP, mmHg	17.1 ± 1.4	26.3 ± 1.8*†	17.1 ± 0.8
Pulmonary diastolic BP, mmHg	7.0 ± 1.0	6.3 ± 1.0	5.9 ± 0.6
Pulmonary mean BP, mmHg	13.8 ± 1.3	17.7 ± 0.5*†	13.3 ± 0.8

BP, blood pressure; CTL, control; ETX, endotoxin; VD, vitamin D. Values are mean ± SE. *$P < 0.05$ vs. CTL, †$P < 0.05$ vs. ETX+VD.

(4–12%), separated by electrophoresis, and transferred to a nitrocellulose membrane. Primary antibodies for specific proteins of interest include collagen type I (1:1,000, Abcam), collagen type III (1:1,000, Abcam), tropo-elastin (1:1,000, Abcam), α-elastin (1:25, Abcam), and β-actin (1:10,000, Cell Signaling). Bands were analyzed with ImageJ software (NIH), normalized to β-actin, and expressed relative to the control group.

Statistical analysis. Results are expressed as means ± SE. A one-way ANOVA test that used a Bartlett's test and corrected for unequal variances was used to compare differences between the three groups (control, ETX, ETX+vit D), and Tukey's multiple comparisons test was used to compare differences between individual groups. Statistical significance was defined as a two-tailed adjusted P value of less than 0.05. All statistics were done with GraphPad Prism Software Version 6 (La Jolla, CA).

RESULTS

Table 1 describes the effects of intra-amniotic ETX and vit D treatment with ETX exposure on blood pressures. Intra-amniotic ETX increased pulmonary systolic and mean arterial pressures ($P < 0.05$) and treatment of ETX with vit D lowered PA pressure ($P < 0.05$) to levels similar to the control rats. Heart rates showed no change between groups. ETX exposure had limited effects on systemic arterial pressure, but ETX exposure with vit D treatment increased diastolic blood pressure compared with the control group ($P < 0.05$).

As shown previously, lung histology from ETX-exposed pups demonstrated alveolar simplification and decreased vessel

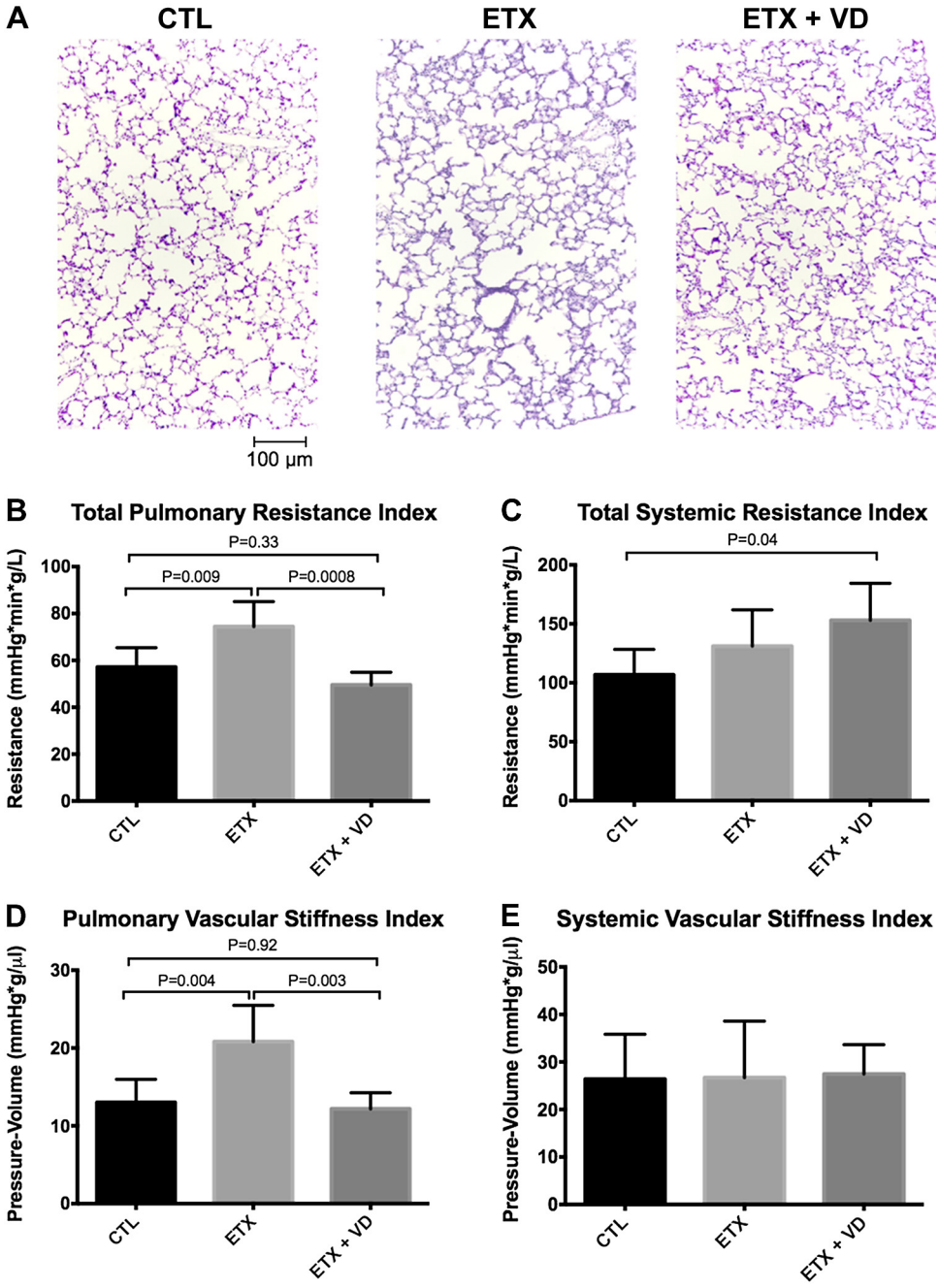

Fig. 1. Altered pulmonary vascular density, pulmonary vascular resistance index, and pulmonary vascular stiffness index. *A*: lung histology from endotoxin (ETX)-exposed in 2-wk rat pups (center) demonstrate decreased distal vasculature and alveolar simplification compared with saline control (CTL; *left*). Lung histology of pups with vitamin D (vit D; VD) treatment (*right*) shows lung structure similar to that of control pups. Prenatal ETX increases total pulmonary vascular resistance index (PVRI) in pups (*B*) and has limited effects on total systemic vascular resistance index (SVRI) (*C*). Vit D reduces PVRI following ETX exposure but elevates SVRI. Prenatal ETX increases total pulmonary vascular stiffness index (PVSI) in pups (*D*) and has no effects on total systemic vascular stiffness index (SVSI) (*E*). Vit D reduces PVSI following ETX exposure and does not alter SVRI ($n = 6$ for each group).

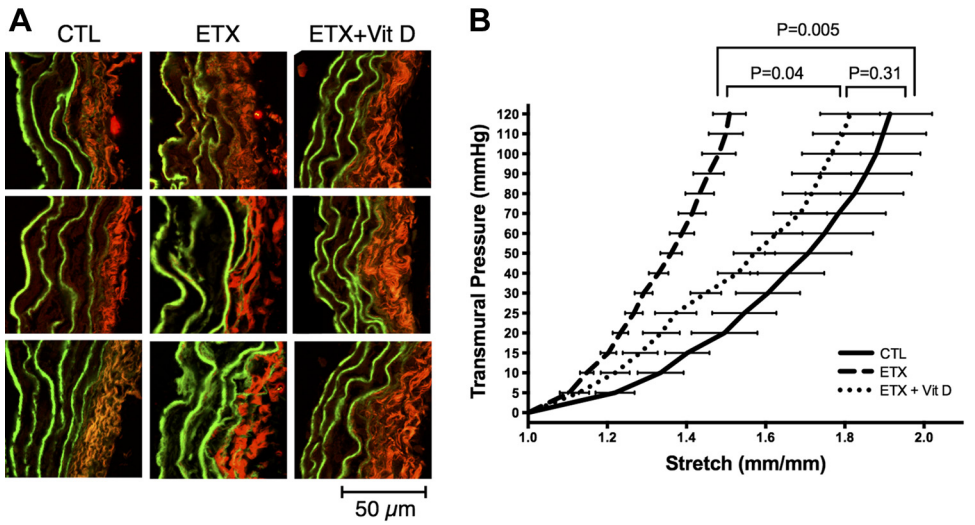

Fig. 2. Vascular remodeling. *A*: second harmonic generation histology shows prenatal ETX remodels collagen (red) and elastin (green) left pulmonary artery (LPA) structure in 2-wk rat pups. Vit D treatment restores LPA collagen and elastin structure. *B*: prenatal ETX increases functional LPA stiffness compared with the control arteries. Vit D prevents proximal artery stiffness ($n = 6$ for each group).

density compared with saline controls, which are markedly improved with vit D treatment (Fig. 1). Systemic vascular resistance index (SVRI) was not different after ETX treatment but was increased in the ETX group receiving vit D treatment ($P < 0.05$). Intra-amniotic ETX increased the total PVR indexes (PVRI) at 2 wk of age. Treatment with vit D during ETX exposure significantly lowered PVRI compared with the ETX group to levels similar to that of the control group. Systemic and pulmonary vascular stiffness indexes followed a similar pattern (Fig. 1). ETX and ETX with vit D treatment had limited effects on total SVSI. The pulmonary vascular stiffness index (PVSI) increased after ETX exposure compared with the control ($P < 0.05$) and ETX with vit D treatment restored PVSI to control values ($P < 0.05$).

ETX treatment increased proximal LPA stiffness compared with the control group ($P < 0.05$; Fig. 2). Vit D prevented increases in LPA stiffness after antenatal ETX exposure ($P < 0.05$). Assessment of proximal artery hydroxyproline and desmosine content showed no differences between groups (data not shown). However, histology showed altered organization of collagen and elastin with ETX samples having decreased numbers but thicker and less continuous medial elastic lamellae and thicker adventitial collagen fibers leading to increased stiffness with intra-amniotic ETX compared with the control LPA. Vit D therapy prevented increased stiffness ($P < 0.05$) of the proximal artery and restored LPA collagen and elastin organization.

At 2 wk of age, fetal ETX exposure decreased body weights of infant rats, indicating a postnatal failure to thrive compared with the control rats (control: 32.67 ± 0.33 g vs. ETX: 21.33 ± 1.52 g; $P < 0.05$). Body weights in the ETX rats receiving vit D treatment had body weights similar to that of the control rats and significantly greater than the ETX group (ETX+VD: 33.5 ± 0.62; $P < 0.05$). Because of the significant difference in body weights between groups all measurements including volumes were normalized by body weight, as an index, to compare across treatments.

Intrauterine ETX not only increased proximal PA stiffness and remodeling but also led to RV dysfunction, representative plots shown in Fig. 3 and recorded averages and standard error of the mean in Table 2. ETX increased afterload through increased end-systolic pressure and end-systolic volume index compared with the control RV ($P < 0.05$). Workload was also increased in ETX as measured in increased maximum power index, stroke work index, pressure-volume area index, and elastic potential energy index ($P < 0.05$). Intra-amniotic ETX increased isovolumic relaxation time, preload recruitable stroke work index, and End-diastolic pressure-volume relationship peak index (Emax) time index ($P < 0.05$) compared with the control RV. Vit D treatment reduced end-systolic pressure, stroke work index, maximum power index, pressure volume area index, and elastic potential energy index ($P < 0.05$) compared with ETX treatment alone. Vit D treatment reduced RV workload and pressure in the presence of intra-amniotic ETX.

RV histology showed structural alterations that correlate with measured functional changes (Fig. 4). Intrauterine ETX increased RV wall thickness compared with the control ($P < 0.05$). Vit D treatment prevented wall thickening with ETX exposure ($P < 0.05$) to preserve the wall thickness similar to

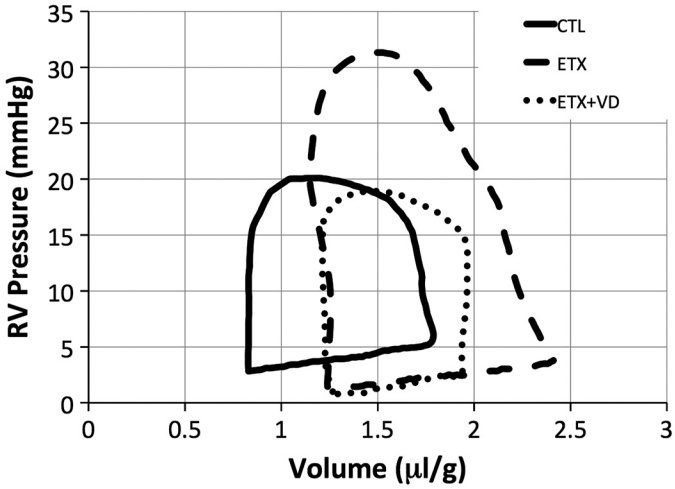

Fig. 3. Representative pressure-volume curves. ETX 2-wk rat pups show right ventricular (RV) dysfunction with increased afterload, workload, end-diastolic volume, and pressure. Vit D treatment reduces afterload, workload, and pressure.

Table 2. *Right ventricle pressure-volume measurements*

	CTL	ETX	ETX + VD
ESP, mmHg	17.88 ± 1.37	24.84 ± 1.68*†	18.28 ± 0.92
EDP, mmHg	2.04 ± 0.28	2.73 ± 0.38	2.95 ± 0.49
ESV, μl/g	0.85 ± 0.17	1.41 ± 0.23	1.10 ± 0.16
EDV, μl/g	1.64 ± 0.18	2.37 ± 0.24*	2.03 ± 0.16
SV, μl/g	0.79 ± 0.07	0.96 ± 0.03	0.93 ± 0.08
CO, $\mu l \cdot min^{-1} \cdot g^{-1}$	247.18 ± 32.21	240.42 ± 10.47	258.18 ± 20.47
EF, %	50.77 ± 5.79	42.32 ± 3.83	48.21 ± 3.90
Stroke work index, mJ/g	10.73 ± 1.66	19.93 ± 1.63*†	13.61 ± 0.96
Maximum power index, mW/g	293.61 ± 69.35	573.96 ± 80.63*†	288.97 ± 25.70
Preload power index, $mW \cdot g^{-1} \cdot \mu l^{-1} \cdot \mu l^{-1}$	160.37 ± 58.33	111.25 ± 25.19	79.23 ± 15.46
E_a, mmHg·g/μl	23.68 ± 2.06	24.33 ± 0.82	21.20 ± 2.17
PVA, $mmHg \cdot \mu l \cdot g^{-1}$	16.09 ± 1.13	33.29 ± 3.60[a],†	15.80 ± 1.96
PE, $mmHg \cdot \mu l \cdot g^{-1}$	7.29 ± 1.21	16.17 ± 3.03*†	6.46 ± 1.21
Efficiency, %	52.40 ± 9.16	52.58 ± 4.94	58.84 ± 4.25
Tau, Weiss method, ms/g	0.41 ± 0.10	0.88 ± 0.12*	0.54 ± 0.04
E_{es}, mmHg·g/μl	13.68 ± 3.23	12.53 ± 1.46	19.11 ± 3.63
V_0, μl/g	−0.69 ± 0.28	−0.91 ± 0.12	−0.35 ± 0.16
EDPVR, stiffness, $\mu l/mmHg^{-1} \cdot g^{-1}$	0.0160 ± 0.0038	0.0177 ± 0.0055	0.0252 ± 0.0044
PRSW, $mmHg \cdot \mu l^{-1} \cdot g^{-1}$	0.29 ± 0.07	0.58 ± 0.08*	0.35 ± 0.04
PRSW volume axis intercept index, μl/g	0.55 ± 0.07	0.19 ± 0.33	0.55 ± 0.20
Emax, $mmHg \cdot g^{-1} \cdot \mu l^{-1}$	28.00 ± 13.34	26.13 ± 4.47	49.42 ± 9.40
Emax time index, ms/g	2.88 ± 0.25	5.13 ± 0.40*†	3.07 ± 0.05

ESP, end-systolic pressure; EDP, end-diastolic pressure; ESV, end-systolic volume index; EDV, end-systolic volume index; SV, stroke volume index; CO, cardiac output index; EF, ejection fraction; E_a, effective arterial elastance index; PVA, pressure-volume area index; PE, elastic potential energy index; tau, isovolumic relaxation time; E_{es}, end-systolic pressure-volume relationship slope index; V_0, theoretical no-pressure-volume index; EDPVR, end-diastolic pressure-volume relationship; PRSW, preload recruitable stroke work index; Emax, end-diastolic pressure-volume relationship peak index. *$P < 0.05$ vs. CTL, †$P < 0.05$ vs. ETX+VD.

that of the control. Colorimetric morphometric analysis showed ETX exposure decreased the ratio of cardiomyocytes to collagen compared with the control ($P < 0.05$). Vit D treatment increased the ratio of cardiomyocytes to collagen ($P < 0.05$), but the ratio following vit D was still decreased compared with the control ($P < 0.05$). ETX exposure decreased RV cell density ($P < 0.05$) compared with the control and vit D treated groups.

Physiological RV measurement changes were related to changes in the lung ECM structural components with intra-amniotic exposure to ETX (Fig. 5). ETX exposure had no effect on tropoelastin but decreased α-elastin, the mature form of elastin, and decreased the ECM cross-linking protein lysyl oxidase. ETX exposure decreased the content of both collagen I and collagen III. However, vit D treatment with ETX exposure restored α-elastin, lysyl oxidase, collagen I, and collagen III in the infant rat lung model of chorioamnionitis.

DISCUSSION

We found that intra-amniotic ETX in the infant rat causes pulmonary hypertension that is associated with altered ECM deposition in the proximal pulmonary arteries and decreases ECM deposition in the distal lung. We further report that RV-PA coupling is abnormal following intrauterine injury, as characterized by increases in afterload and workload through increases in the proximal artery stiffness and increases in PVR, respectively, that are associated with a decrease in RV isovolumic relaxation compared with the control rats. Antenatal vit D treatment prevents ETX-induced alterations of ECM deposition in the infant rat. We further report that vit D prevents pulmonary hypertension and prevents RV afterload and workload compared with ETX exposure alone and is comparable to the control group.

These findings along with our previous findings in this model (23, 37) demonstrate the important balance between proximal and distal artery development in infant pulmonary hemodynamics. Intra-amniotic ETX elevates pulmonary mean and systolic pressures, leading to increased proximal artery stiffness, total PVRI, and total PVSI. Furthermore, we extend our previous studies by defining the role vit D may play as an important nutrient in preventing BPD through preserving ECM development in the experimental model of chorioamnionitis. Vit D treatment protects against pulmonary hypertension in part through preserving lung vascular development.

Furthermore, these findings show altered RV performance and remodeling in an experimental model of BPD. Intrauterine ETX exposure increases afterload and workload on the RV with increased isovolumic relaxation, suggesting increased stiffness, and Emax, suggesting a slower contraction and a labored heart. However, other clinical measures of RV performance such as cardiac output index and ejection fraction, effective arterial elastance index (E_a), and end-systolic pressure-volume relationship slope index (E_{es}) remain unaffected, or only slightly elevated ($P > 0.05$). Histology confirms remodeling corresponding to structural changes with increased thickness, increased collagen content, and hypertrophy shown through decreased cellular density compared with the control RV. Vit D treatment with ETX exposure restores most functional parameters but trends to have higher volume indexes, lower isovolumic relaxation, and increased E_{es} index compared with the control ($P > 0.05$). Histology shows vit D treatment preserves wall thickness and cellularity but has decreased cardiomyocyte to collagen ratio. Vit D treatment may protect the RV by preventing RV-PA dysfunction and promoting vascular development in this experimental model of BPD.

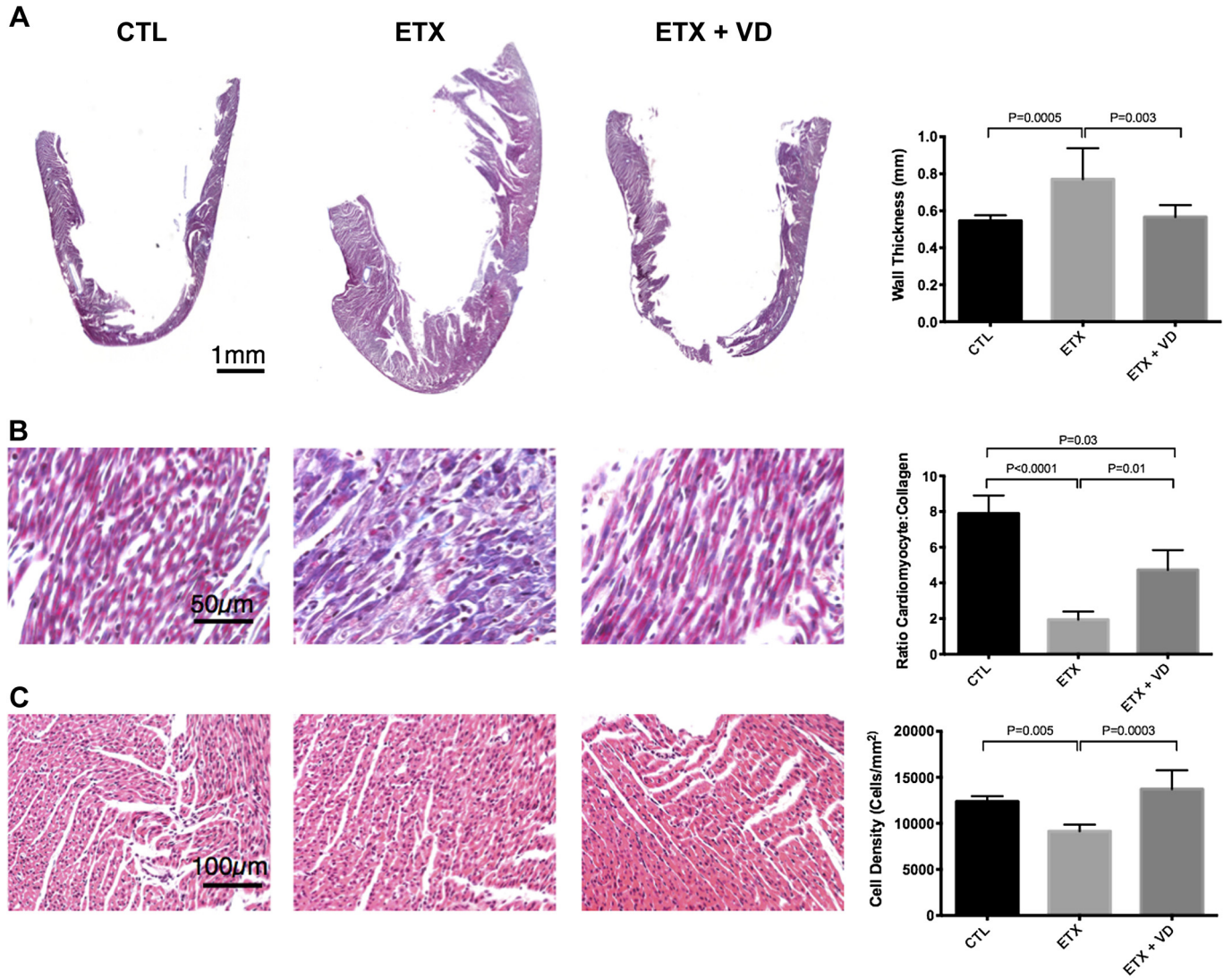

Fig. 4. Right ventricle remodeling. *A*: intrauterine ETX increases RV wall thickness compared with control and vit D-treated RVs. *B*: intrauterine ETX decreases the ratio of cardiomyocytes to collagen in the RV compared with the control. Vit D increases the ratio in the RV compared with ETX exposure but is decreased compared with the control. *C*: compared with the control and vit D-treated RVs, ETX exposure reduces RV cellular density ($n = 4$ for each group).

Other studies have shown RV-PA coupling as a sensitive predictor of PH disease progression in animals (14, 35) and clinically (6, 8, 14). We tested this hypothesis in our model of chorioamnionitis in infant lung development by measuring antenatal ETX effects on RV and PA dysfunction. Our results show that chorioamnionitis leads to pulmonary hypertension characterized by increased impedance due to increased proximal stiffening and increased PVR due to previously shown decreased distal vasculature (23, 37) causing RV afterloads and workloads. Furthermore, we studied the effects of vit D therapy with ETX exposure. Within the literature the effects of vit D on cardiovascular physiology and ECM development still remain poorly defined (27). Vit D deficiency has been correlated with poor cardiovascular disease outcomes. In a transgenic mouse model, the selective elimination of vit D receptors from endothelial cells provides evidence of the importance of vit D in regulating endothelium-dependent vasoreactivity, regulating arterial blood pressure, and alteration of the arterial ECM (25). Furthermore, low levels of vit D have been linked to inflammation, increases in oxidative stress, profibrotic vascular effects, increased vascular stiffness, and decreased vasoreactivity (4, 7, 17, 19). However, modest supplementation of vit D in healthy neonates has also been correlated with negative impacts vascular remodeling. Vit D diet supplementation in healthy maternal and postnatal rats reduces elastin content, elastin lamellae, and contractility forces in aortic rings (26) express similar remodeling and functional characteristics of the elastin haploinsufficiency mouse model (10). In the setting of the chorioamnionitis due to ETX exposure, vit D therapy promotes ECM deposition and alleviates PH in the neonate.

Mechanisms responsible for the improved vit D RV-PA coupling after intra-amniotic ETX exposure may occur through changes in the distal and proximal artery development. First, vit D preserves distal lung vasculature maintaining whole lung structural ECM production and, as our group has shown previously (23), increasing vascular growth. These changes reduce PVR and workload on the heart. Second, vit D preserves proximal PA compliance reducing stiffness after ETX

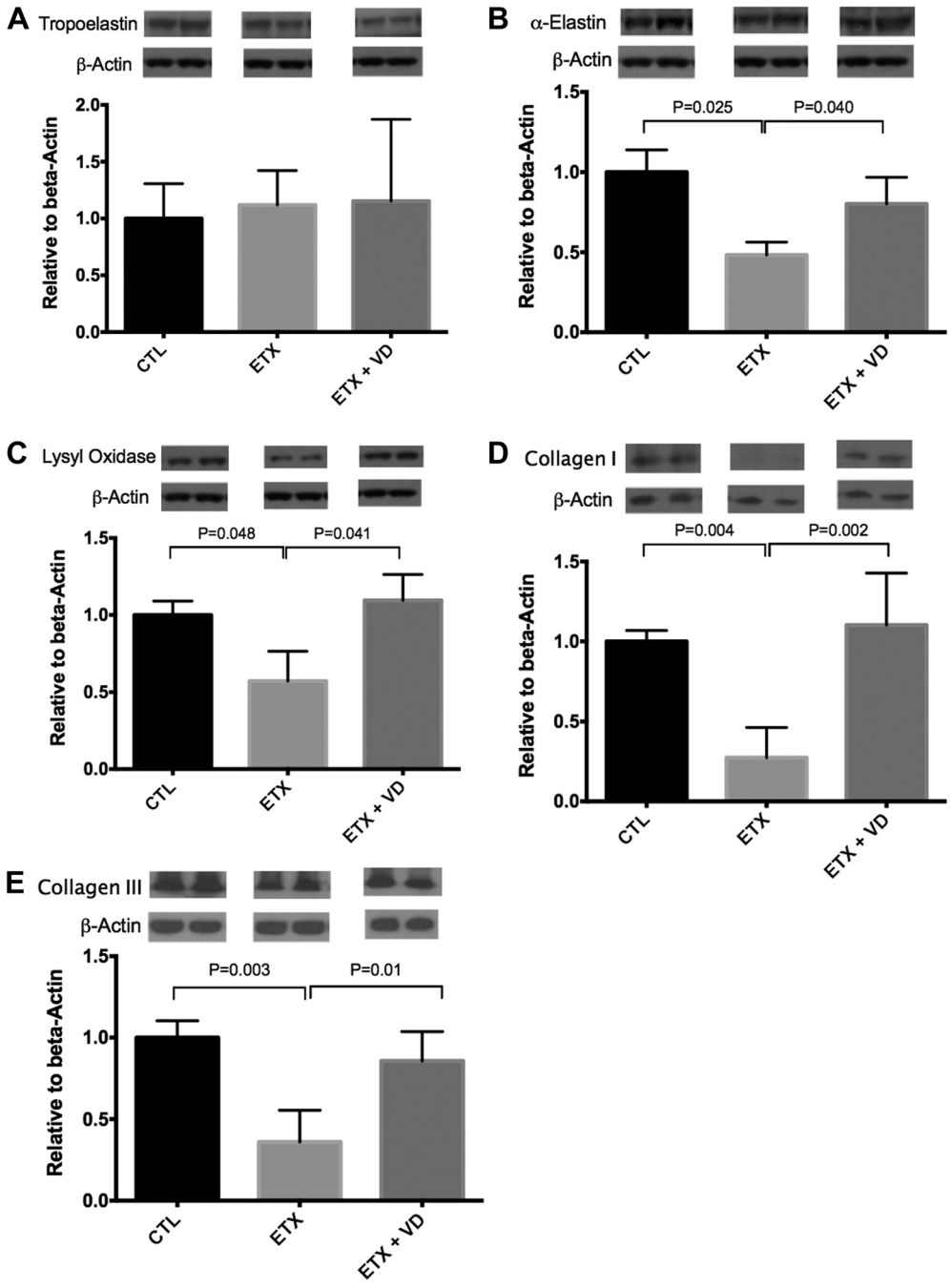

Fig. 5. Decreased extracellular matrix (ECM) content in the lung. Intrauterine ETX decreases structural ECM proteins in the lung including α-elastin, collagen I, and collagen III and also decreases the extracellular cross-linking protein lysyl oxidase. Vit D treatment maintains ECM development in the lung with ETX exposure ($n = 4$ for each group).

exposure by maintaining ECM collagen and elastin organization. These changes reduce afterload and improve RV performance. Finally, recent work in our laboratory has shown that ETX disrupts lung vit D metabolism favoring less vit D conversion and VDR activation, and treatment of ETX with exogenous vit D preserves CYP27B1 and VDR expression in the fetal PA endothelial cells (24). Together, these data and studies by others (4, 7, 17, 19, 25) suggest the vit D may play an important role in vascular development through regulating ECM production and organization.

Several potential limitations in the study design and the interpretation of the results must be acknowledged. The significant reduction in animal weight both at birth and remaining until the 2-wk time point shows other organs and systems may be affected by the postnatal failure to thrive. The specific aim of this study was to understand the cardiovascular effects of ETX and ETX with vit D treatment, and because of the significant size differences all measurements were indexed to account for smaller pup size in the ETX group. In addition, we examined pup weight as a function of vascular stiffness and RV function and found no correlation, demonstrating that pup growth retardation is an independent factor. Furthermore, we carefully measured the systemic hemodynamics through LV and aortic blood pressures and found little cardiovascular effect of the ETX or ETX with vit D. The increase in SVRI in the ETX with vit D group we speculate may be due to increased

systemic vascular tone, possibly as a result of hypercalcemia but will require further future studies. Additionally, the resolution in quantification of protein ECM proteins distinguished proximal (the main pulmonary artery and the right pulmonary artery) and distal lung. Future studies are required to dive deeper into differences in distal and proximal ECM development and their respective contribution to pulmonary development and hemodynamics. Pressure-volume measurements were taken at 2-wk time point. This time point was chosen to obtain the most accurate, highest fidelity hemodynamic measurements at the end of neonatal lung development and was due to size limitations to accommodate the pressure-volume transducer in the RV. Future studies will use lower-resolution Doppler ultrasound echocardiography to allow noninvasive and serial measurements of the heart for remodeling and function to track PH progression in the fetus, at birth, and postnatally. Finally, because of the size of the RV, histology was completed to study the remodeling. Histology provided the best way to study RV structure and organization that could be related to physiological function, more thoroughly than by Western blot.

In summary, we report that exposure of fetal rats to intra-amniotic ETX during late gestation reduces ECM deposition and alters RV-PA coupling, as assessed by proximal PA stiffness and RV performance. Fetal vit D treatment decreases PA stiffness and preserves RV function partly by changes in ECM after antenatal ETX exposure. We speculate that prenatal events can cause long-term changes in RV-PA coupling through remodeling of the proximal pulmonary arteries and RV function, which are sustained during postnatal life. Vit D therapy may preserve RV-PA coupling in infants who may be at risk for late pulmonary vascular disease due to antenatal stress, such as chorioamnionitis.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

E.M., K.S.H., S.H.A., and R.B.D. conception and design of research; E.M., K.N.P., J.W.H., G.J.S., K.S.H., S.H.A., and R.B.D. analyzed data; E.M., K.S.H., S.H.A., and R.B.D. interpreted results of experiments; E.M. and R.B.D. prepared figures; E.M., S.H.A., and R.B.D. drafted manuscript; E.M., K.N.P., J.W.H., G.J.S., K.S.H., S.H.A., and R.B.D. edited and revised manuscript; E.M., K.N.P., J.W.H., G.J.S., K.S.H., S.H.A., and R.B.D. approved final version of manuscript; K.N.P., J.W.H., G.J.S., and R.B.D. performed experiments.

REFERENCES

1. **Abman SH, Accurso FJ.** Acute effects of partial compression of ductus arteriosus on fetal pulmonary circulation. *Am J Physiol Heart Circ Physiol* 257: H626–H634, 1989.
2. **Abman SH, Shanley PF, Accurso FJ.** Failure of postnatal adaptation of the pulmonary circulation after chronic intrauterine pulmonary-hypertension in fetal lambs. *J Clin Invest* 83: 1849–1858, 1989.
3. **Andrukhova O, Slavic S, Zeitz U, Riesen SC, Heppelmann MS, Ambrisko TD, Markovic M, Kuebler WM, Erben RG.** Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice. *Mol Endocrinol* 28: 53–64, 2014.
4. **Artaza JN, Norris KC.** Vitamin D reduces the expression of collagen and key profibrotic factors by inducing an antifibrotic phenotype in mesenchymal multipotent cells. *J Endocrinol* 200: 207–221, 2009.
5. **Baraldi E, Filippone M.** Chronic lung disease after premature birth. *N Engl J Med* 357: 1946–1955, 2007.
6. **Champion HC, Michelakis ED, Hassoun PM.** Comprehensive invasive and noninvasive approach to the right ventricle-pulmonary circulation unit: state of the art and clinical and research implications. *Circulation* 120: 992–1007, 2009.
7. **Cumhur Cure M, Cure E, Yuce S, Yazici T, Karakoyun I, Efe H.** Mean platelet volume and vitamin D level. *Ann Lab Med* 34: 98–103, 2014.
8. **Di Maria MV, Younoszai AK, Mertens L, Landeck BF, 2nd Ivy DD, Hunter KS, Friedberg MK.** RV stroke work in children with pulmonary arterial hypertension: estimation based on invasive haemodynamic assessment and correlation with outcomes. *Heart* 100: 1342–1347, 2014.
9. **Dodson RB, Morgan MR, Galambos C, Hunter KS, Abman SH.** Chronic intrauterine pulmonary hypertension increases main pulmonary artery stiffness and adventitial remodeling in fetal sheep. *Am J Physiol Lung Cell Mol Physiol* 307: L822–L828, 2014.
10. **Faury G, Pezet M, Knutsen RH, Boyle WA, Heximer SP, McLean SE, Minkes RK, Blumer KJ, Kovacs A, Kelly DP, Li DY, Starcher B, Mecham RP.** Developmental adaptation of the mouse cardiovascular system to elastin haploinsufficiency. *J Clin Invest* 112: 1419–1428, 2003.
11. **Fleenor BS, Sindler AL, Eng JS, Nair DP, Dodson RB, Seals DR.** Sodium nitrite de-stiffening of large elastic arteries with aging: role of normalization of advanced glycation end-products. *Exp Gerontol* 47: 588–594, 2012.
12. **Hadi HA, Carr CS, Al Suwaidi J.** Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc Health Risk Manag* 1: 183–198, 2005.
13. **Hartling L, Liang Y, Lacaze-Masmonteil T.** Chorioamnionitis as a risk factor for bronchopulmonary dysplasia: a systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal Ed* 97: F8–F17, 2012.
14. **Hunter KS, Lee PF, Lanning CJ, Ivy DD, Kirby KS, Claussen LR, Chan KC, Shandas R.** Pulmonary vascular input impedance is a combined measure of pulmonary vascular resistance and stiffness and predicts clinical outcomes better than pulmonary vascular resistance alone in pediatric patients with pulmonary hypertension. *Am Heart J* 155: 166–174, 2008.
15. **Ivy DD, Kinsella JP, Abman SH.** Physiologic characterization of endothelin A and B receptor activity in the ovine fetal pulmonary circulation. *J Clin Invest* 93: 2141–2148, 1994.
16. **Ivy DD, Le Cras TD, Parker TA, Zenge JP, Jakkula M, Markham NE, Kinsella JP, Abman SH.** Developmental changes in endothelin expression and activity in the ovine fetal lung. *Am J Physiol Lung Cell Mol Physiol* 278: L785–L793, 2000.
17. **Jablonski KL, Chonchol M, Pierce GL, Walker AE, Seals DR.** 25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults. *Hypertension* 57: 63–69, 2011.
18. **Jobe AH.** Blood cytokines and BPD. *J Pediatr* 154: A2, 2009.
19. **Kunadian V, Ford GA, Bawamia B, Qiu W, Manson JE.** Vitamin D deficiency and coronary artery disease: a review of the evidence. *Am Heart J* 167: 283–291, 2014.
20. **Laurent S, Boutouyrie P, Asmar R, Gautier I.** Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 29: 1034–1042, 2001.
21. **Lewandowski AJ, Bradlow WM, Augustine D, Davis EF, Francis J, Singhal A, Lucas A, Neubauer S, McCormick K, Leeson P.** Right ventricular systolic dysfunction in young adults born preterm. *Circulation* 128: 713–720, 2013.
22. **Lykkedegn S, Sorensen GL, Beck-Nielsen SS, Christesen HT.** The impact of vitamin D on fetal and neonatal lung maturation. A systematic review. *Am J Physiol Lung Cell Mol Physiol* 308: L587–L602, 2015.
23. **Mandell E, Seedorf G, Gien J, Abman SH.** Vitamin D treatment improves survival and infant lung structure after intra-amniotic endotoxin

exposure in rats: potential role for the prevention of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 306: L420–L428, 2014.
24. **Mandell E, Seedorf GJ, Ryan SL, Gien J, Cramer SD, Abman SH.** Antenatal endotoxin disrupts lung vitamin D receptor and 25-hydroxyvitamin D 1α hydroxylase expression in the developing rat. *Am J Physiol Lung Cell Mol Physiol* 309: L1018–L1026, 2015.
25. **Ni W, Watts SW, Ng M, Chen S, Glenn DJ, Gardner DG.** Elimination of vitamin D receptor in vascular endothelial cells alters vascular function. *Hypertension* 64: 1290–1298, 2014.
26. **Norman P, Moss I, Sian M, Gosling M, Powell J.** Maternal and postnatal vitamin D ingestion influences rat aortic structure, function and elastin content. *Cardiovasc Res* 55: 369–374, 2002.
27. **Norman PE, Powell JT.** Vitamin D and cardiovascular disease. *Circ Res* 114: 379–393, 2014.
28. **Paananen R, Husa AK, Vuolteenaho R, Herva R, Kaukola T, Hallman M.** Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. *J Pediatr* 154: 39–43.e3, 2009.
29. **Pittas AG, Chung M, Trikalinos T, Mitri J, Brendel M, Patel K, Lichtenstein AH, Lau J, Balk EM.** Systematic review: Vitamin D and cardiometabolic outcomes. *Ann Intern Med* 152: 307–314, 2010.
30. **Plakkal N, Soraisham AS, Trevenen C, Freiheit EA, Sauve R.** Histological chorioamnionitis and bronchopulmonary dysplasia: a retrospective cohort study. *J Perinatol* 33: 441–445, 2013.
31. **Rosenberg AA, Kennaugh J, Koppenhafer SL, Loomis M, Chatfield BA, Abman SH.** Elevated immunoreactive endothelin-1 levels in newborn infants with persistent pulmonary hypertension. *J Pediatr* 123: 109–114, 1993.
32. **Safar M.** Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. *Circulation* 107: 2864–2869, 2003.
33. **Sims TJ, Bailey AJ.** Quantitative analysis of collagen and elastin cross-links using a single-column system. *J Chromatogr* 582: 49–55, 1992.
34. **Starcher B, Conrad M.** A role for neutrophil elastase in the progression of solar elastosis. *Connect Tissue Res* 31: 133–140, 1995.
35. **Su Z, Tan W, Shandas R, Hunter KS.** Influence of distal resistance and proximal stiffness on hemodynamics and RV afterload in progression and treatments of pulmonary hypertension: a computational study with validation using animal models. *Comput Math Methods Med* 2013: 618326, 2013.
36. **Tan W, Madhavan K, Hunter KS, Park D, Stenmark KR.** Vascular stiffening in pulmonary hypertension: cause or consequence? (2013 Grover Conference series). *Pulm Circ* 4: 560–580, 2014.
37. **Tang JR, Seedorf GJ, Muehlethaler V, Walker DL, Markham NE, Balasubramaniam V, Abman SH.** Moderate postnatal hyperoxia accelerates lung growth and attenuates pulmonary hypertension in infant rats after exposure to intra-amniotic endotoxin. *Am J Physiol Lung Cell Mol Physiol* 299: L735–L748, 2010.
38. **Weng S, Sprague JE, Oh J, Riek AE, Chin K, Garcia M, Bernal-Mizrachi C.** Vitamin D deficiency induces high blood pressure and accelerates atherosclerosis in mice. *PloS One* 8: e54625, 2013.