MONASH University

Investigating Pre and Post-Natal Therapies in the Altered Cardiovascular System of Fetal Growth Restricted Lambs

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The Ritchie Centre, Hudson Institute of Medical Research
Obstetrics and Gynaecology, Medicine, Nursing and Health Sciences.

Supervisors:
Associate Professor Graeme Polglase and Doctor Beth Allison

'You know, I guess one person can make a difference"

Stan Lee, Spider-man 3, 2007

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Thesis Abstract

Fetal growth restriction (FGR) is the failure of a fetus to reach their genetic growth potential. FGR commonly occurs secondary to placental insufficiency, which results in impaired delivery of oxygen and nutrients to a developing fetus. Consequently, fetuses develop in a low-nutrient, adverse intrauterine environment. Amazingly, to survive, fetuses mount cardiovascular adaptations in order to send blood flow (and substrates) to key organs, such as the brain and heart, to maintain maximal perfusion.

Long-term maintenance of these cardiovascular adaptations (coupled with the adverse intrauterine environment) results in impaired cardiovascular structure and function. FGR offspring have increased risk of life-long adverse consequence. It is accepted that vascular alterations contribute towards development of cardiovascular, metabolic and neurological sequale. After birth, FGR infants require greater post-natal support than their appropriately grown (AG) counterparts. Additional interventions in early life can further exacerbate the developmentally programmed changes in the cardiovascular, respiratory and neurological systems.

To date, there have been no clinically successful therapies to improve fetal growth and development following FGR diagnosis. Given that FGR and all subsequent adversity begins at placental dysfunction, it is an obvious therapeutic target. Sildenafil Citrate (SC) is a potent vasodilator and recent studies have aimed to investigate its ability to increase placental blood flow, and thereby function, to improve fetal growth. Despite several preclinical studies supporting the use of SC in the context of FGR, recent outcomes of the STRIDER (Sildenafil Therapy in Dismal Prognosis Early-onset Fetal Growth Restriction) trial suggests fetal exposure to SC resulted in unexpected neonatal death and adverse pulmonary effects.

The failure of large clinical trials highlights the importance of conducting relevant animal studies to fully understand all potential side effects before attempting clinical translation. In the context of the STRIDER trial, I believe that investigation into the interaction between the altered vasculature of the FGR fetus and SC exposure will help determine some mechanisms behind the unexpected adversity observed clinically.

In chapter 3, we induced placental insufficiency, via single-umbilical artery ligation, to cause FGR in fetal sheep. Ewes bearing FGR fetuses were treated with SC to mimic the treatment regimen conducted in clinical studies. Ex-vivo peripheral and central vascular function was assessed in these

fetuses and we found that SC exposure during gestation resulted in functional alterations to blood vessels, changes in function were seen both within vascular endothelium and vascular smooth muscle dysfunction. Unexpectedly we also found that SC exacerbated, rather than improved FGR. These results support the potential for SC to impair vascular development.

In chapter 4, the physiological cardiovascular response to chronic maternal SC treatment was assessed in FGR bearing ewes and their fetuses. Chronic SC administration to the ewe had minimal effects on maternal physiology. However, the fetus demonstrated impairments in the traditional redistribution of blood flow, normally mounted in response to placental insufficiency. The findings from this chapter offer insights into the potential mechanisms by which chronic exposure to SC in utero may impair fetal cardiovascular development.

FGR infants require greater post-natal support as compared to their appropriately grown counterparts. Hypotension is a common complication observed in preterm neonates, and dopamine is a commonly used neonatal treatment for hypotensive support. In chapter 5, we compared the therapeutic benefit of SC and dopamine at providing cardiovascular support in FGR lambs. Dopamine acts on β-adrenergic receptors on the heart and vessels to increase heart contractility and peripheral vascular resistance in an effort to increase cardiac output and blood pressure. However, the vasoconstrictive effects of dopamine in the FGR neonate be impaired due to already vasoconstricted vascular beds. It may be possible that vasodilation, via SC, will increase venous return and cardiac output to better stabilize blood pressure in the FGR neonate. We demonstrate that dopamine treatment is potentially harmful for FGR lambs. Our results suggest that current neonatal hypotensive treatments require re-evaluation in the context of FGR.

In conclusion, the studies in this thesis demonstrate that FGR, following placental insufficiency-drives vascular alterations, not only to the structure but also the function of blood vessels. Importantly, these changes also impact on the ability of blood vessels to respond to vasoactive drugs which can further impair cardiovascular function. I show that therapeutics have the potential to impact cardiovascular function both in the developing growth restricted baby, or after birth in the postnatal period. occur if therapeutics are given antenatally or postnatally. Increased research is needed to understand the optimal therapies for a pregnancy affected by FGR as well as the FGR newborn.

~ this thesis is dedicated to my family, my friends and my love ~

Declarations

I 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, this thesis contains no maternal previously published or written by another person, except where reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals, 2 submitted publications (chapters 3 and 4) and 1 narrative manuscript (Chapter 5). The core theme of the thesis is perinatal therapies for fetal growth restriction.

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 Gynecology, Monash University, under the supervision of Graeme Polglase, Beth Allison and Suzie Miller.

In the case of chapters 3,4,and 5, my contribution to the work is described in the following manuscript declarations.

Name: Ishmael Miguel Inocencio

Date: 07.11.2019

Thesis including published works 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 *1* original paper published in peer reviewed journals and 1 submitted publications. The core theme of the thesis is vascular therapies for fetal growth restriction.

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 Gynecology, Monash University, under the supervision of Graeme Polglase, Beth Allison and Suzie Miller.

In the case of chapters 3 and 4, my contribution to the work involved the following:

Assistance and performance of fetal surgery in both chapters. Assistance and performance of myography analysis in chapter 3. Performance of maternal drug delivery in chapter and *inutero* physiological recordings in 4. Performance of post-mortem collection and analysis in both chapters. Performance of analysis of data and manuscript drafting of all chapters.

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
Chapter 3	The Effects of Maternal Sildenafil Treatment on Vascular Function in Growth Restricted Fetal Sheep	Published	50% Initial concept, study design, data collection, analysis, and writing of first draft.	Graeme R. Polglase 10% Initial concept, study design and analysis.	No
				Suzanne L. Miller 10% Initial concept, study design and analysis.	No
				Arvind Sehgal 2.5% input into manuscript.	No
				Amy Sutherland 2.5% data collection, input into manuscript.	No

				Jamie Mihelakis 2.5% data collection, input into manuscript.	No
				Anqi Li 2.5% data collection, input into manuscript.	No
				Beth J. Allison 20% Initial concept, study design, data collection and analysis.	No
Chapter 4	Maternal Sildenafil Impairs the Cardiovascular Adaptations to Chronic Hypoxia in Growth Restricted Fetal Sheep.	Submitted	60% Initial concept, study design, data collection, analysis, and drafting.	Graeme R. Polglase 10% Initial concept, study design and analysis.	No
				Ilias Nitsos 5% data collection, input into manuscript.	No
				Suzanne L. Miller 10% Initial concept, study design and analysis.	No
				Beth J. Allison 15% Initial concept, study design, data collection and analysis.	No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Ishmael Inocencio

Student signature: **Date:** 07.11.19

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions 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 the authors.

Main Supervisor name: Graeme Polglase

Main Supervisor signature: **Date:** 07.11.19

Contents

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I have always looked up to superheroes in comic books. Now look up to one in real-life.

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To Ben

My poke-bro. Thank you for being my work wife. I will always cherish our long walks in the park and coffee dates. For real though, you have made sticking around this place so easy. Having

someone to laugh with, dnm with and someone to just escape it all and do ridiculous stuff with. Thanks for all of it man, all of it.

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My original little sister! Thank you for being my Asian friend! I can't believe that we have made it this far. Thank you for going through all of it with me! You have become such an amazing scientist and I'm so proud of you!

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There will no doubt be a few hundred people that I haven't thanked by name. But you know how much you mean to me. Thank you for being a part of this story. I love you all very much.

The new generation

Whether it is 10, 20, 50 years from now, it is with no doubt that this book will end up collecting dust on the desk of a new Ritchie center student. You're probably reading this because you're

painstakingly trying to put together your own thesis. To you, I leave this: Love this place. Cherish every moment. This place can be home, if you let it.

Good luck!
Mikee

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Research Dissemination

Manuscripts from Thesis Chapters

Chapter 1:
Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A, Miller S and Allison B. The Effects of Maternal Sildenafil Treatment on Vascular Function in Growth Restricted Fetal Sheep. Arterioscler. Thromb. Vasc. Biol.

Chapter 2:
Inocencio IM, Polglase G, Miller S, Nitsos I, Miller S and Allison B. Maternal Sildenafil Treatment Alters Cardiovascular Adaptations and Exacerbates Growth Restriction in Fetal Growth Restricted Lambs.

Chapter 3:
Inocencio IM, Polglase G, Miller S, Nitsos I, Sutherland A, Miller S and Allison B. Comparison Between Dopamine and Sildenafil at improving Cardiovascular support in FGR lambs

International Conference Abstracts

2017

- Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A and Allison B. Long term maternal sildenafil treatment increases endothelium-independent mediated cerebral vasodilation in both fetal growth restricted (FGR) and appropriately grown (AG) lambs. Perinatal Academic Society, San Fransisco, USA and Fetal and Neonatal Workshop of Australia and New Zealand, Canberra, Australia.

National Conference Abstracts

2019

- Inocencio IM, Polglase G, Sehgal A, Sutherland A, Mihelakis J, Li A Nitsos I, Zahra V, Miller S and Allison B. The Effects of Maternal Sildenafil Treatment on Vascular Function and Physiology in Growth Restricted Fetal Sheep.Perinatal Society of Australia and New Zealand, Gold Coast, Queensland.

2018

- Inocencio IM, Polglase G, Sehgal A, Sutherland A, Mihelakis J, Li A Nitsos I, Zahra V, Miller S and Allison B. The Effects of Maternal Sildenafil Treatment on Vascular Function and Physiology in Growth Restricted Fetal Sheep. Monash Health and Translational Research Week Symposium, Melbourne, Australia.

- Inocencio IM, Polglase G, Sehgal A, Sutherland A, Mihelakis J, Li A Nitsos I, Zahra V, Miller S and Allison B. The Effects of Maternal Sildenafil Treatment on Vascular Function and Physiology in Growth Restricted Fetal Sheep. Hudson Institute Student Society Student Retreat, Melbourne, Australia.

- Polglase G, Nitsos I, Zahra V, Miller S and Allison B. Comparison Between Dopamine and Sildenafil for Neonatal Cardiovascular Support in Fetal Growth Restricted lambs. Australian Society of Medical Research Student Symposium, Melbourne, Australia.

- Polglase G, Nitsos I, Zahra V, Miller S and Allison B. Comparison Between Dopamine and Sildenafil for Neonatal Cardiovascular Support in Fetal Growth Restricted lambs. Australian Society of Medical Research Student Symposium, Melbourne, Australia. Fetal and Neonatal Workshop of Australia and New Zealand, Queenstown, New Zealand.

2017

- Inocencio IM, Polglase G, Nitsos I, Zahra V, Miller S and Allison B. Maternal Sildenafil Treatment Alters In-Utero Cardiovascular Function and Exacerbates Growth Restriction in Lambs Following Single Umbilical Artery Ligation. Australian Society of Medical Research Student Symposium, Melbourne, Australia.

- Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A and Allison B. Long term maternal sildenafil treatment increases endothelium-independent mediated cerebral vasodilation in both fetal growth restricted (FGR) and appropriately grown (AG) lambs. Perinatal Academic Society, San Fransisco, USA.

- Inocencio IM, Polglase G, Nitsos I, Zahra V, Miller S and Allison B. Maternal Sildenafil Treatment Alters In-Utero Cardiovascular Function and Exacerbates Growth Restriction in Lambs Following Single Umbilical Artery Ligation. Fetal and Neonatal Workshop of Australia and New Zealand, Canberra, Australia.

2016

- Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A and Allison B. Sildenafil to Reduce Morbidity in Growth Restricted Preterm Infants. Australian Society of Medical Research Student Symposium, Melbourne, Australia.

- Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A and Allison B. Cardiovascular Function in Growth Restricted Infants – Sildenafil as a Potential Treatment. Centre of Research Excellence Annual Meeting, Perth, Australia.

- Inocencio IM, Polglase G, Miller S, Sehgal A, Sutherland A, Mihelakis J, Li A and Allison B. Sildenafil to Reduce Morbidity in Growth Restricted Preterm Infants. Fetal and Neonatal Workshop of Australia and New Zealand, Canberra, Australia

Prizes and Awards

2018

- Hudson Institute of Medical Research 2018 Student Retreat. 2nd Prize – Senior Student Presentation
- School of Clinical Sciences at Monash Health. Three Minute Thesis Competition. People's Choice Award.
- The Ritchie Centre. Three Minute Thesis Competition. 1st Place – Senior Category.

2017

- Australian Society of Medical Research Student Symposium. Best Junior Presentation
- Fetal and Neonatal Workshop of Australia and New Zealand. Best Junior Presentation

2016

- Ritchie Centre Three Minute Thesis Competition. 3rd place Junior Category

Abbreviations and Symbols

%	Percent
±	Plus or Minus
AA	Arachidonic Acid
AEDF	Absent End Diastolic Flow
AG	Appropriately Grown
AIx	Aortic Pressure Augmentation Index
ANS	Autonomic Nervous System
cGMP	Cyclic Guanosine Monophosphate
cm	Centimeter
CMC	Cardiogenic Mesoderm Cells
CNCC	Cardiac Neural Crest Cells
COX	Cyclooxygenase
CVD	Cardiovascular Disease
DA	Ductus Arteriosus
EDHF	Endothelium-Derived Hyper Polarizing Factor
eNOS	Endothelial Nitric Oxide Synthase
FGR	Fetal Growth Restricted
GC	Guanylyl Cyclase
GRIT	Growth Restriction Intervention Trial
GTP	Guanosine Triphosphate
HIF1α	Hypoxia Inducible Factor -1α
HSP70	Heat Shock Proteins-70
K+	Potassium
kg	Kilogram
MCA	Middle Cerebral Artery
mg	Milligram
ml	Milliliter
mm	Millimeter
MV	Mechanical Ventilation
n=	Number of Animals
NICU	Neonatal Intensive Care Unit
NO	Nitric Oxide

NOS	Nitric Oxide Synthase
ºC	Degrees Celsius
OD	Outer Diameter
OI	Oxygenation Index
$PaCO_2$	Partial Pressure of Carbon Dioxide
PaO_2	Partial Pressure of Oxygen
P_{AW}	Airway Pressure
PDE5	Phosphodiesterase 5
PE	Propericardium
PEDF	Present End Diastolic Flow
PEEP	Positive End Expiratory Pressure
PG	Prostaglandin
PGL_2	Prostacyclin
PGlS	Prostacyclin Synthase
pH	Log of the concentration of hydrogen ions
PIP	Peak Inspiratory Pressure
PSNS	Parasympathetic Nervous System
qPCR	Quantitative Polymerase Chain Reaction
R_{AW}	Airway Resistance
REDF	Reversed End Diastolic Flow
ROS	Reactive Oxygen Species
SaO_2	Arterial Oxygen Saturation
SC	Sildenafil Citrate
SEM	Standard Error of the Mean
SGA	Small for Gestational Age
SNS	Sympathetic Nervous System
SpO_2	Oxygen Saturation
STRIDER	Sildenafil Therapy in Dismal Prognosis Early-onset Fetal Growth Restriction
UA	Umbilical Artery
UVC	Unventilated Control
VEGF	Vascular Endothelial Growth Factor
VEI	Ventilation Efficiency Index
VENT	Ventilation

VILI	Ventilator Induced Lung Injury
V_T	Tidal Volume
µg	Microgram
µL	Microliter
µM	Micrometer

Chapter 1

Literature Review

This chapter has not been published or presented elsewhere and has been formatted as a traditional thesis chapter

1.1. Fetal Growth Restriction: An Introduction.

Fetal Growth Restriction (FGR) is defined as the failure of a fetus to reach their genetic growth potential. Despite advancements in obstetric care, FGR remains a large contributor toward both fetal mortality as well as short and long-term morbidity [1,2]. FGR is the second highest cause of perinatal death and the strongest predictor for stillbirth[1,3,4]. Globally, 30 million FGR infants are born each year, however the incidence varies throughout countries. In developed countries, such as Australia, FGR affects ~7% of all pregnancies[5], whereas in low-income countries the incidence is up to six times higher; 10-20% in Latin America; 15-25% in Africa and 30-55% in South Central Asia[6].

FGR infants have worse postnatal outcomes compared to their appropriately grown (AG) counterparts, irrespective of gestational age. Further, FGR neonates require greater post-natal support [7–10], have increased rates of admission to the neonatal intensive care unit (NICU)[11,12] and are at greater risk of neonatal morbidities such as meconium aspiration syndrome, respiratory distress, hypotension, hypothermia, hypoglycemia and bronchopulmonary dysplasia[13].

FGR commonly occurs due to impaired blood flow within the placenta, resulting in a decreased delivery of oxygen and nutrients to the fetus[14]. This is termed 'placental insufficiency' and can be diagnosed via Doppler ultrasound to identify reduced, absent or reversed end-diastolic flow in the uterine artery. Consequently, these fetuses develop in a low-nutrient, adverse intrauterine environment[15]. These adverse conditions drive a decrease in growth and size of FGR fetuses. In response to fetal hypoxia, fetuses mount cardiovascular (CV) adaptations to maintain perfusion and nutrient supply to key organs[16] such as the brain, heart and adrenal glands. However, redistribution of blood flow occurs at the expense of non-essential organs including the gut, kidneys and peripheral circulation (arms and legs)[17]. When maintained long-term, cardiovascular redistribution alters normal structure and function in many organ systems, including the cardiovascular system. Indeed, the hypoxia-induced changes to the cardiovascular system are a vital mechanism underlying developmental programming for long-term cardiovascular, metabolic and neurological disease[7] in FGR adults[18] and children[19].

The unique physiological consequences of FGR within specific organs still remains largely unknown. Understanding these consequences is critical, as without this knowledge we are unable to provide targeted antenatal therapies or postnatal treatments for FGR, and to organ specific pathologies. A growing body of work now shows that FGR and AG offspring have different responses to many common therapies[20–22], resulting in physiologically diverse responses which

may further injure organs. Clinically used treatment may be less efficient or even detrimental to the FGR neonate. At the time of writing this thesis, The Sildenafil Therapy in Dismal Prognosis Early-onset Fetal Growth Restriction (STRIDER) a large multi-centered, multinational clinical trial was halted[22]. This trial began in 2014 following evidence for improved placental blood flow and fetal growth in several pre-clinical studies[23–31]. However, the STRIDER trial was halted following unexpected pulmonary complications and death[22]. The outcomes of the STRIDER trial has highlighted the need for a greater understanding of i) the altered physiology of the FGR neonate and ii) the potential for different therapeutic responses in FGR offspring. As discussed above, the cardiovascular system is heavily impacted in the context of FGR. Therefore, investigation into how vascular therapies (such as Sildenafil Citrate (SC) and dopamine) affect the FGR perinate is warranted.

To understand the effects of FGR on the development and function of the cardiovascular system, this review will discuss the development of this system, placental function, how placental insufficiency results in impairment of key cardiovascular functions and how these changes could impact on potential therapeutics. Specifically, this thesis will investigate the antenatal and neonatal effects of SC in FGR lambs to highlight potential mechanisms behind the failure of the STRIDER trial.

1.1.A Fetal Cardiovascular Development and Function

The cardiovascular system (CVS) is comprised of a four-chambered heart connected to a vascular network spanning the entire body. The role of the CVS is to perfuse organs around the body with blood, supply substrates and remove waste products. Cardiovascular development, which encompasses the formation of a functional heart and vascular network, begins during the third week of gestation. The development of the cardiovascular system involves several distinct fetal events; cardiogenesis, vasculogenesis and angiogenesis. Cardiogenesis describes the process in which the heart grows and develops, vasculogenesis describes the formation of new blood vessels from endothelial precursor cells and angiogenesis describes the growth of blood vessels from existing blood vessels.

1.1.B Cardiogenesis - Development of the Heart

All organs and tissues arise from the mesoderm, one of the three primary germ layers that form following conception. The heart is comprised of several different types of cells that come from different embryonic progenitor cells, including cardiogenic mesoderm cells (CMCs), proepicardium cells (PEs) and cardiac neural crest cells (CNCCs). These cells differentiate to give

rise to the cells that constitute the CVS; CMCs give rise to cardiomyocytes as well as endocardial cells and conduction system cells[32], PEs give rise to a endothelial and smooth muscle cells, cardiac fibroblasts and the epicardial lining[33] and CNCCs have multipotent properties and can give rise to many different cells within the heart including; the vessel walls of large arteries, the cardiac septum and the carotid body[34].

The heart is the first functional organ to develops in vertebrates[35] and development begins with the formation of the cardiogenic plate at around day 15 of gestation[36]. At day 18 of gestation the outer form of the future heart develops[37] (Fig 1). The cardiogenic plate is comprised of a mass of mesodermal cells and following a complex cascade of cellular signaling from the endoderm[37,38], this forms two tubular structures called the endocardial tubes. These tubes fuse and at day 22 of gestation to form the 'heart tube', a primitive but functional precursor of the heart[37].

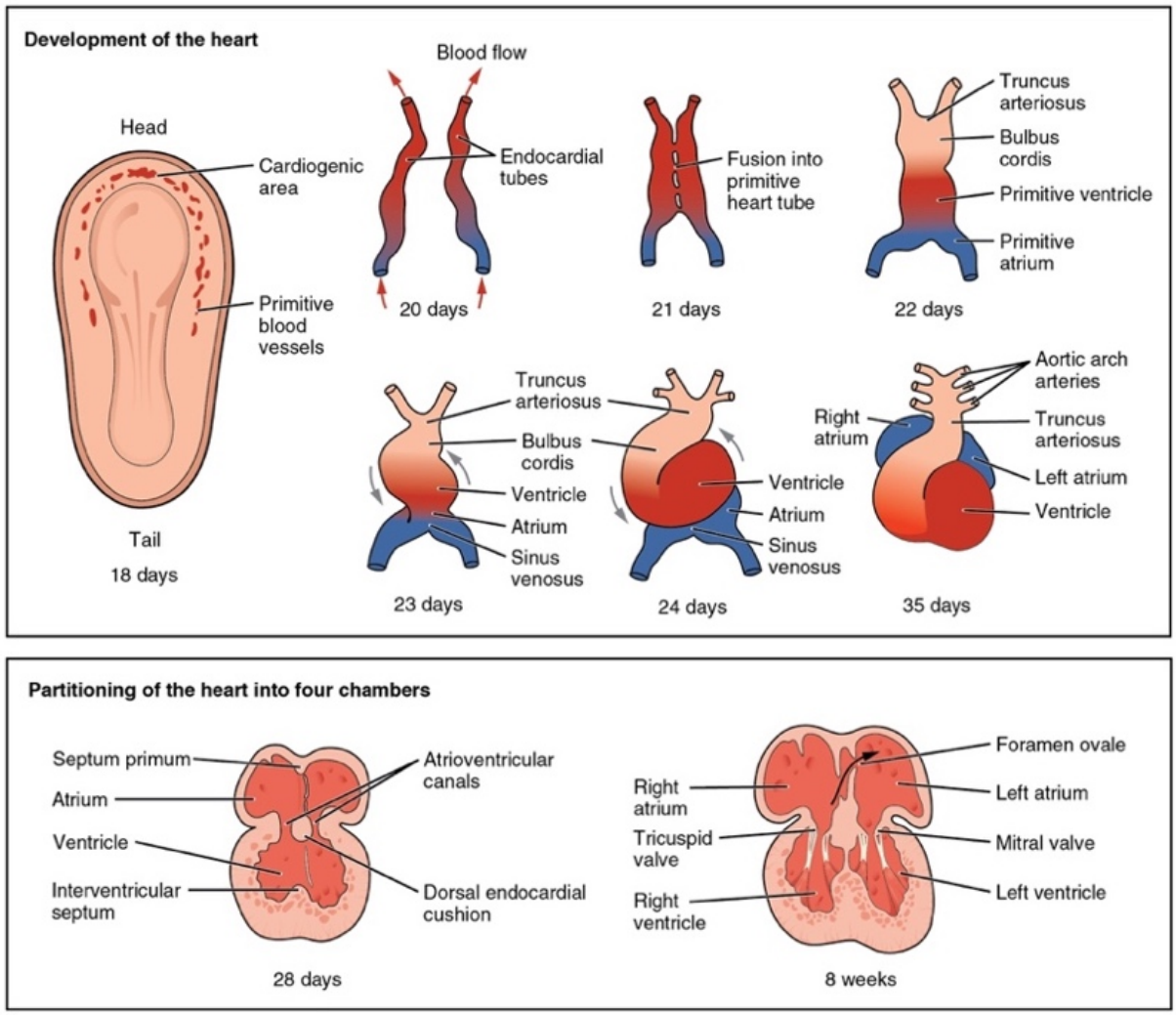

Figure 1. Embryonic development of the heart and formation of the four heart chambers. Image adapted from "*A new hypothesis for foregut and heart tube formation based on differential growth and actomyosin contraction"*[89]

The heart tube displays peristaltic beats at day 28 of gestation, but blood flow within the tube moves back and forth and is not pumped anywhere other than the primitive heart itself[39]. The development of the four chambered heart begins with a looping stage, forming a 'heart loop structure'. This structure undergoes a series of looping and folding due to differential growth of the heart tube, mediated by the expression of several cardiac regulatory genes[40] (Fig 1). The folding of the heart tube ultimately gives rise to the four chambers of the heart. As seen in Fig 1, the heart tube can be segmented into the primitive structures of the heart and S-shaped looping of the heart tube results in the folding of the 'atria' to lie behind the ventricles[41] and give rise to the early ventricular structures of the heart. The atrial and ventricular segments of the heart experience increased cardiomyocyte differentiation relative to the other segments of the heart tube[36].

Following the development of the heart tube the heart undergoes septation, which involves the remodeling of the heart tube into the four chambers of the heart that resemble that of the adult heart[42]. Tissue from the roof of the atria forms the septum primum (Fig 1) resulting in the division of the left and right atria. Additionally, as the ventricle walls develop, their medial walls fuse and form an intraventricular septum (Fig 1). These structures begin to form the future septum between the heart segments.

At week 5 of gestation, the atrioventricular valves (tricuspid and mitral valves) begin to develop between the upper and lower chambers of the heart (Fig 1). The formation of these valves maintains and promotes the movement of blood from atria to ventricle. The top of the heart tube forms a structure called the truncus arteriosus, which are the primitive structures of the aorta and pulmonary artery. These key vessels also develop alongside the folding of the heart tube, and the aortic and pulmonary valves also develop and maintain the outflow tract of the heart in parallel with the development of the atrioventricular valves.

The growth of the heart during gestation is regulated by the division of cardiomyocytes, which are the functional units of the heart[43]. Cardiomyocytes make up the majority of the heart tissue, including the heart walls and valves[44]. During early fetal life, cardiomyocytes proliferate and effectively drive the growth of the heart during gestation. The proliferation of cardiomyocytes and development of cardiac structures is regulated by the expression of several regulatory genes such as hypoxia inducible factor -1 (HIF-1) and vascular endothelial growth factor (VEGF)[45]. Peak differentiation of cardiomyocytes occurs at ~20 weeks of gestation, but decreases as gestation

progresses[46]. Further cardiac development involves a switch from hyperplasia (increased reproduction of cells) to hypertrophy (increase in cell size) which occurs soon after birth[47]. In the early stages of neonatal life, mammalian cardiomyocytes exit cell cycling and division stops[48]. Hereafter, heart growth occurs exclusively through cardiac hypertrophy[49] as the heart continues to mature and contract[46].

An important step in cardiogenesis is the development of the conduction system and innervation of the heart, which controls the contraction of the cardiomyocytes and effectively, the heartbeat of the fetus. The human autonomic nervous system is comprised of two opposing arms; the sympathetic and parasympathetic nervous systems. As previously discussed, at ~4 weeks of gestation the heart tube 'beats', via peristaltic contractions and occurs independently of cardiac innervation. During these early stages of gestation, heart rate is regulated by the autonomic nervous system via sympathetic nerve fibers acting on arterial chemoreceptors[50]. These fibers arise from thoracic neural crest cells and result in positive inotropisim of the cardiac muscle during early development. This suggests that during these early stages of fetal development, the development of these systems and receptors occurs in parallel to heart development.

As fetal development progresses, so does the opposing arm of the autonomic nervous system; the parasympathetic nervous system. Parasympathetic control of the heart is regulated by branches of the vagus nerve which originate from the medulla oblongata within the brain[51]. Several studies have demonstrated that the switch between the control of fetal heart rate from the sympathetic to parasympathetic nervous system occurs during ~ 21 weeks of gestation, indicated by a slowing in fetal heart rate[52]. Examination of human fetal brains by Cheng et al have shown that vagal development was detectable at 8 weeks GA and matures at ~24 weeks of GA[53]. These findings agree with the observations of preclinical and clinical studies, observing a decrease in fetal heart rate as gestation progresses. In humans, fetal heart rate, measured by Doppler, was 175-180 beats per minute at week 9 of GA and by week 14 of GA, heart rate decelerated to 140-145 beats per minute, where it remains until 21 weeks of GA[52]. This suggests that vagal maturation in the fetus occurs between the 11th and 21st week of gestation[52]. Additionally, studies in fetal and neonatal lambs have demonstrated that the sympathetic influence declines after 120 days of GA, coinciding with a decrease in fetal heart rate[54]. The decrease in fetal heart rate is attributed to increasing parasympathetic input[54] and beyond this stage the levels of sympathetic and parasympathetic inputs are stable.

1.2. Vasculogenesis and Angiogenesis- Development of the blood vessels

The vasculature of an organism is the conduit via which organs receive nutrients, oxygen, chemical signals and have wastes removed. The delivery of blood to all fetal organs is particularly crucial as metabolic demand increases with increasing organ development. As gestation progresses, so too does oxygen requirement. Therefore maintaining adequate oxygen delivery is essential to achieve optimal fetal development and growth[35]. As such, the development of the vascular network is integral for fetal survival and development. Development of this network involves two distinct mechanisms; vasculogenesis and angiogenesis.

Vasculogenesis is the formation of a de novo vascular network in the embryo via differentiation of endothelial precursor cells (EPCs) or angioblasts into endothelial cells[55]. Similarly to the heart, development of the vascular system begins early in gestation to allow delivery of blood to the growing fetus. The process of vasculogenesis begins in the 3rd week of gestation in the mesodermal layer of the embryo. Mesodermal cells differentiate into haemangioblasts and migrate into clusters, forming blood islands[56]. Hemangioblasts, are multipotent precursor cells that differentiate into ECPs and hematopoietic stem cells, forming both the vessel and blood that flows within it. Blood islands are cell aggregates that appear at days 13-15 of gestation and formation of these aggregates mark the initiation of vasculogenesis[56] (Fig 2A). Position of the cells within the blood islands are important for the future lineage of the cell. ECPs are located on the periphery of the clusters, whereas cells in the center of the blood islands differentiate into hematopoietic stem cells[56]. As growth of multiple blood islands progress, they fuse to form the primitive capillary network. A key event in the development of blood cells is the formation of the lumen to allow for the flow of blood though the blood vessel. Maturation of the blood vessel involves tissue-specific differentiation of EMCs with functional roles within the vasculature[56]. These include endothelial cells (ECs), which regulate blood vessel function. ECs cell-surface receptors for various growth factors that perpetuate the development of the vascular network[56]. Following the development of the initial vessel structure, growth and development of the vascular network relies on sprouting angiogenesis. By the fourth week of gestation, circulatory patterns can be observed via ultrasound[57].

Angiogenesis is the growth of blood vessels from an existing vessel and perpetuates the development of the vascular network by ongoing sprouting and splitting[56] (Fig 2B). This process involves several key steps. The first of these steps is the degradation of the basement membrane of the pre-existing vessel. The vessel's basement membrane is tasked with holding ECs in place

and proteolytic breakdown of this layer allows the liberation of ECs[56]. ECs are 'activated' by molecular signals to initiate the stimulation of new vessel growth. The activated cells form a tip cell and bud away from the existing vessel, forming a capillary sprout. The tip cell continues to migrate away from the existing vessel, along with the EC behind it which proliferate to form a stalk[56]. As the capillary sprout develops, vacuoles within the EC begin to form and coalesce. This then forms a lumen allowing for the flow of blood within the newly formed blood vessel.

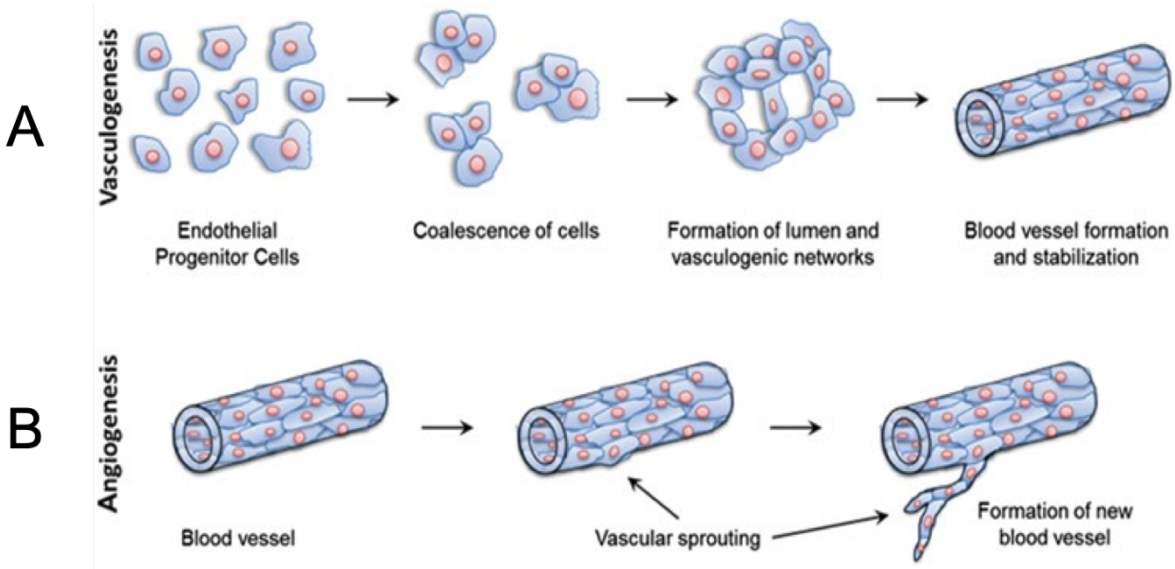

Figure 2. Angiogenesis and Vasculogenesis. Endothelial progenitor cells coalesce to form new blood vessels via vasculogenesis (A). From these established vessels, vascular sprouting can occur, resulting the in the development of new vessels via angiogenesis (B). *Image adapted from Microscale Technologies for Engineering Complex Tissue Structure*[58].

1.3. Normal Cardiovascular Function in Fetal Life

The heart and CVS develop in an environment which is distinct from the one it will operate in postnatally. In the adult/neonatal circulation (Fig 3B) blood moves from the right side of the heart, to the lungs for oxygenation, to the left side of the heart and is then pumped into the systemic ciruclation[59]. A key difference between fetal and neonatal life is the presence of the placenta. Oxygenation of fetal blood is one of the main placental functions (discussed below). The placenta contains a substantial reservoir of fetal blood and makes up ~30% of total fetal blood volume[60]. The placenta also provides ~50% of left ventricular output via two fetal shunts[60], the ductus venosus and foreman ovale.

Another key difference between the fetal and neonatal circulation is the vasculature of the lung. Contrary to the placenta, the vasculature of the lung is highly resistant and consequently has low blood flow[61]. High pulmonary vascular resistance in the fetal lung is driven by the fluid filled airways[62] and thick vessels walls with a high vasomotor tone [63,64]. Two fetal structures; the foramen ovale and ductus arteriosus, allow for the majority of fetal blood to bypass the lung entirely. The foramen ovale is an opening between the left and right atria of the heart and allows for oxygenated blood from the placenta to move from the right side of the heart, into the left side, bypassing the pulmonary circulation completely[65]. The ductus arteriosus is a fetal vessel that connects the pulmonary artery to the descending aorta and allows for blood from the right ventricle to again bypass the pulmonary circulation and directly enter the systemic circulation[65]. Together, these structures enable 92% of the fetal blood to bypass the pulmonary circulation[65] and in combination with the high pulmonary vascular resistance, contributes to the low blood flow within the lung. Consequently, the fetal lungs are not used for oxygenation, for which the placenta is solely responsible.

Fetal cardiac output is made up of blood flow from two inputs; the placenta and the lower body of the fetus. Highly oxygenated blood from the placenta travels to the fetal heart via the umbilical vein and enters the right atrium of the heart. The majority of blood that enters the right atrium moves into the left atrium, via the foramen ovale. Blood then moves from the left atria and ventricle following contraction into the aorta. This pathway of blood flow allows for highly oxygenated blood to be directed towards the brain. Relatively desaturated blood from the lower body of the fetus also re-enters the fetal circulation via the ductus venosus, a fetal-shunt that enables bypass of the hepatic circulation. Blood through the ductus venosus flows into the inferior vena cava and into the right ventricle of the heart. Right ventricular output moves into the pulmonary artery, however due to the presence of the ductus arteriosus 88% of this output enters the descending pulmonary artery to supply the lower body of the fetus. Movement of blood into the main pulmonary artery does results in blood flow into the lung. However, due to the high resistance of the fluid filled fetal lung, this blood is reflected from the pulmonary circulation. Only 8% of the total cardiac output enters the pulmonary circulation, meaning that the pulmonary circulation only provides a small amount of left ventricular output in the fetus[65]. This circulation can be visualised in Fig 3A below.

As discussed, adequate cardiac and vascular development are key events during fetal life. The heart and the blood vessels into which they pump have a synergistic relationship. Compromise in the

cardiac and/or vascular system can lead to and/or exacerbate compromise in the other. The consequences of impaired fetal cardiovascular development and function is discussed in depth below.

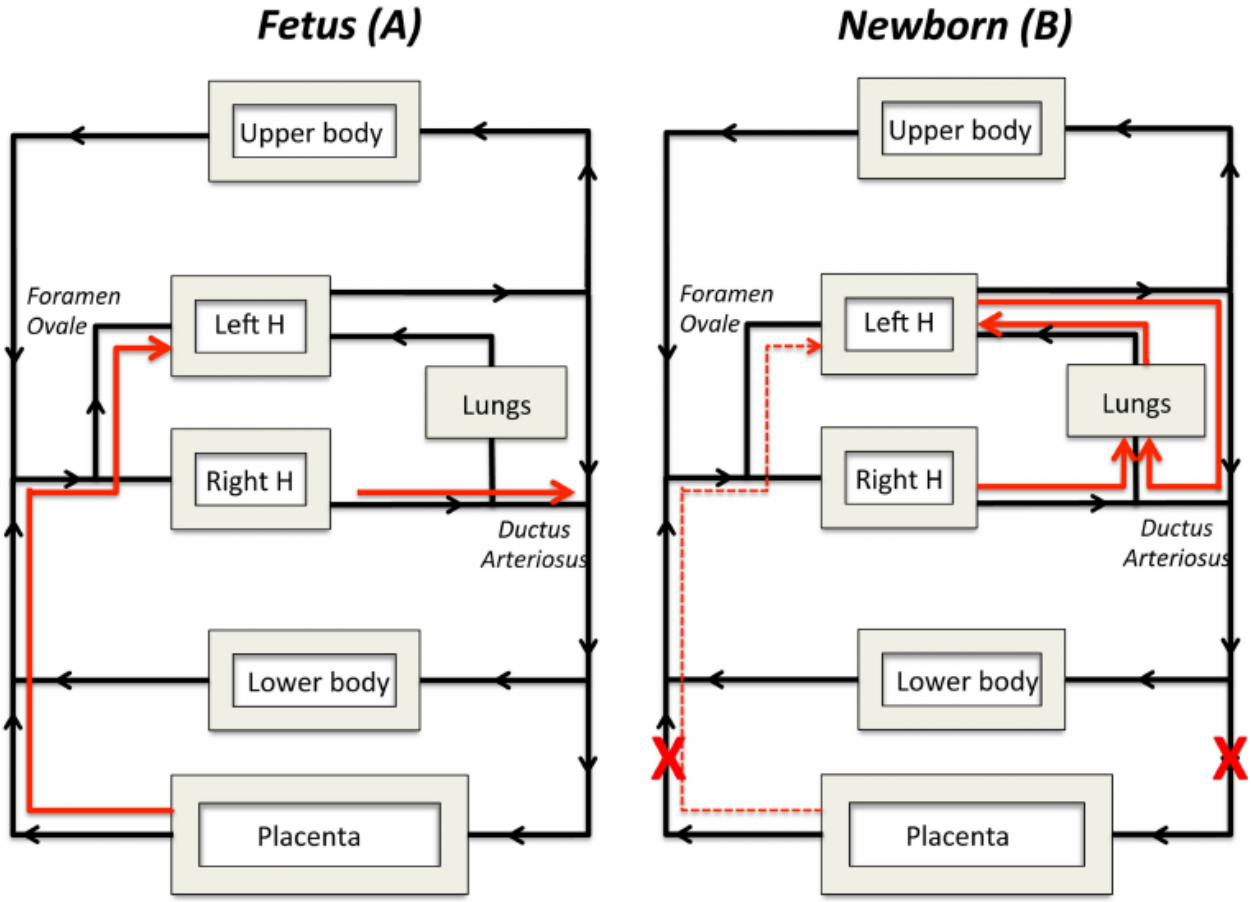

Figure 3. Fetal and Neonatal Circulation. The fetal circulation (A) is vastly different to the newborn (B) due to the presence of fetal shunts; foramen ovale and ductus arteriosus results in the majority of venous return entering the left side of the heart, bypass of the lung and flow of blood to the placenta for oxygenation. Removal of the placenta and closure of these shunts results in blood moving into the right side of the heart, to the lung for oxygenation, to the left side of the heart and into the body. *Image adapted from A physiological approach to the timing of umbilical cord clamping at birth*[59]

1.4. The Endothelium and Normal Vascular Function

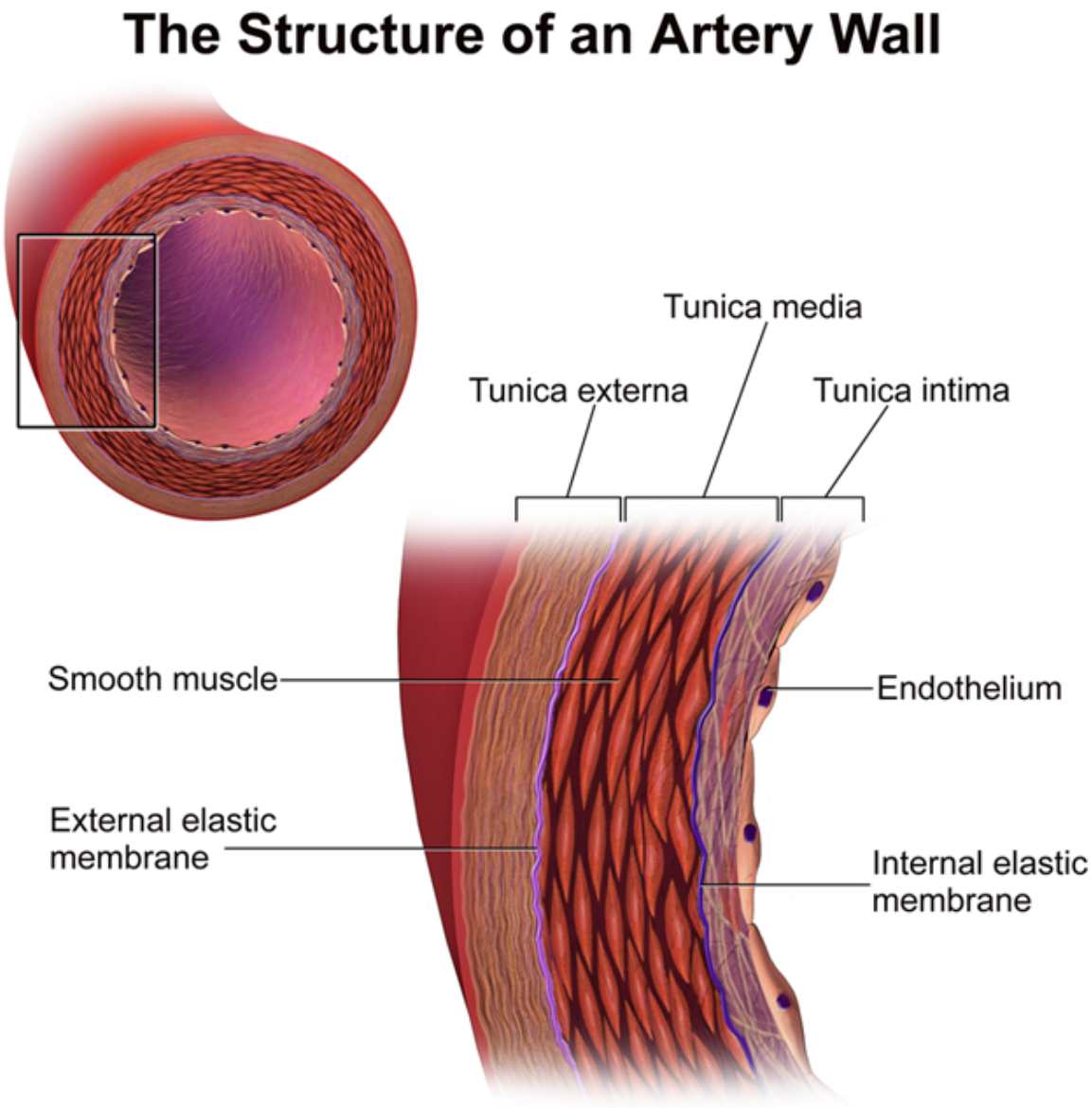

Figure 4. The Structure of an Artery Wall. The structure of a blood vessel can be divided in to three segments; the tunica external, tunica media and tunica intima. *Image adapted from Medical gallery of Blausen Medical 2014*[66].

As with all organs, function is dictated by structure. As seen above (Fig 4) the tunica externa, tunica media and tunica intima are three regions within a blood vessel which house specific cells that make up the vessel. The arrangement of these cells determines its structure and roll each layer will play in vascular function.

The tunica externa is the outermost layer of the blood vessel and is comprised mainly of collagen and elastin fibers. These fibers provide protection and stability to the vessel[67]. The tunica media is comprised mainly of smooth muscle cells, as well as collagen and elastin. Smooth muscle cells

within this layer respond to vasoactive signals received from the endothelium. Through communication between the endothelium and the smooth muscle, this layer dictates the vasodilation or vasocontraction of the blood vessel. The tunica intima is made up of two layers, the internal elastic membrane and the endothelium. The internal elastic membrane acts to provide strength to the endothelium[67]. The endothelium is the most important layer of the blood vessel. The endothelium is the only layer of the blood vessel that comes into contact with the blood that flows within it and responds to stimuli from the blood to regulate vessel diameter[67].

The vascular endothelium is one of the largest organs in the body and interacts with nearly every bodily system[68]. The endothelium is a continuous single vessel endothelial layer, which separates the smooth muscle layers of the circulatory system and the blood that flows within it[69]. Control of vascular tone is mediated by the endothelium via the release of vasoactive substances, which can alter blood vessel diameter and increase (vasodilate) or decrease (vasoconstrict) blood flow. Function of the vascular endothelium is critical for homeostasis as control of local vascular tone maintains appropriate organ perfusion.

A key regulator of vascular tone is the autonomic nervous system (ANS). As previously mentioned, the ANS is comprised of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). Both the SNS and PNS are a key regulators of the fetal heartbeat and can regulate homeostasis and the physiological response to stress[70]. Activation of these systems can occur via external stimuli such as sheer stress from blood flow, vascular stretch from increased cardiac output and intrinsic regulation via the local environment. Activation of the ANS can result in the release of vasodilative agents such as acetylcholine, nitric oxide (NO), prostacyclin and endothelium derived hyperpolarizing factor, and vasoconstrictive factors such as thromboxane and endothelin-1. Through the release of vasoactive agents by the endothelium[71], the ANS modulates vascular diameter to regulate blood flow and organ perfusion[70].

External stressful stimuli, recognized by the SNS, can induce vasocontraction via activation of α1-adrenoceptors by noradrenaline located on the smooth muscle of blood vessels[70]. Conversely, the SNS can also induce vasodilation via the release of neurotransmitters, such as acetylcholine, into the blood stream[70]. Binding of acetylcholine to cell surface receptors induces vasodilation by inducing the synthesis of vasoactive agents such as nitric oxide, prostacyclin and endothelial derived hyper-polarizing factor.

Nitric oxide (NO) is a key component of normal vascular function and is the main mediator of vasodilation throughout the entire body. Under homeostatic conditions, NO is continuously produced by nitric oxide synthase (NOS) to maintain a basal level of vasodialtion[72]. Three isoforms of NOS exist; neuronal NOS, inducible NOS and endothelial NOS.

All isoforms of NOS have role in blood pressure and flow regulation, via action on local vascular smooth muscle. However, the role of each isoform is dictated by the location of expression. For examples, inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) are both described to have roles in the regulation of blood flow. However, the function of nNOS is primarily localized to nervous tissue within the central and peripheral nervous system and has been identified in neurons, astrocytes and brain blood vessels[73]. Accordingly, nNOS is important regulation of blood flow within the brain and for cognitive function. nNOS has roles in synaptic plasticity, learning and memory. iNOS is expressed only under conditions of inflammation and injury and triggered by immunological or microbial stimuli[73]. iNOS expression is absent in the healthy state. The role is iNOS is not well understood. Studies suggest that iNOS expression is pathological and results in cardiovascular damage, while others have shown that iNOS may have a compensatory role in compromised NOS activity by maintaining NO production.

Endothelial nitric oxide synthase (eNOS), is expressed within the endothelial layer of all blood vessels and play an integral role in the maintenance of normal blood flow and pressure. Activity of eNOS is dependent upon a calcium and calmodulin mediated pathway[74,75] (Fig 5) which can be initiated via shear stress from an increase in blood flow or via ligand interaction with receptors located on the surface of endothelial cells. These include, but are not limited to, acetylcholine, bradykinin and adenosine [75]. NO is catalysed within the endothelium via conversion of L-arginine into NO by endothelial nitric oxide synthase (eNOS). The uptake of L-arginine into the cells is mediated by cationic amino acid transporters[76]. The half-life of NO is only a few seconds and rapidly diffuses from endothelial cells and into the adjacent smooth muscle cells[75]. This initiates the pathway of NO mediated vasodilation, beginning with NO binding to guanylyl cyclase (GC) resulting in dephosphorylation of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). Increase in cGMP results in the inhibition of calcium entry into the cell and activation of K+ channels to dephosphorylate myosin-like chains of the smooth muscle[75]. Through this pathway, NO induces smooth muscle relaxation, vasodilation to ultimately increase blood flow. Although NO is recognized as the key mediator of vascular tone, other pathways, including prostacyclin and endothelium-derived hyper polarising factor also induce vasodilation.

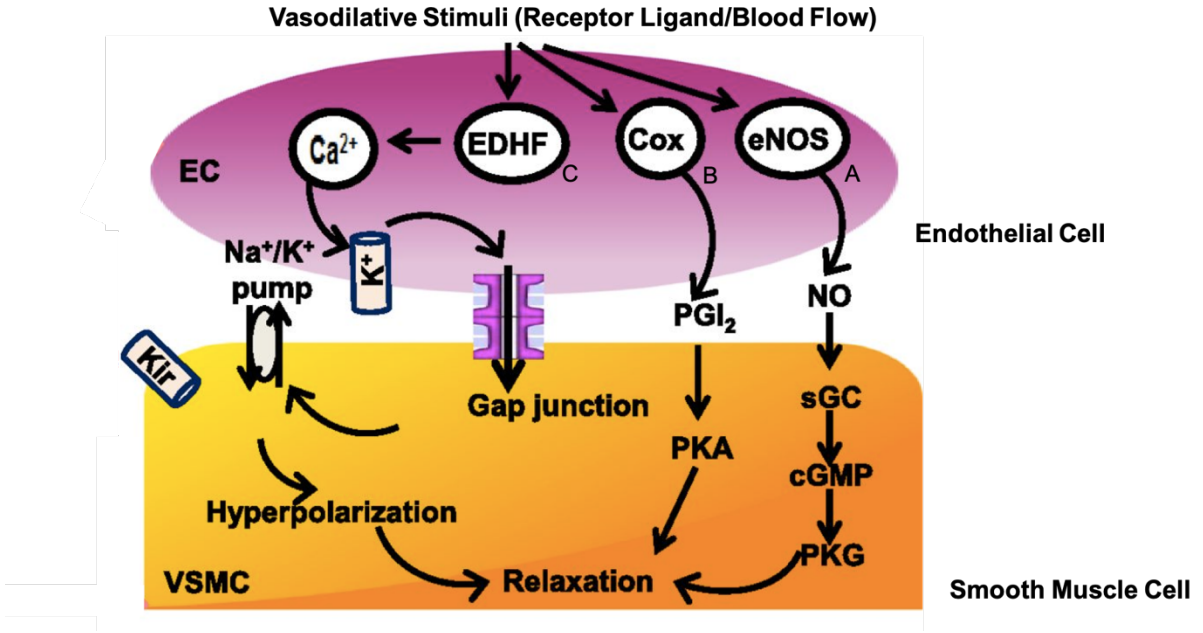

Figure 5. Smooth muscle relaxation from nitric oxide (NO), prostacyclin (PGl2) and endothelial derived hyperpolarizing factor (EDHF) synthesis following blood flow and/or receptor-ligand binding. Blood flow increase can result in the release of vasodilators, such as acetylcholine, which bind to receptors on the surface of endothelial cells and ultimately result in nitric oxide (A), prostacyclin (B) and endothelial derived hyperpolarizing factor (EDHF) synthesis and ultimately smooth muscle relaxation. eNOS; endothelial nitric oxide synthase; COX: cyclooxygenase; Ca^{2+}: Calcium Ion; sGC: Guanylyl Cyclase; GTP; cGMP: Cyclic Guanosine Monophosphate; PKG: protein kinase G, PKA: protein kinase A. *Image adapted from Endothelium-Derived Hyperpolarizing Factors: A Potential Therapeutic Target for Vascular Dysfunction in Obesity and Insulin Resistance*[77].

The activity of prostacyclin (PGl_2) is similar to that of NO (Fig 5). However, unlike NO, synthesis of prostacyclin is not continuous and release is initiated by membrane ligand-receptor binding rather than sheer-stress[78]. Prostacyclin synthesis begins with the removal of arachidonic acid (AA) from membrane-bound phospholipids following an influx of intracellular calcium. AA is metabolized by cyclooxygenase (COX) to form prostaglandin (PG), which in turn is peroxided into PGH2. This acts as substrate for prostacyclin synthase (PGlS) to result in the synthesis of PGl_2[78]. Similar to PGL_2, endothelium-derived hyper polarizing factor (EDHF is also released from the endothelium following cell surface ligand binding. The function of EDHF is most prominent in small arteries (internal diameters <300μm) and therefore plays in key role in organ perfusion and peripheral vascular resistance[79]. Similarly to cGMP, EDHF results in the influx of calcium ions into the cell and causes smooth muscle relaxation[79]. The release of EDHF occurs following hyperpolarization of the endothelial cell, to then activate potassium channels on the surface of smooth muscle cells.

The controlled release of NO, PGI_2 and EDHF by the endothelium is an integral component of appropriate vascular response and blood flow[69,80] and impaired endothelial function can result in inappropriate release of vasoactive factors. The placenta is a highly vascular organ and function relies on the maintenance of low vascular resistance. Unsurprisingly, endothelial dysfunction is known to contribute to placental insufficiency[81], the consequences of which are described below.

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1.5. The Relationship Between Placental Function and Fetal Development

Fetal growth and development are at the mercy of nutrient and oxygen availability during gestation. The primary role of the placenta is to facilitate adequate transfer of substrates from the maternal into the fetal circulation. Substrates required for fetal development originate from the maternal circulation are obtained from the maternal environment, diet and/or via maternal synthesis. Oxygen, glucose, lactate, and amino acids are the main substrates required by the fetus for normal growth and development[82]. The transfer of these substrates into the fetal circulation occur at the functional units of the placenta, termed the chorionic villi. As placenta-derived nutrient transfer is essential for sustaining fetal life and fetal development, it goes without saying that compromise of placental function, impairs fetal development and compromises fetal life[83]. Poor placental function is termed placental insufficiency and is the greatest cause of fetal growth restriction (FGR). To understand placental insufficiency, we must first understand normal placental function.

1.5.A. The Placenta; Structure and Function

Placental development occurs in parallel to fetal development and initiation of development starts from the moment of conception. Fertilization of the human oocyte forms a blastocyst, a multi-cellular structure, containing cells destined to differentiate into the fetus and placenta. The blastocyst's outer cell layer is termed the trophoblast and are the cells of the future placenta. The inner-cell mass is destined to become the fetus.

1.5.B. The Functional Units of the Placenta

As discussed above, the function of the placenta is blood-flow dependent. Successful development of the fetoplacental vascular structures and remodeling of the maternal endometrium is integral

for placental function. These processes give rise to the two distinct units of the placenta; 1) the fetoplacental side, which originates from the chorionic sac and 2) the uteroplacental (maternal) side, comprised of maternal endometrium[84]. Essentially, placental function is regulated by the action of these units, through which substrates pass between the maternal and fetal circulations.

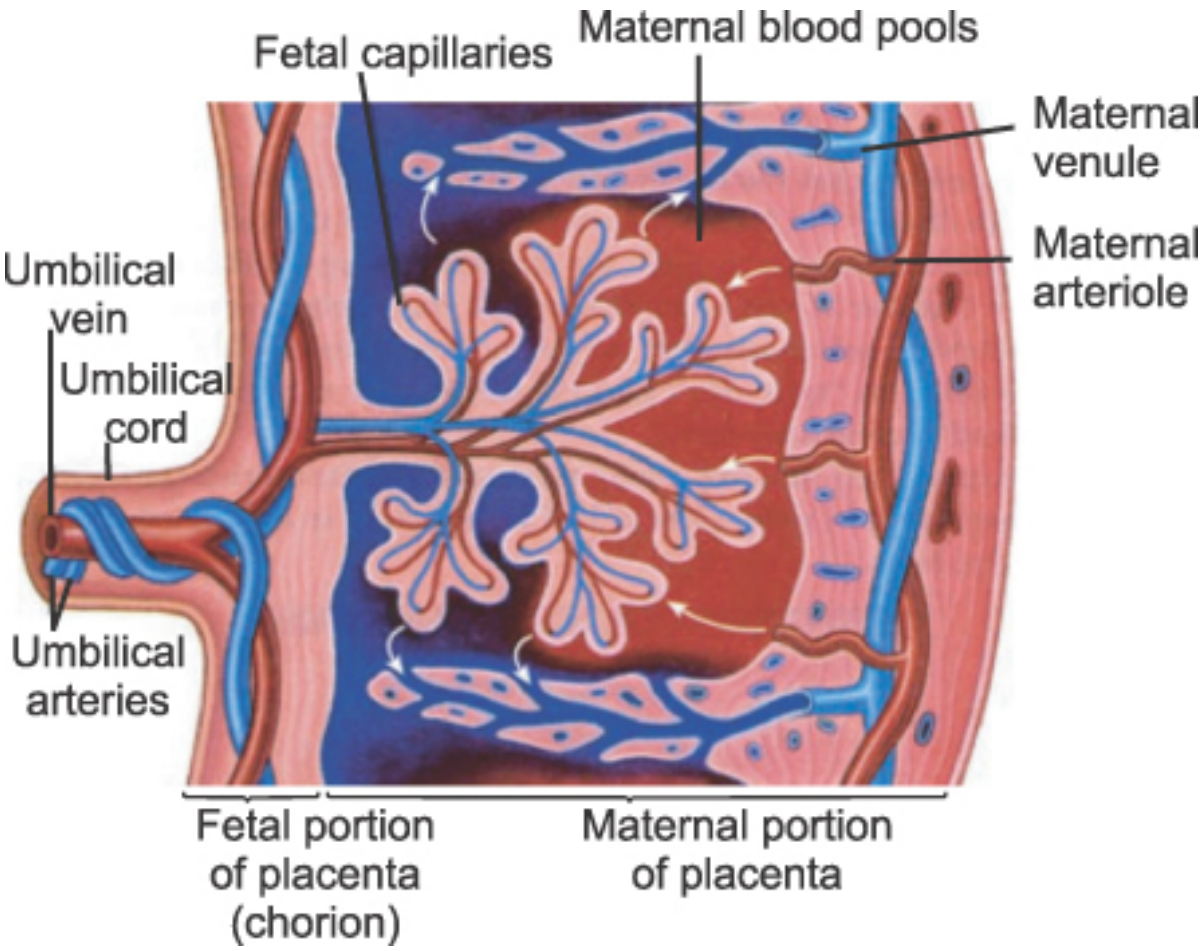

Figure 6. Units of the Uteroplacental and Fetoplacental Circulation. The umbilical vein and arteries comprise the fetoplacental side of the placental circulation. The umbilical vein is tasked with supplying oxygen and nutrients to the fetus, while the umbilical arteries remove carbon dioxide and waste. The maternal arterioles from the maternal circulation supply the uteroplacental circulation, filling the intervillous space with nutrient rich oxygenated blood, while maternal venules transfer fetal waste into the maternal circulation for excretion. *Image adapted from Cornell University Department of Biology*

In humans, the fetoplacental side of the placental circulation is comprised of one umbilical vein and two umbilical arteries. Together these blood vessel form villous protrusions, termed 'chorionic villi', which fill the intervillous space (Fig 6)[84,85]. The intervillous space is the interface between the fetal and maternal placental structures and is supplied with blood by eroded uterine spiral arteries. The intervillous space allows for transfer of substances between the fetal and maternal circulations,

while allowing them to remain separate. Hypercapnic, hypoxic and nutrient poor fetal blood moves into arteriole component of the chorionic villi from the umbilical arteries. Waste products from the fetal circulation diffuse into the intervillous space and into the maternal circulation via maternal venules. Fetal waste products are then excreted via the maternal portal and pulmonary circulation[86]. Conversely, oxygen and nutrients diffuse into venous component of the chorionic villi from the maternal blood in the inter villous space. These processes allow for nutrient rich, oxygenated blood to flow though the umbilical vein and supply the fetal circulation[87].

1.5.C. The Regulation of Placental Blood Flow

As previously discussed, placental function is reliant on consistent blood flow. This is integral to maintain both placental function and fetal life. Control of placental blood flow at the vascular level is described by Poiseuille's Law, which takes into account: 1) the vessel diameter, 2) the vessel length and 3) the viscosity of the blood[88].

The viscosity describes the thickness of the blood, which is influenced by the concentration of cells and other substrates within the blood[89]. High blood viscosity results in decreased velocity of blood flow, which in turn results in decreased perfusion. During pregnancy, maternal haematocrit and whole blood viscosity decreases during weeks 20-31[90]. Decreased maternal blood viscosity is driven from a maternal increase in plasma volume, resulting in haemodilution[91]. Conversely, fetal haematocrit increases during gestation to meet requirement by developing organs. Normal fetal haematocrit is ~40% at 22 weeks of gestation percentage and increases by ~%0.64 weekly until 40 weeks of gestation[92]. Although fetal haematocrit increases, fetuses have lower protein fractions in the plasma, thereby maintaining relatively low viscosity of fetal blood[93]. As per Poiseuille's Law, the low viscosity of the fetal and maternal blood results in decreased vascular resistance, maximizing blood flow for adequate substrate transfer to the fetus.

The remaining factors that dictate blood flow; vessel diameter and length, are determined by the physical structure of the blood vessel. Prior to pregnancy, the spiral arteries that supply the uterine lining with blood, are small and constricted. Thereby, in the non-pregnant state vascular resistance of these arteries is high. Conversely, in the pregnant state, spiral arteries undergo morphologic and physiologic changes, as depicted below in Fig 7. Placental vessels undergo trophoblast-associated remodeling during expansion of the trophoblast cells into the decidua of the uterus[94]. This process involves invasion of endovascular trophoblasts into the spiral arteries which interlock between endothelial cells. Eventually, these trophoblast cells replace the most of the musco-elastic tissue within the vessel walls. Through this process, trophoblasts transform these spiral arteries and

increase diameter and size, which ultimately decreases vascular resistance. Establishment of this circulation marks the development of the 'low-resistance' uteroplacental circulation, which is maintained by the structure of placental vessels and hormonal agents that regulate vessel diameter. The "low-resistance" vasculature of the placenta is also mediated by increased vasodilation of the placental arteries to increase blood vessel diameter. This is discussed in greater detail below.

Taken together with the low blood viscosity and remodeling of the spiral arteries, placental blood flow is high and increases during pregnancy to keep up with the metabolic needs of the developing fetus[86]. Placental function can be compromised by several factors including 1) increased blood viscosity; 2) poor development of placental functional units and 3) dysfunctional placental vasculature. These factors can result in placental insufficiency and impaired delivery of substrates to the developing fetus.

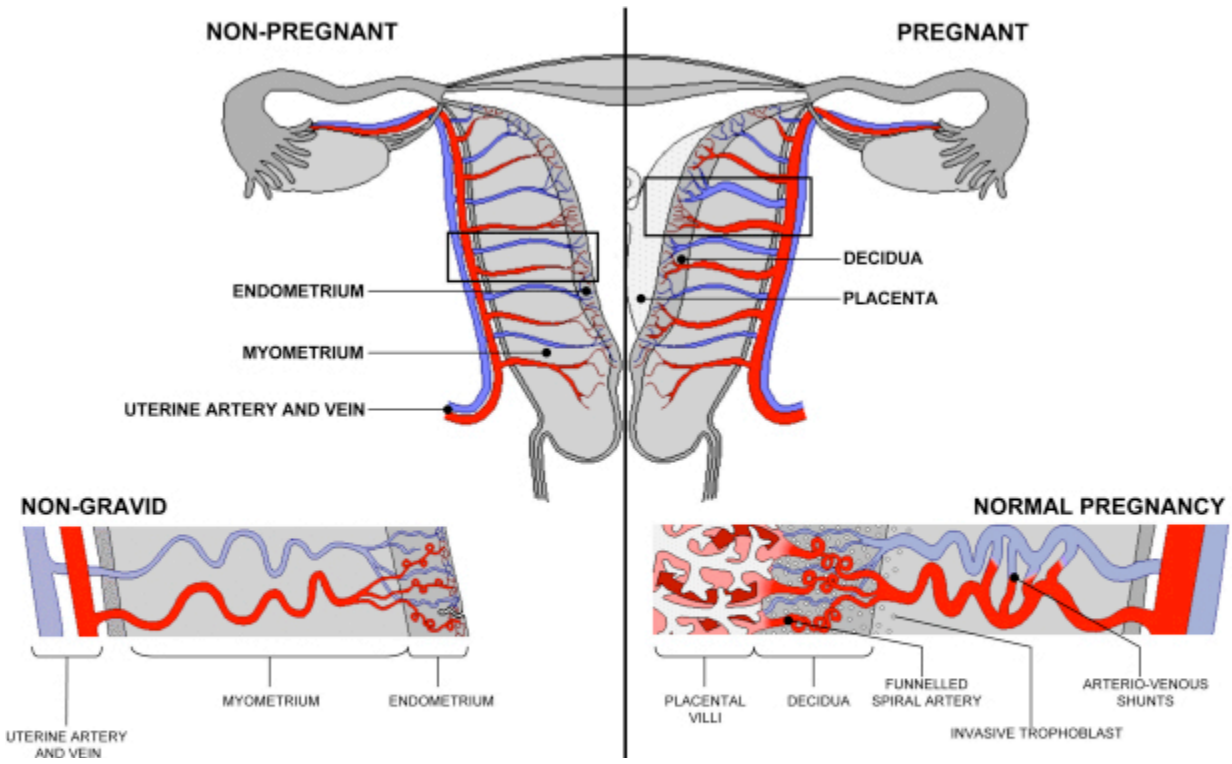

Figure 7. Uterine Circulation in the Setting of Non-pregnant and Normal Pregnancy. *Image adapted from Rheological and Physiological Consequences of Conversion of the Maternal Spiral Arteries for Uteroplacental Blood Flow during Human Pregnancy*[95].

1.6. Placental Insufficiency

By definition, placental insufficiency is the inability to deliver adequate nutrients and oxygen to the developing fetus [96,97] and the fetus that fails to reach its growth potential. This is termed fetal growth restriction (FGR). The origins of FGR are complex and can be maternal, fetal and/or placental in origin. Common amongst all causes of FGR is the development of an adverse intrauterine environment[96,97].

1.6.A The Placental Vascular System and Fetal Growth Restriction

As discussed above, placental perfusion underpins placental function and impaired uterine vascular development and vasodilation can result in decreases to fetal substrate delivery[98]. Similarly, to the developing cardiovascular circulation, both angiogenesis and vasculogenesis are crucial in the development of the placental vasculature.

In the placenta, vascular growth is regulated by the action of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Binding of VEGF to VEGF receptors on endothelial cells within the placental vasculature initiates the development of new blood vessels[55]. In the context of placental development, VEGF has both vasculogenic and angiogenic capability. VEGF and PlGF are angiogenic factors that stimulate the growth of blood vessels[99] and impaired expression of these factors is implicated in decreased placental function and FGR[99–102]. Regnault et al have shown decreased VEGF receptor expression in the placenta of FGR lambs[99]. Studies in mice have shown that blocking of VEGF receptors VEGFR1 and VRGFR2 result in embryonic lethality[74] and the blocking of VEGF signaling mid-pregnancy results in preterm birth[103]. These results suggest that VEGF plays a key role in establishment of the placental vasculature and is a factor that is required for both the initiation of placental development and the development of the placental vasculature as gestation progresses.

Placental growth factor (PlGF) is an angiogenic factor part of the VEGF family that binds to receptor fms tyrosine kinase (Flt-1) on endothelial cells to induce angiogenesis[104]. In the context of FGR, decreased placental PlGF is associated with abnormal fetal blood flow indicative of increased placental vascular resistance[102]. Additionally, PlGF knockout mice have impaired placental angiogenesis. Clinical studies by Semvzuk-Sikora and Cetin et al have shown that PlGF concentration is decreased in the serum of mothers, pregnant with a FGR fetus [100,101]. Taken together, these studies demonstrate the role of PIGF in normal placental vascularisation[105].

Interestingly, Azliana et al have also shown that overexpression of VEGF is associated with hypertensive disorders in pregnancy[106]. These results suggest that overexpression of VEGF, and subsequent blood vessel overgrowth, can also lead to pathologic pregnancy. Taken together these results show the development of the placental vascular system requires tight regulation. Pathologic pregnancy occurs when the development of the placental vascular system does not occur properly.

1.6.B Oxygenation and the Placental Vascular System

The relationship between FGR and oxygenation is complex. While impaired placental function results in fetal hypoxia, an initial state of hypoxia is required to properly initiate embryonic development. Therefore, fine control of oxygen levels is required throughout development. Oxygen is a key mediator for the expression of vascular growth factors and local oxygen levels can influence placental vascular development[107]. Shore et al have shown that hypoxic conditions upregulate VEGF expression, while hyperoxia down regulate VEGF in human placental trophoblast cells[108].

During early development, prior to establishment of blood flow, the placental vasculature develops within a hypoxic environment. In human cytotrophoblast culture studies, Genbacev et al have shown that prior to week 7 of gestation, hypoxic conditions allow for cytotrophoblast differentiation, but is inhibited at week ~10-12 of gestation[109]. Towards the end of the first trimester (weeks ~10-12) placental blood flow and oxygen content increases as maternal blood flow increases from cytotrophoblast invasion and breakdown of the uterine endothelium[96]. Genbacev hypothesize that the hypoxic conditions in early pregnancy (week ~7) allows for trophoblast differentiation and invasion and the initial establishment of the early structures of the placental circulation. These data highlight the regulatory role of hypoxia/hyperoxia on placental development during different stages of pregnancy.

The transition from the hypoxic state, to the now relative hyperoxic state, triggers trophoblast cells to invade and remodel the maternal spiral arteries[110]. As discussed above, remodeling of the maternal spiral arteries results in increased growth and size, allowing for the development of the low resistance placental vasculature[109].

1.6.C Control of Vascular Tone and Fetal Growth Restriction

In normal pregnancy, the placental vasculature is maximally dilated to ensure maximal perfusion and thereby, placental vessels experience forward-flow, even during diastole [111,112]. The "low resistance" placental circulatory unit ensures constant and continuous perfusion (both fetal and

maternal sides) to enable maximal substrate transfer. In several mammalian species (sheep[113] and cows[114]) as well as in humans[115], umbilical blood flow increases as umbilical vessel resistance decreases over gestation. As these increases in blood flow are in line with the increasing development and size of the fetus, it is reasonable to suggest that increased blood supply is required to maintain and promote adequate fetal development.

The placenta is not regulated by adrenergic and cholinergic innervation[116] and the low vascular tone is regulated by vasodilative factors, such as NO. As discussed above, NO is a potent vasodilator and integral for normal vascular function. In the context of placental function, NO plays key roles in the initiation and maintenance of placental function and fetal life. This is evident by expression of placental eNOS increasing dramatically from the start of pregnancy and peaking at the second trimester[117].

Inappropriate expression of NO, receptors and synthases are seen in pathologic pregnancy and both inappropriate increase and decrease of NO are associated with the development of FGR. Studies by Dicket et al have shown that inhibition of NO in rats during the last trimester induces FGR[118]. Impairment of NO likely decreases fetal growth due to placental dysfunction caused by increased vascular tone. Studies by Lyall et al have shown that NO concentrations are increased in placental and umbilical venous blood complicated with FGR[119]. These results highlight the need for tight NO regulation.

1.6.D. Maternal Factors causing Fetal Growth Restriction

Environmental factors that affect maternal oxygen and nutrient levels such as; high altitude, malnutrition and drugs are known to increase the risk of FGR.

1.6.D.i. Maternal Oxygenation

As discussed above, fetal oxygenation is only possible via delivery of oxygen from the maternal circulation. Investigation into pregnancy in high-altitude environments has allowed for examination into the effects of sustained low-maternal oxygen levels on fetal development. Partial pressure of inspired oxygen decreases as altitude increases due to the fall in atmospheric pressure[120]. Pregnant women of populations who live at high-altitudes, such as Tibet, the Andes and Han China, live is a constant state of hypoxia relative to sea-level. Consequently, so too does their developing fetus. The low atmospheric oxygen available at high-altitudes has a two-fold impact on the developing fetus; 1) the partial pressure of oxygen available for transfer to the fetus is low and 2) as oxygen (which as discussed is a key regulator in placental development and

angiogenesis) is low, the placenta does not grow to its full capacity[121]. Consequently, there is a 100g fall in birth weight with every 1000m elevation in which the mother lives[122]. Interestingly, adaptation to high-altitude has been demonstrated in populations over generations of high-altitude dwelling, which gives some clues to the mechanisms underlying altitude-associated growth restriction.

Tibetans and Andeans have had long-term high-altitude settlements (~20,000 and 10,000 years respectively) whereas the Han Chinese have had relatively short high-altitude settlement (<500 years)[123]. Offspring of populations with long-term high-altitude settlements appear to be less affected by FGR and have birthweights a third greater than of populations of short-term settlements. Doppler ultrasound in Andean pregnancies have shown a two-fold increase in uterine artery flow and oxygen delivery in compared to Han counterparts[124]. Comparison between high-altitude populations demonstrate that maternal adaptations (of the Andean population) which increase blood flow and oxygen delivery to the fetus can decrease the severity of FGR. The lack of maternal adaptation (in the Han population) further demonstrates that fetal hypoxia is a key driver of FGR.

1.6.D.ii. Maternal Nutrition

Nutrition is essential for both fetal and maternal health. Maternal malnutrition results in the decreased substrate availability for the placenta and fetus[125]. The Dutch Potato Famine of 1944-1946 demonstrated a correlation between maternal starvation and reduced placental and fetal weight[126]. This effect is conserved amongst species and has been observed in feed restricted rats[127], sheep[128] and humans[129]. Undernutrition challenges the fetuses with an inadequate supply of substrates required for organ development resulting in growth restriction[130].

Interestingly, maternal malnutrition that occurred during the first trimester does not decrease placental and fetal weights relativel[129]. However, malnutrition during the third trimester of pregnancy significantly reduces both placental and fetal size. This suggest that insults occurring in early pregnancy occur when the fetus has reduced metabolic need allow for placental adaptation to meet fetal nutrient demands. However, insult occurring when fetal nutrient demands are increasing, i.e. after the first trimester, is more detrimental to fetal growth[129].

1.6.D.iii. Maternal Exposure to Toxic Substances

Teratogenesis is an altering of normal gene expression, resulting in physical or functional defects. In the context of fetal development, teratogenic substances can cause the disruption of normal

fetal and placental development[131]. Maternal drug use is a common mechanism of fetal/placental exposure to toxic substances. A common and well-studied substance implicated with adverse fetal development is cigarette smoke, which contains over 4000 different chemicals. Cadmium is a toxic heavy metal and maternal exposure is common in? with? cigarette smoke[132]. In large cohort studies[133], cadmium exposure has been shown to be associated with FGR. Studies in mice by Wang et al have shown that maternal cadmium exposure results in impaired placental vascularity and placental apoptosis[134]. Everson et al have suggested that this is driven by altered TNF and steroidogenic gene expression following cadmium exposure[135]. Another common teratogenic substance is benzo(a)pyrene, which is a compound that can be found in the air, water and soil[136]. Placental cell exposure to benzo(a)pyrene has been shown by Drutekenis et al to induce phosphorylation of the p53-dependent cell death pathway[137]. Activation of the p53 pathway results in the arrest of the cell cycle and apoptosis [138]. Inappropriate activation of the p53 pathway and subsequent organ compromise and dysfunction likely drives the placental dysfunction by benzo(a)pyrene.

Taken together, these studies demonstrate the potential effects teratogenic substances have on the progression of FGR. The consequences of improper expression of fetal and placental genes during gestation are vast. However, common to all previously described causes of placental insufficiency, teratogenic substances ultimately impair the development of the fetal and placental vasculature[139], decrease the flow of blood through this circulation to drive impaired placental function and adverse fetal development[140,141].

Smoking is also associated with increased blood viscosity[142]. As previously discussed, increased blood viscosity increases vascular resistance. Furthermore, smoking can also reduce the oxygen carrying capacity in the maternal blood, effectively resulting in a state of maternal hypoxia. Hemoglobin is a protein in the blood, which binds to oxygen enabling systemic delivery[143]. Carbon monoxide is a molecule with a similar structure to oxygen and can preferentially bind to hemoglobin, resulting in a decreased amount of oxygen carried in the blood[143]. Cigarette smoke contains up to 4.5% carbon monoxide. Maternal smoking can thereby disrupt maternal oxygen binding[144,145] and reduce diffusion of oxygen from the uteroplacental circulation into the fetoplacental circulation to cause in fetal hypoxia[146]. Several studies have shown that maternal smoking is a strong, independent risk factor for FGR, and increases the risk of FGR[147] in both healthy and compromised pregnancies[148,149]. Given that maternal smoking increases blood viscosity, impairs vascular development and decreases oxygen concentration, it is not surprising

that smoking during pregnancy is associated with FGR as all these factors can impair placental development and blood flow, resulting in impaired function and fetal substrate delivery.

1.6.E. Fetal factors causing Growth Restriction

Fetal factors of FGR are non-modifiable and include genetic or congenital malformations and infection. A considerable portion of fetal growth is determined by fetal genetics[150] and FGR derived from fetal genetics is associated with more severe FGR. Genetic-driven FGR is associated with symmetrical decrease in growth as impairment has been present since conception[151]. Genetic abnormalities are commonly associated with chromosomal anomalies such as trisomy 21 and 18[152], though the precise mechanisms behind how these altered genetic conditions result in FGR are not well understood. Studies suggest that these genetic conditions result in impaired cell division from inappropriate expression imprinted genes[153]. This may underlie the phenotypic changes associated with genetic anomaly driven FGR[153].

Fetal infection can be attributed to 5-15% of all FGR cases[154]. *Inutero* infection results in a subsequent inflammatory cascade and exposes the fetus to a pro-inflammatory environment[154]. This is well established to cause oxidative stress and cytokine injury to the fetus[155,156]. Infection can also have teratogenic effects and impair normal fetal development in similar mechanisms to toxic substance exposure. Further, infection may impair placental development or damage the placenta, thereby reducing placental function and substrate delivery to the fetus[157].

1.7. Clinical Identification of Fetal Growth Restriction

Potential for FGR can be determined *inutero* by observing fetal size over gestation. Ultrasound can be used to measure the size and length of arms, legs, abdomen and cranium of the fetus during gestation. From these measurements, fetal size can be tracked to determine the change in fetal biometry against normal percentiles is used to inform whether fetal growth is occurring as normal. If there is a falling in fetal growth velocity, a fetus can then be considered at risk of being growth restricted and clinical monitoring will be increased[158]. Fetal growth data can then be considered alongside ultrasound blood flow observations. The patterns of blood flow though the fetoplacental circulation have been well described and the observation of impaired or irregular placental blood flow (though blood vessels such as the umbilical artery (UA) and the middle cerebral artery (MCA)) can be diagnosed via Doppler ultrasound. Assessment of blood flow though the UA is particularly important as impaired flow is indicative of increased resistance within this blood vessel and can suggest placental insufficiency. Taken together, delayed fetal growth and impaired placental blood flow can be used to diagnose FGR. Additionally, increase blood flow in the MCA can indicate

brain-sparing in response to impaired UA flow (which is discussed in greater depth below) and increasing severity of FGR.

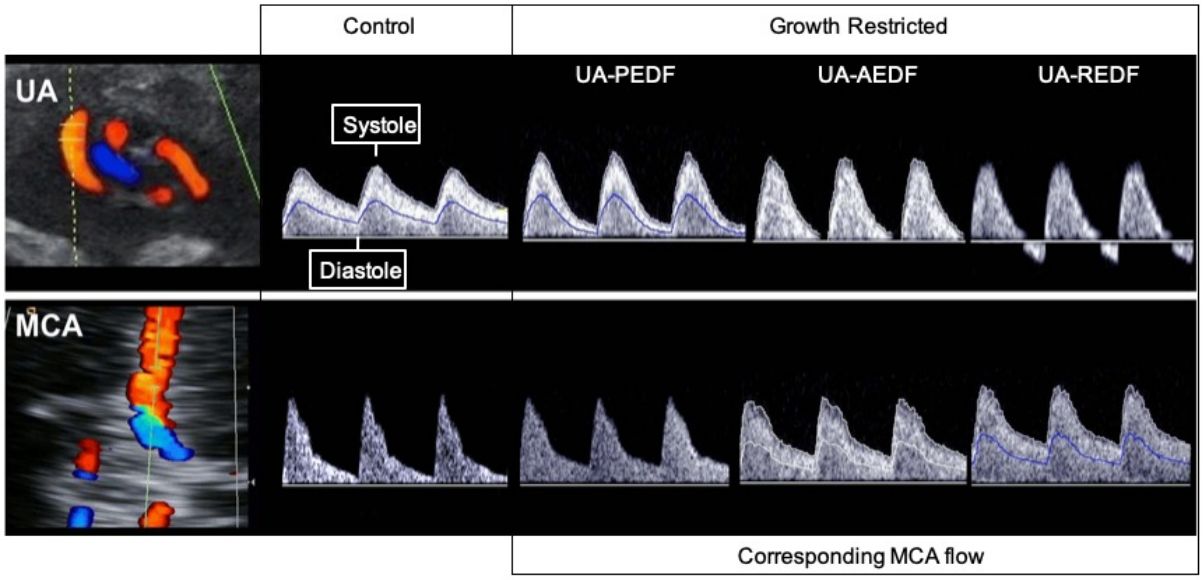

Figure 7. Doppler recordings from umbilical artery (UA) and corresponding middle cerebral artery (MCA) flow for control (appropriately grown) and growth restricted (FGR, and increasing severity of impaired umbilical artery end diastolic blood flow; present (PEDF), absent (AEDF) or reversed (REDF). Adapted from *A Computational Model of the Fetal Circulation to Quantify Blood Redistribution in Intrauterine Growth Restriction*[159].

The "low resistance" of the placental vasculature can be visualized in Fig. 7 as) blood flow within the umbilical artery (UA) in a healthy fetus (control) displays an increase in velocity at systole and remains positive even during diastole[159]. Corresponding MCA flow also peaks during systole but is then followed by dramatic decrease during diastole. The worsening of placental insufficiency can be observed by decreased UA flow during diastole, indicative of increasing placental resistance[159]. The presence of reversed end-diastolic flow indicates that placental vascular resistance is greater than diastolic pressure[111]. Retrograde UA flow, i.e. backwards blood flow (UA-REDF) is a clinical indicator of potential fetal growth restriction. The corresponding MCA flow also increases during this diastole, suggesting increased vasodilation within the cerebral circulation[159].

In the event of fetal hypoxia, the fetal vasculature redistributes cardiac output from non-essential to essential vascular beds for example the brain, these mechanisms will be described in further detail later in this review. This is a well-described and robust mechanisms in hypoxic fetuses and information from essential organs, such as the brain, can give further detail regarding the health

status of the fetus. Using these techniques, doppler ultrasound waveform of UA and MCA blood flow can be indicative of fetal health[111] and these waveforms have become an essential part of clinical diagnosis of FGR fetuses in utero.

1.7.A. Fetal Growth Restriction versus Small for Gestational Age

Fetal size and birthweight are commonly used to determine appropriate fetal growth. However, distinction between the definition of FGR and fetuses that are constitutionally small has been difficult. Fetuses that are constitutionally small are better defined as small for gestational age (SGA) and are infants with a birthweight that falls below the 10th percentile of population norms for that gestational age[160]. SGA and FGR are two separate subgroups where SGA infants are constitutionally small and FGR are pathologically small[160]. The key distinction between FGR and SGA is the deceleration of fetal development in FGR due to a reduction of adequate *inutero* substrate availability. Evaluation of FGR defined by ultrasound compared to neonatal growth curves, revealed that 57% of FGR infants had a birthweight greater than the 10th percentile[161]. Importantly, this group of infants (classified as AG-FGR) had the same clinical severity as FGR with birthweight below the 10th percentile[161].

Identification between FGR and SGA is important as SGA infants may be low in birthweight but healthy. Further, SGA is often confused with FGR resulting in unnecessary clinical intervention. The lack of distinction between FGR and SGA results in difficulty interpreting clinical data and has clouded our understanding of the consequences and responsivity to treatment of the FGR population.

1.8. Cardiovascular Adaptations to Placental Insufficiency

As mentioned above, FGR is associated with 'brain-sparing'. Brain-sparing involves an increased cerebral blood flow that mirrors decreased umbilical blood flow. This is an example of a cardiovascular adaptation to FGR, which aims to increase substrate supply to key organs in the face of decreased substrate availability.

1.8.A The Effects of Chronic Hypoxia.

Chronic hypoxia, following placental insufficiency, is one of the most common pathological mechanisms in growth restriction and is known to result in neural, endocrine and metabolic changes within the fetus[162].

Chronic hypoxia is a key mediator in cardiovascular adaptations that occur in FGR and in response cardiac output is redistributed to vital organs such as the brain, heart and adrenal glands[162]. Studies by Allison et al have shown, in fetuses of chronically-hypoxic sheep, an increased aortic pressure, decreased heart rate and increased femoral and cerebral blood flow[163]. Additionally, these studies demonstrate an increased oxygen and glucose delivery to the brain compared to the peripheral circulation, indicative of blood flow redistribution in aims to maximize substrate delivery to key organs[163,164]. Redistribution of blood flow occur at the expense of 'non-important organs' such as the gut[165] and peripheral circulation (arms and legs[166]) where reduction of metabolic demand and blood flow is decreased. The preservation of perfusion to essential organs is aimed at providing an uninterrupted supply of oxygen to preserve function and development[16]. Proper development of these key organs is vital (in particular the brain and heart) as metabolic demand is high and thus are sensitive to damage. Damage in these systems can result in fetal, neonatal and long-term complications. This adaptive process is termed "brain sparing". Despite the terminology of "sparing" used to describe this adaptive process, studies continue to demonstrate these organs are not 'spared' from adverse consequence, and dysfunction exists throughout the essential organs of FGR fetuses and offspring.

A state of fetal hypoxia is detected by carotid chemoreceptors[167] and firing of carotid chemoreceptors initiates the brain-sparing response[168]. Chemoreceptors are cells located in the carotid bodies that respond to chemical stimuli to relay information via signaling pathways to the brain for a physiological response[169]. In response to hypoxia, carotid chemoreceptors decrease firing to the glossopharyngeal nerve within the central nervous system[169]. Decreased firing to the glossopharyngeal nerve results in a reduction of fetal heart rate. Giussani et al have demonstrated bradycardia in fetal sheep during induced hypoxia[167]. Bradycardia allows for greater ventricular filling during diastole, increasing end-diastolic volume and increased cardiac output[168]. Increasing cardiac output aims to maintain tissue perfusion to counteract the decrease in blood oxygenation. Additionally, the decrease in heart rate also reduces blood velocity to enable increased time for gas exchange between the tissues and blood[170]. This allows for the maintenance of tissue oxygenation despite the hypoxic environment. This response is mediated by carotid chemoreceptors, as confirmed by Giussani et al whereupon carotid denervation results in prevention of fetal bradycardia following a hypoxic insult[167]. Further studies by Green et al[171] and Moore et al[172] have shown that bilateral section of the carotid sinus in sheep impairs the redistribution of blood flow and brain sparing response to hypoxia. These data show that the carotid chemoreceptors drive the brain-sparing response.

Detection of hypoxia by central chemoreceptors also activates neuronal control within the nucleus tractus solitarus to increase stimulation of both the sympathetic and parasympathetic arms of the autonomic nervous system[169]. SNS activation can stimulate α1-adrenoceptors to cause increased vascular resistance within non-essential vascular beds via increased vasoconstriction[167,173]. These mechanisms enable fetal cardiac output to be shunted away from less essential vascular beds such as the gut and peripheral circulations, as discussed above[165]. Additionally, following the initial autonomic reflex, there is an increase in vasoactive circulating hormones; catecholamines[174], vasopressin and angiotensin II[175]. These hormones act to maintain the high vascular resistance in these non-essential vascular beds to maintain the redistribution of cardiac output and perfusion to the central circulation[169].

Concurrently in the cerebral circulation, decreased vascular resistance is mediated by the release; adenosine, NO and prostanoids[169]. Maintenance of cerebral blood flow volume is assisted by right-to-left shunting of fetal blood toward the coronary circulation and the fetal brain resulting from increased right ventricular afterload[176] supplied by the placental and fetal peripheral vascular bed. Doppler studies have shown that vascular size in the middle cerebral artery increases with increasing hypoxemia[16]. It is likely that the combined effects of increased peripheral vasoconstriction and increased cerebral vasodilation drive the increased flow in the MCA. These effects results in the centralization of combined cardiac output and directing blood to the cerebral and coronary circulations.

1.8.B. Hypoxia induced Reactive Oxygen Species Release

In mammalian cells, reactive oxygen species (ROS) are produced in mitochondria, as a by-product of ATP production[177]. ROS are ubiquitous, chemically reactive compounds that have key biological roles in the regulation of the cell death cycle[178]. Antioxidants are enzymes that can scavenge ROS and work to maintain homeostasis of ROS levels. ROS levels are tightly controlled as high levels result in cell damage [179] and cellular damage can occur when the production of ROS outweighs local antioxidant capabilities[180]. This is termed oxidative stress.

In periods of fetal hypoxia, decreased levels of oxygen reduces the activity of the electron transport chain within the mitochondria, consequently increasing fetal ROS levels[181]. Increased ROS leaves the fetus vulnerable to cellular damage and interference with normal cellular signaling[182]. During fetal life antioxidant production is seen to increase over gestation[183] suggesting capability to combat

ROS by the fetus develop over time. Therefore, as there is decreased ability for the fetus to defend against ROS induced cell damage early in gestation, hypoxia can result in critical injury to the fetus.

ROS also has a strong interaction with ubiquitous gaseous transporter and vasodilator, nitric oxide[184,185]. ROS are commonly found as superoxide anion O_2-, following the reduction of O_2[184]. The negative charge of ROS results in reactivity with oxygen molecules and therefore, ROS can react with NO to form peroxynitrite[184]. The binding of ROS to NO reduces NO bioavailability and impairs vasodilative action within blood vessels, leading to increased vasoconstriction.

There is strong evidence in the chronically hypoxic fetus that decreased NO bioavailability perpetuates peripheral vasoconstriction[186] initiated by the autonomic nervous system. Giussani et al suggest that the ratio of ROS to NO regulates local vascular tone, whereby increased NO favors vasodilation, and vice versa[186]. In pre-clinical models, fetal blockade of NO production induces peripheral vasocontraction[185], whereas potent antioxidants (at levels pharmacologically able to reduce ROS levels) increase peripheral vasodilation[187]. These studies suggest that the peripheral vasculature vasoconstricts in response to high ROS. As previously discussed, the cerebral vasculature dilates in the event of chronic hypoxia, which suggests that within the action of NO is greater within the cerebral vasculature than the periphery. Overall, these data demonstrate how the interplay between these two factors maintain the 'brain sparing' response and highlight the crucial role that NO and ROS play in the regulation of vascular tone.

1.9. Pathology of Fetal Growth Restriction

This literature review has so far discussed; 1) placental and fetal cardiovascular development, 2) the potential mechanisms that underlie placental insufficiency to cause FGR and 3) the fetal response to an adverse intrauterine environment. In order to investigate the potential for FGR treatment, we must now discuss the increased morbidity and mortality experienced by FGR neonates. For the purposes of this literature review, I will be focusing on how these adaptations increase cardiovascular morbidity in FGR.

FGR neonates have increased risk of morbidity and mortality compared to AG infants, regardless of gestational age[188]. Neonatal consequences of FGR include, retinopathy of prematurity[189], necrotizing entrocolotis[190] and bronchopulmonary dysplasia[191]. In the longer term, FGR increases the risk of type 2 diabetes, insulin resistance, polycystic ovarian syndrome, as well as neurological sequalae such as motor and cognitive delay[17,192,193].

Large epidemiological data sets have demonstrated that the increased risk of cardiovascular disease in the FGR adult is developmentally programmed in utero[194]. Although the complete mechanisms underlying the increased risk of cardiovascular disease are poorly understood, several key contributors, such as the vascular consequences from chronic hypoxia and increased oxidative stress, have been well studied.

1.9.A. Cardiovascular Consequences of Growth Restriction

As discussed, the fetus mounts cardiovascular adaptations to intrauterine hypoxia. While these adaptations are potentially lifesaving *inutero*, long term maintenance of *inutero* cardiac redistribution results in impaired and altered cardiovascular development. It is well accepted that FGR-driven cardiovascular adaptations underlie the increased risk of cardiovascular disease (CVD) in both childhood and adult life [2,17,20,195–198]. In 1990, the link between FGR and adult CVD was suggested by David Barker[199]. Barker's epidemiological findings showed a causal link between FGR and hypertension and coronary heart disease in adulthood. "The Barker Hypothesis" was the first to suggest that a sub-optimal uterine environment had the potential for a lasting influence on adult health. Clinical long-term follow-up of FGR affected individuals have supported this hypothesis[200] and the nomenclature 'developmental programming' was coined to describe the potential for physiological changes occurring in utero to have life-long impact on offspring health.

1.9.B. Altered Cardiac Mechanics and Cardiovascular Consequence

Cardiac alteration in FGR can be observed *inutero*, supporting the hypothesis of developmental programming. Doppler assessment of the FGR fetal heart throughout gestation has shown that altered vascular mechanics, from increased/decreased vasoconstriction, results in significant changes to cardiac structure. As previously discussed, increased peripheral vascular resistance results in centralization of fetal blood flow. This also increases ventricular filling, end-diastolic volume and cardiac afterload, which consequently increases the pressure the heart beats against during systole[10]. This can result in structural changes to the heart. The comparison of heart shape between AG and FGR fetuses can provide insight to cardiac function. Appropriate cardiac shape and size can also be used as determinant of appropriate cardiac development and *inutero* development can be observed via Doppler ultrasound.

Cardiomegaly is an abnormal enlargement of the heart[201], is driven by increased cardiac demand and is seen in conjunction with impaired myocardial relaxation and decreased functional efficacy. Cardiomegaly can be diagnosed via echocardiography during fetal life from as early as 20 weeks of gestation[202] and is a characteristic cardiac change associated with FGR. Progression of

cardiomegaly in FGR can be identified by three phenotypic stages; 1) elongation, classified as elongation of the one ventricle, 2) globular, classified as elongation of both ventricles and 3) hypertrophy, which is an increase in the cardiac muscle mass driven by increased contractility requirement[200]. Increased FGR severity is associated with less elongated and more globular ventricles[203]. Sphericity index is a measure of normal heart shape. Using echocardiography and Doppler ultrasound, Cruz et al al have shown that the growth restricted heart has significant structural alterations including decreased sphericity index of both the left and right ventricles, suggesting abnormal heart shape in the human fetus[204].

Cardiomyocytes are the functional units of the heart and are responsible for enabling heart contraction. Though FGR results in increased cardiomyocyte hypertrophy[205], impaired maturation results in reduction of cardiomyocyte number[206]. Taken together, these findings demonstrate that FGR results in hearts with too large and too few functional units, likely impairing the ability of the heart to correctly contract.

Inutero calculations of myocardial performance index, using diastolic filling ratios and cardiac output by Crispi et al demonstrate that cardiac dysfunction occurs during *inutero* life[207]. Additionally, Rizzo et al have shown that FGR fetuses have delayed and reduced aortic and pulmonary peak velocities, resulting in decreased aortic peak blood flow velocities and cardiac output over gestation[97]. These results are important as aortic peak velocity is used as a measure of aortic stenosis and these findings suggest an increased narrowing of the aortic valve[20897]. Structural changes to vasculature such as thickening of the aorta and pulmonary arteries likely underlie the deterioration in cardiac function observed in FGR fetuses[194].

Collectively, these studies suggest that cardiovascular adaptations mounted to assist fetal survival, result in significant cardiovascular impairment and cardiac damage. Crispi et al have shown that the umbilical cord blood of FGR human fetuses have increased biomarkers of cardiac damage; blood B-type natriuretic peptide and heart fatty acid binding protein[207]. Additionally, cardiac dysfunction worsens as gestation progresses, which likely underlies the increased cardiac sequalae experienced in post-natal life.

1.9.C. Molecular Responses to Hypoxia and Cardiovascular Consequence

Alongside the mechanically driven cardiovascular consequences of FGR, molecular responses to fetal hypoxia also contribute to cardiovascular alterations. Pre-clinical models of FGR demonstrate increased expression of hypoxic injury markers such as heat shock proteins-70 (HSP70)[209] and

Hypoxia-inducible factor-1α (HIF-1α)[210]. These proteins are involved in cellular response to hypoxia and are key regulators in cell growth and survival. Studies suggest that FGR may alter appropriate expression of these factors which contribute towards increased cardiovascular dysfunction.

Heat shock proteins-70 (HSP70) is a common marker of hypoxic stress [211] and cardiac injuy[209] as they are a family of proteins that are expressed in stressful conditions such as hypoxia[212]. HSP70 have anti-inflammatory effects and can protect against cell apoptosis and organ damage[213214]. Hypoxia-inducible factor-1α (HIF-1α) is a protein complex that is also expressed in response to hypoxia. HIF-1α regulates the cellular adaptation to hypoxia[215] and promotes angiogenesis and erythropoiesis to improve oxygenation in hypoxic regions[216]. Interestingly, despite the protective role of these factors, studies have shown FGR may drive inappropriate expression resulting in increased detriment rather than benefit. Li et al have shown that HSP70 is downregulated in adult rats that faced a hypoxic challenge *inutero*[217] suggesting increased susceptibility to hypoxic injury in later life. Additionally, Zhang et al have shown that offspring of high-altitude pregnant sheep have an increased fetal expression of HIF-1α[210], but have increased susceptibility to cardiac ischemic/reperfusion injury[210]. Taken together, these studies show that despite increased expression of HIF-1a and/or decreased expression of HSP70, fetal hypoxia results in increased susceptibility to cardiac damage.

1.10. Blood Vessels in FGR

The consequences of FGR on vascular structure has been well investigated in both clinical and pre-clinical studies. Basu et al have shown, in healthy rats, that increased arterial blood flow results in increased elastin to collagen ratio and increased thickness within the medial layer of the vessel[218]. This study is important as it demonstrates that vascular remodeling can occur following altered blood flow. The centralization of blood flow is therefore a key mechanism which likely underlies the altered central and peripheral vascular structure observed in FGR affected individuals[219,220].

FGR-driven vascular alterations demonstrated in both preclinical and clinical studies. Canas et al have shown increased thickness and stiffness in the femoral artery of fetal FGR guinea pigs compared to AG[221]. Additionally, Dodson et al have shown increased carotid collagen and elastin content in FGR lambs[222]. Clinically, these studies are supported by Cosmi and Zandro et al[223,224] who have shown that increased aortic thickness inversely correlates to estimated fetal weight[223,224]. Overall, FGR results in increased vascular stiffness and thereby decreases vascular compliance to ultimately increase vascular resistance. Increased arterial thickness is important as this will increase

the force at which the already compromised FGR fetal heart needs to pump. Additionally, increased peripheral vascular resistance is likely to impair the flow of blood though this vascular bed, resulting in reduced venous return to the heart, which can also contribute to further cardiac dysfunction[225]. These studies demonstrate the perpetual cycle of cardiovascular alteration to FGR, increased cardiac work and increased vascular resistance.

1.10.A. Vascular function in FGR

Endothelial dysfunction is a hallmark of cardiovascular adaptations to FGR and is recognized as an early indicator of future cardiovascular disease[197,226,227]. As discussed above, NO production is a key determinant of normal vascular function. Similarly to NO dysfunction within placental vasculature that can cause FGR, FGR offspring have been shown to have vascular NO impairment[228]. This likely underlies the endothelial dysfunction has been shown to occur in FGR offspring and is a likely contributor toward cardiovascular dysfunction observed during post-natal life and into adulthood[226,229].

The potential mechanisms behind FGR-driven endothelial dysfunction observed in humans have been investigated in animal models. In a ovine model of hypoxic pregnancy, Allison et al have shown that offspring of hypoxic mothers have decreased sensitivity to acetylcholine within isolated femoral arteries[230]. Given, that acetylcholine is a key mediator of endothelium-induced vasodilation, these results suggest FGR causes in impairment of this pathway. Similarly, Macuza et al, have shown impaired response to endothelium-derived hyperpolarizing factor (EDHF) in isolated femoral arteries of FGR rats[231]. These findings of these studies are supported by Polglase et al (Figure 8)[20], who found impairment in the vasodilation of femoral arteries via endothelium-dependent mechanisms in the FGR lamb[20]. Interestingly, these studies also show an increased sensitivity to NO donor sodium nitroprusside and PDE5 inhibitor sildenafil citrate[20]. These results are important as they not only support endothelial dysfunction within the peripheral vasculature observed in previous studies, but also suggest altered reactivity of the peripheral circulation to NO and components of this pathway (PDE5)[20].

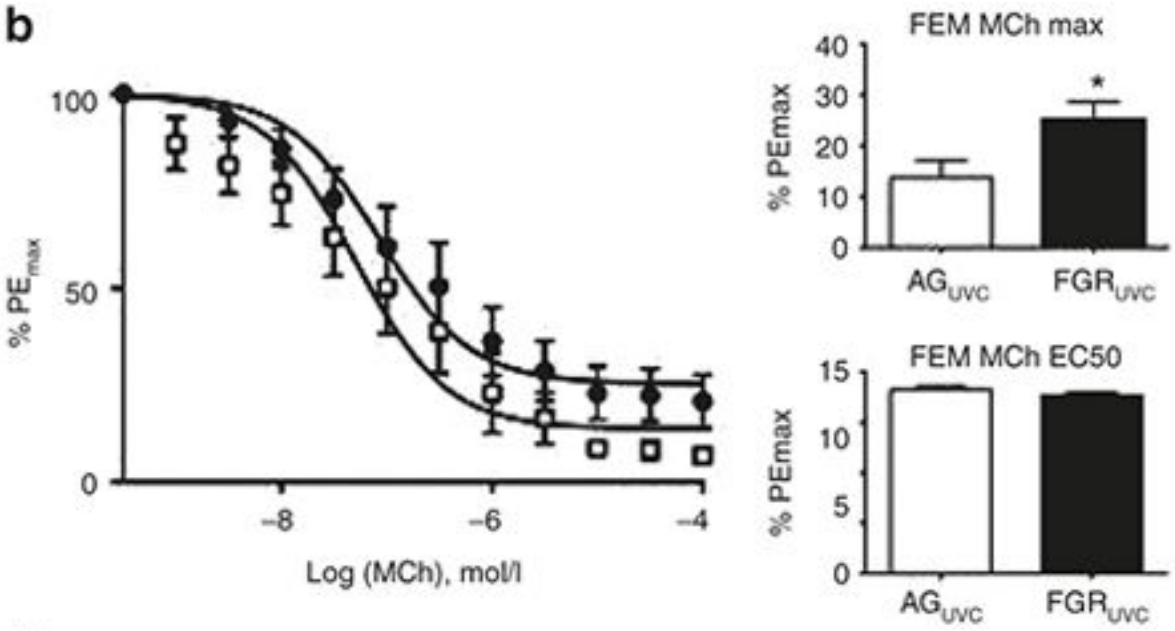

Figure 8. Response of the femoral artery of appropriately grown (AG; white) and fetal growth restricted (FGR; black) lambs to endothelium-dependent vasodilation via methacholine (MCh). Adapted from *Altered cardiovascular function at birth in growth-restricted preterm lambs*[20].

Collectively these pre-clinical studies support the potential for central and peripheral vascular dysfunction to occur following FGR. Particularly, dysfunction occurs in endothelial function and the NO pathway. These studies suggest NO bioavailability may be impaired by altered synthesis or increased NO scavenging by competing molecules, such as ROS, and alterations to normal endothelial function in FGR.

1.11. Postnatal Effects of FGR on Cardiac and Vascular Function

The postnatal consequences following the synergistic relationship between altered cardiovascular function and FGR-driven cardiovascular alterations has been studied extensively in preclinical models and supported by clinical observations. Neonatal FGR lambs have been shown to have increased systemic vascular resistance, lower left ventricular output and lower cerebral blood flow compared to their AG counterparts[20]. These findings mirror clinical observations by Seghal et al who has shown increased cardiac hypertrophy and globularization correlates to impaired diastolic function and ejection fraction in FGR neonates[220]. Multiple studies also demonstrate that FGR newborns have reduced right ventricular output, decreased heart compliance and contractile and dilatory ability[10,196,203,219,232]. Taken together, these studies demonstrate the post-natal cardiovascular dysfunction driven by FGR.

Impaired cardiac dysfunction does not diminish over time. Cosmi and Zandro et al[223,224] have shown the maintenance of aortic thickness in FGR children at 18 months of age, which correlated with an increased systolic blood pressure[224]. Further, Cheung et al have shown increased resistance within the peripheral vasculature (brachioradial artery)[233] and Gailliard et al have shown higher systemic blood pressure and a lower left ventricular mass[234] in FGR born children. Importantly, cardiovascular dysfunction does not resolve, this is evident by the findings of Brodszki, et al who have shown reduced aortic diameter[235] and Goodfellow et al who have shown endothelial dysfunction in young, in adults born FGR[226].

The persistence of cardiovascular dysfunction through childhood and into adulthood is likely underlain by altered vascular structure and endothelial dysfunction, the mechanisms of which have been discussed at length previously. Further, these studies demonstrate that cardiovascular alterations from FGR can be sub-clinical and appear in relatively "healthy" individuals[226] and highlight the lasting impact fetal growth has on the lasting effects FGR has on vascular structure and function. These studies support the observations by David Barker and support the idea of FGR alterations 'program' for cardiovascular disease in adulthood[236].

The relationship between the FGR heart and vasculature is an area of research interest and is not fully understood. However, understanding of how these alterations occur may help to give insight into the mechanisms that contribute to long term risk of cardiovascular disease[237]. With improved Doppler assessment, evidence now suggests that cardiac alterations exist early in fetal life and persist postnatally. The focus of FGR therapies have therefore centered on reducing placental insufficiency and improving fetal growth, in order to reduce or prevent the lasting consequences to adaptations that occur early in FGR.

1.11.A. Fetal Growth Restriction: Management and Potential Therapies

Currently there are no therapies for FGR. Induction of preterm delivery is currently the only method of intervention available and despite best clinical efforts, the risk of post-natal sequela has not improved. The decision for preterm delivery is made upon antenatal surveillance, when the risks of prematurity outweigh the risks of continued development/exposure to an adverse intrauterine environment[238–241] . While life-saving, the induction of early delivery exposes the already compromised FGR fetuses to the complications associated with premature birth[7]. These complications include; apnoea, intraventricular haemorrhage, thrombocytopenia, sepsis and hypoglycaemia. Importantly, risk of all preterm complications are increased in FGR[242,243]. Therefore, the induction and timing of preterm delivery remains controversial.

The 'Growth Restriction Intervention Trial (GRIT)" has investigated the long-term outcomes of immediate and deferred delivery of FGR fetuses[244]. Immediate or early delivery was defined as infants delivered at the time of abnormal Doppler diagnosis, and delayed delivery was defined as a time of delivery when the obstetrician no longer had uncertainty of fetal outcome[244]. 367 infants were involved in the GRIT study and offspring were followed for 2 years[244]. Overall, no difference in survival, morbidity, cognitive function, motor skills and behavior were seen between those where delayed or immediately delivered[245]. This outcome suggests that the adverse post-natal outcomes occur early in gestation and are irreversible. These findings support the need for treatment options to be implemented *inutero* in order to reduce the potential for developmental programming.

1.11.B. Antenatal FGR Treatments

Many different therapies have been investigated in experimental models of FGR with the aim to improve growth and/or associated morbidities. In many of these potential therapies, despite promising pre-clinical data, no improvement to FGR has been observed.

One such avenue of therapy that failed to translate clinically has been the use of antioxidants to target ROS and oxidative stress[246–249]. In pre-clinical experiments, Miller et al have shown treatment of FGR lambs with melatonin during gestation have potential for neuroprotective benefits via reduction of oxidative stress[249]. Additionally, Tare et al have shown that antenatal melatonin treatment in sheep improved heart contractility, NO availability and reduced coronary artery stiffness[250]. Further, Thankor et al have shown that fetal i.v. treatment with melatonin and vitamin C in FGR lambs can increase umbilical blood flow[187], suggesting potential for improvement of placental function. Similarly, recuse of endothelial function has also been shown in a rodent model of prenatal hypoxia following treatment with antioxidant allopurinol[230]. These data highlight the potential restorative neurological and cardiovascular benefits from antioxidant treatment when targeting the detrimental effects of ROS.

Unfortunately, despite the promising results in preclinical models of FGR, no improvement in clinical studies investigating treatment of FGR with vitamin C[246,247] or allopurinol[248] has been seen. Vitamin C supplements may not have any therapeutic benefit as circulating concentration of vitamin C was far below the concentration needed to have biological effect in vivo, despite maximal daily ingestion [246,251]. Additionally, it is also important to note that Gonzales-Candia et al have shown the potential for antenatal melatonin treatment to exacerbate FGR in sheep[252]. This

finding is important as while some studies show that melatonin is safe to use during pregnancy[253], potential for fetal harm exists.

Taken together, these clinical failures demonstrate that while results of pre-clinical studies may demonstrate potential for therapeutic benefit, they highlight the difficulty in reproducing these effects when taken into the clinical context. A possible cause for the difficulty in translation from bench to bedside, may be due to further complexity in the consequences of FGR and unknown or unexpected interaction between these well studied therapies and the altered FGR vasculature. I believe that similarly to melatonin and vitamin C, unknown and unexpected response to sildenafil citrate resulted in the failure of the STRIDER (Sildenafil Therapy in Dismal Prognosis Early-onset Fetal Growth Restriction) Trial

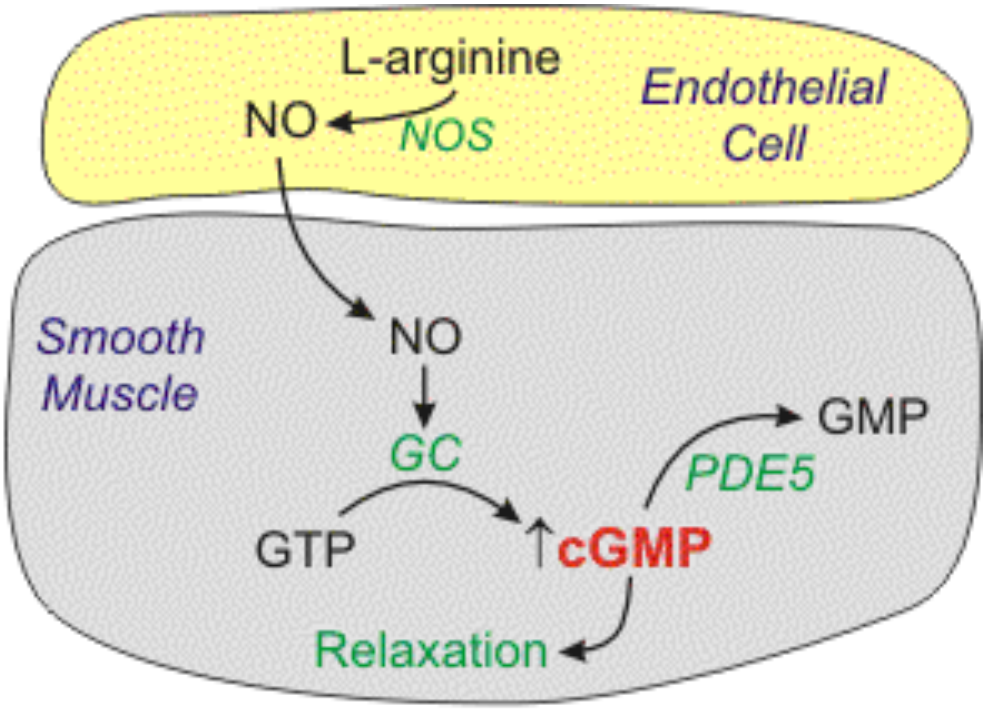

Figure 8. Schematic of Nitric Oxide mediated and breakdown of cGMP via PDE5 activity [75]***.*** *Cyclic guanosine monophosphate (cGMP) is broken down by enzyme phosphodiesterase`5 (PDE5). Resulting in intracellular levels of cGMP and allowing again for vasoconstriction*

1.12. Sildenafil Citrate as an Antenatal Therapy

Sildenafil citrate (SC) is a phosphodiesterase 5 (PDE5) inhibitor which induces vasodilation by decreasing vascular resistance via modulation of the NO vasodilation pathway[254]. The pathway of NO mediated vasodilation is described above. PDE5 is an enzyme responsible for the breakdown of cGMP to GMP, which enables vasoconstriction (Fig 7)[75]. cGMP increase, as mediated via action of NO, causes an increase in vasodilation and blood flow. Sildenafil blocks the PDE5 enzyme

preventing the breakdown of cGMP, resulting in increased intracellular cGMP and prolonged vasodilation [254]. SC has shown clinical benefit in cardiovascular complications such as pulmonary hypertension[255] and stroke [256]. Furthermore SC therapy has neuroprotective effects in a mouse model of inflammatory demyelination[257], increases eNOS activation in mice with gentamicin-induced nephrotoxicity[258] and improves endothelial function in smokers[259] and diabetics[260]. PDE5 is abundant in the placenta and therefore a key regulator in placental vasodilation and vasocontraction.

As discussed previously, placental insufficiency is driven by an inappropriate increase in placental vascular resistance. Therefore, given is potent vasodilative action, PDE5 has been investigated as a potential therapeutic target for placental insufficiency, FGR and fetal hypoxia. These studies have been undertaken in both small and large animal models[23–28], as well as in small human trials[29–31] and similarly, to allopurinol and vitamin C, pre-clinical investigation into SC treatment for FGR had some promising pre-clinical results.

In FGR mice[23], guinea pigs[26], rats[25] and sheep[27], maternal SC treatment increased fetal growth, increased body weight and improved nutrient profile in offspring. These studies demonstrate the antenatal SC treatment has the potential to improve placental function (evident by increased nutrient transfer) and improve fetal growth. Antenatal SC treatment was also shown to have additional benefits. Guinea pigs fetuses exposed to SC also had an improved tolerability to asphyxia at birth[26] and studies by Itani et al have shown SC treatment in hypoxia induced FGR chick embryos resulted in improved nitric oxide bioavailability and protected against oxidative stress[261].

Maternal SC treatment has potential benefits in FGR within the clinical context. Ex-vivo treatment of FGR placental vessels with SC has been shown to improve endothelial function[31] and in a single case study reported by Panda et al, maternal SC treatment was described to improve uterine blood flow[29]. These studies demonstrate that SC has the potential to improve placental function. This is supported by Dadelszen et al who have shown that in a small-cohort study (10 FGR, 17 AG) oral maternal SC treatment was associated with an increase in abdominal circumference growth. These studies demonstrate the ability of SC to fetal growth. Collectively these animal studies and small human trials support the idea that SC treatment has the potential to improve fetal growth in FGR, as well as protect against the cardiovascular effects of fetal hypoxia.

The demonstrated therapeutic benefits of SC for FGR supported the initiation of the STRIDER trial, which was a large multicenter, multinational clinical trial that began in 2014. It was aimed at investigating the potential of SC to improve placental substrate transport by improving placental blood flow[262]. In the STRIDER trial, oral tablets are taken by mothers with fetuses diagnosed with severe early onset growth restriction. Once metabolized, SC enters the maternal circulation and the placental circulation.

Unfortunately, similarly to vitamin C and allopurinol, the promising pre-clinical data surrounding SC also failed to translate clinically. At the time of writing this review the UK arm of the STRIDER has been published (February 2018). The overall outcome of this arm suggested that maternal SC treatment had minimal effects to fetal size and gestational length[262]. The conclusions drawn from the UK results suggested that there was neither harm or nor benefit from maternal SC treatment, suggesting that while SC may not be an appropriate therapy for FGR, there was no significant adverse effects[262,263]. However, in July 2018, the Dutch arm of this trial was halted early due to predisposition toward persistent pulmonary hypertension[22] and neonatal death[22].

Miller et al previously investigated the fetal physiological response to acute maternal SC administration in pregnant sheep. This study found that SC failed to improve uterine blood flow and conversely, these studies report a decrease in umbilical blood flow[28]. Worryingly, this was mirrored by a decrease in fetal pO_2, hypotension, and tachycardia[28]. They concluded that maternal SC can elicit a fetal response. Though Miller et al demonstrate data contradictory to that of those supporting the STRIDER, the adverse fetal response to SC may explain the unexpected mortality seen in the Dutch arm of the trial. Additionally, Refuerzo et al showed that maternal SC treatment in rats resulted in a decrease in fetal size[25]. It is also important to note that, despite not explicitly investigating fetal response, several studies demonstrate that it is possible that SC exposure may be detrimental to the NO pathway. SC has been shown to have inhibitory effects on iNOS expression[264] and does not increase NO secretion from endometrial epithelial cells [265] nor improve NO-mediated vasodilation in healthy men [266]. These findings suggest that maternal SC may have unexpected side effects, and use in treatment should be approached with caution.

1.13. The future of SC in Antenatal treatment.

The failure of the STRIDER trial is an important event in the history of medical research. Further, the response of the medical and scientific research communities may set a precedence for how potential therapies are studied in the future. Following the outcome of this trial, it is likely that all use of SC in perinatal therapy will be stopped[267]. However, it is important to note that SC has

shown benefits in other perinatal diseases such as congenital diaphragmatic hernia[268,269] and fetal distress during labor[270]. Therefore, cessation of SC in all avenues of perinatal treatment may prevent the development of life-saving therapy.

Prior to the halting of the STRIDER (2017), Paauw et al published a review stating the maternal SC treatment had potential to improvement fetal growth in FGR, at higher dosages than that used in the STRIDER trial[271]. Additionally, Dunn et al also published a review stating that that SC had no maternal of fetal side effects[272]. Despite these studies, the poor outcomes of the STRIDER highlight the crucially important need to understand why early pre-clinical studies, conducted predominantly in small animal models, did not predict the poor outcomes in the STRIDER trial. Symonds et al suggest that incomplete referencing to previous animal studies was the weakening point of the STRIDER[273]. I believe that to correctly develop clinical therapies, comprehensive animal studies must be performed that aim to investigate outcomes specific to the condition in question.

The impact of chronic exposure of the fetus to SC on fetal physiology has not yet been described. SC not only reaches the placental circulation but can cross it and enter the fetal circulation[274]. There is currently a paucity of data regarding the effects of SC on the developing fetus, let alone in FGR. I suggest that the failure of the STRIDER trial was underlain by unexpected interaction between SC and the altered vasculature of the FGR perinate. Although SC is unlikely to be used clinically as an antenatal treatment for FGR, however there is still great knowledge to be gained from the cohorts of lambs we have which were exposed to chronic sildenafil.

An important outcome following the unsuccessful results of the STRIDER trial, is that there still remains a lack of treatment for FGR. As FGR neonates require greater postnatal care, research focus should also be aimed at addressing potential improvements in care, tailored to address the altered cardiovascular system.

1.14. Consequences of FGR to Neonatal Transition.

The transition from intrauterine to extra-uterine life involves a complex cascade of vascular adaptations and changes, resulting in shifting from a fetal to neonatal circulatory system (Fig 3B). This is regarded as the most complex and difficult physiological adaptation a human will undergo[275,276] . As previously discussed, FGR fetus has an altered cardiovascular structure and function. Little is known about how these alterations affect the ability of the FGR neonate to undergo transition into extra-uterine life. However, it is well reported that FGR preterm infants

require increased support during this period[277], including greater need for cardiovascular and respiratory support [277–279]. It is likely that the increased need for support is driven by impaired cardiovascular function, secondary to FGR cardiovascular alterations.

A potential contributor towards the difficulty in successful neonatal transition is the persistence of the fetal circulation observed in FGR neonates[280]. The persistence of the fetal circulation describes a failure of the foramen ovale and ductus arteriosus (DA) to close, resulting in blood moving directly from the right side of the heart to the left (right to left shunting) and bypassing the pulmonary circulation[280]. Rakza et al have shown that FGR infants have increased flow though the DA compared to appropriately grow infants[281]. Persistence of this circulation results in abnormal heart contractility and increased requirement for vasopressor use[280]. NO is a key mediator in DA closure[282], it is therefore likely that the previously described dysfunction in the NO pathway contributes towards persistence of the fetal circulation in FGR. These events impair the neonatal transition and results in cardiovascular dysfunction, decreased blood pressure, cardiac output and subsequent decrease in organ perfusion resulting in ischemic injury[280].

Another key contributor towards increased cardiovascular morbidity in FGR is the vasculature of the lung. Pulmonary vascular resistance in fetal high and blood flow is low. Transition from the fetal to neonatal circulation is dependent on a dramatic decrease in pulmonary vascular resistance to enable increased pulmonary blood flow. Persistent pulmonary hypertension of the newborn (PPHN) is the maintenance of a high pulmonary vascular resistance in neonatal life and impairs pulmonary blood flow, results in respiratory failure, impairment in arterial oxygenation and increase risk of neonatal mortality and morbidity[283]. In line with FGR, PPHN is driven by abnormally constricted pulmonary vasculature and therefore, it is unsurprising that FGR increases the risk of PPHN[284]. This suggests vascular alterations in FGR also affect the pulmonary vasculature.

The relationship between FGR and pulmonary function has been well described in FGR neonates developed at high-altitude. Several human studies have shown that in the neonatal period, infants born at high altitude experience higher pulmonary artery pressure in early life[285]. Furthermore, infants developed at high altitudes have decreased oxygenation status (Colorado[285], Peru[286], Tibet[287]) compared to those developed at sea level. Interestingly, their relative hypoxia persists during the first few weeks of life. Seghal et al have also shown that approximately 10 days after birth, FGR infants have increased right pulmonary artery thickness and reduced pulsatility index[288],

which may explain the prolonged hypoxia observed in high-altitude FGR populations. Taken together, these data show FGR can impair the ability for the neonate to oxygenate its blood. Additionally, decrease in right pulmonary artery pulsatility can cause right ventricular hypertrophy and contribute to cardiac dysfunction. This highlights that impairment in pulmonary vascular development can exacerbate cardiac injury. Potential molecular mechanism underlying increased PPHN in FGR have been shown by Rozance et al, demonstrating impaired pulmonary endothelial function and decreased eNOS synthesis in FGR lambs[289]. Given the vasodilative effect of NO, it is likely impaired NO mediated vasodilation underlies the increased pulmonary vascular tone seen in FGR.

FGR can result in impaired cardiovascular and pulmonary structure. It is likely that these impairments underlie the morbidity faced in early life, which results in increased requirement for cardiovascular and respiratory support compared to AG counterparts[219,220]. Postnatal clinical support for preterm neonates centers around increasing heart rate and blood pressure in-order to maintain adequate organ perfusion.

1.13.A. Clinical management of FGR During Transition

Current clinical management for blood pressure support involves the use of inotropes such as dopamine and dobutamine to increase blood pressure and cardiac output[290,291]. Dopamine is an endogenous catecholamine and when delivered intravenously can act on β-adrenoreceptors on the heart[292] to increase heart rate, contractility and cardiac output. Dopamine can also act on α-adrenoreceptor within the peripheral circulation to increase vasocontraction[292]. Increased peripheral vasoconstriction will increase blood within the central circulation, increase preload and cardiac output[293]. These effects of dopamine are utilised to increase neonatal blood pressure and thereby, organ perfusion.

The use of vasopressors such as dopamine and dobutamine are commonly used for hypotensive support in preterm neonates. In a study investigating vasopressor support, Seri et al showed that 50% of very-low birth weight infants require dopamine treatment[295]. However, many of these very-low birth weight infants develop vasopressor resistant hypotension and require increased vasopressor support. Further, in a study by Osborn et al 40% of hypotensive preterm infants in their study failed to increase superior vena cava flow in response to dopamine or dobutamine[296]. These findings are important as they suggest that in a proportion of the preterm population, vasopressor treatment is ineffective. I suggest that it is likely that this sub-population of infants

are FGR. It is likely that the previously described cardiovascular alterations underlie this impaired response.

As discussed, FGR results in an increased arterial thickness and stiffness. I believe that these FGR-driven vascular alterations underlie the increased requirement for cardiovascular support in FGR neonates[277,288]. Further, due to the already increased vasoconstricted state and reduced pulsatility observed in FGR vessels, it is possible that vasoconstrictors such as dopamine may be ineffective or possibly detrimental. A potential mechanism by which dopamine may be harmful is by increasing peripheral vascular resistance to drive a decrease in venous return, rather than increase cardiac output. Additionally, as dopamine acts to increase heart contractility[292], contraction of the heart against the highly resistant vessels may contribute to the ventricular hypertrophy also observed in FGR.

Although the relationship between dopamine and cardiovascular function in FGR has not been investigated clinically, several animal studies support the potential inefficacy of dopamine at improving cardiovascular function in FGR newborns. Black et al have found that β-adrenergic stimulation does not effectively improve cardiovascular function in FGR rats[297] and Tare et al have shown that FGR increases β-adrenergic activation in FGR lambs increases contractile force and heart rate, resulting in increased susceptibility to ischemia-reperfusion injury[250]. Hutchinson et al have shown that hypoxia during fetal life results in increased cardiac β-adrenoreceptors[298], which may explain the increased β-adrenergic response observed by Tare et al[297]. Additionally, Hutchinson et al have also shown that prolonged exposure to dopamine resulted in downregulation of β-adrenoreceptors, which may explain failure of dopamine therapy in FGR[298]. Chen et al have also shown that β-adrenergic receptors are down regulated in the peripheral adipose tissue of FGR sheep fetuses and that these changes persists in FGR lambs[299]. Similarly, Yates et al have shown FGR sheep fetuses have a decreased β-adrenergic expression in skeletal muscle[300]. Though these studies are not in cardiac tissue, they suggest that the conditions of FGR are capable of resulting in change to normal β-adrenergic receptor expression and function. Collectively, these animal studies demonstrate the potential impairment in the β-adrenergic pathway from FGR and highlight the dire need for FGR-targeted cardiovascular support.

1.15. Conclusion

In conclusion, FGR causes alteration to normal cardiovascular function and structure, with effects lasting from fetal to adult life. Currently antenatal and neonatal management for FGR are lacking.

Sildenafil citrate was a promising candidate for FGR therapy but has recently been shown to have adverse effects. SC can cross the placenta and enter the fetal circulation and the effects to fetal exposure have not been extensively investigated. To understand the adverse outcomes that occurred in the STRIDER, it is imperative we determine the effect that fetal SC exposure has on fetal development. FGR also results in increased requirement for cardiovascular support in early life. Dopamine is a common therapy for preterm cardiovascular support, however, some studies suggest that the use of dopamine in FGR is ineffective and potentially detrimental. Sildenafil and dopamine are therapies that alter vascular function. While deemed effective in AG fetuses and neonates, the cardiovascular alterations that occur in FGR may alter the function of these treatments. Given that cardiovascular dysfunction has a significant impact on health, quality of life and overall public health costs, preventative strategies and improved neonatal treatment of FGR are of dire importance.

1.16. Global Aims and Hypothesis.

This thesis aimed to investigate the antenatal and post-natal interaction between clinically used vascular therapies, such as sildenafil and dopamine, and the altered vasculature of the FGR perinate.

I hypothesize that FGR-driven vascular alterations will result in altered responses to sildenafil and dopamine compared to appropriately grown counterparts.

1.16.1 Thesis Aims and Hypothesis.

The three studies that comprise this thesis aimed to investigate;

1) The effect of maternal SC treatment on fetal vascular structure and function in growth restricted fetuses
2) Investigated the effects of maternal SC administration on maternal and fetal cardiovascular physiology
3) Investigate the therapeutic efficacy of SC for hypotension during the first hours of life in FGR lambs, compared to current gold standard treatment, dopamine

I hypothesized in each study that;

1) Maternal SC improves fetal growth and results in functional improvement of NO-mediated vasodilation within the peripheral vasculature.
2) Antenatal SC administration would induce peripheral vasodilation in the developing

FGR fetus, altering the cardiovascular adaption to chronic hypoxia.

3) Dopamine will have decreased efficacy to improve blood pressure in growth-restricted lambs compared to sildenafil citrate, due to FGR-driven vasoconstriction.

Chapter 2

General Methods

2.1. Ethics Statement

Experiments were approved by the Monash Medical Centre Animal Ethics Committee A (MMCA2016/01) at Monash University and run in accordance with guidelines established by the National Health and Medical Research Council of Australia.

2.2. Animals

Date-mated pregnant Border-Leicester ewes were delivered from Monash Animal Research Platform (MARP) Gippsland Field station to MARP Clayton at least two weeks prior to movement to Monash Medical Centre to allow acclimatization. Ewes were housed with other ewes, fed lucerne hay and had constant access to water. Ewes were housed with a natural light cycle (12-hour light/dark cycle). 7 days prior to the experimental/surgical date, ewes were moved to Monash Medical Centre animal house and housed in metabolic cages or floor pens in rooms maintained at 22°C and 44-55% humidity. All ewes were fed ~1kg lucerne chaff daily and had free access to water daily. Food intake and general well-being of the ewe was monitored and noted daily and any abnormal behavior or symptoms were treated accordingly.

2.3. Fetal and Maternal Surgical Interventions.

2.3.A. Surgical Preparation

Ewes were fasted for a minimum of 16 hours prior to surgery. Aseptic surgery was performed at either 89 (Aims 1, 2b and 3) or 104 (Aim 2a) days gestation (term is 145-147 days gestation). In preparation of sterile surgery, ewes received intravenous (i.v) prophylactic antibiotics (1g ampicillin sodium; Aspen Paramacare Australia, Australia and 5ml Engemycin; Coopers Animal Health, Australia). General anaesthesia of the ewe was initiated via i.v. injection of sodium thiopentone (20ml Pentothal i.v.; Boeringer Ingelheim Australia) into the jugular vein. Once sedated, the ewe was placed in a supine position and intubated via an endotracheal tube (ETT; internal diameter (ID) 8.0mm, outer diameter (OD) 10.9 mm, Portex, England). Anaesthesia was maintained via isoflurane (1.5-2.5% in 10/30% O_2/N_2O; Bomac Animal Health, NSW, Australia). Respiration was maintained using positive pressure ventilation (EV500 Anaesthesia Ventilator, Campbell, UCLO Engineering, NSW, Australia). The wool at the abdomen and neck was shorn and these areas were cleaned with an initial wash of aqueous chlorhexidine gluconate (0.5% w/v in 70% ethanol; Johnson % Johnson, Australia) followed by triplicate washes with betadine surgical scrub (7.5% w/v povidine-iodinel Fauldings Australia). Surgical areas were then sprayed with betadine antiseptic solution (10% povidine-iodinel Fauldings Australia) and incision sites further cleaned with chlorhexidine gluconate.

2.3.B Fetal Sterile Surgery

The ewe was placed on the operating table and covered in sterile drapes leaving the intended incision site exposed. Local anaesthetic (Marcaine, 12.5mg; Astra Zeneca, North Ryde, NSW) was subcutaneously injected at the incision site prior to incision. Laparotomy was performed on pregnant ewes to expose the fetus. Incision to the maternal abdomen; maternal skin, subcutaneous fat and linea alba were made to these layers in order to access the uterus. The fetal head was identified by palpation and an incision was made through the uterine wall and fetal membranes, avoiding blood vessels and cotyledons, to exteriorise the fetal head and neck. To minimise loss of amniotic fluid, the uterine wall and fetal membranes were clamped around the skin of the fetal neck with Babcock clamps.

2.3.C. Fetal Instrumentation (Aim 2)

An incision to the fetal neck was performed to expose the right carotid artery and right jugular vein. The jugular vein was catheterized (heparinised polyvinyl catheters ID 0.86mm, OD 1.52 mm Dural Plastics Australia) to allow for administration of antibiotics. A transonic flow probe (Transonic Size 3PD, ADInstruments, Bella Vista, Australia) was placed around the carotid artery to allow for measurement of carotid blood flow. The head of the fetus was then returned to the uterus and the fetus was gently manipulated to expose its hindquarters. An incision was made to the left and right legs to expose both femoral arteries. The right femoral artery was catheterised ((heparinised polyvinyl catheters ID 0.86 mm, OD 1.52 mm Dural Plastics Australia) and a flow probe (Size 3) was placed around the left femoral artery. An additional catheter (heparinised polyvinyl catheters ID 2.6 mm, OD 4.2 mm Dural Plastics Australia) was secured to the exterior of the fetal hip to allow for sampling of amniotic fluid, administration of antibiotics and to correct fetal pressure for physiological recordings.

2.3.D. Single Umbilical Artery Ligation (all Aims)

If the ewe was carrying a twin pregnancy, the first fetus exposed was assigned as the control (sham surgery) to produce an appropriately grown (AG) lamb. In the control fetus, the umbilical cord was located and manipulated but not ligated. To create the single umbilical artery ligation (SUAL), the second fetus (or the SUAL fetus in the instance of a singleton pregnancy) had its umbilical cord located and dissected to expose one of two umbilical arteries. A single umbilical artery was then ligated via application of two ties (Polysorb Suture, Covidien - Metronic, Minneapolis) to induce fetal growth restriction (FGR) in this lamb. Ligation of the umbilical artery results in acute

atrophy and subsequent elimination of half the placental mass. This results in a period of hypoxia, acidosis and hypercapnia and aimed to replicate the effects of chronic placental insufficiency[301].

2.3.E. Closure of incision sites.

Once fetal surgery had been completed, the fetus was returned to the uterus. All incision sites were then closed. The uterus was sutured closed in two layers. The incision site of the uterus was initially closed via a continuous running stitch (Polysorb Suture, Covidien - Metronic, Minneapolis). The incision site was then internalized via a Cushing's stitch. Catheters and flow probe cables from instrumented fetuses were externalized via the upper right flank of the ewe. Sterile 3-way taps (Discofix, Germany) were affixed to each catheter. The maternal linea alba and subcutaneous fat were sutured together via a continuous running stitch (Maxon Loop Suture, Covidien - Metronic, Minneapolis). The maternal flank and abdominal skin were closed via a horizontal mattress stitch.

2.3.F. Maternal Sterile Surgery.

During fetal surgery, the maternal neck was incised to expose the right maternal carotid artery and jugular vein. Vessels were exposed via blunt dissection and catheterized (heparinised polyvinyl catheters ID 2.6mm, OD 4.2 mm Dural Plastics Australia) to allow for collection of maternal blood samples (artery) and administration of antibiotics (vein). Catheters were inserted towards the direction of the heart within the artery (20-23cm) and vein (10-15cm). The catheters were then secured in place with silk sutures and flushed with heparinised saline to ensure patency before the maternal neck was closed via a horizontal mattress stitch.

During surgery physiological parameters; heart rate, respiratory rate, oxygen saturation level, carbon dioxide concentration was monitored constantly but recorded every 10 min. Tongue skin colour and corneal reflex was recorded every 10 min during surgery to ensure well-being. When all surgical procedures were complete, the anaesthetic was removed and mechanical ventilation ceased once the ewe resumed spontaneous breathing. All incision sites were sprayed with iodine solution then covered with a sterile pad. The endotracheal tube was removed upon return of the ewe's swallowing reflex.

2.4. Post-Surgical Care

The ewe was returned to her cage and given food immediately. Water was withheld until the ewe had recovered from anaesthesia. Oral paracetamol (1g Panadol, GSK, Australia) was administered for post-surgical pain relief.

Well-being of the ewe was monitored daily. Ewes with non-instrumented fetuses (Aims 1 and 3) were administered antibiotics (5ml of engemycin and 5 ml of ampicillin) via the maternal jugular catheter for 3 days post-surgery. Either oral or suppository paracetamol (1g; Panadol) was given to the ewe for pain relief daily.

Ewes with instrumented fetuses (Aim 2) received 5 ml engemycin via the maternal catheter, while the fetus was delivered antibiotics via both the fetal jugular vein (200mg ampicillin) and amniotic (800mg ampicillin) catheters.

Well-being of the ewe was monitored daily. In Aim 2a, fetal arterial blood (0.5mL) was collected and analysed (ABL800 Blood Gas Analyser; Radiometer, Denmark) daily for the first three post-surgical days and every other day after that to monitor partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), pH, O_2 saturation (SaO_2), haematocrit, glucose and lactate. Additional blood samples were taken from the fetus (Aim 2a) and ewe (all aims) on alternate days and centrifuged (3000g, -4, 10 min), plasma was then collected and stored at -80°C for later analysis of hormones and sildenafil citrate concentration.

2.4.A Maternal Sildenafil Citrate Administration (Aim 1 and 2)

On day 3 post-surgery, after completion of the antibiotic regimen, ewes in Aims 1 and 2 begun sildenafil citrate (SC; 2mg/ml, Monash Health Pharmacy Department) or saline (vehicle) infusion. SC (36ml/day) or vehicle was administered via a pump (CADD-Legacy 1 Pump; Smiths Medical, Australia) connected to the maternal jugular vein catheter and fastened to the ewes back. SC administration continued until post-mortem (125 days gestation). SC treatment regimen was chosen to replicate the clinical dosage used in the STRIDER trial[302]. Clinically 75mg was ingested orally daily. SC taken orally has a bioavailability of ~41 %[303], thus in my studies we gave a dose of 36 mg per day to replicate this level.

2.4.B Animal Housing During Gestation

Animal preparations without fetal instrumentation (Aims 1 and 3) were transferred back to MARP Clayton platform 12 days post-surgery. Ewes were housed in large open floor pens with open access to hay and water. Daily monitoring of ewes was maintained. Ewes were then returned to Monash Medical Centre 7 days before post-mortem or delivery.

2.4.C. In-utero physiological recordings (Aim 2)

On the day before SC administration begun fetal and maternal catheters as well as fetal flow probes were connected to monitor baseline fetal and maternal mean arterial blood pressure and blood flow as well as fetal heart rate. The maternal carotid artery as well as the fetal femoral artery and amniotic catheters were connected to pressure transducers (ADIntruments, Castle Hill, Australia) sterilised with aqueous chlorhexidine gluconate before being rinsed and filled with sterile saline, ensuring no air bubbles are present, and flow probes were connected to a flow meter (Transonic systems, Ithica, NY). Real-time *in vivo* outputs were recorded on LabChart Pro (ADIntruments, Castle Hill, Australia). Fetal and maternal recordings were initiated post antibiotic regimen and were collected from 9am – 12pm for 5 continuous days, then on alternate days until gestational age 125 days. The amniotic catheter was used as a reference for fetal position and was therefore subtracted from the fetal arterial pressure recording to achieve a corrected fetal blood pressure.

At the 125d of gestation, ewe and fetuses from aim 1 and 2 euthanized via pentobarbital sodium overdose (100 mg/kg i.v. 153 Valabarb; Jurox, Rutherford, Australia) and dead fetuses exposed for collection of fetal organs for analysis (described below).

2.4.D. Ventilation of FGR lambs and treatment with Sildenafil or Dopamine (Aim 3)

At 126 days of gestation, fetuses in Aim 3 were delivered, dried and ventilated. As per SUAL surgery, maternal anaesthesia and sterilization of incision sites were performed as described above (section 2.3. Maternal sedation was maintained with 2-2.5% isoflurane in room air oxygen. Both FGR and AG fetuses were partially exteriorised, in a random order, for instrumentation. Following partial exposure, transonic flow probes were placed around the carotid, femoral and main pulmonary artery (Transonic Size 3/4PD, ADInstruments, Bella Vista, Australia) for measurement of blood flow. Catheters (heparinised polyvinyl catheters ID 0.86mm, OD 1.52 mm Dural Plastics Australia) were implanted into the brachial artery for measurement of blood pressure and collection of blood samples for plasma and blood gas analysis,and the jugular vein for administration of treatment (SC, dopamine or saline). Additionally, a near-infrared spectroscopy (NIRS, CASMED ForeSight) sensor was attached to the left side of the head in order to measure cerebral oxygenation. A pulse oximeter was attached to the forelimb in order to measure peripheral oxygen saturation (SpO_2, MASIMO).

Catheters were each connected to a calibrated pressure transducer (ADIntruments, Castle Hill, Australia) and flow probes were connected to a flow meter (Transonic systems, Ithica, NY). Outputs were recorded in real-time on LabChart Pro (ADIntruments, Castle Hill, Australia).

After instrumentation, connection to transducers and flow meter and a 10 min control recording period of fetal physiology, the fetuses were ventilated. Ventilation was initiated with a sustained inflation (30cmH_2O for 30 seconds). The fetuses were ventilated for 1min 30s prior to clamping of the umbilical cord. Once the umbilical cord was clamped and cut, ventilation was continued using positive pressure ventilation using volume guaranteed mode at 7ml/kg, positive end expiratory pressure of 5cmH_2O, 60 breaths per min, inspiratory time of 0.5s and a fraction of inspired oxygen (FiO_2) at 0.21 (Draeger Babylog 8000+ ventilator, Draeger, Lubeck, Germany). Arterial blood gases were taken at 5, 10, 15min and every subsequent 30min until the end of the experimental period. Ventilator and FiO_2 adjustments were made to maintain SpO_2 between 85-95%. Sedation was maintained via constant infusion of Alfaxane (5-15ml/kg/hr) in 5% dextrose. Ewes were euthanized after delivery of all lambs.

Lambs were ventilated for a total of 4 hours. After 1 hour of ventilation, lambs were randomized to receive i.v. conventional therapy dopamine (0.6mg/kg/hr; DBL Dopamine Concentrate 200mg/5ml, Auckland, New Zealand)[304] or SC (0.07mg/kg/hour; Revatio (SC) Injection, Pfizer Labs, New York)[305] following neonatal care guidelines. At the end of the ventilation period, lambs were euthanized via pentobarbital sodium (100 mg/kg i.v. 153 Valabarb; Jurox, Rutherford, Australia) overdose for post-mortem.

2.5. Post-Mortem

Dissection of the fetus or lamb included collection of brain, lung and vessels (carotid, femoral, pulmonary and aorta) and measurement of brain, lung and fetal body weight and biparietal diameter, abdominal circumference and crown rump and lower limb length. The left side of the brain was frozen in oct and the right half immersion fixed in formalin for histological analysis. The left lung was snap frozen in liquid nitrogen for molecular analysis and right lung pressure fixed in formalin for histological analysis. A section of each vessel was collected for histological analysis (medial) and frozen (ventral) for molecular analysis.

For aim 1, third-order femoral and middle cerebral arteries were carefully dissected for in vitro functional testing via in vitro wire myography. For aim 3, lamb's hearts were also dissected and immersion fixed with 10% buffered formalin. Hearts were then cut into transverse 2 mm sections for measurement of ventricle thickness.

2.5.A. In Vitro Wire Myography

Solutions for myography.

Krebs stock solution (NaCl 173.75g, KCL 8.75g, $MgSO_4.7H_2O$ 7.225g, KH_2PO_4 4.025g in 1L dH_2O) and K+ Depolarisation Stock Solution (KCL 9.22g, $MgSO_4.7H_2O$ 0.289g, KH_2PO_4 0.161g, $C_6H_{12}O_6$ 1.091 g in 1L dH_2O) was made up prior to each myography session.

On the day of the myography session Krebs Solution (80ml stock solution, 4.2g $NaHCO_3$, $C_6H_{12}O_6$ 4g) and K+ solution (300ml stock solution, 630mg $NaHCO_3$, 750ul 1M $CaCl_2$, 150ul EDTA) were made up from respective stock solutions and bubbled with carbogen.

At post-mortem middle cerebral artery (MCA) and femoral vessels from fetuses were dissected and collected for functional analysis. Vessels were first dissected from the carcass and kept in chilled krebs solution. Under a bifocal dissecting microscope (Thomas Scientific, Swedesboro, USA), the first branch from the femoral artery of the left hind limb was excised and placed in ice cold krebs solution. The vessel was carefully cleaned of excess connective tissue and cut to a 2 mm long ring. Two 40 μm diameter stainless steel wires were threaded through the lumen of the femoral sections, maintaining the endothelium intact. The wires were then placed between the mounting support jaws of a 4-chamber small-vessel wire myograph (DMT wire pressure myograph; DMMT-USA, USA). Throughout experimentation, krebs solution was replaced every 20 minutes continually bubbled with carbogen. Force data from the myograph were recorded at 4 Hz (Labchart 6.0, Powerlab 8/30; AD Instruments, Chalgrove, UK), and vessels were normalised to 5.3kpa using the DMT normalisation module of Chart as previously described in detail[306]. To test smooth muscle function vessels were exposed to K+ depolarising solution and to test endothelial function vessels were exposed to phenylephrine and acetylcholine, once both smooth muscle and endothelial function were confirmed, vessels were rested for 20 minutes and the experimental protocol begun. Cumulative concentration-response curves to the α_1- adrenergic agonist phenylephrine (PE; 10^{-10}-10^{-5} mol^{-1}) were determined in half-log increments. The relaxant effects of sodium nitroprusside (SNP; 10^{-10}-10^{-6} mol^{-1}) and acetylcholine (ACh; 10^{-10}-10^{-6} mol^{-1}) were determined after pre-contraction with phenylephrine (10^{-5} mol^{-1}). Throughout experimentation outputs were recorded on LabChart Pro (ADIntruments, Castle Hill, Australia).

2.6. Maternal and Fetal Data Analysis

2.6.A. Physiological Analysis

Maternal, fetal and neonatal blood pressure was record via pressure transducers (ADIntruments, Castle Hill, Australia). Carotid, femoral (aims 2 and 3) and pulmonary (aim 3) blood flow were

measured via implanted transonic flow probes and recorded via a flow meter (Transonic systems, Ithica, NY). Data obtained from physiological readings including fetal and maternal pressures as well as fetal flows were continuously recorded on LabChart Pro (ADIntruments, Castle Hill, Australia). Raw data from aims 2 and 3 collected from LabChart Pro are as follows in Table 1:

Table 1. Data collected from LabChart Pro

Aim 2	Collection Method	Aim 3	Collection Method
Fetal Blood Pressure (FBP)	Femoral Artery Catheter	Blood Pressure (BP)	Brachial Artery Catheter
Raw Fetal Carotid Blood Flow (CBF)	Size 3 Transonic Flow Probe	Raw Carotid Blood Flow (CBF)	Size 3 Transonic Flow Probe
Raw Fetal Femoral Flow (FBF)	Size 3 Transonic Flow Probe	Raw Femoral Flow (FBF)	Size 3 Transonic Flow Probe
Maternal Blood Pressure (MBP)	Carotid Artery Catheter	Raw Pulmonary Flow (PBF)	Size 4 Transonic Flow Probe

Raw physiological measurements, collected as above (Table 1), were analysed to determine physiological parameters as described below in table 2.

Table 2. Physiological calculations from raw physiological measurements.

Aim 2		Aim 3	
Collected Data	**Calculation/Collection**	**Collected Data**	**Calculation/Collection**
Fetal Data		**Fetal Data**	
Fetal Diastolic Pressure (FDP)	FBP Minimum	Diastolic Pressure (DP)	BP Minimum
Fetal Systolic Pressure (FSP)	FBP Maximum	Systolic Pressure (SP)	BP Maximum
Fetal Pulse Pressure (FPP)	FBP Maximum – FBP Minimum	Pulse Pressure (FPP)	BP Maximum – BP Minimum
Fetal Heart Rate (FHR)	Cyclic Trigger Count of Systolic CBF	Heart Rate (FHR)	Cyclic Count of Carotid Flow

Estimated Fetal Weight During Gestation (EFBW)	Y= A + Bx	Lamb Body Weight	Lamb Body Weight at Delivery (LBW)
Fetal Femoral Flow per kg Bodyweight (FBF/Kg)	CBF/EFBW	Femoral Flow per kg Bodyweight	FBF/LBW
Fetal Carotid Flow per kg Bodyweight (CBF/Kg)	CBF/EFBW	Carotid Flow per kg Bodyweight	CBF/LBW
Femoral Vascular Resistance (FVR)	FVR=(FBF/kg)/FBP	Pulmonary Flow per kg Bodyweight	CBF/LBW
Femoral Vascular Conductance (FVR)	FVC=FBP/(FBF/kg)	Femoral Vascular Resistance (FVR)	FVR=FBF/BP
Carotid Vascular Resistance (CVR)	CVR=(CBF/kg)/FBP	Femoral Vascular Conductance (FVR)	FVC=BP/FBF
Carotid Vascular Conductance (CVR)	CVC=FBP/(CBF/kg)	Carotid Vascular Resistance (CVR)	CVR=CBF/BP
Carotid to Femoral Flow Ratio (CBC:FBF)	CBF:FBF=(CBF/Kg)/(FBF/kg)	Carotid Vascular Conductance (CVR)	CVC=BP/CBF
Maternal Data		Pulmonary Vascular Resistance (PVR)	PVR=PBF/BP

Maternal Diastolic Pressure (MDP)	MBP Minimum	Pulmonary Vascular Conductance (PVR)	PVC=BP/PBF
Maternal Systolic Pressure (MSP)	MBP Maximum		
Maternal Pulse Pressure (FPP)	MBP Maximum – MBP Minimum		
Maternal Heart Rate (MHR)	Cyclic Trigger Count of Systolic MBP		

Area under the curve (AUC) was calculated by GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) from graphs made from mean data of all physiological measurements.

Weight during gestation was derived from linear equation drawn from weight prior to SUAL[307] and at post-mortem. This was used for carotid and femoral blood flow per kg calculations over time.

2.6.D. Statistical Analysis

D'Agostino-Pearson normality test was used to assess normality of data. If data was not normal, data was normalized via analysis software GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Normalized data; fetal size and biometry in all studies were analysed and compared using a one-way ANOVA. *In vitro* wire myograph results were compared using non-linear dose response curves and using a two-way repeated measures ANOVA. From these the EC_{50} and maximal response were determined and these were compared between groups using a two-way ANOVA. Maternal, fetal and neonatal physiological data collected *in utero* and during ventilation experiments was compared between groups using two-way repeated measures ANOVA.

Following each statistical analysis, multiple comparisons post-hoc Tukey's test was used to determine if significant difference was found between groups. p value of <0.05 was used to determine significance. Data are presented as mean±SEM unless otherwise stated.

Chapter 3

The Effects of Maternal Sildenafil Treatment on Vascular Function in Growth Restricted Fetal Sheep

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3.1 Abstract

Objective – The objective of this study was to investigate the effect of intravenous maternal sildenafil citrate (SC) administration on vascular function in growth restricted fetal sheep.

Approach and Results - Fetal growth restriction (FGR) results in cardiovascular adaptations that redistribute cardiac output to optimize suboptimal intrauterine conditions. These adaptations result in structural and functional cardiovascular changes, which may underlie postnatal neurological and cardiovascular sequale. Evidence suggests SC, a potent vasodilator, may improve FGR. In contrast, recent clinical evidence suggest potential for adverse fetal consequence. Currently, there is limited data regarding SC effects in the developing fetus. We hypothesized that SC in-utero would improve vascular development and function in an ovine model of FGR. Preterm lambs (0.6 gestation) underwent sterile surgery for single umbilical artery ligation (SUAL) or sham (control, AG) surgery to replicate FGR. Ewes received continuous intravenous SC (36mg/24hrs) or saline from surgery until 0.83 gestation. Fetuses were delivered and immediately euthanized for collection of femoral and middle cerebral artery vessels. Vessel function was assessed via in vitro wire myography. SC exacerbated growth restriction in growth restricted fetuses and resulted in endothelial dysfunction in the cerebral and femoral vasculature, irrespective of growth status. Dysfunction in the cerebral circulation is endothelial, while smooth muscle in the periphery is the origin of the deficit.

Conclusions: SC crosses the placenta and alters key fetal vascular development. Extensive studies are required to investigate the effects of SC on fetal development to address safety prior to additional use of SC as a treatment.

3.2 Introduction

Fetal growth restriction (FGR) is the failure of a fetus to reach their genetic growth potential[308]. FGR is the second highest cause of perinatal death, complicating ~5-10% of all pregnancies[309] and increasing stillbirth risk 20-fold[3]. After birth, FGR infants have an increased risk of both short and long term disease[8]. FGR most commonly occurs due to placental insufficiency, causing impaired placental blood flow and fetal hypoxia. In response to hypoxia the fetus mounts a compensatory cardiovascular response to redistribute blood supply to important organs (e.g. brain) at the expense of the periphery[9]. Despite the redistribution of blood flow, FGR infants are at greater risk of developing neurological and cardiovascular sequelae compared to their appropriately grown (AG) counterparts[219].

Brain sparing is achieved via vasoconstriction of non-essential vascular beds such as the periphery, this mechanism is initiated by chemoreflexes and sustained by reduced NO bioavailability[169]. Long term chronic fetal hypoxia and persistent alteration in vascular resistance also cause vascular developmental deficits, such as increased thickening and stiffness of the aorta and carotid vessels and endothelial dysfunction[220], an early marker of cardiovascular disease[197]. Nitric oxide (NO) is produced by endothelial nitric oxide synthase (eNOS) located within the endothelial layers of blood vessels and is integral to local blood pressure control, acting as an endogenous vasodilator to mediate vascular diameter and blood flow[75]. It is well described that the FGR fetus has impaired NO-mediated vasodilation which can be attributed to NOS deficiency and decreased NO bioavailability[197].

Despite significant perinatal complications associated with FGR, there is currently no routine therapy to improve outcomes. Sildenafil Citrate (SC) is a phosphodiesterase (PDE)5 inhibitor which causes smooth muscle relaxation in blood vessels to increase vasodilation and subsequently, blood flow. SC has been proposed as a therapy in FGR pregnancies by acting to vasodilate placental blood vessels, which are abundant in PDE5, and increase placental blood flow[310]. In turn, improved placental blood flow is hypothesized to increase oxygen and nutrient transfer to the fetus, increasing fetal weight. Promising results from pre-clinical studies[24,29] have underpinned a set of worldwide multi-center clinical trials involving the UK/Ireland, The Netherlands, Australia/New Zealand and Canada. The Sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction (STRIDER) consortium aimed to determine if maternal SC treatment increases gestation length, placental function and fetal growth[302]. While results from some countries have now been reported and show no benefit of SC (AusNZ), the Dutch

STRIDER was recently terminated prior to completion due to increased neonatal mortality in SC-treated infants compared to control[22].

Maternally-administered SC crosses the placenta into the fetal circulation[311], where SC has potential to mediate direct cardiovascular effects. We and others have demonstrated dysfunction of the NO pathway in FGR when compared to AG[20,250]. Therefore, given the vascular adaptations associated with FGR, predisposition toward endothelial dysfunction and the NO mediated mechanism of SC action, we believe investigation into potential fetal effects of SC exposure is a critical unknown. Herein, we utilized our established ovine model of FGR, which involves single umbilical artery ligation (SUAL) to determine fetal effects of maternal sildenafil administration. SUAL results in placental atrophy, subsequent reduction of placental function and the induction of clinical FGR features (decreased weight, brain sparing)[312]. We aimed to investigate the effect of maternal SC treatment on fetal vascular structure and function in growth restricted fetuses. We hypothesized that maternal SC crosses the placenta into the fetal circulation, improves fetal growth and results in functional improvement of NO-mediated vasodilation within the peripheral vasculature.

3.3. Materials and Methods

Experiments were approved by the Monash Medical Centre animal ethics committee (A) (MMCA2016/01) in accordance with guidelines established by the National Health and Medical Research Council of Australia.

3.3.A. Animals

Twin bearing Border-Leicester pregnant ewes (n=13) underwent surgery on day 88-90 days gestation (dGA, term approximately 148dGA) to induce severe, early onset FGR via SUAL, as previously described[312]. Previous studies by our group have investigated differences between early (89d) and late (105d) SUAL and have found that early SUAL resulted in increased FGR and brain sparing[312]. As criteria for the STRIDER was diagnosis of early and/or severe FGR[313] we chose to use the early SUAL model in the current study. Briefly, anesthetized ewes underwent surgery in which one fetus (randomly selected) underwent SUAL to induce FGR. The umbilical cord of the twin fetus was manipulated but not ligated (control, AG). The fetuses were returned to the uterus and catheters inserted into a maternal jugular vein for antibiotic and treatment administration and a carotid artery for blood sampling.

3.3.B. Experimental procedures

Antibiotics were administered to the ewe (Ampicillin, 1g and Engemycin 5mL i.v) for 3 days after surgery via the maternal jugular vein catheter.

3.3.C. Maternal Sildenafil/Saline Administration

On day 3-post surgery, after completion of the antibiotics regimen, ewes were randomized to receive either sildenafil citrate (SC, n=7, 36mg/day) or saline (n=6) infusion via a pump (CADD-Legacy 1 Pump; Smiths Medical, Australia) connected to the maternal jugular vein catheter and fastened to the back of the ewe under netting. Twin fetuses within the ewes were subsequently randomized into groups; fetal growth restricted (FGR) and appropriately grown (AG) or SC treated fetal growth restricted (FGR_{SC}) and SC treated appropriately grown (AG_{SC}). SC and saline administration occurred continuously for ~5 weeks, until post-mortem (126 dGA). This treatment regimen was used to reflect the dose chosen and used in the STRIDER trial[302].

3.3.D. Post-Mortem

At 126 dGA, fetuses from all groups were euthanized via pentobarbital sodium overdose to the ewe (100 mg/kg i.v. 153 Valabarb; Jurox, Rutherford, Australia). Third-order middle cerebral and femoral arteries were carefully and immediately dissected for functional assessment (in vitro wire myography, see below).

3.3.E. Soluble guanylate cyclase protein level

Soluble guanylate cyclase (sGC) is an enzyme present in smooth muscle cells and converts guanosine triphosphate (GTP) into active cyclic guanosine monophosphate (cGMP). cGMP is the key intracellular molecule that is ultimately responsible for smooth muscle relaxation and subsequent vasodilation. We quantified sGC protein levels via western blot.

Snap frozen femoral vessel samples were homogenized in 0.1M Tris/HCL lysis buffer with 0.5% Triton X with 10μL/ml phenylmethylsulphonyl fluoride. Total protein content of the supernatant was determined via the Lowry method using a Bio-Rad DC (detergent compatible) Protein Assay kit (Bio-Rad, California, USA). Protein samples (30μg) in 4x SDS sample buffer (EMD Millipore, California, USA) were heat denatured, separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Protran BA-85 nitrocellulose membrane, Schleicher; Schuell, Germany). Membranes were then incubated in primary antibody (rabbit anti-sGCb1 antibody, 1:500; Cayman or b-actin 1:1000, Cell Signalling Technologies) with 5% skim milk block overnight at 4°C. The protein was then detected by goat anti-rabbit IgG-HRP, 1:10,000 (Santa Cruz Biotechnology), and visualized with Chemiluminescence reagent

LumiGLO (Cell Signalling Technologies, Danvers, USA) using Chemidoc XRS (Biorad, California, USA). The membrane image was analyzed using ImageJ analysis software[314]. The protein expression level was calculated from the average density of the sGC band divided by the average density of the beta-actin band.

3.3.F. In Vitro Wire Myography

Wire myography allows for the interrogation of vascular response and reactivity to vasoactive agents[315]. Third-order middle cerebral arteries (MCA) and femoral (FEM) arteries were dissected and placed in chilled Krebs Solution: mmol/L: NaCl 118.845, KCl 4.69, $MgSO_4$ 1.156, KH_2PO_4 1.175, $NaHCO_3$ 25.2, D-glucose 12, EDTA 0.03 and $CaCl_2$ 2.5, and bubbled with carbogen (95% O2 and 5% CO2). 2 mm dissections of each vessel were threaded with a 40 μm diameter wire, with care to avoid touching the endothelium, and mounted onto a four-chamber mire myograph (Multi-Wire Myograph System 610M, DMT, Denmark). The force of arterial resistance was normalized (0.9 @ 5.3kPa) to mimic fetal lamb blood pressure (~50mmHg)[316]. Initial contractile capacity was assessed in response to Potassium (K+) Physiological Saline (0.12 M) and endothelial vasodilation ability was assessed with acetylcholine (ACh; Sigma A6625, Sigma Aldrich). Collection and mounting of 2mm dissections were repeated until viability of the vessel was determined via response to K+ solution and ACh.

3.3.G. Assessment of atrial vasodilation via Myography:

Wire-mounted arteries were pre-constricted with K^+ Physiological Saline (0.6M). K^+ Physiological Saline allowed for assessment of smooth muscle contractility and subsequent assessment of vasodilation of both MCA and FEM arteries in response to acetylcholine (ACh; Sigma A6625, Sigma Aldrich; 10^{-9}-10^{-4} mol/L), sodium nitroprusside (SNP, Sigma 71778, Sigma-Aldrich; 10^{-10} – 10^{-4} mol/L). Assessment of vasodilation in response to sildenafil citrate was also performed on MCA (SC, Sigma PZ0003, Silgma-Aldrich; 10^{-9}-10^{-4} mol/L). Response to drugs were performed via a similar method. After plateau of a sub-maximal contractile response from vasodilator, an initial dose was added to the bath to a concentration of 10^{-10} mol/L or 10^{-9} mol/L depending on the curve. Vessels were then exposed to progressively higher concentration (increased by a factor of ten with each administration) until a final bath concentration of 10^{-4} mol/L was achieved. SNP, ACh and sildenafil bath concentration was increased after plateau. Vasodilation response curves were performed on the same vessel. Vessels were rested at baseline for 20 min rest between each response curve.

Average force exerted by the artery during each incubation was recorded. Individual results were averaged for drug concentration to create dose-response dilation curves. Relaxation curves were

analyzed using an agonist line of best-fit assessments to determine overall maximal relaxation (%Rmax) and sensitivity (pEC_{50}).

To asses the contribution of PDE5 to vasodilation within the MCA, vessels were constricted pre-constricted with K^+ and dilation curves assed in response to ACh and SC. Activity of sGC was calculated by assessing the area under the curve in response to SC[306].

3.3.H. Plasma Sildenafil Measurement

Baseline maternal plasma samples were collected from day 1 post-surgery. Sildenafil administration begun on day 4-post surgery, after this time maternal blood samples were taken between 9-10am on alternate days until post mortem. In a sub-group of animals exposed to SC treatment, fetal plasma samples were collected at post-mortem. ~1ml of plasma samples were collected and analyzed for SC concentration via liquid chromatography-mass spectrometry by ThermoFisher Australia using a protocol established by Vos et al[317]. SC is a phosphodiesterase (PDE) 5 inhibitor, which blocks the breakdown of cyclic guanosine monophosphate (cGMP) to GMP in blood vessels. Increased intracellular smooth muscle cGMP mediates effects on downstream pathways to ultimately cause decreased vascular resistance[254], increased vasodilation and subsequently, increased blood flow.

3.3.I. Statistical Analysis:

Data are for animal weight, ratios and sildenafil concentration presented as mean±SD. Data for myography analysis are presented as mean±SEM. Maximal contraction to high K^+ was analysed using a one-way ANOVA and a Tukey's post-hoc test was used to compare between groups (Prism 7 for Mac OS X, GraphPad Software, USA). Body weight, brain to body weight ratio, were analysed using a two-way ANOVA and Tukey's post-hoc test to compare differences between groups. Myography results from isolated MCA and FEM were analysed using a two-way repeated measures ANOVA and Tukey's post-hoc test was used to compare between groups and/or doses. Normality was calculated using D'Agostino & Pearson and KS normality test (Prism 7 for Mac OS X, GraphPad Software, USA).

3.4. Results

3.4.A. Plasma sildenafil concentrations.

Maternal i.v. infusion of SC resulted in detectable levels of SC within fetal (3.05±1.50pg/μl) and maternal (16.72±3.56pg/μl) plasma (Table 1). SC was undetectable in untreated animals (data not shown).

3.4.B. Fetal Birthweight and Brain: Body weight

Comparisons were made between saline-treated AG (n=7) and FGR (n=6) fetuses, and SC-treated AG_{SC} (n=5) and FGR_{SC} (n=6) fetuses, Table 1. One FGR and one AG_{SC} fetus were not included in analysis due to complications after surgery. Fetal birthweight shows single umbilical artery ligation resulted in a 25% reduction in birthweight compared to AG ($p=0.03$). Surprisingly, treatment with SC exacerbated FGR resulting in a ~50% reduction in birthweight compared to AG ($p<0.001$) and a 30% reduction compared to FGR ($p=0.02$). No significant difference in birthweight was seen between saline treated and AG_{SC} fetuses. Brain weight was similar between saline treated and AG_{SC} fetuses, but significantly lower in FGR_{SC} compared to AG control. Brain/body weight ratio was significantly increased in FGR_{SC} compared AG, $p=0.003$ and $AG_{SC,}$ $p=0.0024$. No other significant differences in brain/body weight was seen between groups.

3.4.C. Myography Analysis

3.4.C.i. Arterial Contractile Ability

Maximal contraction to high potassium solution (0.6M) was not different in the middle cerebral artery between any groups (Fig 1A). In contrast, in the femoral artery maximal contractile force was significantly reduced ($p<0.05$) in FGR, FGR_{SC} and AG_{SC} groups compared to AG fetuses (Fig 1B). Contractile force within the femoral artery was not different between FGR, AG_{SC} and FGR_{SC}.

3.4.C.ii. Vasodilation in Response to SNP and ACh in Middle Cerebral Arteries and Femoral Arteries

SNP induces NO-mediated, endothelium independent vasodilation. In the middle cerebral arteries (MCA), no difference in SNP mediated vasodilation was seen between groups of the same treatment (AGvsFGR or AG_{SC}vsFGR_{SC}). Respective increasing vasodilation occurred from increasing doses of SNP (10^{-10}to10^{-4} mol/L) in all groups and final SNP dose (10^{-10} mol/L) resulted in greater vasodilation compared to initial SNP dose (10^{-4} mol/L). MCA of both SC treated groups vasodilated to lower concentrations of SNP compared to saline treated. MCA vasodilation was significantly greater between SNP concentrations of 10^{-7} – $10^{-5.5}$ mol/L (Fig 2A, $p<0.05$) of SC compared to control. Increased overall vasodilatory response to SNP (smaller area under the curve) was observed in MCA of SC treated compared to saline (Fig 3A, $p<0.05$). Overall vasodilation to SNP was not seen within groups.

ACh mediates ligand-activated endothelium dependent vasodilation. In MCA, there was no difference in vasodilation to ACh between groups of the same treatment (AGvsFGR or AG_{SC}vsFGR_{SC}). Vasodilation occurred from increasing doses of ACh (10^{-10} to 10^{-4} mol/L) in both saline treated groups and final ACh dose (10^{-10} mol/L) resulted in greater vasodilation compared to initial SNP dose (10^{-4} mol/L). Vasodilatory response was significantly greater at ACh

concentrations 10^{-6} mol/L and 10^{-4} mol/L of both saline treated compared to SC treated groups (AG_{SC} and FGR_{SC}, Fig 2B, $p<0.05$). In contrast, vasodilation of SC treated was not different between any ACh dose. Overall vasodilatory response to ACh (smaller area under the curve) was greater in MCA of saline treated compared to SC (Fig 3B, $p<0.05$). Overall differences in vasodilation to ACh was not seen within groups.

SNP mediated vasodilation of femoral arteries occurred from increasing doses of SNP (10^{-10} to 10^{-4}mol/L) in of AG, FGR and AG_{SC} and final SNP dose (10^{-10}mol/L) resulted in greater vasodilation compared to initial SNP dose (10^{-4}mol/L) (Fig 2C, $p<0.001$, $p<0.001$ and $p=0.0074$ respectively). Vasodilation in femoral arteries of FGR_{SC} was not different between starting and final SNP doses. Femoral response to SNP was significantly greater in saline treated groups compared to SC treated groups, between SNP doses 10^{-8}mol/L to 10^{-4}mol/L (Fig 2C, $p<0.05$). Increased overall vasodilatory response to SNP (smaller area under the curve) was observed in femoral arteries of saline treated compared to SC (Fig 3C, $p<0.05$). Femoral arteries of FGR saline controls had greater overall vasodilation compared to AG. (Fig 3C, $p<0.05$). No difference in overall vasodilation to SNP was seen in SC treated.

ACh mediated vasodilation of femoral arteries was not different within groups of the same treatment (AG vs FGR or AG_{SC} vs FGR_{SC}). Vasodilation in femoral arteries from increasing doses of ACh (10^{-10} to 10^{-4}mol/L) occured in AG, FGR and FGR_{SC} and final ACh dose (10^{-10}mol/L) resulted in greater femoral vasodilation compared to initial ACh dose (10^{-4}mol/L) (Fig 2D, $p<0.001$, $p<0.001$ and $p=0.0258$ respectively). Vasodilation in femoral arteries of AG_{SC} was not different between starting and final Ach doses. Vasodilation was significantly greater in saline treated groups compared to SC treated groups. This difference was significant between ACh concentrations 10^{-6}mol/L to 10^{-5}mol/L (Fig 2D, $p<0.05$). Significantly greater overall vasodilation (smaller area under the curve) occurred in saline treated compared to saline (Fig 3D, $p<0.05$). Overall differences in vasodilation to ACh was not seen within groups.

3.4.D Vascular Sensitivity and maximal relaxation of Middle Cerebral Arteries and Femoral Arteries in response to SNP and ACh.

SC treated MCA had a greater sensitivity to SNP as shown by a significantly greater pEC_{50} compared to saline treated (Fig 4A, $p<0.001$). Maximal relaxation (%Rmax) was greater in control lambs compared to SC treated (Fig 4A, $p=0.0169$). MCA sensitivity to ACh (pEC_{50}) was not different between groups (Fig 4B). Maximal relaxation (%Rmax) was significantly impaired in AG_{SC} and FGR_{SC} vessels compared to saline treated groups. %Rmax was significantly lower in

AG_{SC} and FGR_{SC} compared to saline treated (Fig 4B, $p<0.001$). No difference in pEC50 and %Rmax was seen within treatment groups.

Femoral vascular sensitivity (pEC_{50}) to SNP was significantly lower in SC treated lambs compared to control (Fig 4C, $p=0.0255$). Maximal relaxation (%Rmax) was significantly greater in control lambs compared to SC treated (Fig 4C. $p>0.001$). Femoral vascular sensitivity (pEC_{50}) to ACh was not different between groups. Femoral maximal relaxation to ACh (%Rmax) was significantly greater in control lambs compared to SC treated (Fig 4D. $p>0.001$). No difference in pEC50 and %Rmax was seen within treatment groups.

3.4.E. Contribution of sGC to relaxation

To determine the relative contribution of sGC to overall relaxation, PDE5 inhibitor sildenafil citrate was used. These data reveal a significantly greater sGC capacity of sildenafil treated fetuses, compared to saline treated fetuses ($p=0.0059$, Fig 5).

3.4.F. Protein levels of soluble guanylate cyclase

Protein levels of sGC within the femoral artery were not different between groups (data not shown).

3.5. Discussion

FGR increases the risks of long-term dysfunctions[7]. There is no cure for FGR, however maternal administration of sildenafil citrate (SC) has suggested improvement in some animal studies of FGR [25–27] and in small human trials[29,318]. In turn, SC has been the subject of the worldwide STRIDER clinical trial as a potential therapy for FGR. However, reports suggest that antenatal SC may increase mortality for the growth restricted infant after birth[22]. There is currently limited data to show effects of SC on the already compromised cardiovascular structure and function in the setting of FGR. Assessment of vascular contractile ability is indicative of vascular health, integrity and can inform of potentially dysfunctional vascular pathways[319]. In the current study, we have validated the transfer of SC from the maternal circulation into the fetal circulation. Further, we show that maternal SC administration exacerbated growth restriction and results in vascular dysfunction in the developing peripheral and cerebral cardiovascular system of FGR and AG fetuses. These differences are very important. It has long been demonstrated that homeostasis within the peripheral vascular system is vital for blood pressure control. Of equal concern is dysfunction in the cerebral circulation, wherein exposure to SC caused endothelial dysfunction of

cerebral vessels in FGR fetuses, which has implications for the brain sparing adaptation mounted by the growth restricted fetus, and acute cerebral responses to challenges after birth.

SC has been used clinically for many years. SC is a phosphodiesterase type 5 (PDE5) inhibitor with various applications, including erectile dysfunction (Viagra) and persistent pulmonary hypertension of the newborn[320]. SC induces vasodilation through the NO-cyclic guanosine monophosphate (cGMP) pathway. PDE5 regulates intracellular levels of cGMP by hydrolyzing cGMP to guanosine monophosphate (GMP). Modulation of cGMP levels is critical for basal vascular tone and reactivity, where PDE5 promotes increased breakdown of cGMP and therefore vasoconstriction. SC inhibition of PDE5 thus promotes vasodilation.

Animal and human studies have shown that SC crosses the placenta to enter the fetal circulation[311]. Itani et al have demonstrated protective cardiovascular effect of SC in hypoxic chicken embryos[261]. While, this study did not demonstrate an increase in fetal growth, SC treatment prevented oxidative stress, enhanced NO bioavailability and restored peripheral endothelial function[261]. Our study is the first to demonstrate SC crosses the ovine placenta. SC concentrations of 3.5 nmol/L is considered clinically relevant and has the ability to inhibit 50% of PDE5 activity[321]. The difference in fetal to maternal SC concentrations is likely due to limitations in ovine placental transfer. However, the dose given to ewes resulted in fetal SC concentrations sufficient to inhibit greater than 50% of fetal PDE5 activity.

3.5.A. Exacerbation of FGR

An unexpected but striking observation of this study is the exacerbation of FGR in fetal sheep following SC treatment. In this study, as in previous studies, SUAL resulted in asymmetrical growth restriction[312]. Antenatal SC exacerbated body weight deficits in FGR, resulting in a 49% reduction in FGR_{SC} compared to AG fetuses, a finding accompanied by brain sparing in FGR_{SC} fetuses. No other studies have reported such striking decreases in body weight, but not all studies describe improved body weight. While rodent studies have demonstrated increased fetal body weight[24], other human and sheep studies do not show improvement in body weight[27,262,313].

The mechanisms underlying increased growth restriction were not interrogated in this study, but it is likely that maternal hemodynamics play a central role. Miller et al previously found maternal SC administration to ovine pregnancies complicated by FGR had the potential to cause detrimental utero-placental flow[28]. This study also reported fetal hypotension and tachycardia after maternal SC treatment[28].

It is important to note that while decrease in body weight in AG_{SC} compared to AG lambs was not significant, body weights were ~900g lighter and similar to that of FGR. We speculate that the reduction in body weight observed in both SC treated groups may have occurred in either one, or a combination, of two ways;

1) The proposed mechanism of benefit of SC is through the increase in vasodilation of the placental vasculature and subsequent increase in blood flow. As previously discussed, our model recreates FGR through SUAL, which results in subsequent atrophy to half the placental surface area[301]. In our study, we believe that any increase in blood flow by SC, could only supply the still functional placenta regions. It is unknown if SC has the ability to repair malformed blood vessels and therefore, in the context of impaired spinal artery remodeling, should similar placental vasodilation occur clinically, benefit of SC will also be limited. Given the observed reduction in fetal size following SC treatment seen in this study, we believe that caution surrounding SC treatment is warranted.

2) It is possible that long-term maternal SC administration may lead to chronic peripheral vasodilation within the maternal circulation, resulting in shunting of blood away from the feto-placental circulation, further diminishing placental perfusion. Though not assessed in the current study, the increased abnormality in ductus venosus a-wave (UK STRIDER) may support our finding[262]. Reversal of flow through the ductus venosus may occur due to a decrease in pressure driven by vasodilation of the maternal and placental circulation.

While several clinical studies have demonstrated the potential placental benefits of SC, differences in these studies, regarding timing of treatment, dosage and context of use limit the ability to draw comparisons to the STRIDER trial.

Dunn et al, investigated the utility of SC treatment in the context of intrapartum hypoxia, where pregnant women received 50mg oral SC eight hourly and only when admitted into the birth suite[272]. In a separate study by Sayed et al, 54 FGR-pregnant women were treated with a single dose oral dose of SC, prior to investigation of placental and fetal blood flow via Doppler ultrasound[322]. Unlike the STRIDER trial, SC treatment in these studies were relatively short and did not expose the fetus to chronic SC levels as performed in the STRIDER. Therefore, fetal development did not occur with exposure to SC.

Another important distinction to make between the study by Dunn et al and the STRIDER was that this study was conducted in AG fetuses[272]. As discussed, FGR-driven vascular alterations likely underlie the exacerbation of vascular dysfunction observed in our study. Therefore, comparison between fetal outcome between these two dissimilar populations are limited. These studies demonstrate the difference in outcomes that can occur following different treatment regimens to the same therapy and the highlight the importance for comparison of therapeutic effects between relevant cohorts.

3.5.B. Vascular effects of FGR and SC

To interrogate vascular function, we measured vascular ability to constrict to K+, endothelial-mediated vasodilation using acetylcholine (ACh) and smooth muscle mediated vasodilation using sodium nitroprusside (SNP). We chose to investigate central and peripheral vascular beds, as these are believed to be spared (middle cerebral artery-central) and non-spared (femoral-peripheral) from the vascular consequences of FGR. FGR-associated endothelial dysfunction in the periphery is well describe and found to be NO dependent[197], with restoration possible via treatment with antioxidants such as Vitamin C[323] and melatonin[324]. Additionally Itani et al show cardiovascular protection in chicken embryos following SC, suggesting potential benefit of SC treatment. FGR is associated with heterogeneous vascular remodeling in FGR offspring, where central vessels are 'favored' or 'less affected' compared to the vasculature of peripheral organs[221]. Our findings also support this study as FGR alone did not alter vascular responses in the cerebral circulation.

Sildenafil treatment impaired endothelial-mediated vasodilation in the MCA but enhanced the smooth-muscle mediated vasodilation. The difference in response may be due to potential compensatory mechanisms in the NO pathway of the MCA smooth muscle, following SC exposure. Increased sensitivity to SNP after SC treatment is indicative of developmental re-setting of the NO pathway. This compensation was not observed in the peripheral circulation. Renshall et al have also previously shown impaired endothelium-dependent and -independent vasodilation in SC treated FGR mouse aorta[325], considered to be a central vascular bed. As both direct administration of NO to the smooth muscle and inhibition of cGMP degrader, PDE5, resulted in enhanced vasodilation, it is clear that the vascular smooth muscle is intact. In contrast, endothelial-dependent vasodilation is nearly entirely attenuated, thus SC administration is having its effects within the endothelium, likely due to decreased NO bioavailability or altered agonist receptor population. The potential cerebrovascular dysfunction may be described as a decrease in endothelial function and vascular reactivity observed in both FGR and AG following SC exposure. These results are concerning as they suggest exposure to SC in utero, rather than placental

insufficiency, results in endothelial dysfunction. Interestingly, Kruuse et al found that middle cerebral arteries from adult rats do not vasodilate to SC administration[326].

The maintenance of cerebral blood flow in FGR is a well described response in FGR[16], further studies by Poudel et al have shown that carotid blood flow between FGR and AG lambs is similar, despite increased systemic vascular resistance[327]. We have furthered these findings to show that there is little difference in vasodilation of MCA between FGR and AG fetuses in our current study. Thus during brain sparing blood vessel function within spared beds is not altered.

However, despite attempts at maintaining cerebral perfusion, the FGR brain is susceptible to injury following FGR[312]. Given this, the diminished functionality of the cerebral vasculature, following SC exposure is concerning, considering impaired autoregulation is associated with an increased risk of brain injury[328]. Further studies to interrogate mechanism on how antenatal SC exposure alters cerebrovascular development, and potential long-term effects, would provide greater insight to chronic SC exposure during pregnancy.

Whilst in the dysfunction in the cerebral vasculature are endothelial in origin, in the femoral vascular bed, SC administration decreased the vasodilatory response of the vascular smooth muscle to SNP, a NO donor that binds to sGC to cause vascular relaxation. Vascular smooth muscle relaxation occurs as a result of decreased intracellular calcium, and there are numerous pathways, such as the adenylate cyclase-cGMP pathway which also play a role in vasodilation. In the current study, we focused on activation of sGC, but it is also possible that other pathways may be upregulated following antenatal treatment with SC. We observed that SC treatment impaired fetal peripheral smooth muscle function resulting in endothelial dysfunction. Although the peripheral vascular effects may result from fetal exposure to SC *per se,* it is also possible that a worsening of growth restriction may contribute to peripheral vascular deficits. Endothelial dysfunction is a well-established consequence of FGR[324], the exacerbation of FGR observed in our study is of particular importance to vascular function.

PDE5 inhibition has been associated with smooth muscle preservation[329] and may explain the increased sensitivity to SNP observed in MCA. However, in contrast drastically lower maximal constriction was observed in FGR peripheral vessels. These differences are likely due to the hemodynamic adaptations mounted during brain sparing in FGR. Unexpectedly, similar responses were seen within the femoral of both SC treated groups, regardless of growth status. These findings

are concerning as they suggest that independent SC exposure during development has the ability to disrupt smooth muscle and K^+ channel activity. Studies investigating SC actions on pulmonary hypertension have shown that SC exposure reduces smooth muscle deposition[330] and has anti-proliferative properties within the smooth muscle via the eNOS-NO-cGMP pathway[331].

The pathway of NO mediated vasodilation within the smooth muscle involve several key mediators. cGMP causes smooth muscle relaxation, proliferation and differentiation[332] and is broken down by PDE5, the target of SC action. We and other have shown that SC exposure increases cGMP levels[333]. Of particular importance to this study, increased levels of cGMP and cGMP elevating agents can suppress proliferation of smooth muscle[334]. The observed decrease in smooth muscle response seen in SC treated fetuses supports the potential for cGMP to disrupt smooth muscle proliferation. Furthermore, although SC exposure increased sGC-contribution to vasodilation, overall dilation was reduced in SC treated fetuses (Fig5). These data together support the hypothesis that SC treatment *in utero* reduces NO bioavailability. cGMP curves were only conducted in MCA vessels, and therefore it is not possible to extrapolate this data to the femoral arteries.

3.6. Limitations

In this study, we aimed to mimic the human dose of SC administration given in the STRIDER trials. In order to provide a controlled administration of SC to the pregnant ewe, SC was given intravenously to the ewe. This is in contrast to the STRIDER trial in which SC was taken orally in three tablets over the course of the day. Thus, the exposure to SC is likely to be different between our study and the human trial. SC taken orally has a bioavailability of ~41 %[303], thus in the current study we gave a dose of 36 mg per day to replicate this level. Further, we commenced SC treatment at 3 days after inducing placental insufficiency, which is rather early compared to the human situation in which placental insufficiency and chronic hypoxia are likely to be well progressed. The delay in response to SNP, relative to that of ACh within the MCA of control animals may suggest alteration of MCA smooth muscle from our SUAL intervention. Future studies should aim at interrogating the NO-mediated signaling pathways in this vessel. We also do not have measurements of uterine flow in this study and future studies should aim at investigating the effect of long-term SC treatment on uterine flow to better allude to the increased FGR observed. Additionally, due to the low numbers used in these studies it was not possible to separately analyze the effects of fetal sex.

3.7. Conclusion

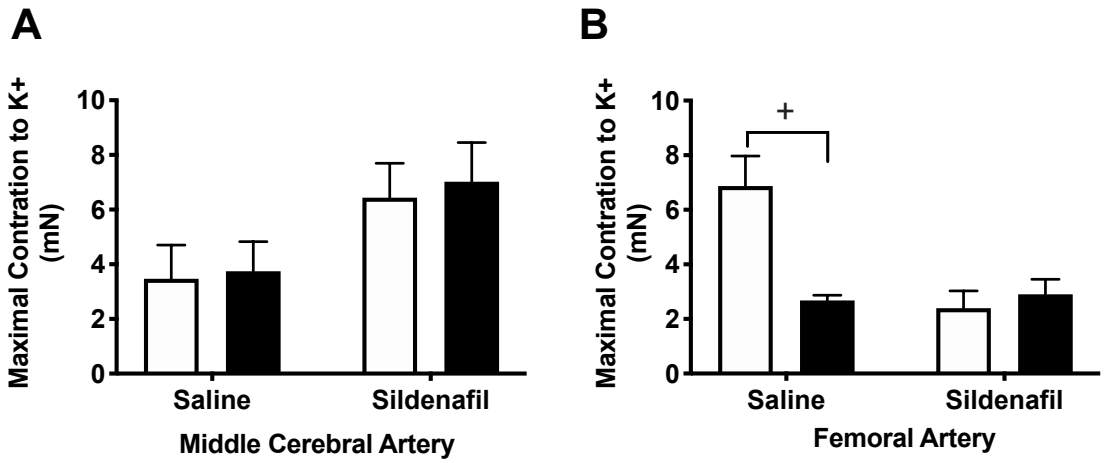
Although previous animal studies have shown SC to improve growth in FGR, the current study did not support those findings. Rather, SC resulted in exacerbation of growth restriction and additionally, was detrimental to vascular function. Cerebral vascular function is normally preserved in growth restricted offspring due to brain sparing, but here we show that antenatal SC exposure impaired vascular function in this vital vascular bed. In turn, this has significant implications for the infant after birth, potentially limiting cerebral vascular reactivity to acute challenge and increasing the risk of neurological compromise. This is the first study to show specific effects of SC exposure on the developing cardiovascular system of the fetus, which may in turn have longer-term detrimental effects on cardiovascular health. SC crosses the ovine and human placenta and accordingly, we suggest that the safety of SC should be considered with long-term cardiovascular health of the offspring in mind.

3.8. Table and Figure Legends

	AG	***FGR***	***AG_{SC}***	***FGR_{SC}***
N	7	6	5	6
Sex (M)	6/7	3/6	2/5	2/6
Weight (kg)	3.7 ± 0.31	2.7 ± 0.72"	2.8 ± 0.46	1.9 ± 0.48"†
Brain Weight (g)	50.37±1.71	49.01±5.09	45.66±2.55	40.01±2.55*
Brain: Body Weight (g/kg)	14.0 ± 1.0	18.56 ± 4.27	16.64 ± 2.67	22.61 ± 4.89*
	Fetal		***Maternal***	
Plasma Sildenafil Concentration (pg/ul)	3.05± 1.50		16.72± 3.56	

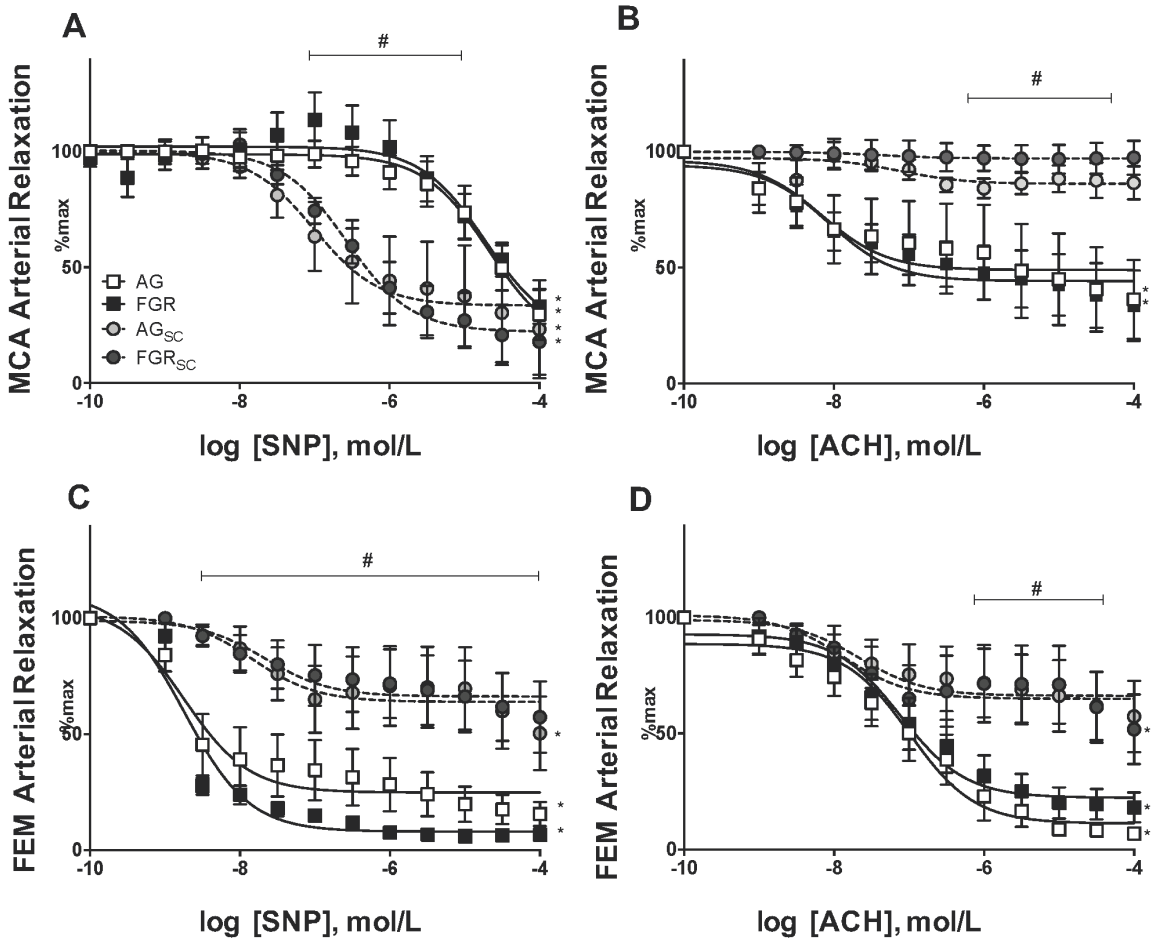
3.8.A. Table 1. Characteristics of fetuses at post-mortem delivery of appropriately grown (AG), growth restricted (FGR), appropriately grown treated with sildenafil (AG_{SC}) and growth restricted treated with sildenafil (FGR_{SC})

Weights and SC concentration are mean ± SD. Data were compared using a two-way ANOVA to determine difference between treatments. Average maternal SC concentration was taken from samples collected over the treatment period and fetal at post-mortem." signifies significant difference compared to AG and † signifies significant difference to FGR ($p<0.05$). Multiple comparisons were performed via a Tukey's test.



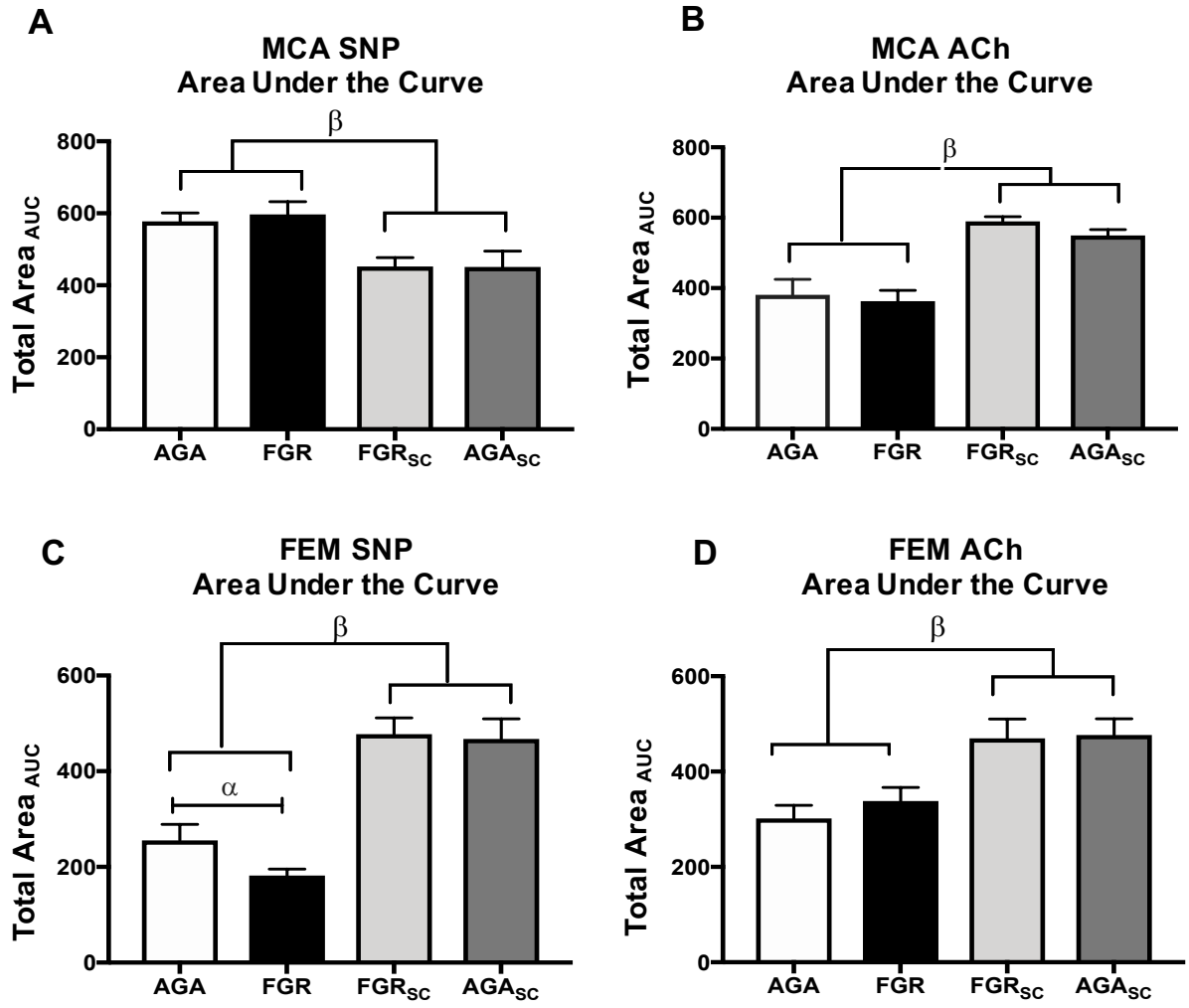
3.8.B. Figure 1. Middle cerebral artery (MCA) and femoral artery response high K+ physiological saline solution (0.1M)

Data are mean ± SEM contractile force (mN) response of middle cerebral artery (MCA, A) and femoral artery (FEM, B) in appropriately grown (AG, white, n=7), growth restricted (FGR, black, n=6), sildenafil treated appropriately grown (AG_{SC}, white, n=5) and sildenafil treated growth restricted (FGR_{SC}, black, n=6) fetuses. Data were compared using a two-way ANOVA to determine difference between treatments. + signifies significant difference between groups ($p<0.05$). Multiple comparisons were performed via a Tukey's test.



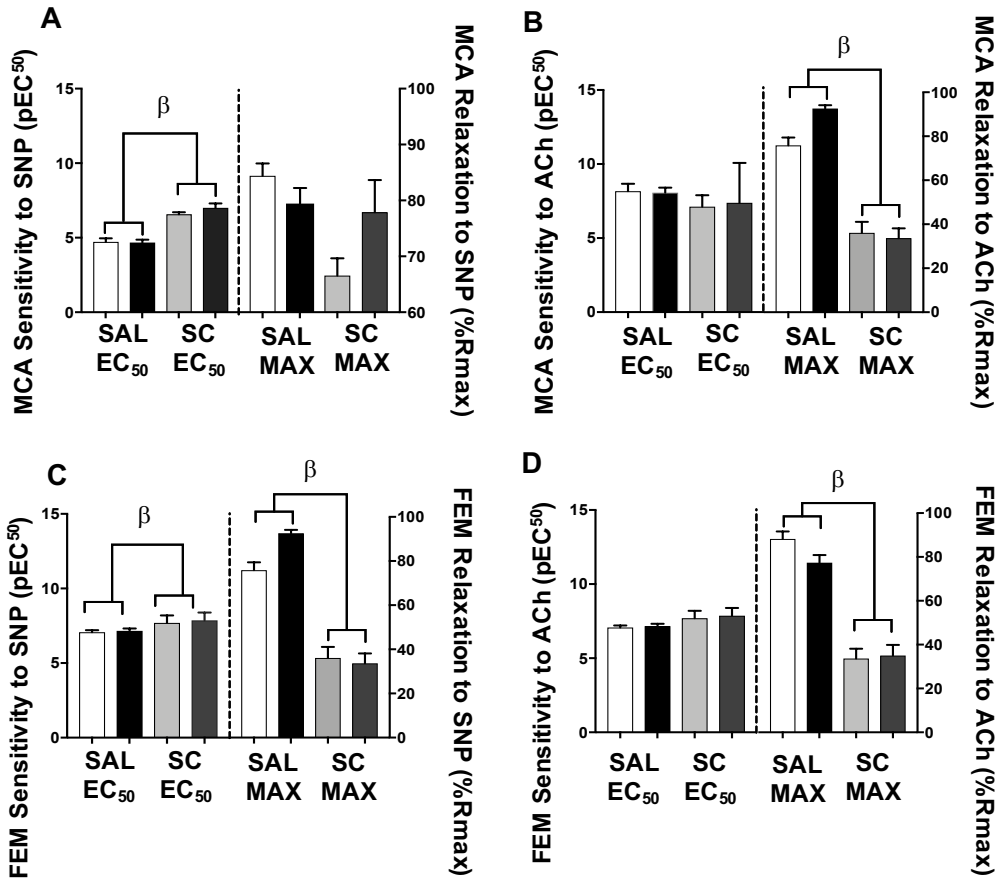
3.8.C. Figure 2. Middle Cerebral Artery (MCA) and Femoral Artery (FEM) response to sodium nitroprusside (SNP) and Acetylcholine (ACh)

Data are mean ± SEM vasodilation from maximal contraction (% max) of middle cerebral artery in response to SNP and ACh (A and B respectively) and femoral artery in response to SNP and ACh (C and D respectively) of appropriately grown (AG, white, n=7), growth restricted (FGR, black, n=6), sildenafil treated appropriately grown (AG_{SC}, grey, n=5) and sildenafil treated growth restricted (FGR_{SC}, dark grey, n=6). Data were compared using a two-way ANOVA to analyze myography results between treatment groups. # signify a significant difference in vasodilation between saline and SC treated groups and * signify a significant dose difference compared to 10^{-10} mol/L within group.



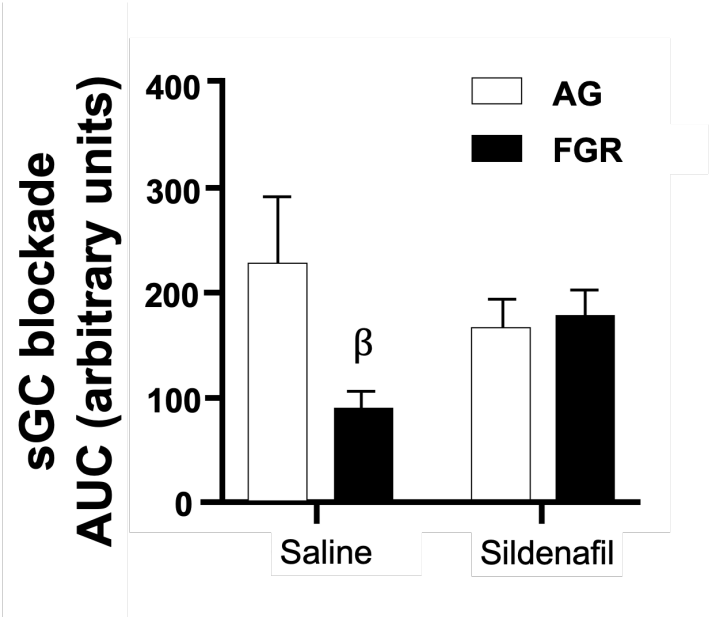
3.8.D. Figure 3. Area Under the Curve (AUC) of Middle Cerebral Artery (MCA) and Femoral Artery (FEM) response to sodium nitroprusside (SNP) and Acetylcholine (ACh)

Data are mean ± SD of area under the curve from middle cerebral artery in response to SNP and ACh (A and B respectively) and femoral artery in response to SNP and ACh (C and D respectively of appropriately grown (AG, white, n=7), growth restricted (FGR, black, n=6), sildenafil treated appropriately grown (AG_{SC}, grey, n=5) and sildenafil treated growth restricted (FGR_{SC}, dark grey, n=6). α signifies significant difference between AG and FGR and β signify significant difference between treatment groups ($p<0.05$). Data were compared using a two-way ANOVA.



3.8.E. Figure 4. Half Maximal Effective Concentration (pEC_{50}) and Total Maximal Dilation Percentage (%Rmax) of middle cerebral artery (MCA) and Femoral Artery (FEM) Response to Sodium Nitroprusside (SNP) and Acetylcholine (ACh)

Data are mean ± SEM vasodilation of half maximal effective concentration (pEC_{50}) and total maximal relaxation (%Rmax) of middle cerebral artery in response to SNP and ACh (A and B respectively) and femoral artery in response to SNP and ACh (C and D respectively) of appropriately grown (AG, white, n=7), growth restricted (FGR, black, n=6), sildenafil treated appropriately grown (AG_{SC}, grey, n=5) and sildenafil treated growth restricted (FGR_{SC}, dark grey, n=6). β signify significant difference between treatment groups ($p<0.05$) Data were compared using a two-way ANOVA.



3.8.F. Figure 5. sGC contribution to vasodilation of the middle cerebral artery (MCA).
Data are mean±SEM area under the curve (AUC) of vasodilation of the MCA in appropriately grown following SC (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), sildenafil citrate–treated AG (n=5) and sildenafil citrate–treated FGR (n=6) fetuses. Data were compared using a 2-way ANOVA to determine difference between treatments and growth status. β signify significant difference between treatment groups ($p<0.05$). Multiple comparisons were performed via a Tukey test.

Chapter 4

Maternal Sildenafil Impairs the Cardiovascular Adaptations to Chronic Hypoxia in Fetal Growth Restricted Fetal Sheep.

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This chapter has been submitted to the journal (The Journal of Physiology), is pending review comments and has been reformatted for consistency in this thesis

4.1 Abstract

Background: Fetal growth restriction (FGR) commonly occurs due to inadequate delivery of nutrients and oxygen to the fetus. In response, fetal cardiovascular adaptations redirect cardiac output to essential organs to maintain oxygen delivery and sustain fetal development. However, FGR infants remain at risk for cardiovascular and neurological sequelae. Sildenafil citrate (SC) has been examined as a clinical therapy for FGR, but also crosses the placenta and may exert direct effects on the fetus. We investigated the effects of maternal SC administration on maternal and fetal cardiovascular physiology in growth restricted fetal sheep.

Methods: Fetal sheep (0.7 gestation) underwent sterile surgery to induce growth restriction by single umbilical artery ligation (FGR) or sham surgery (control, AG). Fetal catheters and flow probes were implanted to measure carotid and femoral arterial blood flows. Ewes containing FGR fetuses were randomized to receive either maternal administration of saline or SC (36 mg i.v. per day) beginning 3 days after surgery, and continuing for 20 days. Physiological recordings were obtained throughout the study.

Results: Antenatal SC treatment reduced body weight by 32% and oxygenation by 18% in FGR compared to AG. SC did not alter maternal or fetal heart rate and blood pressure. Femoral blood flow and peripheral oxygen delivery were increased by 49% and 30% respectively in FGR_{SC} compared to FGR, indicative of impaired cardiovascular adaptation to chronic hypoxia.

Conclusions: Antenatal SC directly impairs the fetal haemodynamic response to chronic hypoxia. Consideration of the consequences upon the fetus should be paramount when administering interventions to the mother during pregnancy.

4.2 Introduction

Fetal growth restriction (FGR) complicates ~5-10% of all pregnancies[309] and is defined as the failure of a fetus to reach their genetic growth potential[308]. FGR increases the risk of stillbirth 20-fold and is a principal cause of perinatal death[3]. Infants born growth restricted have an increased risk of both short[8] and long term neurological and cardiovascular sequelae[219], compared to their appropriately grown (AG) counterparts[335]. FGR most commonly occurs secondary to placental insufficiency, resulting in reduced delivery of nutrients and oxygen to the fetus which compromises normal fetal growth and organ development[308].

Impaired oxygen transfer across the placenta causes chronic fetal hypoxia. The physiological response of the fetus to chronic hypoxia has been well documented, including cardiac output redistribution in an effort to maintain perfusion of key organs (brain, heart, adrenal glands) at the expense of organs such as the gut and periphery; this adaptation is termed 'brain sparing'[169]. Brain sparing is induced by detection of hypoxia within the fetal circulation by chemoreceptors which stimulate an increase in peripheral vasoconstriction[167], concurrent with vasodilation in the vital organs to maintain adequate oxygen delivery. This process is mediated by the release of adenosine, nitric oxide, and prostanoids[169]. However, while brain-sparing and subsequent growth restriction ensure fetal survival, it does not ensure normal postnatal development, with growth restricted newborns at increased risk of death as well as cardiovascular and neurological deficits compared to AG infants[9,19,335].

Placental insufficiency commonly occurs due to abnormally high placental vascular resistance, thus placental vasodilation is a key target for therapeutic intervention to increase placental blood flow. Sildenafil citrate (SC) inhibits phosphodiesterase (PDE) 5, causing vasodilation and increased blood flow[336]. The placenta is rich in PDE5 and there has been considerable interest in targeting this pathway to improve blood flow in pregnancies complicated by placental insufficiency [29,337,338]. Indeed, promising results from animal preclinical and case-control clinical studies have underpinned the initiation of the multinational, multicentre randomized placebo-controlled *Sildenafil TheRapy in Dismal prognosis Early-onset fetal growth Restriction* (STRIDER) trial[302]. This set of four trials within the STRIDER Consortium examined the effect of maternal SC (25mg oral tablets, three times daily) on fetal growth and gestational length[302] in pregnancies complicated by severe placental insufficiency and FGR. Results from the STRIDER trial have been mixed. The UK and New Zealand/Australian arms of the trial demonstrate no positive benefit of antenatal SC on birth weight and gestational length, but also no harm[262,339]. In contrast, the Netherlands consortium of the STRIDER trial was prematurely halted due to an interim analysis which found

evidence of potential harm. In this cohort, SC administration increased rates of persistent pulmonary hypertension and neonatal death (non-significant) in newborns[340]. Subsequently, the STRIDER Consortium recommended the cessation of SC treatment for women with placental insufficiency and growth restricted fetuses[267]. The cause of neonatal demise following antenatal SC is not yet characterized.

SC crosses both the human and sheep placenta[341–343] and PDE5 receptors are found in the developing fetus[344]. We have previously shown that SC administration to fetal vessels *ex-vivo* can alter vascular tone[20,343], therefore, antenatal SC treatment may have direct effects on fetal blood vessels [254]. We propose that it is important to consider the hemodynamic effects of SC in the developing fetus given its strong vasodilator actions[341]. Accordingly, in this study, we investigated the effects of maternal SC administration on maternal and fetal cardiovascular physiology in growth restricted fetal sheep. We hypothesized that antenatal SC administration would induce peripheral vasodilation in the developing FGR fetus, altering the cardiovascular adaption to chronic hypoxia.

4.3. Materials and Methods

Experiments were approved by the Monash Medical Centre Animal Ethics Committee (A) (MMCA2016/01) under guidelines established by the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes (8th Edition, 2013).

4.3.A. Animals

Singleton bearing Border-Leicester pregnant ewes (n=20) underwent sterile surgery on day 105 of pregnancy (term 148-150 days gestation, d GA) to induce FGR via single umbilical artery ligation (SUAL), as previously described[312]. Briefly, anesthetized ewes were randomly allocated for SUAL to induce FGR or sham surgery where the umbilical cord of control fetuses was exposed and handled but not ligated (control, AG). SUAL results in placental atrophy, and subsequent disruption of placental function with chronic hypoxia, and brain-sparing[312].

All fetuses were instrumented with right femoral artery and jugular vein catheters (1.52 mm outer diameter [OD], 0.86 mm inner diameter [ID]) for blood gas sampling and pressure recording, and administration of antibiotics, respectively. Flow probes (Size 3, Transonic Systems, Ithaca, NY) were placed around the left femoral and right carotid arteries for measurement of arterial blood flow. An amniotic catheter (2.70 mm [OD], 1.50 mm [ID]) was also secured to the hindquarters

of the fetus for access to the amniotic cavity for antibiotic administration and to correct fetal pressure recordings. The fetus was returned to the uterus and catheters and flow probes were exteriorized through the right flank of the ewe.

Catheters were inserted into the maternal jugular vein for administration of antibiotics and SC treatment, and the maternal carotid artery for blood sampling and pressure recording (2.70 mm OD, 1.50 mm ID). Fetal, maternal and amniotic catheters were filled with heparinised saline (0.9% NaCl; 25,0000 IU heparin/L).

4.3.B. Experimental procedures

Ewes were placed into mobile cages and, following recovery, were fed twice daily with lucerne chaff and water was available ad libitum. Antibiotics were administered to the ewe (Engemycin 5mL i.v.; Coopers, Macquarie, NSW, Australia) via the maternal jugular vein catheter and the fetus (Ampicillin 1ml:1g/5ml heparinised saline; Austrapenics; CSL, Victoria, Australia) via the jugular vein and amniotic catheter (Ampicillin 4ml:1g/5ml heparinised saline) for 3 days after surgery.

4.3.C. Recording of Maternal Physiology and Fetal In-Utero Physiology.

On day 4 post-surgery, prior to initiation of SC treatment, fetal and maternal mean arterial pressure (DTX Plus Transducer; Becton Dickinson, Singapore) as well as fetal femoral and carotid blood flows (Transonic Systems, Ithaca, NY) were monitored and recorded (AD Instruments, Sydney, Australia) for 2 hours. Fetal and maternal heart rate was derived from the arterial signal. Physiological recordings were conducted from ~9 am to 12 pm from days 4-9 post-surgery and thereafter on alternate days until the fetus was 125 d GA. Ewes had constant access to food and water during the recording period.

4.3.D. Maternal Sildenafil/Saline Administration

On day 4 post-surgery, ewes with a FGR fetus were randomized to receive either sildenafil citrate (SC: 36 mg/day) or saline infusion via a pump (CADD-Legacy 1 Pump; Smiths Medical, Australia) connected to the maternal jugular vein catheter and fastened to the back of the ewe under netting. Thereafter, the groups of interest were; FGR with SC treatment (FGR_{SC}, n=7), FGR with saline (FGR, n=7) and appropriately grown with saline (AG, n=6). SC and saline administration occurred continuously for 20(+/-)1 days until post-mortem (125 d GA). This treatment regime was chosen to reflect the dose of SC in the STRIDER trial[302] where the oral dose has a 50% bioavailability[321].

4.3.E. Fetal Blood Samples

Fetal arterial blood samples (~200 μl) were collected at the beginning of each recording day for the assessment of fetal pH, haematocrit (Hct), oxygen saturation (SaO_2), the partial pressure of arterial oxygen, (PaO_2), the partial pressure of arterial carbon dioxide ($PaCO_2$), lactate and glucose.

4.3.F. Post-Mortem

At 125 d GA, ewes and fetuses from all groups were euthanized via pentobarbital sodium overdose to the ewe (100 mg/kg i.v. Valabarb; Jurox, Rutherford, Australia). Fetuses were exteriorized for measurement of brain, lung and fetal body weight and biparietal diameter, abdominal circumference, crown rump and lower limb length.

4.3.G. Data Analysis

Data are presented as mean ± SEM unless otherwise stated. Normality of data was assessed and normalized as required via GraphPad Prism (Prism 7, GraphPad Software, USA). Data analysis was performed using SigmaStat (Systat Software Inc, San Jose, CA, USA). Mean data were analysed in two epochs; weeks 1 (Week 1) and 2 (Week 2) post-SC administration. When no difference was seen between treatment weeks, data is presented as total time post-SC administration. Fetal weight, biometry and mean physiological parameters were analysed using a one-way analysis of variance (ANOVA). Blood gas parameters and mean daily physiological data were analysed using a two-way repeated measures ANOVA, and Tukey's post-hoc test was used to compare between groups. Significance was accepted at $p \leq 0.05$.

4.4. Results

4.4.A. Fetal Characteristics

Table 1 summarises the fetal characteristics at post-mortem. There was no difference in the numbers of males and females per group. At 125d GA, body weight in the FGR group was reduced by 11% compared to AG (not significant). Treatment with SC exacerbated growth restriction resulting in a 32% reduction in birthweight compared to the AG group ($p=0.003$) and by 23% compared to FGR ($p=0.01$). Brain:body weight ratio was not different between the FGR and AG groups, but was significantly increased in FGR_{SC} compared to both AG ($p=0.007$) and FGR ($p=0.02$). Lung:body weight ratio was not different between groups. Crown rump length was significantly decreased in FGR_{SC} compared to AG ($p= 0.04$) but was not different to FGR. No significant differences were seen in any other measure of fetal biometry (biparietal diameter, crown rump length, abdominal circumference, lower limb length).

4.4.B.Fetal pH, Partial Pressure of Carbon Dioxide and Arterial Saturation

The fetal pH, the partial pressure of CO_2 and arterial saturation over the ~2.5-week duration of the experiment are shown in Figure 1. pH (Figure 1A, B) was not different between groups throughout the experimental period. Mean $PaCO_2$ over the two-week treatment period was significantly greater in FGR compared to AG and FGR_{SC} (Figure 1D; p=0.001 and 0.01 respectively). Twenty-four hours post-SUAL (106 d GA), SaO_2 was significantly lower in FGR and FGR_{SC} fetuses (Figure 1E; p=0.004 and p=0.01) compared to AG fetuses. FGR and FGR_{SC} fetuses remained hypoxic with significantly lower arterial saturations compared to AG at 112 (Figure 1E; p=0.006 and p=0.02 respectively) and 117 d GA (Figure 1E; p=0.007 and p=0.04 respectively). Mean SaO_2 over the two-week treatment period was significantly lower in FGR and FGR_{SC} fetuses compared to AG fetuses (Figure 1F; p=0.001 and 0.01 respectively).

4.4.C. Fetal Arterial Partial Pressure of Oxygen

The fetal arterial partial pressure of O_2 (PaO_2) over the ~2.5 weeks of the experiment is shown in Figure 2. Twenty-four hours post-SUAL (106 d GA), PaO_2 was significantly decreased in FGR and FGR_{SC} fetuses (Figure 2A: p=0.05 and 0.02) compared to AG fetuses. PaO_2 recovered to control values in both FGR groups by the time SC treatment began. At 123 d GA, PaO_2 was significantly lower in FGR_{SC} (Figure 2A, p=0.005) compared to AG and FGR fetuses. Mean PaO_2 after one week of saline/SC treatment was significantly lower in both FGR and FGR_{SC} compared to AG (Figure 2B; p=0.001) and no difference in PaO_2 was seen between FGR and FGR_{SC} fetuses. After two weeks of saline/SC treatment, PaO_2 remained significantly lower in FGR and FGR_{SC} compared to AG (Figure 2B; p=0. 01), however at this time PaO_2 was significantly lower in FGR_{SC} compared to FGR (Figure 2B; p=0. 04)

4.4.D. Fetal Glucose and Lactate

FGR and FGR_{SC} fetuses were hypoglycaemic compared to AG fetuses at 107 and 108 d GA (Figure 3A; $p<0.05$). FGR_{SC} were hypoglycaemic at 112 and 121 d GA (Figure 3A; $p<0.05$) compared to AG, but not FGR fetuses. Mean fetal glucose over the two-week treatment period was not different between FGR and FGR_{SC} fetuses, but both were significantly lower than AG fetuses (Figure 3B; $p<0.0001$). Lactate was significantly higher in FGR_{SC}, compared to AG fetuses, at 119 d GA (Figure 3C; p=0.02) and no differences were seen between FGR and AG at individual timepoints. Mean lactate over the two-week treatment period was significantly higher in FGR_{SC} compared to AG fetuses during SC treatment period (Figure 3D, p=0.007) and no difference was seen between AG and FGR.

4.4.E. Maternal Blood Pressure and Heart Rate Response to Sildenafil Treatment

In all groups, maternal blood pressure decreased throughout the experiment (p=0.03; Figure 4A). Mean maternal blood pressure over the two-week treatment period was significantly reduced in ewes carrying FGR compared to AG fetuses (Figure 4B, p=0.03) but no difference was seen compared to FGR_{SC}. Mean maternal diastolic blood pressure over the two-week treatment period was significantly reduced in FGR and FGR_{SC} compared to AG (Figure 4F, p=0.002 and p=0.04 respectively); no difference was seen between FGR and $FGR_{SC.}$ Maternal systolic blood pressure (Figure 4E/F) and maternal heart rate were not different between groups at any time during the study (Figure 4G/H).

4.4.F. Fetal Blood Pressure and Heart Rate Response to Sildenafil Treatment

Mean fetal arterial (Figure 5B) and systolic (Figure 5D) blood pressure over the two-week treatment period was significantly greater in FGR fetuses compared to AG and FGR_{SC} (Figure 5B/D, p<0.05) but no difference was seen between FGR_{SC} and AG. Mean fetal diastolic blood pressure (Figure 5F) over the two-week treatment period was not different between groups. Mean fetal heart rate was significantly lower in FGR_{SC} fetuses compared to AG and FGR (Figure 5H, p<0.05) during SC treatment.

4.4.G. Carotid and Femoral Blood Flow and Oxygen Delivery

Mean fetal carotid blood flow over the two-week treatment period was significantly greater in AG fetuses compared to FGR (Figure 6B, p<0.04), but no difference was seen compared to FGR_{SC}. Mean fetal femoral blood flow over the two-week treatment period was significantly lower in FGR fetuses compared to AG and FGR_{SC} (Figure 6D, p=0.002 and p=<0.001 respectively) but no difference was seen between AG and FGR_{SC} fetuses. Carotid oxygen delivery at 106 d GA was significantly greater in AG fetuses compared to FGR_{SC} before SC treatment begun, however was not different thereafter (Figure 6E, p=0.01). Mean carotid oxygen delivery over the two-week treatment period was significantly lower in FGR and FGR_{SC} fetuses compared to AG fetuses (Figure 6F; p=0.04) but not between FGR and FGR_{SC} fetuses. Mean fetal femoral oxygen delivery over the two-week treatment period was significantly lower in FGR compared to AG fetuses (Figure 6H; p=0.008) but no difference was seen between AG and FGR_{SC}.

4.5. Discussion

FGR is most commonly caused by impaired placental function resulting in a hypoxic fetal environment. Currently there is no cure or treatment options available for women with suspected FGR. Potential treatments to improve placental function, such as SC[302], are an important area of

investigation. This study aimed to investigate the effect of maternal SC administration on fetal and maternal cardiovascular physiology in ovine pregnancies complicated by FGR. FGR resulted in reduced femoral blood flow and oxygen delivery consistent with brain-sparing. Our key finding was that antenatal treatment with SC altered fetal cardiovascular redistribution, coupled with increased fetal distress and hypoxia. SC exposure resulted in widespread fetal vasodilation which counteracts the normal fetal cardiovascular adaptation (brain sparing response) in the setting of chronic hypoxia.

4.5.A. Impact of SC on Fetal Oxygenation and Growth

An important finding was the effect of SC on fetal oxygenation and growth. SUAL induces FGR via placental insufficiency and thereby creating a chronic fetal hypoxic environment[312] which was observed in both FGR groups in this study. Zhang et al have discussed the potential for the placental vascular bed to mount a vasodilative compensatory response to placental insufficiency in an attempt to modulate the degree of FGR[345] and Kitanaka et al have shown uterine artery blood flow increases gradually in pregnant sheep exposed to chronic hypoxia[346]. These studies suggest in adverse pregnancy, the placental vasculature has some vasodilative ability to increase blood flow and oxygen carrying capacity for the fetus. Accordingly, in the current study we observe a return to normoxia within 4 days of SUAL in saline treated FGR fetuses.

In contrast, SC treated FGR fetuses became progressively more hypoxic which likely resulted in the reduced growth observed. The increased hypoxia in FGR_{SC} fetuses suggests impairment, or failure, to potential placental adaptation to hypoxia as described above. We have previously discussed the potential SC to decrease maternal systemic resistance, resulting in a subsequent 'steal' of blood from the uteroplacental circulation as it flows to a vascular unit with lower resistance[28,343]. It is reasonable to assume the increased fetal hypoxia observed in our current study may have also been driven by similar alterations in maternal and/or placental blood flow by SC. Given we also observe effects of SC on the fetal circulation, we suggest that this too may contribute to the increased fetal distress observed.

The normalisation of peripheral blood flow in FGR lambs observed in our current study may be an alternative mechanism behind the exacerbation of fetal hypoxia observed. We measured femoral blood flow as an indication of peripheral circulation in our study. As SC crosses the placenta and enters the fetal circulation[343], the vasodilative effects of SC are likely global within the fetus. Thereby in the face of fetal hypoxia, global vasodilation by SC may enable perfusion of all organs, rather than preferential distribution to the brain. This would result in increased oxygen

consumption by less-important organs at the expense of critical organs such as the heart and brain. We suggest that this may have contributed to the increased hypoxia and the subsequent further impairment of growth in FGR fetuses exposed to SC.

Lactate is a marker of fetal distress and tissue hypoxia[347,348]. The increased lactate in FGR_{SC} compared to FGR and AG fetuses we observed is likely to reflect increased fetal stress caused by increased hypoxia and nutrient deprivation resulting in anaerobic glycolysis in FGR_{SC} fetuses[300]. Lactate is the main substrate for brain development[349] and Mann et al have shown an increased lactate uptake by the brain following lactate infusion into the fetal circulation[350]. If lactate can act as an energy source for the brain, the increased lactate seen in the growth restricted fetuses exposed to SC may have contributed to the increased fetal brain weight relative to the FGR and AG lambs. As increased brain sparing is associated with worsening placental function[180,188]and adverse fetal outcomes[351], we do not believe that the increased brain weight observed is associated with improved brain development. However, analysis of the brain, which is beyond the scope of this study, will be useful to determine the effects of SC on the developing brain.

4.5.B. Sildenafil Citrate Impairs Cardiovascular Adaptations to FGR

In FGR fetuses, mean arterial blood pressure increased in response to SUAL, driven by vasoconstriction of the periphery, evidenced by a widening pulse pressure (increased systolic BP) also shown in our study. This fetal cardiovascular response to chronic hypoxia is mediated by fetal endocrine and metabolic adaptations and are aimed at maintaining maximal organ perfusion[169,352]. Peripheral vasoconstriction results in a redistribution of blood flow away from the periphery, evidenced by a significant reduction in femoral blood flow and oxygen delivery (calculated from oxygen concentration and arterial blood flow) in FGR, whereas cerebral oxygen delivery was maintained. These observations describe key mechanisms employed by the chronically hypoxic fetus to facilitate the redistribution of cardiac output from the periphery to the cerebral circulation[17] and show that these adaptations are present in our model. However, SC administration normalised mean and systolic fetal blood pressure, and in concert with restoration of femoral blood flow in FGR fetuses, demonstrates interference by SC to this well-described cardiovascular adaptation to hypoxia. Further, peripheral oxygen delivery was also normalised after SC. SC induces vasodilation via inhibition of enzyme PDE5, normally responsible for the degradation of cGMP which enables smooth-muscle vasoconstriction. We have previously shown that femoral arteries of fetal sheep vasodilate in response to SC ex-vivo, which demonstrates the presence of PDE5 within this vascular bed[20,261]. We suggest that SC counteracts the vasoconstrictive adaptation in FGR fetuses by inducing peripheral vasodilation, resulting in the

normalization of perfusion (BP, Resistance) and restoration of femoral oxygen delivery and blood flow. The consequences of FGR-driven cardiovascular adaptation are complex. While redistribution of fetal cardiac output, and subsequent brain-sparing, is a well-accepted adaptation to preserve perfusion of key organs and maintain fetal life[16], an increased degree of brain-sparing is also strongly associated with poorer neonatal outcome[335]. We are unable to determine if the normalization of femoral blood flow observed in our study is detrimental or beneficial to postnatal life. However, in the context of SC, we suggest that benefit is unlikely given the negative effects observed in this study including exacerbation of growth restriction, hypoxia and fetal stress evident by higher lactate levels.

Interestingly, despite the potential for widespread vasodilation, SC treatment did not increase carotid blood flow or oxygen delivery. As adaptation to hypoxia aims to maximally perfuse key organs such as the brain, it is possible that, following cardiovascular adaptation to FGR, the cerebral circulation is maximally dilated to facilitate maximal oxygen delivery to the brain. The cerebral vasculature may therefore have limited ability to further vasodilate in response to a vasodilator such as SC.

4.5.C. Effects of SC on Maternal Heart Rate and Blood Pressure.

Maternal arterial and diastolic blood pressure decreased following SUAL. Whilst fetal growth restriction is not traditionally associated with decreased blood pressure, Steer and colleagues found an association between maternal hypotension and infants that were small for gestational age in their population consisting of >500,000 birth records[353]. As we did not observe a difference between FGR and FGR_{SC} bearing ewes, we suggest that decreased maternal blood pressure in FGR bearing ewes reflects a maternal vascular response to the induction of SUAL. Up-regulation of placental NO occurs as a compensatory mechanism in FGR[354]. Therefore, a potential cause behind the decreased maternal blood pressure in FGR bearing ewes may be increased release of placental vasoactive factors entering the maternal circulation. Action of NO may be impaired by circulating SC and may underlie the absence of hypotension in SC treated ewes.

4.6. Limitations

In this study we aimed to mimic the human dose of SC administration used in the recent STRIDER trials. To provide a controlled administration of SC to the pregnant ewe, SC was given as a continuous i.v. infusion to the ewe. In consideration of the different dosing routes, we decreased the dosage of SC to match the bioactivity of the drug when taken orally (i.e. 50% bioactive via oral route). We acknowledge that the pharmacokinetic profile of SC is likely to be

different between our study and the women in STRIDER, in which oral administration would result in cyclic[355] levels of SC, as opposed to our constant SC levels.

Statistically, saline treated FGR fetuses were not growth restricted compared to AG. However, FGR fetuses did have a reduction in weight of ~400g, which represents a reduction of 11.1% in body weight. This is similar to previously reported FGR weights by our group[356]. This difference in body weight likely represents a decrease in growth velocity, which is associated with an increased risk of postnatal complications and stillbirth[357]. Interestingly, FGR_{SC} fetuses had a reduction of 32% in body weight, which highlights the exacerbation of FGR by maternal SC treatment.

The rationale for the clinical use of SC was undertaken with the expectation that SC would improve placental blood flow and thereby, fetal growth[313]. Although we observed a decreased growth and increased hypoxia observed in FGR_{SC} fetuses, it is important to note there are key differences between our model and the human pathology underlying fetal growth restriction. Further, there are key difference between the aetiology of clinical placental insufficiency and our SUAL model. In contrast to abnormal placental vasodilation causing a decrease in placental perfusion, SUAL induces placental atrophy to mimic placental insufficiency[301]. Increase in umbilical flow may be limited in the SUAL model. Notwithstanding, the ability of SC to improve placental blood flow is contentious, with some studies demonstrating benefit[27,338] and others demonstrating impairment[28,343] of placental function.

4.7. Conclusions.

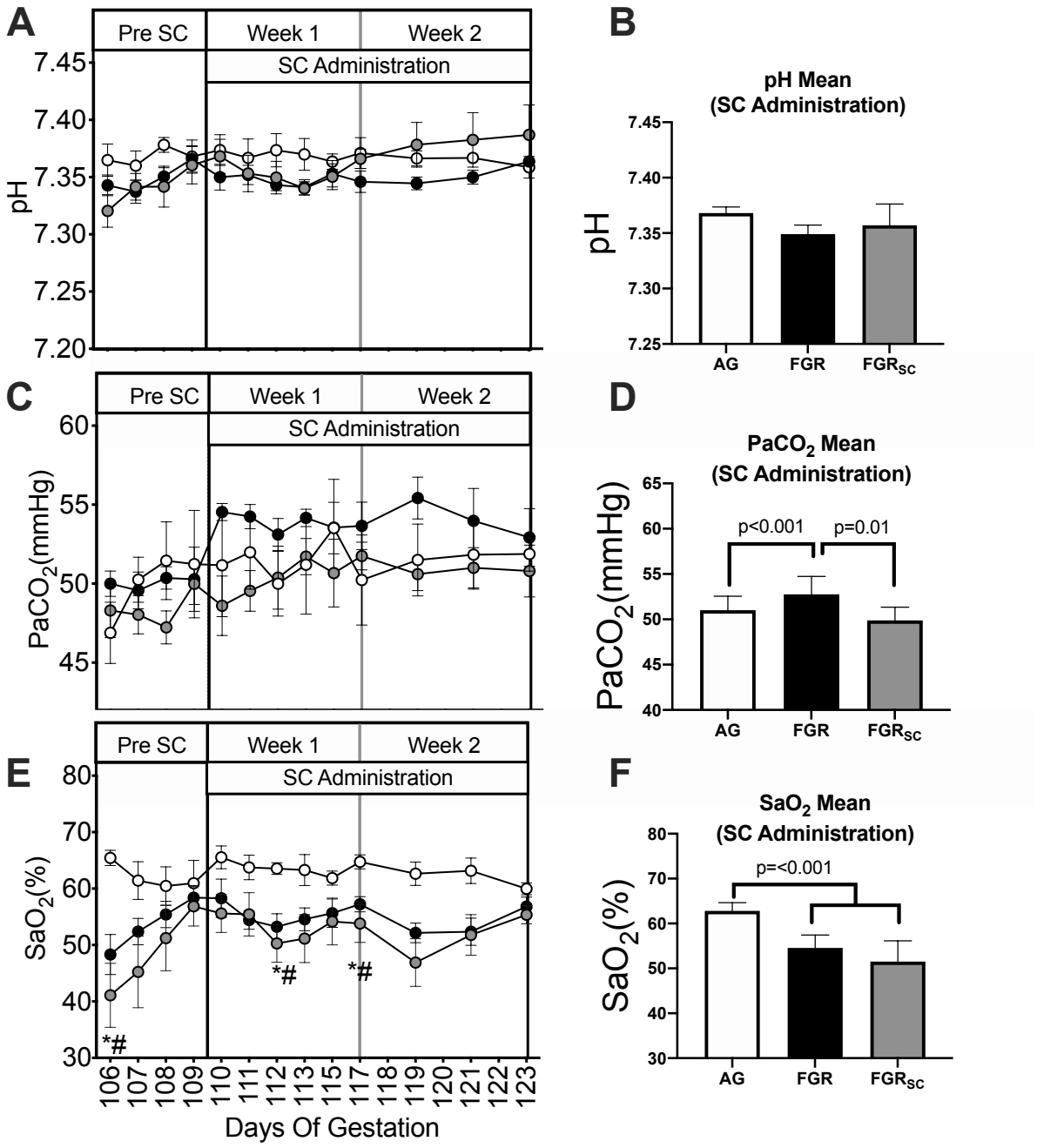
We demonstrated that chronic administration of SC to FGR fetal sheep altered the cardiovascular adaptation to chronic hypoxia. These adaptations aim to assist with in-utero survival, and inhibition of this adaptive mechanism by SC resulted in increased fetal hypoxia and exacerbation of growth restriction. This is the first study to demonstrate the decentralization of blood flow in the developing, hypoxic fetus by sildenafil citrate. Whilst the developmental consequences of altered adaptation to FGR are unknown, the increased hypoxia, distress and reduced fetal growth suggest outcomes are likely to be worsened.

Importantly, our study highlights the potential of therapies administered to pregnant women to have significant implications upon the developing fetus. We believe SC is not an appropriate therapy for FGR and warn against its use due to evidence that chronic SC administration can interfere with key survival mechanisms, such as the conservation of oxygen delivery to critical organs during chronic hypoxia.

4.8. Tables and Figures

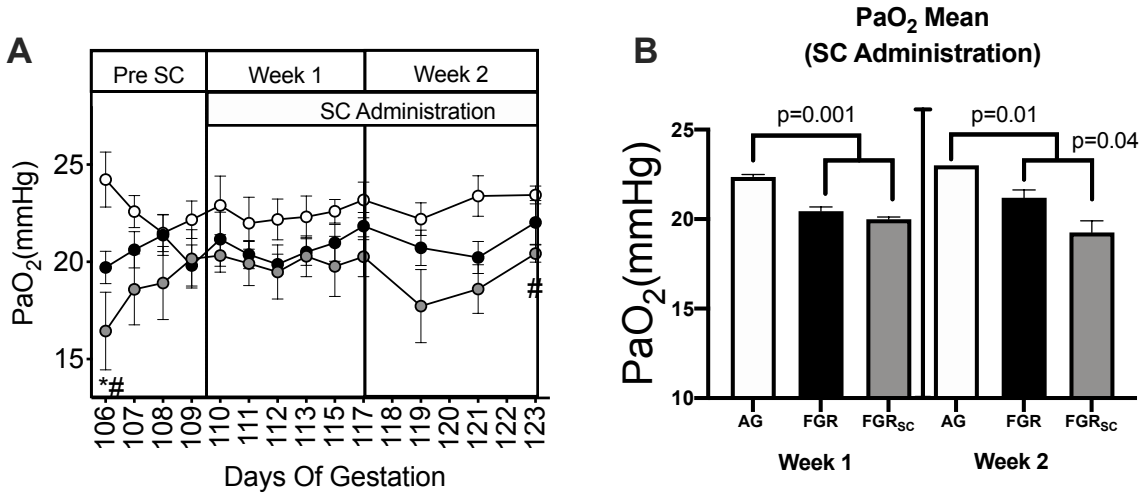
	AG	FGR	FGR_{SC}
Male/ Total Births	5/6	3/7	4/7
Birth Weight	3.4 ± 0.3	3.0 ± 0.3	2.3± 0.5*#
Brain/Body Weight	14.2 ± 0.8	16.0 ± 1.2	19.7 ± 3.2*#
Lung/Body Weight	31.8 ± 4.1	32.1 ± 5.1	38.1 ± 4.9
Biparietal Diameter	11.1 ± 1.3	10.1 ± 0.3	10.3 ± 0.9
Crown rump length	43.6 ± 2.9	41.1 ± 2.3	37.5 ± 4.3*
Abdominal Circumference	33.3 ± 1.9	32.5 ± 1.1	28.8 ± 4.8
Lower Limb Length	17.4 ± 3.1	14.8 ± 3.5	13.1 ± 2.9

4.8.A. Table 1. Characteristics of fetal sheep at post-mortem delivery.
Weights and biometry are mean ± SD of appropriately grown lambs (AG), fetal growth restricted lambs treated with saline (FGR) and fetal growth restricted lambs treated with sildenafil (FGR_{SC}). * indicates significant difference to AG and #indicates significant difference to FGR (p=0.05).



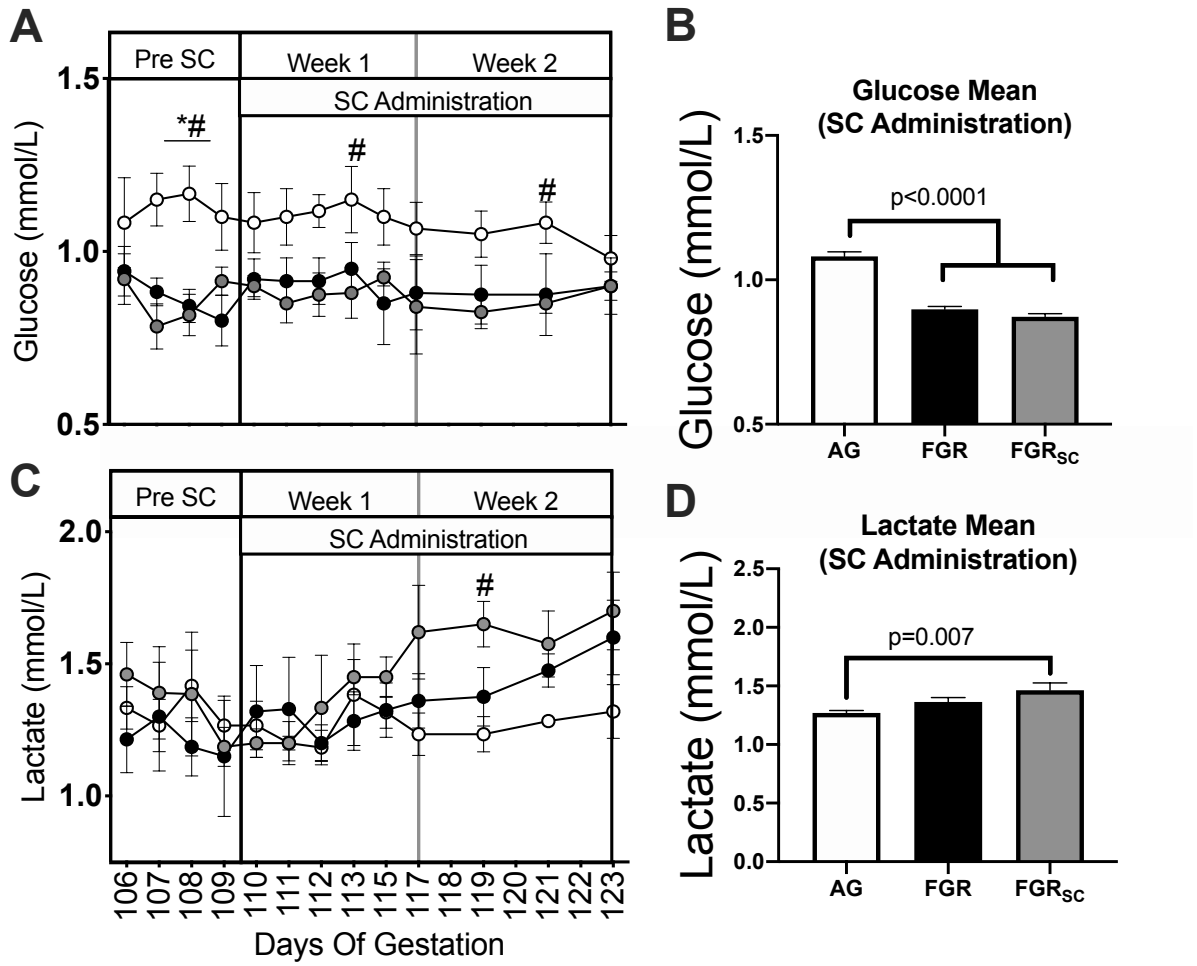
4.8.B. Figure 1. Fetal ph, Partial Pressure of CO_2 and Arterial Saturation

Data are daily (A, C, E) pH (A), partial pressure of arterial oxygen (PaO_2, C) and arterial oxygen saturation (SaO_2, C). Mean values for the duration of the treatment period of all parameters are also shown (right B, D, F). Groups are appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. * signifies significant difference between FGR and AG and # signifies significant difference between FGR_{SC} and AG. Data are mean ± SEM.



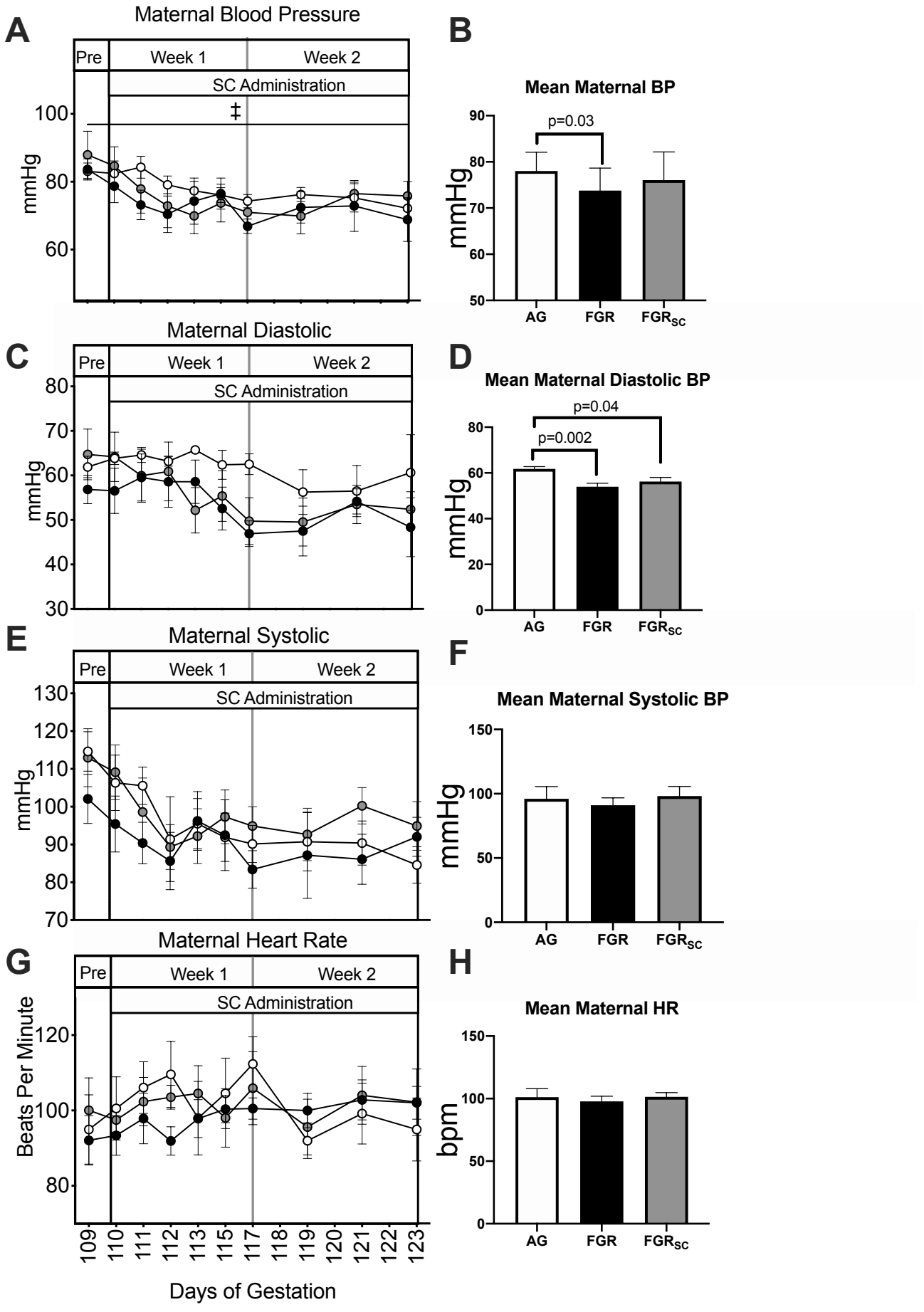
4.8.C. Figure 2. Fetal Partial Pressure of Oxygen.

The partial pressure of oxygen taken at individual time points (PaO_2) (A) and weekly means during the first and second week of SC treatment (B). Groups are appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. * signifies significant difference between FGR and AG and # signifies significant difference between FGR_{SC} and AG. Data are daily mean ± SEM.



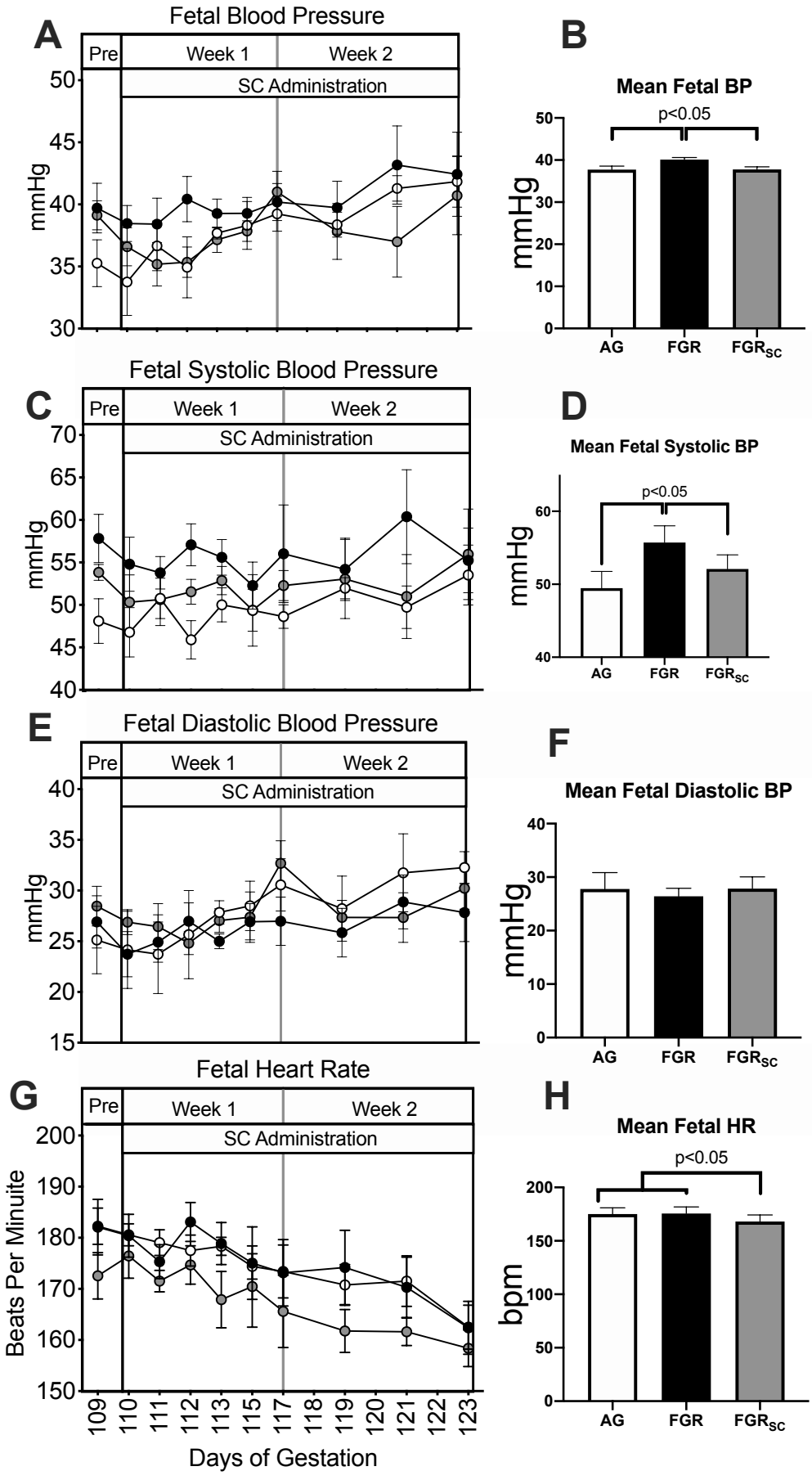
4.8.D. Figure 2. Fetal Glucose and Lactate.

Individual daily means of glucose (A) and lactate (C). Total means throughout the treatment period glucose (B) and lactate (D) are also shown. Groups are appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. * signifies significant difference between FGR and AG and # signifies significant difference between FGR_{SC} and AG. Data are mean ± SEM.


A
Maternal Blood Pressure
Pre
Week 1
Week 2
SC Administration
‡
mmHg
100
80
60
B
Mean Maternal BP
p=0.03
mmHg
90
80
70
60
50
AG
FGR
FGRSC
C
Maternal Diastolic
Pre
Week 1
Week 2
SC Administration
mmHg
80
70
60
50
40
30
D
Mean Maternal Diastolic BP
p=0.04
p=0.002
mmHg
80
60
40
20
0
AG
FGR
FGRSC
E
Maternal Systolic
Pre
Week 1
Week 2
SC Administration
mmHg
130
120
110
100
90
80
70
F
Mean Maternal Systolic BP
mmHg
150
100
50
0
AG
FGR
FGRSC
G
Maternal Heart Rate
Pre
Week 1
Week 2
SC Administration
Beats Per Minute
120
100
80
109
110
111
112
113
115
117
118
119
120
121
122
123
Days of Gestation
H
Mean Maternal HR
bpm
150
100
50
0
AG
FGR
FGRSC

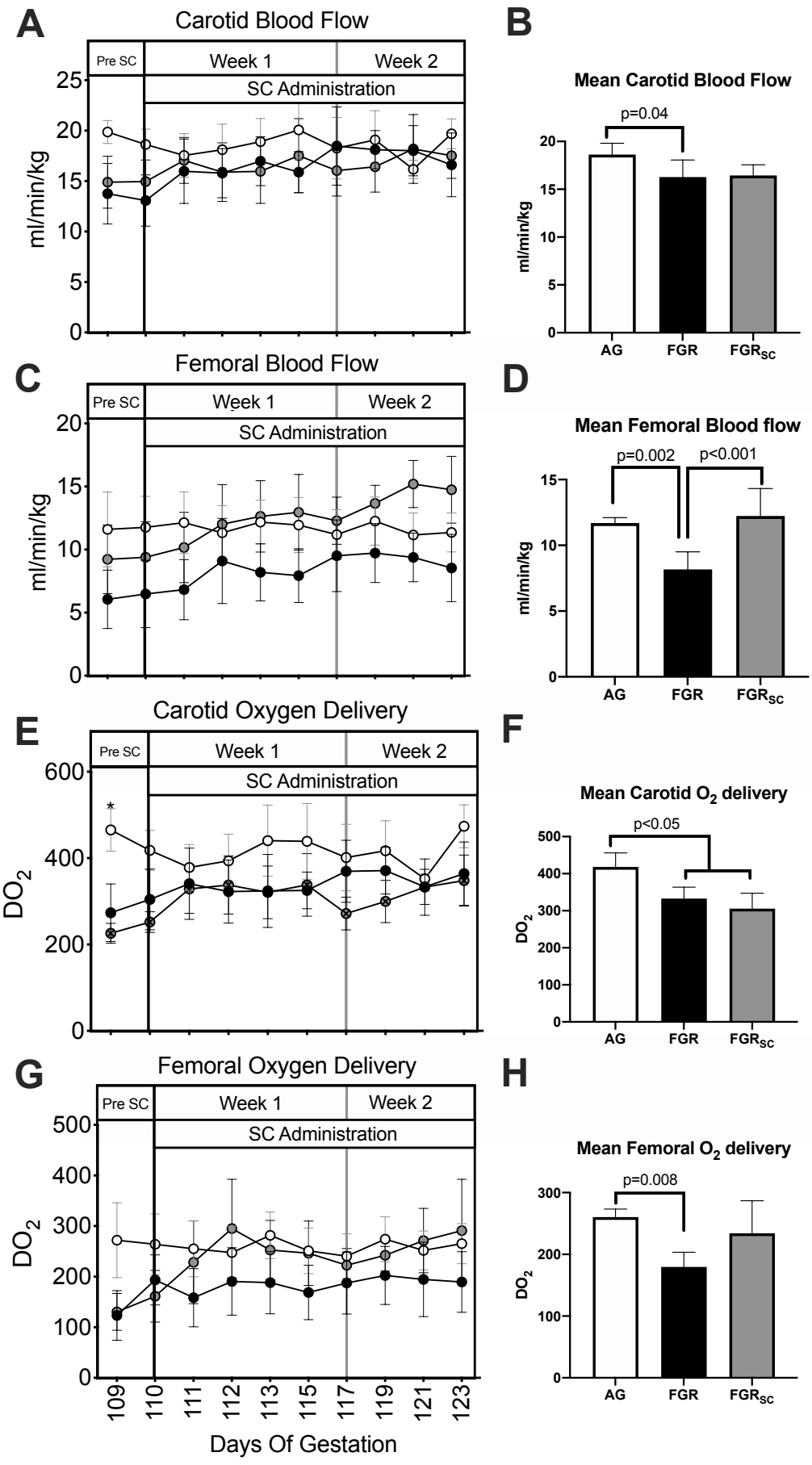
4.8.E. Figure 4. Maternal Cardiovascular Effects of Antenatal SC administration

Individual daily mean maternal blood pressure (BP, A), diastolic blood pressure (C), systolic blood pressure (E) and heat rate (G). Total mean BPs and Heart Rate throughout the treatment period are also shown (right B, D, F, H). Groups are appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. ‡ indicates significant difference over time. Data are mean ± SEM.


A
Fetal Blood Pressure
Pre
Week 1
Week 2
SC Administration
mmHg
B
Mean Fetal BP
p<0.05
mmHg
AG
FGR
FGRSC
C
Fetal Systolic Blood Pressure
D
Mean Fetal Systolic BP
E
Fetal Diastolic Blood Pressure
F
Mean Fetal Diastolic BP
G
Fetal Heart Rate
Beats Per Minuite
Days of Gestation
H
Mean Fetal HR
bpm

4.8.G. Figure 5. Fetal Cardiovascular Responses to Antenatal SC administration

Daily mean (A,C,E,G) blood pressure (BP) (A), diastolic pressure (C), systolic pressure (E) and heat rate (G). Total means throughout the treatment period of all parameters are also shown (right B, D, F, H). Groups are of appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. Data are mean ± SEM.


A
Carotid Blood Flow
Pre SC
Week 1
Week 2
SC Administration
ml/min/kg
B
Mean Carotid Blood Flow
p=0.04
AG
FGR
FGRSC
C
Femoral Blood Flow
D
Mean Femoral Blood flow
p=0.002
p<0.001
E
Carotid Oxygen Delivery
DO2
F
Mean Carotid O2 delivery
p<0.05
G
Femoral Oxygen Delivery
H
Mean Femoral O2 delivery
p=0.008
109
110
111
112
113
115
117
119
121
123
Days Of Gestation

4.8.H. Figure 6. Fetal Carotid and Femoral Blood Flow and Oxygen Delivery

Mean daily (A,C,E,G) carotid blood flow (A), femoral blood flow (C,D), carotid oxygen delivery (E) and femoral oxygen delivery (G). Total weekly means of the treatment period of all parameters are also shown (right B, D, F, H). Groups are appropriately grown (AG, white, n=6), saline treated fetal growth restricted (FGR, black, n=7) and sildenafil treated fetal growth restricted (FGR_{SC}, grey, n=7) fetuses. Duration of treatment is indicated by SC administration bar. * signifies significant difference between FGR and AG. Data are mean ± SEM.

Chapter 5

A comparison of the Cardiovascular effects of Dopamine and Sildenafil between Growth Restricted and Appropriately Grown Preterm Lambs

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This chapter has been not been submitted and is written as a traditional thesis chapter

5.1. Abstract

Introduction:

Fetal growth restriction (FGR) increases the risk of cardiovascular (CV) complications in early life, secondary to prolonged CV adaptations following chronic placental insufficiency and fetal hypoxia in utero. Growth-restricted infants require greater postnatal support compared to appropriately grown (AG) counterparts[335]. Dopamine (DOP), a β-agonist, is commonly used to treat hypotension in preterm infants. However, its efficacy has been challenged in FGR neonates[295]. We have previously demonstrated that targeting the NO pathway may have greater therapeutic potential in FGR neonates. We aimed to investigate the therapeutic efficacy for hypotension of NO pathway modulator sildenafil (SC) compared to dopamine. We hypothesized that SC would provide greater hypotensive support, compared to dopamine.

Methods:

Preterm lambs (0.6GA) underwent sterile surgery for single umbilical artery ligation (SUAL) to induce FGR or sham (AG). Fetuses were exposed via caesarean section at 0.8GA to implant monitoring equipment. Lambs were delivered and ventilated for 4h. 1h post-delivery, lambs received i.v. SC (FGR_{SC}, n=6/AG_{SC}, n=8), dopamine (FGR_{DOP}, n=6/AG_{DOP}, n=7) or saline (FGR, n=6/AG, n=7). Blood pressure (BP), heart rate (HR) and regional blood flows were continuously monitored. Data were compared using a mixed-effects analysis and multiple comparisons were performed via a Tukey's test to determine the difference between treatments.

Results:

SUAL resulted in asymmetrically growth restriction (18% smaller than AG lambs, p=0.04). SC administration significant, but transiently, reduced BP in FGR_{SC} but not AG_{SC} lambs. Dopamine treatment led to a progressive and significant decrease in BP and HR in FGR_{DOP} but not AG_{DOP} lambs. Plasma troponin significantly increased in FGR_{DOP} compared to all other groups.

Conclusions:

Preterm FGR lambs had a distinct CV response to both SC and dopamine, compared to AG lambs, highlighting key differences in the sensitivity to therapies following placental insufficiency. Our findings suggest that neonatal hypotensive support require re-evaluation in the context of growth-restriction.

5.2. Introduction

Fetal growth restriction (FGR) occurs in ~8% of all pregnancies in developed countries[358]. FGR describes impairment of a fetus to reach its genetic growth potential. Currently, there are no antenatal treatments able to restore growth or increase gestational length in pregnancies complicated by FGR. The only clinical intervention is premature delivery, which further exacerbates complications experienced by growth-restricted newborns[220,232].

FGR commonly occurs secondary to placental insufficiency, which reduces oxygen and substrate delivery to the developing fetus[14]. Reduced oxygen can lead to fetal hypoxia and signals carotid chemoreceptors to initiate a cardiovascular (CV) response known as brain sparing[16]. When brain sparing is maintained long term, chronic CV redistribution results in structural alterations to the heart[359,360] and blood vessels[219,237,288] in FGR fetuses and newborns, and subsequent diminished CV function. Consequently, infants born following FGR are at a greater risk of neonatal morbidity and often require intensive postnatal care. However, there is currently a paucity of data on how the uterine environment, and subsequent CV alterations, will impact the efficacy of common neonatal treatments during the perinatal period.

Early neonatal hypotension has been demonstrated in animal models of FGR[361] and is accepted as a common early complication in FGR neonates[335] following myocardial dysfunction[362]. Clinical treatment of hypotension centers around the improvement of heart rate, blood pressure, cardiac output (CO), and oxygenation[363,364] in-order to maintain tissue perfusion. As in adults, hypotensive support in neonates involves the use of inotropes such as dopamine, dobutamine and adrenaline[365]. Dopamine is one of the most common vasopressors used in neonatal inotropic therapy[366] and works by increasing blood pressure by inducing vasoconstriction in peripheral blood vessels, which subsequently increases venous return, CO and thereby blood pressure (BP)[292]. In the context of FGR, the use of dopamine is particularly interesting as its vasoconstrictive action on the FGR-driven vasoconstricted vessels may be ineffective or potentially detrimental.

It is important to note that despite the clinical use of dopamine for over 40 years, little evidence has shown any short- or long-term benefit of inotropic treatment in preterm neonates[367]. This may be due to large variation in clinical practice[368], which does not take into account the organ specific deficiencies in prematurity or FGR. Indeed, evidence suggests growth-restricted and very-low birth weight infants have a reduced response to dopamine resulting in the need for additional vasopressor support[295]. It is likely that the structural alterations in blood vessels resulting from chronic placental insufficiency, such as; increased blood vessel wall thickness, reduced blood

vessel lumen size, and altered β-adrenoceptor function[303,306,188], play a key role in the altered efficacy of dopamine in this population which likely underlie decreased efficacy of vasoconstrictive therapy in the FGR population.

Sildenafil citrate (SC) is a potent vasodilator and is used clinically to treat persistent pulmonary hypertension of the newborn[344]. SC modulates downstream components of the nitric oxide (NO) pathway causing vasodilation[254]. We have previously shown mRNA expression of endothelial nitric oxide synthase is decreased in FGR lambs, demonstrating deficiency within the NO pathway[20]. Importantly, we also found that supplementation of NO, via inhaled NO, improves HR, CO, and BP in FGR but not AG lambs[20]. Therefore, while it may appear counterintuitive to treat hypotension with a potent vasodilator, SC may be a viable alternative therapeutic option. Growth restricted fetuses and newborns are known to have a vasoconstrictive phenotype[219,237], it is possible therefore that peripheral vasodilation will reduce peripheral vascular resistance and subsequently decrease cardiac afterload, improve venous return and ultimately increase CO and increase BP. We have recently demonstrated that SC not only causes peripheral vasodilation in growth-restricted fetal sheep (Inocencio et al in press), but does so with a greater sensitivity than appropriately grown lambs. SC increases the bioactivity of endogenous NO[370], taken together SC may have therapeutic benefit for hypotension in FGR neonates.

Here, we aimed to investigate the therapeutic efficacy of SC for hypotension during the first hours of life in FGR lambs, compared to current gold standard treatment, dopamine. We hypothesized that CV adaptations occurring in response to chronic placental insufficiency would result in decreased efficacy of dopamine to improve blood pressure in growth-restricted lambs. In contrast the vasodilative action of SC, will increase peripheral blood flow, reducing afterload and stabilize BP in FGR lambs.

5.3. Materials and Methods

Experiments were approved by the Monash Medical Centre animal ethics committee (A) (MMCA2016/01) following guidelines established by the National Health and Medical Research Council of Australia.

5.3.A. Animals

The surgical procedure used to induce growth restriction have been previously described in detail[312]. Briefly, aseptic surgery was performed on twin bearing mixed-breed ewes (n=22) at 89-

90 days of gestation (dGA, term being 145-147 dGA). Anesthesia of the ewe and fetuses was induced via intravenous (i.v.) bolus of 5% sodium thiopentone and following intubation maternal sedation was maintained via maternal delivery of isoflurane (1.5-3%). Before the induction of surgery all ewes received prophylactic antibiotics; Ampicillin 1g in 5ml saline (Jurox) and Engemycin 5 ml (Coopers Animal Health, Bendigo East, Australia) i.v. The fetus was exposed via caesarean section, and single umbilical artery ligation (SUAL) was performed on 22 fetuses to induce FGR by placing two silk ligatures tightly around one of the umbilical arteries. In control twin pregnancies, the umbilical artery was handled but not ligated (AG, control).

5.3.B. Post-Surgical Care

Following surgery i.v. antibiotics (5ml of Engemycin and 1g of Ampicillin) and analgesia (oral Panadol, 1g) were administered for 3 days post-operatively.

5.3.C. Instrumentation and delivery

At 127 dGA ewes and fetuses underwent a second surgery to implant catheters and flow probes required for monitoring CV physiology as previously described[20]. Induction and maintenance of anesthesia and lamb exteriorisation were the same as described above. Ultrasonic flow probes were placed around the left main pulmonary artery (4mm), right carotid artery (3mm) and right femoral artery (3mm; Transonic Systems, Ithaca, NY, USA) to measure regional blood flow. Catheters (polyvinyl catheters ID 0.86mm, OD 1.52 mm Dural Plastics Australia) filled with heparinized saline were inserted into the brachial artery and the jugular vein. Arterial access was required for measurement of BP, blood gas analysis and collection of blood samples whilst venous access was used to administer the therapeutics (SC, dopamine or saline). Incisions were closed after instrumentation, the fetal trachea intubated with a 4.0 mm cuffed endotracheal tube and lung liquid was passively drained. A transcutaneous arterial oxygen saturation sensor (Massimo, Radical 4, CA, USA) was placed around the base of the tail for measurement of transcutaneous arterial oxygen saturation (SpO_2). A near-infrared spectroscopy (NIRS) sensor (Casmed Foresight, CAS Medical Systems, Brandford, CT, USA) was attached to the left side of the forehead for measurement of cerebral oxygenation (ctTOI).

5.3.D. Physiological Measurements and Calculations

To monitor CV variables the brachial artery catheter was connected to a pressure transducer (ADIntruments, Castle Hill, Australia), the flow probes were connected to a flow meter (Transonic Systems, Ithica, NY) and NIRS was connected to a FORE-SIGHT tissue oximeter (Casmed Foresight, CAS Medical Systems, Brandford, CT). BP, heart rate (HR), regional blood flows

(carotid, pulmonary and femoral), transcutaneous arterial oxygen saturation (SpO_2) and cerebral oxygenation (ctTOI) were all measured as digital outputs and recorded in real-time in LabChart Pro (ADInstruments, Castle Hill, Australia). Recording of all variables began immediately following instrumentation.

Arterial blood samples were taken before ventilation (fetal), regularly during the first hour of ventilation and hourly thereafter. Blood gasses were analysed via a blood gas machine (ABL90 FLEX PLUS, Radiometer Medical, Denmark) for determination of fetal pH, hematocrit (hct), oxygen saturation (SaO_2), the partial pressure of oxygen, (PaO_2), the partial pressure of carbon dioxide ($PaCO_2$), lactate and glucose.

5.3.E. Delivery

Following instrumentation, a 10-min control period of fetal physiology was recorded before ventilation. Ventilation of fetuses was initiated with a sustained inflation (30/5 cmH_2O for 30 seconds, Neopuff) followed by 1 min and 30 secs of positive pressure ventilation before clamping the umbilical cord. Once the umbilical cord was clamped and cut, ventilation was continued using volume guarantee mechanical ventilation at 7 ml/kg, positive end-expiratory pressure of 5 cmH_2O, 60 breaths per min, inspiratory time of 0.5 s and an initial fraction of inspired oxygen (FiO_2) of 0.21 (Draeger Babylog 8000+ ventilator, Draeger, Lubeck, Germany) which was maintained for a total of 4 hours. Ventilator and FiO_2 adjustments were made to maintain SpO_2 between 85-95% and $PaCO_2$ between 45-55mmHg. Lambs were sedated throughout the study via constant infusion of Alfaxane (5-15 ml/kg/hr) in 5% dextrose. Following stabilization of the first lamb, the twin lamb underwent surgery and delivery as above.

5.3.F. Randomization of CV Treatment

A total of 22 ewes were used in this experiment. Complications from SUAL and/or instrumentation resulted in the loss of 3 FGR lambs and 1 AG lamb. After 1 hour of ventilation, lambs were allocated a code which was blindly drawn to randomize treatment to either: dopamine (0.6mg/kg/hr; DBL Dopamine Concentrate 200mg/5ml, Auckland, New Zealand); sildenafil (SC, 0.07mg/kg/hour; Revatio Injection, Pfizer Labs, New York) or saline, all delivered intravenously. The dose of SC and dopamine was determined by following Monash Newborn[371,372] care guidelines. A total of 40 lambs were available for this study and successful n of each group are as follows; AG_{SC}: n=8, FGR_{SC}: n=6, AG_{DOP}: n=7, FGR_{DOP}: n=6, AG: n=7, FGR: n=6.

5.3.G. Post-Mortem and Heart Morphology Measurements

At 4 hours, lambs were euthanized via pentobarbital sodium overdose (100 mg/kg i.v. 153 Valabarb; Jurox, Rutherford, Australia) and a post-mortem conducted. At post-mortem fetal biometric measurements were taken and organ weights were noted. Lamb's hearts were also dissected and immersion fixed with 10% buffered formalin. Hearts were then cut into transverse 2 mm sections. Ventricle thickness was measured and recorded.

5.3.H. Serum Troponin Analysis

Plasma from blood samples collected at fetal, 1h, 2h, 3h and 4h time points was submitted to Monash Medical Centre Pathology for troponin concentration analysis.

5.3.I. Statistics

Data are presented as mean ± SEM unless otherwise stated. Normality of data was assessed and normalized as necessary via GraphPad Prism (Prism 8 for Mac OS X, GraphPad Software, USA). Fetal weight, biometry, serum troponin levels, and ventricle thickness were analysed using a two-way analysis of variance (ANOVA) and blood gas data and physiological data was analysed using a mixed-effects analysis with repeated measures design. Tukey's posthoc test was used to compare between groups. Data analysis was performed using Prism 8 for Mac OS X, GraphPad Software, USA.

5.4. Results

5.4.A. Baseline Characteristics and Physiological Parameters

Baseline fetal blood gas variables (Table 1) were not different between groups. Order of birth and maternal status were analysed as covariates and no within-subjects significance was found (data not shown).

5.4.B. Ventilation and Oxygenation Parameters

Total mean FiO_2 required during ventilation was greater in FGR_{DOP} compared to AG (FGR_{DOP}; 54.8% vs AG; 36.8%, Table 2, p=0.002). No difference in FiO_2 was seen between any other group. Total mean airway pressure, tidal volume, oxygen saturation, partial pressure of carbon dioxide and partial pressure of oxygenation of AG_{SC}, FGR_{SC}, AG_{DOP}, FGR_{DOP}, FGR and AG was not different within or between groups (mean data; table 2),

5.4.C. Lamb Biometry, Weight and Brain: Body Weight

Lamb body weight (Figure 1A) was significantly reduced by 553g in FGR lambs compared to AG lambs (FGR; 2.6kg vs AG; 3.1kg; Figure 1A, p=0.04). FGR lambs were asymmetrically grown as

evidenced by reduced abdominal circumference (FGR; 31.3cm vs AG 33.8cm; Figure 1B, p=0.01) and increased brain:body weight ratio (FGR; 19.01g/kg vs AG 15.5g/kg; Figure 1C, p=0.01) compared to AG lambs.

5.4.D. Physiological Response to Saline

Cerebral oxygen saturation (ctTOI), arterial saturation, blood pressure (BP), and heart rate (HR) was not different over the 4-hour ventilation period (Figure 2.1A, B, C, D).

Carotid, femoral and pulmonary blood flow was not different over the 4-hour ventilation period (Figure 2.2A, B, C).

5.4.E. Physiological Response to Sildenafil

BP was not different between AG_{SC} and FGR_{SC} lambs prior to SC treatment. Infusion of SC in growth-restricted lambs (Fig 3.1C) caused a transient decrease of mean blood pressure at 15 minutes (34 ± 5.7 mmHg vs. 53 ± 4.6 mmHg, at -30mins, p=0.04) and 30min compared to time 0 (p=0.007). BP was restored by 1 hr. SC administration did not alter BP in AG lambs. Cerebral oxygen saturation (ctTOI), arterial saturation and heart rate was not different over the 4-hour ventilation period (Figure 3.1A, B, D).

While carotid, femoral and pulmonary blood flow was not significantly different over time or between FGR_{SC} and AG_{SC} lambs over the 4-hour ventilation period, femoral flow tended to be lower in FGR_{SC} compared to AG_{SC} at 15-30 minutes of treatment (Figure 3.2B, p=0.07).

5.4.F. Physiological Response to Dopamine

BP was not different between AG_{DOP} and FGR_{DOP} lambs prior to dopamine treatment Dopamine treatment in growth-restricted lambs resulted in a progressive decrease in BP over the experimental period, such that BP was significantly decreased at 2 and 3 hours of dopamine treatment compared to time 0 (Figure 4.1C; p=0.006 and 0.01 respectively) and to AG_{DOP} lambs at the same time points (Figure 4.1C; p=0.002 and 0.02 respectively). No change in BP was seen in AG_{DOP} lambs.

Dopamine treatment resulted in a transient, significant, increase in heart rate at 15 mins in FGR_{DOP} (Figure 4.1.D, p=0.01) lambs compared to baseline values. Thereafter, HR in FGR_{DOP} progressively declined and at 2 and 3 hrs (vs 15 mins, p <0.04) HR was significantly decreased to 32% lower than baseline. AG_{DOP} lambs also had a transient increase in HR at 30 min (Figure 4.1.D, p=0.04) which returned to baseline levels by 1h.

Cerebral oxygen saturation (ctTOI) and arterial saturation was not different between FGR_{DOP} or AG_{DOP} following dopamine treatment over the 4-hour ventilation period (Figure 4.1A, B).

Carotid, femoral and pulmonary blood flow was not significantly different over the course of the experiment or between FGR_{DOP} and AG_{DOP} lambs (Figure 4.2A,B, C).

5.4.G. Serum Troponin levels

Serum troponin levels increased in FGR lambs 3h after treatment with dopamine (FGR_{DOP}) (0.32ul/ml) and these levels were significantly higher than all other groups ($p<0.001$). Troponin levels did not change in the other treatment groups throughout the study (Figure 5).

5.4.G. Left Ventricular Thickness

Growth-restricted lambs had significantly (Figure 6, $p=0.03$) increased left ventricular thickness compared to AG lambs. Although no individual differences in groups reached significance, left ventricular thickness tended to be increased in FGR_{DOP} compared to AG (0.37cm/g vs 0.20cm/g; $p=0.07$).

5.5. Discussion

Chronic placental insufficiency results in cardiovascular remodeling which has been shown in both blood vessels and the heart. There is also increasing evidence that vascular alterations resulting from placental insufficiency alters the responsivity of the blood vessels in FGR fetuses and newborns[20,343]. Many therapies, including those used to control blood pressure, target pathways which have previously been shown to be deficient following placental insufficiency[197,300]. The β-adrenergic pathway, which is a target for the most common inotrope, dopamine, has been shown to be dysfunctional following growth restriction[300]. Dysfunction in the β-adrenergic pathway likely underlies the decreased efficacy of dopamine in low birth weight infants[295].

In our current study, we aimed to investigate the therapeutic efficacy of SC for hypotension during the first hours of life in FGR lambs, compared to commonly used inotrope dopamine. While hypotension was not evident in FGR lambs, treatment with both SC and dopamine resulted in decreased blood pressure and heart rate. Importantly, these effects where only observed in FGR and not appropriately grown lambs, highlighting the potential for placental insufficiency to affect the cardiovascular response to therapies.

5.5.A. Sildenafil in AG and FGR preterm lambs

In contrast to our hypothesis, we did not observe an increase in blood pressure following SC treatment. Instead we observe an immediate decrease to blood pressure in FGR lambs. These results suggest that SC at this dosage and timing may not be an appropriate therapy for hypotension in the FGR neonate. The decrease in blood pressure was not unexpected, given the vasodilative action of SC. As SC entered the systemic circulation, it is likely to have caused vasodilation and a subsequent decrease to vascular resistance and thereby blood pressure. However, it is important to note that the transience to SC-induced hypotension is only seen the FGR group, whereby BP returns to control values 1 hr post initiation of SC treatment. Importantly, this was observed despite continuous systemic infusion of SC. As discussed above, FGR results in an increased arterial thickness and stiffness, which may result in decreased peripheral blood flow, and subsequently, venous return, cardiac output and blood pressure. As SC traveled to the peripheral circulation over time, the return to normal BP values seen in FGR lambs may have been driven by decreased vascular resistance of the vasoconstricted FGR vasculature, resulting in increased peripheral blood flow, venous return, cardiac output and ultimately blood pressure.

The response to SC occurred only in growth restricted lambs and appropriately grown lambs remained unaffected by SC. The dosages used in our study were determined relative to body weight[372], and so the finding of decreased BP in FGR, but not AG, supports our contention that placental insufficiency increases the sensitivity of the offspring to SC. Literature regarding the effect of SC on neonatal hemodynamics in growth-restricted neonates is limited. Miller et al have previously used the SUAL model to demonstrate the ability for SC to decrease fetal HR and BP[28], and excised FGR peripheral blood vessels dilate to a greater degree in response to SC compared to AG blood vessels[20]. In addition we have shown an increased sensitivity to NO donors, ex-vivo in peripheral arteries in the same model[343] and postnatally via inhaled NO in growth-restricted lambs[20]. The current study is the first to show that sensitivity to SC persists during the neonatal period, in vivo.

It is well accepted that growth-restricted fetuses have decreased NO bioavailability[197], primarily due to the presence of high level of systemic oxidants[373]. Previous studies have speculated that the chronic deficiencies in NO bioavailability may also alter downstream targets within the NO-cGMP

pathway to vasodilation. Indeed, the findings from the current study would support such a contention. SC targets PDE5, which is able to breakdown cyclic GMP allowing the blood vessel dilation to stop, however in the presence of SC, PDE5 is inhibited and cyclic GMP is left intact and the blood vessel is able to remain dilated. In the context of FGR, cyclic GMP levels have been shown to be decreased[374]. Without adequate cyclic GMP, vasodilation will remain ineffective, even if NO was able to be increased. Indeed, serum levels of nitrite and s-nitroshemoglobin, NO precursors, have been found to be increased in FGR fetuses and neonates[354]. SC has the potential to circumvent low levels of cyclic GMP as it potentiates the signaling molecule by decreasing its degradation. Thus, in the current study, SC may improve cyclic GMP levels allowing vasodilation resulting in a decreased blood pressure. Further, this response is likely to be transient as it will only be able to occur until the stores of nitrite and s-nitroshemoglobin precursors are used up.

While the findings from our current study do not suggest SC can be used as hypotensive therapy for FGR, it does support investigation into therapy that targets and/or supplements the deficient NO pathway of growth restricted newborns. Importantly, our results add to the growing body of evidence from us and other that chronic placental insufficiency changes the function of the NO pathway[20,197].

5.5.B Dopamine in AG and FGR preterm lambs

Dopamine treatment led to a progressive decline in a number of cardiovascular variables measured in growth restricted lambs in the current study including heart rate, blood pressure and a need for increasing levels of inspired oxygen (FiO_2). Following dopamine treatment, both FGR and AG lambs became bradycardic indicative of an increase heart contractility[366]. Whilst no other cardiovascular variables were altered in AG lambs, FGR lambs treated with dopamine had progressive hypotension and elevated troponin levels.

Cardiac troponin levels are used clinically to assess myocardial injury[375]. Increased levels occur in neonates with respiratory distress[376] and is associated adverse cardiovascular events and death[377]. Therefore, the results of study 3 suggest that dopamine exposure has potential for cardiac damage effect in growth restricted neonates. However, it is important to note that studies have also shown that higher troponin levels did not correlate to greater severity of disease outcome in neonate[375] and some healthy newborns have detectable troponin levels, despite the absence of cardiovascular injury[378]. Given troponin levels only increased in dopamine treated lambs, I believe that future studies should investigate whether increased troponin following dopamine exposure correlates to cardiac muscle injury.

Further, although pulmonary blood flow did not change in FGR_{DOP} lambs these lambs required increasing amounts of inspired oxygen over the period of experimentation reflective of a worsening pulmonary pathology. These results suggest that dopamine treatment is not only ineffective for CV control in FGR newborns, but also puts the growth-restricted heart under stress potentially causing cardiac muscle injury.

The progressive hypotension in FGR lambs in response to dopamine is an important finding, given it is the most widely used therapy for hypotension and bradycardia in preterm neonates[364]. Dopamine targets the β-adrenoceptors on the heart to cause contraction and is regarded as the safest hypotensive treatment for neonates[379]. However, studies detailing the efficacy of inotrope therapy in FGR do not support these findings and suggest that dopamine efficacy is attenuated in growth-restricted neonates. Previous studies have demonstrated that β-adrenergic stimulation does not effectively improve in vivo CV function in FGR rats[21]. This is supported by small retrospective analysis by Issa et al who has shown that FGR neonates often fail to respond to dopamine treatment[380]. Furthermore, Seri et al show very-low birth weight infants develop vasopressor resistant hypotension and require increased vasopressor support following dopamine treatment[295]. Despite mounting evidence, the current clinical management of neonatal hypotension is not tailored to growth-restricted offspring. Exposure to a placental insufficiency is known to result in cardiovascular remodeling[381] and result in stiff and vasoconstricted peripheral blood vessels. The chronically constricted peripheral vessels of a growth restricted newborn are likely to be unable to further constrict in response to dopamine, resulting in decreased treatment efficacy.

The progressive decline in dopamine treated FGR lambs, observed in the current study, supports our hypothesis and adds to the body of evidence suggesting decreased efficacy of dopamine in FGR neonates. In concert with altered structure, growth-restricted hearts also have altered function[220] and Malhotra et al describes FGR-driven cardiac remodeling as similar to that of cardiomyopathy[335]. Dopamine administration has been associated with increased troponin levels in very low birth weight newborns[376]. Indeed, our finding of increased troponin levels, a measure of acute heart failure, cardiac injury and predictor of myocardial damage[382], supports this contention. The falling HR and BP in FGR lambs in response to dopamine may indicate progressive heart injury and potential heart failure. Our findings warrant the need for caution when treating FGR neonates with dopamine, particularly given that cardiac injury is possible. Future

studies should aim to determine mechanisms behind the ineffectively and injury of dopamine in the FGR neonate.

5.6. Limitations

Dopamine is used to treat hypotension, however, before drug administration, neither FGR or AG lambs were hypotensive. Further, BP and HR did not differ throughout our experiment in control FGR and AG lambs. Therefore, while our results allow us to observe differential effects of sildenafil and dopamine between FGR and AG lambs, we cannot comment on treatment for hypotension. The decision to initiate treatment 1 hour after birth was made based on previous work from our group where, in the same model, we found blood pressure began to decline at this time in FGR lambs[20], however, this was not evident in the current study. To investigate the efficacy of SC and dopamine at treating hypotension in FGR, future studies should aim to investigate treatments when hypotension is evident. Further, we chose to ventilate our lambs for 4 hours. At this gestational age (0.8% GA) maintaining the viability of preterm lambs is difficult without requiring the use of additional treatments that would confound our observations to SC and dopamine.

In our current study, there were more males in the AG and AG_{SC} groups compared to all other groups. While we have previously shown no sex-related cardiorespiratory differences in the first hours of life in preterm lambs[383], this has not been investigated in FGR.

The use of Alfaxane for sedation is routine for all our studies, and is an ethical requirement. Despite being a general anesthetic, Alfaxane was chosen as it has been shown to have minimal depressive effects on the cardiovascular system. However, these studies remain limited by the need to have lambs sedated throughout the experimental period, in particular as this would not be the practice in standard neonatal intensive care unit care.

5.7. Conclusion

Overall, these results highlight that exposure to placental insufficiency can have a dramatic influence on vasculature function of the newborn. We have highlighted key differences in the therapeutic potential of common neonatal drugs, Dopamine and Sildenafil, in growth-restricted offspring. Our findings suggest growth-restricted neonates have unique cardiovascular environment, driven in part by altered vascular tone and sensitivity in the blood vessels. Future studies are required to identify optimal treatment for growth-restricted neonates in days after birth.

5.8. Tables and Figures

	AG			FGR		
	SAL	**SC**	**DOP**	**SAL**	**SC**	**DOP**
Male n/Total	4/7	6/8	3/7	1/6	1/6	2/6
GA (d)	130±0.7	128±0.9	128±0.7	130.3±0.7	128.6±1.2	128.7±0.8
Fetal pH	7.33±0.01	7.25±0.05	7.29±0.01	7.24±0.09	7.33±0.02	7.30±0.01
Fetal PaO_2 (mmHg)	21.1±2.7	23.8±3.7	20.6±3.5	20.8±3.6	21.6±1.8	20.7±3.5
Fetal $PaCO_2$ (mmHg)	64.2±10.7	59.42±2.7	60.01±3.8	55.6±1.5	65.7±6.9	62.58±1.6
Fetal SaO_2 (mmHg)	58.6±6.8	58.51±8.3	65.7±7.4	45.0±9.7	54.1±11.7	52.4±8.1

5.8.A. Table 1. Birth and Fetal Characteristics.

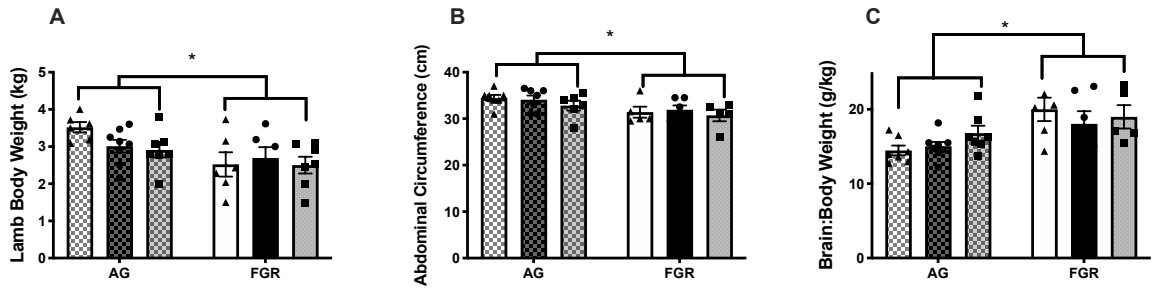
Data are mean ± SEM male number/total), gestational age (GA) in days (d), fetal pH, fetal partial pressure of oxygen (PaO_2), fetal partial pressure of carbon dioxide ($PaCO_2$) and fetal arterial oxygen saturation (SaO_2), significant difference to AG ($p<0.05$). Multiple comparisons were performed via a Tukey's test.

	AG			FGR		
	SAL	**SC**	**DOP**	**SAL**	**SC**	**DOP**
Total Mean Airway Pressure	11.76 ± 0.70	11.92 ± 0.40	13.34 ± 0.42	11.46 ± 0.43	12.27 ± 0.42	13.08 ± 0.35
Total Mean Tidal Volume (V_T)	6.81 ± 0.05	6.45 ± 0.20	7.02 ± 0.13	6.58 ± 0.06	6.83 ± 0.17	6.85 ± 0.17
Total Mean FiO_2 (%)	36.85 ± 2.12	49.52 ± 5.67	46.96 ± 3.41	41.15 ± 4.46	47.94 ± 4.24	54.88 ± 4.61*
Total Mean SaO_2 (mmHg)	75.91 ± 3.79	80.47 ± 4.49	76.8 ± 3.13	78.64 ± 3.70	84.57 ± 3.15	81.17 ± 5.10
Total Mean $PaCO_2$ (mmHg)	41.80 ± 1.87	48.26 ± 1.08	47.24 ± 1.65	40.32 ± 3.03	41.36 ± 2.25	44.04 ± 2.46
Total Mean PaO_2 (mmHg)	31.39 ± 2.78	32.01 ± 1.78	26.48 ± 1.16	30.88 ± 1.66	28.37 ± 1.85	32.42 ± 1.54

5.8.B. Table 2. Ventilation and Oxygenation Parameters.

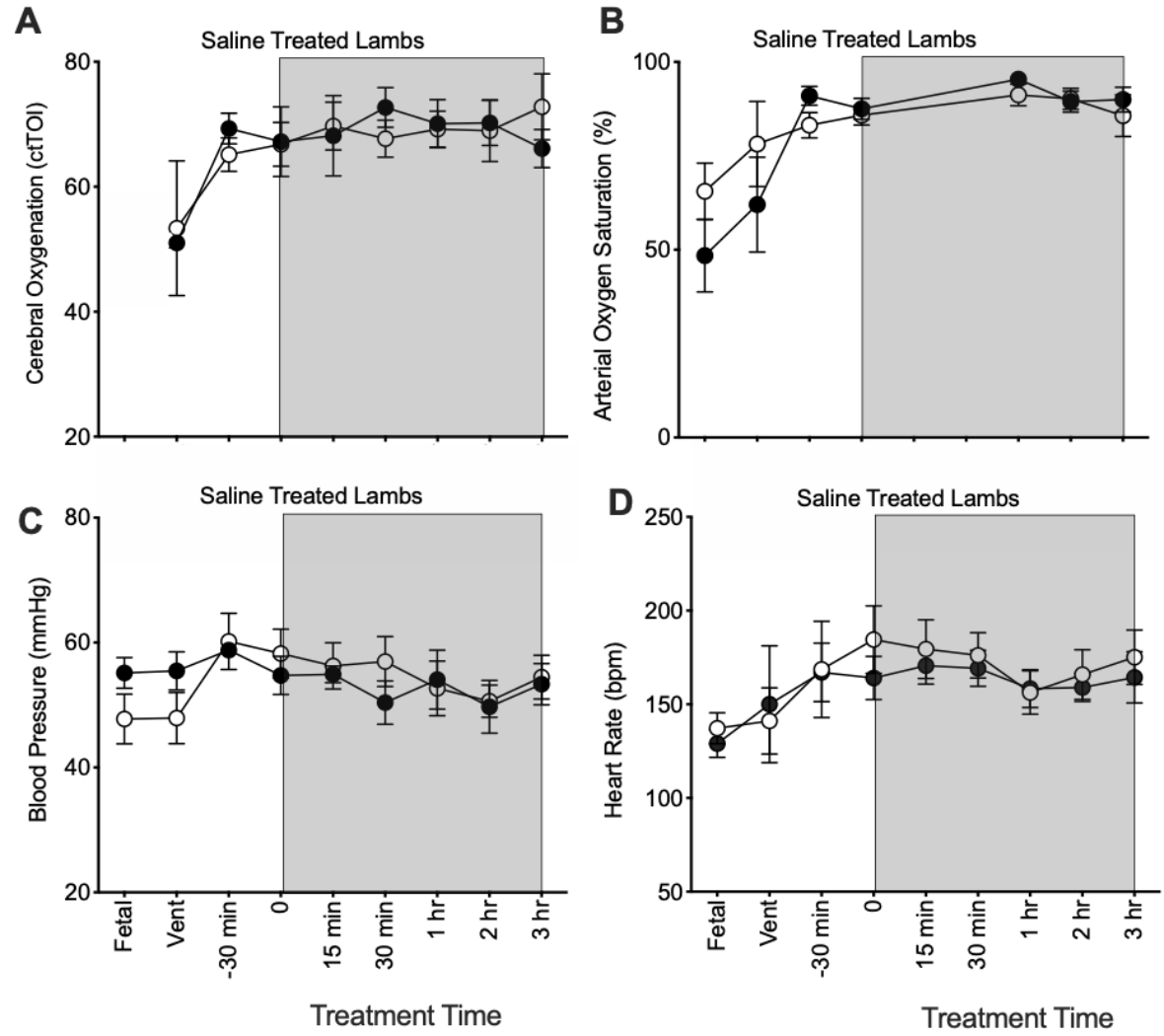
Data are total mean ± SEM (taken over the entire ventilation period) ventilation parameters; airway pressure, tidal volume, fraction of inspired oxygen (FiO_2), and oxygenation parameters; arterial saturation (SaO_2), partial pressure of carbon dioxide ($PaCO_2$) and partial pressure of oxygen (PaO_2). Multiple comparisons were performed via a Tukey's test.

Figures



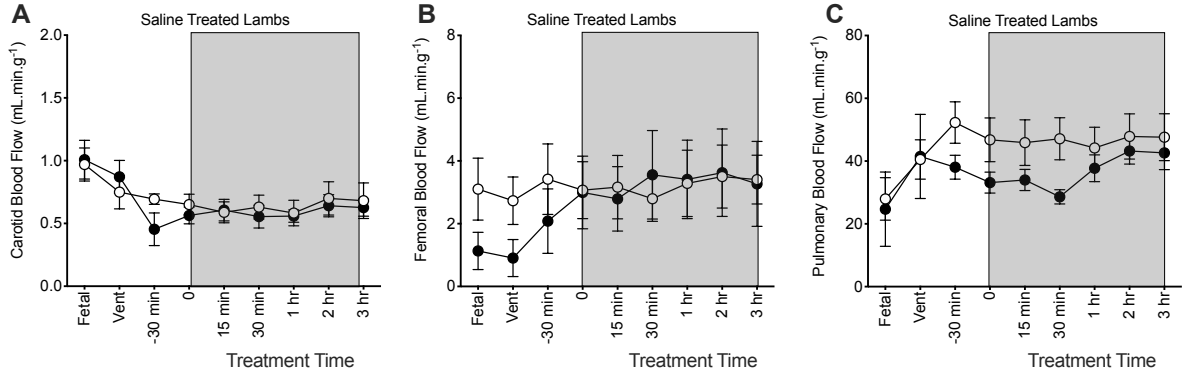
5.8.C. Figure 1. Fetal biometry.

Data are mean ± SEM fetal weight (A, kg), abdominal circumference (B, cm) and corrected brain weight (C, g/kg) in appropriately grown (checkerboard) and growth restricted (solid) lambs treated postnatally with either saline (white), sildenafil (black) or dopamine (grey). * indicates significant difference between FGR and AG ($p<0.05$).



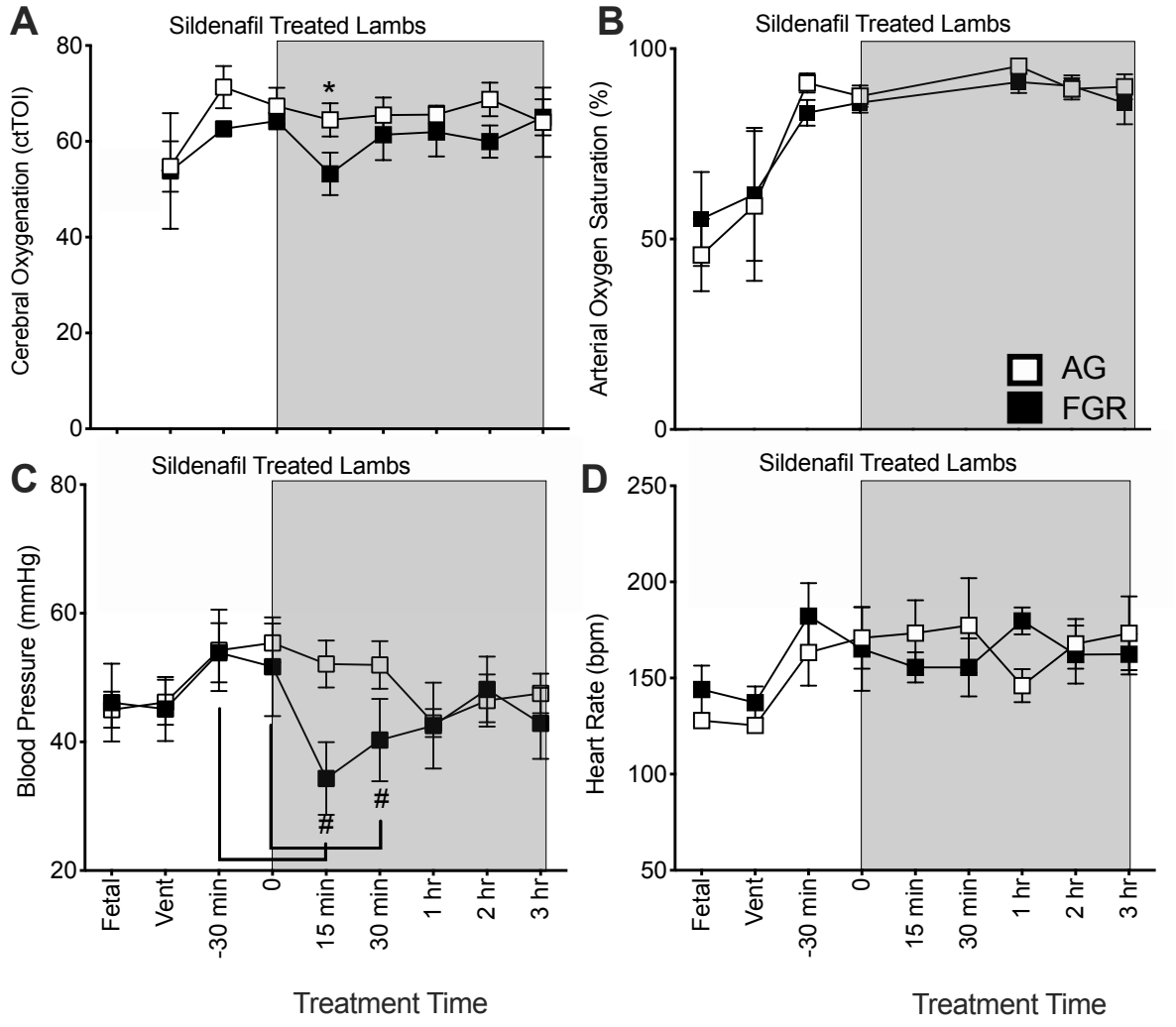
5.8.D. Figure 2.1. Physiological Responses Following Saline Administration.

Data are mean ± SEM cerebral oxygenation (A, ctTOI), arterial saturation (B, %), blood pressure (C, mmHg) and heart rate (D, bpm) in growth restricted (black) and appropriately grown (white) lambs treated with saline (AG, n=6; FGR,n=6; circle). Treatment time is indicated by grey area.



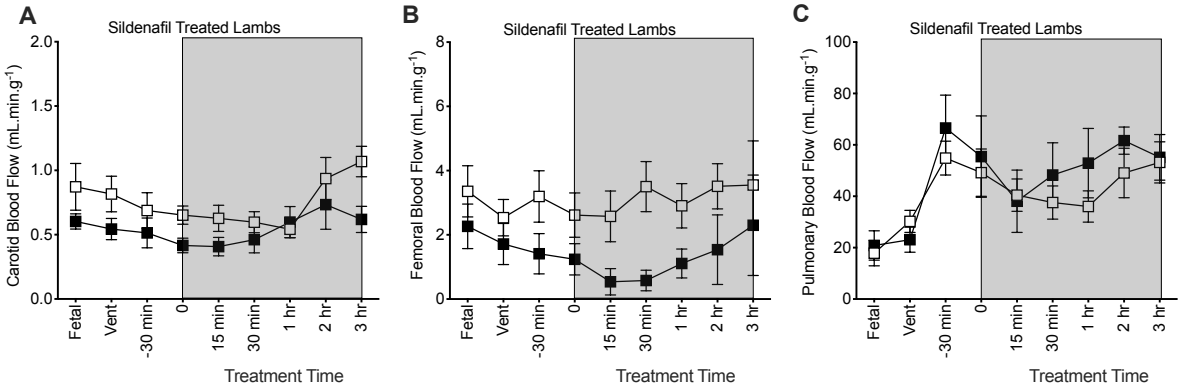
5.8.E. Figure 2.2. Regional Blood Flow Responses Following Saline Administration.

Data are mean ± SEM carotid (A, mL.min.g^{-1}), femoral (B, mL.min.g^{-1}) and pulmonary blood flow (C, mL.min.g^{-1}) of growth restricted (solid) and appropriately grown (white) lambs treated with saline (AG, n=6; FGR, n=6; circle). Treatment time is indicated by grey area.



5.8.E. Figure 3.1. Physiological Responses Following Sildenafil Administration.

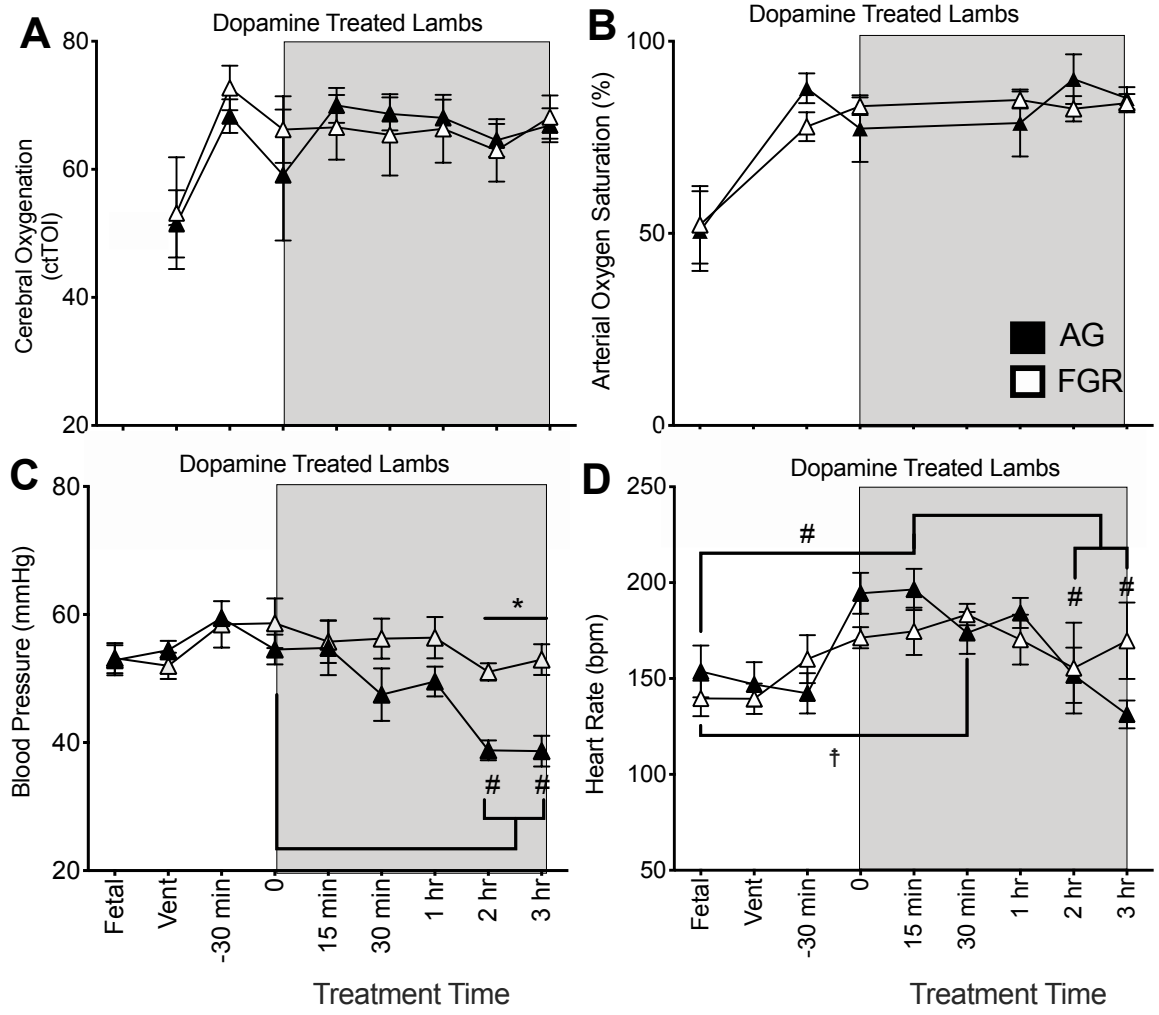
Data are mean ± SEM cerebral oxygenation (A, ctTOI), arterial saturation (B), blood pressure (C, mmHg) and heart rate (D, bpm) in growth restricted (solid) and appropriately grown (white) lambs treated with sildenafil (AG_{SC}, n=6; FGR_{SC}, n=6, square). Treatment time is indicated by grey area. * indicates a significant difference between FGR and AG and # indicates a significant difference within FGR, $p<0.05$.



5.8.F. Figure 3.2. Regional Blood Flow Following Sildenafil Administration.

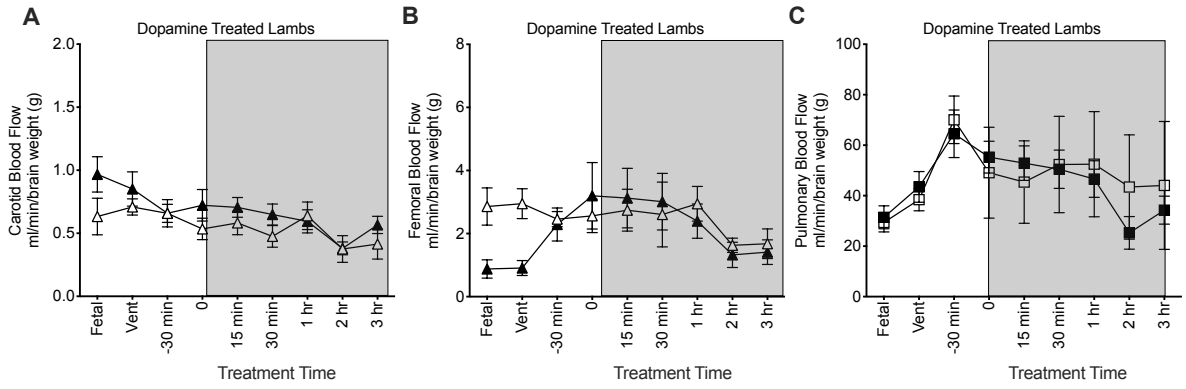
Data are mean ± SEM carotid (A, mL.min.g^{-1}), femoral (B, mL.min.g^{-1}) and pulmonary blood flow (C, mL.min.g^{-1}) corrected for body weight in growth restricted (black) and appropriately grown

(white) lambs treated with sildenafil. Treatment time is indicated by grey shading, AG_{SC}, n=6; FGR_{SC}, n=6, square).



5.8.G. Figure 4.1. Physiological Responses Following Dopamine Administration.

Data are mean ± SEM cerebral oxygenation (A, ctTOI), arterial saturation (B, %), blood pressure (C, mmHg) and heart rate (D, bpm) in growth restricted (black) and appropriately grown (white) lambs treated with dopamine (AG_{DOP}, n=6; FGR_{DOP}, n=6; triangle). Treatment time is indicated by grey area. * indicates a significant difference between FGR and AG and # indicates a significant difference within a group, $p<0.05$. # indicates a significant difference within FGR and † indicates a significant difference within AG, $p<0.05$.



5.8.H. Figure 4.2. Regional Blood Flow Responses Following Dopamine Administration.
Data are mean ± SEM carotid (A, mL.min.g^{-1}), femoral (B, mL.min.g^{-1}) and pulmonary blood flow (C, mL.min.g^{-1}) of growth restricted (solid) and appropriately grown (white) lambs treated with dopamine (AG_{DOP}, n=6; FGR_{DOP}, n=6; triangle). Treatment time is indicated by grey area.

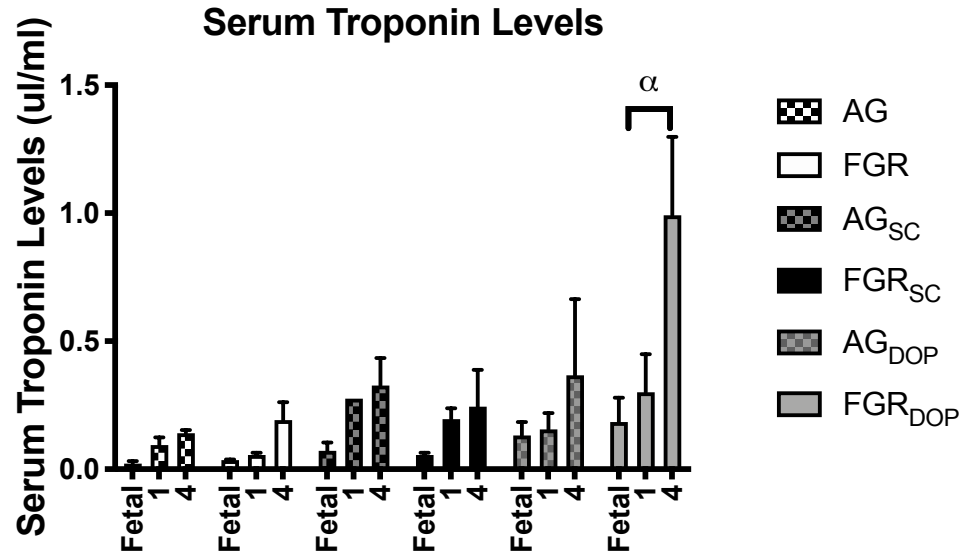
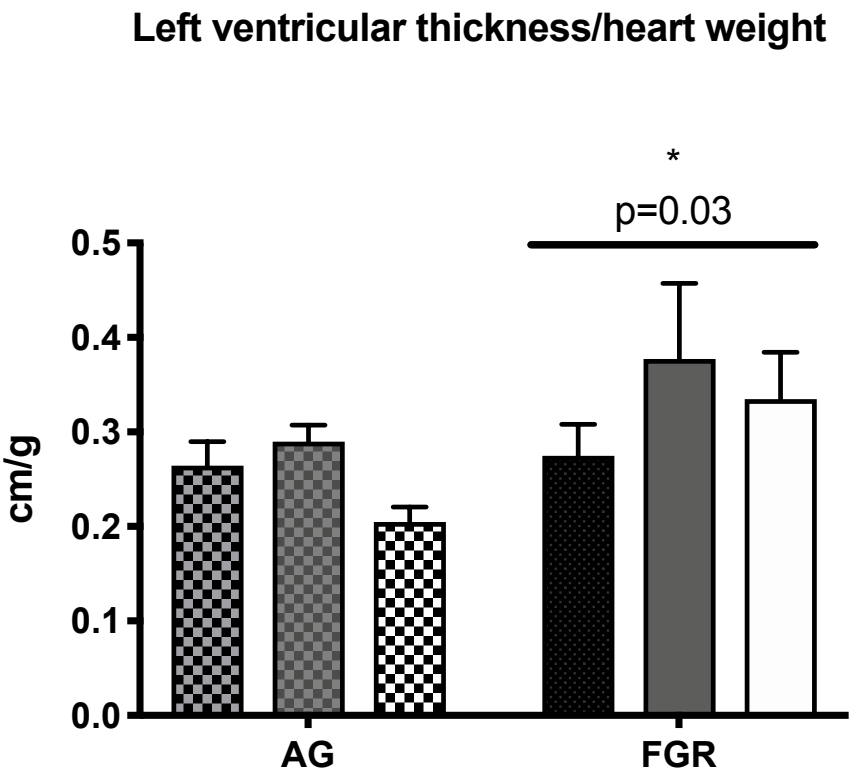


Figure 5. Serum Troponin Levels. Data are mean ± SEM serum troponin levels in growth restricted (FGR, solid) and appropriately grown (AG, checkerboard) lambs treated with either sildenafil (black), dopamine (grey) or saline (white). α indicates significant difference within group over time ($p<0.05$).



5.8.I. Figure 6. Left Ventricular Wall Thickness.

Data are mean ± SEM left ventricular wall thickness corrected for heart weight in growth restricted (solid) and appropriately grown (checkerboard) lambs treated with either sildenafil (black), dopamine (grey) or saline (white). * indicates significant difference between FGR and AG ($p<0.05$).

Chapter 6

General Discussion

6.1. Thesis Aims Overview

Fetal growth restriction (FGR) describes the impairment of the fetus to reach its genetic growth potential. FGR is the second highest cause of perinatal death and the strongest predictor for stillbirth[1,3,4]. Placental insufficiency, the major cause of FGR, and subsequent development of an adverse intrauterine environment, initiates cardiovascular adaptations that aid in fetal survival. However, these fetal adaptations result in an altered cardiovascular structure which contributes to impaired cardiovascular function both before and after birth, resulting in increased morbidity and mortality. Those that survive suffer a greater requirement for postnatal support and have an increased risk of adverse long-term outcomes.

There are no treatments for improving or treating FGR. Further, current postnatal therapeutics targeting cardiovascular dysfunction after birth (i.e. hypotension) are largely ineffective for growth restricted neonates. Previous work by our laboratory have highlighted deficiencies in the nitric oxide (NO) pathway of FGR fetal and newborn lambs[20]. Deficiency in the NO pathway are known to contribute to both the development of FGR and postnatal cardiovascular impairment.

Sildenafil citrate (SC) modulates the NO pathway and may have both antenatal and post-natal therapeutic benefit in the context of FGR. While several studies have demonstrated that SC[27,338] can improve placental perfusion and fetal growth, following increased vasodilation of placental vessels, results of recent clinical trials demonstrate ability to impair pulmonary function and cause death, suggesting that unknown interaction between the altered FGR vasculature and SC result in unknown side effects. Current inotropic therapies used for the management hypotension have shown little benefit in preterm neonates[368]. NO supplementation has also been shown to improve post-natal cardiovascular function[20]. As SC modulated this pathway, SC may provide improved cardiovascular support in the growth restricted offspring.

The key aim of my thesis was to investigate the potential for maternal SC treatment to restore fetal growth and improve postnatal support in a pre-clinical model of FGR. In contrast to the initial hypothesis of my thesis, my results demonstrate that antenatal SC treatment results in exacerbation of growth restriction, impairment of vascular function and dysfunction of the fetal cardiovascular adaptation to hypoxia (*study 1 and 2*).

FGR-driven cardiovascular maladaptation increases the need for cardiovascular support in the neonatal period. Additionally, altered cardiovascular structure, evidenced by increased vascular

thickness and stiffness[194], may underlie the decreased effectiveness of current hypotensive treatments in FGR newborns compared to their AG counterparts. In *study 3*, I compared the efficacy of dopamine, a commonly used treatment for hypotension in preterm neonates, to SC, for improving cardiovascular stability in growth restricted newborns. While limitations in our pre-clinical model impaired any interpretation of therapeutic benefits of both drugs, I found that significant differences in the cardiovascular response to dopamine and SC exists depending on the growth status at birth.

6.2. The outcomes of STRIDER Trial and the Importance of Animal Research.

Prior to the cessation of the STRIDER trial, several studies investigated and supported the use of SC in pregnancy. A recent systematic review published by Dunn et al examined 16 different studies investigating maternal SC treatment. Aside from reports of headache, visual issue and indigestion, they concluded that maternal SC did not cause any significant maternal side effects[272]. In *study 2*, I also observe limited physiological response in ewes treated with SC. These results may suggest that the maternal vascular response to chronic SC is limited and does not have long term side effects. Several studies even demonstrate benefits of SC treatment to vascular health in adults[104,384]. Similarly, studies investigating the vascular effects in the placenta, show improved vasodilation and blood flow[338], again suggesting improvement of placental function and fetal growth by SC. Taken together, these results suggest that exposure to SC in established vasculature does not impair function. However, data regarding SC exposure to the developing fetal vasculature is limited, in particular the vasculature of the FGR. This was not taken into account in the design of the STRIDER trail, which I believe led to the failure of this trial.

The results of the STRIDER trial from UK and Australia/New Zealand participants demonstrated neither harm or benefit of SC at improving birth weight or gestational length[262,339]. However, prior to the completion of the study, the Dutch arm of the STRIDER trial was halted following speculation that antenatal SC was not only ineffective, but impaired postnatal pulmonary function and increased potential for neonatal death[22,267,340].

The failure of the STRIDER trial demonstrates yet again the difficulty in pre-clinical to clinical translation. The failure of this trial may be at fault of choosing the correct pre-clinical models in which to investigate potential harm and/or benefit. The limitations of both preclinical and clinical trials provided the impetus to the body of work contained within this thesis and highlights the importance of my findings.

6.3. Fetal Effects of Sildenafil Citrate on Growth and Cardiovascular Function

As discussed at length in this thesis, SC is a modulator of the NO-cGMP pathway. This pathway is integral for normal vascular function, which is particularly important for organs such as the lung. The results of chapter 3 show all SC treated fetuses have impaired femoral vasodilation to both ACh and SNP, suggesting SC exposure causes both peripheral endothelial and smooth muscle dysfunction. Although we did not specifically investigate endothelial function in pulmonary vessels, the increase in PPHN observed clinically suggest SC exposure had similar effect in the lung. It is well established that impaired vascular function, particularly endothelial dysfunction, plays a key role in PPHN[385,386]. These results are contradictory to the literature, which has shown chronic PDE5 inhibition can improve and restore endothelial function[104,384]. However, it is important to note that the majority of SC investigation has been conducted in adults. Currently, there is very limited data regarding the effect of SC on the developing NO-cGMP pathway in the fetus and the molecular consequences are unknown.

The impaired ability to vasodilate observed in *study 1* and the increase pulmonary resistance observed clinically, suggest that endothelial dysfunction has occurred following fetal exposure to SC. A potential cause for the decreased ability to vasodilate may be due to upregulation of reactive oxygen species (ROS). As previously discussed, NO is a free radical and can react with ROS to form peroxynitriate[184]. The enzyme NADPH oxidase is a major source of ROS in the vasculature[387] and hypoxia can upregulate NADPH activity[388], resulting in reduced NO bioavailability and impaired vasodilative action. Therefore, by upregulation of ROS, the increase in fetal hypoxia as observed in *study 2* may underlie the PPHN observed clinically. Another potential cause for the impairment of vasodilation may be due to altered activity and/or expression of molecular components downstream of PDE5. Prolonged exposure to SC resulting in chronic inhibition of cGMP degradation, may have caused desensitization to cGMP to downstream mediators of this pathway resulting in reduced ability of the smooth muscle to vasodilate.

The physiological responses to SC was the key are of focus *chapters 3 and 4* and unfortunately, I cannot comment on precise molecular alteration to the NO-cGMP pathway. Therefore, further investigation should focus on FGR-driven alterations to the NO-cGMP. This will give better insight to mechanisms underlying vascular dysfunction and better predict response to therapies, such as SC.

The pulmonary circulation is crucially important to the transition from fetal to neonatal life as, following the loss of the placenta, the lungs become the primary organ for gas exchange and oxygenation of the blood[61]. Any impairment in this system is likely to dramatically limit the ability of the newborn to adequately transition from the fetal to neonatal circulation[61]. Therefore, further investigation into the vasoreactivity of the pulmonary circulation, in particular in growth restricted fetuses/newborns, should be a specific area of focus.

Based on the findings in *study 1*, *study 2* investigated 1) the effect of SC exposure on fetal and maternal physiology and 2) the potential physiological mechanisms that may have contributed to the unexpected fetal outcomes observed in *study 1*.

The redistribution of blood flow from the peripheral to the central circulation is a key and well described cardiovascular adaptation to chronic hypoxia, which underlies FGR[16]. Redistribution of blood flow did occur in control FGR fetuses in *study 2*. An important observation in SC treated FGR fetuses was an increased fetal peripheral blood flow and maintenance of cerebral blood flow, despite worsening fetal hypoxia. This suggests disruption to the normal fetal cardiovascular adaptation to hypoxia. The ability of the fetus to survive acute and chronic hypoxic insults is critically dependent upon its ability to mount a fetal cardiovascular response. Importantly, this is the mechanism by which 'brain-sparing' occurs[16] and is associated with improved neurological outcomes and survival compared to symmetrical (non-spared) FGR[389]. This finding highlights that maternal administration of vascular drugs can deleteriously affect fetal wellbeing and development. Going forward, future clinical trials focusing on maternal administration of drugs to pregnant women should consider drug mechanisms, targets and importantly, off target effects, particularly to the developing fetus.

Overall, *studies 1 and* 2 caution against the use of SC to improve growth and development of the growth restricted fetus secondary to placental insufficiency and highlight the critical need and importance of physiological studies in appropriate large animal models to ensure safety and efficacy before translation to human studies.

6.3.A. Exacerbation of Fetal Growth Restriction by SC

A key, albeit unexpected result of my thesis, is the exacerbation of FGR following SC treatment, especially given the improvement of growth shown in studies supporting the implementation of the STRIDER trials[25,27,29,271,318].

Given we observed the same effect of SC on birthweight in FGR fetuses in both *studies 1 and 2*,

we conclude that growth is impaired by maternal SC. Further, these effects occurred despite different lengths of time (36-day SUAL vs 20-day SUAL) and in different pregnancies (twins vs. singletons) between the two studies. Decreased fetal weight and increased brain:body weight ratio show that maternal SC exacerbates growth restriction and brain sparing in a singleton model of late FGR as in the twin model of early FGR. The increase in brain-sparing in SC treated lambs was an interesting finding as it occurred despite increased peripheral blood flow and without changes to cerebral blood flow. This is an important observation as it suggests increased brain-sparing can occur by mechanisms other than increased blood flow. As discussed in *study 2*, lactate can act as an energy source for the brain and increased levels may have driven increased brain growth[349]. However, it is unknow if lactate is a sufficient energy source for altering fetal brain growth in FGR and should be a key focus of future studies.

The demonstration of exacerbated growth restriction following maternal SC across two separate studies does suggest that maternal SC impairs fetal growth. Although we were unable to determine the mechanism behind SC-induced exacerbation of FGR, we believe that a potential worsening of placental dysfunction is a likely cause. We did not measure placental flow, however clinical results by Sharp et al suggest potential impairment in placental blood flow by SC[262]. We suggest SC may drive maternal hypotension secondary to global vasodilation resulting in shunting of blood away from the placental circulation, leading to a decrease in placental perfusion. This theory has been discussed in previous studies by Miller et al[28] and could be validated via the measurement of blood flow though the uterine vasculature and should be a focus of future studies.

Gonzalez-Candia et al have also shown that maternal melatonin treatment can also exacerbate growth restriction[252]. Similarly to SC, this is the only study to find adverse effects of melatonin, whereas several studies have demonstrated improvement of cardiovascular development[250], protection against oxidative stress[249] and increased fetal oxygenation and body weight[390] in the context of FGR. However, the findings of Gonzalez-Candia et al may provide some insight into the decreased fetal weight observed in *studies 3 and 4*. A potential reason proposed behind the exacerbation of FGR, is the ability of melatonin to increase maternal cortisol levels. This may induce fetal hyperglycemia and hyperinsulinemia and cause an increase in umbilical glucose uptake. Increased maternal cortisol may therefore reduce fetal nutrient transfer. Socala et al have shown that SC treatment in mice upregulates expression of corticosterone[391], the mouse analogue to cortisol in humans. In addition to altered maternal hemodynamics, the increase in maternal cortisol following SC treatment may be another driving force behind increased FGR observed in our

current study. Future studies should therefore focus on the relationship between maternal SC treatment and changes to cortisol levels.

6.4. Use of Vascular Therapies in Preterm Infants

FGR increases the risk of neonatal hypotension, secondary to myocardial impairment following adaptation to chronic placental insufficiency and hypoxia[392]. Consequently, growth restricted infants require greater postnatal support compared to appropriately grown (AG) counterparts[335]. Dopamine, a β-agonist, is commonly used to treat hypotension in preterm infants, however evidence of efficacy in preterm neonates is lacking[368]. Further, there is a paucity in evidence regarding efficacy of inotropic treatment of growth restricted neonates and some clinical evidence does suggest inefficiency of dopamine in this population[380]. Additionally, Seri et al have shown that extremely low birthweight (ELBW) develop vasopressor-resistant hypotension and require adjunct therapy, suggesting limited effect in the growth restricted neonate[295]. We also suggest that inotrope treatment may be damaging to neonates born following FGR. In a large cohort study in extremely low birthweight (ELBW) infants, Faust et al inotrope treatment was strongly associated with increased risk of intraventricular haemorhage[393], a condition associated with an abnormal increase in blood pressure[394,395]. Therefore, it is reasonable to predict dopamine therapy in newborns born classified with FGR may also increase the risk of cardiovascular or neurovascular dysfunction.

Consistent with previous investigations regarding the cardiovascular impact of FGR[194], we have shown in *studies 1* and *2* that growth restricted newborns are born with a completely different underlying cardiovascular physiology. However, newborn infants presenting with adverse cardiovascular complications, including hypotension, are all treated similarly and without any consideration of the underlying health of the uterine environment. As a consequence, growth restricted newborns may be exposed to drugs which are ineffective or potentially detrimental to them.

In study 3 we compared the response of FGR and AG newborns to dopamine, to determine whether the responsiveness was different depending on growth status *in utero*. We then investigated whether postnatal treatment with vasodilator SC was more effective for FGR newborns to improve BP than dopamine. The key finding of *study 3* was the demonstration of a different physiological response to the current mainstay of clinical treatment, dopamine, between growth restricted and AG newborns, resulted in a gradual decline in heart rate and blood pressure in growth restricted lambs. We further demonstrated that SC caused a transient blood pressure

decrease in growth restricted preterm lambs, which normalized 1-hour post treatment. This was in contrast to the sustained decrease in blood pressure and heart rate after dopamine treatment. Importantly, none of the drugs tested had any apparent effect on cardiovascular parameters in AG lambs. These results highlight that the cardiovascular impairments induced by FGR result in a differential cardiovascular response to postnatal drugs in the neonatal period as compared to those not exposed to placental insufficiency. My results highlight the critical importance of considering the underlying physiology of the patient before giving any clinical treatment, and support the movement in the clinical landscape towards personalized medicine.

6.4.A. The use of dopamine in FGR

Dopamine works to increase blood pressure via stimulation adrenergic receptors within the heart and blood vessels to increase heart contractility and vasocontriction[366]. However, as blood vessels of growth restricted neonates are stiff and hyperconstricted[381], further constriction by dopamine is unlikely to increase blood pressure. We found dopamine increased indices of acute heart failure, likely driven by inappropriate contractive force of the cardiac muscle. These findings are important as it suggests dopamine treatment is less effective, and worse, potentially injurious in the growth restricted population. These findings from dopamine exposed growth restricted lambs highlight the need for targeted therapy for growth restricted neonates as well as the urgent re-evaluation of clinical guidelines recommending administration of dopamine, or other B-adrenergic stimulants like dobutamine, to growth restricted neonates.

6.4.B. The Use of Sildenafil in FGR

Previous studies by my group have demonstrated NO deficiency in growth restricted preterm lambs and further that subsequent NO supplementation (with inhaled NO) improved cardiac output and blood pressure[20]. As SC modulates the NO pathway and increases endogenous NO[370], in *study 3* we expected an increase in blood pressure, following increased venous return and cardiac output in growth restricted lambs. In contrast, we observed a transient hypotension in FGR lambs, likely caused by a global vasodilative response to SC. However, despite continuous infusion of SC, the hypotensive episode was transient and blood pressure gradually rose to control levels in FGR_{SC} lambs within 1h post treatment. The restoration of blood pressure to control levels may have been caused by vasodilation to the vasoconstricted vasculature in the periphery of growth restricted lambs, increasing peripheral blood flow, venous return, cardiac output and thereby blood pressure. Alternatively, This initial hypotension may have been detected and corrected through typical baroreflex mechanisms to correct blood pressure[396]. However, this study is limited as the control lambs were not hypotensive, and therefore it is difficult to draw conclusions on the potential for SC to provide hypotensive support in FGR neonates. Future studies should focus on the

investigating hypotensive treatment when hypotension is evident. However, our results do demonstrate a difference in drug responses between FGR and AG lambs, which highlight the care that must be taken when choosing hypotensive treatment and suggests that treating the peripherally constricted vasculature of growth restricted neonates has therapeutic potential.

6.4.C. Therapy for Cardiovascular instability in Growth Restricted Neonates.

It is well accepted that FGR-driven vascular alterations result in a greater requirement for hypotensive therapy during early life[220,335] however the efficacy of inotropic therapy is debated in the preterm population[368]. Further, evidence suggests that inotropic support is not only ineffective[295], but potentially detrimental in FGR neonates[21]. We and other have shown that FGR results in differences in the NO pathway[20,197,343,397]. I suggest that targeting these differences may provide therapeutic benefit to the FGR neonate.

The results of study 3 suggest that neither dopamine or SC provide hypotensive support to the FGR neonate. However, Polglase et al have shown that cardiovascular function improves in FGR, but not AG lambs following inhaled NO[20]. Impaired NO production is known to contribute to vascular dysfunction[197]. Indeed, impaired endothelial function and NO production is reduced in FGR offspring[36], which may underlie the cardiovascular dysfunction observed later in life[220].

The results of Polglase et al suggest NO supplementation may counter the decreased bioavailability in FGR offspring to provide cardiovascular benefit. The lack of response seen in *study 3* following SC treatment suggests that impairment in the NO-cGMP pathway, that underlies vascular dysfunction in FGR, may be upstream of PDE5 and is therefore not a valid therapeutic target. Sodium Nitroprusside (SNP) is a NO donor and, similarly to inhaled NO, has been shown to be beneficial at treating PPHN and systemic hypertension[398]. Therefore, treatment with SNP may provide better cardiovascular support in FGR neonates by increasing NO bioavailability and should be the focus of future studies.

6.5. Limitations and Future Directions

A key limitation of *study 1 and 2* was the route of administration of SC compared to clinical administration. Clinically, mothers received SC orally, however for pragmatic reasons in my studies I chose to administer SC intravenously. One key reason underpinning this decision was the differences in the gastrointestinal system between the human and sheep. In order to deliver a comparable dose of SC to our pregnant ewes we investigated the pharmacokinetics of oral SC.

Oral SC has a bioavailability of ~41 %[303] and we therefore delivered a continuous i.v. dose of 36 mg per day to mimic this dose in our ewes. Further, as pregnant women were taking tablets three times daily the clinical pharmacokinetic profile is cyclic, whereas ours was constant. It is unknown how the difference between a cyclic and constant exposure to SC would alter the fetal cardiovascular response to SC, but given the increased growth restriction, hypoxia and fetal stress in my studies, it is reasonable to consider that future studies may be required to investigate different routes and exposures of SC administration to consolidate our understanding.

Another limitation in my antenatal studies is the underlying cause of placental insufficiency between our SUAL model and the clinical aetiology of FGR. Clinically, placental insufficiency commonly occurs secondary to abnormally high placental vasoconstriction resulting in reduced placental perfusion and function[14]. Whereas single umbilical artery ligation induces placental insufficiency by restriction of feto-placental blood flow, resulting in placental atrophy[301]. Given this, it is important to consider the possibility that SC may have had limited ability to increase flow in atrophied vessels, where clinically it is hypothesized that SC would improving placental flow via vasodilation. Additionally, although it has not been previously shown, the surviving placental vasculature after SUAL may be maximally dilated to compensate for the atrophied placenta. Maintained uterine flow post SUAL supports this contention[28] and therefore the ability for SC to increase umbilical blood flow is likely to be limited on in this model. While we are still able to investigate the key aims of my thesis, which was to determine the effect of SC exposure on the fetus and neonate, future studies aimed at improving placental function via vasodilation of the placental vascular bed should use models of FGR where placental vasoconstriction is evident. This will allow for investigation towards the potential for vasodilative therapies to improve FGR. Placental vasoconstriction occurs in the rodent placental under-perfusion model, and may therefore be appropriate for this goal[399].

Another limitation in our studies in the inability to address potential sex-specific dimorphisms in our studies. My current studies were not powered to investigate sex differences. However, there is a significant body of evidence suggesting a difference in consequence to FGR between males and females.

Large cohort studies by Melamed and Aibar et al suggest that females are more likely to develop FGR[400,401], and being born male is an independent risk factor for worse neonatal outcomes[46]. However, Quninones et al have also shown that in the context of FGR, gender does not predispose

to worse perinatal outcome[402]. This demonstrates that the contribution of fetal sex to FGR outcomes is an area of contention that requires further investigation. Indeed, several animal studies have shown differential cardiovascular effects of FGR, suggesting that sex-specific differences do exist. Therefore, investigation into these differences is important, particularly when investigating targeted therapy.

Allison et al have shown that male preterm fetal sheep have increased blood pressure and an impaired baroreflex response, suggesting being born male predisposes to cardiovascular dysfunction[396]. Souza et al have also shown that FGR male rats have metabolic alterations that predispose towards the development of obesity, whereas although females have increased fat deposits but normal glucose and insulin levels[403]. Roghair et al have shown that male offspring of nutrient-restricted mice have increased blood pressure and ex-vivo vascular dysfunction, whereas females had NO-synthase dependent hypotension and enhanced ex-vivo vasodilation[404]. These results suggest that males FGR mice are more likely to develop hypertension, while the female hypotension. Taken together, these studies suggest a potential protective female benefit in FGR.

A potential mechanism behind cadiovascaulr protection in females following FGR, is the increased expression of estrogen compared to males. Patterson et al have shown that oxidative stress, following a hypoxic insult repressed cardiac expression of protein kinase C[405]. Protein kinase C is an enzyme the has roles in cardiac development, function and protection[406]. Therefore, decreased expression predisposes to ischemic injury[405]. Early-growth response factor-1 (EGR1) can bind to the promotor region of protein kinase C and up regulate its activity[407]. Estrogen can also bind to EGR-1 and increase protein kinase C activity and therefore via this mechanism, females have greater protection to hypoxia-driven cardiac injury than males. An important area of investigation may be to determine if these differences are observed clinically and are potential therapeutic targets.

Due to the low numbers in my 3 studies, I was underpowered to assess differences between males and females and cannot comment on the contribution of sex-hormones to vascular deficiencies and/or response to SC or dopamine. As large animal models limit the ability to general large cohorts for studies, small animal models may be more appropriate to investigate sex-specific effects in FGR.

The exacerbation of growth restriction by SC was a central, but unexpected finding in this thesis. As such, our studies were not designed to investigate why exacerbation of growth in FGR lambs

occurred in *studies 1 and 2*. Though the difference in growth between saline treated and SC treated AG lambs was not significant, *study 1* demonstrated that AG lambs still experienced 40% reduction in growth and significant vascular deficits following exposure to SC. Further, given that growth restriction contributes to impaired vascular function, it was not possible to determine the contribution of increased FGR or SC exposure towards vascular dysfunction observed in *study 1*. Further studies should aim to investigate the mechanisms through which SC decreases fetal growth and investigate whether SC exposure alone impairs endothelial and smooth muscle function. Additionally, investigation for potential placental therapies should focus on substances that specifically target the placenta without crossing into the fetal circulation.

Clinical data shows treatment for hypotension commonly occurs within the first 24 h of life[408,409]. Previous studies by our group have shown increased indices of neonatal demise, such as decreased pulmonary and cerebral blood flow, in the FGR lamb within 10 min of ventilation onset. This suggests immediate need of cardiorespiratory support in FGR lambs[410]. We therefore decided to initiate treatment 1-hour post ventilation onset. A limitation of my postnatal study is the absence of hypotension, particularly as treatment with hypertensive drugs would only be given following cardiovascular demise. While our results demonstrate important effects regarding the response to dopamine and SC in growth restricted newborn lambs, interpretation into treatment efficacy is limited. To determine the potential beneficial/detrimental effects of hypotensive treatment in the FGR population, future studies should aim to initiate treatment 1) following the diagnosis of cardiovascular dysfunction and/or 2) increase the experimental period for a longer than 4 hours, with particular focus on dosage and timing of treatment. The completion of these studies would uncover the potential to support cardiovascular function in the growth restricted newborn.

6.6. Conclusions

In conclusion, my studies have demonstrated the unique physiology of the FGR fetus and newborn compared to their AG counterparts. The growth restricted cardiovascular physiology imparts significant differences in their response to pre- and post-natal stressors, including physiological stress or pharmacological stress may inadvertently exacerbate the detrimental consequences of FGR.

It is therefore overwhelmingly clear from my studies that:

~ The growth restricted fetal cardiovascular system is unique, and needs to be treated as such! ~

Therefore, investigation into therapies aiming to improve newborn or postnatal outcomes in FGR newborns should consider the underlying physiology which I have demonstrated to be critically different.

Chapter 7

References:

1. Alberry, M. & Soothill, P. Management of fetal growth restriction. *Arch. Dis. Child. Fetal Neonatal Ed.* 92, F62-7 (2007).
2. Byrne, C. D. & Phillips, D. I. Fetal origins of adult disease: epidemiology and mechanisms. *J. Clin. Pathol.* 53, 822–828 (2000).
3. Figueras, F. & Gratacós, E. Update on the diagnosis and classification of fetal growth restriction and proposal of a stage-based management protocol. *Fetal Diagn. Ther.* 36, 86–98 (2014).
4. Mongelli, M. & Gardosi, J. Symphysis-fundus height and pregnancy characteristics in ultrasound- dated pregnancies. *Obstet. Gynecol.* 94, 591–594 (1999).
5. Australian Institute of Health and Welfare. *Australia's mothers and babies 2013. Perinatal statistics series* 31, (2015).
6. Gaccioli, F. & Lager, S. Placental nutrient transport and intrauterine growth restriction. *Frontiers in Physiology* (2016). doi:10.3389/fphys.2016.00040
7. Ismail, H. & Chang, Y.-L. Sequelae of Fetal Growth Restriction. *J. Med. Ultrasound* 20, 191–200 (2012).
8. Sehgal, A., Skilton, M. R. & Crispi, F. Human fetal growth restriction: a cardiovascular journey through to adolescence. *J. Dev. Orig. Health Dis.* 1–10 (2016). doi:10.1017/S2040174416000337
9. Miller, S. L., Huppi, P. S. & Mallard, C. The consequences of fetal growth restriction on brain structure and neurodevelopmental outcome. *J. Physiol.* 00, 1–17 (2015).
10. M. C. Cruz Lemini, B. Bijnens, B. Valenzuela-Alcaraz, M. Sitges, F. Figueras, F. Crispi, E. G. Cardiac remodeling in utero in early- and late-onset intrauterine growth restriction. *22nd World Congr. Ultrasound Obstet. Gynecol.* 40, 66–67 (2012).
11. A El-Sheikh, A Francis, J. G. Comparative analysis of length of stay in neonatal intensive care after 34 weeks in singleton babies with and without intrauterine growth restriction. 96, 2011 (2011).
12. McGuire, S. F. Understanding the Implications of Birth Weight. *Nurs. Womens. Health* 21, 45–49 (2017).
13. Dashe, J. S., McIntire, D. D., Lucas, M. J. & Leveno, K. J. Effects of symmetric and asymmetric fetal growth on pregnancy outcomes. *Obstet. Gynecol.* (2000). doi:10.1097/00006250-200009000-00001
14. Rosenberg, A. The IUGR Newborn. *Seminars in Perinatology* 32, 219–224 (2008).
15. Sharma, D., Shastri, S. & Sharma, P. Intrauterine Growth Restriction : Antenatal and Postnatal Aspects. 67–83 (2016). doi:10.4137/CMPed.S40070.TYPE
16. Cohen, E., Baerts, W. & Van Bel, F. Brain-Sparing in Intrauterine Growth Restriction: Considerations for the Neonatologist. *Neonatology* 108, 269–276 (2015).
17. Cosmi, E., Fanelli, T., Visentin, S., Trevisanuto, D. & Zanardo, V. Consequences in infants that were intrauterine growth restricted. *J. Pregnancy* 2011, 364–381 (2011).
18. Barker, D. J. P. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* 13, 807–813 (1997).
19. Levine, T. A. *et al.* Early Childhood Neurodevelopment After Intrauterine Growth Restriction: A Systematic Review. *Pediatrics* (2015). doi:10.1542/peds.2014-1143
20. Polglase, G. R. *et al.* Altered cardiovascular function at birth in growth-restricted preterm lambs. *Pediatr. Res.* 80, 538–546 (2016).
21. Zohdi, V., Jane Black, M. & Pearson, J. T. Elevated vascular resistance and afterload reduce the cardiac output response to dobutamine in early growth-restricted rats in adulthood. *Br. J. Nutr.* 106, 1374–1382 (2011).
22. Hawkes, N. Trial of Viagra for fetal growth restriction is halted after baby deaths. *BMJ* 362, k3247 (2018).
23. Stanley, J. L. *et al.* Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase knockout mouse model. *Hypertension* (2012).

doi:10.1161/HYPERTENSIONAHA.111.186270
24. Dilworth, M. R. *et al.* Sildenafil citrate increases fetal weight in a mouse model of fetal growth restriction with a normal vascular phenotype. *PLoS One* 8, e77748 (2013).
25. Refuerzo, J. S. *et al.* Sildenafil citrate and fetal outcome in pregnant rats. *Fetal Diagn. Ther.* 21, 259–263 (2006).
26. Sánchez-Aparicio, P. *et al.* Effects of sildenafil on the fetal growth of guinea pigs and their ability to survive induced intrapartum asphyxia. *Am. J. Obstet. Gynecol.* 198, (2008).
27. Satterfield, M. C., Bazer, F. W., Spencer, T. E. & Wu, G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. *J. Nutr.* 140, 251–258 (2010).
28. Miller, S. L., Loose, J. M., Jenkin, G. & Wallace, E. M. The effects of sildenafil citrate (Viagra) on uterine blood flow and well being in the intrauterine growth-restricted fetus. *Am. J. Obstet. Gynecol.* 200, (2009).
29. Panda, S., Das, A. & Md Nowroz, H. Sildenafil citrate in fetal growth restriction. *J. Reprod. Infertil.* 15, 168–9 (2014).
30. Von Dadelszen, P. *et al.* Sildenafil citrate therapy for severe early-onset intrauterine growth restriction. *BJOG An Int. J. Obstet. Gynaecol.* 118, 624–628 (2011).
31. Wareing, M., Myers, J. E., O'Hara, M. & Baker, P. N. Sildenafil citrate (viagra) enhances vasodilatation in fetal growth restriction. *J. Clin. Endocrinol. Metab.* 90, 2550–2555 (2005).
32. Van Vliet, P., Wu, S. M., Zaffran, S. & Pucéat, M. Early cardiac development: A view from stem cells to embryos. *Cardiovascular Research* (2012). doi:10.1093/cvr/cvs270
33. Winter, E. M. & Gittenberger-de Groot, a C. Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell. Mol. Life Sci.* (2007). doi:10.1007/s00018-007-6522-3
34. Kirby, M. L. Cardiac morphogenesis—recent research advances. *Pediatr. Res.* (1987). doi:10.1203/00006450-198703000-00001
35. Udan, R. S., Culver, J. C. & Dickinson, M. E. Understanding vascular development. *Wiley Interdisciplinary Reviews: Developmental Biology* (2013). doi:10.1002/wdev.91
36. Schleich, J. M., Abdulla, T., Summers, R. & Houyel, L. An overview of cardiac morphogenesis. *Arch. Cardiovasc. Dis.* (2013). doi:10.1016/j.acvd.2013.07.001
37. Moorman et al. Development of the heart (1) formation of the cardiac chambers and arterial trunks. *Heart* (2003). doi:10.1136/heart.89.7.806
38. Brand, T. Heart development: Molecular insights into cardiac specification and early morphogenesis. *Developmental Biology* (2003). doi:10.1016/S0012-1606(03)00112-X
39. Hosseini, H. S., Garcia, K. E. & Taber, L. A. A new hypothesis for foregut and heart tube formation based on differential growth and actomyosin contraction. *Development* (2017). doi:10.1242/dev.145193
40. Mably, J. D. & Liew, C. C. Factors involved in cardiogenesis and the regulation of cardiac-specific gene expression. *Circ. Res.* 79, 4–13 (1996).
41. Männer, J. The anatomy of cardiac looping: A step towards the understanding of the morphogenesis of several forms of congenital cardiac malformations. *Clinical Anatomy* (2009). doi:10.1002/ca.20652
42. Lin, C.-J., Lin, C.-Y., Chen, C.-H., Zhou, B. & Chang, C.-P. Partitioning the heart: mechanisms of cardiac septation and valve development. *Development* (2012). doi:10.1016/j.ijsu.2017.06.083
43. Peter, A. K., Bjerke, M. A. & Leinwand, L. A. Biology of the cardiac myocyte in heart disease. *Mol. Biol. Cell* (2016). doi:10.1091/mbc.E16-01-0038
44. Woodcock, E. A. & Matkovich, S. J. Cardiomyocytes structure, function and associated pathologies. *Int. J. Biochem. Cell Biol.* (2005). doi:10.1016/j.biocel.2005.04.011
45. J. Patterson, A. & Zhang, L. Hypoxia and Fetal Heart Development. *Curr. Mol. Med.* (2010). doi:10.2174/156652410792630643
46. Zebrowski, D. C. *et al.* Cardiac injury of the newborn mammalian heart accelerates

cardiomyocyte terminal differentiation. *Sci. Rep.* (2017). doi:10.1038/s41598-017-08947-2
47. Oparil, S. Pathogenesis of ventricular hypertrophy. *J. Am. Coll. Cardiol.* 5, 57B-65B (1985).
48. Naqvi, N., Li, M., Yahiro, E., Graham, R. M. & Husain, A. Insights into the characteristics of mammalian cardiomyocyte terminal differentiation shown through the study of mice with a dysfunctional c-kit. in *Pediatric Cardiology* (2009). doi:10.1007/s00246-008-9366-1
49. Günthel, M., Barnett, P. & Christoffels, V. M. Development, Proliferation, and Growth of the Mammalian Heart. *Mol. Ther.* 26, 1599–1609 (2018).
50. Khandoker, A. H., Karmakar, C., Kimura, Y. & Palaniswami, M. Development of Fetal Heart Rate Dynamics before and after 30 and 35 Weeks of Gestation. in *Computing in Cardiology 2013* (2013).
51. Kawashima, T. The autonomic nervous system of the human heart with special reference to its origin, course, and peripheral distribution. *Anat. Embryol. (Berl).* (2005). doi:10.1007/s00429-005-0462-1
52. Wladimiroff, J. W. & Seelen, J. C. Fetal heart action in early pregnancy. Development of fetal vagal function. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 2, 55–63 (1972).
53. Cheng, G., Zhou, X., Qu, J., Ashwell, K. W. S. & Paxinos, G. Central vagal sensory and motor connections: Human embryonic and fetal development. *Auton. Neurosci. Basic Clin.* (2004). doi:10.1016/j.autneu.2004.06.008
54. Walker, A. M., Cannata, J., Dowling, M. H., Ritchie, B. & Maloney, J. E. Sympathetic and parasympathetic control of heart rate in unanaesthetized fetal and newborn lambs. *Neonatology* (1978). doi:10.1159/000241063
55. Breier, G. Vasculogenesis. in *Cell Signaling and Growth Factors in Development: From Molecules to Organogenesis* (2008). doi:10.1002/9783527619689.ch24
56. Kolte, D., McClung, J. A. & Aronow, W. S. *Vasculogenesis and Angiogenesis. Translational Research in Coronary Artery Disease: Pathophysiology to Treatment* (Elsevier Inc., 2015). doi:10.1016/B978-0-12-802385-3.00006-1
57. Kaur, A. & Kaur, A. Transvaginal ultrasonography in first trimester of pregnancy and its comparison with transabdominal ultrasonography. *J. Pharm. Bioallied Sci.* (2011). doi:10.4103/0975-7406.84432
58. Peak, C. W., Cross, L., Singh, A. & Gaharwar, A. K. Microscale technologies for engineering complex tissue structures. in *Microscale Technologies for Cell Engineering* (2015). doi:10.1007/978-3-319-20726-1_1
59. Hooper, S. B., Polglase, G. R. & Te Pas, A. B. A physiological approach to the timing of umbilical cord clamping at birth. *Archives of Disease in Childhood: Fetal and Neonatal Edition* (2015). doi:10.1136/archdischild-2013-305703
60. Acharya, G., Sonesson, S. E., Flo, K., Räsänen, J. & Odibo, A. Hemodynamic aspects of normal human feto-placental (umbilical) circulation. *Acta Obstetricia et Gynecologica Scandinavica* 95, 672–682 (2016).
61. Gao, Y. & Ray, J. Regulation of the Pulmonary Circulation in the Fetus and Newborn. *Circulation* (2010). doi:10.1152/physrev.00032.2009.
62. Adamson, T. M. *et al.* Lung liquid production and composition in the 'in utero' foetal lamb. *Aust. J. Exp. Biol. Med. Sci.* (1975). doi:10.1038/icb.1975.7
63. Hislop, A. A. Airway and blood vessel interaction during lung development. *Journal of Anatomy* (2002). doi:10.1046/j.1469-7580.2002.00097.x
64. Heymann, M. A. Control of the pulmonary circulation in the fetus and during the transitional period to air breathing. in *European Journal of Obstetrics Gynecology and Reproductive Biology* (1999). doi:10.1016/S0301-2115(98)00321-2
65. Murphy, P. J. The fetal circulation. *Contin. Educ. Anaesthesia, Crit. Care Pain* 5, 107–112 (2005).
66. Muniyappa, R., Montagnani, M., Koh, K. K. & Quon, M. J. Medical gallery of Blausen

Medical 2014. *WikiJournal Med.* 1, (2014).
67. Tennant, M. & McGeachie, J. K. Blood Vessel Structure and Function: A Brief Update on Recent Advances *Aust. N. Z. J. Surg.* (1990). doi:10.1111/j.1445-2197.1990.tb07468.x
68. Augustin, H. G., Kozian, D. H. & Johnson, R. C. Differentiation of endothelial cells: Analysis of the constitutive and activated endothelial cell phenotypes. *BioEssays* 16, 901–906 (1994).
69. Galley, H. F. & Webster, N. R. Physiology of the endothelium. *British Journal of Anaesthesia* 93, 105–113 (2004).
70. Sheng, Y. & Zhu, L. The crosstalk between autonomic nervous system and blood vessels. *Int J Physiol Pathophysiol Pharmacol* (2018).
71. Sandoo, A., Veldhuijzen van Zanten, J. J. C. S., Metsios, G. S., Carroll, D. & Kitas, G. D. The Endothelium and Its Role in Regulating Vascular Tone. *Open Cardiovasc. Med. J.* (2010). doi:10.2174/1874192401004010302
72. Tousoulis, D., Kampoli, A.-M., Tentolouris, C., Papageorgiou, N. & Stefanadis, C. The role of nitric oxide on endothelial function. *Curr. Vasc. Pharmacol.* 10, 4–18 (2012).
73. Chatterjee, A. & Catravas, J. D. Endothelial nitric oxide (NO) and its pathophysiologic regulation. *Vascular Pharmacology* 49, 134–140 (2008).
74. Krause, B. J., Hanson, M. A. & Casanello, P. Role of nitric oxide in placental vascular development and function. *Placenta* (2011). doi:10.1016/j.placenta.2011.06.025
75. Klabunde, R. E. Cardiovascular Physiology Concepts. *Lippincott Williams & Wilkins* 256 (2004). doi:citeulike-article-id:2086320
76. Casanello, P., Escudero, C. & Sobrevia, L. Equilibrative Nucleoside (ENTs) and Cationic Amino Acid (CATs) Transporters: Implications in Foetal Endothelial Dysfunction in Human Pregnancy Diseases. *Curr. Vasc. Pharmacol.* (2007). doi:10.2174/157016107779317198
77. Jia, G., Durante, W. & Sowers, J. R. Endothelium-Derived Hyperpolarizing Factors: A Potential Therapeutic Target for Vascular Dysfunction in Obesity and Insulin Resistance. *Diabetes* 65, 2118–2120 (2016).
78. Mitchell, J. A., Ali, F., Bailey, L., Moreno, L. & Harrington, L. S. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. in *Experimental Physiology* (2008). doi:10.1113/expphysiol.2007.038588
79. Luksha, L., Agewall, S. & Kublickiene, K. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis* (2009). doi:10.1016/j.atherosclerosis.2008.06.008
80. Jufri, N. F., Mohamedali, A., Avolio, A. & Baker, M. S. Mechanical stretch: Physiological and pathological implications for human vascular endothelial cells. *Vascular Cell* (2015). doi:10.1186/s13221-015-0033-z
81. Schneider, D. *et al.* Oxidative stress as common trait of endothelial dysfunction in chorionic arteries from fetuses with IUGR and LGA. *Placenta* (2015). doi:10.1016/j.placenta.2015.02.003
82. Aldoretta, P. W. & Hay Jr., W. W. Metabolic substrates for fetal energy metabolism and growth. *Clin Perinatol* (1995).
83. Bauer, M. K. *et al.* Fetal growth and placental function. in *Molecular and Cellular Endocrinology* 140, 115–120 (1998).
84. Gude, N. M., Roberts, C. T., Kalionis, B. & King, R. G. Growth and function of the normal human placenta. *Thrombosis Research* 114, 397–407 (2004).
85. Bryant-Greenwood, G. D. The extracellular matrix of the human fetal membranes: Structure and function. *Placenta* 19, 1–11 (1998).
86. Wang, Y. & Zhao, S. *Vascular Biology of the Placenta. Colloquium Series on Integrated Systems Physiology: From Molecule to Function* 2, (2010).
87. Kiserud, T. Physiology of the fetal circulation. *Seminars in Fetal and Neonatal Medicine*

(2005). doi:10.1016/j.siny.2005.08.007
88. Roberts, J. M. & Escudero, C. The placenta in preeclampsia. *Pregnancy Hypertension* (2012). doi:10.1016/j.preghy.2012.01.001
89. Pop, G. A. M. *et al.* The clinical significance of whole blood viscosity in (cardio)vascular medicine. *Neth. Heart J.* 10, 512–516 (2002).
90. K., S. *et al.* How blood viscosity influences changes in circulation during pregnancy? *Fukuoka Igaku Zasshi* (1992).
91. Soma-Pillay, P., Nelson-Piercy, C., Tolppanen, H. & Mebazaa, A. Physiological changes in pregnancy. *Cardiovasc. J. Afr.* (2016). doi:10.5830/CVJA-2016-021
92. Jopling, J., Henry, E., Wiedmeier, S. E. & Christensen, R. D. Reference Ranges for Hematocrit and Blood Hemoglobin Concentration During the Neonatal Period: Data From a Multihospital Health Care System. *Pediatrics* (2009). doi:10.1542/peds.2008-2654
93. Reinhart, W. H., Danoff, S. J., King, R. G. & Chien, S. Rheology of fetal and maternal blood. *Pediatr. Res.* 19, 147–153 (1985).
94. Pijnenborg, R., Vercruysse, L. & Hanssens, M. The Uterine Spiral Arteries In Human Pregnancy: Facts and Controversies. *Placenta* (2006). doi:10.1016/j.placenta.2005.12.006
95. Burton, G. J., Woods, A. W., Jauniaux, E. & Kingdom, J. C. P. Rheological and Physiological Consequences of Conversion of the Maternal Spiral Arteries for Uteroplacental Blood Flow during Human Pregnancy. *Placenta* (2009). doi:10.1016/j.placenta.2009.02.009
96. Krishna, U. & Bhalerao, S. Placental insufficiency and fetal growth restriction. *Journal of Obstetrics and Gynecology of India* 61, 505–511 (2011).
97. Rizzo, G. & Arduini, D. Fetal cardiac function in intrauterine growth retardation. *Am. J. Obstet. Gynecol.* 165, 876–882 (1991).
98. Lapidus, B. A. M. Effects of preeclampsia on the mother, fetus and child. *Pediatrics* 57, 411–413 (1976).
99. Regnault, T. R. H. *et al.* Placental expression of VEGF, PlGF and their receptors in a model of placental insufficiency - Intrauterine growth restriction (PI-IUGR). *Placenta* (2002). doi:10.1053/plac.2001.0757
100. Semczuk-Sikora, A. *et al.* [Maternal serum concentration of angiogenic factors: PIGF, VEGF and VEGFR-1 and placental volume in pregnancies complicated by intrauterine growth restriction]. *Ginekol. Pol.* (2007).
101. Cetin, I. *et al.* PlGF in a clinical setting of pregnancies at risk of preeclampsia and/or intrauterine growth restriction. *J. Matern. Neonatal Med.* (2017). doi:10.3109/14767058.2016.1168800
102. Molvarec, A. *et al.* Comparison of placental growth factor and fetal flow Doppler ultrasonography to identify fetal adverse outcomes in women with hypertensive disorders of pregnancy: An observational study. *BMC Pregnancy Childbirth* (2013). doi:10.1186/1471-2393-13-161
103. Wada, Y. *et al.* Role of vascular endothelial growth factor in maintenance of pregnancy in mice. *Endocrinology* 154, 900–910 (2013).
104. Teloken, P. E. & Mulhall, J. P. Impact of phosphodiesterase type 5 inhibitors on endothelial function. *Rev. Urol.* 10, 26–30 (2008).
105. Rätsep, M. T., Carmeliet, P., Adams, M. A. & Croy, B. A. Impact of placental growth factor deficiency on early mouse implant site angiogenesis. *Placenta* (2014). doi:10.1016/j.placenta.2014.07.006
106. Azliana, A. *et al.* Vascular endothelial growth factor expression in placenta of hypertensive disorder in pregnancy. *Indian J. Pathol. Microbiol.* 60, 515–520 (2017).
107. Kingdom, J. C. P. & Kaufmann, P. Oxygen and placental villous development: Origins of fetal hypoxia. *Placenta* 18, 613–621 (1997).
108. Shore, V. H. *et al.* Vascular endothelial growth factor, placenta growth factor and their

receptors in isolated human trophoblast. *Placenta* (1997). doi:10.1016/S0143-4004(97)90007-2
109. Genbacev, O., Joslin, R., Damsky, C. H., Polliotti, B. M. & Fisher, S. J. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J. Clin. Invest.* (1996). doi:10.1172/JCI118447
110. Castillo-Melendez, M. *et al.* Cerebrovascular adaptations to chronic hypoxia in the growth restricted lamb. *Int. J. Dev. Neurosci.* 45, 55–65 (2015).
111. Poston, L. The control of blood flow to the placenta. *Exp Physiol* 82, 377–387 (1997).
112. Boura, A. L. A., Leitch, I. M., Read, M. A. & Walters, W. A. W. The control of fetal vascular resistance in the human placenta. *Placenta* 19, 299–313 (1998).
113. Meschia, G. Circulation to female reproductive organs. *Handb. Physiol.* 241–269 (1983). doi:10.1002/cphy.cp020308
114. Reynolds, L. P., Ferrell, C. L., Robertson, D. A. & Ford, S. P. Metabolism of the gravid uterus, foetus and utero-placenta at several stages of gestation in cows. *J. Agric. Sci.* (1986). doi:10.1017/S0021859600063309
115. Da Costa, A. G., Mauad Filho, F., Spara, P., Gadelha, E. B. & Santana Netto, P. V. Fetal hemodynamics evaluated by Doppler velocimetry in the second half of pregnancy. *Ultrasound Med. Biol.* (2005). doi:10.1016/j.ultrasmedbio.2005.04.009
116. Reilly, F. D. & Russell, P. T. Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. *Anat. Rec.* (1977). doi:10.1002/ar.1091880302
117. Kwon, H., Wu, G., Meininger, C. J., Bazer, F. W. & Spencer, T. E. Developmental Changes in Nitric Oxide Synthesis in the Ovine Placenta1. *Biol. Reprod.* (2004). doi:10.1095/biolreprod.103.023184
118. Diket, A. L. *et al.* Nitric oxide inhibition causes intrauterine growth retardation and hind-limb disruptions in rats. *Am. J. Obstet. Gynecol.* (1994). doi:10.1016/0002-9378(94)90141-4
119. Lyall, F., Greer, I. A., Young, A. & Myatt, L. Nitric oxide concentrations are increased in the feto-placental circulation in intrauterine growth restriction. *Placenta* 17, 165–168 (1996).
120. Peacock, A. J. ABC of oxygen: oxygen at high altitude. *BMJ* 317, 1063–6 (1998).
121. Keyes, L. E. *et al.* Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. *Pediatr. Res.* 54, 20–25 (2003).
122. Moore, L. G. Fetal Growth Restriction and Maternal Oxygen Transport during High Altitude Pregnancy. *High Alt. Med. Biol.* 4, 141–156 (2003).
123. Moore, L. G. *et al.* Maternal adaptation to high-altitude pregnancy: An experiment of nature - A review. *Placenta* (2004). doi:10.1016/j.placenta.2004.01.008
124. Julian, C. G. *et al.* Augmented uterine artery blood flow and oxygen delivery protect Andeans from altitude-associated reductions in fetal growth. *AJP Regul. Integr. Comp. Physiol.* (2009). doi:10.1152/ajpregu.90945.2008
125. Belkacemi, L., Nelson, D. M., Desai, M. & Ross, M. G. Maternal Undernutrition Influences Placental-Fetal Development1. *Biol. Reprod.* 83, 325–331 (2010).
126. Lindeboom, M., Portrait, F. & van den Berg, G. J. Long-run effects on longevity of a nutritional shock early in life: The Dutch Potato famine of 1846-1847. *J. Health Econ.* (2010). doi:10.1016/j.jhealeco.2010.06.001
127. Doherty, C. B., Lewis, R. M., Sharkey, A. & Burton, G. J. Placental composition and surface area but not vascularization are altered by maternal protein restriction in the rat. *Placenta* (2003). doi:10.1053/plac.2002.0858
128. Wallace, J. M., Aitken, R. P., Milne, J. S. & Hay, W. W. Nutritionally Mediated Placental Growth Restriction in the Growing Adolescent: Consequences for the Fetus1. *Biol. Reprod.* (2004). doi:10.1095/biolreprod.104.030965
129. Lumey, L. H. Compensatory placental growth after restricted maternal nutrition in early

pregnancy. *Placenta* (1998). doi:10.1016/S0143-4004(98)90105-9
130. Fall, C. H. D. Fetal malnutrition and long-term outcomes. in *Nestle Nutrition Institute Workshop Series* (2013). doi:10.1159/000348384
131. Stewart, C. Recreational drug use in pregnancy. *Int. J. Gynecol. Obstet.* S80 (2009). doi:http://dx.doi.org/10.1016/S0020-7292%2809%2960323-4
132. Bernhoft, R. A. Cadmium toxicity and treatment. *The Scientific World Journal* (2013). doi:10.1155/2013/394652
133. Wang, H. *et al.* Maternal serum cadmium level during pregnancy and its association with small for gestational age infants: A population-based birth cohort study. *Sci. Rep.* 6, 1–7 (2016).
134. Wang, Z. *et al.* Cadmium-induced teratogenicity: Association with ROS-mediated endoplasmic reticulum stress in placenta. *Toxicol. Appl. Pharmacol.* (2012). doi:10.1016/j.taap.2012.01.001
135. Everson, T. M. *et al.* Maternal exposure to selenium and cadmium, fetal growth, and placental expression of steroidogenic and apoptotic genes. *Environ. Res.* (2017). doi:10.1016/j.envres.2017.06.016
136. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical agents and related occupations. *IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer* (2012). doi:now it's officially a carcinogen...
137. Drukteinis, J. S., Medrano, T., Ablordeppey, E. A., Kitzman, J. M. & Shiverick, K. T. Benzo[a]pyrene, but not 2,3,7,8-TCDD, induces G2/M cell cycle arrest, p21CIP1 and p53 phosphorylation in human choriocarcinoma JEG-3 cells: a distinct signaling pathway. *Placenta* (2005). doi:10.1016/j.placenta.2005.01.013
138. Kadyrov, M., Kingdom, J. C. P. & Huppertz, B. Divergent trophoblast invasion and apoptosis in placental bed spiral arteries from pregnancies complicated by maternal anemia and early-onset preeclampsia/intrauterine growth restriction. *Am. J. Obstet. Gynecol.* 194, 557–563 (2006).
139. Burton, G. J., Palmer, M. E. & Dalton, K. J. Morphometric differences between the placental vasculature of non-smokers, smokers and ex-smokers. *BJOG An Int. J. Obstet. Gynaecol.* (1989). doi:10.1111/j.1471-0528.1989.tb03344.x
140. Van der Velde, W. J., Copius Peereboom-Stegeman, J. H. J., Treffers, P. E. & James, J. Structural changes in the placenta of smoking mothers: A quantitative study. *Placenta* (1983). doi:10.1016/S0143-4004(83)80002-2
141. Kawashima, A. *et al.* Effects of maternal smoking on the placental expression of genes related to angiogenesis and apoptosis during the first trimester. *PLoS One* (2014). doi:10.1371/journal.pone.0106140
142. Shimada, S. *et al.* High Blood Viscosity Is Closely Associated With Cigarette Smoking and Markedly Reduced by Smoking Cessation. *Circ. J.* (2011). doi:10.1253/circj.CJ-10-0335
143. Vavilala, M. S. *et al.* Impaired Cerebral Autoregulation in Infants and Young Children Early after Inflicted Traumatic Brain Injury: A Preliminary Report. *J. Neurotrauma* 24, 87–96 (2007).
144. Grote, J., Dall, P., Oltmanns, K. & Stolp, W. The effect of increased blood carbon monoxide levels on the hemoglobin oxygen affinity during pregnancy. *Adv. Exp. Med. Biol.* 345, 145–150 (1994).
145. Reeves, S. & Bernstein, I. Effects of maternal tobacco-smoke exposure on fetal growth and neonatal size. *Expert Review of Obstetrics and Gynecology* 3, 719–730 (2008).
146. Socol, M. L., Manning, F. A., Murata, Y. & Druzin, M. L. Maternal smoking causes fetal hypoxia: Experimental evidence. *Am. J. Obstet. Gynecol.* (1982). doi:10.1016/S0002-9378(16)32339-0
147. Cliver, S. P. *et al.* The Relationships Among Psychosocial Profile, Maternal Size and

Smoking in Predicting Fetal Growth Retardation.. *Obstet. Gynecol.* (1992).
148. Pipkin, F. B. Smoking in moderate/severe preeclampsia worsens pregnancy outcome, but smoking cessation limits the damage. *Hypertension* (2008). doi:10.1161/HYPERTENSIONAHA.107.106559
149. Aagaard-Tillery, K. M., Porter, T. F., Lane, R. H., Varner, M. W. & Lacoursiere, D. Y. In utero tobacco exposure is associated with modified effects of maternal factors on fetal growth. *Am. J. Obstet. Gynecol.* (2008). doi:10.1016/j.ajog.2007.06.078
150. Tibiletti, M. G., Bulgheroni, C., Scorbati, E., Balestrieri, D. & Bolis, P. F. Confined placental mosaicism and intrauterine growth retardation. *Ital. J. Gynaecol. Obstet.* 6, 93–96 (1994).
151. Puccio, G. *et al.* Intrauterine growth restriction and congenital malformations: A retrospective epidemiological study. *Ital. J. Pediatr.* (2013). doi:10.1186/1824-7288-39-23
152. Robinson, W. P. *et al.* Assessing the role of placental trisomy in preeclampsia and intrauterine growth restriction. *Prenat. Diagn.* 30, 1–8 (2010).
153. Monk, D. & Moore, G. E. Intrauterine growth restriction - Genetic causes and consequences. *Semin. Fetal Neonatal Med.* 9, 371–378 (2004).
154. Longo, S., Borghesi, A., Tzialla, C. & Stronati, M. IUGR and infections. *Early Human Development* 90, (2014).
155. Lanté, F. *et al.* Neurodevelopmental damage after prenatal infection: Role of oxidative stress in the fetal brain. *Free Radic. Biol. Med.* (2007). doi:10.1016/j.freeradbiomed.2007.01.027
156. Salvemini, D. & Cuzzocrea, S. Oxidative stress in septic shock and disseminated intravascular coagulation. *Free Radical Biology and Medicine* (2002). doi:10.1016/S0891-5849(02)00961-9
157. Adams Waldorf, K. M. & McAdams, R. M. Influence of infection during pregnancy on fetal development. *Reproduction* 146, R151-62 (2013).
158. Owen, P. & Khan, K. S. Fetal growth velocity in the prediction of intrauterine growth retardation in a low risk population. *BJOG An Int. J. Obstet. Gynaecol.* (1998). doi:10.1111/j.1471-0528.1998.tb10155.x
159. Garcia-Canadilla, P. *et al.* A Computational Model of the Fetal Circulation to Quantify Blood Redistribution in Intrauterine Growth Restriction. *PLoS Comput. Biol.* (2014). doi:10.1371/journal.pcbi.1003667
160. Iams, J. D. Small for gestational age (SGA) and fetal growth restriction (FGR). *American Journal of Obstetrics and Gynecology* (2010). doi:10.1016/j.ajog.2009.10.890
161. Marconi, A. M. *et al.* Comparison of fetal and neonatal growth curves in detecting growth restriction. *Obstet. Gynecol.* 112, 1227–1234 (2008).
162. Ojeda, N. B., Grigore, D. & Alexander, B. T. Intrauterine Growth Restriction: Fetal Programming of Hypertension and Kidney Disease. *Adv. Chronic Kidney Dis.* (2008). doi:10.1053/j.ackd.2008.01.001
163. Allison, B. J. *et al.* Fetal in vivo continuous cardiovascular function during chronic hypoxia. *J. Physiol.* (2016). doi:10.1113/JP271091
164. Roza, S. J. *et al.* What is spared by fetal brain-sparing? Fetal circulatory redistribution and behavioral problems in the general population. *Am. J. Epidemiol.* 168, 1145–1152 (2008).
165. Dong, L. *et al.* Intrauterine Growth Restriction Impairs Small Intestinal Mucosal Immunity in Neonatal Piglets. *J. Histochem. Cytochem.* (2014). doi:10.1369/0022155414532655
166. Brown, L. D. & Hay, W. W. Impact of placental insufficiency on fetal skeletal muscle growth. *Mol. Cell. Endocrinol.* (2016). doi:10.1016/j.mce.2016.03.017
167. Giussani, D. A., Spencer, J. A., Moore, P. J., Bennet, L. & Hanson, M. A. Afferent and efferent components of the cardiovascular reflex responses to acute hypoxia in term fetal sheep. *J. Physiol.* (1993). doi:10.1113/jphysiol.1993.sp019521

168. Anderson, B. Y. P. A. W., Glick, K. L., Killam, A. P. & Mainwaring, R. D. On in utero. 557–573 (1986).
169. Giussani, D. A. The fetal brain sparing response to hypoxia: Physiological mechanisms. *Journal of Physiology* 594, 1215–1230 (2016).
170. Boudoulas, H., Rittgers, S. E., Lewis, R. P., Leier, C. V. & Weissler, A. M. Changes in diastolic time with various pharmacologic agents. Implication for myocardial perfusion. *Circulation* (1979). doi:10.1161/01.CIR.60.1.164
171. Green, L. R., Bennet, L., Robson, S. & Hanson, M. A. The role of carotid chemoreceptors in the effects of hypoxia on renal blood flow in the late gestation sheep fetus. *Exp. Physiol.* (1997). doi:10.1113/expphysiol.1997.sp004007
172. Moore, P. J. & Hanson, M. A. The role of peripheral chemoreceptors in the rapid response of the pulmonary vasculature of the late gestation sheep fetus to changes in PaO2. *J Dev Physiol* (1991). doi:10.1080/03461238.1951.10432139
173. Iwamoto, H. S., Rudolph, A. M., Mirkin, B. L. & Keil, L. C. Circulatory and humoral responses of sympathectomized fetal sheep to hypoxemia. *Am. J. Physiol. Circ. Physiol.* (1983). doi:10.1152/ajpheart.1983.245.5.H767
174. Jensen, A. & Hanson, M. A. Circulatory responses to acute asphyxia in intact and chemodenervated fetal sheep near term. *Reprod. Fertil. Dev.* (1995). doi:10.1071/RD9951351
175. Green, L. R., McGarrigle, H. H. G., Bennet, L. & Hanson, M. A. Angiotensin II and cardiovascular chemoreflex responses to acute hypoxia in late gestation fetal sheep. *J. Physiol.* (1998). doi:10.1111/j.1469-7793.1998.857bs.x
176. Baschat, A. A. *et al.* Coronary artery blood flow visualization signifies hemodynamic deterioration in growth-restricted fetuses. *Ultrasound Obstet. Gynecol.* 16, 425–431 (2000).
177. Venditti, P., Di Stefano, L. & Di Meo, S. Mitochondrial metabolism of reactive oxygen species. *Mitochondrion* (2013). doi:10.1016/j.mito.2013.01.008
178. Simon, H.-U., Haj-Yehia, A. & Levi-Schaffer, F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* (2000). doi:10.1023/A:1009616228304
179. Gamaley, I. A. & Klyubin, I. V. Roles of reactive oxygen species: signaling and regulation of cellular functions. *Int. Rev. Cytol.* 188, 203–55 (1999).
180. Thompson, L. P. & Al-Hasan, Y. Impact of oxidative stress in fetal programming. *Journal of Pregnancy* (2012). doi:10.1155/2012/582748
181. Simmons, R. A. Developmental origins of diabetes: The role of oxidative stress. *Best Pract. Res. Clin. Endocrinol. Metab.* 26, 701–708 (2012).
182. Bell, E. L., Klimova, T. A., Eisenbart, J., Schumacker, P. T. & Chandel, N. S. Mitochondrial Reactive Oxygen Species Trigger Hypoxia-Inducible Factor-Dependent Extension of the Replicative Life Span during Hypoxia. *Mol. Cell. Biol.* (2007). doi:10.1128/MCB.02265-06
183. Frank, L. Development of the antioxidant defences in fetal life. *Semin. Neonatol.* 3, 173–182 (1998).
184. Zhao, J. Interplay among nitric oxide and reactive oxygen species: a complex network determining cell survival or death. *Plant Signal. Behav.* 2, 544–7 (2007).
185. Gardner, D. S., Fletcher, A. J. W., Fowden, A. L. & Giussani, D. A. A novel method for controlled and reversible long term compression of the umbilical cord in fetal sheep. *J. Physiol.* (2001). doi:10.1111/j.1469-7793.2001.00217.x
186. Giussani, D. A. *et al.* Developmental programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress. *PLoS One* 7, (2012).
187. Thakor, A. S., Herrera, E. A., Serón-Ferré, M. & Giussani, D. A. Melatonin and vitamin C increase umbilical blood flow via nitric oxide-dependent mechanisms. *J. Pineal Res.* 49, 399–406 (2010).
188. Garite, T. J., Clark, R. & Thorp, J. A. Intrauterine growth restriction increases morbidity

and mortality among premature neonates. *Am. J. Obstet. Gynecol.* (2004). doi:10.1016/j.ajog.2004.01.036
189. Alshaikh, B., Salman, O., Soliman, N., Ells, A. & Yusuf, K. Pre-eclampsia and the risk of retinopathy of prematurity in preterm infants with birth weight <1500 g and/or <31 weeks' gestation. *BMJ Open Ophthalmol.* 1, e000049 (2017).
190. Manogura, A. C. *et al.* Predictors of necrotizing enterocolitis in preterm growth-restricted neonates. *Am. J. Obstet. Gynecol.* (2008). doi:10.1016/j.ajog.2007.11.048
191. Lal, M. K., Manktelow, B. N., Draper, E. S. & Field, D. J. Chronic Lung Disease of Prematurity and Intrauterine Growth Retardation: A Population-Based Study. *Pediatrics* (2003). doi:10.1542/peds.111.3.483
192. Lithell, H. O. *et al.* Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. *Bmj* (1996). doi:10.1136/bmj.312.7028.406
193. Abbott, D. H., Bruns, C. M., Barnett, D. K. & Dumesic, D. A. Fetal programming of polycystic ovary syndrome. in *Polycystic Ovary Syndrome, Second Edition* (2007). doi:10.1017/CBO9780511545191.016
194. Cohen, E., Wong, F. Y., Horne, R. S. & Yiallourou, S. R. Intrauterine growth restriction: impact on cardiovascular development and function throughout infancy. *Pediatr. Res.* 1–10 (2016). doi:10.1038/pr.2016.24
195. Zohdi, V., Lim, K., Pearson, J. T. & Jane Black, M. Developmental programming of cardiovascular disease following intrauterine growth restriction: Findings utilising a rat model of maternal protein restriction. *Nutrients* 7, 119–152 (2015).
196. Demicheva, E. & Crispi, F. Long-term follow-up of intrauterine growth restriction: Cardiovascular disorders. *Fetal Diagnosis and Therapy* 36, 143–153 (2014).
197. Yzydorczyk, C. *et al.* Endothelial dysfunction in individuals born after fetal growth restriction: cardiovascular and renal consequences and preventive approaches. *J. Dev. Orig. Health Dis.* 1–17 (2017). doi:10.1017/S2040174417000265
198. Bjarnegård, N., Morsing, E., Cinthio, M., Länne, T. & Brodszki, J. Cardiovascular function in adulthood following intrauterine growth restriction with abnormal fetal blood flow. *Ultrasound Obstet. Gynecol.* 41, 177–184 (2013).
199. Barker, D. The fetal and infant origins of adult disease. *BMJ Br. Med. J.* 301, 1111 (1990).
200. Crispi, F., Miranda, J. & Gratacós, E. Long-term cardiovascular consequences of fetal growth restriction: biology, clinical implications, and opportunities for prevention of adult disease. *Am. J. Obstet. Gynecol.* 218, S869–S879 (2018).
201. Martinez-Payo, C., Bernabeu, R., Villar, I. & Goy, E. Intrauterine Growth Restriction Associated with Hematologic Abnormalities: Probable Manifestations of Placental Mesenchymal Dysplasia. *Am. J. Perinatol. Reports* (2015). doi:10.1055/s-0034-1394152
202. Chaoui, R., Bollmann, R., Goldner, B., Heling, K. S. & Tennstedt, C. Fetal cardiomegaly: Echocardiographic findings and outcome in 19 cases. *Fetal Diagn. Ther.* (1994). doi:10.1159/000263915
203. Crispi, F. *et al.* Fetal growth restriction results in remodeled and less efficient hearts in children. *Circulation* 121, 2427–2436 (2010).
204. Pérez-Cruz, M. *et al.* Fetal cardiac function in late-onset intrauterine growth restriction vs small-for-gestational age, as defined by estimated fetal weight, cerebroplacental ratio and uterine artery Doppler. *Ultrasound Obstet. Gynecol.* (2015). doi:10.1002/uog.14930
205. Martin, C. *et al.* Cardiac hypertrophy in chronically anemic fetal sheep: Increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. *Am. J. Obstet. Gynecol.* (1998). doi:10.1016/S0002-9378(98)70433-8
206. Corstius, H. B. *et al.* Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatr. Res.* (2005). doi:10.1203/01.PDR.0000157726.65492.CD

207. Crispi, F. *et al.* Cardiac dysfunction and cell damage across clinical stages of severity in growth-restricted fetuses. *Am. J. Obstet. Gynecol.* (2008). doi:10.1016/j.ajog.2008.06.056
208. Kamimura, D. *et al.* Delayed time to peak velocity is useful for detecting severe aortic stenosis. *J. Am. Heart Assoc.* (2016). doi:10.1161/JAHA.116.003907
209. Dybdahl, B. *et al.* Myocardial ischaemia and the inflammatory response: Release of heat shock protein 70 after myocardial infarction. *Heart* (2005). doi:10.1136/hrt.2003.028092
210. Zhang, P. *et al.* Long-term exposure to high altitude hypoxia during pregnancy increases fetal heart susceptibility to ischemia/reperfusion injury and cardiac dysfunction. *International Journal of Cardiology* (2018). doi:10.1016/j.ijcard.2018.07.046
211. Baird, N. A., Turnbull, D. W. & Johnson, E. A. Induction of the heat shock pathway during hypoxia requires regulation of heat shock factor by hypoxia-inducible factor-1. *J. Biol. Chem.* (2006). doi:10.1074/jbc.M608013200
212. Ritossa, F. A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia* (1962). doi:10.1007/BF02172188
213. Pockley, A. G. Heat shock proteins as regulators of the immune response. *Lancet* (2003). doi:10.1016/S0140-6736(03)14075-5
214. Molvarec, A. *et al.* Circulating heat shock protein 70 (HSPA1A) in normal and pathological pregnancies. *Cell Stress and Chaperones* (2010). doi:10.1007/s12192-009-0146-5
215. Semenza, G. L. Hypoxia-inducible factor 1: Master regulator of O2 homeostasis. *Current Opinion in Genetics and Development* (1998). doi:10.1016/S0959-437X(98)80016-6
216. Lee, J.-W., Bae, S.-H., Jeong, J.-W., Kim, S.-H. & Kim, K.-W. Hypoxia-inducible factor (HIF-1)α: its protein stability and biological functions. *Exp. Mol. Med.* (2004). doi:10.1038/emm.2004.1
217. Li, G., Bae, S. & Zhang, L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am. J. Physiol. Circ. Physiol.* (2004). doi:10.1152/ajpheart.00898.2003
218. Basu, P., Sen, U., Tyagi, N. & Tyagi, S. C. Blood flow interplays with elastin: Collagen and MMP: TIMP ratios to maintain healthy vascular structure and function. *Vasc. Health Risk Manag.* 6, 215–228 (2010).
219. Sehgal, A., Doctor, T. & Menahem, S. Cardiac function and arterial biophysical properties in small for gestational age infants: postnatal manifestations of fetal programming. *J. Pediatr.* 163, 1296–300 (2013).
220. Sehgal, A. *et al.* Cardiac Morphology and Function in Preterm Growth Restricted Infants: Relevance for Clinical Sequelae. *J. Paediatr. Child Health* 53, 3–3 (2017).
221. Cañas, D., Herrera, E. A., García-Herrera, C., Celentano, D. & Krause, B. J. Fetal growth restriction induces heterogeneous effects on vascular biomechanical and functional properties in guinea pigs (Cavia porcellus). *Front. Physiol.* 8, (2017).
222. Dodson, R. B. *et al.* Increased arterial stiffness and extracellular matrix reorganization in intrauterine growth-restricted fetal sheep. *Pediatr. Res.* 73, 147–154 (2013).
223. Cosmi, E., Visentin, S., Fanelli, T., Mautone, A. J. & Zanardo, V. Aortic intima media thickness in fetuses and children with intrauterine growth restriction. *Obstet. Gynecol.* 114, 1109–1114 (2009).
224. Zanardo, V. *et al.* Fetal aortic wall thickness: A marker of hypertension in IUGR children. *Hypertens. Res.* (2013). doi:10.1038/hr.2012.219
225. TRAPOLD, J. H. Role of venous return in the cardiovascular response following injection of ganglion-blocking agents. *Circ. Res.* 5, 444–450 (1957).
226. Goodfellow, J. *et al.* Endothelial function is impaired in fit young adults of low birth weight. *Cardiovasc. Res.* 40, 600–6 (1998).
227. Pallares, P. & Gonzalez-Bulnes, A. Intrauterine Growth Retardation in Endothelial Nitric Oxide Synthase-Deficient Mice Is Established from Early Stages of Pregnancy. *Biol. Reprod.* 78, 1002–1006 (2008).

228. Payne, J. A., Alexander, B. T. & Khalil, R. A. Reduced Endothelial Vascular Relaxation in Growth-Restricted Offspring of Pregnant Rats With Reduced Uterine Perfusion. *Hypertension* 42, 768–774 (2003).
229. Leeson, C. P. M., Kattenhorn, M., Morley, R., Lucas, A. & Deanfield, J. E. Impact of Low Birth Weight and Cardiovascular Risk Factors on Endothelial Function in Early Adult Life. *Circulation* 103, 1264–1268 (2001).
230. Beth, J. A. *et al.* Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease. *FASEB J.* (2016). doi:10.1096/fj.201500057
231. Mazzuca, M. Q., Wlodek, M. E., Dragomir, N. M., Parkington, H. C. & Tare, M. Uteroplacental insufficiency programs regional vascular dysfunction and alters arterial stiffness in female offspring. *J. Physiol.* 588, 1997–2010 (2010).
232. Fouzas, S. *et al.* Neonatal cardiac dysfunction in intrauterine growth restriction. *Pediatr. Res.* 75, 651–7 (2014).
233. Cheung, Y. F., Wong, K. Y., Barbara, C. C., Lam, B. C. C. & Tsoi, N. S. Relation of arterial stiffness with gestational age and birth weight. *Arch. Dis. Child.* (2004). doi:10.1136/adc.2003.025999
234. Gaillard, R., Steegers, E. A. P., Tiemeier, H., Hofman, A. & Jaddoe, V. W. V. Placental vascular dysfunction, fetal and childhood growth, and cardiovascular development: The generation r study. *Circulation* (2013). doi:10.1161/CIRCULATIONAHA.113.003881
235. Brodszki, J., Länne, T., Maršál, K. & Ley, D. Impaired vascular growth in late adolescence after intrauterine growth restriction. *Circulation* (2005). doi:10.1161/CIRCULATIONAHA.104.490326
236. Ross, M. G. & Beall, M. H. Adult Sequelae of Intrauterine Growth Restriction. *Seminars in Perinatology* 32, 213–218 (2008).
237. Skilton, M. R., Evans, N., Griffiths, K. A., Harmer, J. A. & Celermajer, D. S. Aortic wall thickness in newborns with intrauterine growth restriction. *Lancet* 365, 1484–1486 (2005).
238. Fisk, N. M. & Atun, R. Market failure and the poverty of new drugs in maternal health. *PLoS Med.* 5, 0022–0028 (2008).
239. Shavit, T. *et al.* Is it necessary to induce labor in cases of intrauterine growth restriction at term ? *J. Perinat. Med.* 40, 539–543 (2012).
240. Boers, K. E. *et al.* Induction versus expectant monitoring for intrauterine growth restriction at term: Randomised equivalence trial (DIGITAT). *BMJ* 342, 35 (2011).
241. Lee, V. R. *et al.* When is the optimal time to deliver late preterm IUGR fetuses with abnormal umbilical artery Dopplers? *J. Matern. Neonatal Med.* 29, 690–695 (2016).
242. Ortigosa Rocha, C., Bittar, R. E. & Zugaib, M. Neonatal Outcomes of Late-Preterm Birth Associated or Not with Intrauterine Growth Restriction. *Obstet. Gynecol. Int.* (2010). doi:10.1155/2010/231842
243. Barker, D. J. P. *et al.* The early origins of chronic heart failure: Impaired placental growth and initiation of insulin resistance in childhood. *Eur. J. Heart Fail.* 12, 819–825 (2010).
244. Thornton, J. G. *et al.* Infant wellbeing at 2 years of age in the Growth Restriction Intervention Trial (GRIT): Multicentred randomised controlled trial. *Lancet* (2004). doi:10.1016/S0140-6736(04)16809-8
245. Walker, D.-M. *et al.* The Growth Restriction Intervention Trial: long-term outcomes in a randomized trial of timing of delivery in fetal growth restriction. *Am. J. Obstet. Gynecol.* 204, 34.e1-34.e9 (2011).
246. Poston, L. *et al.* Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet* 367, 1145–1154 (2006).
247. Villar, J. *et al.* World Health Organisation multicentre randomised trial of supplementation with vitamins C and e among pregnant women at high risk for pre-eclampsia in populations of low nutritional status from developing countries. *BJOG An Int. J. Obstet.*

Gynaecol. 116, 780–788 (2009).
248. Kaandorp, J. J. *et al.* Maternal allopurinol administration during suspected fetal hypoxia:A novel neuroprotective intervention? A multicentre randomised placebo controlled trial. in *Archives of Disease in Childhood: Fetal and Neonatal Edition* (2015). doi:10.1136/archdischild-2014-306769
249. Miller, S. L. *et al.* Antenatal antioxidant treatment with melatonin to decrease newborn neurodevelopmental deficits and brain injury caused by fetal growth restriction. *J. Pineal Res.* 56, 286–294 (2014).
250. Tare, M. *et al.* Maternal melatonin administration mitigates coronary stiffness and endothelial dysfunction, and improves heart resilience to insult in growth restricted lambs. *J. Physiol.* 00, 1–15 (2014).
251. Jackson, T. S., Xu, a, Vita, J. a & Keaney, J. F. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ. Res.* 83, 916–922 (1998).
252. González-Candia, A. *et al.* Potential adverse effects of antenatal melatonin as a treatment for intrauterine growth restriction: findings in pregnant sheep. *Am. J. Obstet. Gynecol.* (2016). doi:10.1016/j.ajog.2016.02.040
253. Jahnke, G. *et al.* Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats. *Toxicol. Sci.* 50, 271–279 (1999).
254. Francis, S. H. & Corbin, J. D. Sildenafil: efficacy, safety, tolerability and mechanism of action in treating erectile dysfunction. *Expert Opin. Drug Metab. Toxicol.* 1, 283–293 (2005).
255. Ghofrani, H. A. *et al.* Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. *Ann. Intern. Med.* 136, 515–522 (2002).
256. Zhang, R. *et al.* Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke* 33, 2675–2680 (2002).
257. Rapôso, C., Luna, R. L. de A., Nunes, A. K. S., Thomé, R. & Peixoto, C. A. Role of iNOS-NO-cGMP signaling in modulation of inflammatory and myelination processes. *Brain Res. Bull.* 104, 60–73 (2014).
258. Musicki, B., Bivalacqua, T. J., Champion, H. C. & Burnett, A. L. Sildenafil promotes eNOS activation and inhibits NADPH oxidase in the transgenic sickle cell mouse penis. *J. Sex. Med.* 11, 424–430 (2014).
259. Vlachopoulos, C. *et al.* Type 5 phosphodiesterase inhibition by sildenafil abrogates acute smoking-induced endothelial dysfunction. *Am. J. Hypertens.* 17, 1040–1044 (2004).
260. Deyoung, L., Chung, E., Kovac, J. R., Romano, W. & Brock, G. B. Daily use of sildenafil improves endothelial function in men with type 2 diabetes. *J. Androl.* 33, 176–180 (2012).
261. Itani, N., Skeffington, K. L., Beck, C. & Giussani, D. A. Sildenafil therapy for fetal cardiovascular dysfunction during hypoxic development: studies in the chick embryo. *J. Physiol.* 595, 1563–1573 (2017).
262. Sharp, A. *et al.* Articles Maternal sildenafil for severe fetal growth restriction (STRIDER): a multicentre, randomised, placebo-controlled, double-blind trial. *Lancet child Adolesc. Heal.* 5, 17–20 (2017).
263. Smith, G. C. S. The STRIDER trial: one step forward, one step back. *Lancet Child Adolesc. Heal.* 2, 90 (2018).
264. Morsy, M. A. *et al.* Sildenafil ameliorates gentamicin-induced nephrotoxicity in rats: Role of iNOS and eNOS. *J. Toxicol.* 2014, (2014).
265. Khazaei, M., Roshankhah, S., Ghorbani, R. & Chobsaz, F. Sildenafil Effect on Nitric Oxide Secretion by Normal Human Endometrial Epithelial Cells Cultured In vitro. *Int J Fertil Steril* 5, 142–147 (2011).
266. Dishy, V. *et al.* The effect of sildenafil on nitric oxide-mediated vasodilation in healthy men. *Clin. Pharmacol. Ther.* 70, 270–279 (2001).
267. Groom, K. M. *et al.* Clinicians should stop prescribing sildenafil for fetal growth

restriction (FGR): comment from the STRIDER Consortium. *Ultrasound Obstet. Gynecol.* (2018). doi:10.1002/uog.19186
268. Bialkowski, A., Moenkemeyer, F. & Patel, N. Intravenous sildenafil in the management of pulmonary hypertension associated with congenital diaphragmatic hernia. *Eur. J. Pediatr. Surg.* (2015). doi:10.1055/s-0033-1357757
269. Kipfmueller, F. *et al.* Continuous intravenous sildenafil as an early treatment in neonates with congenital diaphragmatic hernia. *Pediatr. Pulmonol.* (2018). doi:10.1002/ppul.23935
270. Dunn, L., Flenady, V. & Kumar, S. Reducing the risk of fetal distress with sildenafil study (RIDSTRESS): A double-blind randomised control trial. *J. Transl. Med.* (2016). doi:10.1186/s12967-016-0769-0
271. Paauw, N. D. *et al.* Sildenafil during Pregnancy: A Preclinical Meta-Analysis on Fetal Growth and Maternal Blood Pressure. *Hypertension* 70, 998–1006 (2017).
272. Dunn, L., Greer, R., Flenady, V. & Kumar, S. Sildenafil in Pregnancy: A Systematic Review of Maternal Tolerance and Obstetric and Perinatal Outcomes. *Fetal Diagnosis and Therapy* (2017). doi:10.1159/000453062
273. Symonds, M. E. & Budge, H. Comprehensive literature search for animal studies may have saved STRIDER trial. *Bmj* 4007, k4007 (2018).
274. Pellicer, B. *et al.* Haemodynamic effects of long-term administration of sildenafil in normotensive pregnant and non-pregnant rats. *BJOG An Int. J. Obstet. Gynaecol.* 118, 615–623 (2011).
275. Wu, T. W., Azhibekov, T. & Seri, I. Transitional hemodynamics in preterm neonates: Clinical relevance. *Pediatrics and Neonatology* (2016). doi:10.1016/j.pedneo.2015.07.002
276. Hillman, N. H., Kallapur, S. G. & Jobe, A. H. Physiology of transition from intrauterine to extrauterine life. *Clinics in Perinatology* (2012). doi:10.1016/j.clp.2012.09.009
277. Sasi, A. *et al.* Impact of intrauterine growth restriction on preterm lung disease. *Acta Paediatr. Int. J. Paediatr.* 104, e552–e556 (2015).
278. Sehgal, A. *et al.* Cardiac Morphology and Function in Preterm Growth Restricted Infants: Relevance for Clinical Sequelae. *J. Paediatr. Child Health* 53, 3–3 (2017).
279. Grant, A. & Glazener, C. M. Elective caesarean section versus expectant management for delivery of the small baby. *Cochrane Database Syst. Rev.* CD000078 (2001). doi:10.1002/14651858.CD000078
280. Turan, S. *et al.* Cardiovascular transition to extrauterine life in growth-restricted neonates: Relationship with prenatal doppler findings. *Fetal Diagn. Ther.* (2013). doi:10.1159/000345092
281. Rakza, T. *et al.* Early hemodynamic consequences of patent ductus arteriosus in preterm infants with intrauterine growth restriction. *J. Pediatr.* (2007). doi:10.1016/j.jpeds.2007.04.058
282. Clyman, R. I. *et al.* Regulation of ductus arteriosus patency by nitric oxide in fetal lambs: The role of gestation, oxygen tension, and vasa vasorum. *Pediatr. Res.* (1998). doi:10.1203/00006450-199805000-00012
283. Fike, C. D., Kaplowitz, M., Zhang, Y., Dantuma, M. & Madden, J. A. Effect of a phosphodiesterase 5 inhibitor on pulmonary and cerebral arteries of newborn piglets with chronic hypoxia-induced pulmonary hypertension. *Neonatology* (2012). doi:10.1159/000326270
284. Check, J. *et al.* Fetal growth restriction and pulmonary hypertension in premature infants with bronchopulmonary dysplasia. *J. Perinatol.* (2013). doi:10.1038/jp.2012.164
285. Niermeyer, S., Shaffer, E. M., Thilo, E., Corbin, C. & Moore, L. G. Arterial oxygenation and pulmonary arterial pressure in healthy neonates and infants at high altitude. *J. Pediatr.* (1993). doi:10.1016/S0022-3476(05)80857-1
286. Reuland, D. S. *et al.* Prevalence and prediction of hypoxemia in children with respiratory infections in the Peruvian Andes. *J. Pediatr.* (1991). doi:10.1016/S0022-3476(05)83040-9

287. Niermeyer, S. *et al.* Arterial Oxygen Saturation in Tibetan and Han Infants Born in Lhasa, Tibet. *N. Engl. J. Med.* (1995). doi:10.1056/NEJM199511093331903
288. Sehgal, A. *et al.* Preterm growth restriction and bronchopulmonary dysplasia: the vascular hypothesis and related physiology. *J. Physiol.* (2018). doi:10.1113/JP276040
289. Rozance, P. J. *et al.* Intrauterine growth restriction decreases pulmonary alveolar and vessel growth and causes pulmonary artery endothelial cell dysfunction in vitro in fetal sheep. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* (2011). doi:10.1152/ajplung.00197.2011
290. Ezaki, S. & Tamura, M. Evaluation and Treatment of Hypotension in Premature Infants. *Cardiovasc. Syst. Physiol. Diagnostic Clin. Implic.* (2012).
291. Subhedar, N. V & Shaw, N. J. Dopamine versus dobutamine for hypotensive preterm infants. *Cochrane Database Syst. Rev.* CD001242 (2003). doi:10.1002/14651858.CD001242
292. Habuchi, Y. *et al.* The mechanisms underlying heart stimulation by dopamine, with special reference to direct and indirect β adrenoceptor stimulation. in *Clinical and Experimental Hypertension* (1997). doi:10.3109/10641969709080811
293. Imai, Y. *et al.* Factors that Affect Blood Pressure. *Physiology* (1997).
294. Bhayat, S. I., Gowda, H. M. & Eisenhut, M. Should dopamine be the first line inotrope in the treatment of neonatal hypotension? Review of the evidence. *World J. Clin. Pediatr.* 5, 212 (2016).
295. Seri, I. Management of hypotension and low systemic blood flow in the very low birth weight neonate during the first postnatal week. *J. Perinatol.* 26, S8–S13 (2006).
296. Osborn, D., Evans, N. & Kluckow, M. Randomized trial of dobutamine versus dopamine in preterm infants with low systemic blood flow. *J. Pediatr.* (2002). doi:10.1067/mpd.2002.120834
297. Tare, M. *et al.* Glucocorticoid treatment does not alter early cardiac adaptations to growth restriction in preterm sheep fetuses. *BJOG An Int. J. Obstet. Gynaecol.* (2012). doi:10.1111/j.1471-0528.2012.03309.x
298. Wong, F. Y. *et al.* Effects of hypoxia-ischemia and inotropes on expression of cardiac adrenoceptors in the preterm fetal sheep. *J. Appl. Physiol.* (2018). doi:10.1152/japplphysiol.00472.2018
299. Chen, X. *et al.* ??2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. *J. Physiol.* 588, 3539–3549 (2010).
300. Yates, D. T. *et al.* Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. *Journal of Pregnancy* (2012). doi:10.1155/2012/631038
301. Emmanouilides, G. C., Townsend, D. E. & Bauer, R. A. Effects of single umbilical artery ligation in the lamb fetus. *Obstet. Gynecol. Surv.* 24, 1068–1070 (1969).
302. Ganzevoort, W. *et al.* STRIDER: Sildenafil Therapy In Dismal prognosis Early-onset intrauterine growth Restriction--a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. *Syst. Rev.* 3, 23 (2014).
303. Nichols, D. J., Muirhead, G. J. & Harness, J. A. Pharmacokinetics of sildenafil after single oral doses in healthy male subjects: absolute bioavailability, food effects and dose proportionality. *Br. J. Clin. Pharmacol.* 53 Suppl 1, 5S-12S (2002).
304. Safer Care Victoria. Dopamine. 4–7
305. Neonatal Medicines Formulary Consensus Group. Sildenafil. 1.1, 1–5 (2017).
306. Herrera, E. A., Verkerk, M. M., Derks, J. B. & Giussani, D. A. Antioxidant treatment alters peripheral vascular dysfunction induced by postnatal glucocorticoid therapy in rats. *PLoS One* (2010). doi:10.1371/journal.pone.0009250
307. Greenwood, P. L., Slepetis, R. M., McPhee, M. J. & Bell, A. W. Prediction of stage of pregnancy in prolific sheep using ultrasound measurement of fetal bones. *Reprod. Fertil.*

Dev. (2002). doi:10.1071/RD01047
308. Nardozza, L. M. M. *et al.* Fetal growth restriction: current knowledge. *Archives of Gynecology and Obstetrics* 295, 1061–1077 (2017).
309. MacHado Nardozza, L. M. *et al.* Fetal growth restriction: Current knowledge to the general Obs/Gyn. *Arch. Gynecol. Obstet.* 286, 1–13 (2012).
310. Maharaj, C. H. *et al.* Effects and mechanisms of action of sildenafil citrate in human chorionic arteries. *Reprod. Biol. Endocrinol.* 7, 34 (2009).
311. RUSSO, F. M. *et al.* 474: Placenta transfer of sildenafil citrate in the ex-vivo human cotyledon perfusion model. *Am. J. Obstet. Gynecol.* 216, S280 (2017).
312. Alves de Alencar Rocha, A. K. *et al.* Early- versus Late-Onset Fetal Growth Restriction Differentially Affects the Development of the Fetal Sheep Brain. *Dev. Neurosci.* (2017). doi:10.1159/000456542
313. Pels, A. *et al.* STRIDER (Sildenafil TheRapy in dismal prognosis early onset fetal growth restriction): An international consortium of randomised placebo-controlled trials. *BMC Pregnancy Childbirth* (2017). doi:10.1186/s12884-017-1594-z
314. Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development* 82, 518–529 (2015).
315. Spiers, A. & Padmanabhan, N. A guide to wire myography. *Methods Mol. Med.* 108, 91–104 (2005).
316. Anderson, D. F. & Faber, J. J. Regulation of fetal placental blood flow in the lamb. *Am J Physiol* 247, R567-74 (1984).
317. Vos, R. M., Chahbouni, A., Sinjewel, A. & Swart, E. L. Quantitative analysis of sildenafil and desmethylsildenafil in human serum by liquid chromatography-mass spectrometry with minimal sample pretreatment. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 876, 283–287 (2008).
318. Mohammed, E. E., Alashkar, O. S., Abdeldayem, T. M. & Mohammed, S. A. The use of low dose sildenafil citrate in cases of intrauterine growth restriction. 3, 1–5 (2017).
319. Alford, P. W., Nesmith, A. P., Seywerd, J. N., Grosberg, A. & Parker, K. K. Vascular smooth muscle contractility depends on cell shape. *Integr. Biol. (Camb).* 3, 1063–70 (2011).
320. Baquero, H., Soliz, A., Neira, F., Venegas, M. E. & Sola, A. Oral sildenafil in infants with persistent pulmonary hypertension of the newborn: a pilot randomized blinded study. *Pediatrics* 117, 1077–1083 (2006).
321. Jaillard, S. *et al.* Effects of phosphodiesterase 5 inhibitor on pulmonary vascular reactivity in the fetal lamb. *Ann. Thorac. Surg.* 81, 935–942 (2006).
322. El-Sayed, M. A., Saleh, S. A. A., Maher, M. A. & Khidre, A. M. Utero-placental perfusion Doppler indices in growth restricted fetuses: effect of sildenafil citrate. *J. Matern. Neonatal Med.* (2018). doi:10.1080/14767058.2017.1306509
323. Kane, A. D., Herrera, E. A., Camm, E. J. & Giussani, D. A. Vitamin C Prevents Intrauterine Programming of in vivo Cardiovascular Dysfunction in the Rat. *Circ. J.* 77, 2604–2611 (2013).
324. Itani, N., Skeffington, K. L., Beck, C., Niu, Y. & Giussani, D. A. Melatonin rescues cardiovascular dysfunction during hypoxic development in the chick embryo. *J. Pineal Res.* 60, 16–26 (2016).
325. Renshall, L. J., Dilworth, M. R., Greenwood, S. L., Sibley, C. P. & Wareing, M. In Vitro Asessment of Mouse Fetal Abdominal Aortic Vascular Function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* ajpregu.00058.2014- (2014). doi:10.1152/ajpregu.00058.2014
326. Kruuse, C., Gupta, S., Nilsson, E., Kruse, L. & Edvinsson, L. Differential vasoactive effects of sildenafil and tadalafil on cerebral arteries. *Eur. J. Pharmacol.* 674, 345–351 (2012).
327. Poudel, R., McMillen, I. C., Dunn, S. L., Zhang, S. & Morrison, J. L. Impact of chronic

hypoxemia on blood flow to the brain, heart, and adrenal gland in the late-gestation IUGR sheep fetus. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* (2015). doi:10.1152/ajpregu.00036.2014
328. Pryds, O., Greisen, G., Lou, H. & Frils-Hansen, B. Heterogeneity of cerebral vasoreactivity in preterm infants supported by mechanical ventilation. *J. Pediatr.* 115, 638–645 (1989).
329. Sirad, F. *et al.* Sildenafil Promotes Smooth Muscle Preservation and Ameliorates Fibrosis Through Modulation of Extracellular Matrix and Tissue Growth Factor Gene Expression After Bilateral Cavernosal Nerve Resection in the Rat. *J. Sex. Med.* 8, 1048–1060 (2011).
330. Zhao, L. *et al.* Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation* 104, 424–428 (2001).
331. Tantini, B. *et al.* Antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells. *Basic Res. Cardiol.* 100, 131–138 (2005).
332. Murad, F., Rapoport, R. M. & Fiscus, R. Role of cyclic-GMP in relaxations of vascular smooth muscle. *J Cardiovasc Pharmacol* 7 Suppl 3, S111–S118 (1985).
333. Hassan, M. A. H. & Ketat, A. F. Sildenafil citrate increases myocardial cGMP content in rat heart, decreases its hypertrophic response to isoproterenol and decreases myocardial leak of creatine kinase and troponin T. *BMC Pharmacol.* 5, (2005).
334. Yu, S.-M., Hung, L.-M. & Lin, C.-C. cGMP-Elevating Agents Suppress Proliferation of Vascular Smooth Muscle Cells by Inhibiting the Activation of Epidermal Growth Factor Signaling Pathway. *Circulation* 95, 1269–1277 (1997).
335. Malhotra, A. *et al.* Neonatal Morbidities of Fetal Growth Restriction: Pathophysiology and Impact. *Front. Endocrinol. (Lausanne).* 10, 1–18 (2019).
336. McCullough, A. R. Four-year review of sildenafil citrate. *Rev. Urol.* (2002).
337. Perez, K. M. & Laughon, M. Sildenafil in Term and Premature Infants: A Systematic Review. *Clin. Ther.* 37, 2598–2607 (2015).
338. Dastjerdi, M. V., Hosseini, S. & Bayani, L. Sildenafil citrate and uteroplacental perfusion in fetal growth restriction. *J. Res. Med. Sci.* 17, 632–636 (2012).
339. Groom, K. M. *et al.* STRIDER NZAus: a multicentre randomised controlled trial of sildenafil therapy in early-onset fetal growth restriction. *BJOG An Int. J. Obstet. Gynaecol.* (2019). doi:10.1111/1471-0528.15658
340. Al-Nasiry, S. *et al.* LB 2: Maternal sildenafil for severe early-onset fetal growth restriction: the Dutch multicentre placebo-controlled double-blind STRIDER-trial. *Am. J. Obstet. Gynecol.* (2018). doi:10.1016/j.ajog.2018.12.005
341. Deprest, J. & Conings, S. Modeling the Transfer of Sildenafil Across the Placenta with Data of an Ex-Vivo Human Cotyledon Perfusion Experiment Observed Data of SIL. 2018 (2018).
342. Russo, F. M. *et al.* Sildenafil crosses the placenta at therapeutic levels in a dually perfused human cotyledon model. *Am. J. Obstet. Gynecol.* (2018). doi:10.1016/j.ajog.2018.08.041
343. Inocencio, I. M. *et al.* Effects of Maternal Sildenafil Treatment on Vascular Function in Growth-Restricted Fetal Sheep. *Arterioscler. Thromb. Vasc. Biol.* 27–31 (2019). doi:10.1161/ATVBAHA.119.312366
344. Luong, C. *et al.* Antenatal sildenafil treatment attenuates pulmonary hypertension in experimental congenital diaphragmatic hernia. *Circulation* 123, 2120–2131 (2011).
345. Zhang, S. *et al.* Placental adaptations in growth restriction. *Nutrients* (2015). doi:10.3390/nu7010360
346. Kitanaka, T., Gilbert, R. D. & Longo, L. D. Maternal responses to long-term hypoxemia in sheep. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* (1989). doi:10.1152/ajpregu.1989.256.6.r1348
347. Borruto, F., Comparetto, C., Wegher, E. & Treisser, A. Screening of foetal distress by assessment of umbilical cord lactate. *Clin. Exp. Obstet. Gynecol.* (2006).

348. Kastendieck, E., Paulick, R. & Martius, J. Lactate in fetal tissue during hypoxia; correlation to lactate, pH and base deficit in the fetal blood. *Eur. J. Obstet. Gynecol. Reprod. Biol.* (1988). doi:10.1016/0028-2243(88)90166-9
349. Medina, J. M. & Tabernero, A. Lactate utilization by brain cells and its role in CNS development. in *Journal of Neuroscience Research* (2005). doi:10.1002/jnr.20336
350. Mann, L. I., Solomon, G., Carmichael, A. & Duchin, S. The effect of metabolic acidosis on fetal brain function and metabolism. *Am. J. Obstet. Gynecol.* (1971). doi:10.1016/0002-9378(71)90776-9
351. Cheema, R., Dubiel, M. & Gudmundsson, S. Fetal brain sparing is strongly related to the degree of increased placental vascular impedance. *J. Perinat. Med.* (2006). doi:10.1515/JPM.2006.061
352. Newby, E. A., Myers, D. A. & Ducsay, C. A. Fetal endocrine and metabolic adaptations to hypoxia: The role of the hypothalamic-pituitary-adrenal axis. *American Journal of Physiology - Endocrinology and Metabolism* (2015). doi:10.1152/ajpendo.00126.2015
353. Steer, P. J., Little, M. P., Kold-Jensen, T., Chapple, J. & Elliott, P. Maternal blood pressure in pregnancy, birth weight, and perinatal mortality in first births: Prospective study. *Br. Med. J.* (2004). doi:10.1136/bmj.38258.566262.7C
354. Pisaneschi, S. *et al.* Compensatory Feto-Placental Upregulation of the Nitric Oxide System during Fetal Growth Restriction. *PLoS One* (2012). doi:10.1371/journal.pone.0045294
355. Wang, C. K. & Craik, D. J. Cyclic peptide oral bioavailability: Lessons from the past. *Biopolymers* (2016). doi:10.1002/bip.22878
356. Malhotra, A. *et al.* Neuropathology as a consequence of neonatal ventilation in premature growth-restricted lambs. *Am. J. Physiol. Integr. Comp. Physiol.* (2018). doi:10.1152/ajpregu.00171.2018
357. MacDonald, T. M. *et al.* Reduced growth velocity across the third trimester is associated with placental insufficiency in fetuses born at a normal birthweight: A prospective cohort study. *BMC Med.* (2017). doi:10.1186/s12916-017-0928-z
358. Liu, J., Wang, X. F., Wang, Y., Wang, H. W. & Liu, Y. The incidence rate, high-risk factors, and short- and long-term adverse outcomes of fetal growth restriction. *Med. (United States)* 93, 1–5 (2014).
359. Jonker, S. S., Louey, S., Giraud, G. D., Thornburg, K. L. & Faber, J. J. Timing of cardiomyocyte growth, maturation, and attrition in perinatal sheep. *FASEB J.* (2015). doi:10.1096/fj.15-272013
360. Gonzalez-Tendero, A. *et al.* Intrauterine growth restriction is associated with cardiac ultrastructural and gene expression changes related to the energetic metabolism in a rabbit model. *Am. J. Physiol. Circ. Physiol.* (2013). doi:10.1152/ajpheart.00514.2013
361. Louey, S., Cock, M. L., Stevenson, K. M. & Harding, R. Placental insufficiency and fetal growth restriction lead to postnatal hypotension and altered postnatal growth in sheep. *Pediatr. Res.* (2000). doi:10.1203/00006450-200012000-00018
362. Noori, S. & Seri, I. Pathophysiology of newborn hypotension outside the transitional period. *Early Human Development* 81, 399–404 (2005).
363. Evans, K. Cardiovascular Transition of the Extremely Premature Infant and Challenges to Maintain Hemodynamic Stability. *J. Perinat. Neonatal Nurs.* (2016). doi:10.1097/JPN.0000000000000156
364. Evans, J. R., Lou Short, B., Van Meurs, K. & Cheryl Sachs, H. Cardiovascular support in preterm infants. *Clin. Ther.* 28, 1366–1384 (2006).
365. Joynt, C. & Cheung, P. Y. Treating hypotension in preterm neonates with vasoactive medications. *Front. Pediatr.* (2018). doi:10.3389/fped.2018.00086
366. Burns, M. L. *et al.* Inotropic Therapy in Newborns, A Population-Based National Registry Study*. *Pediatr. Crit. Care Med.* (2016). doi:10.1097/PCC.0000000000000898
367. Lingwood, B. E., Eiby, Y. A., Bjorkman, S. T., Miller, S. M. & Wright, I. M. R. Supporting

preterm cardiovascular function. *Clin. Exp. Pharmacol. Physiol.* (2019). doi:10.1111/1440-1681.13044
368. Garvey, A. A., Kooi, E. M. W. & Dempsey, E. M. Inotropes for preterm infants: 50 years on are we any wiser? *Front. Pediatr.* (2018). doi:10.3389/fped.2018.00088
369. Cohen, E., Wong, F. Y., Horne, R. S. C. & Yiallourou, S. R. Intrauterine growth restriction: Impact on cardiovascular development and function throughout infancy. *Pediatr. Res.* 79, 821–830 (2016).
370. Bigatello, L. M., Hess, D., Dennehy, K. C., Medoff, B. D. & Hurford, W. E. Sildenafil can increase the response to inhaled nitric oxide. *Anesthesiology* (2000). doi:10.1097/00000542-200006000-00044
371. Neonatal Medicines Formulary Consensus Group. *DOPamine Monash Newborn / NeoMed Medication Profile DOPamine Monash Newborn / NeoMed Medication Profile*. (2017).
372. Paediatrics, E. & Health, S. Sildenafil (oral) Monash Newborn Medication Profile Monash Newborn Medication Profile. 11–12 (2018).
373. Hracsko, Z., Orvos, H., Novak, Z., Pal, A. & Varga, I. S. Evaluation of oxidative stress markers in neonates with intra-uterine growth retardation. *Redox Rep.* (2008). doi:10.1179/135100008X259097
374. Payne, J. A., Alexander, B. T. & Khalil, R. A. Decreased endothelium-dependent no-cgmp vascular relaxation and hypertension in growth-restricted rats on a high-salt diet. *Hypertension* (2004). doi:10.1161/01.HYP.0000111832.47667.13
375. Clark, S. J. *et al.* Myocardial injury in infants ventilated on the paediatric intensive care unit: A case control study. *Crit. Care* (2006). doi:10.1186/cc5040
376. Lopes, D. N. *et al.* Cardiac Troponin T and Illness Severity in the Very-Low-Birth-Weight Infant. *Int. J. Pediatr.* (2012). doi:10.1155/2012/479242
377. De Lemos, J. A. *et al.* Association of troponin T detectedwith a highly sensitive assay and cardiac structure and mortality risk in the general population. *JAMA - J. Am. Med. Assoc.* (2010). doi:10.1001/jama.2010.1768
378. Arias-Camison, J. M. & Fiascone, J. M. Cardiac Troponin I (CTI) Elevation in Newborns May Be a Poor Prognostic Factor. *Pediatr. Res.* (1999). doi:10.1203/00006450-199904020-01082
379. Seri, I. & Evans, J. Controversies in the diagnosis and management of hypotension in the newborn infant. *Current Opinion in Pediatrics* 13, 116–123 (2001).
380. Issa, M., Job, S. & Curley, A. 350 Neonatal Inotropic Support: The Role of Antenatal Risk Factors. *Pediatr. Res.* (2010). doi:10.1203/00006450-201011001-00350
381. Sehgal, A. *et al.* Vascular aging and cardiac maladaptation in growth-restricted preterm infants. *J. Perinatol.* (2018). doi:10.1038/jp.2017.135
382. Peacock, W. F. *et al.* Cardiac Troponin and Outcome in Acute Heart Failure. *N. Engl. J. Med.* (2008). doi:10.1056/NEJMoa0706824
383. Polglase, G. R. *et al.* The cardiopulmonary haemodynamic transition at birth is not different between male and female preterm lambs. *Reprod. Fertil. Dev.* 24, 510–516 (2012).
384. Kimura, M. *et al.* PDE5 inhibitor sildenafil citrate augments endothelium-dependent vasodilation in smokers. *Hypertension* (2003). doi:10.1161/01.HYP.0000068202.42431.CC
385. Fuloria, M. & Aschner, J. L. Persistent pulmonary hypertension of the newborn. *Seminars in Fetal and Neonatal Medicine* (2017). doi:10.1016/j.siny.2017.03.004
386. Busè, M., Graziano, F., Lunetta, F., Sulliotti, G. & Duca, V. Inhaled nitric oxide as a rescue therapy in a preterm neonate with severe pulmonary hypertension: A case report. *Ital. J. Pediatr.* (2018). doi:10.1186/s13052-018-0494-9
387. Konior, A., Schramm, A., Czesnikiewicz-Guzik, M. & Guzik, T. J. NADPH oxidases in vascular pathology. *Antioxidants and Redox Signaling* (2014). doi:10.1089/ars.2013.5607
388. Rathore, R. *et al.* Hypoxia activates NADPH oxidase to increase [ROS]i and [Ca2+]i through the mitochondrial ROS-PKCe{open} signaling axis in pulmonary artery smooth

muscle cells. *Free Radic. Biol. Med.* (2008). doi:10.1016/j.freeradbiomed.2008.06.012
389. Fleiss, B. *et al.* Knowledge gaps and emerging research areas in intrauterine growth restriction-associated brain injury. *Frontiers in Endocrinology* (2019). doi:10.3389/fendo.2019.00188
390. Sales, F. *et al.* Rapid Communication: Maternal melatonin implants improve fetal oxygen supply and body weight at term in sheep pregnancies. *J. Anim. Sci.* (2019). doi:10.1093/jas/sky443
391. Fahmey, S. S., Fathy, H., Gabal, K. A. & Khairy, H. Cardiac troponin T in neonates with respiratory distress. *Egypt. Pediatr. Assoc. Gaz.* 66, 100–102 (2018).
392. Gandhi, R. & Marlow, N. Fetal Growth Restriction and Neonatal Outcomes. 237–245 (2019).
393. Faust, K. *et al.* Short-term outcome of very-low-birthweight infants with arterial hypotension in the first 24 h of life. *Arch. Dis. Child. Fetal Neonatal Ed.* (2015). doi:10.1136/archdischild-2014-306483
394. Johnson, V. Systemic Hypertension in Infants with Severe Bronchopulmonary Dysplasia. *Am. J. Perinatol.* 10, 260–261 (1993).
395. Ballabh, P. Intraventricular hemorrhage in premature infants: Mechanism of disease. *Pediatric Research* (2010). doi:10.1203/PDR.0b013e3181c1b176
396. Allison, B. J. *et al.* The effect of sex and prematurity on the cardiovascular baroreflex response in sheep. *Exp. Physiol.* 103, 9–18 (2018).
397. Casanello, P. & Sobrevia, L. Intrauterine growth retardation is associated with reduced activity and expression of the cationic amino acid transport systems y+/hCAT-1 and y+/hCAT-2B and lower activity of nitric oxide synthase in human umbilical vein endothelial cells. *Circ. Res.* (2002). doi:10.1161/01.RES.0000027813.55750.E7
398. Benitz, W. E. *et al.* Use of sodium nitroprusside in neonates: Efficacy and safety. *J. Pediatr.* (1985). doi:10.1016/S0022-3476(85)80477-7
399. Bibeau, K. *et al.* Placental underperfusion in a rat model of intrauterine growth restriction induced by a reduced plasma volume expansion. *PLoS One* (2016). doi:10.1371/journal.pone.0145982
400. Melamed, N., Yogev, Y. & Glezerman, M. Fetal gender and pregnancy outcome. *J. Matern. Neonatal Med.* (2010). doi:10.3109/14767050903300969
401. Aibar, L., Puertas, A., Valverde, M., Carrillo, M. P. & Montoya, F. Fetal sex and perinatal outcomes. *J. Perinat. Med.* (2012). doi:10.1515/jpm-2011-0137
402. Quiñones, J. N., Stamilio, D. M., Coassolo, K. M., Macones, G. A. & Odibo, A. O. Is fetal gender associated with adverse perinatal outcome in intrauterine growth restriction (IUGR)? *Am. J. Obstet. Gynecol.* 193, 1233–1237 (2005).
403. de Souza, A. P. *et al.* Gender-specific effects of intrauterine growth restriction on the adipose tissue of adult rats: A proteomic approach. *Proteome Sci.* (2015). doi:10.1186/s12953-015-0088-z
404. Roghair, R. D. *et al.* Vascular nitric oxide and superoxide anion contribute to sex-specific programmed cardiovascular physiology in mice. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* (2009). doi:10.1152/ajpregu.90756.2008
405. Patterson, A. J., Xiao, D., Xiong, F., Dixon, B. & Zhang, L. Hypoxia-derived oxidative stress mediates epigenetic repression of PKCε gene in foetal rat hearts. *Cardiovasc. Res.* (2012). doi:10.1093/cvr/cvr322
406. Akita, Y. Protein kinase C-ε (PKC-ε): Its unique structure and function. *J. Biochem.* (2002). doi:10.1093/oxfordjournals.jbchem.a003296
407. Chen, M., Xiong, F. & Zhang, L. Promoter methylation of Egr-1 site contributes to fetal hypoxia-mediated PKCε gene repression in the developing heart. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* (2013). doi:10.1152/ajpregu.00461.2012
408. Alderliesten, T. *et al.* Hypotension in preterm neonates: Low blood pressure alone does

not affect neurodevelopmental outcome. *J. Pediatr.* (2014). doi:10.1016/j.jpeds.2013.12.042
409. Logan, J. W. *et al.* Early postnatal hypotension and developmental delay at 24 months of age among extremely low gestational age newborns. *Arch. Dis. Child. Fetal Neonatal Ed.* (2011). doi:10.1136/adc.2010.183335
410. Malhotra, A. *et al.* Fetal growth restriction is associated with an altered cardiopulmonary and cerebral hemodynamic response to surfactant therapy in preterm lambs. *Pediatr. Res.* (2019). doi:10.1038/s41390-019-0398-4