Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognize the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
Sildenafil citrate: A novel therapy for intrauterine growth restriction

Charlotte Oyston

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Obstetrics and Gynaecology, The University of Auckland, 2016
Abstract

Introduction

Intrauterine growth restriction (IUGR) is associated with increased perinatal morbidity, mortality and adverse long term health consequences, yet no treatments are available which can improve fetal growth and wellbeing in utero. As utero-placental insufficiency (increased resistance to uterine artery blood flow with reduced utero-placental perfusion) is an important cause of IUGR, agents that vasodilate the utero-placental circulation are potential therapies. Sildenafil citrate, a phosphodiesterase inhibitor with vasodilatory properties, is a potential new therapy for IUGR. Small animal studies suggest that sildenafil can increase fetal growth, but presently evidence of its efficacy in larger animals, and an understanding of the mechanism/s through which sildenafil affects growth are lacking.

The two aims of this thesis are to assess the effect of sildenafil on

1) fetal growth in large and small animal models of IUGR; and

2) maternal myometrial / uterine artery ex vivo function in animal models of IUGR and human pregnancies complicated by IUGR.

Methods

Two different animal models of IUGR were used: uterine artery embolisation in the pregnant ewe, and gene knockout (endothelial nitric oxide synthase knockout and catechol-o-methyltransferase knockout) ± maternal weight gain in mice. In both studies, pregnant animals were randomised to sildenafil or vehicle treatment during pregnancy. Myometrial arteries were collected from uterine biopsies taken at delivery from women randomised to
sildenafil or placebo as part of the ‘sildenafil treatment for dismal prognosis early onset IUGR’ trial. Wire myography was used to assess \textit{ex vivo} perfusion function in uterine (mouse) or myometrial arteries (sheep, human).

\textbf{Results}

The lambs of sildenafil treated ewes had a weight that was intermediate between the IUGR vehicle treated and the normal growth control lambs. In the murine study, sildenafil was associated with increased fetal growth. Sildenafil treatment was not associated with changes in uterine or myometrial artery function in animal studies. Data pertaining to human myometrial artery \textit{ex vivo} could not be analysed at the time of thesis submission due to treatment concealment in an ongoing clinical trial.

\textbf{Conclusion}

Sildenafil increases fetal growth independent of changes in uterine or myometrial artery function in animal models of IUGR. This suggests that in human pregnancy, sildenafil treatment will not only be useful for the treatment of IUGR caused by utero-placental insufficiency, but also of IUGR due to other causes.
For my parents: Kate Ellis and Martin Oyston
The journey from clinical care to basic scientific research has been exhilarating, tumultuous, all-consuming, but a lot of fun. I am extremely grateful for the support of many, without whom I would not have been able to traverse this divide. I would like to thank my supervisor, Professor Philip Baker, for his guidance, support and encouragement. Under his supervision I have learned the excitement of discovery, the joy of critical analysis, and had the privilege of many great opportunities. I have also been extremely privileged to have the support of a fantastic group of co-supervisors: Professor Frank Bloomfield, Dr Katie Groom, and Dr Joanna Stanley. Frank, your enthusiasm for research, critical thinking and attention to detail are truly something to aspire to. Katie, thank-you for keeping my eyes and mind on the bigger picture; your clinical work and research are an inspiration. Jo, despite seeing the things no one should ever have to see, and nights huddled over a dissecting microscope, you have remained calm, kind, and supportive; thank-you for your wisdom, mentorship and friendship.

I would also like to acknowledge and thank the support and technical staff of the Liggins Institute and Ngapouri research farm, where the research for this thesis was carried out. I thank Dr. Mark Oliver for his teaching and practical assistance in the ovine study. I thank Laura McKay and Mariska Terbals (clinical trial manager extraordinaires) for their competent, cheerful assistance in all things related to the STRIDER study.

Thank-you to the women recruited to STRIDER, who, even in difficult circumstances, selflessly donated tissue for the experiments described in chapter 5.

While carrying out the research described in this thesis, I have been generously supported by stipends from The Mercia Barnes Trust, GRAVIDA (National Centre for Growth and Development), and The University of Auckland. In addition, I would like to thank and
acknowledge The Mercia Barnes Trust, GRAVIDA, and Lotteries Health Research, for funding towards the studies contained in this thesis.

To my beautiful colleagues Jasmine, Jo, Jo, Jamie, Veronica, Valerie, Anna and Emily; thank-you for being part of the journey. The friendships built have been an unexpected, awesome bonus, and I look forward to celebrating your successes, research and otherwise, in the future.

Finally, thank-you to my husband Chris, who has unfailingly supported me through this journey in every way. Somehow you knew everything would end up alright, and it was. Thank-you for your love, thank-you for believing in me.
Table of Contents

ABSTRACT ................................................................................................................................................ III
ACKNOWLEDGEMENTS .......................................................................................................................... VII
TABLE OF CONTENTS ........................................................................................................................... IX
LIST OF TABLES .......................................................................................................................................... XIII
LIST OF FIGURES ......................................................................................................................................... XV
LIST OF ABBREVIATIONS ........................................................................................................................ XVII
CO-AUTHORSHIP FORMS ....................................................................................................................... XXIII

1 INTRODUCTION ........................................................................................................................................... 1

1.1 Big problems for small babies ........................................................................................................... 1
1.2 Current treatment strategies ............................................................................................................. 2
1.3 Terminology, definitions and diagnosis of IUGR ............................................................................. 3
1.4 The aetiologies of IUGR ..................................................................................................................... 8
1.5 Adaptation of the uterine circulation to pregnancy ........................................................................... 12
  1.5.1 Doppler ultrasound for the evaluation of the utero-placental circulation ................................. 12
  1.5.2 Spiral artery modification .......................................................................................................... 15
  1.5.3 Vascular function - beyond physical modifications of the spiral artery ................................. 20
  1.5.4 Uterine vascular function in normal pregnancy .................................................................... 26
  1.5.5 Uterine vascular function in IUGR pregnancy ....................................................................... 28
  1.5.6 Adaptation of the uterine circulation to pregnancy - summary ............................................. 30
1.6 Potential new treatments for IUGR .................................................................................................... 30
  1.6.1 Sildenafil citrate – a novel therapy for intrauterine growth restriction .................................. 33
1.7 Can sildenafil citrate improve fetal growth? A review of the literature ....................................... 34
  1.7.1 Studies in mice and rats ............................................................................................................. 35
  1.7.2 Studies in other animals ........................................................................................................... 44
  1.7.3 Clinical studies .......................................................................................................................... 45
  1.7.4 The effect of sildenafil on fetal growth - summary ................................................................. 50
1.8 Mechanisms that may mediate changes in growth with sildenafil treatment ..................................... 50
1.9 Animal models of IUGR .................................................................................................................... 56
  1.9.1 Using mice to study IUGR ....................................................................................................... 57
  1.9.2 Using sheep to study IUGR ...................................................................................................... 63
1.10 Summary of introduction, thesis aims and hypotheses ..................................................................... 73

2 METHODS .................................................................................................................................................. 75

2.1 Ovine study (chapter 3) .................................................................................................................... 75
  2.1.1 Ethics statement ........................................................................................................................ 75
3 MATERNAL ADMINISTRATION OF SILDENAFIL CITRATE ALTERS FETAL AND PLACENTAL GROWTH AND FETAL-PLACENTAL VASCULAR RESISTANCE IN THE GROWTH RESTRICTED OVINE FETUS

3.1 Preface

3.1.1 Rationale

3.2 Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in the growth restricted ovine fetus

3.2.1 Abstract

3.2.2 Introduction
### 4 SILDENAFIL CITRATE INCREASES FETAL GROWTH IN MICE, INDEPENDENT OF UTERINE ARTERY FUNCTION

#### 4.1 Preface

- **Preface** .................................................................................. 149
  - **Rationale** ........................................................................... 149

#### 4.2 Sildenafil citrate increases fetal growth in mice, independent of uterine artery function

- **Abstract** .............................................................................. 151
- **Introduction** ........................................................................ 152
- **Methods** ............................................................................. 154
- **Fetal pup measurements** ..................................................... 155
- **Ex vivo vascular function** ..................................................... 155
- **RNA extraction and real-time PCR** .................................... 156
- **Statistical analysis** .............................................................. 157
- **Results** .............................................................................. 158
- **Effects of diet** .................................................................... 163
- **Effects of sildenafil** ............................................................ 166
- **Discussion** ......................................................................... 171
- **Conclusion** ......................................................................... 176
- **Additional information (competing financial interests)** .......... 176

#### 4.3 Supplementary material

- **S1. Sex specific differences in pup and placental growth** .... 177
- **S2. Sildenafil increased fetal abdominal circumference and crown-rump length in high fat diet but not normal diet groups** ........ 183
- **S3. Uterine artery ex vivo function: dose response curves for sodium nitroprusside** .................................................. 185

#### 4.4 Effect of sildenafil on maternal blood pressure and mesenteric vessel function

- **Background** ....................................................................... 186
- **Methods** ............................................................................ 187
- **Results** .............................................................................. 187
- **Additional discussion and conclusion** ................................ 193

### 5 THE EFFECT OF SILDENAFIL CITRATE ON MYOMETRICAL AND CHORIONIC SMALL ARTERY EX VIVO FUNCTION

#### 5.1 Preface

- **Preface** .............................................................................. 195

#### 5.2 Introduction

- **Introduction** ....................................................................... 195
List of Tables

Table 1.1 – Proposed consensus-based definitions for diagnosis of early- and late–onset intrauterine growth restriction ................................................................. 7

Table 1.2 – Maternal risk factors associated with IUGR ............................................. 10

Table 1.3 – Fetal and placental risk factors associated with IUGR ............................. 11

Table 1.4 - Summary of the effects of sildenafil on fetal growth in animal studies ........ 38

Table 1.5 - Studies of sildenafil for the treatment of IUGR in human pregnancy ............ 48

Table 1.6 - Randomised controlled trials of sildenafil for the treatment of pre-eclampsia .49

Table 2.1 - Primer sequences and amplicon size of genes studied ............................. 100

Table 2.2 - Volume of stock solutions required and final chamber concentrations achieved for constriction dose-response curves .............................................. 111

Table 2.3 - Volume of stock solutions required and final chamber concentrations achieved for endothelium-dependent vasodilatation dose-response curves .................. 114

Table 2.4 - Volume of stock solutions required and final chamber concentrations achieved for endothelium-independent vasodilatation dose-response curves .................. 115

Table 3.1 - Fetal measurements and organ weights .................................................... 133

Table 3.2 - Placentome weights and morphology ..................................................... 136

Table 3.3 - Maternal uterine artery blood flow (mL/min) ........................................ 148

Table 4.1 - eNOS+/mice had smaller pups with smaller placentae, and demonstrated altered uterine artery function ................................................................. 161

Table 4.2 - Maternal high fat diet reduced fetal size but did not alter uterine artery ex vivo function .................................................................................................... 164

Table 4.3 - Sildenafil increased fetal size but did not alter uterine artery ex vivo function 167

Table 4.4 - Main effects of strain on fetal and placental variables, separated by sex ........ 178
Table 4.5 - Main effects of diet on fetal and placental variables, separated by sex ............ 180
Table 4.6 - Main effects of treatment on fetal and placental variables, separated by sex ... 182
Table 5.1 - Characteristics and obstetric details of study participants ......................... 199
Table 5.2 - Characteristics of myometrial arteries..................................................... 200
Table 5.3 - Characteristics of chorionic plate arteries ............................................. 201
Table 7.1 - Statistical properties for main effects of diet or strain on maternal weight, litter size and sildenafil intake................................................................. 221
Table 7.2 - Statistical properties for main effects of strain on fetal and placental measures, and uterine artery ex vivo function............................................................ 222
Table 7.3 - Statistical properties for main effects of diet on fetal and placental measures ................................................................. 223
Table 7.4 - Statistical properties for main effects of treatment on fetal and placental measures ........................................................................................................ 223
Table 7.5 - Statistical properties for main effects of diet strain or treatment on placental mRNA expression................................................................. 224
List of Figures

Figure 1.1 - Spiral artery modification in non-pregnant, IUGR and normal pregnancy......18

Figure 1.2 - Nitric oxide triggers vasodilatation through the second messenger cGMP ......23

Figure 1.3 – Sheep placentome .........................................................................................67

Figure 1.4 – Multiple discrete placentomes demonstrated in the uterine cavity of an ovine (twin) pregnancy. ........................................................................................................68

Figure 1.5 - Relationship between trophoblast, fetal and maternal circulations in different types of placentation ..................................................................................................................69

Figure 2.1 - Construction of a vascular catheter ................................................................76

Figure 2.2 - Catheterisation of the fetal hind limb ................................................................80

Figure 2.3 - Blood pressure recordings and estimation of systolic pressure........95

Figure 2.4 - Multi-wire myograph ......................................................................................106

Figure 2.5 - Vessel normalization .......................................................................................109

Figure 2.6 - Examples of vessel dose-response curves ......................................................112

Figure 3.1 – Fetal weight was reduced compared to controls for IUGR + V, but not for IUGR + SC ........................................................................................................................................132

Figure 3.2 - Maternal uterine artery embolisation reduced fetal arterial glucose, and increased fetal arterial pCO₂ ..................................................................................................................138

Figure 3.3 - Maternal uterine artery embolisation resulted in a reduced fall in umbilical artery vascular resistance that was partly ameliorated with sildenafil treatment ......................140

Figure 3.4 – Sildenafil did not significantly alter myometrial artery ex vivo function ......141

Figure 4.1 - Strain, but not diet or treatment, altered uterine artery ex vivo function ......160

Figure 4.2 - COMT⁻/⁻ mice had increased placental GLUT4 expression compared to mice from the other strains. ........................................................................................................162
Figure 4.3 - HFD was associated with upregulation of expression of PlGF, TLR-4 and IL-6 in the placenta .......................................................... 165

Figure 4.4 - Sildenafil increased fetal weight in high fat diet groups ......................... 168

Figure 4.5 - Sildenafil reduced rates of IUGR in 4 out of 6 study groups ....................... 169

Figure 4.6 - Sildenafil treatment was associated with increased placental mRNA expressions of VEGFR-2 and TNF-α. Sildenafil also had diet specific effects on placental expression of IL-6 and IL-1β .............................................................................................................. 170

Figure 4.7 - Sildenafil increased fetal abdominal circumference in high fat diet groups .... 183

Figure 4.8 - Sildenafil increased fetal crown-rump length in high fat diet groups ........... 184

Figure 4.9 - Strain, but not diet or treatment, altered uterine artery ex vivo response to endothelium-independent vasodilation .......................................................................................... 185

Figure 4.10 - eNOS+/− strain and HFD in C57 BL/6J mice was associated with increased maternal blood pressure in mid-pregnancy ........................................................................................................... 188

Figure 4.11 - Maternal high fat diet increased maternal systolic blood pressure and sildenafil treatment reduced systolic blood pressure in late pregnancy ..................................................... 189

Figure 4.12 - Dose –response curves for mesenteric arteries ......................................... 191

Figure 4.13 - Mesenteric arteries from pregnant eNOS+/− mice and sildenafil treated HFD were mice had reduced sensitivity to constrictor U4 ................................................................................................. 192

Figure 7.1 – Permission for use of figure 1.1 ...................................................................... 215

Figure 7.2 – Permission for use of figure 1.3 ...................................................................... 216

Figure 7.3 Distribution of mean residuals for average pup weight (COMT+/− HFD + SC) . 219

Figure 7.4 Distribution of mean residuals for crown-rump length (COMT+/− HFD + SC) .. 219

Figure 7.5 – Distribution of mean residuals for average pup weight (eNOS+/− HFD +C) .... 220
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-ME₂</td>
<td>2-methoxyoestradiol</td>
</tr>
<tr>
<td>AC</td>
<td>Abdominal circumference</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AEC</td>
<td>Animal ethics committee</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BPD</td>
<td>Biparietal diameter</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
</tr>
<tr>
<td>C57</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36 (also known as fatty acid translocase)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary double-stranded DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHMP2-A</td>
<td>Chromatin-modifying protein/charged multivesicular body protein 2a</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CPR</td>
<td>Cerebroplacental ratio</td>
</tr>
<tr>
<td>CRL</td>
<td>Crown-rump length</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>dGA</td>
<td>Days gestational age</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DLK1</td>
<td>Delta-like non-canonical notch ligand 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DPEC</td>
<td>Diethylpyrocarbonate treated</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium derived hyperpolarising factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFW</td>
<td>Estimated fetal weight</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FA</td>
<td>Fetal artery</td>
</tr>
<tr>
<td>FV</td>
<td>Fetal vein</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>Glucose transporter 2</td>
</tr>
<tr>
<td>GLUT-3</td>
<td>Glucose transporter 3</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>Internal circumference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IC&lt;sub&gt;100&lt;/sub&gt;</td>
<td>Internal circumference of a relaxed vessel under a transmural pressure of 100 mmHg</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IQ</td>
<td>Interquartile</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>KPSS</td>
<td>High potassium physiologic saline solution</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MA</td>
<td>Maternal artery</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MV</td>
<td>Maternal vein</td>
</tr>
<tr>
<td>ND</td>
<td>Normal diet</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>OU</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>P0</td>
<td>Placental-specific insulin-like growth factor 2 P0 knockout</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Partial pressure of carbon dioxide dissolved in blood (mmHg)</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of oxygen dissolved in blood (mmHg)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDE-5</td>
<td>Phosphodiesterase type 5</td>
</tr>
<tr>
<td>Pe</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PI</td>
<td>Pulsatility index</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PSI</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiologic saline solution</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinylchloride</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RI</td>
<td>Resistance index</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RUPP</td>
<td>Reduced uterine perfusion pressure</td>
</tr>
<tr>
<td>SC</td>
<td>Sildenafil citrate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
</tbody>
</table>
sFLT-1  Soluble fms-like tyrosine kinase-1
SGA  Small for gestational age
SNAT-1  Sodium-coupled neutral amino acid transporter 1
SNAT-2  Sodium-coupled neutral amino acid transporter 2
SNAT-4  Sodium-coupled neutral amino acid transporter 4
SNP  Sodium nitroprusside
STRIDER NZAus  Sildenafil treatment in dismal prognosis early onset IUGR study, New Zealand, Australia
TLR-4  Toll-like receptor 4
TNF-α  Tumour necrosis factor alpha
U4  U46619 (thromboxane A2 mimetic)
UmA  Umbilical artery
UOV  Utero-ovarian vein
UtA  Uterine artery
V  Vehicle
VEGF  Vascular endothelial growth factor
VEGFR-1  Vascular endothelial growth factor receptor 1
VEGFR-2  Vascular endothelial growth factor receptor 2
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3 Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in the growth restricted ovine fetus.

As outlined in the prelude to chapter 3, this study has been published, with the publication details as follows:


| Nature of contribution by PhD candidate | Obtained funding for the project, involved in experimental design, animal husbandry, performed 90% of the fetal surgeries, all ultrasound examinations and other experimental work. Analysed data, wrote manuscript and thesis chapter. |
| Extent of contribution by PhD candidate (%) | 85% |

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joanna L Stanley</td>
<td>Involved in experimental design, assisted / supervised myography experiments, reviewed and provided feedback on published manuscript and thesis chapter.</td>
</tr>
<tr>
<td>Mark H Oliver</td>
<td>Involved in experimental design, obtained ethical approval, performed remainder of surgeries, provided onsite practical support for experiments, reviewed and provided feedback on published manuscript.</td>
</tr>
<tr>
<td>Frank H Bloomfield</td>
<td>Developed the concept of the study, obtained funding for the project, involved in experimental design, provided supervisory support and feedback on published manuscript and thesis chapter.</td>
</tr>
<tr>
<td>Philip N Baker</td>
<td>Developed the hypothesis and concept of the study, obtained funding for the project, involved in experimental design, provided supervisory support and feedback on published manuscript and thesis chapter.</td>
</tr>
</tbody>
</table>

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joanna L Stanley</td>
<td>[Signature]</td>
<td>7/12/2016</td>
</tr>
<tr>
<td>Mark H Oliver</td>
<td>[Signature]</td>
<td>7/12/2016</td>
</tr>
</tbody>
</table>

Last updated: 19 October 2015
This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 4, sildenafil citrate increases fetal growth in mice, independent of uterine artery function

Submitted to Scientific Reports on 25/11/2016

| Nature of contribution by PhD candidate | Assisted in obtaining funding, involved in experimental design, performed experimental work, data collection and analysis, and wrote the manuscript/chapter. |
| Extent of contribution by PhD candidate (%) | 85 |

<table>
<thead>
<tr>
<th>CO-AUTHORS</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anna Ponnampalam</td>
<td>supervised molecular experimental work</td>
</tr>
<tr>
<td>Valene Mills, Jasmine Pows</td>
<td>assisted with experimental work and animal care</td>
</tr>
<tr>
<td>Emily Rutherford</td>
<td>optimised the primers for gene expression and assisted with experimental work</td>
</tr>
<tr>
<td>Huan Zhao</td>
<td>assisted with experimental work and animal care</td>
</tr>
<tr>
<td>Joanna L Stanley</td>
<td>obtained ethical approval, experimental design, obtained funding for the project, assisted with experimental work and provided supervisory support</td>
</tr>
<tr>
<td>Philip N Baker</td>
<td>involved in experimental design, obtained funding for the project, assisted with experimental work and provided supervisory support</td>
</tr>
</tbody>
</table>

**Certification by Co-Authors**

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anna Ponnampalam</td>
<td></td>
<td>9/12/2016</td>
</tr>
<tr>
<td>Valene Mills</td>
<td></td>
<td>11/12/2016</td>
</tr>
<tr>
<td>Jasmine F Pows</td>
<td></td>
<td>9/12/2016</td>
</tr>
<tr>
<td>Emily Rutherford</td>
<td></td>
<td>10/12/2016</td>
</tr>
<tr>
<td>Huan Zhao</td>
<td></td>
<td>Dec/12/16</td>
</tr>
<tr>
<td>Joanna L Stanley</td>
<td></td>
<td>7/12/2016</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Big problems for small babies

Intrauterine growth restriction (IUGR; synonymous with fetal growth restriction) occurs when fetal growth does not reach its genetically determined potential. A growth restricted fetus is at greater risk of intrauterine death and perinatal mortality (Bernstein, Horbar et al. 2000, Simchen, Beiner et al. 2000, Pilliod, Cheng et al. 2012, Piper, Xenakis et al. 1996). An estimated 80,000 neonatal deaths worldwide are attributable to IUGR each year (Lawn, Cousens et al. 2005). Compared to their appropriately grown gestation-matched peers, IUGR neonates have increased rates of serious morbidity, including respiratory distress syndrome (Bernstein, Horbar et al. 2000, Gilbert, Danielsen 2003), necrotising enterocolitis (Bernstein, Horbar et al. 2000, Gilbert, Danielsen 2003), intraventricular haemorrhage (Gilbert, Danielsen 2003) and cerebral palsy (Jacobsson, Ahlin et al. 2008). The consequences of IUGR persist beyond the neonatal period, and those born growth restricted have increased rates of neurocognitive impairment during childhood (Chen, Chen et al. 2016). Abnormal antenatal growth is also associated with adverse effects on long-term health. Epidemiologic studies demonstrate an association between low birthweight and an increased risk of developing type II diabetes, hypertension, obesity and cardiovascular disease (Barker DJ 2004, Reynolds, Borowicz et al. 2010, Barker, Eriksson et al. 2002, Jaddoe, de Jonge et al. 2014). The link between IUGR and these conditions is further supported by animal studies that show molecular, metabolic, neuroendocrine, and physiological adaptations to IUGR in utero that result in permanent changes in metabolic function (Mcmillen, Robinson 2005).

Despite the significant consequences of IUGR, there are presently no clinically available treatments proven to improve fetal growth or in utero wellbeing, once a diagnosis of IUGR
has been made. When IUGR is severe and of early-onset, indicated preterm delivery is the only ‘treatment’ available that can prevent fetal demise in utero. Therefore, as a consequence of IUGR, these babies will also be exposed to the short and long-term risks of pre-term delivery, including neurocognitive impairment, chronic lung disease, and death (Glass, Costarino et al. 2015). These pregnancies are also associated with increased healthcare related costs. Costs of healthcare per infant are inversely proportionate to birthweight (Petrou 2003, Petrou, Sach et al. 2001, Stevenson, McCabe et al. 1996), although the financial impact of IUGR alone has not been quantified. This is because most studies report the costs attributable to low birthweight, and do not differentiate between the effects of prematurity and IUGR (Kramer 1987). In addition to financial costs, IUGR that necessitates pre-term delivery is associated with adverse psychologic sequelae for parents, particularly where delivery is required at very pre-term (i.e. < 32 weeks) gestations. Increased rates of stress, depression and psychologic sequelae have been identified amongst parents of very-low birthweight infants (Helle, Barkmann et al. 2015, Singer, Salvator et al. 1999), as well as in parents of babies born at very preterm gestations (Carson, Redshaw et al. 2015, Kaaresen, Ronning et al. 2006, Helle, Barkmann et al. 2015).

1.2 Current treatment strategies

The current standard of care of the IUGR pregnancy consists of close surveillance and premature delivery if the risks of continuing with pregnancy outweigh risks of premature delivery (American College of Obstetricians and Gynecologists 2013, Lausman, Kingdom et al. 2013, Royal College of Obstetricians and Gynaecologists 2013). Therefore, when severe or of early onset (when the risks of prematurity are highest), IUGR represents a significant clinical challenge. Over the past 50 years, there have been several developments which have improved outcomes for IUGR babies. These include the development of high resolution real-
time ultrasound (enabling better detection of IUGR and stratifying severity for optimal timing of delivery), the use of antenatal corticosteroids (reducing mortality and severe morbidity associated with preterm delivery (Roberts, Dalziel 2006)), the use of antenatal magnesium sulphate \((\text{MgSO}_4)\); reducing the risk of cerebral palsy associated with preterm delivery (Costantine, Weiner et al. 2009)), and advances in neonatal care (Battin, Knight et al. 2012, Gregory, Kitterman et al. 1971, The OSIRIS Collaborative Group 1992). Although these developments have improved outcomes, they do not address the underlying pathology of IUGR. While survival and morbidity are improved, the emotional, social, and financial burden of disease remains. A treatment that could improve intrauterine growth and/or wellbeing would revolutionise care for these pregnancies and improve quality of life for infants and their families, and reduce healthcare-associated costs. In particular, a treatment that resulted in delay in delivery at extreme pre-term gestations \((< 28 \text{ weeks})\) could dramatically reduce the major morbidity, mortality and the associated costs of IUGR.

### 1.3 Terminology, definitions and diagnosis of IUGR

It is useful to clarify some of the terminology used to describe and diagnose IUGR. The ideal definition of IUGR is a ‘fetus that fails to reach its growth potential’. This, however, is a conceptual definition, and does not provide a measure that can be used to determine which fetuses are IUGR for clinical or research purposes. In many cases, small for gestational age (SGA): a fetus whose birthweight (or estimated felt weight (EFW) based on ultrasound measurements) falls below the 10\textsuperscript{th} percentile for that gestation, is used as a surrogate for IUGR. The two conditions are, however, distinct. In IUGR there is always a pathologic process contributing to reduced growth. SGA also encompasses fetuses that, in contrast, are small but healthy and these pregnancies have a lower risk of adverse perinatal outcomes. Furthermore, using SGA as a surrogate for IUGR will fail to identify growth restricted fetuses
whose weights fall within the normal range, but are still at increased risks of adverse outcomes (Vasak, Koenen et al. 2015).

A variety of attempts have been made to more accurately detect and diagnose IUGR distinct from fetuses that are constitutionally small. Using a lower estimated fetal weight (EFW) cut-off (for example, the 5th or 3rd centile) is more predictive of fetuses at risk of adverse outcome, and therefore may be better at excluding those fetuses that are constitutionally small. Retrospective analyses suggest that a significant increase in adverse perinatal outcome only occurs for those with a birthweight less than the 5th centile (relative risk of perinatal adverse outcome for EFW <5th vs <10th centile: 2.7 (95% CI 2.0 – 3.6) vs 1.9 (95% CI 0.8 - 4.8) (Zhang, Mikolajczyk et al. 2011)). Other studies suggest that increased rates of neonatal death and perinatal morbidity only occur when the EFW < 3rd centile (Unterscheider, Daly et al. 2013, McIntire, Bloom et al. 1999). Irrespective of the cut-off used, this approach lacks sensitivity, as it fails to recognise those fetuses who have a weight within the normal range, but whose growth is less than their genetically determined potential (Vasak, Koenen et al. 2015).

Evaluating fetal proportions and assessment of growth trajectory also appears to be helpful in distinguishing IUGR fetuses from those that are constitutionally small. Fetal growth can be described as ‘symmetric’ where head and abdominal circumference (AC) are proportionately small. Alternatively, ‘asymmetric’ IUGR occurs when AC growth is disproportionately less than head measurements; this is thought to be due to a relative sparing of brain growth (Campbell, Thoms 1977), at the expense of fat deposition (Lapillonne, Peretti et al. 1997). In addition to EFW, a fetal AC < 10th centile is also used clinically to diagnose IUGR (Royal College of Obstetricians and Gynaecologists 2013); and has a similar sensitivity (72.9 – 94.5%) and specificity (50.6 – 83.8%) to EFW for predicting a birthweight <10th centile.
Serial measurements showing a slowing of growth velocity - and in particular slowing of the growth of the fetal abdominal circumference (AC) - appear to be better at identifying IUGR compared with a single measurement (Chang, Robson et al. 1993, Sovio, White et al. 2015). In a prospective study of pregnancies where EFW was <10th centile, the only predictor of increased neonatal morbidity was when fetal abdominal circumference growth velocity was in the lowest decile (relative risk of adverse perinatal outcome 3.9 (95% CI 1.9 – 8.1) (Sovio, White et al. 2015)).

Customised centiles differ from standard birthweight or growth centiles as they incorporate maternal physiologic factors known to influence fetal growth (fetal sex, maternal height, weight, ethnicity, and parity) in their model for expected fetal weight. SGA as determined by customised centiles appears to correlate more strongly with perinatal morbidity and mortality than SGA based on non-customised centiles (Gardosi, Francis 2009, McCowan, Harding et al. 2005, Claussen, Gardosi et al. 2001). A fetus who is SGA based on a customised centile is more likely to suffer adverse perinatal events, even if they are appropriately grown on non-customised centiles (Ego, Subtil et al. 2006, Gardosi, Francis 2009). However, it is not universally accepted that use of customised centiles to detect SGA or IUGR during pregnancy improves the prediction of adverse perinatal outcomes (Hutcheon, Zhang et al. 2011, Carberry, Raynes-Greenow et al. 2013).

Finally, Doppler ultrasound can be used to measure blood velocity in vivo. The relationship between IUGR and increased resistance to flow in the uterine artery has long been recognised (Campbell, Diaz-Recasens et al. 1983), although when used alone uterine artery Doppler studies are poorly predictive of IUGR (see below, section 1.5.2.1). Haemodynamic changes in the umbilical and cerebral circulations have also been shown to be predictive of fetuses that are at greater risk of neonatal morbidity. Doppler ultrasound assessment showing increased
resistance to blood flow in the umbilical artery (Spinillo, Montanari et al. 2005, Rochelson, Schulman et al. 1987), reduced resistance to flow in the middle cerebral artery, or an increase in the ratio between the two (cerebroplacental ratio (CPR) (Hernandez-andrade 2013)) are associated with increased morbidity or the need for caesarean delivery, and therefore may be useful in identifying true IUGR from constitutionally small fetuses (Berkowitz, Mehalek et al. 1988, Rochelson, Schulman et al. 1987, Spinillo, Montanari et al. 2005, Hernandez-andrade 2013).

Recently, consensus based diagnostic criteria for IUGR have been formulated based on the opinions of fifty-one experts (Gordijn, Beune et al. 2016). The consensus-based definitions (Table 1.1) illustrate the perceived importance of low EFW, AC, growth trajectory and umbilical and uterine artery blood flow for the diagnosis of IUGR.
Early-onset IUGR (<32 weeks)  
EFW or AC < 3rd centile  
\textit{or}  
Absent umbilical artery end diastolic flow  
\textit{or}  
EFW or AC < 10th centile, \textit{with one of:}  
UtA PI > 95th centile  
UmA PI > 95th centile  

Late-onset IUGR (>32 weeks)  
EFW or AC is < 3rd centile  
\textit{or}  
Two of the three criteria are met:  
EFW or AC < 10th centile  
AC/EFW crossing 2 growth quartiles  
CPR < 5th or UmA PI > 95th centile  

\textit{Table 1.1 – Proposed consensus-based definitions for diagnosis of early- and late – onset intrauterine growth restriction}

Table adapted from (Gordijn, Beune et al. 2016). All centiles used are non-customised. EFW estimated fetal weight; AC abdominal circumference; UtA PI uterine artery pulsatility index; UmA PI umbilical artery pulsatility index; CPR cerebroplacental ratio.

The criteria proposed by Gordijn et al. (2016) provide an opportunity to unify diagnostic definitions that will hopefully lead to consistency in reported outcomes of future studies. However, the ability of the proposed definitions to predict adverse outcomes, and their predictive power compared to other definitions (such as SGA alone) has not been assessed. Therefore, there are no clinical diagnostic criteria that capture all cases of IUGR currently available. Much of the literature regarding human pregnancy still uses SGA as a surrogate for IUGR. Therefore, in this thesis the term SGA will be used in the understanding that within
SGA although some of the fetuses will be IUGR, a proportion of these will be constitutionally small and not growth restricted.

1.4 The aetiologies of IUGR

Factors causing or associated with impaired growth are traditionally divided into maternal, fetal or placental in origin, and examples of these are summarised in Table 1.2 and Table 1.3. It is impossible to state the exact contribution that each pathology makes to the overall incidence of IUGR, as this varies with gestation and population studied. It is clear, however, that placental pathology underpins many deficits in growth, even in disease processes classified as ‘maternal’ or ‘fetal’. For example, chronic hypertension (Maloney, Heller et al. 2012), maternal undernutrition (Roberts, Sohlstrom et al. 2001, Belkacemi, Chen et al. 2009, Han, Mulla et al. 2011), obesity (Higgins, Mills et al. 2013, Roberts, Riley et al. 2011) and some congenital infections (Catalano, Fuccillo et al. 1971, Tondury, Smith 1966, Hamilton, Scott et al. 2012, Umbers, Aitken et al. 2011) result in impaired placental development and function which are likely to cause or significantly contribute to the degree of growth restriction. These impairments may be described collectively as ‘placental insufficiency’ - an umbrella term used to define conditions in which the placenta is unable to supply the fetus with the oxygen/nutrients required to support normal fetal growth.

Utero-placental vascular insufficiency (utero-placental insufficiency) is an important subset of placental insufficiency. This refers to placental insufficiency resulting from placental hypoperfusion secondary to abnormal uterine artery vascular adaption to pregnancy. Utero-placental insufficiency is an important cause of abnormal growth, as in the absence of fetal infection or chromosomal abnormalities, it accounts for the major proportion of IUGR (Cox, Marton 2009). The placentae from these pregnancies show characteristics of impaired
placental vascular development: the absence of physiologic change in the deeper myometrial portions of spiral arteries (Olofsson, Laurini et al. 1993, Lin, Shimizu et al. 1995, Sagol, Ozkinay et al. 1999, Robertson, Brosens et al. 1975), atherosis, thrombosis, or obliteration of the small artery lumens (Madazli, Somunkiran et al. 2003, Aardema, Oosterhof et al. 2001). Therapeutic strategies targeted to the treatment of utero-placental insufficiency are of particular interest as this pathology is the largest identifiable cause of growth restriction. As these pregnancies are otherwise normal, they would be expected to have a good outcome if fetal growth could be improved.
### Maternal risk factors associated with IUGR

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 40</td>
<td>(Odibo, Nelson et al. 2006)</td>
</tr>
<tr>
<td>Black race</td>
<td>(Odibo, Nelson et al. 2006)</td>
</tr>
<tr>
<td>Social deprivation</td>
<td>(Vos, Posthumus et al. 2014)</td>
</tr>
<tr>
<td>Maternal under-nutrition/starvation</td>
<td>(Lumey 1992)</td>
</tr>
<tr>
<td>Obesity</td>
<td>(Anderson, Sadler et al. 2013)</td>
</tr>
<tr>
<td>Hypoxia <em>e.g.</em> high altitude</td>
<td>(Zamudio, Baumann et al. 2006)</td>
</tr>
<tr>
<td>Diabetes with nephropathy or retinopathy</td>
<td>(Haeri, Khoury et al. 2008, Howarth, Gazis et al. 2007)</td>
</tr>
<tr>
<td>Congenital cardiovascular disease</td>
<td>(Thompson, Kuklina et al. 2015)</td>
</tr>
<tr>
<td>Chronic renal impairment</td>
<td>(Fink, Schwartz et al. 1998), (Zhang, Ma et al. 2015)</td>
</tr>
<tr>
<td>Antiphospholipid syndrome</td>
<td>(Yasuda, Takakuwa et al. 1995)</td>
</tr>
<tr>
<td>Recreational / illicit drugs</td>
<td>(Jaddoe, Troe et al. 2008, Quesada, Gotman et al. 2012, Wright, Schuetter et al. 2015, O'Leary, Nassar et al. 2009)</td>
</tr>
<tr>
<td><em>e.g.</em> cigarettes, cocaine, methamphetamine, ethanol</td>
<td></td>
</tr>
<tr>
<td>Prescription pharmaceuticals <em>e.g.</em> anticonvulsants, beta-blockers</td>
<td>(Veiby, Daltveit et al. 2014, Bayliss, Churchill et al. 2002)</td>
</tr>
</tbody>
</table>

*Table 1.2 – Maternal risk factors associated with IUGR*
### Fetal

- **Aneuploidy / chromosomal abnormalities**

- **Infection** *e.g.* malaria, cytomegalovirus, rubella, syphilis

- **Genetic syndromes** *e.g.* Roberts syndrome, Roifman syndrome
  - (Afifi, Abdel-Salam et al. 2016, Merico, Roifman et al. 2015)

- **Genetic imprinting disorders** *e.g.* Silver-Russell syndrome
  - (Wakeling, Brioude et al. 2016, Monk 2015)

- **Multiple pregnancy**
  - (Breathnach, Malone 2012)

### Placental

- **Utero-placental vascular insufficiency**

- **Chorionic haematoma**
  - (Nagy, Bush et al. 2003)

- **Major uterine malformation**
  - (Zyla, Wilczynski et al. 2015)

- **Umbilical cord anomalies** *e.g.* hypercoiling, abnormal insertion
  - (Tantbirojn, Saleemuddin et al. 2009)

- **Advanced placental maturation**
  - (Kazzi, Gross et al. 1983)

### Table 1.3 – Fetal and placental risk factors associated with IUGR
1.5 Adaptation of the uterine circulation to pregnancy

A series of dramatic haemodynamic changes are required to provide sufficient oxygen and nutrients to meet the demands of the growing fetus. In normal pregnancy, the fraction of cardiac output directed to the uterus rises, uterine artery blood flow increases, and the mean diameter of the uterine arteries increase (Palmer, Zamudio et al. 1992, Flo, Wilsgaard et al. 2010). These changes are facilitated by both systemic circulatory changes – such as increased plasma volume, reduced systemic resistance, and increased cardiac output (Ouzounian, Elkayam 2012) - and changes to the uterine vasculature. Although failure of either systemic or local adaptation is associated with the development of IUGR, this section will focus on the adaptations of the uterine vasculature.

1.5.1 Doppler ultrasound for the evaluation of the utero-placental circulation

Doppler ultrasound provides a non-invasive means of evaluating the utero-placental circulation in pregnancy. This technique allows measurement of relative blood flow velocity within a vessel, and by comparing flow in systole and diastole it is possible to infer information regarding resistance downstream to the vessel imaged. In clinical practice, the relationship between flow velocity in systole and diastole is described as an index. For example:

\[
\text{Resistance Index (RI)} = \frac{\text{Peak systolic velocity} - \text{End diastolic velocity}}{\text{Peak systolic velocity}}
\]

Or

\[
\text{Pulsatililty Index (PI)} = \frac{\text{Peak systolic velocity} - \text{End diastolic velocity}}{\text{mean velocity over the cardiac cycle}}
\]
In the uterine artery, downstream resistance will reflect resistance to flow through the vessels feeding into the placenta and the placenta itself. In a placenta where resistance is low, diastolic flow will be high and Doppler indices low. If resistance to flow through the placenta increases, velocity of blood flow during diastole may slow, resulting in a higher RI and PI.

1.5.1.1 The relationship between Doppler flow studies and down-stream resistance

Doppler ultrasound can be used to measure velocity of blood, and where the cross-sectional vessel area is also measured, volumetric flow can be calculated. Volumetric flow within the uterine and umbilical arteries is challenging to measure accurately. Difficulties with measuring volumetric flow arise because the angle of beam insonation and measurement of the vessel radius must be known precisely (Nilsson, Olofsson et al. 1997). These measurements may be especially difficult in tortuous vessels, and in arteries where flow is pulsatile, and the vessel calibre constantly changing. An alternative means of studying flow dynamics is to analyse the Doppler waveform shape, which may be quantified using empirical indices such as the resistance index (RI) and pulsatility index (PI). The Doppler indices are independent of the angle of insonation, and vessel calibre. Most importantly, in high risk pregnancies use of Doppler indices to monitor fetal condition has been associated with improved outcomes (Alfirevic, Stampalija et al. 2017).

Studies using ovine models (Adamson, Morrow et al. 1990, Trudinger, Stevens et al. 1988), the ex vivo study of perfused human placentae (Gordon, Glaubach et al. 2016, Mills, Wareing et al. 2005), and mathematical models of the umbilical-placental circulation (Thompson, S. 1989, Thompson, Trudinger 1990, van den Wijngaard, Westerhof et al. 2006, Kleiner-Assaf, Jaffa et al. 1999) have shown that Doppler indices rise with increasing placental vascular resistance. In ovine studies where pressures are measured invasively, placental embolization results in increased vascular resistance, increased Doppler indices and reduced blood flow
(Trudinger, Stevens et al. 1988, Acharya, Erkinaro et al. 2004). Fetal inflow hydrostatic pressure measured during *ex vivo* perfusion of placentae from healthy human pregnancies is inversely proportional to umbilical artery Doppler indices taken *in vivo*, where the highest inflow pressures recorded are obtained from pregnancies with the highest Doppler indices (Jones, Bischof et al. 2015). Similarly, flow mediated vasodilation is inversely correlated with Doppler indices, suggesting that these indices reflect increased resistance to flow (Jones, Bischof et al. 2015).

The relationship between increasing placental pressure and rise in Doppler indices is non-linear. In mathematical models, terminal branches of vasculature are eradicated to model placental insufficiency. In these models, Doppler indices initially increase slowly (Thompson, Trudinger 1990), but increase rapidly and exponentially when a critical level (estimated to be 60 – 70% eradication of terminal branches), is reached (Thompson, Trudinger 1990, Thompson, Stevens 1989). The requirement for at least 60% obliteration before large rises in Doppler flow occur is supported by studies in perfused human placentae where a similar proportion of chorionic arteries must be ligated sequentially before a rapid rise in Doppler indices occurs (Gordon, Glaubach et al. 2016).

Despite a strong interdependence between placental resistance, Doppler indices, and volumetric flow, the relationship between the three appears to be complex, with increase in Doppler indices not always equating to reductions in flow. In an ovine model where umbilical flow was altered by an infusion of vasoconstrictors, Doppler indices did not correlate with invasively measured umbilical artery blood flow, with indices remaining normal even when flow was dramatically reduced (Adamson, Morrow et al. 1990, Irion, Clark 1990). Mathematical modelling also suggests that Doppler indices do not always correlate with volumetric flow. When placental resistance (and Doppler indices) is substantially increased,
umbilical blood flow can be maintained in a model where fetal blood pressure is increased (Thompson, Trudinger 1990), a phenomenon that is known to occur in response to hypoxia in vivo (Assali, Holm et al. 1962). Finally, in contrast to their studies of healthy placentae, Jones and colleagues (2015) found that in the ex vivo perfused IUGR placentae, there was no correlation between fetal inflow perfusion pressures and Doppler indices (Jones, Bischof et al. 2015). Mathematical models predict that many variables - including blood viscosity, arterial thickness and elasticity and blood density – can alter both Doppler indices and blood flow (Kleiner-Assaf, Jaffa et al. 1999, Thompson, Trudinger 1990). It is not understood how a change in a combination of these variables will affect Doppler indices and blood flow, and it is possible that the complex pathophysiologic changes to these parameters that occur in IUGR pregnancy may further obscure the relationship between Doppler indices, pressure, and volumetric flow.

These examples illustrate that although there is an interdependence between placental resistance, volumetric flow and doppler indices, caution should be applied when using Doppler studies to infer information about resistance and flow. Furthermore, the usefulness of Doppler indices to reflect temporal changes in downstream resistance may also be dependent on the steady state of other parameters such as the fetal blood pressure, and other anatomic and physiologic factors.

1.5.2 Spiral artery modification

The utero-placental circulation is characterised by low resistance and high volume flow. In human pregnancy this is especially important due to the structure of the placenta. Maternal blood flows into intervillous spaces – lake-like structures which surround and perfuse networks of fetal vessels. Blood must enter at a low pressure and velocity, otherwise the delicate fetal vessels will become damaged and their ability to transfer oxygen will be
impaired. As in humans, placentation in rats, guinea pigs and rabbits is haemochorial. In these animals, pressures within the arterial system supplying the placenta have been measured directly, with the major pressure drop occurring in small arteries outside of the placenta, rather than within the placenta (Moll, Kunzel 1973). Although the same studies have not been performed in humans, it is reasonable to assume that, given similarities in the vascular supply to the uterus and the type of placentation, a similar pattern of pressure drop occurs.

Increasing blood vessel calibre is a very efficient way of reducing resistance and increasing flow. In human pregnancy, this is at least partly achieved through the process of spiral artery remodelling (Figure 1.1). From the early first trimester, placental trophoblasts (cytotrophoblasts) migrate from the placenta and invade and replace the walls of the spiral arteries supplying the placental bed (Pijnenborg, Vercruysse et al. 2011, De Wolf, De Wolf-Peeters et al. 1980). The modified arteries lose the musculo-elastic tissue normally found in the vessel wall, which is replaced with fibrinoid material. The vessel lumen becomes distended, increasing from a diameter of around 200 µm pre-pregnancy to 2,000 µm in the remodelled vessels at the point of discharge into the intervillous space (Benirschke 2006). The transformed vessels are flaccid and funnel shaped and lack vasoconstrictor ability. Functionally, this will allow large volumes of blood to pool in the intervillous space (Robertson, Brosens et al. 1975, Pijnenborg, Bland et al. 1983, De Wolf, De Wolf-Peeters et al. 1980). The more proximal radial uterine arteries do not undergo these changes – they retain their reactive ability and are an important site for regulating vascular resistance and supply to the utero-placental circulation (Gokina, Mandalà et al. 2003, Moll, Kunzel 1973, Osol, Moore 2014). Following placental implantation, trophoblast cells of the placenta differentiate into cytotrophoblast and syncytiotrophoblast. Cytotrophoblast invade the decidua and myometrium (interstitial trophoblast) and migrate up the spiral arteries retrograde to blood flow (endovascular trophoblast) (Pijnenborg, Bland et al. 1983). Interstitial trophoblast
migrate through endometrial stroma, penetrating spiral arteries from the outside, and extravillous trophoblast migrate through the lumen of spiral arteries, replacing vascular endothelium of the spiral arteries. As a result, the smooth muscle and elastic lamina of the artery wall are lost, resulting in dilated and flaccid utero-placental vessels (Burton, Woods et al. 2009, Brosens, Pijnenborg et al. 2002). In normal pregnancy, invasion occurs to a depth of the inner third of the maternal myometrium (Pijnenborg, Bland et al. 1983).
Figure 1.1 - Spiral artery modification in non-pregnant, IUGR and normal pregnancy

Pre-pregnancy, the maternal spiral arteries within the uterus are narrow and highly coiled (left panel). In normal pregnancy (right panel) endovascular trophoblast (ENVT) and extravillous trophoblast (EVT) replace the endothelium of the spiral arteries to the inner third of the myometrium. As a result, vessels become thin walled and dilated. In IUGR (middle panel), trophoblast invasion does not occur to the same extent, with reduced depth of invasion and reduced dilatation. This results in increased resistance to flow and reduced flow of maternal blood to the placenta.

Figure republished with permission of Rockefeller University press from “NK cells and trophoblasts: partners in pregnancy” by P Parham, 2004, The Journal of experimental medicine, 200 (8). Copyright © 2004 Permission conveyed through Copyright Clearance Centre, Inc. (Appendix A).
The first changes in the Doppler ultrasound resistance pattern appear around the same time as the first morphological changes are seen in the spiral arteries. Doppler studies of the uterine and spiral arteries suggest that pressure in the human placenta falls as gestation advances; a fall in impedance in the radial or spiral arteries occurs as early as 5 weeks gestation, with reduced impedance and increased flow in the uterine arteries observed at around 8 -10 weeks (Mäkikallio, Tekay et al. 2004, Tamura, Miwa et al. 2008). The fall in resistance in the larger arteries may appear later, as early in gestation the absolute increase in flow in large vessels is likely to be very small, and increase in flow may only become detectable once a critical level of uterine growth / placental mass - and therefore increase in blood flow - is achieved (Mäkikallio, Tekay et al. 2004, Tamura, Miwa et al. 2008).

1.5.2.1 Abnormalities of spiral artery modification in utero-placental insufficiency

Abnormal spiral artery modification (as demonstrated by histomorphology of term placental bed biopsies) is associated with the development of IUGR. Pathologic findings include the absence of physiologic changes in the deeper myometrial portions of spiral arteries (Olofsson, Laurini et al. 1993, Lin, Shimizu et al. 1995, Robertson, Brosens et al. 1975), and acute atherosis, thrombosis, or obliteration of the spiral artery lumen (Madazli, Somunkiran et al. 2003, Aardema, Oosterhof et al. 2001). Raised spiral (Matijevic, Johnston 1999) and uterine (Lin, Shimizu et al. 1995, Matijevic, Johnston 1999, Olofsson, Laurini et al. 1993) artery Doppler indices in either the first or mid trimesters are associated with the development of IUGR (Dugoff, Lynch et al. 2005, Plasencia, Maiz et al. 2008, Melchiorre, Wormald et al. 2008, Gaillard, Arends et al. 2013). A high resistance flow pattern observed in the uterine artery in the first trimester is associated with reduced trophoblast invasion at the placental implantation site, as observed in first trimester pregnancy specimens compared with women with normal uterine artery waveforms (Prefumo, Sebire et al. 2004). Trophoblast invasion into the myometrium is found less often in women with raised uterine artery Doppler indices,
and raised uterine artery Doppler indices are predictive of findings of abnormal infiltration of trophoblast to utero-placental arteries in placental bed biopsy at caesarean section (Lin, Shimizu et al. 1995).

There is not a straight cause and effect relationship between raised impedance in the utero-placental circulation and the development of pregnancy complications. Used alone in a low risk population, first trimester uterine artery Doppler is a poor predictor of growth restriction. Large meta-analyses estimate the sensitivity of a first trimester RI or PI >90th centile to be 15% - 62% (Velauthar, Plana et al. 2014, Cnossen, Morris et al. 2008), with sensitivities of 75% - 93% (Cnossen, Morris et al. 2008, Velauthar, Plana et al. 2014). Doppler indices also perform poorly as predictive tools in the second trimester, where specificity and sensitivity are estimated to be 53% and 87% respectively (Cnossen, Morris et al. 2008). Similarly, the absence of physiologic changes to the spiral arteries occurs in up 40% of normal pregnancies (Sagol, Ozkinay et al. 1999), and “pathologic” findings have been identified in almost all placental bed biopsies from pregnancies with normal outcomes, and do not correlate with the absence of physiologic change, or high resistance to flow in the uterine arteries (Aardema, Oosterhof et al. 2001). These observations suggest that other factors or pathways beyond the physical modification of spiral arteries are also important in placental perfusion, fetal growth, and the development of IUGR. Compensatory pathways may mitigate the effects of raised uterine artery resistance via other mechanisms, and only once these are impaired IUGR develops.

1.5.3 Vascular function - beyond physical modifications of the spiral artery

From the mid to late second trimester there is no further significant fall in resistance in the spiral arteries (Matijevic, Johnston 1999, Hsieh, Chang et al. 2000), suggesting that the majority of spiral artery transformation is completed by this time. There is also limited in vitro
evidence that spiral artery remodelling does not occur at late gestation, with first but not third trimester trophoblast cells participating in endovascular remodelling (Kalkunte, Lai et al. 2008, Matijevic, Johnston 1999). In contrast, over the same timeframe, resistance to flow in the main uterine artery falls (Bower, Vyas et al. 1992, Gaillard, Arends et al. 2013) and placental perfusion increases (Noguchi, Hata et al. 2009, Mercé, Barco et al. 2005, Zalud, Shaha 2007). This disparity implies that spiral artery modification is not the sole cause of reduced resistance to flow in the utero-placental circulation. Similarly, in pregnancies where there has been no trophoblast invasion of the spiral arteries (abdominal pregnancy where the embryo implants outside the uterus somewhere in the abdominal cavity), normal low resistance waveforms in both uterine arteries are seen even where one (Collins, Grant et al. 2011) or both (Acácio 2002) uterine arteries do not feed the placental bed at all.

Alterations in vascular tone – specifically an increase in vasodilatation - may explain the continued reduction in utero-placental resistance which cannot be explained by changes to the physical structure of the spiral artery. Changes in vascular tone are mediated through shifts in the balance of constricting and relaxing factors produced by the vascular endothelium. Vasoactive factors known to be produced by the vascular endothelium include vasodilators nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarising factors (EDHF), and vasoconstrictors thromboxane A2, angiotensin II and endothelin I. The relative contribution these molecules make differs with size of the vessel, vascular bed, and presence of disease (Luksha, Agewall et al. 2009, Bryan, You et al. 2005, Urakami-Harasawa, Shimokawa et al. 1997). In the ascending uterine (Nelson, Steinsland et al. 1998) and myometrial (Kenny, Baker et al. 2002, Luksha, Luksha et al. 2010) arteries of pregnant women, endothelium-dependent vasodilatation appears to be predominantly mediated by NO and EDHF, with PGI₂ having minimal contribution.
1.5.3.1 Assessing vascular function

Vascular function can be assessed *ex vivo*, using the technique of myography. The force exerted by the blood vessel wall can be measured in isolated small arteries that are mounted on parallel wires (wire myography) or cannulated (pressure myography). Wire myography examines vessels that are under a constant tension; pressure myography examines vessels that have a constant intraluminal pressure. The two techniques should be considered as representing measurements of different aspects of vessel function, with neither system giving a perfect representation of conditions *in vivo*, where flow may be turbulent and / or pulsatile and conditions will be neither isobaric nor isometric. In both wire and pressure myography, the force that the vessel exerts in response to different doses of constricting or relaxing agents, or variations in flow, provides insight into the changes in vessel function that occur in both healthy pregnancy and utero-placental insufficiency. Importantly, endothelium-dependent relaxation can be assessed by the use of vasodilators such as acetylcholine (ACh) and bradykinin (BK) that are known to elicit relaxation through endothelium-dependent pathways.

1.5.3.2 Mediators of vasodilatation in the utero-placental circulation

1.5.3.2.1 Nitric oxide

Nitric oxide (NO) is produced in the vascular endothelium via the enzyme endothelial nitric oxide synthase (eNOS). The normal cascade of the NO pathway is as follows: within the vascular endothelium L-arginine is converted to NO via the enzyme nitric oxide synthase (NOS). Nitric oxide diffuses into adjacent vascular smooth muscle where it actives guanylate cyclase, resulting in the enzymatic conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). cGMP provides the signal to the smooth muscle for relaxation and therefore vasodilatation (Figure 1.2).
**Figure 1.2 - Nitric oxide triggers vasodilatation through the second messenger cGMP**

Nitric oxide produced in the vascular endothelium diffuses into adjacent vascular smooth muscle and activates guanylate cyclase. sGC catalyses the formation of 3’,5’-cyclic GMP which triggers smooth muscle relaxation and vessel vasodilatation, resulting in increased blood flow. Phosphodiesterase catalyses breakdown of 5’-GMP.

Nitric oxide (NO); soluble guanylate cyclase (sGC); L-arginine (L-arg); guanosine triphosphate (GTP); guanosine monophosphate (GMP); cyclic guanosine monophosphate (cGMP); phosphodiesterase (PDE).
Agonists (bradykinin, acetylcholine, 5-hydroxytryptamine, and histamine) trigger production of NO (Poston, McCarthy et al. 1995), as does shear stress in vessels with continuous production of NO occurring in vessels with pulsatile flow (Bevan, Laher 1991). NO is important in the generalised vasodilatation of pregnancy (Ahokas, Mercer et al. 1991, Umans, Lindheimer et al. 1990); however, it plays a particularly important role in increasing blood flow in the utero-placental circulation (Anumba, Robson et al. 1999, Sladek, Magness et al. 1997). In pregnancy, there is an increase in protein expression of endothelial NOS in the uterine artery endothelium, while systemic vessels show relative minor changes (Magness, Shaw et al. 1997). This suggests that NO may play a particularly important role in facilitating changes in vascular function occurring within the uterine circulation. The importance of NO is further underlined in animals that lack the ability to produce NO (Baylis, Engels 1992, Kualndavelu, Qu et al. 2006, Kualndavelu, Whiteley et al. 2012, Kusinski, Stanley et al. 2012); these animals have both structural (shorter and narrower) and functional abnormalities of the uterine artery, reduced uterine blood flow and produce smaller offspring (Kualndavelu, Whiteley et al. 2012, Kusinski, Stanley et al. 2012). In human pregnancy, NO is an important mediator of endothelium-dependent vasodilatation and myogenic tone in myometrial arteries. Inhibition of NO production consistently enhances constriction in response to increasing pressure, flow (Kublickien, Nisell et al. 2000, Eckman, Gupta et al. 2012) or norepinephrine stimulation (Kublickien, Cockell et al. 1997), and reduces endothelium-dependent relaxation in the uterine and myometrial arteries (Ashworth, Baker et al. 1999, Wimalasundera, Thom et al. 2005, Nelson, Steinsland et al. 1998).

1.5.3.2.2 Prostacyclin

Prostacyclin (PGI2) is a prostaglandin with a potent vasodilatory effect. It is thought to be important in mediating the reduction in peripheral resistance seen in pregnancy, as urinary metabolites of PGI2 increase with gestation, with lower levels reported in women who have
developed or will eventually develop pre-eclampsia (Ylikorkala, Pekonen et al. 1986). In sheep, both systemic and uterine arteries are able to synthesise PGI₂; in pregnancy, the production of PGI₂ becomes markedly greater in the uterine than systemic vessels (Magness, Rosenfeld et al. 1992). This suggests that in addition to NO, PGI₂ may contribute to the differential responsiveness in uterine compared to systemic arteries. In both human and sheep gestation, the utero-placental beds are refractory to the pressor effects of infused angiotensin II (Erkkola, Pirhonen, Naden, Rosenfeld 1981) However, inhibition of PGI₂ production increases uterine artery resistance measured following angiotensin infusion in chronically catheterised sheep (Magness, Rosenfeld et al. 1992), suggesting PGI₂ may play a role in attenuating the response to vasopressors in pregnancy. Given these findings, it would be expected that inhibition of PGI₂ synthesis would increase constriction or reduce vasodilatation in myometrial resistance vessels. However, inhibition of PGI₂ synthesis makes no difference to endothelium-dependent relaxation in human myometrial resistance vessels (Kenny, Baker et al. 2002), or ascending branches of the uterine artery (Nelson, Steinsland et al. 1998). This is in contrast to inhibition of NOS or endothelium derived hyperpolarising factor, which results in significantly reduced relaxation (Kenny, Baker et al. 2002, Luksha, Luksha et al. 2010, Nelson, Steinsland et al. 1998). Therefore, PGI₂ may be relatively less important in modulating the vascular function of uterine resistance vessels in humans, and therefore may explain why many studies have demonstrated a minimal effect of aspirin prophylaxis (which results in a proportionate increase of PGI₂ in comparison to constrictor thromboxane A2) on the prevention of IUGR (The CLASP collaborative group 1994, Askie, Duley et al. 2007).

1.5.3.2.3 Endothelium derived hyperpolarising factors

Even after inhibiting production of NO and PGI₂, a blood vessel still exhibits some residual endothelium-dependent vasodilatation. Residual non-NO, non-prostanoid dilatation appears especially prominent in the more distal resistance arteries, and this is attributed to the actions
of endothelium derived hyperpolarising factors (EDHF; (Urakami-Harasawa, Shimokawa et al. 1997)). The identity of EDHF is uncertain, and it is generally considered that it is representated by different molecules in different vascular beds (Urakami-Harasawa, Shimokawa et al. 1997, Morton, Davidge 2013). Despite uncertainty of its nature, EDHF function is consistently linked to intermediate and slow conductance calcium-activated potassium channels in almost all human vascular beds studied (Yang, Yim et al. 2007). The physiologic and pathophysiologic roles of EDHF are poorly understood. A popular theory is that EDHF serves as a back-up pathway for NO. NO has been observed to inhibit EDHF (Bauersachs, Popp et al. 1996, Nishikawa, Stepp et al. 2000), so in diseases associated with a reduction in NO bioavailability, EDHF may play a role in maintaining endothelium-dependent vasodilatation (Yang, Yim et al. 2007, Bellien, Thuillez et al. 2008, Nishikawa, Stepp et al. 2000). As the identity/s of EDHF is unknown, ex vivo work is based on an inferred presence - the identification of a dilatory capacity independent of NO and PGI$_2$ synthesis that is inhibited by blockade of slow and intermediate calcium-dependent potassium channels. The presence of non-NO, non-PGI$_2$ mediated vasodilatation has been identified in the myometrial arteries from healthy pregnancies (Luksha, Luksha et al. 2010, Kenny, Baker et al. 2002), where it has been observed to contribute up to 78% of bradykinin-induced endothelial dependent vasodilatation (Luksha, Luksha et al. 2010).

1.5.4 Uterine vascular function in normal pregnancy

Many animal studies suggest that in pregnancy, the uterine resistance vessel response shifts to favour dilatation over constriction. This is demonstrated by either enhanced response of the vessels to vasodilators or blunted response to vasoconstrictors (Dalle Lucca, Adeagbo et al. 2000, Anwar, Docherty et al. 1999, Mandala, Gokina et al. 2002, Weiner, Martinez et al. 1989, Ni, Meyer et al. 1997, Cooke, Davidge 2003, Gokina, Kuzina et al. 2010). These studies
have not been replicated to the same extent in humans. Studies comparing myometrial arteries from normal pregnancy with those from non-pregnant women are limited, due to difficulties obtaining appropriate non-pregnant control vessels; in non-pregnant women, these specimens must be taken from fresh hysterectomy specimens. In four studies comparing vessels from pregnant and non-pregnant women, the mean age of the non-pregnant women was significantly higher than the pregnant group studied. By virtue of their age and requirement for hysterectomy, these women may have suffered comorbid conditions (for example hypertension (Hongo, Nakagomi et al. 1988, Koga, Takata et al. 1988)), or taken medications that affect vascular function (for example oestrogen (Schiffrin 1997, Tagawa, Shimokawa et al. 1997, Kostrzewska, Laudánski et al. 1993) and comparisons should only be made with carefully selected controls.

1.5.4.1 Response to vasoactive agents - vasodilators
Arteries taken from the lateral surface of the uterus show a concentration-dependent relaxation to endothelium-dependent vasodilator acetylcholine (ACh), with significantly greater dilatation in arteries from pregnant compared to non-pregnant women (Nelson, Steinsland et al. 1998). In contrast, other studies using wire (Kenny, Baker et al. 2002) or pressure (Ashworth, Warren et al. 1997) myography found that response to endothelium-dependent vasodilator bradykinin did not differ between pregnant and non-pregnant groups. These discrepancies could be due to differences in the vessel size studied (arteries taken at the point where they enter the uterine serosa are likely to be much smaller than those dissected from myometrial biopsies (Nelson, Steinsland et al. 1998)), or differences in vasoconstrictors used, as the response to a vasodilator is affected by the choice of vasoconstrictor used to pre-constrict the vessel (Plane, Garland 1996).
1.5.4.2 Response to vasoactive agents - vasoconstrictors

Most studies have found no significant difference between the contractile response of pregnant and non-pregnant myometrial arteries to vasoconstriction agents such as 50 mM potassium chloride solution, (Eckman, Gupta et al. 2012, Nelson, Steinsland et al. 1998), angiotensin (Svane, Skajaa et al. 1991), thromboxane A2 mimetic U46619 (Svane, Skajaa et al. 1991), or norepinephrine (Nelson, Steinsland et al. 1998, Svane, Skajaa et al. 1991).

1.5.5 Uterine vascular function in IUGR pregnancy

Two studies have demonstrated greater maximal constriction in the myometrial arteries from women with IUGR pregnancies compared with those from normal pregnancies (Wareing, Myers et al. 2004, Sweeney, Wareing et al. 2008), although sensitivity to vasoconstrictors appears to be unchanged. In contrast, a third study found no difference in maximum constriction (Ong, Moore et al. 2003). The discrepancy between these studies could possibly be due to differences in size of the vessels studied (although information on vessel size is not available for all studies), or the more stringent criteria for defining IUGR in the first two studies (individualised birthweight ratio < 5<sup>th</sup> centile) compared to the other study (individualised birthweight ratio <10<sup>th</sup> centile). In contrast, attenuated responses to endothelium-dependent vasodilators in IUGR compared to normal pregnancy have been demonstrated consistently (Ong, Moore et al. 2003, Wareing, Myers et al. 2005a). The myometrial arteries taken from women with IUGR pregnancies relax significantly less than the same arteries from those with normal pregnancies. Even at the highest dose of BK used, myometrial arteries only relax to approximately a third of their pre-constriction tone, compared to 80% relaxation in the normal growth group (Ong, Moore et al. 2003, Wareing, Myers et al. 2004). The sensitivity of myometrial arteries to BK is impaired in IUGR compared to normal pregnancy, with greater concentrations of BK required to elicit half of
the maximal response (Wareing, Myers et al. 2004). There are limited published data on the mechanisms underlying impaired endothelium-dependent vasodilatation in IUGR, but further insight may be possible through the study of vessels from pregnancies affected by pre-eclampsia. The study of vessels from pregnancies affected by pre-eclampsia is relevant to IUGR, as both conditions share a common aetiology of aberrant spiral artery modification and placental hypoperfusion. As in IUGR, the myometrial vessels from pregnancies complicated by pre-eclampsia have impaired endothelium-dependent relaxation (Ashworth, Warren et al. 1997, Kenny, Baker et al. 2002). Therefore, it is possible that the mechanisms underlying impaired endothelium-dependent relaxation maybe common to both conditions. In pregnancies complicated by pre-eclampsia, myometrial vessels appear to not only be less responsive to vasodilators, but more sensitive to NO inhibition. Inhibition of NO production significantly attenuates the degree of relaxation to bradykinin in myometrial vessels of non-pregnant and pre-eclamptic pregnant women, but not of healthy pregnant women (Ashworth, Baker et al. 1999, Kenny, Baker et al. 2002). The contribution of EDHF to vasodilatation also appears to be altered in pre-eclampsia. In normal pregnancy, inhibition of NO or EDHF alone does not alter the dilatory response of myometrial vessels to bradykinin although when both were inhibited simultaneously, vasodilatation was abolished (Kenny, Baker et al. 2002). In pregnancies complicated by pre-eclampsia, when NO is inhibited, vasodilatation is significantly reduced even when EDHF is not inhibited (Kenny, Baker et al. 2002, Luksha, Luksha et al. 2010). This suggests that in pre-eclampsia, the myometrial arteries are more dependent on NO mediated vasodilatation, possibly due to a lack of EDHF as a backup mechanism. Similar studies have not been performed in the myometrial vessels from IUGR pregnancies. Given that the myometrial vessels of pre-eclampsia and IUGR share the same pathognomonic changes, it is highly probable that similar findings – a greater importance of NO and lesser contribution from EDHF – will be made.
Adaptation of the uterine circulation to pregnancy - summary

Pregnancy is associated with both an increase in placental perfusion and a fall in placental resistance as gestation advances, and failure of these changes to occur is associated with adverse pregnancy outcomes such as IUGR. Spiral artery transformation may be the major contributing factor to the early fall in placental resistance, but does not completely explain the reduced resistance or why resistance continues to fall as pregnancy progresses beyond the second trimester. Alterations in vascular function, specifically increased sensitivity to endothelium-dependent vasodilatation, also contributes to the fall of resistance within the utero-placental circulation during pregnancy. IUGR is associated with impairment of endothelium-dependent vasodilatation of the myometrial arteries. Therefore, pathways involving key mediators of endothelium-dependent vasodilatation – such as NO synthesis and metabolism – may provide therapeutic targets for the treatment of IUGR.

Potential new treatments for IUGR

When used in appropriately selected populations, prophylactic therapies such as aspirin (Dodd, McLeod et al. 2013, Bujold, Roberge et al. 2014) and heparin (Dodd, McLeod et al. 2013, Rodger, Carrier et al. 2014) are associated with small reductions in risk of IUGR. However, there are no treatments presently available that improve fetal growth or wellbeing once a diagnosis of IUGR has been made. There are several agents that show promise in the treatment of IUGR which are in varying stages of translation into the clinical setting. These include insulin-like growth factor supplementation (Eremia, de Boo et al. 2007), 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins; (Saad, Kechichian et al. 2014, Fox, Longo et al. 2011, Wyrmoll, Noble et al. 2016)), vascular endothelial growth factor treatment (Swanson, Rossi et al. 2016, Woods, Hoffmann et al. 2011), melatonin (Alers,

Insulin-like growth factor

Insulin-like growth factor-1 (IGF-1) plays a key role in the regulation of fetal growth. As IGF-1 may have significant effects on maternal physiology, it is preferable to administer the treatment directly to the fetus, for example via injection/infusion into the amniotic fluid (Spiroski 2014). In growth restricted ovine pregnancies, IGF-1 given weekly by amniotic fluid injection significantly improved fetal growth compared with saline controls (Eremia, de Boo et al. 2007). While the treatment appears efficacious, it is in an early phase of development, and further studies are required to investigate its efficacy at improving medium and long-term outcomes. Furthermore, the invasive means of delivery may limit the acceptability of this treatment to women, and therefore hamper its translation into clinical studies.

Statins

Statins are primarily prescribed in a non-pregnant population for their lipid lowering and cardio-protective properties (Cholesterol Treatment Trialists' (CTT) Collaboration, Baigent et al. 2010, Yusuf, Bosch et al. 2016). However, they also have a broad range of effects that make them promising candidates for the treatment of IUGR, such as vasodilatory and anti-inflammatory effects, up-regulation of angiogenic factors and endothelial nitric oxide synthase activation (Kumasawa, Ikawa et al. 2011, Saad, Kechichian et al. 2014, Fox, Longo et al. 2011, Garg, Krishan et al. 2015). There are a small number of animal studies demonstrating a beneficial effect of pravastatin on fetal growth (Wyrwoll, Noble et al. 2016), and maternal pathologies associated with pre-eclampsia (Ahmed, Singh et al. 2010, Kumasawa, Ikawa et al. 2011). In addition, a small case control study of women with IUGR or pre-eclampsia demonstrated improved maternal blood pressure and uterine artery blood
flow, prolonged pregnancy and increased birthweight following treatment with pravastatin (Lefkou, Mamopoulos et al. 2016). Whilst these results are suggestive of benefit, the study was not blinded, participants were not randomised and there was no placebo control. Therefore, the results should be interpreted with caution until replicated in well-designed randomised controlled trials.

**Vascular endothelial growth factor**

Vascular endothelial growth factor (VEGF) is a growth factor with angiogenic properties and a potent vasodilator action. VEGF plays a significant role in virtually all stages of placental development (Chen, Zheng 2014, Ku, Zaleski et al. 1993). Maternal serum levels of VEGF are lower in IUGR pregnancies compared to gestation matched controls (Savvidou, Yu et al. 2006). It has therefore been hypothesised that increasing VEGF might improve placental development and utero-placental blood flow, resulting in improved fetal growth (Mehta, Abi-Nader et al. 2012). To limit the effects of VEGF to the utero-placental vascular bed, this therapy is delivered via viral vectors suspended in a thermosensitive gel that is injected directly into the uterine artery. Using this technique, a beneficial effect of VEGF on fetal growth has been demonstrated in both small and large animal models (Swanson, Rossi et al. 2016, Carr, Wallace et al. 2014), and a phase I clinical trial has been proposed, to assess the therapy in a clinical setting (Sheppard, Spencer et al. 2016). As for intra-amniotic IGF-1 therapy, the invasive nature of this therapy may limit its translation into clinical trials.

**Melatonin**

Melatonin is a hormone produced predominantly by the pineal gland. Melatonin is renowned for its involvement in the regulation of circadian and seasonal rhythms, but also has potent antioxidant actions (Reiter, Tan et al. 2000). Reduced maternal serum melatonin and placental melatonin receptor concentrations have been demonstrated in women with pre-eclampsia and
IUGR pregnancies (Marseglia, D'Angelo et al. 2016, Lanoix, Guerin et al. 2012). It is hypothesised that this results in increased oxidative stress and endothelial dysfunction, and that supplementing with melatonin may reverse these changes and improve fetal growth (Alers, Jenkin et al. 2013). Although animal studies have demonstrated a neuroprotective effect of melatonin (Miller, Yan et al. 2005, Welin, Svedin et al. 2007), evidence of a beneficial effect on fetal growth is minimal. Pilot studies of melatonin for the treatment of IUGR are currently underway (Alers, Jenkin et al. 2013).

1.6.1 Sildenafil citrate – a novel therapy for intrauterine growth restriction

Of the potential new therapies for IUGR, sildenafil citrate (sildenafil) has been the most studied. Sildenafil is a phosphodiesterase – 5 (PDE-5) inhibitor that augments NO mediated vasodilatation. PDE catalyses the breakdown of NO second messenger cGMP, therefore inhibition of this enzyme via agents such as sildenafil increase exposure of vascular smooth muscle to cGMP resulting in smooth muscle relaxation and vasodilatation. Over 60 isoforms of PDE have been described, each with different properties and tissue distributions (Manallack, Hughes et al. 2005, Wallis, Corbin et al. 1999, Lin, Lin et al. 2006). The selectivity and specificity of pharmacologic agents for different PDE isoforms has important therapeutic implications, as targeting specific isoforms will limit PDE inhibition to specific vasculature beds. Sildenafil is a potent selective inhibitor of PDE-5 (Wallis, Corbin et al. 1999), an isoform which is found in organs that contain substantial amounts of smooth muscle, such as the uterus and corpus cavernosum (Lin, Lin et al. 2006, Belmonte, Ticconi et al. 2005). PDE-5 is the predominant isoform of the corpus cavernosum, and sildenafil has been extremely successful commercially as a treatment for erectile dysfunction, as it increases penile blood flow and erectile activity with minimal systemic effects (Boolell, Allen et al. 1996, Corbin, Francis et al. 2002). The success of sildenafil as a treatment for erectile
dysfunction led to a parallel hypothesis: that sildenafil could also increase utero-placental blood flow, and therefore be an effective therapeutic option for the treatment of pregnancy complications caused by utero-placental vascular insufficiency. Early studies lent biologic plausibility to this theory. *Ex-vivo* studies of myometrial small arteries provided evidence that sildenafil altered the response of these vessels to vasoconstrictors and vasodilators. In wire myography experiments, when myometrial arteries from healthy pregnancies were pre-incubated with sildenafil, they exhibited increased sensitivity to endothelium-dependent vasodilator bradykinin (Wareing, Myers et al. 2006). These findings were also replicated in the myometrial small arteries from women with IUGR pregnancies (individualised birthweight ratio $<5^{\text{th}}$ centile). Although the arteries from growth restricted pregnancy showed impaired relaxation and increased constriction compared to healthy pregnancy, when pre-incubated with sildenafil, constriction was reduced and relaxation significantly improved (Wareing, Myers et al. 2004). Around the same time, several small studies of non-pregnant women demonstrated a reduction in uterine artery PI and RI after one hour (Sher, Fisch 2000) and 7 days (Alatas, Yagci 2004) of sildenafil treatment, suggesting that sildenafil increased uterine blood flow. Invasive studies using flow transducers to calculate blood flow directly confirmed this in a sheep study. In oophorectomised ewes, sildenafil treatment was associated with a 33% increase in uterine artery volumetric flow (Zoma, Baker et al. 2004). The first (animal) studies of the use of sildenafil use in pregnancy were published shortly after (Refuerzo 2006).

### 1.7 Can sildenafil citrate improve fetal growth? A review of the literature

There are now more than 14 published animal studies that have assessed sildenafil as a treatment for IUGR (six of which have been published since experimental work for this thesis
began). The findings of studies that assessed the efficacy of sildenafil on fetal growth are summarised in Table 1.4.

1.7.1 Studies in mice and rats

Four studies in mice and five in rats have assessed the efficacy of sildenafil at improving fetal growth. Three of the four mouse studies have used genetically modified mouse strains (Catechol-O-methyl transferase, endothelial nitric oxide synthase, and placental IGF2 knockouts) as models for IUGR pregnancy. In this section, a brief description of the phenotype of the model is provided for each strain; the use of catechol-o-methyl transferase and endothelial nitric oxide synthase knockout mice for the study of IUGR is discussed in more detail in section 1.9.1, below.

The catechol-O-methyl transferase knockout (COMT\(^{-/-}\)) mouse lacks an enzyme involved in metabolism of 17β-oestradiol, and is deficient in 2-methoxyoestradiol (2-ME\(_2\)). 2-ME\(_2\) (and 4-ME\(_2\), another product of COMT metabolism) may be important in development of the utero-placental circulation (Lee, Wong et al. 2010). In humans, deficiencies of both 2- and 4-ME\(_2\) have been associated with pre-eclampsia and IUGR (Kanasaki, Palmsten et al. 2008, Sata, Yamada et al. 2006, Jobe, Tyler et al. 2013). In a study by Stanley et al (2012), COMT\(^{-/-}\) mice had significantly smaller pups compared to wild-type controls. Treatment with 0.2 mg/mL sildenafil in the drinking water (treatment intake not reported) increased pup weight and abdominal circumference, although there was no difference in fetal growth in the control group. The COMT\(^{-/-}\) mouse also displayed an abnormal umbilical artery Doppler waveform from mid-gestation, indicative of increased resistance to fetal-placental flow. This waveform was normalised following treatment with sildenafil, suggesting that sildenafil was reducing feto-placental resistance. The uterine arteries of COMT\(^{-/-}\) mice showed greater constriction when assessed by wire myography; however, they constricted less and showed increased...
sensitivity to methacholine following maternal sildenafil treatment. Despite these differences in vessel function, no differences in uterine artery waveforms were observed (Stanley, Andersson et al. 2012).

The placental-specific insulin-like growth factor 2 P0 knockout mouse (P0 mouse) lacks placental-specific insulin-like growth factor 2. P0 dams produce smaller pups and placentae compared to wild-type controls. In addition, placentae have reduced surface area and thickening of the placental exchange barrier, although utero-placental blood flow is normal. When P0\(^{-/-}\) dams were treated with 0.4 mg/mL sildenafil via drinking water (intake not reported) there was a 9% increase in average pup weight, an increase in pup abdominal circumference, and a trend toward increased placental weight (treated vs untreated: 0.072 ± 0.002 vs 0.065 ± 0.002 g, \(p = 0.056\)). Sildenafil had no effect on pup or placental growth in the wild-type control group. Sildenafil had no effect on umbilical artery waveform or system-A amino acid transport efficacy; however, the larger placentae in the sildenafil-treated group resulted in an increase in estimated amino-acid delivery to the fetus (Dilworth, Andersson et al. 2013).

The third mouse strain used to study the effect of sildenafil on fetal growth was the endothelial nitric oxide synthase knockout (eNOS\(^{-/-}\)). These mice lack the NOs isoform responsible for production of NO by the endothelium. They are hypertensive, have abnormal placental vasculature and growth restricted offspring (Kulandavelu, Whiteley et al. 2013, Kusinski, Stanley et al. 2012). In this study, female eNOS\(^{-/-}\) mice were mated with wildtype males, and dams were treated with sildenafil 0.4 mg/ml day (average sildenafil intake of 2 mg per day) for the entire duration of pregnancy (Roberts, Refuerzo et al. 2016). Pups from eNOS\(^{-/-}\) mothers treated with sildenafil were significantly heavier than those born to untreated mothers; however, there was no effect of sildenafil in the control strain. Sildenafil reversed
the greater constriction and impaired sensitivity to endothelium-dependent vasodilator that was seen in the carotid arteries from eNOS$^{-/-}$ dams. However, changes in uterine artery function were not assessed, and the relevance of the change in carotid artery function to pregnancy outcomes is unclear.

Pharmacologic inhibition of NO synthesis with NG-nitro-L-arginine methyl ester (L-NAME) has been used in several studies (1 mouse, 3 rat), as a model of IUGR. L-NAME competitively inhibits production of NO from L-arginine. In contrast to the eNOS$^{-/-}$ model, L-NAME inhibits all 3 isoforms of NOS, so outcomes for eNOS$^{-/-}$ and L-NAME treated animals may differ. As discussed below, the effects of sildenafil on fetal growth in L-NAME induced IUGR are highly variable. This is likely due to heterogeneity in timing of L-NAME in relation to sildenafil treatment, as well as differences in sildenafil dosing and route of administration.
<table>
<thead>
<tr>
<th>Species</th>
<th>Model of IUGR</th>
<th>Treatment duration</th>
<th>Treatment route</th>
<th>Fetal weight</th>
<th>Placental weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>12.5 – 18.5 dGA</td>
<td>Drinking water (0.2 mg/ml, intake not stated)</td>
<td>↑ in IUGR; ↔ in control</td>
<td>↔</td>
</tr>
<tr>
<td></td>
<td>(Stanley, Andersson et al. 2012)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>P0&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>12.5-18.5 dGA</td>
<td>Drinking water (0.4 mg/ml; intake not stated)</td>
<td>↑ in IUGR; ↔ in control</td>
<td>Trend to ↑</td>
</tr>
<tr>
<td></td>
<td>(Dilworth, Andersson et al. 2013)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>L-NAME</td>
<td>8-16 dGA</td>
<td>Drinking water (10 mg/kg/day)</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td></td>
<td>(Motta, Grosso et al. 2015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1-18 dGA</td>
<td>Drinking water (average intake 2 mg per day)</td>
<td>↑ in IUGR; ↔ in control</td>
<td>↔</td>
</tr>
<tr>
<td></td>
<td>(Roberts, Refuerzo et al. 2016)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Maternal hypoxia</td>
<td>18-21dGA</td>
<td>Water “orally” (45 mg/kg/12 hr)</td>
<td>↑ in IUGR; ↓ in control</td>
<td>↔</td>
</tr>
<tr>
<td></td>
<td>(Refuerzo, Sokol et al. 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>L-NAME</td>
<td>7-19 dGA</td>
<td>Subcutaneous injection (10 mg/kg/day)</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td></td>
<td>(Ramesar, Mackraj et al. 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4 - Summary of the effects of sildenafil on fetal growth in animal studies

(continued next page)
<table>
<thead>
<tr>
<th>Species</th>
<th>Model of IUGR</th>
<th>Treatment duration</th>
<th>Treatment route</th>
<th>Fetal weight</th>
<th>Placental weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>None</td>
<td>4-20 dGA</td>
<td>Gel diet (10, 50, or 90 mg/kg/day)</td>
<td>↔</td>
<td></td>
</tr>
<tr>
<td>(Sasser, Baylis 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>None</td>
<td>0 dGA until end of pregnancy</td>
<td>Drinking water (4 mg/kg/day)</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>(Pellicer, Herraiz et al. 2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>L-NAME</td>
<td>14-21 dGA</td>
<td>Gavage (15 mg/kg/day)</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>(Nassar, Masrouha et al. 2012)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>L-NAME</td>
<td>0 dGA until end of pregnancy</td>
<td>Drinking water (4 mg/kg/day)</td>
<td>↔ in IUGR; ↑ in control</td>
<td>↑</td>
</tr>
<tr>
<td>(Herraiz, Pellicer et al. 2012)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>RUPP at 14 dGA</td>
<td>14 dGA to end of pregnancy</td>
<td>Drinking water (45 mg/kg/day)</td>
<td>↔</td>
<td>↔ in IUGR; ↑ in control</td>
</tr>
<tr>
<td>(George, Palei et al. 2013)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Dahl salt- sensitive rat</td>
<td>10-20 dGA</td>
<td>Gel diet (50 mg/kg/day)</td>
<td>↑ in IUGR</td>
<td>↓</td>
</tr>
<tr>
<td>(Gillis, Mooney et al. 2016)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4 (continued) - Summary of the effects of sildenafil on fetal growth in animal studies (continued next page)
<table>
<thead>
<tr>
<th>Species</th>
<th>Model of IUGR</th>
<th>Treatment duration</th>
<th>Treatment route</th>
<th>Fetal weight</th>
<th>Placental weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea Pig</td>
<td>None</td>
<td>35 dGA until end of pregnancy</td>
<td>Syringe fed (50 -500 µg/kg/day)</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>(Sánchez-Aparicio, Mota-Rojas et al. 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Caloric restriction</td>
<td>22 dGA until end of pregnancy</td>
<td>Syringe fed (5 mg/kg/day)</td>
<td>↔, ↓ pups &lt; 10th centile</td>
<td>↔</td>
</tr>
<tr>
<td>(Lopez-Tello, Arias-Alvarez et al. 2016)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Caloric restriction</td>
<td>28-115 dGA</td>
<td>Sub-cutaneous injection</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>(Satterfield, B. 2010)</td>
<td></td>
<td></td>
<td>(75 - 150 mg/day)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.4 (continued) - Summary of the effects of sildenafil on fetal growth in animal studies*
In a mouse study where L-NAME was given at 7-18 dGA, there was no difference in fetal or placental weights following sildenafil treatment (given from 8 dGA (Motta, Grosso et al. 2015)). This result is not entirely unexpected: in this model L-NAME did not reduce fetal or placental size compared to control, therefore the lack of change in growth is similar to that observed in control mice in other studies (Gillis, Mooney et al. 2016, Dilworth, Andersson et al. 2013, Stanley, Andersson et al. 2012). Despite the lack of effect on fetal or placental growth, L-NAME reduced fetal vascularity within the placental labyrinth (the zone where nutrient exchange occurs between maternal and fetal circulations). This effect was reversed with sildenafil treatment, suggesting sildenafil altered angio- or vasculogenesis within the fetal aspect of the placenta. In rats, L-NAME administered from the beginning of pregnancy reduced fetal growth. Sildenafil did not improve growth in the L-NAME group, however, sildenafil increased growth in the control group, and increased placental growth in both groups. Paradoxically, L-NAME was associated with reduced uterine artery PI compared to controls, and sildenafil was associated with increased uterine artery PI, although there was no difference amongst groups in systolic velocity (Herraiz, Pellicer et al. 2012). L-NAME administered to pregnant rats from 7 dGA also resulted in smaller pups (Ramesar, Mackraj et al. 2010). Although the addition of sildenafil treatment did not significantly increase weight compared to L-NAME alone, pups in the sildenafil + L-NAME group were intermediate in weight between the L-NAME and wild-type control groups suggesting a beneficial effect on growth. Sildenafil was also associated with an increased number of live pups at delivery (Ramesar, Mackraj et al. 2010). A third study, which used L-NAME from mid pregnancy (14 dGA) showed fetal growth was reduced in those treated with sildenafil from day 14 of pregnancy (with or without L-NAME) compared to the control. However, in this study both L-NAME and sildenafil were administered via gavage, with the control group receiving no interventions. Gavage is a stressful event for the mother, and this could negatively influence
fetal growth and explain why pups from control groups were heavier (Nassar, Masrouha et al. 2012).

Dahl salt-sensitive rats have been selectively bred to produce a phenotype of hypertension and renal impairment following even moderate dietary intake of sodium chloride, although the underlying cellular and genetic aetiologies of the disorder is unknown. Dahl salt-sensitive rats produce growth restricted offspring, and exhibit abnormal uterine artery Doppler waveforms with elevated uterine artery RI throughout pregnancy. In these rats, maternal sildenafil treatment from mid pregnancy (10 dGA) increased pup weight and length, and reduced maternal uterine artery RI in late pregnancy, suggesting an increased uterine artery blood flow (Gillis, Mooney et al. 2016). Sildenafil had several other favourable effects including reducing maternal blood pressure and improving maternal renal function (Gillis, Mooney et al. 2016). In addition, plasma TNF-α, urinary endothelin-1 and isoprostane were raised in Dahl salt-sensitive rats but reduced with sildenafil treatment, suggesting a more favourable angiogenic profile, and reduced oxidative stress.

Intermittent maternal hypoxia was used in late gestation (18-20 dGA) rats to induce growth restriction. Sildenafil was administered 12 hourly for 7 doses from 18 dGA. In the hypoxia group, sildenafil exposed pups were significantly heavier than controls. However, in the absence of maternal hypoxia, sildenafil was associated with reduced pup weight (Refuerzo, Sokol et al. 2006). A limitation of this study is that a significant (16%) proportion of the study animals were excluded due to incorrect pregnancy dating, and it is unclear whether incorrect dating influenced the growth outcomes. Furthermore, it appears that the individuals within the litter, rather than the overall average of the litter were used for statistical analysis, which may have confounded results (Refuerzo, Sokol et al. 2006).
Reduced uterine perfusion pressure (RUPP) is a model for utero-placental vascular insufficiency / IUGR that is achieved through the surgical clipping of the aorta and ovarian arteries. Pregnant rats underwent a RUPP procedure at 14 dGA then commenced sildenafil treatment following the procedure. Sildenafil did not result in changes in fetal or placental growth in the RUPP group; however, placental growth was increased in normal pregnant rats treated with sildenafil (George, Palei et al. 2013). In other models of utero-placental vascular insufficiency (for example eNOS−/− mice or Dahl salt-sensitivity in rats) changes in uterine blood flow are relatively subtle. In contrast RUPP results in a sudden and severe reduction in uterine blood supply to a normally grown placenta and fetus. In this respect, rather than IUGR, RUPP may better serve as a model for sudden unexpected reductions to fetal oxygenation - such as profound maternal hypotension (for example following pulmonary embolism, amniotic fluid embolus or sepsis) or placental abruption. Studies that have shown increases in fetal growth with sildenafil suggest a relatively modest effect (Stanley, Andersson et al. 2012, Dilworth, Andersson et al. 2013, Gillis, Mooney et al. 2016); even if sildenafil does increase fetal growth, the insult produced by RUPP may be too severe for it to overcome (George, Palei et al. 2013).

Finally, two studies have assessed the efficacy of sildenafil in improving fetal growth in the absence of growth restriction. In the first study, rats treated with sildenafil from the start of pregnancy had heavier and longer pups with increased fetal liver weight at mid- and late pregnancy, and placental weight was also increased. In this study, sildenafil also reduced uterine artery PI and increased uterine artery end-diastolic velocity in later pregnancy (Pellicer, Herraiz et al. 2011). In contrast, in rats treated with sildenafil from 4-19 dGA, pup size was unaltered, even when high doses of sildenafil were administered (Sasser, Baylis 2010).
1.7.2 Studies in other animals

In addition to studies of fetal growth in small rodents, three studies have assessed the efficacy of sildenafil in increasing fetal growth in the rabbit, guinea pig and sheep. In the guinea pig, sildenafil was administered to normal pregnant sows from day 35 (term = 50-75 dGA), in two doses (50 or 500 µg/kg/day). Sildenafil exposure was for approximately three weeks. Sows were examined twice daily, and when labour appeared to be initiated, caesarean section was undertaken. The umbilical cord was clamped for five minutes to induce hypoxia. There was a dose dependent effect of sildenafil on birthweight; those exposed to the highest dose of sildenafil were 50% heavier than controls. Pups born to mothers treated with sildenafil also had heavier kidneys, hearts, and lungs compared to the other groups. However, sildenafil exposure at both doses resulted in improved tolerance to the hypoxic insult, as demonstrated by a reduced metabolic disturbance (Sánchez-Aparicio, Mota-Rojas et al. 2008).

In rabbits, caloric deprivation from early pregnancy was used to induce growth restriction, then dams were treated with sildenafil 5 mg/kg/day from day 22 (term = 31 dGA). Compared to caloric deprivation alone, there was no significant increase in fetal weight with sildenafil treatment; however, morphometric measurements (biparietal diameter, crown-rump length, thoracic diameter) were all increased. The weights of pups from the normal-growth control group were used to calculate a 10th centile, and IUGR defined as a pup with a weight below this. Sildenafil treatment was associated with a reduction in proportion of IUGR pups, suggesting a beneficial effect on fetal growth. Total placental weight was similar between groups; however, placenta: fetal and labyrinth weight: fetal weight ratios were higher following sildenafil treatment suggesting greater placental efficiency. Sildenafil treatment was also associated with changes in placental structure, including: thickening of the decidual
zone, and a trend towards thickening of the labyrinth zone \( (p = 0.08) \), and an increase in vascularity of the junctional zone (Lopez-Tello, Arias-Alvarez et al. 2016).

In an ovine study, IUGR was induced by restricting nutrition to pregnant ewes from day 28 (term = 147 dGA). Ewes were treated with sildenafil 75 mg or 150 mg per day given as a subcutaneous injection in 3 divided doses, or a vehicle solution. Sildenafil increased fetal weight at 115 dGA in a dose dependent manner, in both normal pregnancy and nutrient restricted groups. There was an increase in weight of the fetal left ventricle (restricted) and pancreas (adequately fed) and reduced spleen size (adequately fed) in the highest dose groups; placental weight was unaffected. Sildenafil treatment had a dose dependent effect whereby it increased total amino acids in the fetal serum and amniotic fluid, however maternal levels were unaffected (Satterfield, Bazer et al. 2010).

### 1.7.3 Clinical studies

The data pertaining to the use of sildenafil use in human pregnancy as a treatment for IUGR is limited to case reports, and a small case-control study. However, two randomised controlled trials of its use as a treatment for pre-eclampsia have recently been published (Table 1.5). Two case reports have been published where sildenafil was administered to women with suspected IUGR, both with absent umbilical artery EDF at 26 weeks’ gestation (Lin, S. 2012, Panda, Das et al. 2014). In both cases, there was continued growth and reduction in uterine artery Doppler resistance indices, and in one case improvements in umbilical artery flow (Panda, Das et al. 2014). In both reports, after 3-4 weeks of treatment, deterioration in umbilical artery Dopplers necessitated delivery of the infant.
In a case control study, women with severely growth restricted pregnancies were offered treatment with sildenafil 25 mg three times daily until delivery. Their outcomes were compared with women who fulfilled entry criteria but who declined or were not offered this treatment. The mean gestation at enrolment was between 21+1 and 22+4 weeks gestation, and the degree of growth restriction was extremely severe (estimated rate of intact survival <50%). Most women enrolled had uterine artery notching at enrolment with the fetal abdominal circumference below the first centile, and half of the sildenafil treated group had absent or reversed umbilical artery end-diastolic flow. Pre-treatment growth velocity of the abdominal circumference was compared to growth velocity post treatment. There was a significant increase in the proportion of mothers whose fetus’s had an increase in abdominal circumference growth velocity with sildenafil treatment (rates of increased abdominal circumference growth for control vs sildenafil: 41 vs 90%, p = 0.02 (von Dadelszen, Dwinnell et al. 2011). Whilst the findings from this study appear promising, caution is necessary, due to the study design. The women exposed to sildenafil may have differed systematically from women not offered or who declined treatment. For example, a greater proportion of women who had taken sildenafil developed severe pre-eclampsia, and had absent or reversed umbilical artery EDF at recruitment; they are thus likely to have represented more severe placental pathologies (von Dadelszen, Dwinnell et al. 2011). The heterogeneity in gestational age, severity, and co-morbidities (particularly pre-eclampsia) amongst previous studies is such that a definitive answer would only be possible from a rigorously designed randomised controlled trial.

Two randomised controlled trials of sildenafil in human pregnancy as a treatment for pre-eclampsia have been conducted (Table 1.6). In the first study, the average gestation at recruitment was 31+4 weeks’ in sildenafil treated vs 29 weeks’ in the control subjects. Approximately a third in each group had suspected IUGR. There was no significant
prolongation of pregnancy with sildenafil treatment, nor was there increased fetal weight or reduction in proportion of indicated deliveries for suspected fetal compromise (Samangaya, Mires et al. 2009). In the second study, the mean gestation at enrolment was 29-30 weeks’, and just over half the pregnancies recruited were complicated by IUGR. Sildenafil treatment 50 mg eight hourly was associated with a 4 day prolongation of pregnancy. There were no significant differences between groups in fetal weight, or delivery indication of fetal distress, however, the study was not powered to determine these outcomes. In this study, sildenafil treatment was associated with significant reductions in umbilical artery and uterine artery resistance 24 hours after the first sildenafil dose (Trapani, Goncalves et al. 2016). These findings are similar to those of a double-blind placebo controlled study where women with severe IUGR between 24-37 weeks gestation received a single dose of sildenafil 50 mg, or placebo. This study also showed sildenafil reduced umbilical artery PI two hours post ingestion (mean difference 0.12; \( p = 0.019 \)) (Dastjerdi, Hosseini et al. 2012). No measurements of uterine artery blood flow were performed in this study, and in both studies it is unclear whether reductions in umbilical or uterine artery resistance indices were maintained over time.
<table>
<thead>
<tr>
<th>Case characteristics (case study) or inclusion criteria (trial)</th>
<th>Treatment dose</th>
<th>Key findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case study</strong> (Lin, S. 2012)</td>
<td>25 mg TDS</td>
<td>Normal fetal growth over 4 weeks of pregnancy with improved uterine artery Doppler indices.</td>
</tr>
<tr>
<td>26 weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fetal weight &lt;5th centile, absent EDF umbilical artery, raised uterine artery PI</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Case study</strong> (Panda, Das et al. 2014)</td>
<td>50 mg TDS</td>
<td>Initially improved umbilical and uterine artery Doppler indices followed by deterioration of umbilical artery Doppler waveform after 3 weeks.</td>
</tr>
<tr>
<td>26 weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>absent EDF umbilical artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Case-control</strong> (von Dadelszen, Dwinnell et al. 2011)</td>
<td>25 mg TDS</td>
<td>Increased abdominal circumference growth compared to controls.</td>
</tr>
<tr>
<td>&lt;25 weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC&lt;5th centile or EFW&lt;600 g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.5 - Studies of sildenafil for the treatment of IUGR in human pregnancy*
### Inclusion criteria

**Randomised controlled trial**  
(Samangaya, Mires et al.
2009)  
Pre-eclampsia at 24-34 weeks  
(stratified for FGR)

<table>
<thead>
<tr>
<th>Key findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy duration no different to controls.</td>
</tr>
</tbody>
</table>

**Randomised controlled trial**  
(Trapani, Goncalves et al.
2016)  
Pre-eclampsia at 24-33 weeks  
(not stratified for FGR)

<table>
<thead>
<tr>
<th>Key findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced uterine and umbilical artery Doppler indices, but no change in fetal cerebral Doppler indices. Pregnancy prolongation by 4 days compared to placebo.</td>
</tr>
</tbody>
</table>

---

**Table 1.6 - Randomised controlled trials of sildenafil for the treatment of pre-eclampsia**
1.7.4 The effect of sildenafil on fetal growth - summary

In summary, there is insufficient evidence available from human trials to determine whether sildenafil can improve fetal growth. A variety of experimental models of IUGR have been used to study the effect of sildenafil on fetal growth. Even within species, differences in the method of inducing growth restriction, treatment dosing, timing and route of administration mean that direct comparisons between studies are difficult. The balance of published evidence suggests sildenafil can have a beneficial effect on fetal growth, with nine of the fifteen animal studies showing an increase in growth in at least one experimental group. It is clear that benefit is not universal, as in eight studies there was no effect of sildenafil on fetal growth in at least one experimental group. There is no obvious explanation (for example differences in study design, treatment initiation or species differences) as to why some groups have shown increased fetal growth with sildenafil treatment and others have not. Although a lack of benefit is often seen in groups where fetal growth was not impaired to begin with, this is not invariably true. Furthermore, in some instances sildenafil has been associated with reduced fetal growth, both in animals where growth restriction has been induced, and in controls. Although significant increases in infant mortality have not been reported in any study, changes in organ growth (spleen, pancreas and left ventricle) have been reported in fetal lambs exposed to sildenafil in utero (Satterfield, Bazer et al. 2010) and the implications of these changes in later life are unknown.

1.8 Mechanisms that may mediate changes in growth with sildenafil treatment

It is unclear from the literature as to the mechanisms by which sildenafil treatment may affect fetal growth (Table 1.6). Understanding these mechanisms may aid understanding of why benefit exists in some groups and not others. In practical terms this may allow the targeting
of a treatment - whose long-term outcomes are unclear - to the group it is most likely to benefit, and avoidance of treatment in those for whom it has potentially detrimental effects. Should sildenafil be proven effective at improving fetal growth in human IUGR, understanding the mechanism of action may also help clinicians use this treatment most effectively and efficiently. For example, if sildenafil works through a vasodilatory effect on the uterine resistance vessels, treatment could be targeted to pregnancies where there is evidence of high resistance to flow in the uterine or radial arteries. In contrast, if sildenafil is shown to work through increasing angiogenesis, it may be more effective when started as a prophylaxis in women at high risk of placental disease, or women who show an impaired anti-angiogenic profile in early pregnancy.

The primary hypothesis as to the mechanism through which sildenafil increases fetal growth has been that sildenafil increases the myometrial small arteries sensitivity to NO induced vasodilatation, and that utero-placental blood flow is increased as a result. Early ex-vivo studies of myometrial arteries from human IUGR pregnancies supported this, demonstrating increased sensitivity to endothelium-dependent vasodilatation after pre-incubation with sildenafil (Wareing, Myers et al. 2004). However, these findings have not been replicated consistently when sildenafil exposure has occurred in vivo. In a study of women with severe early onset pre-eclampsia randomised to sildenafil treatment or control, sildenafil treatment did not affect myometrial artery ex vivo vasoreactivity, although limited time of treatment exposure, and a delay between treatment and assessment, may have contributed to the lack of difference (Samangaya, Mires et al. 2009). In the COMT+/- mouse, although an increase in sensitivity to endothelium-dependent vasodilatation was observed in the uterine arteries of mothers treated with sildenafil, there was no difference in uterine artery Doppler flow indices. This suggests that despite the changes observed in uterine vessel function, uterine flow was not increased. In sheep, a bolus of sildenafil given subcutaneously was associated with a
significant decrease in uterine blood flow, with accompanied reduction in fetal oxygenation in both growth restricted and control animals (Miller, Loose et al. 2009). It is unclear why uterine blood flow was reduced in this study. It is possible that this is due to vasodilation in systemic vascular beds, resulting in blood flow “steal” resulting in reduced uterine perfusion pressures. These studies cast doubt on the primary hypothesis that sildenafil increases fetal growth through increased myometrial artery relaxation and subsequent increase in uterine blood flow. Furthermore, this hypothesis does not explain why in some studies sildenafil increased fetal growth in the absence of utero-placental vascular pathology (Dilworth, Andersson et al. 2013, Nassar, Masrouha et al. 2012, Sánchez-Aparicio, Mota-Rojas et al. 2008).

Some studies have reported findings that suggest sildenafil may increase fetal growth through mechanisms other than (or in addition to) changes in uterine resistance vessel function. One study has suggested changes in inflammation pathways, oxidative stress and balance between angiogenic and anti-angiogenic factors (Gillis, Mooney et al. 2016). Several studies have suggested changes to placental growth and development following maternal sildenafil treatment; these include an increase (Herraiz, Pellicer et al. 2012, Pellicer, Herraiz et al. 2011) - or a trend to increase (Dilworth, Andersson et al. 2013) - in placental weight, and changes to vascularity within the labyrinth (exchange) zone in rodents (Lopez-Tello, Arias-Alvarez et al. 2016, Motta, Grosso et al. 2015). Again, these findings have not been consistently reported or demonstrated across studies, and given differences in placental structure between species, their relevance to human pregnancy is unclear. Sildenafil also has the potential to effect fetal growth through changes to the fetal-placental circulation and is known to cross the placenta. Chorionic (from normal pregnancy) and umbilical arteries (from preeclamptic and normal pregnancies) show dose-dependent relaxation following direct application of sildenafil (Karasu, K. 2012, Wareing, Myers et al. 2005b). Sildenafil has also been shown to reduce
umbilical artery resistance indices in both humans (Trapani, Goncalves et al. 2016, Dastjerdi, Hosseini et al. 2012) and mice (Stanley, Andersson et al. 2012), but no studies have examined the effect of sildenafil on umbilical or placental arteries following in vivo exposure.
<table>
<thead>
<tr>
<th>Species</th>
<th>Model of IUGR</th>
<th>Findings associated with sildenafil treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>COMT&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Reduced constriction and increased vasodilator sensitivity in uterine arteries. Minimum diastolic velocity was increased in umbilical artery. No change observed in uterine artery Doppler indices.</td>
</tr>
<tr>
<td>(Stanley, Andersson et al. 2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>P0&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>No change in uterine and umbilical artery Doppler indices. Placental system A transporter activity unaltered.</td>
</tr>
<tr>
<td>(Dilworth, Andersson et al. 2013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>L-NAME</td>
<td>Increased markers for fetal endothelium identified within the placenta.</td>
</tr>
<tr>
<td>(Motta, Grosso et al. 2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Normal pregnancy</td>
<td>Reduced uterine artery Doppler indices.</td>
</tr>
<tr>
<td>(Pellicer, Herraiz et al. 2011)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>L-NAME</td>
<td>Increased uterine artery Doppler indices.</td>
</tr>
<tr>
<td>(Herraiz, Pellicer et al. 2012)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.6 – Findings reported in association with sildenafil treatment in pregnancy*  
*(continued next page)*
<table>
<thead>
<tr>
<th>Species</th>
<th>Model of IUGR</th>
<th>Findings associated with sildenafil treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>RUPP</td>
<td>No change in placental sFLT-1 or VEGF, or maternal plasma VEGF. Increased maternal renal cGMP.</td>
</tr>
<tr>
<td>(George, Palei et al. 2013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Dahl salt-sensitivity</td>
<td>Reduced TNFα in placenta and maternal plasma, reduced endothelin-1 urinary excretion. Reduced uterine artery Doppler indices.</td>
</tr>
<tr>
<td>(Gillis, Mooney et al. 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Caloric restriction</td>
<td>Increased vascularity of placental labyrinth zone with a trend toward increased thickness of labyrinth.</td>
</tr>
<tr>
<td>(Lopez-Tello, Arias-Alvarez et al. 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Caloric restriction</td>
<td>Increased fetal amino acids and polyamines in fetal serum and amniotic fluid.</td>
</tr>
<tr>
<td>(Satterfield, B. 2010)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Pre-eclampsia</td>
<td>Reduced uterine and umbilical artery Doppler indices.</td>
</tr>
<tr>
<td>(Trapani, Goncalves et al. 2016)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.6 (continued) - Findings reported in association with sildenafil treatment in pregnancy*
1.9 Animal models of IUGR

While aspects of fetal and placental growth and placental function can be studied in human pregnancy or by analysing human samples _ex vivo_, there are significant limitations in using these methods alone. It is difficult, time-consuming and expensive to study uncommon pathologies, and it is unethical to study treatments in human pregnancy which are theoretically promising in the absence of any safety information. Therefore, animal studies are important for the study of mechanisms and functional aspects underpinning the changes seen in both normal and abnormal pregnancy, as well as the development of new therapies for pregnancy complications.

Although the placenta performs common physiologic roles, there are important differences in placental structure amongst species. Knowledge of these similarities and differences is important when designing and interpreting studies, and extrapolating results to human pregnancy. There are also a variety of methods which can be used to model intrauterine growth restriction, including maternal hyperthermia (Galan, Hussey et al. 1999, Regnault, Orbus et al. 1999, Thureen, Trembler et al. 1992), hypoxia (Brain, Allison et al. 2015), over-feeding (Wallace, Bourke et al. 1999, Wallace, Da Silva et al. 1997, Wallace, Bourke et al. 2002), nutrient restriction (Edwards, McMillen 2001, Vonnahme, Lemley 2011, Redmer, Aitken et al. 2005, MacLaughlin, Walker et al. 2005), administration of drugs (e.g. L-NAME (Herraiz, Pellicer et al. 2012)), genetic manipulation (Oyama, Padbury et al. 1992, Supramaniam, Jenkin et al. 2006, Bertucci, Loose et al. 2011) or ligation of the umbilical (Oyama, Padbury et al. 1992, Supramaniam, Jenkin et al. 2006, Bertucci, Loose et al. 2011) or uterine artery (Jansson, Persson 1990, Lafeber, Rolph et al. 1984), restriction of the space available for placental implantation (Giles, Trudinger et al. 1989, Meyer, Koch et al. 2010), and embolisation of the uterine (Bloomfield, Bauer et al. 2002, Clapp III, McLaughlin et al.)
1982) or fetal-placental circulations (Gagnon, Challis et al. 1994). Just as no species has a placenta identical to humans, no model will exactly replicate the pathophysiology of human IUGR. A complete discussion on the advantages and disadvantages of each of the different animal models is beyond the scope of this chapter. Some of the strengths and challenges of the models used in this thesis are outlined in the following section. As this thesis aims to study sildenafil as a treatment of IUGR caused by utero-placental vascular insufficiency, the focus is on animal models that exhibit reduced utero-placental blood flow, and/or endothelial dysfunction in the uterine arteries.

1.9.1 Using mice to study IUGR

General advantages of using mice to study pregnancy complications include the ease of handling, the affordability of housing, and the short oestrus and consequent rapid conception. Disadvantages of mice include their small size (meaning that manipulations or in vivo measurements may be more challenging), the production of litters (another variable affecting fetal weight), and the timeframe of fetal development that is different to humans. In humans, organogenesis is completed in the first quarter of pregnancy (10 weeks’ gestation) compared to being complete at almost three-quarters of the way through mouse gestation (14.5 dGA (Mu, Adamson 2006)) with mice producing altricial young. These differences may mean it is more difficult to translate the effects of pregnancy exposures on developmental processes in fetal mice to human pregnancy.

There are many similarities between the cardiovascular adaptions to pregnancy in mice and humans. In both species, maternal cardiac output increases (Hefler, Tempfer et al. 2001) and blood pressure falls in early to mid- pregnancy, returning to pre-pregnancy values in late gestation (Wong, Kulantavelu et al. 2002, Hefler, Tempfer et al. 2001). As in humans, this fall in peripheral resistance is likely due to generalised changes in vascular function, as
pregnant mice show a blunted pressor response in late pregnancy (Wong, Kulandavelu et al. 2002) similar to that seen in pregnant women (Gant, Daley et al. 1973). The patterns of flow in the utero-placental and umbilical arteries of mice are also similar to those seen in human pregnancy (Schulman, Fleischer et al. 1986), characterised by large reductions in uterine artery resistance as gestation advances. Umbilical artery resistance also falls with gestation; however, in mice, positive end-diastolic flow does not occur until relatively late in gestation (Mu, Adamson 2006). Uterine systolic and end diastolic velocities increase with a disproportionately greater rise in diastolic velocity so that the uterine artery resistance index decreases with advancing gestation (Mu, Adamson 2006). As in humans, this fall appears to be due to a combination of vascular remodelling and altered vascular function. In both species, diameters of uterine, radial and spiral arteries become markedly dilated and dilatation of spiral arteries is associated with a loss of elastin and muscle in the vessel walls (Burton, Woods et al. 2009, Croy, Yamada et al. 2014). In mice the maternal immune system appears to play a more important role in vessel remodelling (Monk, Leonard et al. 2005), and trophoblast invasion is more shallow and limited to the decidua basalis (Croy, Yamada et al. 2014). This contrasts to human pregnancy where trophoblast invades deeply and extensively, replacing the maternal vascular endothelium to the inner third of the myometrium. Finally, pregnancy induces altered vascular function of the murine uterine arteries (Veerareddy, Cooke et al. 2002, Cooke, Davidge 2003), reminiscent of the changes seen in myometrial small arteries in humans (Wareing, Myers et al. 2004). The main uterine artery shows an increased sensitivity to endothelium-dependent and independent vasodilatation (Cooke, Davidge 2003). In mice, the increase in endothelium-dependent vasodilatation appears to be mediated by endothelium derived NO, and PGI2 (Veerareddy, Cooke et al. 2002, Cooke, Davidge 2003), with a reduced contribution from EDHF compared to non-pregnant uterine arteries (Cooke, Davidge 2003).
The mouse and human placentae share important commonalities, such as the overall placental structure and mechanisms that underpin placental development (Rossant, Cross 2001). Both are discoid with the umbilical cord carrying fetal vessels to the fetal surface. In both, physiologic exchange of nutrients and waste occurs where maternal blood directly contacts chorionic trophoblast (haemochorial placentation). However, in mice there are 3 layers of trophoblast separating maternal blood from the fetal villi (haemotrichorial), whereas in humans there is only 1 layer (haemomonochorial). Differences in the number of cell layers in the placental exchange barrier may affect permeability and transport efficiency. Another major difference is that mice have an inverted yolk sac placenta that is primarily responsible for fetal growth and development until mid-pregnancy, and functions in parallel with the chorioallantoic placenta thereafter (Jollie 1990, Mu, Adamson 2006). Therefore, particularly in early pregnancy, differences in placental transfer may not occur in a way that is comparable to human pregnancy. Finally, steroidogenesis differs in rodents and humans. Synctiotrophoblast from human pregnancy is able to synthesis oestrogen, whereas the rodent placenta is unable to synthesise oestrogen de novo. Instead, in rodents progesterone is synthesised by the corpus luteum, converted to androgens in the placenta, then transported back to the corpus luteum where it is metabolised to oestrogen (Malassine, Frendo et al. 2003). The physiologic relevance of this difference is unclear. However, given the influence of estrogen (and its metabolites) on uteroplacental blood flow, it is possible that there are differences in the paracrine regulation of blood flow within the uteroplacental circulation differs between rodents and human.

1.9.1.1 Endothelial nitric oxide synthase knockout

As described earlier (section 1.5.3.2.1), NO plays a critical role in modulating vascular tone and facilitates maternal cardiovascular adaption required for normal pregnancy. NOS catalyses the formation of NO through the conversion of arginine to citrulline. There are three
isoforms; the endothelial nitric oxide synthase (eNOS) isoform is responsible for the production of NO in the vessel endothelium. A null mutation for the eNOS enzyme (eNOS$^{-/-}$) results in a mouse with global systemic vascular dysfunction and mild hypertension (Kusinski, Stanley et al. 2012, Chataigneau, Feletou et al. 1999). As NO is critical to mediating the normal cardiovascular adaptations to healthy pregnancy, it is not surprising that these mice also have growth restricted offspring, with pup weights 10-14% lower than wildtype control fetuses (Kulandavelu, Whiteley et al. 2013, Kulandavelu, Whiteley et al. 2012, Hefler, Reyes et al. 2001, Kusinski, Stanley et al. 2012), although placental weight has generally been reported to be similar to wild-type. Although arteries are of similar size in non-pregnant animals, eNOS$^{-/-}$ mice pregnancies are associated with smaller increases in the radius and cross-sectional area in the uterine artery. The uterine artery only becomes larger than non-pregnant mice at 17 dGA, whereas in wildtypes it is larger by 11 dGA (van der Heijden, Olivier W.H., Essers et al. 2005). The main uterine arteries also show impaired relaxation and enhanced constriction to constrictor phenylephrine, compared to control mice, mirroring the changes seen in the myometrial small vessels in human IUGR. eNOS$^{-/-}$ mice have impaired remodelling of resistance arteries (van der Heijden, Olivier W.H., Essers et al. 2005). At 14.5 and 17.5 dGA, spiral arteries show abnormal morphology with significantly reduced length and tortuosity, and at 17.5 dGA, there is evidence of retained muscle elements in the vessel wall, similar to the retained muscular elements seen in incompletely transformed vessels that are associated with human IUGR. Likely as a result of these differences, eNOS$^{-/-}$ mice exhibit dramatically reduced uterine artery blood flow in mid (14.5 dGA) and late (17.5 dGA) pregnancy, with increased Doppler resistance indices, smaller mean uterine arterial diameter and uterine blood flow only half of that seen in wild-type (Kulandavelu, Whiteley et al. 2012), and the late gestation eNOS$^{-/-}$ placenta shows increased markers of hypoxia. Changes in the fetal circulation have also been demonstrated: at 14.5 and
17.5 dGA eNOS<sup>−/−</sup> fetuses show reduced umbilical vein blood flow, and a blunting of the normal increase in umbilical artery end diastolic velocity, with the Doppler indices higher at 17.5dGA in eNOS<sup>−/−</sup> compared to wildtype fetuses (Kusinski, Stanley et al. 2012, Kulantavelu, Whiteley et al. 2013). Placental capillary density is reduced and the labyrinth showed a reduction in fraction occupied by fetal blood spaces, suggesting that fetoplacental vascularisation is impaired. In addition, amino acid transport is also impaired, with reduced system-A activity observed in late gestation (Kusinski, Stanley et al. 2012).

The eNOS<sup>−/−</sup> mouse shares many features of utero-placental vascular insufficiency with human pregnancies complicated by IUGR. However, when using this mouse as a model to study the effect of sildenafil on fetal growth, the fact that these mice are deficient in the same pathway that sildenafil augments must be considered. Therefore, changes or lack of change found in association with sildenafil treatment in this model must be interpreted conservatively, and attempts should be made to replicate findings using other models.

### 1.9.1.2 Catechol O-methyltransferase knockout

Estrogen and its metabolites have important role in mediating cardiovascular adaptions to pregnancy. (Dubey, Tofovic et al. 2004) The human placenta synthesises primary estrogens (estrone, 17-β estadiol, estriol), which are then converted via the cytochrome P450 enzymes into hydroxylated metabolites (eg such 2-hydroxyestradiol and 4-hydroxyestradiol). These hydroxylated metabolites undergo O-methylation by the catechol-o-methyltransferase (COMT) enzyme to form methoxyoestrogens. Abberent synthesis and metabolism of esttrogen and its metabolites is associated with pre-eclampsia. Jobe et. al (2013) described abberent levels of 15 different estrogens and their metabolites assocciated with preeclampsia, including reductions in COMT metabolites 2-Methoxyestradiol and 4-Methoxyestradiol (2-ME<sub>2</sub> and 4-ME<sub>2</sub>). 2-ME<sub>2</sub> and 4-ME<sub>2</sub> appear to have a role in the development of the normal
uteroplacental circulation, and to influence vascular responsiveness during pregnancy. Both 2- and 4-ME2 stimulate PGI2 production in uterine artery endothelial cells from pregnant ewes at low physiologic concentrations (Jobe, Tyler et al. 2013), and 4ME2 increases the proliferation cultured ovine uterine artery endothelial cells from pregnant, but not non-pregnant ewes (Jobe, Ramadoss et al. 2010). Although 2-ME2 is a potent inhibitor of angiogenesis in some vascular beds (Klauber, Parangi et al. 1997, Mabjeesh, Escuin et al. 2003), in pregnancy, uterine artery endothelial cells are protected from this effect (Salih, Kapur et al. 2011), and 2-ME2 instead facilitates endovascular cytotrophoblast invasion under hypoxic conditions. The importance of 2-ME2 for a normal pregnancy outcome must extend beyond initial placental implantation, as levels continue to rise long after the uteroplacental circulation has been established, reaching a plateau in the third trimester (Berg, Sonsalla et al. 1983). The significance of 2-ME2 (and other oestrogen metabolites) is highlighted by cases where it is deficient: concentrations are significantly lower in women with pre-eclampsia, in which they are negatively correlated with severity of illness (Kanasaki, Palmsten et al. 2008, Pertegal, Fenoy et al. 2015). Other physiologically important metabolites such as 2-methoxyestrone and 4-ME2 are decreased in severe pre-eclampsia (Jobe, 2013) COMT gene polymorphism resulting in reduced enzymatic activity is associated with an increased risk of birthweight below the 10th centile (Sata, Yamada et al. 2006). Possibly the significance of 2-ME2 in later pregnancy is mediated by other known effects of 2-ME2, such as inhibition of endothelin-1 (a potent vasoconstrictor) synthesis, promotion of eNOS expression (potentially enhancing endothelium-dependent dilatation), and its potent antioxidant effects (Dubey, Jackson 2009).

In COMT knockout mice (COMT-/-), the gene for the enzyme COMT has been disrupted, making mice deficient in COMT and unable to produce 2-ME2. COMT−/− mice have growth restricted fetuses in late pregnancy (Stanley, Andersson et al. 2012), with smaller placentae
also described (Kanasaki, Palmsten et al. 2008) – although these effects are not consistent across all published reports (Kanasaki, Palmsten et al. 2008, Rueda-Clausen, Stanley et al. 2014, Poudel, Stanley et al. 2013). The COMT<sup>-/-</sup> mice develop thrombosis in the arteries of the decidua and placenta, reminiscent of the decidual vascular lesions seen in women with pre-eclampsia or IUGR pregnancies (Kanasaki, Palmsten et al. 2008), and exhibit features in common with pre-eclampsia (and IUGR) in human pregnancy, including increased markers of hypoxia within the decidua and placenta, increased levels of anti-angiogenic molecule sFLT-1, reduced eNOS expression, pregnancy induced hypertension, proteinuria and renal glomerular endotheliosis (Kanasaki, Palmsten et al. 2008). Despite the thrombosis demonstrated within the decidua and placenta, these mice lack changes in uterine artery Doppler wave form (Stanley, Andersson et al. 2012). However, fetal-placental vascular resistance is abnormal, with absent umbilical artery end diastolic flow exhibited in late gestation (Stanley, Andersson et al. 2012). This is a significant finding, as it suggests aberrant vascularisation or pathology on the fetal aspect of the placenta, and similar findings in human pregnancy are associated with severe growth restriction and poor perinatal outcome.

Although the knockout is associated with reduced eNOS expression, the COMT<sup>-/-</sup> mice feature pathology additional to that seen in eNOS<sup>-/-</sup> such as pathologic umbilical artery changes. This suggests that the deficits in growth and pathology observed are not merely consequences of altered eNOS expression, and therefore studying both strains together is likely to result in further insight into the mechanisms through which sildenafil may improve fetal growth, as compared studying either model in isolation.

1.9.2  Using sheep to study IUGR

Sheep are one of the most utilised species for the study of pregnancy physiology, and much of the current knowledge surrounding fetal and placental physiology has come from the study
of pregnant ewes (Barry, Anthony 2008). There are many advantages of using sheep over other species in pregnancy studies. Ewes used in pregnancy studies have generally been selected or bred to carry singleton pregnancies; this avoids the need to account for variations in litter size when studying fetal growth in rats and mice. Fetal lambs undergo a similar pattern of organogenesis and development to humans and therefore provide a better means of studying the consequences of in utero exposures. The relatively long gestation of sheep (term = approximately 147 days) compared to small rodents means that the in utero effects of longer exposures can be studied. The term fetal lamb has a birthweight similar to a human infant; the size of the ewe and her lamb mean that it is easier to collect repeated biological samples such as plasma. However, perhaps the biggest advantages of the sheep over other species is that the ewe and fetal lamb are tolerant of invasive procedures, allowing for surgical manipulations during pregnancy. Vascular catheters, flow transducers and amniotic catheters can be inserted into the ewe/lamb, allowing invasive monitoring or fetal blood sampling to be carried out relatively easily throughout gestation.

There are similarities between the cardiovascular adaptations to pregnancy in sheep and humans. As in humans, sheep pregnancy is associated with an increase in cardiac output and a generalised blunted response to vasopressors (Rosenfeld, Gant 1981); however, in contrast to humans, they undergo minimal plasma volume expansion in pregnancy (Metcalf, Parer 1966). As in humans, there is an increase in the uterine artery diameter (Griendling, Fuller et al. 1985, Konje, Howarth et al. 2003) reduced resistance / impedance to flow (Sprague, Phernetton et al. 2009, Magness, Mitchell et al. 1990), and a dramatic increase in uterine blood flow with gestation (Rosenfeld, Morriss et al. 1976, Konje, Howarth et al. 2003, Magness, Mitchell et al. 1990), and fetal-placental (umbilical artery) blood flow also increases as pregnancy progresses (Leiser, Krebs et al. 1997).
As in human pregnancy, NO and PGI\textsubscript{2} appear to be important in mediating increased uterine blood flow in pregnancy (Magness, Shaw et al. 1997, Rosenfeld, Cox et al. 1996), although the role of EDHF remains unclear. Plasma levels of NO, PGI\textsubscript{2} and their metabolites increase in ovine pregnancy (Magness, Rosenfeld et al. 1992, Magness, Mitchell et al. 1990) as does protein expression of NOS and PGI\textsubscript{2} synthase (PGIS) in the pregnant uterine artery endothelium (Bird, Sullivan et al. 2000, Magness, Shideman et al. 2000). Uterine artery endothelium also shows increased levels of basal and provoked production of PGI\textsubscript{2} and NO (Magness, Rosenfeld et al. 1996, Magness, Rosenfeld 1993). This is in contrast to systemic (omentum) vessels, where NOS and PGIS expression is relatively unchanged (Magness, Shaw et al. 1997, Magness, Shideman et al. 2000), and suggests a specific role for NO and PGI\textsubscript{2} in modulating uterine flow. The relative contribution of each is unclear: when either NOS or PGI\textsubscript{2} production is inhibited \textit{in vivo}, uterine blood flow is reduced, but not completely or consistently, suggesting both mediators (and potentially others) are important (Van Buren, Yang et al. 1992, Rosenfeld, Cox et al. 1996). The limited published data on uterine artery \textit{ex vivo} function also supports the importance of NO in endothelium-dependent vasodilatation. NO donor sodium nitroprusside dilates pre-constricted ovine pregnant uterine arteries. (Sladek, Magness et al. 1997). Compared to non-pregnant ewes, pregnancy is associated with reduced vasoconstriction in the uterine arteries, which increases following inhibition of NOS (Sladek, Magness et al. 1997). The influence of PGI\textsubscript{2} and EDHF on resistance vessel function is uncertain.

An obvious difference between sheep and human pregnancy is the appearance of the placenta. Rather than a single discoid placenta, the ovine placenta is made up of a number of units called placentomes (Figure 1.3, Figure 1.4). Each placentome consists of a maternal portion (caruncle) and a fetal portion (cotyledon) lying in apposition. The sheep uterus contains many discrete areas of well vascularised non-glandular endometrial projections called caruncles.
Chorionic epithelium from the fetus forms the cotyledonary portion of the placentome in close apposition with the endometrium at the caruncles (Figure 1.3 (Stegeman 1974)). As gestation advances, caruncles develop crypts, into which fetal villi project and interdigitate with resulting in close association between trophoblast and uterine cells, facilitating nutrient transport (Leiser, Krebs et al. 1997). Microscopic placental structure is different between sheep and humans. In sheep, the placenta is epitheliochorial – a superficial implantation where the trophoblast layer is separated from maternal blood by both maternal epithelium and endothelium (Figure 1.4). As there are a greater number of cell layers separating maternal and fetal circulations, the efficiency of maternal-fetal exchange may be less in sheep than in humans (where only one cell layer separates the circulations), although other factors including the permeability of each cell layer, and arrangements of maternal and fetal vasculature are likely to influence efficiency (Borowicz, Hafez et al. 2008). Therefore findings related to placental transport efficiency may not translate to humans.
Figure 1.3 – Sheep placentome

Single sheep placentome demonstrating separate fetal and maternal portions in close proximity.

Figure 1.4 – Multiple discrete placentomes demonstrated in the uterine cavity of an ovine (twin) pregnancy.

Figure republished from Experimental ovine toxoplasmosis: influence of the gestational stage on the clinical course, lesion development and parasite distribution by P Castaño, M Fuertes, J Regidor-Cerrillo et al., Veterinary Research 47 (43) Copyright © (2016). Used under Creative Commons Attribution-4.0 International public license.
Figure 1.5 - Relationship between trophoblast, fetal and maternal circulations in different types of placentation

Ovine placentation is epitheliochorial (diagram A), and maternal blood (MB) does not directly contact trophoblast. In contrast, murine and human placentation is haemochorial (diagrams C), with trophoblast bathing in maternal blood. In human pregnancy, only one cell of trophoblast – the syncytiotrophoblast (Sy) separates maternal and fetal circulations (diagram C, left panel). In mice, three trophoblast layers (two syncytiotrophoblast (Sy) and one cytotrophoblast (Cy) separates maternal and fetal circulations (diagram C, right panel). Maternal blood (MB); Fetal blood (FB); Syncytiotrophoblast (Sy); Cytotrophoblast (Cy); Maternal vessel (MV), basement membrane (BM), maternal vascular endothelium (MV). Figure republished from A Comparison of the Histological Structure of the Placenta in Experimental Animals, by S Furukawa; Y Kuroda, and A Sugiyama, Journal of Toxicologic Pathology 27 (1) Copyright © (2014). Used under Creative Commons Attribution-4.0 international public license.
Despite these differences, parallels can be drawn between placentae of sheep and humans. Maternal vasculature supplying the placenta shows some similarities, with the spiralling of maternal vessels seen in humans is also present in ruminants, and terminal maternal arteries within the myometrium demonstrate pregnancy-induced sinusoidal dilatation, although this is much less pronounced in sheep compared to humans (Leiser, Krebs et al. 1997). The discrete attachment sites of the cotyledons have been compared to the structural divisions of the human placenta into cotyledons (Barry, Anthony 2008, Leiser, Krebs et al. 1997). The placental villous tree is also similar in shape and structure in humans and sheep (Leiser, Krebs et al. 1997, Hafez, Borowicz et al. 2010), and in both species, the villous trees consisting of stem, intermediate (these larger vessels contain muscular and elastic elements, with stem villi thought to offer both mechanical support and have blood flow regulating properties) and terminal villi that build up to fetal placental cotyledons (Leiser, Krebs et al. 1997, Demir, Kayisli et al. 2004).

1.9.2.1 Uterine artery embolisation

Uterine artery embolisation involves injection of micro-particles or microspheres into the utero-placental circulation. It requires vascular catheterisation of both ewe and fetal lamb; therefore, its use is limited to facilities which have access to the appropriate equipment, housing and trained personnel. It is a costlier and more labour-intensive method of inducing IUGR compared to some other means (for example nutrition modulation). As surgical manipulation and catheter insertion is only possible once the fetus reaches a certain size, it is only possible to study the latter half of pregnancy. Advantages of this model include similarities in reduced uterine perfusion and placental pathology with utero-placental vascular insufficiency in human pregnancy (see below), and - as vascular catheters are inserted as part
of the experimental paradigm - the ability to take longitudinal/ repeated blood samples from both ewe and lamb.

Emboli\sion of the utero-placental circulation in ewes was first described in 1972 (Creasy, Barrett et al. 1972). In this study, uterine artery catheters were inserted between 96-125 dGA. Microspheres 15 µm in diameter in solution were injected daily until 132 dGA, or until fetal blood gas criteria were met (fetal pH < 7.3, pAO\textsubscript{2} < 20 mmHg and pACO\textsubscript{2} < 52 mmHg (Creasy, Barrett et al. 1972)). This protocol resulted in a 29% reduction in fetal weight. Subsequent studies using similar protocols have replicated these outcomes with a 20-33% reduction in fetal weight (Creasy, De Swiet et al. 1973, Clapp, Szeto et al. 1980, Clapp III, Szeto et al. 1981) and a reduction in placental weight (Wali, de Boo et al. 2012). Studies where slightly larger particles have been used to embolise (20-60 µm) have also shown similar reductions (Wali, de Boo et al. 2012, de Boo, Eremia et al. 2008, Bloomfield, Bauer et al. 2002). In all studies, repeated embolisation is required to achieve growth restriction. This is because although an acute reduction in uterine blood flow occurs following a single embolisation (Wali, de Boo et al. 2012, Clapp III, McLaughlin et al. 1982), uterine blood flow then rebounds toward normal, possibly due to the resolution of vasospasm, or recruitment of previously non perfused vessels (Boyle, Lotgering et al. 1984).

Emboli\sion causes IUGR by blockage of the small maternal arterioles supplying the placenta. This results in increased resistance to flow in the uterine artery (as evidenced by diastolic notching), in the absence of changes to flow in other areas of the maternal circulation (Ochi, Suginami et al. 1995, Nakabayashi, Negoro et al. 1997). Emboli\sion is associated with a significant reduction in cotyledon weight, and pathological findings of acute atherosis, thrombosis, obliteration of the maternal artery lumen, and infarction (Aardema, Oosterhof et al. 2001, Madazli, Somunkiran et al. 2003), thereby displaying similarities to human
pregnancies affected by IUGR (Creasy, Barrett et al. 1972). Following embolisation, resistance to flow in the umbilical artery also increases, as seen in human IUGR pregnancies, although the mechanism for this is unclear (Clapp, Szeto et al. 1980, Creasy, De Swiet et al. 1973). Embolisation results in an acute fall in fetal arterial glucose concentration, and polycythaemia (Wali, de Boo et al. 2012, Bloomfield, Bauer et al. 2002) similar to changes that are observed in IUGR neonates (Sharma, Shastri et al. 2016).

Compared to other models of IUGR, embolisation has the potential to produce a more variable growth restriction phenotype, as many experimental variables can influence the degree of growth restriction observed. The severity of growth restriction depends on the fetal blood gas parameters used to titrate embolisation against, and whether one or both uterine arteries are embolised (Clapp, Szeto et al. 1980, Clapp III, McLaughlin et al. 1982). The quantity of beads required to successfully embolise also varies between animals, as it is affected by both the distribution of cotyledons and patterns of vascular branching in an individual animal, and where the catheter is placed within the uterine artery (for example catheter proximity to the internal iliac artery (Boyle, Lotgering et al. 1984)). The changes in uterine artery blood flow in response to embolisation are also highly variable, possibly as a result of redistribution of blood flow to undamaged cotyledons (Clapp, Szeto et al. 1980). It is important to pre-define fetal blood gas targets, the protocol for uterine catheter placement and the protocol of embolisation frequency and dosing, in order to try and maximise consistency between animals.
1.10 Summary of introduction, thesis aims and hypotheses

IUGR causes significant perinatal morbidity and mortality, and is associated with adverse long term health outcomes; however, there are currently no treatments available to improve fetal growth and wellbeing in utero. Utero-placental insufficiency is an important cause of IUGR, and is associated with alterations in both physical structure and function of the maternal resistance arteries supplying the placenta, and increased resistance to blood flow in the maternal uterine arteries. Of note, in IUGR, the maternal resistance arteries supplying the placenta exhibit impaired endothelium-dependent relaxation. Therefore, in IUGR pregnancies, improving vasodilatation in these arteries is a potential mechanism through which placental perfusion, and therefore fetal growth, may be increased. Sildenafil citrate, a phosphodiesterase inhibitor with vasodilator properties, is a potential new therapy for IUGR. The efficacy of sildenafil at improving fetal growth has been predominantly studied in rodents, where to date, five studies have shown it to be effective at increasing fetal growth in models of IUGR.

There are two important but largely unanswered questions regarding the efficacy of sildenafil at increasing fetal growth. Firstly, it is unclear whether sildenafil can improve fetal growth when used over a longer and more clinically relevant time frame than has been studied in rodents. Secondly, the mechanism/s through which sildenafil may alter fetal growth remain unclear. Although it has previously been suggested that sildenafil treatment improves vasodilatation of the uterine resistance vessels resulting in increased placental blood flow and fetal growth, there is limited and conflicting published data to support this. This thesis will use animal models of utero-placental vascular insufficiency, and resistance vessels collected from IUGR pregnancies in humans, in attempt to answer these questions.
Specifically, the aims of this thesis are to

1. Assess the efficacy of maternal sildenafil treatment at increasing fetal growth in a large animal model of utero-placental insufficiency / IUGR (chapter 3)
2. Assess the efficacy of sildenafil at increasing fetal growth in small animal models of utero-placental insufficiency / IUGR (chapter 4)
3. Assess the effect of sildenafil on uterine / myometrial resistance vessel function
   a. in animal models of IUGR (chapters 3 and 4)
   b. in IUGR human pregnancies (chapter 5)

The hypotheses of this research is that sildenafil treatment will be associated with increased fetal growth in both animal models, and that maternal sildenafil treatment will increase either maximal vasodilatation or sensitivity to endothelium-dependent vasodilatation in the uterine / myometrial resistance arteries in both animal models and human pregnancies complicated by IUGR.
2 Methods

2.1 Ovine study (chapter 3)

2.1.1 Ethics statement

The experiment was approved by the University of Auckland Animal Ethics Committee (approval number AEC 001101, Appendix B).

2.1.2 Animals

Five year old multiparous Romney ewes were time-mated with Dorset rams to ensure known gestation. Viability, dating and singularity were confirmed by an ultrasound performed at 49 and 70 days gestational age (dGA, term = 147 dGA) by the farm veterinarian. Ewes were acclimatised to the feedlot from 86 dGA. Ewes were housed in individual pens, in sight of others. Daily pelleted stock feed (65% Lucerne, 30% barley, limestone, molasses, and trace elements) was given at 3-4% of body weight / day as per farm protocol, and water was freely available. At 96 dGA, ewes were weighed and an ultrasound scan was performed to assess umbilical artery blood flow (see 2.1.7).

2.1.3 Surgery

2.1.3.1 Catheter construction

As described previously (Bloomfield 2000), vascular catheters were constructed by joining lengths of either polyvinylchloride (PVC; Tyco Electronics/Biocorp Aust Pty Ltd, Huntingdale, Victoria, Australia) or silastic tubing (Kempthorne Medical Supplies, Auckland) to 2 m lengths of vinyl tubing of a slightly larger bore. Silastic tubing was used in the construction of catheters for the utero-ovarian vein to minimise the risk of rupturing these thin-walled vessels on catheter insertion. All other catheters were PVC, with variable
diameters of the tubing used, as was appropriate for the size of the vessel to be catheterised. To construct the PVC catheters, one end of the “outer” PVC tubing was stretched open with forceps, then the “inner” PVC tubing dipped in cyclohexanone (Scientific supplies, Auckland) and inserted into the outer tubing with forceps. To construct the silastic catheters, one end of the silastic tubing was soaked in xylene, causing the catheter to swell; the vinyl tubing was then inserted into the swollen end of the silastic tubing. For all catheters, two small rings of PVC tubing were placed over the catheter just above the junction between inner and outer tubing, and fixed with cyclohexanone. These rings acted as a cuff to which a suture could be used to secure the cannula to fetal or maternal tissues.

Figure 2.1 - Construction of a vascular catheter

Catheters were constructed of an inner PVC tube that had been inserted into an outer PVC tube. The tubes were ‘glued’ together with cyclohexanone. Two small PVC rings (cuffs) were fixed over the junction of the two tubes and were used for securing the catheter in vivo.
**Fetal tarsal artery and vein**

A 0.15 m length of #sv35 PVC tubing (inner diameter (ID) 0.5 mm, outer diameter (OD) 0.9 mm) was inserted into a 2 m length of #sv74 PVC tubing (ID 1 mm, OD 2 mm).

**Amniotic fluid catheter**

A 2 m length of #sv74 PVC tubing was used, with perforations cut in the distal 0.15 m portion.

**Uterine artery**

A 0.2 m length of #sv55 PVC tubing (ID 0.6 mm OD 1.3 mm) was inserted into a 2 m length of #sv74 PVC tubing.

**Utero-ovarian vein**

A 0.3 m length of silastic tubing (ID 2 mm, OD 2.25 mm) was joined to a 1.2 m length of #sv74 PVC tubing.

**Maternal carotid/tarsal artery and vein**

Single 1.4 m lengths of #sv74 PVC tubing were used with two rings of #sv110 PVC tubing (ID 1.5 mm, OD 2.5 mm) placed 0.4 m from the tip (tarsal vessels) or 0.2 m from the tip (carotid vessels).
2.1.3.2 Anaesthesia and laparotomy

Prior to surgery, ewes were randomised using computer generated random numbers to a control group (non-embolised) or a 5-fold larger group (an IUGR group that received uterine artery embolisation). Surgeries were performed at 96-100 dGA. Feed was withheld from the ewes from the afternoon prior to surgery, but access to water was maintained. To induce anaesthesia, propofol (Health Support Limited, Auckland, NZ) 5 mg/kg was injected via the jugular vein. The ewe was then transferred to and secured to the surgical table in a supine position, and intubated. Following intubation, anaesthesia was maintained with continuous inhalation of 2% isoflurane (MedSource, Auckland, NZ) in 100% oxygen. Depth of anaesthesia was considered appropriate when the palpebral reflex was absent; this reflex was monitored intermittently throughout surgery. Maternal heart rate, oxygen saturation and expired CO$_2$ was continuously monitored to ensure adequate ventilation. Prior to the abdominal incision, ewes were given an intramuscular injection of of 450,000 IU benzathine penicillin (Intervet, Auckland, NZ). The maternal abdomen was shorn, cleaned with iodine (Provet, Auckland, NZ) and hibitaine (Johnson & Johnson, Auckland, NZ), and then draped with sterile surgical linen. A vertical infra-umbilical midline incision was made, the maternal abdomen opened in layers and the uterus palpated to confirm the presence of a single lamb. Following confirmation of a singleton pregnancy, a 20 mm trocar was inserted through the ewes left flank into the abdominal cavity under direct vision. The obturator was removed, the fetal and maternal uterine catheters threaded into the maternal abdominal cavity, and the trocar removed.
2.1.3.3 Catheterisation

All catheters were flushed with 0.9% NaCl to ensure patency prior to placement in vessels.

Fetal tarsal artery and vein

The fetal lamb was gently manipulated to position a fetal hind limb in an area free of placentomes or large vessels, and a small (< 4 cm) incision made in the uterus over the fetal hind limb. In turn, each hind limb was extruded with the edges of the hysterotomy clamped together around the leg to minimise amniotic fluid loss. The fetal leg was wrapped in sterile saline-soaked gauze and fixed in a stable position (Figure 2A). The hock-toe length was measured with a sterile tape measure, and catheters trimmed to this length. Previous experience has shown that at this length, when catheters are inserted via the tarsal vessels, the tips lie in the descending aorta and inferior vena cava (Bloomfield 2000). The appropriate vessels were exposed and opened, and the catheters were inserted and fully advanced (Figure 2.2B). Catheters were secured by tying the segment of vessel distal to the catheter to the catheter cuff using two 4-0 silk ties. Three drops of superglue were placed into the wound to help further secure the catheter, and the skin closed with 3-0 silk. If tarsal artery cannulation was unsuccessful, the catheter was trimmed to 3.5 cm and catheterisation of the femoral vessel of that limb was performed at the femoral triangle.
Figure 2.2 - Catheterisation of the fetal hind limb

The fetal hind limb is extruded from the hysterotomy and the tarsal vessels exposed (A). The fetal hind limb with both tarsal artery and vein catheters in situ (B).
**Umbilical vein catheterisation**

The hysterotomy was extended to allow extrusion of the fetus to the level of the abdomen. A small incision was made above and to the right of the umbilical cord, and a 4-0 nylon purse string suture placed into the wall of the vessel. A hole was made with a needle in the middle of the purse string, and the catheter advanced approximately 3.2 cm so the tip lay in the common umbilical vein. The catheter was secured with the purse-string suture and superglue, and the skin closed with 3/0 silk. It was technically difficult to catheterise this vessel; the vessel calibre was small and wall extremely fragile. In more than half of the surgeries, the vessel would tear when placing the purse-string suture, meaning that the vessel could not be cannulated and the lamb rapidly exsanguinated. The rationale for placing the umbilical venous catheter was that samples drawn from this vessel could be used to calculate fetal-placental blood flow using antipyrine, and the Fick principle (similar to that described for the calculation of uterine artery blood flow below, section 2.1.7.2). However, the alternative means of assessing fetal-placental blood flow (Doppler ultrasound) was straightforward. Due to ongoing fetal losses stemming from difficulties placing the umbilical venous catheter, a decision was made to abandon this procedure, in order to optimise the number of pregnancies that could enter the embolisation and treatment phase of the experiment.

### 2.1.3.4 Amniotic fluid catheter placement and hysterotomy closure

The lamb was returned to its original position in the uterine cavity. Warm sterile 0.9% NaCl (approximately 250 mL) was added to the amniotic cavity to replace lost amniotic fluid. The amniotic fluid catheter was left free floating in the amniotic cavity, and 80 mg gentamicin sulphate (Pfizer, Auckland, NZ) antibiotic prophylaxis was added to the amniotic cavity prior to closing. The hysterotomy was then closed before inserting maternal catheters.
**Uterine artery and utero-ovarian vein**

Only ewes in the IUGR group had uterine artery catheterisation, but all ewes had utero-ovarian vein catheterisation. All vessels were catheterised bilaterally. For each vessel, a distal branch was identified within approximately 30 cm of the main vessel, and the catheter lengths trimmed accordingly. Vessels were cleared of connective tissue, cut, and the catheters were inserted and fully advanced so the tips lay in the main vessels. Catheters were then tied to the distal (uncatheterised portion) vessel with 2-0 silk, and further secured by over-sewing visceral perineum and fixing the catheter to the tip of the horn with a single stitch.

**Maternal systemic vascular catheters**

Maternal systemic arterial and venous catheters were inserted via the tarsal and carotid / jugular vessels. Catheters were secured via the cuff to a distal portion of uncatheterised vessel, using 2-0 silk.

Following insertion, all catheters were sampled and flushed with heparinised saline to ensure patency.

2.1.3.5 **Closure**

The hysterotomy was closed in two layers with 2-0 silk. The amniotic membranes were included in the closure, and the second layer was inverted to minimise further fluid leakage. The rectus sheath was closed in a continuous fashion using cotton umbilical tape. Maternal skin was closed with a continuous subcutaneous suture of 2-0 silk. A purse-string suture using 2-0 silk was placed around the trocar entry site/ catheter exit site in the maternal flank. Where maternal systemic catheters had been placed (maternal neck and leg), the skin was closed with interrupted sutures (2-0 silk). All wounds were injected with subcutaneous 0.25%
bupivacaine (AstraZeneca, Auckland, NZ). Abdominal and maternal tarsal catheters were placed in a clean plastic bag that was tied to the ewe’s back. Maternal neck catheters were placed into a separate bag secured to the back of the ewe’s neck. A tubular bandage was then placed over the ewe’s neck and flank to further secure the catheters.

2.1.4 Postoperative and daily cares

Isoflurane was discontinued at the end of the surgery; the ewe was extubated once licking and chewing. Following removal of the endotracheal tube, ewes were returned to their pen and monitored until independently mobile.

Maternal wounds were checked daily, and cleaned with sterile 0.9% NaCl as required. All maternal and fetal catheters were checked and flushed with heparinised saline for the first three postoperative days, then every other day for the remainder of the experiment. Fetal samples were collected (via fetal catheters) to assess fetal blood gas status for the first three days postoperatively, twice daily during the embolisation period, then twice weekly for the remaining duration of the experiment. Fetal blood samples were collected in heparinised syringes on ice before performing blood gas analysis and glucose concentrations (arterial blood gas: Alere, Waltham, MA; lactate, glucose: Yellow Springs Instruments, Dayton, OH). In order to prevent dead-space contamination, the first 2 mL of blood was drawn into a separate syringe prior to collecting the sample. The “dead space” sample was re-injected following completion of sample collection. Collection was always performed prior to the administration of morning treatment, morning feeding and catheter flushing, and analysis was performed within 15 minutes of sample collection.
2.1.5 Uterine artery embolisation

IUGR was induced via utero-placental embolisation, using a modification of previously described protocols (Wali, de Boo et al. 2012, Bloomfield 2000). Sterile sepharose microspheres 20-50 µm diameter (Superose 12, Pharmacia Biotech, Uppsala, Sweden) were diluted 1:100 in 0.9% NaCl to give a solution of 1-2 x 10^5 beads/mL. Before each embolisation, a fetal arterial sample was analysed for pH, PaO_2 and lactate. Embolisation was performed up to twice daily from 102 - 107 dGA. Up to 2 mL of microspheres in solution were injected into each uterine artery, with the volume of injections titrated to fetal blood gas parameters. Embolisation was withheld when fetal PaO_2 < 16 mmHg, or fetal arterial lactate > 2.5 mmol/L. After approximately half the planned surgeries, interim analysis showed that the weights of lambs born to embolised mothers were statistically no different from non-embolised mothers. Following discussion with senior investigators, the threshold for withholding embolisation was changed, so that embolisation was withheld when fetal PaO_2 < 14 mmHg or arterial lactate was > 4 mmol/L; only lambs embolised under these new criteria were included in analysis.

2.1.6 Sildenafil treatment

Following completion of embolisation, ewes in the IUGR group were randomised to treatment with sildenafil citrate (Zhuhai Jiacheng Bio-Tech, Zhuhai City, China) 150 mg/day dissolved in 54 mL sterile water, or a visually indistinguishable infusion of 54 mL sterile water. Control sheep received no infusion. As no information was available regarding the pharmacokinetics of sildenafil in sheep, the dose was chosen as it is similar to that used in clinical studies of sildenafil in pregnancy (Samangaya, Mires et al. 2009), and the same as used in a previous ovine study where a biologic effect of sildenafil was apparent (Satterfield, Bazer et al. 2010). Sildenafil solution was prepared fresh every 3 days and administered as a 12 hour continuous
subcutaneous infusion via a portable infusion pump secured to the ewe’s back (WalkMed Infusion, Centennial, CO, USA). The infusion bag of the pump was checked and refilled at the same time each day, and any residual fluid was given as a slow subcutaneous bolus. The infusion site, subcutaneous needle and infusion tubing was changed every third day to minimise the risk of infection and localised pooling of the infusate.

2.1.7 Assessment of uterine and umbilical blood flow

2.1.7.1 Doppler ultrasound studies

Ultrasound assessment of blood flow was performed prior to surgery (96 dGA), and at 107, 119 and 128 dGA. Ultrasound was performed at the same time each day, with the ewe standing and non-sedated. The operator was blinded to whether animals were in the sildenafil or vehicle treatment groups, but it was not possible to blind for the control group, as these sheep had a different numbers of catheters, and did not have an infusion pack.

Initially attempts were made to assess both uterine and umbilical artery circulations. However, due to the mobility of the ovine uterus, the ewe’s standing position and movement, it was extremely difficult to obtain acceptable recordings of uterine artery blood flow from a consistent location, and this procedure was abandoned. To measure umbilical artery blood flow, a free floating segment of umbilical cord was identified with pulsed wave colour Doppler. During fetal quiescence, keeping the angle of insonation to < 50°, Doppler waveforms were recorded and stored for offline analysis. The umbilical artery resistance index was later calculated using an average of 3 consecutive waveforms, using the formula:

\[
Resistance\ Index\ (RI) = \frac{peak\ systolic\ velocity - end\ diastolic\ velocity}{peak\ systolic\ velocity}
\]
2.1.7.2 Assessment of uterine blood flow using antipyrine infusion and the Fick principle

Antipyrine is an inert molecule that rapidly diffuses across the placenta. At a steady state, the rate of diffusion across the placenta equals the rate of infusion. When the concentration of antipyrine in the vessels supplying and draining the placenta are known, then the blood flow to the placenta can be calculated according to the Fick principle (Meschia, Battaglia et al. 1967, Bloomfield 2000).

Antipyrine 160 mg was dissolved in 20 mL sterile saline. The infusate was given via 0.2 µm filter (Millipore Corp., Bedford, MA USA) into a fetal tarsal vein as a 4 mL bolus followed by 0.4 mg/min (3 ml/hr) infusion. This rate was used as higher infusion rates can affect regional blood flows in the fetus (Gull, Charlton 1993), whereas a flow of <1 mg/min do not (Pimentel, Figueroa et al. 1986). Previous studies have shown that using this protocol, a steady state is achieved after 90 minutes (Harding 1982). At 90 minutes, samples were drawn simultaneously from a maternal artery (the carotid or tarsal artery both representative of the uterine artery), and the uterine ovarian vein (representative of the mixed blood draining the placenta (Huckabee, Crenshaw et al. 1972)) at 15 minute intervals for 4 samples. All samples were frozen as aliquots of whole blood and stored at -80 °C for future analysis. The syringe containing the infusate was weighed before and after the experiment to quantify the amount infused. The residual infusate was stored at -80 °C and subsequently analysed (see section 2.1.7.2.2) to enable accurate calculation of the quantity of antipyrine infused.

2.1.7.2.1 Calculation of placental blood flow

Once a steady state is reached, the rates of antipyrine infusion, loss from the umbilical circulation and uptake by the uterine circulation are approximately equal (Meschia, Battaglia
et al. 1967). Although some antipyrine accumulates and is metabolised by the fetus and utero-placental tissues, these amounts are small (Meschia, Battaglia et al. 1967).

Uterine blood flow was calculated by the following equation:

\[
Uterine \text{ blood flow (mL/min)} = \frac{R}{[UOV] - [MA]}
\]

Where R was the rate of uptake by the uterine circulation (equal to rate of infusion at a steady state); [UOV] and [MA] were antipyrine concentrations (µg/mL) in the uter-o-ovarian vein and maternal artery.

2.1.7.2.2 Antipyrine Assay

Concentrations of antipyrine in maternal blood were determined by reverse phase high performance liquid chromatography (HPLC) following solvent extraction. All samples were prepared and run in duplicate.

Blood Standards

Standards were prepared by mixing antipyrine stock solution (1 mg/mL; Sigma Aldrich, Auckland, NZ) with blank ovine blood to make a 10 µg/mL standard. Half of this solution was serially diluted with blank ovine blood to make 5 and 2 µg/mL standards. All standards were divided into aliquots and stored at -80 °C; these were thawed as required and processed alongside maternal samples, as described below.

Sample preparation
Thawed maternal blood samples (500 µL) were mixed with 500 µL Milli-Q water and 50 µL phenacetin internal standard (phenacetin 1 mg/mL; Sigma Aldrich, Auckland, NZ) in 10 mL glass test tubes. Samples were extracted with 3 mL dichloromethane / pentane (1:1) (both obtained from Mallinckrodt pharmaceuticals, Dublin, Ireland), capped, and mixed for 10 minutes using a tube rotator. Samples were centrifuged for 15 minutes at 4 °C, 3000 rpm then placed at -80 °C for at least 30 minutes to freeze the aqueous (blood) layer. Once the aqueous layer was completely frozen, the organic layer was decanted off into a 5 mL glass test tube and evaporated using a refrigerated SpeedVac concentrator (Savant™ SC250EXP, ThermoFisher scientific, Waltham, MA), to dry for 4 hours (no heating, pressure 0.08 Torr, vacuum rate 70 torr/min). Dried samples were covered and stored at -20 °C.

Infusate preparation

50 µL of infusate was mixed with 4,450 µL of Milli-Q water and 500 µL phenacetin internal standard in 10 mL glass test tubes (dilution 1). For HPLC, 20 µL of dilution 1 was mixed with 180 µL of MilliQ water.

HPLC preparation

HPLC was performed using a Phenomenex Kinetex column (1.7 µm C18(2) 100 Å), dimensions 150 × 2.1 mm (Phenomenex, Torrance, USA). Dried samples were reconstituted with 200 µL of mobile phase. The mobile phase consisted of a 70:30 mixture of 6.7 mM phosphate buffer (6.7 mM Na₂HPO₄.2H₂O, pH 7.2) and acetonitrile (Mallinckrodt pharmaceuticals). A volume of 20 µL was injected onto the column, run time was 10 minutes, flow rate 400 µL/min, UV detector wavelength 254 nm and the back pressure approximately 1800 – 2300 psi. Retention times were 2.15 minutes (antipyrine) and 3.59 minutes (phenacetin). Data were captured and analysed using Chromeleon© software version 7.1.2
(ThermoFisher Scientific). A standard curve was constructed from the blood standards, and for each sample the ratio of peak heights of antipyrine and phenacetin were compared with the standard curve to determine the concentration of antipyrine in that sample. Duplicates with a high CV (>15%) were excluded from analysis. The inter-assay and intra-assay CVs were 16% and 4.2% respectively.

2.1.8 Post mortem

At 132 – 134 dGA, ewes were weighed, then euthanised with an overdose of sodium pentobarbitone injection (30 mL of 300 mg/mL). Immediately following loss of consciousness, a midline incision was made, and the entire uterus removed. The uterus and its contents were placed in a container on tared scales with two incontinence sheets and the total weight (uterus, fluids and fetus) recorded. The uterus was opened and the lamb euthanised with an overdose of pentobarbitone if signs of life were present. The umbilical cord was cut close to the fetal insertion site and the fetus dried with incontinence sheets and weighed. Biometric measurements were taken of the fetal lamb, based on a previously described protocol (Spiroski 2014):

- **Crown-rump length:** Length from mid-orbital peak to ischial tuberosity
- **Biparietal diameter:** Width of head immediately posterior to orbits
- **Chest circumference:** Circumference of chest immediately posterior to forelimb
- **Abdominal circumference:** Circumference of abdomen at the widest point between the last rib and hind leg
**Hind limb length:** Total length from the femoral trochanter to the tibiofemoral joint to the hock to the toe

**Hock-toe length:** Length from hock to toe

**Front limb length:** Length from the joint space between the glenoid cavity and greater tubercle of the ulnar tuberosity to the toe

The fetus was opened, catheter placement checked, and the organs dissected out and weighed. Placentomes were dissected from the uterus, categorised based on their morphological appearance into “A”, “B”, “C” and “D”, based on the appearance / degree of eversion of the haemophagous zone (Vatnick, Schoknecht et al. 1991), counted and weighed.

### 2.1.9 Myometrial artery collection

Immediately following removal of the lamb from the uterus, a full-thickness biopsy of the uterine wall was taken from an area of myometrium near (within a 15 cm radius) to where placentomes were supplied by the first/main branches of umbilical artery vessels. Biopsies were placed in ice cold physiologic saline solution (PSS; in mmol/L: NaCl 142, KCl 4.7, CaCl₂ 4.7, MgSO₄ 1.17, HEPES 10, KH₂PO₄ 1.18 and glucose 5.5; pH 7.4). Myometrial small arteries were micro-dissected within 30 minutes of euthanasia, and kept in ice cold PSS for evaluation by wire myography (see section 2.4 for myography methods and protocol).

### 2.1.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism (v 6.03) software. Results are presented as mean ± SEM or median (interquartile range) as appropriate. Where comparisons were made amongst three groups, statistical significance was determined by one-way
ANOVA and Tukey’s multiple comparisons test if data were normally distributed, or by the Kruskall-Wallis test and Dunn’s multiple comparisons test if data were not normally distributed. For comparison of fetal arterial blood gas and glucose concentrations, prior to treatment commencing, comparisons were made between two groups (control vs IUGR) and amongst three groups (control vs IUGR+V vs IUGR+SC) following completion of embolisation (110 – 129 dGA) using the appropriate non-parametric test. A p value of <0.05 was considered statistically significant.

2.2 Murine study (chapter 4)

2.2.1 Ethical statement

This study was approved by The University of Auckland Animal Ethics Committee (Ref AEC 001097; Appendix C).

2.2.2 Animals

Female mice from control C57BL/6J (C57; purchased from Jackson Laboratories, Bar Harbor, Maine, USA), catechol-O-methyl-transferase knockout (COMT−/− obtained under a material transfer agreement from Professor J Gogos Columbia university), and endothelial nitric oxide synthase knockout (eNOS−/−; purchased from Jackson laboratories) strains were placed on a high fat diet (HFD; 45% kcal from fat; 35% kcal from carbohydrate; 20% kcal from protein; D12451, Research Diets Inc., New Brunswick, New Jersey USA) or continued on a standard laboratory rodent chow (normal diet (ND); 6% kcal from fat, 44 % kcal from carbohydrate; 19% kcal from protein; Teklad Global Rodent Diets ® 2018, Envigo, Indiana, United States) for at least four weeks prior to mating. Female mice were housed in group cages with others of their corresponding genotype, and had ad libitum access to water and
food. The animal laboratory was humidity-controlled with a temperature of 20°C and a 12 hour light-dark cycle.

Overnight, females were placed in cages with males of the corresponding genotype and all females checked and returned to group cages the following morning. Detection of a mucus plug denoted 0.5 days gestational age (DGA). Pregnant females had an identifying mark drawn on their tail with a permanent marker, then were weighed and returned to group cages.

2.2.3 Sildenafil treatment

When a vaginal mucus plug was identified, mice were re-weighed at 10.5 dGA and, if they had gained >1 g in weight, were randomised to sildenafil treatment (SC) or control (C; no treatment) group. Treatment was initiated at 12.5 dGA and continued until the mice were euthanised at 18.5 dGA (term = 19.5 dGA). An initiation point of 12.5 dGA was chosen, as at this point in gestation, the basic three layer structure of the definitive placenta is present, the placenta is fully functional and the utero-placental circulation open, and it is from this point that utero-placental perfusion is the primary driver of fetal growth (Adamson, Lu et al. 2002).

Sildenafil was administered via the drinking water, with sildenafil citrate powder (Pfizer, New York, New York) added to make a 0.2 mg/mL solution. The control group received normal drinking water. A dose of 0.2 mg/mL was chosen as this is equivalent to a high dose in humans (300 mg/day in a 70 kg human after making the appropriate adjustments for altered pharmacokinetics in mice (Walker, Ackland et al. 1999)) and has previously been shown to improve fetal growth in pregnant mice (Stanley, Andersson et al. 2012). After 72 hours of treatment, the residual volume of sildenafil solution was measured, and replaced with freshly made solution. Residual sildenafil solution was also measured at the end of the experiment
(18.5 dGA). Sildenafil intake for each mouse was estimated by subtracting the volume of sildenafil solution remaining from the volume of solution initially supplied, and dividing this total by the number of mice in the cage. Mice that were untreated remained in group cages; where possible, treated mice were pair-housed with others who were also on treatment.

2.2.4 Blood pressure recording

Maternal blood pressure was recorded at 10.5 and 17.5 dGA using tail cuff plethysmography. Preparatory pre-warming and blood pressure measurements were performed at the same time each day (1400-1800), in a quiet enclosed area away from other mice, using Model 179 mouse/rat blood pressure analyser (IITC Life science, Woodland hills, CA). At all stages there was great care and gentle handling of the mouse in order to minimise maternal stress and movement artefact during recordings. Mice were placed in an appropriately sized restraint tube (model 84 or 84XL mouse restrainer, IITC Life science), and the tail passed through an integrated sensor-cuff occluder (model B60-1/4, IITC Life science) which was then fastened to the endplate of the restrainer as per the manufacturer’s instructions. The mouse was kept for approximately 15 minutes in a warming cabinet (ambient temperature 28-30 °C). Following pre-warming, the mouse/restrainer/occluder unit was placed in a large opaque pre-warmed paper bag (left open at the tail end for ventilation), in order to minimise extraneous stimuli during blood pressure recording. Machine settings were as follows: filter off, band pass on, pulse gain set to a minimum of 8, offset 4. For each mouse, the filter gain was adjusted as required to obtain an adequate recording. Maximum cuff insufflation pressure was 180 mmHg (20-40 mmHg above maximum anticipated systolic pressure), with a deflation of 4 mmHg/second with approximately 10 seconds rest period between each recording. A manometer connected to the pressure source was used to check maximum cuff pressure for each inflation. The first recording for each mouse was discarded, then recordings repeated
until three were obtained where detectable pulsations appeared to be present at a similar pressure, or 10 minutes of recording had occurred.

Print-outs of the blood pressure recordings were collected, then reviewed and analysed at a later date by a single investigator. To calculate the systolic blood pressure in mmHg, a ruler was used to measure from the graph baseline to the highest point of the recording (i.e. maximum cuff insufflation), the resulting measurement was then divided by 180 (maximum insufflation pressure), and the quotient multiplied by the height of the recording at the point where the earliest point where a clear pulsatile pattern in recording was evident (Figure 2.3).
Figure 2.3 - Blood pressure recordings and estimation of systolic pressure

Examples of blood pressure recordings taken from separate mice (A,B). Systolic blood pressure was estimated by measuring the maximum height of the graph (max) and dividing this by maximum cuff insufflation pressure (180 mmHg). The quotient was then multiplied by the height of the graph at the point where regular pulsatile activity was visible in the recording (sys), to obtain an estimate of maternal systolic blood pressure.
Where there was only one recording of acceptable quality, this was discarded; where only two recordings were of acceptable quality, the mean of these readings was used if the recordings differed by no more than 10%. Where three or more readings were obtained, the median value was used for analysis.

2.2.5 Fetal measurements

At 18.5 dGA, mice were euthanised by cervical dislocation. The maternal uterus and mesentery were dissected out immediately and placed in ice cold physiologic saline (composition as before; section 2.1.9) and transported from the animal unit to the laboratory on ice. In the laboratory, the pregnant uterus was placed in a petri dish with a clear silicone gel base, submerged in ice-cold PSS and pinned out in the anatomical position. Pups and placentae were dissected from the uterus, and the umbilical cord cut close to the fetal abdomen. Placentae were trimmed to remove the umbilical cord and membranes, then one placenta was selected at random, snap frozen and stored at -80 °C for future analysis. The pups and placentae were blotted dry with tissue paper and weighed.

Pups were sexed by visual inspection for the presence of scrotal spots and the anogenital distance. To measure fetal crown-rump length and abdominal circumference, the pups were placed in a neutral position (spine straight, with the head slightly flexed) so that the line from fontanelle to base of tail was as straight as possible. The crown-rump length was measured by running a length of cotton from the fontanelle, along the fetal spine to the base of the tail. Forceps were used to firmly grasp the cotton at both ends, then the length of cotton between the forceps was measured using a ruler. The abdominal circumference was measured in a similar fashion; a length of cotton was wrapped around the abdomen at the level of the umbilical cord, taking care not to use undue pressure, and to keep the circumference perpendicular to the long axis of the fetus.
2.2.6 Uterine and mesenteric small artery ex vivo function

Vessel preparation began as soon after euthanasia as possible. Tissues were pinned in a silicone-based petri dish and kept submerged in cold PSS with care was taken to avoid stretching the vessels during dissection. Using a dissection microscope, fine forceps and microdissection scissors, the maternal uterine artery (main loop) and 2nd order mesenteric arteries were cleaned of fat and connective tissue and cut into 2 mm (approximate) lengths. Dissected vessels were stored in clean ice cold PSS prior to mounting. At least four segments from the main uterine artery loop and at least two segments of second order mesenteric vessels were used in each experiment (see section 2.4 for detailed myography methods and protocol).

2.2.7 Placental gene expression

2.2.7.1 mRNA extraction

For each sample, a sterile stainless steel grinding ball and 1 mL Trizol® reagent (Life Technologies, Carlsbad, Ca) were added to a 2 mL microcentrifuge tube. Snap frozen placental tissue was fragmented in liquid nitrogen with a sterile mortar and pestle, and approximately ¼ of the placental tissue was added to each tube. A TissueLyzer (QIAGEN, Hilden, Germany) was used to completely disrupt and homogenate the placental tissue in Trizol®. RNA grade glycogen (ThermoFisher Scientific) 0.01 µg was added to each sample and incubated at room temperature for 5 minutes. Chloroform 0.2 mL (Sigma-Aldrich) was then added to each sample, and the tubes shaken vigorously by hand for 15 seconds. Following a further 2 minutes incubation at room temperature, tubes were centrifuged at 20,817 rcf for 15 minutes at 4 °C. The aqueous phase was transferred to a 1.5 mL microcentrifuge tube, 0.5 mL isopropyl alcohol (Sigma-Aldrich) added, and the tubes incubated at -20 °C for 2 hours. Tubes were then centrifuged at 20,817 rcf for 15 minutes at 4 °C, the supernatant discarded, the pellet washed with 1 mL of 75% ethanol, and centrifuged at 20,817 rcf for 10 minutes at
4°C. The supernatant was discarded, the pellet re-washed with 75% ethanol and then centrifuged at 20,817 rcf for 10 minutes at 4°C. The supernatant was discarded and the pellet air dried for 15 minutes.

2.2.7.2 mRNA quantification and integrity

The dried pellet was reconstituted with 20-40 µL of nuclease-free water (Life-technologies, Auckland, NZ) and total RNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific). RNA purity was considered acceptable if absorbance ratios 260/280 and 280/230 were between 1.8 and 2.1. TAE-based 1% agarose gel electrophoresis was used to visually assess the quality of RNA. The presence of two distinct bands (28S and 18S rRNA) with the absence of smearing indicated high-quality RNA. Samples that produced smeared bands (indicating RNA degradation) were excluded from further study. Following quantification, RNA was stored at -80°C until required for cDNA synthesis.

2.2.7.3 Synthesis of cDNA

RNA 1 µg was incubated with RNase-free DNase I (Invitrogen, Carlsbad, Ca) at room temperature for 15 minutes to eliminate potential DNA contamination. Ethylenediaminetetraacetic acid (EDTA) 1 µL was added to each sample, and incubated at 65°C for 10 minutes to inactivate the DNase. Complementary DNA (cDNA) was synthesised using a high capacity cDNA reverse transcription kit (Life Technologies). This involved adding to each 1 µg of DNase treated RNA: 0.8 µL of dNTP mix, 2 µL of RT buffer, 2 µL of 10x RT primers, 1 µL of MultiScribe Reverse Transcriptase 3.2 µL of DPEC treated water (total reaction volume 20 µL). Reverse transcription was performed on a DNA engine Thermal Cycler (Bio-Rad, Auckland, NZ) under the following conditions: 10 minutes at 25
°C, 30 minutes at 55°C, 5 minutes at 85°C, then cooled to 4 °C. The resulting cDNA was diluted 10 fold and stored at -20 °C until required.

An additional sample of cDNA was reverse transcribed from a randomly selected sample from each of the 12 experimental groups. The cDNA from these samples was pooled then serially diluted to 1:10, 1:100, and 1:1000 with diethylpyrocarbonate treated (DPEC) water to create standard solutions; these were stored at -20 °C until required.

2.2.7.4 Quantitative polymerase chain reaction

The relative transcript abundance of 17 genes was assessed using quantitative real-time PCR (q-PCR). Target genes with their primer and sequence and amplicon size are shown in Table 2.1. Amplification was carried out in triplicate, in 384 well plates. For each gene on each plate, four standard solutions (H2O, and standard solutions 1:10, 1:100, 1:1000) underwent identical processing as the experimental samples; these were used to construct standard curves. For each sample, the total reaction volume was 10 µL, consisting of 5 µL of SYBR green MasterMix (ThermoFisher Scientific), 3 µL cDNA, 0.5 µL forward and reverse primers and 1 µL DPEC treated water.

Quantitative Real-Time PCR (qRT-PCR) using the QuantStudio™ 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA) and SYBR® Select Master mix (Thermo Fisher Scientific) under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes 40 repeat cycles of 95 °C for 15 seconds and 60 °C for 1 minute.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Amplicon Size (BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36</td>
<td>AGA TGA CGT GGC AAA GAA CAG</td>
<td>CCT TGG CTA GAT AAC GAA CTC TG</td>
<td>82</td>
</tr>
<tr>
<td>DLK1</td>
<td>AGT GCG AAA CCT GGG TGT C</td>
<td>GCC TCC TTG TTG AAA GTG GTC A</td>
<td>147</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>CAG TTC GGC TAT AAC ACT GGT G</td>
<td>GCC CCC GAC AGA GAA GAT G</td>
<td>155</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>TCA GAA GAC AAG ATC ACC GGA</td>
<td>GCT GGT GTG ACT GTA AGT GGG</td>
<td>214</td>
</tr>
<tr>
<td>GLUT-3</td>
<td>CTT TGG CAG ACG CAA CTC TAT</td>
<td>ACC AGA ATC CCA ACA ACG ATG</td>
<td>242</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>ACA CTG GTC CTA GCT GTA TTC T</td>
<td>CCA GCC ACG TTG CAT TGT A</td>
<td>117</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GAA ATG CCA CCT TTT GAC AGT G</td>
<td>TGG ATG CTC TCA TCA GGA CAG</td>
<td>115</td>
</tr>
<tr>
<td>IL-6</td>
<td>TCT ATA CCA CTT CAC AAG TCG GA</td>
<td>GAA TTG CCA TTG CAC AAC TCT TT</td>
<td>87</td>
</tr>
<tr>
<td>PIGF</td>
<td>TCT GCT GGG AAC AAC TCA ACA</td>
<td>GTG AGA CA CCT CAT CAG GGT AT</td>
<td>126</td>
</tr>
<tr>
<td>SNAT-1</td>
<td>AAA TGC GAC GAG TAT ATT CCA GG</td>
<td>TGT CAC CGA AGT CAG AAG TAT CA</td>
<td>152</td>
</tr>
<tr>
<td>SNAT-2</td>
<td>TAA TCT GAG CAA TGC GAT TGT GG</td>
<td>AGA TGG ACG GAG TAT AGC GAA AA</td>
<td>128</td>
</tr>
<tr>
<td>SNAT-4</td>
<td>GCG GGG ACA GTA TTA AGG AC</td>
<td>GGA ACT TCT GAC TTT CAG CAT</td>
<td>101</td>
</tr>
<tr>
<td>TLR-4</td>
<td>ATG GCA TGG CTT ACA CCA CC</td>
<td>GAG GCC AAT TTT GTC TCC ACA</td>
<td>128</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCT GTA GCC CAC GTC GTA G</td>
<td>GGG AGT AGA CAA GGT ACA ACC C</td>
<td>147</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>TGG CTC TAC GAC CTT AGA CTG</td>
<td>CAG GTT TGA CTT GTC TGA GTT T</td>
<td>246</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>TTT GGC AAA TAC AAC CCT TCA GA</td>
<td>GCA GAA GAT ACT GTC ACC ACC</td>
<td>132</td>
</tr>
<tr>
<td>CHMP2-A</td>
<td>GTC GCT TAA TCC GGA AAC GG</td>
<td>GAA CTA GCG GAG CCG AGA GA</td>
<td>138</td>
</tr>
</tbody>
</table>

**Table 2.1 - Primer sequences and amplicon size of genes studied**
Following q-PCR, QuantStudio™ software was used to calculate the cycle threshold for each gene from the slope of the standard curve. Cycle threshold (CT) data for each target gene were then exported to a Microsoft excel spreadsheet, where they were normalised to the expression of reference gene CHMP2a using the double delta CT method of calculating relative mRNA expression (Livak, Schmittgen 2001). CHMP2a was used as the reference gene, as previous experiments in our laboratory had demonstrated that expression was stable across the strains and dietary groups used in this experiment.

2.2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS statistics v22. Data are presented as mean ± SEM or median (IQ range) as appropriate. Data were visually inspected on a box-plot; extreme outliers, defined as any value lying more than three box lengths (i.e. three inter-quartile ranges) from the edge of the box, were replaced with imputed values of the next highest or lowest value as appropriate. The Shapiro-Wilk test was used to assess the normality of residuals of the mean, and a square-root or log transformation was applied where appropriate to ensure normality of distribution and variances. Unless otherwise stated, data or transformed data were normally distributed. A three-way ANOVA was conducted to assess the main effects and the presence of two- or three-way interactions between independent variables strain (C57BL6/J vs eNOS−/− vs COMT−/−), diet (ND vs HFD), and treatment (C vs SC). Statistical significance was accepted at the $p < 0.05$ level. Where a statistically significant three-way interaction was identified, this was followed by testing for a simple two-way interaction at all levels of the third independent variable. Where a statistically significant two-way interaction was identified, this was followed by testing for simple main effects and simple-simple comparisons. All simple pairwise comparisons were made using a Bonferroni adjustment.
2.3 Myometrial and chorionic small artery ex vivo function in severe early onset IUGR (chapter 5)

2.3.1 Ethics statement

Full ethics approval for the sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction New Zealand and Australia (STRIDER NZAus) trial was provided by the Northern A Health and Disability Ethics Committee (CEN/12/06/028; Appendix F). This included approval for placental and myometrial tissue collection used in the studies described below.

2.3.2 Study participants

The STRIDER NZAus trial is a bi-national multicentre placebo controlled study of sildenafil as a treatment for severe early onset IUGR (Australia New Zealand Clinical trials registry number ACTRN12612000584831). Women with a singleton pregnancy are eligible for enrolment at 22 – 27+6 weeks gestation if the fetal abdominal circumference is below the 3rd centile, or at 28-29+6 weeks gestation if fetal weight is estimated to be < 700 g. Women are ineligible if the pregnancy is affected by aneuploidy, major anomaly, syndrome or congenital infection, or if delivery is likely to be required within 48 hours of enrolment (for example, in the case of severe pre-eclampsia). Women enrolled in the study are randomised to sildenafil citrate 25 mg, or placebo, orally three times daily until 32 weeks gestation, delivery or intrauterine death (whichever occurs first). Maternal assessment and fetal ultrasound assessment (including growth measurements and Doppler waveform assessment of umbilical, middle cerebral vessels, ductus venosus and maternal uterine artery) are performed at baseline, 48 hours, days 5, 10, 14 then weekly. The remainder of antenatal care, including plan for surveillance and timing of delivery, remains at the discretion of the treating clinician.
2.3.3 Recruitment to the sub-study

After consenting to be part of the STRIDER NZAus study, women enrolled at the Auckland site were counselled regarding the collection of myometrial and placental biopsies at delivery, and given the option of joining the sub-study described in this thesis. Counselling and consent was performed by local site investigators; a copy of the patient information sheet provided to women is included in the appendix. Women who consented to being part of this sub-study signed a consent form separate to the consent for participation in the main STRIDER NZAus study; a copy of this consent form is included in Appendix G.

2.3.4 Sample collection

Mode and timing of delivery was decided by the clinical team and was independent of the women’s consent for myometrial biopsy/enrolment in the sub-study.

2.3.4.1 Myometrial biopsy

Myometrial biopsy was only possible if caesarean section was performed. At caesarean, following delivery of the placenta, the attending surgeon removed a full-thickness strip (approximately 1.5 cm wide and 3 cm long) of myometrium from the middle/superior aspect of the uterine incision. The myometrial biopsy was immediately immersed in ice cold PSS (constitution as before). Using a dissecting microscope, fine forceps and microdissection scissors, myometrial small arteries were identified and dissected clean of connective tissue and trimmed to 2 mm lengths. Cleaned segments were kept in ice cold PSS before mounting onto a wire myograph for analysis of ex vivo function (see section 2.4 for detailed myography methods and protocol).
2.3.4.2 Placental biopsy

Placentae were collected following vaginal delivery or caesarean. As soon after delivery of the placenta as possible, a superficial biopsy of approximately 2 cm diameter was taken from the fetal surface, within 2 cm of the edge of the placenta. The biopsy was immediately immersed in ice cold PSS. Using a dissecting microscope, fine forceps and microdissection scissors, small second or third order chorionic plate arteries were identified and dissected free of connective tissue. Cleaned segments were trimmed to 2 mm lengths and kept in ice cold PSS before mounting onto a wire myograph for analysis of *ex vivo* function (see section 2.4 for detailed myography methods and protocol).

2.4 Assessment of small artery *ex vivo* function using wire myography

For all three studies, small artery *ex vivo* function was assessed. The vessels assessed were:

- maternal myometrial arteries (ovine study)
- maternal main loop uterine and second order mesenteric arteries (murine study)
- maternal myometrial and fetal chorionic small arteries (humans)

All studies were performed using a multi-wire myograph (610M Danish Myo Technology, Aarhus, Denmark), connected to a Powerlab (ADI Instruments, Dunedin, NZ) and computer with all experiments recorded using LabChart software (ADI Instruments). The following section outlines the methodology/protocol for the largest of the myography studies (the murine study), with modifications to the protocol made for the sheep and human studies outlined separately (sections 2.4.8, 2.4.9).
2.4.1 Multi-wire myograph

The multi-wire myograph consists of 4 individual units (Figure 2.4 A-C). Each unit contains a central 8 mL bath in which two jaws are situated, one of which is attached to a micrometer and the other attached to a force transducer (Figure 2.4 B). During experiments, the vessel segment was mounted on two wires which were held between the two jaws (Figure 2.4 C,D). The central chamber was filled with 5 mL of PSS and the vessel was kept submerged throughout the experiment. The myograph was kept at 37 °C by an inbuilt heater, and air (or other gas mixtures) could be bubbled through the chamber to maintain physiologic conditions and encourage mixing of any substances added to the PSS, such as vasoactive drugs.

Following mounting and equilibration, vessels were normalised (see section 2.4.5) to ensure a consistent starting tension and experimental reproducibility. After normalisation, the vessel circumference was constant, so that all experiments were performed under isometric conditions. During experiments, compounds were added to the PSS within the chamber and subsequent force exerted by the vessels on the wire was recorded to assess vessel contractility and reactivity.
The individual myograph units consist of two jaws (J) in a central chamber. One jaw was connected to a transducer (T) and the other to a micrometer (M); adjusting the micrometer altered the distance between the two jaws (A). A close-up of the two jaws in the central chambers is shown in (B). Four individual units make up the wire myograph (C), which was connected to a PowerLab (ADI Instruments, Dunedin, New Zealand) and computer for data recording (not shown). Schematic of a vessel (D) mounted on two wires (dashed lines). Wires exiting the vessel (arrowheads) were secured to the jaws by screws.
2.4.2 Chemicals

All chemicals were purchased from Sigma Aldrich (Sigma Aldrich, Auckland, NZ). Phenylephrine (Pe), U46619 (U4; a thromboxane A2 analogue), Acetylcholine (ACh), and Sodium nitroprusside (SNP) solutions were prepared at dilutions of $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$ M each day from a new pre-frozen aliquot of $10^{-2}$ M stock solution. All dilutions were made with fresh PSS and kept on ice for the duration of the experiment.

2.4.3 Vessel preparation

Vessel preparation is described separately for each experiment in sections 2.1.9 (ovine), 2.2.6 (murine), and 2.3.4 (human).

2.4.4 Mounting

Myograph chambers were filled with 5 mL of fresh PSS, and the chambers warmed to 37°C. In each chamber, one end of a 25 µm tungsten wire was secured to the left jaw using the far fixing screw. Using forceps, the vessel segment was threaded onto and along the free end of the wire, being careful not to stretch or damage the vessel. The (near) free end of the wire was then secured under the near fixing screw of the left jaw. Using forceps, a second 25 µm wire was passed through the vessel lumen and secured to the right jaw using both fixing screws. Any excess vessel extending beyond the jaws was dissected and removed, and the two wires confirmed to be parallel. The length of vessel between the jaws was measured using a calibrated eyepiece attached to the dissection microscope.

2.4.5 Normalisation

The sensitivity and response of blood vessels is dependent on the degree of baseline stretch in the vessel (McPherson 1992). Therefore, it was important to obtain a standardised stretch
of vessels before commencing an experiment. This was achieved through vessel normalisation, a process which determines the separation of the two mounting wires (and therefore the internal circumference (IC) of the blood vessel) for the duration of the experiment (Mulvany, Halpern 1977). Previous studies have shown that the optimal IC for obtaining maximal vessel response in rodent vessels is 90% of the IC of a relaxed vessel under a transmural pressure of 100 mmHg (13.3 kPa, (Mulvany, Halpern 1977)); this is denoted as 0.9 IC\textsubscript{100}. Vessels were also normalised to 0.9 IC\textsubscript{100} in the study of myometrial vessel function in sheep and humans, as this pressure is similar to mean arterial pressures in pregnant ewes and women, and therefore likely to represent physiologic conditions (Sanghavi, Rutherford 2014, Blea, Barnard et al. 1997, Gaynor, Wertz et al. 1998).

Normalisation was performed using the DMT Normalisation module for LabChart (ADI Instruments, Dunedin, NZ). The baseline micrometer reading, vessel length and wire thickness were entered into the computer programme. Following the computer prompts, the vessel was incrementally distended using the micrometer. For each increase in stretch, LabChart recorded the force exerted by the vessel wall, and calculated wall tension and the IC of the vessel. LabChart used these data to calculate the micrometer setting required to place the vessel at 0.9IC\textsubscript{100} (Figure 2.5). The micrometer was then adjusted appropriately for each vessel. Following normalisation, the vessels were rinsed and bathed in fresh PSS, and air continuously bubbled through each chamber. Vessels were left to rest for at least 20 minutes prior to the wake-up.
2.4.6 Wake-up

The “wake-up” is a standardised constriction and relaxation protocol that was performed prior to the dose-response experiments. The purpose of stimulating the vessel to constrict was to re-activate the mechanical properties of the vessel, to ensure the vessel was functional. Following constriction, the integrity of the vascular endothelium was assessed by stimulating a pre-constricted vessel with Acetylcholine (ACh). ACh stimulation results in release of NO from the endothelium and subsequent vessel relaxation. If the vessel was undamaged, substantial relaxation should have occurred; if the endothelium was damaged, partial or no relaxation will have occurred.

Figure 2.5 - Vessel normalization

Screenshot from LabChart following successful normalization. Vessel length and wire diameter were entered (top right), then the micrometer adjusted incrementally to generate a stretch – tension curve. LabChart then calculated the micrometer reading required to place the vessel at 0.9 IC₁₀₀. In this example, the desired micrometer reading was 5240.
Vessel segments were stimulated with alpha agonist phenylephrine (Pe) 10 µmol/L (5 µL of Pe $10^{-2}$ M was added to the bath containing 5 mL PSS). The vessels were allowed to constrict and plateau before vessels were rinsed and baths refilled with fresh PSS. After a 10 minute rest period, a repeat dose of Pe 10 µmol/L was given; once constriction had plateaued, a dose of acetylcholine 10 µmol/L was given (5 µL of ACh $10^{-2}$ M was added to the bath containing 5 mL PSS). Following relaxation, vessels were washed with fresh PSS and given a 20 minute rest period prior to any experiments.

2.4.7 Dose-response curves

2.4.7.1 Constriction

Constriction response curves were performed for uterine and mesenteric arteries using Pe and thromboxane A2 mimetic U46619 (U4) respectively. For mesenteric vessels, U4 was used in preference to Pe, as previous experiments in our laboratory had demonstrated difficulty in consistently obtaining prolonged constriction with Pe, but not with U4. After ensuring the vessel was fully relaxed – washing the vessel with fresh PSS if necessary – increasing concentrations of constrictor were added to the bath containing 5 mL of PSS to obtain a constriction response curve in 9 steps for Pe (0.1 nmol/L to 10 µmol/L; Table 2.2) and in 7 steps for U4 (0.1 nmol/L to 1.0 µmol/L; Table 2.2). For each step of the curve, at least 2 minutes had passed and a stable constriction had been achieved before the next dose of constrictor was given (Figure 2.6 A). The difference in the final concentration of constrictor for mesenteric and uterine arteries reflected the greater sensitivity of the mesenteric vessels to U4 and therefore the lower concentration required to achieve maximum constriction. Following completion of this experiment, vessels were washed with PSS and given a 20 minute rest period prior to performing the ACh dose response curve.
<table>
<thead>
<tr>
<th>Volume of stock solution added to chamber</th>
<th>[Pe] or [U4] in chamber µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µL of $10^{-8}$ M</td>
<td>0.0001</td>
</tr>
<tr>
<td>45 µL of $10^{-7}$ M</td>
<td>0.001</td>
</tr>
<tr>
<td>45 µL of $10^{-6}$ M</td>
<td>0.01</td>
</tr>
<tr>
<td>10 µL of $10^{-5}$ M</td>
<td>0.05</td>
</tr>
<tr>
<td>35 µL of $10^{-5}$ M</td>
<td>0.1</td>
</tr>
<tr>
<td>10 µL of $10^{-4}$ M</td>
<td>0.5</td>
</tr>
<tr>
<td>35 µL of $10^{-4}$ M</td>
<td>1.0</td>
</tr>
<tr>
<td>10 µL of $10^{-3}$ M (Pe only)</td>
<td>5</td>
</tr>
<tr>
<td>35 µL of $10^{-3}$ M (Pe only)</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.2 - Volume of stock solutions required and final chamber concentrations achieved for constriction dose-response curves

When calculating [Pe] or [U4] in the chamber bath, applied volume of [Pe] or [U4] was ignored.

Pe phenylephrine, U4 thromboxane A2 analogue U46619
Figure 2.6 - Examples of vessel dose-response curves

Vessel responses are shown to constrictor phenylephrine (Pe; A); endothelium-dependent vasodilator acetylcholine (ACh; B) and endothelium-independent vasodilator sodium nitroprusside (SNP; C). Due to space constraints, not all doses of drug administered are shown.
2.4.7.2 *Endothelium-dependent relaxation*

For all vessels, the EC 80 (dose of constrictor required to elicit a constriction response approximately 80% of maximum) for U4 or Phenylephrine as appropriate was estimated from the constriction dose-response curves, and used to pre-constrict the arteries. Endothelium-dependent relaxation was assessed by a cumulative dose-response curve to ACh in 9 steps (0.1 nmol/L to 10 µmol/L; Table 2.3), with a standard time of 2 minutes between each application given (Figure 2.6 B). Following completion of this experiment, vessels were washed with PSS and given a 20 minute rest period prior to performing the sodium nitroprusside dose response curve.

2.4.7.3 *Endothelium independent relaxation*

Vessels were pre-constricted using the previously estimated EC 80 for U4 or Phenylephrine as appropriate. Endothelium independent relaxation was then assessed by a cumulative dose response curve to sodium nitroprusside in 7 steps (0.1 nmol/L to 10 µmol/L, Table 2.4), with a standard time of 2 minutes between each application (Figure 2.6 C).

Following completion of the SNP dose-response experiment, vessels were washed with PSS, then bathed in a high potassium solution, (KPSS; in mmol/L: 10 HEPES, 24 NaCl, 124 KCl, 2.4 MgSO₄, 4.9 CaCl₂, 1.18 KH₂PO₄, 5.5 glucose; pH 7.4) and constriction allowed to plateau.
<table>
<thead>
<tr>
<th>Volume of stock solution added to chamber</th>
<th>[ACh] in chamber µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µL of 10⁻⁸ M</td>
<td>0.0001</td>
</tr>
<tr>
<td>45 µL of 10⁻⁷ M</td>
<td>0.001</td>
</tr>
<tr>
<td>45 µL of 10⁻⁶ M</td>
<td>0.01</td>
</tr>
<tr>
<td>10 µL of 10⁻⁵ M</td>
<td>0.05</td>
</tr>
<tr>
<td>35 µL of 10⁻⁵ M</td>
<td>0.1</td>
</tr>
<tr>
<td>10 µL of 10⁻⁴ M</td>
<td>0.5</td>
</tr>
<tr>
<td>35 µL of 10⁻⁴ M</td>
<td>1.0</td>
</tr>
<tr>
<td>10 µL of 10⁻³ M</td>
<td>5</td>
</tr>
<tr>
<td>35 µL of 10⁻³ M</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.3 - Volume of stock solutions required and final chamber concentrations achieved for endothelium-dependent vasodilatation dose-response curves

When calculating [ACh] in the chamber bath, the applied volume of [ACh] was ignored.

*ACh* acetylcholine
<table>
<thead>
<tr>
<th>Volume of stock solution added to chamber</th>
<th>[SNP] in chamber µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µL of $10^{-8}$ M</td>
<td>0.0001</td>
</tr>
<tr>
<td>45 µL of $10^{-7}$ M</td>
<td>0.001</td>
</tr>
<tr>
<td>45 µL of $10^{-6}$ M</td>
<td>0.01</td>
</tr>
<tr>
<td>45 µL of $10^{-5}$ M</td>
<td>0.1</td>
</tr>
<tr>
<td>45 µL of $10^{-4}$ M</td>
<td>1.0</td>
</tr>
<tr>
<td>45 µL of $10^{-3}$ M</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.4 - Volume of stock solutions required and final chamber concentrations achieved for endothelium-independent vasodilatation dose-response curves

When calculating [SNP] in the chamber bath, the applied volume of [SNP] was ignored.

*SNP* sodium nitroprusside
2.4.8  Analysis of myography data

2.4.8.1  Data selection and adjustment for baseline

Recorded experimental data were saved as LabChart data files for offline analysis. Files were reviewed using LabChart 8.1.2. Reader (AD Instruments, Dunedin, New Zealand) and Microsoft Excel 2013 (Microsoft Corporation, Washington, USA). For each experiment, the curves of an individual vessel were visually inspected, an area of interest selected, and selected data copy-pasted to a table in LabChart using the “add to data pad” function. Each selection contained approximately 10-30 s of data, and excluded any obvious artefacts. Relevant data on vessel force (in mN) were then transferred from the LabChart table to an Excel spreadsheet for cleaning. Because each vessel exerted a slightly different force at baseline, raw data from different vessels could not be directly compared. To enable comparison between vessels, the baseline force of each vessel was subtracted from the raw data for each experiment before further calculations or analyses were performed.

2.4.8.2  Assessment of vessel viability

The constriction and relaxation curves from the wake-up were inspected, and an area that demonstrated a plateau of constriction and an area that included the maximal relaxation selected. Following correction for baseline, the percentage relaxation was then calculated:

\[
\text{Vessel relaxation (\%)} = \left( \frac{\text{Force}^{\text{constriction}} - \text{Force}^{\text{relaxation}}}{\text{Force}^{\text{constriction}}} \right) \times 100
\]

where Force^{constriction} was the mean force in mN during pre-constriction, and Force^{relaxation} was the minimum force in mN detected for a given dose of dilator. Vessels were excluded from further analysis if total constriction was less than 2 mN above baseline. Vessels were excluded from analysis of endothelium-dependent relaxation if they exhibited impaired relaxation (<30% of pre-constriction).
2.4.8.3 Dose-response curves

For the analysis of the constriction curves, a representative area where the curve was stable / plateaued was selected for each dose of agonist, and the mean value of the selection used for further analysis. The final plateau of constriction to KPSS was selected for each vessel, and the mean value used for analysis. The response of each vessel to each dose of agonist was then expressed as a percentage of the mean constriction to KPSS. In the murine study, the vessel response to KPSS was up to 10 times higher than the maximum agonist induced constriction. This disproportionate response to KPSS appeared to be more common in the eNOS$^{-/-}$ strain, but did not occur consistently in this group, and was also seen in mice of other strains. Neither normal distribution of mean residuals or equality of variances could be obtained when maximum constriction was calculated this way, so data could not be analyzed by 3-way ANOVA. Therefore, the absolute force that a vessel exerted (mN) for a given dose of agonist was used to assess constriction in the murine study.

For analysis of the relaxation curves, any vessel that exhibited spontaneous loss of tone was excluded from analysis. For all other vessels, an area of plateau during pre-constriction was selected and the mean value of the selection used for further analysis. Then, for each dose of vasodilator, the area of maximum relaxation response was selected, and the minimum value of the selected data used. The percentage relaxation was then calculated for each dose of vasodilator.

As each experiment involved multiple vessel segments from the same animal, data from vessels of the same type from the same animal were averaged for sigmoidal curve fitting and statistical analysis.
2.4.8.4 Calculation of the EC50

Graphpad prism v 6.03 (GraphPad Software Inc., California, USA) was used to calculate the EC50. For each dose-response experiment, an XY table was generated for each experimental group. Response data for individual animals (absolute force of constriction in mN for constriction, or % relaxation for dilators) were pasted as Y values, with the x-values given as the log [constrictor or dilator]. Non-linear regression was used to fit a sigmoidal curve using the ‘Dose-response – Stimulation, log (agonist) vs response- Variable slope’ equation, and generate the EC50 for each vessel.

2.4.9 Modifications of the myography protocol for the ovine study

2.4.9.1 Vessel mounting

Myometrial arteries from the ovine study were mounted using 40 µm wire instead of 25 µm wire used in the murine study. Ovine vessels had thicker walls and were more tortuous than the murine vessels, the 40 µm wire was sturdier and less likely to bend and damage the vessel when mounting.

2.4.9.2 Dose-response curves

Phenylephrine was used to constrict and pre-constrict the myometrial arteries for the dose-response curves. Inhibitor data (L-NAME) was not obtained.

2.4.10 Modifications of the myography protocol for the study of myometrial and chorionic arteries in pregnancies complicated by severe early onset IUGR

2.4.10.1 Vessel mounting

Myometrial and chorionic plate arteries from the human study were mounted using 40 µm wire instead of the 25 µm wire used in the murine study. The human vessels had thicker walls
and were more tortuous than the murine vessels; the 40 µm wire was sturdier and less likely to bend and damage the vessel when mounting.

2.4.10.2 Chorionic vessel oxygen tension and normalisation

Previous studies have demonstrated that oxygen tension modifies chorionic plate artery vascular reactivity (Wareing, Greenwood et al. 2006). Following normalisation of chorionic vessels, the central chamber was continuously perfused with a mixture of 5% CO₂ in Nitrogen (BOC special gases, Auckland, NZ). This is equivalent to 2% oxygenation (15 mmHg) (Wareing, Greenwood et al. 2006), and was chosen as it closely represents umbilical artery oxygenation at term (16 mmHg, (Lackman, Capewell et al. 2001)). Vessel stretch is also known to affect vascular reactivity (McPherson 1992). Intravascular pressures are considerably lower in the fetus than in the mother; therefore, as previously described, the chorionic arteries were normalised to 90% of 5.1 kPa – this is equivalent to 18-26 mmHg, and more similar to the pressures occurring in vivo (Wareing, Greenwood et al. 2006, Cooper, Wareing et al. 2005).

2.4.10.3 Wake-up

U4 was used to constrict both myometrial and chorionic vessels as it has previously been shown to produce the largest and most consistent constrictive effect in these vessels (Wareing, Greenwood et al. 2006). U4 0.05 – 0.1µM was used to constrict both chorionic and myometrial arteries in the wake-up. Following the initial dose of U4, both myometrial and chorionic vessels showed a prolonged constriction and were resistant to endothelium-dependent vasodilators, with tone returning to baseline over a period of approximately 10 minutes and repeated wash-outs of the drug. Due to concerns that vessel tone would not return to baseline after repeated doses of U4, the wake-up protocol was modified to include only one dose of U4 followed by a single dose of endothelium-dependent vasodilator.
2.4.10.4 Endothelium-dependent vasodilatation in myometrial arteries

The endothelium-dependent vasodilator bradykinin (BK) has been used in previous studies of human myometrial artery ex vivo function, and causes dose-dependent relaxation of pre-constricted myometrial arteries (Wareing, Myers et al. 2005b, Samangaya, Wareing et al. 2011, Ong, Moore et al. 2003). Therefore, BK was used to assess the endothelium-dependent relaxation in the myometrial small arteries in studies for this thesis (doses as for ACh - Table 2.3).

2.4.10.5 Endothelium-dependent vasodilatation in chorionic arteries

Previous studies have demonstrated that maximally constricted chorionic arteries show a poor response to endothelium-dependent vasodilators (Hull, White et al. 1994, Wareing, Myers et al. 2006, Ong, Moore et al. 2003). However, BK has been shown to induce changes in vascular tone of chorionic arteries that have been sub-maximally constricted to evoke oscillatory tone (Sweeney, Wareing et al. 2008, Hayward, Higgins et al. 2013). Therefore, in the studies for this thesis, chorionic arteries were pre-constricted with U4 EC80 and left for 15 minutes to develop tone oscillations before commencing a dose-response curve with BK (doses as for ACh - Table 2.3).
3 Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in the growth restricted ovine fetus

3.1 Preface

This chapter consists of the published manuscript:

OYSTON, C., STANLEY, J.L., OLIVER, M.H., BLOOMFIELD, F.H., BAKER, P.N.

Heading numberings, caption numberings and the referencing style of the original manuscript have been reformatted to maintain consistency with the rest of the thesis. With the exception of section 3.3.1 (discussion of supplementary material), the written content including text, figures and tables remain unaltered from that submitted for publication. Because of this, the methods section (3.2.3) contains some repetition of the methods reported in expanded form in chapter 2 of this thesis.

3.1.1 Rationale

The majority of studies of the effect of sildenafil on fetal growth have been in rats and mice (Roberts, Refuerzo et al. 2016, Refuerzo, Sokol et al. 2006, Pellicer, Herraiz et al. 2011, Herraiz, Pellicer et al. 2012, Stanley, Andersson et al. 2012, Dilworth, Andersson et al. 2013, Gillis, Mooney et al. 2016, Nassar, Masrouha et al. 2012). Due to the short gestations of these species, study of the effect of sildenafil on fetal growth, umbilical and uterine artery blood flow, and myometrial artery ex vivo function has been limited to experiments where treatment duration is less than a week. Although treatments that increase fetal growth and result in
pregnancy prolongation of any length would be potentially beneficial, ideally, sildenafil would increase fetal growth sufficiently that pregnancy could be extended over several weeks. In order to ensure ongoing benefit (and an absence of detrimental effects), it is important to study the effects of treatment over a longer period of time than is possible in rodents. The sheep has a gestation of approximately 147 days, and is one species where such a study is possible. The fetal lamb is also remarkably resistant to surgical manipulation including vascular catheterisation in utero. Insertion of fetal vascular catheters enables repeated fetal blood samples to be drawn throughout the experiment. The use of chronically catheterised fetal lambs in this study provides an opportunity to gain a novel insight into utero-placental and fetal-placental haemodynamics and fetal metabolic changes that are associated with medium-term sildenafil exposure in IUGR pregnancies. Finally, a potential limitation of many previous studies of sildenafil has been the use of models where IUGR has been induced by inhibition of NO production (Gillis, Mooney et al. 2016, Motta, Grosso et al. 2015, Ramesar, Mackraj et al. 2010, Nassar, Masrouha et al. 2012, Herraiz, Pellicer et al. 2012). As the action of sildenafil occurs via a downstream upregulation of the same pathway that was inhibited to induce fetal growth, these models may be too simplistic to assess the effects of sildenafil on fetal growth. The following study uses an established model of IUGR that does not involve direct manipulation of the NO pathway: maternal uterine artery embolisation (Eremia, de Boo et al. 2007, Bloomfield, Bauer et al. 2002, Jensen, Harding et al. 1999). Uterine artery embolisation causes obstruction of maternal arterioles supplying the placenta, resulting in placental infarction (Creasy, Barrett et al. 1972). This is therefore highly relevant to IUGR in human pregnancies, where placentae demonstrate infarction, with atherosis, thrombosis, and obliteration of maternal arterial lumens (Aardema, Oosterhof et al. 2001, Madazli, Somunkiran et al. 2003).
3.2 Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in the growth restricted ovine fetus

3.2.1 Abstract

Intrauterine growth restriction (IUGR) causes short- and long-term morbidity. Reduced placental perfusion is an important pathogenic component of IUGR; substances which enhance vasodilatation in the uterine circulation, such as sildenafil citrate (sildenafil), may improve placental blood flow and fetal growth. This study aimed to examine the effects of sildenafil in the growth-restricted ovine fetus.

Ewes carrying singleton pregnancies underwent insertion of vascular catheters, then were randomised to receive uterine artery embolisation (IUGR), or to a control group. Ewes in the IUGR group received a daily infusion of sildenafil (IUGR + SC; n = 10) or vehicle (IUGR + V; n = 8) for 21 days. The control group received no treatment (n = 9). Umbilical artery blood flow was measured using Doppler ultrasound and the resistive index (RI) calculated. Fetal weight, biometry, and placental weight were obtained at post-mortem following treatment completion.

Umbilical artery RI in IUGR+V fell less than in controls; the RI of IUGR + SC was intermediate to that of the other two groups (mean ± SEM for control vs IUGR + V vs IUGR + SC: ∆RI, 0.09 ± 0.03 vs -0.01 ± 0.02 vs 0.03 ± 0.02; F(2,22) = 4.21, p = 0.03. Compared to controls, lamb and placental weights were reduced in IUGR + V but not IUGR + SC (control vs IUGR + V vs IUGR + SC: fetal weight, 4381 ± 247 vs 3447 ± 235 vs 3687 ± 129 g; F(2,24) = 5.49, p = 0.01; placental weight: 559.7 ± 35.0 vs 376.2 ± 32.5 vs 475.2 ± 42.5 g; F(2,24) = 4.64, p = 0.01).
Sildenafil may be a useful adjunct in the management of IUGR. An increase in placental weight and fall in fetal-placental resistance suggests that changes to growth are at least partly mediated by changes to placental growth, rather than alterations in placental efficiency.

3.2.2 Introduction

Intrauterine growth restriction (IUGR) occurs when a fetus fails to achieve its genetic growth potential. These fetuses have an increased risk of significant perinatal morbidity and mortality (Gardosi, Madurasinghe et al. 2013, Baschat, Cosmi et al. 2007), neurodevelopmental impairment in childhood (Chen, Chen et al. 2016), and an increased risk of adult onset chronic diseases (Barker DJ 2004). Currently, there are no clinically available treatments that can improve fetal growth. The management of IUGR pregnancies is limited to intense fetal monitoring in an attempt to determine when the fetus has maximised its time in utero and when the risks of hypoxia and death are so high that early delivery is indicated. When IUGR is severe or of early onset, delivery may be indicated at extremely preterm gestations when the risk of perinatal complications are especially high (Baschat, Cosmi et al. 2007). Of infants with birth weight <10th centile, those delivered at 30-32 weeks have a more than threefold greater chance of survival free of major morbidity, compared with those delivered at 24-28 weeks (Shah, Ye et al. 2012). A therapy facilitating expectant management and safe pregnancy prolongation has the potential to reduce dramatically both short- and long-term health and societal costs.

There are many causes of IUGR; however, the predominant etiology is placental insufficiency, caused by a failure of placental trophoblast to adequately invade and transform maternal spiral arteries in early pregnancy (Olofsson, Laurini et al. 1993, Lin, Shimizu et al. 1995, Sagol, Sagol et al. 2002). The net result of this abnormal transformation is increased resistance to maternal blood flow to the placenta resulting in placental under-perfusion (Lin,
In normal pregnancy, the vasodilator nitric oxide (NO) contributes to the increased vasodilatation and reduced vascular resistance seen in the utero-placental circulation (Anumba, Robson et al. 1999, Sladek, Magness et al. 1997). The NO second messenger cGMP is enzymatically degraded by phosphodiesterases. Sildenafil citrate (sildenafil), an inhibitor of phosphodiesterase-5 (PDE-5; an isoform found extensively throughout the reproductive tract (Lin, Lin et al. 2006, Coppage, Sun et al. 2005)), is able to enhance the vasodilatory action of NO. Thus, it is hypothesised that sildenafil will improve uterine and placental perfusion in compromised pregnancies, increasing placental exchange and fetal growth. This hypothesis is supported by ex-vivo studies of myometrial resistance arteries from growth restricted pregnancies which showed reduced constriction and enhanced relaxation following pre-incubation with sildenafil.

There is accumulating evidence of efficacy of sildenafil at improving fetal growth, particularly in rodents; (Dilworth, Andersson et al. 2013, Stanley, Andersson et al. 2012, Refuerzo, Sokol et al. 2006, Herraiz, Pellicer et al. 2012) however, evidence of an effect of sildenafil treatment on fetal growth in larger species is limited (Satterfield, Bazer et al. 2010). It is important to study efficacy in larger species, as duration of pregnancy (and therefore timeframe for treatment) and organ development are more akin to that seen in humans, thereby increasing the potential for identifying detrimental effects, as well as any beneficial effects. The current study was designed to investigate whether maternal administration of sildenafil citrate improves growth of lambs where growth restriction had been induced by uterine artery embolisation, and to explore potential mechanisms underlying any improvement in fetal growth.
3.2.3 Methods and Materials

3.2.3.1 Ethics Statement

All experimentation was conducted in accordance with accepted standards of humane care, with all experiments approved by the University of Auckland Animal Ethics Committee (approval number AEC 001101; Appendix B).

3.2.4 Animals

Time-mated multiparous Romney-cross ewes carrying singleton pregnancies were acclimatised to indoor individual pens. Ewes were randomised preoperatively to a control group (no uterine artery embolisation) or an IUGR group that received embolisation. Surgery was performed between 96 – 100 days’ gestational age (dGA, term = 147 dGA). After an overnight fast, general anesthesia was induced with 30 mL intravenous propofol and maintained with inhaled isoflurane. As previously described (Wali, de Boo et al. 2012), a midline laparotomy and hysterotomy were performed and polyvinyl catheters were placed into both fetal femoral arteries and veins via the tarsal vessels. A single free-floating amniotic fluid catheter was inserted prior to closing the hysterotomy. Maternal uterine veins were catheterised bilaterally with polyvinyl catheters with silicone tips. In the IUGR groups, the main uterine arteries were catheterised via a distal arterial branch. Following closure of the maternal abdomen, a maternal femoral artery and vein were catheterised via the tarsal vessels. The ewe received a single IM dose of 450,000 IU benzathine penicillin and the fetus received 80 mg gentamicin sulphate into the amniotic fluid prior to closing the hysterotomy.

All maternal and fetal catheters were checked and flushed for the first three postoperative days, then every other day for the remainder of the experiment. Fetal arterial blood samples were collected in heparinised syringes on ice before performing blood gas analysis and glucose concentrations (arterial blood gas: Alere, Waltham, MA; glucose: Yellow Springs
Instruments, Dayton, OH). Fetal samples were collected for the first three days postoperatively, then twice daily during the embolisation period, then twice weekly for the remaining duration of the experiment.

3.2.4.1 Uterine artery embolisation

From 102 – 107 dGA, growth restriction was induced by up to twice daily embolisation of the uterine arteries with polystyrene microspheres 20 - 50 µm diameter (Superose 12, 1:100 dilution, Pharmacia Biotech, Uppasala, Sweden) as described previously (Wali, de Boo et al. 2012). The frequency and volume of injections was titrated against fetal PaO\(_2\) and lactate levels, with embolisation withheld if fetal PaO\(_2\) was < 14 mmHg, or fetal arterial lactate was > 4 mmol/L.

3.2.4.2 Experimental period

Following completion of embolisation, ewes in the IUGR group were randomised to treatment with sildenafil citrate (Zhuhai Jiacheng Bio-Tech, Zhuhai City, China) 150 mg/day dissolved in 54 mL sterile water (IUGR+SC group), or a visually indistinguishable infusion of 54 mL vehicle sterile water (IUGR+V group). As there are no published data on the pharmacokinetics of sildenafil citrate in the pregnant ewe, the dose of sildenafil was chosen in line with previous studies where biologic effect of sildenafil was apparent (Satterfield, Bazer et al. 2010). Sildenafil was administered via a continuous subcutaneous infusion over 12 hours, via a portable infusion pump secured to the ewe's back (WalkMed Infusion, Centennial, CO, USA). The infusion bag of the pump was checked and refilled at the same time each day, and any residual fluid was given as a slow subcutaneous bolus. The infusion site, subcutaneous needle and infusion tubing was changed every third day to minimise the risk of infection and localised pooling of the infusate.
3.2.4.3 Assessment of umbilical and uterine artery blood flows

Ultrasound assessment of umbilical artery blood flow was performed prior to surgery (96 dGA), and at 107, 119 and 128 dGA. With the ewe non-sedated and standing, a free floating segment of umbilical cord was identified with pulsed wave color Doppler. Waveforms were recorded during fetal quiescence with the angle of insonation kept to less than 50°. Recorded images were reviewed offline and umbilical artery resistance index was calculated using an average of 3 consecutive waveforms, using the formula Resistive Index (RI) = (S-D)/S, where S is the peak systolic velocity, and D the height of the end diastolic trough. Ultrasound images were obtained and analyzed by an investigator blinded to treatment group for the IUGR animals.

At 107, 119, and 128 dGA assessment of uterine artery blood flow was performed via infusion of antipyprine using the Fick principle. A tracer solution containing 160 mg of antipyprine in 20 mL of saline was infused into a fetal vein at 3 mL/hr following a 4 mL bolus. Under these conditions, previous studies have shown that antipyprine reaches a steady state at 90 minutes (Jensen, Harding et al. 1999). From 90 minutes, 4 sets of paired blood samples drawn from a maternal artery and the utero-ovarian vein were collected at 15 minute intervals. Antipyrine concentration was measured by HPLC as described previously (Pimentel, Figueroa et al. 1986), with a Phenomenex Kinetex column (1.7 μm C18(2) 100 Å), dimensions 150 × 2.1 mm (Phenomenex, Torrance, USA). Uterine artery blood flow was then calculated using the antipryine steady state diffusion method with the application of the Fick principle.

3.2.4.4 Tissue collection

At 132-133 dGA ewes were euthanised with an overdose of intravenous pentobarbitone. The uterus was removed and opened. The fetus was removed, dried, weighed and measured, fetal organs dissected and weighed. Immediately after removing the fetus, a full thickness uterine
biopsy was taken from a site proximate to umbilical cord insertion, and myometrial resistance vessels were dissected clean and stored in ice cold physiological saline solution (PSS; in mmol/L: NaCl 136.9, KCl 2.7, CaCl₂ 1.8, MgSO₄ 0.6, NaHCO₃ 11.9, KH₂PO₄ 0.5 and glucose 11; pH 7.4). The ruminant placenta is made up of a number of placentomes that develop at uterine attachments sites called caruncles. These placentomes can take a variety of morphological appearances and have been classified as A-D accordingly (Vatnick, Schoknecht et al. 1991). Placentomes were dissected from the uterus, sorted into categories A, B, C or D, counted and weighed.

3.2.4.5 *Ex vivo vascular function*

Segments of myometrial resistance vessels from each sheep were mounted on a wire myograph system (Multi Myograph System 610 M, Danish Myo Technology A/S, Aarhus, Denmark) and normalised within 8 hours of dissection, as previously described (Stanley, Sankaralingam et al. 2010). Vessels were constricted with phenylephrine (PE; 10 μmol/l; Sigma-Aldrich, New Zealand) to confirm viability, washed and equilibrated with PSS for 20 minutes. A second dose of PE 10 μmol/l was given and constriction allowed to plateau before giving a single dose of acetylcholine (Ach; 10 μmol/l; Sigma-Aldrich) to confirm endothelial integrity. Following a further washout and 30 minute equilibration period, a concentration-response curve to PE was constructed (0.1-10 μmol/l). The EC₈₀ concentration was calculated for individual vessels and used to preconstrict the arteries to construct concentration-response curves to Ach (0.1-10 μmol/l) and then sodium nitroprusside (SNP; Sigma-Aldrich 0.1-10 μmol/l). Finally, a 120 mmol/l potassium solution (KPSS; in mmol/l: 10 HEPES, 24 NaCl, 124 KCl, 2.4 MgSO₄, 4.9 CaCl₂, 1.18 KH₂PO₄, 5.5 glucose; pH 7.4) was added to each vessel and the constriction allowed to plateau.
3.2.4.6 Data analysis

Statistical analysis was performed using GraphPad Prism (v 6.03) software. Data are presented as mean ± SEM, or median (interquartile range) with significance determined by one-way ANOVA and Tukey’s multiple comparisons test or by the Kruskall-Wallis test and Dunn’s multiple comparisons test as appropriate. For all analyses, a p value of <0.05 was considered statistically significant.

Fetal weights were normally distributed; histograms were constructed for each group and non-linear regression performed to obtain Gaussian distributions. The 5th percentile of the control group fetal weight was calculated and used to define fetal growth restriction.

Fetal arterial blood gas and glucose concentrations were compared between two groups (control vs IUGR) during embolisation and prior to the onset of treatment (102 – 107 dGA), and then three groups (control vs IUGR+V vs IUGR+SC) following completion of embolisation (110 – 129 dGA) using the appropriate non-parametric test.

For analysis of wire myography data, sigmoidal curve fitting was used to determine EC50 for each substance.

3.2.4.7 Results

Forty-nine ewes underwent surgery (8 controls vs 41 IUGR). Of these, 20 were euthanised prior to the completion of the study: 14 due to perioperative fetal losses (IUGR group only), 4 fetal losses during embolisation (IUGR group only), and 2 fetal losses occurred during the treatment period (one each from control and IUGR+SC group). The IUGR+SC lamb that died during the treatment period had extremely poor blood gases post embolisation (pH 7.2, paCO2 69 mmHg, paO2 8.4 mmHg prior to commencing treatment) and died on the first day of treatment administration. Two ewes in the embolisation group were excluded as
embolisation could not be adequately performed due to technical problems with laboratory equipment in the week of embolisation. This left a total of 27 animals in the final analysis: 9 control, 8 IUGR+V and 10 IUGR+SC. There were no differences amongst groups in maternal weight at the beginning of the experimental period or gestational age at surgery.

3.2.4.8  **Fetal growth**

At post-mortem, mean fetal weight was significantly different amongst groups ($F(2, 24) = 5.48, p = 0.01$). Mean fetal weight in the IUGR+V group was significantly less than controls ($p = 0.01$); however, IUGR+SC treated fetuses were not significantly different from controls or IUGR+V (control vs IUGR+V vs IUGR+SC: 4,381 ± 247 vs 3,448 ± 236 vs 3,687 ± 129 g, Figure 3.1 A). The fifth centile of the control group was 3,162 g; 38% of the IUGR+V lambs and 10% of the IUGR+SC group had a weight below this at post-mortem (Figure 3.1 B).

Fetal crown rump length differed amongst groups ($F(2, 24) = 4.11; p = 0.03$), with lambs in the IUGR+V group significantly shorter than those in the control group ($p = 0.04$). The CRL of lambs in the IUGR+SC group did not differ from that of either of the other two groups (control vs IUGR+V vs IUGR+SC: 57.8 ± 0.6 vs 45.1 ± 1.0 vs 45.5 ± 0.7 cm). There were no other significant differences in lamb measurements or organ weights between any of the groups when expressed as either absolute weight (Table 3.1) or as a proportion of body weight (data not shown).
Figure 3.1 – Fetal weight was reduced compared to controls for IUGR + V, but not for IUGR + SC

Fetal weight at post-mortem was significantly reduced for IUGR + V compared to controls, but not for IUGR + SC compared to controls (A), suggesting a beneficial effect of sildenafil treatment on lamb weight. Distribution of lamb weights for each of the three groups (B); the dashed vertical line represents the 5th centile of fetal weight in the control group. Mean ± SEM; n = 9, 8, 10 (control, IUGR + V, IUGR + SC); *p < 0.05 (1-way ANOVA with Tukey’s multiple comparisons test). —, control; —, IUGR + V; ---, IUGR + SC
<table>
<thead>
<tr>
<th>Measure</th>
<th>Control n = 9</th>
<th>IUGR + V n = 8</th>
<th>IUGR + SC n = 10</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal biometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biparietal diameter (mm)</td>
<td>61.2 ± 0.1</td>
<td>58.3 ± 0.1</td>
<td>59.0 ± 0.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Abdominal circumference (cm)</td>
<td>32.8 ± 1.4</td>
<td>29.9 ± 1.3</td>
<td>30.0 ± 0.4</td>
<td>0.12</td>
</tr>
<tr>
<td>Crown-rump length (cm)</td>
<td>47.8 ± 0.6</td>
<td>45.1 ± 1.0*</td>
<td>45.5 ± 0.7</td>
<td>0.03</td>
</tr>
<tr>
<td>Front limb length (cm)</td>
<td>30.3 ± 0.8</td>
<td>29.2 ± 0.5</td>
<td>29.0 ± 0.5</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus, placenta, liquor</td>
<td>2,925 (2,273 – 4,333)</td>
<td>2,238 (1,731 – 2,749)</td>
<td>2,585 (1,813 – 3,020)</td>
<td>0.23</td>
</tr>
<tr>
<td>Fetus</td>
<td>4,381 ± 247</td>
<td>3,447 ± 235*</td>
<td>3,687 ± 129</td>
<td>0.01</td>
</tr>
<tr>
<td>Brain</td>
<td>51.0 ± 1.3</td>
<td>47.3 ± 1.2</td>
<td>48.4 ± 1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>30.0 (26.5 – 35.0)</td>
<td>24.5 (23.1 – 29.8)</td>
<td>29.7 (23.5 – 32.5)</td>
<td>0.13</td>
</tr>
<tr>
<td>Lungs</td>
<td>102.2 ± 9.4</td>
<td>85.6 ± 7.5</td>
<td>92.0 ± 4.2</td>
<td>0.22</td>
</tr>
<tr>
<td>Liver</td>
<td>153.5 (102.8 – 189.3)</td>
<td>118.9 (100.9 – 139.5)</td>
<td>123.9 (87.1 – 150.5)</td>
<td>0.30</td>
</tr>
<tr>
<td>Kidneys</td>
<td>28.4 ± 2.9</td>
<td>22.6 ± 1.9</td>
<td>24.0 ± 1.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.50 ± 0.07</td>
<td>0.36 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Spleen</td>
<td>10.5 (7.0 – 22.5)</td>
<td>8.1 (5.5 – 9.3)</td>
<td>7.8 (6.0 – 12.1)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*Table 3.1 - Fetal measurements and organ weights*  
(continued next page)
<table>
<thead>
<tr>
<th>Measure</th>
<th>Control (n = 9)</th>
<th>IUGR + V (n = 8)</th>
<th>IUGR + SC (n = 10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid</td>
<td>1.5 (1.4 – 1.8)</td>
<td>1.3 (1.2 – 1.5)</td>
<td>1.6 (1.3 – 1.9)</td>
<td>0.18</td>
</tr>
<tr>
<td>Neck thymus</td>
<td>13.2 ± 2.2</td>
<td>12.4 ± 2.0</td>
<td>14.8 ± 1.8</td>
<td>0.70</td>
</tr>
<tr>
<td>Chest thymus</td>
<td>7.5 (3.3 – 8.6)</td>
<td>4.4 (3.8 – 7.1)</td>
<td>5.4 (4.3 – 6.2)</td>
<td>0.63</td>
</tr>
<tr>
<td>Pancreas</td>
<td>4.7 (3.7 – 5.0)</td>
<td>3.8 (3.5 – 4.3)</td>
<td>4.1 (3.0 – 4.7)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Table 3.1 (continued) - Fetal measurements and organ weights**

Mean ± SEM, or median (inter-quartile range) as appropriate; n = 9, 8, 10 (control, IUGR + V, IUGR + SC). *p < 0.05 compared with controls (1-way ANOVA with Tukey’s multiple comparisons test).
Placental weight differed significantly amongst groups ($F(2,24) = 5.52, p = 0.01$). Placental weight was significantly reduced in the IUGR+V group compared with controls ($p < 0.01$), but in the IUGR+SC was not significantly different to either of the other two groups (placental weight control vs IUGR+V vs IUGR+SC: 560 ± 35 vs 376 ± 33 vs 475 ± 43 g) (Table 3.2). The absolute number of placentomes differed between groups ($F(2,24) = 5.22, p = 0.01$), with the IUGR+SC group having significantly more placentomes than IUGR+V ($p = 0.01$), although neither group differed significantly from control (number of placentomes for control vs IUGR+V vs IUGR+SC: 70 ± 5 vs 57 ± 4 vs 80 ± 5). Mean placentome weight differed amongst groups ($F(2,24) = 4.64, p = 0.02$) with placentome weight significantly reduced in IUGR+SC compared to controls ($p = 0.02$), but not compared to IUGR+V (mean placentomes weight control vs IUGR+V vs IUGR+SC: 8.2 ± 0.6 vs 6.8 ± 0.7 vs 5.9 ± 0.3 g. The IUGR+SC group had significantly lesser proportion of placentomes weighing >4 g compared to the control group ($p = 0.02$, Table 3.2), but the proportion of large placentomes did not differ significantly from that in the controls.

All groups had similar proportions of A, B and C placentome types (Table 3.2). The IUGR+V group had significantly fewer D type placentomes compared with the control group ($p = 0.01$), but not the IUGR+SC group.
<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>IUGR + V</th>
<th>IUGR + SC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental weight (g)</td>
<td>559.7 ± 35.0</td>
<td>376.2 ± 32.5†</td>
<td>475.2 ± 42.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Fetal weight: placental weight</td>
<td>7.9 (7.1 – 8.3)</td>
<td>9.1 (6.7 – 10.1)</td>
<td>8.8 (7.9 – 9.8)</td>
<td>0.3</td>
</tr>
<tr>
<td>Mean placentome weight (g)</td>
<td>8.2 ± 0.6</td>
<td>6.8 ± 0.7</td>
<td>5.9 ± 0.3*</td>
<td>0.02</td>
</tr>
<tr>
<td>Placentome number</td>
<td>70 ± 5</td>
<td>57 ± 4</td>
<td>80 ± 6†</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Distribution of placentomes by morphologic subtype

<table>
<thead>
<tr>
<th>%</th>
<th>Control</th>
<th>IUGR + V</th>
<th>IUGR + SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% A</td>
<td>33 (17 – 45)</td>
<td>41 (28 – 62)</td>
<td>26 (7 – 84)</td>
</tr>
<tr>
<td>% B</td>
<td>26 (20 – 39)</td>
<td>47 (31 – 58)</td>
<td>21 (15 – 43)</td>
</tr>
<tr>
<td>% C</td>
<td>19 (8 – 27)</td>
<td>10 (0 – 20)</td>
<td>31 (0 – 57)</td>
</tr>
<tr>
<td>% D</td>
<td>11 (1 – 34)</td>
<td>0† (0 – 0)</td>
<td>1 (0 – 9)</td>
</tr>
</tbody>
</table>

Distribution of placentomes by weight

<table>
<thead>
<tr>
<th>%</th>
<th>Control</th>
<th>IUGR + V</th>
<th>IUGR + SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Small (&lt;2 g)</td>
<td>7 (4 – 10)</td>
<td>10 (2 – 17)</td>
<td>16 (8 – 25)</td>
</tr>
<tr>
<td>% Medium (2-4 g)</td>
<td>15 (8 – 19)</td>
<td>15 (13 – 25)</td>
<td>23 (19 – 24)</td>
</tr>
<tr>
<td>% Large (&gt;4 g)</td>
<td>79 (75 – 84)</td>
<td>70 (60 – 77)</td>
<td>63* (57 – 68)</td>
</tr>
</tbody>
</table>

**Table 3.2 - Placentome weights and morphology**

Mean ± SEM, or median (inter-quartile range) as appropriate; n = 9, 8, 10 (control, IUGR + V, IUGR + SC). *p < 0.05, † p < 0.01 vs controls (1-way ANOVA with Tukey’s multiple comparisons test, or Kruskall Wallis with Dunn’s multiple comparisons test as appropriate). ‡p < 0.05 vs IUGR + V (1-way ANOVA with Tukey’s multiple comparisons test).
3.2.4.9 *Fetal arterial blood gas and glucose concentration*

Blood sampling from fetal catheters became less reliable with increasing gestation. Due to intermittent catheter function, blood gas and glucose concentrations were not available for every animal at every time point. Prior to the onset of embolisation there were no differences amongst groups in fetal arterial blood gas or glucose concentrations (Figure 3.2). Embolisation resulted in a tendency to increased fetal PaCO$_2$ and reduced fetal PaO$_2$ compared to controls; the differences in PaO$_2$ and PaCO$_2$ were statistically significant on days 105 and 106 (p < 0.05 vs controls day 105; p < 0.01 vs controls day 106) (Figure 3.2 C). During this period, fetal arterial glucose concentrations were also intermittently lower in the embolised group with significantly lower concentrations on day 105 (p < 0.05) and 107 (p < 0.01) (Figure 3.2 D). Fetal arterial pH was significantly lower in the embolised group on day 106 (p < 0.05), but otherwise was similar amongst groups (Figure 3.2 A). There was no significant effect of sildenafil treatment on fetal arterial blood gas parameters or glucose concentrations (Figure 3.2).

3.2.4.10 *Uterine artery blood flow*

Due to complications arising with venous catheter occlusions, only a small number of ewes completed the antipyrine protocol. Median or individual values of blood flow calculated for each of the time points are included as supplementary material (section 3.3).
Figure 3.2 - Maternal uterine artery embolisation reduced fetal arterial glucose, and increased fetal arterial pCO₂

Fetal arterial pH (A), PaO₂ (B), PaCO₂ (C), and glucose concentrations (D) during maternal uterine artery embolisation and treatment periods. Median ± interquartile range. For the embolisation period, IUGR + V and IUGR + SC are combined into a single IUGR group (n = 15-18) vs control (n = 5-6). For the treatment period, control (n = 1-6) vs IUGR + V (n = 4-8) vs IUGR + SC (n = 4-9). * p < 0.05, † p <0.01 vs control (Mann-Whitney Test); ‡ p < 0.05 for IUGR + V vs control (Kruskal-Wallis with Dunn’s multiple comparisons test). ● control; ■ IUGR (IUGR + V for treatment period); ▲ IUGR + SC.
3.2.4.11 Umbilical artery blood flow

Mean umbilical artery RI did not differ amongst groups at any individual time point (figure 3A); however, the mean change in RI for each fetus from 107 dGA (end of embolisation) to 128 dGA (last measurement recorded) differed significantly amongst groups ($F(2,22) = 4.21$, $p = 0.03$) (figure 3B). Over this time period, RI fell significantly less during the treatment period in the IUGR+V group compared to controls (mean change in RI (107-128dGA) control vs IUGR+V vs IUGR+SC: -0.09 ± 0.03 vs 0.01 ± 0.02 vs -0.03 ± 0.02; $p = 0.03$). The change in RI for the lambs of sildenafil treated mothers did not differ significantly from either of the other two groups.

3.2.4.12 Ex vivo vascular function

There was no difference amongst groups in myometrial artery maximum constriction to PE, maximal relaxation to Ach or SNP or sensitivity to any of the 3 agents (as assessed by calculation of the $EC_{50}$; figure 4).
Figure 3.3 - Maternal uterine artery embolisation resulted in a reduced fall in umbilical artery vascular resistance that was partly ameliorated with sildenafil treatment

Fetal Umbilical artery RI fell as gestation advance (A). The fall in RI between the end of the embolisation period (107 dGA) and the last point measured (128 dGA) was significantly less for IUGR +V compared to controls ($p < 0.05$) (B). The IUGR+ SC group did not differ significantly from either of the other groups. Mean ± SEM, $n = 9, 8, 9$ (control, IUGR + V, IUGR + SC); *$p < 0.05$ vs control (1-way ANOVA with Tukey’s multiple comparisons test).

● control; ■ IUGR (IUGR + V for treatment period); ▼ IUGR + SC
**Figure 3.4 – Sildenafil did not significantly alter myometrial artery ex vivo function**

There were no differences amongst groups in maternal myometrial artery response to phenylephrine (A), acetylcholine (B) or sodium nitroprusside (C). Mean ± SEM, n = 3-5, 3-5, 4-7 (control, IUGR + V, IUGR + SC). ● control; ■ IUGR (IUGR + V for treatment period); ▼ IUGR + SC.
3.2.5 Discussion

Our study adds to the growing body of evidence that sildenafil can improve fetal growth, and suggests that changes in growth are at least partly mediated by increased placental growth and reduced fetal-placental vascular resistance, as opposed to changes in maternal myometrial resistance vessel function.

Lamb weights of the IUGR+V group were significantly less than control group, whereas lamb weight from the IUGR +SC group were not, suggesting maternal sildenafil administration had a beneficial effect on fetal growth. Our study was powered to detect a relatively large (25%) difference in fetal weight between vehicle and sildenafil treated groups; however, the difference in lamb weights in the IUGR+V and IUGR+SC group in our study was only 7%. Although this increase is modest, in the absence of any alternative therapies even a treatment that produced small increases in fetal weight would be clinically useful. Clinically, the babies at the greatest risk of serious morbidity and mortality are those that are the most growth restricted (Bukowski, Hansen et al. 2014). Perhaps most importantly, sildenafil reduced the proportion of fetuses with a weight less than the 5th centile of the control group, suggesting that treatment may have had a greater impact on the growth of the smallest fetuses.

Placental size is a determinant of adequate maternal-fetal exchange capacity, and small placentae are associated with both IUGR and adverse pregnancy outcomes (Toal, Keating et al. 2008, Higgins, Rey de Castro et al. 2015). Placentae from IUGR pregnancies show pathological findings of acute atherosis, thrombosis, obliteration of the maternal artery lumen, and infarction (Aardema, Oosterhof et al. 2001, Madazli, Somunkiran et al. 2003). Ovine uterine artery embolisation results in blockage of the small maternal arterioles supplying the placenta and results in placental infarction, and thereby provides a paradigm with relevance to human pregnancies affected by IUGR (Creasy, Barrett et al. 1972). We observed a large
drop in total placental weight in the IUGR+V group, whereas placental weight from the IUGR +SC was intermediate to the other two groups, implying that sildenafil increased placental growth. The weight of fetal tissue produced per unit of placental weight was similar amongst groups, consistent with findings in small animal studies (Dilworth, Andersson et al. 2013, Refuerzo, Sokol et al. 2006), suggesting that changes in fetal growth are due to an increase in placental mass rather than an increase in placental efficiency. We also observed a relative increase in number of placentomes, and an increase in proportion of smaller placentomes, in the sildenafil treated ewes. We speculate that this could represent sildenafil having a selective effect on growth at new or relatively underdeveloped sites of placentome attachment, as opposed to increased growth of all placentomes including those that are already well established. From the findings of the present study alone, it is not possible to determine the mechanism through which sildenafil might increase placental (and therefore fetal) weight. However, there is some evidence from in vitro and in vivo small animal work that sildenafil has a pro-angiogenic function: endothelial cells show increased endothelial cell proliferation migration and organisation following culture with sildenafil (Pyriochou, Zhou et al. 2007), and sildenafil promotes angiogenesis and increased blood flow in cardiomyocytes following cardiac ischemia reperfusion injury in rats (Koneru, Varma Penumathsa et al. 2008). These changes were associated with an upregulation of the vascular endothelial growth factor (VEGF) system – a family of growth factors which play a critical role in all stages of placental development. It is feasible that an upregulation of the VEGF system in the placenta could promote angiogenesis, and result in the increase in placental mass observed in this study.

Our other finding of note is the changes in umbilical artery RI; these suggest that maternal sildenafil administration reduced feto-placental resistance. In human pregnancies affected by IUGR, a plateau or increase in the umbilical artery RI is a marker for fetal compromise and correlates with pathologic placental findings such as reduced volume of intermediate and
terminal villi, reduced number and diameter of fetal capillaries (Giles, Trudinger et al. 1985, Kuzmina, Hubina-Vakulik et al. 2005). In this study, the fall in RI was greatest in the control group and least for the IUGR+V group, with IUGR+SC RI intermediate to the other groups, suggesting that sildenafil may help normalise placental resistance within the fetal – placental circulation. Similar observations have been made in murine studies, where pathologic UmA resistance indices were normalised when dams were treated with sildenafil during pregnancy (Stanley, Andersson et al. 2012). The mechanism behind a reduction in fetal-placental resistance is not clear, but could arise from either an increased sensitivity of the feto-placental vasculature to dilators or via a pro-angiogenic effect on the fetal vasculature, resulting in increased villous growth.

Contrary to our hypothesis, we did not observe an alteration in myometrial resistance vessel sensitivity to endothelium-dependent vasodilators. This is similar to observations in a small study of preeclamptic women (Samangaya, Mires et al. 2009), but differs from that observed in mice (Stanley, Andersson et al. 2012). It is possible that sildenafil may have an effect on resistance vessel function that subsides as tissue levels of sildenafil fall. The half-life of sildenafil is relatively short (approximately 4 hours in humans); median collection time for arteries was over 5 hours after last treatment administration in the human study, and over 24 hours in our study. This is in contrast to the murine study where treatment (administered in drinking water) was available up to the point of tissue collection.

Our results differ somewhat from the only other published report of chronic maternal sildenafil exposure in pregnant sheep. Satterfield et al. (2010) used caloric restriction to induce growth restriction, treating pregnant ewes with sildenafil in thrice daily subcutaneous injections from 28 – 115 dGA. A 14% increase in fetal growth was seen with sildenafil treatment 150 mg/day, with no difference in placental weights between groups (Satterfield,
Bazer et al. 2010). The difference in findings between our studies may be consequent upon the different methods of inducing growth restriction, durations of treatment (87 days in Satterfield et al. (2010) vs 21 days in our study), or the different timings of treatment in relation to ovine placental development. It is also possible that the slow subcutaneous infusion used in our experimental design did not result in sufficiently high peak plasma sildenafil concentrations for maximum effect. Irrespective of these differences, in the context of developing treatments for human pregnancy, it is worthwhile noting that neither study has shown a detrimental effect on fetal growth or fetal organ development. In particular, our study did not show a detrimental effect on fetal oxygenation. This is in contrast to the observed reduction in fetal oxygenation and MAP accompanying a reduction in uterine blood flow seen in another study following a large intravenous bolus of sildenafil in the pregnant sheep (Miller, Loose et al. 2009).

PDE-5 is expressed in tissues other than the uterus (Lin, Lin et al. 2006), and the effect of sildenafil on other organ systems should also be considered. In women, reduced plasma volume increase is associated with IUGR (Salas, Rosso et al. 1993, Hytten, Paintin 1964, Pirani, Campbell et al. 1973, Hays, Cruikshank et al. 1985). A selective increase in PDE-5 activity has been demonstrated in the inner renal medulla of the pregnant rat (Knight, Snellen et al. 2007, Ni, Safai et al. 2004), where it appears to play an important role in increasing sodium and water retention, resulting in increased plasma volume. In rodents, renal infusion of sildenafil has been associated with blunted anti-natriuresis and increased diuresis (Ni, Safai et al. 2004), and oral administration has been associated with reduced maternal plasma volume (Sasser, Baylis 2010). Changes in blood and plasma volumes are minimal in normal ovine gestation (Rumball, Bloomfield et al. 2008), so the changes described in rats may not be present in sheep. However, as we did not measure the ewe’s blood or plasma volume during this study we are unable to clarify the effect of chronic sildenafil administration on maternal
fluid homeostasis. Any future studies should consider incorporating measurements of blood or plasma volume to clarify this point.

3.2.6 Perspectives

This study suggests that maternal sildenafil treatment may be associated with a small increase in fetal weight and a modest increase in placental weight in fetal lambs where IUGR has been induced by uterine artery embolisation. Treatment with sildenafil was not associated with changes in myometrial resistance artery function, but was associated with a fall in umbilical artery resistance indices, suggesting increased fetal-placental perfusion. These findings, together with the absence of detrimental effect on organ growth, suggest that sildenafil may be a useful adjunct to the management of growth restricted pregnancies.

3.2.7 Acknowledgements

We gratefully acknowledge the technical assistance of the Auckland University research farm technicians Travis Gunn, Maggie Worthington, Gregg Pardoe and Anita Wylie.

Sources of funding

This research was supported by GRAVIDA: National centre for growth and development scholarship, and funding from the Mercia Barnes Trust.

3.2.8 Novelty and Significance

3.2.8.1 What is new?

Rodent studies have suggested a positive effect of the phosphodiesterase inhibitor sildenafil citrate on fetal growth, but the mechanism(s) of action remain unclear. Furthermore, the duration of treatment in these studies was considerably less than would pertain in human pregnancies, so a continued beneficial effect and the absence of any detrimental effects on
organ growth and development have not been proven. We have used a clinically relevant paradigm of IUGR to show a positive effect of sildenafil on fetal growth over a longer (and clinically appropriate) timeframe, and for the first time, we describe effects of medium-term sildenafil exposure on fetal-placental blood flow and myometrial resistance vessel function.

3.2.8.2 What is relevant?

There are no clinically available treatments that can improve intrauterine growth. This study provides important evidence in support of the efficacy and safety of sildenafil as a new treatment for intrauterine growth restriction.

3.2.9 Summary

In pregnant ewes, uterine artery embolisation resulted in reduced fetal and placental weights, and an increase in fetal-placental vascular resistance (as assessed by Doppler Ultrasound). These changes were all partially ameliorated when ewes were treated with sildenafil. Furthermore, no changes in resistance vessel function were observed, suggesting that changes in growth are at least partly mediated by increased placental growth and reduced fetal-placental vascular resistance, as opposed to changes in maternal myometrial resistance vessel function.
3.3 Supplementary material

<table>
<thead>
<tr>
<th>Day gestational age</th>
<th>Control</th>
<th>IUGR + V</th>
<th>IUGR + SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>107</td>
<td>983</td>
<td>1090</td>
<td>1159</td>
</tr>
<tr>
<td>(n = 1)</td>
<td>(674-1627)</td>
<td>(745-1574)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>119</td>
<td>2618</td>
<td>1161</td>
<td>1459</td>
</tr>
<tr>
<td>(n = 1)</td>
<td>(728-1593)</td>
<td>(797-2634)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>128</td>
<td>2439</td>
<td>914</td>
<td>1681</td>
</tr>
<tr>
<td>(n = 1)</td>
<td>(n = 1)</td>
<td>(1649-1925)</td>
<td>(n = 3)</td>
</tr>
</tbody>
</table>

Table 3.3 - Maternal uterine artery blood flow (mL/min)

Data shown are median (range) or individual values.

3.3.1 Discussion of supplementary material

Due to occlusions of the utero-ovarian venous catheters, it was only possible to obtain data on uterine artery blood flow for a limited number of ewes, and it is not possible to statistically analyze these data (Table 3.3). In future studies, the use of implantable uterine artery flow transducers could be considered to maximise the chance of accurately quantifying uterine artery blood flow across gestation (Abi-Nader, Mehta et al. 2010).
4 Sildenafil citrate increases fetal growth in mice, independent of uterine artery function

4.1 Preface

The following chapter consists of the manuscript “Sildenafil citrate increases fetal growth in mice, independent of uterine artery function” and associated supplementary material. This manuscript was submitted to the journal *Scientific Reports* in November 2016, and at the time of thesis submission was under peer review.

For the purpose of this thesis, the manuscript has been reordered so that the methods section is now placed second (4.2.3), and reformatted so that it is consistent with the rest of the thesis. The written content including text, figures and tables remains unaltered from that submitted for review. It includes some repetition of experiment methodology that has been reported in expanded form in chapter 2 of this thesis. In addition to the submitted manuscript and supplementary material, this chapter also contains original and unpublished findings on the effect of sildenafil treatment on maternal blood pressure and mesenteric vessel function (section 4.4).

4.1.1 Rationale

Endothelial dysfunction occurs in the myometrial or uterine resistance arteries from IUGR pregnancies and may contribute to the increased uterine artery vascular resistance and reduced placental perfusion seen in these pregnancies (Wareing, Myers et al. 2005b, Ong, Moore et al. 2003). In the previous chapter, in an ovine model of IUGR where IUGR was induced by uterine artery embolisation, lambs from embolised mothers treated with sildenafil had a mean weight intermediate to those born to embolised then vehicle treated and normal growth control groups, suggesting a possible beneficial effect of sildenafil on fetal growth. Despite these
findings, there were no changes in myometrial artery ex vivo function. One limitation of the ovine study was that unlike human IUGR, myometrial vessels from growth restricted ovine pregnancies did not exhibit a change in vascular function compared to those taken from normal pregnancies. As it is possible that sildenafil may only improve vasodilatation in arteries where there is impaired endothelial function, it is necessary to study the effect of sildenafil on small artery function in a different model. Vascular dysfunction has been demonstrated previously in two gene-knockout mouse strains that have growth restricted offspring: the endothelial nitric oxide synthase knockout (eNOS−/−; (Kulandavelu, Whiteley et al. 2012, Kusinski, Stanley et al. 2012)) and the catechol-O-methyltransferase knockout (COMT−/−; (Stanley, Andersson et al. 2012)). Therefore, the experiments described in this chapter used these strains in order to determine whether sildenafil can ameliorate pre-existing endothelial dysfunction in the uterine arteries, and improve fetal growth.

Maternal obesity is associated with a generalised endothelial dysfunction (Jonk, Houben et al. 2007), and this includes endothelial dysfunction of the myometrial arteries (Myers, Hall et al. 2006). Endothelial dysfunction in myometrial resistance arteries may partly contribute to the increased rates of IUGR seen in the pregnancies of obese women (Anderson, Sadler et al. 2013). Therefore, in an attempt to exacerbate both uterine artery endothelial dysfunction and worsen the IUGR phenotype seen in the gene knock-out strains, maternal weight gain was induced pre-pregnancy with a high fat diet. Maternal obesity has undergone a dramatic increase in global prevalence (Poston, Caleyachetty et al. 2016), and contributes increasingly to the burden of pregnancy complications including IUGR. Therefore, investigating the effect of sildenafil on fetal growth in a maternal obesity paradigm is both important and extremely clinically relevant.
4.2 Sildenafil citrate increases fetal growth in mice, independent of uterine artery function

4.2.1 Abstract

Intrauterine growth restriction (IUGR) is associated with short and long term adverse health consequences. Sildenafil citrate (sildenafil) is a phosphodiesterase-5 inhibitor which has been shown to improve fetal growth, possibly through vasodilatation and increased blood flow in the uterine arteries. However, there is minimal published evidence to support this mechanism.

To test the hypothesis that sildenafil increases fetal growth through increased uterine artery endothelium-dependent vasodilatation, we used murine knockout models (endothelial nitric oxide synthase (eNOS\textsuperscript{-/-}) and catechol-O-methyltransferase (COMT\textsuperscript{-/-}) knockout) in combination with high-fat diet (HFD) induced maternal weight gain. We hypothesised that maternal HFD induced weight gain would further impair endothelium-dependent vasodilatation in the maternal uterine artery and reduce fetal growth, and that sildenafil treatment administered from 12.5-18.5 days gestational age would reverse these changes.

Pups were lighter when from eNOS\textsuperscript{-/-} strain or HFD fed mothers, and sildenafil treatment increased fetal weight. However, neither diet nor sildenafil altered endothelium-dependent relaxation in the uterine artery, suggesting that changes in growth were mediated by another mechanism. Sildenafil treatment increased placental gene expression of VEGFR-2 and altered expression of inflammatory mediators TNF\textalpha, IL-1\beta and IL-6.

These findings suggest sildenafil increases fetal growth through mechanisms other than altered uterine artery function.
4.2.2 Introduction

Intrauterine fetal growth restriction (IUGR) occurs when a fetus fails to grow to its full genetic potential. These pregnancies are at greater risk of serious perinatal morbidity and mortality (Simchen, Beiner et al. 2000, Bernstein, Horbar et al. 2000), and those born growth restricted have an increased risk of adverse health consequences in later life (Barker DJ 2004). Although there are no clinically available treatments proven to improve fetal growth, several agents – such as sildenafil citrate (von Dadelszen, Dwinnell et al. 2011), melatonin (Richter, Hansell et al. 2009), and HMG-CoA reductase inhibitors (Costantine 2016) – appear to be promising candidates. Of these, sildenafil citrate (sildenafil) is the most widely / comprehensively studied.

Sildenafil is a phosphodiesterase inhibitor, which inhibits the breakdown of nitric oxide second messenger cyclic guanosine monophosphate (cGMP). As cGMP plays a key role in mediating relaxation of vascular smooth muscle, increased tissue exposure to cGMP enhances vasodilatation and blood flow. IUGR is associated with both increased resistance to blood flow in the uterine arteries (Ferrazzi, Rigano et al. 2011), and an increase in constriction and impaired sensitivity to vasodilatation in the maternal myometrial (uterine) small arteries that supply the placenta (Wareing, Myers et al. 2005a). It has been hypothesised that the vasodilatory action of sildenafil could ameliorate these defects and improve fetal growth. Indeed, when administered in pregnancy, sildenafil appears to increase fetal growth in a variety of animal models (Stanley, Andersson et al. 2012, Oyston, Stanley et al. 2016, Satterfield, Bazer et al. 2010). Early non-randomised studies in human IUGR pregnancies also appear promising (von Dadelszen, Dwinnell et al. 2011), and a network of multicentre randomised trials are underway, to determine whether beneficial effects on fetal growth (and other outcomes) are elicited (Ganjevoort, Alfirevic et al. 2014).
Whilst results from randomised clinical trials to assess the efficacy of sildenafil as a treatment for IUGR are awaited, it is important to consider the mechanisms through which sildenafil may increase growth. Greater knowledge of these mechanisms will assist the translation from animal studies to a clinical treatment, with the potential to optimise timing and application of the treatment. Early studies demonstrated that ex vivo myometrial small arteries showed increased sensitivity to endothelium-dependent vasodilators following pre-incubation with sildenafil (Wareing, Myers et al. 2005a). However, this effect does not always translate to altered function when sildenafil exposure occurs in vivo (Samangaya, Wareing et al. 2011, Oyston, Stanley et al. 2016). Consequently, the first aim of this study was to assess the effect of sildenafil on vascular function of the uterine artery, using mouse models of IUGR that are associated with utero-placental vascular insufficiency. We hypothesised that maternal sildenafil treatment in pregnancy would increase endothelial dependent vasodilatation, and increase fetal growth.

There are many risk factors for IUGR, however, maternal obesity (maternal body-mass index of >30 kg/m²) (Anderson, Sadler et al. 2013, Gardosi, Francis 2009) holds particular clinical significance due to its increasing global prevalence (Poston, Caleyachetty et al. 2016). The mechanism whereby increased maternal weight contributes to impaired fetal growth is unclear, however, endothelial dysfunction has been implicated. Impaired vasodilatation in the myometrial resistance vessels of obese pregnant women (Myers, Hall et al. 2006) is reminiscent of that seen in IUGR pregnancies (Wareing, Myers et al. 2004). If the same effect pertains in obese pregnant women, then sildenafil could reverse this dysfunction and ameliorate the burden of IUGR associated with obesity. Therefore, a second aim of our study was to assess the impact of a high-fat diet induced maternal weight gain on both fetal growth and maternal uterine artery function. We hypothesised that high fat diet induced maternal
weight gain would be associated with both impaired maternal uterine artery function and reduced fetal growth, and that these changes could be reversed with sildenafil treatment.

4.2.3 Methods

4.2.3.1 Ethics statement
All experiments were conducted in accordance with the University of Auckland code of ethical conduct and the New Zealand Animal Welfare Act (1999). All of the protocols used were approved by The University of Auckland Animal ethics committee (Ref 001097; Appendix C).

4.2.3.2 Animal care
Female mice from control C57BL/6J (purchased from Jackson Laboratories, Bar Harbor, Maine, USA), catechol-O-methyl-transferase knockout (COMT<sup>-/-</sup> obtained under a material transfer agreement from Professor J Gogos Columbia university), and endothelial nitric oxide synthase knockout (eNOS<sup>-/-</sup>; purchased from Jackson laboratories) strains were placed on a high fat diet (HFD - 45% kcal from fat; D12451, Research Diets Inc., New Brunswick, New Jersey USA) or continued on a standard laboratory rodent chow (normal diet – ND - 4% kcal from fat) for at least four weeks prior to mating. Mice had ad libitum access to water and food. The animal laboratory was humidity-controlled with a temperature of 20°C and a 12 hr light-dark cycle.

Females were placed in cages overnight with males of the corresponding genotype, and checked the following morning; detection of a mucus plug denoted 0.5 days gestational age (DGA). At 12.5dGA, pregnant mice were randomly allocated to treatment with sildenafil citrate (+SC, Pfizer, New York, New York) 0.2 mg/ml in the drinking water, or control (+C, normal drinking water). Treatment was replaced with fresh solution every three days. The
treatment starting point was chosen on the basis that the basic structure of the definitive placenta is present, the placenta is fully functional and the utero-placental circulation open (Adamson, Lu et al. 2002).

4.2.4  Fetal pup measurements

At 18.5 dGA mice were euthanised by cervical dislocation. The maternal uterus and mesentery were dissected out immediately and placed in ice cold physiologic (PSS; in mmol/L: NaCl 142, KCl 4.7, CaCl2 4.7, MgSO4 1.17, HEPES 10, KH2PO4 1.18 and glucose 5.5; pH 7.4). Pups and placentas were dissected from the uterus, and the umbilical cord cut close to the fetal abdomen. Placentae were trimmed to remove the umbilical cord and membranes, then one placenta was selected at random, snap frozen and stored at -80°C for future analysis. The pups and placentae were blotted dry, sexed, weighed and measured.

4.2.5  Ex vivo vascular function.

The maternal main loop uterine artery was dissected out and cleaned of fat and connective tissue. Vessel segments approximately 2 mm in length were mounted using 20 µm tungsten wire on a multi wire myograph (610M Danish Myo Technology, Aarhus, Denmark), connected to a Powerlab and computer with all experiments recorded using LabChart software (ADI Instruments, Dunedin, New Zealand).

All chemicals used were purchased from Sigma Aldrich (Sigma Aldrich, Auckland, NZ). Prior to dose-response experiments, vessel viability and endothelial integrity was assessed using a standardised protocol as previously described (Stanley, Sankaralingam et al. 2010). Vascular function was assessed by determining dose-responses to vasoconstrictors phenylephrine (Pe), then endothelium-dependent vasodilator acetylcholine (ACh), and finally endothelium-independent vasodilator and NO donor sodium nitroprusside (SNP). Vessels
were washed and equilibrated for 20 minutes in fresh PSS between each experiment. For assessment of vasodilatation, the constriction-response curve was used to estimate the EC\textsubscript{80} for each vessel, and this dose used to obtain a stable constriction before starting relaxation dose-response experiments.

### 4.2.6 RNA extraction and real-time PCR

Total RNA was isolated from frozen placental tissue using high throughput disruption via TissueLyzer (QIAGEN, Hilden, Germany) and TRIZOL® reagent (Life Technologies, Carlsbad, CA) as per manufacturer’s instructions. Total RNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer (Thermoscientific, USA). RNA purity was considered acceptable if absorbance ratios 260/280 and 280/230 were between 1.8 and 2.1. RNA integrity was assessed by a TAE-based agarose gel electrophoresis; the presence of two distinct bands (28S and 18S rRNA) with the absence of smearing indicating high-quality RNA. Samples that produced smeared bands (indicating RNA degradation) were excluded from further study.

RNA was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) then reverse transcription was performed with the high capacity cDNA reverse transcription kit (Life Technologies), using 1 µg of total RNA for each preparation. The resulting cDNA was diluted 10 fold and stored at -20\textdegree C until required.

Gene expression was analysed by Quantitative Real-Time PCR (qRT-PCR) using the QuantStudio\textsuperscript{TM} 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA) and SYBR® Select Master mix (Thermo Fisher Scientific). Specific primer sequences for the target genes are included as supplementary material. Each reaction was performed in triplicate.
and the average transcript quantity in placental tissues was calculated using the relative standard curve and delta-delta CT method, normalised to the expression of *CHMP2a*.

### 4.2.7 Statistical analysis

Statistical analysis was performed using IBM SPSS statistics v22. Data are reported as mean ± SEM, or as back-transformed mean (95% confidence intervals) if a transformation had been applied. P-values are reported with statistical significance accepted at the *p* < 0.05 level. All graphed data depicts untransformed values, or back-transformed means with back-transformed upper limit of the SEM. For all significant findings, the *F* statistic is reported separately in Appendix E.

Data were visually inspected on a boxplot, and extreme outliers (lying more than 3 interquartile-ranges from the median) were replaced with imputed values from the next highest or lowest value as appropriate. Normality of mean residuals were assessed with the Shapiro-Wilk test, and square-root or log transformations were applied where necessary to ensure a normal distribution. In three instances, the normality of residuals could not be achieved; this was for the COMT+/− HFD +SC group in the analysis of fetal weight and fetal crown-rump length and the eNOS+/− HFD+SC group for the analysis of maximum uterine artery constriction to Pe. In all cases, the deviation from the mean appeared mild, so we proceeded with analysis, as ANOVA is considered reasonably robust to deviations from normality. Box-plots of mean residuals from these three groups are included in Appendix D.

A three-way ANOVA was conducted to assess the main effects and the presence of 2- or 3-way interactions between the independent variables strain (C57BL/6J vs eNOS+/− vs COMT+/−), diet (normal diet vs high fat diet), and treatment (control vs sildenafil treatment). Where a statistically significant 3-way interaction was identified, this was followed by testing
for a simple 2-way interaction between diet and treatment at the level of strain. Where there were no significant 3-way interaction, but a significant (or trend toward) a 2-way interaction, this was followed by testing for simple main effects. All pairwise comparisons were made using a Bonferroni adjustment.

4.2.8 Results

4.2.8.1 Maternal weight, litter size and sildenafil intake

One-hundred and twenty mice were studied, with experimental groups of $n = 7$-$13$. Mice fed a high-fat diet (HFD) were heavier at the beginning of pregnancy (ND vs HFD: $21.5 \pm 0.4$ vs $25.2 \pm 0.4g, p < 0.0005$); neither strain nor treatment affected maternal weight. eNOS$^{−/−}$ mice had a smaller litter size than the other strains (C57BL/6J vs eNOS$^{−/−}$ vs COMT$^{−/−}$: 8 vs. 6 vs 7 pups/litter, $p = 0.0008$); diet and treatment did not affect litter size. The C57BL/6J strain consumed less sildenafil treatment compared to other groups (C57BL/6J vs eNOS$^{−/−}$ vs COMT$^{−/−}$: $0.13 (0.11 - 0.14)$ vs $0.16 (0.15 - 0.17)$ vs $0.16 (0.14 - 0.18)$ mg/day $p = 0.005$), but intake was not affected by diet.

The effects of strain, diet and treatment on fetal growth were similar for males and females, therefore data were combined for the main results section, with sex-specific findings included as supplementary material.

4.2.8.2 Effects of strain

4.2.8.2.1 Uterine artery ex vivo function

Dose-response curves are shown in Figure 4.1 with summary statistics in Table 4.1. The uterine arteries from eNOS$^{−/−}$ pregnancies exhibited lower maximum constriction ($p < 0.0005$) and were less sensitive to vasoconstrictor phenylephrine (Pe; $p < 0.0005$). They also showed impaired relaxation ($p < 0.0005$) and a reduced sensitivity to endothelium-dependent
vasodilator acetylcholine (ACh; \( p < 0.0005 \)), but an increased sensitivity to endothelium-independent vasodilator sodium nitroprusside (SNP; \( p < 0.0005 \)).

4.2.8.2.2 Fetal and placental growth

Compared to the other strains eNOS\(^{-/-}\) pups were significantly lighter \( (p < 0.0005) \) and had lighter placentae \( (p < 0.0005) \); fetal: placental ratio was unaltered (Table 4.1).
Figure 4.1 - Strain, but not diet or treatment, altered uterine artery ex vivo function

Uterine arteries from eNOS−/− mice showed reduced constriction and sensitivity to vasoconstrictor Pe (A), reduced maximum relaxation and reduced sensitivity to endothelium-dependent vasodilator ACh (B). Diet and sildenafil treatment had no effect on the maximum constriction or relaxation of vessels, or the sensitivity of the uterine artery to constricting or relaxing agents. Graphs show dose response curves (mean ± SEM) for Pe (A) and ACh (B) in mouse uterine arteries. For Pe curves, n (C57, eNOS−/−, COMT−/−) = 6-10, 8-12, 7-8. For ACh curves, n (C57, eNOS−/−, COMT−/−) = 6-10, 6-10, 6-8: • ND; □ HFD; — control; ---- sildenafil treatment.
<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>COMT&lt;sup&gt;+-&lt;/sup&gt;</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal and placental measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Abdominal circumference (mm)</td>
<td>24.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>29.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.077 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.069 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.083 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Fetal: placental ratio</td>
<td>13.6 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.9 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Uterine artery <em>ex vivo</em> function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constriction&lt;sub&gt;max&lt;/sub&gt; Pe (mN)</td>
<td>6.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; Pe (μMol/L)</td>
<td>0.4</td>
<td>2.2</td>
<td>1.1</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Relaxation&lt;sub&gt;max&lt;/sub&gt; ACh (%)</td>
<td>94 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>ACh EC&lt;sub&gt;50&lt;/sub&gt; (μMol/L)</td>
<td>43</td>
<td>109</td>
<td>17</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Relaxation&lt;sub&gt;max&lt;/sub&gt; SNP (%)</td>
<td>91 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td>SNP EC&lt;sub&gt;50&lt;/sub&gt; (μmol/L)</td>
<td>76 (51 - 105)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 (10 - 36)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51 (30 - 78)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Table 4.1 - eNOS<sup>-/-</sup> mice had smaller pups with smaller placentae, and demonstrated altered uterine artery function*

Data presented are mean ± SEM or back-transformed mean (95% confidence intervals), p calculated using 3-way ANOVA and describes the simple main effect of strain. <sup>a,b,c</sup> values containing the same superscript are not statistically different.
4.2.8.2.3 Placental gene expression

GLUT4 mRNA expression was upregulated in COMT<sup>-/-</sup> mice compared to C57BL/J6 (<i>p</i> = 0.003) or eNOS<sup>-/-</sup> (<i>p</i> = 0.009) (Figure 4.2).

![Graph showing GLUT4 expression in C57, eNOS<sup>-/-</sup>, and COMT<sup>-/-</sup> mice.

**Figure 4.2 - COMT<sup>-/-</sup> mice had increased placental GLUT4 expression compared to mice from the other strains.**

Graph shows back-transformed mean and upper limit of SEM for GLUT4 mRNA expression normalised to CHMP2A. n (c57, eNOS<sup>-/-</sup>, COMT<sup>-/-</sup>) = 39, 41, 31. **<i>p</i> < 0.01 (3-way ANOVA, significant main effect of strain).
4.2.9 Effects of diet

4.2.9.1.1 Uterine artery ex vivo function

Uterine artery response to Pe, ACh or SNP was not affected by maternal diet (Table 4.2).

4.2.9.1.2 Fetal and placental growth

Pups born to mothers on HFD were lighter than those fed ND ($p < 0.0005$), with a smaller abdominal circumference ($p < 0.0005$) and a shorter crown-rump length ($p < 0.0005$). There was no effect of HFD on average placental weight or fetal: placental ratio (Table 4.2).

4.2.9.1.3 Placental gene expression

mRNA expressions of $IL-6$ ($p = 0.007$) $TLR-4$ ($p = 0.01$) and $PIGF$ ($p < 0.0005$) were upregulated in response to HFD (Table 4.3).
<table>
<thead>
<tr>
<th></th>
<th>Normal diet</th>
<th>High fat diet</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal and placental measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.04 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Abdominal circumference (mm)</td>
<td>24.5 ± 0.2</td>
<td>23.5 ± 0.2</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>29.5 ± 0.2</td>
<td>28.6 ± 0.2</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.078 ± 0.002</td>
<td>0.074 ± 0.002</td>
<td>NS</td>
</tr>
<tr>
<td>Fetal: placental ratio</td>
<td>13.3 ± 0.4</td>
<td>12.8 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Uterine artery ex vivo function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constriction_{\text{max}} to Pe (mN)</td>
<td>6.15 (5.66 – 6.67)</td>
<td>5.79 (5.28 – 6.32)</td>
<td>NS</td>
</tr>
<tr>
<td>EC_{50} Pe (\mu\text{Mol/L})</td>
<td>0.9 (0.7 – 1.3)</td>
<td>1.1 (0.8-1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Relaxation_{\text{max}} ACh (%)</td>
<td>85.9 ± 2.2</td>
<td>91.3 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>EC_{50} ACh (\eta\text{mol/L})</td>
<td>53 (37 – 75)</td>
<td>35 (25 – 49)</td>
<td>NS</td>
</tr>
<tr>
<td>Relaxation_{\text{max}} SNP (%)</td>
<td>96 ± 2</td>
<td>91 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>EC_{50} SNP (\eta\text{mol/L})</td>
<td>44 (29 – 63)</td>
<td>48 (32 – 67)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 4.2 - Maternal high fat diet reduced fetal size but did not alter uterine artery ex vivo function**

Data presented are mean ± SEM or back-transformed mean (95% confidence intervals).

\( p \) calculated using 3-way ANOVA and describes the simple main effect of diet.
Figure 4.3 - HFD was associated with upregulation of expression of PIGF, TLR-4 and IL-6 in the placenta

Graphs show back transformed data (mean and upper limit of SEM) for simple main effect of diet on placental mRNA expressions of PIGF, IL-6 and TLR-4 normalised to CHMP2A. n (ND, HFD) = 58, 53. * p < 0.05; ** p < 0.01; *** p < 0.0005 for significant main effect of diet. ● ND; □ HFD.
4.2.10 Effects of sildenafil

4.2.10.1.1 Uterine artery ex vivo function

Uterine artery response to Pe, ACh and SNP was not affected by maternal sildenafil treatment (Table 4.3).

4.2.10.1.2 Pup and placental growth

Sildenafil treatment was associated with increased pup weight \( (p = 0.0008) \), crown-rump length \( (p = 0.006) \), and abdominal circumference \( (p = 0.011; \) Table 4.3). For all growth measures, the \( p \)-value for a 2-way interaction between diet and treatment fell short of statistical significance (diet-treatment interaction for fetal weight, crown-rump length and abdominal circumference: \( p = 0.056, p = 0.065, p = 0.061 \)). However, when dietary groups were separated, sildenafil significantly increased fetal weight in dams fed a HFD \( (p = 0.001) \) but not the ND group \( (p = 0.6; \) Figure 4.4). Similarly, sildenafil increased crown-rump length \( (p = 0.001) \) and abdominal circumference \( (p = 0.002) \) in HFD but not ND groups (figures included as supplementary material) suggesting that a diet-treatment interaction was present.

The 5\textsuperscript{th} centile for fetal weight of the designated control group C57BL/6J mice on a normal diet receiving normal drinking water was 0.938 g; any fetal pup with a weight below this value was categorised as IUGR. Sildenafil treatment was associated with a reduction in the proportion of IUGR fetuses in 4 out of 6 treatment-control comparisons. It should be noted, however, that the proportion of IUGR was increased in sildenafil treated COMT\textsuperscript{−/−} mice fed a normal diet (Figure 4.5).
### Table 4.3 - Sildenafil increased fetal size but did not alter uterine artery ex vivo function

There was a significant main effect of sildenafil on fetal weight, abdominal circumference and crown-rump length. Placental growth and uterine artery ex vivo function was unaltered. Data presented are mean ± SEM or back-transformed mean (95% confidence intervals). \( p \) calculated using 3-way ANOVA and describes the simple main effect of sildenafil; * indicates trend towards a statistically significant diet-treatment interaction (3-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sildenafil</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal and placental measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)*</td>
<td>0.96 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>(&lt; 0.001)</td>
</tr>
<tr>
<td>Abdominal circumference (mm)*</td>
<td>23.7 ± 0.2</td>
<td>24.3 ± 0.2</td>
<td>(0.011)</td>
</tr>
<tr>
<td>Crown-rump length (mm)*</td>
<td>28.7 ± 0.1</td>
<td>29.3 ± 0.2</td>
<td>(0.006)</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.076 ± 0.002</td>
<td>0.077 ± 0.002</td>
<td>(NS)</td>
</tr>
<tr>
<td>Fetal: placental ratio</td>
<td>12.8 ± 0.4</td>
<td>13.4 ± 0.4</td>
<td>(NS)</td>
</tr>
<tr>
<td><strong>Uterine artery ex vivo function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constriction(_{max}) Pe (mN)</td>
<td>5.84 (5.33 –</td>
<td>6.11 (5.61 – 6.63)</td>
<td>(NS)</td>
</tr>
<tr>
<td></td>
<td>6.36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC(_{50}) Pe ((\mu)mol/L)</td>
<td>1.0 (0.7 – 1.4)</td>
<td>1.0 (0.7 – 1.5)</td>
<td>(NS)</td>
</tr>
<tr>
<td>Relaxation(_{max}) ACh (%)</td>
<td>88.5 ± 3.1</td>
<td>88.6 ± 3.1</td>
<td>(NS)</td>
</tr>
<tr>
<td>EC(_{50}) ACh ((\eta)mol/L)</td>
<td>38 (27 – 52)</td>
<td>49 (34 – 70)</td>
<td>(NS)</td>
</tr>
<tr>
<td>Relaxation(_{max}) SNP (%)</td>
<td>94 ± 2</td>
<td>93 ± 2</td>
<td>(NS)</td>
</tr>
<tr>
<td>SNP EC(_{50}) ((\eta)mol/L)</td>
<td>47 (31 – 65)</td>
<td>46 (30 – 65)</td>
<td>(NS)</td>
</tr>
</tbody>
</table>
There was a significant main effect of sildenafil on fetal growth. When dietary groups were separated, sildenafil treatment was associated with increased fetal weight in HFD (B), but not ND groups (A), suggesting a diet-treatment interaction was present. ** p < 0.01 effect of sildenafil at the level of HFD; ● ND; □ HFD; C control; SC sildenafil citrate.
Figure 4.5 - Sildenafil reduced rates of IUGR in 4 out of 6 study groups

IUGR calculated as a weight below the 5th centile of the C57BL/6J ND+C group (A) – was significantly reduced in C57BL/6J, eNOS−/−+HFD and COMT−/−+HFD groups (p value calculated for 2-tailed Chi-square test) (B). In COMT−/− ND mice, sildenafil was associated with an increased risk of IUGR.

4.2.10.1.3 Placental gene expression

Sildenafil treatment was associated with an upregulation of transcription of VEGFR-2 (p = 0.038) and TNFα (p = 0.037). Significant diet-treatment interactions were identified for 2 genes: there was a trend toward sildenafil-associated reduced mRNA expression of IL-1β in HFD (p = 0.056) but not ND groups (p = 0.35). Sildenafil treatment was associated with increased mRNA expression of IL-6 in ND (p = 0.02) but not HFD groups (p = 0.4; Figure 4.6).
Sildenafil increased placental mRNA expressions of VEGFR-2 and TNF-α (A, B). Sildenafil increased placental IL-6 expression in mice fed normal but not high fat diet (C). The mRNA expression of IL1-β was increased in high fat diet groups (D). The ANOVA statistic was significant for a diet-treatment interaction ($p < 0.05$), and there was a trend towards reduction of IL-1β mRNA expression in high fat fed mice in response to sildenafil ($p = 0.056$) (D). Graph shows back-transformed mean and upper limit of SEM for mRNA expression normalised to CHMP2A. $n$ (ND+C, ND+SC, HFD+C, HFD+SC) =29, 29, 26, 27. * $p < 0.05$ for significant simple main effect of sildenafil; ** $p < 0.05$ for effect of sildenafil in normal diet group. + $p = <0.05$ for simple main effect of diet (all analyses performed using 3-way ANOVA); ● ND; □ HFD; C control; SC sildenafil citrate.
4.2.11 Discussion

We have demonstrated an overall increase in fetal growth and a reduction in proportion of IUGR fetuses with maternal sildenafil treatment in pregnancy. This effect was present across strains, but appeared to be limited to groups where growth restriction had been caused or exacerbated by a maternal high fat diet prior to and throughout pregnancy. Contrary to our initial hypothesis, the changes in growth occurred in the absence of changes in uterine artery function. This suggests that high fat diet reduced and sildenafil increased fetal growth through a mechanism other than changes in uterine artery function.

Sildenafil has been shown to increase fetal growth in a range of animal models of IUGR (Satterfield, Bazer et al. 2010, Oyston, Stanley et al. 2016, Stanley, Andersson et al. 2012, Lopez-Tello, Arias-Alvarez et al. 2016, Dilworth, Andersson et al. 2013). The overarching hypothesis as to the mechanism underlying increased growth has been that sildenafil increases the myometrial small arteries sensitivity to nitric oxide (NO) induced vasodilatation, and that utero-placental blood flow is increased as a result. This hypothesis was supported by early ex-vivo work, where increased sensitivity to endothelium-dependent vasodilatation was observed in myometrial arteries from (human) IUGR pregnancies following incubation with sildenafil (Wareing, Myers et al. 2004). However, these observations have not been demonstrated consistently when sildenafil exposure has occurred in vivo. The lack of change in uterine artery function following treatment with sildenafil in our study echoes the lack of change seen in myometrial artery function with sildenafil treatment in sheep carrying growth restricted lambs (Oyston, Stanley et al. 2016) and in women with pre-eclampsia (Samangaya, Mires et al. 2009). Sildenafil has a reasonably short half-life (3-5 hours) and in both of the prior studies sildenafil dosing did not occur proximate to the time of the vessel study (>24 hours in the sheep and a median of 5.3 hours in the human study). Therefore, it is possible that any acute effects had dissipated by the time of vessel assessment. The present study has the advantage
that treatment was available for consumption until tissue collection, so it is more likely that our results reflect vessels that have been recently exposed to or are still under the influence of sildenafil at the time of assessment. Our results contrast with the findings in a similar study of COMT<sup>−/−</sup> mice, where sildenafil was associated with an increase in uterine artery sensitivity to the endothelium-dependent vasodilator methacholine (Stanley, Andersson et al. 2012). The reasons for this disparity are unclear; the sildenafil dosing regimen was the same in both studies, and there was no significant effect of diet or the COMT<sup>−/−</sup> strain on uterine artery sensitivity to ACh in the current study (suggesting that neither diet nor strain differences explain the disparity between the two studies). In this previous study, Doppler ultrasound assessments of blood flow were also reported, with uterine artery resistance and flow indices similar between experimental groups, implying that utero-placental resistance was unaltered. This suggests the changes observed in vasodilator sensitivity had minimal functional impact on blood flow; in this regard, we believe both this prior and the present study support the hypothesis that sildenafil results in increased fetal growth through a mechanism other than increased uterine artery dilatation.

In the present study, sildenafil had no effect on mRNA expressions of placental amino acid or glucose transporters. Although placental expression of these transporters following sildenafil treatment has not previously been reported, this finding is in keeping with lack of change in placental system A amino acid transport activity observed following maternal sildenafil treatment (Dilworth, Andersson et al. 2013). The changes observed in VEGF-R2 expression seen with sildenafil hint at a possible mechanism through which sildenafil may improve fetal growth. VEGFs are a family of glycoproteins that play an important role in the regulation of angiogenesis (the formation of new from pre-existing blood vessels) and vasculogenesis (formation of blood vessels <i>de novo</i>). VEGF-A is the major VEGF for angiogenesis, and stimulates cellular response by binding to one of two structurally similar
tyrosine kinase receptors – *VEGFR-1* (Flt-1) or *VEGFR-2* (KDR/Flk-1). Both *VEGFR-1* and –*R2* are required for normal development and angiogenesis. However, *VEGFR-2* appears to mediate the major growth actions of *VEGF-A*, with *VEGFR-1* having more of a modulatory role mediated through ligand-trapping and receptor hetero-dimerisation with *VEGFR-1* (Rahimi 2006). We identified an increase in *VEGFR-2* mRNA with sildenafil treatment, and suggest that this could represent a pro-angiogenic effect of sildenafil on the placenta. Interestingly, increased *VEGFR-2* expression has also been demonstrated following sildenafil treatment in lung tissue of rat pups following hyperoxic lung injury. In this model, *VEGFR-2* upregulation was associated with increased blood vessel density, and reduced inflammation (de Visser, Walther et al. 2009). The suggestion that sildenafil increases fetal growth through a pro-angiogenic effect on placenta is supported by several studies which have suggested an increase in placental growth following sildenafil treatment (Lopez-Tello, Arias-Alvarez et al. 2016, Pellicer, Herraiz et al. 2011, Herraiz, Pellicer et al. 2012, Oyston, Stanley et al. 2016), although this finding is certainly not consistent across all studies (Gillis, Mooney et al. 2016, Oyston, Stanley et al. 2016). Furthermore, studies in rabbits and mice have shown increased in labyrinthine vascularity (Lopez-Tello, Arias-Alvarez et al. 2016) or fetal vascularity (Motta, Grosso et al. 2015) and vascular density (Stanley, Andersson et al. 2012) within the placenta following sildenafil treatment, suggesting a pro-angiogenic or vasculogenic effect.

A general dysregulation of inflammatory pathways occurs in human pregnancy complicated by obesity, with activated macrophages accumulating within the placenta, and an increased expression of inflammatory molecules such as *TNF-α*, *IL-6* and *IL-1* (Yang, Li et al. 2016, Challier, Basu et al. 2008, Hauguel-de Mouzon, Guerre-Millo 2006). We observed an increase in placental expression of genes associated with inflammation in the HFD groups. It is plausible that increased inflammation impaired placental function, and contributed to the reduced fetal growth. We also observed a tendency for sildenafil to normalise the increase in
IL-1β expression observed in the HFD groups. The anti-inflammatory effects of sildenafil have been described previously in the lung (de Visser, Walther et al. 2009), and brain (Peixoto, Nunes et al. 2015). More recently, in a LPS model of early-pregnancy loss in mice, pre-treatment with sildenafil treatment was associated with reductions of IL-1β and TNF-α protein within the placenta (Luna, Nunes et al. 2015). Collectively, these results imply that modulation of inflammatory pathways represents a mechanism through which sildenafil improved fetal growth and may partly explain why there was a trend for stronger effect in the HFD groups (which displayed greater expression of genes associated with inflammation).

However, we also observed an increase in placental TNF-α expression following sildenafil therapy, and an increase in IL-6 limited to the ND groups treated with sildenafil. The increase in IL-6 may be directly linked to the increase in TNF-α, as TNF-α is known to induce secretion of pro-inflammatory cytokines including IL-6 (Prins, Gomez-Lopez et al. 2012). The rise in expression is at odds with observations in other rodents studies which have demonstrated reduced placental TNF-α following sildenafil treatment in a rat model of pre-eclampsia (Gillis, Mooney et al. 2016) and a mouse model of recurrent pregnancy loss (Luna, Nunes et al. 2015). There is no clear explanation as to why our results differ from previously published studies. Experiments were performed in each group contemporaneously and under identical conditions, so the possibility of additional stressors causing inflammation in these groups seems unlikely. We believe that the observed divergent effects of sildenafil on different inflammatory mediators emphasises the need to gain a better understanding the mechanisms through which this treatment works, and the effects that it has on placental function. This means that when translated to the clinical setting it is offered to those most likely to benefit and its use avoided in groups where it is more likely to cause harm.

Finally, a limitation of this study was the lack of growth restriction observed in the COMT+/− strain. We included this strain in the experimental design as it appeared to offer an opportunity
to study the effect of sildenafil in a model of IUGR that did not directly involve the NO pathway. The COMT−/− mouse has previously been shown to exhibit a preeclampsia-like phenotype, which included growth restricted pups, increased uterine artery constriction, increased fetal placental resistance, (Stanley, Andersson et al. 2012) as well as hypertension and proteinuria in late gestation (Kanasaki, Palmsten et al. 2008). Unfortunately, in this study, these features were not observed. The increased expression of GLUT4 in COMT−/− mice may partially explain the absence of IUGR in this strain, as an increase in the expression of this glucose transporter could result in an increase in substrate supply to the fetus. However, it is unclear why GLUT4 was upregulated, and this finding alone is unlikely to explain the lack of difference in blood pressure and uterine artery dysfunction the strain previously exhibited. It is interesting that sildenafil appeared to have a detrimental effect in the non-growth restricted COMT−/− mice, as in the previous study (where IUGR was present) it appeared to improve fetal growth (Stanley, Andersson et al. 2012). As discussed above, the potential for sildenafil to have an adverse effect on growth impresses the importance of understanding the mechanisms through which it is working.

The results from the first randomised clinical trials of sildenafil as a treatment for severe early onset IUGR are expected soon. If these studies show benefit, in the absence of any other available treatment options there is likely to be a push to extend the use of sildenafil to cases of growth restriction that are less severe, or for other purposes. A deeper knowledge of the effects of sildenafil may help clinicians avoid offering a treatment to groups of women where it may be potentially detrimental, and allow the targeting of a treatment - whose long-term effects on health are unclear - to a group which is most likely to gain significant short term benefits.
4.2.12 Conclusion

In this study we observed an overall increase in fetal growth with sildenafil treatment. Sildenafil did not alter uterine artery vascular function, but did upregulate placental mRNA expression of VEGFR-2. Sildenafil treatment was associated with a detrimental effect on fetal growth in one (non-growth restricted) group, and both increased and decreased placental mRNA expressions of inflammatory cytokines. This suggests a need for careful selection of candidates when translating this treatment into clinical practice, and the need for further research to elucidate the exact mechanisms of action to enable targeting of the treatment to those who will benefit most and avoid unintended harm.

4.2.13 Additional information (competing financial interests)

Nil to declare.
4.3 Supplementary material

4.3.1 S1. Sex specific differences in pup and placental growth

Pups were sexed at delivery, then weighed and measured; the average male and female weights and measures for each litter were then calculated. Litter averages were used to test for significant effects of strain, diet, treatment and the presence of interactions amongst these variables using 3-way ANOVA, as described in the main methods section.

4.3.1.1 Effects of strain

Effects of strain on fetal and placental measurements are summarised in Table 4.4. There was a significant simple main effect of strain on fetal growth for both males in females. Pups from the eNOS$^{-}$ strain were lighter ($p<0.0005$), with a shorter crown-rump length ($p<0.0005$ (males); $p=0.001$ (females)) compared to the other two strains. Main effects of strain on abdominal circumference in male pups could not be analysed due to strong skewing of data in four experimental groups. Female pups from eNOS$^{-}$ strains had a smaller abdominal circumference than females from other strains ($p<0.001$).

Placental weight was lighter in the eNOS$^{-}$ strain for both females and males. There was a significant strain effect on fetal: placental efficiency. Females, but not males, from the C57BL/6J strain had a higher fetal: placental ratio compared to other strains ($p=0.004$).
### Table 4.4 - Main effects of strain on fetal and placental variables, separated by sex

Data presented are mean ± SEM or back-transformed mean (95% confidence intervals), p calculated using 3-way ANOVA and describes the simple main effect of strain. Abdominal circumference data from males could not be analysed due to strong skewing of data in four experimental groups. a,b values containing the same superscript are not statistically different (p = 0.05)

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>COMT&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pup weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Female</td>
<td>1.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td><strong>Abdominal circumference (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Female</td>
<td>24.4</td>
<td>23.1</td>
<td>24.2</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(23.5 – 24.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(22.7 – 23.6)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(23.6 – 24.7)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Crown-rump length (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29.8 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Female</td>
<td>29.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Placenta (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.079 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.068 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.082 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Female</td>
<td>0.072 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.068 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.079 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>Fetal: placental ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13.9 ± 0.5</td>
<td>13.2 ± 0.5</td>
<td>13.2 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>15.3</td>
<td>12.9</td>
<td>12.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(14.1 – 16.4)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(11.7 – 14.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(11.2 – 14.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
4.3.1.2 Effects of diet

Effects of diet on fetal and placental measurements are summarised in Table 4.5. In both males and females, high fat diet reduced fetal weight ($p < 0.0005$), and crown-rump length ($p = 0.008$ (males); $p < 0.0005$ (females)). Abdominal circumference data from males could not be analysed; in females, high fat diet reduced fetal abdominal circumference ($p = 0.006$). High fat diet was associated with a reduced placental size in males ($p = 0.01$), but not females. There was no effect of diet on fetal: placental ratio in either sex.
<table>
<thead>
<tr>
<th></th>
<th>Normal diet</th>
<th>High fat diet</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pup weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.05 ± 0.01</td>
<td>0.97 ± 0.01</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Female</td>
<td>1.04 ± 0.02</td>
<td>0.92 ± 0.02</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td><strong>Abdominal circumference (mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Female</td>
<td>24.3</td>
<td>23.5</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>(23.9 – 24.7)</td>
<td>(23.1 – 23.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Crown-rump length (mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29.6 ± 0.2</td>
<td>28.9 ± 0.2</td>
<td>0.008</td>
</tr>
<tr>
<td>Female</td>
<td>29.5 ± 0.2</td>
<td>28.4 ± 0.2</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td><strong>Placenta (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.080 ± 0.002</td>
<td>0.073 ± 0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Female</td>
<td>0.074 ± 0.002</td>
<td>0.072 ± 0.002</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Fetal: placental ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13.2 ± 0.5</td>
<td>14.0 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>13.4 (12.3 – 14.4)</td>
<td>13.9 (12.9-14.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Table 4.5 - Main effects of diet on fetal and placental variables, separated by sex*

Data presented are mean ± SEM or back-transformed mean (95% confidence intervals), $p$ calculated using 3-way ANOVA and describes the simple main effect of strain. Abdominal circumference data from males could not be analysed due to strong skewing of data in four experimental groups.
4.3.1.3 Effects of treatment

Effects of sildenafil treatment on fetal and placental measurements are summarised in Table 4.6. There was a significant main effect of sildenafil increasing fetal weight in both males \((p = 0.01)\) and females \((p = 0.036)\); however this appeared to be limited to the high fat diet groups. There was a significant diet-treatment interaction for female mice (diet-treatment interaction \(p = 0.046)\). Sildenafil increased pup weight in high fat diet groups \((0.88 \pm 0.02 \text{ vs } 0.97 \pm 0.02 \text{ g}; p = 0.005)\), but not normal diet groups \((p = 0.9)\). Although the \(p\)-value for diet-treatment interaction was not significant in males \((p = 0.1)\), when groups were separated by diet, sildenafil increased weight in high fat diet groups \((0.91 \pm 0.02 \text{ vs } 1.01 \pm 0.02 \text{ g}; p = 0.005)\) but not normal diet groups \((p = 0.5)\), suggesting that as for females, a diet-treatment interaction was present. As before, abdominal circumference data from males could not be analysed; in females there was a significant main effect of sildenafil increasing fetal abdominal circumference \((p = 0.006)\). There was a significant main effect of sildenafil increasing crown-rump length in males. In females, sildenafil only increased crown-rump length in the eNOS\(^{-/-}\) HFD \((26.9 \pm 0.4 \text{ vs } 28.5 \pm 0.4 \text{ mm}; p = 0.011)\) and COMT\(^{-/-}\) HFD groups \((27.9 \pm 0.5 \text{ vs } 29.6 \pm 0.5 \text{ mm}; p = 0.018)\) (strain-diet-treatment interaction \(p = 0.031)\).

Sildenafil did not affect placental weight or efficiency in males or females.
Table 4.6 - Main effects of treatment on fetal and placental variables, separated by sex

Data presented are mean ± SEM or back-transformed mean (95% confidence intervals), p calculated using 3-way ANOVA and describes the simple main effect of strain. Abdominal circumference data from males could not be analysed due to strong skewing of data in four experimental groups.
4.3.2 Sildenafil increased fetal abdominal circumference and crown-rump length in high fat diet but not normal diet groups

When dietary groups were separated, sildenafil treatment was associated with increased fetal abdominal circumference (AC) in HFD (B), but not ND groups (A), suggesting a diet-treatment interaction was present. **p < 0.01 effect of sildenafil at the level of HFD. ● ND; □ HFD; C control; SC sildenafil citrate.

Figure 4.7 - Sildenafil increased fetal abdominal circumference in high fat diet groups
Figure 4.8 - Sildenafil increased fetal crown-rump length in high fat diet groups

When dietary groups were separated, sildenafil treatment was associated with increased fetal crown-rump length (CRL) in HFD (B), but not ND groups (A), suggesting a diet-treatment interaction was present. ** p < 0.01 effect of sildenafil at the level of HFD; ● ND; □ HFD; C control; SC sildenafil citrate.
4.3.3 S3. Uterine artery *ex vivo* function: dose response curves for sodium nitroprusside

![Graph showing dose response curves for sodium nitroprusside in C57, eNOS−/−, and COMT−/− mouse uterine arteries.](image)

**Figure 4.9 - Strain, but not diet or treatment, altered uterine artery ex vivo response to endothelium-independent vasodilatation**

Uterine arteries from eNOS−/− mice showed increased sensitivity to endothelium-independent vasodilator sodium nitroprusside (SNP). Neither diet nor sildenafil treatment affected endothelium-independent vasodilatation. Graphs show dose response curves (mean ± SEM) for SNP in mouse uterine arteries. n (C57, eNOS−/−, COMT−/−) = 6-9, 6-10, 5-8. ● ND; □ HFD; — control; ----- sildenafil treatment.
4.4 Effect of sildenafil on maternal blood pressure and mesenteric vessel function

4.4.1 Background

Pre-eclampsia and IUGR share a common aetiology of utero-placental insufficiency, commonly associated with abnormal placentation or reduced trophoblast remodelling of the spiral arteries (Brosens, Pijnenborg et al. 2011). Many of the signs and symptoms of pre-eclampsia – such as maternal hypertension - are thought to be caused by systemic endothelial dysfunction (Spradley et al. 2015). The cause of endothelial dysfunction is unclear, but it may result from the effects of factors released by the placenta in response to placental hypoperfusion (Spradley, Palei et al. 2015, Brosens, Pijnenborg et al. 2011). Due to its vasodilator properties, sildenafil treatment could potentially ameliorate the endothelial dysfunction associated with pre-eclampsia, and improve maternal signs and symptoms of the disease.

The COMT−/− mouse (Kanasaki, Palmsten et al. 2008) and inhibition of NOS in rodents (Molnar, Suto et al. 1994, Ramesar, Mackraj et al. 2010) have both been used previously as models of pre-eclampsia, as they demonstrate maternal hypertension, proteinuria and growth restricted offspring. In the study described in section 4.2, in addition to the effects of sildenafil on fetal growth and uterine artery function, the effect on maternal blood pressure and maternal mesenteric ex vivo function was also assessed. The mesenteric vascular bed was chosen as it appears to play an important role in determining total peripheral vascular resistance in rodents (Christensen, Mulvany 1993) and hypertension is associated with altered mesenteric artery function (Christensen, Mulvany 1993, Naito, Yoshida et al. 1998). In the present study, it was hypothesised that COMT−/−, eNOS−/−, and HFD would be associated with impaired mesenteric
artery function and maternal hypertension, and that maternal sildenafil treatment would ameliorate these changes.

4.4.2 Methods

Full methodology is described in chapter 2.

4.4.3 Results

4.4.3.1 Maternal systolic blood pressure

For a number of reasons, it was not possible to obtain useable recordings from every mouse in the study at every time point. There were, however, no significant differences in pregnancy outcomes, such as fetal weight, between mice where blood pressure recordings were not obtained compared to those where one, two or any recordings had been obtained.

At mid-pregnancy (10.5 dGA) there was a significant effect of strain on systolic blood pressure; eNOS<sup>−/−</sup> mice had a higher systolic blood pressure compared to other strains (p <0.0005; Figure 4.10 B). C57BL/6J mice on HFD had a higher blood pressure compared to ND (p = 0.006), but a diet effect was not apparent for other groups (Figure 4.10 C). Systolic blood pressure was similar between those mice that went on to start sildenafil treatment, and those who continued on control. In contrast to mid-pregnancy, at the end of pregnancy (17.5dGA) systolic blood pressure was similar amongst strains. There were, however, significant main effects of treatment and diet on maternal systolic blood pressure at 17.5 dGA. Maternal HFD was associated with higher blood pressure (p = 0.003; Figure 4.10 B) compared to ND, and sildenafil with lower blood pressure compared to untreated mice (p = 0.04; Figure 4.11 C).
Figure 4.10 - eNOS<sup>−/−</sup> strain and HFD in C57 BL/6J mice was associated with increased maternal blood pressure in mid-pregnancy.

Systolic blood pressures at 10.5 dGA are shown for all experimental groups in (A). At 10.5 dGA eNOS<sup>−/−</sup> dams had significantly higher systolic blood pressures compared to other strains (B) and HFD significantly increased maternal systolic blood in the C57BL/6J strain (C). Graphs B and C show mean + SEM for median maternal systolic blood pressures. ***p < 0.0005 for significant main effect of diet (B) or p < 0.0005 for significant effect of HFD at level of strain (C) ● ND; □ HFD; ■ ND and HFD groups combined.
Maternal high fat diet increased maternal systolic blood pressure and sildenafil treatment reduced systolic blood pressure in late pregnancy

Systolic blood pressures at 17.5 dGA are shown for all experimental groups in (A). At 17.5 dGA, dams fed HFD had significantly higher systolic blood pressure than those fed normal diet (B). Maternal systolic blood pressure at 17.5 dGA was significantly lower when dams were treated with sildenafil (C). Graphs B and C show mean + SEM for back-transformed median maternal systolic blood pressures **p < 0.01 for significant main effect of diet; * p < 0.05 for significant effect of treatment (3-way ANOVA with Bonferroni adjustment). ● ND; □ HFD; ■ ND and HFD groups combined.
4.4.3.2 **Effect of sildenafil on maternal mesenteric artery ex vivo function**

Mesenteric artery dose response curves are shown in Figure 4.12. There was no significant effect of strain, diet or treatment on maximal constriction of the mesenteric arteries. There were, however, effects of strain, diet and treatment on mesenteric artery sensitivity to the vasoconstrictor U46619 (U4). Mesenteric arteries from eNOS\(^{-/-}\) mice were less sensitive to U4 compared to arteries from C57BL/6J mice \((p = 0.02; \text{Figure } 4.13 \text{ A})\). There was also a significant diet - treatment interaction for eNOS\(^{-/-}\) mice, where sildenafil treatment reduced sensitivity of mesenteric arteries to U4 in HFD groups \((p = 0.008)\); there was also a trend toward reduced sensitivity to U4 with sildenafil treatment in the COMT\(^{-/-}\) HFD group \((p = 0.08; \text{Figure } 4.13 \text{ B})\).

In general the mesenteric vessels – especially those from the eNOS\(^{-/-}\) strains – did not maintain their tone, and therefore reliable relaxation-response data were not obtained. Due to small numbers in some groups we were unable to analyse data for ACh and SNP for mesenteric arteries. Dose-response curves for mesenteric artery response to ACh and SNP are shown in Figure 4.12 B and C.
Figure 4.12 - Dose–response curves for mesenteric arteries
Mesenteric arteries from eNOS<sup>−/−</sup> mice increased sensitivity to vasoconstrictor U4 (A). Due to missing data in some groups, statistical analysis of ACh (B) and SNP (C) curves was not possible. Graphs show mean ± SEM (or mean values only where n < 3) for dose-response curves to U4 (A) and ACh (B) and SNP (C) in mesenteric arteries. n (C57, eNOS<sup>−/−</sup>, COMT<sup>−/−</sup>) U4 = 5-10, 4-11, 4-6; ACh = 3-9, 1-7, 1-4; SNP = 2-9, 3-6, 2-5. ● ND; □ HFD; — control; --- sildenafil treatment.
Figure 4.13 - Mesenteric arteries from pregnant eNOS⁻/ mice and sildenafil treated HFD mice had reduced sensitivity to constrictor U4.

Graphs show mean + SEM for back-transformed mesenteric artery EC₅₀ for response to constrictor U4. Mesenteric arteries from eNOS⁻/ mice had reduced sensitivity to U4 compared to mesenteric arteries from C57BL/6J mice (A). n (C57, eNOS⁻/, COMT⁻/) = 28, 22, 18. In HFD mice, sildenafil treatment significantly reduced sensitivity to U4 in eNOS⁻/ mice and there was a trend toward reduced sensitivity with sildenafil treatment in COMT⁻/ HFD mice (B). Open bars HFD+C, patterned bars HFD+SC. n (HFD+C, HFD+SC) = 8, 4 (C57), 6, 10 (eNOS⁻/), 4, 5 (COMT⁻/) * p < 0.05 for significant main effect of strain; **p < 0.01 pairwise comparison (3-way ANOVA with Bonferroni adjustment).
4.4.4 Additional discussion and conclusion

In this study, sildenafil treatment was associated with a small reduction in maternal systolic blood pressure at 17.5 dGA. A reduction in mean arterial blood pressure with sildenafil treatment has previously been demonstrated in women with pre-eclampsia; when compared to placebo, sildenafil treatment reduced mean arterial pressure and reduced the requirement for additional anti-hypertensive medication (Trapani et al., 2016). In the present study, a reduced sensitivity of mesenteric vessels to the vasoconstrictor U4 in the HFD groups suggests that reduced maternal blood pressure may be at least partly mediated by changes in vascular function in systemic resistance vessels. The difference in sensitivity to U4 was greatest in the mesenteric arteries from eNOS−/− mice, with a trend toward decreased sensitivity in the COMT+/− mice. The lack of difference in sensitivity to U4 in the mesenteric arteries from the C57BL/6J strain, may be due to a dosing effect, as this strain had significantly lower sildenafil intake compared to other strains.

There are several of limitations of this study. Missing data meant that it was not possible to analyse wire myography data on mesenteric artery ex vivo response to vasodilators, and that other results must be interpreted cautiously. Neither the COMT+/− nor eNOS−/− strains provided an adequate model of hypertension / preeclampsia, as in late pregnancy maternal systolic blood pressure was similar to that seen in the C57BL/6J control strain. Finally, tail-cuff plethysmography was used to measure maternal systolic blood pressure. This method was used due to its non-invasive methodology, ease of use, and relative low cost. The pulse-based tail-cuff plethysmography system used in this study has previously been shown to have excellent agreement with invasively monitored systolic blood pressure in non-pregnant animals (Whitesall et al, 2004). The main disadvantage of this method is that it involves both restraint and warming of mice. Recordings obtained may not be representative of normal blood pressure, but rather show the blood pressure in response to stressful stimuli; therefore
measuring blood pressure invasively via radio-telemetry would be preferable. Radio-telemetry requires surgical implantation of intravascular catheters and transmitters under general anaesthesia, followed by 5-7 days recovery post-surgery before accurate cardiovascular phenotyping is possible (Butz, Davisson et al., 2001). Due to the large size of the study and resource constraints, this was not a feasible means of assessing blood pressure in this study.

In conclusion, this study suggests that in addition to increasing fetal growth, sildenafil may reduce maternal blood pressure, and improve endothelial function of systemic resistance vessels. Sildenafil may therefore be of therapeutic benefit in pregnancies complicated by pre-eclampsia, which is characterised by both maternal hypertension and systemic endothelial dysfunction. However, due to low sample sizes and methodological limitations, these findings should be confirmed in studies that use more robust models of preeclampsia, where blood pressure is invasively monitored via radio-telemetry.
5 The effect of sildenafil citrate on myometrial and chorionic small artery ex vivo function

5.1 Preface

This chapter contains an original and unpublished study of ex vivo function of myometrial and chorionic plate arteries obtained from pregnancies complicated by severe, early onset IUGR. The arteries studied were obtained from women participating in the Sildenafil treatment in dismal prognosis early onset IUGR study, New Zealand, Australia (STRIDER NZAus), as part of a sub-study. The STRIDER NZAus trial commenced in 2014 and was initially projected to complete recruitment in 2016. Due to delays in commencing the study and slower than expected recruitment, it is now estimated that recruitment will be completed in mid-2017. To maintain the integrity of STRIDER NZAus, investigators must remain blinded to treatment allocation. Therefore, at the time of thesis submission treatment allocation was not revealed for analysis of this sub-study. Instead, the individual characteristics of the vessels in the study are described.

5.2 Introduction

Changes to the in utero nutritional environment can alter development and differentiation of fetal organ systems, resulting in pathologic consequences that may only be identifiable in later life. Although the studies in this thesis have not observed any detrimental outcomes in the offspring following in utero sildenafil exposure, the long-term effects of sildenafil on health remain unclear and, if present, are unlikely to be known for many years. Therefore it is important to attempt to limit treatment exposure to the pregnancies that are most likely to
benefit, for the shortest period of time possible. Understanding the mechanism/s through which sildenafil may improve fetal growth is key to achieving this.

Sildenafil has been considered as a potential treatment for IUGR primarily because of its vasodilatory properties. It has been hypothesised that sildenafil would vasodilate maternal resistance vessels supplying the placenta, increasing blood flow and placental perfusion. Despite the increase in fetal growth observed in the studies in this thesis (chapters 3 and 4), no change in uterine vessel \textit{ex vivo} function has been observed. There are however features of these studies that may partly explain these findings. Firstly, it is possible that sildenafil may increase endothelium-dependent vasodilatation only when there is pre-existing endothelial dysfunction. With the exception of the eNOS\textsuperscript{−/−} mouse, the animal models in this thesis (COMT\textsuperscript{−/−}, maternal high fat diet induced weight gain, uterine artery embolisation in sheep) did not display endothelial dysfunction \textit{per se} in the uterine arteries. Secondly, there may be differences between species’ metabolism and vessel responsiveness to sildenafil. These, as well as differences in experimental design (for example treatment route and dose), may mean that the vascular response to sildenafil in these studies does not accurately model the vascular response to sildenafil in vessels from human IUGR. These matters can only be clarified by replicating studies of myometrial vessel function in a relevant clinical population. Therefore, the first aim of the present study was to assess the effect of sildenafil treatment in pregnancy on the \textit{ex vivo} response of myometrial small arteries from pregnancies affected by severe early onset IUGR.

The second aim of this study was to assess the effect of \textit{in vivo} sildenafil exposure on chorionic vessel \textit{ex vivo} function. IUGR pregnancies are associated with increased resistance to flow in the fetoplacental circulation, as demonstrated by high umbilical artery Doppler resistance indices \textit{in vivo}, and increased pressures in the \textit{ex vivo} perfused placentae (Jones,
Bischof et al. 2015, Dicke, Huettner et al. 2009, Reuwer, Bruinse et al. 1984, Mills, Wareing et al. 2005). The aetiology of increased resistance to flow is not well understood, although abnormal vascular morphology and reduced villous vascularity may contribute (Higgins, Rey de Castro et al. 2015, Junaid, Brownbill et al. 2014). Altered vascular function of the chorionic vessels may also contribute, as the chorionic arteries from IUGR pregnancies exhibit endothelial dysfunction. NO plays an important role in the regulation of fetal-placental blood flow (McCarthy, Woolfson et al. 1994, Myatt, Brewer et al. 1992, Learmont, Poston 1996) but, in IUGR, chorionic arteries exhibit impaired eNOS dependent vasodilatation (Krause, Carrasco-Wong et al. 2013, Schneider, Hernández et al. 2015, Mills, Wareing et al. 2005). Changes to vascular function in the chorionic arteries therefore represent another potential target for the treatment of IUGR. It is hypothesised that sildenafil could dilate these arteries, increasing volumetric blood flow, and increasing the capacity for nutrient exchange to the fetus. Although sildenafil has been shown to have a vasodilatory effect when applied directly to chorionic vessels (Wareing, Myers et al. 2005b), there are no reports of chorionic vessel function following maternal treatment with sildenafil. In addition to assessing the effect of maternal sildenafil treatment on myometrial vessel ex vivo function, the effect on chorionic vessel ex vivo function will also be assessed. Alterations in function of either vascular bed represent possible pathways through which sildenafil may alter fetal growth.

5.3 Methods

Full methodology has been described in chapter 2.

5.4 Results

Viable myometrial and/or chorionic vessels were obtained from 16 women recruited to the sub-study. The obstetric characteristics of these pregnancies are described in Table 5.1.
Women were an average of 33 years old, with an average BMI of 24.6. The average gestation at delivery was 33\(\pm\)4 weeks’ gestation, with six (38\%) delivering whilst still taking sildenafil (i.e. <32 weeks’ gestation, see section 2.3). Three-quarters of the women were delivered by caesarean section. The average fetal weight was 1506 g, and a greater proportion (69\%) were female.

5.4.1 Myometrial arteries

Myometrial vessels were obtained from uterine biopsies taken at the time of caesarean (see section 2.3.4). Therefore, myometrial vessels could not be obtained from women who delivered vaginally. In total, myometrial samples were obtained from 10 women. Characteristics of these vessels are shown in Table 5.2.

5.4.2 Chorionic plate arteries

Chorionic small arteries were obtained from 16 placentae. Characteristics of these vessels are shown in (Table 5.3). Constriction data were obtained from all vessels. Following each experiment and washout, vessels showed poor responses to repeated doses of thromboxane A2 mimetic U46619 (U4), or constricted well initially but did not maintain tone. Therefore, vasodilatation responses could only be obtained for a limited number of vessels. Of the vessels that did maintain their tone, tone oscillations were demonstrated in all but one vascular segment; however, some groups also had at least one arterial section that displayed tonic constriction at the same dose of U4.
<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>BMI</th>
<th>Delivery gestation</th>
<th>Mode of delivery</th>
<th>Pre-eclampsia</th>
<th>Fetal sex</th>
<th>Fetal weight (g)</th>
<th>Placental weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>23.26</td>
<td>40+1</td>
<td>Vaginal birth</td>
<td>No</td>
<td>Female</td>
<td>3370</td>
<td>510</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>26.8</td>
<td>30+0</td>
<td>Caesarean section</td>
<td>Yes</td>
<td>Male</td>
<td>795</td>
<td>210</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>24.2</td>
<td>33+4</td>
<td>Caesarean section</td>
<td>No</td>
<td>Female</td>
<td>1495</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>29.5</td>
<td>36+2</td>
<td>Caesarean section</td>
<td>No</td>
<td>Male</td>
<td>2170</td>
<td>295</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>19.6</td>
<td>38+0</td>
<td>Vaginal birth</td>
<td>No</td>
<td>Female</td>
<td>2145</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>25.5</td>
<td>36+4</td>
<td>Caesarean section</td>
<td>No</td>
<td>Female</td>
<td>1710</td>
<td>350</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>23.8</td>
<td>33+6</td>
<td>Caesarean section</td>
<td>No</td>
<td>Male</td>
<td>1170</td>
<td>260</td>
</tr>
<tr>
<td>11</td>
<td>34</td>
<td>22.6</td>
<td>36+1</td>
<td>Caesarean section</td>
<td>No</td>
<td>Female</td>
<td>2130</td>
<td>390</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td>28.6</td>
<td>26+0</td>
<td>Caesarean section</td>
<td>Yes</td>
<td>Female</td>
<td>560</td>
<td>210</td>
</tr>
<tr>
<td>14</td>
<td>28</td>
<td>21.6</td>
<td>35+5</td>
<td>Caesarean section</td>
<td>No</td>
<td>Male</td>
<td>1620</td>
<td>300</td>
</tr>
<tr>
<td>15</td>
<td>34</td>
<td>23.1</td>
<td>28+0</td>
<td>Caesarean section</td>
<td>No</td>
<td>Male</td>
<td>630</td>
<td>140</td>
</tr>
<tr>
<td>16</td>
<td>25</td>
<td>24.5</td>
<td>37+3</td>
<td>Caesarean section</td>
<td>No</td>
<td>Female</td>
<td>2180</td>
<td>380</td>
</tr>
<tr>
<td>17</td>
<td>36</td>
<td>25.4</td>
<td>36+1</td>
<td>Vaginal birth</td>
<td>No</td>
<td>Female</td>
<td>1900</td>
<td>340</td>
</tr>
<tr>
<td>18</td>
<td>36</td>
<td>28.0</td>
<td>29+3</td>
<td>Caesarean section</td>
<td>Yes</td>
<td>Female</td>
<td>810</td>
<td>127</td>
</tr>
<tr>
<td>20</td>
<td>33</td>
<td>24.3</td>
<td>28+6</td>
<td>Caesarean section</td>
<td>No</td>
<td>Female</td>
<td>840</td>
<td>210</td>
</tr>
<tr>
<td>22</td>
<td>35</td>
<td>22.1</td>
<td>30+4</td>
<td>Vaginal birth</td>
<td>No</td>
<td>Female</td>
<td>570</td>
<td>270</td>
</tr>
</tbody>
</table>

Table 5.1 - Characteristics and obstetric details of study participants

Data for ID 5, placental weight, was not available.
<table>
<thead>
<tr>
<th>ID</th>
<th>Average vessel diameter (µm)*</th>
<th>Max constriction mN</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; U4 ηmol/L</th>
<th>Max relaxation BK (%)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; BK ηmol/L</th>
<th>Max relaxation SNP (%)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; SNP ηmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>366</td>
<td>11.9</td>
<td>3.3</td>
<td>108.7</td>
<td>1.3</td>
<td>CNC</td>
<td>CNC</td>
</tr>
<tr>
<td>3</td>
<td>495</td>
<td>7.9</td>
<td>23.4</td>
<td>78.7</td>
<td>1205.0</td>
<td>0.2</td>
<td>5.7</td>
</tr>
<tr>
<td>4</td>
<td>229</td>
<td>10.9</td>
<td>24.0</td>
<td>88.9</td>
<td>6.0</td>
<td>80.6</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>368</td>
<td>2.6</td>
<td>57.5</td>
<td>127.3</td>
<td>117.2</td>
<td>92.0</td>
<td>4.0</td>
</tr>
<tr>
<td>7</td>
<td>112</td>
<td>13.9</td>
<td>28.8</td>
<td>103.3</td>
<td>1.1</td>
<td>97.1</td>
<td>CNC</td>
</tr>
<tr>
<td>11</td>
<td>594</td>
<td>3.6</td>
<td>16.2</td>
<td>87.1</td>
<td>8.9</td>
<td>60.2</td>
<td>197.2</td>
</tr>
<tr>
<td>12</td>
<td>148</td>
<td>4.3</td>
<td>195.0</td>
<td>55.0</td>
<td>CNC</td>
<td>58.7</td>
<td>837.5</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>24.1</td>
<td>30.2</td>
<td>146.8</td>
<td>295.8</td>
<td>87.1</td>
<td>77.6</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>5.6</td>
<td>25.7</td>
<td>105.0</td>
<td>53.82</td>
<td>90.0</td>
<td>CNC</td>
</tr>
<tr>
<td>20</td>
<td>464</td>
<td>18.1</td>
<td>128.8</td>
<td>125.1</td>
<td>35.3</td>
<td>94.7</td>
<td>149.3</td>
</tr>
</tbody>
</table>

*Table 5.2 – Characteristics of myometrial arteries

*The calculated internal circumference (produced by Labchart software as part of the vessel normalisation procedure) was used to estimate the vessel diameter using the equation: \( \text{diameter} = \frac{\text{Internal circumference}}{\pi} \). Up to four vessels were studied from each study participant and the reported average diameter is the average of all vessels studied. For two samples (15, 18) vessel data were not saved, and is not available. In some instances, due to the characteristics of a dose-response curve, the EC50 could not be calculated (CNC).
## Table 5.3 - Characteristics ofchorionic plate arteries

(Continued over page)
<table>
<thead>
<tr>
<th>ID</th>
<th>Average vessel diameter (µm)*</th>
<th>Max constriction (mN)</th>
<th>EC50 U4 (ηmol/L)</th>
<th>Oscillatory tone</th>
<th>Tonic constriction</th>
<th>Max relaxation SNP (%)</th>
<th>EC50 SNP (ηmol/L)</th>
<th>Pre-constriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>218</td>
<td>0.57</td>
<td>295.1</td>
<td>Yes</td>
<td>No</td>
<td>Inadequate pre-constriction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>75</td>
<td>1.90</td>
<td>234.4</td>
<td>Inadequate pre-constriction</td>
<td>Inadequate pre-constriction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>65</td>
<td>2.55</td>
<td>CNC</td>
<td>Inadequate pre-constriction</td>
<td>Inadequate pre-constriction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>213</td>
<td>4.84</td>
<td>195.0</td>
<td>Yes</td>
<td>No</td>
<td>Inadequate pre-constriction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>473</td>
<td>3.17</td>
<td>311.2</td>
<td>No</td>
<td>Yes</td>
<td>62.8</td>
<td>251.2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.3 - Characteristics of chorionic plate arteries**

* The calculated internal circumference (produced by Labchart software as part of the vessel normalisation procedure) was used to estimate the vessel diameter using the equation: diameter = \( \frac{\text{Internal circumference}}{\pi} \). Up to four vessels were studied from each placenta, the reported average diameter is the average of all vessels studied. In some instances, due to the characteristics of a dose-response curve, the EC50 could not be calculated (CNC).
5.5 Discussion

In this study, the response of myometrial arteries to U4 was reproducible in repeated studies of the same vessel. Just over half of the arteries studied showed complete (>100%) relaxation to endothelium-dependent vasodilator BK. Myometrial arteries that showed complete relaxation to BK also showed the greatest response to endothelium-independent vasodilator SNP. When STRIDER treatment allocations are revealed, the EC50 for U4, BK and SNP will be compared between treatment and non-treatment groups. Comparisons will be made between groups for all samples, and then in subgroups by gestation < 32 weeks’ gestation (treatment exposed) and > 32 weeks’ gestation (treatment completed). If there is no significant effect of sildenafil on myometrial artery ex vivo function, this will confirm the findings from animal studies (chapter 3 and 4) and strongly support the hypothesis that sildenafil increases fetal growth through mechanisms other than a change in resistance vessel function. Conversely, if there are differences between treatment groups < 32 weeks’ but not >32 weeks’ gestation, this will suggest that sildenafil has an effect on artery function when exposure is recent, but not when exposure has been remote.

Chorionic arteries constricted in response to initial doses of U4, however, most of the arteries did not maintain constriction following a wash and repeated dose of U4. Therefore there is only limited data on the response of the chorionic vessels to vasodilators. When STRIDER treatment allocations are revealed, the EC50 for U4 will be compared between groups - as for the myometrial arteries. Previous studies have demonstrated that NO attenuates the response of chorionic vessels to constrictors (Myatt, Brewer et al. 1992, McCarthy, Woolfson et al. 1994, Learmont, Poston 1996), that U4 induced tension is increased (Mills, Wareing et al. 2005), and that sensitivity to U4 is reduced in chorionic vessels from IUGR pregnancies (Wareing, Crocker et al. 2002). Therefore, it is plausible that sildenafil treatment may result
in changes to the constriction and sensitivity of the vessels to U4. Unfortunately, it is unlikely that the subjects thus far studied will constitute sufficient numbers in each group to allow for any meaningful comparison of the chorionic vessels response to vasodilators, and the number of additional future subjects is unclear. Based on the observations of others, it may not be possible to demonstrate significant differences in endothelium-dependent relaxation with sildenafil treatment. This is because the *ex vivo* response of chorionic vessels to receptor mediated endothelium-dependent vasodilators appears to be minimal in both normal pregnancy, and pregnancies complicated by pre-eclampsia and IUGR (Ong, Moore et al. 2003, Wareing, Myers et al. 2005b, Wareing, Crocker et al. 2002). Secondly, unlike myometrial vessels, vasodilatation was not altered in chorionic vessels following co-incubation with sildenafil (Wareing, Myers et al. 2005b).

### 5.5.1 Limitations

The main limitation of this study was the inability to study chorionic vessel responses to vasodilators. This was due to an inability to obtain a sustained baseline constriction in these vessels following the initial constriction-response experiment and vessel washout. As the chorionic vessels display a sustained constriction at the time of the first experiment, future experiments may be more successful if the relaxation-response experiment commences immediately following the maximum dose of U4 (i.e. avoiding washout and vessel rest period between experiments). Another limitation was the inability to directly measure the diameter of the vessels studied. Instead, the estimated internal circumference was calculated by the LabChart computer software, and the diameter calculated from this. This may lead to an under- or over-estimation of the size of the vessels studied. Describing vessel size is important for this study, as there was a greater variation in vessel size compared to the animal studies (where vessels could be obtained from the same anatomic location, and therefore were similar.
in size). Finally, due to the specific entry criteria for STRIDER, the overall sample size of the sub-study was small. This means that the ability to detect a true difference in vessel response will be limited, especially if that difference is small.

5.5.2 Future directions

Collection and study of myometrial and chorionic vessels is ongoing. When STRIDER NZAus treatment allocations are known, the data will be analysed (as described above) to determine whether in vivo sildenafil exposure can alter resistance vessel function in IUGR pregnancies. Additionally, ex vivo function of both myometrial and chorionic vessels could be reconciled with Doppler ultrasound data collected as part of STRIDER NZAus. This could deepen the understanding of the relationship between ex vivo small artery function and in vivo measurements of larger vessels. This relationship would be particularly important to define if sildenafil were to alter both fetal growth and vascular function in IUGR pregnancies. If raised Doppler indices correlate with endothelial dysfunction in resistance arteries, then ultrasound could be used as a screening tool to identify the pregnancies that are were most likely to benefit from sildenafil treatment.
6 Discussion and conclusion

6.1 Preface

The experiments in this thesis were designed to address the question of whether sildenafil could improve fetal growth in animal models of IUGR caused by utero-placental insufficiency, and to gain a better understanding of the mechanisms through which changes in growth occurred through the study of maternal uterine artery ex vivo function.

Specifically, the aims of this thesis were to:

1. Assess the efficacy of maternal sildenafil treatment at increasing fetal growth in a large animal model of utero-placental insufficiency / IUGR (chapter 3)
2. Assess the efficacy of sildenafil at increasing fetal growth in small animal models of utero-placental insufficiency / IUGR (chapter 4)
3. Assess the effect of sildenafil on uterine / myometrial resistance vessel function
   a. in animal models of IUGR (chapters 3 and 4)
   b. in IUGR human pregnancies (chapter 5)

6.2 Sildenafil may increase fetal growth in animal models of IUGR, but may have a detrimental effect on growth in some instances

The studies in this thesis demonstrate that sildenafil may increase fetal growth in animal models of IUGR, albeit modestly. Lambs from sildenafil treated mothers had a mean weight 7% greater than those born to vehicle treated ewes. Although this difference was not statistically significant, vehicle treated, but not sildenafil treated, IUGR lambs were significantly lighter than normal growth controls, suggesting a beneficial effect of sildenafil on fetal growth (chapter 3). In the murine study (chapter 4) maternal sildenafil treatment increased fetal growth by 10% where IUGR had been induced with maternal HFD. A similar
increase in fetal weight of 6-10% has been observed in some studies (Dilworth, Andersson et al. 2013, Refuerzo, Sokol et al. 2006, Sánchez-Aparicio, Mota-Rojas et al. 2008), although others have shown a larger increase in fetal weight (14 - 35% (Sánchez-Aparicio, Mota-Rojas et al. 2008, Roberts, Refuerzo et al. 2016, Satterfield, Bazer et al. 2010, Herraiz, Pellicer et al. 2012)). The disparity between the increases in growth observed may be explained by differences in sildenafil dose: Sánchez-Aparicio and colleagues (2008) demonstrated a dose dependent increase in fetal weight from 6% to 35% increase in fetal weight with a ten-fold increase in sildenafil dose. Variations in treatment duration, and the nature and timing of the insult causing IUGR may also contribute to the differences observed. While the overall increases in growth observed in chapters 3 and 4 are small, currently, delivery is the only option for preventing in utero demise in pregnancies with severe IUGR. Gestational age at birth is the most significant predictor of survival free of major morbidity at very early gestations. At these very early gestations, even small increases in fetal growth may permit prolongation of pregnancy, and this may result in improved neonatal and longer term outcomes. For example, neonates delivered at 30-32 weeks’ gestation have a three-fold greater (78%) chance of survival free of major morbidity, compared to those born at 24-28 weeks’ gestation (Shah, Ye et al. 2012).

Although sildenafil may have a beneficial effect on fetal growth, as suggested in the ovine study, and demonstrated in the murine study, chapter 4 also suggest that in some circumstances, sildenafil may be associated with reduced fetal growth. Sildenafil treatment of pregnant COMT<sup>-/-</sup> ND mice was associated with increased rates of IUGR. As a reduction in IUGR was demonstrated in the COMT<sup>-/-</sup> HFD group (and an increase in fetal growth demonstrated in previous studies using the COMT<sup>-/-</sup> mouse (Stanley, Andersson et al. 2012)), it is unlikely that the detrimental effect of sildenafil relates specifically to the absence of the COMT enzyme. It should be recognised that in contrast to previous studies (Stanley,
Andersson et al. 2012), the COMT<sup>−/−</sup> mice used in the studies for this thesis did not provide a model of IUGR, as fetuses were similar in weight to those from the C57BL/6J strain. Therefore, one possible explanation is that in the COMT<sup>−/−</sup> strain, maternal sildenafil treatment disrupted adaptive processes that had initially resulted in improved fetal weight. The mechanism/s through which sildenafil reduced fetal growth in COMT<sup>−/−</sup> mice (and other animal models where reduced growth was observed (Refuerzo, Sokol et al. 2006, Nassar, Masrouha et al. 2012)) is not clear. This point warrants further investigation, as there are possibly human pregnancies where sildenafil will also have a detrimental effect on fetal growth. If clinical trials of sildenafil’s effect in severe early onset IUGR show a beneficial effect, it is likely that there will be a clinical push to use sildenafil in a broader clinical context, for example IUGR that is less severe, or of later onset. If more pregnancies will be exposed to sildenafil, determining why a reduction in growth occurs in some instances, and which pregnancies are at risk of this effect will be crucial to minimising harm.

6.3 Sildenafil can increase fetal growth through mechanism/s other than changes in uterine resistance vessel function

In both ovine and murine studies, sildenafil treatment was not associated with a change in myometrial or uterine artery ex vivo function. The lack of change observed in the uterine artery ex vivo function following sildenafil treatment implies that improved fetal growth is not dependent on increased vasodilatation in the uterine or myometrial arteries. This suggests that sildenafil may also increase fetal growth where IUGR is caused by pathologies other than utero-placental vascular insufficiency. As STRIDER NZAus is ongoing, it cannot be ascertained whether human myometrial artery ex vivo function is also unaltered following sildenafil treatment (chapter 5). It is possible that human myometrial arteries from IUGR pregnancies will exhibit increased vasodilatation / a change in ex vivo function following
sildenafil. If this occurs, these findings will not negate the previous conclusions from the ovine and murine studies. Instead, this suggests that in humans, there may be additional mechanisms through which sildenafil has its effect.

The studies within this thesis also hint at alternative and novel hypotheses for the mechanisms through which sildenafil may improve fetal growth: enhancing placental development, and the modulation of inflammatory pathways. In the ovine study, sildenafil treatment resulted in a substantial (26%) increase in mean placental weight, as well as a greater reduction in umbilical artery vascular resistance. Previous studies have also described increased placental growth, as well as increased placental vascularisation / vascular density (Motta, Grosso et al. 2015, Lopez-Tello, Arias-Alvarez et al. 2016), and reduced feto-placental vascular resistance following sildenafil treatment (Stanley, Andersson et al. 2012). Together, these results suggest that sildenafil may improve fetal growth through increased placental growth and vascularity. This hypothesis is also supported by the results of the murine study (chapter 4). Although placental weight was not significantly altered, sildenafil treatment increased expression of placental VEGFR-2, suggesting a pro-angiogenic effect. The next step in this research will be to assess the protein expression of VEGF receptors within the placentae, as well as histologic studies of placentae collected from the studies performed in this thesis, to determine whether structural changes are present.

In the murine study, sildenafil treatment altered placental expression of inflammatory mediators. The immunology of pregnancy is complex; however, it is clear that adverse pregnancy outcomes including IUGR and pre-eclampsia are associated with exaggerated placental and systemic inflammation. Furthermore, inflammatory cytokines are able to cross the placenta, exposing the fetus to an inflammatory environment which may alter fetal development and metabolism, and through developmental programming, predispose the
offspring to metabolic disorders later in life. Given the broad range of pregnancy complications associated with aberrant inflammation, the ability of sildenafil to modulate inflammatory pathways is potentially exciting, but requires further investigation. In particular, future research should attempt to clarify which pathways are modulated by sildenafil treatment, and whether these impact – either positively or adversely – on long-and short term outcomes.

6.4 What is new

The ovine study in this thesis has demonstrated for the first time that medium-term sildenafil exposure reduces fetal-placental vascular resistance, but does not alter myometrial resistance vessel function. The murine study in this thesis is the largest reported study of uterine artery ex vivo function following maternal sildenafil treatment. This study was also the first to report increased placental expression of VEGFR-2 following maternal sildenafil treatment, lending support to a new hypothesis: that sildenafil mediates increased fetal growth through an effect on placental growth and development. The study of human myometrial and chorionic vessels in this thesis is the first description of vascular function in human IUGR pregnancies following sildenafil treatment, and may provide further insight into additional mechanisms (such as altered endothelial function in chorionic and myometrial vessels) through which sildenafil may increase fetal growth.

6.5 Future directions

Further studies are planned to further assess the effect of maternal sildenafil treatment on placental growth and structure, and inflammatory pathways. In addition to these mechanistic questions, future research will be required to assess the effect of sildenafil on fetal wellbeing, long-term outcomes, and to optimise the timing of treatment. A limitation of the work presented in this thesis is that the study endpoints used were preterm fetal weights. It is
assumed that increased fetal weight correlates with improved perinatal outcomes; however, it is also possible that in an environment of nutritional restriction, sildenafil may drive fetal growth at the expense of other developmental processes, or disrupt fetal compensatory mechanisms, resulting in adverse outcomes including those that become evident only later in life. Therefore, it is critical that future studies (in both animals and humans) assess the effect of sildenafil treatment on outcomes such as fetal wellbeing, neonatal morbidity, and long-term health. Long-term cardiovascular and neurocognitive outcomes are of particular interest. The hypoxic fetus redistributes blood flow away from peripheral circulations to maintain brain perfusion, resulting in increased cardiac afterload. Increased afterload may result in structural and functional changes to the developing heart, leading to cardiac dysfunction in the adult offspring (Veille, Hanson et al. 1993, Giussani, Davidge 2013). As sildenafil has been shown to induce changes to flow in the umbilical and middle cerebral circulations (chapter 3, (Lopez-Tello, Arias-Alvarez et al. 2016, Trapani, Goncalves et al. 2016)), it is also likely to alter cardiac afterload, impacting cardiac development and consequently long-term cardiovascular function. Neurocognitive outcomes are important as the fetal brain undergoes massive growth and development in the second half of pregnancy (Dobbing, Sands 1973, Dobbing 1974, Andescavage, du Plessis et al. 2016), so may be especially vulnerable to the direct effects of, or haemodynamic changes associated with, sildenafil treatment. Current clinical trials and their associated follow-up studies are likely to be able to address some of these areas. However, due to the length of follow-up required (and for the purposes of assessing structural changes at a tissue and cellular level), further animal studies will also be required. Examples of outcome measures that could be assessed in both humans and animals (where appropriate/possible) include short-term variability of the fetal heart rate, fetal biophysical profile, acid-base status at delivery; rates of major neonatal morbidity, duration of neonatal unit stay; neurocognitive function, systolic and diastolic pump function, and
markers of metabolic health (glucose tolerance, blood pressure, lipid profiles) in the adult offspring.

The optimal timing for commencing sildenafil treatment is also unclear. In both animal studies in this thesis, sildenafil treatment was commenced mid-pregnancy. As these studies suggest that sildenafil treatment may increase fetal growth through its effect on placental growth, it is feasible that sildenafil may also work prophylactically, and even more effectively, if given during early placental development (i.e. the late first, early second trimester). This may be more a challenging question to answer for two reasons. Firstly, there are a lack of robust animal models for studying implantation and early placental development, as no other species exhibits the depth of haemochorial placental invasion that is seen in human pregnancy. Furthermore, studies in human pregnancy will be challenging, due to the paucity of highly-predictive early pregnancy biomarkers for IUGR. Ongoing research is required in both these areas: to develop better animal models of placental disease, and to identify new biomarkers for IUGR that have a high positive predictive value and a low false positive rate. Advances in these areas will be essential for facilitating the testing of new prophylactic therapies, including sildenafil.

6.6 Conclusion

IUGR contributes to the deaths of thousands of babies every year, with many more developing life-long disabilities. A treatment that improves in utero growth has the potential to reduce perinatal mortality and morbidity, as well as the emotional and financial costs associated with IUGR. The work presented in this thesis has used two clinically relevant animal models of IUGR to demonstrate that maternal sildenafil treatment can increase fetal growth. Increased fetal growth occurred in the absence of altered uterine or myometrial artery ex vivo function. This suggests that sildenafil will be a useful treatment adjunct in the management of IUGR in
human pregnancy, and that it may also be effective for the treatment of IUGR due to causes other than utero-placental vascular insufficiency. There are, however, further questions to be answered in regards to the effect of sildenafil on fetal wellbeing and long-term health of the offspring, optimal timing of treatment, and the potential for adverse effects in a broader (for example, non-growth restricted) demographic; these questions must be answered in order to optimise the translation of this promising new treatment into clinical practice.
7 Appendices

Appendix A - Permissions for use of figures

Figure 7.1 – Permission for use of figure 1.1
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. (“CCC”), at the time that you opened your Rightslink account and that are available at any time at.

Figure 7.2 – Permission for use of figure 1.3

216
Appendix B – Ethical approval for ovine study

UNIVERSITY OF AUCKLAND ANIMAL ETHICS COMMITTEE

19-Feb-2013

MEMORANDUM TO:

Dr Mark Oliver
Liggins Institute

Re: Research application approved (Our Ref. 001101)

The Committee considered your application for animal ethics approval for your project titled Sildenafil treatment of ewes carrying growth restricted fetuses. The Committee is pleased to advise you that this application has now been approved for a period of three years.

The approval date is 19-Feb-2013.

The expiry date is 19-Feb-2016.

Conditions of approval

All deaths which occur prior to the planned end of experiment must be notified to the AEC so that a post mortem may be performed by the Animal Welfare Officer if considered necessary. This includes all animals that are found dead or moribund, or are killed due to abnormalities which make them not fit for purpose.

Please note the requirement regarding the reporting of animal use under the Animal Welfare Act 1999. As Responsible Investigator it is your statutory responsibility to provide to this office:

- Annual Animal Usage figures for incorporation into the University consolidated return to MPI.
- An End of Approval Report along with Final Animal Usage figures for the whole project on completion of the project.

All required forms, general information on the animal ethics procedures, and information on training can be found at www.auckland.ac.nz/ae or can be provided by the Animal Ethics Administrator on request.

If you have any queries regarding your ethics application or wish to discuss general matters relating to ethics approvals, please contact the Animal Ethics Administrator
Appendix C – Ethical approval for murine study

UNIVERSITY OF AUCKLAND ANIMAL ETHICS COMMITTEE

17-Dec-2012

MEMORANDUM TO:

Dr Mark Vickers
Liggins Institute

Re: Research application approved (Our Ref. 001097)

The Committee considered your application for animal ethics approval for your project titled Therapies for Preeclampsia and Fetal Growth Restriction: Effects on Offspring. The Committee is pleased to advise you that this application has now been approved for a period of three years.

The approval date is 17-Dec-2012.

The expiry date is 17-Dec-2015.

Conditions of approval

All deaths which occur prior to the planned end of experiment must be notified to the AEC so that a post mortem may be performed by the Animal Welfare Officer if considered necessary. This includes all animals that are found dead or moribund, or are killed due to abnormalities which make them not fit for purpose.

Please note the requirement regarding the reporting of animal use under the Animal Welfare Act 1999. As Responsible Investigator it is your statutory responsibility to provide to this office:

- Annual Animal Usage figures for incorporation into the University consolidated return to MPI.
- An End of Approval Report along with Final Animal Usage figures for the whole project on completion of the project.

All required forms, general information on the animal ethics procedures, and information on training can be found at www.auckland.ac.nz/ae or can be provided by the Animal Ethics Administrator on request.

If you have any queries regarding your ethics application or wish to discuss general matters relating to ethics approvals, please contact the Animal Ethics Administrator at animalethics@auckland.ac.nz or +64 9 373 7599 ext 86356.

All communication with the AEC regarding this application should include this reference number: 001097.

(This is a computer generated letter. No signature required.)

Fliona Cheal
Animal Ethics Administrator
University of Auckland Animal Ethics Committee
Appendix D - Distribution of mean residuals for groups that failed normality distribution testing in murine study (Chapter 4)

Figure 7.3 Distribution of mean residuals for average pup weight (COMT\textsuperscript{-/-} \textit{HFD + SC})

Figure 7.4 Distribution of mean residuals for crown-rump length (COMT\textsuperscript{-/-} \textit{HFD + SC})
Figure 7.5 – Distribution of mean residuals for average pup weight (eNOS<sup>−/−</sup> HFD +C)
Appendix E - Statistical properties of reported statistically significant findings reported in the murine study (chapter 4)

<table>
<thead>
<tr>
<th></th>
<th>$DF$</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fat diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$21.5 \pm 0.4$</td>
<td>$25.2 \pm 0.4$</td>
<td>$1, 110$</td>
</tr>
<tr>
<td>Litter size (number of pups)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$8 (7 - 9)$</td>
<td>$6 (5 - 7)$</td>
<td>$2, 110$</td>
</tr>
<tr>
<td></td>
<td>$7 (6-8)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment intake (mg/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$0.13 (0.11 - 0.14)$</td>
<td>$0.16 (0.15 - 0.17)$</td>
<td>$0.16 (0.14 - 0.18)$</td>
</tr>
</tbody>
</table>

*Table 7.1 - Statistical properties for main effects of diet or strain on maternal weight, litter size and sildenafil intake*
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Fetal and placental measures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>1.07 ± 0.02</td>
<td>2, 109</td>
<td>37.8</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.88 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1.02 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal circumference -mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>24.5 ± 0.2</td>
<td>2, 106</td>
<td>13.9</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>23.2 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>24.3 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>29.4 ± 0.2</td>
<td>2, 106</td>
<td>7.7</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>28.5 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>29.2 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>0.077 ± 0.002</td>
<td>2, 94</td>
<td>9.8</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.069 ± 0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.083 ± 0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine artery &lt;i&gt;ex vivo&lt;/i&gt;  function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constriction&lt;sub&gt;max&lt;/sub&gt; Pe (mN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>6.83 (6.15-7.53)</td>
<td>2, 93</td>
<td>31.8</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>4.18 (3.70-4.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>7.16 (6.46-7.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; Pe(µMol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>0.4 (0.3 -0.7)</td>
<td>2, 80</td>
<td>14.7</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>2.2 (1.5 – 3.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1.1 (0.7 – 1.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxation&lt;sub&gt;max&lt;/sub&gt; ACh (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>94 ± 2</td>
<td>2, 81</td>
<td>27.1</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>75 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>96 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh EC&lt;sub&gt;50&lt;/sub&gt; (ηmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>43 (28 – 67)</td>
<td>2, 74</td>
<td>19.6</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>109 (73 – 164)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>17 (11 – 25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNP EC&lt;sub&gt;50&lt;/sub&gt; (ηmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>76 (51 –105)</td>
<td>2, 73</td>
<td>7.8</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>21 (10 - 36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>51 (30 - 78)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2 - Statistical properties for main effects of strain on fetal and placental measures, and uterine artery <i>ex vivo</i> function
### Table 7.3 - Statistical properties for main effects of diet on fetal and placental measures

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal and placental measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>1.04 ± 0.01</td>
<td>1, 109</td>
<td>37.8</td>
</tr>
<tr>
<td>High fat diet</td>
<td>0.94 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal circumference (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>24.5 ± 0.2</td>
<td>1, 106</td>
<td>18.7</td>
</tr>
<tr>
<td>High fat diet</td>
<td>23.5 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>29.5 ± 0.2</td>
<td>1, 106</td>
<td>28.7</td>
</tr>
<tr>
<td>High fat diet</td>
<td>28.6 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 7.4 - Statistical properties for main effects of treatment on fetal and placental measures

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal and placental measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.96 ± 0.01</td>
<td>2, 108</td>
<td>37.6</td>
</tr>
<tr>
<td>Sildenafil citrate</td>
<td>1.02 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal circumference (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23.7 ± 0.2</td>
<td>1, 106</td>
<td>6.7</td>
</tr>
<tr>
<td>Sildenafil citrate</td>
<td>24.3 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28.7 ± 0.1</td>
<td>1, 106</td>
<td>7.9</td>
</tr>
<tr>
<td>Sildenafil citrate</td>
<td>29.3 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DF</td>
<td>F</td>
</tr>
<tr>
<td>------</td>
<td>----------------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>GLUT4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>3.3 (2.7 – 3.9)</td>
<td>2.99</td>
<td>5.4</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>3.5 (2.9 – 4.1)</td>
<td>1.99</td>
<td>7.6</td>
</tr>
<tr>
<td>COMT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>5.1 (4.2 – 6.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>1.9 (1.6 – 2.1)</td>
<td>1.99</td>
<td>7.6</td>
</tr>
<tr>
<td>High fat diet</td>
<td>2.5 (2.2 – 2.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>1.0 (0.8 – 1.1)</td>
<td>1.99</td>
<td>20.6</td>
</tr>
<tr>
<td>High fat diet</td>
<td>1.4 (1.2 – 1.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>1.2 (1.1 – 1.4)</td>
<td>1.99</td>
<td>11.7</td>
</tr>
<tr>
<td>High fat diet</td>
<td>1.6 (1.4 – 1.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.4 (1.3 – 1.5)</td>
<td>1.99</td>
<td>4.4</td>
</tr>
<tr>
<td>Sildenafil citrate</td>
<td>1.6 (1.5 – 1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.6 (0.6 – 0.7)</td>
<td>1.99</td>
<td>4.5</td>
</tr>
<tr>
<td>Sildenafil citrate</td>
<td>0.7 (0.7 – 0.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet - control</td>
<td>1.6 (1.3 – 1.9)</td>
<td>1.99</td>
<td>6.0</td>
</tr>
<tr>
<td>Normal diet - sildenafil citrate</td>
<td>2.2 (1.8 – 2.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fat diet - control</td>
<td>2.6 (2.1 – 3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fat diet – sildenafil citrate</td>
<td>2.4 (2.0 – 3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet – control</td>
<td>1.1 (0.9 – 1.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet - sildenafil citrate</td>
<td>1.0 (0.9 – 1.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fat diet - control</td>
<td>1.3 (1.1 – 1.5)</td>
<td>1.99</td>
<td>3.7</td>
</tr>
<tr>
<td>High fat diet - sildenafil citrate</td>
<td>1.0 (0.9 – 1.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.5 - Statistical properties for main effects of diet strain or treatment on placental mRNA expression
Appendix F - Ethical approval for STRIDER study

2 October 2012

Dr Katie Groom
University of Auckland
Dept of Obstetrics & Gynaecology
Lvl 12, Auckland City Hospital
Private Bag 92 019
Auckland 1142

Dear Dr Groom

Re: Ethics ref: CEN/12/06/028 (please quote in all correspondence)
Study title: STRIDER: A Randomized Controlled Trial of Sildenafil Therapy in Dismal Prognosis Early-Onset Intrauterine Growth Restriction (New Zealand and Australia)

I am pleased to advise that this application has been approved by the Northern A Health and Disability Ethics Committee.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study’s sponsor, to ensure that these conditions are met. No further review by the Northern A Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at any locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at any locality in New Zealand, it must be registered in a WHO-approved clinical trials registry (such as the Australia New Zealand Clinical Trials Registry www.anzctr.org.au).

3. Before the study commences at a given locality in New Zealand, it must be authorised by that locality, and this authorisation recorded as soon as possible in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the Standard Operating Procedures for Health and Disability Ethics Committees (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Participant access to ACC

The Northern A Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the
STRIDER (NZAus): A Randomised Controlled Trial of Sildenafil Therapy in Dismal Prognosis Early-Onset Intrauterine Growth Restriction (New Zealand and Australia).

Investigators

**Auckland**
- Dr Katie Groom, Lead Principal Investigator
- Department of Obstetrics & Gynaecology
- University of Auckland, Level 12
- Auckland City Hospital, Private Bag 92019
- Mobile: 021 245 9622
- Email: k.groom@auckland.ac.nz
- Professor Lesley McCowan
- Professor Philip Baker
- Professor Peter Stone
- Dr Ariel Lee, Statistician

**Local Centre**
- Dr Katie Groom
- Mobile: 021 245 9622
- Professor Peter Stone
- Mobile: 021 864736
- Laura Mackay, Clinical Trial Manager
- Phone: 09 3737599 ext 81366
- Research Midwife: 021 083 14624

---

You are being invited to take part in this research study. Before you decide, it is important for you to understand why this research is being done and what it will involve. Please read through this leaflet and feel free to discuss it with others if you wish. Please ask if there is anything that is not clear or if you would like more information. Take time to decide if you would like to take part in the study. Your participation is entirely voluntary (your choice). You do not have to take part in this study and if you choose not to take part you will receive the standard care available. Thank you for your interest in the study and for taking the time to think about whether you wish to take part.
What is the purpose of the study?
Intrauterine growth restriction (IUGR) occurring very early in pregnancy is rare, affecting only 0.2% of pregnancies. Unfortunately due to the severity of disease and extreme prematurity the risks to the baby are very high. Babies may die in-utero before delivery without ever getting big enough to have a chance of survival and even if they do reach an age where they may survive they are susceptible to the effects of hypoxia (lack of oxygen) which may result in poor health in the neonatal period, as children and through to adulthood. There is currently no treatment to improve outcome once growth restriction has occurred and therefore the only treatment doctors are able to offer is timely delivery. Intense monitoring of mother and baby aims to predict when the fetus has maximised its time in-utero and the risk of hypoxia and death is so high that early delivery is indicated. However, this results in very early (premature) birth and its inherent risks including death, severe neonatal morbidity and again potential life-long health consequences.

The purpose of this study is to investigate a drug called sildenafil that may improve blood supply to babies with severe IUGR occurring early in pregnancy. It is possible that by improving the blood supply to the baby that it grows better and can remain in-utero for longer and so less susceptible to the risks of prematurity.

The STRIDER NZAus study will assess whether, in babies with severe early onset IUGR, sildenafil increases the growth rate of the baby’s abdominal circumference whilst it is still in-utero. We will also use the results of the study in collaboration with similar studies being done across the world to find out if, by improving baby’s growth in-utero, sildenafil reduces the number of babies that die or survive with major handicap as a consequence of being IUGR and premature i.e. do more babies survive free of major handicap?

The study is a randomised double-blind placebo controlled trial. This means that half the women in the study will be randomly assigned to sildenafil treatment and the other half will be randomly assigned to a placebo tablet that is identical to the sildenafil tablet and taken in the same way. Whilst the study is on-going neither the women taking part in the study or the doctors caring for the women will know who is on which treatment. At the end of the study we will find out if sildenafil affects fetal growth and ultimately improves outcomes for babies affected by severe IUGR.

Why is this study suitable for me?
You have been invited to take part in this study because you have a singleton pregnancy (one baby) with a baby that is very small for gestational age measured by:
1. At ≥22 weeks + 0 days and ≤27 weeks + 6 days: fetal abdominal circumference ≤3rd percentile;
2. At ≥28 weeks + 0 days and ≤29 weeks + 6 days: an ultrasound estimate of fetal weight (EFW) <700g.

This means your baby is at very high risk of a poor outcome.

We intend to enrol 122 women at similar risk to take part in the study.

Women will be invited to take part in the study across New Zealand and Australia.
Are there any reasons why some women should not take part in the study?
Some women who meet the criteria listed above can not take part in the study.
Reasons include:
1. Known major fetal anomaly/syndrome/congenital infection (such as Toxoplasmosis and Parvovirus) that is deemed to be the likely cause for the baby being small.
2. Known fetal aneuploidy e.g. Down syndrome.
3. Already made plan for termination of pregnancy due to the poor prognosis for their baby.
4. Your doctors expect that your baby will need to be delivered within the next 48 hours.
5. Treatment with sildenafil would not be suitable for you e.g. due to a known allergy.

Do I have to take part?
It is up to you to decide whether you take part in the study. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form that says that you agree to take part in the study. Even after deciding to take part you can withdraw from the study at any time and you don’t have to give a reason. A decision at any time to withdraw from the study or a decision not to take part will not affect the standard of care you receive.

Women who do not wish to take part in the study will receive the same standard of care we provide for all with this condition. Sildenafil will not be available as part of this standard care.

What is standard treatment for women with this condition?
Once a baby is found to be much smaller than expected early in pregnancy, doctors will consider possible causes of IUGR (this includes aneuploidy such as Down syndrome, fetal structural abnormalities not related to chromosome problems and fetal infections such as Toxoplasmosis and Parvovirus). However, most cases of IUGR are related to problems with how the placenta (whenua/after-birth) plants into the wall of the uterus (womb) and in this situation unfortunately there is no current proven medicine or treatment.

The aim of management is to watch mother and baby closely to determine when the baby is at a high risk of dying in-utero or being severely affected by lack of oxygen. This is done by ultrasound scans and cardiotocograph (CTG) monitoring of baby’s heart rate. Mothers also have regular review of blood pressure and urine to assess for possible development of preclampsia. Once it has been decided that baby and/or mother are too sick to continue monitoring, and as long as the baby has reached an age and a site where it may have a chance of survival, delivery is arranged. As babies are so premature, treatments to mothers, such as corticosteroid injections to help the baby’s lungs develop and a magnesium sulphate infusion to protect baby’s brain are considered prior to these planned births (these treatments have been proven to be effective in randomised controlled trials). As the babies are
so small and at risk, delivery is almost always by caesarean section and usually before 30 weeks gestation.

In some cases babies are unable to grow to a size that may result in a live-birth or any chance of the baby surviving without major handicap, or the mother may develop the additional complication of preeclampsia (as can occur in 40% of these cases) when the baby is still too small to survive. In these situations termination of pregnancy on health safety grounds may be discussed with women and their families.

With early onset IUGR care is provided at the hospital by a ‘high risk’ care team including doctors specially trained in maternal and fetal medicine (MFM), specialised obstetricians, specialised midwives, senior sonographers and neonatologists. With such intense monitoring of mother and baby sometimes it is necessary to admit women to hospital for in-patient care.

What additional things will happen to me if I take part in this study?

If you agree to take part in the study we will ask you to complete the consent form. You will then be randomly assigned (selected by chance by a computer) to either sildenafil or an identical looking placebo (inactive ‘dummy’ tablet) treatment. You will have a 50:50 chance of sildenafil or placebo treatment. These will be taken as an oral tablet (by mouth) three times a day from when you start the trial until baby is born (or you reach 32 weeks gestation, whichever occurs first). Neither you nor the doctors caring for you will know which treatment you are receiving. We will ask you to complete a subject medication diary to record your medication use and any side effects that may be related to the treatment.

You will be cared for by the Maternal Fetal Medicine High Risk team. Blood tests and a urine sample will be taken when you join the study and around days 5, 10, 14 and then at least once a week while you are on the study treatment. In the majority of cases these tests are required as part of normal care given to women in these situations. Ultrasound monitoring of your baby will occur at the time you join the study and then at around 14 weeks, 5 days, 10 days and 14 days after you join the study (and will continue at least weekly until the baby is born). It is likely these scans would be performed regardless of your involvement in the study (and may need to occur more frequently depending on the clinical situation). Once your baby is big enough we will do regular CTG monitoring of your baby’s heart beat, this is also part of standard care for very small babies.

If you stop the study treatment (sildenafil or placebo) and you have not yet delivered your baby we would like to carry out a post-treatment assessment between 2 and 10 days after you take the last study drug tablet. This assessment will include an Ultrasound scan, blood and urine tests which may be part of your normal care even if you were not involved with the study.

All other care during the pregnancy will be similar to that given to women not taking part in the study who have comparable high-risk pregnancies and the doctors and midwives caring for you will advise you about this. We will collect information about any additional therapy (such as hospital stays and use of blood pressure tablets if you develop preeclampsia) as your pregnancy proceeds, details of your baby’s birth and any complications that arise until you and your baby are discharged from hospital. This information will be collected from your and your baby’s medical
notes. If your baby is discharged before their Estimated Delivery Date (EDD) a
research midwife or one of the study Investigators will contact you by phone around
the time of EDD to ask about your baby’s progress.

We will ask you to complete a questionnaire after the birth of your baby
telling us about how happy you were with the treatment. We would also like to
make contact with you when your child is about two years old to find out how well
he or she is developing.

For Auckland Study Centre Participants only (voluntary addition).

At the time of delivery we would like to take a sample from the placenta
(whenua/after-birth), and if you deliver by caesarean section, a small sample from
the myometrium (muscle) of your uterus (womb). If you would like to take your
placenta (whenua/after-birth) home after delivery this will still be possible after the
study sample has been taken.

Most of your sample will be studied on the day it is collected. A small amount
will be frozen and stored within The University of Auckland for a period of up to five
years to allow the final studies to be performed once enrolment to STRIDER NZAUs is
complete. Once these planned studies are complete your tissue sample will be will
be destroyed following University approved guidelines for safe and appropriate
disposal of tissue.

Myometrial sampling has been performed for many years for research
purposes. It causes no extra risks, pain or increased operating time for the caesarean
section and has no long term consequences.
Placenta (whenua/after-birth) and myometrial sampling is an optional part of this
study, you may still take part in the study but decline these samples.

What is the drug being tested?

Sildenafil (also known by the trade name Viagra®) is a ‘nitric oxide donor’
drug. This means it acts on some blood vessels within the body to cause
vasodilatation (relaxation of the blood vessel walls). Due to the inadequate changes
in the uteroplacental vessels that occur with IUGR (and preeclampsia) we believe the
blood vessels from the maternal side feeding the placenta and the placental vessels
may still respond to nitric oxide beyond the second trimester and by dilating these
vessels we may increase blood supply to the baby and improve fetal growth.

Sildenafil has been used in pregnancy in women with a severe lung condition
called pulmonary hypertension. In these cases it has a positive effect on the
mother’s heart and lung function and does not appear to cause harm to babies. It
has also been used across the world in a few women with preeclampsia and severe
early onset IUGR with no suggestion of an adverse effect on babies and a possible
effect improving fetal growth. We will be using the drug at similar doses to these
reported cases.

What are the side effects of the drug?

The relatively commonly reported side effects of sildenafil include; headache,
flushing, dyspepsia (indigestion), nasal congestion and impaired vision, including
photophobia and blurred vision. However, none of these side effects were reported
when used by pregnant women in the cases mentioned above.
You will be given a subject medication diary to record your medication use and report any side effects you may have. If you were to have any serious effects that may be related to the drug we are able to ‘break the code’ and find out what treatment you have been receiving before the trial is complete.

What are the alternatives for treatment?
If you choose not to take part in the study you will be offered the standard care provided for pregnancies at such high risk. This includes care provided by a ‘high risk’ team, very close surveillance of mother and baby with timely delivery (with antenatal corticosteroids and magnesium sulphate where appropriate). Sildenafil is not part of standard high-risk care and will not be offered to women not taking part in the study.

What are the blood and urine tests for?
The blood tests from you will measure your blood count, liver and kidney function. The urine sample will be collected to measure the amount of protein in your urine. These tests are done routinely in women with preeclampsia or at high risk of it (as occurs with severe early onset IUGR). The blood and urine tests required for study purposes may be carried out more frequently than is required for clinical reasons if you have IUGR only.

What are the ultrasound scans for?
The scans will include growth measurements of your baby, measurement of fluid surrounding your baby and Doppler blood flow measurements to and from the placenta, in the maternal vessels supplying the uterus (womb) and in the baby itself. These measurements are standard care for IUGR babies and will not cause harm to you or your baby. These scans are performed routinely with IUGR babies to decide when is the best time to deliver them. For the study we will also use this information to find out if sildenafil works to help improve blood flow and growth for these very small babies delivered so early and if it does how the exact mechanism of action might work.

What are the placental and myometrial studies for? (Auckland Study Centre only)
We will use these samples in the laboratory to compare the effects of sildenafil and placebo treatment on blood vessel development and function. This will help us work out the exact mechanism of action of drug and its influence on the utero-placental circulation.

What are the possible benefits of taking part in the study?
The study is designed to find out whether the use of sildenafil will reduce the risk of babies dying or having major handicap as a consequence of IUGR and being born early. However, we do not know if it will make a difference. The information from this study will help us find out whether the drug improves fetal growth. By combining our results with similar studies taking place across the world we will find out if this leads to an improved outcome for these babies and so help us to plan care for women in the future with pregnancies affected by the same condition.

STRIDER NZAus – Information sheet/Consent version 5. 05 July 2016 6

231
Women taking part in the study will not receive any financial remuneration but there is no cost to the women who take part in the study. If we find that sildenafil does improve outcome there may be some advantage to the women who have received the active drug.

What are the possible disadvantages of taking part in the study?

We will not know whether taking sildenafil will make a difference to the in-utero growth of babies until the study is completed. Possible side effects of the drug include headache, flushing, dyspepsia (indigestion), nasal congestion and impaired vision. There is also the possibility that if sildenafil is effective at improving growth a baby that would otherwise have died due to hypoxia in-utero, grows sufficiently to be born alive but then as a consequence of its small size and prematurity has significant handicap. (However, if this were the case then it is likely the drug will also reduce handicap in babies that are slightly older and/or larger that would otherwise have had major handicap).

What if I join the study but change my mind later?

Participation in this study is entirely voluntary. You can decide to stop taking the study drug (sildenafil or placebo) or withdraw from the study at any time. If you decide to stop taking the study drug your study investigator may invite you to attend a post treatment assessment if you have not yet delivered your baby; this may be part of or in addition to your normal care.

If you decide to stop taking the study drug or to withdraw your consent you may still wish to contribute to the study at a reduced level, for example you may still agree to some follow up contact and/or allow the Investigator and study staff to obtain information from your and your baby’s medical records after this point (this decision will be your choice). If you decide to completely withdraw your consent from further trial participation no further information will be collected about you or your baby, however, any information that has been collected about you and your baby to that point will be retained and any publically available information about you or your baby may still be collected, such as information from the Birth Register.

A decision to withdraw from the study at any time will not affect the standard of your continued medical care.

Compensation

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation, and Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Injury Prevention, Rehabilitation, and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.
You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.

What if new information becomes available?

Sometimes in the course of a research project new information becomes available about the drug that is being studied. If we become aware of significant new information one of the study investigators will tell you about it. If you are still in the treatment phase of the study the investigator will discuss with you whether you want to continue taking the study medication. If you decide to withdraw from the study the investigators will arrange for your care to continue. If you decide to continue taking the study medication you will receive an updated information sheet and consent form to sign. Also, on receiving new information, the investigators may consider it best to withdraw you from the study or discontinue the study itself. In this situation the investigator will explain the reasons and arrange for your care to continue.

Will my GP be told I am in the study?

Yes, if you agree, we will tell your GP that you are taking part in the study.

If I need an interpreter, can one be provided?

Yes, if you require an interpreter we will provide one.

Will my taking part in the study be kept confidential?

If you consent to take part in this study your medical records will be reviewed by the research team and study investigators for the purposes of analysing the results. All information collected about you during the research will be kept strictly confidential. No material that could potentially identify you will be used in any reports of this study.

Data will be transmitted overseas and entered into an electronic data collection system managed by University of British Columbia, Vancouver, Canada. Data in this electronic system will be stored under your unique study ID number only. Identifying details such as your name, address and national hospital number will be stored separately to your study ID number and will only be known to the research staff at your hospital and at the Auckland coordinating centre, Department of Obstetrics and Gynaecology, University of Auckland, New Zealand. All hard copy data will be stored at your hospital and in the Department of Obstetrics and Gynaecology, University of Auckland, New Zealand. Records will be stored in a locked room and will be kept for a minimum of 10 years. The Principal Investigators will be responsible for their safe keeping. Access to your personally identifying information will be restricted to the research staff at your hospital, the Lead Principal and Co-investigators at the Auckland coordinating centre and research staff at the Auckland coordinating centre appointed by the Lead Principal Investigator.

Your data may also be accessed by an approved auditor appointed by an Ethics Committee or regulatory authority responsible for overseeing this research, or their approved representative, as required by law for the sole purpose of checking
the accuracy of the information recorded for this study or to ensure the rights and wellbeing of study participants are protected.

Can I find out the results of the study and what drug I received?

If you would like to know the results of the study and whether you received the active or placebo drug we will send you a summary of the study and your treatment allocation once the trial is complete (this is not expected to occur until 2016 or 2017).

Who is organising and funding the research?

The study is being organised by the Department of Obstetrics and Gynaecology, University of Auckland. The study is being funded by a Health Research Council (HRC) project grant (13/242). Each recruiting site will be provided with funding to support recruitment and data entry. Doctors conducting the research will not receive any money for recruiting women to the study or for looking after women in the study.

Where can I get more information about the study?

If you need more information about the study you can contact the Lead Principal Investigator Dr Katie Groom, your local study Investigator and/or the local research midwife (contact details on page 1). You may wish to have a friend, family or whānau support person to help you ask questions and understand the study.

If you require Māori cultural support talk to your whānau in the first instance. Alternatively you may contact the administrator for He Kamaka Waiora (Māori Health Team) by telephoning 09 486 8324 ext 2324.

If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact and independent health and disability advocate:

Freephone: 0800 555 050
Freephone: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

Thank you for taking the time to read this and for thinking about being involved in the study. Please feel free to contact the researchers if you have any questions about this study.

YOU WILL BE PROVIDED WITH A COPY OF THIS FORM TO KEEP

This study has been reviewed and received ethical approval from the Northern A Health and Disability Ethics Committee (CEN/12/06/028).
## Consent form

Name of Study: STRIDER (NZAuss): A Randomised Controlled Trial of Sildenafil Therapy In Dismal Prognosis Early-Onset Intraterine Growth Restriction (New Zealand and Australia).

### Request for interpreter

<table>
<thead>
<tr>
<th>Language</th>
<th>Statement</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>English</td>
<td>I wish to have an interpreter</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Māori</td>
<td>E hiaha ana ahau ki tetahi kaiwhaka Māori/kaiwhaka pakeha korero</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cook Island Māori</td>
<td>Ka inangaro au i tetai tangata uri reo</td>
<td>Ae</td>
<td>Kae</td>
</tr>
<tr>
<td>Fijian</td>
<td>Au gadreva me dua e vakadewa vosa vei au</td>
<td>Io</td>
<td>Seg</td>
</tr>
<tr>
<td>Niuean</td>
<td>Fia manako au ke fakaega e taha tagata fakahokohoko kupu</td>
<td>E</td>
<td>Nakai</td>
</tr>
<tr>
<td>Sāmoan</td>
<td>Ou te mana'oa ia i ai se fa'amataia upu</td>
<td>Io e</td>
<td>Leai</td>
</tr>
<tr>
<td>Tokelau</td>
<td>Ko au e fofou ki he tino ke fakaliflu te gagana Pelecania ki na gagana o na motu o te Fafetika</td>
<td>Io e</td>
<td>Leai</td>
</tr>
<tr>
<td>Tongan</td>
<td>Oku ou fiema'au he fakatoru'ea</td>
<td>Io</td>
<td>Ikei</td>
</tr>
</tbody>
</table>

1. I have read and I understand the information sheet dated 5th July 2016 for volunteers taking part in the STRIDER NZAuss study designed to compare the efficacy of sildenafil tablets to placebo tablets in improving fetal growth velocity in severe early onset intruterine growth restriction (IUGR). I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

2. I have had the opportunity to use whānau/family support or a friend to help me ask questions and understand the study.
3. I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will in no way affect my continuing health care.

4. I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.

5. I understand that the research staff will collect and process information about myself and my baby, including information about my health and the health of my baby.

6. I understand that the treatment, or investigation, will be stopped if it should appear harmful to me.

7. I understand the compensation provisions for this study.

8. I have had time to consider whether to take part in the study.

9. I know who to contact if I have any side effects from the study.

10. I know who to contact if I have any questions about the medication used in this study or about the study in general.

---

I consent to blood tests to check my blood count, kidney and liver function. I understand these samples will not be stored for any further research purposes.

[ ] Yes  [ ] No

I consent to a placental biopsy being taken after the delivery of my baby. I understand that this sample will be stored for a period of up to 5 years. *(Auckland Study Centre only)*

[ ] Yes  [ ] No

I consent to a myometrial (uterine muscle wall) biopsy being taken at the time of delivery if I need a caesarean section. I understand that this sample will be stored for a period of up to 5 years. *(Auckland Study Centre only)*

[ ] Yes  [ ] No

I consent to the study investigators contacting me when my child is around two years old.

[ ] Yes  [ ] No

I wish to receive a copy of the results when they are available.

Please note that a significant delay may occur between data collection and publication of the results.

[ ] Yes  [ ] No

I agree to my GP or other current provider being informed of my participation in this study.

[ ] Yes  [ ] No
Declaration by participant:

I hereby consent to take part in this study.

Participant’s name: ____________________________________________

Signature: __________________________ Date: ________________

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant’s questions about it. I believe that the participant understands the study and has given informed consent to participate.

Researcher’s name: __________________________________________

Signature: __________________________ Date: ________________

<table>
<thead>
<tr>
<th>Lead Principal Investigator</th>
<th>Local Hospital Investigators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Katie Groom</td>
<td>Dr Katie Groom</td>
</tr>
<tr>
<td>Senior Lecturer in Obstetrics and Gynaecology</td>
<td>Mobile: 021 245 9622</td>
</tr>
<tr>
<td>Department of Obstetrics and Gynaecology</td>
<td>Professor Peter Stone</td>
</tr>
<tr>
<td>University of Auckland, New Zealand</td>
<td>Mobile: 021 864726</td>
</tr>
<tr>
<td>Level 12, Support Building</td>
<td>Laura Mackay, Clinical Trial Manager</td>
</tr>
<tr>
<td>Auckland City Hospital</td>
<td>Phone: 09 3737599 ext 81366</td>
</tr>
<tr>
<td>Phone number: 09 3737599 ext 98923</td>
<td>Research Midwife: 021 003 14824</td>
</tr>
<tr>
<td>or 021 245 9622</td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1. A copy of the consent form is to be provided to each participant and a copy is to be placed in the medical file.
8 References


OYSTON, C., STANLEY, J., OLIVER, M., BLOOMFIELD, F. and BAKER, P., 2016. Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-


PIMENTEL, G., FIGUEROA, J.P., MITCHELL, M.D., MASSMANN, A. and
NATHANIELSZ, P.W., 1986. Effect of fetal and maternal intravascular antipyrine infusion
on maternal plasma prostaglandin concentrations in the pregnant sheep at 104 to 127 days'

PIPER, J.M., XENAKIS, E.M., MCFARLAND, M., ELLIOTT, B.D., BERKUS, M.D. and
LANGER, O., 1996. Do growth-retarded premature infants have different rates of perinatal
 morbidity and mortality than appropriately grown premature infants? Obstetrics and

normal first pregnancy. The Journal of obstetrics and gynaecology of the British
Commonwealth, 80(10), pp. 884.

of endothelium-dependent relaxation in rat isolated mesenteric artery. British journal of

artery Doppler at 11 + 0 to 13 + 6 weeks and 21 + 0 to 24 + 6 weeks in the prediction of pre-
eclampsia. Ultrasound in Obstetrics and Gynecology, 32, pp. 138-146.

POSTON, L., CALEYACHETTY, R., CNATTINGIUS, S., CORVALAN, C., UAUY, R.,

the maternal and feto-placental arterial beds. Pharmacology & therapeutics, 65(2), pp. 215-
239.

POUDEL, R., STANLEY, J.L., RUEDA-CLAUSEN, C.F., ANDERSSON, I.J., SIBLEY,
C.P., DAVIDGE, S.T. and BAKER, P.N., 2013. Effects of resveratrol in pregnancy using
murine models with reduced blood supply to the uterus. PloS one, 8(5), pp. e64401.

trophoblast invasion in first trimester pregnancies with high-resistance uterine artery Doppler


PYRIOCHOU, A., ZHOU, Z., KOIKA, V., PETROU, C., CORDOPATIS, P., SESSA, W.C.
and PAPAPETROPOULOS, A., 2007. The phosphodiesterase 5 inhibitor sildenafil stimulates

QUESADA, O., GOTMAN, N., HOWELL, H.B., FUNAI, E.F., ROUNSAVILLE, B.J. and
YONKERS, K.A., 2012. Prenatal hazardous substance use and adverse birth outcomes. The
journal of maternal-fetal & neonatal medicine : the official journal of the European
Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 25(8), pp. 1222-1227.


ROYAL COLLEGE OF OBSTETRICIANS AND GYNAECOLOGISTS, 22/03/2013, 2013-last update, Small for Gestational Age Fetus, Investigation and Management (Green-top Guideline No. 31) [Homepage of Royal College of Obstetricians and Gynaecologists],


