Antenatal Sildenafil Citrate Treatment in a Mouse Model of Fetal Growth Restriction: Effects on Fetus and Offspring.

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SCHOOL OF MEDICINE
# Chapter 1 Introduction

1. **Overview**

2. **Fetal Growth Restriction**
   - 1.2.1.1 Definition of FGR
   - 1.2.1.2 Diagnosis of FGR
   - 1.2.1.3 Classification of FGR infant
   - 1.2.1.4 Aetiology of FGR
   - 1.2.2 Fetal Vascular Blood Flow in Normal and Complicated Pregnancies
   - 1.2.3 Long-Term Health Implications of Poor Growth In Utero

3. **Placental Function**
   - 1.3.1.1 Vascular function
   - 1.3.1.2 Nutrient transport

4. **Placental Dysfunction in FGR**
   - 1.4.1 Altered Uterine Blood Flow Velocity in Human FGR
   - 1.4.2 Altered FetoPlacental Vascular Structure and Placental Villous Development in Human FGR
   - 1.4.3 Altered Myometrial and Chorionic Plate Artery Vascular Reactivity in Human FGR
   - 1.4.4 Altered Nutrient Exchange in Human FGR
   - 1.4.5 Altered Nutrient Exchange in Mouse FGR

5. **Cardiovascular Adaptations to Pregnancy**
   - 1.5.1 Cardiovascular Adaptations in Human Pregnancy
   - 1.5.2 Cardiovascular Adaptations in Mouse Pregnancy

6. **Consequences of Suboptimal Intrauterine Conditions; Long-Term Programming of Adult Disease**
   - 1.6.1 Causes of Intrauterine Programming of Adult Disease
   - 1.6.1.1 Diseases as causes of long-term programming?
   - 1.6.1.2 Placental insufficiency, hypoxia and growth restriction: causes of long-term programming?
   - 1.6.1.3 Diet and environment; association with long-term programming.
   - 1.6.2 Intrauterine Programming of Cardiovascular Disease
   - 1.6.2.1 Coronary heart disease
   - 1.6.2.2 Hypertension
   - 1.6.2.3 Mechanisms underlying pathogenesis of cardiovascular disease
   - 1.6.3 Intrauterine Programming of Metabolic Diseases
   - 1.6.3.1 Impaired glucose tolerance and insulin resistance
   - 1.6.3.2 Non-insulin dependent diabetes
   - 1.6.3.3 Mechanisms underlying pathogenesis of metabolic disease

7. **Mouse Models of Human Pregnancy Complications**
   - 1.7.1 Insulin-Like Growth Factor 1 Knockout Mouse
   - 1.7.2 Igf2 P0 and IGF2-Total Knockout Mouse
   - 1.7.2.1 Placental development and function in Igf2 P0 and Igf2-total knockout mice
   - 1.7.2.2 Postnatal observations in Igf2-P0 and Igf2-total knockout mice
   - 1.7.3 Endothelial Nitric Oxide Synthase
   - 1.7.4 Catechol-O-Methyltransferase

8. **Treatments for FGR**
   - 1.8.1 Low Molecular Weight Heparin
Chapter 2  Methods

2.1  GENERAL METHODS  
2.1.1  THE PLACENTAL-SPECIFIC Igf2 P0+/− MOUSE  
2.1.2  STANDARD HOUSING AND HUSBANDRY  
2.1.3  GENOTYPING  
2.1.4  GENDER GENOTYPING  
2.2  TREATMENT  
2.2.1  SILDENAFIL CITRATE ADMINISTERED VIA DRINKING WATER  
2.2.2  SILDENAFIL CITRATE ADMINISTERED VIA SUBCUTANEOUS INJECTION  
2.3  BLOOD VESSEL DISSECTION AND NORMALISATION.  
2.3.1  FETAL ABDOMINAL AORTA  
2.3.2  ADULT ABDOMINAL AORTA  
2.3.3  ADULT MESENTERIC ARTERY  
2.4  WIRE MYOGRAPHY  
2.4.1  CHEMICALS AND SOLUTIONS  
2.4.2  WIRE MYOGRAPHY PROTOCOL  
2.5  ASSESSMENT OF PROGENY FROM SC TREATED AND UNTREATED PREGNANCIES  
2.5.1  BODYWEIGHT  
2.5.2  NON-INVASIVE SYSTOLIC BLOOD PRESSURE MEASUREMENTS USING TAIL-CUFF PLETHYSMOGRAPHY  
2.5.3  GLUCOSE TOLERANCE TESTS  
2.5.4  ABDOMINAL AORTA AND MESENTERIC ARTERY VESSEL FUNCTION  
2.5.5  TISSUE COLLECTION  
2.6  STATISTICAL ANALYSES  
2.6.1  CALCULATIONS FOR WIRE MYOGRAPHY  
2.6.2  DATA ANALYSIS  

Chapter 3  In Vitro Assessment of Mouse Fetal Abdominal Aortic Vascular Function: Effect of Sildenafil Citrate
3.2.3 Relaxation responses of fetal abdominal aortas to acetylcholine and sodium nitroprusside

3.2.4 Effect of maternal sildenafil citrate treatment on prenatal weight measurements in WT and P0 knockout mice at embryonic day 18.5

3.2.5 Effect of maternal sildenafil citrate treatment on contraction responses of fetal abdominal aortas

3.2.6 Effect of maternal sildenafil citrate treatment on relaxation responses of fetal abdominal aortas

3.3 Discussion

3.3.1 P0 mouse phenotype

3.3.2 Myography studies of mouse fetal abdominal aortic function

3.3.3 Fetal abdominal aortic function in appropriate weight and growth restricted fetuses

3.3.3.1 Effect of potassium-, phenylephrine- and U46619- induced contraction of fetal abdominal aortas from appropriate weight and growth restricted fetuses

3.3.3.2 Acetylcholine- and sodium nitroprusside-induced endothelial-dependent and independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses.

3.3.4 Effect of maternal sildenafil citrate treatment in appropriate weight and growth restricted fetuses

3.3.5 Effect of maternal sildenafil citrate treatment on fetal abdominal aortic function in appropriate weight and growth restricted fetuses

3.3.5.1 Effect of sildenafil citrate on potassium-, phenylephrine- and U46619- induced contraction of fetal abdominal aortas from growth restricted and appropriate weight fetuses

3.3.5.2 Effect of sildenafil citrate on acetylcholine- and sodium nitroprusside-induced endothelial-dependent and independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses

3.3.6 Summary

Chapter 4 Subcutaneous Injection of Sildenafil Citrate: Effects on Fetal Growth and Fetal Vascular Function

4.1 Introduction

4.2 Results

4.2.1 Effect of a subcutaneous injection of saline on contraction responses of fetal abdominal aortas

4.2.2 Relaxation responses of fetal abdominal aortas to acetylcholine and sodium nitroprusside from pregnancies administered a subcutaneous injection of saline.

4.2.3 Effect of a subcutaneous injection of SC on prenatal weight measurements in wild type and P0 knockout mice at embryonic day 18.5

4.2.4 Effect of a subcutaneous injection of SC on contraction responses of fetal abdominal aortas

4.2.5 Effect of subcutaneous injection of SC on relaxation responses of fetal abdominal aortas

4.3 Discussion

4.3.1 P0 mouse phenotype from saline-injected pregnancies
4.3.2 Myography Studies of Mouse Fetal Abdominal Aortic Function from Saline-Injected Pregnancies

4.3.2.1 Potassium-, phenylephrine- and U46619-induced contraction of fetal abdominal aortas from appropriate weight and growth restricted fetuses whose mothers were injected with saline during pregnancy

4.3.2.2 Acetylcholine- and sodium nitroprusside-induced endothelial-dependent and -independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses whose mothers were injected with saline during pregnancy

4.3.3 Differential Effects of Maternal Exposure to a Saline-Injection or to Water on Fetal Vascular Function

4.3.4 Effect of Maternal Sildenafil Citrate Treatment via Subcutaneous Injection in Appropriate Weight and Growth Restricted Fetuses

4.3.5 Effect of Maternal Sildenafil Citrate Treatment via Subcutaneous Injection on Potassium-, phenylephrine- and U46619-induced contraction of fetal abdominal aortas from growth restricted and appropriate weight fetuses

4.3.6 Differential Effects of Maternal Exposure to a Sildenafil Citrate-Injection or to Sildenafil Citrate in Drinking Water on Fetal Vascular Function

4.3.7 Mechanisms of Action of Sildenafil Citrate

4.3.8 Summary

Chapter 5 Long-Term Effects of Antenatal Sildenafil Citrate Treatment on Offspring Health

5.1 Introduction

5.2 Results

5.2.1 Effect of an Antenatal Subcutaneous Injection of Sildenafil Citrate on Expected: Observed Ratios of Fetuses and Offspring

5.2.2 Growth Trajectories of Igf2 P0+/− and WT Offspring from Control C57BL6/J Dams; Effect of Sex

5.2.3 Effect of Antenatal Sildenafil Citrate on Growth Trajectories of Igf2 P0+/− and WT Offspring

5.2.4 Systolic Blood Pressure in Igf2 P0+/− and WT Offspring from Control C57BL6/J Dams; Effect of Sex

5.2.5 Effect of Antenatal Sildenafil Citrate on Systolic Blood Pressure in Igf2 P0+/− and WT Offspring

5.2.6 Blood Glucose Concentration in Igf2 P0+/− and WT Offspring from Control C57BL6/J Dams; Effects of Sex

5.2.7 Effect of Antenatal Sildenafil Citrate on Blood Glucose Concentration in Igf2 P0+/− and WT Offspring

5.2.8 Offspring Organ Weight in Igf2 P0+/− and WT Mice from Control C57BL6/J Dams; Effect of Gender
5.2.9 Effect of antenatal Sildenafil citrate on offspring organ weight in \( \text{Igf2 P0}^{+/} \) and WT mice

5.2.10 Effect of an antenatal subcutaneous injection of saline on contraction responses of offspring abdominal aortas

5.2.11 Relaxation responses of offspring abdominal aortas to Acetylcholine and Sodium nitroprusside from pregnancies administered a subcutaneous injection of saline

5.2.12 Effect of an antenatal subcutaneous injection of Sildenafil citrate on contraction responses of offspring abdominal aortas

5.2.13 Effect of antenatal subcutaneous injection of Sildenafil citrate on relaxation responses of offspring abdominal aortas

5.2.14 Effect of an antenatal subcutaneous injection of saline on contraction responses of offspring abdominal aortas

5.2.15 Relaxation responses of offspring mesenteric arteries to Acetylcholine and Sodium nitroprusside from pregnancies administered a subcutaneous injection of saline.

5.2.16 Effect of an antenatal subcutaneous injection of Sildenafil citrate on contraction responses of offspring mesenteric arteries

5.2.17 Effect of an antenatal subcutaneous injection of Sildenafil citrate on relaxation responses of offspring mesenteric arteries

5.3 Discussion

5.3.1 Neonatal and postnatal expected and observed ratios of \( \text{Igf2 P0}^{+/} \) and WT mice from control C57BL6/J dams

5.3.2 Effect of antenatal Sildenafil citrate on neonatal and postnatal expected and observed ratios in \( \text{Igf2 P0}^{+/} \) and WT mice

5.3.3 Growth trajectories of \( \text{Igf2 P0}^{+/} \) and WT offspring from control C57BL6/J dams; effect of sex

5.3.4 Effect of antenatal Sildenafil citrate on growth trajectories of \( \text{Igf2 P0}^{+/} \) and WT offspring

5.3.5 Systolic blood pressure in \( \text{Igf2 P0}^{+/} \) and WT offspring from control C57BL6/J dams; effect of sex

5.3.6 Effect of antenatal Sildenafil citrate on systolic blood pressure in \( \text{Igf2 P0}^{+/} \) and WT offspring

5.3.7 Blood glucose concentration in \( \text{Igf2 P0}^{+/} \) and WT offspring from control C57BL6/J dams; effects of sex

5.3.8 Effect of antenatal Sildenafil citrate on blood glucose concentration in \( \text{Igf2 P0}^{+/} \) and WT offspring

5.3.9 Offspring organ weight in \( \text{Igf2 P0}^{+/} \) and WT mice from control C57BL6/J dams; effect of sex

5.3.10 Effect of antenatal Sildenafil citrate on offspring organ weight in \( \text{Igf2 P0}^{+/} \) and WT mice

5.3.11 Offspring vasoconstriction and vasorelaxation in \( \text{Igf2 P0}^{+/} \) and WT mice from control C57BL6/J dams; effect of sex

5.3.12 Effect of antenatal Sildenafil citrate on offspring vasoconstriction and vasorelaxation in \( \text{Igf2 P0}^{+/} \) and WT mice

5.4 Summary

Chapter 6 General Discussion
6.1 DISCUSSION

6.1.1 METHODS TO STUDY FETAL VASCULAR FUNCTION IN MOUSE PREGNANCY AND ASSESS THE IMPACT OF ANTENATAL TREATMENT WITH SILDENAFIL CITRATE ON FETAL VASCULAR FUNCTION.

6.1.2 IMPACT OF ANTENATAL TREATMENT WITH SILDENAFIL CITRATE ON OFFSPRING GROWTH, CARDIOVASCULAR AND METABOLIC FUNCTION

6.1.3 EFFECT OF FGR ON PROGRAMMING OF ADULTHOOD DISEASE IN THE IGF2 POLYCOMBO/MOUSE.

6.1.4 EFFECTS OF A MATERNAL SUBCUTANEOUS INJECTION OF SALINE OR SILDENAFIL CITRATE

6.1.5 CLINICAL PERSPECTIVE

6.2 CONCLUSIONS

6.3 FUTURE WORK

Chapter 7 References

Chapter 8 Appendix

8.1 ADDITIONAL DATA

8.2 PUBLICATIONS

8.3 PRESENTATIONS

FINAL WORD COUNT: 63,279
LIST OF FIGURES

CHAPTER 1
Figure 1.1 Second trimester umbilical (A-D; left) and uterine (E-F; right) artery Doppler waveforms in normal and complicated pregnancies............................26

CHAPTER 2
Figure 2.1 Study design for Sildenafil citrate administered via drinking water.........................75
Figure 2.2 Study design for Sildenafil citrate administered via subcutaneous injection....... 76

CHAPTER 3
Figure 3.1. Fetal and placental weights from WT and P0 knockout mice.........................91
Figure 3.2. Fetal weight frequency distribution curve from individual fetuses; WT (black solid curve) and P0 (black dashed curve)........................................92
Figure 3.3. Fetal:placental weight ratios from WT and P0 knockout mice..........................93
Figure 3.4. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619..................................................................................96
Figure 3.5. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS.................................97
Figure 3.6. Dose response curves of fetal abdominal aortas to increasing doses of ACH........99
Figure 3.7. Dose response curves of fetal abdominal aortas to increasing doses of SNP......100
Figure 3.8. Fetal and placental weights from WT and P0 knockout mice following sildenafil citrate treatment.................................................................103
Figure 3.9. Fetal weight frequency distribution curve from individual fetuses following sildenafil citrate treatment.................................................................104
Figure 3.10. Fetal:placental weight ratios from WT and P0 mice of dams following sildenafil citrate treatment.................................................................105
Figure 3.11. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 following sildenafil citrate treatment.................................108
Figure 3.12. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following sildenafil citrate treatment.................................................................109
Figure 3.13. Dose response curves of fetal abdominal aortas to increasing doses of ACH following sildenafil citrate treatment.................................................111
Figure 3.14. Dose response curves of fetal abdominal aortas to increasing doses of SNP following sildenafil citrate treatment................................................112

CHAPTER 4
Figure 4.1. Dose response curves of fetal abdominal aortas from saline-injected pregnancies in response to increasing doses of U46619...............................133
Figure 4.2. Figure 4.2 Dose response curves of fetal abdominal aortas from saline-injected pregnancies in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS.................................................................134
Figure 4.3. Dose response curves of fetal abdominal aortas from saline-injected pregnancies to increasing doses of ACH. ................................................................. 136
Figure 4.4. Dose response curves of fetal abdominal aortas from saline-injected pregnancies to increasing doses of SNP. ................................................................. 137
Figure 4.5. Fetal and placental weights from WT and P0 knockout mice following a subcutaneous injection of sildenafil citrate. ................................................................. 140
Figure 4.6. Fetal weight frequency distribution curve from individual fetuses following a subcutaneous injection of sildenafil citrate. ................................................................. 141
Figure 4.7. Fetal:placental weight ratios from WT and P0 mice from dams administered a subcutaneous injection of sildenafil citrate. ................................................................. 142
Figure 4.8. Dose response curves of fetal abdominal aortas from in response to increasing doses of U46619 following a subcutaneous injection of saline or sildenafil citrate. ................................................................. 145
Figure 4.9. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following sildenafil citrate treatment ................................................................................................................................. 146
Figure 4.10. Dose response curves of fetal abdominal aortas to increasing doses of ACH following sildenafil citrate treatment ................................................................................................................................. 148
Figure 4.11. Dose response curves of fetal abdominal aortas to increasing doses of SNP following sildenafil citrate treatment ................................................................................................................................. 149

Chapter 5
Figure 5.1. Chart illustrating the ratios of WT and Igf2 P0+/− fetuses (left column) and offspring (right column) from saline-treated (A and B) and sildenafil citrate-treated pregnancies (C and D) ................................................................................................................................. 169
Figure 5.2. Mean weekly body weight of offspring from saline-treated pregnancies (C and D) ................................................................................................................................. 170
Figure 5.3. Comparison of mean weekly body weight of WT (top) and Igf2 P0+/− (bottom) offspring from saline-treated (N = 9) and sildenafil citrate-treated pregnancies (N = 11) ................................................................................................................................. 171
Figure 5.4. Systolic blood pressure of WT and Igf2 P0+/− offspring from saline-treated pregnancies ................................................................................................................................. 173
Figure 5.5. Comparison of systolic blood pressure of WT (top) and Igf2 P0+/− (bottom) offspring from saline-treated and sildenafil citrate-treated pregnancies ................................................................................................................................. 174
Figure 5.6. Comparison of glucose tolerance profiles of WT (top) and Igf2 P0+/− (bottom) offspring from saline-treated pregnancies ................................................................................................................................. 176
Figure 5.7. Comparison of glucose tolerance profiles of WT (top) and Igf2 P0+/− (bottom) offspring from saline-treated and sildenafil citrate-treated pregnancies ................................................................................................................................. 177
Figure 5.8. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of U46619 ................................................................................................................................. 182
Figure 5.9. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of U46619 as a % of maximal contraction to KPSS ................................................................................................................................. 183
Figure 5.10. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of ACH ................................................................................................................................. 185
Figure 5.11. Dose response curves of offspring abdominal aortas from saline-treated pregnancies in response to increasing doses of SNP ................................................................................................................................. 186
Figure 5.12. Dose response curves of offspring abdominal aortas in response to increasing doses of U46619 following an antenatal subcutaneous injection of saline or sildenafil citrate. .................................................................190

Figure 5.13. Dose response curves of offspring abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following an antenatal subcutaneous injection of saline or sildenafil citrate. .................................................................191

Figure 5.14. Dose response curves of offspring abdominal aortas to increasing doses of ACH following sildenafil citrate treatment. .................................................................193

Figure 5.15. Dose response curves of offspring abdominal aortas to increasing doses of SNP following sildenafil citrate treatment. .................................................................194

Figure 5.16. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of U46619. .................................................................199

Figure 5.17. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS. .................................................................200

Figure 5.18. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of ACH. .................................................................202

Figure 5.19. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies to increasing doses of SNP. Panel A-D; Arteries were pre-contracted with an EC₈₀ dose of U46619. .................................................................203

Figure 5.20. Dose response curves of offspring mesenteric arteries in response to increasing doses of U46619 following an antenatal subcutaneous injection of saline or sildenafil citrate. .................................................................207

Figure 5.21. Dose response curves of offspring mesenteric arteries in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following an antenatal subcutaneous injection of saline or sildenafil citrate. .................................................................208

Figure 5.22. Dose response curves of offspring mesenteric arteries to increasing doses of ACH following sildenafil citrate treatment. .................................................................210

Figure 5.23. Dose response curves of offspring mesenteric arteries to increasing doses of SNP following sildenafil citrate treatment. .................................................................211

Chapter 7 Appendix

Figure 7.1. Offspring plasma corticosterone concentrations. .................................................................274
LIST OF TABLES

CHAPTER 1
Table 1.1. Maternal hemodynamic changes during pregnancy, peripartum and postpartum in the human. .................................................................42
Table 1.2. Common animal models of human pregnancy illustrating advantages and disadvantages of each. .................................................................53
Table 1.3. Mouse models of FGR; comparison with human FGR with arrows indicating trends.................................................................56

CHAPTER 2
Table 2.1. Reagents for a 1 X PCR reaction using the Expand High Fidelity PCR system.........72
Table 2.2. Reagents for a 1 X PCR reaction using the Expand High Fidelity PCR system.........73

CHAPTER 3
Table 3.3. Physiological salt solutions used in wire myography experiments (in mM per litre). .......................................................................................................80
Table 3.1. Fetal abdominal aorta diameter, basal tone and max contraction data. KPSS (120 mM high potassium salt solution); PE (10^{-5} M phenylephrine); U46619 (2x10^{-6} M thromboxane-A2 mimetic).................................................................95
Table 3.2. Summary table comparing fetal/placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619; expressed as kPa) and relaxation responses (ACH, SNP).........................................................................................101
Table 3.3. Fetal abdominal aorta diameter, basal tone and maximal contraction data following sildenafil citrate treatment. .................................................................107
Table 3.4. Summary table demonstrating the effect of maternal sildenafil citrate treatment (0.8 mg.ml^{-1}) on fetal / placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619; expressed as kPa) and relaxation responses (ACH, SNP) for each group when compared with drinking water controls..........................113
Table 3.5 Fetal abdominal aorta sensitivity to U46619, ACH and SNP........................................114

CHAPTER 4
Table 4.1. Fetal abdominal aorta diameter, basal tone and max contraction data from saline injected pregnancies. .................................................................132
Table 4.2. Summary table comparing fetal / placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619) and relaxation responses (ACH, SNP) from saline-injected pregnancies (chapter 4) and water control pregnancies. ..................138
Table 4.3. Comparison of fetal abdominal aortic diameter, basal tone and max contraction from saline and sildenafil citrate (10 mg.kg^{-1})—injected pregnancies. KPSS (120 mM high potassium salt solution); PE (10^{-5} M phenylephrine); U46619 (2x10^{-6} M thromboxane-A2 mimetic). .................................................................144
Table 4.4. Fetal abdominal aorta sensitivity to U46619, ACH and SNP following subcutaneous injection of saline or SC........................................................................................................................................150
TABLE 4.5. SUMMARY TABLE DEMONSTRATING THE EFFECT OF MATERNAL SILDENAFIL CITRATE TREATMENT (10 mg.kg⁻¹) ON FETAL/PLACENTAL WEIGHT, BASAL TONE, ABDOMINAL AORTA DIAMETER, CONTRACTION (KPSS, PE, U46619) AND RELAXATION RESPONSES (ACH, SNP) FOR EACH GROUP, COMPARED WITH EQUIVALENT FETUSES FROM SALINE-INJECTED PREGNANCIES. ........................................151

TABLE 4.6. SUMMARY TABLE DEMONSTRATING THE EFFECT OF ROUTE OF ADMINISTRATION OF SILDENAFIL CITRATE COMPARED WHEN COMPARED WITH EQUIVALENT CONTROLS........................................152

CHAPTER 5

TABLE 5.1. OFFSPRING ORGAN ALLOMETRY FROM SALINE- AND SILDENAFIL-TREATED PREGNANCIES. ORGAN WET WEIGHTS WERE MEASURED BETWEEN POSTNATAL WEEK 14 AND WEEK 16.................................179

TABLE 5.2. OFFSPRING ABDOMINAL AORTIC DIAMETER, BASAL TONE AND MAX CONTRACTION DATA FROM SALINE-TREATED PREGNANCIES. .............................................................................181

TABLE 5.3. SUMMARY TABLE COMPARING FETAL (DATA FROM CHAPTER 4) AND OFFSPRING (CHAPTER 5) ABDOMINAL AORTIC BASAL TONE, DIAMETER, MAXIMAL CONTRACTION (KPSS, PE), CONTRACTION DOSE RESPONSE (U46619) AND RELAXATION DOSE RESPONSES (ACH, SNP) FROM SALINE-TREATED DAMS. ..................................................................................................................187

TABLE 5.4. COMPARISON OF OFFSPRING ABDOMINAL AORTIC DIAMETER, BASAL TONE AND MAX CONTRACTION DATA FROM SALINE AND SILDENAFIL CITRATE (10 mg.kg⁻¹) –TREATED PREGNANCIES. KPSS (120 mM HIGH POTASSIUM SALT SOLUTION); PE (10⁻³ M PHENYLEPHRINE); U46619 (2x10⁻⁶ M THROMBOXANE-A₂ MIMETIC).............................................................189

TABLE 5.5. ADULT ABDOMINAL AORTA SENSITIVITY TO U46619, ACH AND SNP FOLLOWING SUBCUTANEOUS INJECTION OF SALINE OR SC.................................................................195

TABLE 5.6. SUMMARY TABLE DEMONSTRATING THE EFFECT OF MATERNAL SILDENAFIL CITRATE TREATMENT (10 mg.kg⁻¹) ON FETAL AND OFFSPRING ABDOMINAL AORTIC BASAL TONE, DIAMETER, MAXIMAL CONTRACTION (KPSS, PE), CONTRACTION DOSE RESPONSES (U46619) AND RELAXATION DOSE RESPONSES (ACH, SNP) FOR EACH GROUP, COMPARED WITH EQUIVALENT FETUSES AND OFFSPRING AORTAS FROM SALINE-TREATED PREGNANCIES. .........................................................196

TABLE 5.7. OFFSPRING MESENTERIC ARTERY DIAMETER, BASAL TONE AND MAXIMAL CONTRACTION (KPSS, PE, U46619) DATA FROM SALINE TREATED PREGNANCIES. .................................................................198

TABLE 5.8. SUMMARY TABLE COMPARING OFFSPRING MESENTERIC AND AORTIC BASAL TONE, DIAMETER, MAXIMAL CONTRACTION (KPSS, PE), CONTRACTION RESPONSE (U46619) AND RELAXATION RESPONSES (ACH, SNP) FROM SALINE INJECTED DAMS. ................................................................198

TABLE 5.9. COMPARISON OF OFFSPRING MESENTERIC ARTERY DIAMETER, BASAL TONE, MAXIMAL CONTRACTION (KPSS, PE, U46619) DATA FROM SALINE AND SILDENAFIL CITRATE (10 mg.kg⁻¹) – TREATED PREGNANCIES.................................................................204

TABLE 5.10. ADULT MESENTERIC ARTERY SENSITIVITY TO U46619, ACH AND SNP FOLLOWING SUBCUTANEOUS INJECTION OF SALINE OR SC..........................206

TABLE 5.11. SUMMARY TABLE COMPARING THE EFFECT OF MATERNAL SILDENAFIL CITRATE TREATMENT (10 mg.kg⁻¹) ON OFFSPRING MESENTERIC AND AORTIC BASAL TONE, DIAMETER, MAXIMAL CONTRACTION (KPSS, PE), CONTRACTION RESPONSE (U46619) AND RELAXATION RESPONSES (ACH, SNP) FOR EACH GROUP, COMPARED WITH EQUIVALENT OFFSPRING ARTERIES FROM SALINE-TREATED PREGNANCIES. ..............................................................................208
ABSTRACT

Fetal growth restriction (FGR), when a fetus fails to reach its genetic growth potential, affects up to 10% of pregnancies and is a major risk factor for both neonatal and adulthood morbidity and mortality. There are currently no treatments for FGR except for delivery of the fetus; resulting in premature delivery which, in itself, is linked to poor outcome. Therefore, the focus of current research is to examine whether therapies successfully used to treat diseases with similar aetiologies to FGR can also be used to treat FGR. Sildenafil citrate (SC), a selective phosphodiesterase-5 inhibitor, is one such candidate. With the recent announcement of the STRIDER international clinical trial for the treatment of severe FGR with SC, it is imperative to determine the efficacy and safety of SC treatment on both fetus in utero and long-term adult health. Mouse models that mimic characteristics of human FGR represent an attractive model to perform pre-clinical studies. Recent studies in mice have demonstrated that SC increased fetal and placental weight and normalised umbilical artery blood flow velocity in FGR but no studies have assessed effects of antenatal SC on offspring health. The aims of this study were to assess the effect of antenatal SC treatment on a) fetal weight b) fetal vascular reactivity b) pup viability and d) long-term effects on postnatal development / physiology in a mouse model of FGR.

All experiments were performed in the placental-specific insulin-like growth factor 2 knockout mouse (Igf2 P0+/− mice) which have mixed litters of wild-type (WT) and growth restricted (P0) mice. It has been reported that SC administered in the drinking water was able to increase P0 fetal weight and thus this mouse model was chosen to assess the effects of SC on the fetus and offspring. SC was administered to pregnant dams in two regimens; orally (120 – 160 mg.kg⁻¹) and subcutaneously (10 mg.kg⁻¹) between E12.5 and E18.5. WT and P0 fetal abdominal aortas were isolated at E18.5 and ex vivo vascular function was assessed using wire myography. Fetal abdominal aortas demonstrated reliable and reproducible vasocontraction and vasorelaxation; there were some sex- and genotype-specific differences. SC demonstrated dose-dependent effects on fetal aortic function. Offspring from dams treated with a subcutaneous injection of SC or saline were assessed for postnatal growth (week 5 – week 12), systolic blood pressure (week 8 and week 13), glucose tolerance (week 12) and mesenteric / aortic vascular function (week 14 – week 16). These experiments demonstrated that;

- A supratherapeutic concentration of antenatal SC (120 – 160 mg.kg⁻¹) did not increase fetal weight but significantly blunted relaxation responses of fetal abdominal aortas at E18.5.
- A subcutaneous injection of antenatal SC (10 mg.kg⁻¹) did not increase fetal weight or alter fetal abdominal aortic function in mice but led to increased systolic blood pressure in both WT and P0 offspring. Additionally, glucose sensitivity was significantly reduced in female offspring from SC treated dams.

In conclusion, the studies outlined in this thesis have demonstrated that antenatal SC treatment can cause alterations in fetal blood vessel function and also lead to changes in metabolic and cardiovascular function in mouse offspring. Using ex vivo wire myography, mouse fetal abdominal aortas were able to be assessed at E18.5. This methodological advance will be beneficial as it can be applied to assessing putative treatments in mice that show characteristics of human FGR. In addition, this technique will allow for investigation of the underlying mechanisms of in utero programming of adulthood cardiovascular diseases such as hypertension. Future work must focus on the mechanisms leading to increased systolic blood pressure in offspring from SC treated dams and whether such effects are noted in other animal models of FGR using a variety of SC dosing regimens. These studies will provide information with which to increase efficacy, and ensure the safety, of SC treatment in pregnancy complications.
DECLARATION

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-methoxyoestriol</td>
</tr>
<tr>
<td>ACH</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AREDV</td>
<td>Absent or reversed end-diastolic velocity</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CD-1</td>
<td>Cluster of differentiation-1</td>
</tr>
<tr>
<td>CESDI</td>
<td>Confidential Enquiry into Stillbirths and Deaths in Infancy</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanylate monophosphate</td>
</tr>
<tr>
<td>COMT</td>
<td>Catetchol-O-methyl transferase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental Origins of Health and Disease</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPREDA</td>
<td>Essai Pre-eclampsie Dipyridamole Aspirine</td>
</tr>
<tr>
<td>F:P</td>
<td>Fetal:placental weight ratio</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal growth restriction</td>
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<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GC</td>
<td>guanylate cyclase</td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational diabetes mellitus</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>ICR</td>
<td>Imprinting control region</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1 gene</td>
</tr>
<tr>
<td>IGF2</td>
<td>Insulin-like growth factor 2 protein</td>
</tr>
<tr>
<td>igf2</td>
<td>Insulin-like growth factor 2 gene</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IVS</td>
<td>Inter villous space</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KPSS</td>
<td>High potassium salt solution</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MeAIB</td>
<td>Methylaminoisobutyric acid</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
</tr>
<tr>
<td>MVM</td>
<td>Microvillous membrane</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>P0</td>
<td>Igf2 P0+/− mouse</td>
</tr>
<tr>
<td>P0 F</td>
<td>Igf2 P0+/− female</td>
</tr>
<tr>
<td>P0 M</td>
<td>Igf2 P0+/− male</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PET</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PI</td>
<td>Pulsatility index</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PJ</td>
<td>Pomegranate juice</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Cyclic guanylate monophosphate-dependent protein kinase</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SC</td>
<td>Sildenafil citrate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sflt-1</td>
<td>Soluble fms-like tyrosine-1</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneous hypertensive rat</td>
</tr>
<tr>
<td>SNAT</td>
<td>Sodium dependent neutral amino acid transporter</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SUAL</td>
<td>Single umbilical artery ligation</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tissue necrosis factor-α</td>
</tr>
<tr>
<td>u₂</td>
<td>Upstream exon 2</td>
</tr>
<tr>
<td>U46619</td>
<td>Thromboxane-A₂ mimetic</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>WT F</td>
<td>Wild type female</td>
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<tr>
<td>WT M</td>
<td>Wild type male</td>
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</table>
CONTRIBUTIONS FROM COLLABORATORS

All experiments performed by LJR other than:

Mounting of fetal abdominal aortas and adult mesenteric arteries onto the wire myograph system was performed by Lewis J Renshall and Dr. Mark Wareing. Genotyping was performed with the technical assistance of Elizabeth Cowley. *Igf2 P0*+/− knockout mice were originally a kind gift from Professor W Reik and Dr. M Constância.

Lewis Renshall
October 2014
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DEDICATION
For Mum and Dad.
Chapter 1  Introduction
1.1 Overview

Complications of pregnancy represent a major challenge to the National Health Service. Fetal growth restriction is a complication that occurs in up to 10% of pregnancies and is defined as a condition where the fetus fails to achieve its predefined growth potential. FGR is responsible for over a quarter of all stillbirths and accounts for 1 in 6 sudden infant deaths (Cantwell et al., 2011). These statistics emphasise the severity of FGR and provide evidence that this condition alone is responsible for a large proportion of pregnancy-related mortality and morbidity. Thus, it is estimated that, in the UK the NHS spends over £400 million annually on neonatal care of growth restricted babies (Cantwell et al., 2011). A number of large cohort epidemiological studies have demonstrated that, in addition to the immediate consequences to the fetus, being born small is linked with an increased risk of adulthood diseases such as hypertension and non-insulin dependent diabetes (Barker et al., 1989, Barker et al., 2010).

The most common aetiology of FGR is placental insufficiency, a term used to describe the inability of the placenta to adequately supply the fetus with the oxygen and nutrients necessary for it to reach its genetically predetermined growth potential. Evidence suggests that a major contributor to placental insufficiency is aberrant utero- and fetoplacental blood flow, leading to decreased oxygen and nutrient transport to the developing fetus. There are no therapies for FGR and in cases of severe early-onset FGR the only clinical option is premature delivery of the fetus (Fisk and Atun, 2008). Early delivery can often have adverse effects on maternal and/or fetal health and preterm babies are at greater risk of developing hypertension and metabolic disease in adulthood than babies delivered at term (Barker et al., 2010).

Few therapies have been identified for use in obstetrics over the past twenty years and there are no new therapies in clinical trials that have been designed specifically for use in obstetrics. The developmental abnormalities seen in babies whose mothers received thalidomide during their pregnancies in the 1950s / 1960s highlight the possible consequences of maternal drug exposure on the developing fetus. Safety concerns for fetal development are often the reason for the pharmaceutical industries lack of enthusiasm toward investment and research of obstetric drugs (Fisk and Atun., 2008). However, genetic mouse models have been utilised to test potential therapies; one such therapy is the vascular smooth muscle vasodilator Sildenafil Citrate (SC). SC was designed to treat adults with pulmonary hypertension but recently it has been demonstrated to improve fetal growth and blood supply in mouse models of FGR (Stanley et al., 2012, Dilworth et al., 2013). Data from the mouse and other animal models has culminated in a clinical trial (commencing in 2014) using SC to treat severe early-onset FGR (Ganzevoort et al., 2014).
It is essential that any treatments for FGR do not harm mother and fetus in the short-term and, importantly, do not increase risk of disease in either the mother or the progeny. The effects of SC treatment on the health of the fetus and offspring have yet to be determined. Therefore, utilising a mouse model of FGR, the main objectives of this thesis are to determine the effect of maternal SC administration (a) on fetal growth and fetal vascular function and (b) on the health of offspring as determined by postnatal growth trajectory, glucose tolerance, systolic blood pressure and arterial function.

1.2 Fetal Growth Restriction

FGR is a condition with multiple phenotypes; it is generally described as the inability of the fetus to reach its true genetic growth potential. It is important to note that there is an important distinction between a baby that may have been born fundamentally small but has reached their growth potential and a baby that has truly reduced / restricted growth. The former suggests that the baby has followed a normal growth trajectory and normal development whereas the latter suggests a restriction in growth pattern due to a pathological progression (Gardosi et al., 2006).

A UK report conducted in 2005 into maternal and child health, highlighted that two thirds of stillbirths and over three quarters of neonatal deaths occurred in babies whose birthweight was less than the 10th percentile, i.e. babies that were born small for gestational age (SGA), irrespective of whether or not there was underlying pathology (Weindling 2003). The 8th annual Confidential Enquiry into Stillbirths and Deaths in Infancy (CESDI) found that FGR was responsible for over a quarter of all stillbirths and accounted for 1 in every 6 sudden infant deaths (Cantwell et al., 2011). There were 698,512 live births in England and Wales in 2012 (www.ons.gov.uk). The incidence of stillbirths in the UK is approximately 1 in 200 pregnancies (3,274 in 2013) and this figure has not significantly altered in two decades. FGR therefore constitutes the largest single risk factor for stillbirth, largely due to the fact that FGR is not detected or treated adequately (Gardosi et al., 2013). These statistics highlight the significance of FGR on short-term fetal and neonatal health.

FGR infants are also at greater risk of long-term health complications such as cardiovascular disease (Barker et al., 1989). This was first identified by Barker and Osmond (1989), when evidence provided a link between low birth weight and an increased incidence of mortality from ischaemic heart disease at age 50 in men. Furthermore, studies have shown that being born SGA pre-disposes the infant to diseases such as chronic hypertension, type II diabetes, obesity and Alzheimer’s disease later in life (Barker, Osmond et al., 1989; Osmond and Barker 2000, Curham et al., 1996, Boney et al., 2005). These early studies developed the hypothesis that suboptimal growth in utero may permanently alter the structure and function of specific organs in the fetus.
and “programme” adulthood disease. The term Developmental Origins of Health and Disease (DOHaD) is now used to encompass the concepts that environmental insults during early development in utero alter the capacity to adapt to the environment in later life, thus placing an individual at greater risk of disease.

There are currently no treatments for FGR. In cases of severe early-onset FGR (i.e. onset of less than 32 weeks), early delivery of the fetus is often the only option available to clinicians; but premature delivery is often associated with poor outcome and risk of adulthood disease (Hofman et al., 2004). Therefore, to overcome the pharmaceutical industry’s lack of enthusiasm towards assessing therapies in pregnant women, as a first step, animal models of FGR are an appropriate option to test novel therapies. The sheep has often been used to assess the effect of placental nutrient transport on the fetus. Over-nourished adolescent ewes demonstrate reduced placental growth through reductions in placental angiogenic factors leading to growth restricted lambs (Wallace et al., 2004). Single umbilical artery ligation (SUAL) in the sheep, which reduces blood flow to the uterus, also leads to a significant reduction in fetal weight and increased fetal hypoxaemia (Supramaniam et al., 2006, Miller et al., 2009). However, sheep placental structure (epitheliochorial type interhaemal barrier) and function (such as substrate transfer) are markedly different to the human (Björkman et al., 1965, Boyd et al., 1976, Faber and Anderson., 1995). The ovine placenta is less permeable to ions than human placenta (Atkinson et al., 2006) and ion transport in rat and mouse are thought to more closely mimic human placental ion transport (Sibley., 1994). However, the transport capacity (e.g. sodium or calcium) in rodent placentae compared with human is much greater in late gestation to sustain exponential fetal growth in the former (Atkinson et al., 2006). A major advantage to using mouse models is that many of the same maternal cardiovascular adaptations to pregnancy occur in mice as they do in human (discussed in section 1.5) (Veerareddy et al., 2002, Cooke and Davidge., 2003). Furthermore, the mouse demonstrates similar placental transport function when compared to human (see section 1.3; Kusinski et al., 2010, Kusinski et al., 2011). However, in contrast to humans, mice have large litter sizes (between 6 - 12 pups per litter, dependent on strain) and have a haemotrichorial placenta rather than haemomonochorial (see section 1.3.2.3). Other advantages include a short gestation period, the ability to manipulate the genome (both specific knockout and over-expression models are available) and the capability to assess placental function in vivo. Therefore, mouse models represent a powerful tool to trial new therapies for FGR and assess their safety.

1.2.1.1 Definition of FGR

Human birth weight in a whole population follows a normal distribution. From the normal distribution curve, using basic statistical methods, the lowest 10th percentile of body weight for fetuses at any particular gestational age can be determined; babies lower than this percentile are
deemed to be small for gestational age (SGA). However, as noted above, due to natural variation within the population, some SGA babies are constitutionally small rather than their small size / weight being due to a pathological process. FGR has also been defined as a fetus whose weight falls below the 10th percentile for gestational age (Wollmann et al., 1998); using such a definition again ignores the constitutionally small baby (i.e. it is unlikely that 10% of new-born babies have succumbed to growth restriction in utero). As these observations of size / weight are gestational age-specific, the correct gestational age must also be known for accurate assignment of FGR (Bernstein et al., 1997). Within this 10% of the population there are fetuses which are appropriately grown and those which are FGR due to environmental or genetic factors. Hence, all FGR fetuses will be SGA but not all SGA fetuses will be classed as FGR. To try to dissociate between FGR and SGA, the 3rd percentile measurement could be utilised. However, if the 3rd percentile is used in the clinical setting it is likely that it will be too stringent (i.e. there will be some unidentified cases of FGR which may not be given appropriate clinical attention). Therefore, many scientific and clinical groups use the 5th percentile for gestational age as a suitable intermediate for defining FGR.

### 1.2.1.2 Diagnosis of FGR

Fetal weight can be estimated using ultrasound sonography between 16 and 40 weeks gestation. Fetuses at risk of FGR may be identified using customised antenatal growth charts. Customised antenatal growth charts reduce misdiagnosis of FGR and give a more accurate customised weight range by considering maternal characteristics such as gestational age, height, weight, ethnicity and parity (Gardosi et al., 1992, Gardosi et al., 2006). There is also evidence to suggest no further predictive benefit of customised antenatal growth charts over non-customised population-based charts (Carberry et al., 2013). However, customised antenatal growth charts (individualised birthweight centile charts) are the most common clinical tool used to define FGR (Gardosi et al., 2006, Gardosi et al., 1992). For higher risk pregnancies, women may undergo serial sonography throughout the second and third trimester to assess the fetal growth trajectory.

Adequate uteroplacental and fetoplacental blood flow are required to optimise fetal growth and development by maximising oxygen and nutrient delivery from mother to placenta and from placenta to fetus. FGR can often be associated with abnormal blood flow in the maternal uterine and fetal umbilical blood vessels. Uterine artery blood flow is monitored clinically \textit{in-situ} using non-invasive Doppler velocimetry, a measure of blood flow velocity at the site of the probe (Bower et al., 1992). However, a recent systematic review of 61 studies involving 41,131 patients demonstrated use of uterine artery Doppler ultrasonography was associated with poor prediction of FGR (Cnossen et al., 2008). Doppler velocimetry has also been utilised to monitor fetoplacental blood flow; Stuart and colleagues (1980) demonstrated a progressive fall in umbilical artery
resistance during the course of normal human pregnancy suggesting a high flow / low resistance circulation (Stuart et al., 1980). The characteristic “saw toothed” umbilical artery Doppler waveform in normal pregnancy is illustrated in Figure 1.1; it can clearly be seen that flow occurs in the vessel at all stages of the cardiac cycle with significant levels occurring in diastole. In severe cases of FGR, the umbilical artery Doppler waveform may show reduced, absent or reversed end diastolic flow suggestive of reduced blood flow and oxygen and nutrient delivery to the fetus (see figure 1.1).
Figure 1.1 Second trimester umbilical (A-D; left) and uterine (E-F; right) artery Doppler waveforms in normal and complicated pregnancies. Umbilical artery blood flow increases until term; significant blood flow is apparent throughout the cardiac cycle (A). In FGR pregnancies, the umbilical artery Doppler waveform is modified leading to elevated (B; also known as reduced), absent (C) or reversed (D) end diastolic flow. Uterine artery remodelling results in a high flow low resistance Doppler waveform (E); significant flow can be seen throughout the cardiac cycle. In some cases of FGR with inadequate spiral artery remodelling, there is a relatively reduced increase in blood flow (F). Key; s = maximum systolic, d = minimum diastolic. PI = pulsatility index, RI = resistance index. Adapted from Baschat., 2011.

- PI = S - D / Average flow velocity
- RI = S - D / D
1.2.1.3 Classification of FGR infant

FGR infants can be further classified into two subcategories; “symmetrical” and “asymmetrical” FGR. These distinct anthropometric phenotypes present at birth are thought to be the result of differential growth patterns that occur at various different stages of gestation (Hindmarsh et al., 2001). Fetuses which experience growth restriction early on in pregnancy demonstrate overall or “global” growth restriction; these babies are characterized by reduced body length, bodyweight and head size / circumference; this is defined as symmetrical growth restriction and occurs in up to 25 % of clinical cases. Growth restriction that occurs nearer to term often culminates in a baby born with a normal head size at birth but the abdomen, as measured by circumference, is reduced in size. This asymmetrical growth restriction, often described as “brain sparing”, is thought to represent a physiological response which occurs in order to protect brain growth / development. Asymmetrical growth restriction is associated with abnormalities in placental function which lead to reduced oxygen and nutrient delivery at a period of increasing substrate demand for the fetus (Wollmann et al., 1998). Thus, fetal nutrient demand outstrips placental supply and the fetus fails to achieve its growth potential as a result. However, fetuses that display poor growth in utero often display accelerated postnatal (“catch up”) growth (Hack et al., 1996).

1.2.1.4 Aetiology of FGR

Restricted fetal growth is the clinical end point of the condition; the aetiologies can be genetic (Howley et al., 2005), environmental (Castro and Avina, 2002), related to disease (e.g. obesity and preeclampsia), or idiopathic (i.e. no known cause). All can be associated with placental dysfunction. There are estimates that almost 40 % of FGR cases have no detectable underlying pathology (RCOG, 2014); however of the other 60 % of FGR cases genetic and environmental influences are thought to be the main contributory factors. Genetic factors account for around one third of all known causes whilst environmental factors account for two thirds of cases of FGR with an underlying aetiology. Genetic karyotypic abnormalities such as trisomy 21 have been estimated to account for 5 % of all cases of FGR; other congenital abnormalities such as chromosomal deletions also account for the genetic component of FGR (Monk et al., 2004). Interestingly, cases of FGR with a genetic cause often lead to early-onset FGR. A study by Ounsted et al., (1981) previously demonstrated that 7 % of FGR infants with chromosomal or congenital abnormalities (i.e. a genetic component) accounted for almost two thirds of fetal deaths.

Environmental factors are suggested to account for approximately 40 % (2/3 of the 60 % of known causes) of FGR cases. Maternal undernutrition is a contributing factor to FGR but only in cases of severe malnutrition. During the Dutch famine of 1944 - 1945 an average maternal intake of less than 1500 calories during the third trimester led to a reduction in birth weight, body length and head circumference by 10 %, 2.5 % and 2.7 %, respectively (Stein et al., 1975). Although 10 % may
only seem a modest reduction in fetal weight, this would result in a far greater proportion of fetuses below the 10\textsuperscript{th} percentile for weight at birth. Importantly, fetuses which are below the 10\textsuperscript{th} percentile of weight at any particular gestational age, whether pathologically small or genetically small, have increased risk of disease in the long-term (see section 1.6). The Dutch famine studies also demonstrated that the period of gestation when malnutrition occurred was important in the outcome of the fetus. There was no effect on birthweight in mothers who were subjected to undernutrition in the first or both the first and second trimesters but there was a reduction in birthweight in women who had been subjected to the famine in the third trimester of pregnancy (Stein et al., 1975). However, Smith et al., (1947) demonstrated there was a trend towards increased prematurity if mothers were subjected to nutrient restriction due to the famine in the first trimester.

1.2.2 Fetal vascular blood flow in normal and complicated pregnancies

In cases of FGR and preeclampsia (PET), longitudinal monitoring of fetal health is essential in determining relative risk of adverse outcomes and timing of delivery in the fetus. Ultrasound sonography is routinely used in pregnancy to assess fetal wellbeing and is helpful in determining fetal biometry measures such as fetal weight, size and symmetry of growth. In a pathologically small fetus, metabolic processes are reduced and cardiovascular function is altered to adapt to placental insufficiency and hypoxaemia (Baschat, 2006). Doppler velocimetry can be used to assess altered fetal cardiovascular physiology in cases of pregnancy complications such as FGR (Figueras and Gratacós, 2014, Hecher et al., 2001). Functional evidence for altered vascular function using Doppler velocimetry is therefore effective in determining those fetuses at greater risk of poor outcome. In addition, collecting functional evidence from Doppler allows longitudinal monitoring of the fetus across gestation and permits determination of the appropriate gestation to intervene when benefits of early delivery outweigh risk to the neonate. In high-risk pregnancies with placental dysfunction, detected by abnormal umbilical artery Doppler, middle cerebral artery and ductus venosus Doppler measurements allow obstetricians to monitor and determine appropriate intervention (Baschat, 2006).

Doppler velocimetry observations in the middle cerebral artery and ductus venosus of high-risk pregnancies are important predictors of fetal wellbeing. Although Doppler measurements are useful tools for the obstetrician, a number of other studies in mammals have assessed how vascular function in the fetus relates to growth restriction (Poudel et al., 2015, Tare et al., 2014, Miller et al., 2009, Morrison et al., 2007). In rodents, ex vivo studies showed that there was a reduction in fetal aortic vasoconstriction and vasorelaxation when dams were maintained in hypoxic chambers for 14 days of gestation (Herrera et al., 2012). Hypoxia also led to an increased
wall thickness in fetal abdominal aortas without alterations in cardiac volume, but prenatal undernutrition caused alterations in cardiac morphology without changes in vascular structure (Camm et al., 2010). In fetal sheep, single umbilical artery ligation led to FGR with significant fetal hypoxaemia and a reduced response of isolated neonatal coronary arteries to the endothelial-dependent vasodilator bradykinin (Tare et al., 2014). A recent study by Poudel and colleagues (Poudel et al., 2015) also demonstrated that FGR in fetal sheep resulted in reduced femoral artery blood flow as a consequence of increased blood flow to the adrenal gland and temporal lobe. No studies have yet determined the effect of placental insufficiency on mouse fetal vascular function; such experiments would be useful in determining the effects of placental insufficiency and FGR on fetal vascular function in a species with similar placentation to human. Furthermore, environmental and genetic manipulation of the mouse is more easily achieved compared with other species such as the sheep, making the mouse a useful tool in assessing the effects of pregnancy interventions on fetal vascular function.

1.2.3 Long-term health implications of poor growth in utero

Studies have shown that being born SGA pre-disposes the infant to diseases such as chronic hypertension (Barker et al., 1989, Osmond and Barker, 2000, Law et al., 2002), type II diabetes (Phillips et al., 2005), obesity (Ong et al., 2000) and could be associated with Alzheimer’s disease later in life (Ross et al., 2007). These studies have revealed the direct association between impaired fetal / placental growth and poor health outcomes in both childhood and adulthood. Barker and Osmond (1989) first provided evidence for a link between a cohort of Hertfordshire men with low birth weight and an increased incidence of mortality from ischaemic heart disease between the ages of 59 and 78. Although controversial at the time, these findings have been validated by a more recent study which demonstrated that an adverse intrauterine environment leads to adult hypertension and glucose intolerance; both important risk factors of cardiovascular disease (Osmond and Barker, 2000). Furthermore, Barker, Gelow and co-workers (2010) have suggested that chronic heart failure in adult life is initiated by impaired placental growth, which adversely affects cardiac development. The effects of suboptimal intrauterine growth and its association with long-term diseases are discussed in section 1.6.

Importantly, abnormal placental development and function culminate in abnormal growth of the fetus, thus these events precede the long-term consequences. Therefore, strategies to improve fetal growth and the health of the individual in the longer term should target the placenta. A number of potential therapies to improve uteroplacental and fetoplacental blood flow have been tested in mouse models of human pregnancy disease, including FGR (Stanley et al., 2012, Stanley et al., 2012b, Dilworth et al., 2013, Poudel et al., 2013). Mouse models of FGR allow for the
assessment of the effects of antenatal therapies, which often target placental function, on fetal growth. In the next section, the structure, development and function of the human and mouse placenta are compared.
1.3 Placental function
In mammals the placenta acts as an immunological barrier between the mother and fetus whilst promoting growth of the fetus without being detrimental to maternal health. It assists in the exchange of substances between mother and fetus and is the first organ to form in the mammalian embryo (Simmons and Cross, 2005). The placenta also releases hormones such as human placental lactogen, into the maternal blood that can alter the maternal environment (for review see Newbern and Freemark, 2011). Adequate blood flow, oxygenation, nutrient transport and an efficient immunological barrier are all required for a healthy pregnancy; if one or a number of these functions fail pathologies such as FGR and PET may ensue.

1.3.1.1 Vascular function

Adequate uterine blood flow and adaptation of the maternal myometrial arteries is required for sufficient supply and subsequent transfer of nutrients and oxygen by the placenta to the growing fetus. Providing ample blood supply to the uterus / placental bed requires major maternal cardiovascular adaptations to pregnancy often referred to as the “haemodynamic response” (see section 1.5) (Chapman et al., 1998). Pregnancy is associated with maintained or reduced mean arterial blood pressure, increased circulating volume of maternal blood, increased cardiac output and altered capillary permeability. It is well documented that mice exhibit similar cardiovascular changes during pregnancy (Kulandavelu et al., 2006, Wong et al., 2002, Cooke and Davidge, 2003). For example, radiotelemetry studies in pregnant mice have demonstrated similar maintenance of mean arterial blood pressure even with significant increases of heart rate (Butz and Davisson, 2001).

1.3.1.2 Nutrient transport

The placenta supplies oxygen and the nutrients required for normal fetal growth, whilst also transferring waste products of fetal metabolism back to the mother. There are a number of specific mechanisms through which oxygen and nutrients can pass from maternal circulation to fetal circulation via the syncytiotrophoblast. Regulation of placental nutrient transfer is not well understood, although it is clear that solute transfer involves transport across two physiological barriers and / or by an as yet uncharacterised paracellular route. The syncytiotrophoblast MVM and BM are barriers to transport in the human placenta (Bell and Ehrhardt, 2002, Jones et al., 2007). As pregnancy proceeds, the placental barrier begins to thin and the total villous surface area increases in readiness for the increased maternofetal exchange. This morphological change within the placenta is necessary in order to meet the nutrient requirements of the rapidly growing fetus. The mean villous diameter also decreases with every new villous branch formation. As a consequence fetal capillaries come into closer contact with maternal blood in the IVS. These physical changes result in the mean maternofetal diffusion distance decreasing from 100µm to 5µm between 2 months gestation and term (Benirschke, 2000). Similar placental adaptations also
occur in mice with the labyrinthine zone expanding in size in proportion to the junctional zone (Coan et al., 2004).

Transport across the MVM and BM are assumed to be the rate-limiting steps in substrate transfer, whereas the fetal endothelial layer is only rate-limiting for larger molecules e.g. immunoglobulins (Firth and Leach, 1996). If a concentration gradient exists and the molecule is lipophilic (e.g. free fatty acids; Kamp et al., 1995) then a simple diffusional process will occur from the IVS through the syncytiotrophoblast layer and into the fetal capillary. The rate at which diffusion occurs is dependent on the surface area of exchange, the maternofetal concentration difference and the permeability of the placenta (e.g. histological type of placenta and membrane thickness) (Coan et al., 2008, Sibley et al., 2004). Diffusion of hydrophilic substances (such as ethylenediaminetetraacetic acid; EDTA, which does not enter cells) also occurs as molecules with no specific transporters are able to cross the placenta via an as yet uncharacterised paracellular route (Štulc., 1989). Studies have not yet conclusively shown / visualized the existence of such microscopic channels (Kaufmann., 1985).

Glucose is the primary source of energy for cells and fetal demand for glucose is directly related to gestational age. Glucose is transported across the MVM and BM by facilitative glucose transporter (GLUT) proteins (Jansson et al., 1993). Amino acids are transferred across the placenta by facilitated diffusion and secondarily active transport via proteins present in the syncytiotrophoblast MVM and BM. Like glucose, amino acids are essential for appropriate growth of the fetus and for tissue development (Yudilevich and Sweiry, 1985).
1.4 Placental dysfunction in FGR

Idiopathic FGR is a condition associated with placental insufficiency. This term encompasses abnormal development, aberrant blood flow and reduced nutrient transport by the placenta resulting in an undernourished fetus. The evidence for each will now be discussed and comparisons made between human studies and mouse models of altered growth in utero.

1.4.1 Altered uterine blood flow velocity in human FGR

Trophoblast invasion into the maternal myometrium is essential for transforming maternal spiral arteries into high conductance low impedance vessels which supply the placenta and fetus an ever increasing demand for oxygen and nutrients. However, it is suggested that in some cases of FGR trophoblast migration does not occur, or occurs to a lesser extent, and the endothelium surrounding the maternal spiral arteries is insufficiently remodelled. A study by Lin and colleagues (1995) demonstrated that placental bed biopsies with absent trophoblast migration were most commonly associated with infants below the 10th centile for fetal weight. In some cases of human FGR, abnormal uterine artery pulsatility index (PI) can be detected using Doppler velocimetry (Cnossen et al., 2008) (section 1.2; figure 1.1). Within these cases there was a 50 % reduction in uterine artery blood volume when compared to normal weight term pregnancies (Ferrazzi et al., 2011). Multiphasic contrast-enhanced magnetic resonance imaging in cases of FGR with abnormal uterine blood flow also demonstrated hypoperfusion in placentas with a slow intervillous blood flow (Brunelli et al., 2010). However, not all cases of FGR demonstrate abnormal PI values and these Doppler velocimetry indices are poor predictors of outcome (Ferrazzi et al., 2011).

1.4.2 Altered fetoplacental vascular structure and placental villous development in human FGR

As pregnancy progresses, in order to meet the increasing demand for oxygen and nutrients from the fetus, the placental barrier begins to thin and the total villous surface area increases. The mean villous capillary diameter also decreases with every new villous branch formation and as a consequence the fetal capillaries come into closer contact with the IVS. These physical changes result in the mean maternofetal diffusion distance decreasing from 100µm to 5µm between 2 months gestation and term (see section 1.3.2) (Benirschke, 2000). Stereological analysis by Mayhew and colleagues (2003) demonstrated an overall decrease in villous volume (stem, intermediate and terminal villi) in 5 cases of FGR, as well as an overall decrease in IVS volume. Further, a reduced villous surface area for exchange was attributed to a decrease in the hypothetical maternofetal diffusion capacity by up to 60 %, with decreased diffusion of oxygen
leading to hypoxic stress. As of yet there is little physiological data to support these estimations in human FGR (Burton, Mayhew et al. 1989). Thus, changes in vascularity (i.e. altered angiogenesis / vasculogenesis) in the fetoplacental vasculature of FGR pregnancies may contribute to the increase resistance to flow demonstrated in FGR. Therefore, promoting fetoplacental vascular growth and / or development and increasing blood flow velocity (e.g. with a vasodilator) may be beneficial in cases of FGR.

Structural abnormalities in the placental villi are well established in FGR. Morphometric analyses have revealed there is reduced trophoblast and stromal cell proliferation in FGR pregnancies with abnormal umbilical artery Doppler indices (Chen et al., 2002). However, the volumes and surface areas of intermediate and terminal villi were reduced in cases of growth restriction associated with abnormal umbilical Doppler indices (Jackson et al., 1995). In addition, stereological examination of placentas from FGR pregnancies also demonstrated that there was a significant reduction in vascular surface density of stem, intermediate and terminal villi and reduced volume of intermediate and terminal villi in cases of FGR with normal umbilical blood flow indices (Sağol et al., 2002).

Apoptosis is increased in FGR, an observation that may be the explanation for a disturbance in the epithelial steady state (Mayhew, 2001) leading to a decrease in thickness and volume of trophoblast (Teasdale, 1984; Smith et al., 1997; Mayhew et al., 2003). It is well documented that FGR is associated with increased levels of oxidative stress in maternal plasma, umbilical cord blood and placental tissue compared to normal pregnancies (Morris et al., 1998; Karowicz-Bilinska et al., 2002; Biri et al., 2007). One stereological study has concluded that post-placental oxidative stress originates from a reduced diffusive conductance of oxygen at the villous membrane (Mayhew, Ohadike et al. 2003) whilst in normal pregnancies an increase in fetal weight is proportional to the diffusive conductance of oxygen (Mayhew et al., 1993; Mayhew, 2006). Conversely, earlier stereological studies had demonstrated that the thinning of intervascular tissue layers of the human placenta in response to fetal hypoxia (Mayhew, 1998) was an adaptive response that may be absent in FGR. However, most studies agree that FGR-associated fetal hypoxia and oxidative stress is due to abnormal villous formation and maladaptation of the placenta (Kingdom and Kaufmann, 1997).

1.4.3 Altered myometrial and chorionic plate artery vascular reactivity in human FGR

Myometrial arteries assessed from pregnancies with FGR show significant increases in agonist-induced contraction and reduction in endothelial-dependent relaxation (Wareing et al., 2005). Myometrial arteries from FGR pregnancies also show a decreased relaxation to the endothelial-dependent vasodilator bradykinin (Ong et al., 2003) suggesting that, as in PET, endothelial
function may be compromised. Myography studies have also been performed on human placental blood vessels of the chorionic plate (Wareing et al., 2002, Wareing et al., 2004, Wareing et al., 2006). These vessels demonstrated constriction to the thromboxane-A₂ mimic U46619 in normal and complicated pregnancies with maximal constriction to U46619 reduced in PET and FGR pregnancies (Wareing and Baker, 2004). However, more recent studies have shown increased constriction to U46619 in chorionic plate arteries (Wareing et al., 2006, Mills et al., 2005) and veins (Wareing et al., 2006) but increased endothelium-independent relaxation (Mills et al., 2005) in FGR pregnancies. Chorionic plate arteries from uncomplicated pregnancies were incubated for 24 hours with glucocorticoids (mediators of vascular resistance associated with FGR) and demonstrated increased vasoconstriction in response to U46619 (Nugent et al., 2013). In addition, U46619-induced tone oscillations (i.e. a series of contractions and relaxations) were reduced in amplitude over a one-hour period in chorionic plate arteries from FGR pregnancies (Sweeney et al., 2008). These alterations may be a contributing factor or a compensatory mechanism in FGR. In addition to altered vascular responsiveness and blood flow, FGR is also associated with altered placental nutrient transport.

1.4.4 Altered nutrient exchange in human FGR

One of the first studies to compare amino acid transport in placentas from normal and growth restricted fetuses demonstrated a reduction in transport of a substrate specific for an amino acid transporter now referred to as the system A transporter (Dicke and Henderson, 1988). The System A proteins transport small neutral amino acids, such as alanine, into the placenta in a sodium-dependent manner. A number of in vitro studies have demonstrated the reduced capacity for placental amino acid transport in FGR. For example, the system A neutral amino acid transporters (SNATs) located on the MVM are responsible for the transfer of glycine, serine and alanine and activity is inversely related to birth weight in normal pregnancies (Godfrey et al., 1998) and reduced in FGR pregnancies (Dicke and Henderson, 1988, Glazier et al., 1997). Amino acids such as leucine and phenylalanine are reduced in umbilical plasma of FGR fetuses (Paolini et al., 2001, Jansson et al., 1998, Norberg et al., 1998). In contrast, other amino acids and amino acid transporters such as the glucose and GLUT1, respectively, appear unaffected (Jansson et al., 1998, Jansson et al., 1993).

1.4.5 Altered nutrient exchange in mouse FGR

In normal mouse pregnancy a decrease in the thickness of the mouse placental barrier between E14 and E16 (Coan et al., 2004) is attributed to thinning of vascular endothelium and cytotrophoblast cells. Conversely, the placental-specific insulin-like growth factor 2 (Igf2) knockout (P0) mouse, a commonly used mouse model of FGR (see section 1.7.2) exhibits an
increased interhaemal membrane thickness with a decreased passive permeability to radiolabelled tracers; suggesting less diffusive conductance of the placenta of the growth restricted fetuses (Coan et al., 2008, Sibley et al., 2004, Constancia et al., 2002), akin to studies in the human (Mayhew, 1998). The P0 knockout mouse also displays other characteristics that are present in human FGR. For example, P0 litters have a twenty five percent reduction in fetal weight and display asymmetry at birth (Mikaelsson et al., 2013). However, unlike in the human where there is reduced system A activity (Jansson et al., 2002), placental transfer of $^{14}$C-methylaminoisobutyric (MeAIB) acid, a non-metabolisable substrate for the system A amino acid transporter, in the P0 mouse is increased and able to maintain an adequate growth trajectory until E16 (Constância et al., 2005, Kusinski et al., 2011). However, this up regulation of $^{14}$C-MeAIB transfer in P0 placentas is unable to sustain fetal growth near term. Studies in haemotrichorial rat placenta have demonstrated the main barrier to transport is the syncytiotrophoblast layer II (Glazier et al., 1990), which also possesses the Na$^+$/K$^+$ ATPase exchanger present in human syncytiotrophoblast (Glazier et al., 1996). More recently, Novak and colleagues demonstrated an increase in mRNA expression of the sodium dependent neutral amino acid transporters 1,2 and 4 (SNAT1, 2 and 4) towards the end of gestation in rat, which contribute to System A activity, is similar to that found in human (Novak et al., 2006). Reductions in placental system A amino acid transport is associated with FGR in pups from rat dams fed a low protein diet (Jansson et al., 2006) and inhibition of system A by administering MeAIB to rats caused severe fetal growth restriction (Cramer et al., 2002).

Although relatively few studies have been performed to investigate mouse placental transport (reviewed in Dilworth and Sibley, 2013), Kusinski and colleagues demonstrated the ability to purify and quantify (using alkaline phosphatase cytochemistry) syncytiotrophoblast layer II of the murine placenta and utilise this to assess system A transporter activity in a similar manner to the methods of human placental syncytiotrophoblast MVM isolation (Kusinski et al., 2009, Glazier and Sibley, 2006). Subsequently, studies on these isolated vesicles, together with studies of the unidirectional flux of radiolabelled substrates from mother to fetuses across the placenta, were performed in the endothelial nitric oxide synthase (eNOS) deficient mouse model of human FGR. In common with human FGR, system A activity was significantly reduced in both the in vitro and in vivo experiments (Kusinski et al., 2012). Vesicle studies have confirmed that substrates of system A are transported in a Na$^+$- dependent manner across what is assumed to be the transporting membranes of the syncytiotrophoblast in the mouse. These data demonstrate functional overlap between human and mouse placental transport systems and gives confidence that transporter defects typical of pregnancy complications in women can be assessed in mouse models of FGR (Dilworth and Sibley, 2013).
1.5 Cardiovascular adaptations to pregnancy

Cardiovascular adaptations in pregnancy show a number of similarities between the mouse and human; these are described below and summarised in table 1.2.

1.5.1 Cardiovascular adaptations in human pregnancy

The physiological determinants of blood pressure, will be considered prior to introducing cardiovascular adaptations of human pregnancy. There are two main physiological determinants of blood pressure; cardiac output and arterial resistance (Despopoulos and Silbernag, 2001). Cardiac output can be influenced by both heart rate and stroke volume, i.e. the number of heartbeats per minute and the amount of blood pumped out of heart during each contraction, respectively. The balance of sympathetic and parasympathetic activity from the autonomic nervous system determines heart rate; neurones from both systems innervate the cardiac sinoatrial node (Lewis et al., 2001). Parasympathetic control of blood pressure by the vagal nerve leads to muscarinic receptor stimulation in the sinoatrial node and a reduction in heart rate. Sympathetic activity, caused by endogenous catecholamines such as norepinephrine and epinephrine, stimulates the beta-adrenergic receptors in the heart to increase heart rate, increasing cardiac output and blood pressure. Stroke volume is influenced by 1) end diastolic volume, which is proportional to the amount of blood returning from the veins to the heart (venous return), and 2) end systolic volume a main determinant of which is the force of cardiac contraction. An increase in stroke volume and blood pressure can be due to either an increased venous return, as a consequence of hypervolaemia, or via increased contractility of the ventricle induced by positive inotropes or simply by an increased volume of blood in the ventricle (Starlings Law; Despopoulos and Silbernag, 2001).

Poisseuilles Law and Darcys Law are the basis for understanding how arterial resistance can significantly alter blood pressure (Starlings Law; Despopoulos and Silbernag, 2001). Arterial length, blood viscosity and the velocity of blood within an artery are linearly related to arterial resistance e.g. a doubling of the viscosity of blood would increase arterial resistance and blood pressure by a factor of 2. However, the radius of the vessel can also significantly alter arterial resistance with this resistance being inversely related to radius raised to the power four; for every doubling of radius of the vessel, resistance can be reduced by a factor of 8, i.e. a parabolic relationship. Therefore, the main mechanism for altering blood pressure within the body is to manipulate / fine tune the calibre / diameter of blood vessels by either vasodilation or vasoconstriction; arterial resistance changes in line with these changes in the physical nature of the artery (Despopoulos and Silbernag, 2001). Blood pressure is sensed by baroreceptors present within the aortic arch;, if there is a sudden fall in blood pressure the aortic baroreceptors sense
this change and induce a physiological response, increasing adrenergic activity within the heart and vasculature to increase the heart rate, stroke volume and peripheral vascular resistance and thus increase blood pressure rapidly (Despopoulos and Silbernag, 2001).

Pregnancy induces major hemodynamic changes in the mother. There is an increased heart rate leading to increased cardiac output and stroke volume early in gestation (Chapman et al., 1998). Van Oppen and colleagues (1996) measured an increased heart rate from an average of 87 beats per minute in the first trimester to 92 beats per minute in the third trimester. Others have also noted a similar increase between 5 - 15 beats per minute (Robson et al., 1989). There is also a large expansion in plasma volume and red cell mass during pregnancy. However, the red cell mass expansion is proportionately less than the expansion in plasma volume leading to an apparent anaemia of pregnancy (Taylor and Lind, 1979). A recent Cochrane review of 60 clinical trials using iron supplementation (with or without folic acid) during pregnancy demonstrated a reduction in the risk of delivering a low birthweight infant and reduced maternal anaemia and iron deficiency (Peña-Rosas et al., 2012).

In pregnant women, increased plasma volume results in physiological hypervolaemia which continues until the end of the second trimester with an almost 50 % increase in blood volume compared to non-pregnant women. In European women this equates to an average increased in 1300 ml of blood (Pritchard, 1965, Pirani et al., 1973) and is related to the size and number of fetuses (Hyttten, 1985, Cavill, 1995, Carlin and Alfrevic, 2008). Some studies have demonstrated a continuous increase in blood volume until the end of pregnancy (Lund and Donovan, 1967) but others have suggested a plateau in the third trimester (Taylor and Lind, 1979). Hypervolaemia is therefore a physiological adaptation that ensures adequate blood supply to the uteroplacental circulation, enhancement of maternal-fetal nutrient and oxygen transfer and allows women to tolerate the blood loss associated with delivery (Silversides et al., 2007). An inadequate expansion of the red cell mass has also been associated with low birthweight and FGR (Salas et al., 1993). The mechanisms of hypervolaemia are poorly understood but some have suggested oestrogen plays an important role in up-regulating concentrations of renin, altering the balance of the renin-angiotensin-aldosterone axis resulting in increased sodium retention and total body water (Atherton et al., 1988, Churchill et al., 1980).

Studies using echocardiography have shown a larger left ventricular mass due to an increase in wall thickness in response to increased heart rate and cardiac output (Mabie et al., 1994, Geva et al., 1997, Gilson et al., 1997, Schannwell et al., 2002). These anatomical adaptations are essential to supply the developing fetus with adequate nutrients and oxygen. There is still debate as to whether cardiac output is maintained or reduced within the third trimester and conflicting reports tend not to take into account inter-individual differences (Van Oppen et al., 1996). Post-partum,
cardiac output and stroke volume are significantly reduced due to a subsequent reduction in heart rate (Myhrman et al., 1982) with all values returning to normal 6 months after delivery (Robson et al., 1989, Robson et al., 1987). It has been demonstrated that the increased left ventricular mass seen in pregnancy regresses by 24 weeks post-delivery (Umar et al., 2012, Campos., 1996).

During pregnancy systemic vascular resistance is decreased from 6 weeks gestation (Chapman et al., 1998) until at least 20 weeks of gestation (Robson and Hunter, 1989, Duvekot et al., 1993). This fall in peripheral vascular resistance is a consequence of altered resistance and flow in multiple vascular beds. For example, clinical assessments using colour Doppler ultrasound noted a significant increase (500 ml/minute near term) in blood flow to the uteroplacental circulation during pregnancy (Jurkovic et al., 1991). There is also significantly increased renal blood flow during normal pregnancy which can reach 180 % of that seen in non-pregnant women by the third trimester and leads to a 50 % increase in glomerular filtration rate (Sims and Krantz, 1958). The increased glomerular filtration rate in pregnancy is required to clear the increased concentrations of fetal waste such as urea, creatinine, urate and bicarbonate in the maternal blood. The mechanisms surrounding reduced peripheral vascular resistance are still debated but some have suggested a role for the vasodilators relaxin (Novak et al., 2002, for review see Conrad, 2010), endothelin (Gandley et al., 2001), nitric oxide (Conrad et al., 1993) and activation of the renin-angiotensin-aldosterone system (Chapman et al., 1998). A further study demonstrated an increased endothelial dependent vasodilatation amongst 71 women; thus these data suggest NO activity is enhanced in normal pregnancies and is an important contributor to the decreased systemic vascular resistance (Dørup et al., 1999). Systemic vascular resistance increases by a third within the first 2 weeks postpartum and continues to rise until reaching pre-pregnancy levels (Robson et al., 1987).

Bearing in mind the fall in systemic vascular resistance, it is unsurprising that pregnancy is associated with a reduction in both systolic and diastolic blood pressure (Wilson et al., 1980, Grindheim et al., 2012), with most studies showing a larger decrease in diastole (Atkins et al., 1981, Wilson et al., 1980). There is a small reduction in mean arterial blood pressure (MAP) from 14 weeks gestation until mid-gestation when a progressive increase occurs until parturition (Chapman et al., 1998, Van Oppen et al., 1996, Grindheim et al., 2012). Blood pressure returns to normal levels as early as six weeks (Wilson et al., 1980) but can remain refractory up to 6 months (Grindheim et al., 2012) postpartum.

1.5.2 Cardiovascular adaptations in mouse pregnancy
Kulandavelu and colleagues (2006) previously demonstrated that the increased cardiac output in human pregnancy is also seen in C57BL/6J pregnant mice; at E9.5 cardiac output increased by almost 30% with a concurrent 25% increase in stroke volume. These alterations in physiological function are maintained throughout pregnancy so that by E17.5 cardiac output and stroke volume increase by 48% and 41% respectively, compared to non-pregnant values. At this late stage of gestation increased stroke volume was associated with an increase in plasma volume of approximately 30%. Using the tail cuff plethysmograph, heart rate was not shown to be increased during pregnancy in the inbred C57BL/6J mouse strain (Kulandavelu et al., 2006, Eghbali et al., 2005) but was increased when assessed with radiotelemetry (approximately 100 beats per minute increase) (Butz and Davisson, 2001). Heart rate was also increased in the outbred cluster of differentiation-1 (CD-1) strain (Wong et al., 2002). Cardiac output, stroke volume and heart rate returned to pre-pregnancy levels 17 days postpartum in the CD-1 strain highlighting that there may be strain-specific cardiovascular adaptations during mouse pregnancy.

As with human pregnancy, increased cardiac output in the mouse results in eccentric hypertrophy of the left ventricle (Kulandavelu et al., 2006, Eghbali et al., 2005, Xiao et al., 2014) and a total increase in heart weight (Iorga et al., 2012). It has been hypothesised that the altered physiology of the heart is a result of mechanical stress and increased oestrogen resulting in an increased activity of the stretch-response c-Src tyrosine kinase (Eghbali et al., 2005). Maternal heart mass returns to near normal weight in mice post-pregnancy, which is similar to that seen in human (Iorga et al., 2012).

Using ultrasound biomicroscopy, Doppler blood flow velocity waveforms have also been used to assess the uteroplacental circulation of pregnant C57BL/6J mice (Mu and Adamson, 2006). As with human pregnancy (Burton et al., 2009), there was a significant increase in the diameter of uterine and radial arteries with a concomitant 8-fold decrease in vascular resistance in the C56BL/6J mouse. Systemic resistance (mesenteric) and uterine arteries from pregnant C57BL/6J mice demonstrated increased vasodilatation in response to endothelial derived NO when compared with arteries from non-pregnant mice (Veerareddy et al., 2002) suggesting NO plays a significant role in reducing peripheral vascular resistance in mouse pregnancy. Kulandavelu and colleagues also demonstrated a 15% reduction in mean arterial pressure in pregnant C57BL/6J mice when compared with non-pregnant C57BL/6J animals (Kulandavelu et al., 2006). Indeed, inhibition of NO biosynthesis using the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) during rat pregnancy led to an increased arterial pressure in dams compared with those without L-NAME treatment (Tsukimori et al., 2008). Furthermore, pregnant rats administered monoclonal antibodies which neutralize the vasodilator relaxin did not demonstrate gestational-
induced decreases in systemic vascular resistance suggesting relaxin also plays an important role in the normal pregnancy-induced cardiovascular changes in rodents (Debrah et al., 2006).

Using radiotelemetry, MAP in pregnant C57BL/6J mice was reduced in comparison to non-pregnant controls between E11- E13 (Butz and Davisson, 2001); similar results have also been noted using the “tail cuff” method (Hefler et al., 2001). In agreement with these studies, there were no differences in arterial blood pressure in C57BL/6J mice at E17.5 when compared with non-pregnant mice (Wong et al., 2002). As seen in human studies (Chapman et al., 1998, Clapp and Capeless, 1997) there was a reduction in arterial pressure in pregnant imprinted control region (ICR) mice during early gestation (E3.5) (Wong et al., 2002) but not in C57BL/6J mice (E6 – E8) (Butz and Davisson, 2001).

In summary, it is clear that women and mice undergo similar cardiovascular adaptations to pregnancy. These similarities are summarised in table 1.1.

Table 1.1. Maternal hemodynamic changes during pregnancy, peripartum and postpartum in the human. Key: BP; blood pressure, LV; Left ventricular, *Vascular, ↑; increased, ↓; decreased. See text for references.
1.6 Consequences of suboptimal intrauterine conditions; long-term programming of adult disease

The term “programming” is given to the situation where environmental insults in utero result in permanent structural and functional changes in the behaviour / reactivity of tissues through physiological and epigenetic modifications. Distinct populations from the USA (Boney et al., 2005, Li et al., 2001), UK (Whincup et al., 1997, Law et al., 2002) and India (Bhargava et al., 2004) provide support to the hypothesis that insults during the in utero period are associated with adulthood disease. Human epidemiological studies and animal models demonstrate that physiological outcome is dependent upon the type of insult as well as the timing, length of duration and severity.

1.6.1 Causes of intrauterine programming of adult disease

The aetiologies of intrauterine programming can be separated into four main groups; disease, diet, stress and environmental (Fowden et al., 2006), all of which may affect fetal growth and development. Examples of how diseases, diet and the maternal environment affect fetal / placental growth and the mechanism associated with each are now introduced.

1.6.1.1 Diseases as causes of long-term programming?

PET is associated with intrauterine programming of adult disease. A small retrospective cohort study demonstrated the direct link between fetuses from PET pregnancies and an increased risk of stroke in adulthood (Kajantie et al., 2009). Although fetal weight was not altered there was a small but significant reduction in head circumference of babies whose mothers experienced severe PET. PET was also associated with elevated blood pressure in the offspring of affected pregnancies at 12 years of age (Tenhola et al., 2006).

Fetuses whose mothers had gestational diabetes mellitus (GDM) are at increased risk of type 2 diabetes in adulthood; maternal obesity alone and maternal obesity with type-1 diabetes produced a similar increased risk of type 2 diabetes in the adult (Clausen et al., 2007). The authors suggested prenatal exposure to high concentrations of glucose in the maternal circulation in the third trimester led to increased prevalence of type 2 diabetes in the offspring. Furthermore, a recent study demonstrated that children whose mothers developed GDM were more likely to be overweight and have greater insulin resistance in early childhood (Boerschmann et al., 2010). Taken together, these studies suggest that maternal pathology can influence offspring long-term health.
1.6.1.2 Placental insufficiency, hypoxia and growth restriction: causes of long-term programming?

In a Bolivian cohort, pregnancies at high altitude (3649m) had increased frequencies of low birth weight babies compared with those from a low altitude (437m) city (Giussani et al., 2001). There are currently no human epidemiological data which demonstrate a direct link between prenatal exposure to high altitude (and thus fetal hypoxia) and postnatal development of cardiovascular disease. However, animal studies inducing fetal hypoxia (whether that be the result of maternal high altitude or not) suggest an overarching mechanism of developmental programming in cases of FGR (see section 1.6.4 for further experimental data) (for review see Giussani and Davidge, 2013). In the chick embryo, chronic hypoxia induces asymmetric growth restriction and culminates in hypertrophic growth of the heart and aorta (Ruijtenbeek et al., 2000); increased thickness in the latter is associated with increased cardiovascular risk in the human (McEniery and Wilkinson, 2005). In addition, chronic hypoxia culminated in sympathetic hyper-innervation of chick arteries (Ruijtenbeek et al., 2000) presumably through alterations in the beta-adrenoceptors function of the vasculature (Lindgren and Altimiras., 2009). When chicks were supplemented with oxygen, these effects were reversed which suggests hypoxia contributes directly to the programmed cardiovascular alterations (Salinas et al., 2010). Ruijtenbeek and colleagues demonstrated reduced fetal weight in both the chick model of protein restriction and chronic hypoxia (Ruijtenbeek et al., 2003). This study was able to differentiate between the effects of both insults and demonstrated that only chronic hypoxia culminated in endothelial dysfunction through reduced NO production. Hypoxia-induced cardiovascular alterations have also been demonstrated in sheep (Thompson et al., 2011) and rat (Herrera et al., 2012, Xu et al., 2006, Giussani et al., 2012) but have not yet been fully assessed in mice with recent studies examining only fetal / placental weight and placental transporter / renin-angiotensin system expression (Cuffe et al., 2014, Cuffe et al., 2014b). However, chronic hypoxia in the catechol-O-methyltransferase knockout and the eNOS knockout mouse models of PET/FGR demonstrate an additional effect of hypoxia in elevating blood pressure thus suggesting a further reduced vasorelaxation induced by hypoxia (Rueda-Clausen et al., 2014). The mechanisms by which vascular dysfunction develops in relation to small size at birth is unknown. Researchers have suggested an accelerated ageing process through vascular telomere shortening (Tarry-Adkins et al., 2008), but more recent findings did not note significant associations between telomere shorting and vascular dysfunction (Allison et al., 2014). In addition, others have demonstrated prenatal hypoxia and oxidative stress in rats leads to altered vascular structure and function in male offspring (Giussani et al., 2012), possibly through alterations in epigenetic regulation of gene expression (Patterson et al., 2012).
1.6.1.3 Diet and environment; association with long-term programming.

Other environmental factors which may be associated with intrauterine programming are prenatal exposure to alcohol and drugs. In a small cohort of infants exposed to cocaine *in utero*, there was a significant increase in cortisol concentration at 7 months of age compared to those infants whose mothers who were not recreational drug users (Eiden et al., 2010). Prenatal alcohol exposure can also lead to increased cortisol and elevated heart rates in infants; however, the birth weight of these infants was not documented by the authors of this study (Haley et al., 2006).

Epidemiological data from a period of the Dutch famine first demonstrated that the timing of the insult is key in determining the effects to the fetus (Stein et al., 1975, Lumey., 1992). Stein first reported that there were no effects of first and second trimester maternal malnutrition on birthweight indices (Stein et al., 1975). However, a later study by Lumey and colleagues suggested first and second trimester maternal malnutrition led to smaller, shorter babies (Lumey et al., 1992). Lumey also demonstrated third trimester maternal under-nutrition was not associated with reductions in birth weight (Lumey et al., 1992) but this was in direct contradiction earlier reports from Stein and colleagues who demonstrated a reduction in birthweight when mothers were exposed to third trimester malnutrition. Nevertheless, these studies do demonstrate that timing of maternal undernutrition can reduce birthweight. More recent data have confirmed that the timing of the insult can lead to differential disease in later life (Ravelli et al., 1998, Roseboom et al., 2000). Exposure to the famine in mid and late gestation led to reduced glucose tolerance in adults. However, prevalence of coronary heart disease was higher in adults that had early prenatal exposure to the famine and not in those exposed in mid or late gestation (Roseboom et al., 2000).

1.6.2 Intrauterine programming of cardiovascular disease

A number of studies have demonstrated there are strong associations between impaired fetal growth and intrauterine programming of cardiovascular disease. Examples of such studies will now be discussed, with a later emphasis on the potential mechanisms associated with long-term programming of cardiovascular disease.

1.6.2.1 Coronary heart disease

In a Hertfordshire cohort of 5654 men born between 1911 and 1930, those who had the lowest weight at birth and at one year of age had the highest incidence of death from ischaemic heart disease (Barker et al., 1989). Similar supporting data was published by a study on the Helsinki birth cohort; development of coronary heart disease was associated with reduced growth *in utero* with rapid postnatal growth (Eriksson et al., 2001). Importantly, these results have been
replicated in a number of populations including Europe, North America and India (Forsén et al., 1997, Frankel et al., 1996, Leon et al., 1998, Stein et al., 1996).

1.6.2.2 Hypertension

Epidemiological evidence from the UK first demonstrated that increased systolic blood pressure in both 10 year olds and adults was associated with low birth weight (Barker et al., 1989). This study also demonstrated that, in areas of high cardiovascular mortality, children who were born small were much shorter and had an increased resting pulse rate. More recent studies have extended this initial observation to further associate low placental weight with risk of hypertension in adulthood (Campbell et al., 1996, Eriksson et al., 2000). A study of 71,100 mainly white European women who were between age 30-55 demonstrated that the odds ratio of hypertension in women who were born less than 5 lbs was 1.39 – 1.43 (Curhan et al., 1996). Women who were born over 10 lb also had a high odds ratio of hypertension (1.62). Therefore, when risk of hypertension is plotted against birth weight there is a “U – shaped” relationship. Recent evidence has also confirmed these earlier findings in females but also suggest high birth weight in males is associated with an increased risk of hypertension in adulthood (Tian et al., 2006).

A systematic review of 80 individual studies assessing the relationship between size at birth with blood pressure in adulthood found a significant inverse correlation. For each additional kg of birth weight, there was a reduction of approximately 2 mmHg in mean systolic blood pressure (Huxley et al., 2000). In addition to the association with birth weight, skeletal and non-skeletal accelerated postnatal growth has been positively correlated with systolic blood pressure. In a Swedish cohort, men at age 50 had an increased likelihood of hypertension for every kg reduction in birth weight, but only if they were above median adult height (Koupilová et al., 1997). Fat mass (i.e. non skeletal postnatal growth) has also been linked with risk of adult hypertension (Law et al., 2002). In a cohort of 346 British men and women, those who had accelerated postnatal growth (between 1 and 5 years, independent of first year growth) had the highest blood pressures. Thus, a reduction in obesity in childhood may prevent the increased risk of hypertension in adult life.

A meta-regression analysis on 20 studies from Norway, Finland, Sweden and Denmark demonstrated that there were sex specific differences in the association of low birth weight with systolic blood pressure (Gamborg et al., 2007). For every kg reduction in birth weight, females had an average increased systolic blood pressure of 2.8 mmHg whereas males had an increase of only 1.5 mmHg. An earlier meta-regression analysis of 57 observational studies showed no difference between sexes and concluded that previous findings were merely due to chance (Lawlor et al., 2002). Two systematic reviews of the literature have also demonstrated a stronger association between low birth weight and adult hypertension than low birth weight and childhood
hypertension, suggesting hypertension is a progressive disease exacerbated with age (Lawlor et al., 2002, Huxley et al., 2000).

1.6.2.3 Mechanisms underlying pathogenesis of cardiovascular disease

The mechanism(s) associated with low birth weight and hypertension could be due in part to alterations in the development of the kidney. An inverse relationship in renal filtration surface area and the risk of hypertension in adulthood has been proposed by Brenner and colleagues (Brenner et al., 1988). Those patients with reduced renal filtration surface also had fewer glomeruli, which impaired the excretion of sodium and regulation of blood pressure in the renal system. Further evidence was gained by Keller and colleagues (2003) using stereological techniques in kidney at post mortem; kidneys from patients with primary hypertension had a reduced number of glomeruli when compared to normotensive controls. Therefore, abnormal kidney development may be a precursor for development of cardiovascular disease in later life.

Poor / underdevelopment of the fetal kidney is also associated with low birth weight (Mañalich et al., 2000) and it has been suggested, in experimental models, as a possible mechanism leading to hypertension in the offspring (Woods et al., 2004). In 35 human neonates who died before birth, the number of glomeruli per mm² was reduced in the kidneys of low birth weight neonates compared to kidneys harvested from normal birth weight neonates (Mañalich et al., 2000). In a cohort of African Americans and Caucasians, birth weight was a strong determinant of the number of glomeruli (i.e. a low birth weight was associated with reduced number of glomeruli (Hughson et al., 2003)). Furthermore, in low birthweight infants that succumb to sudden infant death syndrome (SIDS), there were a reduced number of glomeruli when compared with non-SIDS normal birth weight infants (Beech et al., 2000).

Experimental models of FGR have also shown similar associations between low birth weight and nephrogenesis. In the rat, reduced maternal protein intake was associated with low birth weight (Langley-Evans and Nwagwu., 1998) and reduced nephrogenesis (Langley-Evans et al., 1999). The reduced nephrogenesis seen in growth restricted neonates persisted at 4 weeks of age with hypertension and reduced renal mass / size at 19 weeks of age. Increased blood pressure or reduced renal size was not associated with reduced glomerular filtration rate (Langley-Evans et al., 1999) in these animals. In addition, maternal protein restriction led to hypertension in the offspring and this was also associated with reduced number of glomeruli (Woods et al., 2004). However, these effects were sex-specific and there was no reduction in glomerular number in female offspring that were hypertensive suggesting that abnormal nephrogenesis alone is not adequate to induce hypertension. Furthermore, modest maternal protein restriction in the rat induced hypertension (with reduced glomeruli number) in males but did not affect females (Woods et al., 2005). In a model of reduced uterine perfusion, low birth weight rat pups
developed prepubertal hypertension but, in the first instance, only male mice continue to demonstrate hypertension post puberty (Alexander et al., 2005). At 4 (Ojeda et al., 2007) and 10 weeks of age (Alexander et al., 2005), denervation of both renal arteries in offspring that were growth restricted significantly reduced systolic blood pressure, but did not affect systolic blood pressure in normally grown offspring. Male offspring from growth restricted pregnancies also demonstrated increased oxidative stress in the kidneys (Ojeda et al., 2012). Chronic treatment with the antioxidant Tempol normalised both systolic blood pressure and renal oxidative stress. Although female rat offspring from growth restricted pregnancies are not hypertensive in the early post puberty period, aging increased systolic blood pressure in 12, but not 6 month old females from growth restricted pregnancies by mechanisms of increase renal vascular resistance (Intapad et al., 2013). Bilateral renal denervation normalised the elevated systolic blood pressure in aged female offspring from growth restricted pregnancies. Thus, these data provide support for the hypothesis that prenatal insults program sex-specific differences in blood pressure.

Others have sought to determine possible mechanisms aside from changes in renal function, by which low birth weight could programme adult hypertension. A study by Linder and colleagues (1990) demonstrated that, in adult patients with essential hypertension, there was a reduction in endothelial-dependent acetylcholine-induced vasodilatation of the forearm when compared to normotensive patients; thus demonstrating endothelial dysfunction in hypertensive patients. Later, Taddei and colleagues demonstrated that offspring from at least one hypertensive parent had reduced acetylcholine-induced vasodilatation in the forearm and that this effect preceded the appearance of hypertension (Taddei et al., 1992). The authors therefore suggested that rather than a consequence of hypertension, endothelial dysfunction could indeed be a cause. Vasodilatation in the coronary artery of patients with essential hypertension was also reduced (Egashira et al., 1995). Relaxation of the coronary artery was impaired when acetylcholine, but not sodium nitroprusside (a direct vascular smooth muscle cell dilator) was administered. Additionally, reduced endothelium-dependent vasodilatation could be associated with a thickening of the vascular wall in hypertensive patients (Ghiadoni et al., 1998).

Using Doppler velocimetry, children who were born small for gestational age had aortic vessel wall diameters that were smaller than those of normal birth weight children (Ley et al., 1997). Endothelial dysfunction has also been demonstrated in low birth weight babies at 3 months of age (Norman and Martin., 2003). Additionally, reduced endothelial flow-mediated dilatation of brachial arteries was associated with low birth weight in children and adults between the ages of 9 and 11 or 20 and 28, respectively (Leeson et al., 1997, Leeson et al., 2001). This may be an early indicator of the pathogenesis of cardiovascular disease in adulthood.
Experimental models have also demonstrated the role of the endothelium in the developmental programming of hypertension. In the rat, maternal undernutrition is associated with hypertension in the offspring (Franco et al., 2002). Male offspring demonstrated more severe hypertension in response to maternal undernutrition compared with females. In addition, there was a sex-independent reduction in endothelial-dependent vasodilatation (aorta) to acetylcholine of rat offspring from dams with restricted diets. As with human studies of associations between low birth weight and endothelial dysfunction (Leeson et al., 1997, Leeson et al., 2001) there were no differences in endothelial-independent vasodilation. Furthermore, Payne and colleagues demonstrated that offspring from dams with reduced uteroplacental perfusion, which were growth restricted at birth, had reduced endothelial-dependent relaxation to acetylcholine in thoracic aortas. The mechanisms by which low birth weight induces endothelial dysfunction in offspring are not clear (Payne et al., 2003). However, a reduced activity of eNOS, the enzyme responsible for the production of the vasodilator NO, could be one possibility (Franco et al., 2002). There is also evidence to suggest that in FGR, elevated oxidative stress \textit{in utero} may lead to impaired endothelial dysfunction and adulthood disease (Franco et al., 2002b). Indeed, intrauterine malnutrition was associated with increased superoxide production in the mesenteric arterial bed of both male (Franco et al., 2003) and female (Franco et al., 2007) rat offspring. Giussani and colleagues (Giussani et al., 2012) have also demonstrated that hypoxia and oxidative stress may play an important role in the development of cardiovascular disease. Male offspring from hypoxic pregnancies in rats demonstrated reduced NO-dependent relaxation in resistance arteries which was associated with aortic remodelling and increased cardiac sympathetic sensitivity. The authors concluded that prenatal hypoxia results in oxidative stress in the fetal heart and vasculature that, in turn, programmes cardiovascular dysfunction in adulthood.

1.6.3 Intrauterine programming of metabolic diseases

1.6.3.1 Impaired glucose tolerance and insulin resistance

Impaired glucose tolerance and increased concentrations of fasting glucose are risk factors for non-insulin dependent diabetes (Edelstein et al., 1997). An analysis of 6 studies investigating the role of impaired glucose tolerance in development of non-insulin dependent diabetes demonstrated individuals with impaired glucose tolerance had an 8% increased risk of developing the disease (Edelstein et al., 1997). Furthermore, impaired glucose tolerance was associated with an increased risk of developing cardiovascular disease in later life (Tominaga et al., 1999). The association between low birth weight and glucose intolerance is well established. In a study of UK men at age 50 there was an increased 2-hr plasma glucose concentration after a glucose tolerance test in those which had reduced weight compared to normal weight at birth (Phipps et al., 1993). A later study in a Swedish cohort of men at age 70 demonstrated that those born small for
gestational age had increased likelihood of impaired glucose tolerance and non-insulin dependent diabetes (McKeigue et al., 1998). Furthermore, this study demonstrated that obesity in adulthood also predicted the risk of impaired glucose tolerance and non-insulin dependent diabetes.

Insulin resistance is a physiological condition in which cells are unable to respond to insulin. As a consequence, plasma glucose concentration increases and in response to this hyperglycaemia, the pancreatic beta cell production of insulin is increased leading to hyperinsulinemia. In a cohort of 1,151 of children who were examined between the ages of 5 and 11 years of age, those that had a lower birth weight that normal had reduced fasting insulin and post-load insulin. After adjustment for childhood height and ponderal index, for each kg increase in birth weight fasting insulin fell by almost 17 % (Whincup et al., 1995) and post-load insulin concentrations were reduced by approximately 12 %. As these observations were only demonstrated after adjustment for childhood height and ponderal index, the authors conclude that birth weight is not associated with insulin resistance but rather childhood accelerated growth is a stronger determinant. Fasting plasma concentrations of glucose, insulin and pro-insulin were highest in women who were small a birth and were associated with raised blood pressure in adulthood (Fall et al., 1995). Furthermore, abnormal growth in utero was also associated with a reduced final height and increased concentrations of plasma insulin in a study of 236 singleton pregnancies in France (Leger et al., 1997).

### 1.6.3.2 Non-insulin dependent diabetes

In 91 men from the Hertfordshire cohort, there was a strong link between low birth weight and incidence of both impaired glucose tolerance and non-insulin dependent diabetes in adulthood (Hales et al., 1991). A later study by Lithell and colleagues (1996) further confirmed that small size at birth, especially thinness (small abdominal circumference), increased insulin resistance and increased the risk of non-insulin dependent diabetes in later life. Furthermore, the authors suggested that obesity in adulthood could further exacerbate this risk. A later study confirmed this finding and demonstrated that small for gestation age infants who had high growth rates from 7 years of age were much more likely to develop non-insulin dependent diabetes, compared with small babies with normal postnatal growth (Forsén et al., 2000). As with hypertension, it was demonstrated that in a cohort of Pima Indians the relationship between prevalence of diabetes and weight at birth was U shaped, i.e. those small for gestational age and large for gestational age were at increased risk of non-insulin dependent diabetes (McCance et al., 1994). A large cohort study of US births (1921 to 1946) also showed a strong link between low birth weight and non-insulin dependent diabetes in adult women (Rich-Edwards et al., 1999). This study also controlled for postnatal environmental factors such as socioeconomic factors, smoking and alcohol.
consumption and demonstrated that birth weight alone determined risk of non-insulin dependent diabetes.

1.6.3.3  Mechanisms underlying pathogenesis of metabolic disease

Studies investigating the mechanisms by which low birth weight programs impaired glucose tolerance, insulin resistance and non-insulin dependent diabetes have mainly utilised the rat. Importantly, in the low protein diet model of maternal undernutrition, young adult rats which were born small for gestational age had enhanced glucose tolerance compared with controls (Langley et al., 1994, Holness., 1996, Shepherd et al., 1997). However, this difference was diminished by 44 - 52 weeks after birth (Langley et al., 1994, Petry et al., 1997) and impaired glucose tolerance was noted by 15 months of age in rats with that had reduced birth weight (Hales et al., 1996). In male offspring by 17 months of age, diabetes was evident and was likely due to insulin resistance; however females demonstrated insulin deficiency (Hales et al., 1996).

Early investigations into the mechanisms surrounding impaired glucose tolerance and insulin resistance in the “low protein diet fed” rat focussed on the development and function of the pancreas. Snoeck and colleagues demonstrated that there was a reduction in vascularisation in the islets of the pancreas from neonates whose mothers were administered a low protein diet (Snoeck et al., 1990). There was also a reduction in the proliferation of pancreatic beta cells and total islet size was reduced. A more recent study demonstrated that low protein diet does indeed reduce the proliferation of fetal pancreatic beta cells and increases the apoptosis associated with these cells (Petrik et al., 1999). The timing of the insult was important in determining the reduction in beta cell number and area (Chamson-Reig et al., 2006). There were also gender specific effects; females were more susceptible to protein restriction in mid-gestation whereas males were more susceptible in late gestation. Oxidative stress within the pancreas may also contribute to altered glucose tolerance, insulin resistance and non-insulin dependent diabetes. Indeed, recent evidence has demonstrated that rat offspring from dams administered a low protein diet had increased expression of factors associated with oxidative stress and reduced expression of antioxidants within the islets of the pancreas (Tarry-Adkins et al., 2010).

Taken together, these data illustrate morbidity and mortality associated with low birth weight is not only confined to perinatal life but are also apparent in adulthood. The wealth of evidence that shows small size at birth is associated with elevated risk of cardiovascular and metabolic disease in later life emphasises the need to develop therapies to improve the in utero environment and fetal growth in FGR. Well characterised mouse models of human FGR can be used to develop and assess the efficacy and safety of such therapies.
1.7 Mouse models of human pregnancy complications

Various animal species are used to mimic human pregnancy but, for reasons outlined in section 1.3, 1.4 and 1.5 above, there are many advantages of using murine models (see table 1.2 for summary). A variety of mouse strains have been used to model pregnancy complications in women (see table 1.3 for overview). Maternal dietary modification (for review see Sinclair and Watkins., 2013), reduced uterine perfusion through surgical ligation (Janot et al., 2014), hypoxia (Ream et al., 2008) and genetic manipulation (Constância et al., 2002, Kanasaki et al., 2008, Dilworth et al., 2012) have been used to induce growth restriction in mice. Well-characterised genetic mouse models of pregnancy complications such as FGR and PET are the focus of this chapter.

1.7.1 Insulin-like growth factor 1 knockout mouse

Baker and colleagues were the first to illustrate the role of insulin-like growth factor 1 (Igf1) in fetal development (Baker et al., 1993). At E13.5 and until late gestation there was a significant reduction in weight of Igf1−/− knockout fetuses compared with wild type; placental weight was not altered. Postnatal growth rates of Igf1−/− knockout offspring were reduced compared with wild type with Igf1−/− 30 % smaller than controls. Insulin-like growth factor 2 (Igf2) knockout mice demonstrate placental growth restriction from E14 with fetal weight significantly reduced from E12 when compared to wild type controls (see section 1.7.2, Constância et al., 2005).

1.7.2 Igf2 P0 and Igf2-total knockout mouse

Igf2 is an imprinted gene with the majority (approximately 95 %) of gene expression from the paternal mouse chromosome 7. There are four main Igf2 transcripts P0, P1, P2 and P3. In Igf2-total KO mice all four promoter regions are replaced with a LacZ vector which specifically targets the exon containing P0, P1, P2 and P3. Deletion of the four main promoter regions completely abolishes expression of IGF2 in both fetus and placenta and leads to severe growth restriction with no postnatal catch up growth (Constância et al., 2002, Mikaelsson et al., 2013).

The P0 transcript of Igf2 is expressed only in the mouse placenta. Deletion of a 5 kb region of the U2 exon led to a complete reduction in labyrinthine IGF2 but no change in fetal IGF2 concentration or production. Male Igf2-P0+/− mice mated with female Igf2-P0+/+ produce litters with both Igf2-P0+/− (referred to as P0) and Igf2-P0+/+ (referred to as wild type) fetuses.
Table 1.2. Common animal models of human pregnancy illustrating advantages and disadvantages of each. Where applicable, comparisons are made between animal and human. *Carter., 2007, †Atkinson et al., 2006.

<table>
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<th></th>
<th>Mice</th>
<th>Rats</th>
<th>Sheep</th>
<th>Non-human Primate</th>
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<tr>
<td>Genetic divergence</td>
<td>Relatively similar</td>
<td>Relatively similar</td>
<td>Distant (Ruminants)</td>
<td>Very similar</td>
</tr>
<tr>
<td>Genetic manipulation</td>
<td>Relatively easy</td>
<td>Moderate difficulty</td>
<td>Difficult</td>
<td>Difficult</td>
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<tr>
<td>Surgical manipulation</td>
<td>Requires expertise</td>
<td>Requires expertise</td>
<td>Requires expertise</td>
<td>Requires expertise</td>
</tr>
<tr>
<td>Gestation</td>
<td>~19-21 days</td>
<td>~21-24 days</td>
<td>~152</td>
<td>~180 days</td>
</tr>
<tr>
<td>Development <em>in utero</em></td>
<td>Underdeveloped</td>
<td>Underdeveloped</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Placentation*</td>
<td>Similar (Haemotrichorial)</td>
<td>Similar (Haemotrichorial)</td>
<td>Distinct (Epitheliochorial)</td>
<td>Very similar (Hemochorial)</td>
</tr>
<tr>
<td>Placental transfer†</td>
<td>Similar for some solutes</td>
<td>Similar for some solutes</td>
<td>Less permeable to solutes</td>
<td>Similar</td>
</tr>
<tr>
<td>Cost</td>
<td>Relatively inexpensive</td>
<td>Relatively inexpensive</td>
<td>Expensive</td>
<td>Very expensive</td>
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1.7.2.1 Placental development and function in Igf2 P0 and Igf2-total knockout mice

At E12.5 placentae from P0 mice are significantly smaller than their wild type (WT) littermates and this differential remains until term. Interestingly, the onset of growth restriction only occurs in late gestation in the P0 mice (Constância et al., 2002). Thus, this timeline suggests that the FGR in this mouse model is due to placental dysfunction. By E19, P0 placental weight is reduced by approximately 30% compared with WT placentas and P0 fetal weight is reduced by up to 25% (Constância et al., 2002, Sibley et al., 2004).

Sibley and co-workers demonstrated a reduced diffusional exchange capacity in the P0 mouse (Sibley et al., 2004). Studies utilizing radiolabelled tracers of increasing size (mannitol, EDTA and inulin) illustrated that the diffusion capacity of the P0 placentas could be reduced by 40% in P0 placentas compared to WT placentas. These experiments support an earlier hypothesis of Mayhew and colleagues that diffusion capacity may be reduced up to 60% in human FGR, further showing how the P0 mouse lends itself as a model of human FGR (Mayhew et al., 2003). This led to the hypothesis that IGF2 is essential for the maintenance of a healthy placental phenotype and for normal control of diffusional characteristics within the mouse.

The reason for reduced diffusional exchange in Igf2 total knockout and P0 placentas was later investigated by Coan and colleagues (Coan et al., 2005). Using stereological analysis both the Igf2 total KO and P0 placentas exhibited reduced diffusional exchange capacity as a result of a thickening of the interhaemal membrane (Coan et al., 2005). However, the Igf2 total KO demonstrated a significantly lower theoretical diffusing capacity compared to P0 placentas. The authors suggest this is a consequence of differential placental development; P0 placentas demonstrate a proportional reduction in each of the placental compartments (Jz, Lz and decidua) but the Igf2 total KO placentas show reduced labyrinthine trophoblast and an increased volume of the junctional zone.

In the Igf2 total KO mouse, fetal and placental growth restriction occur at the same point in gestation (Coan, Fowden et al. 2008), whereas in the P0 mouse placental growth restriction precedes fetal growth restriction (Sibley, Coan et al., 2004). The P0 mouse exhibits reduced placental growth before the onset of growth restriction in the fetus. Thus, the P0 model displays an increased fetal: placental weight ratio, which is suggestive of increased placental efficiency. By using 14C-radiolabelled glucose and methylaminoisobutyric acid (MeAIB), an amino acid analogue transported by the System A amino acid transport system, Constância and colleagues (2005) demonstrated placentas from P0 mice had increased transfer of both solutes at E16 but not at E19. Increased placental glucose transport in P0 was in part due to an increase in the GLUT3
transporter. Increased MeAIB transport was partly due to an increase in the imprinted System A gene *Slc38a4*. In contrast, there was no change in placental glucose or MeAIB transport at either E16 or E19 in the *Igf2* total KO placentas. More recent data from Dilworth and colleagues demonstrated a reduction in P0 fetal calcium content at E17 but not at E19, compared with WT littermates (Dilworth et al., 2010). The authors demonstrated similar unidirectional maternofetal calcium transport across the P0 placenta at E17 but an increased calcium clearance at E19 in P0 compared to WT placenta. These data suggest that placental *Igf2* is essential for maintaining appropriate fetal growth. Furthermore, in the absence of placental *Igf2* placental nutrient transfer is up-regulated to meet fetal demand but this up-regulation fails near term and thus adequate fetal growth is unable to be maintained.

### 1.7.2.2 Postnatal observations in *Igf2*-P0 and *Igf2*-total knockout mice

Consistent with findings at E19 (Coan et al., 2008), P0 mice are 25 % smaller than their WT littermates at birth and demonstrate asymmetric FGR (Dilworth et al., 2013). In the P0 offspring, rapid postnatal growth between postnatal day 25 and 50 led to a near normal weight by postnatal day 100 (Mikaelsson et al., 2013). In contrast, *Igf2* total KO birth weight was reduced by around 45 % compared to WT littermates but these mice demonstrated accelerated postnatal growth within the first 25 days of postnatal life. At postnatal day 100 *Igf2* total KO mice are 40 % of the body weight of WT control littermates. These size differences continue to be apparent at postnatal day 400. In P0, but not *Igf2* total KO offspring, there an increased sensitivity to “anxiety provoking stimuli” such as noise and novel foodstuffs. The authors suggest that an *in utero* mismatch of placental supply and fetal demand in the P0 mouse may somehow programme the fetus for increased anxiety in adulthood (Mikaelsson et al., 2013).

Unlike other genetic mouse models of growth restriction, the P0 model is placental specific and does not affect concentrations of *Igf2* within the fetus or offspring. The *Igf2* total KO mouse model is not placental-specific and there is no *Igf2* present within the fetus or offspring thus, although growth restricted at birth, fetuses do not display postnatal catch up growth which is the case in the majority of human FGR cases. The P0 model FGR phenotype is more moderate and is placental in origin. In addition, P0 mice demonstrate significant postnatal catch up growth. The P0 therefore represents a good model for assessing the impact of placental insufficiency on programming of postnatal disease. However, there have yet to be any studies which have focussed on the impact of the placental-specific *Igf2*, the subsequent fetal growth and how this relates to cardiovascular or metabolic consequences on the offspring. A summary of the *Igf2* P0<sup>+/–</sup> mouse model can be found in table 1.3.
Table 1.3. Mouse models of FGR; comparison with human FGR with arrows indicating trends. Key: *conflicting evidence from Stanley et al., 2012 and Poudel et al., 2013), #myometrial arteries from FGR pregnancies, ^from Lembo et al., 1996. All other data references found in the text.

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<tr>
<th></th>
<th>Igf1/−</th>
<th>Igf2 P0/−</th>
<th>eNOS/−</th>
<th>eNOS +/− P0</th>
<th>COMT/−</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth restriction</strong></td>
<td>↓65 %</td>
<td>↓22 %</td>
<td>↓10 %</td>
<td>↓20 %</td>
<td>Yes*</td>
<td>Yes (varies)</td>
</tr>
<tr>
<td>&lt;5th Percentile of WT</td>
<td>?</td>
<td>43 %</td>
<td>50 %</td>
<td>&gt;90 %</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>weight</td>
<td></td>
<td>(E16.5)</td>
<td>(E18.5)</td>
<td>(E17.5-E18.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Placental weight</strong></td>
<td>↔</td>
<td>↓30 %</td>
<td>↔</td>
<td>↓30 %</td>
<td>↑15 %</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(E13.5-E18.5)</td>
<td></td>
<td>(E17.5-E18.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fetal:placental ratio</strong></td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>?</td>
</tr>
<tr>
<td><strong>Asymmetric FGR</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes (majority)</td>
</tr>
<tr>
<td><strong>Uterine artery</strong></td>
<td>?</td>
<td>↓</td>
<td>↑</td>
<td>?</td>
<td>↔</td>
<td>↓#</td>
</tr>
<tr>
<td>constriction</td>
<td>(Phenylephrine^)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Phenylephrine)</td>
<td></td>
<td>(hypertensive^)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Uterine artery</strong></td>
<td>?</td>
<td>↔</td>
<td>↓</td>
<td>?</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>relaxation to ACH</td>
<td>(Phenylephrine^)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>System A activity</strong></td>
<td>↑</td>
<td>↓</td>
<td>↔</td>
<td>?</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>(MeAIB transfer per g of placenta)</td>
<td></td>
<td></td>
<td>(E17.5-E18.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.7.3 Endothelial nitric oxide synthase

The eNOS enzyme plays an important part in the control of vascular tone. In endothelial cells, activated eNOS is responsible for converting L-arginine to L-citrilline and the potent vasodilator NO (Kulandavelu et al., 2013). Unsurprisingly, mice deficient in eNOS are hypertensive (approximately 140 mmHg systolic blood pressure; Shesely et al., 1996) and this hypertension persists throughout pregnancy (Hepler et al., 2001). Knockout animals show intrauterine growth restriction with a 10% reduction in fetal weight at E17.5 (Hepler et al., 2001b, Kusinski et al., 2012). Thus, eNOS mice have been described as a model of pre-pregnancy hypertension with FGR.

As hypertension is maintained throughout pregnancy, it is perhaps not surprising that reduced uterine artery maximal and minimal velocity is evident in eNOS-/- pregnant dams (Kulandavelu et al., 2006, Poudel et al., 2013). In addition, reduced endothelial-dependent relaxation was noted in the uterine artery from eNOS dams using wire myography (Kusinski et al., 2012). Impaired function of, and reduced blood flow in, the uterine artery may be a possible mechanism for the increased placental hypoxia and reduced system A transport associated with eNOS placentas (Kusinski et al., 2012). Recently, eNOS+/ mice have been crossed with male placental-specific Igf2+/ (P0) knockout mice to observe whether a more severe phenotypic could be demonstrated (Dilworth et al., 2012). eNOS+/ P0 fetuses were no more growth restricted than either eNOS-/- fetuses or P0 fetuses. However, the reduced system A activity in eNOS-/- placentas was attenuated in eNOS+/ P0 placentas. Although the eNOS-/- mouse model is of use in understanding the role of NO during pregnancy, FGR is not often associated with pre-pregnancy hypertension and this mouse model more mimics cases of PET (see table 1.3).

1.7.4 Catechol-O-methyltransferase

Catechol-O-methyltransferase (COMT) generates 2-Methoxyoestradiol (2-ME), an oestradiol metabolite which is thought to play an important role in the regulation of the expression of a number of hypoxia-sensitive genes (Kanasaki et al., 2008). In human pregnancy, levels of 2-ME fluctuate throughout gestation and peak (96 nmol/L) within the third trimester. In women whose pregnancies are affected by PET, COMT expression and 2-ME levels are significantly reduced (Wentz et al., 2007).

The phenotype displayed by mice deficient in COMT (COMT-/- knockout mice) highlight the importance of this enzyme to normal vascular function. Pregnant mice deficient in COMT-/- were proteinuric, demonstrated elevated systolic blood pressure, arteriopathy, preterm delivery (E19) and increased fetal wastage compared to normotensive controls. COMT-/- mice also show a reduction in the expression of eNOS in placentas (Kanasaki et al., 2008). Doppler waveform
analyses have also illustrated reversed end diastolic flow in the umbilical artery of COMT\(^{-}\) mice compared to WT controls at E18.5 (Stanley et al., 2012). Reduced maximal and reduced minimal uterine artery blood flow velocity was also demonstrated in COMT\(^{-}\) dams (Poudel et al., 2013). There was also a small but significant reduction in fetal weight in COMT\(^{-}\) mice when compared to wild type fetuses (Stanley et al., 2012) which was no longer apparent in a later study (Poudel et al., 2013). Coupled to the data suggesting reduced 2-ME in PET pregnancies, these data suggested a role for COMT and 2-ME in the control of normal vascular function during healthy pregnancy in humans.

There are a number of mouse models which mimic human pregnancy complications (see table 1.3) which could be useful in determining the effects of treatments in cases of FGR and PET. Of the mouse models of FGR, the P0 mouse has so far been studied most and shows similarities compared with the majority of cases of late onset human FGR (e.g. asymmetric, reduced placental weight and normal uterine blood flow; highlighted in table 3.1). Therefore, assessing therapeutics in the P0 mouse will provide evidence for the efficacy and safety of therapies in human pregnancy complications such as FGR.
1.8 Treatments for FGR

Treatments that may be of use in pregnancy complications such as FGR must be assessed for safety and efficacy in experimental animal models of pregnancy complications. Moreover, for treatments to be of use in particular clinical situations it is important to demonstrate positive effects on clinical criteria such as abnormal umbilical / uterine artery Doppler waveforms or abdominal growth velocity. As such, studies characterising fetal and postnatal vascular phenotype in mice are essential to appropriately assess effects of treatments.

1.8.1 Low molecular weight heparin

Heparin, a type of glycosaminoglycan (GAG), has been utilised in medicine for over half a century. GAGs are linear polysaccharides which consist of a mixture of glucosamine, glucuronic acid or iduronic acid (Lever and Page, 2002). Unfractionated high molecular weight heparins (UFH) demonstrate non-specific binding, inhibit the effects of platelets and increase the likelihood of haemorrhage (Hirsh et al., 2001). UFH has poor bioavailability, is rapidly cleared and can lead to the degradation of bone (Muir et al., 1996). Therefore, much shorter fractionated compounds began to be synthesised. These compounds are known as the low molecular weight heparins (LMWH). Importantly LMWH is unable to cross the placenta and thus absent in the fetal circulation (Chan and Ray, 1999).

Small scale non-randomised trials have shown enoxaparin to be a safe and effective prophylactic therapy for pregnant women at increased risk of venous thromboembolism (VTE) (Rowan et al., 2003). The bioavailability of enoxaparin is close to 100 % which is likely due to reduced nonspecific binding to plasma proteins (Young et al., 1994, Al-Ansari et al., 2007). In vitro studies in aged and aged-diabetic hamsters have shown enoxaparin improves the altered reactivity of the vasculature by reducing contractility and increasing endothelial-dependent relaxation of the microvasculature (Georgescu et al., 2003). A recent study has demonstrated that the effect of enoxaparin on improving the endothelial dysfunction does not involve the nitric oxide synthase (NOS) pathway, but instead shows a reduction in the mitogen-activated protein (MAP) kinase pathway (Georgescu et al., 2011). Therefore, enoxaparin may be useful for the treatment of widespread endothelial dysfunction in PE or in FGR cases where uterine artery blood flow is reduced.

Hypoxia-induced pulmonary remodelling demonstrated in a guinea pig model of pulmonary arterial hypertension (PAH) was shown to be significantly reduced when animals were treated with dalteparin (a similar LMWH) suggesting that a LMWH was able to reduce the vascular remodelling associated with pulmonary hypoxia (Al-Ansari et al., 2007). Dalteparin, but not
enoxaparin, was able to reduce the vascular remodelling, hypertrophy and hyperplasia associated with PAH. Dalteparin is an effective treatment for thromboembolic diseases associated with pregnancy due to its antithrombotic properties (Rey et al., 2009, Rey and Rivard., 2000). A small scale non-randomised trial of 276 pregnant women with thrombophilia and a history of PET / FGR found LMWH combined with aspirin to increase favourable pregnancy outcomes (Riyazi et al., 1998) with no observed side effects. A significant reduction in uterine artery resistance index was observed in a cohort of women with gestational hypertension who were administered enoxaparin (Torricelli et al., 2006). These studies also demonstrated that enoxaparin could increase the predictive performance of uterine artery Doppler for PET. In agreement with these data, Mello and colleagues (2005) have demonstrated enoxaparin improved uterine artery blood flow and reduced the incidence of PET in a cohort of women with a genetic polymorphism known to increase the risk of PET.

1.8.2 Low dose aspirin

The Essai Pre-eclampsie Dipyridamole Aspirine (EPREDA) trial demonstrated that low dose aspirin led to a reduction in the incidence of fetal growth restriction in pregnant women and significantly increased mean birth weight (Uzan et al., 1991). There were no measured detrimental side effects to fetuses but some mothers did suffer from acute headaches. In addition birth weight, head circumference and placental weight were all increased in patients receiving aspirin with high umbilical artery systolic / diastolic ratios (i.e. those that may have reduced umbilical blood flow) (Trudinger et al., 1988). The mechanism(s) by which aspirin elicits these pharmacological actions is likely to be via an action on prostaglandin synthesis. An in vitro study by Thorp and colleagues (1988) demonstrated thromboxane (a contractile derivative of the prostaglandin synthesis process) production in human placental arteries was inhibited with the addition of aspirin, but that of the closely related dilator prostacyclin was unaffected. In PET placentas, aspirin was also shown to inhibit thromboxane production but no effect on prostacyclin production / levels was observed (Wang et al., 1995); other groups have also demonstrated that low-dose aspirin led to a reduction in thromboxane B2 (Feldman et al., 2001). Furthermore, a recent meta-analysis on the use of low dose aspirin concluded that risk of PET, FGR and preterm delivery were reduced in pregnancies associated with abnormal placentation but only if treatment with aspirin was initiated prior to 16 weeks gestation (Bujold et al., 2014). Although the positive effects on fetal weight have been demonstrated, aspirin is only used in at-risk populations and not routinely used in low-risk pregnancies due to the risk of prolonged bleeding in the mother and new-born (Visintin et al., 2010).
In a mouse model of fetal alcohol exposure, aspirin was able to reduce the birth defects in a dose dependent manner (Randall et al., 1984, Randall et al., 1991). More recently, aspirin prevented hypertension and reduced proteinuria in a mouse model of PET (Doridot et al., 2013).

### 1.8.3 Antioxidants

In cases of FGR with increased uterine artery Doppler PI, it is thought that the changes in Doppler waveform are indicative of hypoperfusion of the fetoplacental vasculature (Brunelli et al., 2010). Thus, it is hypothesised placental hypoxia will lead to fetoplacental oxidative stress and the production of reactive oxygen species (ROS). ROS are cytotoxic at higher concentrations and elicit a number of detrimental effects at lower concentrations (Hubel, 1999). Platelet-derived ROS can damage endothelial cells thus increasing the recruitment of immune foam cells as well as additional platelet cells. It is hypothesised that this leads to endothelial dysfunction and a shift towards an oxidative, rather than a more balanced oxidative / antioxidant phenotype (Iuliano et al., 1997, Kitiyakara and Wilcox, 1998). Superoxide (a ROS) is also able to bind directly to the potent vasodilator nitric oxide (NO) forming peroxynitrite and nitrotyrosine which ultimately reduces the vasodilatory effects of NO (Beckman and Koppenol, 1996). This imbalance of vasodilators and vasoconstrictors caused by increased ROS could be overcome by the addition of antioxidants.

The antioxidant Tempol (a superoxide dismutase mimetic) has been utilized in the spontaneous hypertensive rat (SHR) model. Tempol treated animals exhibited reduced blood pressure and reduced renal vascular resistance (Schnackenberg et al., 1998) by a mechanism involving the increased production or bioavailability of NO (Schnackenberg et al., 1998). Chronic application of Tempol has also been used in a mouse model of hypertension with growth restriction (BPH/5 mouse); treatment increased fetal growth and survival (Hoffmann et al., 2008). In the eNOS mouse model of FGR Tempol was able to significantly increase fetal weight and fetal body length presumably via increasing nutrient supply to the placental bed since uterine artery blood flow velocity was increased in treated dams (Stanley et al., 2012b).

Pomegranate juice (PJ), a natural source of polyphenols with three times the antioxidant activity of green tea extract and red grapes, offers an alternative source of antioxidants. De Nigris and colleagues (2006) demonstrated that addition of PJ to an endothelial cell line reduced the down-regulation effect of oxidised low density lipoproteins (oxLDLs) on NO synthesis, thus increasing the availability of NO. More recent data from the same laboratory has demonstrated a direct effect on expression of eNOS; PJ and pomegranate juice extract were able to increase the expression of eNOS in obese Zucker rats (De Nigris et al., 2007). In humans PJ has beneficial effects in elderly hypertensive patients by lowering blood pressure and inhibiting the effects of...
angiotensin converting enzyme (ACE), an enzyme involved in the cleavage of angiotensin I to the potent vasoconstrictor, angiotensin II (Aviram and Dornfeld, 2001). A small scale human trial in patients with atherosclerosis demonstrated a decrease in systolic blood pressure after one year’s consumption of PJ. This is likely due to a reduction in the levels of oxLDLs and increase of total antioxidant status by 130% over one year, compared to the placebo control (Aviram et al., 2004).

Resveratrol, a compound found in PJ, reduced blood pressure in obese Zucker rats and increased eNOS expression; likely due to a down regulation of tissue necrosis factor-α (TNFα), an inhibitor of eNOS expression (Rivera et al., 2009). Resveratrol also stimulates NO production in a rat model of myocardial ischaemia-reperfusion injury (Hung et al., 2004) and is a potent vasorelaxant in guinea pig mesenteric and uterine arteries (Naderali et al., 2000). Furthermore, studies have suggested endothelial-independent effects of resveratrol on rat mesenteric artery are due to interactions with voltage-gated calcium channels (Gojkovic-Bukarica et al., 2008). It is hypothesised that the blood-pressure lowering effects of resveratrol may be due to the increase in eNOS expression as well as the antioxidative effects. In support of this, resveratrol treatment in the eNOS+/− mouse did not improve uterine artery blood flow or fetal weight (Poudel et al., 2013). However, resveratrol was able to increase uterine artery blood flow and fetal weight in the COMT+/− mouse (Poudel et al., 2013).

Although animal studies have shown promising results, antioxidant therapy has variable outcomes in human diseases such as hypertension and PET. A small-scale trial using the antioxidants vitamin C, thiopronine and glutathione revealed the latter two antioxidants had blood pressure lowering effects whereas randomised double-blinded studies using vitamin C and vitamin E showed no effects on blood pressure in cases of severe PET (Gulmezoglu et al., 1997). The results from vitamin C and E human trials have been largely disappointing with only one positive result from a trial in 2002 (Chappell et al., 2002). This study demonstrated that with the addition of vitamin C and E, indicators of placental dysfunction (e.g. soluble fms-like tyrosine-1; Sflt-1) in high-risk pregnancies such as PET could be reduced to levels of that of low risk cohorts. Furthermore, a small scale trial in women without any pregnancy complications, 8 oz / day of PJ led to a significant reduction in placental oxidative stress (Chen et al., 2012)

1.8.4 Sildenafil citrate

SC (SC, marketed as Viagra®) is a selective phosphodiesterase type-5 (PDE-5) inhibitor whose action results in inhibition of the hydrolysis of cyclic guanosine monophosphate (cGMP) and thus increases the bioavailability of the vasodilator nitric oxide (NO). Sildenafil (UK-92,480), first developed to reduce blood pressure and treat angina patients, demonstrated a mild hypotensive
response following drug administration. It was noted during early trials of the drug that PDE-5 inhibition led to an enhanced response in patients with erectile dysfunction (Boolell et al., 1996).

The free radical NO is produced in the corpus cavernosum of the penis upon sexual stimulation and binds directly to heme within the soluble form of guanylate cyclase (sGC) (Hille et al., 1979). When bound, NO produces a 200 fold activation of sGC which then leads to the catalysis of guanosine triphosphate (GTP) to cGMP (Lucas et al., 2000). cGMP is able to induce smooth muscle cell relaxation by reduction in intracellular calcium either via interaction with cGMP-binding proteins, protein kinase G or cGMP-gated ion channels. (Lincoln et al., 2001).

The PDE-5 enzyme is highly expressed in the corpora cavernosa of the penis but is also expressed in other tissues such as the lung (Corbin et al., 2005) and heart (Nagayama et al., 2008). When bound to cGMP, PDE-5 is able to catalyse the hydrolysis of cGMP to the relatively inert GMP. SC demonstrates a structure similar to cyclic guanosine monophosphate (cGMP) and is thus able to reduce the degradation of cGMP through selective binding and inhibition of phosphodiesterase type 5 (PDE-5). Thus, SC is able to modulate the intracellular concentration of cGMP and prolongs the vasorelaxant (or vasodilator) effects of the NO / cGMP pathway.

### 1.8.4.1 Effects of Sildenafil citrate on maternal vasculature

SC has been effective in reducing the elevated blood pressure associated with Eisenmenger syndrome-induced pulmonary hypertension in pregnant women (Lacassie et al., 2004, Molelekwa et al., 2005). SC has also been used in studies focussed on assistive reproductive therapy (ART); the rationale being that SC might improve localised blood flow to the uterus and thus aid endometrial development and implantation. In a small initial study, vaginal SC application led to a decreased PI (i.e. increased blood flow) in the uterine artery in women undergoing ART (Sher and Fisch., 2000). A larger study by the same group demonstrated that vaginal SC administration for 3 - 10 days led to an overall enhanced endometrial development and an increased implantation rate in poor prognosis pregnancies (Sher and Fisch., 2002). However, this effect was not reproduced by Check and colleagues (2004) who showed no effect of 25 mg vaginal SC four times daily on endometrial thickness or uterine artery blood flow (Check et al., 2004). Similarly, two patients with a thin endometrial layer, who were unable to conceive following treatment for fibrosis of the endometrium, were administered SC vaginally for up to 14 days. In both cases SC increased endometrial thickness and resulted in healthy singleton pregnancies to term (Zinger et al., 2006). Intravaginal SC treatment also led to an increased endometrial thickness but without any reduction in uterine artery PI indices (i.e. there was no change in blood flow) (Paulus et al., 2001), suggesting SC may have beneficial effects on vascular development / function without effects on blood flow. Similar observations were found in cohort of 36 women with a history of recurrent miscarriage (Jerzak et al., 2008). In addition to the increased endometrial thickness,
there was a reduction in peripheral, but not endometrial, natural killer cell activity; a predictor of subsequent miscarriage (Aoki et al., 1995, Quenby et al., 1999). Recently, a small pilot study investigating the role of SC on promoting endometrial growth also demonstrated SC increased uterine radial artery blood flow using Doppler velocimetry (Takasaki et al., 2010). Together these studies suggest SC may be promoting growth of the endometrial layer via increased blood flow and other unrelated mechanisms.

Using colour Doppler ultrasound, Hale and colleagues were able to demonstrate increased uterine volumetric blood flow and reduced vascular impedance in non-pregnant nulliparous women who received either 25 mg or 100 mg of oral SC, compared with placebo (Hale et al., 2010). This study also demonstrated that there was a greater SC-induced increase in blood flow from those women in the treatment group who had a higher basal uterine volumetric flow compared to those with lower uterine volumetric flow. The authors concluded that in some cases of FGR, where there is apparent endothelial dysfunction with altered NO signalling and reduced uteroplacental perfusion, inhibition of PDE-5 with SC and a subsequent increase in cellular cGMP would not lead to increased blood flow, as downstream targets of cGMP would be unresponsive (Hale et al., 2010). Even so, addition of the SC to myometrial arteries from FGR pregnancies did lead to an increase in vasodilatation within this vascular bed (Wareing et al., 2005).

1.8.4.2 Evidence from clinical studies of FGR and PET

Given the evidence for SC-induced increased uterine artery perfusion in cases of infertility, it is unsurprising that clinicians began to look at other pregnancy complications in which reduced maternal and fetoplacental perfusion may play a role in the pathogenesis of disease. Small scale clinical trials have demonstrated an increased abdominal growth velocity (Von Dadelszen et al., 2011) and improved uteroplacental perfusion (Dastjerdi et al., 2012) in growth restricted fetuses whose mothers received SC. One report has since highlighted that SC administration led to a reduction in an abnormally high PI and in uterine artery impedance in a case of severe early onset FGR (Lin et al., 2012). A small scale randomised, double-blinded, placebo-controlled clinical trial did not demonstrate any beneficial effect of SC in prolonging PET-compromised pregnancies (Samangaya et al., 2009). The lack of beneficial effect of SC on primary outcome may have been due to the SC treatment regimen (i.e. the timing [too late in the disease process] and dosage [insufficient initial dose] of SC). Others have suggested a much earlier intervention “no later than 25 weeks gestation, to have the best chance of enhancing the prognosis of the baby” (Downing., 2010). Thus, the timing and dosage of SC administration in high risk pregnancies may well be important in maximising any potential beneficial effects of SC.
1.8.4.3 Evidence from experimental animals

There are several animal studies that have demonstrated the potential beneficial effects of SC for the treatment of PET and FGR. A recent publication by Stanley and colleagues (2012) demonstrated that addition of SC to the drinking water of the pregnant dam (0.2 mg/ml) increased fetal weight in the COMT-/- KO mouse model of FGR. Furthermore, SC improved umbilical blood flow to the fetus as determined by Doppler ultrasonography (Stanley et al., 2012). The authors suggest SC led to increased placental vascularisation and in turn increased blood flow to the placenta and nutrient supply to the fetus. More recently, Dilworth and colleagues (2013) have demonstrated that in the P0 mouse model of FGR, SC added to the dam’s drinking water (0.4 mg/ml) also increased the weight of FGR fetuses. It is interesting to note that the P0 mouse has no detectable vascular abnormalities and therefore SC could be beneficial in cases where reduced growth has been detected but uterine / umbilical artery Doppler indices are within the normal range.

In a rat model of congenital diaphragmatic hernia, 100mg/kg/day of SC was administered subcutaneously to the dam between E11.5 to E20.5 (Luong et al., 2011). Maternal and fetal SC concentration peaked 6 hours post injection and was cleared by 24 hours but there was no effect of SC on fetal body weight. There were no adverse effects noted following SC administration in either the dam or fetuses. These data demonstrate that, at least in the rat, SC is able to cross the placenta through, as yet unknown, cellular pathways. As SC increases weight in mouse models of FGR (e.g. Dilworth et al., 2013), increased fetal weight could be due to SC affecting the fetus directly and / or be due to increasing nutrient supply via increased maternal uterine perfusion (e.g. Stanley et al., 2012).

In an experimental ovine model, FGR was induced by a decreased nutrient supply to the pregnant ewe (Satterfield et al., 2010). At day 115 of gestation concentrations of amino acids within the fetal umbilical venous and maternal uterine artery serum were reduced when compared to the adequately nourished control ewes. Confirming the model’s efficacy, fetuses from nutrient restricted dams had decreased fetal weight compared to those from the control arm of the study. However, SC administered subcutaneously to the dam between days 28 to 115 of gestation led to a significant increase in fetal weight and fetal umbilical venous serum amino acids in a dose-dependent manner, without affecting concentrations of amino acids in maternal serum. SC also increased the weight of fetuses from nutrient replete ewes. However, detrimental effects, such as a reduction in fetal spleen weight and an increase in fetal pancreas weight, were noted in fetuses delivered from adequately nourished ewes which were administered 150 mg.dl⁻¹ SC. Despite this, the authors suggested that SC may be used to increase the nutrient supply of amino acids and polyamines in human FGR.
Other studies have been performed in sheep but use different interventions to induce growth restriction. Miller and colleagues (2009) demonstrated that SC may have a detrimental effect and reduce uterine blood flow in sheep. In their study, FGR was induced by ligating the uterine artery of the pregnant ewe between day 105 and 115. In both control and uterine artery ligated ewes, SC treatment significantly decreased uterine blood flow and both groups displayed hypotension and tachycardia in the fetus. Furthermore, FGR fetuses exhibited hypoxia compared with controls pre SC treatment; a difference that was magnified following initiation of SC treatment. The authors suggested caution when utilising SC as a treatment in growth restricted pregnancies and that further studies must elucidate the mechanisms of action of SC (Miller et al., 2009). In ovariectomised sheep, IV administration of SC increased uterine blood flow, reduced MAP but showed a trend towards reduced coronary blood flow (Zoma et al., 2004). It must be noted however, that these latter studies administered SC intravenously for 24 hours and 5 minutes, respectively. SC also had differential effects on fetal weight and length from dams exposed to a period of hypoxia. Refuerzo and colleagues induced hypoxia by placing dams in a specially designed chamber with a reduced oxygen (9 % O₂) atmosphere for two hours each day from gestational day 18 to 20 (Refuerzo et al., 2006). Although no FGR was associated with hypoxia at birth, oral treatment with SC led to an increase in birth weight, in common with the results in sheep (Satterfield et al., 2010). However, dams administered SC, which did not receive a hypoxic insult, delivered lambs that were significantly smaller and had reduced length when compared to lambs of dams which had no hypoxia and no SC treatment. These data imply that timing, dosage and route of administration of SC may be important in clinical cases of FGR.

The positive preliminary data in experimental sheep models coupled to the data suggesting improved blood flow in a range of human studies have culminated in the initiation of the multicentre clinical trial investigating the efficacy of SC in severe early-onset FGR (Ganzevoort et al., 2014). However, despite the wealth of information regarding the beneficial effects of SC in vivo and in vitro, relatively little is known about fetal toxicity and the long-term effects of SC on the offspring of treated pregnancies.

1.9 Summary

FGR is an important pregnancy complication. FGR is associated with short-term (increased incidence of fetal mortality and morbidity) and long-term (increased risk of cardiovascular disease and diabetes) health problems. There are currently no treatments for FGR. Treatment strategies are hindered by the nature of pregnancy; there are two individuals, both mother and baby, at risk of side effects in both the short- and long-term.
Experimental animal models represent an appropriate way to assess possible treatments. Pregnant mice have been well characterised and show considerable overlap with human pregnancy particularly in terms of their vascular physiological adaptations to pregnancy and placental transport processes. The placental-specific *Igf2* knockout (P0) mouse exhibits FGR and represents a model in which to further assess the potential benefits of putative treatments, especially as individual pregnancies contain appropriately grown and FGR pups. Another benefit of using the P0 genetic mouse model of FGR is that, unlike the eNOS knockout model or COMT knockout models of FGR, the growth restriction in the P0 mouse is induced solely by reduction of placental IGF2. Thus, this mouse model represents a tool to assess the effects of placental insufficiency and subsequent FGR on fetal and offspring wellbeing. There are little or no data assessing the long-term effects of SC on offspring health and development. Considering that SC has now progressed to a clinical trial for severe early onset FGR, it is imperative that the short- and long-term effects of SC treatment are evaluated. SC can be assessed in a mouse model of FGR without any apparent vascular phenotype (the P0 mouse) to determine whether there are any detrimental effects of SC. A study focusing on fetal and offspring vascular function, postnatal growth and metabolism would be extremely informative in this regard.
1.10 Hypothesis

In the placental-specific Igf2 KO mouse model of FGR, sildenafil citrate treatment will, in addition to increasing growth of growth restricted fetuses, not have detrimental effects on fetal vascular physiology or alter offspring growth, blood pressure or metabolic processes in either appropriately grown or growth restricted fetuses.

1.11 Aims and objectives

To test this hypothesis the specific aims and objectives were:

Chapter 3

Aim: To develop a wire myography technique to determine the vascular reactivity of the mouse fetal aorta.

Objective: To apply the above technique to determine whether there is altered fetal aortic function in relation to growth and sex of the fetus using the placental-specific Igf2 KO mouse.

Chapter 3 and 4

Aim: To administer sildenafil citrate to the placental-specific Igf2 KO mouse model of FGR.

Objective: To determine the effects of maternal sildenafil citrate treatment on mouse fetal growth and vascular function in the placental-specific Igf2 KO mouse.

Chapter 5

Aim: To characterise growth, blood pressure and metabolic function in relation to size at birth in male and female offspring of the placental-specific Igf2 KO mouse.

Objective: To determine the effects of maternal sildenafil citrate treatment on growth, blood pressure and metabolic function in offspring of the placental-specific Igf2 KO mouse.
Chapter 2  Methods
2.1 General Methods

2.1.1 The placental-specific \textit{Igf2} P0\textsuperscript{+/−} mouse

Insulin-like growth factor 2 (\textit{Igf2}) is an imprinted gene expressed on the paternal allele of mouse chromosome 7. The P0 promoter region of the \textit{Igf2} gene is found exclusively on the paternal allele (Moore et al., 1997). A 5-kb region, representing upstream exon 2 (u2) and encoding the P0 promoter region of the \textit{Igf2} gene, was deleted as previously described (Moore et al., 1997). This deletion prevents production of the placenta-specific P0 \textit{Igf2} gene transcript in the labyrinthine trophoblast and leads to complete depletion of IGF2 in the labyrinthine zone of the mouse placenta (Constancia et al., 2002). Expression of other fetal and placental gene transcripts (P1 - P3) were not affected by this deletion and thus fetal IGF2 concentrations were similar between heterozygote and wild type fetuses (Constância et al., 2002). Deletion of the P0 transcript and transmission through the paternal line leads to placental growth restriction at E11.5 with fetal growth restriction at near term. These mice were originally a kind gift from Professor W Reik and Dr. M Constância (Babraham Institute, UK). All mice were derived from the C57Bl/6J strain.

2.1.2 Standard housing and husbandry

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986; all work was carried out under a Home office project licence awarded to Professor Colin Sibley (PPL number 40/3385). Mice were supplied with nesting material, standard pellet mouse chow (Beekay Rat and Mouse Diet; Bantin & Kingman; Hull, UK), water \textit{ad libitum} and caged under a 12 hour light/dark cycle at 21-23 °C with 65 % humidity. Mice were housed in individually ventilated cages. Ear-notch tissue was used to determine the genotype of individual mice at week 4 of age. For timed matings, males heterozygous for the deletion of the P0 transcript were mated with 8 - 12 week old virgin C57Bl6/J females. Identification of a vaginal plug the following morning was deemed the beginning of pregnancy and designated embryonic day 0.5 (E0.5; term = E19.5). Upon identification of a vaginal plug female mice were, where possible, singly housed and body weight measured. To identify pregnant females, weight was also measured at E12.5 prior to commencing dosing regimens. Dams were euthanased at E18.5 by cervical dislocation. A surgical laparotomy was performed on the dam to expose the uterine horn. Fetuses were identified by position in the uterine horn. Maternal weight gain (in grams) between E12.5 and E18.5 was measured to assess the effects of maternal treatment. Fetal and placental tissues were carefully removed from surrounding tissue using jeweller’s forceps. Fetal tail tips were retrospectively genotyped and fetuses were either \textit{Igf2} P0\textsuperscript{+/+} (wild type; WT) or \textit{Igf2} P0\textsuperscript{−/−} (mutant; P0). All experiments on fetuses were performed at E18.5.
2.1.3 Genotyping

Fetuses were genotyped by using extracted genomic DNA (gDNA) from tail tips (Constância et al., 2005) using a DNeasy extraction kit (Qiagen, UK). Fetal tail tissue was incubated with 180 µl Buffer ATL and 20 µl Proteinase K for 6 hours at 55 °C in a hybridisation oven (Hybaid, VWR, UK). Tissue was then mixed using a vortex and centrifuged at 13,000 rpm for 1 minute with 120 µl of the supernatant transferred to a clean 1.9 ml Eppendorf tube. 400 µl buffer AL-ethanol mixture was added to the supernatant, mixed with a vortex, and then transferred to a clean DNeasy mini column sitting in a 2 ml collection tube. The sample was centrifuged at 8,000 rpm for 1 minute at room temperature and the flow through was discarded. After addition of 500 µl of buffer AW1 (wash buffer) the sample was centrifuged for 1 minute at room temperature and the flow through discarded. The DNeasy mini column was then transferred to a new 2 ml collection tube and 500 µl AW2 (wash buffer) added. The DNeasy mini column was then centrifuged (13,000 rpm for 3 minutes at room temperature) and the collection tube discarded. The DNeasy mini column was placed in a 1.9 ml Eppendorf tube with 200 µl buffer AE (elution buffer) and incubated at room temperature for 1 minute. The Eppendorf containing the DNeasy mini column was then centrifuged at 8,000 rpm for 1 minute. Extracted gDNA was stored at -20 °C for no longer than 12 weeks before polymerase chain reaction (PCR).

Adult mice were genotyped using tissue from ear-notching. A tri-primer system was used to amplify the 495-base pair (bp) WT fragment and a 740-bp fragment from the 5-kb deletion site (P0 deletion). The primer sequences were as follows:

- **d-F 5’-TCCTGTACCTCCTAACTACCAC-3’**
- **d-R 5’-GAGCCAGAAGCAAATC-3’**
- **WT 5’-CAATCTGCTCCTGCTG-3’**

Genomic DNA (gDNA) was amplified using a Techne Prime thermal cycler (Bibby scientific, UK) and the Expand High Fidelity PCR system (table 1; Roche, UK). The PCR conditions were as follows:

- step 1: 94 °C for 4 minutes
- step 2: 94 °C for 1 minute
- step 3: 56 °C for 1 minute
- step 4: 72 °C for 1 minute
- step 2-4: repeat for 40 cycles
- step 5: 72 °C for 10 minutes.

18 µl of PCR product mixed with 2 µl bromophenol blue loading dye was loaded onto a 1.5 % agarose gel. HyperLadder IV molecular weight marker (Bioline, UK) was added to lane 1 and the
gel was visualised using the Syngene UV transilluminator (Cambridge, UK) after 40 minutes at 120 volts.

**Table 2.1. Reagents for a 1 X PCR reaction using the Expand High Fidelity PCR system.**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X PCR buffer</td>
<td>20 mM Tris-HCL, pH 8.4, 50 mM KCl</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>200 µM</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>5 µM d-F primer</td>
<td>300 nM</td>
</tr>
<tr>
<td>5 µM d-R primer</td>
<td>300 nM</td>
</tr>
<tr>
<td>5 µM WT primer</td>
<td>300 nM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/µl</td>
</tr>
<tr>
<td>gDNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>PCR H₂O</td>
<td>Final volume 20 µl</td>
</tr>
</tbody>
</table>

**2.1.4 Gender genotyping**

Sex-specific genes were used to determine the gender of WT and P0 fetuses according to previous reports (Kunieda et al., 1992). Primers specific for the male SRY gene (Sry2/JG and Sry4/JG) and primers for the DXNds3 locus on the mouse X chromosome (NDS3 and NDS4) were used. The Sry product amplified at 404-bp and NDS product at 244-bp. The primer sequences are detailed below;

SRY-F 5’-TCTTAAACTCTGAAGAAGAGAC-3’
SRY-R 5’-GTCTTGCTGTATGTGATGG-3’
DXNds-F 5’-GAGTGCCCTCATCTATACGACAG-3’
DXNds-R 5’-TCTAGTTCATTGTTGATTTGC-3’

gDNA was amplified with the thermal cycler and Expand High Fidelity PCR system (see 2.1.3 for details). Specific concentrations of primers and reagents can be found in table 2. The following PCR cycling conditions were used;

step 1: 94 °C for 5 minutes
step 2: 94 °C for 1 minute
step 3: 55 °C for 1 minute
step 4: 72 °C for 1 minute
step 2-4: repeat for 35 cycles
step 5: 72 °C for 10 minutes.

18 µl of DNA product was mixed with 2 µl Xylene Cyanol (XC) loading dye (Sigma-Aldrich, UK) and loaded onto a 2 % agarose gel.

Table 2.2. Reagents for a 1 X PCR reaction using the Expand High Fidelity PCR system.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X PCR buffer</td>
<td>20 mM Tris-HCL, pH8.4, 50 mM KCl</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>200 µM</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>5 µM Sry2/JG primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>5 µM Sry4/JG primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>5 µM NDS3 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>5 µM NDS4 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/µl</td>
</tr>
<tr>
<td>gDNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>PCR H₂O</td>
<td>Final volume 20 µl</td>
</tr>
</tbody>
</table>

2.2 Treatment

Two dosing regimens were used in the present study to understand the importance of both route of administration (orally or subcutaneous injection) and the concentration of Sildenafil citrate (SC) administered per dam. Dams in the oral dosing regimen may have variable concentrations of SC in the plasma dependent on water intake. The circulating maternal concentration of SC that fetuses were exposed to in utero would be more consistent in the subcutaneous dosing study.

2.2.1 Sildenafil Citrate administered via drinking water

Dams received either 0.8 mg.ml⁻¹ of SC in drinking water or drinking water alone (control) between E12.5 and E18.5 (figure 1). Upon completion of the dosing period, bottles were changed at E15.5. Dams were euthanased by cervical dislocation. Fetal and placental tissues were removed following a surgical laparotomy of the dam. Fetal and placental wet weights were recorded before a surgical laparotomy was performed on the fetus. Fetal abdominal aortas were dissected free.
from surrounding tissue and assessed ex vivo for vascular response (see 2.3). The number of resorptions and total number of fetuses per litter were recorded. Fetal weight histograms were formulated for individual fetal weights. The 5th percentile of fetal weight was calculated using equation 1. Average litter fetal weight, placental weight and fetal: placental weight ratios were calculated for both WT and P0 fetuses per litter. Litter average data were assessed for normality using the D’Agostino-Pearson omnibus normality test. Litter average data were normally distributed thus the mean litter averages for each group were used in statistical analyses. Litters were only included in analysis if there were at least 2 fetuses of each genotype per litter.

\[ (-z_{\text{critical value}} \times SD) + \text{Mean} \]

\[ z_{\text{critical value}} = 1.645, \text{SD} = \text{Standard deviation}. \]

**Equation 1.** There is a range of 1.96 standard deviations on either side of the mean in a normal distribution which covers 95% of the population. Similarly, a range of 1.645 standard deviations on either side of the mean will cover 90% of the population (with 5% of the population in each tail of the normal distribution). (Dilworth et al., 2011, Kusinski et al., 2012).
Figure 2.1 Study design for Sildenafil citrate administered via drinking water. Number of litters and corresponding number of pups for each treatment arm are shown in the primary tier. Sildenafil citrate was administered in drinking water (0.8mg/ml⁻¹) from embryonic day (E) 12.5. All measurements were taken at E18.5. The secondary tier demonstrates the number of wild type (WT), mutant (P0), male (M) and female (F) mice that were used for ex vivo wire myography studies. Not all fetal descending abdominal aortas were assessed for vascular function.
2.2.2 Sildenafil Citrate administered via subcutaneous injection

Using a 27 gauge needle, SC (10 mg.kg$^{-1}$) or saline (control) was administered to dams via a daily subcutaneous injection in the skinfold between the scapula from E12.5 to E17.5 (figure 2). At the end of the administration period, dams were euthanased by cervical dislocation. Fetal and placental tissues were removed following a surgical laparotomy of the dam. The number of resorptions and total number of fetuses per litter were recorded. Fetal and placental wet weights were recorded before a surgical laparotomy was performed on the fetus. Vascular responses of fetal abdominal aortas were assessed using wire myography (see 2.3). Fetal weight histograms were created using equation 1. Average litter fetal weight, placental weight and fetal: placental weight ratios were calculated for both WT and P0 fetuses per litter. Litter average data were assessed for normality using the D’Agostino-Pearson omnibus normality test. Litter average data were normally distributed thus the mean litter averages for each group were used in statistical analyses. Litters were only included in analysis if there were at least 2 fetuses of each genotype per litter.
Figure 2.2. Study design for sildenafil citrate administered via subcutaneous injection. Sildenafil citrate (10 mg.kg$^{-1}$) or saline (0.9 %) was administered daily via subcutaneous injection from embryonic day (E) 12.5. The number of litters, corresponding number of pups and offspring (combined) for each treatment arm are shown in the primary tier. The secondary tier demonstrates the number of wild type (WT) and mutant (P0) pups used for fetal and placental weights at E18.5 and the number of offspring used in the long-term study. The tertiary tier demonstrates the number of WT, P0, male (M) and female (F) mice that were used for ex vivo wire myography studies. Not all fetal descending abdominal aortas were assessed for vascular function. Not all offspring were used for systolic blood pressure and ex vivo vascular function studies. SBP; systolic blood pressure, GTT; glucose tolerance test.
2.3 Blood vessel dissection and normalisation.

2.3.1 Fetal abdominal aorta

Pregnant female mice following mating with P0 heterozygotes (see 2.1.1) were euthanased and a surgical laparotomy performed. The uterine horn was taken whole and placed directly into ice-cold PBS (table 3). There were four distinct groups within the litter; WT M, P0 M, WT F and P0 F. It was not possible to accurately determine genotype or sex visually thus fetuses were chosen at random. Gender and genotype (WT or P0) were confirmed retrospectively following genotyping of fetal tail tips (as described previously in sections 2.1.3 and 2.1.4). The intact uterine horn was pinned out (Kusinski et al., 2009); individual fetuses/placentas were randomly selected, dissected free from the tissue and kept moist with cold PSS. Fetal and placental wet weights were recorded before a surgical laparotomy was performed on the fetus. Using a dissection microscope (model S6E, Leica, UK), the abdominal aorta was exposed within the peritoneal cavity following excision of the liver and intestines. The aorta was then dissected free from surrounding tissues from the level of the renal artery to the femoral arterial bifurcation; care was taken not to physically manipulate the area of the aorta that was to be used for the assessment of vascular function. Tissues excised at these points were transferred to cold PSS prior to mounting on the wire myograph.

Dissected arterial rings were then mounted onto two 40 µm steel wires and secured in the jaws of a Multi Myograph System 610M (Danish Myo Technology A/S, Denmark) as described previously (Wareing et al., 2002, Kusinski et al., 2012). Once mounted, the length of each fetal abdominal aorta was measured using a calibrated eyepiece graticule. Equilibration of aortas to physiological temperature and oxygen condition was achieved by warming 6 ml PSS to 37 °C under 5 % O₂ / 5 % CO₂ / balance N₂ (BOC Gases, Worsley, UK). 5 % O₂ was used to replicate the in vivo oxygen conditions of the fetal descending aorta at 40 weeks of gestation (Struijk et al., 2008). Classical normalisation of fetal vessels at 0.9 of L₁₃.₃kPa (L being the internal diameter of the vessel) resulted in significant damage to the fetal abdominal aortas (Mulvany and Halpern., 1976, Mulvany et al., 1979). Therefore, mounted vessels were normalised to 0.9 of L₅.₁kPa using the Laplace relationship (Wareing et al., 2002). Normalisation was achieved by stretching vessels to produce a measurable amount of tension, dependent on length and diameter of the vessel, to achieve a transmural pressure equivalent to that seen in vivo. This was to ensure that any experimental results were not due to the degree of stretch in the vessel and to ensure maximal response from vessels (Kusinski et al., 2009). Fetal aortas demonstrated median basal tone equivalent to 25 - 35 mmHg. There are no data on fetal aortic blood pressure in mice; however these values are similar to the
blood pressure in human fetal aorta between 20 weeks (20 mmHg) and 40 weeks of gestation (45 mmHg) (Struijk et al., 2008).

2.3.2 Adult abdominal aorta

Offspring from pregnancies treated with either a subcutaneous dose of saline of SC (10 mg.kg$^{-1}$) were sacrificed by cervical dislocation at 14 weeks of age. As with fetal aortas (section 2.2.1) offspring abdominal aortas from each mouse were dissected free from surrounding tissue but (in contrast to fetal abdominal aortas) were then cut into duplicate rings, both mounted separately on two 40µm steel wires and secured in the jaws of a Multi Myograph System 610M (Danish Myo Technology A/S, Denmark). Equilibration of aortas was achieved by warming 6 ml PSS to 37 °C under 20 % O$_2$ / 5 % CO$_2$ / balance N$_2$. 20 % O$_2$ is the physiological oxygen condition for systemic arteries in rodents (Herrera et al., 2012). Mounted vessels were normalised to 0.9 of L$_{13.3}$kPa using the Laplace relationship as this tension elicits the greatest vascular responses to agonists for this systemic artery.

2.3.3 Adult mesenteric artery

Mesentery along with supporting jejunum and ileum were also dissected free from the abdominal cavity and surrounding tissues. The mesentery was then pinned out and second order mesenteric arteries isolated from surround tissues; care was taken not to touch arteries. As with adult abdominal aortas, mesenteries were cut into duplicate rings and both mounted separately on two 40 µm steel wires and secured in the jaws of the myograph. Equilibration of mesenteric arteries was achieved by warming 6 ml PSS to 37 °C under 20 % O$_2$ / 5 % CO$_2$ / balance N$_2$. As with adult aortic arteries, mesentery followed the classical normalisation protocol and were normalised to 0.9 of L$_{13.3}$kPa (Aalkjaer and Mulvany., 1979). The diameters of mesenteric arteries were reduced compared with abdominal aortas and, as resistance vessels, mesenteric arteries would require less transmural pressure to elicit the greatest agonist-induced responses (Cooke and Davidge., 2003).
2.4 Wire Myography

2.4.1 Chemicals and solutions

All chemicals, including ACH and Phenylephrine (PE), were purchased from Sigma-Aldrich Co Ltd (Dorset, UK) except for U46619 which was purchased from Calbiochem Co Ltd (Nottingham, UK). Solutions were prepared daily and stored at 4°C.

Table 3.3. Physiological salt solutions used in wire myography experiments (in mM per litre).

<table>
<thead>
<tr>
<th>Physiological salt solution (PSS)</th>
<th>High potassium salt solution (KPSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>119 mM NaCl</td>
<td>12.45 mM NaCl</td>
</tr>
<tr>
<td>4.69 mM KCl</td>
<td>120 mM KCl</td>
</tr>
<tr>
<td>2.4 mM MgSO₄</td>
<td>2.4 mM MgSO₄</td>
</tr>
<tr>
<td>25 mM NaHCO₃</td>
<td>25 mM NaHCO₃</td>
</tr>
<tr>
<td>1.18 mM KH₂PO₄</td>
<td>1.18 mM KH₂PO₄</td>
</tr>
<tr>
<td>6.05 mM Glucose</td>
<td>6.05 mM Glucose</td>
</tr>
<tr>
<td>0.034 mM EDTA</td>
<td>0.034 mM EDTA</td>
</tr>
<tr>
<td>1.6 mM CaCl₂</td>
<td>1.6 mM CaCl₂</td>
</tr>
<tr>
<td>pH 7.4 (HCl)</td>
<td>pH 7.4 (HCl)</td>
</tr>
<tr>
<td>95 % O₂/ 5 % CO₂ 10 min</td>
<td>95 % O₂/ 5 % CO₂ 10 min</td>
</tr>
</tbody>
</table>

2.4.2 Wire myography protocol

Figure 3 demonstrates a schematic of a typical myography experiment. Post-equilibration (20 minutes), vessel contractility was assessed using two 120 mmol KCl (KPSS; table 3) washes added to the myograph bath for a maximum of 8 minutes, each separated by a 10 minute physiological salt solution (PSS; table 3) wash stage. Post-wash in PSS agonist-induced contraction was assessed using a single dose of 10 μM PhE for a maximum of 10 minutes. Post-wash in PSS a dose-response curve was created to the thromboxane-A₂ mimetic U46619 (10⁻¹⁰ - 2x10⁻⁶ M at 2 minute intervals). To determine the effective concentration of U46619 to contract the vessels to 80 % of the Vmax (EC₈₀ dose of U46619), contraction data were loaded into GraphPad Prism. Non-linear regression (curve fit) was used to determine the logECF value for EC₈₀. LogECF value was then converted to effective concentration to produce 80 % contraction (i.e. EC₈₀) using the following equation:

\[ \text{LogECF} \times 10^{-5} \div 6000 \]
Equation 2. Where $10^5$ is used to convert to µM and 6000 (µl) is the volume of buffer in myograph bath.

Post-wash in PSS, endothelial-dependent relaxation was assessed in vessels pre-contracted with an EC₈₀ dose of U46619 and exposed to ACH ($10^{-10}$ - $2 \times 10^{-6}$ M at 2 minute intervals). Finally, post-wash in PSS endothelial-independent relaxation was assessed in vessels pre-contracted with an EC₈₀ dose of U46619 and exposed to the nitric oxide donor sodium nitroprusside (SNP; $10^{-10}$ - $2 \times 10^{-6}$ M at 2 minute intervals). The specific components of endothelial-dependent relaxation were not assessed due to the length time taken to perform the initial experiment (approximately 5 - 7 hours); viability of tissue could not be guaranteed after this point.
Figure 2.3. Schematic representation of a wire myography experimental protocol. The X axis denotes time and the Y axis denotes tension (mN/mm). Vertical grey lines denote the addition of a drug into the myograph bath. High potassium salt solution (KPSS) and phenylephrine (PhE) were used to assess activation of arteries. U46619 dose response curves were created to determine the 80 % maximal effective dose (EC80) of U46619 on blood vessels. Pre-constricted blood vessels (EC80 U46619) were then administered increasing concentrations of acetylcholine or sodium nitroprusside to assess endothelial-dependent and –independent vasodilation responses, respectively. Total time of experiment is approximately 7 hours.
2.5 Assessment of progeny from SC treated and untreated pregnancies

Offspring from the subcutaneous dosing regimen were used to determine the effects of SC in utero on bodyweight, systolic blood pressure, glucose tolerance, ex vivo vascular reactivity of mesenteric arteries and abdominal aortas and organ growth.

2.5.1 Bodyweight

Offspring from subcutaneous dosing studies were housed as described in section 2.1.1. Offspring were caged with dams until weaning at 4 weeks of age. At 4 weeks of age offspring were ear-notched to aid identification of individual mice. Genotyping was performed on tissue taken from ear notches. A maximum of 4 female mice were housed in one cage. Male mice were separated and singly housed from 8 weeks of age. Bodyweights were measured each week between weeks 5 and 12. Terminal bodyweights (between weeks 14 and 16) were also measured. The mean bodyweight at each age was calculated for individual groups. The level of significance was determining using mean bodyweight for each group with repeated-measures two-way ANOVA and Sidak’s post hoc test.

2.5.2 Non-invasive systolic blood pressure measurements using tail-cuff plethysmography

Systolic blood pressure (SBP) was measured using the non-invasive blood pressure system (model LE5001, PanLab, Spain) at weeks 8 and 13. Mice were transferred to a room with a temperature between 22-24 °C and left to acclimatise for 45 minutes before measurements were recorded. First, a small rodent restraining tube was placed on a Thermopad heated mat (Harvard Apparatus, Kent, UK). Mice were not forced to enter the rodent restrainer but when entered were secured with the tail exposed for measurements. The tail-cuff occlusion device and pulse transducer apparatus were placed on the mouse tail for at least 15 minutes prior to recording SBP measurements (Whitesall et al., 2004). Heart rates were monitored and no SBP measurements were taken when the heart rate exceeded 600 beats per minute. Mice were not conditioned or exposed to the restraining tube on days prior to measurement. Thus, to obtain reliable data twenty SBP measurements were taken in total in each conscious restrained mouse (for a maximum of 15 minutes). The mean data of the middle 12 SBP readings were calculated (adapted from Watkins et al., 2007). Mean SBP for each group was then calculated and used for statistical analyses. Data were analysed using two-way ANOVA with Bonferroni post hoc test.

2.5.3 Glucose tolerance tests
Glucose tolerance tests (GTTs) were performed at 12 weeks of age (Andrikopoulos et al., 2008). Mice were fasted overnight for 16 hours between 18:00hrs and 10:00hrs, with ad libitum access to water. Mice were transferred to single cages and weights recorded. One small incision was made at the base of the tail to venepuncture using a 25 gauge needle and baseline blood glucose concentrations were measured using the ACCU-CHEK® Aviva (Roche Diagnostics, UK) blood glucose monitoring system with disposable microchip test strips. Immediately afterwards mice were administered a freshly prepared filter-sterilised 1 g.kg⁻¹ glucose (0.9 % NaCl, 10 % glucose) solution via intraperitoneal injection. Blood glucose concentrations at 15, 30, 60, 90 and 120 minutes were recorded by gentle massage of the tail to encourage small quantities of blood on to the microchip test strips. Total area under the curve (AUC) values were calculated for each individual mouse using GraphPad Prism. Mean area under the curve values in each particular group (e.g. WT M, P0 F etc.) were then calculated and used in statistical analyses. Data were not normally distributed (D’Agostino-Pearson omnibus normality test) therefore statistical differences between the mean AUC values for two populations (e.g. mean AUC for WT M vs. mean AUC for WT F) were measured using the Mann-Whitney U test.

2.5.4 Abdominal aorta and mesenteric artery vessel function

Contraction and relaxation responses of both aortic and mesenteric mounted vessels were then assessed as described in 2.3.2 and 2.3.3, respectively. Analyses were performed on the average data from duplicate vessels from abdominal aortas and mesenteric arteries as described in 2.6.1.

2.5.5 Tissue collection

Immediately after cervical dislocation trunk bloods were collected by decapitation. Blood was collected in Microvette lithium-heparinised 2 ml tubes (Scientific Laboratory Supplies, UK) and centrifuged at 5,000 RPM for 5 minutes until a clear separation of plasma. Plasma samples were immediately snap-frozen in liquid nitrogen. Heart, kidneys, spleen, lungs and brain were dissected free from surrounding tissue and weighed. For each individual, total heart weight was used in statistical analyses. For kidneys, the average weight of both kidneys was used in analyses. Data were not normally distributed (D’Agostino-Pearson omnibus normality test); therefore the levels of significant difference between the mean values were measured using the Mann-Whitney U (2 populations) or the Kruskal-Wallis (3 or more populations) test.

2.6 Statistical analyses

2.6.1 Calculations for wire myography
Contraction data were calculated as active effective pressure using the equation 3 below. Active effective pressure is a measurement of the force required to maintain a particular vessel circumference.

\[
\text{Active Effective Pressure} = \left( \frac{\text{Wall Tension}}{2\pi r} \right) / \text{Vessel Internal Circumference}
\]

Relaxation data were expressed as percent of EC\textsubscript{80} pre-contraction to U46619. Data were expressed as mean ± S.E.M (unless noted) where in the case of fetal studies \(n\) = number of fetuses and \(n\) = number of offspring in offspring myography studies. Myodata 2.02 (Myonic Software, National Instruments Corporation, USA), Excel and GraphPad Prism version 6.0 (GraphPad Software, USA) were used to analyse the data. An assessment of whether data were normally distributed was performed using the D’Agostino-Pearson omnibus normality test prior to statistical analysis. Statistical comparisons (vascular diameter; maximal contraction; basal tone) were not normally distributed and data were therefore analysed using either the Kruskal-Wallis test (for non-treatment group when assessing genotype and gender) or the Mann-Whitney \(U\) test (for treatment groups when assessing a treated vs. untreated group). Dose-response curves were compared using two-way ANOVA followed by a Bonferroni \textit{post hoc} test where appropriate. EC values were calculated using GraphPad Prism software for U46619, ACH and SNP dose-response curves. For U46619, the Hill slope was set at 1 and the minima of the curve was constrained to equal 0. For ACH and SNP, the Hill slope was set at -1 and maximum value of the curve constrained to equal 0 % residual contraction / 100 % relaxation. EC values were compared using Mann-Whitney \(U\) test. Blood vessels that did not conform to classical dose response curves were not included in the analysis.

Previous studies from our group have determined a sample size of 8 - 10 vessels to provide sufficient power (of 90 % with 5 % level of confidence) to demonstrate significant differences between groups (Kusinski et al., 2009, Kusinski et al., 2012).

### 2.6.2 Data analysis

All data were analysed using GraphPad Prism statistical software version 6.0 (GraphPad Software, USA). Unless otherwise stated analyses of data were performed using two-way ANOVA with Bonferroni \textit{post hoc} test for comparisons between specific groups. Data which did not conform to a normal distribution were analysed using the Kruskal-Wallis (3 or more groups) or Mann-Whitney \(U\) test. All data are expressed as mean ± SEM unless otherwise stated. \(P < 0.05\) was taken to be indicative of statistical significance.
Chapter 3  *In Vitro* Assessment of Mouse Fetal Abdominal Aortic Vascular Function: Effect of Sildenafil Citrate
3.1 Introduction

FGR is a major pregnancy complication affecting 10% of births in the UK (Figueras et al., 2014). Fetuses that do not reach their predetermined genetic growth potential are at increased risk of mortality and morbidity in the short and longer term (Barker et al., 2002). FGR fetuses are 10 times more likely to be stillborn than fetuses that are normal birthweight (Ashworth, 1998) and infants that survive are at increased risk of poor health (e.g. increased incidence of cardiovascular disease) in later life including (Osmond and Barker, 2000). There are no proven treatments for FGR. The link between poor growth in utero and risk of cardiovascular disease in later life is unknown. However, alterations in the structure and function of the fetoplacental vasculature are thought to be the likely causes.

In FGR, it is thought that there is an increased vascular resistance within both the placental and the fetal vasculature contributing to an inadequate supply of nutrients and oxygen to the fetus. In vivo measurements of umbilical artery blood flow suggest increased resistance to flow in the fetoplacental vasculature of FGR pregnancies (Karsdorp et al., 1994) and fetuses with reduced umbilical placental perfusion in vivo, show signs of increased aortic stiffness post-delivery (Akira and Yoshiyuki, 2006). Thromboxane-A2- and endothelin-induced vascular contraction of term placental chorionic plate arteries was increased in cases of FGR (Mills et al., 2005, Liu et al., 1995). Thus, increased resistance in the placental and the fetal vasculature is most likely a cause vascular dysfunction (i.e. increased contraction / reduced relaxation).

Doppler velocimetry has been used in the fetus to assess whether, in addition to reduced blood flow in the umbilical artery, there are other alterations in fetal vascular resistance in FGR. Low PI values in the middle cerebral artery of the fetus indicate increased cerebral blood flow known as the “brain sparing” effect often seen in asymmetric FGR (Wladimiroff et al., 1986). Doppler measurements of blood flow-velocity in this “central” artery can be used to assess the health of the fetus and whether early delivery is necessary (Hershkovitz et al., 2000).

Doppler measurements in the ductus venosus may also be indicative of abnormal vascular reactivity. In cases of hypoxia, the ductus venosus shunts blood from the fetal liver to the fetal brain to ensure adequate oxygenation (Baschat et al., 2011). An absent “A wave” in ductus venosus Doppler waveforms is indicative of severe fetal compromise. Although important in the clinical setting, Doppler indices provide only some information on the progression of FGR and do not provide any functional evidence for the association of FGR and altered vascular reactivity. Such experiments on fetal vasculature would be unfeasible in the human.
Although there is no direct functional evidence for the association of FGR and altered vascular reactivity of fetal blood vessels numerous studies have assessed morphology of the vasculature in neonates, children and adolescents as a proxy for altered fetal vascular function. Abdominal aortic wall thickness (Skilton et al., 2005, Koklu et al., 2006, Zanardo et al., 2011) was increased in newborn infants who were classified as FGR. In adolescents who demonstrated altered fetal aortic blood flow and were growth restricted at birth, there are reductions in the diameter of the abdominal aortic lumen (Brodszki et al., 2005) compared to adolescents who had normal birth weight. These studies in neonates, children and adolescents did not assess whether there was any sexual dimorphism in these subjects but experimental studies in mice show hypertension and impaired vasodilatation in male, but not female, offspring that were growth restricted at birth (Roghair et al., 2009). Collectively, these studies suggest there may be a potential link between maternal and fetoplacental altered vascular structure and function, poor growth in utero and the development of long-term cardiovascular disease.

It is important to note that a large proportion of clinical cases of FGR do not display vascular abnormalities when assessed by Doppler ultrasound. However, FGR fetuses that do not present with abnormal umbilical blood flow velocities still demonstrate increased risk of cardiovascular disease in adulthood. Whether there is an association between fetal growth and fetal vascular dysfunction alone is unknown, however previous studies in rats have shown an association between suboptimal fetal growth (due to hypoxia or undernutrition) and vascular dysfunction in adulthood (Camm et al., 2010, Williams et al., 2005). The placental-specific insulin-like growth factor 2 (Igf2 P0+/−) knockout mouse model of FGR was therefore utilised to assess whether poor growth in utero could be directly associated with vascular dysfunction in the developing fetus.

In a mouse model of programmed cardiovascular disease, maternal antioxidant treatment (Tempol) was able to reduce adulthood cardiovascular abnormalities (Roghair et al., 2011). In a rat nutrient restriction model of FGR, increased systolic blood pressure in offspring of experimental animals was reduced when dams were supplemented with folate (Torrens et al., 2006). Giussani and colleagues (2012) also showed that maternal vitamin C supplementation was able to reduce the adverse effects of maternal hypoxia on fetal / offspring vascular structure and function. These studies suggest that treatments during pregnancy may in fact be able reduce or reverse the association between small size at birth and cardiovascular disease later in adulthood. Thus, there exists the potential to intervene with a therapeutic in the antenatal period and reverse the poor outcomes associated with growth restriction. One such therapeutic could be sildenafil citrate (SC).

SC increases vasodilatation of small myometrial arteries from FGR pregnancies (Wareing et al., 2005). SC also increases fetal weight in the COMT−/− mouse model of FGR by improving umbilical
artery blood flow (Stanley et al., 2012). In the P0 mouse, a model of FGR without any apparent vascular abnormalities, SC was also able to increase fetal weight (Dilworth et al., 2013) through as yet unknown mechanisms. A small scale non-randomised clinical trial in severe early-onset FGR demonstrated increased abdominal growth velocity in fetuses from pregnancies treated with SC (Von Dadelszen et al., 2011). A multicentre international clinical trial for the use of SC in severe early-onset FGR is underway (Ganjevoort et al., 2014) and if the trial is successful in improving fetal outcome in these severe cases, without side effects, it is possible that in the future SC might be used in less severe cases of FGR. Whether increasing fetal weight with maternal treatment of SC will improve any putative vascular abnormalities associated with FGR is unknown and such studies cannot be assessed in the human fetus. In addition, it will be important to assess the effects of SC on vascular function in fetuses of appropriate birth weight as in the future SC may be used in less severe cases of FGR or as a prophylaxis in high risk groups.

Accordingly, given the potential link between altered vascular structure / function and associated risk of long-term cardiovascular disease, it first seems logical to assess what effects maternal administration of SC may have on fetal vascular function in relation to fetal growth. In human, assessing effects of SC on fetal vascular function ex vivo is unfeasible and thus another focus of this study was to assess SC effects on vascular function in mice. To directly assess the effect of SC on fetal vascular function in FGR, the Igf2 P0+/− knockout mouse model (herein referred to as “P0”) of FGR was used. It is important to reiterate that the P0 mouse model of FGR contains a mixed litter of appropriately grown (wild type; WT) and growth restricted (P0) fetuses.

The aims of this particular study were to 1) develop a myography technique using the P0 mouse model of FGR to assess fetal artery function at E18.5 and 2) to assess fetal vascular function after administration of SC in the drinking water of the pregnant dam. Furthermore, this study aimed to analyse the data according to sex, as the literature suggests differential effects of sex on vascular function and sex-specific programming of cardiovascular disease in growth restricted pregnancies (Kublickiene and Luksha., 2008, Roghair et al., 2009).

These aims will provide information on the following unknowns; a) is systemic vascular reactivity altered in a growth restricted fetus compared to one which is appropriately grown and is this dependent upon sex? If so b) can vascular function in the growth restricted fetus be improved via a maternally administered therapy? Finally, c) does SC increase fetal weight in growth restricted fetuses without detrimental effects on vascular function of the normally grown fetus?
3.2 Results

The first aim of the study was to assess fetal / placental weight and aortic function in fetuses from the P0 mouse. These results will now be presented in 3.2.1 and will be later used to directly assess the effect of treatment on fetuses exposed to SC in utero. For effects of SC on weight and vascular function in the P0 mouse model of FGR see section 3.2.4.

3.2.1 Prenatal weight measurements in WT and P0 knockout mice at embryonic day 18.5

P0 fetal and placental weight was significantly lower at E18.5, compared with WT littermates (figure 3.1, \( P<0.0001 \)). When plotted as a frequency distribution curve, the 5\(^{th}\) percentile of WT fetal weight was 1.07g (figure 3.2). >76 % of P0 fetuses fell below the 5\(^{th}\) percentile of WT fetal weight. There was a significantly greater fetal:placental weight ratio in P0 versus WT (figure 3.3).
Figure 3.1. Fetal and placental weights from WT and P0 knockout mice regardless of sex. Fetal weights (A,B) and placental weights (C,D) at embryonic day 18.5 (N = 10 pregnant dams). In figure 2A and 2C individual fetal/placental weights are shown. In figure 2B and 2D each symbol represents an average litter weight for a particular phenotype. Horizontal line denotes mean ± SEM.
Figure 3.2. Fetal weight frequency distribution curve from individual fetuses; WT (black solid curve) and P0 (black dashed curve). Vertical red dashed line denotes the 5th centile of WT fetal weights (1.07g). Individual pup n’s: WT n = 26, P0 n = 43.
Figure 3.3. Fetal:placental weight ratios from WT and P0 knockout mice; individual fetuses (left) and litter average for individual phenotypes (right) are presented. Horizontal line denotes mean ± SEM.
3.2.2 Contraction responses of the fetal abdominal aorta (drinking water controls)

Fetal aortas were assessed for genotype and sex-specific effects on vascular function. There was no significant difference in the abdominal aortic diameter or basal tone (0.9 of L5.1kPa) between WT M, P0 M, WT F, P0 F (table 3.1). The median basal tone for the control group (N = 43) was 33 (min 11 – max 67) mmHg. Median basal tone (min - max) for individual groups was; WT M 33 (23 – 52) mmHg, P0 M 34 (11 – 44) mmHg, WT F 32 (21 – 67) mmHg, P0 F 36 (12 – 50) mmHg. Maximal contraction to KPSS (120 mM), PE (10\(^{-5}\) M) and U46619 (2x10\(^{-6}\) M) was not significantly different in all four control groups (table 3.1). However, U46619 elicited the greatest agonist-induced responses when compared with PE and KPSS.

U46619 dose-response curves were constructed for fetal abdominal aortas (figure 3.4). U46619-induced contraction of fetal aortas was reduced in P0 M vs. WT M mice (figure 3.4A, P<0.01, two-way ANOVA). However, there were no differences in EC\(_{20}\), EC\(_{50}\) or EC\(_{80}\) values (table 3.5). U46619-induced contraction of fetal aortas of P0 F mice was similar to that of WT F (figure 3.4B). When fetal sex was considered, WT F mice had reduced U46619-induced fetal aortic contraction vs. WT M mice but this difference was not apparent in the in the P0 genotype (figure 3.4C and 3.4D, P<0.01). There were no differences in EC\(_{20}\), EC\(_{50}\) or EC\(_{80}\) values in WT M vs. WT F mice (table 3.5).

U46619-induced contraction responses are also presented as a percentage of maximal KPSS contraction to account for any changes in vascular smooth muscle mass or function (figure 3.5). U46619-induced contraction of fetal aortas (as % KPSS) was lower in P0 M vs. WT M mice (figure 3.5A, P<0.01); however, there were no differences in EC\(_{20}\), EC\(_{50}\) or EC\(_{80}\) values (table 3.5). When expressed as a % of KPSS maximal contraction, fetal aortas from P0 F mice had significantly lower U46619-induced contraction compared with WT F mice (figure 3.5B, P<0.05); however, there were no differences in EC\(_{20}\), EC\(_{50}\) or EC\(_{80}\) values (table 3.5). There were no significant differences in U46619-induced fetal aortic contraction (as % of KPSS) between M and F mice, when comparing within each individual genotype (figure 3.5C and 3.5D).
Table 3.1. Fetal abdominal aorta diameter, basal tone and max contraction data. KPSS (120 mM high potassium salt solution); PE (10^5 M phenylephrine); U46619 (2x10^-6 M thromboxane-A2 mimetic). Data are shown as median [min - max]; number of fetuses per group in parenthesis. A Kruskal-Wallis statistical test was performed on four groups within the same column.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Fetal ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
<th>Maximal Contraction (kPa)</th>
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<tr>
<td></td>
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<tr>
<td>Control</td>
<td>WT Male (11)</td>
<td>668 [552 - 737]</td>
<td>4.4 [3.0 - 6.9]</td>
<td>0.34 [0.19 - 0.51]</td>
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<td></td>
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<td></td>
<td>0.18 [0.03 - 0.45]</td>
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<td>1.13 [0.42 - 2.10]</td>
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<td></td>
<td>WT Female (11)</td>
<td>650 [560 - 717]</td>
<td>4.5 [2.9 - 8.9]</td>
<td>0.35 [0.07 - 0.79]</td>
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<td>0.25 [0.03 - 0.60]</td>
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<td>0.80 [0.57 - 1.30]</td>
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<td>P0 Male (10)</td>
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<td>4.3 [1.5 - 5.9]</td>
<td>0.32 [0.10 - 0.96]</td>
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<td>0.10 [0.00 - 0.30]</td>
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<td></td>
<td></td>
<td>0.80 [0.16 - 1.83]</td>
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<td></td>
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<td>0.22 [0.02 - 0.48]</td>
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<td></td>
<td>0.82 [0.32 - 1.56]</td>
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</table>
Figure 3.4. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 3.5. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
3.2.3 Relaxation responses of fetal abdominal aortas to acetylcholine and sodium nitroprusside

Aortas were pre-contracted with an EC₈₀ dose of U46619 before ACH (endothelial-dependent, figure 3.6) and SNP (endothelial-independent, figure 3.7) -induced relaxation was measured. ACH-induced relaxation responses of fetal abdominal aortas were lower in P0 M vs. WT M mice (figure 3.6A, P<0.05). However, ACH-induced relaxation responses in P0 F vs. WT F mice were not significantly different (figure 3.6B). When assessing the effect of sex, ACH-induced relaxation responses in M vs. F fetal aortas were similar in WT mice (figure 3.6C) but reduced in P0 mice (figure 3.6D, P<0.001).

SNP-induced relaxation responses of fetal abdominal aortas were similar in P0 M vs. WT M mice (figure 3.7A). There was no difference in SNP relaxation responses in WT F vs. P0 F mice (figure 3.7B). When assessing the effect of sex, SNP-induced relaxation responses of fetal aortas were similar in M vs. F from WT (figure 3.7C) and P0 mice (figure 3.7D). There were no statistical differences between any of the four control groups in the effective concentration of either ACH or SNP required to produce 20 %, 50 % or 80 % relaxation of fetal abdominal aortas (table 3.5). There were also no significant differences in maximal response of fetal abdominal aortas to U46619 (see table 3.3), ACH or SNP (data not shown) between WT M, WT F, P0 M or P0 F.
Figure 3.6. Dose response curves of fetal abdominal aortas to increasing doses of ACH. Panel A-D; Arteries were pre-contracted with an EC$_{80}$ dose of U46619. ACH dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: ACH; acetylcholine, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 3.7. Dose response curves of fetal abdominal aortas to increasing doses of SNP. Panel A-D; Arteries were pre-contracted with an EC$_{80}$ dose of U46619. SNP dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: SNP; sodium nitroprusside, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Table 3.2. Summary table comparing fetal/placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619; expressed as kPa) and relaxation responses (ACH, SNP). Comparisons were made between genotypes (WT and P0) and sex. ↔ arrow denote no change, ↓ arrow denotes a decrease in a parameter compared to another relevant group e.g. U46619 constriction in P0 M reduced compared to WT M. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

<table>
<thead>
<tr>
<th></th>
<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
</tr>
</thead>
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<tr>
<td>Fetal weight</td>
<td>↑ vs. P0</td>
<td>↓ vs. WT</td>
<td>↓ vs. WT</td>
<td>↓ vs. WT</td>
</tr>
<tr>
<td>Placental weight</td>
<td>↑ vs. P0</td>
<td>↓ vs. WT</td>
<td>↓ vs. WT</td>
<td>↓ vs. WT</td>
</tr>
<tr>
<td>Basal tone</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
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<tr>
<td>Aortic diameter</td>
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<td>↔</td>
<td>↔</td>
<td>↔</td>
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<tr>
<td>Maximal KPSS constriction</td>
<td>↔</td>
<td>↓ vs. WT M</td>
<td>↓ vs. WT M</td>
<td>↔</td>
</tr>
<tr>
<td>PE constriction</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
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<tr>
<td>U46619 constriction</td>
<td>↔</td>
<td>↓ vs. WT M</td>
<td>↓ vs. WT M</td>
<td>↔</td>
</tr>
<tr>
<td>ACH relaxation</td>
<td>↔</td>
<td>↔</td>
<td>↓ vs. WT M</td>
<td>↓ vs. P0 F</td>
</tr>
<tr>
<td>SNP relaxation</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
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</tbody>
</table>
3.2.4 Effect of maternal sildenafil citrate treatment on prenatal weight measurements in WT and P0 knockout mice at embryonic day 18.5

The number of fetuses per litter was not significantly different between control and treated groups. SC had no effect on the number of resorbed fetuses per litter (data not shown). Within the SC treatment group, fetal weight was significantly reduced in P0 vs. WT mice. SC had no overall effect on fetal weight in either WT or P0 mice compared with controls (figure 3.8A and 3.8B). Within the SC treatment group, placental weight was significantly reduced in P0 vs. WT mice (figure 3.8C and 3.8D, P<0.0001). SC did not affect the placental weight in either WT or P0 mice compared with controls (figure 3.8C and 3.8D). A frequency distribution curve of fetal weights from control and SC-treated pregnancies is shown in figure 3.9. The 5th percentile of WT control fetal weights was 1.07g with 77% of P0 control fetal weights measured less than 1.07g. 81% of P0 mice from SC-treated pregnancies measured less than 1.07g. Fetal:placental weight ratios from control and SC-treated pregnancies are shown in figure 3.10. There was a significant increase in fetal:placental weight ratio in P0 mice from both control and SC-treated pregnancies.
Figure 3.8. Fetal and placental weights from WT and P0 knockout mice following sildenafil citrate treatment. Fetal weights (A,B) and placental weights (C,D) at embryonic day 18. Pregnant dams were allowed access to water with (N = 9) or without (N = 10) sildenafil citrate (0.8 mg.ml⁻¹). In figure 2A and 2C individual fetal/placental weights are shown. In figure 2B and 2D each symbol represents an average litter weight for a particular phenotype. Horizontal line denotes mean ± SEM. Statistical analyses for genotype and treatment were performed using two-way ANOVA with Bonferroni post hoc test. **** P<0.0001 using Bonferroni post hoc test.
Figure 3.9. Fetal weight frequency distribution curve from individual fetuses following sildenafil citrate treatment. Pregnant dams were allowed access to water with (N = 9) or without (N = 10) sildenafil citrate (0.8 mg.ml⁻¹). WT (black solid curve), WT SC (grey solid curve), P0 (black dashed curve) and P0 SC (grey dashed curve). Vertical red dashed line denotes the 5th centile of WT fetal weights (1.08g). Untreated individual pup n’s: WT n = 26, WT SC n = 41, P0 n = 43, P0 SC n = 31.
Figure 3.10. Fetal:placental weight ratios from WT and P0 mice of dams following Sildenafil citrate treatment. Pregnant dams were allowed access to water with (N = 9) or without (N = 10) sildenafil citrate (0.8 mg.ml^-3). Individual fetuses (left), litter average for each group (right). Horizontal line denotes mean ± SEM.
3.2.5 Effect of maternal sildenafil citrate treatment on contraction responses of fetal abdominal aortas

SC had no effect on fetal abdominal aortic intraluminal diameter when comparing SC-treated vs. equivalent groups e.g. the abdominal aortic diameters of WT M fetuses from the water control group were not significantly different to aortic diameters of WT M from the SC-treated group (table 3.3). There was no effect of SC on basal tone (the degree of contractile tension within the walls of the blood vessel) in any of the individual groups; the median basal tone for SC-treated groups (N = 31) was 35 (min 17 – max 67) mmHg. Median basal tone (min-max) for individual groups were; WT M 37 (30 – 52) mmHg, P0 M 29 (22 – 54) mmHg, WT M 40 (29 – 67) mmHg, P0 M 28 (17 – 37) mmHg.

Maximal contraction responses to KPSS (120 mM), PE (10⁻⁵ M) and U46619 (2x10⁻⁶ M) were not significantly different between SC-treated and control groups (table 3.3). In the SC-treatment group, fetal abdominal aortas had the greatest response to U46619, when compared with PE and KPSS (see table 3.3).

U46619 dose response curves were constructed and are shown in figure 3.11. Comparisons were made to assess the effect of treatment only. There was no effect of SC treatment on U46619-induced contraction of aortas from M mice of either WT or P0 genotypes (figure 3.11A and 3.11C). Fetal aortas of WT F mice from SC-treated pregnancies had significantly lower U46619-induced contraction when compared with fetal aortas of WT F mice receiving drinking water (figure 3.11B, P<0.05); however, there was no effect of SC on fetal aortic contraction in P0 F mice (figure 3.11D). There were no statistically significant differences between SC-treated and control groups in the effective concentration of U46619 required to produce 20 %, 50 % or 80 % contraction of fetal abdominal aortas (table 3.5).

U46619-induced contraction was also expressed as a % of maximal KPSS contraction (figure 3.12) to account for changes in vascular smooth muscle cell mass. SC did not alter the U46619-induced contraction (% KPSS) of WT fetal abdominal aortas from either M (figure 3.12A) or F mice (figure 3.12B). U46619-induced contraction (% KPSS) of fetal abdominal aortas was significantly increased in P0 mice from SC-treated pregnancies in both M and F (figure 3.12C and 3.12D, respectively. P<0.05).
Table 3.3. Fetal abdominal aorta diameter, basal tone and maximal contraction data following sildenafil citrate treatment. KPSS (120 mM high potassium salt solution); PE (10⁻⁵ M phenylephrine); U46619 (2x10⁻⁶ M thromboxane-A₂ mimetic). Data are shown as median [min - max]; number of fetuses per group in parenthesis. Control data are those previously presented in table 3.1. A Mann-Whitney U test was performed to assess the effect of treatment on each group (e.g. for aortic diameter; WT M vs WT M SC).

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Fetal ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
<th>Maximal Contraction (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KPSS</td>
</tr>
<tr>
<td>Control</td>
<td>WT Male (11)</td>
<td>668 [552 - 737]</td>
<td>4.4 [3.0 - 6.9]</td>
<td>0.34 [0.19 – 0.51]</td>
</tr>
<tr>
<td></td>
<td>WT Female (11)</td>
<td>650 [560 - 717]</td>
<td>4.5 [2.9 - 8.9]</td>
<td>0.35 [0.07 – 0.79]</td>
</tr>
<tr>
<td></td>
<td>P0 Male (10)</td>
<td>696 [592 - 798]</td>
<td>4.3 [1.5 - 5.9]</td>
<td>0.32 [0.10 – 0.96]</td>
</tr>
<tr>
<td></td>
<td>P0 Female (11)</td>
<td>668 [629 - 882]</td>
<td>4.5 [1.6 - 6.6]</td>
<td>0.35 [0.12 – 0.60]</td>
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<tr>
<td>Sildenafil citrate (0.8 mg.ml⁻¹)</td>
<td>WT Male (10)</td>
<td>715 [575 - 794]</td>
<td>4.9 [4.0 - 6.9]</td>
<td>0.31 [0.06 – 0.68]</td>
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<tr>
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<td>WT Female (7)</td>
<td>717 [499 - 989]</td>
<td>5.3 [3.9 - 8.9]</td>
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<td>P0 Male (6)</td>
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</table>
Figure 3.11. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 following sildenafil citrate treatment. Panel A-D; Pregnant dams were allowed access to water with (N = 9) or without (N = 10) sildenafil citrate (0.8 mg.ml⁻¹). U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: Fetuses from pregnant dams allowed access to water with (grey solid curves) or without (black solid curves) sildenafil citrate.
Figure 3.12. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following sildenafil citrate treatment. Panel A-D; Pregnant dams were allowed access to water with (N = 9) or without (N = 10) sildenafil citrate (0.8 mg.ml\(^{-1}\)). U46619 dose response curves were compared using two-way ANOVA for treatment and U46619 concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: Fetuses from pregnant dams allowed access to water with (grey solid curves) or without (black solid curves) sildenafil citrate.
3.2.6 Effect of maternal sildenafil citrate treatment on relaxation responses of fetal abdominal aortas

Aortas from SC-treated pregnancies were pre-contracted with an EC_{50} dose of U46619 before ACH (endothelial-dependent, figure 3.13) and SNP (endothelial-independent, figure 3.14) -induced relaxation was measured.

SC treatment led to a blunted relaxation of fetal abdominal aortas in response to ACH in both WT M (figure 3.13A, *P*<0.01) and WT F mice (figure 3.13B, *P*<0.001). For WT M mice only, SC treatment led to a significant increase in the concentration of ACH required to produce 20 % and 50 % relaxation of fetal abdominal aortas (see table 3.5). There was no effect of SC on relaxation of P0 M fetal abdominal aortas (figure 3.13C) but a significant reduction in relaxation response of P0 F fetal abdominal aortas (figure 3.13D, *P*<0.001). The concentration of ACH required to produce 20 %, 50 % and 80 % relaxation of fetal abdominal aortas was significantly increased in P0 F mice from SC-treated pregnancies when compared with P0 F fetal aortas from water control mice (table 3.5).

When compared with equivalent controls SC treatment did not alter relaxation in response to SNP in WT M fetal abdominal aortas (figure 3.14A) but significantly reduced the relaxation response of WT F fetal abdominal aortas (figure 3.14B, *P*<0.05). SC treatment significantly reduced the relaxation response of P0 fetal abdominal aortas to SNP in both M (figure 3.14C, *P*<0.01) and F (figure 3.14D, *P*<0.001). The concentration of SNP required to elicit 20 %, 50 % and 80 % relaxation of P0 F fetal abdominal aortas was increased in SC-treated groups when compared to water controls (table 3.5).
Figure 3.13. Dose response curves of fetal abdominal aortas to increasing doses of ACH following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC50 dose of U46619. ACH dose response curves were compared using two-way ANOVA for treatment and ACH concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: ACH; acetylcholine, fetuses from pregnant dams allowed access to water with (grey solid curves) or without (black solid curves) sildenafil citrate. ****P<0.0001, ***P<0.001, **P<0.01.
Figure 3.14. Dose response curves of fetal abdominal aortas to increasing doses of SNP following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC₈₀ dose of U46619. SNP dose response curves were compared using two-way ANOVA for treatment and SNP concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: SNP; sodium nitroprusside, fetuses from pregnant dams allowed access to water with (grey solid curves) or without (black solid curves) sildenafil citrate. ***P<0.001, **P<0.01, *P<0.05.
Table 3.4. Summary table demonstrating the effect of maternal sildenafil citrate treatment (0.8 mg.ml⁻¹) on fetal/placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619; expressed as kPa) and relaxation responses (ACH, SNP) for each group when compared with drinking water controls. ↔ arrow denote no change, ↓ arrow denotes a decrease in a parameter compared to water control group, ↑ denotes an increase in a parameter compared to water control group e.g. ↔ denotes no change in U46619 constriction in WT M from sildenafil citrate treated pregnancies compared to WT M controls. Key: KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

<table>
<thead>
<tr>
<th></th>
<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
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<tr>
<td>Placental weight</td>
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<tr>
<td>Basal tone</td>
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<tr>
<td>Aortic diameter</td>
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<td>Maximal KPSS constriction</td>
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<td>U46619 constriction</td>
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<tr>
<td>U46619 sensitivity (EC₅₀)</td>
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<td>SNP sensitivity (EC₅₀)</td>
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Table 3.5 Fetal abdominal aorta sensitivity to U46619, ACH and SNP. Key: All data for WT / P0 fetuses in control and SC-treated mice expressed as effective concentration (EC in nM). A= Reduced ACH sensitivity in SC-treated WT male vs. control WT male (EC$_{20}$, EC$_{50}$; P<0.05; Mann-Whitney U Test). B/C= Reduced ACH sensitivity in SC-treated P0 female vs. control P0 female (EC$_{20}$, EC$_{50}$, P<0.01, EC$_{80}$; P<0.001, Mann-Whitney U Test). D/E= Reduced SNP sensitivity in SC-treated P0 female vs. control P0 female (EC$_{20}$, EC$_{50}$, P<0.01, EC$_{80}$; P<0.05, Mann-Whitney U Test). All data are mean±SEM with number of fetuses in parenthesis.

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<th>Effective concentration</th>
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<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
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<td>U46619 EC$_{20}$</td>
<td>4.5±0.9 (11) 18.9±3.6 (11) 49.0±10.7 (11) 2.7±0.5 (11) 10.8±1.9 (11) 43.4±7.7 (11) 4.5±0.9 (10) 17.9±3.6 (10) 71.7±14.6 (10) 4.7±1.3 (11) 18.9±5.0 (11) 75.6±19.9 (11)</td>
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<td></td>
<td>EC$_{50}$</td>
<td>75.2±39.1 (11) 300±156 (11) 515±101 (11) 56.1±21.8 (11) 224±87.3 (11) 844±300 (11) 42.2±13.7 (6) 169±54.8 (6) 858±253 (6) 28.7±12.0 (11) 115±48.0 (11) 397±155 (11)</td>
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<tr>
<td></td>
<td>EC$_{80}$</td>
<td>60.1±8.6 (11) 240±34.5 (11) 1030±193 (11) 60.2±10.7 (11) 241±42.6 (11) 1045±189 (11) 104±31.5 (9) 417±126 (9) 1612±481 (9) 40.1±4.8 (11) 161±19.3 (11) 637±118 (11)</td>
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<tr>
<td></td>
<td>Sildenafil citrate</td>
<td>U46619 EC$_{20}$</td>
<td>8.00±1.0 (10) 32.0±3.9 (10) 128±15.5 (10) 8.4±2.6 (6) 33.7±10.2 (6) 135±41.0 (6) 8.3±1.4 (7) 33.2±5.5 (7) 133±21.9 (7) 7.35±1.5 (8) 29.4±5.8 (8) 118±23.2 (8)</td>
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<tr>
<td></td>
<td>EC$_{50}$</td>
<td>248±96.8 (8) 990±387 (8) 4329±1767 (8) 106±25.2 (6) 425±101 (6) 1463±466 (6) 67.1±16.7 (6) 268±66.6 (6) 1133±277 (6) 127±33.6 (8) 509±134 (8) 2851±1046 (8)</td>
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<tr>
<td></td>
<td>EC$_{80}$</td>
<td>130±45.4 (10) 521±181 (10) 2748±1422 (10) 88.9±20.2 (6) 356±80.7 (6) 1955±651 (6) 136±36.8 (7) 544±147 (7) 3272±122 (7) 179±64.5 (7) 715±258 (7) 4781±2216 (7)</td>
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3.3 Discussion

This study utilised a well characterised mouse model of FGR (Constancia et al., 2002, Dilworth et al., 2010) to determine fetal vascular function using isolated abdominal aortas. Contraction of vessels in the presence of U46619, PE and KPSS was measured and compared between sexes and genotypes. Endothelial-dependent and –independent relaxation was assessed in pre-contracted vessels. The effect of 0.8 mg.ml$^{-1}$ of SC in the drinking water of the dam on prenatal outcome and fetal vascular function was also assessed. P0 fetal and placental weights were significantly reduced compared to their WT littermates. Growth restricted male (i.e. P0 M) fetal abdominal aortas demonstrated significantly reduced U46619-induced contraction and reduced endothelial-dependent relaxation; however, growth restricted female (i.e. P0 F) fetal abdominal aortas demonstrate neither of these characteristics. The addition of SC in the drinking water of the dam did not increase fetal or placental weight of either the growth restricted P0 fetuses or WT fetuses. In general, fetal abdominal aortas from SC-treated pregnancies demonstrated significantly reduced endothelial-dependent and –independent relaxation with minimal alterations in contraction induced by the thromboxane A$_2$ mimetic U46619.

3.3.1 P0 mouse phenotype

Previous studies suggest placental-specific IGF2 is a foremost determinant of fetal growth and leads to a reduction in both fetal and placental weight near term (Constância et al., 2002). In the present study we confirmed this phenotype; there was a decrease in both fetal and placental weight in P0 mice at E18.5 when compared with WT littermate controls. The reduction in fetal weight in P0 mice was much larger than the reduction in placental weight, thus fetal to placental weight ratio was significantly higher in P0 mice compared with wild type littermates (figure 3.3 and figure 3.10). A higher fetal to placental weight ratio suggests an increased fetal weight per gram of placenta; some authors suggest that this is a sign of a more efficient placenta. Fetuses from these experiments were not routinely genotyped for sex and thus there may be sex-specific differences in fetal weight that were not detected in WT and P0 fetuses.

3.3.2 Myography studies of mouse fetal abdominal aortic function

The first aim of this study was to develop a methodology to assess fetal vascular function that could be used to determine whether vascular function is altered in growth restriction and / or following administration of drugs to the dam to treat FGR. Mesenteric arteries have been the vessel of choice to determine the vascular function in mouse models of diseases such as hypertension. The reasoning for using these so called “resistance vessels” is that these vessels, and not the systemic vasculature (such as the aorta), regulate peripheral vascular resistance and
thereby arterial blood pressure. In hypertensive patients, pre-capillary blood pressure is normal when compared to normotensive controls, thus leading to the conclusion that peripheral resistance small arteries and small arterioles account for this increased blood pressure (Williams et al., 1990). In conscious unrestrained rats, it was demonstrated that mesenteric arcade arteries were responsible for the majority of peripheral vascular resistance (Christensen and Mulvany, 1993). In rats infused with the vasoconstrictor angiotensin-II, systemic pressure (as measured by a pressure catheter) increased by 40% but mesenteric arcade artery pressure increased by almost 90%, thus suggesting blood pressure is regulated by peripheral small arteries (i.e. mesenteric) rather than large conductance arteries (i.e. aortic). Studies in adult mice have demonstrated reproducible and reliable endothelial-dependent and –independent relaxation after pre-contraction with PE in mouse mesenteric arteries (Cooke and Davidge, 2003) and thus it seemed sensible to dissect mesentery and assess function of such vessels in the WT and P0 fetuses. Mesenteric arteries were dissected from mice at E18.5; however, the process was difficult as these vessels were extremely delicate. The small numbers of fetal mesenteric arteries mounted on to the wire myograph were likely to be physically damaged both by the dissection and the mounting procedure (as placing both wires into the lumen was problematic). Thus, reliable and reproducible results from these wire myography studies would be difficult and would lead to an unnecessary usage of mice.

It was necessary to identify a vessel in which vascular function could be assessed in the fetus which could later be related to the vascular function in the same vessel of the adult offspring; the fetal abdominal aorta was therefore chosen. The primary reason for choosing fetal abdominal aortas was that recent evidence from Herrera and colleagues (Herrera et al., 2012) and Camm and colleagues (Camm et al., 2010) demonstrate aortic vascular function can be reliably and reproducibly assessed in fetuses at E20 from rat dams, which were undernourished or hypoxic during pregnancy. Thus, experiments were aimed at assessing whether growth restriction in P0 mice through placental-specific deletion of Igf2 (and placental insufficiency) would alter fetal abdominal aortic vascular reactivity and whether this was sex-specific. As the previous studies in rat fetal aortas highlighted, it is also possible to manipulate the external environment of the dam (e.g. with hypoxia) and detect changes in abdominal aortic vascular reactivity. Thus, the effect of maternal SC treatment on fetal vascular function was measured in fetal abdominal aortas (see section 3.3.5).

As has previously been noted with mouse umbilical arteries and veins, fetal abdominal aortas were damaged using the classical normalisation at 0.9 of L_{12.1kPa} (Kusinski et al., 2009, Wareing et al., 2002). However, when fetal abdominal aortas were normalised in the same manner as umbilical arteries and veins (0.9 of L_{5.1kPa}) reproducible contraction and relaxation responses to
endothelial-dependent and -independent stimuli were noted. Using this adapted classical normalisation method the basal tone of the vessels were approximately 32 mmHg. Unfortunately, no data are available for the fetal aortic blood pressure in mice, but when comparing to a similar stage in human fetal development, fetal aortic blood pressure was estimated to be 28 mmHg at 20 weeks gestation and increased to 45 mmHg at 40 weeks gestation (Struijk et al., 2008). Mouse fetal aortas were therefore maintained at a physiological pressure comparable to that of the human fetal aortic blood pressure. Therefore, mouse fetal aortas were normalised to a set basal tone and vascular contractility / dilatation were then assessed in vessels which were maintained under this physiological pressure. Functional responses of the fetal aortas to vasoconstrictors and endothelial-dependent and -independent vasodilators could therefore be assessed independent of the length and diameter of the vessel. To further try to mimic physiological conditions, experiments were performed on fetal abdominal aortas using the $0.9 \times L_{5.1 \text{kPa}}$ normalisation criteria at 5 % $O_2$ and 37°C. 5 % oxygen tension (physiological) was chosen rather than 20 % oxygen tension (atmospheric) as oxygen tension in the fetal circulation is lower than in the mother. However, fetal haemoglobin has a higher affinity for oxygen and can therefore provide the fetus with adequate oxygen (Manca and Masala, 2008). These conditions therefore make it possible to assess functional responses of the fetal abdominal aorta at E18.5.

3.3.3 Fetal abdominal aortic function in appropriate weight and growth restricted fetuses

There were no differences in fetal abdominal aortic internal diameter in WT M, WT F, P0 M or P0 F mice. This was an expected result as all vessels were normalised to $0.9 \times L_{5.1 \text{kPa}}$ and basal tone was not significantly different between sexes or genotype. When assessing the vasoactive response of aortas, significant contraction was noted with KPSS, PE and U46619 in WT and P0 fetuses of dams receiving drinking water (i.e. controls). Endothelium-dependent relaxation post-application of ACH demonstrated a fully functioning endothelium with no damage from the dissection or mounting processes, as shown by consistent relaxation (>30 %) of aortic rings to U46619.

3.3.3.1 Potassium-, phenylephrine- and U46619- induced contraction of fetal abdominal aortas from appropriate weight and growth restricted fetuses

Depolarisation of the cell membrane in the presence of hyperosmotic extracellular potassium (120 mM), influx of potassium into the cell, the consequent influx of extracellular calcium and contraction of the vascular smooth muscle cell, was not significantly different between WT and P0 fetuses of either sex (table 3.1). These results suggest the mechanism by which depolarisation
induces contraction is conserved between sexes and that growth restriction does not alter the contraction association with either potassium channels or the opening of voltage dependent calcium channels in the smooth muscle of fetal abdominal aortas. In rat fetal aortas, wire myography was able to show that fetuses exposed to prenatal hypoxia had reduced maximal responses to potassium compared with fetal aortas from normoxic animals (Herrera et al., 2012). Taken together these data may suggest that P0 fetuses develop under normoxic conditions. Recent data from Dilworth and colleagues supports this theory as umbilical Doppler blood flow measurements were not altered in P0 mice (Dilworth et al., 2012), thus it is likely oxygenation of the fetus is adequate. The lack of any differences in maximal response to 120 mM depolarising potassium solution also suggests vascular smooth muscle cell content / development is similar between WT and P0 mice. However, a limitation of the current study is that molecules associated with potassium depolarisation (e.g. voltage gated calcium channels / calmodulin / myosin light chain kinase) and morphology of the smooth muscle cell were not assessed and therefore cannot conclusively state that these systems were unaffected.

Contraction was consistently lower in response to PE when compared to U46619 in WT and P0 fetuses of dams receiving drinking water (table 3.1). A previous study also demonstrated a reduced contraction in response to PE compared with U46619 in umbilical arteries and veins (Kusinski et al., 2009). Contraction responses have also been reported in adult aortic rings with PE (Zemse et al., 2010) and U46619 (Swafford et al., 2007). Responses of these aortas to PE were far greater in adult abdominal aortas (Zemse et al., 2010) when compared to the responses of fetal aortas to PE in the current study. PE is a selective α₁-adrenergic agonist, which promotes vascular smooth muscle cell contraction through degradation of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) into inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ promotes increased intracellular release of calcium from organelles and contraction in vascular smooth muscle cells (Somlyo and Somlyo., 1994). Reduced contraction of umbilical arteries (Kusinski et al., 2009) and fetal aortas (this study) in response to PE the fetus when compared with the adult imply vascular smooth muscle cells of the fetus do not possess a fully functional α₁-adrenergic system. This could be a protective mechanism in the developing fetus as smooth muscle cell vasoconstriction could increase blood pressure, redistribute blood flow and may be detrimental to fetal development. In support of this concept, a study examining the role of α₁-adrenergic receptors in neurodegeneration demonstrated that overexpression of this protein in pregnant mice was associated with reduced numbers of fetuses when compared to WT controls (Zuscik et al., 2000). Fetal aortic responsiveness to PE was not altered in P0 mice of either sex when compared with WT mice. In contrast, a reduced and increased response of carotid arteries and femoral arteries, respectively, to PE was recently demonstrated in neonatal rats from hypoxic pregnancies but there were differential effects from under nourished pregnancies (Williams et al., 2005). These
data suggest that there could be regional changes in vascular contraction dependent on the particular insult (e.g. placental insufficiency, hypoxia or undernutrition) and that P0 fetal aortas maintain responsiveness to PE when compared with WT controls.

U46619-induced contraction was reduced in P0 M vs. WT M fetal abdominal aortas (figure 3.4A); this was also true when expressing U46619 contraction as a % of maximal KPSS contraction (figure 3.5A). Therefore, P0 M fetal aortas have reduced agonist (U46619) -induced contraction and maintain adequate responses to depolarising potassium solution as the maximal response to depolarisation is similar in P0 M vs WT M fetal aortas (see table 3.1). A similar reduced response to U46619 was observed in fetal aortas from males of undernourished dams (Camm et al., 2010). A previous study indicated reduced fetal calcium content in P0 fetal blood at E17 (Dilworth et al., 2010). In addition, calbindin-D\textsubscript{9K}, which binds to intracellular calcium, is up-regulated in P0 placentas between E17 and E19. Therefore, there may be two possibilities as to why P0 M fetal aortas have reduced U46619-induced contraction. Firstly, the reduced extracellular calcium content may cause a reduction in calcium-dependent U46619-induced contraction. Evidence from fetal sheep exposed to hypoxia during pregnancy suggests intracellular calcium concentrations of fetuses exposed to hypoxia are reduced (Maruko et al., 2009) and that maximal contraction responses to calcium (induced by U46619) can be reduced or increased in fetal coronary arteries, depending on the specific area of the coronary artery (Garcia et al., 2000). Although extracellular calcium was present in the myograph bath in the current study, P0 M fetal aortas may have down regulated responses to extracellular calcium influx and thus produce smaller contraction in response to U46619. That being said, it is unlikely that down regulation of calcium-dependent contraction occurs in P0 M fetal aortas as the responses to depolarising potassium solution (which also lead to increased intracellular calcium) are not different between WT M and P0 M fetal aortas (table 3.1). A second mechanism could be that U46619-induced extracellular influx of calcium may be sequestered by increased calcium binding proteins (as is the case with calbindin-D\textsubscript{9K} in the P0 placenta; Dilworth et al., 2010) in P0 M fetal aorta. Whether this is the case in P0 M fetal aortas is not known but identifying expression of calcium-associated proteins within aortas may explain reduced U46619-induced contraction in P0 M vs. WT M fetuses.

In contrast to males, U46619-induced contractions of fetal aortas from females were not different between P0 and WT mice when expressed as active effective pressure (figure 3.4B). When this contraction was expressed as a % KPSS maximal contraction (to normalise for vascular smooth muscle cell mass) there was a subtle reduction in P0 F fetal aortas (figure 3.5B), similar to that seen in P0 M fetal aortas compared with WT M fetal aortas. This suggests there may be more vascular smooth muscle cell mass in P0 F vs. WT F. U46619-induced contraction was greater in WT M than WT F but only when expressed as a dose-response curve with active effective pressure of
the vessel and not as a percentage contraction in response to KPSS. When the data are expressed as a % of KPSS this normalises the data to account for any changes in smooth muscle cell mass and contractility. Thus, based on these observations WT F fetal aortas may exhibit reduced sensitivity to U46619-induced contraction but maintain the normal contractile response to cell membrane depolarisation (i.e. smooth muscle cell mass may be similar in WT F vs WT M aortas). U46619-induced contraction in vascular smooth muscle causes production of IP₃ and DAG. DAG is responsible for the translocation of protein kinase C (PKC) to the plasma membrane, which in turn activates pathways which make actin available and increase the contraction of the cell to calcium; male aortic strips from rats demonstrate increased expression of PKC. Phorbol esters, which activate PKC, also produce greater contraction in male vs. female aortas (Kanashiro and Khalil, 2001). Increased PKC expression in male aortas suggests a sex-specific PKC-mediated mechanism for vascular smooth muscle cell contraction. In addition, there is also evidence that differences in the regulation and expression of thromboxane receptors between males and females (Higashiura et al., 1997), which may explain the increased contraction in response to U46619 in WT M, compared with WT F fetal aortas. Together, this evidence suggests that WT M fetal aortas could have increased numbers or activity of thromboxane receptor or increased sensitivity in downstream signalling pathways associated with the thromboxane receptor and that this could promote increase contraction in WT M vs. WT F fetal aortas. In contrast to the reduced U46619-induced contraction of WT F vs. WT M fetal aortas, there were no differences in contraction of P0 F vs. P0 M fetal aortas (figure 3.4D). There were also no differences in the ability of the smooth muscle cell to contract upon depolarisation in P0 M vs. P0 F aortas (table 3.1) or contraction when expressed as % KPSS (figure 3.5D). These data suggest similarities between smooth muscle mass and function between P0 F and P0 M fetal aortas and thus give further support to the theory that P0 M fetal aortic vascular smooth muscle may be less sensitive to pathways associated with agonist induced contraction.

### Acetylcholine- and sodium nitroprusside-induced endothelial-dependent and -independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses.

There was a subtle but significant reduced relaxation response to ACH in P0 M fetal aortas when compared with WT M littermates (figure 3.6A); but no difference in sensitivity to ACH was noted. In contrast, male fetal aortas from rats whose mothers were either hypoxic or undernourished during pregnancy demonstrated similar relaxation in response to metacholine (Herrera et al., 2012). However, relaxation in response to ACH in fetal femoral arteries from sheep whose mothers were given 50 % restricted diet were significantly blunted when compared to those fetal arteries from nourished ewes (sex was not specified; Ozaki et al., 2000). It is important to note
that neither of these two studies demonstrated reduced birthweight but both highlight that endothelial dysfunction may be dependent on sex, environmental insult (e.g. hypoxia / undernutrition) and species. Taken together, P0 M fetal aortas demonstrate reduced U46619-induced contraction and a blunted relaxation response to ACH, which may suggest defective systemic arterial function. P0 F fetal aortas however, respond similarly to endothelial-dependent vasodilatation by ACH when compared to WT F. Similar U46619-induced contraction and ACH-induced relaxation in P0 F when compared with WT F are in complete contrast to the reduced contraction and reduced relaxation demonstrated in P0 M vs. WT M fetal aortas. It could be that there are sex-specific alterations in vascular smooth muscle and endothelial function as a result of low birth weight. In addition, females may be less likely to develop dysfunction in smooth muscle and / or endothelium due to sex-hormones (e.g. oestrogen). A good example of this is in the spontaneously hypertensive rat model of hypertension. Female rats develop hypertension at a much slower rate and do not develop as severe a hypertensive phenotype than their male littermates (Iams and Wexler, 1979). Female rats that were ovariectomised (thus removing oestrogen) escaped this beneficial effect and demonstrated as severe a hypertensive phenotype as males. When oestradiol (an oestrogen precursor) was administered to ovariectomised females, blood pressure was significantly lowered compared to ovariectomised rats alone (Iams and Wexler, 1979). Further investigation of these mechanisms revealed reduced endothelial-dependent relaxation in male vs. female rats (Kauser and Rubanyi, 1995). Although the majority of oestrogen present in the fetus is produced by the placenta, it is possible that there is an increased placental oestrogen production in females compared with males which could alter fetal vascular function and alleviate the endothelial dysfunction associated with low birth weight in P0 F fetal aortas.

In mice, female femoral arteries demonstrate greater endothelial-dependent relaxation in response to ACH when compared with males (Luksha et al., 2006). However, this is completely dependent on whether the proximal or distal femoral arteries are assessed, suggesting the sex-specific effects may be different in different vascular beds. In the current study, there was a trend towards increased relaxation responses between WT F vs. WT M (figure 3.6C) but this was not significant. A limitation of the current study was that there were no assessments of the relative components (i.e. NO, prostacyclin and endothelium-dependent hyperpolarising factor) of endothelium-dependent relaxation in fetal abdominal aortas which could have proven useful in dissecting out any differences between males and females. When assessing the effect of sex in P0 fetal aortas, P0 F fetal aortas demonstrated increased relaxation in response to ACH when compared with P0 M fetal aortas (figure 3.6D). As suggested previously, this is more likely due to the fact that P0 M fetal aortas show blunted endothelium-dependent vasodilation (figure 3.6A) in response to ACH rather than an increase in relaxation in P0 F aortas.
Endothelium-independent relaxation using SNP produced relaxation of the vessels in all groups; however, there were no significant effects of genotype or sex in any of the comparisons (figure 3.7). Endothelium-independent vasodilation of fetal aortas from hypoxic dams was reduced or unaltered in response to SNP, depending on the length of hypoxia (Camm et al., 2010). In support of the current study finding that there were no differences in endothelium-independent relaxation, human adults who had a low birth weight show alterations in endothelium-dependent but not –independent vasodilation (Leeson et al., 2001). These data suggest a specific alteration in P0 male mice in endothelial but not smooth muscle cell function and are summarised in table 3.2.

The reduction in U46619-induced contraction and blunted endothelial-dependent relaxation in response to ACH in P0 M vs. WT M fetal aortas could be a consequence of growth restriction in a mouse model of placental insufficiency. Altered smooth muscle and endothelial cell function could be a prerequisite for hypertension and cardiovascular disease in later life in P0 male mice. Young adults of low birth weight demonstrate early signs of endothelial-dysfunction (Goodfellow et al., 1998) as early as 8 years of age (Halvorsen et al., 2006). In this study low birth weight was associated with an increase in blood pressure at 8 years of age, and suggests alterations in vascular function may predispose to hypertension (Halvorsen et al., 2006). It is possible then, that P0 male fetuses may be developing the early signs of endothelial dysfunction which may lead to adulthood hypertension. It has also been suggested that hypertension occurs in both sexes through sex-specific mechanisms (McMullen and Langley-Evans., 2005). The fact that blunted endothelium-dependent relaxation is not apparent in P0 female mice either suggests growth restriction alone is not sufficient to promote vascular dysfunction or that female fetuses are somehow protected from vascular dysfunction and develop hypertension through other mechanisms.

To summarise, this study demonstrates it is possible to assess fetal vascular function of the mouse using the fetal descending abdominal aorta. It is important to note that this vessel is a conduit artery; ideally fetal mesenteric arteries would be assessed as these arteries contribute to vascular total peripheral vascular resistance (and increased blood pressure) in adulthood and have been extensively studied (Christensen and Mulvany., 1993). Nevertheless, fetal aortas from rats that were growth restricted at E20 (due to maternal hypoxia) have increased wall thickness, altered responses to vasoactive substance and fetal hearts have increased wall thickness (Camm et al., 2010). These studies indicate that the fetal aorta may be a good substitution to determine the effects of growth restriction on fetal vascular function of the P0 mouse. In addition, this method was sensitive enough to detect differences between sex and genotype in this mouse model of FGR.
3.3.4 Effect of maternal sildenafil citrate treatment in appropriate weight and growth restricted fetuses

The second aim of this study was to assess whether a maternally targeted treatment could affect fetal vascular function. Previous studies from Dilworth and Stanley have determined SC to increase fetal weight in both the P0 and COMT−/− mouse models of FGR (Dilworth et al., 2013, Stanley et al., 2012). As proof of principle, the dosage of SC was increased to maximise the potential effects of SC on fetal vascular reactivity. SC (0.8 mg.ml⁻¹) was administered in the drinking water of the pregnant dam from E12.5 to E18.5; thus utilising a similar dosing regimen (but with a doubling of SC concentration) to the recent study by Dilworth and colleagues (Dilworth et al., 2013). There were no differences in resorption number or litter size in SC-treated dams when compared to controls. In contrast to the study by Dilworth and colleagues performed in the P0 mouse, SC did not increase fetal weight in WT or P0 fetuses (figure 3.8A and 3.8B). These data suggest that the optimal concentration of SC required to increase fetal weight in this mouse model of FGR is lower than what was used in the current study. However, it should also be noted that there were fewer litters (nearly 50%) administered this dose of SC compared with the earlier study by Dilworth and colleagues and that this study may therefore not be sufficiently powered to detect differences in fetal weight. In accordance with Dilworth and colleagues (Dilworth et al., 2013) placental weight in both WT and P0 mice was unaffected by SC treatment (figure 3.8C and 3.8D); thus fetal:placental weight ratios were not altered in the treatment group (figure 3.10). Furthermore, SC did not affect the average number of pups per litter or the number of resorbed fetuses per litter. These observations suggest no obvious effects of SC on fetal or placental growth. It is still possible that in the absence of growth promoting effects, SC could have effects on fetal vascular function. No other studies have assessed the effects of SC directly on mouse fetal vascular function (i.e. the abdominal aorta). However, studies have previously assessed the effects of SC on umbilical artery function in humans (Karasu et al., 2012) and chicken egg embryos (Sawatdee et al., 2014); other researchers have also shown a significant reduction in peripheral vascular resistance and increased pulmonary blood flow in fetal and neonatal lambs (Deruelle et al., 2005) after administration of SC.

3.3.5 Effect of maternal sildenafil citrate treatment on fetal abdominal aortic function in appropriate weight and growth restricted fetuses

As discussed previously, SC selectively inhibits the action of cGMP-dependent phosphodiesterase type 5 (PDE-5), which under normal physiological conditions binds to cGMP in vascular smooth muscle cells and catalyses the hydrolysis of cGMP to GMP (Lincoln et al., 2001). cGMP is able to
reduce smooth muscle cell contraction through phosphorylation of cGMP-dependent protein kinase (PKG) and reduce the increased intracellular calcium associated with contraction. In addition, cGMP is able to promote relaxation through increased activity of myosin light chain phosphatase (see section 1.8.4). Therefore, SC, by inhibiting PDE-5 is able to prolong the dilator effects of cGMP (via PKG and other mechanisms) in the vascular smooth muscle cell.

3.3.5.1 Effect of sildenafil citrate on potassium-, phenylephrine- and U46619- induced contraction of fetal abdominal aortas from growth restricted and appropriate weight fetuses

There was no effect of SC on the intraluminal diameter of aortas, nor were there any significant differences in basal tone or maximal contraction to KPSS, PE or U46619 between SC-treated and water control groups. In general, there were minimal effects of SC on U46619-induced contraction responses of fetal abdominal aortas. However, in both P0 M and P0 F fetuses exposed to SC in utero there was an subtle increased contraction in response to U46619 when expressed as % KPSS, this may suggest altered smooth muscle mass and seems to be specific to growth restricted fetuses. These data are in contrast to an earlier study which demonstrated 100 nmol/litre of SC blunted contraction to U46619 in small myometrial arteries from both normal pregnancy and FGR pregnancies (Wareing et al., 2005). However, a selective PDE-5 inhibitor (UK-343664) was not able to reduce the increased contraction to U46619 or arginine vasopressin (AVP; a vasoconstrictor) associated with PET pregnancies (Wareing et al., 2004). It is important to note that in the P0 uterine artery branches (those which supply individual placentas and fetuses) do not display altered contraction responses to vasoconstrictors (Kusinski et al., 2011), nor were there any differences in blood flow velocity using ultrasound biomicroscopy in either uterine or umbilical arteries (Dilworth et al., 2013). Therefore, the lack of effect of SC on fetal aortic contraction is not a surprising result. That said, SC does elicit differential effects on coronary (reduced) and uterine artery (increased) blood flow in non-pregnant sheep, suggesting SC may indeed increase contraction of specific vascular beds in pregnant and non-pregnant animals (Zoma et al., 2004). In addition, in sheep that were administered a bolus injection of 100 mg SC and a consequent hourly infusion of 4.1 mg for 24 hours, there was a reduced uterine and umbilical blood flow in both control dams and ewes with FGR fetuses (Miller et al., 2009). Together these data suggest SC may alter contraction or dilatation of specific vascular beds depending on a number of criteria including concentration of SC, length of administration and the species assessed.
3.3.5.2 Effect of sildenafil citrate on acetylcholine- and sodium nitroprusside-induced endothelial-dependent and -independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses

SC treatment led to a reduced relaxation capacity of fetal abdominal aortas in response to the endothelial-dependent agonist ACH (figure 3.13); with the exception of P0 male mice (figure 3.13C). ACH is proposed to contribute to endothelium dependent relaxation through three distinct mechanisms; NO-, EDHF- and prostacyclin-mediated relaxation (Morton et al., 2010). NO increases activity of soluble guanylate cyclase (sGC) which in turn leads to increased concentrations of cGMP in vascular smooth muscle (Ohashi et al., 1998, Yamashita et al., 2000). PDE-5 catalyses the hydrolysis of cGMP to inactive GMP therefore, it was hypothesised that SC, a selective PDE-5 inhibitor, will promote the effects of the NO component of ACH-induced endothelium-dependent relaxation. Reduced endothelium-dependent relaxation of aortas from fetuses exposed to SC in utero was contrary to what was expected. Fetal lungs from rat pregnancies that were injected with 100 mg/kg/day of SC from E11.5 to E20.5 reduced concentrations of PDE-5 but have similar concentrations of cGMP in response to SC when compared with non-treated controls (Luong et al., 2011). Thus, this suggests that SC crosses the placenta and is biologically active but that fetal lungs are able to regulate concentrations of cGMP, possibly through altered GC activity and sensitivity to NO. Addition of SNP (a nitric oxide donor) to gastric smooth muscle cells increased GC activity and increased the activity of PDE-5 (Murthy, 2001, Murthy, 2004). When PDE-5 was inhibited, cGMP and PKG concentrations were elevated but this elevation reduced the activity of GC. GC was then increased after inhibition of PKG suggesting feedback inhibition occurs in situations where there an excess of cGMP / PKG. Mice that overexpress eNOS have higher basal concentrations of NO and cGMP (Ohashi et al., 1998) but have 50 % reduced activity of GC (Yamashita et al., 2000) providing further support of feedback inhibition in situations of cGMP / PKG excess. The most interesting observation from mice overexpressing eNOS was an attenuated endothelium-dependent and –independent relaxation, mimicking results from similar experiments presented in this chapter. More recent evidence suggests a direct role of GC in the reduced responsiveness of vasculature to NO (Van Deel et al., 2007). Therefore, chronic exposure of fetuses to SC in utero may in fact reduce activity of GC and reduce the responses of fetal vasculature to the NO-component of ACH-induced endothelium-dependent relaxation. In addition, fetal vasculature exposed to SC may be programmed to elicit a reduced response to NO in general as a result of altered activity of molecules associated with the NO / cGMP pathway (termed nitrate tolerance). There was also a decrease in SNP-induced relaxation responses of fetal abdominal aortas in the SC treatment group; with the exception of WT M mice. This effect was most pronounced in the P0 F mice where the effective concentration of SNP required to elicit 20 %, 50 % and 80 % relaxation was
significantly increased (table 3.5). Thus SC led to a significant desensitisation of vascular smooth muscle cells to NO. These data are summarised in table 3.3.

SC did not alter fetal aortic vasorelaxation in response to ACH in P0 M mice (figure 3.13C). It is worth reiterating that P0 M fetal abdominal aortas demonstrate an inherent inability to respond to ACH when compared with WT M controls but respond appropriately with direct addition of NO (figure 3.6A and figure 3.7A). These data give evidence for the hypothesis that there is a dysfunctional endothelium in P0 M fetal aortas and an inability to produce NO through ACH mediated pathways; this hypothesis could be tested by direct inhibition of nitric oxide synthase with L-NAME or similar inhibitors of NO synthase.

### 3.3.6 Summary

P0 mice exhibit FGR and reduced placental weight at E18.5. Fetal aortic function at E18.5, assessed by wire myography, revealed subtle genotype and sex-specific differences in vasoconstriction and vasodilatation responses to agonists. Fetal aortas from P0 male mice exhibit reduced contraction in response to the thromboxane A2 mimetic U46619 and a reduced response to the endothelium-dependent vasodilator ACH but no difference in relaxation in response to direct addition of NO. Taken together, these results suggest there may be a vascular defect in the endothelium in P0 mice but that this is sex-specific. In humans, reduced endothelium-dependent relaxation is associated with increased risk of hypertension and cardiovascular disease (Linder et al., 1990, Taddei et al., 1992). Therefore, further studies assessing cardiovascular physiology in P0 mice may prove to be of interest and may provide evidence for the association between low birth weight, vascular dysfunction and hypertension in later life (see chapter 5).

The effect of SC on fetal and placental weight and fetal vascular function was determined. In contrast to previous studies in mice (Dilworth et al., 2013, Stanley et al., 2012) and sheep (Satterfield et al., 2010) models of FGR SC (0.8 mg.ml⁻¹) administered to the drinking water of the dam between E12.5 and E18.5 did not increase fetal or placental weight or alter litter size or total number of resorptions. Even in the absence of any gross changes in placental or body weight, fetal vascular function was impaired in both WT and P0 mice, from SC-treated dams and this was independent of sex. Fetal aortas demonstrated blunted relaxation in response to ACH and direct addition of NO. It is hypothesised that these alterations are due to changes in the NO / cGMP pathway and could be specifically associated with reduced GC activity; future experiments should focus on maternal and fetal measurements of PDE-5, GC, cGMP and NO in vascular tissues. These experiments will also indirectly measure the effectiveness of maternofetal transfer of SC. The long-term implications of SC-induced endothelial dysfunction are unknown but human studies
suggest a strong link between endothelial dysfunction and hypertension in adulthood (Egashira et al., 1995, Leeson et al., 2001).

It is important to note that the concentration of SC administered in the current study equates to double (Dilworth et al., 2013) and quadruple (Stanley et al., 2012) that used in earlier studies in mouse models of FGR but there was no effect of this dose on the number of resorptions or litter size. However, positive effects on fetal weight may be lost at higher concentrations of SC and could be concentration dependent. Further studies should try to assess the vascular effects of a dose of SC in mice which is similar to that currently administered in an on-going clinical trial for severe early onset FGR.
Chapter 4  Subcutaneous Injection of Sildenafil Citrate: Effects on Fetal Growth and Fetal Vascular Function
4.1 Introduction

The data presented in Chapter 3 suggest that an oral dose of SC at double the concentration administered (0.8 mg.ml\(^{-1}\)) previously used (Dilworth et al., 2013) did not increase fetal weight in the P0 mouse model of FGR. In addition, there were no deleterious effects of SC on litter characteristics such as number of fetuses per litter and number of resorptions per litter. When assessing fetal vascular function, SC led to a blunted agonist-induced relaxation when endothelial-dependent and -independent vasodilatation was assessed. It could be argued that the supratherapeutic dosage of SC (approximately 120 - 160 mg.kg\(^{-1}\)) could be the reason for effects seen on fetal vascular function and the lack of effect on fetal / placental weight. Therefore, assessing the effects of a reduced concentration of SC on fetal / placental weight and fetal vascular function in the P0 would be useful.

Studies reporting beneficial effects in growth restricted animals have employed a variety of concentrations, routes of administration and dosing regimens to investigate the effects of SC treatment on fetal outcomes. However, there remains a lack of consensus as to which is the most clinically relevant. Ideally, experimental studies in animals should mimic criteria such as the route of administration and concentration of SC likely to be given in human clinical trials for FGR and PET. This in itself is quite difficult as human trials have utilised a variety of concentrations of SC and given treatment at different stages in gestation. In addition, although pharmacokinetic data for SC have demonstrated specific differences between human and rodents (Walker et al., 1999) only one study has addressed the pharmacokinetics of SC in human pregnancy (Samangaya et al., 2009). This study utilised a maximum oral dose (based on a 70kg woman) of 3.2 mg.kg\(^{-1}\) which reached concentrations of 271 ng.ml\(^{-1}\) in maternal blood and had no adverse effects on fetal morbidity or mortality. A recent non-randomised clinical trial administered an oral dose of 1 mg.kg\(^{-1}\) SC daily (based on 70kg woman) to patients with severe early onset FGR which led to increased abdominal growth velocity of FGR fetuses (Von Dadelszen et al., 2011). Studies in rats have demonstrated beneficial effects of SC on fetal outcomes, such as increased birth weight and reduced fetal mortality using a concentration of 10 mg.kg\(^{-1}\) (Ramesar et al., 2010) whilst others have demonstrated no detrimental effects on fetal growth in normal rat pregnancy at 90 mg.kg\(^{-1}\) (Sasser et al., 2010). In mice, a maternal injection of 100 mg.kg\(^{-1}\) of SC led to a maximum concentration of 2300 ng.ml\(^{-1}\) (approximately 10 times as much seen in maternal blood from Samangaya and colleagues (Samangaya et al., 2009) within maternal blood within 6 hours. However, there are differences in pharmacokinetics in mice when compared to humans; a higher liver clearance rate of SC in rodents vs. humans results in a metabolic half-life of SC of 1.3 hr and 3.7 hr, respectively (Walker et al., 1999).
Based on human and experimental data from animals, a daily subcutaneous injection of SC (10 mg.kg\(^{-1}\)) was administered to pregnant P0 dams between E12.5 and E17.5. This concentration was selected as it was hypothesised that, given the pharmacokinetic data in mice, SC concentrations should reach between 250 to 300 ng.ml\(^{-1}\) within 6 hours of injection in the mouse, as previous studies in pregnant rats with 100 mg.kg\(^{-1}\) (i.e. 10 X that of 10 mg.kg\(^{-1}\)) have demonstrated maternal blood contains 2500 – 3000 ng.ml\(^{-1}\) 6 hours after subcutaneous injection. 271 ng.ml\(^{-1}\) was the peak concentration of SC in maternal blood in a clinical trial of SC in PET (Samangaya et al., 2009). A similar dosing regimen is also currently used in the STRIDER multi-centre international trial for SC use in cases of early-onset FGR (Ganjevoort et al., 2014) and thus it is expected that maximal concentrations of SC in these cases will reach between 250 – 300 ng.ml\(^{-1}\).

Rather than using 3.2 mg.kg\(^{-1}\) which was used in the PET clinical trial and is currently used in the early onset FGR clinical trial, 10 mg.kg\(^{-1}\) of SC was selected to account for increased metabolism and higher clearance rates of SC in mouse vs. human (Walker et al., 1999). Another important consideration was the method of delivery (i.e. subcutaneous or oral). The subcutaneous injection method was chosen to give reproducible plasma concentrations of SC and to increase bioavailability of SC in the dam, as it was suggested that when administered in drinking water, plasma concentrations of SC are variable depending on water consumption in the mouse (Pfizer®, personal communication). Control mice were administered a volume-matched daily subcutaneous injection of saline (0.9 % NaCl) to control for any potential stress of the injection.

The primary hypothesis of this study was that a lower dose of 10 mg.kg\(^{-1}\) of SC administered subcutaneously to the pregnant dam once daily between E12.5 and E17.5 will increase fetal / placental weight in P0 fetuses without having detrimental effects on fetal vascular function at E18.5. The secondary hypothesis of this study was that a subcutaneous sham injection of saline once daily to the pregnant dam between E12.5 and E17.5 will have no effect on WT or P0 fetal / placental weight or fetal vascular function at E18.5.
4.2 Results

4.2.1 Effect of a subcutaneous injection of saline on contraction responses of fetal abdominal aortas

There was no significant difference in fetal abdominal aortic diameter between all four saline control groups (WT M, WT F, P0 M and P0 F). There was no effect of a subcutaneous dose of saline on basal tone in any of the individual groups (table 4.1); the median basal tone for SC-treated groups (N = 33) was 25 (min 17 – max 44) mmHg. Median basal tone (min - max) for individual groups was; WT male 30 (17 – 40) mmHg, P0 male 25 (18 – 40) mmHg, WT female 21 (18 – 42) mmHg, P0 female 25 (20 – 44) mmHg.

Contraction to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) was not significantly different between all four saline control groups (table 4.1). However, U46619 elicited the greatest agonist-induced responses when compared with PE and KPSS.

U46619 dose response curves were constructed for fetal abdominal aortas (figure 4.1) from subcutaneous saline-treated pregnancies. U46619-induced contraction of aortas from WT M and WT F mice were similar to their P0 littermates (figure 4.1A and 4.1B, respectively). When fetal sex was considered, WT F and P0 F mice had similar U46619-induced contraction compared to WT M (figure 4.1C) and P0 M (figure 4.1D). There were no differences in EC_{20}, EC_{50} or EC_{80} values across all saline control groups (table 4.4).

U46619-induced contraction of aortas (as % KPSS) from WT M mice were similar to P0 M (figure 4.2A). P0 F U46619-induced contraction (as % KPSS) was significantly increased when compared to WT females (figure 4.2B; \( P<0.05 \)). When fetal sex was considered, female mice had similar U46619-induced contraction compared to WT M (figure 4.2C) and P0 M mice (figure 4.2D).
Table 4.1. Fetal abdominal aorta diameter, basal tone and max contraction data from saline injected pregnancies. KPSS (120 mM high potassium salt solution); PE (10^{-5} M phenylephrine); U46619 (2x10^{-6} M thromboxane-A2 mimetic). Data are shown as median [min-max]; number of fetuses per group in parenthesis.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Fetal ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
<th>Maximal Contraction (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KPSS</td>
</tr>
<tr>
<td>Saline</td>
<td>WT Male (10)</td>
<td>670 [550 - 762]</td>
<td>4.0 [2.3 – 5.3]</td>
<td>0.32 [0.13 – 0.99]</td>
</tr>
<tr>
<td></td>
<td>P0 Male (7)</td>
<td>632 [557 - 667]</td>
<td>3.3 [0.4 – 5.4]</td>
<td>0.38 [0.13 – 0.60]</td>
</tr>
<tr>
<td></td>
<td>WT Female (5)</td>
<td>644 [482 - 668]</td>
<td>2.8 [2.4 – 5.7]</td>
<td>0.41 [0.36 – 0.75]</td>
</tr>
<tr>
<td></td>
<td>P0 Female (11)</td>
<td>631 [491 – 680]</td>
<td>3.3 [2.6 – 5.9]</td>
<td>0.51 [0.04 – 0.74]</td>
</tr>
</tbody>
</table>
Figure 4.1. Dose response curves of fetal abdominal aortas from saline-injected pregnancies in response to increasing doses of U46619. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: WT male (blue closed circles), P0 male (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 4.2. Figure 4.2 Dose response curves of fetal abdominal aortas from saline-injected pregnancies in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: WT male (blue closed circles), P0 male (black open circles), WT F (cyan closed squares), P0 F (black open squares).
4.2.2 Relaxation responses of fetal abdominal aortas to Acetylcholine and Sodium nitroprusside from pregnancies administered a subcutaneous injection of saline.

Aortas were pre-contracted with an EC$_{80}$ concentration of U46619 before ACH (endothelial-dependent, figure 4.3) and SNP (endothelial-independent, figure 4.4) induced relaxation was measured.

ACH-induced relaxation responses of fetal abdominal aortas were similar in P0 M vs. WT M mice (figure 4.3A) and P0 F vs. WT F mice (figure 4.3B). When assessing the effect of sex, ACH-induced relaxation response in male vs. female was similar in WT mice (figure 4.3C) and P0 mice (figure 4.3D).

SNP-induced relaxation responses of fetal abdominal aortas were similar in P0 M vs. WT M mice (figure 4.4A). There was no difference in SNP relaxation response in WT F vs. P0 F mice (figure 4.4B). When assessing the effect of sex, SNP-induced relaxation responses were similar in male vs. female from WT mice (figure 4.4C) and P0 mice (figure 4.4D). There were no significant differences between any of the four saline control groups in the effective concentration of either ACH or SNP required to produce 20 %, 50 % or 80 % relaxation of fetal abdominal aortas (table 4.4).
Figure 4.3. Dose response curves of fetal abdominal aortas from saline-injected pregnancies to increasing doses of ACH. Panel A-D: Arteries were pre-contracted with an EC₈₀ dose of U46619. ACH dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: ACH; acetylcholine, WT male (blue closed circles), P0 male (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 4.4. Dose response curves of fetal abdominal aortas from saline-injected pregnancies to increasing doses of SNP. Panel A-D; Arteries were pre-contracted with an EC_{80} dose of U46619. SNP dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parentheses. Key: SNP; sodium nitroprusside, WT male (blue closed circles), P0 male (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Table 4.2. Summary table comparing fetal / placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619) and relaxation responses (ACH, SNP) from saline-injected pregnancies (chapter 4) and water control pregnancies (chapter 3). Comparisons were made between genotypes (WT and P0) and sex within each control group and are presented here to highlight differences between the two control regimens. ↔ arrow denote no change, ↓ or ↑ arrow denotes a decrease or increase in a parameter compared to another relevant group e.g. U46619 contraction in WT female reduced compared to WT male from the water control group. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside).

<table>
<thead>
<tr>
<th></th>
<th>WT Male</th>
<th>P0 Male</th>
<th>WT Female</th>
<th>P0 Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Saline</td>
<td>Water</td>
<td>Saline</td>
</tr>
<tr>
<td>Fetal weight</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
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<tr>
<td>Placental weight</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Basal tone</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Aortic Diameter</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Maximal KPSS contraction</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>PE contraction</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>U46619 contraction</td>
<td>↑ vs. P0 M</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>ACH Relaxation</td>
<td>↑ vs. P0 M</td>
<td>↔</td>
<td>↓ vs. P0 F</td>
<td>↔</td>
</tr>
<tr>
<td>SNP Relaxation</td>
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</table>
4.2.3 Effect of a subcutaneous injection of SC on prenatal weight measurements in wild type and P0 knockout mice at embryonic day 18.5

The number of fetuses per litter was not significantly different between control and treated groups ([mean±SEM]; 8.3±1 vs. 7.8±1.8, respectively). SC had no effect on the percentage of resorbed fetuses per litter ([mean±SEM]; 0.75±0.9 control vs. 0.35±0.5 SC). For the SC treatment group, fetal weight was significantly reduced in P0 vs. WT mice ($P<0.0001$). SC had no overall effect on fetal weight in either WT or P0 mice (figure 4.5A and 4.5B). Subcutaneous SC did not affect the placental weight in either WT or P0 mice (figure 4.5C and 4.5D). Fetal:placental weight ratios from control and SC-treated pregnancies are shown in figure 4.7. There was a significant increase in fetal:placental weight ratio in P0 mice from both control and SC-treated pregnancies ($P<0.01$). A frequency distribution curve of fetal weights from control and SC-treated pregnancies is shown in figure 4.6. The 5th percentile of WT control fetal weights was 1.08 g with 70 % of P0 control fetuses below this 5th centile. 70 % of P0 mice from subcutaneous SC-treated pregnancies fell below this 5th centile of WT weight.
Figure 4.5. Fetal and placental weights from WT and P0 knockout mice following a subcutaneous injection of sildenafil citrate. Fetal weights (A,B) and placental weights (C,D) at embryonic day 18. Pregnant dams were allowed access to water with (N = 12) or without (N = 10) sildenafil citrate (10 mg.kg\(^{-1}\)). In figure 2A and 2C individual fetal/placental weights are shown. In figure 2B and 2D each symbol represents an average litter weight for a particular phenotype. Horizontal line denotes mean ± SEM. Statistical analyses for genotype and treatment were performed using two-way ANOVA with Bonferroni post hoc test. **** P<0.0001, ** P<0.01 using Bonferroni post hoc test.
Figure 4.6. Fetal weight frequency distribution curve from individual fetuses following a subcutaneous injection of sildenafil citrate. Pregnant dams were allowed access to water with (N = 12) or without (N = 10) sildenafil citrate (10 mg.kg$^{-1}$). WT (black solid curve), WT SC (grey solid curve), P0 (black dashed curve) and P0 SC (grey dashed curve). Vertical red dashed line denotes the 5th centile of WT fetal weights (1.08g). Untreated individual pup n's: WT saline n = 31, WT SC n = 42, P0 saline n = 47, P0 SC n = 48.
Figure 4.7. Fetal:placental weight ratios from WT and P0 mice from dams administered a subcutaneous injection of sildenafil citrate. Pregnant dams were allowed access to water with (N = 12) or without (N = 10) sildenafil citrate (10 mg·kg⁻¹). Individual fetuses (left), litter average for each group (right). Horizontal line denotes mean ± SEM.
4.2.4  Effect of a subcutaneous injection of SC on contraction responses of fetal abdominal aortas

SC had no effect on fetal abdominal aortic diameter when comparing aortas from SC-injected vs. saline-injected pregnancies except in P0 F i.e. a subcutaneous injection of SC increased the fetal abdominal aortic diameter when compared to P0 F saline-injected controls (table 4.3; $P<0.05$). There was no effect of SC on basal tone in any of the individual groups; the median basal tone for SC-treated groups ($N = 37$) was 29 (min 9 – max 54) mmHg. Median basal tone (min-max) for individual groups was; WT M 30 (17 – 38) mmHg, P0 M 28 (15 – 54) mmHg, WT F 29 (18 – 37) mmHg, P0 F 25 (9 – 35) mmHg.

Maximal contraction responses to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) were not significantly different between SC-treated and equivalent saline control group (table 4.3) except in the case of P0 F SC which had significantly reduced maximal contraction response to KPSS compared with P0 F saline controls ($P<0.01$). In the subcutaneous SC-treated group, fetal abdominal aortas had the greatest response to U46619, when compared with PE and KPSS.

U46619 dose response curves were constructed and are shown in figure 4.8. Comparisons were made to assess the effect of a subcutaneous injection of SC against the equivalent saline control group (e.g. WT M SC vs. WT M from saline control group). There was no effect of SC treatment on U46619-induced contraction of aortas from male mice of WT or P0 genotypes (figure 4.8A and 4.8C), but SC treatment did lead to a significant increase in sensitisation to U46619 in both WT M and P0 M mice (table 4.4, $EC_{50}; P<0.05$). WT F mice from SC-treated pregnancies had similar U46619-induced contraction when compared with WT F control aortas however; there was a significant reduction in contraction of P0 F fetal abdominal aortas from SC-treated groups when compared with P0 female saline controls. However, there were no differences in sensitivity to U46619 in P0 female fetal aortas from SC-treated pregnancies compared with those from saline treated pregnancies (table 4.4).

U46619-induced contraction was also expressed as a % of maximal KPSS contraction to account for small changes in vascular smooth muscle cell mass (figure 4.9). A subcutaneous injection of SC increased U46619-induced contraction (% KPSS) of WT fetal abdominal aortas from male (figure 4.9A; $P<0.05$) but not female mice (figure 4.9B). U46619-induced contraction (% KPSS) of fetal abdominal aortas was significantly increased in P0 mice of SC-treated pregnancies in females (figure 4.9D; $P<0.05$) but not males (figure 4.9C).
Table 4.3. Comparison of fetal abdominal aortic diameter, basal tone and max contraction from saline and sildenafil citrate (10 mg.kg\(^{-1}\))–injected pregnancies. KPSS (120 mM high potassium salt solution); PE (10\(^{-5}\) M phenylephrine); U46619 (2x10\(^{-6}\) M thromboxane-A2 mimetic). A denotes \(P<0.05\) and B denotes \(P<0.01\) when comparing saline control group with equivalent sildenafil citrate group. Data are shown as median [min-max]; number of fetuses per group in parenthesis.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Fetal ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
<th>Maximal Contraction (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KPSS</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT Male (10)</td>
<td>670 [550 - 762]</td>
<td>4.0 [2.3 – 5.3]</td>
<td>0.32 [0.13 – 0.99]</td>
<td>0.09 [0.03 – 0.29]</td>
</tr>
<tr>
<td>P0 Male (7)</td>
<td>632 [557 - 667]</td>
<td>3.3 [0.4 – 5.4]</td>
<td>0.38 [0.13 – 0.60]</td>
<td>0.12 [0.06 – 0.29]</td>
</tr>
<tr>
<td>WT Female (5)</td>
<td>644 [482 - 668]</td>
<td>2.8 [2.4 – 5.7]</td>
<td>0.41 [0.36 – 0.75]</td>
<td>0.06 [0.03 – 0.10]</td>
</tr>
<tr>
<td>P0 Female (11)</td>
<td>631 [491 – 680]</td>
<td>3.3 [2.6 – 5.9]</td>
<td>0.51 [0.04 – 0.74]</td>
<td>0.19 [0.03 – 0.41]</td>
</tr>
<tr>
<td>Sildenafil citrate (10mg.kg(^{-1}))</td>
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</tr>
<tr>
<td>WT Male (10)</td>
<td>640 [573 - 764]</td>
<td>3.9 [2.2 – 5.0]</td>
<td>0.26 [0.17 – 0.83]</td>
<td>0.03 [0.00 – 0.21]</td>
</tr>
<tr>
<td>P0 Male (13)</td>
<td>665 [539 - 771]</td>
<td>3.8 [2.0 - 7.2]</td>
<td>0.35 [0.12 – 1.16]</td>
<td>0.09 [0.00 – 0.30]</td>
</tr>
<tr>
<td>WT Female (7)</td>
<td>589 [482 - 668]</td>
<td>3.8 [2.4 – 4.9]</td>
<td>0.34 [0.21 – 1.24]</td>
<td>0.07 [0.00 – 0.37]</td>
</tr>
<tr>
<td>P0 Female (7)</td>
<td>677 [593 - 778]</td>
<td>3.4 [1.2 – 4.7]</td>
<td>0.15 [0.04 – 0.51]</td>
<td>0.03 [0.00 – 0.47]</td>
</tr>
</tbody>
</table>
Figure 4.8. Dose response curves of fetal abdominal aortas from in response to increasing doses of U46619 following a subcutaneous injection of saline or sildenafil citrate. Panel A-D; Pregnant dams were administered a subcutaneous injection of saline (N = 7) or sildenafil citrate (10 mg.kg⁻¹; N = 10). U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: Fetuses from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (blue solid curves). ****P<0.0001, **P<0.01.
Figure 4.9. Dose response curves of fetal abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following sildenafil citrate treatment. Panel A-D: Pregnant dams were administered a subcutaneous injection of saline (N = 7) or sildenafil citrate (10 mg·kg⁻¹; N = 10). U46619 dose response curves were compared using two-way ANOVA for treatment and U46619 concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: Fetuses from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (blue solid curves).
4.2.5 Effect of subcutaneous injection of SC on relaxation responses of fetal abdominal aortas

Aortas from SC-treated pregnancies were pre-contracted with an EC_{80} concentration of U46619 before ACH (endothelial-dependent, figure 4.10) and SNP (endothelial-independent, figure 4.11) induced relaxation was measured.

SC-treatment did not alter relaxation of fetal abdominal aortas in response to ACH in either WT M (figure 4.10A) or WT F mice (figure 4.10B). There was no effect of SC on relaxation responses of P0 M fetal abdominal aortas (figure 4.10C) but there was a significant reduction in sensitivity of P0 M fetal aortas from SC-treated pregnancies in response to ACH, compared with P0 M fetal aortas from saline-treated pregnancies (table 4.4, EC_{50}; P<0.01). There was also a significant reduction in relaxation response of P0 F fetal abdominal aortas (figure 4.10D; P<0.05) but no change in sensitivity to ACH (table 4.4).

A subcutaneous injection of SC did not alter relaxation responses of fetal abdominal aortas to SNP in either WT mice (figure 4.11A and 4.11B) or P0 mice (figure 4.11C and figure 4.11D).
Figure 4.10. Dose response curves of fetal abdominal aortas to increasing doses of ACH following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC$_{80}$ dose of U46619. ACH dose response curves were compared using two-way ANOVA for treatment and ACH concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: ACH; acetylcholine, fetuses from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg.kg$^{-1}$; blue solid curves).
Figure 4.11. Dose response curves of fetal abdominal aortas to increasing doses of SNP following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC$_{80}$ dose of U46619. SNP dose response curves were compared using two-way ANOVA for treatment and SNP concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of fetuses per group in parenthesis. Key: SNP; sodium nitroprusside, fetuses from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg.kg$^{-1}$; blue solid curves).
Table 4.4. Fetal abdominal aorta sensitivity to U46619, ACH and SNP following subcutaneous injection of saline or SC. Key: All data for WT / P0 fetuses in control and SC-treated mice expressed as effective concentration (EC in nM). A= increased U46619 sensitivity in SC-treated WT male vs. control WT male (EC$_{20}$, EC$_{50}$, EC$_{80}$; P<0.05; Mann-Whitney U Test). B= Increased U46619 sensitivity in SC-treated P0 male vs. control P0 male (EC$_{20}$, EC$_{50}$; P<0.05, EC$_{80}$; P<0.01, Mann-Whitney U Test). C= Reduced ACH sensitivity in SC-treated P0 male vs. control P0 male (EC$_{50}$; P<0.01, Mann-Whitney U Test). D= Reduced ACH sensitivity in SC-treated P0 male vs. control P0 male (EC$_{80}$; P<0.001, Mann-Whitney U Test). All data are mean±SEM with number of fetuses in parenthesis.

<table>
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<tr>
<th>Maternal Treatment</th>
<th>Agonist</th>
<th>Effective concentration</th>
<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
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<tr>
<td>Control</td>
<td>U46619</td>
<td>EC$_{20}$</td>
<td>27.4±6.6 (10)$^A$</td>
<td>25.8±8.0 (5)</td>
<td>32.2±13.4 (7)$^B$</td>
<td>17.9±6.6 (11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC$_{50}$</td>
<td>106±26 (10)$^A$</td>
<td>103±32.2 (5)</td>
<td>128.7±53.4 (7)$^B$</td>
<td>71.6±26.5 (11)</td>
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<tr>
<td></td>
<td></td>
<td>EC$_{80}$</td>
<td>439±105 (10)$^A$</td>
<td>413±129 (5)</td>
<td>515±214 (7)$^B$</td>
<td>286±106 (11)</td>
</tr>
<tr>
<td></td>
<td>ACH</td>
<td>EC$_{20}$</td>
<td>264±110 (8)</td>
<td>302±199 (4)</td>
<td>101±74.8 (7)</td>
<td>207±85.7 (8)</td>
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<tr>
<td></td>
<td></td>
<td>EC$_{50}$</td>
<td>447±112 (8)</td>
<td>492±182 (4)</td>
<td>159±90.9 (7)$^C$</td>
<td>570±166 (8)</td>
</tr>
<tr>
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<td></td>
<td>EC$_{80}$</td>
<td>1104±229 (8)</td>
<td>1088±115 (4)</td>
<td>275±93.9 (7)$^D$</td>
<td>2417±1201 (8)</td>
</tr>
<tr>
<td></td>
<td>SNP</td>
<td>EC$_{20}$</td>
<td>269±87 (9)</td>
<td>43.0±13.5 (5)</td>
<td>61.2±10.6 (7)</td>
<td>167±77.3 (11)</td>
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<tr>
<td></td>
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<td>EC$_{50}$</td>
<td>580±106 (9)</td>
<td>249±49.3 (5)</td>
<td>231±46.8 (7)</td>
<td>488±123 (11)</td>
</tr>
<tr>
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<td>EC$_{80}$</td>
<td>1156±127 (9)</td>
<td>1829±587 (5)</td>
<td>907±225.8 (7)</td>
<td>1589±337 (11)</td>
</tr>
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<td>Sildenafil citrate</td>
<td>U46619</td>
<td>EC$_{20}$</td>
<td>11.9±1.3 (10)$^A$</td>
<td>12.0±1.6 (7)</td>
<td>15.8±7.2 (13)$^B$</td>
<td>13.0±2.3 (7)</td>
</tr>
<tr>
<td>(10mg.kg$^{-1}$)</td>
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<td>EC$_{50}$</td>
<td>47.7±5.3 (10)$^A$</td>
<td>48.0±6.4 (7)</td>
<td>68.1±28.9 (13)$^B$</td>
<td>52.0±9.1 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC$_{80}$</td>
<td>191±21.3 (10)$^A$</td>
<td>192±25.7 (7)</td>
<td>253±116 (13)$^B$</td>
<td>208±36.3 (7)</td>
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<tr>
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<td>ACH</td>
<td>EC$_{20}$</td>
<td>539±166 (9)</td>
<td>359±125 (7)</td>
<td>308±104 (12)</td>
<td>479±186 (5)</td>
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<td>750±154 (9)</td>
<td>629±138 (7)</td>
<td>492±123 (12)$^C$</td>
<td>692±195 (5)</td>
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<td>EC$_{80}$</td>
<td>1718±488 (9)</td>
<td>1591±579 (7)</td>
<td>1040±270 (12)$^D$</td>
<td>1226±233 (5)</td>
</tr>
<tr>
<td></td>
<td>SNP</td>
<td>EC$_{20}$</td>
<td>143±28.2 (10)</td>
<td>62.1±25.4 (6)</td>
<td>122±50.0 (12)</td>
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<td>629±183 (10)</td>
<td>290±116 (6)</td>
<td>327±62.4 (12)</td>
<td>508±127 (7)</td>
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<td>3316±1381 (10)</td>
<td>1403±544 (6)</td>
<td>1134±101 (12)</td>
<td>2077±718 (7)</td>
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</table>
Table 4.5. Summary table demonstrating the effect of maternal sildenafil citrate treatment (10 mg.kg$^{-1}$) on fetal/placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619) and relaxation responses (ACH, SNP) for each group, compared with equivalent fetuses from saline-injected pregnancies. ↔ arrow denote no change, ↓ and ↑ arrows denote a decrease or increase, respectively in a parameter compared to saline control group e.g. ↔ denotes no change in U46619 constriction in WT males from sildenafil citrate treated pregnancies compared to WT male controls. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

<table>
<thead>
<tr>
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<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal weight</td>
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<tr>
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</tr>
<tr>
<td>Basal tone</td>
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</tr>
<tr>
<td>Aortic diameter</td>
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<td>↑</td>
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<td>Maximal KPSS contraction</td>
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<td>PE contraction</td>
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<td>U46619 contraction</td>
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<td>ACH relaxation</td>
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<td>ACH sensitivity (EC$_{50}$)</td>
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<td>↔</td>
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<tr>
<td>SNP relaxation</td>
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<tr>
<td>SNP sensitivity (EC$_{50}$)</td>
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Table 4.6. Summary table demonstrating the effect of route of administration of sildenafil citrate compared when compared with equivalent controls. The effect of dosing regimen (oral; 0.8 mg.ml\(^{-1}\) or subcutaneous injection; 10 mg.kg\(^{-1}\)) on fetal / placental weight, basal tone, abdominal aorta diameter, contraction (KPSS, PE, U46619) and relaxation responses (ACH, SNP) can therefore be compared. ↔ arrow denote no change, ↓ and ↑ arrows denote a decrease or increase, respectively, in a parameter compared to control group. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

<table>
<thead>
<tr>
<th></th>
<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oral</td>
<td>Subcut</td>
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<td>Subcut</td>
</tr>
<tr>
<td>Fetal weight</td>
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<td>↔</td>
</tr>
<tr>
<td>Placental weight</td>
<td>↔</td>
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</tr>
<tr>
<td>Basal tone</td>
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<tr>
<td>Aortic diameter</td>
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<td>↔</td>
</tr>
<tr>
<td>Maximal KPSS contraction</td>
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<td>↔</td>
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<tr>
<td>PE contraction</td>
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<td>↔</td>
</tr>
<tr>
<td>U46619 contraction</td>
<td>↔</td>
<td>↔</td>
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<td>↔</td>
</tr>
<tr>
<td>U46619 sensitivity (EC(_{50}))</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>ACH relaxation</td>
<td>↓</td>
<td>↔</td>
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<tr>
<td>ACH sensitivity (EC(_{50}))</td>
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<td>SNP relaxation</td>
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<tr>
<td>SNP sensitivity (EC(_{50}))</td>
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</tbody>
</table>
4.3 Discussion

A subcutaneous injection of 10 mg.kg\(^{-1}\) of SC did not increase fetal / placental weight of growth restricted fetuses in the P0 mouse model of FGR. Fetal aortic vascular function was assessed in pups at E18.5 using wire myography. SC caused a profound decrease in contraction responses of growth restricted females to U46619 and high potassium depolarising solution, but did not affect contraction in males or WT females. SC blunted the endothelial-dependent relaxation of fetal aortas from growth restricted females but did not affect males or WT females. SC did not elicit any effects on endothelial-independent relaxation of fetal aortas. A daily sham injection of saline in the dam between E12.5 and E17.5 did not alter fetal or placental weight when indirectly compared with WT and P0 fetal weights from oral untreated pregnancies. In addition, a subcutaneous injection of saline did not seem to alter fetal vascular function when sex and genotype differences were assessed.

4.3.1 P0 mouse phenotype from saline-injected pregnancies

Results from chapter 3 of this thesis and other studies (Constância et al., 2002, Dilworth et al., 2013) have demonstrated a reduction in fetal and placental weight and increase in fetal:placental weight ratio at E18.5 in P0 mice when compared to WT mice. The current study, using 10 litters, demonstrated P0 fetuses (expressed as mean±SEM, grams) from dams administered a subcutaneous injection of saline were significantly lighter than WT fetuses (1.04±0.02 and 1.22±0.02, respectively) and had reduced placental weight compared with their WT littermates (0.067±0.008 vs 0.098±0.004, respectively).

In a previous study, mean WT and P0 fetal weight from 23 litters were 1.20 grams and 0.94 grams, respectively (Dilworth et al., 2013). An earlier study also demonstrated mean P0 fetal weight at E18.5 from 8 litters was 0.89 grams (Constância et al., 2002). Thus, although WT fetal weight data are similar between the data from saline-injected pregnancies and by Dilworth and colleagues (1.22 and 1.20 grams, respectively) there seems to be a subtle increase in P0 mean fetal weight in the current study to what has recently been reported by Dilworth and colleagues (i.e. 1.04 vs 0.94 or a 10 % increase). In the study by Dilworth et al., 75 % of P0 mice had a fetal weight which was below the 5\(^{th}\) percentile of WT fetal weights (Dilworth et al., 2013) which is similar to the 70 % of P0 mice below the 5\(^{th}\) percentile of WT fetal weights in the current study.

Placental weight in the P0 mouse was previously noted to be between 0.065 (Dilworth et al., 2013) and 0.077 grams (Constância et al., 2002) with WT mouse placental weight between 0.092 (Dilworth et al., 2013) and 0.112 grams (Constância et al., 2002). Both studies demonstrated a significant reduction in placental weight in P0 vs. WT mice at E18.5. Similarly, the data from
saline-injected pregnancies reveal a mean placental weight of 0.067 grams in P0 mice which was significantly lighter than WT saline control placental weight (0.098 grams). In support of previous findings, the fetal:placental weight ratio was also significantly increased in P0 mice when compared with WT littermates (14.97±0.8 and 12.99±0.5 arbitrary units, respectively). Therefore, a subcutaneous injection of saline does not affect P0 placental weight or the number of P0 mice with a fetal weight below the 5th percentile of WT mouse fetal weight. The mean P0 fetal weight from the current study is at least 10 % larger than previously reported (Dilworth et al., 2013, Constância et al., 2002). It could be that a subcutaneous injection of saline increases P0 fetal weight; however, this is unlikely as there are no changes in P0 or WT placental weight or WT fetal weight. Therefore, further interpretation of effects of SC on P0 fetal weight may be difficult as the difference between WT and P0 fetal weight in the current study is less pronounced compared with what other studies have reported (Dilworth et al., 2013, Constância et al., 2002; see section 4.1.5). However, it is important to reiterate that the previous study which assessed the effect of SC in P0 mice utilised significantly more animals (N = 23 vs. N = 10, respectively) and was adequately powered to assess the effect of genotype on fetal weight.

4.3.2 Myography studies of mouse fetal abdominal aortic function from saline-injected pregnancies

Fetal aortic vascular function was assessed at E18.5 to determine whether there were sex- and genotype-specific differences in contraction and relaxation responses. Using an adapted classical normalisation method (0.9 of L50kPa) the median basal tone of isolated fetal abdominal aortas was 25 mmHg. At a similar stage of human fetal development (20 weeks gestation) fetal aortic blood pressure was estimated to be 28 mmHg (Struijk et al., 2008); similar data were seen with dams treated with SC / water controls.

4.3.2.1 Potassium-, phenylephrine- and U46619-induced contraction of fetal abdominal aortas from appropriate weight and growth restricted fetuses whose mothers were injected with saline during pregnancy

There were no differences between the four saline control groups (WT M, P0 M, WT F, P0 F) in aortic diameter, basal tone or maximal contraction responses to KPSS, PE or U46619. Maximal contraction in response to U46619 was consistently greater than in response to either KPSS or PE. In the isolated fetal abdominal aortas U46619-induced contraction was not different amongst the four saline control groups when genotype and sex were taken into account (see table 4.2). This is in contrast to a study by Karanian and colleagues which demonstrated that U46619-induced contraction was significantly reduced in female vs. male adult rat aortas; although, differences
were age-dependent and became more pronounced in older animals (Karanian et al., 1981). The mechanism for increased U46619 sensitivity in males vs. females is thought to be due to an increased expression of thromboxane-A$_2$ receptor in vascular smooth muscle cells of males (Higashiura et al., 1997). Testosterone is able to increase thromboxane-A$_2$ receptor expression in vitro (Masuda et al., 1991) and increased the contraction response to U46619 in female carotid arteries incubated with testosterone from canines (Karanian and Ramwell., 1996). Therefore, it may be that male and female mouse fetal aortas do not show differences in sensitivity or maximal contraction in response to U44619 as testosterone concentrations in the male fetus may not be sufficient enough to increase thromboxane-A$_2$ receptor expression. In support of this, a recent study by Poling and Kauffman demonstrated that C57BL/6J male mice have ~40 ng.dl$^{-1}$ of serum testosterone at birth but that during adulthood testosterone concentration is increased to ~120 ng.dl$^{-1}$ (Poling et al., 2012) which suggests that at lower concentrations of testosterone at E18.5 (40 ng.dl$^{-1}$) there may be less pronounced sex-specific differences in U46619-induced contraction. When fetal aortic contraction data were expressed as U46619-induced responses as a % of KPSS contraction, P0 F had increased response compared with WT F, which may suggest greater smooth muscle cell mass in P0 F abdominal aortas compared with WT F fetal aortas. However, there were no significant differences in EC$_{50}$ values between WT F and P0 F fetal aortas when expressed as U46619-contraction as % KPSS contraction and nor where there any differences in maximal contraction to KPSS. These data may suggest a lack of biological significance of this result.

4.3.2.2 Acetylcholine- and sodium nitroprusside-induced endothelial-dependent and -independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses whose mothers were injected with saline during pregnancy

Endothelial-dependent ACH-induced relaxation responses of isolated fetal aortas were not different when comparing the effect of genotype and sex separately. ACH (10$^{-5}$ M) induced 40 to 60 % average relaxation in WT M, P0 M, WT F and P0 F, suggestive of an appropriately functioning endothelium with little or no damage in the dissection and mounting processes. A study assessing the effect of hypoxia on rat fetal vascular function previously demonstrated that rat fetal aortas from normoxic dams, which were dissected at E20 and mounted on the wire myograph, relaxed up to 80 % with methacholine after pre-contraction with high potassium solution (Camm et al., 2010). A later study by Herrera and colleagues also reported that rat fetal abdominal aortas pre-contracted with a high potassium solution demonstrate 40 – 50 % relaxation to 10$^{-5}$ M methacholine (Herrera et al., 2012). Relaxation responses to SNP from U46619 pre-contracted fetal abdominal aortas were not different in any of the four saline control groups (WT M, P0 M,
WT F, P0 F). Data within this chapter demonstrated SNP induced between 40 to 60 % relaxation at maximal concentrations of ACH (i.e. $10^{-5}$ M). A relaxation response of 60 - 70 % at the equivalent concentrations of SNP has been demonstrated in pre-contracted rat fetal abdominal aortas (Herrera et al., 2012). Therefore, although not directly comparable due to the differences in pre-contraction agonists (i.e. high potassium solution-induced pre-contraction [Camm et al., 2010, Herrera et al., 2012] vs. U46619-induced pre-contraction in this thesis), fetal aortic responses in mice at E18.5 show similar endothelial- dependent and -independent relaxation responses compared with rat fetal aortas at E20.

4.3.3 Differential effects of maternal exposure to a saline-injection or to water on fetal vascular function

Previous studies have demonstrated that pre-natal exposure to stress, via a maternal injection of saline can induce long-term behavioural changes in rat offspring (Grimm et al., 1987, Slamberova et al., 2002). Therefore, it seems prudent to relate the effects of a daily injection of saline in the dam (E12.5 – E17.5) with the effect of ad libitum access to water (without injection or handling) on fetal outcome such as fetal / placental weight and fetal vascular function. Although no direct comparisons can be made between water controls and saline-injected controls due to the timing of experiments, similarities and differences will now be discussed between the two control groups (i.e. water vs. saline injection; see summary table 4.2).

In both saline-injected and water control pregnancies, there were significant reductions in P0 fetal and placental weight compared with controls (table 4.2). In water control pregnancies (N = 10 litters) WT and P0 fetal weight (expressed as mean±SEM, grams) was 1.21±0.02 and 1.01±0.02. In saline-injected pregnancies (N = 10 litters) WT and P0 fetal weight was 1.25±0.02 and 1.05±0.03, respectively. Thus, an injection with saline in the pregnant dam does not seem to significantly alter fetal weight in either WT or P0 fetuses when compared to fetuses from pregnant dams given water ad libitum. In water control pregnancies, WT and P0 placental weight was 0.105±0.003 and 0.079±0.002, respectively. In saline-injected pregnancies WT and P0 placental weight was 0.098±0.004 and 0.067±0.008. Thus, there is also no significant effect of a daily subcutaneous saline injection to the dam on placental weight. In both saline-injected and water control pregnancies, basal tone, aortic diameter, maximal contraction in response to KPSS and PE were not different when assessing for sex- and genotype-specific effects (table 4.2).

There were no differences in fetal aortic responses to U46619 in saline-injected pregnancies when comparing sex and genotype. In contrast, P0 M fetal aortas from water control pregnancies had significantly increased U46619-induced contraction and WT F fetal aortas had significantly decreased U46619-induced contraction compared to WT M mice. The reason for discrepancies in
sex- and genotype-specific U46619-induced contraction between the two control groups could be due to the fact that maximal contraction to U46619 seems to be higher in the saline treated group (table 4.1; average maximal contraction to U46619 from WT M, P0 M, WT F and P0 F mice = 1.45 kPa) compared with the water treated group (table 3.1; mean maximal contraction to U46619 from WT M, P0 M, WT F and P0 F mice = 0.89 kPa). Recent data from Nugent and colleagues has demonstrated that exposure to glucocorticoids, which are increased in cases of stress, led to an increased contraction of chorionic plate arteries in response to U46619 in normal pregnancies (Nugent et al., 2013). It may be that in saline injected dams, raised concentrations of glucocorticoids could contribute to an increased maximal contraction of mouse fetal aortas when compared with fetal aortas from water control animals. Further work assessing the effect of a saline injection during pregnancy on the concentration of glucocorticoids in maternal / fetal plasma would be of interest.

In saline injected pregnancies, there were no differences in fetal aortic responses when assessing the effect of sex and genotype on endothelial-dependent relaxation (figure 4.3 and table 4.2). In contrast, in the water control fetal aortas, relaxation in P0 M was reduced compared with relaxation responses in WT M and P0 F (figure 3.6 and table 4.2). The average maximal % relaxation responses to ACH in fetuses from saline injected pregnancies were between 40 – 60 %, whereas fetal aortas from water control pregnancies demonstrated average maximal % relaxation response to ACH of approximately 60 - 90 % (see figure 4.3 and figure 3.6). There were no sex- or genotype-specific differences in endothelial-independent relaxation of fetal aortas in either the saline control group or water control group. However, average maximal relaxation responses in fetal aortas from the water control group were between 60 – 90 % and from the saline control group were between 40 – 60 % (see figure 4.4 and figure 3.7). The reason as to why fetal aortas from saline injected pregnancies have seemingly reduced relaxation responses to ACH and SNP when indirectly compared with fetal aortas from water control pregnancies is unknown but there are at least two possibilities. Firstly, these two studies were not performed in parallel and thus variations could be due to an alteration in phenotype by genetic inbreeding of the P0 mouse. Secondly, mild maternal stress with a daily subcutaneous injection of saline may induce changes in fetal vascular smooth muscle and endothelial cell function. To elucidate whether this effect is due to genetic inbreeding it would be useful to repeat earlier studies using water control animals to see if fetal responses were identical in WT and P0 fetal aortas. This may also suggest whether reduced maximal endothelial-dependent and –independent relaxation in fetal aortas of saline injected pregnancies is a consequence of maternal stress.

4.3.4 Effect of maternal sildenafil citrate treatment via sub cutaneous injection in appropriate weight and growth restricted fetuses
Pregnant dams were administered a subcutaneous injection of 10 mg.kg⁻¹ SC to determine whether SC could increase fetal / placental weight in the P0 mouse. SC has previously increased fetal weight in two mouse models of FGR (Dilworth et al., 2013, Stanley et al., 2012). In the P0 mouse model of FGR SC was able to significantly increase P0 fetal weight (Dilworth et al., 2013). SC treatment did not affect P0 fetal weight (expressed as mean±SEM, grams) when compared to P0 saline fetal weight (1.04±0.01 vs. 1.04±0.02, respectively). Treatment with SC did not alter P0 placental weight when compared to P0 saline placental weight (0.72±0.004 vs. 0.67±0.008, respectively) nor did SC treatment alter fetal:placental weight ratios in P0 mice compared to P0 saline mice (15.07±0.8 vs. 14.97±0.8, respectively). A subcutaneous injection of 10 mg.kg⁻¹ SC in the dam did not increase the number of resorptions or reduce the litter size in the P0 mouse. In the recent study by Dilworth and colleagues, mean fetal weight of P0 mice was 0.94 grams, SC treatment (0.4 mg.ml⁻¹) administered to the drinking water of 19 pregnant dams significantly increased P0 fetal weight to 1.04 grams. In this chapter, P0 saline mean fetal weight was 1.04 grams and a subcutaneous injection of SC (10 mg.kg⁻¹) to 12 pregnant dams did not alter P0 fetal weight (1.04 vs. 1.04 grams, respectively). In addition, Dilworth and colleagues demonstrated that 75 % of P0 fetal weights were lower than the 5th percentile of WT water control fetal weights and that SC reduced this to value to 51 %. In the current study, 70 % of P0 mice presented with a fetal weight below the 5th percentile of WT saline fetal weight. SC did not affect the percentage of P0 mice with a fetal weight below the 5th percentile of WT saline fetal weight. Collectively, the current data presented in this chapter suggests that growth restriction in this smaller cohort (N = 12 litters) of P0 mice is not as severe as which has previously been reported by Dilworth and colleagues (N = 19 litters) and that perhaps SC is more effective in fetuses that are the most severely growth restricted.

Treatment with SC in the drinking water of the dam was previously shown to increase placental weight in the COMT⁻¹ mouse model of FGR (Stanley et al., 2012). There was also a subtle increase in mean P0 placental weight (Dilworth et al., 2013) from dams which were given access to SC in water (0.4 mg.ml⁻¹) compared with water controls (0.072±0.002 vs. 0.065±0.002, respectively, P=0.056). In contrast, using a subcutaneous injection of 10 mg.kg⁻¹ SC, there was no change in P0 placental weight when compared to P0 saline placental weight. In addition, there were no changes in fetal:placental weight ratio in P0 mice when comparing treated with untreated controls. Thus, previous studies suggest it is likely that SC treatment is increasing P0 fetal weight (Dilworth et al., 2013, Stanley et al., 2012) through increasing placental size (and presumably function) and that the concentration of SC (10 mg.kg⁻¹) or the route of administration to the dam is unsuitable to elicit the beneficial increase on placental and fetal weight.
4.3.5 Effect maternal sildenafil citrate treatment via subcutaneous injection on fetal abdominal aortic function in appropriate weight and growth restricted fetuses.

The effect of a subcutaneous injection of 10 mg.kg$^{-1}$ to the dam on fetal vascular function was also assessed. When isolated fetal abdominal aortas were assessed from SC-treated pregnancies the median basal tone was 29 mmHg, which was not significantly different to the overall median basal tone of fetal aortas from saline controls (25 mmHg). Fetal abdominal aortas from the SC treatment group were maintained at a basal tone similar to that seen at 20 weeks gestation (28 mmHg) in the human (Struijck et al., 2008).

4.3.5.1 Effect of maternal sildenafil citrate treatment via subcutaneous injection on potassium-, phenylephrine- and U46619- induced contraction of fetal abdominal aortas from growth restricted and appropriate weight fetuses

SC treatment had no overall effect on fetal abdominal aortic diameter (except in P0 females where there was a small but significant increase, $P = 0.04$), basal tone or maximal contraction responses to PE or U46619 when compared to relevant saline control groups. P0 F fetal aortas from dams injected with SC had reduced KPSS-induced contraction compared with P0 F fetal aortas from dams injected with saline (see table 4.5). An increase in intraluminal diameter of the P0 F fetal aorta of SC treated pregnancies was unexpected as one would expect similar intraluminal diameters when vessels are set at a standard pressure. However, upon closer inspection of the data, an outlier (> 2 standard deviations from the mean) was found in the P0 F saline group (diameter 491 µm) which may be the reason for differences in vessel diameter in P0 F from saline- and SC-injected pregnancies. Vascular contraction and relaxation responses of this particular vessel were indicative of a fully functioning endothelium and vascular smooth muscle cell and therefore these data were included within the results. Reduced KPSS-induced contraction of P0 F fetal aortas compared with P0 F fetal aortas from saline injected dams may suggest SC is altering the entry of calcium into the smooth muscle cell (upon depolarisation with high potassium solution), that entry of calcium into the smooth muscle cell is normal but that there is reduced sensitivity to calcium in the smooth muscle or that smooth muscle cell content is reduced.

U46619-induced contraction of fetal abdominal aortas from subcutaneous SC-treated pregnancies was similar when compared with saline controls; except in the case of P0 females (figure 4.8 and table 4.5). U46619-induced contraction was significantly reduced in P0 F mice from SC-treated pregnancies which gives further support to the argument that P0 F fetal aortic vascular smooth muscle cell function is altered in response to SC treatment; although, the effective concentration
of U46619 required to elicit 50 % and 80 % contraction in P0 F fetal aortas from SC treatment was not altered when compared with P0 F from saline treated pregnancies. When U46619-induced contraction was expressed as a % of maximal KPSS-induced contraction there were subtle but significant increases in both WT M and P0 F fetal aortic contractions in SC treated groups compared with WT M and P0 F saline groups, respectively (figure 4.9). Given that there are no differences in U46619-induced contraction in WT M fetal aortas between SC and saline-treated groups, the increase in U46619-induced contraction as a % KPSS contraction suggests there may be reduced smooth muscle cell mass in WT M fetal aortas from SC-treated pregnancies (figure 4.8A and figure 4.9A). In contrast, P0 F fetal aortas from SC-treated pregnancies demonstrated reduced U46619-induced contraction but an increased U46619-induced contraction when expressed as a % of KPSS contraction compared with P0 F fetal aortas from saline injected pregnancies. This suggests that, P0 F fetal aortas, in addition to altered vascular smooth muscle cell function, may have reduced vascular smooth muscle cell mass. A limitation of the current study was that there was no morphological assessment of mouse fetal aortas which has been previously performed in rat fetal aortas at E20 (Herrera et al., 2012, Camm et al., 2010). This would have proven useful in determining whether there were alterations in the density of smooth muscle as a percent of total fetal aortic area.

4.3.5.2 Effect of maternal sildenafil citrate treatment via subcutaneous injection on acetylcholine- and sodium nitroprusside-induced endothelial-dependent and -independent relaxation of fetal abdominal aortas from growth restricted and appropriate weight fetuses

Pre-contracted fetal abdominal aortas from dams treated with SC demonstrate maximal relaxation responses of 50 – 60 % in response to 10⁻⁵ M ACH. There were no differences in endothelial-dependent relaxation of fetal aortas from SC-treated and saline-treated pregnancies except in the case of P0 F from SC-treated pregnancies which had significantly reduced maximal endothelial-dependent relaxation; however sensitivity to ACH was not altered. This suggests that P0 F fetal aortas from SC-treated pregnancies may have impaired endothelial function through, as yet, unknown mechanisms.

SNP stimulated 50 – 70 % maximal relaxation in fetal aortas from SC-treated pregnancies that were pre-contracted with U46619 (figure 4.11). There were no differences in relaxation responses of fetal aortas from SC-treated pregnancies from any of the experimental groups when compared with fetal aortas from saline treated pregnancies. Although endothelial-dependent relaxation is impaired in P0 F fetal aortas from SC-treated pregnancies, direct addition of NO and thus, endothelial-independent relaxation, was not altered when compared with P0 F fetal aortas from
saline treated pregnancies. P0 F fetal aortas from SC treated pregnancies therefore have reduced contraction responses in the vascular smooth muscle and dysfunction in endothelial-dependent but not –independent relaxation. Given that oral and subcutaneous antenatal administration of SC seems to affect fetal abdominal aortic vascular function, it will be important to assess the effects of antenatal SC on vascular function in those dams treated with SC.

4.3.6 Differential effects of maternal exposure to a sildenafil citrate-injection or to sildenafil citrate in drinking water on fetal vascular function

There were observable differences in fetal aortic function in dams that were treated with SC in the drinking water (0.8 mg.ml\(^{-1}\)) compared to subcutaneous injection (10 mg.kg\(^{-1}\); see table 4.4). It is important to note that it is not possible to tell whether these differences are due to the dosage of SC and / or the route of administration.

The concentration of SC used in small scale clinical trials and cohort studies for pregnancy complications has not yet been standardised. In 2009, Samangaya and colleagues led a small scale clinical trial which administered up to 240 mg daily (orally) to try to prolong pregnancies associated with early onset PET (Samangaya et al., 2009). SC was not able to prolong gestation or increase fetal weight when reaching concentrations of 271 ng.ml\(^{-1}\) in maternal blood. In addition, vascular function of omental and myometrial arteries was assessed from women who had participated in the clinical trial. SC did not alter vasoconstriction or vasorelaxation in either omental or myometrial small arteries when compared to those from SC-naïve mothers (Samangaya et al., 2011). The authors suggested that acute effects of SC on vascular responses may not be evident as the time since sildenafil administration was many hours prior to in vitro myography experiments. A small cohort study also assessed the effects of SC on severe early onset FGR with the primary aim of increasing fetal growth (Von Dadelszen et al., 2011). Women were given 75 mg SC daily (orally) which increased abdominal growth velocity in fetuses from the SC-treated but not SC-naïve mothers. These two small scale intervention studies demonstrate that it is difficult to determine whether the dosage of SC is high enough and whether SC was administered at the appropriate time. Therefore, a number of animal models have used a variety of SC concentrations and routes of administration to try to ascertain optimal regimens for SC administration.

In mice, addition of SC to the drinking water of the dam at concentrations between 0.2 mg.ml\(^{-1}\) and 0.4 mg.ml\(^{-1}\) from E12.5 to E18.5 can significantly increase fetal weight in two separate mouse models of FGR (Dilworth et al., 2013, Stanley et al., 2012). However, administration of 15 mg.kg\(^{-1}\) of SC by gavage from E14 to E21 in a rat model of FGR (induced by inhibiting nitric oxide synthase) augmented a reduction in pup birth weight when compared with untreated FGR pups (Nasser et
Therefore in the current study, the lack of effect of 10 mg.kg\(^{-1}\) SC on increasing fetal weight in the P0 mouse compared with studies performed in mouse (Dilworth et al., 2013, Stanley et al., 2012) and in human FGR (Von Dadelszen et al., 2011) could be due to differences in concentration of SC and routes of administration. It is also important to reiterate that the rate of metabolism of SC in the mouse is increased compared with the human (Walker et al., 1999).

In both human and mouse, the major metabolite of SC is N-desmethyl-sildenafil; the effects of N-desmethyl-sildenafil in mouse vs. human are unknown. The elimination half-life of N-desmethyl-sildenafil in man is similar to SC (approximately 2.3 hours) but the elimination half-life in rodents is relatively longer than the parent compound. Whether N-desmethyl-sildenafil or any SC metabolites are able to cross the placental barrier and promote growth in the fetus has yet to be determine in either mice or humans. In fact, there are relatively few studies at all (all in animals) which directly demonstrate that SC alone crosses the placental barrier and has direct effects on the fetus (Luong et al., 2011, Pellicer et al., 2011). Thus, it will be important to assess the concentration of SC and the major metabolite in the dam and the fetus after subcutaneous injection of 10 mg.kg\(^{-1}\). This will determine whether SC / N-desmethyl-sildenafil are able to cross the placenta and enter fetal circulation and whether concentrations in the mouse maternal / fetal plasma are comparable to maternal / fetal SC concentrations from previous (Samangaya et al., 2009) and current (Ganzevoort et al., 2014) clinical trials. In the P0 mouse, maternal and fetal plasma samples were taken at 1, 6 and 24 hours post subcutaneous injection with 10 mg.kg\(^{-1}\) SC. At the time of writing, these samples are in the process of being analysed by our group's collaborators in New Zealand using high performance liquid chromatography; such experiments will provide information on how well a 10 mg.kg\(^{-1}\) injection of SC in mouse can match earlier human studies demonstrating maximum SC concentrations of 271 ng.ml\(^{-1}\) in maternal blood (Samangaya et al., 2009).

**4.3.7 Mechanisms of action of sildenafil citrate**

Irrespective of route of administration, effects of SC treatment are most prominent on female fetuses that would have been born growth restricted (P0) and had adequate weight at E18.5 (WT) (table 4.6). In females, a subcutaneous injection of SC in the dam caused reduced contraction of fetal aortas in response to U46619 in P0 mice, whereas an oral dose of SC caused reduced contraction of fetal aortas in response to U46619 in WT mice. A subcutaneous injection of SC in the dam also caused reduced endothelium-dependent relaxation of P0 fetal aortas in female mice, whereas oral SC led to a marked reduction in endothelial-dependent relaxation of both WT and P0 fetal aortas in female mice. In addition, there was a significant reduction in endothelial-independent relaxation of fetal aortas in WT and P0 female mice from the oral SC study (chapter
3, figure 3.13B and D). Effects of SC on male fetal vascular function were less pronounced compared with effects on females. In male fetuses, oral administration of SC did lead to a reduction in endothelial-dependent relaxation in aortas from WT but not P0 mice. Endothelial-independent relaxation in P0 (but not WT) male mice was blunted in response to maternal oral administration of SC. In general, oral administration of SC had greater effects on fetal vascular function than subcutaneous administration of SC but there were reductions in contraction / relaxation responses in both dosing regimens (table 4.6).

The mechanism(s) associated with the sex-specific effects of SC are unknown but these data warrant further investigation. In response to an adverse environment, such as maternal glucose intolerance, there is a greater incidence of macrosomia (Ricart et al., 2009) and stillbirth (Engel et al., 2008) in male compared with female fetuses. In addition, male fetuses in from mild PET pregnancies demonstrate normal fetal growth but females reduce their growth, possibly to enable females to survive additional insults in utero (Stark et al., 2009). Thus, these studies highlight that male and female fetuses adapt differently to environmental insults and that females may adapt to reduce growth and survive further insults whereas males adapt to increase growth and risk additional insults such as reduced nutrient or oxygen supply. This could be the reason for the greater effects of SC on female fetal vascular function. Excess NO bioavailability, and presumably greater vasodilatation of the vasculature, could be deemed an adverse environment and could lead to females down regulating molecules associated with the NO / cGMP mediated pathways (e.g. GC) such that, when isolated fetal aortas are assessed using wire myography, responses to endothelial-dependent and –independent vasodilators are reduced to a greater extent in females vs. males. If true, one would perhaps expect a down regulation of these pathways may lead to long-term alterations in offspring vascular function.

In addition to sex-specific effects, there were differential effects of SC dependent on the route of administration to the dam. Based on the 0.8 mg.ml⁻¹ SC dosing regimen, a pregnant dam weighing between 30 and 38 grams drinking 6 ml of water per day would have a daily dose of 120 – 160 mg.kg⁻¹ of SC. Clearly, this is a much larger dose than the daily subcutaneous injection of 10 mg.kg⁻¹ SC, but it is important to remember that a dosage of 10mg.kg⁻¹ SC given subcutaneously will have a greater bioavailability than that of an equivalent oral dose. Additionally, maternal and fetal plasma concentration of SC from an oral dosage will be much more variable and will depend on water consumption of the mother. In contrast, a subcutaneous injection of SC will lead to a peak concentration of SC over 6 hours and will be eliminated from the body within 24 hours (as seen in pregnant rats; Luong et al., 2011). It is likely that SC metabolites will have effects on mother and fetus and that there will be differences in concentrations of metabolites between the oral and subcutaneous dosing regimens. Additionally, fetuses from both oral and subcutaneous SC dosing
Regimens were harvested at E18.5 however; those from the oral SC dosing regimen had free access to water up until time of culling, whereas the last subcutaneous injection of SC was administered to dams at E17.5 (i.e. 24 hours before). Therefore, it is likely that the effects of SC exposure are more pronounced in the oral SC study as the time between fetal exposure to SC and wire myography experiments is much shorter compared with the subcutaneous SC study.

4.3.8 Summary

The data from within this chapter provide further evidence that at E18.5 P0 fetal and placental weight is significantly reduced compared to WT fetal weight. A daily subcutaneous injection of saline (E12.5 – E17.5) did not alter WT or P0 fetal / placental weight when compared with WT / P0 fetal weights from water control pregnancies. Sex-specific differences in U46619-induced contraction responses of male and female mice were not evident in fetal aortas from mothers who had been injected with saline from E12.5 to E17.5 but were evident in WT male and female fetuses from water control pregnancies.

Subcutaneous administration of 10 mg.kg\(^{-1}\) SC to the P0 mouse model of FGR did not increase fetal or placental weight in growth restricted fetuses. When assessing vascular function, SC administration significantly reduced contraction and endothelial-dependent relaxation of fetal aortas from growth restricted females. These effects show similarities with the effects of a supratherapeutic oral dosage of SC on fetal aortas from both WT and P0 female mice, suggesting females may either adapt pathways associated with control of vascular contraction / relaxation in response to SC, or that females are more susceptible to damage of the endothelium / vascular smooth muscle. Whether alterations in vascular function at E18.5 persist into adulthood is unknown. Therefore, it will be important to determine whether growth restriction or exposure to SC in utero permanently alters vascular function in offspring and whether such alterations contribute to disease in later life.
Chapter 5  Long-Term Effects of Antenatal Sildenafil Citrate Treatment on Offspring Health
5.1 Introduction

Small body size at birth, whether pathological or not, can lead to adulthood diseases such as ischaemic heart disease (Barker et al., 1989), hypertension (Barker et al., 1990, Barker et al., 1992) and non-insulin dependent diabetes (Phillips et al., 2005). Low birth weight has also been previously associated with endothelial dysfunction in early childhood (Franco et al., 2006) and adulthood (Leeson et al., 2001). In support of this, children with impaired endothelial function also demonstrated an increased systolic blood pressure as early as 8 years of age, when compared with normal birth weight children, independent of sex (Franco et al., 2006). Therefore, it has been hypothesised that endothelial dysfunction, as a result of poor growth in utero, is the mechanism for increased risk of adulthood hypertension in individuals who had a low birthweight. Animal studies have also demonstrated impaired endothelial function is often more pronounced in male vs. female offspring (Ozaki et al., 2001, Franco et al., 2002). In addition, hypoxia (Hemmings et al., 2005) and maternal undernutrition (Kwong et al., 2000) cause impaired vascular reactivity and increased blood pressure, respectively, in male but not female offspring. Therefore, assessing sex differences in fetal programming of adult disease in the Igf2 P0+/− mouse model of FGR will be informative.

Treatments which increase fetal weight and which may reduce or abolish the risk of disease associated with intrauterine programming would be beneficial in identifiable cases of FGR. Most research has focussed on using genetic manipulation, maternal dietary restriction and maternal hypoxia in rodents to induce FGR and promote long-term programming of adulthood cardiovascular and metabolic dysfunction. Vitamin C (Kane et al., 2013, Giussani et al., 2012) and folate (Torrens et al., 2006) supplementation both reduce the long-term programming effects of small size at birth in animal models of FGR. Thus, although a subcutaneous injection of a 10 mg.kg⁻¹ of SC did not alter fetal weight (see chapter 4) it could be that SC reduces the putative long-term programming effects associated with small size at birth in the Igf2 P0+/− mouse model of FGR.

It could also be true that antenatal SC treatment may induce or exacerbate adulthood disease associated with low birth weight. SC did result in a reduced U46619-induced contraction and reduced endothelial-dependent relaxation of P0 female fetal aortas at E18.5. SC was also associated with an increase in sensitivity of fetal aortas to U46619 from both WT male and P0 male mice, suggesting effects of SC may not be only confined to growth restricted fetuses. Thus it is possible that, although there was no effect of a lower concentration of SC on fetal weight but effects on endothelial function, SC may actually lead to intrauterine programming that could result in cardiovascular and metabolic dysfunction in WT and P0 offspring. Given the fact that there is currently a multicentre international clinical trial assessing the beneficial effects of SC
treatment in cases of severe early-onset FGR (Ganzevoort et al., 2014), understanding the effects of antenatal SC on the offspring health is imperative. It may also be the case that a reduction in contraction of P0 F fetal aortas at E18.5 could be a beneficial effect of SC specifically on growth restricted females which could prevent growth restricted female offspring from developing adulthood disease. Therefore, in this chapter, WT and P0 offspring phenotypic observations were made to assess both the effect of small size at birth and the effect of a subcutaneous injection of SC during pregnancy in offspring.

The objectives of this chapter were to determine whether;

- In the P0 mouse model of FGR, offspring that are growth restricted at birth show sex-specific differences in growth, glucose tolerance, systolic blood pressure and / or arterial function in adulthood, compared to offspring that are of normal birth weight.
- SC treatment has beneficial or detrimental effects on postnatal growth, glucose tolerance, systolic blood pressure and / or arterial function in adulthood, compared to offspring from dams treated with a subcutaneous injection of saline.
5.2 Results

5.2.1 Effect of an antenatal subcutaneous injection of Sildenafil citrate on expected: observed ratios of fetuses and offspring

Figure 5.1 shows the observed ratios of fetuses at E18.5 (left) and offspring at week 5 of age (right) from saline- (figure 5.1A and 5.1B) and SC-treated (figure 5.1C and 5.1D) pregnancies. The expected 1:1 ratio of WT and P0 fetuses was observed in fetuses from both saline- and SC-treated fetuses at E18.5 (figure 5.1A and 5.1C). This expected 1:1 ratio was not observed in either saline- or SC-treated offspring at week 5 of age (figure 5.1B and 5.1D). In offspring from saline-treated pregnancies there was a significantly greater ratio of WT mice compared to P0 mice (figure 5.1B) and this was also evident in offspring from the SC-treated dams (figure 5.1D).

5.2.2 Growth trajectories of Igf2 P0+/− and WT offspring from control C57BL6/J dams; effect of sex

Body weight of WT F mice was significantly lower than that of WT M mice between week 5 and week 12 of age; P0 F mice were also significantly lighter compared with P0 Ms (figure 5.2). Irrespective of genotype there was no difference in body weight of male mice between week 5 and week 12 of age. P0 F body weight was also not significantly different at any recorded age when compared with WT F body weight.

5.2.3 Effect of antenatal Sildenafil citrate on growth trajectories of Igf2 P0+/− and WT offspring

Body weights from offspring of SC-treated pregnancies were compared with offspring from saline-treated pregnancies (figure 5.3). SC treatment did not affect body weight of male offspring between week 5 and week 12 of age, irrespective of genotype (figure 5.3A and 5.3B). Female P0 offspring which were exposed SC in utero were similar in body weight at each recorded age to female offspring from saline-treated pregnancies (figure 5.3D). However, WT F offspring from SC-treated pregnancies were significantly heavier than WT F offspring from saline-treated pregnancies (figure 5.3B, P<0.05); this difference was most apparent (expressed as mean±SEM) at postnatal week 5 (17.3±0.3 vs. 15.8±0.4 grams, respectively) and week 6 (19.0±0.3 vs. 17.3±0.4 grams, respectively).
Figure 5.1. Chart illustrating the ratios of WT and Igf2 P0+/− fetuses (left column) and offspring (right column) from saline-treated (A and B) and sildenafil citrate-treated pregnancies (C and D). Panel A-D; Expected vs. observed ratios were evaluated using the binomial test (two tailed). Key: WT (black) and Igf2 P0+/− (grey) from saline-treated pregnancies. WT (dark blue) and Igf2 P0+/− (light blue) from SC-treated pregnancies.
Figure 5.2. Mean weekly body weight of offspring from saline-treated pregnancies (N = 9). Offspring body weight was measured between postnatal week 5 and week 12. Data are shown as mean ± SEM; number of offspring per group in parenthesis.
Figure 5.3. Comparison of mean weekly body weight of WT (top) and *Igf2* P0+/− (bottom) offspring from saline-treated (N = 9) and sildenafil citrate-treated pregnancies (N = 11). Panel A-D; Offspring body weight was measured between postnatal week 5 and week 12 and compared with equivalent offspring from saline-treated pregnancy. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key; light blue line denotes average body weight of offspring from SC-treated pregnancy, *P<0.05, **P<0.01.
5.2.4 Systolic blood pressure in \textit{Igf2} P0\textsuperscript{+/−} and WT offspring from control C57BL6/J dams; effect of sex

At week 8 and week 13 of age, SBP was not different between WT and P0 offspring from saline-treated pregnancies, irrespective of sex (figure 5.4A and 5.4B). At week 8 of age there was no difference in SBP in offspring from P0 mice irrespective of sex. However amongst WT offspring SBP was significantly higher in females than males (figure 5.4A, \(P<0.05\)). At week 13 of age there was no significant difference in SBP between any of the four groups of offspring from saline-treated pregnancies (figure 5.4B).

5.2.5 Effect of antenatal Sildenafil citrate on systolic blood pressure in \textit{Igf2} P0\textsuperscript{+/−} and WT offspring

Figure 5.5 shows SBP data from WT (top) and P0 (bottom) offspring from saline- and SC-treated pregnancies. SBP was measured at week 8 (figure 5.5A and 5.5C) and week 13 (figure 5.5B and 5.5D). At week 8 and week 13 of age, SBP in WT offspring from SC-treated pregnancies was significantly higher than offspring from saline-treated pregnancies; these results were independent of sex (figure 5.5A and 5.5B, \(P<0.0001\)). SBP was also significantly higher in P0 offspring when dams had been treated with SC compared with those treated with saline; these results were also independent of sex (figure 5.5C and 5.5D, \(P<0.0001\)).
Figure 5.4. Systolic blood pressure of WT and Igf2 P0+/− offspring from saline-treated pregnancies. Blood pressure was measured at postnatal week 8 (A) and week 13 (B). Genotype and gender were compared using one-way ANOVA with Tukey’s multiple comparisons post hoc test. Data are mean ± SEM; N = number of offspring per group. *P<0.05 for post hoc.
Figure 5.5. Comparison of systolic blood pressure of WT (top) and 
Igf2 P0+/− (bottom) offspring from saline-treated and sildenafil citrate-treated pregnancies. Blood pressure was measured at postnatal week 8 (A and C) and week 13 (B and D). Systolic blood pressures were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are mean ± SEM; N = number of offspring per group. **P<0.01, ***P<0.001, ****P<0.0001 for post hoc.
5.2.6 Blood glucose concentration in Igf2 P0+/− and WT offspring from control C57BL6/J dams; effects of sex

The concentration of glucose in blood collected from the tail vein was measured in offspring at 12 weeks of age from saline-treated dams (figure 5.6). Fasting blood glucose concentration was increased in WT M vs. WT F offspring but was not different between P0 M vs. P0 F offspring (figure 5.6A [P<0.0001] and figure 5.6D, respectively). When assessing the effect of sex on blood glucose concentration following an injection of glucose, there was a significantly higher blood glucose in male offspring from WT (figure 5.6A) and P0 (figure 5.6D) genotypes when compared with female equivalents (P<0.0001 and P<0.01, respectively). At each time point there was no significant effect of the P0 genotype on the blood glucose concentration of female offspring when compared with WT equivalent (figure 5.6B). In contrast, P0 M offspring had reduced blood glucose concentrations when compared with WT M offspring (P<0.05); however, there was no difference in fasting blood glucose concentration (T = 0; figure 5.6C) between WT M and P0 M.

5.2.7 Effect of antenatal Sildenafil citrate on blood glucose concentration in Igf2 P0+/− and WT offspring

Blood glucose concentrations of offspring from SC-treated and equivalent saline-treated controls are shown in figure 5.7. Antenatal SC treatment had no effect on fasting blood glucose concentration of male offspring when compared with those offspring of saline-treated dams, irrespective of genotype (figure 5.7A and 5.7C). Additionally, at all time-points following glucose injection, blood glucose concentration in male offspring from saline- and SC-treated dams was not significantly different. Conversely, antenatal SC treatment significantly increased blood glucose concentrations in female offspring, irrespective of genotype (figure 5.7B and 5.7D, P<0.01 and P<0.05, respectively) when compared with control offspring from saline-treated dams. In WT F offspring this increase was most significant at T = 30 (P<0.01) and T = 60 (P<0.05) but there was no difference in fasting blood glucose concentration (figure 5.7B). In P0 F offspring this increase was most significant at T = 30 (P<0.01) and T = 90 (P<0.05) but there was no difference in fasting blood glucose concentration (T = 0; figure 5.7D).
Figure 5.6. Comparison of glucose tolerance profiles of WT (top) and Igf2 P0\textsuperscript{+/−} (bottom) offspring from saline-treated pregnancies. Offspring were fasted overnight for 16 hours, injected with 1 g.kg\textsuperscript{−1} intraperitoneal glucose and serial blood samples taken between 0 and 120 mins. Mann-Whitney U test was used to assess area under the curve values between genotypes and genders. At each individual time point blood glucose concentrations from offspring were also compared using Mann-Whitney U test. Key: mean ± SEM. *P<0.05, **P<0.01, ****P<0.0001.
Figure 5.7. Comparison of glucose tolerance profiles of WT (top) and Igf2 P0+/− (bottom) offspring from saline-treated and sildenafil citrate-treated pregnancies. Offspring were fasted overnight for 16 hours, injected with 1 g.kg⁻¹ intraperitoneal glucose and serial blood samples taken between 0 and 120 mins. Mann-Whitney U test was used to assess area under the curve values between saline-treated and sildenafil citrate-treated groups. At each individual time point blood glucose concentrations from saline-treated and Sildenafil citrate-treated offspring were also compared using Mann-Whitney U test. Key: mean ± SEM. *P<0.05, **P<0.01.
5.2.8 Offspring organ weight in Igf2 P0+/− and WT mice from control C57BL6/J dams; 
effect of gender

Average organ weights from the offspring of saline-treated dams are shown in table 5.1. There was no significant difference in spleen, lung or brain weight in offspring from saline-treated pregnancies. Heart weight was not significantly different in P0 offspring from saline-treated pregnancies. WT M offspring heart weight was significantly increased when compared with WT F heart weight (P<0.05) however, when expressed as a percentage of total body weight these differences were no longer significant (data not shown). There was no effect of genotype on average kidney weight in either male or female mice; although average kidney weight was significantly increased in male offspring when compared with females, regardless of WT or P0 genotype (P<0.0001 and P<0.05, respectively; table 5.1). This effect was also significant when data were expressed as a percentage of total body weight (data not shown).

5.2.9 Effect of antenatal Sildenafil citrate on offspring organ weight in Igf2 P0+/− and 
WT mice

Average organ weights from offspring of SC-treated pregnancies were also compared with equivalent offspring from saline-treated pregnancies (table 5.1). There was no effect of antenatal SC on weight of the heart, spleen, lung or brain when compared with weight of equivalent offspring from saline-treated pregnancies. WT offspring average kidney weight was affected by antenatal SC treatment; average kidney weight in WT M offspring was increased but reduced in WT F offspring compared with WT M and WT F offspring, respectively, from saline-treated dams (P<0.05, table 5.1). When organ weight was expressed relative to total body weight these differences were no longer present (data not shown).
Table 5.1. Offspring organ allometry from saline- and sildenafil-treated pregnancies. Organ wet weights were measured between postnatal week 14 and week 16. Organ weights were assessed for effect of genotype and sex within saline-treated groups using the Kruskal Wallis statistical test with letters denoting significance from Dunns multiple comparisons post hoc test. Mann-Whitney U test was used to assess the effect of treatment between two groups e.g. WT M from saline-treated pregnancy vs. WT M from Sildenafil citrate-treated pregnancy. A,C,D,E \(P<0.05\), B \(P<0.0001\) for post hoc test. Data are expressed as median [min - max].

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5.2.10 Effect of an antenatal subcutaneous injection of saline on contraction responses of offspring abdominal aortas

Adult abdominal aortic diameter was significantly larger in P0 Ms compared with P0 Fs ($P<0.05$). However, there was no significant difference in adult abdominal aortic diameter between any other groups. The basal tone of adult abdominal aortas was significantly higher in WT compared to P0 Ms ($P<0.05$), although there was no effect of a subcutaneous dose of saline on basal tone in any other groups (table 5.2). The median aortic basal tone for offspring from saline-treated pregnancies (N = 33) was 59 (min 46 – max 89) mmHg. Median basal tone (min - max) for individual groups was; WT M 63 (50 – 68) mmHg, P0 M 58 (48 – 58) mmHg, WT F 57 (52 – 89) mmHg, P0 F 56 (46 – 56) mmHg.

Contraction to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) was not significantly different between all four control groups (table 5.2). U46619 elicited the greatest agonist-induced responses when compared with PE and KPSS (table 5.2).

U46619 dose response curves were constructed for adult abdominal aortas (figure 5.8) from subcutaneous saline-treated dams. U46619-induced contraction of aortas from WT M offspring was similar to P0 M offspring but WT F offspring had reduced U46619-induced contraction compared with P0 F offspring (figure 5.8A and 5.8B [P<0.05], respectively). When sex was considered, female mice had similar U46619-induced contraction vs. male mice irrespective of genotype (figure 5.8C and figure 5.8D). There were no differences in EC_{20}, EC_{50} or EC_{80} values of abdominal aortas from WT M, WT F, P0 M and P0 F mice from saline-treated dams (table 5.3, for data see table 5.5).

U46619-induced contraction of adult aortas (as % KPSS) from WT M offspring were similar to P0 M offspring (figure 5.9A). P0 F U46619-induced aortic contraction (as % KPSS) was also not different when compared to WT F aortas (figure 5.9B). When offspring sex was considered, WT and P0 F mice had similar U46619-induced aortic contraction vs. WT M (figure 5.9C) and P0 M mice (figure 5.9D), respectively.
Table 5.2. Offspring abdominal aortic diameter, basal tone and max contraction data from saline-treated pregnancies. KPSS (120 mM high potassium salt solution); PE (10^{-5} M phenylephrine); U46619 (2x10^{-6} M thromboxane-A_2 mimetic). Data are shown as median [min - max]; number of offspring per group in parenthesis. Letters denote $P<0.05$ between groups with the same letter in each column.

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<td>KPSS</td>
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Figure 5.8. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of U46619. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 5.9. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
5.2.11 Relaxation responses of offspring abdominal aortas to Acetylcholine and Sodium nitroprusside from pregnancies administered a subcutaneous injection of saline

Aortas were pre-contracted with an EC\textsubscript{80} concentration of U46619 before ACH (endothelial-dependent, figure 5.10) and SNP (endothelial-independent, figure 5.11) induced relaxation was measured.

ACH-induced relaxation responses of adult abdominal aortas were similar in P0 M vs. WT M offspring (figure 5.10A) and P0 F vs. WT F mice (figure 5.10B). When assessing the effect of offspring sex, ACH-induced relaxation of aortas was greater in males than females in both WT (\(P<0.0001\), figure 5.10C) and P0 (\(P<0.0001\), figure 5.10D) mice.

SNP-induced relaxation responses of adult abdominal aortas were greater in P0 M vs. WT M offspring (\(P<0.05\), figure 5.11A) and P0 F vs. WT F offspring (\(P<0.01\), figure 5.11B). When assessing the effect of sex, SNP-induced relaxation responses of aortas were greater in male vs. female WT (\(P<0.0001\), figure 5.11C) and P0 mice (\(P<0.0001\), figure 5.11D). However, there were no significant differences between WT M, WT F, P0 M and P0 F mice, from saline-injected dams, in the effective concentration of either ACH or SNP required to produce 20 %, 50 % or 80 % relaxation of adult abdominal aortas (table 5.3, for data see table 5.5).

Table 5.3 summarises the effects of a saline-injection in the dam on fetal aortic vascular function at E18.5 (chapter 4) and offspring aortic vascular function between postnatal week 14 and 16 (chapter 5).
Figure 5.10. Dose response curves of offspring abdominal aortas from saline-treated dams in response to increasing doses of ACH. Panel A-D; Arteries were pre-contracted with an EC80 dose of U46619. ACH dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: ACH; acetylcholine, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).

185
Figure 5.11. Dose response curves of offspring abdominal aortas from saline-treated pregnancies in response to increasing doses of SNP. Panel A-D; Arteries were pre-contracted with an $EC_{80}$ dose of U46619. SNP dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: SNP; sodium nitroprusside, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Table 5.3. Summary table comparing fetal (data from chapter 4) and offspring (chapter 5) abdominal aortic basal tone, diameter, maximal contraction (KPSS, PE), contraction dose response (U46619) and relaxation dose responses (ACH, SNP) from saline-treated dams. Comparisons were made between genotypes (WT and P0) and sex. ↔ arrow denote no change, ↓ or ↑ arrow denotes a decrease or increase in a parameter compared to relevant group e.g. ACH relaxation in WT F offspring reduced compared to WT M offspring.

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5.2.12 Effect of an antenatal subcutaneous injection of sildenafil citrate on contraction responses of offspring abdominal aortas

SC had no effect on offspring abdominal aortic diameter when comparing aortas from SC-treated vs. saline-treated pregnancies except in P0 M. P0 M aortic diameter from saline-treated dams was smaller than P0 M aortas from SC-treated dams (P<0.05, table 5.4). There was no effect of SC on basal tone in any of the individual groups; the median basal tone for SC-treated groups (N = 44) was 59 (min 41 – max 110) mmHg. Median basal tone (min-max) for individual groups was; WT M 59 (44 – 76) mmHg, P0 M 56 (41 – 110) mmHg, WT F 61 (53 – 74) mmHg, P0 F 63 (54 – 88) mmHg.

Contraction responses to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) were not significantly different between offspring from SC-treated and saline-treated pregnancies (table 5.4). Offspring abdominal aortas from SC-treated pregnancies had the greatest response to U46619, when compared with PE and KPSS (table 5.4).

U46619 dose response curves were constructed and are shown in figure 5.12. Comparisons were made to assess the effect of a subcutaneous injection of SC against the equivalent control group. There was no effect of SC treatment on U46619-induced contraction of aortas from male offspring of WT or P0 genotypes (figure 5.12A and 5.12C). WT F offspring from SC-treated pregnancies had elevated U46619-induced contraction when compared with WT F control aortas (P<0.05, figure 5.12B). In contrast, there was no significant difference in contraction of P0 F offspring abdominal aortas from SC-treated groups when compared with P0 F saline controls (figure 5.12D). There were was a significant increase in the effective concentration of U46619 required to produce 20 %, 50 % or 80 % relaxation in adult abdominal aortas from WT M and P0 mice from SC-treated dams, when compared with WT M and P0 M mice from saline-treated dams (see table 5.5).

U46619-induced contraction was also expressed as a % of maximal KPSS contraction (figure 5.12). Antenatal SC treatment did not alter U46619-induced contraction (% KPSS) of abdominal aortas in WT M and female (figure 5.13A and 5.13B, respectively) or P0 M offspring (figure 5.13C). However, a subcutaneous injection of SC significantly reduced U46619-induced contraction (% KPSS) of P0 F offspring abdominal aortas compared with the equivalent P0 F offspring from saline-treated pregnancies (P<0.05, figure 5.13D).
Table 5.4. Comparison of offspring abdominal aortic diameter, basal tone and max contraction data from saline and sildenafil citrate (10 mg.kg\(^{-1}\)) – treated pregnancies. KPSS (120 mM high potassium salt solution); PE (10\(^{-5}\) M phenylephrine); U46619 (2x10\(^{-6}\) M thromboxane-A\(_2\) mimetic). \(A^p\) denotes \(P<0.05\) when comparing saline controls group with other saline control groups with identical letter. \(A^c\) denotes \(P<0.05\) when comparing saline control group with equivalent sildenafil citrate group. Data are shown as median [min-max]; number of offspring per group in parenthesis.

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<th>Maternal Treatment</th>
<th>Offspring ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
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Figure 5.12. Dose response curves of offspring abdominal aortas in response to increasing doses of U46619 following an antenatal subcutaneous injection of saline or sildenafil citrate. Panel A-D: Pregnant dams were administered a subcutaneous injection of saline (N = 9) or sildenafil citrate (10 mg.kg⁻¹; N = 11). Offspring aortas were harvested between postnatal week 14 and week 16. U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: Offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (blue solid curves).
Figure 5.13. Dose response curves of offspring abdominal aortas in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following an antenatal subcutaneous injection of saline or sildenafil citrate. Panel A-D; Pregnant dams were administered a subcutaneous injection of saline (N = 9) or sildenafil citrate (10 mg·kg⁻¹; N = 11). Offspring aortas were harvested between postnatal week 14 and week 16. U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: Offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or Sildenafil citrate (blue solid curves).
5.2.13 Effect of antenatal subcutaneous injection of Sildenafil citrate on relaxation responses of offspring abdominal aortas

Aortas from SC-treated pregnancies were pre-contracted with an EC\textsubscript{80} concentration of U46619 before ACH (endothelial-dependent, figure 5.14) and SNP (endothelial-independent, figure 5.15) induced relaxation was measured.

There was a reduction in relaxation response to ACH in WT ($P<0.0001$, figure 5.14A) and P0 ($P<0.0001$, figure 5.14C) male offspring from SC-treated pregnancies when compared with equivalent male offspring from saline-treated pregnancies. Antenatal SC-treatment did not alter relaxation of abdominal aortas in response to ACH in WT F offspring (figure 5.14B). However, there was a significant increase in relaxation response of P0 F offspring abdominal aortas from SC-treated pregnancies compared with P0 Fs from saline treated pregnancies ($P<0.01$, figure 5.14D).

A subcutaneous injection of SC during pregnancy significantly reduced relaxation responses of offspring abdominal aortas to SNP in WT M and P0 M offspring (figure 5.15A [$P<0.001$] and 5.15C [$P<0.001$], respectively). Conversely, WT F offspring from SC-treated pregnancies had increased endothelial-independent relaxation (to SNP) of abdominal aortas when compared with WT F offspring from saline-treated pregnancies ($P<0.05$, figure 5.14B). There was no effect of antenatal SC on P0 F offspring abdominal aortic response to SNP when compared with comparable controls.

There was a significant increase (i.e. desensitisation) in the concentration of ACH required to produce 20%, 50% and 80% relaxation in abdominal aortas from WT M mice of SC-treated dams, when compared with abdominal aortas from WT M mice of saline-treated dams (see table 5.5).

Table 5.6 summarises the effects of a subcutaneous dose of 10 mg.kg\textsuperscript{-1} of SC to the dam on fetal aortic vascular function at E18.5 (chapter 4) and offspring aortic vascular function between postnatal week 14 and 16 (chapter 5).
Figure 5.14. Dose response curves of offspring abdominal aortas to increasing doses of ACH following sildenafil citrate treatment. Panel A-D; Arteries were precontracted with an EC₈₀ dose of U46619. ACH dose response curves were compared using two-way ANOVA for treatment and ACH concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: ACH; acetylcholine, offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg·kg⁻¹; blue solid curves). *P<0.05, ****P<0.0001 for post hoc.
Figure 5.15. Dose response curves of offspring abdominal aortas to increasing doses of SNP following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC$_{80}$ dose of U46619. SNP dose response curves were compared using two-way ANOVA for treatment and SNP concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: SNP; sodium nitroprusside, offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg.kg$^{-1}$; blue solid curves).
Table 5.5. Adult abdominal aorta sensitivity to U46619, ACH and SNP following subcutaneous injection of saline or SC. Key: All data for WT / P0 offspring from saline control and SC-treated dams expressed as effective concentration (EC in nM). A = Reduced U46619 sensitivity in SC-treated WT male vs. control WT male offspring (EC<sub>20</sub>, EC<sub>50</sub>, EC<sub>80</sub>; P<0.05; Mann-Whitney U Test). B/C = Reduced ACH sensitivity in SC-treated WT male and P0 male vs. control WT male and P0 male, respectively. All data are mean±SEM with number of animals in parenthesis.

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<th>WT Female</th>
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<td>616±105 (10)</td>
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Table 5.6. Summary table demonstrating the effect of maternal sildenafil citrate treatment (10 mg.kg⁻¹) on fetal and offspring abdominal aortic basal tone, diameter, maximal contraction (KPSS, PE), contraction dose responses (U46619) and relaxation dose responses (ACH, SNP) for each group, compared with equivalent fetuses and offspring aortas from saline-treated pregnancies. ↔ arrow denote no change, ↓ and ↑ arrows denote a decrease or increase, respectively. E.g. ↔ denotes no change in U46619 constriction in WT M fetal aortas from Sildenafil citrate-treated pregnancies compared to WT M fetal aortas from saline-treated pregnancies. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

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5.2.14 Effect of an antenatal subcutaneous injection of saline on contraction responses of offspring mesenteric arteries

There was no significant difference in offspring mesenteric artery diameter between all four control groups (table 5.6). There was no effect of a subcutaneous dose of saline on basal tone amongst the four groups except for an increase in P0 F vs. WT F offspring. The median basal tone for saline-treated groups (N = 33) was 41 (min 14 – max 79) mmHg. Median basal tone (min - max) for individual groups was; WT M 40 (19 – 79) mmHg, P0 M 35 (14 – 77) mmHg, WT F 32 (25 – 32) mmHg, P0 F 63 (42 – 63) mmHg.

Contraction to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) was not significantly different between all four control groups (table 5.6). However, U46619 elicited the greatest agonist-induced responses when compared with PE and KPSS (table 5.6).

U46619 dose response curves were constructed for offspring mesenteric arteries (figure 5.16) from subcutaneous saline-treated pregnancies. U46619-induced contraction of mesenteric arteries from WT M and WT F offspring were similar to their P0 littermates (figure 5.16A and 5.16B, respectively). When sex was considered, WT F mice had reduced U46619-induced contraction vs. WT M mice (P<0.01, figure 5.16C) but P0 F mice had similar U46619-induced contraction compared to P0 M mice (figure 5.16D). There were no differences in EC_{20}, EC_{50} or EC_{80} values when comparing the effect of antenatal SC treatment on offspring mesenteric artery U46619-induced contraction (table 5.8, for data see table 5.10).

There was no difference in U46619-induced contraction of mesenteric arteries (as % KPSS) from WT M and female offspring when compared with P0 equivalent (figure 5.17A and 5.17B, respectively). When offspring gender was considered, female mice had similar U46619-induced contraction vs. male mice from WT (figure 5.17C) and P0 litters (figure 5.17D).
Table 5.7. Offspring mesenteric artery diameter, basal tone and maximal contraction (KPSS, PE, U46619 data from saline treated pregnancies. KPSS (120 mM high potassium salt solution); PE (10⁻⁵ M phenylephrine); U46619 (2x10⁻⁶ M thromboxane-A₂ mimetic). Data are shown as median [min-max]; number of offspring per group in parenthesis.  A denotes P<0.05 for groups with same letter in columns.

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<td></td>
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<td>Saline</td>
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Figure 5.16. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of U46619. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 5.17. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS. Panel A-D; U46619 dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
5.2.15 Relaxation responses of offspring mesenteric arteries to Acetylcholine and Sodium nitroprusside from pregnancies administered a subcutaneous injection of saline.

Mesenteric arteries were pre-contracted with an EC$_{80}$ concentration of U46619 before ACH (endothelial-dependent, figure 5.18) and SNP (endothelial-independent, figure 5.19) induced relaxation was measured.

ACH-induced relaxation response of adult mesenteric arteries was increased in P0 M vs. WT M mice ($P<0.05$, figure 5.18A) but not in P0 F vs. WT F offspring arteries (figure 5.18B). When assessing the effect of gender, ACH-induced relaxation response of offspring mesenteric arteries in male vs. female was similar in WT (figure 5.18C) but increased in P0 offspring ($P<0.05$, figure 5.18D).

SNP-induced relaxation responses of offspring mesenteric arteries were similar in P0 M vs. WT M mice (figure 5.19A) and P0 F vs. WT F arteries (figure 5.19B). When assessing the effect of sex, SNP-induced relaxation response of mesenteric arteries in male vs. female was increased in WT offspring ($P<0.01$, figure 5.19C) but similar in P0 offspring (figure 5.19D). There were no significant differences between any of the four control groups in the effective concentration of either ACH or SNP required to produce 20 %, 50 % or 80 % contraction of offspring mesenteric arteries (table 5.8, for data see table 5.10).

Table 5.7 summarises the differences in aortic and mesenteric vascular function of offspring from saline-treated dams.
Figure 5.18. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies in response to increasing doses of ACH. Panel A-D: Arteries were pre-contracted with an EC\textsubscript{80} dose of U46619. ACH dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: ACH; acetylcholine, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares).
Figure 5.19. Dose response curves of offspring mesenteric arteries from saline-treated pregnancies to increasing doses of SNP. Panel A-D; Arteries were pre-contracted with an EC\textsubscript{80} dose of U46619. SNP dose response curves were compared using two-way ANOVA with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: SNP; sodium nitroprusside, WT M (blue closed circles), P0 M (black open circles), WT F (cyan closed squares), P0 F (black open squares). **P<0.01.
Table 5.8. Summary table comparing offspring mesenteric and aortic basal tone, diameter, maximal contraction (KPSS, PE), contraction response (U46619) and relaxation responses (ACH, SNP) from saline injected dams. ↔ arrow denotes no change, ↓ and ↑ arrows denote a decrease or increase, respectively. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

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</table>
5.2.16 Effect of an antenatal subcutaneous injection of Sildenafil citrate on contraction responses of offspring mesenteric arteries

SC had no effect on offspring mesenteric artery diameter when comparing arteries from SC-treated vs. saline-treated pregnancies (table 5.8). There was an increase in mesenteric artery basal tone of WT M offspring from SC-treated dams and a reduction in P0 F offspring from SC-treated dams when compared with equivalent saline control (P<0.05, table 5.8). The median basal tone for SC-treated groups (N = 44) was 49 (min 12 – max 106) mmHg. Median basal tone (min-max) for individual groups was; WT M 54 (34 – 106) mmHg, P0 M 52 (26 – 98) mmHg, WT F 48 (12 – 77) mmHg, P0 F 46 (28 – 61) mmHg.

Contraction responses to KPSS (120 mM), PE (10^{-5} M) and U46619 (2x10^{-6} M) were not significantly different between SC-treated and equivalent saline control group; except in WT Ms from SC-treated dams, where there was a reduction in the maximal response of mesenteric arteries to PE (P<0.05, table 5.8). In the SC-treated group, offspring mesenteric arteries had the greatest response to U46619, when compared with PE and KPSS (table 5.8).

U46619 dose response curves were constructed and are shown in figure 5.20. Comparisons were made to assess the effect of an antenatal subcutaneous injection of SC against the equivalent saline control group in offspring mesenteric arteries. There was a significant reduction in contraction of WT M offspring mesenteric arteries from SC-treated pregnancies when compared with WT M offspring from saline-treated pregnancies (P<0.01, figure 5.20A). However, P0 M offspring from SC-treated pregnancies had similar U46619-induced contraction when compared with P0 M control arteries (figure 5.20C). There was no effect of SC treatment on U46619-induced contraction of mesenteric arteries from female offspring of WT or P0 genotypes (figure 5.20B and 5.20D). There were no significant differences between any of the four control groups in the effective concentration of U46619 required to produce 20 %, 50 % or 80 % contraction of offspring mesenteric arteries (table 5.10).

U46619-induced contraction was also expressed as a % of maximal KPSS contraction (figure 5.21). U46619-induced contraction (% KPSS) of offspring mesenteric arteries was not altered in male mice of SC-treated pregnancies from either WT (figure 5.21A) or P0 genotype (figure 5.21C). An antenatal subcutaneous injection of SC increased U46619-induced contraction (% KPSS) of female offspring mesenteric arteries from P0 (P<0.05, figure 5.21D) but not WT genotype (figure 5.21B).
Table 5.9. Comparison of offspring mesenteric artery diameter, basal tone, maximal contraction (KPSS, PE, U46619) data from saline and sildenafil citrate (10 mg.kg\(^{-1}\))–treated pregnancies. KPSS (120 mM high potassium salt solution); PE (10\(^{-5}\) M phenylephrine); U46619 (2x10\(^{-6}\) M thromboxane-A\(_2\) mimetic). \(^{A}\) denotes \(P<0.05\) when saline control group is compared with another saline control group with identical letter. \(^{B,C,D}\) denote \(P<0.05\) when comparing saline control group with equivalent sildenafil citrate group. Data are shown as median [min-max]; number of offspring per group in parenthesis.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Fetal ID (n)</th>
<th>AA Diameter (µm)</th>
<th>Basal Tone (kPa)</th>
<th>Maximal Contraction (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KPSS</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT Male (10)</td>
<td>184 [140 – 194]</td>
<td>5.30 [2.59 – 10.5](^{B})</td>
<td>21.8 [17.4 – 30.1]</td>
<td>29.7 [27.8 – 42.9]</td>
</tr>
<tr>
<td>P0 Female (5)</td>
<td>159 [129 – 227]</td>
<td>8.40 [5.52 – 10.5](^{A,C})</td>
<td>30.8 [16.0 – 32.9]</td>
<td>29.2 [24.6 – 39.5]</td>
</tr>
<tr>
<td><strong>Sildenafil citrate (10 mg.kg(^{-1}))</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0 Male (10)</td>
<td>171 [127 – 204]</td>
<td>6.91 [3.51 – 13.1]</td>
<td>22.7 [6.30 – 35.0]</td>
<td>32.9 [10.2 – 49.3]</td>
</tr>
<tr>
<td>P0 Female (9)</td>
<td>193 [133 – 226]</td>
<td>6.11 [3.77 – 8.07](^{C})</td>
<td>20.0 [18.4 – 31.0]</td>
<td>23.9 [22.9 – 35.9]</td>
</tr>
</tbody>
</table>
Figure 5.20. Dose response curves of offspring mesenteric arteries in response to increasing doses of U46619 following an antenatal subcutaneous injection of saline or sildenafil citrate. Panel A-D; Pregnant dams were administered a subcutaneous injection of saline (N = 9) or sildenafil citrate (10 mg.kg⁻¹; N = 11). Offspring aortas were harvested between postnatal week 14 and week 16. U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: Offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (blue solid curves).
Figure 5.21. Dose response curves of offspring mesenteric arteries in response to increasing doses of U46619 as a percentage of maximal contraction to KPSS following an antenatal subcutaneous injection of saline or sildenafil citrate. Panel A-D: Pregnant dams were administered a subcutaneous injection of saline (N = 9) or sildenafil citrate (10 mg.kg⁻¹; N = 11). Offspring aortas were harvested between postnatal week 14 and week 16. U46619 dose response curves were compared using two-way ANOVA for treatment and concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: Offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (blue solid curves).
5.2.17 Effect of an antenatal subcutaneous injection of Sildenafil citrate on relaxation responses of offspring mesenteric arteries

Mesenteric arteries from offspring of SC-treated pregnancies were pre-contracted with an $EC_{80}$ concentration of U46619 before ACH (endothelial-dependent, figure 5.22) and SNP (endothelial-independent, figure 5.23) induced relaxation was measured.

There was an increased ACH-induced relaxation of WT M and P0 F mesenteric arteries in offspring from SC-treated pregnancies (figure 5.22A [P<0.05] and figure 5.22D [P<0.05], respectively). Conversely, SC led to a reduced ACH-induced relaxation of P0 M mesenteric arteries when compared to P0 M offspring from saline-treated pregnancies (P<0.05, figure 5.22C). However, antenatal SC-treatment did not alter relaxation of offspring mesenteric arteries in response to ACH in WT F offspring (figure 5.22B).

SNP-induced relaxation of offspring mesenteric arteries was reduced in WT M and WT F mice from SC-treated pregnancies when compared with equivalent saline controls (figure 5.23A [P<0.01] and 5.23B [P<0.05], respectively). In contrast, a subcutaneous injection of SC during pregnancy did not alter relaxation responses of offspring mesenteric arteries to SNP in P0 mice, regardless of gender (figure 5.23C and 5.23D). In WT M mice from SC-treated dams, there was an increase in the concentration of SNP required to produce 20 %, 50 % or 80 % relaxation of mesenteric arteries, compared with WT M mice from saline-treated dams (table 5.10). There were no other significant differences when assessing the effect of SC on the effective concentration of either ACH or SNP required to produce 20 %, 50 % or 80 % relaxation of offspring mesenteric arteries (table 5.10).

Table 5.9 summarises the effects of a subcutaneous injection of 10 mg.kg⁻¹ SC to the dam on offspring aortic and mesenteric vascular function.
Figure 5.22. Dose response curves of offspring mesenteric arteries to increasing doses of ACH following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC₈₀ dose of U46619. ACH dose response curves were compared using two-way ANOVA for treatment and ACH concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: ACH; acetylcholine, offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg.kg⁻¹; blue solid curves). *P<0.05.
Figure 5.23. Dose response curves of offspring mesenteric arteries to increasing doses of SNP following sildenafil citrate treatment. Panel A-D; Arteries were pre-contracted with an EC₉₀ dose of U46619. SNP dose response curves were compared using two-way ANOVA for treatment and SNP concentration with Bonferroni post hoc test, where applicable. Data are shown as mean ± SEM; number of offspring per group in parenthesis. Key: SNP; sodium nitroprusside, offspring from pregnant dams given a subcutaneous injection of saline (black solid curves) or sildenafil citrate (10 mg.kg⁻¹; blue solid curves). **P<0.01.
Table 5.10. Adult mesenteric artery sensitivity to U46619, ACH and SNP following subcutaneous injection of saline or SC. Key: All data for WT / P0 offspring from saline control and SC-treated dams expressed as effective concentration (EC in nM). A = Reduced SNP sensitivity in SC-treated WT male vs. control WT male offspring (EC_{20}, EC_{50}, EC_{80}; P<0.05; Mann-Whitney U Test). All data are mean±SEM with number of animals in parenthesis.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>Agonist</th>
<th>Effective concentration</th>
<th>WT Male</th>
<th>WT Female</th>
<th>P0 Male</th>
<th>P0 Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>U46619</td>
<td>EC_{20}</td>
<td>2.39±0.7 (10)</td>
<td>4.22±1.0 (11)</td>
<td>7.31±2.2 (7)</td>
<td>4.77±1.8 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC_{50}</td>
<td>9.57±2.7 (10)</td>
<td>16.9±3.9 (11)</td>
<td>29.2±8.7 (7)</td>
<td>19.1±7.1 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC_{80}</td>
<td>38.3±10.7 (10)</td>
<td>67.5±15.6 (11)</td>
<td>117±34.8 (7)</td>
<td>76.3±28.5 (5)</td>
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<tr>
<td></td>
<td>ACH</td>
<td>EC_{20}</td>
<td>72.8±20.2 (7)</td>
<td>163±50.0 (11)</td>
<td>27.5±12.6 (6)</td>
<td>163±72.2 (5)</td>
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<tr>
<td></td>
<td></td>
<td>EC_{50}</td>
<td>291±80.8 (7)</td>
<td>654±200 (11)</td>
<td>110±50.4 (6)</td>
<td>652±289 (5)</td>
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<tr>
<td></td>
<td></td>
<td>EC_{80}</td>
<td>1165±323 (7)</td>
<td>2616±801 (11)</td>
<td>440±202 (6)</td>
<td>2609±1154 (5)</td>
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<tr>
<td></td>
<td>SNP</td>
<td>EC_{20}</td>
<td>18.0±4.6 (10)</td>
<td>76.1±21.9 (11)</td>
<td>35.0±9.0 (7)</td>
<td>54.0±33.5 (5)</td>
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<td>EC_{50}</td>
<td>721±18.5 (10)</td>
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<td>140±36.1 (7)</td>
<td>216±134 (5)</td>
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<td>EC_{80}</td>
<td>288±73.9 (10)</td>
<td>1218±350 (11)</td>
<td>559±144 (7)</td>
<td>864±536 (5)</td>
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<td>Sildenafil citrate (10 mg.kg^{-1})</td>
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<td>5.01±1.4 (12)</td>
<td>3.23±1.0 (13)</td>
<td>4.34±0.9 (10)</td>
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<tr>
<td></td>
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<td>EC_{50}</td>
<td>20.1±5.6 (12)</td>
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<td>80.2±22.5 (12)</td>
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<tr>
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<td>ACH</td>
<td>EC_{20}</td>
<td>27.0±12.4 (8)</td>
<td>74.1±43.0 (12)</td>
<td>69.9±20.3 (7)</td>
<td>181±41.5 (8)</td>
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<td>EC_{50}</td>
<td>108±49.8 (8)</td>
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<td>280±81.3 (7)</td>
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<td>433±199 (8)</td>
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<td>2893±664 (8)</td>
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<td>113±26.5 (13)</td>
<td>27.2±9.7 (10)</td>
<td>32.1±9.5 (9)</td>
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<td>EC_{50}</td>
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<td>452±106 (13)</td>
<td>109±38.8 (10)</td>
<td>128±38 (9)</td>
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<td></td>
<td>EC_{80}</td>
<td>1333±571 (9)</td>
<td>1809±424 (13)</td>
<td>434±155 (10)</td>
<td>513±152 (9)</td>
</tr>
</tbody>
</table>
Table 5.11. Summary table comparing the effect of maternal sildenafil citrate treatment (10 mg.kg⁻¹) on offspring mesenteric and aortic basal tone, diameter, maximal contraction (KPSS, PE), contraction response (U46619) and relaxation responses (ACH, SNP) for each group, compared with equivalent offspring arteries from saline-treated pregnancies. ↔ arrow denotes no change, ↓ and ↑ arrows denote a decrease or increase, respectively. E.g. ↔ denotes no change in U46619 constriction of aortae in WT M offspring from sildenafil citrate treated pregnancies compared to WT M offspring from saline-treated pregnancies. Key; KPSS; high potassium salt solution, PE; phenylephrine, ACH; acetylcholine, SNP; sodium nitroprusside.

<table>
<thead>
<tr>
<th></th>
<th>WT M Aorta</th>
<th>Mesentery</th>
<th>P0 M Aorta</th>
<th>Mesentery</th>
<th>WT F Aorta</th>
<th>Mesentery</th>
<th>P0 F Aorta</th>
<th>Mesentery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal tone</td>
<td>↔</td>
<td>↑</td>
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<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
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<tr>
<td>Aortic diameter</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>KPSS contraction</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
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<td>PE contraction</td>
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<tr>
<td>U46619 contraction</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>U46619 sensitivity (EC₅₀)</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
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<tr>
<td>ACH relaxation</td>
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<td>↓</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
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<tr>
<td>ACH sensitivity (EC₅₀)</td>
<td>↓</td>
<td>↔</td>
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<tr>
<td>SNP relaxation</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↔</td>
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<tr>
<td>SNP sensitivity (EC₅₀)</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
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</tbody>
</table>
5.3 Discussion

There were two main objectives of this study. The first was to assess whether mice that are growth restricted at birth, due to a placental-specific deletion of \textit{Igf2}, develop impaired metabolic / cardiovascular function in adulthood when compared with those who were appropriate weight at birth and whether these differences were sex-dependent. The second objective was to assess whether antenatal SC treatment was associated with beneficial or detrimental effects on the metabolic / cardiovascular function of offspring from SC-treated dams.

There was a significant reduction in P0 offspring survival when compared with WT littermates. In this chapter, the data presented demonstrate that there was postnatal catch up growth by 5 weeks of age in both male and female offspring which were growth restricted at birth. However, it is important to note that birthweight of individual pups was not measured in the current study but previous data do support the fact that P0 mice have a 25 \% reduction in birthweight compared to WT mice (e.g. Constância et al., 2002, Mikaelsson et al., 2013), thus, it is not too presumptuous to suggest postnatal catch up growth has occurred in P0 mice. Low birth weight in P0 mice was not associated with hypertension at 8 or 13 weeks of age when compared with WT littermates. Female offspring, whether growth restricted or not, showed increased glucose sensitivity compared with males. Growth restricted females showed similar glucose tolerance profiles compared with females with adequate birthweight however, growth restricted males had significantly increase glucose sensitivity when compared with adequate birthweight males.

Abdominal aortas from male offspring, whether growth restricted at birth or not, demonstrate significantly increased endothelial-dependent and -independent relaxation in response to ACH and donated NO when compared with female offspring. Abdominal aortas from P0 mice demonstrated similar endothelial-dependent relaxation in response to ACH when compared with WT aortas but both growth restricted male and female abdominal aortas had increased endothelial-independent relaxation in response to donated NO compared with WT males and females, respectively. Mesenteric arteries from growth restricted males demonstrated increased endothelial-dependent but not -independent relaxation in response to ACH and SNP, respectively, compared with growth restricted females. However, mesenteric arteries from WT males show similar endothelial-dependent relaxation in response to ACH but increased endothelial-independent relaxation in response to SNP, when compared with appropriate birthweight females. Growth restriction led to a significant increase in endothelial-dependent relaxation of male, but not female, mesenteric arteries compared with appropriately grown male and female mesenteric arteries, respectively.
In the \textit{Igf2 P0}^{+/−} and COMT\textsuperscript{−/−} mouse models of FGR, SC treatment led to an increase fetal weight (Dilworth et al., 2013, Stanley et al., 2012) and normalised abnormal umbilical artery Doppler waveforms in the COMT\textsuperscript{−/−} mouse (Stanley et al., 2012). In this current study, treating the \textit{Igf2 P0}^{+/−} dam with SC at 10 mg.kg\textsuperscript{−1} (daily subcutaneous; E12.5 − E17.5) did not increase fetal weight or have any deleterious effects on litter size or number of litter resorptions (chapter 4). However, results presented in this current chapter demonstrate antenatal SC did increase postnatal growth but only in female offspring who had a normal birthweight (WT F). In addition, antenatal SC treatment led to a reduction in glucose sensitivity of female offspring and a significant increase in SBP in both WT and P0 male and female offspring compared with offspring from saline-treated pregnancies. Increased SBP in female offspring was present regardless of relaxation capacity in isolated aorta and mesenteric arteries.

5.3.1 Neonatal and postnatal expected and observed ratios of \textit{Igf2 P0}^{+/−} and WT mice from control C57BL6/J dams

Close to term (E18.5) there was no difference between the observed and expected 1:1 ratio of WT to P0 mice present in the uterine horns of pregnant dams. There was a significant difference between the observed and expected 1:1 ratio of WT to P0 mice at postnatal week 5 of age, suggesting that there was a greater loss of P0 offspring within the first 5 weeks of life (figure 5.1A). This study did not directly examine the reason for this effect but one could speculate that this could be due to either fetal death from undernourishment or maternal infanticide. The loss of P0 progeny has been previously observed in other studies using P0 mice (Miguel Constância, personal communication). Although the current study was not powered to assess expected and observed ratios of both sexes, there did not seem to be an increased fetal demise associated with individual sexes (data not shown). Weber and colleagues utilised video recordings of C57BL6/J females from two independent studies to assess maternal infanticide but never observed an infanticidal dam in either set of recordings; fetuses were only eaten when presumed dead (Weber et al., 2013). It could be that P0 fetuses, which weigh 25 % less at birth compared with WT littermates (Mikaelsson et al., 2013), are either at increased risk of stillbirth or are weaker in the first few days of postnatal life and are unable to attain appropriate nutrients from dams, thus resulting in death.

5.3.2 Effect of antenatal sildenafil citrate on neonatal and postnatal expected and observed ratios in \textit{Igf2 P0}^{+/−} and WT mice

The administration of 10 mg.kg\textsuperscript{−1} to the dam did not affect litter size, number of fetal resorptions or fetal weight (see chapter 4). In addition, SC did not affect the expected genotypic ratio at E18.5
A significant reduction in the ratio of P0 offspring at postnatal week 5, similar to that seen with saline-treated dams, was noted in SC-treated pregnancies. Thus, P0 offspring from SC-treated dams showed a similar pattern of death in the first few days of postnatal life as those from saline-treated pregnancies.

5.3.3 Growth trajectories of Igf2 P0+/− and WT offspring from control C57BL6/J dams; effect of sex

This study demonstrated that P0 mice, irrespective of sex at birth, have similar growth trajectories from postnatal week 5, when compared with WT littermates (figure 5.2). A recent publication by Mikaelsson and colleagues (2013) demonstrated that P0 mice were 75% of the WT littermate control fetal weight at birth but had accelerated growth between 25 and 50 days of age (Mikaelsson et al., 2013). This led to P0 mice having a near normal body weight at postnatal day 100. However, Mikaelsson and colleagues did not assess the effect of gender on postnatal growth and thus comparisons between the current study and Mikaelsson et al., can only be made amongst male mice. It is also important to note that the aforementioned study utilised the CD1 genetic strain of mouse, which have a larger litter size resulting in reduction in birth weight and increased competition during the lactation period but this work in this thesis utilised a C57BL6/J background strain. Thus, differences between the current study and that of Mikaelsson and colleagues (2013) may be due to the genetic strain of mouse used.

In the current study it was not possible to ascertain weight measurements between birth and postnatal week 5. This was primarily due to the increased fetal demise in P0 progeny and thus it was important not to interfere with normal lactation and maternal husbandry during the first 5 weeks of postnatal life as this may increase maternal stress and lead to greater fetal demise. It is also important to note that it is unknown whether for example P0 F fetuses are more growth restricted at birth compared with P0 M fetuses. Such a situation would suggest even greater postnatal growth in growth restricted females when compared with growth restricted males. Nevertheless, it is evident from the data in this chapter that there has been a period of accelerated growth between E18.5 and postnatal week 5 in both male and female growth restricted fetuses; the data in this study are therefore consistent with the small amount of published data in the P0 mouse (Mikaelsson et al., 2013). In humans, postnatal catch up growth results in arterial remodelling in young children (Evelein et al., 2013) and is associated with increased systolic blood pressure (Huxley et al., 2000) and obesity (Ong et al., 2000) in adulthood (discussed in chapter 5.3.5 and 5.3.6). Confirming previous findings from Mikaelsson et al., the current study suggests that a placental-specific deficiency in Igf2, subsequent growth restriction and postnatal catch up growth, are not associated with increases in bodyweight in P0 mice.
compared with WT litters (Mikaelsson et al., 2013). However, it is important to note that there may be relative changes in fat depositions within the P0 mouse which are not detected by bodyweight measurements.

5.3.4 Effect of antenatal sildenafil citrate on growth trajectories of Igf2 P0+/− and WT offspring

The results in this chapter show no effect of SC on postnatal growth of male mice, irrespective of genotype (figure 5.3A and 5.3C). Postnatal growth in female mice from SC-treated dams was increased in WT mice (figure 5.3B) compared with WT F mice from saline-treated dams. Mean body weight of P0 F mice from SC-treated dams was not different at any age when compared with P0 F mice from saline treated dams. Therefore, SC is promoting growth at least in the first 6 weeks in WT F but not P0 F mice.

The mechanisms for increased body weight in WT F mice from SC-treated dams are unknown. In the current study, there was no effect of SC on key organ weights and thus the most plausible explanation for increased body weight could be an increase in visceral and / or subcutaneous fat stores through increased calorific intake. Excessive early postnatal weight gain has been associated with increased visceral and abdominal subcutaneous adipose tissue and obesity in adulthood (Demerath et al., 2009) as well as insulin resistance as early as 3 years of age in humans (Mericq et al., 2005, Soto et al., 2003). Measurements of food intake may therefore help elucidate increased calorie intake as a potential mechanism for the increased body weight in female offspring. It is also important to note that there could be another argument as to why SC increases bodyweight in WT F mice from SC vs. saline treated dams. The daily subcutaneous injection of saline in the dam during pregnancy could in fact induce a reduction fetal birthweight due to stress, but exposure to SC could normalise birthweight. However, if this were the case it may be expected that SC would also significantly increase P0 F postnatal weight. Further studies would need to be conducted in offspring from water-treated (i.e. no injection) dams to elucidate the effect of an antenatal injection of saline in the dam on postnatal weight.

5.3.5 Systolic blood pressure in Igf2 P0+/− and WT offspring from control C57BL6/J dams; effect of sex

Longitudinal studies in humans have demonstrated that rapid weight gain after year 1 of age in males who were thin at birth led to thicker arterial walls and increased arterial stiffness in children (Evelein et al., 2013) as well as increased risk of heart disease in later life (Eriksson et al., 2001). An increase in arterial wall thickness and stiffness are associated with an increase in
systolic blood pressure and risk of cardiovascular disease in adulthood (Eriksson et al., 2001). The present study did not assess the effect of fetal weight at birth and postnatal growth on arterial wall morphology or arterial stiffness. However, systolic blood pressure was measured in offspring at postnatal week 8 and postnatal week 13 (figure 5.4).

There was a subtle but significant increase in SBP at postnatal week 8 in female mice when compared with male mice; this effect was most significant between WT F and WT M mice. Deschepper and colleagues (2004) also demonstrated a subtle but significant increase in SBP in female C57BL6/J mice compared with male C57BL6/J mice (Deschepper et al., 2004). However, earlier studies using tail cuff plethysmography did not show any sexual dimorphism in SBP (Hoit et al., 2002) and there was no difference in SBP between WT M and WT F mice at postnatal week 13. In contrast to human studies (Nilsson et al., 1997), a reduced fetal weight at birth was not associated with increased SBP in either P0 M or P0 F mice at postnatal week 8 or week 13. These results are supported by an as yet unpublished study demonstrating no increase in SBP in P0 M mice compared with WT M littermates at 5, 12 and 17 months of age (Susan Ozanne, personal communication). In a rodent model of maternal low protein diet, birth weight was significantly lower in both male and female offspring from dams fed a low protein diet vs. normal protein diet (Woods et al., 2005). However, only male mice demonstrated a hypertensive phenotype at postnatal week 20 (Woods et al., 2005). Therefore, the lack of programmed hypertension in P0 offspring is not unique and could be helpful in distinguishing the mechanisms related to intrauterine programming of cardiovascular disease. It could be that, given the lack of vascular phenotype in the P0 mouse during pregnancy (i.e. normal uterine and umbilical blood flow / vascular function; Kusinski et al., 2011, Dilworth et al., 2013) and the lack of endothelial dysfunction near term in P0 vs. WT fetal aortas (see chapter 3 and 4), P0 mice do not develop hypertension in adulthood; such a phenomenon may be the case in some sub-sets of clinical cases of FGR.

5.3.6 Effect of antenatal sildenafil citrate on systolic blood pressure in Igf2 P0+/− and WT offspring

Collectively the results of this study demonstrate that irrespective of whether fetuses were smaller at birth, SC treatment during mid-late mouse gestation led to an elevated SBP in both male and female offspring. Several studies have focused on manipulating the maternal environment in the pre- and post-implantation periods to assess the consequences on the offspring. In particular, dietary, oxygen tension and therapeutic manipulations have been routinely used to study effects on offspring cardiovascular and metabolic homeostasis (Langley-Evans et al., 1999, Williams et al., 2005, De Vries et al., 2007, Tain et al., 2014).
Dietary intervention with moderate maternal under-nutrition in the pregnant rat led to an increased systolic blood pressure in the offspring; however this increase (5 – 8 mm Hg) was more subtle than the increase in SBP caused by antenatal SC treatment herein (Woodall et al., 1996). In a rat pregnancy model, maternal protein restriction has been shown to result in elevated blood pressure in offspring (increase from 108 mmHg to 124 mmHg, in control vs. protein restriction offspring, respectively) and endothelial dysfunction in both dam and offspring (Torrens et al., 2006). Folate supplementation was able to reduce endothelial dysfunction and ablated the elevated SBP (reduced to 113 mmHg) present in male offspring (Torrens et al., 2006). More recent data in mice have also confirmed elevated SBP in offspring of maternal low protein diet (increase from 110 mmHg to ~115 mmHg in control vs. low protein offspring, respectively; Watkins et al., 2008). Furthermore, this study demonstrated that, akin to a previous study in humans (Stein et al., 1975), the gestational time point at which the dietary intervention took place was important in the development of cardiovascular dysfunction in the offspring (Watkins et al., 2011). These studies suggest the timing of SC administration may well be important, as organ development is ongoing during the last third of pregnancy (in both mouse and human). Further studies are therefore warranted to assess the effect of timing of SC intervention to balance the positive benefit of increasing fetal growth against any possible adverse long-term effects that are apparent in offspring from SC-treated dams.

The mechanism(s) by which antenatal SC led to increased SBP in offspring is unknown. It is possible that increased weight gain in early life could influence SBP however; increased SBP was independent of sex i.e. only female mice from SC-treated pregnancies demonstrated an increase in weight. These data suggest the observed hypertension is not as a result of diet and / or weight. The elevated SBP present in offspring from SC-treated pregnancies cannot be explained by the restraint involved with the tail-cuff measurement as control offspring (handled / monitored under the same conditions) had normal SBP values. Additionally, my preliminary studies show that there are no alterations in basal concentrations of the stress hormone corticosterone in (male and female) offspring from SC-treated pregnancies when compared with offspring from saline-treated pregnancies (see appendix). Nevertheless, measurements using radiotelemetry on conscious unrestrained offspring would be beneficial as offspring from SC-treated dams may be more susceptible to stress provoking stimuli- such as the restraint associated with the tail-cuff method. Radiotelemetry measurements would also allow for the measurement of indices associated with the control of blood pressure, such as cardiac output and heart rate. In addition, measurements of PDE-5, sGC, cGMP, NO and oxidative stress in fetal and offspring tissues would inform of any possible mechanisms underlying the effects of maternal SC treatment.
There was a reduction in endothelial-dependent and –independent relaxation responses of fetal aortas from dams that were given access to 0.8 mg.ml⁻¹ SC in the drinking water, suggestive of vascular dysfunction in the fetus (see chapter 3). This constrictor phenotype is similar to that found in the hypertensive eNOS⁻/⁻ mouse model, which lacks the eNOS enzyme and has a phenotype that includes increased systolic blood pressure (Huang et al., 1995) and also mimics previous data demonstrating that antioxidant treatment in pregnancy alters vascular function in offspring not exposed to in utero hypoxia (Giussani et al., 2012). Therefore, it is likely that results in isolated vessels (using wire myography) are akin to vascular dysfunction in vivo which, in turn, is associated with adulthood hypertension (Leeson et al., 2001).

Fetal arteries from pregnancies treated with a subcutaneous dose of SC only demonstrated a subtle degree of endothelial dysfunction. For example, P0 F fetal aortas from dams treated with SC (chapter 4) demonstrated reduced ACH-induced relaxation responses when compared with P0 F fetal aortas from saline-treated dams. WT M, WT F and P0 M fetal aortas from SC-treated dams demonstrated similar endothelial-dependent and –independent relaxation responses when compared to saline-treated pregnancies. However, it could be possible that such dysfunction may be exacerbated with age and may only be present in adulthood. In support of this, WT M and P0 M mice from SC-treated dams show similar endothelial-dependent and –independent relaxation at E18.5 when compared with WT M and P0 M mice, respectively, from saline-treated pregnancies (chapter 4); however, WT M and P0 M offspring from dams treated with SC have impaired endothelial-dependent and –independent aortic relaxation in adulthood (figure 5.14A and figure 5.14C) which suggests age may exacerbate any effects of SC on vascular function.

A study published by Ohashi and colleagues (1998) demonstrated that in transgenic mice that over express eNOS, aortas had attenuated relaxation to ACH and SNP (Ohashi et al., 1998). The authors suggest that this attenuated response was due to a reduced response of cGMP to stimulation by ACH and SNP (i.e. a desensitization effect). Yamashita and colleagues (2000) demonstrated that within transgenic aortas there was a 50 % reduction in activity of sGC, a second messenger involved in NO-dependent vasodilation and a reduction in protein and activity of PKG. The authors therefore suggest these two molecules may be involved in the generation of “nitrate tolerance” in situations where there is excess NO bioavailability. Systemic vascular resistance was also assessed in eNOS over-expressor mice using isoflurane-anesthetised open-chest surgery (Van Deel et al., 2007). Systemic vascular resistance was increased in transgenic mice with a reduced response to SNP. There was no further reduced systemic vascular resistance when blocking PDE-5 (using EMD-360527 rather than SC) and 8-bromo-cGMP pathways, thus there is a direct blunted response of GC to NO. Therefore, it could be possible that antenatal SC has led to desensitisation of the fetal vasculature to NO as a compensatory mechanism for the
increased bioavailability of NO. Additional studies are required to assess expression and activity of cGMP, sGC, PKG and related proteins in fetal and offspring vasculature.

5.3.7 Blood glucose concentration in \textit{Igf2} P0\textsuperscript{+/−} and WT offspring from control C57BL6/J dams; effects of sex

In the current study there were sexually dimorphic responses to a glucose tolerance test. Irrespective of genotype, female mice were more sensitive to a glucose challenge than male equivalents (figure 6A and 6D). Female mice are known to have a greater deposition of adipocytes but unlike male mice these fat deposits are mainly subcutaneous rather than abdominal (Chen et al., 2012). Additionally, adipocytes from female mice are more sensitive to insulin and this is likely due to increased circulating concentrations of oestrogen compared with male mice (Guerre-Millo et al., 1985; Macotela et al., 2009). Therefore, female mice have a greater uptake of glucose (as seen in figure 6A and 6D) in response to insulin. P0 F mice demonstrated similar glucose tolerance profiles compared with WT F littermates suggestive of normal glucose metabolism in response to insulin (figure 6B). Although blood glucose concentrations can be useful in understanding insulin sensitivity, more insight could have been gained with direct insulin measurements, however, this was not assessed in the current study.

When male mice received explants of subcutaneous fat implanted into regions of visceral fat there was an overall reduction in body weight, total fat mass and reduced plasma glucose and increased sensitivity to insulin (Tran et al., 2008). P0 M mice were similar in body weight when compared to WT M equivalents. However, it is not improbable that P0 M mice may have a greater deposition of subcutaneous rather than visceral fat and thus have increased glucose sensitivity (figure 6C). A thesis by Keiran Matharu (University of Cambridge) studying the impact of P0 transcript deletion on offspring longevity and metabolic function did not reveal any difference in glucose tolerance between WT and P0 M mice (Susan Ozanne, personal communication). This inconsistency may well be explained by differences in methodologies and the age at which the glucose tolerance test was performed (postnatal 17 months); further interrogation of metabolic functions in the P0 M is therefore required.

Although visceral fat is thought to be important in the development of type 2 diabetes there is now contradictory evidence to suggest that the role of visceral fat may not be as significant as previously thought. Male mice transplanted with visceral fat from male littermates did not demonstrate increased plasma glucose but instead this procedure led to reduced plasma glucose concentrations and improved insulin sensitivity (Konrad et al., 2007). In addition to a lack of insulin tolerance assessment, another limitation of the current study was a lack of measurement of visceral fat and/or subcutaneous fat using dual-energy X-ray absorptiometry (Chen et al.,
2012). These data would prove useful and are required for further characterisation of the \textit{Igf2 P0}^{+/-} phenotype.

5.3.8 Effect of antenatal sildenafil citrate on blood glucose concentration in \textit{Igf2 P0}^{+/-} and WT offspring

In WT and P0 female offspring from SC-treated pregnancies, AUC for blood glucose was higher than the equivalent WT and P0 female controls, respectively, from saline-treated pregnancies. Unlike previous studies purporting to show females are protected against metabolic syndrome in cases of high fat diet (Pettersson et al., 2012), treatment of dams with SC selectively reduced glucose sensitivity in female but not male offspring. The increased glucose sensitivity in P0 M was also not reversed by antenatal SC. Combined with the effect of antenatal SC on female offspring growth, these data suggest female mice are more susceptible to the effects of antenatal SC.

Previous studies suggest female mouse offspring are protected against obesity-induced metabolic dysfunction when fed a high fat diet (Pettersson et al., 2012). These results were also confirmed by further work demonstrating maternal high fat diet led to insulin resistance and altered pancreatic \(\beta\) cell function in male but not female mouse offspring (Yokomizo et al., 2014). Therefore, reduced glucose metabolism in female mice is likely not an effect of increased body weight but rather both are a direct effect of SC-treatment \textit{in utero}. The mechanism(s) behind this effect is not known though altered function of pancreatic beta cells could represent a possible explanation. Further insight could have been gained from the measurement of offspring plasma insulin concentration following a glucose challenge and morphological examination of alpha and beta pancreatic cells.

5.3.9 Offspring organ weight in \textit{Igf2 P0}^{+/-} and WT mice from control C57BL6/J dams; effect of sex

The results of the current study show genotype did not affect offspring body weight between 5 and 12 weeks. There was also no effect of genotype on any of the observed organ weights, suggesting there is no effect of a reduction in placental IGF-2 on organ weight. This effect is not unexpected as \textit{Igf2 P0}^{+/-} led to a reduction in placental IGF-2 but did not affect other \textit{Igf2} transcripts (Constância et al., 2002); thus circulating concentrations of IGF-2 within the fetus were unaltered. There was a significant decrease in heart (WT) and average kidney weights (WT and P0) in females when compared with males; however when these data were assessed as a percentage of body weight there was no significant difference (data not shown).
5.3.10 Effect of antenatal sildenafil citrate on offspring organ weight in *Igf2* P0+/− and WT mice

Although organ weight does give insight into the development of the organ, it does not allow for functional assessment. For example, it has recently been reported that although a maternal low protein diet lead to elevated SBP (Watkins et al., 2008) there was no overall effect on organ (e.g. heart, liver, lungs and kidney) weight in offspring from low protein diet fed compared with offspring from dams fed a normal diet (Watkins et al., 2011). Furthermore, in mice, altered embryo culture followed by embryo transfer leads to increased SBP in subsequent offspring (Watkins et al., 2007), with minor differences in organ weight between control and treatment groups. Although heart weight was not altered, it may have proved useful to assess the morphology of the ventricles as left ventricular hypertrophy is a common response to hypertension. For example, left ventricular hypertrophy and increased systolic blood pressure were observed in WT mice infused with angiotensin II but not in transgenic mice lacking the angiotensin II receptor gene (Ichihara et al., 2001).

Fetal exposure to SC led to an increased and decreased average kidney weight in WT M and WT F offspring, respectively, however when these data were expressed relative to body weight there were no differences (data not shown). Langley and colleagues (1999) previously demonstrated that in a rat maternal dietary protein restriction model, reduced kidney size and a reduction in nephron number was likely to be the mechanism for the onset of hypertension (Langley-Evans et al., 1999). Although kidney organ weight was differentially altered, further studies are required to assess nephrogenesis in offspring. Additionally, alterations in renal artery morphology have been suggested to be a key cause of secondary hypertension. Ligation of the mouse renal artery led to an elevated SBP (Stouffer et al., 2010) via increased cardiac output. Collectively the results herein suggest raised blood pressure is perhaps not related to organ allometry but is occurring through other mechanisms.

5.3.11 Offspring vasoconstriction and vasorelaxation in *Igf2* P0+/− and WT mice from control C57BL6/J dams; effect of sex

There was a significant reduction in P0 fetal weight at E18.5 when compared to WT littermates (see chapter 4). P0 offspring, which presumably had a significantly reduced body weight at E18.5, did not have altered mesenteric artery contractile response to U46619. These observations are in contradiction to those reported by Anderson and colleagues (2006) in mesenteric arteries of male and female rat offspring (Anderson et al., 2006). Offspring subjected to placental insufficiency (through surgical ligation of the abdominal aorta) in late gestation, had increased vascular
contraction to PE compared with control rats; this effect was augmented in the second generation. However, ligation of the abdominal aorta results in a severe FGR phenotype with a considerable reduction in litter size and thus differences between Anderson et al., (2006) and the current study may be due to the severity of the method used to induce FGR.

There were no gender differences in mesenteric contraction responses to PE or U46619, except in WT F whereby there was a reduced contraction of mesenteric arteries to U46619. In other studies, female offspring were shown to have reduced myogenic tone in response to increased isobaric pressure (Chan et al., 2012, Gros et al., 2002) but there is also conflicting evidence to suggest no overall gender differences in mesenteric response to PE or depolarising potassium solution (McKee et al., 2002).

P0 F offspring mesentery demonstrated reduced propensity to relax in response to ACH but showed no difference with endothelial-independent stimulation with NO which suggests endothelial dysfunction in P0 F but not vascular smooth muscle cell desensitisation to NO. These data are unlike those obtained from the rat model of placental underperfusion (Anderson et al., 2006) where female offspring, which were growth restricted at birth, did not show any altered mesenteric artery response to ACH. In the current study, WT F offspring mesenteric arteries demonstrated reduced relaxation in response to SNP (but normal ACH responses). Collectively, these results are contradictory to other studies in rat (Tatchum-Talom et al., 2011) which suggest female mesenteric arteries are more sensitive to ACH and direct addition of NO. However, recent mouse studies using endothelium-dependent stimulation and direct addition of NO have demonstrated reduced endothelium-derived relaxation response of female mesenteric arteries compared with male mesenteric arteries (Chan et al., 2012). The authors conclude that mesenteric arteries from female mice are less sensitive to sGC-dependent pathways and that EDHF is far more important in regulating vascular tone. Experiments using other endothelium-dependent agonists that preferentially stimulate non-NO pathways would be informative.

Offspring aortic vascular contraction to U46619, PE and KPSS was not altered when comparing genotype or gender. An earlier study, in rats, attempted to assess the effect of growth restriction using maternal dietary restriction on offspring vascular function (Ozaki et al., 2001). Although not directly comparable to abdominal aortas, femoral arteries from both male and female rat offspring, which were growth restricted at birth, demonstrated no difference in maximal contraction to PE or noradrenaline in adulthood. However, male offspring which were growth restricted at birth demonstrated an increased contraction to U46619 at 200 days of age; in contrast P0 M offspring (~98 – 112 days of age) did not show altered U46619-induced contraction compared with WT M offspring in the current study. These results suggest that either the method
by which FGR is induced (e.g. maternal dietary restriction or genetic manipulation) or age may affect results obtained from *ex vivo* vascular function studies.

Although a direct comparison was not made between sexes, Fulton and colleagues (2002) demonstrated a reduced response of male rat aortas to PE when compared with female rat aortas; this effect was not seen in arteries in either WT M or P0 M offspring when comparing with WT F or P0 F offspring, respectively. Additionally, Li and co-workers (2005) have also shown female rat aortas demonstrate greater contraction in response to U46619 when compared with male rat aortas; this effect was also not seen in either WT or P0 female offspring aortas when compared with WT or P0 male offspring aortas, respectively (Li et al., 2005). Thus, there seem to be sex- and species-specific differences in vascular responses to vasoconstrictors.

Adult male offspring from hypoxic pregnancies that were significantly smaller at birth do not show long-term endothelial-dependent or -independent vascular dysfunction (Williams et al., 2005). Furthermore, both male and female rat offspring from dams that were fed a 70 % restricted diet, did not demonstrate any altered response to endothelial-dependent or -independent vasodilators but did demonstrate hypertension in male, and later female offspring (Ozaki et al., 2001). This study did however demonstrate an overall age-dependent blunted response to ACH in offspring; therefore it may have proved useful in the current study to observe vascular responses in later adulthood.

The data in the current chapter therefore suggests that growth restriction, due to placental insufficiency, was not associated with widespread endothelial dysfunction in either conduit or resistance arteries of P0 offspring compared with WT offspring. Given the fact that umbilical artery and uterine artery blood flow (Dilworth et al., 2013) and function (Kusinski et al., 2011) are not altered in the P0 mouse, it could be that this mouse represents a specific subset of FGR cases which are not at increased risk of hypertension or metabolic disease in adulthood.

**5.3.12 Effect of antenatal sildenafil citrate on offspring vasoconstriction and vasorelaxation in Igf2 P0+/- and WT mice**

Antenatal SC led to a subtle reduction in maximal contraction of WT M mice mesenteric arteries to PE when compared to equivalent untreated WT M. However, antenatal SC did not lead to increased contraction of mesenteric or aortic offspring arteries in response to U46619 or KPSS. Therefore, antenatal SC did not lead to an overall increase in contraction in offspring mesentery and is unlikely to be the mechanism associated with the increased SBP in offspring from SC-treated dams. Observations of increased BP without altered *ex vivo* vascular function have been previously reported in mice. For example, offspring from dams that were fed a low protein diet
exhibited adulthood hypertension without altered vasoconstriction (Watkins et al., 2008). Aortas from male mice, but not females, were less responsive to ACH-induced and SNP-induced relaxation. Interestingly, these alterations in aortic vascular function were not consistent in peripheral resistance arteries. Together, these data suggest that although antenatal SC treatment is able to alter vascular function in male and female mice of both WT and P0 mice, hypertension cannot be explained by these alterations alone.

5.4 Summary

In humans, growth restriction in utero is associated with increased risk of hypertension in adulthood (Barker et al., 1989). Mechanisms involved in fetal programming of adulthood disease have been examined in animal models of FGR. Endothelial dysfunction in humans (Leeson et al., 2001) and animals (Ozaki et al., 2001, Roberts et al., 2005) is thought to be one of the causes of the development of hypertension in offspring which were growth restricted in utero. Surprisingly, the placental-specific Igf2 P0+/− mouse model of FGR, which demonstrates postnatal catch up growth in both sexes, failed to show any consistent abnormalities in endothelial function in male or female offspring which were growth restricted at birth, and were not hypertensive at 8 and 13 weeks of age. These data therefore suggest that the P0 mouse model may only represent a good model to assess in utero growth restriction but not long-term programming effects associated with poor growth in utero.

Sildenafil citrate (10 mg.kg−1), administered subcutaneously to the dam, did not increase fetal or placental weight at E18.5 in the P0 mouse model of FGR. The ratio of P0 offspring compared with WT offspring was significantly reduced suggesting postnatal death in P0 mice. Antenatal SC did not normalise the ratio of P0 to WT offspring; however, maternal SC administration did show effects on growth, cardiovascular / metabolic function and vascular function in offspring. Bodyweight of WT female mice was increased in the offspring of SC-treated dams compared with those of saline-treated dams. SC treatment was also associated with decreased sensitivity to glucose in both WT and P0 females (i.e. irrespective of whether they were born growth restricted or not). In addition, antenatal SC was associated with hypertension in offspring, irrespective of gender or growth in utero. Altered endothelial-dependent and –independent relaxation responses were noted in aorta and mesenteric arteries from offspring of SC-treated dams but these responses were not always consistent and suggest other mechanisms may be responsible for the widespread hypertension seen in offspring of dams treated with SC.
Chapter 6  General Discussion
6.1 Discussion

FGR affects between 5 - 10 % of pregnancies in the UK and is the single largest cause of stillbirth and neonatal death. There are no treatments for FGR and often the only solution is to deliver the baby and placenta pre-term, which in turn, is associated with neonatal mortality and morbidity. Therefore, due to the importance of FGR and the clear lack of treatments, developing the ability to assess the efficacy and safety of possible treatments in animal models of FGR is extremely important.

In addition to the short-term poor prognosis, fetuses which are small at birth are at increased risk of cardiovascular and metabolic diseases in later life (Barker et al., 1989, Phillips et al., 2005). Thus, current research is focussed on finding treatments which may increase fetal growth in FGR and in turn also reduce the risk of adulthood disease associated with small size at birth. In rat models of FGR, antenatal melatonin, vitamin C and folate have all been shown to prevent programmed adulthood disease associated with small size at birth (Tain et al., 2014, Kane et al., 2013, Torrens et al., 2006). Developing a method to assess the effects of antenatal treatments on fetal vascular function in mice, thus exploiting genetic models of FGR, is important to determine whether antenatal treatments have affected the fetus in utero.

Recently, antenatal SC treatment has been shown to increase abdominal growth trajectory in severe early onset human FGR fetuses (Von Dadelszen et al., 2011) and animal studies suggest antenatal SC increases placental and fetal blood flow (Stanley et al., 2012), total nutrient transfer (Dilworth et al., 2013) and amino acid bioavailability in the fetus (Satterfield et al., 2010). The placental-specific \(\text{Igf2}^\text{P0}\) mouse, a mouse model of FGR with growth restricted and appropriate birthweight littermates, which has previously been used to assess the effects of antenatal SC treatment on fetal growth (Dilworth et al., 2013), represented an excellent model to develop a methodology; firstly to assess fetal vascular function in response to antenatal treatment with SC and secondly to assess fetal vascular function in a mouse model of growth restriction. As it has been well documented that sex-specific differences are apparent with respect to vascular reactivity (Chan et al., 2012, Luksha et al., 2006, Fulton et al., 2002), fetal vascular responses in both sexes were assessed in the \(\text{Igf2}^\text{P0}\) mouse.

Currently, no studies have been performed to determine the long-term effects of antenatal SC treatment on offspring health. With the commencement of the STRIDER multicentre international clinical trial assessing the effect of antenatal SC treatment in severe early onset cases of FGR (Ganzevoort et al., 2014), this thesis aimed to determine the effects on the fetus of two distinct regimens of antenatal SC treatment in a mouse model of FGR and additionally, to phenotype offspring from one regimen to assess effects of antenatal SC treatment on long-term health.
There were two main findings from this project. Firstly, antenatal SC treatment in the *Igf2* P0+/− mouse model of FGR led to a significant increase in systolic blood pressure in both male and female mice. Antenatal SC treatment also reduced glucose sensitivity, a marker of metabolic disease, in female but not male mice. Importantly, the effects of antenatal SC treatment on offspring systolic blood pressure and glucose tolerance were independent of size at birth. Secondly, The P0 mouse model of FGR was not associated with fetal or offspring vascular dysfunction and did not result in hypertension or alterations in markers indicative of metabolic disease in either male or female mice that were growth restricted at birth. These main observations will now be discussed with specific consideration to the relevance of such observations to human FGR.

6.1.1 A methodology to study fetal vascular function in mouse pregnancy and assess the impact of antenatal treatment with sildenafil citrate on fetal vascular function.

This study has shown that mouse fetal abdominal aortas can be successfully isolated and that blood vessel function can be assessed using ex vivo wire myography. Fetal vascular reactivity in response to endothelium-dependent and endothelium-independent agonists was assessed. Future studies may now be able to draw comparisons between fetal vascular function ex vivo and vascular function in vivo. In addition, researchers now have the ability to assess the effect of antenatal treatments on fetal vascular function ex vivo in mice; these measures will provide understanding of the effect of antenatal treatments on fetal vascular reactivity in vivo.

SC increases fetal weight in mouse models of FGR by improving umbilical artery blood flow (Stanley et al., 2012) and increasing placental size and total nutrient transfer (Dilworth et al., 2013). The current study extended the previous approaches and examined the effects of a supraphysiological dose of antenatal SC (120 - 160 mg.kg⁻¹) to assess whether there were measurable effects on fetal vascular function. Fetal weight was not altered with this higher concentration of SC but widespread endothelial dysfunction in fetal abdominal aortas from both male / female growth restricted and appropriate weight fetuses at E18.5 was detected. Therefore, although the high dose of antenatal SC treatment was ineffective with regards to altering primary outcome (birth weight), fetal vascular function was altered independent of effects on fetal weight. A subcutaneous dose of 10 mg.kg⁻¹ antenatal SC, presumably a more stable dose than those previously administered in drinking water (Dilworth et al., 2013, Stanley et al., 2012), in the dam, did not increase fetal weight in P0 fetuses and did not cause widespread endothelial dysfunction in either growth restricted or appropriate weight fetuses. It must be noted that although the dose of SC used in the subcutaneous injection study (10 mg.kg⁻¹ SC) was
hypothesised to give similar maternal plasma concentrations of SC as previously achieved in clinical trials of PET with FGR (Samangaya et al., 2009), there is currently no data to confirm that similar plasma concentrations were achieved in the mice. However, maternal and fetal plasma samples were collected and these are currently being assessed for the concentrations of SC. Whether there are differences in placental transport and metabolism of SC in human and mouse pregnancy is also unknown and further work should focus on addressing these issues.

Overall, these data suggest that determining the dosage and the route of administration of SC will be important criteria to maximise SC efficacy in cases of human FGR. The STRIDER trial will be assessing the effect of SC treatment in cases of severe early onset FGR on neonatal survival (primary outcome) and fetal growth (secondary outcome) between 20 and 32 weeks gestation (Ganzevoort et al., 2014). SC will be administered in tablet form and the dosage will be 25 mg, three times daily, in pregnancies which fulfil the criteria for the clinical trial. Thus, it is likely that SC will reach concentrations in the mother of similar proportions to that of the earlier trial of antenatal SC treatment in pregnancies affected by PET (271 ng/ml; Samangaya et al., 2009). Importantly, SC treatment will mainly be started in the second trimester and will continue until delivery / neonatal death or 32 weeks gestation, whichever is first. With regards to this issue of timing of dosing regimen, we utilised E12.5 as a point at which to commence administration of SC. This time point equates to the second trimester in mouse pregnancy and is equivalent to the earliest possible point in which severe FGR could be detected in the human and therefore when SC treatment would likely be commenced.

The current study did not assess whether the timing of antenatal SC treatment has differential effects on fetal weight or fetal vascular function. Further work in other mouse models of FGR could investigate the effect of different dosages / timings of antenatal SC treatment and these data will provide evidence to maximise SC efficacy in human cases of FGR. Conversely, it could also be the case that clinical observations of SC in cases of FGR or PET determine when dosing would have to occur for maximal efficacy; various scenarios could be replicated in animal models of FGR to assess the effect of antenatal SC treatment on offspring health.

This study has also provided evidence that agonist-induced mouse fetal vascular function was not consistently altered in the P0 mouse when compared to WT mice (chapter 3 and chapter 4). These data are similar to previous findings in uterine and umbilical arteries, which demonstrated that there were no differences in ex vivo vascular function between P0 and WT littermates (Kusinski et al., 2011). Correlations of fetal weight against indices of vascular function were also performed. However, there was no association between fetal weight, gender or treatment on fetal or offspring vascular function (data not shown). Taken together, normal P0 fetal, umbilical and uterine artery vascular function confirm that, as previously reported, the key mechanism for
growth restriction in the P0 mouse model is altered placental transport due to a thickening of the placental interhaemal membrane and not vascular dysfunction / reduced utero or fetoplacental blood flow (Kusinski et al., 2011, Dilworth et al., 2010, Sibley et al., 2004, Constância et al., 2002). Given the fact that uterine, umbilical and fetal blood flow and vascular function is unaltered in P0 mice, this mouse model may represent the majority of late onset human FGR cases which, of those pregnancies that are assessed with Doppler ultrasound, do not demonstrate abnormalities in uterine or umbilical artery blood flow (Cnossen et al., 2008). Therefore the P0 mouse remains an appropriate model with which to assess antenatal treatments.

6.1.2 Impact of antenatal treatment with sildenafil citrate on offspring growth, cardiovascular and metabolic function

There was significant neonatal mortality in P0 mice when compared to WT littermates, akin to human FGR. In human FGR the risk of stillbirth is greater in male fetuses (Gardosi et al., 2013). Further insight could have been gained from assessing the relative risk of death in male and female P0 neonates when compared to their appropriate birthweight littermates. Antenatal SC treatment did not reduce neonatal mortality in P0 offspring but did increase offspring weight in a sex-dependent manner. WT female offspring had increased bodyweight from week 5 to week 12 of age. Females from SC-treated dams, irrespective of genotype and birthweight, also demonstrated reduced glucose sensitivity in adulthood. These latter data suggest an alteration in glucose metabolism or insulin sensitivity. Future work should determine if circulating levels of insulin, cellular insulin sensitivity and pancreatic β cell function are altered in offspring of treated dams; these data would provide further information on the mechanisms by which antenatal SC impairs glucose tolerance in female offspring.

The most important finding of this study was that antenatal SC treatment led to an increase in systolic blood pressure in offspring of both WT and P0 offspring, irrespective of sex. This evidence of a programming effect of SC is of note since it occurred in an animal model that is otherwise normotensive. It will be of interest to determine if antenatal SC treatment in animal models which do demonstrate programmed hypertension may have even greater effects on fetal / offspring morbidity / mortality. The most obvious mechanism for increased systolic blood pressure would be altered vascular function. However, neither male nor female mice demonstrated consistent reductions in endothelial-dependent or –independent relaxation responses of mesenteric or aortic arteries, suggesting blood pressure differences may be attributed to other mechanisms, such as altered nephrogenesis or increased cardiac output. It is possible that altered nephrogenesis may lead to a reduction in nephron endowment which can lead to increased salt retention, water retention and an increase in total blood volume. The physiological response to
an increase in blood volume is an increase in cardiac output. As cardiac output is controlled by heart rate and stroke volume, any increases in either of these two components will have effects on cardiac output and thus blood pressure (Despopoulos and Silbernag, 2001). An increase in heart rate in offspring from SC treated pregnancies may arise as a consequence of increased norepinephrine release from the adrenal medulla or postganglionic noradrenergic neurons supplying the heart. Norepinephrine release has a positive chronotropic effect on the heart through beta 1 adrenergic receptors and therefore would cause an increase in heart rate, cardiac output and blood pressure. It is also important to note that an increased blood volume would increase the amount of blood entering the left ventricle of the heart, which, in turn, stretches the myocytes and causes an increase in the force of contraction (positive inotropic effect) and the amount of blood ejected from the left ventricle during each heartbeat (Despopoulos and Silbernag, 2001). It is likely that the increased SBP in offspring from SC treated dams could be caused by raised peripheral vascular resistance as small changes in resistance have profound effects on blood pressure. The raised resistance could reside in vascular beds not investigated in the current study. Therefore, future studies should focus on assessing total peripheral vascular resistance in offspring from SC treated pregnancies. In addition, blood flow indices using the Doppler ultrasound technique would have proven useful in assessing whether there were alterations in resistance to flow in vasculature of offspring from SC treated pregnancies.

As there were similar increases in systolic blood pressure in both male and female offspring from SC treated dams, it is probable that the mechanism by which SC increases systolic blood pressure is likely the same in male and female mice. It could be that the timing of the SC treatment in this particular case has direct effects on the development of the kidney in utero culminating in an elevated systolic blood pressure in offspring due to increased blood volume and cardiac output. Alternatively, in utero SC treatment may have a direct effect on long-term cardiac function. A study by Borlaug and colleagues (2005) demonstrated that in healthy adult volunteers SC treatment led to a reduction in the cardiac systolic response to the beta-adrenergic stimulator dobutamine, thus the authors suggested PDE-5 plays a role in the response to stimulated cardiac function. Beta-adrenergic agonists such as norepinephrine ensure cardiac contractility is balanced by blunting the response with NO synthase, leading to increased cGMP synthesis and activity of PKG (Takimoto et al., 2005). Inhibition of PDE-5 by SC in mouse hearts led to increased cGMP synthesis and activity of PKG and a reduction in the cardiac contractility to the beta-adrenergic agonist isoproterenol (Takimoto et al., 2005), once again demonstrating the important role of PDE-5 in modulation of cardiac contractility. It is worth reiterating that in transgenic mice overexpressing eNOS, there are increased concentrations of cardiac cGMP but a 50% reduction sGC and a significant reduction in PKG protein in isolated vessels (Ohashi et al., 1998, Yamashita et al., 2000). It is possible that in utero SC treatment in mice could reduce systolic responsiveness
to beta-adrenergic stimulation in the fetal heart, through increases in cGMP concentration and PKG activity. An excess of fetal cardiac cGMP and PKG could in turn lead to a reduction in cardiac sGC activity in the fetus, which may persist into adulthood. If so, a reduction in cardiac sGC and PKG activity in the absence of SC could lead to an increased contractility in response to beta-adrenergic agonists such as norepinephrine in adulthood and a blunted cardiac response to NO relaxation, thus leading to an increase in heart rate, cardiac output and systolic blood pressure. To test this hypothesis, future studies would need to determine cardiac sGC and PKG activity in the fetus and in offspring, and assess in vivo and ex vivo cardiac function in response to beta-adrenergic agonists as well as inhibitors and activators of sGC / PKG. These experiments would reveal whether antenatal SC treatment is detrimental cardiac function in the long term, through the NO directed pathway.

Taken together, and given the fact that no studies have assessed the longitudinal effects of antenatal SC treatment, these findings on the detrimental effects of antenatal SC on offspring cardiovascular and metabolic functions are extremely important and warrant further investigation using other dosing regimens and / or animal models of FGR. Of particular interest would be to assess the effects of antenatal SC in a model of FGR with a well-defined vascular phenotype. In such a model, antenatal SC treatment may increase uteroplacental blood flow and increase fetal weight (as is the case in the COMT⁻/⁻ mouse model of FGR; Stanley et al., 2012) but could alter cardiovascular / metabolic function in offspring. Thus, future studies should consistently assess not just the effects of antenatal treatments on fetal weight, but also the long-term consequences of treatment on offspring health.

6.1.3 Effects of FGR on programming of adulthood disease in the Igf2 P0⁺/⁻ mouse.

A secondary finding of this thesis was that, of those P0 offspring that survived, systolic blood pressure in males and females did not differ from the blood pressure of the WT (appropriate birthweight) offspring. Additionally, in support of recent findings (Mikaelsson et al., 2013), P0 mice also demonstrate accelerated postnatal catch-up growth and reached WT littermate bodyweight by postnatal week 5. It was also obvious from the growth trajectories of both male and female mice that there was a clear separation of bodyweight between the two sexes (chapter 5), irrespective of size at birth. In humans, postnatal catch up growth in small for gestational age babies is associated with alterations in morphology and function of arteries in young children (Evelein et al., 2013, Brodzszi et al., 2005), which in turn, is associated with hypertension (Leeson et al., 2001) and death from coronary heart disease (Eriksson et al., 2001) in adulthood. The data presented in the current study show that in the P0 offspring, regardless of sex, mesenteric artery
and abdominal aortic artery vascular reactivity were normal and probably reflect normal vascular physiology in vivo.

In addition to an increased risk of cardiovascular disease, growth restriction is associated with the development of reduced glucose sensitivity, reduced insulin sensitivity (i.e. increased plasma concentrations of glucose and insulin) and type 2 diabetes (Phillips et al., 2005, Newsome et al., 2003) in adulthood. I aimed to assess glucose tolerance, a marker of metabolic function, in the P0 mouse and found that neither males nor females demonstrated impaired glucose sensitivity; unexpectedly, P0 males showed increased glucose sensitivity. In chapter 5 it was also demonstrated that there are differences in glucose tolerance between male and female mice, irrespective of size at birth. These observations are supported by previously published data (Macotela et al., 2009). The fact that P0 mice do not demonstrate impaired glucose sensitivity contradicts evidence in animal models of FGR induced by undernutrition which suggest that reduced dietary intake by the dam and subsequent FGR leads to reduced glucose sensitivity in the offspring (Phillips et al., 2005). However, examination of insulin sensitivity and glucose tolerance in later adulthood in P0 mice (of both sexes) would provide further support to the finding that, in the P0 mouse, growth restriction is not associated with the development of impaired glucose / insulin metabolism or type 2 diabetes. Whether ageing exacerbates the effects of growth restriction on blood pressure may also be informative in the P0 mouse. It could be that, as measurements of blood pressure and glucose tolerance were only performed in early adulthood (corresponding to 20 – 30 years of age in humans), P0 mice may go on to develop hypertension and insulin resistance in later life. Additionally, in humans, the risk of type 2 diabetes is highest in those who were born small and are obese in adulthood (Hales et al., 1991); therefore P0 offspring fed a healthy nutritional diet may only develop cardiovascular / metabolic dysfunction when fed a diet high in fat (i.e. if they are exposed to a second stressor).

6.1.4 Effects of a maternal subcutaneous injection of saline or sildenafil citrate

Another interesting finding of this study was that a daily subcutaneous injection with saline or SC led to greater U46619-induced contractions of fetal aortas when compared with U46619-induced contractions in isolated fetal aortas from water controls. It is possible that the injection caused stress in the animal and elevated blood corticosterone concentrations. A previous study by Nugent and colleagues (2013) showed that when chorionic plate arteries were exposed to glucocorticoids there was an increased vasoconstriction to U46619. A number of animal studies have also demonstrated that prenatal overexposure to glucocorticoids either through exogenous addition of glucocorticoids or by inducing stress in the mother (Langley-Evans et al., 1998, Cuffe et al., 2014) is a prerequisite for adult cardiometabolic disease (for review see Drake et al., 2007).
Although maternal corticosterone was not measured in the current study, it could be that the stress of a subcutaneous injection alters sensitivity of the fetal and offspring vasculature to vasoconstrictors such as U46619. In addition, maternal glucocorticoid overexposure in the last week of rat pregnancy lead to hypertension in offspring through a reduction in renal 11β-hydroxysteroid dehydrogenase type 2, an enzyme which oxidises cortisol into inactive cortisone (Tang et al., 2011). Therefore, fetal glucocorticoid exposure could lead to altered vascular sensitivity and functional deficits in other organs important in maintaining cardiovascular homeostasis. These are important considerations as it could be maternal stress in human pregnancy, irrespective of environmental stressors such as hypoxia and starvation, could lead to early life programming of adult disease.

The data presented within this thesis suggest that, even though there could be an effect of a maternal injection on fetal glucocorticoid exposure, the injection per se could not contribute to the increased blood pressure in offspring as those offspring from saline-injected pregnancies had normal SBP. Whether or not an injection exacerbates the effects of SC on adulthood disease is unknown but this could be answered by assessing offspring from dams treated with SC in the drinking water.

6.1.5 Clinical perspective

To date, there have been a number of experimental and clinical studies assessing the effects of antenatal SC treatment during pregnancy. A systematic review assessing the experimental and clinical studies of SC for FGR (Villanueva-Garcia et al., 2007) highlighted only one study where maternal administration of SC had detrimental effects on fetal wellbeing (a decrease in fetal weight). Two recent studies in mice have both shown antenatal SC led to increased fetal weight (Stanley et al., 2012, Dilworth et al., 2013) and when SC was given to women with severe early onset FGR there was a significant increase in fetal abdominal growth velocity (von Dadelszen et al., 2011). The focus of these studies has been to assess maternal, fetal and in some cases neonatal outcomes but none have examined the effects of antenatal SC in the long term. This thesis provided a different approach and demonstrated that although there were no beneficial or detrimental effects of SC on fetal weight or fetal viability, maternal SC treatment altered offspring cardiometabolic function as early as 8 weeks of age in the mouse (approximately 11 - 15 years of age in the human), through as yet unknown mechanisms. The current randomised clinical trial is assessing the use of SC in severe early-onset FGR with very dismal prognosis (Ganjevoort et al., 2014). Neonatal follow up will be assessed in these children from SC treated pregnancies; however, the data provided herein suggest it is important to continue follow up into at least early adulthood (10 – 30 years of age) as effects of SC are seen as early 8 weeks of age in mice.
Nevertheless, given the data suggesting antenatal SC is beneficial in the treatment of FGR, the potential beneficial effects of SC treatment in very dismal prognosis pregnancies should outweigh the possible long term cardiometabolic dysfunction associated with antenatal SC treatment.
6.2 Conclusions

The principle conclusions from the present study are that:

- Mouse fetal abdominal aortic vascular function can be assessed at E18.5 using *ex vivo* wire myography; sex- and genotype-specific differences in vascular function may be apparent as early as E18.5.

- Although there may be subtle sex-specific differences, growth restriction *per se*, due to placental insufficiency in the *Igf2* P0\(^{+/-}\) knockout mouse model of FGR, is not associated with altered fetal vascular function at E18.5.

- A 10 mg.kg\(^{-1}\) antenatal dose of SC did not increase fetal weight in the P0 mouse, a model of growth restriction with no abnormalities in vascular function.

- Antenatal SC led to sex-specific effects on postnatal growth and glucose tolerance. Female mice from SC treated dams were more susceptible to impaired glucose tolerance.

- Irrespective of size at birth and sex, antenatal SC treatment increased systolic blood pressure in the offspring. Increased systolic blood pressure was not directly associated with endothelial or vascular dysfunction and mechanisms may be related to altered nephrogenesis, increased stress responses, increased cardiac output / stroke volume or increased total peripheral vascular resistance.

- Placental insufficiency is associated with increased neonatal / postnatal death in growth restricted mice but those which do survive show no alterations in cardiovascular or metabolic function in adulthood.

- P0 mice represent a sub-set of clinical cases of human FGR whereby a lack of vascular abnormalities *in utero* are not associated with long-term programming of adulthood disease. Furthermore, the early postnatal environment of the P0 mouse, in which there is a high degree of developmental plasticity, does not promote and may perhaps reduce the risk of long-term disease.
6.3 Future work

In order to extend and confirm the current findings the following experiments should be performed:

- Ascertain the concentration of SC in maternal and fetal plasma using HPLC. These studies will not only allow comparisons between maternal SC concentration in mouse and human but will also investigate the relative clearance of SC and its major metabolite over the 24 hour period following subcutaneous injection.

- Assess the transfer of SC in both human and mouse placentae. *Ex vivo* dual perfusion of human placentae and *in situ* perfusion of mouse placentae following injection of SC and measurement in perfusate / fetuses, respectively, will begin to answer the question as to whether there are differences in mouse and human placental transport of SC.

- Perform morphological analysis on fetal aorta and offspring aortas and mesenteric arteries from SC treated dams. These studies will provide evidence as to whether there have been changes in the structure of the vasculature (e.g. smooth muscle cell content) as a consequence of antenatal SC treatment.

- Assess cardiovascular parameters using radiotelemetry in offspring from SC treated dams. These studies will answer whether, in conscious freely-moving animals, there are differences in basal systolic / diastolic and mean arterial blood pressure or cardiac output and whether offspring from SC-treated dams are more susceptible to anxiety provoking stimuli such as the tail cuff restraint.

- Assess mechanisms for increase blood pressure in offspring from SC treated dams with molecular indices of NO biology and oxidative stress and morphological assessment of the kidney (to determine nephron number; glomerular abnormalities) and assessment of renal artery vascular function. These studies will provide evidence as to whether antenatal SC alters NO biology in the offspring and whether this is associated with oxidative stress. In addition these studies will highlight whether there is a reduction in nephron number associated with antenatal SC treatment and / or whether renal vascular function (and thus renal blood flow) is reduced.

- Use the developed methodologies to screen the effects of appropriate SC treatment regimens on fetal growth and postnatal cardiovascular / metabolic function in other animal models of FGR, particularly those that do show a postnatal phenotype associated with growth restriction. These studies will provide information to maximise efficacy and reduce any side effects of SC when translating to the clinical cases of human FGR or PE.

- Examine whether ageing is associated with endothelial dysfunction, hypertension or metabolic dysfunction in P0 offspring of both sexes. These studies will either confirm or refute the suggestion that placental insufficiency and the associated growth restriction within the *Igf2* P0+/− knockout mouse model of FGR, does not increase the risk of adulthood disease.
Chapter 7 References


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8.1 Additional data

Figure 8.1. Offspring plasma corticosterone concentrations. Plasma corticosterone concentrations from saline-treated pregnancies (A) and sildenafil citrate-treated pregnancies (WT mice; B and P0 mice; C). Offspring were euthanased by cervical dislocation between 9am and 1am and blood plasma samples were assessed for corticosterone concentrations.
8.2 Publications

Publications of work contained herein;


8.3 Presentations


