Metabolic Phenotyping of Young Adults and Mice

Born Through In Vitro Fertilization (IVF)

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This thesis is dedicated to my parents and my wife for their love.

My dream will not come true without you.
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Abstract

In vitro fertilisation (IVF) has been widely used to treat infertility since 1978. Worldwide, there are over 5 million children who have been born following assisted reproduction, mostly by IVF. However, the long term health implications of IVF are unknown. This thesis focuses on the metabolic risks of IVF in adult humans and mouse offspring.

A suboptimal in vivo environment during pregnancy, and the early postnatal period, predisposes offspring to chronic diseases later in life. Preimplantation embryos are also sensitive to adverse environmental insults in vivo or in vitro. Emerging evidence suggests that IVF children may be at an increased risk of developing metabolic and cardiovascular diseases. However, it is unclear if increased risk is related to the underlying genetics of the parents, environmental factors, or the treatment procedures per se which include both ovarian stimulation and embryo culture.

This is the first study to show that IVF adult humans were insulin resistant, by using gold standard assessment hyperinsulinemic-euglycemic clamp, compared to BMI and aged matched naturally conceived individuals after 3 days of a baseline energy balanced diet (30% fat), and that they tended to be more susceptible to the metabolic consequences of 3 days of high-fat overfeeding (+1250 kcal/day, 45% fat) as evidenced by a greater increase in systolic blood pressure.

To separate out potential genetic and environmental confounders as well as the effects of ovarian stimulation versus embryo culture, we developed an IVF mouse model using inbred C57BL/6J mice. Here, we examined glucose metabolism in adult offspring conceived by natural conception (NC), by ovarian stimulation alone (OS) or by IVF, and then fed a chow or high-fat diet (60% fat) for 8 weeks. Our data suggest it is the process of
IVF itself that contributes to impaired glucose metabolism in the adult mouse, which was more prominent in males. Moreover, we show that ovarian stimulation impairs fetal growth, and also results in glucose intolerance in offspring, which was unmasked by a high-fat diet in adult females. This study suggests that ovarian stimulation alone and IVF may program distinct metabolic effects in the offspring, but that high fat diet may be required to uncover these differences.

Our data shows that the preimplantation period is a critical stage for development and later adult health. The mechanisms underlying these differences are unclear, but may involve epigenetic modifications and/or changes in mitochondrial numbers and function. We initially examined whether altered DNA methylation and expression of key genes PPARGC1A and IGF2 occurs in peripheral insulin sensitive tissues of morbidly obese individuals with or without type 2 diabetes. Our data showed that obese patients with and without type 2 diabetes displayed tissue specific DNA methylation of PPARGC1A and IGF2, highlighting the importance of measuring individual tissues in this response in humans and controlling for adiposity. Whether these alternations are evident in IVF conceived adults requires further study.

In conclusion, this study highlights an increased risk of developing type 2 diabetes and cardiovascular disease in IVF offspring later in life in an obesogenic environment.
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in 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.

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Name: Miaoxin Chen

Signed:                                                           Date:   11th April 2014
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**Conference Proceedings**

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Miaoxin Chen, Junli Zhao, Campbell Thompson, Gary Wittert, Robert Norman, Leonie Heilbronn. Metabolic Phenotyping of Young Adults born through In Vitro Fertilisation (IVF). The Second World Congress in Reproductive Biology (WCRB) and annual meeting of Society for Reproductive Biology (SRB), Cairns, Queensland, Australia (2011 Oct). Poster presentation.
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# List of Abbreviations

AKT: Protein Kinase B  
ART: Assisted reproduction technology  
ATP: Adenosine Triphosphate  
AUC: Area under the curve  
B2M: Beta-2 microglobulin  
BMI: Body mass index  
BWS: Beckwith-Wiedemann syndrome  
CEBPα: CCAAT-enhancer-binding protein alpha  
COC: Cumulus–oocyte complex  
COX7A1: Cytochrome c oxidase (COX) subunit 7A, polypeptide 1  
CpG: Cytosine-phosphate-guanine  
Cpt1a: Carnitine palmitoyltransferase 1a  
DEPC: Diethylpyrocarbonate  
DMRs: Differentially methylated regions  
DNA: Deoxyribonucleic acid  
Dnmts: DNA methyltransferases  
eCG: Equine chorionic gonadotropin  
EDTA: Ethylenediaminetetraacetic acid disodium salt dihydrate  
EGTA: Ethylene glycol-bis (2-aminoethylether) –N,N,N’,N’-tetraacetic acid  
ER: Endoplasmic reticulum  
ERK: Extracellular signal-regulated kinase  
FAT/CD36: Fatty acid translocase/Cluster of Differentiation 36  
FFAs: Free fatty acids
FFM: Fat free mass
FOXO1: Forkhead box O1
FTO: Fat mass and obesity associated
Gapdh: Glyceraldehyde-3-phosphate dehydrogenase
Gck: Glucokinase
GIR: Glucose infusion rate
GNASAS: Guanine Nucleotide Binding Protein Antisense RNA
GnRH: Gonadotrophin releasing hormone
G6pc: Glucose-6-phosphatase catalytic subunit
hCG: human chorionic gonadotropin
HDL: Higher high-density lipoprotein
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD: High fat diet
HOMA-IR: Homeostasis model of assessment - insulin resistance
Hprt: Hypoxanthine phosphoribosyltransferase
ICM: Inner cell mass
ICSI: Intracytoplasmic sperm injection
IGF: Insulin-like growth factor
IGF2: Insulin-like growth factor 2
IKK-β: IkB kinase-β
IL-6: Interleukin 6
IL10: Interleukin-10
INSIGF: Insulin – IGF
IR: Insulin resistance
IRR: Insulin receptor-related receptor
IRS: Insulin receptor substrate
i.p.: Intraperitoneal
IPGTT: Intraperitoneal glucose tolerance test
IPITT: Intraperitoneal insulin tolerance test
IVF: In vitro fertilization
i.v.: Intravenous
IVGTT: Intravenous glucose tolerance test
JNK-1: Jun kinase-1
MEG3: Maternally Expressed 3 (Non-Protein Coding)
MEST: Mesoderm-specific transcript
MtDNA: Mitochondrial DNA
mTOR: Mammalian target of rapamycin
NaF: Sodium fluoride
NaPPi: Sodium pyrophosphate tetrabasic decahydrate
NC: Natural conception
Ndufb5: NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5
NGAL: Neutrophil gelatinase-associated lipocalin
NNAT: Neuronatin
nPCKCs: Novel protein kinase Cs
OS: Ovarian stimulation
OXPHOS: Oxidative phosphorylation
PAI-1: Plasminogen activator inhibitor-1
PBS: Phosphate buffered saline
PC-1: Plasma cell membrane glycoprotein-1
Pck1: Phosphoenolpyruvate carboxykinase 1, cytosolic
PCR: Polymerase chain reaction
PDK: Phosphoinositide-dependent kinase
PI3K: Phosphoinositol 3-kinase
PIP3: Phosphatidylinositol 3,4,5-trisphosphate
PKC: Protein kinase C
PTPase: Protein-tyrosine phosphatase
PPARγ: Peroxisome proliferator activated receptor gamma
PPARGC1A: Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; PGC1a; Pgc1a
Ppia: Cyclophilin-A
PVDF: Polyvinylidene difluoride
PVP: Polyvinylpyrrolidone
PWS: Prader-Willi Syndrome
RBP: Retinol-binding protein
RBP-4: Lipocalins retinol-binding protein 4
RNA: Ribonucleic acid
Rn18s: 18S ribosomal RNA
SAM: S-adenosylmethionine
SBTI: Soybean trypsin inhibitor
SCD1: Stearoyl-CoA desaturase-1
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERPINF1: Pigment epithelium-derived factor; PEDF
Srebf1: Sterol regulatory element-binding transcription factor 1
SRS: Silver-Russell Syndrome
TBS: Tris-buffered saline
TBST: Tris-buffered saline with Tween 20
T2DM: Type 2 diabetes
TE: Trophoderm
Tfam: Mitochondrial transcription factor A
TG: Triglycerides
TGFβ: Transforming growth factor β
ten-α: Tumour neorosis factor α
UBE3A: E6-AP ubiquitin-protein ligase
WHO: World Health Organization
1.1 Infertility

1.1.1 Definition

In humans, the pregnancy rate in couples with proven fertility is about 20-40% in each menstrual cycle, and the accumulated pregnancy rate is about 90% and 95% after 12 cycles or 2 years respectively [1-3]. Female fertility generally starts to decline after age 30 and falls dramatically by the age of 35 [4].

There are three definitions of infertility in the medical literature [5]. Clinically, infertility is defined by the International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization as ‘a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse’ [6]. The epidemiological definition extends the period of unprotected intercourse to 2 years, whereas the demographic definition of infertility is the inability to have a live birth over a longer period of time (5 years) in non-contracepting, sexually active women [7].

1.1.2 Prevalence

It is estimated that the overall median prevalence rate of infertility at 12 months is 9% around the world [8]. For instance, the estimated prevalence rate ranged from 11.5% to 15.7% in Canada [9], and was 10.2% in males and 9.7% in females respectively in the USA [10]. On the other hand, the lifetime prevalence of infertility varied globally from 1.3 to 32.6% [8, 11-14]. Take the UK as an example, one study reported that the lifetime prevalence of infertility in 6584 women investigated was 16% and 8% of them received treatment to help them to conceive [12].
1.1.3 Causes

The causes of infertility include male specific factors, female specific factors, common factors in either gender and unexplained factors. For instance, the major factors in male are impaired sperm production, function and transport, and dysfunction of sperm-oocyte fusion [15]. In females, ovulatory disorders, oocyte factor, tube infertility, endometriosis and implantation failure are major problems [16]. Chromosomal abnormalities, environmental and lifestyle factors are observed in both genders [15, 16]. Importantly, 40% of infertile couples display a combination of factors and about 15% of couples display unexplained infertility, with no detectable causes [3].

1.1.4 Impact

Infertile couples experience psychological distress such as frustration, confusion, anger and even anxiety and depressive disorders [17-19]. Infertile couples may experience social isolation and culture discrimination, which can result in violence and suicide [17], poor marital adjustment, decreased quality of life and sexual dysfunction [20-22]. Treatments for infertility generally require life style adjustment and may influence career development [23] and are a huge financial burden for the infertile couple [24].

1.1.5 Treatment

There are several approaches to treat infertility, including lifestyle modification, medication, surgery, artificial insemination and assisted reproduction technologies (ART). Lifestyle modifications such as an improved diet and exercise regimes are not only critical to help infertile couples to conceive naturally, but also to improve the outcome of assisted reproduction [25]. Clomiphene citrate, gonadotrophin releasing hormone (GnRH) and gonadotropins are used for anovulatory disorders in females and gonadotrophin deficiency
in males. Antibiotics are used to treat genital tract infection. Surgery is used to treat endometriosis and tubal disorders in females and obstructive azoospermia in males. In addition, artificial insemination is a potential choice for male infertility and unexplained infertility. While 85% to 90% infertility cases can be treated with these approaches, there still are a large number of patients who require assisted reproduction technologies (ART) to conceive a child.

Assisted reproduction technologies are defined as ‘all treatments or procedures that include the in vitro handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy’ [6]. There are several types of assisted reproduction technologies which are widely used globally nowadays, including in vitro fertilization and embryo transfer (IVF), intracytoplasmic sperm injection (ICSI) whereby a single sperm is injected into an oocyte, gamete and embryo cryopreservation, preimplantation genetic diagnosis (PGD), preimplantation genetic screening (PGS).
1.2 In Vitro Fertilization (IVF)

The first human IVF baby Louise Joy Brown was born in 1978 in England [26]. IVF was initially designed to overcome tubal disorders of women. However, in the last three decades, IVF has been used to treat other causes of female infertility, unexplained infertility and some cases of male infertility. The reported number of IVF cycles in Western countries has continued to increase [27-29]. ART now accounts for up to 4.5% of all live births in developed countries, with an estimated 5 million individuals conceived through ART worldwide, mostly by IVF and ICSI which is used for severe cases of male infertility [30, 31]. IVF is regarded as one of the most important breakthroughs in medicine in the 20th century.

Briefly, the routine IVF procedure includes ovarian hyperstimulation, in vitro fertilization and embryo culture, and embryo transfer. Firstly, the ovaries are stimulated with gonadotropins to induce development of multiple follicles. The commonly prescribed medications are gonadotropin-releasing hormone (GnRH) agonists, GnRH Antagonists, follicle stimulating hormone (FSH) and human menopausal gonadotropin (hMG). Human chorionic gonadotropin (hCG) is administered for the final maturation of the oocytes when certain criteria are met based on hormone tests and serial ultrasound examinations. Then, the oocytes are retrieved from the ovaries 36 hours after the hCG injection using a transvaginal ultrasound-guided fine needle. Next, oocytes are inseminated with the prepared sperm in vitro to achieve fertilization. Embryos are cultured in a prepared medium in the incubator for three days while they divide to the 8 cell stage or five days to develop to the blastocyst stage prior to transfer. Finally, the embryos are transferred into the uterus or frozen for future use.
1.2.1 Health of IVF offspring

Although the use of IVF is increasing, the long term health of IVF offspring is still unclear due to limited follow-up data to date. Concerns about the potential implications of IVF on babies’ health have been raised since the introduction of this technology [32, 33]. It is well accepted that risks increase as a consequence of multiple pregnancies after multiple embryos transfer, which are clearly associated with low birth weight and preterm delivery [33-39]. Therefore, I focus solely on the health outcomes of IVF singleton babies below.

1.2.2 Perinatal outcomes

IVF singleton pregnancies are associated with more adverse obstetric and perinatal outcome as compared with spontaneous conception [40]. This includes an increased risk of low birth weight, preterm birth, small for gestational age, stillbirth, congenital abnormalities, perinatal mortality, requirement for neonatal intensive care, antepartum haemorrhage, hypertensive disorders of pregnancy, preterm rupture of membranes, gestational diabetes, induction of premature labour and the need for Caesarean section in IVF singleton pregnancies [38, 40-47]. Of note, vanishing twin pregnancies, which contribute to about 10% of IVF singletons pregnancies, are also associated with an additional increase in perinatal risk in IVF [48, 49].

An increased risk of adverse perinatal outcomes was also observed in humans conceived by ovarian stimulation alone as compared to naturally conceived individuals and even in naturally conceived siblings from subfertile parents [50-53]. In addition, a systematic review and meta-analysis showed that singletons born after the transfer of frozen and then thawed embryos also have better obstetric and perinatal outcome as compared with those after the transfer of fresh IVF embryos [54]. This data suggest that some risk of IVF may
be attributable to suboptimal endometrial development induced by hormone stimulation [37, 47]. Parental characteristics may also contribute to the increased risk of poor perinatal outcomes in IVF [33, 55], since subfertile women also have increased risk versus women with normal fertility [56, 57]. Taken together, both embryo culture, hormonal stimulation alone and parental characteristics may all contribute to the adverse outcome of pregnancies after IVF.

**1.2.3 Metabolic health of IVF offspring**

The long term health implications of IVF are unknown since the majority of IVF offspring are still in their childhood. Accumulating evidence suggests that IVF children may be at an increased risk of developing metabolic and cardiovascular diseases later in life [39, 58]. This work is discussed in more detail in Chapters 3 to 5.

There are only a few studies in mouse models to examine the effects of IVF on metabolic risks in adult offspring. These studies are also discussed in the review article and in the introduction and discussion of chapters 4 and 5.

To date, it is unclear if increased risks observed in IVF children are related to underlying genetics of the parents, environmental factors, or the treatment procedures per se. Furthermore, it is unclear whether any differences in outcomes are due to the process of ovarian stimulation and/or the embryo culture. Of note, no studies have been conducted to investigate metabolic health in human adults.
1.3 Developmental origins of health and disease

The developmental origins of health and disease (DOHaD) hypothesis or Barker hypothesis [59, 60] proposes that impaired fetal and postnatal growth, caused by suboptimal periconceptional and perinatal environments, combined with rapid childhood weight gain predisposes offspring to increased risk of developing hypertension, obesity, type 2 diabetes and coronary heart disease in later life [61-69].

1.3.1 Metabolic programming

Developmental plasticity is the basic assumption of the Barker hypothesis and is defined as “the phenomenon by which one genotype can give rise to a range of different physiological or morphological states in response to different environmental conditions during development” [70, 71]. This enables the developing fetus to make short-term adaptive responses to its environment in utero to maintain the tissue function and ensure survival following environmental insults such as nutrition factors [71-73]. In addition to the uterine period, the preconception period and early postnatal life also are critical windows of developmental plasticity [74, 75].

These early adaptive responses to a stress in the periconceptional period, gestation, and early postnatal period may irreversibly change the physiology, for instance the metabolism, of the organism and continue to be expressed even in the absence of the stress later in life, and such processes are referred to metabolic programming [76, 77]. Consequently, these alterations induced by metabolic programming predispose the offspring to disease later in life [73].

Evidence suggests that the regulation systems that are plastic during early development such as blood pressure, insulin and deposits of body fat, are strongly associated with adult
disease [71]. For instance, maternal undernutrition during pregnancy results in hypertension, permanent changes in insulin metabolism and body fat distribution in adult offspring [78, 79].

The consequences of metabolic programming depend on the stage of developmental plasticity during which in utero stress occurs. Evidence from Dutch famine cohort studies is a good example to demonstrate the different effects of maternal undernutrition during different stages of pregnancy. Individuals exposed to famine during early gestation had an increased risk of a more atherogenic lipid profile, stress responsiveness, obesity, coronary heart disease, and in women, breast cancer [80]. Exposed to famine in mid gestation was associated with increased risk of microalbuminuria and obstructive airways disease, whereas maternal undernutrition in late gestation was related to low birth weight [80, 81]. Of note, exposure to famine was associated with glucose intolerance, independently of the stage of gestation [80]. These results suggest that early gestation seems to be a particularly vulnerable period [80].

The preimplantation period is now emerging as a critical window for development and later adult health [82, 83]. Mammalian preimplantation embryos manipulated in vitro are sensitive to their environment and are predisposed to aberrant embryo gene expression and programming which results in subsequent prenatal and postnatal developmental consequences [84]. For instance, maternal exposure to low protein diet during only the preimplantation stage led to reduced inner cell mass and trophectoderm cell numbers in the embryos, altered birthweight and postnatal growth rate, and increased blood pressure in the offspring [82]. This stress also results in increased blood pressure in adult mice [85], and cardiovascular dysfunction in adult sheep [83].
Sexually dimorphic effects have been identified in several developmental programming models of adult diseases both in humans and animals although the mechanism is not fully understood [86, 87]. For instance, males grow faster than females in utero and thus are more vulnerable to malnutrition [88]. Men had greater risk of chronic disease and shorter lives than women in response to maternal undernutrition [89]. However, the prevalence of obesity was higher in females than males conceived during the Dutch famine [90]. In rodent models, glucose intolerance, increased blood pressure and impaired renal function were more severe in male offspring than female offspring with several prenatal insults [86]. Increased triglycerides and altered hepatic fatty acid enzymes were only found in adult male offspring in rats after uterine artery ligation [91]. Males are also more susceptible to deleterious effects of high fat diet [92-94]. In contrast, increased blood pressure was only observed in female offspring of lard-fed pregnant rats [95]. In addition, female mice offspring are more vulnerable than males exposed to a hypercholanemic intrauterine environment, which was further uncovered by a high-fat diet [96]. Generally, males are more susceptible to adverse metabolic consequences than females, but it depends on the type of in utero or postnatal stress [86].

1.3.2 Epidemiological evidence

Barker et al. [97] observed a strong positive correlation between ischaemic heart disease mortality rates in 1968-78 and infant mortality in 1921-25 in the least affluent areas of England and Wales, suggesting that malnutrition in early life increases susceptibility to cardiovascular disease in later life.

While low birth weight is an indication of poor fetal growth in utero, further studies by Barker et al. in 10,141 men and 5585 women born during 1911–1930 in Hertfordshire of UK reported low birth weight was associated with increased risk of cardiovascular disease
[63, 98]. Similar findings have been replicated by a number of studies [65, 66, 99]. In addition, a large number of studies have shown that low birth weight increased the risk of developing obesity, metabolic syndrome, glucose intolerance and type 2 diabetes [64, 67, 100-102].

Accelerated growth in infancy and childhood also exacerbates the risk of developing obesity, cardiovascular disease and type 2 diabetes [61, 64, 103, 104]. However, timing of the catch up growth may also be important. Low infant weight gain at age of 1 year with subsequent catch-up growth has been associated with increased risk of coronary heart disease, independently of size at birth [63, 104]. Conversely, accelerated early postnatal growth in the first year also strongly predicts later obesity, insulin resistance and cardiovascular disease [67, 105-107].

Normally, BMI increases rapidly in children during the first year of life and subsequently declines and reaches a minimum around 6 years of age, before a gradual increase again through puberty. The point of minimal BMI has been called the adiposity rebound [108]. Early adiposity rebound is related to obesity in later childhood and in adulthood, independently of parent obesity and the individual’s BMI at the point of adiposity rebound [109, 110]. Early adiposity rebound is also associated with small size at birth and at one year, and increased risk of type 2 diabetes in later life [111, 112].

1.3.3 Mechanisms of metabolic programming

A number of mechanisms have been proposed to contribute to metabolic programming and these include tissue and systemic responses, organ alterations, cellular responses, and a combination of the above [75, 113]. At the organ level, alterations of morphology and cell number were observed in adult organs such as reduced nephron mass in kidney and
beta-cell mass in pancreas, that typically have lesser energy allocations during
development [78, 114]. Hormones and metabolism may also be altered in a thrifty way
[71], promoting insulin resistance in muscle in order to maintain glucose concentrations
for the brain [115]. Tissue or systemic responses include abnormal placentation and
changes in hypothalamic-pituitary-adrenal axis and hypothalamic appetite regulatory
networks. Cellular responses to stress involve epigenetic modifications, mitochondrial
dysfunction, endoplasmic reticulum stress and oxidative stress.

1.3.4 DNA Methylation and IVF

For evidence examining the potential link between IVF and alterations in DNA
methylation I refer you to the published review in Chapter 3. Initially, we intended that
examination of epigenetic changes were to form one part of my thesis, to potentially
explain the metabolic phenotypes we hypothesised would be present in our IVF cohorts.
However, recruiting for the human study and set up of the IVF animal models took longer
than anticipated and I have ended up only examining this pathway cursorily. Below I
establish the two genes we chose to examine here, IGF2 and PGC1a (peroxisome
proliferator activated receptor gamma co-activator 1a).

1.3.4.1 Imprinted gene IGF2

IGF2 is a key factor in human growth and development. The IGF2 gene is maternally
imprinted through the differentially methylated region (DMR), thus only the paternal
allele is expressed in most tissues. Aberrant imprinting of IGF2 gene is associated with the
overgrowth disorder Beckwith-Wiedemann syndrome [116] and the growth restriction
disorder Silver-Russell syndrome [117]. Hypomethylation of IGF2 DMR0 is associated
with human colorectal cancer, Wilms tumour [118-120], and has also been linked to later
development of obesity and insulin resistance in individuals exposed to famine in utero [80].

The epigenetic defect found most often following on from IVF in animal models is loss of DNA methylation at the maternal allele of the imprinted gene insulin like growth factor 2 (IGF-2), this leads to large offspring phenotype “macrosomia” [121]. A mice study [122] reported IVF-derived mouse embryos and embryonic stem (ES) cells derived from IVF blastocysts demonstrated abnormal IGF2-H19 gene region imprinting. In humans, Turan et al. [123] reported aberrant methylation of the maternal IGF2/H19 differentially methylated regions was more common in children conceived by IVF, and the overall variance was also significantly greater in the in vitro group. Consequently, the developmental difference may have an effect on placental and fetal growth.

1.3.4.2 Mitochondrial gene PGC1a

PGC1a (also known as PPARGC1A) is a transcriptional coactivator of nuclear receptors that regulates the genes involved in mitochondrial energy metabolism, and is linked to development of insulin resistance and type 2 diabetes [124]. A twofold increase in DNA methylation of the PGC1a gene promoter was observed in pancreatic islets from 10 type 2 diabetic subjects as compared with that from 9 control donors [125]. A recent study reported 20 young healthy men with low birth weight have higher PGC1a methylation than 26 normal birth weight controls during a controlled diet. Moreover, another study reported an association between PGC1a promoter DNA methylation in umbilical cord and maternal BMI of pregnancy [126].
1.4 Metabolic risk factors

1.4.1 Obesity

It is estimated that more than 400 million adults were obese worldwide in 2005 [127]. The prevalence of obesity has increased significantly the world over in the last three decades [127, 128]. Obesity was recognized as an independent risk factor for metabolic syndrome, type 2 diabetes, and cardiovascular disease [129-131], and increasing obesity may reduce the overall life expectancy in developed countries for the first time in recent history [132]. Therefore, obesity is a global issue and a major public health problem.

The majority of obese individuals develop insulin resistance, which links obesity to metabolic and cardiovascular disease. A number of mechanisms have been proposed that promote insulin resistance, including elevated plasma fatty acids concentrations and adipokines, and chronic inflammation [133].

1.4.2 Obesity and Insulin resistance

Insulin is released from the pancreas in response to glucose and stimulates glucose uptake into peripheral tissues (primarily skeletal muscle) and splanchnic tissues (liver and gut) and by inhibiting hepatic glucose production. Insulin also inhibits fatty acid release from adipose tissue and promotes lipid synthesis in liver and adipocytes [134]. In addition, insulin promotes protein synthesis, cell growth and differentiation and inhibits protein breakdown [135]. Insulin resistance is defined as the inability of peripheral target tissues to respond appropriately to insulin and is characterized by a decreased glucose uptake and glycogen synthesis in skeletal muscle, and a failure of insulin to inhibit hepatic glucose production.
Insulin resistance is associated with dyslipidemia, and hypertension, and increases the risk of developing a number of disorders such as type 2 diabetes, atherosclerosis, cancer, and nonalcoholic fatty liver disease [136-139]. For instance, insulin resistance plays a key role in the development of type 2 diabetes since it is present 10–20 years before diagnosis of the disease [140] and is a major risk factor which predicts the development of the disease [141]. It is generally accepted that resistance to insulin in target tissues (liver, muscle and adipose tissue) develops initially, followed by decreased insulin secretion as a result of progressive pancreatic β-cell dysfunction [142].

Of note, insulin resistance may occur in selective tissues or pathways and hyperinsulinemia can promote the insulin action of metabolic or non-metabolic regulation such as increased sodium retention in the kidney and hyperandrogenism in the ovary [136]. However, the mechanism of insulin resistance is still poorly understood. The potential pathways include ectopic lipid accumulation, endoplasmic reticulum stress and inflammation [139, 143-145].

1.4.2.1 Insulin signalling

There are many excellent reviews of the insulin signalling pathways and how each of these pathways may contribute to insulin resistance however this is not the focus of this thesis. Briefly and as shown in Figure 1, insulin binding to the insulin receptors results in its autophosphorylation at sites on the intracellular β subunit, followed by phosphorylation of the insulin-receptor substrates (IRS). Once phosphorylated, IRS proteins activate phosphatidylinositol 3-kinase (PI3K). Activated PI3K generates the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3). Increased PIP3 leads to the activation of a protein kinase cascade; first stimulating the protein kinase PDK, which phosphorylates and activates serine/threonine kinases Akt (also known as protein kinase B, PKB). Then
Akt2 induces phosphorylation and inhibition of glycogen synthase kinase 3 (GSK-3),
promoting glycogen synthesis. In skeletal muscle and fat, activated Akt phosphorylates its
160 kDa substrate AS160 resulting in release of glucose transporter type 4 (GLUT4)
vesicles that subsequently undertake translocation to the cell surface membrane, following
glucose uptake into the cell. PDK also phosphorylates the atypical protein kinase C
isoforms ζ and λ (PKC ζ/λ) which stimulate GLUT4 vesicles translocation in response to
insulin. In the liver, phosphorylation of the forkhead transcription factor FOXO1 by Akt2
decreases gluconeogenesis by inhibiting the expression of phosphoenolpyruvate
carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase).

Impaired phosphorylation of the insulin receptor in response to insulin has been observed
in skeletal muscle, adipose tissue and the liver of obese patients with type 2 diabetes [146-
148]. Defects in insulin-stimulated phosphorylation of IRS-1 and decreased PI 3-kinase
activity has also been reported in skeletal muscle and adipocytes of type 2 diabetic
patients [149-151]. Impaired Akt phosphorylation was observed in skeletal muscle and
adipocytes of type 2 diabetic patients [151, 152]. Conversely, defects in insulin activation
of PKC-ζ [153, 154] but not Akt [149, 154] have been reported in biopsies of skeletal
muscle from obese subjects and type 2 diabetic patients, and in cultured myocytes and
adipocytes from obese subjects [155]. Reduced glucose uptake in skeletal muscle may also
result from impaired function or distribution of GLUT4 itself [156] or impaired docking
and fusion with the membrane [134].

1.4.2.2 Inhibitors of insulin signalling

As shown in Figure 1, elevated fatty acid metabolites such as fatty acyl CoAs,
diacylglycerol, and ceramides trigger a serine/threonine kinase cascade stimulated by
novel protein kinase Cs (PKC 0, δ, βII) and IKK-β or by JNK in liver, muscle and fat. This
inhibits insulin signalling transduction [157]. IKK-β and JNK were also activated by endoplasmic reticulum stress and inflammatory cytokines such as TNFα, IL6 or CRP. Acquired or inherited defects in mitochondrial fatty acid oxidation, defects in adipocyte lipid metabolism or increased energy intake may all contribute to increased fatty acid metabolites and insulin resistance [157].
Figure 1 Insulin signalling pathway in muscle and fat, and liver.
1.5 Key tissues of insulin resistance

1.5.1 Liver

The liver plays a key role in maintaining glucose homeostasis in healthy individuals. In the fasting state, the liver produces glucose by glycogenolysis and gluconeogenesis to maintain circulating glucose levels, and is responsible for about 25% whole body glucose disposal [158]. In the postprandial state, insulin promotes glycogen synthesis and inhibits hepatic glucose production, and the liver accounts for about 30% of whole body insulin-stimulated glucose disposal [158].

A number of metabolic enzyme genes play a key role in the fasted to fed transition induced by glucose and insulin. Glucokinase (GCK) is the major glucose-phosphorylating enzyme in the liver and it promotes the uptake of glucose by phosphorylation of the hexose. Glucokinase deficiency is related to type 2 diabetes in humans and rodents [159, 160]. In contrast, phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are the key enzymes to stimulate gluconeogenesis and promote hepatic glucose output. Obese and type 2 diabetes patients have increased hepatic glucose production due to impaired suppression of gluconeogenesis by insulin [161, 162].

Hepatic gluconeogenesis is inhibited by insulin via the interaction of PGC1α and FoxO1 [163]. PGC1α binds FoxO1 and activate the expression of PEPCK and G6Pase that contributes to circulating hyperglycemia, however this process is inhibited by insulin stimulated phosphorylation of FoxO1 [163, 164]. Conversely, liver Pgc1α-deficient mice have reduced PEPCK and G6Pase expression and consequently mild hypoglycaemia [165].
Insulin also stimulates fatty acid and triglyceride synthesis in the liver [166]. Fatty acids are esterified into triglycerides which are stored in the liver or delivered to fat for storage and to muscle for oxidation as a very low density lipoprotein (VLDL). The transcription factor sterol regulatory element binding transcription factor 1 (SREBP-1c, also known as SREBF-1C), is a master regulator of de novo lipogenesis. Once activated by insulin, SREBP-1c promotes transcription of downstream genes required for fatty acid and triglyceride biosynthesis such as acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1) and diacylglycerol acyltransferase-1 (DGAT1). Alternatively, fatty acids can be transported into mitochondrial for oxidation, which provides substrates for gluconeogenesis during fasting [167]. FAT/CD36 is a transporter of long chain fatty acids to the mitochondrial membrane, whereas carnitine palmitoyltransferase (CPT1) is a rate limiting enzyme in carnitine transport of long chain fatty acids across the mitochondrial membrane. Enhanced hepatic Cpt1a expression improved glucose tolerance in obese mice [168].

The liver is one of the first organs to display insulin resistance in response to an overfeeding diet. In young healthy men, impaired suppression of hepatic glucose production as assessed by the hyperinsulinemic-euglycemic clamp was observed within 5-11 days of high fat overfeeding [169, 170]. However, longer periods are necessary to induce peripheral insulin resistance following high fat overfeeding [171, 172]. This was also observed in rats as evidenced by insulin resistance in the liver and adipose tissue induced by a high fat diet prior to a significant reduction of insulin-mediated peripheral glucose disposal in skeletal muscle [173].

Hepatic insulin resistance is likely to be selective in both humans and rodents, since the suppression of gluconeogenesis through FoxO1 pathway is impaired while the activation
of lipogenesis through SREBP1-c pathway is not altered [174]. This results in the combination of hyperglycemia, hyperinsulinemia, and hypertriglyceridemia.

1.5.2 Skeletal muscle

As shown in Figure 1, insulin stimulates glucose uptake and glycogen synthesis. Skeletal muscle accounts for about 75% of whole body insulin-stimulated glucose uptake [142]. One mechanism through which insulin resistance may occur in skeletal muscle is through increased intramyocellular fatty acid metabolites such as fatty acyl CoAs, diacylglycerol, and ceramides accumulation [142, 157]. This may result from acquired or inherited defects in mitochondrial fatty acid oxidation, or impaired fatty acid uptake into the mitochondria or increased delivery of fatty acids [157]. Patients with type 2 diabetes also had increased accumulation of fatty acids metabolites and reduced lipid oxidation in skeletal muscle [175-177].

Mitochondria controls energy homeostasis by metabolizing nutrients such as glucose and fatty acids, and producing ATP through the oxidative metabolism. This ability to switch appropriately between carbohydrate and fat is termed “metabolic flexibility” and there is large variation in this parameter between individuals [178]. In healthy individuals, during an overnight fast or increasing the proportion of dietary fat rapidly switches on fatty acid oxidation as evidenced by a drop in respiratory quotient (RQ), whilst the infusion of glucose and insulin rapidly promotes carbohydrate oxidation as shown by increase in RQ [178, 179].

An impaired fat oxidative capacity and metabolic inflexibility are key features of skeletal muscle insulin resistance and may play a major role in the development of type 2 diabetes [180, 181]. For instance, the ability to switch from fat to carbohydrate oxidation was
impaired in insulin-resistant subjects during a hyperinsulinemic clamp, and it is most likely due to impaired glucose uptake of skeletal muscle [178, 179, 182, 183]. Similarly, it has also been reported that obese and weight-reduced obese individuals as well as patients with type 2 diabetes have impaired metabolic flexibility [178, 184], which was characterized by a reduced fat oxidative capacity and high levels of circulating free fatty acids (FFAs) [176]. A study by Heilbronn et al. showed that even a single high-fat meal impaired fatty acid oxidation in skeletal muscle of healthy individuals with a family history of type 2 diabetes [185]. This occurred before the onset of insulin resistance and was related to impaired activation of genes involved in lipid metabolism [185].

Mitochondrial function is determined by genetic factors, oxidative stress, mitochondrial biogenesis, and aging [186]. A number of genes have been linked to mitochondrial dysfunction in humans and animals. Peroxisome proliferator activated receptor gamma co-activator 1a (PGC1a) is a master regulator of mitochondrial biogenesis and oxidative phosphorylation by activating nuclear transcription factors and promoting expression of many mitochondrial genes, including those involved with fatty acid oxidation and oxidative phosphorylation. Decreased PGC1a gene expression results in impaired oxidative phosphorylation and ATP production in muscle obtained from patients with type 2 diabetes and family history positive nondiabetic subjects [125, 187]. Mitochondrial transcription factor A (TFAM) plays an important role in the maintenance of mitochondrial DNA as it stabilizes mitochondrial DNA through the formation of mitochondromesomes and regulates the amount of mitochondrial DNA [188]. Reduced expression of TFAM and genes involved in oxidative phosphorylation (OXPHOS) and impaired oxidative capacity of the mitochondria were reported in skeletal muscle of patients with type 2 diabetes and prediabetic individuals [125, 187, 189].
1.5.3 Adipose tissue

Adipocytes store excess lipid, thereby protecting other tissues and organs from the deleterious effects of abnormal ectopic fat deposition [190]. Insulin stimulates fatty acid synthesis along with formation and storage of triglycerides in adipose tissue and inhibits lipolysis [131, 134]. Over the past decade, adipose tissue has been increasingly recognized as not only an inert storage depot for fat, but an active endocrine organ that secretes a number of metabolic hormones [190]. Collectively, these hormones have been termed “adipokines”. Leptin, a major critical hormone in central control of energy balance, is secreted by adipose tissue. In addition to leptin, there are other cytokines as well as hormones directly involved in lipid metabolism, in the complement system and in vascular haemostasis, such as adiponectin, monocyte chemoattractant protein-1 (MCP-1), retinol-binding protein (RBP), tumour neorosis factor α (TNF-α), interleukin 6 (IL-6) [191].

As shown in Figure 1, increased inflammatory cytokines such as TNF-α and IL-6 in adipose tissue of obese subjects have been implicated as causal in the development of insulin resistance and type 2 diabetes [137, 192-194]. In contrast, the plasma concentration of anti-inflammatory adiponectin is inversely related to the degree of obesity, insulin resistance and type 2 diabetes [195-197].
1.6 Research questions

This research aims to answer the following questions:

1. Are there differences in insulin sensitivity and metabolic risk factors between IVF and naturally conceived (NC) human adults at baseline or in response to a high fat overfeeding challenge (45% fat)?

2. Are there differences in glucose metabolism between IVF adult mice versus natural conception (NC) and ovarian stimulation alone (OS) fed a chow or high fat diet (60% fat) for 8 weeks, and are any gender differences in phenotypes of IVF mice offspring?

3. Are DNA methylation and/or the expression of PGC-1α and IGF2 altered in subcutaneous fat, visceral fat and skeletal muscle tissue from obese patients with or without type 2 diabetes compared with lean individuals?
1.7 Aims and hypotheses

1.7.1 Study 1: IVF humans

The aim of this study is to metabolically phenotype young adults born following IVF versus controls at baseline as well as in response to high fat (45%fat) overfeeding challenge.

Hypothesis: Young adults conceived following IVF will have insulin resistance and have greater responses to high fat dietary challenge as compared with naturally conceived individuals.

1.7.2 Study 2: IVF mice study

The aim is to metabolically phenotype adult mice conceived by IVF, ovarian stimulation alone or natural conception; following chow as well as high fat diet (60%fat), and separate out the effects of ovarian stimulation and embryo culture in the offspring.

Hypothesis 1: Mice born following IVF or by ovarian stimulation (OS) alone will have impaired glucose metabolism as compared to natural conception (NC) and this effect will be exacerbated by high fat diet for 8 weeks.

Hypothesis 2: Ovarian stimulation and embryo culture will result in distinct metabolic effects in mice offspring, including gender differences, following chow or high fat diet.
1.7.3 Study 3: Epigenetic study of obesity and type 2 diabetes

The aim is to determine the tissue specificity of DNA methylation and expression of PGC-1α and IGF2 and their association with obesity, in the presence or absence of type 2 diabetes compared with lean individuals, and to examine the relationships between DNA methylation patterns and gene expression.

Hypothesis: Tissue specific DNA methylation and gene expression of PGC-1α and IGF2 will be associated with obesity and type 2 diabetes.
1.8 Outline of the publications

In addition to the introduction and methodology (Chapter 2), this thesis is organised into a series of published and unpublished articles, including a review of the current literature (published in 2011) and three journal articles (one published, two submitted).

Chapter 3 is entitled “Does in vitro fertilisation increase type 2 diabetes and cardiovascular risk?” and was published in Current Diabetes Review in 2011. This review summarizes the existing evidence as to whether IVF increases diabetes and cardiovascular disease risk in animals and in human models and discusses the epigenetic mechanisms that may underpin this increase in risk. We proposed that these risks need to be urgently assessed in IVF born adult humans and animal models.

Chapter 4 is the manuscript entitled “Altered glucose metabolism in mouse and humans conceived by in-vitro fertilization (IVF)” and was submitted to Diabetes (accepted for publication). In this paper, glucose metabolism in young adult humans and adult male C57BL/6J mice conceived by IVF versus natural conception was examined, under energy balance and high-fat overfeeding conditions. We show that IVF conceived human individuals were more insulin resistant and tended to be more susceptible to the metabolic consequences of high-fat overfeeding for 3 days. Our data in mice support these findings, and further delineate the effects of embryo culture versus ovarian stimulation. These suggest that it is the process of embryo culture itself rather than genetic and/or environmental differences that contributes to impaired glucose metabolism and that ovarian stimulation impaired fetal growth, at least in male mice. This paper highlights an increased risk of developing metabolic and cardiovascular disease in IVF offspring later in life.
Chapter 5 is the manuscript entitled “Increased susceptibility to high fat diet in female mice conceived by in vitro fertilisation (IVF) or ovarian stimulation alone” and was submitted to Biology of Reproduction. Gender differences in phenotypes have been identified in several models both in humans and animals although the mechanism is not fully understood. Whether IVF female mice offspring display a similar phenotype is unknown. We examined glucose metabolism in adult female C57BL/6J mice conceived by natural conception, by ovarian stimulation (OS) alone or by IVF following chow or high fat diet (HFD) for 8 weeks. Interestingly, in contrast to male offspring, chow fed adult females only displayed increased fasting glucose in IVF females. However, in response to high fat diet, both IVF and OS females were more susceptible to high fat diet with increased adiposity and impaired glucose tolerance. This paper suggests that in normal weight conditions, females may be protected from deleterious consequences of manipulation of the preimplantation embryo but that metabolic defects are unmasked following exposure to high fat diet.

Chapter 6 is the manuscript published in Diabetes Research & Clinical Metabolism. Increasing evidence has shown epigenetic modifications in certain genes are associated with disease susceptibility in later life, both in humans and animal models. In this study, we focus on two genes, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) and imprinted insulin-like growth factor 2 (IGF2). As expected, our data showed tissue specific DNA methylation and gene expression of PPARGC1A and IGF2. Moreover, altered DNA methylation and expression of both genes may also be associated with obesity and type 2 diabetes. Therefore, this paper shows epigenetic modification may play a role in the development of obesity and type 2 diabetes in a tissue specific manner in humans.
1.9 Unpublished data

There are additional unpublished data listed in the Appendices as some experiments are still ongoing. Listed below are the examples.

In the IVF human study, we examined the gender differences of glucose metabolism in young adults on energy balanced diet and in response to high fat overfeeding challenge.

In the IVF mouse study, we examined the DNA methylation of Pgc1α and IGF2 in liver of male mice on chow or high fat diet.
Chapter 2: Materials and Methods
2.1 IVF human study

2.1.1 Study subjects

The details are described in Chapter 4.

Subject inclusion criteria (Aug 2010 - Aug 2012)

- Non-obese individuals born as singletons (n=20 per group, BMI and sex matched), either following IVF or natural conception (NC) were studied. The number of subjects to be included was based on power calculations from our previous work.
- aged 18 – 26 years.
- weight-stable (< 5 % fluctuation in their body weight for past 6-months at study entry).

Changes to inclusion criteria (Mar 2012 - Mar 2013)

After 18 months recruiting, 18 subjects (NC 14; IVF 6) participated in this study. Thus, we widened the criteria to reduce the inclusion age from 18 years to 16 years and we also allowed twins to enrol. We removed collection of the fat and muscle biopsies reducing the study burden. Then, we were able to recruit 16 subjects (NC 8; IVF 8) including 3 IVF twins and 1 NC twin.

Subject exclusion criteria

Each subject was questioned prior to the study to exclude:

- individuals with significant disease or surgery (e.g. diabetes mellitus, epilepsy, cardiovascular or respiratory diseases), any other illnesses as assessed by the investigator (including chronic illnesses not explicitly listed above)
- individuals born prematurely (<37 weeks gestational age)
• individuals born with low (<2.5kg) or high birth weights (>4.0 kg)
• individuals born from mothers who had significant health problems during pregnancy (e.g. gestational diabetes, pre-eclampsia)
• individuals who use prescribed or non-prescribed medications (including vitamins and herbal supplements) which may affect energy metabolism, gastrointestinal function, body weight or appetite
• individuals with intolerance/allergy to paracetamol
• individuals with lactose intolerance / other food allergy(s)
• individuals who regularly perform high intensity exercise (>2 week)
• current intake of > 2 standard drinks on > 5 days per week
• current smokers of cigarettes/cigars/marijuana
• current intake of any illicit substance
• those who experience claustrophobia in confined spaces
• subjects who are unable to comprehend study protocol

Recruitment strategy from the IVF database

The ACN database was searched by the data custodians to identify women who gave birth through IVF only between 1986 and 1994, and restricting this search to women who had singleton births, within normal birth weights (2.5 - 4 kg) and gestation lengths (37 – 42 weeks). Names and addresses were cross-checked against current electronic white pages and past and present electoral rolls and an information pack of this study was sent to the patient by the data custodians if a current address was verified. Then, once the child’s contact details were obtained from the parent, an enrolment package was posted or emailed to the child. Finally, following contact with this individual, we invited them in for a screening visit to determine eligibility and on subsequent visits if they would like to
participate in the full study. Then eligible individuals were scheduled for this study after the screening. 437 patients were sampled from the database. 115 mails were sent to patients who were identified. 40 patients replied and 23 patients forwarded the study information to their IVF children. 8 individuals conceived by IVF contacted me for the details and 2 IVF adults participated in the study.

**Subject recruitment summary**

480 individuals completed a questionnaire for this study through advertising. 54 individuals underwent the screening process and 36 young adults were determined to be eligible and took part in this study. 2 NC subjects were subsequently excluded as they did not fast during the first study visit (Figure 2).

![Diagram of subject recruitment](image)

**Figure 2 Diagram of subject recruitment**
2.1.2 Study design

The scheduling of this study is shown in Figure 3. Briefly, following screening, eligible participants were provided with 3 days of energy balanced diet or high fat diet (detailed in Chapter 4) prior to metabolic tests. The two visits were identical. The protocol for the metabolic testing day is depicted in Figure 4. Two sequential blood pressure were taken after a seated 10 minute rest and the results were averaged, if the two measurements were > 5% different, a third measurement was taken. Diets were analyzed for macronutrients using Food-Works 2007 based on the Australian foods database (Xyris Software). Please refer to Chapter 4 for in depth description of the study design and methodology.
Additional Tests

Additional assessments performed, but not included in Chapter 4, were assessment of resting metabolic rate (RMR), activity by an accelerometer and serum adipokines. Following a supine 30min rest, RMR and respiratory quotient (RQ) were measured by indirect calorimetry in semi-supine position for 30 minutes at rest and during the final 30-minutes of the insulin clamp (TrueOne, ParvoMedics, UT, USA) as described previously [198]. Total daily energy expenditure (TEE) was measured by Sensewear Pro armband monitors (SWA; Bodymedia, PA, USA) for 6 consecutive days from day-3 to day+3. This validated monitor integrates motion data from two orthogonal accelerometers plus data from several heat related sensors and was comfortably worn over the subjects’ triceps. The blood lipids and serum insulin assays are detailed in Chapter 4. Additionally, serum high molecular weight adiponectin (HMW adiponectin) was measured by an enzyme-linked immunosorbent assay (ELISA) (Millipore, St Charles, MO, USA). Serum monocyte chemotactic protein-1 (MCP-1), C-reactive protein (CRP) and insulin like growth factor 1 (IGF1) were examined by Quantikine® ELISA (R&D Systems Europe, Ltd., Abingdon, UK). The intra-assay and inter-assay coefficients of variation (CV) were 3.5%~6.2% and 2.2%~5.3% for HMW adiponectin, 4.2%~7.4% and 5.2%~6.8% for MCP-1, 4.6%~8.6% and 6.2%~8.8% for CRP, as well as 3.1%~4.5% and 6.9%~8.5% for IGF1 respectively. The minimum detectable dose of HMW adiponectin, MCP-1, CRP and IGF1 were 0.5 ng/mL, 1.7 pg/mL, 0.01 ng/mL and 0.026 ng/mL respectively. All assays were performed in duplicate. The results are shown in the Appendix 1.
2.2 IVF mouse model

The methodology for generating mouse offspring for this study is described briefly in Chapters 4 and 5. This section describes in more detail the procedures undertaken to set up an inbred IVF mouse model in our laboratory.

2.2.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Consumable materials for embryo culture were purchased from Calbiochem (Merck Pty. Ltd., VIC, Australia).

Culture medium

Embryo handling medium was prepared by using αMEM powder (minimal essential medium, Gibco 12000-022, Invitrogen Australia Pty. Ltd.) according to the manufacturer’s instructions.

Human IVF commercial medium was purchased from CooperSurgical (Trumbull, CT, USA), including Quinn’s Advantage® Medium with HEPES (ART-1023), Quinn’s Advantage® Fertilization Medium (ART-1020), and Quinn’s Advantage® Cleavage Medium (ART-1026).

Mouse research medium was purchased from Cook Medical (William A. Cook Australia Pty. Ltd., QLD, Australia), including Research Vitro Wash (K-RVWA-50), Fertilization (K-RVFE-50A) and Cleave (K-RVCL-50)

Anesthetic
Avertin (20mg/ml) was prepared as per manufacturer’s instructions and kept in a dark bottle at 4°C. The usage dosage was 0.25-0.5mg/g body weight in mice once by intraperitoneal injection. Pentobarbital (60mg/ml solution in saline) was prepared as per manufacturer’s instructions and filtered before use. The usage dosage was 60 mg/kg body weight in mice once by intraperitoneal injection.

**Analgesic**

Carprofen was purchased from Pfizer (Rimadyl ®). The working solution (50mg/ml) was prepared as per manufacturer’s instructions and kept at 4°C. The usage dosage was 2.5-5.0 mg/kg body weight in mice once by subcutaneous injection.

**Gonadotropins**

Equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) were purchased from Calbiochem (Merck Pty. Ltd., VIC, AU).

**Diet**

Rodent chow diet was SF06-105 from Specialty Feeds (Glen Forrest, Australia). High fat diet (HFD) was made up in house using the same recipe of D12492 of Research Diets (New Brunswick, NJ) (Fat: lard 91%, soybean oil 9%).
Table 1 Nutrient composition of diets for mouse

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Chow (SF06-105)</th>
<th>HFD (D12492)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>16% 19.6%</td>
<td>20% 26.2%</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>64% 48.3%</td>
<td>20% 26.3%</td>
</tr>
<tr>
<td>Fat</td>
<td>20% 10%</td>
<td>60% 34.9%</td>
</tr>
<tr>
<td>Energy</td>
<td>3.57 kcal/g</td>
<td>5.24 kcal/g</td>
</tr>
</tbody>
</table>

Table 2 HFD recipe of D12492 (1Kg)

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight (g)</th>
<th>Component</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>258.44</td>
<td>Vitamin Mix</td>
<td>12.92</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>161.5</td>
<td>Calcium Carbonate</td>
<td>7.106</td>
</tr>
<tr>
<td>Sucrose</td>
<td>88.88</td>
<td>L-Cystine</td>
<td>3.876</td>
</tr>
<tr>
<td>Cellulose</td>
<td>64.6</td>
<td>Choline Bitartrate</td>
<td>2.589</td>
</tr>
<tr>
<td>Potassium Citrate</td>
<td>21.318</td>
<td>Soybean Oil</td>
<td>32.3</td>
</tr>
<tr>
<td>DiCalcium Phosphate</td>
<td>16.796</td>
<td>Lard</td>
<td>316.54</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>12.92</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 Animals

All mice were maintained at the medical school animal house of the University of Adelaide at 24°C on a 12-h light, 12-h dark cycle, with standard rodent chow (SF06-105, Specialty Feeds, Glen Forrest, Australia) and water available ad libitum. All mice were acclimatised for 2 week on chow before experimentation. All experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2.2.1 Donor mice for IVF

Stage 1: (CBA x C57BL/6) F1 mouse

To set up the IVF mouse model, (CBA x C57BL/6) F1 males and females at 6-weeks of age were obtained from the University of Adelaide Laboratory Animal Services (LAS, Adelaide, South Australia), since the embryo quality of this strain was well established in the laboratory. After successfully generating 2 litters of F1 mice for three study groups (detailed below), we moved to establish an inbred mouse model.

Stage 2: C57BL/6 mouse

As designed, inbred strain C57BL/6 mice were initially chosen as a model that is susceptible to high fat diet and so we could control genetic background. C57BL/6 males and females at 6-weeks of age were purchased from the LAS (Adelaide, South Australia). This strain was imported from Harlan Laboratories in the USA and does not have a spontaneous deletion mutation in nicotinamide nucleotide transhydrogenase. However, we were unable to generate good quality blastocysts (see section optimizing culture system).
Stage 3: C57BL/6J mouse

I decided to switch to C57BL/6J mice, and males and females at 6-weeks of age were purchased from the Animal Resources Centre (ARC, Perth, West Australia). This mouse varies from the C57BL/6 mice by having a spontaneous deletion mutation in nicotinamide nucleotide transhydrogenase discovered in the year 2006 [199, 200].

2.2.2.2 Recipient mice

(CBA X C57BL/6) F1 female mice 6-weeks of age were obtained from the LAS (Adelaide, South Australia).

2.2.2.3 Vasectomized mice

Vasectomized (CBA X C57BL/6) F1 male mice at 8-weeks of age were obtained from the ARC (Perth, West Australia).

Table 3 Summary of mice used to produce offspring

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sex</th>
<th>Age</th>
<th>Number</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasectomized (CBA X C57BL/6) F1</td>
<td>Male</td>
<td>3-10 months</td>
<td>14</td>
<td>Mating</td>
</tr>
<tr>
<td>(CBA X C57BL/6) F1</td>
<td>Female</td>
<td>8-12 weeks</td>
<td>144</td>
<td>Recipients</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Male</td>
<td>8-16 weeks</td>
<td>32</td>
<td>Donors</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Female</td>
<td>8-12 weeks</td>
<td>156</td>
<td>Donors</td>
</tr>
</tbody>
</table>
2.2.2.4 Power Calculations of sample size

Sample size calculation of mice offspring is supported by the professional biostatistician of the CRE in Nutritional Physiology, Ms Kylie Lange. The primary outcomes are glucose area under the curve (AUC) assessed by glucose tolerance test and insulin sensitivity examined by insulin tolerance test. The study is powered to detect a 25% difference between groups in glucose AUC and insulin sensitivity at $\alpha < 0.05$, $(1-\beta)>0.8$ with 6 and 8 mice/group respectively. Thus 10 pups for each gender were generated per group to ensure sufficient numbers for each test required.
2.2.3 Mouse model design

![Mouse blastocyst donation and transfer model.](image)

Briefly, mouse blastocysts were generated by natural conception (NC group), or by ovarian hormonal stimulation followed by either mating (OS group) or by IVF and embryo culture (IVF group), followed by blastocyst transfer to pseudopregnant mice (7-10 blastocysts/recipient). Genetics, embryo transfer, maternal uterine environment and litter size were thus controlled in this model.
2.2.4 Generating blastocysts

2.2.4.1 Natural conception (NC) group

2.2.4.1.1 Natural mating

Female mice were placed with male mice overnight at 8-10 weeks of age. The following morning, female mice with the presence of vaginal plugs were considered to be pregnant and were humanely sacrificed by cervical dislocation 3 days later (86-88 hours post mating).

2.2.4.1.2 Dissection of uteri

A 35-mm petri dish containing 2 ml of handling medium was placed on a warm stage at 37°C and instruments were disinfected with 70% ethanol. Following sacrifice, the mouse was placed ventral side up on an absorbent sheet. Then the abdomen was disinfected with 70% ethanol and the peritoneum was cut to expose the body cavity. Next, the coils of gut were moved to one side to expose the uterus. The cervix, the mesometrium and the ovary were cut with fine scissors, keeping the utero-tubal junction intact.

2.2.4.1.3 Collection of blastocysts from uteri

A 30G blunted needle was connected to a sterile 1-ml syringe containing handling medium, and was inserted into the cervix and up into one of the uterine horns. The blastocysts were flushed through the uterine horn. The process was repeated for the other uterine horn. The blastocysts were picked up by a pipette, washed through fresh culture medium and transferred to a culture dish which was placed in a modular incubator chamber at 37°C in 6% CO₂, 5% O₂, 89% N₂ for no more than 1 hour prior to being transferred to uteri of pseudopregnant mice.
2.2.4.2 Ovarian stimulation (OS) group

2.2.4.2.1 Hormone stimulation

Female mice at 8-10 weeks of age were superovulated with consecutive injections 7.5 IU of eCG and hCG administered intraperitoneally 48 h apart. After injection with hCG, these females were placed with male mice overnight. The following morning, female mice with the presence of vaginal plugs were considered to be pregnant and were humanely sacrificed by cervical dislocation 3 days later (86-88 hours post hCG injection).

2.2.4.2.2 Collection of blastocysts from uteri

Blastocysts were collected by flushing dissected uteri with the handling medium as described above.

2.2.4.3 IVF group

2.2.4.3.1 Hormone stimulation

The protocol was same as with the OS group above.

2.2.4.3.2 Collection of ovulated oocytes

Mouse oocytes were collected at 13 hours post hCG injection. Once the abdominal cavity was exposed as described above, the utero-tubal junction and the oviduct - ovary junction were cut with fine scissors. The oviduct was transferred to a petri dish with handling medium. The swollen ampulla of the oviduct was teased with fine forceps to release the cumulus mass. The procedures were repeated until the cumulus masses from each oviduct were released.

2.2.4.3.3 Sperm collection
On the day of fertilization, males were sacrificed humanely by cervical dislocation 12 hours after administering the hCG injection to females. The male was placed on its back, and the abdomen was disinfected with 70% ethanol. The peritoneum was cut to expose the body cavity. Then the testes and the epididymus were exposed. Next, the vas deferens was dissected around 1.5 cm from the epididymis which was removed from the fat pad and the testes, and transferred into a dish of warmed handling medium.

The epididymus with attached vas deferens was placed into the sperm dish with the fertilization media. Forceps were slid along the vas deferens to remove the sperm through the cut end and the epididymus was punctured to release sperm. The dish was placed in a modular incubator chamber at 37°C in 6% CO₂, 5% O₂, 89% N₂ for 1 hour and then 10 ul of sperm suspension was added to each 90 ul drop of mouse fertilization medium.

2.2.4.3.4 In vitro fertilization of oocytes

In vitro fertilizations were performed using a modified version of that described previously [201-203]. Handling medium with 5 mg/ml human serum albumin, sperm capacitation dish, oocytes wash and fertilization dishes were prepared the day before fertilization. The liquid in all dishes was covered with paraffin oil and equilibrated at 6% CO₂ in an incubator.

Mouse oocytes were fertilized by sperm at around 13 hours post hCG injection. Collected oocytes were washed well using pasteur pipettes in warmed handling medium, and then washed three times in mouse fertilization medium prior to placing them into the fertilization drops containing the sperm (1 cumulus mass per drop). Then the dish was placed back into the modular incubator chamber.
After 5-6 hour of incubation, oocytes were washed three times in fertilization medium, and transferred into the culture medium drop (10-20 oocytes per drop). Then the culture dish was returned to the modular incubator chamber. The next morning, 2 cell embryos were transferred to a new culture medium dish and incubated in the modular incubator chamber to blastocyst stage (92-96 hours post hCG injection) at 37°C in 6% CO₂, 5% O₂, 89% N₂.

2.2.4.3.5 Optimizing culture system

2.2.4.3.5.1 Culture medium

We initially chose a commercial human IVF culture medium (Quinn’s Advantage®) and the Cook mouse research medium for the study. Then we did the experiments to compare the two media side by side using the (CBA X C57BL/6) F1 mice and C57BL/6 mice (also known as B6 mice) from the University of Adelaide Laboratory Animal Services (LAS) at 12 weeks of age. Embryos were cultured in an incubator at 37°C with 6% CO₂ in air. As shown in Table 4 below, Cook media produced embryos of higher quality and thus was selected for use in this study.

**Table 4 Comparison of the effects of two culture media on embryo development**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Source</th>
<th>Culture media</th>
<th>Oxygen</th>
<th>Oocytes</th>
<th>2-Cell embryo (%)</th>
<th>4-Cell embryo (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 x F1</td>
<td>LAS</td>
<td>Quinn's</td>
<td>Air</td>
<td>27</td>
<td>8 (29.6%)</td>
<td>8 (29.6%)</td>
<td>6 (22.2%)</td>
</tr>
<tr>
<td>F1 x F1</td>
<td>LAS</td>
<td>Cook</td>
<td>Air</td>
<td>29</td>
<td>29 (100%)</td>
<td>28 (96.5%)</td>
<td>28 (96.5%) *</td>
</tr>
<tr>
<td>B6 x B6</td>
<td>LAS</td>
<td>Quinn's</td>
<td>Air</td>
<td>18</td>
<td>2 (11.1%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B6 x B6</td>
<td>LAS</td>
<td>Cook</td>
<td>Air</td>
<td>27</td>
<td>6 (22.2%)</td>
<td>5 (18.5%)</td>
<td>5 (18.5%) *</td>
</tr>
</tbody>
</table>

Chi-square test, * P<0.001; # P=0.05.
2.2.4.3.5.2 Oxygen concentration

There is evidence showing that atmospheric oxygen is detrimental to embryo development in vitro and low oxygen concentrations (i.e. 5% O₂) is more optimal for mouse embryo development [204-206]. To improve the embryo development, again we used C57BL/6 mice (B6) from the LAS at 8-10 weeks of age but embryos were cultured in a modular incubator chamber at 37°C with 6% CO₂, 5% O₂, and 89% N₂.

As shown in Table 5 below, embryo development was improved with 5% O₂, thus we decided to use the modular incubator chamber for embryo culture. However, the blastocyst development rate was still quite low.

**Table 5 Comparison of the effects of atmospheric O₂ and 5% O₂ on embryo development in C57BL/6 mice from LAS**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Source</th>
<th>Culture media</th>
<th>Oxygen</th>
<th>Oocytes</th>
<th>2-Cell embryo (%)</th>
<th>4-Cell embryo (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 x B6</td>
<td>LAS</td>
<td>Cook</td>
<td>Air</td>
<td>32</td>
<td>6 (18.8%)</td>
<td>3 (9.4%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>B6 x B6</td>
<td>LAS</td>
<td>Cook</td>
<td>5% O₂</td>
<td>81</td>
<td>29 (35.8%)</td>
<td>24 (29.6%)</td>
<td>24 (29.6%) *</td>
</tr>
</tbody>
</table>

Chi-square test, *P=0.02.

2.2.4.3.5.3 Animal source

The C57BL/6J mice from Jackson Laboratories in the USA were used in IVF models [207, 208]. Of note, C57BL/6J mice have a spontaneous deletion mutation in nicotinamide nucleotide transhydrogenase [199, 200]. In contrast, the C57BL/6 mice imported by the
LAS from Harlan Laboratories in the USA do not have the mutation. Thus we purchased the C57BL/6J mice (B6J) from the Animal Resources Centre (ARC, Perth, West Australia) and performed IVF and embryo culture using the two strains side by side at 8 weeks of age.

The blastocyst development rate was significantly higher in the C57BL/6J mice from the ARC than the C57BL/6 mice from the LAS (Table 6). Thus, we selected the C57BL/6J mice for the remainder of experimentation.

**Table 6 Side-by-side comparison of two mouse strains**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Source</th>
<th>Culture media</th>
<th>Oxygen</th>
<th>Oocytes</th>
<th>2-Cell embryo (%)</th>
<th>4-Cell embryo (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 x B6</td>
<td>LAS</td>
<td>Cook</td>
<td>5% O₂</td>
<td>26</td>
<td>8 (30.8%)</td>
<td>7 (26.9%)</td>
<td>7 (26.9%)</td>
</tr>
<tr>
<td>B6J x B6J</td>
<td>ARC</td>
<td>Cook</td>
<td>5% O₂</td>
<td>12</td>
<td>9 (75%)</td>
<td>9 (75%)</td>
<td>9 (75%) *</td>
</tr>
</tbody>
</table>

Chi-square test, * P=0.005.

Again, we compared the effect of atmospheric oxygen and 5% O₂ on embryo development in the C57BL/6J mice at 8-10 weeks of age (Table 7). In contrast to the C57BL/6 mice, the blastocyst rate was comparable with either of the two oxygen concentrations in the C57BL/6J mice (Chi-square test, P=0.75).
Table 7 Comparison of the effects of atmospheric O₂ and 5% O₂ on embryo development in C57BL/6J mice from ARC

<table>
<thead>
<tr>
<th>Mice</th>
<th>Source</th>
<th>Culture media</th>
<th>Oxygen</th>
<th>Oocytes</th>
<th>2-Cell embryo (%)</th>
<th>4-Cell embryo (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6J x B6J</td>
<td>ARC</td>
<td>Cook</td>
<td>Air</td>
<td>30</td>
<td>19 (63.3%)</td>
<td>19 (63.3%)</td>
<td>19 (63.3%)</td>
</tr>
<tr>
<td>B6J x B6J</td>
<td>ARC</td>
<td>Cook</td>
<td>5% O₂</td>
<td>17</td>
<td>10 (58.8%)</td>
<td>10 (58.8%)</td>
<td>10 (58.8%)</td>
</tr>
</tbody>
</table>

2.2.5 Preparation of pseudopregnant recipients

The sterility of the vasectomized (CBA X C57BL/6) F1 males was confirmed by mating them three times with superovulated (CBA X C57BL/6) F1 females before experimentation. Prior to embryo transfer, unstimulated (CBA X C57BL/6) F1 females at 8–12 weeks of age were mated with the vasectomized (CBA X C57BL/6) F1 males, and the next morning mating is checked by the presence of a vaginal plug. The morning of plug is designated day 0.5 of pseudopregnancy.

Usually, 7-10 females are housed in one cage and are exposed to the urine produced by a male mouse 2 days before mating to stimulate synchronous estrus. The status of the vagina can be examined to select females.
2.2.6 Uterine blastocyst transfer

Blastocyst transfer was performed using a modified version of that described previously [202, 203, 209].

Shortly prior to transfer, the embryos were placed into 500ul handling medium in a petri dish that had been warmed to 37°C on a warm stage. On day 2.5 of pseudopregnancy CBA X C57BL/6F1 females were anesthetized by Avertin ip injection (0.5 mg/g) and placed face down on the microscope stage. After being disinfected with 70% ethanol, a 3cm incision was made along the midline of the back which is adjacent to the hips of the mouse and the body cavity was exposed on the left side. The uterus was stabilized and a 30-G needle was used to make a hole in the uterus adjacent to utero-tubal junction. Then the pipette loaded with 3-5 blastocyst was inserted into the hole and the blastocysts were expelled into the uterus.

The procedure was repeated for the right side of uterus. The incision in the skin was closed using wound clips. Analgesics Carprofen (2.5-5mg/kg/mouse) was injected subcutaneously once. The mouse was placed on the warm stage until it recovered. All recipients were fed with a standard rodent chow diet until being sacrificed at weaning.
2.2.7 Challenges and strategies

2.2.7.1 To set up the IVF mouse model (5 months)

I initially practiced experiments using the robust strain CBA F1 mice to master all the techniques for this blastocyst donation and transfer model.

2.2.7.2 To obtain good quality of IVF blastocysts (5 months)

As described above, I used the Cook research media and a modular incubator chamber, and switched to C57BL/6J mice from the Animal Resources Centre (Perth) instead of C57BL/6 from the Laboratory Animal Services (Adelaide).

2.2.7.3 To obtain sufficient numbers of blastocysts from NC group for transfer

Generally, about 10-30% of C57BL/6J females presented a vaginal plug in the following morning after natural mating, and we could only get blastocysts from 20-50% of females with a vaginal plug. Thus we had to increase the number of C57BL/6J female mice to generate a sufficient number of blastocysts.

2.2.7.4 To obtain sufficient numbers of plugged recipients for transfer

Similarly, only 10-30% of (CBA X C57BL/6J) F1 females presented a vaginal plug in the following morning after natural mating. Thus we increased the number of (CBA X C57BL/6J) F1 female mice to induce pseudopregnancy.
2.2.8 Metabolic tests in adult offspring

Figure 6 Metabolic tests in mouse adult offspring.

The details of metabolic testing undertaken for male and female mice offspring are described in Chapter 4 and 5 respectively.

Additional testing performed that is not included in submitted manuscripts include DNA Methylation assays, additional proteins by western blotting and mtDNA copy number in blastocysts.
2.2.9 DNA methylation assays

Initially, I chose to examine Pgc1α and Igf2 DRM2 DNA methylation in liver tissue of male mice since higher fasting glucose levels and impaired glucose tolerance were observed in males on either chow or HFD.

Genomic DNA extraction and bisulphite modification, nested touchdown PCR, and pyrosequencing were performed by using a similar protocol to the human DNA methylation assays detailed in Chapter 6.

Primers for mouse Pgc1α and Igf2 DRM2 are shown in Table 8. For mouse Pgc1α, 2 PCRs and 4 sequencing primers were used for 6 CpG’s including 3 homologous in human, mouse and rat. CpG 1: chr5 (- strand) 51554591; CpG 6: chr5 (- strand) 51554002. For mouse Igf2, 14 CpG sites in the DMR2 were examined. CpG 1: chr 7 (-) 142653993; CpG 8: chr 7 (-) 142653941; CpG 14: chr 7 (-) 142653841.

Firstly, we tested all PCR primers which were designed in our laboratory. A single round of PCR and two rounds of PCR were performed for Pgc1α and Igf2 respectively to amplify bisulphite treated genomic DNA. We detected the target size bands for each PCR (Figure 7).

Only samples with single target size bands without primer dimers were used for pyrosequencing. The assay controls included: PCR negative control with sequencing primer, PCR negative control with no sequencing primer, PCR positive (single band) with no sequencing primer, and water with sequencing primer.
In the first run of pyrosequencing, we used 5 µl PCR products which are the same volume as the human pyrosequencing assay (detailed in Chapter 6), but the signals were very low so the results were not reliable. Then in the second run we tested 3 different volumes of 4 PCR products (10ul, 15ul and 20ul) for the assays. We found all the signals were normal in each assay. The results of some CpG sites, including 4 CpG sites of Pgc1α and 7 CpG sites of Igf2 DRM2, were reliable since the coefficient of variation (CV) was below 10% (shown in Table 9 and Table 10). Thus, we selected 10ul of PCR products for the remainder of experimentation performed in triplicate and the data analysis only included 4 CpG sites of Pgc1α and 7 CpG sites of Igf2 DRM2. The results are listed in the Appendix 4.

**Figure 7 2% agarose gel electrophoresis of PCR products**

![Figure 7 2% agarose gel electrophoresis of PCR products](image)
Table 8 Primers and sequences of mouse Pgc1α and Igf2 DRM2 for pyrosequencing

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* denotes 5’ biotinylated primer
Table 9 Mouse Pgc1α DNA methylation assays of three volumes of PCR products

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Std: standard deviation; CV: coefficient of variation.
Table 10 Mouse Igf2 DMR2 DNA methylation assays of three volumes of PCR products

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Std: standard deviation; CV: coefficient of variation.
2.2.10 Protein immunoblotting

Methodological details for immunoblotting are provided in Chapters 4 and 5. I tested a number of primary antibodies for the insulin signalling pathway, including IRβ (BD Biosciences, Cat. No 611276), IR/IGF1R phospho-Tyr1158/1162/1163 (Invitrogen, Cat. No 44806G), IRS-1 (Cell Signaling, Cat. No 2382), Phospho-Tyr612 IRS-1 (Sigma-Aldrich, Cat. No 2658), FoxO1 (C29H4) (Cell Signaling, Cat. No 2880), Phospho- Ser256 FoxO1 (Cell Signaling, Cat. No 9461), GSK-3α/β (Santa Cruz, Cat. No sc-7291), Phospho-Ser21/9 GSK-3α/β (Cell Signaling, Cat. No 9331), Akt (Cell Signaling, Cat. No 9272), Phospho-Ser473 Akt (Cell Signaling, Cat. No 9271), Phospho-Thr308 Akt (Cell Signaling, Cat. No 9275), β -Tubulin (Cell Signaling, Cat. No 2146), PGC1α (Abcam, Cat. No ab54481), total OXPHOS (Abcam, Cat. No ab 110413). However, only Akt and phospho-Ser473 Akt, β –Tubulin, PGC1α and total OXPHOS antibody were found to work reliably and tested on the whole cohort.

2.2.11 Quantification of mtDNA copy number

2.2.11.1 mtDNA copy number in blastocysts

The mtDNA copy number in individual blastocysts was quantified absolutely in triplicate as described in [210]. Briefly, day 5 blastocysts were washed with PBS + PVP (1mg/ml PVP in PBS), collected individually into 1.5ml siliconised low retention microcentrifuge tubes (Fisher Scientific) with 5µl of PBS+PVP and stored at -80°C until use.

Genomic DNA was isolated from each sample using a QIAamp DNA Micro Kit (Qiagen), according to the manufacturer’s protocol. Carrier RNA (1µg; Qiagen) was added to each
sample during DNA extraction. Genomic DNA was eluted twice with 50 µl of water and
diluted 10 times for quantitative polymerase chain reaction (PCR). To prepare the
quantification standards, a long 1186 bp fragment of the 12S rRNA region of mtDNA was
amplified from mouse liver by PCR using the primer pair 5’-ACA CCT TGC CTA GCC
A-3’ and 5’-TTT GCC ACA TAG ACG AGT T-3’ by LongRange PCR Kit (Qiagen) and
then purified by QIAquick PCR Purification Kit (Qiagen) and cloned by using the Qiagen
PCR Cloning Kit (Qiagen). The plasmid DNA was purified by using Plasmid Maxi kit
(Qiagen), quantified by using a Nanodrop ND-1000 Spectrophotometer (Biolab).

The approach to generate the plasmid DNA standard curves was based on the Applied
Biosystems “Creating Standard Curves with Genomic DNA or Plasmid DNA templates

The mass of the plasmid containing the copy numbers of interest was calculated using
formula: Copy number of interest × mass of single plasmid = mass of plasmid DNA
needed.

Thus a standard curve was generated by using seven 10-fold serial dilutions (10- 1x10^7
copies) for each run. Quantitative PCR using the mtDNA 12S rRNA primer pair 5’-CGT
TAG GTC AAG GTG TAG CC-3’ and 5’-CCA GAC ACA CTT TCC AGT ATG-3’ was
performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Foster
City, CA) and a Rotor-Gene 6000 (Corbett, Valencia, CA) real-time rotary analyzer. The
standard regression analysis was performed during each run. The Ct values were accepted
in each quantitative PCR run when the standard curve correlation coefficient was greater
than 0.98. Under such conditions, Ct values could be used to measure the copy number of
mtDNA by standard regression analyses. The results are listed in the Appendix.
2.2.11.2 mtDNA copy number in muscle tissue

DNA was extracted from quadriceps (10mg) using a QIAamp® DNA Micro Kit (Qiagen) following manufacturer’s instructions. The concentration and purity of DNA was determined by Nanodrop (Thermo Fisher Scientific). DNA was then used to estimate average mtDNA copy number/cell as described in [211]. The primers for amplification of the mitochondrial gene (12S rRNA) were 5’-CGTTAGGTCAAGGTGTAGCC-3’ and 5’-CCAGACACACTTTCCAGTATG-3’. The primers for amplification of the nuclear gene (β-actin) were 5’-GGAAAAGAGCCTCAGGGCAT-3’ and 5’-CTGCCTGACGCGCCAGG-3’. PCR amplification of mitochondrial DNA and nuclear DNA was performed simultaneously in each sample in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) and a Rotor-Gene 6000 (Corbett, Valencia, CA) real-time rotary analyzer. The Ct value for β-actin was subtracted from that for 12S rRNA to give the value ΔCt value. mtDNA copy number per nuclear genome (two actin gene copies) is calculated as $2 \times 2^{-(\Delta Ct)}$.

2.3 Epigenetic study of obesity and type 2 diabetes

The methodology for this study is described in Chapter 6.
Chapter 3: Does in vitro fertilisation increase type 2 diabetes and cardiovascular risk?

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2 Discipline of Medicine, The University of Adelaide, SA, 5005

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Email: leonie.heilbronn@adelaide.edu.au
Phone: +61 8 8222 4900
Fax: +61 8 223 3870
Statement of Authorship

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Author Contributions

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

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<th>Name of Co-Author</th>
<th>Robert Norman</th>
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<td>Contribution to the Paper</td>
<td>Critically reviewed and revised the manuscript, and approved the final manuscript.</td>
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<th>Name of Co-Author</th>
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3.1 Abstract

Since the first in-vitro fertilisation (IVF) birth in 1978, the number of children born by assisted reproductive technologies (ART) continues to increase worldwide. However, the safety issues surrounding these procedures remain controversial, and the long term impact on human health is unknown. There is emerging evidence to indicate that IVF may predispose individuals to increased incidence of obesity, elevated blood pressure, fasting glucose and triglycerides and subclinical hypothyroidism. However, few studies have been conducted to date and the underlying mechanisms are unclear. This review will summarize the existing evidence in animal models and in humans, and will discuss epigenetic alterations, which may link manipulation of the pre-implantation embryo with increased risk of the later development of obesity, insulin resistance, type 2 diabetes and cardiovascular disease in offspring. Since these diseases are the leading cause of mortality and can be delayed or prevented by lifestyle modification, prospective follow up studies in IVF born adults are now urgently required to determine the degree of risks utilizing gold standard measures in human and animal models.

Keywords: In Vitro Fertilisation (IVF); Assisted Reproductive Technologies (ART); Diabetes; Cardiovascular Disease; Epigenetic; DNA Methylation.
3.2 Introduction

Infertility has been calculated to affect up to 10% of couples (see Glossary Box) [10]. With the birth of the first baby, Louise Joy Brown, by in-vitro fertilization (IVF) in 1978, assisted reproduction technologies (ART) have been successfully used worldwide to treat infertility. The reported number of ART cycles in the western world continues to rise with an estimated 3.5 million individuals conceived through ART [212, 213]. However, concerns over the safety of ART remain, with some studies reporting an increased risk of birth defects, paediatric cancers, growth and development disorders, human imprinting disorders, physical, psychological and behavioral health issues, and some studies reporting no difference. These studies themselves are the subject of large systematic reviews, but even these reports are conflicting [35, 214, 215]. Notably, one systematic review of 25 studies showed a 30% increased risk of birth defects in ART conceived children [216]. However, others have concluded that the perinatal outcomes of singletons born at term following ART are reassuring for the general health, growth, mental and neurological development [35, 214, 217, 218].

Until recently, the longer term health implications of ART have largely been ignored. However, there are emerging reports that show IVF may predispose individuals to an increased risk of obesity, insulin resistance, type 2 diabetes and cardiovascular disease later in life (summarized in Table 11). Some of this risk may be due to the consequences of multiple pregnancies and pre-term births, since low birth weight is associated with increased rates of type 2 diabetes, hypertension and cardiovascular disease in adulthood [219]. It is recognised that even singleton ART offspring may be more at risk of preterm delivery, and low birth weight [34, 35, 220]. However, many studies have allowed for this by only studying children who were born as singletons, at term, in adequate weight ranges,
or by including controls who were born from sub-fertile parents. We must also consider whether the increased risk observed in ART offspring is related to underlying genetics of the infertile couple, or the treatment procedures *per se* [33, 34, 220] and thus studies of appropriate animal models are also essential. Below we will summarize the existing evidence, from studies pertaining to animal and human offspring, as to whether IVF predisposes to insulin resistance, diabetes and cardiovascular risk and will discuss the potential epigenetic mechanisms that may underlie this response.

### 3.3 Does IVF alter body composition?

Obesity is associated with insulin resistance, cardiovascular disease, certain type of cancers and type 2 diabetes. Although animal studies have shown that in vitro embryo culture contributes to significantly heavier offspring than spontaneous conceptions [221, 222], including large offspring syndrome [121], few studies have assessed body fat composition. Through carcass analysis, Tamashiro *et al.* [222] reported that 12-month old mice generated by in vitro embryo culture had a trend towards an increased percentage of body fat, and significantly higher serum leptin concentrations. Increased body fat was also observed by Sjoblom *et al.* [221], who showed that this effect was more pronounced in males. In that study, body weight of male offspring of in vitro culture was 11% higher, with a 36% increase in abdominal fat mass, and a 47% increase in retroperitoneal fat mass compared with control males at 12-weeks of age [221]. However, Scott *et al.* [223] were unable to demonstrate body composition differences by MRI or leptin between ART-generated mice and controls at 8 weeks of age in either gender. These inconsistent results indicate further studies in animal models at older ages are necessary.

Inconsistent results have also been documented in human studies. One study [224] reported no difference in fat percentage by Dual-energy x-ray absorptiometry (DXA)
between IVF children and controls at 4-14 years of age. However, since the IVF group in that study had significantly lower body mass index (BMI), it is unclear why this was not translated to a lower fat mass by DXA. Conversely, Ceelen et al. [225] demonstrated an increase in body fat as assessed by skinfold thickness in IVF children who were matched for BMI. Post-pubertal children in that study also had body fat assessed by DXA, and a trend towards increased body fat was also observed in the post-pubertal IVF children. Importantly, in this study the control children were born to sub-fertile parents and the divergence in results could not be explained by current size, birth weight, gestational age or any parental characteristics [225]. No studies to date have assessed for differences in abdominal fat distribution by computerized tomography or magnetic resonance imaging. This may also be of value since visceral fat is clearly associated with increased risk of type 2 diabetes and cardiovascular diseases [226]. Moreover, no studies have assessed older age groups, and since the obesity incidence as well as the amount of visceral fat increases from adolescence into adulthood, especially in males, this is a major gap.

3.4 Does IVF associate with impaired glucose metabolism and insulin resistance?

Recent studies in humans [227] and animals [222, 223] have also shown that ART may be associated with impaired glucose metabolism and insulin resistance in offspring [223]. Adult mice conceived in-vitro had a hyperinsulinaemic response to intraperitoneal glucose tolerance test (IPGTT) as compared with mice conceived in-vivo, despite similar body weights [223]. These results indicate that adult mice born by ART, including IVF were insulin resistant, independently of obesity [223]. Similarly, Tamashiro et al. [222] reported that mice derived from in vitro cultured embryos had significantly higher fasting insulin
compared with control mice. However, neither study examined mechanisms through which glucose metabolism may be impaired in these animals.

Ceelen et al. [227] reported elevated fasting glucose levels in IVF adolescents compared with controls, independently of any early life factors, or parental characteristics. No significant differences were seen in fasting insulin levels, and insulin sensitivity as measured by the glucose to insulin ratio and the homeostasis assessment model (HOMA). Whilst HOMA-IR often correlates with insulin sensitivity, it is more an estimate of hepatic and not peripheral insulin sensitivity [228]. In contrast, Sakka et al. [229] did not detect any differences in weight, glucose, fasting insulin, fasting glucose-to-insulin ratio, leptin, adiponectin, interleukin-6 or C-reactive protein in IVF children and controls. No differences in fasting glucose and insulin were detected in a New Zealand cohort, although the IVF children, were taller with significantly higher IGF1 serum levels [224]. No studies have performed oral glucose tolerance tests, or other gold standard assessments such as euglycemic hyperinsulinemic clamp to assess peripheral insulin sensitivity and β-cell capacity in ART-conceived offspring, and thus it is unknown if IVF born individuals are more insulin resistant.

3.5 Does IVF increase cardiovascular risk?

Increased blood pressure has been reported in human and animal models of ART [227, 229-231]. In mice, Watkins et al. [231] found increased systolic blood pressure at 21 weeks in offspring whose embryos were cultured in vitro from two cells to early blastocyst, compared with mice whose embryos were developed in vivo following transfer and naturally mated controls whose litter size was matched after birth. It should be noted that no difference in systolic blood pressure was found between mice derived from in vitro-cultured embryos and naturally mated controls whose litter size was not reduced at
birth, after statistical adjustment for litter size. Further studies, controlling for pup
numbers in-utero, are required to determine if ART procedures result in increased blood
pressure in mice offspring.

In humans, Celeen et al. [227] reported that systolic and diastolic blood pressure levels
were higher in IVF children than control children who were conceived naturally from sub-
fertile parents, independently of early life factors and parental characteristics. More
recently, Sakka et al. [229] also reported that children born by IVF had significantly
higher systolic and diastolic blood pressure than controls. These differences remained
significant even after dividing IVF children into small for gestational age (SGA) and
adequate for gestational age (AGA). This study may be confounded because the IVF
conceived children had significantly smaller birth weight and length, more multiple births
and preterm delivery, and were mostly firstborn and less frequently breastfed. Moreover,
the mothers of IVF children were older, and had significantly higher BMI, polycystic
ovary syndrome (PCOS) and gestational diabetes mellitus (GDM).

To date, research about the effects of ART on lipid metabolism is scarce. Rerat et al. [232]
reported that in vitro fertilised calf embryos had higher triglycerides on day 28 after birth
than calves derived from artificial insemination. More recently, Sakka et al. [229] found
that children conceived by IVF had significantly higher triglycerides, but no differences
were seen in total cholesterol, HDL, low-density lipoprotein (LDL), uric acid,
apolipoprotein-A1, apolipoprotein-B, or lipoprotein(a) values [229]. Conversely, Miles et
al. [224] found more favourable lipid profiles in a prepubertal IVF children with higher
high-density lipoprotein (HDL) levels and lower triglyceride levels than in controls. More
prospective follow-up studies in ART offspring are required before firm conclusions can
be drawn in this area.
There is also some evidence that IVF may be associated with alterations in thyroid function. Rerat et al. [232] reported that calves derived from in vitro fertilized embryos had lower T3, T4 and potassium concentrations at birth than calves derived from artificial insemination, but no differences were observed 4 months later. In humans, Sakka et al. [233] reported seven IVF children (and none of the naturally conceived children) had elevated thyroid-stimulating hormone (TSH) (7.7±1.2 mIU/L). Since these children had normal T3 and T4 and did not have detectable anti-thyroid antibodies, this is defined as subclinical primary hypothyroidism [234]. It should be noted that 4 of these children were born prematurely (<2kg), although statistically it was reported to be independent of birth weight and gestational ages. Further study is necessary to ascertain whether subclinical hypothyroidism is prominent in offspring born by ART.

Subclinical hypothyroidism is linked to cardiovascular disease [235-237]. In particular, an increased risk of coronary heart disease events and total mortality is reported in patients with a TSH concentration higher than 7 mIU/L (the hazard ratio is 1.17 and 1.42 respectively) [234]. Moreover, higher serum total cholesterol, low-density lipoprotein cholesterol and triglycerides were observed in 38 patients with subclinical hypothyroidism than in 44 BMI and gender matched controls [236]. Interestingly, there is some evidence that both subclinical hypothyroidism and hypothyroidism are associated with insulin resistance since hormone replacement therapy improves insulin sensitivity [238, 239] and patients with hypothyroidism (n=12) and subclinical hypothyroidism (n=13) had higher fasting plasma insulin, increased insulin resistance by HOMA-IR and by oral glucose tolerance test [237].
3.6 How could in-vitro culture influence adult health?

As described in Box 2, epigenetic mechanisms [240] play a key role in normal development and function via the regulation of gene expression, not only of imprinted genes such as IGF2 but also of many other non-imprinted genes. Moreover, research has shown that epigenetic modifications are sensitive to various environmental factors in early embryogenesis [241, 242]. Importantly, persistent epigenetic changes induced by environmental factors in-utero have been associated with the later development of obesity, insulin resistance and cardiovascular disease in humans and animal offspring [243, 244]. The agouti mouse is an example of this, in that reducing maternal intake of folate alters DNA methylation of the agouti gene, resulting in a yellow coat and obesity in offspring [245, 246]. Similarly, in sheep, maternal restriction of folate did not alter birth weight, but produced epigenetic changes that were associated with obesity and insulin resistance in male offspring at 22 months of age [244]. In humans, epidemiological studies also show individuals conceived during the “Dutch Hunger Winter” famine six decades ago have reduced methylation of imprinted gene insulin like growth factor 2 (IGF2) and INS-IGF2 and hypermethylation of non-imprinted genes leptin and interleukin-10 [247, 248]. These individuals had a significantly higher prevalence of obesity and cardiovascular disease, compared with their unexposed same-sex sibling [80].

3.7 Altered DNA methylation reprogramming by ART?

DNA methylation is established during the development of germ cells and is maintained at pre-implantation stage in all somatic cells. Unfaithful maintenance of the epigenetic modifications will contribute to cancer and human imprinting diseases. There is some evidence that periconceptual manipulation of oocyte or blastocyst during IVF and ICSI
may disrupt the establishment of the DNA methylation in gametes and/or with the maintenance of DNA methylation within preimplantation embryos [249]. The epigenetic defect found most often after ART in animal models is loss of DNA methylation at the maternal allele of the imprinted gene insulin like growth factor 2 receptor (IGF-2R), thereby resulting in large offspring phenotype “macrosomia” [121]. Since 2002, there have been a number of case reports which suggested an increased risk of human imprinting disease such as Beckwith–Wiedemann syndrome and Angelman syndrome associated with ART [36]. However, recent large epidemiological studies showed no correlation between ART and genomic imprinting disorders [250, 251].

Research has reported that there is an association between ART and aberrant DNA methylation both in animals and humans [252, 253]. In vitro culture of zygotes from agouti mice induced hypomethylation of viable yellow allele and increased the percentage of pups with yellow coat compared with embryos transfer without culture or natural matings [254]. A recent study has examined DNA methylation at more than 700 genes (1536 CpG sites) in placenta and cord blood of 10 IVF children and 13 controls and measured gene expression levels of several genes [253]. The results suggested that IVF was associated with lower mean methylation at CpG sites in placenta and higher mean methylation at CpG sites in cord blood. These differences were associated with gene expression differences and several of the genes identified such as peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (CEBPα), Mesoderm-specific transcript (MEST), neuronatin (NNAT) and pigment epithelial derived factor (PEDF) are associated with obesity and type 2 diabetes [253]. However, a recent larger study of 112 phenotypically normal children conceived by ART did not show a higher degree of imprint variability in comparison with 73 controls, although only 10 differentially methylated regions in DNA from maternal peripheral blood, umbilical cord
blood and amnion/chorion tissue were analysed [255]. Collectively, no studies to date have been performed to determine the association of abnormal DNA methylation and cardiometabolic disease in ART offspring.

3.8 Epigenetic candidate genes for future research?

Insulin resistance plays a key role in the development of metabolic syndrome, type 2 diabetes mellitus (T2DM) and cardiovascular diseases and may underlie the increased risk reported in some IVF studies. IVF was associated with increased DNA methylation and reduced gene expression of PPARγ and CEBPα and reduced methylation and increased gene expression of PEDF in human placental or cord blood samples [253]. These genes are involved in regulating adipogenesis. Many genes that control adipose tissue development are under epigenetic regulation [256, 257], and impaired adipogenesis has been implicated in the development of insulin resistance and type 2 diabetes [258]. PPARγ is a key regulator of adipocyte differentiation, and the glitazone class of drugs act via the PPARγ receptor to improve insulin sensitivity. Recently Fujiki et al. [256] showed that treatment of adipocytes with an inhibitor of DNA methylation increased PPARγ expression and hypermethylation of the promoter region was associated with reduced expression of PPARγ in visceral adipose tissue in rodent models of obesity. CEBPα is another master regulatory adipogenic transcription factor that interacts with PPARγ, and is required in late adipogenesis [259]. DNA hypermethylation of CEBPα has been reported in acute myeloid leukemia [257] but to our knowledge no studies have investigated this in obesity. PEDF is a neurotrophic and anti-angiogenic protein that is one of the most abundant proteins secreted by adipose tissue. PEDF is upregulated in type 2 diabetes, and induces insulin resistance in vitro and in vivo [260, 261]. The exact role of PEDF in
adipogenesis is under debate, but there is some evidence that PEDF can suppress CEBPα and inhibit adipogenesis [262].

Gene targets in skeletal muscle may also be important target of future studies as an impaired capacity to oxidize dietary fat and mitochondrial dysfunction has been described as a feature of many models of insulin resistance [263]. A master regulator of mitochondrial biogenesis is peroxisome proliferator activated receptor gamma co-activator 1a (PGC1a). Decreased PGC1a gene expression has been observed in patients with type 2 diabetes, as well as non-diabetic individuals with a family history of this disease [187]. A twofold increase in DNA methylation of the PGC1a gene promoter has been observed in pancreatic islets from 10 type 2 diabetics as compared with that from 9 control donors [125]. PGC1a promoter methylation was inversely correlated with PGC1a gene expression in liver [264]. Mitochondrial transcription factor A (TFAM) plays an important role in the stabilizing mitochondrial DNA through formation of mitochromosome, and regulates mitochondrial DNA transcription [188]. A recent study [265] reported that DNA methylation of TFAM gene promoter was associated with insulin resistance in adolescents. To our knowledge no studies have investigated whether DNA methylation of these gene candidates is altered in IVF, and further research in this area is required.

### 3.9 Conclusion

The number of children born by assisted reproductive technologies (ART) continues to increase worldwide, but the potential long term health consequences of IVF have largely been ignored. There is now emerging evidence to indicate that IVF may predispose individuals to increased incidence of obesity and associated co-morbidities. However, this issue remains controversial, because inconsistent results have been reported by the relatively small numbers of studies that have been conducted. No studies have
investigated adults, and few have utilized gold standard measures and although research has shown that manipulation of preimplantation embryos may lead to epigenetic alterations, no studies have simultaneously investigated mechanisms that may link aberrant DNA methylation to cardiometabolic risk. Since these diseases are the leading cause of mortality and can be delayed or prevented by lifestyle modification, prospective follow up studies in IVF born adults are now urgently required.
**Box 1 A glossary of relative terminology**

**Assisted reproductive technology (ART)**

All treatments or procedures for initiating pregnancy that include the in vitro handling of both human oocytes and sperm or embryos, predominantly in vitro fertilization (IVF) and embryo transfer and intracytoplasmic sperm injection (ICSI) [66].

**In vitro fertilization (IVF)**

an ART procedure that involves extracorporeal fertilization.

**Intracytoplasmic sperm injection (ICSI)**

A procedure in which a single spermatozoon is injected into the oocyte cytoplasm [66].

**Infertility**

The failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [66]. Approximately 10 percent of couples are infertile [1], and 10-15 percent of these require ART to conceive successfully.

**Preterm birth**

A live birth or stillbirth that takes place after at least 20 but before 37 completed weeks of gestational age [66].

**Low birth weight**

Birth weight less than 2,500 grams [66].

**Epigenetics**

“An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.” [34]

**DNA methylation reprogramming**

Genomewide demethylation followed by remethylation several days later, occurs early in the development of primordial germ cells and preimplantation stage in mammalian embryos [67].
Box 2 The role of epigenetic mechanisms and DNA methylation in early embryogenesis

Epigenetic ("epi" meaning on top of) code adds an essential layer of complexity to the primary genetic DNA code imposing a higher level of ‘gene regulation’, without alterations in DNA sequence. These modifications include DNA methylation, histone modifications, micro-RNAs and higher order packaging of DNA around nucleosomes [271]. Epigenetic modifications regulate the temporal and spatial gene expression patterns and are essential in embryonic, fetal and postnatal development, thereby resulting in the imprinted genes expressed only from one allele, either from paternal or maternal, whereas autosomal genes maintain a bi-allelic expression pattern. It is estimated that 1% of all human genes are imprinted, but only approximately 50 imprinted genes have been identified to date [272]. Moreover, CpG islands are found in 72% of promoters in human genes [273], suggesting that DNA methylation is important in regulating not only imprinted genes, but also in regulating the expression of many other non-imprinted genes (18).

DNA methylation is the most widely studied epigenetic mechanism and occurs via the enzymatic addition of a methyl group from S-adenosylmethionine (SAM) to the carbon-5 position of the cytosine of the Cytosine-phosphate-guanine (CpG) dinucleotide sequence. This process is catalysed by DNA methyltransferases (Dnmts) [253]. There are two major categories of Dnmts in mammalian cells on the basis of their characteristics: maintenance methyltransferase (Dnmt1) and de novo methyltransferases (Dnmt3a, Dnmt3b, and Dnmt3L) [253]. The methyl group interferes with the binding of particular transcription factors to DNA and attracts methyl binding proteins and the methyl-binding proteins also regulate transcriptional repression [274]. Hence, gene expression is generally inhibited by DNA methylation, whereas unmethylated genes are usually active in gene expression [275]. DNA methylation is also responsible for the preservation of chromosomal integrity and the inactivation of X-chromosome [253]. There are four types of DNA methylation, including hypermethylation and hypomethylation occurring on either the maternal or paternal allele.
Box 3 Key points

- Over 3.5 million individuals conceived through ART to date.
- The long term impact of IVF on human health is unknown, but increasing evidence indicates that IVF may predispose individuals to increased risk of metabolic syndrome, type 2 diabetes and cardiovascular disease.
- Persistent epigenetic changes induced by environmental factors *in-utero* have been associated with the development of obesity, insulin resistance and cardiovascular disease in humans and animal offspring.
- IVF is associated with altered DNA methylation of several genes in humans and animal models, some of these genes have been linked to development of diabetes and cardiovascular disease.
Table 11 Studies of Metabolic Health in IVF Versus Naturally Conceived Offspring

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<td>8-18 years</td>
<td>233 IVF (139 pubertal) vs. 233 controls (143 pubertal) born from sub-fertile parents</td>
<td>↑ Body fat</td>
<td>[225, 227]</td>
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<td>↑ Blood pressure</td>
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<td>↑ Fasting glucose</td>
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<tr>
<td>4-14 years</td>
<td>106 IVF (39 pubertal) vs. 68 controls (30 pubertal)</td>
<td>↑ Blood pressure</td>
<td>[229, 233]</td>
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<td>↑ Triglycerides</td>
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<td>4-10 years</td>
<td>69 IVF (34 ICSI) vs. 71 controls (friends and family members)</td>
<td>↑ TSH</td>
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<td>↑ HDL</td>
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<td>Animal Studies</td>
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<td>↓ Triglycerides</td>
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<td>8-week mice</td>
<td>IVF (n=22) and ICSI (n=12) vs. naturally mated</td>
<td>↑ Insulin AUC</td>
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<td>↑ Glucose AUC (females only).</td>
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<td>21-week</td>
<td>In-vitro cultured embryo (IV-CE, n=52), In-vivo embryo, blastocyst transferred (IV-BT, n=48), naturally mated (NM, n=154).</td>
<td>↑ Systolic blood pressure (IV-CE).</td>
<td>[231]</td>
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<tr>
<td>mice</td>
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<td>10-12 mo. Mice</td>
<td>IV-CE (n=7) versus naturally mated (n=7).</td>
<td>↑ Fasting insulin</td>
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<td>↑ Body fat</td>
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<td>12-week</td>
<td>IV-CE (n=15) versus naturally mated (n=15).</td>
<td>↑ Carcass fat</td>
<td>[221]</td>
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<td>Calves</td>
<td>IVF (n=11) vs. Artificial insemination (n=8)</td>
<td>↓ T3 and T4 at birth</td>
<td>[232]</td>
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<td>(birth and 1-mo.)</td>
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<td>↑ Triglycerides at 1-mo.</td>
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IV-CE: In-vitro cultured embryo
Chapter 4: Altered glucose metabolism in mouse and humans conceived by in-vitro fertilization (IVF)

Running title: Altered glucose metabolism in IVF offspring

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**Author Contributions**

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

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<td>Gary A. Wittert</td>
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<td>Rebecca L. Robker</td>
<td>Designed and supervised the mouse study, interpreted data, critically reviewed and revised the manuscript, and approved the final manuscript.</td>
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<td>Leonie Heilbronn</td>
<td>Conceived, designed and supervised the human and mouse study, interpreted data, critically reviewed and revised the manuscript, approved the final manuscript and acted as corresponding author.</td>
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Chapter 5: Increased susceptibility to high fat diet in female mice conceived by in vitro fertilisation (IVF) or ovarian stimulation alone

Running title: Metabolic phenotyping of female mice born by assisted reproduction.

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## Statement of Authorship

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### Author Contributions

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

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<td>Performed the mouse experiments, acquired and analyzed the data, wrote the manuscript, critically reviewed the manuscript and approved the final manuscript.</td>
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<td>Leonie Heilbronn</td>
<td>Conceived, designed and supervised the study, interpreted data, critically reviewed and revised the manuscript, approved the final manuscript, and acted as corresponding author.</td>
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5.1 Abstract

Individuals conceived by in vitro fertilisation (IVF) may be at increased risk of obesity and associated metabolic disorders. We have recently reported that male mice conceived by IVF, but not ovarian stimulation alone (OS), had impaired glucose metabolism. We now examine glucose metabolism in adult female C57BL/6J mice conceived by natural conception (NC), by OS or by IVF following chow or high-fat diet (HFD) for 8 weeks. Litter size was comparable between groups, but birth weight of IVF and OS females was lower than NC females (p≤0.001). Catch up growth was observed in OS pups by 1 week, and by 4 weeks in IVF pups. Chow fed adult females that were conceived by IVF displayed increased fasting glucose compared to NC and OS females. In response to a HFD, both IVF and OS females had increased adiposity for their size, and impaired glucose tolerance (p≤0.01). Interestingly, impaired insulin response to glucose and decreased hepatic expression of Cpt1α, a key regulator for fatty acids oxidation, were observed in OS females only (p<0.05), whereas peripheral insulin resistance and increased hepatic expression of gluconeogenic genes Pgc1α, Pck1 and G6pc were exhibited in IVF mice only (p<0.05). This study suggests that ovarian stimulation alone and IVF may program distinct metabolic effects in the offspring, but that high fat diet is required to unmask these differences. Thus IVF procedures may increase the risk of developing type 2 diabetes, in obesity prone environments.

Key words: IVF; Ovarian stimulation; Embryos; High fat diet; (DOHaD).
5.2 Introduction

Adverse prenatal environments are linked to development of cardiovascular disease and type 2 diabetes in adult human, rodent and sheep offspring [63, 86, 273], collectively known as the developmental origins of health and disease (DOHaD) hypothesis. Manipulation of only the preimplantation environment has also been shown to increase the risk of these diseases in adulthood [82, 83, 85, 289, 290]. In particular, maternal low protein diets that are given exclusively during the preimplantation period led to hypertension in adult rats [82, 289] and mice [85], and cardiovascular dysfunction in adult sheep [83].

In vitro fertilisation (IVF) exposes the preimplantation embryo to a non-physiological environment during culture, and has been associated with changes in embryo growth and development in animal models and humans [282, 285]. An increased risk of metabolic and cardiovascular diseases has been reported in IVF children and mouse models [90, 223, 225, 227, 229, 267, 268]. We have recently demonstrated that IVF young adult humans were more insulin resistant than controls, and were more susceptible to adverse outcomes of short term high fat overfeeding. Similarly, we observed that adult IVF male mice displayed impaired glucose tolerance under chow and high fat fed conditions.

Evidence from humans and animal models showed that male embryos grow faster than female embryos [88], and metabolic differences are generally more profound in male than female offspring in response to an adverse periconceptional maternal environment [86, 87]. A previous mouse study [223] reported IVF female offspring were glucose intolerant, but males maintained normal glucose tolerance although both genders displayed evidence of insulin resistance. However, this study utilised outbred mice and maternal environment and litter size were also different between groups, which may have confounded the
outcomes. Thus, it is unclear whether IVF impairs glucose metabolism in female offspring.

The process of IVF involves not only embryo culture, but ovarian hyperstimulation, which itself has been associated with poor perinatal outcomes, such as low birth weight and preterm birth [50, 51, 91], and increased systolic blood pressure in 4-year-old IVF children [291]. In our hands, reduced fetal growth was observed in males generated by either ovarian stimulation (OS) alone or by IVF, whereas differences in glucose metabolism were evident only in male mice generated by IVF. It is unknown whether ovarian stimulation alone will impact fetal growth or impair adult glucose metabolism in female offspring.

The aim of the current study was to examine whether adult female C57BL/6J mice conceived by IVF or by ovarian stimulation alone have impaired glucose metabolism compared with naturally conceived female mice following chow or high fat diet (HFD) for 8 weeks.
5.3 Materials and Methods

Mice

C57BL/6J male and female mice were obtained at 6-weeks of age, and vasectomized (CBA X C57BL/6) F1 male mice were obtained at 8-weeks of age from the Animal Resource Centre (Perth, Western Australia). (CBA X C57BL/6) F1 female mice were obtained at 6-weeks of age from the University of Adelaide Laboratory Animal Services (Adelaide, South Australia). All mice were maintained at 24°C on a 12-h light, 12-h dark cycle, with standard rodent chow diet with 20% of calories from fat, 16% of calories from protein, and 64% of calories from carbohydrate (SF06-105, Specialty Feeds, Glen Forrest, Australia) and water available ad libitum. All mice were acclimatised for 2 weeks on chow before experimentation. All experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Generating blastocysts

Mouse blastocysts were generated by natural conception (NC group), or by ovarian hormonal stimulation followed by either mating (OS group) or by IVF and embryo culture (IVF group) as reported (manuscript submitted). For the NC group, female C57BL/6J mice were placed with male C57BL/6J mice overnight. For the OS group, female C57BL/6J mice were superovulated with consecutive intraperitoneal injections of 7.5 IU equine chorionic gonadotropin (Calbiochem, San Diego, CA) followed by 7.5 IU human chorionic gonadotropin (hCG; Calbiochem) 48 h later. After injection with hCG, these females were placed with male C57BL/6J mice overnight. The following morning, female mice (NC and OS groups) with the presence of vaginal plugs were considered to be
pregnant and were humanely sacrificed by cervical dislocation 3 days later. Blastocysts were collected by flushing dissected uteri with pre-warmed HEPES-buffered minimal essential medium (Invitrogen Australia Pty. Ltd.) supplemented with 5 mg/ml human serum albumin (SAGE® Media ART-3001, CooperSurgical, USA). Blastocysts from NC and OS group were placed in Cook Research Cleave media (Cook Medical; QLD, Australia) for no more than 1 hour prior to being transferred to uteri of pseudopregnant mice (see below).

For the IVF group, the ovarian stimulation protocol was the same as for the OS group. In vitro fertilizations were performed using a modified version of that described previously [201]. At 13 hours after injection with hCG, females C57BL/6J mice were humanely killed by cervical dislocation. Cumulus-oocyte complexes were placed in Cook Research Fertilization media (Cook Medical) under paraffin oil (Calbiochem) and incubated in a modular incubator chamber at 37°C in 6% CO₂, 5% O₂, 89% N₂ for 5-6 hours with sperm collected from the cauda epididymis of male C57BL/6J mice that had been previously incubated for 1 hour in Cook Research Fertilization media (Cook Medical) for sperm capacitation. The putative zygotes were then placed in Cook Research Cleave media (Cook Medical) and checked for fertilization/cleavage the next morning. Embryos were then transferred to a new drop of Cleave media and cultured in the modular incubator chamber at 37°C in 6% CO₂, 5% O₂, 89% N₂ for a further 2 days to the blastocyst stage (90-96 hours post-hCG injection).

**Blastocysts transfer**

The sterility of the vasectomized (CBA X C57BL/6) F1 males was confirmed by mating them three times with superovulated (CBA X C57BL/6) F1 females before experiments were conducted. To generate pseudopregnant recipient females for embryo transfer,
unstimulated (CBA X C57BL/6) F1 female mice were mated with vasectomized (CBA X C57BL/6) F1 male mice and those with copulatory plugs the next morning were considered as day 0.5 of pseudopregnancy. At day 2.5 of pseudopregnancy, 7 to 10 blastocysts were transferred to uteri of each pseudopregnant mouse anesthetized by i.p. injection of Avertin (0.5 mg/g body weight, Sigma-Aldrich, St. Louis, MO). Analgesia Carprofen (5 mg/kg) (Rimadyl® Pfizer) was injected subcutaneously once after the surgery.

**Pups and diets**

Pups were born on day 19.5 of pregnancy and birth weights measured the next morning. Body weights of pups were measured weekly. Growth rate was calculated as described previously [292]. Pups were weaned at 3-weeks of age onto chow diet or high-fat diet (HFD) for 8 weeks. High-fat diet was made in house using the same recipe of D12492 of Research Diets (New Brunswick, NJ) (60% energy from fat with 91% lard and 9% soybean oil, 20% energy from protein, and 20% energy from carbohydrate). Only female offspring were examined in this study.

**Glucose and insulin tolerance tests**

At 11 weeks of age, mice were fasted for 6 hours (from 8am to 2pm) and were then challenged with either an intraperitoneal injection of glucose (2g/kg) or insulin (Actrapid®, 0.75U/kg, Novo Nordisk Australasia) for glucose or insulin tolerance tests respectively. Blood samples were obtained from tail tip for assessment of glucose at 0, 15, 30, 60, 120 minutes with a glucometer (Accu Chek Performa Monitor, Roche Diagnostics), and for insulin assays at 0, 30, 60, 120 minutes during glucose tests only by ultra-sensitive ELISA kits (Millipore). At 12 weeks of age, mice were sacrificed by
cervical dislocation; quadriceps, inguinal fat, parametrial fat and liver were immediately excised, weighed and snap frozen. Tissue samples were frozen at -80°C for later assessment.

**Quantitative real-time PCR**

Total RNA was extracted from liver and muscle using Trizol (Invitrogen, USA) following manufacturer’s instructions. The concentration and purity of RNA were determined by Nanodrop (Thermo Fisher Scientific, California, USA). cDNA was synthesized from 1μg of each RNA sample in 20μl reactions using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA). Standard control samples (25ng/μl) pooled from each cDNA sample were diluted to create a standard curve as described previously [271].

Quantitative real-time PCR was performed with the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan® Fast Universal Master Mix and TaqMan primers and probes (Applied Biosystems) for Pgc1α (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, Mm01208835_m1), Tfam (Mitochondrial transcription factor A, Mm00447485_m1), Ndufb5 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, Mm00452592), Cpt1a (carnitine palmitoyltransferase 1A, Mm01231183_m1), G6pc (Glucose-6-phosphatase catalytic subunit, Mm00839363_m1), Pck1 (phosphoenolpyruvate carboxykinase 1, cytosolic, Mm01247058_m1), Gck (Glucokinase, Mm00439129_m1), Srebf1 (Sterol regulatory element-binding transcription factor 1, Mm00550338_m1) and reference genes Gapdh (glyceraldehyde-3-phosphate dehydrogenase, Mm99999915_g1), Ppia (Cyclophilin-A, Mm02342430_g1), Rn18s (18S ribosomal RNA, Mm03928990_g1), Hprt (hypoxanthine phosphoribosyltransferase, Mm01545399_m1), and B2M (Beta-2 microglobulin, Mm00437762_m1) as per manufacturer’s instructions. The NormFinder program was used as described previously
and Hprt and Ppia out of 7 potential reference genes were identified as the best combination of reference genes. Data were analyzed using the \(2^{-\Delta\Delta CT}\) method and expressed as the fold change relative to a calibrator sample, which was included in each run.

**Western immunoblotting**

Liver and quadriceps tissues were lysed and protein concentration was determined by bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific). Lysates (20 μg protein) were resolved by SDS-PAGE (Criterion XT 10% Bis Tris precast gels, Bio-Rad, Australia) and transferred onto PVDF membranes (criterion gel blotting sandwiches, Bio-Rad, Australia). Membranes were blocked in Tris-buffered saline with Tween 20 (TBST) [10 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5)] containing 5% (wt/vol) Amersham ECL blocking agent (GE Healthcare Australia Pty. Ltd., NSW, Australia) for 1 hour at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C in 5% bovine serum albumin/TBST. Primary antibodies used were Pgc1α antibody at 1:1000 (Abcam, MA, USA, Cat. No ab54481) and Total OXPHOS (oxidative phosphorylation) antibody at 1:1000 (Abcam, Cat. No ab110413). Beta-tubulin antibody at 1:1000 (Cell Signaling, Cat. No 2146) was used as loading control. Membranes were then washed in TBST and incubated with sheep anti-rabbit IgG alkaline phosphatase conjugated secondary antibody at 1:2500 (Millipore, Temecula, CA) or goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody at 1:2000 (Millipore, Temecula, CA) for 1 hour. ECF substrate (GE Healthcare) was prepared and applied to the blots and then all blots were scanned for fluorescence by the Typhoon Trio+ (GE Healthcare) following the manufacturer’s instructions. The band intensity was measured using Image J software (The National Institutes of Health, USA).
Statistical analysis

Data are shown as mean ± the standard error of the mean, unless otherwise stated. Data were analyzed statistically with SPSS 20 (SPSS, Chicago, IL, USA) and log-transformed for analysis if not normally distributed. Single comparisons were performed with one-way or two-way ANOVA, whereas time-courses were analyzed by repeated-measures ANOVA and Bonferroni post hoc analysis. Nonparametric tests (Mann-Whitney U test or Kruskal-Wallis test) were used when required. Chi square tests were used for nominal data. Differences were considered statistically significant at P<0.05.
5.4 Results

Reduced fetal growth and postnatal catch-up growth in OS and IVF mice

Litter size and sex ratio were not different between groups (Both P=0.5, Table 13). Despite this, the birth weight of IVF pups and OS pups was significantly lower than NC pups (P≤0.001, Table 13). This difference in body weight was maintained in IVF pups until weaning (P=0.04, Figure 13A), whereas OS pups displayed rapid catch-up growth as shown by increased growth rate as compared with NC pups by 1 week (P=0.004; Figure 13A,C). After weaning, IVF mice displayed catch-up growth, and significantly increased growth rate on chow diet at 4 weeks of age and 8 weeks of age on HFD (P<0.04, Figure 13B,D). OS mice weighed significantly less than IVF and NC mice from 4 weeks after exposure to HFD (P<0.01, Figure 13B).

Increased body fat gain in HFD fed OS and IVF mice

As expected the liver weight ratio was decreased and adipose tissue weight ratio increased in mice fed a HFD (P<0.001, Figure 14). However, OS and IVF mice fed a HFD had a significantly higher percentage of body weight in the inguinal and parametrial fat pad than HFD fed NC mice (P≤0.001, Figure 14B and C).

Impaired glucose metabolism in OS and IVF mice following HFD

As expected, HFD increased fasting glucose, fasting insulin, glucose area under curve (AUC), insulin AUC and reduced insulin sensitivity (diet effect, P<0.05, Figure 15). Fasting glucose was significantly higher in IVF versus NC and OS mice, independently of diet (P<0.05, Figure 15A). No other phenotypes were noted in IVF or OS females fed a chow diet. Following a HFD, impaired glucose tolerance as assessed by glucose tolerance
test was noted in OS and IVF versus NC mice (P≤0.01, Figure 15D), and insulin sensitivity as assessed by insulin tolerance test was significantly lower in IVF mice versus NC mice only (P<0.001, Figure 15H). Lower fasting insulin (P=0.03, Figure 15B) and an impaired insulin response to glucose tolerance test as evidenced by reduced insulin AUC was observed in OS mice as compared with NC and IVF mice (P<0.05, Figure 15F).

**Altered gene expression in IVF mice**

We next examined the mRNA expression of genes involved in glucose and lipid metabolism in liver. HFD increased the expression of the genes Srebf1, Cpt1a, Pgc1α, Tfam, and Gck, but not gluconeogenic genes Pck1 and G6pc (Figure 16). Significant differences were also noted between groups, with increased hepatic expression of Pgc1α and Pck1 in IVF mice and decreased expression of Cpt1a in OS mice independently of diet. The expression of G6pc and Tfam was also higher in IVF versus NC or OS mice respectively (Figure 16). However, no difference was observed in protein levels of mitochondrial biogenesis markers Pgc1α and total OXPHOS in liver (N=4, data not shown).
5.5 Discussion

Children conceived by IVF have altered cardio-metabolic outcomes [225, 227, 229, 267] and we recently showed that IVF young adult humans display peripheral insulin resistance, an early risk factor in the development of type 2 diabetes [140, 141], and significantly greater increases in systolic blood pressure when exposed to a high fat overfeeding diet for 3 days. In this carefully controlled mouse study, we show that female mice conceived by IVF exhibit higher fasting glucose levels, independently of diet. Female mice fed chow diet, did not display any other adverse risk factors, whereas impaired glucose tolerance was unmasked by HFD, in both IVF and OS females. This suggests that manipulation of the preimplantation embryo may predispose adult female offspring to increased risk of developing type 2 diabetes, under an obesity prone environment.

In this study, birth weight was lower in female mice conceived either by ovarian stimulation alone or by IVF. This is consistent with the results we have recently reported in male mice. Lower weights have also been observed in mouse fetuses at day 14 or day 18 of gestation following ovarian stimulation [293, 294] and in human infants conceived by ovarian stimulation alone, and by IVF [40, 41, 50-53]. Furthermore, one study demonstrated that embryo culture without ovarian stimulation did not affect human birth weights [51]. Collectively, these results suggest that ovarian stimulation, rather than embryo culture, impairs fetal growth, and that is independent of fetal sex. In this study, while female donor mice for both the IVF group and OS groups underwent ovarian hyperstimulation, blastocysts for the OS group were developed in the stimulated uterine environment, whereas the IVF blastocysts were developed in the culture media prior to transfer. Previous studies have shown that both stimulated uterine and in vitro culture
environments are suboptimal and impair embryo and fetal development [206, 295]. The mechanism underlying the association between ovarian stimulation and low birth weight was not a focus of this study. However, since the embryos were transferred to unstimulated recipients, this suggests that differences are due to impaired oocyte quality and/or subsequent embryonic and fetal development, rather than changes in endometrial receptivity [293-296].

Accelerated postnatal catch-up growth in infancy and early childhood are independent risk factor of cardiovascular disease and type 2 diabetes in humans [63, 64, 67, 104, 105, 112, 273] and rodents [292, 297, 298], and having a higher BMI in late childhood and or adulthood further increases these risks [69, 99, 299]. Of note, an accelerated weight gain in late infancy was reported in IVF children as compared with spontaneously conceived controls born to subfertile parents, and early childhood growth was related to blood pressure in IVF children only [230]. In this study, we observed that OS female pups had rapid catch-up growth within 1 week after birth, whereas IVF female pups displayed catch-up growth after weaning. Both IVF and OS females had relatively higher adiposity and displayed impaired glucose tolerance following HFD. We did not observe a phenotype in chow fed females. In contrast, Scott et al. reported glucose intolerance, and a compensatory insulin response to glucose in chow fed IVF versus NC B6C3F1 female mice at 8 weeks of age [223]. A more recent study reported no evident differences in glucose tolerance in chow fed CF1xB6D2F1 female mice conceived by ovarian stimulation alone versus IVF [204], which is in line with our findings but notably this study did not test relative to an unstimulated control. Ovarian stimulation has been linked to increased systolic blood pressure percentiles and subscapular skinfold thickness in 4-year-old IVF children since these parameters were normal in children born by modified natural IVF, without ovarian stimulation [291]. Thus, we propose that ovarian stimulation
results in low birth weight, and when coupled with accelerated catch up growth, results in impaired glucose metabolism in female mice following HFD.

Interestingly, the pathways underlying differences in glucose tolerance observed in OS and IVF females may be distinct. In OS females, impaired glucose tolerance was observed in conjunction with lower insulin, indicating there may be impaired β-cell function. We did not collect pancreas however, and thus cannot assess islet numbers or size, and similarly we did not sample insulin rapidly enough during the glucose test to confirm this result. In IVF females fed HFD, impaired glucose tolerance was related to peripheral insulin resistance as assessed by insulin tolerance test. This data supports our previous findings of peripheral insulin resistance in IVF young adults, of whom 70% were women, but we did not detect any differences in β-cell secretory capacity in response to iv injection of glucose in humans.

Higher fasting blood glucose was observed in IVF females on either diet, which we have previously observed in IVF males. We speculate this may be due to increased hepatic gluconeogenesis, since we observed increased expression of key gluconeogenic genes including Pgc1α, Pck1 and G6pc in livers of IVF females. Pgc1α is a key regulator of hepatic gluconeogenesis that contributes to circulating hyperglycemia [164], and liver-specific Pgc1α-deficient mice have reduced Pck1 and G6pc expression and mild hypoglycaemia [165]. On the other hand, we have previously observed that IVF males display increased hepatic expression of key lipogenic gene Srebf1 and impaired hepatic insulin sensitivity as evidenced by reduced Akt phosphorylation in liver following an insulin stimulation test. Thus, whilst male and female IVF offspring both display hyperglycemia, this may be due to alternative activation of Pgc1α or Srebf1pathway [167]. Importantly, our findings support previous results in humans that have shown higher
fasting glucose levels in pubertal IVF children that could not be explained by confounding factors, including parental subfertility [227]. Both OS males and females displayed normal fasting glucose levels, suggesting that this may be a consequence of embryo culture, rather than ovarian stimulation per se. In addition, we have previously reported that IVF males also displayed impaired glucose tolerance independently of diet, but the OS males did not display any phenotype. Taken together, the data suggest that in vitro culture induces more profound alternations of metabolic pathways as compared with in vivo tract environment.

Sex differences in phenotypes have also been identified in several developmental programming models of adult diseases both in humans and animals [86, 87]. Some of these differences may be because males grow faster than females in utero and thus are more vulnerable to malnutrition [89]. Furthermore, marked difference in the gene expression patterns are noted in male and female preimplantation embryos [80]. In line with this, our data show sexual dimorphism in the offspring conceived by IVF or OS. Interestingly, impaired glucose tolerance was found in chow fed IVF males but not in IVF females on chow, and was observed in OS females following HFD while OS males exhibited no phenotypes up to 11 weeks of age. This supports different molecular pathways may be altered in response to distinctive stresses in utero [80]. However, further studies are required to determine the underlying mechanisms.

Only a handful of studies have examined the long term effects of IVF to date [90, 204, 223, 268, 269, 300]. However, none have previously controlled for maternal environment and/or genetics. Environmental differences have included differences in litter sizes between IVF and control groups [223], which may have confounded outcomes [301], or have compared to models of ovarian stimulation alone [204], which we and others [293-296] have shown impairs fetal growth, and may alter metabolic outcomes [204].
Furthermore, outbred mice are also less suited for metabolic studies [204], which may have contributed to some of the discrepancies observed in male mice generated by IVF with lower systolic blood pressure reported in CF1xB6D2F1 males [204], whereas increased systolic blood pressure was reported in (CBA x C57/BL6)F1 and FVB [90, 268]. Our study highlights that inbred NC mice conceived following embryo donation from non-stimulated donors are necessary to properly study the long-term effects of IVF.

In conclusion, ovarian stimulation reduced fetal growth in utero, but adult female mice were only susceptible to metabolic consequences of manipulation of the preimplantation environment, under obesity prone conditions. Further, in vitro culture of embryos uniquely increased fasting glucose and markers of hepatic gluconeogenesis, suggesting that IVF in particular, may increase the risk of developing type 2 diabetes, later in life.
Acknowledgments

No potential conflicts of interest relevant to this article were reported.

M.C. performed the mouse experiments, acquired and analysed the data, wrote the manuscript, critically reviewed the manuscript and approved the final manuscript. L.W. contributed to acquiring data, critically reviewed the manuscript and approved the final manuscript. F.W. contributed to acquiring data, critically reviewed the manuscript and approved the final manuscript. G.A.W. contributed to interpretation of the data and helped to draft the manuscript, critically reviewed the manuscript and approved the final manuscript. R.J.N. contributed to interpretation of the data and helped to draft the manuscript, critically reviewed the manuscript and approved the final manuscript. R.L.R. designed and supervised the study, interpreted data, critically reviewed and revised the manuscript, and approved the final manuscript. L.K.H. conceived, designed and supervised the study, interpreted data, critically reviewed and revised the manuscript, and approved the final manuscript.

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Preliminary results of this study were presented at the Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology, Sydney, Australia, 25 - 28 August, 2013.
Table 13 Litter characteristics and birth weight of female pups

<table>
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<tr>
<th>Group</th>
<th>Pups</th>
<th>Litters</th>
<th>Litter size</th>
<th>Range of litter size</th>
<th>Sex ratio (M/F)</th>
<th>Females</th>
<th>Birth weight</th>
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<tr>
<td>NC</td>
<td>49</td>
<td>8</td>
<td>6.2 ± 0.6</td>
<td>4 - 8</td>
<td>1 : 1.33</td>
<td>28</td>
<td>1.69 ± 0.04</td>
</tr>
<tr>
<td>OS</td>
<td>51</td>
<td>7</td>
<td>7.4 ± 0.7</td>
<td>5 - 10</td>
<td>1 : 0.82</td>
<td>23</td>
<td>1.54 ± 0.03  (\Delta)</td>
</tr>
<tr>
<td>IVF</td>
<td>54</td>
<td>8</td>
<td>6.9 ± 0.5</td>
<td>5 - 9</td>
<td>1 : 1.08</td>
<td>28</td>
<td>1.52 ± 0.03  *</td>
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NC: natural conception; OS: ovarian stimulation; IVF: in vitro fertilization. Data presented as mean ± SEM; Litter size (P=0.5); M/F: male/female (P=0.5); \(\Delta\) P=0.001, * P<0.001.
Figure 13 Body weight and weight growth in female mice.

Specific growth rate was calculated as follow: \((W_n - W(n-1)) / W(n-1)\). \(W\) is body weight, and \(n\) is age in weeks. (A), * IVF vs NC, \(P=0.04\); (n=21-26). (B), # IVF vs OS, \(P<0.001\); * IVF & NC vs OS; \(P<0.01\); ** Diet effect, \(P<0.001\). (C),* OS vs NC, \(P=0.004\); # IVF vs OS, \(P=0.006\). (n=21-26). (D), Chow: * IVF vs NC, \(P=0.03\); HFD: # IVF vs OS & NC, \(P<0.01\).
Figure 14 Weight ratio of liver, inguinal fat and parametrial fat in female mice.

Tissue weight was normalized to body weight. ** Diet effect, P<0.001. (B), * OS vs NC, P=0.02; IVF vs NC, P=0.001; (C), * OS vs NC, P=0.01; IVF vs NC, P<0.001.
Figure 15 Fasting glucose, fasting insulin, glucose and insulin response to glucose tolerance tests (C-F), and insulin tolerance tests (G-H) in female mice.

(A), * IVF vs NC, P=0.01; IVF vs OS, P=0.03; ** Diet effect, P=0.01. (B), * IVF vs OS, P=0.03; ** Diet effect, P=0.002. (D), * OS vs NC, P=0.01; IVF vs NC, P<0.001; ** Diet effect, P<0.001; AU, arbitrary unit. (F), * OS vs IVF, P=0.001. OS vs NC, P=0.04; ** Diet effect, P<0.001. (H), * IVF vs NC, P<0.001; ** Diet effect, P=0.02.
Figure 16 Hepatic gene expression of female mice.

White bars represent NC group, grey bars represent OS group and black bars represent IVF group (n=6). (A), ** Diet effect, P=0.009. (B), * OS vs IVF & NC, P<0.01; ** Diet effect: P<0.001. (C), * IVF vs NC, P=0.03; IVF vs OS, P<0.001; ** Diet effect, P=0.005. (D), *IVF vs OS, P=0.003; ** Diet effect, P=0.003. (E), * IVF vs NC, P=0.05; IVF vs OS, P=0.02. (F), * IVF vs NC, P=0.03. (G), ** Diet effect, P<0.001.
Chapter 6: Obesity alone or with type 2 diabetes is associated with tissue specific alterations in DNA methylation and gene expression of \textit{PPARGC1A} and \textit{IGF2}

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Author Contributions

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

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<td>Carried out the epigenetic experiments; participated in the acquisition, analysis and interpretation of the data and drafted the manuscript, and approved the final manuscript.</td>
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<th>Anne Macpherson</th>
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<td>Designed the pyrosequencing assays, participated in the acquisition of pyrosequencing data, and helped to draft the manuscript, and approved the final manuscript.</td>
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<th>Gary A. Wittert</th>
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<td>Participated in the conception and design of the study, revised the manuscript critically for important intellectual content and gave final approval of the version to be published, and acted as corresponding author.</td>
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6.1 Abstract

**Background:** Epigenetic modifications of key genes have been linked to the development of aging related diseases, such as type 2 diabetes, with increased DNA methylation of the transcriptional co-activator, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) in islets and skeletal muscle of patients with type 2 diabetes. Here, we examined DNA methylation and gene expression of PPARGC1A and insulin like growth factor-2 (IGF2) in adipose tissue and skeletal muscle of lean and morbidly obese individuals with or without type 2 diabetes.

**Methods:** Adipose tissue and skeletal muscle biopsies were collected from 24 lean, obese, and obese patients with type 2 diabetes (n=8/group). DNA methylation and gene expression of PPARGC1A and IGF2 were measured using pyrosequencing and quantitative real-time PCR respectively.

**Results:** DNA methylation and expression of both genes varied in a tissue specific manner (P<0.05). The highest levels of PPARGC1A methylation were observed in subcutaneous adipose tissue and lowest in muscle (P≤0.001), whereas IGF2 methylation was lowest in subcutaneous adipose tissue as compared with visceral adipose tissue and muscle (P≤0.04). Expression of PPARGC1A and IGF2 was highest in muscle and lowest in subcutaneous adipose tissue (P≤0.001) and PPARGC1A expression was conversely correlated with DNA methylation in skeletal muscle (r=-0.54, P=0.008). Obese patients with type 2 diabetes had higher PPARGC1A methylation in subcutaneous adipose tissue (P=0.01) and lower IGF2 DNA methylation in muscle (P=0.01) as compared with lean individuals. Obese patients with and without type 2 diabetes had reduced expression of both genes in subcutaneous adipose tissue (P≤0.04) as compared to lean individuals.
Conclusions: This study showed tissue specific DNA methylation and gene expression of \textit{PPARGC1A} and \textit{IGF2}, which may also be associated with obesity and type 2 diabetes. Further study of the effects of tissue specific DNA methylation on risk of obesity and type 2 diabetes in a larger cohort is now warranted.

Keywords: DNA methylation; Gene expression; \textit{PPARGC1A}; \textit{IGF2}; Type 2 diabetes; Obesity.
6.2 Introduction

An increasing number of studies suggest that epigenetic modifications in certain genes are associated with subsequent disease susceptibility later in life, both in humans and in animal models [80, 244, 302, 303]. For instance, Godfrey et al. reported that lower maternal carbohydrate intake in early pregnancy was associated with increased methylation of retinoid X receptor-α gene in umbilical cord tissue, and higher incidence of childhood adiposity at age 9 years [304]. Supporting this, Relton et al. showed the association between altered DNA methylation patterns in cord blood at birth and later adiposity in childhood, with perturbed gene expression [305]. Individuals conceived during the “Dutch Hunger Winter” famine also had an elevated prevalence of obesity, insulin resistance and cardiovascular disease [80], with reduced DNA methylation of imprinted gene, insulin-like growth factor 2 (IGF2), and increased DNA methylation of non-imprinted genes leptin and interleukin-10 as adults, as compared with their unexposed same-sex siblings [247, 248]. Whether altered DNA methylation and expression of key genes occurs in peripheral insulin sensitive tissues is a source of current investigation.

Recent studies have demonstrated increased promoter DNA methylation and decreased gene expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) in pancreatic islets [306], in skeletal muscle from patients with type 2 diabetes [307], and in liver biopsies from individuals with non alcoholic fatty liver disease [264]. Low birth weight, potentially reflecting in utero deprivation, was also associated with increased PPARGC1A DNA methylation in skeletal muscle in adults at increased risk of diabetes [308]. PPARGC1A is a master regulator of mitochondrial biogenesis and oxidative phosphorylation [309], and is linked to development of insulin resistance and type 2 diabetes [124]. Although reduced PPARGC1A expression in adipose tissue has
been reported in insulin resistance [310-312], whether DNA methylation is also altered in this tissue is unknown. Furthermore, whether similar methylation changes in peripheral tissues exist in obese individuals without type 2 diabetes is also unclear.

*IGF2* is a key factor in human growth and development and is imprinted through the differentially methylated region (DMR), thus only the paternal allele is expressed. Aberrant imprinting of *IGF2* gene is associated with the overgrowth disorder Beckwith-Wiedemann syndrome [116] and the growth restriction disorder Silver-Russell syndrome [117]. Hypomethylation of *IGF2* DMR0 which is located 5′ to the main *IGF2* promoters in humans [313] leads to bi-allelic expression of *IGF2* [118] and is associated with human colorectal cancer, Wilms tumour [118-120], and has also been linked to later development of obesity and insulin resistance in individuals exposed to famine in utero [80]. To date, no studies have examined *IGF2* DMR0 methylation and gene expression in adipose tissue and skeletal muscle from patients with obesity and type 2 diabetes.

The present study aimed to determine the tissue specificity of DNA methylation and expression of *PPARGC1A* and *IGF2* and their association with obesity, in the presence and absence of type 2 diabetes, compared with lean individuals, and also, to examine the relationships between DNA methylation patterns and gene expression. The data showed that DNA methylation and gene expression of *PPARGC1A* and *IGF2* occurs in a tissue specific fashion, and that altered DNA methylation and expression of both genes appears to be associated with obesity and type 2 diabetes.
6.3 Methods

Subjects

The characteristics of 24 subjects are presented in Table 14. Rectus abdominus muscle, subcutaneous adipose tissue and omental adipose tissue biopsies were obtained from eight obese individuals without type 2 diabetes and eight diet controlled obese patients with type 2 diabetes (four females and four males for each group) who were undergoing gastric bypass surgery for the treatment of morbid obesity. Rectus abdominus muscle and abdominal subcutaneous adipose tissue were obtained from four lean males who were undergoing hernia surgery. Vastus lateralis muscle and abdominal subcutaneous adipose tissue were collected from four lean female volunteers [270, 314]. All tissues were snap frozen for later analysis. The study was approved by the human research ethics committees of the University of Adelaide, Calvary Hospital, and Burnside Hospital. Informed consent was obtained from all participants.

DNA and RNA extraction

Genomic DNA and total RNA were extracted from adipose tissue (120-150 mg) and muscle tissue (10-30mg) using a QIAamp DNA Mini kit (Qiagen, Valencia, CA) and Trizol (Invitrogen, USA) respectively following manufacturer’s instructions. The concentration and purity of DNA and RNA were determined by Nanodrop (Thermo Fisher Scientific, California, USA).

Bisulphite modification

0.4 - 1μg genomic DNA was bisulphite treated to convert unmethylated cytosines to uracil using the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic
Signatures Pty Ltd, NSW, Australia) as per the manufacturer’s instructions. All samples were bisulphite treated simultaneously to prevent possible batch effects. There was insufficient subcutaneous adipose tissue sample in one lean and one obese muscle sample for bisulphite conversion.

**Nested touchdown PCR**

A nested touchdown PCR strategy was used to amplify bisulphite treated genomic DNA. PCR primers (shown in Supplementary Table 3) were designed to amplify regions containing specific CpG sites using Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden).

5 CpG dinucleotides sites in the promoter of the PPARGC1A gene were examined (Supplementary Figure 1). The outer primers amplify the region on the minus strand of chromosome 4:23892115-23892603 (UCSC BLAT results from Assembly Feb 2009 (GRCh37/hg19)) to yield a 489 bp product, and the inner primers with biotinylated reverse primer and first-round product as template amplify the region minus strand chromosome 4:23892285-23892570 to yield a 286 bp product. The first 4 of these CpG sites were previously measured by Ling et al. in human islets [306] and Brons et al. in human muscle [308]. The 5th CpG site is identical to that measured by Gemma et al. in umbilical cord blood [126].

5 CpG sites in the IGF2 DMR0 (chr11:2,126,035-2,126,372 in NCBI build 36.1) [315] were also examined (Supplementary Figure 1). The outer primers amplify the region on Chromosome 11(+ strand): 2169429-2169753 (UCSC BLAT results from Assembly Feb 2009 (GRCh37/hg19)) to yield a 325 bp product. The inner primers with biotinylated reverse primer and first-round product as template amplify the region Chromosome
11:2169452-2169751 to yield a 300 bp product. These sites have been previously quantified by others: all 5 on the minus strand by Steegers-Theunissen et al. [315], and the first 3 on the plus strand by Murrell et al [120] both in peripheral blood.

All PCR reactions for one tissue were completed simultaneously with the ABI 7500 sequence detection system using two MicroAmp® fast 96-well reaction plates (Applied Biosystems, Foster City, CA). Each PCR reaction also included a no template negative control and unmodified DNA control (Qiagen, Valencia, CA). Only samples with single target size bands without primer dimers were used for pyrosequencing.

**Pyrosequencing**

Quantification of cytosine methylation percentage was carried out on a PyroMark MD Pyrosequencing System (Biotage, Uppsala, Sweden) using PyroMark Gold Q96 CDT Reagents 972824 (Qiagen, Valencia, CA). Specific pyrosequencing primers (Supplementary Table 3) were designed using Biotage Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden). For both genes, two different pyrosequencing assays utilized different forward sequencing primers, with the first to sequence the region containing the first 3 CpG’s of interest, and the second to analyse the 4th and 5th CpG’s of interest. Methylation was quantified using Pyro Q-CpG 1.0.9 Software (Biotage, Uppsala, Sweden) which calculated the percentage of methylated cytosines to total cytosines at each CpG site. The mean of five methylated sites was calculated for each sample.

**Quantitative real-time PCR**

cDNA was synthesized from 1μg of each RNA sample in 20ul reactions using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA) consistent with the manufacturer’s protocol. Standard control samples (25ng/μl) pooled from each cDNA
were diluted to create a standard curve. There was insufficient subcutaneous adipose tissue sample in two obese patients, and one visceral adipose tissue and one muscle sample in the obese group.

Quantitative real-time PCR was performed with the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan primers and probes for \textit{IGF2} (Hs00171254\_m1), \textit{PPARGC1A} (Hs01016719\_m1) and Beta-actin (Hs99999903\_ml) (Applied Biosystems) according to manufacturer’s instructions. To calculate the normalized relative expression levels, the individual expression levels of each sample were divided by the expression values of the beta-actin gene, which was not different by groups.

\textbf{Statistics}

Quantitative data were presented as mean ± SEM unless indicated. Differences between groups were assessed by one-way ANOVA followed by a Fisher’s LSD post hoc test. The effect of obesity on methylation was investigated by linear mixed models, with tissue, individual site methylation, group and sex as between factors and age as a covariate, Bonferroni post hoc test as appropriate. Correlations were calculated using Pearson correlation coefficients for normally distributed values and Spearman correlation coefficients when normality was rejected. Statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). All P values were 2-sided, and P≤0.05 was considered significant.
6.4 Results

Clinical data

Lean patients were aged between 18-63 years, while obese and obese patients with type 2 diabetes were between 41-56 years of age (Table 14). Obese and obese patients with type 2 diabetes had higher body mass index (BMI) than those who were lean, whereas obese patients with type 2 diabetes had increased fasting plasma glucose compared to lean and obese patients (Table 14). Plasma lipids, including total cholesterol and triglycerides did not vary between groups.

DNA methylation and gene expression of PPARGC1A

DNA methylation of PPARGC1A varied significantly between tissues, with the highest DNA methylation in subcutaneous adipose tissue and lowest in muscle (Figure 17A). We also observed the highest expression of PPARGC1A in muscle and the lowest expression in subcutaneous adipose tissue, consistent with these tissue specific patterns of methylation (Figure 17B). Overall, there was also a significant negative relationship between average DNA methylation and gene expression of PPARGC1A gene in muscle (Figure 17C), but not in either visceral or subcutaneous adipose tissue (data not shown). DNA methylation of PPARGC1A in muscle was negatively correlated with age (Figure 17D).

In subcutaneous adipose tissue, obese patients with type 2 diabetes had increased DNA methylation of PPARGC1A compared with lean individuals (P=0.01, Figure 17E), and decreased PPARGC1A expression was also observed in obese patients with or without diabetes as compared to lean individuals (P=0.001, P=0.04, respectively) (Figure 17F).
PPARGC1A DNA methylation or gene expression in visceral adipose tissue or muscle tissue did not vary between groups however (data not shown).

**DNA methylation and gene expression of IGF2**

As expected for an imprinted gene, DNA methylation of IGF2 was higher than that of PPARGC1A. IGF2 methylation was lowest in subcutaneous adipose tissue as compared to visceral adipose tissue or skeletal muscle (Figure 18A). The expression of IGF2 was lower in visceral adipose tissue than in muscle (P≤0.001) (Figure 18B). IGF2 methylation and expression in muscle were also significantly correlated with age (Figure 18C and D). In skeletal muscle, obese patients with type 2 diabetes had lower DNA methylation than lean individuals (59±2% vs 69±2%, P=0.01), but this was not significant between obese and lean individuals (P=0.1) (Figure 18E). IGF2 methylation in adipose tissue was not different between groups (data not shown), but IGF2 expression was lower in subcutaneous adipose tissue in obese patients with or without type 2 diabetes as compared with lean individuals (Figure 18F). There was no association between DNA methylation and expression of IGF2 in any tissue examined (data not shown).
6.5 Discussion

Epigenetic modifications in key genes may be important in the development of obesity and type 2 diabetes. In this study, we investigated the expression and DNA methylation of \textit{PPARGC1A} and \textit{IGF2} in subcutaneous and visceral adipose tissues and skeletal muscle from lean, obese and obese individuals with type 2 diabetes. Interestingly, we observed that \textit{PPARGC1A} and \textit{IGF2} gene had tissue specific differences in DNA methylation and expression, and that these may be influenced by obesity and aging.

DNA methylation plays a key role in regulating gene expression via the addition of a methyl group to the carbon-5 position of the Cytosine-phosphate-guanine (CpG) dinucleotide sequence in promoter regions, which interferes with the binding of particular transcription factors to DNA [316]. Our results indicate that higher DNA methylation was related to lower gene expression of \textit{PPARGC1A} in a tissue specific manner. To our knowledge, this is the first study to demonstrate that both subcutaneous and visceral adipose tissue had significant higher DNA methylation of \textit{PPARGC1A} compared with muscle in humans. Supporting this, our data also show a significant negative correlation between average DNA methylation and gene expression of \textit{PPARGC1A} across the whole cohort in muscle, although this was not observed in adipose tissue. This is broadly consistent with Barres et al. who showed methylation levels of \textit{PPARGC1A} promoter were negatively correlated with mRNA expression and mitochondrial DNA in skeletal muscle that was collected from patients with or without type 2 diabetes [307]. Of interest, Ling et al. observed a similar correlation between \textit{PPARGC1A} promoter methylation and mRNA expression in islets from patients with type 2 diabetes [306]. It is not clear why this association is also not seen in adipose tissue in our study, and may be a reflection of tissue specific factors that separately influence expression.
Previous studies have shown increased promoter DNA methylation of *PPARGC1A* in skeletal muscle and islets in patients with type 2 diabetes [306, 307]. Our data also showed this association, but only in subcutaneous adipose tissue. This may be related to different CpG sites being selected for study and the different DNA methylation detection methods employed. We did observe reduced *PPARGC1A* expression in subcutaneous adipose tissue in obese patients with type 2 diabetes. Reduced *PPARGC1A* expression has been reported in both visceral and subcutaneous adipose tissues as well as muscle from patients with type 2 diabetes in many studies previously [125, 187, 307, 311]. It is not clear why these associations were not observed in visceral adipose tissue, and again may be a reflection of size of cohort or other tissue specific factors that influence expression.

We next examined DNA methylation and expression patterns of *IGF2*. Firstly and as expected, we detected higher DNA methylation in paternally imprinted *IGF2* gene as compared to the non-imprinted *PPARGC1A* gene [120, 248, 306, 308, 317]. We also observed visceral adipose tissue and muscle had higher DNA methylation than subcutaneous adipose tissue. However the changes in methylation were not accompanied by inverse changes in expression of *IGF2*, in that muscle also had higher expression of *IGF2*. However, this is not necessarily contradictory, as shown by Murrell et al. [120], who reported that DNA methylation of the *IGF2* DMR0 examined here, may play a role in activation and not suppression of gene expression. This relationship is complex and still requires further investigation, as the expression of the human *IGF2* gene is controlled at least by 5 promoters [313, 318, 319].

To date, few studies have examined associations between DNA methylation and gene expression of *IGF2* in obesity and type 2 diabetes. Heijmans et al. showed individuals who were prenatally exposed to the Dutch famine had lower methylation of *IGF2* in blood
and increased prevalence of obesity, insulin resistance and type 2 diabetes in later life [81, 248, 320, 321]. In our study, patients with type 2 diabetes also had lower IGF2 DNA methylation in muscle as compared to lean individuals. We also observed lower expression of IGF2 in subcutaneous adipose tissue in obese and obese patients with type 2 diabetes as compared to those who were lean. Together these results suggest that DNA methylation and gene expression of IGF2 may be associated with obesity and type 2 diabetes, but this result needs to be confirmed in a larger, age-matched cohort.

Interestingly, our data showed that aging may be associated decreased DNA methylation of both genes and increased IGF2 expression in muscle. Supporting this, Heijmans et al. demonstrated IGF2 methylation in blood cells decreased 4% in 10 years within the age range of 43–70 years [248]. In contrast, Issa et al. have reported increased methylation of the IGF2 promoter which switched from monoallelic to biallelic methylation during aging in adult human colon, breast and lung tumors [322]. However, Cui et al. reported leukocyte IGF2 methylation was stable to middle age [323, 324]. A recent longitudinal study also showed DNA methylation levels in five of eight imprinted loci were stable over 2–20 years in blood and buccal cell samples [325]. These results indicate tissue specific difference with aging in humans. Similarly, genome-wide analysis of DNA methylation in rats demonstrated significant tissue differences and age-related tissue specific changes, including hypomethylation in intra-abdominal tissues and hypermethylation in liver [326]. However, care should be taken when comparing results of these various studies in humans, since different assays, tissues, and the examined loci varied by study [327].
Conclusions

This study demonstrates tissue specific DNA methylation and gene expression of *PPARGC1A* and *IGF2*, which may be related to aging, obesity and type 2 diabetes. The strong negative correlation between DNA methylation and gene expression of *PPARGC1A* indicates epigenetic regulation may play a key role in expression of this gene, at least in muscle. These outcomes provide the rationale for further study of a larger cohort and emphasize the importance of assessing DNA methylation of these genes at least, in a tissue specific manner in humans.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors acknowledge the Channel 7 Children’s Research Foundation for funding to conduct this study.
Table 14 Clinical characteristics of the subjects

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<th>Obese with diabetes</th>
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<tr>
<td>Age (years)</td>
<td>31 ± 6*</td>
<td>49 ± 3</td>
<td>48 ± 2</td>
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<tr>
<td>BMI</td>
<td>22.3 ± 0.6*</td>
<td>47.6 ± 1.8</td>
<td>46.3 ± 1.8</td>
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<td>Fasting Glucose (mmol/L)</td>
<td>5.2 ± 0.6</td>
<td>4.9 ± 0.3</td>
<td>8.5 ± 1*</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
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<td>5.2 ± 0.3</td>
<td>5.4 ± 0.4</td>
<td>0.76</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>2.6 ± 0.8</td>
<td>0.37</td>
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Data are presented as mean ± SEM. * denotes P<0.01 of the asterisked marked group as compared to both other groups.
Figure 17 PPARGC1A DNA methylation and gene expression in muscle, subcutaneous and visceral adipose tissue.
(1A) PPARGC1A DNA methylation levels, ** denotes significance between subcutaneous and visceral adipose tissue at P=0.001; # denotes significance between muscle and both subcutaneous and visceral adipose tissues at P<0.001; (1B) PPARGC1A expression in muscle is significantly higher than subcutaneous and visceral adipose tissue at P<0.001; (1C-1D) depicts the inverse relationship between PPARGC1A average DNA methylation and (1C) PPARGC1A expression and (1D) age in muscle; (1E) Obese patients with type 2 diabetes had higher DNA methylation of PPARGC1A in subcutaneous adipose tissue as compared to lean and (1F) obese patients with and without diabetes had lower PPARGC1A expression in subcutaneous adipose tissue as compared with lean individuals. *P=0.01; **P=0.001; # P<0.001; † P=0.04. AU: arbitrary units.
Figure 18 *IGF2* DNA methylation and gene expression in muscle, subcutaneous and visceral adipose tissue.
(2A) Lower DNA methylation was observed in subcutaneous adipose tissue than visceral adipose tissue and muscle; (2B) Lower IGF2 expression was observed in subcutaneous adipose tissue and visceral adipose tissue versus muscle; (2C-2D) Age was negatively correlated with IGF2 DNA methylation (2C) and positively correlated with IGF2 expression (2D) in muscle; (2E) Patients with type 2 diabetes had significant lower IGF2 DNA methylation in muscle; (2F) IGF2 expression was decreased in obese and obese patients with type 2 diabetes in subcutaneous adipose tissue.

*P=0.01; **P=0.001; # P<0.001; † P=0.04; § P=0.1. AU: arbitrary units.
Supplementary Table 3 Primers and sequences of *PPARGC1A* and *IGF2* for pyrosequencing

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<td>AAGGTATTITTAAGGTAGGAGGAG AAATAATAAACAACCTCTCCACC</td>
<td>286bp</td>
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<td>PyroSequencing 1</td>
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<td>IGF2</td>
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* denotes 5’ biotinylated primer
PPARGC1A

#1

5’-CCCTAAGGCAGTTAGGAGAAAAGCTACATGTATGAAACGAGCCGGAATCA

#2

AAGCTGATCTGACAGCAGCGACGAGACGACTGTATTTACTAACCATTGTTTCTGGGAGCCT

ATGAGAGAAAATGAAAATAATTGAAGGAAAGCTGAAAGGATGGGGTTTTGTGGCTTG

#3

TGGGGGCAGC

CG

AATTCTGGGTGGAGGAGTTTGTTTATACCTT

#4

CCTTATATGGAGCAAAGAAAACTGCAGCAACTCTT

CG

GGAGCTGGTATTCCCTACTGCCA

#5

TGGGGGACGCGAAATTCGCTGGAGAGGAGTTTTGGTTTATACCTT-3’

IGF2

#1

5’-AAGGAGGAGGCCAGCACAAGCAGACACAGCTGGTAGAGCGAGCCAGTGAGGGACG

#2

CGCTGGCTGTGCTTCCTGGGAATGAACCCCCTCCCCGCGGGAGGCTGGGAGAGCAGCAAGGGGGACCAGGTG

#3

CGCTGGCTGGTTCTGGGAAATGAACCCCCTCCCCCGCG

CG

G

AATTCTGGGTGGAGGAGTTTGTTTATACCTT

#4

GGGGCCCAGCGAAATTCGCTGGAGAGGAGTTTTGGTTTATACCTT

CG

CCATCAGGAGGAGAG

AAGCTAAACCTAGGGGGGACATGGAG

#5

GAGCCCTCTCTGTGCCAGGGAGGCTGGGAGAGCAGCAAGGGGGACCAGGTG

CG

CCATCAGGAGGAGAG

AAGCTAAACCTAGGGGGGACATGGAG
Supplementary Figure 1 The wild-type DNA sequence and methylation target sites of the **PPARGC1A** and **IGF2**.

The upper graph shows three methylation sites (#1, #2, and #3) in the promoter of **PPARGC1A** assessed in this study. The lower graph shows three methylation sites (#1, #2, and #3) in the **IGF2** DMR0 assessed in this study.
Chapter 7: Conclusions and future directions
7.1 Conclusions

The IVF human study demonstrates for the first time that young adults conceived by IVF were more insulin resistant and tended to be more susceptible to the metabolic consequences of high fat overfeeding. This finding confirms our hypothesis and supports the accumulating evidence from previous studies in IVF children, and suggests that IVF adults may have increased risk of developing metabolic syndrome, type 2 diabetes and cardiovascular disease later in life. This is of importance, since the number of IVF cycles has been steadily increasing in developed countries and the increased risk of metabolic disease may be modifiable with early lifestyle interventions.

Of note, embryo culture system has been improved in the last two decades. For instance, most IVF units used to generate their own culture media and for a period of time, included human serum in the media. This was shown to increase the risk of infectious disease transmission and inhibit human embryo growth [328]. In the last 10 years, most IVF have switched to commercially produced IVF media, which have undergone strict quality control programs and do not generally include human serum. Differences in the metabolic health may occur in individuals conceived by using different culture system as compared with naturally conceived controls, thus this needs to be investigated in further studies.

However, changes in IVF practice over the sampling timeframe studied here were infrequent, with only one culture change in SA. Changes in recent years have been more frequent, but perinatal outcomes continue to be compromised [39, 41], thus we expect this phenotype will be apparent even utilising optimised culture conditions, as we and others [329] observed in the mouse studies.

Our data in mice support the findings in the human study, and further suggest that it is the process of IVF itself, rather than genetic and/or environmental differences, that contribute
to the metabolic phenotype, although sexual dimorphism may be evident. As shown in the phenotype summary in Table 15, both males and females conceived by IVF displayed higher fasting glucose. Additionally, glucose intolerance was observed in IVF male mice fed chow and HFD and IVF female mice fed HFD. Interestingly, we observed that the mechanisms underlying these phenotypes may be distinct between genders. IVF male mice display increased hepatic lipogenic gene expression and hepatic insulin resistance at normal and high body weight, whereas IVF female mice exhibit elevated hepatic gluconeogenic gene expression, and peripheral insulin resistance when fed a HFD only. This is in line with the literature that metabolic differences are generally more profound in male than female offspring in response to an adverse periconceptional maternal environment [86, 87]. Some of these differences may be because males grow faster than females in utero and thus are more vulnerable to stresses in utero [89]. In addition, marked difference in the gene expression patterns are noted in male and female preimplantation embryos [80]. However, further studies are required to determine the underlying mechanisms.

The process of IVF involves ovarian hyperstimulation and embryo culture. In this study, while female donor mice for both the IVF group and OS groups underwent ovarian hyperstimulation, blastocysts for the OS group were developed in the stimulated uterine environment, whereas the IVF blastocysts were developed in the culture media prior to transfer. Previous studies have shown that both stimulated uterine and in vitro culture environments are suboptimal and impair oocyte quality and/or subsequent embryonic and fetal development [206, 293-296]. Our work in mice also delineates the effects of embryo culture versus ovarian stimulation. Ovarian stimulation impaired fetal growth, since all the mice conceived by ovarian stimulation alone or by IVF had significantly lower birth weight as compared with controls. Male mice conceived by OS did not display any
metabolic phenotype, however ovarian stimulation increased susceptibility to HFD in females, since both OS and IVF females displayed increased adiposity and glucose intolerance. In addition, we observed that HFD fed OS females had lower insulin response to glucose injection, indicating there may be impaired pancreatic beta cell function. This finding is of importance since up to 7% of children are conceived by ovarian stimulation alone or ovulation induction in developed countries, which is nearly twice the percentage of children conceived by IVF [212].

The results obtained using the mouse model suggest that in vitro culture induces more profound alternations of metabolic pathways as compared with an in vivo environment following ovarian stimulation as the phenotypes were more severe in IVF mice than OS mice of either gender. The results also revealed sexual dimorphisms in offspring conceived by IVF or OS, including postnatal growth pattern, body fat percentage, hepatic gene expression, glucose tolerance as well as insulin sensitivity. This suggests the metabolic phenotype depends on the type of stress and gender.

Our data shows that the preimplantation period is a critical stage for development and later adult health. The mechanisms underlying these differences are unclear, but may involve epigenetic modifications and/or changes in mitochondrial numbers and function. Our data also showed that obese patients with and without type 2 diabetes displayed tissue specific DNA methylation of PGC1a and IGF2, highlighting the importance of measuring multiple individual tissues in humans and controlling for adiposity when utilising these measures.

In conclusion, this study highlights an increased risk of developing type 2 diabetes and cardiovascular disease in IVF offspring later in life.
### Table 15 Phenotype summary of OS and IVF mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chow</td>
<td>HFD</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td>No phenotype No phenotype</td>
<td>↓ Body fat No phenotype</td>
</tr>
<tr>
<td></td>
<td>↓ fasting insulin ↓ hepatic Cpt1a gene expression</td>
<td>↓ fasting insulin ↓ hepatic Cpt1a gene expression</td>
</tr>
<tr>
<td><strong>IVF</strong></td>
<td>↑Fasting glucose Glucose intolerant Hepatic insulin resistant</td>
<td>↑Fasting glucose Glucose intolerant Hepatic insulin resistant</td>
</tr>
<tr>
<td></td>
<td>↑ lipogenic gene expression in liver ↑ lipogenic gene expression in liver</td>
<td>↑ lipogenic gene expression in liver</td>
</tr>
<tr>
<td></td>
<td>↓ fasting insulin Beta cell dysfunction?</td>
<td></td>
</tr>
</tbody>
</table>

- **Chow** indicates a normal diet.
- **HFD** indicates a high-fat diet.
- **↓** indicates a decrease.
- **↑** indicates an increase.
- **No phenotype** indicates no observable phenotype difference.
- **Beta cell dysfunction?** indicates uncertainty regarding beta cell dysfunction.

**Note:** The table compares the phenotype summary of OS (Obese Sprague-Dawley) and IVF (In Vitro Fertilization) mice under normal and high-fat diet conditions.
7.2 Future directions

1. **Are gender differences apparent in hepatic and peripheral insulin sensitivity in IVF versus naturally conceived humans?**

   We showed peripheral insulin resistance in IVF conceived individuals, although no difference in fasting glucose or insulin. This is similar to observations in cohorts of young non-diabetic individuals, whom have a strong family history of type 2 diabetes, and whom are up to 6 times more likely to develop type 2 diabetes [270, 274]. Our mouse data suggests that hepatic insulin sensitivity is perturbed in both IVF males and females. In our human study, we did not perform the 2-step clamp with tracers and thus cannot assess hepatic insulin resistance and only 4 IVF males were included, and thus we cannot separate out results by gender. Therefore it remains currently unknown whether there is sexual dimorphism in hepatic and peripheral insulin sensitivity in IVF versus naturally conceived adult individuals.

2. **Is insulin sensitivity, mitochondrial metabolism and DNA methylation altered in IVF conceived male and female mice at distinct stages of development?**

   Hepatic and peripheral insulin resistance were not assessed by gold standard methodology (hyperinsulinemic euglycemic glucose clamp) in the mouse study. Our work also suggests that there may have differences in mitochondrial copy number in blastocysts between groups (Appendix 2) and reduced mitochondrial content and activity was observed in porcine embryos that were cultured in vitro [330]. A number of studies have reported an association between IVF and aberrant DNA methylation both in animal models and humans [122, 123, 180, 249, 252, 331], although whether this is transmitted into IVF offspring is unclear, at this stage.
3. **Are metabolic risks in IVF offspring mitigated by natural IVF?**

The world’s first IVF baby was conceived by natural IVF without hormone stimulation, and natural IVF is performed when clinically warranted, and on patient request. To our knowledge, no studies have examined the long term health outcomes of natural versus stimulated IVF. Our mouse data suggests that it is the process of ovarian stimulation, rather than IVF per se, that is responsible for producing smaller size at birth, and this may also underlie metabolic susceptibility to HFD in IVF females. The effect of in vitro culture of embryos generated without the use of gonadotropin stimulation on subsequent glucose metabolism in humans is unknown.

4. **Is shorter embryo culture better (i.e. 3 days versus 5 days)?**

Advances in cell culture media have led to a shift in practice in recent times from transferring the embryo at cleavage stage (Day 3) to the blastocyst stage (D5/6). This is recognized as preferable since this is the more biologically correct stage for the embryo to be in the uterus and this process enhances self-selection of viable embryos. However, transfer of the cleavage stage embryo is still the preferred time-point in some IVF centers. Meta-analysis of 12 RCTs shows that pregnancy rates are higher when cleavage stage embryo transfers are performed, but live birth rates are higher when blastocyst transfer is performed [332, 333]. Transfer at the blastocyst stage may increase the risk of preterm birth (<37 weeks) in singletons [334]. Comparison studies on the effects of transfer at cleavage versus blastocyst on glucose metabolism in IVF offspring have not been performed.
5. **What is the effect of embryo cryopreservation (vitrification versus slow freezing)?**

Whilst embryo cryopreservation is standard procedure in the practice of IVF, the effects of this on mechanisms surrounding embryo development are less clear. Data concerning infant outcomes following embryo cryopreservation are reassuring [335], pregnancy rates are increased [332] and the mean birth weight of babies is higher from cryopreserved embryos as compared with fresh embryo transfer [336]. However, a recent study suggests that whilst cleavage stage embryos had similar mitochondrial activity following cryopreservation, blastocyst mitochondrial activity was reduced. The effects of vitrification versus slow freezing on glucose metabolism in IVF offspring, are still unknown.

6. **What is the effect of intracytoplasmic sperm injection (ICSI) on glucose metabolism?**

The number of ICSI cycles has continued to increase in recent years and now account for about two thirds of all ART cycles in western countries [31]. Children conceived as a result of ICSI treatment have an increased risk of congenital abnormality [43]. Increased adiposity was also reported in adolescent girls conceived by ICSI [337]. Higher blood pressure was observed in 8-year-old children but not in adolescents born after ICSI as compared with spontaneously conceived controls [338, 339]. Scott et al. reported both ICSI and IVF female mice offspring fed chow at 8 week of age displayed glucose intolerance as compared with NC females while no differences were observed between ICSI and IVF females, but this study did not control genetics, litter size and maternal environment. To date, no studies have examined the effects of ICSI on glucose metabolism in human offspring. Thus it is unclear whether individuals conceived by ICSI have more profound metabolic phenotype than IVF conceived individuals.
Appendices
Appendix 1 Changes of metabolic risk factors in male and female participants following 3 days high fat overfeeding

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male (n=10)</th>
<th>Female (n=18)</th>
<th>$p$ value</th>
<th>Diet</th>
<th>Sex</th>
<th>interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Overfeeding</td>
<td>Baseline</td>
<td>Overfeeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>70.8 ± 8.9</td>
<td>71.4 ± 8.8</td>
<td>60.6 ± 8.8</td>
<td>61.4 ± 8.7</td>
<td>&lt;0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>113 ± 7</td>
<td>113 ± 8</td>
<td>108 ± 5</td>
<td>107 ± 7</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>59 ± 4</td>
<td>59 ± 6</td>
<td>61 ± 7</td>
<td>60 ± 7</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/L)</td>
<td>4.1 ± 0.2</td>
<td>4.2 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>0.04</td>
<td>0.8</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>11.3 ± 3.0</td>
<td>12.4 ± 4.1</td>
<td>11.8 ± 2.3</td>
<td>12.9 ± 2.7</td>
<td>0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose, clamp (mmol/L)</td>
<td>5.2 ± 0.6</td>
<td>5.1 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>5.1 ± 0.3</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Insulin, clamp (μU/mL)</td>
<td>175 ± 48</td>
<td>193 ± 42</td>
<td>210 ± 44</td>
<td>212 ± 53</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1 ± 0.6</td>
<td>2.3 ± 0.8</td>
<td>2.2 ± 0.5</td>
<td>2.4 ± 0.6</td>
<td>0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>TEE (KCal/day)</td>
<td>2840 ± 151</td>
<td>2611 ± 260</td>
<td>2153 ± 308</td>
<td>2067 ± 256</td>
<td>0.07</td>
<td>0.003</td>
</tr>
<tr>
<td>RMR (KCal/day)</td>
<td>1667 ± 190</td>
<td>1659 ± 229</td>
<td>1463 ± 180</td>
<td>1483 ± 159</td>
<td>0.9</td>
<td>0.07</td>
</tr>
<tr>
<td>RQ</td>
<td>0.79 ± 0.06</td>
<td>0.84 ± 0.05</td>
<td>0.81 ± 0.07</td>
<td>0.85 ± 0.08</td>
<td>0.007</td>
<td>0.6</td>
</tr>
<tr>
<td>Δ RMR,clamp (KCal/day)</td>
<td>278 ± 100</td>
<td>219 ± 108</td>
<td>223 ± 142</td>
<td>206 ± 105</td>
<td>0.16</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Data are presented as mean ± Standard deviation. BP, blood pressure; HOMA-IR, homeostasis model of assessment - insulin resistance; TEE: Total energy expenditure; RMR, resting metabolic rate; RQ, respiratory quotient; GIR, glucose infusion rate; FFM, fat free mass; iAUC, incremental area under the curve; IGF1, insulin like growth factor 1; HMW, high molecular weight; CRP, C-reactive protein; MCP-1, monocyte chemotactic protein-1. RMR was adjusted for fat free mass. GIR/FFM was adjusted for clamp insulin levels.

As shown in the Appendix 1, there was no evident gender difference in response to 3 days high fat overfeeding. Male participants had higher systolic blood pressure, lower insulin sensitivity (GIR/FFM) and HMW adiponectin levels. This is consistent with the findings in the literature, highlighting the importance of controlling gender in metabolic phenotyping study.
Appendix 2 Mitochondrial DNA copy number of blastocysts

This figure shows that mitochondrial DNA copy number was significantly lower in IVF blastocysts than OS blastocysts. This suggests the in vitro environment impaired mitochondrial biogenesis as compared with the in vivo environment following ovarian stimulation. However embryo developmental was not closely controlled in this case and further experiments are needed to confirm this finding and determine whether alterations in mitochondrial biogenesis may be associated with differences in offspring metabolic phenotypes.
Appendix 3 Tissue weight of liver and adipose tissues

Male (A-C) and female (D-F) mice.

Of note, in response to a HFD, OS and IVF mice displayed sexual dimorphism in body fat composition.
Appendix 4 Liver Pgc1α and Igf2 DMR2 DNA methylation of male mice

Igf2 DMR2 (differentially methylated region 2) DNA methylation in liver of male mice increased following a HFD.
Appendix 5 Hepatic expression of Igf2 in male and female mice

Of note, in response to a HFD, mice displayed sexual dimorphism in hepatic Igf2 expression.
Appendix 6 mitochondrial biogenesis markers Pgc1α, Tfam and Ndufb5 and mitochondrial DNA copy number in muscle of female mice fed a HFD

White bars represent NC group, grey bars represent OS group and black bars represent IVF group. A: n=6; B: n=8.
Appendix 7 Protein levels of PGC1α and total OXPHOS in liver of mice

Male mice (A-B) and Female mice (C-D).

Of note, in response to a HFD, mice displayed sexual dimorphism in PGC1α protein expression in liver.
Appendix 8 Protein levels of PGC1α and total OXPHOS in muscle of mice

Male (A-B) and female mice (C-D).

Of note, in response to a HFD, mice displayed sexual dimorphism in PGC1α protein expression in quadriceps muscle.
Bibliography


