Complementary Effect of Maternal Sildenafil and Fetal Tracheal Occlusion Improves Lung Development in the Rabbit Model of Congenital Diaphragmatic Hernia

Francesca Maria Russo, PhD,†✉ 0000-0002-5029-7899 Marina Gabriela Monteiro Carvalho Mori Da Cunha, PhD,* 0000-0001-6658-7797 Julio Jimenez, PhD,*‡ 0000-0001-6260-2814 Flore Lesage, PhD,* 0000-0001-8578-9650 Mary Patrice Eastwood, PhD,* 0000-0002-6437-8972 Jaan Toelen, PhD,* 0000-0001-9339-5408 and Jan Deprest, PhD*†§✉ 0000-0002-4920-945X*

Objective: To evaluate the effect of combining antenatal sildenafil with fetal tracheal occlusion (TO) in fetal rabbits with surgically induced congenital diaphragmatic hernia (CDH).

Background: Although antenatal sildenafil administration rescues vascular abnormalities in lungs of fetal rabbits with CDH, it only partially improves airway morphometry. We hypothesized that we could additionally stimulate lung growth by combining this medical treatment with fetal TO.

Methods: CDH was created on gestational day (GD)23 (n=54). Does were randomized to receive either sildenafil 10 mg/kg/d or placebo by subcutaneous injection from GD24 to GD30. On GD28, fetuses were randomly assigned to TO or sham neck dissection. At term (GD30) fetuses were delivered, ventilated, and finally harvested for histological and molecular analyses. Unoperated littermates served as controls.

Results: The lung-to-body-weight ratio was significantly reduced in sham-CDH fetuses either ($1.2 \pm 0.3\%$ vs $2.3 \pm 0.3\%$ in controls, $P=0.0003$). Sildenafil had no effect on this parameter, while CDH fetuses undergoing TO had a lung-to-body-weight ratio comparable to that of controls ($2.5 \pm 0.8\%$, $P<0.0001$). Sildenafil alone induced an improvement in the mean terminal bronchiolar density (2.5 ± 0.8 br/mm^2 vs 3.5 ± 0.9 br/mm^2, $P=0.043$) and lung mechanics (static elastance 61 ± 36 cmH_2O /mL vs 113 ± 40 cmH_2O/mL, $P=0.008$), but both effects were more pronounced in fetuses undergoing additional TO (2.1 ± 0.8 br/mm^2, $P=0.001$ and 31 ± 9 cmH_2O/mL, $P<0.0001$ respectively). Both CDH-sham and CDH-TO fetuses treated with placebo had an increased medial wall thickness of peripheral pulmonary vessels ($41.9 \pm 2.9\%$ and $41.8 \pm 3.2\%$, vs $24.0 \pm 2.9\%$ in controls, $P<0.0001$). CDH fetuses treated with sildenafil, either with or without TO, had a medial thickness in the normal range ($29.4\% \pm 2.6\%$). Finally, TO reduced gene expression of vascular endothelial growth factor and surfactant protein A and B, but this effect was counteracted by sildenafil.

Conclusion: In the rabbit model for CDH, the combination of maternal sildenafil and TO has a complementary effect on vascular and parenchymal lung development.

Keywords: congenital diaphragmatic hernia, fetal surgery, fetal therapy, lung development, tracheal occlusion

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Congenital diaphragmatic hernia (CDH) is a developmental anomaly that affects 1:2500 liveborns.[1] As a consequence of the defect, the abdominal viscera occupy the thoracic cavity and create a mass effect on the growing lung. This results in impaired development of both the airways and vasculature, since the growth of the pulmonary vessels is inextricably linked with that of the airways.[2] Therefore, newborns with CDH suffer from a combination of respiratory insufficiency and pulmonary hypertension (CDH-associated pulmonary hypertension (CDH-PH)),[3] which leads to postnatal death in 30% of cases and to significant morbidity in the survivors, despite optimal postnatal care.[4] Such a poor postnatal outcome prompts the need for new strategies that can effectively treat or prevent pulmonary complications in newborns with CDH. This would ideally already start in utero, to prevent the structural developmental changes that lead to such complications after birth.

Animal studies demonstrated that transplacental sildenafil promotes pulmonary angiogenesis and inhibits pulmonary artery remodeling in CDH fetuses.[5–7] Despite the remarkable functional and morphologic effect on the vascular compartment, sildenafil only partially improves airway morphometry and lung function, and does not increase lung size. Fetal lung growth can be stimulated by tracheal occlusion (TO), which clinically seems to improve survival rates in fetuses with severe pulmonary hypoplasia.[8] TO is currently being investigated in a randomized clinical trial,[9] though early data question its efficacy in reducing the risk for CDH-PH.[10] We hypothesized that we could further stimulate parenchymal and vascular lung development by combining maternal sildenafil and fetal TO in fetal rabbits with surgically induced CDH.

METHODS

Study Design

This study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine, KU Leuven, Belgium (Project number: P014/2013) and follows the ''Animal Research: Reporting of In Vivo Experiments'' (ARRIVE) guidelines for reporting on animal research.

From the *Cluster Woman and Child, Department of Development and Regeneration, Biomedical Sciences, KU Leuven, Leuven, Belgium; †Department of Obstetrics and Gynecology, University Hospitals of Leuven, Leuven, Belgium; ‡Department of Obstetrics and Gynaecology, Clínica Alemana University Desarrollo, Santiago, Chile; and §Department of Obstetrics and Gynecology, Institute of Women's Health, University College London, London, UK.

✉francescamaria.russo@kuleuven.be, jan.deprest@uzleuven.be.

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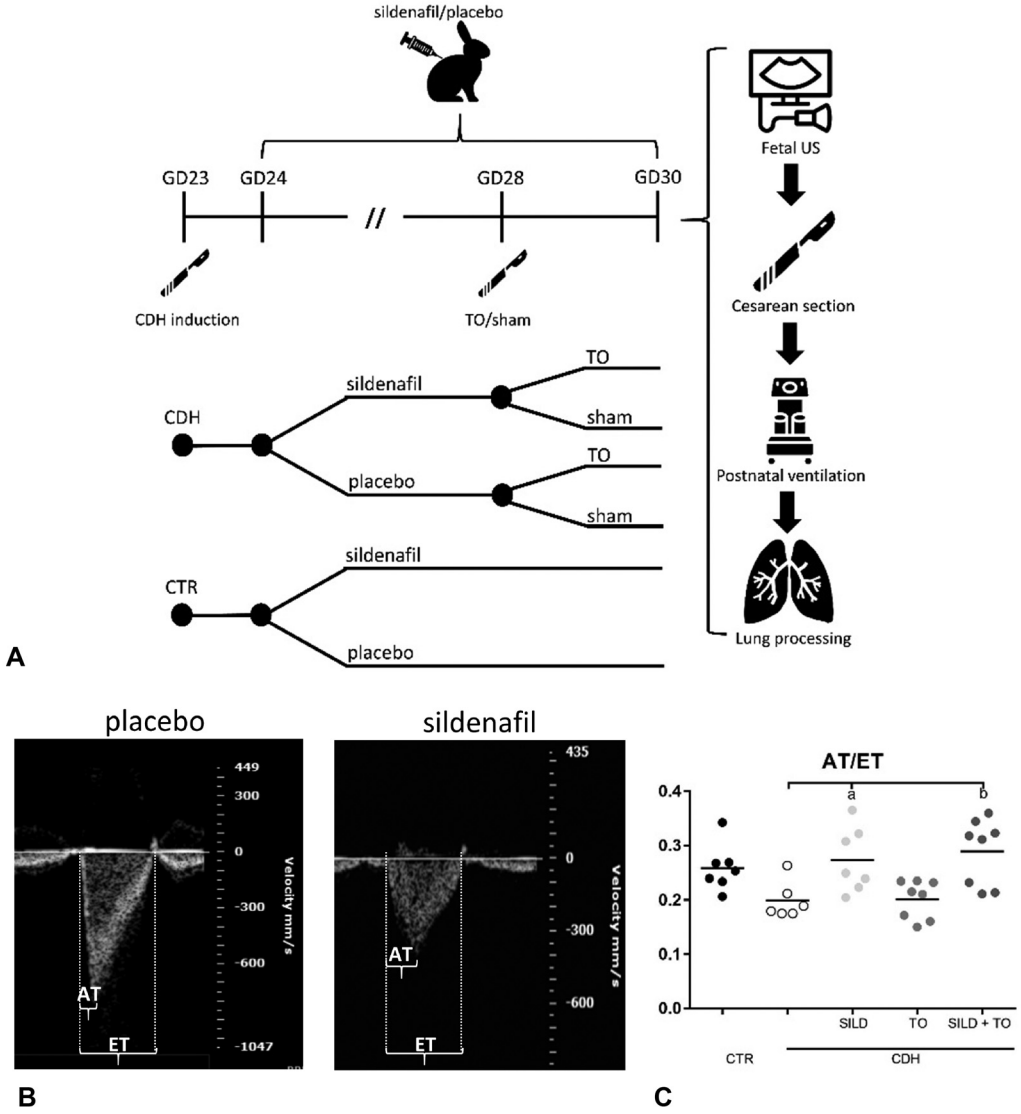

FIGURE 1. Feto-maternal interventions and their functional effect on the fetal pulmonary circulation. A, Overview of the study design and of the study groups. B, Representative images of the Doppler waveforms of the main pulmonary artery in a fetus treated with placebo and in one treated with sildenafil. C, Main pulmonary artery acceleration time over ejection time (AT/ET) ratio in the different study groups. Large horizontal bars in the middle indicate the mean. (A) $P=0.03$, (B) $P=0.006$. US indicates ultrasound; SILD: sildenafil.

The study design is displayed in Figure 1A. Time mated pregnant hybrid Denderdmonde-New Zealand white rabbits underwent maternal-fetal surgery on gestational day (GD) 23 for induction of left-sided CDH, as previously described[11] (Supplemental Methods, http://links.lww.com/SLA/C150). In each doe, a maximum of 3 fetuses were operated. Briefly, after hysterotomy, the fetal left foreleg was exteriorized and a left thoracotomy performed. The left hemi-diaphragm was then incised, allowing intrathoracic liver herniation. The fetus, uterus, and at the end the maternal abdominal wall were closed. After surgery, does were randomly assigned to receive a daily subcutaneous injection of either placebo (sterile saline, 7 mL) or sildenafil citrate (10 mg/kg) (Teva, Antwerpen, Belgium)[7] from GD24 until GD30 by using randomization software (GraphPad Software). A second fetal surgery was performed on CDH fetuses on GD28. After confirmation of viability, CDH-fetuses were randomized to TO or sham neck dissection. As previously described,[12] the fetal head and neck were exposed through hysterotomy and the fetal trachea was carefully dissected. For fetuses randomized to TO, a double ligature of the trachea was performed. In both the groups, the skin was then closed and the fetal head was repositioned inside the uterus, which was closed.

On GD30, following laparotomy, fetal echocardiography was performed as detailed below. Pups were then delivered and ventilated for invasive lung function testing. To avoid potential confounders, we compared ventilatory parameters in the first 5 to 10 minutes after intubation, once a steady state was confirmed by 3 consecutive measurements in the same range. After this, neonatal and maternal euthanasia was performed. The fetal lungs were collected for (immuno)-histological and PCR analysis. Fetal body weight and

total fetal lung weight were measured, allowing calculation of the lung-to-body-weight ratio (LBWR), which is indicative of the degree of lung growth.

Nonoperated littermates of comparable size to the operated ones served as controls (CTR), both in the sildenafil and placebo group. This design led to 6 different study groups: CDH-placebo-sham (further called CDH), CDH-sildenafil-sham (CDH-sildenafil), CDH-placebo-TO (CDH-TO), CDH-sildenafil-TO, CTR-sildenafil, and CTR-placebo.

All analyses were performed by an experienced observer (F.M.R.) who was as much as possible blinded to the nature of the experimental procedure and who was familiar with measuring these outcomes. Complete blinding is anatomically impossible for fetuses who had a CDH induced and those not.

Fetal Echocardiography Measurements

Fetal echocardiography was performed prior to cesarean section on GD30, 60 to 90 minutes after the last injection of sildenafil or placebo (Supplemental Methods, http://links.lww.com/SLA/C150), to determine the functional effect of sildenafil and/or TO on the prenatal pulmonary circulation. As a proxy of vasodilation of the downstream circulation, we measured the acceleration-over-ejection time (AT/ET) ratio of the main pulmonary artery and the pulmonary artery branch. AT was defined as a period from the onset to the peak of the rapid ''spike'' blood flow. ET was measured from the onset of the rapid ''spike'' to the onset of the brief reverse blood flow. The AT/ET ratio was calculated to eliminate the influence of HR[13] (Fig. 1B). This parameter is inversely related to the right ventricle systolic pressure.[14] The velocity time integral of the blood flow and HR were evaluated in the spectral Doppler waveform of the main pulmonary artery, allowing the calculation of the right cardiac output: $RCO = (\pi/4) \times D^2 \times VTI \times HR$.[15]

Measurements of Ventilatory Mechanics

Immediately after cesarean section, the pups were anesthetized and underwent invasive lung function testing using a forced oscillation technique with the FlexiVent system. The following parameters were assessed using the pressure–volume perturbation: total lung capacity (estimation of inspiratory capacity), static compliance (elastic recoil pressure of the lung at a given lung volume), and static elastance (1/compliance).[16]

Lung Histology

The following indices of airway morphometry were measured (Supplemental Methods, http://links.lww.com/SLA/C150): mean terminal bronchiolar density (MTBD, inversely related to the number of alveoli supplied by each bronchiole), mean linear intercept (Lm, related to alveolar size), mean wall transection length (Lmw, index of the thickness of alveolar septa), and mean linear intercept of parenchymal airspace (Lma, index of size of the airspaces).[17,18] For vascular morphometry, the proportionate medial thickness (%MT) and proportionate adventitial thickness (%AT) were calculated.[17]

Lung Immunohistochemistry

A costaining for CD31 and α-smooth muscle actin was used to calculate the percentage of muscularized vessels for vessels with ED $\leq$100 μm. Immunoreactivity for vascular endothelial growth factor (VEGF) and its receptor Flk in lung parenchyma was determined with ImageJ software (1.48 v, Wayne Rasband, National Institutes of Health, Bethesda, USA). For each slide, 20 nonoverlapping ×400 fields not including vessels were randomly selected for quantification. The digital color images were segmented (color deconvolution plug in) and further binarized to measure the percentage of the area stained in brown with an automated algorithm. The results were expressed as percentages of brown-stained area per field. In sections stained for surfactant protein B (SP-B), the number of positive cells over the total number of cells was counted in 20 nonoverlapping ×400 fields not including vessels or conducting airways. Further details on immunohistochemistry are provided in the Supplemental Methods, http://links.lww.com/SLA/C150.

qRT-PCR

The expression of VEGF, Flk, SP-A, B, and C was analyzed in triplicate for 5 rabbits per treatment group (Supplemental Methods and Supplemental Table 1, http://links.lww.com/SLA/C150). Relative quantitation was determined using the comparative Ct method.

VEGF Protein Quantification in Lung Tissue

Enzyme-linked immunoassay assay (ELISA) was carried out to quantify rabbit VEGF-A protein in lung homogenates (Supplemental Methods, http://links.lww.com/SLA/C150).

Statistical Analysis

Lung function was defined as primary outcome. Sample size calculation according to the previously published literature revealed that a total of 7 CDH fetuses per treatment group would provide a power of $\geq$80% with a 2-sided type I error of 5% to detect a 40% increase in lung compliance. This sample size is also powered to detect a 15% decrease in %MT (secondary outcome).[17] The D'Agostino-Pearson normality test was used to assess the distribution of variables. Since all variables were normally distributed, results are presented throughout the text as mean ± standard deviation. One-way analysis of variance was used to evaluate differences between treatment groups; multiplicity-adjusted P values were calculated using the Dunnett test. Fisher exact test was used for dichotomous variables. A 2-tailed $P<0.05$ was considered significant. Throughout the manuscript, comparisons are presented between CDH-placebo fetuses and the other study groups. Comparisons with CTR-placebo fetuses are displayed in Supplemental Table 2, http://links.lww.com/SLA/C150. All comparisons were performed using Prism for Windows V.6.0 (GraphPad software, San Diego, CA).

RESULTS

Sildenafil Does Not Affect Fetal Survival

We surgically induced CDH in 64 rabbit fetuses on GD23. An overview of the study groups is provided in Supplemental Table 3, http://links.lww.com/SLA/C150. In total, 39 CDH fetuses (61%) survived until delivery with no difference between placebo- and sildenafil-exposed pups (61% in both the groups, $P=1$). In all, the presence of a diaphragmatic defect and liver herniation was confirmed at autopsy. Survival rates in controls were also independent of maternal treatment (71% vs 70% for placebo and sildenafil respectively, $P=1$). Out of a total of 114 unoperated littermates alive at harvesting, 17 (8 placebo exposed and 9 sildenafil exposed) were used as CTR.

Sildenafil, But Not TO, Has a Measurable Functional Effect on the Fetal Pulmonary Vasculature

The AT/ET ratio of the main pulmonary artery was not different between CDH and CTR fetuses. However, sildenafil exposure induced an increase in the AT/ET ratio in all the groups, as compared with placebo exposure (Fig. 1B, C). TO was not associated with measureable changes in AT/ET. No difference in right cardiac output and in the Doppler waveform of the right pulmonary artery branch was observed among study groups (Supplemental Figures 1A and 1B, http://links.lww.com/SLA/C151).

Sildenafil Partially Improves Lung Function and Parenchymal Histology, But the Effect Is More Pronounced After TO

In CDH fetuses, static compliance was significantly reduced whereas static elastance significantly increased compared with CTR (0.009 ± 0.003 vs 0.056 ± 0.011 mL/cmH_2O, $P<0.0001$ and 113.8 ± 40.3 vs 18.4 ± 3.6 mH_2O/mL, $P<0.0001$). CDH fetuses also had a significantly decreased total lung capacity (0.22 ± 0.08 vs 1.41 ± 0.25 mL, $P<0.0001$). Exposure of CDH fetuses to sildenafil led to a significant improvement in all these parameters. However, such improvement was more pronounced in fetuses treated with TO, with or without sildenafil (Fig. 2A).

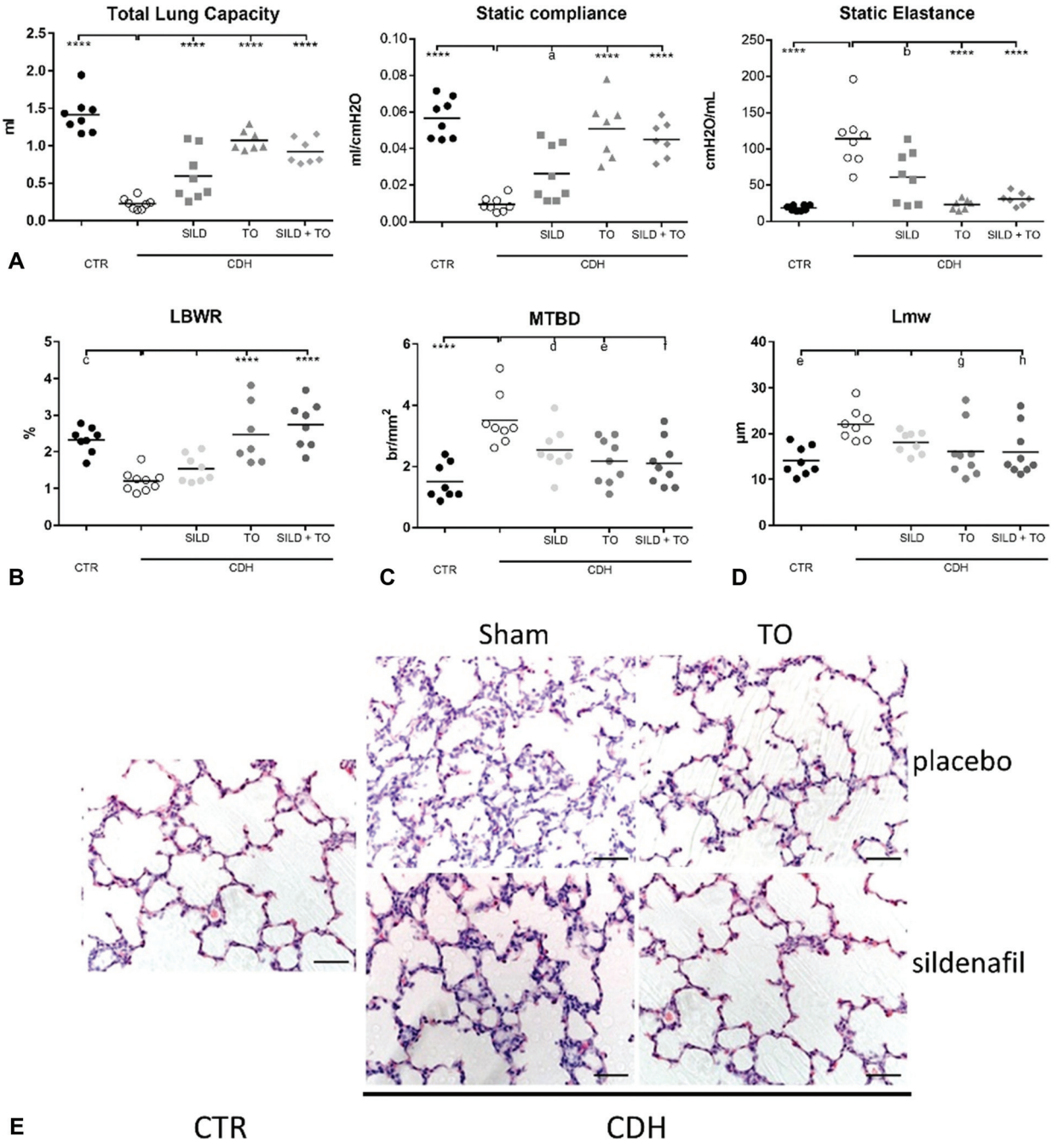

FIGURE 2. Effect of sildenafil and TO on parenchymal development. A, Measures of lung mechanics. B, Lung-to-body weight-ratio (LBWR). C, Mean terminal bronchiolar density (MTDB). D, Mean wall transection length (Lmw). E, Representative H&E stained sections showing the differences in lung parenchyma among the 6 study groups. Scale bars: 50 um. In the graphs, large horizontal bars in the middle indicate the mean. (A) $P = 0.031$, (B) $P = 0.008$, (C) $P = 0.0003$, (D) $P = 0.043$, (E) $P = 0.003$, (F) $P = 0.001$, (G) $P=0.026$, (H) $P=0.024$, $****P<0.0001$. SILD indicates sildenafil.

Untreated CDH fetuses had a significantly lower LBWR compared with CTR (1.2% ± 0.3% vs 2.3 ± 0.3%, P=0.0003). Sildenafil alone had no effect on this parameter, while TO, alone, or in combination with sildenafil, led to a LBWR comparable to that of CTR fetuses (Fig. 2B). There was no difference in fetal body weight among different study groups (Supplemental Figure 1C, http://links.lww.com/SLA/C151).

Functional changes caused by CDH were paralleled by histologic findings in the lung parenchyma. The MTBD was significantly higher in CDH fetuses as compared with CTR (3.51 ± 0.30 br/mm^2 vs 1.49 ± 0.21 br/mm^2, P<0.0001). Sildenafil treatment alone significantly improved this parameter (2.53 ± 0.76 br/mm^2, P=0.043 compared with CDH), even though this effect was more pronounced after TO, with or without sildenafil (Fig. 2C, E). CDH fetuses also had increased Lmw (22.0 ± 3.5 μm vs 14.1 ± 3.2 μm, P=0.003). The Lmw was not affected by sildenafil, but it was significantly reduced by TO, alone or in combination with sildenafil (Fig. 2D, E). The other airway morphometric measures were comparable in the 6 study groups (Supplemental Figures 2A and 2B, http://links.lww.com/SLA/C152).

Sildenafil treatment had no effect on parenchymal structure and lung function in CTR fetuses (Supplemental Table 2, http://links.lww.com/SLA/C150).

Sildenafil Rescues the Effect of TO on Surfactant Protein Expression

In TO-placebo-CDH fetuses, the expression of SP-A and SP-B was significantly reduced as compared with sham. However, this change was reverted by sildenafil (Fig. 3A). Immunohistochemistry for SP-B confirmed a reduced expression in TO-placebo-CDH fetuses at the protein level, and normal expression in CDH fetuses treated with sildenafil (Fig. 3B, C). There was no difference in RNA expression of SP-C among CDH groups.

Sildenafil, But Not TO, Prevents Pulmonary Vascular Remodeling

CDH fetuses had a significantly thicker media as compared with CTR (%MT 41.9 ± 2.9% vs 24.0 ± 2.9%, P<0.0001). TO alone had no effect on %MT, while sildenafil, alone or in combination with TO, reverted this parameter to the normal range (Fig. 4A, C). We further categorized the vessels into 3 groups according to the external diameter (ED), ie, intra-acinar vessels with ED<30 μm and ED between 30 and 60 μm, and preacinar with ED between 60 and 100 μm, to assess effects on different size of vessels.[19] Even though present in all categories of vessels, the difference between CDH and CTR fetuses and the effect of sildenafil were more pronounced in the intra-acinar vessels (Supplemental Figure 2C, http://links.lww.com/SLA/C152).

No difference in the %AT was observed among study groups (Fig. 4B).

In line with observations on increased medial thickness, the proportion of muscularized vessels was significantly higher in CDH fetuses (43 ± 3% vs 21 ± 8% in CTR, P<0.0001). Both sildenafil and TO alone significantly decreased this parameter, but only sildenafil treatment, with or without TO, led to a degree of muscularization

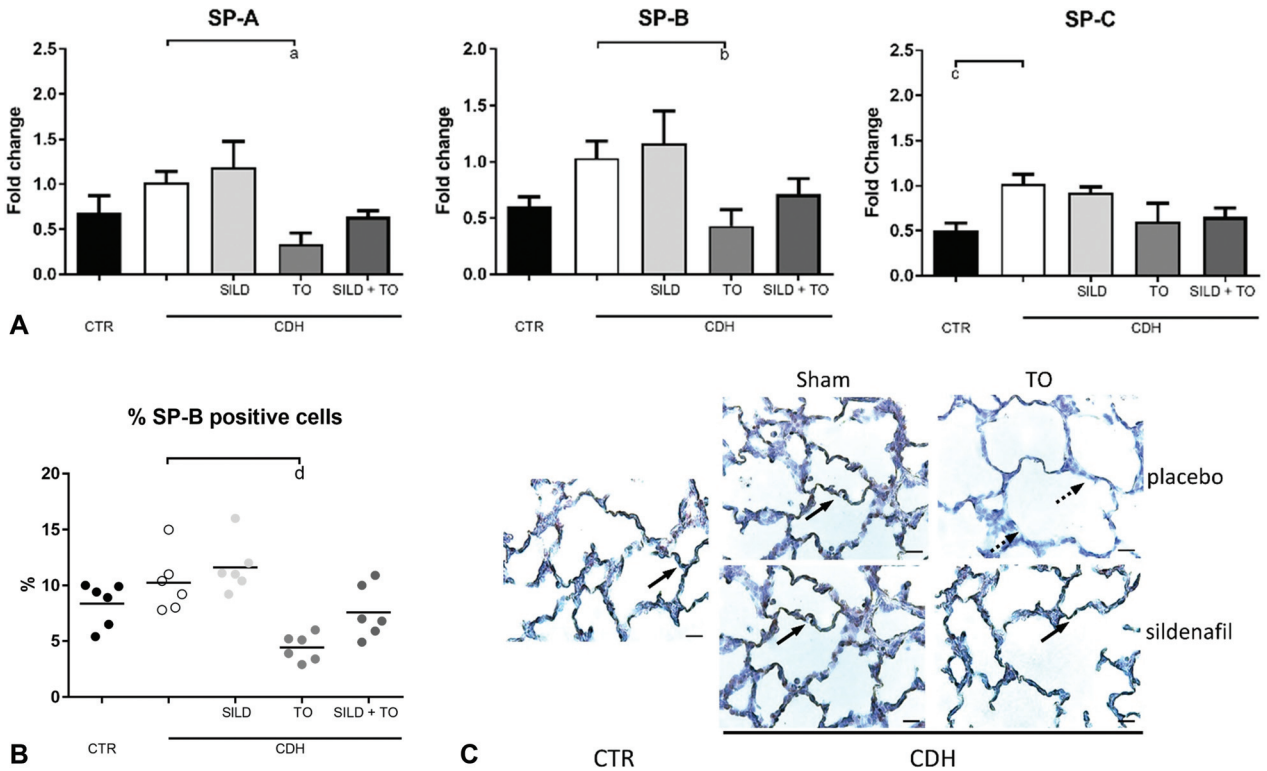

FIGURE 3. Effect of sildenafil and TO on surfactant production. A, Gene expression for surfactant proteins (SP); histograms indicate the mean, error bars indicate standard deviation; n=4 per group. B, Percentage of SP-B positive cells in lung parenchyma. Large horizontal bars in the middle indicate the mean. C, Representative immunohistochemical staining for SP-B (in black, full arrow): lung parenchyma is extensively positive for SP-B in all groups with the exception of CDH-TO fetuses, where SP-B negative areas (dotted arrow) are frequently observed. Scale bars: 20 μm. (A) P=0.042, (B) P=0.029, (C) P=0.023, (D) P=0.0003. SILD indicates sildenafil.

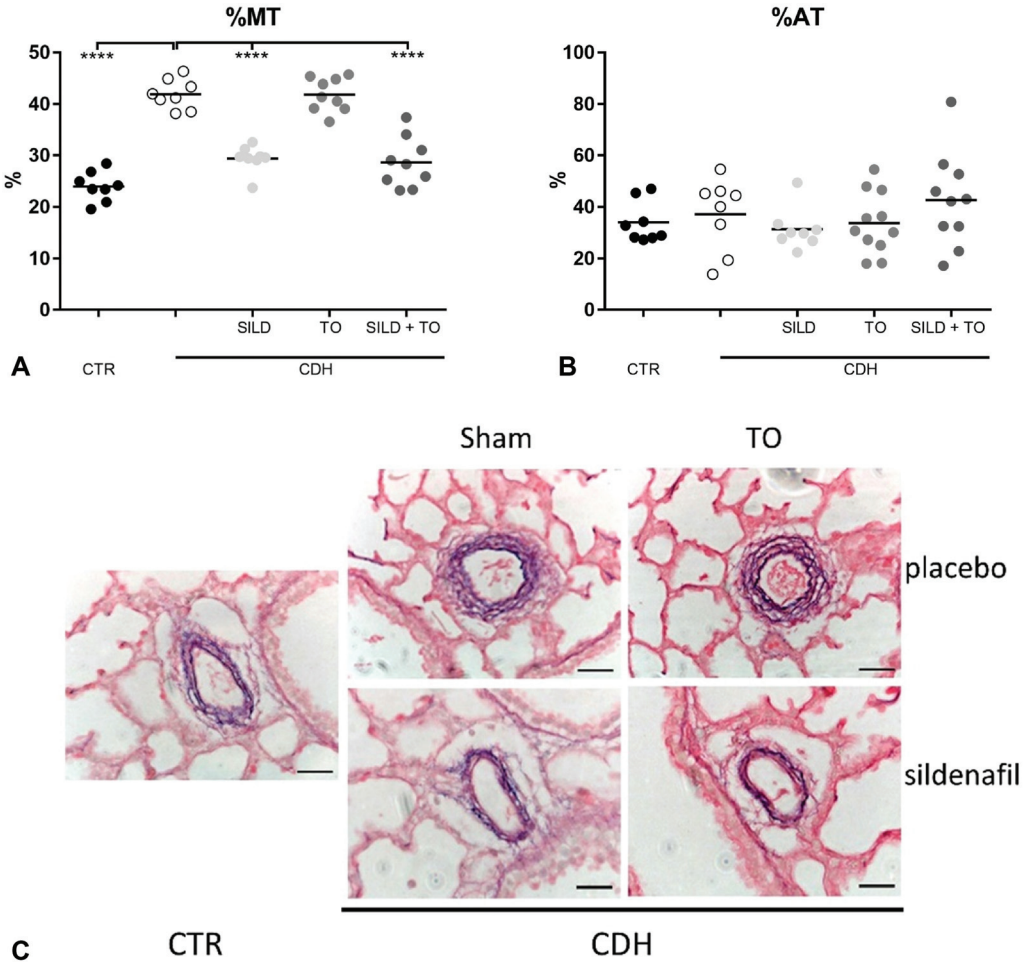

FIGURE 4. Effect of sildenafil and TO on vascular morphometry for vessels with external diameter <100 μm. A, Proportionate medial thickness (%MT). B, Proportionate adventitial thickness (%AT). C, Representative Miller stained sections showing the differences in %MT among the study groups. Scale bars: 20 μm. In the graphs, large horizontal bars in the middle indicate the mean. ****$P<0.0001$. SILD indicates sildenafil.

comparable to CTR (Fig. 5A, C). In CDH, muscularization extended to more peripheral vessels. The mean diameter of the smallest muscularized vessel was significantly lower in CDH than in CTR (12 ± 2 vs 21 ± 3 μm, $P<0.0001$). Again, an improvement was observed in CDH fetuses undergoing TO (16 ± 3 μm, $P=0.04$), but the improvement was more evident in fetuses treated with sildenafil, with or without TO (Fig. 5B).

Sildenafil and TO Have a Synergistic Effect in Stimulating VEGF Expression

qRT-PCR showed a significant reduction in VEGF expression in TO-placebo-CDH fetuses as compared with CDH. However, when sildenafil was combined with TO, VEGF expression was increased to levels significantly higher than those in CDH (Fig. 5D). At the protein level, there was no difference among the study groups in the expression of VEGF in the whole lung, as evidenced by ELISA (Fig. 5E). However, CDH was associated with a decrease in the immune-reactivity for VEGF in pulmonary parenchyma compared with CTR ($1.6 \pm 0.5\%$ vs $11.4 \pm 9\%$, $P=0.026$). Sildenafil alone significantly increased the percentage of VEGF-positive tissue ($7.0 \pm 5.3\%$, $P=0.049$). This percentage was even higher in fetuses treated with the combination of sildenafil and TO ($11.4 \pm 9.5\%$, $P<0.0001$) (Fig. 5F). Sildenafil was also associated with an increase in the immunoreactivity for VEGF receptor Flk in the lung parenchyma, both in sham fetuses and in fetuses undergoing TO ($56.3 \pm 2.3\%$ and $55.4 \pm 7.3\%$ respectively vs $19.5 \pm 2.8\%$, $P<0.0001$). However, no difference in Flk RNA expression was observed between the study cohorts (Supplemental Figure 3, http://links.lww.com/SLA/C153).

DISCUSSION

This study demonstrates that maternal sildenafil and fetal TO have a complementary effect on lung development in fetal rabbits with surgically induced CDH. Sildenafil reverts the vascular changes to normal but has a limited effect on the airways, whereas TO mainly acts on lung parenchyma.

In line with previous studies in humans and in animal models,[13,20–22] in our study TO increased lung size (as evidenced by the increased LBWR) and induced maturation of the lung parenchyma (as evidenced by the lower MTBD and Lmw). Functionally, this led to a striking improvement in passive pulmonary mechanical properties. However, this intervention alone had only a mild effect on lung

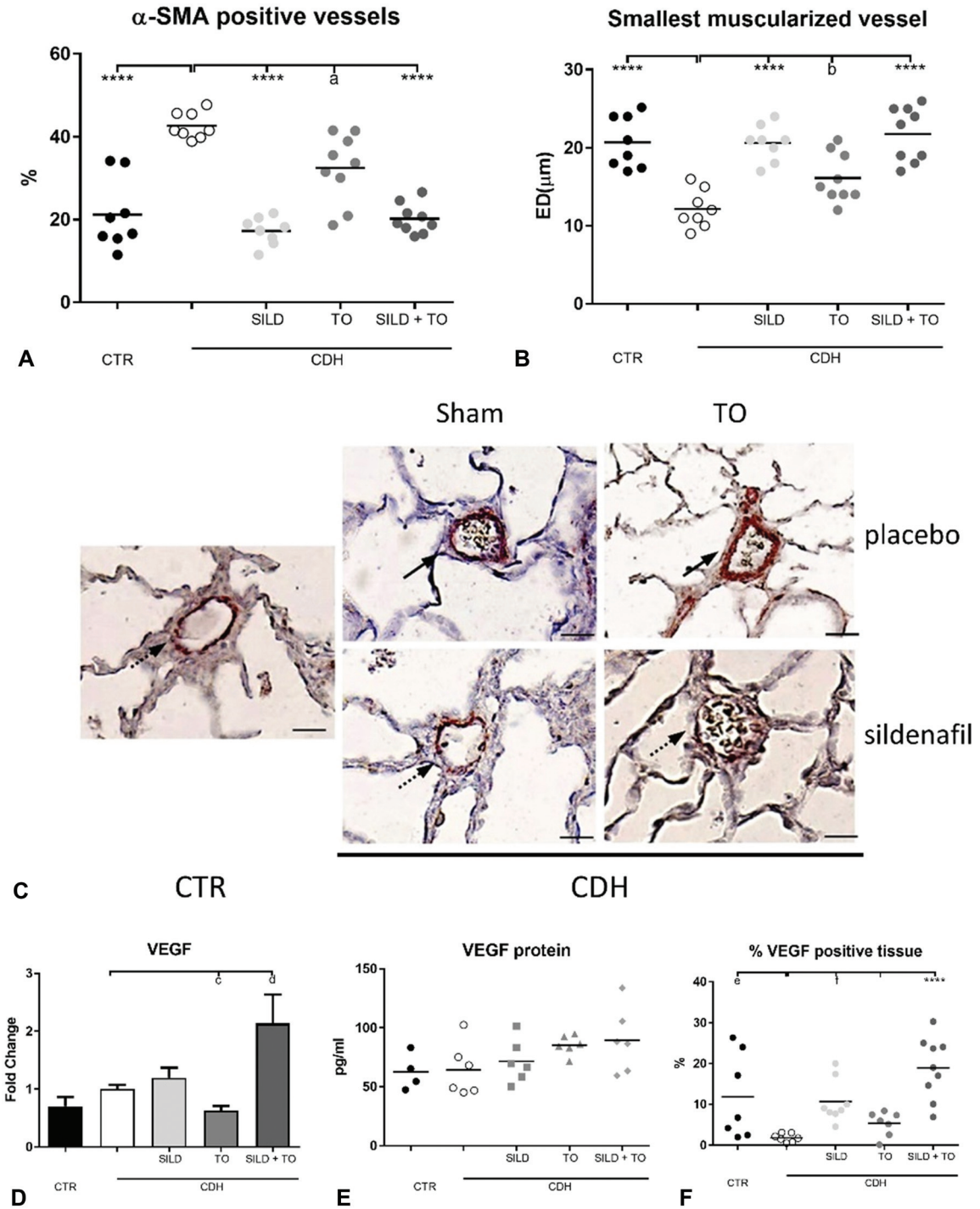

FIGURE 5. Effect of sildenafil and TO on the muscularization of vessels with external diameter <100 μm and on VEGF expression. A, Percentage of α smooth muscle actin (α-SMA) positive vessels. B, Mean ED of the smallest muscularized vessel. C, Representative sections stained for α-SMA and CD31 showing muscularized (full arrow) or partially/nonmuscularized (dotted arrow) vessels. Scale bars: 20 μm. D, qRT-PCR analysis of VEGF expression in lung tissue, n=4/group. E, ELISA analysis of VEGF protein concentration in lung homogenates. F, Quantification of the immunoreactivity for VEGF in lung parenchyma. In the graphs, histograms or large horizontal bars indicate the mean, error bars indicate standard deviation. (A) P=0.004, (B) P=0.038, (C) P=0.012, (D) P=0.021, (E) P=0.026, (F) P=0.049, $^{****}P$<0.0001. SILD indicates sildenafil.

vascular development. TO reduced the degree and extension of muscularization of peripheral vessels, but not at levels comparable to CTR. Also, it was not associated with a change in the wall thickness of resistance vessels. The effect of TO on lung vascular development in CDH is controversial. While some animal studies claim TO reverses vascular changes,[23,24] others failed to demonstrate any effect.[25,26] That controversy may be explained by morphometric differences between ventilated (as in our study) and nonventilated lungs, by the differences in duration or the sustained nature of the occlusion, and by different gestational age at assessment (full term or late preterm). The available clinical studies also yield conflicting results. In the trial by Ruano et al,[27] TO decreased the risk of severe CDH-PH in fetuses with severe CDH (n=41). Conversely, in another series (n=131) the incidence of CDH-PH was comparable in expectantly managed fetuses and in fetuses undergoing TO.[10]

The novelty of our study consists in the combination of TO with a medical therapy that can be administered to the mother, ie, sildenafil. Apart from having a vasodilatory effect, sildenafil also counteracts the vascular remodeling that characterizes pulmonary hypertension.[28] This led to the concept of prenatal administration to CDH fetuses for the prevention of CDH-PH. Studies in rats and rabbit already proved the potential of transplacental sildenafil administration.[5,7] Consistent with those studies, we observed a normal vascular wall thickness and peripheral vessels muscularization in CDH fetuses exposed to sildenafil. The biological effect of sildenafil on fetal lungs was proven by an increase in the AT/ET ratio of the main pulmonary artery, indicating vasodilation in the downstream circulation. However, in contrast with observations in rats,[29] we did not document differences in the Doppler waveform of the pulmonary artery branches, either between CDH and CTR fetuses, or between different fetal treatment groups. One potential explanation is the technical complexity of such measurements in the rabbit, due to smaller dimensions and marked displacement of the major pulmonary vessels, typical for CDH.[30] This might be partially responsible for the high variability of measurements within groups.

In line with previous results from us and others,[5,6,7] CDH fetuses treated with sildenafil alone also presented a reduction in the MTBD as compared with the untreated ones, indicating an increased distal airway complexity. Functionally, we observed lower resistances to the expansion of pulmonary parenchyma, as shown by a decrease in static elastance and an increase in compliance, and an increased expiration capacity. Despite such improvement, complete rescue of parenchymal function and morphometry was only observed in TO-fetuses.

As a result, CDH fetuses undergoing both sildenafil and TO had a remarkable improvement in all outcome measures analyzed.

Intriguingly, sildenafil also restored normal surfactant protein expression, thereby correcting a major side effect of sustained TO. Despite promoting compensatory lung growth, prolonged TO decreases surfactant production through loss of type II pneumocytes.[31] Experimental data suggest benefit of temporary tracheal occlusion, which seems to stimulate lung maturation and pneumocytes differentiation.[32] For this reason, timely in-utero reversal of the occlusion is currently practiced in the clinical setting. However, in more than 20% of cases, patients present earlier than planned with ruptured membranes or preterm delivery.[33] Based on the present animal data, additional antenatal treatment with sildenafil might be beneficial, stimulating surfactant production already before the reversal of the occlusion.

The sildenafil-induced improvement in vascularization and alveolar maturation was paralleled by an increase in VEGF RNA, and in VEGF and flk-1 protein expression in parenchymal cells. While TO alone reduced VEGF RNA expression, this effect was abolished by the administration of sildenafil. VEGF is of paramount importance in lung development, as it contributes to pulmonary angiogenesis and vessel growth, with subsequent alveolar maturation and adequate matching of ventilation and perfusion.[34] The decreased VEGF expression in the lung parenchyma is a consistent finding in CDH, both in humans[35] and in animal models.[36] Sildenafil might upregulate VEGF expression by activation of endothelial nitric oxide synthase.[37] The effect of TO on VEGF is less investigated. In a study on murine non-CDH lung explants, analysis by RT-PCR showed an increase in VEGF expression by TO after 48 hours of culture.[38] However, the stretching stimuli induced by TO might have different effects at different stages of lung development. In that study by Unbekandt et al,[38] lungs were occluded in the early pseudoglandular stage, while TO in rabbits as well as in humans is performed in the saccular phase. Furthermore, different effects might be observed in lungs that are normally (as in the murine study) or abnormally developed (as in CDH). Finally, long-lasting TO might induce changes that are not seen in a shorter term.

Of note, consistent with our previous results,[7] at the protein level the differences in VEGF expression between the study cohorts were not obvious when assessing the whole lung tissue, but only at analysis of the alveolar parenchyma.

Our study has a number of limitations, which have to be considered in perspective. First, the rabbit model does not allow comprehensive functional testing with postnatal ventilation nor direct measurements of neonatal pulmonary pressures and blood gasses, because of the dimensions of the pups and the severe degree of pulmonary hypoplasia in selected groups. Furthermore, in the rabbit, CDH is surgically created during the pseudoglandular stage of lung development. Therefore, this model cannot mimic the early embryologic disturbances in parenchymal and vascular development, and the potential effects of antenatal treatment on those changes cannot be assessed. The paucity of reliable antibodies is another deficiency of the rabbit model, as many antibodies are prepared in rabbits and few validated staining protocols are available. This limits the possibility to obtain mechanistic data in this species. However, the rabbit has several advantages over other models. In rabbits, alveolization starts prior to birth, to be completed postnatally. Therefore, pulmonary development mimics to a larger extent that of the human lung than other animal models frequently used to study CDH, such as rodents and lambs.[17] Furthermore, the rabbit is an ideal model to study transplacental therapy, because placental transfer in the latter half of pregnancy mirrors that in humans.[39]

Another limitation of this model, as in rodents, is that prenatal re-establishment of the airways is not possible. Consequently, the effects induced by the ''plug-unplug sequence,'' as clinically used, on lung development cannot be assessed. In sheep, in utero reversal of TO significantly reduced the %MT of peripheral vessels.[24] However, as stated above, in the clinical setting timely reversal of TO is not always possible or practiced,[33] and our results indicate a potential benefit for fetuses with TO sustained till birth. In conclusion, this study provides relevant information towards the further preclinical and clinical exploration of a combined fetal treatment for CDH. The TOTAL trial will provide a definite answer of the efficacy of TO in fetuses with left-sided CDH and severe or moderate pulmonary hypoplasia.[9] Under the reasonable assumption that TO has a beneficial effect,[8] we and others are conducting studies to investigate the safety and efficacy of maternally administered sildenafil in selected women with a fetus with CDH. Since we could demonstrate sufficient transplacental transfer in ex vivo perfused human placentas,[40] we moved toward a clinical phase I/IIB study investigating in vivo transplacental transfer and feto-maternal tolerance of maternally administered sildenafil at the gestational age window of interest.[41] Demonstration of fetal safety has gained particular relevance after an alert following the interim analysis on another study investigating the

use of sildenafil in fetuses with growth restriction (Dutch STRIDER). That study was suspended because of the lack of benefit yet ''potential signal of harm relating to an increased incidence of persistent pulmonary hypertension.''[42] We and others believe that this suspicion should not lead to the suspension of all studies on sildenafil in pregnancy.[43] There are several arguments for that. First, the increased incidence of pulmonary hypertension in the Dutch trial is not consistent with the results of the other STRIDER-studies in the UK and New Zealand/Australia, nor with other studies on antenatal sildenafil administration. Second, assuming there would be an association between sildenafil and pulmonary hypertension in growth-restricted fetuses, this should not automatically be extrapolated to other clinical conditions, such as CDH. In the case of CDH, fetal lung development is impaired hence not comparable to that in case of growth restriction. The effect of sildenafil on the lung vasculature is therefore expected to be different. The different effects of sildenafil on normal and abnormal lungs have already been shown in animal studies, including our previous study in the rabbit model.[7,44] However, based on this alert, we have initiated additional preclinical studies on fetal safety. When shown safe and having a biological effect on the pulmonary circulation, we should move toward a clinical efficacy trial.

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