Prenatal treprostinil reduces the pulmonary hypertension phenotype in the rat model of congenital diaphragmatic hernia

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Summary
Background Persistent pulmonary hypertension (PH) causes significant mortality and morbidity in infants with congenital diaphragmatic hernia (CDH). Since pulmonary vascular abnormalities in CDH develop early during foetal development, we hypothesized that prenatal maternal administration of treprostinil, through its anti-remodelling effect, would improve the PH-phenotype in the nitrofen rat model of CDH.

Methods In a dose-finding study in normal, healthy pregnant rats, we demonstrated target-range foetal plasma treprostinil concentrations without signs of toxicity. Next, an efficacy study was performed assessing the effects of treprostinil administration at 900 and 1500 ng/kg/min from gestational day (GD) 16 until term (GD 21) in CDH and control pups. Pulmonary vascular and airway morphometry, lung mechanics, and expression patterns of genes implicated in the prostaglandin vasoactive pathway were studied.

Findings In rats maternal administration of 1500 ng/kg/min treprostinil reached target foetal concentrations, with no detrimental maternal or foetal side-effects. Prenatal exposure to 900 and 1500 ng/kg/min treprostinil reduced the medial wall thickness (%MWT) (CDH 900, 38.5 ± 8.4%; CDH 1500, 40 ± 9.7%; CDH, 46 ± 8.2%; both p < 0.0001) in rat pups with CDH, however increased the %MWT in normal foetuses (C.T.900, 36 ± 6.1%; C.T.1500, 36 ± 9.3%; C.P., 26 ± 6.2%; both p < 0.001). Pulmonary airway development, lung hypoplasia and pulmonary function were unaffected by drug exposure.

Interpretation In pregnant rats maternally administered treprostinil crosses the placenta, attains foetal target concentrations, and is well tolerated by both mother and foetuses. This report shows a significant reduction of pulmonary arteriole muscularization with prenatal treprostinil in a nitrofen rat model, supporting the promise of this treatment approach for PH of CDH.

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Introduction
Congenital diaphragmatic hernia (CDH) occurs in 1 in 2000 to 4000 live births and is characterized by a diaphragmatic defect allowing herniation of the abdominal viscera into the chest, resulting in compression of the developing lungs and heart.1 The pathophysiology of CDH is characterized by pulmonary airway hypoplasia, pulmonary vascular remodelling and cardiac dysfunction.2 Clinically, this results in a challenging perinatal transition and a neonatal course complicated by impaired gas exchange, pulmonary hypertension (PH) and cardiac left- and right ventricular dysfunction. Despite sustained improvements in neonatal intensive care, the persistence of PH continues to represent the major source of CDH-related mortality and morbidity.3

CDH is most commonly diagnosed during routine prenatal ultrasonography and has been the focus of
therapeutic strategies targeting the affected foetus. Foetal endoluminal tracheal occlusion (FETO) is currently the only validated foetal therapy for CDH. Both TOTAL (Tracheal Occlusion To Accelerate Lung growth) randomized controlled trials (RCT) demonstrated improved survival in foetuses with moderate and severe lung hypoplasia who underwent FETO, however higher rates of preterm delivery were also observed in the FETO cohorts due to the invasive nature of fetoscopy. One single-centre retrospective study observed increased resolution of PH at one year of age in CDH survivors who underwent FETO, but this effect was inconsistently reported in other reports.

In animal models of CDH, tracheal occlusion (TO) restores normal lung size and airway structure, however this intervention has not been shown to reverse pathologic changes in the peripheral pulmonary vessels.

Prenatal interventions targeting pulmonary vascular development would be anticipated to have an additive therapeutic benefit in CDH. The ideal strategy would be non-invasive, with proven safety and efficacy for PH postnatally, and with no significant adverse effects on the mother and the foetus. We hypothesized that treprostinil, a prostacyclin-analogue which is clinically approved for the treatment of PH, may represent a promising candidate for prenatal treatment of CDH. Treprostinil has been shown to mediate its antihypertensive effects through regression of pulmonary vascular remodelling, raising the possibility of reversing the pathogenesis of PH during pulmonary development. Treprostinil is not teratogenic and is clinically used in neonatal CDH patients, making it a potential good candidate for clinical translation if proven safe and effective.

In this study, we assessed maternal and foetal tolerance to maternally administered treprostinil, defined the pharmacokinetics of transplacental passage of the drug, and characterized the effect of treprostinil on the pulmonary vasculature in the nitrofen rat model of CDH.

Method

We hereunder provide a summary of the Methods with additional details available in the online supplementary material.

Ethics

This study was approved by the Institutional Animal Care and Use Committee of Children’s Hospital of Philadelphia Research Institute (IACUC protocol number: 20-001374) and followed the ‘Animal Research: Reporting of In Vivo Experiments’ (ARRIVE) 2.0 guidelines for reporting on animal research. The animal vendors and facilities operated under national guidelines put forth by the United States Department of Agriculture (USDA).

Dose-finding and tolerance study

Time-dated pregnant Sprague-Dawley rats underwent olive oil (=placebo) gavage at gestational day (GD) 9. On GD 16, under general gas anaesthesia (Isoflurane) an osmotic pump (2ML4 ALZET®, Durect Corporation, Cupertino, California, USA) was implanted subcutaneously in the maternal neck scuff for continuous administration (Figure 1). We investigated four doses, i.e. 100, 400, 900 and 1500ng/kg/min. The first three doses were based on demonstrable tolerance in adult rats, while the 1500ng/kg/min dose was selected to maximize the likelihood of reaching target foetal concentrations of 0.5ng/mL, which is the lowest reported active concentration of treprostinil in neonates. Pregnant dams undergoing olive oil gavage (GD 9) and sham surgery (GD 16) were used as controls.

Dose-finding study outcomes

Dams and litters were euthanized after 120 hours (h) of treprostinil infusion (Figure 1). Maternal blood was collected via cardiac puncture, and foetal blood via decapitation using heparinized capillary tubes. Blood samples
were kept in EDTA tubes and centrifuged at 2000g, 4°C for 15 min. All foetal samples were pooled per litter. Supernatant plasma was harvested and stored at -80°C for concentration analysis with high-performance liquid chromatography and mass-spectrometry analysis in two batches (Centre for Clinical Pharmacology, Children’s Hospital of Philadelphia, Philadelphia, USA). The transplacental transfer ratio was calculated as the \[\frac{\text{average foetal concentration}}{\text{average maternal concentration}} \times 100\] at the 120h timepoint.

Tolerance study outcomes
Dams were monitored every other day for maternal tolerance, including the following: maternal weight gain per pup (body weight after therapy – body weight before therapy)/size of litter), pain using the validated rodent grimace score (RGS)\(^{15}\) and inspection for adverse effects specific to treprostinil administration, i.e. pump site induration and/or infection, diarrhoea and acral redness or flushing (pinnae, nose, paws, tail).\(^{16}\) Foetal tolerance indices were survival, foetal body weight (FBW) and organ weights (lung, heart, kidney and placenta), pulmonary alveolar morphometry and qualitative morphology of brain and placenta by a pathologist (A. Radu).

Efficacy study
To induce CDH in part of the offspring, pregnant Sprague-Dawley rats were gavage fed 100mg of nitrofen dissolved in 1mL of olive oil on GD 9, as previously described\(^{17}\) (Figure 2a). Only animals with a left-sided CDH were considered for the efficacy study, body weights and organ weight ratios of nitrofen-exposed CDH-negative pups were reported in online supplemental Figure 1. Control animals (WT) were gavaged with olive oil only. On GD 16 dams underwent either a sham surgery, or received a subcutaneous osmotic pump programmed to deliver either a lower dose of 900ng/kg/min or a higher dose of 1500ng/kg/min of treprostinil. On GD 21 (term GD 20-22), pups were delivered via C-section to undergo different outcome assessments (Figure 2b).

Gross anatomy
Foetuses destined for histological and molecular analysis were euthanized, inspected for gross developmental anomalies, and weighed. Lungs, placentas, hearts and left kidneys were also weighed and expressed relative to the FBW.

Histology & morphometry
Lungs were instilled with formalin (20cmH\(_2\)O pressure) through tracheal cannulation, processed, paraffin-embedded, sectioned and stained with haematoxylin-eosin (H&E) and \(\alpha\)-smooth muscle actin (\(\alpha\)-SmA). Vascular morphometry consisted of manual measurement of the proportionate medial wall thickness (%MWT) of pulmonary arterioles <100µm internal diameter, a surrogate marker of PH and the primary outcome of this study.\(^{18}\) Airway morphometry was assessed using a validated semi-automated software measuring alveolar septal volume density (V\(_{\text{sep}}\)), alveolar mean linear intercept (Lm), mean transsectional wall length (Lmw) and alveolar surface area density (S\(_{\text{air}}\)).\(^{19}\) Placentas, fetal brains and left kidneys were immersion-fixed in formalin, stained with H&E and processed for qualitative pathological evaluation.

Molecular analyses
Upon foetal euthanasia, left lungs were harvested, snap frozen and prepared for analysis. A TaqMan™ Array Rat Inflammation Panel was analysed with quantitative polymerase chain reaction (qPCR) on pooled samples (\(n = 4\)) per group. Individual samples were used to quantify prostaglandin E2-receptor (PTGER2), prostacyclin-receptor (PTGIR) and peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) using western blot (WB), and cyclic adenosine monophosphate (cAMP) with enzyme-linked immunosorbent assay (ELISA).

Ventilation
To assess pulmonary function, selected pups were sequentially ventilated for 20 min (Harvard Apparatus 683, Holliston, MA, USA) using a volume-controlled,
pressure-limited method as previously described. Airway pressure was continuously measured and the volume delivery adjusted to maintain the peak inspiratory pressure at 20 cmH₂O. After 20 min, tidal volumes were measured and pressure-volume curves constructed. Following foetal euthanasia, the presence of left-sided CDH was assessed.

Statistics
Continuous variables are reported as medians and range or means and standard deviations depending on the distribution of data assessed with D’Agostino-Pearson’s test. Groups were compared using one-way ANOVA and Dunnett’s multiple comparisons test. To adjust for litter-specific effects, organ and body weights were compared using nested analysis. Statistical significance was defined as \( p < 0.05 \). We determined that a sample size of 9 foetuses/group would provide a power of \( \geq 90\% \) with a two-sided type I error of 5% to detect a 20% reduction in %MWT. All analyses were performed using Prism (V9.2.0, Graphpad Software, San Diego, CA, United States). Numbers at the bottom of the bar graphs represent sample sizes (n) and error bars standard deviations unless stated otherwise in the figure legends.

Role of the funder
United Therapeutics Corporation provided the investigated drug and financial support for the study (ISS-2020-10879). The funder had no role in the design or conduct of the study, including the collection, analysis, and preparation of the data or the drafting, editing, review or approval of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funder.

Results
Treprostinil crosses the placenta and therapeutic foetal concentrations were not associated with toxicity
Figure 3 displays foetal and maternal plasma concentrations after 120h of continuous subcutaneous administration of treprostinil starting from GD16. Both maternal and foetal concentrations were dose-dependent. Only the highest dose (1500 ng/kg/min) attained foetal concentrations above the lower limit of the target range (>0.5 ng/mL) with an average transplacental transfer of 11.9% (± 4.1).

Weight gain per pup was not different for pregnant dams exposed to treprostinil (Figure 4a). At no point dams exposed to treprostinil experienced severe pain.
Figure 3. Pharmacokinetics. Treprostinil concentrations after 120h administration of 100, 400, 900 and 1500 ng/kg/min. Bar graphs depict median concentrations. N = 3 dams and litters per tested dose. The black dotted line (at 0.5ng/mL) represents the lowest reported active neonatal concentration. Mat = maternal compartment, Fet = fetal compartment.

Figure 4. Maternal and fetal tolerance. (a) Maternal weight gain corrected for litter size during late-phase gestation, displayed per group. Comparison did not reveal a difference between groups (P = 0.460, One-way ANOVA). (b) Maternal Rodent Grimace Scores (RGS) per group. The RGS-score assesses pain observing orbital tightening, nose/cheek flattening, ear changes and whisker changes. Datapoints represent scores per dam throughout the five days of treprostinil administration. RGS-scores were significantly increased only for the C.T.900 group (P = 0.002, marked ** on the graph, nested ANOVA with multiple comparison test [Dunnett’s test]). (c) Total body weight, (d) lung-to-body weight, (e) heart-to-body weight and (f) placenta-to-body weight per group. Absolute weights per group are detailed in online supplemental Figure 2.
Treprostinil rescues pulmonary vascular hyper muscularization, but does not impact airway morphometry or lung volumes in CDH

The pulmonary arteriolar proportionate medial wall thickness (%MWT) was higher in CDH than control foetuses receiving placebo (C.P.) (46.6±8.2% vs 26.9±6.2%, p < 0.0001, Dunnett’s test). Prenatal exposure to treprostinil reduced %MWT in CDH pups to 38.5±8.4% and 40.2±9.7% for the 900 and 1500ng/kg/min doses respectively (p < 0.0001, Dunnett’s test). In control pups prenatally exposed to treprostinil, an increase of %MWT was noted: 36.6±11.1% and 36.9±9.3% for the 900 and 1500ng/kg/min doses respectively (p < 0.0001, Dunnett’s test) (Figure 5a & b). The effect of treprostinil on %MWT was not dose-dependent. Lung-to-body weight ratios were significantly lower in all CDH groups, without change after treprostinil exposure (Figure 5c). Similarly, airway morphometric measures were different between CDH and control groups, regardless of treatment with treprostinil: alveolar septal volume density and mean transsectional wall length were higher in CDH pups, whereas the air space surface area and airspace intercept were lower (Figure 5d-h).

Treprostinil does not improve pulmonary mechanics in CDH

In all pups with CDH, tidal volumes were lower than control pups, regardless of treprostinil exposure (Figure 6a). Prenatal treprostinil did not result in decreased static elastance or increased static compliance in foetuses with CDH (Figure 6b & c).

Treprostinil upregulates pulmonary prostaglandin pathways in CDH

Levels of PTGER2, PTGIR and PPARγ tended to be lower in lungs of foetuses with CDH, and were markedly increased when exposed to 1500ng/kg/min treprostinil in both CDH and control lungs (Figure 7a-c, e). Similarly, mRNA-expression of PTGER2 and its upstream synthases (PTGIS&2) were lower in pups with CDH and restored to normal levels when treated with treprostinil (Figure 7f). In contrast to its synthase (PTGIS) which seemed unaffected, PTGIR’s mRNA expression increased with treprostinil. Cyclic adenosine monophosphate (cAMP), a secondary messenger in the prostaglandin pathway was upregulated in CDH, however normalized by prenatal treprostinil 1500ng/kg/min exposure (Figure 7d).

Discussion

This study demonstrates transplacental passage of maternal treprostinil into the foetal circulation, with reduction of pulmonary arteriolar muscular wall thickness in foetal rats with nitrofen-induced CDH. No adverse effects were observed in treated dams or pups. In foetuses with normal lung development however, prenatal exposure to treprostinil increased pulmonary arteriolar muscularization.

We first assessed transplacental transfer of treprostinil in normal pregnant dams. The variability of maternal treprostinil concentrations in our study has also been demonstrated clinically and characterized as a combination of inter-subject (14-26%) and diurnal variation (20-30%). Treprostinil is primarily metabolized by CYP2C8, a hepatic enzyme already expressed in first trimester human foetuses. The initial tested doses of treprostinil (100, 400 & 900ng/kg/min) were chosen based on previous studies in adult rats with pulmonary arterial hypertension, reporting good tolerance and clinical-range maternal plasma concentrations. Since treprostinil is clinically titrated to effect (pulmonary vasodilation) and not to specific concentrations, there is no formal target concentration range established in the neonate or foetus. We therefore decided to target foetal concentrations above 0.5ng/mL, which is the lowest neonatal therapeutic concentration reported in the literature. To attain such foetal concentrations, we additionally trialled a 1500ng/kg/min dose, while closely monitoring maternal toxicity signs. Only in the 900ng/kg/min dose, higher RGS scores were noted, however these scores were transient, occurred immediately after pump implantation and reassuringly were not found in the higher dosage group.

We then investigated the effect of prenatal treprostinil in rats with nitrofen-induced CDH, starting drug administration during the pseudoglandular phase of lung development (GD16). In humans, this phase coincides with the time point of diagnosis in two out of three cases. The pulmonary vascular changes associated with CDH (i.e. pulmonary arteriolar hyper muscularization), known to induce persistent PH after birth, are already present in utero. Given treprostinil’s strong anti-remodelling effect on cultured pulmonary arteriolar smooth muscle cells, we hypothesized it might attenuate CDH-related PH features in an...
Figure 5. Pulmonary morphology. (a) Proportionate Medial Wall thickness (%MWT) in pulmonary arterioles with an internal diameter <100µm. The %MWT in foetuses with CDH was higher than controls (46.6±8.2% vs 26.9±6.2%, p < 0.0001, Dunnett’s test). In CDH-foetuses exposed to treprostinil, there was a reduction of the %MWT (CDH.900: 38.5±8.4% (P < 0.0001), CDH.1500: 40.2±9.7% (P < 0.0001), Dunnett’s test). In normal pups exposed to treprostinil, the %MWT increased compared to C.P. (CT.900: 36.6±11.1% (P < 0.001), CT.1500: 36.9±9.3% (P < 0.001), Dunnett’s test). Black asterisks designate the degree of significance of difference in comparison to the C.P. group. Blue asterisks designate significance of difference compared to the CDH group. (b) Representative images of tracheally-instilled lungs stained with Haematoxylin-Eosin (H&E) and alpha Smooth muscle Actin (α-SmA). Scale bar = 100µm. (c) Lung-to-body weight ratio. Lung-to-body weight ratios were lower in pups with CDH and did not increase with treprostinil (CDH.900: P = 0.956, CDH.1500: P = 0.824, Dunnett’s test). Similarly, exposure of normal pups to treprostinil did not change lung-to-body weight ratios (CT.900: P = 0.746, CT.1500: P = 0.978, Dunnett’s test). Effects of treprostinol on total body weight, heart-to-body weight ratio, placenta-to-body weight ratio and kidney-to-body-weight ratio were reported in online supplementary Figure 5. (d) Quantiﬁed pulmonary airway morphology. V_{vsep} = volume density of alveolar septa, Lm = mean linear intercept of airspaces, Lm = mean transsectional wall length, S_{aer} = surface area density of air spaces. Statistical comparison of groups (one-way ANOVA, with multiple comparison test (Dunnett)) revealed signiﬁcant differences between all CDH groups when compared to a healthy control group (C.P.). Exposure of pups with CDH to treprostinol did not generate a statistically signiﬁcant difference compared to the untreated diseased pups (CDH). (e) Representative images of tracheally-instilled lungs stained with Haematoxylin-Eosin (H&E). Scale bar = 50µm. Levels of signiﬁcance were indicated on the graphs as follows: * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.
in-vivo setting. At the structural level, treprostinil indeed did reduce muscularization of pulmonary arterioles, as evidenced by the lower %MWT. This effect was observed with both the high and low treprostinil dose and was not dose/concentration-dependent, suggesting that foetal concentrations lower than 0.5 ng/mL are already sufficient to exert an anti-remodelling effect. Conversely, in healthy pups with normal lung development, prenatal exposure to treprostinil increased %MWT and thus the hypertensive phenotype. Although this seems counterintuitive, such phenomenon has been observed before. Sildenafil (a phosphodiesterase 5 inhibitor) exposure of foetal rats and rabbits with normal lung development, also holding an anti-remodeling and vasodilatory effect, increased %MWT and reduced pulmonary vascular branching. While the pathophysiological mechanism is not elucidated, that observation may provide an explanation for the findings of the Sildenafil TheRapy In Dismal prognosis Early onset fetal growth Restriction (STRIDER) trial. In that multicentre-RCT, which investigated the effect of prenatal sildenafil on uterine vasodilation and foetal growth in the setting of early-onset and severe intra-uterine growth restriction, one study arm (Dutch trial) was concluded early due to an observed association between sildenafil exposure and severe persistent PH of the new-born. While this effect was not confirmed in other trial arms, prenatal use of sildenafil was since banned from further application despite our appeal to keep its indication for CDH. Similarly, we conclude that based on our

![Figure 6. Pulmonary function.](image-url)
findings any investigation of prenatal treprostinil in conditions with normal lung development should be avoided, at least until the pathophysiologic mechanism is better understood.

Despite the assumption of a close interaction between airway and blood vessels and therefore the potential of prenatal interventions to improve vascular and airway development,17,35,36 as was observed with sildenafil,17,37,38 we did not observe an impact on the pulmonary parenchyma in our study. While treprostinil and sildenafil act on different pathways (prostacyclin & nitric oxide pathways respectively), the timing of administration may also have played a role. Studies assessing prenatal prostacyclin-analogues in the nitrofen-rat model have demonstrated improved airway development only when administered early, i.e. during the embryonic phase (GD 9–5).39,40

On a molecular level, treprostinil binds to PTGER2 and PTGIR with very high affinity.41 Both receptors mediate the anti-remodelling effect of treprostinil on pulmonary arteriolar smooth muscle cells.42 While protein levels of the two receptors were expressed at lower levels in CDH lungs, increased expression was observed in treprostinil-treated lungs. Similarly, protein levels of PPARγ, a downstream transcription factor implicated in treprostinil-induced anti-remodelling, increased following treprostinil exposure.43 Levels of cAMP, another key downstream factor in the prostaglandin pathway, were upregulated in CDH and normalized by prenatal treatment with treprostinil. In vitro studies have suggested that cAMP exerts an antiproliferative effect on pulmonary arterial smooth muscle cells through activation of Smad1/5, inhibition of Smad6 and platelet derived growth factor (PDGF)-BB.44 In addition, cAMP also reduces extracellular matrix deposition, another hallmark of pulmonary arterial hypertension, through reduction of lung fibroblast secretion of tumor growth factor (TGF)-β and reduction of collagen type I and fibronectin deposition.45

Collectively, the molecular data suggests that treatment with treprostinil helps reverse the biochemical changes characteristic of CDH, however it may disrupt
homeostasis itself in normal lungs as supported by the structural, morphometric vascular pulmonary findings observed in this study.

Several other drugs have demonstrated potential to improve PH-features in different animal models of CDH.1,46 There are however many concerns, either because of teratogenicity (imatinib), immaturity of the foetal metabolizing pathways (tadalafil), or more generic lack of safety information (ONO-1301SR, BAY41-2272 & BAY60-2770). Treprostinil however does not have teratogenic effects in several animal studies, even when administered during embryogenesis, which is much earlier than the envisioned prenatal use for CDH.10 There is also increasing experience and safety data available regarding neonatal use of treprostinil, notably in CDH patients.14,47-50 Furthermore, prenatal treprostinil has not yet been investigated for any indication, allowing targeted research and avoiding undesirable side effects such as those reported in the STRIDER-trial, with administration of sildenafil in foetuses with normal lung development resulting in increased pulmonary vascular complications. These features make treprostinil a promising candidate for prenatal clinical application.

Caveats and limitations
This study does have several limitations deserving acknowledgement. Firstly, although important, the pharmacokinetic section of our study succinctly demonstrating transplacental transfer at different maternal concentrations. In anticipation of clinical translation, more in-depth pharmacokinetic profiling should be performed, ideally using a human placenta perfusion model and/or a clinical phase I study.23,24 Furthermore, although the experimental nitrofen-induced CDH model recapitulates the pulmonary abnormalities present in human CDH, the mechanism by which nitrofen induces the diaphragmatic defect is not fully understood.16-17 The herbicide also affects overall foetal growth suggesting potential systemic effects (Online supplemental Figure 1).17 Therefore, our results should be validated in higher animal models such as the rabbit and sheep model of diaphragmatic hernia, which mimic human lung development more closely. Additionally, there is no long-term toxicity data on treprostinil, and in this study direct measurements of neonatal pulmonary pressures and blood gasses were not possible due to the severe degree of pulmonary hypoplasia impeding postnatal survival as well as the small dimensions of the pups. Finally, we opted for continuous subcutaneous drug administration for practical reasons, as it has the same bioavailability as intravenous administration, however that will not be practical clinically. An extended-release oral compound (orenitram) of treprostinil is approved and available for eventual clinical use, and the effects of such a dosing regimen will have to be documented in future studies.31

In conclusion, this study demonstrates that treprostinil crosses the placenta, is well tolerated by both the mother and the foetus, and effectively attenuates pulmonary vascular hypermuscularization in rats with nitrofen-induced CDH. While treprostinil holds promise as a prenatal treatment strategy in CDH, we think it should be avoided in normal lungs.

Contributors
Design of the study: FRDB, CGH, EAP
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Critical review of the manuscript: FRDB, CGH, FMR, HH, JDP, EAP
Decision to submit the manuscript: FRDB, EAP
All authors read and approved the final version of this manuscript.

Data sharing statement
All original data are available on request from the corresponding author.

Declaration of interests
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Supplementary materials
Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104106.
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