THE UNIVERSITY OF ADELAIDE

The Effect of Prenatal Hypoxia on Cardiomyocyte Development and Postnatal Heart Health

Kimberley J. Botting
B. Biomedical Sci. (Hons)

Discipline of Physiology
School of Medical Sciences
Faculty of Health Science

September 2013

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy to The University of Adelaide

Table of contents

ABSTRACT vi

DECLARATION viii

ACKNOWLEDGEMENTS ix

RELATED PUBLICATIONS x

LIST OF FIGURES AND TABLE xii

COMMONLY USED ABBREVIATIONS xv

1. CHAPTER ONE – LITERATURE REVIEW 5

1.1 Published review: Botting K.J., Wang K.C.W., Padhee M, McMillen I.C., Summers-Pearce B., Rattanatray L., Cutri N., Posterino G.S., Brooks D. A., Morrison J.L. Early Origins of Heart Disease: Low birth weight and determinants of cardiomyocyte endowment. Clinical and Experimental Pharmacology and Physiology. 2012;39:814-823[1] 5

1.1.1 Summary (Abstract) 5

1.1.2 Introduction 6

1.1.3 Cardiomyocyte development 6

1.1.4 Cardiomyocyte turnover and polyploidization in postnatal life 18

1.1.5 The effect of IUGR in species where cardiomyocyte maturation occurs after birth 19

1.1.6 The effect of IUGR in species where cardiomyocyte maturation occurs before birth 20

1.1.7 Postnatal cardiac consequences of IUGR 26

1.1.8 Concluding remarks 26

1.2 Review for submission: Early Origins of Heart Disease: IUGR and postnatal cardiac metabolism 27

Introduction....27

1.2.1 Cardiac metabolism....28

1.2.2 Effect of IUGR on postnatal cardiac metabolism 37

1.2.3 The effect of obesity and insulin resistance on cardiac metabolism....38

1.2.4 The effect of cortisol on cardiac metabolism....42

1.2.5 The effect of cardiac hypertrophy on cardiac metabolism....46

1.2.6 Conclusion 49

1.3 Experimental hypotheses 50

2. CHAPTER TWO....57

2.1 Abstract....57

2.2 Introduction....58

2.3 Methods....60

2.3.1 Animal model and surgical procedures....60

2.3.2 Arterial blood gas measurements....61

2.3.3 Tissue collection....61

2.3.4 Total number of cardiomyocytes and capillary length density....62

2.3.5 TUNEL....64

2.3.6 Measurement of mRNA expression....64

2.3.7 Quantification of protein abundance....69

2.3.8 Statistical analysis....69

2.4 Results ... 70

2.5 Discussion ... 79

2.6 Conclusion ... 82

3. CHAPTER THREE ... 87

3.1 Abstract ... 87

3.2 Introduction ... 88

3.3 Methods ... 91

3.3.1 Animal model ... 91

3.3.2 Experimental protocol ... 92

3.3.3 Blood pressure analysis ... 93

3.3.4 Post mortem collection of tissue ... 93

3.3.5 Total number of cardiomyocytes ... 94

3.3.6 Measurement of mRNA expression ... 96

3.3.7 Quantification of protein abundance ... 97

3.3.8 Statistical analysis ... 100

3.4 Results ... 101

3.4.1 Maternal data ... 101

3.4.2 Birth and postnatal growth ... 101

3.4.3 Blood pressure ... 106

3.4.4 Organ weights ... 106

3.4.5 Cardiomyocyte number ... 106

3.4.6 Markers of cardiac hypertrophy ... 107

3.5 Discussion 115

3.6 Conclusion 123

4. CHAPTER FOUR 127

4.1 Abstract 127

4.2 Introduction 129

4.3 Methods 132

4.3.1 Animal model 132

4.3.2 Experimental protocol 132

4.3.3 Post mortem collection of tissue 133

4.3.4 Quantification of plasma substrate and hormone concentrations 133

4.3.5 Measurement of mRNA expression 134

4.3.6 Quantification of protein abundance 138

4.3.7 Statistical analysis 139

4.4 Results 139

4.4.1 Plasma analysis 139

4.4.2 Expression of genes involved in fatty acid metabolism 139

4.4.3 Expression and abundance of glucose transporters 146

4.4.4 Abundance of regulators of fatty acid and glucose metabolism 146

4.5 Discussion 151

4.6 Conclusion 156

5. CHAPTER FIVE 159

5.1 Overall discussion 159

5.2 Overall Conclusion 166

6. REFERENCES 168

ABSTRACT

Environmental factors can act in early life to increase the risk of disease in adulthood. Animal models demonstrate that intrauterine growth restriction (IUGR) results in a greater susceptibility to cardiac ischaemia/reperfusion injury and reduced cardiac power during reperfusion than Control offspring in postnatal life. Despite having an equivalent utilisation of fatty acids and glucose for cardiac ATP production prior to ischaemia/reperfusion, IUGR offspring have decreased utilisation of fatty acids and increased reliance on glycolysis for ATP production compared to Control offspring during reperfusion. We therefore aimed to determine if IUGR reduces cardiomyocyte endowment and alters the expression of cardiometabolic genes in postnatal life. We determined that IUGR due to placental restriction from conception, which causes chronic fetal hypoxaemia and hypoglycaemia, reduced the number of cardiomyocytes in the heart of sheep in late gestation. In addition, IUGR fetuses had the same percentage of apoptotic cardiomyocytes, length of coronary capillaries and expression of the majority of genes whose upregulation occurs during hypoxia, compared to Controls. Furthermore, we found that IUGR reduced cardiomyocyte endowment in adolescent guinea pigs if they were exposed to Maternal Hypoxia (MH) and were female, but not if they were male or if IUGR was induced by Maternal Nutrient Restriction (MNR). IUGR offspring exposed to MH had increased expression of the transcriptional regulator of fatty acid metabolism, *PPAR*α, and increased expression of fatty acid transporters, *FATP1, FAPT6* and *FABPpm*, but offspring exposed to MNR only had an increased expression of *FATP6*, compared to Control. Interestingly, IUGR male offspring, but not female offspring, had decreased expression of factors in the sarcoplasm that regulate fatty acid activation (*FACS*) and transport of active fatty acids into the mitochondria for fatty acid β-oxidation (*AMPK*α_2 and *ACC*) if exposed to MNR, but a decrease in only *FACS* and *AMPK*α_2 if exposed to MH. Interestingly, only IUGR females exposed to MH had increased activity of the metabolic fuel gauge, AMPK, suggesting that a decrease in ATP may be related to the deficit in

cardiomyocyte endowment. In conclusion, we have shown that in response to placental restriction, reducing cardiomyocyte endowment whilst maintaining the total length of coronary capillaries, results in the heart being normoxic, despite chronic hypoxaemia, in late gestation. Furthermore, this data suggests that females are more likely to have reduced cardiomyocyte endowment, following IUGR, in adolescence than males and that cardiomyocytes may be influenced by hypoxia more than nutrient restriction. Furthermore, we have demonstrated that IUGR programs changes in cardiometabolic gene expression in the absence of other IUGR pathologies such as cardiac hypertrophy, hypertension and increased plasma fatty acid and cortisol concentrations.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in my name, in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Kimberley Botting

September 2013

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my principle supervisor A/Prof Janna Morrison for her support throughout my PhD. You had confidence in me and allowed me to contribute to the group, both in my own research and the research of others. Your knowledge, guidance, patience and perseverance have made this experience rewarding and I am very grateful. Thank you for always acting in my best interest and in particular for continuing to support me after the flood.

I would like to thank the members of the Early Origins of Adult Health Research Group, both past and present. In particular I would like to thank my co-supervisor Professor Caroline McMillen for her support, wealth of knowledge and passion for research; Darran Tosh, Robb Muirhead, Bernard Chuang and Bang Hoang for their assistance in gene analysis; Stacey Dunn, Robb Muirhead and Rajan Poudel for their assistance with protein analysis; Song Zhang for her assistance with plasma assays; Bang Hoang, Poh Song, Michelle Lloyd, Melissa Walker, Dr. Kimberley Wang, Rajan Poudel and Long Nguyen for their assistance with maintenance of the guinea pig cohort and post mortems; and Melissa Walker for her surgical skills. I am very grateful for each of your contributions and I could not have done it without you. I am also extremely grateful for the animal care and support provided by Jayne Skinner and the staff at the IMVS, without which the guinea pig cohorts would not have been possible.

I would like to sincerely thank Prof Jens Nyengaard and his staff at the University of Aarhus for all of their help and kindness during my visit to count cardiomyocytes.

I would especially like to thank my laboratory manager and friend, Stacey Dunn, for her continued support over my entire PhD. It’s been a long journey and you were always there to make the experience easier and enjoyable.

To my family, thank you for your guidance and support throughout my studies and for your constant love and encouragement. I am grateful for everything you have done for me.

RELATED PUBLICATIONS

List of Publications from Other Work Performed during Candidature

Original Manuscripts

1. Wang KC, Brooks DA, **Botting KJ**, Morrison JL, IGF-2R-Mediated Signaling Results in Hypertrophy of Cultured Cardiomyocytes from Fetal Sheep. *Biology of Reproduction*, 2012; 86(6), 183.
2. Soo PS, Hiscock J, **Botting KJ**, Roberts CT, Davey AK, Morrison JL, Maternal undernutrition reduces P-glycoprotein in guinea pig placenta and developing brain in late gestation. *Reproductive Toxicology*., 2012; 33(3), 374-81.
3. Wang KC, Zhang L, McMillen IC, **Botting KJ**, Duffield JA, Zhang S, Suter CM, Brooks DA and Morrison JL, Fetal growth restriction and programming of heart growth and cardiac insulin-like growth factor 2 expression in the lamb, *Journal of Physiology,* 2011; 589(Pt 19), 4709-22.
4. Posterino G, Dunn SL, **Botting KJ**, Wang W, Gentili S, and Morrison JL, Changes in cardiac troponins with gestational age explain changes in cardiac muscle contractility in the sheep fetus, *Journal of Applied Physiology*, 2011; 111(1), 236-43.
5. Nguyen LT, Muhlhausler BS, **Botting KJ**, Morrison JL, Maternal undernutrition alters fat cell size distribution, but not lipogenic gene expression, in the visceral fat of the late gestation guinea pig fetus, *Placenta,* 2010; 31(10) 902-9.
6. Wallace SJ, Morrison JL, **Botting KJ** and TW Kee, Second harmonic generation and two-photon excited autofluorescence microscopy of cardiomyocytes: quantification of cell volume and myosin filaments, *Journal of Biomedical Optics*, 2008; 13(6) art. no. 064018.

Reviews

7. Morrison JL, **Botting KJ**, Soo PS, McGillick EV, Hiscock J, Zhang S, McMillen IC and Orgeig S, Antenatal steroids and the IUGR fetus: Are exposure and physiological effects on the lung and cardiovascular system the same as in normally grown fetuses?, *Journal of Pregnancy*, Epub November 2012.

8. **Botting KJ***, Wang KC*, Padhee M, Zhang S, Caroline McMillen I, Suter CM, Brooks DA, Morrison JL, Early origins of heart disease: Low birth weight and the role of the insulin-like growth factor system in cardiac hypertrophy, *Clinical and Experimental Pharmacology and Physiology,* 2012; 39(11), 958-64.

9. **Botting KJ**, Wang KC, Padhee M, McMillen IC, Summers-Pearce B, Rattanatray L, Cutri N, Posterino GS, Brooks DA, Morrison JL., Early origins of heart disease: Low birth weight and determinants of cardiomyocyte endowment, *Clinical and Experimental Pharmacology and Physiology*, 2011; 30(9), 814-823.

Invited Editorials

10. Morrison JL, Souter C, **Botting KJ**, Nyengaard JR, Does maternal obesity change cardiomyocyte endowment? *Expert Review in Obstetrics and Gynecology*, 2013; 8(1), 1-3.

11. Morrison JL, **Botting KJ**, Does a growth-restricted fetus have fewer cardiomyocytes than a normally grown fetus? *Expert Review in Obstetrics and Gynecology,* 2012; 7(4), 301-303.

12. Morrison JL, Wang KCW, Brooks DA, **Botting KJ**, Fetal heart growth: IGFs and Sex, *Expert Reviews in Obstetrics and Gynecology,* 2009*;* 4(3), 255-259.

LIST OF FIGURES AND TABLE

Chapter 1.1

Figure 1. Proliferation of fetal mononucleated cardiomyocytes is regulated by multiple signalling pathways that stimulate or inhibit cyclins and cytokinesis…………………………7

Figure 2. Apoptosis is critical for cardiac development and can be mediated by the mitochondrial dependent intrinsic pathway and the death receptor mediated extrinsic pathway. 13

Table 1. Timing of multinucleation and proportion of mononucleated and multinucleated cardiomyocytes in adult life in a range of species. 17

Figure 3. Mean gestational PaO_2 is positively related to fetal body weight and heart weight in the late gestation sheep fetus. 22

Figure 4. Regardless of the model of IUGR employed, IUGR in sheep results in an increased percentage of mononucleated cardiomyocytes across late gestation.......................................24

Chapter 1.2

Figure 1. Cardiac fatty acid and glucose metabolism. 31

Figure 2. The balance between fatty acid and glucose metabolism.......................................36

Chapter 2

Table 1. Primer sequences used in quantitative real-time reverse transcription-PCR to measure genes of interest. 66

Table 2. Fetal arterial blood gas measurements. 71

Table 3. Fetal body and heart weight measurements. 72

Figure 1. Placental restriction reduced the total number of cardiomyocytes in the right ventricle. 73

Figure 2. The effect of IUGR on cardiomyocyte apoptosis. 74

Table 4. mRNA expression of HIFs, genes with hypoxia response elements, and genes involved in cardio-protection and HIF-α stability. 76

Figure 3. The effect of IUGR on the length of capillaries.. 77

Figure 4. Placental restriction does not change the protein abundance of PHD-1, but increases the protein abundance of PHD-2 in the left ventricle.. 78

Chapter 3

Table 1. Primer sequences used in Quantitative Real-Time Reverse Transcription-PCR to measure genes of interest………………………………………………………………..94

Figure 1. Maternal body weight weight prior to treatment. 102

Figure 2. Maternal body weigt and food intake per body weight during treatment 103

Figure 3. Gestational age at birth, litter size and birth weights 104

Figure 4. Postnatal growth 105

Figure 5. Basal blood pressure. 108

Table 2. Postnatal body weight, absolute organ weights and relative organ weights. 109

Figure 6. Heart weight and left ventricular weights 110

Figure 7. Cardiomyocyte number 111

Figure 8. Cardaic mRNA expression of insulin-like growth factors. 112

Figure 9. Cardiac abundance and activity of Atk. 113

Figure 10. Cardiac abundance and activity of CaMKII. 114

Chapter 4

Table 1. Primer sequences used in Quantitative Real-Time Reverse Transcription-PCR to measure genes of interest. 136

Figure 1. Plasma concentrations of gluocse, non-esterified fatty acids and cortisol 141

Figure 2. Cardiac mRNA expression of transcriptional regulators of fatty acid metabolism. 142

Figure 3. Cardiac mRNA expression of fatty acid tranporters 143

Figure 4. Cardiac mRNA expression of genes involved in transport of fatty acids into the mitochondria and fatty acid oxidation 144

Figure 5. Cardiac mRNA expression of genes involved in fatty acid activation and regulation of mitochondrial uptake.. 145

Figure 6. Cardiac mRNA expression and abundance of glucose transporters......................147

Figure 7. Cardiac abundance and activity of AMPK 148

Figure 8. Cardiac adundance and activity of ACC 149

Figure 9. Cardiac abundance and activity of AS160 150

Chapter 5

Figure 1. We speculate that the heart of the chronically hypoxaemic fetus is not hypoxic in late gestation due to programmed cell death and decreased cardiomyocyte proliferation in response to cardiomyocyte hypoxia earlier in gestation. 161

Figure 2. The present study demonstrates the IUGR programs changes in cardiometabolic genes, which we speculate underlie the pathological metabolism that occurs in response to secondary insults, such as ischaemia/reperfusion, obesity and hypertrophy in postnatal life. Secondary insults may also be caused by IUGR. 165

COMMONLY USED ABBREVIATIONS

A-C

ACADL	Long chain acyl-CoA dehydrogenase
ACADM	Medium chain acyl-CoA dehydrogenases
ACADVL	Very Long chain acyl-CoA dehydrogenase
ACC	Acetyl-CoA Carboxylase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
Adm	Adrenomedullin
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
Ang-II	Angiotensin-II
ANGPT	Angiopoietin
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
AT-R	Angiotensin receptor
β-AR	Adrenergic receptor - beta
CAs	Catecholamines
CD36	Fatty acid translocase
CDK	Cyclin dependent kinase
CPT-Iβ	Carnitine palmatonyl transport protein –I beta
CRH	Corticotrophin-releasing hormone
CVD	Cardiovascular disease

D-H

d	Day
DRs	Death receptors
ERK	Extracellular signal-related kinase
ETC	Electron transport chain
FABPpm	Plasma membrane specific fatty acid binding
FACS	Fatty-acyl CoA synthetase
FADD	Fas-Associated protein with Death Domain
$FADH_2$	Flavin adenine dinucleotide
FATP	Fatty acid transport protein
FGF2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor receptor
Flk-1	Vascular endothelial growth factor receptor 1
G_0	Cell cycle - gap zero phase (resting/quiescent)
G_1	Cell cycle - first gap phase
G_2	Cell cycle - second gap phase
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GS	Glycogen synthase
GSK-3β	Glycogen synthase kinase-3 beta
H-FABP	Heart-type fatty acid binding protein
HIF	Hypoxia inducible factor
HK	Hexokinase
HPA	Hypothalamic-pituitary adrenal
HRE	Hypoxia response element

IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGF-2	Insulin-like growth factor-2
IGF-2R	Insulin-like growth factor-2 receptor
iNOS	Inducible nitric oxide synthase
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LDH	Lactate dehydrogenase
LV	Left ventricle
LVH	Left ventricular hypertrophy
M	Cell cycle - mitosis
MCD	Manalyl CoA dehydrogenase
MH	Maternal hypoxia
miR	MicroRNA
NADH	Nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acid
NRG1	Neuregulin 1
PDH	Pyruvate dehydrogenase (PDH)
PFK	Phospho-6-fructose kinase I
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PR	Placental restriction

R-Z

Rb	Retinoblastoma protein
RV	Right ventricle
RXR	Retinoid X receptor
S	Cell cycle - DNA synthesis phase
T_3	Thyroid hormone
TCA	Tricarboxylic acid
Tie-2	Tyrosine-protein kinase receptor
UPE	Umbilicoplacental embolization
VEGF	Vascular endothelial growth factor

CHAPTER 1

Statement of Authorship

Title of Paper	Early origins of heart disease: low birth weight and determinants of cardiomyocyte endowment.
Paper Status	Published
Publication Details	Botting KJ, Wang KC, Padhee M, McMillen IC, Summers-Pearce B, Rattanatray L, Cutri N, Posterino GS, Brooks DA, Morrison JL. Early origins of heart disease: low birth weight and determinants of cardiomyocyte endowment. Clin Exp Pharmacol Physiol. 2012; 39: 814-823

Author contribution

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Kimberley Botting
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Wrote drafts of manuscript Created figures Edited manuscript Approved final version of the manuscript

Name of Co-Author	Dr. Kimberley Wang
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Monalisa Padhee
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Prof Caroline McMillen
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Dr. Brooke Summers-Pearce
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Dr. Leewen Rattanatray
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript

Name of Co-Author	Dr. Natalie Cutri
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Dr. Giuseppe Posterino
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	Prof Doug Brooks
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Edited manuscript Approved final version of the manuscript

Name of Co-Author	A/Prof Janna Morrison
Contribution to the Paper	Participated in discussions surrounding concepts addressed in the manuscript Involved in construction of the manuscript Edited manuscript Approved final version of the manuscript Acted as corresponding author

1. CHAPTER ONE – LITERATURE REVIEW

1.1 Published review: Botting K.J., Wang K.C.W., Padhee M, McMillen I.C., Summers-Pearce B., Rattanatray L., Cutri N., Posterino G.S., Brooks D. A., Morrison J.L. Early Origins of Heart Disease: Low birth weight and determinants of cardiomyocyte endowment. Clinical and Experimental Pharmacology and Physiology. 2012;39:814-823[1]

1.1.1 Summary (Abstract)

1. World-wide epidemiological and experimental animal studies demonstrate that adversity in fetal life, resulting in intrauterine growth restriction (IUGR), programs the offspring for a greater susceptibility to ischemic heart disease and heart failure in adult life.
2. After cardiogenesis, cardiomyocyte endowment is determined by a range of hormones and signalling pathways that regulate cardiomyocyte proliferation, apoptosis and the timing of multinucleation/terminal differentiation.
3. The small fetus may have reduced cardiomyocyte endowment due to the impact of suboptimal intrauterine environment on the signalling pathways that regulate cardiomyocyte proliferation, apoptosis and the timing of terminal differentiation.

1.1.2 Introduction

World-wide epidemiological and experimental animal studies demonstrate that adversity in fetal life, resulting in intrauterine growth restriction (IUGR), programs the offspring for a greater susceptibility to ischemic heart disease and heart failure in adult life.[2-10] It is currently not clear how exposure to reduced substrate supply *in utero* can alter heart health some fifty years later. However, the human heart undergoes considerable maturation *in utero*, such that the majority of cardiomyocytes, present shortly after birth, beat for a lifetime.[11-14] This has led to a significant body of research focusing on the regulation of cardiomyocyte maturation, endowment and growth *in utero*, particularly in the last half of pregnancy; and how specific insults at critical periods of development can alter the profile of cardiomyocytes present before and after birth. This review will focus on new insights into the regulation and consequences of IUGR on cardiomyocyte endowment.

1.1.3 Cardiomyocyte development

After cardiogenesis, the fetal heart initially grows as a consequence of mononucleated cardiomyocyte proliferation. In the last trimester and shortly after birth, these mononucleated cardiomyocytes cease proliferating, due to the absence of karyokinesis and/or cytokinesis.[15] The final endowment of cardiomyocytes in the newborn heart is the result of a highly orchestrated balance between the creation of cardiomyocytes from cardiac progenitor cells in early gestation, subsequent cardiomyocyte proliferation across gestation, apoptosis and the critical timing of terminal differentiation. Typically, perturbations during pregnancy result in IUGR in the second half of pregnancy,[16] therefore this review will focus on the regulation of cardiomyocyte endowment after cardiogenesis.

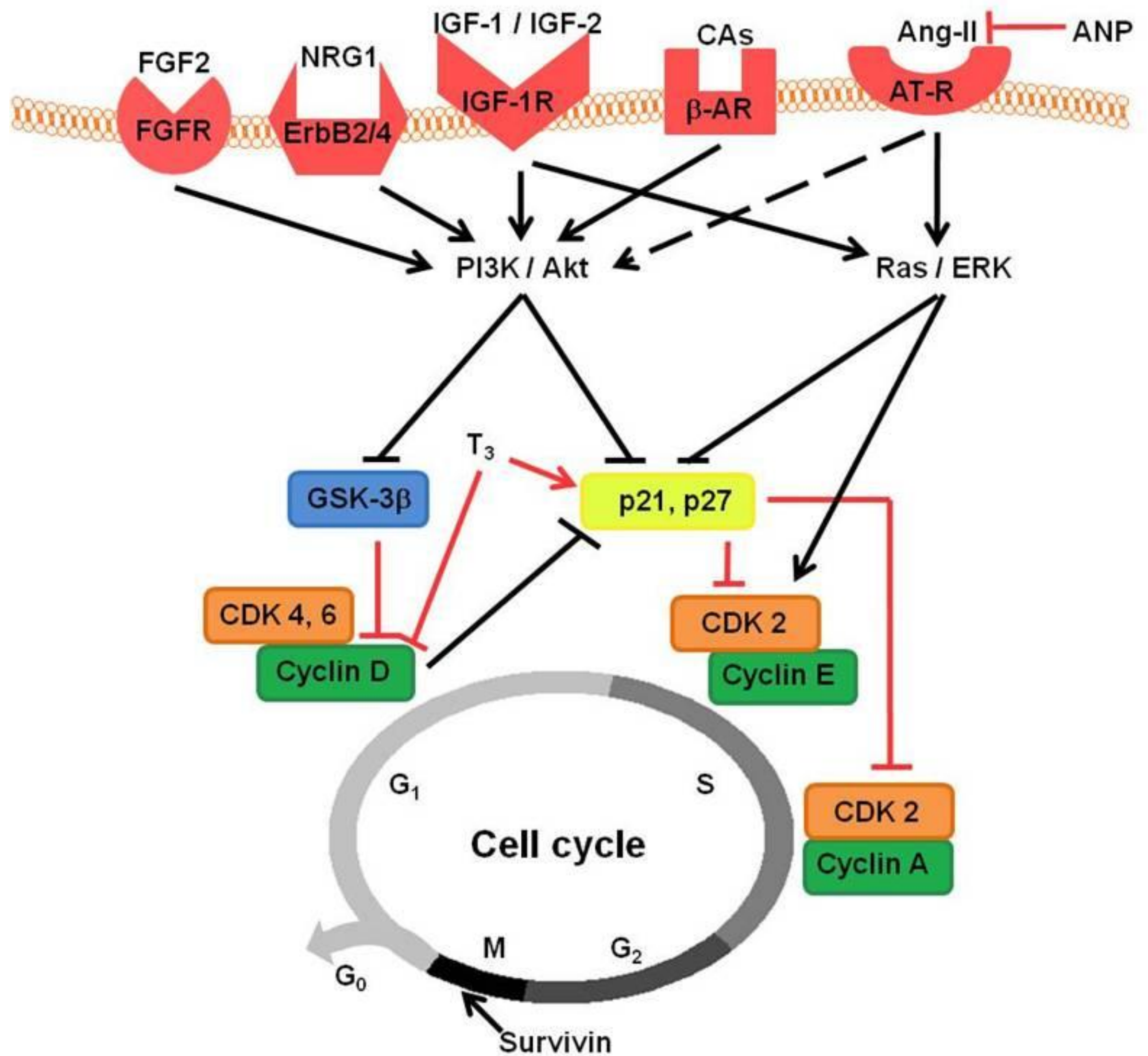

Figure 1. Proliferation of fetal mononucleated cardiomyocytes is regulated by multiple signalling pathways that stimulate or inhibit cyclins and cytokinesis. Promotion of cell cycle progression is indicated by black lines and inhibition is indicated by red lines. FGF2: fibroblast growth factor 2, FGFR: fibroblast growth factor receptor, NRG1: neuregulin 1, ErbB2/4: heterodimer of ErbB2 and ErbB4, IGF-1: insulin-like growth factor-1, IGF-2: insulin-like growth factor-2, IGF-1R: insulin-like growth factor-1 receptor, CAs: catecholamines, β-AR: beta adrenergic receptor, Ang-II: angiontensin II, AT-R: angiotensin receptor, ANP: atrial natriuretic peptide, PI3K: phosphoinositide-3 kinase, Akt: protein kinase B, ERK, extracellular signal-related kinase, GSK-3β: glycogen synthase kinase-3β, T3: thyroid hormone, CDK: cyclin dependent kinase. G1: first gap phase, S: DNA synthesis phase, G2: second gap phase, M: mitosis, G0: gap zero phase (resting/quiescent).

1.1.3.1 *Cardiomyocyte proliferation*

During development, cardiomyocyte proliferation is regulated by paracrine factors secreted from the epicardium,[17] endocardium[17, 18] and fibroblasts,[19] in addition to endocrine growth hormones.[12] In response to mitotic stimuli, D-type cyclins and their catalytic partners CDK4 and CDK6 accumulate in the nucleus, which then phosphorylate and deactivate retinoblastoma protein (Rb), enabling cell cycle progression from the first gap phase (G_1) to initiate DNA synthesis (S phase).[20] Cyclin D/CDKs facilitate the cell cycle by sequestering CDK inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, allowing S phase initiation and progression through the activity of cyclin E/CDK2 and cyclin A/CDK2, respectively[21] (Figure 1). Cardiomyocyte proliferation is initiated through mainstream canonical mitogenic signalling pathways; such as the phosphoinositide 3-kinase (PI3K)/Akt and Ras/extracellular signal-related kinase (ERK) pathways.[15, 17, 22, 23] Specifically, the PI3K/Akt pathway promotes proliferation by phosphorylating and deactivating glycogen synthase kinase-3β (GSK-3β). GSK-3β acts as a negative regulator of proliferation by phosphorylating cyclin D1,[24] causing its nuclear export and proteasomal degradation, thereby preventing progression from G_1 to S phase. Activation of Akt also reduces the expression of $p21^{Cip1}$ and $p27^{Kip1}$ through FOXO transcription factors,[25] which in turn promote both CDK2 activity and progression to S phase. The Ras/ERK pathway increases expression of cyclin D1 both directly[26] and indirectly through down-regulation of anti-proliferative genes such as Tob1 and JunD.[27-30] Recent data suggests that ERK and Akt activation leads to phosphorylation and inhibition of $p27^{Kip1}$.[31] ERK is also required for the translocation of CDK2 to the nucleus[32] and its subsequent phosphorylation,[33] which results in cyclin E association and cell cycle progression from G_1 to S phase. There is potential cross talk between the PI3K/Akt and Ras/ERK mitogen signalling pathways.[28]

Studies involving mice, rats and chickens have contributed greatly to the understanding of cardiomyocyte proliferation. *In vitro* administration of extracellular mitogens such as fibroblast growth factor (FGF) -2[34] and neuregulin (NRG) -1[35] stimulate

proliferation of cardiomyocytes from embryonic chickens and fetal rats, respectively. The mitotic actions of both FGF-2 and NRG-1 are dependent on the activation of the PI3K/Akt pathway.[36, 37] Additionally, *in vitro* exposure to insulin-like growth factor (IGF) -1[38] and IGF-2[39] results in greater DNA synthesis in fetal cardiomyocytes from mice and rats, respectively. Furthermore, conditional knockout of their receptors, insulin receptor (*INSR*) and IGF1 receptor (*IGF1R*), in the myocardium of embryonic mice, results in decreased ventricular cardiomyocyte proliferation in the first half of gestation.[40] Activation of IGF1R leads to activation of both the PI3K/Akt and Ras/ERK signalling pathways[41], however, less is known about the specific mitotic signalling pathways downstream of the INSR in cardiomyocytes. Catecholamines have also been implicated in cardiomyocyte proliferation, such that blocking β-adrenergic receptor (β-AR) activation *in vivo,* in neonatal rats, decreases cardiomyocyte mitosis and deactivates p70 ribosomal protein S6 kinase, which is downstream of PI3K/Akt[42].

In addition to the activation of mitotic signalling pathways, proliferation can be regulated by cytokinetic mechanisms. Embryonic mice with cardiac specific deletion of Survivin and neonatal rats with Survivin small interfering RNA (siRNA) knockdown have fewer cardiomyocytes, due to a reduction in proliferation.[43] Survivin is a key regulator of mitosis and cytokinesis because it is a component of the chromosomal passenger complex, essential for appropriate chromosomal separation and cytokinesis (reviewed[44]). It is currently unknown if IUGR influences the regulation of components of the chromosomal passenger complex.

Recent studies by Heallen *et al* demonstrated that the canonical Wnt signalling pathway, which is an essential regulator of pre-cardiac mesoderm cell proliferation and differentiation into cardiomyocytes, promotes cardiomyocyte proliferation.[45] Through the use of conditional knockouts of *Salv* to selectively inhibit Hippo signalling in mice, Haellen *et al* demonstrated that Hippo signalling is essential for the appropriate control of cardiomyocyte proliferation and heart size; due to its inhibition of transcription factors that are promoted by

the Wnt/β-catenin signalling pathway. Ablating Hippo signalling led to embryos with larger hearts containing more cardiomyocytes. Interestingly, this cardiomegaly was specifically due to exaggerated cardiomyocyte proliferation and was not associated with altered fibroblast, smooth muscle cell or cardiac progenitor cell proliferation; and was observed in the left and right ventricle, despite these cardiomyocytes originating from different heart fields.[45]

Pathological conditions in fetal life that result in fetal growth restriction occur predominantly in late gestation[16] and often lead to changes not only in the endocrine environment, but also in both preload and afterload. Therefore, the effects of these changes on cardiomyocyte proliferation have been studied in sheep, where cardiomyocyte maturation begins in late gestation as in humans, unlike postnatal life such as rats, mice and chickens. The endocrine effects have been studied in sheep models of IUGR (reviewed[46]) and show that there is a decrease in plasma IGF-1,[47] glucose[48] concentrations and an increase in plasma cortisol,[48] noradrenaline[49] and adrenaline[49] concentrations in late gestation. Different models of IUGR in sheep cause no change[50] or an increase[51-53] in mean arterial pressure.

In late gestation, increased cardiac systolic load[54, 55] and a range of hormonal and growth factors including IGF-1[41] and angiotensin II (Ang-II)[56] have been shown to stimulate proliferation of mononucleated cardiomyocytes (reviewed[12]). Ang-II acts through the Ras/ERK pathway in cardiomyocytes,[56] but evidence from mouse embryonic stem cells suggests it may also activate the PI3K/Akt pathway.[57]

Studies in sheep fetuses provide conflicting results regarding the regulation of cardiomyocyte proliferation by cortisol. Increased cortisol concentrations in late gestation led to maturation of fetal organs prior to birth, but a comprehensive study in sheep fetuses identified cortisol as a potent cardiomyocyte mitogen.[58] In contrast, a similar intrafetal infusion of cortisol has been reported to decrease DNA content in the left ventricle[59] and adrenalectomized sheep fetuses exhibit greater cardiomyocyte proliferation, thus suggesting

cortisol inhibits progression through the cell cycle.[60] The signalling pathway that links cortisol to proliferation of cardiomyocytes, however, remains unclear.

In the late gestation sheep fetus, cardiomyocyte proliferation is inhibited in the presence of reduced cardiac systolic load,[61] thyroid hormone (T_3)[62] and atrial natriuretic peptide (ANP)[63] (reviewed[12]). Specifically, T_3 increases the protein abundance of the CDK inhibitor p21^{Cip1}, while simultaneously decreasing the protein abundance of cyclin D1.[62] ANP does not inhibit basal rate of proliferation, however, Ang-II stimulated proliferation is inhibited due to reduced Akt and ERK activity[63] (Figure 1).

1.1.3.2 Apoptosis of cardiomyocytes

Apoptosis is critical for appropriate cardiovascular development (reviewed[64, 65]) and is tightly regulated and controlled by two main pathways; the intrinsic and extrinsic pathways, which regulate apoptosis through mitochondrial activity and death receptors, respectively.[66] Upon release of cytochrome C from the mitochondria or ligation of the death receptors (DRs), 'initiator' caspases such as procaspase 9 and procaspase 8, are cleaved to their active form (Figure 2). Activation of 'initiator' caspases results in the cleavage and activation of 'effector/executioner' caspases (e.g. 3, 6 and 7), which cause the biochemical and morphological changes associated with apoptotic cell death.[67, 68] The apoptotic pathway is regulated at various levels by members of the Bcl-2 family of proteins, consisting of both pro-apoptotic (Bad, Bax, Bak, tBid, Bim and Bmt) and anti-apoptotic (Bcl-2 and Bcl-X_L) elements.[66] Anti-apoptotic proteins of the Bcl-2 family act at the mitochondrial level to block the release of cytochrome C, whereas pro-apoptotic proteins inhibit the action of the anti-apoptotic proteins.[66] The cleavage and activation of anti-apoptotic Bid by caspase 8 allows for the extrinsic pathway to initiate the intrinsic (mitochondrial dependent) apoptosis pathway.[69]

In the rat heart, there is very little cardiomyocyte apoptosis either prenatally (1.4-2%)[70, 71] or at 21days postnatal life.[72] However, in the first day of postnatal life, there is

considerable apoptosis,[72] especially in the right ventricle, which undergoes extensive remodelling due to the abrupt changes in blood flow patterns and circulatory resistance shortly after birth (reviewed[73]). It is important to note that in the rat heart, this period of cardiac remodelling and apoptosis corresponds to a period where the majority of cardiomyocytes are mononucleated and still capable of proliferating[74] (Table 1). In the sheep, this remodelling and apoptosis occurs at a time when the majority of cardiomyocytes are terminally differentiated.[75] Apoptosis in the late gestation sheep heart is also minimal (<0.05% of cardiomyocytes).[76] At birth, the majority of human cardiomyocytes are mononucleated[77] (Table 1), however, they lose the ability to proliferate shortly after birth. It is currently unclear if the remodelling of the right ventricle occurs when human cardiomyocytes are still capable of proliferating.

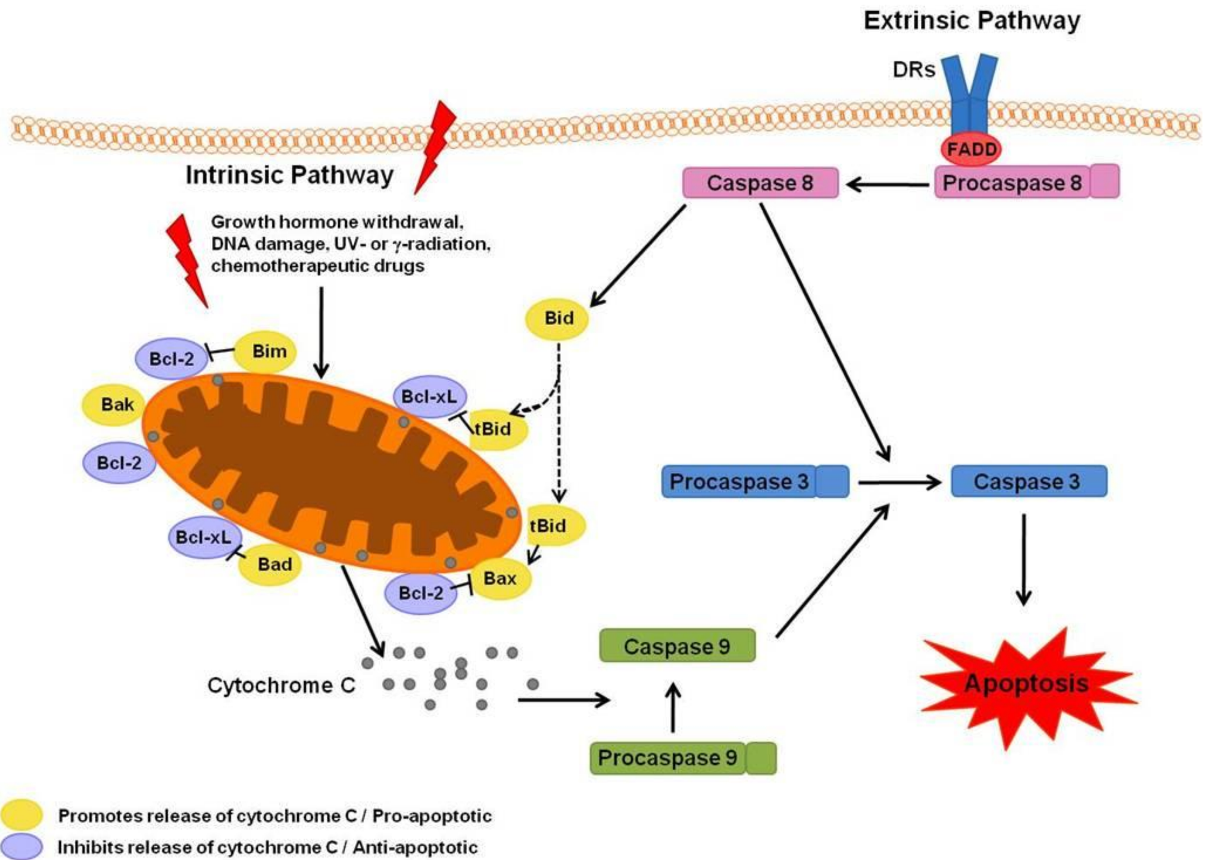

Figure 2. Apoptosis is critical for cardiac development and can be mediated by the mitochondrial dependent intrinsic pathway and the death receptor mediated extrinsic pathway. DRs: death receptors, FADD: Fas-Associated protein with Death Domain.

1.1.3.3 Multinucleation and terminal differentiation of cardiomyocytes

In late gestation and shortly after birth, mononucleated cardiomyocytes cease proliferating due to the absence of karyokinesis and/or cytokinesis. If a cardiomyocyte undergoes karyokinesis in the absence of cytokinesis, it becomes a multinucleated (predominantly binucleated) cardiomyocyte and is terminally differentiated [11, 78] (Table 1). Unlike humans, mice and rats are born with hearts comprised of mononucleated cardiomyocytes that do not undergo binucleation or cease proliferating until after birth.[74, 79] Consequently, injuries to the neonatal mouse heart before 7 days of age can result in cardiomyocyte regeneration due to the proliferation of existing mononucleated cardiomyocytes.[80] To date the signals that prevent mononucleated cardiomyocytes from undergoing karyokinesis to become polyploid, or cytokinesis to become multinucleated, are not well understood. Studies in rats demonstrate that during binucleation there is a simultaneous downregulation of cyclins and CDKs associated with G_1/S and G_2/M transition and an upregulation of cyclins and CDKs associated with G_1 phase.[81] Recent studies by Di Stefano *et al* demonstrate that simultaneous knockdown of CDK inhibitors p21^{Cip1}, p27^{Kip1,} and p57^{Kip2} by siRNAs in cultured neonatal and adult rat cardiomyocytes can result in entry to S-phase and a proportion of cardiomyocytes completing karyokinesis.[82] Transgenic mouse studies have demonstrated that overexpression of CyclinD1 and CyclinG1 induce multinucleation,[83, 84] but it has been suggested by Naqvi *et al*[85] that terminal differentiation may not be simply due to altered expression of genes that regulate the cell cycle.

Recent studies by Porrello *et al* demonstrate that cell cycle withdrawal and multinucleation may be regulated by microRNAs (miRs)[86]. By comparing the expression of miRs in cardiomyocytes from mice before (1 day of age) and after (10 days of age) the transition of the cardiomyocyte pool from proliferative mononucleated cardiomyocytes to non-proliferative multinucleated cardiomyocytes, Porrello *et al* identified the miR-15 family member, miR-195, as the most upregulated miR during this period. Furthermore, premature

overexpression of miR-195 in embroyonic hearts resulted in smaller hearts that had a reduced number of cells in the cell cycle and a greater percentage of multinucleated cardiomyocytes at postnatal day 1, suggesting premature cell cycle arrest. Furthermore, postnatal knockdown of the miR-15 family resulted in a greater number of mitotic cardiomyocytes at 12 days of age, however, this did not involve an increase in the number of cardiomyocytes undergoing cytokinesis and suggests that the miR-15 family is not alone in preventing cardiomyocyte proliferation in postnatal hearts.

Despite the absence of proliferation in the adult heart under basal conditions,[87] studies of human hearts after myocardial infarction suggest that a small proportion of cardiomyocytes are capable of cytokinesis,[88] albeit insufficient to maintain/repair heart function. Similarly, *in vitro* stimulation of adult rat cardiomyocytes with NRG1 causes DNA synthesis followed by completion of cytokinesis in approximately 0.6% of previously quiescent mononucleated cardiomyocytes.[37] Engel *et al*[89] have identified the signalling molecule, p38 mitogen-activated protein (MAP) kinase (p38), as an inhibitor of adult cardiomyocyte cytokinesis. Inhibiting p38 *in vitro* results in an approximate 3.8–fold increase in adult cardiomyocytes undergoing cytokinesis after stimulation with FGF1 compared to FGF1 stimulation alone.[89] p38 has also been implicated as a potential regulator of cytokenetic genes such as components of the chromosomal passenger complex (Aurora B, INCENP and Survivin) and actin assembly genes such as Anillin.[89, 90] In addition, Engel *et al*[90] have identified Anillin recruitment and localization during anaphase and late cytokinesis as being essential for the completion of cytokinesis and its absence as a cause of binucleation.

It is not clear why mature mammalian cardiomyocytes have a limited capacity to proliferate, whilst cardiomyocytes from species such as newts and zebrafish retain the ability to replicate DNA and divide.[91, 92] The physiological benefit of multinucleated cardiomyocytes is uncertain, but it has been proposed to be an adaptive response in cells with a high metabolic demand, such as skeletal muscle cells, where the capacity to generate twice the ribonucleic

acid (RNA) for protein synthesis might be advantageous.[15] Studies in sheep, where binucleation begins prenatally, as it does in humans, demonstrate that the maximum Ca^{2+}-activated force and adult cardiac troponin I and C protein expression increase with the decrease in the percentage of mononucleated cardiomyocytes in late gestation.[93] The timing of binucleation can be accelerated or delayed by alterations to the fetal environment, as discussed below.

Table 1. Timing of multinucleation and proportion of mononucleated and multinucleated cardiomyocytes in adult life in a range of species.

Species	Length of gestation/ incubation (days)	Timing of binucleation (days)		Proportion of mononucleated cardiomyocytes (%)		Proportion of binucleated cardiomyocytes (%)		Proportion of cardiomyocytes with >2 nuclei (n) (%)
		Before birth/ hatching	After birth/ hatching	At birth/hatching	Adult	At birth	Adult	Adult
Human	280	Begins by ~224[78]	unknown	~ 90%[77]	74 ± 8[94]	8.8 ± 5.3[77]	25.5 ± 8[94] 57[77] 54-63[95]	~0.5 (3n and 4n)[94]
Rat	21	n/a	4-12[74]	> 95[74]	10-14[96]	2.9 ± 1.8[74]	85-89[96]	1-5% (3n and 4n)[96]
Mouse	19	n/a	3-10[97]	~ 98[97]	< 8.5[97]	1.5 ± 0.3[97]	91.5[97]	unknown
Chicken	21	n/a	Begins by 15[98]	100[98]	43.6 ± 4.6 at 42 days[98]	0[98]	44.2 ± 2.8 at 42 days[98]	~ 11.5 at 42 days[98]
Sheep	145-150	Begins 100[76]-110[75]	Ends ~ 4[75]	Between 16.8 ± 2.6 and 21.1± 2.2[75]	8 ± 1.5 at 4-6 weeks[75]	Between 78.9 ± 2.2 and 83.2 ± 2.6[75]	92.0 ± 1.5 at 4-6 weeks[75]	unknown
Pigs	114	unknown	unknown	Unknown	~5 at 6 mos[99]	unknown	~12 at 6 mos[99]	~83 (3n-16n) at 6 mos[99]

1.1.4 Cardiomyocyte turnover and polyploidization in postnatal life

It appears that terminal differentiation of cardiomyocytes is complete soon after birth in humans and therefore it has been proposed that the human heart lacks the capacity to generate more cardiomyocytes postnatally. Although it is still accepted that adult cardiomyocytes, unless experimentally stimulated, do not undergo cytokinesis,[15] studies carried out in several laboratories have identified the presence of human cardiac stem cells (hCSCs) that have the ability to generate new cardiomyocytes.[100-102] Currently, the contribution of hCSCs to the replacement and turnover of cardiomyocytes after birth is contentious. Some studies have suggested that ~50% of cardiomyocytes are replaced in a normal life span,[13, 14] whereas others suggest that the entire cardiomyocyte pool is replaced 11 to 15 times in the life of men and women respectively, with the maximum age of cardiomyocytes being 23 years.[103] Studies in mice demonstrate that the annual renewal rate of cardiomyocytes is approximately 1.1%,[97, 104] which is similar to an estimation made from carbon dating studies in humans.[13, 14] Regardless of the actual rate of cardiomyocyte turnover, hCSCs are not capable of replacing enough of the cardiomyocytes lost due to aging and injury (e.g., due to myocardial infarction) to maintain cardiac function and prevent heart failure. Therefore, the impact of restrictions to fetal substrate supply on cardiomyocyte endowment at birth has implications for the individuals' vulnerability to cardiovascular disease in adult life.

Despite the inability of adult cardiomyocytes to undergo cell division, they do retain the ability to undergo DNA replication. In the human heart, 90% of cardiomyocyte nuclei are diploid (2c) shortly after birth, whereas the majority of adult cardiomyocyte nuclei are tetraploid (4c).[105] Ploidy is positively related to heart weight[106, 107] and increases further in response to injury.[107, 108] Studies in sheep, where the majority of binucleation and terminal differentiation occurs prenatally, identify that premature lambs have a greater percentage of tetraploid mononucleated cardiomyocytes compared with term controls at two months of age.[109] Studies in rats, where gastroenteritis was induced during the period of binucleation and

terminal differentiation (4-12d postnatal), demonstrated that polyploidization can also be increased.[110] The regulation and advantage/disadvantage of polyploidization is not well understood. Cardiomyocyte polyploidization is suggested to be protective against hypoxia induced apoptosis, but maladaptive for aerobic metabolism.[110] Consequently, alterations to cardiomyocyte ploidy during development may have implications for adult heart health.

1.1.5 The effect of IUGR in species where cardiomyocyte maturation occurs after birth

Maternal protein restriction during pregnancy results in reduced birth weight, heart weight and number of cardiomyocytes at birth in rats.[111] Lim and colleagues extended maternal protein restriction during pregnancy to the lactation period, because cardiomyocyte binucleation in rats occurs postnatally,[112] but found no difference in the total number of cardiomyocytes in the offspring compared with controls at four weeks of age.[112] These studies are important because they suggest the presence of a critical window during cardiomyocyte maturation when cardiomyocyte endowment can be rescued. In rats, they also suggest that matching the prenatal and postnatal environment until cardiomyocyte maturation is complete, may be beneficial, but the opposite is true for nephron number.[113] Moreover, these studies demonstrate that heart weight and cardiomyocyte number are positively related.[111, 112]

Maternal hypoxia during late gestation results in reduced birth weight in rats and offspring who have a greater susceptibility to ischemia/reperfusion injury in adulthood.[114-116] Furthermore, ischaemia/reperfusion injury in these adult offspring results in a larger infarct and diminished post-ischemic recovery of left ventricular function when compared to controls,[115, 116] which is accompanied by an increase in caspase 3 activity and cardiomyocyte apoptosis.[115] This vulnerability may be due, in part, to decreased capillary density[117] and cardiac remodelling that includes increased collagen I and III and fibrillar thickness and density.[116] Interestingly, prior to ischaemia/reperfusion injury, these rats have the same body weight, heart weight and left ventricle weight compared to controls, but their cardiomyocytes have a larger cross sectional area.[114] These data suggest that rats, whose mothers were

exposed to hypoxia during pregnancy, have fewer cardiomyocytes and a heart weight that has been maintained by greater hypertrophy of the remaining cardiomyocytes. This premise is supported by studies of the hypertrophic heart rat (HHR) model, which develops cardiac hypertrophy in the absence of increased blood pressure, by 2 months of age.[118] At 2 days of age, these rats have smaller hearts containing smaller and fewer cardiomyocytes, which have become prematurely binucleated and exited the cell cycle.[119] Unlike maternal protein restriction studies, IUGR caused by maternal hypoxia can disrupt the positive relationship between heart weight and cardiomyocyte number in the postnatal heart.[114]

Fetuses of dams exposed to hypoxia have increased cardiomyocyte apoptosis and premature binucleation and exit from the cell cycle in the heart in late gestation.[71] One would therefore presume that maternal hypoxia would result in decreased numbers of cardiomyocytes in offspring at birth. In the rat, based on the observed ‘catch up’ of cardiomyocyte endowment in offspring exposed to maternal protein restriction,[112] it is not known if a proposed deficit in cardiomyocytes at birth will be corrected after birth, before cardiomyocytes have completed terminal differentiation.[74] Similarly, it is not known if the proposed deficit in cardiomyocyte number in the heart of postnatal offspring exposed to maternal hypoxia is a direct consequence of fewer cardiomyocytes at birth or if it is due to greater apoptosis in the heart of the IUGR offspring compared to control offspring post weaning. Furthermore, it has yet to be answered if IUGR, in an animal model where binucleation occurs prenatally, or in fact in humans, results in a deficit in the number of cardiomyocytes before or after birth.

1.1.6 The effect of IUGR in species where cardiomyocyte maturation occurs before birth

To date, there have been no studies published identifying the effect of IUGR on cardiomyocyte endowment in a species where binucleation of cardiomyocytes begins before birth. A study in sheep, a species where cardiomyocyte maturation occurs prenatally,

demonstrates that naturally occurring variation in birth weight changes cardiomyocyte endowment. Specifically, birth weight and body and heart weight at nine weeks of age were positively correlated with the number of left ventricular cardiomyocytes.[120] These data support evidence from maternal protein restriction studies in rats, which show heart weight is positively related to total cardiomyocyte number.[111, 112]

The use of a sheep model of chronic fetal hypoxemia, caused by fetal anemia, has identified alterations in fetal heart growth and poor cardiovascular outcomes in the adult sheep.[121, 122] Sheep models of placental insufficiency result in chronic fetal hypoxemia, hypoglycemia, hypercortisolemia, low birth weight and reduced heart weight in late gestation, which are endocrine and growth changes that also occur in human pregnancies of IUGR.[123-125] Studies in our laboratory have shown that in a model of chronic placental restriction, fetal arterial PO_2 (PaO_2) across late gestation is correlated with fetal body weight and heart weight, such that the greater the degree of hypoxemia, the greater the fetal growth restriction and reduction in heart growth (Figure 3). There is, however, no change in heart weight relative to body weight in IUGR compared to control fetuses.[123]

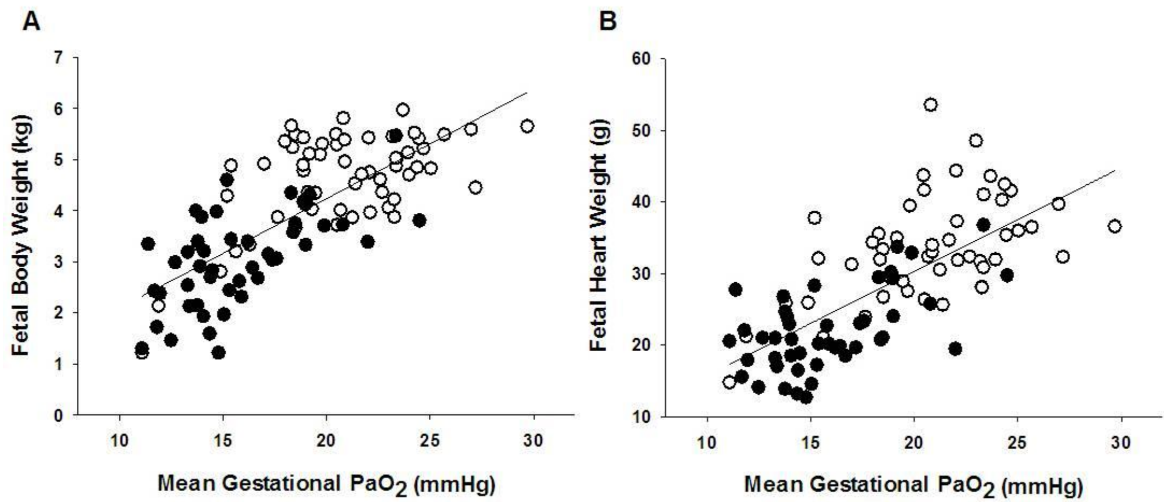

Figure 3. Mean gestational PaO_2 is positively related to fetal body weight (A; $r^2 = 0.553$, $P < 0.001$, $y = 0.21x - 0.05$) and heart weight (B; $r^2 = 0.501$, $P < 0.001$, $y = 1.45x + 1.20$) in the late gestation sheep fetus (137-145 days; term ~ 150 days). Open circles are control fetuses, filled circles are fetuses exposed to placental insufficiency.

Studies using two different sheep models of IUGR, induced by placental insufficiency, have investigated cardiomyocyte development. One involves surgical removal of endometrial caruncles in the non-pregnant ewe to reduce placental size (placental restriction, PR),[123] while the other involves embolization of the uterine artery of the pregnant horn to reduce umbilicoplacental blood flow in late gestation (UPE).[124, 125] In both models, placental insufficiency caused a delay in the transition of mononucleated cardiomyocytes to binucleated cardiomyocytes (Figure 4). This delay in maturation is in direct conflict with the results from maternal hypoxia studies in rats, which demonstrated an acceleration of binucleation,[71] reflecting the importance of cardiomyocyte maturation timing differences between species.

A reduction in fetal substrate supply changes cardiomyocyte growth patterns, but these changes are dependent on the timing, duration and severity of the placental insufficiency. For example, placental insufficiency by UPE for up to 20 days is associated with a decrease in the percentage of mononucleated cardiomyocytes undergoing proliferation,[124] however this is not observed in PR, where placental insufficiency has occurred over at least the last half of gestation.[123] Interestingly, the reverse is true for cardiomyocyte size, where PR results in a decrease in the absolute size of cardiomyocytes,[123] with no change in absolute cardiomyocyte size observed after UPE.[124] Despite a reduction in absolute cardiomyocyte size in PR fetuses, the size of each cardiomyocyte is larger in relation to heart weight when compared to controls. Studies in sheep demonstrate that the size of cardiomyocytes relative to heart weight decreases with gestation in the normally grown fetus, but that this is delayed in the IUGR fetus suggesting that there may be fewer cardiomyocytes in the heart of the IUGR fetus (Figure 5). The use of both sheep models of placental insufficiency highlights how differences in the degree and timing of fetal insults can result in different cardiomyocyte phenotypes.

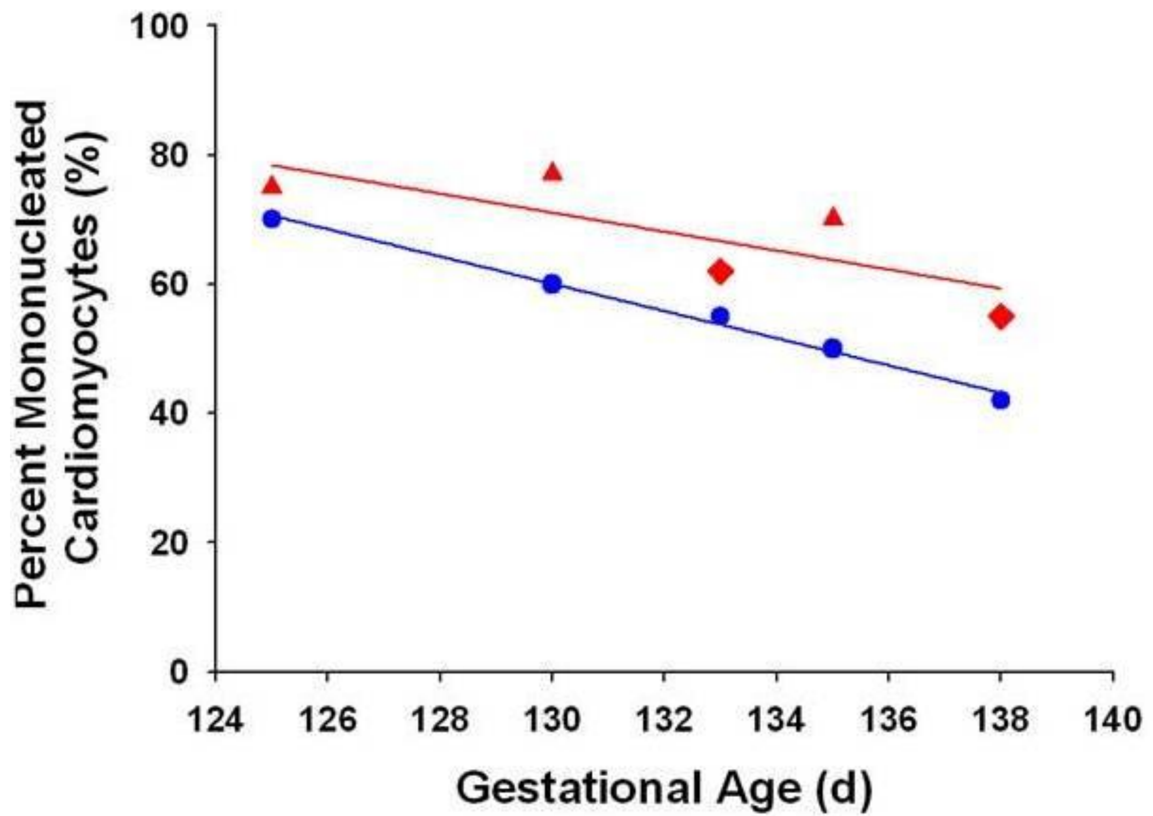

Figure 4. Regardless of the model of IUGR employed, IUGR in sheep results in an increased percentage of mononucleated cardiomyocytes across late gestation.[123-125] Control, blue circles; umbilicoplacental embolization (UPE),[124, 125] red triangles; placental restriction (PR),[123] red diamonds.

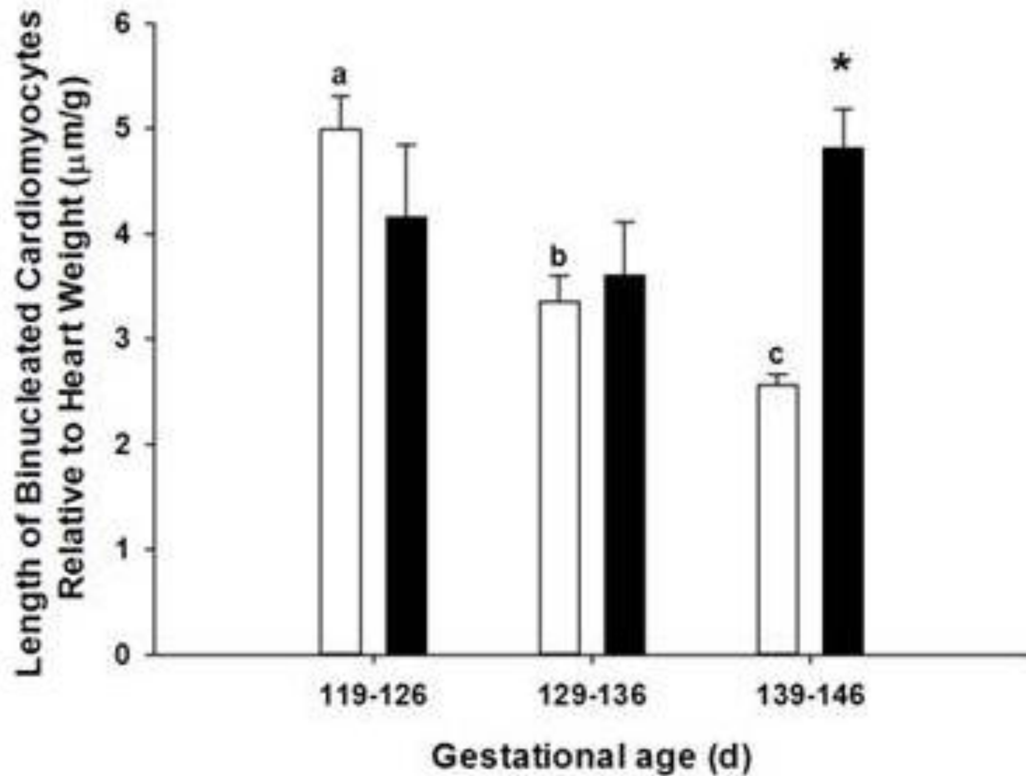

Figure 5. In the normally grown sheep fetus (open bars) the length of binucleated cardiomyocytes relative to heart weight decreases with increasing gestational age, however, this does not occur in the PR fetus (solid bars), such that there is an increase in the relative length of binucleated cardiomyocytes in the PR fetus compared to the normally grown fetus in late gestation.[93, 123] Different superscripts (*e.g.* a, b, c) denote a significant difference between gestational ages in the normally grown fetus. * denotes a significant difference between normally grown fetuses and PR fetuses at 139-146 days gestation ($P < 0.05$).

1.1.7 Postnatal cardiac consequences of IUGR

The adaptation of the fetal heart to a period of reduced substrate supply and decreased body growth has critical consequences for heart health in later life because at birth, the human heart contains most of the cardiomyocytes it will have for life.[11-14] Consequently, in cases where the endowment of cardiomyocytes is reduced, the remaining cells will be required to increase in size in order to increase their capacity for contractile force generation, with a consequent increased risk of coronary heart disease.[12, 65, 126] A reduction in cardiomyocyte endowment is not the only consequence of IUGR that may change the profile of cardiomyocytes present in the postnatal heart. Increasing evidence suggests the regulation of cardiomyocyte metabolism,[127, 128] contractility,[129] protection/survival[114, 115, 130, 131] and hypertrophy[114, 123, 132] may each be affected by a reduced substrate supply *in utero*.

1.1.8 Concluding remarks

The data discussed in this review suggest that IUGR induced by reduced substrate supply in different species leads to alterations in cardiomyocyte development and may lead to reduced cardiomyocyte endowment. It is not known, however, if the observed changes to cardiomyocyte development are all induced by a common mechanism evoked by suboptimal substrate supply, or whether deficiencies in specific substrates, such as protein or oxygen, induce specific consequences to cardiomyocytes. This is important for the understanding of the mechanisms that regulate cardiomyocyte endowment. It is also not clear, if a window of time exists when cardiomyocyte endowment can be rescued. Studies in rats suggest that the early postnatal period may represent such a window, but it is not clear if this is due to the remodelling of the heart that occurs during postnatal changes in the circulatory system or due to the postnatal timing of cardiomyocyte terminal differentiation in rats. It is clear that further studies are required to address these critical issues and to determine whether or not intervention strategies are likely to be beneficial in restoring cardiomyocyte endowment.

1.2 Review for submission: Early Origins of Heart Disease: IUGR and postnatal cardiac metabolism

Introduction

Cardiovascular disease (CVD) currently affects over 3 million Australians,[133] and the incidence is expected to rise, with the World Health Organisation predicting that by 2030, 23.3 million people worldwide will die annually from CVD.[134] Although a range of lifestyle risk factors have been identified, it has also been shown that growth patterns in early life contribute to CVD risk.[135] Together with accelerated growth in childhood, slow growth *in utero* results in an increased risk of hypertension and a ~50% greater risk of coronary heart disease in adult life.[136-138] In Australia, ~6% of babies are born with intrauterine growth restriction (IUGR),[139, 140] defined as a birth weight less than the 10th centile.[46, 135, 141] Placental insufficiency is the leading cause of IUGR in the developed world and results in decreased oxygen and nutrient supply to the fetus.[142] This results in a number of adaptive responses in the fetus, including a redistribution of cardiac output to key organs such as the brain and a decrease in fetal growth rate.[143]

The programming of CVD is multifaceted and is likely to result from, but not be exclusive to, a deficit of cells, such as cardiomyocytes, and alteration to cardiac hypertrophy and cardiac energy production. Changes to these systems allow the fetus to survive an adverse environment *in utero*, but renders them vulnerable to CVD in adulthood, especially if faced with a secondary insult, such as obesity, in postnatal life. This review will focus on the effect of IUGR on cardiac metabolism and the interaction of this initial cardiac effect with other IUGR-induced pathologies such as obesity, insulin resistance, increased activation of the hypothalamic-pituitary adrenal (HPA) axis and left ventricular hypertrophy (LVH), which can also cause cardiometabolic dysfunction.

1.2.1 Cardiac metabolism

In humans, cardiomyocytes present at birth constitute the majority of the cardiomyocytes an individual will have for a lifetime, therefore, alterations to cardiomyocytes *in utero* can be the basis of altered function in adulthood.[11] IUGR results in fewer cardiomyocytes (for review, see [1]) that may be primed for pathological hypertrophy (for review, see [144]), however, emerging evidence indicates that IUGR offspring also have altered regulation of cardiac energy production,[145] especially if faced with a secondary insult such as obesity.[127]

Within weeks of human conception, cardiomyocytes beat and require a continuous supply of adenosine triphosphate (ATP) for contraction, requiring an estimated 6kg of ATP daily.[146] Considering the importance of continued contraction and that almost the entire supply of cardiac ATP is turned over every ten seconds,[147] it is imperative that cardiomyocyte metabolism is flexible and rapidly adapts to alterations in substrate supply and stimuli in order to efficiently produce ATP. In postnatal life, fatty acid β-oxidation followed by glucose oxidation are the predominant generators of cardiac ATP (105mol ATP per 1mol palmitate and 31mol of ATP per 1mol glucose, respectively).[148] *In utero*, however, fatty acids and oxygen are limited and anaerobic glycolysis, albeit less efficient, is the chief source of ATP (2mol ATP per 1mol of glucose; for review, see [148]). Loss of metabolic flexibility and a greater reliance on glucose for ATP production is observed in postnatal pathologies such as cardiac hypertrophy and congestive heart failure.[149]

1.2.1.1 Fatty acid β-oxidation

Fatty acid β-oxidation occurs in the mitochondrial matrix (Figure 1) and results in the production of acetyl-CoA and electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle resulting in the generation of ATP and additional NADH and $FADH_2$, which donate their electrons to the electron transport chain (ETC) and ultimately synthesise ATP. Long

chain fatty acids are the predominant fatty acid oxidised by the heart to generate ATP and cross the sarcolemma via fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATP; isoforms FATP1 and FATP6 expressed in the heart[150, 151]). Within the sarcoplasm, fatty acids are activated via esterification by fatty acyl-CoA synthetase (FACS) and bound to cytoplasmic acyl-CoA binding proteins, specifically, heart-type fatty acid binding protein (H-FABP),[152] before being shuttled into the mitochondrial matrix via carnitine palmitonyl transferases on the outer (CPT-I; CPT-Iβ is the predominant cardiac isoform in the adult heart[153, 154]) and inner (CPT-II) mitochondrial membrane. Once in the mitochondrial matrix, fatty acid *β*-oxidation is initiated by acyl-CoA dehydrogenases, specific to the type of fatty acid being metabolised (medium chain acyl-CoA dehydrogenase (ACADM), long chain (ACADL) and very long chain (ACADVL); Figure. 1; for review, see [155]).

1.2.1.1.1 Regulation of fatty acid metabolism by PPARα and PPARδ

Fatty acid metabolism only contributes a small fraction of ATP produced in fetal life, presumable due to the limited supply of circulating fatty acids.[148] Shortly after birth, there is a switch from glycolysis as the predominant source of ATP to fatty acid *β*-oxidation, central to which is the increased abundance of peroxisome proliferator-activated receptor alpha (PPARα),[148] the key transcriptional regulator of the fatty acid *β*-oxidation pathway and cardiac lipid homeostasis.[156, 157] PPARα is a member of a family of ligand-activated transcription factors that belong to the super family of nuclear hormone receptors.[158] There are three subtypes of PPARs, PPARα, PPARδ/β and PPARγ, and their expression is tissue specific. PPARα is abundant in tissues with high rates of mitochondrial fatty acid *β*-oxidation, including heart, liver, kidney, brown fat and slow-twitch skeletal muscle; PPARδ, for which there is less known, appears ubiquitously expressed; while PPARγ is predominantly expressed in adipose tissue.[159] PPARα forms heterodimers with retinoid X receptor (RXR) and binds to

peroxisome proliferator response elements (PPREs)[160] located within the promoter region of genes involved in different steps of fatty acid metabolism, such as fatty acid transport into the sarcoplasm (*FATP*), fatty acid activation (*FACS*), transport within the sarcoplasm (*H-FABP*), mitochondrial uptake of fatty-acyl CoA (*CPT-I* and *CPT-II*), upregulation of CPT-Iβ activity (malonyl-CoA decarboxylase (*MCD*)[161]) and fatty acid *β*-oxidation (*ACADM, ACADL* and *ACADVL*; Figure 1; for review, see [162, 163]). In addition to upregulating the transcription of genes involved in fatty acid metabolism, PPARα has also been associated with promoting the translocation of fatty acid transporter CD36 to the sarcolemma.[164]

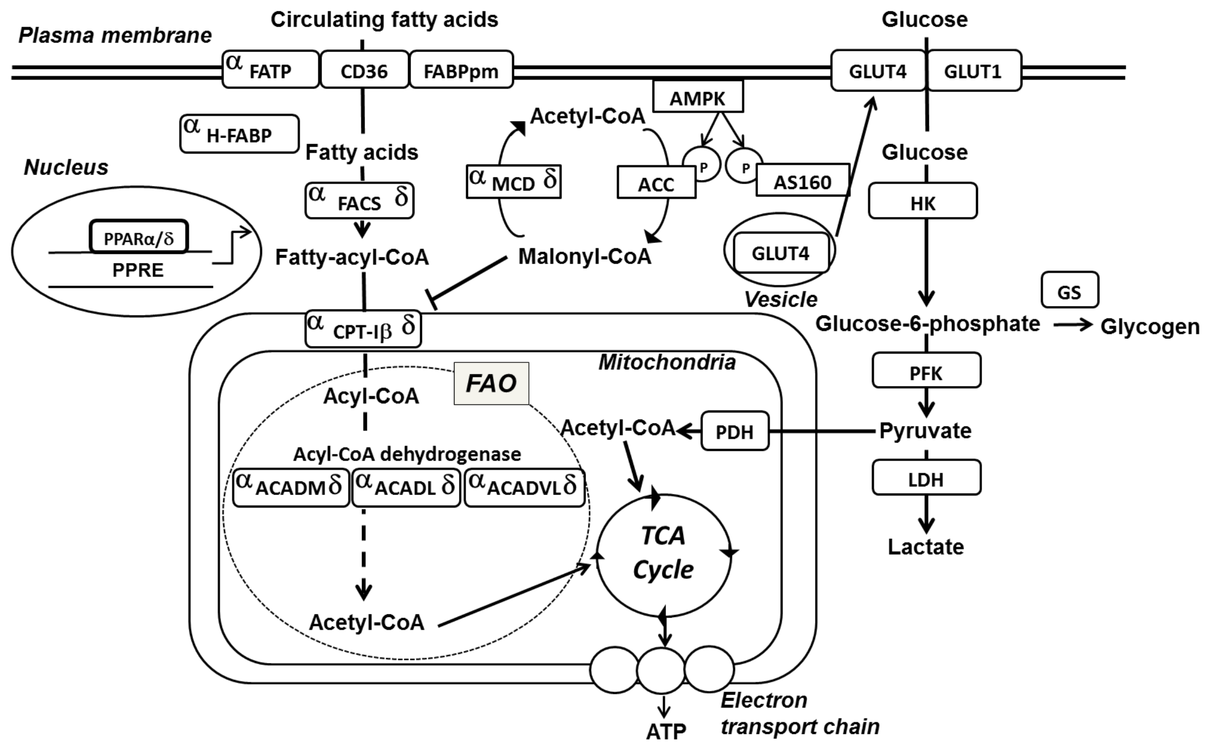

Figure 1. Cardiac fatty acid and glucose metabolism. Circulating fatty acids enter cardiomyocytes through the actions of fatty acid transport protein (FATP), fatty acid translocase (CD36) and plasma membrane specific fatty acid binding (FABPpm). Fatty acids are transported within the sarcoplasm by binding to heart-type fatty acid binding protein (H-FABP) and activated by fatty-acyl CoA synthetase (FACS). Activated fatty acids enter the mitochondria via carnitine palmatonyl transport proteins (CPT) and are oxidised by acyl-CoA dehydrogenases (ACAD) that are specific for the size of the fatty acid (medium ACADM, long (ACADL) and very long (ACADVL) chain). Glucose metabolism is predominantly regulated by glucose uptake into the sarcoplasm. In adult cardiomyocytes, glucose transporter 4 (GLUT4) is the predominant transporter followed by GLUT1. Within the sarcoplasm, glucose is immediately phosphorylated by hexokinase (HK) to glucose-6 phosphate, which can either by stored as glycogen through the actions of glycogen synthase (GS) or converted to pyruvate by 6-phosphofructo-1-kinase-1 (PFK). Under aerobic conditions pyruvate enters the mitochondria and is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) and under anaerobic conditions is converted to lactate by lactate dehydrogenase (LDH). Fatty acid β-oxidation in cardiomyocytes is under the transcriptional regulation of peroxisome proliferator-activated receptor alpha (PPARα) and PPARδ (specific genes signified by PPAR isoform symbol). AMPK is a metabolic ‘fuel gauge’, that in the presence of a high AMP:ATP ratio results in increased ATP production via fatty acid β-oxidation and glucose metabolism. Specifically, this is achieved by phosphorylation and inhibition of acetyl-CoA Carboxylase (ACC), which reduces the production of malonyl CoA, which continues to be degraded by malonyl CoA dehydrogenase (MCD), resulting in reduced inhibition of mitochondrial uptake of fatty acids through CPT-Iβ and promotion of glucose uptake into the sarcoplasm by phosphorylating AS160, which promotes the translocation of encapsulated GLUT4 to the sarcolemma.

The essential role of PPARα in the upregulation of fatty acid metabolism is evident in genetic knockout studies. *PPARα*$^{-/-}$ mice have decreased fatty acid metabolism, which is associated with decreased expression of *MCD*, and a secondary increase in both glucose oxidation and glycolysis.[161] Interestingly, *PPARα*$^{-/-}$ mice are asymptomatic until faced with a metabolic challenge, such as fasting, which leads to lipid accumulation in cardiomyocytes and hepatocytes as well as hypoglycaemia due to a compensatory increase in cardiac glucose metabolism.[161, 165] Chronic exposure to a PPARα agonist *in vitro*, however, leads to increased fatty acid *β*-oxidation, which has deleterious effects on cardiomyocytes such as insulin resistance.[166] Chronic exposure to fatty acids, such as occurs in obesity, may also result in insulin resistant cardiomyocytes.[166]

Most likely due to its ubiquitous expression, less focus has been placed on the regulation of cardiac fatty acid metabolism by PPARδ. Interesting, PPARδ is associated with the transcriptional regulation of numerous genes that are also regulated by PPARα, such as *FACS, CPT-Iβ, MCD, MCAD, LCAD* and *VLCAD*.[167, 168] *PPARδ*$^{-/-}$ results in placental malformation and subsequent mid-gestation lethality in greater than 90% of mice and severe IUGR in those that survive.[169] Cardiac specific knockout of *PPARδ* results in decreased fatty acid *β*-oxidation, increased glucose transport and increased cardiac lipid accumulation by 10 weeks of age.[168] By 4 months of age, cardiac *PPARδ*$^{-/-}$ mice had cardiac hypertrophy and in some mice dilated cardiomyopathy, which resulted in premature death,[168] highlighting the interplay between cardiac metabolism and hypertrophy.

1.2.1.2 Glucose metabolism

Despite contributing only a fraction of the total ATP produced in healthy normoxic hearts, glycolysis appears to be the preferential pathway for generation of ATP for sarcolemmal and sarcoplasmic ATPases that are required for maintaining ionic homeostasis.[170-172] This is in addition to the essential role glycolysis plays in providing ATP

during periods of insufficient oxygen such as hypoxaemia and ischaemia (for review, see [173]). Equally important is the coupling of glycolysis with glucose oxidation in order to maintain appropriate shuttling of pyruvate into the mitochondrial matrix, which takes with it protons generated from lactate production. A build-up of protons in the cytoplasm leads to acidosis and Ca^{2+} overload, which is exaggerated during periods of ischemia, due to the accumulation of metabolic waste products within the cell.[174]

Glucose metabolism is facilitated upon translocation of glucose transporters, GLUT1 and GLUT4, from vesicles in the sarcoplasm to the sarcolemma. In postnatal life, GLUT4 is the predominant cardiac glucose transporter and its translocation is initiated by insulin, AMPK, catecholamines, increased mechanical load and ischemia.[175-178] GLUT1, despite being the predominant cardiac glucose transporter in fetal life, has a low abundance in postnatal life, and its translocation can also be stimulated by insulin, increased mechanical load, catecholamines and ischaemia.[175, 177, 179] Upon entry into the sarcoplasm, glucose is rapidly phosphorylated by hexokinase (HK) to glucose-6-phosphate and is either stored as glycogen or catabolised by 6-phosphofructo-1-kinase-1 (PFK; glycolysis) to form pyruvate (for aerobic metabolism) or lactate (anaerobic metabolism). Pyruvate is shuttled into the mitochondrial matrix, whereupon it undergoes oxidative catabolism by pyruvate dehydrogenase (PDH) to acetyl-CoA, which, similar to the acetyl-CoA produced from fatty acid β-oxidation, enters the TCA cycle, generating ATP and electron carriers that through the ETC ultimately produces ATP (Figure 1; for review, see [180]).

1.2.1.3 Regulation of metabolism by AMPK

Fatty acid β-oxidation is negatively regulated by the presence of malonyl-CoA due to its inhibition of the shuttle protein CPT-Iβ leading to a reduction of fatty acid uptake into the mitochondria (Figure 1).[148] In the immediate newborn period, cardiac malonyl-CoA levels rapidly decline coinciding with the upregulation of fatty acid β-oxidation.[181] Malonyl-CoA can be degraded by MCD to acetyl-CoA and synthesised by acetyl-CoA carboxylase

(ACC),[182-184] whose activity is regulated through the reversible phosphorylation/inhibition by adenosine monophosphate-activated protein kinase (AMPK).[185] AMPK is highly active in the postnatal heart and has been implicated in the upregulation of fatty acid *β*-oxidation through its phosphorylation/deactivation of ACC[181] and the regulation of fatty acid transport proteins CD36 and FABPpm.[186] AMPK acts as a metabolic fuel gauge, which is activated during periods of low ATP due to the inhibition of AMPK dephosphorylation by the binding of either adenosine monophosphate (AMP) or adenosine diphosphate (ADP) to protein phosphatases. A build-up of the phosphorylated catalytic α-subunit of AMPK at Thr-172 during periods of high ratios of AMP:ATP leads not only to the upregulation of fatty acid *β*-oxidation, but also to the upregulation of glucose metabolism due to AMPK stimulated translocation of GLUT4 to the sarcolemma.[176] AMPK has also been implicated in the upregulatiuon of glycolysis due to the phosphorylation and activation of 6-phosphofructo-2-kinase (PFK2), a rate limiting glycolytic enzyme that converts fructose-6-phosphate to fructose 2, 6-bisphosphate.[187] In addition to promoting ATP production, AMPK assists in restoring energy balance by switching off ATP consuming processes such as glycogen and protein synthesis.[188]

1.2.1.4 Competitive metabolism

In cardiomyocytes, as in most mammalian cells, there is a dynamic interaction between fatty acid and glucose metabolism centred on the shared use of the TCA cycle. The inhibition of glucose metabolism by fatty acids and vice versa was first described by Randle and colleagues in the 1960's[189] and the specific mechanisms involved have since been explained (for review, see [190]). Given that fatty acid *β*-oxidation is the most efficient source of cardiac ATP and that acetyl-CoA produced from both fatty acid *β*-oxidation and glucose oxidation require the TCA cycle, it is critical that the acetyl-CoA produced from fatty acids is preferentially used to generate ATP. The increase in acetyl-CoA and NADH from fatty acid

β-oxidation inhibits glucose oxidation at multiple levels and in a graded way, with the greatest inhibition being of PDH,[191] followed by PFK then HK,[192] which results in the inhibition of pyruvate oxidation and glycolysis. In order to allow cardiac metabolic flexibility, glucose metabolism is also capable of inhibiting fatty acid metabolism, whereby the citrate produced from pyruvate derived acetyl-CoA accumulates in the sarcoplasm and is converted to malonyl-CoA,[193] which inhibits CPT-Iβ and subsequently the shuttling of activated fatty acids into the mitochondrial matrix, thus preventing fatty acid β-oxidation. Importantly, however, metabolic stress, such as low ATP and increased mechanical load activates AMPK, which overrides the inhibition of fatty acid β-oxidation by glucose oxidation (Figure 2).[194]

Despite the highly orchestrated regulation of cardiac metabolism and the preference for fatty acid β-oxidation in the postnatal heart, chronic exposure to fatty acids results in deleterious effects on both cardiac ATP production and contractile function. In diabetes, the heart is exposed to high concentrations of circulating fatty acids,[195] which subsequently results in increased expression of PPARα, transcriptional upregulation of genes involved in fatty acid metabolism, permanent translocation of CD36 to the sarcolemma and further inhibition of glucose metabolism (Figure 2; for review, see [196]).

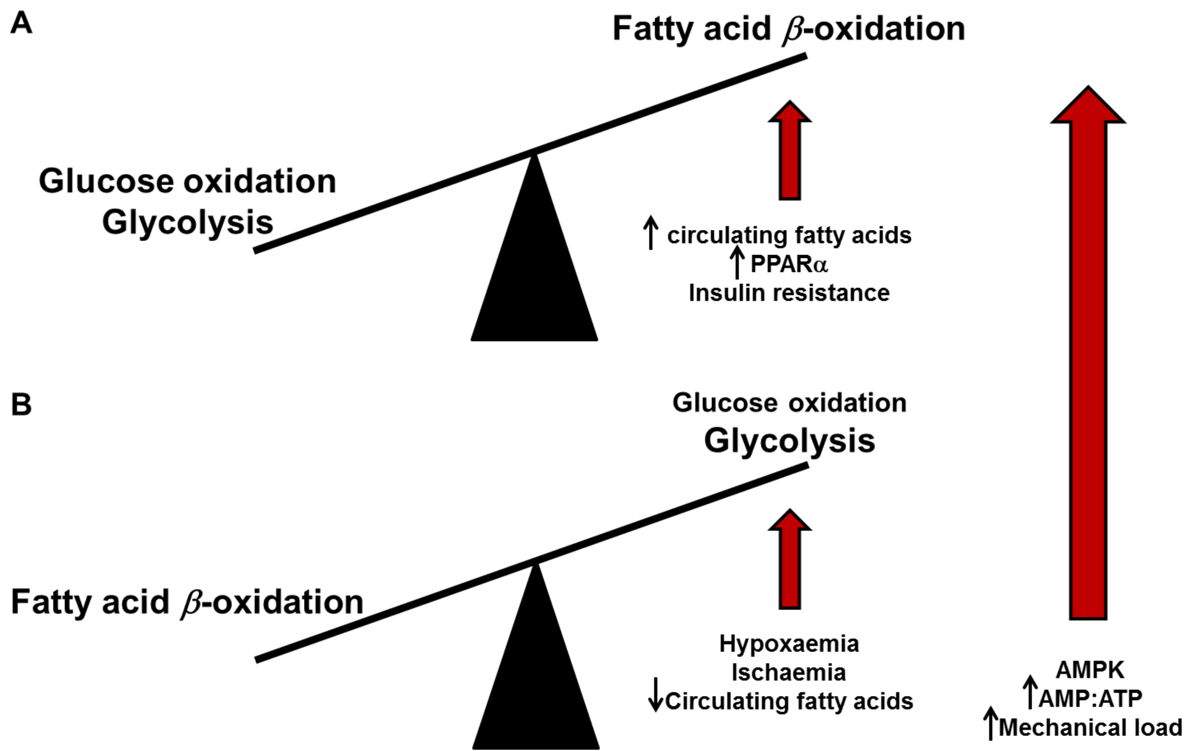

Figure 2. The balance between fatty acid and glucose metabolism. In postnatal life, fatty acid β-oxidation is the predominant source of cardiac ATP and can be upregulated by increased concentrations of circulating fatty acids, increased abundance of transcriptional regulator, PPARα, and insulin resistance (A). During periods of low oxygen, such as hypoxaemia and ischaemia, anaerobic glycolysis is upregulated, more so than glucose oxidation (B). Furthermore, during periods of low circulating fatty acids, glucose metabolism is increased (B). Due to the common use of the tricarboxylic acid cycle, metabolic substrates from fatty acid β-oxidation can inhibit glucose metabolism and vice versa. Importantly, in times of metabolic stress, such as a high AMP:ATP ratio or increased mechanical load, both fatty acid β-oxidation and glucose metabolism can be upregulated by AMPK.

1.2.2 Effect of IUGR on postnatal cardiac metabolism

Animal models demonstrate that fetal substrate restriction and IUGR can alter the regulation of cardiac metabolism in postnatal life. IUGR due to maternal protein restriction across pregnancy in rats is associated with altered cardiac fatty acid content, specifically decreased saturated and increased unsaturated fatty acids, coupled with increased cardiac mRNA expression of *PPARα* in week old offspring.[128] In a similar study, maternal protein restriction across pregnancy did not alter the mRNA expression of *PPARα* in hearts of day old offspring, however, *PPARα* mRNA expression was increased in adult offspring.[197] Interestingly, in both day old and adult offspring, there was decreased methylation of the *PPARα* promoter compared to Control offspring, suggesting that the increased expression in adulthood was epigenetically programmed *in utero*.[197]

In rats, IUGR due to hypoxia in the last week of pregnancy results in offspring with a greater susceptibility to ischaemia/reperfusion injury in adulthood.[115, 198] Furthermore, despite an equivalent cardiac performance prior to ischaemia/reperfusion, IUGR offspring have diminished cardiac power during reperfusion, defined as the difference between peak systolic pressure and maximum preload multiplied by cardiac output and adjusted for heart weight.[198] Interestingly, despite an equivalent contribution of substrates to ATP production prior to ischaemia/reperfusion, IUGR offspring produced a greater proportion of ATP from both glycolysis and glucose oxidation and a reduced proportion from fatty acid β-oxidation during reperfusion.[198] Furthermore, hearts from IUGR offspring metabolised more glucose via glycolysis than glucose oxidation relative to hearts from Control offspring.[198] Subsequently, the uncoupling of glucose metabolism in IUGR hearts resulted in an increased production of protons, which is known to lead to inefficient energy utilisation because correcting the proton imbalance is an ATP-dependent process.[199-201] Additionally, exposure to a high fat diet in postnatal life exacerbates the effect of hypoxia-induced LWB on the susceptibility to ischaemia/reperfusion injury in adulthood.[202]

Changes in cardiac metabolism have also been observed in large animal models of human cardiac development and function such as sheep. Lambs exposed to maternal nutrient restriction from early to mid-gestation have an increased prevalence of ectopic lipid deposition in myocardial tissue if they developed obesity in later life.[127] Elevated lipid deposition subsequently increased the risk of heart failure with increasing age.[127] Maternal hyperthermia from 35-120d gestation (term, 147d), which results in placental restriction and subsequent fetal hypoxaemia, hypoglycaemia and hypoinsulinaemia in late gestation (135d), causes increased sarcolemma GLUT4 and insulin receptor-β protein abundance. These cardiac changes are likely adaptions to the decreased plasma glucose and insulin concentrations to maintain cardiac glucose transport and ATP production, which is supported by increased cardiac glycogen storage and plasma lactate in these animals. Evidence from 21 day old lambs who were IUGR due to placental restriction indicates that there is no difference in the amount of glycogen stored in cardiomyocytes compared to Control lambs.[145] This suggests that fetal storage of glycogen may be utilised for cardiac ATP production in early postnatal life, potentially to fuel the increase in left ventricular hypertrophy observed in the IUGR lamb.[203] Interestingly, these lambs also have an increased abundance of cardiac insulin signalling molecules and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), which inhibits PDH and thus the oxidation of pyruvate, suggesting an increase in glycolysis in early postnatal life. If this observation were to persist into later life, it provides the molecular potential for increased glycolysis in response to ischaemia/reperfusion as occurs in IUGR rat in adulthood.[198]

1.2.3 The effect of obesity and insulin resistance on cardiac metabolism

White adipose tissue is a dynamic metabolic organ that regulates fatty acid flux by storing circulating triglycerides as triglycerols during feeding and releasing them into the circulation as free fatty acids during fasting for uptake and oxidation by other tissues to maintain ATP production. Maintaining optimal levels of circulating free fatty acids is essential because fatty

acids are the predominant substrate used for cardiac ATP production and elevated free fatty acids can result in insulin resistance and eventual type II diabetes mellitus. The association between obesity and insulin resistance is postulated to be caused by increased concentrations of portal vein circulating free fatty acids, increased ectopic lipid storage in muscle and liver, and/or the dysregulation of hormones secreted from adipose tissue (for review, see [204]).

As discussed previously with regards to the heart, increased concentrations of circulating free fatty acids, explained by the Randle cycle, can inhibit glucose metabolism in favour of fatty acid β-oxidation preventing insulin mediated glucose uptake. It is postulated that increased circulating free fatty acids may be due to inefficient storage in adipocytes. As an individual becomes overweight, adipocytes become dysfunctional with regards to metabolic activity and morphology. Obese individuals have larger adipocytes that are less sensitive to insulin and despite their size, inadequately store excess lipid.[205] Hypertrophy of adipocytes may be a consequence of inefficient adipocyte proliferation and/or differentiation. The consequent spill over of free fatty acids into the circulation results in greater uptake into other tissues such as liver, muscle and heart, however, it is currently unclear if ectopic lipid storage is caused by impaired actions of enzymes involved in metabolising these fatty acids or inadequate transport of activated fatty-acyl CoA from the cytosol into the mitochondria for oxidation. Ectopic cardiac lipid storage can interrupt insulin signalling, impair insulin-dependent translocation of glucose transporters to the plasma membrane, impair glycogen synthesis in muscle and promote glucose production in the liver.[206] In patients with insulin resistance and type II diabetes mellitus, cardiac CD36 becomes permanently located on the sarcolemma and GLUT4 is internalised within vesicles promoting incorporation of excess circulating free fatty acids into cardiomyocytes, termed diabetic cardiomyopathy.[207]

Adipose tissue secretes various endocrine hormones (adipokines), such as adiponectin and leptin, which can upregulate fatty acid and glucose metabolism, potentially having an insulin-sensitising effect in other tissues through activation of AMPK.[208, 209] Obese

individuals have increased circulating leptin concentrations[210] and have lower circulating adiponectin concentrations.[211] Despite evidence that obese individual are leptin resistant at the level of appetite regulation, recent evidence in obese mice suggests that the heart is still responding to both circulating and local leptin and that leptin receptor signalling is a major contributor to obesity-mediated LVH.[212] This data may explain why in humans, obesity, independent of hypertension, is a risk factor for LVH.[213] Cardiac hypertrophy causes metabolic dysfunction in the myocardium (please see 1.2.6 “The effect of cardiac hypertrophy on cardiac metabolism”). In addition to direct effects on cardiac metabolism, adiponectin protects against ischaemia/reperfusion injury[209] and pathological cardiac remodelling after myocardial infarction[214] and pressure overload.[215] This is may be due to the promotion of angiogenesis and inhibition of apoptosis, hypertrophy and oxidative stress by adiponectin (for review, see [216]).

Adipocytes can also affect cardiac metabolism as a consequence of inducing hypertension and thus LVH. In order to normalise an increase in wall stress, similar to that observed in hypertension, the ventral wall becomes thicker and thus hypertrophic.[217] The adipokine, angiotensinogen, has been implicated in obesity induced hypertension. Obese individuals have elevated concentrations of angiotensinogen and vasoconstrictive angiotensin II.[218, 219] Angiotensinogen is predominantly produced in the liver is converted to angiotensin I by renin then to angiotensin II by angiotensin converting enzyme (ACE) in the lung. By selectively blocking adipocyte production of angiotensinogen, Yiannikouris and colleagues demonstrated a normalisation of plasma angiotensin II concentrations and systolic blood pressure in diet-induced obese male mice.[220]

1.2.3.1 IUGR and obesity and insulin resistance

The association between IUGR and postnatal obesity, insulin resistance and type-2 diabetes mellitus has been demonstrated in epidemiological studies and numerous animal models.[135] The mismatch between low nutrient availability *in utero* and adequate/excessive

nutrition in postnatal life underpins the programming of obesity and is exaggerated if coupled with accelerated postnatal 'catch up' growth.

IUGR infants have reduced plasma leptin concentrations at birth followed by elevated concentrations at one year of age, coinciding with the period of accelerated growth, compared to normally grown infants.[221-223] IUGR fetuses have a similar plasma concentration of adiponectin compared to normally grown fetuses in late gestation[224] and at birth.[223] However, in childhood (~9 years of age), infants that were born IUGR have decreased plasma concentrations of adiponectin compared to normally grown infants.[225] Furthermore, the concentration of adiponectin in childhood is negatively correlated with waist circumference and systolic blood pressure,[225] indications of an early emergence of the metabolic syndrome.

In late gestation, IUGR due to placental restriction in humans, results in the fetus being exposed to chronic hypoxaemia,[226] hypoglycaemia,[226] hypercortisolaemia[227] and decreased plasma IGF-1 concentrations.[228] This profile is also observed in the placentally restricted and IUGR fetal sheep.[48, 229-231] In the early newborn period, IUGR lambs have accelerated growth compared to Controls, such that by 6 weeks of age have normalised body weight, but have increased visceral adiposity compared to Controls.[232] This altered growth is accompanied by increased insulin sensitivity with regards to circulating fatty acid uptake into adipose tissue.[233] At 6 weeks of age, IUGR lambs also have decreased expression of insulin signalling molecules, such as the insulin receptor, insulin receptor substrate-1, Akt2 and GLUT4 in skeletal muscle[234] suggesting the early onset of insulin resistance. Likewise, maternal undernutrition across pregnancy in guinea pigs results in increased visceral adiposity and decreased glucose tolerance in 4 month old male offspring (females not reported),[235] with increased lipid locule size observed in both male and female offspring in late gestation.[236, 237] The increase in adipocyte size may be explained by inadequate adipocyte proliferation and differentiation, which may be a result of decreased expression of *IGF1* and leptin (*ob*) mRNA, as is observed in perirenal fat of IUGR sheep in late gestation.[231] Day old piglets

exposed to maternal protein restriction across gestation have increased abundance of proteins responsible for fatty acid synthesis from glucose and increased activity of FACS in subcutaneous adipose tissue, suggesting that IUGR offspring are primed for increased fat deposition in this depot from birth.[238]

Evidence from humans and animal models indicate that IUGR infants have impaired insulin sensitivity, adipocyte morphology, secretion of adipokines and circulating free fatty acid concentrations, all of which are factors with the potential to alter cardiac metabolism. Further research is required to decipher the independent effects of IUGR on altered cardiac metabolism and those that are secondary consequences of alteration in global metabolism due to IUGR.

1.2.4 The effect of cortisol on cardiac metabolism

When the body is faced with stress it triggers a response via the hypothalamic-pituitary adrenal (HPA) axis to increase plasma glucose concentrations, maximise metabolism and suppress immune function. Similarities in the profile of disorders within the metabolic syndrome, which includes central obesity, insulin resistance, elevated triglycerides, hypertension and the hypercortisolaemic condition, Cushing's syndrome, have led to the suggestion that cortisol, albeit moderately increased compared to those with Cushing's syndrome, may be at the centre of the metabolic syndrome.[239]

Cortisol is the predominant form of active glucocorticoid in humans, guinea pigs and sheep. The paraventricular nucleus of the hypothalamus secretes arginine vasopressin (AVP) and corticotrophin-releasing hormone (CRH), which act on the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH) into the peripheral circulation. Circulating ACTH exerts its effects on the adrenal, via the melanocortin type 2 receptor (MC2R), stimulating steroidogenesis and secretion of cortisol. The HPA axis is under negative feedback control because cortisol acts through glucocorticoid receptors to inhibit the stimulation of ACTH synthesis and secretion from the pituitary.[240] The majority of circulating

cortisol is bound to corticosteroid-binding globulin, which prevents cortisol from acting on tissues. Unbound cortisol passively diffuses into the cytoplasm, where its concentration is tightly regulated by 11β-hydoxysteroid dehydrogenase (11β-HSD) enzymes. Specifically, 11β-HSD2 converts cortisol to inactive cortisone and 11β-HSD1 converts inactive cortisone to cortisol. Cortisol exerts its actions on energy metabolism through binding to the glucocorticoid receptor (GR). Binding of cortisol to the GR in the cytoplasm results in its translocation to the nucleus, whereupon it binds to glucocorticoid responsive elements in the promoter region of target genes and through recruitment of co-factors, either initiates or inhibits transcription.[241]

Excess glucocorticoid can decrease the expression of signalling proteins downstream of the insulin receptor, such as insulin receptor substrate-1, phosphoinositide-3- kinase (PI3K) and protein kinase B (Akt), which inhibit the translocation of glucose transporters to the plasma membrane and subsequent uptake of glucose into the cell.[242] Furthermore, there is evidence that glucocorticoid administration stimulates lipolysis in adipocytes,[243, 244] which results in increased concentrations of circulating free fatty acids, promoting fatty acid metabolism and inhibiting glucose uptake and metabolism in tissues including the heart. In addition to cardiac metabolic consequences, patients with Cushing's syndrome have LVH and hypertension.[245] Interestingly, correcting the elevated cortisol concentrations in individuals with Cushing's syndrome normalised left ventricular size wihtout normalising blood pressure, suggesting that cortisol has an independent effect on LVH.[246]

1.2.4.1 Effect of IUGR on the HPA axis

There is evidence that exposure of the embryo, fetus or neonate to a range of environmental stressors such as undernutrition, placental dysfunction, excess glucocorticoids or poor maternal care alters the development of the HPA axis and stress responsiveness of the offspring for life.[135, 247-250] It has therefore been proposed that developmental programming of the HPA axis is a conserved evolutionary response to prepare an individual to face a lifetime

of continuing adversity.[251] When this prediction fails, however, as occurs when there is a mismatch between a poor prenatal and abundant postnatal nutritional environment, the individual is at risk of hypercortisolism, central obesity, hypertension and metabolic disease.[135, 252] For example, plasma cortisol concentrations are higher in small for gestational age (SGA) fetuses compared with appropriate for gestational age fetuses.[227]

Restriction of maternal food intake by 50% in the last week of pregnancy in rats results in increased maternal and fetal plasma corticosterone concentrations, the main glucocorticoid in rats, decreased expression of placental 11β-HSD2, which plays a role in protecting the fetus from maternal cortisol by converting it to inactive cortisone, and reduced fetal weight on the last day of gestation.[253] IUGR offspring had an altered HPA axis demonstrated by decreased glucocorticoid and mineralocorticoid mRNA expression in the hippocampus, CRH expression in the hypothalamic paraventricular nucleus and plasma ACTH concentrations.[253] Subsequently, despite an elevated corticosterone concentration at birth, IUGR offspring had decreased plasma corticosterone concentrations compared to Control offspring two hours after birth.[253] Interestingly, when maternal plasma corticosterone concentrations were maintained at basal levels during feed restriction by adrenalectomy with corticosterone supplementation, IUGR and decreased placental 11β-HSD2 expression still occurred, however, there were no alterations to the fetal HPA axis.[253]

In sheep, it is well established that prepartum activation of the fetal HPA axis is essential for the normal timing of parturition and a successful transition from intrauterine to extrauterine life.[254] We have demonstrated that exposure of the sheep oocyte and embryo to a period of moderate maternal undernutrition extending from around a month before conception and for only one week after conception resulted in changes in the development of the fetal HPA axis and cardiovascular systems in late gestation.[255, 256] Since then, it has been shown that maternal undernutrition extending from before conception and into the preimplantation period only, or including the period of early placentation results in altered relationships

between adrenal growth, insulin-like growth factors and steroidogenic enzyme expression during the first 55 days of gestation,[257] an earlier prepartum activation of the fetal HPA axis,[255, 258] an increased risk of premature delivery[259] and increased basal plasma cortisol concentrations in the postnatal lamb.[260, 261]

In sheep, IUGR induced by placental restriction from conception (chronic fetal hypoxaemia and hypoglycaemia) results in increased adrenal weight and plasma cortisol concentrations compared to the normally grown fetus in late gestation.[48] Similarly, IUGR in the sheep fetus resulting from single uterine artery ligation (decreased placental efficiency; chronic fetal hypoxaemia and hypoglycaemia) or umbilicoplacental embolisation (UPE; decreased placental efficiency; chronic intermittent fetal hypoxaemia and hypoglycaemia) from 109d gestation is associated with increased plasma ACTH and cortisol concentrations in late gestation.[262, 263] Interestingly, when UPE occurred later in gestation, from 120d, plasma cortisol concentrations were not different compared to Control lambs in late gestation and after birth at 8 weeks of age.[264] Interestingly, chronic fetal hypoxaemia in sheep due to pregnancy at high altitude does not alter plasma cortisol concentrations under basal conditions compared to Control fetuses in late gestation, however, results in alterations to the HPA axis despite fetuses not being IUGR. Specifically, long term hypoxia results in increased expression of AVP and CRH in the paraventricular nucleus, increased production of ACTH in the anterior pituitary and increased plasma ACTH concentrations (for review, see [265]). Subsequently, in response to a secondary stressor such as hypotension[266] or umbilical cord occlusion[267] the fetus exposed to long term hypoxia produces a greater concentration of cortisol. Recent studies in primates indicate that the increased fetal plasma ACTH and cortisol concentrations due to IUGR are driven by increased CRH, but not AVP.[268] The elevated plasma cortisol concentrations present in IUGR fetuses imply that the fetal HPA axis is operating at a new central set point in the IUGR fetus. However, naturally or spontaneously

occurring differences in fetal growth[269] or modest decreases in birth weight due to either maternal undernutrition or overnutrition[270] do not alter the HPA axis in late gestation.

There is evidence that the alteration in the HPA axis of the fetus continues into postnatal life. Low birth weight is associated with raised fasting plasma cortisol concentrations,[250] enhanced adrenal secretion of cortisol in response to ACTH and increased total urinary cortisol metabolite excretion[271] in human populations. Furthermore, a meta-analysis of human data published between 1998 and 2005, which excluded individuals who were born preterm, demonstrated that a 1kg decrease in birth weight was associated with a 24.2nmol/L elevation in plasma cortisol concentrations in both men and women.[272] Considering that cortisol can influence cardiac metabolism and that IUGR may alter the HPA axis, further research is required to decipher the independent effect of IUGR on cardiac metabolism.

1.2.5 The effect of cardiac hypertrophy on cardiac metabolism

Dysregulation of cardiac metabolism results in disturbances to ion homeostasis, oxidative stress and promotes cardiomyocyte remodelling such as pathological hypertrophy. Likewise, hypertrophy can disturb the regulation of cardiac metabolism and as such determining what is the upstream cause and what is the downstream consequence for a given patient with cardiac hypertrophy is difficult to determine (for review, see [273]). LVH occurs in response to increased workload, such as hypertension, in order to normalise wall stress.[217] Physiological hypertrophy is coupled with an appropriate increase in capillary density and thus gas and nutrient exchange, efficient ATP production and maintenance of contractile function. Pathological hypertrophy, however, is decompensating and associated with a reversion to the fetal profile of expression for many genes, such as increased atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and α-skeletal actin. The altered expression of genes results not only in altered metabolism, but a decreased density of capillaries and assumed poor gas

and nutrient exchange, fibrosis and poor contractile function and leads to heart failure and eventual demise (for review, see [274]).

Fatty acid metabolism in hypertrophied hearts is reduced compared to non-hypertrophied hearts, whilst glycolysis is increased.[275] In hypertrophied hearts of spontaneously hypertensive rats, CD36,[276] H-FABP[277] and FACS[278] are decreased, which may lead to a decrease in the incorporation and transport of fatty acids into the sarcoplasm and decreased activation. Hypertrophied hearts also have less carnitine,[275, 279] which may reduce the ability to shuttle activated fatty acids via CPTs into the mitochondria. Furthermore, in hypertrophied hearts due to pressure overload there is decreased mRNA expression of β-oxidative enzymes such as *ACADM* and *ADADL*, which when hypertrophy is prolonged and decompensation is apparent, result in a decrease in protein abundance.[278, 280] Hypertrophied hearts also have decreased mRNA expression of *PPAR*α, and this is key to the observed downregulation of its fatty acid metabolic target genes.[281] Interestingly, however, supplementing hypertrophied hearts with PPARα leads to a worsening of contractile function, suggesting that the decrease in fatty acid metabolism is a necessary adaption during hypertrophy.[281]

In hypertrophied hearts there is an increased uptake of glucose into the sarcoplasm and increased glycolysis[275, 282] with either no change[275] or decreased glucose oxidation.[279] Due to hypertrophied hearts having a similar expression of glucose transporters and many other genes involved in glucose metabolism,[283] it is proposed that the mechanism for increased glucose uptake is via activation of AMPK, most likely triggered by the decrease in cardiac ATP produced from fatty acids.[282] Likewise, the increased activity of AMPK may also explain the increase in glycolysis in hypertrophied hearts since AMPK phosphorylates and activates PFK, which is increased in hypertrophied hearts.[282] Hypertrophied hearts are more susceptible to ischaemia/reperfusion injury than non-hypertrophied hearts[284] and metabolic dysregulation is believed to be a contributing factor.

1.2.5.1 The effect of IUGR on cardiac hypertrophy

Animal models demonstrate that in late gestation or at birth, IUGR fetuses have a smaller heart than normally grown fetuses.[111, 123-125] In postnatal life, however, the heart has accelerated growth such that in postnatal life it is the equivalent weight[114, 285] or there is LVH[203] compared to the normally grown fetuses. Hypoxia in the last week of pregnancy in rats results in smaller offspring at birth that by 2 months of age have a similar body weight, heart weight and left ventricular weight compared to controls.[114] Interestingly, despite an equivalent ventricular weight, the cardiomyocytes of offspring exposed to hypoxia are hypertrophic, indicated by a larger cross sectional area.[114] This data suggests that offspring of dams exposed to hypoxia during pregnancy have fewer cardiomyocytes and that heart weight and therefore cardiac output has been maintained by greater hypertrophy of the remaining cardiomyocytes. This proposed mechanism is supported by studies of the hypertrophic heart rat (HHR) model, which at 2 days of age have smaller hearts that contain fewer cardiomyocytes compared to Controls,[119] but by 2 months of age develop cardiac hypertrophy in the absence of increased blood pressure.[118] Interestingly, despite an equivalent heart weight at 2[114] and 4 months of age,[286] at 12 months of age rats exposed to hypoxia in the last week of gestation have an increased left ventricle weight relative to heart weight (LVH) and evidence of left ventricular dysfunction,[286] which suggest the emergence of pathological hypertrophy.

At 21d of age, lambs that were IUGR due to placental restriction from conception have a larger left ventricle relative to heart weight compared to Controls.[203] Moreover, IUGR lambs have increased expression of the key regulators of cardiac growth, insulin-like growth factor (IGF) -1 (IGF-1) and IGF-2 and their hypertrophy related receptors IGF-1R and IGF-2R..[203] Both IGF-1 and IGF-2 bind and activate the IGF-1R signalling pathway, which promotes physiological hypertrophy through increased protein synthesis via activation of elongation factors eIF2B and eIF4E and ribosomal protein S6 (for review, see [274]). IGF-2R was initially known as a clearance receptor, which under physiological conditions binds and

subsequently removes IGF-2 from the circulation, thus preventing IGF-2 from binding to the IGF-1R and mediated hypertrophy. More recently, however, it has been demonstrated that IGF-2R can itself promote hypertrophy via the pathological Gαq signalling pathway, which is mediated by phosphorylation/activation of calcium/calmodulin-dependent protein kinase II (CaMKII).[287, 288] In 21d old normally grown lambs, the cardiac IGF-2R abundance is negatively correlated to cardiomyocyte size, which suggests that IGF-2R is acting in the classical role of IGF-2 clearance.[203] Interestingly, in 21d old IUGR lambs, there is a positive correlation between IGF-2R abundance and the size of cardiomyocytes, which suggests that the pathological pathway has been activated.[203]

Considering that IUGR may result in LVH and that hypertrophied hearts have altered metabolism, it is important to consider the size of hearts and cardiac expression of hypertrophic genes when concluding that there is altered cardiac metabolism in IUGR offspring.

1.2.6 Conclusion

From the literature published to date, it is evident that IUGR results in changes in the regulation of both fatty acid and glucose metabolism in the heart. Despite the interplay between these systems, it is not currently known if the observed alterations are due to dysregulation of one or both substrates. Furthermore, it is unclear if the observed differences in postnatal life are a direct result of *in utero* programming of cardiac metabolism or if they are secondary responses to other IUGR induced alterations, such as obesity, insulin resistance[289] and cardiac hypertrophy.[203] Intriguingly, for the most part, the observed changes in the expression of metabolic genes do not result in pathology until faced with a secondary insult such as changes in global metabolism, afterload or ischaemia. Considering that IUGR will also result in a greater risk of obesity, insulin resistance, HPA activity, hypertension, and LVH, it is clear that a systems approach will be required to alleviate the effect of IUGR on altered postnatal cardiac metabolism to restore optimal metabolic flexibility.

1.3 Experimental hypotheses

Epidemiological studies show that IUGR infants have a greater risk of CVD in adulthood.[135] Furthermore, animal models demonstrate that IUGR, due to maternal hypoxia, results in a greater susceptible to ischaemia/reperfusion injury,[115, 198] cardiomyocyte apoptosis[115] and altered cardiac metabolism[198] in postnatal life. Considering that the cardiomyocytes present at birth constitute the majority of cardiomyocytes an individual will have for a lifetime,[13, 14] the general aims of this study were to determine if:

1. IUGR in the presence of chronic hypoxaemia results in decreased cardiomyocyte endowment in late gestation in a large animal model where, as in humans, cardiomyocyte binucleation begins *in utero*.
2. IUGR offspring have reduced cardiomyocyte endowment in adolescence and whether or not the cause of IUGR, be it maternal hypoxia or maternal nutrient restriction, and sex of the offspring, influence cardiomyocyte endowment.
3. both male and female IUGR offspring, exposed to either maternal hypoxia or maternal nutrient restriction, have altered expression of cardiometabolic genes in adolescence.

Chapter 2

It is known that IUGR, due to maternal protein restriction in rats, results in fewer cardiomyocytes in the heart of the fetus compared to Controls on the last day of gestation.[111] Furthermore, IUGR, due to maternal hypoxia in rats, results in a greater percentage of apoptotic cardiomyocytes compared to Controls on the last day of gestation.[71] Unlike humans, rats are born with immature cardiomyocytes, which are mononucleated and retain the ability to proliferate until approximately 12 days of age.[74] Currently there are no studies that determine the effect of IUGR on the number of cardiomyocytes in an animal model where, like humans, have diminished proliferative capacity before birth because cardiomyocyte

maturation/binucleation begins *in utero*.[78] In sheep, cardiomyocyte binucleation begins in the last third of gestation and evidence from lambs with naturally occurring variations in birth weights demonstrates there is a positive correlation between body weight and cardiomyocyte endowment.[120] IUGR, due to placental restriction from conception and thus chronic fetal hypoxaemia and hypoglycaemia in lambs in late gestation, results in smaller hearts that contain cardiomyocytes that are larger relative to the weight of the heart compared to normally grown fetuses in late gestation.[123] This suggests that the heart of the IUGR fetus contains fewer cardiomyocytes. Interestingly, it is known that IUGR fetus exposed to placental restriction from conception has an equivalent percentage of cardiomyocytes in the cell cycle compared to Controls in late gestation.[123] **Therefore, I hypothesised that IUGR, due to placental restriction and the presence of chronic hypoxaemia, will result in fewer cardiomyocytes in the heart of fetal sheep in late gestation due to an increased percentage of hypoxia mediated apoptosis.**

Chapter 3

With the knowledge that IUGR due to placental restriction and thus exposure to chronic hypoxaemia and hypoglycaemia result in fewer cardiomyocytes in late gestation (Chapter 2), I therefore aimed to determine whether maternal hypoxia and/or maternal nutrient restriction results in fewer cardiomyocytes in adolescent guinea pigs, who, like humans, are more mature than rats at birth. It is known that IUGR, due to maternal nutrient restriction in rats, results in fewer cardiomyocytes at birth,[111] however, if maternal nutrient restriction continues throughout pregnancy and lactation, IUGR offspring have an equivalent number of cardiomyocytes to Control offspring at 4 weeks of age.[112] In contrast, IUGR, due to maternal hypoxia in rats, results in an equivalent heart weight at 2 months of age, but results in cardiomyocytes with a larger cross sectional area compared to Controls. This suggests that maternal hypoxia results in fewer cardiomyocytes in rat offspring at 2 months of age.[114] These data suggest that nutrient restriction alone does not maintain a deficit in cardiomyocyte

endowment into postnatal life, but that exposure to maternal hypoxia can. **Therefore, I hypothesise that IUGR due to maternal hypoxia, but not maternal nutrient restriction will result in fewer cardiomyocytes compared to Controls in adolescence.** To date, no study has compared the effect of IUGR on cardiomyocyte endowment between male and female offspring. Considering that male offspring are more susceptible to a reduction in the number of nephrons due to IUGR,[290, 291] **I, therefore, hypothesise that male offspring, but not female offspring exposed to maternal hypoxia will have fewer cardiomyoytes compared to Controls in adolescence.**

With the knowledge that IUGR, due to placental restriction, results in fewer cardiomyocytes compared to Control lambs in late gestation (Chapter 2) and that these IUGR lambs have a greater left ventricular (LV) weight relative to heart weight, LV hypertrophy, compared to Controls at 21 days of age,[203] I further aimed to determine if IUGR, due to maternal hypoxia and maternal nutrients restriction results in LVH in adolescent guinea pigs. Studies of the hypertrophic heart rat (HHR) model demonstrate that cardiac hypertrophy at 2 months of age, in the absence of hypertension,[118] is preceded by reduced cardiomyocyte endowment at 2 day of age.[119] **Therefore, I hypothesise that IUGR offspring with a reduction in the number of cardiomyocytes compared to Controls will have left ventricular hypertrophy in adolescence.**

Chapter 4

IUGR due to maternal hypoxia in rats, results in an equivalent cardiac power and cardiac metabolism compared to Control offspring in adulthood.[198] Interestingly, when the heart of IUGR male offspring is subjected to ischaemia followed by reperfusion, cardiac power is diminished, cardiomyocyte apoptosis is increased, fatty acid metabolism is decreased and glucose metabolism is increased, compared to Control offspring.[198] This altered metabolic response to stress suggests that the basal expression of genes involved in fatty acid

metabolism are decreased and/or those involved in glucose metabolism are increased. IUGR due to maternal nutrient restriction in rats, results in an epigenetically programmed increase in cardiac $PPAR\alpha$ expression, the key transcriptional upregulator of genes involved in fatty acid metabolism, in adulthood.[197] This result is in contrast to the down regulation in fatty acid metabolism observed after ischaemia/reperfusion in rat offspring that were exposed to maternal hypoxia,[198] but supports the premise that IUGR may alter the basal expression of cardiometabolic genes in postnatal life. IUGR due to placental restriction, which causes chronic fetal hypoxaemia and hypoglycaemia, results in increased abundance of proteins that promote glycolysis in the absence of a change $PPAR\alpha$ expression, compared to Control lambs at 21 days of age.[145] Oxygen in essential for aerobic metabolism and as such, hypoxia results in increased glycolysis for ATP production. I, therefore, aimed to determine the effect of IUGR due to maternal hypoxia and maternal nutrient restriction on the basal expression of metabolic genes and abundance of regulatory factors. **I hypothesise that IUGR due to maternal hypoxia, will result in an increased abundance of proteins that promote glycolysis and, alternatively, that IUGR due to maternal nutrient restriction, will result in an upregulation of genes involved in fatty acid metabolism in the adolescent heart.**

CHAPTER 2

Statement of Authorship

Title of Paper	Chronic hypoxaemia in late gestation is associated with decreased cardiomyocyte endowment, but does not change expression of hypoxia responsive genes
Paper Status	Paper for submission

Author contribution

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Kimberley Botting		
Contribution to the Paper	Drove the study and interpretation of data Collected hearts for analysis Determined the number of cardiomyocyte, capillary density and protein abundance Designed primers for real-time analysis Analysed all data and created figures Wrote drafts of manuscript Edited manuscript Approved final version of the manuscript		
Signature		Date	20/12/13

Name of Co-Author	Prof Caroline McMillen		
Contribution to the Paper	Established the animal model used in this study Provided intellectual interpretation of data Edited manuscript Approved final version of the manuscript		
Signature		Date	20/12/13

Name of Co-Author	Heather Forbes		
Contribution to the Paper	Performed final TUNEL analysis Involved in discussions regarding interpretation of data Approved final version of the manuscript		
Signature		Date	20/12/13

<table>
<tr><td>Name of Co-Author</td><td colspan="3">Prof Jens Nyengaard</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Provided training and deigned method for determining cardiomyocyte number and capillary density.
Provided intellectual interpretation of data
Edited manuscript
Approved final version of the manuscript</td></tr>
<tr><td>Signature</td><td></td><td>Date</td><td>Dec.20-2013</td></tr>
</table>

<table>
<tr><td>Name of Co-Author</td><td colspan="3">A/Prof Janna Morrison</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Designed and lead the study including running of the animal cohort, surgical procedures, collection of tissues and data analysis and presentation
Provided intellectual interpretation of data
Involved in construction of the manuscript
Edited manuscript
Approved final version of the manuscript</td></tr>
<tr><td>Signature</td><td></td><td>Date</td><td>20/12/13</td></tr>
</table>

2. CHAPTER TWO

Experimental paper for submission: Chronic hypoxaemia in late gestation is associated with decreased cardiomyocyte endowment, but does not change expression of hypoxia responsive genes

2.1 Abstract

Placental insufficiency is the leading cause of intrauterine growth restriction (IUGR) in the developed world and results in chronic hypoxaemia in the fetus. Oxygen is essential for fetal heart development, but a hypoxaemic environment *in utero* can permanently alter development of cardiomyocytes. We induced experimental restriction of placental growth (PR) from conception by removing the majority of uterine caruncles from ewes before conception. Caruncles are the maternal attachment site of cotyledons, which make up the sheep placenta, and as such we reduced placental weight and induced IUGR and chronic hypoxaemia in the fetus. Fetuses in the PR group had fewer cardiomyocytes, but interestingly, there was no difference in the percentage of apoptotic cardiomyocytes in the free wall of the right ventricle (RV), or the abundance of pro-apoptotic transcription factor p53 or expression of *Bax* in the free wall of the left ventricle (LV). Furthermore, fetuses exposed to normoxaemia (Control) or chronic hypoxaemia (PR) had a similar mRNA expression of HIF target genes, which are essential for angiogenesis (*VEGF, Flt-1, ANGPT-1, ANGPT-2* and *Tie-2*), vasodilation (*iNOS* and *Adm*) and glycolysis (*GLUT-1* and *GLUT-3*) in both ventricles. In addition, there was no change in the expression of *PKCε*, a cardio-protective gene whose transcription is regulated by hypoxia in a HIF-independent manner. There was, however, an increased capillary length density, but no difference in the total length of capillaries, in the RV of the chronically hypoxaemic fetuses. The lack of an upregulation of hypoxia target genes in response to chronic hypoxaemia in the fetal heart in late gestation may be due to a decrease in the number of cardiomyocytes and the maintenance of the total length of capillaries. These adaptive responses in the fetal heart may therefore maintain a normal

oxygen tension within the cardiomyocyte of the chronically hypoxaemic fetus in late gestation.

2.2 Introduction

At sea level, air is composed of 21% oxygen, which results in a pressure of oxygen in arterial blood (PaO_2) of 75-100mmHg in adults. Hypoxaemia is an inadequate PaO_2, which without a compensatory increase in oxygen delivery to tissues, results in a decrease in oxygen supply at the cellular level (hypoxia). *In utero*, a fetus's sole source of oxygen is delivered across the placenta from maternal arterial blood and as such, fetal PaO_2 is approximately 20-25mmHg. Despite a low PaO_2, the fetus has an appropriate supply of oxygen to tissues due to an alternate haemoglobin molecule that has a greater affinity for oxygen and a greater combined ventricular output. Interestingly, hypoxia during early fetal life is essential for normal heart growth, especially for embryonic outflow track remodelling[292] and coronary vessel growth.[293] Oxygen homeostasis is tightly regulated at the tissue level by hypoxia inducible factors (HIFs). During acute hypoxia, HIFs recruit mechanisms to increase oxygen supply (via erythropoesis, angiogenesis and vasodilation), decrease oxygen demand (via increased glycolysis coupled with decreased oxidative metabolism), and regulate both the cell cycle and apoptosis (for review, see [294]). HIFs function in a heterodimeric complex consisting of an oxygen regulated α isoform (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β isoform. Together HIFs act as a transcription factor that binds to a hypoxia response element (HRE) upstream of the promoter of genes required for the response to cellular hypoxia. The degradation of HIF-α subunits is oxygen dependent as prolyl hydroxylases (PHD1-3) require oxygen as a co-substrate. During acute hypoxia, PHDs cannot hydroxylate HIF-α leading to increased HIF-α protein stability and translocation to the nucleus, whereupon they form a heterodimer with HIF-1β and recruit co-activators CBP/p300 to induce gene transcription (for review, see [295]). Additionally, the stabilisation of HIF-1α protein also directly stabilises and

causes the accumulation of tumour suppressor p53 protein,[296] a transcription factor that regulates cell cycle activity and apoptosis, thus mediating hypoxia induced apoptosis.

A lower than normal oxygen supply *in utero* can result in lifelong consequences.[297] Exposure to chronic hypoxaemia *in utero* results in intrauterine growth restriction (IUGR) and low birth weight (LBW).[298] A series of worldwide epidemiological studies have demonstrated that LBW is a predictor of ischaemic heart disease and heart failure in adulthood and this association is supported by studies in a range of animal models of fetal hypoxaemia (for review, see [135]). The cardiomyocytes present at birth constitute the majority of the cardiomyocytes that an individual will have for a lifetime,[14] and thus considerable focus has been placed on understanding the impact of reduced oxygen *in utero* on cardiomyocyte development.

Chronic hypoxaemia in the fetus can have different effects on cardiomyocyte development depending on the timing in relation to cardiac development, duration and degree of the insult.[46] In this context, “chronic” has been used to define periods of exposure to hypoxia for periods ranging from 24 hours to several weeks. Interpretation of experimental studies on the impact of fetal hypoxaemia is further complicated by the fact that exposure to hypoxaemia for one week constitutes a third of gestation in rats, but only 5% of gestation in sheep and less in humans. Maternal hypoxia during the last week of gestation in rats increases cardiomyocyte apoptosis and accelerates cardiomyocyte maturation.[71] In adulthood, these rats have an increased susceptibility to ischaemia/reperfusion injury,[115] which is partly due to decreased expression of cardio-protective protein kinase C-epsilon (PKCε).[130] Interestingly, the hypoxia induced decrease in *PKCε* mRNA expression is independent of HIFs, instead it is mediated by intracellular reactive oxygen species.[299] Unlike humans, however, rats are born with an immature cardiovascular system, with all cardiomyocytes capable of proliferating.[74] Terminally differentiated human cardiomyocytes, in the form of binucleated cardiomyocytes have been observed at 0.8 of gestation, which is similar to sheep, where binucleation begins at

0.7 of gestation (for review, see [1]). Studies of placental insufficiency in sheep, which results in chronic fetal hypoxaemia, hypoglycaemia and LBW, report altered fetal heart growth in late gestation.[123-125] Placental insufficiency, resulting in chronic hypoxemia for the last third of gestation[49, 123] or for 20 days (d) in late gestation,[124, 125] causes a delay in the transition of proliferating mononucleated cardiomyocytes to terminally differentiated binucleated cardiomyocytes. Chronic hypoxaemia for up to 20d is associated with a decrease in the percentage of mononucleated cardiomyocytes undergoing proliferation[124] (term, ~150d in sheep), however, this is not observed in late gestation when chronic hypoxaemia has occurred over at least the last third of gestation[123]. Interestingly, the reverse is true for cardiomyocyte size, where chronic hypoxaemia for at least the last third of gestation results in decreased cardiomyocyte size,[123] but chronic hypoxaemia for up to 20d in late gestation does not.[124]

It is currently not known if the chronically hypoxaemic and growth restricted fetus has a cardiac response to hypoxia in late gestation. We hypothesise that fetal hearts exposed to chronic hypoxaemia for at least the last third of gestation will have fewer cardiomyocytes, greater cardiomyocyte apoptosis, greater length of coronary capillaries and increased expression of genes that are upregulated in response to acute hypoxia.

2.3 Methods

2.3.1 Animal model and surgical procedures

All procedures were approved by the University of South Australia and the University of Adelaide Animal Ethics Committees. Carunclectomy was performed in 19 non-pregnant ewes whereby the majority of the endometrial caruncles were removed from the uterus prior to conception, which resulted in placental restriction (PR)[123] and chronic hypoxaemia, which has been measured previously from 102d gestation.[49] All surgery was performed under aseptic conditions with general anesthesia induced by sodium thiopentone (1.25g/ml, intravenous, Boehringer Ingeheim, Australia) and maintained with 3-4% halothane in oxygen. At surgery, antibiotics were administered to the ewe (153.5mg Procaine penicillin, 393mg benzathine

penicillin; 500mg dihydrostreptomycin, Lyppards, Australia). Ewes recovered from surgery for >12 weeks prior to entering a mating program.

Vascular surgery was performed at 119±1d gestation with general anaesthesia induced by sodium thiopentane (1.25g; Pentothal, Rhone Merieux, Australia) and maintained by inhalation of halothane (2.5-4%) in oxygen, as described previously.[123] Briefly, vascular catheters (Critchley Electrical Products, Australia) were inserted in the maternal jugular vein, fetal carotid artery and jugular vein, and the amniotic cavity. Fetal catheters were exteriorised through a small incision in the ewe's flank. Ewes were administered antibiotics during surgery and 3d post-surgery (as above, intramuscularly). Fetuses were administered antibiotics during surgery (150mg Procaine penicillin, 112.5mg benzathine penicillin; 250mg dihydrostreptomycin, Lyppards, Australia; intramuscularly) and 4d post-surgery (500mg ibimicyn, GenePharm, Australia; intraamniotically).

2.3.2 Arterial blood gas measurements

Fetal carotid artery blood samples were collected daily to monitor fetal health by measuring blood gases (PaO_2, $PaCO_2$, oxygen saturation, pH, haemoglobin and arterial oxygen content[123]) at 39°C with an ABL 520 analyzer (Radiometer, Denmark). Animals recovered for 4d post-operatively before blood samples were recorded for experimental comparisons.

2.3.3 Tissue collection

At 140±1d gestation, ewes and fetuses were humanely killed with an overdose of sodium pentobarbitone (8.2g; Vibrac Aus, Australia) and fetuses were delivered by hysterotomy, weighed and exsanguinated. The fetal heart was dissected and weighed. The free wall of the right ventricle (RV) was either dissected, weighed and stored in 4% formaldehyde to determine the total number of cardiomyocytes and capillary length density, frozen for gene analysis or enzymatically digested using a reverse Langendorff apparatus as previously described[123] to determine the average number of nuclei per cardiomyocyte and the percentage

of apoptotic cardiomyocytes. The RV was chosen because in the fetus, the majority of cardiac output is from the RV and hence, if oxygen delivery through the coronary vasculature was insufficient, the greater demand of the RV may make an effect of chronic hypoxaemia more pronounced. Since it is unknown whether or not chronic hypoxaemia causes asymmetric delivery of cardiac output into the left and right coronary artery from the aortic root, gene expression was also performed in the free wall of the left ventricle (LV). Due to limited frozen RV tissue and that neither the left nor right ventricle had an upregulation of hypoxic response genes, protein abundance was analysed in the LV.

2.3.4 Total number of cardiomyocytes and capillary length density

The estimation of total cardiomyocyte number and capillary length were performed using design-unbiased stereological techniques.[300]

Tissue sampling: RV samples were fixed in 4% formaldehyde and serially sectioned into 2mm slices. 5-6 slices were selected using the smooth fractionator principle[301] and further cut to create cubes of $\leq$ 2mm^3. Using the same principle, tissue cubes were divided into groups of 8-12, with one group being embedded in glycolmethacrylate (Technovit 7100, Ax-lab, Denmark) for cardiomyocyte number estimation and the other group becoming isotropic with the isector[302] and embedded in paraffin for capillary length analysis.

Cardiomyocyte number estimation: The average number of nuclei per cardiomyocyte ($\overline{N_N}(nuclei/cm)$) was determined from isolated cardiomyocytes from the RV using Eq.1. Briefly, isolated cardiomyocytes were stained with methylene blue to visualise cardiomyocyte nuclei. The number of mononucleated ($\Sigma Q^{-}(mono)$) and binucleated ($\Sigma Q^{-}(bi)$) cardiomyocytes in a total of 300 cardiomyocytes was determined.

Equation 1.

$$\overline{N_N}(nuclei/cardiomyocyte) := \frac{\Sigma Q^{-}(mono)+2(\Sigma Q^{-}(bi))}{\Sigma[Q^{-}(mono+Q^{-}(bi)]}$$

The numerical density of cardiomyocyte nuclei was determined using the optical disector technique on glycolmethacrylate embedded sections.[303] From the centre of each

glycomethacrylate block, a 30µm thick section was cut and mounted on Superfrost plus slides (Menzel-Gläser, Germany). In order to visualise cardiomyocyte nuclei, sections were stained with Mayer's haematoxylin and 0.15% basic fuchsine. ~Twenty 2D unbiased counting frames of surface area 500µm^2 were systematic, uniformly randomly assigned by newCAST software (Visiopharm, Denmark) to each ventricle piece. A disector height 10µm in the centre of each section determined after a z-axis analysis was used to determine the numerical density of nuclei in a minimum of six ventricle pieces per animal. The numerical density of nuclei in the RV ($N_V(nuclei/rv)$) was determined using Eq.2, where $\Sigma Q^{-}(nuc)$ is the number of nuclei, h is the Z height analysed, a/p is the area of ventricle each point represents and $\Sigma P(rv)$ is the sum of points that hit ventricle tissue.

Equation 2.

$$N_V(nuclei/rv) := \frac{\Sigma Q^{-}(nuc)}{h \cdot (a/p) \cdot \Sigma P(rv)}$$

The number of cardiomyocytes in the RV (*N(cm,rv)*) was determined by dividing the $N_V(nuclei/rv)$ by the average number of nuclei per cardiomyocyte and multiplying by the volume of the RV (volume of RV = post mortem wet weight ÷ 1.06g/cm^3).[304]

Length density and total length of capillaries: The length density of coronary capillaries in the RV ($L_v(cap/rv)$) was determined in 5µm thick paraffin sections on Superfrost plus slides. Non-specific antibody binding was blocked by 1% bovine serum albumin (BSA; 0.2% gelatine, 0.05% saponin in phosphate buffered saline (PBS)). The primary antibody anti-α-smooth muscle actin (1:4000, Sigma-Aldrich, A2547) was used to identify pericytes that wrap around the endothelial cells of capillaries. Primary and secondary antibody anti-mouse-HRP (1:200, Cell Signaling, 7076) were diluted in PBS containing 0.1% BSA and 0.3% Triton X. Antigen location was visualised with DAB (3,3'-Diaminobenzidine tetrahydrochloride; Kem-en-tec Diagnostics, 4170) and nuclei were visualised with Mayer's haematoxylin.

Capillaries were visualised at a magnification of 600X with a BX53 microscope (Olympus, Australia) equipped with a motorised stage (ProScan™III Motorized Stage Systems, Prior, USA) and digital camera (DP72, Olympus, Australia). ~Twenty 2D unbiased counting frames of surface area 10,000μm^2 were uniformly randomly assigned by newCAST software (Visiopharm, Denmark) to each ventricle piece. $L_v(cap/rv)$ was calculated using Eq.3,[304] where ($\Sigma Q^{-}(cap)$) was the number of capillary profiles within the counting frame.

Equation 3. $$L_V(cap/rv) := \frac{2 \cdot \Sigma Q^{-}(cap)}{(a/p) \cdot \Sigma P(rv)}$$

The total length of capillaries in the RV (*L(cap,rv)* was calculated by multiplying the *Lv(cap/rv)* by the volume of the right ventricle.

2.3.5 TUNEL

Apoptosis was measured by the presence of DNA fragmentation with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL; Invitrogen) and visualised with DAB (Metal Enhanced DAB Substrate Kit, Thermo-Fischer Scientific). Isolated, paraformaldehyde fixed cardiomyocytes from the RV were dried onto polylysine coated Superfrost plus slides and fixed with acetone. Positive control slides were generated by inducing DNA nicks with DNAse I (Sigma-Aldrich, AMPD1) and negative control slides were generated by the absence of either terminal deoxynucleotidyl transferase or nucleotides. The percentage of apoptotic cardiomyocytes was determined by the presence of positive TUNEL staining in at least one nuclei of 200 mononucleated and 200 binucleated cardiomyocytes.

2.3.6 Measurement of mRNA expression

RNA was isolated from the LV and RV (~100mg) of each fetus and cDNA was synthesized as previously described.[203] Controls containing no Superscript III (NAC) and no RNA transcript (NTC) were used to test for genomic DNA and reagent contamination, respectively.

The reference genes tyrosine 3-monooxygenase (*YWHAZ*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*)[305] and phosphoglycerate kinase 1 (*PGK1*)[305] were chosen from a suite of reference genes based on expression analysis using the geNorm component of the qBase relative quantification analysis software,[306] because their expression was stable across samples.[307] The expression of target and reference mRNA transcripts were measured by qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystems, USA) in a final volume of 6μL on a ViiA7 Fast Real-time PCR system (Applied Biosystems, USA) as described previously.[307]

Primers were validated to generate a single transcript as confirmed by the presence of one double stranded DNA product of the correct size and sequence (Table 1). Controls containing no cDNA were included for each primer set on each plate to test for reagent contamination. Melt curve/dissociation curves were also run to check for non-specific product formation. Amplification efficiency reactions were performed on 5 triplicate serial-dilutions of cDNA template for each primer set. Amplification efficiencies were determined from the slope of a plot of C_t (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (1-100ng). The amplification efficiency was close to 100%. Each sample was run in triplicate for target and reference genes. The reactions were quantified by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding C_t values. The abundance of each transcript relative to the abundance of the three stable reference genes was calculated using DataAssist Software v3.0 (Applied Biosystems, USA) and expressed as mean normalised expression (MNE).[306]

Table 1. Primer sequences used in quantitative real-time reverse transcription-PCR to measure genes of interest.

Gene	Primers	Accession Number
Reference genes		
Tyrosine 3-monooxygenase (*YWAHZ*)	Fwd 5'-CCTGGAGAAACCTGCCAAGT-3' Rev 5'-GCCAAATTCATTGTCGTACCA-3'	AY970970
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	Fwd 5'-TGTAGGAGCCCGTAGGTCATCT-3' Rev 5'- TTCTCTCTGTATTCTCGAGCCATCT-3'	DQ152956.1
Phosphoglycerate kinase 1 (*PGK1*)	Fwd 5'-ACTCCTTGCAGCCAGTTGCT-3' Rev 5'-AGCACAAGCCTTCTCCACTTCT-3'	NM_001034299
Apoptosis		
B-cell CLL/lymphoma 2 (*Bcl-2*)	Fwd 5'-GTGGAGGAGCTCTTCAGGGA-3' Rev 5'-GTTGACGCTCTCCACACACA-3'	HM630309.1
Bcl-2 associated protein (*Bax*)	Fwd 5'-CAGGATGCATCCACCAAGAAGC-3' Rev 5'-TTGAAGTTGCCGTCGGAAAACATT-3'	AF163774.1
Oxygen sensing		
Hypoxia Inducible Factor -1 alpha (*HIF-1α*)	Fwd 5'-TGAGCTTGCTCATCAGTTGCCA-3' Rev 5'-ACGCAAATAGCTGATGGTGAGCCT-3'	AY485676.1
Hypoxia Inducible Factor–2 alpha (*HIF-2α*)	Fwd 5'-TACAGGTTCCTCCCCGTCAC-3' Rev 5'-CTTGTCAGCTGTCATTGTCGC-3'	NM_174725.2
Hypoxia Inducible Factor–3 alpha (*HIF-3α*)	Fwd 5'-GTGGAGTTCCTGGGCATCAG-3' Rev 5'-CCCGTCAGAAGGAAGCTCAG-3'	EU340262.1
Hypoxia Inducible Factor–1 beta (*HIF-1β*)	Fwd 5'-AGGTGTGGCAATAGCTCTGTGGAT-3' Rev 5'-AGGCCTTGATATAGCCTGTGCAGT-3'	NM_173993.1

Angiogenesis		
Vascular Endothelial Growth Factor-A (*VEGF-A*)	Fwd 5'-TGTAATGACGAAAGTCTGGAG-3' Rev 5'-TCACCGCCTCGGCTTGTCACA-3'	AF071015.1
VEGF Receptor 1 (*Flt-1*)	Fwd 5'-CCGAAGGGAAGAAGGTGGTC-3' Rev 5'-GACTGTTGTCTCGCAGGTCA-3'	NM_001191132.2
Angiopoietin-1 (*ANGPT-1*)	Fwd 5'-TGCAAATGTGCCCTCATGCT-3' Rev 5'-TTCCATGGTTCTGTCCCGCT-3'	AY881028.1
Angiopoietin–2 (*ANGPT-2)*	Fwd 5'-AGAACCAGACCGCTGTGATG-3' Rev 5'-TGCAGTTTGCTTATTTCACTGGT-3'	AY881029.1
Tyrosine-protein kinase receptor (*TEK / Tie-2*)	Fwd 5'-CAGTTTACCAGGTGGACATC-3' Rev 5'-ACATTTTGGAAGGCTTGGGC-3'	AY288926.1
Vasodilation		
Inducible Nitric Oxide Synthase (*iNOS*)	Fwd 5'-AAGGCAGCCTGTGAGACATT-3' Rev 5'-CAGATTCTGCTGCGATTTGA-3'	AF223942.1
Adrenomedullin (*Adm*)	Fwd 5'-GGGGTGCAAGCCTCACTATT-3' Rev 5'- CACATTCCACGCAGCAAACA-3'	NM_173888.3
Glucose metabolism		
Solute carrier family 2 (facilitated glucose transporter), member 1 (*SLC2A1 / GLUT-1*)	Fwd 5'-ATCGTGGCCATCTTTGGCTTTGTG-3' Rev 5'-CTGGAAGCACATGCCCACAATGAA-3'	U89029.1
Solute carrier family 2 (facilitated glucose transporter), member 3 (*SLC2A3 / GLUT-3*)	Fwd 5'-AGAGTATGCGGATGTCGCAG-3' Rev 5'-CACCGATAGTGGCGTAGACC-3'	NM_001009770.1
Cardioprotection		
Protein kinase C-epsilon (PKC*e*)	Fwd 5'-AGCACCCGTTCTTCAAGGAG-3' Rev 5'-TGCTTGCAGCATCACCAAAC-3'	XM_004005978.1

HIF-α stability		
Egl nine homolog 2 (*EGLN2 / PHD-1*)	Fwd 5’-ATGGTGGCATGTTACCCAGG-3’ Rev 5’-AGGGGCTCAATGTTGGCTAC-3’	NM_001102193.1
Egl nine homolog 1 (*EGLN1 / PHD-2*)	Fwd 5’-TGGAGATGGAAGATGTGTGA-3’ Rev 5’-TTGGGTTCAATGTCAGCAAA-3’	NM_001206046.2
Egl nine homolog 3 (*EGLN3 / PHD-3*)	Fwd 5’-TGCTACCCAGGAAATGGAACAGGT-3’ Rev 5’-GCTTGGCATCCCAGTTCTTGTTCA-3’	NM_001101164.1

2.3.7 Quantification of protein abundance

Proteins were extracted from the LV by sonication in lysis buffer (50mM Tris-HCL (pH 8), 150mM NaCl, 1% NP-40, 1mM Na orthovanadate, 30mM Na fluoride, 10mM Na pyrophosphate, 10mM EDTA, and a protease inhibitor tablet (cOmplete Mini; Roche)). Total protein concentration was determined by microBCA assay (Thermo-Fisher Scientific). Protein was diluted to a concentration of 5mg/ml in 1X SDS sample buffer (containing 75 mM DL-Dithiothreitol) and Coomassie blue stain used to confirm equal protein loading on SDS-PAGE before diluted protein was used for experimental blots. Proteins were transferred to a nitrocellulose membrane (Amersham Hybond-C extra; GE Healthcare Life Sciences) using boric acid transfer buffer. Non-specific antibody binding was blocked with 5% skim milk in TBST (Tris buffered saline with 1% Tween-20) or 5% BSA in TBST. Primary antibodies, anti-p53 (1:200, mouse mAb, OP104L, MERCK), anti-PHD-1 (1:500, rabbit pAb, NB100-310, Novus Biological) and anti-PHD-2 (1:500, rabbit pAb, NB100-137, Novus Biological) were incubated overnight. Secondary antibodies, mouse-HRP (1:2000, #7075, Cell Signaling) and rabbit-HRP (1:2000, #7076, Cell Signaling), were incubated for one hour at room temperature. SuperSignal West Pico chemiluminescent substrate (Thermo-Fisher Scientific) and an ImageQuant LAS 4000 (GE Healthcare, Australia) were used to detect and image antigens of interest. ImageQuantTL Analysis Toolbox (GE Healthcare, Australia) was used to quantify the protein bands and the ratio of band density from a 50% and 100% of the loading control constituted from an equal amount of protein extracts from each animal was used to ensure linearity of density measurement. Each antibody was repeated on new blots to ensure reproducibility of result.

2.3.8 Statistical analysis

Fetuses were included in the Control group if the ewe did not undergo carunclectomy surgery and they had a mean gestational PaO_2 >17mmHg and in the PR group if they ewe underwent carunclectomy surgery and they were chronically hypoxaemic, defined as a mean gestational

PaO_2 <17mmHg.[123, 308] Normality was determines using Shapiro-Wilk-test (swilk) in STATA10. Student's unpaired *t*-test was used to determine the effects of PR compared to Control unless otherwise stated. A probability level of 5% ($P<0.05$) was considered significant. In tables, data is presented as mean ± standard error of the mean (SEM). In figures, data is presented as boxplots in order to describe the data in more detail for the reader.

2.4 Results

Placental restriction (PR) resulted in reduced PaO_2, oxygen saturation and arterial oxygen content, but did not alter the concentration of haemoglobin compared to Controls (Table 2). PR and Control fetuses had an equivalent $PaCO_2$ and base excess, but PR fetuses had lower pH (Table 2). Fetuses exposed to chronic hypoxemia had a reduced body and heart weight, but an increase in heart weight relative to body weight in late gestation (Table 3).

Fetuses exposed to chronic hypoxemia had a decreased total number of cardiomyocytes, mononucleated and binucleated cardiomyocytes in the RV (Figure 1A-1C). The total number of cardiomyocytes in the RV was positively correlated with fetal body weight ($P<0.001$; $R^2=0.968$; $y = 1.95 - 0.70x + 0.17x^2$; Figure 1D). Despite a reduction in the total number of cardiomyocytes, there was no effect of being exposed to chronic hypoxaemia on the percentage of apoptotic cardiomyocytes or the mRNA expression of the pro-apoptotic gene *Bax* or the anti-apoptotic gene *Bcl-2* in the RV (Figure 2A-C). Furthermore, fetuses exposed to chronic hypoxaemia had an equivalent abundance of p53 protein in the LV (Figure 2D), which is responsible for hypoxia induced apoptosis. Paradoxically, fetuses exposed to chronic hypoxaemia had a decrease in the mRNA expression of pro-apoptotic *Bax*, however, there was also a decrease in the mRNA expression of anti-apoptotic *Bcl-2* in the LV (Figure 2E-F).

Table 2. Fetal arterial blood gas measurements.

	Control (n=26)	PR (n=20)
PaO_2 on day of post mortem (mmHg)	20.8±0.7	12.7±0.5*
Mean gestational PaO_2 (mmHg)	21.9±0.5	13.7±0.4*
Mean gestational O_2 saturation (%)	65.8±1.7	37.5±1.7*
Mean gestational Haemoglobin (g/dL)	10.5±0.22	11.3±0.61
Mean gestational O_2 content (ml/dL)	9.6±0.2	5.8±0.3*
Mean gestational $PaCO_2$ (mmHg)	49.2±0.6	50.4±1.2
Mean gestational pH	7.384±0.005	7.369±0.005*
Mean gestational base excess (mEq/L)	2.8±0.3	3.5±0.5

PR, placental restriction; values are mean ± SEM; *, $P<0.05$.

Table 3. Fetal body and heart weight measurements.

	Control (*n*=32) (Includes 21 fetuses that were twins)	PR (*n*=22) (Includes 10 fetuses that were twins)
Fetal weight (kg)	4.68±0.12	2.52±0.16*
Heart weight (g)	32.17±0.79	18.77±0.81*
Relative heart weight (g/kg)	6.69±0.09	7.19±0.19*

PR, placental restriction; values are mean ± SEM; Data was analysed by 2-way ANOVA for treatment group and fetal number and determined no interaction; * Treatment $P<0.05$.

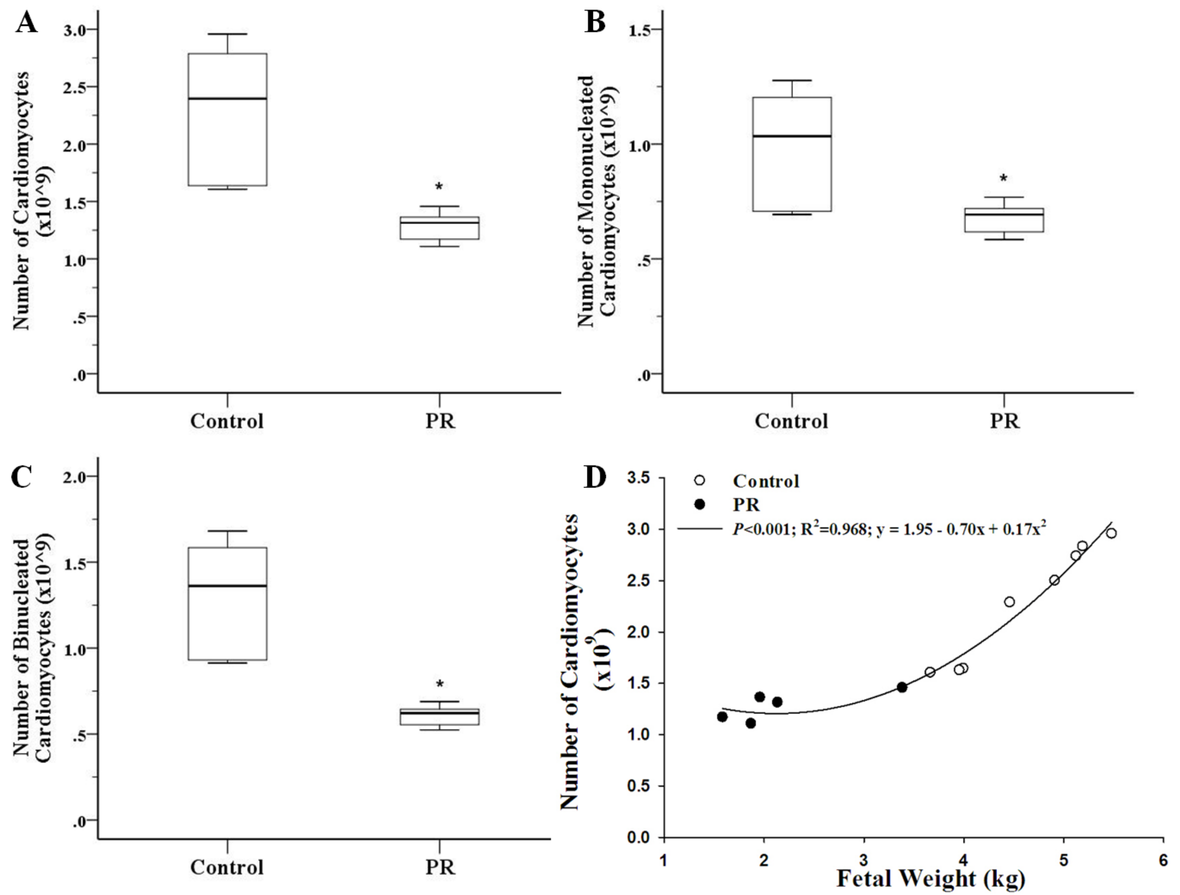

Figure 1. Placental restriction (PR) resulting in chronic hypoxaemia reduced the total number of cardiomyocytes (A), mononucleated cardiomyocytes (B) and binucleated cardiomyocytes (C) in the right ventricle. The total number of cardiomyocytes is positively correlated with fetal weight (D).*, $P<0.05$; Control, n=8, ○; PR, n=5, ●.

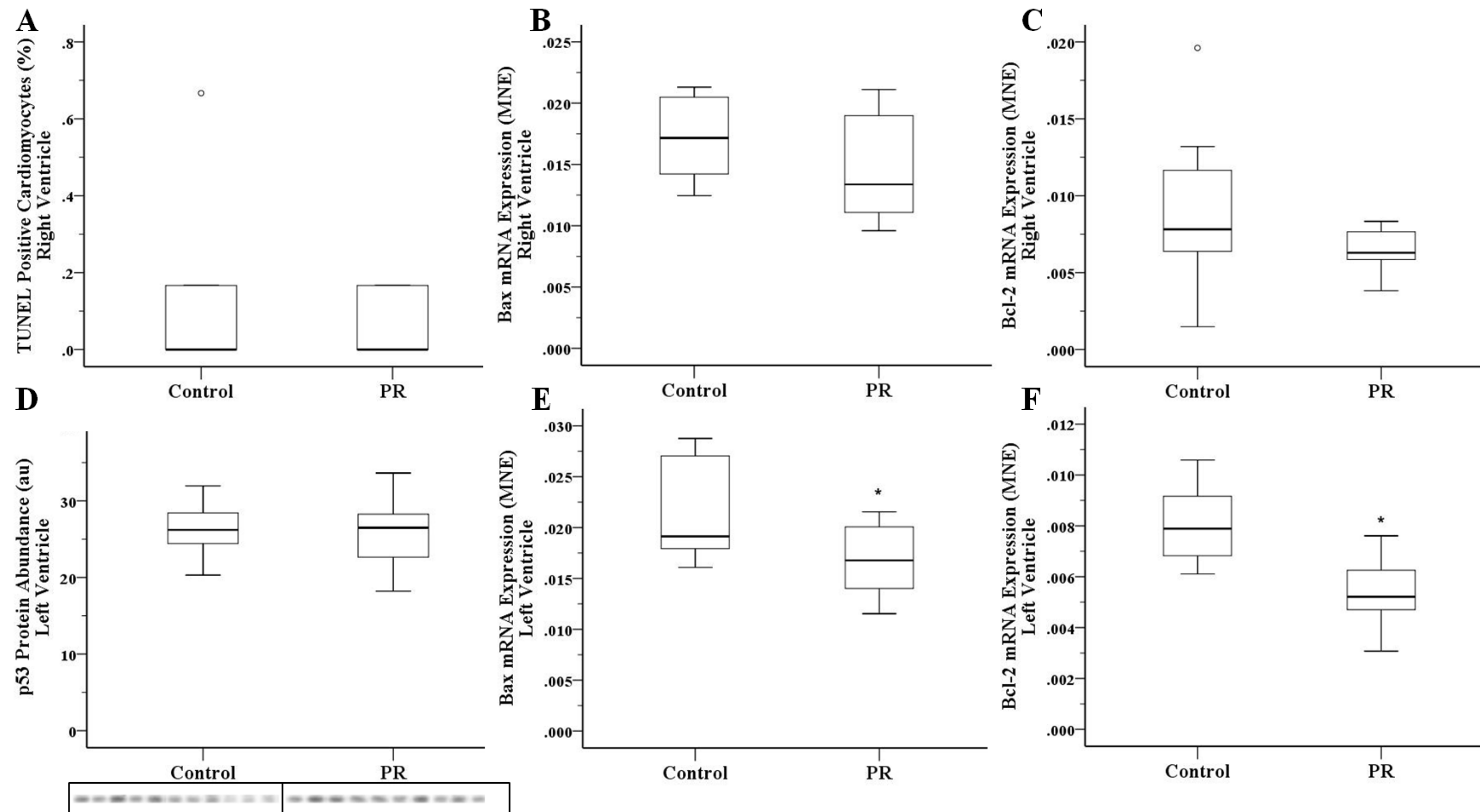

Figure 2. Placental restriction (PR) resulting in chronic hypoxaemia did not alter the percentage of apoptotic cardiomyocytes (A; Control, n=6; PR, n=6) or the mRNA expression of pro-apoptotic gene *Bax* (B) or anti-apoptotic gene *Bcl-2* (C) in the right ventricle (Control, n=9; PR, n=8). PR did not affect the protein abundance of hypoxia-mediated apoptosis regulator p53 (D), but resulted in decreased mRNA expression of both *Bax* (E) and *Bcl-2* (F; Control, n=15; PR, n=12) in the left ventricle. Open circle represents an outlier; *, $P<0.05$.

There was no difference in the mRNA expression of *HIF-1*α, *HIF-2*α, *HIF-3*α, *HIF-1*β and genes with HREs that are involved in angiogenesis (*VEGF, Flt-1, ANGPT-2* and *Tie-2*), vascular tone (*iNOS*) and glycolysis (*GLUT-1* and *GLUT-3*), or of the hypoxia regulated cardio-protective gene *PKC*ε in either the LV or RV of fetuses exposed to chronic hypoxemia compared to Controls (Table 4). Fetuses exposed to chronic hypoxaemia had a decrease in the mRNA expression of the angiogenic gene *ANGPT-1* and the vasoactive gene *Adm* in the LV, but there was no change in the RV. Fetuses exposed to chronic hypoxaemia had an increased capillary length density (Figure. 3A), but a similar total length of capillaries in the RV compared to Controls (Figure. 3B). Interestingly, Control fetuses maintained a positive relationship between the number of cardiomyocytes and the length of capillaries ($y = 3.68x - 4.55$; Figure. 3C), but PR fetuses did not. A similar length of capillaries with a reduction in the number of cardiomyocytes would indicate that there was a greater length of capillaries per cardiomyocyte in the RV of the PR fetus (Figure. 3D).

Exposure to chronic hypoxaemia resulted in increased mRNA expression of the HIF destabilising gene *PHD-3,* but only in the LV (Table 4), coupled with an increased abundance of PHD-2 protein (Figure. 4). There was, however, no change in the mRNA expression of *PHD-1* or *PHD-2* in either ventricle (Table 4). Similarly, there was no change in the protein abundance of PHD-1 (Figure. 4).

Table 4. mRNA expression of HIFs, genes with hypoxia response elements, and genes involved in cardio-protection and HIF-α stability.

	Left Ventricle		**Right Ventricle**	
Gene	Control (n = 15)	PR (n = 12)	Control (n = 9)	PR (n = 8)
Oxygen sensing				
Hif-1α	0.390±0.035	0.343±0.036	0.348±0.022	0.285±0.018
Hif-2α	0.389±0.026	0.499±0.083	0.487±0.090	0.387±0.047
Hif-3α	0.073±0.010	0.061±0.013	0.065±0.006	0.093±0.013
Hif-1β	0.060±0.010	0.049±0.008	0.056±0.005	0.076±0.010
Angiogenesis				
VEGF	0.611±0.054	0.637±0.122	0.436±0.076	0.603±0.065
Flt-1	0.102±0.009	0.108±0.011	0.115±0.006	0.140±0.012
ANGPT-1	0.018±0.002	0.009±0.002*	0.047±0.016	0.019±0.004
ANGPT-2	0.016±0.001	0.015±0.003	0.018±0.003	0.021±0.002
Tie-2	0.075±0.009	0.055±0.011	0.054±0.005	0.085±0.014
Vasodilation				
iNOS	0.010±0.002	0.006±0.001	0.006±0.001	0.007±0.001
Adm	0.013±0.001	0.009±0.001*	0.007±0.001	0.006±0.001
Glucose metabolism				
GLUT-1	0.050±0.006	0.056±0.010	0.042±0.010	0.053±0.009
GLUT-3	1.020±0.158	0.931±0.072	1.384±0.327	1.729±0.271
Cardio-protection				
PKCε	0.053±0.006	0.048±0.007	0.072±0.011	0.050±0.008
HIF-α stability				
PHD-1	0.081±0.007	0.077±0.006	0.071±0.007	0.072±0.009
PHD-2	0.391±0.026	0.344±0.048	0.379±0.029	0.418±0.055
PHD-3	0.284±0.020	0.450±0.047*	0.340±0.036	0.436±0.058

PR, placental restriction; values are mean ± SEM; *, P<0.05.

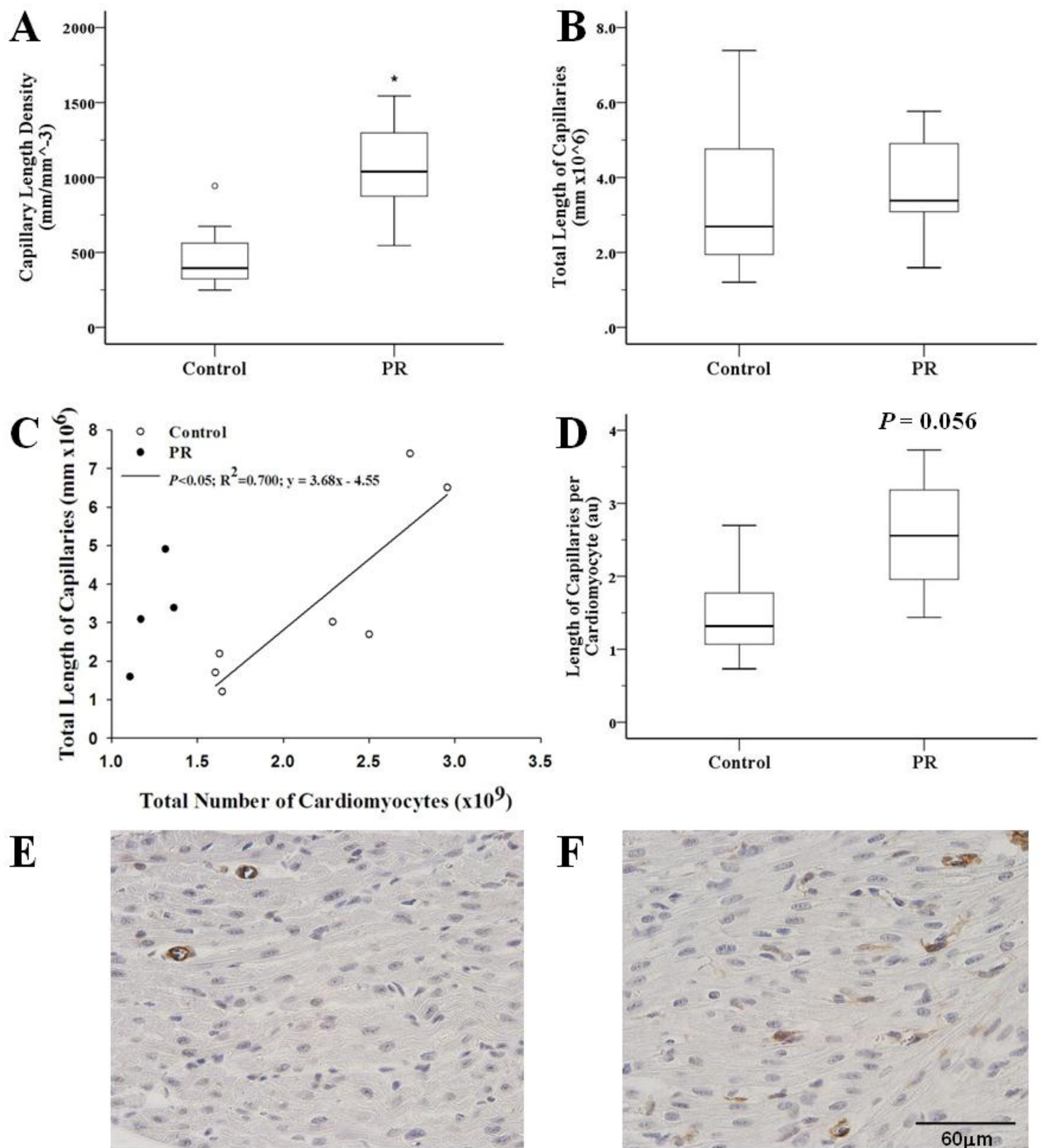

Figure 3. The effect of IUGR on the length of capillaries. Placental restriction (PR; A and F; n=5) resulting in chronic hypoxaemia increased capillary length density in the right ventricle (RV) compared to Controls (A and E; n=7), however, there was a similar total length of capillaries (B). In the Control RV, there was a significant positive correlation between the total number of cardiomyocytes and the total length of capillaries, but not in PR (C). PR hearts have a suggested increase in the length of capillaries per cardiomyocyte (D; presented as arbitrary units (au) due to the two analyses being performed in sections embedded in different compounds; Control, n=7; PR, n=4). Coronary capillaries were identified with immunohistochemistry for α-SMA in the pericytes that surround the capillaries (brown) and counter-stained with Mayer's haematoxylin. Open circle represents an outlier; *, $P<0.05$.

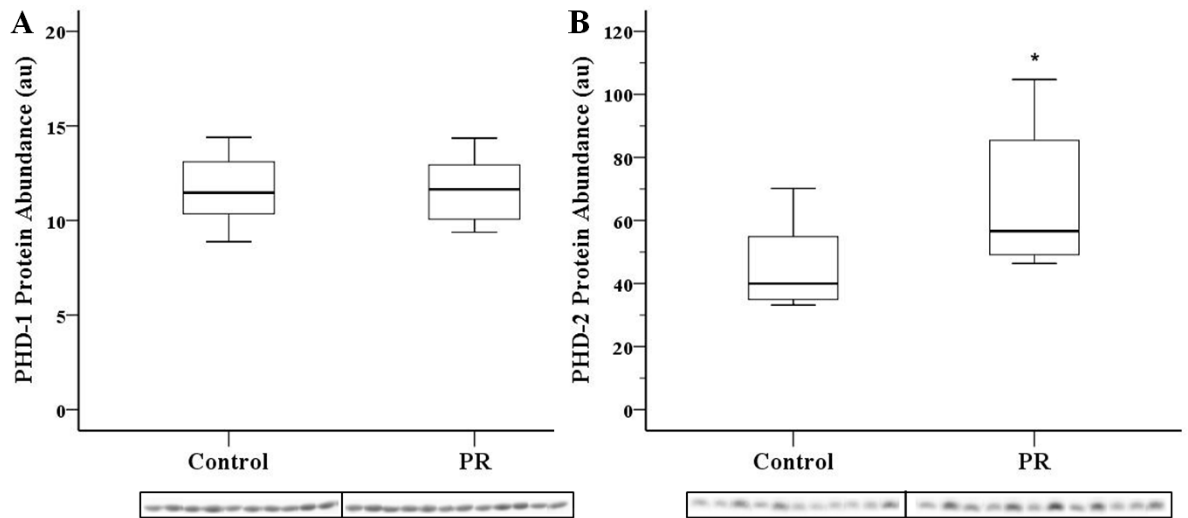

Figure 4. Placental restriction (PR) resulting in chronic hypoxaemia does not change the protein abundance of PHD-1 (A), but increases the protein abundance of PHD-2 (B) in the left ventricle. Treatment groups were alternated across the Western blot to minimise transfer bias; *, $P<0.05$.

2.5 Discussion

In the present study, experimental induction of placental restriction in the sheep from conception resulted in chronic fetal hypoxaemia, reduced fetal growth and fewer cardiomyocytes in the fetal RV. This study is the first to demonstrate that there is a reduction in the number of cardiomyocytes in a large animal model of IUGR where the timing of cardiomyocyte maturation is similar to humans. Consistent with the current findings, maternal protein restriction in rats reduces fetal growth, heart weight and total number of cardiomyocytes at birth.[111] Furthermore, rats exposed to maternal hypoxia during the last week of pregnancy may also have a lower number of cardiomyocytes, since adult offspring have the same heart weight as Controls, but larger individual cardiomyocytes.[114] A reduction in the number of cardiomyocytes, particularly in the number of mononucleated cardiomyocytes may have critical consequences for heart health in later life because at birth, the heart of both the sheep and the human contains the majority of the cardiomyocytes it will have for life.[14, 75] Consequently, the remaining cardiomyocytes will be required to increase in size in order to increase their capacity for contractile force generation, rendering the individual vulnerable to heart disease.[65, 126] This premise is supported by studies in the hypertrophic heart rat (HHR) model, which have smaller bodies from 2 days of age and have hearts that contain smaller and fewer cardiomyocytes that have prematurely exited the cell cycle,[119] and develop cardiac hypertrophy in the absence of hypertension by 2 months of age.[118] Interestingly, in the present study the linear relationship between the number of cardiomyocytes and fetal weight, as previously reported in sheep with naturally occurring variations in birth weight,[120] only holds true for fetuses with a body weight greater than 3kg. It appears that there may be a critical threshold for the number of cardiomyocytes required to maintain function in fetuses less than 3kg. A reduction in cardiomyocyte endowment, as observed in the present model of chronic hypoxaemia and IUGR, provides a potential link for

the epidemiological association between IUGR and an increased incidence of ischemic heart disease and heart failure in adult life.

Maternal hypoxia from 15 to 21d of gestation in rats results in an increase in HIF-1α protein abundance in the fetal heart at 21d gestation.[71] Furthermore, fetal rats exposed to maternal hypoxia had a greater percentage of binucleated cardiomyocytes, which were also larger in size. This is in contrast to previous studies in fetal sheep where hypoxaemia due to umbilicoplacental embolisation[124] or PR[123] reduced the percentage of binucleated cardiomyocytes and PR also reduced their size.[123] Furthermore, in contrast to the present study, fetal rats exposed to maternal hypoxia in the last week of gestation had a greater percentage of TUNEL positive/apoptotic cardiomyocytes, possibly due to a decrease in anti-apoptotic Bcl-2 protein abundance and an increase in the pro-apoptotic protein Fas.[71] In the present study, chronic hypoxaemia did not alter the mRNA expression of the anti-apoptotic gene *Bcl-2* or the pro-apoptotic gene *Bax* in the RV, whereas in the LV, exposure to chronic hypoxaemia decreased the mRNA expression of *Bcl-2* and *Bax*. A decrease in both anti- and pro-apoptotic factors may suggest that there is a similar Bcl-2:Bax ratio, an index of mitochondrial-mediated apoptosis, which is trigged when the ratio favours Bax.[309] Although HIFs do not transcribe *Bax*, its expression can be regulated by hypoxia due to a HIF-1α dependent interaction with the transcription factor p53.[296, 310] Tumour suppressor p53 protein is stabilised in response to cellular stressors, including hypoxia,[311] to arrest the cell cycle, induce apoptosis, inhibit autophagy, alter metabolism and other cellular processes.[312] HIF-1α promotes apoptosis in conditions of chronic cellular hypoxia (24h),[313] however, in the present study chronic hypoxaemia for at least the last third of gestation in fetal sheep did not lead to an accumulation of p53 protein, an increase in *Bax* transcription or an increase in apoptosis, suggesting that in late gestation the heart is not experiencing hypoxia.

Despite PR fetuses being hypoxaemic in late gestation, we did not observe an increase in the cardiac mRNA expression of genes with HREs, which are crucial for a cell’s response

to hypoxia to increase oxygen supply by angiogenesis (*VEGF, Flt-1, ANGPT-2* and *Tie*-2) and vasodilatation (*iNOS* and *Adm*) or decrease oxygen demand by increasing anaerobic metabolism (*GLUT-1* and *GLUT-3*). An alternative interpretation of the results presented in this study is that chronic hypoxia is unable to upregulate hypoxia responsive processes due to the "desensitisation" and destabilisation of HIF-α subunits. In the present study, we were unable to detect HIF-1α protein, despite testing every HIF-1α antibody previously published in sheep tissue. Ginouvès and colleagues suggest that a reduction in mitochondrial respiration during hypoxia, results in an increase in intracellular oxygen, enabling PHDs to be active despite hypoxia.[314] Mice exposed to chronic hypoxia (24h; 8% O_2 in air) do not have stabilised HIF-1α protein in the kidney, brain and thymus, despite HIF-1α being present after 6h of hypoxia (acute).[314] After acute hypoxia, there was a decrease in PHD activity, which was re-activated after 24h of hypoxia. Furthermore, silencing all PHDs using small interfering RNA (siRNA) after 24h of hypoxia resulted in a stabilisation of HIF-1α protein, providing evidence that it is PHD mediated. The theory of HIF-1α desensitisation is supported by studies in the skeletal muscle of exercising mice exposed to one day of hypobaric hypoxia (acute), where HIF-1α protein was stabilised, versus one week of hypobaric hypoxia (chronic), where no HIF-1α protein was measured.[315] In the present study, chronic hypoxaemia resulted in an increase in the mRNA expression of *PHD-3*, but only in the LV, as well as an increase in the abundance of PHD-2 protein. The transcription of *PHD-2* and *PHD-3* is regulated by HIFs, possibly to promote a rapid degradation of HIF-α once normoxia is achieved. Therefore, the increase in PHDs and the absence of increased transcription of hypoxia responsive genes in our study present several interpretations, either that the heart is chronically hypoxic and HIF-1α has been desensitised or the heart is not hypoxic. Considering that we did not see a difference in the mRNA expression of *PKCε*, whose transcription due to prenatal hypoxia in cardiomyocytes is inhibited by intracellular reactive oxygen species and is independent of HIFs, is evidence that the heart is not hypoxic.[130, 299]

This lack of transcriptional activation in the heart of PR fetuses suggests that, despite chronic hypoxaemia, the heart is not experiencing cellular hypoxia potentially due to a lower demand for oxygen since it contains fewer cardiomyocytes that are smaller.[123] Considering that there is also no difference reported in the percentage of cardiomyocytes in the cell cycle,[123] we suggest that earlier in gestation, there has either been a reduction in the rate of proliferation (as observed in fetal sheep studies where hypoxaemia has been present for up to 20d)[124] or an increase in cardiomyocyte apoptosis (as observed in rat offspring of maternal hypoxia in the last week of gestation)[71] that has resulted in the reduced number of cardiomyocyte observed in late gestation in the present study.

Considering that we did not observe a change in the mRNA expression of the angiogenic genes *VEGF, Flt-1, ANGPT-1, ANGPT-2* or *Tie-2* in the RV, it was surprising to observe an almost doubling of capillary length density in the RV of the chronically hypoxaemic fetus. An increase in capillary density (angiogenesis) in response to acute hypoxia has been well documented and is essential to increase oxygen supply (for review, see [316]). Interestingly, however, there was no difference in the total length of capillaries in the RV of fetuses exposed to chronic hypoxaemia compared to Controls, which suggests an increased length of capillaries per cardiomyocyte. We speculate, therefore, that cardiomyocytes and capillaries are differentially sensitive to the early environment in the PR fetus, which ensures each cardiomyocyte may have a normal oxygen tension.

2.6 Conclusion

In the present study, chronic hypoxaemia for at least the last third of gestation[49] resulted in growth restricted fetuses, with smaller hearts that contained fewer cardiomyocytes in the RV. Despite a reduction in the number of cardiomyocytes, there is no difference in the percentage of TUNEL positive/apoptotic cardiomyocytes or the protein abundance of the hypoxia-mediated apoptosis regulator p53. Furthermore, there was either no difference or a decrease in the mRNA expression of the pro-apoptotic gene *Bax*, whose transcription is typically

upregulated during hypoxia by p53.[310] Interestingly, there was no difference in the mRNA expression of HIFs (*HIF-1*α, *HIF-2*α, *HIF-3*α and *HIF-1*β) or target genes with HREs that are central to hypoxia mediated angiogenesis (*VEGF, Flt-1, ANGPT-2* or *Tie-2*), glycolysis (*Glut-1* or *Glut-3*) and vasodilation (*iNOS* and *Adm*). Furthermore, we did not observe a decrease in the mRNA expression of *PKC*ε, whose transcriptional regulation due to prenatal hypoxia is independent of HIFs. Despite chronic hypoxaemia, PR fetuses had a similar length of capillaries compared to Control in the RV, which suggests an increased length of capillaries per cardiomyocyte. Together, this suite of data suggests that in late gestation the heart of the chronically hypoxaemic fetus is not experiencing cellular hypoxia, potentially due to a decrease in oxygen demand (fewer and smaller cardiomyocytes) and an appropriate oxygen supply (maintenance of the total length of capillaries despite a smaller heart).

CHAPTER 3

Statement of Authorship

Title of Paper	IUGR due to maternal hypoxia, but not maternal nutrient restriction reduces the number of cardiomyocytes in female, but not male, adolescent offspring
Paper Status	Paper for submission

Author contribution

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Kimberley Botting		
Contribution to the Paper	Drove the study and interpretation of data Ran the animal model Determined blood pressure Collected hearts for analysis Determined the number of cardiomyocytes Designed primers for real-time analysis Determined protein abundance Analysed all data and created figures Wrote drafts of manuscript Edited manuscript Approved final version of the manuscript		
Signature		Date	20/12/13

Name of Co-Author	Prof Jens Nyengaard		
Contribution to the Paper	Provided training and deigned method for determining cardiomycoyte number Provided intellectual interpretation of data Edited manuscript Approved final version of the manuscript		
Signature		Date	Dec 20 - 2013

Name of Co-Author	A/Prof Janna Morrison
Contribution to the Paper	Designed and lead the study including running of the animal cohort, surgical procedures, collection of tissues and data analysis and presentation Provided intellectual interpretation of data

	Involved in construction of the manuscript Edited manuscript Approved final version of the manuscript		
Signature		Date	20/12/13

3. CHAPTER THREE

Experimental Paper for submission: IUGR due to maternal hypoxia, but not maternal nutrient restriction reduces the number of cardiomyocytes in female, but not male, adolescent offspring

3.1 Abstract

Placental insufficiency is the leading cause of intrauterine growth restriction (IUGR) in the Western world and burdens the offspring with a greater risk of cardiovascular disease in adulthood. In order to distinguish the separate effects of fetal hypoxaemia and hypoglycaemia on the programming of cardiomyocyte endowment, cardiac hypertrophy and hypertension, Maternal Hypoxia (MH; 12% oxygen) and Maternal Nutrient Restriction (MNR; ~22% reduction in food intake per body weight; matched to the daily reduction in food intake per body weight of MH) was induced in guinea pigs from mid-gestation. MH female, but not male, offspring at 120d of age had fewer cardiomyocytes in the left ventricle compared to both MNR and Control offspring. There was no effect of MNR or MH on heart weight and left ventricle weight. Despite this, MH offspring had increased cardiac mRNA expression of the physiological hypertrophy receptor *IGF-1R*, despite no difference in the abundance of the downstream mediator, Akt1 or phosphorylation/activation of Akt. MNR offspring had reduced cardiac mRNA expression of *IGF-2R*, but no difference in cardiac abundance of phosphorylated/activated pathological hypertrophy mediator CaMKII or mRNA expression of the pathological hypertrophy marker, *ANP*. Additionally, there was no effect of MH or MNR on basal blood pressure in males. In conclusion, the present study suggests that IUGR can result in a permanent reduction in the number of cardiomyocytes and importantly, the cause of IUGR and sex interact in their impact on cardiomyocyte endowment in adolescence.

3.2 Introduction

Epidemiological studies of populations from a range of continents and ethnicities demonstrate that poor fetal growth programs the offspring for a greater susceptibility to ischemic heart disease and heart failure in adult life.[2-10] Placental insufficiency is the leading cause of intrauterine growth restriction (IUGR) in the developed world and results in decreased oxygen and nutrient supply to the fetus.[142] This results in a number of adaptive responses in the fetus, including a redistribution of cardiac output to key organs like the brain and a decrease in fetal growth rate.[143] These adaptions may allow the fetus to survive an adverse environment *in utero*, however, they also result in a greater risk of cardiovascular disease and the metabolic syndrome in adulthood.[135]

Animal models provide insight into the intricacies of how exposure to an adverse environment *in utero* alters heart development and the mechanisms that underlie why heart function is compromised in postnatal life. IUGR rats exposed to maternal protein restriction have fewer cardiomyocytes at birth[111] and IUGR rats exposed to maternal hypoxia have increased cardiomyocyte apoptosis in late gestation.[71] Likewise, IUGR lambs exposed to placental restriction from conception (fetal hypoxaemia and hypoglycaemia) have fewer cardiomyocytes in late gestation (Chapter 2), but no difference in the percentage of apoptotic (Chapter 2) or proliferative cardiomyocytes[123] and an equivalent length of coronary capillaries compared to normally grown fetuses (Chapter 2). These data suggest that a reduction in the number of cardiomyocytes is an adaptation to the reduction in global substrate supply, but that when oxygen and nutrient delivery is matched to demand, through an increase in capillary length per cardiomyocyte, cardiomyocyte apoptosis ceases.

Due to limited cardiomyocyte proliferation in the postnatal heart,[13, 14, 317] the cardiomyocytes present shortly after birth will constitute the majority of cardiomyocytes an individual will have for a lifetime.[13, 14] Consequently, a reduction in the total number of cardiomyocytes at birth, due to IUGR, may be permanent and leave the remaining

cardiomyocytes to generate the equivalent force of contraction as hearts of individuals who were born with a full endowment of cardiomyocytes. IUGR rats exposed to maternal hypoxia are more vulnerable to ischaemia/reperfusion injury[115, 198] and experience greater cardiomyocyte apoptosis[115] compared to offspring that were normally grown at six months of age. Furthermore, despite an equivalent cardiac power prior to ischaemia/reperfusion, IUGR offspring have decreased cardiac power during reperfusion.[198] Therefore, a reduction in the number of cardiomyocytes may be one cause for the increased incidence of ischemic heart disease and heart failure in adults that were born IUGR.[2-10]

To date, no studies have measured the cardiomyocyte endowment of IUGR offspring in later life. At two months or age, IUGR rats exposed to maternal hypoxia, have larger cardiomyocytes, but an equivalent heart weight compared to Control rats.[114] Combined with evidence of increased cardiomyocyte apoptosis shortly before birth,[71] this suggests that maternal hypoxia may result in a permanent reduction in the number of cardiomyocytes throughout life. Interestingly, however, maternal protein restriction results in fewer cardiomyocytes at birth,[111] but if maternal protein restriction is continued throughout lactation, until the majority of cardiomyocyte proliferation has ceased in rats,[74] IUGR rats have a normal endowment of cardiomyocytes.[112] These data suggest that exposure to hypoxia *in utero* may have more permanent effects on cardiomyocyte endowment than protein restriction.

In response to increased cardiac workload and wall stress, as occurs in hypertension, cardiomyocytes undergo hypertrophy. This mechanism is typically physiological, but if sustained, may be maladaptive and become pathological. Pathological hypertrophy, unlike physiological hypertrophy, is associated with interstitial fibrosis, re-activation of fetal genes and cardiomyocyte apoptosis (for review, see [274]). In the IUGR fetus exposed to placental restriction from conception, the fewer cardiomyocytes that remain are smaller, but when expressed relative to heart weight are larger compared to the normally grown fetus in late

gestation.[123] This suggests that the regulation of cardiomyocyte hypertrophy is altered either as a direct result of substrate restriction or in response to a reduction in the number of cardiomyocytes. The hypertrophic heart rat (HHR) model, which develops cardiac hypertrophy in the absence of increased blood pressure by 2 months of age,[118] has smaller hearts containing fewer cardiomyocytes at 2 days of age, which have become prematurely binucleated and exited the cell cycle.[119] The HHR model supports the premise that postnatal cardiac hypertrophy in IUGR offspring may be a consequence of fewer cardiomyocytes.

At 21 days of age, IUGR lambs have a larger left ventricle weight when expressed relative to heart weight compared to normally grown offspring, indicating left ventricular hypertrophy (LVH).[203] Furthermore, at 21 days of age, IUGR lambs have increased cardiac mRNA expression of insulin-like growth factor (IGF) 2 (IGF-2), and its receptors, IGF-1R and IGF-2R. IGF-2, in addition to IGF-1, acts on the IGF-1R to initiate physiological hypertrophy through the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathway. IGF-2R has been traditionally viewed as a clearance receptor, whereby binding removes IGF-2 from the circulation and prevents it from binding to IGF-1R. In the 21d old normally grown lamb, the IGF-2R is negatively correlated to cardiomyocyte size,[203] which suggests that IGF-2R is acting in the classical role of an IGF-2 clearance receptor. More recently, however, IGF-2R has been implicated in pathological hypertrophy, whereupon IGF-2 binding activates the Gαq pathway,[288, 318-320] which mediates pathological hypertrophy through the activation of calcium-dependent protein kinase II (CaMKII)[319] and increases the expression of fetal genes such as atrial natriuretic peptide (*ANP*).[287] Interestingly, in the 21d old IUGR lamb, IGF-2R is positively correlated with cardiomyocyte size,[203] which suggests that the IGF-2R is activating a pathological hypertrophy signalling pathway.

In order to determine the independent contributions of reduced oxygen versus reduced nutrition on cardiomyocyte endowment and cardiac hypertrophy in later life, we induced maternal hypoxia (MH) or maternal nutrient restriction (MNR) in guinea pigs in the second

half of gestation. We hypothesised that IUGR due to MH, but not MNR, would result in a permanent deficit in cardiomyocyte number, such that MH offspring would have fewer cardiomyocytes in adolescence compared to both MNR and Control offspring. Furthermore, we hypothesise that offspring with a deficit in cardiomyocyte endowment will have LVH and increased activity of the pathological hypertrophy mediator, CaMKII, and the pathological hypertrophy marker, *ANP,* in adolescence.

3.3 Methods

3.3.1 Animal model

All procedures were approved by the Animal Ethics Committees at both the University of South Australia (IMVS) and The University of Adelaide. IMVS tri-coloured guinea pigs were individually housed at 18-22°C in plastic bottomed cages with sawdust bedding and a 12/12 light/dark cycle. All animals were fed standard laboratory rabbit/guinea pig chow (Laucke Mills, Daveyston, Australia) and had *ad libitum* access to water that was supplemented with 0.5g/L vitamin C. Breeding male and nulligravida female guinea pigs were acclimatised to individual cage living for two weeks prior to commencement of the study. Each female was weighed and given a known weight of food three times weekly. This set weight was in excess of consumption and therefore, on the following weighing day, the *ad libitum* daily food intake per body weight of each female was determined.

Detection of oestrous: The oestrous cycle of each female was determined by checking for an opening in the vaginal membrane. The first day that an opening was observed in the vaginal membrane was defined as day 0. Preliminary studies identified the average oestrous cycle length to be 17.2±0.2d. The average standard deviation for an individual female’s cycle length was 1.2±1.0d; however, the length of an oestrous cycle across the cohort could range from 13-21d. Therefore, females were checked for an opening in the vaginal membrane from day 12 to day 21 of their oestrous cycle to determine day 0.

Time Date Mating: In order to achieve a known day of conception, a single female in oestrous was placed in a males' cage for 24h. Copulation plugs proved to be an unreliable marker of conception, therefore, pregnancy was confirmed by the failure to come into oestrous within 21 days of mating.

3.3.2 Experimental protocol

At 35d gestation, pregnant females (dams) were randomly assigned to one of three treatment groups; Control (21% oxygen, *ad libitum* food), Maternal Nutrient Restriction (MNR; 21% oxygen, food intake matched daily to food intake per body weight of MH) or Maternal Hypoxia (MH; 12% oxygen, *ad libitum* food but ate less than Controls). Dams in the MH group were placed into a glass fronted temperature controlled chamber that was maintained at 12% oxygen by the infusion of nitrogen (Roxy-4, Sable Systems International, USA). To minimize environmental stress, a minimum of two individually housed females were inside the chamber at any given time and the remaining colony was visible. In order to record body and residual food weight, MH dams were removed from the chamber for a maximum of one minute and the total time that the hypoxia chamber was away from 12% oxygen was no greater than 30min. In the time that dams were outside the chamber, a clean cage, fresh water with vitamin C, and a pre-weighed amount of food were provided. Spherasorb with colour indicator (Intersurgical, UK) was used to absorb carbon dioxide in the chamber and was replenished at these times.

MH dams were removed from the hypoxia chamber at 65d gestation to ensure pups were born into a normoxic environment (term, 69d gestation). All dams had *ad libitum* access to food and 21% oxygen throughout lactation. Litters ranged from one to five offspring. Postnatal litters were culled to three pups at 1d of age. Pups were weighed every day for the first two weeks of life and then three times a week. Offspring were weaned at 28d and housed in same sex pairs until humanely killed at ~120d of age (119.4±0.2 d).

3.3.3 Blood pressure analysis

Male offspring were chosen for blood pressure analysis due to the protective effects of estrogen in female offspring and since it was previously published that male, but not female guinea pig offspring exposed to maternal nutrient restriction across all of pregnancy are hypertensive in adolescence.[321] In one male offspring per litter, surgery for the implantation of a catheter in the jugular vein (Silastic; id, 0.051 mm; od, 0.94 mm) and carotid artery (polyvinyl; id, 0.4mm; od, 0.8mm) was performed under general anaesthesia (Atropine (0.05mg/kg, subcutaneously (SC)); ketamine (75mg/kg, intramuscularly (IM) (~100μl) and then intraperitoneally (IP)) and xylazil (6mg/kg, IM))[321] at 110d of age. Catheters were tunnelled under the skin and secured to the back of the neck. Patency of the jugular vein catheter was maintained by flushing with heparinised saline daily (50,000 IU/L heparin). Initial studies identified that male offspring exposed to MH had a greater tendency for blood clotting in the catheter, therefore, all carotid artery catheters contained nylon fishing wire that was pre-soaked in heparin and remained present from surgery until the day of recording. Animals recovered from surgery for 4d before baseline recording of blood pressure and heart rate using a PowerLab data acquisition system (ADInstruments, Australia). Basal values for systolic (SBP) and diastolic (DBP) blood pressure were calculated as the maximum and minimum pressure, respectively. Mean arterial blood pressure (MAP; 2/3 DBP + 1/3 SBP) and heart rate (HR) was derived from the blood pressure signal. SBP, DBP, MAP and HR were calculated by averaging each minute for 136±16 minutes. Due to a period of reduced food intake after surgery, body and organ weight data from males who underwent surgery are not included in data collected after 110d.

3.3.4 Post mortem collection of tissue

Offspring were humanely killed at 119.4±0.2d of age with an overdose of sodium pentobarbitone (200mg/kg IP; Lethobarb, Lyppards, Australia). Crown-rump length and

abdominal circumference were measured and organs (brain, heart, lungs, liver, adrenal glands, perirenal fat, kidneys, epigonadal fat and gonads) were dissected and weighed. For one animal in each litter, the heart was perfused through the aorta with 4% formaldehyde for estimation of cardiomyocyte number. For another animal in a litter, the left ventricle and septum was dissected and snap frozen in liquid nitrogen for analysis of gene and protein expression. In litters where there were 3 pups, the additional animal was only processed in either of the two methods if a sibling of the same sex was not already included, therefore, only one pup of each sex from a given litter is represented in cardiomyocyte number, gene and protein analysis. The left ventricle was selected for analysis in order to determine if LVH is associated with a decrease in cardiomyocyte endowment in the left ventricle.

3.3.5 Total number of cardiomyocytes

The estimation of total cardiomyocyte number was performed using design-unbiased stereological techniques in the free wall of the LV.[300]

Tissue Sampling: The LV was dissected, weighed and serially sectioned into 2mm slices. Using the smooth fractionator principle,[301] 4-5 slices were selected and further cut to create pieces of ≤ 2mm^3. Using the same principle, tissue pieces were divided into groups of 8-12, with one group being embedded in glycolmethacrylate (Technovit 7100, Ax-lab, Denmark) for cardiomyocyte nuclei estimation and the other group becoming isotropic with the isector[302] and embedded in paraffin for determining the average number of nuclei per cardiomyocyte.

Numerical density of cardiomyocyte nuclei: The numerical density of cardiomyocyte nuclei was determined using the optical disector technique on glycolmethacrylate embedded sections.[303] From the centre of each glycomethacrylate block, a 30μm thick section was cut and mounted on Superfrost plus slides (Menzel-Gläser, Germany). In order to visualise cardiomyocyte nuclei, sections were stained with Mayer's haematoxylin and 0.15% basic fuchsine. About twenty 2D unbiased counting frames of surface area 800μm^2 were

systematically, uniformly and randomly assigned by newCAST software (Visiopharm, Denmark) to each tissue piece. A disector height of 10μm in the centre of each section was determined after a z-axis analysis was used to determine the numerical density of nuclei in a minimum of six ventricle pieces per animal. The numerical density of nuclei in the left ventricle ($N_V(nuclei/lv)$) was determined using Eq.1, where $\Sigma Q^{-}(nuc)$ is the number of nuclei, h is the Z height analysed, a/p is the area of ventricle each point represents and $\Sigma P(rv)$ is the sum of points that hit ventricle tissue.

Equation 1.

$$N_V(nuclei/lv) := \frac{\Sigma Q^{-}(nuc)}{h \cdot (a/p) \cdot \Sigma P(lv)}$$

Average number of nuclei per cardiomyocyte: The average number of nuclei per cardiomyocyte was determined in 16 serial 2.5μm paraffin sections, with 2 sections mounted on each Superfrost plus slide (Menzel-Gläser, Germany). In order to identify individual cardiomyocytes, immunohistochemistry for cadherin (1:5000; Sigma Aldrich; C1821; monoclonal mouse) and dystrophin (1:800; Abcam; AB15277; polyclonal rabbit) followed by hydrogen peroxidase-linked secondary antibodies (goat anti-mouse (P0447) and goat anti-rabbit (P0448), DAKO, Denmark) and DAB (3,3'-Diaminobenzidine tetrahydrochloride; Kem-en-tec Diagnostics, 4170) were used to visualise intercalated discs and the sarcolemma, respectively. Nuclei were visualised by counterstaining with Mayer's haematoxylin.

Through the use of an 8-slide motorised stage (ProScan™II Motorized Stage Systems, Prior, England), a BX51 light microscope (Olympus, Germany), digital camera (DP70, Olympus, Germany) and NewCAST software (Visiopharm, Denmark), cardiomyocytes from each section were reconstructed as described previously.[322] Briefly, longitudinal cardiomyocytes were selected in the middle section (section 7) for analysis to ensure that the entire cardiomyocyte and all of its nuclei could be visualised. Cardiomyocytes were selected based on the physical disector[323] and nuclei within 800μm^2 unbiased counting frames were

sampled. By following the cardiomyocyte both up and down the reconstructed stack of sections, the number of nuclei within ~100 cardiomyocytes was determined.

Calculation: The number of cardiomyocytes in the left ventricle (*N(cm,lv)*) was determined by dividing the N_V *(nuclei/lv)* by the average number of nuclei per cardiomyocyte and multiplying by the volume of the left ventricle (volume = post mortem wet weight ÷ 1.06g/cm^3).[304]

3.3.6 Measurement of mRNA expression

RNA was isolated from the left ventricle (~100mg) and cDNA was synthesized as previously described.[203] Controls containing no Superscript III (NAC) and no RNA transcript (NTC) were used to test for genomic DNA and reagent contamination, respectively. The reference genes cyclophilin, tyrosine 3-monooxygenase (*YWHAZ*) and ribosomal protein P0 were chosen from a suite of reference genes based on expression analysis using the geNorm component of the qBase relative quantification analysis software,[306] because their expression was stable across samples.[307] The expression of target and reference mRNA transcripts were measured by qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystems, USA) in a final volume of 6μL on a ViiA7 Fast Real-time PCR system (Applied Biosystems, USA) as described previously.[307]

Primers were designed and validated to generate a single transcript as confirmed by the presence of one double stranded DNA product of the correct size and sequence based on the sequence information for *cavia porcellus* on the National Centre for Biotechnology Information (NCBI) database (Table 1). Controls containing no cDNA were included for each primer set on each plate to test for reagent contamination. Melt curve/dissociation curves were also run to check for non-specific product formation. Amplification efficiency reactions were performed on 5 triplicate serial-dilutions of cDNA template for each primer set. Amplification efficiencies were determined from the slope of a plot of C_t (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template

concentration (1-100ng). The amplification efficiency was close to 100%. Each sample was run in triplicate for target and reference genes. The reactions were quantified by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding C_t values. The abundance of each transcript relative to the abundance of the three stable reference genes was calculated using DataAssist Software v3.0 (Applied Biosystems, USA) and expressed as mean normalised expression (MNE).[145, 306]

3.3.7 Quantification of protein abundance

Proteins were extracted from the left ventricle by sonication in lysis buffer (50mM Tris-HCL (pH 8), 150mM NaCl, 1% NP-40, 1mM Na orthovanadate, 30mM Na fluoride, 10mM Na pyrophosphate, 10mM EDTA, and a protease inhibitor tablet (cOmplete Mini; Roche)). Total protein concentration was determined by microBCA assay (Thermo-Fisher Scientific). Protein was diluted to a concentration of 5mg/ml in 1X SDS sample buffer (containing 75 mM DL-Dithiothreitol) and Coomassie blue stain used to confirm equal protein loading on SDS-PAGE before diluted protein was used for experimental blots. Proteins were transferred to a nitrocellulose membrane (Amersham Hybond-C extra; GE Healthcare Life Sciences) using boric acid transfer buffer. Non-specific antibody binding was blocked with 5% BSA in TBST (Tris buffered saline with 1% Tween-20). Since there are only commercially available antibodies to detect phosphorylation of total Akt and not Akt1 (isoform involved in physiological hypertrophy) and Akt2 (isoform involved in metabolic signalling) separately, primary antibodies for Akt1 (mouse mAb, 2967, Cell Signaling), Akt2 (rabbit pAb, 3063, Cell Signaling), phospho-Akt (thr-308; rabbit pAb, 9275, Cell Signaling) and phospho-Akt (ser-473; rabbit pAb, 4060, Cell Signaling) were used to extrapolate phosphorylation of Akt1. In addition to Akt antibodies, primary antibodies for CaMKII (rabbit pAb, 3362, Cell Signaling), phospho-CaMKII (thr-286; mouse mAb, sc32289, Santa Cruz) and phospho-CaMKII (thr-305; rabbit pAb, ab22183, Abcam) were incubated overnight at 4^oC. Secondary antibodies, rabbit-HRP (7076, Cell Signaling) and mouse-HRP (7075, Cell Signaling) were incubated for

one hour at room temperature. For each membrane GAPDH-HRP (8884, Cell signalling) was incubated overnight at 4°C and served as a loading control (abundance stable across samples). SuperSignal West Pico chemiluminescent substrate (Thermo-Fisher Scientific) and an ImageQuant LAS 4000 (GE Healthcare, Australia) were used to detect and image antigens of interest. ImageQuantTL Analysis Toolbox (GE Healthcare, Australia) was used to quantify the protein bands. The abundance of each protein of interest is expressed relative to its loading control.

Table 1. Primer sequences used in Quantitative Real-Time Reverse Transcription-PCR to measure genes of interest.

Gene	Primers	Accession Number
Tyrosine 3-monooxygenase (*YWAHZ*; Reference gene)	Fwd 5'-TGTAGGAGCCCGTAGGTCATCT-3' Rev 5'-TTCTCTCTGTATTCTCGAGCCATCT-3'	XM_003479856.2
Ribosomal protein P0 (*RpP0*; Reference gene)	Fwd 5'-CAACCCTGAAGTGCTTGACAT-3' Rev 5'-AGGCAGATGGATCAGCCA-3'	XM_003478381.2
Peptidyl-prolyl cis-trans isomerase A/Cyclophilin (*Cyclo*; Reference gene)	Fwd 5'-CCTGCTTTCACAGAATAATTCCA-3' Rev 5'-CATTTGCCATGGACAAGATGCCA-3'	XM_003465805.2
Insulin-like growth factor-1 (*IGF-1*)	Fwd 5'-GCACGCGGTGTCCTCTTCCC-3' Rev 5'- GTGGTGCCCTCCGACTGCTG -3'	NM_001172966.1
Insulin-like growth factor-2 (*IGF-2*)	Fwd 5'-TCTTGGCCTTCGCCTCGTGC-3' Rev 5'-CTCACACGGCTTGCGGGTCT-3'	XM_003468138.2
Insulin-like growth factor-1 receptor (*IGF-1R*)	Fwd 5'-AGCTGGACCTGGAGCCCGAG-3' Rev 5'-GTTCGTGCGGCCTCCGTTCA-3'	XM_003475268.2
Insulin-like growth factor-2 receptor (*IGF-2R*)	Fwd 5'-TGGCGAACGACTGCAGCCTG-3' Rev 5'-AAGTGTCCGGGTCGGAGTCA-3'	XM_003466364.2
Atrial natriuretic peptide (*ANP*)	Fwd 5'-CAATGTCGTGTCCAACGCAG-3' Rev 5'-GCAGATCGATCGGAGGAGTC-3'	XM_003471420.1

3.3.8 Statistical analysis

Normality of data was determined using Shapiro-Wilk-test (swilk) in STATA10. Maternal data was analysed using 2-way analysis of variance (ANOVA) (Stata 10, StataCorp Statistical Analysis Software, Texas, USA). Specifically, the variation between treatment group, litter size and their interaction were tested against the variation within animals by treatment group and litter size. Across pregnancy, data was tested with week as a repeated measure. When an interaction between treatment group and week was observed, a 2-way ANOVA for treatment group and litter size was performed for each week separately with post-hoc Bonferroni tests applied where appropriate.

For body weights and organ weights, a 2-way ANOVA was applied to test the variation between treatment group, sex and their interaction against the variation within litter by treatment group and sex. In this analysis the values of each offspring are represented and nested within their litter.

Body weight across postnatal life was tested with week as a repeated measure. An interaction between treatment group and week was observed, therefore, a 2-way ANOVA for the variation between treatment group, sex and their interaction was tested against the variation within litter for each week separately with post-hoc Bonferroni tests applied where appropriate.

For cardiomyocyte number, gene and protein analysis, only one sex from each litter was analysed, therefore, a 2-way ANOVA for the variation between treatment group, sex and their interaction was tested against the variation within animal by treatment group and sex with post-hoc Bonferroni tests applied where appropriate.

Statistical significance was assumed if $P<0.05$. In tables, data is presented as the mean of each treatment group ± the standard error of the mean (SEM). In figures, data is presented as boxplots.

3.4 Results

3.4.1 Maternal data

MNR dams entered pregnancy at a lower weight than Control and MH dams (Figure 1A), but were of a similar weight at the start of treatment (35d gestation; Figure 1B). Pregnant dams across all treatment groups had a similar body weight across the experimental protocol (35d gestation - term; Figure 2A). MH dams had decreased food intake per body weight compared to Control dams (Figure 2B). MNR dams had a similar food intake per body weight to the MH and significantly less than Control dams during the experimental protocol (Figure 2B). Dams pregnant with a greater number of fetuses had a greater body weight, however, there was no interaction between treatment group and litter size (data not shown).

3.4.2 Birth and postnatal growth

Litters in all treatment groups had a similar gestational age at birth, however, MH resulted in greater variability (Figure 3A). There was no effect of MNR or MH on litter size (Figure 3B). Being exposed to MNR and MH reduced birth weight compared to Controls (Figure 3B). Of note, some offspring did not survive the birthing process and were born dead. This observation has been previously reported in guinea pigs.[324] Interestingly, in the present study, both MNR and MH resulted in a decreased percentage of deaths at birth (13% and 17%, respectively, compared to 29% of Control offspring), which may be due to their reduced size.

MH offspring remained smaller than Control offspring until 16wks of age, however, MNR offspring reached the weight of Controls by 4wks of age (Figure 4). MH offspring weighed less than MNR offspring from 6wks of age (Figure 4). Male offspring weighed more than female offspring from 5wks of age, which was consistent across all treatment groups.

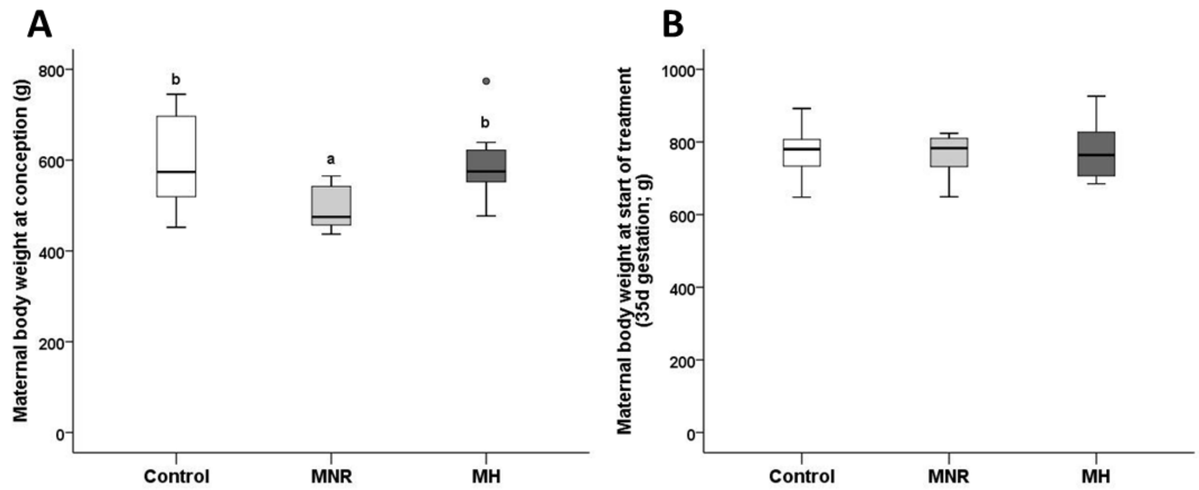

Figure 1. At 35d gestation, pregnant dams were randomly assigned to treatment groups. By chance, Maternal Nutrient Restriction (MNR; A; n=9) dams weighed less than Maternal Hypoxia (MH; n=9) and Control (n=11) dams at conception, however, all dams had an equivalent body weight at the start of treatment (B; 35d gestation). Data anlysed by 2-way ANOVA for treatment group and litter size with post-hoc Bonferroni tests (no interaction between treatment group and litter size); different letters indicate a significant difference between treatment groups; $P < 0.05$.

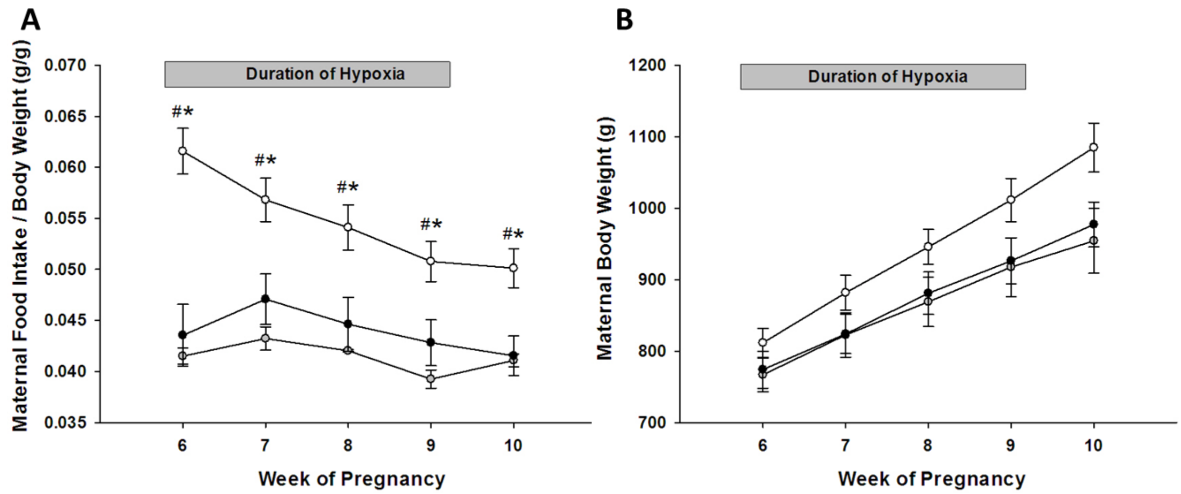

Figure 2. Maternal Hypoxia (● ; MH; n=9; A; 35d gestation to term) resulted in decreased food intake per body weight compared to Control (○; n=11), which was matched to the Maternal Nutrient Restriction group (● ; MNR; n=9). MNR and MH dams had a similar body weight across the treatment period compared to Control. Data was analysed using a 2-way repeated measures ANOVA for treatment group and litter size, repeated for week. There was an interaction between treatment group and week, therefore a 2-way ANOVA for treatment and litter size followed by post-hoc Bonferroni test was performed for each week separately (# MNR vs Control; * MH vs Control; $P < 0.05$). Dams pregnant with a greater number of fetuses had a greater body weight (no interaction between treatment group and litter size).

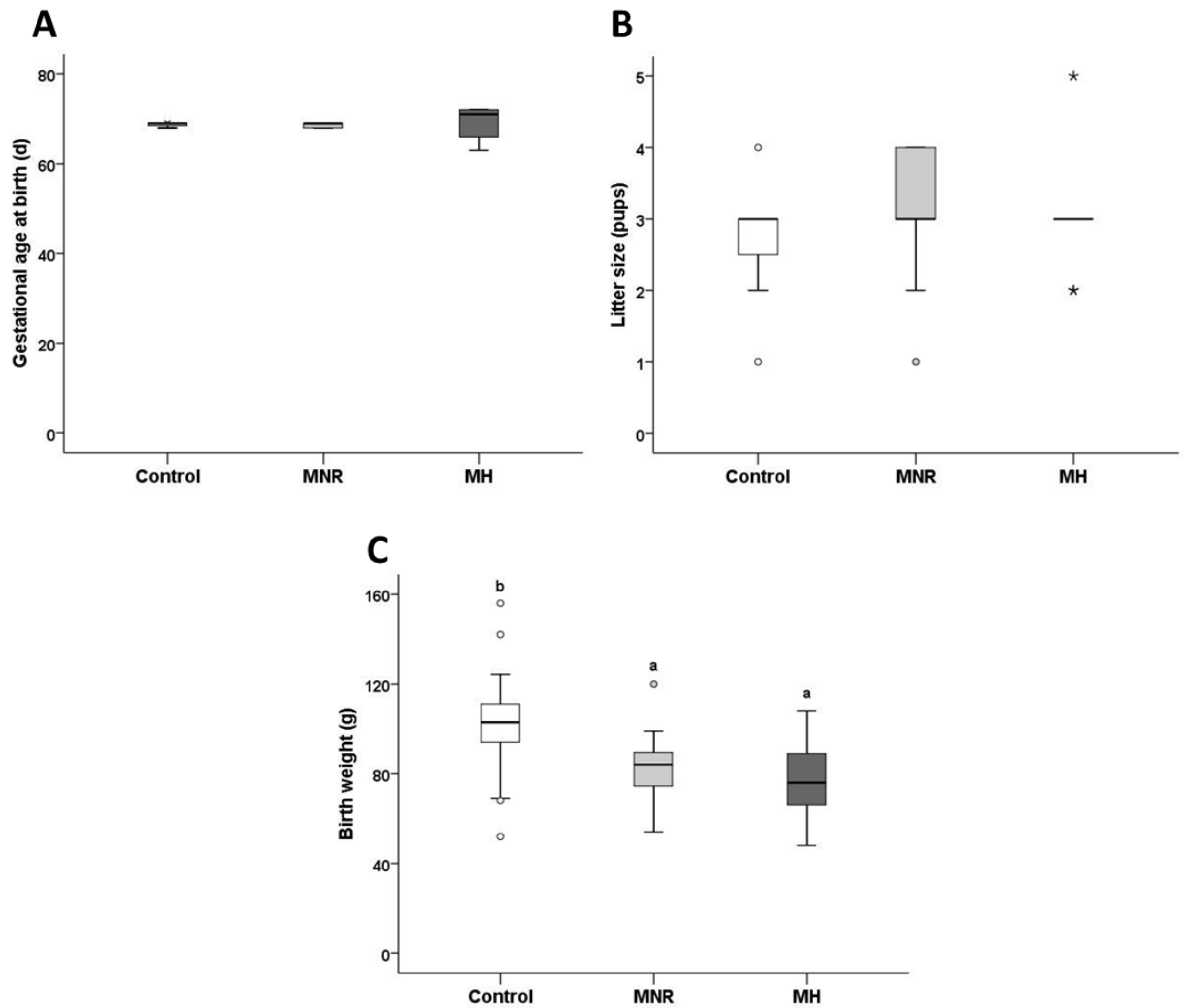

Figure 3. Litters in all treatment groups had a similar gestational age at birth, however, Maternal Hypoxia (MH) resulted in greater variability (A; Control n=11; MNR n=9; MH n=9 litters). There was no effect of Maternal Nutrient Restriction (MNR) or MH on litter size at birth (B). Being exposed to MNR (C; n=27 pups) and MH (n=26 pups) reduced birth weight compared to Controls (n=37 pups). Data was analysed by 2-way ANOVA for treatment group and sex, nested for litter with post-hoc Bonferroni tests where appropriate; different letters indicate a significant difference between treatment groups; $P < 0.05$.

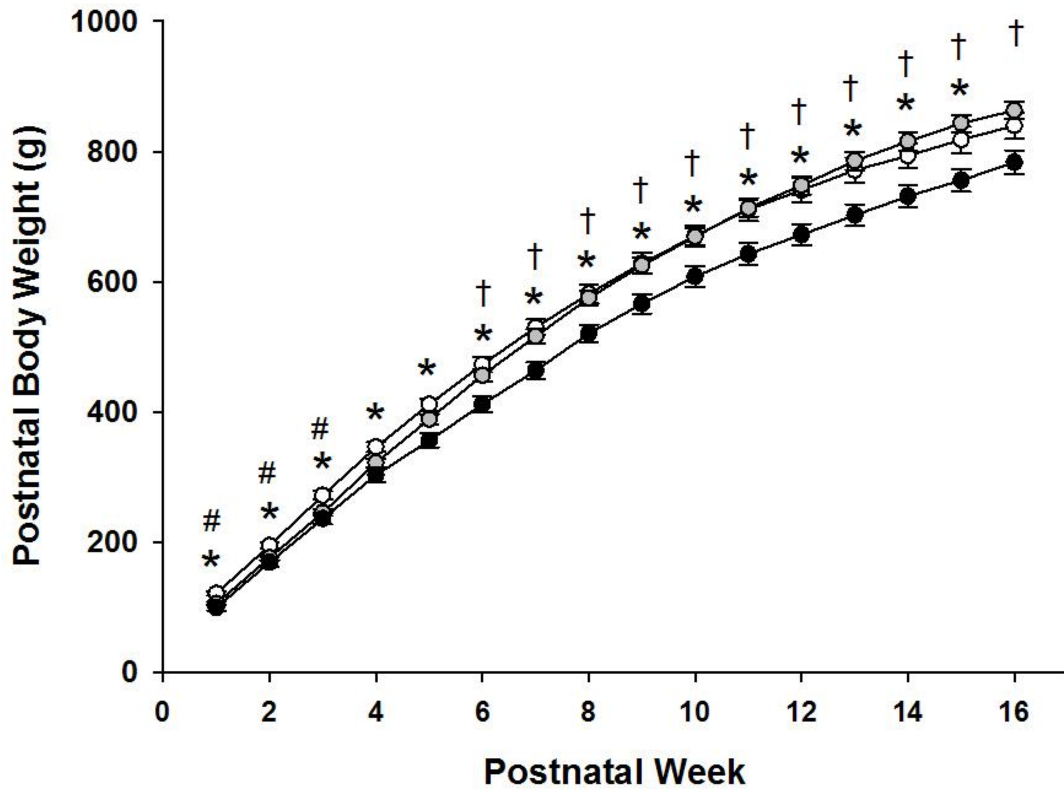

Figure 4. Offspring exposed to Maternal Nutrient Restriction (MNR; ◉; n=25 pups) and Maternal Hypoxia (MH; ●; n=19 pups) had altered postnatal growth compared to Control offspring (○; n=25 pups). Specifically, MNR offspring were born lighter than Control, but had accelerated growth and weighed the same as Controls by 4 weeks of age and were heavier than MH offspring by 6 weeks of age. MH offspring were lighter than Control offspring form birth until 16 weeks of age. Males weighed more than females by 5 weeks of age (2-way repeated measures ANOVA for treatment group and sex repeated for week, treatment * week $P < 0.05$ therefore a 2-way ANOVA for treatment and sex followed by post-hoc Bonferroni tests where appropriate was performed for each week separately. Treatment group * sex = NS; # MNR v Control; * MH v Control; † MH v MNR; $P < 0.05$).

3.4.3 Blood pressure

Male offspring exposed to MNR and MH had a similar basal systolic blood pressure (Figure 5A), diastolic blood pressure (Figure 5B), mean arterial blood pressure (Figure 5C) and heart rate (Figure 5D) in adolescence compared to Controls.

3.4.4 Organ weights

MH offspring had a smaller liver and perirenal fat depot compared to Control and MNR offspring in adolescence (Table 2). MH offspring had a greater adrenal weight compared to Control offspring (Table 2). Length from crown to rump, abdominal circumference, brain, lung, spleen and kidney weight were unchanged across all treatment groups (Table 2). MH offspring had an increased crown to rump length and adrenal weight relative to body weight compared to Control and MNR offspring, but decreased relative perirenal fat weight (Table 2). MH offspring had an increased abdominal circumference and brain weight relative to body weight compared to MNR offspring (Table 2). Lung, liver, spleen and kidney weights relative to body weight were not changed by treatment (Table 2).

MNR offspring had a larger heart weight compared to MH offspring (Figure 6A), whilst heart weight relative to body weight was not different between treatment groups (Figure 6B). MH and MNR did not alter the weight of the left ventricle (Figure 6C) or left ventricle relative to heart weight (Figure 6D).

3.4.5 Cardiomyocyte number

Only mononucleated (6.3±1.3%) and binucleated (93.7±1.3%) cardiomyocytes were observed in the LV of adolescent guinea pig and there was no effect of treatment on the percentage of each cell type (Control: 6.3±0.3% and 93.7±0.3%; MNR: 6.7±1.0% and 92.3±1.0%; MH: 5.8±1.0% and 94.2±1.0%, mononucleated and binucleated, respectively). MH decreased the number of cardiomyocytes in the left ventricle of female, but not male offspring, compared to MNR and Control offspring (Figure 7).

3.4.6 Markers of cardiac hypertrophy

MH and MNR did not alter the cardiac mRNA expression of hypertrophic genes IGF-1 (Figure 8A) and IGF-2 (Figure 8B). MH offspring had increased mRNA expression of the physiological hypertrophy receptor, *IGF-1R* (Figure 8C), compared to MNR and Control offspring In contrast, MNR offspring had decreased cardiac mRNA expression of the pathological hypertrophy receptor, *IGF-2R* (Figure 8D), compared to MH and Control offspring. There was, however, no difference in the abundance of the physiological hypertrophy mediator, Akt-1 (Figure 9A), the alternate metabolic isoform of Akt, Akt-2, (Figure 9B) or the phosphorylation/activity of Akt at site thr-308 (Figure 9C) or ser-478 (Figure 9D). Likewise, there was no difference in the abundance of the pathological hypertrophy mediator, CaMKII (Figure 10A), the phosphorylation/activity of CaMKII at site thr-286 (Figure 10B) and thr-305 (Figure 10C) or the mRNA expression of pathological hypertrophy marker, *ANP* (Figure 10D).

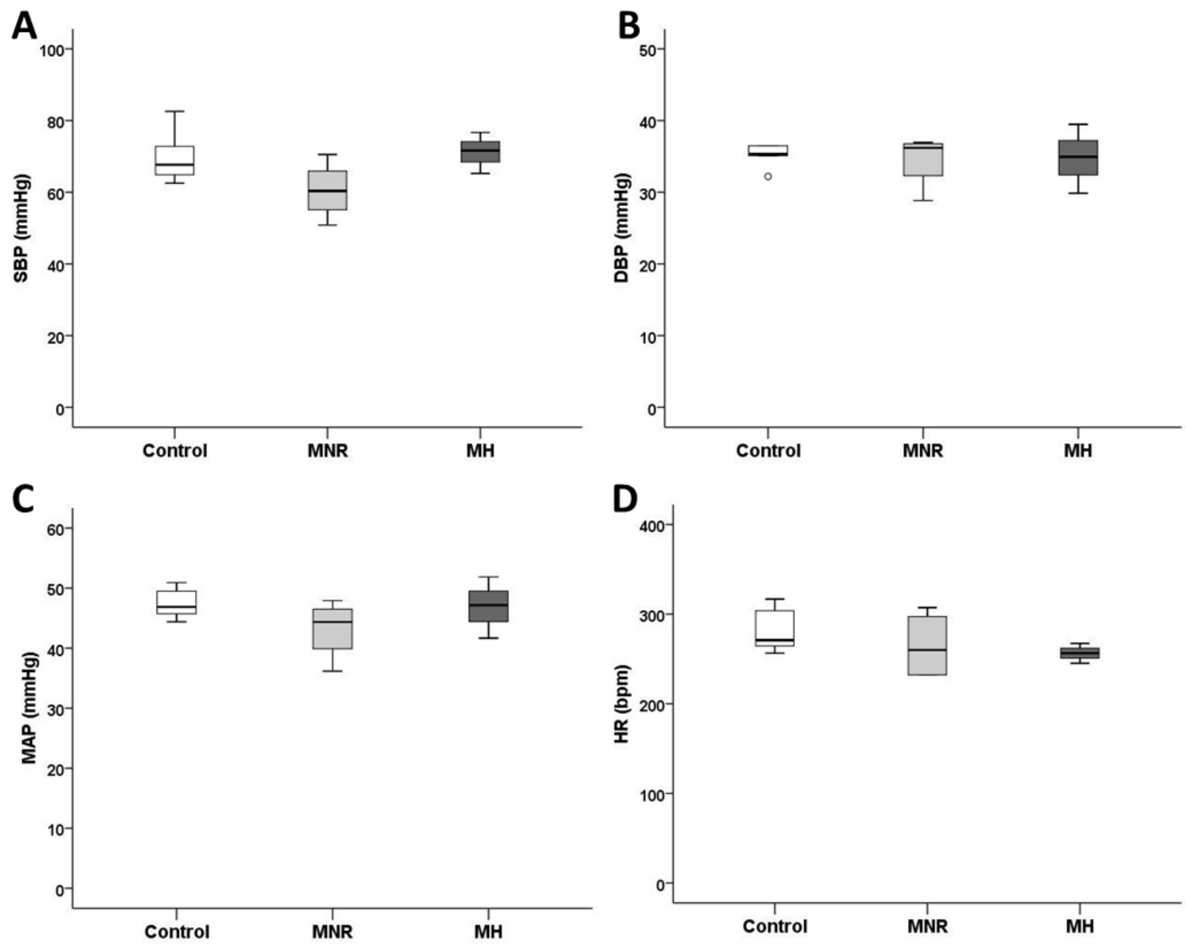

Figure 5. Male offspring exposed to Maternal Nutrient Restriction (MNR; n=4) and Maternal Hypoxia (MH; n=3) had a similar basal systolic blood pressure (SBP; A), diastolic blood pressure (DBP; B), mean arterial pressure (MAP; C) and heart rate (HR; D) in adolescence compared to Control offspring (n=5). Data analysed by 1-way ANOVA for treatment group; $P<0.05$.

Table 2. Postnatal body weight, absolute organ weights and relative organ weights at 120d.

	Control (n = 11 litters) (n = 17)	MNR (n = 9 litters) (n = 15)	MH (n = 9 litters) (n = 12)
Absolute values			
Age at *post mortem* (d)	119.8 ± 0.2	119.3 ± 0.6	119.2 ± 0.5
Body weight (BW) (g)	876.2 ± 22.7[b]	894.3 ± 16.9[b]	811.0 ± 25.3[a]
Crown rump length (cm)	33.4 ± 0.5	34.2 ± 0.5	33.7 ± 0.5
Abdominal circumference (cm)	27.7 ± 0.4	27.5 ± 0.5	26.8 ± 0.4
Brain weight (g)	4.04 ± 0.08	3.96 ± 0.06	3.96 ± 0.06
Lung weight (g)	4.27 ± 0.24	4.58 ± 0.21	3.91 ± 0.21
Liver weight (g)	40.38 ± 1.53[b]	41.04 ± 1.52[b]	34.65 ± 1.38[a]
Spleen weight (g)	1.33 ± 0.09	1.15 ± 0.05	1.23 ± 0.09
Adrenal weight (g)	0.33 ± 0.01[a]	0.36 ± 0.02[ab]	0.39 ± 0.02[b]
Kidney weight (g)	5.80 ± 0.28	6.09 ± 0.26	5.18 ± 0.17
Perirenal fat weight (g)	10.54 ± 0.59[b]	10.68 ± 0.57[b]	7.74 ± 0.81[a]
Relative values			
BW / birth weight (g/g)	8.63 ± 0.30	10.34 ± 0.37	10.0 ± 0.5
Crown rump length / BW (cm/kg)	38.41 ± 0.80[a]	38.35 ± 0.67[a]	41.74 ± 0.74[b]
Abdominal circumference / BW (cm/kg)	31.83 ± 0.57[ab]	30.80 ± 0.56[a]	33.31 ± 0.88[b]
Brain weight / BW (g/kg)	4.66 ± 0.15[ab]	4.44 ± 0.08[a]	4.94 ± 0.18[b]
Lung weight / BW (g/kg)	4.96 ± 0.25	5.07 ± 0.16	4.73 ± 0.28
Liver weight / BW (g/kg)	46.27 ± 1.63	45.84 ± 1.38	42.49 ± 1.37
Spleen weight / BW (g/kg)	1.54 ± 0.12	1.28 ± 0.06	1.54 ± 0.14
Adrenal weight / BW (g/kg)	0.38 ± 0.01[a]	0.40 ± 0.02[a]	0.48 ± 0.02[b]
Kidney weight / BW (g/kg)	6.60 ± 0.22	6.77 ± 0.20	6.40 ± 0.14
Perirenal fat weight / BW(g/kg)	12.01 ± 0.61[b]	11.88 ± 0.53[b]	9.38 ± 0.71[a]

2-way ANOVA for treatment group and sex, nested for litter with post-hoc Bonferroni tests where appropriate. No interaction between treatment group and sex was observed. Different letters indicate significant difference between treatment groups; $P < 0.05$.

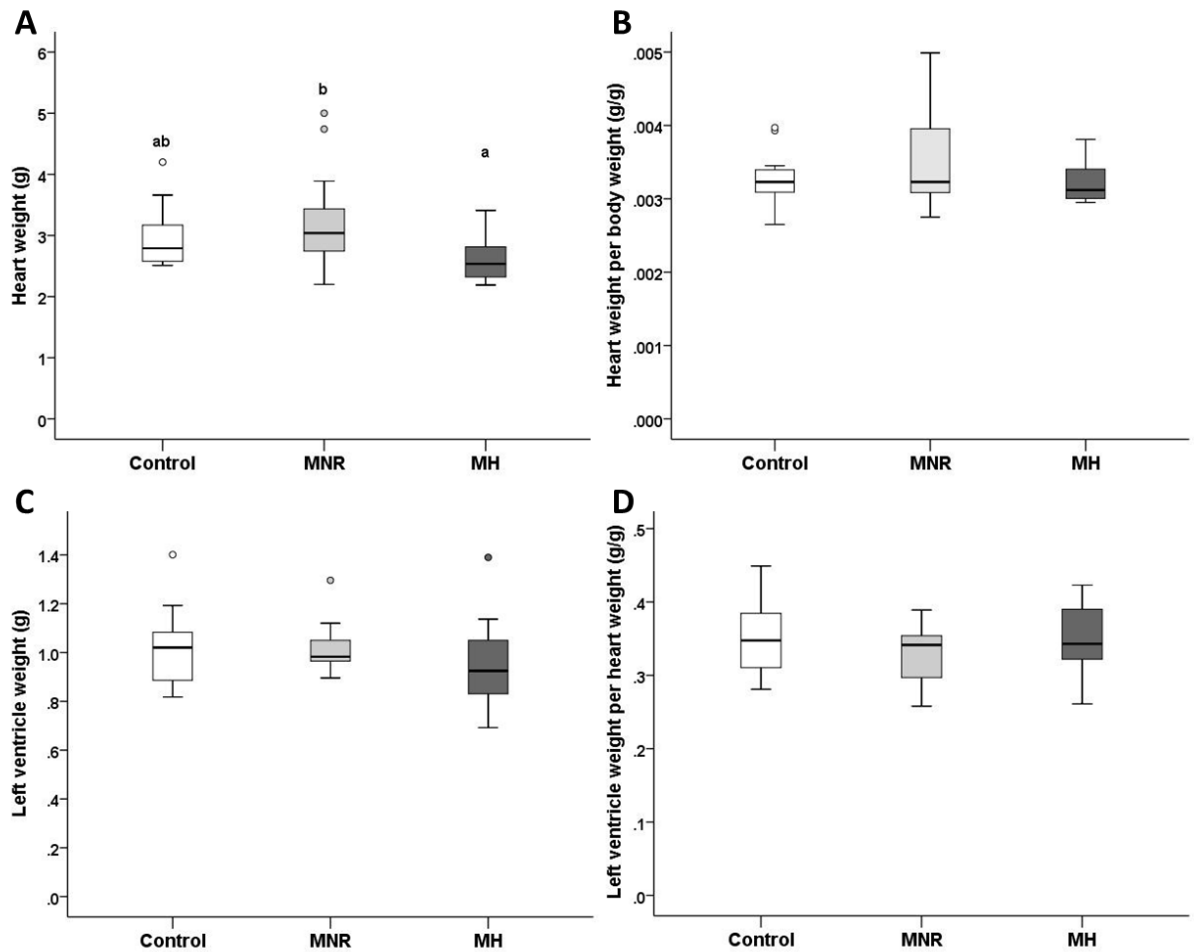

Figure 6. Maternal Nutrient Restriction (MNR; A; n=15) offspring had a greater heart weight in adolescence compared to Maternal Hypoxia (MH; n=12) offspring, but MNR and MH had a similar heart weight compared to Control (n=17) offspring. MNR and MH had a similar heart weight relative to body weight compared to Control offspring. MNR (n=9) and MH (n=11) had a similar left ventricle weight (C) and left ventricle weight relative to heart weight as Control (n=12) offspring. Data was analysed by 2-way ANOVA for treatment group and sex, nested for litter, with post-hoc Bonferroni tests where appropriate. No interaction between treatment group and sex was observed. Different letters indicate a significant difference between treatment groups; $P < 0.05$.

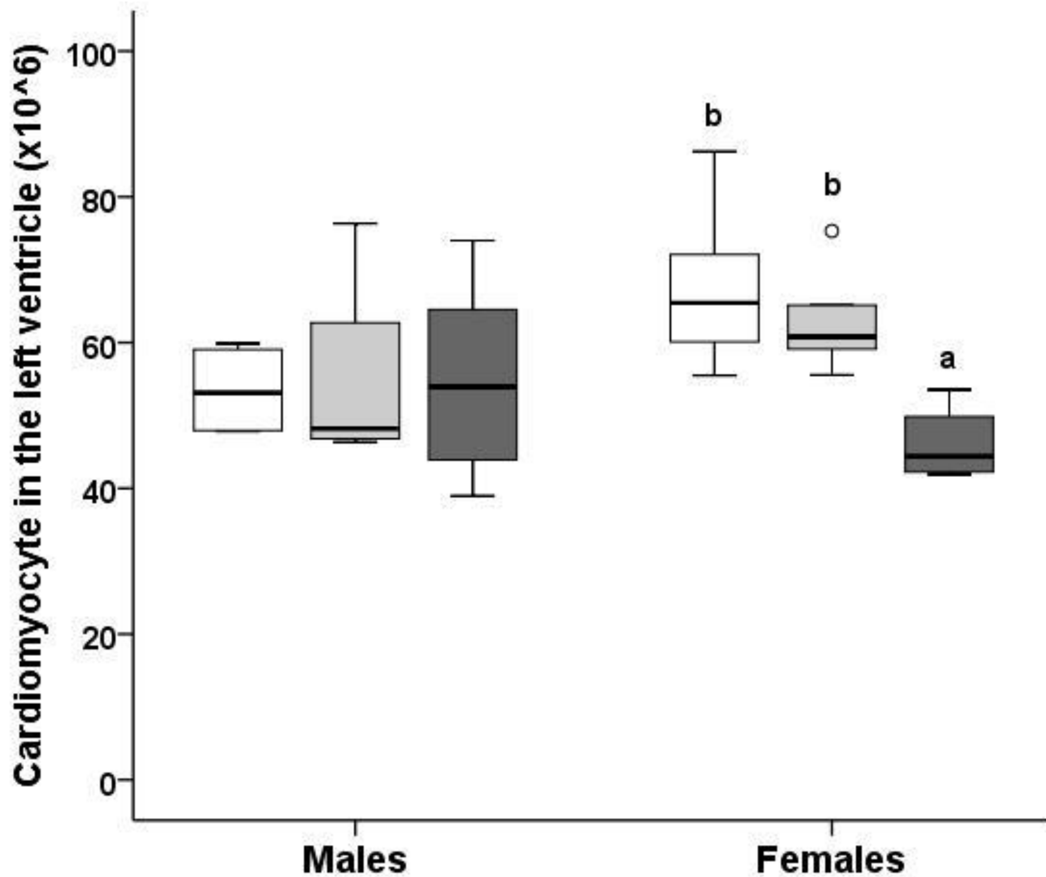

Figure 7. Maternal Hypoxia (MH, dark grey bar) reduced the number of cardiomyocytes in the left ventricle of female (n=4), but not male (n=7) offspring compared to Maternal Nutrient Restriction (MNR; male, n=4; female, n=5; light grey bar) and Control (white bar; male, n=4; female, n=8) offspring in adolescence. Data was analysed by 2-way ANOVA for treatment group and sex. An interaction between treatment group and sex was observed, therefore, a 1-way ANOVA for treatment group was applied to each sex separately with post-hoc Bonferroni tests; $P < 0.05$.

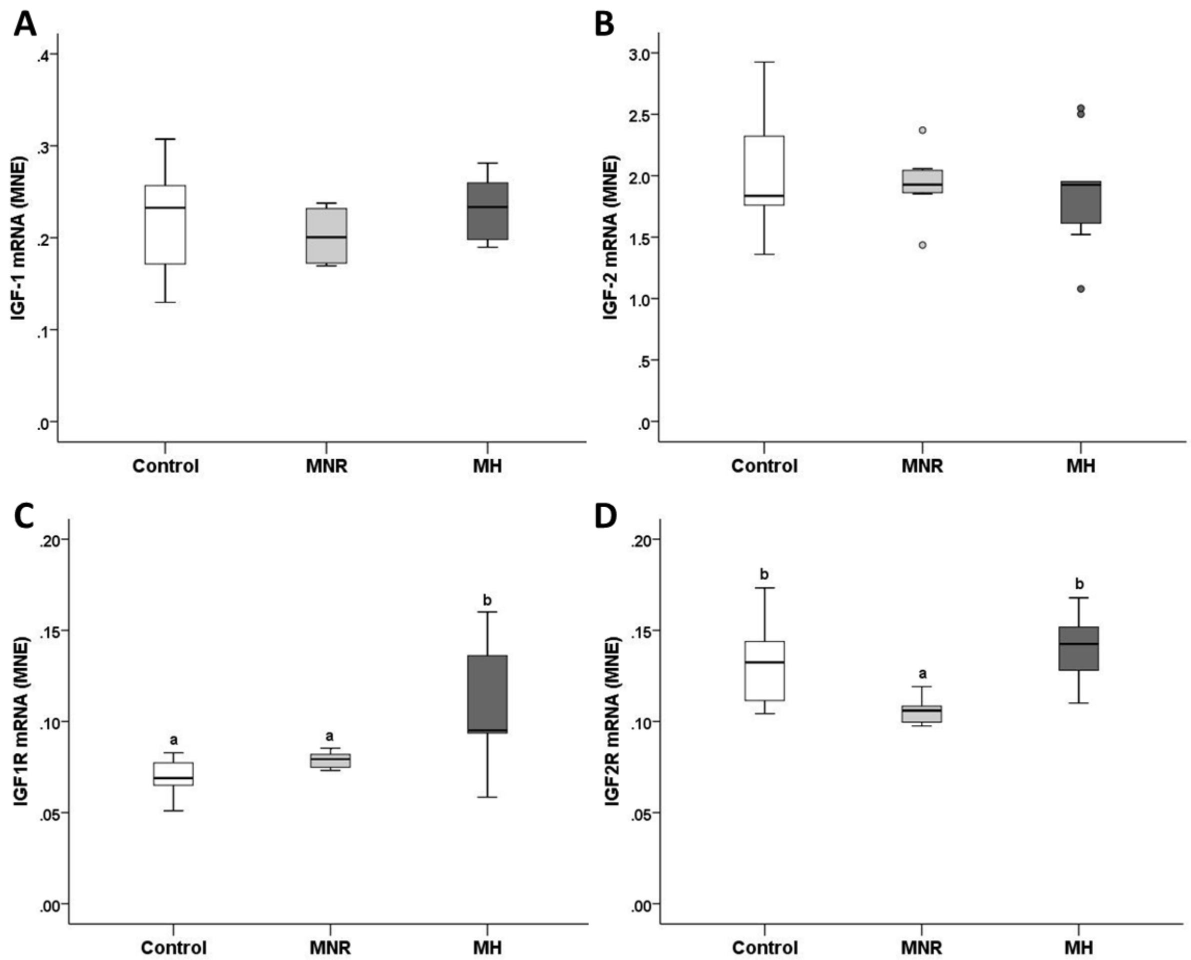

Figure 8. Maternal Nutrient Restriction (MNR; n=7) and Maternal Hypoxia (MH; n=10) offspring had similar expression of *IGF-1* (A) and *IGF-2* (B) mRNA compared to Control offspring (n=13). MH offspring had a greater expression of *IGF-1R* mRNA compared to both MNR and Control offspring. MNR offspring had reduced expression of *IGF-2R* mRNA compared to both MH and Control offspring. Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance; $P < 0.05$.

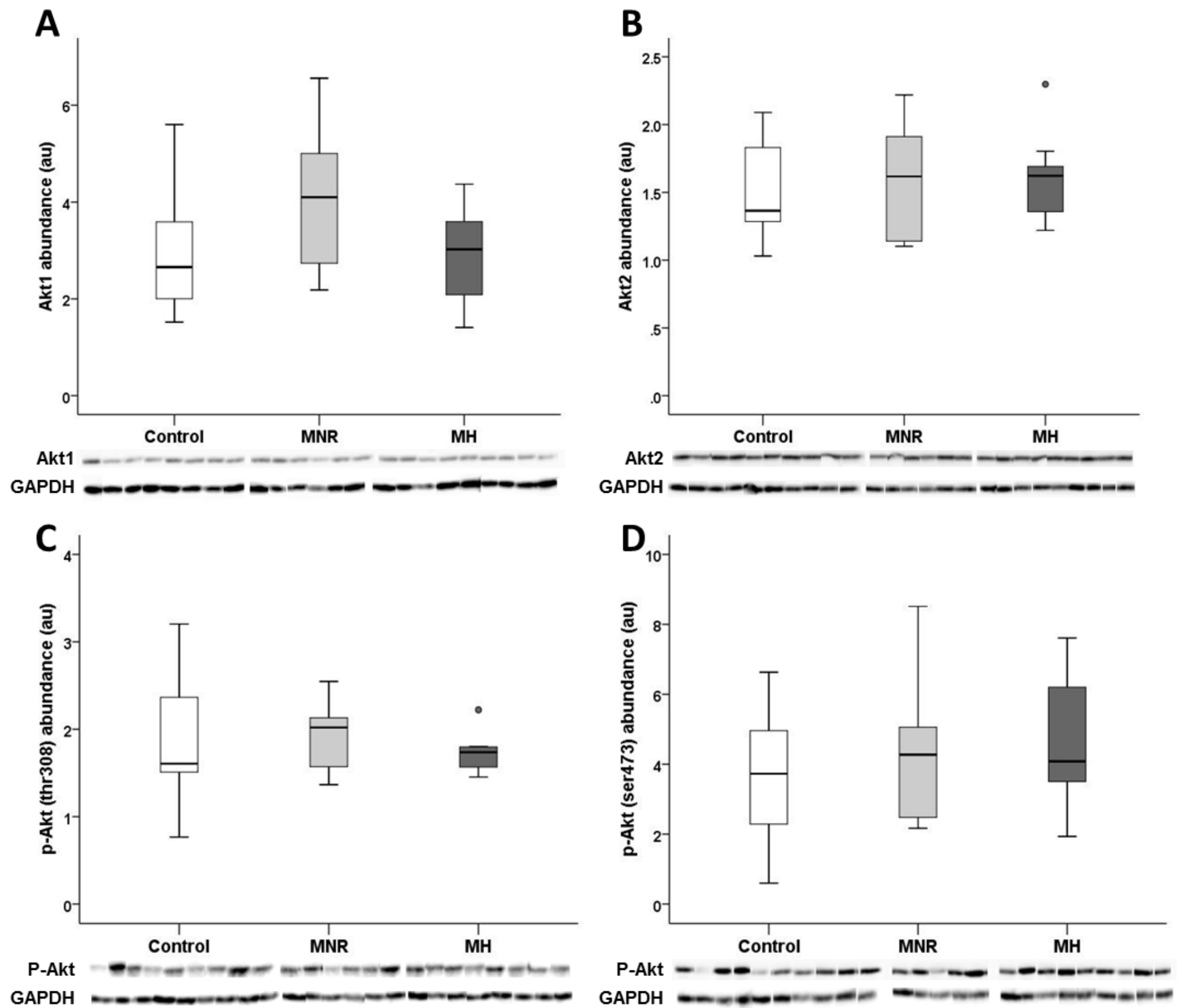

Figure 9. Maternal Nutrient Restriction (MNR) and Maternal Hypoxia (MH) offspring had similar abundance of Akt-1 (A), Akt-2 (B) and phosphorylation/activity of Akt at amino acid Thr-308 (C) and ser-473 (D). The abundance of each protein is expressed relative a loading control (GAPDH, bottom blot of each graph; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex, $P < 0.05$.

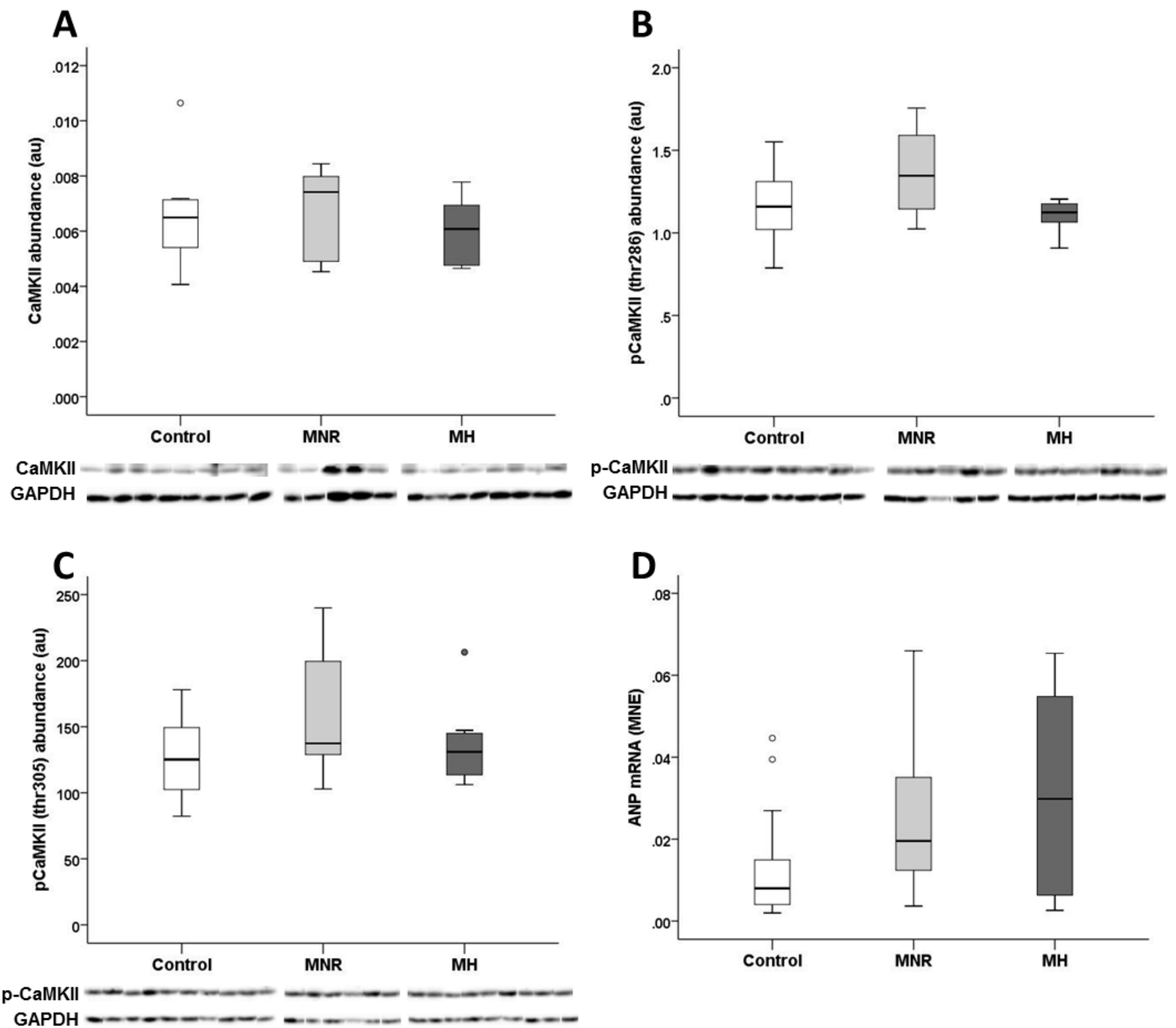

Figure 10. Maternal Nutrient Restriction (MNR; A) and Maternal Hypoxia (MH) offspring had a similar abundance of a mediator of pathological hypertrophy, calcium/calmodulin-dependent protein kinase II (CaMKII; B) and phosphorylation/activity of CaMKII at thr-286 (C) and thr-305 (D) compared to Control offspring in adolescence. MNR (D; n=7) and MH (n=10) offspring had similar expression of atrial natriuretic protein (*ANP*) mRNA compared to Control offspring (n=13). The abundance of each protein is expressed relative a loading control (GAPDH, bottom blot of each graph; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex, $P < 0.05$.

3.5 Discussion

This study demonstrates that IUGR due to MH results in fewer cardiomyocyte in the adolescent female, but not male offspring. This is the first study to determine the effect of sex on cardiomyocyte number on IUGR offspring and is the first to demonstrate that IUGR can result in a deficit in cardiomyocytes that persists into later life. IUGR in sheep due to placental restriction results in fewer cardiomyocytes in late gestation, but the effect of sex was not determined (Chapter 2). IUGR due to maternal protein restriction results in fewer cardiomyocytes at birth in female offspring, but male offspring were not reported.[111]

To our knowledge, this is the first study to report the percentage of mononucleated and binucleated cardiomyocytes in the postnatal guinea pig. Like mice, rats and sheep, guinea pigs have a high percentage of binucleated cardiomyocytes and only 8-14% mononucleated cardiomyocytes in adolescence (for review, see [1]; Chapter 1). This is unlike humans who retain approximately 74% mononucleated and 26% binucleated cardiomyocytes.[94] It is currently not known whether binucleation in guinea pigs begins in late gestation as in humans[78] and sheep[75, 76] or if it occurs within approximately the first 3-12 days of postnatal life as in mice[97] and rats.[74] Considering that guinea pigs are born with mature organ systems like humans and sheep and unlike mice and rats, which are immature at birth, we speculate that binucleation begins in late gestation in guinea pigs. It is generally accepted that adult mononucleated, but not binucleated cardiomyocytes are capable of proliferating if stimulated appropriately[15] and that the majority of cardiomyocytes present at birth represent the majority of cardiomyocytes present in the heart throughout life.[13, 14] In humans[13, 14] and mice,[97, 104] the generation of cardiomyocytes throughout life is estimated at approximately 1% per annum. Therefore, IUGR male offspring that were exposed to MH may have had an appropriate number of cardiomyocytes in adolescence due to either having an appropriate number of cardiomyocytes throughout life or, alternatively, have replaced the cardiomyocytes lost in postnatal life.

Olivetti and colleagues identified the number and volume of cardiomyocytes in human hearts at post mortem across a range of ages, excluding individuals who had cardiac pathologies such as LVH, myocardial infarction and severe atherosclerosis, and found that the heart volume and the number of cardiomyocytes decreased with age in males, but was maintained in females.[325] Furthermore, in males, the volume of cardiomyocytes increased with age, but was maintained in females,[325] which supports the premise that cardiomyocyte hypertrophy is a compensation for fewer cardiomyocytes. In the present study, if we examine the number of cardiomyocytes in only Control offspring we find that males have fewer cardiomyocytes than females (Males: $54.5 \pm 3.2 \times 10^6$; Females $67.1 \pm 3.5 \times 10^6$; Student's t-test; $P=0.03$). Post mortem analysis of human hearts from individuals who died without cardiac pathologies, found that men have a 3-fold higher percentage of apoptotic cardiomyocytes.[326] Furthermore, older male monkeys have a 4-fold higher percentage of apoptotic cardiomyocytes than older female monkeys, but no difference in the percentage of cardiomyocytes in the cell cycle.[327] Like humans, cardiomyocyte size increased with age in male monkeys, but was unchanged with age in females.[327] Moreover, after myocardial infarction, females exhibit less cardiomyocyte apoptosis than men.[328] Together these data suggest that the maintenance of cardiomyocyte number in MH males is more likely due to MH not effecting cardiomyocyte endowment *in utero*. This finding itself is interesting, since with regards to the kidney, a decreased number of nephrons is observed in IUGR male rat offspring as a result of moderate maternal protein restriction,[329, 330] but only occurs in female offspring if exposed to a more severe maternal protein restriction.[331] Likewise, females tend to be protected from pathological cardiac remodelling compared to males due to the effect of estrogen (for review, [332]). Interestingly, there is no effect of IUGR, induced in rats by reduced uterine artery blood flow, on plasma estradiol concentration in female offspring at 4-16 weeks of age compared to Controls.[333] Therefore, the mechanism behind why cardiomyocytes of

females exposed to MH are more susceptible than cardiomyocytes of males needs further investigation.

Considering that both MH and MNR caused an equivalent reduction in birth weight compared to Controls, it is interesting that MNR offspring had an equivalent number of cardiomyocytes compared to Control offspring. Maternal protein restriction in rats results in fewer cardiomyocytes at birth,[111] but if protein restriction continues across pregnancy and lactation, cardiomyocyte number is the same as Controls at 4 weeks of age.[112] Considering that rats retain mononucleated cardiomyocytes[74] and undergo cardiomyocyte proliferation in the first weeks of life,[86] it suggests that a window of time exists that cardiomyocyte number can be rescued *ex utero,* even with continued maternal protein restriction, suggesting the substrate(s) required for cardiomyocyte proliferation are adequate during lactation. Recent evidence suggests that human hearts contain a low percentage of cardiomyocyte that undergoing cytokinesis in early life, which decreases in adolescence and is absent by 20 years of age.[317] It is unclear, however, if guinea pigs and humans retain the ability for considerable proliferation shortly after birth to repair a deficit in cardiomyocyte endowment at birth. Due to the immaturity of fetal rat cardiomyocytes at birth, we postulate that reductions in cardiomyocyte endowment due to nutrient deprivation may only occur in guinea pigs if nutrient restriction occurs earlier in gestation than the present study. Alternatively, the substrate(s) required to reduce cardiomyocyte endowment are ample in the MNR offspring, but not in the protein restricted rat in late gestation. Further information regarding placental transport of substrates in these models and MH are required to better understand the regulation of cardiomyocyte endowment in late gestation.

Female offspring exposed to MH have fewer cardiomyocytes in adolescence, which may be a result of MH induced cardiomyocyte apoptosis or decreased proliferation *in utero*. Maternal hypoxia in the last week of pregnancy in rats results in an increased percentage of apoptotic cardiomyocytes on the last day of gestation.[71] Fetal sheep exposed to

umbilicoplacental embolization (UPE) for 20d, which causes chronic fetal hypoxaemia and hypoglycaemia, have a decreased percentage of proliferating cardiomyocytes in late gestation.[124] Interestingly, when fetal sheep are exposed to placental restriction from conception, there is no difference in the percentage of apoptotic (Chapter 2) or proliferative cardiomyocyte[123] in late gestation, despite a decrease in the number of cardiomyocytes (Chapter 2). The appropriate level of apoptosis and proliferation may be due to the observed maintenance of capillary length compared to normally grown fetuses (Chapter 2) and potentially an adequate gas and nutrient exchange between the remaining cardiomyocytes and the circulation. Considered together, these data suggest that a reduction in cardiomyocytes is an adaptive response to low oxygen and/or nutrient delivery, which is controlled, such that cardiomyocyte number is maintained once appropriate oxygen and/or nutrient delivery is achieved.

An alternate explanation for the reduced number of cardiomyocytes in MH female offspring in adolescence, however, may be due to increased cardiomyocyte apoptosis in postnatal life. The number of cardiomyocytes in the fetal heart should be confirmed in late gestation, but prior to returning the MH dams to normoxia at 65d gestation, to confirm if observations in postnatal life originated *in utero*.

In the present study, males were chosen for analysis of blood pressure due to evidence from several models of IUGR, such as maternal nutrient restriction in guinea pigs,[321] uteroplacental insufficiency in rats,[290, 291] and modest maternal protein restriction in rats,[329, 330] which indicates that male offspring, but not female offspring develop hypertension in postnatal life. Androgens play a key role in the regulation of blood pressure, with testosterone having blood pressure raising effects[334, 335] and estrogen being cardioprotective.[336, 337] Studies of IUGR due to reduced uterine blood flow in rats demonstrate that male offspring have elevated plasma testosterone compared to Control rats.[338] Furthermore, castration of IUGR male rats abolishes hypertension, but does not alter blood pressure in Control rats.[338] Blocking

the renin-angiotensin system (RAS) with enalapril in intact IUGR males lowers blood pressure to a similar level as blocking the RAS in Control males, suggesting that testosterone is mediating its effect on blood pressure through the RAS.[338] At 16 weeks of age, IUGR female rats that were exposed to reduced uterine blood flow have an equivalent blood pressure and plasma estradiol concentrations to Control offspring, however, ovariectomised IUGR offspring are hypertensive.[333] Interestingly, ovariectomised Control females retain normal blood pressure.[333] Treating ovariectomised females with estrogen lowers blood pressure in both IUGR and Control offspring and a similar blood pressure lowering effect is seen when blocking the RAS with enalapril compared to estrogen treatment[333] suggesting that, like testosterone, oestrogen effects blood pressure through the RAS. In the present study, sexually mature male offspring were not hypertensive,[339] leading to the speculation that from the literature discussed above that sexually mature female offspring exposed to MH and MNR were not hypertensive.

Intriguingly, in the same strain of guinea pig as was used in the present study, maternal nutrient restriction to a comparable degree (15% reduction in food intake per body weight), but for a longer duration (a month before conception and throughout pregnancy) results in IUGR male offspring (similar reduction in birth weight to present study) that are hypertensive at 4 months of age (same postnatal age as the present study).[321] This data suggests that either insults earlier in pregnancy or for a longer duration results in altered blood pressure in adolescence. Male guinea pigs exposed to a 30% reduction in maternal food intake in the first half of gestation have increased MAP and LVH at 3 months of age, but offspring exposed to maternal nutrient restriction in the second half of pregnancy are normotensive and have a comparable left ventricle mass to Control offspring.[340] Interestingly, offspring exposed to maternal nutrient restriction in the first half of pregnancy had an equal birth weight to Control offspring, but those exposed to nutrient restriction in the second half of pregnancy had a reduced birth weight.[340] This data suggests that hypertension can be programmed in the

first half of pregnancy and persists even if fetal growth is maintained or restored to that of normally grown offspring later in gestation.

IUGR induced by placental restriction from conception results a greater dependence on the RAS[341] and the sympathetic nervous system,[50] but not endothelial nitric oxide,[342] for the maintenance of basal arterial blood pressure in the late gestation sheep fetus. Furthermore, IUGR is associated with increased activation of the hypothalamic-pituitary-adrenal (HPA) axis.[48] The paraventricular nucleus of the hypothalamus secretes arginine vasopressin (AVP) and corticotrophin-releasing hormone (CRH), which act on the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH) into the peripheral circulation. Circulating ACTH exerts its effects on the adrenal, via the melanocortin type 2 receptor (MC2R), stimulating steroidogenesis and secretion of cortisol. In humans, low birth weight is associated with elevated fasting plasma cortisol concentrations,[250] enhanced adrenal secretion of cortisol in response to ACTH and increased total urinary cortisol metabolite excretion in adult men.[271] Furthermore, a meta-analysis of data from humans published between 1998 and 2005, which excluded individuals who were born preterm, demonstrated that a 1kg decrease in birth weight was associated with a 20.6nmol/L and 30.9nmol/L elevation in plasma cortisol concentrations in men and women, respectively.[272] Interestingly, long term fetal hypoxaemia due to pregnancy at high altitude does not alter plasma cortisol concentrations under basal conditions compared to normally grown fetal sheep in late gestation, however, there is increased expression of AVP and CRH in the paraventricular nucleus, increased production of ACTH in the anterior pituitary and increased plasma $ACTH_{1-39}$ concentrations.[265] Consequently, in response to stress, the hypoxaemic fetus produces greater cortisol.[266, 267] Interestingly, despite chronic hypoxaemia, these fetuses are not IUGR,[343] indicating that exposure to hypoxaemia, not necessarily being IUGR mediates alterations in the HPA axis. Together, these studies suggest that there are a range of neuroendocrine adaptations in response to a decrease in substrate supply *in utero*, which maintain arterial blood pressure, but

may program an elevated response to stress in postnatal life. Unfortunately, we were unable to measure the regulation of blood pressure in male offspring and subsequently do not know if their blood pressure response to stress was altered. In the present study, however, MH resulted in increased adrenal weight both in absolute terms and relative to body weight, which may suggest an increased capacity for cortisol production.

LVH is recognised as the major risk factor for cardiovascular disease[344-346] and has been reported in IUGR offspring.[132, 203, 285, 286, 347] LVH occurs in response to an increase in work load and wall stress and is subsequently present in those with hypertension[348, 349] and obesity.[350] IUGR, too, results in an increased incidence of hypertension and obesity,[135] therefore, deciphering if LVH is a direct consequence of IUGR or if it is secondary to essential hypertension or obesity is difficult. To date, there is only one study that has observed LVH as a consequence of IUGR where blood pressure was recorded and was similar to Controls.[116] In the aforementioned study, IUGR male offspring exposed to maternal hypoxia in the last week of pregnancy had LVH at both 4 and 7 months of age coupled with increased abundance of fibrotic proteins, collagen I and collagen II, and an increased ratio of the β−myosin heavy chain (MHC) isoform to the α−MHC isoform.[116] In rats, like *ANP*, *β-MHC* transcription is increased in response to stimulation of the pathological hypertrophy signalling pathway.[351, 352] More interestingly, IUGR rats exposed to maternal nutrient restriction also have increased abundance of collagen I and collagen III and an increased β-MHC:α-MHC in the absence of both LVH and hypertension at 7 months of age.[116] This study suggests that the origins of pathological cardiac remodelling can be programmed *in utero*, independent of the development of postnatal hypertension.

In the present study, MH and MNR did not affect heart weight or heart weight relative to body weight compared to Control offspring, however, MNR offspring had a greater heart weight compared to MH offspring. MH and MNR did not affect left ventricle weight or left ventricle weight relative to heart weight compared to Control. Despite this, MH offspring had

an increased expression of *IGF-1R* mRNA in the left ventricle, however, this did not result in a difference in the activation of the physiological hypertrophy mediator, Akt1. An increase in *IGF-1R* mRNA is also observed in IUGR lambs at 21d of age who display LVH.[203] Furthermore, IUGR lambs have an increased abundance of Akt1 and phosphorylation of Akt,[203] which suggest that the physiological hypertrophy signalling pathway may be contributing to the relative increase in relative left ventricular weight compared to normally grown offspring. In the present study, MNR offspring have decreased expression of *IGF-2R* mRNA compared to MH and Control offspring. IGF-2R has been identified as anti-hypertrophic due to its ability to bind and remove IGF-2 from the circulation and subsequently to prevent IGF-2 binding to the IGF-1R to activate physiological hypertrophy. Considering that MNR offspring have a greater heart weight than MH offspring, suggests the increase in growth may be a result of increased available IGF-2. The absence of an increase in *IGF2R* mRNA in both MNR and MH IUGR offspring is in contrast to 21d old IUGR lambs,[203] but may explain why MNR and MH offspring displayed no evidence of LVH or activation of the pathological hypertrophy signalling pathway.

It is interesting to note that the female offspring of MH have fewer cardiomyocytes in the left ventricle, but an equivalent left ventricle weight. This suggests that the remaining cardiomyocytes are larger compared to those of Control offspring, but that the absence of a difference in Akt and CaMKII phosphorylation suggests it may not be the case. The cardiomyocytes in the heart of IUGR rats exposed to maternal hypoxia have a greater cross sectional area and an equivalent heart weight at 2 months of age,[114] which the authors suggest is due to fewer cardiomyocytes. The present data in MH females supports this hypothesis. Alternatively, heart weight may be explained by pathological cardiac remodelling such as increased collagen I and III and fibrillar thickness and density as is the case in IUGR rat offspring exposed to maternal hypoxia and maternal nutrient restriction at 7 months of age.[116]

Both MH and MNR caused an equivalent reduction in birth weight compared to Control offspring, however, both male and female MNR offspring experienced accelerated postnatal growth, such that they weighed the same as Control offspring by 4 weeks of age and weighed more than MH offspring by 6 weeks of age. Accelerated postnatal growth following IUGR is associated with a greater risk of coronary heart disease,[353] hypertension[354] and obesity[355] than IUGR alone. It may be, therefore, that pathological consequences associated with IUGR and accelerated postnatal growth in the MNR offspring are not present at 4 months of age, but may present beyond the age investigated in this study.

3.6 Conclusion

In summary, this study is the first to demonstrate that IUGR due to MH results in a reduced number of cardiomyocytes in the adolescent offspring. Furthermore, this study demonstrates that female offspring are more susceptible to MH induced cardiomyocyte deficit than male offspring. This is in contrast to studies of kidneys where male offspring are more susceptible to IUGR induced nephron deficit than females.[329-331] Furthermore, this is in contrast with the cardioprotective effects of estrogen that confers a lower rate of cardiac pathology and hypertension in pre-menopausal women than men.[332] MH females with fewer cardiomyocytes had an equivalent heart weight, but no evidence of pathological hypertrophy such as an increase in activation of the pathological signalling mediator, CaMKII, or expression of the pathological marker, *ANP*, suggesting that the deficit in cardiomyocytes is not detrimental under basal conditions.

CHAPTER 4

Statement of Authorship

Title of Paper	IUGR effects the expression of cardiac metabolic genes in adolescent offspring dependent on the cause of IUGR and sex
Paper Status	Paper for submission

Author contribution

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author	Kimberley Botting		
Contribution to the Paper	Drove the study and interpretation of data Ran the animal model Collected hearts for analysis Designed primers for real-time analysis Determined protein abundance Analysed all data and created figures Wrote drafts of manuscript Edited manuscript Approved final version of the manuscript		
Signature		Date	20/12/13

Name of Co-Author	Dr Song Zhang		
Contribution to the Paper	Determined cortisol and glucose concentrations Provided intellectual interpretation of data Edited manuscript Approved final version of the manuscript		
Signature		Date	22/12/2013

Name of Co-Author	Xin Yee Loke (Signed for on her behalf by A/Prof Janna Morrison)		
Contribution to the Paper	Designed primers for real-time analysis Provided intellectual interpretation of data Approved final version of the manuscript		
Signature		Date	20/12/13

<table>
<tr><td>Name of Co-Author</td><td colspan="3">A/Prof Janna Morrison</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Designed and lead the study including running of the animal cohort, surgical procedures, collection of tissues and data analysis and presentation
Provided intellectual interpretation of data
Involved in construction of the manuscript
Edited manuscript
Approved final version of the manuscript</td></tr>
<tr><td>Signature</td><td></td><td>Date</td><td>20/12/13</td></tr>
</table>

4. CHAPTER FOUR

Experimental paper for submission: IUGR effects the expression of cardiac metabolic genes in adolescent offspring dependent on the cause of IUGR and sex

4.1 Abstract

IUGR results in an increased risk of ischaemic heart disease in adulthood. Animal models demonstrate that hearts from IUGR offspring are more susceptible to ischaemia/reperfusion injury and experience greater cardiomyocyte apoptosis than Control offspring in postnatal life. Despite utilising fatty acids and glucose for cardiac ATP production prior to ischaemia/reperfusion, IUGR rats have decreased utilisation of fatty acids and increased reliance on glycolysis for ATP production compared to Control offspring during reperfusion. Furthermore, studies of offspring of nutrient restricted rats demonstrate that the key regulator of cardiac fatty acid metabolism, PPARα, is epigenetically upregulated in postnatal life. Therefore, we aimed to determine the expression of PPARα, genes involved in fatty acid metabolism and glucose transport and the regulation of cardiac metabolism by the metabolic 'fuel gauge,' AMPK, in adolescent guinea pigs that were IUGR due to either Maternal Hypoxia (MH) or Maternal Nutrient Restriction (MNR). In the present study, we determined that MH, but not MNR increased the expression of *PPARα* and the mRNA expression of two fatty acid transporters, *FATP1* and *FABPpm,* as well as glucose transporter, *GLUT4*. Furthermore, both MH and MNR increased the expression of *FATP6*. Interestingly, in male, but not female offspring, MH and MNR decreased the expression of long chain *FACS* and $AMPK\alpha_2$ compared to Control. Likewise, MNR males, but not females, had reduced expression of *ACC*. Surprisingly, MH females had increased phosphorylation of $AMPK\alpha_2$, but there was no effect of IUGR on the phosphorylation of ACC or AS160. These findings suggest that IUGR programs increased fatty acid metabolism through increased fatty acid transporters, but that deregulation of sarcoplasmic regulators of fatty acid metabolism in male offspring may render IUGR males more vulnerable to ectopic cardiac lipid accumulation.

Considering that we have previously demonstrated that MH females, but not MH males or MNR offspring have fewer cardiomyocytes in adolescence, these data suggest that AMPK activation may be associated with a deficit in cardiomyocyte endowment. We therefore suggest that changes in gene expression observed in IUGR offspring do not result in pathology unless faced with a secondary insult such as ischaemia, hypertension, LVH or obesity.

4.2 Introduction

It is predicted that by 2030, annual deaths from cardiovascular disease (CVD) will reach 23.3 million people worldwide.[134] Comprehensive epidemiological studies demonstrate that restriction of growth *in utero* (intrauterine growth restriction; IUGR), which results in low birth weight (LBW) is a predictor of ischaemic heart disease and heart failure in adulthood.[2-10] In Australia, ~6% of babies are born IUGR, defined as a body weight below the tenth centile.[318] Placental insufficiency is the leading cause of IUGR in the developed world and results in decreased oxygen and nutrient supply to the fetus.[142] This results in a number of adaptive responses in the fetus, including a redistribution of cardiac output to key organs like the brain and decreased fetal growth rate.[143]

IUGR rats exposed to maternal hypoxia in the last week of gestation are more susceptible to ischaemia/reperfusion injury and undergo greater cardiomyocyte apoptosis as a result of ischaemia/reperfusion injury in adulthood.[115, 198] Despite an equivalent cardiac power before ischaemia/reperfusion, IUGR rats have decreased cardiac power during reperfusion.[198] One mechanism responsible for poor recovery from ischaemia/reperfusion may be due to altered cardiac metabolism. In spite of a similar contribution of substrates used for cardiac adenosine triphosphate (ATP) production before ischaemia/reperfusion, IUGR rats have an increased contribution of glucose metabolism via glycolysis and a reduced contribution of fatty acid metabolism to cardiac ATP production during reperfusion compared to Controls.[198] Furthermore, the IUGR heart produces more protons, which may be explained by an uncoupling of glycolysis with glucose oxidation,[198] which requires ATP for correction. Failure to correct the proton imbalance may result in acidaemia leading to Ca^{2+} accumulation and contractile dysfunction.[199-201] Additionally, exposure to a high fat diet in postnatal life exacerbates the effect of hypoxia-induced IUGR on the susceptibility to ischaemia/reperfusion injury in adulthood.[202] Considering the importance of continued contraction and that almost the entire supply of cardiac ATP is turned over every ten

seconds,[147] it is imperative that cardiomyocyte metabolism is flexible and rapidly adapts to alterations in substrate supply and stimuli in order to efficiently produce ATP, which it appears to be altered in IUGR offspring.

In postnatal life, fatty acids are the predominant cardiac fuel and are metabolised via fatty acid β-oxidation (105mol ATP per 1mol palmitate), followed by glucose metabolism via glucose oxidation (31mol of ATP per 1mol glucose), glycolysis (2mol ATP per 1mol of glucose) and lactate oxidation (15mol ATP per 1mol of lactate).[148] This is in contrast to fetal life, when fatty acids and oxygen are in limited supply, and thus glycolysis is the predominant source of cardiac ATP.[148] In the weeks after birth, cardiac metabolism switches from glycolysis to fatty acid β-oxidation, central to which is increased cardiac abundance of peroxisome proliferator-activated receptor alpha (PPARα),[148] the transcriptional regulator of genes involved in multiple steps of fatty acid metabolism and cardiac lipid homeostasis.[156, 157] PPARα binds to peroxisome proliferator response elements (PPREs)[160] located within the promoter region of genes involved fatty acid transport into the sarcoplasm (fatty acid transport proteins; *FATP1* and *FATP6*), fatty acid activation (fatty acyl-CoA synthetase; *FACS*), transport within the sarcoplasm (heart-type fatty acid binding protein; *H-FABP*), mitochondrial uptake of fatty-acyl CoA (carnitine palmitonyl transferases; *CPT-I* and *CPT-II*), upregulation of CPT-Iβ activity (malonyl-CoA decarboxylase (*MCD*)[161]) and fatty acid β-oxidation (acyl-CoA dehydrogenases specific to the size of the fatty acid; medium chain (*ACADM*), long chain *(ACADL)* and very long chain (*ACADVL)* (for review, see [162, 163]). In addition to upregulating the transcription of genes involved in fatty acid metabolism, PPARα has also been associated with promoting the translocation of fatty acid transporter FAT/CD36 to the sarcolemma.[164]

IUGR due to maternal protein restriction across pregnancy in rats results in increased expression of *PPAR*α, in 1 week old[128] and adult offspring.[197] Interestingly, IUGR offspring have decreased methylation of the *PPAR*α promoter compared to Control offspring,

suggesting that the increased expression in adulthood was epigenetically programmed.[197] In addition to PPARα, PPARδ (known also as PPARβ), can regulate the transcription of cardiac fatty acid genes such as *FACS, CPT-Iβ, MCD, MCAD, LCAD* and *VLCAD*,[167, 168] however, the effect of IUGR on PPARδ has not been determined.

In addition to PPARs, cardiac metabolism is regulated by AMPK, which is activated when cardiomyocyte ATP is depleted. Phosphorylation/activation of the catalytic α_2-subunit of AMPK results in phosphorylation of acetyl-CoA carboxylase (ACC), which promotes transport of fatty acids into the mitochondria, and phosphorylation of AS160, which promotes glucose transporter 4 (GLUT4)'s translocation to the sarcolemma and glucose transport into the sarcoplasm. Interestingly, when lambs are exposed to an obesogenic environment, AMPKα_2 activity is decreased, however if lambs were exposed to maternal nutrient restriction from early to mid-gestation, AMPKα_2 activity is maintained despite an obesogenic environment. Furthermore, lambs exposed to maternal nutrient restriction have an increased prevalence of ectopic cardiac lipid deposition if they developed obesity in the later life and subsequently an increased risk of heart failure with increasing age.[127]

Loss of metabolic flexibility and a greater reliance on glucose for ATP production is observed in postnatal pathologies, such as cardiac hypertrophy and congestive heart failure.[149] IUGR lambs exposed to placental restriction from conception, which results in fetal hypoxaemia and hypoglycaemia, have an increased cardiac phosphorylation of the insulin receptor, increased GLUT1 and increased pyruvate dehydrogenase kinase (PDK) -4 compared to Controls at 21 days of age,[145] which suggests greater glycolysis. Interestingly, this altered metabolism is coupled with greater left ventricle weight relative to body weight (left ventricular hypertrophy; LVH) compared to Controls.[203] Likewise, IUGR due to maternal hypoxia in rats, which is associated with increased glycolysis after ischaemia/reperfusion, results in cardiomyocytes with a greater cross sectional area compared to Controls.[114] Considering that IUGR is associated with cardiomyocyte hypertrophy and LVH, determining

the contribution of IUGR to alterations in postnatal cardiac metabolism are difficult. Furthermore, it appears that exposure to maternal nutrient restriction results in increased expression of fatty acid genes, whereas exposure to hypoxia results in increased reliance on glycolysis. Therefore, we aimed to determine the effect of maternal hypoxia and maternal nutrient restriction in adolescence, in an animal model we have previously determined does not result in cardiac hypertrophy.

4.3 Methods

4.3.1 Animal model

All procedures were approved by the Animal Ethics Committees at both the University of South Australia (IMVS) and The University of Adelaide. IMVS tri-coloured guinea pigs were individually housed at 18-22°C in plastic bottomed cages with sawdust bedding and a 12/12 light cycle. All animals were fed standard laboratory rabbit/guinea pig chow (Laucke Mills, Daveyston, Australia) and had *ad libitum* access to water that was supplemented with 0.5g/L vitamin C. Breeding male and nulligravida female guinea pigs were acclimatised to individual cage living for two weeks prior to the commencement of the study. Each female was weighed and given a known weight of food three times weekly. In order to achieve a known day of conception a single female in oestrous was placed in a males' cage for 24h. Pregnancy was confirmed by the failure to come into oestrous within 21 days (maximum length of oestrous; Chapter 3) of mating.

4.3.2 Experimental protocol

At 35d gestation, pregnant females were randomly assigned to one of three treatment groups; Control (21% oxygen (O_2), *ad libitum* food), Maternal Nutrient Restricted (MNR; 21% O_2, food intake matched daily to food intake per body weight of Maternal Hypoxia dams) or Maternal Hypoxia (MH; 12% O_2, *ad libitum* food but ate ~22% less than Controls; Chapter 3). Pregnant females in the MH group were placed into a glass fronted temperature controlled

chamber that was maintained at 12% oxygen by the infusion of nitrogen (Roxy-4, Sable Systems International, USA). To minimize environmental stress, a minimum of two individually housed females were inside the chamber at any given time and the colony was visible. To ensure all pups were born into a normoxic environment, MH dams were removed from the hypoxia chamber at 65d (term, 68.8±0.4d gestation; Chapter 3). All litters were exposed to normoxia and had *ad libitum* access to food. The average litter size was 3.0±0.2 pups and did not vary between treatment groups (Chapter 3). Both MH and MNR resulted in decreased birth weight (Chapter 3). Litters that contained more than 3 pups were culled to three pups on the first day of life. Offspring were weaned at 28d after birth and were housed in same sex pairs until humanely killed at 4 months of age.

4.3.3 Post mortem collection of tissue

Offspring were humanely killed at 119.4±0.2d of age with an overdose of sodium pentobarbitone (200mg/kg IP; Lethobarb, Lyppards, Australia). Cardiac puncture was immediately performed to collect blood from the left ventricle to determine plasma glucose, non-esterified fatty acid (NEFA) and cortisol concentrations. For one male and one female in a litter, selected at random, the left ventricle and septum was dissected and snap frozen in liquid nitrogen for analysis of gene and protein expression.

4.3.4 Quantification of plasma substrate and hormone concentrations

Plasma glucose concentration: Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (Konelab 20, Program Version 6.0 automated analysis system, Thermo Fisher Scientific, USA).[308] The sensitivity of the assay was 0.5mmol/l and the intra- and inter-assay coefficients of variation (CV) were < 5%.[356]

Plasma non-esterified fatty acid (NEFA) concentration: Plasma NEFA concentrations were measured *in vitro* using an enzymatic colourimetric method, which relies

on the acylation of coenzyme-A (CoA) by fatty acids in the presence of added acyl-CoA synthase (Wako Pure Chemical Industries Ltd., Japan). Oxidation of acyl-Co-A by added acyl-CoA oxidase produces hydrogen peroxide, which aids in the formation of a purple-colored adduct, which can be measured colorimetrically at 550 nM using a Konelab 20XTi automated sample analyzer (Thermo Electron). The intra- and inter-assay CV were < 10%.[356]

Plasma cortisol concentration: Total plasma cortisol concentration was measured in extracts, using an ^{125}I radioimmunoassay kit (GE Healthcare, Sydney, Australia) as previously described [357]. The average efficiency of recovery of ^{125}I cortisol using dichloromethane extraction was 90%. The sensitivity of the assay was 0.39nmol/l. The rabbit anti-cortisol antibody cross-reacted <1% with cortisone and 17-hydroxyprogesterone and <0.01% with aldosterone, pregnenolone, estradiol and progesterone. The inter- and intra- assay CV was less than 10%.

4.3.5 Measurement of mRNA expression

RNA was isolated from the left ventricle (~100mg) and cDNA was synthesized as previously described (Chapter 3 and [203]). Controls containing no Superscript III (NAC) and no RNA transcript (NTC) were used to test for genomic DNA and reagent contamination, respectively. The reference genes cyclophilin, tyrosine 3-monooxygenase (*YWHAZ*) and ribosomal protein P0 were chosen from a suite of reference genes based on expression analysis using the geNorm component of the qBase relative quantification analysis software,[306] because their expression was stable across samples.[307] The expression of target and reference mRNA transcripts were measured by qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystems, USA) in a final volume of 6μL on a ViiA7 Fast Real-time PCR system (Applied Biosystems, USA) as described previously.[307]

Primers were designed and validated to generate a single transcript as confirmed by the presence of one double stranded DNA product of the correct size and sequence (Supplementary data, Table 1). Controls containing no cDNA were included for each primer

set on each plate to test for reagent contamination. Melt curve/dissociation curves were also run to check for non-specific product formation. Amplification efficiency reactions were performed on 5 triplicate serial-dilutions of cDNA template for each primer set. Amplification efficiencies were determined from the slope of a plot of C_t (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (1-100ng). The amplification efficiency was close to 100%. Each sample was run in triplicate for target and reference genes. The reactions were quantified by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding C_t values. The abundance of each transcript relative to the abundance of the three stable reference genes was calculated using DataAssist Software v3.0 (Applied Biosystems, USA) and expressed as mean normalised expression (MNE).[145, 306]

Table 1. Primer sequences used in Quantitative Real-Time Reverse Transcription-PCR to measure genes of interest.

Gene	Primers	Accession Number
Reference genes		
Tyrosine 3-monooxygenase (*YWAHZ*)	Fwd 5'-TGTAGGAGCCCGTAGGTCATCT-3' Rev 5'-TTCTCTCTGTATTCTCGAGCCATCT-3'	XM_003479856.2
Ribosomal protein P0 (*RpP0*)	Fwd 5'-CAACCCTGAAGTGCTTGACAT-3' Rev 5'-AGGCAGATGGATCAGCCA-3'	XM_003478381.2
Peptidyl-prolyl cis-trans isomerase A/Cyclophilin (*Cyclo*)	Fwd 5'-CCTGCTTTCACAGAATAATTCCA-3' Rev 5'-CATTTGCCATGGACAAGATGCCA-3'	XM_003465805.2
Regulation of transcription		
Peroxisome proliferator-activated receptor alpha (*PPARα*)	Fwd 5'-TCCACCATGAACAAGGACGG-3' Rev 5'-TTGCAGGTGGAGTTTGAGCA-3'	NM_001173004.1
Peroxisome proliferator-activated receptor delta/beta (*PPARδ/β*)	Fwd 5'-GTTCATCGCGGCCATCATTC-3' Rev 5'-GCTTGGGGAAGAGGTACTGG-3'	XM_003473900.2
Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*PGC-1α*)	Fwd 5'-GGATTGCCCTCATTTGACGC-3' Rev 5'-GTGCTGGGTACTGAGACCAC-3'	XM_003467408.2
Fatty acid transport into the sarcoplasm		
Fatty acid transport protein 1 (*FATP1*)	Fwd 5'-GCAGATCGGGGAGTTCTACG-3' Rev 5'-GTTGACCTTTACCAGCCGGA-3'	XM_003464194.2
Fatty acid transport protein 6 (*FATP6*)	Fwd 5'-TCCTCAACTCCAACATCCGC-3' Rev 5'-GGAGGCTGGCAAGGATTTCT-3'	XM_005007003.1
Plasma membrane fatty acid binding protein (*FABPpm*)	Fwd 5'-GGCCGACCGTATCATTAGCA-3' Rev 5'-CTACCTGCTCGGGCTTTAGG-3'	XM_003472049.2
Fatty acid translocase (*FAT/CD36*)	Fwd 5'-TCTCTCCTATTGGCCAGGCT-3' Rev 5'-ACCGTACGATGTGCAGTTGT-3'	XM_003469814.2

Regulation of fatty acids in the sarcoplasm		
Long chain fatty acyl-CoA synthetase (*FACS*)	Fwd 5’-CCCTTGGAGCAGATGCCATCACG-3’ Rev 5’-TGCCTTGTTCCACGAGTTCACTGC-3’	NM_001172908.1
Heart-type fatty acid binding protein (*H-FABP*)	Fwd 5’-GTGGCCTGCATGACCAAGCCT-3’ Rev 5’-ACAAGTTTTCCGCCATCCAGCGT-3’	XM_003471234.2
Fatty acid transport into mitochondria		
Carnitine palmitonyl transferase I beta (*CPT-I*β)	Fwd 5’-AGATCGCCCTGCAGCTGGCT-3’ Rev 5’-GCCTGCACAAAGGCTGTGGACT-3’	XM_003461559.1
Malonyl-CoA decarboxylase (*MCD*)	Fwd 5’-GCATTCGCCGTGTGAGGTGC-3’ Rev 5’-GCTCCCCGGGCGTTGAACAA-3’	XM_003461961.2
Acetyl-CoA Carboxylase (*ACC*)	Fwd 5’-CAGCTGGTCCACATGAACAGGCT-3’ Rev 5’-AGCAAATCCTCCTGGGGCCCA-3’	XM_005003514.1
Catalytic α-2 subunit of adenosine monophosphate-activated protein kinase ($AMPK\alpha_2$)	Fwd 5’-GGCACATGGTTGTTCATCGTGACCT-3’ Rev 5’-CCTGAGATGACTTCAGGTGCTGCG-3’	XM_003464067.2
Fatty acid oxidation		
Medium chain acyl-CoA dehydrogenase (*ACADM*)	Fwd 5’-ACATGGGCCAGCGATGTTCAGA-3’ Rev 5’-TGCTAATCCAATTGCACCAGCTGC-3’	XM_003479087.2
Long chain acyl-CoA dehydrogenase (*ACADL*)	Fwd 5’-TCGATCCCCTGCCCATGGCA-3’ Rev 5’-GGGCACTTGCTGGCAATCGGA-3’	XM_003474475.2
Very long chain acyl-CoA dehydrogenase (*ACADVL*)	Fwd 5’-AGAATGTGCTGGGTGAGGTG-3’ Rev 5’-TCTGTGTATCCCCGGTCCAT-3’	XM_003466183.2
Glucose transport into the sarcoplasm		
Glucose transporter 1 (*GLUT1*)	Fwd 5’-GTGCCCATGTATGTTGGGGA-3’ Rev 5’-ATTGCGGTTGATGAGCAGGA-3’	XM_003462510.2
Glucose transporter 4 (*GLUT4*)	Fwd 5’-ATTGCTCCCACTCACCTTCG-3’ Rev 5’-AGCTTGCGCTTCTCATCCTT-3’	XM_003466265.2

4.3.6 Quantification of protein abundance

Proteins were extracted from the left ventricle by sonication in lysis buffer (50mM Tris-HCL (pH 8), 150mM NaCl, 1% NP-40, 1mM Na orthovanadate, 30mM Na fluoride, 10mM Na pyrophosphate, 10mM EDTA, and a protease inhibitor tablet (cOmplete Mini; Roche)). Total protein concentration was determined by microBCA assay (Thermo-Fisher Scientific). Protein was diluted to a concentration of 5mg/ml in 1X SDS sample buffer (containing 75 mM DL-Dithiothreitol) and Coomassie blue stain used to confirm equal protein loading on SDS-PAGE before diluted protein was used for experimental blots. Proteins under 250kDa were transferred (wet tank system) to a nitrocellulose membrane (Amersham Hybond-C extra; GE Healthcare Life Sciences) using boric acid transfer buffer. Proteins over 250kDa were transferred (semi-dry system) to a PVDF membrane (PolyScreen® PVDF Hybridization Transfer Membrane; Perkin Elmer) in tris-glycine transfer buffer. Non-specific antibody binding was blocked with 5% skim milk in TBST (Tris buffered saline with 1% Tween-20) or 5% BSA in TBST. Since primary antibodies specific for phosphorylated AMPKα_2 are not commercially available, antibodies for phospho-AMPKα-total (thr-172; rabbit pAb, 2535, Cell Signaling) and the alternate alpha substrate, phospho-AMPKα_1 (ser-485; rabbit pAb, 4184, Cell Signaling) were used to extrapolate phosphorylation of AMPKα_2. These, in addition to other primary antibodies: AMPKα_2 (rabbit pAb, 2757, Cell Signaling), ACC (rabbit pAb, 3662, Cell Signaling), phospho-ACC (ser-79; rabbit pAb, 3661, Cell Signaling), AS160 (rabbit pAb, 2670, Cell Signaling), phospho-AS160 (thr-642; rabbit pAb, 4288, Cell Signaling), GLUT1 (rabbit pAb, CBL242, Millipore) and GLUT4 (rabbit pAb, ab654, Abcam) were incubated overnight at 4°C. Secondary antibody raised against rabbit (horse radish peroxidase (HRP) linked; 7076, Cell Signaling) was incubated for one hour at room temperature. For each membrane GAPDH-HRP (8884, Cell signalling) was incubated

overnight at 4°C and served as a loading control (abundance stable across samples). SuperSignal West Pico chemiluminescent substrate (Thermo-Fisher Scientific) and an ImageQuant LAS 4000 (GE Healthcare, Australia) were used to detect and image antigens of interest. ImageQuantTL Analysis Toolbox (GE Healthcare, Australia) was used to quantify the protein bands. The abundance of each protein of interest is expressed relative to its loading control.

4.3.7 Statistical analysis

Normality was determines using Shapiro-Wilk-test (swilk) in STATA10. A 2-way ANOVA for the variation between treatment group, sex and their interaction was tested against the variation within animal by treatment group and sex with post-hoc Bonferroni tests applied where appropriate. Statistical significance was assumed if $P < 0.05$. In tables, data is presented as the mean of each treatment group ± the standard error of the mean (SEM). In figures, data is presented as boxplots to provide a greater description of the data.

4.4 Results

4.4.1 Plasma analysis

MNR offspring had increased plasma glucose concentrations compared to both MH and Control offspring (Figure 1A), but there was no change in the plasma concentration of NEFA (Figure 1B) or cortisol (Figure 1C) in MNR or MH compared to Control offspring in adolescence. Females had a higher plasma cortisol concentration than male offspring, which was not affected by treatment (Figure 1C).

4.4.2 Expression of genes involved in fatty acid metabolism

IUGR offspring that were exposed to MH, but not MNR, had an increased cardiac expression of the key transcriptional regulator of fatty acid metabolism, $PPAR\alpha$ (Figure 2A). There was

no effect, however, of MH or MNR on the cardiac expression of *PPAR*δ (Figure 2B) or the transcriptional co-activator *PGC-1*α (Figure 2C) compared to Control offspring in adolescence. IUGR resulted in the upregulation of 3 of the 4 genes responsible for the transport of fatty acids into cardiomyocytes, specifically, MH increased the expression of *FATP1* (Figure 3A) and *FABPpm* (Figure 3B), MH and MNR increased the expression of *FATP6* (Figure 3C), but IUGR did not alter the expression of *CD36* (Figure 3D). Additionally, MH and MNR did not alter the cardiac expression of mitochondrial transporter, *CPT-I*β (Figure 4A) or the three isoforms of acyl-CoA dehydrogenase that are responsible for oxidating medium chain fatty acid, *ACADM* (Figure 4B), long chain fatty acids, *ACADL* (Figure 4C), or very long chain fatty acids, *ACADVL* (Figure 4D). Interestingly, IUGR males, but not females, exposed to either MH or MNR had decreased cardiac expression of long chain *FACS* (Figure 5A) and the catalytic-α_2 subunit of metabolic regulator AMPK (Figure 5B). Similarly, male, but not female, offspring exposed to MNR had decreased cardiac expression of ACC (Figure 5C) compared to Controls, but MCD expression was not different between treatment groups (Figure 5D).

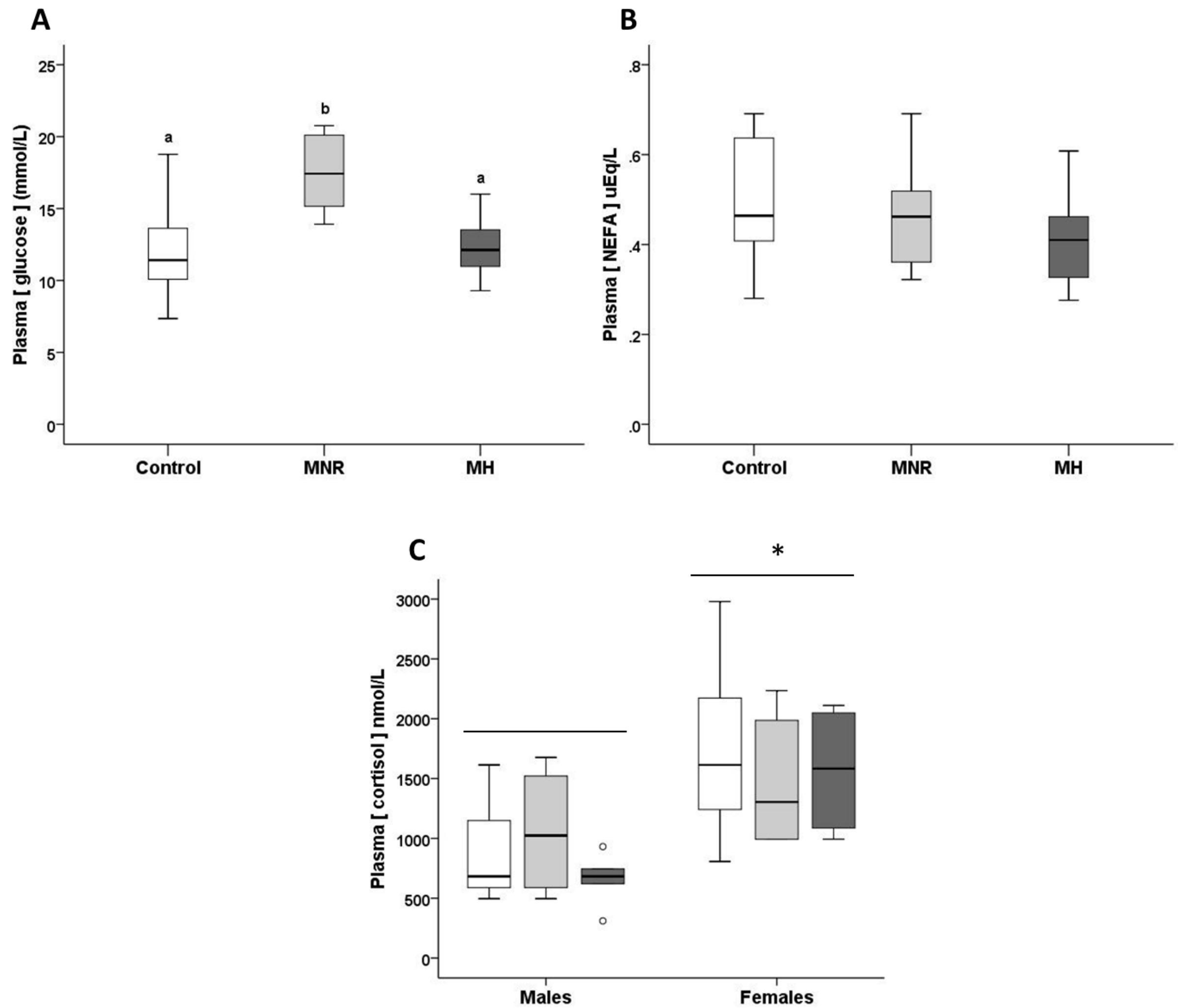

Figure 1. Maternal Nutrient Restriction (MNR; ▩; n=14) offspring had a greater plasma glucose concentration in adolescence compared to Maternal Hypoxia (MH; ■; n=9) and Control (□; n=9) offspring. MNR and MH had a similar plasma non-esterified fatty acid (NEFA; B) and cortisol (C) concentration compared to Control offspring in adolescence. Female offspring (Control, n=11; MNR, n=5, MH, n=4) had a higher plasma cortisol concentration than males (Control, n=3, MNR, n=4, MH, n=5; C), but there was no interaction between treatment and sex. Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni tests; different letters indicate a significant difference between treatment groups; * denotes significant difference between males and females; $P < 0.05$.

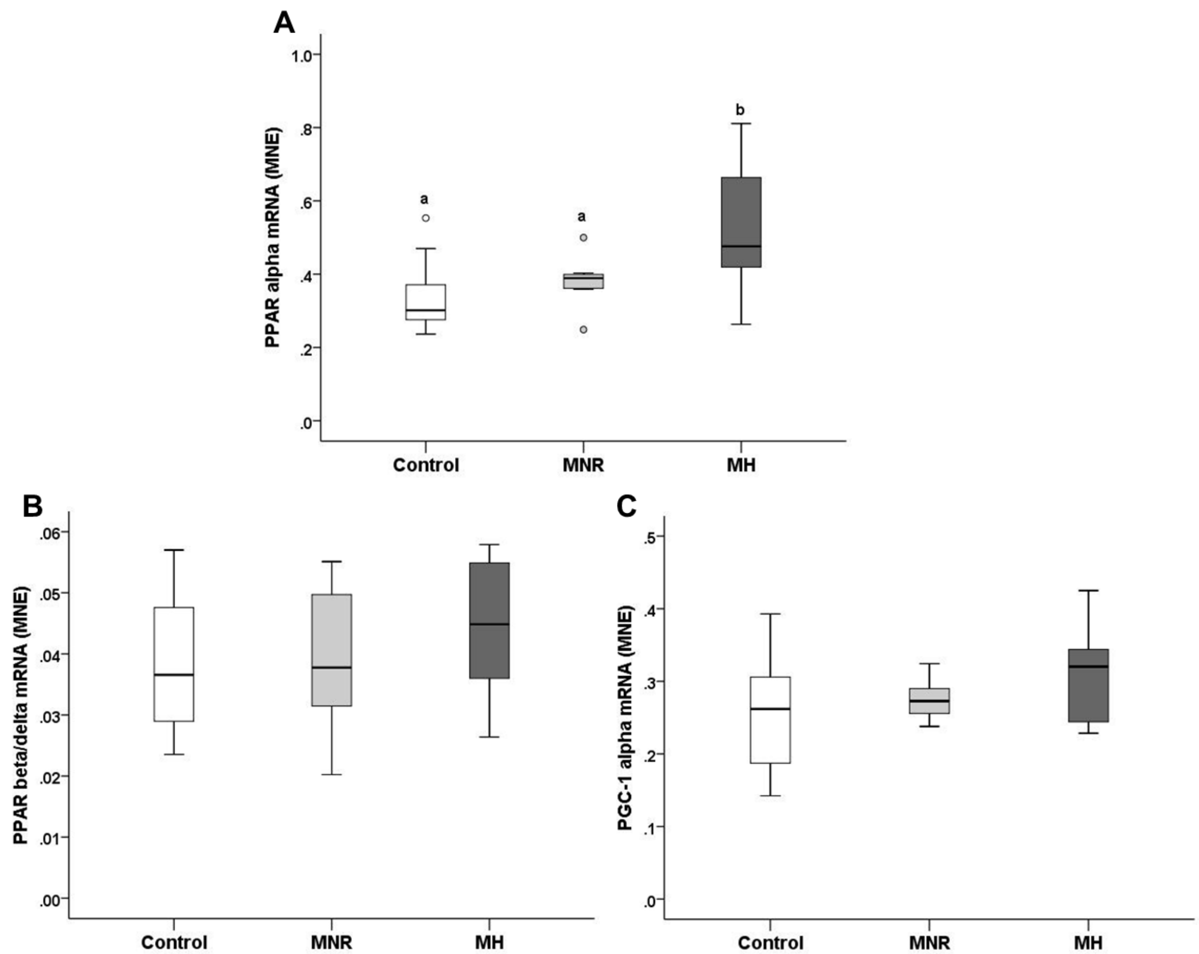

Figure 2. Offspring exposed to Maternal Hypoxia (MH; A; n=10) had increased mRNA expression of the key transcriptional regulator of cardiac fatty acid metabolism, peroxisome proliferator-activated receptor alpha (*PPARα*) compared to offspring exposed to Maternal Nutrient Restriction (MNR; n=7) and Control (n=13). MH and MNR offspring had a similar expression of *PPARβ/δ* (B) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*PGC-1α;* C) compared to Control. Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance between groups; $P < 0.05$.

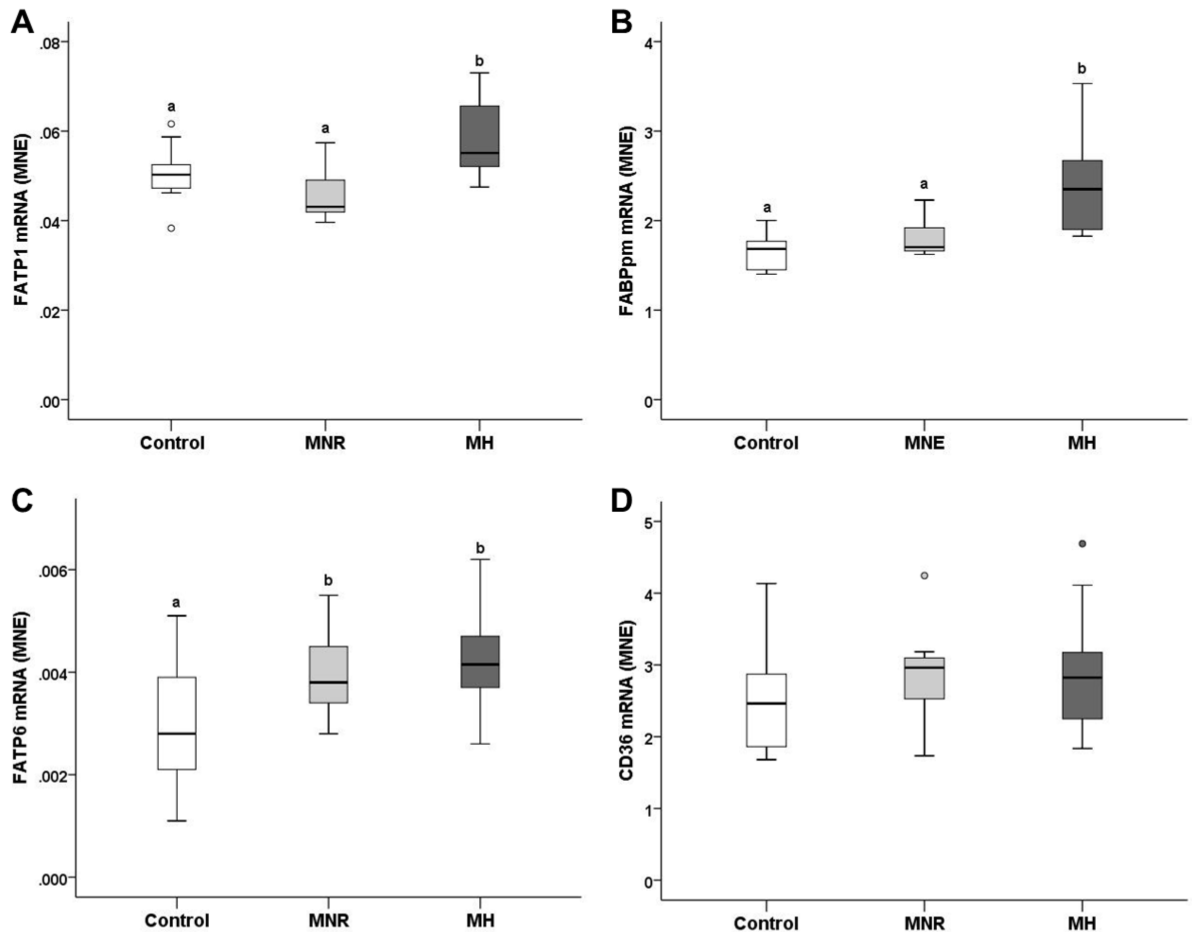

Figure 3. Offspring exposed to Maternal Hypoxia (MH; A; n=10) had increased mRNA expression of fatty acid transporters, fatty acid transport protein 1 (FATP1; A) and plasma membrane fatty acid binding protein (FABPpm; B) compared to offspring exposed to Maternal Nutrient Restriction (MNR; n=7) and Control (n=13). MH and MNR offspring had a greater expression of FATP6 (C) compared to Control offspring, however, there was no effect of MH or MNR on the expression of fatty acid translocase (CD36; D). Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance between groups; $P < 0.05$.

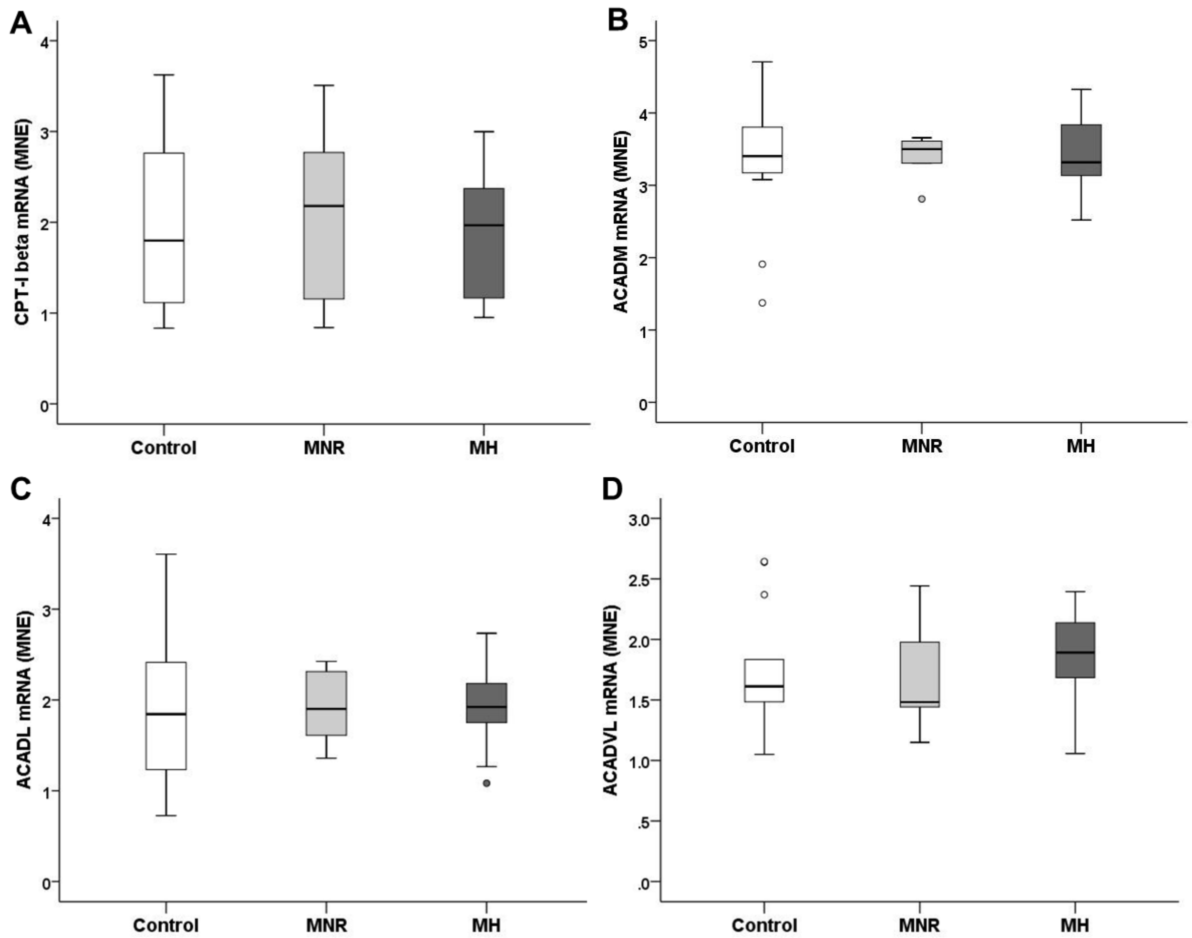

Figure 4. Maternal Hypoxia (MH; n=10) and Maternal Nutrient Restriction (MNR; n=7) did not alter the cardiac expression of genes involved in transport of fatty acids into the mitochondria, carnitine palmitonyl transferase-Iβ (CPT-Iβ; A), and fatty acid oxidation, medium chain acyl-CoA dehydrogenase (ADADM; B), long chain (ACADL) and very long chain (ACADVL) compared to Control (n=13). Data was analysed by 2-way ANOVA for treatment group and sex.

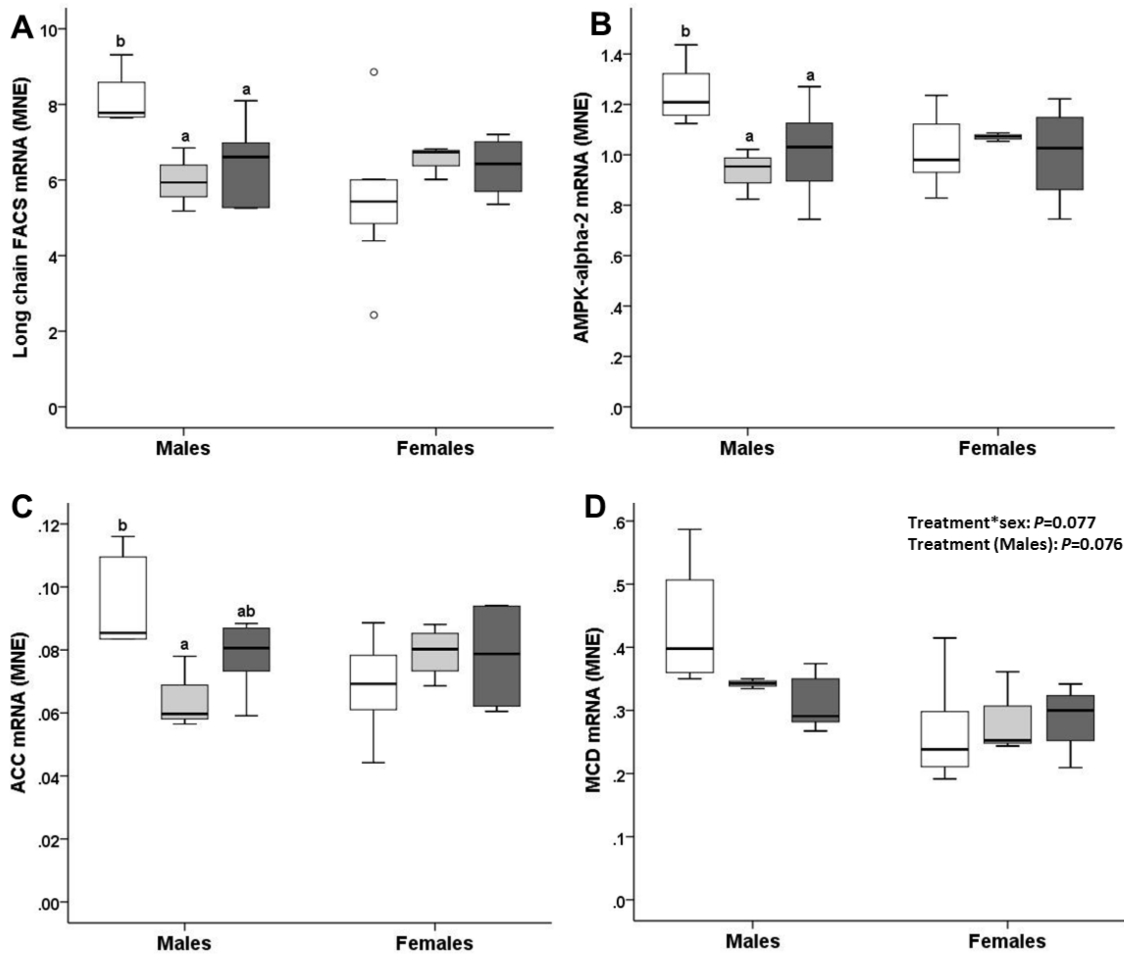

Figure 5. Male offspring, but not female offspring, exposed to Maternal Hypoxia (MH; A;■; males, n=6; females, n=4) and Maternal Nutrient Restriction (MNR; ■; males, n=3; females, n=4) had a reduced expression of the gene responsible for esterification/activation of long chain fatty acids in the sarcoplasm, fatty acyl-CoA synthetase (FACS; A), and the catalytic subunit-2 of the metabolic 'fuel gauge', adenosine monophosphate-activated protein kinase (AMPKα_2; B), compared to Control (□ males, n=5; females n=8). MNR males had a reduced expression of acyl-CoA carboxylase (*ACC*; C), but not (*MCD*; D), which are involved in the regulation of fatty acid uptake into the mitochondria, compared to MH and Control males. There was no effect of treatment group on the expression of *ACC* (C) and *MCD* (D) in female offspring. Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance; $P < 0.05$.

4.4.3 Expression and abundance of glucose transporters

There was no effect of IUGR due to either MH or MNR on the cardiac expression of glucose transporter, GLUT1, (Figure 6A) compared to Controls in adolescence. MH, but not MNR, however, resulted in the increased expression of cardiac GLUT4 (Figure 6B). Despite this, there was no effect of MH or MNR on the abundance of either GLUT1 (Figure 6C) or GLUT4 (Figure 6D) protein compared to Control in adolescence.

4.4.4 Abundance of regulators of fatty acid and glucose metabolism

Despite IUGR resulting in a decreased expression of cardiac $AMPK\alpha_2$ in male offspring, there was no effect of MH or MNR on the abundance of cardiac AMPKα_2 protein (Figure 7A) in either sex compared to Control offspring in adolescence. Interestingly, female offspring exposed to MH had increased abundance of phosphorylated total-AMPKα (Figure 7B), but an equivalent abundance of phosphorylated AMPKα_1 (Figure 7C). Due to their being only two isoforms of the catalytic subunit, this suggests that MH female offspring have increased phosphorylation of AMPKα_2 compared to MNR and Control females. There was no effect of MH or MNR on the phosphorylation of either AMPKα in male offspring (Figure 7B-C). Despite the increased phosphorylation of AMPK in MH females, there was no effect of MH or MNR on the abundance of total (Figure 8A) or phosphorylated (Figure 8B) ACC. Likewise, there was no effect of MH or MNR on the abundance of total (Figure 9A) or phosphorylated (Figure 9B) AS160.

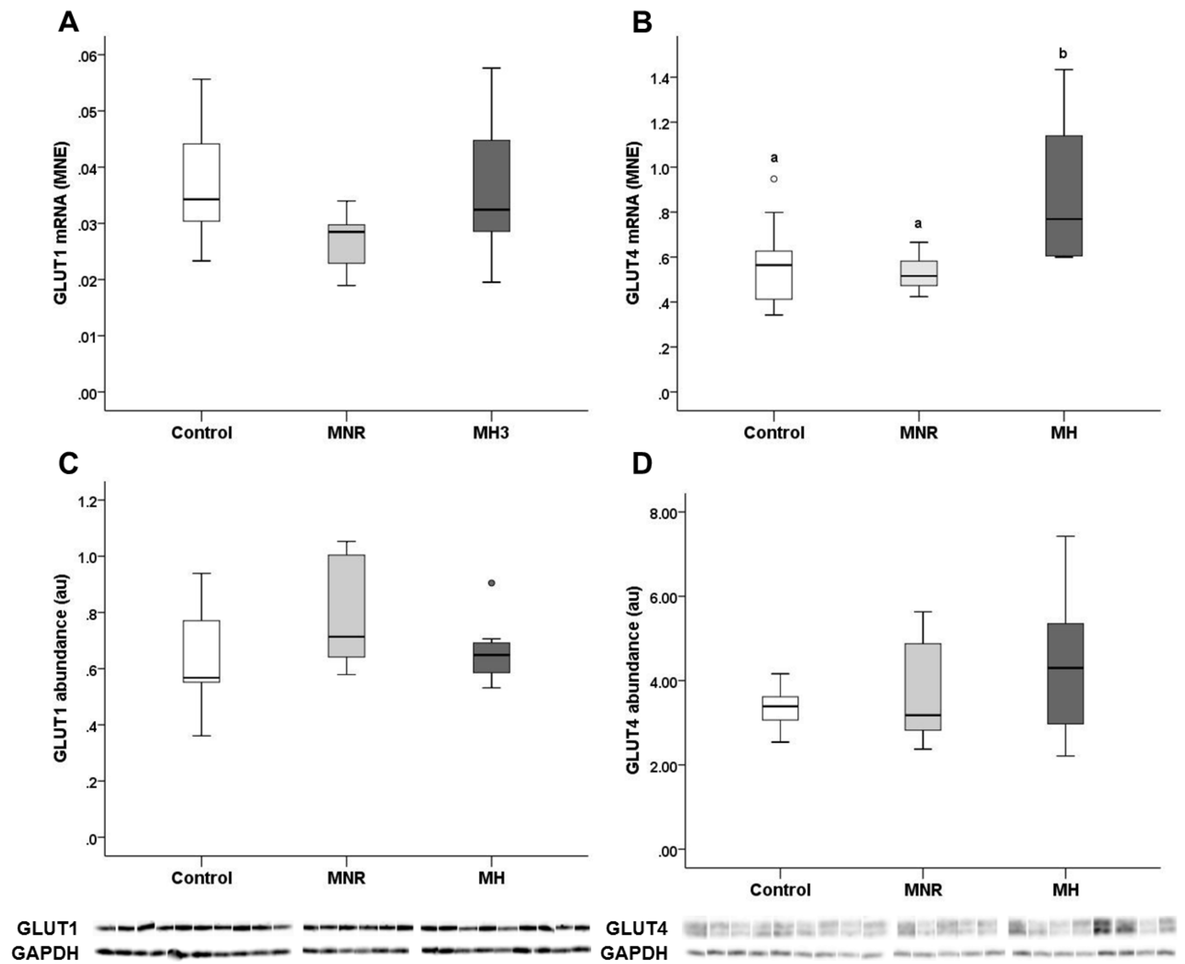

Figure 6. Maternal Hypoxia (MH; A; n=10) and Maternal Nutrient Restriction (MNR; n=7) did not alter the cardiac expression of glucose transporter 1 (GLUT1) compared to Control (n=13), however, MH offspring had an increased cardiac expression of GLUT4 (B). Despite this, neither MH nor MNR altered the cardiac abundance of GLUT1 (C) or GLUT4 (D) protein compared to Control in adolescence. The abundance of each protein is expressed relative a loading control (GAPDH; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance between groups; $P < 0.05$.

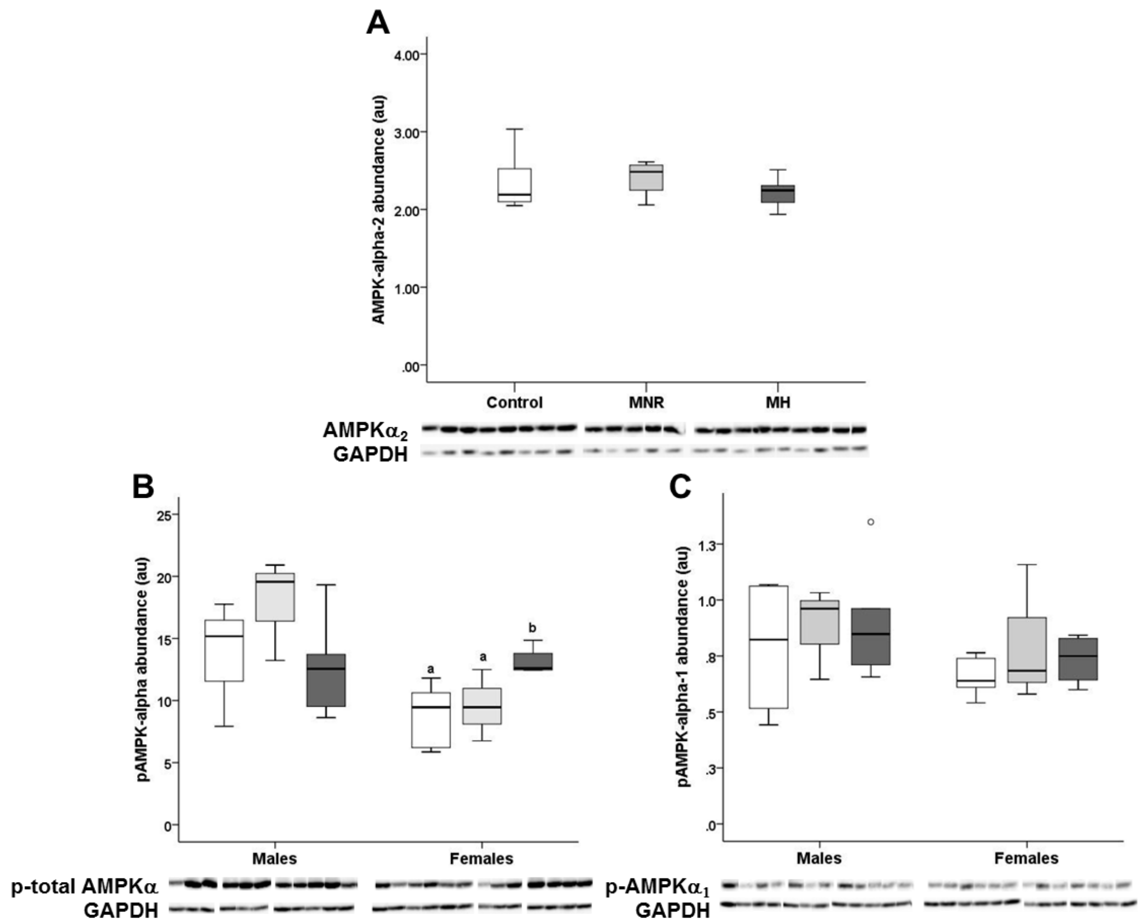

Figure 7. Maternal Hypoxia (MH; A; ■) and Maternal Nutrient Restriction (MNR; ■) did not alter the cardiac abundance of the metabolic 'fuel gauge', adenosine monophosphate-activated protein kinase (AMPKα_2) in adolescence compared to Control (□). Female offspring, but not male offspring, exposed to MH had a greater abundance of phosphorylated total-AMPKα (B), but no difference in the abundance of phosphorylated AMPKα_1 (C) compared to MNR and Control offspring. The abundance of each protein is expressed relative a loading control (GAPDH; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex with post-hoc Bonferroni test where appropriate. Different letters indicate significance between groups; $P < 0.05$.

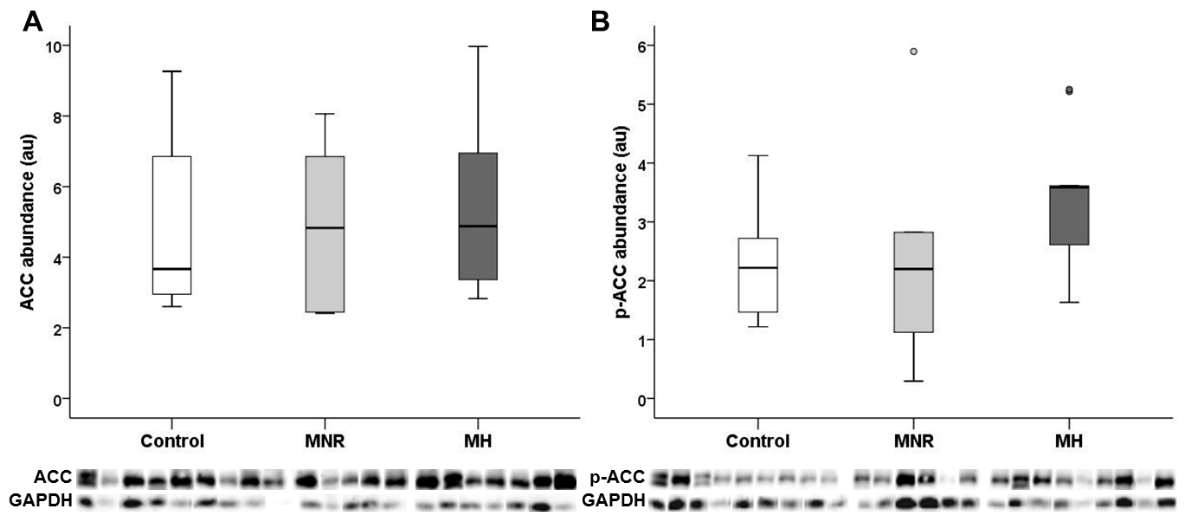

Figure 8. Maternal Hypoxia (MH) and Maternal Nutrient Restriction (MNR) did not alter the cardiac abundance of acyl-CoA carboxylase (ACC; A) or phosphorylation/activity of ACC (B) in adolescent offspring compared to Controls. The abundance of each protein is expressed relative a loading control (GAPDH; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex; $P < 0.05$.

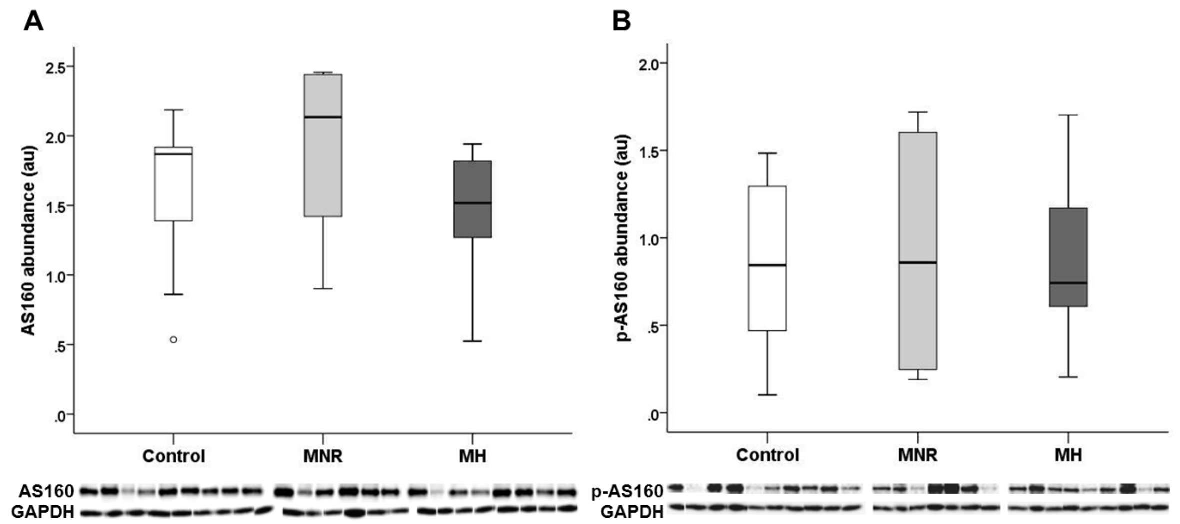

Figure 9. Maternal Hypoxia (MH) and Maternal Nutrient Restriction (MNR) did not alter the cardiac abundance of AS160 (A) or phosphorylation/activity of AS160 (B) in adolescent offspring compared to Controls. The abundance of each protein is expressed relative a loading control (GAPDH; each band represents an animal). Data was analysed by 2-way ANOVA for treatment group and sex; $P < 0.05$.

4.5 Discussion

This study demonstrates that IUGR alters the expression of genes involved in cardiac metabolism specific to the cause of IUGR and sex of the offspring in adolescence. It is critically important that cardiac metabolism is tightly regulated to ensure the continued production of ATP for effective cardiomyocyte contractility. Alterations in the regulation of cardiac metabolism may explain some of the association between IUGR and the elevated risk of ischaemic heart disease and heart failure in adulthood.[2-10]

IUGR induced by MH, but not MNR, results in increased expression of the central transcriptional regulator of cardiac fatty acid metabolism, *PPARα*. The increased expression in MH offspring is consistent with studies of IUGR rats exposed to maternal protein restriction across pregnancy.[128, 197] Tappia and colleagues demonstrate that at 1, 3, 5, 7 and 9, but not 21 days of age, IUGR offspring have increased cardiac expression of *PPARα*.[128] In contrast, Slater-Jefferies and colleagues demonstrate that, compared to Control offspring, IUGR rats have a similar cardiac expression of *PPARα* at 1 day of age, but have increased cardiac expression of *PPARα* in adulthood.[197] Further to this, Slater-Jefferies and colleagues demonstrated that at both 1 day of age and in adulthood, IUGR offspring have decreased methylation of the *PPARα* promoter compared to Control offspring, which suggests the increased expression of *PPARα* in adulthood was epigenetically programmed *in utero*.[197] Interestingly, exposure to a low fat or high fat diet post weaning did not alter the adult expression of *PPARα* or the methylation of its promoter.[197]

In addition to epigenetic regulation, PPARα expression is upregulated by glucocorticoids through direct action of the glucocorticoid receptor.[358, 359] We have previously reported that MH offspring have increased adrenal weight, both in absolute terms and relative to body weight, compared to Control and MNR offspring in adolescence (Chapter 3). The

adrenal gland secretes cortisol in response to activation of the hypothalamic-pituitary-adrenal (HPA) axis to maximise metabolism and suppress immune function during stress. Therefore, an increase in adrenal weight may signify an increased capacity for cortisol production. Studies of adult men demonstrate that low birth weight is associated with elevated fasting plasma cortisol concentrations[250] and enhanced secretion of cortisol in response to adrenocorticotropic hormone (ACTH), which is released from the pituitary upon activation of the HPA axis.[271] Furthermore, a 1kg decrease in birth weight is associated with a 20.6nmol/L and 30.9nmol/L elevation in plasma cortisol concentrations in men and women, respectively.[272] In the present study, however, IUGR due to MH and MNR did not alter the concentration of plasma NEFA under basal conditions, but female offspring had a higher plasma NEFA concentration than male offspring. This suggests that plasma cortisol concentrations do not explain the increased expression of *PPAR*α observed in IUGR offspring.

A programmed increase in *PPAR*α expression may be a mechanism to deal with continued nutrient depravation or periods of fasting in postnatal life. PPARα regulates fatty acid metabolism by increasing the transcription of genes involved in fatty acid transport into the sarcoplasm (*FATP*) and within the sarcoplasm (*H-FABP*), fatty acid activation (*FACS*), mitochondrial uptake of fatty-acyl CoA (*CPT-I* , *CPT-I* and *MCD*) and fatty acid β-oxidation (*ACADM, ACADL* and *ACADVL*; for review, see [162, 163]). Interestingly, in the present study, despite *PPAR*α expression being greater in MH offspring, the only PPARα target genes with increased mRNA expression were *FATP1* and *FATP6*. This suggests that despite the increased mRNA expression of *PPAR*α, there is not a corresponding increase in PPARα induced transcription of target genes in adolescence. This may be due to an equivalent PPARα protein abundance or an equivalent efficiency of PPARα induced transcription compared to Controls. In the present study, we could not determine the abundance of PPARα

using Western Blotting due to variations between guinea pig sequence and commercially available antibodies. PPARα induces transcription by first heterodimerising with retinoid X receptor (RXR), then binding to peroxisome proliferator response elements (PPRE) which are present in either in single or multiple copies in the promoter region of target genes.[160] Transcriptional control also requires the recruitment of various co-factors, which can either promote or inhibit transcription.[360] Therefore, promotion of PPARα mediated transcription is dependent on the availability of activating ligands, RXR and co-activators. In the heart, peroxisome proliferator-activated gamma coactivator 1 alpha (PGC-1α) can promote the transcription of PPARα gene targets and its expression is influenced by environmental stimuli.[360, 361] In the present study we determined that the expression of *PGC-1α* was similar between MH, MNR and Control offspring. Likewise, the expression of PPARδ was also similar between treatment groups. Further studies into the effect of IUGR on the efficiency and regulation of PPARα induced transcription are required.

An increase in *PPARα* expression may also be explained by an increase in circulating NEFA concentrations,[166] which can occur in obesity and insulin resistance. The association between IUGR and postnatal obesity, insulin resistance and type-2 diabetes mellitus has been demonstrated in epidemiological studies and numerous animal models.[135] In the present study, however, IUGR due to MH and MNR did not alter the plasma concentration of NEFA. Likewise, we have previously reported that MH and MNR offspring do not weigh more than Control offspring or have increased visceral fat deposition (Chapter 3), suggesting that neither MH or MNR offspring are obese. The mismatch between low nutrient availability *in utero* and adequate/excessive nutrition in postnatal life underpins the programming of obesity and is exaggerated if coupled with accelerated postnatal 'catch up' growth. We have previously reported that MNR offspring have accelerated postnatal growth (Chapter 3) and data from the current study demonstrates that MNR offspring have increase plasma glucose concentrations.

The increase in glucose concentrations may support the accelerated growth of MNR offspring after birth. An increase in basal glucose concentration is also a sign of a dysregulation of global glucose homeostasis, which may be caused by insulin resistance and diabetes. Glucose metabolism is facilitated upon translocation of glucose transporters, GLUT1 and GLUT4, from vesicles in the sarcoplasm to the sarcolemma. In postnatal life, GLUT4 is the predominant cardiac glucose transporter and its translocation is initiated by insulin, AMPK, catecholamines, increased mechanical load and ischemia.[175-178]. We have previously reported that MNR offspring have an equivalent heart weight, left ventricle weight, number of cardiomyocytes, blood pressure and activity of insulin signalling molecule, Akt (Chapter 3). Likewise, in the present study, MNR offspring have an equivalent abundance of GLUT1, GLUT4, activity of AMPK and AS160. We therefore speculate that in adolescence, hearts of MNR offspring may not have altered cardiac metabolism in the presence of higher plasma glucose concentrations.

Despite having a normal birth weight, lambs exposed to maternal nutrient restriction have an increased prevalence of ectopic cardiac lipid deposition if they developed obesity in the later life and subsequently an increased risk of heart failure with increasing age.[127] Furthermore, IUGR rats exposed to maternal protein restriction across gestation have altered cardiac lipid accumulation, specifically of triacylglycerol (TAG), according to sex.[197] In males, maternal protein restriction did not alter the accumulation of cardiac TAG in adulthood and this was irrespective of whether they were fed a low or high fat diet post weaning.[197] In females, however, IUGR rats on a low-fat diet post weaning had an increased cardiac accumulation of TAG compared to Control offspring and cardiac TAG accumulation was exaggerated if exposed to a high-fat diet post weaning.[197] In the present study, IUGR male, but not female, offspring have decreased expression of long chain *FACS*, *AMPK*α_2 and *ACC*, which suggest that males may be genetically predisposed to altered lipid homeostasis.

Considering that in the present study neither male nor female IUGR offspring were obese or had increased circulating NEFA concentrations, suggests that at 4 months of age the alteration in gene expression may not result in an altered cardiometabolic phenotype.

IUGR lambs exposed placental restriction from conception have an increased phosphorylation of the insulin receptor, increased GLUT1 protein and increased pyruvate dehydrogenase kinase (PDK) -4 protein abundance in the heart compared to Control lambs at 21 days of age.[145] These changes suggest that at 21d of age, IUGR lambs are utilising glycolysis, more so than Control offspring, for cardiac ATP production. Interestingly, IUGR lambs have a similar expression of $PPAR\alpha$ compared to Control lambs, which is in contrast to IUGR offspring in the present study and the majority of data from IUGR rats exposed to maternal protein restriction.[128, 197] This data suggests that there may have been a delay in the downregualtion of glycolysis, which occurs shortly after birth.[274] In the present study, MH increased the expression of *GLUT4*, however, there was no difference in the protein abundance of GLUT4 or mRNA and protein levels of GLUT1. Since glucose transporters reside in sarcoplasmic vesicles unless stimulated, we determined the phosphorylation of AS160, whose activation results in GLUT4 translocation to the sarcolemma.[362] In the present study, MH and MNR did not alter the phosphorylation of AS160 and furthermore, previous studies demonstrate that downstream insulin signalling molecule, Akt, which phosphorylates AS160, is also similarly phosphorylated compared to Control offspring in adolescence (Chapter 3). Data in the present study suggests that IUGR offspring do not have an increased transport of glucose into the sarcoplasm, however, we did not determine the effect of IUGR on the abundance of enzymes that regulate glycolysis. Furthermore, IUGR lambs have LVH and evidence of pathological hypertrophy,[145] which also results in increased glycolysis.[275] We have previously reported that MH and MNR offspring do not have LVH or evidence for

pathological hypertrophy (Chapter 3), therefore, we speculate that the increase in glycolysis in IUGR lambs is associated with LVH.

IUGR rats exposed to maternal hypoxia have an equivalent contribution of fatty acids (~92%), lactate (~5%), glucose via glycolysis (~2%) and glucose via oxidation (~1%) to cardiac ATP production compared to Control offspring at 4 and 12 months of age.[198] After experimentally induced ischaemia/reperfusion, however, IUGR males had a decreased contribution of fatty acids and an increased contribution of glucose to ATP production, coupled with decreased cardiac power, compared to Control offspring.[198] Furthermore, hearts from IUGR male offspring had increased production of protons, which may be explained by an uncoupling of glycolysis with glucose oxidation,[198] which requires ATP to correct.[199-201] Since we did not measure the contribution of each substrate to cardiac ATP production, we do not know if MH and MNR offspring had a similar contribution of substrates to ATP production compared to Control offspring in adolescence. We do, however, speculate that programmed change in genes expression may result in an altered metabolism if exposed to secondary insults such as ischaemia. Further to this, only IUGR female offspring that were exposed to MH had an increased phosphorylation of AMPKα_2, which suggest that only MH female offspring are responding to a deprivation in ATP in adolescence. We have previously demonstrated that MH females, but not MH males or MNR males or females, have fewer cardiomyocytes compared to Control offspring (Chapter 3), which may be a sufficient secondary insult to trigger altered cardiac metabolism.

4.6 Conclusion

In summary, this study demonstrates that IUGR due to MH and MNR alters the expression of genes involved in cardiac metabolism. These changes occur in the absence of associated pathologies of IUGR that can alter cardiac metabolism, such as increased cortisol, obesity,

cardiac hypertrophy (Chapter 3) and hypertension (Chapter 3). We postulate, therefore, that the observed changes in cardiac gene expression in the present study are a direct result of IUGR. Interestingly, the expression of genes involved in fatty acid metabolism were sexually dimorphic and suggest that male offspring are more susceptible to cardiac lipid accumulation than females in postnatal life. Furthermore, MH female offspring, but not male offspring, had increased phosphorylation/activity of metabolic fuel gauge, AMPKα_2, compared to MNR and Control offspring. This suggests that MH females are the only offspring responding to a deprivation in ATP in adolescence, which may be due to MH females having fewer cardiomyocytes in adolescence (Chapter 3).

CHAPTER 5

5. CHAPTER FIVE

5.1 Overall discussion

In early life, environmental factors can act to increase the risk of disease in adulthood. This association was termed the 'Barker Hypothesis' by the British Medical Journal in 1995[363] in honour of Professor David Barker and colleagues who published epidemiologic evidence from British men born between 1911-1930, which demonstrated that those born with the lowest birth weights and body weight at 1 year of age had the highest death rates from ischaemic heart disease.[364] In the past 20 years there has been a wealth of epidemiological studies and animal models that demonstrate that intrauterine growth restriction (IUGR) is associated with a higher risk of cardiovascular disease (CVD), hypertension, insulin resistance, type-II diabetes, obesity and greater activation of the hypothalamic-pituitary-adrenal (HPA) axis in postnatal life (for review, see [135]). The effect of IUGR on cardiomyocyte development and the altered programming of cardiac metabolism as a potential cause of increased vulnerability of cardiomyocytes in postnatal life was the focus of this thesis.

We had previously demonstrated,[123] as had others,[124, 125] that IUGR due to placental insufficiency and consequent chronic fetal hypoxaemia and hypoglycaemia, reduces overall heart growth and delays cardiomyocyte maturation in fetal sheep in late gestation. Further to this, we had demonstrated that placental insufficiency, caused by a restriction of placental growth from conception (placental restriction; PR), results in cardiomyocytes that are larger relative to heart weight compared to Controls.[123] In the present thesis, I have determined that IUGR due to PR results in fewer cardiomyocytes in the right ventricle in late gestation, which was not due to increased apoptosis. Considering that we had previously demonstrated that IUGR sheep had an equivalent percentage of cardiomyocytes in the cell cycle,[123] this led us to speculate that a reduction in cardiomyocyte number was a consequence of an earlier adaption to poor fetal substrate supply; and that despite chronic hypoxaemia, the heart is not hypoxic in

late gestation. This premise was supported in the present study by a lack of hypoxia mediated transcription in IUGR fetuses who were hypoxaemic. Interestingly, when placental insufficiency is induced only in late gestation, by umbilico-placental embolization, the percentage of cardiomyocytes in the cell cycle is reduced,[124] suggesting that these cardiomyocytes may be experiencing hypoxia. Additionally, IUGR rat offspring exposed to maternal hypoxia in the last week of gestation have an increased percentage of apoptotic cardiomyocytes. Together, these data suggest that a reduction in cardiomyocyte number *in utero* is a regulated mechanism that is initiated in response to a deprivation in oxygen and/or nutrient supply, but is suppressed when supply and demand are restored. This assertion was supported by the present study, which demonstrates that IUGR fetuses exposed to PR have an equivalent length of coronary capillaries in the right ventricle compared to Control offspring, suggesting an increased delivery of substrates to the fewer cardiomyocytes that remain (Figure 1).

Interestingly, despite an increased capillary length density, there was no difference in the mRNA expression of the principal regulator of angiogenesis, VEGF in either ventricle. The absence in an increased mRNA expression may be due to a hypoxia mediated increase in the half-life of target genes. This has been demonstrated for VEGF mRNA in human M21 melanoma cells.[365] Hypoxia has also been shown to enhance translation efficiency of VEGF mRNA, by a protein interaction with the intra-ribosomal entry site in the 5' untranslated region.[366] Confirmation of the abundance of VEGF protein and of other genes with HRE should be performed to determine unequivocally that the heart is not hypoxic. Likewise, to understand the mechanism for why IUGR fetuses have fewer cardiomyocyte in late gestation, alternate methods of cell death, such as necrosis and autophagy should be analysed.

The study presented in Chapter 2 was the first to demonstrate in a large animal model, where, as in humans,[78] cardiomyocytes mature *in utero* and have minimal proliferative

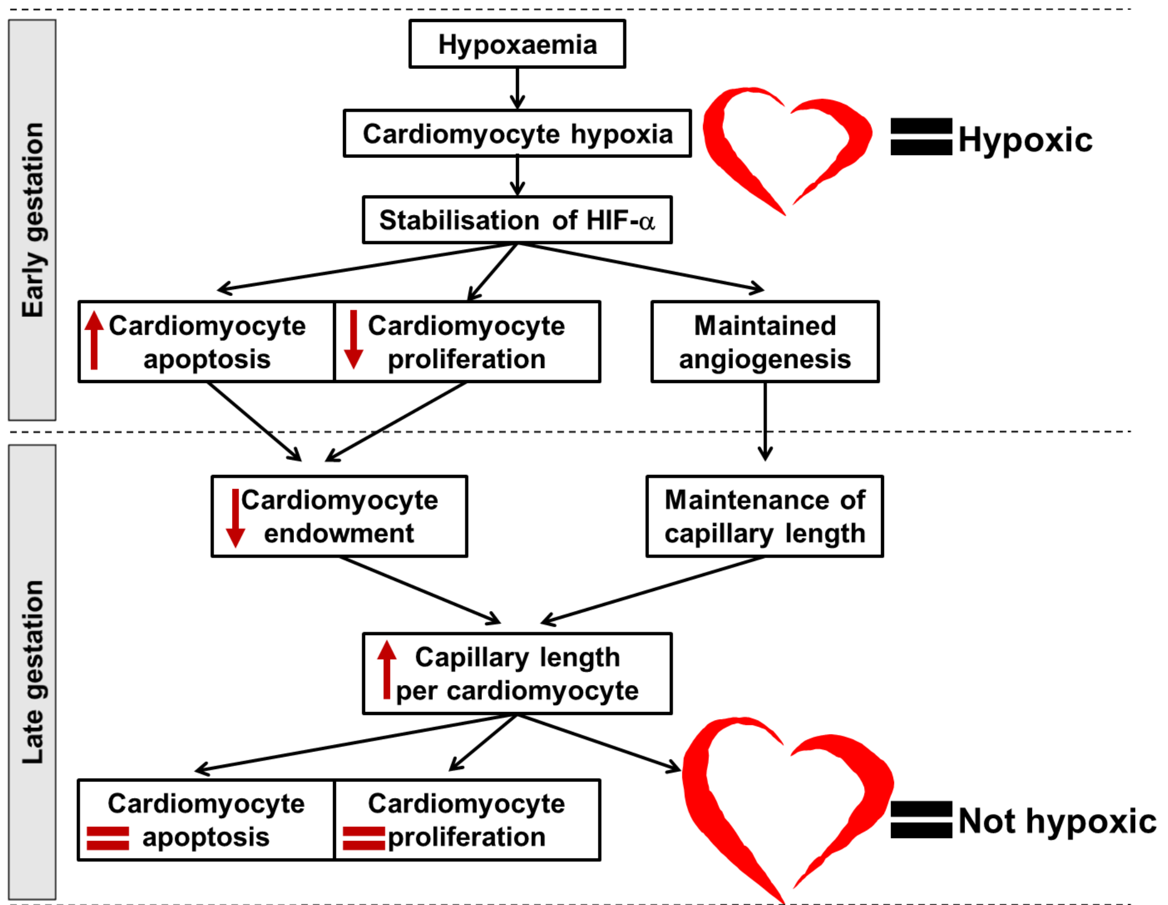

Figure 1. We speculate that the heart of the chronically hypoxaemic fetus is not hypoxic in late gestation due to programmed cell death and decreased cardiomyocyte proliferation in response to cardiomyocyte hypoxia earlier in gestation.

potential after birth, that IUGR can reduce the number of cardiomyocytes.[75, 76] Previous studies in IUGR rats had demonstrated that maternal protein restriction across pregnancy results in fewer cardiomyocytes at birth,[111] but that if maternal protein restriction continued through lactation, the period of cardiomyocyte maturation in rats,[74] that the number of cardiomyocytes after lactation was the same as Control.[112] This led us to determine if a deficit in cardiomyocytes due to either maternal hypoxia (MH) or maternal nutrient restriction (MNR), in a species that is born mature, the guinea pig, results in fewer cardiomyocytes in adolescence. In the present study I determined that IUGR female offspring exposed to MH have fewer cardiomyocytes in the left ventricle in adolescence. Interestingly, IUGR male offspring exposed to MH and IUGR offspring exposed to MNR had an equivalent number of cardiomyocyte to Control. This increased vulnerability of cardiomyocytes in females to MH was unexpected, since previous literature demonstrates that male offspring are more vulnerable to loss of nephrons than females if exposed to maternal protein restriction.[290, 291] Once sexually mature, females are less susceptible to pathologic cardiac remodelling in postnatal life due to the protective effects of estrogen,[332] however, lower concentrations *in utero* may not provide protection. Cardiomyocytes are also targets of testosterone, which has been implicated in cardiomyocyte differentiation,[367] hypertrophy[368, 369] and glucose uptake.[370] It has also been shown that treating female embryonic heart cells (H9c2) with testosterone protects against metabolic stress through a mechanism involving estrogen receptor signalling.[371] Further research into the effect of IUGR on the cardiac expression of androgens, their receptors and receptor signalling *in utero* is required.

In Chapter 3, IUGR females exposed to MH had fewer cardiomyocytes, but those exposed to MNR had an equivalent number of cardiomyocytes as Controls. This suggests that oxygen is an important regulator of cardiomyocyte endowment in females. Studies in maternal protein restricted rats suggest that nutrition alone is capable of reducing cardiomyocyte endowment in the fetus, but it is important to note that maternal protein

restriction from conception can also reduce placental growth[372] and may therefore result in poor oxygen delivery and fetal hypoxaemia. In the present study, it is also plausible that there was an additive effect of reduced maternal food intake during hypoxia on cardiomyocyte endowment in the offspring. Further investigations into the specific mechanism behind reduced cardiomyocyte endowment due to maternal hypoxia and maternal protein restriction are required.

Male IUGR rats exposed to maternal hypoxia have increased cardiomyocyte apoptosis,[115] decreased cardiac power[198] and altered cardiac metabolism[198] after ischaemia/ reperfusion injury in adolescence. We, therefore, aimed to determine if IUGR offspring exposed to MH and MNR have altered expression of cardiometabolic genes, specifically those regulating the predominant contributor to cardiac ATP production in postnatal life: fatty acid metabolism. In Chapter 4, I determined that IUGR offspring exposed to MH had increased expression of the transcriptional regulator of fatty acid metabolism, *PPAR*α, and increased expression of fatty acid transporters, *FATP1, FAPT6* and *FABPpm*, but that of these genes, MNR only increased the expression of *FATP6*, compared to Control. Interestingly, IUGR male offspring, but not female offspring, had a decreased expression of factors in the sarcoplasm that regulate fatty acid activation (*FACS*) and transport of active fatty acids into the mitochondria for fatty acid β-oxidation (*AMPK*α*2* and *ACC*) if exposed to MNR, but a decrease in only *FACS* and *AMPK*α*2* expression if exposed to MH. Interestingly, only IUGR females exposed to maternal hypoxia had increased activity of the metabolic fuel gauge, AMPK, suggesting that regulation of energy utilisation may be related to the reduction in cardiomyocyte endowment in the left ventricle. Since I did not quantify the abundance or activity of enzymes involved in fatty acid metabolism or the contribution of metabolised substrates to ATP production, I do not know, however, if the observed gene changes result in altered ATP production compared to Control. Based on evidence that prior to ischemia/reperfusion IUGR rats exposed to maternal hypoxia have similar metabolism and

cardiac power to Controls, I hypothesise that a secondary insult, such as ischaemia, hypertension, hypertrophy or insulin resistance, is required for an altered cardiometabolic phenotype to develop. Likewise, lambs exposed to maternal nutrient restriction have an increased prevalence of ectopic cardiac lipid deposition if they developed obesity in the later life and an increased incidence of heart failure compared to Control lambs that were also exposed an obesogenic environment.[127] This suggests that a programmed alteration in cardiometabolic gene expression may underlie the pathological phenotype that develops in a postnatal environment of chronically elevated free fatty acids.

Fetal hypoxia is one of the most common consequences of complicated pregnancies and may be fetal, placental or maternal in origin.[373] Clinically, maternal hypoxia is a consequence of pregnancy at high altitude, maternal respiratory illnesses such as asthma, pneumonia and cystic fibrosis, maternal anaemia and sleep apnoea and likely cause fetal hypoxaemia due to decreased uteroplacental blood flow.[374] Maternal hypoxia also results in a greater dependence on glucose for anaerobic ATP production in both the mother and placenta, which combined with decreased uteroplacental blood flow may result in decreased glucose delivery to the fetus.[375] Likewise, decreased maternal nutrition may reduce placental growth or transport efficiency,[372] which may decrease oxygen delivery to the fetus. Consequently, isolating the specific cause of fetal abnormalities associated with IUGR is difficult. For this reason, the use of multiple animal models of IUGR, each with a different profile of insults that lead to IUGR allow us to tease out the profile of pathologies an IUGR infant may exhibit. Data from this thesis highlights that the consequences of being IUGR are most likely specific to the cause of IUGR, not a generalised outcome from being IUGR.

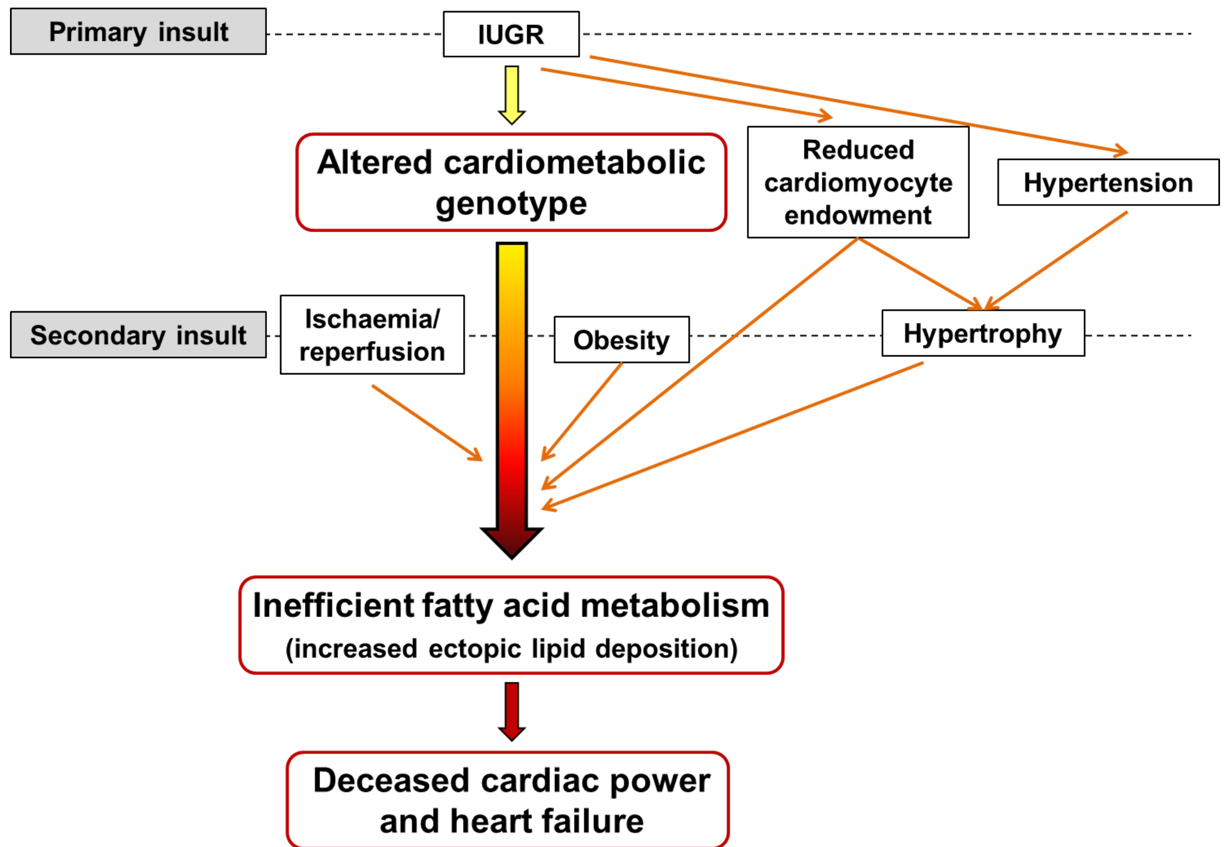

Figure 2. The present study demonstrates that IUGR programs changes in cardiometabolic genes, which we speculate underlie the pathological metabolism that occurs in response to secondary insults, such as ischaemia/reperfusion, obesity and hypertrophy in postnatal life. Secondary insults may also be caused by IUGR.

5.2 Overall Conclusion

In conclusion, this thesis demonstrates that IUGR offspring can have reduced cardiomyocyte endowment and altered expression of cardiometabolic genes, which is specific to the cause of IUGR and sex of the offspring. Furthermore, these data suggests that females may be more vulnerable to IUGR associated deficits in cardiomyocyte endowment and that males may be more vulnerable to inefficient fatty acid metabolism due to IUGR associated changes in gene expression. Importantly, this study demonstrates that changes in the expression of cardiometabolic genes occurs in the absence of other IUGR associated pathologies that can influence cardiac metabolism, such as hypertrophy, hypertension, insulin resistance, obesity and increased cortisol concentrations, suggesting that cardiac metabolism may be programmed in IUGR offspring.

REFERENCES

6. REFERENCES

1. Botting KJ, Wang KC, Padhee M, McMillen IC, Summers-Pearce B, Rattanatray L, Cutri N, Posterino GS, Brooks DA, Morrison JL. Early origins of heart disease: Low birth weight and determinants of cardiomyocyte endowment. *Clin Exp Pharmacol Physiol*. 2012;39:814-823
2. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet*. 1989;2:577-580
3. Fall CHD, Osmond C, Barker DJP, Clark PMS, Hales CN, Stirling Y, Meade TW. Fetal and infant growth and cardiovascular risk factors in women. *BMJ*. 1995;310:428-432
4. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD. Birthweight, body-mass index in middle age, and incident coronary heart disease. *Lancet*. 1996;348:1478-1480
5. Forsen T, Eriksson JG, Tuomilehto J, Teramo K, Osmond C, Barker DJ. Mother's weight in pregnancy and coronary heart disease in a cohort of finnish men: Follow up study. *Bmj*. 1997;315:837-840
6. Leon DA, Lithell HO, Vagero D, Koupilova I, Mohsen R, Berglund L, Lithell UB, McKeigue PM. Reduced fetal growth rate and increased risk of death from ischaemic heart disease: Cohort study of 15 000 swedish men and women born 1915-29. *Bmj*. 1998;317:241-245
7. Stein CE, Fall CH, Kumaran K, Osmond C, Cox V, Barker DJ. Fetal growth and coronary heart disease in south india. *Lancet*. 1996;348:1269-1273
8. Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. *Bmj*. 1993;307:1519-1524
9. Rich-Edwards JW, Stampfer MJ, Manson JE, Rosner B, Hankinson SE, Colditz GA, Willett WC, Hennekens CH. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *Bmj*. 1997;315:396-400
10. Barker DJ, Gelow J, Thornburg K, Osmond C, Kajantie E, Eriksson JG. The early origins of chronic heart failure: Impaired placental growth and initiation of insulin resistance in childhood. *Eur J Heart Fail*. 2010;12:819-825
11. Woodcock EA, Matkovich SJ. Cardiomyocytes structure, function and associated pathologies. *Int J Biochem Cell Biol*. 2005;37:1746-1751
12. Thornburg K, Jonker S, O'Tierney PO, Chattergoon N, Louey S, Faber J, Giraud GG. Regulation of the cardiomyocyte population in the developing heart. *Progress in Biophysics and Molecular Biology*. 2010;doi:10.1016:1-11
13. Bergmann O, Zdunek S, Alkass K, Druid H, Bernard S, Frisen J. Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. *Exp Cell Res.* 2011;317:188-194. Epub 2010 Sep 2017.
14. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. *Science*. 2009;324:98-102
15. Ahuja P, Sdek P, MacLellan WR. Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol Rev*. 2007;87:521-544
16. Evans S, Newnham J, MacDonald W, Hall C. Characterisation of the possible effect on birthweight following frequent prenatal ultrasound examinations. *Early Hum Dev*. 1996;45:203-214
17. Sucov HM, Gu Y, Thomas S, Li P, Pashmforoush M. Epicardial control of myocardial proliferation and morphogenesis. *Pediatr Cardiol*. 2009;30:617-625
18. Smith TK, Bader DM. Signals from both sides: Control of cardiac development by the endocardium and epicardium. *Semin Cell Dev Biol*. 2007;18:84-89

19. Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell*. 2009;16:233-244
20. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995;81:323-330
21. Sherr CJ, Roberts JM. Cdk inhibitors: Positive and negative regulators of g1-phase progression. *Genes Dev*. 1999;13:1501-1512
22. Kang JO, Sucov HM. Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signaling in fetal heart development. *Mech Dev*. 2005;122:57-65
23. Tseng YT, Yano N, Rojan A, Stabila JP, McGonnigal BG, Ianus V, Wadhawan R, Padbury JF. Ontogeny of phosphoinositide 3-kinase (pi3k) signaling in developing heart: Effect of acute {beta}-adrenergic stimulation. *Am J Physiol Heart Circ Physiol*. 2005;8:8
24. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin d1 proteolysis and subcellular localization. *Genes Dev*. 1998;12:3499-3511
25. Evans-Anderson HJ, Alfieri CM, Yutzey KE. Regulation of cardiomyocyte proliferation and myocardial growth during development by foxo transcription factors. *Circ Res*. 2008;102:686-694
26. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin d1 expression is regulated positively by the p42/p44mapk and negatively by the p38/hogmapk pathway. *J Biol Chem*. 1996;271:20608-20616
27. Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous erk activation downregulates antiproliferative genes throughout g1 phase to allow cell-cycle progression. *Curr Biol*. 2006;16:1171-1182
28. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. Erk implication in cell cycle regulation. *Biochim Biophys Acta*. 2007;1773:1299-1310
29. Yoshida Y, Nakamura T, Komoda M, Satoh H, Suzuki T, Tsuzuku JK, Miyasaka T, Yoshida EH, Umemori H, Kunisaki RK, Tani K, Ishii S, Mori S, Suganuma M, Noda T, Yamamoto T. Mice lacking a transcriptional corepressor tob are predisposed to cancer. *Genes Dev*. 2003;17:1201-1206
30. Weitzman JB, Fiette L, Matsuo K, Yaniv M. Jund protects cells from p53-dependent senescence and apoptosis. *Mol Cell*. 2000;6:1109-1119
31. Lee JG, Kay EP. Pi 3-kinase/rac1 and erk1/2 regulate fgf-2-mediated cell proliferation through phosphorylation of p27 at ser10 by kis and at thr187 by cdc25a/cdk2. *Invest Ophthalmol Vis Sci*. 2011;52:417-426
32. Keenan SM, Bellone C, Baldassare JJ. Cyclin-dependent kinase 2 nucleocytoplasmic translocation is regulated by extracellular regulated kinase. *J Biol Chem*. 2001;276:22404-22409
33. Lents NH, Keenan SM, Bellone C, Baldassare JJ. Stimulation of the raf/mek/erk cascade is necessary and sufficient for activation and thr-160 phosphorylation of a nuclear-targeted cdk2. *J Biol Chem*. 2002;277:47469-47475
34. Kardami E. Stimulation and inhibition of cardiac myocyte proliferation in vitro. *Mol Cell Biochem*. 1990;92:129-135
35. Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, Kelly RA. Neuregulins promote survival and growth of cardiac myocytes. Persistence of erbb2 and erbb4 expression in neonatal and adult ventricular myocytes. *J Biol Chem*. 1998;273:10261-10269
36. Kuhn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, Arab S, Keating MT. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nat Med*. 2007;13:962-969

37. Bersell K, Arab S, Haring B, Kuhn B. Neuregulin1/erbb4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell*. 2009;138:257-270
38. Hertig CM, Kubalak SW, Wang Y, Chien KR. Synergistic roles of neuregulin-1 and insulin-like growth factor-i in activation of the phosphatidylinositol 3-kinase pathway and cardiac chamber morphogenesis. *J Biol Chem*. 1999;274:37362-37369
39. Liu Q, Yan H, Dawes NJ, Mottino GA, Frank JS, Zhu H. Insulin-like growth factor ii induces DNA synthesis in fetal ventricular myocytes in vitro. *Circ Res*. 1996;79:716-726
40. Li P, Cavallero S, Gu Y, Chen THP, Hughes J, Hassan AB, Bruning JC, Pashmforoush M, Sucov HM. Igf signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. *Development*. 2011;138:1795-1805
41. Sundgren NC, Giraud GD, Schultz JM, Lasarev MR, Stork PJ, Thornburg KL. Extracellular signal-regulated kinase and phosphoinositol-3 kinase mediate igf-1 induced proliferation of fetal sheep cardiomyocytes. *Am J Physiol*. 2003;285:R1481-1489
42. Tseng YT, Kopel R, Stabila JP, McGonnigal BG, Nguyen TT, Gruppuso PA, Padbury JF. Beta-adrenergic receptors (betaar) regulate cardiomyocyte proliferation during early postnatal life. *Faseb J*. 2001;15:1921-1926.
43. Levkau B, Schafers M, Wohlschlaeger J, von Wnuck Lipinski K, Keul P, Hermann S, Kawaguchi N, Kirchhof P, Fabritz L, Stypmann J, Stegger L, Flogel U, Schrader J, Fischer JW, Hsieh P, Ou YL, Mehrhof F, Tiemann K, Ghanem A, Matus M, Neumann J, Heusch G, Schmid KW, Conway EM, Baba HA. Survivin determines cardiac function by controlling total cardiomyocyte number. *Circulation*. 2008;117:1583-1593
44. Lens SM, Vader G, Medema RH. The case for survivin as mitotic regulator. *Curr Opin Cell Biol*. 2006;18:616-622
45. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, Martin JF. Hippo pathway inhibits wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science*. 2011;332:458-461
46. Morrison JL. Sheep models of intrauterine growth restriction: Fetal adaptations and consequences. *Clin Exp Pharmacol Physiol*. 2008;35:730-743
47. Owens JA, Kind KL, Carbone F, Robinson JS, Owens PC. Circulating insulin-like growth factors-i and -ii and substrates in fetal sheep following restriction of placental growth. *J Endocrinol*. 1994;140:5-13
48. Phillips ID, Simonetta G, Owens JA, Robinson JS, Clarke IJ, McMillen IC. Placental restriction alters the functional development of the pituitary-adrenal axis in the sheep fetus during late gestation. *Pediatr Res*. 1996;40:861-866
49. Simonetta G, Rourke AK, Owens JA, Robinson JS, McMillen IC. Impact of placental restriction on the development of the sympathoadrenal system. *Pediatr Res*. 1997;42:805-811
50. Danielson L, McMillen IC, Dyer JL, Morrison JL. Restriction of placental growth results in greater hypotensive response to alpha-adrenergic blockade in fetal sheep during late gestation. *J Physiol*. 2005;563:611-620
51. Murotsuki J, Challis JR, Han VK, Fraher LJ, Gagnon R. Chronic fetal placental embolization and hypoxemia cause hypertension and myocardial hypertrophy in fetal sheep. *Am J Physiol*. 1997;272:R201-207.
52. Regnault TR, de Vrijer B, Galan HL, Wilkening RB, Battaglia FC, Meschia G. Development and mechanisms of fetal hypoxia in severe fetal growth restriction. *Placenta*. 2007;28:714-723
53. Galan HL, Anthony RV, Rigano S, Parker TA, de Vrijer B, Ferrazzi E, Wilkening RB, Regnault TR. Fetal hypertension and abnormal doppler velocimetry in an ovine model of intrauterine growth restriction. *Am J Obstet Gynecol*. 2005;192:272-279

54. Barbera A, Giraud GD, Reller MD, Maylie J, Morton MJ, Thornburg KL. Right ventricular systolic pressure load alters myocyte maturation in fetal sheep. *Am J Physiol*. 2000;279:R1157-1164
55. Jonker SS, Faber JJ, Anderson DF, Thornburg KL, Louey S, Giraud GD. Sequential growth of fetal sheep cardiac myocytes in response to simultaneous arterial and venous hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2006
56. Sundgren NC, Giraud GD, Stork PJ, Maylie JG, Thornburg KL. Angiotensin ii stimulates hyperplasia but not hypertrophy in immature ovine cardiomyocytes. *J Physiol*. 2003;548:881-891
57. Han HJ, Han JY, Heo JS, Lee SH, Lee MY, Kim YH. Ang ii-stimulated DNA synthesis is mediated by ang ii receptor-dependent ca(2+)/pkc as well as egf receptor-dependent pi3k/akt/mtor/p70s6k1 signal pathways in mouse embryonic stem cells. *J Cell Physiol*. 2007;211:618-629
58. Giraud GD, Louey S, Jonker S, Schultz J, Thornburg KL. Cortisol stimulates cell cycle activity in the cardiomyocyte of the sheep fetus. *Endocrinology*. 2006;147:3643-3649
59. Rudolph AM, Roman C, Gournay V. Perinatal myocardial DNA and protein changes in the lamb: Effect of cortisol in the fetus. *Pediatr Res*. 1999;46:141-146
60. Jonker SS, Scholz TD, Segar JL. The effect of adrenalectomy on the cardiac response to subacute fetal anemia. *Can J Physiol Pharmacol*. 2011;89:79-88
61. O'Tierney PF, Anderson DF, Faber JJ, Louey S, Thornburg KL, Giraud GD. Reduced systolic pressure load decreases cell-cycle activity in the fetal sheep heart. *Am J Physiol Regul Integr Comp Physiol*. 2010;299:R573-578
62. Chattergoon NN, Giraud GD, Thornburg KL. Thyroid hormone inhibits proliferation of fetal cardiac myocytes in vitro. *J Endocrinol*. 2007;192:R1-8
63. O'Tierney PF, Chattergoon NN, Louey S, Giraud GD, Thornburg KL. Atrial natriuretic peptide inhibits angiotensin ii-stimulated proliferation in fetal cardiomyocytes. *J Physiol*. 2010;588:2879-2889
64. Poelmann RE, Gittenberger-de Groot AC. Apoptosis as an instrument in cardiovascular development. *Birth Defects Res C Embryo Today*. 2005;75:305-313
65. Porrello ER, Widdop RE, Delbridge LM. Early origins of cardiac hypertrophy: Does cardiomyocyte attrition programme for pathological 'catch-up' growth of the heart? *Clin Exp Pharmacol Physiol*. 2008;35:1358-1364
66. Gustafsson AB, Gottlieb RA. Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol*. 2007;292:C45-51
67. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res*. 2000;45:528-537
68. van Heerde WL, Robert-Offerman S, Dumont E, Hofstra L, Doevendans PA, Smits JF, Daemen MJ, Reutelingsperger CP. Markers of apoptosis in cardiovascular tissues: Focus on annexin v. *Cardiovasc Res*. 2000;45:549-559
69. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of bid by caspase 8 mediates the mitochondrial damage in the fas pathway of apoptosis. *Cell*. 1998;94:491-501
70. Xiao Y, Xiao D, He J, Zhang L. Maternal cocaine administration during pregnancy induces apoptosis in fetal rat heart. *J Cardiovasc Pharmacol*. 2001;37:639-648
71. Bae S, Xiao Y, Li G, Casiano CA, Zhang L. Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. *Am J Physiol Heart Circ Physiol*. 2003;285:H983-990
72. Kajstura J, Mansukhani M, Cheng W, Reiss K, Krajewski S, Reed JC, Quaini F, Sonnenblick EH, Anversa P. Programmed cell death and expression of the protooncogene bcl-2 in myocytes during postnatal maturation of the heart. *Exp Cell Res*. 1995;219:110-121

73. Smolich JJ. Ultrastructural and functional features of the developing mammalian heart: A brief overview. *Reprod Fertil Dev*. 1995;7:451-461
74. Li F, Wang X, Capasso JM, Gerdes AM. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol*. 1996;28:1737-1746
75. Burrell JH, Boyn AM, Kumarasamy V, Hsieh A, Head SI, Lumbers ER. Growth and maturation of cardiac myocytes in fetal sheep in the second half of gestation. *Anat Rec A Discov Mol Cell Evol Biol*. 2003;274:952-961
76. Jonker SS, Zhang L, Louey S, Giraud GD, Thornburg KL, Faber JJ. Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart. *J Appl Physiol*. 2006
77. Schmid G, Pfitzer P. Mitoses and binucleated cells in perinatal human hearts. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1985;48:59-67
78. Kim HD, Kim DJ, Lee IJ, Rah BJ, Sawa Y, Schaper J. Human fetal heart development after mid-term: Morphometry and ultrastructural study. *J Mol Cell Cardiol*. 1992;24:949-965
79. Clubb FJ, Jr., Bishop SP. Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest*. 1984;50:571-577
80. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. *Science*. 2011;331:1078-1080
81. Poolman RA, Brooks G. Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy. *J Mol Cell Cardiol*. 1998;30:2121-2135
82. Di Stefano V, Giacca M, Capogrossi MC, Crescenzi M, Martelli F. Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. *J Biol Chem*. 2011;286:8644-8654
83. Soonpaa MH, Koh GY, Pajak L, Jing S, Wang H, Franklin MT, Kim KK, Field LJ. Cyclin d1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. *J Clin Invest*. 1997;99:2644-2654
84. Liu Z, Yue S, Chen X, Kubin T, Braun T. Regulation of cardiomyocyte polyploidy and multinucleation by cycling1. *Circ Res*. 2010;106:1498-1506
85. Naqvi N, Li M, Yahiro E, Graham RM, Husain A. Insights into the characteristics of mammalian cardiomyocyte terminal differentiation shown through the study of mice with a dysfunctional c-kit. *Pediatr Cardiol*. 2009;30:651-658
86. Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW, 2nd, van Rooij E, Olson EN. Mir-15 family regulates postnatal mitotic arrest of cardiomyocytes. *Circ Res*. 2011;109:670-679
87. Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle regulation. *Circ Res*. 2002;90:1044-1054
88. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med*. 2001;344:1750-1757
89. Engel FB, Schebesta M, Duong MT, Lu G, Ren S, Madwed JB, Jiang H, Wang Y, Keating MT. P38 map kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev*. 2005;19:1175-1187
90. Engel FB, Schebesta M, Keating MT. Anillin localization defect in cardiomyocyte binucleation. *J Mol Cell Cardiol*. 2006;41:601-612
91. Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. *J Exp Zool*. 1974;187:249-253
92. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science*. 2002;298:2188-2190

93. Posterino GS, Dunn SL, Botting KJ, Wang W, Gentili S, Morrison JL. Changes in cardiac troponins with gestational age explain changes in cardiac muscle contractility in the sheep fetus. *J Appl Physiol*. 2011
94. Olivetti G, Cigola E, Maestri R, Corradi D, Lagrasta C, Gambert SR, Anversa P. Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. *J Mol Cell Cardiol.* 1996;28:1463-1477.
95. Brodsky V, Chernyaev AL, Vasilyeva IA. Variability of the cardiomyocyte ploidy in normal human hearts. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1991;61:289-294
96. Kellerman S, Moore JA, Zierhut W, Zimmer HG, Campbell J, Gerdes AM. Nuclear DNA content and nucleation patterns in rat cardiac myocytes from different models of cardiac hypertrophy. *J Mol Cell Cardiol*. 1992;24:497-505
97. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am J Physiol.* 1996;271:H2183-2189.
98. Li F, McNelis MR, Lustig K, Gerdes AM. Hyperplasia and hypertrophy of chicken cardiac myocytes during posthatching development. *Am J Physiol*. 1997;273:R518-526
99. Grabner W, Pfitzer P. Number of nuclei in isolated myocardial cells of pigs. *Virchows Arch B Cell Pathol*. 1974;15:279-294
100. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation*. 2007;115:896-908
101. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. *Proc Natl Acad Sci U S A*. 2007;104:14068-14073
102. Itzhaki-Alfia A, Leor J, Raanani E, Sternik L, Spiegelstein D, Netser S, Holbova R, Pevsner-Fischer M, Lavee J, Barbash IM. Patient characteristics and cell source determine the number of isolated human cardiac progenitor cells. *Circulation*. 2009;120:2559-2566
103. Kajstura J, Gurusamy N, Ogorek B, Goichberg P, Clavo-Rondon C, Hosoda T, D'Amario D, Bardelli S, Beltrami AP, Cesselli D, Bussani R, del Monte F, Quaini F, Rota M, Beltrami CA, Buchholz BA, Leri A, Anversa P. Myocyte turnover in the aging human heart. *Circ Res*. 2010;107:1374-1386
104. Porrello ER, Olson EN. Building a new heart from old parts: Stem cell turnover in the aging heart. *Circ Res*. 2010;107:1292-1294
105. Adler CP. Polyploidization and augmentation of heart muscle cells during normal cardiac growth and in cardiac hypertrophy. In: Oberpriller JO, Oberpriller JC, Mauro A, eds. *The development and regenerative potential of cardiac muscle*. New York: Harwood Academic Publishers; 1991:227–252.
106. Sandritter W, Scomazzoni G. Deoxyribonucleic acid content (feulgen photometry) and dry weight (interference microscopy) of normal and hypertrophic heart muscle fibers. *Nature*. 1964;202:100-101
107. Adler CP, Neuburger M, Herget GW, Muhlbach D. Regeneration processes in human myocardium after acute ischaemia--quantitative determination of DNA, cell number and collagen content. *Virchows Arch*. 1997;430:149-153
108. Yan SM, Finato N, Di Loreto C, Beltrami CA. Nuclear size of myocardial cells in end-stage cardiomyopathies. *Anal Quant Cytol Histol*. 1999;21:174-180

109. Bensley JG, Stacy VK, De Matteo R, Harding R, Black MJ. Cardiac remodelling as a result of pre-term birth: Implications for future cardiovascular disease. *Eur Heart J*. 2010;31:2058-2066
110. Anatskaya OV, Sidorenko NV, Beyer TV, Vinogradov AE. Neonatal cardiomyocyte ploidy reveals critical windows of heart development. *Int J Cardiol*. 2010;141:81-91
111. Corstius HB, Zimanyi MA, Maka N, Herath T, Thomas W, van der Laarse A, Wreford NG, Black MJ. Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatr Res.* 2005;57:796-800. Epub 2005 Mar 2017.
112. Lim K, Zimanyi MA, Black MJ. Effect of maternal protein restriction during pregnancy and lactation on the number of cardiomyocytes in the postproliferative weanling rat heart. *Anat Rec (Hoboken)*. 2010;293:431-437
113. Wlodek ME, Mibus A, Tan A, Siebel AL, Owens JA, Moritz KM. Normal lactational environment restores nephron endowment and prevents hypertension after placental restriction in the rat. *J Am Soc Nephrol*. 2007;18:1688-1696
114. Li G, Bae S, Zhang L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol*. 2004;286:H1712-1719
115. Li G, Xiao Y, Estrella JL, Ducsay CA, Gilbert RD, Zhang L. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig*. 2003;10:265-274
116. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253
117. Hauton D, Ousley V. Prenatal hypoxia induces increased cardiac contractility on a background of decreased capillary density. *BMC Cardiovasc Disord*. 2009;9:1
118. Harrap SB, Danes VR, Ellis JA, Griffiths CD, Jones EF, Delbridge LM. The hypertrophic heart rat: A new normotensive model of genetic cardiac and cardiomyocyte hypertrophy. *Physiol Genomics*. 2002;9:43-48.
119. Porrello ER, Bell JR, Schertzer JD, Curl CL, McMullen JR, Mellor KM, Ritchie RH, Lynch GS, Harrap SB, Thomas WG, Delbridge LM. Heritable pathologic cardiac hypertrophy in adulthood is preceded by neonatal cardiac growth restriction. *Am J Physiol Regul Integr Comp Physiol.* 2009;296:R672-680. doi: 610.1152/ajpregu.90919.92008. Epub 92009 Jan 90917.
120. Stacy V, De Matteo R, Brew N, Sozo F, Probyn ME, Harding R, Black MJ. The influence of naturally occurring differences in birthweight on ventricular cardiomyocyte number in sheep. *Anat Rec (Hoboken)*. 2009;292:29-37
121. Jonker SS, Giraud MK, Giraud GD, Chattergoon NN, Louey S, Davis LE, Faber JJ, Thornburg KL. Cardiomyocyte enlargement, proliferation and maturation during chronic fetal anaemia in sheep. *Exp Physiol*. 2010;95:131-139
122. Yang Q, Hohimer AR, Giraud GD, Van Winkle DM, Underwood MJ, He GW, Davis LE. Effect of fetal anaemia on myocardial ischaemia-reperfusion injury and coronary vasoreactivity in adult sheep. *Acta Physiol (Oxf)*. 2008;194:325-334
123. Morrison JL, Botting KJ, Dyer JL, Williams SJ, Thornburg KL, McMillen IC. Restriction of placental function alters heart development in the sheep fetus. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R306-313
124. Louey S, Jonker SS, Giraud GD, Thornburg KL. Placental insufficiency decreases cell cycle activity and terminal maturation in fetal sheep cardiomyocytes. *J Physiol*. 2007
125. Bubb KJ, Cock ML, Black MJ, Dodic M, Boon WM, Parkington HC, Harding R, Tare M. Intrauterine growth restriction delays cardiomyocyte maturation and alters coronary artery function in the fetal sheep. *J Physiol*. 2007;578:871-881
126. Thornburg KL, Louey S, Giraud GD. The role of growth in heart development. *Nestle Nutr Workshop Ser Pediatr Program*. 2008;61:39-51

127. Chan LL, Sebert SP, Hyatt MA, Stephenson T, Budge H, Symonds ME, Gardner DS. Effect of maternal nutrient restriction from early to midgestation on cardiac function and metabolism after adolescent-onset obesity. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:R1455-1463
128. Tappia PS, Nijjar MS, Mahay A, Aroutiounova N, Dhalla NS. Phospholipid profile of developing heart of rats exposed to low-protein diet in pregnancy. *Am J Physiol Regul Integr Comp Physiol*. 2005;289:R1400-1406
129. Tintu A, Rouwet E, Verlohren S, Brinkmann J, Ahmad S, Crispi F, van Bilsen M, Carmeliet P, Staff AC, Tjwa M, Cetin I, Gratacos E, Hernandez-Andrade E, Hofstra L, Jacobs M, Lamers WH, Morano I, Safak E, Ahmed A, le Noble F. Hypoxia induces dilated cardiomyopathy in the chick embryo: Mechanism, intervention, and long-term consequences. *PLoS One*. 2009;4:e5155
130. Patterson AJ, Chen M, Xue Q, Xiao D, Zhang L. Chronic prenatal hypoxia induces epigenetic programming of pkc{epsilon} gene repression in rat hearts. *Circ Res*. 2010;107:365-373
131. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: Role of protein kinase c epsilon. *J Pharmacol Exp Ther*. 2009;330:624-632
132. Battista MC, Calvo E, Chorvatova A, Comte B, Corbeil J, Brochu M. Intra-uterine growth restriction and the programming of left ventricular remodelling in female rats. *J Physiol.* 2005;565:197-205. Epub 2005 Mar 2017.
133. The shifting burden of cardiovascular disease in australia. 2005:103
134. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med*. 2006;3:e442
135. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. *Physiol Rev*. 2005;85:571-633
136. Fall CH, Vijayakumar M, Barker DJ, Osmond C, Duggleby S. Weight in infancy and prevalence of coronary heart disease in adult life. *Bmj*. 1995;310:17-19
137. Barker DJ, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Trajectories of growth among children who have coronary events as adults. *N Engl J Med*. 2005;353:1802-1809
138. Barker DJ, Eriksson JG, Forsen T, Osmond C. Fetal origins of adult disease: Strength of effects and biological basis. *Int J Epidemiol*. 2002;31:1235-1239
139. Laws PJ, Sullivan EA. Australia's mothers and babies 2002. *PERINATAL STATISTICS SERIES*. 2004:1-93
140. Li Z, Zeki R, Hilder L, Sullivan E. Australia's mothers and babies 2010. *PERINATAL STATISTICS SERIES*. 2012:1-122
141. Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaidy N, Patel FA, Whittle W, Fraser M, Moss TJ, Newnham J. The fetal placental hypothalamic-pituitary-adrenal (hpa) axis, parturition and post natal health. *Mol Cell Endocrinol*. 2001;185:135-144
142. Ghidini A. Idiopathic fetal growth restriction: A pathophysiologic approach. *Obstet Gynecol Surv*. 1996;51:376-382
143. McMillen IC, Adams MB, Ross JT, Coulter CL, Simonetta G, Owens JA, Robinson JS, Edwards LJ. Fetal growth restriction: Adaptations and consequences. *Reprod*. 2001;122:195-204
144. Wang KC, Botting KJ, Padhee M, Zhang S, McMillen IC, Suter CM, Brooks DA, Morrison JL. Early origins of heart disease: Low birth weight and the role of the insulin-like growth factor system in cardiac hypertrophy. *Clin Exp Pharmacol Physiol*. 2012;39:958-964
145. Wang KC, Lim CH, McMillen IC, Duffield JA, Brooks DA, Morrison JL. Alteration of cardiac glucose metabolism in association to low birth weight: Experimental evidence in lambs with left ventricular hypertrophy. *Metabolism*. 2013;5:00194-00197

146. Neubauer S. The failing heart--an engine out of fuel. *N Engl J Med*. 2007;356:1140-1151
147. Neely JR, Morgan HE. Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annu Rev Physiol*. 1974;36:413-459
148. Lopaschuk GD, Jaswal JS. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol*. 2010;56:130-140
149. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev*. 2005;85:1093-1129
150. Gimeno RE, Ortegon AM, Patel S, Punreddy S, Ge P, Sun Y, Lodish HF, Stahl A. Characterization of a heart-specific fatty acid transport protein. *J Biol Chem*. 2003;278:16039-16044
151. Heather LC, Cole MA, Lygate CA, Evans RD, Stuckey DJ, Murray AJ, Neubauer S, Clarke K. Fatty acid transporter levels and palmitate oxidation rate correlate with ejection fraction in the infarcted rat heart. *Cardiovasc Res*. 2006;72:430-437
152. Binas B, Danneberg H, McWhir J, Mullins L, Clark AJ. Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. *FASEB J*. 1999;13:805-812
153. Brown NF, Weis BC, Husti JE, Foster DW, McGarry JD. Mitochondrial carnitine palmitoyltransferase i isoform switching in the developing rat heart. *J Biol Chem*. 1995;270:8952-8957
154. Cook GA, Edwards TL, Jansen MS, Bahouth SW, Wilcox HG, Park EA. Differential regulation of carnitine palmitoyltransferase-i gene isoforms (cpt-i alpha and cpt-i beta) in the rat heart. *J Mol Cell Cardiol.* 2001;33:317-329.
155. Jaswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD. Targeting fatty acid and carbohydrate oxidation--a novel therapeutic intervention in the ischemic and failing heart. *Biochim Biophys Acta*. 2011;1813:1333-1350
156. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc Natl Acad Sci U S A.* 1994;91:11012-11016.
157. Brandt JM, Djouadi F, Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase i gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. *J Biol Chem*. 1998;273:23786-23792
158. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocr Rev*. 1999;20:649-688
159. Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (ppars): Tissue distribution of ppar-alpha, -beta, and -gamma in the adult rat. *Endocrinology*. 1996;137:354-366
160. Issemann I, Prince RA, Tugwood JD, Green S. The retinoid x receptor enhances the function of the peroxisome proliferator activated receptor. *Biochimie*. 1993;75:251-256
161. Campbell FM, Kozak R, Wagner A, Altarejos JY, Dyck JR, Belke DD, Severson DL, Kelly DP, Lopaschuk GD. A role for peroxisome proliferator-activated receptor alpha (pparalpha) in the control of cardiac malonyl-coa levels: Reduced fatty acid oxidation rates and increased glucose oxidation rates in the hearts of mice lacking pparalpha are associated with higher concentrations of malonyl-coa and reduced expression of malonyl-coa decarboxylase. *J Biol Chem*. 2002;277:4098-4103
162. Barger PM, Kelly DP. Ppar signaling in the control of cardiac energy metabolism. *Trends Cardiovasc Med*. 2000;10:238-245
163. Finck BN. The ppar regulatory system in cardiac physiology and disease. *Cardiovasc Res*. 2007;73:269-277

164. Kalinowska A, Gorski J, Harasim E, Harasiuk D, Bonen A, Chabowski A. Differential effects of chronic, in vivo, ppar's stimulation on the myocardial subcellular redistribution of fat/cd36 and fabppm. *FEBS Lett*. 2009;583:2527-2534
165. Leone TC, Weinheimer CJ, Kelly DP. A critical role for the peroxisome proliferator-activated receptor alpha (pparalpha) in the cellular fasting response: The pparalpha-null mouse as a model of fatty acid oxidation disorders. *Proc Natl Acad Sci U S A*. 1999;96:7473-7478
166. Asrih M, Lerch R, Papageorgiou I, Pellieux C, Montessuit C. Differential regulation of stimulated glucose transport by free fatty acids and pparalpha or -delta agonists in cardiac myocytes. *Am J Physiol Endocrinol Metab*. 2012;302:E872-884
167. Gilde AJ, van der Lee KA, Willemsen PH, Chinetti G, van der Leij FR, van der Vusse GJ, Staels B, van Bilsen M. Peroxisome proliferator-activated receptor (ppar) alpha and pparbeta/delta, but not ppargamma, modulate the expression of genes involved in cardiac lipid metabolism. *Circ Res*. 2003;92:518-524
168. Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y, Chen YE, Yang Q. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. *Nat Med*. 2004;10:1245-1250
169. Barak Y, Liao D, He W, Ong ES, Nelson MC, Olefsky JM, Boland R, Evans RM. Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc Natl Acad Sci U S A*. 2002;99:303-308
170. Jeremy RW, Koretsune Y, Marban E, Becker LC. Relation between glycolysis and calcium homeostasis in postischemic myocardium. *Circ Res*. 1992;70:1180-1190
171. Jeremy RW, Ambrosio G, Pike MM, Jacobus WE, Becker LC. The functional recovery of post-ischemic myocardium requires glycolysis during early reperfusion. *J Mol Cell Cardiol*. 1993;25:261-276
172. Kockskamper J, Zima AV, Blatter LA. Modulation of sarcoplasmic reticulum ca2+ release by glycolysis in cat atrial myocytes. *J Physiol*. 2005;564:697-714
173. Montessuit C, Lerch R. Regulation and dysregulation of glucose transport in cardiomyocytes. *Biochim Biophys Acta*. 2013;1833:848-856
174. Hool LC. What cardiologists should know about calcium ion channels and their regulation by reactive oxygen species. *Heart Lung Circ*. 2007;16:361-372
175. Wheeler TJ, Fell RD, Hauck MA. Translocation of two glucose transporters in heart: Effects of rotenone, uncouplers, workload, palmitate, insulin and anoxia. *Biochim Biophys Acta*. 1994;1196:191-200
176. Russell RR, 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial glut-4 and increased glucose uptake through activation of ampk by aicar. *Am J Physiol*. 1999;277:H643-649
177. Egert S, Nguyen N, Schwaiger M. Contribution of alpha-adrenergic and beta-adrenergic stimulation to ischemia-induced glucose transporter (glut) 4 and glut1 translocation in the isolated perfused rat heart. *Circ Res*. 1999;84:1407-1415
178. Sun D, Nguyen N, DeGrado TR, Schwaiger M, Brosius FC, 3rd. Ischemia induces translocation of the insulin-responsive glucose transporter glut4 to the plasma membrane of cardiac myocytes. *Circulation*. 1994;89:793-798
179. Egert S, Nguyen N, Schwaiger M. Myocardial glucose transporter glut1: Translocation induced by insulin and ischemia. *J Mol Cell Cardiol*. 1999;31:1337-1344
180. Dale E, Abel. Glucose transport in the heart. *Frontiers in Bioscience*. 2004;9:201-215
181. Lopaschuk GD, Witters LA, Itoi T, Barr R, Barr A. Acetyl-coa carboxylase involvement in the rapid maturation of fatty acid oxidation in the newborn rabbit heart. *The Journal of biological chemistry*. 1994;269:25871-25878

182. Bianchi A, Evans JL, Iverson AJ, Nordlund AC, Watts TD, Witters LA. Identification of an isozymic form of acetyl-coa carboxylase. *The Journal of biological chemistry*. 1990;265:1502-1509
183. Thampy KG. Formation of malonyl coenzyme a in rat heart. Identification and purification of an isozyme of a carboxylase from rat heart. *The Journal of biological chemistry*. 1989;264:17631
184. Saddik M, Gamble J, Witters LA, Lopaschuk GD. Acetyl-coa carboxylase regulation of fatty acid oxidation in the heart. *The Journal of biological chemistry*. 1993;268:25836-25845
185. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD. Characterization of 5'amp-activated protein kinase activity in the heart and its role in inhibiting acetyl-coa carboxylase during reperfusion following ischemia. *Biochim Biophys Acta*. 1996;1301:67-75
186. Chabowski A, Momken I, Coort SL, Calles-Escandon J, Tandon NN, Glatz JF, Luiken JJ, Bonen A. Prolonged ampk activation increases the expression of fatty acid transporters in cardiac myocytes and perfused hearts. *Mol Cell Biochem*. 2006;288:201-212
187. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart pfk-2 by ampk has a role in the stimulation of glycolysis during ischaemia. *Curr Biol*. 2000;10:1247-1255
188. Hardie DG. Minireview: The amp-activated protein kinase cascade: The key sensor of cellular energy status. *Endocrinology*. 2003;144:5179-5183
189. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet*. 1963;1:785-789
190. Hue L, Taegtmeyer H. The randle cycle revisited: A new head for an old hat. *Am J Physiol Endocrinol Metab*. 2009;297:E578-591
191. Garland PB, Newsholme EA, Randle PJ. Effect of fatty acids, ketone bodies, diabetes and starvation on pyruvate metabolism in rat heart and diaphragm muscle. *Nature*. 1962;195:381-383.
192. Garland PB, Randle PJ, Newsholme EA. Citrate as an intermediary in the inhibition of phosphofructokinase in rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes, and starvation. *Nature*. 1963;200:169-170.
193. Poirier M, Vincent G, Reszko AE, Bouchard B, Kelleher JK, Brunengraber H, Des Rosiers C. Probing the link between citrate and malonyl-coa in perfused rat hearts. *Am J Physiol Heart Circ Physiol*. 2002;283:H1379-1386. Epub 2002 Jun 1313.
194. Goodwin GW, Taylor CS, Taegtmeyer H. Regulation of energy metabolism of the heart during acute increase in heart work. *J Biol Chem*. 1998;273:29530-29539.
195. Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with niddm. *Diabetes*. 1988;37:1020-1024.
196. Heather LC, Clarke K. Metabolism, hypoxia and the diabetic heart. *J Mol Cell Cardiol*. 2011;50:598-605. doi: 510.1016/j.yjmcc.2011.1001.1007. Epub 2011 Jan 1022.
197. Slater-Jefferies JL, Lillycrop KA, Townsend PA, Torrens C, Hoile SP, Hanson MA, Burdge GC. Feeding a protein-restricted diet during pregnancy induces altered epigenetic regulation of peroxisomal proliferator-activated receptor-alpha in the heart of the offspring. *J Dev Orig Health Dis*. 2011;2:250-255
198. Rueda-Clausen CF, Morton JS, Lopaschuk GD, Davidge ST. Long-term effects of intrauterine growth restriction on cardiac metabolism and susceptibility to ischaemia/reperfusion. *Cardiovasc Res*. 2011;90:285-294

199. Hata K, Takasago T, Saeki A, Nishioka T, Goto Y. Stunned myocardium after rapid correction of acidosis. Increased oxygen cost of contractility and the role of the na(+)-h+ exchange system. *Circ Res*. 1994;74:794-805
200. Kihara Y, Sasayama S, Inoko M, Morgan JP. Sodium/calcium exchange modulates intracellular calcium overload during posthypoxic reoxygenation in mammalian working myocardium. Evidence from aequorin-loaded ferret ventricular muscles. *J Clin Invest*. 1994;93:1275-1284
201. Talukder MA, Kalyanasundaram A, Zhao X, Zuo L, Bhupathy P, Babu GJ, Cardounel AJ, Periasamy M, Zweier JL. Expression of serca isoform with faster ca2+ transport properties improves postischemic cardiac function and ca2+ handling and decreases myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2007;293:H2418-2428
202. Rueda-Clausen CF, Morton JS, Dolinsky VW, Dyck JR, Davidge ST. Synergistic effects of prenatal hypoxia and postnatal high-fat diet in the development of cardiovascular pathology in young rats. *Am J Physiol Regul Integr Comp Physiol*. 2012;303:R418-426
203. Wang KC, Zhang L, McMillen IC, Botting KJ, Duffield JA, Zhang S, Suter CM, Brooks DA, Morrison JL. Fetal growth restriction and the programming of heart growth and cardiac insulin-like growth factor 2 expression in the lamb. *J Physiol*. 2011;589:4709-4722
204. Heilbronn L, Smith SR, Ravussin E. Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type ii diabetes mellitus. *Int J Obes Relat Metab Disord.* 2004;28:S12-21.
205. Frayn KN, Fielding BA, Karpe F. Adipose tissue fatty acid metabolism and cardiovascular disease. *Curr Opin Lipidol.* 2005;16:409-415.
206. Shulman GI. Cellular mechanisms of insulin resistance. *J Clin Invest.* 2000;106:171-176.
207. Schwenk RW, Luiken JJ, Bonen A, Glatz JF. Regulation of sarcolemmal glucose and fatty acid transporters in cardiac disease. *Cardiovasc Res*. 2008;79:249-258
208. Palanivel R, Vu V, Park M, Fang X, Sweeney G. Differential impact of adipokines derived from primary adipocytes of wild-type versus streptozotocin-induced diabetic rats on glucose and fatty acid metabolism in cardiomyocytes. *J Endocrinol.* 2008;199:389-397. doi: 310.1677/JOE-1608-0336. Epub 2008 Sep 1611.
209. Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through ampk- and cox-2-dependent mechanisms. *Nat Med*. 2005;11:1096-1103
210. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med.* 1996;334:292-295.
211. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med.* 2001;7:941-946.
212. Leifheit-Nestler M, Wagner NM, Gogiraju R, Didie M, Konstantinides S, Hasenfuss G, Schafer K. Importance of leptin signaling and signal transducer and activator of transcription-3 activation in mediating the cardiac hypertrophy associated with obesity. *J Transl Med.* 2013;11:170.:10.1186/1479-5876-1111-1170.
213. Woodiwiss AJ, Libhaber CD, Majane OH, Libhaber E, Maseko M, Norton GR. Obesity promotes left ventricular concentric rather than eccentric geometric remodeling and hypertrophy independent of blood pressure. *Am J Hypertens.* 2008;21:1144-1151. doi: 1110.1038/ajh.2008.1252. Epub 2008 Aug 1128.

214. Shibata R, Izumiya Y, Sato K, Papanicolaou K, Kihara S, Colucci WS, Sam F, Ouchi N, Walsh K. Adiponectin protects against the development of systolic dysfunction following myocardial infarction. *J Mol Cell Cardiol.* 2007;42:1065-1074. Epub 2007 Mar 1020.
215. Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel DR, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci WS, Walsh K. Adiponectin-mediated modulation of hypertrophic signals in the heart. *Nat Med.* 2004;10:1384-1389. Epub 2004 Nov 1321.
216. Shibata R, Ouchi N, Murohara T. Adiponectin and cardiovascular disease. *Circ J.* 2009;73:608-614. Epub 2009 Mar 2003.
217. Ganau A, Devereux RB, Pickering TG, Roman MJ, Schnall PL, Santucci S, Spitzer MC, Laragh JH. Relation of left ventricular hemodynamic load and contractile performance to left ventricular mass in hypertension. *Circulation.* 1990;81:25-36.
218. Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, Quignard-Boulange A, Negrel R, Ailhaud G, Seydoux J, Meneton P, Teboul M. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J.* 2001;15:2727-2729. Epub 2001 Oct 2715.
219. Ruano M, Silvestre V, Castro R, Garcia-Lescun MC, Rodriguez A, Marco A, Garcia-Blanch G. Morbid obesity, hypertensive disease and the renin-angiotensin-aldosterone axis. *Obes Surg.* 2005;15:670-676.
220. Yiannikouris F, Karounos M, Charnigo R, English VL, Rateri DL, Daugherty A, Cassis LA. Adipocyte-specific deficiency of angiotensinogen decreases plasma angiotensinogen concentration and systolic blood pressure in mice. *Am J Physiol Regul Integr Comp Physiol.* 2012;302:R244-251. doi: 210.1152/ajpregu.00323.02011. Epub 02011 Nov 00329.
221. Cetin I, Morpurgo PS, Radaelli T, Taricco E, Cortelazzi D, Bellotti M, Pardi G, Beck-Peccoz P. Fetal plasma leptin concentrations: Relationship with different intrauterine growth patterns from 19 weeks to term. *Pediatr Res.* 2000;48:646-651.
222. Jaquet D, Leger J, Tabone MD, Czernichow P, Levy-Marchal C. High serum leptin concentrations during catch-up growth of children born with intrauterine growth retardation. *J Clin Endocrinol Metab.* 1999;84:1949-1953.
223. Martinez-Cordero C, Amador-Licona N, Guizar-Mendoza JM, Hernandez-Mendez J, Ruelas-Orozco G. Body fat at birth and cord blood levels of insulin, adiponectin, leptin, and insulin-like growth factor-i in small-for-gestational-age infants. *Arch Med Res.* 2006;37:490-494.
224. Sivan E, Mazaki-Tovi S, Pariente C, Efraty Y, Schiff E, Hemi R, Kanety H. Adiponectin in human cord blood: Relation to fetal birth weight and gender. *J Clin Endocrinol Metab.* 2003;88:5656-5660.
225. Strufaldi MW, Puccini RF, Silverio OM, do Pinho Franco MC. Association of adipokines with cardiovascular risk factors in low birth weight children: A case-control study. *Eur J Pediatr.* 2013;172:71-76. doi: 10.1007/s00431-00012-01846-x. Epub 02012 Sep 00427.
226. Economides DL, Nicolaides KH. Blood glucose and oxygen tension levels in small-for-gestational-age fetuses. *Am J Obstet Gynecol*. 1989;160:385-389
227. Economides DL, Nicolaides KH, Linton EA, Perry LA, Chard T. Plasma cortisol and adrenocorticotropin in appropriate and small for gestational age fetuses. *Fetal Ther*. 1988;3:158-164
228. Ostlund E, Bang P, Hagenas L, Fried G. Insulin-like growth factor i in fetal serum obtained by cordocentesis is correlated with intrauterine growth retardation. *Hum Reprod*. 1997;12:840-844
229. Orgeig S, Crittenden TA, Marchant C, McMillen IC, Morrison JL. Intrauterine growth restriction delays surfactant protein maturation in the sheep fetus. *Am J Physiol Lung*

Cell Mol Physiol. 2010;298:L575-583. doi: 510.1152/ajplung.00226.02009. Epub 02010 Jan 00222.

230. Owens JA, Kind KL, Carbone F, Robinson JS, Owens PC. Circulating insulin-like growth factors-i and -ii and substrates in fetal sheep following restriction of placental growth. *J Endocrinol.* 1994;140:5-13.
231. Duffield JA, Vuocolo T, Tellam R, Yuen BS, Muhlhausler BS, McMillen IC. Placental restriction of fetal growth decreases igf1 and leptin mrna expression in the perirenal adipose tissue of late gestation fetal sheep. *Am J Physiol Regul Integr Comp Physiol.* 2008;294:R1413-1419. doi: 1410.1152/ajpregu.00787.02007. Epub 02008 Feb 00713.
232. De Blasio MJ, Gatford KL, Robinson JS, Owens JA. Placental restriction of fetal growth reduces size at birth and alters postnatal growth, feeding activity, and adiposity in the young lamb. *Am J Physiol Regul Integr Comp Physiol.* 2007;292:R875-886. Epub 2006 Oct 2005.
233. De Blasio MJ, Gatford KL, McMillen IC, Robinson JS, Owens JA. Placental restriction of fetal growth increases insulin action, growth, and adiposity in the young lamb. *Endocrinology.* 2007;148:1350-1358. Epub 2006 Nov 1316.
234. De Blasio MJ, Gatford KL, Harland ML, Robinson JS, Owens JA. Placental restriction reduces insulin sensitivity and expression of insulin signaling and glucose transporter genes in skeletal muscle, but not liver, in young sheep. *Endocrinology.* 2012;153:2142-2151. doi: 2110.1210/en.2011-1955. Epub 2012 Mar 2120.
235. Kind KL, Clifton PM, Grant PA, Owens PC, Sohlstrom A, Roberts CT, Robinson JS, Owens JA. Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol*. 2003;284:R140-152
236. Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS, Owens JA. Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R119-126
237. Nguyen LT, Muhlhausler BS, Botting KJ, Morrison JL. Maternal undernutrition alters fat cell size distribution, but not lipogenic gene expression, in the visceral fat of the late gestation guinea pig fetus. *Placenta.* 2010;31:902-909. doi: 910.1016/j.placenta.2010.1007.1014. Epub 2010 Aug 1021.
238. Sarr O, Louveau I, Kalbe C, Metges CC, Rehfeldt C, Gondret F. Prenatal exposure to maternal low or high protein diets induces modest changes in the adipose tissue proteome of newborn piglets. *J Anim Sci.* 2010;88:1626-1641. doi: 1610.2527/jas.2009-2542. Epub 2010 Jan 1615.
239. Anagnostis P, Athyros VG, Tziomalos K, Karagiannis A, Mikhailidis DP. Clinical review: The pathogenetic role of cortisol in the metabolic syndrome: A hypothesis. *J Clin Endocrinol Metab.* 2009;94:2692-2701. doi: 2610.1210/jc.2009-0370. Epub 2009 May 2626.
240. Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. Fetal programming of hypothalamo-pituitary-adrenal function: Prenatal stress and glucocorticoids. *The Journal of Physiology*. 2006;572:31-44
241. Schoneveld OJ, Gaemers IC, Lamers WH. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta*. 2004;1680:114-128
242. Pivonello R, De Leo M, Vitale P, Cozzolino A, Simeoli C, De Martino MC, Lombardi G, Colao A. Pathophysiology of diabetes mellitus in cushing's syndrome. *Neuroendocrinology*. 2010;92:77-81. doi: 10.1159/000314319. Epub 000312010 Sep 000314310.
243. Gravholt CH, Dall R, Christiansen JS, Moller N, Schmitz O. Preferential stimulation of abdominal subcutaneous lipolysis after prednisolone exposure in humans. *Obes Res.* 2002;10:774-781.

244. Krsek M, Rosicka M, Nedvidkova J, Kvasnickova H, Hana V, Marek J, Haluzik M, Lai EW, Pacak K. Increased lipolysis of subcutaneous abdominal adipose tissue and altered noradrenergic activity in patients with cushing's syndrome: An in-vivo microdialysis study. *Physiol Res*. 2006;55:421-428. Epub 2005 Oct 2017.
245. Muiesan ML, Lupia M, Salvetti M, Grigoletto C, Sonino N, Boscaro M, Rosei EA, Mantero F, Fallo F. Left ventricular structural and functional characteristics in cushing's syndrome. *J Am Coll Cardiol.* 2003;41:2275-2279.
246. Pereira AM, Delgado V, Romijn JA, Smit JW, Bax JJ, Feelders RA. Cardiac dysfunction is reversed upon successful treatment of cushing's syndrome. *Eur J Endocrinol.* 2010;162:331-340. doi: 310.1530/EJE-1509-0621. Epub 2009 Nov 1523.
247. de Vries A, Holmes MC, Heijnis A, Seier JV, Heerden J, Louw J, Wolfe-Coote S, Meaney MJ, Levitt NS, Seckl JR. Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal axis function. *J Clin Invest.* 2007;117:1058-1067. Epub 2007 Mar 1022.
248. Levitt NS, Lindsay RS, Holmes MC, Seckl JR. Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology*. 1996;64:412-418
249. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. Epigenetic programming by maternal behavior. *Nat Neurosci.* 2004;7:847-854. Epub 2004 Jun 2027.
250. Phillips DI, Walker BR, Reynolds RM, Flanagan DE, Wood PJ, Osmond C, Barker DJ, Whorwood CB. Low birth weight predicts elevated plasma cortisol concentrations in adults from 3 populations. *Hypertension.* 2000;35:1301-1306.
251. Worthman CM, Kuzara J. Life history and the early origins of health differentials. *Am J Hum Biol*. 2005;17:95-112
252. Phillips DI. Programming of the stress response: A fundamental mechanism underlying the long-term effects of the fetal environment? *J Intern Med.* 2007;261:453-460.
253. Lesage J, Blondeau B, Grino M, Breant B, Dupouy JP. Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary adrenal axis in the newborn rat. *Endocrinology*. 2001;142:1692-1702
254. Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, Lye SJ, Gibb W, Challis JR. Glucocorticoid regulation of human and ovine parturition: The relationship between fetal hypothalamic-pituitary-adrenal axis activation and intrauterine prostaglandin production. *Biol Reprod*. 2001;64:1019-1032
255. Edwards LJ, McMillen IC. Impact of maternal undernutrition during the periconceptional period, fetal number, and fetal sex on the development of the hypothalamo-pituitary adrenal axis in sheep during late gestation. *Biol Reprod*. 2002;66:1562-1569
256. Edwards LJ, McMillen IC. Maternal undernutrition during the periconceptional period has long term effects on development of the fetal cardiovascular system in the sheep. *Am J Phyiol*. 2002;283:R669-679
257. MacLaughlin SM, Walker SK, Kleemann DO, Sibbons JP, Tosh DN, Gentili S, Coulter CL, McMillen IC. Impact of periconceptional undernutrition on adrenal growth and adrenal insulin-like growth factor and steroidogenic enzyme expression in the sheep fetus during early pregnancy. *Endocrinology*. 2007;148:1911-1920
258. Bloomfield FH, Oliver MH, Hawkins P, Holloway AC, Campbell M, Gluckman PD, Harding JE, Challis JR. Periconceptional undernutrition in sheep accelerates maturation of the fetal hpa axis in late gestation. *Endocrinology*. 2004;145:4278-4285

259. Bloomfield FH, Oliver MH, Hawkins P, Campbell M, Phillips DJ, Gluckman PD, Challis JR, Harding JE. A periconceptional nutritional origin for non-infectious preterm birth. *Science*. 2003;300:561-562
260. Chadio SE, Kotsampasi B, Papadomichelakis G, Deligeorgis S, Kalogiannis D, Menegatos I, Zervas G. Impact of maternal undernutrition on the hpa axis responsiveness in sheep at different ages postnatal. *J Endocrinol*. 2007;192:495-503
261. Gardner DS, Van Bon BW, Dandrea J, Goddard PJ, May SF, Wilson V, Stephenson T, Symonds ME. Effect of periconceptional undernutrition and gender on hypothalamic-pituitary-adrenal axis function in young adult sheep. *J Endocrinol*. 2006;190:203-212
262. Sutherland AE, Crossley KJ, Allison BJ, Jenkin G, Wallace EM, Miller SL. The effects of intrauterine growth restriction and antenatal glucocorticoids on ovine fetal lung development. *Pediatr Res*. 2012;71:689-696
263. Murotsuki J, Gagnon R, Matthews SG, Challis JR. Effects of long-term hypoxemia on pituitary-adrenal function in fetal sheep. *American Journal of Physiology - Endocrinology And Metabolism*. 1996;271:E678-E685
264. Louey S, Cock ML, Stevenson KM, Harding R. Placental insufficiency and fetal growth restriction lead to postnatal hypotension and altered postnatal growth in sheep. *Pediatr Res*. 2000;48:808-814
265. Myers DA, Ducsay CA. Adrenocortical and adipose responses to high-altitude-induced, long-term hypoxia in the ovine fetus. *J Pregnancy*. 2012;2012:681306.:10.1155/2012/681306. Epub 682012 May 681314.
266. Adachi K, Umezaki H, Kaushal KM, Ducsay CA. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *Am J Physiol Regul Integr Comp Physiol.* 2004;287:R209-217. Epub 2004 Mar 2011.
267. Imamura T, Umezaki H, Kaushal KM, Ducsay CA. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *J Soc Gynecol Investig.* 2004;11:131-140.
268. Li C, Ramahi E, Nijland MJ, Choi J, Myers DA, Nathanielsz PW, McDonald TJ. Up-regulation of the fetal baboon hypothalamo-pituitary-adrenal axis in intrauterine growth restriction: Coincidence with hypothalamic glucocorticoid receptor insensitivity and leptin receptor down-regulation. *Endocrinology*. 2013;26:26
269. Probyn ME, Stacy V, Desai M, Ross M, Harding R. Spontaneously occurring differences in fetal weight do not affect blood pressure, the hypothalamic–pituitary–adrenal axis or the renin–angiotensin system in the late-gestation ovine fetus. *Reproduction, Fertility and Development*. 2008;20:451-459
270. Wallace JM, Milne JS, Green LR, Aitken RP. Postnatal hypothalamic-pituitary-adrenal function in sheep is influenced by age and sex, but not by prenatal growth restriction. *Reprod Fertil Dev*. 2011;23:275-284
271. Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Whorwood CB, Phillips DIW. Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors. *Journal of Clinical Endocrinology & Metabolism*. 2001;86:245-250
272. van Montfoort N, Finken MJ, le Cessie S, Dekker FW, Wit JM. Could cortisol explain the association between birth weight and cardiovascular disease in later life? A meta-analysis. *Eur J Endocrinol*. 2005;153:811-817
273. Nickel A, Loffler J, Maack C. Myocardial energetics in heart failure. *Basic Res Cardiol.* 2013;108:358. doi: 310.1007/s00395-00013-00358-00399. Epub 02013 Jun 00396.
274. Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies. *Pharmacol Ther.* 2010;128:191-227. Epub 2010 May 2012.

275. Allard MF, Schonekess BO, Henning SL, English DR, Lopaschuk GD. Contribution of oxidative metabolism and glycolysis to atp production in hypertrophied hearts. *Am J Physiol.* 1994;267:H742-750.
276. Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. Identification of cd36 (fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet.* 1999;21:76-83.
277. van der Vusse GJ, van Bilsen M, Glatz JF. Cardiac fatty acid uptake and transport in health and disease. *Cardiovasc Res.* 2000;45:279-293.
278. Sack MN, Disch DL, Rockman HA, Kelly DP. A role for sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. *Proc Natl Acad Sci U S A*. 1997;94:6438-6443
279. El Alaoui-Talibi Z, Guendouz A, Moravec M, Moravec J. Control of oxidative metabolism in volume-overloaded rat hearts: Effect of propionyl-l-carnitine. *Am J Physiol.* 1997;272:H1615-1624.
280. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation.* 1996;94:2837-2842.
281. Young ME, Laws FA, Goodwin GW, Taegtmeyer H. Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart. *J Biol Chem.* 2001;276:44390-44395. Epub 42001 Sep 44326.
282. Nascimben L, Ingwall JS, Lorell BH, Pinz I, Schultz V, Tornheim K, Tian R. Mechanisms for increased glycolysis in the hypertrophied rat heart. *Hypertension.* 2004;44:662-667. Epub 2004 Oct 2004.
283. Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H. Metabolic gene expression in fetal and failing human heart. *Circulation.* 2001;104:2923-2931.
284. Gaasch WH, Zile MR, Hoshino PK, Weinberg EO, Rhodes DR, Apstein CS. Tolerance of the hypertrophic heart to ischemia. Studies in compensated and failing dog hearts with pressure overload hypertrophy. *Circulation.* 1990;81:1644-1653.
285. Lim K, Zimanyi MA, Black MJ. Effect of maternal protein restriction in rats on cardiac fibrosis and capillarization in adulthood. *Pediatr Res*. 2006;60:83-87
286. Rueda-Clausen CF, Morton JS, Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. *Cardiovasc Res*. 2009;81:713-722
287. D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, Dorn GW, 2nd. Transgenic galphaq overexpression induces cardiac contractile failure in mice. *Proc Natl Acad Sci U S A.* 1997;94:8121-8126.
288. Wang KC, Brooks DA, Botting KJ, Morrison JL. Igf-2r-mediated signaling results in hypertrophy of cultured cardiomyocytes from fetal sheep. *Biol Reprod*. 2012;86:183
289. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol.* 2010;25:669-677. doi: 610.1007/s00467-00009-01407-00463. Epub 02009 Dec 00422.
290. Moritz KM, Mazzuca MQ, Siebel AL, Mibus A, Arena D, Tare M, Owens JA, Wlodek ME. Uteroplacental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats. *J Physiol.* 2009;587:2635-2646. doi: 2610.1113/jphysiol.2009.170407. Epub 172009 Apr 170409.

291. Wlodek ME, Westcott K, Siebel AL, Owens JA, Moritz KM. Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats. *Kidney Int.* 2008;74:187-195. doi: 110.1038/ki.2008.1153. Epub 2008 Apr 1023.
292. Sugishita Y, Leifer DW, Agani F, Watanabe M, Fisher SA. Hypoxia-responsive signaling regulates the apoptosis-dependent remodeling of the embryonic avian cardiac outflow tract. *Dev Biol*. 2004;273:285-296
293. Yue X, Tomanek RJ. Stimulation of coronary vasculogenesis/angiogenesis by hypoxia in cultured embryonic hearts. *Dev Dyn*. 1999;216:28-36
294. Semenza GL. Hif-1: Mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol*. 2000;88:1474-1480
295. Kaelin WG, Jr., Ratcliffe PJ. Oxygen sensing by metazoans: The central role of the hif hydroxylase pathway. *Mol Cell*. 2008;30:393-402
296. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature*. 1998;392:405-408
297. Patterson AJ, Zhang L. Hypoxia and fetal heart development. *Curr Mol Med*. 2010;10:653-666
298. Gagnon R. Placental insufficiency and its consequences. *Eur J Obstet Gynecol Reprod Biol*. 2003;110 Suppl 1:S99-107
299. Patterson AJ, Xiao D, Xiong F, Dixon B, Zhang L. Hypoxia-derived oxidative stress mediates epigenetic repression of pkcepsilon gene in foetal rat hearts. *Cardiovasc Res*. 2012;93:302-310
300. Muhlfeld C, Nyengaard JR, Mayhew TM. A review of state-of-the-art stereology for better quantitative 3d morphology in cardiac research. *Cardiovasc Pathol*. 2010;19:65-82
301. Gundersen HJ. The smooth fractionator. *J Microsc*. 2002;207:191-210
302. Nyengaard JR, Gundersen JG. The isector: A simple and direct method for generating isotropic, uniform random sections from small specimen. *J. Microsc*. 1992;165:427-431
303. Gundersen HJ. Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of william r. Thompson. *J Microsc*. 1986;143:3-45
304. Bruel A, Oxlund H, Nyengaard JR. The total length of myocytes and capillaries, and total number of myocyte nuclei in the rat heart are time-dependently increased by growth hormone. *Growth Horm IGF Res*. 2005;15:256-264
305. Passmore M, Nataatmadja M, Fraser JF. Selection of reference genes for normalisation of real-time rt-pcr in brain-stem death injury in ovis aries. *BMC Mol Biol*. 2009;10:72
306. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. Qbase relative quantification framework and software for management and automated analysis of real-time quantitative pcr data. *Genome Biol*. 2007;8:R19
307. Soo PS, Hiscock J, Botting KJ, Roberts CT, Davey AK, Morrison JL. Maternal undernutrition reduces p-glycoprotein in guinea pig placenta and developing brain in late gestation. *Reprod Toxicol*. 2012;33:374-381
308. Gentili S, Morrison JL, McMillen IC. Intrauterine growth restriction and differential patterns of hepatic growth and expression of igf1, pck2, and hsdl1 mrna in the sheep fetus in late gestation. *Biol Reprod*. 2009;80:1121-1127
309. Takagi-Morishita Y, Yamada N, Sugihara A, Iwasaki T, Tsujimura T, Terada N. Mouse uterine epithelial apoptosis is associated with expression of mitochondrial voltage-dependent anion channels, release of cytochrome c from mitochondria, and the ratio of bax to bcl-2 or bcl-x. *Biol Reprod*. 2003;68:1178-1184

310. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Dis*. 2011;2:e164
311. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*. 1996;379:88-91
312. Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol*. 2008;9:402-412
313. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E. Role of hif-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*. 1998;394:485-490
314. Ginouves A, Ilc K, Macias N, Pouyssegur J, Berra E. Phds overactivation during chronic hypoxia "desensitizes" hifalpha and protects cells from necrosis. *Proc Natl Acad Sci U S A*. 2008;105:4745-4750
315. Le Moine CM, Morash AJ, McClelland GB. Changes in hif-1alpha protein, pyruvate dehydrogenase phosphorylation, and activity with exercise in acute and chronic hypoxia. *Am J Physiol Regul Integr Comp Physiol*. 2011;301:R1098-1104
316. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: Role of the hif system. *Nat Med*. 2003;9:677-684
317. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S, Kuhn B. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci U S A.* 2013;110:1446-1451. doi: 1410.1073/pnas.1214608110. Epub 1214602013 Jan 1214608119.
318. Huang CY, Hao LY, Buetow DE. Hypertrophy of cultured adult rat ventricular cardiomyocytes induced by antibodies against the insulin-like growth factor (igf)-i or the igf-i receptor is igf-ii-dependent. *Mol Cell Biochem.* 2002;233:65-72.
319. Chu CH, Tzang BS, Chen LM, Kuo CH, Cheng YC, Chen LY, Tsai FJ, Tsai CH, Kuo WW, Huang CY. Igf-ii/mannose-6-phosphate receptor signaling induced cell hypertrophy and atrial natriuretic peptide/bnp expression via galphaq interaction and protein kinase c-alpha/camkii activation in h9c2 cardiomyoblast cells. *J Endocrinol.* 2008;197:381-390. doi: 310.1677/JOE-1607-0619.
320. Wang KC, Brooks DA, Thornburg KL, Morrison JL. Activation of igf-2r stimulates cardiomyocyte hypertrophy in the late gestation sheep fetus. *J Physiol*. 2012;590:5425-5437
321. Kind KL, Simonetta G, Clifton PM, Robinson JS, Owens JA. Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Exp Physiol*. 2002;87:469-477
322. Bruel A, Nyengaard JR. Design-based stereological estimation of the total number of cardiac myocytes in histological sections. *Basic Res Cardiol*. 2005;100:311-319
323. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc.* 1984;134:127-136.
324. Rocca MS, Wehner NG. The guinea pig as an animal model for developmental and reproductive toxicology studies. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:92-97
325. Olivetti G, Giordano G, Corradi D, Melissari M, Lagrasta C, Gambert SR, Anversa P. Gender differences and aging: Effects on the human heart. *J Am Coll Cardiol.* 1995;26:1068-1079.
326. Mallat Z, Fornes P, Costagliola R, Esposito B, Belmin J, Lecomte D, Tedgui A. Age and gender effects on cardiomyocyte apoptosis in the normal human heart. *J Gerontol A Biol Sci Med Sci.* 2001;56:M719-723.
327. Zhang XP, Vatner SF, Shen YT, Rossi F, Tian Y, Peppas A, Resuello RR, Natividad FF, Vatner DE. Increased apoptosis and myocyte enlargement with decreased cardiac

mass; distinctive features of the aging male, but not female, monkey heart. *J Mol Cell Cardiol.* 2007;43:487-491. Epub 2007 Jul 2021.

328. Biondi-Zoccai GG, Abate A, Bussani R, Camilot D, Giorgio FD, Marino MP, Silvestri F, Baldi F, Biasucci LM, Baldi A. Reduced post-infarction myocardial apoptosis in women: A clue to their different clinical course? *Heart.* 2005;91:99-101.
329. Woods LL, Ingelfinger JR, Nyengaard JR, Rasch R. Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res.* 2001;49:460-467.
330. Woods LL, Ingelfinger JR, Rasch R. Modest maternal protein restriction fails to program adult hypertension in female rats. *Am J Physiol Regul Integr Comp Physiol.* 2005;289:R1131-1136. Epub 2005 Jun 1116.
331. Woods LL, Weeks DA, Rasch R. Programming of adult blood pressure by maternal protein restriction: Role of nephrogenesis. *Kidney Int.* 2004;65:1339-1348.
332. Du XJ, Fang L, Kiriazis H. Sex dimorphism in cardiac pathophysiology: Experimental findings, hormonal mechanisms, and molecular mechanisms. *Pharmacol Ther.* 2006;111:434-475. Epub 2006 Jan 2024.
333. Ojeda NB, Grigore D, Robertson EB, Alexander BT. Estrogen protects against increased blood pressure in postpubertal female growth restricted offspring. *Hypertension.* 2007;50:679-685. Epub 2007 Aug 2027.
334. Martin DS, Biltoft S, Redetzke R, Vogel E. Castration reduces blood pressure and autonomic venous tone in male spontaneously hypertensive rats. *J Hypertens.* 2005;23:2229-2236.
335. Fortepiani LA, Reckelhoff JF. Treatment with tetrahydrobiopterin reduces blood pressure in male shr by reducing testosterone synthesis. *Am J Physiol Regul Integr Comp Physiol.* 2005;288:R733-736. Epub 2004 Dec 2016.
336. Grodstein F, Stampfer MJ, Manson JE, Colditz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *N Engl J Med.* 1996;335:453-461.
337. Brower GL, Gardner JD, Janicki JS. Gender mediated cardiac protection from adverse ventricular remodeling is abolished by ovariectomy. *Mol Cell Biochem.* 2003;251:89-95.
338. Ojeda NB, Grigore D, Yanes LL, Iliescu R, Robertson EB, Zhang H, Alexander BT. Testosterone contributes to marked elevations in mean arterial pressure in adult male intrauterine growth restricted offspring. *Am J Physiol Regul Integr Comp Physiol.* 2007;292:R758-763. Epub 2006 Aug 2017.
339. Richardson VCG. The reproductive system. *Diseases of domestic guinea pigs*. Blackwell Science Ltd; 2008:14-38.
340. Bertram C, Khan O, Ohri S, Phillips DI, Matthews SG, Hanson MA. Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic-pituitary-adrenal function. *J Physiol*. 2008;586:2217-2229
341. Edwards L, Simonetta G, Owens J, Robinson J, McMillen I. Restriction of placental and fetal growth in sheep alters fetal blood pressure responses to angiotensin ii and captopril. *J Physiol*. 1999;515:897-904
342. Dyer J, McMillen I, Warnes K, Morrison J. No evidence for an enhanced role of endothelial nitric oxide in the maintenance of arterial blood pressure in the iugr sheep fetus. *Placenta*. 2009;30:705-710
343. Harvey LM, Gilbert RD, Longo LD, Ducsay CA. Changes in ovine fetal adrenocortical responsiveness after long-term hypoxemia. *Am J Physiol.* 1993;264:E741-747.
344. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the framingham heart study. *N Engl J Med.* 1990;322:1561-1566.

345. Brown DW, Giles WH, Croft JB. Left ventricular hypertrophy as a predictor of coronary heart disease mortality and the effect of hypertension. *Am Heart J.* 2000;140:848-856.
346. Kannel WB. Epidemiological aspects of heart failure. *Cardiol Clin.* 1989;7:1-9.
347. Leipala JA, Boldt T, Turpeinen U, Vuolteenaho O, Fellman V. Cardiac hypertrophy and altered hemodynamic adaptation in growth-restricted preterm infants. *Pediatr Res.* 2003;53:989-993. Epub 2003 Mar 2005.
348. Devereux RB, Pickering TG, Harshfield GA, Kleinert HD, Denby L, Clark L, Pregibon D, Jason M, Kleiner B, Borer JS, Laragh JH. Left ventricular hypertrophy in patients with hypertension: Importance of blood pressure response to regularly recurring stress. *Circulation.* 1983;68:470-476.
349. Verdecchia P, Schillaci G, Borgioni C, Ciucci A, Gattobigio R, Zampi I, Reboldi G, Porcellati C. Prognostic significance of serial changes in left ventricular mass in essential hypertension. *Circulation.* 1998;97:48-54.
350. Abel ED, Litwin SE, Sweeney G. Cardiac remodeling in obesity. *Physiol Rev.* 2008;88:389-419. doi: 310.1152/physrev.00017.02007.
351. Waspe LE, Ordahl CP, Simpson PC. The cardiac beta-myosin heavy chain isogene is induced selectively in alpha 1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes. *J Clin Invest.* 1990;85:1206-1214.
352. Dorn GW, 2nd, Robbins J, Ball N, Walsh RA. Myosin heavy chain regulation and myocyte contractile depression after lv hypertrophy in aortic-banded mice. *Am J Physiol.* 1994;267:H400-405.
353. Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJ. Catch-up growth in childhood and death from coronary heart disease: Longitudinal study. *BMJ.* 1999;318:427-431.
354. Huxley RR, Shiell AW, Law CM. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: A systematic review of the literature. *J Hypertens*. 2000;18:815-831
355. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: Prospective cohort study. *BMJ.* 2000;320:967-971.
356. Muhlhausler BS, Adam CL, Findlay PA, Duffield JA, McMillen IC. Increased maternal nutrition alters development of the appetite-regulating network in the brain. *FASEB J.* 2006;20:1257-1259. Epub 2006 May 1259.
357. Warnes KE, Coulter CL, Robinson JS, McMillen IC. The effect of intrafetal infusion of metyrapone on arterial blood pressure and on the arterial blood pressure response to angiotensin ii in the sheep fetus during late gestation. *J Physiol*. 2003;552:621-633
358. Lemberger T, Saladin R, Vazquez M, Assimacopoulos F, Staels B, Desvergne B, Wahli W, Auwerx J. Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm. *J Biol Chem.* 1996;271:1764-1769.
359. Garratt ES, Vickers MH, Gluckman PD, Hanson MA, Burdge GC, Lillycrop KA. Tissue-specific 5' heterogeneity of pparalpha transcripts and their differential regulation by leptin. *PLoS One.* 2013;8:e67483. doi: 67410.61371/journal.pone.0067483. Print 0062013.
360. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: Cellular and molecular biology. *Endocr Rev.* 1999;20:321-344.
361. Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest.* 2000;106:847-856.
362. Samovski D, Su X, Xu Y, Abumrad NA, Stahl PD. Insulin and ampk regulate fa translocase/cd36 plasma membrane recruitment in cardiomyocytes via rab gap as160

and rab8a rab gtpase. *J Lipid Res.* 2012;53:709-717. doi: 710.1194/jlr.M023424. Epub 022012 Feb 023426.

363. Paneth N, Susser M. Early origin of coronary heart disease (the "barker hypothesis"). *BMJ.* 1995;310:411-412.

364. Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Bmj.* 1989;298:564-567

365. Claffey KP, Shih SC, Mullen A, Dziennis S, Cusick JL, Abrams KR, Lee SW, Detmar M. Identification of a human vpf/vegf 3' untranslated region mediating hypoxia-induced mrna stability. *Mol Biol Cell.* 1998;9:469-481.

366. Stein I, Itin A, Einat P, Skaliter R, Grossman Z, Keshet E. Translation of vascular endothelial growth factor mrna by internal ribosome entry: Implications for translation under hypoxia. *Mol Cell Biol.* 1998;18:3112-3119.

367. Hashimoto H, Yuasa S. Testosterone induces cardiomyocyte differentiation from embryonic stem cells. *J Mol Cell Cardiol.* 2013;62:69-71.:10.1016/j.yjmcc.2013.1005.1008. Epub 2013 May 1024.

368. Marsh JD, Lehmann MH, Ritchie RH, Gwathmey JK, Green GE, Schiebinger RJ. Androgen receptors mediate hypertrophy in cardiac myocytes. *Circulation.* 1998;98:256-261.

369. Altamirano F, Oyarce C, Silva P, Toyos M, Wilson C, Lavandero S, Uhlen P, Estrada M. Testosterone induces cardiomyocyte hypertrophy through mammalian target of rapamycin complex 1 pathway. *J Endocrinol.* 2009;202:299-307. doi: 210.1677/JOE-1609-0044. Epub 2009 May 1627.

370. Wilson C, Contreras-Ferrat A, Venegas N, Osorio-Fuentealba C, Pavez M, Montoya K, Duran J, Maass R, Lavandero S, Estrada M. Testosterone increases glut4-dependent glucose uptake in cardiomyocytes. *J Cell Physiol*. 2013;11:24413

371. Ballantyne T, Du Q, Jovanovic S, Neemo A, Holmes R, Sinha S, Jovanovic A. Testosterone protects female embryonic heart h9c2 cells against severe metabolic stress by activating estrogen receptors and up-regulating ies sur2b. *Int J Biochem Cell Biol.* 2013;45:283-291. doi: 210.1016/j.biocel.2012.1010.1005. Epub 2012 Oct 1022.

372. Rosario FJ, Jansson N, Kanai Y, Prasad PD, Powell TL, Jansson T. Maternal protein restriction in the rat inhibits placental insulin, mtor, and stat3 signaling and down-regulates placental amino acid transporters. *Endocrinology*. 2011;152:1119-1129

373. Koos BJ. Adenosine a(2)a receptors and o(2) sensing in development. *Am J Physiol Regul Integr Comp Physiol*. 2011;301:R601-622

374. Moore LG, Shriver M, Bemis L, Hickler B, Wilson M, Brutsaert T, Parra E, Vargas E. Maternal adaptation to high-altitude pregnancy: An experiment of nature--a review. *Placenta*. 2004;25 Suppl A:S60-71

375. Zamudio S, Torricos T, Fik E, Oyala M, Echalar L, Pullockaran J, Tutino E, Martin B, Belliappa S, Balanza E, Illsley NP. Hypoglycemia and the origin of hypoxia-induced reduction in human fetal growth. *PLoS One*. 2010;5:e8551