PERINATAL PROGRAMMING OF METABOLIC HEALTH IN GUINEA PIGS

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DANE MICHAEL HORTON B. SC. (HONS)

DISCIPLINE OF OBSTETRICS & GYNAECOLOGY
ADELAIDE MEDICAL SCHOOL
THE UNIVERSITY OF ADELAIDE
AUSTRALIA

2017

Table of Contents

1.1 Table of Contents

TABLE OF CONTENTS II

1.1 TABLE OF CONTENTS II
1.2 TABLE OF ABBREVIATIONS VI
1.3 LIST OF FIGURES VIII
1.4 LIST OF TABLES IX

THESIS ABSTRACT X

DECLARATION XII

ACKNOWLEDGEMENTS XIII

CHAPTER 1 LITERATURE REVIEW 1

1.1 SCOPE OF THE LITERATURE REVIEW 1
1.2 CONCEPT OF DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE (DOHAD) 2
1.3 INTRAUTERINE GROWTH RETARDATION (IUGR) 3
1.4 MECHANISMS AND RISK FACTORS FOR SMALL SIZE AT BIRTH OR CLINICAL IUGR4
1.4.1 Fetal Factors 4
1.4.2 Maternal Factors 4
1.4.3 Uterine and Placental Factors 6
1.4.4 Environmental Factors 7
1.5 CATCH-UP GROWTH 9
1.5.1 Definition and Incidence 9
1.5.2 Underlying Mechanisms of Catch-up Growth Following IUGR 9
1.5.2.1 Increased insulin action -- 9
1.5.2.2 Down-regulation of thermogenesis -- 10
1.5.2.3 Partitioning of nutrient flow from lean to adipose tissue -- 10
1.5.2.4 Hyperphagia -- 11
1.5.3 Catch-up Growth is an Independent Risk Factor for Adult Disease 11
1.6 DETERMINANTS OF GLUCOSE HOMEOSTASIS: GLUCOSE EFFECTIVENESS, INSULIN SECRETION AND INSULIN SENSITIVITY 12
1.6.1 Glucose Effectiveness 12
1.6.2 Secretion of Insulin 13
1.6.3 The Insulin Signalling Pathway 17
1.6.4 Insulin Action in Key Target Tissues 17
1.6.4.1 Insulin action in skeletal muscle -- 17
1.6.4.2 Insulin action in liver -- 20
1.6.4.3 Insulin action in kidney -- 22
1.6.4.4 Insulin action in adipose tissue -- 22
1.6.4.5 Insulin action in the brain -- 22
1.6.5 Insulin-Stimulated Glucose Disposition 23
1.7 FAILURE OF GLUCOSE HOMEOSTASIS - TYPE 2 DIABETES MELLITUS 25
1.8 MEASURING INDICES OF GLUCOSE HOMEOSTASIS IN VIVO 26
1.8.1 Insulin Tolerance Test 26
1.8.2 Hyperinsulinaemic-Euglycaemic Clamp 27

1.8.3 Hyperglycaemic Clamp 28
1.8.4 Glucose Tolerance Test 28
1.8.4.1 Oral glucose tolerance test ----- 29
1.8.4.2 Frequently sampled intravenous glucose tolerance test ----- 29
1.8.5 Indices Based on Fasting Insulin and Glucose 30
1.8.5.1 Fasting insulin and fasting insulin to glucose ratios ----- 30
1.8.5.2 Homeostatic model assessment (HOMA) ----- 31
1.8.5.3 Quantitative insulin sensitivity check index (QUICKI) ----- 31

1.9 PRENATAL PROGRAMMING OF GLUCOSE METABOLISM IN HUMANS 31
1.9.1 Prenatal Programming of Impaired Glucose Homeostasis and T2DM 31
1.9.2 Prenatal Programming of Glucose Effectiveness 34
1.9.3 Prenatal Programming of Insulin Sensitivity 34
1.9.4 Prenatal Programming of Pancreatic Function and Insulin Secretion 35
1.9.5 Mechanisms for Prenatally-Induced Insulin Resistance 37
1.9.6 Neonatal Catch-Up Growth as an Independent Mechanism for Impaired Insulin Action 38
1.9.7 Obesity as Contributing Factor to Metabolic Disease after IUGR 39
1.9.8 Sex Differences in Perinatal Programming 39

1.10 NEED FOR ANIMAL MODELS TO INVESTIGATE PERINATAL PROGRAMMING OF GLUCOSE HOMEOSTASIS 42

1.11 ANIMAL MODELS OF IUGR AND IMPAIRED GLUCOSE HOMEOSTASIS 42
1.11.1 Restricted Placental Size and/or Function 43
1.11.1.1 Uterine artery ligation/occlusion in the guinea pig and the rat ----- 43
1.11.1.2 Reduced uterine placental pressure (RUPP) in the rat ----- 45
1.11.1.3 Surgical placental restriction in sheep ----- 46
1.11.1.4 Embryo transfer into overnourished adolescent sheep ----- 46
1.11.1.5 Heat –induced placental restriction in sheep ----- 47
1.11.2 Maternal Malnutrition 48
1.11.2.1 Global maternal nutrient restriction ----- 48
1.11.2.2 Maternal glucose restriction ----- 52
1.11.2.3 Maternal protein restriction in the rat ----- 53
1.11.2.4 Maternal hypoxia in the rat ----- 57
1.11.3 Spontaneous Growth Restriction 58
1.11.3.1 Variations within a litter ----- 58
1.11.3.2 Litter size effects ----- 59
1.11.4 Gaps in Existing Knowledge 62
1.11.5 The Guinea Pig as a Potential Species for Studying Developmental Programming by IUGR 64
1.11.6 Spontaneous Growth Restriction in the Guinea Pig as a Potential Model for Studying Developmental Programming of Metabolic Dysfunction 64

1.12 HYPOTHESIS AND AIMS 66

CHAPTER 2 SPONTANEOUS INTRAUTERINE GROWTH RESTRICTION DUE TO INCREASED LITTER SIZE IN THE GUINEA PIG PROGRAMS POSTNATAL GROWTH, APPETITE AND ADULT BODY COMPOSITION 67

2.1 OVERVIEW 67

2.2 STATEMENT OF AUTHORSHIP 68

2.3 APPENDIX 1 70

2.4 ABSTRACT 71

2.5 INTRODUCTION 72

2.6 MATERIALS AND METHODS 74
2.6.1 Animals 74
2.6.2 Adult Body Composition 75
2.6.3 Statistical Analysis 76

2.7 RESULTS ... 77
2.7.1 Maternal Outcomes ... 77
2.7.2 Litter Outcomes ... 80
2.7.3 Birth Phenotype ... 80
2.7.4 Postnatal Growth ... 83
2.7.4.1 Neonates --- 83
2.7.4.2 Juveniles --- 83
2.7.4.3 Adolescents --- 84
2.7.5 Feed Intake ... 88
2.7.6 Adult Phenotype and Body Composition ... 90
2.7.6.1 Body size --- 90
2.7.6.2 Body composition --- 90

2.8 DISCUSSION ... 94
2.8.1 Maternal Outcomes ... 94
2.8.2 Birth Phenotype ... 95
2.8.3 Neonatal Phenotype ... 97
2.8.4 Post-weaning phenotype ... 97
2.8.5 Adult Body Size and Composition ... 99

2.9 CONCLUSION ... 101

CHAPTER 3 INSULIN SENSITIVITY OF GLUCOSE METABOLISM IN YOUNG ADULT GUINEA PIGS - VALIDATION OF THE HYPERINSULINAEMIC EUGLYCAEMIC CLAMP METHOD ... 102

3.1 OVERVIEW ... 102

3.2 APPENDIX 2 ... 103

3.3 ABSTRACT ... 104

3.4 INTRODUCTION ... 105

3.5 MATERIALS AND METHODS ... 106
3.5.1 Animals ... 106
3.5.2 Surgery: Insertion of Catheters ... 107
3.5.3 Hyperinsulinaemic-Euglycaemic Clamps (HEC) ... 107
3.5.4 Analysis of Human and Guinea Pig Insulin ... 109
3.5.5 Plasma D-[3-^{3}H]-Glucose and $^{3}H_2O$ for Partitioned Glucose Metabolism ... 109
3.5.6 Calculations ... 110
3.5.6.1 Glucose utilisation --- 111
111
3.5.6.2 Glucose production --- 111
3.5.6.3 Glycolysis and glucose storage --- 112
3.5.7 Statistical Analysis ... 113

3.6 RESULTS ... 113
3.6.1 Insulin Dose Response of Whole Body Glucose Metabolism ... 113
3.6.2 Whole Body Insulin Sensitivity and Partitioned Glucose Metabolism at ~Half-Maximal Insulin Dose (7.5 $mU.min^{-1}.kg^{-1}$) ... 116
3.6.3 Whole Body Insulin Sensitivity and Partitioned Glucose Metabolism at Near Maximal Insulin Dose (30 $mU.min^{-1}.kg^{-1}$) ... 117

3.7 DISCUSSION ... 121

3.8 CONCLUSION ... 126

CHAPTER 4 SEX-SPECIFIC PERINATAL PROGRAMMING OF INSULIN SENSITIVITY IN THE GUINEA PIG. ... 127

4.1 OVERVIEW ... 127

4.2 ABSTRACT 128

4.3 INTRODUCTION 129

4.4 MATERIALS AND METHODS 131

4.4.1 Animals 131
4.4.2 Surgery: Insertion of Catheters 132
4.4.3 Hyperinsulinaemic-Euglycaemic Clamps 132
4.4.4 Insulin and Metabolite Analyses 133
4.4.5 Plasma D-[3-^{3}H]-Glucose and ^3H_2O 133
4.4.6 Calculations 134
4.4.7 Adult Body Composition 134
4.4.8 Statistical Analysis 135

4.5 RESULTS 136

4.5.1 Birth and Neonatal Phenotype 136
4.5.2 Litter Size, Perinatal Growth and Whole Body Metabolic Outcomes 139
4.5.3 Perinatal Growth and Partitioned Glucose Metabolism 144
4.5.3.1 Endogenous glucose production -------- 144
4.5.3.2 Glucose utilisation -------- 144
4.5.3.3 Glucose storage -------- 144
4.5.3.4 Glycolysis -------- 145
4.5.4 Adult Body Composition does not Explain Perinatal Programming of Insulin Sensitivity 147

4.6 DISCUSSION 149

4.6.1 Perinatal Growth and Whole Body Insulin Sensitivity 149
4.6.2 Perinatal Growth and Components of Glucose Metabolism 150
4.6.3 Impaired Insulin Sensitivity in the Low Birth Weight Male is not Related to Body Composition 152
4.6.4 Sex Differences 153

4.7 CONCLUSION 154

CHAPTER 5 GENERAL DISCUSSION 155

5.1 SUMMARY 155

5.2 EFFECTS OF LITTER SIZE, SPONTANEOUS IUGR AND NEONATAL GROWTH ON POSTNATAL PHENOTYPE (CHAPTERS 2 AND 4) 157

5.3 SEX-SPECIFIC OUTCOMES (CHAPTERS 2, 3 AND 4) 161

5.4 STRENGTHS AND LIMITATIONS 165

5.5 FUTURE DIRECTIONS 168

5.6 INTERVENTIONS 170

5.7 CONCLUSION 171

CHAPTER 6 REFERENCES 172

1.2 Table of Abbreviations

AGA	Appropriate for gestational age
AGR	Absolute growth rate
AGR_{10-28}	Absolute growth rate for weight (neonatal) from day 10 to 28
AGR_{30-60}	Absolute growth rate for weight (juvenile) from day 30 to 60
AGR_{60-90}	Absolute growth rate for weight (adolescent) from day 60 to 90
AKT	Product of the AKT protooncogene, serine/threonine-protein kinase or protein kinase B
p-AKT/AKT	phosphorylated to non-phosphorylated AKT (or protein kinase B)
AMPK	5' adenosine monophosphate-activated protein kinase
p-AMPK/AMPK	phosphorylated to non-phosphorylated 5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of Variance (statistical test)
ATP	Adenosine triphosphate
$AUC_{insulin}$	Area under the curve for insulin
$AUC_{glucose}$	Area under the curve for glucose
BMI	Body mass index
CoA	Coenzyme A
DOHaD	Developmental origins of health and disease
dpm	Disintegrations per minute
$FADH_2$,	Flavin adenine dinucleotide (hydroquinone form)
FAT/CD36	Fatty Acid Translocase/Cluster of Differentiation 36
FFA	Free fatty acid concentration
FGR	Fractional growth rate
FGR_{10-28}	Fractional growth rate for weight (neonatal) from day 10 to 28
FGR_{30-60}	Fractional growth rate for weight (juvenile) from day 30 to 60
FGR_{60-90}	Fractional growth rate for weight (adolescent) from day 60 to 90
G0	Gestational age day 0 (day of mating)
G0-G40	Gestational age day 0 to day 40
G0-G60	Gestational age day 0 to day 60
GIR	Glucose infusion rate
GLUT 1/2/3	Glucose transporter 1/2/3 isoforms
GLUT4	Glucose transporter 4 isoform
HEC	Hyperinsulinaemic euglycaemic clamp
ID	Internal diameter

IMVS	Institute of Medical and Veterinary Science
IRS 1/2	Insulin receptor substrate isoforms 1 and 2
IUGR	Intrauterine growth restriction (or retardation)
IVGTT	Intravenous glucose tolerance test
L0	Day 0 of lactation (day of birth)
L0-L30	Day 0 to day 30 of lactation (day of birth to day of weaning)
LBW	Low birth weight
LPL	Lipoprotein lipase
LS	Litter size
MCR	Metabolic clearance rate
meq	Milli equivalent
mRNA	Messenger ribonucleic acid
µU	Micro unit
NAD^+/NADH	Nicotinamide adenine dinucleotide (oxidised and reduced form)
NCD	Non-communicable diseases
n.d.	Not determined
NSD	Not significantly different
NIDDM	Non-insulin dependent diabetes mellitus
OD	Outer diameter
OGTT	Oral glucose tolerance test
$p70^{S6K}$	Ribosomal protein S6 kinase beta-1
PI3K	Phosphatidylinositol-3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PKCζ,	Protein kinase C zeta isoform
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
SPSS	Statistical Package for Social Sciences
ssGIR	Steady state glucose infusion rate achieved during the second hour of the hyperinsulinaemic euglycaemic clamp
T2DM	Type 2 Diabetes Mellitus
%CV	Coefficient of variance
Ψ	Membrane Potential

1.3 List of Figures

Figure 1-1 Mechanisms of Stimulus-Secretion Coupling of Insulin in Humans 14
Figure 1-2 Summary of the Insulin Signalling Pathway 16
Figure 1-3 Mechanism of Insulin Action in Skeletal Muscle 19
Figure 1-4 Mechanism of Insulin Action in the Liver 21
Figure 1-5 Mechanism of Insulin Action in Adipose Tissue.................................. 24
Figure 2-1 Maternal weight in pregnancy and lactation according to litter size. 78
Figure 2-2 Birth weight distribution and litter size. 82
Figure 2-3 Growth of progeny as neonates, juveniles and during adolescence 85
Figure 3-1 Insulin dose response curve for glucose infusion rates during hyperinsulinaemic-euglycaemic clamps in the guinea pig............... 115
Figure 4-1 Correlation between whole body insulin sensitivity and birth weight in male and female guinea pigs.. 143

1.4 List of Tables

TABLE 1-1 FACTORS CONTRIBUTING TO SMALL SIZE AT BIRTH IN HUMANS 8

TABLE 2-1 EFFECT OF LITTER SIZE ON MATERNAL WEIGHTS, ABSOLUTE GROWTH RATES AND FEED INTAKE DURING PREGNANCY. 79

TABLE 2-2 EFFECT OF LITTER SIZE ON BIRTH PHENOTYPE OF LIVE-BORN PUPS 81

TABLE 2-3 EFFECT OF LITTER SIZE AND SEX ON POSTNATAL GROWTH RATES AND FOOD INTAKE. 86

TABLE 2-4 RELATIONSHIPS BETWEEN BIRTH WEIGHT AND NEONATAL FGR AND SUBSEQUENT POSTNATAL GROWTH RATES AND FOOD INTAKE.............. 89

TABLE 2-5 EFFECT OF LITTER SIZE AND SEX ON ADULT SIZE AND BODY COMPOSITION 92

TABLE 2-6 RELATIONSHIPS BETWEEN BIRTH WEIGHT, NEONATAL FGR AND ADULT PHENOTYPE IN THE GUINEA PIG. 93

TABLE 3-1 METABOLIC RESPONSES TO DIFFERING INFUSION RATES DURING THE HYPERINSULINAEMIC EUGLCAEMIC CLAMP IN YOUNG ADULT GUINEA PIGS.. 114

TABLE 3-2 WHOLE BODY INSULIN SENSITIVITY AND PARTITIONED GLUCOSE METABOLISM AT HALF-MAXIMAL INSULIN DOSE IN THE GUINEA PIG (7.5 $MU.MIN^{-1}.KG^{-1}$). 118

TABLE 3-3 WHOLE BODY INSULIN SENSITIVITY AND PARTITIONED GLUCOSE METABOLISM AT MAXIMAL INSULIN DOSE IN THE GUINEA PIG (30 $MU.MIN^{-1}.KG^{-1}$). 120

TABLE 4-1 EFFECT OF LITTER SIZE ON LITTER OUTCOMES 136

TABLE 4-2 EFFECT OF LITTER SIZE AND SEX ON BIRTH PHENOTYPE AND NEONATAL GROWTH RATES 138

TABLE 4-3 EFFECT OF LITTER SIZE AND SEX ON WHOLE BODY GLUCOSE METABOLISM... 140

TABLE 4-4 WHOLE BODY METABOLIC OUTCOMES BEFORE AND DURING HEC AT 7.5 MU $INSULIN.MIN^{-1}.KG^{-1}$ IN THE GUINEA PIG 142

TABLE 4-5 RELATIONSHIPS OF PERINATAL GROWTH AND PARTITIONED GLUCOSE METABOLISM AT 7.5 MU $INSULIN.MIN^{-1}.KG^{-1}$ INSULIN INFUSION IN THE GUINEA PIG. 146

TABLE 4-6 EFFECT OF SEX ON ADULT SIZE AND BODY COMPOSITION.................. 148

Thesis Abstract

Intrauterine growth restriction (IUGR) and neonatal catch-up growth are risk factors for the development of metabolic disease in later life. Developmental programming of insulin resistance is hypothesised to underpin many of these disorders. Animal models are required to investigate the mechanisms underlying this programming of insulin resistance and metabolic disease. Therefore, the current study assessed the effects of spontaneous growth restriction due to natural variation in litter size in the guinea pig on programming of adult metabolic outcomes.

Increasing litter size reduced birth weight, birth length and birth weight to length ratio while head dimensions at birth were relatively conserved, indicating head sparing and asymmetrical IUGR. Offspring from larger litters displayed faster neonatal fractional growth and faster absolute and fractional juvenile growth. Relative feed intake in juveniles was increased in offspring from larger litters, and increased neonatal growth also predicted hyperphagia in juveniles. Rapid neonatal growth also correlated with increased visceral adiposity in adult males, but not females, suggesting sex-specific programming of postnatal phenotype. Thus, the spontaneously IUGR guinea pig exhibits key features of human IUGR including neonatal catch-up growth, postnatal hyperphagia and increased fat deposition (Chapter 2).

To enable further study of the effects of litter size and neonatal growth on insulin sensitivity, methodology for the hyperinsulinaemic euglycaemic clamp (HEC) was validated for use in the guinea pig. The dose-response curve for whole-body glucose uptake using recombinant human insulin was characterised and HECs with D-[3-^{3}H]-glucose infusion were performed to characterise insulin sensitivities of whole body glucose uptake and partitioning of glucose metabolism in males and females at ~half maximal and near maximal insulin doses. Insulin infusion at 7.5 mU.min^{-1}.kg^{-1} increased glucose utilisation and storage, while suppressing glucose production, while insulin at 30 mU.min^{-1}.kg^{-1} also increased the rate of glycolysis. Fasting plasma glucose, metabolic clearance of insulin and rates of glucose

utilisation, storage and production during insulin stimulation were higher in female than male guinea pigs, but insulin sensitivity of these and whole body glucose uptake did not differ between sexes (Chapter 3).

HEC was then used to assess whole body insulin sensitivity and partitioned glucose metabolism in young adult offspring from varying litter sizes. In males, insulin sensitivities of whole body glucose uptake and glucose utilisation correlated positively, while that of endogenous glucose production tended to correlate positively with birth weight, and these associations were independent of neonatal catch-up growth, adult adiposity and muscle mass. Whole body and partitioned glucose metabolism in young adult females were not related to birth weight, however, the insulin sensitivity of endogenous glucose production correlated negatively with neonatal catch-up growth independently of birth weight. These results suggest a contribution of intrinsic deficits in skeletal muscle and liver to sex-specific perinatal programming of insulin resistance in this species (Chapter 4).

Overall, these studies demonstrate that increasing litter size in the guinea pig results in asymmetrical IUGR. The spontaneously growth restricted guinea pig exhibits sex-specific programming of postnatal growth, appetite, adiposity and insulin sensitivity, occurring primarily in males, not unlike that in humans and other animal models. This therefore provides a model for investigating the causal mechanisms and effects of ageing on the perinatal programming of obesity and insulin resistance in liver and skeletal muscle.

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 in my name, for any other degree or diploma 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

Signature__ __ Date: 28th of June 2017

Dane M Horton

Acknowledgements

Firstly, I would like to sincerely thank Professor Julie Owens, Professor Jeffery Robinson and Dr Karen Kind for their academic and financial support of the project during my postgraduate degree. I am sincerely thankful for the academic support that Associate Professor David Saint, Dr Kathy Gatford and Dr Karen Kind have provided to see this through to the finished stage. Many thanks to all the other members of the Centre for Fetal Growth and Development, the University of Adelaide, Dr Prema Thavaneswaran, Dr Miles De Blasio, Dr Arkadi Katsman and Ms Mellisa Walker for making the years both productive and an enjoyable experience.

I acknowledge the financial support of the Faculty of Science, The University of Adelaide for the Faculty Scholarship I received during my postgraduate years. I acknowledge the financial support from project grants from the Heart Foundation. I thank the Endocrine Society of Australia, Australia Society for Medical Research and the Scholarships Branch at The University of Adelaide for the support with travel grants enabling me to present my studies at both National and International Conferences.

Finally, I would like to thank my dear wife Kathryn who has been my steadfast foundation when pressures and stressors took hold. To my parents, Michael and Marcia, my sisters, Claire and Mia, and friends for making all the “I think I cans” into a reality.

CHAPTER 1
Literature Review

1.1 Scope of the Literature Review

In epidemiological and geographical studies, intrauterine growth restriction (IUGR) and faster rates of neonatal growth, or "catch-up" growth, following perturbed growth *in utero* are risk factors for the development of numerous cardio-metabolic disease states later in life. This is an example of developmental programming of adult disease, the concept that events or exposures in early life can alter the developmental trajectory of the individual and alter their health and disease risk in later life. This literature review focuses on the relationship between IUGR and metabolic dysfunction, with specific emphasis on evidence for prenatal programming of insulin resistance, type 2 diabetes mellitus (T2DM), the metabolic syndrome, and obesity in later life in human studies and in experimental animal models. Neonatal accelerated or catch-up growth following IUGR in humans is an additional risk factor for impaired glucose homeostasis and obesity later in life and the evidence for effects of neonatal catch-up growth on the perinatal programming of metabolic dysfunction and obesity in humans and animal models is therefore also considered in this review. Because there is increasing evidence for sex-specific programming of metabolic disease in humans and in animal models with IUGR and neonatal catch-up growth, this review also assessed the evidence for sex-specific perinatal programming of metabolic dysfunction.

1.2 Concept of Developmental Origins of Health and Disease (DOHaD)

According to the World Health Organisation Global Status Report on Non-Communicable Disease 2010, non-communicable diseases (NCD) accounted for 63% of all deaths in 2008 with nearly 80% of deaths from NCDs coming from low and middle income countries[1]. With an ageing population the annual NCD death rate is likely to be the leading cause of death in all countries by 2030[1]. These NCDs include cardiovascular disease, hypertension, stroke, metabolic diseases, hyperlipidaemia, central obesity, insulin resistance, glucose intolerance, T2DM, respiratory disease and some infection-related cancers[1]. Over the last several decades historical and epidemiological studies have consistently demonstrated that IUGR, as indicated by a low birth weight or being disproportionately short or thin for gestational age, is associated with an increased risk of developing NCDs later in life[2-4]. These findings led to the concept of the "Fetal Origins of Adult Disease", also known as the "Thrifty Phenotype Hypothesis"[5-7] or "Barker Hypothesis" as Professor David Barker was one of the principal pioneers of the field[8]. The hypothesis has since been expanded to the "Developmental Origins of Health and Disease" (DOHaD) hypothesis, as peri-conception environment, size at birth, accelerated growth trajectories after birth and subsequent exposures are now all recognised as contributing to the risk of NCDs[8-13].

The DOHaD hypothesis states that a fetus growing in a sub-optimal environment makes physiological and biochemical adaptions to survive[14]. This plasticity of fetal tissues induced by sub-optimal conditions *in utero* results in "programmed" changes, at a phenotypic level by the alteration of organs, tissues and cell signalling pathways [2, 3, 14]. These adaptations through developmental plasticity ensure survival in the short-term; however, a mismatch between the *in utero* conditions and the individual's postnatal life-course may expose dysfunction of key tissues involved in homeostatic control [2, 14-16]. Therefore, the growing field of DOHaD investigates the programming of specific physiological and biochemical systems due to sub-optimal pre-conception or *in utero* environment, sometimes reflected by being small

for gestational age[2, 14, 17-19]. Further, the hypothesis now also considers subsequent postnatal exposures, including how the accelerated neonatal growth following exposure to *in utero* constraint leads to increased risk of chronic disease states, such as stroke, cardiovascular disease and T2DM[2, 3, 14].

1.3 Intrauterine Growth Retardation (IUGR)

According to American Congress of Obstetricians and Gynecologists guidelines and the World Health Organization Report 2006, IUGR is defined as a fetus with a weight below the 10th percentile for any given gestational age[20, 21]. Similarly, an IUGR infant is born with a birth weight below the 10th percentile for gestational age. However, this definition, which is also used to define small size at birth for gestational age (SGA), can overestimate IUGR pregnancies as infants with incorrect estimates of gestational age and those infants who are proportionally small, but have not suffered an insult in an adverse *in utero* environment will be included[22]. Low birth weight (LBW = birth weight <2500 g) is also often used as a surrogate marker for exposure to a restricted *in utero* environment, particularly in historical human cohorts, but is even more limited because it does not differentiate effects of IUGR from those of gestational age. IUGR pregnancies have a prevalence of 5–8% in the general population, whilst IUGR is a contributing factor in 10-15% of all pregnancies with complications[20].

IUGR has been further classified into two sub-classes based on phenotype at birth - symmetrical (20-30%) and asymmetrical (70-80%) growth restriction[22, 23]. Symmetrical, type I or proportionate IUGR refers to a proportionate growth restriction of all organs of the fetus, perhaps suggesting mild growth restriction throughout gestation, as growth potential for these individuals is normal[22]. Asymmetrical, type II or disproportionate IUGR reflects more severe growth restriction predominantly in late gestation where fetal growth has exceeded placental capacity[23]. This asymmetrical growth is due to fetal adaptation to the mismatched nutrient requirement/delivery by shifting the cardiac output to more vital organs, such as the brain and

adrenals at the expense of visceral organs such as the pancreas, liver and skeletal muscle as well as limiting long bone growth[23, 24]. Thus, the asymmetrical IUGR infant is indicated by being light, short or thin at birth, with the common phenomenon of brain sparing indicated by increased head circumference relative to birth weight and to abdominal circumference[25, 26]. These asymmetrical IUGR infants have a higher risk of morbidity and mortality after birth[27] throughout childhood[28, 29] and later in life[2, 3, 8].

1.4 Mechanisms and Risk Factors for Small Size at Birth or Clinical IUGR

1.4.1 Fetal Factors

Although factors of fetal origin, such as intrauterine infections and congenital abnormalities (Table 1-1), can result in profound growth retardation, the incidences of these are rare at 7% and 10% of all IUGR births respectively[23]. Congenital malformations, genetic errors in metabolism or rhesus-induced anaemia account for only a small percentage of IUGR cases; however, approximately 30-40% of all fetuses with major structural disorders or chromosomal abnormalities have IUGR[21, 30, 31]. The sex of the fetus also influences size at birth with males approximately 4% heavier than females[32].

1.4.2 Maternal Factors

Growth *in utero* is largely determined by a complex interaction between the fetal genome and the availability of nutrients supplied to the fetus[33]. Although both the father and mother make equal contributions to the fetal genome, and hence growth potential, birth weight is largely determined by maternal constraints to fetal growth mediated by the *in utero* environment and maternal nutrient supply[34-36]. This is clearly illustrated by results of experiments crossing large Shire horses and Shetland ponies. Lower birth weight of Shetland-Shire cross offspring born to Shetland mothers when compared to those born to Shire mothers, demonstrated the impact of maternal constraint of growth[37].

Maternal factors (Table 1-1) may account for approximately 50% of all cases of IUGR in developing countries[22], with most ultimately leading to a reduction in supply of nutrients to the fetus[36]. Therefore, factors that reduce available maternal nutrient availability, such as malnutrition and multiple concepti, account for most of the IUGR cases in developed countries. Severe nutrient deficiency during pregnancy, as occurs in times of famine or seasonally in developing countries, results in reduced weight at birth, especially if nutrient deficiency occurs in later gestation where fetal demand for nutrients is at its greatest[38-40]. Exposure to the famine in Holland (Dutch Famine) during World War II in the first, second or third trimesters decreased birth weight by 0.2%, 6.6% and 8.1% respectively, demonstrating the impact of inadequate nutrition in later pregnancy[38]. Similarly, exposure to a low protein, low calorie (~ 300 calories/day) diet *in utero* during the Leningrad siege in 1942 reduced average birth weights by 18% and 16% in males and females respectively[41].

Multiple gestation pregnancy is associated with a higher risk of IUGR. In humans, appropriate for gestational age (AGA) twins and triplets have -24% and -35% lower mean birth weights compared to AGA singletons[42]. Even in singletons regarded as SGA, twins and triplets are on average -4.5% and -18% lighter[42]. Reductions of other measures of size at birth are observed in AGA twins and triplets in abdominal circumference and crown to heel length compared to AGA and SGA singletons, while head circumference remains relatively conserved across all groups[42]. In addition to the effects of maternal constraint, individual twins within monochorionic twin pregnancies are at risk of IUGR due to the unequal sharing of placental surface area, blood flow, and therefore nutrient supply[20]. The growth of twins *in utero* deviates from that observed in singletons at 32 weeks[31] while that of triplets deviates at 26 weeks[42] suggesting that nutrient availability, transfer or placental sufficiency in multiple births is impaired in late gestation. These observations suggest that offspring from multiple births resemble asymmetrical IUGR.

Maternal condition and phenotype before conception can also alter size at birth. Birth weight is reduced by 2.5% for every 10 kg decrease in maternal pre-pregnancy weight and by 1-2% for every 10 cm decrease in maternal height, presumably due to maternal growth competing for nutrients or increased uterine constraint[32]. Other maternal factors that may contribute to small size at birth or IUGR are previous history of an IUGR pregnancy, maternal age less than 16 years, ethnicity and stress during pregnancy[22, 25, 32, 33, 43].

Smoking is estimated to account for 40% of growth-restricted newborns, with one study reporting a decrease in birth weight by 5% for each pack of 25 cigarettes smoked per day during gestation[32]. Other drug use, prescription, illicit drugs, or alcohol can also lead to a reduced weight at birth; however, in developed countries these do not have as great an effect as that of smoking[22, 36] and the effects may be mediated in part by other factors such as low/poor nutrition or disruption of placental function[33, 36].

1.4.3 Uterine and Placental Factors

Uterine infections and diseases such as endometriosis limit the potential uterine endometrial implantation area and therefore subsequent placental function. Damage to the uterine endometrium can also disrupt placental function later in the pregnancy.

The major extrinsic determinant of fetal growth is nutrient transfer by the placenta[44]. Placental factors (Table 1-1) contribute to most IUGR in developed countries where no pathological cause is identified[21, 31, 45, 46]. The placenta is the lifeline of the fetus, allowing oxygen, amino acids, glucose and other nutrients to be transferred from, and by-products of metabolism to be transferred to, the maternal circulation[47]. Placental growth and metabolism also utilises maternal nutrients, and hence is a further drain on maternal supplies[48]. In many mammalian species size at birth correlates positively with placental weight, therefore placental size has been suggested as an index of the placental capacity for nutrient supply[46, 49]. Most

IUGR babies have a small placenta[46, 50] and placental transfer of nutrients across the placenta is reduced in IUGR pregnancies[46].

Pregnancy-induced hypertension (preeclampsia) can reduce placental surface area and therefore these pregnancies are at up to 4-fold increased risk of IUGR[33, 51-53]. Preeclampsia reduces birth weight by approximately 5% in its milder forms but the effect can be as great as 10-12% in more severe cases[22, 32, 51, 52], and in early-onset preeclampsia, birth weight is reduced by up to 23%[52]. Preeclampsia induces asymmetrical IUGR, with reductions of 4.4% in birth weight but less substantial effects on crown-heel length (0.8%) and ponderal index (2.6%) reported in one study[51].

The placenta also produces hormones and growth factors that govern metabolism of mother and fetus during pregnancy[49, 54]. Disruption to the normal functioning of the placenta through exposure to challenges such as low oxygen, malnutrition or toxins, or through physical effects, such as lesions, infarcts, abnormal placentation, or placental abruption therefore also have an impact on production of placental growth factors and hormones[49, 54]. Hence, placental development and function are major determinants of fetal growth and resultant size at birth.

1.4.4 Environmental Factors

Most environmental factors only account for a small proportion of all IUGR. These include high altitude, pollution (smoking, heavy metals and other toxins), hyperthermia, quality of drinking water and irradiation[21, 31, 55]. Secondary or passive smoking decreased birth weight by -7.0% compared to -6.3% for maternal smoking[56], indicating that environmental smoke exposure has as significant an impact on fetal growth as maternal smoking itself. Psychosocial environmental factors such as family size, education and access to health care largely impact fetal growth through other factors previously discussed[21].

Table 1-1 Factors contributing to small size at birth in humans

Fetal Factors	Chromosomal abnormalities (Trisomy)
	Congenital malformation
	Genetic metabolic errors
	Fetal infections
	Sex of the fetus
	Rhesus induced anaemia (hypoxia)
Maternal Characteristics	Race/ethnicity
	Small/short stature
	Pregnancy (parity, multiple conceptus, interval)
	Low pre-pregnancy weight, body mass index
	Overweight/Obesity at conception
	Delivery at age <16 or >35 years
Maternal Lifestyle	Malnutrition (amino acids, lipids, iron, iodine)
	Low pregnancy weight gain (2nd and 3rd trimesters)
	Drug use (smoking, alcohol, illicit and prescription drugs)
	Severe chronic disease, infection or anaemia
	Socio-economic disadvantage
	Emotional, physical stress
Uterine Factors	Abnormalities (fibroids, malformation)
	Disease (intrauterine infection, endometriosis)
Placental Factors	Abnormal placentation
	Small placental size
	Low uteroplacental blood flow
	Impaired placental transport of nutrients
	Placental pathologies (infarcts, haematomas)
	Antepartum haemorrhage
	Pre-eclampsia
Environmental Factors	High altitude
	Hyperthermia
	Quality of drinking water
	Pollutants (smoking, irradiation, heavy metals, toxicants)
	Other (family size, education, health care support)

Table adapted from World Health Organization 2006[21].

1.5 **Catch-Up Growth**

1.5.1 Definition and Incidence

Most infants undergo accelerated growth termed "catch-up" after IUGR which occurs in the first few months of life[57-59]. This accelerated growth is primarily a gain in fat mass rather than long bone growth. While most IUGR children catch-up to some degree, approximately 15% of infants do not undergo this accelerated growth and 50% of all IUGR infants have short stature as adults[57]. Children who fail to catch-up or "fail to thrive" after IUGR have increased perinatal morbidity and mortality[60], and neurodevelopmental delay during childhood[61-63], compared to those with catch-up growth, which may persist into adult life. Despite the advantageous effects of catch-up growth following IUGR, catch-up is a risk factor for the development of adult metabolic disease later in life[64-66].

1.5.2 Underlying Mechanisms of Catch-up Growth Following IUGR

1.5.2.1 *Increased insulin action*

Recent studies in humans show that insulin sensitivity at 48 hours of age[67] and at 1 year of age[68] is greater in IUGR/SGA children than infants born with appropriate size at birth. Furthermore, infant insulin sensitivity is positively associated with growth in terms of weight[68], suggesting that increased insulin action may stimulate growth in this catch-up growth period. In neonatal lambs, insulin sensitivity during catch-up growth is positively associated with growth in terms of both birth weight, and long bones[69]. Insulin stimulates growth during early postnatal life, acting on both the chondritic growth plate in long bones and on skeletal muscles and other soft tissues[70, 71]. Therefore, high insulin sensitivity and/or secretion during early postnatal life following IUGR is a plausible mechanism driving the faster rates of growth in IUGR neonates.

1.5.2.2 *Down-regulation of thermogenesis*

Down-regulation of thermogenesis has been suggested as a means by which catch-up growth, especially that of fat, can occur with or without hyperphagia, by creating a "thrifty metabolism"[72, 73]. In the longer-term, however, this thrifty metabolism and increased fuel conservation lead to insulin resistance and associated disease[72].

1.5.2.3 *Partitioning of nutrient flow from lean to adipose tissue*

The DOHaD hypothesis states that fetal development has plasticity and therefore organs of higher immediate importance, like the brain and adrenals, are conserved over organs of lower importance for immediate survival. This redistribution of blood flow and therefore nutrients is achieved by the endocrine signalling of cortisol and catecholamines[24], both of which reduce size at birth when delivered to fetuses experimentally[74, 75]. The reduction of blood flow limits glucose utilisation and growth of somatic tissues while increasing glucose uptake in adipose tissue and hence promoting relatively faster growth of adipose depots than skeletal muscle[64, 76]. Further insulin resistance of skeletal muscle and relative insulin hyper-responsiveness in fat redirects storage of excess fuels to adipose tissue after IUGR which leads to increased fat gain during catch-up[72]. Consistent with these differential effects of prior restriction between tissues, adipose tissue has a greater insulin sensitivity than skeletal muscle under hyperinsulinaemic-euglycaemic clamp conditions in animal models of catch-up growth[76].

1.5.2.4 *Hyperphagia*

Catch-up growth often occurs when restricted nutrient availability, as occurs in IUGR, is followed by hyperphagia in a relatively nutrient-rich environment. Experimental IUGR alters the balance of orexigenic and anorexigenic neuropeptides in the brain which tend to increase feed intake later in life[77, 78]. Later, insulin and leptin resistance also follow IUGR which further impairs the negative feedback for feed intake. Although evidence for this is limited in humans[79], offspring of small animal models of maternal undernutrition, obesity and diabetes have altered hypothalamic control of energy balance, leading to hyperphagia and obesity[78, 80].

1.5.3 Catch-up Growth is an Independent Risk Factor for Adult Disease

Systematic reviews have implicated catch-up growth following IUGR as an additional risk factor for the development of hypertension[81], cardiovascular disease[66], obesity[64] and dysfunction in glucose homeostasis such as insulin resistance[65, 82], especially in individuals then challenged with a high caloric/fat diet as occurs in developed countries. Poor growth *in utero* and catch-up growth appear to induce adverse cardio-metabolic outcomes in adulthood, including insulin resistance[81, 83-89], via independent mechanisms[65, 82, 90]. Catch-up growth may also be a mechanism for gender-specific programming as early life factors contributed to more of the total variance in insulin resistance in 49-51 year-old males than in females[65].

1.6 Determinants of Glucose Homeostasis: glucose effectiveness, insulin secretion and insulin sensitivity

Blood glucose concentrations are controlled by insulin secretion, insulin sensitivity of the target tissues, and by glucose effectiveness, the intrinsic action of glucose on its own metabolism[91]. In individuals with insulin resistance, the pancreas initially hyper-secretes insulin to compensate for the impaired responsiveness at the target tissue[92], usually due to impaired post-receptor signalling. Hyperglycaemia and overt T2DM occur when the pancreas can no longer sustain this compensatory hypersecretion of insulin[92]. Humans with T2DM have reduced glucose effectiveness in addition to their insulin resistance and impaired insulin secretion which together impair glucose homeostasis[93, 94].

1.6.1 Glucose Effectiveness

Glucose effectiveness is the ability of glucose to auto-regulate the suppression of endogenous glucose production and enhance glucose uptake to control its intrinsic metabolism under basal insulin conditions (independent of insulin's actions)[95, 96]. Increased glucose availability under basal or steady insulin concentrations increases glucose storage in the liver by the induction of glucokinase and glycogen synthase while impairing enzymes of gluconeogenesis[96]. Additionally, GLUT4 translocation in skeletal muscle can be induced by increased glucose concentrations, independent of insulin action[96].

1.6.2 Secretion of Insulin

Insulin is secreted by β-cells in the pancreas in response to a high nutrient concentration in blood (summarised in Figure 1-1). Insulin is contained in vesicles within the β-cell as a prohormone containing C-peptide. After a meal, circulating concentrations of nutrients including glucose, amino acids and free fatty acids are high and these are taken up into the β-cell to stimulate insulin and C-peptide secretion. The major stimulus for the release of insulin is increased concentrations of glucose in blood. In rodents, glucose enters the β-cell through the high capacity glucose transporter type 2 (GLUT2)[97]; however, other glucose transporters may also be involved, as fetal islets of GLUT2 knockout mice do not have impaired insulin secretion[98]. In the human pancreas, GLUT1 and GLUT3 are the main glucose transporters in the β-cell[99]. Once glucose is phosphorylated to glucose-6-phosphate, it is then metabolised down the glycolytic, citric acid cycle to produce the high redox potential molecules NADH and $FADH_2$. These enter the electron transport system leading to an increase in the intracellular ATP/ADP ratio. Metabolism of amino and keto acids in the citric acid cycle and free fatty acids (FFA) via β-oxidation also increase ATP concentrations. The increase in ATP activates the prohormone convertase required for the cleavage of insulin from proinsulin. This relative increase in ATP also deactivates (closes) the ATP sensitive potassium channels, resulting in a depolarisation of the β-cell. This in turn activates voltage-gated calcium channels allowing extracellular Ca^{++} to enter the cell and act as a second messenger to initiate the exocytosis of insulin from its vesicles. Intracellular conversion of FFA to malonyl CoA may also act directly on protein kinase C and diacylglycerol, which in turn act to release Ca^{++} from intracellular stores initiating the exocytosis of insulin-containing vesicles[100].

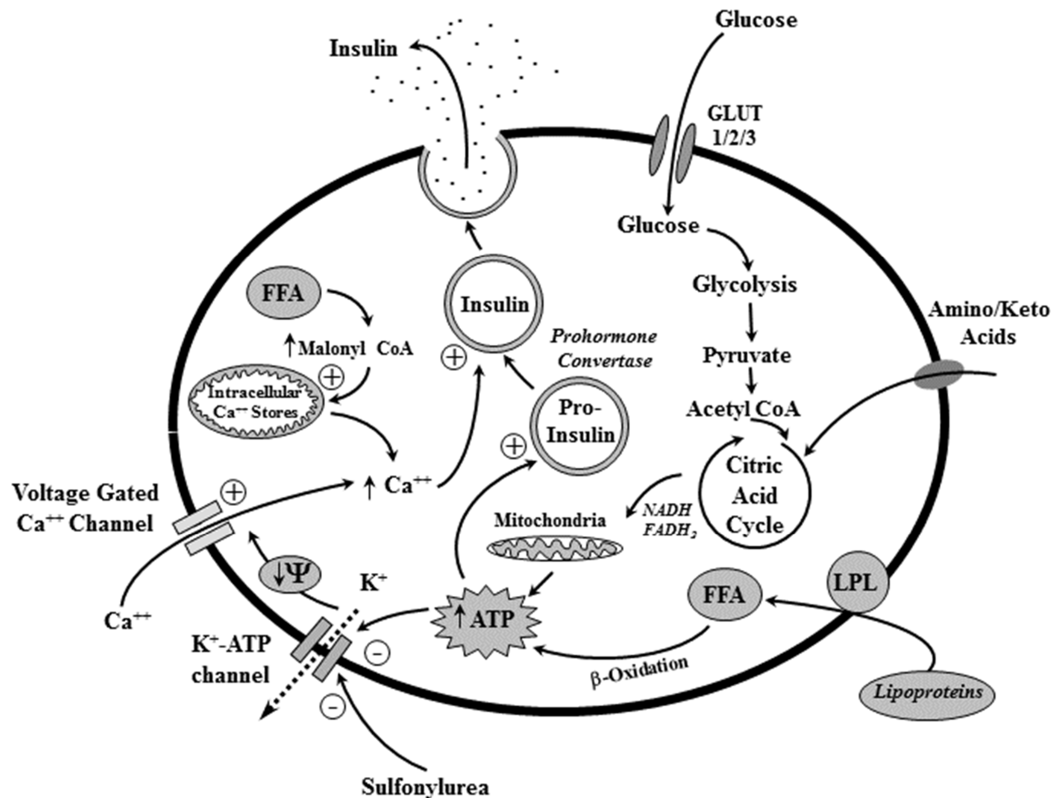


Figure 1-1 Mechanisms of Stimulus-Secretion Coupling of Insulin in Humans

GLUT 1/2/3, Glucose transporter 1/2/3 isoforms;
NADH, Nicotinamide adenine dinucleotide (reduced form)
$FADH_2$, Flavin adenine dinucleotide (hydroquinone form)
ATP, Adenosine triphosphate;
LPL, Lipoprotein lipase;
CoA Coenzyme A;
FFA, Free Fatty Acids;
Ψ Membrane Potential

⊕ Stimulatory ⊖ Inhibitory

(Adapted from Fowden et al. 2001[98])

Other stimuli for insulin secretion are gastrointestinal hormones such as GLP-1[101], acetylcholine[102, 103], and drugs including the sulfonylureas[104, 105]. Acetylcholine and the sulfonylureas increase intracellular Ca^{++} to initiate exocytosis of insulin via differing mechanisms. Acetylcholine activates inositol triphosphate-dependent mobilization of intracellular Ca^{++} [99, 100] while the sulfonylureas inhibit the K^{+}-ATP channels leading to activation of the voltage gated Ca^{++} channels and influx of Ca^{++} from extracellular fluid[104, 105].

Glucose supply from the diet varies substantially throughout 24-hours from low concentrations during periods of complete fasting to high concentrations during postprandial glucose loads. In the basal (fasted state) the brain is the primary user of glucose, with other tissues using alternative fuels such as lipids, amino acids and proteins. With high insulin concentrations, as occur after a meal, whole body glucose utilisation is increased with skeletal muscle contributing substantially to the rise in glucose utilisation[106]. Glucose concentrations in blood are controlled primarily by insulin. The diverse actions of insulin that range from growth and differentiation in fetal life to carbohydrate, lipid and protein metabolism in postnatal life, are made possible by the extensive post-receptor signalling cascades in insulin sensitive tissues (Figure 1-2). Insulin's main actions are to inhibit endogenous glucose production by the liver and to stimulate glucose utilisation in peripheral tissues like skeletal muscle and adipose tissue[107, 108].

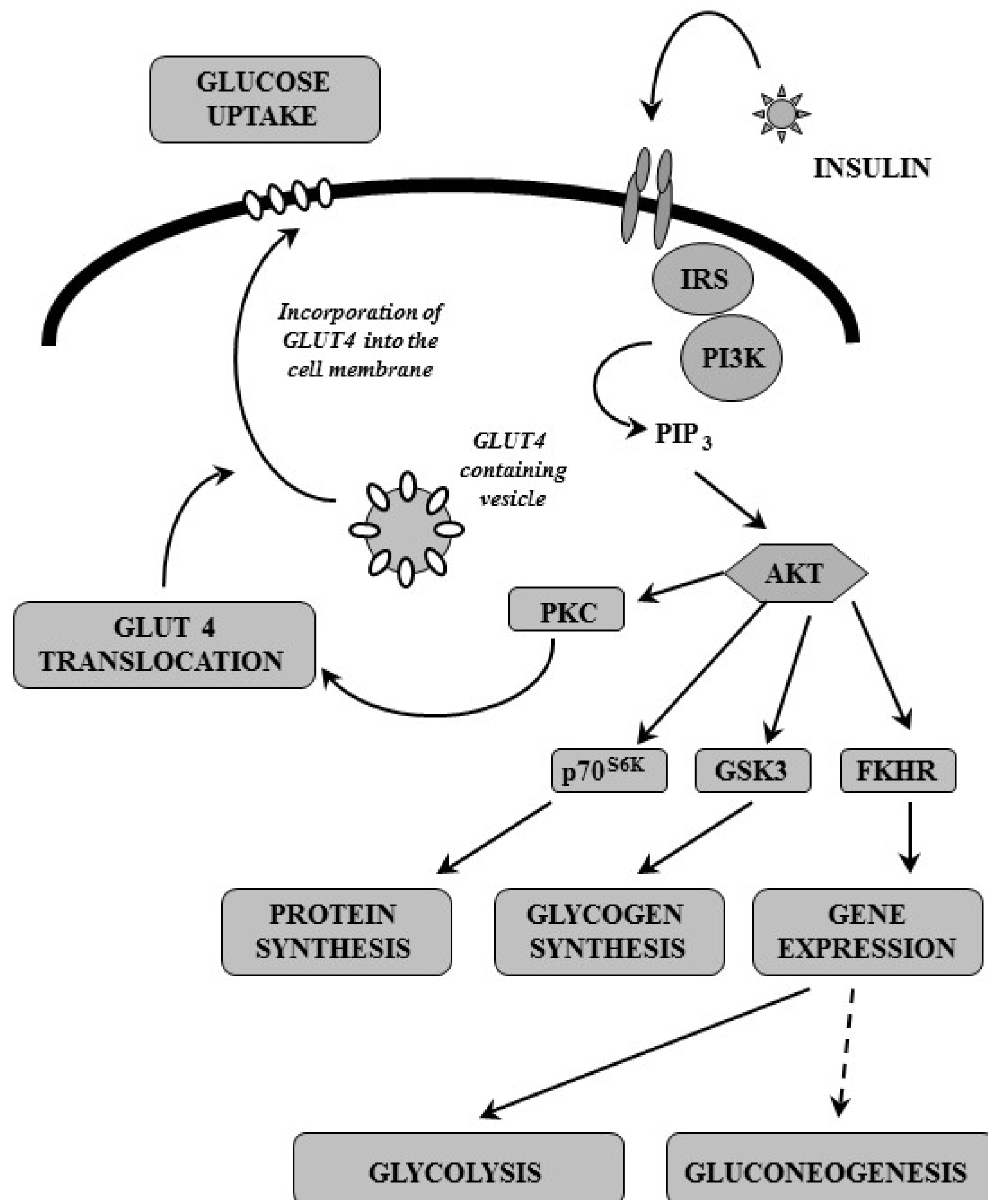


Figure 1-2 Summary of the Insulin Signalling Pathway

IRS, Insulin Receptor Substrate; PI3K, phosphatidylinositol 3-Kinase,
PIP_3, Trisphosphorylated inositol; AKT, product of the AKT protooncogene;
PKC, Protein Kinase C; GSK3, Glycogen Synthase Kinase 3;
FKHR, Forkhead in Human Rhabdomyosarcoma; GLUT4, Glucose Transporter 4.
$p70^{S6K}$, Ribosomal protein S6 kinase beta-1
Solid line: stimulated by insulin. Dashed line: Inhibited by insulin.

1.6.3 The Insulin Signalling Pathway

When insulin binds to its tyrosine kinase receptor, the receptor dimerises to initiate the phosphorylation of the tyrosines on the intracellular domain of the receptor. This in turn, initiates the post-receptor insulin signalling pathway by the phosphorylation of the insulin receptor substrate (IRS), first the protein of insulin transduction signalling (Figure 1-2). IRS activates the p85a and p110 subunits of phosphatidylinositol 3-Kinase (PI3K) to activate Product of the AKT protooncogene (AKT) or protein kinase B (Figure 1-3). This initiates the translocation of GLUT4 to increase glucose uptake in insulin responsive tissues like skeletal muscle (major, Figure 1-3) and adipose tissue (minor, Figure 1-5). Phosphorylation of AKT activates other downstream cascade pathways increasing protein (skeletal muscle), and glycogen (skeletal muscle and liver) synthesis while suppressing endogenous glucose production by the liver (major, Figure 1-4) and kidney (minor).

1.6.4 Insulin Action in Key Target Tissues

Insulin’s primary tissue targets are skeletal muscle, adipose tissue and the liver. In muscle, the primary action of insulin is to activate glucose, amino acid and fat uptake to increase utilisation for ATP production and storage of glucose as glycogen, protein synthesis, and intramuscular fat deposition. In adipose, insulin acts to store glucose, FFA and amino acids as fats and triglycerides. In liver, insulin acts to inhibit glucose production via gluconeogensis and glycogenolysis while stimulating glycogen synthesis. Insulin receptors are also found in the brain and in the kidney.

1.6.4.1 *Insulin action in skeletal muscle*

Skeletal muscle is one of the major target tissues of insulin (Figure 1-3). Insulin’s main action in muscle is to increase the rate of glucose uptake by stimulating translocation of the insulin sensitive glucose transporter 4 (GLUT4), increasing glucose clearance from

blood[109]. On binding to its receptor, insulin stimulates the formation of the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) through the interaction of IRS and PI3K. PIP_3 initiates many phosphorylation cascades resulting in a net increase in glucose uptake and utilisation for energy and storage within the muscle cells (Figure 1-3). PIP_3 activates protein kinase C (PKC) facilitating the translocation of intracellular vesicles containing GLUT4 to the cell membrane, where GLUT4 can facilitate glucose uptake into the cell. Insulin also influences metabolic processes within skeletal muscle through the up-regulation and/or activation of signalling pathways, through the dephosphorylation of key rate limiting enzymes involved in both storage of glucose as glycogen and utilisation of glucose for energy through glycolysis. In addition, other enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), which is involved in the production of glucose-6-phosphate via gluconeogenesis, are inhibited by insulin. Insulin and the increased intracellular glucose-6-phosphate concentration also lead to activation of fatty acid synthesis and an increased intracellular pool of FFA. PIP_3 also acts on lipoprotein lipases to cleave and facilitate the uptake of FFAs from circulating lipoproteins in the blood. In addition, other fatty acid transporters like fatty acid translocase/cluster of differentiation 36 (FAT/CD36) can be translocated to the cell membrane from intracellular stores in response to insulin, or exercise-induced increases in fatty acid demand[110].

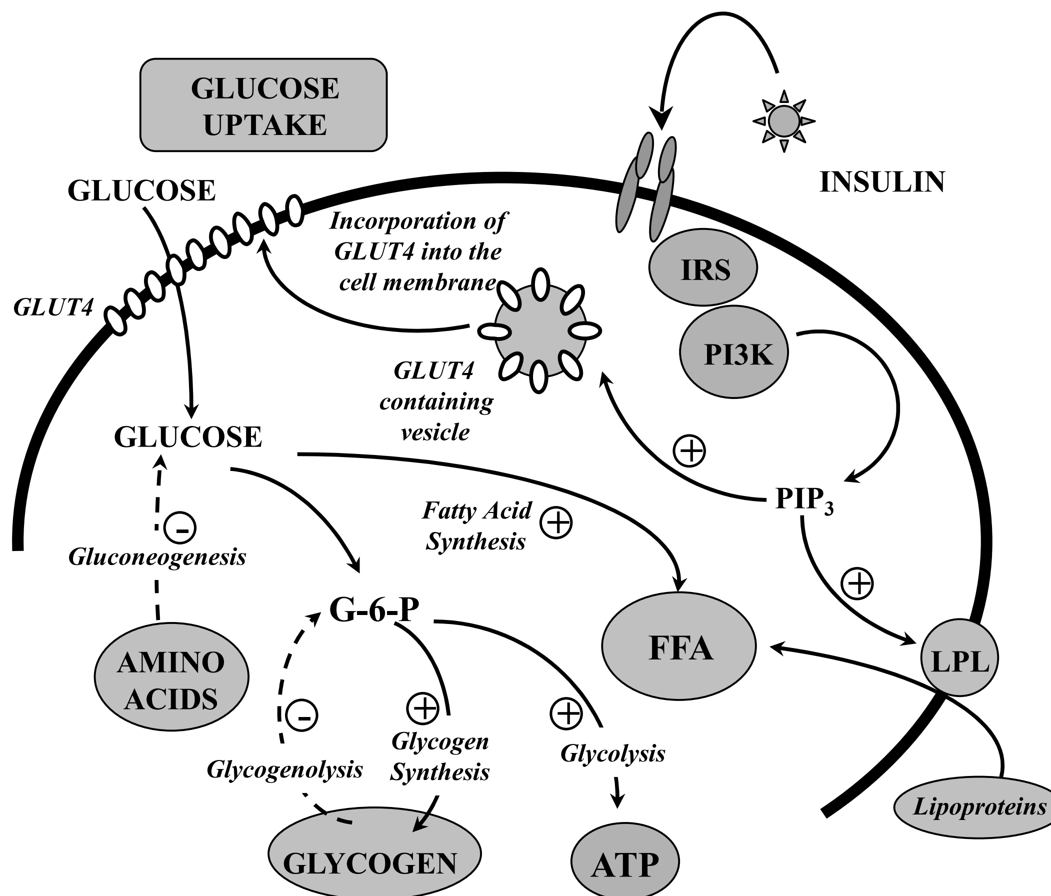


Figure 1-3 Mechanism of Insulin Action in Skeletal Muscle

IRS, Insulin Receptor Substrate; PI3K, phosphatidylinositol 3-Kinase;

PIP_3, Trisphosphorylated inositol; LPL, Lipoprotein Lipase;

FFA, Free Fatty Acids; ATP, Adenosine Triphosphate;

G-6-P, Glucose-6-Phosphate; GLUT4, Glucose Transporter 4.

⊕ Stimulated by the action of insulin. ⊖ Inhibited by the action of insulin.

1.6.4.2 *Insulin action in liver*

Liver and kidney are the only two tissues that contain significant amounts of glucose-6-phosphatase, the enzyme responsible for the conversion of glucose-6-phosphate to glucose[111]. Liver is also a major site of storage of glucose as glycogen. Therefore the liver is able to regulate the concentrations of blood glucose by its storage via glycogenesis and its release via gluconeogenesis and glycogenolysis[97].

Glucose transport into the liver is not controlled by insulin as the major glucose transporter in liver is GLUT2, and to a minor extent GLUT1, and these are not insulin responsive[109]. GLUT2 is a low affinity, but high capacity, glucose transporter making it well suited to a tissue such as the liver, where the role is to facilitate both uptake and efflux of glucose depending on hormonal and nutritional status[97]. Insulin increases storage of glucose as glycogen in the liver, by the activation of glycogen synthase and deactivation of glycogenolysis within the hepatocytes (Figure 1-4). Similar to skeletal muscle, insulin binding to its receptor stimulates the formation of the second messenger PIP_3 through the interaction of the IRS and PI3K, which initiates many phosphorylation cascades resulting in a net increase in glucose storage and inhibition of glucose production by the hepatocytes. Insulin sensitive enzymes like fructose-1,6-phosphatase and PEPCK, the rate limiting enzyme in the gluconeogenesis pathway, are inhibited by insulin to reduce the conversion of amino acids to glucose-6-phosphate[111]. By inhibiting glucose-6-phosphatase, insulin inhibits the formation of glucose and thus decreases hepatic glucose production. In addition, insulin stimulates fatty acid synthesis, converting glucose to FFAs, which are then mobilised to lipoproteins in the circulation, and transported to adipose tissue in the periphery.

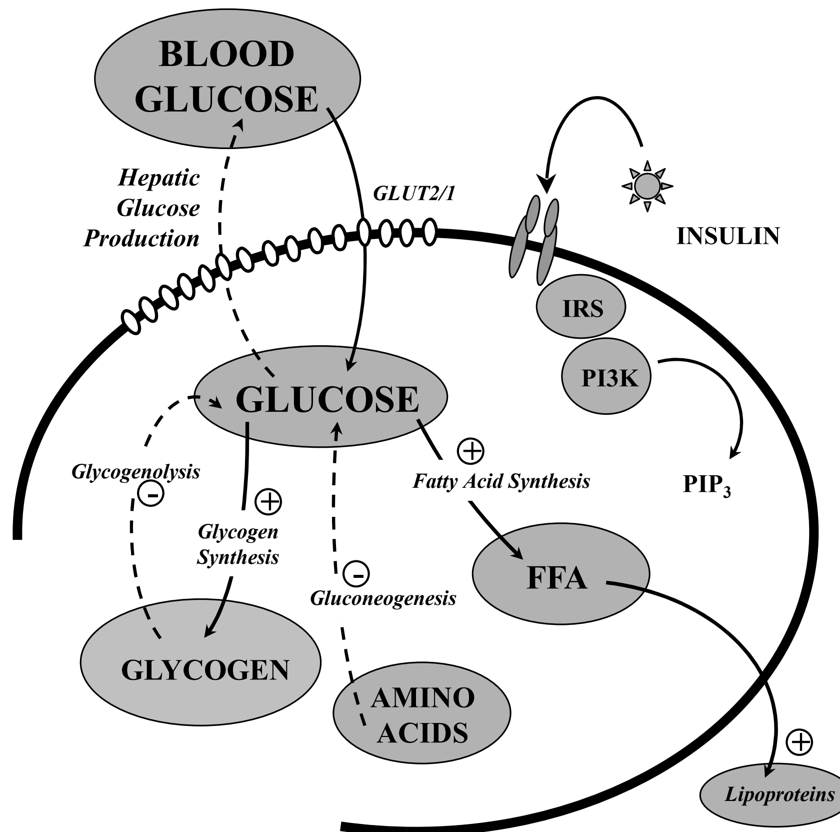


Figure 1-4 Mechanism of Insulin Action in the Liver

IRS, Insulin Receptor Substrate; PI3K, phosphatidylinositol 3-Kinase,

PIP_3, Trisphosphorylated inositol; FFA, Free Fatty Acids;

GLUT2/1 Glucose transporter isoforms 2 and 1

⊕ Stimulated by the action of insulin. ⊖ Inhibited by the action of insulin.

1.6.4.3 *Insulin action in kidney*

Like the liver, the kidney contains glucose-6-phosphatase, the enzyme responsible for the conversion of glucose-6-phosphate to glucose, and is the main extra-hepatic site of gluconeogenesis[111, 112]. However, the kidney only produces a significant amount of glucose after prolonged starvation, providing about 10% of total blood glucose[111], while others report figures from 5%-30% in the postprandial state[112]. Therefore, in physiological conditions the liver is the primary source of endogenous glucose production[111]. In addition, the production of glucose by the kidney is increased in the postprandial state when insulin and glucose concentrations are high, suggesting that, unlike the liver, insulin does not have a major role in suppressing glucose production in this tissue[113].

1.6.4.4 *Insulin action in adipose tissue*

Insulin's primary action in adipose tissue is to increase the rate of glucose uptake by the increased translocation of the insulin-sensitive GLUT4 to the cell surface[109]. Insulin also acts on lipoprotein lipases in the membrane, which increases the uptake of FFA into the cells. The increased intracellular glucose and FFA are stored as triglycerides in adipose cells (Figure 1-5). The action of insulin on adipocytes leads to decreased circulating FFA, which in turn has an indirect effect on the liver to reduce hepatic glucose production[97].

1.6.4.5 *Insulin action in the brain*

The brain contains insulin receptors, with high concentrations reported in the olfactory bulbs, hypothalamus and hippocampus of the guinea pig, rat and human brain[114-116]. Insulin has many actions as a neuropeptide in the brain, including modulation of appetite, peripheral metabolism, and behaviour[117, 118]. Central administration of insulin into the ventricular space acts as a positive feedback for pancreatic insulin release[119, 120]. One important role of insulin in the brain is its effect on food intake, body weight and energy balance, where

centrally-available insulin acts to suppress appetite and therefore reduces fat mass[117]. In rats, direct infusion of an antisense oligodeoxynucleotide targeting mRNA of the insulin receptor into the third ventricle of the brain specifically reduces hypothalamic insulin receptor number, causes hyperphagia and obesity and attenuates hepatic but not peripheral insulin sensitivity[121]. Therefore, insulin resistance of the brain may also act indirectly on the liver via increased glucose and FFA availability in the blood leading to the accumulation of further adipose tissue as well as the direct central actions on nutrient intake, obesity and impaired positive feedback on insulin secretion.

1.6.5 Insulin-Stimulated Glucose Disposition

Insulin action on glucose metabolism has two determinants; the amount of available insulin at the receptor (insulin secretion) and the sensitivity of the receptor and post-receptor events to initiate insulin's actions in its target tissue (insulin sensitivity). Failure of both factors are known contributors to the risk of the development of T2DM[122]. Multiplication of insulin secretion and insulin sensitivity indices gives the insulin-stimulated glucose disposition index (DI) which is constant for individuals with the same degree of glucose tolerance. The DI can also be used to evaluate the efficacy of glucose homeostasis via assessment of an individual's ability to increase β-cell insulin secretion as insulin sensitivity decreases[123]. The DI can determine if an individual has impaired insulin secretion relative to their insulin sensitivity, therefore can assess their risk of T2DM[122].

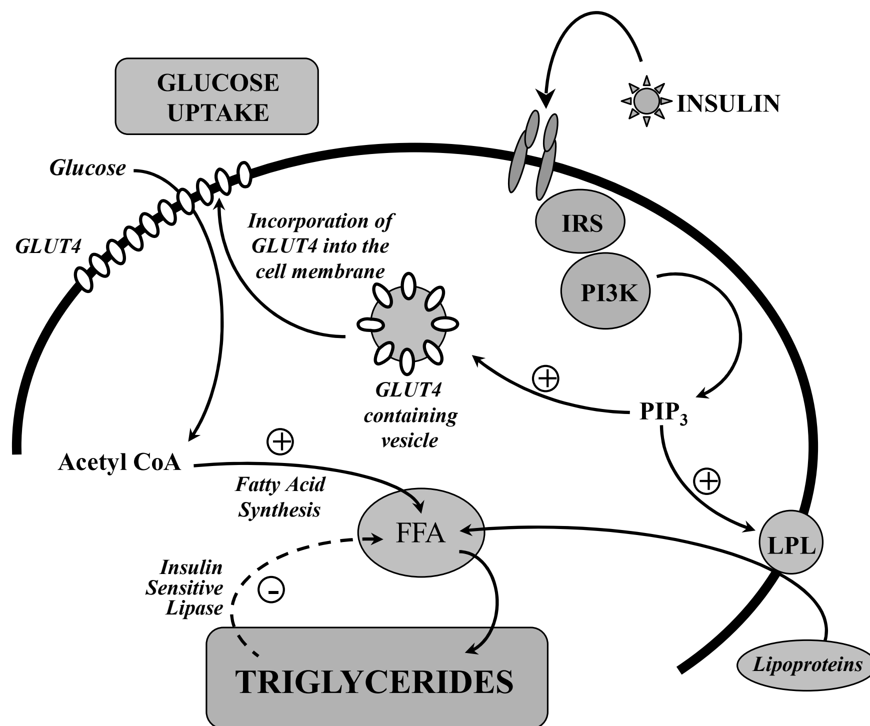


Figure 1-5 Mechanism of Insulin Action in Adipose Tissue

IRS, Insulin Receptor Substrate; PI3K, phosphatidylinositol 3-Kinase,

PIP_3, Trisphosphorylated inositol; LPL, Lipoprotein Lipase;

FFA, Free Fatty Acids; GLUT4, Glucose Transporter 4;

Acetyl CoA, Acetyl Coenzyme A.

⊕ Stimulated by the action of insulin. ⊖ Inhibited by the action of insulin.

1.7 Failure of Glucose Homeostasis - Type 2 Diabetes Mellitus

T2DM is an inter-organ disease where failure of insulin action (insulin resistance) at skeletal muscle, adipose tissue and liver, impaired insulin secretion from the pancreas and impaired glucose effectiveness are all involved in the loss of glucose homeostatic control[107, 124, 125]. Although insulin resistance in skeletal muscle is the primary defect and may develop decades prior to the total failure of insulin secretion it is estimated that those with T2DM have a reduction in their insulin secretion output by up to 50%, independent of their current insulin resistance[126, 127]. Therefore, failure to maintain an appropriate insulin secretion response, relative to the sensitivity of the insulin target tissues, or the insulin disposition index, is crucial to the pathogenesis of T2DM[122].

Obesity due to a sedentary lifestyle and high caloric diets are the major risk factors for the metabolic syndrome of which T2DM is one of the primary components[124, 128]. FFA release from the increased adipose depots leads to an accumulation in skeletal muscle and liver which can down regulate insulin receptor numbers[126]. FFAs are an alternate fuel source for skeletal muscle and therefore high circulating FFAs impairs glucose utilisation by muscle[128-130]. In addition, elevated FFAs induce apoptosis of β-cells which reduces the compensatory hyperinsulinaemia associated with insulin resistance[128, 130, 131]. FFAs further impair insulin sensitivity in skeletal muscle, adipose tissue and liver by impairing peripheral glucose uptake and inhibiting endogenous glucose production, stimulating hepatic gluconeogenesis flux rate and reducing glucose-induced glucose uptake in skeletal muscle, assessed during a hyperinsulinaemic-euglycaemic clamp[132-135]. In addition, adipokines such as resistin and TNF-α, which are elevated in obesity, alter insulin signalling in target tissues perhaps via downregulation of tyrosine kinase activity[107, 136, 137]. Therefore, high adiposity especially central adiposity and FFA leads to a resultant systemic insulin resistance by reducing insulin secretion and interfering with insulin signalling[107, 126, 128, 129, 136, 137].

Due to chronic hyperglycaemia, even in the fasted state, glucose uptake is high into cells that express glucose transporters that are non-insulin dependent glucose transporters (not GLUT4) including, smooth muscle, cardiac muscle, macrophages, and endothelium[138]. This leads to increased levels of pyruvate entering the citric acid cycle and thus over-production of the high redox potential molecule NADH. This in turn elevates H_2O_2 and other reactive oxygen species in the mitochondria of these tissues[138]. Hyperglycaemia also increases advanced glycation end products as up to 35% of glucose is metabolised using alternate pathways such as the polyol pathway[138]. Once oxidised, these reactive oxygen species and advanced glycation end products further cause oxidative stress to tissues, contributing to the mitochondrial oxidative stress of T2DM and the associated vascular disease, poor endothelial function and low immune function[138].

1.8 Measuring Indices of Glucose Homeostasis *in vivo*

1.8.1 Insulin Tolerance Test

Probably the simplest way to directly assess insulin action *in vivo* is the insulin tolerance test (ITT)[139], which involves a bolus intravenous (*i.v.*) injection of insulin to induce glucose disappearance from the blood[139, 140]. The ITT has been criticised as a clinical measure of insulin sensitivity due to the unpleasant symptoms of hypoglycaemia, as well as the potential for counter-regulatory effects of the hypothalamic-pituitary-adrenal axis via the release of cortisol and growth hormone to suppress glucose disappearance rates[139]. Importantly this method has been modified to include intraperitoneal injections of insulin enabling its use in small animals such as mouse, and results show good agreement with insulin sensitivity measured by other methods, such as the hyperinsulinaemic-euglycaemic clamp[141].

1.8.2 Hyperinsulinaemic-Euglycaemic Clamp

The hyperinsulinaemic-euglycaemic clamp (HEC) is considered the gold-standard method to assess insulin sensitivity *in vivo*. The method utilises a continuous infusion of insulin, either at a single or in a sequential series of infusion rates, with a variable glucose infusion rate (GIR) to maintain glucose levels at the basal (fasting) concentration measured prior to the insulin infusion. The GIR required at steady state to maintain euglycaemia during the insulin infusion (calculated using a previously published algorithm) provides an index of insulin sensitivity[142].

One advantage of the HEC over the insulin tolerance test as a method to measure response to insulin is that by maintaining plasma glucose concentrations during insulin stimulation the side effects of hypoglycaemia are alleviated[142]. In addition, the neuroendocrine homeostatic mechanisms that occur in response to hyperglycaemia are also reduced[142]. The HEC is an accurate measure to quantify insulin sensitivity and since only small blood samples are required for checking of euglycaemia it has been established and used extensively in a number of experimental animal models, including rats[143-145] and mice[146-148] and in non-human primates such as rhesus monkeys[149-153] and baboons[154]. Continuous infusion of [3-^{3}H]-glucose tracers prior to and during HEC allows assessment of rates of glucose utilisation, glycolysis and of storage, primarily by skeletal muscle, as well as the rate of endogenous glucose production, primarily by the liver, in the basal and insulin-stimulated states[142, 145, 152].

The main disadvantages of the HEC is that due to its complexity, it is a costly, labour and time intensive test[155]. When glucose tracers are used, larger blood volumes are required, which, when combined with the frequent blood samples required for monitoring blood glucose levels, can be a disadvantage for use in small animal models. In addition, surgical catheterisation is required therefore, experiments immediately following surgery may induce stress responses, or acute experiments are performed under anaesthesia both of which alter insulin sensitivity in rodents (reviewed in [156]).

1.8.3 Hyperglycaemic Clamp

The hyperglycaemic clamp is similar to that of the HEC except that a bolus of glucose is infused acutely to achieve a rise in plasma glucose levels of 125 mg/dl above basal levels[142]. This raised glucose set point is then maintained by a variable glucose infusion throughout the clamp[142]. Because the glucose concentrations are constant during the clamp the amount of glucose infused is a measure of glucose metabolism[142]. The hyperglycaemic clamp can quantify the response and therefore the sensitivity of the β-cell to a rise in glucose, the early and late phases of insulin release can be measured independently and whole body glucose metabolism can be assessed[142]. The main disadvantages of the hyperglycaemic clamp are those of the HEC. The hyperglycaemic clamp like the HEC is a costly, labour and time intensive test as well as posing problems for small animal models as significant blood volumes may be required in assessing glucose metabolism over a time course[142, 155].

1.8.4 Glucose Tolerance Test

The glucose tolerance test (GTT) is a simple *in vivo* test used clinically to determine if an individual is glucose tolerant ($x < 7.8$ mmol/l), intolerant (7.8 mmol/l $> x <$ 11.1 mmol/l) or diabetic ($x >$ 11.1 mmol/l) where x is the glucose concentration 2-hours post the glucose load[157]. Glucose is given as a bolus load, either intravenously (IVGTT) or orally (OGTT). Measurement of insulin concentrations during a GTT allows assessment of insulin secretion in response to a glucose challenge. This provides an index of pancreatic and therefore β-cell responsiveness to high circulating plasma glucose. The GTT operates on the principle that insulin will be released in a homeostatic manner to counter rises in blood glucose concentration.

1.8.4.1 *Oral glucose tolerance test*

The OGTT is a simple test that involves administration of an oral glucose load, typically of 75 g in humans, following an overnight fast[158]. Blood samples are collected at 0 (basal), 30, 60, and 120 minutes to determine profiles of glucose clearance and insulin responsiveness[158]. The glucose load is sometimes given as a meal, known as a meal tolerance test (MTT), which mimics more closely the physiological, postprandial condition[155]. The oral minimal model is a computation method that enables the estimation of insulin sensitivity, β-cell function and hepatic insulin extraction based on the results from the OGTT[158]. A disadvantage of the OGTT and MTT is the assumption that there is low inter- and intra-individual variation in the absorption of glucose[159]. In addition, due to variations in oral glucose loading regimens used experimentally between species and within the same species comparisons of studies that report glucose tolerance outcomes based on this test may be more difficult[158, 160-163].

1.8.4.2 *Frequently sampled intravenous glucose tolerance test*

The frequently sampled IVGTT typically involves a schedule of either fifteen samples to 180 minutes or twenty-six samples to 240 minutes after an *i.v.* bolus of glucose at 0.3-0.5 $g.kg^{-1}$[164]. The 15-sample schedule has become more accepted in large cohort experiments in humans[164]. The frequently sampled IVGTT utilises the glucose and insulin profiles over the 3 to 4-hour glucose tolerance test to indirectly calculate glucose effectiveness and insulin sensitivity using the minimal model technique as described by Bergman *et al*[91, 159, 165, 166]. The main advantage of the IVGTT over the OGTT is it avoids the uncertainty of gastric emptying and thus reduces the effects of variable gastric/intestinal motility rates, and therefore glucose absorption rates, on outcome measures[159]. However, this test requires additional care due to the use of *iv* glucose and heparinised saline infusions as well as insertion of a catheter for frequent *i.v.* blood sampling[159].

1.8.5 Indices Based on Fasting Insulin and Glucose

1.8.5.1 *Fasting insulin and fasting insulin to glucose ratios*

Fasting insulin concentration and the fasting glucose to insulin ratio are also utilised as measures of glucose homeostasis and insulin sensitivity *in vivo*[91, 167]. A general advantage of these indices is that they rely on measurement of glucose and insulin in a single blood sample following an overnight fast, hence they provide a simple, inexpensive assessment of insulin resistance especially in large clinical studies where direct measures such as the HEC may not be feasible. In the fasting state glucose is in homeostasis, with endogenous glucose production by the liver, and to a small degree by the kidney, balanced with whole-body glucose disposal. Therefore, the level of insulin secreted under fasting conditions reflects the sensitivity of the peripheral tissues for insulin dependent glucose disposal (mainly skeletal muscle). Calculation of fasting glucose to insulin ratio is also used as a measure of insulin resistance as it gives a measure of glucose control for a given insulin secretion.

However, calculating insulin action indirectly using fasting concentrations does not differentiate low insulin secretion due to a relatively high insulin sensitivity of the tissues from individuals with failing β-cells and therefore reduced insulin secretion. Therefore, as glucose tolerance and insulin sensitivity decreases with ageing and the progression of β-cell exhaustion leads to diminished compensatory hyperinsulinemia the correlation of insulin sensitivity measured directly by HEC with that assessed by fasting insulin loses strength. Independent measures of both insulin secretion and sensitivity are required to determine relative secretion to sensitivity or insulin-stimulated glucose DI as calculated by the multiplication of insulin's sensitivity and its secretion[122].

1.8.5.2 *Homeostatic model assessment (HOMA)*

The homeostatic model assessment (HOMA) or the updated HOMA2 uses paired fasting insulin and glucose concentrations to calculate insulin sensitivity and insulin secretion through a computer derived simulation based on feedback mechanisms[167, 168]. The HOMA insulin resistance (HOMA-IR) model correlates reasonably well with insulin sensitivities derived from HEC in adults without severe impairment of glucose homeostasis[167, 168]. However, this correlation becomes skewed when selecting those with either high insulin sensitivity or with severe impairment such as in T2DM[155].

1.8.5.3 *Quantitative insulin sensitivity check index (QUICKI)*

The quantitative insulin sensitivity check index (QUICKI) is a computer modelled calculation of insulin sensitivity using the inverse of the sum of the logarithms of basal glucose and log of insulin concentrations[169]. With ageing and in diabetic patients the glucose to insulin ratio begins to rise due to the loss of compensatory insulin secretion. Using the inverse of the sum of the logarithms reduces these standard errors of the high glucose to insulin ratio measures in these cohorts[169].

1.9 **Prenatal Programming of Glucose Metabolism in Humans**

1.9.1 Prenatal Programming of Impaired Glucose Homeostasis and T2DM

Historical and geographical evidence provided the initial evidence for the prenatal origin of impaired glucose homeostasis and subsequent disease[170, 171]. Offspring subjected to the Dutch Winter famine in late gestation, when fetal growth is at its greatest, are small at birth and as adults have increased risk of impaired glucose tolerance, T2DM and the metabolic syndrome compared to those not affected by famine or exposed in early gestation only[172]. This suggests that the timing of the insult is also crucial to the programming of metabolic dysfunction

later in life. These effects of famine on later glucose homeostasis have been supported by studies of other war-time and famine cohorts[173].

Geographical studies predominantly carried out in England and Wales have shown that areas of low socio-economic status, in industrial areas to the north of England and lower socio-economic status to the west, had the highest rates of cardiovascular disease in adults aged 35-71 years in 1968-1978[171]. When compared to the infant death rate (1901-1910) in the same geographical zones, areas of higher rates of infantile death had higher rates of cardiovascular disease in later life[171]. This is consistent with correlation analysis of areas of high perinatal death rates today and mortality from stroke in the USA[174, 175]. This “Stroke Belt” occurs in the lower southern states, which are the lower socio-economic areas of the USA[174, 175].

These studies of famine and effects of socio-economic status on both perinatal outcomes and adult disease rates have given insight into the link between associations of early environment and diseases in later life[171-175]. Several studies since have assessed individual measures of size at birth in numerous cohorts around the world and have confirmed these associations across the normal spectra of size at birth. This has led to systematic reviews[65, 176-178] assessing the overall impact of small size at birth on later insulin sensitivity and glucose metabolism. Several systematic reviews have now shown that small size at birth is associated with increased risk of insulin resistance, T2DM and the metabolic syndrome later in life[65, 176-178].

The systematic review carried out by Newsome *et al* in 2003[176] showed that the link between low birth weight and a risk of impaired glucose homeostasis was consistent across multiple published studies, however, this review did not discriminate between small size at birth due to prematurity and that due to IUGR[176].

A systematic review by Harder *et al* in 2007 examined the shape of the association of birth weight and subsequent risk of T2DM[178]. Some previous studies report a linear relationship between small size at birth and T2DM risk implying that high birth weight offers

protection against such risk. However, Harder et al (2007) report a J- or U-shaped relationship with both extremes in birth weight having increased risk of T2DM[178] and this has also been supported in other systematic reviews[177, 178]. Most recently, Whincup *et al* 2008 confirmed that the adverse effects of low birth weight on risk of T2DM persisted even after correction for gestational age and current socioeconomic status[177].

These systematic reviews reveal that the associations of insulin resistance, glucose intolerance, T2DM or the metabolic syndrome with measures of small size at birth occur across the normal range in size at birth, and not only in IUGR individuals[176-178]. The systematic reviews by Whincup *et al* 2008 and Harder *et al* 2007 also give information on the shape of the resultant J or U-shaped relationship[177, 178]. This implies that being at the high extreme of birth weight is a risk factor rather than protective against metabolic disease. Exposure to maternal diabetes or obesity *in utero* are risk factors for the development of T2DM, obesity and the metabolic syndrome in childhood[179-182], thus being large for gestational age due to nutrient excess *in utero* may also contribute to developmental programming of T2DM and metabolic dysfunction.

Other hypotheses linking low birth weight to later adult disease propose a genetic link. As insulin is a potent growth factor *in utero*, genetically determined insulin resistance would be expected to result in both a reduced size at birth and a predisposition to T2DM later in life. However, although in Pima Indians, a population “genetically” prone to T2DM, LBW individuals are thinner at ages 5-29 years, they are more insulin resistant relative to their body size than those of normal birth weight[181]. Studies in twins have also given some insight as to the genotype versus phenotype contributions to T2DM, although the use of twins in such studies has received some criticism[183]. In monozygotic twins discordant for T2DM, the twin with adult T2DM had a lower birth weight compared to the one without[184]. In dizygotic twins where both twins have T2DM as adults the twin with the lowest birth weight had the highest fasting plasma glucose concentration after OGTT[184]. This supports the suggestion that genetic factors do not

solely account for the incidence of T2DM, and that intrauterine events, in part, program glucose metabolism in later life. Overall, these studies demonstrate that low birth weight is an important contributor to the risk of developing diseases later in life. Therefore, the underlying mechanisms that mediate the effects of early life environmental determinants of impaired glucose homeostasis and T2DM require further investigation so that interventions can be developed.

1.9.2 Prenatal Programming of Glucose Effectiveness

Few studies have examined the effects of IUGR on glucose effectiveness. In young adults, glucose effectiveness was increased in males who were light or short at birth but no association with size at birth was found in females[185]. In pre-pubertal children, glucose effectiveness was unaltered in short children following IUGR compared to controls[186]. Therefore, these studies suggest that reduced glucose effectiveness is not contributing to impaired glucose homeostasis following IUGR, at least in younger cohorts.

1.9.3 Prenatal Programming of Insulin Sensitivity

Many studies to date have utilised the gold standard HEC[187-199], minimal model techniques[185, 199-208], basal plasma insulin to glucose concentrations[208, 209], or the ITT[210] to examine associations between size at birth and insulin sensitivity. Overall, systematic reviews have shown that in the majority of studies there is a positive correlation between adult insulin sensitivity and size at birth[176]. In individuals over 50 years of age, the development of obesity strengthens the association of size at birth with insulin sensitivity[187, 201, 210]. In the age group from 30-50 years, two studies have shown a reduced insulin sensitivity with decreasing size at birth using the minimal model[202] and HEC[188] techniques. Another study showed no association between birth weight and insulin sensitivity in this age group using the minimal model approach, however[204]. Similar associations of impaired insulin action with reduced size at birth have been reported in 20-30 year-old adults[189, 206, 207, 209], although other studies have shown

associations in only females[205], or only males[185] or have found no effects of size at birth on insulin sensitivity in this age group[199]. Insulin sensitivity in IUGR children is enhanced after birth and up to 1 year of age, which may explain in part the increased rates of growth or catch-up[67, 68]. After the catch-up period, a positive correlation of insulin sensitivity with size at birth following IUGR is also evident as early as four years of age suggesting that there is an early switch in insulin sensitivity[85, 192, 208, 211, 212]. However, one study in Indian neonates showed impairment in insulin sensitivity and increased adiposity present at the time of birth rather than a switch initiated by catch-up[213]. Therefore, the variability in the direction of the correlation of size at birth with insulin sensitivity depends on the age group of the children tested and ethnicity. In IUGR neonates, insulin sensitivity may still be enhanced when insulin sensitivity is assessed in the catch-up growth phase[67, 68]. In contrast when catch-up growth is complete, which would be the case in studies of four-year-old children, IUGR consistently predicts impaired insulin sensitivity directly using HEC[192, 212] or indirectly using HOMA[85] or insulin and /or glucose to insulin rations[208, 211]. One longitudinal study in children from birth to three years showed that IUGR infants transition from an insulin sensitivity to an insulin resistant state in their first three years of life[214]. This suggests that at birth and in the early catch-up growth phase insulin sensitivity is high in IUGR infants; however, in childhood with the effects of ageing and other lifestyle events, the associations are reversed such that associations between impaired insulin sensitivity and IUGR are enhanced.

1.9.4 Prenatal Programming of Pancreatic Function and Insulin Secretion

In young adults to 30 years, six of nine studies[185, 205, 207, 215-217] describe a negative association between fasting insulin concentrations and size at birth, while one showed no relationship[199] and two showed a positive relationship[206, 218]. One study in young adults (20-21 years) examined males and females separately[185] and showed that fasting insulin concentrations tended to decrease with decreasing birth weight in males, but not in females[185]. In addition,

insulin secretion was negatively correlated with birth weight and length in males but not in females suggesting a sex-specific onset of alterations in insulin secretion and action[185]. Three of seven studies directly measuring insulin secretion found no association with size at birth[199, 200, 217], two described a negative association[189, 205] and two a positive association[206, 218]. Most of the studies measuring insulin secretion have not measured insulin resistance independently. In those studies where insulin sensitivity has also been measured, IUGR predicts impaired insulin secretion relative to insulin sensitivity[195]. Therefore, the variability between these studies may reflect changes in insulin resistance and the capacity for compensatory increases in insulin secretion between cohorts.

With ageing the correlations between insulin secretion and size at birth begin to become more consistent. In adults with mean ages of 30 to 50 years, all five studies describe a negative association between fasting insulin concentrations and size at birth[203, 219-222]. Four studies assessed glucose stimulated insulin secretion in this age group (30 to 50 years) and all found negative associations between insulin secretion and size at birth[201, 203, 223, 224], suggesting that the pancreas is compensating for an impaired insulin sensitivity in this age group. These negative associations of size at birth with basal insulin concentrations and insulin secretion after a glucose load in the 30 to 50-year-old age group are more consistent with prenatal programming of insulin resistance rather than β-cell insufficiency and reduced insulin secretion.

Many studies have assessed both basal insulin concentrations and insulin secretion directly during OGTT/IVGTT to examine the associations with size at birth. In studies of adults with mean ages from 50 to 70 years, six of seven studies[65, 184, 201, 225-227] described negative associations between fasting insulin concentrations and size at birth while only one[228] showed no association. In studies where the mean age tended to be older (~65 years) the negative associations of birth size with fasting insulin were stronger suggesting that the hyperinsulinaemia was still present and that IUGR adults maintained hypersecretion of insulin, due to insulin resistance, later in life[184, 225, 226].

Nevertheless, in IUGR children where insulin-stimulated glucose disposition has been measured, insulin secretion relative to insulin sensitivity is reduced, suggesting that the ability of the β-cell to compensate for the insulin resistance adds to the risk of T2DM later in life[212, 214]. Therefore, the effects of insulin resistance and relative β-cell secretory capacity appears early in life following IUGR and is evident even in children.

1.9.5 Mechanisms for Prenatally-Induced Insulin Resistance

Skeletal muscle is the major tissue that determines glucose uptake[92] and primarily utilises glucose by storage to glycogen rather than oxidation to ATP with increased insulin stimulus[106]. The majority of human studies demonstrate a positive correlation between size at birth and whole body insulin sensitivity[176]. However, although IUGR reduces lean tissue, an index of skeletal muscle mass[229], muscle mass itself does not correlate with insulin sensitivity[230]. This suggests that the effects of small size at birth on insulin sensitivity involve intrinsic alterations in insulin responses of skeletal muscle, rather than simply being mediated by changes in total muscle mass[230].

In IUGR humans, insulin sensitivity of skeletal muscle is impaired[189-191, 194-199] and altered expression of insulin signalling genes in skeletal muscle may in part contribute to this decline in insulin action[190, 196, 198, 229, 231-238]. Genes that have reduced expression in human skeletal muscle following IUGR include GLUT4, and the post-receptor signalling proteins protein kinase C-ζ, and the p85α and p110β subunits of phosphoinositol 3-kinase[190, 196, 198, 229, 231-238]. These studies further support a role for intrinsic alterations in skeletal muscle following IUGR leading to impaired insulin action in this tissue.

Increased abdominal fatness in adult men is associated with restricted growth in fetal life[239]. Obesity, especially increases in central or visceral fat, increases circulating FFA, which impair insulin sensitivity[240-242] and lead to diabetes[243, 244] and the metabolic syndrome[244]. This

increased central obesity and associated rise in circulating lipids has been hypothesised to be a primary cause of the insulin resistance seen in centrally obese adults who were small at birth. However, the relationship between insulin sensitivity and size at birth is independent of circulating lipid levels in men and women aged 50 years[245] and of the amount of visceral or subcutaneous fat assessed by computed tomography scanning[200]. In addition, insulin resistance is evident in younger cohorts in their twenties who were IUGR, who still have similar lean mass, total fat and abdominal fat mass as controls[229]. Reduced insulin sensitivity in the liver, resulting in increased endogenous glucose output, can also contribute to development of T2DM. However, human studies have not consistently shown impairment in insulin-suppression of endogenous glucose output following IUGR[190, 197, 246]. In young adults, endogenous glucose production rate was equally suppressed in both IUGR and a control AGA group during HEC suggesting that, at this age, reduced insulin sensitivity of liver, and perhaps kidney, are not major determinants of whole body insulin resistance observed following IUGR[190]. Conversely, insulin suppression of endogenous glucose production is impaired after IUGR in young adults[197] and in the Pima Indians, a population genetically-susceptible to T2DM[246]

Therefore, impaired insulin sensitivity following IUGR cannot be fully explained by effects on mass of the three primary insulin sensitive tissues - skeletal muscle, adipose and liver. In addition, the association of impaired glucose tolerance, T2DM or the metabolic syndrome with small size at birth is also not fully explained by genetic predisposition, decreased insulin secretion, altered glucose effectiveness, impaired insulin action in the liver or circulating lipid concentrations.

1.9.6 Neonatal Catch-Up Growth as an Independent Mechanism for Impaired Insulin Action

Neonatal catch-up growth is also implicated in the programming of impaired insulin sensitivity, via independent mechanisms to the prenatal environment[65, 82, 90]. Catch-up growth

may also be a mechanism contributing to gender-specific programming by early life factors in males as it contributed to more of the total variance in insulin resistance in 49 to 51-year-old males compared to females[65]. Each of the three main mechanisms of the catch-up fat hypothesis potentially contribute to insulin resistance following IUGR: compensatory hyperphagic drive, re-partitioning of nutrients from lean to fat tissue or alteration to metabolism such that thermogenesis is suppressed leading to fuel accumulation[72].

1.9.7 Obesity as Contributing Factor to Metabolic Disease after IUGR

Obesity is a known risk factor for the development of the metabolic syndrome, T2DM, cardiovascular disease, gestational diabetes and reduced life expectancy[247-250]. There is accumulating evidence that small size at birth and catch-up growth are independent risk factors for the development of obesity in childhood which persists into adulthood[72, 251-259]. This increased adiposity after IUGR and catch-up growth occurs primarily in visceral deposits which contribute greater risk for metabolic dysfunction than subcutaneous depots[80]. This increase in fat deposition may in part be due to perinatal programming of both increased feed intake and reduced exercise activity[260].

In addition, others show that muscle mass decreases following IUGR producing a “metabolically dangerous”, high adipose:muscle ratio phenotype in later life[261, 262]. As skeletal muscle is the primary tissue of glucose disposal[107] and adiposity can impair whole body insulin action[263] a high adipose to lean tissue mass would be expected to reduce insulin sensitivity. Hence, it is important to also consider the role of altered body composition in prenatal programming of insulin resistance.

1.9.8 Sex Differences in Perinatal Programming

Many studies in humans have demonstrated sex-specific programming of cardio-metabolic adult outcomes. Systematic reviews suggest that males are more susceptible to

perinatal programming of cardio-metabolic disease in later life compared to females[176, 264, 265]. Consistent with this, in human studies including both sexes, impaired insulin sensitivity was associated with declining size at birth in males but not in females[65, 185, 203]. Conversely, in a large Danish cohort which included 113,801 men and 109,298 women born between 1936-1983, the associations differed with sex, such that in females the relationship was U-shaped with risk of T2DM increased at both the lower and higher birth weight ranges[266]. In men, the relationship between risk of diabetes and birth weight was near inverse throughout the birth weight range to 4 kg and then levelled off thereafter[266]. While associations with risk of T2DM at the extremes in birth weight were stronger in females, within any birth weight category the percentage risk of T2DM was higher in men compared to women, except in the highest birth weight range where percentage risk was comparable between the sexes[266]. Other studies report that impaired insulin action or measures of T2DM correlate with reduced size at birth in males, but do not report outcomes in females[187, 189, 190, 193-195, 197, 198, 201, 229]. When positive correlations between insulin sensitivity and size at birth are evident in both males and females the strength of the associations are approximately 2.5-fold greater in males than in females suggesting an increased impact or susceptibility of males to a sub-optimal intrauterine environment[65, 176]. In 50-year-old men and women, both fasting plasma insulin and insulin during an OGTT correlated negatively with small size at birth in terms of weight and abdominal circumference[227]. Although the associations between size at birth and insulin concentrations were stronger in men than women, when the sexes were analysed separately no significant interaction of sex and size at birth was detected in multiple linear regression analyses, hence not providing sufficient evidence to support a difference between sexes within that cohort[227].

The increased susceptibility to prenatal programming in males may be due to the faster rates of growth earlier in gestation in males with less investment into placental growth than females[267, 268]. This may lead to later adaptations of the placenta to increase its overall size and expand surface area late in gestation. This late gestation adaptation could increase the

metabolic demand of the placenta at the expense of the nutrient supply available to the male fetus[267, 268]. In sheep, the cotyledons of male fetuses are larger in weight and surface area[269]. The faster rates of growth in males *in utero* and the greater demand for nutrients in later gestation may in part put them at higher risk of susceptibility to IUGR if the placental or maternal nutrient supply cannot meet their growth potential. Therefore, in the male fetus this may increase the risk of programmed future dysfunction such as insulin resistance and cardio-metabolic disease.

Another mechanism of the observed sex-specific difference in programmed metabolism of glucose homeostasis is via postnatal actions of sex steroids[270, 271], however, the mechanisms by which sex steroids modulate insulin action are poorly understood[272]. Postnatally, oestrogen deficiency impairs insulin sensitivity and action, and hormone replacement therapy improves insulin sensitivity in postmenopausal women[270]. However, in premenopausal women, basal glucose and FFA concentrations do not differ in the follicular and luteal phases of the oestrous cycle[273]. Sex hormones may be acting to alter insulin sensitivity via the effect of testosterone to decrease adiponectin secretion, which then increases insulin sensitivity and lowers circulating lipids[271]. Women have higher adiponectin levels compared to men with similar body mass index and fat mass[271]. The absence of female sex hormones appears to decrease insulin-mediated whole-body glucose uptake via an impaired insulin-stimulated translocation of GLUT4 to the plasma membrane and by decreased protein expression of glycogen synthase[274]. Testosterone treatment further impairs whole-body insulin-mediated glucose uptake, presumably by additional impairment of glycogen synthase expression[274] or testosterone's inhibition of adiponectin[271]. Androgen excess in Polycystic Ovarian Syndrome is associated with impaired insulin sensitivity, however, IUGR does not raise androgens nor does impaired insulin sensitivity following IUGR correlate with androgen concentration in females[209]. Overall, this evidence does not support a role for androgens as a mediator for programmed insulin resistance following IUGR.

1.10 Need for Animal Models to Investigate Perinatal Programming of Glucose Homeostasis

Human studies have consistently shown a negative relationship of small size at birth with insulin resistance, T2DM or metabolic syndrome[176-178]. Furthermore faster rates of growth in the early postnatal period are positively related to these metabolic diseases[65]. Many human studies are based on size at birth as a proxy measure of *in utero* experience rather than being able to look specifically at types of exposures and their timings, however. Furthermore, the mechanisms that drive these pathophysiological changes before birth and in the early neonatal period and how they are influenced by postnatal life are not fully understood. Therefore, experimental models of IUGR are now utilised in a range of species to assess the effects of specific perturbations at critical windows of development on fetal development, size at birth, growth during the early neonatal period and metabolic outcomes later in life[18].

Assessment of adult outcomes in animal models of IUGR is required to control or reduce confounders such as incorrect estimates of gestational age, maternal diets, and other lifestyle factors such as smoking. Animal models allow control of maternal factors such as parity and maternal phenotype and assessment of insulin sensitivity in models with known size at birth measures and accurate postnatal growth data. Species with shorter gestations have the advantage of allowing transgenerational effects to be assessed, while species with longer gestations allow more accurate assessment of the effects of timing of gestational insults on the programming of adult disease[18].

1.11 Animal Models of IUGR and Impaired Glucose Homeostasis

This section focuses on experimental and naturally occurring IUGR in animal models that have been used to assess the perinatal programming of glucose homeostasis, and models in which relationships of neonatal catch-up growth, appetite and body composition with glucose homeostasis have been described.

1.11.1 Restricted Placental Size and/or Function

As placental insufficiency is the most common cause of human IUGR and even in idiopathic IUGR it is expected that the placenta plays an important part in the reduced size at birth[31], several animal models have been utilised to investigate the role of placental dysfunction in metabolic programming.

1.11.1.1 *Uterine artery ligation/occlusion in the guinea pig and the rat*

Occlusion of the uterine arteries in small animal models of IUGR such as the rat[275-282] and the guinea pig[283-286] have been achieved either by ligating (suturing)[275-282, 286], diathermy ablation (burning)[283, 284] or clamping (ameroid constrictor)[285] placental blood flow and therefore placental nutrient supply to the fetus. This is achieved early in pregnancy and the vessels are occluded under general anaesthesia.

In the guinea pig, uterine artery ligation from 30–35 days of gestation, using diathermy ablation of the uterine arteries in one horn also reduces size at birth, and leads to a disproportionate birth phenotype[283]. Low birth weight offspring (<80 g) combined from both control pregnancies and those with unilateral ablation of the uterine arteries remained smaller than normal birth weight offspring (>90 g) to 98 days postnatal age, but the groups did not differ in weight by 145 days. Offspring were weaned at day 14 and from day 16 to 60 the low birth weight offspring were hyperphagic compared to those of normal birth weight[284]. Although bodyweight did not differ at 145 days of age, low birth weight guinea pigs had a disproportionately greater epididymal white adipose tissue mass, fewer adipocytes, greater adipocyte diameter, and greater lipid content than the high birth weight offspring[284].

Unilateral uterine artery ligation in the rat at day 18 or 19 of gestation (term ~ 22 days) is a commonly used model to reduce size at birth (10%-30% compared to controls) via reduced placental sufficiency (reviewed in[275]). In the rat, low birth weight offspring, following

control pregnancy or unilateral uterine artery ligation, have an increased brain to body weight ratio at 3-4 months of age, suggesting maintenance of an asymmetric IUGR phenotype into adult life[276]. Furthermore, low birth weight was associated with increased blood pressure, increased fasting blood glucose, impaired glucose tolerance and reduced insulin secretion during an IVGTT at 3-4 months[276]. Despite the asymmetrical IUGR following unilateral uterine artery ligation no postnatal catch-up growth was evident[276], unlike that found following IUGR in humans[22]. Furthermore, associations between low birth weight and postnatal blood pressure and glucose metabolism were only found in female rat offspring, unlike the majority of human studies where males are at greater risk of cardio-metabolic programming[65, 185, 203].

Bilateral uterine artery ligation at 19 days gestation in the rat reduces size at birth to a similar degree to that observed following unilateral ligation (reviewed in[275]) and results in a "brain sparing" phenotype in the offspring[275, 280-282]. Bilateral uterine ligation is often associated with a high fetal loss (30% compared to 3% in control or sham operated)[282]. Some studies report comparable body, liver and brain weights by 7 days after birth in IUGR offspring of bilateral uterine artery ligated dams and controls[282], while others report a delay in the acceleration of growth at 7-10 weeks postnatal age[280]. During the accelerated growth phase these restricted rat offspring become hyperglycaemic and hyperinsulinaemic at weeks 15 and 28[280] and progressively more obese, especially in visceral depots, later in life at 28 weeks[280]. At birth, β-cell volume, pancreatic mass and mass/kg body weight are decreased in the IUGR fetuses, and this reduction in β-cell number persists to adult life[280, 281, 287]. In a study where the 6 smallest pups from a ligated pregnancy were compared with the heaviest 12 control pups at 15 weeks of age, levels of HbA1c% were raised, suggesting poorly controlled glycaemia and by week 25 offspring from bilaterally-ligated dams were hyperphagic compared to controls[277]. Therefore, in the rat, bilateral uterine artery ligation, which limits supply of oxygen and nutrients to the fetus, reduces size at birth, and is associated with accelerated postnatal growth, increased adiposity, and impaired glucose tolerance with compensatory hyperinsulinaemia in adults[280].

This adverse phenotype is further amplified with ageing, perhaps in part via hyperphagia and increasing adiposity, leading to progressive β-cell loss, raised HbA1c% and overt T2DM[275, 277, 280-282] resembling that seen in human studies[250]. Therefore, restricting both oxygen and nutrients in gestation via restricting placental function, does produce offspring that are insulin resistant and glucose intolerant in this species[275, 277, 280-282].

Another study showed that in fetal life, skeletal muscle mitochondrial mRNA levels of NADH ubiquinone-oxidoreductase, subunit C of the F_1-F_0 ATP synthase and ADP/ATP translocator were all reduced in IUGR rats, suggesting impaired glucose utilisation[278]. In 28-day old IUGR offspring, the oxidation of $NADH/H^+$ in the electron transport system of skeletal muscle remains reduced[278]. This in turn inhibits the utilisation of glucose via the negative feedback of high $NADH/H^+$ on isocitrate dehydrogenase in the citric acid cycle. Therefore, the programming of glucose metabolism via placental insufficiency in the rat may be due to impaired glucose utilisation in skeletal muscle.

In addition, GLUT2 protein and mRNA levels are unaltered in the livers of IUGR rat offspring generated by maternal unilateral uterine ligation, compared to controls, suggesting that IUGR does not alter the capacity of the liver to remove glucose from circulation[279].

1.11.1.2 *Reduced uterine placental pressure (RUPP) in the rat*

In the rat, reduced uterine placental pressure (RUPP) throughout the entire last trimester (day 14, term being 22 days) also induces changes in glucose homeostasis[288]. RUPP involves the clipping of the uterine arteries to reduce blood flow to the uterus. RUPP reduces size at birth by 9% and the restricted offspring exhibit accelerated growth post-weaning such that their weight does not differ from control by 9 weeks[288]. At 9 weeks, RUPP progeny had higher fasting glucose and insulin concentrations, impaired glucose tolerance (IVGTT) and were insulin resistant (HOMA-IR) compared to controls[288].

1.11.1.3 *Surgical placental restriction in sheep*

In sheep, surgical removal of the majority of the endometrial caruncles (placental attachment sites) prior to pregnancy reduces total placental size and therefore the efficacy of oxygen and nutrient transfer[269, 289, 290]. Surgically induced placental restriction in the ewe reduces fetal growth, impairs β-cell function and insulin secretion in late gestation (day 140 of ~ 147-150 days gestation for term) and reduces size at birth of their offspring[291]. Placentally restricted fetuses have increased abundance of insulin receptor protein in skeletal muscle, but reduced abundance of PKCζ, and GLUT4 protein[292]. At birth, placentally restricted offspring have asymmetrical IUGR with smaller size at birth in terms of weight (-21 to 25%), crown-rump length (-7 to 9%), abdominal circumference (-10%), and body mass index (-12%), and relative sparing of skull width (-5%) and length (-4%)[69, 293], resembling the phenotype observed with placental insufficiency in human studies[22, 23].

Placental restriction increased fractional growth rates of lambs in terms of weight (+24 to 29%) and various growths of long bone lengths (+15 to 23%), increased feed intake to 45 days of age, increased relative visceral adiposity and reduced muscle mass[69, 293]. At 30 days of age, placentally restricted lambs have high basal glucose, higher fasting insulins, lower post-prandial insulin secretion, assessed after an IVGTT, and are insulin resistant as assessed by HEC[69, 294]. Expression of GLUT2 is increased in the liver of placentally restricted males while expression of insulin receptor, IRS-1, AKT-2, GLUT4, GSK3a and GYS1 are all reduced in skeletal muscle of placentally restricted male and female offspring, suggesting that impairment to skeletal muscle signalling is the primary defect underlying whole-body insulin resistance in these IUGR lambs[294].

1.11.1.4 *Embryo transfer into overnourished adolescent sheep*

Overnourished adolescent ewes partition nutrients to the growth of the mother and away from the developing placenta[295, 296]. Therefore, placental restriction can be induced by

embryo transfer into young ewes, followed by overfeeding during gestation. A variable response is observed in this model, with approximately 50% of lambs born IUGR, with birth weights less than two standard deviations below the mean birth weight of lambs born to control pregnancies (<4 kg), and an overall reduction in birth weight in this IUGR group of approximately 35%[295]. IUGR offspring of adolescent pregnancies have increased fasting glucose concentration at 48 days and 6 months of age and fasting glucose is negatively associated with birth weight at both ages[296]. IUGR offspring also undergo increased neonatal fractional growth, which correlates positively with fasting glucose concentration at 48 days and 6 months of age. Glucose tolerance, assessed by IVGTT, is not altered in IUGR offspring at 48 days, but fasting glucose to insulin concentrations and insulin secretion during the first 20 minutes of the IVGTT were reduced, suggesting attenuated insulin production when compared to normal-weight offspring of adolescent pregnancies[295]. At 6 months of age glucose tolerance during the IVGTT was impaired in offspring of adolescent pregnancy, compared to offspring of control pregnancies, however, fasting insulin, insulin area under the curve, and insulin secretion during the initial phase of the IVGTT did not differ between birth groups[296]. While this model provides evidence for prenatal programming of glucose metabolism, the overnutrition model is associated with a preterm delivery[295, 296] making it difficult to differentiate effects of IUGR and gestational age.

1.11.1.5 *Heat –induced placental restriction in sheep*

Exposing ewes to increased temperature (40°C for 12 h/day, 35°C for 12 h/day) from day 40 to typically day 95-120 of gestation redistributes blood away from the placenta and induces IUGR in the fetus[297]. Heat stress, from day 45 to day 120 of gestation, increases maternal body temperature to 40°C, 1°C higher than controls[297]. This reduces fetal and placental size by 50%, uterine blood flow by 33% and umbilical blood flow by 52% at 135 days gestation[297].

At approximately 90% of term (131-138 days; term ~ 147-150 days gestation) fetuses of heat stressed mothers had a 58% reduction in whole pancreatic β-cell mass, and a 47% reduction in β-cell number, with insulin mRNA levels 66% less than controls[298]. In addition, these IUGR fetuses had increased insulin sensitivity of glucose utilisation, partitioning their glucose to glucose storage during a hyperglycaemic clamp[299].

Heat-induced placental restriction reduces muscle fibre size and the proportion of Type 1 muscle fibres in fetal skeletal muscle near term[300]. *In vitro* analysis of *M. semitendinosus* strips showed a lower glucose uptake under insulin-stimulated conditions in muscle from IUGR lambs of one month of age compared with controls[301]. If these changes to muscle fibre types persisted into later life the lower percentage of type I fibres in IUGR sheep may lead to insulin resistance, as in adult humans the percentage of type I fibres correlates positively with insulin sensitivity and individuals with the metabolic syndrome and T2DM have less type 1 fibres than controls[302].[300]

1.11.2 Maternal Malnutrition

1.11.2.1 *Global maternal nutrient restriction*

Maternal feed restriction has also been utilised in various species, with differing timing and magnitude of restrictions, to examine the fetal origins of T2DM and insulin resistance in a malnourished models such as rats[303-307], guinea pigs[308-317], sheep[318-321] and primates[154].

In baboons, maternal feed restriction to 70% of *ad libitum* feeding from 30 days gestation (term ~184 days) and throughout lactation tended (P<0.06) to reduce birth weight (-11%)[154]. At 3.5 years (juveniles), fasting plasma insulin and glucose concentrations were higher in the restricted offspring compared to control offspring[154], suggesting insulin resistance and glucose intolerance. The $AUC_{glucose}$ in an IVGTT did not differ between the two groups;

however, the offspring of feed restricted mothers secreted more insulin to maintain similar glucose levels compared to controls[154]. In feed restricted offspring, the glucose disposal rate during the HEC was lower compared to controls suggesting insulin resistance. No sex differences were found in any measure of glucose homeostasis in this study[154].

Feed restriction of ewes to 50% *ad libitum* feeding from early to mid-gestation (day 28-78, term ~ 147-150 days gestation), followed by *ad libitum* feeding to lambing did not alter birth weight or length of singleton male lambs (females not assessed), compared to *ad libitum* fed controls[321]. Feed restriction also had no effect on birth weight of twin lambs, although birth weight of control and restricted twins was 22% lower than that of singletons overall[321]. At 63 days of age, weaned offspring of feed restricted ewes had higher basal glucose, and increased $AUC_{glucose}$ and $AUC_{insulin}$ during an IVGTT compared to controls. At 250 days of age, young adult progeny from restricted ewes had a higher $AUC_{glucose}$ compared to controls suggesting glucose intolerance[321], but $AUC_{insulin}$ was lower than controls suggesting that insulin compensation for any given glucose load was impaired[321]. In addition, restricted progeny had a greater live weight, increased absolute and relative adiposity, and tended to have a reduced muscle mass at 280 days of age[321].

In contrast, feed restriction of ewes at 50% *ad libitum* feeding in twin pregnancies in the last 6 weeks of gestation reduces size at birth by 11% compared to non-restricted twins[318]. However, at 6 months of age glucose tolerance did not differ in offspring of feed restricted mothers, compared to non-restricted twins[319]. A more severe feed restriction of 0.3 MJ/d from day 105-115 then 0.5 MJ/d until 125 days, compared to controls fed at 13 MJ/d from day 105-115 then 15 MJ/d until 125 days, followed by a return of both groups to pasture for lambing (term ~147-150 days) also reduced size at birth[320]. In this severe maternal feed restriction model, low birth weight and current weight, but not undernutrition *per se,* impaired glucose tolerance in 5-month-old lambs[320]. This suggests that size at birth may be part of the causal

pathway linking fetal nutritional environment and later adult disease. Contrasting outcomes in these studies may come from differences in singleton versus twin pregnancies.

In rats, maternal feed restriction to 50% *ad libitum* intake from days 1 to 14 of gestation, followed by *ad libitum* feeding to term, did not alter basal glucose or insulin concentrations in male offspring at 15-16 weeks, while female offspring were not studied[303]. Further these male offspring did not have altered glucose tolerance or insulin secretory responses following an IVGTT, or impaired glucose production or glucose utilisation during HEC[303]. In contrast, maternal feed restriction to 50% *ad libitum* from day 15 of gestation to weaning reduced glucose tolerance in male rat offspring at 1 year of age compared to controls[304]. This suggests that perturbations occurring during late gestation and early postnatal development in the rat may have greater impact on the programming of glucose tolerance than those occurring during early-mid gestation. Adult female offspring of rats that were restricted to 50% *ad libitum* feeding during late gestation and throughout lactation had impaired whole body insulin sensitivity and impaired suppression of endogenous glucose production but peripheral glucose utilisation was not altered[305]. Therefore, in studies where endogenous glucose production and peripheral glucose utilisation were measured, maternal feed restriction reduces the insulin sensitivity of liver in female offspring with no change in skeletal muscle glucose utilisation[305]. However, these studies in offspring of maternal feed restriction in the rat contrast human observations where insulin resistance of skeletal muscle is the primary defect more generally in the pathogenesis of whole body insulin resistance and T2DM[92, 236, 322].

In the pig, unlike many other species, size at birth is not consistently affected by various maternal feed restriction magnitudes or timings during gestation (reviewed in[323]). One study, however, did show that reducing feed intake to one-third of *ad libitum* feeding in the first two trimesters results in offspring that tend to have increased rates of growth postnatally compared to controls[324].

In the guinea pig, severe maternal feed restriction (50%) from day 30 to term (~67-70 days) reduced birth weight (-30%), while early feed restriction from conception to day 30 did not reduce size at birth[325]. Even at 8 weeks postnatal age, the restricted group remained smaller (-22%) than the controls suggesting catch-up had not occurred to restore growth in these offspring of severely feed restricted guinea pigs[325]. Weights of adipose depots were reduced in pups of restricted mothers at 8 weeks and for subcutaneous depots such as shoulder fat and groin fat, this decrease was proportional to the reduction in body weight[325]. In contrast, the relative weights of the interscapular and retroperitoneal fat depots at 8 weeks of age correlated negatively with birth weight[325]. This suggests that either some fat depots were programmed differently *in utero* by maternal feed restriction or different fat depots are more prone to deposition during catch-up following IUGR.

Moderate global restriction, in guinea pigs, by feeding 70% *ad libitum* intake to day 35 gestation followed by 90% *ad libitum* intake to term, reduces size at birth in male (-12%) but not female offspring, however, only limited numbers of female offspring from moderately feed-restricted mothers were examined in this study[310]. Maternal feed restriction did not alter neonatal fractional growth rate across the maternal diets or in either sex [310]. Moderate feed restriction induced hyperphagia, increased visceral (retroperitoneal) adiposity and reduced relative *M biceps brachii* mass in young adult males but not in females. Also in males only, moderate feed restriction increased the fasting insulin and fasting insulin:glucose ratio compared to controls, but this effect was lost when birth weight was included in statistical analyses. This suggests that in the guinea pig growth *in utero* is a greater determinant of the programming of glucose homeostasis than maternal feed restriction treatment itself. When outcome data were pooled (sex and treatment) and analysed according to birth weight tertiles, the lowest birth weight offspring had the highest food intakes and were hyperinsulinaemic as adults suggesting that growth *in utero* may program glucose homeostasis[310].

Mild global restriction, induced by feeding pregnant guinea pigs 85% *ad libitum* intake throughout gestation, also decreases size at birth in terms of weight (male -10 to -14%; female -8 to -11%), without increasing neonatal fractional growth rates from birth to weaning at 28 days[310, 312]. Young adult males from undernourished mothers had higher cholesterol levels prior to and post feeding a high-cholesterol diet for 6 weeks, which was not observed in females[312]. Systolic blood pressure and insulin secretion measured during an IVGTT (as area under the insulin profile relative to area under the glucose profile) were both higher in restricted male, but not female, offspring compared to controls at 100 days[310, 311]. This effect on insulin secretion was not evident when correcting for litter size or birth weight, again suggesting that the effect may be more strongly related to fetal growth rather than the maternal feed restriction itself. These studies demonstrate that IUGR induced by maternal feed restriction does in fact program many of the features of the metabolic syndrome in the guinea pig and in a sex-specific fashion[309-312]. Males appear to be more at risk of the adverse effects of IUGR and neonatal catch-up compared to females in this species, resembling findings reported in systematic reviews in humans[65, 176-178].

1.11.2.2 *Maternal glucose restriction*

Feeding pregnant rats isoenergetic diets containing 0% glucose from day 2 of gestation reduces size at birth (-30%) compared to a control diet containing 60% glucose[326]. In contrast, diets containing 12 or 24% glucose did not affect weight at birth. Percentage mortality in offspring by postnatal day 1 was 100%, 11% and 4% for the 0%, 12% and 24% glucose diets, respectively[326]. Feeding a 12% glucose diet throughout pregnancy and lactation reduced the plasma insulin to glucagon ratio in the first 12-16 hours of postnatal life, due to both a marked reduction in insulin and raised glucagon concentration in maternal glucose-restricted offspring. Despite the lower insulin levels offspring from mothers whose glucose intake was restricted during gestation and lactation did not have enhanced glycogen mobilisation. This was most

likely due to an impaired activation of glycogen phosphorylase in the maternal glucose-restricted offspring compared to the control glucose diet. Six-day-old neonates from dams fed a 12% glucose diet had higher expression of PEPCK mRNA compared to those from dams fed a 24% glucose diet suggesting that maternal diets low in glucose have the potential to increase gluconeogenesis in the liver[326]. However, by day 15 hepatic PEPCK mRNA did not differ between these treatment groups[326]. No sex effects or adult outcomes were reported in this study[326]

1.11.2.3 *Maternal protein restriction in the rat*

Restriction of maternal protein intake, by feeding a 5%-8% protein diet, compared to 15%-20% protein, throughout gestation and lactation does not consistently reduce birth weight of the offspring[327-336]. Some studies do not report size at birth[327-330], others report decreases in fetal weight at 19 days of gestation[331] with a few reporting a reduced size in a least one sex with maternal feed restriction[332-336].

Maternal protein restriction at 7% compared to control of 25% in the rat, throughout gestation but not during lactation improves glucose tolerance in offspring at 4 months of age[337]. At 9 weeks (young adults), blood glucose was normalised faster, had a smaller rise in maximum glucose concentration, and a more rapid glucose clearance to basal levels following an intraperitoneal glucose tolerance test (IGTT) in the offspring of protein-restricted dams, suggesting glucose tolerance was improved at this age[330]. In contrast, a more severe maternal protein restriction of 5% protein diet, compared to control diets of 15% protein in the last trimester of gestation, does not alter glucose tolerance in offspring of protein-restricted dams compared to control at 8 weeks of age (only females studied)[335].

However, glucose intolerance does develop with ageing (15-21 months) in offspring of protein restricted mothers in some[327, 332, 338] but not (44 or 52 weeks) all studies[330, 333, 334]. Interestingly, males develop hyperglycaemia at 15 months[334] while females do not develop

hyperglycaemia until 21 months[332] suggesting males are at greater risk of developing T2DM at an earlier age after IUGR. Together these studies suggest that protein restriction does not result in an impaired glucose tolerance in young offspring but this develops with ageing in offspring of the maternal protein restricted rat[327, 332, 338]. Supporting this, glucose intolerance did not develop until 130 days in female offspring of maternal protein restricted dams suggesting that ageing is required to reveal the programmed dysfunction in insulin sensitive organs leading to metabolic dysfunction[338].

Severe maternal protein restriction in rats at 5% also reduced fetal pancreas weight, islet cell number, and insulin content in the β-cells compared to control at 21 days gestation[339]. This effect on the pancreas was still seen at postnatal 8 weeks suggesting that the impact of maternal protein restriction in the rat may be in part via reduced insulin secretory capacity[335, 339]. Consistent with this, maternal protein restriction at 8% increased the rate of apoptosis and reduced the rate of proliferation of β-cells compared to control in 19.5 to 21.5-day-old fetuses (only females examined) which persisted 21 days after birth[340]. Four-month-old female offspring of dams that were protein restricted during pregnancy and lactation have reduced pancreatic islet number, islet size and overall β-cell mass, but no effects were observed in male offspring[338]. In four-month-old females, the basal insulin concentration was half that of males, showing that impairment in β-cell function in offspring of protein restricted mothers at this age is sex-specific[338]. Overall, insulin secretion in male and female offspring of protein restricted mothers also tends to be lower compared to controls at 1 year of age[333].

In the rat, some studies report that maternal protein restriction during gestation[337] or throughout gestation and lactation[331] enhances insulin sensitivity at 4 months of age. In contrast, others report that maternal protein restriction during gestation and lactation reduces insulin sensitivity in males at 4 months[338]. Insulin sensitivity measured by GTT in 110-day-old males, but not females, is enhanced by maternal protein restriction in gestation and lactation combined or lactation alone[336]. In contrast, protein restriction in gestation alone impaired insulin

sensitivity in 110-day-old males, but not females, compared to controls[336]. Restriction of maternal protein to 5% of the total diet, compared to 15%, in the last trimester of gestation, does not alter submaximal glucose utilisation or production rates in progeny at 8 weeks (only females studied)[335].

Although experimental *in vivo* studies in the rat have not consistently shown links between maternal protein deprivation and altered glucose homeostasis postnatally, some *in vitro* studies do report alterations in the structure and functional development of key tissues for insulin action, including skeletal muscle, liver and adipose tissue, in adult offspring following maternal protein restriction[328, 331, 338, 341-347]. In male rats at 130 days of age, phosphorylation of AKT in skeletal muscle and visceral adipose tissue increased 4-6 fold with insulin-stimulation in controls, but did not change in tissues from male offspring of protein restricted mothers suggesting peripheral insulin resistance in the signalling pathway[338]. Insulin-stimulated phosphorylation of AKT in skeletal muscle and visceral adipose tissue did not differ between 130-day-old female offspring from control and protein restricted mothers, however, implying sex-specific effects of maternal protein restriction on insulin sensitivity in the peripheral tissues[338]. *In vitro* insulin sensitivity of skeletal muscle strips from three-month-old offspring of protein restricted mothers is increased in association with increased insulin-stimulated glucose uptake, insulin receptor concentration, and GLUT4 protein within plasma membranes in skeletal muscle[328]. These effects of maternal protein restriction differ from those observed following uterine artery ligation, where GLUT4 protein concentration in fetal and postnatal skeletal muscle were not altered[348]. Decreased membrane-associated GLUT4 in skeletal muscle after maternal protein-restriction[328] is however consistent with the finding that 18-week-old offspring of dams fed an 8% protein diet during gestation and lactation, compared to 20% protein, have impaired glucose turnover and glucose utilisation in *M. soleus*, a slow twitch oxidative muscle[331].

Protein restriction leads to reduced liver size and increased visceral fat in both absolute and relative terms in 130-day-old male rats, compared to controls. However, these effects were not observed in females at the same age[338]. Structural and functional development of the liver is altered in adult offspring of protein restricted pregnant rats. In offspring of mothers fed diets containing 8% protein, compared to 20% protein, insulin stimulation *in vitro* in isolated perfused liver cells increased glucose output [329]. This is in contrast to the expected action of insulin in the liver to suppress endogenous glucose output[349]. This was in conjunction with a three-fold increase in the number of insulin receptors, increased insulin uptake and increased insulin degradation in the livers of protein-restricted offspring. This suggests that protein restriction increases hepatic insulin sensitivity, but alters the post-receptor signalling mechanisms [329].

Many metabolic processes are known to be zoned within specific areas of the liver. Glucokinase (GK), the insulin sensitive enzyme that converts glucose to glucose-6-phosphate and the rate-limiting step in the glycolytic pathway, is essentially located in the perivenous region of liver parenchyma[345]. Fructose 1,6-bisphosphatase, another glycolytic and insulin sensitive enzyme, and phosphoenolpyruvate carboxykinase (PEPCK), an enzyme involved in gluconeogenesis, are predominantly located in the periportal regions[343, 344]. Offspring of rats that were protein restricted (6% protein diet) during pregnancy have 50% lower expression of GK in the perivenous parenchymal regions of the liver, compared to offspring of control fed (20% protein) animals, whereas PEPCK activity was increased by 100% in the periportal regions[346]. Therefore, there is impairment in the ability of the liver to utilise glucose as a fuel through glycolysis, and an increase in activity of enzymes, like PEPCK, responsible for glucose production, resulting in a net potential to over-produce glucose. Such changes could in part account for the hyperglycaemia found in humans who were small at birth. More recently, the lobular volume in livers of offspring from 8% protein restricted mothers was shown to be half that of control offspring (maternal 25% protein feed)[347].

In the rat, adipocytes isolated from offspring from protein-restricted dams have significantly higher *in vitro* basal and insulin-stimulated glucose uptakes than controls, with a three-fold increase in insulin receptors and significantly higher basal and insulin-stimulated IRS associated PI3K activity[341]. In contrast, insulin-enhanced glucose uptake and inhibited lipolysis in adipocytes from control offspring, but not in offspring of protein-restricted mothers[342].

Chickens have also provided an alternative model in which to study the effects of protein restriction, independent of maternal and placental factors, through the *in ovo* removal of albumin[163]. Albumin removal tends to reduce weight at hatching ($p<0.06$)[350], and reduces breast muscle and adipose mass in absolute but not relative terms at 25, 40 and 55 weeks[163]. At 10 weeks, plasma glucose remained higher at 30 minutes after an oral glucose bolus in the albumin-deprived group compared to control suggesting glucose intolerance[163]. However, glucose tolerance was not altered at later ages in these protein-deprived chickens, and insulin sensitivity measured by ITT in older adult hens at 51 weeks also showed no effects of albumin removal[163].

1.11.2.4 *Maternal hypoxia in the rat*

In the rat, maternal hypoxia (10% O_2 compared to 21% for control) in the last two-thirds of gestation (day 7-21), reduces size at birth (-12%) and at 28 days of postnatal age, and reduces insulin sensitivity as measured by homeostatic model assessment (HOMA) without changes in plasma glucose or insulin concentrations[351]. However, levels of PEPCK protein were increased, and levels of GLUT2, AKT and the IRS-2 were reduced in the liver at birth and at 28 days of age in offspring of hypoxic mothers[351]. At 11 weeks, triglycerides were increased and phosphorylated AKT (p-AKT/AKT) and AMPK (p-AMPK/AMPK) in skeletal muscle were reduced in the pups from the hypoxic dams[352]. Insulin infusion, however, did not alter GLUT4 translocation in maternal hypoxia-exposed pups compared to a two-fold increase seen in the controls suggesting a defect in signalling of glucose uptake in muscle[352].

A shorter period of maternal hypoxia (10% O_2 compared to 21% for control) throughout the last third of gestation (day 15-21) reduces size at birth by 12%. At 4 months of age, protein expression of IRS-1 and p-AKT, which are involved in glucose metabolism, was reduced in the liver of offspring of hypoxic mothers[353]. Levels of PKCζ, a kinase involved in lipid metabolism, and Akt-1 and Akt-2 were also reduced in the liver of offspring of hypoxic mothers, however, similar decreases were observed in offspring of mothers pair-fed during pregnancy, to account for the decreased feed intake during hypoxia, suggesting a contribution of maternal undernutrition. Protein levels of the insulin signalling molecules IRS-1, p85α, and Akt-1 were not altered in skeletal muscle following maternal hypoxia, but levels of Akt-2 were reduced. In addition, GLUT4 expression in skeletal muscle was reduced in four-month-old offspring of hypoxic mothers to a greater degree that observed in offspring of pair-fed pregnancies, suggesting reduced potential for insulin uptake in this target tissue following maternal hypoxia[353].

1.11.3 Spontaneous Growth Restriction

1.11.3.1 *Variations within a litter*

Naturally occurring variation in size at birth or spontaneous IUGR due to litter size has long been known in the pig with “runt piglets” often born to larger litters[354]. This natural variation can result in 2-3 fold variations in size at birth within the same litter and is partly due to the competition for space for implantation and limited growth of placentas and fetuses in larger litters[354, 355]. This reduction in placental growth impairs oxygen and nutrient transfer to the fetuses limiting their growth. Overall these IUGR pigs are asymmetrically growth-restricted and generally thin at birth[354], have reduced muscle mass and fibre number, increased adiposity, and decreased lean-to fat-ratio compared to larger litter mates[355, 356], consistent with observations in IUGR humans[229, 239, 357].

Pigs that are categorised as low birth weight grow faster in the first month of age compared to the larger littermates suggesting neonatal catch-up growth after spontaneous IUGR[358]. Spontaneous IUGR in pigs, as indicated by thinness at birth, and fractional neonatal catch-up in the first month, both independently predicted increased insulin sensitivity at 3 months in males, while thinness at birth but not fractional neonatal growth rate predicted impaired insulin sensitivity in females[358]. At 12 months of age catch-up growth predicted impaired insulin sensitivity in both sexes[358]. Other components of the metabolic syndrome such as raised mean atrial pressure and adiposity are also increased in spontaneous IUGR piglets[359, 360]. Hence, natural variation of fetal growth induces programming of several components of the metabolic syndrome in this species.

1.11.3.2 *Litter size effects*

Twinning in sheep is relatively common with the majority being dizygotic[361]. Therefore, each placenta is distinct and due to reduced area of placentation the availability of nutrient supply is reduced. Twins are often born premature compared to singleton births and therefore the combination of nutrient competition and premature delivery may both contribute to their fetal growth restriction[361]. At approximately day 120 of gestation, glucose (-22%) and insulin (-20%) are reduced in twins compared to singletons, likely contributing to the reduction in fetal weight (-4%)[362]. In triplet lambs, fetal weight at 120 days is 23% less than that of singletons, although not significantly different from that of twins[363]. By day 140 of gestation (term at ~145 days), fetal twins have fewer placentomes (-35%) and reduced weight of the caruncular tissues (-28%) and of total placentomes (-33%), compared to singletons[364]. Together this reduces nutrient supply, especially when the demand is increased in late gestation, and reduces fetal weight by 16% at 140 days[364]. Differences in singleton and twin growth trajectory *in utero* appear to be determined early in gestation and may reflect placental capacity[362-364]. In near-term fetuses the liver:brain weight ratio is reduced in the lighter compared to the heavier

twin suggesting head sparing in the greater restricted of the two[365]. Birth weight is reduced in twins (-17 to 30%) compared to singletons, as are other measures of size at birth such as crown rump length (-8 to -10%), chest girth (-7 to -8%) and abdominal circumference (-6%); also consistent with asymmetrical IUGR[362, 366-369], not unlike that seen in human studies[23, 24].

Despite the differences in size at birth, there is no difference in neonatal growth rates (0-3 months) between twin and singleton lambs[368]. Others show that the growth rates from birth to 10 months are stronger predictors of glucose and insulin responsiveness to an IVGTT than birth weight, however, these associations were not different in singletons or in twins separately[367]. In addition, glucose or insulin responsiveness after an IVGTT did not differ between the lighter and larger twin[365]. Similarly at 11 months, the expression of p85α and PI3K in perirenal fat or skeletal muscle and GLUT4 expression in skeletal muscle do not differ between single and twin lambs[368].

Hancock *et al* (2006) euthanised one twin at 42-43 days gestation (after the period of placental attachment in sheep) and allowed the other ("reduced" twin) to birth naturally and followed growth to 24 months[366]. These "reduced" twins had a lower birth weight compared to singletons (-13%) but were heavier than control twins (+9%). Growth rates from birth to weaning tended to be higher in the reduced twin lambs, compared to both singletons and control twins, whereas growth rates from weaning to 12 months were greater in control twins compared to singletons and reduced twin progeny[366]. At 12, 18 and 24 months, body weights were not different between the three groups, but control twins and reduced twins had increased fat mass (+33%, +40%) and reduced lean mass (-5%, -6%) respectively compared to singletons at 24 months[366]. At 3 years of age reduced twins had a greater percentage fat mass and lower lean weight than singletons, whereas the control twins were intermediate between the singleton and reduced twins for both fat mass and lean weight[369]. Basal and IVGTT challenged glucose and insulin levels did not differ at 11 months and at 3 years between control twins, reduced twins or singletons or between the sexes, suggesting no impairment of glucose tolerance[368, 369].

However, the insulin responsiveness relative to glucose levels was higher in twins compared to singletons suggesting that twins release more insulin for a given plasma glucose concentration achieved[368]. Further, 3-year-old sheep conceived as twins (control and reduction twins combined) had a lower insulin sensitivity assessed by HEC compared to singletons[369]. Overall when insulin sensitivity data for female singletons and female control twins were pooled and compared to the corresponding pool of males, males had a higher insulin sensitivity than females at 3 years of ages. This contrasts with human studies where males appear to be the most sensitive to the programming of metabolic disease[65, 176-178]. However in a more recent study in humans, females who were at the extremes of the birth weight range (both low and high) had the highest risk of T2DM compared to males perhaps suggesting that females at the ends of the birth weight ranges have the highest risk of insulin resistance[266]. In sheep, insulin sensitivity was not different in singletons, reduced twins or control twins at 1 year of age, suggesting that the impairment in insulin sensitivity does not become apparent until later life[369]. The loss in insulin sensitivity at 3 years of age was independent of current bodyweight, adiposity and lean mass, but was not significant when corrected for birth weight. This suggests that the effect of litter size on insulin sensitivity in adult sheep is due to differences in prenatal environment and not an indirect consequence of altered adult body composition.

In ewes selected for their ability to conceive litters of multiple lambs (singletons to quintuplets), a series of experiments run by Greenwood *et al* 1998-2004 examined postnatal outcomes in male sheep of low (<2.9 kg) or high (>4.3 kg) birth weight[370-373]. These low and high birth weight male lambs were reared on milk replacer either *ad libitum* to enable rapid postnatal growth (>300 g/d), or at a feeding level sufficient for the maintenance of a slower growth rate of 150 g/d. Only males were studied and litter size effects were only reported for size at birth[370-373]. Although not reported, these low birth weight male lambs, appeared to come from ewes carrying larger litters, grew faster to 20 kg live weight, and were hyperphagic for any given live weight with a 13% higher total feed intake from birth to 20 kg live weight[370, 371].

High birth weight lambs had a greater weight of *M. semitendinosus*, *M. extensor digitorum lateralis* and *M. extensor carpi radialis* muscles than offspring of low birth weight at 20 kg live weight[371]. However, the number and proportion of muscle fibre types did not differ between the low and high birth weight groups[371]. Low birth weight lambs reared on the rapid growth diet had higher plasma insulins than high birth weight reared slowly[372].

1.11.4 Gaps in Existing Knowledge

Small animal models have many logistical advantages for studies into the mechanisms that underpin the DOHaD hypothesis. These include rapid development and shorter lifespans such that intergenerational and ageing effects can be examined more readily than in humans or larger animals. To date, much of the evidence for programming of insulin sensitivity has been generated in rodents. These offspring of maternal feed-restricted[310-312, 335, 374] and protein-restricted mothers[332, 335, 375-377] display some of the elements of programming of glucose homeostasis such as impaired insulin secretion and insulin resistance. However, in human populations, much of variance in size at birth is due to placental insufficiency rather than malnutrition[378, 379]. In addition, reducing nutrient intake of the mother does not alter oxygen levels, another important determinant of fetal growth[46, 47]. Placental restriction induced by uterine ligation in rats causes IUGR and programs many components of the metabolic syndrome[276, 280, 380, 381]. However, many of these IUGR models in the rat do not exhibit neonatal catch-up growth[276, 280, 381], which is an important and independent risk factor for the development of cardio-metabolic disease in humans[83, 382-384].

Species differences in the timing of development also limit translation of some models. In the human, the endocrine pancreas develops in late gestation[98]. This contrasts with pancreatic development in the altricial species commonly used as animal models for programming studies, such as the rat and mouse[98]. In rats and mice, the pancreas continues to develop into the lactation period and therefore intrauterine insults are likely to produce different

effects on glucose homeostasis in these altricial species than those observed in the human. Therefore, more precocial small animal models in which the timing of organogenesis and pancreatic development more closely resemble the human fetus are required. In addition, placental restriction/insufficiency accounts for the majority of IUGR, even in under-developed countries where malnutrition is common[31]. Hence, animal models of low birth weight based on placental restriction of oxygen and nutrient supply, rather than maternal undernutrition, that more closely resemble human IUGR, are required.

A significant gap in both the studies in IUGR humans and in animal models is that in most studies only one sex is assessed, often due to researchers wishing to avoid variation due to effects of the hormone cycle of females. There is compelling evidence that there are sex-specific programming effects for cardiovascular and now for metabolic diseases[266, 385, 386], indicating that consideration of both sexes is required.

In litter bearing species, spontaneous fetal growth restriction is commonly observed in larger litters. Spontaneous fetal growth restriction based on litter size has advantages as a model as it utilises natural variation in litter size without the need for dietary or surgical interventions. Surgical models involve the additional stress of surgery, anaesthesia and are often associated with increased rates of spontaneous fetal loss (as little as 14% survival)[283]. Dietary restriction models vary in the degree, severity and timing of the restriction and can also impact on placental development and function[387]. These surgical and feed restriction models often have abrupt/acute onsets in mid-late pregnancy, which differs from IUGR in humans. In addition, animal models with smaller litter sizes have an advantage in that there is no need to cull pups postnatally to “standardise” the postnatal environment, which can be associated with variation in nutrient intake by the pups during the lactation period.

1.11.5 The Guinea Pig as a Potential Species for Studying Developmental Programming by IUGR

The guinea pig is a precocial species[308], with a mean litter size of 3-4 pups[388] with the IMVS Tri-colour strain used in this thesis having litter sizes of 1-5 pups. The guinea pig has a relatively long gestation period compared to other small animals such as the rat and mouse therefore several developmental events that occur *in utero* are similar to that of the human[308, 389]. The fat content of guinea pig pups at birth is 10-20%[325] similar to that in humans (16%), in contrast to other species such as the sheep (2%, in the fetus near term at 140 days), pig (1%), rat (2%) and mouse (1%)[363, 390]. Development of the pancreas, liver, kidney and skeletal muscle are mostly complete at, or soon after, birth in the guinea pig[308, 388, 389] suggesting this species is an appropriate animal in which to investigate prenatal programming of metabolic dysfunction.

In addition, uteroplacental insufficiency via unilateral uterine artery ligation in the guinea pig increases appetite, and increases epididymal (visceral) adiposity in young adult male (females not assessed) offspring[284]. As previously discussed, maternal feed restriction at either 70% or 85% *ad libitum* feeding from day 30 to day 60 of gestation impairs cholesterol and glucose homeostasis, increases appetite, and increases adiposity in guinea pig progeny, primarily in males[309-312]. Therefore, maternal feed restriction[309-312] and uteroplacental insufficiency[284] in the guinea pig program much of the later dysfunction associated with the metabolic syndrome. However, in maternal feed restriction studies size at birth was a stronger predictor of many of these adult outcomes than the maternal nutritional treatment suggesting that the effects are programmed by size at birth rather than nutrition itself.

1.11.6 Spontaneous Growth Restriction in the Guinea Pig as a Potential Model for Studying Developmental Programming of Metabolic Dysfunction

In the guinea pig, with increasing litter size from one to five pups there is a decrease in mean birth weight in males by 31% and females by 34% in litters of five pups compared to

mean birth weight of singleton pups of the same sex[391-394]. Average placental size is decreased by 28% at day 65 gestation (term ~ 70 days) in litters of five compared to litters of one suggesting that spontaneous fetal growth restriction due to increased litter size in the guinea pig has a substantial placental component[391-393]. Like IUGR in humans, spontaneous IUGR guinea pig offspring from larger litters undergo neonatal catch-up growth with percentage weight gain from birth to day 28 (weaning) being 308% and 319% in males and females respectively in litters of five, compared to 267% and 247% in males and females from litters of one[394]. Therefore, these larger litters are producing offspring with IUGR with a substantial placental component and these offspring undergo catch-up growth in the neonatal period in both sexes[391-394]. Thus, the consequences of spontaneous IUGR due to natural variation in litter size provides a potential model where catch-up growth and its impact on perinatal programming of glucose metabolism in both sexes can be examined.

1.12 **Hypothesis and Aims**

To address these gaps in knowledge the studies in this thesis were designed to test the following general hypothesis:

Spontaneous growth restriction due to increasing litter size in the guinea pig induces neonatal catch-up growth, postnatal hyperphagia, and obesity and impaired insulin sensitivity in young adults.

To test this hypothesis, studies were conducted to address the following aims:

1/ To characterise the effects of natural variation of litter size on birth phenotype, neonatal and postnatal growth, postnatal appetite and adult body composition in male and female guinea pigs. (Chapter 2)

2) To develop and validate methodology for measurement of whole body and tissue specific insulin action in male and female guinea pigs. (Chapter 3)

3) To characterise the effects of natural variation in litter size, birth phenotype and catch-up growth on insulin sensitivity and sites of insulin action in adult male and female guinea pigs. (Chapter 4)

CHAPTER 2
Spontaneous intrauterine growth restriction due to increased litter size in the guinea pig programs postnatal growth, appetite and adult body composition

---

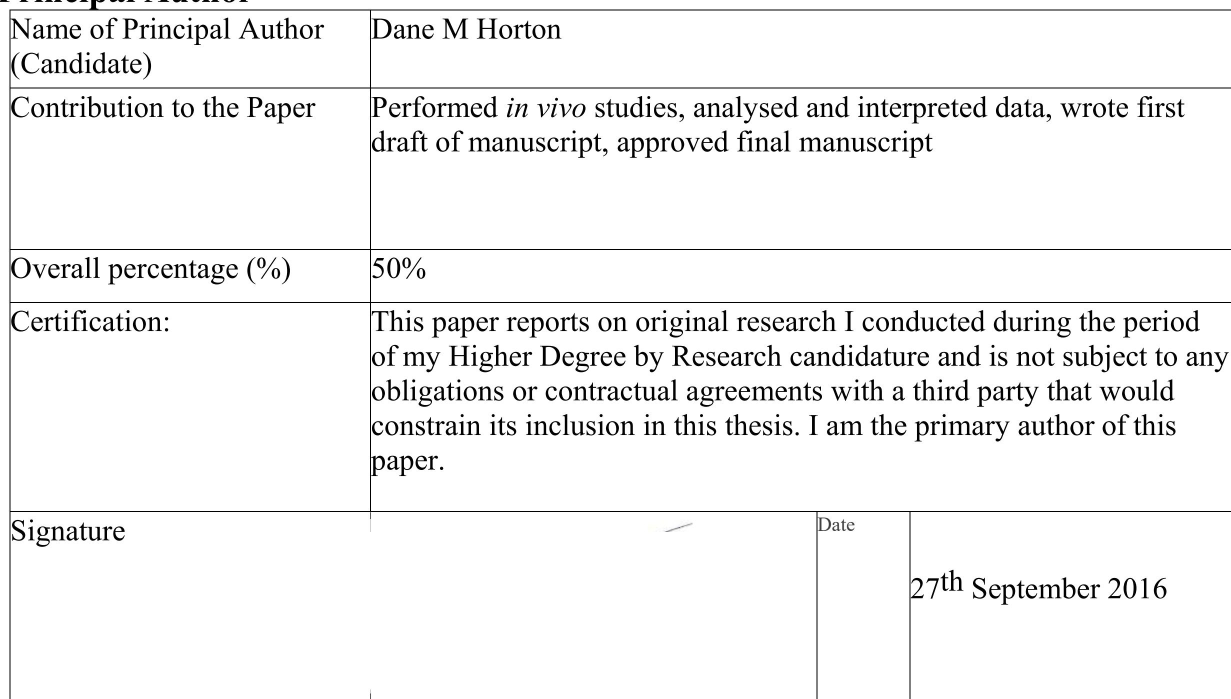
2.1 Overview

The following study used 68 pregnant dams to produce the offspring (*n*=158, males: *n*=78, females: *n*=80). Of these 158 animals, approximately 20 were from control pregnancies in another study investigating the effects of maternal feed restriction on postnatal cholesterol metabolism. These animals had size at birth, feed intake and postnatal growth measures recorded, but their body composition could not be included in the data analysis for this chapter, as they were fed a high-cholesterol diet as adults. I conducted all remaining animal work, including managing the mating program, monitoring growth and wellbeing of dams during pregnancy, assessing birth parameters, monitoring postnatal growth and feed intake, all tissue dissections and all data analysis. This enabled me to assess the first main aim of my thesis to determine the effects of spontaneous IUGR due to natural variation in litter size on postnatal growth, appetite and adult body composition in the guinea pig. This chapter has been published in the Journal of Developmental Origins of Health and Disease ([395]Appendix 1), and has been reproduced exactly as published according to The University of Adelaide guidelines.

2.2 Statement of Authorship

Title of Paper	Spontaneous intrauterine growth restriction due to increased litter size in the guinea pig programs postnatal growth, appetite and adult body composition.
Publication Status	☑ Published
Publication Details	Journal of Developmental Origins of Health and Disease, volume 7 (5) pp. 548-562. doi: 10.1017/S2040174416000295. Published online: 23 June 2016

Principal Author



Name of Principal Author (Candidate)	Dane M Horton		
Contribution to the Paper	Performed *in vivo* studies, analysed and interpreted data, wrote first draft of manuscript, approved final manuscript		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27th September 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:
the candidate's stated contribution to the publication is accurate (as detailed above);
permission is granted for the candidate in include the publication in the thesis; and
the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David A Saint		
Contribution to the Paper	Interpreted data, edited manuscript, approved final manuscript		
Signature		Date	7th October 2016
Name of Co-Author	Julie A Owens		
Contribution to the Paper	Designed study, interpreted data, edited manuscript, approved final manuscript		
Signature		Date	05/10/2016
Name of Co-Author	Karen L. Kind		
Contribution to the Paper	Designed study, assisted with in vivo studies, analysed and interpreted data, assisted in manuscript writing, edited manuscript, approved final manuscript		
Signature		Date	6 October 2016
Name of Co-Author	Kathryn L Gatford		
Contribution to the Paper	Analysed and interpreted data, assisted in manuscript writing, edited manuscript, approved final manuscript		
Signature		Date	30 September 2016

Please cut and paste additional co-author panels here as required.

2.3 Appendix 1

Journal of Developmental Origins of Health and Disease (2016), 7(5), 548–562.
© Cambridge University Press and the International Society for Developmental Origins of Health and Disease 2016
doi:10.1017/S2040174416000295

ORIGINAL ARTICLE

Spontaneous intrauterine growth restriction due to increased litter size in the guinea pig programmes postnatal growth, appetite and adult body composition

D. M. Horton[1,2], D. A. Saint[2], J. A. Owens[1,2], K. L. Kind[1,3] and K. L. Gatford[1,2]*

[1]*Robinson Research Institute, The University of Adelaide, SA, Australia*
[2]*School of Medicine, The University of Adelaide, SA, Australia*
[3]*School of Animal and Veterinary Sciences, The University of Adelaide, SA, Australia*

Intrauterine growth restriction (IUGR) and subsequent neonatal catch-up growth are implicated in the programming of increased appetite, adiposity and cardiometabolic diseases. Guinea pigs provide an alternate small animal model to rodents to investigate mechanisms underlying prenatal programming, being relatively precocial at birth, with smaller litter sizes and undergoing neonatal catch-up growth after IUGR. The current study, therefore, investigated postnatal consequences of spontaneous IUGR due to varying litter size in this species. Size at birth, neonatal, juvenile (post-weaning, 30–60 days) and adolescent (60–90 days) growth, juvenile and adolescent food intake, and body composition of young adults (120 days) were measured in 158 male and female guinea pigs from litter sizes of one to five pups. Compared with singleton pups, birth weight of pups from litters of five was reduced by 38%. Other birth size measures were reduced to lesser degrees with head dimensions being relatively conserved. Pups from larger litters had faster fractional neonatal growth and faster absolute and fractional juvenile growth rates ($P < 0.005$ for all). Relationships of post-weaning growth, feed intakes and adult body composition with size at birth and neonatal growth rate were sex specific, with neonatal growth rates strongly and positively correlated with adiposity in males only. In conclusion, spontaneous IUGR due to large litter sizes in the guinea pig causes many of the programmed sequelae of IUGR reported in other species, including human. This may therefore be a useful model to investigate the mechanisms underpinning perinatal programming of hyperphagia, obesity and longer-term metabolic consequences.

Received 5 April 2016; Revised 17 May 2016; Accepted 20 May 2016; First published online 23 June 2016

Key words: adiposity, appetite, guinea pig, litter size, sex differences

Introduction

Small size at birth, following intrauterine growth restriction (IUGR) and/or subsequent neonatal catch-up growth are implicated in the initiation of permanent metabolic and/or physiological adaptations that persist through adult life. This 'programming' may lead to increased appetite, adiposity (particularly of visceral depots) and cardiovascular and metabolic diseases including insulin resistance and glucose intolerance in adults.[1,2] Therefore, animal models that mimic human IUGR aetiology, develop cardiovascular and metabolic sequelae with ageing after IUGR and/or catch-up growth and are comparable with the human in their relative maturity at birth are required so that underlying mechanisms and intervention strategies can be investigated.

Small animal models have logistical advantages for such studies, including relatively rapid development, and short lifespans facilitating study of the development of progeny with ageing and intergenerational effects. Many small animal models of IUGR have used maternal feed[3–7] or protein restriction[6,8–11] to restrict fetal growth and investigate long-term outcomes. In developed countries, however, placental insufficiency rather than maternal undernutrition accounts for the majority of human IUGR.[12,13] Placental restriction induced by uterine ligation in rats causes IUGR and programmes many components of the metabolic syndrome.[14–17] These IUGR rat models often lack catch-up growth in the early neonatal period,[14,16,17] which is an independent risk factor for the development of adult metabolic disease in humans.[18–21]

Guinea pigs provide an alternate species to investigate developmental programming of health and disease. The guinea pig has a smaller litter size than the rat,[7,14,22,23] is relatively precocial at birth and resembles the human fetus in having a body fat composition of around 10% at term.[24] In addition, and unlike the rat, the guinea pig spontaneously develops a phenotype resembling type 2 diabetes and including hyperglycaemia at 4 months of age[25,26] making it a good species to investigate whether IUGR accelerates the development of metabolic disease with ageing. Maternal feed restriction in the guinea pig at 85 or 70% of *ad libitum* intake reduces fetal and placental weights, increases visceral adiposity in late gestation fetuses and alters placental structure impairing function.[27–29] Offspring of feed-restricted mothers do not undergo neonatal catch-up growth, but are hyperphagic post-weaning, and as adults have impaired glucose tolerance and cholesterol homoeostasis, increased blood pressure and visceral adiposity,[3–5] demonstrating that prenatal restriction programmes metabolic

*Address for correspondence: Dr K. L. Gatford, Discipline of Obstetrics and Gynaecology, School of Medicine, University of Adelaide, SA 5005, Australia. (Email Kathy.gatford@adelaide.edu.au)

2.4 Abstract

Intrauterine growth restriction (IUGR) and subsequent neonatal catch-up growth are implicated in the programming of increased appetite, adiposity and cardiometabolic diseases. Guinea pigs provide an alternate small animal model to rodents in which to investigate mechanisms underlying prenatal programming, being relatively precocial at birth, with smaller litter sizes, and undergoing neonatal catch-up growth after IUGR. The current study therefore investigated postnatal consequences of spontaneous IUGR due to varying litter size in this species. Size at birth, neonatal, juvenile (post-weaning, 30-60 d) and adolescent (60-90 d) growth, juvenile and adolescent food intake and body composition of young adults (120 d) were measured in 158 male and female guinea pigs from litter sizes of one to five pups. Compared to singleton pups, birth weight of pups from litters of five was reduced by 38%. Other birth size measures were reduced to lesser degrees with head dimensions being relatively conserved. Pups from larger litters had faster fractional neonatal growth and faster absolute and fractional juvenile growth rates (P<0.005 for all). Relationships of post-weaning growth, feed intakes and adult body composition with size at birth and neonatal growth rate were sex-specific, with neonatal growth rates strongly and positively correlated with adiposity in males only. In conclusion, spontaneous IUGR due to large litter sizes in the guinea pig causes many of the programmed sequelae of IUGR reported in other species, including human. This may therefore be a useful model in which to investigate the mechanisms underpinning perinatal programming of hyperphagia, obesity and longer-term metabolic consequences.

Keywords: adiposity, appetite, guinea pig, litter size, sex differences

2.5 **Introduction**

Small size at birth, following intrauterine growth restriction (IUGR), and/or subsequent neonatal catch-up growth are implicated in the initiation of permanent metabolic and/or physiological adaptations that persist through adult life. This "programming" may lead to increased appetite, adiposity (particularly of visceral depots) and cardiovascular and metabolic diseases including insulin resistance and glucose intolerance in adults[16, 396]. Therefore, animal models that mimic human IUGR aetiology, develop cardiovascular and metabolic sequelae with ageing after IUGR and/or catch growth and are comparable to the human in their relative maturity at birth are required so that underlying mechanisms and intervention strategies can be investigated.

Small animal models have logistical advantages for such studies, including relatively rapid development, and short lifespans facilitating study of the development of progeny with ageing and intergenerational effects. Many small animal models of IUGR have used maternal feed[310-312, 335, 374] or protein restriction[332, 335, 375-377] to restrict fetal growth and investigate long-term outcomes. In developed countries, however, placental insufficiency rather than maternal undernutrition accounts for the majority of human IUGR[378, 379]. Placental restriction induced by uterine artery ligation in rats causes IUGR and programs many components of the metabolic syndrome[276, 280, 380, 381]. These IUGR rat models often lack catch-up growth in the early neonatal period[276, 280, 381], which is an independent risk factor for the development of adult metabolic disease in humans[83, 382-384].

Guinea pigs provide an alternate species in which to investigate developmental programming of health and disease. The guinea pig has a smaller litter size than the rat[280, 374, 392, 397], is relatively precocial at birth, and resembles the human fetus in having a body fat composition of around 10% at term[398]. In addition, and unlike the rat, the guinea pig spontaneously develops a phenotype resembling type 2 diabetes and including hyperglycaemia at 4 months of age[399, 400] making it a good species in which to investigate whether IUGR accelerates the development of

metabolic disease with ageing. Maternal feed restriction in the guinea pig at 85% or 70% of *ad libitum* intake reduces fetal and placental weights, increases visceral adiposity in late gestation fetuses and alters placental structure impairing function[309, 401, 402]. Offspring of feed restricted mothers do not undergo neonatal catch-up growth, but are hyperphagic post-weaning, and as adults have impaired glucose tolerance and cholesterol homoeostasis, increased blood pressure and visceral adiposity[310-312], demonstrating that prenatal restriction programs metabolic dysfunction in this species. These adverse effects are mostly seen in males, suggesting developmental programming in the guinea pig is sex-specific as described in other species, including humans[385]. Similarly uterine artery ablation in the mid-gestation guinea pig produces offspring with disproportionate IUGR (also known as asymmetrical IUGR), IUGR, and male offspring are hyperphagic post-weaning and have increased epididymal adiposity (postnatal outcomes were not assessed in females)[284, 403]. Furthermore, these surgical models impose sudden restriction on normal fetal growth at ~0.5 of term, and induce fetal death of between ~50 and 70% of pups[283]. Interestingly, at least in the case of the maternal undernutrition studies where correlation analyses were reported, size at birth was a stronger predictor of adult outcomes than was nutritional group[310-312]. In the present study, we therefore chose to investigate the effects of spontaneous fetal growth restriction that occurs with variation in litter size in the guinea pig.

Spontaneous variation in litter size in the guinea pig also restricts fetal and placental growth in larger litters[391, 392]. Importantly, IUGR progeny from large litters undergo neonatal catch-up growth[394]. Consequences of this spontaneous IUGR due to litter size for subsequent postnatal growth, appetite and adult body composition have not been assessed. We therefore characterized the effects of variation in litter size and hence size at birth on these outcomes in the guinea pig. Because developmental programming is sex-specific in other species and in the guinea pig following maternal feed restriction, we investigated outcomes in both male and female progeny.

2.6 **Materials and Methods**

2.6.1 Animals

All guinea pigs had *ad libitum* access to a commercially-prepared guinea pig and rabbit ration (diet L102, Milling Industries Stockfeeds, Blair Athol, SA, Australia) containing 2640 kcal/kg digestible energy, 19.0% crude protein and 2.5% crude fat, and supplemented with an increased content of vitamin E (165 mg/kg). All guinea pigs had *ad libitum* access to tap water with added ascorbic acid (400 mg/l, Ace Chemical Company, Camden Park, S.A. Australia). Nulliparous female guinea pigs were obtained at 3-4 months of age (Institute of Medical and Veterinary Science Tri-coloured, Gilles Plains Resource Centre, Gilles Plains SA, Australia) and housed in a 12:12 h day:night cycle throughout the experiment. Females were individually housed in wire cages and after 2-4 weeks acclimatisation were weighed three times per week, and checked for oestrus daily as indicated by a rupture of the vaginal membrane[404]. A single male was placed with the female during her oestrus (2-3 days of ~15 days cycle) and pregnancy was detected by the presence of a copulatory plug on the following morning. Pregnancy was confirmed (n=68) if the animal failed to return to oestrus in the subsequent cycle. At day 60 of gestation dams were transferred to individual housing in plastic tubs with paper bedding.

After spontaneous delivery at term (range 65-74 days, mean ± S.E.M. 69.7 ± 0.1 days), numbers, sex, weights, abdominal circumference and nose to rump lengths of all liveborn offspring were measured and recorded on the day of birth or following morning if delivered overnight (n=158 offspring, males: n=78, females: n=80). Head widths (n=148, males: n=71, females: n=77) and lengths (n=144, males: n=69, females: n=75) were also measured in a subset of the progeny. Each dam was housed with her offspring and provided with *ad libitum* lucerne chaff in addition to the standard diet. Litters were weighed at least five times per week, until weaned at days 28-30 of age. Absolute growth rates (AGRs) for weight were calculated from the slope of the growth curve

from day 10 until weaning (AGR_{10-28}), with data from birth to day 10 excluded due to non-linear growth over this period (Figure 2-2). After weaning all guinea pigs were housed individually and were weighed at least three times per week from day 30 until post-mortem at day 115 ± 1. Puberty in the guinea pig occurs between day 56-60 in males, and between day 30 and day 134 in females, with a mean age at puberty for females of 68 ± 22 days (mean ± SD)[404]. AGRs were, therefore, also calculated for juvenile (weaning - day 60, AGR_{30-60}), and adolescent (days 60-90, AGR_{60-90}) periods. Current fractional growth rate (FGR) for weight for each stage was calculated as AGR divided by the animal's weight at the beginning of that stage. Feed intakes were recorded daily from day 40 to day 100 (61 males, 58 females) by weighing the filled feed hopper at 9 am, and then again before refilling at 9 am the following day. Relative feed intakes were calculated by dividing daily feed intake by body weight. Average feed intakes for each animal were then calculated for juvenile (days 40-60) and adolescent (days 60-90) periods, and feed efficiency was calculated as average daily weight gain divided by average daily absolute food intake over each period.

2.6.2 Adult Body Composition

At day 115 ± 1, a subset of animals (n=41, 22 males, 19 females) selected randomly within each sex were humanely killed between 2 pm and 4 pm by lethal injection of sodium phenobarbitone. Fat depots (interscapular, omental and right side of the neck as well as bilateral axillary, retroperitoneal, perirenal, and groin depots) and bilateral skeletal muscles (hindlimb: *M. biceps femoris, M.* sem*itendinosus, M. gastrocnemius, M. plantaris, and M. tibialis; forelimb: M. biceps brachii*) were dissected and weighed. Visceral adipose weight was calculated as the sum of weights of the left and right perirenal and retroperitoneal fat depots. The omental fat associated with the gastrointestinal tract is highly insulin resistant, drains directly into the portal vein and is strongly associated with hepatic insulin resistance[405] and was, therefore, weighed and analysed

separately. Subcutaneous adipose weight was calculated as the sum of weights of left and right axillary and groin fat, right side of the neck fat and interscapular fat depots. Visceral and subcutaneous fats were summed to give a measure of combined adiposity. The weights of dissected skeletal muscles were summed to obtain combined skeletal muscle mass. A high adiposity to lean tissue ratio is a risk factor for many cardiovascular and metabolic diseases[406]. A ratio of the combined adiposity to the combined muscle mass was, therefore, calculated as an index of adiposity relative to lean tissue.

2.6.3 Statistical Analysis

Data were analysed using SPSS 23.0 for Windows (IBM, Armonk, NY, USA). The effects of litter size on weight before mating, weight gain during gestation and change in weight during lactation were analysed by repeated-measures ANOVA. Effects of litter size on proportions of liveborn and stillborn progeny were analysed by χ^2 test. The effects of litter size and sex on birth phenotype and postnatal outcomes were analysed by mixed models ANOVA, including the dam as a random variable to account for effects of a common maternal environment. Bonferroni's *post-hoc* comparisons were used to compare differences in maternal and offspring outcomes between litter sizes. Where effects of litter size differed between sexes, outcomes were analysed separately in each sex. Relationships between size at birth and growth rates were examined by Pearson's correlation analysis, separately in each sex. Because birth weight ranges overlapped between litter sizes, and in order to assess the effect of catch-up growth on postnatal phenotypes, the independent effects of birth weight and neonatal growth rate for weight on post-weaning outcomes were examined by multiple linear regression separately in each sex. Gestation length was not correlated with outcomes when included in initial models and was, therefore, excluded from final multiple linear regression models. A P-value of $\leqslant 0.05$ was accepted as statistically significant. All results are expressed as mean ± S.E.M.

2.7 **Results**

2.7.1 Maternal Outcomes

Maternal weight at the oestrus before mating did not differ between the litters, however, weight over the 10 days prior to mating differed over time (*P*<0.001) and differed between the litter sizes over time (*P*=0.048, Figure 2-1). Dam weight on the day of mating (G0) correlated positively with subsequent litter size (*r*=0.280, *P*=0.025, data not shown).

Weight increased with time over gestation, and differed between litter size groups, and the change in weight with time also differed between litter sizes (*P*<0.001 for all, Figure 2-1). The change in maternal weight in absolute terms from mating to day 60 of gestation (G60) increased with litter size (*P*<0.001, Table 2-1). Dams with litter size of three, four and five gained more weight over the first 60 days of gestation than those with litter sizes of one or two (Table 2-1). In contrast, change in maternal weight from mating to the day after delivery, reflecting weight of the dam herself, was lower in dams that delivered five pups than in all other groups (Table 2-1). Maternal feed intakes in mid gestation were greater in dams carrying five fetuses than in those with smaller litters (Table 2-1). In late gestation, dams carrying four or five fetuses ate more than dams carrying one or two fetuses (Table 2-1).

Weight during lactation also differed between dams that delivered different litter sizes (*P*=0.036), changed with day (*P*<0.001), and the change in weight over time differed between the litter sizes (*P*=0.023, Figure 2-1). Dams that gave birth to litters of five gained more weight during lactation than all other litter sizes (Table 2-1).

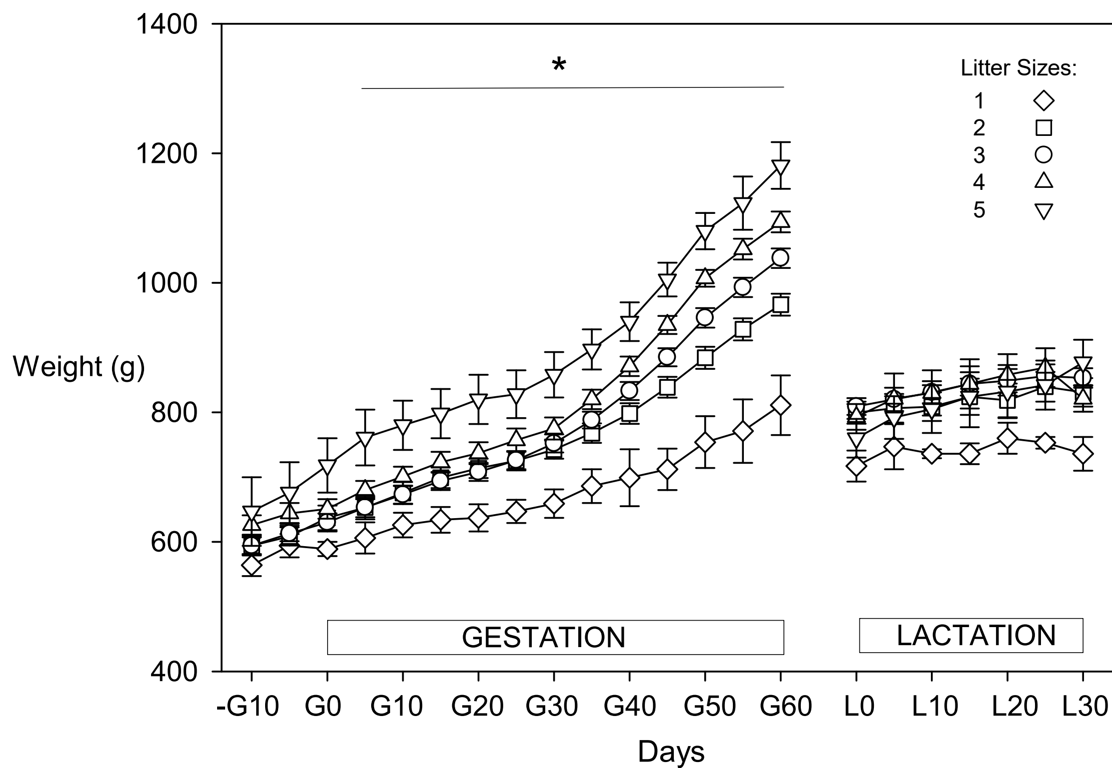


Figure 2-1 Maternal weight in pregnancy and lactation according to litter size. Maternal weight during the first 60 days of gestation (G) and throughout lactation (L). Dams carrying litters of five are shown in downwards triangles, four in upward triangles, three in circles, two in squares and singletons in diamonds. * $P< 0.05$ for litter size effects at these time points.

Table 2-1 Effect of litter size on maternal weights, absolute growth rates and feed intake during pregnancy.

	Litter Size					Significance
Outcome	One	Two	Three	Four	Five	LS
Number of Litters	5	18	25	15	4	
Surviving:stillborn pups at birth	5:0	36:0	71:4	49:11	10:10	<0.001
Total Pup weight per litter (g)	120 ± 2^{a}	221 ± 5^{b}	295 ± 6^{c}	367 ± 8^{d}	384 ± 31^{d}	<0.001
Gestation length (days)	71 ± 1^{a}	71 ± 0^{a}	$70 \pm 0^{a,b}$	$70 \pm 0^{a,b}$	68 ± 1^{b}	0.012
Maternal Weights						
Pre-mating Weight (g)	589 ± 11	637 ± 19	630 ± 14	651 ± 15	718 ± 41	0.091
Δ Weight through gestation, G0-G60 (g)	217 ± 35^{a}	329 ± 16^{a}	408 ± 13^{b}	440 ± 17^{b}	442 ± 20^{b}	<0.001
Δ Maternal weight, G0-L0 (g)	$128 \pm 17^{a,b}$	165 ± 22^{a}	173 ± 14^{a}	$153 \pm 19^{a,b}$	42 ± 29^{b}	0.025
Δ Maternal weight, L0-L30 (g)	26 ± 20^{a}	44 ± 13^{a}	51 ± 7^{a}	21 ± 14^{a}	117 ± 21^{b}	0.003
Maternal Feed Intake						
Mid-gestation, G20-G40 (g/day)	31 ± 3^{a}	40 ± 2^{a}	41 ± 1^{a}	43 ± 2^{a}	59 ± 4^{b}	<0.001
Late-gestation, G40-G60 (g/day)	38 ± 3^{a}	42 ± 1^{a}	$46 \pm 1^{a,b}$	47 ± 1^{b}	50 ± 3^{b}	0.001

Data are expressed as mean ± S.E.M. for dams carrying each of the litter sizes (LS). [a,b,c,d] Means with different superscripts differ, *P*<0.05.

2.7.2 Litter Outcomes

Gestation length differed between litter sizes, and was shorter in dams that delivered five pups compared to those with one or two (Table 2-1). Gestation length correlated negatively with the total pup weight in the litter (r= -0.268, P=0.035, n=67). The proportion of pups born alive decreased with increasing litter size (P<0.001), falling from 100% in litter sizes of one and two, to 94% in litters of three pups, 82% in litters of four pups, and 50% in litters of five pups. Stillbirths were unevenly distributed between litters, and were not consistently smaller than liveborn litter mates (data not shown). Total litter weight increased with increasing litter size (P<0.001), and was greater than three-fold higher in litters of four or five than in singleton litters (Table 2-1).

2.7.3 Birth Phenotype

Size at birth of liveborn pups included in later studies in terms of weight, length, abdominal circumference and weight:length ratio decreased as litter size increased (Table 2-2). Birth weight averaged 97 ± 1 g and ranged from 57 to 134 g across the cohort. Pups from singleton litters were all heavier than pups from litters of five, whereas some overlap in the range of individual birth weights was seen between pups from other litter sizes (Figure 2-2). Average birth weight of pups from a litter size of five was 38% lower than those from litter size of one, whereas other birth size measures were reduced to a lesser degree (length 18%; abdominal circumference 17%; weight:length 26%, Table 2-2). Head lengths and widths were relatively conserved in large litters (Table 2-2). Weight:length ratio and body mass index, measures of disproportionate growth, decreased with increasing litter size (Table 2-2).

Table 2-2 Effect of litter size on birth phenotype of live-born pups

Outcome	Litter Size					ANOVA		
	One	Two	Three	Four	Five	LS	Sex	LS × Sex
Number of litters	5	18	25	15	4			
Number of offspring	5	33	65	46	9			
Male:female Ratio	1:4	18:15	30:35	21:25	3:6			
Size at Birth								
Weight (g)	121 ± 2[a]	111 ± 2[a]	96 ± 1[b]	90 ± 2[b]	73 ± 6[c]	<0.001	NSD	NSD
Length (mm)	173 ± 3[a]	163 ± 2[a,b]	158 ± 1[a,b,c]	154 ± 1[c]	141 ± 4[d]	<0.001	NSD	NSD
Abdominal circumference (mm)	117 ± 4[a]	111 ± 2[a]	104 ± 1[b]	103 ± 1[b]	96 ± 2[b]	<0.001	NSD	NSD
Head length (mm)[e]	47.1 ± 0.9[a]	43.8 ± 0.6[a]	42.2 ± 0.5 [a,b]	40.4 ± 0.7[b]	42.7 ± 0.7[a,b]	0.004	NSD	NSD
Head width (mm)[f]	23.5 ± 0.7[a]	22.0 ± 0.2[a,b]	22.1 ± 0.2[a,b]	21.1 ± 0.2[b]	21.2 ± 0.3[a,b]	0.024	NSD	NSD
Weight:length (g/mm)	0.70 ± 0.02[a]	0.68 ± 0.01[a]	0.61 ± 0.01[b]	0.58 ± 0.01[b]	0.51 ± 0.02[c]	<0.001	NSD	NSD
Body mass index (kg/m^2)	4.05 ± 0.16[a,b]	4.16 ± 0.08[a]	3.89 ± 0.04 [a,b]	3.81 ± 0.06[b]	3.61 ± 0.13[a,b]	0.008	NSD	NSD

LS × sex, interaction between litter size and sex. NSD: not significantly different, *P*>0.1
Data are expressed as actual means ± S.E.M. of offspring in each of the litter sizes (LS) for pups that survived to adulthood and were included in growth measures. Statistical models included dam to correct for the common intrauterine environment in multiple birth litters.
[a,b,c,d]Means with different superscripts differ, *P*<0.05.
[e]Head lengths were not measured in 4 offspring from litter size of three and 10 offspring from litter size of four.
[f]Head widths were not measured in 4 offspring from litter size of three and six from litter size of four.

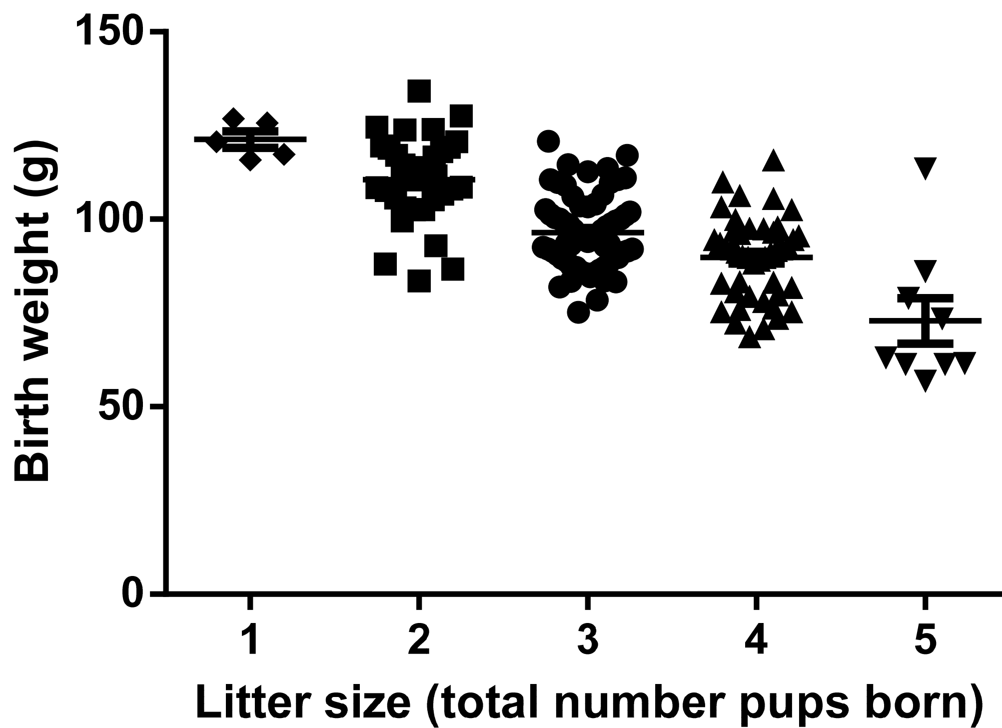


Figure 2-2 Birth weight distribution and litter size.
Litter size and birth weights of individual guinea pig pups included in postnatal studies. Each symbol indicates an individual pup that survived to young adulthood, lines and whiskers indicate means and S.E.M. for each litter size group.

2.7.4 Postnatal Growth

2.7.4.1 *Neonates*

Growth rates were non-linear in the first 10 days of life in both sexes (Figure 2-3A and B). In repeated-measures analysis of weight measures during the neonatal period, weight decreased with increasing litter size in both sexes, and this difference in weight amplified with ageing in males but not females (Figure 2-3A and Figure 2-3B). $AGR_{10\text{-}28}$ did not differ between litter sizes. Effects of litter size on neonatal FGR differed between sexes, such that in males $FGR_{10\text{-}28}$ increased with each increase in litter size from two to four pups (Table 2-3). In females, $FGR_{10\text{-}28}$ was greater in pups from a litter size of four than those from litter sizes of two or three (Table 2-3). Neonatal AGR and FGR were higher in males than females (Table 2-3). $AGR_{10\text{-}28}$ correlated positively with birth weight across the full range of litter sizes (r=0.296, P<0.001) and in males and females separately (r=0.323, P=0.003; r=0.303, P=0.001, respectively, Figure 4A). $FGR_{10\text{-}28}$ correlated negatively with birth weight overall (r= - 0.525, P<0.001) and in males and females separately (r= -0.522, P<0.001; r= -0.701, P<0.001 respectively, Figure 2-4B).

2.7.4.2 *Juveniles*

Similar to the neonatal period, body weights during the juvenile period did not differ between litter size groups in females (Figure 2-3D). In males, juvenile body weights increased more rapidly in those from larger litters during this period (Figure 2-3C). AGR and FGR of juvenile guinea pigs were higher in those from litter sizes of four compared to those in litter sizes of two, and higher in males than females (Table 2-2). $AGR_{30\text{-}60}$ was not correlated with birth weight overall, or in males or females separately. $FGR_{30\text{-}60}$ correlated negatively with birth weight overall (r= -0.371, P<0.001), and in males (r= -0.398, P<0.001) and females (r= -0.452,

P<0.001) separately. In males, AGR_{30-60} was independently and positively correlated with neonatal FGR, but not with birth weight (Table 2-4). AGR_{30-60} was not independently correlated with birth weight or neonatal FGR in females (Table 2-4). In both males and females, FGR_{30-60} was negatively correlated with birth weight but not neonatal FGR (Table 2-4).

2.7.4.3 *Adolescents*

In adolescents, the change in body weight with age differed between litter sizes in males (Figure 2-3E), but litter size did not affect weights in females (Figure 2-3F). During the adolescent period, neither AGR nor FGRs differed between litter sizes (Table 2-3). AGR_{60-90} was higher in males than females, and FGR_{60-90} did not differ between sexes. AGR_{60-90} was not correlated with birth weight, in males or in females, and was independently and positively correlated with neonatal FGR in females only (Table 2-4). Similarly, FGR_{60-90} was not correlated with birth weight, in males or in females, and was independently and positively correlated with neonatal FGR in females only (Table 2-4).

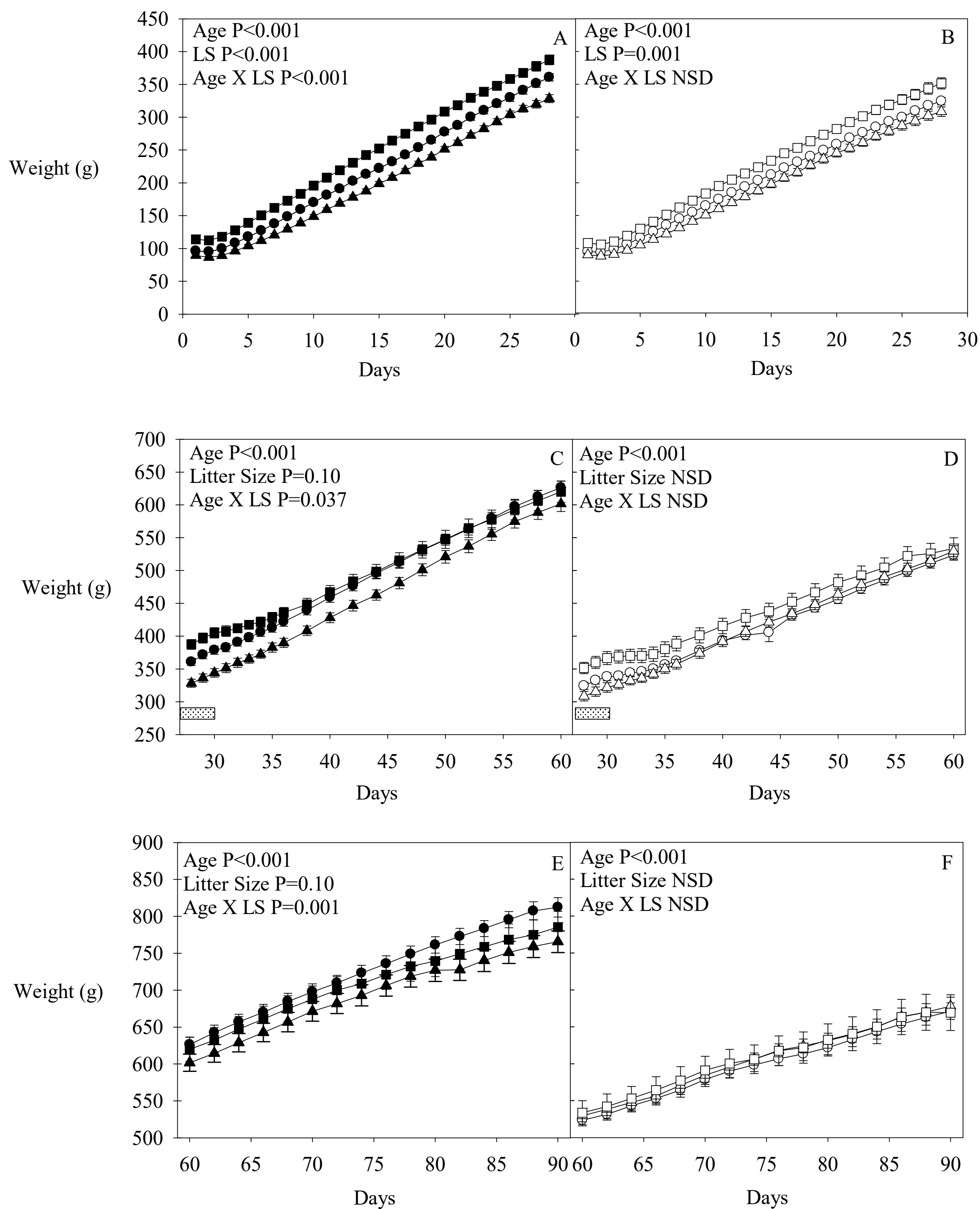


Figure 2-3 Growth of progeny as neonates, juveniles and during adolescence

Body weights of male (A, C, E) and female (B, D, F) progeny that survived to adulthood and were included in growth measures, from birth to weaning (A, B), during the juvenile period (C, D) and during adolescence (E, F). Males are shown in closed symbols and females in open symbols with offspring from litters of four in upward triangles, three in circles and two in squares. Bar in panels C and D indicate weaning of offspring from the dam between 28 and 30 days after birth.

Table 2-3 Effect of litter size and sex on postnatal growth rates and food intake.

	Males from Litter Size			Females from Litter Size			ANOVA		
Outcome	Two	Three	Four	Two	Three	Four	LS	Sex	LS × sex
Postnatal Growth Rates									
Number of Offspring	18	35	21	15	30	25			
Neonatal									
AGR_{10-28} (g/d)	10.7 ± 0.3	10.7 ± 0.2	10.2 ± 0.2	9.4 ± 0.3	9.0 ± 0.2	8.9 ± 0.2	NSD	<0.001	NSD
FGR_{10-28} (%)	5.5 ± 0.1[a]	6.3 ± 0.1[b]	6.9 ± 0.2[c]	5.2 ± 0.2[a]	5.5 ± 0.1[a]	6.0 ± 0.1[b]	<0.001	<0.001	<0.008[1]
Juvenile									
AGR_{30-60} (g/d)	7.5 ± 0.4[a]	8.7 ± 0[a,b]	8.9 ± 0.3[b]	6.0 ± 0.3[a]	6.2 ± 0.2[a]	7.1 ± 0.2[b]	0.005[2]	<0.001	NSD
FGR_{30-60} (%)	1.86 ± 0.09[a]	2.29 ± 0.07[b]	2.59 ± 0.08[c]	1.66 ± 0.08[a]	1.85 ± 0.07[b]	2.24 ± 0.08[c]	<0.001[3]	<0.001	NSD
Adolescent									
AGR_{60-90} (g/d)	5.5 ± 0.3	6.4 ± 0.2	5.6 ± 0.2	4.9 ± 0.3	4.9 ± 0.2	5.1 ± 0.2	NSD	<0.001	0.046[4]
FGR_{60-90} (%)	0.89 ± 0.04	1.01 ± 0.03	0.94 ± 0.04	0.92 ± 0.06	0.95 ± 0.03	0.97 ± 0.04	NSD	NSD	NSD
Feed Intakes									
Number of Offspring	15	28	16	12	21	17			
Juvenile (40-60 days)									
Absolute (g/day)	40 ± 2	43 ± 1	43 ± 1	34 ± 1	36 ± 1	37 ± 1	NSD	<0.001	NSD
Relative (g/kg/day)	73 ± 2	78 ± 1	78 ± 1	70 ± 2[a]	77 ± 2[b]	79 ± 1[b]	0.003[5]	NSD	NSD
Adolescent (60-90 days)									
Absolute (g/day)	45 ± 2	50 ± 1	50 ± 2	41 ± 3	40 ± 1	42 ± 2	NSD	<0.001	NSD
Relative (g/kg/day)	65 ± 2	70 ± 1	72 ± 3	70 ± 3	68 ± 2	69 ± 2	NSD	NSD	NSD
Feed Efficiency									
Juvenile (40-60 days)	0.184 ± 0.008	0.197 ± 0.005	0.204 ± 0.006	0.163 ± 0.008	0.177 ± 0.006	0.186 ± 0.008	0.060[6]	<0.001	NSD
Adolescent (60-90 days)	0.177 ± 0.010	0.189 ± 0.006	0.168 ± 0.008	0.155 ± 0.007[a]	0.179 ± 0.004[b]	0.173 ± 0.002[a,b]	0.040[7]	NSD	NSD

LS × sex , interaction between litter size and sex; AGR, absolute growth rate; NSD: not significantly different, $P>0.1$; FGR, fractional growth rate.
Feed efficiency was calculated as average daily weight gain over each period divided by average daily feed intake. Data are expressed as means ± S.E.M. Statistical models included dam to correct for common intrauterine environment in multiple births. [a,b,c] Means with different superscripts differ, $P<0.05$.
[1]In males, neonatal FGR_{10-28} differed between all litter sizes ($P<0.01$ for all), while in females, offspring of litters of four grew faster than litter of two and three only ($P<0.02$ for both).
[2]Overall, juvenile AGR_{30-60} was greater in offspring from litters of four compared to litters of two ($P=0.004$) only.
[3]Overall, juvenile FGR_{30-60} differed between all litter sizes ($P<0.02$ for all). [4]Adolescent growth rates did not differ between litter sizes in males or females analysed separately.
[5]Relative juvenile feed intake was greater in offspring from litters of three and four than in offspring from litters of two ($P<0.02$ for both).
[6] Juvenile (40-60 days) feed efficiency did not differ between litter sizes in males or females analysed separately. [7] Adolescent feed efficiency was greater in females, and tended to be greater overall, in progeny from litters of three pups than in those from litters of two pups (females: $P=0.013$, overall: $P=0.066$), and did not differ between litter sizes in males.

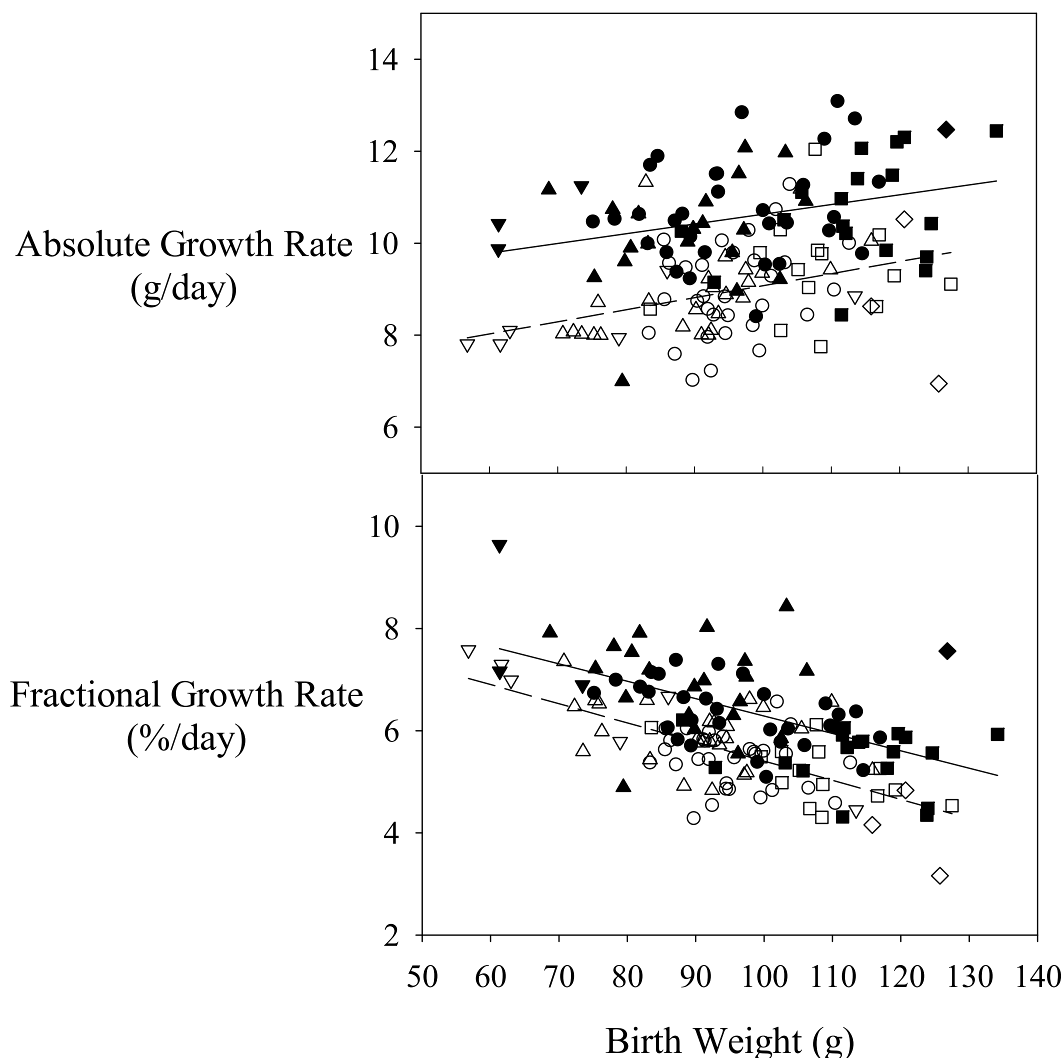


FIGURE 2-4 RELATIONSHIPS BETWEEN ABSOLUTE AND FRACTIONAL NEONATAL GROWTH RATE AND BIRTH WEIGHT IN MALE AND FEMALE GUINEA PIGS.

Relationships between absolute (A) and fractional (B) neonatal growth rates from days 10-28 and birth weight for male (n=78 closed symbols, solid regression line) and female (n=80, open symbols, dashed regression line) guinea pigs. A. Absolute neonatal growth rate correlated positively with birth weight in males (r=0.323, P=0.003) and females (r=0.303, P=0.006). B. Fractional neonatal growth rate correlated negatively with birth weight in males (r=-0.522, P<0.001) and females (r=-0.701, P<0.001). Offspring from litters of five are shown in downwards triangles, four in upward triangles, three in circles, litters of two in squares and singletons in diamonds.

2.7.5 Feed Intake

Feed intake in juvenile progeny did not differ between litter sizes, and was greater in males than females, in absolute terms (Table 2-3). In contrast, relative feed intake in juveniles differed between litter sizes (Table 2-3). Overall, and in females, progeny from litters of three or four pups ate more in relative terms than those from litters of two pups (Table 2-3). Feed efficiency (weight gain per intake) in juvenile guinea pigs did not differ between litter sizes and was higher in males than in females (Table 2-3). In males, absolute juvenile feed intake was independently and positively predicted by birth weight and neonatal FGRs; these correlations were not significant in females, or for relative feed intake in the juvenile period (Table 2-4). Feed efficiency was correlated negatively with birth weight in juveniles of both sexes (Table 2-4).

Feed intake of adolescent progeny did not differ between litter size groups, and was higher in absolute but not relative terms in males than females (Table 2-3). Feed efficiency (weight gain per intake) in adolescent guinea pigs varied with litter size and was higher in progeny from litters of three than those from litters of two in females, with a similar trend overall, but not in males (Table 2-3). In males, adolescent absolute feed intake was predicted independently and positively by neonatal FGR, but not by birth weight (Table 2-4). In females, adolescent absolute feed intake was predicted by the overall model and independently and positively by both birth weight and neonatal FGR (Table 2-4). Relative adolescent feed intake was not correlated with birth weight or neonatal growth rate in males. In females, however, relative adolescent feed intake was predicted independently and positively by neonatal FGR, but not by birth weight (Table 2-4). Feed efficiency in adolescent males was not correlated with either birth weight or neonatal FGR (Table 2-4). Feed efficiency in adolescent females was correlated independently and negatively with birth weight (Table 2-4).

Table 2-4 Relationships between birth weight and neonatal FGR and subsequent postnatal growth rates and food intake

Measure	Overall and Partial Correlation (*r, P*)					
	Males			Females		
	Overall	Birth Weight	FGR_{10-28}	Overall	Birth Weight	FGR_{10-28}
Postnatal Growth Rates		*n*=76			*n*=80	
Juvenile						
AGR_{30-60} (g/day)	0.240, 0.115	0.099, 0.397	**0.238, 0.040***	0.128, 0.531	0.008, 0.944	0.097, 0.396
FGR_{30-60} (%)	**0.412, 0.001***	**-0.297, 0.010***	0.114, 0.330	**0.452, 0.001***	**-0.334, 0.003***	0.010, 0.929
Adolescent						
AGR_{60-90} (g/day)	0.208, 0.204	0.047, 0.693	*0.193, 0.093*	**0.408, 0.001***	*0.189, 0.099*	**0.390, 0.001***
FGR_{60-90} (%)	*0.272, 0.063*	-0.185, 0.115	0.081, 0.494	**0.494, 0.001***	-0.052, 0.650	**0.344, 0.002***
Feed Intakes		*n*=61			*n*=58	
Juvenile (40-60 days)						
Absolute (g/day)	**0.396, 0.007***	**0.350, 0.006***	**0.354, 0.006***	0.253, 0.161	0.188, 0.162	*0.289, 0.057*
Relative (g/kg/day)	0.221, 0.234	-0.025, 0.850	0.176, 0.178	**0.351, 0.027***	-0.159, 0.236	0.127, 0.347
Adolescent (60-90 days)						
Absolute (g/day)	**0.344, 0.026***	*0.255, 0.055*	**0.336, 0.009***	**0.434, 0.004***	**0.327, 0.014***	**0.434, 0.001***
Relative (g/kg/day)	*0.282, 0.090*	-0.100, 0.445	0.180, 0.168	**0.507, 0.001***	0.124, 0.361	**0.445, 0.001***
Feed Efficiency						
Juvenile (40-60 days)	*0.295, 0.072*	**-0.281, 0.021***	-0.072, 0.587	**0.323, 0.049***	**-0.269, 0.043***	-0.056, 0.679
Adolescent (60-90 days)	0.106, 0.729	-0.021, 0.878	-0.100, 0.457	**0.339, 0.037***	**-0.285, 0.033***	-0.063, 0.645

n: Total number of offspring from all litters. FGR_{10-28}, fractional growth rate for weight in neonates from 10-28 days of age. AGR, absolute growth rate
*Significant correlations ($P < 0.05$) are shown in **bold** and trends ($P < 0.1$) in *italics.*
Feed efficiency was calculated as average daily weight gain over each period divided by average daily feed intake.

2.7.6 Adult Phenotype and Body Composition

2.7.6.1 *Body size*

Adult body size did not differ between litter size groups in males or females (Table 2-5). Males were heavier, longer and had a higher weight:length ratio than females, but body mass index did not differ between sexes (Table 2-5). In males, adult body weight and weight:length ratio correlated independently and positively with neonatal FGR with similar trends for birth weight (Table 2-6). In females, adult body weight correlated independently and positively with birth weight with a similar trend for neonatal FGR (Table 2-6). Other measures of adult size were not correlated (P>0.05 for all) with size at birth or neonatal FGR in either sex.

2.7.6.2 *Body composition*

Relative weights of subcutaneous, visceral, omental and total dissected fat depots and absolute weight of visceral and omental fat differed between litter sizes overall, with similar trends for absolute weights of subcutaneous and total dissected fat depots, and effects of litter size were similar in each sex (Table 2-5). Nevertheless, absolute weight of visceral and total dissected fat and relative weights of subcutaneous and visceral depots did not differ between any two litter sizes. Overall, offspring from litters of three had higher absolute and relative omental fat weights (P=0.049 and P=0.013 respectively) and higher relative total dissected fat (P=0.038) than those from litters of two; absolute and relative weights of fat depots did not differ between litter size pairs in sex-specific analyses. Absolute but not relative weights of subcutaneous, visceral and total dissected fat depots were greater in males than females (Table 2-5). Skeletal muscle weights did not differ between litter sizes, whilst absolute skeletal muscle weights were higher in males than females (Table 2-5). The ratio of dissected fat:skeletal muscle weights did not differ between litter size groups or sexes (Table 2-5). In males, absolute

and relative weights of multiple fat depots, but not absolute or relative skeletal muscle weights, were independently and positively correlated with birth weight and neonatal FGR (Table 2-6). These correlations were strongest for weights of visceral fats and with the ratio of dissected fat to skeletal muscle weights (Table 2-6). In females, in contrast, absolute skeletal muscle weight was independently and positively correlated with birth weight but not neonatal FGR, and fat depot weights were not correlated with either birth weight or neonatal FGR (Table 2-6).

Table 2-5 Effect of litter size and sex on adult size and body composition

	Males from Litter Size			Females from Litter Size			ANOVA		
	Two	Three	Four	Two	Three	Four	LS	Sex	LS × Sex
Number of litters	6	5	3	2	5	3			
Number of offspring	8	7	7	2	9	3			
Adult Phenotype									
Weight (g)	763 ± 37	838 ± 32	782 ± 34	676 ± 26	679 ± 18	674 ± 41	NSD	0.001	NSD
Length (mm)	342 ± 4	344 ± 10	339 ± 3	330 ± 6	318 ± 4	315 ± 7	NSD	0.001	NSD
Weight:length (kg/m)	2.2 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.2	NSD	0.021	NSD
Body mass index (kg/m^2)	6.5 ± 0.3	7.1 ± 0.3	6.8 ± 0.2	6.2 ± 0.5	6.7 ± 0.2	6.8 ± 0.7	NSD	NSD	NSD
Adult Body Composition									
Subcutaneous fat (g)	34 ± 4	42 ± 3	33 ± 5	26 ± 2	32 ± 2	28 ± 4	0.096	0.028	NSD
Subcutaneous fat (%)	4.3 ± 0.3	5.1 ± 0.2	4.2 ± 0.4	3.8 ± 0.1	4.6 ± 0.3	4.1 ± 0.4	0.035[1]	NSD	NSD
Visceral fat (g)	15 ± 2	19 ± 1	13 ± 2	10 ± 2	14 ± 1	12 ± 3	0.047[1]	0.026	NSD
Visceral fat (%)	1.9 ± 0.1	2.2 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	2.1 ± 0.1	1.7 ± 0.4	0.026[1]	NSD	NSD
Omental fat (g)	15 ± 1	17 ± 1	15 ± 1	12 ± 1	16 ± 1	14 ± 2	0.037[2]	0.098	NSD
Omental fat (%)	1.9 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	0.013[2]	NSD	NSD
Total dissected fat (g)	63 ± 7	78 ± 4	62 ± 8	48 ± 4	62 ± 4	53 ± 9	0.050[1]	0.029	NSD
Total dissected fat (%)	8.1 ± 0.5	9.5 ± 0.2	7.8 ± 0.7	7.0 ± 0.3	9.1 ± 0.4	7.8 ± 1.0	0.017[2]	NSD	NSD
Skeletal muscle (g)	21 ± 1	22 ± 1	21 ± 1	19 ± 3	18 ± 0	19 ± 1	NSD	0.024	NSD
Skeletal muscle (%)	2.7 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	2.6 ± 0.0	2.7 ± 0.1	2.9 ± 0.2	NSD	NSD	NSD
Total dissected fat: Skeletal muscle	3.0 ± 0.2	3.6 ± 0.1	3.0 ± 0.3	2.6 ± 0.2	3.5 ± 0.2	2.8 ± 0.5	0.060	NSD	NSD

LS × sex, interaction between litter size and sex. NSD: not significant different, $P>0.1$.
Data are expressed as mean ± S.E.M. Mean age ± S.E.M. at post mortem was 115 ± 1 days. Statistical models included dam to correct for common intrauterine environment in multiple births.
[1]Relative subcutaneous, absolute and relative visceral, and absolute total dissected fat weights did not differ ($P>0.05$) between any two litter sizes.
[2]Overall, offspring from litters of three had higher absolute and relative omental fat and relative total dissected fat than those from litters of two ($P<0.05$ for all).

Table 2-6 Relationships between birth weight, neonatal FGR and adult phenotype in the guinea pig.

Measure	Overall and Partial Correlation (r, P)					
	Males n=22			Females n=19		
	Overall	Birth Weight	FGR_{10-28}	Overall	Birth Weight	FGR_{10-28}
Adult size						
Weight (g)	**0.542, 0.037***	*0.428, 0.053*	**0.528, 0.014***	*0.522, 0.079*	**0.517, 0.028***	*0.444, 0.065*
Length (mm)	0.244, 0.558	0.186, 0.420	0.234, 0.307	0.119, 0.899	0.051, 0.846	0.112, 0.668
Body mass index (kg/m^2)	0.405, 0.183	0.308, 0.175	*0.392, 0.079*	0.370, 0.332	0.369, 0.144	0.255, 0.322
Ponderal index (kg/m^3)	0.268, 0.493	0.197, 0.391	0.259, 0.256	0.287, 0.526	0.282, 0.273	0.164, 0.528
Weight:length (kg/m)	**0.524, 0.048***	*0.407, 0.067*	**0.510, 0.018***	0.465, 0.160	*0.464, 0.060*	0.368, 0.146
Adult body composition						
Subcutaneous fat (g)	*0.496, 0.079*	*0.436, 0.054*	**0.455, 0.044***	0.396, 0.255	0.339, 0.169	0.389, 0.111
Subcutaneous fat (%)	0.452, 0.128	*0.410, 0.073*	*0.398, 0.082*	0.322, 0.416	0.240, 0.337	0.322, 0.192
Visceral fat (g)	**0.619, 0.010***	**0.571, 0.007***	**0.563, 0.008***	0.396, 0.255	0.396, 0.104	0.292, 0.239
Visceral fat (%)	**0.611, 0.012***	**0.579, 0.006***	**0.532, 0.013***	0.346, 0.361	0.344, 0.162	0.224, 0.371
Omental fat (g)	**0.656, 0.005***	**0.486, 0.025***	**0.652, 0.001***	0.398, 0.252	0.396, 0.103	0.262, 0.293
Omental fat (%)	**0.620, 0.010***	*0.397, 0.078*	**0.620, 0.002***	0.323, 0.414	0.307, 0.215	0.145, 0.566
Total dissected fat (g)	**0.564, 0.032***	**0.490, 0.028***	**0.529, 0.016***	0.397, 0.254	0.384, 0.116	0.351, 0.153
Total dissected fat (%)	**0.542, 0.044***	**0.482, 0.031***	**0.497, 0.026***	0.316, 0.431	0.306, 0.216	0.273, 0.267
Skeletal muscle (g)	0.217, 0.633	0.112, 0.434	0.198, 0.389	**0.657, 0.011***	**0.537, 0.022***	0.021, 0.934
Skeletal muscle (%)	0.348, 0.294	-0.204, 0.376	-0.348, 0.123	0.477, 0.126	*0.452, 0.059*	0.212, 0.399
Total dissected fat: skeletal muscle	**0.604, 0.017***	**0.511, 0.021***	**0.580, 0.007***	0.323, 0.415	0.203, 0.420	0.320, 0.195

n, Number of offspring. FGR_{10-28}: fractional growth rate for weight in neonates from 10-28 days of age.
Adult body composition is expressed as an absolute weight (g) and as a percentage of the body weight at the time of *postmortem* (%).
Age at *postmortem* was 115 ± 1 days. *Significant correlations ($P < 0.05$) are shown in **bold** and trends ($P < 0.1$) in *italics*.

2.8 **Discussion**

In the current study, increasing litter size in the guinea pig induced disproportionate IUGR which was followed by catch-up growth commencing in neonatal life and which persisted post-weaning but not into adolescence. Concomitant with this accelerated growth, offspring from large litters had increased relative feed intakes as juveniles. Increased neonatal growth also predicted hyperphagia in juveniles, which persisted into adolescent life in both sexes. Interestingly, perinatal growth was correlated with adult body composition differently in males and females, predicting visceral adiposity in males, but not females, and consistent with sex-specific relationships between neonatal growth and adiposity in children[12]. Spontaneous fetal growth restriction induced by large litter sizes in the guinea pig, therefore, induces sex-specific programming of postnatal phenotype. As the guinea pig also develops diabetes mellitus spontaneously in adulthood[399, 400], this provides a model in which to investigate developmental programming of susceptibility to metabolic diseases of ageing.

2.8.1 Maternal Outcomes

Mothers who were larger at conception tended to have the larger litter sizes, consistent with previous reports of a positive relationship between weight and number of corpora lutea at conception[397], and suggesting that their greater litter sizes reflect higher ovulation rates. In the present study, the proportions of stillborn pups increased in large litters, particularly in litters of five pups. This may reflect their earlier gestational age at delivery, which is negatively correlated with stillbirth rates in this species[407]. Small size at birth relative to gestational age is also a predictor for stillbirth in the guinea pig[407]. Restricted nutrient supply *in utero* due to competition for maternal nutrients and/or restricted delivery due to placental growth and/or function may, therefore, also have contributed to poorer neonatal outcomes in large litters in the present study, as these pups were substantially smaller at birth[401, 402]. Not surprisingly, mothers carrying larger litters gained more weight during gestation than those with

smaller litters. Nevertheless, the change in maternal weight from conception to the start of lactation, reflecting growth of the mother herself, was lowest in dams that gestated five pups. Guinea pigs give birth to relatively mature pups that account for a significant part of their own weight[398], and pregnancy appears to reduce maternal energy stores in late gestation, reflected in lighter adipose depots[408]. Together with these previous studies, our results suggest that high fetal demand for nutrients plus inability to further increase feed intake in late gestation limits maternal growth in dams carrying the largest number of pups. Compensatory hyperphagia may, therefore, contribute to the subsequent faster lactation growth rates that we observed in dams that gave birth to large litters. It is also likely that nutrient flow to milk production was lower in these mothers that gestated litters of five pups than in those that delivered three or four pups due to lower perinatal survival of pups and/or earlier weaning. Previous studies have reported that although mothers produce higher yields of milk in larger litters the milk yield per pup is reduced, suggesting pups in larger litters may be "force" weaned earlier in comparison to those from smaller litters[409]. Interestingly, dams in all litter size groups gained weight in lactation in the present study, in contrast to a previous report[410].

2.8.2 Birth Phenotype

Although there was some overlap in ranges of individual birth weights between litter size groups, mean birth weight decreased consistently with increasing litter size and singleton pups were 38% heavier than pups from litters of five. This birth weight difference is of a similar magnitude as that reported for weights of pups in late gestation in multiparous females[391]. Most measures of size at birth decreased with increasing litter size in the present study, including weight to length ratio (an index of thinness), and head width to birth weight ratio (an index of head sparing). Thus, spontaneous fetal growth restriction in guinea pigs from large litters induces disproportionate IUGR, which in human epidemiological studies is associated with greater increases in risks in cardiovascular and metabolic diseases than those associated with a

proportionate reduction in size at birth[210, 411]. Asymmetrical growth restriction resulting in a thin neonate and characterised by head sparing usually reflects restriction predominantly in late gestation[26], and is also observed following maternal famine exposure in late gestation in humans[172] and in experimental models of restricted placental capacity in sheep[293].

Increased litter size may reduce fetal nutrient availability and hence size at birth via a reduction in placental size and function and/or competition for maternal nutrients together with physical limitations on maternal feed intake[392, 394, 397]. Fetal weight in guinea pigs diverges between litter sizes from ~55 days of gestation[391], consistent with progressively increasing limitation of nutrient/oxygen supply in large litters through late gestation. Limited maternal nutrient intake may also contribute to reduced fetal growth in large litters, with similar mean birth weights in litters of four and five in the present study as those induced by feed restriction of guinea pigs throughout gestation to either 85% or 70% of *ad libitum* feed intake[310]. Shorter gestation lengths may also contribute to reduced size at birth. Gestation length is reduced in guinea pig litters with high total fetal weights[394], which may have contributed to the 3-4 day reduction in gestation lengths in litters of five compared to those in litters of one or two pups in the present study, and may in turn have contributed at least in part to smaller sizes at birth in this group. Weight of fetal guinea pigs increases by ~10% over the last 3-4 days of gestation, although interestingly weight gain during this period is markedly lower in large litters than in litters with only one or a few pups[391]. Differences in gestation length are thus unlikely to explain the majority of difference in size at birth that we observed in large litters. In recent studies of neuroactive steroids in this species, preterm delivery at 62-63 days of gestation (~12% reduction in gestation length) reduced birth weight by only 17%[412], considerably less than the 38% difference in average birth weight between pups from litters of one and five. In the present study, the negative relationship between size at birth and litter size in most parameters was observed across the full range of litter sizes, further implying that additional factors contribute to small size at birth in pups from large litters.

2.8.3 Neonatal Phenotype

Offspring of larger litters grew faster in fractional terms as neonates, indicating a change in partitioning of nutrient intake towards growth in these spontaneous IUGR guinea pigs. Increased appetite may also contribute to accelerated neonatal growth after IUGR, as occurs in other species including humans[79, 293, 413], but neonatal feed intakes have not as yet been reported in IUGR guinea pigs. Accelerated neonatal growth may program adverse later outcomes in these guinea pigs from large litters, as this is an independent risk factor for the development of cardiovascular and metabolic disease in humans[83, 382-384]. Interestingly, despite the more rapid relative neonatal growth in pups from larger litters, these pups exhibited less growth check after weaning at 28 days than those from smaller litters (Figures 3C and 3D), suggesting that they may have changed their intake from milk to solid feed earlier. This may reflect lower milk production per pup, reported previously in large guinea pig litters[409], and competition between litter mates for the two available teats in this species which only allows pups to suckle periodically[404]. Guinea pigs have been successfully weaned at birth or 8 days of age[409], indicating that there is not an absolute requirement for suckling in this species.

2.8.4 Post-weaning phenotype

Interestingly, the accelerated relative growth in guinea pigs from large litters persisted after the neonatal period, and was also observed in juveniles at 30-60 days of age, but this did not continue subsequently to adolescence. The accelerated juvenile growth does appear to reflect continued catch-up growth following removal of prenatal constraint, since juvenile relative growth rates were negatively correlated with birth weight in both males and females, as were relative growth rates in the neonatal period. The relative duration of catch-up in IUGR guinea pigs thus appears to be somewhat longer than in IUGR humans, where the majority of catch-up growth occurs in the first 6 months of life, and is largely complete in infancy by 2

years of age[57, 414-416]. Juvenile growth rates in guinea pigs from large litters were greater than those from smaller litters, not only in relative, but also in absolute terms. Increased appetite and feed intake probably underlies the accelerated juvenile growth rates of these spontaneously growth-restricted guinea pigs, at least in part, since we also observed increased food intake relative to body size in these animals. This role for increased appetite as a mechanism for early catch-up growth after IUGR is further supported by a lack of difference in absolute or relative feed intakes between litter sizes during adolescence, when the litter sizes also had similar growth rates. Feed efficiency did not differ between litter sizes in the juvenile period in either sex, and was improved in female offspring from litters of three compared to two as adolescents. Although this may suggest decreased relative fat deposition in these adolescent females, since fat has a greater energy content per weight than muscle, this did not reduce fatness in adulthood. Juvenile but not adolescent feed efficiency was negatively correlated with birth weight in both sexes, indicating improved efficiency of conversion of food to weight gain, and thus suggesting greater relative lean tissue deposition in the juvenile period. This pattern of post weaning accelerated growth may also lead to adverse health consequences, since accelerated growth after IUGR in rats; particularly when allowed increased caloric feed intake or fed Western-style diets as adults; is associated with obesity, cardiovascular complications and early death[374, 375, 417, 418]. Despite the fact that effects of litter size on growth were similar in both sexes in the present study, relationships between perinatal growth and both post-weaning growth and feed intake were sex-specific. Relative juvenile growth rates correlated negatively with birth weight in both sexes, as discussed above, but absolute juvenile growth rates were not correlated with size at birth in either sex, and correlated positively with neonatal growth in males only. Juvenile feed intake in absolute terms correlated positively with size at birth and neonatal FGR, but in males only, and may reflect effects of perinatal growth on body size, since relative feed intakes did not correlate with perinatal growth in either sex. In contrast to these largely male-specific relationships in juveniles, the majority of relationships between perinatal growth and adolescent

growth were observed only in females, where rapid neonatal growth predicted faster growth and higher feed intakes in adolescence. These results suggest that programming of postnatal appetite and nutrient partitioning by prenatal and early life exposures is sex-specific in guinea pig, and furthermore that the sex-specific nature of these effects depend on age. Sex-specific programming of later outcomes has been also reported in humans and in animal models, particularly for adult metabolic and body composition outcomes[385]. These results further reinforce the need to study progeny of both sexes in future studies investigating developmental programming of metabolic and other outcomes in the guinea pig.

2.8.5 Adult Body Size and Composition

In the present study, progeny of large litters, despite their smaller size of birth, attained similar adult size as those from small litters who were subject to less restriction *in utero*. This is consistent with findings of human studies, where ~85-90% of individuals who were born IUGR attain a final height within 2 S.D. of their peers[57, 415, 416]. Accelerated neonatal growth was a major determinant of adult size, particularly in male guinea pigs, again consistent with human studies where failure of early catch-up growth is a strong predictor of shorter adult height[57, 415]. Few differences were seen in adult body composition between litter size groups, although we were not able to include progeny of litters of one or five pups in these litter size comparisons due to low numbers of adult pups of each sex available as young adults. We did observe greater weights of omental and total dissected fat in offspring from litters of three than in those from either two or four pups. We would have expected greater fatness with increasing litter size, given that increasing litter size was associated with progressive decreases in size at birth and increases in neonatal growth rates. Interestingly, IUGR induced by chronic maternal ethanol consumption throughout guinea pig pregnancy, a model for fetal alcohol syndrome, is followed by neonatal catch-up growth and increased visceral and subcutaneous adiposity in young adults of both sexes[419]. Thus, sex-specific effects of fetal growth patterns differ between

these causes of IUGR. In male IUGR rats catch-up growth after weaning is followed by development of increased visceral adiposity by 7 weeks and obesity by 26 weeks[280]. IUGR induced by uterine artery ablation in guinea pigs is also associated with increased visceral adiposity in male offspring, although in this model progeny do not undergo catch-up growth, and body composition outcomes in females were not reported[284]. Small size at birth and catch-up growth in early life are independent risk factors for obesity in human adults[382] and similar effects might explain the greater omental and total adiposity we observed in progeny from litters of three compared to two male pups. The reason why these effects were not even more pronounced in progeny from litters of four pups, which had similar fatness as those from litters of two pups, is not clear, given that these pups also experienced catch-up growth and grew faster than progeny of litters of two or three pups during the neonatal and juvenile periods. It is possible that these progeny of litters of four pups will develop central adiposity with further ageing beyond young adulthood, as these animals achieved similar weights to those of pups from litters of two nearly a month later than the pups from litters of three, particularly in males.

Across the entire range of litter sizes, the relationships between size at birth, neonatal growth and adult body composition were sex-specific, as also seen for predictors of post-weaning growth and feed intake. The increased adiposity, particularly visceral adiposity, observed in adult male guinea pigs who had grown rapidly as neonates is likely to be associated with adverse metabolic outcomes in these animals. Visceral fat is insulin resistant and in humans, visceral fat mass is a stronger predictor of cardiovascular and metabolic dysfunction than subcutaneous fat[405, 406]. The association between early life catch-up growth and later adiposity seen here in male guinea pigs is consistent with reports in humans and other species[239, 258, 293, 374, 382, 420]. Interestingly, our results are consistent with reports from several of these studies where the independent associations of small size at birth and neonatal catch-up growth with adult adiposity were investigated, and neonatal catch-up growth was more predictive of adult adiposity than small size at birth[258, 293, 420].

2.9 Conclusion

Spontaneous fetal growth restriction due to litter size in the guinea pig gives rise to offspring with disproportionate IUGR and these offspring undergo catch-up growth which persists from neonatal life post-weaning into the juvenile period. Further programmed adult outcomes described here in progeny of large litters whose growth was restricted *in utero*, such as increased visceral adiposity and hyperphagia, are similar to effects of small size at birth induced in other models of IUGR in guinea pig[310], rat[374] and in humans[421]. The spontaneous IUGR guinea pig may, therefore, be a useful model in which to investigate the mechanisms underpinning perinatal programming of hyperphagia and obesity. Because many of these relationships are sex-specific, it will be critical to include progeny of both sexes in future studies. Further studies are required to determine if the spontaneously growth restricted guinea pig develops insulin resistance and other metabolic and cardiovascular pathologies with ageing.

CHAPTER 3
Insulin sensitivity of glucose metabolism in young adult guinea pigs - validation of the hyperinsulinaemic euglycaemic clamp method

3.1 Overview

The following study was designed to characterise the hyperinsulinaemic-euglycaemic clamp in the chronically catheterised, non-anaesthetised guinea pig. A dose response study for whole body glucose uptake using human insulin was conducted, followed by clamp experiments at two insulin infusion rates; 30 $mU.min^{-1}.kg^{-1}$ (*n*=53, males: *n*=31, females: *n*=22) and 7.5 $mU.min^{-1}.kg^{-1}$ (*n*=38, males: *n*=19, females: *n*=19). Animals studied in this chapter were bred and monitored by myself during pregnancy, postnatal growth and post-surgery for insertion of vascular catheters. I independently performed >80% of the hyperinsulinaemic-euglycaemic clamps, performed the majority of the assays for plasma human insulin and [3-^{3}H]-glucose ^3H_2O, was involved in all post-mortems for the collection of body composition data, and analysed all of the data.. Dr Arkadi Katsman is acknowledged for the dose response studies and Dr Arkadi Katsman and Ms Melissa Walker assisted with catheterisation surgeries, post-mortem tissue collection, human insulin and [3-^{3}H]-glucose ^3H_2O assays. I would like to acknowledge Prof. C.C. Yip (University of Toronto, Canada) for donation of the purified guinea pig insulin and rabbit anti-guinea pig insulin for the measurement of guinea pig insulin in a subset of these animals and Ms Patricia Grant for performing the assays.

Since thesis assessment, the data in Chapter 3 has been published in combination with comparative data (not described in the thesis) and the first page of this manuscript is shown in Appendix 2. The chapter included here is the original version from thesis assessment, with minor revisions as suggested by assessors.

Horton D.M., Saint D.A., Owens J.A., Gatford, K.L. Kind K.L. (2017) Use of the hyperinsulinemic euglycemic clamp to assess insulin sensitivity in guinea pigs: dose response, partitioned glucose metabolism, and species comparisons. American Journal of Physiology – Regulatory, Integrative and Comparative doi:10.1152/ajpregu.00028.2017

3.2 Appendix 2

Am J Physiol Regul Integr Comp Physiol 313: R000–R000, 2017.
First published April 24, 2017; doi:10.1152/ajpregu.00028.2017.

RESEARCH ARTICLE | *Model Systems for the Study of Integrative Physiology: The Rebirth of Translational Biology*

Use of the hyperinsulinemic euglycemic clamp to assess insulin sensitivity in guinea pigs: dose response, partitioned glucose metabolism, and species comparisons

Dane M. Horton,[1,2] David A. Saint,[2] Julie A. Owens,[1,2] Kathryn L. Gatford,[1,2] and Karen L. Kind[1,3]
[1]*Robinson Research Institute, The University of Adelaide, Adelaide, South Australia, Australia;* [2]*School of Medicine, The University of Adelaide, Adelaide, South Australia, Australia; and* [3]*School of Animal and Veterinary Sciences, The University of Adelaide, Adelaide, South Australia, Australia*

Submitted 27 January 2017; accepted in final form 18 April 2017

Horton DM, Saint DA, Owens JA, Gatford KL, Kind KL. Use of the hyperinsulinemic euglycemic clamp to assess insulin sensitivity in guinea pigs: dose response, partitioned glucose metabolism, and species comparisons. *Am J Physiol Regul Integr Comp Physiol* 313: R000–R000, 2017. First published April 24, 2017; doi:10.1152/ajpregu.00028.2017.—The guinea pig is an alternate small animal model for the study of metabolism, including insulin sensitivity. However, only one study to date has reported the use of the hyperinsulinemic euglycemic clamp in anesthetized animals in this species, and the dose response has not been reported. We therefore characterized the dose-response curve for whole body glucose uptake using recombinant human insulin in the adult guinea pig. Interspecies comparisons with published data showed species differences in maximal whole body responses (guinea pig ≈ human < rat < mouse) and the insulin concentrations at which half-maximal insulin responses occurred (guinea pig > human ≈ rat > mouse). In subsequent studies, we used concomitant D-[3-^{3}H]glucose infusion to characterize insulin sensitivities of whole body glucose uptake, utilization, production, storage, and glycolysis in young adult guinea pigs at human insulin doses that produced approximately half-maximal (7.5 mU·min^{-1}·kg^{-1}) and near-maximal whole body responses (30 mU·min^{-1}·kg^{-1}). Although human insulin infusion increased rates of glucose utilization (up to 68%) and storage and, at high concentrations, increased rates of glycolysis in females, glucose production was only partially suppressed (~23%), even at high insulin doses. Fasting glucose, metabolic clearance of insulin, and rates of glucose utilization, storage, and production during insulin stimulation were higher in female than in male guinea pigs ($P < 0.05$), but insulin sensitivity of these and whole body glucose uptake did not differ between sexes. This study establishes a method for measuring partitioned glucose metabolism in chronically catheterized conscious guinea pigs, allowing studies of regulation of insulin sensitivity in this species.

guinea pig; insulin sensitivity; sex differences; species differences

INSULIN SECRETION and its actions on glucose metabolism in vivo have been characterized in a range of species, using chronically catheterized preparations to reduce the impact of the stress of anesthesia and surgery. The hyperinsulinemic euglycemic clamp (HEC, 8) is considered the reference standard method for measurement of insulin sensitivity (39). Concomitant administration of radiolabeled glucose during the HEC also allows the metabolic fate of glucose and insulin actions to be partitioned between central and peripheral actions. This approach has been used extensively in small mammalian species, such as the mouse and rat, to investigate the insulin axis under normal and pathophysiological conditions (1, 20, 29, 43, 52). An alternative animal model is the guinea pig, which resembles the human in its susceptibility to diabetes (32, 33) and diet-induced elevated circulating LDL cholesterol and atherosclerosis (12). However, to date, use of the HEC has only been reported in a single study in guinea pigs, investigating the effects of the chemotherapeutic agent cisplatin on insulin sensitivity (57). Only males were used in this study, and they were assessed at a single insulin dose and under general anesthesia (57), which reduces whole body and hepatic insulin sensitivity in the rat (5). Validation of the methodology for the HEC and characterization of both whole body and partitioned insulin action on glucose metabolism in conscious male and female guinea pigs are required to enable further study of insulin sensitivity and ~~it's~~ regulation in this species.

The sequence of insulin in hystricomorph mammals such as the guinea pig is relatively divergent from that of other mam-mals, sharing ~65% homology across the entire sequence, whereas the sequence of the A and B regions of insulin is tightly conserved (90–95%) in other mammals (61). This difference in sequence translates to lower activity of guinea pig insulin compared with that of other species. For example, guinea pig insulin has only ~10% of the potency of bovine insulin as a stimulus for glucose oxidation in rat tissues (64) and is ~3% as potent as porcine, bovine, or recombinant human insulin in stimulating glucose oxidation in rat adipocytes (25). In contrast, the insulin receptors of guinea pigs, humans, and rats share similar binding affinities for chicken and porcine insulin (37), suggesting that responses to a single type of insulin may be similar across species. In vivo metabolic re-sponses to human insulin have not been compared between guinea pig and other species, and whether the guinea pig responds sufficiently to human insulin to allow assessment of changes in insulin receptor number and downstream signaling has not been established.

Address for reprint requests and other correspondence: K. L. Kind, School of Animal and Veterinary Sciences, The Univ. of Adelaide, SA 5005, Australia (e-mail: karen.kind@adelaide.edu.au).

 0363-6119/17 Copyright © 2017 the American Physiological Society

3.3 Abstract

The guinea pig is an alternate small animal model for the study of metabolic disorders and for investigating the mechanisms underlying prenatal programming of such disorders, including insulin sensitivity. However, only one study to date has reported the use of the hyperinsulinaemic-euglycaemic clamp (HEC) to assess glucose metabolism in anaesthetised animals in this species, and the dose-response has not been reported. We therefore characterised the dose-response curve for whole-body glucose uptake using recombinant human insulin in the adult guinea pig. In subsequent studies, we used concomitant D-[3-^{3}H]-glucose infusion to characterise insulin sensitivities of whole body glucose uptake, glucose utilisation, production, storage and glycolysis in young adult guinea pigs at infusion rates of human insulin that produce ~half maximal and near maximal whole body responses. Clamps were successfully performed in 84% of catheterised guinea pigs. Human insulin infusion at 7.5 mU.min^{-1}.kg^{-1} increased rates of glucose utilisation (*P*<0.001) and storage (*P*<0.01), while suppressing that of glucose production (*P*<0.001). Insulin infusion at 30 mU.min^{-1}.kg^{-1}also increased the rate of glycolysis in females (*P*<0.01). Fasting plasma glucose, metabolic clearance of insulin and rates of glucose utilisation, storage and production during insulin stimulation were higher in female than male guinea pigs (*P*<0.05), but insulin sensitivity of these and whole body glucose uptake did not differ between sexes. This study establishes a method for measuring partitioned glucose metabolism in chronically catheterised conscious guinea pigs, providing the basis for further investigation of developmental programming of insulin sensitivity in this species.

3.4 Introduction

Guinea pigs provide an alternate small animal species to rat and mouse in which to investigate developmental programming of health and disease. The guinea pig has a smaller litter size than the rat[280, 374, 392, 397], is relatively precocial at birth, and resembles the human fetus in having ~10% body fat at term[398]. Restriction of fetal growth in the guinea pig by maternal feed restriction increases visceral adiposity and blood pressure, impairs glucose tolerance and alters cholesterol homoeostasis in adult offspring[310-312], indicating that prenatal restriction can perturb metabolic function in this species. Size at birth was a stronger predictor of adult outcomes than maternal nutritional group in these studies[310-312], suggesting the need for further study of developmental programming in a model of spontaneous fetal growth restriction in this species. Studies described in Chapter 2 demonstrated that increased litter size in the guinea pig is associated with decreases in birth weight, and IUGR progeny from these larger litters exhibit neonatal catch up growth, as well as increased central adiposity as adults, particularly in males[395]. Hence, fetal growth restriction due to increasing litter size in the guinea pig may be a suitable model in which to further study mechanisms underpinning the programming of impaired metabolic function.

In human populations, increased risks of insulin resistance after IUGR have been reported in children and in young and older adults[187, 189, 190, 192-199], and development of insulin resistance in skeletal muscle is suggested as a primary initiating mechanism in the programmed development of diabetes[126]. Animal models enable study of whole body, hepatic and peripheral insulin sensitivity, as well as investigation of the specific tissue and molecular changes that may underlie altered functionality of insulin action in key target tissues. While impaired glucose tolerance has been reported in IUGR guinea pig offspring of feed-restricted mothers[310], the effects of fetal growth restriction on insulin sensitivity have not been reported in this species.

The hyperinsulinaemic-euglycaemic clamp (HEC) is the “gold standard” method for measurement of insulin sensitivity[91, 142, 155]. Concomitant administration of radiolabelled

glucose during the HEC also allows the metabolic fate of glucose and insulin actions in individual tissues *in vivo* to be characterised. This approach has been used extensively in small mammalian species, such as the mouse and rat, to investigate the insulin axis under normal and pathophysiological conditions[143, 144, 147, 422, 423]. However, to date, use of the HEC has only been reported in a single study in guinea pigs, investigating the effects of the chemotherapeutic agent cisplatin on insulin sensitivity[424]. Only males were used in this study and were assessed at a single insulin dose and under general anaesthesia[424], which is known to reduce whole body and hepatic insulin sensitivity in the rat[425]. Validation of the methodology for the HEC and characterisation of both whole body and partitioned insulin action on glucose metabolism in conscious animals of both sexes is required to enable further study of the programming of metabolic function in this species. Hence, the aim of this study was to characterise the insulin dose-response curve for whole body glucose metabolism in chronically catheterised guinea pigs using the HEC. Secondly, we measured whole body glucose utilisation, production, glycolysis and storage, and their responsiveness to human insulin in chronically catheterised young adult guinea pigs and compared these between males and females. Validation of this methodology will provide the basis for further studies into the programming of insulin resistance following fetal growth restriction in the guinea pig.

3.5 **Materials and Methods**

3.5.1 Animals

All animal studies were approved by the Animal Ethics Committee of the University of Adelaide (Approval number M56/96). Adult male guinea pigs (*n*=8, 3-4 months of age, Institute of Medical and Veterinary Science Tri-coloured, Gilles Plains Resource Centre, Gilles Plains South Australia) were used for the initial insulin dose response study. For all additional experiments, animal management was as described in Chapter 2[395]. Nulliparous female guinea pigs (3-4 months of age) were entered into a mating program, and their progeny were studied

at 100 days of age. All animals were housed under 12:12 hour light:dark conditions and had *ad libitum* access to a commercial guinea pig/rabbit ration modified with an increased content of vitamin E (165 mg.kg^{-1}), except when fasted for HEC studies as described below, and free access to tap water supplemented with ascorbic acid (400 mg.L^{-1}).

3.5.2 Surgery: Insertion of Catheters

For these studies, a total of 84 guinea pigs underwent surgery for the insertion of vascular catheters. Body weight in the males ranged from 678 g to 942 g (Mean ± SEM, 806 ± 11 g, *n*=45) and in the females from 562 g to 806 g (686 ± 10 g, *n*=39). Catheters were inserted into the right jugular vein (silastic, 0.51 mm ID, 0.94 mm OD, softened in chloroform, sleeved onto polyvinyl, 0.5 mm ID, 1.00 mm OD) and right carotid artery (polyvinyl, 0.4 mm ID, 0.8 mm OD, sleeved into polyvinyl, 0.58 mm ID, 0.96 mm OD) under general anaesthesia induced by ketamine (75 mg.kg^{-1} body weight, intraperitoneal) and xylazine (6 mg.kg^{-1} body weight, intramuscular). Both carotid and jugular catheters were successfully implanted in 80 animals (95%). Catheters were maintained by flushing daily with heparinised saline (500 U.ml^{-1}). Patent carotid and jugular catheters were present in 78% of the guinea pigs at 5 days after surgery, 68% at 7 days after surgery, 58% at 10 days after surgery and 40% at 14 days after surgery. HECs, which commenced a minimum of 5 days after surgery, were successfully completed in 66 of the 80 catheterised guinea pigs.

3.5.3 Hyperinsulinaemic-Euglycaemic Clamps (HEC)

Guinea pigs were fasted for 16 h prior to the HEC. Extension lines, made from polyvinyl tubing, were attached to the catheters and exteriorised through the top of the cage allowing guinea pigs to remained unrestrained during the experiment.

Recombinant human insulin (Actrapid, Novo Nordisk, A/S, Denmark) was diluted in 0.9% NaCl to the required concentration and infused intravenously at a rate of 25 µl.min^{-1} for

120 minutes. In a subset of eight males, the effect of increasing the rate of insulin infusion was examined by performing HECs at insulin infusion rates of 7.5 (*n*=8), 15 (*n*=8), 30 (*n*=3) and 60 (*n*=4) $mU.min^{-1}.kg^{-1}$. At least 3 days recovery was allowed between each HEC. Remaining HECs were then performed using insulin infusions of either 7.5 $mU.min^{-1}.kg^{-1}$ (*n*=31 male, *n*=22 female) or 30 $mU.min^{-1}.kg^{-1}$ (*n*=16 male, *n*=19 female). Twenty-five of the animals had HECs performed at both 7.5 and 30 $mU.min^{-1}.kg^{-1}$ insulin infusion rates.

Blood glucose was measured by glucometer (HemoCue AB, Sweden) in fasting samples collected 20, 15, 10, 5 and 0 minutes prior to the start of the insulin infusion, and in blood samples (50 - 100 μl) collected every 5 minutes throughout the HEC. Intravenous infusion of glucose (10% glucose, Baxter Healthcare, NSW, Australia) commenced 15 minutes after the start of the insulin infusion. The glucose infusion rate (GIR) was adjusted based on the blood glucose measurements to restore and maintain euglycaemia, defined as the mean fasting blood glucose concentration, using a modified version[426] of the algorithm described by De Fronzo et al.[142].

A subset of the animals were co-infused with D-[3-^{3}H]-glucose (Amersham Pharmacia Biotech, Buckinghamshire, England) to determine insulin sensitivity of individual components of whole body glucose metabolism (peripheral glucose utilisation and endogenous glucose production). D-[3-^{3}H]-glucose was administered as a priming bolus (14.5 $\mu Ci.kg^{-1}$) and as a continuous intravenous infusion (0.45 $\mu Ci.min^{-1}.kg^{-1}$) for two hours prior to and during clamps at insulin doses of 7.5 (*n*=19) or 30 (*n*=19) $mU.min^{-1}.kg^{-1}$.

Larger blood samples (500 μl) were collected at the -20, -15, -10, -5, 0, 60, 75, 80, 85, 90, 95, 105 and 120 minutes from the start of the insulin infusion for subsequent analysis of radiolabelled metabolites, and human and guinea pig insulin. Blood was centrifuged at 3000 rpm for 15 minutes and plasma was removed and stored at -20°C. The total blood volume removed from each guinea pig during the experiment was approximately 7.5 ml (~12% of blood volume in a young adult guinea pig). The average fluid volume infused throughout the 2 hours

of the experiment was 9 ml in the 7.5 mU.min^{-1}.kg^{-1} clamps and 11.5 ml for the 30 mU.min^{-1}.kg^{-1} clamps.

3.5.4 Analysis of Human and Guinea Pig Insulin

Human insulin concentrations were analysed in samples collected prior to and at 60, 75, 90, 105 and 120 minutes of the insulin infusion by radioimmunoassay using a commercially available kit (Insulin-CT, CIS Bio International, France). Cross-reactivity of guinea pig insulin in this assay was <2%.

Guinea pig insulin concentrations were measured by radioimmunoassay, as described previously[310, 427]. In brief, guinea pig insulin was measured in plasma samples collected prior to (-10, -5, 0) and at 60, 75, 90, 105 and 120 minutes of insulin infusion during the dose response studies. Purified guinea pig insulin and rabbit anti-guinea pig insulin were provided by Professor C.C. Yip (University of Toronto, Canada). Guinea pig insulin was iodinated with Na ^{125}I (Amersham Pharmacia-Biotech, Sydney, NSW, Australia) and chloramine T to specific activities of 35-50 Ci.g^{-1}, and separated from reaction components by chromatography on Sephadex G50 (Amersham Pharmacia-Biotech, Sydney, NSW, Australia). Guinea pig insulin was measured in duplicate samples of guinea pig plasma and standards (0.1225 to 31.25 ng.ml^{-1}). The amount of guinea pig insulin that inhibited radioligand binding by 50% averaged 485 pg, while the CV for the same sample assayed on different occasions was 9.6% within assays, and 5.3% between assays.

3.5.5 Plasma D-[3-^{3}H]-Glucose and $^{3}H_2O$ for Partitioned Glucose Metabolism

The specific activities of D-[3-^{3}H]-glucose and of $^{3}H_2O$ were measured in plasma samples collected prior to and during the HEC (-20, -15, -10, -5, 0, 60, 75, 80, 85, 90, 95, 105, 120 minutes), using methods based on previous studies in the rat[428, 429]. To deproteinise the samples, 100 μl 0.3N $Ba(OH)_2$ (Sigma Aldrich, St Louis) was added to 50 μl of plasma,

followed by 30 minutes incubation on ice, and addition of 100 μL of 0.3N $ZnSO_4$ (Sigma Aldrich). Samples were incubated on ice for a further 30 minutes, centrifuged at 4°C for 15 minutes at 3500 rpm, and the supernatant removed and weight determined. Forty μl was assayed in quadruplicate in 10 ml scintillation vials, two samples of which were dried uncovered in an oven at 55ºC for 60 minutes. Water, 0.34 ml and 0.3 ml was added to each of the dried and undried vials respectively. In addition, to each vial 1 ml of Tissue Solubilizer (NCS-II Tissue Solubilizer 0.5N Amersham International Arlington Heights, IL) and 10 ml Ready Organic Scintillant (Beckman Coulter Inc., Fullerton CA) was added. Vials were capped and covered with aluminium foil, and equilibrated at room temperature overnight before counting for 10 minutes using a β-counter (Beckman Coulter Inc.). The activity of the dried (radioactivity of D-[3-^{3}H]-glucose) vials was subtracted from that of the undried vials (radioactivity of D-[3-^{3}H]-glucose + ^3H_2O) to give the activity of ^3H_2O alone.

3.5.6 Calculations

Basal plasma glucose and FFA concentrations were calculated as the mean ± SEM of the five samples collected in the last 20 minutes (-20, -15, -10, -5, 0 minutes) of the 2-hour saline infusion prior to commencing the HEC. Steady-state plasma concentrations of guinea pig and human insulin were calculated as the average of concentrations measured every 15 minutes throughout the final hour of the HEC (60-120 minutes). Steady state glucose infusion rate (ssGIR) was calculated as the average GIR during the final hour of the clamp. Whole body insulin sensitivity was calculated by dividing ssGIR by steady-state plasma concentrations of human insulin. The post-hepatic metabolic clearance rate (MCR) of human insulin was calculated as the insulin infusion rate during the HEC divided by steady-state plasma concentrations of human insulin.

3.5.6.1 *Glucose utilisation*

Plasma D-[3-^{3}H]-glucose specific activity plateaued during the last 20 to 40 minutes of D-[3-^{3}H]-glucose infusion in the basal state and during HEC at insulin infusion of 7.5mU.min^{-1}.kg^{-1}. This is similar to that seen in hyperinsulinaemic-euglycaemic clamp studies in the rat[145]. During this steady-state period, the rate of glucose utilisation or disappearance of glucose (Rd) was calculated (Equation 1) by dividing the D-[3-^{3}H]-glucose infusion rate (dpm.min^{-1}) by the steady-state plasma D-[3-^{3}H]-glucose specific activity (dpm.µmol^{-1}) as previously described[428, 429].

EQUATION 1: Calculating glucose utilisation in the basal and insulin-stimulated state (Rd):

$$Rd_{basal} = \left(\frac{\text{D-[3-}^{3}\text{H]-glucose infusion rate (dpm.min}^{-1}\text{)}}{\text{D-[3-}^{3}\text{H]-glucose specific activity (dpm.}\mu\text{mol}^{-1}\text{)}} \right)$$

3.5.6.2 *Glucose production*

In the basal state, the rate of glucose appearance (Ra) is equal to the basal rate of endogenous whole body glucose production (Rd), which can be calculated as the D-[3-^{3}H]-glucose infusion rate (dpm.min^{-1}) divided by the steady-state plasma D-[3-^{3}H]-glucose specific activity (dpm.µmol^{-1}) as previously described [428, 429](Equation 2) [427, 428].

EQUATION 2: Calculating basal endogenous glucose production (Ra):

$$Ra_{basal} = Rd_{basal} = \left(\frac{\text{D-[3-}^{3}\text{H]-glucose infusion rate (dpm.min}^{-1}\text{)}}{\text{D-[3-}^{3}\text{H]-glucose specific activity (dpm.}\mu\text{mol}^{-1}\text{)}} \right)$$

In the insulin-stimulated state, the rate of whole body glucose utilisation equals the rate of endogenous glucose production plus the exogenous glucose infusion. Therefore, endogenous glucose production was calculated as the rate of whole body glucose utilisation minus exogenous glucose infusion (Equation 3) [428, 429].

EQUATION 3: Calculating insulin-stimulated endogenous glucose production (Ra):

$$Rd_{Insulin} = \left(\frac{\text{D-[3-}^{3}\text{H]-glucose infusion rate (dpm.min}^{-1}\text{)}}{\text{D-[3-}^{3}\text{H]-glucose specific activity (dpm.}\mu\text{mol}^{-1}\text{)}} \right) - \text{GIR}$$

3.5.6.3 *Glycolysis and glucose storage*

The ^{3}H in the C-3 position of D-[3-^{3}H]-glucose is lost selectively to H_2O during glycolysis[430]. Rates of whole body glycolysis were determined from the rate of increase in $^{3}H_2O$ (dpm.min^{-1}) multiplied by the total body water mass and divided by the D-[3-^{3}H]-glucose specific activity (dpm.µmol^{-1}) in both the basal and insulin-stimulated states. Plasma H_2O was assumed to be 93% of plasma volume, and the mass of H_2O in the body was assumed to be 65% of total body weight, based on estimates in rodents (Equation 4) [428, 429]. The rate of whole body glycolysis was determined during the last 15 minutes of D-[3-^{3}H]-glucose infusion in the basal state, and the last 60 minutes of insulin infusion. Glucose storage in each state was calculated as the difference between total glucose utilisation and glycolysis (Equation 5) [427, 428].

EQUATION 4: Calculating basal and insulin-stimulated whole body glycolysis:

$$\text{Rate of Glycolysis} = \left(\frac{\text{Rate of increase of }^{3}\text{H}_2\text{O (dpm.min}^{-1}\text{) x 0.93 x 0.65}}{\text{D-[3-}^{3}\text{H]-glucose infusion rate (dpm.min}^{-1}\text{)}} \right)$$

EQUATION 5: Calculating basal and insulin-stimulated whole body glucose storage:

$$\text{Rate of Glucose Storage} = \text{Rd} - \left(\frac{\text{Rate of increase of } {}^{3}H_2O \text{ (dpm.min}^{-1}\text{) x 0.93 x 0.65}}{\text{D-[3-}^{3}\text{H]-glucose infusion rate (dpm.min}^{-1}\text{)}} \right)$$

3.5.7 Statistical Analysis

Data were analysed using SPSS 24.0 for Windows (IBM, Armonk, USA). For comparison between sexes, due to inclusion of different animals in studies at each insulin dose, the effect of sex was determined by analysis of variance separately at each dose. The effect of recombinant human insulin infusion on fasting plasma guinea pig insulin levels, and on components of glucose metabolism was determined using a paired two-tailed t-test. Significance was accepted at $P < 0.05$, and results are expressed as mean ± S.E.M.

3.6 **Results**

3.6.1 Insulin Dose Response of Whole Body Glucose Metabolism

Blood glucose levels during the steady state period of insulin infusion were similar to the fasting blood glucose levels at all doses of insulin studied (Table 3-1). The coefficient of variation of blood glucose during the 60-120 minute period of the clamp ranged from 6.5% to 10.5% at differing insulin doses (Table 3-1). Circulating human insulin concentration and steady state glucose infusion rate increased with increasing infusion rate of human insulin up to doses of 30 mU.min^{-1}.kg^{-1} (Table 3-1, Figure 3-1). Compared to fasting values, the circulating concentrations of guinea pig insulin were reduced by infusion of human insulin at doses of 7.5 and 60 mU.min^{-1}.kg^{-1} (P<0.05, Table 3-1) and a similar trend was observed at 15 mU.min^{-1}.kg^{-1} (P<0.1, Table 3-1). Insufficient samples were available for analysis of guinea pig insulin in the animals infused with 30 mU.min^{-1}.kg^{-1} insulin. Infusion rates of 7.5 and 30 mU.min^{-1}.kg^{-1} were chosen for further study to measure insulin responses at around half and near maximal human insulin doses.

Table 3-1 Metabolic responses to differing infusion rates during the hyperinsulinaemic euglcaemic clamp in young adult guinea pigs

	Insulin infusion rate (mU.min^{-1}.kg^{-1})			
Outcomes	7.5	15	30	60
Number (*n*)	8	8	3	4
Bodyweight (g)	698 ± 27	695 ± 29	795 ± 53	685 ± 25
Fasting state outcomes (20 min prior to HEC)				
Fasting whole blood glucose (mmol.l^{-1})	7.02 ± 0.29	7.51 ± 0.22	7.22 ± 0.48	7.60 ± 0.46
Fasting plasma guinea pig insulin (ng.ml^{-1})	5.91 ± 0.96	6.06 ± 1.51	nd	6.73 ± 0.88
Steady state outcomes (60-120 min)				
Blood glucose (mmol.l^{-1})	7.06 ± 0.34	7.41 ± 0.23	7.15 ± 0.54	7.50 ± 0.45
Blood glucose %CV	6.5 ± 1.4	6.4 ± 0.8	10.5 ± 6.0	8.1 ± 1.6
Glucose infusion rate (μmol.min^{-1}.kg^{-1})	53.7 ± 3.9	69.4 ± 8.7	82.9 ± 11.1	88.1 ± 12.0
Glucose infusion rate %CV	20.3 ± 5.6	19.5 ± 2.6	23.5 ± 13.4	23.3 ± 6.6
Plasma human insulin (μU.ml^{-1})	291 ± 33	1419 ± 175	10557 ± 2124	15724 ± 1897
Plasma guinea pig insulin (ng.ml^{-1})	4.16 ± 0.49*	3.60 ± 0.48	nd	3.74 ± 0.29*
Whole body insulin sensitivity (μmol.ml.μU^{-1}.min^{-1}.kg^{-1})	0.211 ± 0.037	0.064 ± 0.020	0.0086 ± 0.002	0.0057 ± 0.0007

Values are Mean ± SEM. Steady state outcomes are averaged from 60-120 minutes of insulin infusion.
*P<0.05 compared to fasting levels.
nd= not determined.

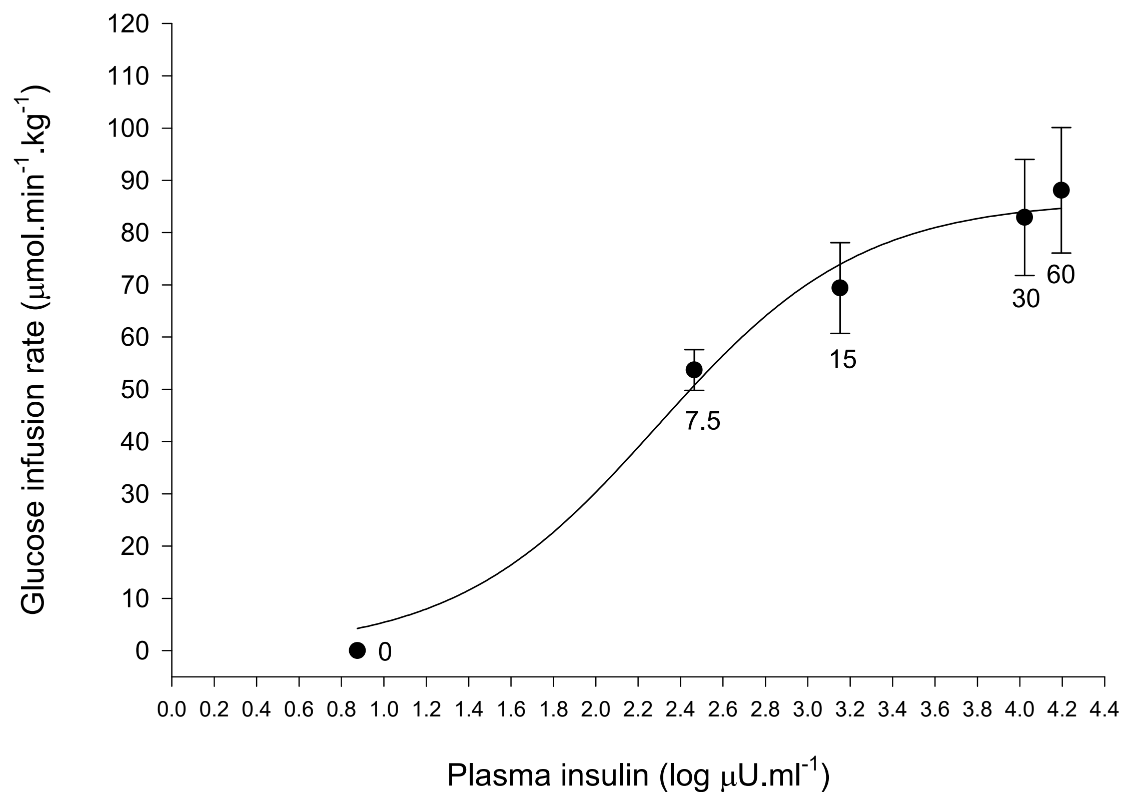


Figure 3-1 Insulin dose response curve for glucose infusion rates during hyperinsulinaemic-euglycaemic clamps in the guinea pig.

Plasma human insulin concentration (log $\mu U.ml^{-1}$) and glucose infusion rate are mean values for 60-120 minutes of insulin infusion, with the insulin infusion dose in $mU.min^{-1}.kg^{-1}$ shown next to each data point. Data are mean ± S.E.M.

3.6.2 Whole Body Insulin Sensitivity and Partitioned Glucose Metabolism at ~Half-Maximal Insulin Dose (7.5 $mU.min^{-1}.kg^{-1}$)

In the cohort of guinea pigs in which whole body insulin sensitivity was assessed at 7.5 $mU.min^{-1}.kg^{-1}$ (n=53), fasting plasma glucose was higher in females than males (P<0.05, Table 3-2). The co-efficient of variation of blood glucose averaged 5.9% during 60-120 minutes of insulin infusion at 7.5 $mU.min^{-1}.kg^{-1}$. Steady state human insulin concentrations achieved during insulin infusion at 7.5 $mU.min^{-1}.kg^{-1}$ averaged 238 ± 13 $\mu U.ml^{-1}$ overall, and were higher in males than females (P<0.007, Table 3-2), whereas metabolic clearance rate of human insulin was higher in females (P<0.02, Table 3-2). Steady state glucose infusion rates averaged 47.8 ± 2.5 $\mu mol.min^{-1}.kg^{-1}$ during the HEC and both ssGIR and whole body insulin sensitivity did not differ between males and females (Table 3-2).

In the cohort of animals in which partitioned glucose metabolism was measured at 7.5 $mU.min^{-1}.kg^{-1}$ (n=19), fasting blood glucose was similarly higher in females than males (P<0.001, Table 3-2), but no sex differences were observed in steady state human insulin concentrations or ssGIR (Table 3-2).

Basal rates of glucose production and utilisation tended to be higher in females compared to males prior to the 7.5 $mU.min^{-1}.kg^{-1}$ clamp (P<0.075 for both). Insulin infusion at 7.5 $mU.min^{-1}.kg^{-1}$ suppressed endogenous glucose production (both P<0.001, Table 3-2) and enhanced whole body glucose utilisation in both males and females (P<0.001, Table 3-2). The insulin-stimulated rates of glucose utilisation at half-maximal insulin dose were higher in females than males (P<0.03, Table 3-2), and glucose production under insulin-stimulated conditions tended to be higher in females than males (P=0.08, Table 3-2).

Basal levels of whole body glycolysis and glucose storage did not differ between sexes prior to the 7.5 $mU.min^{-1}.kg^{-1}$ clamps. Insulin infusion at 7.5 $mU.min^{-1}.kg^{-1}$ tended to enhance the rate of whole body glycolysis in males (P<0.08), but did not alter whole body glycolysis in females (Table 3-2). Whole body glucose storage was increased by insulin

infusion in males (P<0.001) and females (P<0.005), and insulin-stimulated rates of glucose storage were higher in females than males (P<0.05, Table 3-2).

Overall, glycolysis accounted for 43 ± 6% (males: 50 ± 9%; females: 35 ± 7%) of glucose utilisation in the fasting state and 37 ± 4% (males: 43 ± 7%; females: 32 ± 4%) during infusion of human insulin at 7.5 $mU.min^{-1}.kg^{-1}$ (n=19). Therefore, glucose storage accounted for 57 ± 6% (males: 50 ± 9%; females: 65 ± 7%) of glucose utilisation in the fasting state, and 63 ± 4% (males: 58 ± 7%; females: 68 ± 4%) during infusion of 7.5 $mU.min^{-1}.kg^{-1}$ human insulin. The proportions of glucose utilisation accounted for by glycolysis and glucose storage did not differ between males and females.

3.6.3 Whole Body Insulin Sensitivity and Partitioned Glucose Metabolism at Near Maximal Insulin Dose (30 $mU.min^{-1}.kg^{-1}$)

In the cohort of guinea pigs in which whole body insulin sensitivity was assessed at 30 $mU.min^{-1}.kg^{-1}$ (n=38), fasting plasma glucose was higher in females than males (P<0.05, Table 3-3). The co-efficient of variation of blood glucose averaged 6.7% during 60-120 minutes of insulin infusion at 30 $mU.min^{-1}.kg^{-1}$. Steady state human insulin concentrations achieved during insulin infusion at 30 $mU.min^{-1}.kg^{-1}$ averaged 3199 ± 156 $\mu U.ml^{-1}$ overall, and were higher in males than females (P<0.01, Table 3-3); however, metabolic clearance rate of human insulin during near maximal insulin infusion did not differ between sexes. Steady state glucose infusion rates averaged 65.4 ± 3.0 $\mu mol.min^{-1}.kg^{-1}$ during this HEC and both ssGIR and whole body insulin sensitivity did not differ between males and females (Table 3-3).

In the animals in which partitioned glucose metabolism was measured at 30 $mU.min^{-1}.kg^{-1}$ (n=19), fasting blood glucose was also higher in females (P<0.05, Table 3-3), but no differences were observed in steady state human insulin concentrations or glucose infusion rate between sexes (Table 3-3).

Table 3-2 Whole body insulin sensitivity and partitioned glucose metabolism at half-maximal insulin dose in the guinea pig (7.5 $mU.min^{-1}.kg^{-1}$).

	Male	Female
Whole body insulin sensitivity measures		
Number	31	22
Bodyweight (g)	796 ± 15	661 ± 12***
Fasting blood glucose ($mmol.l^{-1}$)	6.77 ± 0.13	7.31 ± 0.19*
Glucose infusion rate $_{(60\text{-}120\ min)}$ ($\mu mol.min^{-1}.kg^{-1}$)	48.7 ± 3.6	46.6 ± 3.2
Plasma human insulin $_{(60\text{-}120\ min)}$ ($\mu U.ml^{-1}$)	266 ± 18	195 ± 14**
Whole body insulin sensitivity ($\mu mol.ml.\mu U^{-1}.min^{-1}.kg^{-1}$)	0.204 ± 0.019	0.261 ± 0.031
MCR human insulin $_{(60\text{-}120\ min)}$ ($ml.min^{-1}.kg^{-1}$)	32.9 ± 2.7	43.7 ± 3.9*
Partitioned glucose metabolism		
Number	10	9
Glucose infusion rate $_{(60\text{-}120\ min)}$ ($\mu mol.min^{-1}.kg^{-1}$)	36.6 ± 3.4	40.2 ± 4.3
Fasting blood glucose ($mmol.l^{-1}$)	6.41 ± 0.22	7.52 ± 0.18**
Plasma human insulin $_{(60\text{-}120\ min)}$ ($\mu U.ml^{-1}$)	203 ± 21	162 ± 11
Whole body glucose production		
Basal ($\mu mol.min\text{-}1.kg^{-1}$)	53.7 ± 5.9	69.3 ± 5.7§
Insulin stimulated ($\mu mol.min^{-1}.kg^{-1}$)	39.6 ± 5.7†††	55.2 ± 6.3††† §
Insulin-stimulated decrease ($\mu mol.min^{-1}.kg^{-1}$)	14.1 ± 2.4	14.1 ± 2.6
Whole body glucose utilisation		
Basal ($\mu mol.min^{-1}.kg^{-1}$)	53.7 ± 5.9	69.3 ± 5.7§
Insulin stimulated ($\mu mol.min^{-1}.kg^{-1}$)	74.7 ± 7.3†††	96.3 ± 5.3††† *
Insulin-stimulated increase ($\mu mol.min^{-1}.kg^{-1}$)	21.0 ± 4.3	27.0 ± 6.3
Whole body glycolysis		
Basal ($\mu mol.min^{-1}.kg^{-1}$)	23.5 ± 3.7	25.7 ± 4.9
Insulin stimulated ($\mu mol.min^{-1}.kg^{-1}$)	29.9 ± 4.4	30.1 ± 3.6
Insulin-stimulated increase ($\mu mol.min^{-1}.kg^{-1}$)	6.3 ± 3.3	4.4 ± 4.9
Whole body glucose storage		
Basal ($\mu mol.min^{-1}.kg^{-1}$)	30.2 ± 7.3	43.6 ± 4.6
Insulin stimulated ($\mu mol.min^{-1}.kg^{-1}$)	44.8 ± 8.2†††	66.2 ± 5.4†† *
Insulin-stimulated increase ($\mu mol.min^{-1}.kg^{-1}$)	14.6 ± 3.2	22.6 ± 6.0

Values are mean ± SEM. Glucose infusion rate, human insulin and metabolic clearance rates (MCR) are averaged from 60-120 minutes of insulin infusion. For whole body insulin sensitivity, human insulin concentrations were available for n=30 males and 20 females.
§P<0.10, *P<0.05, **P<0.01, ***P<0.001 compared to males.
† P<0.05, †† P<0.01, ††† P<0.001 compared to basal level prior to the clamp.

Insulin infusion at 30 $mU.min^{-1}.kg^{-1}$ suppressed endogenous glucose production in males (*P*<0.001, Table 3-3) and females (*P*<0.05, Table 3-3) and enhanced whole body glucose utilisation to a similar degree in both sexes (*P*<0.001 for both, Table 3-3). Glucose production during insulin stimulation at 30 $mU.min^{-1}.kg^{-1}$ was higher in females than males (*P*<0.04), but there was no difference in the insulin-stimulated decrease in endogenous glucose production between the sexes.

Insulin infusion at 30 $mU.min^{-1}.kg^{-1}$ did not alter whole body glycolysis in males, but enhanced glycolysis in females (*P*<0.01, Table 3-3), and therefore the insulin-stimulated change in glycolysis from basal levels was higher in females (*P*<0.04, Table 3-3). Insulin-stimulated rates of glycolysis during the clamp also tended to be higher in females than males (*P*<0.06, Table 3-3). Prior to the 30 $mU.min^{-1}.kg^{-1}$ HEC, basal rates of glucose storage were higher in females than males (*P*=0.05). Insulin infusion at 30 $mU.min^{-1}.kg^{-1}$ increased whole body glucose storage in males (*P*<0.001) and females (*P*<0.04, Table 3-3), and insulin-stimulated glucose storage was similar in males and females.

In the fasting state, prior to insulin infusion at 30 $mU.min^{-1}.kg^{-1}$, glycolysis accounted for 43 ± 6% of glucose utilisation and glucose storage accounted for 57 ± 6%. Glycolysis accounted for a higher percentage of glucose utilisation in males than females (males: 54 ± 9%; females: 31 ± 6%, *P*<0.04) and conversely glucose storage accounted for a lower percentage of utilisation in males (males: 46 ± 9%; females: 69 ± 6%, *P*<0.04). In the insulin infused state, at 30 $mU.min^{-1}.kg^{-1}$ insulin, glycolysis accounted for 38 ± 3% of glucose utilisation (males: 36 ± 4%; females: 40 ± 5%) and glucose storage accounted for 62 ± 3% (males: 64 ± 4%; females: 60 ± 5%), and there were no differences between males and females.

Table 3-3 Whole body insulin sensitivity and partitioned glucose metabolism at maximal insulin dose in the guinea pig (30 mU.min^{-1}.kg^{-1}).

	Male	Female
Whole body insulin sensitivity measures		
Number	19	19
Bodyweight (g)	785 ± 19	658 ± 15***
Fasting blood glucose (mmol.l^{-1})	6.7 ± 0.2	7.3 ± 0.2*
Glucose infusion rate $_{(60\text{-}120\ min)}$ (µmol.min^{-1}.kg^{-1})	66.7 ± 4.3	63.9 ± 3.7
Plasma human insulin $_{(60\text{-}120\ min)}$ (µU.ml^{-1})	3644 ± 289	2878 ± 128**
Whole body insulin sensitivity (µmol.ml.µU^{-1}.min^{-1}.kg^{-1})	0.022 ± 0.0044	0.023 ± 0.0019
MCR Human insulin $_{(60\text{-}120\ min)}$ (ml.min^{-1}.kg^{-1})	9.4 ± 1.3	10.8 ± 0.5
Partitioned glucose metabolism		
Number	10	9
Glucose infusion rate $_{(60\text{-}120\ min)}$ (µmol.min^{-1}.kg^{-1})	66.0 ± 6.3	61.5 ± 4.5
Fasting whole blood glucose (mmol.l^{-1})	6.3 ± 0.3	7.0 ± 0.2*
Plasma human insulin $_{(60\text{-}120\ min)}$ (µU.ml^{-1})	3679 ± 451	3008 ± 205
Whole body glucose production		
Basal (µmol.min^{-1}.kg^{-1})	51.9 ± 5.8	65.1 ± 6.0
Insulin stimulated (µmol.min^{-1}.kg^{-1})	24.3 ± 6.1†††	50.2 ± 9.9† *
Insulin-stimulated decrease (µmol.min^{-1}.kg^{-1})	27.6 ± 5.5	14.9 ± 6.1
Whole body glucose utilisation		
Basal (µmol.min^{-1}.kg^{-1})	51.9 ± 5.8	65.1 ± 6.0
Insulin stimulated (µmol.min^{-1}.kg^{-1})	89.7 ± 7.0†††	109.3 ± 9.9†††
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	37.8 ± 8.0	44.2 ± 8.2
Whole body glycolysis		
Basal (µmol.min^{-1}.kg^{-1})	25.6 ± 2.5	19.6 ± 4.0
Insulin stimulated (µmol.min^{-1}.kg^{-1})	30.7 ± 3.1	41.6 ± 4.5†† §
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	5.1 ± 3.9	22.0 ± 6.7*
Whole body glucose storage		
Basal (µmol.min^{-1}.kg^{-1})	26.3 ± 6.7	45.5 ± 6.3*
Insulin stimulated (µmol.min^{-1}.kg^{-1})	59.1 ± 7.2†††	67.7 ± 9.4†
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	32.7 ± 6.2	22.2 ± 8.7

Values are mean ± sem. Glucose infusion rate, human insulin and metabolic clearance rates are averaged from 60-120 minutes of insulin infusion. For whole body insulin sensitivity, human insulin concentrations were available for n=13 males and 18 females. For partitioned glucose metabolism, human insulin concentrations were available for n=6 males and 5 females.
§*P*<0.10, **P*<0.05, ***P*<0.01, ****P*<0.001 compared to males.
† *P*<0.05, †† *P*<0.01, ††† *P*<0.001 compared to basal level prior to the clamp.

3.7 Discussion

This study has characterised whole body glucose metabolism and its response to insulin in the euglycaemic state in the chronically catheterised guinea pig. Human insulin increased glucose utilisation and suppressed endogenous glucose production in the guinea pig, although sensitivity to the actions of human insulin particularly for suppression of endogenous glucose production was lower in the guinea pig than reported previously in human[431-434], or in other small experimental species, such as rats and mice[143, 144, 147, 422, 423]. Nevertheless, these studies have demonstrated that HEC using human insulin can be used for quantitative assessment of insulin sensitivity in the guinea pig. As the guinea pig resembles the human in its susceptibility to diabetes[435, 436], atherosclerosis and in aspects of cholesterol homeostasis[312, 437], this establishes methodology that will allow this species to be used for further investigation of the mechanistic basis underlying perturbations of glucose metabolism and insulin action.

Perturbations of fetal growth and development in the guinea pig impair postnatal growth, visceral adiposity (Section 2.5.6), and feed intake (Section 2.5.5)[395], and cholesterol[312] and glucose metabolism[310], therefore also establishing it as an excellent animal model for further investigations into the underlying causes of the developmental origins of metabolic disease. While the HEC is well established and used in rats and mice[143, 144, 147, 422, 423], the volume of blood that can be sampled, and the number of experiments and analyses possible is limited in these species, particularly in younger animals. The guinea pig, in contrast, ranges in weight from 60 to 140 g at birth and attains a body weight of 400 g by 3-4 weeks of age, thus avoiding some of the limitations related to size and body weight in other small animal models. In particular, in the present study, we have demonstrated the capacity to perform repeat HEC studies in the chronically catheterised young adult guinea pig.

Glucose concentrations were successfully clamped during the last hour of the HEC in the guinea pig with a covariance of blood glucose of 5.9% ± 0.3% and 6.7% ± 0.7% during the 7.5 and 30 $mU.min^{-1}.kg^{-1}$ insulin infusions respectively. This is comparable to the glucose

variability seen during HEC in rats (5-8%;[143-145]), young lambs (2.6%;[69]), dogs (7%;[438]), baboons (~5%;[154]) and humans (4-6%;[142]). In the guinea pig, circulating insulin concentrations and GIR required to maintain glycaemia increased with increasing insulin dose up to 30 $mU.min^{-1}.kg^{-1}$, but were similar at insulin doses of 30 and 60 $mU.min^{-1}.kg^{-1}$. At near-maximal insulin dose (30 $mU.min^{-1}.kg^{-1}$), plasma human insulin concentrations averaged 3199 $\mu U.ml^{-1}$ and required a ssGIR of 65 $\mu mol.min^{-1}.kg^{-1}$ for maintenance of euglycaemia. In contrast, in young adult humans, dose response studies of whole body insulin sensitivity indicate an insulin ED_{50} of 54 $\mu U.ml^{-1}$ and near maximal actions at >500 $\mu U.ml^{-1}$ insulin, with glucose infusion rates of 62 and 65 $\mu mol.min^{-1}.kg^{-1}$ required to maintain euglycaemia at plasma insulin concentrations of 168 and 666 $\mu U.ml^{-1}$ respectively[439]. In the rat, ssGIRs of 22 and 68 $\mu mol.min^{-1}.kg^{-1}$ are required to maintain glycaemia at human insulin concentrations of 39 and 120 $\mu U.ml^{-1}$ during HEC[440], and similar results are observed with porcine insulin (ssGIRs of 74 $\mu mol.min^{-1}.kg^{-1}$at plasma insulin of 100 $\mu U.ml^{-1}$)[143, 144]. The mouse is more sensitive to insulin compared to these species, as an ssGIR of 227 $\mu mol.min^{-1}.kg^{-1}$ is required to maintain euglycaemia in mice at a human plateau insulin concentration of only 37 $\mu U.ml^{-1}$ [146]. Furthermore, circulating insulin concentrations reported during HEC in other species represent combined levels of infused and endogenous insulin, due to strong cross-reactivity between insulins from different species. In the current study, it was possible to specifically analyse human insulin concentrations, exclusive of the endogenously produced guinea pig insulin, since the assays do not cross-react between these species. These observations in the present study suggest that the insulin responsiveness of the guinea pig to human insulin is low, in comparison with the human, and to other small experimental species, particularly the mouse. However, one other study that utilised HEC in male guinea pigs, with infusion of human insulin at 3 $mU.kg^{-1}$, required glucose infusion rates of approximately 72-83 $\mu mol.min^{-1}.kg^{-1}$ to maintain euglycaemia at a plateau plasma insulin concentration of 100 ± 5 $\mu U.ml^{-1}$ in their control groups[424]. These ssGIR resemble the rates of

65.4 μmol.min^{-1}.kg^{-1} required to maintain glycaemia at human insulin concentrations of ~3200 μU.ml^{-1} in the current study. A number of factors may have contributed to the discrepancies in apparent insulin sensitivity between these two studies. Guinea pigs were anaesthetised during the HEC in the previous study[424]; however, in rats anaesthesia reduces insulin sensitivity[425], suggesting that anaesthesia may not explain the differences. In the previous study, although control groups did not receive the treatments (capsaicin or cisplatin) they were treated with the solvent used to administer these drugs (75 mg.kg^{-1} mannitol *i.p*) for 9 days prior to HEC testing[424]. In addition, body weight of animals studied by Szilvassy et al (2006)[424] ranged from 350-400 g, a considerably lighter weight than the male guinea pigs in the current study, and animals were fasted for 24 hours, compared to 16 hours used in this study. In mice, fasting time can influence insulin sensitivity assessed by HEC, with insulin sensitivity increasing following an 18 hour compared to a 5 hour fast[146]. This is consistent with the greater insulin sensitivity of muscle but not in liver following a 16 hour compared to a 5 hour fast using HEC with D-[^{14}C]-glucose and 2-deoxy-D-[^{3}H]-glucose reported in mice[441]. Insulin sensitivity similarly increases following an overnight fast in humans[442]. Therefore, the use of a 24 h fast by Szilvássy[424], compared to a 16 h fast in our study, may contribute to the higher insulin sensitivity that they observed. The effects of mannitol on insulin sensitivity have not been reported, however the sugar-alcohol is poorly absorbed through the gut[443] so mannitol *per se* is unlikely to act systemically to alter insulin sensitivity. Similarly, although repeated *i.p.* injections for 8 d prior to HEC may induce mild chronic stress, this is associated with decreased rather than increased insulin sensitivity in rats[444], and therefore also seems unlikely to contribute to the greater insulin sensitivity of guinea pigs reported by Szilvássy[424], compared to the present study. Furthermore, details of the assay used to measure plasma insulin in the previous study were not provided[424]. It is therefore not clear why a higher ssGIR at a lower steady-state insulin concentration was observed in this previous guinea pig study[424] compared to our findings.

Guinea pig insulin has a low affinity for many mammalian insulin receptors, including its own, and also has lower affinity for its own receptor when compared to insulins of other species[445]. Previous studies have reported that guinea pig insulin is not as potent as bovine insulin in lowering blood glucose in the guinea pig[446]. The guinea pig appears to compensate for this through an increased plasma insulin concentration[310, 427, 445-447] and possibly an increased tissue abundance of insulin receptors[445]. This study provides evidence that human insulin can bind and act through the guinea pig insulin receptor, as previously reported for insulins from several other species, including bovine and porcine[445].

Use of concomitant radioactively labelled glucose infusion during HEC allows determination of basal and insulin-stimulated rates of glucose utilisation, production, storage and glycolysis. In the guinea pig, as in other species, glucose is utilised for oxidative purposes and carbon dioxide production, with much of the remainder directed to hepatic glycogen storage in the fasted state or to lipid storage in adipose tissue in the fed state[448]. Basal rates of endogenous glucose production in the guinea pig were high (e.g. average of 61 μmol.min^{-1}.kg^{-1} prior to the 7.5 mU.min^{-1}.kg^{-1}clamps) when compared to rates reported for healthy adult humans (11-12 μmol.min^{-1}.kg^{-1})[431-434] and rats (27-38 μmol.min^{-1}.kg^{-1})[428, 449, 450]. Infusion of human insulin suppressed glucose production and stimulated glucose utilisation and storage in the guinea pig, consistent with actions on both hepatic and peripheral glucose metabolism. Overall, human insulin infusion at 7.5 mU.min^{-1}.kg^{-1} suppressed glucose production by 20% and stimulated glucose utilisation by 39% in the guinea pig and infusion of human insulin at 30 mU.min^{-1}.kg^{-1} suppressed glucose production by an average of 23% and increased glucose utilisation by 68%. In contrast, in humans, maximal suppression of endogenous production is achieved at lower insulin concentrations than those required to stimulate maximal rates of glucose utilisation. In humans, dose response studies using porcine insulin [433] show that the plateau insulin concentrations required to achieve half-maximal effects are lower for hepatic glucose production (29 ± 2 μU.ml^{-1}) than for glucose utilisation (55 ± 7 μU.ml^{-1}) . Similar results are reported in

the rat, with lower plasma insulin concentrations required to achieve maximal suppression of hepatic glucose production, compared to those required for stimulation of glucose utilisation [428, 434]. Furthermore, in rats, hepatic glucose production is suppressed by >90% and glucose uptake is stimulated ~5-fold at insulin concentrations of 124 μU.ml^{-1} during HEC with porcine insulin[434]. Similarly, in humans, maximal and complete suppression of glucose production occurs at an insulin concentration of 57 μU.ml^{-1} and a ~5-fold increase in glucose utilisation occurs at plateau plasma insulin levels of 678 μU.ml^{-1} [433]. In the guinea pig, a 37% suppression of glucose production and 68% increase in glucose utilisation during the 30 mU.min^{-1}.kg^{-1} HEC were observed at human insulin concentrations of >3000 μU.ml^{-1}, suggestive of lower activity of human insulin in guinea pig liver and peripheral tissues, when compared to its actions in human tissues.

Sex-specific differences in glucose metabolism were observed in the current study. Compared to males, female guinea pigs had higher fasting plasma glucose, metabolic clearance of insulin and rates of glucose utilisation, storage and production during insulin stimulation. Whole body insulin sensitivity and the insulin sensitivity of glucose production and utilisation did not differ between the sexes, although the insulin-stimulated increase in glycolysis was greater in females than males at high-dose insulin. Numerous studies have examined sex-specific differences in insulin sensitivity in humans[273, 451-455]. Metabolic clearance of insulin is greater in women than men[456], in agreement with the sex-differences observed in guinea pigs. Greater endogenous glucose production in women than men, per kilogram body weight, has been reported in some studies[273, 454], however, others report no differences in glucose production between sexes[273, 453]. Suppression of endogenous glucose production is greater in women than men at low, but not at high, doses of insulin[273, 454]. This contrasts with the results seen here in guinea pigs, where glucose production was higher in females than males during insulin stimulation at the high insulin dose. Greater skeletal muscle sensitivity to insulin has also been observed in women than men[273, 451]. Differences in relative adiposity and lean muscle mass, and

the potential actions of steroid hormones are suggested as potential factors contributing to the sex-specific differences in insulin sensitivity and glucose metabolism[271, 273, 455]. Although insulin sensitivity, measured as the change from basal to insulin-stimulated states, did not differ between young adult male and female guinea pigs in the current study, some measures of glucose metabolism in the fasting and insulin-stimulated states did differ between sexes. The potential for sex-specific effects should be therefore considered in studies investigating perturbations of insulin sensitivity of glucose metabolism in the guinea pig.

3.8 Conclusion

In conclusion, this study has validated the HEC in chronically catheterised male and female guinea pigs, and has confirmed that concomitant tracer infusions can also be utilised to investigate partitioned glucose metabolism in an unanaethetised guinea pig model. Human insulin stimulated glucose utilisation and suppressed endogenous glucose production in the guinea pig, although the relative magnitude of these changes differed from those reported during HEC in the human and rat. Nevertheless, this study demonstrates that quantitative assessment of insulin action is possible in the guinea pig, indicating the guinea pig as a useful model species in which determinants of insulin resistance and diabetes can readily be investigated and from early in life.

CHAPTER 4
Sex-specific perinatal programming of insulin sensitivity in the guinea pig.

4.1 Overview

The following study aimed to characterise the effects of natural variation in litter size, birth phenotype and catch-up growth on insulin sensitivity and sites of insulin action in adult male and female guinea pigs. This utilised the same cohort of animals as described in Chapter 3, and I performed the additional statistical analysis of data for this chapter.

4.2 Abstract

Fetal growth restriction and subsequent neonatal catch-up growth have been implicated in the programming of insulin resistance later in life. Spontaneous fetal growth restriction due to litter size in the guinea pig gives rise to offspring with disproportionate IUGR and these offspring undergo catch-up growth. We therefore hypothesised that spontaneous IUGR due to natural varying litter size and subsequent fractional neonatal growth (FGR_{10-28}) or catch-up growth would impair insulin sensitivity in young adult male and female guinea pigs. Insulin sensitivity of glucose metabolism was determined by hyperinsulinaemic-euglycaemic clamp (HEC) in 38 (male=21, female=17) young adult guinea pigs from litter sizes of two to four pups. A subset (male=10, female=8) were infused with D-[3-^{3}H]-glucose two hours prior to and throughout the HEC to determine rates of basal and insulin-stimulated glucose utilisation, storage, glycolysis, and endogenous glucose production. In males, the insulin sensitivities of whole body glucose uptake (r=0.657, P=0.002) and glucose utilisation (r=0.884, P=0.004) correlated positively and independently with birth weight, and a similar trend was observed with the insulin sensitivity of endogenous glucose production (r=0.621, P=0.074), whereas no associations were observed with FGR_{10-28}. In females, whole body glucose uptake and partitioned glucose metabolism were not related to birth weight, but the insulin sensitivity of endogenous glucose production correlated negatively (r= -0.815, P=0.025) and independently with FGR_{10-28}. Effects of perinatal growth on whole body and partitioned glucose metabolism in the guinea pig are sex-specific, with evidence of impaired insulin action including of glucose utilisation after IUGR in males, and for impaired hepatic insulin sensitivity after rapid neonatal growth in females. Therefore both small size at birth and rapid neonatal growth are independent and sex-specific risk factors for the development of insulin resistance in the guinea pig.

Keywords: guinea pig, insulin sensitivity, glucose uptake, glycolysis, endogenous glucose production, glucose storage, catch-up growth

4.3 Introduction

Numerous epidemiological studies have implicated intrauterine growth restriction (IUGR), as indicated by being light, short or thin at birth, in the initiation of permanent physiological and/or metabolic adaptations[2]. These adaptations induced by sub-optimal conditions *in utero* result in "programmed" changes in determinants of insulin action at the tissue and molecular level, which may alter metabolic signalling, leading to insulin resistance, particularly in skeletal muscle[189, 190, 196, 198, 229, 231, 234, 236]. A mismatch between the environmental conditions experienced *in utero* and those experienced later in the individual's life course may expose these programmed changes and lead to the development of type 2 diabetes mellitus (T2DM) and the metabolic syndrome[2, 13, 65, 176, 457]. Human studies using the hyperinsulinaemic-euglycaemic clamp (HEC), the "gold standard" method to assess insulin sensitivity, have directly demonstrated increased risks of insulin resistance after IUGR, in children and in young and older adult males[187, 189, 190, 192-199].

In human infants, IUGR is often followed by accelerated, or catch-up, growth in the first few months of life[57]. Neonatal catch-up growth following IUGR has been identified as an additional risk factor for the development of insulin resistance[65, 82] Poor growth *in utero* and catch-up growth are independently associated with adverse cardio-metabolic outcomes in adulthood[83-89], including insulin resistance [65, 90]. Catch-up growth often occurs when restricted nutrient availability, as occurs in IUGR, is followed by hyperphagia in a relatively nutrient-rich environment[80, 374]. Down-regulation of thermogenesis, and a redistribution of glucose towards adipose tissue, associated with limited uptake of glucose and growth of skeletal muscle, are also suggested as potential mechanisms contributing to increased risk of obesity and T2DM following catch-up growth[72, 73, 76].

Insulin sensitivity, assessed using the HEC, is variably affected by IUGR in rodent models, including uterine artery ligation and maternal feed or protein restriction[303, 305, 306, 417, 458-

[461]. Maternal feed restriction in rodents has been associated with impaired [305, 417], unchanged[303] or even improved[306] insulin sensitivity. Similarly, maternal protein restriction in the rat appears to induce variable changes in insulin sensitivity of the offspring, with studies reporting no change[459] or improved insulin sensitivity[460, 461].

Evidence from studies of T2DM in humans suggest skeletal muscle as the key site of adult insulin resistance[432, 462]. The increase in glucose uptake in skeletal muscle accounts for approximately 70% of total whole body glucose disposal in the insulin-stimulated state, and the primary fate of this increased muscle glucose uptake is for storage as glycogen[106]. Studies in IUGR humans and animal models of IUGR demonstrate altered tissue development and gene expression which may impair insulin action within skeletal muscle[190, 196, 198, 229, 231-238]. The liver is also involved, as in rats, experimental IUGR permanently reduces its growth and programs reduced hepatic expression of genes in the insulin signalling pathway[232, 326, 377, 463]. In adipose tissue, insulin receptor numbers and glucose uptake are increased after IUGR in humans and in progeny of undernourished rats, suggesting a mechanism for increased adiposity, in turn contributing to increased risks of T2DM following IUGR[189, 341]. This suggests that these tissues are plausible targets for perinatal programming of altered insulin sensitivity later in life.

Guinea pigs provide an alternate species in which to investigate developmental programming of insulin resistance. After spontaneous growth restriction due to increasing litter size, pups are smaller at birth[391, 392] and these IUGR progeny from large litters undergo neonatal catch-up growth[394] (Chapter 2)[395]. Previously we have shown that spontaneous growth restriction in the guinea pig also induces catch-up growth in both absolute and fractional terms that persists post weaning, as well as increased central adiposity in adults (Chapter 2)[395]. In addition, and unlike the rat, the guinea pig can develop a phenotype resembling T2DM and including hyperglycaemia at 4 months of age[399, 400] making it a good species in which to investigate whether IUGR accelerates the risk of development of metabolic disease. Restricted fetal growth in the guinea pig induced by maternal feed restriction programs visceral adiposity, impaired glucose

tolerance, increased blood pressure and altered cholesterol homoeostasis in adult offspring[310-312]. These studies demonstrate that prenatal restriction can perturb metabolic function in the guinea pig; however, insulin sensitivity was not assessed. Furthermore, size at birth was suggested as a stronger predictor of adult outcomes than was maternal nutritional group[310-312], suggesting that further studies should focus on a model of spontaneous fetal growth restriction. In addition, adverse effects of exposure to maternal nutrient restriction were mostly seen in males, suggesting developmental programming of metabolic dysfunction in the guinea pig is sex-specific as described in other species, including humans[65, 185, 336, 464-466].

We therefore hypothesised firstly that small size at birth and spontaneous IUGR due to natural variation in litter size would be associated with reduced whole body, peripheral and hepatic insulin sensitivity. Our secondary hypothesis was that accelerated neonatal growth in terms of weight would independently predict impaired whole body, peripheral and hepatic insulin sensitivity. We therefore measured these outcomes in chronically catheterised young adult guinea pigs from litters of two, three or four pups by HEC with concomitant infusion of D-[3-^{3}H]-glucose tracer. Because developmental programming of glucose metabolism is sex-specific in IUGR humans[185], and other species including the guinea pig[310, 464], we investigated outcomes in both male and female progeny.

4.4 Materials and Methods

4.4.1 Animals

The Animal Ethics Committee of The University of Adelaide approved all animal studies (Approval number M56/96). Animal management was as described previously; the present cohort with insulin sensitivity measures are a subset of the animals described previously (Section 2.4.1)[395]. In brief, the present cohort were progeny of *ad libitum*–fed nulliparous female guinea pigs, which were timed-mated and spontaneously delivered pups in litter sizes of two,

three of four pups at term (range 67-71 days, mean ± S.E.M: 69.5 ± 0.3 days). Litter size, sex, weights, abdominal circumference, nose to rump lengths and head dimensions of all offspring were measured and recorded on the day of birth or following morning if delivered overnight. The cohort in which insulin sensitivity was measured in adulthood consisted of 38 pups (males: *n*=21, females: *n*=17). Each dam was housed with her offspring and provided with *ad libitum* lucerne chaff in addition to the standard diet. Litters were weighed at least 5 times per week until weaning at days 28-30 of age and absolute and fractional growth rates from day 10 until weaning (AGR_{10-28}, FGR_{10-28}), were calculated as described previously (Section 2.4.1, [395].

4.4.2 Surgery: Insertion of Catheters

A total of 38 guinea pigs underwent surgery at 100.1 ± 0.4 days of age for the insertion of vascular catheters. Catheters were inserted into the right jugular vein and carotid artery, as described in Section 3.4.2, under general anaesthesia induced by ketamine (75 $mg.kg^{-1}$ body weight, intraperitoneal) and xylazine (6 $mg.kg^{-1}$ body weight, intramuscular)[312]. Catheters were kept patent by daily flushing with heparinised saline, with patency success as described in the larger cohort (Section 3.4.2).

4.4.3 Hyperinsulinaemic-Euglycaemic Clamps

Six days post-surgery animals were fasted for 16 hours and a HEC was performed to determine whole body insulin sensitivity of glucose metabolism in conscious, unrestrained guinea pigs as previously described (Section 3.4.3). In brief, human insulin was continuously infused at 7.5 $mU.min^{-1}.kg^{-1}$, for 2 hours. Prior to insulin infusion, blood samples (800 µl) were collected at -20, -15, -10, -5 and 0 minutes to determine basal glucose concentration in whole blood measured by glucometer (HemoCue AB, Sweden). These samples were also collected for basal concentrations of free fatty acids (FFA). Blood samples (50-100 µl) were taken every 5 minutes throughout the insulin infusion. Intravenous infusion of glucose (as 10% dextrose

solution) commenced 15 minutes after the start of the insulin infusion. The glucose infusion rate (GIR) was adjusted, based on the blood glucose measurements, to restore and maintain euglycaemia, defined as the mean fasting blood glucose concentration, using a modified version[426] of the algorithm described previously[142]. The mean blood glucose concentration achieved during the last 60 minutes of the HEC was 6.69 ± 0.12 $mmol.l^{-1}$ with co-efficient of variation of 5.9 ± 0.3%.

A subset of the animals (*n* = 18, males = 10; females = 8) were infused with D-[3-^{3}H]-glucose (Amersham Pharmacia Biotech, Buchinghamshire, England) as a priming bolus (14.5 $\mu Ci.kg^{-1}$) followed by a continuous infusion (0.45 $\mu Ci.min^{-1}.kg^{-1}$) for 2 hours prior to and then throughout the HEC in order to determine rates of glucose utilisation, storage, glycolysis, and endogenous glucose production, in the fasting and insulin-stimulated states[428, 429]. Larger blood samples were collected (800 µl) at 60, 75, 80, 85, 90, 95, 105 and 120 minutes after the start of the insulin infusion, centrifuged and plasma stored at -20°C for analysis of radiolabelled metabolites and human insulin.

4.4.4 Insulin and Metabolite Analyses

Plasma concentrations of glucose (Glucose HK, Roche) and free fatty acids (FFA C, Wako Pure Chemical Industries, Japan) were measured by colorimetric enzymatic analysis on a COBAS Mira automated centrifugal analyser. Human insulin concentrations were analysed by radioimmunoassay using a commercially available assay with <2% cross-reactivity with guinea pig insulin (Insulin-CT, CIS Bio International, France).

4.4.5 Plasma D-[3-^{3}H]-Glucose and $^{3}H_2O$

The specific activity of D-[3-^{3}H]-glucose and of $^{3}H_2O$ was measured exactly as described in Section 3.4.5. In brief, samples were deproteinised using 0.3N $Ba(OH)_2$ and 0.3N $ZnSO_4$, and the ^{3}H content of dried and undried aliquots of supernatant was measured by β-

scintillation counting. The DPM of the dried (radioactivity of D-[3-^{3}H]-glucose) vials was subtracted from that of the undried vials (radioactivity of D-[3-^{3}H]-glucose + $^{3}H_2O$) to give the DPM of $^{3}H_2O$ alone.

4.4.6 Calculations

Steady-state plasma concentrations of human insulin were calculated as the average of concentrations every 15 minutes throughout the final hour of the HEC. Steady state glucose infusion rate (ssGIR) was calculated as the average GIR during the final hour of the clamp. Whole body insulin sensitivity was calculated by dividing ssGIR by steady-state plasma concentrations of human insulin. The post-hepatic metabolic clearance rate (MCR) of human insulin was calculated as the insulin infusion rate during the HEC divided by steady-state plasma concentrations of human insulin. Rates of partitioned components of glucose metabolism in fasting and insulin-stimulated states (endogenous glucose production, glucose utilisation, glucose storage and glycolysis) were calculated based on kinetics of D-[3-^{3}H]-glucose metabolism in the steady-state (last 20-40 minutes) for each condition, as described in detail previously Section 3.4.6, [428, 429].

4.4.7 Adult Body Composition

The subset of animals that underwent tracer studies (n=18, 10 male, 8 female) were humanely killed between 2 pm and 4 pm by lethal injection of sodium phenobarbitone immediately after the HEC and a post mortem was performed (age: 115 ± 2 days). Fat depots (interscapular, omental, right side of the neck and bilateral axillary, retroperitoneal, perirenal, and groin depots) and bilateral skeletal muscles (hindlimb: *M. biceps femoris, M. semitendinosus, M. gastrocnemius, M. plantaris, and M. tibialis; forelimb: M. biceps brachii*) were dissected and weighed as described previously Section 2.4.2, [395]. Visceral adipose weight was calculated as the sum of weights of the left and right perirenal and retroperitoneal fat depots. Subcutaneous adipose

weight was calculated as the sum of weights of left and right axillary and groin fat, right side of the neck fat and interscapular fat depots. Visceral and subcutaneous fats were summed to give a measure of combined adiposity. The weights of dissected skeletal muscles were summed to obtain combined skeletal muscle mass. The ratio of the combined adiposity to the combined muscle mass was calculated as an index of relative adiposity.

4.4.8 Statistical Analysis

Data were analysed using SPSS 23.0 for Windows (IBM, Armonk, USA). The effect of litter size on total pup weight and gestational length was assessed by ANOVA. Effects of litter size on proportions of liveborn and stillborn progeny were analysed by chi-squared test. The effects of litter size and sex on birth phenotype, neonatal growth and whole body insulin sensitivity and postnatal outcomes were analysed by mixed models ANOVA, including the dam as a random variable to account for effects of a common maternal environment. Bonferroni's post-hoc comparisons were used to compare differences in maternal and offspring outcomes between litter sizes. Insulin sensitivity had not been measured previously by HEC in the guinea pig nor was tracer analysis to determine the partitioned glucose metabolism assessed therefore a *priori* power analysis was not possible in this instance. Where effects of litter size differed between sexes, outcomes were analysed separately in each sex. Due to limited numbers of animals and therefore power for tracer studies of components of glucose metabolism and post-mortem body composition measures within each litter size group and sex, mixed models analyses could not be performed for these outcomes. The independent effects of birth weight and neonatal growth rate for weight on outcomes were assessed by multiple linear regression separately in each sex for both whole body and partitioned glucose metabolism. As neonatal growth did not affect the insulin sensitivity of whole body glucose uptake, effects of birth weight on this outcome were therefore also subsequently examined by Pearson's correlation analysis, separately in each

sex. A *P*-value of < 0.05 was accepted as statistically significant. All results are expressed as mean ± S.E.M.

4.5 Results

4.5.1 Birth and Neonatal Phenotype

In the litters that generated the animals in which insulin sensitivity was measured, gestation length did not differ between litter sizes (overall mean 69.4 ± 0.2 days, Table 4-1). The proportion of pups born alive tended (P=0.054) to decrease as litter size increased from 2 (100% survival) to three (97% survival) or four (82% survival), and total litter weight increased with increasing litter size (P<0.001, Table 4-1).

Table 4-1 Effect of litter size on litter outcomes

Outcome	Litter Size			Significance
	Two	Three	Four	LS
Number of litters	5	13	7	-
Surviving:Stillborn pups at birth	10:0	35:1	23:5	0.054
Total pup weight per litter (g)	230 ± 7^{a}	286 ± 5^{b}	374 ± 11^{c}	<0.001
Gestation length (days)	69.6 ± 0.7	69.5 ± 0.3	69.6 ± 0.6	NSD

Data expressed as means ± SEM of dams carrying each of the litter sizes (LS).
^{a,b,c}Means with different superscripts differ, P<0.05. NSD: not significantly different, P>0.1.

Average birth weight of pups from a litter size of four was 17% lower than those from litter size of two, while other measures of size at birth were reduced to a lesser degree (Table 4-2). Birth weight and length were higher in males from litters of two when compared to males from litters of three or four pups (P=0.001 for both, Table 4-2). In females, birth weight, length and head length did not differ between litter sizes. Abdominal circumference was higher in males from litters of two compared to those from litters of four pups (P=0.034) and tended to be higher

compared to litters of three pups (P=0.051). Abdominal circumference was higher in females from litters of two compared to litters of three pups (P=0.039), but not compared to offspring of litters of four pups. The weight:length ratio was higher in males of litters of two pups compared to litters of three and four pups (P=0.004 and P=0.003 respectively). Weight:length ratio did not differ with litter size in females. Body mass index at birth was not altered by litter size overall or within each sex.

Neonatal absolute growth rates (AGR_{10-28}) were higher in males (10.5 ± 0.2 $g.day^{-1}$) than females (8.9 ± 0.2 $g.day^{-1}$) (P<0.001) but did not differ with litter size (Table 4-2). Neonatal fractional growth rates (FGR_{10-28}) were also higher in males (6.3 ± 0.2%) than females (5.4 ± 0.1%), whilst effects of litter size on FGR_{10-28} differed between sexes (P=0.027, Table 4-2). In males, FGR_{10-28} differed between litter sizes (P=0.002), with litters of four growing faster than litters of two (P=0.003) or three (P=0.011) pups. In females, FGR_{10-28} did not differ between litter sizes. AGR_{10-28} tended to correlate positively with birth weight overall (r=0.292, P=0.068, n=38), and in males alone (r=0.431, P=0.051, n=21), but not in females. FGR_{10-28} correlated negatively with birth weight overall (r= -0.431, P=0.007, n=38) and in males (r= -0.578, P=0.006, n=21) and females (r= -0.501, P=0.04, n=17) separately.

Table 4-2 Effect of litter size and sex on birth phenotype and neonatal growth rates

Outcome	Males from Litter Size			Females from Litter Size			Significance		
	Two	Three	Four	Two	Three	Four	LS	Sex	LS × sex
Number of Offspring	4	10	7	2	11	4			
Size at Birth									
Weight (g)	119 ± 3[a]	94 ± 3[b]	91 ± 4[b]	106 ± 22	94 ± 2	101 ± 5	0.008	NSD	0.070[1]
Length (mm)	172 ± 3[a]	156 ± 3[b]	155 ± 2[b]	155 ± 16	157 ± 2	161 ± 5	NSD	NSD	0.049[2]
Abdominal circumference (mm)	114 ± 5[a]	103 ± 2[a,b]	101 ± 2[b]	118 ± 7[a]	100 ± 2[b]	111 ± 3[a,b]	0.007	NSD	NSD
Head-length (mm)[†]	47 ± 1[a]	40 ± 2[a,b]	39 ± 2[b]	42 ± 1	40 ± 1	41 ± 3	NSD	NSD	0.016[3]
Head-width (mm)[‡]	23 ± 1	21 ± 1	21 ± 1	22 ± 2	22 ± 0	21 ± 0	NSD	NSD	NSD
Weight:length ($g.mm^{-1}$)	0.69 ± 0.01[a]	0.60 ± 0.01[b]	0.59 ± 0.02[b]	0.67 ± 0.07	0.60 ± 0.02	0.63 ± 0.03	0.028	NSD	NSD
Body mass index ($kg.m^{-2}$)	4.0 ± 0.1	3.9 ± 0.1	3.8 ± 0.2	4.3 ± 0	3.8 ± 0.1	3.9 ± 0.3	NSD	NSD	NSD
Neonatal Growth Rates									
AGR_{10-28} ($g.day^{-1}$)	11.1 ± 0.4	10.2 ± 0.2	10.6 ± 0.3	8.8 ± 0.3	8.8 ± 0.3	9.2 ± 0.3	NSD	<0.001	NSD
FGR_{10-28} (%)	5.7 ± 0.1[a]	6.1 ± 0.2[a]	7.0 ± 0.2[b]	5.3 ± 0.8	5.5 ± 0.2	5.3 ± 0.1	NSD	<0.001	0.027[4]

Data expressed as actual means ± SEM of offspring in each of the litter sizes (LS).
[a,b,c,]Means with different superscripts differ, P<0.05. NSD: not significantly different, P>0.1.
LS × sex = interaction between litter size and sex. Statistical models included dam to correct for common intrauterine environment in multiple births.
[†]Head lengths were not measured in 1 male from litter size of three and 2 males and 1 female from litter size of four.
[‡]Head widths were not measured in 1 male from litter size of three.
[1]In males, birth weight differed across the litter sizes (P=0.001) with litters of two being larger than litters of three and four (P=0.001 for both) while birth weights of offspring of litters three and four were not significantly different. In females, there were no differences in birth weight found across the litters.
[2]In males, birth length differed across the litter sizes (P=0.029) with litters of two being larger than litters of three and four (P<0.05 for both) while birth lengths of offspring of litters three and four did not differ. In females, there were no differences in birth length found across the litters.
[3]In males, birth head-length tended to differ across the litters (P=0.066), with litters of two tending to have larger head-length than litters of three (P=0.097) while birth head-lengths of offspring of litters three and four did not differ. In females, there were no differences in birth head-length found across the litters.
[4]In males, fractional neonatal growth rate differed between litter sizes (P=0.002) with litters of four growing faster than litters of two (P=0.003) and three (P=0.011), whereas in females, fractional neonatal growth rate did not differ between litter sizes.

4.5.2 Litter Size, Perinatal Growth and Whole Body Metabolic Outcomes

Fasting plasma glucose and FFA were not altered by litter size or sex (Table 4-3). Effects of litter size on steady-state plasma concentrations of human insulin differed between sexes, with males (255 ± 23 μU.ml^{-1}) having a higher steady state plasma insulin than females (212 ± 15 μU.ml^{-1}) overall (Table 4-3). Nevertheless, when the sexes were analysed separately steady-state plasma concentrations of human insulin did not differ between litter sizes in either sex. The MCR for human insulin was ~13% lower in males (34 ± 3 ml.kg^{-1}.min^{-1}) than females (39 ± 3 ml.kg^{-1}.min^{-1}, P=0.011), and effects of litter size differed between sexes (Table 4-3). In males, the MCR of human insulin differed between litter sizes (P=0.036) with males from litters of two pups having a higher MCR than those from litters of three pups (P=0.036), whereas in females MCR was not affected by litter size (Table 4-3). Although effects of litter size on insulin sensitivity of whole-body glucose metabolism differed between sexes (P=0.021), the effects of litter size were not significant in either sex analysed separately (Table 4-3).

Table 4-3 Effect of litter size and sex on whole body glucose metabolism.

Outcome	Males from Litter Size			Females from Litter Size			Significance		
	Two	Three	Four	Two	Three	Four	LS	Sex	LS × Sex
Number of Offspring	4	10	7	2	11	4			
Weight (g)	829 ± 29	801 ± 16	790 ± 27	670 ± 20	652 ± 17	692 ± 21	NSD	<0.001	NSD
Fasting Plasma Concentrations									
Glucose (mmol.l^{-1})	6.7 ± 0.2	6.7 ± 0.2	6.7 ± 0.4	6.6 ± 1.1	7.3 ± 0.2	7.2 ± 0.2	NSD	NSD	NSD
Free fatty acids (meq.l^{-1})	2.5 ± 0.1	2.1 ± 0.2	2.5 ± 0.2	1.8 ± 0.3	2.3 ± 0.1	2.4 ± 0.4	NSD	NSD	NSD
Steady State Outcomes									
Glucose infusion rate (μmol.min^{-1}.kg^{-1})	41.1 ± 3.9	41.5 ± 5.1	47.4 ± 9.3	33.9 ± 7.9	43.9 ± 3.9	45.7 ± 6.0	NSD	NSD	NSD
Plasma human insulin (μU.ml^{-1})	155 ± 22	297 ± 40	252 ± 18	273 ± 2	199 ± 20	216 ± 23	NSD	NSD	0.003[1]
MCR human insulin (ml.kg^{-1}.min^{-1})	52 ± 8[a]	30 ± 4[b]	30 ± 2[ab]	28 ± 0	42 ± 5	36 ± 4	NSD	0.011	<0.001[2]
Whole body insulin sensitivity (μmol.ml.μU^{-1}.min^{-1}.kg^{-1})	0.29 ± 0.06	0.15 ± 0.02	0.20 ± 0.04	0.12 ± 0.03	0.24 ± 0.03	0.22 ± 0.03	NSD	NSD	0.021[3]

Data expressed as actual means ± SEM of offspring in each of the litter sizes (LS). [a,b,c]Means with different superscripts differ, *P*<0.05. NSD: not significantly different, *P*>0.1. LS × Sex = interaction between litter size and sex. Statistical models included dam to correct for common intrauterine environment in multiple births.

[1]Plasma human insulin during steady state in the last hour of the HEC did not differ between litter sizes in males or females analysed separately.

[2]In males, the metabolic clearance rate (MCR) of human insulin differed between litter sizes (*P*=0.036) with litters of two having a higher MCR than litters of three (*P*=0.045) and tending to have a higher MCR than litters of four (*P*=0.090), whereas in females, MCR did not differ between litter sizes.

[3]In males, whole body insulin sensitivity tended to differ between litter sizes (*P*=0.083) with litters of three tending to have a lower insulin sensitivity of whole body glucose metabolism than litters of two (*P*=0.077), whereas in females, whole body insulin sensitivity did not differ between litter sizes

In multiple linear regression analyses, fasting plasma glucose concentrations did not correlate with birth weight or FGR_{10-28} in males or females separately. In males, fasting plasma FFA concentrations did not correlate with FGR_{10-28} or birth weight, whereas in females FFA levels tended to correlate negatively with FGR_{10-28}, but not with birth weight (Table 4-4).

In males, birth weight was independently and positively associated with the MCR of insulin and with whole body insulin sensitivity, and tended to correlate negatively with plasma human insulin concentrations ($P<0.07$), while FGR_{10-28} was not associated with any of these measures (Table 4-4). The $ssGIR_{60-120}$, in contrast was positively associated with FGR_{10-28}, but not with birth weight, in males (Table 4-4). In females, whole body insulin sensitivity, ssGIR, plasma human insulin and MCR insulin did not correlate with birth weight or FGR_{10-28} (Table 4-4). These relationships were also evident in simple correlation analyses, such that the whole-body insulin sensitivity of glucose uptake (Figure 4-1) correlated positively with birth weight overall (r=0.417, P=0.007, n=38), and in males (r=0.606, P=0.004, n=21) but not in females (r=0.128, NS, n=17).

Table 4-4 Whole body metabolic outcomes before and during HEC at 7.5 mU insulin.min^{-1}.kg^{-1} in the guinea pig

Whole Body Measure	Overall and Partial Correlation (*r*, *P*)					
	Males n=21			Females n=17		
	Overall	Birth weight	FGR_{10-28}	Overall	Birth weight	FGR_{10-28}
Fasting Plasma Concentrations						
Glucose (mmol.l^{-1})	0.057, 0.972	0.048, 0.841	0.051, 0.832	0.234, 0.674	0.112, 0.680	-0.079, 0.770
Free fatty acids (meq.l^{-1})	0.246, 0.607	0.023, 0.927	-0.199, 0.429	0.476, 0.165	0.338, 0.200	*0.476, 0.062*
Steady State Outcomes						
Glucose infusion rate (μmol.min^{-1}.kg^{-1})	*0.479, 0.096*	0.359, 0.120	**0.465, 0.037***	0.491, 0.145	-0.086, 0.769	-0.423, 0.102
Plasma human insulin (μU.ml^{-1})	**0.554, 0.037***	*-0.413, 0.070*	0.175, 0.460	0.466, 0.180	-0.296, 0.266	*-0.462, 0.071*
MCR human insulin (ml.kg^{-1}.min^{-1})	**0.646, 0.008***	**0.544, 0.013***	0.126, 0.598	0.362, 0.374	0.185, 0.494	0.353, 0.180
Whole body insulin sensitivity (μmol.ml.μU^{-1}.min^{-1}.kg^{-1})	**0.661, 0.006***	**0.657, 0.002***	0.332, 0.152	0.130, 0.888	0.080, 0.769	-0.022, 0.937

n, Number of offspring. FGR_{10-28}: fractional growth rate for weight in neonates from 10-28 days of age.
Age at HEC was 115 ± 1 days. *Significant correlations ($P < 0.05$) are shown in **bold** and trends ($P < 0.1$) in *italics.*

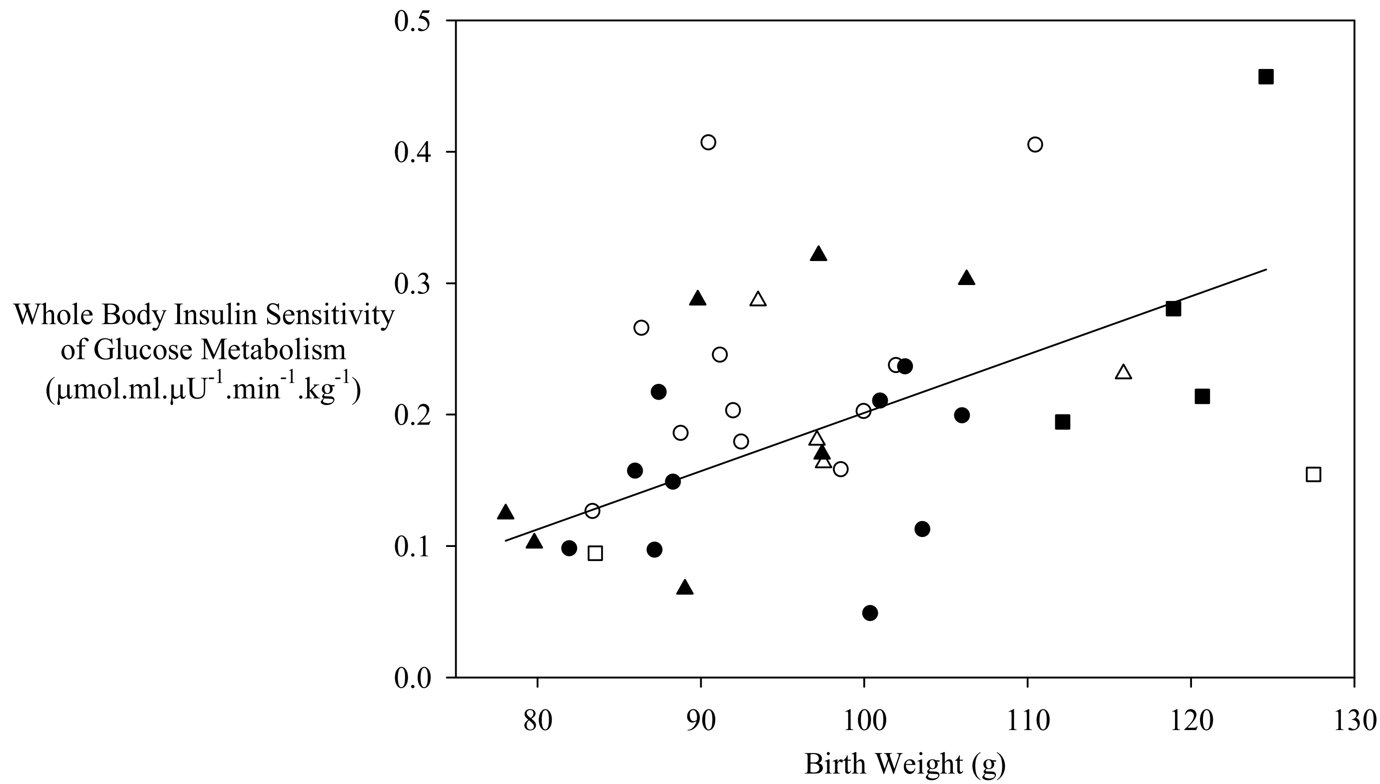


Figure 4-1 Correlation between whole body insulin sensitivity and birth weight in male and female guinea pigs

Relationship of the insulin sensitivity of whole body glucose metabolism and birth weight in males, closed symbols, solid regression line (*r*=0.606, *P*=0.004, *n*=21) and females in open symbols (*r*=0.128, NS, *n*=17). Offspring of (■ males = 4; □ females = 2) litters of two are shown in squares, (● males = 10; ○ females = 11) litters of three in circles and (▲ males = 7; △ females = 4) litters of four in triangles

4.5.3 Perinatal Growth and Partitioned Glucose Metabolism

4.5.3.1 *Endogenous glucose production*

In males, basal and insulin-stimulated rates of endogenous glucose production were not associated with birth weight or FGR_{10-28} (Table 4-5), but the insulin sensitivity of endogenous glucose production tended to correlate positively with birth weight, but not with FGR_{10-28}, in males (Table 4-5). In females, basal endogenous glucose production was not related to birth weight or neonatal growth rates. However, the insulin-stimulated rate, the insulin stimulated decrease and the insulin sensitivity of endogenous glucose production were all positively and independently correlated with FGR_{10-28} in females (Table 4-5). Birth weight was not associated with the insulin stimulated decrease or insulin sensitivity of glucose production in females, but did tend to correlate positively with the insulin stimulated rate of endogenous glucose production (Table 4-5).

4.5.3.2 *Glucose utilisation*

In males, the basal and insulin-stimulated rates of whole-body glucose utilisation and the insulin-stimulated increase in whole-body glucose utilisation did not independently correlate with birth weight or FGR_{10-28} (Table 4-5), whereas the insulin sensitivity of whole-body glucose utilisation correlated independently and positively with birth weight, but not with FGR_{10-28} (Table 4-5). In females, basal and insulin-stimulated rates of whole body glucose utilisation and its insulin sensitivity were not related to birth weight or neonatal FGR (Table 4-5).

4.5.3.3 *Glucose storage*

Basal and insulin-stimulated rates of whole-body glucose storage and its insulin sensitivity were not related to birth weight or FGR_{10-28} in males or females (Table 4-5).

4.5.3.4 ***Glycolysis***

In males, basal and insulin-stimulated rates of whole body glycolysis and its insulin sensitivity did not independently correlate with birth weight nor with FGR_{10-28} (Table 4-5). In females, the basal rate of glycolysis correlated independently and positively with birth weight and with FGR_{10-28} (Table 4-5), whereas the insulin-stimulated rate of glycolysis correlated positively with FGR_{10-28}, but not with birth weight (Table 4-5). In females, the insulin-stimulated increase and the insulin sensitivity of the rate of glycolysis did not independently correlate with birth weight or with FGR_{10-28} (Table 4-5).

Table 4-5 Relationships of perinatal growth and partitioned glucose metabolism at 7.5 mU insulin.min^{-1}.kg^{-1} insulin infusion in the guinea pig.

Partitioned Glucose Metabolism Measure	Overall and Partial Correlation (*r*, *P*)					
	Males *n*=10			Females *n*=8		
	Overall	Birth weight	FGR_{10-28}	Overall	Birth weight	FGR_{10-28}
Endogenous glucose production						
Basal (µmol.min^{-1}.kg^{-1})	0.153, 0.920	0.084, 0.830	-0.067, 0.864	0.621, 0.296	0.604, 0.151	0.564, 0.187
Insulin stimulated (µmol.min^{-1}.kg^{-1})	0.260, 0.783	-0.170, 0.662	0.086, 0.826	*0.813, 0.067*	*0.750, 0.052*	**0.807, 0.028***
Insulin-stimulated decrease (µmol.min^{-1}.kg^{-1})	0.332, 0.152	0.130, 0.888	0.080, 0.769	**0.948, 0.003***	-0.556, 0.195	**-0.941, 0.002***
Insulin sensitivity (µmol.ml.µU^{-1}.min^{-1}.kg^{-1})	*0.746, 0.058*	*0.621, 0.074*	-0.198, 0.610	*0.821, 0.061*	-0.597, 0.157	**-0.815, 0.025***
Whole body glucose utilisation						
Basal (µmol.min^{-1}.kg^{-1})	0.153, 0.920	0.084, 0.830	-0.067, 0.864	0.621, 0.296	0.604, 0.151	0.564, 0.187
Insulin stimulated (µmol.min^{-1}.kg^{-1})	0.396, 0.550	0.233, 0.546	-0.176, 0.647	0.598, 0.330	0.598, 0.156	0.433, 0.331
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	0.604, 0.205	0.393, 0.295	-0.296, 0.439	0.473, 0.530	0.254, 0.582	-0.163, 0.727
Insulin sensitivity (µmol.ml.µU^{-1}.min^{-1}.kg^{-1})	**0.918, 0.002***	**0.884, 0.004***	-0.537, 0.136	0.459, 0.554	0.320, 0.483	-0.057, 0.904
Whole body glycolysis						
Basal (µmol.min^{-1}.kg^{-1})	0.234, 0.821	0.050, 0.898	-0.172, 0.659	*0.834, 0.051*	**0.770, 0.043***	**0.830, 0.021***
Insulin stimulated (µmol.min^{-1}.kg^{-1})	0.184, 0.886	0.165, 0.672	0.014, 0.971	**0.904, 0.013***	0.454, 0.306	**0.883, 0.009***
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	0.305, 0.710	0.259, 0.502	0.278, 0.278	0.533, 0.433	-0.461, 0.297	-0.091, 0.846
Insulin sensitivity (µmol.ml.µU^{-1}.min^{-1}.kg^{-1})	0.228, 0.830	0.198, 0.610	0.202, 0.602	0.539, 0.424	-0.516, 0.235	-0.244, 0.598
Whole body glucose storage						
Basal (µmol.min^{-1}.kg^{-1})	0.075, 0.980	-0.014, 0.972	0.056, 0.886	0.228, 0.875	0.201, 0.666	0.056, 0.905
Insulin stimulated (µmol.min^{-1}.kg^{-1})	0.227, 0.756	0.075, 0.848	-0.195, 0.616	0.649, 0.255	0.543, 0.208	0.031, 0.948
Insulin-stimulated increase (µmol.min^{-1}.kg^{-1})	0.587, 0.228	0.173, 0.656	-0.453, 0.221	0.644, 0.262	0.508, 0.245	-0.041, 0.930
Insulin sensitivity (µmol.ml.µU^{-1}.min^{-1}.kg^{-1})	*0.737, 0.064*	0.503, 0.168	-0.435, 0.242	0.670, 0.225	0.594, 0.159	0.120, 0.798

n, Number of offspring. FGR_{10-28}: fractional growth rate for weight in neonates from 10-28 days of age. Age at HEC was 115 ± 1 days.
*Significant correlations ($P < 0.05$) are shown in **bold** and trends ($P < 0.1$) in *italics*.
Basal: prior to insulin infusion, Insulin-stimulated: during insulin infusion, insulin stimulated decrease/increase: change between the basal and insulin-stimulated states: Insulin sensitivity: change between the basal and insulin-stimulated states divided by the steady-state plasma insulin concentration.

4.5.4 Adult Body Composition does not Explain Perinatal Programming of Insulin Sensitivity

Overall males had a greater adult weight, longer length and a greater weight:length than females, while body mass index was not different between the sexes (Table 4-6). Absolute weights of subcutaneous, visceral and total dissected fat were greater in males compared to females (P<0.007 for all), while the absolute weight of omental fat did not differ between sexes (Table 4-6). The relative weight of subcutaneous fat tended to be greater in males compared to females, however, all other measures of adiposity in relative terms did not differ between the sexes.

In males, fasting plasma metabolites were not correlated with adult body weight or body composition. In females, fasting plasma glucose concentration correlated positively with the relative weight of omental fat (r=0.727, P=0.041), but was not related to any other measures of body composition. In males, insulin sensitivity of whole body glucose metabolism was not correlated with adult body weight or body composition. In females, the insulin sensitivity of whole body glucose metabolism correlated positively with the relative weight of omental fat (r=0.731, P=0.039) but was not related to any other measures of body composition. In males, basal rates of glucose utilisation and endogenous glucose production correlated negatively with relative omental fat mass (r= -0.699, P=0.024 for both). In males, the insulin sensitivity of the endogenous glucose production tended to correlate negatively with the relative omental fat mass (r= -0.601, P=0.066) and the total dissected fat to muscle ratio (r= -0.630, P=0.051). In females, the insulin sensitivity of glucose utilisation correlated positively with both the absolute and relative mass of omental and of total dissected fat (P<0.05 for all). Similarly, in females, the insulin sensitivity of glucose storage correlated positively with both the absolute and relative omental fat mass (P<0.025 for both). No correlations were found between the insulin sensitivity of glucose utilisation or of storage and adult weight or body composition in males. No correlations were found between the insulin sensitivity of the rate of glycolysis and adult weight or body composition in either sex.

Table 4-6 Effect of sex on adult size and body composition

Outcome			Significance
	Males (n=10)	Females (n=8)	Sex
Age at Post Mortem	116 ± 3	113 ± 1	NSD
Adult Size			
Weight (g)	840 ± 28	680 ± 22	<0.001
Length (mm)§	349 ± 6	320 ± 5	0.002
Weight:Length ($g.mm^{-1}$) §	2.41 ± 0.07	2.13 ± 0.08	0.021
Body Mass Index ($kg.m^{-2}$) §	6.9 ± 0.2	6.7 ± 0.3	NSD
Adult Body Composition			
Subcutaneous fat (g)	43 ± 3	30 ± 3	0.002
Subcutaneous fat (%)	5.1 ± 0.2	4.4 ± 0.3	0.079
Visceral fat (g)	19 ± 1	13 ± 1	0.007
Visceral fat (%)	2.2 ± 0.1	1.9 ± 0.2	NSD
Omental fat (g)	17 ± 1	15 ± 1	NSD
Omental fat (%)	2.05 ± 0.05	2.22 ± 0.09	NSD
Total dissected fat (g)	79 ± 4	58 ± 5	0.006
Total dissected fat (%)	9.3 ± 0.3	8.5 ± 0.5	NSD
Skeletal muscle (g)	22 ± 1	18 ± 1	0.01
Skeletal muscle (%)	2.64 ± 0.08	2.74 ± 0.09	NSD
Total dissected fat: skeletal muscle	3.5 ± 0.1	3.2 ± 0.2	NSD

Data expressed as means ± SEM of dams carrying each of the litter sizes (LS). NSD: not significantly different, P>0.1.
Weights of all measures of adult body composition are expressed in absolute terms (g) and as a percentage of adult body weight (%).
§Adult lengths were only measured in n=7 females.

4.6 Discussion

We have found for the first time that spontaneous IUGR due to variable litter size in the guinea pig is associated with impaired whole body insulin sensitivity in young adult males. This is consistent with sex-specific programming of insulin sensitivity following IUGR in young adult human males in their twenties[185, 194, 195, 198, 229, 328]. This impaired whole body insulin sensitivity in the spontaneously IUGR male guinea pig appears to reflect primarily impaired insulin sensitivity of glucose utilisation, which decreased with decreasing size at birth. These observations are also consistent with findings in IUGR humans and animal models of IUGR demonstrating impaired insulin signalling in skeletal muscle primarily of glucose uptake[190, 231, 237, 328, 329, 467-469]. The insulin sensitivity of glycolysis, in contrast, was not related to size at birth in young adult guinea pigs of either sex.

While effects of birth weight on whole body insulin sensitivity were not observed in female offspring, rapid neonatal growth predicted impaired hepatic insulin sensitivity but improved basal and insulin-stimulated rates of glycolysis in young adult females only. Thus, in the guinea pig, spontaneous IUGR impairs whole body and peripheral insulin sensitivity in young adult males, with similar trends for hepatic insulin sensitivity, which may lead to overt diabetes and other cardio-metabolic pathology with ageing in this species.

4.6.1 Perinatal Growth and Whole Body Insulin Sensitivity

In the present study, small size at birth was associated with impaired whole body insulin sensitivity in young adult male guinea pigs. Consistent with this relationship, insulin sensitivity tended to be lower in males from litters of three, compared to two pups. In contrast to the larger cohort described in Chapter 2[395], males from litters of three and four pups were of similar weight and size at birth within the subset of animals in which insulin sensitivity was measured, which may explain their similar whole body insulin sensitivity. The negative

association between insulin sensitivity in young adult guinea pigs and their size at birth is consistent with studies in the IUGR human and in other species measured using the HEC[154, 187, 189, 194, 464]. In contrast, IUGR induced by maternal protein restriction in the rat does not consistently impair insulin sensitivity of young adult offspring, also measured by HEC[335, 460]. We found that fasting glucose levels in adult guinea pig offspring were not related to size at birth, and did not differ between litter sizes, in either sex. These observations suggest that the impairment in insulin sensitivity in male guinea pigs who were small at birth precedes any impairment of glucose tolerance or hyperglycaemia. This is consistent with previous reports of insulin resistance without hyperglycaemia in young adulthood in humans after small size at birth or IUGR[185, 189, 217]. Previously we have shown that catch-up following spontaneous IUGR is associated with increased adiposity in visceral compartments in young adult guinea pigs, primarily in males (Chapter 2, [395]). Neonatal growth rates did not predict whole-body insulin sensitivity in young adult guinea pigs. We hypothesise that with further ageing a progressive increase in visceral adiposity, particularly in males after IUGR and rapid neonatal growth, would exacerbate impaired insulin sensitivity, leading to compensatory failure, glucose intolerance and hyperglycaemia. Consistent with this, many studies in humans only report correlations between IUGR and impaired glucose tolerance and overt diabetes in aged cohorts[187, 201, 202].

4.6.2 Perinatal Growth and Components of Glucose Metabolism

Insulin resistance in the male guinea pig of low birth weight was apparent for whole-body glucose utilisation with a tendency for impairment in endogenous glucose production. Impaired glucose utilisation and storage largely reflect impaired insulin action in skeletal muscle[106]. In humans, skeletal muscle is the primary tissue for glucose disposal in the insulin-stimulated state, accounting for approximately 70% of the increase in glucose uptake[106]. Skeletal muscle is also the primary site of insulin resistance in humans with T2DM, reinforcing

the physiological importance of this tissue[432]. Our findings in guinea pig are consistent with those of previous studies in humans where small size at birth reduces expression of key targets in the insulin signalling pathway leading to insulin resistance in young adults. This includes reduced expression of GLUT4 and reduced glucose uptake, as well as decreased expression of the post-receptor signalling protein kinase C-ζ and p85α and p110β subunits of phosphoinositol 3-kinase[190, 196, 198, 229, 231-238].

In the current study, we also observed a tendency for a positive independent correlation between the insulin sensitivity of endogenous glucose production and birth weight in males only. This suggests that both skeletal muscle and liver contribute to the whole-body insulin resistance associated with small size at birth in males in this species. This hepatic contribution is also consistent with studies in humans where insulin suppression of endogenous glucose production is impaired after IUGR[197, 246]. Similarly, insulin signalling in liver is impaired in several animal models of IUGR[326, 329, 346, 463, 470]. Additional studies will be required to determine the underlying mechanisms for insulin resistance in muscle and liver in the prenatally growth restricted guinea pig.

In contrast to previous reports in humans and other species[358], neonatal catch-up growth was not associated with impaired insulin sensitivity of whole-body glucose uptake in either sex in the young adult guinea pig. This lack of relationship is consistent, however, with previous reports in the control and placentally-restricted sheep, where measures of neonatal growth were not correlated with adult insulin sensitivity of whole-body glucose uptake in either sex[464]. The lack of correlation between neonatal growth and insulin sensitivity in the young adult male guinea pigs is particularly interesting given the positive correlations observed between neonatal growth and visceral adiposity (Chapter 2, [395]).

Intriguingly, the insulin sensitivity of endogenous glucose production was correlated negatively with neonatal fractional growth rate but not with birth weight in female guinea pigs only. This implies that in female guinea pigs, fast rates of growth following IUGR may

contribute to the sequelae of insulin resistance later in life. Females in this species also tend to have a higher basal endogenous glucose production rates than males (Section 3.5.2), consistent with reports of higher basal endogenous glucose production rates in women than men of similar age, and suggesting a sex-specific hepatic handling of glucose[454]. Fast neonatal growth also correlated with high circulating FFA in adult female guinea pigs, and elevated FFAs are known to impair insulin action in the liver which leads to increased hepatic glucose production[471]. This suggests that elevated FFA might contribute to the impaired suppression of endogenous glucose production under HEC conditions after accelerated neonatal growth in the female guinea pig.

In females, basal rates of glycolysis correlated positively with birth weight perhaps suggesting a reduced capacity for glycolysis in low birth weight female guinea pigs. The rates of basal and insulin-stimulated glycolysis increased with increasing neonatal fractional growth rate without an increase in the insulin sensitivity of glycolysis with neonatal catch-up. This suggests that neonatal catch-up may enhance the rate limiting step in glycolysis but does not alter the overall insulin sensitivity of the entire pathway. In humans, IUGR indicated by thinness at birth is associated with reduced rates of glycolysis during exercise, measured by ^{31}P magnetic resonance spectroscopy[234]. In these IUGR humans, there was no association between insulin sensitivity and thinness at birth and previous findings suggest insulin mediated glucose uptake into muscle is not the rate limiting step of glycolysis [472]. The observation of reduced glycolytic potential during exercise in IUGR humans did not report effects of sex. Hence, further investigation is required to determine if the impaired rate of basal glycolysis following IUGR is sex-specific in a larger cohort in guinea pigs, other animal models of IUGR and in humans.

4.6.3 Impaired Insulin Sensitivity in the Low Birth Weight Male is not Related to Body Composition

Insulin sensitivity was not correlated with adult body composition in young adult male guinea pigs, suggesting that the insulin resistance in male guinea pigs who were small at birth is not due to central adiposity or reduced lean tissue. This is consistent with studies in

young adult humans showing that adiposity does not explain reductions in insulin sensitivity with small size at birth[200, 206]. In turn, these findings suggest that impaired insulin sensitivity is due to changes in the function of insulin sensitive tissues, rather than the absolute or relative amounts of these.

4.6.4 Sex Differences

In the current study, prenatal programming of insulin resistance was evident primarily in male guinea pigs. This male-specific programming by IUGR was of whole body insulin sensitivity and glucose utilisation, with a similar trend for the suppression of endogenous glucose production. Consistent with this, studies in humans report impaired insulin sensitivity in males but not in females after IUGR[65, 185]. Others report that impaired insulin action or measures of T2DM correlate positively with size at birth in males and do not report outcomes in females[187, 194, 195, 201, 229]. Systematic reviews of the literature suggest that when positive correlations between insulin sensitivity and size at birth are evident in both males and females the strength of the associations are approximately 2.5-fold greater in males than in females suggesting an increased impact or susceptibility of males to a sub-optimal intrauterine environment[65, 176]. In contrast, other studies in humans have not shown sex-specific programming of insulin sensitivity of glucose metabolism[154, 227]. It is possible that greater numbers of progeny and inclusion of the full range of litter sizes, including progeny of the less common extreme litter sizes of one and five pups, might allow us to observe subtler effects of prenatal restriction on insulin sensitivity in female as well as male guinea pigs. In the rat, insulin sensitivity is impaired in male but not female offspring of maternal protein restricted dams[305, 336, 465, 466]. Conversely another study using the same model did report reduced insulin sensitivity in both male and female offspring[305]. One limitation to this study is that because females were age matched, they were not all measured at a specific stage of the oestrous cycle. In rats, glucose tolerance is greatest and plasma insulin concentrations are highest during pro-oestrus (~10%)

and oestrus (~15%) where oestrogen is the major circulating steroid hormone and lower at diestrus where progesterone from the corpus luteum is the dominant steroid hormone [473]. In humans, insulin sensitivity derived from the tolbutamide-modified intravenous glucose tolerance test (IVGTT) is higher in the follicular phase when circulating 17β-oestradiol is high compared to the luteal phase[474], and consistent with this, glucose uptake during hyperglycaemic clamp is also higher in the follicular than luteal phase, despite similar circulating insulin concentrations[475]. Direct measures of whole-body and hepatic insulin sensitivity by HEC in women did not, however, differ between follicular and luteal phases in another study[476]. Glucose tolerance measured by oral glucose tolerance test also did not differ between follicular and luteal phases in healthy, non-obese, regularly menstruating women[477]. Effects of the oestrous cycle on glucose tolerance and insulin sensitivity have not, as yet, been reported in guinea pig, but could potentially have added variability to these outcome measures in the females in this study. Our results do however suggest that sex differences in the impact of prenatal restriction on insulin sensitivity are probably not due to postnatal effects of sex steroids acting indirectly via altered body composition, since fat and lean tissue proportions were not correlated with insulin sensitivity in these animals. Nevertheless, sex steroids may contribute to sex differences in programming and may protect female offspring from insulin resistance, as oestrogen replacement in postmenopausal women increases insulin clearance and sensitivity[270].

4.7 Conclusion

Spontaneous IUGR in the guinea pig provides a model for investigating the causal mechanisms for insulin resistance in muscle and in liver. The associations between spontaneous IUGR and impaired insulin sensitivity of whole body glucose metabolism and utilisation, are sex-specific occurring primarily in males, which is consistent with studies in humans and other animal models. We suggest that further studies in ageing adults are merited, to determine whether the impairments in insulin sensitivity observed in low birth weight young adults, lead to compensatory failure, glucose intolerance and hyperglycaemia with ageing in the guinea pig.

CHAPTER 5
General Discussion

5.1 Summary

Studies in humans have shown that small size at birth is associated with NCD including metabolic diseases such as insulin resistance, T2DM and the metabolic syndrome in later life[2-4]. A fast rate of growth or catch-up growth that often follow times of constraint or nutrient deprivation, as occurs in IUGR, is also an independent risk factor for the development of these NCD later in life[2, 3, 14]. This has brought about the hypothesis of the Developmental Origins of Health and Disease or DOHaD[8-13]. Several small animal models of IUGR have been utilised to investigate the mechanisms underlying the perinatal programming of metabolic disease, however, many models do not undergo catch-up growth immediately after birth or do not induce all the programmed adult outcomes seen in human studies. Therefore, the studies described in this thesis were designed to investigate spontaneous IUGR due to natural variation in litter size in the guinea pig as a potential model of perinatal programming of insulin resistance.

Firstly, I investigated the birth phenotype to determine if guinea pigs of larger litters are disproportionately growth restricted (Chapter 2)[395]. Secondly, we examined their postnatal growth to determine if guinea pigs from larger litter sizes undergo catch-up as both IUGR and neonatal catch-up are independent risks for insulin resistance later in life in humans (Chapter 2)[395]. We also examined if these spontaneous IUGR guinea pigs became hyperphagic and had increased adiposity as young adults (Chapter 2)[395], the latter being a known contributor to T2DM in humans [123, 127]. Both hyperphagia and increased adiposity are early events following IUGR and/or catch-up growth in SGA humans and some experimental animal models of IUGR. I then validated the HEC, the gold standard method of assessing insulin sensitivity with D-[3-

^{3}H]-glucose tracer to determine partitioned glucose metabolism, in unanaesthetised, unrestrained male and female guinea pigs (Chapter 3). Finally, I used an insulin infusion rate of 7.5 mU.kg^{-1}.min^{-1} that was an approximately half maximal insulin doses, determined through a dose response study in Chapter 3, to determine whole body and partitioned insulin sensitivities of male and female guinea pigs of known size at birth and neonatal growth rates (Chapter 4) to determine if these measures of insulin sensitivity are programmed in early life.

In addition to comparisons between groups based on litter size, all outcomes in Chapters 2, 3 and 4 were assessed by multiple linear regression against size at birth and neonatal fractional growth rate to determine independent contributions of pre- and early postnatal growth. These dual approaches allowed me to examine both effects of litter size and how perinatal growth predicts adverse outcome across the entire spectrum. This is important as systematic reviews in humans show the relationship of insulin resistance, the primary defect in syndrome X, with size at birth, is U- or J-shaped[177, 178]. This suggests that both ends of the birth weight spectrum may have risks for the development of metabolic disease via different mechanisms. Others have investigated effects of IUGR in litter-bearing species such as pigs and in sheep selected for variable litter size by comparing outcomes in growth-restricted (runt) or low birth weight littermates to those of littermates in the normal birth weight range [359, 360, 370-373, 478]. These approaches select a more extreme IUGR phenotype, but only allows inclusion of a limited subset of progeny per litter, and does not permit analyses across the birth weight range as used throughout this thesis.

This final chapter summarises the context of the key findings of these studies, discusses the strengths and weakness of the studies conducted as well as the future directions for the guinea pig as a model in further studies into the perinatal programming of metabolic disease.

5.2 Effects of Litter Size, Spontaneous IUGR and Neonatal Growth on Postnatal Phenotype (Chapters 2 and 4)

Pups from larger litter size were not only of low weight at birth, but also exhibited disproportionate growth restriction. This is consistent with multiple births in human[42] and other spontaneous IUGR animal models due to multiple births in sheep[362, 366-369] and pigs[358-360, 479]. Spontaneous IUGR in the guinea pig gave a large natural variation in birth weight where animals from larger litters are thin at birth and have relative sparing of head/brain size and length in relation to soft tissue mass (Section 2.7.3)[395]. This was indicated by a conserved head width across the litter sizes and low birth weight to length ratio an index of thinness at birth (Section 2.7.3)[395]. Disproportionate size at birth is a known risk factor for metabolic disease later in life in human studies[66, 252, 253]. Spontaneous growth restriction in the guinea pig due to larger litter size has a substantial contribution originating from placental insufficiency[391-394]. It is also possible that intrinsic maternal differences that contribute to differences in ovulation and conception rates contribute to differences in progeny phenotypes associated with litter size. Heavier maternal weight rather than age or parity at conception is the greatest predictor for large litter sizes in the guinea pig [397]. Litter size was similarly associated with maternal size in guinea pigs in the present study (Chapter 2)[395], creating the possibility that dams with larger litters also have greater potential food intake and/or maternal nutrient reserves, potentially increasing lactational capacity. Consistent with this hypothesis, in goats, litter size is positively associated with total milk production, nevertheless volume per kid is lower in large litters[480]. We suggest that pups in large litters in the present study may have responded to limited milk availability by earlier commencement of solid feeding on pellets available within the dam's cage, explaining the smaller "check" in their growth trajectories at weaning, compared to guinea pig pups from small litters (Chapter 2)[395]. Altered feed composition in early postnatal life may therefore also have contributed to programming effects associated with litter size. Litter size also increases with maternal age and parity in guinea pig [397], but as all dams were of similar age and first parity in the present study this cannot have contributed to progeny differences here.

Other animal models that specifically induce placental restriction, such as uni- or bi-lateral uterine artery ligation in the rat[275, 276, 280-282], surgical caruncIectomy in the sheep[69, 293], and heat-induced placental restriction[300] also produce offspring with disproportionate IUGR. We expect that in IUGR due to large litter size in guinea pigs, relative placental insufficiency will worsen with advancing gestation as fetal size increases rapidly and demand exceeds placental capacity for nutrient supply[391]. In larger litters although fetal weight gain is slower compared to that of smaller litters in late gestation the gain in length is comparable[481] which gives rise the greater weight to length ratio at birth in larger litters compared to smaller litters in this species (Chapter 2)[395].

In late gestation when nutrient requirement for the fetuses is high, dams carrying litters of three and four pups increased their feed intake in late gestation (40-60 days) compared to mid gestation (20 to 40 days) with no changes in maternal intake over gestation in dams pregnant with one or two pups (Chapter 2)[395]. Paradoxically, litters of five pups had a 15% reduction (ns) in feed intake in late gestation compared to mid-gestation. This suggests that total pup volume in these largest litters interferes with maternal feeding and gastric filling and emptying, and may further reduce nutrient supply to the growing fetuses especially in late gestation. In feed-restricted dams, 60-day-old offspring have reduced liver and muscle weights with heavier adipose tissue deposits compared with progeny of *ad libitum*-fed dams [309], demonstrating programming via maternal feed intake.

Guinea pigs from larger litter sizes were IUGR and underwent neonatal catch-up which persisted after weaning into the juvenile period in both sexes. Catch-up growth is a phenomenon observed in IUGR humans[57-59] and animal models of IUGR in sheep[69, 293, 295], pigs[358], rats[77, 282, 288] and guinea pigs[394] where faster rates of growth occur soon after birth. The IUGR male and female guinea pig offspring were hyperphagic after weaning suggesting that altered appetite regulation is one of the early sequelae of spontaneous IUGR in the guinea pig similar to that

seen in IUGR sheep[293] and following maternal feed restriction in the guinea pig[310] and in the rat[77, 374].

Both neonatal catch-up growth and larger size at birth in males independently predicted increased adiposity, especially in visceral depots, and increased the fat to muscle ratio in adolescence which was not evident in females. Catch-up growth in early life has been associated with increased adiposity in many human studies covered in systematic reviews[66, 252, 253] and this increase in adiposity with catch-up growth is replicated in many IUGR animal models[259, 482, 483].

Small size at birth predicted insulin resistance of whole body glucose uptake independent of neonatal catch-up growth, adiposity and muscle mass in males but not in females in the young adult (Section 4.5.3 and 4.5.4). Many *in vivo* studies in humans have shown, using the HEC, that insulin sensitivity is positively associated with size at birth[187-199]. In the studies described in this thesis programming of insulin resistance due to small size at birth was sex-specific, occurring in males and not in females. This is consistent with reports in young adult humans, where insulin sensitivity correlated positively with birth weight in males but not females[65, 185]. In humans, impaired insulin action or measures of T2DM also correlate positively with size at birth in males; however, outcomes in females are often not reported[187, 194, 195, 201, 229]. In animal models, IUGR in sheep[295, 464], maternal protein restriction in the rat[336, 465, 466] and maternal feed restriction in the guinea pig also program impaired glucose homeostasis in a sex-specific manner, and primarily in males.

Here we have shown for the first time that glucose utilisation was positively correlated with birth weight, independent of neonatal fractional growth rate, adult adiposity and muscle mass in males, while a similar trend was observed for endogenous glucose production. In contrast, no associations of insulin sensitivity of whole body glucose uptake or partitioned glucose metabolism with birth weight were found in young adult females. The impaired insulin sensitivity of glucose utilisation in males predominantly reflects insulin resistance of skeletal

muscle. Skeletal muscle is responsible for approximately 70% of the glucose disposal under insulin stimulation[92] and primarily utilises glucose by storage to glycogen rather than oxidation to ATP with increased insulin stimulus[106]. This relationship is consistent with the majority of *in vivo* and *in vitro* studies in LBW/IUGR humans which show impaired skeletal muscle uptake[189-191, 194-199], and altered expression of components of the insulin signalling pathway in skeletal muscle that would be expected to result in reduced insulin action in this tissue[190, 196, 198, 229, 231-238]. Consistent with this, IUGR in sheep and in rats, altered the expression of insulin signalling proteins such as PKCζ, IRS-1, AKT-2, GSK3a and GYS1 and GLUT4 in skeletal muscle, explaining impairing insulin action in this tissue[231, 292, 294, 328, 338, 353].

The current studies also provided evidence of perinatal programming of hepatic insulin sensitivity. Although human studies[197, 246] and other animal models have shown there is programming of the liver in terms of growth[377], insulin sensitivity[329] and insulin signalling[326, 329, 346, 463, 470, 484] these studies either examined one sex only, did not report differences between males and females, or did not find differences between the sexes. In the young adult guinea pig, perinatal programming of hepatic insulin sensitivity, as indicated by the impaired suppression of endogenous glucose production during HEC was sex-specific. Insulin sensitivity of endogenous glucose production tended to correlate positively with birth weight in males, but not in females, as young adults. In contrast, in females the insulin sensitivity of endogenous glucose production correlated negatively with neonatal fractional growth rate. This impaired responsiveness to insulin in the liver after IUGR or neonatal catch-up growth may also add to the risk of hyperglycaemia and overt T2DM later in life, as observed in both LBW/IUGR humans and animal models of IUGR.

Although we showed that spontaneous IUGR and/or neonatal catch-up growth were associated with increased adiposity, reduced muscle mass or a metabolically adverse high adipose to muscle weight ratio, we did not find consistent associations between insulin resistance of whole body glucose disposal, or peripheral or hepatic glucose action with adult

body composition. This suggests that in male guinea pigs of small size at birth, impaired insulin sensitivity is due to intrinsic alterations in the structure or protein expression within insulin sensitive tissues, particularly skeletal muscle and liver. This is consistent with numerous studies in humans and animal models where IUGR alters, mostly down-regulating, the expression of insulin signalling proteins in liver[197, 246, 326, 329, 346, 463, 470], skeletal muscle[190, 196, 198, 229, 231-238, 278, 294, 485], and in adipose tissue[332, 341, 342, 486, 487] which may ultimately lead to impaired insulin action.

5.3 Sex-Specific Outcomes (Chapters 2, 3 and 4)

The numerous sex-specific programmed outcomes demonstrated in the current thesis highlights the need for both sexes to be assessed in future studies of metabolic programming. It has long been established that there are sex differences in size at birth with males generally having higher birth weights but higher neonatal mortality and morbidity[488]. The very fact that the fetal placenta is derived from the trophoblast cells of the blastocyst which carries the XY or XX sex chromosomes gives the placenta a degree of "sex" and this may induce differing responses to a suboptimal environment [488, 489]

Sex-specific programming by *in utero* events may result from differential adaptations and plasticity at the timing of insult at conception, the placenta and how the fetal and therefore early postnatal growth responds to acute or chronic perturbations of maternal nutrient deprivation, oxidative stress, overnutrition, glucocorticoid and sex steroid supplementation[15, 467, 488-492].

Male embryos are more likely to form at the extremes of the fertility window of the ova than female embryos[490]. This may account for the higher incidence in spontaneous abortions in males suggesting lower viability and notion of the "fragile male embryo" [490]. This may explain the fact that the 5 of 10 surviving guinea pigs in the litters of five were all female in the current study (Chapter 2)[395].

Recent reviews suggest that the placentas of each sex have differing biochemistry which may alter growth *in utero* in sub-optimal conditions[488, 489]. The male fetus continues to grow despite a sub-optimal uterine environment as the male-specific placenta is glucocorticoid resistant and therefore does not allow elevated glucocorticoids, which usually inhibit glucose uptake and fetal growth during maternal or fetal stress, to activate the expression of genes of the glucocorticoid-sensitive IGF axis[488]. Therefore, male fetuses continue to grow and develop during periods of environmental insult *in utero* such as undernutrition and hypoxia through signalling of increased placental growth allowing increased blood flow[489]. During times of sub-optimal nutrient supply, a male fetus ensures short-term survival by increasing placental size and therefore supply of nutrients to the fetus. In later gestation when growth is accelerated the increased placental size of males results in increased competition for nutrients resulting in an asymmetric phenotype at birth[489]. In contrast, the placenta of a female fetus is responsive to glucocorticoid changes in the mother and therefore the IGF-axis, glucose metabolism and growth is inhibited leading to a slowing down of growth in sub-optimal conditions. This ensures in late pregnancy that the placental:fetal ratio is optimal for survival in the female fetus[488]. Nevertheless, in the current study (Chapter 2)[395] we did not see any effect of sex on birth phenotype in the guinea pig. Therefore, the contributions of the uterine environment on the sex-specific programming in this species remains unclear.

Reduced maternal nutrient intake, oxidative stress *in utero* or paternal obesity at the time of conception, can lead to sex-specific alterations in expression of genes postnatally or epigenetic programming, which leads to greater susceptibility to perturbations in males than in female fetuses [15, 467, 488, 489, 491]. This does not alter the overall DNA base sequence itself but can silence the expression of genes in early and in postnatal life. Offspring from obese fathers have hypomethylation, or the loss of suppression of the IGF-2 gene which results in increased growth, insulin secretion and obesity in these offspring[491]. Similarly DNA methylation of corticosteroid 11-beta-dehydrogenase isozyme 2 (11β-HSD-2) gene, the enzyme that

metabolises glucocorticoids from the placenta and therefore the fetus can be silenced by methylation in the placenta[467]. This decreased clearance of glucocorticoids may also act as a mechanism for metabolic dysfunction in later life[467]. There is increasing evidence that DNA methylation accounts for a significant proportion of sex-specific programmed events *in utero* [15, 489]. Based on these studies, approaches to increase fetal nutrition and oxygenation, such as maternal nutrient supplementation, development of anti-epigenetic therapeutics or epigenetic markers to determine fetuses at risk may merit investigation as intervention approaches.

Programming of the HPA axis is also an important mechanism likely contributing to sex-specific disease outcomes in later life. Hypercortisolaemia is associated with increased risks of development of cardio-metabolic disease in later life[493]. In fetal sheep males have a higher cortisol response, despite similar adrenocorticotrophic hormone responses to an acute hypoxic stress compared to females [494]. Consistent with this, in humans, adult males have a higher cortisol release response to psychological stress compared to females [495]. This suggests that sensitivity of the HPA to the environment differs between sexes.

In rats, maternal feed restriction causes IUGR and when *ad libitum* feeding is allowed postnatally rapid catch-up occurs which can be normalised if nutrients are restricted [496, 497]. Further rapid catch-up growth in these studies resulted in impaired metabolic function which was rectified in females but not males when postnatal growth was slowed with undernutrition following IUGR[496, 497]. In the guinea pigs studies reported in Chapter 2, adult adiposity was strongly positively correlated with neonatal FGR in males, but not females. Together, this suggests that catch-up growth in males has more serious consequences in males than females.

Many of the programmed adult outcomes observed in IUGR guinea pigs in the current study, such as adiposity, whole body, peripheral (skeletal muscle: glucose utilisation) and central (liver: endogenous glucose production) insulin resistance, occurred in males but not in females. This is consistent with systematic reviews, where males are at greater risk of developing cardio-metabolic disease than females later in life[176, 264, 265]. Many studies have

assessed insulin sensitivity in males only and shown strong associations between size at birth and insulin resistance later in life[65, 185]. In sheep models of IUGR based on surgical placental restriction or adolescent mothers, male but not female offspring exhibit impaired glucose homeostasis in later life[295, 464]. This male-specific insulin resistance after IUGR in guinea pigs is also consistent with observations following protein-restriction during gestation and lactation in the rat. Protein-restricted male but not female rat offspring were insulin resistant as assessed by IVGTT and HOMA[338]. The insulin-stimulated activation of phosphorylation of AKT was impaired in skeletal muscle and adipose tissue of male offspring of protein-restricted mothers, males compared to controls, however the phosphorylation of AKT did not differ between the protein-restricted or control female progeny[338]. Therefore, in males only both spontaneous IUGR in guinea pigs (Section 4.5.3) and protein-restriction during gestation and lactation in the rat[338], leads to peripheral insulin resistance. In the current thesis, several sex-specific postnatal programmed outcomes were also found such as an association of increased obesity with neonatal catch-up in males, but not females, and reduced muscle mass in IUGR females.

Early postnatal factors that may contribute to altered homeostasis later in life either via prenatal events or as independent factors require further investigation. In females, rapid neonatal growth predicted impaired insulin sensitivity of endogenous glucose production but higher rates of basal and insulin-stimulated rates of glycolysis. In females, rapid neonatal growth also tended to predict raised plasma FFAs (Section 4.5.2), suggesting that circulating concentrations of FFA may contribute to the sex-specific association of neonatal growth with hepatic insulin sensitivity. In males, faster rates of neonatal growth although predicting increased adiposity especially in visceral deposits (Section 2.7.6.2)[395] did not predict higher FFAs or impaired insulin sensitivity of the liver or skeletal muscle (Sections 4.5.2 and 4.5.3). Investigation of the insulin sensitivity of FFAs in male and female guinea pigs following IUGR and neonatal catch-up growth is required to assess this further. However, the combined contributions of the added visceral adiposity due to neonatal catch-up and the effects of aging

on metabolic insulin resistance and overt T2DM needs to be elucidated in this species as in older humans catch-up growth accounted for a large variance in insulin sensitivity in males but not in females[65].

These data demonstrate that spontaneous IUGR and neonatal catch-up growth programmed glucose metabolism in a sex-specific manner. Therefore, could be utilised as a model but to assess the sex-specific mechanisms of insulin resistance and it's cardio-metabolic sequalae known to be programmed by perinatal growth in humans.

5.4 Strengths and Limitations

The spontaneous growth restricted guinea pig is a small animal model of IUGR which has logistical advantages over larger species, such as the sheep and pigs. The guinea pig has smaller litter sizes than rodents but still has a significant spread of mean birth weight across the litter sizes of one (121 ± 2 g) to five (73 ± 6 g) pups and here had a range 57 g to 134 g or a 235% difference between the lightest to the heaviest pup within the total cohort (Chapter 2) allowing for sufficient power to detect programming effects of variable prenatal growth. The guinea pig is also a precocial species, therefore, unlike altricial species like the rat and mouse, most of the development of fat, pancreas, muscle and liver have already occurred at the time of birth when measures of IUGR are assessed[325, 388]. In addition, nutrient gain during lactation impacts the growth and development of organs in species where organogenesis continues through early life. Most of the studies to date in rats have restricted food or protein during both pregnancy and lactation, therefore impacting the early neonatal period which is known to be a contributor to later disease via catch-up growth[12, 65].

We have characterised the HEC in the chronically catheterised, un-anesthetised guinea pig using human insulin (Chapter 3) and demonstrated that the guinea pig responds to human insulin, albeit with lower sensitivity compared to human, rat and mouse, thus validating this method for use in the guinea pig. In the current study, insulin lines were pre-filled with

infusates for at least 20 minutes prior to each clamp commencing, and new lines were used for each clamp. Although it is possible that some insulin may not reach the animal due to adsorption of insulin to the PVC infusion line, this would not affect results, as calculations of insulin sensitivity were based on circulating concentrations of human insulin measured in guinea pig plasma throughout the clamp. Insulin sensitivity of whole body, glucose utilisation and endogenous glucose production was sex-specific such that the associations with birth weight only occurred in males (Chapter 4). In contrast in females, rapid neonatal growth rates predicted impaired endogenous glucose production independent of birth weight. Therefore, the HEC could be used in this model of spontaneous IUGR and subsequent neonatal catch-up for further investigations into the perinatal programming of metabolic disease such as the sequelae of insulin resistance, T2DM and the metabolic syndrome.

One of the limitations of the studies described in Chapter 4 that assessed the associations of size at birth and insulin sensitivity was a smaller range in birth weights in females compared to males. This may not have allowed a significant enough birth weight spectrum to detect changes in insulin sensitivity in this cohort. Additional studies including the full range of birth weights in both sexes are required to confirm the sexual dimorphism in the programming of insulin resistance in this model. In addition, the subset of females in this group did not undergo catch-up growth in the larger litters of four pups. This again may have reduced power at the extremes of birth weight and postnatal growth. Due to the significant restriction in the larger litters, especially that of litters of five, still births were more common than in smaller litters. Therefore, in some of the larger litters the postnatal environment was changed with mothers that had carried larger litter feeding smaller litters during lactation. Whether this impacted our results may need further investigation.

Because insulin sensitivity in the guinea pig using HEC with or without D-[3-^{3}H]-glucose tracer had not been assessed before the studies reported in this thesis, an *a priori* power analysis was not possible to determine required numbers. Low numbers in the analyses of

partitioned glucose metabolism in Chapter 4 (10 males, 8 females) may not have been sufficient to detect with enough power other sex-specific programming effect of prenatal and postnatal growth on partitioned glucose metabolism. Nevertheless, the observed sex-specific outcomes in this cohort were consistent with the fact that impaired insulin sensitivity of whole-body glucose uptake in individuals of low birth weight was only seen in males and not females in the larger cohort (21 males,18 females). Another potential contributing factor to the lack of observed effects of litter size or size at birth on insulin sensitivity in females may be variability due to different oestrous stages when insulin sensitivity was measured. Because outcomes were measured at a set age, females were likely at variable points in their cycles, and there is some evidence from human studies that glucose tolerance and insulin sensitivity vary throughout the cycle by up to 2-fold [474, 475], although these outcomes do not differ with cycle stage in all studies [476, 477], as discussed in Chapter 4.

Guinea pig insulin differs from other mammalian insulins in various key amino acid sequences in the chain which allow dimerisation of the insulin molecule[441, 478]. These changes in the amino acid structure impair the cross reactivity of guinea pig insulin with the insulin receptor of other species and guinea pig insulin also binds with low affinity to its own receptor[446, 498]. Guinea pig insulin has a lower efficacy to reduce blood glucose compared to other mammalian insulins such as bovine insulin[446]. The guinea pig appears to compensate for this through an increased secretion[310, 427, 445-447] and therefore insulin abundance at the receptors on the target tissues or increased tissue abundance of insulin receptors themselves[445]. Despite the unique differences in guinea pig insulin and its receptor and the lower affinity of the guinea pig receptor for most mammalian insulins, this study provides evidence that human insulin can bind and act through the guinea pig insulin receptor. Human insulin increased whole body glucose disposal and glucose utilisation and enhanced the rate of glycolysis when used at a near maximal dose. Human insulin also suppressed endogenous glucose production in the guinea pig, although total suppression of glucose production was not achieved at a near maximal dose.

In conclusion, despite guinea pig insulin and its receptor having unique pharmacokinetics to many other small animal models, human insulin still increased glucose disposal, utilisation and glycolysis and therefore is a viable model for HEC studies using human insulin.

5.5 Future Directions

The findings from this thesis suggest that the spontaneous growth restricted guinea pig due to natural variation in litter size is a potential model for future studies into the metabolic programming of insulin resistance and its sequelae in the development of the metabolic syndrome with ageing. The sex-specific findings highlight the need for all animal model studies to assess programming events in both sexes. The spontaneous growth restricted guinea pig should be further investigated as a model with significant merit to determine the underlying perinatal programmed mechanisms for altered appetite regulation, obesity and insulin resistance in muscle and liver.

Glucose utilisation was impaired in low birth weight male but not female guinea pigs, and endogenous glucose production also tended to be reduced impaired in these IUGR males. Therefore, this would be a good model to investigate insulin signalling pathways in muscle, liver and adipose tissue in both sexes to determine if sex-specific dimorphisms exist in the programming of expression of key proteins in these pathways. Glucose transporters in liver and pancreas, and specifically GLUT4 in skeletal muscle and adipose tissue and its insulin stimulated translocation to the membrane, need to be measured in the spontaneous IUGR guinea pig as this is a feature of many molecular defects associated with small size at birth in humans[190, 198, 231] and other IUGR models[231, 292, 294, 353]. Other signalling proteins such as the insulin receptor, PKCζ, and the p85α and p110β subunits of phosphoinositol 3-kinase, and the phosphorylation of AKT, AMPK and IRS should also be determined for comparisons with the human [190, 196, 198, 229, 231-238] and other animal models of IUGR [294, 328, 331, 338, 346, 347, 351, 352]. This will help determine the likely defects in the signalling pathway so that interventions targeting

these specific pathways can be trialled. Ideally, the effects of 17β-oestradiol and oestrous cycling would need to be controlled as expression and activation of many of these insulin signalling proteins are positively regulated by oestrogen [473, 499].

Once glucose has entered the muscle cell it essentially has two fates: storage as glycogen or the synthesis of ATP with the first pathway that of glycolysis. The accumulation of a tracer of ^{3}H-glucose into glycogen can also be determined in specific muscles of differing fibre composition. Similarly, the accumulation of ^{3}H-glucose and conversion to glycogen in specific zones of the liver could also be examined. This would give detailed measures of glycogenesis in muscles of differing composition to determine if skeletal muscle fibre types or liver zones are programmed differently *in utero*. IUGR lambs due to maternal heat-induced placental restriction have reduced insulin sensitive Type 1 muscle fibres which may predispose to insulin resistance later in life[300] consistent with the muscle fibre changes seen in humans with T2DM and those with metabolic syndrome[302]. In animal models of IUGR the intralobular distribution of periportal enzyme, PEPCK (gluconeogenesis) is increased while perivenous enzyme glucokinase is suppressed following IUGR such that the liver tends to become more proficient at producing rather than storing glucose[347, 500, 501]. In humans, FFA concentrations are known to impair hepatic insulin sensitivity and are a risk factor in the pathogenesis of T2DM[471]. In the current thesis, rapid neonatal growth tended to predict higher fasting FFA in females but not males (Section 4.5.3). The assessment of accumulated fat in the livers of both male and female guinea pigs that were small at birth and/or underwent neonatal catch-up may help determine if hepatic insulin resistance is mediated via increased visceral adiposity, circulating FFAs and/or intrinsic changes to enzyme activity in the lobes of the liver itself. Therefore, the mechanisms by which rapid neonatal or catch-up growth impairs the insulin sensitivity of the liver also requires further investigation.

Studies using 2-deoxy-D-[1-^{14}C]-glucose should also be done in this model to determine the uptake of glucose in specific muscles and adipose tissue. Labelled 2-deoxy-D-

[1-^{14}C]-glucose is a non-metabolisable analogue of glucose that can be taken up by muscle, liver and fat, but cannot be utilised as an energy source[502]. Therefore, a dose of 2-deoxy-D-[1-^{14}C]-glucose during the fasting (no clamp) or insulin stimulated-state during HEC would enable measurement of glucose uptake in specific skeletal muscles, adipose depots and the liver in the basal or insulin-stimulate state. Glucose uptake is one of the rate limiting steps of the signalling pathway so direct measurement with 2-deoxy-D-[1-^{14}C]-glucose will assist in determining location of the site of impairment in these low birth weight males from larger litters.

Cross fostering experiments to control litter size during lactation could help to address any effects of potential differences in feed availability during lactation due to varying litter sizes and stillbirths, as this may also impact on growth rates during the immediate neonatal period. This may be a useful intervention in this model to determine the independent effects of growth *in utero* and postnatal catch-up growth on metabolic outcomes.

5.6 **Interventions**

Intervention studies are the primary goal of future research into the field of DOHaD with increasing placental function a major target[503]. Several interventions in both humans and animal models of IUGR have already investigated possible pharmaceutical agents to increase placental blood flow via increased placental growth, angiogenesis and/or vasodilation to alleviate IUGR. These include the use of maternal IGF-1 and IGF-2 [504-506], VEGF antagonists, Sidenefil [507, 508] and similar PDE5A specific antagonists which block the breakdown of cGMP reducing the clearance of nitric oxide, and L-arginine infusion to increase nitric oxide synthesis [507] in the placenta.

Studies utilising leptin[509], the glucagon-like peptide 1 (GLP-1) analogue exendin-4[510, 511], exercise/training[512], and dietary supplementation (taurine)[513] are examining these

interventions as possible counter-measures for the cardiometabolic consequences of IUGR in animal models.

These interventions could also be examined in the spontaneous growth restricted guinea pig to determine if insulin resistance can be alleviated in this model of IUGR. Studies manipulating individual aspects of the intra-uterine environment suggest that maternal nutrient deprivation or specific nutrient deficiency, oxidative stress with hypoxia (via experimental uteroplacental insufficiency) and exposure to excess glucocorticoids may each impact fetal tissue development directly[467, 514-516] or via epigenetic pathways[15, 467, 488, 489, 491]. Many of these perturbations disrupt placental development especially when severe and chronic, therefore interventions to enhance placental growth and blood flow would be a prime target to elevate programmed metabolic disease. Given sex-specific outcomes after prenatal adversity, efficacy of interventions is likely to differ between sexes and it will be important to include both males and females in such studies.

5.7 Conclusion

In conclusion, spontaneous IUGR due to larger litters in the guinea pig produces a disproportionate fetal growth restriction, which is followed by catch-up growth in the neonatal and juvenile periods in both male and females. Pups from larger litters or with low birth weights exhibit increased feed intake, obesity, and insulin resistance of whole body glucose disposal, utilisation and endogenous glucose production as young adults. This programming due to low birth weight is sex-specific with adverse adult outcomes primarily in male offspring. In contrast, in females, neonatal catch-up growth predicted impaired insulin sensitivity of endogenous glucose production. The primary dysfunction of insulin resistance in young adult guinea pigs that were IUGR and/or underwent rapid growth postnatally may lead to overt T2DM and the metabolic syndrome with ageing. The effects of ageing warrants further investigation in this species.

CHAPTER 6
References

1. World Health Organization. Global status report on noncommunicable diseases 2010: description of the global burden of NCDs, their risk factors and determinants. Geneva: 2011.

2. Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiological Reviews*. 2014;94(4), 1027-1076.

3. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological Reviews*. 2005;85, 571-633.

4. Delisle H. Programming of chronic disease by impaired fetal nutrition: Evidence and implications for policy and intervention strategies. Switzerland: 2001 Contract No.: WHO/NHD/02.3.

5. Hales CN, Barker DJP. The thrifty phenotype hypothesis: Type 2 diabetes. *British Medical Bulletin*. 2001;60, 5-20.

6. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. 1992;35(7), 595-601.

7. Hales CN. Fetal and infant growth and impaired glucose tolerance in adulthood: the "thrifty phenotype" hypothesis revisited. *Acta Paediatrica Supplement*. 1997;422, 73-77.

8. Barker D. *Mothers, Babies and Health in Later Life*, 2 edn, 1998. Churchill Livingstone: Edinburgh.

9. Barker D, Eriksson J, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. *International Journal of Epidemiology*. 2002;31(6), 1235-1239.

10. Ong KK, Dunger DB. Birth weight, infant growth and insulin resistance. *European Journal of Endocrinology*. 2004;151, U131-U139.

11. Ong KK, Dunger DB. Perinatal growth failure: the road to obesity, insulin resistance and cardiovascular disease in adults. *Best Practice and Research Clinical Endocrinology & Metabolism*. 2002;16(2), 191-207.

12. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *British Medical Journal*. 2000;320(7240), 967-971.

13. Silveira VM, Horta BL. Birth weight and metabolic syndrome in adults: meta-analysis. *Revista de Saude Publica*. 2008;42(1), 10-18.

14. Gluckman PD, Buklijas T, Hanson MA. The developmental origins of health and disease (DOHaD) concept: past, present, and future. In "The Epigenome and Developmental Origins of Health and Disease". (ed. Rosenfeld C), 2015; pp. 1-15. Academic Press.

15. Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Carel J-C, Boileau P, Le Bouc Y, Deal CL, Lillycrop K, Scharfmann R, Sheppard A, Skinner M, Szyf M, Waterland RA, Waxman DJ, Whitelaw E, Ong K, Albertsson-Wikland K. Child health, developmental plasticity, and epigenetic programming. *Endocrine Reviews*. 2011;32(2), 159-224.

16. Gluckman PD, Hanson MA. Living with the past: evolution. development, and patterns of disease. *Science*. 2004;305, 1733-1736.

17. Silveira PP, Portella AK, Goldani MZ, Barbieri MA. Developmental origins of health and disease (DOHaD). *Jornal De Pediatria*. 2007;83(6), 494-504.

18. Dickinson H, Moss TJ, Gatford KL, Moritz KM, Akison L, Fullston T, Hryciw DH, Maloney CA, Morris MJ, Wooldridge AL, Schjenken JE, Robertson SA, Waddell BJ, Mark PJ, Wyrwoll CS, Ellery SJ, Thornburg KL, Muhlhausler BS, Morrison JL. A review of fundamental principles for animal models of DOHaD research: an Australian perspective. *Journal of Developmental Origins of Health and Disease*. 2016;7(5), 449-472.

19. Storme L, Luton D, Abdennebi-Najar L, Le Huerou-Luron I. DOHaD: long-term impact of perinatal diseases (IUGR and prematurity). *Medecine Sciences*. 2016;32(1), 74-80.

20. American College of Obstetricians and Gynecologists Committee on Practice Bulletins. Practice Bulletin no. 134: Fetal growth restriction. *Obstetrics and Gynecology*. 2013;121(5), 1122-1133.

21. World Health Organization. Promoting optimal fetal development: report of a technical consultation. Geneva: 2006.

22. Wollmann HA. Intrauterine growth restriction: definition and etiology. *Hormone Research*. 1998;49 Suppl 2, 1-6.

23. Lin C, Santolaya-Forgas J. Current concepts of fetal growth restriction: Part I. Causes, classifcation, and pathophysiology. *Obstetrics and Gynecology*. 1998;92(6), 1044-1055.

24. Lumbers ER, Yu Z, Gibson KJ. The selfish brain and the Barker Hypothesis. *Clinical and Experimental Pharmacology and Physiology*. 2001;28, 942-947.

25. Kramer MS, McLean FH, Olivier M, Willis DM, Usher RH. Body proportionality and head and length 'sparing' in growth-retarded neonates: a critical reappraisal. *Pediatrics*. 1989;84(4), 717-723.

26. Rosenberg A. The IUGR newborn. *Seminars in Perinatology*. 2008;32(3), 219-224.

27. Ashworth A. Effects of intrauterine growth retardation on mortality and morbidity in infants and young children. *Eur J Clin Nutr*. 1998;52 (Suppl 1), S34-S42.

28. Ferro-Luzzi A, Ashworth A, Martorell R, Scrimshaw N. Report of the IDECG Working Group on effects of IUGR on infants, children and adolescents: immunocompetence, mortality, morbidity, body size, body composition, and physical performance. *Eur J Clin Nutr*. 1998;52 (Suppl 1), S97-S99.

29. Levine TA, Grunau RE, McAuliffe FM, Pinnamaneni R, Foran A, Alderdice FA. Early childhood neurodevelopment after intrauterine growth restriction: a systematic review. *Pediatrics*. 2015;135(1), 126-141.

30. Pollack RN, Divon MY. Intrauterine growth retardation: definition, classification, and etiology. *Clinical Obstetrics and Gynecology*. 1992;35, 99-107.

31. Resnik R. Intrauterine growth restriction. *Obstetrics and Gynecology*. 2002;99(3), 490-496.

32. Kramer MS, Olivier M, McLean FH, Dougherty GE, Willis DM, Usher RH. Determinants of fetal growth and body proportionality. *Pediatrics*. 1990;86(1), 18-26.

33. Styne DM. Fetal growth. *Clinics in Perinatology*. 1998;25(4), 917-938.

34. Owens JA, Falconer J, Robinson JS. Effect of restriction of placental growth on oxygen delivery to and consumption by the pregnant uterus and fetus. *Journal of Developmental Physiology*. 1987;9(2), 137-150.

35. Owens JA, Falconer J, Robinson JS. Effects of restriction of placental growth on umbilical and uterine blood flows. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1986;250, R427-R434.

36. Robinson JS, Owens JA. Pathophysiology of intrauterine growth failure. In "Pediatrics and Perinatology: The Scientific Basis". (eds. Gluckman PD, Heymann MA), 1996; pp. 290-297. Arnold: London.

37. Walton A, Hammond J. The maternal effects on growth and conformation in shire horse-shetland pony crosses. *Proceedings of the Royal Society of London Series B, Biological Sciences*. 1938;125(840), 311-335.

38. Roseboom TJ, van der Meulen JH, Ravelli AC, van Montfrans GA, Osmond C, Barker DJ, Bleker OP. Blood pressure in adults after prenatal exposure to famine. *Journal of Hypertension*. 1999;17(3), 325-330.

39. Antonov AN. Children born during the siege of Leningrad in 1942. *Journal of Pediatrics*. 1947;30, 250-259.

40. Strauss RS, Dietz WH. Low maternal weight gain in the second or third trimester increases the risk for intrauterine growth retardation. *The Journal of Nutrition*. 1999;129(5), 988-993.

41. Stanner SA, Bulmer K, Andres C, Lantseva OE, Borodina V, Poteen VV, Yudkin JS. Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. *British Medical Journal*. 1997;315(7119), 1342-1348.

42. Kuno A, Akiyama M, Yanagihara T, Hata T. Comparison of fetal growth in singleton, twin, and triplet pregnancies. *Human Reproduction*. 1999;14(5), 1352-1360.

43. Robinson JS, Moore VM, Owens JA, McMillen IC. Origins of fetal growth restriction. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*. 2000;92, 13-19.

44. Owens J, Owens P, Robinson J. Experimental restriction of fetal growth. In "Fetus and Neonate: Physiology and Clinical Applications". (eds. Hanson MA, Spencer JAD, Rodeck CH), 1995; pp. 139-175. Cambridge University Press: Cambridge.

45. Sankaran S, Kyle PM. Aetiology and pathogenesis of IUGR. *Best Practice & Research Clinical Obstetrics & Gynaecology*. 2009;23(6), 765-777.

46. Pardi G, Marconi AM, Cetin I. Placental-fetal interrelationship in IUGR fetuses - a review. *Placenta*. 2002;23(Supplement A, Trophoblast Research 16), S136-S141.

47. Jones HN, Powell TL, Jansson T. Regulation of placental nutrient transport – a review. *Placenta*. 2007;28(8–9), 763-774.

48. Owens JA, Owens P, Robinson JS. Experimental fetal growth retardation: metabolic and endocrine aspects. In "Advances in Fetal Physiology, Experimental Fetal Growth Retardation". (eds. Gluckman PD, Johnson BM, Nathanielsz PW), 1989; pp. 263-286. Perinatology Press: Ithaca, New York.

49. Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ. Placental efficiency and adaptation: endocrine regulation. *The Journal of Physiology*. 2009;587(Pt 14), 3459-3472.

50. McKeown T, Record RG. The influence of placental size on foetal growth according to sex and order of birth. *The Journal of Endocrinology*. 1953;10, 73-81.

51. Rasmussen S, Irgens LM. Fetal growth and body proportion in preeclampsia. *Obstetrics and Gynecology*. 2003;101(3), 575-583.

52. Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Preeclampsia and fetal growth. *Obstetrics and Gynecology*. 2000;96(6), 950-955.

53. Ros HS, Lichtenstein P, Ekbom A, Cnatitngius S. Tall or short? Twenty years after preeclampsia exposure in utero: comparisons of final height, body mass index, waist-to-hip ratio, and age at menarche among women, exposed and unexposed to preeclampsia during fetal life. *Pediatric Research*. 2001;49(6), 763-769.

54. Sferruzzi-Perri AN, Camm EJ. The programming power of the placenta. *Frontiers in Physiology*. 2016;7(33), doi: 10.3389/fphys.2016.00033.

55. Meas T. Fetal origins of insulin resistance and the metabolic syndrome: A key role for adipose tissue? *Diabetes and Metabolism*. 2010;36(1), 11-20.

56. Luciano A, Bolognani M, Biondani P, Ghizzi C, Zoppi G, Signori E. The influence of maternal passive and light active smoking on intrauterine growth and body composition of the newborn. *Eur J Clin Nutr*. 1998;52(10), 760-763.

57. Karlberg J, Albertsson Wikland K. Growth in full-term small-for-gestational-age infants: from birth to final height. *Pediatric Research*. 1995;38(5), 733-739.

58. Boersma B, Wit JM. Catch-up growth. *Endocrine Reviews*. 1997;18(5), 646-661.

59. Karlberg JP, Albertsson Wikland K, Kwan EY, Lam BC, Low LC. The timing of early postnatal catch-up growth in normal, full-term infants born short for gestational age. *Hormone Research*. 1997;48(Suppl 1), 17-24.

60. Larsen T, Greisen G, Petersen S. Intrauterine growth correlation to postnatal growth - influence of risk factors and complications in pregnancy. *Early Human Development*. 1997;47, 157-165.

61. Stuart CA, McCurry MP, Marino A, South MA, Howell MEA, Layne AS, Ramsey MW, Stone MH. Slow-Twitch Fiber Proportion in Skeletal Muscle Correlates With Insulin Responsiveness *The Journal of Clinical Endocrinology & Metabolism*. 2013;98(5), 2027–2036.

62. Markestad T, Vik T, Ahlsten G, Gebre Medhin M, Skjaerven R, Jacobsen G, Hoffman HJ, Bakketeig LS. Small-for-gestational-age (SGA) infants born at term: growth and development during the first year of life. *Acta Obstetricia et Gynecologica Scandinavica Supplement*. 1997;165, 93-101.

63. Arcangeli T, Thilaganathan B, Hooper R, Khan KS, Bhide A. Neurodevelopmental delay in small babies at term: a systematic review. *Ultrasound in Obstetrics & Gynecology*. 2012;40(3), 267-275.

64. Ong KK. Size at birth, postnatal growth and risk of obesity. *Hormone Research in Paediatrics*. 2006;65(Suppl. 3), 65-69.

65. Parker L, Lamont DW, Unwin N, Pearce MS, Bennett SMA, Dickinson HO, White M, Mathers JC, Albertit KGMM, Craft AW. A lifecourse study of risk for hyperinsulinaemia, dyslipidaemia and obesity (the central metabolic syndrome) at age 49-51 years. *Diabetic Medicine*. 2003;20, 406-415.

66. Kelishadi R, Haghdoost AA, Jamshidi F, Aliramezany M, Moosazadeh M. Low birthweight or rapid catch-up growth: which is more associated with cardiovascular disease and its risk factors in later life? A systematic review and cryptanalysis. *Paediatrics and International Child Health*. 2015;35(2), 110-123.

67. Bazaes RA, Salazar TE, Pittaluga E, Pena V, Alegria A, Iniguez G, Ong KK, Dunger DB, Mericq MV. Glucose and lipid metabolism in small for gestational age infants at 48 hours of age. *Pediatrics*. 2003;111, 804-809.

68. Soto N, Bazaes RA, Pena V, Salazar T, Avila A, Iniguez G, Ong KK, Dunger DB, Mericq MV. Insulin sensitivity and secretion are related to catch-up growth in small-for-gestational-age infants at age 1 year: results from a prospective cohort. *Journal of Clinical Endocrinology and Metabolism*. 2003;88(8), 3645-3650.

69. 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.

70. Davis TA, Reeds PJ. The roles of nutrition, development and hormone sensitivity in the regulation of protein metabolism: an overview. *The Journal of Nutrition*. 1998;128, 340S-341S.

71. Henson FMD, Davenport C, Butler L, Moran I, Shingleton WD, Jeffcott LB, Schofield PN. Effects of insulin and insulin-like growth factors I and II on the growth of equine fetal and neonatal chondrocytes. *Equine Veterinary Journal*. 1997;29(6), 441-447.

72. Dulloo AG, Jacquet J, Seydoux J, Montani JP. The thrifty 'catch-up fat' phenotype: its impact on insulin sensitivity during growth trajectories to obesity and metabolic syndrome. *International Journal of Obesity,*. 2006;30(Supp 4s), S23-S35.

73. Crescenzo R, Samee S, Antic V, Rohner-Jeanrenaud F, Seydoux J, Montani J, Dulloo AG. A role for suppressed thermogenesis favoring catch-up fat in the pathophysiology of catch-up growth. *Diabetes*. 2003;52, 1090-1097.

74. Langley-Evans SC. Intrauterine programming of hypertension by glucocorticoids. *Life Sciences*. 1997;60(15), 1213-1221.

75. Bassett JM, Hanson C. Catecholamines inhibit growth in fetal sheep in the absence of hypoxemia. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1998;274(6), R1536-R1545.

76. Cettour-Rose P, Saunec S, Russell AP, Summermatter S, Mainieri D, Carrillo-Theander C, Montani J, Seydoux J, Rohner-Jeanrenaud F, Dulloo AG. Redistribution of glucose from skeletal muscle to adipose tissue during catch-up fat: A link between catch-up growth and later metabolic syndrome. *Diabetes*. 2005;54, 751-756.

77. Desai M, Gayle D, Han G, Ross MG. Programmed hyperphagia due to reduced anorexigenic mechanisms in intrauterine growth-restricted offspring. *Reproductive Sciences*. 2007;14(4), 329-337.

78. Qasem RJ, Li J, Tang HM, Pontiggia L, D'Mello AP. Maternal protein restriction during pregnancy and lactation alters central leptin signalling, increases food intake, and decreases bone mass in 1year old rat offspring. *Clinical and Experimental Pharmacology and Physiology*. 2016;43(4), 494-502.

79. Ounsted M, Sleigh G. The infant's self-regulation of food intake and weight gain. Difference in metabolic balance after growth constraint or acceleration in utero. *Lancet*. 1975;1(7922), 1393-1397.

80. Dearden L, Ozanne SE. Early life origins of metabolic disease: Developmental programming of hypothalamic pathways controlling energy homeostasis. *Frontiers in Neuroendocrinology*. 2015;39(SI), 3-16.

81. 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. *Journal of Hypertension*. 2000;18(7), 815-831.

82. Ong KK, Petry CJ, Emmett PM, Sandhu MS, Kiess W, Hales CN, Ness AR, Dunger DB, The ALSPAC Study Team. Insulin sensitivity and secretion in normal children related to size at birth, postnatal growth, and plasma insulin-like growth factor-1 levels. *Diabetologia*. 2004;47, 1064-1070.

83. Fagerberg B, Bondjers L, Nilsson P. Low birth weight in combination with catch-up growth predicts the occurrence of the metabolic syndrome in men at middle age: the atherosclerosis and insulin resistance study. *Journal of Internal Medicine*. 2004;256, 254-259.

84. Eriksson JG, Forsen T, Tuomilehto J, Jaddoe VWV, Osmond C, Barker DJP. Effects of size at birth and childhood growth on the insulin resistance syndrome in elderly individuals. *Diabetologia*. 2002;45(3), 342-348.

85. Bavdekar A, Yajnik CS, Fall CH, Bapat S, Pandit AN, Deshpande V, Bhave S, Kellingray SD, Joglekar C. Insulin resistance syndrome in 8-year-old Indian children: small at birth, big at 8 years, or both? *Diabetes*. 1999;48(12), 2422-2429.

86. Yarbrough DE, Barrett Connor E, Kritz Silverstein D, Wingard DL. Birth weight, adult weight, and girth as predictors of the metabolic syndrome in postmenopausal women: the Rancho Bernardo Study. *Diabetes Care*. 1998;21(10), 1652-1658.

87. Rasmussen KM. The "fetal origins" hypothesis: challenges and opportunities for maternal and child nutrition. *Annual Reviews in Nutrition*. 2001;21, 73-95.

88. Ozanne SE, Hales CN. Thrifty yes, Genetic no. *Diabetologia*. 1998;41(4), 485-487.

89. Hales CN, Desai M, Ozanne SE. The thrifty phenotype hypothesis: how does it look after 5 years? *Diabetic Medicine*. 1997;14(3), 189-195.

90. Vanhala MJ, Vanhala PT, Keinanen-Kiukaanniemi SM, Kumpusalo EA, Takala JK. Relative weight gain and obesity as a child predict metabolic syndrome as an adult. *International Journal of Obesity and Related Metabolic Disorders*. 1999;23, 656-659.

91. Bergman RN, Finegood DT, Ader M. Assessment of insulin sensitivity in vivo. *Endocrine Reviews*. 1985;6(1), 45-86.

92. Reaven GM. Pathophysiology of insulin resistance in human disease. *Physiological Reviews*. 1995;75(3), 473-486.

93. Basu A, Caumo A, Bettini F, Gelisio A, Alzaid A, Cobelli C, Rizza RA. Impaired basal glucose effectiveness in NIDDM: contribution of defects in glucose disappearance and production, measured using an optimized minimal model independent protocol. *Diabetes*. 1997;46(3), 421-432.

94. Basu A, Rizza RA. Glucose effectiveness:measurement in diabetic and nondiabetic humans. *Experimental and Clinical Endocrinology & Diabetes*. 2001;109(Supp 2), S157-S165.

95. Tonelli J, Kishore P, Lee DE, Hawkins M. The regulation of glucose effectiveness: how glucose modulates its own production. *Current Opinion in Clinical Nutrition & Metabolic Care*. 2005;8(4), 450-456.

96. Dube S, Errazuriz-Cruzat I, Basu A, Basu R. The forgotten role of glucose effectiveness in the regulation of glucose tolerance. *Current Diabetes Reports*. 2015;15(6), 6.

97. Nordlie RC, J.D. F. Regulation of glucose production by the liver. *Annual Reviews in Nutrition*. 1999;19, 379-406.

98. Fowden AL, Hill DJ. Intra-uterine programming of the endocrine pancreas. *British Medical Bulletin*. 2001;60, 123-142.

99. McCulloch L, van de Bunt M, Braun M, Frayn K, Clark A, Gloyn A. GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus. *Molecular Genetics and Metabolism*. 2011;104(4), 648-653.

100. Prentki M, Vischer S, Clay Glennon M, Regazzi R, Deeney JT, Corkey BE. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *The Journal of Biological Chemistry*. 1992;267(9), 5802-5810.

101. Holst JJ. Enteroglucogon. *Annual Reviews in Physiology*. 1997;59, 257-271.

102. Gilon P, Henquin J. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. *Endocrine Reviews*. 2001;22, 565-604.

103. Niwa T, Matsukawa Y, Senda T, Nimura Y, Hidaka H, Niki I. Acetylcholine activates intracellular movement of insulin granules in pancreatic β-cells via inositol triphosphate-dependent mobilization of intracellular Ca^{2+}. *Diabetes*. 1998;47, 1699-1706.

104. Dunne MJ, Cosgrove KE, Shephard RM, Ammala C. Potassium channels, sulphonlyurea receptors and the control of insulin release. *Trends in Endocrinology and Metabolism*. 1999;10(4), 146-152.

105. de Wet H, Proks P. Molecular action of sulphonylureas on KATP channels: a real partnership between drugs and nucleotides. *Biochemical Society Transactions*. 2015;43(5), 901-907.

106. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose: results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes*. 1981;30(12), 1000-1007.

107. DeFronzo RA. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes*. 1988;37(6), 667-687.

108. Cheatham B, Kahn CR. Insulin action and the insulin signaling network. *Endocrine Reviews*. 1995;16(2), 117-142.

109. Kahn BB. Glucose transport: pivotal step in insulin action. *Diabetes*. 1996;45(11), 1644-1654.

110. Luiken JFP, Dyck DJ, Han X, Tandon NN, Arumugam Y, Glatz JC, Bonen A. Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. *American Journal of Physiology: Endocrinology and Metabolism*. 2002;282, E491-E495.

111. Girard J. Hormonal control of glucose metabolism. In "Diabetes". (ed. Cowett RM), 1995; pp. 1-22. Vevey/Raven Press Ltd: New York.

112. Battezzati A, Caumo A, Martino F, Serent LP, Coppa J, Romito R, Ammatuna M, Regalia E, Matthews DE, Mazzaferro V, Luzi L. Nonhepatic glucose production in

humans. *American Journal of Physiology: Endocrinology and Metabolism*. 2003;286, E129-E135.

113. Meyer C, Dostou JM, Welle SL, Gerich JE. Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *American Journal of Physiology: Endocrinology and Metabolism*. 2002;282, E419-E427.

114. Havrankova J, Roth J, Brownstein M. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature*. 1978;272, 827-829.

115. Hopkins DFC, Williams G. Insulin receptors are widely distributed in human brain and bind human and porcine insulin with equal affinity. *Diabetic Medicine*. 1997;14, 1044-1050.

116. Lowe W, LeRoith D. Insulin receptors from guinea pig liver and brain: structural and functional studies. *Endocrinology*. 1986;118(4), 1669-1677.

117. Kullmann S, Heni M, Hallschmid M, Fritsche A, Preissl H, Haring HU. Brain insulin resistance at the crossroads of metabolic and cognitive disorders in humans. *Physiological Reviews*. 2016;96(4), 1169-1209.

118. Stockhorst U, de Fries D, Steingrueber H, Scherbaum WA. Insulin and the CNS: effects on food intake, memory, and endocrine parameters and the role of intranasal insulin administration in humans. *Physiology & Behavior*. 2004;83, 47-54.

119. Chen M, Woods SC, Porte D. Effect of cerebral intraventricular insulin on pancreatic insulin secretion in the dog. *Diabetes*. 1975;24, 910-914.

120. Chowers I, Lavy S, Halpern L. Effect of insulin administered intracisternally in dogs on the glucose level of the blood and the cerebrospinal fluid. *Experimental Neurology*. 1961;3, 197-205.

121. Obici S, Feng Z, Karkanias G, Baskin DG, Rossetti L. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nature Neuroscience*. 2002;5(6), 566-572.

122. Bergman RN, Ader M, Huecking K, Van Citters G. Accurate assessment of β-cell function: the hyperbolic correction. *Diabetes*. 2002;51(suppl 1), S212-S220.

123. Denti P, Toffolo GM, Cobelli C. The disposition index: from individual to population approach. *American Journal of Physiology: Endocrinology and Metabolism*. 2012;303(5), E576-E586.

124. Bergman R, Ader M. Pathogenesis of non-insulin-dependent diabetes mellitus. In "Diabetes". (ed. Cowett RM), 1995; pp. 99-117. Vevey/Raven Press Ltd: New York.

125. Turner RC, Matthews DR, Clark A, S OR, Rudenski AS, Levy J. Pathogenesis of NIDDM--a disease of deficient insulin secretion. *Bailliere's Clinical Endocrinology and Metabolism*. 1988;2(2), 327-342.

126. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care*. 2009;32(suppl 2), S157.

127. Ritzel RA, Butler AE, Rizza RA, Veldhuis JD, Butler PC. Relationship between β-cell mass and fasting blood glucose concentration in humans. *Diabetes Care*. 2006;29(3), 717-718.

128. Bergman RN, Ader M. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends in Endocrinology and Metabolism*. 2000;11(9), 351-356.

129. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI. Mechanism of free fatty acid-induced insulin resistance in humans. *The Journal of Clinical Investigation*. 1996;97(12), 2859-2865.

130. Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes*. 1997;46(1), 3-10.

131. Shimabukuro MZ, Y. Levi, M. Unger R. Fatty acid-induced β cell apoptosis: a link between obesity and diabetes. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95, 2498-2502.

132. Krebs M, Krssak M, Nowotny P, Weghuber D, Gruber S, Mlynarik V, Bischof M, Stingl H, Furnsinn C, Waldhausl W, Roden M. Free fatty acids inhibit the glucose-stimulated increase of intramuscular glucose-6-phosphate concentration in humans. *Journal of Clinical Endocrinology and Metabolism*. 2001;86(5), 2153-2160.

133. Chu CA, Sherck SM, Igawa K, Sindelar DK, Neal DW, Emshwiller M, Cherrington AD. Effects of free fatty acids on hepatic glycogenolysis and gluconeogenesis in

conscious dogs. *American Journal of Physiology: Endocrinology and Metabolism*. 2002;282, E402-E411.

134. Bessey PQ, Pruitt SK, Wilmore DW. The effect of free fatty acids on insulin-mediated glucose uptake. *The Journal of Surgical Research*. 1984;36(5), 462-469.

135. Van Epps Fung M, Williford J, Wells A, Hardy RW. Fatty acid-induced insulin resistance in adipocytes. *Endocrinology*. 1997;138(10), 4338-4345.

136. Bays H, Mandarino L, DeFronzo RA. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *Journal of Clinical Endocrinology and Metabolism*. 2004;89(2), 463-478.

137. Groop LC, Saloranta C, Shank M, Bonadonna RC, Ferrannini E, DeFronzo RA. The role of free fatty acid metabolism in the pathogenesis of insulin resistance in obesity and noninsulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism*. 1991;72(1), 96-107.

138. Patel TP, Rawal K, Bagchi AK, Akolkar G, Bernardes N, Dias DdS, Gupta S, Singal PK. Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. *Heart Failure Reviews*. 2016;21(1), 11-23.

139. Reaven GM. Insulin resistance in noninsulin-dependent diabetes mellitus. *The American Journal of Medicine*. 1983;74(1), 3-17.

140. Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, Corgnati A, Muggeo M. Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. *Journal of Clinical Endocrinology and Metabolism*. 1989;68(2), 374-378.

141. Codella R, Gabellini D, Luzi L, Caumo A. Validation of the intraperitoneal insulin tolerance test for the measurement of insulin sensitivity in mice. *Diabetologia*. 2015;58(supp 1), S292-S292.

142. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *American Journal of Physiology: Endocrinology and Metabolism*. 1979;237(3), E214-E223.

143. James DE, Jenkins AB, Kraegen EW. Heterogeneity of insulin action in individual muscle in vivo: euglycaemic clamp studies in rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1985;248, E567-E574.

144. Kraegen EW, James DE, Bennett SP, Chisholm DJ. In vivo insulin sensitivity in the rat determined by euglycemic clamp. *American Journal of Physiology: Endocrinology and Metabolism*. 1983;245(1), E1-E7.

145. Koopmans SJ, Mandarino L, DeFronzo RA. Time course of insulin action on tissue-specific intracellular glucose metabolism in normal rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1998;274(4), E642-E650.

146. Ayala JE, Bracy DP, McGuinness OP, Wasserman DH. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes*. 2006;55(2), 390-397.

147. Hughey CC, Wasserman DH, Lee-Young RS, Lantier L. Approach to assessing determinants of glucose homeostasis in the conscious mouse. *Mammalian Genome*. 2014;25(9-10), 522-538.

148. Lee S, Muniyappa R, Yan X, Chen H, Yue LQ, Hong EG, Kim JK, Quon MJ. Comparison between surrogate indexes of insulin sensitivity and resistance and hyperinsulinemic euglycemic clamp estimates in mice. *American Journal of Physiology: Endocrinology and Metabolism*. 2008;294(2), E261-E270.

149. Ortmeyer HK, Sajan MP, Miura A, Kanoh Y, Rivas J, Li Y, Standaert ML, Ryan AS, Bodkin NL, Farese RV, Hansen BC. Insulin signaling and insulin sensitizing in muscle and liver of obese monkeys: peroxisome proliferator-activated receptor gamma agonist improves defective activation of atypical protein kinase C. *Antioxidants & Redox Signaling*. 2010;14(2), 207-219.

150. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, Matsuzawa Y. Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes*. 2001;50(5), 1126-1133.

151. Lee H-W, Muniyappa R, Yan X, Yue LQ, Linden EH, Chen H, Hansen BC, Quon MJ. Comparison between surrogate indexes of insulin sensitivity/resistance and

hyperinsulinemic euglycemic glucose clamps in rhesus monkeys. *Endocrinology*. 2011;152(2), 414-423.

152. Bodkin NL, Metzger BL, Hansen BC. Hepatic glucose production and insulin sensitivity preceding diabetes in monkeys. *American Journal of Physiology: Endocrinology and Metabolism*. 1989;256(5), E676-E681.

153. Tozzo E, Bhat G, Cheon K, Camacho RC. Pioglitazone increases whole body insulin sensitivity in obese, insulin-resistant rhesus monkeys. *PLoS ONE*. 2015;10(5), 14.

154. Choi J, Li C, McDonald TJ, Comuzzie A, Mattern V, Nathanielsz PW. Emergence of insulin resistance in juvenile baboon offspring of mothers exposed to moderate maternal nutrient reduction. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2011;301(3), R757-R762.

155. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *American Journal of Physiology: Endocrinology and Metabolism*. 2008;294(1), E15–E26.

156. Bowe JE, Franklin ZJ, Hauge-Evans AC, King AJ, Persaud SJ, Jones PM. Metabolic phenotyping guildlines: Assessing glucose homeostasis in rodent models. *Journal of Endocrinology*. 2014;222(3), G13–G25.

157. World Health Organization. Definition, diagnosis and classification of diabetes mellitus and its complications. Geneva: World Health Organisation, 1999.

158. Cobelli C, Dalla Man C, Toffolo G, Basu R, Vella A, Rizza R. The oral minimal model method. *Diabetes*. 2014;63(4), 1203-1213.

159. Bergman RN. Toward physiological understanding of glucose tolerance. Minimal-model approach. *Diabetes*. 1989;38(12), 1512-1527.

160. Santos JL, Yevenes I, Cataldo LR, Morales M, Galgani J, Arancibia C, Vega J, Olmos P, Flores M, Valderas JP, Pollak F. Development and assessment of the disposition index based on the oral glucose tolerance test in subjects with different glycaemic status. *Journal of Physiology and Biochemistry*. 2016;72(2), 121-131.

161. Merezak S, Reusens B, Renard A, Goosse K, Kalbe L, Ahn MT, Tamarit-Rogdriguez J, Remacle C. Effect of maternal low-protein diet and taurine on the vulnerability of adult Wistar rat islets to cytokines. *Diabetologia*. 2004;47, 669-675.

162. Siemelink M, Verhoef A, Dormans JAMA, Span PN, Piersma AH. Dietary fatty acid composition during pregnancy and lactation in the rat programs growth and glucose metabolism in the offspring. *Diabetologia*. 2002;45, 1397-1403.

163. Willems E, Koppenol A, De Ketelaere B, Wang YF, Franssens L, Buyse J, Decuypere E, Everaert N. Effects of nutritional programing on growth and metabolism caused by albumen removal in an avian model. *The Journal of Endocrinology*. 2015;225(2), 89-100.

164. Steil GM, Volund A, Kahn SE, Bergman RN. Reduced sample number for calculation of insulin sensitivity and glucose effectiveness from the minimal model: suitability for use in population studies. *Diabetes*. 1993;42(2), 250-256.

165. Bergman RN, Ider YZ, Bowden CR, Cobelli C. Quantitative estimation of insulin sensitivity. *American Journal of Physiology: Endocrinology and Metabolism*. 1979;236(6), E667-E677.

166. Bergman RN, Steil GM, Bradley DC, Watanabe RM. Modeling of insulin action in vivo. *Annual Reviews in Physiology*. 1992;54, 861-883.

167. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7), 412-419.

168. Hill NR, Levy JC, Matthews DR. Expansion of the homeostasis model assessment of β-cell function and insulin resistance to enable clinical trial outcome modeling through the interactive adjustment of physiology and treatment effects: iHOMA2. *Diabetes Care*. 2013;36(8), 2324-2330.

169. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *The Journal of Clinical Endocrinology and Metabolism*. 2000;85(7), 2402-2410.

170. Barker DJP. From birth to death. In "Mothers, Babies and Disease in Later Life". (ed. Barker DJP), 1998; pp. 43-62. Churchill Livingstone: Edinburgh.

171. Barker DJP. Clues from geography. In "Mothers, Babies and Disease in Later Life". (ed. Barker DJP), 1998; pp. 1-12. Churchill Livingstone: Edinburgh.

172. Ravelli ACJ, Vandermeulen JHP, Michels RPJ, Osmond C, Barker DJP, Hales CN, Bleker OP. Glucose tolerance in adults after prenatal exposure to famine. *Lancet*. 1998;351((9097)), 173-177.

173. Keinan-Boker L, Shasha-Lavsky H, Eilat-Zanani S, Edri-Shur A, Shasha SM. Chronic health conditions in Jewish Holocaust survivors born during World War II. *The Israel Medical Association Journal*. 2015;17(4), 206-212.

174. Lanska DJ, Peterson PM. Geographic variation in the decline of stroke mortality in the United States. *Stroke*. 1995;26(7), 1159-1165.

175. Howard G, Evans GW, Pearce K, Howard VJ, Bell RA, Mayer EJ, Burke GL. Is the stroke belt disappearing? An analysis of racial, temporal, and age effects. *Stroke*. 1995;26(7), 1153-1158.

176. Newsome CA, Shiell AW, Fall CHD, Phillips DIW, Shier R, Law CM. Is birth weight related to later glucose and insulin metabolism? - a systematic review. *Diabetic Medicine*. 2003;20, 339-348.

177. Whincup PH, Kaye SJ, Owen CG, Huxley R, Cook DG, Anazawa S, Barrett-Connor E, Bhargava SK, Birgisdottir BSE, Carlsson S, de Rooij SR, Dyck RF, Eriksson JG, Falkner B, Fall C, Forsen T, Grill V, Gudnason V, Hulman S, Hypponen E, Jeffreys M, Lawlor DA, Leon DA, Minami J, Mishra G, Osmond C, Power C, Rich-Edwards JW, Roseboom TJ, Sachdev HS, Syddall H, Thorsdottir I, Vanhala M, Wadsworth M, Yarbrough DE. Birth weight and risk of type 2 diabetes: A systematic review. *Jama-Journal of the American Medical Association*. 2008;300(24), 2886-2897.

178. Harder T, Rodekamp E, Schellong K, Dudenhausen JW, Plagemann A. Birth weight and subsequent risk of type 2 diabetes: a meta-analysis. *American Journal of Epidemiology*. 2007;165(8), 849-857.

179. Boney CM, Verma A, Tucker T, Vohr BR. Metabolic syndrone in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics*. 2005;115(3), e290-e296.

180. Dabelea D, Hanson R, Lindsay R, Pettitt D, Imperatore G, Gabir M, Roumain J, Bennett P, Knowler W. Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. *Diabetes*. 2000;49(12), 2208-2211.

181. Dabelea D, Pettitt DJ, Hanson RL, Imperatore G, Bennett PH, Knowler WC. Birth weight, type 2 diabetes, and insulin resistance in Pima Indian children and young adults. *Diabetes Care*. 1999;22(6), 944-950.

182. Dabelea D, Hanson RL, Bennett PH, Roumain J, Knowler WC, Pettitt DJ. Increasing prevalence of Type II diabetes in American Indian children. *Diabetologia*. 1998;41(8), 904–910.

183. Morley R, Dwyer T, Carlin JB. Studies of twins: What can they tell us about the developmental origins of adult health and disease? In "Early Life Origins of Health and Disease". (eds. Wintour-Coghlan EM, Owens JA), 2006; pp. 29-40. Eurekah.com and Springer Science+Business Media. : Boston, MA.

184. Poulsen P, Vaag AA, Kyvik KO, Moller Jensen D, Beck Nielsen H. Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs. *Diabetologia*. 1997;40(4), 439-446.

185. Flanagan DE, Moore VM, Godsland IF, Cockington RA, Robinson JS, Phillips DIW. Fetal growth and the physiological control of glucose tolerance in adults: a minimal model analysis. *American Journal of Physiology: Endocrinology and Metabolism*. 2000;278, E700-E706.

186. Hofman PL, Cutfield WS, Robinson EM, Bergman RN, Menon RK, Sperling MA, Gluckman PD. Insulin resistance in short children with intrauterine growth retardation. *Journal of Clinical Endocrinology and Metabolism*. 1997;82(2), 402-406.

187. McKeigue PM, Lithell HO, Leon DA. Glucose tolerance and resistance to insulin-stimulated glucose uptake in men aged 70 years in relation to size at birth. *Diabetologia*. 1998;41(10), 1133-1138.

188. Serne EH, Stehouwer CDA, ter Maaten JC, ter Wee PM, Donker JM, Gans ROB. Birth weight relates to blood pressure and microvascular function in normal subjects. *Journal of Hypertension*. 2000;18, 1421-1427.

189. Jaquet D, Gaboriau A, Czernichow P, Levy-Marchal C. Insulin resistance early in adulthood in subjects born with intrauterine growth retardation. *Journal of Clinical Endocrinology and Metabolism*. 2000;85, 1401-1406.

190. Jaquet D, Vidal H, Hankard R, Czernichow P, Levy-Marchal C. Impaired regulation of glucose transporter 4 gene expression in insulin resistance associated with in utero undernutrition. *Journal of Clinical Endocrinology and Metabolism*. 2001;86(7), 3266-3271.

191. Hermann TS, Rask-Madsen C, Ihlemann N, Dominguez H, Jensen CB, Storgaard H, Vaag AA, Kober L, Torp-Pedersen C. Normal insulin-stimulated endothelial function and impaired insulin-stimulated muscle glucose uptake in young adults with low birth weight. *Journal of Clinical Endocrinology and Metabolism*. 2003;88(3), 1252-1257.

192. Veening MA, Van Weissenbruch MM, Delemarre-Van De Waal HA. Glucose tolerance, insulin sensitivity, and insulin secretion in children born small for gestational age. *Journal of Clinical Endocrinology and Metabolism*. 2002;87(10), 4657-4661.

193. Murtaugh MA, Jacobs DR, Moran A, Steinberger J, Sinaiko AR. Relation of birth weight to fasting insulin, insulin resistance, and body size in adolescence. *Diabetes Care*. 2003;26(1), 187-192.

194. Sonne MP, Højbjerre L, Alibegovic AC, Vaag A, Stallknecht B, Dela F. Diminished insulin-mediated forearm blood flow and muscle glucose uptake in young men with low birth weight. *Journal of Vascular Research*. 2010;47(2), 139-147.

195. Jensen CB, Storgaard H, Dela F, Holst JJ, Madsbad S, Vaag AA. Early differential defects of insulin secretion and action in 19-year-old Caucasian men who had low birth weight. *Diabetes*. 2002;51, 1271-1280.

196. Vaag A, Jensen CB, Poulsen P, Brøns C, Pilgaard K, Grunnet L, Vielwerth S, Alibegovic A. Metabolic aspects of insulin resistance in individuals born small for gestational age. *Hormone Research in Paediatrics*. 2006;65(suppl 3), 137-143.

197. Brons C, Jensen CB, Storgaard H, Alibegovic A, Jacobsen S, Nilsson E, Astrup A, Quistorff B, Vaag A. Mitochondrial function in skeletal muscle is normal and unrelated to insulin action in young men born with low birth weight. *Journal of Clinical Endocrinology and Metabolism*. 2008;93(10), 3885-3892.

198. Jensen CB, Martin-Gronert MS, Storgaard H, Madsbad S, Vaag A, Ozanne SE. Altered PI3-Kinase/Akt signalling in skeletal muscle of young men with low birth weight. *PLoS ONE*. 2008;3(11), e3738.

199. Hulman S, Kushner H, Katz S, Falkner B. Can cardiovascular risk be predicted by newborn, childhood, and adolescent body size? An examination of the longitudinal data in urban African Americans. *Journal of Pediatrics*. 1998;132, 90-97.

200. Choi CS, Kim C-H, Lee WJ, Park JY, Hong SK, Lee MG, Park SW, Lee K-U. Association between birth weight and insulin sensitivity in healthy young men in Korea: role of visceral adiposity. *Diabetes Research and Clinical Practice*. 2000;49, 53-59.

201. Lithell HO, McKeigue PM, Berglund L, Mohsen R, Lithell UB, Leon DA. Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. *British Medical Journal*. 1996;312(7028), 406-410.

202. Carlsson S, Persson PG, Alvarsson M, Efendic S, Norman A, Svantrom L, Ostenson CG, Grill V. Low birth weight, family history of diabetes, and glucose intolerance in Swedish middle-aged men. *Diabetes Care*. 1999;22(7), 1043-1047.

203. Fall CHD, Stein CE, Kumaran K, Cox V, Osmond C, Barker DJP, Hales CN. Size at birth, maternal weight, and type 2 diabetes in South India. *Diabetic Medicine*. 1998;15(3), 220-227.

204. Cook JT, Levy JC, Page RC, Shaw JA, Hattersley AT, Turner RC. Association of low birth weight with beta cell function in the adult first degree relatives of non-insulin dependent diabetic subjects. *British Medical Journal*. 1993;306(6873), 302-306.

205. Clausen JO, Borch-Johnsen K, Pedersen O. Relation between birth weight and the insulin sensitivity index in a population sample of 331 young, healthy Caucasians. *American Journal of Epidemiology*. 1997;146(1), 23-31.

206. Levitt NS, Lambert EV, Woods D, Hales CN, Andrew R, Seckl JR. Impaired glucose tolerance and elevated blood pressure in low birth weight, nonobese, young South

African adults: early programming of cortisol axis. *Journal of Clinical Endocrinology and Metabolism*. 2000;85(12), 4611-4618.

207. Bhargava SK, Sachdes HS, Fall CHD, Osmond C, Lakshmy R, Barker DJP, Dey Biswas SK, Ramji S, Prabhakaran D, Reddy KS. Relation of serial changes in childhood body mass index to impaired glucose tolerance in young adulthood. *The New England Journal of Medicine*. 2004;350(9), 865-875.

208. Mohn A, Chiavaroli V, Cerruto M, Blasetti A, Giannini C, Bucciarelli T, Chiarelli F. Increased oxidative stress in prepubertal children born small for gestational age. *The Journal of Clinical Endocrinology & Metabolism*. 2007;92(4), 1372-1378.

209. Jaquet D, Leger J, Chevenne D, Czernichow P, Levy-Marchal C. Intrauterine growth retardation predisposes to insulin resistance but not to hyperandrogenism in young women. *Journal of Clinical Endocrinology and Metabolism*. 1999;84(11), 3945-3949.

210. Phillips DI, Barker DJP, Hales CN, Hirst S, Osmond C. Thinness at birth and insulin resistance in adult life. *Diabetologia*. 1994;37(2), 150-154.

211. Yajnik CS, Fall CH, Vaidya U, Pandit AN, Bavdekar A, Bhat DS, Osmond C, Hales CN, Barker DJ. Fetal growth and glucose and insulin metabolism in four-year-old Indian children. *Diabetic Medicine*. 1995;12(4), 330-336.

212. Veening MA, Van Weissenbruch MM, Heine RJ, Delemarre-Van De Waal HA. Beta-cell capacity and insulin sensitivity in prepubertal children born small for gestational age. *Diabetes*. 2003;52, 1756-1760.

213. Yajnik C, Lubree HG, Rege SS, Naik SS, Deshpande JA, Deshpande SS, Joglekar CV, Yudkin JS. Adiposity and hyperinsulinemia in Indians are present at birth. *Journal of Clinical Endocrinology and Metabolism*. 2002;87(12), 5575-5580.

214. Mericq V, Ong KK, Bazaes R, Peña V, Avila A, Salazar T, Soto N, Iñiguez G, Dunger DB. Longitudinal changes in insulin sensitivity and secretion from birth to age three years in small- and appropriate-for-gestational-age children. *Diabetologia*. 2005;48(12), 2609-2614.

215. Irving RJ, Belton NR, Elton RA, Walker BR. Adult cardiovascular risk factors in premature babies. *Lancet*. 2000;355, 2135-2136.

216. Leeson CPM, Kattenhorn M, Morley R, Lucas A, Deanfield JE. Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life. *Circulation*. 2001;103, 1264-1268.

217. Leger J, Levy Marchal C, Bloch J, Pinet A, Chevenne D, Porquet D, Collin D, Czernichow P. Reduced final height and indications for insulin resistance in 20 year olds born small for gestational age: regional cohort study. *British Medical Journal*. 1997;315(7104), 341-347.

218. Stern MP, Bartley M, Duggirala R, Bradshaw B. Birth weight and the metabolic syndrome: thrifty phenotype or thrifty genotype. *Diabetes/Metabolism Research and Reviews*. 2000;16, 88-93.

219. Shiell AW, Campbell DM, Hall MH, Barker DJP. Diet in late pregnancy and glucose-insulin metabolism of the offspring 40 years later. *British Journal of Obstetrics and Gynaecology*. 2000;107(7), 890-895.

220. Mi J, Law C, Zhang K, Osmond C, Stein CE, Barker DJ. Effects of infant birthweight and maternal body mass index in pregnancy on components of the insulin resistance syndrome in China. *Annals of Internal Medicine*. 2000;132(4), 253-260.

221. Bo S, Cavallo-Perin P, Scaglione L, Ciccone G, Pagano G. Low birthweight and metabolic abnormalities in twins with increased susceptibility to type 2 diabetes mellitus. *Diabetic Medicine*. 2000;17, 365-370.

222. Valdez R, Athens MA, Thompson GH, Bradshaw BS, Stern MP. Birthweight and adult health outcomes in a biethnic population in the USA. *Diabetologia*. 1994;37(6), 624-631.

223. Alvarsson M, Efendic S, Grill VE. Insulin responses to glucose in healthy males are associated with adult height but not with birth weight. *Journal of Internal Medicine*. 1994;236, 275-279.

224. Phillips DI, Hirst S, Clark PM, Hales CN, Osmond C. Fetal growth and insulin secretion in adult life. *Diabetologia*. 1994;37(6), 592-596.

225. Fall CH, Osmond C, Barker DJ, Clark PM, Hales CN, Stirling Y, Meade TW. Fetal and infant growth and cardiovascular risk factors in women. *British Medical Journal*. 1995;310(6977), 428-432.

226. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD. Fetal and infant growth and impaired glucose tolerance at age 64. *British Medical Journal*. 1991;303(6809), 1019-1022.

227. Martyn CN, Hales CN, Barker DJP, Jespersen S. Fetal growth and hyperinsulinaemia in adult life. *Diabetic Medicine*. 1998;15(8), 688-694.

228. Phipps K, Barker DJ, Hales CN, Fall CH, Osmond C, Clark PM. Fetal growth and impaired glucose tolerance in men and women. *Diabetologia*. 1993;36(3), 225-228.

229. Jensen CB, Storgaard H, Madsbad S, Richter EA, Vaag AA. Altered skeletal muscle fiber composition and size precede whole-body insulin resistance in young men with low birth weight. *Journal of Clinical Endocrinology and Metabolism*. 2007;92(4), 1530-1534.

230. Phillips DI. Relation of fetal growth to adult muscle mass and glucose tolerance. *Diabetic Medicine*. 1995;12(8), 686-690.

231. Ozanne SE, Jensen CB, Tingey KL, Storgaard H, Madsbad S, Vaag AA. Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia*. 2005;48, 547-552.

232. Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K, Pak YK, Lee HK. Fetal and early postnatal protein malnutrition cause long-term changes in rat liver and muscle mitochondria. *The Journal of Nutrition*. 2003;133, 3085-3090.

233. Phillips DI, Borthwick AC, Stein C, Taylor R. Fetal growth and insulin resistance in adult life: relationship between glycogen synthase activity in adult skeletal muscle and birthweight. *Diabetic Medicine*. 1996;13(4), 325-329.

234. Taylor DJ, Thompson CH, Kemp GJ, Barnes PR, Sanderson AL, Radda GK, Phillips DI. A relationship between impaired fetal growth and reduced muscle glycolysis revealed by ^{31}P magnetic resonance spectroscopy. *Diabetologia*. 1995;38(10), 1205-1212.

235. Thamotharan M, Shin B, Suddirikku DT, Thamotharan S, Garg M, Devaskar SU. GLUT4 expression and subcellular localisation in the intrauterine growth-restricted adult rat female offspring. *American Journal of Physiology: Endocrinology and Metabolism*. 2005;288, E935-E947.

236. Thompson CH, Sanderson AL, Sandeman D, Stein C, Borthwick A, Radda GK, Phillips DI. Fetal growth and insulin resistance in adult life: role of skeletal muscle morphology. *Clinical Science*. 1997;92(3), 291-296.

237. Ozanne SE, Olsen GS, Hansen LL, Tingey KL, Nave BT, Wang CL, Hartil K, Petry CJ, Buckley AJ, Mosthaf-Seedorf L. Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *The Journal of Endocrinology*. 2003;177, 235-241.

238. Selak MA, Storey BT, Peterside I, Simmons RA. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. *American Journal of Physiology: Endocrinology and Metabolism*. 2003;285, E130-E137.

239. Law CM, Barker DJ, Osmond C, Fall CH, Simmonds SJ. Early growth and abdominal fatness in adult life. *Journal of Epidemiology and Community Health*. 1992;46(3), 184-186.

240. Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J, Rossetti L. Surgical removal of visceral fat reverses hepatic insulin resistance. *Diabetes*. 1999;48(1), 94-98.

241. Barzilai N, Wang J, Massilon D, Vuguin P, Hawkins M, Rossetti L. Leptin selectively decreases visceral adiposity and enhances insulin action. *The Journal of Clinical Investigation*. 1997;100(12), 3105-3110.

242. Barzilai N, Banerjee S, Hawkins M, Chen W, Rossetti L. Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *The Journal of Clinical Investigation*. 1998;101(7), 1353-1361.

243. Gautier JF, Mourier A, de Kerviler E, Tarentola A, Bigard AX, Villette JM, Guezennec CY, Cathelineau G. Evaluation of abdominal fat distribution in non insulin-dependent diabetes mellitus: relationship to insulin resistance. *Journal of Clinical Endocrinology and Metabolism*. 1998;83(4), 1306-1311.

244. Kopelman P, Albon L. Obesity, non-insulin-dependent diabetes mellitus and the metabolic syndrome. *British Medical Bulletin*. 1997;53(2), 322-340.

245. Phillips DI, McLeish R, Osmond C, Hales CN. Fetal growth and insulin resistance in adult life: role of plasma triglyceride and non-esterified fatty acids. *Diabetic Medicine*. 1995;12(9), 796-801.

246. Stefan N, Weyer C, Levy-Marchal C, Stumvoll M, Knowler WC, Tataranni PA, Bogardus C, Pratley RE. Endogenous glucose production, insulin sensitivity, and insulin secretion in normal glucose-tolerant Pima Indians with low birth weight. *Metabolism*. 2004;53(7), 904-911.

247. Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. *Journal of Clinical Endocrinology and Metabolism*. 2004;89, 2595-2600.

248. Bray GA. Medical consequences of obesity. *Journal of Clinical Endocrinology and Metabolism*. 2004;89, 2583-2589.

249. Visscher TLS, Seidell JC. The public health impact of obesity. *Annual Reviews in Public Health*. 2001;22, 355-375.

250. Leong KS, Wilding JP. Obesity and diabetes. *Best Practice and Research Clinical Endocrinology & Metabolism*. 1999;13(2), 221-237.

251. Yajnik CS. The lifecycle effects of nutrition and body size on adult adiposity, diabetes and cardiovascular disease. *Obesity Reviews*. 2002;3, 217-224.

252. Monteiro POA, Victora CG. Rapid growth in infancy and childhood and obesity in later life – a systematic review. *Obesity Reviews*. 2005;6(2), 143-154.

253. Baird J, Fisher D, Lucas P, Kleinjnen J, Roberts H, Law C. Being big or growing fast: systematic review of size and growth in infancy and later obesity. *British Medical Journal*. 2005;331(7522), 929-934.

254. Brands B, Demmelmair H, Koletzko B, for the EarlyNutrition Project. How growth due to infant nutrition influences obesity and later disease risk. *Acta Paediatrica*. 2014;103(6), 578-585.

255. Huang JS, Lee TA, Lu MC. Prenatal programming of childhood overweight and obesity. *Maternal and Child Health Journal*. 2007;11(5), 461-473.

256. Wild SH, Byrne CD. Evidence for fetal programming of obesity with a focus on putative mechanisms. *Nutrition Research Reviews*. 2004;17, 153-162.

257. McMillen IC, Rattanatray L, Duffield JA, Morrison JL, MacLaughlin SM, Gentili S, Muhlhausler BS. The early origins of later obesity: pathways and mechanisms. In "Early

Nutrition Programming and Health Outcomes in Later Life: Obesity and Beyond". (eds. Kolatzko B, Decsi T, Molnar D, DeLaHunty A), 2009; pp. 71-81. Springer: Dordrecht.

258. Rogers I, the EURO-BLCS Study Group. The influence of birthweight and intrauterine environment on adiposity and fat distribution in later life. *International Journal of Obesity and Related Metabolic Disorders*. 2003;27, 755-777.

259. 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. *Pediatric Nephrology*. 2010;25(4), 669-677.

260. Gardner DS, Rhodes P. Developmental origins of obesity: programming of food intake or physical activity? In "Early Nutrition Programming and Health Outcomes in Later Life: Obesity and Beyond". (eds. Koletzko B, Decsi T, Molnár D, de la Hunty A), 2009; pp. 83-93. Springer Netherlands: Dordrecht.

261. Rogers IS, Ness AR, Steer CD, Wells JCK, Emmett PM, Reilly JR, Tobias J, Smith GD. Associations of size at birth and dual-energy X-ray absorptiometry measures of lean and fat mass at 9 to 10 y of age. *American Journal of Clinical Nutrition*. 2006;84(4), 739-747.

262. Kahn HS, Narayan KMV, Williamson DF, Valdez R. Relation of birth weight to lean and fat thigh tissue in young men. *International Journal of Obesity*. 2000;24(6), 667-672.

263. Hocking S, Samocha-Bonet D, Milner KL, Greenfield JR, Chisholm DJ. Adiposity and insulin resistance in humans: the role of the different tissue and cellular lipid depots. *Endocrine Reviews*. 2013;34(4), 463-500.

264. Vos LE, Oren A, Bots LM, Gorissen WHM, Grobbee DE, Uiterwaal CSPM. Birth size and coronary heart disease risk score in young adulthood. the atherosclerosis risk in young adults (ARYA) study. *European Journal of Epidemiology*. 2006;21(1), 33-38.

265. Alexander BT, Dasinger JH, Intapad S. Fetal programming and cardiovascular pathology. *Comprehensive Physiology*. 2015;5(2), 997–1025.

266. Zimmermann E, Gamborg M, Sørensen TIA, Baker JL. Sex differences in the association between birth weight and adult type 2 diabetes. *Diabetes*. 2015;64(12), 4220-4225.

267. Rosenfeld CS. Sex-specific placental responses in fetal development. *Endocrinology*. 2015;156(10), 3422-3434.

268. Eriksson JG, Kajantie E, Osmond C, Thornburg K, Barker DJP. Boys live dangerously in the womb. *American Journal of Human Biology*. 2010;22(3), 330-335.

269. Alexander G. Studies on the placenta of the sheep (Ovis aries l.): placental size. *Journal of Reproduction and Fertility*. 1964;7(3), 289-305.

270. Van Pelt RE, Gozansky WS, Schwartz RS, Kohrt WM. Intravenous estrogens increase insulin clearance and action in postmenopausal women. *American Journal of Physiology: Endocrinology and Metabolism*. 2003;285, E311-E317.

271. Kautzky-Willer A, Harreiter J, Pacini G. Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. *Endocrine Reviews*. 2016;37(3), 278-316.

272. Xita N, Tsatsoulis A. Fetal origins of the metabolic syndrome. *Annals of the New York Academy of Sciences*. 2010;1205(1), 148-155.

273. Magkos F, Wang X, Mittendorfer B. Metabolic actions of insulin in men and women. *Nutrition*. 2010;26(7–8), 686-693.

274. Rincon J, Holmang A, Wahlstrom EO, Lonnroth P, Bjorntorp P, Zierath JR, Wallberg Henriksson H. Mechanisms behind insulin resistance in rat skeletal muscle after oophorectomy and additional testosterone treatment. *Diabetes*. 1996;45(5), 615-621.

275. Gallo LA, Tran M, Moritz KM, Wlodek ME. Developmental programming: Variations in early growth and adult disease. *Clinical and Experimental Pharmacology and Physiology*. 2013;40(11), 795-802.

276. Jansson T, Lambert GW. Effect of intrauterine growth restriction on blood pressure, glucose tolerance and sympathetic nervous activity in the rat at 3-4 months of age. *Journal of Hypertension*. 1999;17(9), 1239-1248.

277. Nüsken K, Dötsch J, Rauh M, Rascher M, Schneider H. Uteroplacental insufficiency after bilateral uterine artery ligation in the rat: Impact on postnatal glucose and lipid metabolism and evidence for metabolic programming of the offspring by sham operation. *Endocrinology*. 2008;149(3), 1056-1063.

278. Lane RH, Chandorkar AK, Flozak AS, Simmons RA. Intrauterine growth retardation alters mitochondrial gene expression and function in fetal and juvenile rat skeletal muscle. *Pediatric Research*. 1998;43(5), 563-570.

279. Lane RH, Crawford SE, Flozak AS, Simmons RA. Localization and quantification of glucose transporters in liver of growth-retarded fetal and neonatal rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1999;276(1 Pt 1), E135-142.

280. Simmons R, Templeton L, Gertz S. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes*. 2001;50(10), 2279-2286.

281. Siebel AL, Guan TC, Owens JA, Westcott KT, Wlodek ME. Uteroplacental insufficiency causes growth restriction which impairs glucose tolerance and insulin secretion, due to compromised pancreatic structure in adult male offspring. *Early Human Development*. 2013;83(1), S75-S76.

282. Sadiq HF, deMello DE, Devaskar SU. The effect of intrauterine growth restriction upon fetal and postnatal hepatic glucose transporter and glucokinase proteins. *Pediatric Research*. 1998;43(1), 91-100.

283. Turner AJ, Trudinger BJ. A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta*. 2009;30(3), 236-240.

284. Sarr O, Thompson JA, Zhao L, Lee TY, Regnault TRH. Low birth weight male guinea pig offspring display increased visceral adiposity in early adulthood. *PLoS ONE*. 2014;9(6), e98433.

285. Herrera EA, Alegria R, Farias M, Diaz-Lopez F, Hernandez C, Uauy R, Regnault TR, Casanello P, Krause BJ. Assessment of in vivo fetal growth and placental vascular function in a novel intrauterine growth restriction model of progressive uterine artery occlusion in guinea pigs. *The Journal of Physiology*. 2016;594(6), 1553-1561.

286. Briscoe TA, Rehn AE, Dieni S, Duncan JR, Wlodek ME, Owens JA, Rees SM. Cardiovascular and renal pathology in the adolescent guinea pig following chronic placental insufficiency. *American Journal of Obstetrics and Gynecology*. 2004;191, 847-855.

287. Styrud J, Eriksson UJ, Grill V, Swenne I. Experimental intrauterine growth retardation in the rat causes a reduction of pancreatic B-cell mass, which persists into adulthood. *Neonatology*. 2005;88(2), 122-128.

288. Heltemes A, Gingery A, Soldner ELB, Bozadjieva N, Jahr KN, Johnson BK, Gilbert JS. Chronic placental ischemia alters amniotic fluid milieu and results in impaired glucose tolerance, insulin resistance and hyperleptinemia in young rats. *Experimental Biology and Medicine*. 2010;235(7), 892-899.

289. Alexander G. Studies on the placenta of the sheep (Ovis aries l.): effect of surgical reduction in the number of caruncles. *Journal of Reproduction and Fertility*. 1964;7(3), 307-322.

290. Robinson JS, Kingston EJ, Jones CT, Thorburn GD. Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *Journal of Developmental Physiology*. 1979;1(5), 379-398.

291. Gatford KL, Mohammad SNB, Harland ML, De Blasio MJ, Fowden AL, Robinson JS, Owens JA. Impaired β-cell function and inadequate compensatory increases in β-cell mass after intrauterine growth restriction in sheep. *Endocrinology*. 2008;149(10), 5118-5127.

292. Muhlhausler BS, Duffield JA, Ozanne SE, Pilgrim C, Turner N, Morrison JL, McMillen IC. The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. *The Journal of Physiology*. 2009;587(17), 4199-4211.

293. 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. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2007;292, 875-886.

294. 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(5), 2142-2151.

295. Wallace JM, Milne JS, Aitken RP, Adam CL. Impact of embryo donor adiposity, birthweight and gender on early postnatal growth, glucose metabolism and body composition in the young lamb. *Reproduction Fertility and Development*. 2014;26(5), 665-681.

296. Wallace JM, Milne JS, Adam CL, Aitken RP. Adverse metabolic phenotype in low-birth-weight lambs and its modification by postnatal nutrition. *British Journal of Nutrition*. 2012;107(4), 510-522.

297. Thureen PJ, Trembler KA, Meschia G, Makowski EL, Wilkening RB. Placental glucose transport in heat-induced fetal growth retardation. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1992;263(3), R578-R585.

298. Limesand SW, Jensen J, Hutton JC, Hay WW. Diminished β-cell replication contributes to reduced β-cell mass in fetal sheep with intrauterine growth restriction. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2005;288(5), R1297-R1305.

299. Limesand SW, Rozance PJ, Smith D, Hay WW. Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction. *American Journal of Physiology: Endocrinology and Metabolism*. 2007;293(6), E1716-E1725.

300. Yates DT, Cadaret CN, Beede KA, Riley HE, Macko AR, Anderson MJ, Camacho LE, Limesand SW. Intrauterine growth-restricted sheep fetuses exhibit smaller hindlimb muscle fibers and lower proportions of insulin-sensitive Type I fibers near term. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2016;310(11), R1020-R1029.

301. Camacho LE, Yates DT, Allen DE, Limesand SW. Reduced insulin-stimulated glucose uptake in skeletal muscle strips from intrauterine growth restricted lambs. *Reproductive Sciences*. 2016;23(Supp 1), S-139.

302. Stuart CA, McCurry MP, Marino A, South MA, Howell ME, Layne AS, Ramsey MW, Stone MH. Slow-twitch fiber proportion in skeletal muscle correlates with insulin responsiveness. *The Journal of Clinical Endocrinology and Metabolism*. 2013;98(5), 2027-2036.

303. Portha B, Kergoat M, Blondel O, Bailbe D. Underfeeding of rat mothers during the first two trimesters of gestation does not alter insulin action and insulin secretion in the progeny. *European Journal of Endocrinology*. 1995;133(4), 475-482.

304. Garofano A, Czernichow P, Bréant B. Effect of ageing on beta-cell mass and function in rats malnourished during the perinatal period. *Diabetologia*. 1999;42(6), 711-718.

305. Holemans K, Verhaeghe J, Dequeker J, Van Assche FA. Insulin sensitivity in adult female rats subjected to malnutrition during the perinatal period. *Journal of the Society for Gynecologic Investigation*. 1996;3(2), 71-77.

306. Agote M, Goya L, Ramos S, Alvarez C, Gavete ML, Pascual-Leone AM, Escriva F. Glucose uptake and glucose transporter proteins in skeletal muscle from undernourished rats. *American Journal of Physiology: Endocrinology and Metabolism*. 2001;281, E1101-E1109.

307. Picarel-Blanchot F, Alvarez C, Bailbe D, Pascual-Leone AM, Portha B. Changes in insulin action and insulin secretion in the rat after dietary restriction early in life: influence of food restriction versus low-protein food restriction. *Metabolism*. 1995;44(12), 1519-1526.

308. Elias AA, Ghaly A, Matushewski B, Regnault TRH, Richardson BS. Maternal nutrient restriction in guinea pigs as an animal model for inducing fetal growth restriction. *Reproductive Sciences*. 2016;23(2), 219-227.

309. Kind KL, Roberts CT, Sohlstrom A, Katsmans AI, Clifton PM, Robinson JS, Owens JA. Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2005;288(1), R119-R126.

310. Kind KL, Simonetta G, Clifton PM, Grant PA, Owens PC, Sohlstrom A, Roberts CT, Robinson JS, Owens JA. Effect of maternal feed restriction on glucose tolerance and insulin secretion in the adult guinea pig. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2003;284, R140-R153.

311. Kind KL, Simonetta G, Clifton PM, Robinson JS, Owens JA. Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Experimental Physiology*. 2002;87(4), 469-477.

312. Kind KL, Clifton PM, Katsman AI, Tsiounis M, Robinson JS, Owens JA. Restricted fetal growth and the response to dietary cholesterol in the guinea pig. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1999;277, R1675-R1682.

313. Dwyer CM, Stickland NC. Does the anatomical location of a muscle affect the influence of undernutrition on muscle fibre number? *Journal of Anatomy*. 1992;181(Pt 2), 373-376.

314. Dwyer CM, Madgwick AJ, Ward SS, Stickland NC. Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. *Reproduction, Fertility, and Development*. 1995;7(5), 1285-1292.

315. Bedi KS, Birzgalis AR, Mahon M, Smart JL, Wareham AC. Early life undernutrition in rats: 1 - Quantitative histology of skeletal muscles from underfed young and refed adult animals. *British Journal of Nutrition*. 1982;47(3), 417-431.

316. Handel SE, Stickland NC. The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight. *Journal of Anatomy*. 1987;152, 107-119.

317. Ward SS, Stickland NC. The effect of undernutrition in the early postnatal period on skeletal muscle tissue. *British Journal of Nutrition*. 1993;69(1), 141-150.

318. Khanal P, Johnsen L, Axel AMD, Hansen PW, Kongsted AH, Lyckegaard NB, Nielsen MO. Long-term impacts of foetal malnutrition followed by early postnatal obesity on fat distribution pattern and metabolic adaptability in adult sheep. *PLoS ONE*. 2016;11(6), e0156700.

319. Khanal P, Axel AMD, Kongsted AH, Husted SV, Johnsen L, Pandey D, Pedersen KL, Birtwistle M, Markussen B, Kadarmideen HN, Nielsen MO. Late gestation under- and overnutrition have differential impacts when combined with a post-natal obesogenic diet on glucose-lactate-insulin adaptations during metabolic challenges in adolescent sheep. *Acta Physiologica*. 2015;213(2), 519-536.

320. Oliver MH, Breier BH, Gluckman PD, Harding JE. Birth weight rather than maternal nutrition influences glucose tolerance, blood pressure, and IGF-1 levels in sheep. *Pediatric Research*. 2002;52(4), 516-524.

321. Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H, Nathanielsz PW. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *Journal of Animal Science*. 2007;85(5), 1285-1294.

322. Vogt B, Muhlbacher C, Carrascosa J, Obermaier Kusser B, Seffer E, Mushack J, Pongratz D, Haring HU. Subcellular distribution of GLUT 4 in the skeletal muscle of lean type 2 (non-insulin-dependent) diabetic patients in the basal state. *Diabetologia*. 1992;35(5), 456-463.

323. Campos PHRF, Silva BAN, Donzele JL, Oliveira RFM, Knol EF. Effects of sow nutrition during gestation on within-litter birth weight variation: a review. *Animal*. 2011;6(5), 797-806.

324. Pond WG, Mersmann HJ, Yen J. Severe feed restriction of pregnant swine and rats: effects on postnatal growth and body composition of progeny. *The Journal of Nutrition*. 1985;115, 179-189.

325. Ashwell M, Purkins L, Cowen T, Day KC. Pre- and postnatal development of adipose tissue at four sites in the guinea pig: effect of maternal diet restriction during the second half of pregnancy. *Annals of Nutrition and Metabolism*. 1987;31(4), 197-210.

326. Lanoue L, Liu XJ, Koski KG. Postnatal profiles of glycogenolysis and gluconeogenesis are modified in rat pups by maternal dietary glucose restriction. *The Journal of Nutrition*. 1999;129(4), 820-827.

327. Hales CN, Desai M, Ozanne SE, Crowther NJ. Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. *Biochemical Society Transactions*. 1996;24(2), 341-350.

328. Ozanne SE, Wang CL, Coleman N, Smith GD. Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1996;271(6 Pt 1), E1128-E1134.

329. Ozanne SE, Smith GD, Tikerpae J, Hales CN. Altered regulation of hepatic glucose output in the male offspring of protein-malnourished rat dams. *American Journal of Physiology: Endocrinology and Metabolism*. 1996;270(4 Pt 1), E559-E564.

330. Langley SC, Browne RF, Jackson AA. Altered glucose tolerance in rats exposed to maternal low protein diets in utero. *Comparative Biochemistry and Physiology*. 1994;109A(2), 223-229.

331. Holness MJ. Impact of early growth retardation on glucoregulatory control and insulin action in mature rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1996;270(6 Pt 1), E946-954.

332. Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, Ozanne SE. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2005;288, 368-373.

333. Petry CJ, Ozanne SE, Wang CL, Hales CN. Early protein restriction and obesity independently induce hypertension in 1-year-old rats. *Clinical Science*. 1997;93(2), 147-152.

334. Petry CJ, Dorling MW, Pawlak DB, Ozanne SE, Hales CN. Diabetes in old male offspring of rat dams fed a reduced protein diet. *International Journal of Experimental Diabetes Research*. 2001;2(2), 139-143.

335. Bertin E, Gangnerau M, Bailbe D, Portha B. Glucose metabolism and beta-cell mass in adult offspring of rats protein and/or energy restricted during the last week of pregnancy. *American Journal of Physiology: Endocrinology and Metabolism*. 1999;277, E11-E17.

336. Zambrano E, Bautista CJ, Deas M, Martinez-Samayoa PM, Gonzalez-Zamorano M, Ledesma H, Morales J, Larrea F, Nathanielsz PW. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. *The Journal of Physiology*. 2006;571(Pt 1), 221-230.

337. Berlez KL, Muller AP, Schweigert ID, Longoni A, Sordi F, de Assis AM, Rotta LN, de Souza DOG, Perry MLS. Gestational and postnatal low protein diet alters insulin sensitivity in female rats. *Experimental Biology and Medicine*. 2009;234(12), 1437-1444.

338. Chamson-Reig A, Thyssen SM, Hill DJ, Arany E. Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by

different mechanisms in males and females. *Experimental Biology and Medicine*. 2009;234(12), 1425-1436.

339. Bertin E, Gangnerau M, Bellon G, Bailbe D, Arbelot de Vacqueur A, Portha B. Development of β-cell mass in fetuses of rats deprived of protein and/or energy in last trimester of pregnancy. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2002;283, R623-R630.

340. Petrik J, Reusens B, Arany E, Remacle C, Coelho C, Hoet JJ, Hill DJ. A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology*. 1999;140(10), 4861-4873.

341. Ozanne SE, Nave BT, Wang CL, Shepherd PR, Prins J, Smith GD. Poor fetal nutrition causes long-term changes in expression of insulin signaling components in adipocytes. *American Journal of Physiology: Endocrinology and Metabolism*. 1997;273(1 Pt 1), E46-E51.

342. Ozanne SE, Dorling MW, Wang CL, Nave BT. Impaired PI 3-kinase activation in adipocytes from early growth-restricted male rats. *American Journal of Physiology: Endocrinology and Metabolism*. 2001;280, E534-E539.

343. Lawrence GM, Jepson MA, Trayer IP, Walker DG. The compartmentation of glycolytic and gluconeogenic enzymes in rat kidney and liver and its significance to renal and hepatic metabolism. *Histochemical Journal*. 1986;18(1), 45-53.

344. Wimmer M, Luttringer C, Colombi M. The development of the acinar heterotopic pattern of phosphoenolpyruvate carboxykinase activity in the newborn rat. *Histochemistry*. 1990;94(1), 55-59.

345. Wimmer M, Luttringer C, Colombi M. Changes in the acinar activity patterns of phosphoenolpyruvate carboxykinase in livers of male and female rats upon feeding a high protein and a high fat diet. *Histochemistry*. 1990;93(3), 257-262.

346. Desai M, Byrne CD, Zhang J, Petry CJ, Lucas A, Hales CN. Programming of hepatic insulin-sensitive enzymes in offspring of rat dams fed a protein-restricted diet. *American Journal of Physiology: Gastrointestinal and Liver Physiology*. 1997;272(5 Pt 1), G1083-G1090.

347. Burns SP, Desai M, Cohen RD, Hales CN, Iles RA, Germain JP, Goinsg TC, Bailey RA. Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *The Journal of Clinical Investigation*. 1997;100(7), 1768-1774.

348. Sadiq HF, Das UG, Tracy TF, Devaskar SU. Intra-uterine growth restriction differentially regulates perinatal brain and skeletal muscle glucose transporters. *Brain Research*. 1999;823(1-2), 96-103.

349. Fisher SJ, Kahn CR. Insulin signaling is required for insulin's direct and indirect action on hepatic glucose production. *The Journal of Clinical Investigation*. 2003;111, 463-468.

350. Willems E, Wang Y, Willemsen H, Lesuisse J, Franssens L, Guo X, Koppenol A, Buyse J, Decuypere E, Everaert N. Partial albumen removal early during embryonic development of layer-type chickens has negative consequences on laying performance in adult life. *Poultry Science*. 2013;92(7), 1905-1915.

351. You JH, Su YM, Zhuang YF, Lin HT, Cheng J, Huang H, Lv GR. Metabolic programming from neonate to adulthood in rats with maternal hypoxia. *International Journal of Clinical and Experimental Medicine*. 2016;9(2), 2921-2928.

352. You J, Zhuang Y, Cheng J, Lv G, Xie J. Juvenile activity levels affect predisposition to metabolic syndrome induced by maternal hypoxia in male offspring rats. *International Journal of Clinical and Experimental Medicine*. 2016;9(2), 1422-1434.

353. Camm EJ, Martin-Gronert MS, Wright NL, Hansell JA, Ozanne SE, Giussani DA. Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. *The FASEB Journal*. 2011;25(1), 420-427.

354. Bauer R, Walter B, Hoppe A, Gaser E, Lampe V, Kauf E, Zwiener U. Body weight distribution and organ size in newborn swine (sus scrofa domestica) — A study describing an animal model for asymmetrical intrauterine growth retardation. *Experimental and Toxicologic Pathology*. 1998;50(1), 59-65.

355. Gonzalez-Bulnes A, Astiz S, Ovilo C, Lopez-Bote CJ, Torres-Rovira L, Barbero A, Ayuso M, Garcia-Contreras C, Vazquez-Gomez M. Developmental Origins of Health

and Disease in swine: implications for animal production and biomedical research. *Theriogenology*. 2016;86(1), 110-119.

356. Du M, Tong J, Zhao J, Underwood KR, Zhu M, Ford SP, Nathanielsz PW. Fetal programming of skeletal muscle development in ruminant animals. *Journal of Animal Science*. 2010;88(13), E51-E60.

357. Wigmore PM, Stickland NC. Muscle development in large and small pig fetuses. *Journal of Anatomy*. 1983;137(2), 235-245.

358. Poore KR, Fowden AL. Insulin sensitivity in juvenile and adult Large White pigs of low and high birthweight. *Diabetologia*. 2004;47(2), 340-348.

359. Poore KR, Forhead AJ, Gardner DS, Giussani DA, Fowden AL. The effects of birth weight on basal cardiovascular function in pigs at 3 months of age. *The Journal of Physiology*. 2002;539(3), 969-978.

360. Poore KR, Fowden AL. The effects of birth weight and postnatal growth patterns on fat depth and plasma leptin concentrations in juvenile and adult pigs. *The Journal of Physiology*. 2004;558(1), 295-304.

361. Clark R. The mode of production of twins in sheep. *Proceedings of the American Society of Animal Nutrition*. 1932;1932(1), 207-209.

362. Rumball CWH, Harding JE, Oliver MH, Bloomfield FH. Effects of twin pregnancy and periconceptional undernutrition on maternal metabolism, fetal growth and glucose–insulin axis function in ovine pregnancy. *The Journal of Physiology*. 2008;586(5), 1399-1411.

363. Rattray PV, Garrett WN, East NE, Hinman N. Growth, development and composition of the ovine conceptus and mammary gland during pregnancy. *Journal of Animal Science*. 1974;38(3), 613-626.

364. van der Linden DS, Sciascia Q, Sales F, McCoard SA. Placental nutrient transport is affected by pregnancy rank in sheep. *Journal of Animal Science*. 2013;91(2), 644-653.

365. Clarke L, Firth K, Heasman L, Juniper DT, Budge H, Stephenson T, Symonds ME. Influence of relative size at birth on growth and glucose homeostasis in twin lambs during juvenile life. *Reproduction, Fertility and Development*. 2000;12(2), 69-73.

366. Hancock SN, Oliver MH, McLean C, Jaquiery AL, Bloomfield FH. Size at birth and adult fat mass in twin sheep are determined in early gestation. *The Journal of Physiology*. 2012;590(5), 1273-1285.

367. Bloomfield FH, M.H. O, Harding JE. Effects of twinning, birth size, and postnatal growth on glucose tolerance and hypothalamic-pituitary-adrenal function in postpubertal sheep. *American Journal of Physiology: Endocrinology and Metabolism*. 2007;292(8), E231–E237.

368. Gardner DS, Tingey KL, Van Bon BWM, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T, Symonds ME. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2005;289, R947-R954.

369. Donovan EL, Buckels EJ, Hancock S, Smeitink D, Oliver MH, Bloomfield FH, Jaquiery AL. Twin conception in sheep leads to impaired insulin sensitivity and sexually dimorphic adipose tissue and skeletal muscle phenotypes in adulthood. *Reproductive Sciences*. 2016;10.1177/1933719116670516.

370. Greenwood PL, Hunt AS, Hermanson JW, Bell AW. Effects of birth weight and postnatal nutrition on neonatal sheep: I Body growth and composition, and some aspects of energetic efficiency. *Journal of Animal Science*. 1998;76, 2354-2367.

371. Greenwood PL, Hunt AS, Hermanson JW, Bell AW. Effects of birth weight and postnatal nutrition on neonatal sheep: II Skeletal muscle growth and development. *Journal of Animal Science*. 2000;78(1), 50-61.

372. Greenwood PL, Hunt AS, Slepetis RM, Finnerty KD, Alston C, Beermann DH, Bell AW. Effects of birth weight and postnatal nutrition on neonatal sheep: III Regulation of energy metabolism. *Journal of Animal Science*. 2002;80(11), 2850-2861.

373. Greenwood PL, Hunt AS, Bell AW. Effects of birth weight and postnatal nutrition on neonatal sheep: IV Organ growth. *Journal of Animal Science*. 2004;82, 422-428.

374. Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *American Journal of Physiology: Endocrinology and Metabolism*. 2000;279(1), E83-E87.

375. Ozanne SE, Hales CN. Poor fetal growth followed by rapid postnatal catch-up growth leads to premature death. *Mechanisms of Ageing and Development*. 2005;126, 852-854.

376. Woods LL, Weeks DA, Rasch R. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney International*. 2004;65, 1339-1348.

377. Desai M, Crowther NJ, Lucas A, Hales CN. Organ-selective growth in the offspring of protein-restricted mothers. *British Journal of Nutrition*. 1996;76(4), 591-603.

378. Bleker OP, Buimer M, van der Post JAM, van der Veen F. Ted G.J. Kloostermon: On intrauterine growth. The significance of prenatal care. Studies on birth weight, placental weight and placental index. *Placenta*. 2006;27(11–12), 1052-1054.

379. Pardi G, Marconi AM, Cetin I. Pathophysiology of intrauterine growth retardation: role of the placenta. *Acta Paediatrica Supplement*. 1997;423, 170-172.

380. Engelbregt MJT, van Weissenbruch MM, Lips P, van Lingen A, Roos JC, Delemarre-van de Waal HA. Body composition and bone measurements in intra-uterine growth retarded and early postnatally undernourished male and female rats at the age of 6 months: comparison with puberty. *Bone*. 2004;34(1), 180-186.

381. Houdijk E, Engelbregt M, Popp-Snijders C, Delemarre-Vd Waal H. Endocrine regulation and extended follow up of longitudinal growth in intrauterine growth-retarded rats. *The Journal of Endocrinology*. 2000;166(3), 599-608.

382. Eriksson JG, Forsen T, Tuomilehto J, Osmond C, Barker DJ. Size at birth, childhood growth and obesity in adult life. *International Journal of Obesity and Related Metabolic Disorders*. 2001;25, 735-740.

383. 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. *British Medical Journal*. 1999;318(7181), 427-431.

384. Law CM, Shiell AW, Newsome CA, Syddall HE, Shinebourne EA, Fayers PM, Martyn CN, de Swiet M. Fetal, infant and childhood growth and adult blood pressure: A longitudinal study from birth to 22 years of age. *Circulation*. 2002;105, 1088-1092.

385. Gilbert JS, Nijland MJ. Sex differences in the developmental origins of hypertension and cardiorenal disease. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2008;295(6), R1941-R1952.

386. Aiken CE, Ozanne SE. Sex differences in developmental programming models. *Reproduction*. 2013;145(1), R1-R13.

387. Dwyer CM, Stickland NC. The effects of maternal undernutrition on the growth and development of the guinea pig placenta. *Journal of Developmental Physiology*. 1992;18(6), 295-302.

388. Wagner JE, Manning P. *The Biology of the Guinea Pig*, 1976. Academic Press Inc: New York.

389. Carter AM. Animal models of human placentation – a review. *Placenta*. 2016;28(Supp A), S41–S47.

390. Widdowson EM. Chemical composition of newly born mammals. *Nature*. 1950;166(4224), 626-628.

391. Ibsen HL. Prenatal growth in guinea-pigs with special reference to environmental factors affecting weight at birth. *Journal of Experimental Zoology*. 1928;51(1), 51-94.

392. Eckstein P, McKeown T, Record RG. Variation in placental weight according to litter size in the guinea-pig. *The Journal of Endocrinology*. 1955;12, 108-114.

393. Saintonge J, Rosso P. Placental blood flow and transfer of nutrient analogs in large, average, and small guinea pig littermates. *Pediatric Research*. 1981;15(2), 152-156.

394. McKeown T, MacMahon B. The influence of litter size and litter order on length of gestation and early postnatal growth in the guinea-pig. *The Journal of Endocrinology*. 1956;13, 195-200.

395. Horton DM, Saint DA, Owens JA, L. KK, Gatford KL. Spontaneous intrauterine growth restriction due to increased litter size in the guinea pig programmes postnatal growth, appetite and adult body composition. *Journal of Developmental Origins of Health and Disease*. 2016;7(5), 548-562.

396. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia*. 1993;36(1), 62-67.

397. Eckstein P, McKeown T. The influence of maternal age, parity and weight on litter size in the guinea-pig. *The Journal of Endocrinology*. 1955;12, 115-119.

398. Engle WA, Lemons JA. Composition of the fetal and maternal guinea pig throughout gestation. *Pediatric Research*. 1986;20(11), 1156-1160.

399. Arbeeny CM, Nordin C, Edelstein D, Stram N, Gibbons N, Eder HA. Hyperlipoproteinemia in spontaneous diabetic guinea pigs. *Metabolism*. 1989;38, 895-900.

400. Vannevel J. Diabetes mellitus in a 3-year-old, intact, female guinea pig. *The Canadian Veterinary Journal*. 1998;39(8), 503.

401. Roberts CT, Sohlstrom A, Kind KL, Earl RA, Khong TY, Robinson JS, Owens PC, Owens JA. Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta*. 2001;22, 177-186.

402. Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC, Owens JA. Altered placental structure induced by maternal food restriction in guinea pigs: a role for circulating IGF-II and IGFBP-2 in the mother? *Placenta*. 2001;22(Supp A), S77-S82.

403. Palliser HK, Kelleher MA, Welsh TN, Zakar T, Hirst JJ. Mechanisms leading to increased risk of preterm birth in growth-restricted guinea pig pregnancies. *Reproductive Sciences*. 2014;21(2), 269-276.

404. Sisk DB. Physiology. In "The Biology of the Guinea Pig". (eds. Wagner J, Manning P), 1976; pp. 63-98. Acedemic Press: New York.

405. Mittelman SD, Van Citters GW, Kirkman EL, Bergman RN. Extreme insulin resistance of the central adipose depot in vivo. *Diabetes*. 2002;51, 755-761.

406. Lim KI, Yang SJ, Kim TN, Yoo HJ, Kang HJ, Song W, Baik SH, Choi DS, Choi KM. The association between the ratio of visceral fat to thigh muscle area and metabolic

syndrome: the Korean Sarcopenic Obesity Study (KSOS). *Clinical Endocrinology*. 2010;73, 588–594.

407. Goy RW, Hoar RM, Young WC. Length of gestation in the guinea pig with data on the frequency and time of abortion and stillbirth. *The Anatomical Record*. 1957;128(4), 747-757.

408. Sohlstrom A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS, Owens JA. Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *American Journal of Physiology: Endocrinology and Metabolism*. 1998;274(3 Pt 1), E410-E416.

409. Davis SR, Mepham TB, Lock KL. Relative importance of pre-partum and post-partum factors in the control of milk yield in the guinea-pig. *Journal of Dairy Research*. 1979;46, 613-621.

410. Mepham TB, Beck NFG. Variation in the yield and composition of milk throughout lactation in the guinea pig (Cavia porcellus). *Comparative Biochemistry and Physiology Part A: Physiology*. 1973;45(2), 273-281.

411. Law CM, Gordon GS, Shiell AW, Barker DJ, Hales CN. Thinness at birth and glucose tolerance in seven-year-old children. *Diabetic Medicine*. 1995;12(1), 24-29.

412. Kelleher MA, Hirst JJ, Palliser HK. Changes in neuroactive steroid concentrations after preterm delivery in the guinea pig. *Reproductive Sciences*. 2013;20(11), 1365-1375.

413. Shin BC, Dai Y, Thamotharan M, Gibson LC, Devaskar SU. Pre- and postnatal calorie restriction perturbs early hypothalamic neuropeptide and energy balance. *Journal of Neuroscience Research*. 2012;90(6), 1169-1182.

414. Tenovuo A, Kero P, Piekkala P, Korvenranta H, Sillanpaa M, Erkkola R. Growth of 519 small for gestational age infants during the first two years of life. *Acta Paediatrica Scandinavica*. 1987;76(4), 636-646.

415. Albertsson Wikland K, Boguszewski M, Karlberg J. Children born small-for-gestational age: postnatal growth and hormonal status. *Hormone Research*. 1998;49(Supp 2), 7-13.

416. Hokken-Koelega AC, De Ridder MA, Lemmen RJ, Den Hartog H, De Muinck Keizer-Schrama SM, Drop SL. Children born small for gestational age: do they catch up? *Pediatric Research*. 1995;38(2), 267-271.

417. Holness MJ, Sugden MC. Antecedent protein restriction exacerbates development of impaired insulin action after high-fat feeding. *American Journal of Physiology: Endocrinology and Metabolism*. 1999;39(1), E85-E93.

418. Benyshek DC, Johnston CS, Martin JF. Post-natal diet determines insulin resistance in fetally malnourished, low birthweight rats (F1) but diet does not modify the insulin resistance of their offspring (F2). *Life Sciences*. 2004;74, 3033-3041.

419. Dobson CC, Mongillo DL, Brien DC, Stepita R, Poklewska-Koziell M, Winterborn A, Holloway AC, Brien JF, Reynolds JN. Chronic prenatal ethanol exposure increases adiposity and disrupts pancreatic morphology in adult guinea pig offspring. *Nutrition and Diabetes*. 2012;2, e57.

420. Kensara OA, Wootton SA, Phillips DI, Patel M, Jackson AA, Elia M, Hertfordshire Study Group. Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen. *American Journal of Clinical Nutrition*. 2005;82, 980-987.

421. Ravelli ACJ, van der Meulen JHP, Osmond C, Barker DJP, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. *American Journal of Clinical Nutrition*. 1999;70, 811-816.

422. Kim JK. Hyperinsulinemic-euglycemic clamp to assess insulin sensitivity in vivo. *Methods in Molecular Biology*. 2009;560, 221-238.

423. Ayala JE, Bracy DP, Malabanan C, James FD, Ansari T, Fueger PT, McGuinness OP, Wasserman DH. Hyperinsulinemic-euglycemic clamps in conscious, unrestrained mice. *Journal of Visualized Experiments*. 2011;57(e3188), doi: 10.3791/3188.

424. Szilvássy J, Sziklai I, Sári R, Németh J, Peitl B, Porszasz R, Lonovics J, Szilvássy Z. Neurogenic insulin resistance in guinea-pigs with cisplatin-induced neuropathy. *European Journal of Pharmacology*. 2006;531(1–3), 217-225.

425. Clark PW, Jenkins AB, Kraegen EW. Pentobarbital reduces basal liver glucose output and its insulin suppression in rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1990;258(4 Pt 1), E701-E707.

426. Poncher M, Heine RJ, Pernet A, Hanning I, Francis AJ, Cook D, Orskov H, Alberti KGMM. A comparison of the artificial pancreas (glucose controlled insulin infusion system) and a manual technique for assessing insulin sensitivity during euglycaemic clamping. *Diabetologia*. 1984;26, 420-425.

427. Gorray KC, Fujimoto WY. "Micro"-insulin radioimmunoassay: measurement of the insulin response during glucose tolerance tests in guinea pigs. *Proceedings of the Society of Experimental Biology and Medicine*. 1980;163(3), 388-392.

428. Koopmans SJ, de Boer SF, Sips HC, Radder JK, Frolich M, Krans HM. Whole body and hepatic insulin action in normal, starved, and diabetic rats. *American Journal of Physiology: Endocrinology and Metabolism*. 1991;260(6 Pt 1), E825-E832.

429. Koopmans SJ, Maassen JA, Radder JK, Frolich M, Krans HM. *In vivo* insulin responsiveness for glucose uptake and production at eu- and hyperglycemic levels in normal and diabetic rats. *Biochimica et Biophysica Acta*. 1992;1115(3), 230-238.

430. Rossetti L, Giaccari A. Relative contribution of glycogen synthesis and glycolysis to insulin-mediated glucose uptake. A dose-response euglycemic clamp study in normal and diabetic rats. *The Journal of Clinical Investigation*. 1990;85(6), 1785-1792.

431. Kruszynska YT, Olefsky JM, Frias JP. Effects of obesity on susceptibility to fatty acid-induced peripheral tissue insulin resistance. *Metabolism*. 2003;52(2), 233-238.

432. DeFronzo RA. Glucose intolerance and aging: evidence for tissue insensitivity to insulin. *Diabetes*. 1979;28(12), 1095-1101.

433. Rizza RA, Mandarino LJ, Gerich JE. Dose-response characteristics for effects of insulin on production and utilisation of glucose in man. *American Journal of Physiology: Endocrinology and Metabolism*. 1981;240, E630-E639.

434. Fukagawa NK, Anderson JW, Hageman G, Young VR, Minaker KL. High-carbohydrate, high-fiber diets increase peripheral insulin sensitivity in healthy-young and old adults. *American Journal of Clinical Nutrition*. 1990;52(3), 524-528.

435. Lang CM, Munger RL, Rapp F. The guinea pig as an animal model of diabetes mellitus. *Laboratory Animal Science*. 1977;27(5 Pt 2), 789-805.

436. Lang CM, Munger BL. Diabetes mellitus in the guinea pig. *Diabetes*. 1976;25(5), 434-443.

437. Chapman MJ. Comparative analysis of mammalian lipoproteins. In "Methods in Enzymology". (eds. Segrest JP, Albers JJ), 1986; pp. 70-144. Acedemic Press Inc.

438. Rebrin K, Steil GM, Mittelman SD, Bergman RN. Causal linkage between insulin suppression of lipolysis and suppression of liver glucose output in dogs. *The Journal of Clinical Investigation*. 1996;98(3), 741-749.

439. Nijs HGT, Radder JK, Poorthuis B, Krans HMJ. Insulin resistance in type-1 (insulin-dependent) diabetes: Dissimilarities for glucose and intermediary metabolites. *Diabetes Research Clinical and Experimental*. 1990;15(1), 15-19.

440. Pagliassotti MJ, Shahrokhi KA, Moscarello M. Involvement of liver and skeletal-muscle in sucrose-induced insulin-resistance: dose-response studies. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1994;266(5), R1637-R1644.

441. Heijboer AC, Donga E, Voshol PJ, Dang Z-C, Havekes LM, Romijn JA, Corssmit EPM. Sixteen hours of fasting differentially affects hepatic and muscle insulin sensitivity in mice. *Journal of Lipid Research*. 2005;46(3), 582-588.

442. Vogt C, Petrides AS. Stimulation of muscle glucose disposal by insulin in humans is a function of the preexisting plasma insulin level. *American Journal of Physiology - Endocrinology And Metabolism*. 1995;268(5), E1031-E1038.

443. Xiao J, Sakaguchi E, Min X, Kawasaki K. Dietary mannitol increased the absorption of calcium and magnesium in rats. *Journal of Animal Physiology and Animal Nutrition*. 2016;100(4), 715-722.

444. Shen J-D, Wei Y, Li Y-J, Qiao J-Y, Li Y-C. Curcumin reverses the depressive-like behavior and insulin resistance induced by chronic mild stress. *Metabolic Brain Disease*. 2017; doi: 10.1007/s11011-017-0017-1, 1-10.

445. Muggeo M, Ginsberg BH, Roth J, Neville DM, De Meyts P, Kahn CR. The insulin receptor in vertebrates is functionally more conserved during evolution than insulin itself. *Endocrinology*. 1979;104(5), 1393-1402.

446. Zimmerman AE, Moule ML, Yip CC. Guinea pig insulin. II. Biological activity. *The Journal of Biological Chemistry*. 1974;249(13), 4026-4029.

447. Pouteau E, Aprikian O, Grenot C, Reynaud D, Pace-Asciak C, Cuilleron CY, Castañeda-Gutiérrez E, Moulin J, Pescia G, Beysen C, Turner S, Macé K. A low α-linolenic intake during early life increases adiposity in the adult guinea pig. *Nutrition and Metabolism*. 2010;7(Artical 8), 1-8.

448. Mullany CJ, Wolfe RR, Burke JF. The fate of a glucose-infusion in fasting and fed guinea-pigs: Glucose-oxidation rates and the distribution of glucose in liver, muscle, and adipose-tissue. *The Journal of Surgical Research*. 1980;29(2), 116-125.

449. Smith D, Rossetti L, Ferrannini E, Johnson CM, Cobelli C, Toffolo G, Katz LD, DeFronzo RA. In vivo glucose metabolism in the awake rat: tracer and insulin clamp studies. *Metabolism*. 1987;36(12), 1167-1174.

450. Jacob R, Barrett E, Plewe G, Fagin KD, Sherwin RS. Acute effects of insulin-like growth factor-I on glucose and amino-acid metabolism in the awake fasted rat. Comparison with insulin. *Journal of Clinical Investigation*. 1989;83(5), 1717-1723.

451. Yki-Järvinen H. Sex and insulin sensitivity. *Metabolism*. 1984;33(11), 1011-1015.

452. Rochlani Y, Pothineni NV, Mehta JL. Metabolic syndrome: does it differ between women and men? *Cardiovascular Drugs and Therapy*. 2015;29(4), 329-338.

453. Frias JP, Macaraeg GB, Ofrecio J, Yu JG, Olefsky JM, Kruszynska YT. Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women. *Diabetes*. 2001;50(6), 1344-1350.

454. Basu R, Dalla Man C, Campioni M, Basu A, Klee G, Toffolo G, Cobelli C, Rizza RA. Effects of age and sex on postprandial glucose metabolism: differences in glucose turnover, insulin secretion, insulin action, and hepatic insulin extraction. *Diabetes*. 2006;55(7), 2001-2014.

455. Varlamov O, Bethea CL, Roberts CT. Sex-specific differences in lipid and glucose metabolism. *Frontiers in Endocrinology*. 2015;5(Artical 241), doi: 10.3389/fendo.2014.00241.

456. Jensen MD, Nielsen S, Gupta N, Basu R, Rizza RA. Insulin clearance is different in men and women. *Metabolism Clinical and Experimental*. 2012;61(4), 525-530.

457. Forsen T, Eriksson JG, Tuomilehto J, Reunanen A, Osmond C, Barker D. The fetal and childhood growth of persons who develop type 2 diabetes. *Annals of Internal Medicine*. 2000;133(3), 176-182.

458. Nilsson C, Larsson B, Jennische E, Eriksson E, Bjorntorp P, York DA, Holmang A. Maternal endotoxemia results in obesity and insulin resistance in adult male offspring. *Endocrinology*. 2001;142, 2622-2630.

459. Agnoux AM, Antignac JP, Simard G, Poupeau G, Darmaun D, Parnet P, Alexandre-Gouabau MC. Time window-dependent effect of perinatal maternal protein restriction on insulin sensitivity and energy substrate oxidation in adult male offspring. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2014;307(2), R184-R197.

460. Escriva F, Kergoat M, Bailbe D, Pascual-Leone AM, Portha B. Increased insulin action in the rat after protein malnutrition early in life. *Diabetologia*. 1991;34(8), 559-564.

461. Escriva F, Rodriguez C, Cacho J, Alvarez C, Portha B, Pascual Leone AM. Glucose utilization and insulin action in adult rats submitted to prolonged food restriction. *American Journal of Physiology: Endocrinology and Metabolism*. 1992;263(1 Pt 1), E1-E7.

462. Krook A, Björnholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Wallberg-Henriksson H, Zierath JR. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes*. 2000;49(2), 284-292.

463. Peterside IE, Selak MA, Simmons RA. Impaired oxidative phosphorylation in hepatic mitochondria in growth-retarded rats. *American Journal of Physiology: Endocrinology and Metabolism*. 2003;285, E1258-E1266.

464. Owens JA, Thavaneswaran P, De Blasio MJ, McMillen IC, Robinson JS, Gatford KL. Sex-specific effects of placental restriction on components of the metabolic syndrome

in young adult sheep. *American Journal of Physiology: Endocrinology and Metabolism*. 2007;292, E1879 –E1889.

465. Sugden MC, Holness MJ. Gender-specific programming of insulin secretion and action. *The Journal of Endocrinology*. 2002;175(3), 757-767.

466. Theys N, Bouckenooghe T, Ahn M-T, Remacle C, Reusens B. Maternal low-protein diet alters pancreatic islet mitochondrial function in a sex-specific manner in the adult rat. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2009;297(5), R1516-R1525.

467. Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. *British Medical Bulletin*. 2001;60(1), 103-121.

468. Holemans K, Aerts L, Van Assche FA. Fetal growth restriction and consequences for the offspring in animal models. *Journal of the Society for Gynecologic Investigations*. 2003;10(7), 392-399.

469. Dunlop K, Cedrone M, Staples JF, Regnault TR. Altered fetal skeletal muscle nutrient metabolism following an adverse in utero environment and the modulation of later life insulin sensitivity. *Nutrients*. 2015;7(2), 1202-1216.

470. Desai M, Byrne CD, Meeran K, Martenz ND, Bloom SR, Hales CN. Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. *American Journal of Physiology: Gastrointestinal and Liver Physiology*. 1997;273(4 Pt 1), G899-G904.

471. Lam TKT, A C, Lewis GF, van der Werve G, Fantus IG, Giacca A. Mechanisms of free fatty acid-induced increase in hepatice glucose production. *American Journal of Physiology: Endocrinology and Metabolism*. 2003;284, E863-E873.

472. Katz A, Sahlin K, Broberg S. Regulation of glucose utilization in human skeletal muscle during moderate dynamic exercise. *American Journal of Physiology: Endocrinology and Metabolism*. 1991;260(3), E411-E415.

473. Bailey CJ, Matty AJ. Glucose tolerance and plasma insulin of the rat in relation to the oestrous cycle and sex hormones. *Hormone and Metabolic Research*. 1972;4(4), 266-270.

474. Pulido JME, Salazar MA. Changes in insulin sensitivity, secretion and glucose effectiveness during menstrual cycle. *Archives of Medical Research*. 1999;30(1), 19-22.

475. Diamond MP, Simonson DC, DeFronzo RA. Menstrual cyclicity has a profound effect on glucose homeostasis. *Fertility and Sterility*. 1989;52(2), 204-208.

476. Diamond MP, Grainger DA, Rossi G, Connolly-Diamond M, Sherwin RS. Counter-regulatory response to hypoglycemia in the follicular and luteal phases of the menstrual cycle. *Fertility and Sterility*. 1993;60(6), 988-993.

477. Bonora E, Zavaroni I, Alpi O, Pezzarossa A, Dall'Aglio E, Coscelli C, Butturini U. Influence of the menstrual cycle on glucose tolerance and insulin secretion. *American Journal of Obstetrics and Gynecology*. 1987;157(1), 140-141.

478. Widdowson EM. Intra-uterine growth retardation in the pig. I. Organ size and cellular development at birth and after growth to maturity. *Biology of the Neonate*. 1971;19(4), 329-340.

479. Poore KR, Fowden AL. The effect of birth weight on hypothalomo-pituitary-adrenal axis function in juvenile and adult pigs. *The Journal of Physiology*. 2003;547(1), 107-116.

480. Hayden TJ, Thomas CR, Forsyth IA. Effect of number of young born (litter size) on milk yield of goats: role for placental lactogen. *J Dairy Sci*. 1979;62(1), 53-63.

481. Thomas CR, Lowy C. The assessment of gestational age and litter size in the guinea pig using a radiographic method. *Placenta*. 1982;3(1), 91-97.

482. Berends LM, Fernandez-Twinn DS, Martin-Gronert MS, Cripps RL, Ozanne SE. Catch-up growth following intra-uterine growth-restriction programmes an insulin-resistant phenotype in adipose tissue. *International Journal of Obesity*. 2013;37(8), 1051-1057.

483. Hales CN, Ozanne SE. The dangerous road of catch-up growth. *The Journal of Physiology*. 2003;547(1), 5-10.

484. Lane RH, Flozak AS, Ogata ES, Bell GI, Simmons RA. Altered hepatic gene expression of enzymes involved in energy metabolism in the growth retarded fetal rat. *Pediatric Research*. 1996;39(3), 390-394.

485. Garvey WT, Maianu L, Zhu JH, Brechtel Hook G, Wallace P, Baron AD. Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *The Journal of Clinical Investigation*. 1998;101(11), 2377-2386.

486. Ozanne SE, Wang CL, Dorling ML, Petry CJ. Dissection of the metabolic actions of insulin in adipocytes from early growth retarded male rats. *The Journal of Endocrinology*. 1999.

487. Shepherd PR, Crowther NJ, Desai M, Hales CN, Ozanne SE. Altered adipocyte properties in the offspring of protein malnourished rats. *The Journal of Nutrition*. 1997;78, 121-129.

488. Clifton VL. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta*. 2010;24, S33–S39.

489. Nugent BM, Bale TL. The omniscient placenta: Metabolic and epigenetic regulation of fetal programming. *Frontiers in Neuroendocrinology*. 2015;39(SI), 28-37.

490. James WH. The variations of human sex ratio at birth with time of conception within the cycle, coital rate around the time of conception, duration of time taken to achieve conception, and duration of gestation: A synthesis. *Journal of Theoretical Biology*. 2008;255(2), 199-204.

491. Dunford AR, Sangster JM. Maternal and paternal periconceptional nutrition as an indicator of offspring metabolic syndrome risk in later life through epigenetic imprinting: A systematic review. *Diabetes Metab Syndr*. 2017;in press(10.1016/j.dsx.2017.04.021).

492. Nugent BM, Tobet SA, Lara HE, Lucion AB, Wilson ME, Recabarren SE, Paredes AH. Hormonal programming across the lifespan. *Hormone and Metabolic Research*. 2012;44(8), 577-586.

493. Rosmond R, Bjorntorp P. The hypothalamic-pituitary-adrenal axis activity as a predictor of cardiovascular disease, type 2 diabetes and stroke. *Journal of Internal Medicine*. 2000;247(2), 188-197.

494. Giussani DA, Fletcher AJW, Gardner DS. Sex differences in the ovine fetal cortisol response to stress. *Pediatric Research*. 2011;69(2), 118-122.

495. Kirschbaum CW, S; Hellhammer, D. Consistent sex differences in cortisol responses to psychological stress. *Psychosomatic Medicine*. 1992;54(6), 648-657.

496. Desai M, Gayle D, Babu J, Ross MG. Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2005;288, R91-R96.

497. Howie GJ, Sloboda DM, Vickers MH. Maternal undernutrition during critical windows of development results in differential and sex-specific effects on postnatal adiposity and related metabolic profiles in adult rat offspring. *British Journal of Nutrition*. 2011;108(2), 298-307.

498. Zimmerman AE, Kells DI, Yip CC. Physical and biological properties of guinea pig insulin. *Biochemical and Biophysical Research Communications*. 1972;46(6), 2127-2133.

499. Narasimhan A, Sampath S, Jayaraman S, Karundevi B. Estradiol favors glucose oxidation in gastrocnemius muscle through modulation of insulin signaling molecules in adult female rats. *Endocrine Research*. 2013;38(4), 251-262.

500. Murphy HC, Regan G, Bogdarina IG, Clark AJL, Iles RA, Cohen RD, Hitman GA, Berry CL, Coade Z, Petry CJ, Burns SP. Fetal programming of perivenous glucose uptake reveals a regulatory mechanism governing hepatic glucose output during refeeding. *Diabetes*. 2003;52.

501. Desai M, Hales CN. Role of fetal and infant growth in programming metabolism in later life. *Biological Reviews of the Cambridge Philosophical Society*. 1997;72(2), 329-348.

502. Wick A, Drury D, Nakada H, Wolfe J. Localization of the primary metabolic block produced by 2-deoxyglucose. *The Journal of Biological Chemistry*. 1957;224(2), 963-969

503. Godfrey KM. The role of the placenta in fetal programming - a review. *Placenta*. 2002;23(supp A), S20-S27.

504. Gluckman PD, Morel PC, Ambler GR, Breier BH, Blair HT, McCutcheon SN. Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of fetal growth by removing maternal constraint. *The Journal of Endocrinology*. 1992;134(1), R1-R3.

505. Roberts CT, Kind KL, Earl RA, Grant PA, Robinson JS, Sohlstrom A, Owens PC, Owens JA. Circulating insulin-like growth factor (IGF)-1 and IGF binding proteins -1 and -3 and placental development in the guinea pig. *Placenta*. 2002;23, 763-770.

506. Sohlstrom A, Fernberg P, Owens JA, Owens PC. Maternal nutrition affects the ability of treatment with IGF-I and IGF-II to increase growth of the placenta and fetus, in guinea pigs. *Growth Hormone & IGF Research*. 2001;11, 392–398.

507. Chen JC, Gong XY, Chen PY, Luo KJ, Zhang XQ. Effect of L-arginine and sildenafil citrate on intrauterine growth restriction fetuses: a meta-analysis. *Bmc Pregnancy and Childbirth*. 2016;16, 7.

508. von Dadelszen P, Dwinnell S, Magee LA, Carleton BC, Gruslin A, Lee B, Lim KI, Liston RM, Miller SP, Rurak D, Sherlock RL, Skoll MA, Wareing MM, Baker PN, for the Research into Advanced Fetal Diagnosis and Therapy (RAFT) Group. Sildenafil citrate therapy for severe early-onset intrauterine growth restriction. *Bjog-an International Journal of Obstetrics and Gynaecology*. 2011;118(5), 624-628.

509. Vickers MH, Sloboda DM. Strategies for reversing the effects of metabolic disorders induced as a consequence of developmental programming. *Frontiers in Physiology*. 2012;3, 11, 10.3389/fphys.2012.00242.

510. Stoffers DA, Desai BM, DeLeon DD, Simmons RA. Neonatal exendin-4 prevents the development of diabetes in the intrauterine growth retarded rat. *Diabetes*. 2003;52(3), 734.

511. Raab EL, Vuguin PM, Stoffers DA, Simmons RA. Neonatal exendin-4 treatment reduces oxidative stress and prevents hepatic insulin resistance in intrauterine growth-retarded rats. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 2009;297(6), R1785-1794.

512. Gatford KL, Kaur G, Falcão-Tebas F, Wadley GD, Wlodek ME, Laker RC, Ebeling PR, McConell GK. Exercise as an intervention to improve metabolic outcomes after intrauterine growth restriction. *American Journal of Physiology: Endocrinology and Metabolism*. 2014;306, E999 –E1012.

513. Hultman K, Alexanderson C, Manneras L, Sandberg M, Holmang A, Jansson T. Maternal taurine supplementation in the late pregnant rat stimulates postnatal growth

and induces obesity and insulin resistance in adult offspring. *The Journal of Physiology*. 2007;579(3), 823-833.

514. Chavatte-Palmer P, Tarrade A, Rousseau-Ralliard D. Diet before and during pregnancy and offspring health: The importance of animal models and what can be learned from them? *International Journal of Environmental Research and Public Health*. 2016;13(6), 586-600.

515. Fernandez-Twinn DS, Ozanne SE. Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. *Physiology & Behavior*. 2006;88(3), 234-243.

516. Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Hammer CJ, Maddock Carlin KR, Grazul-Bilska AT, Redmer DA. Developmental programming: The concept, large animal models, and the key role of uteroplacental vascular development. *Journal of Animal Science*. 2010;88(13), E61-E72.