MONASH University

Fetal therapies and improved perinatal stabilisation to prevent pulmonary hypertension in infants with a congenital diaphragmatic hernia

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Summary

Whilst the transition from fetal to newborn life is uneventful for most babies, some may require help to breathe at birth because their underdeveloped lungs are not ready for life in the outside world. One devastating cause of impaired lung development is congenital diaphragmatic hernia (CDH), which affects 1 in 3000 babies. Early in fetal development, a hole in the diaphragm allows abdominal organs to enter the chest and impair normal lung development. This abnormal development of the airways and pulmonary vasculature is termed lung hypoplasia, and predisposes infants to respiratory insufficiency and pulmonary hypertension after birth.[1] While the hole in the diaphragm can be repaired after birth, survival rates remain less than 15% in babies with the most severely underdeveloped lungs, predominantly due to severe pulmonary hypertension that is resistant to current neonatal therapies.[2, 3]

In this PhD thesis, I aimed to investigate interventions that reduce the severity of pulmonary hypertension, by either protecting the vulnerable pulmonary vascular bed during the neonatal cardiopulmonary transition at birth or attenuating abnormal pulmonary vascular development before birth.

In *Chapter 2*, we established a CDH model in fetal sheep, that was based on a previously described model. In this experiment, we compared lambs with a diaphragmatic hernia (DH) to lambs that underwent a sham operation at the same gestational age (controls). We found that while DH lambs had impaired gas exchange and reduced dynamic lung compliance, they had similar pulmonary blood flows as control lambs after adjusting for lung weight. This unexpected finding prompted us to consider that our neonatal resuscitation protocol, which differed from previous studies, may have resulted in reduced pulmonary vascular resistance. We hypothesised that, instead of immediate cord clamping (ICC) after birth, our approach of deferring umbilical cord clamping until after lung aeration (termed physiologically based cord clamping; PBCC) may protect the pulmonary vasculature from stress induced by hypoxia and high arterial pressures immediately after birth. To further investigate the potential protective effects of PBCC, in *Chapter 3* we compared the effects of PBCC and ICC on cardiopulmonary physiology during the neonatal transition in lambs with a DH. We found that PBCC avoided the

severe hypoxia and high arterial blood pressures associated with ICC and resulted in higher pulmonary blood flows during the first 2 hours after birth.

Even with optimal delivery room management, infants with CDH are predisposed to pulmonary hypertension due to abnormal pulmonary vascular development *in utero*. In countries that routinely perform a mid-trimester morphology ultrasound scan, between 70 – 90% of babies with CDH are diagnosed at 20 weeks of pregnancy, which provides an opportunity to identify the most severe cases.[4, 5] The current antenatal management outside of clinical trials is expectant, however it is possible that the severe cases could benefit from antenatal interventions that rescue the lung from lethal hypoplasia. To specifically target pulmonary hypertension, I aimed to investigate a novel antenatal medical therapy, sildenafil, that had previously been shown to attenuate pulmonary vascular abnormalities in small animal models of CDH. However, it was unclear if the biochemical and structural changes observed in the animal models would translate to improved pulmonary haemodynamics after birth. Before translating these preclinical data into a clinical trial, in *Chapter 4* I aimed to investigate the effect of antenatal sildenafil on cardiopulmonary haemodynamics and lung function during the neonatal transition in lambs with a DH. We found that antenatal sildenafil treatment was associated with lower pulmonary arterial pressure and greater pulmonary blood flow during the early neonatal transition period, in lambs with a DH.

We have demonstrated that DH lambs have abnormal cardiopulmonary haemodynamics during the neonatal transition, and that these abnormalities can be attenuated by either antenatal sildenafil treatment to improve pulmonary vascular development or PBCC to optimise perinatal stabilisation. Our findings suggest that these perinatal interventions may prevent or reduce the severity of pulmonary hypertension in infants with CDH.

Research outputs

Publications arising from the doctoral studies included in the thesis

1. **Kashyap AJ**, Crossley KJ, DeKoninck PLJ, et al. Neonatal cardiopulmonary transition in an ovine model of congenital diaphragmatic hernia. *Arch Dis Child Fetal Neonatal Ed* 2019 doi: 10.1136/archdischild-2018-316045

2. DeKoninck PLJ, Crossley KJ, **Kashyap AJ**, et al. Effects of tracheal occlusion on the neonatal cardiopulmonary transition in an ovine model of diaphragmatic hernia. *Arch Dis Child Fetal Neonatal Ed* 2019 doi: 10.1136/archdischild-2018-316047

3. **Kashyap AJ**, Hodges RJ, Thio M, et al. Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia. *Arch Dis Child Fetal Neonatal Ed* 2019 doi: 10.1136/archdischild-2019-316906

4. **Kashyap AJ**, Dekoninck PLJ, Rodgers KA, et al. Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia. *Ultrasound Obstet Gynecol* 2019;54(4):506-16. doi: 10.1002/uog.20415

5. **Kashyap A**, DeKoninck P, Crossley K, et al. Antenatal Medical Therapies to Improve Lung Development in Congenital Diaphragmatic Hernia. *Am J Perinatol* 2018;35(9):823-36. doi: 10.1055/s-0037-1618603

Research presentations

Invited presentations

1. CDH Australia Annual Forum, 2019
   Future directions for CDH care

2. Australian Association of Obstetrical and Gynaecological Ultrasonologists, 2019
   Fetal therapy for congenital diaphragmatic hernia: future directions

3. Leiden University Medical Centre, Netherlands 2019
   Improving the transition to newborn life for infants with CDH

4. Perinatal Research Society Annual Meeting, 2018
   Antenatal medical therapies for congenital diaphragmatic hernia

5. Children's Hospital of Philadelphia, USA 2018
   Improving the transition to newborn life for infants with CDH

6. Antoine-Béclère Hospital, Paris, France 2018
   Improving the transition to newborn life for infants with CDH

7. Monash Cardiovascular Symposium, 2018
   Diaphragmatic hernia: Exciting antenatal interventions on the horizon

8. CDH Australia Annual Forum, 2018
   What's on the horizon in CDH research?

9. Monash Leadership Development Seminar, 2017
   Taking advantage of research opportunities

Presentations to learned societies

1. Fetal and Neonatal Physiological Society Annual Meeting, 2019
   The effect of antenatal sildenafil on neonatal pulmonary haemodynamics in an ovine model of diaphragmatic hernia.
   Awarded the Julian T Parer Prize for Best Oral Presentation by a PhD Candidate

2. Society for Maternal Fetal Medicine Pregnancy Meeting, 2019
   Effects of antenatal sildenafil on neonatal pulmonary haemodynamics in an ovine model of diaphragmatic hernia.
   Awarded the Best Oral Presentation in Fetal Therapy and Genetics

3. Fetal Neonatal Workshop of Australia and New Zealand, 2019
   Physiologically based cord clamping improves pulmonary hemodynamics during the neonatal transition in lambs with diaphragmatic hernia
   Awarded the Best Oral Presentation

4. International Fetal Medicine and Surgery Society Annual Meeting, 2018
   Does delaying cord clamping improve pulmonary blood flow in congenital diaphragmatic hernia?

5. Perinatal Society of Australia and New Zealand Annual Congress, 2018
   Fetal Therapy for Congenital Diaphragmatic Hernia
   Awarded the Mont Liggins Early Career Researcher Award for Best Oral Presentation

6. The Royal Society of Victoria, 2018
   Improving the transition to newborn life for babies with congenital diaphragmatic hernia
   Awarded the Young Scientist Research Prize in the Biomedical Sciences

7. Perinatal Society of Australia and New Zealand Annual Congress, 2017
   Establishing an ovine model of congenital diaphragmatic hernia to evaluate neonatal transition at birth
   Awarded the President's New Investigator Award for Best Oral Presentation

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original manuscripts published in peer reviewed journals (Chapter 2.1, 3.1 and 4), 1 submitted manuscript (Chapter 3.2) and 1 manuscript that is significant to the work of the thesis presented as a narrative (Chapter 2.2). The core theme of the thesis is fetal and neonatal therapies that may prevent pulmonary hypertension in infants with congenital diaphragmatic hernia. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Obstetrics and Gynaecology under the supervision of Professor Stuart Hooper, Associate Professor Ryan Hodges, Dr Kelly Crossley and Dr Philip DeKoninck.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, 3 and 4, my contribution to the work is described in the table overleaf:

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author names, nature and % of co-authors contribution	Co-author a Monash student:
2.1	Neonatal cardiopulmonary transition in an ovine model of congenital diaphragmatic hernia	Published	Initial concept, data collection, analysis, and drafting; 70%	Kelly J. Crossley; Initial concept, data collection, and input into manuscript; 8% Philip L. J. DeKoninck; Initial concept, data collection, and input into manuscript; 8% Karyn A. Rodgers; Data collection and input into manuscript; 1% Marta Thio; Initial concept, data collection, and input into manuscript; 1% Sasha M. Skinner; Data collection and input into manuscript; 1% Jan A. Deprest; Initial concept and input into manuscript; 1% Stuart B. Hooper; Initial concept, data collection, and input into manuscript; 5% Ryan J. Hodges; Initial concept, data collection, and input into manuscript; 5%	No No No No No No No No

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author names, nature and % of co-authors contribution	Co-author a Monash student:
3.1	Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia	Published	Initial concept, data collection, analysis, and drafting; 70%	Ryan J. Hodges; Initial concept, data collection, and input into manuscript; 5% Marta Thio; Initial concept, data collection, and input into manuscript; 5% Karyn A. Rodgers; Data collection and input into manuscript; 1% Benjamin J. Amberg; Data collection and input into manuscript; 1% Erin V. McGillick; Data collection and input into manuscript; 1% Stuart B. Hooper; Initial concept, data collection, and input into manuscript; 5% Kelly J. Crossley; Initial concept, data collection, and input into manuscript; 6% Philip L. J. DeKoninck; Initial concept, data collection, and input into manuscript; 6%	No No No Yes No No No No

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author names, nature and % of co-authors contribution	Co-author a Monash student:
3.2	Effects of diaphragmatic hernia on the cardiopulmonary transition in newborn lambs that undergo immediate cord clamping	Submitted	Initial concept, data collection, analysis, and drafting; 70%	Erin V. McGillick; Initial concept, data collection and input into manuscript; 5% Indya M. Davies; Data collection and input into manuscript; 5% Benjamin J. Amberg; Data collection and input into manuscript; 2% Philip L. J. DeKoninck; Initial concept and input into manuscript; 2% Karyn A. Rodgers; Data collection, manuscript input; 2% Marta Thio; Initial concept, data collection, and input into manuscript; 2% Ryan J. Hodges; Initial concept, data collection, and input into manuscript; 2% Stuart B. Hooper; Initial concept, data collection, and input into manuscript; 5% Kelly J. Crossley; Initial concept, data collection, and input into manuscript; 5%	No Yes Yes No No No No No No

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author names, nature and % of co-authors contribution	Co-author a Monash student:
4	Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia	Published	Initial concept, data collection, analysis, and drafting; 70%	Philip L. J. DeKoninck; Initial concept, data collection, and input into manuscript; 6% Karyn A. Rodgers; Data collection and input into manuscript; 2% Marta Thio; Initial concept, data collection, and input into manuscript; 1% Erin V. McGillick; Data collection and input into manuscript; 1% Benjamin J. Amberg; Data collection and input into manuscript; 1% Sasha M. Skinner; Data collection and input into manuscript; 1% Alison M. Moxham; Data collection and input into manuscript; 1% Francesca M. Russo; Initial concept and input into manuscript; 1%	No No No No Yes No No No

continued

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author names, nature and % of co-authors contribution	Co-author a Monash student:
4	*continued*			Jan A. Deprest; Initial concept and input into manuscript; 1% Stuart B. Hooper; Initial concept, data collection, and input into manuscript; 5% Kelly J. Crossley; Initial concept, data collection, and input into manuscript; 5% Ryan J. Hodges; Initial concept, data collection, and input into manuscript; 5%	No No No No

I have renumbered sections of submitted and published papers in order to generate a consistent presentation within the thesis.

Candidate name: Aidan Kashyap

Candidate's Signature: **Date:** 19/11/2019

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contribution to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of authors.

Main Supervisor name: Professor Stuart Hooper

Main Supervisor's Signature: **Date:** 19/11/2019

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Abbreviations

CDH	Congenital diaphragmatic hernia
cGMP	Cyclic guanosine monophosphate
ECMO	Extracorporeal membrane oxygenation
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FETO	Fetoscopic endoluminal tracheal occlusion
iNO	Inhaled nitric oxide
IUGR	Intrauterine growth restriction
LBWR	Lung-to-body weight ratio
MRI	Magnetic resonance imaging
NO	Nitric oxide
PBCC	Physiologically based cord clamping
PBF	Pulmonary blood flow
PDE5	Phosphodiesterase-5
PPHN	Persistent pulmonary hypertension of the newborn
PVR	Pulmonary vascular resistance
VEGF	Vascular endothelial growth factor

Abstract

Background

Most infants transition smoothly from life *in utero* to life after birth, but for infants with underdeveloped lungs, such as those with congenital diaphragmatic hernia (CDH), the transition to newborn life is far more difficult. The diaphragmatic defect allows abdominal organs to herniate into the fetal chest, leading to abnormal development of the airways and pulmonary vasculature. This predisposes infants to respiratory insufficiency and pulmonary hypertension after birth. We aimed to investigate interventions that reduce the severity of pulmonary hypertension, by either attenuating abnormal fetal pulmonary vascular development or by protecting the vulnerable pulmonary vascular bed during the perinatal stabilisation period.

Methods

At ≈80 days of gestational age (dGA; term≈147 dGA) a diaphragmatic hernia (DH) was surgically created in fetal lambs (n=35) whereas controls underwent sham surgery (n=6). DH fetuses were untreated (n=17) or treated with either fetoscopic endoluminal tracheal occlusion (FETO; n=8) or sildenafil (n=7). At ≈138 dGA, all lambs were delivered via caesarean section. Immediately before delivery, lambs were instrumented to monitor arterial blood gas status, arterial and cerebral oxygen saturation, as well as carotid and pulmonary artery blood pressures and flows. After delivery, lambs were ventilated for 2 hours using a ventilation protocol designed to reflect clinical practice. All physiological variables were continuously recorded and arterial blood gas status was regularly assessed. In one group of untreated DH lambs, umbilical cord clamping was delayed until after lung aeration was achieved (physiologically based cord clamping, PBCC; n=11), whereas the umbilical cord was immediately clamped in the remaining DH lambs (ICC; n=6).

Results

DH lambs, compared to control lambs, had reduced wet lung-to-body-weight ratio (0.016±0.002 vs. 0.033±0.004; p=0.02), dynamic lung compliance (0.4±0.1 vs. 1.2±0.1 ml/cmH_2O; p=0.001), pulmonary blood flow (PBF; 26±2 vs. 61±8 ml/min/kg; p=0.009)

and cerebral tissue oxygen saturation ($SctO_2$; 56±4 vs. 68±4 %; p=0.039) during the first 2 hours after birth.

Compared to DH lambs, DH+FETO lambs had increased wet lung-to-body-weight ratio (0.031±0.004 vs. 0.016±0.002; p=0.02) and dynamic lung compliance (0.7±0.1 vs. 0.4±0.1 ml/cmH2O; p=0.049), however no difference in PBF corrected for lung weight (4.2±0.6 vs. 4.1±0.7 ml/min/g; p=0.86) or alveolar-arterial difference in oxygen tension (402±41 vs. 401±45 mmHg; p=0.96) during the first 2 hours after birth.

Compared to DH lambs, DH+sildenafil lambs had reduced pulmonary arterial pressure (50±1 vs. 63±2 mmHg; p=0.011), increased PBF (26±3 vs. 13±2 ml/min/kg; p=0.024), and lower partial pressure of arterial carbon dioxide adjusted for minute volume (49±8 vs. 96±24 mmHg·mL·bpm; p=0.045) during the first 2 hours after birth.

Compared to DH+ICC lambs, DH+PBCC lambs had increased $SctO_2$ (59±4 vs. 30±5 %; p<0.001) and similar pulmonary arterial pressures (64±3 vs. 77±5 mmHg; p=0.08) during the first ten minutes after umbilical cord clamping. However, PBF was threefold higher (23±4 vs 8±2 mL/min/kg, p=0.01) and pulmonary vascular resistance was threefold lower (0.6±0.1 vs 2.2±0.6 mm Hg/(mL/min), p<0.001) by 2 hours after birth.

Conclusion

We have demonstrated that lung hypoplasia, secondary to a DH, has a significant effect on cardiopulmonary physiology during the transition from fetal to neonatal life. In lambs with DH, improving pulmonary vascular development by fetal therapy and protecting the vessels by optimising delivery room management improved neonatal cardiopulmonary haemodynamics. In future, infants with CDH may by treated with a bundle of care that incorporates FETO to enhance lung growth, antenatal sildenafil to promote vascular development, and PBCC to provide perinatal cardiopulmonary stability. Together, these therapies may reduce the risk of pulmonary hypertension and ensure that each baby born with a CDH is given the best possible start to life.

Introduction

1

1 Introduction

Most infants transition smoothly at birth from *in utero* to *ex utero* life, but for infants with underdeveloped lungs, such as those with congenital diaphragmatic hernia (CDH), this fetal to neonatal transition is far more difficult.

In infants with a CDH, the diaphragm fails to close during embryogenesis, which allows abdominal organs to herniate into the thorax and impairs fetal lung development (Figure 1). Despite advances in antenatal detection and neonatal management, CDH contributes to more than 1% of the infant mortality rate in developed countries such as the United States.[6, 7]

The morbidity and mortality associated with CDH is predominantly related to abnormal lung development, affecting both the airways and the vasculature.[8] CDH infants have reduced surface area for alveolar gas exchange, reduced pulmonary vascular cross-sectional area, abnormal pulmonary vascular muscularisation, and altered vasoreactivity.[9-11]

Most fetuses with isolated CDH survive until term, because their hypoplastic lungs are not required for gas exchange during fetal life. However, when the umbilical cord is clamped at birth the infant becomes solely dependent on the lungs for gas exchange. If cord clamping occurs before lung aeration, the infant loses placental gas exchange before pulmonary gas exchange can be established. In preterm infants, this results in a prolonged period of hypoxia and reduced cardiac output immediately after birth.[12, 13] An alternative approach to birth, which involves aerating the lungs before clamping the umbilical cord (known as physiologically based cord clamping; PBCC), avoids this cardiopulmonary instability and hence may also improve the fetal to neonatal transition for infants with a CDH.

CDH is usually diagnosed on routine second trimester ultrasound, providing an opportunity to enhance lung growth and development *in utero* to improve subsequent neonatal outcomes.[14-16] Antenatal surgical interventions such as fetoscopic endoluminal tracheal occlusion (FETO) improve outcomes, however are often complicated by preterm birth and have a limited effect on the incidence and severity of

pulmonary hypertension.[17, 18] Less invasive antenatal medical therapies, such as the phosphodiesterase-5 (PDE5) inhibitor sildenafil, therefore represent an important opportunity to reduce the risk of severe pulmonary hypertension in infants with a CDH.

In this thesis, I will first describe the effects of CDH on cardiopulmonary physiology during the neonatal transition, in a sheep model. Using this model, I will then describe the impact of three management strategies:

1. occluding the fetal trachea during gestation, to trap lung liquid and hence stimulate lung growth (FETO);
2. delaying umbilical cord clamping until after the lungs have aerated, to allow a smoother transition to newborn life (PBCC); and
3. maternal administration of sildenafil, which is known to cross the placenta, to improve pulmonary vascular development and hence reduce the risk of developing pulmonary hypertension after birth.

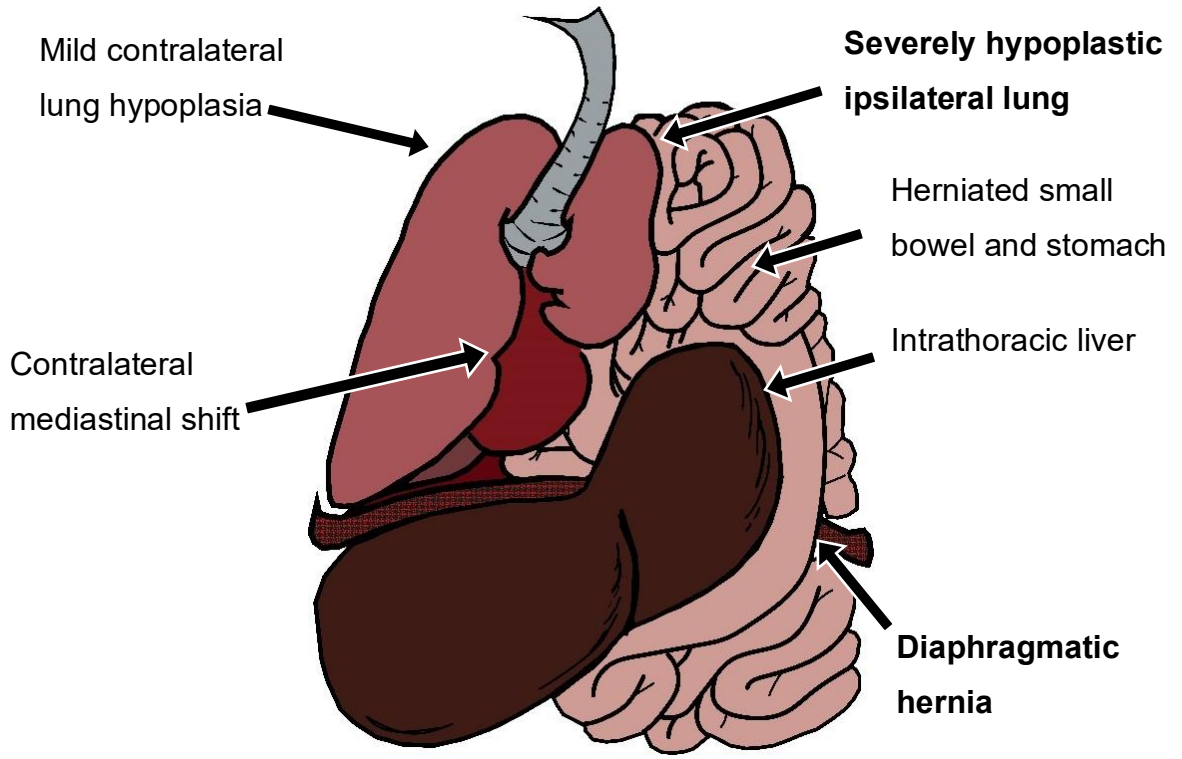

Figure 1 – Congenital diaphragmatic hernia

Diaphragmatic hernia allows abdominal viscera, sometimes including the liver, to herniate into the thoracic cavity. The abdominal viscera occupy space in the thoracic cavity, compressing the heart and lungs. *Original figure.*

1.2 Congenital diaphragmatic hernia

1.2.1 Epidemiology and prognosis

The reported incidence of isolated CDH varies widely, but ranges between 1.6 to 5.7 per 10,000 births.[4, 6, 19-28] The most accurate incidence for the general population is likely 1.6 per 10,000 births, determined using data from the European Surveillance of Congenital Abnormalities (EUROCAT) consortium that combines registries capturing ~30% of European births.[28]

The true mortality of CDH has always been difficult to determine, as historically many severe CDH patients would die before neonatal transfer to specialist hospitals, and hence were not included in institution-based statistics – termed the "hidden mortality" of CDH.[24, 29] In the modern era, improved prenatal diagnosis and centralisation of care has significantly reduced the number of infants with severe CDH who are born outside of specialised hospitals. However, improved prenatal diagnosis has also increased the number of pregnancy terminations in response to a CDH diagnosis with poor prognosis. Hence, while the proportion of CDH *newborns* that survive has increased from 56% to 73% over the past 20 years, when terminations of pregnancy and stillbirths are included the proportion of CDH *fetuses* that survive to 1 month of age remains at 55% in the modern era.[30] The apparent improvement in survival rates has been largely attributed to improvements in neonatal management such as extracorporeal membrane oxygenation (ECMO), high-frequency oscillatory ventilation (HFOV) and permissive hypercapnia. However, given the true mortality has not changed over the past 20 years, there clearly remains scope to improve upon our antenatal and neonatal management strategies for infants with a CDH.

The morbidity of CDH is largely respiratory, with 40% of survivors developing bronchopulmonary dysplasia[31] and 30% developing pulmonary hypertension that persists beyond 2 months of age.[2] Survivors also fail to thrive and have high rates of neurocognitive deficits.[32-34] In adult life, 60% of CDH survivors have impaired pulmonary function and most require structured multidisciplinary follow-up.[35, 36]

Standardisation of care and advances in surgical technique have improved survival rates (albeit not to the degree described in institution-based studies, as described above)[37], however, there remains a population of CDH patients with such severely underdeveloped lungs that they cannot survive *ex utero*. For example, neonates with severe pulmonary hypertension have mortality rates ranging from 56.1% to 100%.[2, 3, 38] Preventing pulmonary hypertension by identifying and treating these patients *in utero* may improve their survival.

1.2.2 Aetiology and pathogenesis

The majority of CDH cases (60-70%) are isolated, however some cases are complicated by additional structural abnormalities. These complex CDH cases have a higher mortality (>80%) and often are associated with genetic abnormalities (such as trisomy 18) or are part of a syndrome (such as Fryns syndrome).[26, 39, 40]

The aetiology of isolated CDH is largely unknown, however appears genetically heterogenous, with potential defects in different developmental pathways.[40] The majority of currently identified genes associated with a CDH are involved in the retinoic acid signalling pathway.[41-43] Retinoic acid signalling is critical for both diaphragm and pulmonary development and appears implicated in CDH pathogenesis.[43-45] In the lungs, retinoic acid signalling integrates the Wnt and TGF-β regulatory pathways that mediate airway branching and pulmonary vasculature muscularisation, both of which are disturbed in CDH.[46, 47]

Dietary deficiency of Vitamin A (retinoic acid precursor) predisposes offspring to diaphragmatic defects in both rodents and humans.[48, 49] Other environmental factors such as maternal smoking and alcohol use may also contribute to the development of CDH.[27]

Diaphragm development in normal infants and infants with a CDH

The diaphragm separates the abdominal and thoracic cavities. From the 4th to 8th week of gestation there is fusion of three separate amuscular mesenchymal structures: the septum transversum, pleuroperitoneal membranes and oesophageal mesentery.[50-53]

Subsequently, muscular progenitors migrate and proliferate across this structure, with later muscular ingrowth from the lateral body wall finalising a tight seal between the thorax and abdomen.[51] In fetuses with CDH, abnormal development of the amuscular mesenchymal component results in an incomplete migration platform for muscular progenitors.[52]

The defect is predominantly left-sided (LCDH; 85%), uncommonly right-sided (RCDH; 13%) and rarely bilateral (<1%).[54] LCDH and RCDH differ in their natural history[55, 56] and right-sided defects are usually associated with worse prognosis, however their rarity makes this difficult to ascertain.[56-60] CDH is predominantly posterolateral (90%), and less commonly anterior or central.[39]

An incomplete diaphragm allows herniation of abdominal contents into the thoracic cavity from the embryonic stage, which then leads to impaired lung development. While the diaphragmatic defect can be repaired postnatally, it is the impaired lung development that underlies the mortality and morbidity associated with a CDH.

1.2.3 Normal lung development and the effects of congenital diaphragmatic hernia

Pulmonary gas exchange requires air to be conducted through the airways into the peripheral lung, respiratory gases to efficiently diffuse between the air-spaces and blood vessels within the respiratory acini, and blood to perfuse these regions at high rates. Hence, the lung consists of conducting airways and respiratory gas exchange regions and a dense network of blood vessels that surround these gas exchange regions.

The conducting (pre-acinar) airways consist of the trachea, bronchi, bronchioles, and terminal bronchioles, and do not participate in gas-exchange. The respiratory (intra-acinar) airways consist of respiratory bronchioles, alveolar ducts, and alveolar sacs.

Alongside the airways, there is a parallel pulmonary vascular system that consists of pre-acinar and intra-acinar vessels. The first six generations of pre-acinar vessels are elastic, with a higher proportion of elastic laminae than circular muscle in the muscular layer

of the vessel wall.[61] The next three generations are transitional, and the remaining generations of pre-acinar vessels are muscular, with a greater proportion of circular muscle than elastic laminae. In contrast, intra-acinar vessels are normally amuscular during fetal life.[62]

Within the respiratory acinus, capillaries and airways are closely aligned in the mature lung, with fusion of the basement membranes underlying the alveolar epithelial cells and capillary endothelial cells for up to 50% of the surface area of the lung. As such, gas exchange occurs across ultrathin membranes (~200 nm) composed of the cytoplasm of type-1 alveolar epithelial cells, a fused basement membrane, and the cytoplasm of the capillary endothelial cells.[63]

To create this structure normal pulmonary development involves a complex process of airway and vascular growth and differentiation. It begins in early embryogenesis and continues well into the postnatal period. It can be classified into five overlapping periods of embryonic, pseudoglandular, canalicular, saccular and alveolar stages, as described in *Figure 2*.

The gross structure of the lung develops during the embryonic and pseudoglandular stages. In the embryonic stage (the first 7 weeks of gestation), the lung bud evaginates from the ventral foregut and divides into two. The close relationship between pulmonary airway and vascular development is already evident during the 5th week of gestation, with each epithelial lung bud being surrounded by a capillary plexus supplied by a pulmonary artery and drained by a pulmonary vein. During the pseudoglandular stage (from 7 – 16 weeks of gestation), the entire conducting airway structure (pre-acinar zone) is formed.[64] This requires continuous dichotomous branching of the airways, from a single lung bud to thousands of terminal bronchioles, mirrored by the formation of all pre-acinar pulmonary arteries.

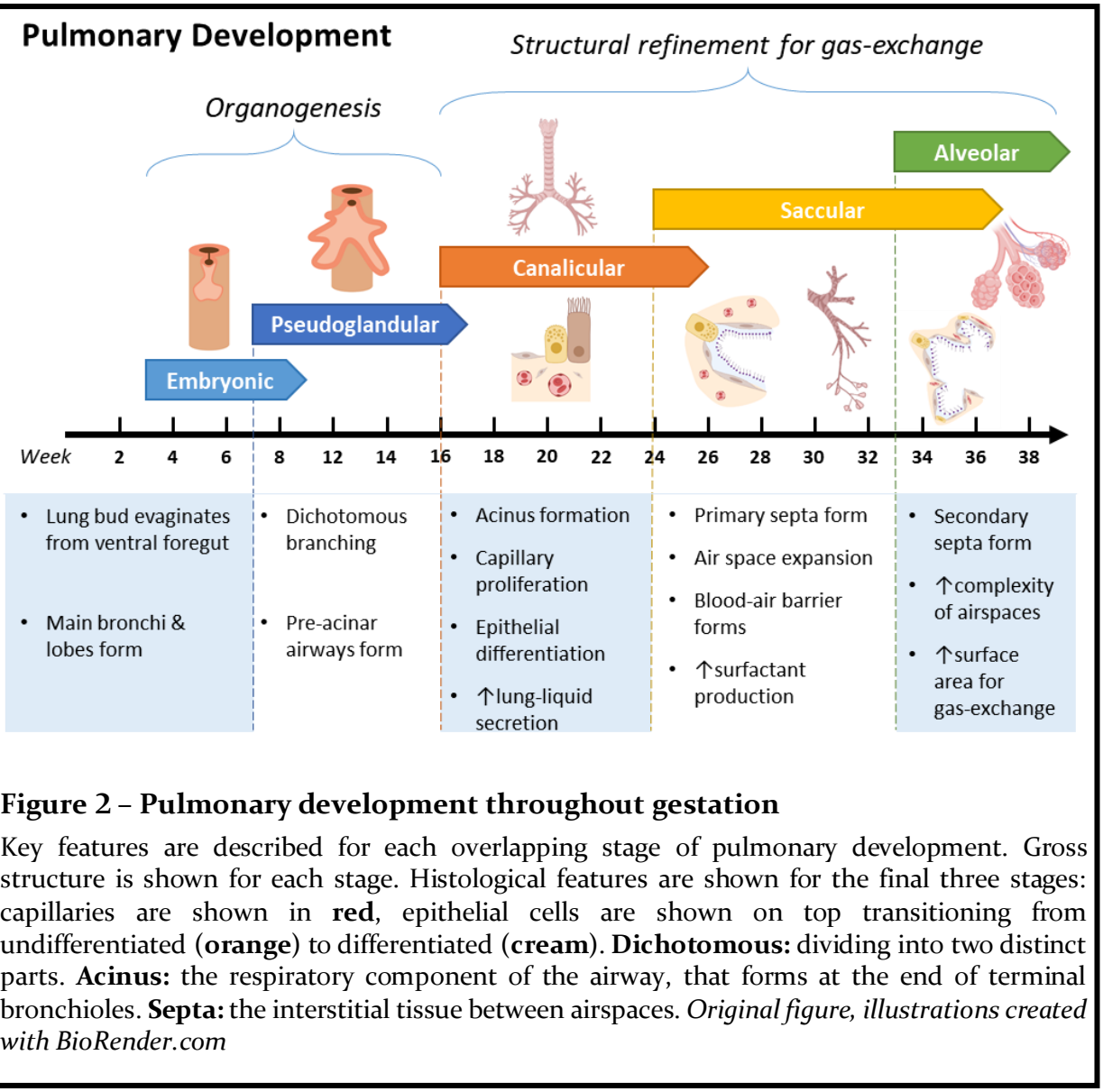

Figure 2 – Pulmonary development throughout gestation

Key features are described for each overlapping stage of pulmonary development. Gross structure is shown for each stage. Histological features are shown for the final three stages: capillaries are shown in **red**, epithelial cells are shown on top transitioning from undifferentiated (**orange**) to differentiated (**cream**). **Dichotomous:** dividing into two distinct parts. **Acinus:** the respiratory component of the airway, that forms at the end of terminal bronchioles. **Septa:** the interstitial tissue between airspaces. *Original figure, illustrations created with BioRender.com*

The canalicular stage (from 17 – 28 weeks of gestation) marks the beginning of structural maturation to prepare for postnatal gas exchange. Throughout the canalicular, saccular and alveolar stages there is progressive thinning of interstitial tissue (septa), capillary proliferation, and air-space expansion. Differentiation of alveolar epithelial cells into type I and II cells also occurs. Large air sacs are ultimately subdivided into numerous, smaller air-spaces (alveoli), between which capillaries mature to form a single layer within thin secondary septa (Figure 3).

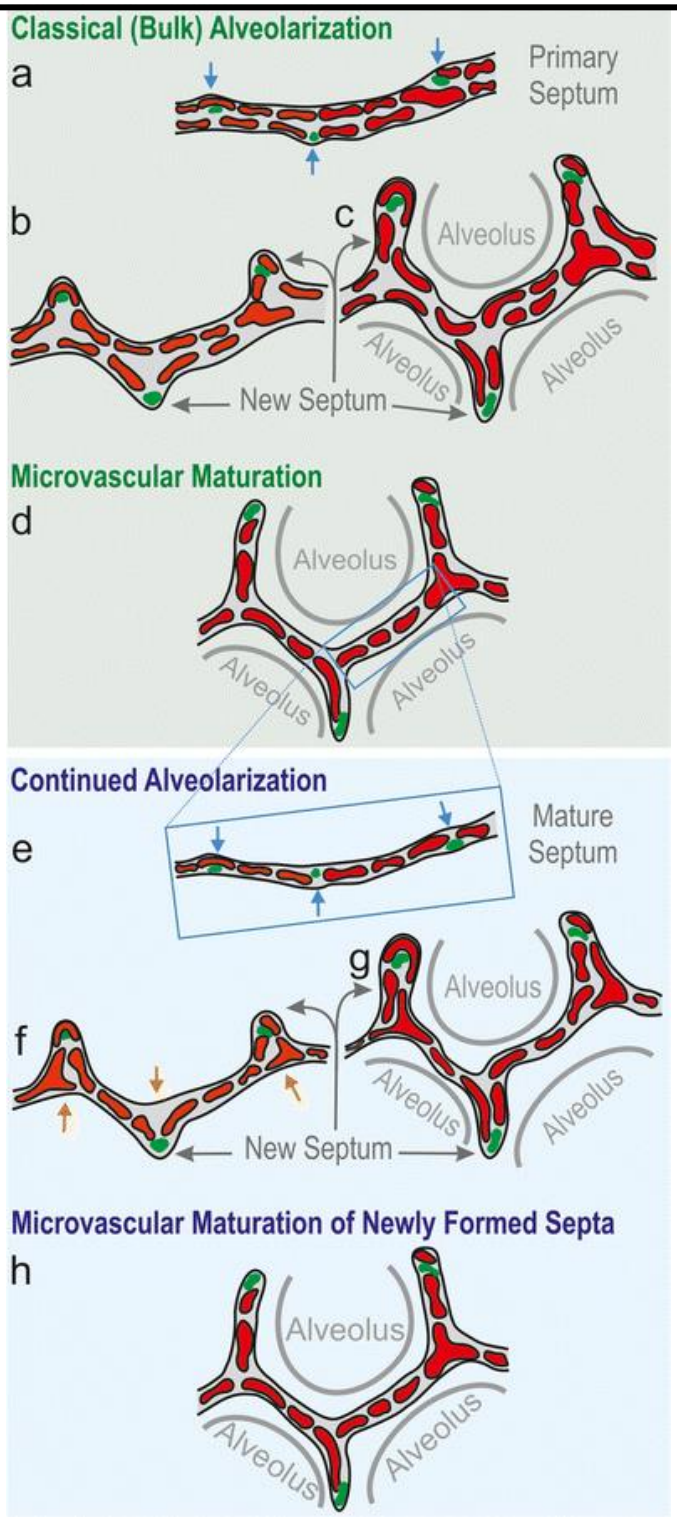

Figure 3 – Process of microvascular maturation during alveolarisation

A: Initially, primary septa separate air sacs (saccules). **B:** Secondary septa begin to form in the alveolar stage. **C:** Initially, secondary septa are thick with a capillary bilayer. **D:** Microvascular maturation leads to thin secondary septa with a single layer of capillaries. **E – H:** Alveolarisation continues into adulthood, however when this occurs the alveolar surface opposing the upfolded capillary layer is left without capillaries; these are immediately replaced via angiogenesis. *Reproduced from Schittny et al.[65] under the terms of the Creative Commons Attribution 4.0 International License.*

Mechanisms underlying pulmonary airway and vascular development

During early embryogenesis, lung growth primarily involves growth and division of the conducting airways. This is driven by growth factors secreted by the mesenchyme, which regulate airway branching and cell differentiation.[66, 67]

Lung liquid also drives pulmonary development by inducing mechanical stretch. Mechanical stretch becomes particularly important during the canalicular stage of lung development, when lung liquid secretion significantly increases.[68] Continued liquid secretion and a high resistance to its efflux via the trachea (the fetal larynx is predominantly adducted) causes liquid to accumulate within the fetal airways. This provides a mechanical stretch that stimulates cell division and tissue remodelling.[68, 69] To prevent lung liquid loss during fetal breathing movements, rhythmic diaphragmatic contractions oppose lung recoil.[70, 71] In CDH, this process may be disturbed.

Deflating the fetal lung by draining it of liquid leads to lung hypoplasia. Conversely, occluding the trachea prevents liquid loss from the lungs causing them to expand (Figure 4).[72] This potent stimulus for lung growth increases alveolar number and surface area, promotes mesenchymal and epithelial cell division, and stimulates elastin synthesis.[73-77] Mechanical stretch induced by tracheal occlusion also stimulates type II alveolar epithelial cells to differentiate into type I alveolar epithelial cells.[78] Thin type I cells allow more efficient gas exchange, however type II cells produce surfactant, which reduces surface tension within the lungs after aeration, preventing alveolar collapse. Tracheal occlusion seems to promote pulmonary vascular development in parallel by increasing vascular endothelial growth factor (VEGF) A mRNA and protein expression.[79] Tracheal occlusion will be described further in *Chapter 1.5.3*.

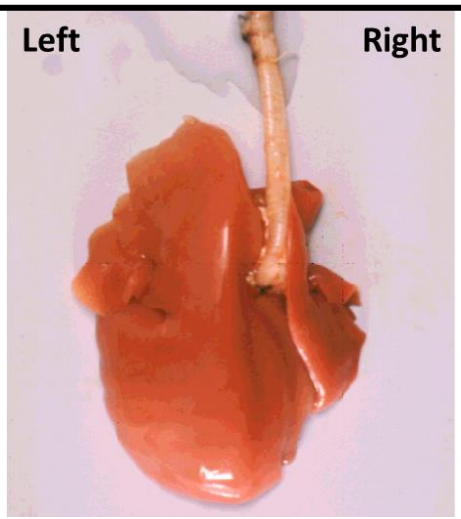

Figure 4 – The effect of lung liquid on lung size

Posterior view of a sheep lung. The right lung is hypoplastic following lung liquid drainage. The left lung is hyperplastic following lung liquid retention. *Original figure, methods described by Moessinger et al.*[72]

The pulmonary vasculature develops by distal angiogenesis (Figure 5), which involves endothelial tubes sprouting from pre-existing vessels at the growing tips of the branching epithelium, before being stabilised by pericytes to become functional capillaries.[80-82] This process is mediated by platelet derived growth factor-β.

In contrast to classical models of pulmonary vascular development, which describe central angiogenesis and distal vasculogenesis (*de novo* formation of blood vessels from endothelial progenitors), distal angiogenesis implies that airways and capillaries proliferate in a co-ordinated manner mediated by complex epithelial-endothelial interactions throughout gestation (Figure 5).[83] Indeed, the pulmonary vasculature is the rate-limiting factor in branching morphogenesis.[84] Vice-versa, any disruption to airway development, such as the mechanical pressure of herniated abdominal organs secondary to CDH, will also affect the pulmonary vasculature.[85] The airways stimulate vascular development by expressing VEGF that acts on capillary VEGF receptors.[85, 86] Mouse studies suggest VEGF is involved in the differentiation and proliferation of endothelial cells and vessel formation.[87, 88] The carefully balanced expression of TGF-β appears to be vital for both airway and vascular development, as alveolarisation is arrested when TGF-β is either upregulated or blockaded.[89, 90]

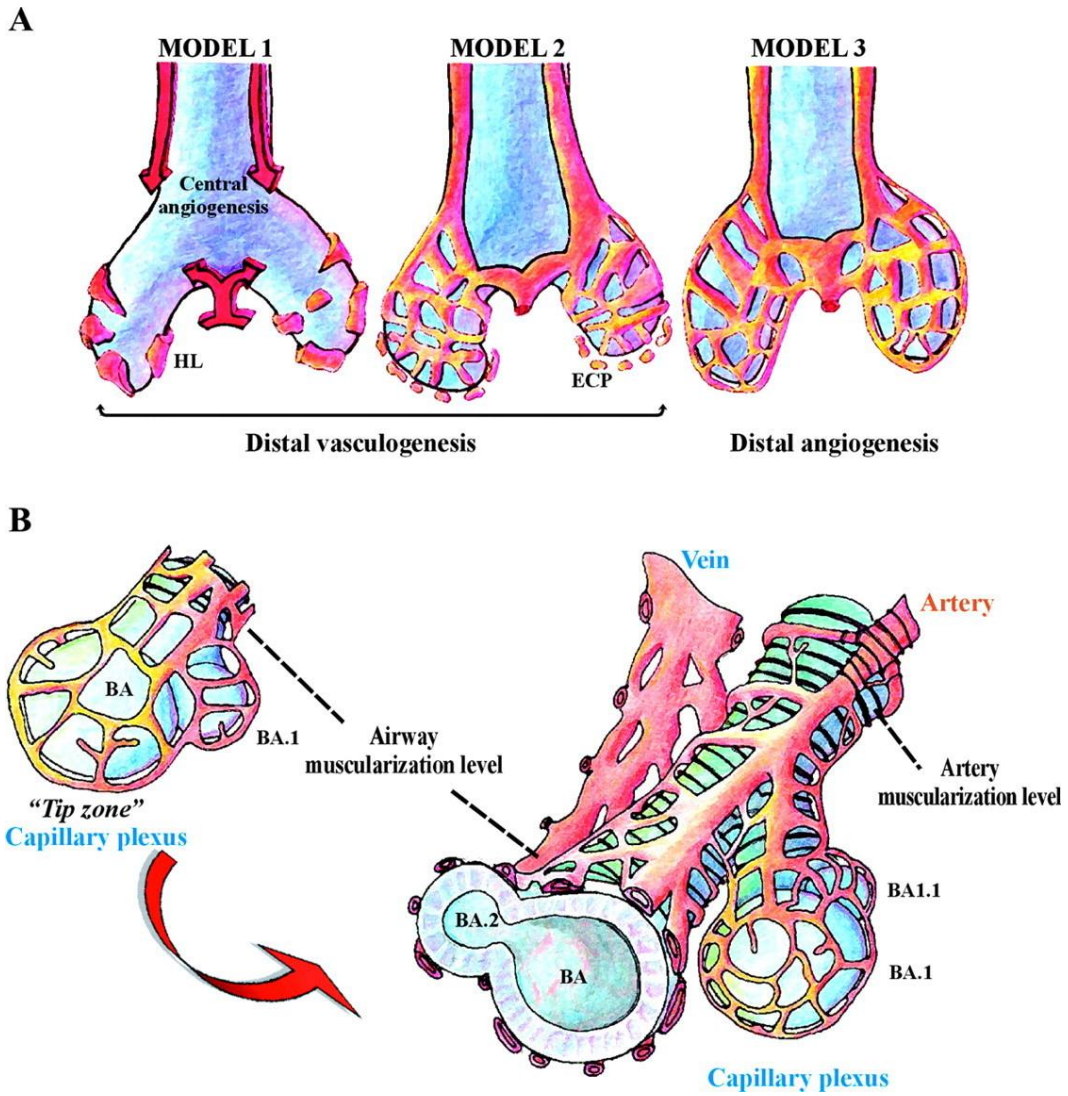

Figure 5 - Distal angiogenesis

A: Lung vascular morphogenesis models. Model 1, proposed by deMello et al.[83], describes 2 mechanisms of lung vascular morphogenesis, central angiogenesis (sprouting of arteries and veins from central vascular trunks) and distal vasculogenesis (development of hematopoietic lakes in the mesenchyme). Connection between the 2 vascular beds would occur at embryonic day (E)13/14. Model 2, proposed by Hall et al.[91], proposes distal vasculogenesis (development of new vessels from endothelial cell precursors) as the mechanism of lung vascularization. Model 3 explains the newly proposed mechanism of distal angiogenesis as the process to develop lung vasculature (formation of new capillaries from preexisting ones). HL, hematopoietic lakes; ECP, endothelial cell precursor. **B:** Distal angiogenesis model. In the model Parera et al.[80] propose, the formation of new capillaries from preexisting vessels takes place at the "tip zone," where airway and capillary network expand in a coordinated way through epithelial-endothelial interactions. Newly formed vessels remodel dynamically as they form part of the afferent or efferent component. BA, airway branch A; BA.1, BA1.1, and BA.2, daughter branches from branch A. *Reproduced with permission from Parera et al.[80]*

Pulmonary development across species

Pulmonary development is similar across mammalian species, however there are important differences in the timing of alveolarisation, as shown in *Figure 6*. In humans, rabbits and sheep alveolarisation begins prior to birth, whereas in rats alveolarisation begins postnatally.[92, 93] Therefore, data from sheep and rabbits are more relevant when translating findings into a clinical situation.

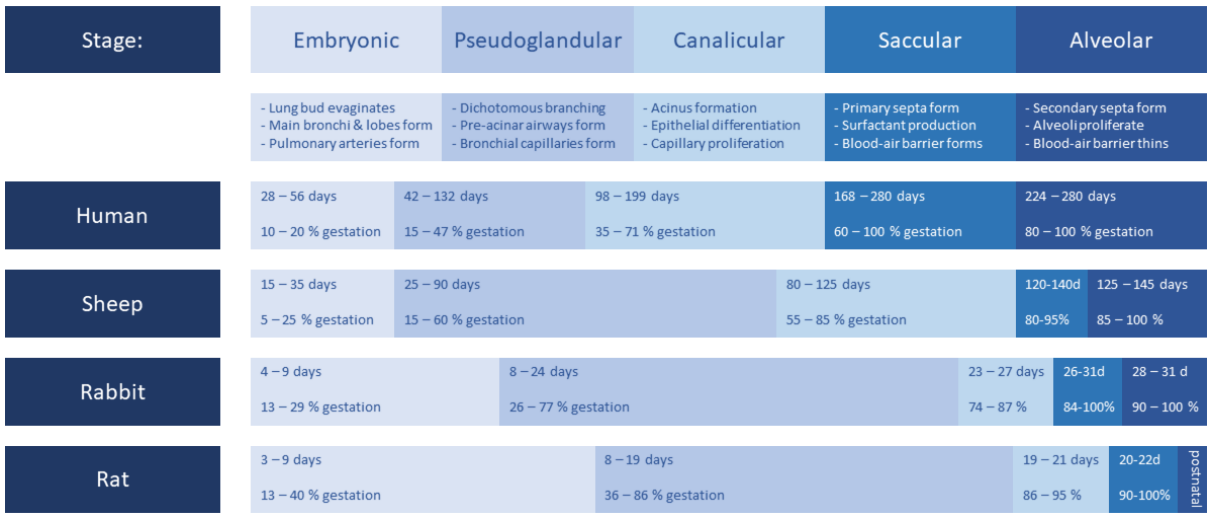

Figure 6 - Normal pulmonary development across species

Pulmonary development in humans, compared to animal models of congenital diaphragmatic hernia. *Original figure, using data from Pringle et al.*[92]

Pulmonary development in congenital diaphragmatic hernia

Mechanisms for impaired pulmonary development in CDH

The mechanisms by which CDH impairs pulmonary development are not entirely understood. The classical hypothesis is that herniating abdominal viscera occupy space in the thoracic cavity, placing external pressure on the developing lungs.[94] This external pressure reduces the capacity of continuous secretion of lung liquid into the airways to maintain lung distension, hence reduces the imposed mechanical stretch and subsequent cell division and tissue remodelling described above.[68, 69] Increased intrathoracic pressure also increases the volume of lung liquid lost during fetal breathing movements, which is further exacerbated by the impaired ability of the

defective diaphragm to oppose passive lung recoil.[70, 71] Hence, lung liquid volumes are significantly reduced in CDH, resulting in significant lung hypoplasia (Figure 4).

Keijzer *et al.*[95] have proposed the "dual-hit" hypothesis: early in fetal development, the same genetic or environmental insult that prevents closure of the diaphragm also impairs bilateral pulmonary branching morphogenesis. Following this initial insult, the diaphragmatic defect and herniating abdominal viscera impose a "second-hit" on the lungs via the mechanisms described above.[95]

The dual-hit hypothesis was conceived based upon observations in a rodent model of CDH, in which the diaphragmatic defect is induced by maternal treatment with nitrofen. Nitrofen is a teratogenic herbicide that causes cardiac, pulmonary and diaphragmatic defects, by interfering with retinoic acid signalling.[52] Interestingly, pulmonary hypoplasia is seen in 100% of the offspring of nitrofen-rats, whereas CDH is only seen in 60-90%.[95, 96] In addition, when nitrofen is applied *in vitro* to rat lungs, there is reduced dichotomous branching and epithelial cell differentiation.[95] Other experiments have demonstrated that administering Vitamin A to nitrofen-rats both improves lung maturation and decreases diaphragmatic hernia incidence.[97-99] Together, these findings suggest that disrupted retinoic acid signalling causes pulmonary hypoplasia independently of the diaphragmatic defect.

Yet whilst the "dual-hit" hypothesis certainly explains the mechanism of nitrofen-induced diaphragmatic hernia, it is unclear whether this applies to naturally occurring CDH in humans. There is growing evidence linking the retinoic signalling pathway with both diaphragmatic and pulmonary development, but it remains unclear whether it is affected in the same way by nitrofen as it is in *de novo* CDH.[45] For example, the expression pattern over time of a crucial protein (ALDH1A2) in the retinoic acid signalling pathway is identical in both human CDH and surgically induced diaphragmatic hernia.[44] These findings demonstrate that at least some disruptions in retinoic acid signalling are actually a result of the hernia, rather than the cause.

It is also important to note that subsequent studies have raised issues with Keijzer's definition of "pulmonary hypoplasia", which is based on lung weight. Whilst lung weight decreases following nitrofen exposure, so too does body weight. Mayer *et al.*[50] found

that lung-body weight ratio is no different between control rats and nitrofen-exposed rats without diaphragmatic hernia, however it is markedly decreased in nitrofen-exposed rats with diaphragmatic hernia. It has also been shown that appropriately-timed surgical induction of diaphragmatic hernia in rabbits and sheep replicates the clinical phenotype of CDH, including pulmonary hypoplasia.[100-103] These results indicate that herniation of abdominal viscera alone is sufficient to cause significantly impaired pulmonary development. Therefore, surgical CDH models such as rabbits and sheep appear relevant to the human disease, with the key limitation that the defect is only created during the late pseudoglandular phase of lung development, so earlier embryonic changes are not replicated.

Some also suggest that decreased pulmonary blood flow (PBF) alone causes lung parenchymal hypoplasia, as fetal sheep with a ligated left or main pulmonary artery develop lung hypoplasia.[104, 105] However, it is difficult to extrapolate the consequences of pulmonary hypoperfusion due to complete pulmonary artery ligation to the potential moderate pulmonary artery compression from herniating abdominal viscera in CDH.

Aberrations in pulmonary development caused by CDH

Regardless of the underlying pathogenesis, CDH severely affects the developing lungs.[8] These effects are apparent in post-mortem reports from human fetuses[9, 38, 106] and also in animal studies.[103, 107-115]

Airway

Arrested pulmonary development begins during the pseudoglandular stage, with a 33-66% decrease in conducting airway branches and reduced lung size.[8, 9, 38, 103, 106, 113-115] Both lungs are affected, probably because of a left-to-right mediastinal shift, however the effects are more severe in the ipsilateral lung.[8] Acinar complexity is also reduced, with a lower overall number and decreased alveolar size.[9, 38, 103, 106, 114, 115] A reduced alveolar surface-area and thickened septa (increased air-blood gas barrier) significantly impairs gas exchange.[103, 106, 112] Increased airway muscularisation has been described in CDH infants but is thought to be iatrogenic, as it commonly occurs in infants who received significant ventilator assistance but is not seen in infants who die before 24 hours of age.[8, 116]

Vascular

The pulmonary vascular abnormalities associated with a CDH were first described by Reid *et al.*, in a series of post-mortem studies of infants who died from pulmonary hypertension.[9, 117] The first post-mortem was performed in a CDH infant that died at 4 hours after birth.[9] Pulmonary arteries were reduced in both size and number, and the ipsilateral lung had a reduced number of elastic arteries (in proportion to muscular and transitional arteries). Muscularisation was observed in smaller arteries than in normal neonates, however the intra-acinar arteries remained amuscular.[9] In contrast, in the second post-mortem, performed on an infant that died at ~10 weeks after birth, muscularised intra-acinar vessels were observed adjacent to the alveolar ducts (distal neomuscularisation).[117] The absence of this intra-acinar muscularisation shortly after birth suggests that neomuscularisation of distal pulmonary blood vessels occurs during the neonatal period, in response to the high pulmonary arterial pressures caused by a high pulmonary vascular resistance (PVR).[118] Hence, preventing increased pulmonary arterial pressures during the neonatal transition may reduce the degree of neomuscularisation and prevent the cycle of persistent pulmonary hypertension from beginning.

Taira *et al.* have suggested that intra-acinar vessel neomuscularisation occurs *in utero* in CDH fetuses, and hence is an irreversible factor contributing to pulmonary hypertension at birth.[119] This is plausible, because the fetal pulmonary vascular endothelium and smooth muscle cells are more susceptible to modulation by exogenous stimuli than neonatal lungs.[120] Hence, a small increase in pulmonary perfusion pressures may trigger over-proliferation of vascular smooth muscle cells. However, this is unlikely because the open fetal shunts between the systemic and pulmonary circulation (ductus arteriosus and foramen ovale) allow any increase in pulmonary blood pressure to simply equilibrate to the systemic circulation. Regardless, compared to newborns that died due to sudden infant death syndrome (SIDS), both newborns and stillborns with a CDH demonstrated evidence of peripheral pulmonary vascular muscularisation.[119]

However, Taira *et al.*[119] categorised vessels based on size, whereas Reid *et al.*[9, 117] categorised vessels based on branching generation number. This is an important

distinction, as the diameter of supernumerary arteries can be substantially smaller than their parent artery, so pulmonary artery branches of any given diameter are distributed over a wide range of generations and small branches are not necessarily intra-acinar.[121] Particularly in CDH infants, the entire pulmonary vascular tree is smaller due to the lung hypoplasia. Hence, by comparing vessels based on size, Taira *et al.* likely compared early generation arteries in CDH infants with late generation arteries in SIDS infants. The greater degree of muscularisation in small vessels in CDH patients may therefore have been appropriate for their position in the pulmonary vascular tree (Figure 7). Subsequent neonatal post-mortem studies have consistently demonstrated hypermuscularisation and neomuscularisation of the pulmonary vascular tree.[122-124] However, there have been no fetal post-mortem studies that analyse vessels by branching generation, so it remains unclear whether neomuscularisation of intra-acinar vessels occurs before birth in CDH.

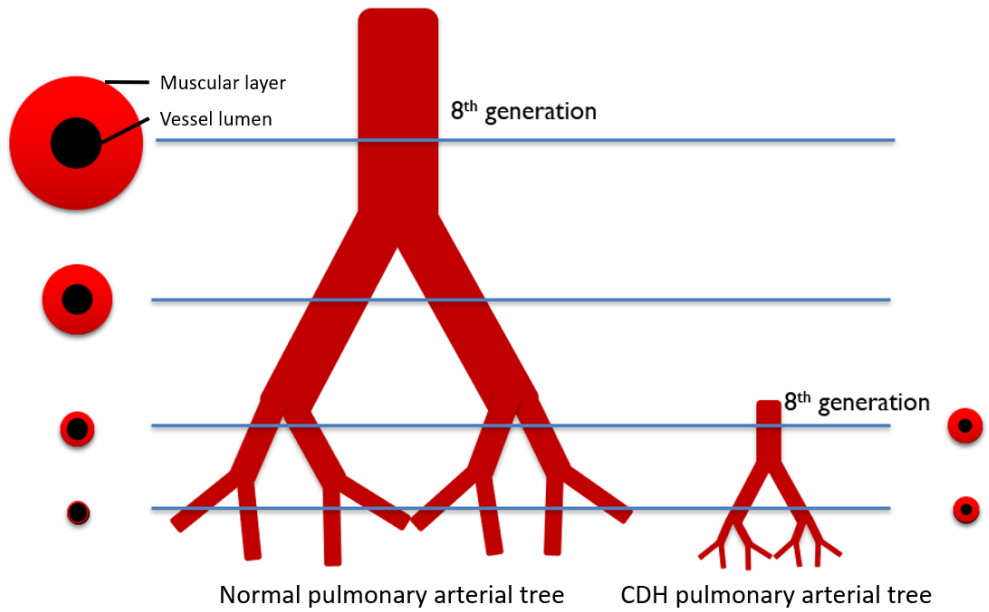

Figure 7 - Comparison of normal and CDH pulmonary arteries by size

Normal and CDH pulmonary arterial trees are depicted, beginning at the 8th generation. Axial sections of arteries depicting the lumens and muscular layers are shown beside each tree. In this diagram, the 8th generation vessels in the CDH lung are as large as the 10th generation vessels in the normal lung. In the normal lung, as generation number increases, vessel and luminal diameter decrease along with the proportion of muscularisation. The same is true for CDH arteries. Comparing CDH and normal pulmonary arteries by size reveals that CDH arteries are more muscular. However, they may be appropriately muscularised for their generation number. *Figure used with permission from Mr Andrew Stainsby.*

In addition to hypermuscularisation of pre-acinar vessels and neomuscularisation of intra-acinar vessels, the number and diameter of distal vessels are reduced in infants with a CDH, which further decreases total vascular cross-sectional area.[115, 125] These structural abnormalities are irreversible factors that contribute to increased PVR in CDH infants.[126] However, there are also reversible factors related to aberrations in the biochemical pathways that regulate pulmonary arterial pressure.[127] To better understand their effect, it is important to first appreciate the mechanisms underlying the greatest physiological challenge that a CDH infant will face. This challenge is the transition from fetal to neonatal life at birth, when placental support is lost and their underdeveloped lungs are required to assume the role of gas exchange for the very first time.

1.3 The fetal to neonatal transition: a physiological challenge

When the fetus is separated from the placenta during the transition from fetal to neonatal life, major physiological changes must occur for the infant to survive. The lungs must be cleared of liquid and filled with air to enable pulmonary gas exchange, the cardiovascular system must rapidly remodel from a parallel to series circulation, and oral energy intake must begin. While breastfeeding (or alternative nutrition) should begin within an hour of birth, the respiratory and cardiovascular transition must occur with far greater urgency.

1.3.1 Respiratory transition at birth

Before birth, the lungs are filled with liquid and do not perform gas exchange.[128] After birth, when the umbilical cord is clamped and placental gas exchange is lost, lung liquid must be rapidly cleared so that pulmonary gas exchange can commence. Lung liquid clearance occurs in two phases, in which liquid is firstly cleared from the airways and enters lung tissue, then is secondly cleared from the tissue (Figure 8). Once lung liquid clearance is complete, the third phase of the respiratory transition, in which gas exchange can be optimised, can begin.

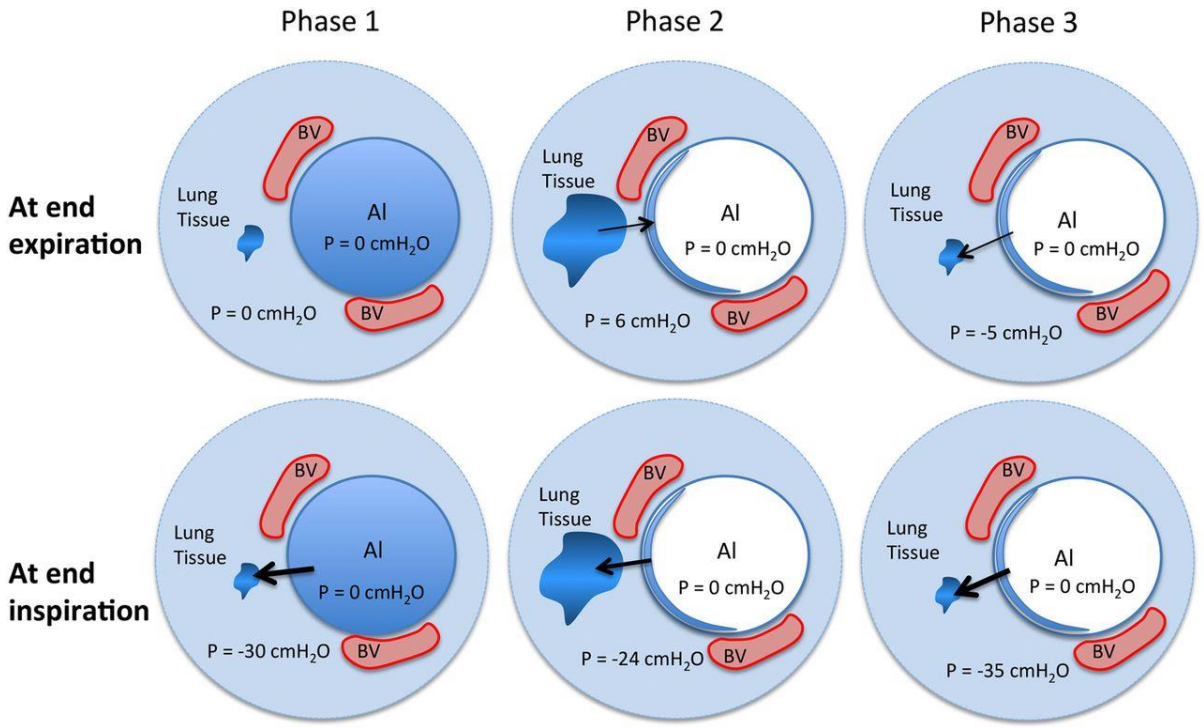

Figure 8 - The three phases of the respiratory transition at birth

The lung passes through three distinct phases as it transitions from a liquid-filled organ with a low blood flow into the sole organ of gas exchange after birth. During the first phase, the liquid-filled airways are cleared of lung liquid so that gas exchange can commence, due to transepithelial pressure gradients generated during inspiration. During the second phase, the liquid cleared from the airways resides within the interstitial tissue, which increases interstitial tissue pressures and increases the likelihood of liquid re-entering the airways at end-expiration (ie, at functional residual capacity). The third phase depicts the lung following all airway liquid clearance from the chest, resulting in subatmospheric interstitial tissue pressures and end-expiratory pressure gradients, which assist in keeping the airways cleared of liquid. Al, alveolus; BV, blood vessels; P, pressure. *Reproduced with permission from Hooper et al.*[129]

During the first phase, liquid is cleared from the airways into the interstitium, which is driven by the transpulmonary pressures generated by inspiration.[129] Most neonates, including very preterm infants, breathe spontaneously at birth, but whether or not they clear their airways of liquid depends on the depth and number of inspiratory efforts.[130-132] It is important to note that when the alveoli remain predominantly liquid-filled, gas exchange is limited and there is a high risk of ventilation induced injury because a seemingly physiologic tidal volume is being forced into only a small proportion of the lungs.

If infants are not breathing at birth, similar pressure gradients to those that occur during breathing can be replicated by applying positive pressure respiratory support, thereby assisting infants to clear their airways of liquid. While the optimal approach for providing this support is unclear, in view of the known science, there are two important considerations.

1. During the first phase the airways are filled with liquid, so gas exchange is not possible. As such it is logical to provide a sustained inflation to optimally clear the airways of liquid, as expiration has no purpose.

   If no CO_2 exchange can occur because the alveoli are liquid-filled then expiration is not required.[131, 132] Considering this, the optimal approach to aerate a liquid-filled lung is to give a sustained inflation (to optimise lung liquid clearance and achieve uniform lung aeration) that is not interrupted to allow for expiration. Some suggest that a sustained inflation imitates normal physiology, as an infant's first breaths after birth are generally long and deep, requiring significant effort by the infant.[133, 134] However, spontaneous deep inspiratory efforts (500 – 800 msec) are considerably shorter than the inflation times required to aerate the lung.[135] Nevertheless, a sustained inflation has been shown to be very efficient at uniformly aerating the lung.[135] Considering this, we provided a sustained inflation to initiate neonatal ventilation in our experiments in the lamb model of CDH. The suitability of this is discussed further in *Chapter 5.3.3*.

2. Pulmonary gas exchange and the supply of oxygen to the newborn cannot commence until after the gas exchange regions of the lung have aerated.

   If air cannot reach the alveoli, then there is no pulmonary oxygen uptake and the infant must continue to rely on placental gas exchange to maintain oxygenation. However, if the umbilical cord is clamped before the lungs have aerated, then the infant is left without an oxygen source. As a result, if lung aeration is delayed (for instance due to severe lung hypoplasia), it is likely that the infants will become hypoxaemic, as has been shown in pre-term lambs.[12]

During the second phase of the respiratory transition, lung liquid has been cleared from the airways and temporarily accumulates within interstitial lung tissue.[129] This has

significant consequences for respiratory function in the newborn period. The presence of this liquid in lung tissue decreases lung compliance, increases airway resistance, reduces functional residual capacity, expands the chest wall and flattens the diaphragm. Management during this phase should focus on maintaining positive end-expiratory pressure to counteract increased interstitial pressure, to prevent alveolar re-flooding and counteract the reduction in functional residual capacity.

The third phase of the respiratory transition occurs once lung liquid has been completely cleared from the airways and tissue, so respiratory support should focus on providing uniform ventilation and optimising gas exchange.[129]

Most infants navigate the first phase of lung liquid clearance within the first minute after birth, however infants with a CDH likely take far longer due to their underdeveloped lungs. Furthermore, the presence of abdominal organs in the intrathoracic space may contribute to increased interstitial pressures, increasing the probability of alveolar re-flooding during expiration and a reduction in functional residual capacity. Indeed, intubated rabbits with a surgically-induced diaphragmatic hernia take longer to achieve lung aeration after birth than rabbits without a diaphragmatic hernia.[136] Hence, it is likely that infants with a CDH cannot rely on pulmonary gas exchange during the first few minutes after birth.

1.3.2 Cardiovascular transition at birth

A successful respiratory transition allows pulmonary gas exchange to replace placental gas exchange after birth. However, simply aerating the lungs is not sufficient; cardiac output must also be redirected through the lungs to facilitate pulmonary gas exchange. Hence, the cardiovascular system must also undergo substantial changes for the infant to successfully transition to newborn life and these changes are intrinsically linked with lung aeration.[137] The cardiovascular transition at birth involves removing the placental circulation, dilating the pulmonary vasculature, and closing the fetal shunts to separate the pulmonary and systemic circulations.

Fetal circulation

During fetal life, PVR is markedly higher than it is after birth, but gradually decreases with increasing gestation. While this gradual reduction in PVR is thought to be due to growth of the pulmonary vascular bed, the underlying pathways driving high fetal PVR are not completely understood. However, they likely relate to a low PO_2, an absent gas-liquid interface, the balance between vasodilatory and vasoconstrictive mediators such as endothelin-1 (ET-1), and the hyper-expanded state of liquid-filled fetal lungs.[138-145] In contrast, systemic vascular resistance is low due to the low resistance placental circulation. Hence, most right ventricular output (88%) bypasses the lungs and flows through the ductus arteriosus into the descending thoracic aorta towards the placenta (Figure 9).[146] Indeed, during late systole and throughout diastole there is retrograde PBF (Figure 10), as blood reflects off the high resistance pulmonary vascular bed and instead passes through the ductus arteriosus to the systemic circulation.[147, 148]

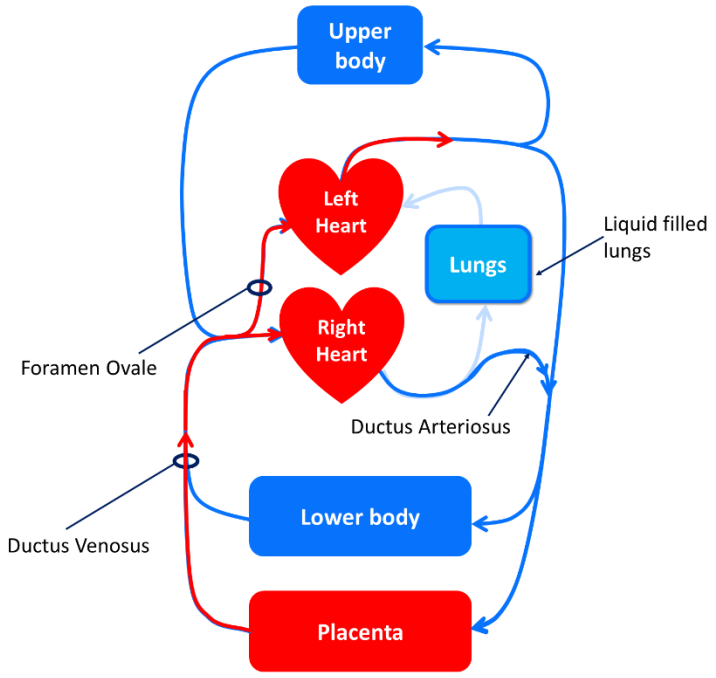

Figure 9 - Fetal circulation

During fetal life, the lungs are filled with liquid and pulmonary vascular resistance is high. Hence, most right ventricular output bypasses the lungs and flows through the ductus arteriosus towards the placenta. The placenta performs gas exchange, then returns oxygenated blood to the fetus via the umbilical vein then ductus venosus. Placental venous return is preferentially streamed through the foramen ovale towards the left atrium, which provides the left ventricle with oxygenated blood to distribute throughout the body. *Original figure.*

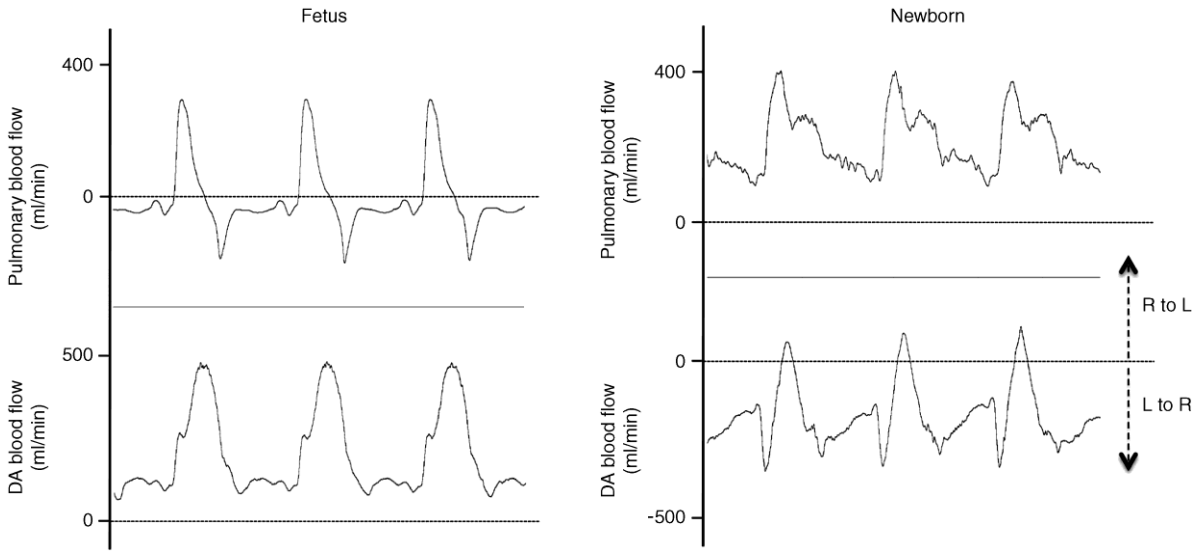

Figure 10 - Blood flow waveforms in the pulmonary artery and ductus arteriosus in the fetal and neonatal lamb

Blood flow waveforms in the left pulmonary artery and ductus arteriosus (DA) in a lamb before (Fetus) and after (Newborn) ventilation onset. PBF oscillates around zero before ventilation onset (negative flows reflect retrograde flow of blood away from the lungs), but is positive after ventilation onset. Before ventilation onset, DA flow is positive. This indicates that blood flows right-to-left (R to L), from the pulmonary circulation into the aorta, continuously throughout the cardiac cycle. R to L DA flow during diastole before ventilation onset is due to retrograde PBF. Following ventilation onset, DA blood flow is predominantly left-to-right (L to R), flowing from the aorta and into the lungs, throughout most of the cardiac cycle, except during early systole. L to R flow in the DA following ventilation onset significantly contributes to PBF and is entirely responsible for PBF during diastole. *Reproduced with permission from Hooper et al.*[149]

While fetal PBF is predominantly low, during accentuated fetal breathing movements there is a transient increase in PBF (by 60%) that is closely associated with the amplitude of the decrease in tracheal pressure associated with inspiratory efforts (only occurring when amplitude >3.5 mm Hg).[150] In CDH, disrupted diaphragmatic function and increased intrathoracic pressure due to herniating abdominal organs may disrupt this normal physiological process. In contrast, when the trachea is occluded, PBF paradoxically decreases during accentuated fetal breathing movements.[151] This likely reflects diaphragmatic eversion related to over-distension of the fetal lungs, which results in inspiratory efforts that compress the lungs and the capillaries within them. Alternatively, it may occur because total lung capacity is reached during inspiratory efforts in an already over-distended lung. When total lung capacity is reached *in utero*, PBF completely ceases.[152]

The shunting of blood from the pulmonary to systemic circulation via the ductus arteriosus also ensures that the placenta receives a high proportion of total fetal cardiac output, with the right ventricle being the main contributor (Figure 9). As such the right ventricle provides most blood flowing through the organ of gas exchange (placenta), just like in the adult. Oxygenated blood returning form the placental circulation flows through the ductus venosus into the foramen ovale (in humans, flowing via the right atrium into the foramen ovale; and in sheep, flowing via the medial wall of the inferior vena cava directly into the foramen ovale, as in *Figure 11*). As very little placental venous return enters the right ventricle and is instead preferentially directed into the left atrium through the foramen ovale, the left ventricle receives most of its preload from the organ of gas exchange (placenta), just like in the adult. These flow dynamics were first described by Rudolph *et al.* using microspheres.[153] More recently, Schrauben *et al.* have utilised 4D magnetic resonance imaging (MRI) to obtain particle traces of flow in fetal sheep that clearly illustrate blood flowing from the ductus venosus through the foramen ovale to the left atrium, without any visible mixing with unoxygenated blood from the distal inferior vena cava (Figure 11).[154]

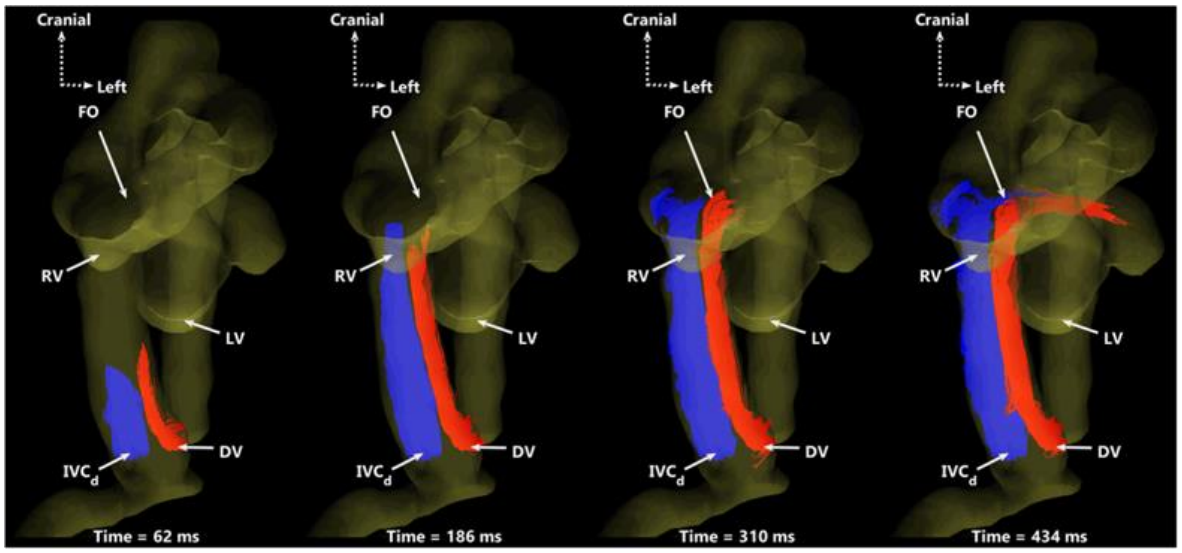

Figure 11 - Flow dynamics directing placental venous return to the left atrium

Particle traces in a ventral view showing preferential delivery of DV blood to the left side of the heart. DV (red) and IVCd (blue) particles are shown at four time-points over one cardiac cycle. The two streams remain well-separated, with blood from the DV primarily entering the left heart while IVCd blood passes into the right ventricle and main pulmonary artery. Particle trace movies over two cardiac cycles are available as supplementary videos. DV: ductus venosus; IVCd: distal inferior vena cava; FO: foramen ovale; RV: right ventricle; LV: left ventricle. *Reproduced from Schrauben et al.*[154] *under the terms of the Creative Commons Attribution 4.0 International License* http://creativecommons.org/licenses/by/4.0/

While the left ventricle receives most of its preload from the placental circulation, with only a small amount from the pulmonary circulation, there is an inverse relationship between PBF and foramen ovale flow. This suggests these two sources of venous return compete to supply the left ventricle with preload.[155] Some hypothesise that in CDH, the herniating abdominal viscera displace and rotate the heart in such a way that preferential flow through the foramen ovale is reduced during gestation.[156] As PBF is also reduced *in utero* in CDH, according to this hypothesis, both sources of left ventricular preload would be reduced, resulting in left ventricular hypoplasia. As a result, they suggest that the underdeveloped left heart cannot accept adequate pulmonary venous return after birth, leading to pulmonary venous hypertension.[157]

Fetal to neonatal cardiovascular transition

Removing the placental circulation by clamping the umbilical cord at birth increases carotid arterial pressure by 30% within 4 heart beats and simultaneously decreases cardiac output by 30-50%.[158] The reduction in cardiac output reflects both increased afterload, due to an increase in systemic vascular resistance when the low-resistance placental circulation is removed, and decreased preload, due to the loss of umbilical venous return. Cardiac output is only restored when the pulmonary circulation vasodilates to accept the entire output of the right ventricle, leading to a large increase in PBF that can sustain left ventricular preload.

This vital increase in PBF at birth is primarily triggered by lung aeration.[159] There are a number of proposed mechanisms, which include increased oxygenation, vasodilator expression, increased lung recoil, and shear stress. Increased oxygenation stimulates pulmonary vasodilation directly via mitochondria and through signalling pathways related to nitric oxide (NO), vasodilatory prostaglandins and platelet-activating factor.[160, 161] Replacing lung liquid with air effectively reduces intraluminal lung volume (from 45 mL/kg lung liquid in fetal lambs, to 20 – 30 mL/kg functional residual capacity in newborn lambs) and creates an air-liquid interface that establishes surface tension. Together, increased intraluminal volume and surface tension increase lung recoil, hence reducing intraluminal pressure and allowing increased expansion of peri-alveolar capillaries.[145] The initial increase in PBF is also thought to result in increased shear

stress that further increases PBF by stimulating and upregulating endothelial NO synthase (eNOS)[139, 162], stimulating inducible NOS (iNOS)[163], and activating potassium channels[164].

Most of these hypotheses are based on mechanisms that regulate regional vascular tone. However, an elegant series of studies by Lang *et al.* suggest that a previously unknown *global* factor is most relevant during the neonatal transition.[137, 165, 166] Using simultaneous phase contrast X-ray imaging and angiography to visualise lung ventilation and perfusion simultaneously, they first demonstrated that the increase in PBF at birth is not spatially related to lung aeration in rabbit kittens.[137] Secondly, a global increase in PBF was induced by partial lung aeration regardless of the oxygen content of the inspired gas.[165] Thirdly, this process was inhibited by vagal denervation.[166] Together, these findings suggest that lung aeration triggers a rapid, global increase in PBF, via a neurally-mediated mechanism that is potentiated by, but not reliant on locally-acting birth-related factors such as increased oxygenation, vasodilator release and increased lung recoil.

The combination of reduced PVR and increased systemic vascular resistance at birth leads to the reversal of blood flow through the ductus arteriosus, with left-to-right shunting a key contributor to the rise in PBF at birth. Indeed, left to right shunting through the ductus arteriosus contributes to 50% of total PBF during the first 30 min after birth and maintains high basal PBF during diastole throughout the early neonatal period.[167] Despite this reversal, there remains some instantaneous right-to-left shunting in neonatal life during early systole, because the right ventricle is closer to the ductus arteriosus than the left ventricle.

When should the umbilical cord be clamped at birth?

The optimal timing of umbilical cord clamping at birth has been debated for millennia, with the weight of scientific opinion oscillating from delaying cord clamping "till all pulsation in the cord ceases" (Darwin, 1796)[168], to immediate clamping with the aim of reducing the risk of post-partum haemorrhage[169]. However, the pendulum has swung again due to more recent scientific evidence, and so now most guidelines recommend delaying cord clamping for at least 60 seconds after birth in infants not requiring

resuscitation.[170] These current recommendations are mostly based on evidence suggesting that delaying umbilical cord clamping results in net placenta-to-infant blood transfusion. If the umbilical cord is clamped immediately after birth, approximately 20-40% of fetal-placental blood remains in the placenta, whereas if cord clamping is delayed then some of this blood is transfused from the placenta to the infant (supposedly at a rate of 2 – 3 mL/kg/min during the first 3 min after delivery).[171, 172] This suggestion is consistent with a systematic review of 18 randomised controlled trials that found delayed cord clamping is associated with increased neonatal haematocrits and a 10% reduction in required blood transfusions for preterm infants.[173]

For the net transfusion of blood to occur from placenta to infant, net umbilical venous flow must be greater than umbilical arterial flow during the period of delayed cord clamping, however it is unclear what factor would differentially affect umbilical arterial and venous flow. Gravity does not differentially affect umbilical arterial and venous flow in lambs[174], and indeed placental transfusion is similar whether a baby is positioned on the maternal abdomen/chest or at the level of the introitus.[175] Some suggest that uterine contractions will drive blood from the placenta to the baby, based on neonatal blood volume estimates.[176]. However, more recent studies that directly assess blood flow have shown that uterine contractions cause both umbilical arterial and venous flows to cease.[177-180] This finding suggests that oxytocin administration, given to stimulate uterine contractions and prevent maternal post-partum haemorrhage, should be delayed until after umbilical cord clamping.

Another factor proposed to result in placental transfusion is neonatal breathing. It is suggested that decreased intrapleural pressures during inspiration will provide a pressure gradient that increases umbilical venous flow while reducing umbilical arterial flow, hence creating net placenta-to-infant transfusion. However, in spontaneously breathing term lambs, the reduced intrapleural pressures during inspiration were associated with *decreased* umbilical venous flows.[181] This suggests that spontaneous breathing does not cause net placenta-to-infant transfusion, which may be due to diaphragmatic contractions during inspiration occluding the ductus venosus. Indeed, the diaphragm is known to constrict the inferior vena cava during fetal breathing movements.[182] The authors hypothesise that given the high compliance of the venous

system, these reductions in flow are slightly delayed relative to the timing of inspiration. This explains why inspiration appeared to be associated with increases in umbilical venous flow in a study that used Doppler ultrasound to assess flows upstream in the umbilical cord.[180]

Given that gravity, uterine contractions, and spontaneous breathing do not lead to higher umbilical venous flow compared to arterial flow, the mechanisms underlying net placenta-to-infant transfusion remain unclear.

An important caveat to the recommendation to delay umbilical cord clamping for at least 60 seconds after birth is that this only applies to vigorously breathing infants who do not require respiratory support. Hence, infants with a CDH are excluded, because they require immediate intubation at birth to provide respiratory support and avoid inadvertent gaseous distension of the intrathoracic stomach.[183]

In order to accelerate the placental transfusion process in infants requiring resuscitation at birth, umbilical cord milking has been suggested as an alternative to delayed cord clamping. This technique involves grasping the unclamped umbilical cord and pushing blood towards the infant multiple times; it is designed to provide rapid placental transfusion without postponing neonatal resuscitation. While umbilical cord milking results in greater systemic blood flow and increased haemoglobin compared to delayed cord clamping[184], it is also associated with a greater risk of intraventricular haemorrhage in extremely premature infants (23-27 weeks GA).[185] This increased risk of intraventricular haemorrhage is likely related to non-physiological large oscillations in cerebral blood flow and pressure associated with each milking action, which have been described in preterm lambs.[186] This lamb study also raised concerns that a milking technique that is commonly performed in clinical trials, repeated milking without allowing for placental refill, results in no net placental transfusion.[186]

An alternative method for extending the benefits of delayed cord clamping to infants requiring resuscitation would be to bring the resuscitation devices to the maternal bedside.[187-190] There is now an emerging body of evidence that such an approach, termed intact-cord resuscitation or physiologically based cord clamping (PBCC), is feasible in preterm[191] and very preterm[13, 192] infants, as well as infants with a CDH[193]. A

remaining barrier is the attitudes of caregivers towards this technique: a study in the United States of America found that 50% of caregivers had concerns regarding access to the infant during neonatal resuscitation at the maternal bedside, and that 16% felt uncomfortable with the family observing the resuscitation.[194] Despite caregiver concerns, neonatal resuscitation at the maternal bedside was a positive experience for most parents.[194] Indeed, allowing earlier mother-to-child contact may reduce parental stress and improve maternal-infant bonding.[195]

Importantly, providing ventilatory support at the maternal bedside allows for a physiological, rather than a time-based, approach to cord clamping. Previous delayed cord clamping strategies have been time-based, in order to optimise placental transfusion. However, the primary benefit of delayed cord clamping may actually relate to a more stable cardiopulmonary transition. This is because cord clamping causes a reduction in cardiac output that is only restored when PBF increases to replace placental venous return as the primary source of preload for the left ventricle. As such, increasing PBF before the placental circulation is removed allows a smoother transition from placental to pulmonary gas exchange and venous return (Figure 12). Indeed, recent evidence suggests that achieving lung aeration before umbilical cord clamping (PBCC) can avoid the increase in arterial pressures and reduction in cardiac output associated with immediate cord clamping, resulting in improved systemic and cerebral oxygenation.[158, 196] In order to achieve these physiological benefits, it is suggested that the timing of cord clamping should be based on the infant's clinical condition and respiratory stability, rather than on an arbitrary timepoint. In an ongoing randomised controlled trial in preterm infants, the definition for "respiratory stability" (lungs ready to replace placental gas exchange and venous return) is taken as regular spontaneous breathing with oxygen saturation above 90%, heart rate > 100, and oxygen requirements < 40%.[197]

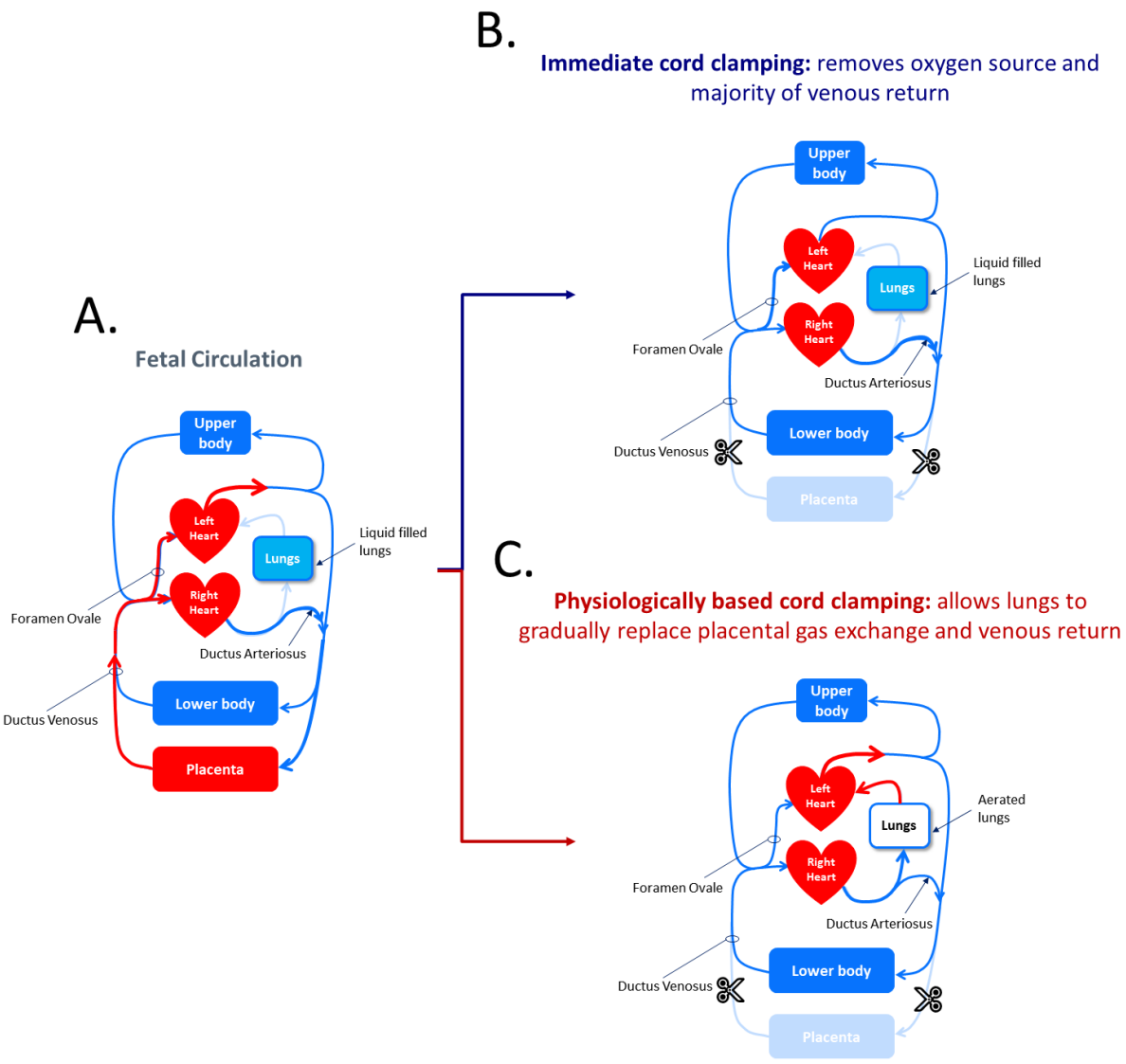

Figure 12 - The transition from fetal to neonatal circulation, with immediate or physiologically based cord clamping

A: In the fetal circulation, the lungs are filled with liquid and pulmonary vascular resistance is high. Hence, most right ventricular output is diverted through the ductus arteriosus towards the placenta. The placenta performs gas exchange, then provides most venous return to the left ventricle via the ductus venosus and foramen ovale. **B:** When immediate cord clamping is performed, the placental circulation may be removed before the lungs aerate and pulmonary blood flow increases. When this occurs, placental gas exchange and venous return are lost before the lungs are ready to take over these roles. **C:** When physiologically based cord clamping is performed, the umbilical cord is clamped only after the lungs are aerated and pulmonary blood flow has increased. This allows pulmonary gas exchange and venous return to gradually replace placental gas exchange and venous return. *Original figure.*

Closure of fetal cardiovascular shunts

As described above, the fetal circulation is reliant on several shunts (ductus arteriosus, ductus venosus and foramen ovale) in order for the right ventricle to provide the majority of blood flow to the organ of gas exchange and, once oxygenated, for this blood to return to the left ventricle.

During fetal life, the ductus arteriosus allows blood to flow from the pulmonary artery into the descending aorta, down the pressure gradient between the two circulations. While the precise mechanism for ductus arteriosus closure at birth is not clear, it is thought that when the umbilical cord is clamped at birth, the placental supply of prostaglandin E_2 is lost. This suggestion is consistent with the finding that prostaglandin synthesis inhibitors (such as indomethacin) can cause ductus arteriosus closure in infants with a patent duct. Furthermore, an increase in oxygenation is associated with vasoconstriction of the ductus arteriosus, which may be mediated by cytochrome P450, ET-1 receptors or voltage-gated K^+-channel inhibition.[198] However, no study has yet been able to determine the direct effect of increased oxygenation on the ductus arteriosus without the confounding effects of pulmonary vasodilation.

It has been suggested that increased oxygenation and reduced circulating PGE_2 levels lead to a significant reduction in ductal patency within 5 min after birth. However, this is clearly not correct as studies in humans[199] and sheep[167] demonstrate very large left-to-right (reversed) shunts over the first 10 min after birth. This results from the haemodynamic changes associated with birth, in particular the increased systemic vascular resistance and decreased PVR, which reverses the pressure gradient across the ductus arteriosus leading to a reversal of ductal flow, rather than cessation. Indeed, left-to-right flow through the ductus arteriosus is an important component of PBF during early neonatal life,[167] which not only helps to improve the efficiency of gas exchange, but also ensures the left ventricle is well supplied with preload, via pulmonary venous return.

Ultimately, functional closure (cessation of ductal flow) is thought to be complete by 27 hours (median) in male infants and 45 hours in female infants, although the reasons for the gender differences are unclear.[200] Permanent structural closure eventually

occurs due to the ingrowth of intimal cushions, integrin-mediated remodelling driven by shear stress stimulation, endothelial cell proliferation driven by hypoxia-inducible agents in the widening vessel wall, and platelet-vessel wall interactions.[198]

While it is widely considered that the foramen ovale and ductus venosus both close with the haemodynamic changes at birth, leading to a cessation rather than a reversal of flow, this has not been well demonstrated. Increased pulmonary venous return increases left atrial volume, increasing left above right atrial pressure, causing the foramen ovale to collapse and fuse with the intra-atrial septa. However, intra-atrial shunting, both left-to-right and right-to-left, is known to occur after birth and are usually indicative of haemodynamic issues such as a large left-to-right ductus arteriosus shunt and/or pulmonary hypertension with a significant right-to-left ductus arteriosus shunt.[201, 202] Little is known about the dynamic flow changes through the ductus venosus at birth, but presumably it ceases immediately after the umbilical circulation is clamped.

1.3.3 Metabolic transition at birth

While the respiratory and cardiovascular components of the neonatal transition are initially the most pressing issues, the metabolic changes must also be considered. During fetal life, the fetus is entirely dependent on transplacental transport of glucose from the maternal into the fetal compartment, which occurs by facilitated diffusion down its concentration gradient. However, after the placental circulation is lost, neonates must meet their metabolic requirements independently via enteral feeding. During the first hour after birth, neonatal blood glucose concentrations decrease, but then blood glucose concentrations quickly recover due to the mobilisation of hepatic glycogen stores.[203] These are rapidly depleted during the first 12 hours after birth, hence ongoing energy homeostasis requires exogenous nutrients (e.g. breastfeeding).[204]

1.3.4 A successful transition to neonatal life

Ultimately, an infant has successfully transitioned to neonatal life if they can deliver sufficient oxygen and nutrients to their tissues to meet the demands of metabolism and growth. Oxygen delivery is the product of cardiac output, which depends upon heart rate and stroke volume (determined by pulmonary venous return), and the oxygen content of blood, which is mainly depicted by O_2 saturation levels resulting from pulmonary gas exchange. Hence, to enable a successful neonatal transition, care in the delivery room must optimise the physiological processes of lung aeration, pulmonary vasodilation, and reorganisation of the cardiovascular system to separate the two circulations.

1.4 Persistent pulmonary hypertension of the newborn (PPHN) in CDH infants

As described above, the transition to newborn life at birth involves the lungs taking over the role of gas exchange from the placenta and the closure of cardiovascular shunts to separate the pulmonary and systemic circulations. Central to these cardiopulmonary changes is a reduction in PVR, which enables the lung to accept the entire output of the right ventricle, while also facilitating a reduction in pulmonary arterial pressure.

However, PVR remains increased at birth in almost all CDH infants and while it is often not clinically obvious immediately after birth, it can be demonstrated on echocardiogram.[205] Increased PVR leads to significantly elevated pulmonary artery pressures (persistently above systemic levels in 20% of CDH infants[2]), hence right ventricular output is preferentially shunted to the systemic circulation via the ductus arteriosus.[206] This shunting causes severe post-ductal hypoxaemia and significantly lower PBF.[113, 126] Reduced PBF leads to reduced left ventricular preload, hence reduced cardiac output, unless the foramen ovale continues to shunt blood from right to left. Together, this physiological maladaptation is referred to as persistent pulmonary hypertension of the newborn (PPHN).

PPHN occurs in CDH infants due to a combination of irreversible abnormalities in pulmonary vascular structure and reversible changes in the vasoreactivity of the pulmonary vascular bed.

1.4.1 Irreversible contributors to PPHN in CDH infants

The irreversible contributors to PPHN in CDH were discussed in *Chapter 1.1.3*. Infants with a CDH are born with fewer and smaller distal pulmonary blood vessels, hypermuscularisation of pre-acinar arteries, and possibly neomuscularisation of intra-acinar arteries. This results in a large reduction in the total cross-sectional area of the pulmonary vascular bed and therefore increased PVR.[9, 115, 117, 122-125]

Recent evidence suggests that abnormal cardiac development may also contribute to PPHN by causing pulmonary *venous* hypertension. Indeed, both humans[207, 208] and animals[111, 209-211] with diaphragmatic hernia demonstrate significant left ventricular hypoplasia and structural immaturity. The most compelling hypothesis is that herniating abdominal contents cause myocardial shift and rotation, altering the flow mechanics of blood returning from the umbilical circulation and preventing oxygenated blood from preferentially flowing through the foramen ovale.[157] In this way, preload to the left ventricle is reduced and left ventricular hypoplasia ensues. The resulting increase in left atrial pressure increases the driving pressure required to adequately perfuse the pulmonary circulation, hence further contributing to PPHN.

1.4.2 Reversible contributors to PPHN in CDH infants

The reversible component of PPHN in CDH describes a hyperresponsiveness of the pulmonary vascular bed in response to vasoconstrictive stimuli. This abnormal vasoreactivity is believed to be reversible because, hypothetically, dominant vasoconstriction can be opposed by pharmacologically-induced vasodilation.

Preclinical models suggest that the vascular dysfunction in CDH is mediated via endothelium-dependent pathways. Firstly, in excised arteries, pulmonary vascular contractility is increased in animal models of CDH to a similar degree as that observed when the pulmonary arterial endothelium has been mechanically removed.[212] Secondly, pulmonary vascular relaxation is impaired in response to ACh, an endothelium-dependent vasodilator, but normal in response to SNP, an endothelium-independent vasodilator.[111, 212]

Endothelin-1 (ET-1)

The most potent vasoconstrictor secreted by endothelial cells is ET-1. ET-1 plays a significant role in regulating PVR, as it mediates both vasoconstriction (via the ET-A receptor, on vascular smooth muscle cells) and vasodilation (via the ET-B receptor, primarily on vascular endothelial cells).[144] ET-A expression is high throughout fetal life, whereas ET-B expression is initially low before significantly increasing during the third

trimester, which suggests that this vasoactive peptide plays an important role in the transition from high fetal PVR to low neonatal PVR.[213, 214] ET-1 appears to adopt a pro-vasoconstrictive profile in both preclinical CDH models and *ex vivo* pulmonary arteries from CDH infants, with increased ET-A mediated pulmonary vasoconstriction and reduced ET-B mediated vasodilation.[215-219] In CDH infants, plasma ET-1 levels correlate with the severity of both pulmonary hypertension and eventual outcome.[127]

An imbalance in ET-1 receptor expression may also mediate the structural remodelling of the pulmonary vasculature *in utero*. ET-1 increases pulmonary vascular smooth muscle cell proliferation by inhibiting apoptosis, blockading ET-A decreases distal pulmonary arterial muscularisation, and blockading ET-B increases muscularisation.[220-222]

Nitric oxide (NO)

ET-1 partially exerts its vasoactive effects via eNOS, which is inhibited by ET-A and stimulated by ET-B. eNOS is an enzyme that catalyses the production of the potent vasodilator NO. NO diffuses from the endothelium into smooth muscle cells and activates guanylate cyclase, which converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP). cGMP exerts its action via a protein kinase (PK-G) that reduces intracellular calcium ion concentration, thereby inducing vasodilation. cGMP is broken down by the enzyme phosphodiesterase-5 (PDE5).

CDH is associated with multiple abnormalities in the eNOS-NO-cGMP system. In preclinical models, eNOS expression is reduced.[111] Interestingly, in CDH infants eNOS expression has been reported as decreased[223, 224], unchanged[225], and increased[226]. However, these studies are likely confounded by the effects of neonatal therapies such as oxygen and mechanical ventilation, which upregulate eNOS expression.[162] PDE5 expression is also increased in preclinical CDH models, leading to rapid degradation of cGMP, which reduces the efficacy of both endogenous and exogenous NO.[111, 227-229]

Neural

There also appears to be a neural component to pulmonary vascular hyperresponsiveness in CDH. CDH infants have decreased peribronchial and perivascular innervation, and in nitrofen-induced CDH in rats, sympathetic tone is

increased, whereas parasympathetic tone is decreased in peripheral airways.[230] This imbalance in autonomic innervation may increase pulmonary arterial hyperreactivity in CDH infants. This study also reported reduced expression of vasoactive intestinal peptide, a vasodilator that is normally present in nerve endings innervating the pulmonary airways and vasculature.[230]

Muscular

The pulmonary vasculature of CDH infants also has a higher proportion of contractile vascular smooth muscle cells, which likely increases vasoconstriction in response to the vasoactive stimuli described above.[10]

1.4.3 Implications for delivery room management

Given this pulmonary vascular hyperreactivity, optimal care of CDH infants in the delivery room should aim to allow a smooth neonatal transition that does not expose these infants to unnecessary hypoxic insults or swings in systemic and pulmonary arterial pressures. Indeed, any hypoxia or high pulmonary arterial pressures during the neonatal transition may potentially trigger pulmonary vasoconstriction, initiating a vicious cycle of PPHN.[231, 232] However, in the current guidelines for the standardised neonatal care of CDH infants, delivery room management is entirely based on expert opinion.[183] Clearly, a better understanding of the optimal support required during the neonatal cardiopulmonary transition in CDH infants is an area of research that has been relatively overlooked.

1.5 Neonatal management of CDH

Given the complex difficulties CDH infants face after birth, the CDH EURO Consortium published a consensus statement in 2010 to standardise care of CDH neonates.[233] After adopting the standardised protocol, two hospitals improved survival rates from 67% to 88%.[37] The 2016 update of this consensus statement recommends that CDH neonates should undergo planned delivery after 39 weeks in a specialised hospital, by either vaginal or caesarean section delivery.[183, 234-236] They are intubated immediately at birth, to avoid inadvertent gaseous distension of the intrathoracic stomach. In practice, immediate intubation usually translates to immediate umbilical cord clamping, in order to move the infant to a resuscitation bed for respiratory support. In normal term infants, lung aeration occurs rapidly after cord clamping. Hence, there is only a limited interruption to cardiac output and gas exchange at birth as the lung takes over the role of gas exchange and provides left ventricular preload. In contrast, CDH infants have stiff, hypoplastic lungs which take longer to aerate, as demonstrated in a preclinical CDH model.[136] As such, immediate cord clamping in infants with a CDH may expose them unnecessarily to a prolonged period of hypoxia, reduced cardiac output and increased arterial pressures immediately after birth. Hypoxia and high pulmonary arterial pressures can both trigger pulmonary vasoconstriction, which may be the first insult initiating the viscous cycle of PPHN.[231, 232]

Gentle ventilation (peak inspiratory pressure <25cm H_2O) with permissive hypercapnia (allowing $PaCO_2$ <70 mmHg) is critical because the reduction in lung growth is not uniform and so excessive ventilation can cause injury in less hypoplastic lung regions.[237-239] Whether this is initially achieved with conventional ventilation or high frequency oscillatory ventilation does not affect morbidity or mortality.[240]

Echocardiography should be performed within the first day of life in order to detect cardiac anomalies, assess right heart function and classify pulmonary hypertension severity.[205, 241] Treatment of pulmonary hypertension begins following signs of inadequate organ perfusion or preductal saturations below 85%, and involves addressing the reversible vasoconstrictive component to reduce pulmonary arterial pressures.[183] It is vitally important to reduce pulmonary arterial pressures during the

neonatal period, as sustained mechanical stretch causes irreversible vascular remodelling and progression to either chronic PPHN or death.[232] In most infants with PPHN due to other causes, this is accomplished with inhaled NO (iNO), however only 30% of CDH infants respond to iNO therapy.[242] Impaired responsiveness to iNO in CDH patients is likely due to irreversible factors, such as a reduced cross sectional area of the pulmonary vascular bed. On the other hand, rapid degradation of cGMP by abnormally high levels of PDE5 may also contribute to the failure of iNO therapy.[227-229] Hence, PDE5 inhibitors such as sildenafil are increasingly being used to promote pulmonary vasodilation, however their efficacy also appears limited by anatomical deficiencies in the pulmonary vascular bed. Intravenous sildenafil and iNO are being compared in a large European randomised controlled trial, with the primary outcome of neonatal mortality and PPHN incidence at 14 days of life (CoDiNOS, Eudra CT: 2017-000421-13).

In CDH infants with PPHN who remain hypoxaemic on maximal respiratory support despite medical therapy, ECMO can be used to reduce right ventricular afterload and maintain adequate tissue oxygen delivery. By reducing ventilation requirements, ECMO also prevents lung injury due to barotrauma and oxidative stress. However, CDH neonates require ECMO more frequently[243] and for longer[244] than other infants with PPHN, and ECMO itself is associated with significant morbidity and increased healthcare costs.[31, 245-247] The risks and benefits of ECMO should be carefully considered, as in the VICI trial there was no difference in survival between centres with and without ECMO.[240] Attenuating abnormal pulmonary vascular remodelling *in utero* and enabling a smoother neonatal transition may reduce the severity of pulmonary hypertension and hence reduce ECMO requirements in the CDH population.

It is recommended that surgical repair be performed electively, following stabilisation of physiological parameters (blood pressure, preductal oxygen saturation, lactate and urine output).[183] Whilst a Cochrane Review published in 2000 found no clear evidence favouring delayed repair of CDH[248], more recent reviews emphasise the survival benefits of an initial stabilisation period.[245, 249] Some evidence suggests that the question of early versus late repair may actually be one of risk stratification; patients with less severe CDH benefit from delayed surgery, whereas patients with more severe CDH may require a more aggressive approach.[250]

1.6 Antenatal management of CDH

CDH is commonly diagnosed on prenatal ultrasound, during which the severity of abnormal lung development can also be quantified.[4] Assessing the severity of CDH prenatally allows healthcare providers to counsel parents on the expected prognosis. It also provides the exciting opportunity to offer antenatal interventions that may modify disease progression *in utero* and hence improve neonatal outcomes.

1.6.1 Antenatal diagnosis

In a population-based study using registers from 20 European countries between 1996 to 1998, CDH was prenatally diagnosed in 59% of cases.[4] However, there was a significant difference in prenatal detection rates between countries with routine second trimester morphology ultrasound screening programs (72%) and those without (30%). Given second trimester ultrasound screening is now performed more widely, it is likely that the current prenatal detection rate is closer to 72%. Indeed, this is supported by data from the European Surveillance of Congenital Anomalies (EUROCAT) register, which reports that 70% of CDH cases between 2013 to 2017 were diagnosed prenatally with some countries reporting rates as high as 86%.[5]

A CDH is diagnosed antenatally by visualising the diaphragmatic defect or by observing the stomach (hypoechogenic) or intestines in the thorax.[251] It is more difficult to detect the liver, as it appears similar to the lungs on ultrasound. Herniating abdominal viscera also lead to a detectable mediastinal and cardiac shift to the contralateral side.

Antenatal diagnosis most commonly occurs between 20-22 weeks of gestational age.[4] However, 40% of CDH cases have increased nuchal translucency on a 10-14 week scan, which is routinely assessed as part of Down syndrome screening.[252] Increased nuchal translucency should therefore prompt sonographers to thoroughly assess the thorax for herniated abdominal viscera, which may allow earlier diagnosis.

Despite allowing centralisation of care, antenatal diagnosis is associated with increased mortality because larger defects that are more easily observed on ultrasound have

poorer survival and 35% of parents elect to terminate the pregnancy following antenatal diagnosis.[4, 26] Antenatal diagnosis is also more likely when there are other associated anomalies (complex CDH), which further increase mortality.[4]

1.6.2 Antenatal prediction of outcome

Following antenatal detection, it is possible to predict neonatal outcomes by evaluating fetal lung size and liver position with ultrasound examination or MRI.[253-255] A combination of liver position and the lung-to-head ratio (adjusted for gestational age) is used to classify CDH into mild, moderate, severe and extreme cases (Figure 13).[253]

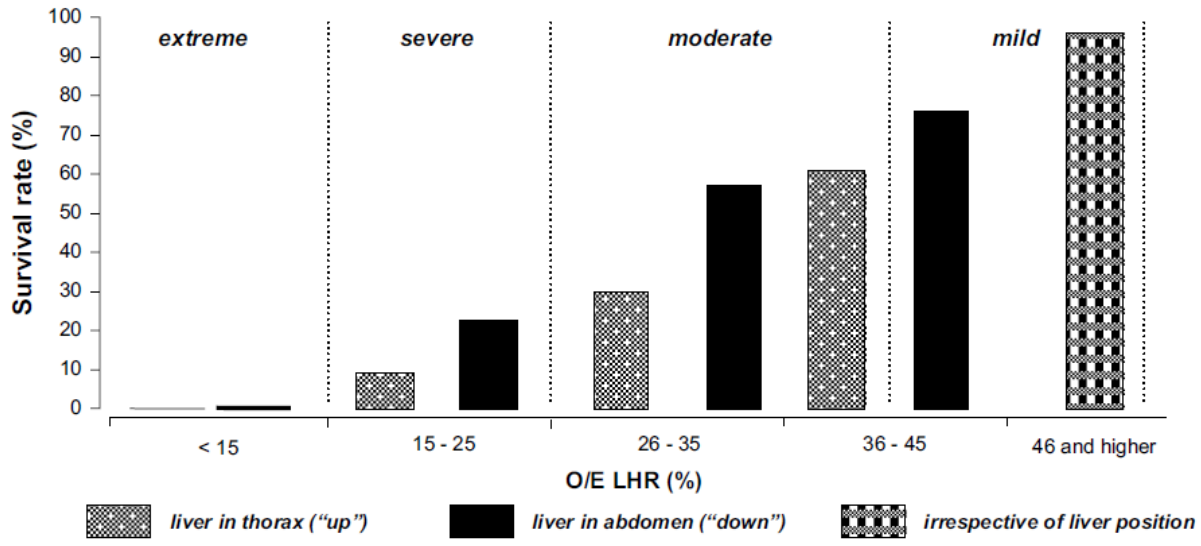

Figure 13 - Classification of congenital diaphragmatic hernia by prognosis

Decreased survival correlates with reduced lung size (as measured by O/E LHR) and liver herniation (liver in thorax). **O/E LHR:** observed/expected lung-to-head ratio. *Reproduced with permission from Deprest et al.*[253]

Whilst the lung-to-head ratio provides an accurate representation of pulmonary hypoplasia, it remains difficult to predict the occurrence and severity of pulmonary hypertension.[254] Measures of pulmonary arterial blood flow and vascularisation such as resistance index, pulsatility index and peak systolic velocity are not used clinically as they do not show predictive value and are dependent on the lung-to-head ratio.[254] However, the *change* in pulsatility index in response to maternal hyperoxygenation can

predict PPHN.[256] The acceleration time/ejection time ratio has also been shown to inversely correlate with risk of pulmonary hypertension, however it lacks reproducibility.[257] Abnormalities of the main pulmonary arteries appear to correlate with pulmonary hypoplasia, rather than pulmonary hypertension.[258] Hence, techniques that evaluate the distal pulmonary vascular bed are likely required to predict PPHN. Evaluation of the vascular bed using 3D power ultrasonography appears promising, with vascular indices offering a better prediction of prognosis than lung volume in an initial prospective observational study.[259] Future advances in this area will help identify which cases would benefit most from antenatal medical therapies that attenuate pulmonary vascular remodelling.

1.6.3 Antenatal surgical management

Antenatal prognostic indicators allow early assessment of severity and prediction of non-survivors, presenting the opportunity for antenatal interventions to reduce morbidity and mortality.

Antenatal repair of the diaphragmatic defect

In 1980, Harrison demonstrated that if space occupying abdominal viscera were reduced from the chest cavity, then the lungs would grow and develop normally in the sheep model.[260] These results led to attempts to conduct patch closure of the diaphragmatic defect *in utero* in humans.[14, 15] The initial trial was conducted on CDH fetuses with liver herniation into the chest (severe CDH).[14] Unfortunately, repositioning the liver into the abdominal cavity led to umbilical vein kinking and fetal death *in utero*.[14] A second clinical trial demonstrated that in fetuses without liver herniation, open fetal repair of CDH *in utero* offered no survival advantage, hence further attempts were abandoned.[15]

Fetoscopic endoluminal tracheal occlusion (FETO)

Given that early attempts to surgically repair the diaphragmatic defect *in utero* were unsuccessful, subsequent fetal interventions have focused instead on enhancing prenatal lung growth. As described in Chapter 1.1.3, lung growth and development are

primarily driven by mechanical stretch exerted by lung liquid, which is continuously secreted throughout gestation. When lung liquid is trapped within the airways, such as in congenital high airway obstruction syndrome (CHAOS), cell growth and division are enhanced, resulting in lung hyperplasia.[261] Replicating this condition by obstructing the trachea also enhances lung growth, as was first described in 1965 during attempts to demonstrate that fetal lung liquid is a secretory product of the lung.[262] Subsequent studies in fetal sheep demonstrated that the lung growth response to tracheal obstruction is rapid, with a peak in DNA synthesis rates (to 777% above baseline) occurring within 2 days, resulting in lung size almost doubling within 7 days.[263, 264] However, the type of growth depends on the stage of lung development when the obstruction occurred[265, 266] and if prolonged, the majority of type-II alveolar epithelial cells (surfactant producing) transdifferentiate into type-I cells.[78] Benachi *et al.* used transmission electron microscopy to perform an ultrastructural evaluation of the lungs of fetal lambs with a DH, and found that the ratio between type-I/type-II cells was ~20-fold greater (9 vs. 0.45) in DH lambs that underwent tracheal occlusion (from 120 – 139d GA) compared to those that did not.[267] In these DH lambs that underwent tracheal occlusion, ~10% of alveolar epithelial cells were in an intermediate state with features of both type-I and type-II cells.[267] This further supports the concept that the reduction in type-II cells is due to transdifferentiation into type-I cells. A reduction in type-II cells would make the lung surfactant deficient, hence raising the need for a period of tracheal release to allow the type-I cells to transdifferentiate back into type-II cells.[75, 78, 268-270]

Tracheal occlusion was first achieved clinically in an open procedure that required hysterotomy and fetal neck dissection.[271] This open procedure was associated with severe neurological morbidity and a survival rate of 33%, so efforts were made to develop less invasive approaches. Initially, this included a maternal laparotomy and multiple uterine trocar insertions, but gradually this evolved to a percutaneous single port procedure in which an inflatable balloon is fetoscopically introduced into the fetal trachea.[272-274] Fetoscopic endoluminal tracheal occlusion (FETO) is now under investigation in two large multicentre randomised trials (www.totaltrial.eu), in which the balloon is inserted at 27-30 weeks gestation in severe cases of CDH, and 30-32 weeks gestation in moderate cases.[275-277]

The current clinical strategy includes reversal of tracheal occlusion, in order to reverse the transdifferentiation of surfactant-producing type II alveolar epithelial cells into non-surfactant producing type I cells. This possibility was first demonstrated in animal studies demonstrating that, at least *in utero*, type-I cells are not terminally differentiated as first thought, but can transdifferentiate into type-II cells in response to lung deflation.[270] Elective balloon removal is typically scheduled for 34 weeks gestation either by ultrasound-guided percutaneous puncture or by fetoscopy, and in experienced centres is successfully performed in 97% of elective cases.[278, 279] In 28-43% of all FETO cases however, obstetrical complications and in particular threatened preterm labour (median GA at delivery: 35.3 weeks), prompt an emergent removal of the balloon.[17, 279]

In this emergency setting, if release cannot be performed by percutaneous puncture or fetoscopy, balloon extraction may be required while the infant remains on placental circulation. Balloon extraction was initially performed as a formal *ex utero* intrapartum procedure, yet now is safely done during a modified caesarean section.[17, 279, 280] As a last resort, the balloon can be removed postnatally with a specially-designed tracheoscope or percutaneous puncture.[278] Difficulties in balloon removal were a major contributor to neonatal death in 10 of the first 210 cases reported by the FETO consortium, so a 24/7 team of experienced clinicians should be on-call to deal with emergency balloon removal.[17, 276] This limits the use of FETO to high-volume tertiary referral centres.

The success of FETO also appears dependent upon pre-existing lung size, most likely because smaller lungs have a reduced epithelial surface area to secrete lung liquid and are less compliant.[17] The osmotic pressure gradient that drives lung liquid secretion equates to a hydrostatic pressure of around 6-7 mmHg.[263] Hence, if the pressure required to expand the hypoplastic lung exceeds 6-7 mmHg, then lung liquid secretion will cease when lung luminal pressure reaches 6-7 mmHg and the lung will not expand further. As a result, despite an overall increase in survival, approximately half of infants with severe lung hypoplasia do not survive even after FETO treatment and there is limited benefit regarding the incidence of PPHN.[256, 275, 281, 282] Hence, antenatal therapies that specifically address the pulmonary vascular abnormalities associated with CDH appear necessary. Preferentially, these should be medical in nature to reduce invasiveness and make prenatal therapy more accessible.

1.6.4 Antenatal medical management

There are currently no antenatal medical therapies for CDH used in clinical practice.[283] A randomised clinical trial of antenatal corticosteroids ceased recruiting after interim analysis suggested that more than 1700 infants would be required to detect a 10% difference in survival.[284] In the trial, only 32 infants were recruited in 4 years at 7 centres, thereby highlighting the difficulties of translating evidence from animal research into clinical trials in rare diseases. Other antenatal medical therapies, including sildenafil and Vitamin A, have only been investigated in animal models. Recently, there have also been promising results using cell-based therapies to improve pulmonary development in CDH.[285-287] In contrast to medical therapies, cell-based therapies may not only prevent further lung injury, but also repair established disease.[288] Regenerative medicine could contribute to the management of CDH on various levels, as recently reviewed by Deprest and De Coppi[289], but it is beyond the scope of this thesis.

My published review of antenatal medical therapies for CDH is attached as Appendix 1. In summary, the majority of antenatal medical therapies for CDH display limited benefit (such as bosentan[110] and Vitamin C[290, 291]), may be teratogenic (Vitamin A[292]) or have failed to demonstrate benefit in humans despite promising animal trials (corticosteroids[284]). A notable, promising exception is the PDE5 inhibitor sildenafil, which will now be discussed.

1.7 Sildenafil

As described above, PPHN in CDH infants is often refractory to treatment with iNO, suggesting either a purely anatomical problem (i.e. reduced cross-sectional area of the pulmonary vascular bed) or abnormalities in endothelium dependent vasodilatory pathways.[293] The latter is supported by the finding that CDH is associated with pulmonary overexpression of PDE5, which suppresses NO-mediated vasodilation by rapidly degrading cGMP.[227] By inhibiting PDE5, the medication sildenafil already improves outcomes in the neonatal care of CDH infants, but correcting this biochemical abnormality *in utero* may provide further benefit.

1.7.1 Mechanism of Action

Phosphodiesterases are a group of enzymes that play an important role in regulating cardiac smooth muscle tone and vascular smooth muscle contraction.[294] Sildenafil is a selective inhibitor of one of these enzymes, PDE5, which acts to degrade and thereby reduce intracellular cGMP concentrations. As such, PDE5 interferes with NO mediated vasodilation and hence results in vasoconstriction, whereas sildenafil-induced PDE5 inhibition increases the half-life of cGMP, leading to vasodilation and increased blood flow (Figure 14).

PDE5 is the most active phosphodiesterase in the pulmonary vasculature, hence sildenafil induces pulmonary vasodilation, a reduction in PVR and an increase in PBF.[296] Indeed, sildenafil is used to treat pulmonary hypertension in adults, children, and neonates, particularly as PDE5 is upregulated in pulmonary hypertension and so is very sensitive to the effects of sildenafil.[296-299]

PDE5 is also upregulated in the lungs of CDH infants at birth, which suggests that CDH fetuses may also be sensitive to sildenafil's effects.[227] Sildenafil's acute vasodilatory effect may appear less useful *in utero*, when the placenta performs gas exchange and PBF is minimal. Furthermore, there appear to be remarkably strong autoregulatory mechanisms that tightly control PBF *in utero*; when fetal lambs were continuously

infused with sildenafil, there was a transient decrease in PVR and increase in PBF, but within 2 hours pulmonary vascular tone had returned to baseline.[300] This autoregulation, which is also seen in response to other pulmonary vasodilators[301, 302], likely acts to prevent the pulmonary circulation from "stealing" blood flow from the placenta, so as not to impair gas exchange.

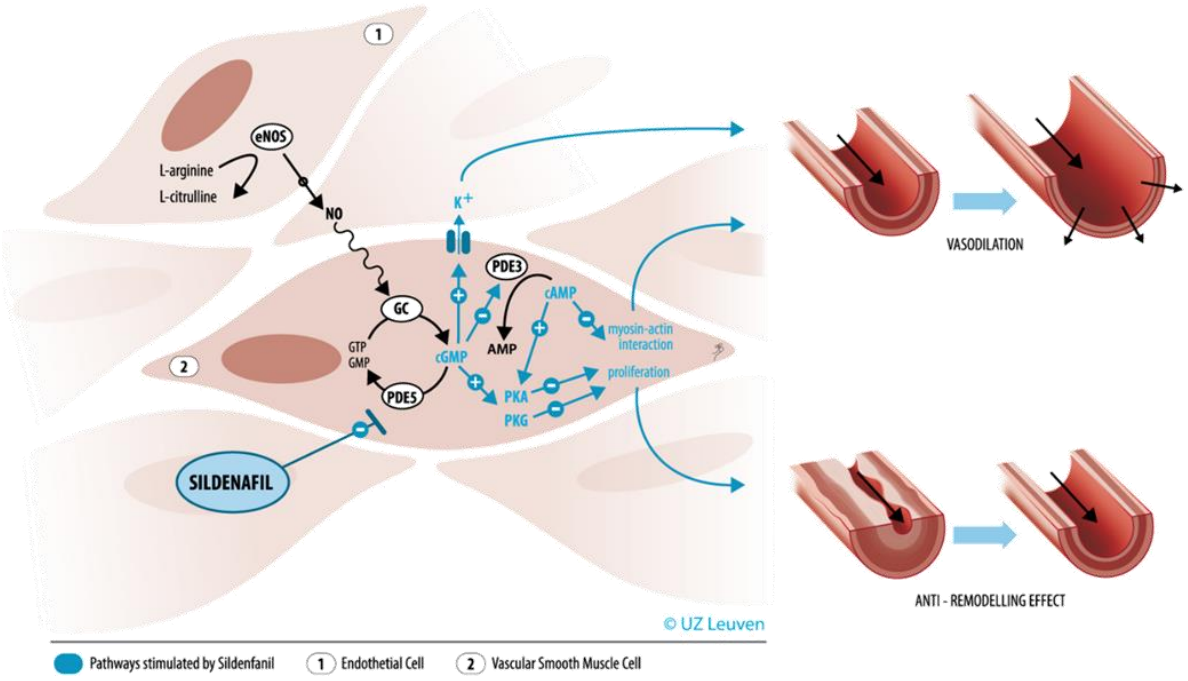

Figure 14 - Mechanism of action of sildenafil

eNOS: endothelial nitric oxide synthase; **NO**: nitric oxide; **GC**: guanylate cyclase; **GTP**: guanosine triphosphate; **GMP**: guanosine triphosphate; **cGMP**: cyclic GMP; **PDE**: phosphodiesterase; **ATP**: adenosine triphosphate; **AMP**: adenosine monophosphate; **cAMP**: cyclic AMP; **PKG**: cGMP dependent protein kinase; **PKA**: cAMP dependent protein kinase. *Reproduced with permission from Kashyap et al.*[295] *Drawing by Myrthe Boymans, UZ Leuven.*

In contrast to its transient effects on PVR and PBF, antenatal sildenafil also mediates a sustained change in vascular reactivity to birth-related stimuli that persists to delivery. For example, increasing PaO_2 does not change PVR in fetal lambs with chronic pulmonary hypertension treated with antenatal saline, but decreases PVR by 60% in lambs that have been treated with antenatal sildenafil.[300, 303]

Sildenafil also inhibits pulmonary vascular smooth muscle cell proliferation, so may attenuate the hypermuscularisation and neomuscularisation of pulmonary blood vessels seen in CDH infants.[304] This anti-proliferative effect appears to be mediated via

cGMP activation of protein kinase A and G (Figure 14), and upregulation of bone morphogenetic protein.[305-307] cGMP also inhibits phosphodiesterase-3, which increases cyclic AMP (cAMP) bioavailability. cAMP is also known to have anti-proliferative effects on vascular smooth muscle cells.[308]

Furthermore, PDE5 suppresses VEGF expression, so by inhibiting PDE5 sildenafil may also improve VEGF-driven angiogenesis and therefore restore the reduced number of distal vessels seen in CDH.[309] VEGF-driven angiogenesis also creates mature alveolocapillary units[310] that will appropriately match ventilation and perfusion during neonatal life, reflecting the complex interactions between airway and vascular pulmonary development discussed earlier.[311]

Ultimately, by potentiating pulmonary vasodilation in response to birth-related stimuli, inhibiting proliferation of pulmonary vascular smooth muscle cells and stimulating pulmonary angiogenesis, antenatal sildenafil treatment may attenuate both the reversible and irreversible components of PPHN in CDH infants.

1.7.2 Pharmacokinetics and bioavailability

The impact of antenatal sildenafil on fetuses with a CDH has been predominantly studied in the nitrofen-rat model[109-112, 114, 312, 313] and also one rabbit study has been published so far.[115] An alternative PDE5 inhibitor, tadalafil, has recently been investigated in the sheep model, however sildenafil is more likely to be used clinically as human neonates are unable to properly metabolise tadalafil due to an immature glucuronidation pathway.[113, 314]

The first important finding from these studies is that maternal sildenafil successfully crosses the placenta in all current animal models. Interestingly, in sheep, fetal tadalafil concentrations remain at a steady state despite fluctuating maternal levels.[113] This steady state may reflect low fetal metabolism of tadalafil in sheep fetuses, as in human neonates.[314] These steady fetal concentrations are not seen when sildenafil is used in the rat[111] and rabbit[115] models. Sildenafil clearance in human neonates increases three-fold (to adult levels) during the first week of postnatal life,[315] so well-designed fetal

pharmacokinetic studies are required to ensure that sildenafil does not accumulate in the fetal circulation when administered antenatally.

It is evident that transplacental sildenafil and tadalafil both exert a biochemical action on the fetal lungs, with an increase in pulmonary cGMP concentration following maternal administration in both the rat and sheep model.[111, 113]

1.7.3 Safety

Human safety data specific to fetuses with a CDH is not available for sildenafil. On the other hand, there have been no adverse effects reported in animal models of CDH.[111]

In the neonatal population, using sildenafil to treat pulmonary hypertension was not associated with higher rates of adverse effects in three small randomised controlled trials.[316-318] However, a randomised controlled trial investigating sildenafil in the paediatric population found that high-dose sildenafil is associated with greater mortality than low-dose sildenafil.[319] This association led to a United States Food and Drug Administration recommendation against the use of sildenafil in the paediatric population[320]. However, a number of expert groups have refuted this recommendation due to limitations of the trial, including inconsistent plasma sildenafil concentrations, no survival data for the placebo group, and a large number of confounding variables.[321, 322] Furthermore, the European Medicines Agency approved sildenafil use in paediatric pulmonary hypertension (while warning against high-doses) based on the same evidence.[323]

Antenatally administered sildenafil has been investigated in randomised controlled trials to determine its effect upon pregnancy duration in women with pre-eclampsia[324], and on amniotic fluid volume in pregnancies complicated by idiopathic oligohydramnios.[325] In both trials no differences in adverse effects between sildenafil and placebo were observed, in either mother or fetus.

At the time this thesis was conceived, antenatal sildenafil was also under investigation to increase placental blood flow to fetuses with severe intrauterine growth restriction (IUGR) in the STRIDER trial, an international consortium of five randomised controlled

trials with a planned individual patient data meta-analysis.[326, 327] STRIDER recruited women with a small for gestational age fetus that had absent or reversed end-diastolic umbilical artery blood flow on Doppler ultrasound examination. These fetuses represent the most severe end of the IUGR spectrum, and indeed 32% of the STRIDER cohort in the United Kingdom (UK) trial died *in utero*.[328] The UK and Australia/New Zealand trials were completed in 2018, and while they demonstrated no beneficial effect on fetal growth, there was also no evidence of maternal, fetal or neonatal harm.[329, 330]

While our experiments were already underway, the Dutch STRIDER trial was suspended after interim analysis raised concerns regarding an increased incidence of PPHN (27% vs 5%) and neonatal mortality (27% vs 14%) in the sildenafil-treated group.[331] Given that these adverse effects were not reported in the completed UK and Australia/New Zealand trials, it appears prudent to await the results of the planned individual patient data meta-analysis before drawing conclusions.[329, 330]

Some animal studies that preceded the STRIDER trials demonstrated that sildenafil has a beneficial effect on the uteroplacental circulation[332], however other animal studies suggested it may be detrimental[333]. A recently published study in the lamb IUGR model suggests that by attenuating the brain-sparing cerebral vasodilation and peripheral vasoconstriction normally associated with IUGR, antenatal sildenafil may limit acute cerebral responses to neonatal challenges.[334] While this offers a possible explanation for the (non-significantly) increased neonatal mortality in the sildenafil-treated group of the Dutch STRIDER trial, the lamb study did not assess the pulmonary vascular bed, so the increased incidence of PPHN remains puzzling. A possible explanation may be found in the preclinical CDH literature, as described below in *Chapter 1.6.4*; control animals (without CDH, or IUGR) treated with antenatal sildenafil have fewer distal pulmonary blood vessels and decreased total vascular volume compared to untreated controls.[111, 115, 313]

While the concerns raised by the Dutch STRIDER trial are not yet verified by the individual patient data meta-analysis and may be specific to IUGR fetuses, it is important that a safety profile is established in preclinical CDH models before sildenafil is considered for clinical trials in CDH infants.

1.7.4 Effect on the lungs in diaphragmatic hernia

The biomolecular effects of sildenafil are described in *Chapter 1.6.1: Mechanism of Action*. In preclinical CDH models, antenatal sildenafil treatment increases vasodilator expression and reduces vasoconstrictor expression[111-113], restores the vasodilatory response to stimuli such as NO and O_2 that is impaired in CDH[111, 114], and enhances anti-proliferative (bone morphogenic protein) and pro-apoptotic (Bax/Bcl-2 ratio) signalling in vascular smooth muscle cells[112] (Table 1). Sildenafil also increases expression of VEGF in the lung parenchyma, which likely improves angiogenesis.[115] Sildenafil has no effect on PDE5 RNA expression, but decreases the distribution of PDE5 particularly in distal pulmonary vessels.[313]

Table 1 - Antenatal sildenafil mediates biomolecular and physiological changes

Study	**Vasodilator expression**	**Vasoconstrictor expression**	**Vasoreactivity to vasodilatory stimuli**
(Luong, 2011)[111]	↑ eNOS ↑ VEGF	NA	↑ vasodilatory response to NO donor
(Yamamoto, 2014)[114]	NA	NA	↑ vasodilatory response to maternal hyperoxia
(Makanga, 2015)[112]	↑ eNOS ↑ iNOS ↑ NO	↓ ET-1 ↓ ETA ↓ PPET-1	NA
(Shue, 2014)[113]	↑ eNOS	NA	NA

NO: Nitric oxide. **eNOS:** endothelial NO synthase. **VEGF:** Vascular endothelial growth factor. **iNOS:** inducible NO synthase. **ET-1:** Endothelin-1. **ETA:** ET-1 receptor A. **PPET-1:** ET-1 precursor. **PBF:** Pulmonary blood flow. **NA:** Not applicable.

Sildenafil improves pulmonary vascular development in preclinical CDH models by increasing distal vessel number[109, 111, 112, 115] and decreasing distal vessel muscularisation (Table 2).[110-112, 115, 313] Sildenafil does not significantly increase total vascular volume, however this is less important than the ratio of distal to proximal vessels (as a larger proportion of distal vessels provides greater cross-sectional area, therefore lower resistance).[313]

Table 2 - Antenatal sildenafil mediates changes in vascular morphology

Study	**Vessel number** (distal vessel number or density)	**Vessel muscularisation** (medial wall thickness)	**Other**
Rat models (sildenafil)			
(Luong, 2011)[111]	in CDH: ↑ in non-CDH: ↓	↓	↓ Right ventricular Hypertrophy
(Kattan, 2014)[109]	↑	Proximal vessels: ↑ Distal vessels: -	*NA*
(Lemus, 2014)[110]	*NA*	↓	*NA*
(Makanga, 2015)[112]	↑	↓	↑ Vessel diameter
(Burgos, 2016)[312]	*NA*	No effect	*NA*
(Mous, 2016)[313]	*NA*	↓	↓ Cellular markers of muscularisation
Rabbit model (sildenafil)			
(Russo, 2016)[115]	in CDH: ↑ in non-CDH: ↓	↓	*NA*
Sheep model (tadalafil)			
(Shue, 2014)[113]	*NA*	*NA*	No effect on right ventricular hypertrophy

It is concerning that when sildenafil was given to control animals (i.e. without CDH), it decreased the number of distal vessels[111, 115] and decreased total vascular volume.[313] The authors hypothesised that sildenafil-mediated vasodilation was beneficial in lungs with increased PVR, but in normal lungs hypoperfusion of the pulmonary vascular bed may impair vessel growth.[115] These findings could alternatively be explained by the fact that while cGMP (increased following sildenafil induced PDE5 inhibition) is important for vasodilation and angiogenesis, sustained exposure at high levels can lead to lung endothelial cell death and apoptosis.[335] While sildenafil may attenuate the reduced cGMP levels characteristic of CDH[111], its use in healthy fetuses may increase cGMP to

toxic levels. These findings may also explain the paradoxical pulmonary hypertension observed in IUGR fetuses exposed to sildenafil during the Dutch STRIDER trial.[331]

Sildenafil also appears to improve airway development (Table 3). The effect of sildenafil on gross lung size is unclear, with most evidence showing no difference[111-113, 115], one rat model demonstrating a significant decrease[312] and two rat models demonstrating a significant increase in lung weight.[114, 313] At a histological level sildenafil appears to increase distal airway complexity.[112, 114, 115, 313] On a functional level, rabbit lungs treated with sildenafil demonstrate improved static compliance and total lung capacity.[115]

Table 3 - Antenatal sildenafil mediates changes in airway histology and function

Study	Gross lung size	Septal thickness	Airway complexity	Functional
Rat models (sildenafil)				
(Luong, 2011)[111]	No effect	↓	*NA*	*NA*
(Lemus, 2014)[110]	*NA*	No effect	No effect	NA
(Yamamoto, 2014)[114]	↑ LBWR	*NA*	↑ RAC	*NA*
(Makanga, 2015)[112]	No effect	No effect	↑ RAC	*NA*
(Burgos, 2016)[312]	↓ LBWR	↓	↑ Alveolar volume density No effect on RAC	↑ PO_2 No effect on tidal volume
(Mous, 2016)[313]	↑ LKWR	No effect	↑ Alveolar airspace diameter	*NA*
Rabbit model (sildenafil)				
(Russo, 2016)[115]	No effect	No effect	↑ Distal airway complexity	↑ Static compliance ↑ Total lung capacity
Sheep model (tadalafil)				
(Shue, 2014)[113]	No effect	*NA*	*NA*	*NA*

LBWR: Lung-to-body weight ratio. **LKWR:** Lung-to-kidney weight ratio (used because body weight was significantly different between groups). **NA:** Not applicable. **PO_2:** Partial pressure of arterial oxygen (from a mixed arteriovenous sample). **RAC:** Radial alveolar count.

1.7.5 Translation from Animal Models to Clinical Practice

Sildenafil's extensive use as a penile vasodilator to treat erectile dysfunction[336] means that it is now available off-label in a much cheaper generic form. Along with consistent oral bioavailability[337], this would allow antenatal sildenafil to be used in low-resource settings, in contrast to the highly-specialised care required for other antenatal CDH interventions such as FETO.[277, 280]

Most experiments on antenatal sildenafil in CDH have been performed in the nitrofen-rat model, which does not allow detailed assessment of neonatal physiology during the transition at birth. Hence, before sildenafil can be investigated in a clinical trial, the results of rodent and rabbit CDH models must be validated in a larger animal model to demonstrate that improvements to pulmonary vascular structure translate to improved pulmonary vascular function after birth.

1.8 Aims

Infants with a CDH face their greatest physiological challenge during the transition from fetal to neonatal life, when placental support is lost and their underdeveloped lungs are required to assume the role of gas exchange. However, very little is understood regarding the impact that lung hypoplasia, secondary to CDH, has on cardiopulmonary physiology during the neonatal transition. Hence, the first aim of this thesis was **to characterise the changes in cardiopulmonary physiology during the neonatal transition in lambs with a DH**. I hypothesised that abnormal lung development would adversely affect the transition to newborn life, particularly the change in respiratory mechanics and the increase in pulmonary blood flow.

Using this sheep CDH model, I then aimed to investigate interventions that may improve neonatal cardiopulmonary haemodynamics and reduce the severity of pulmonary hypertension. This may be achieved by either protecting the vulnerable pulmonary vascular bed during the neonatal transition at birth or attenuating abnormal pulmonary vascular development before birth.

The second aim of this thesis was **to determine the effects of physiologically based cord clamping (PBCC) on neonatal cardiopulmonary physiology in lambs with a DH**. I hypothesised that a physiologically based approach to umbilical cord clamping would avoid a prolonged period of hypoxia and low cardiac output at birth and reduce the risk of developing pulmonary hypertension after birth.

The underlying lung hypoplasia and pulmonary vascular abnormalities that afflict CDH infants develop *in utero*. Recent studies have demonstrated that the PDE5 inhibitor sildenafil attenuates these pulmonary vascular structural abnormalities, in preclinical CDH models. The third aim was **to determine the effects of antenatal sildenafil on neonatal cardiopulmonary physiology in lambs with a DH**. I hypothesised that antenatal sildenafil treatment would reduce PVR and increase PBF during the neonatal transition at birth.

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Chapter 2

Fetal therapy and the neonatal transition in a sheep model of congenital diaphragmatic hernia

2 Fetal therapy and the neonatal transition in a sheep model of congenital diaphragmatic hernia

In this PhD thesis, I aimed to investigate antenatal and postnatal treatments that improve cardiopulmonary haemodynamics during the neonatal transition and reduce the severity of pulmonary hypertension. To describe the effects of a DH, and candidate treatments, on cardiopulmonary physiology during the neonatal transition, the most appropriate animal model is the sheep. Most importantly, the relative large size of lambs allows instrumentation to continuously record arterial blood pressures, flows and oxygenation, and allows ventilation using standard neonatal equipment. Therefore, in Chapter 2.1, I aimed to establish the sheep CDH model at our facility.

As outlined in Chapter 1.2, the effects of CDH on cardiopulmonary physiology during the neonatal transition are not well understood. Hence, in Chapter 2.1 I also aimed to describe cardiopulmonary physiology during the neonatal transition in lambs with a surgically induced left-sided diaphragmatic hernia (DH). Based on the prior experience of the group in delivering and ventilating lambs with lung hypoplasia, our resuscitation strategy, developed in consultation with expert neonatologists, involved physiologically based umbilical cord clamping and a sustained inflation.

As I was ultimately interested in the effects of sildenafil on pulmonary vascular function, the primary outcome for this study was pulmonary blood flow (PBF). Our study shows that despite severe lung hypoplasia and very poor respiratory function, DH lambs had a large increase in PBF in response to lung aeration that, per gram of lung weight, was identical to sham controls. This indicates that, at least initially, the higher pulmonary vascular resistance is simply a consequence of a smaller lung and is not due to abnormal pulmonary vascular function.

I next aimed to use the sheep CDH model to investigate antenatal interventions that may rescue the lung from hypoplasia, and directly measure their effects on the neonatal cardiopulmonary transition. Before investigating antenatal sildenafil therapy, in Chapter 2.2 we first investigated the effect of a fetal intervention that is already under investigation in an international randomised controlled trial, FETO. However, virtually

no scientific information is available on how FETO affects the physiological transition and whether it improves respiratory function postnatally. In this second study, I aimed to determine whether FETO improves the cardiopulmonary transition at birth in lambs with a DH. We demonstrated that FETO improves lung compliance and CO_2 exchange and increases PBF in proportion to lung weight, however oxygen exchange is not improved. This indicates that the gas barrier/surface area for gas exchange has improved sufficiently for CO_2 but not O_2 exchange.

While the studies are clearly very complementary, they each contain very significant amounts of data, the majority of which is relevant and required to fully explain the physiology underpinning the fetal to neonatal transition in these lambs. As such it was not possible to combine the studies into one paper, however we requested that they were peer-reviewed together. Both manuscripts have now been published back-to-back in *Archives of Disease in Childhood: Fetal and Neonatal Edition*, so they are presented in that form in Chapter 2.1 and Chapter 2.2.

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2.1 The neonatal cardiopulmonary transition in an ovine model of congenital diaphragmatic hernia

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Neonatal cardiopulmonary transition in an ovine model of congenital diaphragmatic hernia

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ABSTRACT

Objective Infants with a congenital diaphragmatic hernia (CDH) are at high risk of developing pulmonary hypertension after birth, but little is known of their physiological transition at birth. We aimed to characterise the changes in cardiopulmonary physiology during the neonatal transition in an ovine model of CDH.

Methods A diaphragmatic hernia (DH) was surgically created at 80 days of gestational age (dGA) in 10 fetuses, whereas controls underwent sham surgery (n=6). At 138 dGA, lambs were delivered via caesarean section and ventilated for 2 hours. Physiological and ventilation parameters were continuously recorded, and arterial blood gas values were measured.

Results DH lambs had lower wet lung-to-body-weight ratio (0.016±0.002vs0.033±0.004), reduced dynamic lung compliance (0.4±0.1mL/cmH_2O vs1.2±0.1 mL/cmH_2O) and reduced arterial pH (7.11±0.05vs7.26±0.05), compared with controls. While measured pulmonary blood flow (PBF) was lower in DH lambs, after correction for lung weight, PBF was not different between groups (4.05±0.60mL/min/gvs4.29±0.57 mL/min/g). Cerebral tissue oxygen saturation was lower in DH compared with control lambs (55.7±3.5vs67.7%±3.9%).

Conclusions Immediately after birth, DH lambs have small, non-compliant lungs, respiratory acidosis and poor cerebral oxygenation that reflects the clinical phenotype of human CDH. PBF (indexed to lung weight) was similar in DH and control lambs, suggesting that the reduction in PBF associated with CDH is proportional to the degree of lung hypoplasia during the neonatal cardiopulmonary transition.

What is already known on this topic?

- Lung hypoplasia and pulmonary hypertension associated with congenital diaphragmatic hernia (CDH) contribute to mortality rates that remain between 30% and 40% despite centralised, standardised neonatal care.
- Normally at birth, lung aeration triggers an increase in pulmonary blood flow that enables gas exchange and venous return to the left ventricle via the pulmonary circulation.
- Infants with CDH have small pulmonary vascular cross-sectional area and increased distal vessel muscularisation. These structural changes likely influence cardiopulmonary physiology during the neonatal transition.

What this study adds?

- Lung aeration stimulated a large increase in pulmonary blood flow (PBF) that was proportional to lung size in both DH and control lambs.
- PBF indexed to lung weight was similar between DH and control lambs, indicating that the lower PBF and higher pulmonary vascular resistance seen in DH lambs are mostly because the lung is smaller.
- During hypoxaemia, titrating fraction of inspired oxygen based on oxygen saturation (SpO_2) causes an overshoot in cerebral oxygen delivery due to increased carotid blood flow.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a rare birth defect (1 in 2500 live births) caused by the failed closure of the diaphragm in the embryonic period.[1] CDH allows visceral organs to herniate into the fetal chest, which disrupts lung development. Though surgically correctable, neonates with CDH face significant respiratory challenges after birth.[2] The lungs are smaller and structurally abnormal in both airway and vascular development, resulting in respiratory insufficiency immediately after birth that is often associated with pulmonary hypertension.[3–8] Despite early referral to high volume care centres for intensive neonatal care, neonatal mortality of isolated CDH remains high (30%).[2 9–11]

Standardised neonatal care for CDH infants is largely based on expert opinion, and outside of clinical trials, antenatal management is expectant.[8 12] To further improve antenatal and neonatal management of CDH infants, we require a better understanding of how CDH affects the physiological transition at birth.

The transition to newborn life at birth involves the lungs taking over the role of gas exchange from the placenta and the closure of cardiovascular shunts to separate the pulmonary and systemic circulations. Critical to these cardiopulmonary changes is a reduction in pulmonary vascular resistance (PVR), which enables the lung to accept the entire output of the right ventricle while also facilitating a reduction in pulmonary arterial pressure (PAP). However,

development of the pulmonary vasculature is impaired in infants with CDH, resulting in a small cross-sectional area predominantly in the ipsilateral lung.[4] Combined with this, infants with CDH have abnormal thickening of the smooth muscle surrounding small pulmonary arteries. This abnormal development, together with an increased sensitivity to vasoactive triggers such as hypoxia, are thought to contribute to the maintenance of a high PVR in the newborn period, leading to the development of persistent pulmonary hypertension of the newborn (PPHN). However, it remains unclear what drives this abnormal vascular development and whether maladaptation to the cardiopulmonary changes associated with birth may contribute to the development of PPHN.

Birth-related cardiopulmonary adaptations are not well described in infants with CDH, particularly the capacity for PVR to decrease in response to lung aeration. We aimed to describe the cardiopulmonary physiology during transition to neonatal life in lambs with pulmonary hypoplasia due to a diaphragmatic hernia (DH). In DH rabbit kittens, we have recently found that the increase in pulmonary arterial blood flow (PBF) induced by lung aeration in the normal lung also occurs in the hypoplastic lung.[13] Therefore, in this study, we hypothesised that abnormal lung development may predispose DH lambs to low lung compliance and respiratory insufficiency, but any reduction in pulmonary blood flow during the transition period will be proportional to the reduction in lung size.

METHODS

Animal surgery and treatment groups

All surgical procedures were performed under general anaesthesia using intravenous sodium thiopental (20 mg/kg, Pentothal; Jurox, New Zealand) for induction and inhaled isoflurane (≈2% in room air; Isoflow, Abbot) for maintenance. Ewes were intubated and monitored (ECG, expired CO_2 and oxygenation) during surgery.

After surgery, the ewes received 3 days of analgesia (transdermal fentanyl patch, 75 µg/hour; Janssen Cilag) and were monitored daily until delivery. Before surgery, animals were allocated to control or DH groups.

Ultrasound evaluation

Before every procedure, a fetal ultrasound was performed (CX50 Ultrasound System, Philips) to measure the thoracic and cardiac circumference, right and left lung area, midtracheal diameter and femur length. The shape of the fetal skull and limited visualisation at later gestational ages precluded reproducible images of the fetal head. We calculated the right lung to femur ratio (RLFR).

DH creation

A DH was surgically created at ≈80 days of gestational age (dGA; term≈147 dGA). The ewe was given cefazolin (intravenous) (1 g, AFT Pharmaceuticals) before a midline laparotomy was used to expose the uterus. The fetal head and forelimbs were exteriorised via hysterotomy, taking care to avoid placental cotyledons. The fetal diaphragm was visualised via a thoracotomy through the ninth intercostal space, and the diaphragm was incised and resected, enabling the stomach and bowels to be repositioned into the fetal chest cavity. The thoracotomy incision was sutured closed (Maxon 2–0; Covidien) before the fetus was returned to the amniotic sac, and the uterus was closed in two layers (polysorb 2–0, Covidien); leaked amniotic fluid was replaced using warmed saline, and intra-amniotic cefazolin (1 g) was given. The maternal fascia, subcutaneous tissue and the skin were then closed (Maxon 0, Covidien and Vetafil 2–0; Supramid). In control fetuses the thorax was opened, but there was no incision of the diaphragm.

Delivery and ventilation

At ≈138 dGA, the ewe was anaesthetised (as above), and the fetal head and neck were exposed via hysterotomy. The lamb was intubated using a size 4.0 cuffed endotracheal tube. Polyvinyl catheters were inserted into the jugular vein and carotid artery to allow neonatal drug administration and continuous carotid arterial pressure monitoring, respectively. An ultrasonic flow probe (Transonic Systems, Ithaca, New York, USA) was placed around the contralateral carotid artery to continuously record carotid arterial blood flow (CBF). The fetal chest was exteriorised, and a thoracotomy was performed through the fourth intercostal space. An ultrasonic flow probe was placed around the left pulmonary artery to continuously record PBF. A non-occlusive catheter was implanted in the main pulmonary artery, which was connected to a pressure transducer to continuously record PAP. The thorax was closed (Maxon 2–0) to prevent air leakage during ventilation. Pulse-oximeter, near-infrared spectroscopy (NIRS) and temperature probe were placed on the right forelimb, skull and rectally, respectively. The stomach was drained via an orogastric tube. After instrumentation, the lamb was delivered from the uterus, dried and carefully positioned on the ewe's abdomen to avoid obstruction of umbilical cord blood flow.

After the lung liquid was passively drained, ventilation commenced with a 30 s sustained inflation (35 cmH_2O, 21% O_2) followed by intermittent positive pressure ventilation (iPPV) in volume guarantee mode using tidal volume of 4 mL/kg (Babylog 8000, Dräger, Lübeck, Germany). Positive end-expiratory pressure was set at 5 cmH_2O and peak inspiratory pressure (PIP) was initially limited to 35 cmH_2O. If the target tidal volume was not reached after 30 s, a second sustained inflation was performed followed by iPPV for a total of 120 min. The umbilical cord was clamped when a tidal volume of 4 mL/kg was achieved or at 10 min after ventilation onset. The lamb was then moved to a warming bed, sedated with continuous alfaxalone (10 mg/mL, 5 mL/hour; Alfaxan, Jurox) and covered with plastic drapes to maintain a stable body temperature. Lambs were ventilated with warmed and humidified oxygen. Ventilator rate, inspiratory and expiratory rate and fraction of inspired oxygen (FiO_2) were titrated to achieve gas targets for arterial carbon dioxide tension ($PaCO_2$) 60–80 mm Hg, arterial oxygen tension (PaO_2) >40 mm Hg and oxygen saturation (SpO_2) of 85%–88%. The lamb was euthanised (sodium pentobarbitone intravenous 100 mg/kg) at the end of the 2-hour ventilation protocol or earlier in case of significant pneumothorax or severe acidosis unresponsive to alteration of ventilation parameters.

Outcome measures

Arterial blood samples were analysed every 5 min during the first 30 min of ventilation and every 10 min thereafter.

Physiological data analysis

Tidal volume, airway pressures, cerebral tissue oxygen saturation ($SctO_2$), pulmonary and cerebral perfusion were continuously recorded using LabChart (ADInstruments, New South Wales, Australia) and analysed offline. Ten heartbeat average data points were collected prior to ventilation onset (fetal), immediately at

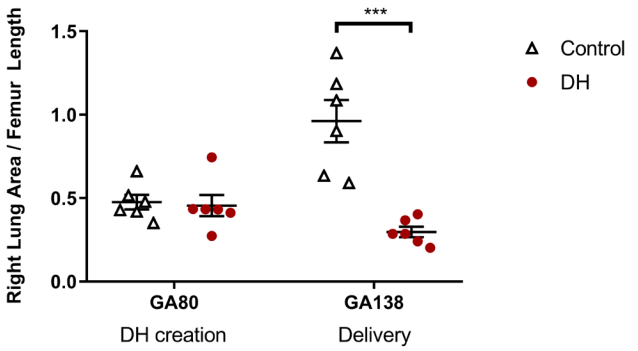

Figure 1 The ratio of fetal right lung area to femur length assessed using ultrasound throughout gestation. Groups shown are control (white triangles, n=6; thoracotomy only at ≈80 dGA, delivery at ≈138dGA) and diaphragmatic hernia (DH) (red circles, n=7; DH creation at ≈80 dGA, delivery at ≈138 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisks (***) indicate significant difference (p<0.001) between control and DH at delivery. dGA, days of gestational age.

the onset of ventilation (t=0) and every 5 min throughout the ventilation period.

Postmortem examination

The presence of a diaphragmatic defect and herniation of visceral organs was confirmed during postmortem examination. The lungs were weighed and expressed as a ratio to the body weight (wet lung-to-body weight ratio [LBWR]). Lung size (LBWR) was used as a proxy for degree of lung hypoplasia, as it has previously been shown to also correlate with radial alveolar count and lung DNA content in a similar ovine CDH model.[14]

Calculations

- PVR (mm Hg/[mL/min]): (pulmonary artery pressure – left atrial pressure)/pulmonary blood flow. Based on previous studies, the left atrial pressure was assumed to equal 9 mm Hg.[15]
- Alveolar-arterial difference in oxygen tension ($AaDO_2$; mm Hg): $(FiO_2*713-PaCO_2/0.8)-PaO_2$.
- Cerebral oxygen extraction (%): $(SaO_2-SctO_2)/SaO_2$, where SaO_2 is the arterial oxygen saturation.[16]
- Arterial oxygen content (CaO_2; mL O_2/L): $[1.39 \bullet Hb \bullet SaO_2/100] + [0.03 \bullet PaO_2]$, where Hb is the haemoglobin concentration (g/L).[17]
- Cerebral oxygen delivery (DO_2; mL/min/kg): $(CBF \bullet CaO_2)$.[17]

Statistical analysis

Based on power analysis from previous studies, we estimated ≈7 animals per group and were required to detect minimal biologically significant differences in PBF and ventilation characteristics with a coefficient of variation of ≈25%, assuming an α value of 0.05 and a power of >0.8.[18] Continuous data are expressed as means±SEM or medians (IQR) if the data were not normally distributed. Dichotomous data are expressed as percentages. Differences between control and DH groups were analysed using two-way repeated measures analysis of variance combined with Holm-Sidak's multiple comparisons test. A p value below 0.05 was considered statistically significant.

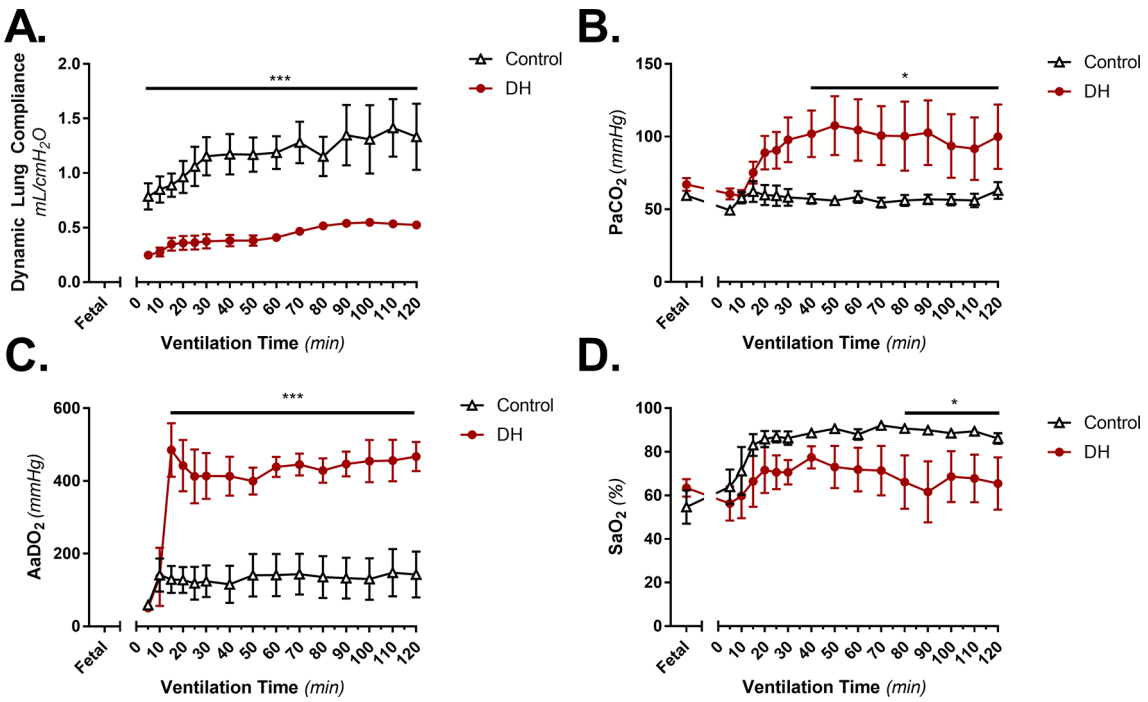

Figure 2 (A) Dynamic lung compliance, (B) partial pressure of arterial carbon dioxide ($PaCO_2$), (C) alveolar-arterial difference in oxygen tension ($AaDO_2$) and (D) arterial oxygen saturation (SaO_2) over the 2 hours following delivery at ≈138 dGA. Groups shown are control (white triangles, n=6; thoracotomy only at ≈80 dGA) and diaphragmatic hernia (DH) (red circles, n=7; DH creation at ≈80 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates significant differences between groups (*p<0.05, ***p<0.001) at all individual timepoints below line. dGA, days of gestational age.

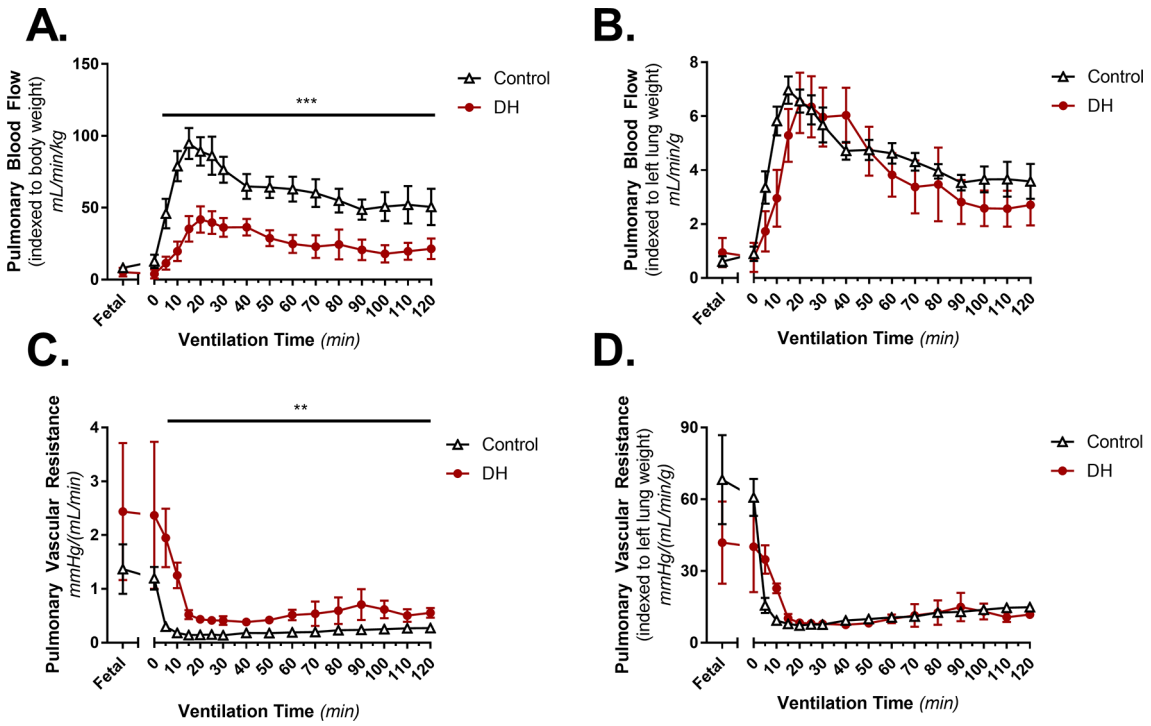

Figure 3 Pulmonary blood flow indexed to (A) body weight and (B) left lung weight, and pulmonary vascular resistance indexed to (C) body weight and (D) left lung weight over the 2 hours following delivery at ≈138 dGA. Groups shown are control (white triangles, n=6; thoracotomy only at ≈80 dGA) and diaphragmatic hernia (DH) (red circles, n=7; DH creation at ≈80 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates significant differences between groups (**p<0.01, ***p<0.001) at all individual timepoints below line. dGA, days of gestational age.

RESULTS

Animal surgery

Thirteen (of 16) fetuses survived to delivery: six controls (100%) and seven DH lambs (70%). All control lambs survived the 2-hour ventilation period; however, for two DH lambs, it was necessary to discontinue the experimental protocol early due to developing treatment-resistant pneumothoraces (at 70 min and 110 min).

Postmortem examination

A diaphragmatic defect with substantial gastrointestinal herniation into the thorax was confirmed at postmortem examination in all DH lambs. The wet LBWR for DH animals was significantly smaller than controls (0.016±0.002 vs 0.033±0.004; p=0.02).

Ultrasound

In control fetuses, RLFR increased between sham surgery at ≈80 dGA and delivery at ≈138 dGA (0.48±0.04 vs 0.96±0.13; p=0.002, figure 1). In contrast, RLFR in DH fetuses decreased between DH creation at ≈80 dGA and delivery at ≈138 dGA (0.46±0.06 vs 0.30±0.03; p=0.044).

Ventilation and oxygenation

Only two DH lambs reached the target tidal volume (V_T; 4 mL/kg) within the 120 min experiment (at 20 min and 25 min), whereas control lambs reached target V_T at approximately 5 min (IQR 5–6.25). Hence, the umbilical cord was clamped at t=10 min in DH lambs and t≈5 min in control lambs. To obtain these tidal volumes, DH lambs required a higher PIP than controls (36.9±2.3 cmH_2O vs 25.3±2.5 cmH_2O; p=0.009); therefore, dynamic lung compliance was significantly lower in DH lambs throughout the 2-hour ventilation period (0.4±0.1 mL/cmH_2O vs 1.2±0.1 mL/cmH_2O; p=0.001, figure 2A).

Before ventilation onset, DH and control lambs had similar $PaCO_2$ and arterial pH and these parameters remained stable in both groups during the initial period of ventilation with an intact umbilical cord (figure 2A). However, in DH lambs, $PaCO_2$ increased and arterial pH decreased immediately after the umbilical cord was clamped (t=10 min). In DH lambs, by 40 min after ventilation onset, $PaCO_2$ was significantly higher (102.0±16.0 vs 57.2±3.4 mm Hg; p=0.029) and pH lower (7.08±0.07 vs 7.28±0.02; p=0.017) than controls, and these differences persisted for the remainder of the 2-hour ventilation period (figure 2B).

The $AaDO_2$ was similar between DH and control lambs, while the umbilical cord remained intact (figure 2C). However, the $AaDO_2$ became markedly increased in DH lambs compared with controls immediately after the umbilical cord was clamped (485±74 mm Hg vs 129±37 mm Hg; p<0.001). Despite having a high FiO_2 throughout the 2-hour ventilation period (0.86±0.08 vs controls 0.36±0.08; p<0.001), DH lambs had a lower SaO_2 than controls from 80 min after ventilation onset and onwards (66.1±12.3 vs 90.7%±1.0%; p=0.025, figure 2D).

Pulmonary perfusion

Mean PBF increased rapidly in both groups, reaching a maximum at 20 min following ventilation onset. The rate of PBF increase (2.06 mL/min/kg per min vs 5.57 mL/min/kg per min; p<0.001) and the maximum PBF reached (41.8±9.1 mL/min/kg vs 94.6±10.8 mL/min/kg; p<0.001) were lower in DH lambs compared with controls. Following this maximum, PBF levels stabilised and remained relatively constant although tended to gradually decrease in both groups during the remainder of the

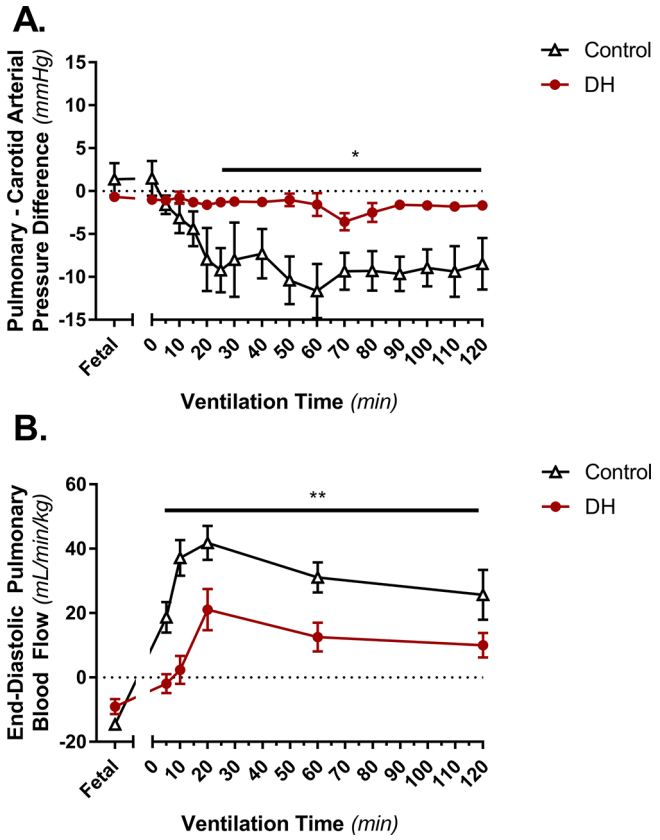

Figure 4 (A) The difference between pulmonary arterial pressure and systemic arterial pressure and (B) end-diastolic pulmonary blood flow over the 2 hours following delivery at ≈138 dGA. Groups shown are control (white triangles, n=6; thoracotomy only at ≈80 dGA) and diaphragmatic hernia (DH) (red circles, n=7; DH creation at ≈80 dGA). Presented as mean±SEM and significance accepted when P<0.05. Asterisk (*) indicates significant differences between groups (*p<0.05, **p<0.01) at all individual timepoints below line. dGA, days of gestational age.

2-hour ventilation period (figure 3A). At 2 hours, PBF was significantly lower in DH compared with control lambs (21.4±7.2 mL/min/kg vs 50.5±12.7 mL/min/kg; p=0.048).

After correcting for lung weight, the increase in PBF was similar in DH and control lambs over the 2-hour ventilation period, although the rate of increase was slower in DH lambs. PBF (per gram of lung weight) was lower at 10 min (2.95±1.06 mL/min/g vs 5.83±0.54 mL/min/g; p=0.009) after ventilation onset in DH lambs compared with controls but not significantly different at any other timepoint (figure 3B).

PAP was similar to systemic arterial pressure in both groups before ventilation onset (figure 4A). In control lambs, by 10 min after ventilation onset PAP was 3.3±1.8 mm Hg lower than systemic arterial pressure and by 60 min was 11.7±3.2 mm Hg lower. In contrast, PAP remained similar to systemic arterial pressure throughout the 2-hour experiment in DH lambs (figure 4A).

End-diastolic PBF was retrograde (assigned a negative value) in both groups before ventilation onset (figure 4B), which is a normal characteristic of PBF in the fetus.[19] At 5 min after ventilation onset, when the umbilical cord had been clamped in controls but not in DH lambs, end-diastolic flow was positive in controls but remained mostly retrograde in DH lambs (18.7±4.8 mL/min/kg vs −1.9±3.0 mL/min/kg; p=0.004). After umbilical cord clamping, end-diastolic flow was positive in both groups throughout the 2-hour experiment; however, it was greater in controls compared with DH lambs (at 2 hours 25.7±7.7 mL/min/kg vs 10.0±3.8 mL/min/kg; p=0.024).

PVR was high in both DH lambs and controls before ventilation onset (3.37±1.30 vs 2.07±0.77 mm Hg/(mL/min); p=0.068) but markedly decreased after ventilation onset in control lambs. In contrast, in DH lambs PVR did not decrease until 15 min after ventilation onset. As such, at 5 min after ventilation onset PVR was significantly greater in DH lambs compared with controls (1.95±0.55 vs 0.30±0.05 mm Hg/(mL/min); p=0.014). While PVR did eventually decrease in DH lambs (figure 3C), from 15 min to 120 min, it remained greater than in controls (0.53±0.06 vs 0.20±0.05 mm Hg/(mL/min); p=0.003). After correcting for lung weight, PVR was not different between DH and control lambs at any timepoint (figure 3D).

Cerebral perfusion

Cerebral DO_2 was similar in DH lambs and controls before ventilation onset. In control lambs, DO_2 remained at ≈2 mL/min/kg throughout the 2-hour experiment (figure 5B). Despite the persistently low SaO_2 described above, in DH lambs, DO_2 initially increased to a maximum at 30 min (3.9±0.5 vs controls 2.1±0.5 mL/min/kg; p=0.015) before returning to similar levels as controls by the end of the 2-hour experiment (figure 5B). Underlying this, in DH lambs CBF initially increased to a maximum at 30 min (30.0±4.5 mL/min/kg vs controls 12.7±3.1 mL/min/kg; p=0.002) and remained greater than controls throughout the 2-hour experiment (at 2 hours 21.0±6.2 mL/min/kg vs 10.5±2.6 mL/min/kg; p=0.038, figure 5A). Cerebral oxygen extraction was not significantly different between groups throughout the 2-hour neonatal ventilation (figure 5C). $SctO_2$ was not significantly different between groups during the first 60 min of ventilation; however, towards the end of the experiment, it was lower in DH lambs compared with controls and remained lower until the end of the experiment (figure 5D).

DISCUSSION

Using this ovine CDH model, we can invasively monitor and directly measure the cardiopulmonary changes that are responsible for the clinical challenges associated with supporting an infant with CDH through the neonatal transition. Our DH lambs demonstrated reduced dynamic lung compliance, low tidal volumes and respiratory acidosis that closely reflect the clinical experience of supporting CDH infants after birth.[20] Following lung aeration, DH was associated with increased PAP compared with controls, and despite higher input pressures, PBF was decreased. Low PBF and high PAP is indicative of a high PVR that likely contributes to impaired gas exchange and subsequent hypoxaemia and hypercapnia observed in DH lambs and also in infants with CDH.[2] However, after correcting for lung weight, we found that PBF was similar in control and DH lambs. These observations are similar to those reported by Hill *et al*,[21] who observed a decrease in PBF proportional to the degree of lung hypoplasia in fetal lambs with DH. Thus, the primary mechanism for the higher PVR and lower uncorrected PBF in DH lambs seems mostly due to a smaller lung rather than an abnormal vasodilatory response. This result is also consistent with our previous finding in DH rabbits and indicates that any pulmonary vessel smooth muscle hypertrophy and neomuscularisation that may have occurred prenatally does not directly impact on the capacity of the vessels to vasodilate in response to lung aeration.[13]

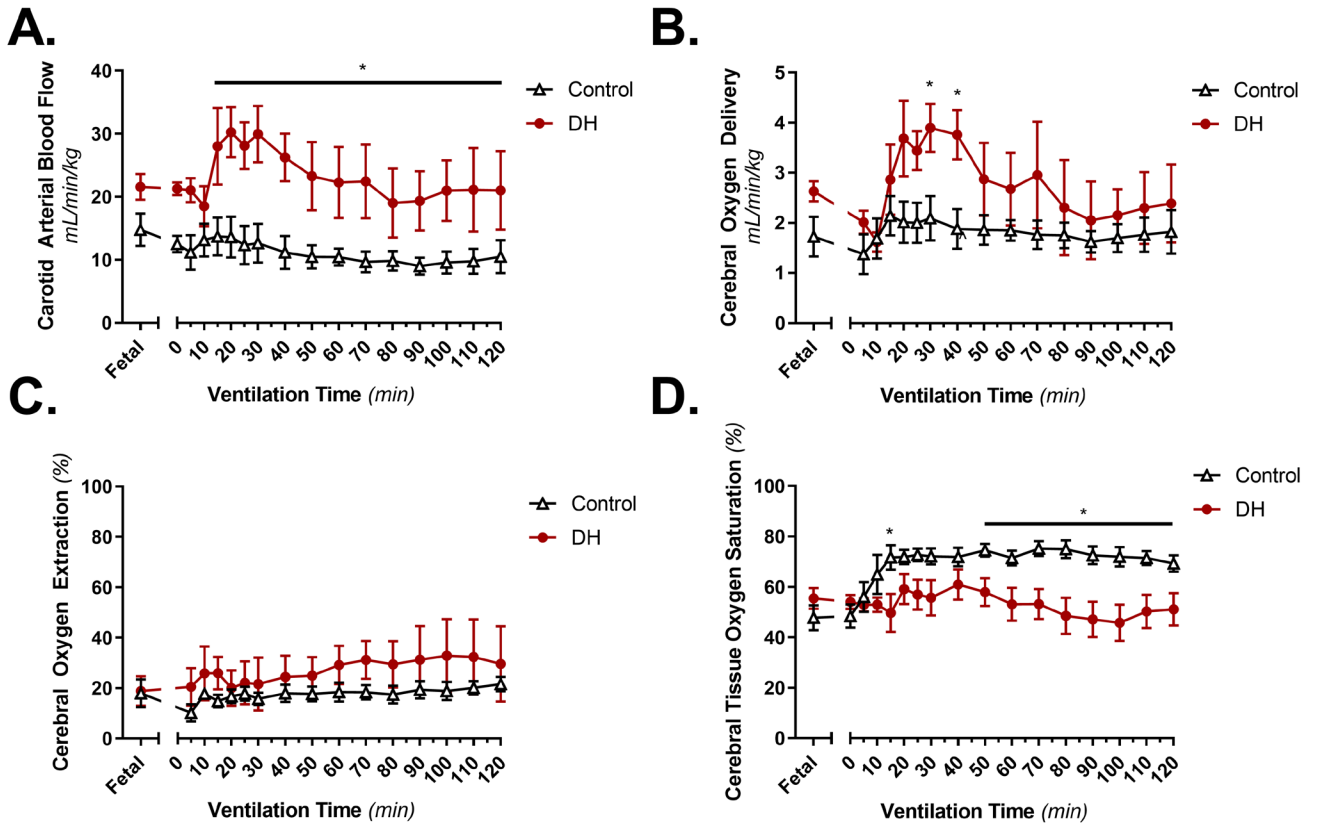

Figure 5 (A) Carotid blood flow (mL/min/kg), (B) cerebral oxygen delivery (mL/min/kg), (C) cerebral oxygen extraction (%) and (D) cerebral tissue oxygen saturation (%) over the 2 hours following delivery at ≈138 dGA. Groups shown are control (white triangles, n=6; thoracotomy only at ≈80 dGA) and diaphragmatic hernia (DH) (red circles, n=7; DH creation at ≈80 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates significant differences between groups (p<0.05) at all individual timepoints below line. dGA, days of gestational age.

It is interesting that O'Toole *et al*[22] previously observed that, when corrected for lung weight, DH lambs had reduced PBF levels compared with controls following ventilation onset. Compared with our DH lambs, their DH lambs were generally more hypoxic, hypercapnic and acidotic despite the DH being created at a similar timepoint (≈78 dGA). One methodological difference between the two studies relates to the timing of umbilical cord clamping. We clamped the umbilical cord after achieving our target tidal volume, whereas they clamped the umbilical cord immediately before ventilation commenced. This methodological difference could explain the discrepancy between the two experiments because immediate cord clamping is known to cause a rapid (within four heart beats) and large (30%) increase in afterload for the heart. Due to the presence of an open ductus arteriosus (DA), this increased afterload is experienced by both the left and right ventricles and must also influence PA pressure. However, cord clamping after the lung has aerated and PBF has increased greatly reduces this increase in afterload, because the pulmonary circulation becomes an alternative low resistance pathway for blood to flow via left to right shunting through the DA.[23 24] While this characteristic is reliant on a reduction in PVR, significant left-to-right shunting through the DA was observed in all DH lambs following lung aeration, as indicated by forward flow into the pulmonary artery throughout diastole (figure 4B). Thus, although the reduction in PVR associated with lung aeration was delayed and diminished in DH lambs, by also delaying cord clamping there was sufficient time for PVR to fall, allowing left-to-right DA shunting and thereby minimising the increase in ventricular afterload caused by cord clamping. These findings suggest that the effect of delayed umbilical cord clamping in infants with CDH certainly warrants further research in experimental models, and feasibility studies for clinical trials have already commenced.[25]

While PBF increased in DH lambs after ventilation onset, the increase occurred later than controls, and the rate of increase was much slower. This finding could be the result of impaired vasoreactivity; however, we speculate that the greatest contribution is resulting from the slower rate of lung aeration due to poor lung compliance. We anticipated this slower rate of lung aeration and tried to mitigate the difference by initiating ventilation with a sustained inflation, as it has been shown to aerate the lungs more rapidly and uniformly and to increase PBF during the transition at birth.[26]

Normally after birth, CBF gradually decreases in response to an increase in oxygenation, which is an autoregulatory process that has been well described.[27 28] In contrast, we observed a marked increase in CBF in our DH lambs immediately after cord clamping as they rapidly became hypoxaemic after losing placental circulatory support. This increase in CBF helped to maintain adequate DO_2; however, at the same time, we were increasing FiO_2 in response to rapidly deteriorating SpO_2. Together, the autoregulatory increased cerebral blood flow and iatrogenically improved oxygenation led to a dramatic overshoot in oxygen delivery to the brain: DO_2 was twofold greater

in DH lambs for 30 min of the 2-hour experiment, which may have predisposed these lambs to hyperoxia-induced brain injury. Clearly, clinicians must consider autoregulatory responses to hypoxaemia during the neonatal transition before increasing FiO_2 in response to low SpO_2, and management may be better guided by the use of NIRS to directly assess cerebral oxygenation.

Our ovine CDH model demonstrates features of severe lung hypoplasia and pulmonary hypertension that are consistent with human CDH, and we report the physiology underpinning these clinical features during the fetal to neonatal cardiopulmonary transition. By understanding these physiological changes, we can better target antenatal and neonatal interventions. A potential limitation of our study is that the DH was surgically induced during the late pseudoglandular/early canalicular stage of lung development. In humans, the potential for pulmonary development to be disrupted starts from the pseudoglandular stage following failure of the diaphragm to close during the embryonic stage. As such, the effects of CDH on vascular and airway development may commence at an early stage when the larger airways and vessels are forming. Nevertheless, we observed significantly reduced LBWR, reduced dynamic lung compliance and impaired cardiopulmonary physiology in DH lambs that is consistent with that observed in humans. While our study is limited by the lack of histological data, Pringle *et al*[29] described both hypoplastic lung morphology and less prominent alveolar capillaries even with a DH creation at ~78 dGA. However, as diaphragmatic defects were created at 80 dGA, the effects on lung airway and vascular development may be less profound than when the DH is induced at ~63 dGA.[30]

In summary, we have unearthed novel findings related to the changes in PBF during the first 2 hours after birth that reshape our understanding of how infants with CDH may transition to life after birth. Despite small, non-compliant lungs and respiratory acidosis, DH lambs experienced an increase in PBF following lung aeration of a similar magnitude to control lambs after correcting for lung weight. We now aim to use this model to investigate antenatal surgical and medical therapies that aim to rescue the lung from hypoplasia, and directly measure their effects on the neonatal cardiopulmonary transition.[31]

Contributors All authors included on this paper fulfil the criteria of authorship, specifically. PLJD, KJC, JAD, SBH, MT and RJH designed the experiments. PLJD, KJC, AJK, SMS, MT, SBH, JAD and RJH were essential for establishing the model. AJK, PLJD, KJC and SH were responsible for data analysis. AJK, KJC and PLJD wrote the first draft of the manuscript. All authors contributed by modifying and editing the manuscript and all approved final version.

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Disclaimer These funders were not involved in the study design; in the collection, analysis and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The experiment was performed in accordance with guidelines established by the National Health and Medical Research Council of Australia. This study was approved by the relevant animal ethics committee at Monash University.

Provenance and peer review Not commissioned; externally peer reviewed.

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2.2 Effects of tracheal occlusion on the neonatal cardiopulmonary transition in an ovine model of diaphragmatic hernia

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ABSTRACT

Objective Fetoscopic endoluminal tracheal occlusion (FETO) aims to reverse pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH) and mitigate the associated respiratory insufficiency and pulmonary hypertension after birth. We aimed to determine whether FETO improves the cardiopulmonary transition at birth in an ovine model of CDH.

Methods In 12 ovine fetuses with surgically induced diaphragmatic hernia (DH; 80 dGA), an endotracheal balloon was placed tracheoscopically at ≈110 dGA and removed at ≈131 dGA (DH+FETO), while 10 were left untreated (DH). At ≈138 dGA, all lambs (survival at delivery: 67% [DH+FETO], 70% [DH]) were delivered via caesarean section and ventilated for 2 hours. Physiological and ventilation parameters were continuously recorded, and arterial blood-gas values were measured.

Results Compared with DH, DH+FETO lambs had increased wet lung-to-body-weight ratio (0.031±0.004 vs 0.016±0.002) and dynamic lung compliance (0.7±0.1 vs 0.4±0.1 mL/cmH_2O). Pulmonary vascular resistance was lower in DH+FETO lambs (0.44±0.11 vs 1.06±0.17 mm Hg/[mL/min]). However, after correction for lung weight, pulmonary blood flow was not significantly different between the groups (4.19±0.57 vs 4.05±0.60 mL/min/g). Alveolar–arterial difference in oxygen tension was not significantly different between DH+FETO and DH (402±41mm Hg vs 401±45 mm Hg).

Conclusions FETO accelerated lung growth in fetuses with CDH and improved neonatal respiratory function during the cardiopulmonary transition at birth. However, despite improved lung compliance and reduced pulmonary vascular resistance, there were less pronounced benefits for gas exchange during the first 2 hours of life.

What is already known on this topic?

- Fetoscopic endoluminal tracheal occlusion (FETO) aims to correct severe lung hypoplasia associated with congenital diaphragmatic hernia (CDH) and hence prevent respiratory insufficiency at birth.
- Infants with severe CDH treated with FETO have higher survival rates than those expectantly managed; however, the underlying mechanisms are unknown, particularly as pulmonary hypertension continues to complicate CDH infants treated with FETO.

What this study adds?

- Increased total pulmonary blood flow in DH lambs treated with FETO correlated with the increase in lung growth, suggesting that the pulmonary vascular bed grows in proportion with the lung following FETO.
- Despite improved respiratory mechanics and reduced pulmonary vascular resistance, FETO did not improve pulmonary gas exchange during the first 2 hours of life in our model.
- Steady improvements in arterial pH, carbon dioxide tension ($PaCO_2$), oxygen tension (PaO_2) and oxygen saturation in FETO lambs towards the end of the 2-hour experiment may reflect either ongoing alveolar recruitment or overcoming an initial period of lung oedema.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is characterised by failed diaphragm closure during embryogenesis, enabling abdominal viscera to herniate and impair lung development.[1] As the lungs are smaller and structurally abnormal, CDH infants often have severe respiratory insufficiency immediately after birth and develop pulmonary hypertension of the newborn, which can be refractory in up to 30% of cases.[2–7] Neonatal mortality of isolated CDH remains high (30%) despite intensive neonatal care.[8–11]

As lung size is a major determinant of outcome in isolated CDH, prenatal lung measurement can identify fetuses at risk of severe hypoplasia with associated high mortality.[8 9 12–15] In fetuses with severe CDH, antenatal surgical approaches are being investigated to improve postnatal outcomes.[16] Current techniques aim to stimulate lung growth by temporary tracheal occlusion, which prevents lung liquid egress, expands the lungs and promotes lung development.[17 18] As sustained occlusion impairs type II alveolar epithelial cell development, a period of lung deflation is necessary before birth to restore type II cell numbers; this is termed the

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'plug and unplug sequence'.[17] Clinically, this can be achieved by inserting a small balloon inside the fetal trachea at 26–28 weeks and removing it at 34 weeks' gestation, all of this being possible by percutaneous techniques.[15] Preliminary outcomes following fetoscopic endoluminal tracheal occlusion (FETO) are promising, and randomised clinical trials are ongoing (the 'Tracheal Occlusion to Accelerate Lung growth' trials for moderate and severe lung hypoplasia; NCT01240057/NCT00763737).[19–22] The downside of this approach is the occurrence of preterm premature rupture of membranes and preterm birth, which are inherent to fetal surgical procedures and offset the possible benefits of FETO.[21] Furthermore, despite an apparent increase in survival, there remains a proportion of neonates not surviving after birth.[23] The reasons for this are unclear, particularly as little is known about how FETO affects the cardiopulmonary changes that underpin the transition to newborn life. In the preceding article in this edition of *Archives*, we have described the effects of lung hypoplasia on cardiopulmonary physiology during the transition to newborn life in an ovine diaphragmatic hernia (DH) model.[24] In this study, we used that DH model to evaluate the effect of FETO on the cardiopulmonary adaptations that occur immediately after birth.

METHODS

Animal surgery and treatment groups

All surgical procedures were performed under general anaesthesia using intravenous sodium thiopental (Pentothal; Jurox, New Zealand) for induction and inhaled isoflurane (≈2% in room air; Isoflow, Abbot) for maintenance. Ewes were intubated and monitored (ECG, expired CO_2 and oxygenation) during surgery. After surgery, the ewes received 3 days of analgesia (transdermal fentanyl patch, 75 µg/hour; Janssen Cilag) and were monitored daily until delivery. Before surgery, animals were allocated to DH or DH+FETO groups.

Ultrasound evaluation

Before every procedure, fetal ultrasound was performed (CX50 Ultrasound System, Philips) to measure thoracic and cardiac circumference, right and left lung area and femur length. The shape of the fetal lamb skull and limited visualisation at late gestational ages precluded reproducible images of the fetal head. We calculated the right lung to femur ratio (RLFR).

DH creation

A DH was surgically created at ≈80 days gestational age (dGA; term≈147 dGA), as previously described.[24] Briefly, a midline laparotomy and hysterotomy allowed exteriorisation of fetal forelimbs and chest. The fetal chest was incised through the ninth intercostal space to expose and incise the diaphragm, allowing the stomach and bowels to be pulled through into the fetal thoracic cavity.

FETO and unplug

At ≈110 dGA, a midline laparotomy was performed using the same incision. The fetal head was identified, and a small uterine incision was made directly over the fetal mouth. The position of the fetal mouth was fixed using two stay sutures (polysorb 2–0). The fetoscope (11530 AA, 1.3 mm, Karl Storz, Tuttlingen, Germany) was placed into the mouth and access to the trachea was obtained under direct fetoscopic vision. A detachable balloon (Goldbal5, Balt, France) loaded on a microcatheter (BALTACCIBDPE100, Balt, France) was positioned above the carina, inflated with 2.5 mL of saline and detached. After the fetoscope was removed, the hysterotomy and laparotomy were closed. The ewe was given tocolysis (medroxyprogesteron acetate 150 mg, Pfizer Australia Pty Ltd.) and antibiotic prophylaxis (cefazolin 1 g, intra-amniotic and intravenous). At ≈131 dGA, the balloon was removed, either by ultrasound guided puncture or direct fetoscopic vision.[15]

Delivery and ventilation

At ≈138 dGA, the ewe was anaesthetised to allow fetal instrumentation as previously described.[24] Briefly, midline laparotomy and hysterotomy exposed the fetal head, neck and chest. The lamb was intubated (size 4.0 cuffed endotracheal tube). Polyvinyl catheters were positioned in the left carotid artery and main pulmonary artery to continuously monitor arterial blood pressure and in the left jugular vein to allow neonatal intravenous sedation. Ultrasonic flow probes (Transonic Systems, Ithaca, New York, USA) were placed around the right carotid and left pulmonary artery to continuously monitor arterial blood flow. Pulse-oximeter, near-infrared spectroscopy and temperature probe were placed on right forelimb, forehead and rectum, respectively. The stomach was drained via an orogastric tube. After instrumentation, the lamb was delivered from the uterus, dried and carefully positioned on the ewe's abdomen to avoid occluding umbilical cord blood flow.

After passively draining lung liquid and recording the volume drained, ventilation commenced with a 30 s sustained inflation (35 cmH_2O, 21% O_2) followed by intermittent positive pressure ventilation (iPPV) in volume guarantee mode using a tidal volume (V_T) of 4 mL/kg (Babylog 8000, Dräger, Lübeck, Germany) and heated and humidified gases. Positive end-expiratory pressure was set at 5 cmH_2O and peak inspiratory pressure (PIP) was initially limited to 35 cmH_2O. If the target V_T was not reached after 30 s, a second 30 s sustained inflation was performed. We then continued with iPPV for a total of 120 min. The umbilical cord was clamped when the target V_T was achieved or at 10 min after ventilation onset. The lamb was then moved to a warming bed, sedated with alfaxalone (50 mg/hr; Alfaxan, Jurox) and covered with plastic drapes to maintain body temperature. Ventilator rates and fraction of inspired oxygen (FiO_2) levels were titrated to achieve gas targets for arterial carbon dioxide tension ($PaCO_2$) 60–80 mm Hg, arterial oxygen tension (PaO_2) >40 mm Hg and arterial oxygen saturation (SaO_2) of 85%–88%. The lamb was euthanised (sodium pentobarbitone intravenous 100 mg/kg) after completing the 2-hour ventilation protocol or earlier due to ethical endpoints (i.e. severe acidosis or pneumothorax).

Outcome measures

Arterial blood samples were analysed every 5 min during the first 30 min of ventilation and every 10 min thereafter.

Physiological data analysis

V_T, airway pressures, cerebral tissue oxygen saturation ($SctO_2$), pulmonary and cerebral perfusion were continuously recorded using LabChart (ADInstruments, New South Wales, Australia) and analysed offline. Ten heartbeat average data points were collected prior to ventilation onset (fetal), immediately at the onset of ventilation (0 min) and every 5 min throughout the ventilation period.

Postmortem examination

Presence of a diaphragmatic defect and herniation of visceral organs was confirmed during postmortem examination. Lungs

were weighed and expressed as a ratio to the body weight (wet lung-to-body weight ratio [LBWR]).

Calculations

- Pulmonary vascular resistance (PVR): (pulmonary artery pressure – left atrial pressure)/pulmonary blood flow. Based on previous studies, the left atrial pressure was assumed to equal 9 mm Hg.[25]
- Alveolar–arterial difference in oxygen tension (A–aDO_2; mm Hg): (FiO_2*713-$PaCO_2$/0.8)−PaO_2.
- Cerebral oxygen extraction (%): (SaO_2−$SctO_2$)/SaO_2.[26]
- Arterial oxygen content (CaO_2; mL O_2/L): [1.39•Hb•SaO_2/100] + [0.03•PaO_2]), where Hb is the haemoglobin concentration (g/L).[27]
- Cerebral oxygen delivery (DO_2; mL/min/kg): (CBF•CaO_2).[27]

Statistical analysis

Based on power analysis from previous studies, we estimated ∼7 animals per group were required to detect minimal biologically significant differences in pulmonary blood flow (PBF) and ventilation characteristics with a coefficient of variation of ∼25%, assuming an α value of 0.05 and a power of >0.8.[28]

Continuous data are expressed as means±SEM, or medians (IQR) if the data were not normally distributed. Dichotomous data are expressed as percentages. Differences between DH and DH+FETO were analysed using two-way repeated measures analysis of variance combined with Holm-Sidak's multiple comparisons test. A p value <0.05 was considered statistically significant.

RESULTS

Animal surgery

Twenty-two fetuses were operated on at ≈80 dGA and allocated to either DH (n=10; data presented in the preceding article in this edition of *Archives*[24]) or DH+FETO (n=12). Intratracheal balloon placement was successful in all DH+FETO animals at ≈110 dGA. Intrauterine fetal death or preterm delivery occurred in three cases at 4, 4 and 15 days after balloon placement (online supplementary figure 1). Of nine fetuses alive for balloon removal, the balloon was successfully punctured under ultrasound guidance in five cases (56%). In one case, the balloon was not visualised during ultrasound, and it was subsequently confirmed at time of delivery that this balloon was dislodged. In three fetuses, ultrasound-guided puncture was unsuccessful, and instead it was removed fetoscopically. One ewe went into early labour 2 days after fetoscopic balloon removal, and the fetus was not viable for experimentation.

Fifteen fetuses survived to delivery at ≈138 dGA: seven DH lambs (70%) and eight DH+FETO lambs (67%). During the 2-hour neonatal ventilation, it was necessary to discontinue the experimental protocol early in two DH lambs (at 70 min and 110 min) due to developing treatment-resistant pneumothoraces and in one DH+FETO lamb (80 min) due to developing severe acidosis.

Postmortem examination

A diaphragmatic defect with stomach herniation was confirmed at postmortem examination in all lambs and was often combined with intrathoracic herniation of small and large intestines. Compared with DH, DH+FETO had increased wet LBWR at postmortem (0.031±0.004 vs 0.016±0.002; p=0.02) and increased lung liquid volume (30 [15–54] vs 10 [2–20] mL; p=0.045).

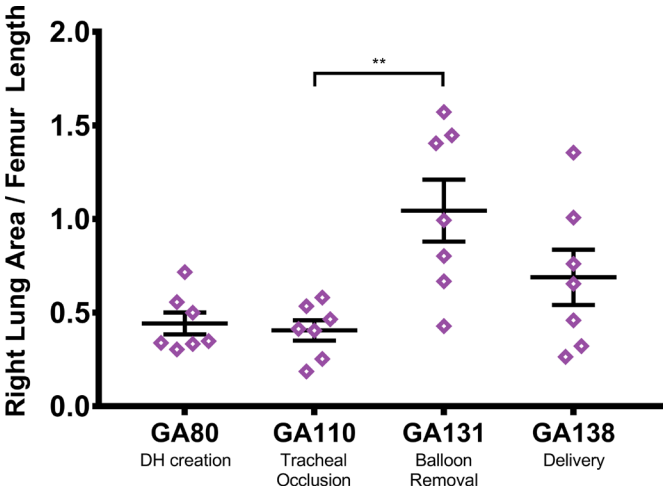

Figure 1 Ratio of right lung area to femur length (RLFR) in fetal lambs with a surgically induced diaphragmatic hernia (DH) treated with fetoscopic endoluminal tracheal occlusion (FETO). Timepoints shown are DH creation at ≈80 dGA, FETO 'plug' procedure at ≈110 dGA, FETO 'unplug' at ≈131 dGA and delivery by caesarean section at ≈138 dGA. Presented as mean±SEM and significance accepted when p<0.05. **RLFR significantly different (p=0.008) between FETO plug and FETO unplug. dGA, days gestational age.

Ultrasound

In DH+FETO fetuses, we observed significant increases in RLFR between balloon placement and removal (0.41±0.05 vs 1.05±0.17; p=0.009), followed by a reduction between balloon removal and delivery (0.69±0.15; figure 1). In contrast, in the DH group, RLFR was significantly lower at delivery compared with baseline (0.297±0.03 vs 0.46±0.06; p=0.044).

Ventilation and oxygenation

Only two DH lambs reached the target V_T (4 mL/kg) during ventilation (at 20 min and 25 min), whereas all DH+FETO lambs reached this target V_T at median of 5 min (range 5–15) after ventilation onset. To achieve these V_T, DH+FETO lambs required a PIP that was consistently below 35 cmH_2O and was significantly lower than the DH group (31.4±2.1 vs 39.8±1.8 cmH_2O; p=0.026) at 60 min onwards. Dynamic lung compliance was higher in DH+FETO compared with DH throughout the 2-hour ventilation period (0.7±0.1 vs 0.4±0.1 mL/cmH_2O; p=0.049, figure 2A).

$PaCO_2$ and arterial pH were not significantly different between DH+FETO and DH lambs before ventilation onset or during the 2-hour ventilation period (figure 2B,C). During the first 30 min of ventilation, $PaCO_2$ increased and pH decreased in both groups. From 40 min to 120 min, this trend continued in DH lambs, whereas in DH+FETO lambs, $PaCO_2$ decreased and pH increased (figure 2B,C). At 2 hours, there were no significant differences between DH+FETO and DH lambs in $PaCO_2$ (73±8 vs 100±22 mm Hg; p=0.075) or pH (7.22±0.04 vs 7.06±0.10; p=0.059).

SaO_2 was 20% higher in DH+FETO compared with DH lambs after 2 hours of neonatal ventilation (85.4±1.6 vs 65.5%±12%; p=0.049). However, to maintain this arterial oxygenation, high FiO_2 levels were required for both DH+FETO and DH (0.82±0.07 vs 0.85±0.08, p=0.741). Hence, the alveolar-arterial difference in oxygen tension was not significantly different between DH+FETO and DH (402±41 vs 401±45 mm Hg, p=0.959; figure 2D).

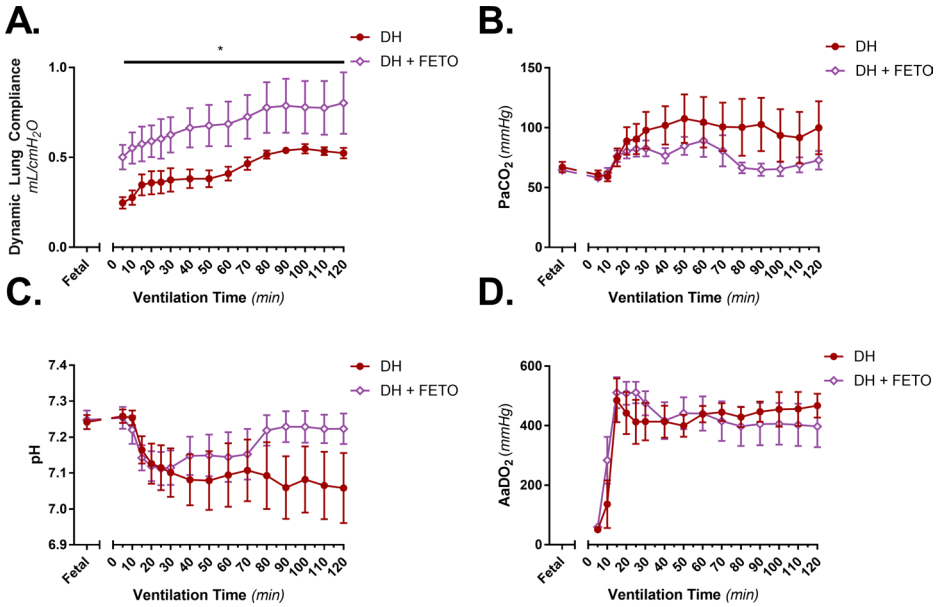

Figure 2 (A) Dynamic lung compliance, (B) arterial carbon dioxide tension ($PaCO_2$), (C) arterial pH and (D) alveolar-arterial difference in oxygen tension ($A–aDO_2$) over the 2 hours following delivery at ≈138 dGA. Groups shown are diaphragmatic hernia (DH) (•, n=7; DH creation at ≈80 dGA) and DH+FETO (◊, n=8; DH creation at ≈80 dGA, FETO plug at ≈110 dGA and unplug at ≈131 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates significant differences between groups (p<0.05) at all individual timepoints below line. FETO, fetoscopic endoluminal tracheal occlusion.

Pulmonary perfusion

PBF increased rapidly in both groups, reaching a maximum at 20 min following ventilation onset (figure 3A). During the first 5 min, the rate of PBF increase tended to be higher in DH+FETO compared with DH lambs; however, this difference was not statistically significant (5.8 vs 1.5 mL/min/kg per min; p=0.077). The maximum PBF reached, at 20 min in both groups, was significantly greater in DH+FETO compared with DH lambs (67.9±11.1 vs 41.8±9.1 mL/min/kg; p=0.038). Following this maximum, PBF levels gradually decreased in both groups during the remainder of the 2-hour ventilation period but to a greater degree in DH+FETO lambs (figure 3A). Hence, at 2 hours, there was no longer a significant difference in PBF between DH+FETO and DH lambs (32.2±6.8 vs 21.4±7.2 mL/min/kg, p=0.414).

After correcting for lung weight, the increase in PBF was similar in DH and DH+FETO lambs over the 2-hour ventilation period, and there was no significant difference at any time (figure 3B).

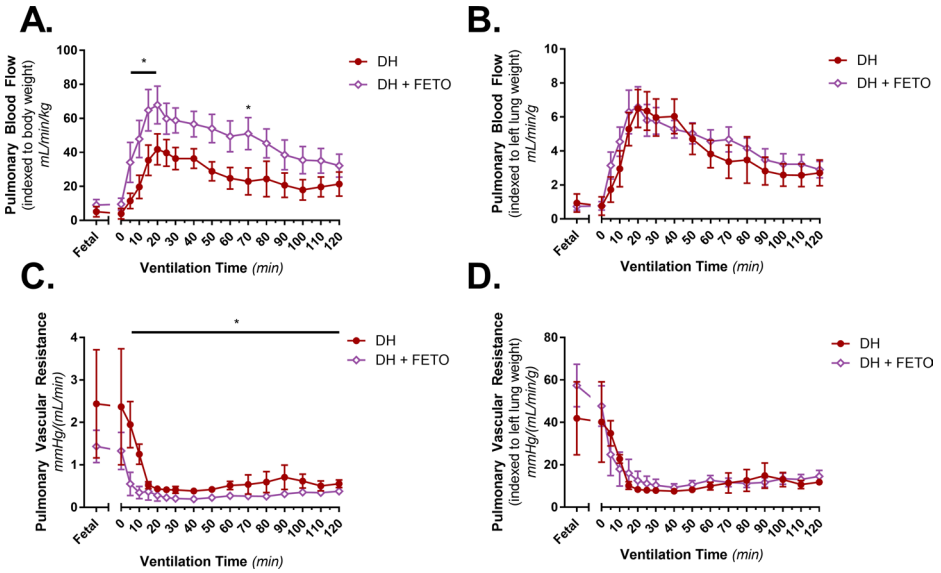

Figure 3 (A) Pulmonary blood flow (PBF) indexed to body weight, (B) PBF indexed to left lung weight, (C) pulmonary vascular resistance (PVR) and (D) PVR indexed to left lung weight over the 2 hours following delivery at ≈138 dGA. Groups shown are diaphragmatic hernia (DH) (•, n=7; DH creation at ≈80 dGA) and DH+FETO (◊, n=8; DH creation at ≈80 dGA, FETO plug at ≈110 dGA and unplug at ≈131 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates significant differences between groups (p<0.05) at all individual timepoints below line. dGA, days gestational age; FETO, fetoscopic endoluminal tracheal occlusion.

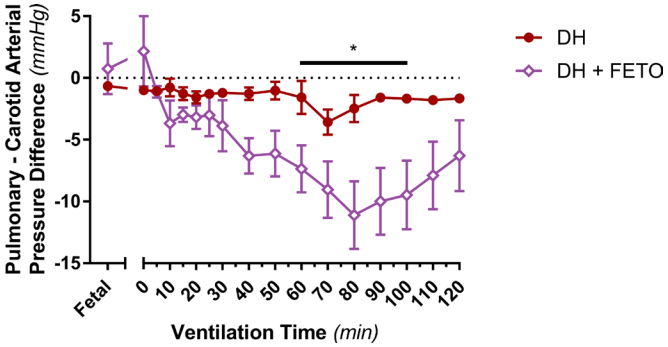

Figure 4 The difference between pulmonary arterial pressure and carotid (systemic) arterial pressure over the 2 hours following delivery at ≈138 dGA. Groups shown are diaphragmatic hernia (DH) (•, n=7; DH creation at ≈80 dGA) and DH+FETO (◊, n=8; DH creation at ≈80 dGA, FETO plug at ≈110 dGA and unplug at ≈131 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates statistically significant differences between DH and DH+FETO at individual timepoints below line. dGA, days gestational age; FETO, fetoscopic endoluminal tracheal occlusion.

Pulmonary arterial pressure was similar to systemic arterial pressure in both groups before ventilation onset (figure 4). In DH lambs, pulmonary arterial pressure remained similar to systemic arterial pressure throughout the 2-hour ventilation period. In contrast, in DH+FETO lambs the pulmonary to systemic pressure gradient increased to a maximum at 80 min (−11.2±2.7 vs DH −2.5±1.1 mm Hg; p=0.007) before decreasing towards the end of the 2-hour experiment (−6.3±2.9 vs −1.7±0.3 mm Hg; p=0.156).

PVR was lower in DH+FETO compared with DH fetuses before ventilation onset (1.43±0.38 vs 3.37±1.30 mm Hg/(mL/min); p<0.001), but relatively high (>1 mm Hg/(mL/min)) in both groups (figure 3C). In DH lambs, PVR did not decrease below 1 mm Hg/(mL/min) until 15 min after ventilation onset. In contrast, in DH+FETO lambs PVR immediately decreased after ventilation onset (figure 3C). Throughout the 2-hour ventilation period, PVR was lower in DH+FETO than DH lambs (0.44±0.11 vs 1.06±0.17 mm Hg/(mL/min); p=0.02).

After correcting for lung weight, PVR was not different between DH+FETO and DH lambs at any time (figure 3D).

Cerebral perfusion

Cerebral DO_2 was similar in DH+FETO and DH lambs before ventilation onset (1.7±0.3 vs 2.6±0.2 mL/min; p=0.247) and throughout the 2-hour ventilation period (figure 5B). In both groups, DO_2 increased to a maximum at 30 min (DH+FETO 3.7±0.4 vs DH 3.9±0.5 mL/min; p=0.661) before returning to fetal levels (figure 5B). Cerebral oxygen extraction was not significantly different in DH+FETO compared with DH lambs

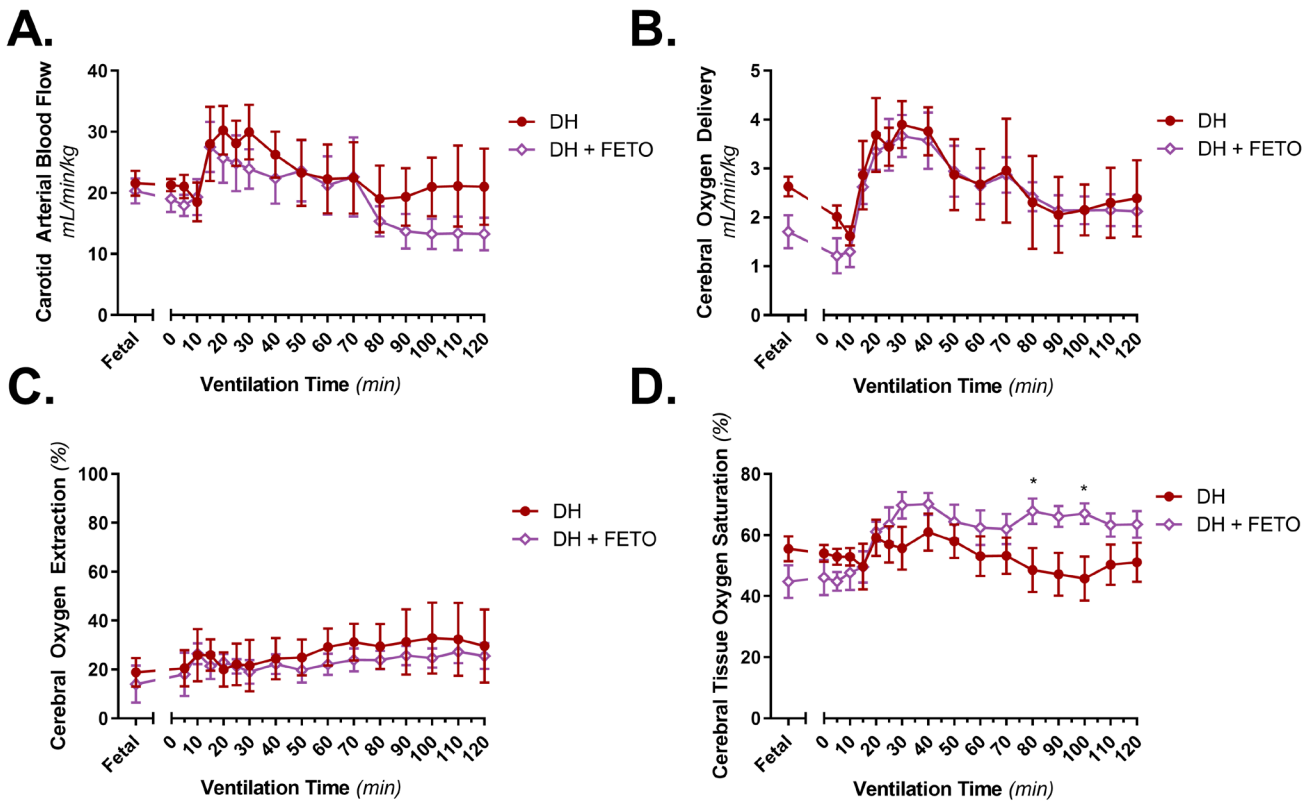

Figure 5 Cerebral haemodynamics and oxygenation during the 2-hour ventilation experiment. (A) Carotid blood flow, (B) cerebral oxygen delivery, (C) cerebral oxygen extraction and (D) cerebral tissue oxygen saturation ($SctO_2$) over the 2 hours following delivery at ≈138 dGA. Groups shown are diaphragmatic hernia (DH) (•, n=7; DH creation at ≈80 dGA) and DH+FETO (◊, n=8; DH creation at ≈80 dGA, FETO plug at ≈110 dGA and unplug at ≈131 dGA). Presented as mean±SEM and significance accepted when p<0.05. Asterisk (*) indicates statistically significant differences between DH and DH+FETO at individual timepoints. dGA, days gestational age; FETO, fetoscopic endoluminal tracheal occlusion.

throughout the 2-hour ventilation period (23±6 vs 22%±7%; p=0.931 figure 5C). $SctO_2$ was greater in DH+FETO compared with DH lambs at 80 min (68±4 vs 49%±7%; p=0.034) and 100 min but at no other timepoints (figure 5D).

DISCUSSION

We describe the effects of FETO on the pulmonary circulation and respiratory function during the neonatal transition in lambs with DH. Despite marked improvements in lung growth, compliance and overall perfusion, persistently high $PaCO_2$ and $A–aDO_2$ suggest that FETO did not significantly improve pulmonary gas exchange in this model of CDH.

In our study, FETO was associated with increased lung size and significant improvements in lung compliance, as described in other animal models of CDH.[18] Improved compliance allowed us to achieve target V_T at lower PIP, reducing the risk of ventilator-induced lung injury (VILI). VILI is an important risk factor for the development of bronchopulmonary dysplasia (BPD) in CDH infants, and before the widespread introduction of 'gentle ventilation', may have contributed to up to 25% of CDH mortality.[29] However, exposure to high levels of supplemental oxygen also plays a role in developing BPD.[30 31] In our study, FETO did not reduce the high FiO_2 requirements associated with DH.

High FiO_2 requirements despite adequate V_T and overall perfusion, along with high $PaCO_2$, suggest there may be persistent problems with the alveolar–capillary interface in DH animals treated with FETO. Increased alveoli septal wall thickness that limits gas exchange in CDH is reversed after 6 weeks of tracheal occlusion in a lamb model, but this was less pronounced after a 2-week occlusion period.[32] Nardo *et al* similarly demonstrated a time-dependent benefit of tracheal occlusion, including alveolar wall thinning and increased luminal surface area for gas exchange.[33] In this experiment, we occluded the trachea for 3 weeks before reversal during a similar stage of lung development to the clinical application of this technique. Nevertheless, this may not be adequate to promote thinning of the alveolar septal walls. Davey *et al* also performed tracheal occlusion and reversal at similar timepoints to our study and also observed persistently increased septal wall thickness with no significant benefits to gas exchange.[34]

Alternatively, poor gas exchange may suggest persistently impaired perfusion of fifth and sixth generation pulmonary vessels that are important for gas exchange. In CDH, reduced pulmonary vascular cross-sectional area in combination with extensive hypermuscularisation and neomuscularisation of distal pulmonary vessels is thought to lead to higher resistance within peripheral pulmonary vessels.[35] Luks *et al* suggested that short-term tracheal occlusion (2 weeks) reduces abnormal muscularisation of pulmonary arterioles; however, their histological analysis defined distal vessels by size, rather than branching generation.[36] Conversely, others have found that releasing the tracheal occlusion 1 week prior to delivery abolishes the tracheal occlusion-induced thinning of the abnormally thick pulmonary artery medial area in DH.[37] Our current study demonstrates that despite the increase in lung size and overall increase in PBF in response to FETO, PBF corrected for lung weight was not different from DH animals. However, this is not surprising as we have previously found that, corrected for lung weight, PBF is also similar in DH and control lambs.[24] This finding indicates that pulmonary vascular growth occurs in proportion with lung tissue growth and that, if present, abnormally thickened pulmonary arterial walls may have little functional consequences, at least not during the first 2 hours after birth. These findings are consistent with the observation that infants with severe CDH (observed/expected lung-to-head ratio (O/E LHR) <25%) treated with FETO had similar rates of pulmonary hypertension to moderate cases (O/E LHR 25%–45%) that were expectantly managed.[38]

An important limitation of our study is that the experiment only lasted for 2 hours after birth. In a similar lamb DH model with tracheal occlusion at 108–129 dGA and delivery at 136 dGA, Bratu *et al* observed improved gas exchange only emerging after 3 hours of neonatal ventilation.[39] These authors hypothesised that ongoing alveolar recruitment eventually matched ventilation and perfusion after prolonged ventilation. This hypothesis is supported by our previous observation that functional residual capacity and V_T recruitment are significantly slower in DH lungs.[40] Furthermore, in this study, we observed a steady improvement in arterial pH values (and declining trend in $PaCO_2$ and $A–aDO_2$) at the end of the experiment in FETO lambs, in contrast to the progressive decline observed in DH lambs. However, if alveolar recruitment was improving over this time, we would also expect a simultaneous increase in lung compliance to reflect the overall increase in lung gas volume. It is also possible that FETO lambs had greater airway liquid volumes at birth, resulting in greater lung oedema following lung aeration.[41 42] We passively drained greater lung liquid volumes in DH lambs treated with FETO, which suggests residual lung liquid volumes may also be greater. Recent studies have demonstrated the adverse effects of elevated airway liquid volumes on lung mechanics and respiratory function following lung aeration at birth.[43]

Our experimental approach was not designed to completely duplicate current clinical practice but was a pragmatic approach to meet feasibility and ethical requirements. In humans, the FETO procedure is performed via a percutaneous approach, whereas we performed a maternal laparotomy and created a small uterine incision in order to introduce the fetal tracheal balloon via fetoscope. While this procedure is more invasive for the ewe, it is not more invasive for the fetus and is unlikely to have influenced fetal development any more than the minimally invasive FETO procedure. In the delivery room, CDH infants usually undergo immediate cord clamping, are resuscitated with higher FiO_2 values and do not receive a sustained inflation. In contrast, we performed delayed cord clamping and began ventilation with a sustained inflation. We have previously shown that cord clamping after the lung is aerated allows PBF to increase before the placental circulation is lost and that a sustained inflation aerates the lungs more uniformly while not affecting the increase in PBF following lung aeration.[44 45] The effects of delayed cord clamping and a sustained inflation in our CDH model are discussed in more detail in our preceding article.[24] Regardless, the approach to neonatal resuscitation was consistent between the two experimental groups.

In conclusion, we found that while FETO increases lung size and PBF in proportion with lung size, it appears to have minimal benefit for pulmonary gas exchange during the cardiopulmonary transition at birth. Clinically, arterial blood gas status during the transition to newborn life is an important predictor of long-term survival.[46] To further improve outcomes for CDH infants, novel therapies, including antenatal medical therapies, should target improvements in pulmonary gas exchange efficiency, such as the stimulation of pulmonary microvascular development.[47]

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Chapter 3

Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia

3 Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia

In Chapter 2.1, despite severe lung hypoplasia and very poor respiratory function in lambs with DH, we found that the increase in PBF per gram of lung tissue at birth was similar to control lambs, suggesting that the overall reduction in PBF was simply due to a reduction in lung size. This finding contradicted the commonly accepted understanding that CDH infants are born with neomuscularisation of the intra-acinar pulmonary vasculature that manifests as pulmonary hypertension after birth, and indeed the anatomical evidence upon which this theory is based is unconvincing (discussed in Chapter 1.1). This result was also inconsistent with previous work done by others using the same sheep CDH model.[338] However, in the previous study they performed immediate cord clamping, whereas in Chapter 2.1 we performed physiologically based cord clamping (PBCC). As discussed in Chapter 1.2, the timing of umbilical cord clamping has a significant impact on cardiopulmonary haemodynamics during the neonatal transition.

Current guidelines recommend CDH infants are intubated immediately after birth, which in practice usually translates to immediate cord clamping (ICC).[183] An alternative strategy, as we used in Chapter 2.1, is to aerate the lung before removing placental support (PBCC). In Chapter 3.1 I aimed to provide a detailed description of the effects of PBCC, compared to ICC, on cardiopulmonary physiology during the fetal to neonatal transition in lambs with a DH. I hypothesised that PBCC would avoid a prolonged period of hypoxaemia and low cardiac output in the newborn period, leading to greatly improved cardiopulmonary haemodynamics and gas exchange immediately after birth.

We found that establishing lung aeration prior to umbilical cord clamping avoids transient, severe hypoxia at birth and enables increased PBF in lambs with a DH for at least the first 120 min after birth. However, despite improved pulmonary perfusion, PBCC lambs had persistent hypoxia and hypercapnia, similar to ICC lambs, suggesting that the underlying lung hypoplasia was primarily responsible for the impaired gas

exchange. Overall, our findings suggest that a physiological approach to umbilical cord clamping will enable a better transition to newborn life for infants with a CDH.

In Chapter 3.2, I compared our lambs with DH that underwent ICC to historical control lambs that also underwent ICC, in order to describe the effects of DH in the current clinical context of ICC.

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3.1 Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia

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Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia

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ABSTRACT

Objective Lung hypoplasia associated with congenital diaphragmatic hernia (CDH) results in respiratory insufficiency and pulmonary hypertension after birth. We have investigated whether aerating the lung before removing placental support (physiologically based cord clamping (PBCC)), improves the cardiopulmonary transition in lambs with a CDH.

Methods At ≈138 days of gestational age, 17 lambs with surgically induced left-sided diaphragmatic hernia (≈d80) were delivered via caesarean section. The umbilical cord was clamped either immediately prior to ventilation onset (immediate cord clamping (ICC); n=6) or after achieving a target tidal volume of 4 mL/kg, with a maximum delay of 10 min (PBCC; n=11). Lambs were ventilated for 120 min and physiological changes recorded.

Results Pulmonary blood flow (PBF) increased following ventilation onset in both groups, but was 19-fold greater in PBCC compared with ICC lambs at cord clamping (19±6.3 vs 1.0±0.5 mL/min/kg, p<0.001). Cerebral tissue oxygenation was higher in PBCC than ICC lambs during the first 10 min after cord clamping (59%±4% vs 30%±5%, p<0.001). PBF was threefold higher (23±4 vs 8±2 mL/min/kg, p=0.01) and pulmonary vascular resistance (PVR) was threefold lower (0.6±0.1 vs 2.2±0.6 mm Hg/(mL/min), p<0.001) in PBCC lambs compared with ICC lambs at 120 min after ventilation onset.

Conclusions Compared with ICC, PBCC prevented the severe asphyxia immediately after birth and resulted in a higher PBF due to a lower PVR, which persisted for at least 120 min after birth in CDH lambs.

What is already known on this topic?

- Umbilical cord clamping stops placental gas exchange and reduces venous return to the heart; lung aeration triggers an increase in pulmonary blood flow (PBF) that restores gas exchange and venous return.
- Infants with congenital diaphragmatic hernia (CDH) have severely hypoplastic lungs and abnormal pulmonary vasculature, which delays the normal increase in PBF after birth, resulting in hypoxia and hypercarbia.
- Physiologically based cord clamping (PBCC) prevents the loss in cardiac output and reduction in oxygenation caused by immediate cord clamping (ICC) in preterm lambs.

What this study adds?

- PBCC in lambs with a diaphragmatic hernia avoided the severe hypoxia associated with ICC and resulted in higher PBFs during the first 2 hours after birth.
- Compared with PBCC, ICC was associated with transient asphyxia, tachycardia and increases in systemic and pulmonary arterial pressures.
- PBCC may improve the cardiopulmonary transition at birth in newborns with a CDH.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a birth defect that affects 1 in 3000 live births and is responsible for >1% of the infant mortality rate in the USA.[1 2] The diaphragm fails to close during embryogenesis, which allows abdominal organs to herniate into the chest and disrupt fetal lung development.

The majority of fetuses with isolated CDH survive until term, because their hypoplastic lungs are not required for gas exchange during fetal life. However, when the umbilical cord is clamped at birth the infant becomes solely dependent on the lungs for gas exchange. The lungs of CDH infants are small, with a reduced surface area for gas exchange and reduced pulmonary vascular development, resulting in respiratory insufficiency and pulmonary hypertension soon after birth.[3]

Current guidelines recommend that CDH infants should be intubated immediately at birth, to avoid inadvertent gaseous distension of the intrathoracic stomach.[4] In practice, immediate intubation usually translates to immediate umbilical cord clamping at delivery, in order to move the infant to a resuscitation bed for respiratory support. However, we have previously shown that in preterm lambs that require respiratory support, immediate cord clamping (ICC) leads to a transient reduction in cardiac output, marked swings in arterial blood pressures and flows as well as hypoxaemia.[5 6]

During fetal life, the placental circulation provides the majority of venous return to the left

heart (preload) via the foramen ovale, as pulmonary blood flow (PBF) is low, due to a high pulmonary vascular resistance (PVR), and unable to sustain left ventricular preload.[7] Removing the low-resistance placental circulation by clamping the umbilical cord removes this important source of preload, and also increases systemic vascular resistance (afterload). The concurrent increase in afterload and decrease in preload immediately after cord clamping therefore reduces cardiac output.[8] Cardiac output is only restored when the pulmonary circulation vasodilates (following lung aeration) to accept the entire output of the right ventricle, leading to a large increase in PBF that can sustain left ventricular preload.[9 10] On the other hand, if lung aeration precedes umbilical cord clamping, PBF increases before umbilical venous return is lost and as a result there is no loss in cardiac output following cord clamping. This has been termed physiologically based cord clamping (PBCC).[11]

In normal term infants, lung aeration occurs rapidly and so ICC results in only a limited interruption to cardiac output and gas exchange at birth. In contrast, CDH infants have stiff, hypoplastic lungs which are likely to take much longer to aerate, as has been demonstrated experimentally.[12] As lung aeration stimulates the increase in PBF, it is not surprising that the increase in PBF is also delayed in newborn lambs with lung hypoplasia.[13] As such, ICC in infants with a CDH may expose them unnecessarily to a prolonged period of hypoxia and reduced cardiac output immediately after birth.

We hypothesise that PBCC in lambs with a diaphragmatic hernia (DH) will avoid a prolonged period of hypoxia and low cardiac output in the newborn period, leading to greatly improved cardiopulmonary haemodynamics and gas exchange immediately after birth. We aimed to provide a detailed description of the effects of PBCC on cardiopulmonary physiology during the fetal to neonatal transition in lambs with a DH.

METHODS

Ethics statement

All procedures in animals were performed in accordance with the National Health and Medical Research Council of Australia guidelines for care and use of experimental animals.

General surgical methods

All surgical procedures were performed under general anaesthesia using intravenous sodium thiopental (Pentothal; Jurox, New Zealand) for induction and inhaled isoflurane (≈2% in room air; Isoflow, Abbot) for maintenance. Ewes (Merino X Border-Leicester) were intubated and monitored (ECG, expired CO_2, arterial oxygen saturation (SaO_2)) during surgery.[14]

Diaphragmatic hernia creation

At ≈80 days gestational age (dGA; term≈147 dGA), a DH was surgically created in fetal lambs as previously described.[14] Following maternal midline laparotomy and hysterotomy, the fetal chest was exteriorised and incised through the left ninth intercostal space. A small incision was made in the fetal diaphragm, through which the stomach and bowels were pulled into the thoracic cavity. Ewes received 3 days of postoperative analgesia (transdermal fentanyl patch, 75 µg/hour; Janssen Cilag) and were monitored daily until delivery.

Delivery and ventilation

Fetal instrumentation

At ≈138 dGA, ewes and their fetuses were anaesthetised and the lambs instrumented prior to caesarean section delivery as previously described.[14] Briefly, lambs were partially exteriorised and intubated with a clamped endotracheal tube (size 4.0) prior to instrumentation to prevent lung aeration. Polyvinyl catheters were positioned in the left carotid artery and main pulmonary artery to allow continuous arterial pressure monitoring, and in the left jugular vein to allow neonatal sedation. Ultrasonic flow probes (Transonic Systems, Ithaca, New York, USA) were placed around the right carotid and left pulmonary artery, to continuously record blood flow. A pulse-oximeter (Masimo, Radical 7, California, USA), near-infrared spectroscopy (Casmed Foresight, CAS Medical Systems, Branford, Connecticut, USA) and temperature probe were placed on right forelimb, forehead and rectum, respectively. The stomach was drained via an orogastric tube. After instrumentation, lambs were delivered from the uterus, dried and carefully positioned on the maternal abdomen to avoid occluding the umbilical cord blood flow. The endotracheal tube was unclamped and lung liquid was passively drained.

Each fetus was allocated to either ICC (n=6) or PBCC (n=11) groups. In ICC lambs, the umbilical cord was immediately clamped and cut before ventilation commenced. In PBCC lambs, ventilation commenced first, and umbilical cord clamping was delayed until after a target tidal volume (V_T) of 4 mL/kg was achieved (maximum delay of 10 min).

Ventilation

In both groups, ventilation commenced with a 30 s sustained inflation (35 cmH_2O, 21% O_2) followed by intermittent positive pressure ventilation (iPPV) in volume guarantee mode using a V_T of 4 mL/kg (Babylog 8000, Dräger, Lübeck, Germany), peak inspiratory pressure (PIP) limit of 35 cmH_2O and positive end-expiratory pressure of 5 cmH_2O. If the target V_T was not reached after 30 s, a second sustained inflation was performed followed by iPPV for a total of 120 min. After umbilical cord clamping, lambs were moved to a warming bed (CosyCot, Fisher and Paykel, Auckland, New Zealand), and sedated with alfaxalone (10 mg/kg/hour; Alfaxan, Jurox). Respiratory support was titrated to achieve $PaCO_2$ 60–80 mm Hg, PaO_2 >40 mm Hg and SaO_2 85%–88% to allow gentle ventilation. Lambs were euthanised (sodium pentobarbitone intravenous 100 mg/kg) after completing the 120 min ventilation protocol or earlier due to ethical end points (ie, treatment-resistant pneumothorax).

Outcome measures

Physiological data collection

Pulmonary and carotid arterial blood flows and pressures, cerebral tissue oxygen saturation ($SctO_2$), V_T and airway pressures were continuously recorded using LabChart (ADInstruments, New South Wales, Australia) and analysed offline in 20 s epochs. Arterial blood gas tensions were assessed every 5 min during the first 30 min of ventilation and every 10 min thereafter. PVR, alveolar-arterial difference in oxygen tension and cerebral oxygen delivery were calculated using equations listed in table 1.

Postmortem examination

Presence of a diaphragmatic defect and herniation of visceral organs were confirmed during post-mortem examination. Lungs were weighed and expressed as a ratio to the body weight (wet lung-to-body weight ratio).

Statistical analysis

Data are expressed as means±SEM, or medians (IQR) if the data were not normally distributed. The effect of group (ICC vs PBCC), time and interaction between these two effects were

Table 1 Calculations for derived measures

Measure	Calculation
Pulmonary vascular resistance	(PAP−LAP)/PBF
Dynamic lung compliance	V_T / (PIP−PEEP)
Arterial oxygen content (CaO_2)[35]	1.39×Hb×SaO_2/100+0.003×PaO_2
Cerebral oxygen delivery[35]	Carotid arterial blood flow×CaO_2

Hb, arterial haemoglobin concentration (g/L); LAP, left atrial pressure, assumed to equal 9 mm Hg based on previous studies[36]; PAP, pulmonary arterial pressure (mm Hg); PaO_2, partial pressure of arterial oxygen (mm Hg); PBF, pulmonary blood flow (mL/min); PEEP, positive end expiratory pressure (cmH_2O); PIP, peak inspiratory pressure (cmH_2O); SaO_2, arterial oxygen saturation; V_T, tidal volume (mL).

analysed using two-way repeated measures analysis of variance with post hoc analysis (Holm-Sidak) determining simple effects if an interaction was present (SigmaPlot V.13.0, Systat Software). Statistical significance was accepted when p value was <0.05.

RESULTS

Baseline fetal characteristics

Fetal body weight (4.1±0.2 vs 4.5±0.3 kg; p=0.21), lung-to-body weight ratio (0.017±0.002 vs 0.013±0.001; p=0.06) and percentage of male lambs (46% vs 50%; p=0.99) were not significantly different between PBCC and ICC groups. Before delivery, SaO_2 was greater in PBCC compared with ICC lambs (61%±3% vs 48%±4%; p=0.02), but both were within normal range. There was no significant difference in any other arterial blood gas or haemodynamic parameter between groups before delivery (table 2).

It was necessary to discontinue the experimental protocol early in one ICC lamb (at 80 min) and two PBCC lambs (at 70 and 100 min) due to developing treatment-resistant pneumothoraces.

Arterial blood gas status and cardiopulmonary physiology in the first 10 min after clamping

In the PBCC group, the median period between ventilation onset and cord clamping was 10 min (range: 3.5–10 min). PaO_2 and SaO_2 values increased following cord clamping in PBCC lambs, whereas values decreased in ICC lambs (figure 1A and B). By 5 min after cord clamping, PaO_2 (45±12 vs 11±1.9 mm Hg, p=0.006) and SaO_2 (70%±8.4% vs 17%±6.6%, p<0.001) were significantly higher in PBCC compared with ICC lambs. $PaCO_2$ increased and arterial pH decreased during the 10 min following cord clamping in both groups (figure 1C and D).

PBF was 19-fold greater in the ventilated PBCC lambs compared with the unventilated ICC lambs at the time of cord clamping (19.0±6.3 vs 1.0±0.5 mL/min/kg, p<0.001). PBF increased following cord clamping in both groups, however remained threefold greater in PBCC compared with ICC lambs at 10 min after cord clamping (51±7.8 vs 18±4.2 mL/min/kg, p=0.009; figure 1E). Pulmonary arterial pressure increased following cord clamping in both groups (figure 1F), and while was not significantly different, tended to be lower in PBCC compared with ICC lambs at 10 min after cord clamping (64±2.6 vs 77±5.1 mm Hg, p=0.08).

Carotid blood flow increased during the 10 min following umbilical cord clamping in ICC lambs (0 min 19±1.7 vs 10 min 33±5.0 mL/min/kg, p=0.002; figure 1G), however did not significantly change in PBCC lambs (0 min 18±2 vs 10 min 24±3.0 mL/min/kg, p>0.99). Carotid arterial pressure increased following cord clamping in both groups, but to a lesser degree in PBCC lambs (figure 1H). By 10 min following cord clamping, carotid arterial pressure was 20 mm Hg lower in PBCC compared with ICC lambs (61±3.8 vs 80±5.2 mm Hg, p=0.02). Heart rate increased following cord clamping in ICC lambs, but did not significantly change in PBCC lambs (figure 1I). At 10 min after

Table 2 Ventilation parameters, arterial blood gas status and cardiopulmonary physiology at fetal baseline and during the 120 min neonatal ventilation

	Fetal			60 min			120 min		
	ICC	PBCC	p value	ICC	PBCC	p value	ICC	PBCC	p value
DLC (mL/cmH_2O)	–	–	–	0.6±0.1	0.5±0.1	0.23	0.7±0.2	0.6±0.1	0.32
FiO_2	–	–	–	0.6±0.2	0.8±0.1	0.34	0.5±0.2	0.7±0.1	0.21
Hb (g/L)	119±7	134±4	0.07	123±8	137±4	0.09	131±10	143±5	0.10
Hct (%)	37±2	41±1	0.11	38±2	42±1	0.09	40±3	44±2	0.08
PaO_2 (mm Hg)	21±2	25±1	0.38	34±7	43±4	0.09	34±7	40±4	0.36
SaO_2 (%)	48±4	61±3	0.02*	58±14	76±7	0.11	59±14	73±10	0.30
$PaCO_2$ (mm Hg)	64±2	66±3	0.95	92±21	95±15	0.90	90±28	95±16	0.92
pH	7.26±0.02	7.24±0.01	0.79	7.10±0.10	7.12±0.06	0.85	7.11±0.15	7.09±0.07	0.85
Heart rate	147±11	144±6	0.95	184±11	168±7	0.23	180±21	171±8	0.38
PAP (mm Hg)	47±3	52±4	0.33	55±6	57±3	0.70	55±5	58±2	0.33
Mean PBF (mL/min/kg)	1±0.3	1.4±1	0.69	12±5	29±5	0.01*	8±2	23±4	0.01*
PBF/g lung weight	0.3±0.1	0.6±0.3	0.81	3.5±1.5	5.2±0.6	0.34	1.8±0.5	4.0±0.4	0.04*
End-diastolic PBF	−9±1	−12±2	0.45	4±4	12±3	0.02*	0.6±1	11±3	0.02*
PVR (mm Hg)/(L/min)	6.1±0.7	5.2±0.7	0.25	1.7±0.6	0.5±0.1	<0.01*	2.2±0.6	0.6±0.1	<0.01*
PVR×g lung weight	123±24	119±17	0.89	30±9	11±2	<0.01*	37±8	15±2	<0.01*
CAP (mm Hg)	46±2	53±3	0.24	56±7	60±3	0.49	56±6	60±3	0.26
CBF (mL/min/kg)	17±2	22±1	0.16	18±3	20±4	0.97	14±3	19±4	0.62
$SctO_2$ (%)	43±6	56±2	0.32	50±10	60±5	0.23	55±10	62±5	0.51

*p<0.05. Two-way repeated measures analysis of variance (group, time) with post hoc analysis (Holm-Sidak) determining differences between groups at each timepoint.
CAP, carotid arterial pressure; ICC, immediate cord clamping; CBF, carotid arterial blood flow; DLC, dynamic lung compliance; FiO_2, fraction of inspired oxygen; Hb, arterial haemoglobin concentration; Hct, haematocrit; $PaCO_2$, partial pressure of arterial carbon dioxide; PaO_2, partial pressure of arterial oxygen; PAP, pulmonary arterial pressure; PBCC, physiologically based cord clamping; PBF, pulmonary arterial blood flow; PVR, pulmonary vascular resistance; SaO_2, arterial oxygen saturation; $SctO_2$, cerebral tissue oxygen saturation.

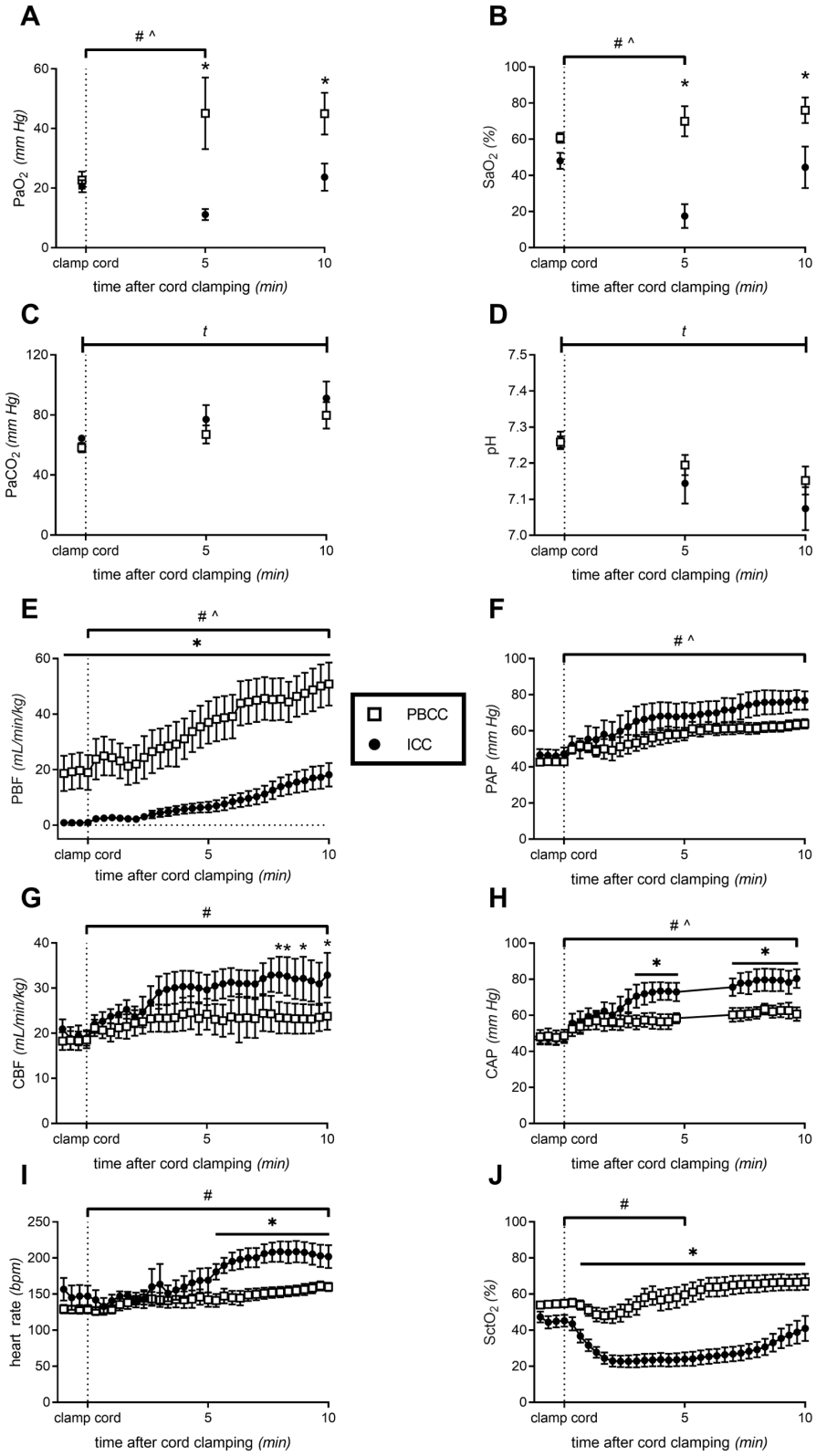

Figure 1 Arterial blood gas status and cardiopulmonary haemodynamics following umbilical cord clamping. (A) Partial pressure of arterial oxygen (PaO_2; mm Hg), (B) arterial oxygen saturation (SaO_2; %), (C) partial pressure of arterial carbon dioxide ($PaCO_2$; mm Hg), (D) arterial pH, (E) pulmonary arterial blood flow (PBF; mL/min/kg), (F) pulmonary arterial blood pressure (PAP; mm Hg), (G) carotid arterial blood flow (CBF; mL/min/kg), (H) carotid arterial blood pressure (CAP; mm Hg, no data obtained while catheter used to obtain arterial blood gas sample), (I) heart rate (bpm) and (J) cerebral tissue oxygen saturation ($SctO_2$; %) during the 10 min following umbilical cord clamping in immediate cord clamping (ICC; black circles, n=6) and physiologically based cord clamping (PBCC; white squares, n=11) groups. Two-way repeated measures analysis of variance (group, time) with Holm-Sidak's multiple comparisons test. *p<0.05 for effect of treatment (ICC vs PBCC) at each timepoint below line. ^{t} p<0.05 for main effect of time. #p<0.05 for effect of time between the two indicated timepoints in ICC lambs, ^p<0.05 for effect of time between the two indicated timepoints in PBCC lambs.

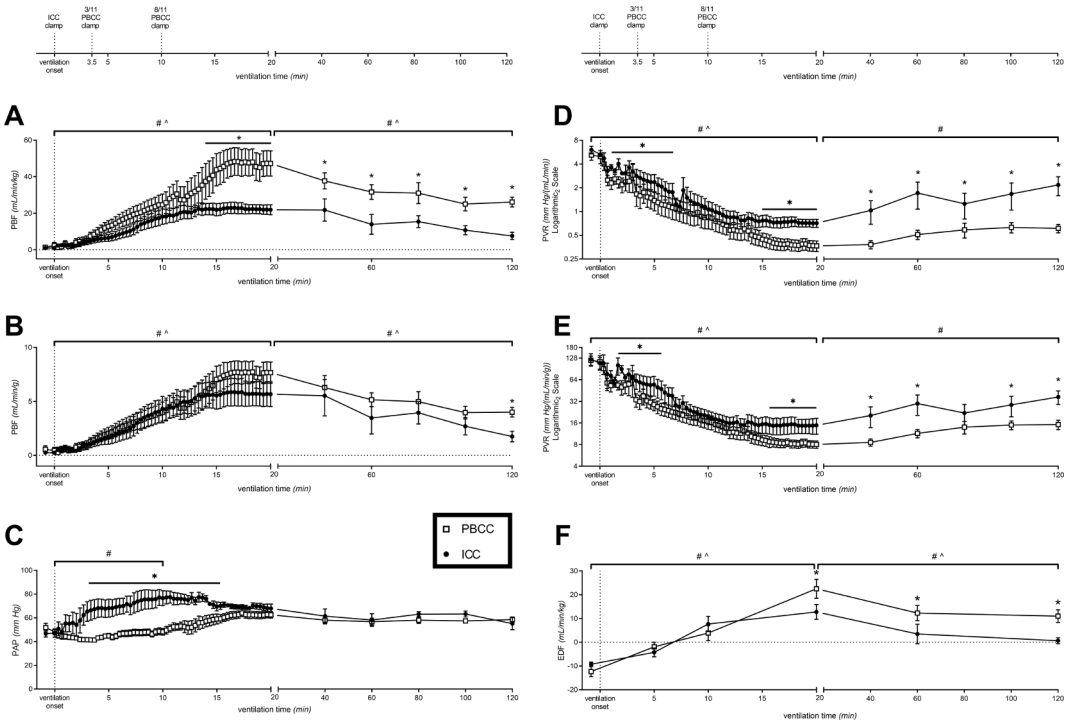

Figure 2 Pulmonary haemodynamics during the 120 min neonatal ventilation. The timing of umbilical cord clamping relative to ventilation onset is shown at the top of the figure, in immediate cord clamping (ICC; cord clamped immediately prior to ventilation onset) and physiologically based cord clamping (PBCC; cord clamped when target tidal volume of 4 mL/kg achieved, up to a maximum of 10 min after ventilation onset) groups. (A) Pulmonary arterial blood flow corrected for body weight (PBF; mL/min/kg), (B) PBF corrected for left lung weight (mL/min/g),(C) pulmonary arterial blood pressure (PAP; mm Hg), (D) pulmonary vascular resistance (PVR; mm Hg/(mL/min)) presented on a logarithmic (base 2) scale, (E) PVR corrected for left lung weight (mm Hg/(mL/min/g)) presented on a logarithmic (base 2) scale and (F) end-diastolic pulmonary blood flow (EDF; mL/min/kg) during the 120 min following ventilation onset in ICC (black circles, n=6) and PBCC (white squares, n=11) groups. Two-way repeated measures analysis of variance (group, time) with Holm-Sidak multiple comparisons test. *p<0.05 for effect of treatment (ICC vs PBCC) at each timepoint below line. #p<0.05 for effect of time between the two indicated timepoints in ICC lambs, ^p<0.05 for effect of time between the two indicated timepoints in PBCC lambs.

cord clamping, heart rate was slower in PBCC compared with ICC lambs (160±6 vs 202±16 bpm, p=0.01).

Cerebral oxygen delivery was greater in PBCC compared with ICC lambs at 5 min following cord clamping (2.6±0.5 vs 0.6±0.2 mL/min/kg, p=0.005), however by 10 min there was no significant difference between groups (3.2±0.5 vs 2.5±0.8 mL/min/kg, p=0.25). In ICC lambs, $SctO_2$ decreased to a minimum of 23% at 2 min following cord clamping, whereas in PBCC lambs there was no significant change in $SctO_2$ following cord clamping (figure 1J). During the first 10 min after cord clamping, mean $SctO_2$ was significantly greater in PBCC compared with ICC lambs (59%±4% vs 30%±5%, p<0.001).

Arterial blood gas status and cardiopulmonary physiology throughout the 120 min neonatal ventilation

The target V_T (4 mL/kg) was achieved in 6 PBCC lambs (55%; median 26 min, range 3–100 min after ventilation onset) and 3 ICC lambs (50%; median 30 min, range 3–80 min after ventilation onset). Dynamic lung compliance, FiO_2 and arterial blood gas parameters were not significantly different between PBCC and ICC lambs after 60 and 120 min of neonatal ventilation (table 2).

PBF (corrected for body weight) increased in both groups following ventilation onset, however to a greater degree in PBCC lambs (figure 2A). PBF was twofold greater in PBCC lambs compared with ICC lambs by 20 min after ventilation onset (47±7 vs 22±3 mL/min/kg, p=0.002) and remained threefold greater at 120 min (23±4 vs 8±2 mL/min/kg, p=0.01).

As PBF was measured in the left pulmonary artery, we also corrected PBF for left lung weight (figure 2B). Despite there being no significant difference in left lung weight between PBCC and ICC lambs (24±6 vs 20±9 g, p=0.28), after correcting for left lung weight, the difference in PBF between PBCC lambs and ICC lambs was no longer significant at 20 min (7.7±1.0 vs 5.7±1.1 mL/min/g, p=0.21), but remained significantly greater in PBCC lambs at 120 min (4.0±0.4 vs 1.8±0.5 mL/min/g, p=0.04).

Pulmonary arterial pressure increased immediately after ventilation onset in ICC lambs (figure 2C), and at 10 min after ventilation onset was significantly lower in PBCC lambs (48±3 vs 77±6 mm Hg, p<0.001). By 20 min after ventilation onset, pulmonary arterial pressure was similar between PBCC lambs and ICC lambs (63±3 vs 68±4, p=0.38).

PVR decreased in both groups following ventilation onset (figure 2D), however to a greater degree in PBCC lambs (at 20 min 0.4±0.1 vs 0.7±0.1 mm Hg/(mL/min), p=0.02). PVR subsequently increased in ICC lambs, yet remained low in PBCC lambs. By 120 min after ventilation onset, PVR was threefold lower in PBCC lambs compared with ICC lambs (0.6±0.1 vs 2.2±0.6 mm Hg/(mL/min), p<0.001). After correcting for left lung weight, PVR remained significantly lower in PBCC lambs

compared with ICC lambs at 120 min (15±2 vs 35±10 mm Hg/(mL/min/g), p<0.001; figure 2E).

End-diastolic PBF, which inversely correlates with PVR, was retrograde (assigned a negative value) in both PBCC and ICC groups before ventilation onset (−12±2 vs −9±1 mL/min/kg, p=0.45). At 60 min, end-diastolic PBF was positive in both groups but greater in PBCC compared with ICC lambs (12±3 vs 4±4 mL/min/kg, p=0.02). By 120 min, end-diastolic PBF remained positive in PBCC lambs but approached 0 in ICC lambs (11±3 vs 0.6±1 mL/min/kg, p=0.02; figure 2F).

DISCUSSION

During the transition from fetal to neonatal life, major physiological changes must occur for the infant to survive, which is particularly challenging for infants with underdeveloped lungs. In this study, we demonstrate that, in DH lambs, establishing lung aeration prior to umbilical cord clamping prevents severe transient hypoxia and facilitates a greater increase in PBF during the first 120 min of neonatal life, compared with ICC. These findings suggest that a physiological approach to umbilical cord clamping may improve the neonatal transition for infants with a CDH.

When umbilical cord clamping occurs before lung aeration, neonates are separated from the fetal oxygen source (placenta) and progressively become more hypoxaemic until adequate pulmonary gas exchange is established. Most infants avoid severe hypoxia at birth, because they rapidly aerate their lungs, which triggers a large increase in PBF. This facilitates a rapid switch to pulmonary gas exchange, and ensures maintenance of cardiac output.[10] All of the DH lambs had severely hypoplastic lungs that were difficult to ventilate, and as a result the increase in PBF was delayed and remained low for the first few minutes after ventilation onset. Thus, during this time, as ICC lambs had lost the placenta as an oxygen source, they gradually became very hypoxaemic with markedly reduced cerebral oxygen delivery. On the other hand, as placental gas exchange continued while lung aeration was gradually established in PBCC lambs, oxygenation levels gradually increased over the same time period and remained stable after cord clamping (figure 1J). The transient severe cerebral hypoxia we observed in ICC lambs is concerning, given the well-described neuropathological impact of perinatal asphyxia.[15 16] Neurodevelopmental dysfunction is increasingly being recognised in the long-term follow-up of infants with CDH, and while there are many potential causative factors, the role of transient neonatal hypoxia related to ICC may warrant further study.[17]

The most striking and unexpected finding of this study was the difference in PBF between ICC and PBCC lambs throughout the ventilation period. This difference was statistically significant from 15 to 120 min after ventilation onset. However, during the first 15 min, ICC lambs required driving pressures (pulmonary arterial pressures) that were 20–25 mm Hg greater than PBCC lambs to achieve similar or slightly reduced PBFs. This finding indicates that PBCC had an immediate beneficial effect on PVR that persisted well into the newborn period. In our previous study, in which all DH lambs had PBCC, we found that the increase in PBF per gram of lung tissue at birth was similar to control lambs, suggesting that the overall reduction in PBF was simply due to a reduction in lung size.[14] As this was not consistent with a previous study in DH lambs that received ICC, we considered that this unexpectedly high PBF in DH lambs might be due to PBCC, which we have now confirmed.

The mechanisms underlying the higher PVR in ICC DH lambs are unknown, but cannot be due to a reduced cross-sectional area of the pulmonary vascular bed as the reduction in lung sizes were similar. It has recently been shown that lung aeration causes a global reduction in PVR that, at least initially, is independent of oxygen and is thought to be mediated by a neural reflex.[18] As this reflex can be activated by partial lung aeration, we would expect a similar response (per gram of tissue) in all lambs. However, after ventilation onset, PVR was different in PBCC and ICC lambs, although PBF was not different due to the higher driving pressure in ICC lambs. Thus, ICC in CDH lambs may activate specific vasoconstrictive responses within the pulmonary vascular bed. Initially, this vasoconstrictive response may be caused by hypoxia, as pulmonary vessels in infants with CDH are thought to be highly responsive to vasoconstrictive stimuli such as hypoxia.[19–21] As such, the difference in PVR would be expected to gradually disappear as oxygenation levels between the two groups equalise, which is consistent with the finding of similar PVRs at 10 min after ventilation onset. However, after 15 min it diverges again, despite oxygenation levels being similar. This secondary phase of higher PVRs may reflect the gradual onset of persistent pulmonary hypertension of the newborn (PPHN) and while the mechanisms are unknown, it is very interesting that it occurred in most ICC lambs but in no PBCC lambs.

Despite improved pulmonary perfusion, PBCC lambs had persistent hypoxia and hypercapnia, similar to ICC lambs, suggesting that gas exchange remained impaired by the underlying lung hypoplasia. Indeed, in infants with CDH with relatively mild lung hypoplasia, resuscitation with an intact umbilical cord for ~7 min increased pH and reduced pCO_2 levels in the first 30 min after birth.[22] While survival rates and length of hospital stay were similar, the study only assessed feasibility in infants with relatively mild lung hypoplasia. In more severe cases, a smoother cardiopulmonary transition may avoid reactive vasoconstriction to the severe asphyxia caused by ICC and reduce the incidence and/or severity of PPHN.

The increase in carotid arterial pressure following ICC results from a sudden increase in systemic vascular resistance caused by removal of the low resistance placental circulation. However, if PVR decreases before cord clamping, the pulmonary circulation becomes an alternative low resistance pathway for blood flow due to the onset of left-to-right flow through the ductus arteriosus.[5 8] As a result, when cord clamping follows lung aeration (PBCC), the rapid increase in arterial pressure caused by cord clamping is greatly reduced. As PVR remained relatively high in ICC, and to a lesser extent, in PBCC lambs, arterial pressures increased in both groups in response to cord clamping, although to a greater degree in ICC lambs. ICC (but not PBCC) lambs also experienced transient tachycardia and hypertension at approximately 2–3 min following cord clamping. These findings likely result from a sympathetic response to low oxygen tensions immediately following cord clamping that was largely absent in PBCC lambs.[23 24] PBCC lambs were able to maintain cerebral oxygen delivery without tachycardia or a pressure-driven increase in carotid blood flow.

End-diastolic PBF, which reflects left-to-right shunting through the ductus arteriosus, was present in PBCC lambs, but largely absent in ICC lambs by the end of the 120 min neonatal ventilation period. The loss of left-to-right shunting and the re-emergence of right-to-left shunting (as occurs prenatally) during diastole, suggests that PVR was progressively increasing back to fetal levels. In contrast, in PBCC lambs end-diastolic PBF remained positive, suggesting that the higher PBF in PBCC

lambs partially reflects a continuing contribution of flow from the systemic circulation, via the ductus arteriosus, at 120 min.

Although our lambs were delivered via caesarean section in a controlled environment, the feasibility of replicating this approach clinically in the delivery room is slowly being realised.[25 26] Several studies have now assessed the feasibility of assessing, resuscitating and ventilating infants at the maternal bedside in preterm[27] and very preterm[28 29] infants, as well as infants with CDH.[22 30] However, other confounding factors such as labour and oxytocin administration and their potential effects on umbilical blood flow and cardiopulmonary haemodynamics have not been assessed.[31] On the other hand, it is possible that our findings underestimate the beneficial effects of PBCC in clinical practice, because we were able to commence ventilation within 10 s of cord clamping in ICC lambs. This is because the lambs had already been intubated (with a clamped endotracheal tube) during fetal instrumentation. Clinically, respiratory support may be delayed for up to 60 s, so the hypoxic period between cord clamping and lung aeration would presumably be much longer than demonstrated in our lamb study.[32]

Our study adds to the growing consensus that a physiological approach to umbilical cord clamping, which includes resuscitation on the cord if required, is preferable to immediate, or even time-based delayed cord clamping.[5 11 22 25–29 33] The majority of our PBCC lambs did not achieve their target V_T, so umbilical cord clamping was delayed until our arbitrary maximum of 10 min. If cord clamping had occurred at 3 min after ventilation onset, merely to optimise placental transfusion, many of the benefits of PBCC would not have been realised, as both V_T and PBF remained low (figure 2B).[34] As our PBCC lambs did not experience transient hypoxia despite not achieving their target V_T, this target may have been an overly conservative target to guide cord clamping. It is possible that similar benefits could have been achieved at an earlier timepoint and so alternative targets, such as exhaled CO_2 may prove more useful for guiding cord clamping time.[27]

In conclusion, we found that establishing lung aeration prior to umbilical cord clamping avoids transient, severe hypoxia at birth and enables increased PBF in lambs with a DH for at least the first 120 min after birth. Our findings suggest that a physiological approach to umbilical cord clamping will enable a better transition to newborn life for infants with a CDH.

Correction notice This paper has been amended since it was published Online First. The statement 'KJC and PLJDeK share joint senior authorship' was erroneously omitted during the production process and this has now been reinstated.

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Contributors All authors included in this paper fulfill the criteria of authorship, specifically: PLJD, AJK, RJH, KJC, MT and SBH designed the experiments. AJK, KJC, MT, KAR, BJA, EVM, PLJD and SBH were essential for undertaking the experiments. AJK, PLJD, KJC and SBH were responsible for data analysis. AJK wrote the first draft of the manuscript; PLJD and KJC provided initial critical feedback. All authors contributed by modifying and editing the manuscript and all approved final version.

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3.2 Effects of diaphragmatic hernia on the cardiopulmonary transition in newborn lambs that undergo immediate cord clamping

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Abstract

Background

Infants with congenital diaphragmatic hernia (CDH) have small, stiff lungs that do not rapidly aerate at birth. In newborn lambs with DH, compared to control lambs, we hypothesised that the period between umbilical cord clamping and achieving lung aeration would be associated with transient severe hypoxaemia and subsequent disturbances to cardiopulmonary physiology.

Methods

A retrospective analysis was performed using two experimental groups previously reported by our laboratory. At ≈138 days of gestational age, 6 lambs with surgically induced left-sided diaphragmatic hernia (≈d80) and 6 control lambs were delivered via caesarean section. After immediate umbilical cord clamping, lambs were ventilated for 120 min with real-time physiological monitoring and intermittent arterial blood-gas measurement.

Results

At 5 min after birth, DH lambs had lower PBF (7 ± 2 vs. 62 ± 9 mL/min/kg, $p<0.001$), arterial oxygen saturation (17 ± 7 vs. 84 ± 8 %, $p<0.001$) and cerebral tissue oxygen saturation (24 ± 4 vs. 59 ± 12 %, $p<0.001$) than control lambs. At 10 min after birth, DH lambs had greater HR (202 ± 16 vs. 145 ± 5 bpm, $p<0.001$), carotid blood flow (33 ± 5 vs. 15 ± 2 mL/min/kg, $p=0.003$) and carotid arterial pressure (78 ± 4 vs. 53 ± 2 mmHg, $p<0.001$) than control lambs. At 120 min after birth, DH lambs had 2-fold lower PBF adjusted for lung weight (2 ± 1 vs. 4 ± 1 mL/min/g, $p=0.03$).

Conclusions

Lambs with DH have hypoplastic lungs that are slow to achieve adequate aeration and perfusion after birth. When placental support is removed by immediate cord clamping, this leads to severe, transient hypoxemia and cardiovascular compromise during the neonatal transition.

Introduction

Congenital diaphragmatic hernia (CDH) affects 1 in 3000 live births and describes incomplete closure of the diaphragm during embryogenesis.[1] The subsequent intrathoracic displacement of abdominal organs disrupts lung development, resulting in smaller lungs with abnormal airway and vascular structure (lung hypoplasia).[2]

Lung hypoplasia does not adversely affect the developing fetus *in utero*, as the placenta provides oxygen and eliminates carbon dioxide. In contrast, when placental support is lost at birth, the incompliant, hypoplastic lungs are frequently unable to support neonatal respiratory needs.[3] The neonatal course of CDH is also complicated by pulmonary hypertension, caused by reduced vascular cross-sectional area and raised vascular tone due to increased vascular muscularisation.[2, 4] The smaller and structurally abnormal pulmonary vascular bed is unable to accept complete right ventricular output and return adequate flows of oxygenated blood to the left atrium. This reduced pulmonary blood flow (PBF) further exacerbates the respiratory insufficiency and forces the infant to rely on a persistent atrial shunt (foramen ovale) to maintain cardiac output during the neonatal transition at birth.

We have previously described respiratory function and cardiopulmonary physiology during the neonatal transition in lambs with and without a diaphragmatic hernia (DH).[5] However, in that study we performed physiologically based cord clamping (PBCC; clamping the umbilical cord after achieving adequate lung aeration). The timing of umbilical cord clamping has a significant impact on cardiopulmonary haemodynamics during the neonatal transition, and current guidelines recommend CDH infants are intubated immediately after birth, which in practice usually translates to immediate cord clamping.[6, 7] In this study, we aimed to describe neonatal cardiopulmonary physiology in lambs with DH that underwent immediate cord clamping. We hypothesised that in addition to respiratory insufficiency, lambs with DH would experience severe hypoxaemia during the period between clamping the umbilical cord and achieving adequate lung aeration, and that this would adversely affect cardiopulmonary haemodynamics during the neonatal transition.

Methods

Ethical Approval

All procedures in animals were performed in accordance with the National Health and Medical Research Council of Australia guidelines for care and use of experimental animals, were approved by the relevant animal ethics committee at Monash University, and comply with the ARRIVE guidelines (Animal Research: Reporting *In Vivo* Experiments).[8]

To reduce animal use, the research question was answered by comparing two animal groups that have been previously reported in separate publications.[9, 10] Both groups were taken from the same cohort of Merino X Border-Leicester ewes, housed in the same animal facility in individual pens with a 12:12 hr light:dark cycle, fed twice daily with water *ad libitum*, and cared for by the same research staff. Lambs allocated to the DH group (n=8) are previously reported by Kashyap *et al.*[9] and lambs allocated to the control group (n=6) are previously reported by McGillick & Davies *et al.*[10] The raw data obtained from physiological recordings was reanalysed in the same manner for both groups, by the same investigator (AK).

Diaphragmatic hernia creation

In lambs allocated to DH, a DH was surgically created at ≈80 days gestational age (dGA; term≈147 dGA) as previously described.[5] Briefly, general anaesthesia of the ewe was induced using intravenous sodium thiopental (Pentothal; Jurox, New Zealand) and maintained with inhaled isoflurane (≈2% in room air; Isoflow, Abbot). Following maternal midline laparotomy and hysterotomy, the fetal chest was exteriorised and a small incision was made through the left ninth intercostal space. The fetal diaphragm was incised, and the stomach and bowels were pulled into the thoracic cavity. Ewes received three days of post-operative analgesia (transdermal fentanyl patch, 75 μg/hr; Janssen Cilag) and were monitored daily until delivery.

Delivery and ventilation

Fetal instrumentation

At ≈138 dGA, ewes were anaesthetised (as above) and a caesarean section was performed. Fetal lambs in both groups were instrumented prior to delivery as previously described.[5] Briefly, exteriorised fetal lambs were intubated with a clamped endotracheal tube (size 4.0). Polyvinyl catheters were positioned in the left carotid artery to allow continuous carotid arterial pressure monitoring, and in the left jugular vein to allow neonatal intravenous sedation. In the DH lambs only, a catheter was also positioned in the main pulmonary artery to allow continuous pulmonary arterial pressure monitoring. Ultrasonic flow probes (Transonic Systems, Ithaca, NY, USA) were placed around the right carotid and left pulmonary artery, to continuously record blood flow. Pulse-oximeter (Masimo, Radical 7, CA, USA), near-infrared spectroscopy (NIRS; Casmed Foresight, CAS Medical Systems Inc., Branford, CT, USA) and temperature probe were placed on right forelimb, forehead and rectum respectively. The stomach was drained via an orogastric tube. After instrumentation, lambs were delivered from the uterus, dried with a warm towel and carefully positioned on the maternal abdomen adjacent to a hot water bottle. The endotracheal tube was unclamped, lung liquid was passively drained and the umbilical cord was immediately clamped and cut. Ventilation commenced on the maternal abdomen, then the lambs were transferred to a warming bed (CosyCot, Fisher and Paykel, Auckland, New Zealand) and sedated with alfaxalone (10 mg/kg/hr; Alfaxan, Jurox).

Ventilation

In both groups, ventilation commenced with a 30 sec sustained inflation (35 cmH_2O, 21% O_2) followed by intermittent positive pressure ventilation (iPPV) in volume guarantee mode using a tidal volume (V_T) of 4 mL/kg for DH lambs and 7 mL/kg for control lambs (Babylog 8000, Dräger, Lübeck, Germany), peak inspiratory pressure (PIP) limit of 35 cmH_2O, and positive end-expiratory pressure (PEEP) of 5 cmH_2O. If the target V_T was not reached after 30 sec, a second sustained inflation was performed followed by iPPV for a total of 120 min. A gentle ventilation strategy was employed for DH lambs, with respiratory support titrated to achieve $PaCO_2$ 60-80 mmHg, PaO_2 >40

mmHg and arterial oxygen saturation (SaO_2) 85-88%. Control lambs were supported to achieve $PaCO_2$ 40-50 mmHg and SaO_2 90-95%. Lambs were euthanised (sodium pentobarbitone i.v. 100 mg/kg) after completing the 2 hr ventilation protocol or earlier due to ethical endpoints (i.e. severe acidosis or pneumothorax).

Physiological data collection

PBF, carotid blood flow, carotid arterial pressure, cerebral tissue oxygen saturation ($SctO_2$), V_T and airway pressures were continuously recorded using LabChart (ADInstruments, NSW, Australia) and analysed offline in 20 sec epochs for the first 20 min after birth, and every 5 min thereafter. In DH lambs only, pulmonary arterial pressure was also recorded. Arterial blood gas tensions were assessed every 5 min during the first 30 min of ventilation and every 10 min thereafter. Pulmonary vascular resistance (PVR), alveolar-arterial difference in oxygen tension ($AaDO_2$) and cerebral oxygen delivery (DO_2) were calculated using equations listed in Table 1.

Table 1 – Calculations for derived measures

Measure	Calculation
Pulmonary vascular resistance	(PAP – LAP) / PBF
Dynamic lung compliance	V_T / (PIP – PEEP)
Arterial oxygen content (CaO_2)[11]	1.39 x Hb x SaO_2/100 + 0.003 x PaO_2
Cerebral oxygen delivery[11]	carotid arterial blood flow x CaO_2

PAP = pulmonary arterial pressure (mmHg); LAP = left atrial pressure, assumed to equal 9 mmHg based on previous studies[10]; PBF = pulmonary blood flow (mL/min); V_T = tidal volume (mL); PIP = peak inspiratory pressure (cmH2O); PEEP = positive end expiratory pressure (cmH2O); PaO2 = partial pressure of arterial oxygen (mmHg); Hb = arterial haemoglobin concentration (g/dL); SaO2 = arterial oxygen saturation.

Postmortem examination

In DH lambs, presence of a diaphragmatic defect and herniation of visceral organs were confirmed during post-mortem examination. Lungs were weighed and expressed as a ratio to the body weight (wet lung-to-body weight ratio; LBWR).

Statistical analysis

Data are expressed as means ± standard error of the mean, or medians (interquartile range) if the data were not normally distributed. Before retrospectively comparing DH and control lambs, we determined that a minimum of five animals per group would provide power of ≥90% (α=0.05) to detect a 20% difference in arterial oxygen tension during the first 20 min after birth, based on our previous experiment.[5, 13] We compared DH and control lambs over time using a mixed model fitted by the restricted maximum likelihood (REML) method with a compound symmetry covariance matrix (GraphPad Prism 8). Pairwise comparisons were made at specific timepoints defined *a priori* (immediately before birth (fetal) and 5, 10, 20, 60 & 120 min after birth) and adjusted for multiple comparisons by controlling the experiment-wise maximum false discovery rate (FDR) to 0.05 using the two-stage linear step-up procedure described by Benjamini, Kreiger and Yekutieli.[14, 15] Statistical significance was accepted when the FDR-adjusted p <0.05.

Results

Morbidity and mortality

Twelve (of 14) fetuses survived to delivery; six controls (100%) and six DH lambs (75%). All control lambs survived the 2 hr ventilation period, however for one DH lamb it was necessary to discontinue the experimental protocol early due to developing treatment-resistant pneumothorax (at 80 min). At post-mortem examination a diaphragmatic defect with substantial gastrointestinal herniation into the thorax was confirmed in all DH lambs, and small pneumothoraces that were not clinically apparent were discovered in three DH lambs.

Lung size and ventilation

The wet LBWR was lower in DH lambs than controls (0.013 ± 0.001 vs 0.032 ± 0.002; $p<0.001$).

After the initial 30 sec sustained inflation, one DH lamb and all control lambs achieved their target V_T within the next 30 sec of iPPV. A second sustained inflation was performed for animals that did not achieve their target V_T; after this, one additional DH lamb achieved target V_T. During the neonatal ventilation, V_T increased more gradually in DH lambs than in control lambs, reaching a plateau in DH lambs within 60 min and control lambs within 10 min (Figure 1a). At 60 min after birth, V_T was 2-fold lower in DH lambs compared to controls (3.6 ± 0.6 vs. 7.1 ± 0.8 mL/kg, $p<0.001$). Peak inspiratory pressure (PIP) was set at 35 cmH_2O at birth and was automatically decreased by the ventilator when a lower pressure could achieve the target volume (Figure 1b). By the end of the 120 min ventilation period, the required PIP was 8 mmHg greater in DH lambs than control lambs (31 ± 5 vs. 23 ± 2 cmH_2O, $p=0.005$).

Dynamic lung compliance gradually increased throughout the neonatal ventilation in both groups, however was 2-fold lower in DH lambs at both 10 min (0.36 ± 0.07 vs. 0.89 ± 0.09, $p<0.001$) and 120 min after birth (0.70 ± 0.17 vs. 1.37 ± 0.17, $p<0.001$; Figure 1c).

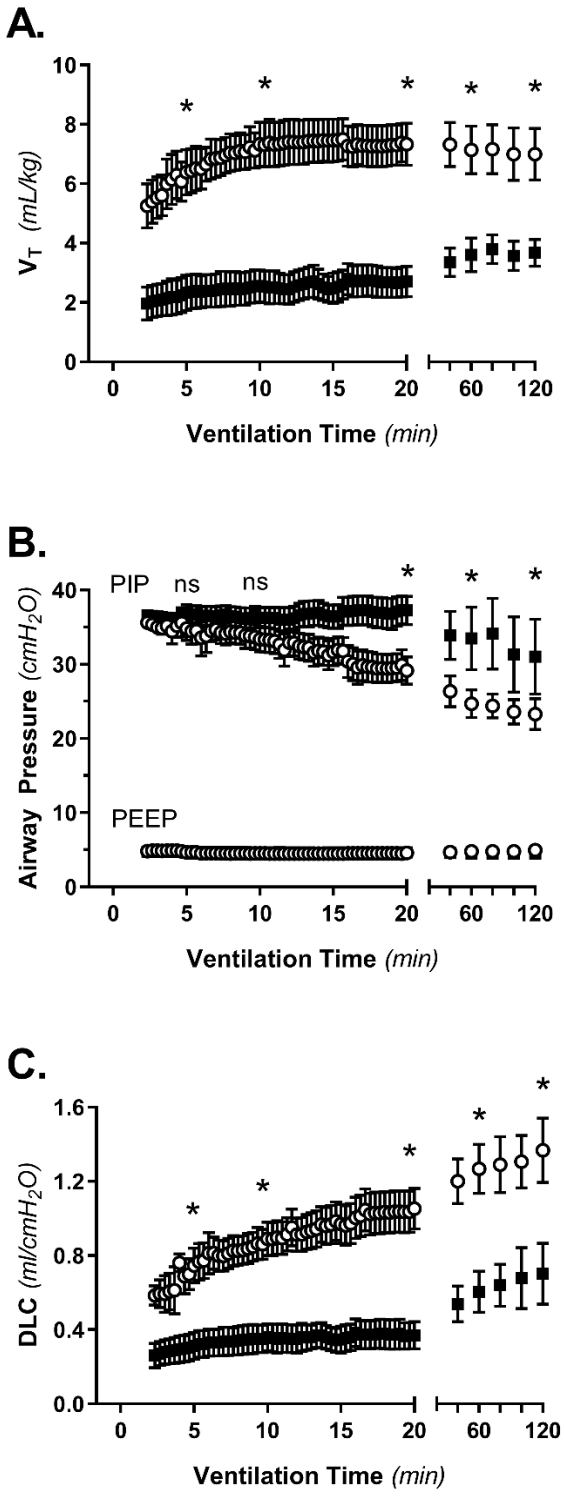

Figure 1

(A) Tidal volume (V_T; mL/kg), (B) peak inspiratory pressure (PIP; cmH_2O) and positive end-expiratory pressure (PEEP; cmH_2O) and (C) dynamic lung compliance (mL/cmH_2O) during the 120 min neonatal ventilation in lambs with a diaphragmatic hernia (DH; black squares, n=6) and control lambs (white circles, n=6). Linear mixed model fit using restricted maximum likelihood. * $p<0.05$ for an *a priori* pairwise comparison between DH and control lamb, after adjustment to control the experiment-wise maximum false discovery rate to 0.05.

Pulmonary perfusion

PBF increased after birth more gradually in DH lambs than control lambs (1.1 vs. 10.5 mL/min/kg per min, p=0.006) and to a lesser degree (*at 20 min* 22 ± 2.7 vs. 73 ± 7.4 mL/min/kg, p<0.001). Indeed, in DH lambs PBF remained ~0 mL/min/kg for the first 2 min after birth (Figure 2a). At 120 min after birth, PBF was 5-fold lower in DH lambs compared to control lambs (9 ± 2 vs. 47 ± 11 mL/min/kg, p<0.001).

PBF corrected for left lung weight was lower in DH lambs compared to control lambs at 5 min (2 ± 1 vs. 7 ± 1 mL/min/g, p<0.001) and 10 min after birth (4 ± 1 vs. 8 ± 1 mL/min/kg, p=0.01), but there was no significant difference between groups at 20 and 60 min after birth (Figure 2b). However, by 120 min after birth PBF corrected for left lung weight was again lower in DH lambs compared to control lambs (2 ± 1 vs. 4 ± 1 mL/min/g, p=0.03).

End-diastolic PBF (EDF), which inversely correlates with pulmonary vascular resistance, was retrograde (assigned a negative value) in both DH and control lambs before birth (Figure 2c). By 5 min after birth, EDF remained retrograde in DH lambs but was positive in control lambs (-4.3 ± 1.9 vs. 39 ± 6.6 mL/min/kg, p<0.001). By 10 min after birth, EDF was positive in both groups but lower in DH lambs (7.7 ± 3.2 vs. 43 ± 6.0 mL/min/kg, p<0.001). By 120 min after birth, EDF approached 0 in DH lambs but remained positive in control lambs (0.6 ± 1.2 vs. 22 ± 7.7 mL/min/kg, p=0.001).

Pulmonary arterial pressure was measured in DH, but not in control lambs. In DH lambs, pulmonary arterial pressure increased between birth and 10 min after birth (47 ± 3 vs. 77 ± 6 mmHg, p<0.0001), then gradually decreased between 10 min and 120 min after birth (77 ± 6 vs. 59 ± 2 mmHg, p=0.0034; Figure 2d). In DH lambs, pulmonary vascular resistance decreased between birth and 20 min after birth (6.5 ± 0.9 vs. 0.7 ± 0.1 mmHg/(mL/min), p<0.0001), then gradually increased again between 20 min and 120 min after birth (0.7 ± 0.1 vs. 2.2 ± 0.6, p=0.031; Figure 2e).

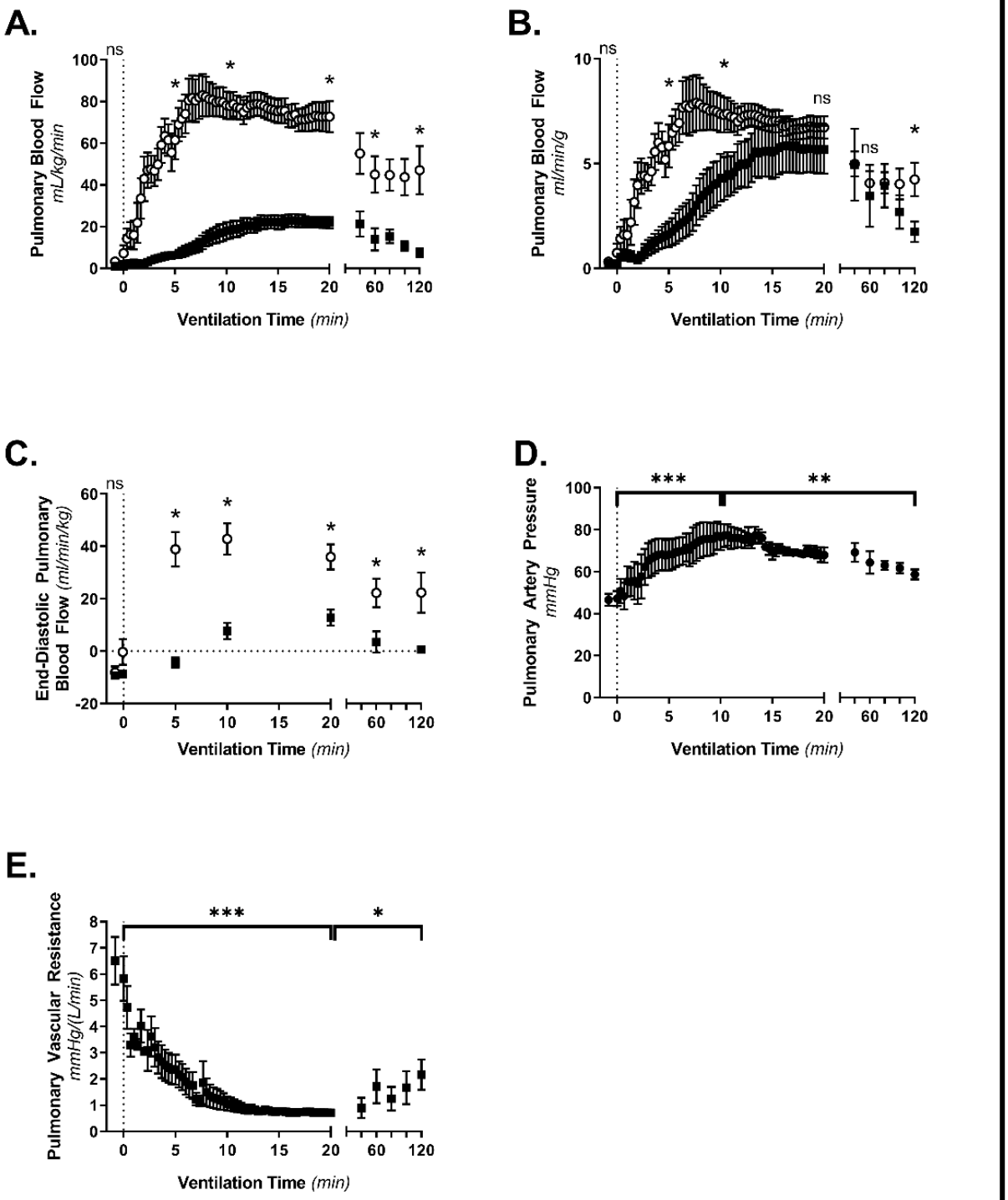

Figure 2

(A) Pulmonary arterial blood flow adjusted for body weight (PBF; mL/min/kg), (B) PBF adjusted for lung weight (mL/min/g), (C) end-diastolic pulmonary blood flow (EDF; mL/min/kg), (D) pulmonary arterial pressure (PAP; mmHg) and (E) pulmonary vascular resistance (PVR; mmHg/(mL/min)) during the 120 min neonatal ventilation in lambs with a diaphragmatic hernia (DH; black squares, n=6) and control lambs (white circles, n=6). Linear mixed model fit using restricted maximum likelihood. * $p<0.05$ for an *a priori* pairwise comparison between DH and control lamb, after adjustment to control the experiment-wise maximum false discovery rate to 0.05.

Gas exchange

$PaCO_2$ was greater in DH lambs than control lambs before birth (64 ± 2 vs. 49 ± 2 mmHg, p=0.4; Figure 3a), however arterial pH was not significantly different (7.26 ± 0.02 vs. 7.25 ± 0.02, p=0.89; Figure 3b). By 10 min after birth, $PaCO_2$ was 60 mmHg greater in DH lambs than control lambs (92 ± 11 vs. 34 ± 6 mmHg, p=0.003) and arterial pH was lower (7.07 ± 0.06 vs. 7.29 ± 0.05, p=0.03). These differences between DH lambs and control lambs persisted until 120 min after birth for both $PaCO_2$ (90 ± 28 vs. 42 ± 6 mmHg, p=0.005) and arterial pH (7.10 ± 0.15 vs. 7.37 ± 0.05, p=0.008).

PaO_2 (Figure 3c) and SaO_2 (Figure 3d) were similar between DH lambs and control lambs before birth (PaO_2: 21 ± 2 vs. 19 ± 1 mmHg, p=0.89 and SaO_2: 48 ± 4 vs. 48 ± 4 %, p=0.99), however both were severely lower in DH lambs at 5 min (PaO_2: 11 ± 2 vs. 49 ± 8 mmHg, p=0.004 and SaO_2: 17 ± 7 vs. 84 ± 8 %, p<0.001) and 10 min after birth (PaO_2: 24 ± 5 vs. 61 ± 12 mmHg, p=0.004 and SaO_2: 44 ± 12 vs. 93 ± 4 %, p<0.001). By 20 min after birth, both PaO_2 and SaO_2 were not significantly different between DH lambs and control lambs (PaO_2: 76 ± 26 vs. 57 ± 10 mmHg, p=0.15 and SaO_2: 81 ± 8 vs. 93 ± 3 %, p=0.3). SaO_2 was again lower in DH lambs compared to control lambs at 60 min (58 ± 14 vs. 92 ± 3 %, p=0.005) and 120 min after birth (65 ± 12 vs. 91 ± 4 %, p=0.02). In contrast, PaO_2 remained not significantly different between DH and control lambs at 120 min after birth (38 ± 7 vs. 48 ± 5 mmHg, p=0.42). However, to maintain these PaO_2, DH lambs required greater FiO_2 than control lambs from 10 min (76 ± 11 vs. 22 ± 2 %, p=0.0005) to 120 min after birth (53 ± 18 vs. 26 ± 5 %, p=0.03). Hence, the $AaDO_2$ was greater in DH lambs compared to control lambs from 10 min (405 ± 74 vs. 65 ± 16 mmHg, p=0.001) to 120 min after birth (280 ± 130 vs. 49 ± 7 mmHg, p=0.03; Figure 3e).

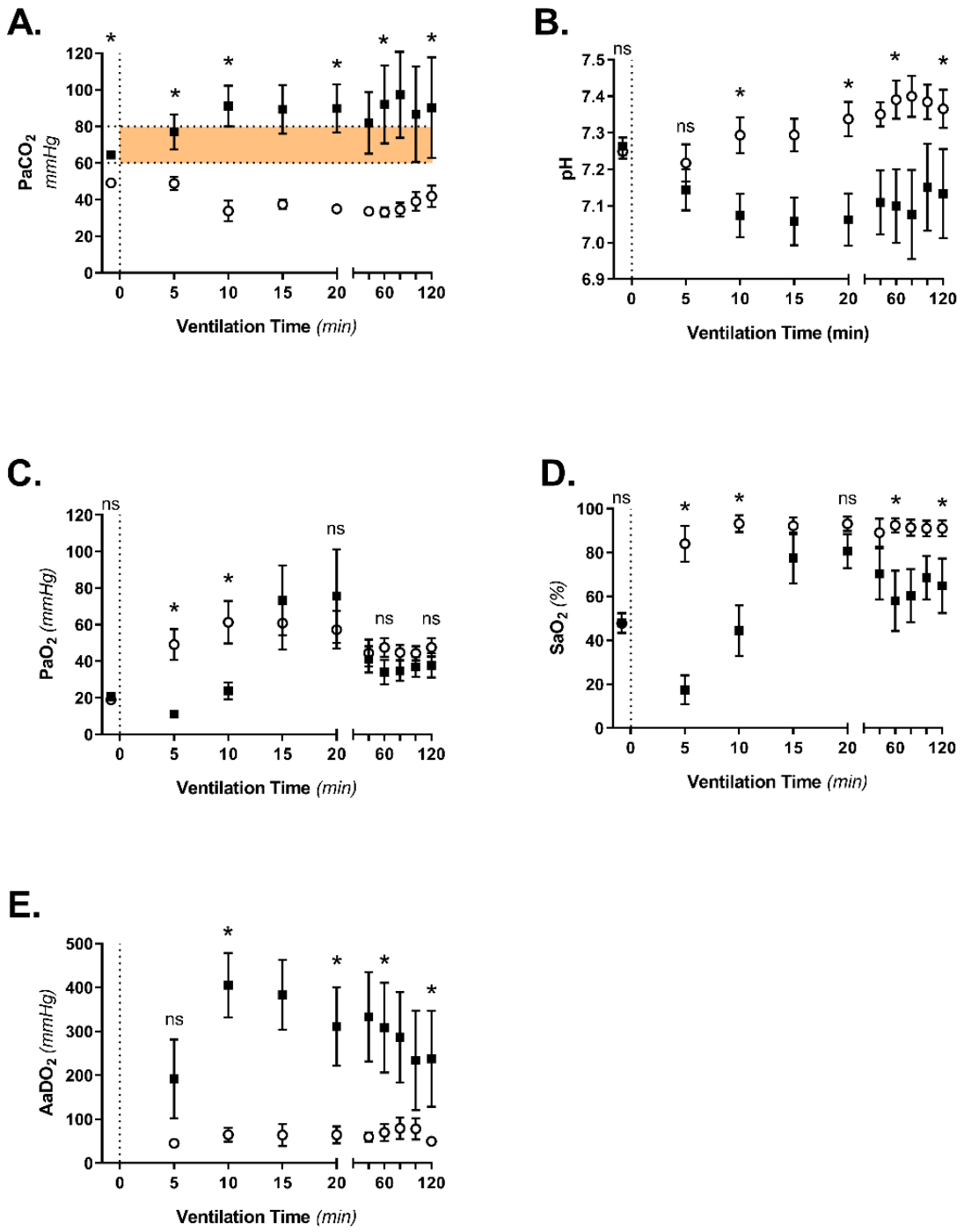

Figure 3

(A) Partial pressure of arterial carbon dioxide ($PaCO_2$; mmHg, with a target range of 60 – 80 mmHg), (B) arterial pH, (C) Partial pressure of arterial oxygen ($PaCO_2$; mmHg), (D) arterial oxygen saturation (SaO_2; %) and (E) alveolar-arterial difference in oxygen tension ($AaDO_2$; mmHg) during the 120 min neonatal ventilation in lambs with a diaphragmatic hernia (DH; black squares, n=6) and control lambs (white circles, n=6). Linear mixed model fit using restricted maximum likelihood. * $p<0.05$ for an *a priori* pairwise comparison between DH and control lamb, after adjustment to control the experiment-wise maximum false discovery rate to 0.05.

Cerebral perfusion

Heart rate remained stable after birth in control lambs (Figure 4a), but varied in DH lambs throughout the 2 hr ventilation period: at 10 min after birth heart rate was greater in DH lambs than control lambs (202 ± 16 vs. 145 ± 5 bpm, $p<0.001$), at 20 min groups were not significantly different (169 ± 12 vs. 146 ± 6 bpm, $p=0.14$), and by 60 min heart rate was again greater in DH lambs (184 ± 11 vs. 149 ± 10, $p=0.02$).

Carotid arterial pressure increased after birth in both groups (Figure 4b), however to a greater degree in DH lambs than control lambs (*at 10 min* 78 ± 4 vs. 53 ± 2 mmHg, $p<0.001$). By 20 min after birth, carotid arterial pressure was not significantly different between DH lambs and control lambs (69 ± 3 vs. 58 ± 4 mmHg, $p=0.1$).

Carotid arterial blood flow increased after birth in both groups (Figure 4c), and at 5 min after birth was not significantly different between DH lambs and control lambs (30 ± 3 vs. 30 ± 6 mL/min/kg, $p=0.92$). However, carotid arterial blood flow remained persistently high in DH lambs and at 10 min after birth was 2-fold greater than in control lambs (33 ± 5 vs. 15 ± 2 mL/min/kg, $p=0.003$). By 20 min after birth, carotid arterial blood flow was not significantly different between DH lambs and control lambs (25 ± 5 vs. 15 ± 2 mL/min/kg, $p=0.09$) and there remained no significant difference at 120 min after birth (14 ± 3 vs. 15 ± 1 mL/min/kg, $p=0.94$).

Cerebral oxygen delivery was not significantly different between DH lambs and control lambs before birth (132 ± 16 vs. 189 ± 23 mL/min/kg, $p=0.40$), however by 5 min after birth was severely lower in DH lambs (61 ± 16 vs. 447 ± 90 mL/min/kg, $p<0.001$; Figure 4d). By 10 min after birth, cerebral oxygen delivery was not significantly different between groups (249 ± 80 vs. 246 ± 39 mL/min/kg, $p=0.96$).

$SctO_2$ sharply decreased after birth in both groups, however rapidly recovered in control lambs (Figure 4e). $SctO_2$ was lower in DH lambs compared to control lambs at 5 min (24 ± 4 vs. 59 ± 12 %, $p<0.001$) and 10 min (41 ± 7 vs. 76 ± 6 %, $p<0.002$). By 20 min after birth, $SctO_2$ was not significantly different between DH lambs and control lambs (69 ± 6 vs. 75 ± 6 %, $p=0.52$). At 60 min after birth, $SctO_2$ was again lower in DH lambs than control lambs (50 ± 10 vs. 70 ± 5 %, $p=0.02$), however this difference was not significant at 120 min after birth (55 ± 10 vs. 71 ± 5 %, $p=0.052$).

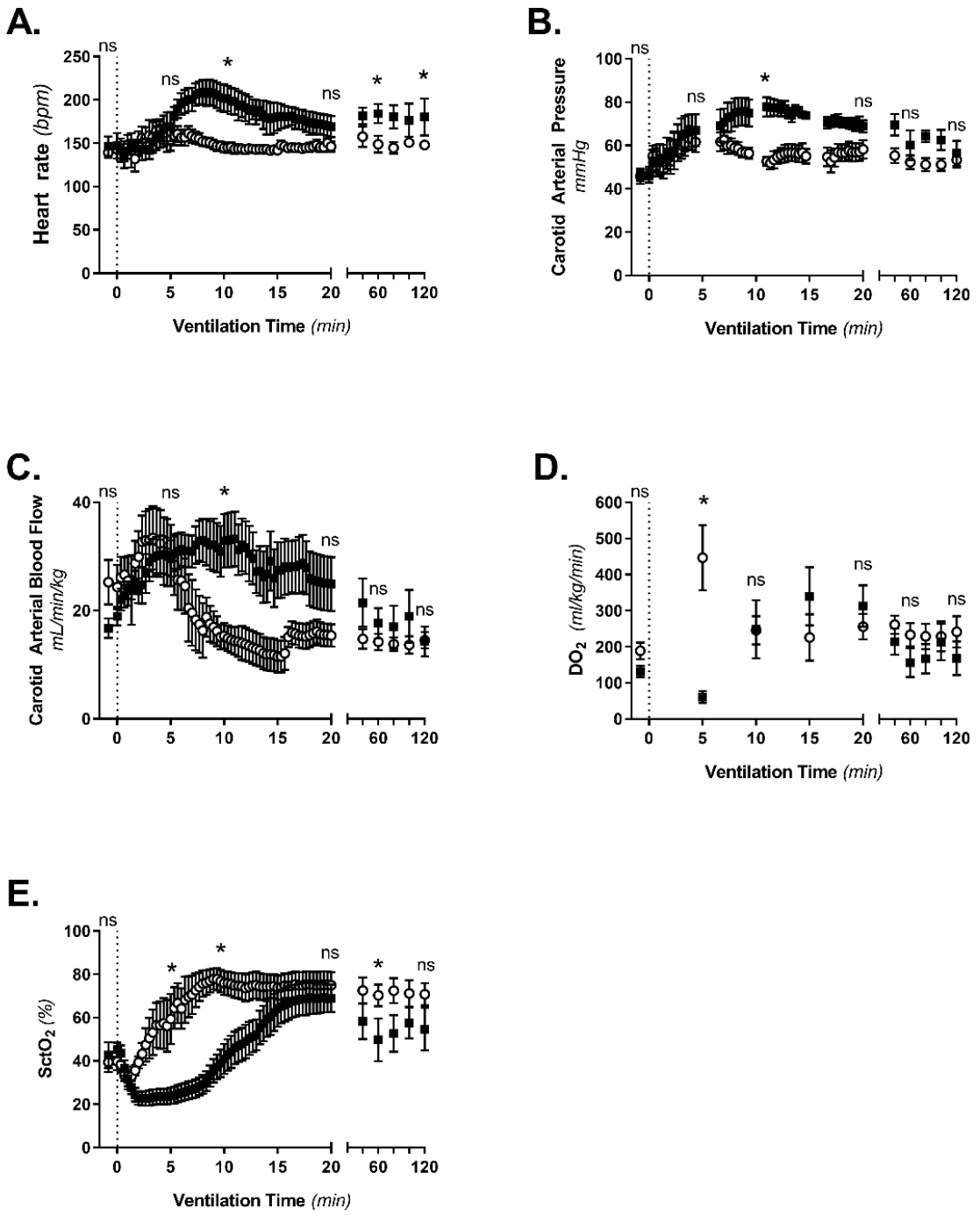

Figure 4

(A) Heart rate (bpm), (B) carotid arterial pressure (CAP; mmHg), (C) carotid arterial blood flow (CBF; mL/min/kg), (D) cerebral oxygen delivery (DO_2; mL/kg/min) and (E) cerebral tissue oxygen saturation ($SctO_2$; %) during the 120 min neonatal ventilation in lambs with a diaphragmatic hernia (DH; black squares, n=6) and control lambs (white circles, n=6). Linear mixed model fit using restricted maximum likelihood. * $p<0.05$ for an *a priori* pairwise comparison between DH and control lamb, after adjustment to control the experiment-wise maximum false discovery rate to 0.05.

Discussion

Our study demonstrates that the neonatal transition at birth is an especially challenging period for infants with a CDH. As our lambs with DH transitioned from placental to pulmonary gas exchange, their hypoplastic and poorly perfused lungs were unable to maintain adequate arterial oxygenation during the first 10 min after birth. Consequently, DH lambs experienced sympathetic tachycardia, hypertension, and an autoregulatory increase in carotid blood flow to maintain cerebral oxygen delivery. We also observed gradually increasing pulmonary vascular resistance in DH lambs during the 2nd hour of neonatal ventilation, that likely reflects vasoconstriction in response to the initial period of transient, severe hypoxia or to the elevated pulmonary arterial pressures experienced during the first 20 min after birth.

Normally at birth, rapid lung aeration triggers an increase in PBF that allows the lungs to begin performing gas exchange and providing left ventricular preload. In our lambs with DH, lung aeration was slow and PBF did not even begin to increase until ~2 min after birth. We observed similarly low PBF at birth in our previous study, however in that study we delayed umbilical cord clamping, so the placenta continued to perform gas exchange and provide preload while PBF gradually increased.[5] In contrast, in this study we clamped the umbilical cord before ventilation began. By removing placental support, we can now describe the true physiological effects of inadequate PBF during the neonatal transition: insufficient gas exchange and impaired cardiovascular function. These results are more relevant to current clinical practice, as most centres continue to perform immediate cord clamping for CDH infants.

After losing placental support and with the lungs not yet adequately perfused, arterial oxygen saturation became severely low (<20%) in DH lambs and did not recover until at least 10 min after birth. This poor oxygenation likely further restricted PBF, as hypoxia is a strong stimulus for pulmonary vasoconstriction, particularly in CDH.[16] Indeed, pulmonary arterial pressure gradually increased in DH lambs during the first 10 min after birth. The extremely low cerebral oxygen delivery at 5 min after birth and subsequent autoregulatory increase in carotid blood flow also raises concerns regarding neurological injury, particularly given recent clinical studies that have identified long-

term neurological dysfunction in CDH survivors.[17] We discuss the impact of this 10 min period of severe cerebral hypoxia ($SctO_2$ <30%) in more detail elsewhere.[9]

Delayed pulmonary vasodilation in DH lambs also had important cardiovascular effects. We have previously shown that removing the low-resistance placental circulation by clamping the umbilical cord rapidly increases afterload for the heart, which is only reduced when the pulmonary circulation vasodilates and allows the ductus arteriosus to act as a pressure relief valve.[18, 19] When this occurs, blood begins to flow from the systemic circulation to the pulmonary circulation at the end of diastole (defined as positive EDF). In control lambs, this occurred within the first 5 min after birth. In DH lambs, EDF only became weakly positive at 10 min after birth. Hence, the high afterload (reflected by high carotid arterial pressure) was sustained for longer in DH lambs than controls.

The tachycardia observed in our DH lambs that underwent immediate cord clamping is interesting, as in preterm lambs immediate cord clamping is associated with profound bradycardia.[19] Indeed, increased afterload should stimulate arterial baroreceptors and hence reduce heart rate (albeit to a lesser degree in the fetus and neonate than in the adult), decreased preload should reduce heart rate via the reverse Bainbridge reflex, and hypoxia should reduce heart rate in the fetus and neonate due to the diving reflex.[20-22] Yet in the setting of increased afterload, reduced preload and severe hypoxia, our neonatal lambs with DH experienced significant tachycardia. On the other hand, we began the neonatal ventilation with one or two 30 sec sustained inflations, which may have increased heart rate via the Hering-Breuer inflation reflex.[23] Hypercapnia, seen to a greater degree in DH lambs, also stimulates an increase in heart rate, likely by provoking catecholamine release from the cardiac sympathetic nerve.[24] The interaction between these factors affecting heart rate during the neonatal transition in DH lambs certainly warrants further study.

PBF was lower in DH lambs throughout the neonatal ventilation period, although after correcting PBF for lung weight there was no difference between groups between 20-60 min after birth. However, during the second hour of ventilation PBF adjusted for lung weight began to decrease in DH lambs while remaining stable in control lambs. In DH lambs, this coincided with an increase in calculated pulmonary vascular resistance.

Unfortunately, we were unable to calculate pulmonary vascular resistance in control lambs because pulmonary arterial pressure was not recorded. However, as described above, EDF reflects shunting from the systemic to pulmonary circulation, which is dependent on pulmonary vascular resistance. Hence, EDF can be used to indirectly reflect pulmonary vascular resistance (for correlation using historical data, see Supplementary Figure 1). In our study, EDF decreases towards 0 as pulmonary vascular resistance rises in DH lambs. In contrast, EDF remains positive in control lambs, which suggests pulmonary vascular resistance remains low in control lambs. Together, these results suggest that DH lambs began to develop pulmonary hypertension between 60 and 120 min after birth. Future animal studies should extend the neonatal ventilation period to determine the extent of this pulmonary hypertension, and should consider incorporating pulmonary vasodilators used in clinical practice such as inhaled nitric oxide and sildenafil.

We did not confirm lung hypoplasia using histology, which may limit the interpretation of our study. However, Pringle *et al.* have previously described hypoplastic lung morphology in a lamb model with DH creation at a similar gestation (~78d GA) with similar lung weights.[25] Furthermore, in normal fetal lamb development, the greatest increase in pulmonary vascular bed cross-sectional area occurs between 85 to 140d GA.[26] Nevertheless, the effects on lung airway and vascular development may be less profound than when the DH is induced at ~63 dGA.[27] The severity of lung hypoplasia may also be reflected by the high rate of (often not clinically apparent) pneumothoraces in our DH lambs, however this also demonstrates the limitations of our ventilation strategy. While our gentle ventilation strategy using conventional iPPV is the recommended starting point in clinical guidelines for CDH infants, high-frequency oscillatory ventilation should be considered when high inspiratory pressures are insufficient to maintain blood gas parameters within the target range.[7] We did not have access to high-frequency oscillatory ventilation, and despite causing pneumothoraces, the inspiratory pressures and V_T delivered by conventional iPPV were not sufficient to maintain $PaCO_2$ within the target range. In DH lambs, $PaCO_2$ increased above our permissive hypercapnia range (>80 mmHg) within 10 min after birth and remained greater throughout the 2 hr ventilation period, as in CDH infants with severe lung

hypoplasia.[28] The inconsistency between normal PaO_2 and low SaO_2 simply reflects increased dissociation of Hb and O_2 due to high CO_2 and low pH (Bohr effect).

In conclusion, lambs with DH have hypoplastic lungs that are slow to achieve adequate aeration and perfusion after birth. When placental support is removed by immediate cord clamping, this leads to severe, transient hypoxia and cardiovascular compromise during the neonatal transition. Future therapies should aim to extend placental support until lung aeration is achieved, by performing physiologically based cord clamping, or improve the capacity of the pulmonary vascular bed to accept cardiac output after birth, by using antenatal therapies such as sildenafil.[9, 29]

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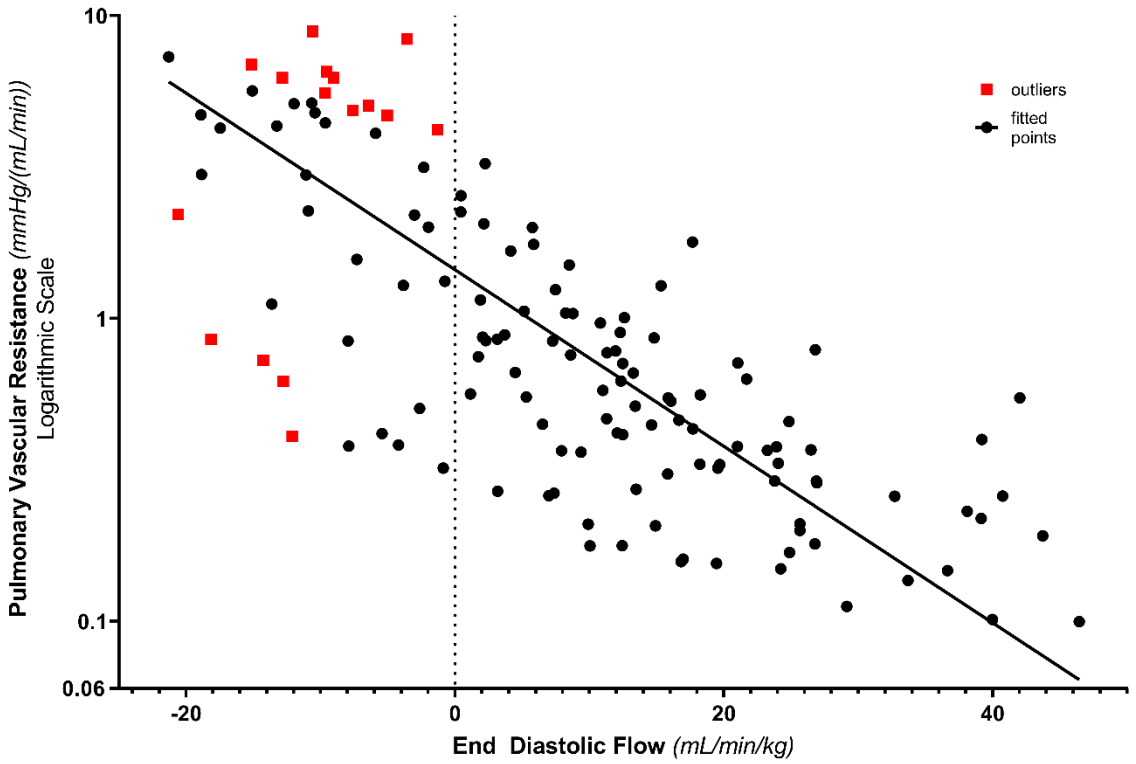

Supplementary Figure 1

Correlation between pulmonary vascular resistance (PVR; mmHg/(mL/min)) and end-diastolic pulmonary blood flow (EDF; mL/min/kg) in newborn lambs with and without diaphragmatic hernia during the first 120 min after ventilation onset. Model: $PVR = 10^{-0.03 * EDF + 0.16}$; $R^2 = 0.68$

Chapter 4

Antenatal sildenafil treatment improves neonatal pulmonary haemodynamics and gas exchange in lambs with diaphragmatic hernia

4 Antenatal sildenafil treatment improves neonatal pulmonary haemodynamics and gas exchange in lambs with diaphragmatic hernia

As shown in Chapter 3.2, when ICC is performed, the hypoplastic and poorly perfused lungs of DH lambs are unable to maintain adequate arterial oxygenation during the first 10 min after birth as they transition from placental to pulmonary gas exchange. We also observed a gradually increasing PVR during the two hour ventilation period after birth. This likely reflects vasoconstriction in response to the transient severe hypoxia that occurred initially or to the high pulmonary arterial pressures experienced during the first 20 min after birth.

These results suggest that our DH lambs were developing pulmonary hypertension toward the end of the 2 hour ventilation period, which is consistent with previous lamb experiments and what is seen clinically.[125, 205] This reinforced our hypothesis that the unexpected findings in our original study (Chapter 2.1) were at least partially due to PBCC. Hence, I decided to perform ICC for our sildenafil study in Chapter 4.1, to isolate the effects of antenatal sildenafil therapy.

As described in Chapter 1.6 and detailed further in my review published in the *American Journal of Perinatology* (Appendix 1), numerous preclinical studies in rodent and rabbit models have demonstrated that antenatal sildenafil treatment attenuates pulmonary vascular abnormalities associated with CDH. However, it is unclear if these biochemical and structural changes will translate to improved cardiopulmonary haemodynamics after birth. Before translating these preclinical data into a clinical trial, I aimed to investigate the effect of antenatal sildenafil on cardiopulmonary haemodynamics and lung function during the neonatal transition in lambs with diaphragmatic hernia. Before beginning this experiment, a significant volume of work was required to optimise the route of administration for antenatal sildenafil in our model. This preliminary work is described in Appendix 2.

Antenatal sildenafil treatment improves neonatal pulmonary haemodynamics and gas exchange in lambs with diaphragmatic hernia

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Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia

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KEYWORDS: congenital abnormality; fetal therapy; neonatal transition; pulmonary hypertension; pulmonary vascular development

CONTRIBUTION

What are the novel findings of this work?
Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in a lamb model of congenital diaphragmatic hernia (CDH), which correlates with previous work in rodents demonstrating that antenatal sildenafil treatment improves pulmonary vascular structure.

What are the clinical implications of this work?
Antenatal sildenafil treatment should be investigated in a clinical trial for fetuses with CDH, in order to reduce the incidence and/or severity of persistent pulmonary hypertension in the newborn.

ABSTRACT

Objectives *Infants with congenital diaphragmatic hernia (CDH) are predisposed to pulmonary hypertension after birth, owing to lung hypoplasia that impairs fetal pulmonary vascular development. Antenatal sildenafil treatment attenuates abnormal pulmonary vascular and alveolar development in rabbit and rodent CDH models, but whether this translates to functional improvements after birth remains unknown. We aimed to evaluate the effect of antenatal sildenafil on neonatal pulmonary hemodynamics and lung function in lambs with diaphragmatic hernia (DH).*

Methods *DH was surgically induced at approximately 80 days' gestation in 16 lamb fetuses (term in lambs is approximately 147 days). From 105 days' gestation, ewes received either sildenafil (0.21 mg/kg/h intravenously) or saline infusion until delivery (*n = *8 fetuses in each group). At approximately 138 days' gestation, all lambs were instrumented and then delivered via Cesarean section. The lambs were ventilated for 120 min with continuous recording of physiological (pulmonary and carotid artery blood flow and pressure; cerebral oxygenation) and ventilatory parameters, and regular assessment of arterial blood gas tensions. Only lambs that survived until delivery and with a confirmed diaphragmatic defect at postmortem examination were included in the analysis; these comprised six DH-sildenafil lambs and six DH-saline control lambs.*

Results *Lung-to-body-weight ratio (*0.016 ± 0.001 vs 0.013 ± 0.001; $P = 0.06$*) and dynamic lung compliance (*0.8 ± 0.2 vs 0.7 ± 0.2 *mL/cm*H_2O; $P = 0.72$*) were similar in DH-sildenafil lambs and controls. Pulmonary vascular resistance decreased following lung aeration to a greater degree in DH-sildenafil lambs, and was 4-fold lower by 120 min after cord clamping than in controls (*0.6 ± 0.1 vs 2.2 ± 0.6 *mmHg/(mL/min);* $P = 0.002$*). Pulmonary arterial pressure was also lower (*46 ± 2 vs 59 ± 2 *mmHg;* $P = 0.048$*) and pulmonary blood flow higher (*25 ± 3 vs 8 ± 2 *mL/min/kg;* $P = 0.02$*) in DH-sildenafil than in*

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DH-saline lambs at 120 min. Throughout the 120-min ventilation period, the partial pressure of arterial carbon dioxide tended to be lower in DH-sildenafil lambs than in controls (63 ± 8 vs 87 ± 8 mmHg; P = 0.057), and there was no significant difference in partial pressure of arterial oxygen between the two groups.

Conclusions *Sustained maternal antenatal sildenafil infusion reduced pulmonary arterial pressure and increased pulmonary blood flow in DH lambs for the first 120 min after birth. These findings of improved pulmonary vascular function are consistent with improved pulmonary vascular structure seen in two previous animal models. The data support the rationale for a clinical trial investigating the effect of antenatal sildenafil in reducing the risk of neonatal pulmonary hypertension in infants with CDH.*

INTRODUCTION

In congenital diaphragmatic hernia (CDH), abdominal organs herniate into the thorax during embryogenesis, interfering with fetal lung development and resulting in severe pulmonary hypoplasia. While CDH is considered a rare disease, it continues to account for more than 1% of infant mortality in the USA[1,2]. This is despite recent advances in the antenatal diagnosis of CDH and neonatal care at birth, and it relates primarily to the degree of lung hypoplasia that affects both airway and vascular development[3]. CDH infants are born with small stiff lungs with a reduced gas-exchange surface area and fewer pulmonary vessels with altered vasoreactivity[4,5]. These changes predispose infants to respiratory insufficiency and pulmonary hypertension immediately after birth.

Early attempts to repair the diaphragmatic defect surgically *in utero* were unsuccessful, so subsequent fetal interventions have instead focused on enhancing prenatal lung growth[6,7]. This approach is feasible owing to improved assessment of fetal morphology on antenatal ultrasound, which allows quantification of the severity of abnormal lung development[8]. One potential strategy for improving lung growth is to increase the volume of lung liquid retained within the future airways by occluding the fetal trachea[9–11]. The clinical evolution of this technique, named fetoscopic endoluminal tracheal occlusion (FETO)[12], has shown some promising preliminary outcomes[13–15], and there are two ongoing randomized clinical trials in CDH fetuses at risk for severe and moderate lung hypoplasia (both known by the acronym Tracheal Occlusion to Accelerate Lung growth, TOTAL)[16]. Nevertheless, despite an apparent increase in survival following FETO, approximately half of the infants with severe lung hypoplasia do not survive, and there is limited benefit regarding the incidence of persistent pulmonary hypertension (PPHN) of the newborn[17–19]. Hence, antenatal therapies that specifically address the pulmonary vascular abnormalities associated with CDH appear necessary[20–22].

PPHN in CDH infants is often refractory to treatment with inhaled nitric oxide (NO), suggesting abnormalities in the endothelium-dependent vasodilatory pathways[23]. This is supported by the finding that phosphodiesterase-5 (PDE-5) is upregulated in neonatal CDH lungs; PDE-5 suppresses NO-mediated vasodilatation by rapidly degrading cyclic guanosine monophosphate (cGMP)[24]. Furthermore, antenatal treatment with the PDE-5 inhibitor sildenafil has been shown to attenuate pulmonary vascular abnormalities in both a nitrofen-induced CDH rodent model and a surgery-induced CDH rabbit model, which is more clinically relevant in terms of lung development[25–33]. In these animal studies, antenatal sildenafil treatment increased cGMP and vascular endothelial growth factor expression in lung tissue, increased distal pulmonary vessel density, decreased pulmonary vascular muscularization, reduced right ventricular wall thickness and increased distal airway complexity[25–33]. However, it is unclear if these improvements in pulmonary vascular structure and biochemistry translate to improved pulmonary hemodynamics after birth.

Our aim in this study was to investigate the physiological effects of antenatal sildenafil on pulmonary hemodynamics and lung function during neonatal transition in lambs with diaphragmatic hernia (DH).

METHODS

Animal model

General surgical methods

This experiment was performed in accordance with guidelines established by the National Health and Medical Research Council of Australia and was approved by the Monash University animal ethics committee. For the surgical procedures, eight Merino X Border-Leicester ewes carrying twin pregnancies were anesthetized by administration of an intravenous bolus of sodium thiopentone (1 g in 20 mL (Pentothal; Jurox, New Zealand)), intubated with an 8-mm endotracheal tube (Portex Ltd, Kent, England), and maintained with inhaled isoflurane (~2% in room air/oxygen (IsoFlo; Abbott Laboratories, North Chicago, IL, USA)) administered via a positive-pressure ventilator (EV500 Anaesthesia Ventilator; ULCO Medical Engineering, NSW, Australia). In addition to absence of a corneal reflex, end-tidal carbon dioxide (CO_2), tidal volume, heart rate and oxygen saturation were continuously monitored (SurgiVet Vital Signs Monitor; Smiths Medical, USA), to ensure adequate analgesia and maternal wellbeing.

Induction of diaphragmatic hernia

At approximately 80 days' gestation (term in lambs is approximately 147 days), a DH was surgically created in 16 lamb fetuses, as described previously[34]. Briefly, maternal midline laparotomy and hysterotomy allowed the fetal chest to be exposed and incised through the

ninth intercostal space. A small segment of the fetal diaphragm was excised and the stomach and bowels were pulled into the thoracic cavity. The amniotic fluid content was restored and 1000 mg Cefazolin was administered directly into the amniotic sac as antibiotic prophylaxis (Cefazolin-AFT 1000 mg in 5 mL sterile water; AFT Pharmaceuticals Pty Ltd, Australia). Ewes received postoperative analgesia (transdermal fentanyl patch, 75 μg/h; Janssen-Cilag Pty Ltd, NSW, Australia) for 3 days and were monitored daily until delivery at 138 days' gestation.

Sildenafil infusion

At 103 days' gestation, catheters were placed in the maternal right jugular vein and carotid artery under general anesthesia. The jugular vein catheter was connected to an infusion pump (CADD-Legacy 1 Ambulatory Infusion Pump Model 6400; Smiths Medical Australasia Pty Ltd, Australia), which was secured to the ewe's back using netting (Tubular-Net; Sutherland Medical Pty Ltd, Australia).

At 105 days' gestation (equivalent to human fetal lung development at 22 weeks), eight ewes carrying twins were allocated to either DH-sildenafil or DH-saline. In four ewes ($n = 8$ fetuses; DH-sildenafil), the infusion pump administered sildenafil (2.8 mg/mL sildenafil citrate in 0.9% sodium chloride; Fagron, Belgium) via continuous intravenous infusion at a dose of 0.21 mg/kg/h until delivery. In the other four ewes ($n = 8$ fetuses; DH-saline), the infusion pump administered an equivalent volume of 0.9% sodium chloride.

Delivery and ventilation

At approximately 138 days' gestation (near-term), the lamb fetuses were instrumented prior to Cesarean delivery, as described previously[34]. Briefly, the fetuses were exteriorized and intubated with a clamped endotracheal tube (size 4.0). Polyvinyl chloride catheters were placed in the left carotid artery and main pulmonary artery to allow continuous arterial pressure monitoring, and in the left jugular vein to allow neonatal drug administration if required. Ultrasonic flow probes (Transonic Systems, Ithaca, NY, USA) were placed around the right carotid and left pulmonary arteries, to allow continuous recording of blood flow. A pulse oximeter (Radical-7; Masimo Corp., Irvine, CA, USA) was placed on the right forelimb, a near-infrared spectroscopy probe (NIRS; Casmed Foresight, CAS Medical Systems Inc., Branford, CT, USA) on the forehead and a temperature probe in the rectum. The stomach was drained via an orogastric tube. After instrumentation, the lambs were delivered from the uterus and the endotracheal tube was unclamped.

The umbilical cord was immediately clamped and ventilation was commenced with 30-s sustained inflation (35 cmH_2O, 21% oxygen) followed by intermittent positive-pressure ventilation (iPPV) in volume guarantee mode using a target tidal volume of 4 mL/kg (Babylog 8000; Dräger, Lübeck, Germany), peak inspiratory pressure limit of 35 cmH_2O, and positive end-expiratory pressure of 5 cmH_2O. If the target tidal volume was not reached after 30 s, a second sustained inflation was performed followed by iPPV for a total of 120 min.

The lambs were moved to a warming bed (CosyCot; Fisher and Paykel, Auckland, New Zealand) and sedated using alfaxalone (10 mg/kg/h (Alfaxan; Jurox)). Respiratory support was titrated to achieve a partial pressure of arterial CO_2 ($PaCO_2$) of 60–80 mmHg, partial pressure of arterial oxygen (PaO_2) of > 40 mmHg and oxygen saturation (SaO_2) of 85–88%, as previously described[34]. The lambs were euthanized (intravenous sodium pentobarbitone, 100 mg/kg) after completing the 120-min ventilation protocol or earlier owing to ethical endpoints (i.e. in case of severe acidosis or pneumothorax).

Outcome measures

Sildenafil concentration in plasma

Maternal arterial blood samples were obtained at 1, 7 and 21 days after the antenatal infusion of sildenafil or saline commenced. At delivery, paired maternal and fetal arterial blood samples were obtained. Plasma from these samples was isolated via centrifugation (3000 rpm for 10 min). Total sildenafil concentration (calculated as sildenafil concentration + 50% of its active metabolite N-desmethyl-sildenafil[35]) was analyzed using ultra-high-performance liquid chromatography (Vanquish Flex Quaternary; ThermoFisher Scientific, Australia) in tandem with mass spectrometry (TSQ Quantiva; ThermoFisher Scientific).

Neonatal cardiopulmonary hemodynamics and lung function

Pulmonary and carotid arterial blood flow and pressure, cerebral tissue oxygen saturation ($SctO_2$), tidal volume and airway pressures were recorded continuously using LabChart data analysis software (ADInstruments, NSW, Australia) and analyzed offline in 20-s epochs. Arterial blood gas tensions were assessed every 5 min during the first 30 min of ventilation and every 10 min thereafter.

Pulmonary vascular resistance (PVR), dynamic lung compliance, alveolar–arterial difference in oxygen tension ($AaDO_2$) and cerebral oxygen delivery were calculated using the equations listed in Table 1.

Postmortem examination

The presence of a diaphragmatic defect and herniation of visceral organs was confirmed during postmortem examination. Both lungs were weighed, and lung weight was expressed as a ratio to body weight (fresh lung-to-body-weight ratio; LBWR).

Table 1 Equations used for calculation of derived measures

Parameter	*Equation*
PVR	(PAP – LAP)/PBF
DLC	V_T/(PIP – PEEP)
$AaDO_2$	$(FiO_2 \times (P_{atm} - P_{H_2O}) - PaCO_2/0.8) - PaO_2$
CaO_2[46]	$(1.39 \times Hb \times (SaO_2/100)) + (0.003 \times PaO_2)$
DO_2[46]	Carotid arterial blood flow × $(CaO_2/100)$

$AaDO_2$, alveolar–arterial difference in oxygen tension; CaO_2, arterial oxygen content; DLC, dynamic lung compliance; DO_2, cerebral oxygen delivery (mL/min/kg); FiO_2, fraction of inspired oxygen (%); Hb, arterial hemoglobin concentration (g/dL); LAP, left atrial pressure, assumed to equal 9 mmHg based on previous studies[47]; $PaCO_2$, partial pressure of arterial carbon dioxide (mmHg); PaO_2, partial pressure of arterial oxygen (mmHg); PAP, pulmonary arterial pressure (mmHg); P_{atm}, atmospheric pressure (760 mmHg); PBF, pulmonary blood flow (mL/min); PEEP, positive end-expiratory pressure (cmH_2O); P_{H_2O}, water vapor pressure (47 mmHg); PIP, peak inspiratory pressure (cmH_2O); PVR, pulmonary vascular resistance; SaO_2, arterial oxygen saturation; V_T, tidal volume (mL).

Statistical analysis

We have previously shown that pulmonary blood flow (PBF) in sham-operated controls is 2.4-fold greater than in DH lambs[34]. Based on the variability in that study, we determined that at least six successful animals per group would provide power of ≥ 80% with a two-sided type-I error of 5% to detect a 1.5-fold increase in PBF in DH-sildenafil lambs compared with DH-saline lambs. The Shapiro–Wilk test was used to assess frequency distributions. Normally distributed data are expressed as mean ± standard error of the mean (SEM), and non-normally distributed data are presented as median (interquartile range). For physiological data, differences between DH-sildenafil and DH-saline lambs were analyzed over time and between groups using two-way repeated-measures ANOVA with *post-hoc* analysis (Holm–Sidak) determining the time that differences were evident (SigmaPlot v13.0; Systat Software Inc., San José, CA, USA); $P < 0.05$ was taken to indicate a statistically significant difference.

Data from historical controls (no DH and ventilated using the same protocol) are displayed in the figures to provide a physiological context for the ovine model; however, no statistical comparisons with these historical controls were made[36].

RESULTS

Sildenafil concentration in plasma

Maternal plasma sildenafil concentration was 68 ± 20 ng/mL after 24 h of continuous intravenous infusion, 69 ± 17 ng/mL after 7 days and 79 ± 9 ng/mL after 21 days. Immediately prior to delivery (~33 days after the infusion began), maternal plasma sildenafil concentration was 118 ± 50 ng/mL and fetal plasma sildenafil concentration was 3 ± 0.5 ng/mL. After 120 min of ventilation, neonatal plasma sildenafil concentration remained at 3 ± 0.5 ng/mL. Sildenafil was not detected in maternal or fetal plasma samples from DH-saline animals.

Animal groups and gross morphology

Fourteen (of 16) fetuses survived to delivery: seven (88%) DH-sildenafil lambs and seven (88%) DH-saline ones. All DH-sildenafil lambs survived the 120-min neonatal ventilation, however, it was necessary to humanely euthanize one DH-saline lamb at 90 min after birth owing to the development of treatment-resistant pneumothorax. Only lambs with a confirmed diaphragmatic defect at postmortem examination were included in the analysis; these comprised six DH-sildenafil lambs and six DH-saline lambs.

Body weight was not significantly different between DH-sildenafil and DH-saline lambs (4.14 ± 0.25 *vs* 4.49 ± 0.34 kg; $P = 0.43$) and neither was LBWR (0.016 ± 0.001 *vs* 0.013 ± 0.001; $P = 0.06$).

Ventilation parameters and arterial blood gas tensions

Peak inspiratory pressure and positive end-expiratory pressure were not significantly different between the two groups throughout the 120-min ventilation period (Figure 1a). However, tidal volume (V_T) was significantly greater in DH-sildenafil lambs than in DH-saline lambs at 15 min after cord clamping (4.1 ± 0.5 *vs* 2.5 ± 0.5 mL/kg; $P = 0.03$) (Figure 1b). At the end of the 120-min ventilation period, there was no significant difference in V_T (4.1 ± 0.4 *vs* 3.7 ± 0.5 mL/kg; $P = 0.45$) or dynamic lung compliance (0.8 ± 0.2 *vs* 0.7 ± 0.2 mL/cmH_2O; $P = 0.72$) between the two groups.

PaO_2 and SaO_2 increased more rapidly after cord clamping in DH-sildenafil lambs. At 10 min after cord clamping, both PaO_2 (57 ± 18 *vs* 24 ± 5 mmHg; $P = 0.02$) and SaO_2 (80 ± 8 *vs* 44 ± 12%; $P = 0.009$) were greater in DH-sildenafil than in DH-saline lambs, despite having similar fraction of inspired oxygen (62 ± 14 *vs* 76 ± 11%; $P = 0.48$). However, after 15 min, PaO_2 and SaO_2 were no longer significantly different between the two groups (Figure 2a,b). $AaDO_2$ was not significantly different between DH-sildenafil and DH-saline lambs throughout the 120-min ventilation period (288 ± 84 *vs* 308 ± 87 mmHg; $P = 0.88$), nor was the oxygenation index (23 ± 10 *vs* 33 ± 10; $P = 0.46$).

Mean $PaCO_2$ tended to be lower in DH-sildenafil lambs than in DH-saline lambs throughout the 120-min ventilation period (63 ± 8 *vs* 87 ± 8 mmHg; $P = 0.057$) (Figure 2c). As minute volume (MV) and $PaCO_2$ are inversely related, we calculated the product of the two ($MV \times PaCO_2$) to help explain any differences in $PaCO_2$ levels. This product was initially not different between the groups, which demonstrates that lower $PaCO_2$ in DH-sildenafil lambs was associated with better ventilation. However, by 60 min after cord clamping $MV \times PaCO_2$ was significantly lower in DH-sildenafil lambs than in DH-saline lambs (49 ± 8 *vs* 96 ± 24 mL × heart rate × mmHg; $P = 0.045$), which

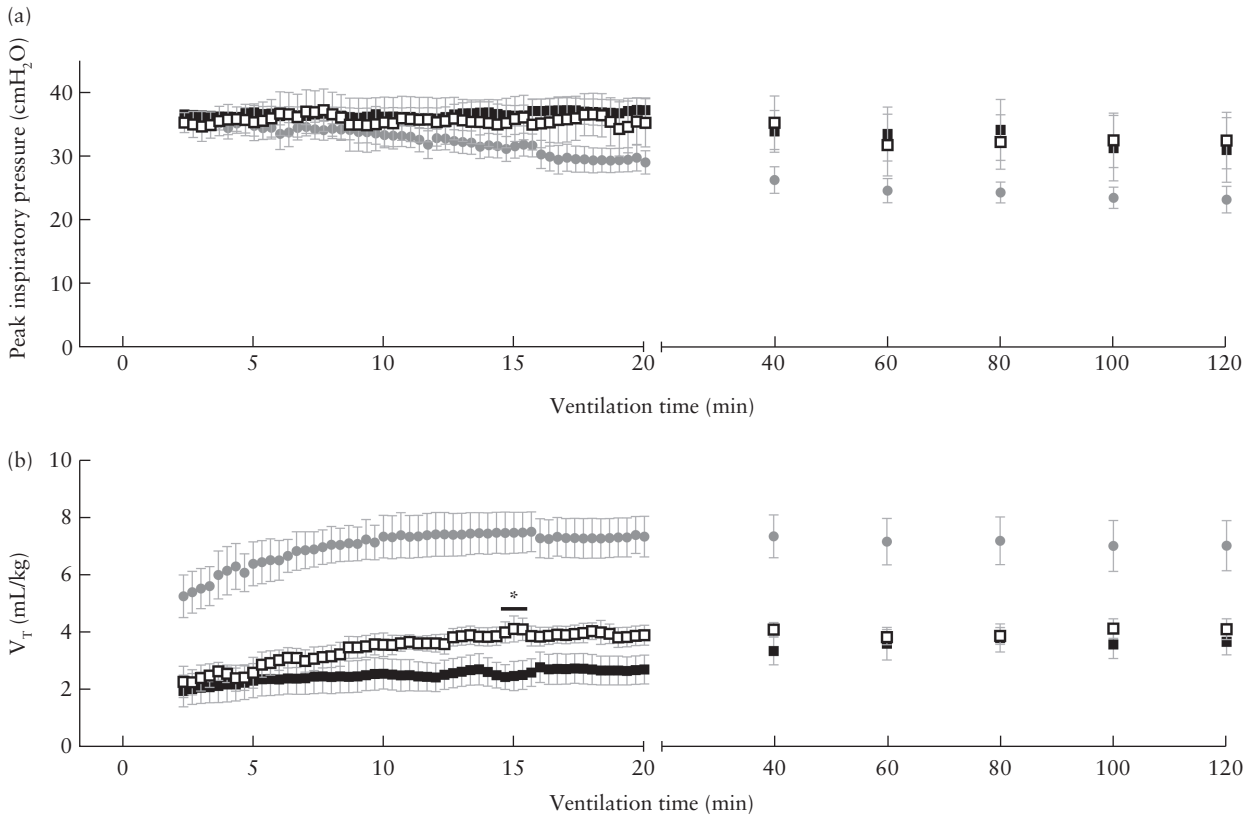

Figure 1 Peak inspiratory pressure (a) and tidal volume (V_T) (b), during 120-min ventilation after cord clamping (timepoint 0) in lambs with diaphragmatic hernia (DH) that received sildenafil (□, $n = 6$) or saline (■, $n = 6$) antenatally. Data are mean ± standard error of mean. Differences between two DH groups analyzed over time and between groups by two-way repeated-measures ANOVA with Holm–Sidak's multiple-comparisons test. *$P < 0.05$ for effect of treatment at timepoint below horizontal line. Data from historical controls without DH (●, $n = 6$) are displayed to provide physiological context for ovine model, but no statistical comparisons with these historical controls were made.

shows that $PaCO_2$ was lower in DH-sildenafil lambs despite similar ventilation. pH was not significantly different between the two groups throughout the 120-min ventilation period (7.22 ± 0.05 *vs* 7.11 ± 0.06; $P = 0.18$) (Figure 2d).

Pulmonary perfusion

PBF increased to a greater degree after cord clamping in DH-sildenafil than it did in DH-saline lambs (Figure 3a), and at 20 min after cord clamping PBF was 2-fold greater (42 ± 4 *vs* 22 ± 3 mL/min/kg; $P = 0.006$) in DH-sildenafil lambs. By the end of the 120-min ventilation period, PBF was 3-fold greater in DH-sildenafil than in DH-saline lambs (25 ± 3 *vs* 8 ± 2 mL/min/kg; $P = 0.02$). Pulmonary arterial pressure increased rapidly after cord clamping in both groups, but to a lesser degree in DH-sildenafil lambs (Figure 3b). By the end of the 120-min ventilation period, pulmonary arterial pressure was significantly lower in DH-sildenafil than in DH-saline lambs (46 ± 2 *vs* 59 ± 2 mmHg; $P = 0.048$).

PVR was lower in DH-sildenafil lambs before cord clamping (3.2 ± 0.7 *vs* 6.5 ± 0.9 mmHg/(mL/min); $P < 0.001$) and throughout the 120-min neonatal ventilation (Figure 3c). The magnitude of the difference in PVR between DH-sildenafil and DH-saline lambs increased between 20 min (0.33 ± 0.08 *vs* 0.71 ± 0.08 mmHg/(mL/min); $P = 0.007$), 60 min (0.45 ± 0.08 *vs* 1.86 ± 0.54 mmHg/(mL/min); $P = 0.008$) and 120 min (0.60 ± 0.13 *vs* 2.17 ± 0.58 mmHg/(mL/min); $P = 0.002$) after cord clamping. PBF at the end of diastole (end-diastolic pulmonary blood flow (EDF)) was negative in both DH-sildenafil and DH-saline lambs before cord clamping (−14.2 ± 4.2 *vs* −8.7 ± 1.3 mL/min/kg; $P = 0.46$), and became positive within 10 min after cord clamping (14.4 ± 6.1 *vs* 7.7 ± 3.2 mL/min/kg; $P = 0.179$). In DH-saline lambs, EDF subsequently decreased during the neonatal ventilation and ultimately returned to about 0 mL/min/kg by 120 min (Figure 3d). In contrast, EDF remained positive in DH-sildenafil lambs, and at the end of the 120-min neonatal ventilation it was significantly greater than in DH-saline lambs (10.2 ± 1.6 *vs* 0.6 ± 1.3 mL/min/kg; $P = 0.036$).

Cerebral perfusion and oxygenation

Carotid arterial blood pressure (CAP) and blood flow (CBF) increased rapidly within 5 min after cord clamping in both groups, although the increase was smaller in DH-sildenafil lambs (Figure 4a,b). At 10 min after cord clamping, DH-sildenafil lambs had ∼20% lower CAP (61 ± 6 *vs* 78 ± 4 mmHg; $P = 0.046$) and ∼33% lower

CBF (20.4 ± 4.5 *vs* 32.9 ± 4.9 mL/min/kg; $P = 0.042$) compared with DH-saline lambs. From 10 to 120 min after cord clamping, both CAP and CBF gradually decreased in both groups. At the end of the experimental period, both CAP (49 ± 6 *vs* 56 ± 6 mmHg; $P = 0.42$) and CBF (12.4 ± 2.0 *vs* 14.3 ± 2.8 mL/min/kg; $P = 0.78$) were similar in both DH-sildenafil and DH-saline lambs.

Heart rate was lower in DH-sildenafil lambs than in DH-saline lambs between 5 min (141 ± 10 *vs* 181 ± 11 beats per min (bpm); $P = 0.02$) and 10 min (153 ± 15 *vs* 202 ± 16 bpm; $P = 0.005$) after cord clamping, but it was not significantly different at 20 min after cord clamping (152 ± 9 *vs* 169 ± 12 bpm; $P = 0.34$) or thereafter (Figure 4c).

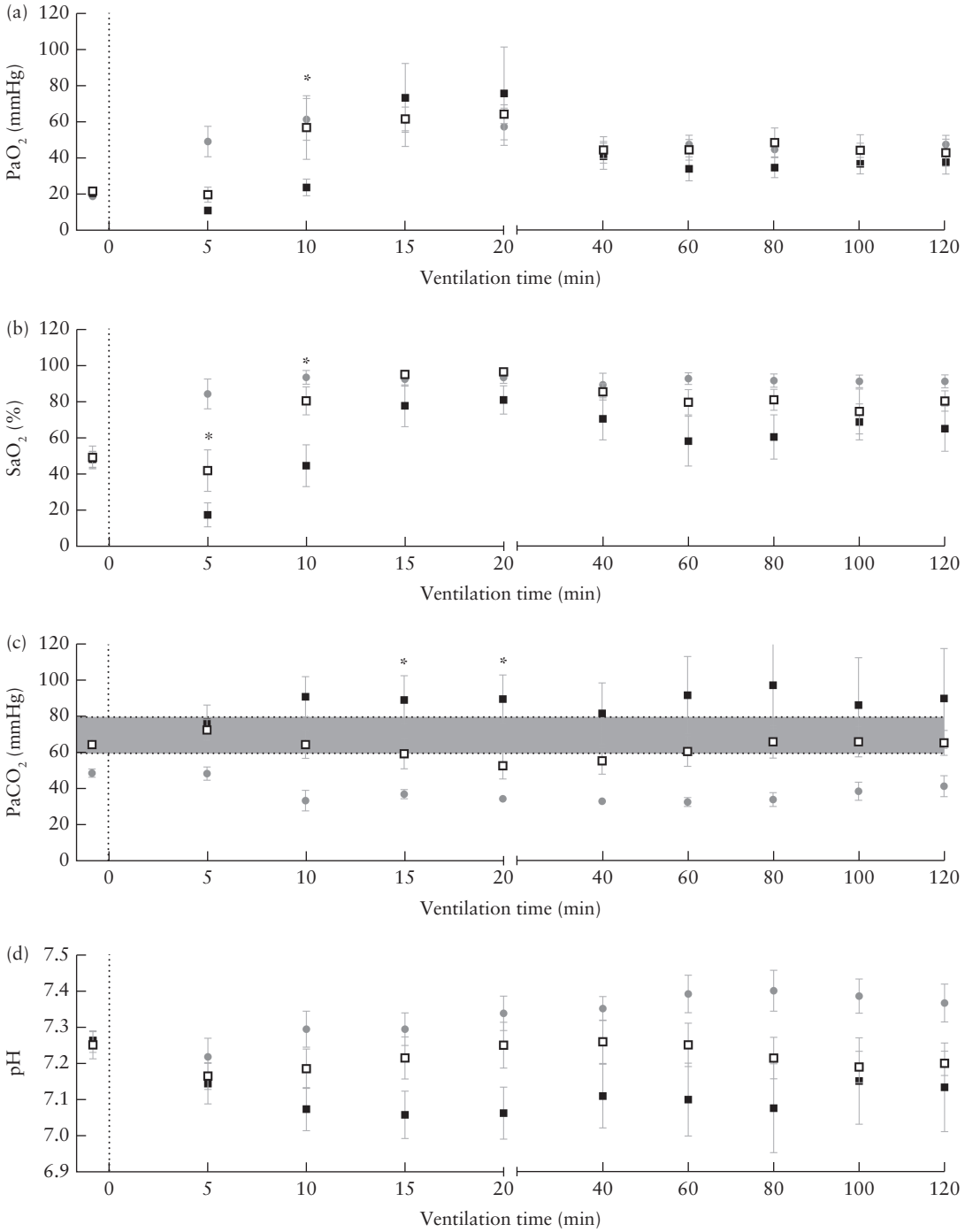

Figure 2 Partial pressure of arterial oxygen (PaO_2) (a), arterial oxygen saturation (SaO_2) (b), partial pressure of arterial carbon dioxide ($PaCO_2$) (c) and arterial pH (d), during 120-min ventilation after cord clamping (timepoint 0) in lambs with diaphragmatic hernia (DH) that received sildenafil (□, $n = 6$) or saline (■, $n = 6$) antenatally. Data are mean ± standard error of mean. Differences between two DH groups analyzed over time and between groups by two-way repeated-measures ANOVA with Holm–Sidak's multiple-comparisons test. $^*P < 0.05$ for effect of treatment. In all DH lambs, respiratory support was titrated to achieve a $PaCO_2$ of 60–80 mmHg (shaded area in (c)). Data from historical controls without DH (●, $n = 6$) are displayed to provide physiological context for ovine model, but no statistical comparisons with these historical controls were made.

Cerebral oxygen delivery was greater in DH-sildenafil lambs than in DH-saline lambs at 5 min after cord clamping (1.7 ± 0.4 *vs* 0.6 ± 0.2 mL/min/kg; $P = 0.04$), but was not significantly different at 10 min after cord clamping (2.6 ± 0.6 *vs* 2.5 ± 0.8 mL/min/kg; $P = 0.89$) or thereafter.

$SctO_2$ decreased rapidly in both groups after cord clamping, but recovered more rapidly in DH-sildenafil lambs than it did in DH-saline lambs (Figure 4d). $SctO_2$ was significantly greater in DH-sildenafil lambs than in DH-saline lambs between 7 and 14 min after cord clamping (at 10 min: 65 ± 5 *vs* $41 \pm 7\%$; $P = 0.007$). At the end

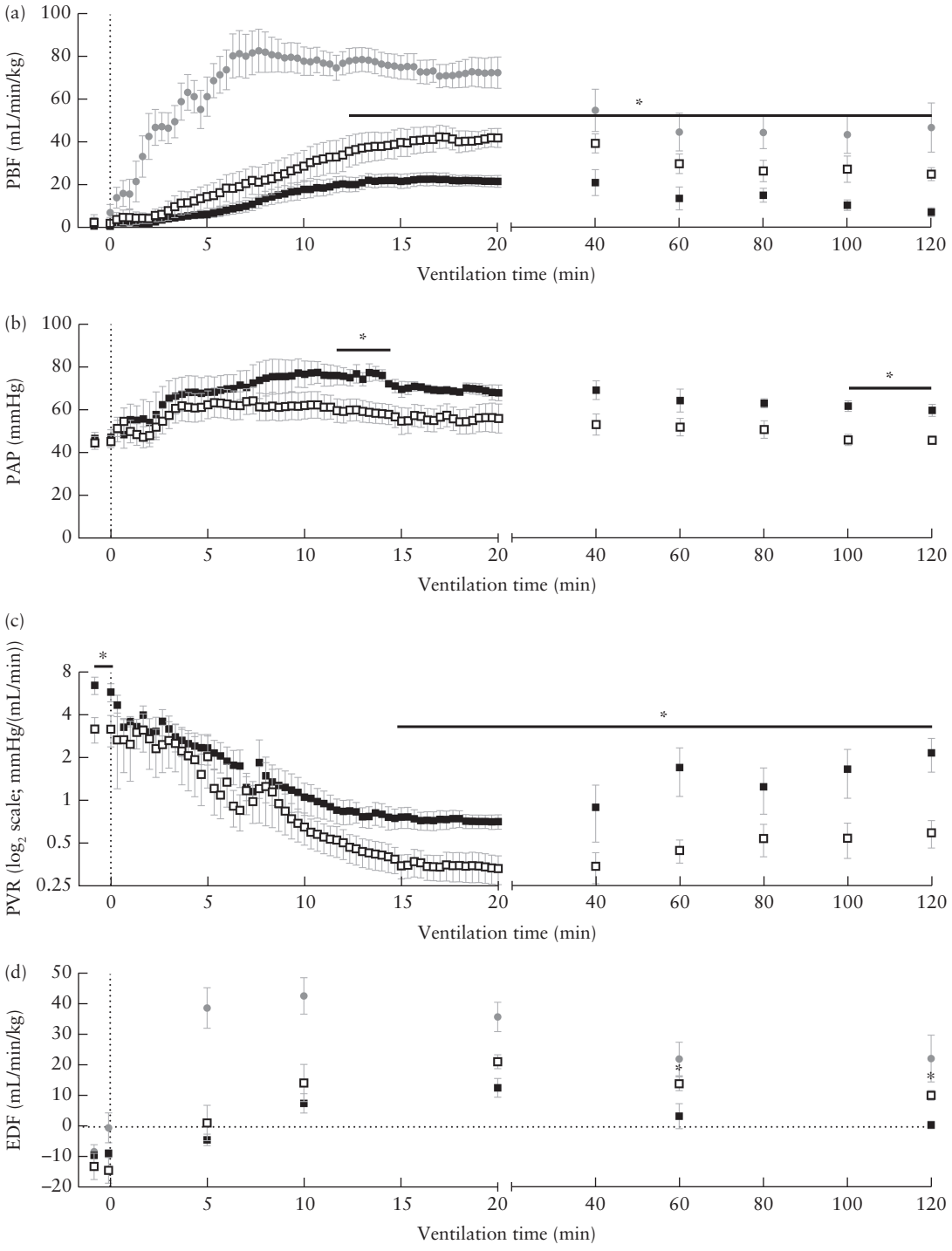

Figure 3 Pulmonary arterial blood flow (PBF) corrected for body weight (a), pulmonary arterial blood pressure (PAP) (b), pulmonary vascular resistance (PVR) presented on $\log_2$ scale (c) and end-diastolic pulmonary blood flow (EDF) corrected for body weight (d), during 120-min ventilation after cord clamping (timepoint 0) in lambs with diaphragmatic hernia (DH) that received sildenafil (□, $n = 6$) or saline (■, $n = 6$) antenatally. Data are mean ± standard error of mean. Differences between two DH groups analyzed over time and between groups by two-way repeated-measures ANOVA with Holm–Sidak's multiple-comparisons test. *$P < 0.05$ for effect of treatment at each timepoint below horizontal line. In (a) and (d), data from historical controls without DH (●, $n = 6$) are displayed to provide physiological context for ovine model, but no statistical comparisons with these historical controls were made.

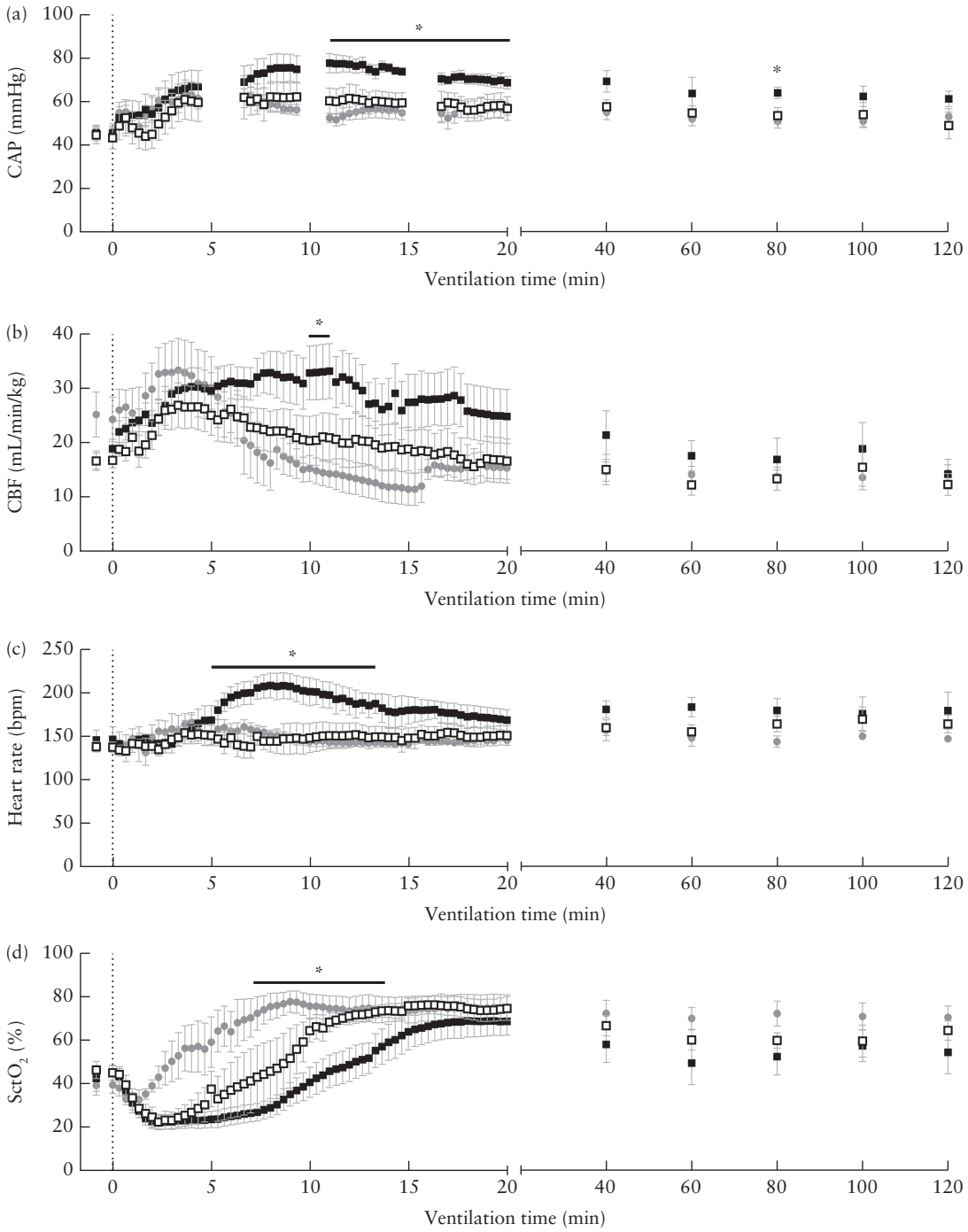

Figure 4 Carotid arterial blood pressure (CAP) (a), carotid arterial blood flow (CBF) corrected for body weight (b), heart rate (c) and cerebral tissue oxygen saturation ($SctO_2$) (d), during 120-min ventilation after cord clamping (timepoint 0) in lambs with diaphragmatic hernia (DH) that received sildenafil (□, $n = 6$) or saline (■, $n = 6$) antenatally. Data are mean ± standard error of mean. Differences between two DH groups analyzed over time and between groups by two-way repeated-measures ANOVA with Holm–Sidak's multiple-comparisons test. $^*P < 0.05$ for effect of treatment at each timepoint below horizontal line. Data from historical controls without DH (●, $n = 6$) are displayed to provide physiological context for ovine model, but no statistical comparisons with these historical controls were made.

of the experimental period, $SctO_2$ was not significantly different between the groups (65 ± 4 *vs* $55 \pm 10\%$; $P = 0.18$).

DISCUSSION

We have shown that antenatal sildenafil treatment causes a greater reduction in PVR after birth in DH lambs, resulting in higher PBF with lower pulmonary arterial pressures.

We have shown previously that DH lambs experience severe hypoxia at birth if the umbilical cord is immediately clamped[37]. This is because the hypoplastic lungs of DH lambs are slow to aerate and perfuse at birth, so they cannot take over the role of gas exchange

from the placenta as rapidly as can age-matched control lambs. Our sildenafil-treated DH lambs also experienced a hypoxic period immediately after birth; however, as tidal volumes and PBF increased more rapidly, cerebral oxygenation recovered considerably faster than in DH lambs that did not receive antenatal sildenafil treatment.

Compared with DH-saline lambs, antenatal sildenafil-treated DH lambs maintained higher PBF throughout the 120-min ventilation period despite reduced pulmonary arterial pressure, which reflects a considerably lower PVR. PVR was lower in DH-sildenafil, compared with DH-saline, lambs during the fetal instrumentation period immediately before birth. This finding is consistent with those of previous studies in DH rabbits receiving antenatal sildenafil treatment[33], in which lower PVR was attributed to an improvement in fixed structural factors such as the density of distal pulmonary blood vessels[25,29,31,33]. However, the magnitude of the difference in PVR between DH-sildenafil and DH-saline lambs increased after birth, which may reflect a greater vasodilatory response to lung aeration and ongoing ventilation. The mechanisms involved are unknown, but may include decreased muscularization of the pulmonary arteries and increased expression of vasodilatory mediators, as shown in rodent DH models[25,26,28,29,33]. Alternatively, antenatal sildenafil may enhance the vasodilatory response to birth-related stimuli such as increased oxygenation[38].

Despite higher PBF in DH-sildenafil lambs, oxygen gas exchange was not different between the two DH groups during the 120-min neonatal ventilation period. However, $PaCO_2$ could be maintained more easily within or below the permissive hypercapnia range (60–80 mmHg), reflected by a significantly lower $MV \times PaCO_2$ product and indicating improved CO_2 gas exchange in antenatal sildenafil-treated DH lambs. This inconsistency in improvements between oxygen and CO_2 exchange probably reflects the higher solubility and hence greater diffusion capacity of CO_2 across the alveolar–capillary membrane[39]. Antenatal sildenafil therapy increases distal airway complexity and gas exchange surface area in rodent DH models, which, combined with higher PBF, may have improved CO_2 exchange without being sufficient to significantly improve oxygen exchange[26,29,30,33].

Reverse (away from the lungs) flow in the pulmonary arteries during diastole is a common feature of PBF before birth, resulting in continuous right-to-left shunting via the ductus arteriosus throughout the cardiac cycle. After lung aeration, the large decrease in PVR rapidly changes the PBF waveform. Reverse-flow PBF is abolished and forward flow into the lung becomes continuous, resulting in positive EDF. In untreated DH lambs, EDF was lower and returned to near 0 towards the end of the ventilation period (Figure 3d), suggesting that right-to-left shunting, a common feature of PPHN, may have soon re-emerged. In contrast, in DH lambs treated with sildenafil, higher EDF and lower PVR make it less likely that right-to-left shunting would have re-emerged.

In rodent models, the effect of antenatal sildenafil on gross lung size is unclear. Most studies have found no difference[25,29,33,40], one reported a decrease[27] and two studies reported an increase in lung weight[26,30]. While there was no significant difference between the two groups in our study, we found that DH lambs treated with sildenafil tended ($P = 0.06$) to have greater LBWR than did untreated DH lambs, but the lungs were still markedly hypoplastic in both treated (48% of historical controls) and untreated (39%) DH lambs[34]. Indeed, while dynamic lung compliance was initially (during the first 20 min) better in sildenafil-treated DH lambs, by the end of the 120-min ventilation period it was no longer different between the two groups. These findings suggest that antenatal sildenafil treatment does not improve lung parenchymal growth to the same degree as it improves pulmonary vascular development and function. Hence, combining antenatal sildenafil therapy with FETO, which significantly improves lung growth but has limited effect on the pulmonary vasculature, could provide a synergistic benefit for fetuses with severe CDH, as demonstrated in the rabbit CDH model[41].

Strengths and limitations

We focused primarily on the physiological effects of antenatal sildenafil treatment in DH lambs, so our study is limited by the absence of histological evidence to confirm the morphological and biochemical features underlying the observed physiology. However, the effects of sildenafil on pulmonary vascular development have been well described in other animal models of CDH and correlate well with our current findings[25–33]. Interestingly, fetal plasma sildenafil concentrations in our lambs immediately prior to delivery (2–5 ng/mL) were similar to those obtained in rats by Mous *et al.*[26], but lower than those obtained in rats by Luong *et al.*[25] and in rabbits by Russo *et al.*[33]. As plasma concentrations of sildenafil persisted in the same range until the end of the ventilation experiment, residual effects of sildenafil on pulmonary arterial pressure and PVR may have confounded our results[42]. However, the magnitude of the reduction in PVR seen in our study (360%) is significantly greater than when sildenafil is administered only after birth to the neonate (18%)[42]. Nevertheless, in future studies it will be important to clarify whether the observed benefits of sildenafil persist once it has been cleared from the circulation.

Another limitation of our study is that we did not investigate the effect of antenatal sildenafil treatment on lambs without DH. When sildenafil was given to rodents and rabbits without DH, it decreased the number of distal vessels and decreased total vascular volume[25,26,33]. Given the recent safety concerns regarding the use of antenatal sildenafil to treat intrauterine growth restriction (STRIDER)[43,44], these worrying findings in rodents and rabbits may require further investigation in the lamb DH model before antenatal sildenafil treatment is considered for mild and moderate CDH cases that may not have significantly abnormal pulmonary vascular development.

Conclusions

We have shown that antenatal sildenafil treatment improves neonatal pulmonary hemodynamics in lambs with DH. These promising findings correlate with previous work demonstrating that sildenafil attenuates abnormal pulmonary vascular development in DH, and provide evidence for investigating sildenafil in a clinical setting for fetuses with severe CDH, as is currently underway in Belgium and France[45].

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General Discussion

5

5 General discussion

5.1 Summary of findings

In this thesis we have demonstrated that lung hypoplasia, secondary to a DH, has a significant effect on cardiopulmonary physiology during the transition from fetal to neonatal life. The development of PPHN is one of the most important factors that influences survival for CDH infants. We observed that improving pulmonary vascular development with fetal therapy or protecting the vessels by optimising delivery room management could prevent setting these infants on a path towards PPHN.

In *Chapter 2*, we found that while our lambs with a DH had similarly poor respiratory outcomes to previous studies using the same model, PBF was similar between DH and control lambs after adjusting for lung weight.[338, 339] We hypothesised that, in contrast to previous studies in which the umbilical cord was clamped immediately at birth, our physiological approach to umbilical cord clamping protected the pulmonary vasculature from stresses induced by hypoxia and high arterial pressures during the fetal to neonatal transition.[338, 339] We also demonstrated that the only fetal intervention for CDH currently in clinical trials, FETO, increases lung size and increases PBF in proportion with lung size, but has minimal benefit on pulmonary gas exchange during the first 2 hours after birth.[340]

In *Chapter 3*, we described the effects of physiologically based cord clamping (PBCC) on neonatal physiology in lambs with DH, compared to immediate cord clamping (ICC). We found that in DH lambs, establishing lung aeration prior to umbilical cord clamping prevents severe transient hypoxia at birth and facilitates a greater increase in PBF during the first 120 min of neonatal life, compared with ICC.[341] However, in *Chapter 3.2*, comparing DH and control lambs that received ICC demonstrated that an irreversible factor, such as reduced cross-sectional area of the pulmonary vascular bed, contributed to the raised PVR in DH lambs. We hypothesised that this irreversible factor reflected abnormal pulmonary vascular development, and hence neonatal PBF may be improved by an antenatal medical therapy that attenuates pulmonary vascular abnormalities associated with CDH.

In *Chapter 4*, we investigated the effect of antenatal sildenafil treatment on neonatal cardiopulmonary physiology in lambs with a DH. We found that antenatal sildenafil treatment was associated with lower pulmonary arterial pressures and greater PBF during the neonatal transition. However, while antenatal sildenafil treatment resulted in a more rapid pulmonary vasodilation, it did not totally prevent the severe transient hypoxia immediately after birth and did not increase lung size. Hence, it is possible that infants with a CDH will receive synergistic benefits from a combination of FETO to increase lung size, sildenafil to improve pulmonary vascular development, and PBCC to provide perinatal cardiopulmonary stability.

5.2 The sheep model of congenital diaphragmatic hernia

In order to investigate novel fetal and neonatal therapies to prevent pulmonary hypertension, I first aimed to establish the sheep CDH model at our institution (*Chapter 2*). Establishing this model also allowed us to characterise the effect of CDH on neonatal cardiopulmonary haemodynamics in more detail than previous studies, given our well-established techniques for physiological monitoring in neonatal lambs.[338, 342, 343]

To model CDH, a diaphragmatic hernia is surgically induced in lambs at ~80d GA (equivalent human fetal lung development to 11 weeks of gestation). We chose to use sheep, rather than the nitrofen-rat or another surgical model such as the rabbit, because their large size permits fetal and neonatal catheterisation, regular arterial blood gas monitoring, and the use of equipment designed for human neonates. This allowed us to invasively monitor and directly measure pulmonary and systemic blood pressures and flows during the cardiopulmonary transition at birth. Furthermore, fetal lung development is similar in sheep and humans, and the associated fetal and neonatal pulmonary physiology has been well characterised.[76, 92, 128, 344]

However, a major limitation of the sheep CDH model is that the hernia is induced during the late pseudoglandular stage of lung development, so any pulmonary changes that occur in human CDH during the embryonic or early pseudoglandular stage are not accounted for. Given the proposed "dual-hit" hypothesis for lung hypoplasia in CDH,

some suggest that surgical models are less useful in studying the aetiology and early pathogenesis of CDH than pharmacological or transgenic models.[95] However, due to its large size and similar pattern of lung development, the sheep CDH model remains useful for assessing the effects of fetal interventions and neonatal ventilation strategies in the context of severe lung hypoplasia before they are considered in humans.

The timing of DH induction in fetal sheep has varied from 63 to 95d GA in different studies.[345-347] In our studies, we surgically induced DHs at 80d GA because at earlier gestational ages the fetal skin was too fragile to safely perform a reproducible procedure. Indeed, our extremely low intraoperative mortality rate across all studies (5%) demonstrates the improved safety associated with later surgery as inducing the DH at 60 – 63d GA reportedly caused a 30% intraoperative mortality rate.[345] While our studies are limited by a lack of histological data, the morphology of the hypoplastic lung secondary to a DH created at ~78 dGA has been described previously.[346] They found less prominent alveolar capillaries, which is consistent with the finding that the greatest increase in pulmonary vascular bed cross-sectional area occurs between 85 to 140d GA in fetal sheep (the number of fifth and sixth generation vessels increases from 7.2×10^3 to 61.8×10^3).[348] Hence, inducing a hernia at ~80d GA is early enough to disrupt this aspect of pulmonary vascular development.

All lambs were delivered by caesarean section at ~138d GA (near-term) to avoid both lung immaturity associated with early delivery and the complications associated with spontaneous vaginal delivery. Indeed, caesarean section delivery allows for the lamb to be exteriorised and catheterised in a controlled environment before it is fully delivered.[349]

Another limitation of the sheep CDH model is that the placenta is synepitheliochorial, whereby the fetal and maternal capillaries are separated by a bilayer of uterine epithelial cells and syncytotrophoblasts. In contrast, the human placenta is haemomonochorial, with only a monolayer of syncytotrophoblasts separating fetal capillaries from maternal blood. Hence, the placental transfer of any maternally administered medical therapy, such as sildenafil, must be investigated in humans before clinical trials are considered. Russo *et al.* have already demonstrated that sildenafil crosses the human placenta *ex vivo* at a therapeutic level (50 ng/mL) with a fetomaternal concentration ratio of 0.95,

and are now investigating the *in vivo* placental transfer and safety in a phase I/IIb study.[350, 351]

A final limitation of our sheep experiments is that the neonatal ventilation component of our study only continued for 2 hours and so we can only speculate as to the long-term effects of our fetal and neonatal therapies. Indeed, in our experiment, FETO did not improve neonatal gas exchange, yet in a similar experiment in the sheep CDH model, FETO was associated with improved gas exchange only after 3 hours of neonatal ventilation.[342] Future experiments should continue the neonatal ventilation period for longer, but this raises additional challenges that include the confounding effects of neonatal therapies that are necessary for survival. Indeed, these include therapies such as ionotropes (to maintain cardiopulmonary perfusion) and high-frequency oscillatory ventilation (to avoid ventilation-induced lung injury and pneumothoraces), which have known independent effects of neonatal physiology. Regardless, some suggest that arterial blood gas status during the neonatal transition may predict long-term survival, hence our results do have some generalisability to longer-term outcomes.[352]

During each neonatal ventilation, a large volume of continuous physiological data was generated. Given respiratory and cardiovascular variables were dynamically changing during the neonatal transition, analysing outcomes solely at one arbitrarily chosen timepoint (such as the end of the experimental period) would not convey the entire physiological picture. Instead, differences between groups for each variable were analysed over time using a 2-way repeated measures analysis of variance, as performed extensively in previous studies.[167, 196, 344, 353, 354] However, this approach is limited because it considers time as a categorical nominal variable (rather than continuous, or at least ordinal), it can only correct for missing timepoints by excluding an entire animal, and sphericity is assumed. In future experiments, as was performed in *Chapter 3.2*, a mixed model fitted by the restricted maximum likelihood method is likely more appropriate.

Given the large number of timepoints and physiological variables analysed, the issue of multiple comparisons also becomes important. If 100 variables are compared in samples taken from two populations that are not truly different in any way, by chance alone there will be a statistically significant difference in 5 variables at the $p<0.05$ level. There

are two approaches to controlling this Type I error. The first, proposed by Bonferroni and subsequently developed by Holm and Sidak, aims to ensure that the chance of making *any* Type I error remains below 5%.[355] However, this approach results in the paradoxical situation in which reporting a continuous variable at a greater number of discrete time points results in a significant reduction in statistical power. Consider the following situation. If PBF is averaged every 10 sec for 20 min, there are 120 data points, and therefore the difference between each group at each time point must have a *p*-value of <0.0005 to be statistically significant after adjusting for multiple comparisons. If PBF is averaged every 2 min for 20 min, there are 10 data points, and therefore the difference between each group at each time point must have a *p*-value of <0.005 to be statistically significant after adjusting for multiple comparisons. Hence, more comparisons are described as significant when fewer time points are assessed, despite the underlying physiological data not changing. To prevent this situation, an alternative approach to correcting for multiple comparisons is required, such as controlling the false-discovery rate (FDR). Controlling the FDR, proposed by Benjamini and Hochberg, aims to ensure that the total number of false-discoveries (Type I errors) remains less than 5% of all discoveries.[356] In this way, increasing the number of sampled time points does not reduce statistical power.

Analysing physiological data at multiple time points also reduces the number of animals required to achieve adequate statistical power. By estimating the variance and correlation patterns not only between subjects, but also of repeated measures of the same physiological variable in the same subject, a given statistical power can be achieved with less subjects than if groups were compared at a single time point.[357, 358] This has important ethical considerations.

5.3 Preventing pulmonary hypertension in congenital diaphragmatic hernia

As described in *Chapter 1.3*, PPHN occurs in CDH infants due to a combination of irreversible abnormalities in pulmonary vascular structure and reversible changes in the vasoreactivity of the pulmonary vascular bed.

Aside from a smaller lung, the irreversible components of PPHN appear less important during the neonatal transition than has previously been assumed, given that there was no difference in PBF corrected for lung weight between lambs with and without a DH when PBCC was performed (Chapter 2). This finding is consistent with our discussion in Chapter 1.1.3. In the post-mortem studies that categorised pulmonary vessels by branching generation rather than size, neomuscularisation of intra-acinar vessels that was observed at 10 weeks of postnatal age was not evident immediately after birth.[9, 117] Together with our findings, this suggests that abnormal structural development and vasoreactivity is more important initially and then the subsequent physiological changes cause pulmonary vascular remodelling and significant neomuscularisation after birth. However, this remains only a hypothesis because histological analysis was not performed in our study. We did not perform histological analysis because we are developing X-ray imaging and analytical techniques to create 3D-reconstructions of the pulmonary vasculature (Figure 15). We will use these reconstructions to investigate the impact of DH on pulmonary vascular morphology and determine whether any abnormalities in vascular branching, arterial generations, cross-sectional area, tortuosity and density are attenuated by antenatal sildenafil. To prepare the lungs for imaging, we perfused the pulmonary vasculature with the contrast agent Microfil immediately at post-mortem, which precludes traditional histology and immunohistochemistry analyses. We have already captured these images at the Australian Synchrotron, as described in Appendix 3, however the reconstruction and analysis of these images is ongoing and will be published separately.

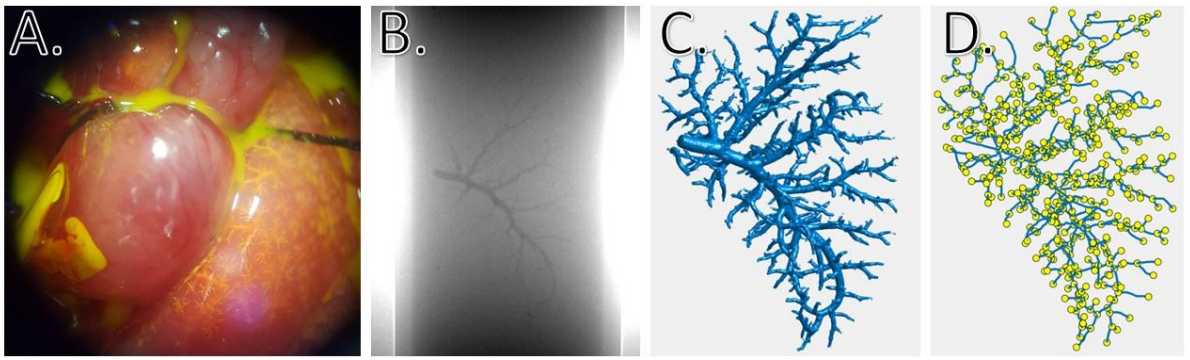

Figure 15 - The process to reconstruct the pulmonary vasculature for analysis, in the left lung of a rabbit kitten with a diaphragmatic hernia.

(A) The contrast agent Microfil is perfused into the pulmonary vasculature immediately at post-mortem. (B) The pulmonary vasculature is imaged using computated tomography to obtain a series of 2D projections. (C) The 2D projections are then segmented, combined and rendered as a mesh in 3D space. (D) The mesh of the pulmonary vasculature tree is then converted to a skeleton, wherein nodes (yellow) and edges (blue) are defined. This allows analysis of vessel generation numbers, branching angles, tortuosity, vessel length, vessel radius and cumulative vessel volume. *Images used with permission from Mr Andrew Stainsby.*

Our experiments suggest that a component of the PPHN that develops after birth is avoidable and potentially commences during the neonatal transition. We found that PVR at 2 hours after birth was lower in DH lambs that underwent PBCC compared to those that underwent ICC. We hypothesise that this lower PVR occurred because PBCC lambs gradually transitioned from placental to pulmonary gas exchange without rapid swings in arterial pressures, blood flows, or oxygenation. A higher PVR in ICC lambs suggests that an initial period of severe hypoxia and elevated pulmonary arterial pressures at birth may be the initial trigger for pulmonary vasoconstriction in infants with a CDH who undergo ICC. Indeed, hypoxia is a strong stimulus for pulmonary vasoconstriction, particularly in CDH infants.[219, 359-361]

If hypoxia and raised intravascular pressure related to ICC is the initial trigger for pulmonary hypertension in CDH infants, this may explain why PPHN is so difficult to predict prenatally. That is, rather than depending on prenatal factors, it may also depend on how the infant is managed at birth, and particularly the delay between cord clamping and establishing pulmonary gas exchange in the delivery room; this is not reported in prenatal prognosis studies.[362] The most promising avenue for future prenatal predictors of pulmonary hypertension may in fact be the fetal response to

maternal hyperoxygenation, as this reflects the response of the pulmonary vasculature to birth-related vasoactive stimuli.[363] While measurements obtained via Doppler ultrasound are too variable to be clinically useful, ongoing advances in phase-contrast MRI imaging may allow more accurate measurements of PBF to be obtained in utero.[364] This technique will also allow operators to determine flow patterns from the umbilical circulation through the foramen ovale to the left ventricle, which may contribute to PPHN in CDH infants by causing left ventricular hypoplasia and subsequent pulmonary venous hypertension.[157]

While the physiological benefits of PBCC appear important, merely avoiding an initial period of hypoxia and raised intravascular pressure at birth is unlikely to completely prevent PPHN. Indeed, the neonate will encounter future vasoconstrictive stimuli and the underlying structural abnormalities remain, although they would be expected to subside as the lung grows postnatally.[9, 117] Hence, antenatal therapies such as sildenafil, that increase pulmonary vascular angiogenesis, reduce pulmonary vascular muscularisation and alter the pulmonary vascular response to vasoactive stimuli, are also required. Indeed, our DH lambs treated with antenatal sildenafil still experienced a hypoxic period with raised intravascular pressures following immediate cord clamping. However, they recovered more rapidly than lambs not exposed to antenatal sildenafil and this initial insult did not appear to trigger the same degree of pulmonary vasoconstriction. Another promising antenatal medical therapy which acts earlier in the endothelium-dependent vasodilation pathway and also has anti-proliferative effects is the ET-1 inhibitor bosentan. This may provide some synergistic benefit in combination with sildenafil, as shown in a rat CDH model.[110] Targeting a different pathway, the prostacyclin receptor agonist selexipag also reduces pulmonary vascular wall thickness in the rat CDH model, however does not have any synergistic effect with sildenafil.[365]

Given our experiments suggest that raised pulmonary arterial pressure during the initial neonatal transition may contribute to pulmonary vascular remodelling, reducing this pressure with early administration of pulmonary vasodilators (immediately at birth) such as iNO, sildenafil and milrinone may also reduce the incidence of subsequent PPHN. A further reduction in pulmonary arterial pressures could be achieved by allowing some degree of right-to-left shunting, as this would reduce the volume of blood

flowing through the high-resistance pulmonary circulation. To achieve this, ductal patency could be maintained using prostaglandin E1.

Throughout this thesis, PVR was calculated based on measured PBF and pulmonary arterial pressure. To determine the driving pressure for this calculation, left atrial pressure was assumed to equal 9 mmHg based on previous studies.[150, 366, 367] However, left atrial pressure is likely to vary between lambs, and also to dynamically change during the neonatal cardiopulmonary transition. Hence, the calculated PVR may not accurately reflect true PVR, particularly in the context of increased PBF flow, such as in lambs that received antenatal sildenafil treatment. Hence, we also assessed PVR by measuring end-diastolic PBF, a sensitive indicator of downstream resistance.[367] Reassuringly, there was a strong correlation between end-diastolic PBF and calculated PVR across all experiments (Chapter 3.2, Supplementary Figure 1).

5.4 Future directions

In this thesis I have described the effects of lung hypoplasia secondary to a DH on cardiopulmonary physiology during the neonatal transition and investigated fetal and neonatal approaches to ensure that PBF and gas exchange is optimised during this period. Our experiments have also suggested future directions for research in this field, regarding case selection for fetal therapy and optimising the neonatal transition in clinical practice.

5.4.1 Determining candidates for fetal therapy

If fetal therapy is to be offered for CDH patients, prenatal imaging should provide an accurate prognosis so that parents can make an informed decision. However, at least 70 scans are required for an inexperienced trainee to become competent in measuring the lung-to-head ratio (the most accurate prenatal predictor of neonatal outcome).[368] Previously, fetal therapy for CDH has been limited to high-resource settings, as FETO requires highly-experienced operators and access to significant medical resources and

equipment. In these settings, there is enough case-volume for sonographers and maternal fetal medicine specialists to become competent in measuring lung-to-head ratio and determining prognosis. In contrast, an antenatal medical therapy such as sildenafil would allow fetal therapy for CDH in low-resource settings, such as developing countries, and countries with low population density, such as Australia. Given the relative rarity of CDH (1 in 3000), many practitioners in these settings may not see 70 cases in their entire career. Hence, a new method for prenatal prognostication with a more rapid learning curve would be desirable. A promising method appears to be grading stomach position on antenatal ultrasound, given the hypoechoic stomach can be easily visualised[369, 370]

5.4.2 Establishing the safety profile of sildenafil during pregnancy

To attenuate the pulmonary vascular abnormalities associated with CDH and hence improve neonatal pulmonary haemodynamics, we investigated antenatal treatment with the PDE5 inhibitor, sildenafil. A number of antenatal medical therapies for CDH have been investigated in pre-clinical models, however, we chose to investigate sildenafil because it was already widely used to treat pulmonary hypertension in adults and children. At the time this thesis commenced the literature suggested that sildenafil could be used in pregnant women without maternal or fetal side effects[298, 319, 324, 371-373] and it had also been used off-label in neonates with a CDH, to treat pulmonary hypertension (CoDiNOS, Eudra CT: 2017-000421-13). There was also excellent placental transfusion data in an *ex vivo* model, with a fetomaternal concentration ratio of 0.95.[350]

The safety of sildenafil in pregnancy was recently called into question following an interim analysis of the Dutch STRIDER trial.[331] As described in *Chapter 1.6.3*, STRIDER is an international consortium of five randomised controlled trials (Netherlands, UK, Australia/New Zealand, Canada, Ireland), that aimed to determine if maternal sildenafil treatment increases gestation length, placental function and fetal growth in pregnancies with dismal prognosis early-onset IUGR. Each site agreed to follow the same protocol and to subsequently conduct a planned individual patient data meta-analysis. After

interim analysis of the Dutch trial, the study was suspended due to a likely lack of efficacy and potential harm relating to an increased incidence of PPHN and neonatal mortality.[331] Of the first 183 patients recruited, 134 survived to birth. Of these neonates, 17 (27%) of 71 in the sildenafil group died prior to discharge compared to only 9 (14%) of 63 in the placebo group, hence the relative risk of neonatal death associated with antenatal sildenafil treatment was 1.87 (95% CI: 0.91 – 3.84).[328, 331] Furthermore, PPHN was observed in 27% of sildenafil neonates compared to only 5% of placebo neonates.[328, 331]

Interestingly, an association between antenatal sildenafil treatment and increased neonatal mortality or PPHN was not observed in the completed UK or Australia/New Zealand trials. In the UK trial, sildenafil and placebo groups had similar rates of fetal mortality (21/70 [30%] vs. 22/65 [34%]), neonatal mortality (10/49 [20%] vs. 7/43 [16%]), and only one neonatal death was attributed to PPHN in each group.[329] In the Australia/New Zealand trial, sildenafil and placebo groups also had similar rates of fetal mortality (7/63 [11%] vs. 12/59 [20%]), neonatal mortality (5/56 [9%] vs. 4/47 [9%]), and PPHN (1/56 [2%] vs. 1/47 [2%]).[330]

While the planned individual patient data meta-analysis of all five sites has not yet been reported, a preliminary report combining the Dutch, UK and Australia/New Zealand trials shows that antenatal sildenafil treatment is associated with a 1.49-fold increased risk of neonatal mortality, but this is not statistically significant (95% CI: 0.90 – 2.47).[328] Despite the lack of statistical significance, caution is clearly required when considering sildenafil for use in any human pregnancy, particularly for fetuses with severe IUGR.

A mechanism for the increased neonatal mortality in the Dutch trial has been proposed in a recently published study in a sheep model of fetal growth restriction. Inocencio *et al.* found that antenatal sildenafil attenuated the brain-sparing cerebral vasodilation and peripheral vasoconstriction that normally follows growth restriction, which may limit acute cerebral responses to neonatal challenges.[334] However, that study did not assess the pulmonary vascular bed. Interestingly, the CDH literature provides some evidence to support the association between antenatal sildenafil and neonatal pulmonary hypertension reported in the Dutch STRIDER trial. In rodents and rabbits with normally developed lungs (control animals for CDH experiments), antenatal

sildenafil treatment reduces pulmonary vascular branching and increases vessel wall thickness.[111, 115, 313]

Together, these studies suggest that sildenafil exerts a different action on the lungs of growth-restricted fetuses, healthy fetuses, and fetuses with a CDH. Given these differential effects, the negative findings in the Dutch STRIDER trial should not be extrapolated to other indications for the use of sildenafil during pregnancy. The significantly improved pulmonary haemodynamics we observed in sildenafil-treated CDH lambs in *Chapter 4* suggest that antenatal sildenafil may decrease the incidence of PPHN in the CDH cohort, rather than increasing risk.

The maternal and fetal safety profile for antenatal sildenafil treatment is also under investigation by Russo *et al.* in a Phase I/IIb clinical trial.[351] The next step will be to translate our findings into a randomised controlled trial that assesses the efficacy of antenatal sildenafil in preventing and/or reducing the severity of PPHN in CDH infants. Given that antenatal sildenafil treatment adversely affected the pulmonary vasculature of control animals in rodent and rabbit studies, such a trial would likely first recruit moderate-severe CDH cases.[111, 115, 313] This raises the question of whether fetuses enrolled in this trial should also be offered FETO (pending TOTAL trial results). As we demonstrated in *Chapter 2.2*, FETO predominantly improves lung size, compliance and ventilation, with limited benefit to neonatal pulmonary haemodynamics. Given that sildenafil primarily improves pulmonary vascular development, without increasing lung size, the two fetal therapies appear likely to have synergistic benefit. Indeed, in rabbit DH kittens the combination of maternal sildenafil treatment and FETO was more effective in improving pulmonary vascular and alveolar development than either therapy alone. [374] In this study, sildenafil also restored normal surfactant protein expression in kittens exposed to FETO, which suggests that adjuvant sildenafil treatment may absolve the need for balloon removal prior to delivery. Sildenafil may mediate this effect by inhibiting TGF-β, as this factor is increased after prolonged tracheal occlusion, inhibits surfactant protein expression, and is inhibited by sildenafil.[375-379] Restoring surfactant expression will significantly improve outcomes for the 20% of CDH fetuses treated with FETO who do not undergo planned balloon removal due to pre-term premature rupture of membranes and/or pre-term delivery.

Furthermore, if PBCC becomes routine care for CDH infants, balloon retrieval in the delivery room can be calmly performed while placental gas exchange continues through the intact umbilical cord.

If antenatal sildenafil is trialled for CDH fetuses, the earliest point at which it could be routinely initiated would be at 22 weeks of gestation, as most CDH cases are diagnosed on routine obstetric morphology ultrasound scans between 18 – 22 weeks.[4] At this gestational age, fetal lung development is midway through the canalicular stage, when progressive thinning of interstitial tissue (septa), capillary proliferation and air-space expansion occurs. The equivalent stage of lung development in the fetal sheep occurs at 105d GA, which is when we began our continuous maternal sildenafil infusion. We continued the infusion until immediately prior to planned delivery by caesarean section at ~138d GA, however did not administer any neonatal sildenafil infusion. A possible explanation for the pulmonary hypertension observed in the Dutch STRIDER trial is that the fetuses became dependent on sildenafil-mediated PDE5 inhibition, so when this was withdrawn after birth they were left in a pro-vasoconstrictive state. Hence, future studies should investigate administering neonatal sildenafil in the delivery room and slowly weaning the dose after birth.

At a maternal dose of 0.21 mg/kg/hour, we achieved a steady state of 3 ng/mL sildenafil in the fetal plasma. This is well below the established therapeutic range (47 – 500 ng/mL), however we did observe a significant improvement in neonatal pulmonary haemodynamics. This suggests that the plasma concentration required for sildenafil to exert chronic anti-proliferative and angiogenic effects may be lower than that required to stimulate acute vasodilation; less than 50% inhibition of PDE5 activity may still improve vascular development, particularly if baseline PDE5 activity is abnormally increased.[380, 381] Our fetal plasma sildenafil concentrations were similar to those obtained in the nitrofen-rat by Mous *et al.*, yet significantly lower than those obtained in rabbits by Russo *et al.*[115, 313] Furthermore, the fetomaternal concentration ratio in our study was 0.03, whereas in *ex vivo* perfused human placental cotyledons it is 0.95.[350] In that placental transfusion study, sildenafil was found to saturate all cotyledon binding sites before fetal transfer began.[350] Given their epitheliochorial placenta and multiple discrete cotyledons, it is possible that sheep have more placental binding sites for

sildenafil than humans, which may explain the reduced placental transfer. Unfortunately, the discrepancy between our fetal plasma sildenafil concentrations in lambs and those obtained by Russo *et al.* in rabbits make it difficult to recommend the ideal target therapeutic range for human trials.[115]

5.4.3 Achieving a physiological approach to birth in clinical practice

Our study in Chapter 3 joins a large body of research suggesting that PBCC is the appropriate strategy for managing neonates requiring resuscitation at birth.[12, 13, 158, 187, 191-193, 354, 382-384] While ICC is standard clinical practice for infants requiring resuscitation at birth, this is merely based on expert opinion.[183] In the absence of high quality clinical evidence, guidelines should consider scientific knowledge from high quality animal research. As such, we suggest that PBCC should now be considered in CDH infants where resuscitation at the maternal bedside is feasible.

Feasibility of delaying cord clamping in infants that require resuscitation

The remaining barrier to performing PBCC in infants with a CDH is the feasibility of providing neonatal respiratory support at the maternal bedside. To achieve this, neonatologists are designing resuscitation equipment that can be used non-obtrusively at the maternal bedside. These range from simple mobile platforms that can be covered with a sterile drape (Baby-DUCC[191]) to complete resuscitation modules with in-built physiological monitoring devices, such as the Concord Birth Flow[197]. We are working closely with the group of Prof Arjan te Pas at Leiden University Medical Centre and Dr Philip DeKoninck at Erasmus MC to develop a clinical trial for PBCC in CDH infants using this Concord Birth Flow device, which has already been successfully used for pre-term infants (NL69575.078.19).[13]

In a small pilot study, Lefebvre *et al.* have already demonstrated that PBCC (termed intact-cord resuscitation in their study) is feasible for CDH infants.[193] Their preliminary results are consistent with our findings; PaCO2 was lower in infants that underwent intact cord resuscitation compared to those that underwent ICC and there was no significant difference in SpO2, however pre- and post-ductal SpO2 were maintained in

the target range in both groups by using high FiO2. As we described in Chapter 3, the discrepancy between O2 and CO2 can be partially explained by the high FiO2 and airway pressures used in all groups to maintain relatively normal PaO2, but also by the higher solubility and hence greater diffusion capacity of CO2 across the alveolar-capillary membrane.[385]

Very recently, Foglia et al. have also demonstrated that PBCC is feasible for CDH infants with more severe prognostic parameters (median observed/expected lung-to-head ratio 30.1%; intrathoracic liver in 70%).[384] The primary outcome of this pilot study was successful intubation prior to umbilical cord clamping within 3 minutes after birth, which occurred in 17 of 20 infants. Intubation was not possible in 3 infants due to infant positioning, so the umbilical cord was clamped, and they were transferred to a warming bed before ventilation commenced. The study was not powered to detect differences in physiological or clinical outcomes. Regardless, compared to matched historical controls, CDH infants that were intubated and ventilated prior to umbilical cord clamping had higher haemoglobin and mean arterial pressure at 1 hour after birth, and no difference in blood gas parameters or oxygenation indices within the first 48 hours after birth. All infants had evidence of pulmonary hypertension on echocardiograms conducted at a mean of 13 hours after birth, with no difference in severity between infants in the trial arm and matched historical controls. In contrast, in our lamb study (Chapter 3), PVR was lower in PBCC lambs at 2 hours after birth, compared to ICC. This discrepancy may be due to the shorter duration of intact-cord resuscitation in the Foglia et al. study compared to our lamb study, as the umbilical cord was clamped after consistent qualitative colorimetric CO2 detection, which occurred after a median ventilation duration of only 14 (IQR 11-19) seconds. Alternatively, it may suggest that the cardiopulmonary benefits we observed are less apparent beyond the first 2 hours after birth. We anticipate that these questions will be answered in an appropriately powered randomised study, as is currently underway in The Netherlands and Belgium led by DeKoninck et al. (NL69575.078.19).

Further investigating the unique impacts of immediate cord clamping on infants with hypoplastic lungs

In Chapter 3.2, we demonstrated that DH lambs responded to ICC with significantly greater physiological disturbances than healthy control lambs, including sympathetic tachycardia, hypertension, and an autoregulatory increase in carotid blood flow to maintain cerebral oxygen delivery.

The tachycardia observed in our DH lambs that underwent ICC is interesting, as in preterm lambs ICC is associated with profound bradycardia.[158] Indeed, increased afterload should stimulate arterial baroreceptors and hence reduce heart rate (albeit to a lesser degree in the fetus and neonate than in the adult), decreased preload should reduce heart rate via the reverse Bainbridge reflex, and hypoxia should reduce heart rate in the fetus and neonate due to the "diving reflex".[386-388] Yet in the setting of increased afterload, reduced preload and severe hypoxia, our neonatal lambs with DH experienced significant tachycardia. However, we began the neonatal ventilation with one or two 30 sec sustained inflations, which may have increased heart rate via the Hering-Breuer inflation reflex.[389] Hypercapnia, seen to a greater degree in DH lambs, also stimulates an increase in heart rate, likely by provoking catecholamine release from the cardiac sympathetic nerve.[390] The interaction between these factors affecting heart rate during the neonatal transition in DH lambs certainly warrants further study.

Sustained inflations to initiate respiratory support for infants with a CDH

In our PBCC strategy, we began neonatal ventilation with a 30 sec sustained inflation, as we have previously shown that this allows more rapid alveolar recruitment and a faster increase in PBF at birth.[391-393] Hence, we may have underestimated the benefits of PBCC, as our ICC lambs reached tidal volume and established pulmonary gas exchange more rapidly than if a sustained inflation had not been used (as occurs clinically). However, the sustained inflation was performed using room air, so ICC lambs did not receive supplemental oxygen until at least 90 sec after the umbilical cord was clamped. In contrast, as PBCC lambs were already on intermittent positive pressure

ventilation at the time of umbilical cord clamping. Hence, PBCC lambs could immediately receive supplemental oxygen when the cord was clamped and they became solely reliant on pulmonary gas exchange. This raises the important question of whether a sustained inflation should be performed with supplemental oxygen, which is particularly relevant after ICC as there is no supplemental placental gas exchange.

While a sustained inflation results in better lung mechanics at birth, a recent study in pre-term lambs suggests that it is associated with long-term lung injury compared to initiating the respiratory transition with conventional ventilation.[394] In that study, Tingay *et al.* propose that a sustained inflation preferentially aerates more compliant regions, creating injury within the non-dependent lung due to continuous stretch at high distending pressure, and leading to atelectasis within the dependent lung.[394] Instead, they suggest that a gradual aeration strategy with dynamic positive end-expiratory pressure is a more appropriate initial ventilation strategy. However, the inflations performed in that study were sustained for up to 180 sec, so our 30 sec sustained inflation likely did not cause the same degree of stretch-related injury.

While the lamb study by Tingay *et al.* raises concerns regarding the use of sustained inflations in CDH infants, the recent SAIL trial (Sustained Aeration of Infant Lungs) appears less relevant to the CDH population.[395] The increased early mortality in the sustained inflation group of the SAIL trial (7.5% vs 1.4% died within 48 hours of birth, p=0.002) likely reflects the failure of non-invasive ventilation against an actively adducted glottis immediately at birth, rather than an inherent failure of sustained inflation. Indeed, a sustained inflation, when delivered non-invasively, is only effective if the infants are breathing.[396] Animal experiments suggest this is due to active glottic adduction, as the glottis and epiglottis are predominantly closed until a stable breathing pattern is established.[397]

Supplemental oxygen requirements during PBCC

Standardised guidelines for neonatal management of infants with a CDH suggest that FiO_2 should begin at 100% and be titrated to achieve preductal oxygen saturations between 80 – 95%.[183] As we demonstrated that PBCC avoids the severe, transient hypoxia associated with ICC, infants managed with PBCC may not require supplemental

oxygen initially. As placental gas exchange continues while the umbilical cord remains unclamped, arterial oxygenation remains at normal fetal levels regardless of alveolar oxygenation. Our group has previously shown that alveolar oxygenation is not required to induce the increase in PBF at birth, despite concerns that alveolar hypoxia may induce local pulmonary vasoconstriction.[165] Indeed, unnecessary alveolar hyperoxia may simply induce reactive oxygen species that damage the pulmonary vasculature. Furthermore, while the normal fetal PaO_2 inhibits respiratory drive, infants with a CDH are intubated and hence are not required to spontaneously breathe at birth.[398] Clearly this is an area that requires further study, and we have already designed preclinical experiments to address the question of supplemental oxygen requirements during PBCC.

The optimal moment of umbilical cord clamping

As discussed in Chapter 1.2, the moment of umbilical cord clamping should not be based on an arbitrary timepoint. Instead, the umbilical cord should be clamped when the infant is ready to transition from placental to pulmonary gas exchange. We used tidal volume as a proxy for this moment, believing that if the lungs are fully aerated, then the lamb is ready to transition to pulmonary gas exchange. However, pulmonary perfusion is also important for gas exchange. 3 of our 11 PBCC lambs reached their target tidal volume within 210 sec, however, all DH lambs had low PBF at this timepoint (Chapter 3, Figure 2b). This suggests that for these 3 animals, we clamped the umbilical cord before their lungs were adequately perfused. On the other hand, the remaining 8 PBCC lambs did not experience hypoxia despite not achieving their target tidal volume when their umbilical cords were clamped at 10 min after birth, so we may have clamped their umbilical cords later than necessary. This experience suggests that determining the moment of cord clamping based on PBF would be superior. Unfortunately, it is difficult to assess PBF in human neonates. Instead, we propose using a combined measure of pulmonary ventilation and perfusion, such as the expired CO2, or waiting until the infant is stable, defined as achieving pre-ductal oxygen saturation of over 85% with less than 40% FiO2.[191, 197, 384]

Regardless, current protocols that emphasise 1 – 3 min of delayed cord clamping clearly need revising. In lambs, our group has shown that it takes up to 20 min to establish a stable breathing pattern and for the PBF waveform to adopt the neonatal phenotype after birth, and that umbilical cord clamping can be safely delayed for this duration.[181] A study of over 15,000 healthy term births in a rural Tanzanian hospital found that the risk of death or admission to the neonatal care unit decreased by 20% for every 10-second delay in umbilical cord clamping after the onset of spontaneous respirations.[399]

The timing of umbilical cord clamping should not be based upon optimising placental transfusion, as emerging evidence suggests that the primary benefit for delaying umbilical cord clamping relates to achieving a more stable cardiopulmonary transition rather than placental transfusion.[382] Indeed, we did not observe any difference in neonatal haemoglobin concentration or haematocrit between DH lambs that underwent PBCC compared to ICC. However, this may be due to our mode of delivery, as all lambs were delivered by caesarean section. When twins are born vaginally, first-born twins have significantly lower haemoglobin levels than second-born twins.[400, 401] This difference is not evident in twins delivered by caesarean section, suggesting that placental transfusion only occurs during spontaneous vaginal births. Alternatively, the absence of an observed placental transfusion may relate to our mechanical ventilation strategy, as intermittent increases in intrathoracic pressure and positive end-expiratory pressures may restrict placental transfusion. However, a recent study in spontaneously breathing preterm lambs also did not find evidence of any placental transfusion.[181]

From the preceding discussion, it is clear that our approach to delivery room management should be re-defined as a period of perinatal stabilisation, rather than neonatal resuscitation. Rather than dictating the time at which a fetus must become a newborn, we should respond to the infant's physiological needs during this complex transition and establish a continuous flow of management that involves collaboration between obstetricians, midwives, neonatologists and neonatal nurses. By enhancing normal physiology, such as providing a sustained inflation to initiate ventilation, and optimising physiological resources, such as allowing placental gas exchange to continue while lung aeration is established, we can provide infants with a CDH a smoother transition to newborn life.

5.4.4 Conclusion

Ultimately, we envisage a future for CDH management in which effective interventions can be offered at each stage of the patient's journey, for each aspect of the infant's condition. After diagnosis on routine obstetric morphology ultrasound at 20 weeks of gestation, the mother could be offered a daily sildenafil tablet to improve fetal pulmonary vascular development. If the severity of CDH is classified as moderate or severe on prenatal imaging, FETO could be employed to improve lung growth and airway development. In the delivery room, all infants with a CDH could remain connected to their mother via the umbilical cord while their lungs are gradually aerated and perfused, so that they experience a smooth transition from placental to pulmonary gas exchange. By addressing both the irreversible and reversible components of PPHN, optimising lung development *in utero* and enabling a smooth transition in the delivery room, we believe that each baby born with a CDH will be given the best possible start to life.

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References

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Appendices

Appendix 1: Antenatal medical therapies for congenital diaphragmatic hernia

Appendix 2: Optimising continuous transplacental antenatal sildenafil treatment for lambs with diaphragmatic hernia

Appendix 3: Reconstructing the pulmonary vascular architecture of lambs with diaphragmatic hernia

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Appendix 1 – Antenatal medical therapies for congenital diaphragmatic hernia

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Antenatal Medical Therapies to Improve Lung Development in Congenital Diaphragmatic Hernia

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Abstract

Congenital diaphragmatic hernia (CDH) is a birth defect characterized by failed closure of the diaphragm, allowing abdominal viscera to herniate into the thoracic cavity and subsequently impair pulmonary and vascular development. Despite improving standardized postnatal management, there remains a population of severe CDH for whom postnatal care falls short. In these severe cases, antenatal surgical intervention (fetoscopic endoluminal tracheal occlusion [FETO]) may improve survival; however, FETO increases the risk of preterm delivery, is not widely offered, and still fails in half of cases. Antenatal medical therapies that stimulate antenatal pulmonary development are therefore interesting alternatives. By presenting the animal research underpinning novel antenatal medical therapies for CDH, and considering the applications of these therapies to clinical practice, this review will explore the future of antenatal CDH management with a focus on the phosphodiesterase-5 inhibitor sildenafil.

Keywords

- congenital diaphragmatic hernia
- antenatal therapy
- fetal
- sildenafil
- pulmonary hypertension of the newborn
- pulmonary hypoplasia

Congenital diaphragmatic hernia (CDH) is a birth defect characterized by failed closure of the diaphragm, allowing abdominal viscera to herniate into the thoracic cavity and subsequently impair pulmonary and vascular development.[1] Normal pulmonary development is predominantly driven by mechanical stretch,[2,3] fetal breathing movements,[4,5] and complex interactions between blood vessels and airways.[6] In CDH, pulmonary development is impaired, causing pulmonary hypoplasia and clinically resulting in respiratory insufficiency and pulmonary hypertension in the newborn,[7]

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which are major determinants of morbidity and mortality in CDH infants.[8]

Despite in utero referral, centralization of care and standardization of postnatal management,[9] there remains a population of severe CDH infants with extremely poor survival chances.[8,10] Overall, 20 to 30% of the 1 in 3,000 live-born neonates with isolated CDH still die, and in severe cases, survival is less than 15%.[11] In these cases, antenatal interventions may improve outcomes by increasing lung growth and possibly improving pulmonary structural development.[12] Until now, most strategies are based on fetal surgical interventions, and the current experimental approach is by fetoscopic endoluminal tracheal occlusion (FETO).[12] Though there is an apparent increase in survival rates, the success of fetal surgery is limited by technical feasibility, the major complication of preterm birth, and the requirement for patient access to experienced fetal surgical centres.[12] Apart from that, in the best case scenario up to 40% of fetuses will eventually not survive because of limited pulmonary response.[13] Because of these limitations, much research effort has been directed toward investigating alternative antenatal medical therapies. In 2015, Eastwood et al published a systematic overview of antenatal medical therapies under investigation in animal models of CDH, predominantly in the nitrofen rat model.[14] Several recent promising findings, particularly in relation to phosphodiesterase-5 inhibitors, warrant an update of this review that will take a more descriptive approach.

By presenting the animal research underpinning novel antenatal medical therapies for CDH, and considering the applications of these therapies to clinical practice, we will explore the future of antenatal CDH management.

Pathophysiology of Congenital Diaphragmatic Hernia

The pathogenesis of CDH remains unclear; however, the leading hypothesis is that abnormal development of the diaphragm's amuscular mesenchymal component results in an incomplete migration platform for muscle precursors.[15] The defect predominantly occurs on the left side (80–90%), the majority of which are located posterolaterally (70%).[1] An incomplete diaphragm allows herniation of abdominal contents into the thoracic cavity from the embryonic phase onward, from then disturbing further lung development.

The process by which CDH impairs pulmonary development is not entirely understood. The classical hypothesis is that herniating abdominal viscera occupies space in the thoracic cavity, placing external pressure on the developing lungs and interfering with fetal breathing movements.[16] Alternatively, Keijzer et al proposed the "dual-hit" hypothesis: early in development a genetic or environmental insult prevents closure of the diaphragm and also impairs bilateral pulmonary branching morphogenesis. Following this initial insult, the herniating abdominal viscera further contributes to pulmonary hypoplasia.[17]

In addition to reduced lung size and airway complexity,[7] increased muscularization and decreased cross-sectional area of distal pulmonary vessels lead to increased pulmonary vascular resistance.[18] Normally at birth, air entry drives lung liquid out of the airways and into the surrounding tissue, which triggers a decrease in pulmonary vascular resistance and subsequent increase in pulmonary blood flow and oxygenation.[19,20] In contrast, pulmonary vascular resistance remains high at birth in most neonates with CDH, often leading to clinically significant pulmonary hypertension.[21] This adds significant postnatal morbidity, as in most cases, infants with pulmonary hypertension need mechanical ventilation to treat pulmonary hypertension-induced hypoxemia, which may contribute to further lung injury. Neonates with CDH and severe pulmonary hypertension have mortality rates ranging from 56.1 to 100%.[8,10,22] Therefore, preventing pulmonary hypertension by identifying and treating these patients in utero may be key to improving their survival.

Limitations of Antenatal Surgical Therapies for Congenital Diaphragmatic Hernia

Tracheal ligation was first described as a method of enhancing lung growth by preventing egress of lung liquid, in 1965.[23] After intensive preclinical experimentation, tracheal occlusion was first achieved clinically in an open procedure that required hysterotomy and fetal neck dissection.[24] This open procedure was associated with severe neurological morbidity and a survival rate of 33%; therefore, efforts were made to investigate less invasive approaches.[24] Initially, this included a maternal laparotomy and multiple trocar insertions, but gradually this evolved to a percutaneous single port procedure that was optimized by the FETO consortium.[25–27]

In 2009, the FETO consortium published its experience of using FETO for isolated CDH in more than 200 cases. Indeed, survival rates were higher than was anticipated based on historical controls. However, despite the use of keyhole surgery (3.3 mm single trocar), preterm premature rupture of membranes (47.1%) and subsequently preterm birth (median gestational age at delivery: 35.3 weeks) are still important complications.[12] Nevertheless, these promising results lead to two large multicenter randomized trials evaluating the efficacy of FETO (www.totaltrial.eu).[28,29] Within these trials, the balloon is inserted at 27 to 30 weeks of gestation in severe cases of CDH and 30 to 32 weeks of gestation in moderate cases.[30]

The current clinical strategy includes reversal of tracheal occlusion because experimental work has shown that if the balloon is not removed, then surfactant deficiency results from transdifferentiation of surfactant-producing type II alveolar epithelial cells into nonsurfactant-producing type I cells.[31–33] Balloon removal also allows the patient to return to the institution where she would normally deliver and potentially have a vaginal delivery. Elective balloon removal is typically scheduled for 34 weeks of gestation either by ultrasound-guided percutaneous puncture or by fetoscopy.[34] A recent series described that in experienced centers in 96.8% of elective procedures, the balloon can be successfully

removed.[35] In 28 to 43% of cases, however, obstetrical complications and, in particular, threatened preterm labor prompted an emergent removal of the balloon.[12,35] In this emergency setting, if release cannot be performed by percutaneous puncture or fetoscopy, extraction may be required on placental circulation. Balloon extraction was initially performed as a formal ex utero intrapartum procedure, yet now is safely done during a modified cesarean section.[12,35,36] As a last resort, the balloon can be removed postnatally with a specially designed tracheoscope or percutaneous puncture.[34] Difficulties in balloon removal played a major contribution to neonatal death in 10 of the 210 cases performed by the FETO consortium;[12] therefore, it is recommended that a 24/7 team of experienced clinicians are on-call to deal with emergency balloon removal.[30] This limits the use of FETO to high-volume tertiary referral centers.

Finally, the success of FETO appears dependent on preexisting lung size, potentially because smaller lungs have less lung liquid secreting epithelium.[12] Furthermore, as lung liquid secretion ceases when the fetal lung's intraluminal pressure reaches ~6 mm Hg,[37] if the pressure required to expand the hypoplastic lung exceeds 6 mm Hg, then FETO will not increase lung expansion. These limitations indicate that further antenatal medical therapies are required to address the remaining morbidity and mortality of severe CDH, preferentially medical in nature to reduce invasiveness and make prenatal therapy more accessible.

Antenatal Medical Therapies for Congenital Diaphragmatic Hernia

This review will predominantly focus on the antenatal medical therapies that show the most promise in correcting pulmonary hypoplasia and pulmonary hypertension in CDH —corticosteroids, retinoids, and phosphodiesterase-5 inhibitors. Several other medical therapies for CDH have been investigated and are extensively reviewed by Eastwood et al.[14]

Recently, there have also been promising results using cell-based therapies to improve pulmonary development in CDH.[38–40] In contrast to medical therapies, cell-based therapies may not only prevent further lung injury but also repair established disease.[41] Regenerative medicine could contribute to the management of CDH on various levels, as recently reviewed by De Coppi and Deprest,[42] but it is beyond the scope of this article.

Corticosteroids

The only antenatal medical therapy for CDH studied in a human randomized controlled trial is the corticosteroid betamethasone, which was administered by intramuscular injection to mothers at 34 weeks of gestation (two doses of 12.5 mg 24 hours apart, then 12.5 mg weekly for 2 weeks).[43] An interim analysis after 32 completed cases (17 steroid, 15 placebo) showed no differences in perinatal mortality, mechanical ventilation time, or days of hospital admission. Based on these preliminary results, the data safety monitoring committee decided to end the study prematurely, as it was determined that to demonstrate any significant difference more than 1,700 fetuses with a prenatally diagnosed CDH would need to be enrolled. The experience of this trial highlights that as a rare condition in which only a subgroup of the most severely afflicted will obtain benefit from antenatal therapy, randomized controlled trials of CDH are only feasible if conducted in a co-ordinated world-wide multicenter approach.

Mechanism of Action

Research efforts initiated by Liggins and Howie[44] and summarized by Ballard and Ballard[45] demonstrated that the timing of fetal lung maturation correlates with a physiological increase in circulating corticosteroids, and that administering antenatal exogenous corticosteroids accelerates lung development. Antenatal corticosteroids improve neonatal lung function by acting at a transcriptional level. The mechanism of action was initially described as accelerating the maturation of alveolar structure, cell differentiation, surfactant production, and lung liquid clearance mechanisms;[46] however, findings from glucocorticoid receptor knockout mice indicate that glucocorticoids predominantly improve lung maturation by restraining hyperproliferation of the interalveolar septum and surrounding mesenchyme.[47,48] The benefits of antenatal corticosteroids to lung development have led to their extensive use to reduce disease associated with developmental immaturity in the setting of threatened preterm delivery, recently summarized by Kemp et al.[49] Observations by George et al that infants with CDH had structurally immature lungs suggested that antenatal corticosteroids may also improve lung development in the setting of CDH.[50]

Pharmacokinetics and Bioavailability

The efficacy of antenatal corticosteroids for CDH was first established in successful rat,[51–54] rabbit,[55–57] and sheep[58,59] models (►**Table 1**). These animal studies predominantly investigated betamethasone and dexamethasone, fluorinated synthetic corticosteroids that cross the placenta from maternal to fetal circulation, have minimal mineralocorticoid activity and are weak immunosuppressants.[49] In the setting of threatened preterm delivery, betamethasone is administered intramuscularly in two 12 mg doses, 24 hours apart.[60] This generates a maximum fetal plasma concentration of ~20 ng/mL, 1 to 2 hours after treatment, with a half-life of 12 hours in the fetal circulation.[45] The maximum maternal plasma concentration of ~100 ng/mL is reached 1 hour after treatment, with a half-life of 6 hours.

Safety

Antenatal corticosteroids are extensively used to improve fetal lung maturation for women with threatened preterm delivery, thereby reducing neonatal mortality and respiratory morbidity. A recent Cochrane review including 30 randomized controlled trials found that antenatal corticosteroid treatment does not increase the risk of neurodevelopmental delay, chorioamnionitis, endometritis, or maternal

Table 1 Antenatal steroids in experimental animal models of congenital diaphragmatic hernia

Domain	Impact of antenatal steroids	
Gross lung size (LBWR)	No significant effect[51–53,55,56,63]	
Airway morphometry	↓ Septal thickness[51,53,58,59]	
	↑ Airspace volume[51,53,58,59]	
	↑ Radial alveolar count[58]	
Vascular Morphometry	↓ Medial wall thickness[53,56,57]	
	↑ Distal vessel number[56]	
Biochemical changes	Unclear effect on surfactant protein expression	No change[51]
		↑[64]
	Unclear effect on eNOS expression	No change[51]
		↑[53]
	Unclear effect on growth factors and proliferation markers	↑ bFGF, PDGF, TGF-β1 expression[54]
		↓ K_i-67 and PCNA mRNA[63]
	Unclear effect on glycogen levels	No change[55]
		↓[59]
	Increases VEGF and VEGF receptor expression	↑[52,67]
		↓[53]
Physiological changes	↑ Postductal PaO_2[59]	
	↑ Dynamic compliance[59]	

Abbreviations: bFGF, basic fibroblast growth factor; eNOS, endogenous nitric oxide synthase; LBWR, lung-to-body weight ratio; mRNA, messenger ribonucleic acid; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor.

death, and reduces the risk of perinatal and neonatal death.[61] It found no definitive evidence suggesting differences between a single course and weekly repeated corticosteroids; however, the Maternal-Fetal Medicine Units Network raises concerns regarding a nonstatistically significant ($p = 0.12$) association between multiple doses of antenatal corticosteroids and cerebral palsy.[62]

Effects on Airways in Diaphragmatic Hernia

Antenatal corticosteroids appear to accelerate lung maturation, but there are concerns that this may be at the expense of lung growth. Accelerated lung maturation is indicated by decreased septal thickness, increased airspace volume, and increased radial alveolar count.[51,53,58,59] On the contrary, lung:body weight ratio appears unaffected by antenatal corticosteroids, and messenger ribonucleic acid (mRNA) expression of proliferation markers K_i-67 and proliferating cell nuclear antigen is reduced.[63] Promisingly, corticosteroids appear to primarily inhibit mesenchymal cell proliferation, which may provide benefit by enhancing perisaccular septation.[47]

The impact of antenatal steroids on surfactant production in CDH is unclear; Davey et al[64] demonstrated that antenatal betamethasone partially restored surfactant protein mRNA expression in sheep, whereas Suen et al[51] found no effect in the rat. It is also unclear whether antenatal corticosteroids enhance[56,65] or reduce[63] the beneficial effects of tracheal occlusion. In current clinical programs, corticosteroids are used due to threatened preterm delivery at around the time of balloon removal (usually at 34 weeks), hence it is often forgotten that they are part of the current clinical prenatal strategy and should be included in experimental models of tracheal occlusion.

Effects on Pulmonary Vasculature in Diaphragmatic Hernia

Antenatal corticosteroids improve the vasodilation response of pulmonary blood vessels by increasing expression of endothelial nitric oxide synthase and K^+ channels;[53,66] however, their effect on vascular proliferation is not yet fully understood. At a biomolecular level, antenatal corticosteroids initially appeared to increase vascular endothelial growth factor (VEGF) receptor expression;[52,67] however, Gonçalves et al found that in ventilated lungs, dexamethasone was associated with decreased expression of VEGF and its vasodilatory receptor Flk-1.[53] Morphologically, antenatal corticosteroids increase distal vessel number and decrease distal vessel muscularization.[53,56,57]

Translation from Animal Models to Clinical Practice

At this time, there does not appear to be additional benefit in providing antenatal steroids to CDH fetuses, other than the well-established benefits of antenatal steroids for infants expected to deliver prior to 34 weeks.[9]

Retinoids

Retinoids are a family of molecules derived from vitamin A, an essential micronutrient required for organogenesis, reproduction, immune competence, cellular differentiation, and vision. The retinoid signaling pathway appears important in normal diaphragmatic and pulmonary development, and it is hypothesized that disruptions in this pathway may contribute to the pathogenesis of CDH.[68]

Pharmacokinetics, Bioavailability, and Mechanism of Action

Vitamin A is obtained from the diet as retinyl esters in meat or β-carotene in vegetables, which are then metabolized to retinol in the liver. After being secreted by the liver and transported by retinol binding protein in the blood, retinol enters cells and is eventually converted to the active metabolite, retinoic acid. Retinoic acid enters the nucleus and regulates gene transcription.

In the lungs, retinoid signaling is an important component of lung budding and branching early in development, and later influences septation and alveolarization.[69]

The retinoid signaling pathway also appears to play an important role in complete closure of the diaphragm. Diaphragmatic defects are present in the offspring of vitamin A–deficient rats[70] and retinoic acid receptor double knockout mice,[71] and infants with CDH have low levels of plasma retinol.[72,73]

In the rat model of CDH, the herbicide nitrofen is used to induce diaphragmatic hernia. Noble et al[74] suggest that nitrofen acts by inhibiting the enzymes that convert retinol to retinoic acid, whereas Nakazawa et al[75] suggest that nitrofen interferes with the cellular uptake of retinol. Importantly, both hypotheses indicate that in nitrofen-induced diaphragmatic hernia, administering vitamin A or retinol will not necessarily increase intracellular levels of the active metabolite, retinoic acid. Considering this, retinoic acid appears to be a more appealing therapeutic agent than vitamin A itself.

Furthermore, while neonatal plasma retinol levels are low in CDH, maternal plasma retinol levels are either equivalent to controls or elevated.[72,73] This suggests impaired placental transfer of retinoids in the setting of CDH; therefore, vitamin A administered to the mother may never reach the fetus. In contrast, retinoic acid appears to cross the placenta in the nitrofen rat model of CDH, as it exerts a therapeutic effect.[76,77]

Safety

The use of exogenous retinoids to prevent pulmonary hypoplasia in CDH requires caution, as retinoid toxicity is associated with teratogenic effects such as spontaneous abortion and cranial neural crest defects.[78] Rothman et al estimate that among mothers who ingest more than 10,000 IU of vitamin A supplements per day, 1 in 57 infants are born with a birth defect attributable to vitamin A.[78]

The dangers of retinoid teratogenicity are demonstrated by cases in which isotretinoin, used to treat severe acne, has inadvertently been taken during pregnancy. Lammer et al[79] determined that of 154 pregnancies exposed to isotretinoin, 95 were aborted electively, 12 spontaneously aborted, and 21 were born with malformations.

Isotretinoin is the 13-cis isomer of retinoic acid and exerts species-dependent teratogenic effects. An extensive body of work undertaken by Nau[80] can be summarized as follows. 13-cis-retinoic acid has a long half-life (16 hours) and extensive access to cell nuclei, however, limited placental transfer. Some, but not all, of the teratogenicity of 13-cis-retinoic acid can be attributed to its continuous isomerization to all transretinoic acid, which has a short half-life (1 hour) but extensive placental transfer and high affinity for retinoic acid receptors. In humans, exogenous 13-cis-retinoic acid is more teratogenic than exogenous all transretinoic acid because continuous isomerization during its long half-life results in higher total fetal exposure to toxic levels of all transretinoic acid.

The teratogenic potency of 13-cis-retinoic acid is more in humans than in rats, likely because the rodent metabolic pathway more efficiently eliminates 13-cis-retinoic acid and placental transfer is almost nonexistent in rodents.[80] In rodents, all transretinoic acid is more teratogenic than 13-cis-retinoic acid.

Studies in the nitrofen rat have administered all transretinoic acid via intraperitoneal injection at a dose of 5 mg/kg/d, however, have not reported rates of craniofacial, thymic, or cardiac malformations.[76,77] Further investigations into potential teratogenicity in the rat, rabbit, and sheep models of CDH are required before antenatal retinoids should be considered for use in humans.

Effect on the Lungs in Diaphragmatic Hernia

The effect of retinoids on lung development varies depending on the time of administration. When administered late in lung development (E16–20 in the nitrofen rat; term = 21 days), antenatal retinoids improved structural maturation without affecting lung growth; the size and number of airspaces were increased (increasing surface area available for gas exchange), distal artery muscularization was decreased, and there was increased expression of VEGF and its receptors, but the lung:body weight ratio was not improved.[76,77] In contrast, when administered early in lung development (E 9– 14 days in the nitrofen rat), antenatal retinoids improved both lung growth and maturation; the lung:body weight ratio was increased and surfactant protein expression was increased.[81,82]

In the nitrofen rat model, a chemical "first-hit" impairs lung growth early in development. Later, once the diaphragmatic defect has allowed herniation of abdominal viscera, a "second-hit" further impairs pulmonary development. Only the "first-hit" is directly related to nitrofen-related impairment of the retinoid signaling pathway, which explains why only early administration of retinoids improves lung growth. Findings from surgical models of CDH are consistent with this hypothesis. In the rabbit model, lung growth was unaffected by antenatal retinoids, and in the sheep model, antenatal retinoids were associated with a decreased lung:body weight ratio.[83,84]

While late antenatal retinoids did not improve lung growth, they did appear to improve lung maturation in nitrofen rats. This likely reflects the importance of retinoid signaling in

septation and alveolarization; thinner interalveolar septa and improved alveolarization were also seen in the sheep model of CDH.[84] These structural changes were associated with improved lung function after delivery of the lambs. On the contrary, septation and alveolarization were unaffected by antenatal retinoids in the rabbit model of CDH.[83]

Translation from Animal Models to Clinical Practice

To improve both lung growth and maturation in the nitrofen rat, antenatal retinoids needed to be administered during the pseudoglandular stage of lung development.[85] In humans, the pseudoglandular stage is complete by ~18 weeks.[85] Given that CDH is predominantly diagnosed at 20 weeks or later in gestation,[86] it may not be possible to administer vitamin A at a stage early enough to improve lung:body weight ratio. However, it is sometimes possible to diagnose CDH in first-trimester screening programs, yet severity assessment has not been validated that early in pregnancy, and the prognosis of these infants is unclear.[87]

Ultimately, vitamin A remains a promising antenatal therapy to improve pulmonary development in CDH that is worthy of further investigation in animal experiments; however, concerns regarding teratogenicity must be addressed and further advances in early antenatal diagnosis are required before it can be considered in a clinical trial.

Phosphodiesterase-5 Inhibitors

The previously discussed antenatal medical therapies display limited benefit, may be teratogenic (retinoic acid), or have failed to demonstrate benefit in humans despite promising animal trials (corticosteroids). In contrast, the phosphodiesterase-5 inhibitor sildenafil has been extensively used in animal and human studies for other maternal–fetal conditions, and has demonstrated promising structural and biochemical pulmonary vasculature changes in small animal models of CDH.[88–95]

Sildenafil's extensive use in erectile dysfunction[96] means that it is now available off-label in a much cheaper generic form. Along with consistent oral bioavailability,[97] this allows sildenafil to be used in low-resource settings—in stark contrast to the highly specialized care required for other antenatal CDH interventions such as FETO.[30,36]

Sildenafil is already used clinically in the postnatal management of persistent pulmonary hypertension of the newborn in CDH neonates,[9] and has been proven to be safe when given to pregnant women for oligohydramnios, or within trials for growth restriction, however, applying its effects antenatally to improve pulmonary development would be an exciting step forward.

Mechanism of Action

Phosphodiesterases are a group of enzymes that play an important role in regulating cardiac smooth muscle tone and vascular smooth muscle contraction.[98] Sildenafil is a relatively selective inhibitor of one of these enzymes, phosphodiesterase-5. By degrading cyclic guanosine monophosphate (cGMP), phosphodiesterase-5 interferes with the nitric oxide–mediated vasodilation process and hence results in vasoconstriction. Sildenafil-induced phosphodiesterase-5 inhibition leads to an increase in the half-life of cGMP, hence vasodilation and increased blood flow (►**Fig. 1**).

Phosphodiesterase-5 is the most active phosphodiesterase in the pulmonary vasculature, hence sildenafil induces pulmonary vasodilation.[99] Pulmonary vasodilation reduces pulmonary vascular resistance, which increases pulmonary blood flow. This mechanism of action allows sildenafil to effectively treat pulmonary hypertension in adults[100] and children,[101] and there is also early evidence in neonatal populations.[102] These patients are particularly sensitive to the effects of sildenafil because phosphodiesterase-5 is upregulated in pulmonary hypertension.[99]

Similarly, phosphodiesterase-5 is upregulated in CDH lungs at birth;[103] therefore, CDH infants are often nonresponsive to inhaled nitric oxide.[104] Sildenafil's acute vasodilatory effect may appear less useful in utero, when gas exchange is not required and pulmonary blood flow is minimal. Yet, in addition to a steady increase in pulmonary blood flow throughout gestation, there are dynamic increases in pulmonary blood flow during accentuated fetal breathing movements.[105] Sildenafil-mediated vasodilation may therefore enhance these episodes by allowing more blood to flow through the lungs during fetal breathing movements, suggesting a potential role in utero for sildenafil's acute vasodilatory effect.

However, in utero the long-term effects of sildenafil are likely more important. While its effects on pulmonary vascular resistance and pulmonary blood flow are transient, antenatal sildenafil mediates a sustained change in vascular reactivity to birth-related stimuli (such as hyperoxemia) that persists to delivery.[106] Sildenafil also exerts an antiproliferative effect on pulmonary artery smooth muscle cells, so may reduce the increased distal artery muscularization characteristic of CDH.[107] Decreased muscularization may be due to upregulation of antiproliferative bone morphogenetic protein.[108] Furthermore, phosphodiesterase-5 suppresses VEGF expression, so by inhibiting phosphodiesterase-5, sildenafil may also improve VEGF-driven angiogenesis and therefore restore the reduced number of distal vessels seen in CDH.[109] VEGF-driven angiogenesis also creates mature alveolocapillary units[110] that will appropriately match ventilation and perfusion during neonatal life, reflecting the complex interactions between airway and vascular pulmonary development discussed earlier.[111]

Sildenafil may exert these long-term effects directly, or they may be secondary to increased cGMP levels.[107] Interestingly, increased cGMP inhibits phosphodiesterase-3 (a cAMP-specific phosphodiesterase) and hence also leads to increased cAMP levels.[112] cAMP is known to have antiproliferative effects on smooth muscle, which may explain sildenafil's effect on distal arterial musculature.[112]

Pharmacokinetics and Bioavailability

The impact of antenatal sildenafil on fetuses with CDH has been predominantly studied in the nitrofen rat model[88,89,91–95] and also one rabbit study has been published so far (►**Table 2**).[90] An alternative phosphodiesterase-5 inhibitor, tadalafil, has recently been investigated in the sheep model.[113]

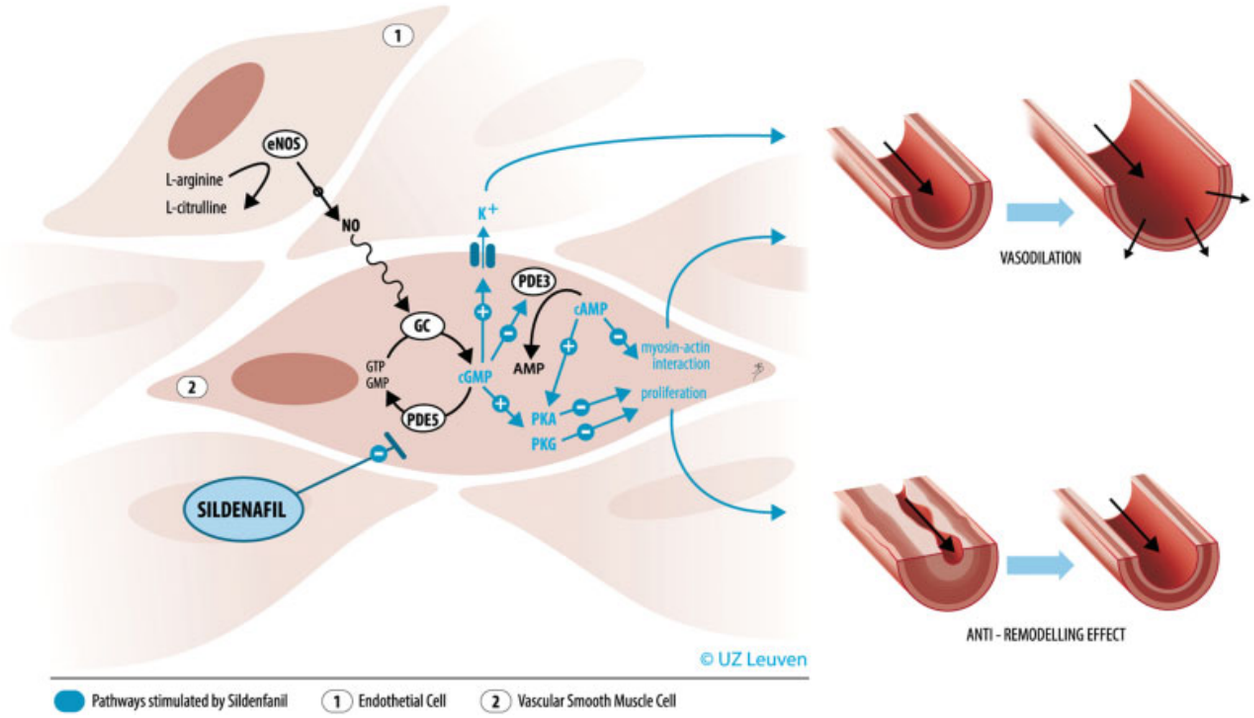

Fig. 1 Schematic representation of the mechanism of action of sildenafil on the pulmonary vasculature. AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic AMP; cGMP, cyclic GMP; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; GMP, guanosine triphosphate; GTP, guanosine triphosphate; NO, nitric oxide; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase. (Reproduced with permission from UZ Leuven, Leuven, Belgium. Drawing by Myrthe Boymans.)

Tadalafil is a more potent and selective phosphodiesterase-5 inhibitor; therefore, a lower dose is required to achieve the same effect relative to sildenafil.[113] Furthermore, its longer half-life means that serum levels would be more consistent with daily oral dosing. Unfortunately, human neonates are unable to properly metabolize tadalafil due to an immature glucuronidation pathway; therefore, it is unlikely to be used clinically as an antenatal therapy.[114] Nevertheless, when sildenafil treatment was compared with tadalafil in neonates with persistent pulmonary hypertension, both were shown to decrease mean pulmonary artery pressure with no significant difference between the two treatments.[115]

The pharmacokinetics of sildenafil differ between species, mostly due to rate of metabolism.[116] To obtain plasma levels within the therapeutic range (47–500 ng/mL[90]), rabbits require 10 mg/kg/d and rodents require 100 mg/kg/d (►**Table 2**).[90–95]

In both species, there is excellent bioavailability following both oral and subcutaneous administration, hence either

Table 2 Antenatal sildenafil in experimental models of congenital diaphragmatic hernia

Author, y	Dose	Route	Timing
Rat models (gestational term: ED 22)			
Luong et al, 2011[92]	100 mg/kg/d Sildenafil	Subcutaneous injection	ED 11.5–20.5
Kattan et al, 2014[95]	45 mg/kg BD Sildenafil	Oral	ED 14–22
Lemus-Varela et al, 2014[91]	100 mg/kg/d Sildenafil	Oral	ED 16–20
Yamamoto et al, 2014[94]	100 mg/kg/d Sildenafil	Subcutaneous injection	ED 11.5–20.5
Makanga et al, 2015[93]	100 mg/kg/d Sildenafil	Oral	ED 11–21
Burgos et al, 2016[88]	100 mg/kg/d Sildenafil	Subcutaneous injection	ED 11.5–20.5
Mous et al, 2016[89]	100 mg/kg/d Sildenafil	Oral	ED 17.5–20.5
Rabbit model (gestational term: GA 31)			
Russo et al, 2016[90]	10 mg/kg/d Sildenafil	Subcutaneous injection	GA 24–30
Sheep model (gestational term: GA 145)			
Shue et al, 2014[113]	2 mg/kg/d Tadalafil	Oral	GA 75–135

Abbreviations: ED, embryonic day; GA, days of gestational age.

method is appropriate.[116] In the majority of rodent models, sildenafil was administered from the pseudoglandular stage onward;[88,91–95] however, in the most recent study, sildenafil treatment only began during the more clinically relevant canalicular stage (the stage during which human CDH is usually detected at 18–20 weeks ultrasound).[89] In rabbits, it was administered from the canalicular stage onward.[90]

The first important finding from these studies is that maternal sildenafil successfully crosses the placenta in all current animal models. Interestingly, in sheep, fetal tadalafil concentrations remain at a steady state despite fluctuating maternal levels (►**Fig. 2**).[113] This steady state may reflect low fetal metabolism of tadalafil in sheep fetuses, as in human neonates.[114] These steady fetal concentrations are not seen when sildenafil is used in the rat[92] and rabbit[90] models. Sildenafil clearance in human neonates increases threefold (to adult levels) during the first week of postnatal life;[117] therefore, well-designed fetal pharmacokinetic studies are required to ensure that sildenafil does not accumulate in the fetal circulation when administered antenatally.

It is evident that transplacental sildenafil and tadalafil both exert a biochemical action on the fetal lungs, with an increase in pulmonary cGMP concentration following maternal administration in both the rat and sheep model.[92,113]

Safety

Human safety data specific to fetuses with CDH are not available for sildenafil, and there have been no adverse effects reported in animal models of CDH.[92]

In the neonatal population, using sildenafil to treat pulmonary hypertension was not associated with higher rates of any adverse effects in three small randomized controlled trials.[118–120] On the contrary, a randomized controlled trial investigating sildenafil in the pediatric population found that high-dose sildenafil is associated with greater mortality than low-dose sildenafil.[121] This association led to the U.S. Food and Drug Administration recommendation against the use of sildenafil in the pediatric population.[122] However, several expert groups have refuted this recommendation due to limitations of the trial, including inconsistent plasma sildenafil concentrations, no survival data for the placebo group, and a large number of confounding variables.[123,124] Furthermore, the European Medicines Agency approved sildenafil use in pediatric pulmonary hypertension (while warning against high doses) based on the same evidence.[125]

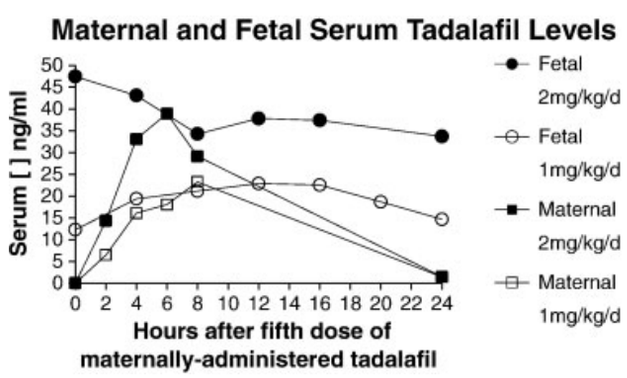

Fig. 2 Maternal and fetal serum tadalafil levels in the sheep model. Tadalafil was given maternally for 5 days, prior to recording data. (Reproduced with permission from Shue et al.)[113]

Antenatally, sildenafil has been investigated in randomized controlled trials to determine its effect upon pregnancy duration in women with preeclampsia,[126] and on amniotic fluid volume in pregnancies complicated by idiopathic oligohydramnios.[127] In both trials, no differences in adverse effects between sildenafil and placebo were observed, in either mother or fetus.

Antenatal sildenafil is also under investigation to increase placental blood flow in intrauterine growth restriction (IUGR) in the international STRIDER trial.[128] A recently published (2016) meta-analysis was unable to draw conclusions about the effect of antenatal sildenafil in IUGR, due to the lack of studies with placebo comparison,[129] but the ongoing randomized controlled trial has not reported any serious adverse effects to date.[128] Animal trials have raised controversy over whether sildenafil displays a beneficial[130] or detrimental[131] effect on the uteroplacental circulation. Concerns regarding adverse fetal effects have also been raised in an ongoing study in a sheep model of IUGR.[132] It is important that a safety profile is established in animal models of diaphragmatic hernia before sildenafil is considered for clinical trials in CDH.

Effect on the Lungs in Diaphragmatic Hernia

Sildenafil appears to be associated with several biomolecular changes, such as supporting a provasodilatory expression profile (►**Table 3**).[92,93,113] Sildenafil also restores the vasodilatory response to stimuli such as nitric oxide and oxygen that is impaired in CDH.[92,94] Furthermore, increases in bone morphogenic protein signaling (antiproliferative) and Bax/Bcl-2 ratio (proapoptotic) may underlie a reduction in vessel muscularization.[93] There is also increased expression of VEGF in the lung parenchyma, which may drive the observed increase in distal vessel number.[90] Sildenafil has no effect on phosphodiesterase-5 RNA expression but decreases the distribution of phosphodiesterase-5 particularly in distal pulmonary vessels.[89]

Sildenafil improves pulmonary vascular development in experimental models of CDH by increasing distal vessel number[90,92,93,95] and decreasing distal vessel muscularization (►**Table 4**).[89–93] Sildenafil did not significantly increase total vascular volume;[89] however, this is less important than the ratio of distal to proximal vessels (as a larger proportion of distal vessels provides greater cross-sectional area, therefore, lower resistance).

A concerning finding is that when sildenafil was given to control animals (i.e., without CDH), it decreased the number of distal vessels[90,92] and decreased total vascular volume.[89] The authors hypothesized that sildenafil-mediated vasodilation was beneficial in lungs with increased pulmonary vascular resistance, but in normal lungs, hypoperfusion of the pulmonary vascular bed may impair vessel growth.[90] These findings could alternatively be explained by the fact that while cGMP (increased following sildenafil-induced

Table 3 Antenatal sildenafil mediates biomolecular and physiological changes

Author, y	Vasodilator expression	Vasoconstrictor expression	Vasoreactivity to vasodilatory stimuli
Luong et al, 2011[92]	↑ eNOS ↑ VEGF	NA	↑ Vasodilatory response to NO donor
Yamamoto et al, 2014[94]	NA	NA	↑ Vasodilatory response to maternal hyperoxia
Makanga et al, 2015[93]	↑ eNOS ↑ iNOS ↑ NO	↓ ET1 ↓ ETA ↓ PPET1	NA
Burgos et al, 2016[88]	NA	NA	NA
Russo et al, 2016[90]	NA	NA	NA
Shue et al, 2014[113]	↑ eNOS	NA	NA

Abbreviations: eNOS, endothelial NO synthase; ET1, endothelin-1; ETA, ET1 receptor A; iNOS, inducible NO synthase; N/A, not applicable; NO, nitric oxide; PBF, pulmonary blood flow; PPET1, ET1 precursor; VEGF, vascular endothelial growth factor.

phosphodiesterase-5 inhibition) is important for vasodilation and angiogenesis, sustained exposure at high levels can lead to lung endothelial cell death and apoptosis.[133] While sildenafil may attenuate the reduced cGMP levels characteristic of CDH,[92] its use in healthy fetuses may increase cGMP to toxic levels.

Sildenafil also appears to have some beneficial effects upon airway development (►**Table 5**). The effect of sildenafil on gross lung size is unclear, with the majority of the evidence showing no difference,[90,92,93,113] one rat model demonstrating a significant decrease[88] and two rat models demonstrating a significant increase in lung weight.[89,94] At a histological level, sildenafil appears to increase distal airway complexity.[89,90,93,94] On a functional level, rabbit lungs treated with sildenafil demonstrate improved static compliance and total lung capacity.[90]

Translation from Animal Models to Clinical Practice

Most investigations into antenatal sildenafil in CDH have been performed in the nitrofen rat model, which does not allow assessment of the physiological transition at birth. Ultrasound assessments suggest that sildenafil reduces pulsatility index, an indicator of pulmonary vascular resistance.[88,90] In the larger sheep model, it was possible to demonstrate that tadalafil improved pulmonary blood flow during neonatal ventilation.[113] Despite no significant difference observed in mean pulmonary artery pressure, tadalafil did appear to improve pulmonary artery pressure response

Table 4 Antenatal sildenafil mediates changes in vascular morphology

Author, y	Vessel number (distal vessel number or density)	Vessel muscularization (medial wall thickness)	Other
Rat models (sildenafil)			
Luong et al, 2011[92]	In CDH: ↑ In non-CDH: ↓	↓	↓ Right ventricular hypertrophy
Kattan et al, 2014[95]	↑	Proximal vessels: ↑ Distal vessels: –	NA
Lemus-Varela et al, 2014[91]	NA	↓	NA
Makanga et al, 2015[93]	↑	↓	↑ Vessel diameter
Burgos et al, 2016[88]	NA	No effect	NA
Mous et al, 2016[89]	NA	↓	↓ Cellular markers of muscularization
Rabbit model (sildenafil)			
Russo et al, 2016[90]	In CDH: ↑ In non-CDH: ↓	↓	NA
Sheep model (tadalafil)			
Shue et al, 2014[113]	NA	NA	No effect on right ventricular hypertrophy

Abbreviations: CDH, congenital diaphragmatic hernia; N/A, not applicable.

Table 5 Antenatal sildenafil mediates changes in airway histology and function

Author, y	Gross lung size	Septal thickness	Airway complexity	Functional
Rat models (sildenafil)				
Luong et al, 2011[92]	No effect	↓	NA	NA
Lemus-Varela et al, 2014[91]	NA	No effect	No effect	NA
Yamamoto et al, 2014[94]	↑ LBWR	NA	↑ Radial saccular count	NA
Makanga et al, 2015[93]	No effect	No effect	↑ Radial alveolar count	NA
Burgos et al, 2016[88]	↓ LBWR	↓	↑ Alveolar volume density No effect on radial alveolar count	↑ PO_2 No effect on tidal volume
Mous et al, 2016[89]	↑ LKWR	No effect	↑ Alveolar airspace diameter	NA
Rabbit model (sildenafil)				
Russo et al, 2016[90]	No effect	No effect	↑ Distal airway complexity	↑ Static compliance ↑ Total lung capacity
Sheep model (tadalafil)				
Shue et al, 2014[113]	No effect	NA	NA	NA

Abbreviations: LBWR, lung-to-body weight ratio; LKWR, lung-to-kidney weight ratio (used because body weight was significantly different between groups in this study); NA, not applicable; PO_2, partial pressure of oxygen (from a mixed arteriovenous sample).

to inhaled nitric oxide.[113] Further experiments should be conducted in the sheep model to verify these results, and assess their relevance to sildenafil. Meanwhile, we are preparing to move to clinical trials and have recently obtained orphan designation for antenatal sildenafil for CDH by the European Medicines Agency.

Future Directions

Although FETO is limited by complications associated with preterm delivery and technical difficulties, it does significantly improve lung growth and may be associated with a significant decrease in morbidity and mortality in CDH.[12,28] To overcome the limitations while retaining the benefits of FETO, antenatal medical therapies should be investigated not only as alternatives to tracheal occlusion but also for potential synergistic effects. Mixed results from animal experiments make it unclear whether betamethasone and vitamin A have synergistic effects with tracheal occlusion;[64,134–136] however, promising early results from a rabbit model of CDH indicate that there may be synergistic effects of antenatal sildenafil and tracheal occlusion on vascular and parenchymal lung development.[137] FETO improves lung growth and sildenafil appears to improve pulmonary vascular development, so further investigating synergistic effects between the two is an important avenue for future research.

Conclusion

Novel approaches to CDH management are urgently required to further reduce the significant mortality associated with severe CDH because postnatal management occurs too late to prevent lung hypoplasia and pulmonary hypertension. Antenatal surgical approaches are limited by high rates of preterm birth and high technical complexity limiting availability. Of the currently investigated antenatal medical therapies for CDH, the phosphodiesterase-5 inhibitor sildenafil appears the most promising candidate for a future clinical trial. Small animal studies have demonstrated that antenatal sildenafil attenuates vascular remodeling in utero;[90–95] however, further research is required to determine if this leads to a functional improvement during the neonatal cardiovascular transition. We are hopeful that one day a novel antenatal medical therapy such as sildenafil will offer a new treatment modality in the care of pregnancies with severe CDH.

Conflict of Interest
None.

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Appendix 2 - Optimising continuous transplacental antenatal sildenafil treatment for lambs with diaphragmatic hernia

Before beginning the study described in *Chapter 4*, a significant volume of work was required to optimise the route of administration and dose to achieve quantifiable fetal plasma sildenafil concentrations.

Study 1 - Subcutaneous infusion

We planned our study after Russo *et al.* demonstrated that antenatal transplacental sildenafil treatment attenuated pulmonary vascular abnormalities in fetal rabbits with a surgically-induced DH. In that study, sildenafil was administered via daily subcutaneous injection at a dose of 10 mg/kg/day (10 mg crushed sildenafil citrate tablets dissolved in 7 mL saline).[1]

After discussion with our local animal ethics committee, a daily subcutaneous injection from 105 – 138 days of gestational age (d GA) was deemed too distressing for maternal ewes. Instead, we initially administered sildenafil via a continuous subcutaneous maternal infusion. To provide a continuous infusion, a reservoir of sildenafil citrate was attached to an infusion pump mounted to the ewe's back using netting (Tubular-Net, Sutherland Medical Pty. Ltd., Australia). To ensure even distribution in this reservoir, the crushed sildenafil citrate tablets were suspended in a solution made up of 50% OraPlus (Perrigo Australia Pty. Ltd., Australia) and 50% sterile water. OraPlus is a suspending vehicle that contains purified water, microcrystalline cellulose, carboxymethylcellulose sodium, xanthan gum, carrageenan, calcium sulfate, trisodium phosphate, citric acid, sodium phosphate and dimethicone antifoam emulsion.

Study 2 - Maternal intravenous infusion

We soon determined that a continuous subcutaneous infusion was not appropriate, as the infused suspension gradually built up at the infusion site. This may have related to the high viscosity of the suspending vehicle, which was necessary to ensure even distribution of sildenafil in the infusion pack. This subcutaneous infusion site collection was not a problem for Russo *et al.*, or indeed for Satterfield *et al.* who performed daily subcutaneous sildenafil injections in sheep, because a new injection site could be used each day and a suspending agent was not required.[1, 2] Hence, we altered our route of administration to intravenous.

Methods

Diaphragmatic hernia creation surgery

A diaphragmatic hernia was created at ≈80d GA, as described in Chapter 2.1.

Sildenafil infusion

To ensure reliable chronic intravenous access, sterile surgery was performed on pregnant ewes at ≈GA103 (term ≈GA145), to place a maternal jugular venous catheter and carotid artery catheter. Following pre-operative preparation and local anaesthesia using subcutaneous bupivacaine with adrenaline, a small incision in the right neck was made through which vinyl catheters (ID 2.6mm, OD 4.2mm, Dural Plastics Inc., Australia) were inserted and introduced ≈12cm into the jugular vein and carotid artery. Ligatures were used to prevent blood loss, and catheters were primed with heparinised saline (50,000 IU Heparin [Heparin sodium 25,000 IU in 5ml sterile water, Pfizer Australia Pty. Ltd., Australia] in 1 L normal saline [0.9% sodium chloride, Fresenius Kabi Australia Pty. Ltd., Australia]). The incision site was then sutured closed with 3.0 non-absorbable silk (DYSILK, Dynek Pty. Ltd., South Australia).

The carotid artery catheter was used to draw blood throughout the infusion period (105 – 138d GA), to assess regular plasma sildenafil concentration. Arterial blood was sampled at baseline (105d GA), 8 hours after the infusion began, 24 hours, day 2, day

7, day 14, day 21, day 28 and at delivery (138d GA). Approximately 5 mL of blood was collected at each timepoint, then transferred into an ethylene tetra-acetic acid (EDTA; an anti-coagulant) spray-coated tube (8 mg EDTA per 5 mL blood; Sarstedt Australia Pty. Ltd., Australia). Plasma from these samples was isolated via centrifugation (3000 revolutions per minute for 10 min at 4°C; Allegra 21R Centrifuge, Beckman Coulter Australia Pty. Ltd., Australia) and aliquoted into cryovials (Cryo.s; Greiner Bio-One International GmbH, Germany) for overnight storage at approximately -20°C (LG ExpressCool, LG Electronics Australia Pty. Ltd., Australia). 24 hours later, samples were transferred to long-term storage at approximately -80°C (Froilabo Bio Memory Ultra Low, Scientific Partners Australia Pty. Ltd., Australia). Total sildenafil concentration (calculated as sildenafil concentration + 50% of its active metabolite *N*-desmethyl-sildenafil) was analysed using ultra high performance liquid chromatography (Vanquish Flex Quaternary, ThermoFisher Scientific, Australia) in tandem with mass spectrometry (TSQ Quantiva, ThermoFisher Scientific, Australia).[3]

The jugular vein catheter was connected to an infusion pump (CADD-Legacy 1 Ambulatory Infusion Pump Model 6400; Smiths Medical Australasia Pty. Ltd., Australia), which was secured to the ewe's back using netting (Tubular-Net, Sutherland Medical Pty. Ltd., Australia). The intravenous infusion rate was set at 0.66 mg/kg/day (0.0275 mg/kg/hour), to reflect the Satterfield *et al.* dose of 150 mg/day in 90 kg ewes given sildenafil's subcutaneous bioavailability in sheep is ≈40%.[2]

Delivery and ventilation

Lambs were delivered and ventilated at ≈138d GA, as described in Chapter 2.1. Of note, physiologically based cord clamping was performed for all groups in this study. Physiological data was analysed as described in Chapter 2.1.

Results

Plasma sildenafil concentrations

At 7 days after the infusion began, maternal plasma sildenafil concentration was 11.4 ng/mL ± 5.7 ng/ml (mean ± SEM). At delivery, this increased to 27.5 ng/mL ± 9.2 ng/ml. At delivery, fetal plasma sildenafil concentration was 2.0 ng/ml ± 1.5 ng/ml. Regression analysis indicated that fetal levels were 15.4% ± 2.7% (slope ± SE) of maternal levels. Two fetuses had plasma sildenafil concentrations below the lower limit of the therapeutic range, and one fetal sample could not be analysed (Table 1).

Table 1 - Plasma Sildenafil Concentrations in Experimental Animals

	Maternal		Fetal
Animal #	**Day 7** *(ng/ml)*	**Delivery** *(ng/ml)*	**Delivery** *(ng/ml)*
16080	*NA*	53.7	6.2
16076	10.1	10.8	Non-quantifiable
16086	2.2	24.5	1.9
16154A	21.8	*NA*	*NA*
16066	*NA*	21.0	Non-quantifiable

Maternal (day 7 post-infusion, and prior to delivery) and fetal (prior to delivery) plasma sildenafil concentrations in the CDH + Sildenafil group. NA = not analysed. Non-quantifiable = Below lower limit of quantification on our assay.

We performed a subgroup analysis using animals with a quantifiable concentration of sildenafil in the fetal plasma at delivery (**quantifiable sildenafil**). This subgroup analysis is presented in a separate graph following each intention-to-treat analysis, and any findings of statistical significance should be interpreted cautiously due to the small number of animals in this subgroup (n=2).

Characteristics of the Diaphragmatic Hernia

A patent diaphragmatic hernia was observed at delivery in all animals in **CDH + sildenafil**, with stomach and intestines present in the thoracic cavity (Figure 1). The liver remained in the abdominal cavity in all **CDH + sildenafil** animals.

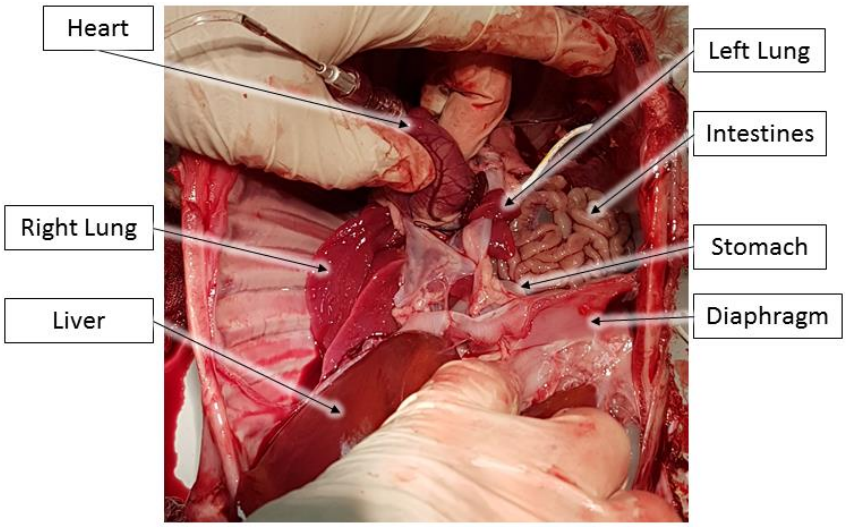

Figure 1- Thoracic Cavity at Post-Mortem Following Delivery at ≈GA138

Superior view of the thoracic cavity at post-mortem (≈GA138) in an animal that had surgical induction of diaphragmatic hernia at ≈84d GA, and then received antenatal sildenafil from GA105. The stomach and intestines are present in the thoracic cavity. A small left-lung is visible, in contrast to the large right lung.

Body and Organ Weights

Body weight was not different between **CDH + sildenafil** and **CDH** (Figure 2). Lung:body weight ratio was 1.7-fold higher in **CDH + sildenafil** as compared to **CDH** (p=0.0168) (Figure 3).

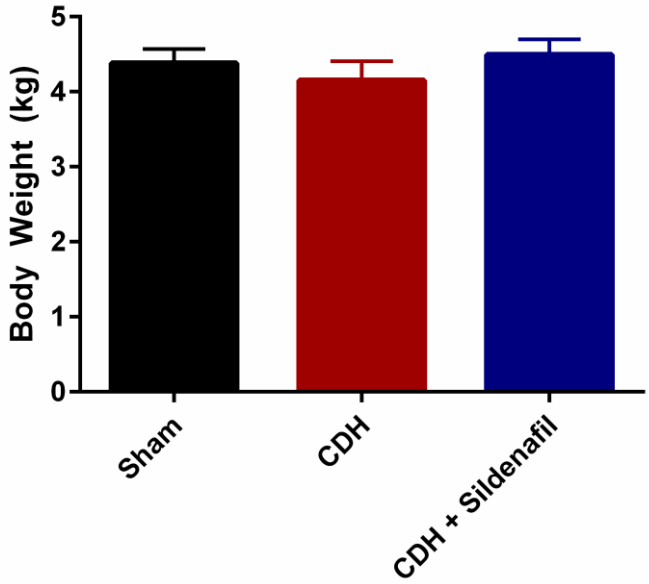

Figure 2 - Lamb Body Weight (kg)

Lamb body weight was determined at post-mortem following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. Groups shown are CDH only (●; diaphragmatic hernia [DH] creation at ≈84d GA) and CDH + Sildenafil (■; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). Sham (▲; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between CDH + sildenafil and CDH only groups.

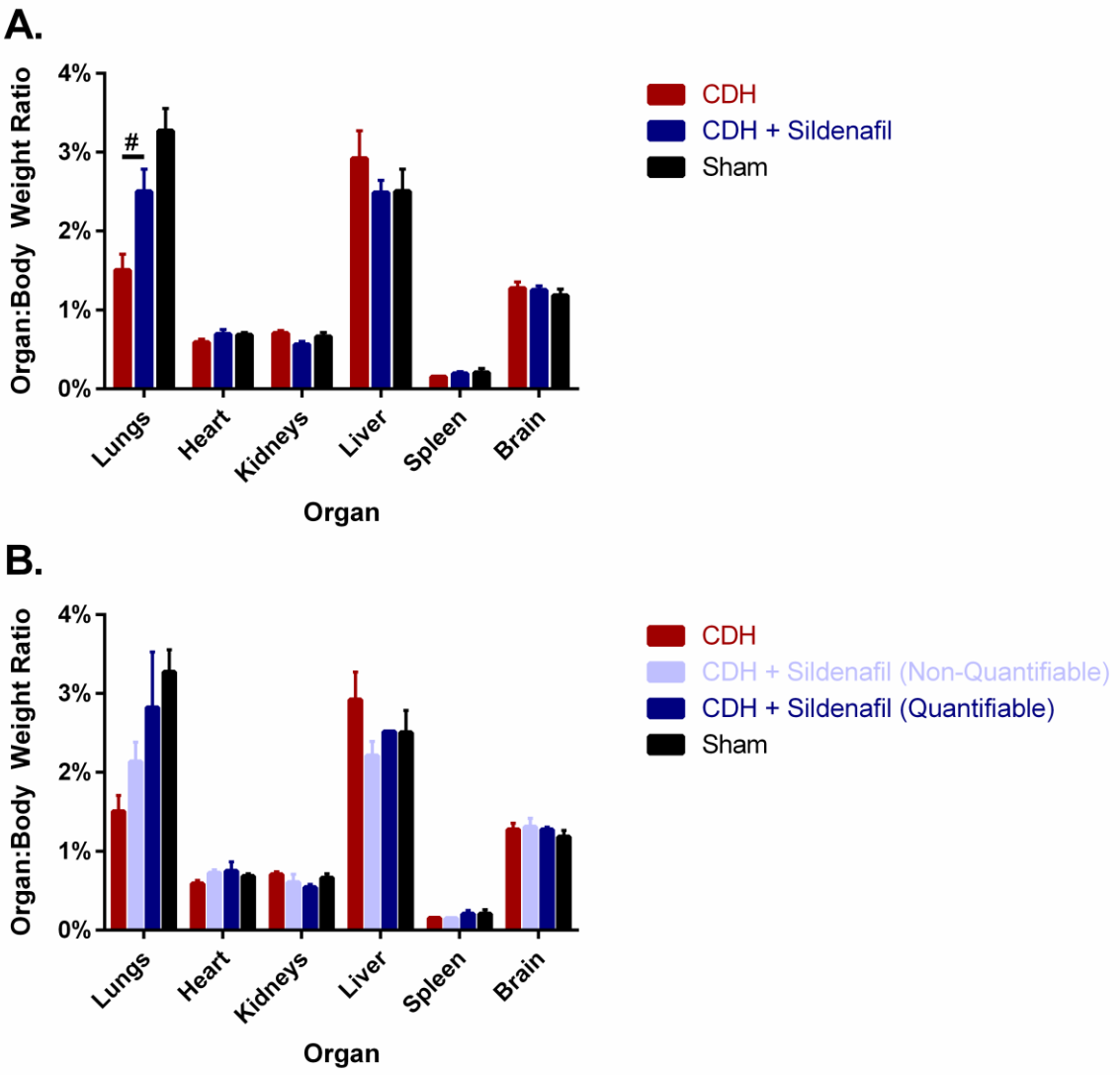

Figure 3 - Organ:Body Weight Ratio (%)

Organ weights were determined at post-mortem following delivery at ≈GA138, and are expressed here as a percentage of lamb body weight. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. **CDH + sildenafil** lung:body weight ratio significantly different to **CDH** ($p=0.0132$). **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

Pulmonary Blood Flow

Total PBF was not different between **CDH + sildenafil** and **CDH**, nor was PBF different at any individual time-point (Figure 4A).

Effect of Quantifiable Sildenafil

Total PBF in **quantifiable sildenafil** was 2.6-fold higher than **CDH** ($p<0.05$) and 2.4-fold higher than **non-quantifiable sildenafil** ($p<0.05$), over the 2-hour ventilation period. There was no difference between **non-quantifiable sildenafil** and **CDH**.

The effect of quantifiable sildenafil was dependent upon ventilation time-point ($p<0.001$). Pulmonary blood flow was significantly increased in **quantifiable sildenafil** compared to **CDH** from 30 – 60 minutes after ventilation onset ($p<0.05$) (Figure 4B). The largest difference in PBF was seen at 40 minutes after ventilation onset, when PBF was 2.5-fold lower in **CDH** compared to **quantifiable sildenafil** ($p=0.002$).

In contrast, PBF was not significantly different between **non-quantifiable sildenafil** and **CDH** at any time-point.

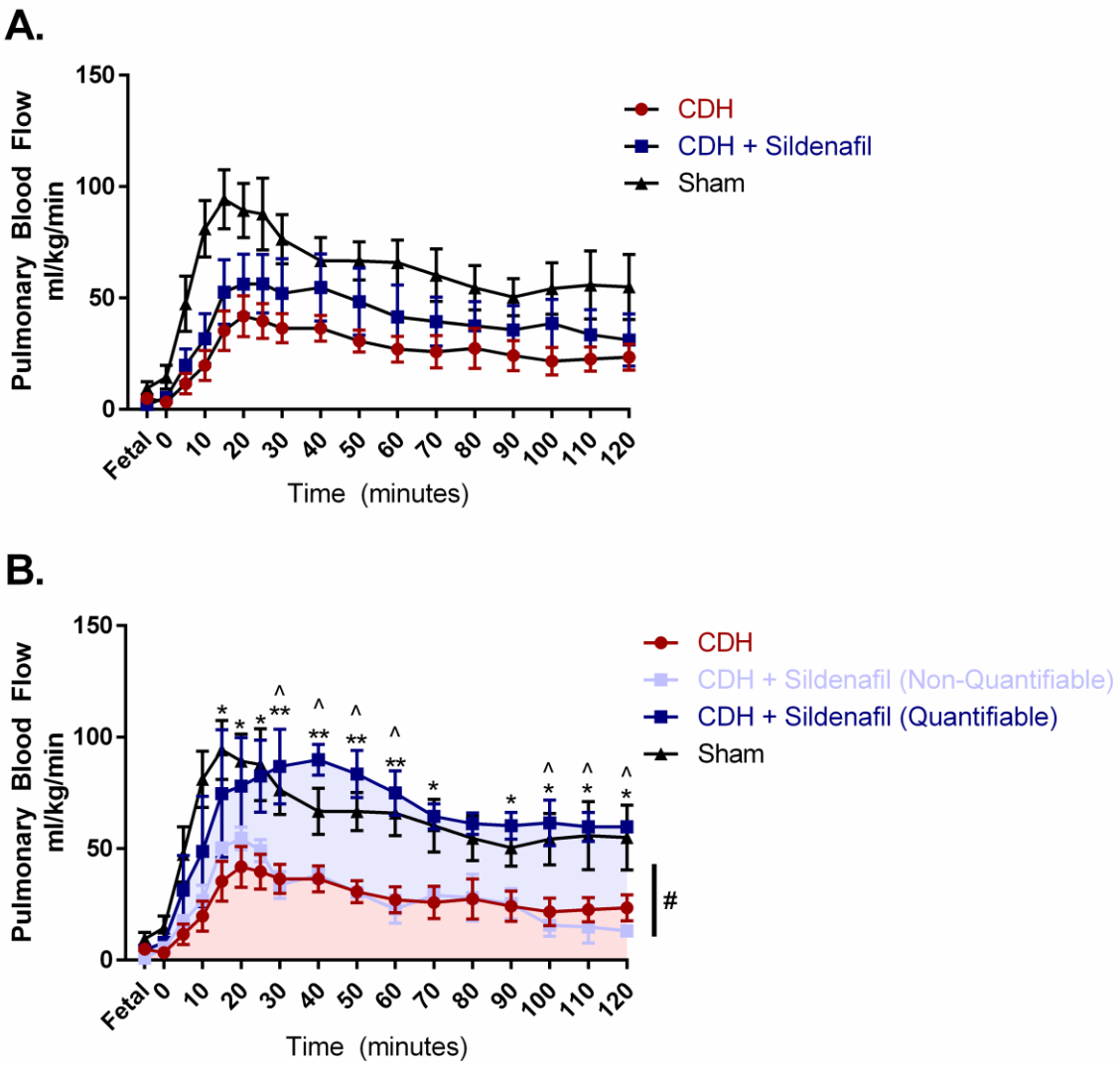

Figure 4 - The effect of antenatal sildenafil on Pulmonary Blood Flow (ml/kg/min) During 2-Hour Neonatal Ventilation

Pulmonary Blood Flow (indexed to body weight) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between **CDH + sildenafil** and **CDH**. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. * **quantifiable sildenafil** significantly different to **CDH** at 15 – 25, 70 and 90 – 120 minutes ($p<0.05$). ** **quantifiable sildenafil** significantly different to **CDH** at 30 – 60 minutes ($p<0.01$). ^ **quantifiable sildenafil** significantly different to **non-quantifiable sildenafil** at 30 – 60 and 100 – 120 minutes ($p<0.05$). # **quantifiable sildenafil** total pulmonary blood flow during 2-hour period (Area Under Curve) significantly different to **CDH** ($p<0.05$).

Pulmonary Blood Flow Waveform

Fetal and neonatal PBF waveforms appeared similar within **CDH**, with the absolute minimum occurring during end-systolic flow, and periods of negative flow (Figure 5). In contrast, fetal and neonatal PBF waveforms appeared different within **quantifiable sildenafil**; during fetal life absolute minimum PBF occurred during end-systolic flow, during neonatal life the absolute minimum occurred during end-diastolic flow. There were also no periods of negative PBF in the representative **quantifiable sildenafil** waveform.

In contrast, **non-quantifiable sildenafil** PBF waveform was similar to **CDH**.

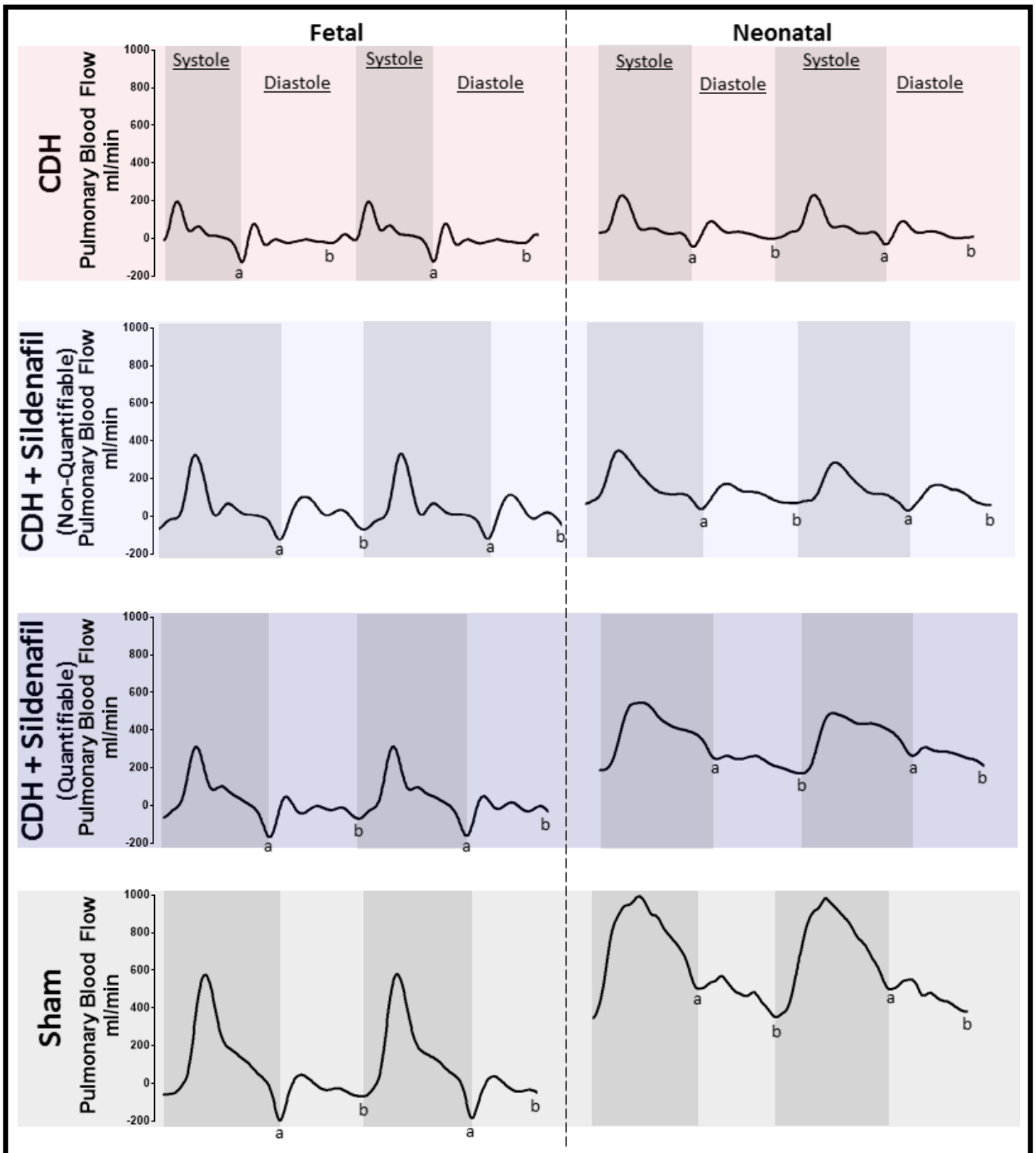

Figure 5 - Pulmonary Blood Flow Waveform During Fetal and Neonatal Life

Representative samples of pulmonary blood flow waveforms obtained throughout 2 consecutive cardiac cycles. Representative fetal and neonatal waveforms are shown from **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA), CDH + sildenafil (non-quantifiable) (■, n=2; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA, non-quantifiable fetal sildenafil concentration), CDH + sildenafil (quantifiable) (■, n=2; non-quantifiable fetal sildenafil concentration) and sham (▲, n=5; thoracotomy only at ≈84d GA). Periods of systolic flow are shaded and periods of diastolic flow are not shaded. (a) End-systolic flow and (b) end-diastolic flow are labelled.

Pulmonary Blood Flow Indexed to Lung Weight

After indexing to lung weight, pulmonary blood flow (both total and at any individual time-point) was not different between **CDH + sildenafil** and **CDH** (Figure 6).

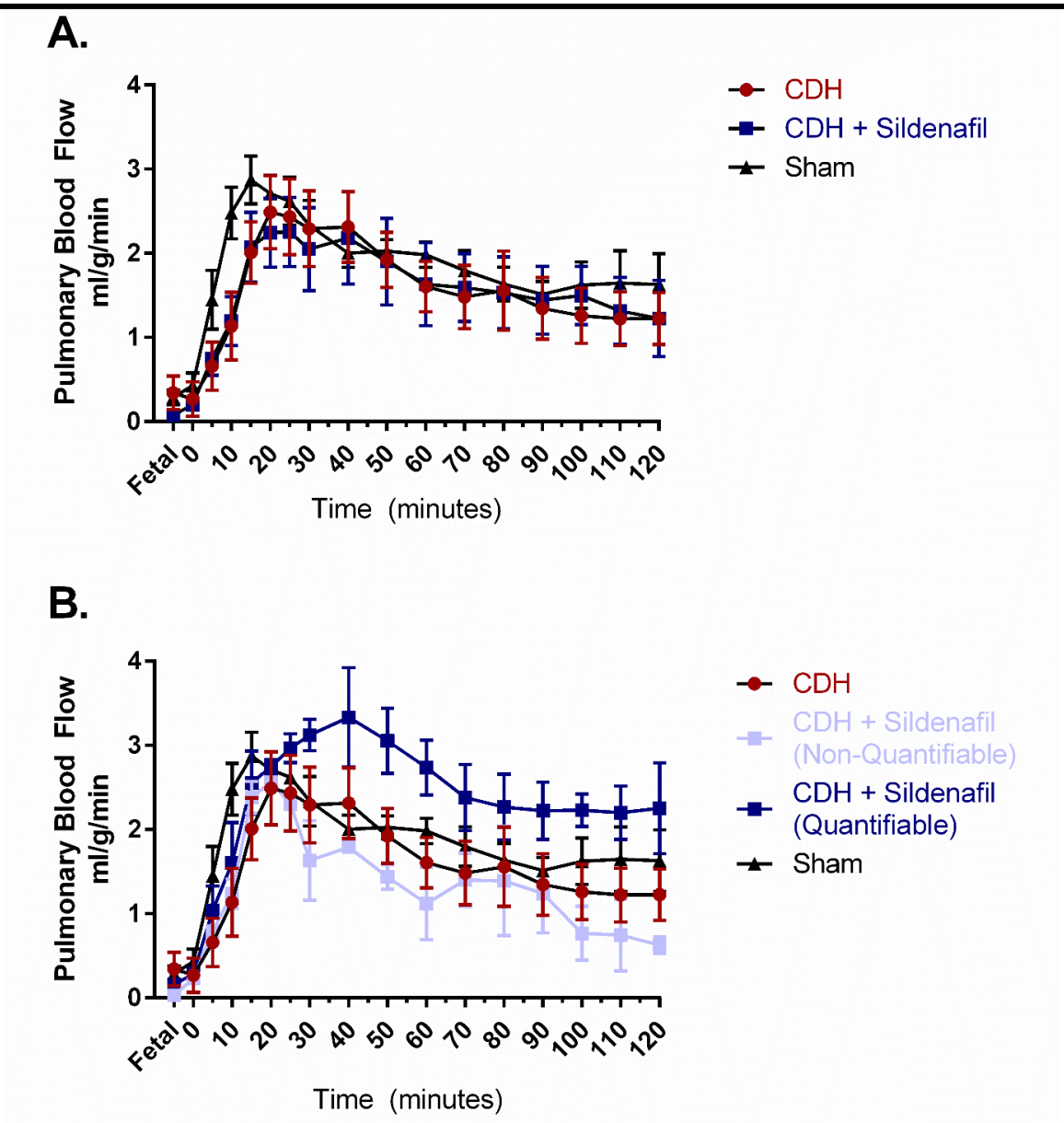

Figure 6 - Effect of Antenatal Sildenafil on Pulmonary Blood Flow Indexed to Lung Weight (ml/g/min) During 2-Hour Neonatal Ventilation

Pulmonary Blood Flow (indexed to lung weight) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between **CDH + sildenafil** and **CDH**. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

Pulmonary Artery Pressure

Pulmonary artery pressure was not different between **CDH + sildenafil** and **CDH** throughout the 2-hour ventilation period, nor was any difference apparent following subgroup analysis (Figure 7).

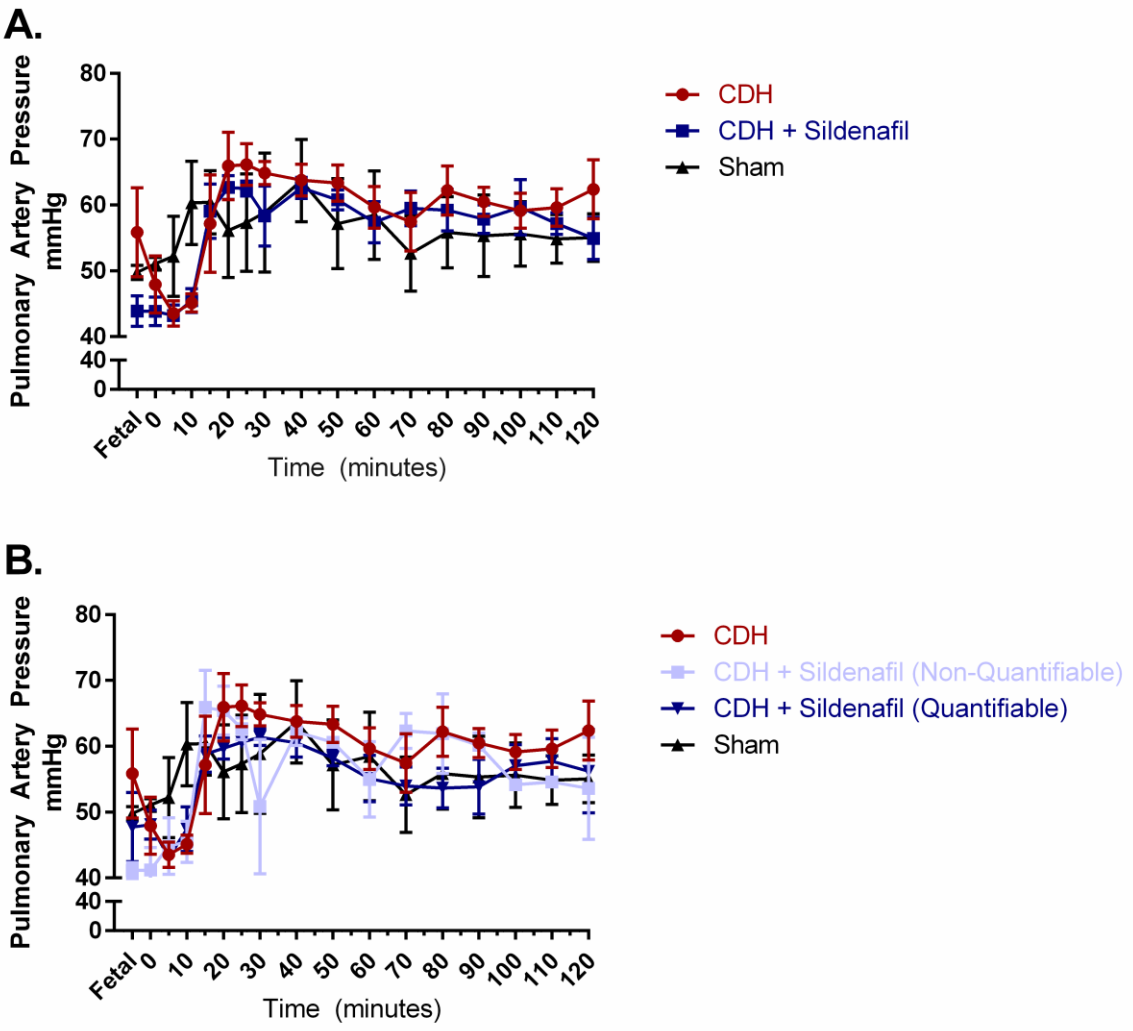

Figure 7 - Effect of Sildenafil on Pulmonary Artery Pressure (mmHg) During 2-Hour Neonatal Ventilation

Pulmonary Artery Pressure over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between **CDH + sildenafil** and **CDH**. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (; n=2) fetal plasma sildenafil concentration. No differences between groups.

Pulmonary Vascular Resistance

Pulmonary vascular resistance was not different between **CDH + sildenafil** and **CDH** from 5 – 120 minutes (Figure 8A), nor was any significant difference apparent upon subgroup analysis (Figure 8B).

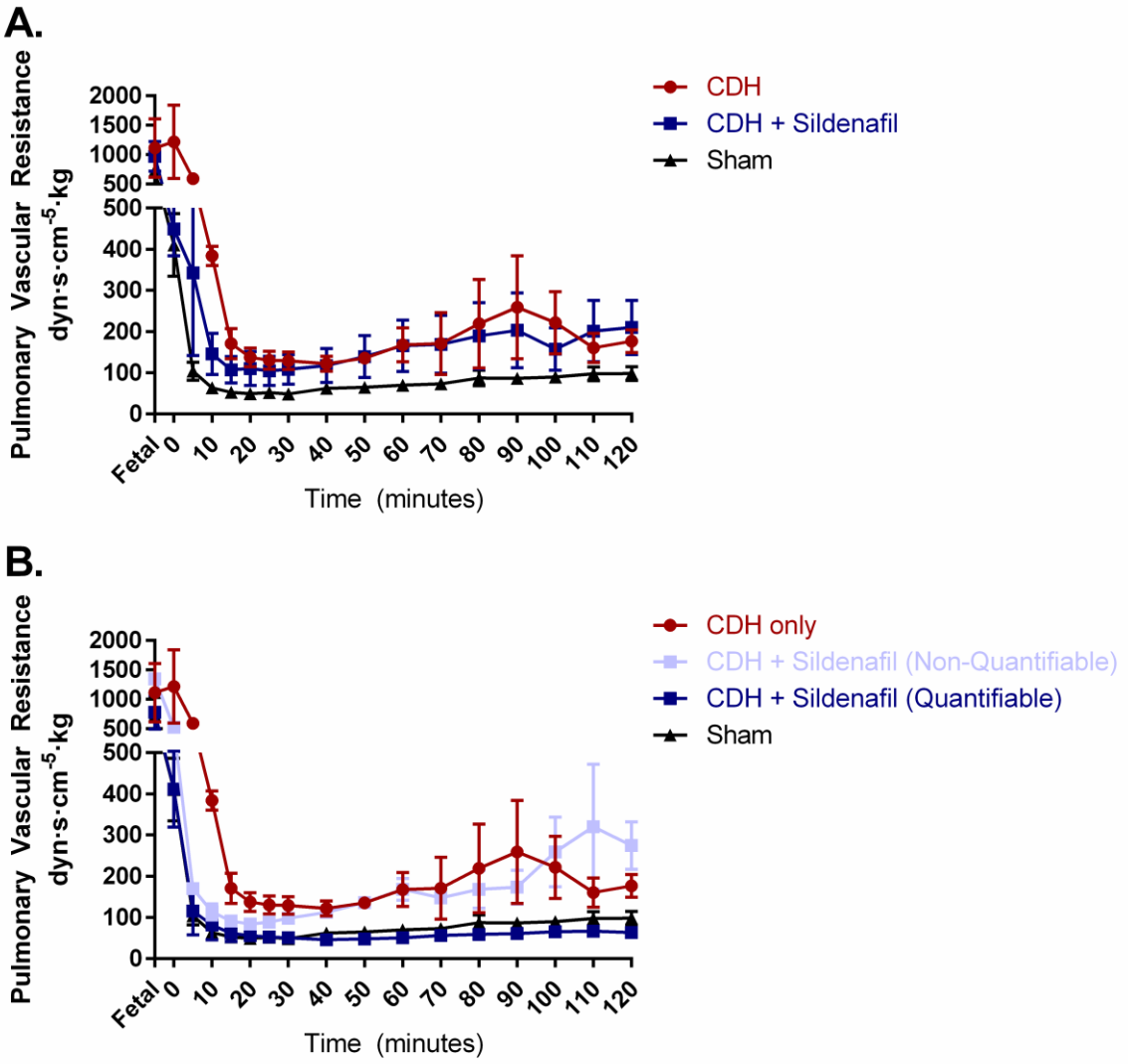

Figure 8 - Effect of Antenatal Sildenafil on Pulmonary Vascular Resistance (dyn·second·cm-5·kg) During 2-Hour Neonatal Ventilation

Pulmonary vascular resistance over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (; n=2) fetal plasma sildenafil concentration. No differences between groups.

Pulmonary Vascular Resistance indexed to lung weight

Pulmonary vascular resistance (indexed to lung weight) was not different between **CDH + sildenafil** and **CDH** during the neonatal period (Figure 9A), nor was there any difference upon subgroup analysis (Figure 9B).

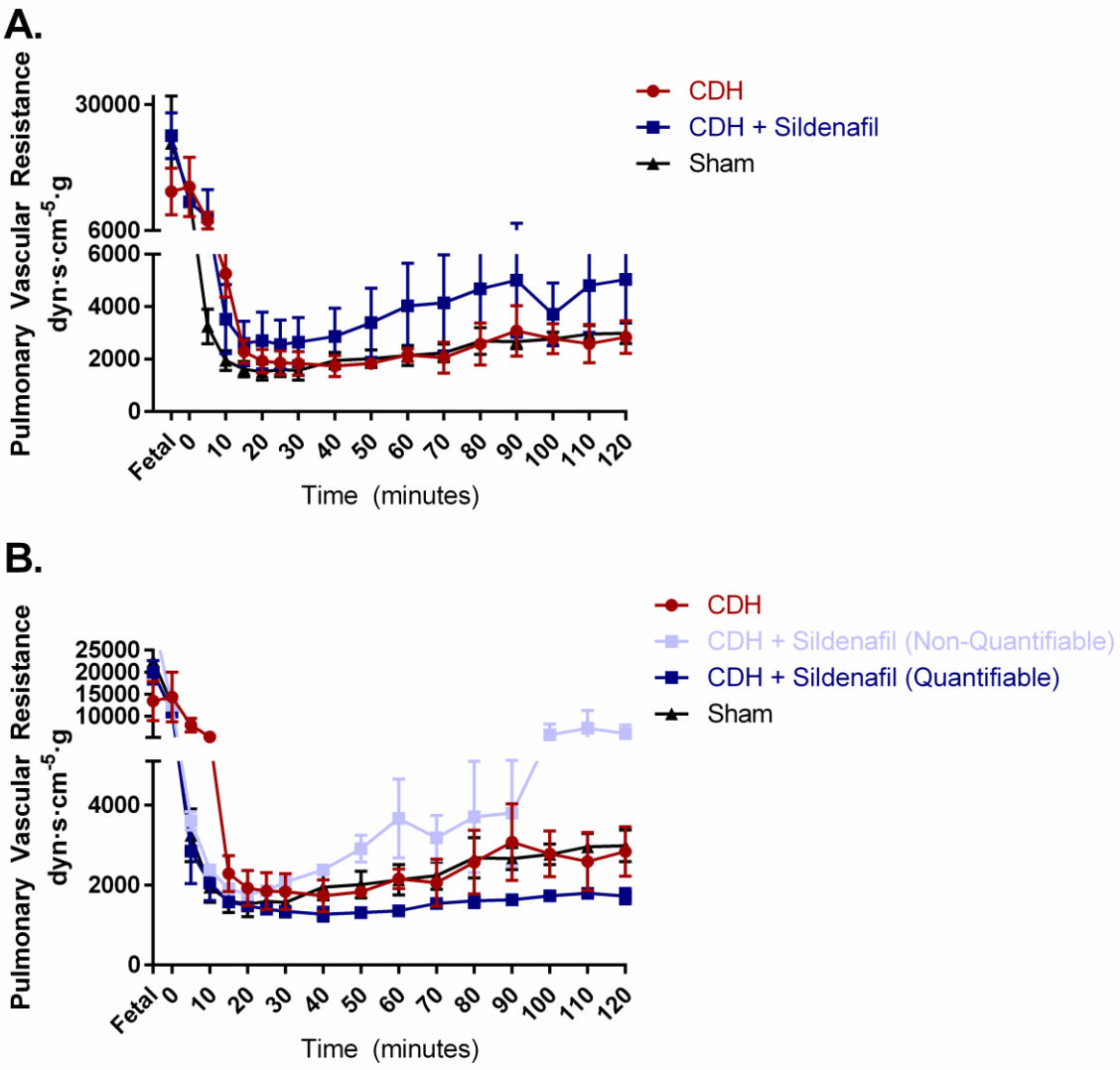

Figure 9 - Effect of Antenatal Sildenafil on Pulmonary Vascular Resistance (dyn·second·cm-5·g) During 2-Hour Neonatal Ventilation

Pulmonary vascular resistance (indexed to lung weight) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (; n=2) fetal plasma sildenafil concentration. No differences between groups.

Pulmonary End Diastolic Flow

Pulmonary end-diastolic flow was not different between **CDH + sildenafil** and **CDH** at any point during the 2-hour ventilation period (Figure 10A). The effect of **quantifiable sildenafil** on pulmonary end-diastolic flow was significantly dependent upon ventilation time-point (p=0.014). Pulmonary end-diastolic flow at 60 minutes was 2.5-fold greater in **quantifiable sildenafil** as compared to **CDH** (p=0.046), however not different at any other time-point (Figure 10B).

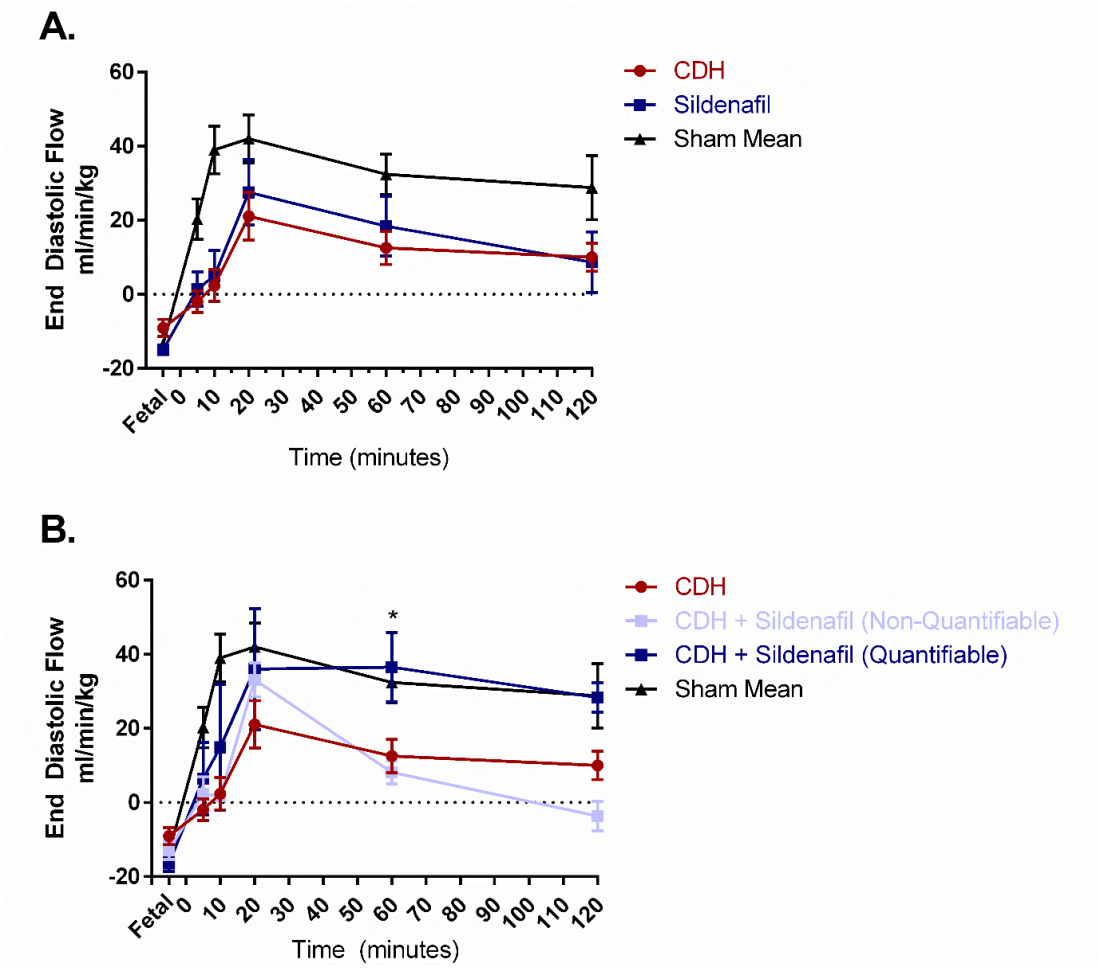

Figure 10 - Effect of Antenatal Sildenafil on End Diastolic Flow (Indexed to Body Weight) Through Left Pulmonary Artery During 2-Hour Neonatal Ventilation

End Diastolic Flow (indexed to body weight) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when p<0.05. Flow less than 0 (dotted line) indicates reversed flow. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (; n=2) fetal plasma sildenafil concentration. * CDH + Sildenafil (Quantifiable) group significantly different to CDH group at 60 minutes (p=0.046).

Effect of Antenatal Sildenafil on Overall Pulmonary Function

Cerebral Oxygen Saturation

Cerebral oxygen saturation was not significantly different between **CDH + sildenafil** and **CDH** (Figure 11), nor were any differences apparent following subgroup analysis of **quantifiable** and **non-quantifiable sildenafil.**

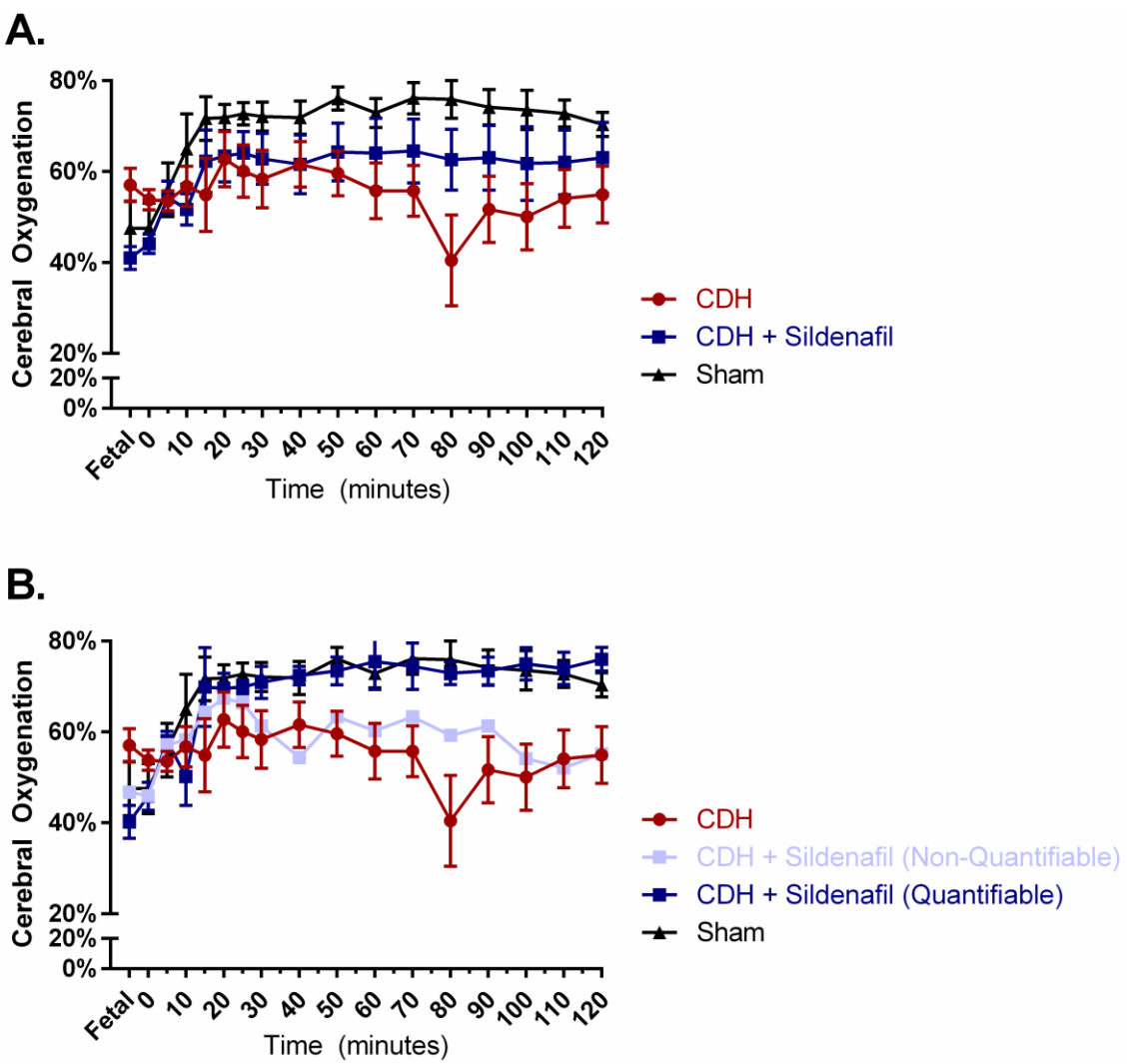

Figure 11 - Cerebral Oxygen Saturation (%) During 2-Hour Neonatal Ventilation

Cerebral oxygen saturation (%) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

Arterial Partial Pressure of Oxygen

Arterial Partial Pressure of Oxygen (PaO_2) was not different between **sildenafil** and **CDH**, nor was any difference apparent upon subgroup analysis of **quantifiable** and **non-quantifiable sildenafil** (Figure 12).

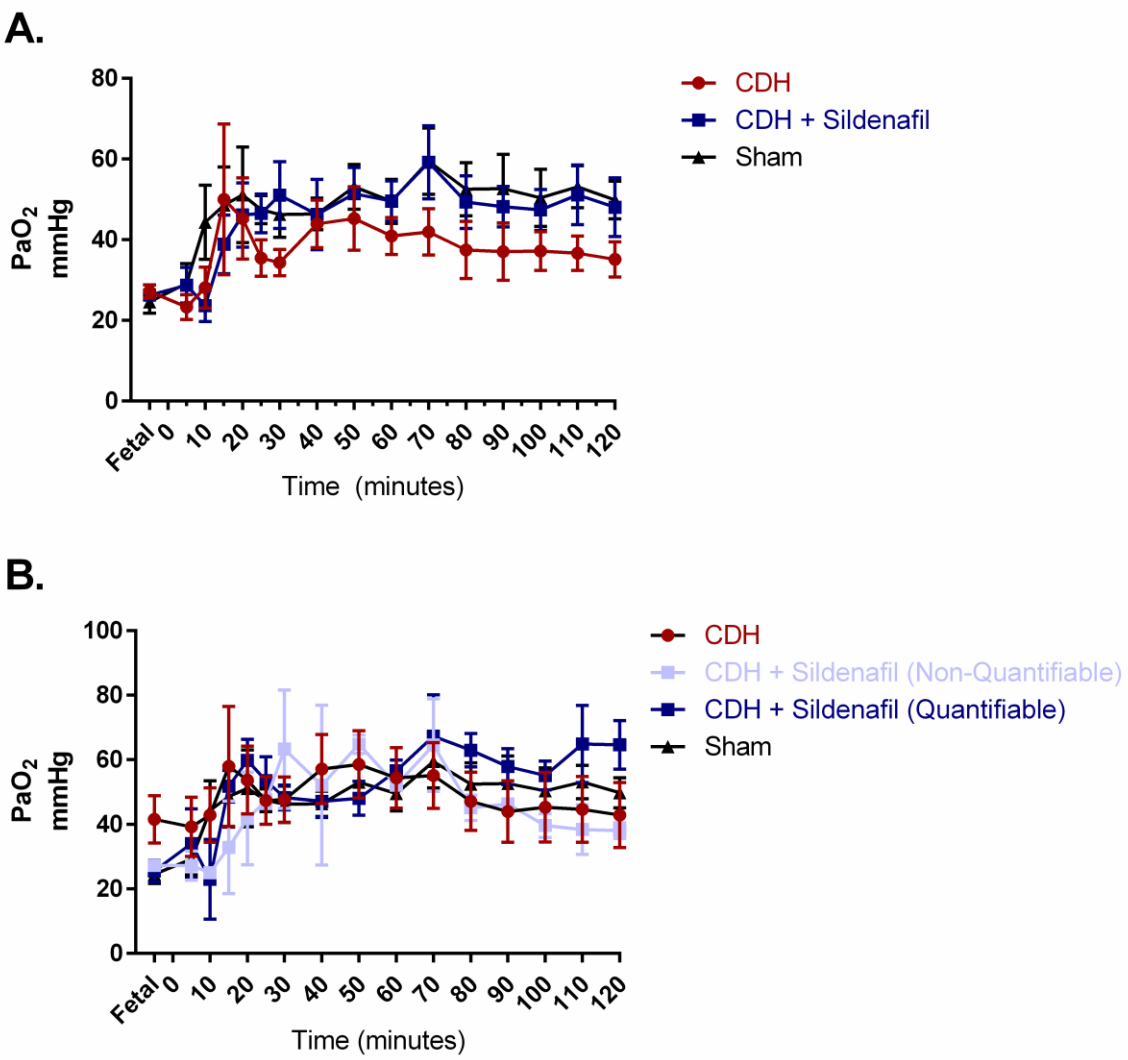

Figure 12 – Effect of Sildenafil on Arterial Partial Pressure of Oxygen (PaO_2; mmHg) During 2-Hour Neonatal Ventilation

Arterial Partial Pressure of Oxygen (PaO_2) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

Arterial Partial Pressure of Carbon Dioxide

$PaCO_2$ was not significantly different between **CDH + sildenafil** and **CDH** at any time-point during the 2-hour ventilation period (Figure 13), nor was any difference apparent following subgroup analysis of **quantifiable** and **non-quantifiable sildenafil**.

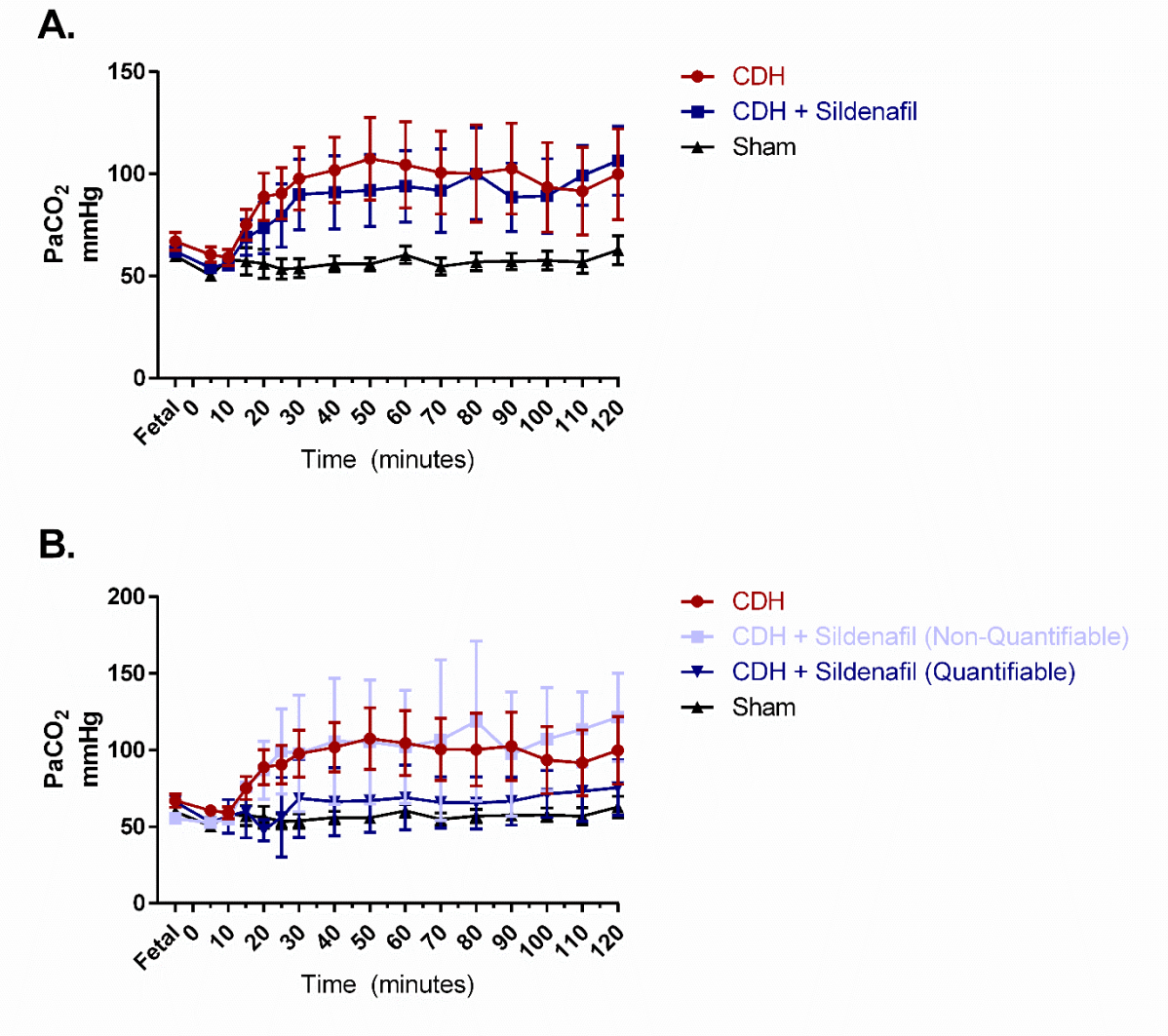

Figure 13 - Effect of Sildenafil on Arterial Partial Pressure of Carbon Dioxide (mmHg) During 2-Hour Neonatal Ventilation

Arterial Partial Pressure of Carbon Dioxide ($PaCO_2$) over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

pH

pH was not different between **sildenafil** and **CDH**, nor was any difference apparent upon subgroup analysis of **quantifiable** and **non-quantifiable sildenafil** (Figure 14).

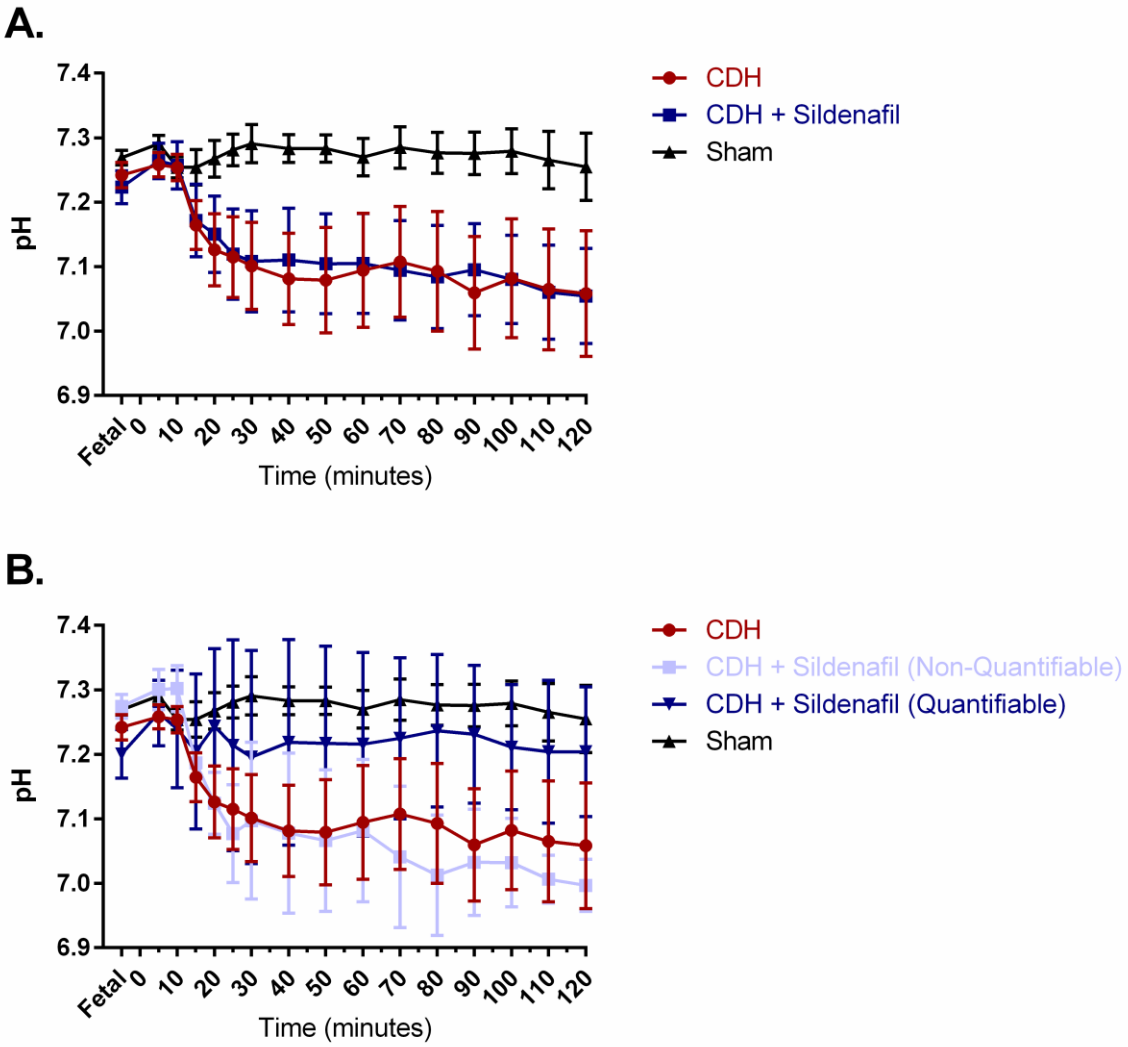

Figure 14 - Effect of Sildenafil on pH During 2-Hour Neonatal Ventilation

pH over the two hours following delivery at ≈GA138. Presented as mean ± SEM and significance accepted when $p<0.05$. **A.** Groups shown are **CDH** (●, n=7; diaphragmatic hernia [DH] creation at ≈84d GA) and **CDH + sildenafil** (■, n=5; DH creation at ≈84d GA, sildenafil infusion ≈105-138d GA). **Sham** (▲, n=5; thoracotomy only at ≈84d GA) group is shown for reference, however no statistical comparisons were made with this group. No differences between groups. **B. CDH + sildenafil** split into two sub-groups: animals with **quantifiable** (■; n=2) and **non-quantifiable** (■ ; n=2) fetal plasma sildenafil concentration. No differences between groups.

Study 3 - Dose optimisation by comparing direct fetal intravenous infusion with transplacental therapy

The results of our initial study suggested that (1) our dose was too low, as even the maternal plasma sildenafil concentrations were below the therapeutic range, and (2) placental transfer of sildenafil in sheep is extremely limited, as the fetomaternal concentration ratio was 0.15. Despite this, there were some promising improvements to neonatal physiology in the 2 animals in which we achieved a quantifiable fetal plasma sildenafil concentration.

As a pre-clinical experiment, it was more important for us to achieve the correct fetal plasma sildenafil concentration than to mirror the clinical situation by providing transplacental therapy. Hence, we performed a small pilot experiment to determine if direct fetal administration was required, or if a higher maternal dose could instead be used to achieve acceptable fetal concentrations.

Clinically, sildenafil is used to treat neonatal pulmonary hypertension with a loading dose of 0.4 mg/kg over 3 hours followed by a maintenance dose of 0.067 mg/kg/hour. In three fetal lambs, we administered this dose directly to the fetus from 129 – 136d GA. We then determined the resultant fetal plasma sildenafil concentration, which would serve as the target for transplacental therapy. We compared this with the fetal concentrations achieved by a higher maternal dose. The maternal dose of 5 mg/kg/day was chosen based on a small pilot study by our collaborators at KU Leuven (Russo *et al.*) who found that this provided safe maternal concentrations near the upper limit of the therapeutic range.

While our previous experiments had used sildenafil in crushed tablet form suspended in the OraPlus vehicle, for this study we sourced pure sildenafil citrate powder (Fagron, Belgium) which could be reliably dissolved in 0.9% sodium chloride at a concentration of 2.8 mg/mL.

Given invasive fetal instrumentation was required regardless, we also used this as an opportunity to assess changes in both maternal and fetal arterial blood pressure during sildenafil infusion.

Methods

Maternal & Fetal Instrumentation

Pre-operative Preparation

Ewes were anaesthetised with an intravenous bolus of 20mg/kg sodium thiopentone (Pentothal, 1g in 20mL; Jurox, NSW, Australia) and intubated using an 8mm endotracheal tube (internal diameter (ID) 8.0mm, outer diameter (OD) 10.9mm; Portex Ltd., Kent, England). Anaesthesia was maintained for the duration of the experiment with inhaled isofluorane (1.5 – 2.5% in room air/oxygen; Isoflow, Abbot Pty. Ltd., Australia) administered via a positive pressure ventilator (EV500 Anaesthesia Ventilator, ULCO Medical Engineering, NSW, Australia). An oxygen saturation probe was placed on the ewe's teat and electrocardiogram leads were placed on the chest and flank. End-tidal CO_2, tidal volume, heart rate and oxygen saturation were continuously monitored (SurgiVet Vital Signs Monitor, Smiths Medial, USA), along with the absence of a corneal reflex to ensure adequate analgesia and maternal wellbeing.

The ewes right flank was sheared (Oster Golden A5, Model 5-55J) and cleaned using aqueous chlorohexidine (gluconate 0.5% w/v in H_2O; Hibiclens, ICI Pharmaceuticals, Australia), Betadine surgical scrub (7.5% w/v povidine-iodine; Fauldings, SA, Australia) and Betadine antiseptic solution (10% w/v povidine-iodine; Fauldings, SA, Australia). The surrounding skin was then rinsed with chlorohexidine in isopropyl-alcohol (gluconate 4% w/v; Hibiclens, ICI Pharmaceuticals, Australia). The ewe was then placed in a supine position and the groin and abdomen sheared, cleaned and scrubbed in the same manner.

Ewes received 1000 mg intravenous Cefazolin (Cefazolin-AFT 1000 mg in 5 ml sterile water, AFT Pharmaceuticals Pty. Ltd., Australia) as antibiotic prophylaxis at the time of surgery.

Fetal Instrumentation

Sterile surgery was performed on pregnant ewes at ≈GA126 (term ≈GA145), to place a fetal jugular venous catheter and carotid artery catheter, and an amniotic catheter. Following pre-operative preparation and local anaesthesia using subcutaneous bupivacaine with adrenaline, maternal abdominal incision (laparotomy) and uterine incision (hysterotomy) allowed the fetal head and forelimbs to be exteriorised. Laparotomy and hysterotomy involved a 12cm incision made just lateral to the midline using a scalpel or diathermy (Elektrotom 80B, KLS Martin Australia Pty. Ltd., Australia), avoiding superficial epigastric and mammary veins. Subcutaneous tissue was then blunt dissected to expose the abdominal fascia. Once haemostasis was ensured, the fascia (linea alba) was incised to expose the uterus. The uterus was delivered through the abdominal incision and isolated from maternal skin using sterile sponges or towels. The fetal head was located, and directly above this site an incision in the stretched uterine wall and membranes was made using scalpel or diathermy, taking care to avoid blood vessels and placentomes. All layers of the uterine wall and membranes were tightly gripped with Babcock forceps to prevent excessive amniotic fluid loss, and the fetal head and forelimbs were exteriorised. To prevent tissues drying out, the uterus and fetus were periodically dampened with warmed sodium chloride (0.9% sodium chloride, Fresenius Kabi Australia Pty. Ltd., Australia). A 5mL sample of amniotic fluid was taken.

A small incision was made in the left fetal neck, lateral to the trachea. The left carotid artery was identified and the cranial end occluded with a ligature using 3.0 non-absorbable silk. A second ligature was loosely tied around the caudal end, and pulled superiorly to prevent blood flow. A small (≈1 mm) incision was made between these two ligatures, and a vinyl catheter (ID 0.86 mm, OD 1.52 mm, Dural Plastics Inc., Australia) introduced 5 cm into the left carotid artery. The caudal ligature was then tightened to secure the catheter. The catheter was then flushed with previously primed heparinised saline. The left jugular vein was catheterised in a similar manner. The catheters were exteriorised and sutured to the fetal neck to prevent dislodgement, and the neck incision was sutured closed using 3.0 non-absorbable silk.

A catheter (1.50mm ID, 2.70mm OD; Dural Plastics Inc., Australia) was also placed in the amniotic cavity and secured to the fetal neck. All catheters were exteriorised from

the uterus before the hysterotomy was closed in two layers in an inverted pattern, in order to bury all cut tissue edges, using Polysorb 2.0 suture (Covidien, Medtronic Australasia, Australia). The catheters were then tunnelled through the abdominal cavity and exteriorised on the ewe's flank. Fascia overlying the uterus (linea alba) and flank wound was sutured closed with a continuous running stitch using Maxon 1.0 suture (Covidien, Medtronic Minneapolis, Australia), and the overlying skin closed using intradermal Vetafil 2.0 suture (Supramid, B. Braun Australia Pty. Ltd., Australia).

Maternal Instrumentation

A small incision was also made in the right maternal neck through which vinyl catheters (ID 2.6mm, OD 4.2mm, Dural Plastics Inc., Australia) were inserted and introduced ≈12cm into the jugular vein and carotid artery. Ligatures were used to prevent blood loss, and catheters were primed with heparinised saline (50,000 IU Heparin [Heparin sodium 25,000 IU in 5ml sterile water, Pfizer Australia Pty. Ltd., Australia] in 1 L normal saline [0.9% sodium chloride, Fresenius Kabi Australia Pty. Ltd., Australia]). The incision site was then sutured closed 3.0 non-absorbable silk (DYSILK, Dynek Pty. Ltd., South Australia).

The ewes recovered for 3 days under close monitoring and received post-operative analgesia (transdermal fentanyl patches, 75 μg/h; Janssen-Cilag, North Ryde, NSW, Australia).

Recovery Period

Daily prophylactic antibiotics will be administered to the fetus and ewe via the jugular vein catheters for 3 days following the operation (Cefazolin; 1000mg maternal intravenous, 100mg fetal intravenous, 400mg amniotic).

Sildenafil Infusion Period

Drug Preparation

The sildenafil infusion solution was prepared by dissolving 33 mg sildenafil citrate (Fagron, Belgium) in 50 mL 0.9% sodium chloride. The solution was vortexed for 2 min at 2000 rpm, then left overnight on a roller at 60 rpm.

Drug Infusion

The fetal jugular vein catheter was connected to an infusion pump (CADD-Legacy 1 Ambulatory Infusion Pump Model 6400; Smiths Medical Australasia Pty. Ltd., Australia), which was secured to the ewe's back using netting (Tubular-Net, Sutherland Medical Pty. Ltd., Australia). The infusion pump was loaded with a 100 mL cassette containing the 0.66 mg/mL sildenafil citrate in 0.9% sodium chloride solution.

A loading dose of 400 µg/kg was given over 3 hours (~0.8 mL/hour) followed by a maintenance dose of 67 µg/kg/hour (~0.4 mL/hour), to reflect local guidelines for continuous IV infusion of sildenafil citrate to neonates with pulmonary hypertension.[4] In our experience, lambs at our facility weigh 3.5 kg at 129d GA and 4.5 kg at 136d GA, so a fetal weight of 4 kg was assumed for this study between 129 – 136d GA.

Maternal and Fetal Blood Pressure recording

The maternal and fetal carotid artery catheters were connected to a pressure transducer (TNF-R, BD DTXPlus, DT Mumbai), which were amplified via bridge amp (Quad Bridge Amps, ADInstruments Pty. Ltd., Castle Hill, Australia) and subsequently connected to a bio-amp (Powerlab 16/35, ADInstruments Pty. Ltd., Castle Hill, Australia) to measure blood pressure. Fetal blood pressure was corrected for amniotic pressure, to account for changes in maternal position.

Fetal Sampling

The fetal carotid artery catheter was used to draw blood throughout the infusion period (≈GA129 – 136), to assess regular plasma sildenafil concentrations. Approximately 5 mL of blood was collected at each timepoint, then transferred into an ethylene tetra-acetic acid (EDTA; an anti-coagulant) spray-coated tube (8 mg EDTA per 5 mL blood;

Sarstedt Australia Pty. Ltd., Australia). Arterial blood samples were centrifuged (Allegra 21R Centrifuge, Beckman Coulter Australia Pty. Ltd., Australia) for 10 minutes at 3000 rpm and 4°C, and the supernatant was aliquoted into cryovials (Cryo.s; Greiner Bio-One International GmbH, Germany) for overnight storage at approximately -20°C (LG ExpressCool, LG Electronics Australia Pty. Ltd., Australia). 24 hours later, samples were transferred to long-term storage at approximately -80°C (Froilabo Bio Memory Ultra Low, Scientific Partners Australia Pty. Ltd., Australia). Plasma concentrations of sildenafil and its active metabolite, N-desmethyl-sildenafil, were analysed using ultra high performance liquid chromatography (Vanquish Flex Quaternary, ThermoFisher Scientific, Australia) in tandem with mass spectrometry (TSQ Quantiva, ThermoFisher Scientific, Australia).[3]

Maternal Sampling

The maternal carotid artery catheter was also used to collect blood in the same manner as for fetal samples throughout the infusion period (129 – 136d GA), to assess regular maternal plasma sildenafil concentrations.

Results

Plasma sildenafil concentrations

Sildenafil concentration rapidly increased in both maternal and fetal plasma within the first 3 hours of administration, before plateauing to a constant level for the remainder of the infusion period (Figure 15). When sildenafil was administered to the maternal ewe (5 mg/kg/day), the fetomaternal concentration ratio at peak concentration was 0.08 (38 vs 321 ng/mL). When sildenafil was administered directly to the fetus (1.6 mg/kg/day), the fetomaternal concentration ratio at peak concentration was 3.42 (28 vs 8 ng/mL) in one animal and 2.01 (22 vs 11 ng/mL) in the other.

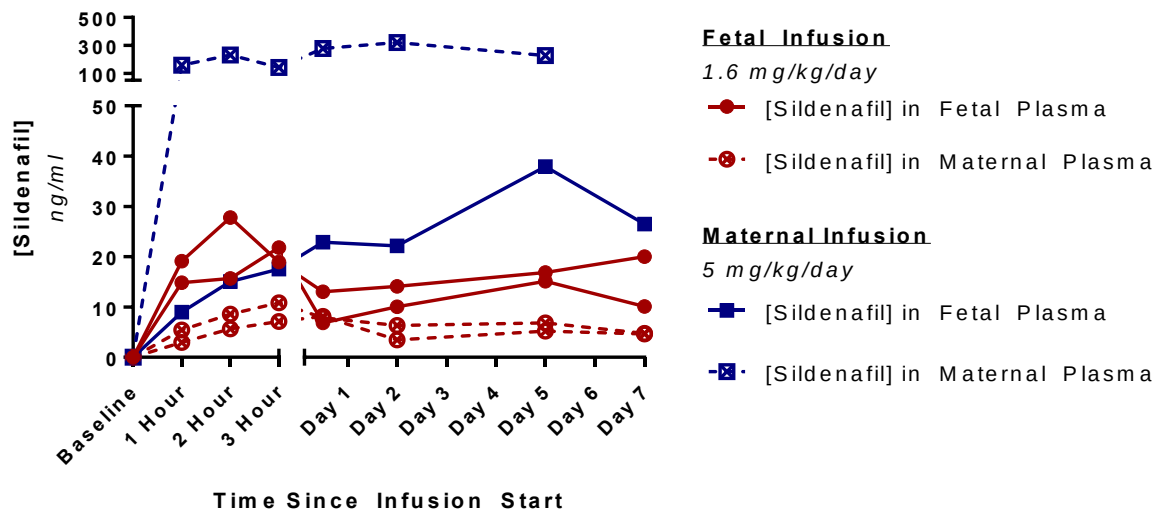

Figure 15 - Plasma sildenafil concentrations throughout the infusion period

Sildenafil concentration (ng/mL) over time in maternal (crossed shapes) and fetal (solid shapes) plasma, in animals that received a continuous sildenafil infusion to the maternal ewe (5 mg/kg/day; blue squares) or fetal lamb (1.6 mg/kg/day; red circles).

Maternal blood pressure

In the ewe that received a continuous intravenous sildenafil infusion, maternal blood pressure decreased from 83 mmHg to 74 mmHg after one hour of sildenafil infusion. For the remainder of the infusion period, maternal blood pressure remained between 71 – 78 mmHg (Figure 16).

In the two ewes carrying fetuses that received direct fetal intravenous sildenafil infusion, maternal blood pressure was 70 mmHg and 51 mmHg before the infusion started. One hour after the infusion started, maternal blood pressure was 78 mmHg and 51 mmHg, respectively. For the remainder of the infusion period, maternal blood pressure varied from 54 – 82 mmHg and 40 – 59 mmHg, respectively (Figure 16).

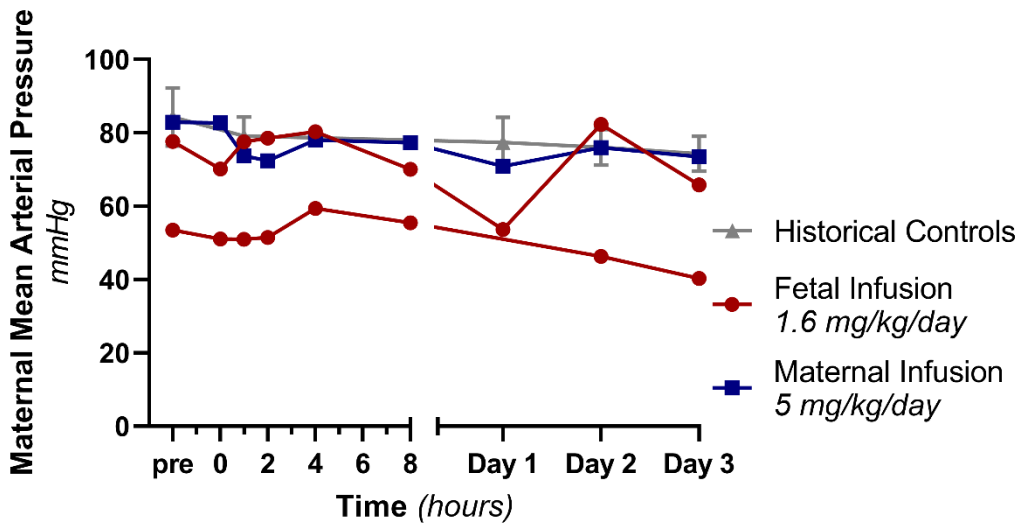

Figure 16 - Maternal blood pressure thoughout the sildenafil infusion period

Maternal blood pressure (mmHg) in the ewe that received a continuous intravenous sildenafil infusion (blue squares) and the two ewes carrying fetuses that received a direct fetal intravenous sildenafil infusion (red circles).

Fetal blood pressure

In the fetal lamb that received transplacental sildenafil (maternal infusion), fetal blood pressure increased from 44 mmHg to 45 mmHg after one hour of sildenafil infusion. For the remainder of the infusion period, fetal blood pressure remained between 36 – 48 mmHg (Figure 17).

In the two fetal lambs that received direct fetal intravenous sildenafil infusion, fetal blood pressure was 40 mmHg and 45 mmHg before the infusion started. One hour after the fetal infusion started, fetal blood pressure was 39 mmHg and 47 mmHg, respectively. For the remainder of the infusion period, fetal blood pressure varied from 35 – 45 mmHg and 41 – 52 mmHg, respectively (Figure 17).

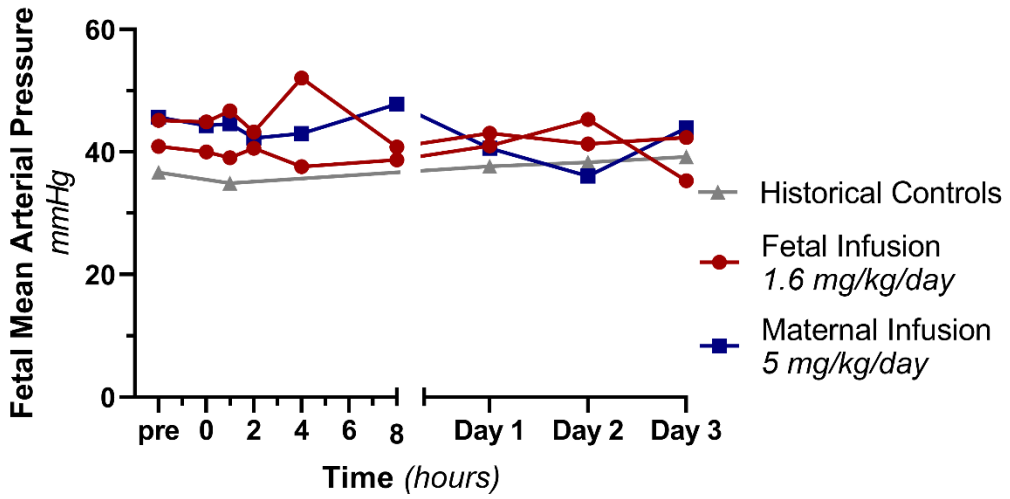

Figure 17 - Fetal blood pressure throughout the sildenafil infusion period

Fetal blood pressure (mmHg) in the fetal lamb that received transplacental sildenafil (maternal infusion; blue squares) and the fetal lambs that received direct fetal intravenous infusion (red circles).

Conclusion

A maternal sildenafil dose of 5 mg/kg/day achieves similar fetal plasma sildenafil concentrations as direct fetal intravenous infusion of the neonatal therapeutic dose (67 µg/kg/hour), without adversely affecting maternal or fetal blood pressure.

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Appendix 3 - Reconstructing the pulmonary vascular architecture of lambs with diaphragmatic hernia

We are developing X-ray imaging and analytical techniques to create and then analyse 3D-reconstructions of the pulmonary vasculature. To achieve this, we perfused the pulmonary vasculature with the contrast agent Microfil (Microfil, Flow Tech, USA) immediately at post-mortem, as described below. Microfil perfusion precluded histological analysis, which was a limitation of each experimental chapter in this thesis.

75 mL of Microfil solution was prepared from 30 mL Microfil Yellow (MV-122, Flow Tech Inc., Massachusetts, U.S.A.), 37.5 mL diluent (MV-Diluent, Flow Tech Inc., Massachusetts, U.S.A.) and 3.375 mL 5% curing agent (MV Curing Agent.).

Immediately following lethal injection with sodium pentobarbitone (i.v. 100 mg/kg), the lamb's or rabbit kitten's heart was exposed via midline thoracotomy and blunt dissection. A 15G blunt needle catheter, primed with heparinised saline containing 2% lignocaine, was inserted into the right ventricle and positioned within the main pulmonary artery, then secured with silk ligature. This catheter was connected via large-bore vinyl catheter (ID 2.6mm, OD 4.2mm, Dural Plastics Inc., Australia) to a peristaltic pump (Masterflex 7554-85, John Morris Scientific Pty. Ltd., Australia) (Figure 1). A three-way tap was placed in this circuit to remove any air bubbles inadvertently added to the line. The ductus arteriosus was clamped using a specially designed bulldog clip. A second vinyl catheter was placed via the aorta into the left ventricle, in order to create a back pressure of 5 cmH_2O.

The pulmonary vasculature was perfused via the catheter inserted into the right ventricle, initially with heparinized saline containing 2% lignocaine (Lignocaine Hydrochloride 100 mg in 5 ml, Pfizer Australia Pty. Ltd., Australia) at a rate of 25 mL/min. Once clear fluid was observed draining from the left atrium, Microfil solution was infused at the slowest rate possible (2 mL/min). The Microfil set within 20 minutes, and the lungs were subsequently pressure-fixed in 4% v/v formaldehyde (Neutral Buffered Formalin, Amber Scientific, Australia) at a constant pressure of 25 cmH_2O via the trachea. The lungs were then stored at 4^oC.

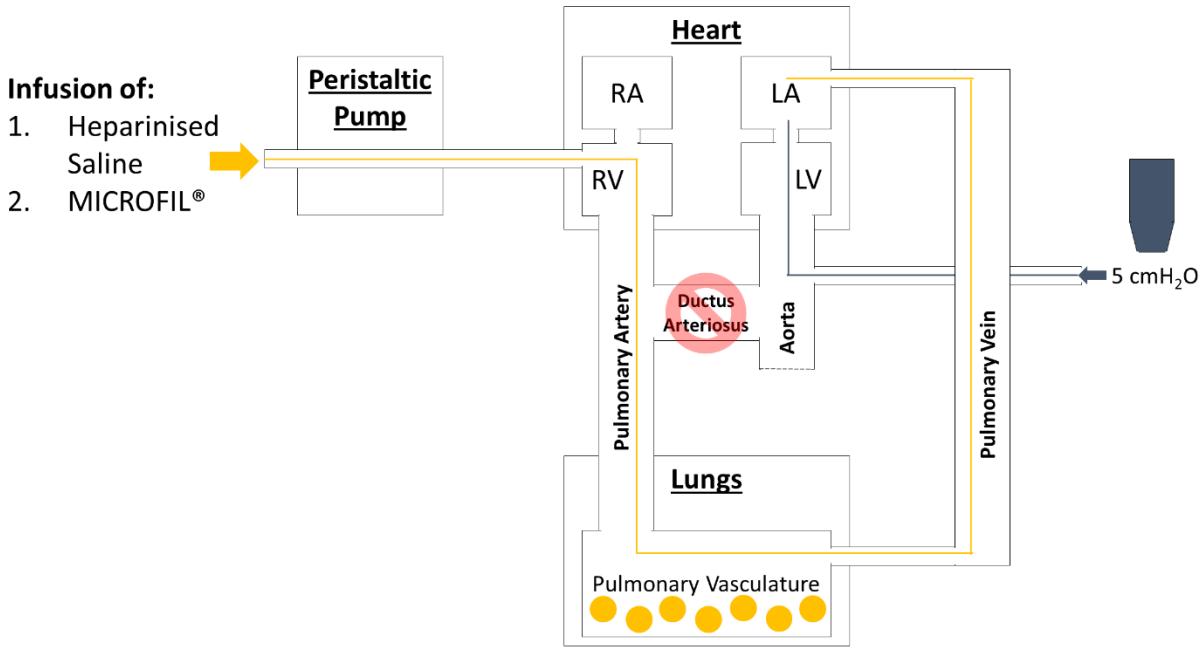

Figure 1 - Schematic of MICROFIL® Infusion into the Pulmonary Vasculature

This schematic shows the experimental set-up for infusion of MICROFIL®, a compound used to opacify intravascular spaces on computed tomography scanning, into the pulmonary vasculature. The ductus arteriosus was first clamped using hemostatic forceps. A back pressure of 5 cmH_2O was applied into the left atrium (LA) via the aorta and left ventricle (LV). The pulmonary circulation was initially primed with heparinised saline. was Finally, MICROFIL® was administered to the pulmonary vasculature via the right ventricle (RV), using a peristaltic pump. RA: Right atrium.

One day prior to imaging, the lungs were removed from formalin and embedded in a 1.5% agarose solution, then set overnight at 4°C. The next day, each lung was imaged in the Imaging and Medical Beamline (IMBL) at the Australian Synchrotron. An X-ray beam with an energy of 40 keV was selected with a double crystal Laue monochromator, and beam intensity was normalised with time. The exposure time for each frame chosen was 200 ms, to produce the best possible quality images without saturating the detector. The specimen to detector distance was 3 meters, and the specimen was approximately 135 meters downstream from the beamline source.

A PCO edge detector was coupled with a Nikkon Micro-Nikor 105mm/f 2.8 macro lens and a 25 micron gadollinium oxysulfide scintillator. The effective pixel size was 16.2 microns, and the field of view was 41.4 mm by 35.0 mm (2560x2160 pixels). In the lamb lungs, but not rabbit lungs, this field of view was smaller than the specimen both horizontally and vertically, so multiple scans were performed. First, a 180 degree CT

scan with 3601 projection images was taken of a column with diameter 25 mm. To accommodate the large diameter of the specimen, the sample was then moved horizontally by 25 mm, and a second scan performed. The sample was again moved horizontally by 25 mm, and a third scan performed. To accommodate the large height of the specimen, the specimen was moved vertically by 20 mm and this procedure was repeated for a variable number of rows dependent on the specimen height. The data was stitched together in post-processing, before reconstructing the final CT slices using XTRACT software run on the MASSIVE supercomputing cluster.

Projection images were corrected for dark current noise as well as beam inhomogeneity and detector artifacts by using the dark and flat field images acquired immediately before and after the CT scan. We used a pixel-wise correction algorithm to correct for variation in detector response and optical lens systems.[1]

We have already used these CT images to reconstruct the pulmonary vascular tree in three dimensions, as shown in *Figure 2*. However, the quantitative analysis of these reconstructions is ongoing. We plan to quantify the diameter of individual branches at each branching generation, branching angles, vessel length, vessel radius and the total vascular volume. The processing power required to quantify these variables for the large lamb lungs at high resolution exceeds the capabilities of any pre-existing software (commercially available or experimental), as these methods have previously only been applied to the renal vasculature of rodents.[2] Hence, we are working closely with the Phenomics and Bioinformatics Research Centre at the University of South Australia to accomplish this.

References

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2. Khan Z, Ngo JP, Le B, Evans RG, Pearson JT, Gardiner BS, Smith DW. Three-dimensional morphometric analysis of the renal vasculature. Am J Physiol Renal Physiol. 2018;314(5):F715-f25.

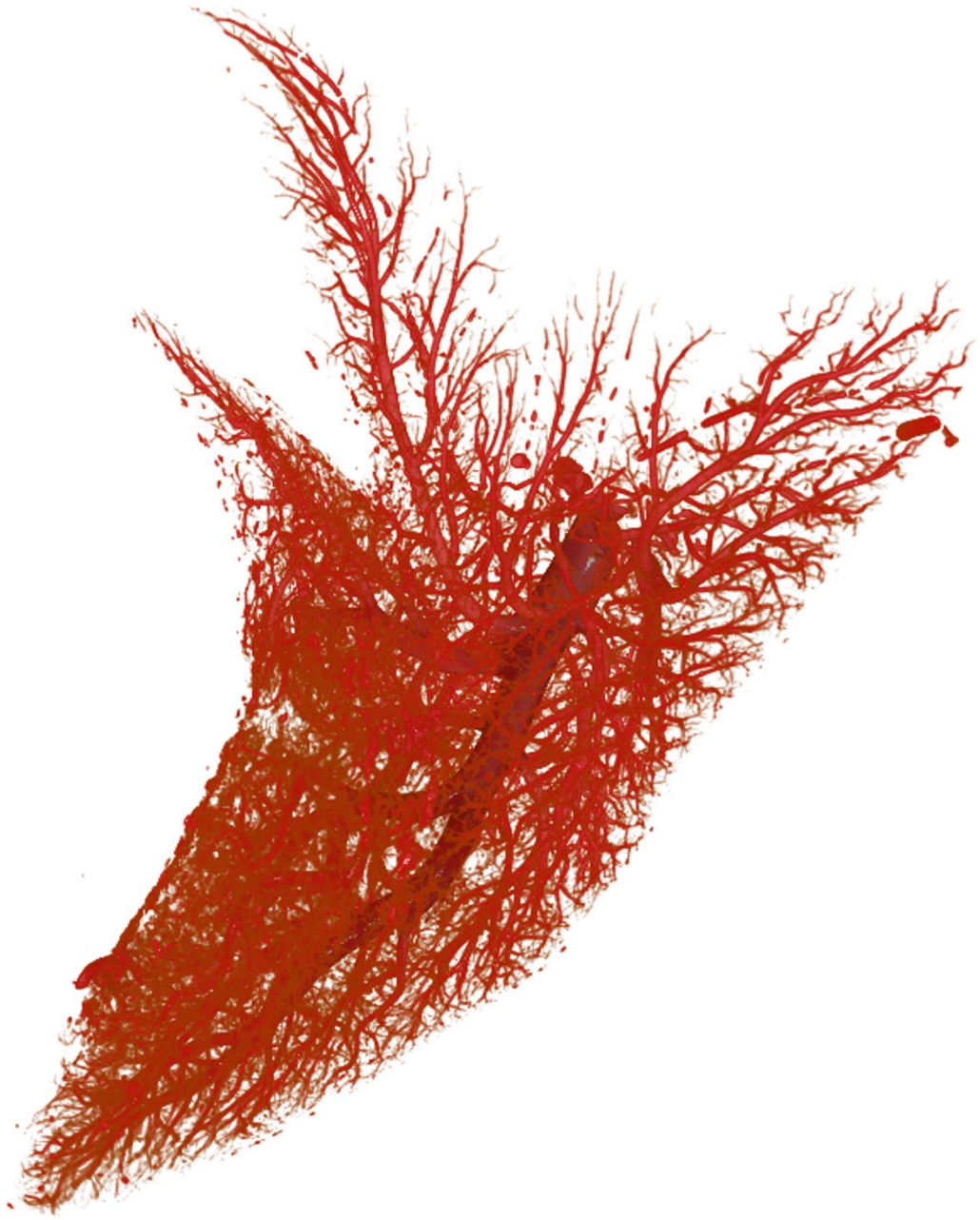

Figure 2 – 3D Reconstruction of the Pulmonary Vasculature

Infusing the pulmonary vasculature with MICROFIL® allowed us to create 3D-reconstructed images of the pulmonary vasculature. The left lung of an animal in **CDH + sildenafil** is shown. Computed tomography scanning was conducted at the Australian Synchnotron, and 3D-reconstruction was performed using XTRACT software run on the MASSIVE supercomputing cluster.