The effect of maternal overweight and obesity on the viability and metabolism of human oocytes and early embryos.

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A thesis submitted for the Degree of Doctor of Philosophy

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August 2015

Abstract

Overweight and obese (OWOB) women are reported to have a lower chance of becoming pregnant. The nutritional enrichment of the periconceptional environment apparent in obese women may be detrimental for embryo viability. This is the first study to examine the development and metabolism of embryos derived from OWOB women compared with those from normal weight women.

Measurements of oocyte quality, embryo development and utilisation of exogenous and endogenous metabolites were performed. Results were compared for normal weight and OWOB and examined in relation to blastocyst development and pregnancy outcome. Patients attending the Hull IVF Unit for fertility treatment (n=176) donated supernumerary embryos (n=808) from day 5 to 7/9 of development. Full ethical approval was obtained.

Oocytes from overweight or obese women were smaller than those from women of healthy weight, yet post-fertilization they reached the morula stage faster and, as blastocysts, showed reduced glucose consumption and elevated endogenous triglyceride levels. There were no differences in the metabolism of pyruvate or lactate. Amino acid metabolism was more active in embryos generated from OWOB women and significant differences were seen in the turnover of individual amino acids that were also predictive of blastocyst formation and pregnancy outcome. The significant findings were independent of male BMI.

In vitro culture supplementation with insulin did not influence glucose consumption, but did appear to modulate developmental progression to the blastocyst stage as well as the utilisation of specific amino acids. Whereas, when the medium was supplemented with l-carnitine (LC), embryos consumed LC from the medium, and at 0.05mM LC addition; embryos showed reduced intracellular triglyceride levels and increased exogenous glucose consumption. However, at 0.5mM LC addition; embryo viability was compromised in cohorts of embryos from women with a BMI <25kg/m^2.

The data support the hypothesis that embryo viability is compromised in OWOB women. This may reduce not only the chances of conception, but have long-term implications for the health of the offspring in later life.

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Abbreviations

ACE	Association of clinical embryologists
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine Diphosphate
AMH	Anti-mullarian hormone
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate-activated protein kinase
ANOVA	Analysis of variance
ART	Assisted reproductive technology
ATP	Adenosine Triphosphate
BCB	Brilliant crestyl blue
BD	Blastocyst development
BMI	Body Mass Index
BSA	Bovine serum albumin
cAMP	cyclic Adenosine Monophosphate
CACT	Carnitine-Acylcarnitine Translocase
COC	Cumulus oocyte complex
COS	Controlled ovarian stimulation
CORE	Consumption, release
CP(R)	Clinical pregnancy (rate)
CPT	Carnitne-Palmitoyl Transferase
DABA	Diaminobutyric Acid
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic Acid
DOHaD	Developmental Origins of Health and Disease
DZT	Dizygotic twinning
EBBS	Earls balanced salt solution
EDTA	Ethylenediaminetetracetic acid
EGA	Embryonic Genomic Activation
EPPS	3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulphonic acid
ER	Endoplasmic Reticulum
ET	Embryo transfer
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide (oxidised)

$FADH_2$	Flavin Adenine Dinucleotide (reduced)
FAO	Fatty Acid β-Oxidation
FCS	Fetal calf serum
FET	Frozen embryo transfer
FF	Follicular fluid
FFA	Free fatty acids
FOAD	Fetal Origins of Adult Disease
FSH	Follicle stimulating hormone
G-6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GC	Granulosa cell
GEE	Generalised estimating equations
GH	Growth Hormone
GI	Glycolytic Index
GLUT	Glucose transporter
GnRH	Gonadotrophin releasing hormone
GTP	Guanosine Triphosphate
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
hCG	human chorionic gonadotropin
HEPES	N-(2-hydroxyethyl)piperazine-N-ethanesulphonic acid
HFEA	Human Fertilisation & Embryology Authority
HK	Hexokinase
hMG	human menopausal gonadotrophin
HPLC	High Pressure Liquid Chromatography
hr/pi	hours post insemination
HSL	Hormone Sensitive Lipase
ICM	Inner cell mass
ICSI	Intra-cytoplasmic sperm injection
IGF	Insulin-like growth factor
IGFR2	Insulin-like growth factor 2 receptor
IR	Implantation rate
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
IU	International units

IVM	In vitro maturation
IVF	In vitro-fertilisation
KMO	Kaiser-Meyer-Olkin Measure of Sampling Adequacy
LB(R)	Live birth (rate)
LC	L-Carnitine
LH	Lutenising hormone
LPL	Lipoprotein lipase
MAPK	Mitogen-activated phosphorylase kinase
MBR	Multiple birth rate
MI	Metaphase I
MII	Metaphase II
MPF	Mitogen promoting factor
MZT	Monozygotic twining
NAD+	Nicotinamide Adenine Dinucleotide (oxidised)
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NADP+	Nicotinamide Adenine Dinucleotide Phosphate (oxidised)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NEFA	Non-esterified fatty acids
NICE	National Institute for Health & Clinical Excellence
OCC	Oocyte cumulus complex
ONS	Office for National Statistics
OPA	o-phthaldialdehyde
Ox-Phos	Oxidative Phosphorylation
OWOB	Overweight and obese
pb	polar body
PBS	Phosphate buffered saline
PC	Principal component
PCOS	Polycystic ovarian syndrome
PDH	Pyruvate Dehydrogenase
PDT	Positive hCG pregnancy determined
PFK	Phosphofructokinase
PGC	Primordial Germ Cell
pi	post insemination / injection
PN	Pronuclei
PPP	Pentose Phosphate Pathway

QA	Quinn's Advantage (series media)
REDOX	Reduction oxidation
RCT	Randomised controlled trial
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SHBG	Sex hormone binding globulin
SSA	Synthetic serum albumin
SD	Standard deviation
SEM	Standard error of the mean
SGLT	Sodium dependent glucose transporter
TAG	Triacylglycerol
TE	Trophectoderm
TCA	Tricarboxylic acid cycle
THF	Tetrahydrofolate
TG	Triglyceride
TTP	Time to pregnancy
WHO	World health organisation
WoW	Well-of-the-well
ZP	Zona Pellucida

Acknowledgements

I would like to thank The Hull IVF Charitable Trust for their funding support and the Directors of East Riding Fertility Services for permitting me leave to undertake this work. I am grateful to my supervisors; Dr Roger Sturmey, Professor Henry Leese, Professor Stephen Killick & Mr Piotr Lesny for their guidance and advice throughout this project. I would also like to thank the current and past members of the HYMS research team and both the staff and patients at the Hull IVF Unit, without whom I would not have been able to complete this project. Finally I would like to acknowledge the encouragement given to me by my family and friends and dedicate this book to my husband for his absolute support and understanding.

Declaration

I confirm that this work is original and that if any passage(s) or diagrams(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain and unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Aspects of this thesis have been published and are detailed below;
Leary C, Killick S, Leese H, Sturmey R (2012) Compromised oocyte quality and embryo development in women with an elevated BMI. Human Fertil, 15,1,p42 (O C15).

Leary C, Killick S, Sturmey R (2012) Comparison of clinical pregnancy rates and embryo cryosurvival in frozen cycles for normal and overweight obese women. Human Fertil, 15,1,p1-26 (Poster 40).

Leary C, Killick S, Sturmey R (2012) Maternal BMI and incidence of dizygotic twining amongst IVF patients. Human Reproduction 12 (S2) p226 (Poster 316).

Leary C, Killick S, Sturmey R (2013) Human embryo developmental dynamics in relation to maternal and paternal BMI. Human Fertil16,3,p218 (Poster)

Leary C, Sturmey R (2014) Evaluation of human embryo metabolism in embryos derived from IVF and ICSI. Human Fertility 17,2,p145 (Poster)

Leary C, Leese H, Sturmey R (2015) Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. Human Reproduction 30,1, p122-132.

Bolton V, Leary C, Harbottle S, Cutting R, Harper J (2015) How should we choose the 'best' embryo? Human Fertility (*in press*)

Chapter 1 : Introduction

1.1Obesity; definition, incidence and relationship with body mass index

The proportion of adults who are overweight or obese (OWOB) has more than doubled since 1980 (World Health Organisation; WHO 2012) and currently stands at approximately 50% of the world's population (Ng et al., 2014). Body mass index (BMI), which is the ratio of body weight in kilograms (kg) to height in metres squared (m^2), is the most widely used assessment criterion. A BMI of <18.5kg/m^2 is defined as underweight, 18.5-24.9 normal weight, 25-29.9 overweight and >30kg/m^2 obese according to the WHO classification.

The increased body mass associated with obesity is predominantly due to an accumulation of fat (Webster et al., 1984). However the relationship between BMI and % fat is not simple and may be described by a linear or curvilinear relationship, with a small, but significant age-gender interaction (Meeuwen et al., 2010; Shah & Braverman et al., 2012). The current cut-offs for BMI classification are based on >25% body fat for men and >35% for women. However, recent studies propose that using cut-offs of >30% body fat may be a more appropriate indicator of adiposity for women; this would equate to a BMI index of 25 being reclassified as obese and conversely for men the index should be increased to 28 to account for differences between the sexes in bone and muscle mass (Okorodudu et al., 2010). Such an adjustment would increase the sensitivity of BMI for detecting body fatness and could limit the misclassification to 16% of women, based on the findings from 13,601 subjects (Romero-Corral et al., 2008).

Despite the shortcomings of BMI, it is a frequently used gauge of normality for a person's weight. Although BMI does not account for lean body mass, nor does it provide any indication of the distribution of body fat it is a standardised and easily reproduced measurement. Furthermore, generally accepted thresholds have been established, originally based on the statistics of Life Insurance Companies and there is a wealth of data with which findings can be compared.

BMI is therefore used as a proxy measure of obesity throughout this thesis, as for most people it correlates with their amount of body fat with sufficient accuracy (NHLBI, 1998). Furthermore as this study primarily focuses on Caucasian women of reproductive age this risk of misclassification is reduced (Pedersen et al., 2011). As the

evidence presented above would suggest that the present cut-offs for BMI provide an underestimate of obesity prevalence, the data have been grouped according to normal (BMI <25) or overweight and obese (OWOB - BMI ≥ 25kg/m^2) further reducing the risk of misclassification.

1.2 The developmental programming of susceptibility to obesity

There is substantial evidence that maternal BMI is a significant early risk factor for obesity in the offspring, which may first be recorded during *in utero* growth (Catalano et al., 2009), is then reflected in birth weight (McDonald et al., 2010; Kulie et al., 2011), during childhood (Reilly et al., 2005) and may persist into adulthood (Yu et al., 2011). Brisbos et al., (2012) identified 17 studies that reported a significant correlation between maternal BMI and both infant and adult obesity in the offspring.

Leese, (2014) provided a succinct historical review of our current understanding of the influence that maternal, gamete, embryo, fetal and infant nutrition has on the health of the offspring in later life. The review highlighted the process of appreciation of knowledge that has lead to the current 'Developmental Origins of Health and Disease' concept (DOHaD); beginning with an understanding of how the uterine environment influences growth (Walton & Hammond 1938) and how fetal birth weight was subsequently shown to be related to the onset of adult disease (Barker 1990, 2007). The current emerging realisation that not only is the embryo especially sensitive to environmental modulation, but that this may be traced back further to the developmental environment of the gametes has highlighted the need for a 'life-course approach to issues of human health' (reviewed by Leese, 2014).

With specific regard to how maternal over nutrition may predispose the conceptus of OWOB women to macrosomia; Pedersen (1967) hypothesised that fetal overgrowth and adiposity occur as a consequence of poor maternal glucose control, resulting in fetal hyperglycemia and excess fetal insulin secretion (reviewed by Catalano & Hauguel-De Mouzon, 2011). In support of this, is the finding that increased fat mass *in utero* is reflected in greater levels of insulin in the cord blood of these babies (Catalano et al., 2009). Conversely, fetal exposure to maternal under nutrition, during a critical period of development could result in fetal programming of metabolism and the downstream development of adult metabolic disorders. Such considerations underlie the initial

Barker Hypothesis of the fetal origins of adult disease (FOAD) (Barker 1990, 1992), a concept which was primarily proposed to explain the inverse relationship between weight at birth and predisposition to type 2 diabetes and cardiovascular disease in later life. Which was later extended to encompass the 'thrifty phenotype hypothesis', which suggests fetal adaptations for survival made in response to maternal under nutrition, predispose the offspring to rapid 'catch-up-growth' if subsequently exposed to a nutrient rich environment after birth (Hales & Barker, 1992). Figure 1.1 aims to show the parallels between these described theories and the relationship between birth weight and later development of obesity linked through *in utero* adaptations to the maternal nutritional status.

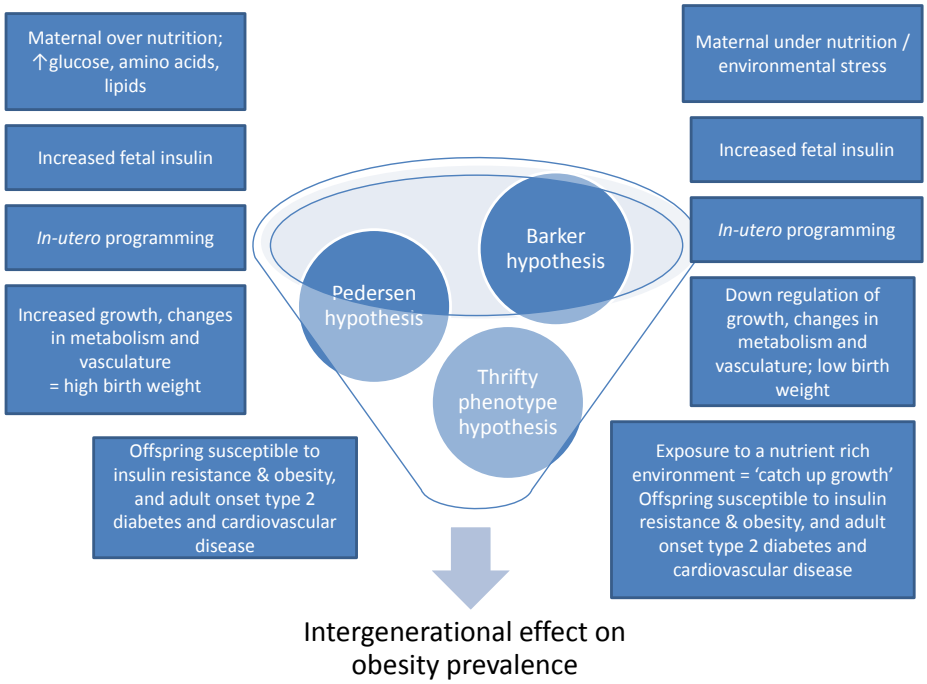

Figure 1.1 The intergenerational effects of obesity.

A summation of several theories (based on text from Hales & Barker, 1992; Catalano & Hauguel-De Mouzon, 2011) proposed to explain the developmental programming of obesity, which may begin *in utero* in response to suboptimal nutrition.

These early theories of FOAD have been modified, as it has become increasingly apparent that adaptations to suboptimal nutrition may begin much earlier than during fetal development and the period around the time of conception is viewed as a particularly vulnerable period of development. The preimplantation embryonic development phase and sensitivity to environmental modulation has become a particular

focus for research. Comprehensive reviews, primarily of experimental animal data have been compiled by Chason et al., (2011); Eckert & Fleming, (2011) and Lucas, (2013), although data from human embryos is currently lacking. Observations in humans have highlighted the possible intergenerational effects of maternal nutrition on oocyte development, another uniquely vulnerable period to environmental modulation (reviewed by Ashworth et al., 2009).

With respect to maternal obesity, weight gain increases slowly between the ages of 25 to 44, (i.e., within the fertile age range) (Bjorntorp et al., 1975). Given the fact that the average age of first time mothers has been gradually increasing (27.9 years in 2011 compared to 25 in 2004) and nearly half of all live births (49%) in 2011 were to mothers aged 30 and over (Office for National Statistics), this may be contributing to the rising rates of pre-pregnancy obesity. The latest Department of Health figures indicate that 53% of pre-gravid women were OWOB and since women who are overweight pre-pregnancy are more vulnerable to gestational weight gain and post-partum weight retention (Adamo et al., 2012), this would increase the risks further in any subsequent maternities, potentiating the obesity cycle (as aimed to illustrate in Figure 1.2). However, it is also very likely that genetic and lifestyle factors will be passed on by the parent and will increase the probability of obesity and metabolic disorders in the offspring and the relative contributions of each remains a matter of controversy (Gluckman et al., 2005).

Collectively these studies and observations serve to highlight how the wider concept of DOHaD may be applied to explain how obesity begets obesity and the need for focused studies exploring the impact of maternal BMI on the uniquely, environmentally sensitive periods of; oocyte and preimplantation embryo development.

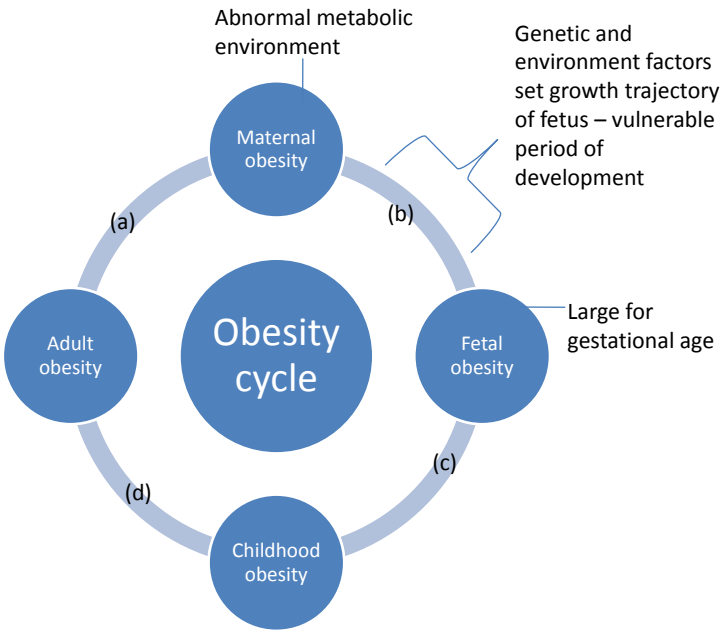

Figure 1.2 Obesity seemingly begets obesity.

(a) Women are increasing delaying pregnancy until their late 20 to early 30's, a time when they are more vulnerable to weight gain. (b) Women who are overweight pre-pregnancy are more vulnerable to gestational weight gain (c) Children born to OWOB mothers are significantly more likely to be large for gestational age. (d) High infant birth weight is correlated with childhood obesity, obesity in adolescence and adulthood.

1.3 Factors that lead to the development of obesity

In simple terms, weight gain occurs when energy intake is greater than energy expenditure. The increased availability of energy dense food that are high in fat, sugar and salt and increasing sedentary lifestyle habits have been implicated as causal factors in the obesity epidemic (Leonard, 2002). Inappropriate weight gain can lead to a variety of health problems and metabolic disturbances. For example, a number of metabolic changes in obesity are associated with insulin resistance. Under normal circumstances insulin, secreted by pancreatic beta cells in response to the absorption of foodstuffs, especially glucose, stimulates the uptake of (1) glucose into target cells where it has numerous actions; notably, as a fuel, biosynthetic intermediate or, and, in liver and skeletal muscle, as a precursor of glycogen (2) lipids into adipose cells to be stored as triacylglycerols (3) uptake and use of amino acids. Insulin also acts on the hypothalamus to reduce appetite and stimulate energy expenditure. The secretion of leptin, which is produced by adipose tissue, is stimulated by insulin and further acts on

the hypothalamus to suppress appetite. In apposition to insulin are glucagon (from the alpha cells of the pancreas), adrenaline (from the adrenal medulla), cortisol and growth hormone. (The interactions of these hormones with those of the reproductive system are discussed in further detail in Chapter 3.)

In obese individuals, both insulin and leptin secretion is increased and cells may become resistant to their action, leading to high blood glucose and lipid levels and lack of appetite regulation. Insulin resistance is thought to occur by consequence of increased total body fat and is associated with a central or android-type fat distribution. In obese individuals elevated plasma non-esterified fatty acid concentrations and a reduction in sensitivity to insulin leads to impaired muscle glucose utilisation and disruption of the glucose-fatty acid cycle, first described by Randle et al., (1963) and recently reviewed by Hue et al., (2009). An overview of the metabolic interactions and responses to high and low blood sugar is provided in Figure 1.3 and is discussed again in more detail in Chapter 5. In obese women, raised levels of triglycerides, glucose and insulin have been reported in the ovarian follicle (Robker et al., 2009) but the consequence this exposure has for oocyte maturation and embryo viability has not been investigated in any detail.

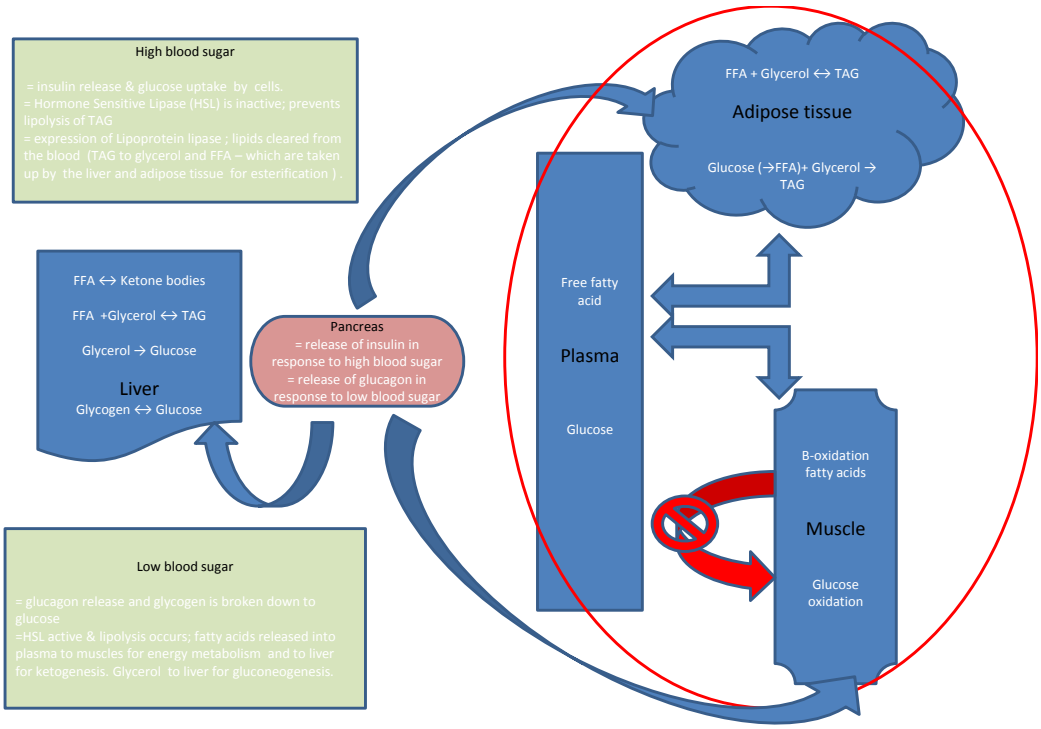

Figure 1.3 The glucose-fatty acid cycle.

It was proposed by Randle et al (1963) that if muscle is able to choose between free fatty acids (FFA) and glucose, fatty acids will be metabolised and glucose uptake is restricted (the specific metabolic interactions that explain how β-oxidation of FFA prevents the oxidation of pyruvate and suppresses glycolysis will be discussed later). The effects of glucose are mediated by insulin (the normal responses to low/ high blood glucose are described in the boxes), but when there is an abundance of glucose and fatty acids, insulin does not stimulate glucose uptake as normal. This cycle can contribute to insulin resistance, decreased utilisation of glucose and increased accumulation in the blood, failure of insulin function to permit activation of hormone sensitive lipase (HSL) leading to increased lipolysis of triacylglycerol (TAG) and increased blood fatty acid and glycerol concentrations. Insulin is also needed for the expression of lipoprotein lipase (LPL), which is required to clear lipids from the blood. (A more comprehensive description of the specific mechanisms in different cell types is provided by Frayn 1996; Hue et al., 2009).

1.4 *In vivo* oocyte and embryo development and manipulations *in vitro*–as 'Assisted Reproductive Technologies'

To provide a context for how maternal overweight and obesity impacts on the reproductive system a brief recap of the stages of oocyte and embryo development is now provided. In addition the assisted reproductive techniques (ART) used to manipulate and direct these events will be discussed, specifically *in vitro* fertilisation

related treatments, which are now estimated to contribute to 1-2% of all births in the UK (HFEA 2011). Around 1 in 7 couples experience difficulty conceiving (failure to become pregnant within 2 years of regular unprotected sexual intercourse; NICE 2013), the cause of which may be diverse and multifaceted. Male factor infertility (i.e. a low sperm count, motility, morphology) accounts for approximately a third of cases, with female factors (i.e. tubal obstruction, ovulatory disorders, endometriosis) accounting for a further third. In 25% of cases no cause can be identified and lifestyle factors may be a significant contributory, specifically the impact of maternal obesity on the time taken to achieve, and ability to sustain a pregnancy has received much attention in recent years.

1.4.1 Oocyte development and maturation *in vivo*

The ovary consists of follicles and interstitial tissue. Granulosa, theca, endothelial cells and supportive connective tissue form the components of the follicle in which the oocyte is enveloped. Follicular and oocyte development (oogenesis & folliculogenesis) occurs in the cortex of the ovary.

Germ cells migrate from the yolk sac into the developing gonad with accompanying somatic cells early in fetal life, with the primordial germ cells (PGC) eventually becoming oocytes and the somatic cells differentiating into the follicular cells. The relevance of this, to the context of this thesis being that these developing cells may be vulnerable to environmental cues that affect their future development in the next generation.

In oogenesis, PGC divide by mitosis and differentiate into oogonia at week 8-13 (Gondos et al., 1986), after which they cease mitotic division before birth and enter into their first meiotic division, becoming primary oocytes and are arrested at the 1st meiotic prophase. The chromosomes, enclosed within the nucleus are referred to as the germinal vesicle (GV) (reviewed by Virant-Klun et al., 2015).

A single layer of ovarian mesenchymal cells (granulosa cells) surround the primary oocytes, resulting in the formation of primordial follicle. The stroma around the follicle (theca) forms a capsule. Those oocytes not incorporated into follicles degenerate and the number of oocytes at birth is 1-2 million. Primordial follicles then remain in their arrested state until puberty (Eichenlaub-Ritter & Peschke, 2002), after which time, a few follicles are recruited regularly to form a pool of growing follicles (20-50 mature

each month). The primordial follicles selected increase in size, as does the primary oocyte. Additional layers of granulosa cells surround the oocyte, as it undergoes hypertrophy and proliferates in response to growth factors, including insulin like growth factor (IGF), epidermal growth factor (EGF) and Anti Mullerian hormone (AMH) (Fair 2003). The growing oocyte accumulates water, lipids, RNA and protein and the number of mitochondria and ribosomes increases (Johnson et al., 2004). The oocyte secretes glycoproteins (ZP1, ZP2, ZP3 and ZP4) that form the surrounding zona pellucida (ZP). Granulosa-derived cumulus cells also surround the oocyte and the innermost layer forms the corona radiata which penetrates the ZP to form gap junctions with the oocyte oolemma (Anderson & Albertini, 1976). At this point the follicle is said to be at the primary / preantral stage.

Increased granulosa cell proliferation occurs in response to endogenous and exogenous autocrine and paracrine effects. The outer stromal cells differentiate into the theca interna, which are vascularised, steroidogenic cells and the theca externa of connective tissue. Follicles become associated with blood vessels and Follicle Stimulating Hormone (FSH) from the pituitary is required for the formation of follicular fluid. The formation of follicular fluid marks the transition to the secondary/ antral follicle stage. The oocyte surrounded by a layer of cumulus cells is suspended in the fluid within the antral cavity and is approximately 120μm in size when the cavity is formed. It is at this stage that the oocyte is capable of resuming meiosis. Figure 1.4 depicts the advancing stages of folliculogenesis and increasing sensitivity of preantral and antral follicles to gonadotrophins, the cyclic activity of which is described in Figure 1.5. These stages of oocyte growth and development and the dependence on appropriate cyclic activity of gonadotrophins are discussed further in Chapter 3, with specific reference to the ovarian pathophysiology associated with obesity.

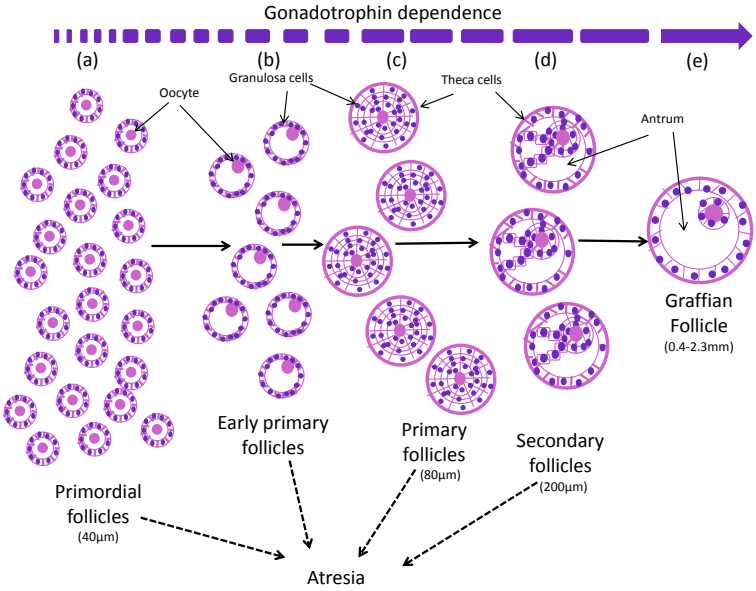

Figure 1.4 Schematic diagram of folliculogenesis.

The increasing follicular size (a-e) and dependence on gonadotrophins and progressive loss of growing follicles from the pool is depicted.

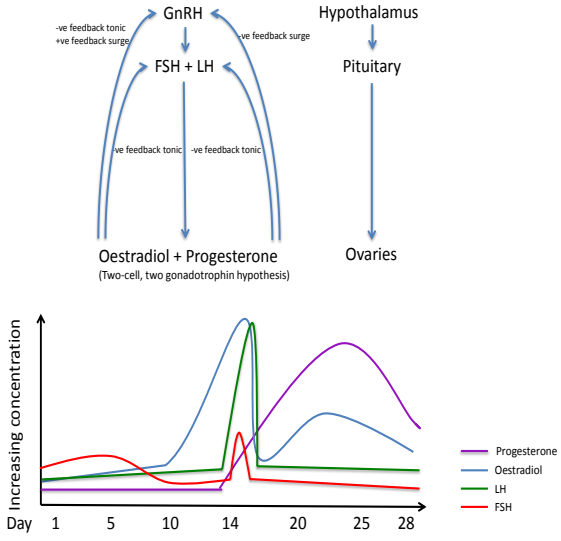

Figure 1.5 Overview of endocrine control of ovulatory cycle.

The pituitary gonadotrophins (FSH & LH), under the control of gonadotrophin releasing hormone from the hypothalamus (GnRH), regulate the ovarian production of sex steroids. Oestradiol and Progesterone exert negative feedback control over FSH/LH and GnRH. Rising Oestradiol also exerts a positive feedback action to induce the ovulatory surge in LH, marking the end of the follicular phase (day 1-14) and the beginning of the luteal phase of the menstrual cycle. Progesterone production (from the corpus luteum) then rises, but in the absence of fertilisation the uterine endothelium (endometrium) is shed and the next phase begins. The impact and causes of higher free circulating estrogen in OWOB women on menstrual cycles is discussed in Chapter 3.

The majority of the group of follicles that began development will have undergone atresia (apoptosis and loss, though not through ovulation). The causes of this phenomenon are not clear, however at the preovulatory stage continued growth is influenced by pituitary gonadotrophin hormones; FSH and lutenizing hormone (LH) (Figure 1.5). These hormones bind to FSH receptors on the granulosa cells and LH receptors expressed on the cells of the theca interna, the synergistic action of which results in Oestradiol production (Figure 1.6). Follicles compete for FSH and a dominant, growing follicle is selected, and increases in size from approximately 7 to 18mm over a period of 10-15 days (de Ziegler et al., 2007). Factors which regulate the expression of FSH and LH receptors will no doubt influence which follicle becomes the dominant tertiary / Graffian follicle (Rawan et al., 2015).

Figure 1.8, shows how LH binds to LH receptors on the thecal cells, causing these cells to synthesize androgens, which diffuse into the granulosa cells where they are aromatized to oestradiol under the influence of FSH. Granulosa cells also acquire LH receptors in the mid to late follicular phase, under the influence of FSH (Erickson et al., 1979) and cooperate to maintain levels of oestradiol. Furthermore, oestradiol stimulates additional granulosa cell proliferation; these cells in turn secrete more oestradiol –in a system of positive feedback. At low levels, oestradiol has a negative feedback effect on the hypothalamus and pituitary (Figure 1.5), but at high maintained levels a threshold is reached and oestradiol then elicits a positive feedback effect resulting in an LH surge, which is the trigger for ovulation, which occurs approximately 38 hours later.

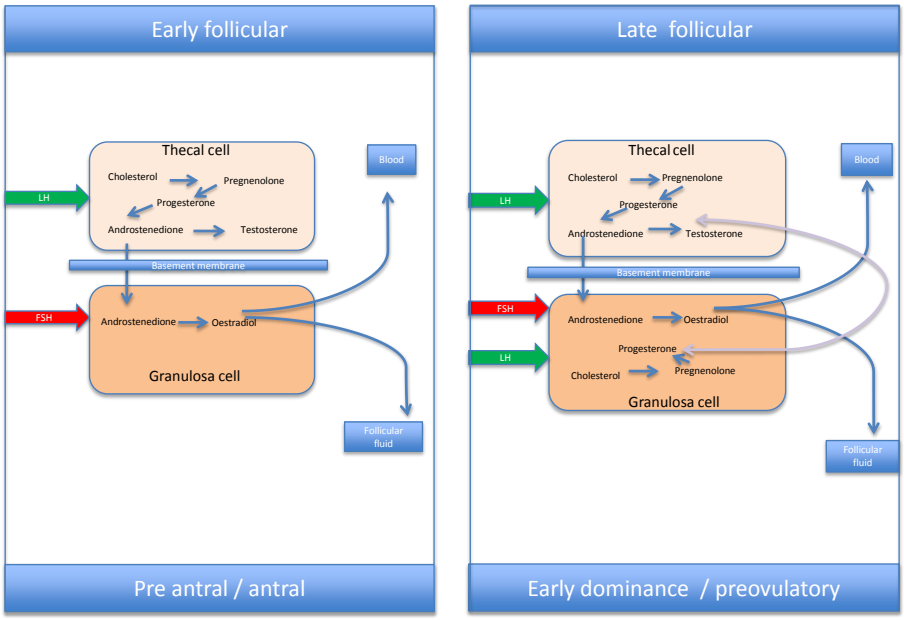

Figure 1.6 The two cell two gonatotrophin theory.

Theory describes the synergistic actions of LH acting on thecal cells to produce androgens, which under the influence of FSH stimulate granulosa cells to produce oestradiol. Granulosa cells also acquire receptors for LH in the mid to late follicular phase and stimulate progesterone production (adapted from Hillier et al., 1994; Nunes et al., 2015). The additional contribution of adipose tissue to the peripheral aromatization of androgens to oestrogen and the impact of this is discussed in Chapter 3.

Nuclear and cytoplasmic oocyte maturation occur in the period post LH surge and prior to ovulation and it is proposed that these events could be impaired in OWOB women, as discussed in Chapter 4. During nuclear maturation the oocyte completes the first meiotic division from prophase I to metaphase II, mediated by paracrine factors produced by the oocyte and granulosa cells acting via gap junctions, of which LH and FSH influence the opening and closing (Nunes et al., 2015). Hence, the actions of LH are indirect and there are no receptors on the oocyte (Dekel et al., 1988). Elevated levels of the inhibitory signal cyclic adenosine monophosphate (cAMP) within the oocyte maintain meiotic arrest (Norris et al., 2009). The surge of LH causes cAMP levels to drop and leads to activation / dephosphorylation of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) leading to GV breakdown (Hashimoto et al., 1988). MPF is a heterodimer of cyclin B and $P34^{cdc2}$ and when cAMP within the oocyte decreases protein kinase A type I (PKA1) is inhibited which in turn, removes the inhibition from cdc2, allowing activation of MPF (Vaccari et al., 2009).

Meiotic maturation is accompanied by modification of centrosomes and microtubules within the spindle and the formation of metaphase spindles and homologous chromosomes cross over at meiosis I. The result of the first meiotic division is two daughter cells via asymmetrical division, a larger cell the secondary oocyte and a smaller cell, the polar body. The oocyte then enters a second round of meiosis, the chromosomes are attached to the spindle metaphase and meiosis is again halted. Activated MPF and cytostatic factor cMos maintain metaphase II arrest (Ya & Downs, 2013).

Cytoplasmic maturation accompanies nuclear maturation and is essential for ongoing oocyte competence. It is characterised by continued oocyte growth and preparation of reserves including; increased ribosomal production, the synthesis and storage of RNA and translation products (Campbell et al., 1996) and the storage of lipids, ions and carbohydrates. Furthermore, in preparation for fertilisation the golgi apparatus synthesises lysosome like cortical granules, which are translocated to the periphery, as are mitochondria in association with smooth endoplasmic reticulum. For this reason, oocyte polarity and spatial patterning of organelles is often used to define cytoplasmic maturation (Hegele-Hartung et al., 1999).

Prior to ovulation the follicle becomes vascularised and swollen, causing increased tension and thinning of the follicular wall. Additionally, LH increases follicular fluid accumulation and the activity of collagenase, cumulus expansion enabling factor and the active secretion of hyaluronic acid. This culminates in the separation of the cumulus from the mural granulosa, facilitating the ovulation of the oocyte and embedded cumulus cells. Once the oocyte is expulsed from the ovary, under the influence of LH the theca and granulosa cells of the ruptured follicle become reorganised and shift to producing significant quantities of progesterone (in addition to some continued oestradiol production) and the collapsed follicle forms a corpus luteum (lutenization of the follicle) and represents the luteal phase of the menstrual cycle.

The ovulated oocyte-cumulus complex (OCC) is picked up by the distended fimbriae of the oviduct Gordts et al., (1998).. The oocyte is then slowly transported by ciliary activity to the ampullary-isthmic junction- the site of fertilisation.

1.4.2 Oocyte development and maturation *in vitro*

During ART the proliferative phase of the menstrual cycle is 'down-regulated', this can be achieved using a so-called 'long' or 'short' protocol and using a pituitary agonist or antagonist (Figure 1.7). Agonists initially cause an increase in oestrogen and testosterone, but sustained use causes their production to stop. Antagonists work by competing with GnRH for receptor binding sites in the pituitary. Based on the studies performed so far, it is not possible to draw any a reliable conclusion about which is the superior protocol (Marcus & Ledger 2001) and there are several formulations of GnRH agonists / antagonists commercially available.

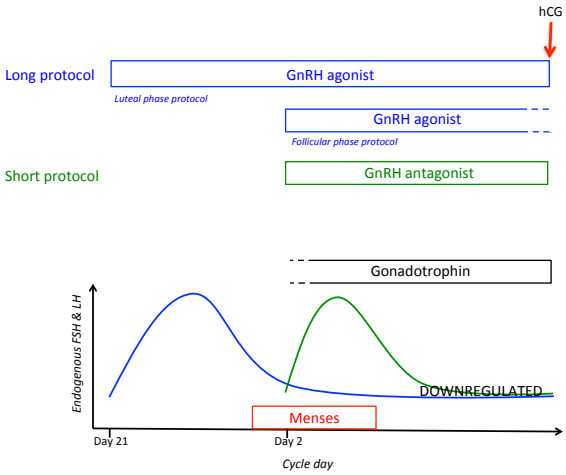

Figure 1.7 Overview of stimulation regimes used in ART.

Various stimulation regimes are available to achieve controlled ovarian hyperstimulation (COS). These include the use of highly purified FSH/ LH urinary preparations (i.e. human menopausal gonadotrophin- hMG) as well as FSH recombinant products (rFSH). Recent publications of large randomised controlled trials (RCTs) have shown urinary products (hMG) to be at least equivalent, if not more effective than recombinant FSH (Van Wely et al., 2003; Coomarasamy et al., 2008; Al-Inany et al., 2008). The use of a FSH-only recombinant may however, have theoretical advantages for patients at risk of hyperstimulation (Ragni et al., 2005).

Follicle development, hormone levels and endometrial thickness are carefully monitored to obtain a cohort of co-dominant follicles containing oocytes capable of responding to human chronic gonadotrophin (hCG) administration, which is

administered to mimic the endogenous surge of LH that would occur in a natural menstrual cycle to initial ovulation.

Oocytes are collected approximately 36 hours post hCG injection. This injection is planned to release oocytes from meiotic arrest, i.e., meiosis is resumed and the oocytes are allowed to reach metaphase of meiosis II. Oocyte retrieval is timed prior to spontaneous ovulation. Routinely >85% of oocytes recovered are graded as mature at the time of the procedure, when they are graded on the basis of their oocyte-cumulus complex morphology/ degree of expansion (Rattanachaiyanant et al., 1999). Oocyte grading is discussed in Chapter 2 and the relationship between oocyte quality, maturation and female BMI is reviewed in Chapter 4.

1.4.3 Fertilisation *in vivo*

Spermatozoa that have been deposited in the female tract reach the oviduct by active (their own motility) and passive (muscular contractions of the female tract and ciliary currents) transport. Suarez et al., (2006) have compiled an extensive review on how sperm transport differs in human to that of other mammalian species from its site of deposition (anterior vagina), coagulation, transport through the cervix, uterus, uterotubal junction and possible entrapment to endosalpingeal epithelium. It is not clear if a distinct sperm reservoir exists in human, as observed in other species (Williams et al., 1993), but it is apparent that the advancement of sperm into the ampulla is somehow prolonged, thus ensuring only a few sperm reach the ampulla at any one time.

At some point in the female tract, sperm undergo a process of hyperactivation and capacitation (Austin 1951). The vigorous 'whiplash' movement of sperm flagellum morphemes hyperactivation and is thought to enable sperm to penetrate viscoelastic solutions of the female tract and detach from the endosalphingeal epithelium and may also aid sperm passage through the corona radiata of the oocyte (Suarez et al., 2006). Only capacitated sperm are able to bind to the zona pellucida surrounding the oocyte (Chang et al., 1984; Lamirande et al., 1997). This essential priming step involves a process of changes to the sperm plasma membrane, including the loss and modification of proteins and cholesterol, elevation of intracellular calcium ion concentration, which enhances the intracellular generation of cAMP (Fraser 1989) and sperm gain the ability to undergo acrosome reaction. Capacitated sperm bind first, to ZP3 on zona pellucida, this receptor protein interaction initiates the release of proteolytic enzymes from the

sperm acrosome and the membrane of the acrosome, fuses with the plasma membrane permitting further additional binding to ZP2. Once the ZP has been fully breached the sperm and oocyte plasma membranes fuse resulting in the passage of the sperm and contents through oolema. The male pronucleus (PN) is formed, as its heterochromatin decondenses and protamines are quickly replaced by histones (Morgan et al., 2005).

This sperm egg fusion is followed by a propagated calcium wave (Jaffe, 1980), as calcium is release from internal oocyte stores and additionally it this point cortical granules are exocytosis from their membrane-bound position at the periphery of the oocyte into the perivitelline space. These changes indicate that the oocyte has been activated and the cortical reaction results in zona hardening to prevent further sperm from entry. The initial rise in calcium is followed by a series of oscillating spikes which promotes the destruction of cyclin B, thus maturation promoting factor activity is lost and the oocyte is able to resume meiosis (reviewed by Whitaker, 2006). This results in the extrusion of the second polar body and the formation of a now true haploid nucleus and the formation of the female PN

The male and female PN align at the periphery of the oocyte and rotate to the centre, approximately 16-18 hours after the initial fusion of the gametes. It is during this migration that chromosomes synthesize their DNA and nucleoli within the PN begin to condense and align. The PN membranes then disintegrate, the mitotic metaphase spindle forms and chromosomes are aligned and come together in syngamy, chromatids separate, a cleavage furrow forms and the first mitotic anaphase and telephase are completed. In humans syngamy of PN occurs approximately 18-24 hours after sperm-oocyte interaction and by enlarge it results from the close alignment of PN membranes (Levron et al., 1995).

It is important to note that during PN maturation the paternal and maternal genomes undergo different DNA methylation patterns; they are epigenetically asymmetrical (Corry et al., 2009). The paternal genomes are packaged differently to the maternal genomes (protasomal and nuclesomal respectively) and whilst the paternal PN undergoes active DNA demethylation, acquisition and modification of histones, the maternal PN lags behind. The maternal PN remains methylated, maintaining imprints post-fertilisation and into early embryo development (reviewed by Inbar-Feigenberg et al., 2013). Prior to this point of fertilisation, the timing of remethylation also varies

between the male and female germline; DNA is globally demethylated in the PGC of both sexes and denovo methylation occurs rapidly in spermatagonium, but is established gradually during the growth phase of oogenesis (this is discussed further in Chapter 4). DNA methylation is the process of gene silencing via methylation at CpG dinucleotides, and histone modifications affect chromatin accessibility to DNA replication and transcription, hence both processes contribute to the epigenetic regulation of gene expression. Much of what is known about epigenetic remodelling has been derived from animal studies, however the processes are believed to be largely conserved in the human (Lucas 2013), although the paternal demethylation may occur to a lesser extent (Santos et al., 2010). The process of epigenetic control and potential consequences of environmental disruption to this process is highly significant and relevant to this thesis.

1.4.4. Fertilisation *in vitro*

Men are asked to produce a semen sample, having observed 3-5 days prior abstinence from sexual activity, to optimize the volume and concentration (Lampe et al., 1956, Poland et al., 1985). The laboratory preparation of sperm is then needed to separate sperm from the seminal fluid – which inhibits capacitation. Semen samples are processed to separate motile sperm, of optimum morphology from the seminal plasma, either by (a) 'swim-up' technique – in which media is layered over seminal plasma and motile sperm swim up into the overlay or (b) density gradient separation –by pipetting semen onto a density column and centrifuging (Boomsma et al., 2007). Prepared samples are re-suspended in culture medium, at a concentration suitable for insemination (IVF) or intra cytoplasmic injection (ICSI) of the collected oocytes.

Oocytes are inseminated in vitro often in 4-well plates with approximately 100,000-150,000 sperm per ml of culture medium. Insemination concentration is optimised to attain successful fertilisation without overexposing oocytes to high concentrations of sperm and risking polyspermia, pH changes and generation of reactive oxygen species (Aitken et al., 1994, Ozgur et al., 1999). The timing of insemination (2 to 4 hours after retrieval) is performed according to the maturity of the oocyte, in order to avoid abnormal fertilisation, which can occur when oocytes are inseminated in an immature or post mature state (Trounson, 1982).

When semen parameters are suboptimal (below WHO manual 2010 reference values), ICSI may be used to facilitate fertilisation (Cooper et al., 2009). In this procedure the

cumulus and corona cells, which surround the oocytes, are removed by a combination of enzymic and mechanical manipulations. A single sperm is then injected through the zona pellucida and the oolemma directly into each MII oocyte (Van Steirtingham et al., 1999).

Oocytes are inspected for pronuclear status approximately 18 hours after insemination (IVF) or injection (ICSI). The presence of two distinct pronuceli (PN) and two polar bodies (pb) is indicative of normal fertilisation.

1.4.5 Embryo cleavage and blastocyst formation *in vivo*

Some 20-24 hours after sperm penetration of the oocyte, the fertilised oocyte (zygote) completes its first cleavage division. Subsequent cell divisions (cleavage) then occur every 15-18 hours, as the dividing embryo moves along the fallopian tube to the uterus. Cleavage cell divisions occur without altering the overall size of the embryo, which is enclosed by the zona pellucida, hence the cytoplasm is divided between daughter cells, known as blastomeres, which become increasingly smaller in size and closer in apposition.

At the 4-8 cell stage in the human (day 2-3 of development), embryonic genome activation (EGA) occurs (Braude et al., 1988). At this point maternal mRNA transcripts are depleted and replaced by those of the zygote, hence zygotic genome expression increases. The transition is believed not to be absolute; as a small amount of maternal mRNA is needed up to the blastocyst stage and therefore environmental factors influencing oocyte development can still affect development post EGA (Moor et al., 1998). Impairments of transition from maternal to embryonic control may have their origins in aberrant gene expression of maternal factors possibly resulting from incomplete oocyte cytoplasmic maturation (Schramm et al., 2003) (discussed further in Chapter 4). The transition to EGA is also phased, as the replacement of maternal transcripts occurs at different rates for different genes, for instance several sex determining genes are thought to be active from the pronuclear stages (Edwards & Beard, 1997). It is also highly probable that blastomeres develop cell automonously, with some cells advancing to EGA and others arresting Wong et al., (2010), possibly explaining why some cells fail to divide.

At about the 8-16 cell stage the increased contact between blastomeres, causes them to compact and flatten. Compaction marks the starting signal for cell differentiation, as intercellular junctional complexes assemble between the blastomeres and form zonal tight junctions composed of occludin and cingulin and gap junctions that permit the flow of second messengers that act to govern cellular diversification (Brison et al., 2014; Sozen et al., 2014). Following compaction the embryo is described as a morula and at this point the cells on the periphery of the morula will have different contacts to those in the middle, which may help explain the onset of the different polarity and differentiation of the cells. Thus, cells on the periphery begin to differentiate into the trophectoderm cells (TE) and those internally form the inner cell mass (ICM). The TE will eventually form the placenta and extra-embryonic tissue, whereas the ICM will generate the embryo proper and represent a pluripotent cell population. It is likely that this idea of cell allocation based on positional relationships within the embryo is an overly simplistic view, as polarisation can occur in cells prevented from compacting (Cockburn et al., 2010). The opposing view is that position determines cell fate and gene expression determines position. This idea was initially presented by Gardner (2005), who has made a cogent argument for the role of pre-patterning in mammalian embryos, being a normal facet of development and dependent on intrinsic organisation of the oocyte.

Coinciding with cell differentiation, de novo methylation begins in the morula and epigenetic differences are seen between the cells of the ICM and TE. Appropriate epigenetic remodelling during early embryonic development is essential and is a critical period of susceptibility to dysregulation, with the human trophectoderm reported to be highly susceptible to epigenetic misregulation (Rugg-Gunn 2012).

From approximately the 32 cell onwards cavitation begins; activation of the Na+, K+, ATPase enzyme drives the movement of Na+ to the basolateral side of the TE creating an osmotic gradient which draws in water to form a fluid filled cavity known as a blastocoel. Tight junctions prevent the fluid from moving out (Krupinski et al., 2011).

In the human the embryo forms an early blastocyst on approximately day5, fluid accumulation continues and the blastocyst expands on day 5-6. This causes the surrounding zona pellucida to thin and blastocyst hatching can be initiated usually on day 6-7. It is not know if this occurs as a result of hydrostatic pressure or through a

small rupture in the zona and given the discourse in the literature (Ebner et al., 2010; Miyata et al., 2010) on this topic, it is tempting to speculate that this is not only species specific but is also influenced by environmental factors. Figure 1.8 provides an illustrative summary of some of the processes as the embryo develops to the blastocyst stage.

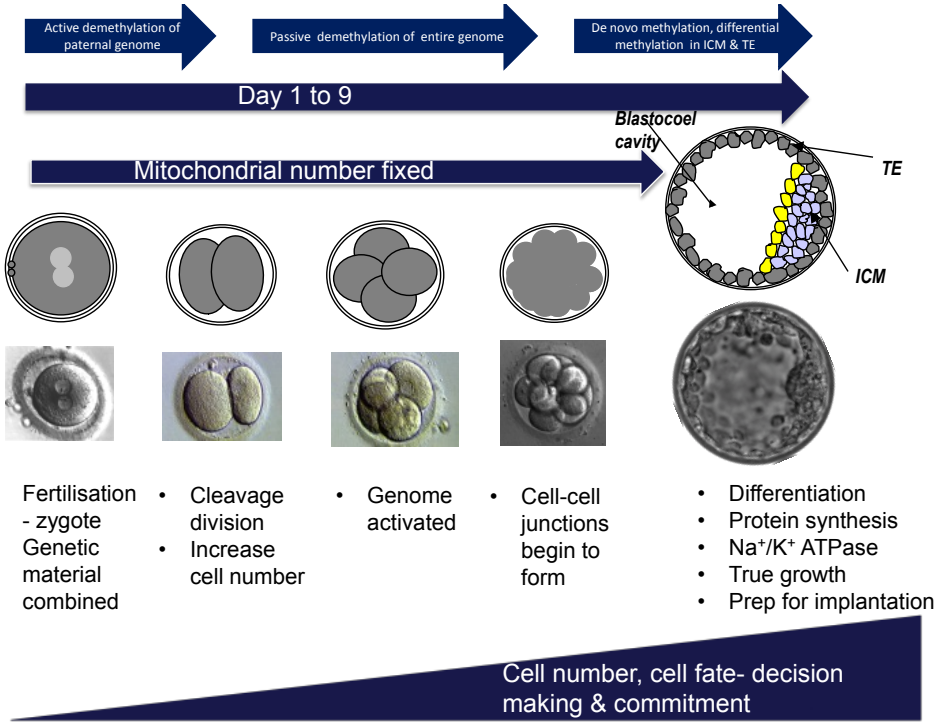

Figure 1.8 Embryo developmental changes from day 1 to 9.

Development from zygote to blastocyst stage is depicted (Figure adapted from Smith & Sturmey 2013).

1.4.6 Embryo cleavage, development and assessment *in vivo*

Assessment of embryo development is discussed in more detail in chapter 2, however it is important to outline the different assessment approaches and criteria which may be applied. Assessments may be conducted at discreet time points throughout development to build up a sequential picture, alternatively in recent years attention has moved to the use of time-lapse imaging systems. Time-lapse imaging permits numerous observations to be made, with images typically being acquired every 10-20 minutes. Whilst the virtues of such analyses are widely extolled, randomised controlled trials demonstrating the efficacy of such an approach are still currently lacking (Bolton et al., 2015).

Traditionally, embryos are checked for development/quality at distinct time points; commonly at 20-24 hours post insemination (hr/pi) for early cleavage division, 46hr/pi (day2) and 68hr/pi (day 3) for cleavage stage assessments and 116hr/pi for blastocyst development (day 5). Embryos may be transferred into the uterus at the cleavage or blastocyst stage. In each case, embryos are checked visually immediately prior to transfer to ensure selection of the best quality embryos.

A number of strategies have been designed for grading and selecting embryo(s) for transfer. These include pronucleate scoring which assesses the symmetry, position and localisation of nucleoli (Scott & Smith 1998), checking for the timing of the first cleavage division (Sakkas et al., 2001), cell scoring on day 2 and day 3 and extended culture which permits selection of blastocysts for transfer (details are provided in chapter 2).

Often embryos are critiqued on their cell number and morphology on day 2/3. The cell number should be appropriate for the time of assessment, with slow growing and unusually fast growing embryos have low implantation potential (Cummins et al., 1986, Puissant et al., 1987, Staessen et al., 1992, Giorgetti et al., 1995) and shown to have higher levels of chromosome aberrations (Van Royen et al., 1999). Whilst gross visual morphology assessment criteria are imprecise (Sharpiro et al., 2000), it has been shown that embryos with higher fragmentation are less likely to implant and develop than unfragmented counterparts (Giorgetti et al., 1995). Furthermore, the presence of multinucleated blastomeres is associated with significantly impaired implantation (Pickering et al., 1995, Jackson et al., 1998, Van Royen et al., 2003), possibly through defective transcription of genes leading to developmental arrest.

There is an established link between the surmised embryo quality score (combined cell number and morphology grade) and the pregnancy outcome (Giorgetti et al., 2006, Ziebe et al., 1997). Gerris et al., (1999), reported that patients who had embryos regarded as 'top quality' ($\geq$7 blastomeres, <20% fragmentation, day 3) had a good prognosis for achieving a pregnancy, however they were significantly more likely to have a multiple implantation if two embryos are transferred. Multiple pregnancies carry a higher risk of miscarriage, maternal complications and premature delivery, therefore when the patient has multiple good quality embryos to select from; the pressure to identify the most viable embryo for transfer is increased. Extending the culture period

until day 5, is one way to facilitate the selection process (Cutting et al., 2008), although the quest to identify putative biomarkers of embryo viability is discussed throughout this thesis.

On day 5 of development blastocysts are selected for transfer or freezing according to presence of cavity, visible inner cell mass, regular trophoblast cells, degree of expansion, and zona thinning. Analpha-numerical score is assigned for the degree of expansion/ hatching status and the visual appearance of the cells of the inner cell mass (ICM) and trophectoderm (TE). Good quality blastocysts are identified by a high number of cells in the ICM and tight knit epitheliem of the TE (Gardner and Schoolcraft et al., 1999).

Studies comparing implantation and pregnancy rates following transfer of blastocyst or cleavage stage embryos have in the past produced conflicting results (Gardner et al., 1998; Schoolcraft et al., 1999; Milki et al., 2000, Coskun et al., 2000; Huisman et al., 2000). However, Blake et al., (2007), reviewed data from 18 randomised controlled trials and concluded that for selected good prognosis patients blastocyst transfer offers a significant advantage. Furthermore, karyotype analysis has shown that whilst 68% of 8 cell embryos were abnormal only 43 % of the cells in the blastocyst were aneuploid (Magli et al., 2001). Taken together, it may be argued that extended culture to the blastocyst allows selection of the best quality embryo for transfer and could reduce the likelihood of cryopreserving non viable embryos. Despite this, safety concerns over blastocyst culture linger and evidence has emerged recently that suggests that prolonged culture in suboptimal conditions could cause long-term reprogramming in the embryo, thus the need for structured long-term follow up studies of children conceived by ART is paramount (Chronopoulou et al., 2015).

The actual embryo transfer procedure requires the selected embryo(s) to be loaded into a transfer catheter, which is fed through the cervix, so the tip is positioned 1cm from the fundus and the embryo(s) expelled. Luteal support (progesterone), is necessary in IVF cycles (Van der Linden et al., 2001) and is continued until pregnancy testing (after 10 days) and often up to the 9th gestational week in the case of pregnancy. Progesterone prepares the endometrium for pregnancy in the luteal phase of the menstrual cycle and is insufficient in ART cycles, possibly due to the supra-physiological concentrations of

steroids secreted by a high number of corpora lutea during the early luteal phase and negative feedback effects (Sofuoglu et al., 2015).

Surplus good quality embryos / blastocysts may be selected for cryopreservation. Cryopreservation coupled with frozen embryo transfer can increase cumulative pregnancy rates (Sharipo et al., 2011) and is also used to reduce the risk of hyperstimulation in vulnerable patients and as a fertility preservation strategy. There are two predominant methods for cryopreservation; slow freezing and vitrification and there is no single optimum protocol for a cryopreservation cycle, however several studies have shown a correlation between embryo quality and freeze tolerance (Karlstrom et al., 1997; Check et al., 2009).

1.5 Energy metabolism

The sequential stages of dynamic, preimplantation development from oocyte maturation to blastocyst formation are obviously energy dependent. Energy metabolism can be described as the liberation of chemical energy from food and its conversion into different forms of chemical energy (as almost exclusively adenosine triphosphate- ATP and reducing equivalents and precursors for macromolecular synthesis) to make energetically unfavourable reactions possible. ATP is central to energy metabolism. The ratio of ATP:AMP or ATP:ADP permits cells to ‘sense’ energy availability and thus regulate metabolic pathways that produce or use the ATP.

The principle means by which ATP may be formed are aerobic oxidation of substrates into carbon dioxide and water or anaerobic glycolysis of glucose to lactic acid. The precise metabolic requirement of oocytes, cleavage stage embryos and blastocysts are met via pathways specific to each cell type, utilising the oxidisable substrates; glucose, pyruvate, amino acids and fatty acids. The common metabolic pathway for each of these substrates is the tricarboxylic acid cycle (TCA), which produces reducing equivalents (NADH and $FADH_2$) for use in the electron transport chain (ETC), which allows oxidative phosphorylation (Ox-Phos) to occur and leads to the generation of ATP.

The regulation of energy production is fundamental for embryo survival and propagation (Gardner et al., 2013) and maybe indicative of embryo viability. Furthermore, the rate of uptake and the metabolic fate of nutrients utilised by early embryos are reported to be associated with developmental outcome (Gardner et al., 2011). This has led to a desire to identify the normal pattern of substrate utilisation, and how the detailed metabolic profile of an embryo may be measured using non-invasive biochemical assays to determine individual embryo viability. Much of the fundamental research, has been conducted in animals and this has facilitated our understanding of the dynamic changes in bioenergetic status that accompany oocyte and embryo development and how the loss of this control may lead to compromised viability. A review of studies attempting to use metabolic activity as a biomarker of individual embryo viability is presented in Chapter 5, but first an account of those studies that have led to our current understanding of normal preimplantation metabolism is provided in the following sections.

The environment in which the oocyte / embryo develops can influence its metabolism, as the developing embryo is exposed to a variety of micro environments as it traverses the female reproductive tract from the oviduct to the uterus (Leese et al., 2008) and the strategies/ pathways utilised by the embryo to derive energy are reflective of this. In the normal course of development embryonic genes are successively turned on, as mitosis and biosynthesis increases after embryonic genome activation and the demand for energy increases (Gardner et al., 2013). The metabolic pathways and 'machinery' available to gametes and embryos are aligned with their energetic demands, substrate availability and crucially, oxygen and the reductive or oxidative (REDOX) state of the cell. Consequently the generally accepted pattern of early embryo metabolism is; low activity and demand for ATP in the cleavage stages, with reliance on pyruvate and less so on glucose, and higher activity at the blastocyst stage with concomitant increases in glucose uptake, initially higher at the blastocyst stage, as energy is required to power the Na^+/K^+ ATPase and for protein synthesis since true growth only begins at this stage (reviewed by Leese, 2012).

The embryonic cells, are the progenitor cells with the potential to express every gene and capacity to differentiate into every cell in the human body and therefore it is not known to what extent metabolic pathways available to specialised cell types (i.e. differences in enzymes expressed in a myocyte, adipocyte, hepatocyte) can be

extrapolated to the embryo. Furthermore, as the cells of the embryo become increasingly differentiated their metabolic properties are likely to change and maybe modulated by prior environmental exposure. It can be inferred that, defined and specific roles for different cell types (oocyte, blastomeres of a cleavage cell embryo, ICM and TE cells of a blastocyst) will be evident, based on their individual needs and adaptation to their environment. Nonetheless, an understanding of cellular metabolism and energy production is valued in terms of whole body health, as control of biochemical metabolic pathways is essential for control of obesity; the central tenant of this thesis. Figure 1.9 highlights how the specific substrate requirements of a mammalian cell maybe met through these interlinked pathways.

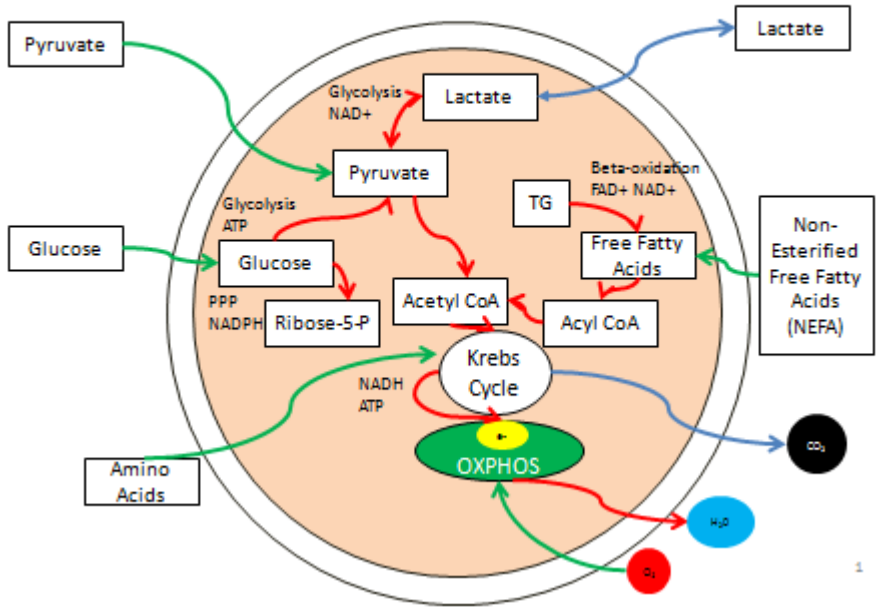

Figure 1.9 Interlinked metabolic pathways in a mammalian cell.

ATP is generated by glycoysis and OxPhos. Glucose is metabolised by glycolysis producing ATP and pyruvate which is converted to lactate (anaerobic) or to acetyl CoA (aerobic) and enters the TCA cycle, similarly β-oxidation of fatty acids generates Acetyl CoA and amino acids can be used as substrates entering the TCA cycle at various points. The TCA cycle generates NADH and $FADH_2$ for use in the ETC and allows OxPhos and generation of ATP. Glucose, once converted to G-6P (first step in glycolysis) may also be directed to the Pentose Phosphate Pathway (PPP), particularly when there is a need for biosynthesis during rapid phases of growth to generate NADPH and ribose moieties. (Figure adapted and reproduced, with the permission of P. McKeegan, 2015).

1.6 Oocyte metabolism

The oocyte metabolises a range of substrates including pyruvate, glucose, oxygen, fatty acids, amino acids, purines and pyrimidines for energy production, nuclear maturation, production of matrices, signalling molecules and maintenance of cellular homeostasis/ REDOX potential (Collado-Fernandez et al., 2012). The oocyte itself has a limited capacity for the uptake of these substrates and is reliant on the bidirectional communication which occurs between it and the granulosa-derived cumulus cells (Dan-Goor et al., 1997). The granulosa cells are metabolically coupled to the oocyte via gap junctions, throughout the initial growth and maturation of the oocyte (de Loos et al., 1991). During folliculogenesis the granulosa cells proliferate, resulting in increased gap junctions and increased coupling due to increased surface area and attachment. The cumulus granulosa cells express growth factor receptors, mRNA for growth factors and provide angiogenic factors (involved in blood vessel formation), prostaglandins and metabolic precursors (amino acids and nucleotides) to the oocyte to facilitate maturation as well as ATP for the reactions involved in nuclear maturation and resumption of meiosis (reviewed by Sutton-Mcdowell et al., 2010). Thus, removing the cumulus cells can disrupt oocyte maturation and compromise viability (Eppig 1991).

The energetic demands for GVBD and resumption of meiosis appear to be met by the oxidative metabolism of pyruvate (Downs et al., 1997, Sutton et al., 2003). Whilst glucose is the preferred metabolic substrate for cumulus cells (Leese & Barton, 1985), the oocyte itself has a poor capacity to utilise glucose (Biggers et al., 1967) and it is the cumulus cell which metabolise glucose to provide a source of pyruvate and lactate for uptake by the mammalian oocyte (Leese & Barton, 1985). It has been shown that oocytes express very few receptors for the uptake of glucose, compared to cumulus cells, which not only express more transporters (Dan-Goor et al., 1997), but also express the facilitative transporter SLC_2A_4, which has a high affinity for glucose and is insulin sensitive (Sutton-McDowall et al., 2010). Furthermore, oocytes have limited expression and activity of glycolytic enzymes such as phosphofructokinase (PFK) (Sutton-McDowall et al., 2010), but their relative expression of glucose-6-phosphate dehydrogenase (G6PDH) is reported to be higher (Cetica et al., 2002). (The high G6PDH activity in developing oocytes is discussed in further detail in Chapter 4). This enzyme regulates the flow of substrates through the Pentose Phosphate Pathway (PPP) and would indicate that the PPP occurs mainly in the oocyte, which has low glycolytic

activity, whereas the converse expression pattern was reported for cumulus cells, which are thought to be primarily glycolytic. To complete maturation the oocyte does therefore, need to metabolise some glucose via PPP for the synthesis of the purine and pyrimidine precursors of DNA and RNA (Sutton et al., 2003) and NADPH for biosynthesis of lipids and complex molecules, which is important during growth.

It is apparent that the metabolic profiles of the follicular cells and the oocyte change during folliculogenesis and oogenesis, as demand for energy increases. In the mouse oocyte growth results in increased pyruvate and oxygen consumption, whereas follicular growth results in increased glucose consumption and lactate production (Harris et al., 2009). This shift from low glycolytic activity in pre-antral follicles to high glycolytic activity in antral follicles has also been reported in the bovine, with mature bovine cumulus-oocyte complexes (COC) reportedly consuming twice as much glucose, pyruvate and oxygen compared to immature COC (Sutton et al., 2003). The increased glycolytic activity of follicular cells, could be related to oxygen availability, with increased size compromising the supply from theca capillaries (Redding et al., 2007). Additionally the initiation of oestrogen synthesis in follicular cells could be creating an increased demand for energy (Harris et al., 2007). Consistent with this is the finding that FSH increases glycolytic activity (Downs et al., 1996), as glucose is required to support FSH-dependent cumulus expansion and oocyte maturation.

During maturation the oocyte requires amino acids for protein and mRNA synthesis (Picton et al., 1998). Specifically, glutamine, aspartate, glycine, together with ribose-5-phosphate from PPP are required for the de-novo synthesis of purine and pyrimidine nucleotides. Different amino acid transport systems have been identified in growing and mature oocytes. Glutamine uptake is reported to increase by 2.5 times during the first 18 hours of maturation (Reiger & Loskutoff 1994) and during GVBD there is an increase in oxidative metabolism of glutamine and glycine, which can feed into the TCA cycle (Harris et al., 2007) to provide ATP. Aspartate uptake by the oocyte is also reported to increase after GVBD (Pelland et al., 2009). Additionally, amino acids maybe taken up by cumulus cells, as these cells poses greater activity of enzymes involved in amino acid metabolism than oocytes (Cetica et al., 2003). The roles of individual amino acids during oocyte development are only just beginning to be understood, however the pattern of amino acid depletion and appearance from culture medium during *in vitro* maturation (IVM) has used to predict the developmental competence of bovine oocytes

(Hemmings et al., 2012), underscoring the importance of amino acid metabolism during acquisition of nuclear and cytoplasmic oocyte maturation.

Once the oocyte has been ovulated and gap junctions coupling the oocyte to granulosa cells have broken down, the oocyte is surrounded by a limited number of corona cells and it is plausible that it will become increasingly reliant on endogenous energy stores of glycogen, fatty acids and protein as a source of ATP. Lipids in particular have been identified as good candidates for energy provision during this phase of development (Sturmey et al., 2009). In part, because lipid droplets have been shown to accumulate during initial oocyte growth and significant stores of triglycerol (TG) have been reported within the ova of pigs, and sheep (McEvoy et al., 2000). In addition, during IVM, TG levels are reported to decrease (Ferguson & Leese, 1999) and significant lipase activity has been reported in bovine oocytes (Cetica et al., 2002). Lipases hydrolyse TG and free fatty acids (FFA) can then be transported into the mitochondrial matrix for β-oxidation and conversion to acetyl CoA for use in the TCA cycle. This transport is facilitated by the 'Carnitine-Acyl Carnitine Shuttle' (Vaz & Wanders, 2002) and this is discussed in further detail in Chapter 6.

The speculative role of endogenous TG stores and FFAs in supporting oocyte development is also born of the fact that fatty acid oxidation can generate a high number of ATP molecules, (given their anhydrous nature and number of carbon-carbon bonds, fatty acids produce more ATP per unit mass/ per mole than glucose) and the observed close association of lipid with mitochondria reported by ultrastructural (Sun et al., 2001) and FRET (Sturmey et al., 2006) analysis – leading authors to propose that these are acting in convoy as 'metabolic units'. However, whilst the inhibition of β-oxidation during IVM results in impaired developmental competence (Dunning et al., 2010), it is unlikely that endogenous sources alone can supply sufficient energy, as development is also limited during culture without external energy substrates (Sutton-McDowall 2012). It is highly probable that the role of endogenous stores will be species specific akin to the significant differences in lipid accumulation reported for a variety of species (reviewed by Sturmey et al., 2009). Similarly on the whole, species specific differences in substrate preference may exist, as shown by Krisher et al., (2007).

In summary; studies on oocyte metabolism have generally revealed that development is supported by low levels of oxidation of pyruvate, lactate, specific amino acids, with possible contribution of endogenous TG, however it is vital to note that metabolic activity will be influenced by the availability of substrates. In *in vitro* studies, this is dictated by culture media, which are relatively simple in composition with a limited number of nutrients; in the ovarian follicle the follicular fluid is the source of energy substrates and the follicular environment that the growing COC is exposed to may affect long term developmental competence post fertilisation. Follicular fluid provides a source of oxygen, metabolites, growth factors, hormones and buffering molecules (Sutton et al., 2003). In general terms the concentration of metabolites within the follicular fluid is believed to be comparable to plasma levels, although individual follicular concentrations are positively correlated with ovarian follicular size (Nandi et al., 2008) and COC metabolism changes as the follicle develops (Harris et al., 2009). Controlled ovarian stimulation for fertility treatment results in the recruitment of a relatively homogenous pool of growing follicles, synchronously triggered to mature, and hence provides an opportunity to study the impact of maternal nutritional status on oocyte phenotypic features and subsequent morphokinetic development, a factor which is explored in detail in chapter 4 of this thesis.

1.7 Embryo metabolism

Embryo metabolic activity can be thought of in two phases; (1) the cleavage stage and (2) the blastocyst stage (Biggers et al., 1967). As previously mentioned, at the early stage of development the embryo's energy demands are lower and similar to those of the oocyte, with ATP produced from pyruvate, lactate or amino acids, whereas at the later blastocyst stage energy requirements are higher primarily met by increased glucose consumption. Oxygen consumption believed to be low throughout embryo cleavage stages reaches a peak at the blastocyst stage (Sturmey et al., 2003). The pattern appears to be relatively conserved across all species (human, bovine, porcine, murine) studied.

Much of what we know about the different metabolic requirements of the early and later stage embryo have been derived from the application of inhibitory / stimulatory treatments- the effects differing according to whether treatment is applied pre or post-compaction. Inhibition of oxidative phosphorylation prior to compaction is highly inhibitory to development (Thompson et al., 2000), whereas suppression of glycolysis at

this stage improves development (Gardner & Lane 1996). The use of radiolabeled substrates and micro florescence analysis has provided data on the precise amount of substrate uptake / production, however these measurements are made under *in vitro* conditions and may not be representative of the *in vivo* situation. Additionally, many of these studies have focused on the murine embryo due to difficulties attaining research licensing and ethical approval to study the metabolism of human embryos.

1.7.1 Embryo metabolism at the cleavage stage

Studies in numerous species have shown that fertilisation and oocyte activation result in an elevation in metabolism (Dumollard et al., 2008), sperm entry causes up-regulation of the PPP (Urner & Sakkas, 1999) and results in an approximate 30% increase in pyruvate uptake (Devreker et al., 2000) and a peak in oxygen consumption has been observed to coincide with the time of the first cell division (Tejera et al., 2012).

During the subsequent cleavage stages, the embryo displays a preference for oxidative (aerobic) metabolism of pyruvate, lactate and amino acids (Brinster et al., 1965). The energy requirements are low at this stage (Leese et al., 2001), as is the rate of oxygen consumption and carbon dioxide production, although the ATP:ADP ratio is high, indicating a degree of metabolic quiescence. At this point the energy needs of the embryo are relatively low, as despite the fact that DNA replication and cell division are occurring, there is no change in the overall embryo size (Leese et al., 2008). Pyruvate enters the cleavage embryo by a facilitated carrier (Gardner & Leese, 1998) and it is used preferentially as a substrate for generating ATP via the TCA cycle and OxPhos and therefore lactate production is relatively low. The conversion of some pyruvate to lactate is however, required to regenerate cystolic NAD^+, for use in glycolyis at the later stages of development (Lane & Gardner, 2000), as an excess of pyruvate to lactate, can disrupt the ratio of NADH:NAD^+ and can impair embryo development (Dumollard et al., 2007). Furthermore, whilst pyruvate uptake is the preferred substrate at the cleavage stage, it's uptake by human embryos has been shown to continue to increase between successive cleavage divisions and the blastocyst stage (Hardy et al., 1989), even once glucose consumption rises post compaction.

Prior to compaction respiration rates are low and there is a high ATP: ADP ratio, post compaction the reverse is true and the low ATP:ADP ratio releases PFK from inhibition permitting an increase in glycolytic flux. By the 8-cell stage in the mouse, glycolysis is

fully functional (Biggers et al., 2008) and the uptake of glucose relative to pyruvate increases after this stage (Leese et al., 1993). There is a shift in the REDOX state to a more reduced state, which may be important in accommodating an increasingly hypoxic environment (Harvey et al., 2002). Embryos are thought to encounter a decreasing oxygen concentration as they traverse down the reproductive tract towards the uterus (Leese, 1995). Exposure to a prolonged oxidized state is thought to be unfavorable for embryo development (Harvey et al., 2002), stimulating the generation of reactive oxygen species (ROS), primarily produced through the ETC. ROS are important signalling molecules, however if produced in excess they can cause cellular damage and compromise mitochondrial function (reviewed by Muoio 2014).

The REDOX state is influenced in part by the ratio of NAD:NADH and the ratio of pyruvate: lactate, which differ between the cleavage stage and blastocyst stage (Lane & Gardner 2000). Rather than pyruvate being utilized in the TCA cycle, as happens at the cleavage stage, as the embryo begins to compact, pyruvate is increasing used to generate of lactate and this regenerates cytosolic NAD^+. A supply of NAD^+ is required for glycolytic flux. During glycolytic metabolism of glucose the pyruvate produced may then be converted into lactate (usually under conditions of low oxygen); this requires NADH and allows the regeneration of NAD^+ and prevents glycolysis from being stalled. This is vital for maintaining the REDOX balance and is vital for maintaining cellular stability (Coffman & Denegre 2007). NAD^+ has also been shown to be regenerated indirectly by the malate-aspartate shuttle and this mitochondrial shuttle transports protons into the mitochondria and regenerates NAD^+ in the cytosol (Newsholme et al., 1983). The mitochondrial structure and distribution are crucial for normal embryonic metabolism, the distribution appearing to be specific for embryo compaction (Harvey et al., 2007) and the number needing to attain a threshold to generate energy production to support further development and growth (Wakefield et al., 2011).

1.7.2 Embryo metabolism at the blastocyst stage

Prior to compaction, levels of biosynthesis of proteins, lipids and complex molecules are low, which increase at the post-compaction and blastocyst stages, hence the demand for energy rises sharply. The demand for ATP production at the blastocyst stage is partly due to the energy requirements of the Na+/K+ ATPase, which facilitates cavity formation (Houghton et al., 2003). Secondly, there is a large increase in carbon

incorporation into protein synthesis for growth at the blastocyst stage (Houghton & Leese 2004). Each of these two processes require approximately 25% of total energy intake (Leese at al., 1991).

Protein synthesis increases at the blastocyst stage, with the onset of true growth (Leese, 1993). In addition, amino acid consumption increases between the cleavage and blastocyst stage, primarily due to their role in protein synthesis. Excess amino acid/ protein is oxidised in preference to carbohydrate as the cell does not have the capacity to store it. Amino acid breakdown products can enter the TCA cycle at various points, as shown in Figure 1.10. In bovine and human embryos the requirements for individual amino acids are shown to change with development (Partridge & Leese 1996, Houghton et al., 2003), reflective of the distinct and varying functions of amino acids within the mammalian embryo. Beyond being utilised in biosynthetic pathways (protein synthesis and nucleotide synthesis) and as energy substrates, amino acids are precursors of signal transducers, they provide a defence against oxidative damage, act as pH zwitterons, osmolarity regulators, heavy metal chelators and donors of methyl groups (reviewed by Leary et al., 2012).

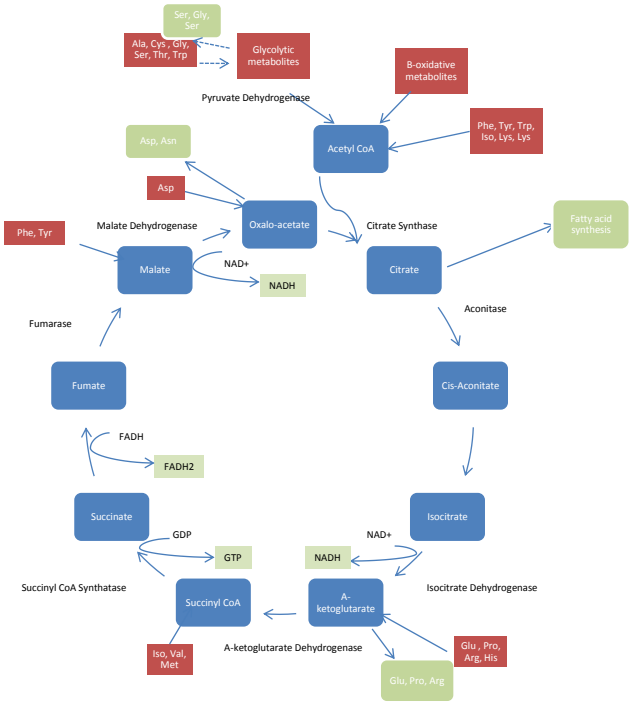

Figure 1.10 Amino acids can be converted to TCA cycle intermediates.

The TCA cycle is required for the stepwise oxidation of Acetyl CoA to produce NADH and $FADH_2$ for use in the ETC. Components of the TCA cycle are also used as precursors in biosynthetic pathways, including the amino acids; glutamate, glutamine, proline and arginine and also in fatty acid synthesis.

The increase in ATP demand at the blastocyst stage is accompanied by an increase in glucose consumption and lactate production (Leese et al., 2001) as well as large rises in oxygen consumption (Houghton et al., 1996). Oxidative phosphorylation of glucose (mitochondrial respiration and the electron transport chain) is believed to satisfy 70-90% of the ATP demand in most cells (Muoio 2014). However aerobic glycolytic activity, the conversion of glucose to lactate in the presence of oxygen, termed; Warburg metabolism (1956), appears to play a significant role in supporting blastocyst development and has been observed in the cow (Thompson et al., 1996), pig (Swain et al 2002) and human (Gardner et al., 2011).The possible reasons for this are described below and are speculated to revolve around glucose being directed through the PPP for biosynthesis of macromolecules (Smith & Sturmey, 2013) required for cellular growth and division which characterise this phase of development.

The increase in glucose uptake and metabolism is believed to be as a consequence of increased glycolytic enzyme activity, as observed in the mouse (Houghton et al., 1996) and reported by Martin et al., (1993), who measured enzyme activity in single human embryos and found that hexokinase activity increased significantly from the 8-16 cell stage to the blastocyst stage. Increased glucose utilisation may also coincide with the expression of transporters responsible for glucose uptake at the blastocyst stage (reviewed by Pantaleon and Kaye 1998). Glucose uptake is regulated and facilitated by specific carrier proteins; the Facilitated Glucose Transporter (GLUT) or Sodium-Dependent Glucose Transporter (SGLT) family proteins and the expression of those important in the embryo is reviewed in Chapter 6. Once inside the cell cytosol, glucose is phosphorylated to glucose-6-phosphate (G-6P) by hexokinase and this ensures that it is retained within the embryo. This is the first step of glycolysis and at this point the fate of G-6P depends on the ATP concentration in the cell and the REDOX equilibrium, it may (a) be metabolised by glycolysis to pyruvate, which is then converted to (i) lactate or (ii) acetyl CoA and used in the TCA cycle or (b) enter the pentose phosphate pathway (PPP).

It would appear that the utilisation of glucose via the PPP is important again at the blastocyst stage, when cells are rapidly dividing. The PPP provides ribose moieties for nucleic acid synthesis and NADPH for biosynthesis of lipids and complex molecules. It has been proposed that the high glycolytic activity displayed by the blastocyst, whilst being energetically relatively inefficient, does ensure sufficient carbon substrate for

biosynthetic pathways, a process akin to that in cancer cells described by the Warburg effect (reviewed by Smith & Sturmey, 2013). High glycolysis ensures that $NADP^+$ is available for PPP thus facilitating biosynthesis and the production of reduced glutathione, which plays an important role in the protection against oxidative stress. The flow of substrates through this pathway is also regulated by G6PDH and a deficiency in G6PDH in the embryo may reduce developmental capacity (Nicol et al., 2000), highlighting the importance of this pathway.

A further advantage of the high glycolytic flux, is that lactate production is high at the blastocyst stage where it may facilitate endometrial tissue breakdown, new blood vessel formation and induction of local immune modulation of the uterine environment (Gardner et al., 2015). It is speculated that the Warburg metabolism is a selective advantage for preparation for invasion of the endometrium –prior to the establishment of a vascular blood supply (Gardner et al., 2015).

Once the blastocyst has completed expansion oxygen consumption is reported to fall to pre-compaction levels (Sturmey et al., 2003). Of note, the metabolic profile of inner cell mass cells is reported to differ from that of trophectoderm cells (Hewitson & Leese, 1993; Gopichandran & Leese 2003; Houghton 2005). Trophectoderm cells reportedly convert approximately 50% of the glucose they consume into lactate, whereas the cells of the ICM were found to be almost entirely glycolytic. It has been postulated that this may arise due to an oxygen gradient from the outer to the centre of the embryo (Byatt-Smith et al., 1991), resulting in the establishment of spatial relationships between blastomeres. This is however, controversial as it assumes O_2 diffuses as it does through water, whereas in the embryo the O_2 may diffuse more readily into lipid within the cytoplasm. A further factor which must be taken into consideration when attempting the metabolic characterization of the ICM, TE, and blastocoel fluid is, that the metabolism of isolated ICM and TE component cells are likely to be very different to the behaviours seen in an intact embryo.

In summary, the pattern of preimplantation embryo metabolism has been established but, it is also striking that the described differences in substrate utilisation at each stage of development coincide with the substrate availability that has been measured in the female reproductive tract. It would appear that the developing embryo is exposed to a prominent substrate gradient as it makes its way towards the uterus. Firstly pyruvate and

lactate levels are significantly more abundant in the oviduct than the uterus, whereas glucose concentrations are significantly higher in the uterus than in the oviduct (Leese et al., 2008). Secondly, reduction in oxygen tension from the oviduct to the uterus has been reported in animal models including the primate (Fischer et al., 1993).

Collectively, these reports suggest that embryos display a degree of plasticity and metabolic adaptation to their environment. While the metabolism of the embryo is well-characterised under normal maternal physiological conditions- we know very little about how it responds to different environments for example how an embryo formed in response to maternal overweight/obesity may respond. The ovarian follicular environment is known to be enriched with metabolites in OWOB women, notably levels of glucose and triacylglycerol are reported to be higher (Robker et al., 2009). A steady influx of competing fuels can this can impair energy homeostasis (Muoio, 2014). Fatty acids and glucose can each be catabolised to Acetyl CoA and via the TCA cycle generate electron donors (NADH and $FADH_2$). If the electron supply exceeds the demand for ATP (i.e. the ratio of ADP to ATP is low), the mitochondrial membrane potential will rise, creating increased back pressure at the pumping complexes and the generation of ROS, which may lead to cellular damage (Fisher-Wellman et al., 2013). Pyruvate dehydrogenase (PDH) complex, has a critical role regulating the flux of glycolitic intermediates into the TCA cycle. PDH activity is decreased when ratios of $NADH/NAD^+$ and acetyl CoA/CoA are high, reducing glucose uptake. The glucose: fatty acid cycle is impaired in obesity resulting in reduced glucose uptake (as described earlier; the Randle cycle) and contributes to insulin resistance. In support of this, levels of insulin have been shown at an elevated concentration in the follicular fluid of obese women (Valckx et al., 2012) and it seems, this may compromise embryo metabolism, which is reviewed in further detail in Chapters 5 and 6.

1.7.3 Embryo metabolism of substrates provided *in vitro*

Providing the optimum culture environment for gametes and embryos is fundamental for viable embryo development and implantation potential. During the 1990's there was a great deal of research in animal systems-biochemical studies on the metabolic pathways which lead to better understanding of the nutritional requirements of gametes and embryos. The commercialisation of culture medium has increased the production quality and testing procedures, compared to those performed 'in-house' (Chronopoulou

et al., 2015), but a pitfall of this has been reduced transparency, regarding the composition of the media, as companies seek to protect their commercial interests.

Various types of commercial culture media are available, which differ in their complexity and their need for; (a) renewal (i.e. continuous culture to day 5 or renewal on day 3) and (b) their stage specificity or (c) universal use throughout development (i.e. single medium protocol or sequential medium protocol). A present a justification can be made for the use of any of these three key approaches to culture media systems, as the specific needs of gametes/embryos are yet to be fully elucidated, as discussed by Biggers & Summers (2008) in a lucid review of culture media protocols. Much of the debate has centred on the optimum concentration of glucose, the complement of amino acids and functions of glutamine and chelators, such as ethylendiaminetetraacetic acid (EDTA). There is plethora of literature examining the efficacy of different culture media formulations, the specific components and rational for inclusion/ exclusion of supplements based on their reported impact on oocyte and embryo development and an exhaustive review is beyond the scope of this thesis.

The formulation of the complete culture medium aims to support the specific *in vitro* developmental needs of gametes and embryos. Culture media primarily consists of water and ionic salts with added carbohydrates (glucose, lactate, pyruvate), amino acids, antibiotics and macromolecules. Macromolecules (human serum albumin, recombinant albumin), play a number of roles acting as a chelator of heavy metals, functioning as a surfactant, increasing cryotolerance and colloidal osmotic pressure; a comprehensive review is provided by Pool et al., (2012). Media formulations require the correct balance electrolytes (to maintain water balance and avoid osmotic shock) and availability of substrates to support development. At the cleavage stage the balance of pyruvate and lactate maybe important, as early stages embryos metabolise pyruvate as the primary substrate (Hardy et al., 1989) and EDTA is often added to culture media that is targeted to support development at this stage on the basis of its ability to inhibit the premature stimulation of glycolysis (Lane & Gardner 2001). L-Lactate is also often included in the formulation, as it is shown to affect pyruvate metabolism and internal pH (Lane et al., 2000). Media developed specifically for development from day 3 onwards often contains a higher concentration of glucose, but pyruvate is also included as it is utilised throughout development (Hardy et al., 1989), as are amino acids, which act not only as an energy source but act as zwitterons to buffer pH and osmolytes to

control cell volume. The amino acids glycine and glutamine in particular have been shown to proffer a protective role (Van Winkle et al., 1990; Lawitts et al., 1992).

A stable incubation environment is necessary; minor changes in both pH and temperature can disrupt embryo metabolism, altering enzyme function (Regula et al., 1981; Lane et al., 1998; Phillips et al., 2002), microtubule assembly and developmental competence (Pickering et al., 1990; Magli et al., 2008). The pH control is accomplished using a bicarbonate buffered media and equilibrium with 5- 6.5% CO_2, at 37^{o}C, or an alternative is offered by the organic zwitterionic buffers such as a 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES).

A wealth of research has been conducted on the benefits of low culture at low oxygen (5%), which has been shown to result in higher rates of live births (difference of 10 – 15% improvement) when compared with rates among women whose embryos were cultured in an atmospheric O_2 environment (Meintjes et al., 2009, Dumoulin et al., 1999 – to list but a few studies). These findings are supported by numerous studies in various mammalian species, in which even a brief exposure to atmospheric O_2 has been reported to reduce blastocyst development and alter metabolism and gene expression (Pabon et al., 1989; Karagenc et al., 2004; Harvey et al., 2004). *In vivo* embryos are not exposed to oxygen concentrations of above 8% (Yedwab et al., 1976), significantly below that present atmospherically (20%). Furthermore the oxygen concentration in the uterus is believed to be lower than that in the oviduct (Fischer et al., 1993) and may explain why blastocysts are particularly sensitive to higher oxygen concentrations and why culture of embryos under 5% O_2 not only results in a higher incidence of blastocyst formation, but higher mean blastocyst cell counts (Waldenström et al., 2009). The possible implications of oxygen concentration and different clinical embryo culture protocols are reviewed briefly in Chapter 7.

1.8 Obesity and infertility

In the UK, 20.2% of women of childbearing age are obese and a further 30% are overweight. The prevalence of obesity amongst women aged 24-28 has risen from 9.9% in 1990 and 16% in 2004 (Health Survey for England). Within the Humber and Yorkshire region, from where participants for the present study reside, 23.8% of

females are obese, making it the second highest across all regions of England (Scarborough & Allender 2008).

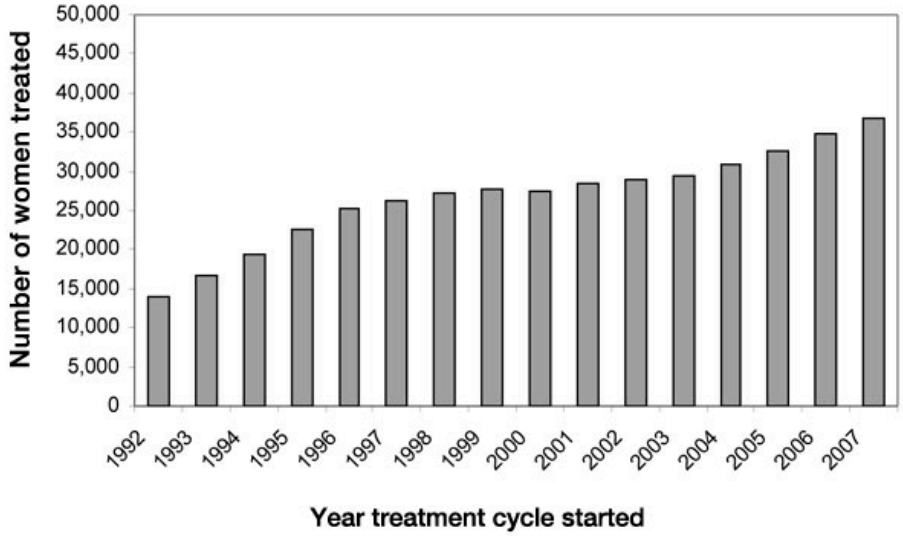

Figure 1.11 Number of women treated at UK fertility clinics, per year.

Data attained from the Human Fertilisation and Embryology Authority (HFEA) database.

In parallel with the rising increase in rates of obesity the number of women seeking fertility treatment is rising annually (Figure 1.11). Obesity impacts on the female reproductive system in a number of ways and is a well-documented cause of female subfertility (Killick et al., 2009). Obese women are less likely to get pregnant, at increased risk of miscarriage and more likely to encounter health problems during pregnancy (Balen & Anderson 2007). Moreover, obese women are at significant risk of suffering gestational hypertension, pre-eclampsia, gestational diabetes later in gestation and have an increased caesarean section rate (Catalano & Ehrenberg 2006, Farquhar & Gillett 2006). Rates of fetal abnormalities including neural tube defects, spina bifida, cardiovascular abnormalities and cleft lip/palate are increased with maternal obesity (reviewed by Kulie et al., 2011). Furthermore, admission to neonatal intensive care, stillbirth and perinatal death are all increased in the infants of obese mothers (Farquhar & Gillett 2006).

The impact of obesity on reproduction starts at a young age. Obese girls often experience the onset of puberty at a younger age than their normal weight peers (Lash et al., 2009). However obesity is frequently associated with disturbances of the menstrual

cycle, with up to 47% of OWOB women reporting menstrual irregularity (Practice Committee of American Society for Reproductive Medicine, 2008). The risk of anovulation, as a result of hyperandrogenism and granulosa cell appoptosis is significantly increased in obese women compared to those of normal weight (Jonard & Dewailly 2004). Polycystic ovarian syndrome (PCOS), a specific metabolic syndrome, is often associated with both annovulation and obesity. It is estimated that approximately 20% of overweight and obese women have PCOS, compared to 9-10% of women who are of normal weight (Yildiz et al., 2008). Many of the hormonal disturbances evident in PCOS women may be consistent with those seen in obese women, linkage between the two conditions has yet to be established. For this reason women with a diagnosis of PCOS have not been included within the scope of this study.

In obese women, metabolic disturbances of the hypothalamic-pituitary ovarian axis may be caused by excessive steroid production from adipose tissue (Gambineri et al., 2002) and by the elevated levels of insulin, leptin (Tamer Erel & Senturk 2009) and other adipokines (further details in Chapter 3). Consequently, the incidence of subfertility (Hassan & Killick 2004, Wise et al., 2010) and menstrual and ovulatory disturbances are higher in these women than in women of normal weight (Clark et al., 1995). Weight loss has been shown to correct some of these abnormalities (Clark et al., 1995), however, weight loss is a long term commitment and any improvements to reproductive outcome as a consequence of decreasing BMI may be offset by the detrimental effects of advancing maternal age on oocyte quality. The impact of obesity and weight loss on pregnancy outcome are discussed further in chapter 3.

1. 8.1 Obesity and oocyte quality.

In assisted conception, oocyte quality plays a critical role in treatment success. For OWOB women, the impact of poor oocyte quality may be magnified since ovulatory problems are circumnavigated by the use of exogenous gonadotrphic stimulation. A number of studies have reported lower IVF treatment success rates in OWOB compared to normal weight and this evidence is reviewed in Chapter 3.

There is ample evidence from animal models to suggest that oocyte quality may be compromised in sub-optimal maternal metabolic conditions (reviewed in Chapter 4). Equivalent data for the human is currently lacking, but the observation of lower oocyte numbers and quality in OWOB women (Maheshwari et al., 2007), combined with a lack

of evidence of any compromised pregnancy outcomes for OWOB donor-oocyte recipients (Styne-Gross et al., 2005) supports the notion that a high BMI is not compromising endometrial receptivity but more likely, is exerting an influence on oocyte development and embryo quality.

Further evidence that oocyte quality may be compromised in OWOB is provided by the finding that the follicular fluid from obese women has been shown to have increased insulin, glucose, lactate, androgen levels (Robker et al., 2009), higher concentrations of non-esterified fatty acids (Valckx et al., 2014), decreased hCG levels (Carrell et al., 2001) and increased leptin (Hill et al., 2007). Further study is required to assess the impact of this pathological environment on the developmental competence of oocytes, ongoing embryo development, quality and implantation potential. However, current methods of assessment of oocyte quality lack sensitivity and are limited to the direct visualisation of nuclear maturation, i.e. development to metaphase II (MII) of meiosis II and extruded the first polar body. The pitfalls of this assessment method and alternative observations that maybe used to compare the quality of oocytes from OWOB and normal weight women is discussed further in chapter 4.

1.8.2 Obesity and embryo quality

A number of reports have indicated that development of the embryo post fertilisation is sub-optimal in the obese (Metwally et al., 2007, Carrell et al., 2001), however others have reported no relationship (Bellver at al., 2010). Such discordance in the literature may well result from lack of comparable objective methods of assessment for embryo quality. Embryo quality assessments are traditionally based on limited observations of appropriate temporal patterns of cleavage division and morphological appearance of blastomeres on day 2/3 of development and blastocyst development on day 5, although advances in time-lapse imaging may strengthen the precision of assessment in the future. The assumption is that the observed development features are affected by intrinsic and environmental factors and as such are a reflection of genetic viability of an embryo.

There is a continuing need for accurate and subjective biomarkers of embryo viability. One possibility is the determination of the consumption and release of metabolites from embryo culture medium (CORE). Compelling evidence has been amassed, demonstrating that the most viable cleavage stage embryos are those that exhibit

a‘quiet’ metabolism (Leese 2002; Leese et al., 2007; 2008). At the blastocyst stage the capacity to metabolise glucose increases significantly at the transition from morula to blastocyst (Devreker & Englert 2000). At this point embryos consuming twice the amount of glucose were more likely to form blastocysts than those that failed (Gardner et al., 2011), those embryos capable of increasing their glucose uptake, with the least expenditure of energy (e.g. measured by oxygen consumption) are postulated to be the most viable (Leese, 2012). The metabolic analysis of substrate utilisation is compared for embryos from women of normal and OWOB BMI in chapters 5 and 6.

Metabolic events not only contribute to embryo viability, but impact on the chances of successful pregnancy and the health of the offspring. The extent to which maternal obesity-induced metabolic disturbances in women impact on embryo metabolism and development has not been studied in any detail but is important because the long-term health of children born to obese mothers is of particular concern. Obesity has been associated with congenital abnormalities in the offspring (Stothard et al., 2009) and the long term consequences of a sub-optimal maternal nutritional environment are only now beginning to be appreciated. The FOHaD suggest a correlation between pre and periconceptional maternal nutrition, embryonic, placental and fetal growth and the development of chronic disease in later life (O’Brien et al., 1999). Embryo metabolism has therefore formed the focus of this research exploiting the unique opportunity to study surplus human embryos created during *in vitro* fertilisation (IVF) treatment cycles

1.9 Working hypothesis and study aims

The primary focus of the study was to elucidate the impact of obesity on reproductive outcome in a subfertile population seeking IVF treatment, with a focus on the periconceptional period. Obesity may be programmed from periconception and the study sought to identify early markers during the critical times of gametogenesis and early embryogenesis that could set a trajectory for future susceptibility to obesity and long-term health issues.

It has been shown in this chapter that the prevalence of obesity is increasing and that rates of subfertility are rising in tandem, suggesting a causal link. It is proposed that this link between obesity and a lower chance of reproductive success (clinical pregnancy and live births) will be replicated in the local population. Contradictions have however,

been highlighted the literature, regarding the impact of obesity on oocyte developmental competence and embryo quality. References to embryo quality and BMI are scarce a, possibly owing to the subjective nature of current methods to assess embryo viability.

The study of embryo metabolism has previously been linked to embryo viability and an overview of the acquisition of oocyte developmental competence the established normal pattern of metabolism has been provided. As the embryo passes through the developmental stages prior to implantation it undergoes very specific changes, reflected in differences in metabolism, hence the embryo displays plasticity and is adaptive to its environment. It is speculated that any inappropriate adaptations to a sub-optimal environment are linked to lower embryo viability. It is the central hypothesis of this thesis that the metabolic phenotype is sensitive to maternal body weight at the time of conception and oocyte and embryo metabolism will be perturbed giving rise to the compromised reproductive outcomes reported for OWOB women. This study will be the first to investigate embryo metabolism from normal and OWOB in human. Proof of this concept for this theory, will be sought though the simultaneous study of the utilisation of available key exogenous metabolites and possible endogenous energy sources by embryos from both normal and OWOB women.

My central argument is that it is the developing oocyte, which adapts to the nutritionally enriched follicular environment reported for OWOB women, resulting in an altered utilisation of substrates for energy metabolism, growth and development. Animal studies have shown that exposure to a high fat diet compromises embryo development, however studies in humans are lacking. It is hypothesised that oocyte maturational events will be compromised, resulting in fewer embryos reaching the blastocyst stage of development and those which do may have a reduced implantation potential, contributing to the poorer reproductive outcomes are reported for OWOB patients. In the short term this results in lower pregnancy rates, increased risks in pregnancy, and possible longer-term adaptations relating to risks of adult onset disease, as discussed previously in this chapter.

The specific aims of each chapter were to compare the outcomes for OWOB women to those of normal weight in terms of:

1) Clinical pregnancy outcomes from natural, IVF fresh and frozen treatment cycles in women of Hull and East Yorkshire.
2) Oocyte developmental competence and morphokinetic parameters.
3) Embryo metabolism of carbohydrates, amino acids and triglyceride content.
4) The influence of *in vitro* manipulation of the developmental environment.

The Hull IVF Unit sees approximately 350 patients each year, 48% of whom are overweight or obese. The reproductive outcomes for these women were studied in detail, in terms of live births, clinical pregnancy and miscarriage rates and with particular focus on the embryo and gamete quality.

In this thesis a range of quantitative metabolic assessments were performed to look at the phenotype and viability of individual human embryos produced by IVF from women with a BMI greater than 25kg/m^2 and compared with those from women, with a BMI in the normal range (below 25kg/m^2). Embryo metabolism provided an objective biomarker of human embryo health and could be measured to examine (a) the relationship with maternal BMI and blastocyst development and (b) whether embryo metabolism is characteristic of a given patient and relates to pregnancy outcome.

The additional information gleaned from this study will enable us to provide information to patients on the effects of maternal lifestyle and how this may influence their prognosis. Furthermore we will be able to advise patients on what positive steps they can take to maximise their chances of pregnancy and ensure their offspring's health.

Chapter 2 : Methods

2.1 Clinical procedures

2.1.1 Clinical Investigation

All patients underwent full clinical assessment prior to treatment to diagnose the cause of infertility. All blood tests (FSH, Oestradiol, LH, Prolactin, Thyroid hormones, Androgens, Progesterone, AMH, viral screen) and semen evaluation were performed by Clinical Pathology Accredited/ ISO15189 laboratories within 12 months of treatment starting. BMI (kg/m^2) was recorded at the initial referral and at the down-regulation appointment prior to treatment to determining 'weight stability', which was defined as maintaining weight over a period of three months.

2.1.2 Down-regulation, stimulation and follicle tracking

Pituitary Regulation was achieved in one of two ways:

(a) *Down regulation with agonist Buserelin acetate*. Buserelin (Suprecur®, supplied by Pharmasure) was commenced on approximately day 21 of the cycle and was given at a dose of 0.5mls once a day, and continued throughout FSH administration;

(b)*GnRH Antagonist; Cetrorelix acetate* (Cetrotide® Merck Serono) was given as an additional injection during the time patients were being given stimulation drugs. Cetrroelix acts directly on the pituitary stopping its production of LH and FSH. The choice of regime was dictated by cost, cycle planning constraints and patients'response to previous regimes.

The patient was said to be "down-regulated" when the endometrium was less than 4mm in width and there were no follicles on either ovary greater than 10mm diameter. If the endometrium was greater than 3mm or a cystic structure was seen on the ovaries (polyps ≥1cm are removed), then the serum oestradiol level was checked and the stimulation cycle was delayed.

FSH stimulation (Menopur® Ferring, Merional® Phamasure, Gonal F® Serono or Puregon® Organon) was started at a dose that was determined by patient age, serum FSH, AMH, response in previous cycles and any history of PCOS (starting dose of 75IU, up to 100 or 150 and increments of 75IU to a possible maximum of 450IU of FSH).

All patients were scanned by ultrasound on Day 6 or 8 of FSH administration at which time endometrial thickness was determined and the sizes of all ovarian follicles above 10mm were recorded. In addition, the number of follicles less than 10mm was recorded. Daily FSH administration was continued at the same dose, if follicular development was seen, and patients scanned at regular intervals. The daily dose of FSH was adjusted according to the total follicle number and size of the second order follicle.

Patients were only accepted for egg collection when the second order follicle was at least 18mm in diameter on ultrasound. Thirty-six hours prior to oocyte retrieval, 10,000IU of human menopausal gonadotrophin (hMG; Pregnyl® Ferring) was administered. Patients stopped Buserelin injections after the hMG was administered, and started taking micronized progesterone (Utrogestan® Besins), six tablets vaginally at night.

2.1.3 Oocyte retrieval and sperm preparation

Oocyte retrieval was performed 36 hours after Pregnyl injection under conscious sedation with a double channel 15-gauge needle. All follicles greater than 15mm were drained. Eggs were placed into commercial culture medium from Copper Surgical (Sage, Quinn's Advantage QA series). Eggs were collected into tubes containing gassed QA Fert medium and incubated at 6% CO_2/ 5%O_2/Bal N_2 at 37^0C for up to 4 hours prior to insemination or injection.

Puresperm (Nidchem) gradients were used to prepare sperm samples. Samples were centrifuged at 200g for 15 minutes. The pellet was washed and spun for a further 5 minutes at 200g. Samples were re-suspended in culture medium and placed into the incubator. Sperm parameters were assessed according to WHO manual criteria (2010) before and after sample work-up. Patients were scheduled for IVF or ICSI accordingly.

2.1.4 Insemination, injection and fertilisation

Where IVF was indicated, eggs were retrieved from tubes 3-4 hours after collection and transferred into 4-well culture dishes (up to 6 eggs per-dish). Oocytes were then graded under a stereomicroscope (x15 to x40 magnification) and assigned a score of 1 to 3 according to the extent of cumulus oocyte expansion, according to previous published criteria (Mikkelsen et al., 2001). Oocytes were co-incubated with 1.0-1.5 x 10^5 sperm per ml of culture media.

Where ICSI was indicated, oocytes were placed into 50ul droplet of cumulase (Origio) for 40 seconds, before being transferred to 50ul droplets of HEPES (QA). After 5 minutes, the cumulus and corona cells were removed by mechanical manipulation. Approximately 3 hours after retrieval, MII oocytes were loaded in individual 4ul droplets of HEPES surrounding a central droplet containing PVP (Origio) and 1ul of sperm. Injections were performed on an Integra Ti micromanipulator (Research Instruments) fitted with Hoffman Modulation Contrast System. Humagen micromanipulation tools were used to immobilise the oocyte with the polar body at the '6 o'clock' position and injection at '3 o'clock' position. Once injected, oocytes were cultured in QA cleavage medium in 4-well culture plates at 6% CO_2/ 5%O_2/Bal N_2 at 37^0C.

2.1.5 Embryo culture and assessments

Approximately 18 hours after insemination or injection, oocytes were assessed for fertilisation and moved to QA cleavage medium. Normally fertilised embryos were assessed on the morning of day 2 and day 3 and were transferred into QA blastocyst medium 112 hours post insemination. Embryos were graded according to the Association of Clinical Embryologists (ACE) national grading scheme, which takes account of cell number, evenness of cells and degree of fragmentation (Table 2.1).

Table 2.1 ACE cleavage stage embryo grading scheme.

Reproduced from Policy and practice document (Cutting et al., 2008).

Blastomere number		
Blastomere size	4 = regular, even division 3 = <20% difference (blast diam) 2 = 20-50% difference 1 = >50% difference	*after Hardarson et al 2001*[33]
Fragmentation	4 = <10% frags by volume 3 = 10-20% 2 = 20-50% 1 = >50%	*after van Royen et al 2003*[35]

Example: The grade is recorded as [cell number] c (size/fragmentation); therefore a 4-cell embryo with slightly uneven cell division (~10% difference in cell size) and around 30% fragmentation by volume will be scored as **4c(3/2)**

On the basis of embryo scores patients were selected to undergo either a day 3 or day 5 transfer. Patients with two or more good quality embryos available on day 3 (6-8 cells and grade 3/3 or above) were selected for blastocyst transfer.

2.1.6 Blastocyst assessments

On the morning of day 5, embryos were graded according to the ACE national grading scheme (Table 2.2), which necessitated the degree of expansion, integrity of ICM and TE to be assessed.

Table 2.2 ACE blastocyst stage grading scheme.

Reproduced from Policy and practice document (Cutting et al., 2008).

Expansion Status	1 = Early blastocyst; blastocoel less than half the volume of the embryo, little or no expansion in overall size, zona pellucida (ZP) still thick
	2 = Blastocyst; blastocoel more than half the volume of the embryo, some expansion in overall size, ZP beginning to thin
	3 = Full blastocyst; blastcoel completely fills the embryo.
	4 = Expanded blastocyst: blastocoel volume now larger than that of the early embryo. ZP very thin
	5= Hatching blastocyst; trophectoderm has started to herniated through the ZP
	6 = Hatched blastocyst; the blastocyst has evacuated the ZP
ICM grading	A = ICM prominent, easily discernible and consisting of many cells, cells compacted and tightly adhered together
	B= Cells less compacted so larger in size, cells loosely adhered together, some individual cells may be visible
	C = Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophectoderm
	D = Cells of the ICM appear degenerate or necrotic
	E = No ICM cells discernible in any focal plane
Trophectoderm	a = Many small identical cells forming a continuous trophectoderm layer
	b = Fewer, larger cells, may not form a completely continuous layer
	c= Sparse cells, may be very large, very flat or appear degenerate

2.1.7 Embryo selection transfer and cryopreservation

One or two of the top scoring embryos were selected for transfer on the basis of female age, overall embryo quality score and past treatment history. Suitable good quality embryos were frozen on day 5 (3Bb or above).

2.1.8 Determination of pregnancy/ data collection

Positive pregnancy was determined by urinary pregnancy test. Fetal heart beat was identified by ultrasound scan at 5 weeks post transfer.

2.2 Donation to research/ extended culture

2.2.1 Consent and donation of fresh embryos

Surplus human embryos not suitable for treatment or cryopreservation may be donated to research if appropriate consents are obtained, or placed into extended culture to permit the study of their developmental kinetics and to provide enhanced feedback at follow-up audit assessment.

All research was carried out according to licence conditions of the Human Fertilisation and Embryology Authority (licence R0067), and with full ethical approval (09/HI304/44). Patients presenting for IVF at the Hull IVF Unit donated embryos with full informed consent. Nursing staff, who were not involved in the research project, obtained patient consent at the down-regulation appointment. Patients were given broad information about the research project aims, i.e. 'The study of the biochemistry of the human embryo'. All patients indicating a willingness to be approached about research were given the opportunity to participate in the study.

Two independent witnesses checked consent forms before any samples or results were coded and released to research. The embryos were anonymised and given a unique "Research Number"; a 5-digit number prefixed with "H", starting at "H00001". This code was recorded next to the patient ID number in a research book, and on the front of a sealed envelope containing a copy of the consent form and on the tube in to which the embryos were be placed for transfer between the IVF Unit and research laboratory.

Patient data were made available from routine clinical investigation and included a description of cause of infertility, age, BMI, smoking status, cycle number, hormonal profile as well as details of treatment, including stimulation, semen parameters, ovarian response, number and quality of eggs retrieved, rates of fertilisation and embryo development and quality, number replaced, number frozen and pregnancy, miscarriage and live birth rates.

The data were retrospectively matched to that generated from the research findings (i.e. patient number to embryo research number) to classify data into embryos that originated from patients who were overweight/obese (OWOB) (BMI $\geq$25kg/m^2) and compared to embryos from women of normal BMI (18.5 to 24.9kg/m^2). Women with polycystic

ovaries were excluded from the study as it was anticipated that these may represent an additional subgroup with a very specific metabolic profile linked with the condition.

2.2.2 Consent and donation of frozen embryos

Frozen embryos that had been donated to research were removed from storage at the monthly cryo-audits and allocated to a dedicated research tank. A detailed log of the storage location, patient details and witnessing procedures was maintained. An anonymised version was made available for research use, detailing the embryo code, numbers, stage of development, freeze details and expiry dates. Embryos were requested for use in advance of the expiration date and were transferred to the research laboratory when as much identifying information as possible had been removed from visitubes. The embryos were thawed using commercial thaw medium appropriate to the stage of development (Origio cleavage stage or blastosyst stage medium) and were cultured and analysed as per fresh embryos donated to research.

2.2.3 Research embryo culture and assessment

Once donated to research, embryo development stage and grade were recorded and embryos placed individually into 4µl drops of Earle's balanced salt solution (EBBS), supplemented with 0.5% (v/v) synthetic serum albumin (SSA, Sage), 1mM glucose, 0.47mM pyruvate, 5mM Lactate and a physiological mixture of amino acids (Houghton et al., 2002) (details given below). Embryos were cultured under light mineral oil (Sage) at 37°C in 6% CO_2/5% O_2/Bal N_2 for 24 hour periods, alongside control drops, maintained in identical conditions, but free of embryos. Micro-droplet culture plates (Falcon 0061) were prepared as displayed in Figure 2.1.

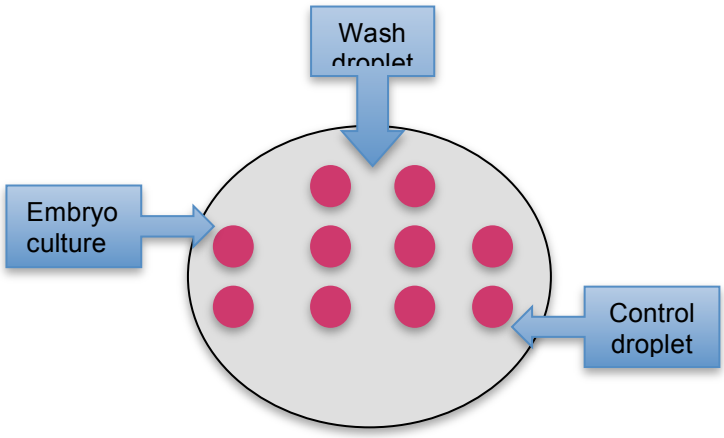

Figure 2.1 Micro-droplet culture set up.

Media were changed every 24hours and plates frozen at ˉ80°C.

Embryos that failed to form a blastocyst, but continued to undergo cell divisions/ organisation were classified as 'slow developing', whereas those that reached the blastocyst stage were classified according to their degree of expansion (unexpanded, expanded, hatched and graded as per clinical protocol). An embryo that failed to change after 48 hours culture was considered arrested. Observations were halted on day 9 or at developmental arrest. After incubation, the spent culture medium was frozen immediately at -80^{0}C for later analysis.

Culture medium was prepared from stock solutions as described in appendix A1, and used to prepare a working solution (Table 2.3). Media were pre-incubated at 37^{o}C, 6% CO_2, 5% O_2 for at least 2 hours to stabilise the pH to 7.3±0.1 prior to use.

Table 2.3 Final working solution of standard embryo culture medium used to culture research embryos.

Component	**MI**
EBBS	1
Sterile water	6.935
NaHCO3	1
Antibiotics	0.1
Pyruvate	0.1 (0.47mM)
Glucose	0.2 (1mM)
Glutamine	0.2 (0.2mM)
SSA	0.25
D-L-Lactate	15µl (5mM)
Amino acids	0.2

2.3 Metabolic profiling

2.3.1 Ultraflourometric methods

Metabolic COnsumption/RElese (CORE) profiles (Guerif et al., 2013) were determined by measuring the depletion and appearance of key compounds from the culture medium, namely glucose, pyruvate, lactate and 18 amino acids, according to established techniques, that may be applied to individual oocytes and embryos (Sturmey et al., 2009) and provide quantitative markers of embryo health, throughout development.

Triglyceride content of embryos was then determined at the end of the culture period from samples of embryos pooled in groups of 2-5 embryos at equivalent development stages for each patient.

Glucose and pyruvate consumption and lactate production were measured using assays based on the ultramicrofluorometric methods described by Leese et al., 1984. The assays are based on the enzymatic phosphorylation of substrate and the subsequent consumption or generation of NADH or NADPH in coupled reactions. This causes an increase in fluorescence that is directly proportional to the concentration of substrate present. Fluorescence was measured using a plate reader (Tecan Infinite M200; Tecan Reading, UK or BMG LabtechFLUOstar Omega Flourometric; BMG Labtech, Buckinghamshire, UK) (excitation 340nm, fluorescence 459nm and above). Reactions were carried out in 96 well assay plates with 'V' shaped wells. Standards were assayed in triplicate – in the layout given below and incubations were at 37^0C unless otherwise stated. The concentration of substrate was related to a series of standards, for each experiment, for sample and control droplets. All values are expressed as pmol per embryo per hour.

2.3.2. Glucose assays

These are based on the enzymatic phosphorylation of glucose and the subsequent reduction of NADP+ to NADPH. This causes an increase in fluorescence which can be measured. The reactions are as follows;

(a) Glucose + ATP --*hexokinase*-→ glucose-6-phosphate

(b) Glucose-6-phosphate + $NADP^+$ --*G6PDH*→ Gluconate-6-phosphate +NADPH + H^+

A reaction mixture was prepared, based on the following; 4-(2-Hydroxyethyl) piperazine-1-propanesulfonic acid (EPPS) buffer (31.25mM) and containing DL-Dithiothreitol (0.42mM), MgSO4 (3.08mM), ATP (0.42mM), NADP+ (1.25mM), Hexokinase (14.17Uml)/ G6PDH (7.08U/ml) (Table 2.4)

Table 2.4 Components of buffer, cocktail and substrate for glucose assays

Chemical	Molecular weight	Supplier	Cat. No.
EPPS	252.33	Sigma	E9502
Penicillin G	372.5	Sigma	P4697
Streptomycin	1457.4	Sigma	S1277
Dithiothreitol	154.2	Sigma	D0632
Magnesium sulphate	246.48	Fisons	M/1050
NADP		Roche	128 040
ATP	551.1	Sigma	A6419
Hexokinase/G-6-P		Roche	127-825
Glucose standard 5mM/L		Analox	GMRD-010
Sodium hydroxide	40.0	Fisher	S4920/60

EPPS buffer was prepared from 2.52g of EPPS dissolved in 150ml of distilled water and adjusted to pH to 8 with 1M NaOH. (4g NaOH in 100ml of distilled water) and made up to 200ml with distilled water.10mg Penicillin G and 10mg of Streptomycin were added to the buffer (Table 2.5).

Table 2.5 Composition of EPPS Buffer

-adjusted to pH8 with 1M NaOH

Chemical	Amount	Final concentration
EPPS	2.52g	50mM
Penicillin G	10mg	50µg/l
Streptomycin	10mg	50µg/l
Water	200ml	-

The glucose assay cocktail was prepared using the stock solutions listed in Table 2.6.

Table 2.6 Stock solutions for Glucose cocktail

Chemical	Weight (mg)	Distilled water (ml)
Dithiothreitol	7.715	10
Magnesium sulphate($MgSO_4$)	91.2	10
NADP	39.37	5
ATP	30.26	5

The glucose cocktail consisted of the following well mixed constituents; 15ml of EPPS buffer,1ml of Hexokinase glucose 6 phosphate dehydrogenase, and from table x, 2ml of Dithiothreitol, 2ml of Magnesium sulphate, 3ml of NADP, and 1ml of ATP. Chemicals were aliquoted into 1.5ml Eppendorf tubes and stored at -20°C for up to 8 weeks.

To prepare substrates to measure a glucose standard curve (Table 2.7), a 1mM stock of glucose solution was prepared (100µl of the 5mM standard glucose solution to 900µl of distilled water). This was diluted further as follows in Eppendorf tubes, which were stored on ice.

Table 2.7 Glucose standards

1mM stockµl	Distilled water µl	STD mM
100	0	1.0
80	20	0.8
60	40	0.6
40	60	0.4
20	80	0.2
0	100	0.0

The florescence of the glucose reaction mixture was measured, then the standard was added (to a ratio of 1:10), after ten minutes incubation at room temperature the fluorescence was measured again and the difference was calculated. The average value for each standard was calculated and plotted.

A standard curve was produced for each assay in the range of 0-0.5mM with only standard curves with a correlation coefficient (r^2) of >0.9 being accepted.

The florescence was measured for sample droplets and control (non-embryo containing) droplets and the changes related to those given by the standards. The values were attained by substituting the linear regression equation.

2.3.3 Lactate assays

These are based on the reduction of NAD^+ to NADPH and the conversion of lactate to pyruvate.

Lactate + NAD^+ --*LDH*→ Pyruvate + NADH + H+

The equilibrium of the reaction is to the left and in order to drive it in the direction of Pyruvate and NADH, hydrazine sulphate is added, which combines with pyruvate to form hydrazine pyruvate and removes it from solution; the reaction is also carried out at pH 9.4 to remove the protons that are formed.

A reaction mixture was prepared based on glycine-hydrazine buffer (0.474mM glycine/ 0.189mM hydrazine), ddH20, LDH (15.79U/ml) and NAD^+ (4.76mM).

Glycine-hydrazine buffer was prepared as listed below (Table 2.8) and used in the reaction cocktail mix as described in Table 2.9.

Table 2.8 Composition of glycine-hydrazine Buffer

- adjusted to pH9.4 with 1M NaOH.

Chemical	Amount	Final concentration
Hydrazine sulphate	5.2g	400mM
Glycine	7.5g	322mM
EDTA	0.2g	5.4Mm
Water	100ml	-

Table 2.9 Stock solutions for lactate cocktail

Chemical	Amount	Final concentration
Glycine-hydrazine Buffer	9ml	0.474mM/ 0.189mM
LDH	0.5ml	15.79U/Mm (at 25^0C)
NAD^+	1.5ml	4.76mM
ddH_2O	8ml	-

The fluorescence was measured as per the procedure for glucose. Standards or samples were added to the cocktail droplet in a 1:10 dilution and the reaction time was 30 minutes at 37^0C. The concentration of lactate present in the samples was compared with the standards and adjusted for non-specific changes in fluorescence using data from control droplets.

2.3.4 Pyruvate assays

This reaction is based on the oxidation of reduced NADH using LDH

Pyruvate + NADH + H^+--*LDH*→ Lactate + NAD^+

As the reaction proceeds readily to the right there is a decrease in fluorescence, as NADH is being oxidised to NAD^+.

A reaction mixture was prepared based on EPPS (47.6Mm), LDH (16.32U/ml) and 0.1mM NADH, pH 8. 1µl sample/ standard was added to 10µl assay mixture and

incubated for 3 minutes at 37^0C. The final concentration of pyruvate in each spent culture sample was determined from a six point standard curve of 0-0.45mM pyruvate.

2.3.5 Determination of triglyceride content

Triglycerides were measured using a coupled colourimetric assay described by Sturmey and Leese (2003). In a dual reagent assay the amount of glycerol present in the sample was quantified by the amount of NADH oxidised to NAD^+.

On day 9 of development embryos were incubated with 0.1 % (w/v) pronase for approximately 30 seconds to remove the zona pellucida allowing the enzymes used in the TG assay access to the intracellular triglyceride. A 0.5 % pronase solution was prepared by dissolving 0.02 g pronase in 20 ml 0.9 % NaCl (0.9g NaCl + 100mls H2O). 500 µl aliquots were stored at $^-20^o$C and pre-warmed prior to use.

Individual embryos were placed into a 1.1µl droplet of freezing buffer (Table 2.10) and aspirated into a 5µl microcap using a syringe (Figure 2.2). The microcaps were then sealed with parafilm, before being coded and stored at -80^0C to allow blind analysis at a later date.

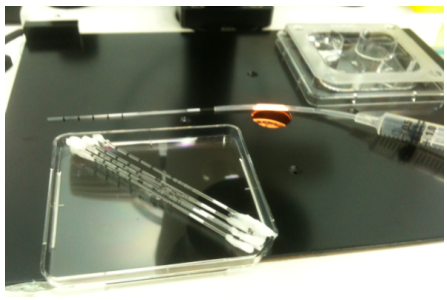

Figure 2.2 Samples frozen in microcaps, sealed with paraffin.

Table 2.10 Composition of Freezing buffer.

*FCS of known TG content, as certified by Sigma. Freezing buffer was stored at $+4^o$C for up to 8 weeks.

Component	Amount
PBS	50 ml
BSA	0.05 g
FCS*	0.15ml

The triglyceride assay (Ferguson & Leese, 1999) is based on the enzymatic hydrolysis of triglyceride into free fatty acids and glycerol.

(a) Triglyceride --*Lipase*→Glycerol + Free fatty acids
(b) Glycerol + ATP –*Glycerol kinase*→Glycerol-1-phosphate + ADP
(c) ADP + Phosphenolpyruvate –*Pyruvate kinase*→Pyruvate + ATP
(d) Pyruvate + NADP + H^+ --*Lactate dehydrogenase*→Lactate +NAD^+

The amount of glycerol present in the sample was quantified in terms of the amount of NADH oxidised to NAD^+. This causes a change in the fluorescence which was measured using a plate reader (Tecan Infinite M200 / BMG Labtech) (excitation 340nm, fluorescence 459nm and above). The reagents necessary for the assay were derived from a triglyceride test kit (Sigma UK) which was scaled down to work at a microlitre level. The assay used two reaction mixtures; mixture A contained ATP, lactate dehydrogenase, lipase, NADH, phosphoenol pyruvate, pyruvate kinase, buffer and non-reactive stabilisers and fillers; mixture B contained glycerol kinase and non-reactive stabilisers and fillers.

A six point standard curve was established before each assay (r^2>0.9), using triglyceride standards in the range of 0- 0.54 µg /ml (Table 2.11). Standards were prepared by diluting certified FBS of known triglyceride concentration with ddH_2O.

Table 2.11 Triglyceride standards

STDµg/ml	Amount of ddH_2O µl	Amount of FCS µl
0	100	0
0.135	75	25
0.27	50	50
0.405	25	75
0.459	15	85
0.54	0	100

Reactions were carried out in 96 well assay plates incubated at 37^0C. 10µl of reagent A was added to each well, the plate was then incubated for 10 minutes prior to recording

the fluorescence. 1µl of standard (or sample, once a suitable standard curve had been established) was then added to each well and the process repeated. Finally 1.1µl of regent B was added to each well and incubation for 7 minutes prior to reading the florescence.

Any endogenous ADP was consumed by the pyruvate kinase and lactate dehydrogenase reactions, prior to the addition of cocktail B. The reactions (b), (c) and (d) could then proceed and any changes in fluorescence that resulted were from the conversion of NADH to NAD^+ derived from ADP resulting from the hydrolysis of TG only.

The amount of triglyceride was calculated by relating changes in fluorescence to those given by the standards. Blank samples containing freezing buffer but no embryos were also assayed and the values subtracted from the unknowns to account for dilution effects and any triglyceride present in the freezing buffer.

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2.3.6 HPLC

Amino acid profiles were determined in spent culture droplets by reverse-phase high performance liquid chromatography (HPLC), as described by Houghton et al.,(2002) and Leary et al.,(2012). Briefly 2µl of sample was diluted 1:12:5 in HPLC grade water. The sample was then diluted into a mobile phase (83mM sodium acetate/Methanol), pumped under high pressure onto a C-18 ODS Hyperclone column (Phenomenex, UK) and the column retention time and signal amplitude was used to determine the identity and concentration of each amino acid. Average sums of amino acid production and depletion, were expressed in pmol/embryo/hour for day 5 to 9 of culture. All data were normalised to a non-metabolisable internal standard. Results were recorded according to stage reached at the end of the period of culture.

Amino acid profiles of spent culture droplets were determined using a Agilent 1100 Series automated HPLC (Santa Clara, CA, USA) system, fitted with a PhenomenexHypoclone 5µm C18 octodecyl silica column 250 x 4.6mm (Phenomenex, Macclesfield, UK). Chromatography was performed at 25^0C.

The reagents required are;

1. OPA (o-phthaldialdehyde) 1ml supplemented with 2µl β-mercaptoethanol and aliquoted into 1.5ml glass HPLC vials and stored at -20^oC. This is used as a pre-

column derivitising reagent, as in the presence of β-mercaptoethanol (reducing agent) it reacts with primary amines to form flouresentthio-substituted isoindoles which can be separated and analysed.

2. Solvent buffers A and B, which are based on sodium acetate (56.5g in 5 litres ddH2O, adjusted to pH 5.9 with glacial acetic acid) and methanol. The composition shown in table 2.12. Buffer A is hydrophilic and buffer B is hydrophobic, both are required to elute the amino acids from the column. The gradient of each was series automated, starting with a high concentration of A and adding B.
3. Amino acid standard solution composed of 1:1 mix of (a) reference amino acid mixture, Sigma AA-S-18 amino acid standard solution and (b) DABA mix – a non-metabolisable internal standard, against which, all peak areas were corrected.

Table 2.12 Composition of solvent buffers

Chemical	Amount- Buffer A	Amount – Buffer B
83mM Sodium Acetate Buffer	800ml	200ml
Methanol	200ml	800ml
Tetrahydrofuran	12ml	-

Samples were prepared for chromatography by diluting 2µl of spent and control media drops with 23µl of ddH_2O (1:12.5). Samples were interspaced with 25µl vials of amino acid standard solution and loaded into the sample tray, with position 1 being occupied by OPA and position 100 with a vial of wash solution. During the analysis, each 25µl sample was mixed with 25µl OPA and after a reaction time of 2 minutes, 25µl of the mixture was loaded onto the column, using the autosampler device. The peaks were eluted with a gradient mobile phase. Using the retention time (column interaction time, prior to being eluted) the peaks were identified. Quantification of amino acid concentration was carried out by comparing peak areas of unknowns against those given from the certified standard solutions (area units per pmol, corrected OPA). To calculate the amino acid concentration, values from peak area were then converted to actual number of moles in the control (from the blank culture droplet) and sample, the difference between these values gave the change attributed to the embryo. Values were corrected for sample volume and dilution and subsequently calculated in terms of pmoles per embryo per hour of culture. A net fall in an amino acid concentration was

termed ‘consumption’, a rise ‘release’ and the sum of these two was termed ‘amino acid turnover’.

2.4 Time lapse recordings and measurements

Embryos at various stages of development were placed into extended culture and observed using time-lapse technology (Primovision).

Well-On-Well (WOW^{TM}) 16 point dishes were prepared in advance. Each individual well was pre-filled with culture medium using a 135µl micropipette aimed at the side of each well to avoid any scratches or air bubbles. A Gilson pipette was then used to deliver an 80µl overlay of media, which was subsequently covered in 2-3mls oil to avoid osmotic changes. Prior to use, trapped air was removed by tapping the base of the dish firmly against the surface of the flow hood and using a pipette remove the air bubbles. When placed into the wells, embryos sank to the bottom. The location of each embryo was recorded, the dish was aligned onto the Primovision camera. The camera was connected and project started and assigned a project name, after which, the duration and frequency of imaging was set. Development was observed and data interrogated using the Primovision Analyser software. Recordings were made of specific developmental timings, blastocyst diameters and hatching patterns. Embryos were cultured until day 9 of development, images were acquired every 20 minutes. Further details are contained in chapter 4.

2.5 Embryo fate; disposal, disruption and freezing & differential cell staining

Embryo development was arrested of on day 9 of development (or day 7 if a weekend). Development was arrested by one of the following;

- The addition of alcohol.
- The disruption of development with pronase and freezing of embryos in microcaps for triglyceride analysis (details included in chapter 5).
- Expanded blastocysts were fixed on day 7 of development using the differential staining technique describes by Thouas et al., 2001. Chromatin-specific dyes were used to determine ICM and TE counts (details included in chapter 4).

In each scenario the removal of embryos from culture was witnessed and recorded by a second operator.

2.6 Statistical analysis

The data from the metabolic profiling of spare embryos was compared between normal and OWOB women and retrospectively correlated to the chosen study end points of (a) blastocyst development and (b) clinical pregnancy outcome of the sibling embryos form transferred sibling embryos (which had not been analysed). Power calculations were performed based on the Birket and Day method (1994), and studies were designed to achieve 80% power, unless otherwise stated. All analyses were performed using SPSS (Statistical Package for Social Sciences v11.5) and Minitab (v17).

Univariate regression analysis was used to compare continuous data and paired t-test chosen to compare grouped two sample data. ANOVA was used to assess intra and inter patient variability within the embryo cohort in combination with multiple linear regression analysis to assess the predictive accuracy of metabolic profile on blastocyst development rate. To account for patient-specific effects, Generalized Estimating Equations (GEEs) were used to model the mean response and within cluster associations separately to reduce the variance and increase the power. Principal component analysis was used to reduce the dimensionality of the individual 18 amino acid measurements and adjust for multiple testing.

Levens test for normality was performed and ANOVA with Tukey Kramer was utilised as indicated. Mann-Whitney U-test (2-groups) and Kruskal-Wallis test (2 or more groups) were used to compare means when homoscedasticity could not be assumed.

In each scenario, the null hypothesis, that all groups are simply random samples from the same population i.e. female BMI has no bearing on embryo viability criteria, was rejected when the probability (p-value) was less than the 95% confidence threshold ($p<0.05$). The statistical design of experiments was changed in each setting to adjust factors and measure responses in an attempt to determine an effect, and further detail is included in each chapter and within the appendix. Throughout the course of this study (from 2010 to 2015) clinical policy and protocol changes may have introduced 'nuisance' variables that need to be characterised and subsequently blocked, randomised or controlled for in the experimental design. The study population and details of extraneous variables and validation of experimental design/ modelling are described in full in the validation section below.

2.7 Method validation / refinement

2.7.1 Sensitivity and specificity of assays

A number of pilot studies were performed to validate and refine the assays. Firstly a monochromator florescent plate reader was used in place of conventional florescence microscopy. This necessitated the use of 'v' bottom transparent plates since fluorescence was read from the bottom and readings taken at 340nm excitation and 460 emission. The increase in fluorescence observed with increasing amounts of glucose was linear. The values of R^2 for linear regression lines for glucose, pyruvate and lactate each exceeded 0.9 and replicate experiments indicated high repeatability of the method.

The accuracy of the assay was determined by comparing values obtained for commercial culture media with those reported by the manufacturer. The average glucose concentration in Quinns advantage blastocyst was determined to be 2.70mM compared to the reported concentration of 2.78mM (Table 2.13).

Table 2.13 Verification of method using media of known metabolite concentration (Quinn's advantage blastocyst media)

Substrate	Calculated value mM	Reported value mM
Glucose	2.70	2.78
Lactate	2.72	2.04
Pyruvate	0.25	0.1

The experiments were repeated using different dilutions; 1 in 10, 1 in 5, 1 in 2 to determine the most appropriate/ accurate method for determining differences due to embryo metabolism. A dilution factor of 1 in 10 was applied. The experiments were repeated using different volumes and with / without oil overlay. The lowest volume permitted for accurate reading is 0.5ul of sample and an oil overlay did not improve the sensitivity of the assay.

To determine the most appropriate sample volume for embryo culture, embryos were cultured in 25µl, 10 µl, 5 µl and 1µl samples of QA blast media for 24 hours and glucose concentrations were analysed against controls. The assay was sufficiently sensitive to determine differences between wells of control and test media when the lowest volume was used, however to reduce the risk of pipetting errors reducing the

accuracy of the analysis- embryos were cultured in 4µl droplets of defined medium containing lower concentrations of substrates than those found in the commercial medium. A working solution of culture media was prepared, in which the concentration of glucose was reduced to 1mM and the concentration of pyruvate was reduced to 0.47Mm, no lactate was added. This ensured that differences in production/ uptake could be readily determined. A volume of 4ul and culture period of 24 hours was sufficient to ensure that substrate availability did not become a limiting factor for embryo development. After 24 hours the culture plate was changed the spent media was frozen at -80^{0}C until assay.

2.7.2. Validity of using supernumerary embryos

To determine how representative the analysis of sibling embryos metabolism is of those transferred, data previously collated and reported by Conaghan et al., (1993) was re-analysed (with the Author's permission). Inter-patient variability was compared in transferred embryos only and it was determined that pyruvate consumption differed amongst groups and could be used to predict pregnancy outcome. Figure 2.3 shows that implantation and pyruvate consumption on day 2 were negatively correlated (t=-2.581, p=0.010). The f value of 6.663 is indicative of significant variability amongst implantation grouping of failed implantation (0), singleton (1) and twin (2) implantation ;p=0.010.

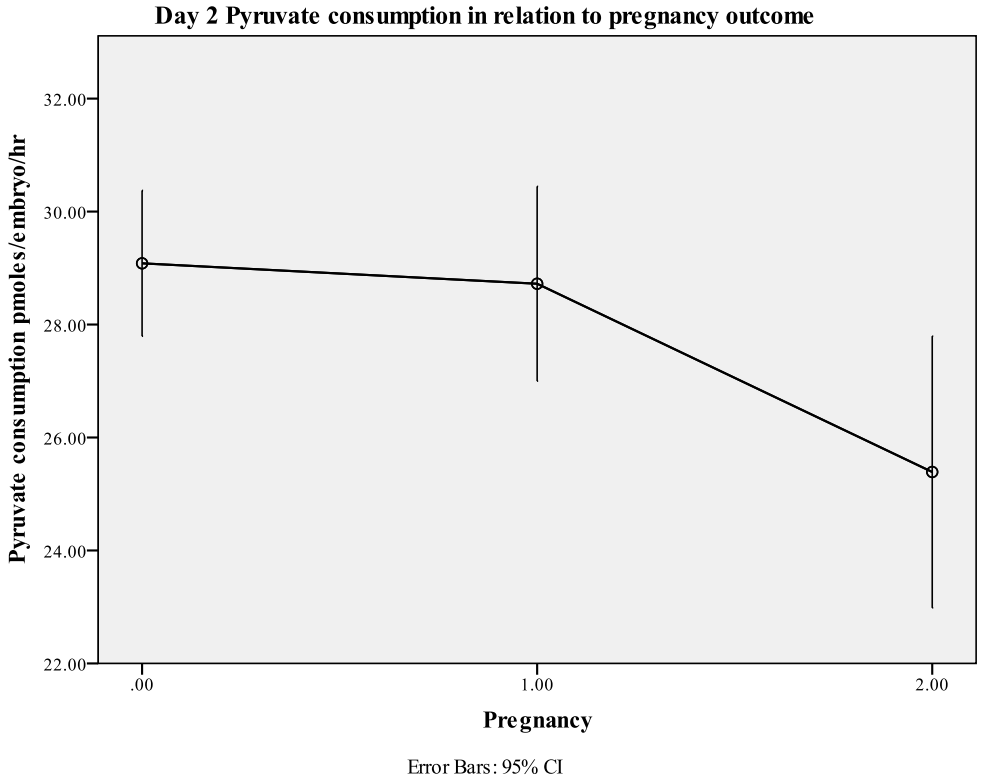
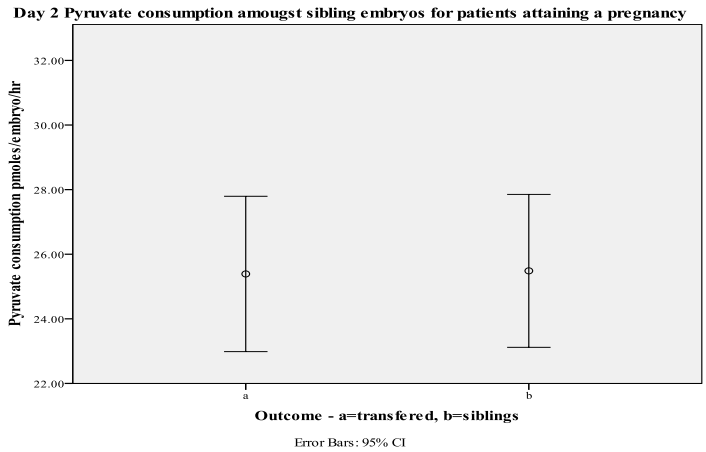


Figure 2.3 Lower pyruvate consumption on day 2 is indicative of higher implantation potential.

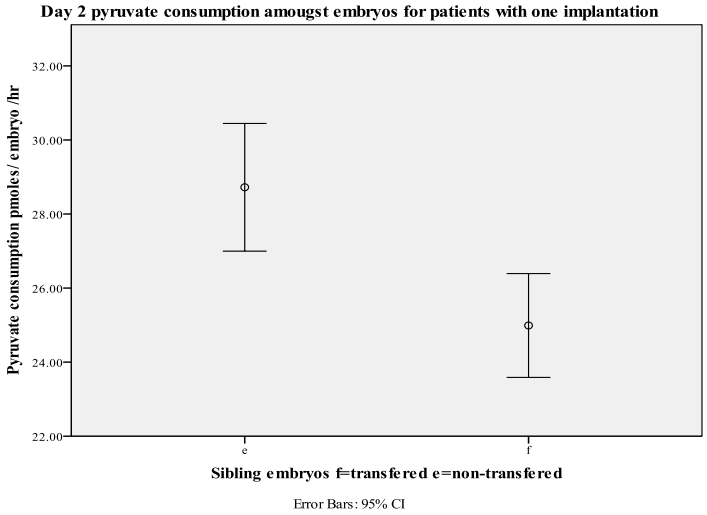
When two embryos were transferred, those with the lowest pyruvate consumption were more likely to implant (p=0.01).

When the data were analysed to assess intra-patient variability by comparing the pyruvate consumption of all embryos in a patient's cohort, rather than just the two transferred, there was no significant variability evident amongst sibling embryos for each implantation category (Figure 2.4A-twin, B- singleton, C-no implantation).

2.4A



2.4B



2.4C

Figure 2.4 Intra patient variability; sibling embryo pyruvate consumption.

(A) For 100% implantation group n=92 embryos transferred, 160 not transferred, from 22 patients. Overall no significant variability amongst sibling embryos p=0.67 (0/22 patients had any significant differences amongst sibling embryos). Figure (B) For the 50% implantation group p=0.148 (2/30 patients had variability), n=126 transferred, 193 not transferred. Figure (C) For the 0% implantation group p=0.422 (16/71 patients had variability), n=283 transferred, 321 not transferred.

The findings presented from this re-evaluation of previous collated data, serve to indicate that the metabolism of sibling supernumerary embryos is reflective of those transferred and is indicative of the probability of pregnancy, thus it is appropriate to relate embryo metabolism of supernumerary embryos to the pregnancy outcome of their transferred sibling embryos for which data is not available.

2.7.3. Validity of using mean CORE profiles determined over a 24 hour period.

To determine if 24 hour sampling and mean values provide a sufficiently accurate representation of metabolic CORE profile in relation to developmental progression, sampling was combined with time-lapse imaging. In a crude experiment, prepared research culture medium was dispensed into alternate wells of the WOW dish to ensure droplets were not in communication, the dome was not filled. Wells were individually selected for analysis. At 4 hour intervals the media was refreshed. Data were collated from 23 embryos (4 patients). Developmental stage was recorded at the start of culture – observation point 1, after 4 hours culture (observation point 2- sample point 1) and 8 hours (observation point 3 – sample point 2). Embryos were subsequently cultured under standard conditions for a further 48 hours to ascertain continued developmental viability or arrest. Real-time developmental activity was correlated to CORE profiles as shown in figure 2.5.

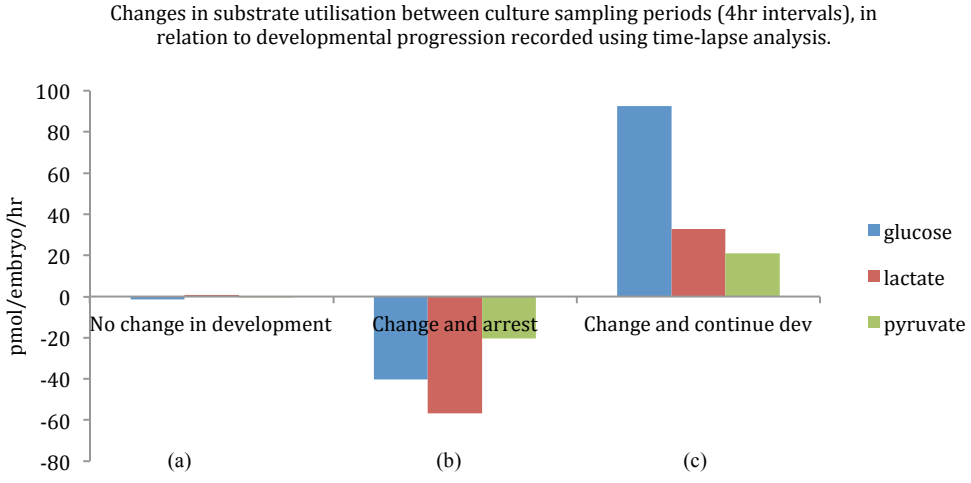


Figure 2.5 CORE profile of embryos coupled with time-lapse observations over an 8 hour period.

(a) Viable embryos that did not divide during the 8 hours utilised substrates at a constant rate. (b) A fall in CORE activity was recorded post- cell cycle arrest. (c) Division activity was associated with an initial increase in CORE of substrates.

The data indicated that if the embryo displayed no change in developmental status throughout the 8 hour evaluation period– but was still viable (i.e. developmental change observed within 48 hours) then the CORE profile did not differ significantly at sample point 1 and 2. Embryos that underwent further development between observation point 1 and 2 and then subsequently arrested between point 2 and 3 and did not change after 48 hours additional culture utilised a lower turnover of metabolites between sample point 1 and 2. The converse was true for those that displayed an active development profile – continuing to develop until culture was terminated.

These findings indicate that developmental and metabolic changes are synchronised and whilst 24 hour sampling and static observations will carry a level of uncertainty, the results should be broadly representative. The findings of this crude experiment must however, be interpreted with caution as sampling errors will be amplified when using the low volumes necessitated in this analysis.

2.7.4. Study population and validation of confounding variables

This thesis has incorporated retrospective clinical data from;

1. Antenatal clinic setting 2001 to 2007 (n=2269 women)
2. Hull and East Yorkshire Sub-fertility clinic setting 2006 (n=345 women)
3. Hull IVF Clinic fresh treatment cycles 2008 to 2010 (n= 709 women)
4. Hull IVF Clinic frozen treatment cycles 2008 to 2011 (n=65 women)

This data has been utilised to provide context for the primary research findings and examine the wider impact of female BMI on reproductive health, the findings are discussed in Chapter 3.

Prospective data have been collected from those women contributing to the primary research project from 2010 to 2015;

1. 29 women attained 218 oocytes and 101 embryos that were observed in studies. (Chapter 4)
2. 176 women donated 808 fresh embryos (Chapter 5 and 6)
3. 26 women donated 106 frozen embryos (Chapter 6).

Figure 2.6 provides a summary of how these donated embryos have been utilised, depicting the experimental design groupings and justification. This figure will appear again in subsequent chapters to highlight the particular study groupings being discussed.

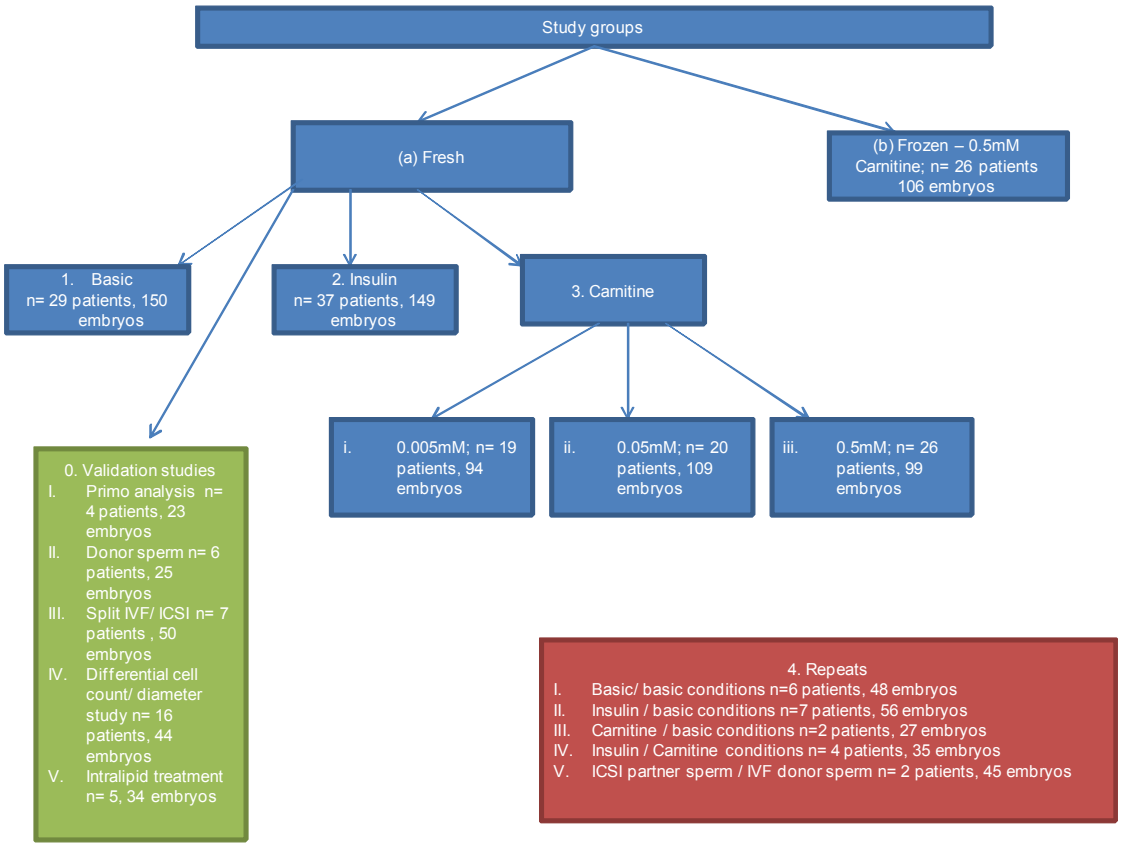


Figure 2.6 Summary of number of patients and embryos included in each study group

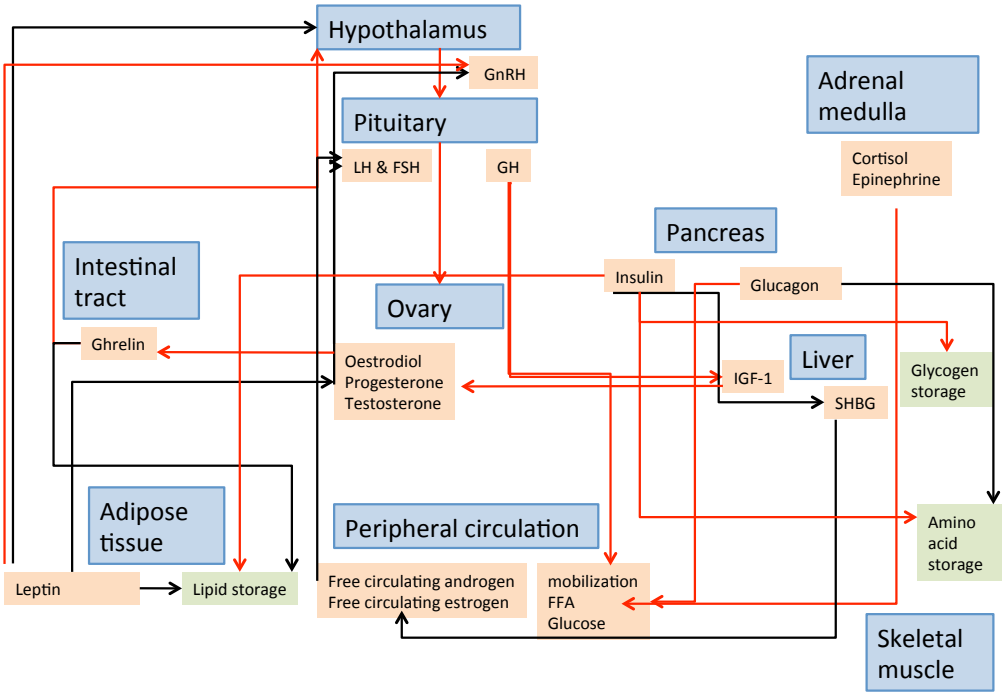
The demographics of each study group are discussed in detail in each study chapter, with specific reference to the matching amongst study groups for confounding variables such as age, male BMI, cause of infertility. Any differences in clinical protocol applied throughout the duration of the study are also accounted for, including; an increase in the use of antagonist stimulation protocol (from August 2013), a change in culture medium from Sage® to Vitrolife® (from July 2014), prior culture in WOW system for a subset of patients (from July 2014) and use of intralipid infusion therapy for a restricted patient set (from August 2013). Differences in research protocol have also been accounted for – specifically the introduction of low oxygen culture for research from day 5-9 (from January 2013) – to align with the prior clinical culture conditions.

Chapter 3 : Obesity and reproductive outcome

3.1 Introduction

3.1.1 OWOB women and rates of natural conception

Infertility in natural cycles is almost three-times higher among obese women (Rich-Edwards et al., 1994). OWOB women take longer to conceive even if they are young and have regular menstrual cycles (Robker et al., 2009). Obesity is however, commonly associated with menstrual disorders, primarily anovulation, implying that excess weight can impact on ovarian function via the endocrine system. Obesity is coupled with changes in the secretion and action of insulin and other adipose hormones including elevated leptin, depressed adiponectin, elevated resistin and depressed ghrelin (reviewed by Eyster 2011). These endocrine parameters may combine, leading to hyperinsulinism and hyperandrogenism (reviewed by Mitchell et al., 2005). The hormonal mechanisms that link nutrition and female fertility are depicted in Figure 3.1 and the metabolic and endocrine related disruption seen in obese women that leads to ovulatory dysfunction is highlighted in Figure 3.2.



→ Stimulates, ⟶ Inhibits

Figure 3.1 Hormonal links between nutrition and fertility.

Insulin promotes uptake and storage of glucose, lipids, amino acids. Leptin reduces appetite and increases energy expenditure and lipolysis. These two hormones alter the bioavailability of oestradiol and testosterone and may also have direct functional actions on the ovary.

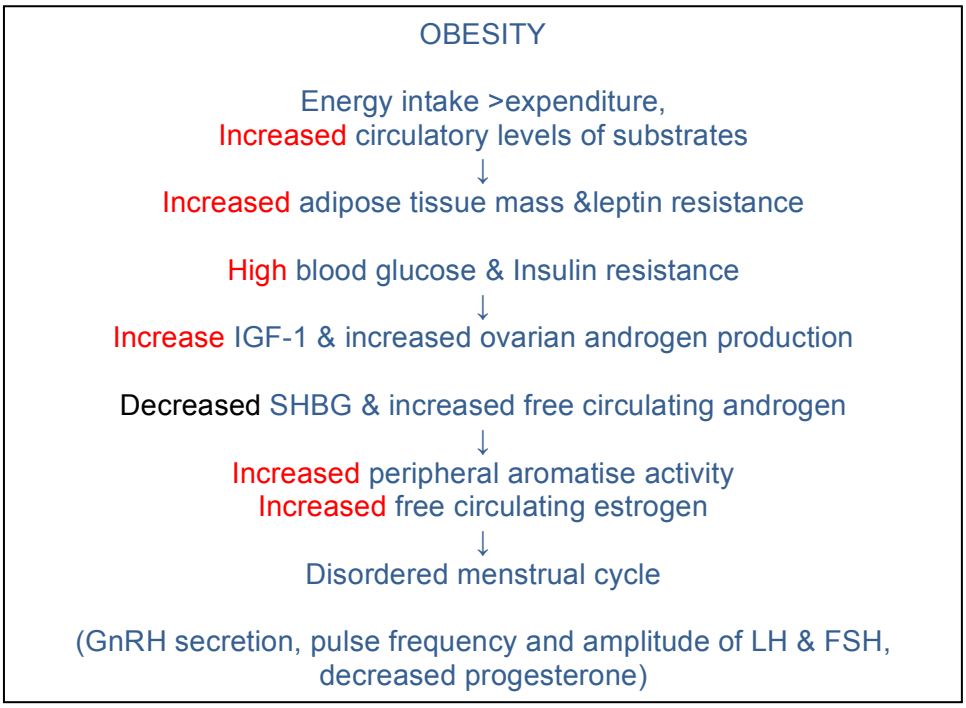


Figure 3.2 Representation of the link between obesity and the endocrine system.

Abdominal obesity in particular is associated with increased insulin production and insulin resistance. Insulin resistance leads to; (1) high blood glucose levels, (2) elevated plasma triacylglycerol and plasma non-esterified fatty acid concentrations (3) suppressed hepatic sex hormone binding globulin synthesis (SHBG), resulting in increased functional androgen levels, (4) increased levels of bioactive insulin growth factor 1 (IGF1), which act on the ovary, resulting in increased androgen production.

An increase in adipose tissue leads to increased oestrogen production, as the enzyme aromatase, found in adipose tissue (Nelson et al., 2001), converts androgens into oestrogens, which suppress the hypothalamic-pituitary axis. There is reduced availability of GnRH, which at the pituitary stimulates the synthesis and secretion of the gonadotrophins; follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The disorderly activity of these two hormones impairs follicular growth, ovulation and corpus luteum maintenance, leading to menstrual irregularities and anovulation (Mitchell et al., 2005). Similarly, Leptin influences GnRH secretion from the hypothalamus and pituitary secretion of LH and FSH and may also act directly to inhibit ovarian follicular development and steroidogenesis (Karamouti et al., 2009).

Hyperandrogenism leads to granulosa cell apoptosis, increased conversion of androgen to oestrogen, and increased negative feedback. These alterations can affect follicle

growth and have adverse effects on the growing oocyte, onward embryo development and implantation (Jungheim & Moley 2010).

3.1.2 OWOB women and ART outcomes

Studies focusing on the impact that raised BMI has on the outcome of assisted conception treatment cycles have yielded conflicting results. It is generally accepted that obese women require a longer duration and dose of ovarian stimulation and that they attain lower oocyte yields (ASRM practice committee report, 2008). However, not all studies are in agreement about the impact of obesity on pregnancy, miscarriage and live birth rates. For example, Ferlitsch et al., (2004) reported that, for each unit that BMI increased, the odds for pregnancy decreased by 0.84 in IVF and similarly, each reduction of BMI by one unit increased the chance of pregnancy by 1.19. Likewise other studies have reported that very obese women have significantly lower clinical pregnancy rates with ART compared to their lean counterparts (Wang et al., 2002; Nichols et al., 2001; Fedorcsak et al., 2004) and higher rates of early miscarriage (Fedorcsak et al., 2000; Wang et al., 2002). Conversely, Dokras et al., 2006 and Maheshwari et al.,(2007), reported no differences in pregnancy rates attributable to BMI. It is noteworthy that many of these studies have been retrospective, and few have commented on live birth rates or have been unable to adjust for key confounding factors such as female age. Taking account of these factors and sample sizes the majority of the evidence does indicate lower pregnancy rates and increased rates of miscarriage for obese women following IVF treatment compared to normal weight women.

3.1.3 OWOB women and frozen embryo transfer outcomes

There is even less consensus in the scientific literature about the impact that obesity has on the outcome of frozen embryo transfer (FET) cycles. In prospective randomized trials, Shapiro et al., (2011) reported that clinical pregnancy rates are improved when cryopreserved embryos are transferred compared to fresh transfers. It is believe that this is because controlled ovarian stimulation (COS) is avoided/minimized during frozen cycles. It is widely reported that COS is associated with impaired endometrial receptivity in animal models. A longer duration of COS has also been associated with precocious progesterone elevation and impaired endometrial receptivity in women (Valbuena et al., 1999). The more intense controlled ovarian stimulation (COS) required for OWOB women (Crosignani et al., 1994) may be detrimental for implantation. However links between female BMI, endometrial sensitivity to COS and embryo

freezing sensitivity have not previously been reported. The reproductive environment is likely modified in OWOB women, which may lead to adverse consequences both to the embryo and endometrium. This is an area meriting further investigation.

3.1.4 BMI and the relationship with dizygotic twinning

In twin pregnancies, conversely, a higher plane of nutrition may offer an advantage – direct evidence for this is currently lacking, but data from animal models and epidemiological studies suggest that maternal nutritional status is linked to twinning rates (Hoekstra et al., 2008). It has been proposed that reduced maternal nutrition may account for the decreased incidence of dizygotic twins (DZT) conceived during time of food scarcity (Hoekstra et al., 2008). However, since the mid-1960's multiple birth rates (MBR) have increased, even after adjustments for maternal age, use of fertility treatment and incidence monozygotic twining (MZT) (Derom et al., 2011). One possibility is that the rising incidence of DZT is linked to rising female body weight. Women with a higher BMI may have an elevated daily food intake and may be better able to meet the increased nutritional demands of carrying a twin pregnancy or, alternatively, be more likely to ovulate more than one oocyte. DZT occurs as a result of multiple follicle rupture in a natural cycle or the transfer of multiple embryos in an IVF cycle. In each scenario the embryos must successfully implant and be sustained to term. It is possible to interrogate IVF data to investigate whether BMI, a proxy for nutritional intake, is a contributing factor in the rising incidence of DZT.

3.1.5 Outcomes in repeat cycles

Weight loss may modulate treatment cycle parameters such as ovarian responsiveness and chances of successful IVF cycle pregnancy outcome. It is widely believed that ovarian dysfunction and the follicular microenvironment may be corrected once a healthy weight is attained (Clark et al., 1998; Norman et al., 2004). It is not clear from the literature if embryo quality in successive cycles of fertility treatment is similar for the same couple and if it is influenced by weight change. Inconsistent embryo quality grading systems have prevented such analyses, it may be more informative to compare embryo metabolism in successive treatment cycles. This is particularly important, in view of the strong supporting evidence for embryo metabolic assessment as an objective indicator of embryo viability – as reviewed briefly in Chapter 1 and a focus of Chapter 5.

If the general pattern of substrate utilization is not only inherent to the cohort of developing sibling embryos belonging to a couple (as demonstrated in Chapter 2 of the method validation), but can be demonstrated to correlate to the pattern of nutrient utilization in subsequent cycles of treatment in the same couples this will be a significant finding. Furthermore if the existence of patient specific inter-cycle homogeneity in embryo development and metabolism can be established this could have foreseeable possibilities for assessing if embryo viability might be improved by weight loss.

Aims and objectives

The literature on the impact that female BMI has on pregnancy outcome is inconclusive, and the aim of this chapter was to review treatment outcomes for consecutive patients attending the Hull IVF Unit, based on female BMI.

The specific objectives were;

a) To investigate the extent of combined obesity and fertility problems within the study population (i.e. women in the Humber and Yorkshire region) using data collected in a previous local population survey.

b) To compare clinical pregnancy (CPR), implantation (IR) and miscarriage rates and live birth data from OWOB and normal weight women in both fresh and frozen transfer cycles (FET).

c) To discover if a raised BMI is linked to the incidence of dizygotic-twinning (DZT), using data from IVF patients who had two embryos replaced, thus enabling controlled investigation, taking account of other known specific risk factors.

d) To compare subsequent treatment cycle outcomes for patients returning for a repeat cycle of IVF treatment who had remained weight stable with those who had lost or gained weight over a 3 year period.

e) To discover if embryo development and metabolism of substrates are matched in successive treatment cycles for a given patient.

3.2 Materials and methods

3.2.1 Assessment of study population; natural conception rates

A survey conducted in Hull and East Yorkshire and Sheffield, asked consecutive women attending the antenatal clinics between 2001 and 2007 to self-complete a questionnaire inquiring about their Time To Pregnancy (TTP: the interval of exposure to unprotected intercourse from discontinuing all birth control methods until conception); female height and weight were also collected. As the study of current pregnancies will exclude those who failed to conceive or gave up the pregnancy attempt, data were also collected from women attending the Subfertility Clinic at the Women and Children's Hospital, Hull Royal Infirmary and used in comparison.

Data were reviewed from 2269 consecutive women, attending the antenatal clinic. 1460 (64.3%) conceived within 6 months rising to 1780 (78.4%) by the end of the first year, and 489 couples (21.6%) were sub-fecund. Patients who had fertility treatment, unplanned pregnancies and missing BMI results were excluded from further analysis. This significantly reduced the data set and complete records were available for 196 women.

Data were also reviewed from 345 consecutive couples referred to the Subfertility Clinic at the Women and Children's Hospital, Hull Royal Infirmary. Obese patients were subsequently identified from the notes of those referred to the clinic in 2006. The majority of patients would have subsequently been referred to the IVF clinic for further investigation or treatment, however women who were obese (BMI>30kg/m^2) will have been advised to lose weight prior to IVF referral for NHS funded treatment. Full notes were obtained to verify this and to permit an assessment of weight loss success rates. The appropriateness of the advice and support was determined by whether the patient lost weight to such an extent that they subsequently conceived or were offered NHS funded fertility treatment.

3.2.2 Assessment of study population; ART conception rates from fresh and frozen cycles

Data were recorded from 709 consecutive IVF / ICSI patients attending the Hull IVF Unit between 2007 and 2011 for fresh treatment cycles. Patients receiving donated oocytes were excluded from the analysis; the outcome of donor and recipient cycles was

assessed separately (n=40). The outcomes from weight discordant (i.e. normal/OWOB) paired, heterosexual couples were assessed to determine any impact of male BMI on treatment outcome. BMI was recorded at the start of treatment and only those considered weight stable were included in the study.

Data collected from any frozen embryo transfer (FET) cycles performed for the study participants, were also retrospectively interrogated. Treatment outcomes in OWOB and normal weight women were compared using data from first their fresh and then their FET cycle (n=65). The women served as their own control, meaning that any difference in clinical pregnancy rates (CPRs) could be attributed to endometrial receptivity and embryo quality rather than to inter-individual variation between treatment groups.

In fresh cycles, patients underwent a standard long-protocol agonist IVF cycle. Embryos, selected on the basis of morphology were transferred on day 3 (prior to 2009) or on day 5 (post 2009). Supernumerary embryos of suitable quality were cryopreserved according to a slow-freeze protocol.

In frozen cycles, patients were down-regulated and given oestradiol supplementation 10–14 days before thaw to achieve a target endometrial thickness of 8 mm. Progesterone supplementation began on the day preceding thaw. Patients received similar progesterone supplements in their fresh cycle, beginning 1 day after retrieval. Thawed cleavage-stage embryos were cultured *in vitro* overnight to confirm onward developmental potential; thawed blastocysts were cultured for 1-2 hours.

3.2.3 Assessment of maternal outcome

In order to discover whether the prevalence of MBRs had risen over the past 30 years, data obtained from the UK Office for National Statistics (ONS), for all maternities in England and Wales between 1985 and 2008 were compared to data recorded for patients undergoing fertility treatment both nationally (using data from the Human Fertilisation and Embryology Authority- HFEA) and specifically; those attending the Hull IVF Clinic.

Using the data from IVF patients attending the Hull IVF Clinic the impact of obesity on multiple pregnancies of known dizygosity- (monozygotic pregnancies were excluded) was compared for study participants. The incidence of DZT in women who received a

transfer of two embryos was 107/233 (45.9%) in normal weight women who achieved a positive pregnancy (PP) result compared with 74/155 (47.7%) overweight and 43/100 (43%) obese women.

3.2.4 Weight change and assessment of repeat cycle outcome

A retrospective study was designed to compare cycle outcome in patients receiving two cycles of IVF / ICSI treatment between 2008 and 2011 at the Hull IVF Unit (n= 709 cycles).

Data were reviewed from first and second treatment cycles. Clinical pregnancy outcomes were compared for those that had remained weight stable and those who had gained or lost weight between cycles.

In an effort to isolate the influence of BMI on subsequent treatment outcome the BMI change between cycles (stable, gain or loss) was also evaluated. A total of 186 patients returned for a second treatment cycle in the duration of the study. The majority had remained weight stable between treatment cycles (n= 158), 8.6% of patients had lost weight between cycles (n= 16) and 6.5% had gained weight (n= 12).

Regression analysis was used to control for confounding variables, such as the impact of increased maternal age between cycles. The likelihood of successful treatment outcome does not differ over 4 cycles (NICE 2007), however women returning for a subsequent treatment cycle within 1-2 years of their last cycle are likely to be those who have experienced a negative outcome. A duel regression logistic sub-model was created using cycle as an indicator variable to control for this.

To review the impact of weight change on repeat cycle embryo development and substrate metabolism; data were reviewed from weight stable women, who underwent a first and second treatment cycle within a 12 month period. The number of fertilised embryos obtained in the 2nd treatment (mean 5.8) cycle did not significantly differ to the number obtained in the 1st cycle (mean 4) and therefore the proportion of embryos forming a blastocyst was compared. The quality of blastocysts (the proportion grade 3Bb or above- assessed according to grading criteria described in chapter 2) was compared in each cycle for individual patients, as was the utilisation of glucose, lactate and pyruvate using methods described previously in chapter 2.

3.2.5 Statistical analysis

Data analysis was carried out using SPSS (Statistical Package for Social Sciences v11.5). Outcome measures (mean TTP, PP, CP, miscarriage, LB, DZT) were calculated for each BMI grouping, using the non-parametric Kruskal–Wallis test, Fisher's exact test or chi squared. Continuous data was assessed using ANOVA and paired t-tests. A general linear logistic regression model was used to examine the relationship between maternal BMI and the likelihood of live birth per treatment cycle and adjust for potential applicable confounders (e.g. age, cause of infertility, smoking and parity, ovarian response and embryo transfer data). An alpha of $p \leq 0.15$ was used for adding or removing predictors from the model. Statistical significance was indicated by $p < 0.05$. The odds ratios (OR) and 95% confidence interval (CI) are given for each significant independent variable's contribution to the model.

3.3 Results

3.3.1 Obesity and natural conception

The data from antenatal clinics indicates that 39% of the women who had achieved a pregnancy were overweight or obese (77/196). There were no significant differences in the proportion of women and their partners who smoked, amongst women of normal weight and OWOB attending the clinic. In addition OWOB women did not report a significantly higher incidence of menstrual infrequency (23.4% verses 16.7%), acne or hirsutism (1.9% verses 8.8%) compared to those of normal weight. The average age of onset of menarche was comparable for normal and overweight women (12.6 years and 12.5 years respectively), however it was significantly lower for obese women (12.1 years $p<0.05$). Female age at the time of first pregnancy was comparable, although time taken to achieve a pregnancy tended to be longer in OWOB women averaging 7.92 months compared to 6.99 months for normal weight women, despite comparable reported frequency of intercourse. The adjusted regression analysis showed a negative relationship (β-0.015, t-1.55, *p*0.06) between BMI and TTP.

The data from the subfertility clinic shows that 48.1% (166/345) of patients attending the clinic were be classified as overweight or obese. 15.1% of female partners were classified as morbidly obese (BMI > 35).

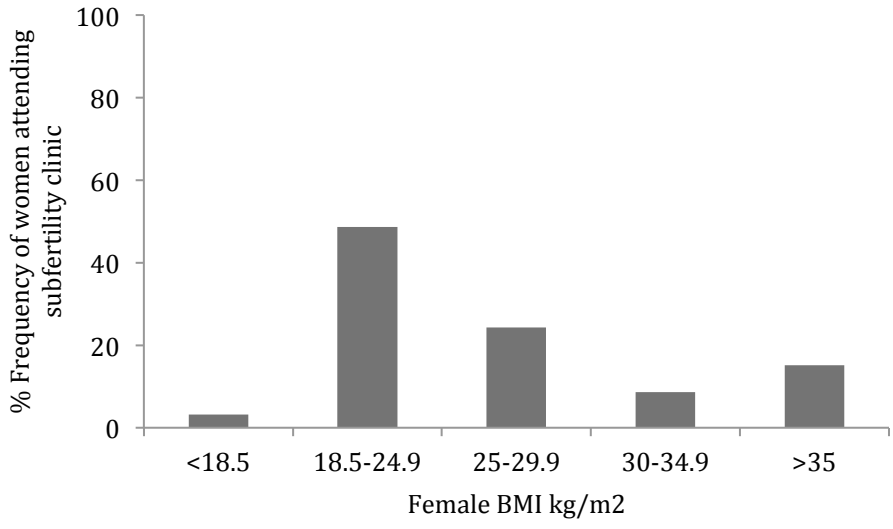


Figure 3.3 Female obesity rates amongst patients attending the subfertility clinic in Hull, in 2006. Over half of all patients seen were overweight or obese (n = 345).

In total, 74% (64/82) of obese women were given advice on the effect of their body fat on their subfertilty and subsequent treatment. One year later 24% (16/64) of obese women had lost a sufficient amount of weight in order to be given NHS-funded IVF treatment, 12.5% continued to receive support, however the majority (62.5%) had stopped attending the subfertility clinic and discontinued their treatment, although they had not yet become pregnant.

3.3.2 Obesity and assisted conception

3.3.2.1 Fresh cycles

The percentage of patients attending the Hull IVF Unit classified as overweight or obese was 48%; this is directly comparable to the findings reported in the previous section, for women who attended the subfertility clinic. Notably, non-treated were morbidly obese, compared to 15.2% of women who attended the subfertility clinic.

The data in table 3.1 shows that clinical pregnancy outcomes did not differ significantly according to BMI classification, although pregnancy rates were lowest in obese patients. In a univariate analysis live birth rates, per cycle started were significantly lower for women with a BMI >25kg/m^2 compared to those with a BMI <25kg/m^2 (p<0.01), indicative of the higher rates of miscarriage in the OWOB, which included two prenatal losses.

Table 3.1 Pregnancy and live birth outcomes for women of differing BMI (kg/m^2).

Live birth rates shown to be lowest for obese patients. Values (± SD) or (n) for percentages, * p<0.05 for ANOVA / Kruskal-Wallis test as appropriate.

BMI kg/m^2	< 18.5 n=10	18.5-24.9 n=361	25-29.9 n=192	30-35 n=146
Mean female age (SD)	29.8 (4.7)	33 (3.6)	33.6 (5.3)	33 (4.3)
% positive pregnancy test (n)	36.4 (4)	40.4 (146)	44.3 (85)	39 (57)
% clinical pregnancy (n)	36.4 (4)	33.5 (121)	35.4 (68)	31.5 (46)
% early loss <6wks	0	6.9	8.9	7.5
% miscarriage >6 wks	0	3.1	10.4	5.6
% Live birth rate (n)	36.4 (4)	32.4 (117)	31.7 (61)	29.4 (43)*

There were no significant differences in female age (mean 33.4years ±4.6), cause or duration of infertility (mean 3.9years ±1.6), ethnicity (96.4% white, British) or number of smokers (18.5%) among the groupings. The duration and dose of stimulation was significantly higher for OWOB (12.35 days, 1979.63 total FSH IU) compared to normal weight women (11.22 days, 1746.71 total FSH IU; $p<0.05$) and average oocyte numbers were significantly lower (9.6 compared to 10.6 $p<0.01$), although endometrial thickness on the day of oocyte retrieval was comparable (10.6 verses 10.8mm). The number of embryos replaced was comparable (average of 1.61 and 1.53), as were the rates of multiple births (16.1 for OWOB and 14.1 for normal weight group). Each exploratory variable was included in a stepwise logistic regression and significant predictor variables of the likelihood of live birth were; female age (OR 0.94, 95% CI 0.93-0.95) and number of oocytes (OR 1.02, 95% CI 1.01-1.02). In this adjusted model BMI remained a significant predictor of live birth (OR 0.36, 95% CI 0.23-0.69).

The data from patients attending the IVF clinic shows that the mean female BMI was 22.6 (±3.22 SD) whereas mean male BMI was 26.7 (±3.99 SD, full data available for n=88 pairings). Regression analysis showed a weak trend for assortive mating amongst couples, (r =0.11, p=0.3, Figure 3.4), which was more apparent when couples were categorized into normal (BMI 18.5-24.9) or OWOB (BMI>25) and 83% of the couples fell into matching categories. Male obesity was associated with significantly lower blastocyst development (BD), compared to all other discordant pairings of male and female BMI (p=0.01; Figure 3.5), however there was no significant impact on clinical pregnancy rates. Interestingly, this finding was independent of sperm quality.

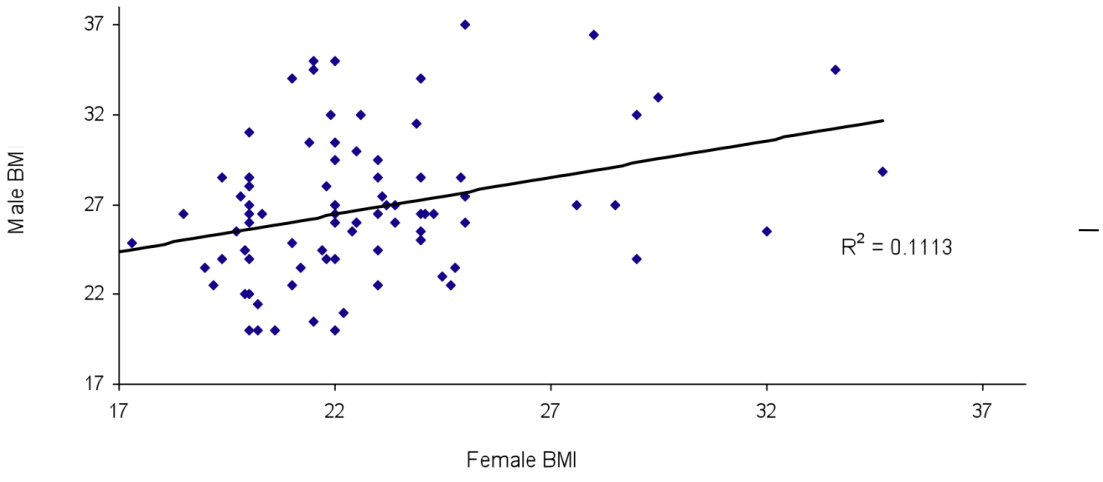


Figure 3.4 The relationship between male and female spouses in BMI (kg/m^2) is not significant.

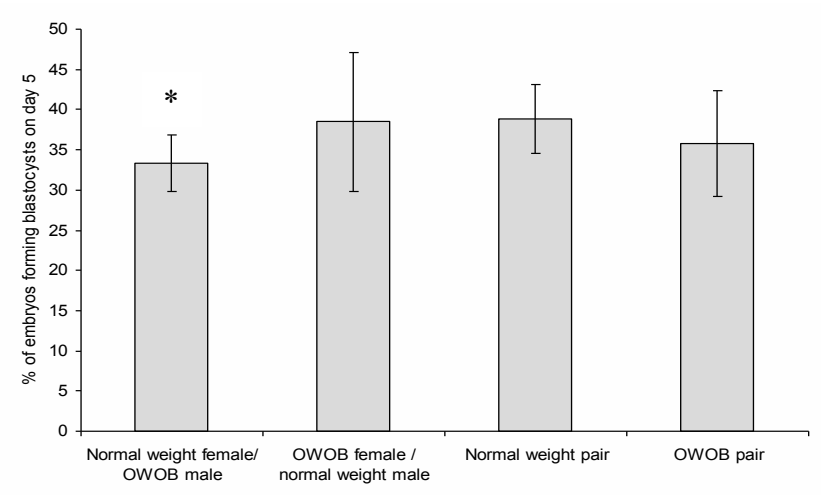
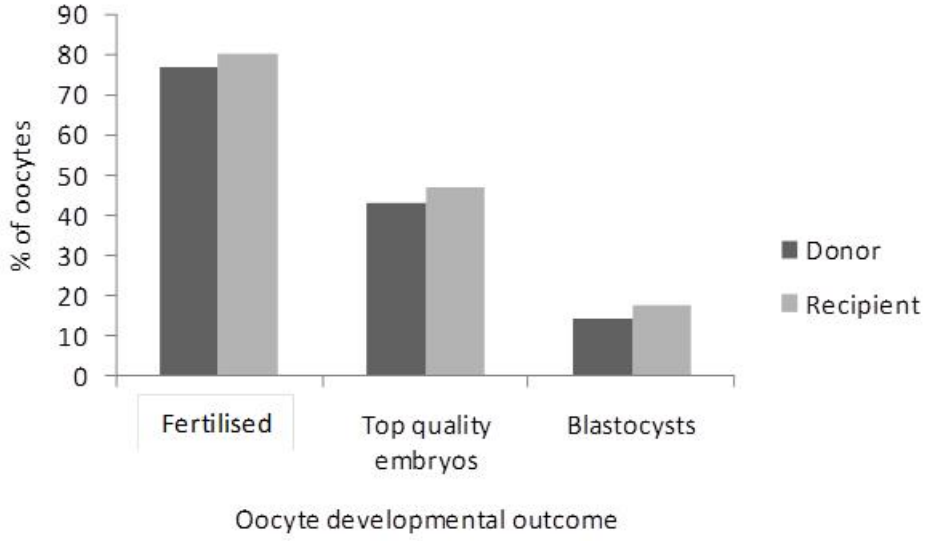


Figure 3.5 The relationship between male and female spouses and the impact of pairing on embryo development to blastocyst on day 5.

Male OWOB appeared to contribute to higher rates of early embryo arrest (Data expressed as mean +/- SD * $p<0.05$), n=518 embryos, n=88 patients.

An additional subgroup of 40 patients took part in an egg-sharing scheme and donated half of their retrieved oocytes to a matched recipient (n=40). Differences in fertilisation, embryo quality and blastocyst development, pregnancy, miscarriage and live birth rates were compared using a paired t-test and found to be non-significant.

3.6 a



3.6 b

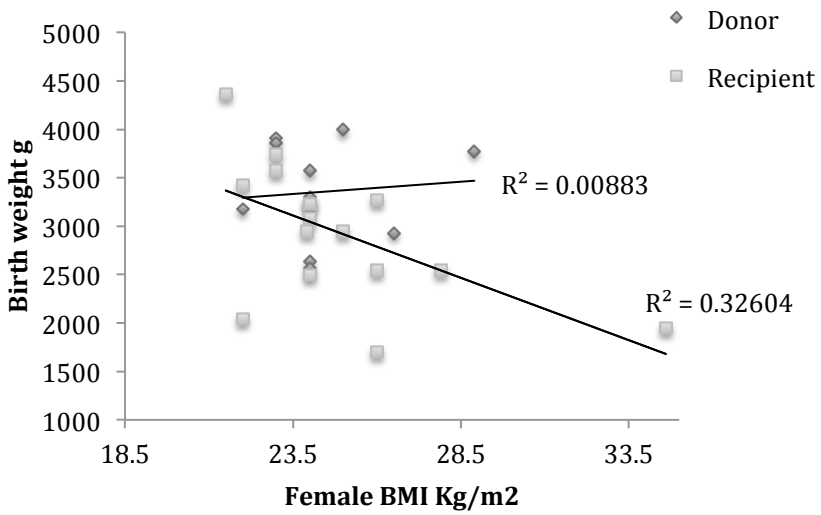


Figure 3.6 Egg sharer (donor) and recipient treatment cycle outcomes.

3.6a Embryo developmental competence did not significantly differ between egg donors and recipients despite significant differences in sperm donor BMI. **3.6b** Birth weights in relation to BMI of birth mother show differing trends for recipients (n=16 live births) and donors (n=11 live births).

The mean female BMI of matched pairs was statistically comparable, although did show a trend for higher BMIs in the recipient group (23.5±0.3 SEM for donors compared to 25.6±0.6 for recipients p=0.06). Egg donors were required to have a BMI of less than 25, although an exception was made for a known donor. The BMI of egg sharers who achieved clinical pregnancy and live birth was comparable to that of successful recipients (p=0.34). Likewise the birth weight and gestation period was analogous for

singleton births (all received a single embryo transfer) in the recipient and donor groupings. When the data were analysed according to female BMI of the birth mother a significant inverse relationship was apparent between increasing recipient BMI and birth weight of newborn (Figure 3.4b; r^2 -0.57, p=0.02), whereas in donors there was no such relationship. When the data were stratified into BMI classifications it was determined via ANOVA and post-hoc Tukey-Kramer method that birth weights were significantly lower when eggs from a normal weight donor had been received by an OWOB recipient (p=0.02). However, this study is underpowered and outlier observations may have influenced the test statistics.

Sperm counts also showed considerable variance amongst pairings (p=0.005). When the data were assessed according to male BMI, the male partner of recipients had a significantly higher BMI than that of the partner of the paired donor (24.1 verses 25.1, p=0.05).

3.3.2.2 Frozen cycles

For the 65 patients included in this portion of the analysis, there were no differences in female age, cause of infertility, dose and duration of stimulation, day of transfer, proportion of elective single embryo transfer, treatment type, between the OWOB and normal patients. However women with normal BMI, tended to produce more eggs (mean 10.6) than OWOB (mean 10.1, p=0.07), although this did not result in a significantly different number of embryos available for cryopreservation for OWOB and normal weight women (3.8 verses 4.17 respectively).

As reported in the previous section there were no significant differences in pregnancy or miscarriage rates for fresh cycles and this was also the case when the data was limited to those patients with embryos available for freezing (as shown in figure 3.7a). The clinical pregnancy rate (CPR) for normal weight women was 10% per embryo transferred compared to 8.6% for the OWOB. However, in frozen cycles, significantly lower CPRs were obtained for OWOB compared to normal weight women (CPR 20% v 42% p=0.049). Rates of pregnancy losses in frozen cycles were also significantly higher for this group (13 v 6.9% p=0.049), as indicated in figure 3.7b.

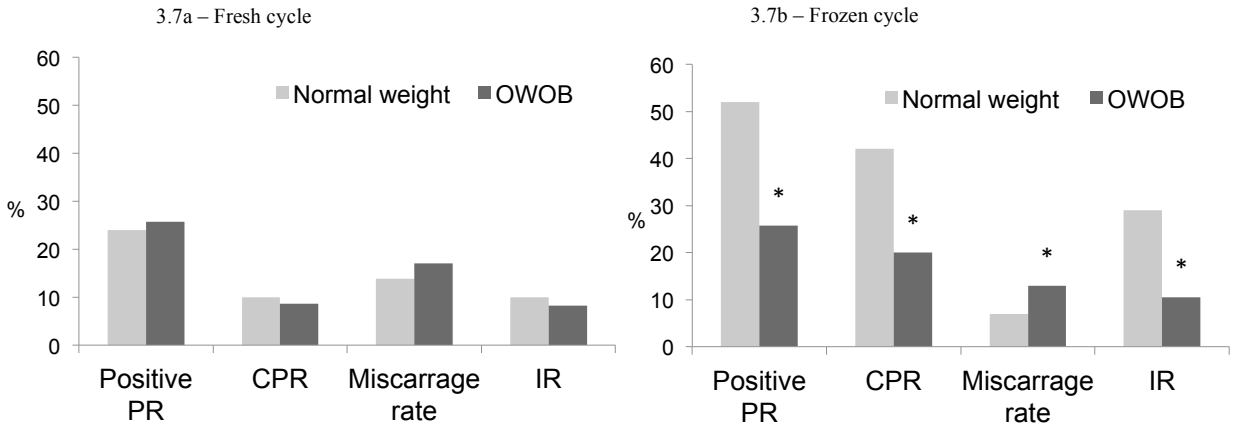


Figure 3.7 Pregnancy outcomes from fresh and frozen treatments.

Pregnancy outcomes for normal and OWOB women were comparable in fresh cycles (a), however outcomes were significantly poorer in frozen cycles (b) for OWOB compared to normal weight women. **p*<0.05

In women of normal weight, rates of pregnancy and miscarriage were significantly higher in their subsequent frozen cycles. Figure 3.8 shows an increase in CPRs from 10% to 42%. In the OWOB group outcomes were improved in their frozen cycle, however results did not significantly differ from those of their fresh cycle (8.6% to 20% CPR).

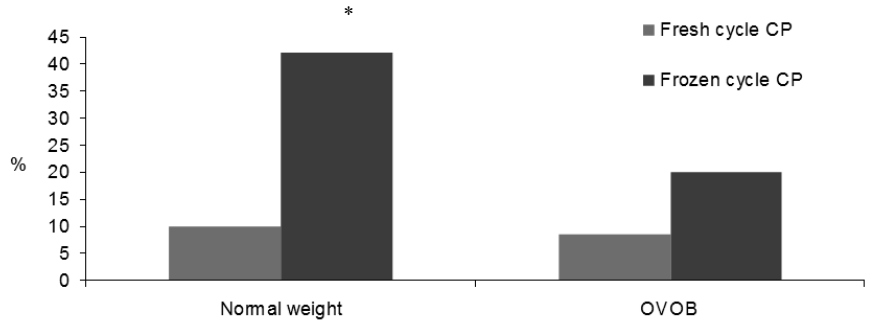


Figure 3.8 Pregnancy rates were improved in frozen cycles

The improvement was significant in the normal weight group. **p*<0.05

There were no significant differences in cryosurvival or onward development/viability between the embryos from normal weight or OWOB (n=254 embryos), as shown in figure 3.9. When the evaluation was limited to blastocysts only (n=156) rates of cryosurvival tended to be lower for the OWOB group (44% v 59%; *p*=0.06).

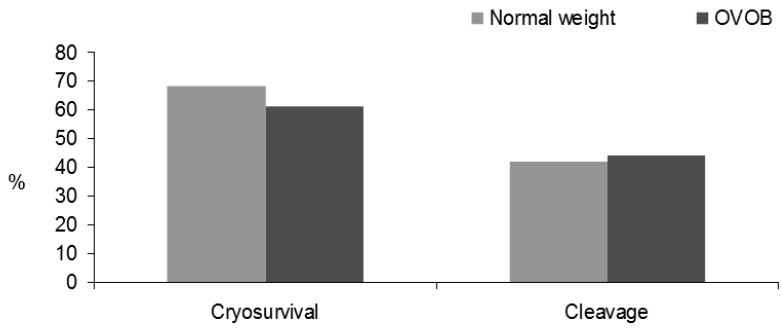


Figure 3.9 Marginally poorer embryo cryosurvival is reported for OWOB compared to the normal weight grouping.

There were no differences in the percentage of embryos resuming ongoing cleavage development.

3.3.3 Obesity and birth-outcome -dizygotic twinning

The data from the ONS shows that multiple births have risen consistently in the UK from 1980 to 2008 (R^2=0.93, p=<0.01) (figure 3.10). MBR associated with IVF have remained above 25% from 1990 onwards both nationally and locally. ARTs have accounted for only 2% of all multiple pregnancies in 2008, suggesting other social and biological factors must be causal.

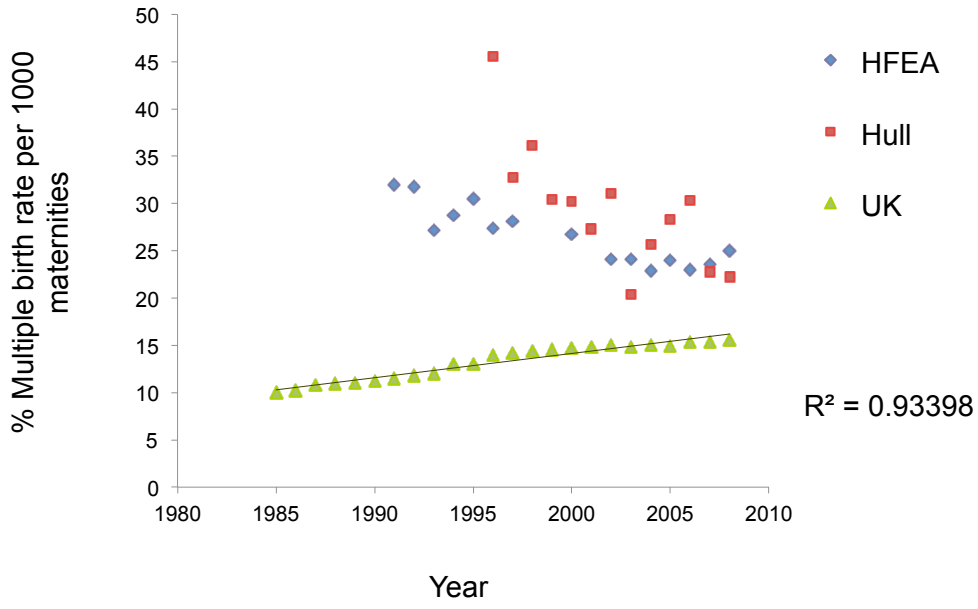


Figure 3.10 The rising incidence of multiple pregnancies across the UK.

Factors previously reported to influence the incidence of multiple births are listed in Figure 3.11. Multiple regression was used to assess the impact of these known risk factors on implantation outcome (i.e. implantation of -2, -1 (miscarriage), 0 (no pregnancy), 1 (singleton) 2 (dizygotic twins) fetal hearts, following the transfer of two embryos in Hull IVF patients (n=224), using the forward enter stepwise method, a significant model emerged (F=3.048, p=0.02. Adjusted R square = 0.52). Female age and blastocyst development were independent predictors of implantation outcome. BMI as a continuous variable was not significant, however, the data were suggestive of a non-linear relationship between BMI and DZT.

Smoking, maternal age, paternal age, parity and ethnicity were not independent predictors of implantation outcome. Gamete and embryo development / quality may be compromised in OWOB women, and were therefore entered into the model. Only blastocyst development was found to significantly influence implantation outcome.

Significant variables are shown below:

Predictor Variable	Beta	t	p
Female age	-.224	-3.650	<0.01
No. blastocysts on day 5	.113	2.042	0.042

The beta weights reflect standardized coefficents from final regression models when all simple effects and interactions have been included.

ANOVA was used to examine the relationship between predictor variables within and between the single verses double implantation groups.
Female age emerged as the only significant (F 10.663, p 0.01) independent predictorof dizygotic twinning probability.
BMI was not a significant predictor of pregnancy and did not significantly differ amongst those achieving a singleton pregnancy verses those with a twin pregnancy (F= 2.35, P=0.13). However the variance amongst BMI in the two outcome groups was not equal –Levenes test for equality of variance F 3.6, p 0.059. This is suggestive of a non-linear relationship, meriting further investigation – by BMI categorization, rather than as a continuous variable.

Figure 3.11 Factors influencing DZT pregnancy.

Literature review findings and regression model evidence using Hull IVF Unit patients. Regression results for prediction of implantation outcome are displayed for significant predictor variables p<0.05

Clinical pregnancy was confirmed for 185 of the 224 patients who achieved a positive pregnancy (PP); DZTs accounted for 53 of these pregnancies (28.6% MBR). The LB rate per cycle was 37.3% (n=183). There was no difference between normal weight and OW and OB women in baseline characteristics, including age (Table 3.2). There were no significant differences in rates of PP, CP or LB (37.9% normal weight verses 37.2% OWOB). The incidence of MZT was low (1.8%) and did not differ significantly between groups.

	Maternal body mass index			
	<24.99	25-29.99	>30	*p*
n	242	153	100	
Age (years)	33.6	34.1	33.3	0.49
Cause of infertility				0.27
Male factor	73	52	30	
Tubal	41	29	10	
Unexplained	59	47	18	
Ovulatory	26	10	16	
Uterine	1	0	0	
Endometriosis	7	1	3	
Multiple factors	35	14	23	
% Multiparous	11.2	10.4	10.9	0.94
% Smokers	20	18.8	20	0.13
% ICSI	30.2	35.9	38.6	0.25
Endom thickness	10.8	10.8	11.3	0.55
Oocyte number	10.6	9.6	9.1	0.06
Number fertilised	6.7	6.2	5.8	0.02
Number top scoring embryos	3.1	2.8	3.2	0.62
Blastocyst development, day 5	0.9	1	0.7	0.79
Male age	34.8	36.8	34.7	0.08
Male BMI	22	28.1	30.6	0.03
p value based on ANOVA and Kruskal Wallis tests				

Table 3.2 Demographic and embryological characteristics of normal, OWOB groupings.

Each displayed no significant differences.

The primary outcome measure for this study was DZT birth rate, which did not differ between women of differing BMI (28.1% normal weight verses 31.2% p =0.98; 0.81 power). There were no significant differences when the data were further classified to normal verses overweight (22/58; 37.9% DZT) and obese (4/35; 11.4% DZT).

BMI and Birthweight of DZTs

The average birth weight of the larger twin was 2401.4g and 2116.8g for the smaller twin. There was no significant difference in birth weights with female BMI and weight discordance amongst twins was comparable amongst the BMI groupings. A weight difference of greater than 18% between twins is considered to be clinically significant (Breathnach et al., 2011) and there were an equal number of incidences of this amongst groups (r=0.13 , p=0.4). The data indicate a very weak inverse trend between female BMI and average birth weight (r= -0.13, p=0.4).

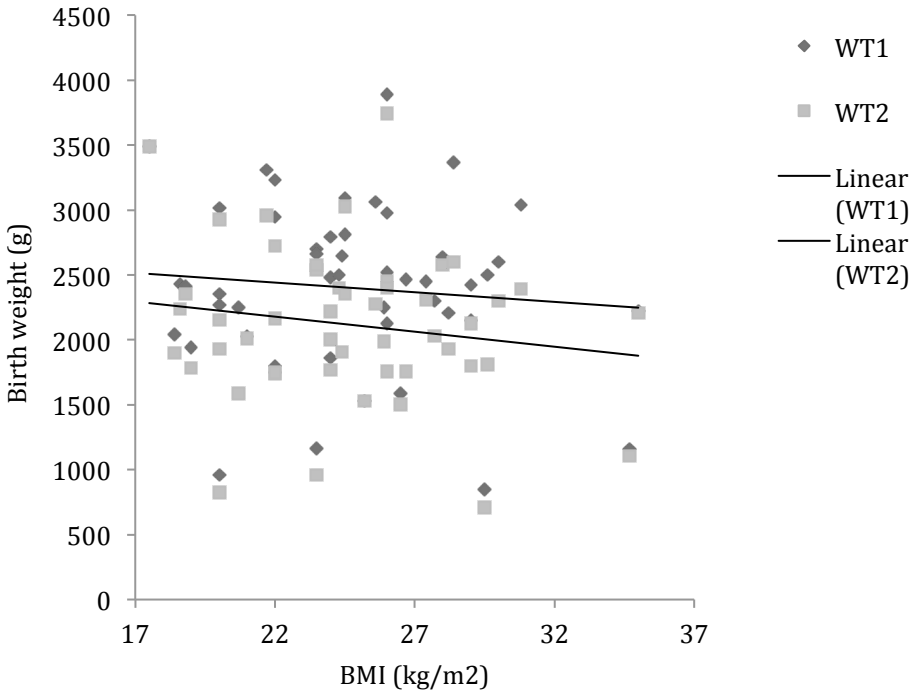


Figure 3.12 Female BMI and twin birth weight.

Female BMI did not significantly correlate with twin birth weight (WT of twin 1 /2). Twin weight discordance (indicated by the distance between the parallel lines) was statistically comparable for women of differing BMI (n=48).

Figure 3.13 shows the wide range in mean birth weights of twin maternities – the range is most evident for the OWOB grouping and data are skewed to the lower end of the scale. Similarly, whilst the average gestation period was 35.23 weeks for the normal weight group and 34.59 weeks (t=0.72, *p*=0.5), the data are skewed to the lower end of the scale for OWOB.

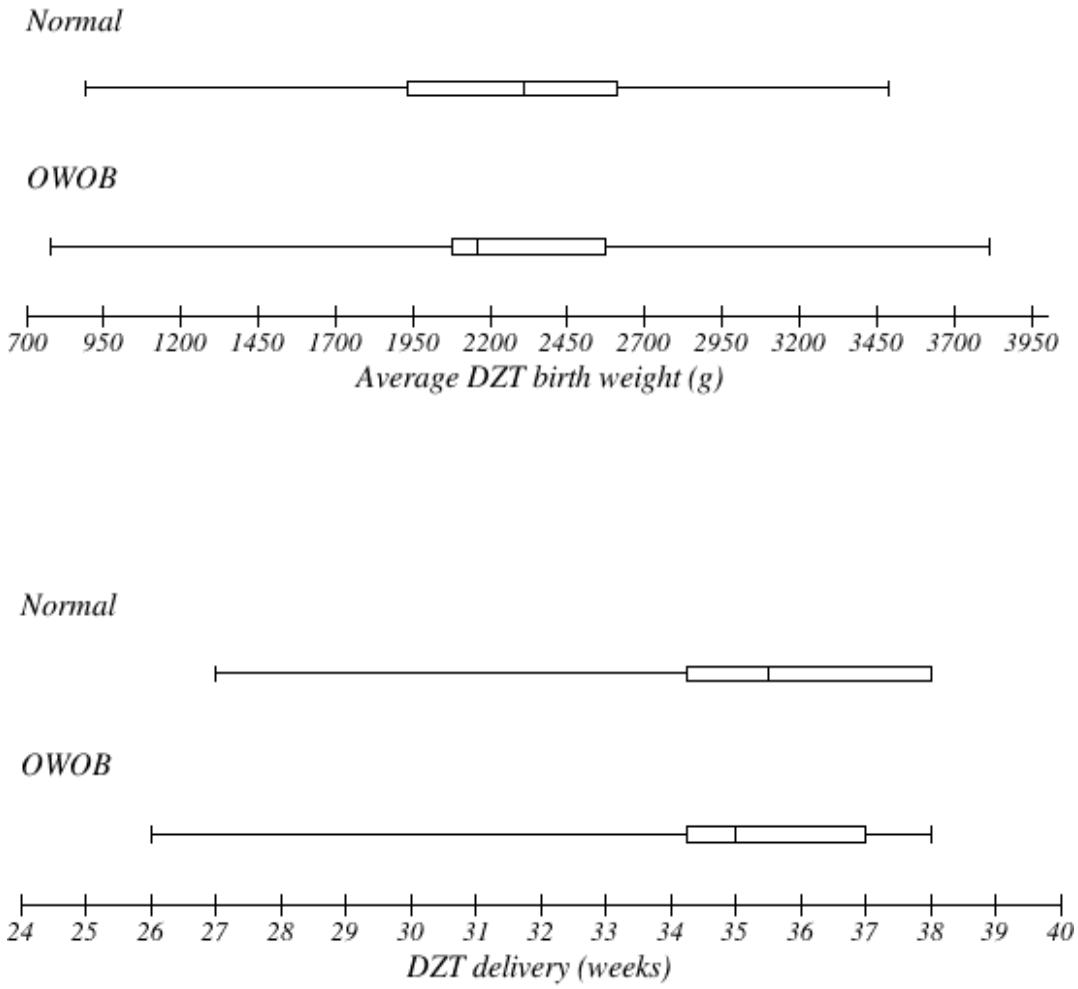


Figure 3.13 Birth weights and delivery times.

Boxplots of the median, inter quartile and range indicate the spread and skew of data, particularly for the OWOB group- towards the lower values. Analysis based on n=26 normal and n=22 OWOB live birth events.

When analysis was limited to include the transfer of two embryos of the same quality score, the data hinted at differences in birth rates of; 17% for normal weight women, verses 44% for overweight women and 10% for the obese group, (Figure 3.14, p=0.07). This association did not change upon adjustment of the regression analysis to consider embryo quality.

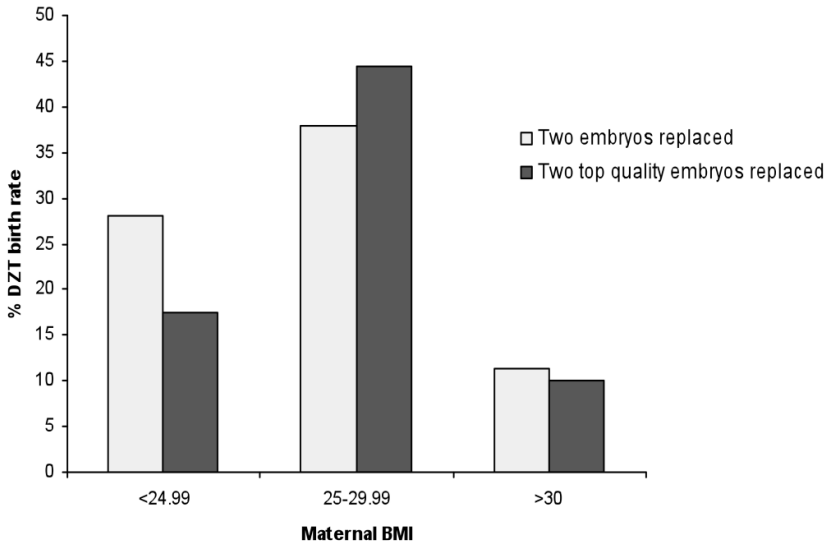


Figure 3.14 DZT rates presented according to female BMI and embryo quality.

The data shows a trend for higher twin rates in the overweight grouping when two top quality embryos were replaced (ANOVA p=0.07). Analysis of 186 pregnancies achieved from the transfer of two embryos and 51 clinical pregnancies achieved from the transfer of two top quality embryos.

The power calculation assumed that a quarter of all IVF pregnancies conceived from the transfer of two embryos would be multiple births (validated by HFEA annual figures 2008). The study was adequately powered to make general inferences about the lack of differences in DZT rates amongst BMI groups. The possibility of a type II error cannot be excluded when the restricting the analysis to the transfer of top scoring embryos and further study is required to confirm or refute the reported trend.

3.3.4 Repeat cycles;

Weight change and pregnancy rates

Clinical pregnancy rates were significantly better in patients who returned for a second treatment cycle (p<0.05, paired t-test), however there is no evidence to suggest that other confounding variables differed between cycles sufficiently to influence the probability of success. BMI *per se* was not a significant predictor of cycle outcome, nor did it differ significantly between cycles for the majority of patients (Table 3.3 – regression model). Likewise, oocyte number and embryo developmental competence, as assessed by morphology at the cleavage stage and the ability to form a blastocyst, did not significantly differ between successive cycles and could not be used to determine the probability of success in the first and second treatment attempts.

Table 3.3 Duel regression model (cycle 1 and 2).

Using cycle as an indicator variable to determine the influence of prognostic variables on cycle outcome.

	Beta	T	Signif
Female BMI- kg/m^2	-.085	-.757	.451
Number of eggs	-.121	-.641	.523
Fertilization – no. 2PN	-.122	-.552	.582
Top quality embryos day 3	.152	.924	.359
Blastocyst development day 5	-.156	-.905	.368
Number embryos replaced	-.136	-1.036	.303

CPRs in the first cycle of treatment were significantly lower than those expected (data not shown- based on annual audit results for clinic) however CPRs achieved in the 2nd cycle of treatment for those who remained weight stable were significantly improved (p<0.01) and were in the expected range (Table 3.4). Those who gained weight also saw an improvement in CPRs but this was not statistically significant, probably due to the sample size. Those who lost weight had CPRs on their first cycles that were closer to the expected values and there was no significant difference in the outcome of the second cycle (Figure 3.15).

In the weight stable group the overall CPR for cycle 1 was 5.7% (9/158) and for cycle 2 this was 33.5% (53/158) (p<0.01). Differences in treatment outcome between cycle 1 and 2 were comparable amongst the BMI sub-groupings (Table 3.5 -Kruskal-Wallis H=0.5, p=0.9) (i.e. the proportion seeing an improvement in outcome in each BMI subgroup).

In the group who lost weight between cycles the overall CPR for cycle 1 was 25% (4/16) and for cycle 2 this was 18.8% (3/16). Differences in treatment outcome between cycle 1 and 2 were again comparable amongst the BMI sub-groupings (Table 3.5- Kruskal-Wallis H=1.8, p=0.4). The average weight loss was indicated by a fall of -0.98kg/h2 (±1.83 SD). The average BMI for cycle 1 was 28.4 and the average for cycle 2 was 26.2.

In the group who gained weight between cycles the overall CPR for cycle 1 was 8.3% (1/12) and for cycle 2 this was 33.3% (4/12). Differences in treatment outcome between

cycle 1 and 2 were again comparable amongst the BMI sub-groupings (table 3.5 - Kruskal-Wallis H=2.0, p=0.6). The average weight gain was +2.3 BMI (± 2.3 SD). The average BMI for cycle 1 was 24.7 and the average for cycle 2 was 27.1 kg/m^2.

Similarly, there were no significant differences in rates of miscarriage, either within or between all groupings.

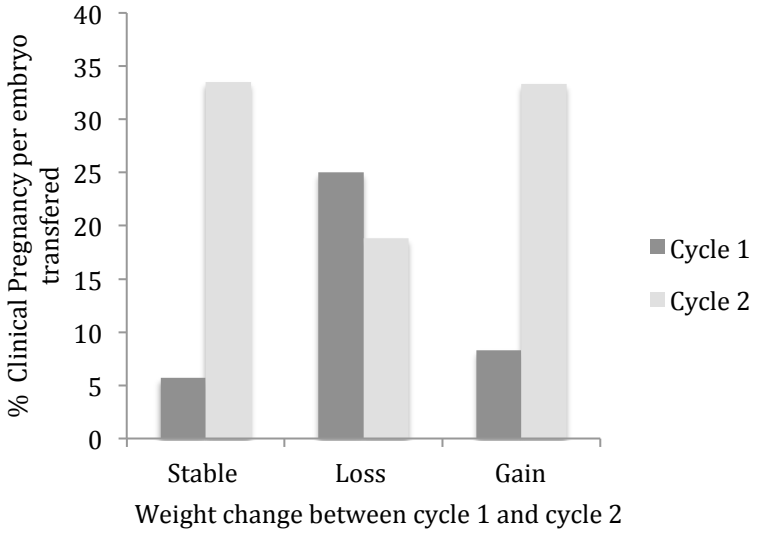


Figure 3.15 Clinical pregnancy rates were compared in first and second treatment cycles according to weight change.

Table 3.4 Breakdown according to BMI classification, cycle CPR and weight change.

All groups showed significantly increased rates of CPR in second cycle, this was not influenced by BMI (kg/m^2).

	Weight stable		Weight loss		Weight gain	
	BMI<24.9	BMI>25	BMI<24.9	BMI>25	BMI<24.9	BMI>25
CPR cycle 1	2/81	7/78	0/3	4/13	1/7	0/5
CPR cycle 2	26/81	28/78	1/6	2/11	0/3	4/8

Repeat cycles; embryo development and substrate metabolism

The proportion of embryos capable of undergoing blastocyst formation was comparable amongst the cohort of zygotes generated from a patient's first and second treatment cycle (paired t-test p=0.19). In addition the quality of those blastocyst was not

significantly different, as indicated by the proportion of blastocysts of 3bb quality or above (Figure 3.16)

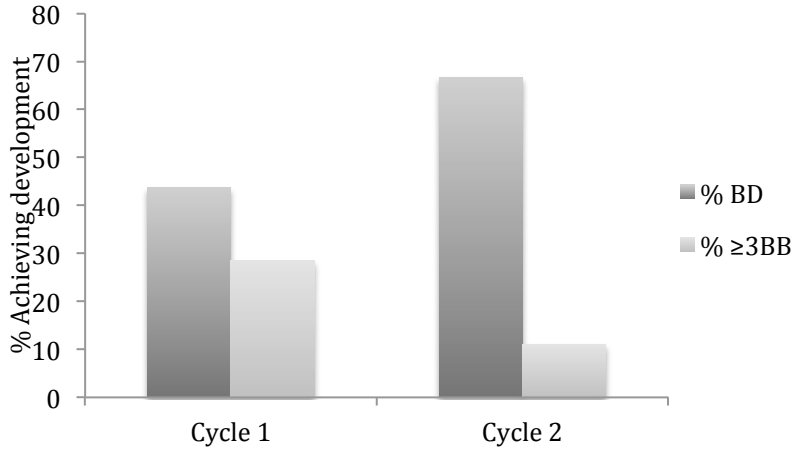


Figure 3.16 Blastocyst development in successive cycles.

No significant differences in blastocyst development or quality in embryos generated from a first (n= 16) and second treatment cycle (n=21). Analysis based on paired t-test.

The uptake of glucose and pyruvate and production of lactate was compared for embryos generated from individual patients in successive treatment cycles. As stated above there were no significant differences in developmental rates or quality and similarly substrate utilisation of those remaining at the cleavage stage and those reaching the blastocyst stage was comparable and intrinsic to the cohort of embryos produced from a patient. Figure 3.17 shows that embryo metabolism is patient specific and repeatable patterns of uptake and release are displayed in subsequent treatment cycles. In this weight stable population, the relationships observed between BMI and embryo development and metabolism in a first cycle of treatment were similarly observed in a second cycle ($p > 0.05$ paired t-test).

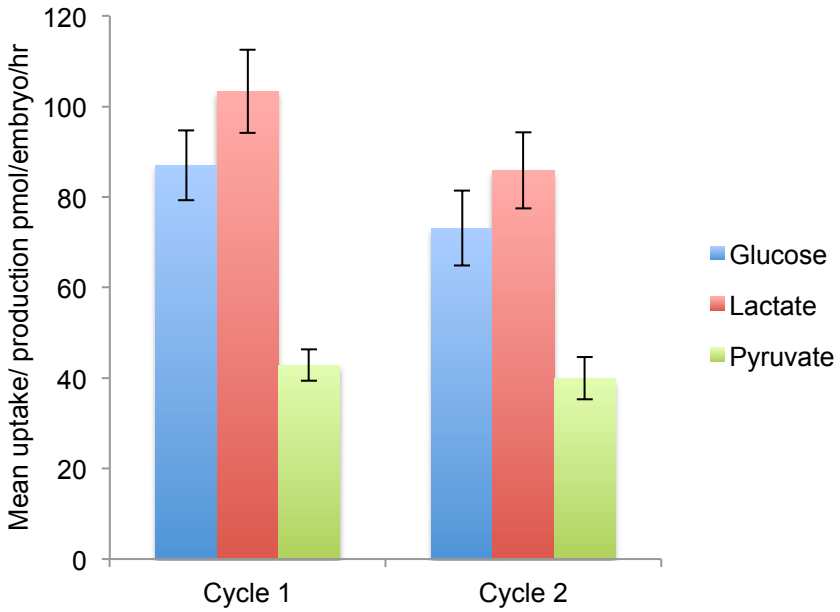


Figure 3.17 Inter-cycle variability in blastocyst metabolism was determined for weight stable women.

The mean uptake/ production of substrate per embryos recorded from a first cycle (n=16) was comparable to that of an embryo produced in a woman's second treatment cycle (n=21). Analysis based on paired t-test, values depicted as mean ±SEM.

Pyruvate uptake was most closely comparable for cohorts of embryos of individual patients in each successive cycle (p=0.64), whereas values for lactate and glucose were more widely dispersed (p=0.17 and 0.19 respectively).

3.4 Discussion

3.4.1 Summary of key findings

The data from the antenatal clinic showed that the average age of menarche was significantly lower for OWOB women compared to those of normal weight, suggesting an early origin for body weight disorders in this cohort. The subfertility clinic data revealed high rates of infertility among OWOB women, this was supported by the significantly lower live birth rates among OWOB women compared to normal weight women undergoing ART. Increased BMI may however increase the likelihood of a dizygotic pregnancy (p=0.07).

In ART cycles OWOB women required a higher dose and duration of ovarian stimulation and produced significantly fewer oocytes. Male BMI contributed to poorer blastocyst developmental progression, although this was not observed in the ovum donation subgroup analysis. In general, the blastocyst quality data was comparable in subsequent treatment cycles, in terms of both morphological grade and biomarkers of viability, for a population of weight stable women.

Reproductive outcomes were also poorer for OWOB women post FET, compared normal weight women (significantly lower pregnancy rates and a trend for reduced cryosurvival of blastocysts; p0.06).

3.4.2 OWOB women and rates of natural conception

The antenatal clinic data indicates that not all obese women have problems with their fertility; however it would appear that BMI does have an appreciable impact on fecundity. The mechanisms remain unclear and the lack of any significant difference in reported menstrual irregularities in this data set, suggests that the divergence in reproductive outcomes may not be simply due to endocrine induced ovulatory factors.

Previous studies have reported that 30-47% of OWOB women have menstrual irregularities (Castillo-Martinez et al., 2003, Douchi et al., 2002). Whilst the data from the antenatal clinic did show a trend for increasing TTP and increasing BMI, this was not significant. As this new analysis focused on natural conceptions only, those with server ovulatory dysfunction were not included within the cohort – possibly explaining the lower than expected incidence of menstrual irregularities. A notable observation was

that obese women reported a significantly earlier age of onset of menarche. This finding is consistent with that widely reported in the scientific literature and was first proposed by Frisch and Revelle (1971) to be linked to the age at which a 'critical body mass' is achieved. Leptin, produced by adipocytes provides a signal to the hypothalamic-pituitary with the amount produced being proportional to the size of the fat mass. Obesity occurring in the teenage years has been associated with a higher incidence of infertility in adulthood (Jokela et al., 2007). On the strength of this, one likely interpretation of these new data is that body weight disorders may have begun early for this cohort of reporting women.

A significant number of women who are of reproductive age are overweight or obese, as illustrated from the antenatal, subfertility and IVF Unit findings. The proportion of OWOB women in the subfertility patient study group was higher than in the group attending the antenatal clinic, indicating a high prevalence of infertility and lower natural conception rates. The data also support the narrative that higher rates of subfertility are evident amongst overweight obese (Jenson et al., 1999, Hassan et al., 2004) and there is an increased time to conceive for women with and without reported ovulatory dysfunction (Gesink et al., 2007). The high rate of treatment drop-out for obese patients underlines the profound psychological consequences that may incur as a result of obesity stigmatisation and serves to highlight the poor-success rates associated with meeting weight-loss targets through dietary advice (Kulie et al., 2011). This is further supported by the finding of higher rates of depression amongst obese women attempting to conceive reported by Roberts et al., (2002).

3.4.3 OWOB women and ART outcomes

Over 24 studies, including more than 78,000 cycles have thus far reviewed the evidence of association between ART success rates and female BMI. Of these, 14 studies have reported no association with CPRs, although a meta-analysis has been used to demonstrate lower live birth rates (Metwally et al., 2008) and a linear increase in rates of fetal loss with increasing BMI (Luke et al., 2011). In the present study, there were no significant differences in pregnancy rates amongst women of differing BMI classification attending the Hull IVF Unit for IVF / ICSI treatment. There was, however, a trend for increasing early pregnancy loss (biochemical and ectopic), miscarriage (6-22wks) and neonatal loss (>22wks) with elevated female BMI. Consequently, subsequent live birth rates were significantly lower for both overweight

and obese women. This is in contrast to earlier studies that have reported lower birth rates in those classified as obese but not in women who are classified as overweight (Lake et al., 1997, Bolumar et al., 2000). These new data do reflect the findings of Wise et al., (2010), who reported a negative linear relationship with female BMI and birth rates.

OWOB women attending for ART at the Hull IVF Unit received a higher dose and duration of stimulation. Endometrial preparation was comparable among BMI groupings, however, importantly, OWOB women yielded significantly fewer oocytes. This finding is consistent with those of Fedorcsak et al., (2004), who in a review of 5,019 IVF cycles, noted a similar distinction in response to ovarian stimulation among lean and obese women. The relationship between oocyte yield and BMI has been reviewed in 21 prior studies, 11 of which support the finding of lower oocyte yield – ranging from 7-25% lower yields for OWOB (Reviewed by Chaverro & Toth 2012), although evidence for differences in fertilisation or embryo development is less convincing. Three studies have reported lower fertilisation rates (Orvieto et al., 2009, Matalliotakis et al., 2008, Sneed et al., 2008), whilst five refuted any association with BMI (Lashen et al., 1999; Fredorcsak et al 2004; Spandorfer et al., 2004; Dokras et al., 2006; Metwally et al., 2007; Bellver et al., 2010). Embryo score was found not to correlate with BMI in three studies (Dechaud et al., 2006; Bellver et al., 2010; Shah et al., 2011) and lower embryo quality was reported for OWOB women by Carrell et al., (2001) and Metwally et al., (2007).

It is possible that failing to account for the impact of male BMI may be associated with the lack of clear correlation with female obesity and embryo development. In the present study, it has been observed that body mass index is generally positively correlated within a couple. When the data were analysed according to both female and male BMI, male BMI did appear to have an effect on blastocyst formation, suggesting a paternal effect on early embryo development. It has previously been reported that rates of blastocyst formation are lower for ICSI derived embryos compared to those generated through IVF (Grifiths et al., 2000) and also that OWOB males have lower sperm counts (Jensen et al., 2004). This finding reported here, was however, independent of sperm count. Likewise, in the analysis of donor oocyte cycles, significant differences in sperm counts did not result in differences in embryo development or implantation. In contrast to the earlier findings, despite the higher male BMI in the recipient group, this did not result in lower rates of blastocyst development

when compared to embryos generated from males of normal BMI using eggs from the same female donor.

The analysis of egg sharer (donor) / recipient studies highlights the importance of oocyte quality on subsequent embryo development, implantation and risk of miscarriage. Clinical pregnancy and miscarriage rates did not differ for discordantly weight matched oocyte donor and recipients, suggesting that the endometrial/ uterine receptivity is less influential on establishment of pregnancy than the oocyte physiology. This is consistent with the findings of Styne-Gross et al (2005) and would suggest that it is the exposure of the developing oocyte to suboptimal follicular conditions that is leading to poorer outcomes in OWOB. The follicular environment has been demonstrated to vary according to female BMI (Robker et al., 2009). However not all studies have reported similar results; in a study of 9,587 cycles using donor eggs from normal weight women Bellver et al., (2013) reported significantly lower success rates in obese recipients. Similarly, Metwally et al., (2008) reported increased rates of miscarriage in OWOB recipients following egg donation. The lack of clear evidence for higher rates of aneuploidy in 1st trimester miscarriages (using own eggs) from OWOB women (Landres et al., 2010; Bellver et al., 2011; Kroon et al., 2011) would also suggest a uterine component associated with pregnancy loss. Endometrial function, specifically endometrial glandular Leukemia Inhibitory Factor (LIF) concentrations are reported to be negatively correlated with higher BMI levels (Metwally et al., 2007), but the impact of differing response to COS was not accounted for in this study. Taken together, the data from this chapter and the extensive published literature would suggest that egg quality/follicular environment is an important determining component of pregnancy outcme in OWOB women, although the overall likelihood of pregnancy is multifactorial and is affected by, amongst others, uterine factors and maternal metabolic physiology.

3.4.4 OWOB women and frozen embryo transfer cycles

The successful implantation of a transferred embryo is clearly dependent on a complex series of interactions with the hormonally primed endometrium. The study of FET cycle outcomes permitted some inferences to be made about the BMI mediated response to COS which may influence endometrial receptivity. It would appear that in the subgroup of women studied who had embryos available to freeze, the potentially detrimental effects of COS may have been more acute in the normal weight women compared to the OWOB group. In this subgroup of patients, regression analysis of the

data demonstrated that COS duration and total dose was comparable for each patient, implying that a similar dose was adequate across the groups. However, the OWOB group may have had a reduced response to a fixed regimen of stimulation which may explain the observation that fewer oocytes were retrieved. Furthermore, these findings could explain the significant improvement in pregnancy outcome in frozen cycles compared to fresh in the normal weight group but not in the OWOB group. Replacing cryopreserved embryos into a minimally stimulated uterus was associated with improved CPR in women of normal weight, but not in OWOB women. The absence of ovarian stimulation may improve endometrial receptivity, but poorer initial oocyte quality might negate this in OWOB.

An alternative hypothesis for the improvement in outcomes for FET for normal weight women, but not OWOB findings, centres on the observed subtle difference in cryosurvival rates. A modest reduction of cryosurvival of blastocysts obtained from OWOB women was observed. It is plausible that this occurs because oocytes from obese women may have increased sensitivity to cryopreservation due to altered lipid content resulting from development in hyperlipidemic follicular environment. Nagashima et al., (1996) reported in the pig that elevated levels of intracellular triglyceride reduces cryotolerance. The embryo lipid content can be modified by the culture environment (Ferguson et al., 2006, Aardema et al., 2013) and the presence of triglycerides in the follicular fluid may result in a series of potentially deleterious events; the oocyte could be induced to absorb lipoproteins and perform neo-synthesis of triglycerides (Romek et al., 2010); increased lipid peroxidation could result in the imbalance of oxidation-reduction metabolism and increased production of free radicals leading to increased sensitivity to cryopreservation (Sudano et al., 2011). Conversely, essential and polyunsaturated fatty acids can alter the membrane lipid composition and fluidity (reviewed in Sampath et al., 2005). This may be especially relevant since physical changes in lipids during freezing are a major cause of cellular cryo-damage which can have significant impact of ongoing embryo viability (reviewed by Seidel, 2006).

3.4.5 BMI and relationship with DZT

The case for compromised oocyte quality and subsequent diminished embryo developmental viability in OWOB women is clearly strong, however it is important to define the circumstances under which increased adipose may actually confer an

advantage in sustaining a pregnancy. Paradoxically, a nutrient-enriched uterine milieu that may arise in OWOB women may favour multiple implantations and an association between DZT and increased BMI has been highlighted in previous studies (Basso et al., 2004, Reddy et al., 2005). In the present study, the incidence of DZT was not significantly influenced by maternal BMI in IVF treatment cycles although there was a trend for higher rates amongst the overweight group. DZT rates in the obese group were lower suggesting a cut-off at which point rising BMI becomes detrimental to treatment outcome. Likewise, Groeneveld et al., (2010), reported that DZT was higher in mildly overweight women and theorised, but could not substantiate, that the adipose secreted angiogenic factor VEGF-A, could be increasing the microvasculature at the implantation site and thus helping to sustain a twin pregnancy in the overweight group.

It was observed that when transferring two top quality embryos of high implantation potential, the trend for increased DZT in the overweight group became more pronounced. One attractive explanation for this observation is that this group of women are better equipped to support a twin pregnancy. However the lack of any significant difference in rates of DZT births and incidence of miscarriages amongst women undergoing IVF treatment suggests that the higher probability of DZT in overweight women previously reported in the literature is also influenced by multiple ovulation rather than body reserves alone and ability to maintain the pregnancy.

The birth weight data provide a crude measure of the mother's nutritional status during pregnancy. McDonald et al., (2010) reported that OWOB women have a decreased risk of having an infant of low birth weight. By contrast, numerous studies have shown that undernourished women are more likely to have small for gestational age infants and intrauterine growth restricted infants (reviewed by King et al., 2006). Interestingly, the data from the present study shows no significant differences in birth weights or gestation periods of DZT offspring born to normal and OWOB women; an observation that provides further support to suggest that the association between DZT and BMI is more complex than a simple reduced maternal constraint and increased capacity to meet the higher nutritional demands of a twin pregnancy.

Deviations in fetal growth rates are more commonly reported among OWOB women compared to those of normal weight; the offspring of OWOB women are at increased risk of macrosomia (>4000g; Kulie et al., 2011) and large for gestational age birth weight, but are also at increased risk of extreme low birth weight <1000g (McDonald et

al., 2010). Moreover, OWOB women are more likely to have a pre-term delivery, either spontaneously, or electively delivered due to increased risks of pregnancy complications (Guelinckx et al., 2008). Hypertension and diabetes, which are both elevated in OWOB women, influence gestational age at delivery (Luke et al., 2009). The box-plots show the observed range in fetal growth and development and indicate a trend for lower birth weights in OWOB in the DZT group. This difference was found to be significant in the singleton births among OWOB women who received donor oocytes, compared to their normal weight counterparts. Brooks et al (1995) reported that after ovum donation, small women tend to have babies with lower birth weight, even when the woman donating the egg is large, suggesting the *in utero* environment directs growth and by virtue of this, the association reported in the present study was unexpected.

Epidemiological studies indicate that the health implications of low or high birth weight in childhood persist into adulthood. An inverse relationship has been reported between weight at birth and predisposition to type 2 diabetes and cardiovascular disease in later life (Barker et al., 1990, 1992), although risk may also be increased at the upper end of the scale too (Alfaradhi & Ozanne, 2011). Low birth weight is associated with obesity in childhood; an observation on which the 'thrifty phenotype' hypothesis of Hales and Barker (1992) is based; thus, it is proposed to reflect fetal programming mechanisms in utero which promote 'catch-up growth' when nutrients are in excess. Additionally, higher birth weight is also associated with a higher BMI mediated by increased fetal insulin in response to chronic maternal hyperglycemia (Hatersley& Tooke, 1999). Often maternal blood glucose concentrations are poorly control in OWOB (Villamor et al 2006), although it is also possible that that OWOB women transmit a greater number of susceptibility genes to their offspring and that the genetic variants that reduce insulin sensitivity / secretion might also reduce birth weight and predispose to type 2 diabetes (Hatersley & Tooke 1999). It is clear that fetal growth and development is influenced by both genetics and the environment (Gluckman et al., 2005) and the role of each remains a matter unclear.

3.4.6 Outcomes in repeat cycles

Pregnancy and miscarriage outcomes were not significantly different between the first and second cycles in either the weight gain or loss groups. The degree of weight change in these women was relatively modest, and likely to be insufficient to influence the outcome of pregnancy. It is unsurprising that benefits on fertility of steady gradual

weight loss may not be immediately apparent. For example, Chavarro et al., (2012) reported that an average of 3.1kg loss was required to improve oocyte quality (% MII cycle 1 verses 2), but this was unrelated to CPRs or LBRs.

Patients whose embryos formed blastocysts in a previous cycle were more likely to reach blastocysts in a subsequent cycle and additionally, the pattern of substrate utilization was comparable in embryos of each successive cycle cohort. This extends the findings reported in Chapter 2, that substrate utilization was comparable amongst sibling embryos of the same cycle cohort. Whilst collectively this data appears to demonstrate that embryo substrate utilization is intrinsically characteristic to each patient, it is not known how changes to the environmental conditions to which the developing oocytes are exposed may influence this. It is speculated that oocytes from OWOB women will have developed in a nutritionally enriched environment and thus specific patterns of embryo substrate uptake and use may correlate with BMI and associated viability and birth outcomes.

Weight loss may improve ovarian physiology, however it is a long term commitment and any improvements to reproductive outcome as a consequence of decreasing BMI may be offset by the known detrimental effects of advancing maternal age on oocyte quality. Furthermore the conclusions which can be drawn from this aspect of study are limited due to the following; it is unknown if the women who had changed weight between cycles were still in a state of flux and without performing dietary assessments it was not possible to ascertain the influence of dietary change on treatment outcome. It was assumed that weight changes were as a consequence of altered food consumption, but nothing is known about how the balance of consumption of macronutrients, such as carbohydrate and protein differed between cycles.

3.5 Conclusion

This chapter has set the context for this thesis – as it has attempted to quantify the impact that obesity has on the relative contributions of the oocyte, sperm, embryo and uterine aspects of the female reproductive system.

There is compelling evidence from the literature and the study of the local population to suggest that reproductive outcomes are poorer for OWOB women, compared to those of

normal weight, in both natural (antenal and subfertility clinic data) and assisted conception cycles (including fresh and frozen cycles, Hull IVF Unit). Maternal nutrition in the periconceptional period appears to have an appreciable impact on ovarian responsiveness, resulting in significantly lower oocyte numbers in OWOB compared to normal weight. The chances of attaining a live birth were shown to be significantly lower for OWOB, compared to normal weight women.

There is ample evidence in the literature to suggest that embryo metabolism is an objective and quantifiable measure of embryo viability. Furthermore it is reported that if the environmental conditions are altered the metabolism of the embryo can adapt to the available metabolic substrates in order to maximise its survival; i.e. Gardner et al., (1996) observed an up regulated glycolytic rate in in-vitro mouse embryos compared to in-vivo controls. There is a clear need to determine if and indeed how maternal over-nutrition is impacting on embryo metabolism. Crucially, in this chapter it was demonstrated that embryo metabolism is repeatable between cycles under standardised conditions.

It is clear that the impact of maternal BMI on oocyte and subsequent embryo development is an area requiring further detailed investigation; as here lower blastocyst development rates have been observed with raised male BMI in discordant couples, but male BMI appeared to exert little influence on development when oocyte donor and recipent cycles were compared. Cryosurvival rates tended to be lower in blastocysts from OWOB and maybe indicative of differences in lipid content compared to those from normal weight women.

Obesity has a multifactoral impact on the chances of achieving and sustaining a pregnancy and the findings from this chapter are highlighted in the hypothetical model depicted in Figure 3.18. The next stage of this thesis will be to determine answers to the as yet undefined preconceptional factors.

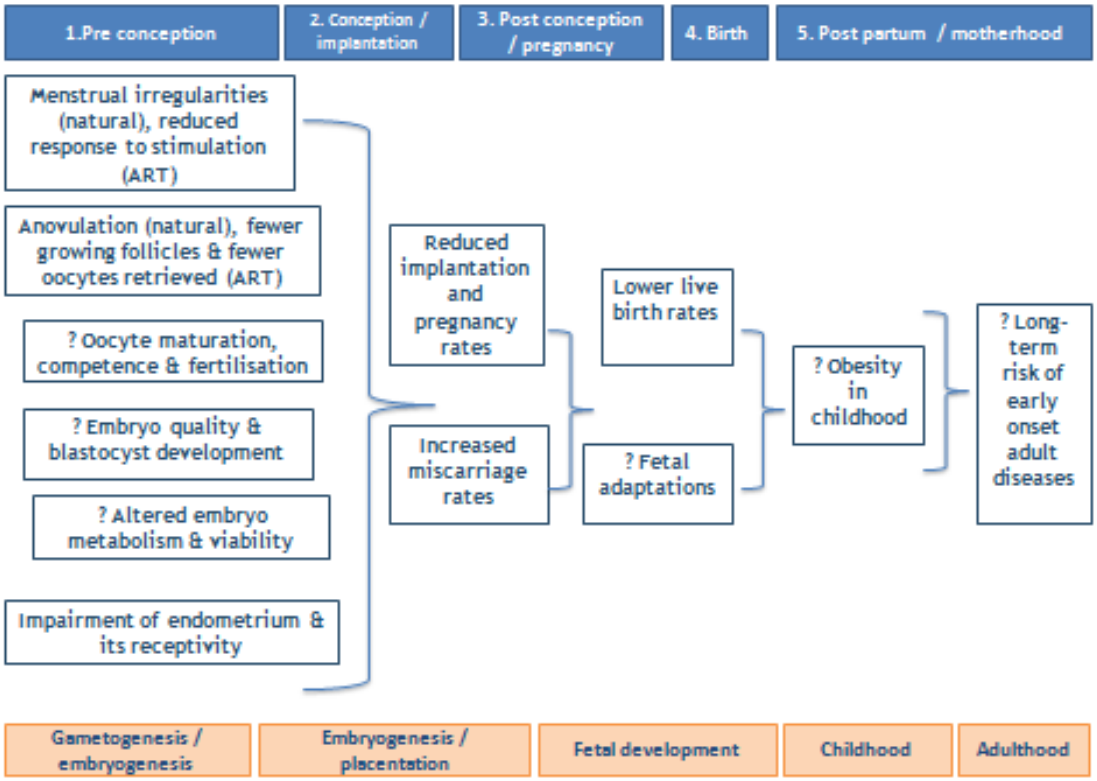


Figure 3.18 Hypothetical impact of maternal obesity.

1= Increased time to pregnancy, infertility, 2 = Failed implantation, early loss (biochemical), misscarage, 3 = Pre-eclampsia, diabetes, hypertension, alterations in fetal growth, 4 = pre-term delivery, perinatal deaths, macrosomia, fetal abnormalities, 5 = short and longterm adaptations to adverse conditions.

Chapter 4 : Markers of oocyte and embryo quality, metabolism and morphokinetics

4.1 Introduction

Oocyte developmental competence is progressively acquired during follicular growth in the ovary. Oocyte quality maybe assessed in a number of ways. The simplest method is light microscopic evaluation of cumulus oocyte complex morphology. Veeck (1988), described a method which is commonly employed in IVF laboratories to assess cumulus dispersion; the homogeneity of the cytoplasm and visualisation of the polar body. However the accuracy of this method is low and nuclear maturation can only be truly assessed by the denudation of oocytes (figure 4.1), which is an invasive technique and is therefore only be performed in ICSI cycles. Studies suggest that higher rates of oocyte immaturity are associated with obese and morbidly obese women (Wittemer et al., 2000; Dokras et al., 2006). It is reasonable to postulate from this, that oocyte developmental potential may be altered in these women.

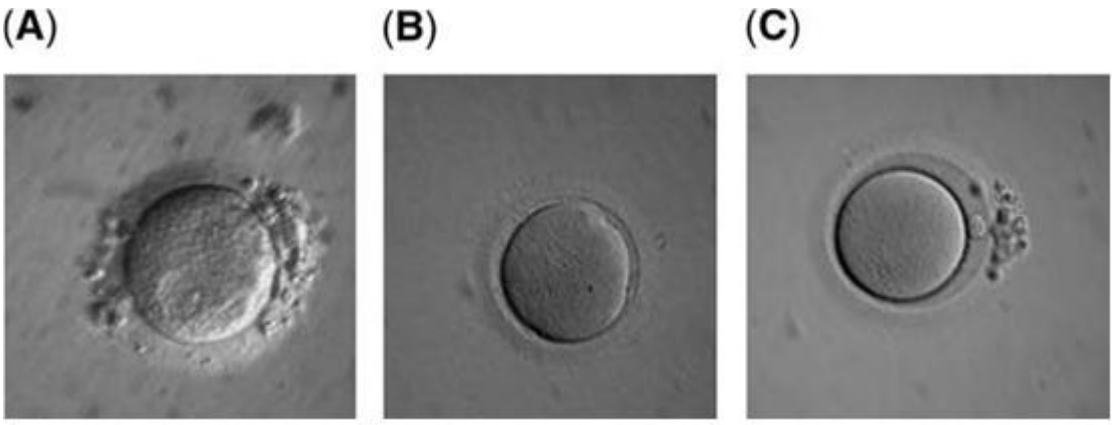


Figure 4.1 Oocytes at different stages of nuclear maturation.

(a) Germinal vesicle stage, (b) metaphase I (c) metaphase II.

The ability of the oocyte to undergo meiotic maturation is a poor marker of oocyte developmental capacity (Moor & Trounson, 1977) and oocyte developmental competence is therefore best described by the capacity of the oocyte to sustain early development. It is during folliculogenesis that the growing oocyte acquires the necessary cellular machinery to support this development (i.e. cytoplasmic maturation in addition to nuclear maturation at the point of ovulation (as discussed in chapter 1).

Currently, our ability to predict the developmental competence of MII oocytes remains poor, though the diameter of a denuded oocyte has been correlated to developmental competence to form a blastocyst in bovine oocytes (Otoi et al., 1997). Smaller oocytes

may resume maturation and have the ability to support pronuclear development, however Otoi et al (1997), found that oocyte diameter was a critical determinant of the ability of a zygote to undergo cleavage. This is an interesting finding, as during *in vitro* maturation delaying nuclear maturation to permit cytoplasmic maturation to occur first, has been shown to improve fertilization and cleavage competence (Hegele-Hatrung et al., 1999). *In vivo*, the dominant follicle regulates the timing of key components of oocyte maturation. By contrast, in an IVF treatment cycle, multiple follicles are recruited and therefore some oocytes may be recovered from smaller follicles. Once extracted, these could spontaneously undergo premature nuclear maturation leading to lower cleavage competence rates.

Few studies have examined the relationship between diameter and cleavage competence in humans; fewer still have sought to determine a possible link with female BMI. Based on a retrospective analysis of 48 IVF cycles, Marquard et al., (2011) reported that oocytes from obese women had a lower diameter than those from control subjects, independent of PCOS. The study did not however report on embryo development. It could therefore be argued that the oocytes were still growing and had not reached full maturational state.

Oocytes that have a larger diameter are known to have decreased Glucose-6-phosphate dehydrogenase (G6PDH) activity. G6PDH is an early enzyme in the pentose phosphate pathway, involved in maintenance of the level of NADPH. G6PDH activity has been reported to be high in developing oocytes and low in fully grown oocytes. Such knowledge has been obtained by the use of the dye brilliant cresyl blue (Ericsson et al., 1993). The dye is reduced to become colourless through the action of G6PDH, and so oocytes with high G6PDH activity appear clear; low G6PDH activity is associated with a blue appearance. In humans it is not possible to measure this, as it may be detrimental to expose oocytes to such chemical dyes, however using an animal model (bovine) it may be possible to establish what proportion of MII oocytes are yet to complete cytoplasmic maturation and how this may relate to diameter and other possible physical differences such as density, which may provide an indication of the oocytes endogenous lipid content.

The relationship between the ovarian and follicular environment and *in vitro* developmental competence of the oocyte is well established. It is clear that the quality /

nurturing capacity of the follicular environment from which the oocyte originates is a major determining factor that will impact on its subsequent development. There are extensive studies correlating follicular fluid (FF) composition with that of the serum and during follicular growth the follicular epithelium becomes increasingly porous in nature, resulting in a greater equilibrium between the two (Leroy et al., 2004). Furthermore, it is becoming increasingly accepted that diet induced metabolic changes are reflected in the composition of follicular fluid (Robker et al., 1999). Obese women are in a high plane of nutritional enrichment and it is possible that the level of metabolites could reach levels that impact negatively on oocyte quality. The metabolism of cumulus cells is adapted to control the flow of metabolites toward the oocyte, however previous studies have shown that elevated non-esterified fatty acid levels are detrimental to oocyte developmental capacity and granulosa cell function in the bovine (Leroy et al., 2005). Lower oocyte viability could in turn lead to a diminished embryo development capacity. A number of reports have indicated that early embryo development is sub-optimal in women who are OWOB (Carrell et al., 2001; Metwally et al., 2007,), however others have reported no relationship (Bellver at al., 2010).

The growth and development of oocytes is dependent on their energy / nutrient supply. This may be the exogenous energy supply (the extracellular milieu) or the endogenous. There are well established differences in the follicular environment of oocytes from obese and normal weight donors, but differences in endogenous supplies have not previously been studied. Mammalian oocytes are known to contain endogenous lipid. Intracellular triglycerides constitute over 50% of all lipid material in mammalian oocytes (McEvoy et al., 2000; Kim et al., 2001). Large amounts of intracellular lipids can compromise embryo quality through impaired mitochondrial function (Abe et al., 2002), as demonstrated in bovine embryos cultured using serum containing media. A greater content of intracellular lipids increases their sensitivity to oxidative stress. Increased lipid accumulation has been shown in the embryos of high yielding dairy cows (Leroy et al., 2005) and in the embryos of diabetic rats (Sinner et al., 2003). In both models energy and lipid metabolism is disturbed and oocyte quality is said to be reduced.

It is possible to measure the triglyceride content of individual oocytes using the method described by Sturmey et al.,(2003) to establish if differences exist between oocytes from normal weight and obese patients. However the examination of lipid content is invasive

and renders oocytes non-viable and therefore it would only be possible record findings from failed to fertilise oocytes and it would not be possible to correlate any differences in content with any ongoing embryo developmental competence. A non-invasive method based on density- could facilitate this analysis, if proven to be sensitive enough.

Embryo developmental competence is typically described by quality assessment at key stages of development. The quality of the embryo selected for transfer is obviously a key factor influencing the success of assisted conception. Currently such observations are made at static time points, however, advances in time-lapse imaging now permit the recording of more precise information and may strengthen these methods of assessment. Embryo development is a dynamic process and scoring can change markedly within a few hours (Montag et al., 2011). The importance of maternal and paternal weight in relation to embryo quality is relatively unknown and it is possible that subtle differences in embryo development could contribute to poorer reproductive outcomes reported for OWOB patients. In this chapter, this is investigated by comparing the BMI of patients at the time of IVF to the developmental kinetics of supernumerary embryos.

Time-lapse imaging systems enable multiple observations to be made without disturbing the culture environment. The Primovision system (Vitrolife, Sweden) permits the use of a compact time-lapse microscope system, which can be placed inside a regular incubator. Embryos are cultured in funnel-shaped microwells, which are 350μm wide at the opening x 150μm deep (3x3 configuration) set in a dome shaped inset, meaning that the contents of each well are openly connected with the medium above (total volume 80μl^3). The system, termed the Well of the Well (WOW) system creates a microenvironment for each embryo, but allows them to share in a larger common culture media reservoir (Swain & Smith, 2011). Higher rates of blastocyst development have been reported for this system in comparison to traditional microdroplet culture systems: using sibling human oocytes Vajta et al.,(2008) reported blastocyst rates of 56% vs 37% respectively.

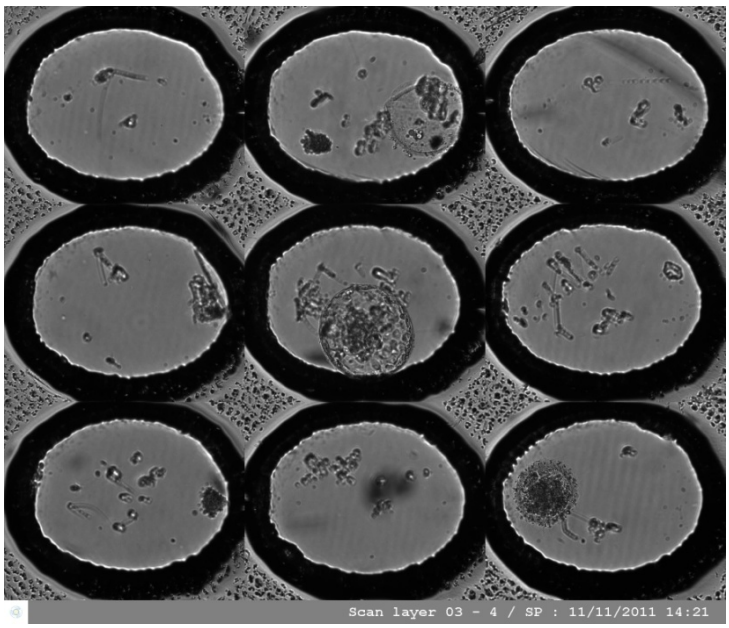


Figure 4.2 WOW dishes are used in combination with time-lapse technology

The camera scans each of the wells in turn and acquires images of each embryo collected at 5-minute intervals. The analysis of data obtained has been used to generate an algorithm to select the embryo with the highest implantation potential (Meseguer et al., 2011). It is widely accepted that implantation prediction models based on time-lapse analysis require validation within each laboratory as a number of factors, such as the culture medium and incubator conditions may subtly alter the prediction parameter timing thresholds. It is thus apparent that morphokinetic development features are affected by intrinsic oocyte factors and environmental factors. The effects of the follicular environment on embryo development may be resolved with increased confidence when data are drawn from a series of multiple observations rather than infrequent static observation techniques which characterise conventional embryo assessments.

Thus far implantation prediction models have been based on early embryo developmental activities and little attention has been focused on blastocyst development. Obesity may contribute to reduced rates of blastocyst formation however once formed, the impact of BMI on blastocyst expansion and hatching may be key to determining differences in viability amongst embryos generated from OWOB women and those of normal weight.

Aims and objectives

a) To investigate the relationship between oocyte diameter and female BMI.
b) To examine the relationship between oocyte diameter and developmental competence.
c) To discover if full growth capacity is likely to have been reached prior to nuclear maturation, in a bovine model of *in vitro* maturation.
d) To ascertain if oocytes differ in lipid content using non-invasive methods of analysis, in a bovine model of *in vitro* maturation.
e) To compare developmental rates and timing differences amongst embryos generated from normal weight and OWOB women in extended culture up to day 9 of development.
f) To record measurements of embryo diameter at specific time points and critical developmental events.
g) To ascertain if differences in blastocyst size correlate with cell counts.

4.2 Materials and methods

4.2.1 Oocyte diameter studies; ICSI patients

The proportion of mature oocytes retrieved after oocyte retrieval was reviewed using retrospective data from ICSI patients attending the Hull IVF Unit (2008-2010).

To assess for differences in the quality of mature oocytes collected from normal weight and OWOB women, oocyte diameters were prospectively measured at x400 magnification using an ocular micrometer. Prior to ICSI, two perpendicular measurements were taken of the ooplasm of 218 oocytes from 29 patients participating in the observational study arm of this thesis. As these measurements were taken prospectively, it was possible to track the onward developmental competence of the oocytes, based on their fertilisation, cleavage division to form embryos, development to form high scoring cleavage embryos (designated as having 6-8 cells on day 3 and a morphology score of grade 3 or above) and blastocyst formation on day 5.

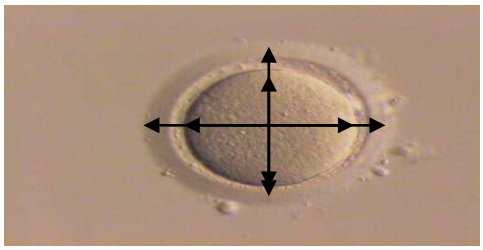


Figure 4.3 Measurements of oocyte dimensions.

Two perpendicular oocyte measurements were taken and used to calculate diameter, radius and volume. Measurements including the zona (a) and to the edge of the ooplasm (b).

The method for recording oocyte diameters was first validated by recording pilot measurements on bovine oocytes. There were significant correlations between all measurements, and increasing zona radius was correlated with a proportional increase in oolemma radius, although the ratio was not significant. Diameter and volume of oolemma were found to be the most repeatable measurements, confirmed by Shapiro-Wilk normality test: the pass level was p=0.290. The diameter of the oolemma was hence measured prior to ICSI for all 29 patients participating in this study.

Embryo and blastocyst diameters were recorded from outer zona to outer zona (due to anticipated considerable variation in zona thickness) and at the longest and widest cellular cross-section. The inner cell mass was assumed to be rounded.

4.2.2 Nuclear and cytoplasmic maturation; Bovine model

To gauge the synchronicity of nuclear and cytoplasmic maturation in smaller oocytes a bovine model was used (due to the ethical constants, restricting the use of using human oocytes). Brilliant cresyl blue (BCB) uptake was recorded in 54 bovine oocytes to assess for continued Glucose-6-phosphate dehydrogenase (G6PDH) activity, an enzyme which is active during maturation.

The method was validated to determine the most appropriate concentration and duration of staining. Oocytes were denuded since cumulus cells were shown to neither facilitate nor interfere with staining. Oocytes were incubated in a HEPES buffer without Phenol red (SynVitro, Origio-Denmark) containing 26 μM BCB for 90 min at 37 °C in humidified air atmosphere (-a concentration and duration of BCB staining, previously shown to be optimum- Goovaerts et al., 2010). After staining, oocytes were washed in HEPES and examined under a stereomicroscope at magnification 50×. They were classified into different groups according to BCB coloration in the ooplasm.

In order to determine if oocytes which had undergone complete maturation differed in density, a continuous gradient method was used, using a 50/50 mix of PureSperm (Nidacon) and HEPES (Sage, Cooper Surgical), centrifuged to give a continuous gradient, in which oocyte descent could be measured. Differences in relative densities for a fixed volume were determined to assess the use of this method for future comparative studies of human oocyte quality.

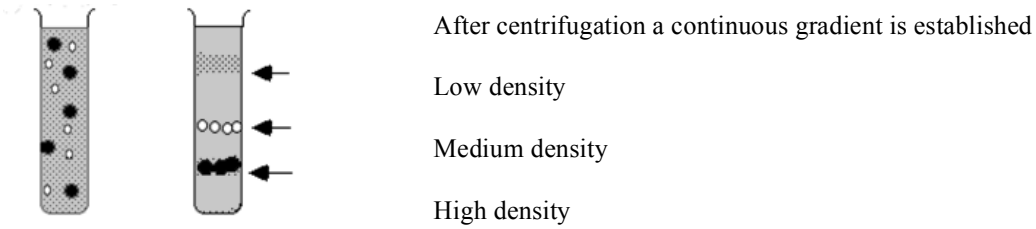


Figure 4.4 Recording oocyte density.

A continues gradient was set up and used to determine relative differences in oocyte density.

The method was validated to ensure that the solution was pH neutral and iso-osmotic (i.e. non-toxic, did not exert an osmotic influence on the oocyte resulting in shrinkage/ swelling). 5 minutes of centrifugation was sufficient to create a continuous gradient sufficient to establish a useful density range based on colour distribution/ assimilation

of the two solutions. A 6ml falcon tube, containing a continuous gradient was clamped in a vertical position and an oocyte which had been stained and measured for diameter was added. Its descent down the column was recorded using a horizontal view microscope. To confirm the accuracy of the recorded depth of descent, the amount of medium that needed to be removed from the tube prior to oocyte recovery was compared to the measured depth to ensure agreement.

In order to determine the triglyceride (TG) content of the oocytes, the same samples were incubated with 0.1% (w/v) pronase for 30 seconds. Individual oocytes were placed in 1.1ul droplet of freezing buffer and frozen in microcaps (as described in Chapter 2.5.6), before being coded and stored at -80^{0}C to permit blind analysis at a later date.

Method development studies were conducted using two enzymatic assays kits both of which use a coupled colorimetric assay to measure triglycerides and relied on the hydrolysis of triglycerides by a lipoprotein lipase to generate glycerol, which is then phosphorylated by glycerol kinase forming glycerol-1-phosphate and ADP. One method (hereafter referred to as Method A- Reagents from Sigma UK) then measures the oxidation of glycerol by glycerol phosphate oxidase to produce hydrogen peroxide, which is in turn catalysed by peroxidase to generate quinoneimines that absorb light between 450 and 600nm. In the second method (reagents from Abcam UK), ADP reacts with phosphophenolpyruvate to liberate pyruvate. Lactate dehydrogenase then catalyses the oxidation of pyruvate and NADH (measured at 340nm) to lactate and NAD. Method A was employed with limited success – the test is designed for determining triglyceride levels in blood or serum and as such measures values in the mg/dl range rather than the μg/ml range required. Dilution of the assay to construct a standard curve within the required range resulted in poor reproducibility (high intra and inter assay variance). It was concluded that this method lacked the sensitivity to determine the triglyceride content of individual oocytes. Method B resulted in more satisfactory standard curves and values could be determined within the desired range of specificity for bovine oocytes. (Further detail of this method is included in the methods Chapter 2.5.6)

4.2.3 Time-lapse imaging; Supernumerary human ICSI embryos

A total of 25 of the 29 patients consented to the extended culture and observation of their surplus embryos using time-lapse technology (Primovision technology system).

WOW dishes were prepared in advance; as described in Chapter 2.6. Recordings were made of specific developmental timings, blastocyst diameters and hatching patterns.

At 68 hours post insemination a total of 101 surplus embryos at various stages of development were placed into extended culture. Embryos were cultured in WOW dishes (Primovision, supplied by Vitrolife, Sweden), in culture conditions as described in section 2.1.5. Embryos were cultured without media change until 116 hours post insemination. At this time embryos were moved into a new WOW dish in which wells were filled with pre-equilibrated QA Blastocyst medium (Sage, USA) supplemented with 10% SSA. Embryo culture was terminated on day 9.

Recordings were made of specific developmental timings/events, using techniques described by Kirkegaard et al., 2012. The time to reach (1) morula stage was defined as when all cells have fused, (2) unexpanded blastocyst; the first time a blastocoel cavity was visible, (3) expanded blastocyst when the blastocoel expands (4) hatching; when the embryo escapes from the zona. The diameter of the blastocoel following collapse, recovery and hatching were also recorded. Collapse was defined as the time point at which the measured diameter is smaller than the previous time point and recovery when the diameter is identical to that just before collapse. The extent of collapse was noted from the largest diameter minus the smallest and the number of collapses was defined as the number per 24 hours from the appearance of blastocoel until the end of culture or hatching.

4.2.4 Blastocyst differential staining

Expanded blastocysts donated to research by 16 patients were fixed on day 7 of development using the differential staining technique based on that described by Thouas et al.,(2001) for mouse and bovine blastocysts. Chromatin-specific dyes were used to determine ICM and TE counts.

Expanded blastocysts (n=44) were incubated in 500µl of solution A (Table 4.1) for 30-45 seconds until the trophectoderm visibly changed colour and shrank slightly, as monitored visually using a dissecting microscope. Blastocysts were then immediately transferred into 500µl of solution B (Table 4.1) and stored for 5 hours at 4^{o}C. Blastocysts were mounted onto a glass slide in a drop of glycerol, gently flattened with a coverslip and visualised for cell counting.

Prior validation studies were performed to empirically define the staining kinetics. Staining is dependent on temperature, reagent concentration, exposure times, cell permeability and density. Using reagents at the concentrations suggested by Thouas et al., (2001) 30-45 seconds was found to be the optimum time for exposure to solution 1, timings were adjusted according to zona and blastocyst diameters. More than 45 seconds incubation caused non-specific cell staining. 5 hours at 4^{o}C was found to be sufficient to permit staining and fixative with solution 2, this could be reduced to 1 hour if performed at room temperature- although longer than 1 hour resulted in signal degradation.

Table 4.1 Composition of solutions 1 and 2 for differential staining of blastocysts.

Component	Quantity	Solution A
1% Triton X-100	0.1ml	to 10ml HEPES (Synvitro,
Propidium iodide (100μg/ml)	1mg	Orgio, Denmark)

Component	Quantity	Solution B
Hoechst 33258 (25μg/ml)	50μl	to 9950μl 100% ethanol

Cell counting was performed directly on an inverted microscope (Vickers, UK), fitted with a UV lamp and excitation filters (460nm for total staining and 560nm for TE red only). Propidium iodide is unable to penetrate tight gap junctions between cells and therefore does not reach the ICM and stains only the permeabilised TE cells.

Slides were coded and stored at 4^{o}C away from light to permit repeat cell counts to be performed on a separate occasion to reduce operator bias.

4.2.5 The study groupings

The patient profile of those donating to each study was compared and can be summarised as follows;

a) Retrospective data from Hull IVF Clinic fresh treatment cycles 2008 to 2010 (n= 709)
b) Prospective data (2010-2015) from 29 women who attained 218 oocytes and 101 embryos that were observed in studies (diameter and time-lapse; table 4.2a & b)

c) Data from 16 women who donated 44 embryos to research – used for differential cell counts (see table 4.2c).

Table 4.2 Overview of study groupings

Table 4.2a Characteristics of patients included in the observational study (± SEM)

	Normal weight	OWOB	p value
Female age (years)	31.27 (1.04)	34.67 (0.96)	>0.05
Male age (years)	35.25 (1.27)	39.43 (1.64)	>0.05
Cycle number	1.58 (0.19)	1.71 (0.31)	>0.05
Female BMI (kg/m^2)	21.41 (0.59)	28.37 (0.92)	<0.001
Male BMI (kg/m2)	25.58 (0.66)	26.0 (0.36)	>0.05
Av. Follicles	15.67 (1.55)	10.53 (1.02)	<0.05
Av. Oocytes	12.67 (1.83)	8.82 (0.87)	0.05

Table 4.2b Details of oocytes and embryos observed.

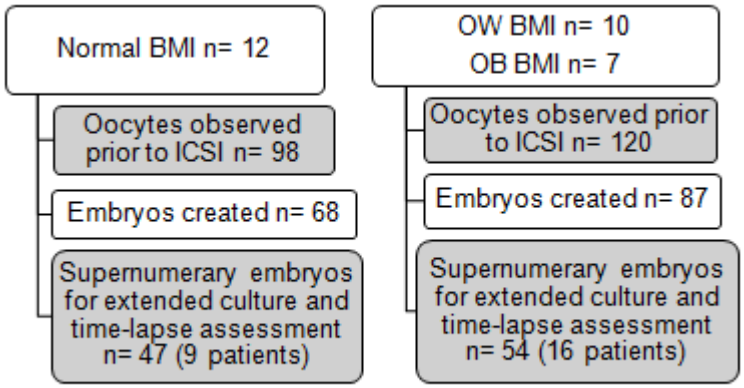
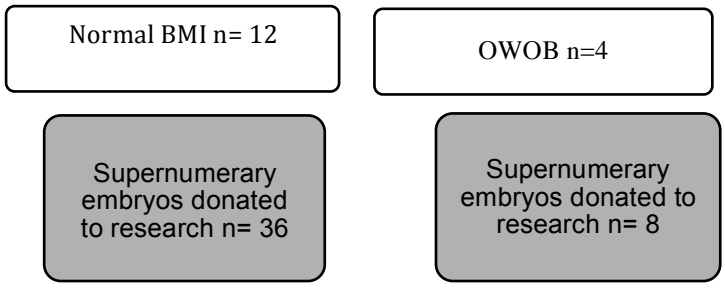


Table 4.2c Details of embryos used in research for differential staining of blastocysts on day 7 of development.



4.2.6 Statistical Analysis

Outcome measures (oocyte numbers, MII and endometrial thickness) were calculated for each BMI grouping, using ANOVA with Tukey Kramer due to unequal sample size groups, to reduce the risk of a type 1 error. Multiple regression was subsequently used

to assess the interrelationship among variables predictive of oocyte numbers and adjusted *p* values are given.

Data distributions (bovine model oocyte diameter and volume measurements) were checked for normality using Shapiro-Wilk test with $p>0.05$ being indicative of a normally distributed sample and confirmation of reputability of measurements. Volume was calculated from the equation $4/3\ \pi\ (\text{diameter}/\ 2)^3$. Relative density was based on volumetric mass density (measured descent within a continuous column) and multiplied by measured volume to give relative total weight. The relationship between size and density was established based on ranks using Kruskal Wallis test and the *H* statistic and associated *p* value determined.

Accuracy of test measures for triglyceride assays were assessed based on the calculated error to signal ratio, with relative error being determined from the coefficient of variance (values >10% were rejected) and absolute accuracy from the standard deviation.

The measure of linear association between univariates (i.e. oocyte / embryo diameter measurements and female BMI) was ascertained by correlation coefficient r= -1 to 1 and the *p* value associated with r ascertained according to sample size and distribution. Generalized Estimating Equations (GEEs) were used to account for patient-specific effects. Data was categorised into inter quartile ranges and groups compared using ANOVA or Kruskal–Wallis test as indicated.

Embryo kinetic data recordings were validated using ImageJ software (nih.gov) to calculate area and pixel value statistics. To ensure impartiality and accuracy, Primovison™ videos were coded by a third party and re-run and re-annotated to determine the confidence of variance in measurements (values >10% were rejected). Continuous data was assessed using a two-sample, unpaired t-test (Welch's t-test due to differences in sample size and variance). Non-parametric data (i.e. hatching pattern) was assessed using Fishers exact test. Multivariate regression analysis was used to determine the influence of explanatory variables on cell count.

All power calculations were performed as previously described and sample sizes were sufficient to achieve 80% power unless otherwise stated. In all cases statistical significance was indicated by $p< 0.05$.

4.3 Results

4.3.1 Patient assessments (ICSI patients, observational measurements)

The number of oocytes retrieved from OWOB women was significantly lower than for normal weight women (9.6 verses 10.6 $p<0.01$), however the proportions of oocytes that had reached metaphase II did not differ significantly (Table 4.3).

Table 4.3 Oocyte development and quality in normal weight and OWOB women.

The retrospective comparison of data (n=709 patients) shows significantly lower oocyte numbers (**$p<$ 0.01), but similar rates of egg immaturity from OWOB compared to normal weight women. ANOVA with Tukey kramer – (standardized for female age)

BMI (kg/m2)	*<18.5*	*18.5-24.9*	*25-29.9*	*>30*
n	10	361	192	146
Mean age (years)	29.8	33	33.6	33
Endometrial thickness (mm)	12	10.6	10.8	10.8
Number of OCC (mean)	101 (10.1)	3809 (10.6)	1897 (9.9)	1316 (9.0)**
% mature - MII	98	92.6	92.3	92.3

The mean oocyte diameter of the 29 ICSI patients who participated in the observational study was 120.34±0.7µm. The mean diameter of OWOB oocytes was 118.29±1.0µm compared to those of a healthy BMI 121.49±1.4µm. Figure 4.5 shows a significant inverse relationship between maternal BMI and mean oocyte size ($p<0.001$).

Figure 4.6 shows that as female BMI increases, there tends to be a reduced capacity for embryo development post-fertilisation. All oocytes generated from normal weight women went on to cleave and form embryos, compared to 94.3% of those from OWOB women ($p=0.06$). In addition the blastocyst development rates of 33.9% and 62.1% for OWOB and normal weight groups respectively ($p<0.01$) are of particular note.

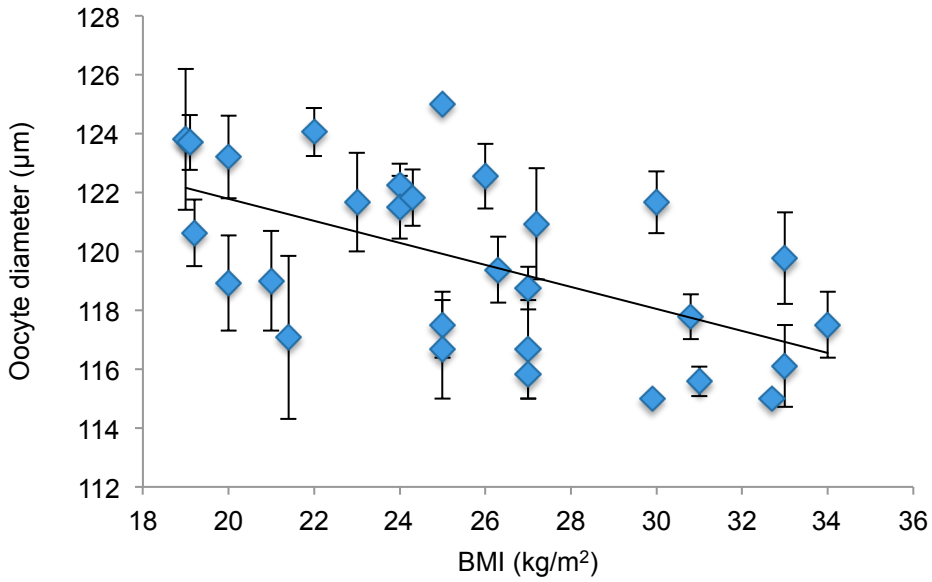


Figure 4.5 Relationship between oocyte diameter and female BMI.

Mean oocyte diameters (n=218), recorded from women of differing BMI classification (n=29), prior to ICSI. The R^2 value (-0.45) indicates a negative relationship between diameter and increasing BMI (p<0.001). Error bars represent ±SEM

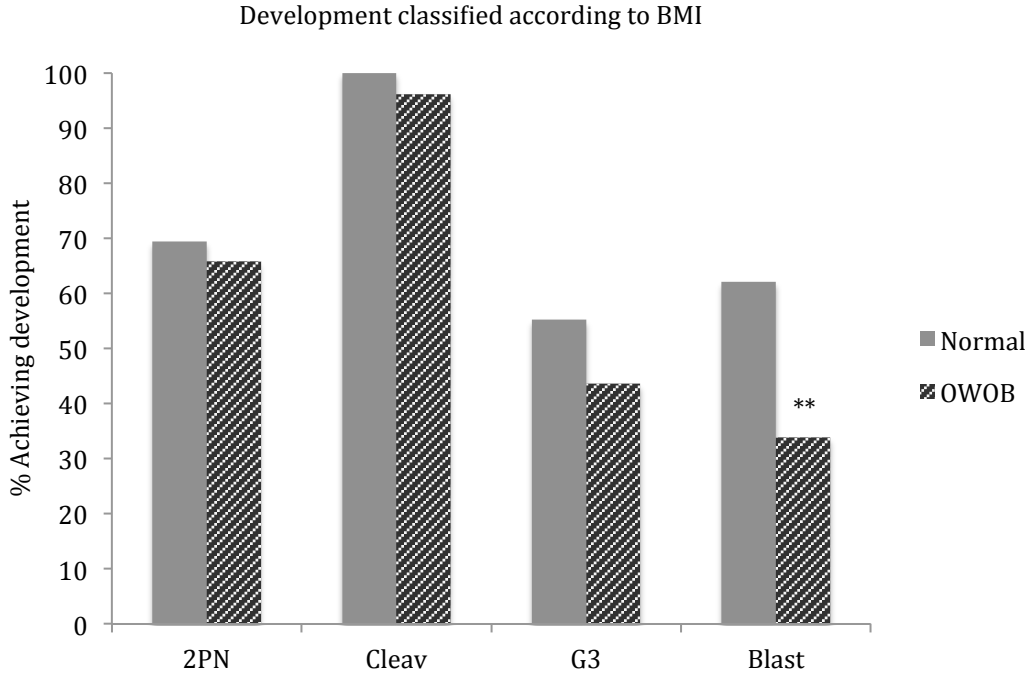
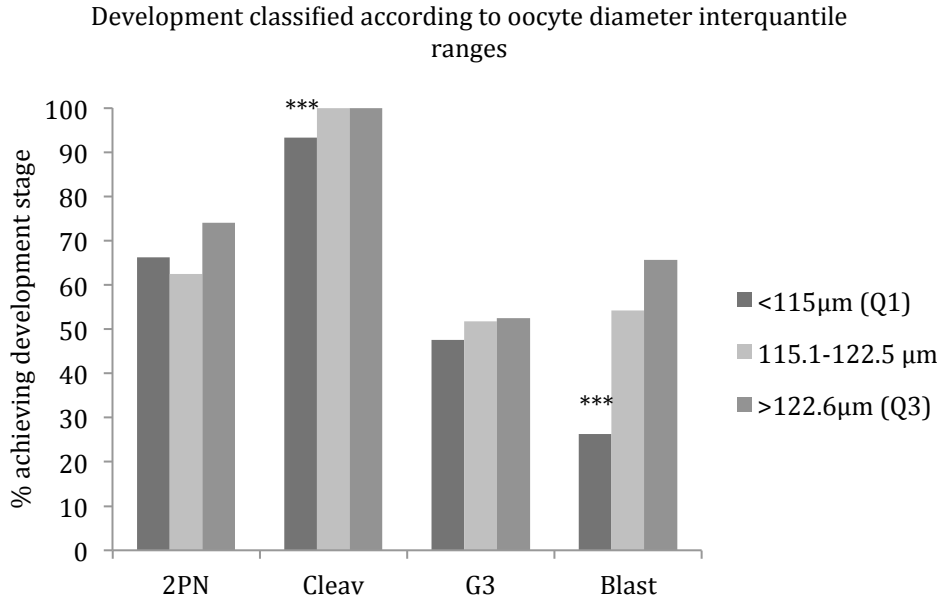


Figure 4.6 Overview of oocyte development and female BMI.

Percentage of oocytes (n=218) that fertilised normally (2PN), percentage of 2PN that cleaved to form embryos and the proportion of top scoring embryos (grade 3) on day 3 of development and the proportion of 2PN embryos forming blastocysts in extended culture. Significantly fewer embryos formed blastocysts in the OWOB group compared to the normal group (p<0.01).

Oocytes from women of higher BMI were significantly more likely to be in the lower quantile range for diameter; these smaller oocytes were significantly less likely to undergo a cleavage division, arresting at the one cell stage (Figure 4.7).



	Normal fertilisation (2PN)	Zygote cleavage (Cleav)	Day3 embryo quality (G3)	Day5 Blastocyst develop. (Blast)	BMI (kg/m^2)
Oocyte diameter (µm)					
Correlations (*r*)	0.13	0.23***	0.05	0.28***	-0.46***

Figure 4.7 Development according to oocyte diameter classification.

The smallest oocytes, were significantly less likely to cleave and to form blastocysts ***p<0.001 and smaller oocytes were significantly more likely to have originated from women with a higher BMI ***p<0.001.

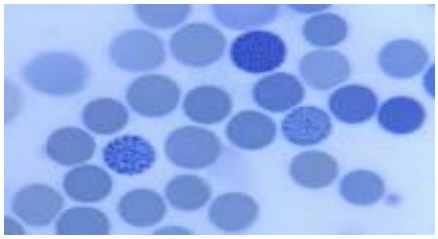
Embryo progression to the blastocyst stage was significantly poorer for the smaller oocytes and the arresting embryos were more likely to have been generated from OWOB women.

4.3.2 Oocyte diameter as a marker of maturation status (in a bovine proxy model)

Using bovine oocytes, as a mammalian model for human oocyte maturation, G6PDH activity was used as an indirect marker of ongoing maturation, (n=54) to determine the relationship between diameter and both cytoplasmic and nuclear maturation.

Of the 54 bovine oocytes assessed, only 20.1% of MII oocytes retained G6PDH activity and these oocytes were significantly smaller (p=0.002) than those which lost G6PDH activity.

A minimum volume of 261,000µm^{3}was indicated as necessary for oocytes to have complete nuclear and cytoplamic maturation. Volumes ranged from 215,262 µm^3 to 881,712µm^3. For any given oocyte volume, significant differences were observed in oocyte relative densities (p<0.05 Kruskal-Wallis analysis of variance ranks). The overall range in column descent was 0.1 to 2.8µl surface depth.



A minimum volume of 261,000µm3 was necessary for completion of nuclear and cytoplasmic maturation.

Figure 4.8 Assessment of oocyte cytoplasmic maturation.

All immature oocytes took up BCB and appeared clear; these were significantly smaller than MII oocytes (p= 0.002, n =54). At a volume of 255,300µm^3- 75% completed nuclear maturation. 20.1% of MII retained G6PDH activity; these were significantly smaller (p=0.002) than MIIs with no G6PDH activity, a minimum volume of 261,000 *µm3* was required to complete both nuclear and cytoplasmic maturation.

Bovine oocytes of comparable dimensions/volume (calculated volume from $4/3\pi r^3$), displayed significant differences in density - those with a larger volume also had a higher density (p=0.004). The trend was however reversed when results were controlled for cytoplasmic and nuclear maturation – (i.e. limited to those above the identified critical volume -261,000µm^3*)*. Kruskal Wallis test indicates a tendency for an inverse relationship between size and density (p=0.07).

The triglyceride content of each oocyte was subsequently determined via enzymatic assay and plotted against the relative weight for each oocyte (volume x relative density). Figure 4.9 shows that relative weight is not indicative of oocytes triglyceride content

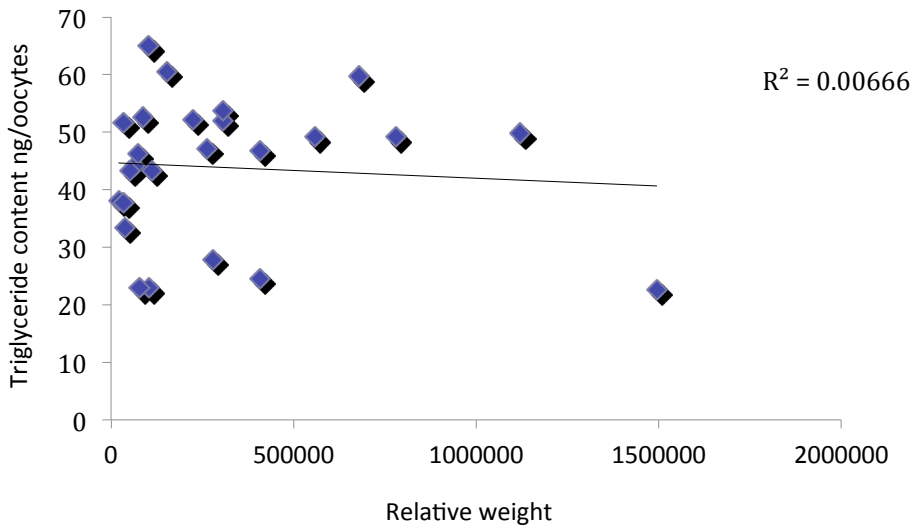


Figure 4.9 Measured triglyceride content compared to measured volume/relative density.

No significant relationship between the parameters.

4.3.3 Time-lapse observations from the fertilised oocytes of the ICSI patient group

The time-lapse developmental kinetic data recorded for 101 embryos, showed that 61 arrested with no further development between days 5 and 7; 39 had reached the morula stage by 115.29±14.22 hours post insemination (hpi) and; 35 had reached the blastocyst stage by 128.76±12.67 hpi. An expanded blastocyst was formed at around 137.37 ±12.08 hpi in 21 embryos, 13 of which underwent hatching from the zona pellucida (ZP), at approximately 154.99±19.25 hpi.

The time-lapse data showed significant differences in the timings of initial blastulation, (103.92±4.6 OWOB, 120.98±1.7 hpi ±SEM normal weight; $p<0.05$) and expansion in relation to female BMI, with embryos from OWOB women developing faster (Figure 4.10). Intriguingly, despite higher rates of cleavage-stage arrest, embryos from oocytes from OWOB women that were capable of reaching the morula stage did so 17 h earlier than counterparts from women with a BMI < 25 ($p< 0.001$). The time intervals between stages did not significantly differ between embryos generated from normal weight and OWOB women.

The ability to form a blastocyst could be predicted by female BMI, oocyte diameter and embryo quality at the cleavage stage and a higher proportion of embryos failed to hatch from OWOB women.

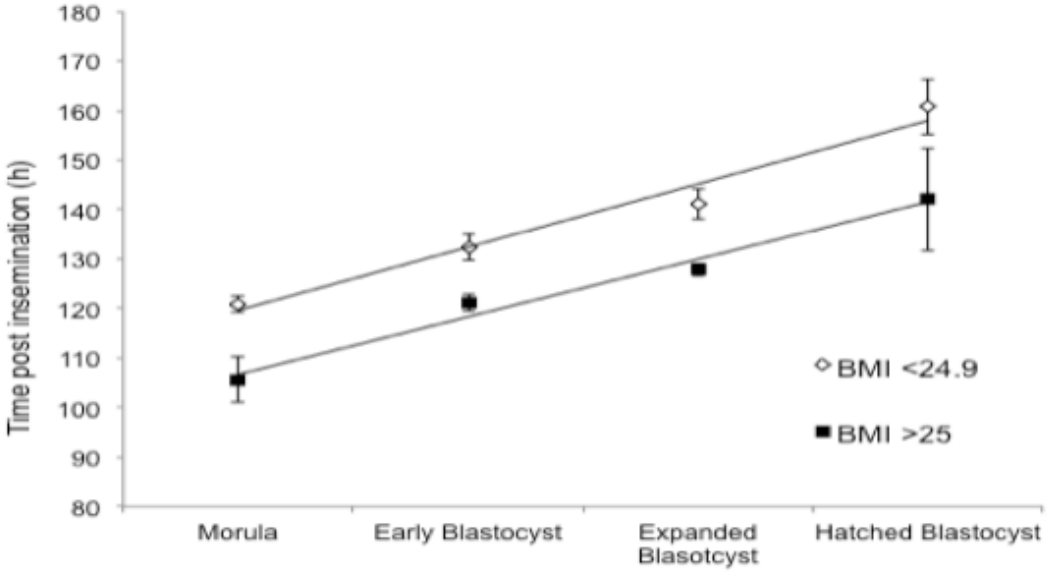


Figure 4.10 Mean time elapsed post insemination for each embryonic development stage to be reached in normal and OWOB women (values ±SEM, n= 101 embryos observed).

The embryo size recorded at a static time point (116 hpi), displayed a weak correlation with female BMI (Figure 4.11a) and was not correlated with the initial recording of the oocyte diameter. Size was dependent on stage attained at the specific time point, however equivalent stage embryos were consistently smaller if generated from OWOB women (figure 4.11b).

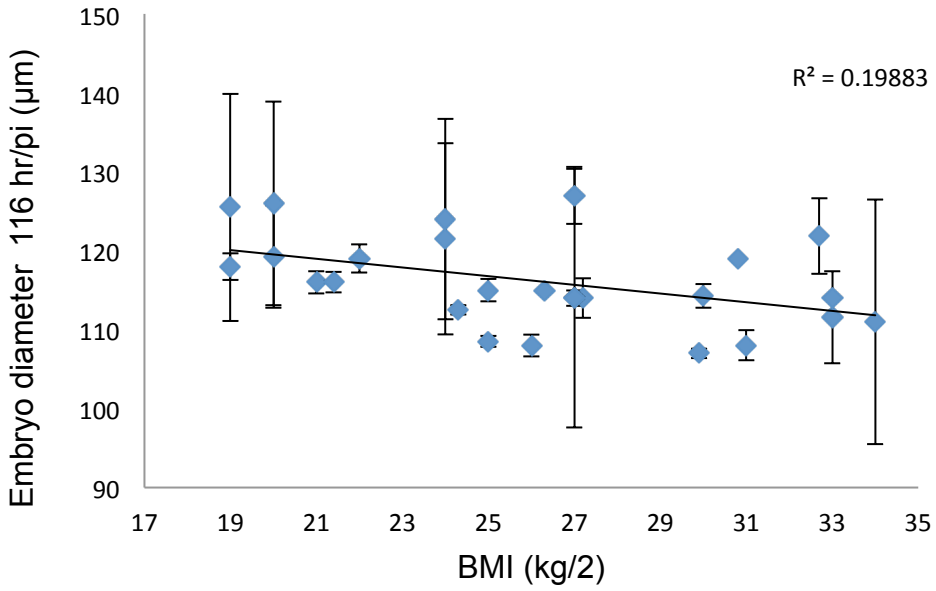


Figure 4.11a Mean embryo diameters (n=101), recorded from women of differing BMI classification at 116 hpi. The R^2 value indicates a weak negative relationship between diameter and increasing BMI.

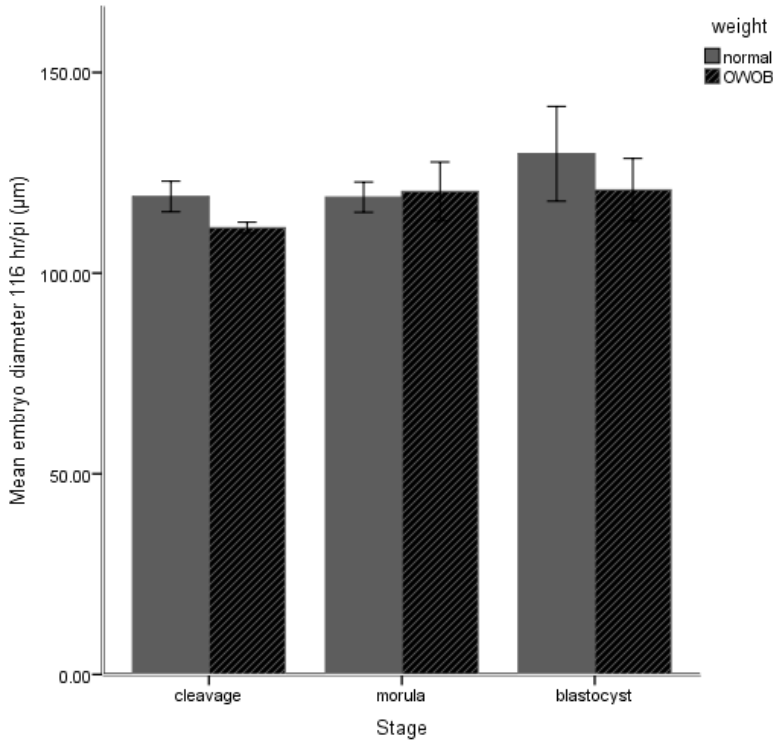


Figure 4.11b Mean embryo diameters (n=101), recorded from women of differing BMI classification at 116 hpi, according to development stage attained.

Figure 4.11 Overview of relationship between female BMI, embryo diameter and development.

Blastocysts of obese women tended to have smaller diameters at the time of hatching (196μm verses 214.3μm), increased zona thickness (6.75μm verses 6.44μm) and a significantly altered pattern of hatching, favouring projected escape, over hydrostatic pressure, compared to normal weight controls ($p<0.05$). The findings were independent of age and cause of infertility.

Embryos from OWOB couples had an aberrant pattern of hatching from the zona. In embryos generated from normal weight couples, blastocyst expansion was predominantly followed by alternating expansion and contraction and it appeared that the hydrostatic pressure exerted by the increasingly expanding blastocyst caused a break in the ZP and the tight junctions, as the volume alternated permitting water to move in and out (Fig 4.12A and B). The amplitude and frequency of contraction cycles averaged 2.6 per hour with a mean change in volume of 73%. Hatching occurred both adjacent and opposite to the inner cell mass (ICM) with equal frequency. In OWOB couples, blastocyst hatching was disrupted and mainly achieved via the projection of undulating trophectodermal extensions that pierced the ZP (Fig 4.12C). Cyclic expansion and contraction occurred more frequently in this group; occurring at an average frequency of 3.2 per hour, but the amplitude of contraction was lower; a mean change in volume of 54%. Hatching occurred adjacent to the ICM in 2 cases and opposite in 8 cases (Fig 4.12D).

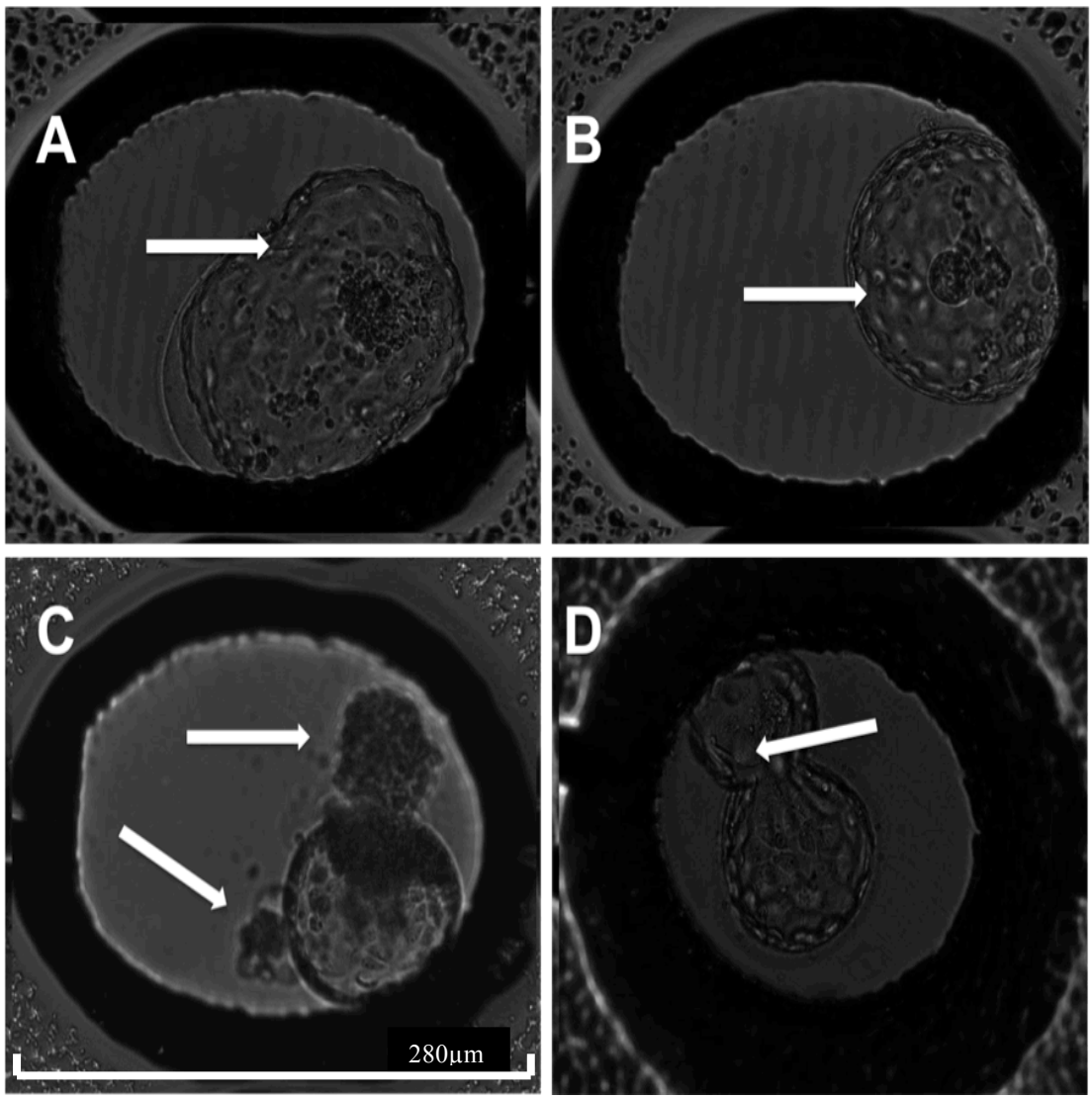


Figure 4.12 Hatching patterns observed.

A shows hatching opposite ICM

B shows an expanded blastocyst that failed to hatch, although the hatching site is visible.

C shows multiple hatching sites

D shows hatching adjacent to the ICM

4.3.4 Differential cell counts from embryos donated by couples for research

Figure 4.13 shows the differential cell counts for expanded blastocysts fixed on day 7 of development (n=44). Embryos from oocytes collected from OWOB women had significantly lower total cell counts and a proportional decline in ICM and TE cell counts compared to those of healthy BMI.

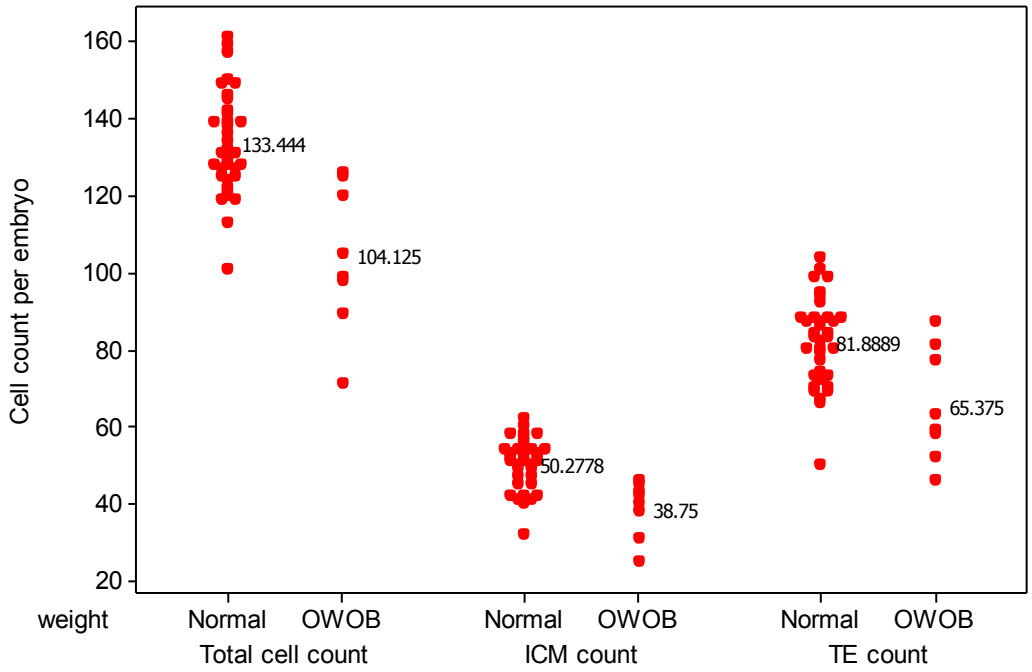


Figure 4.13 Overview of blastocyst cell counts and female BMI.

Total blastocyst cell counts, inner cell mass and trophectoderm cell counts for expanded blastocysts on day 7 of development from 8 embryos of OWOB women and 36 embryos from normal weight. Mean values displayed.

Total, ICM and TE counts were significantly lower for OWOB women (total counts 104.1± 6.8 SEM verses 133.4± 2.2 for OWOB and normal weight women respectively p=0.01). There was no significant relationship with male BMI.

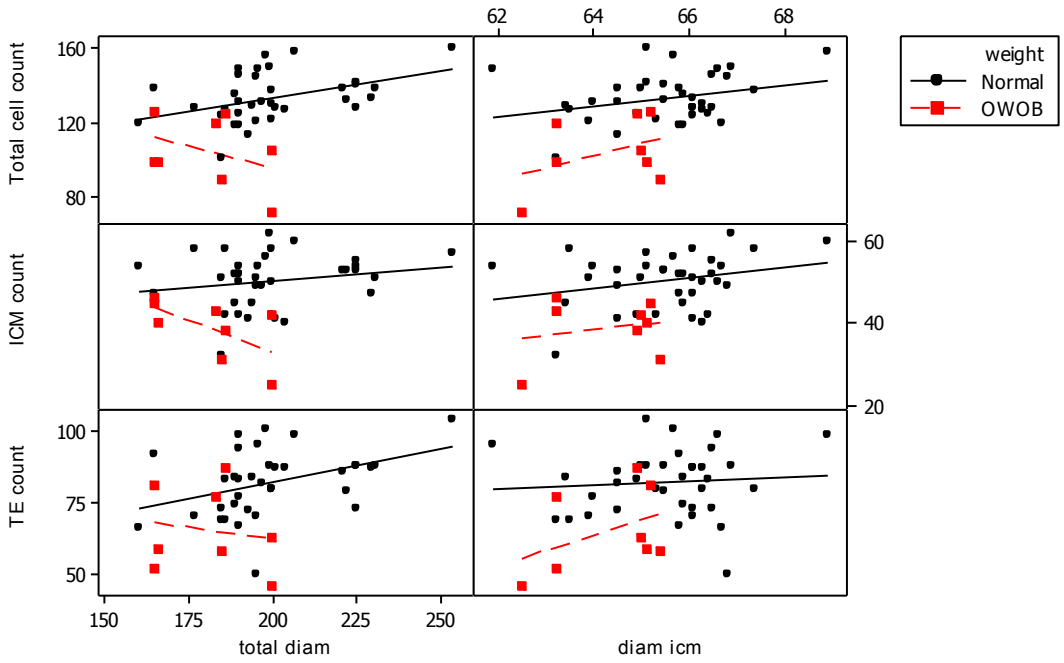


Figure 4.14 Overview of blastocyst cell counts and measurements.

Total blastocyst cell counts, inner cell mass and trophectoderm cell counts for expanded blastocysts on day 7 of development from 8 embryos of OWOB women and 36 embryos from normal weight, according to measures of total blastocyst diameter (µm) and ICM diameter (µm).

The relationship with expanded blastocyst diameter at the point of maximum expansion and ICM diameter is depicted in Figure 4.14. As shown in the previous dataset blastocyst diameter again shows a weak inverse relationship with female BMI. The resulting blastocysts from women with a BMI > 25 kg/m2 at equivalent time points, tended to be smaller (p= 0.07) at the point of maximum expansion, and had significantly lower cell counts (Fig. 4.14).

In a multivariate analysis of the expanded blastocyst data, only female BMI was shown to be a significant predictor of cell count, independent of embryo diameter, female age, cause of infertility and male BMI. Furthermore, at equivalent time points, embryos from OWOB mothers had fewer trophectoderm cells (p=0.001). The ICM count is predicted by female BMI and there is a trend for increased cell count with ICM diameter (p=0.08). The diameter of the ICM does not correlate with total cell count or total blastocyst diameter. There is a weak correlation between the diameter of the ICM and male BMI (p=0.07).

4.4 Discussion

4.4.1 Summary of key findings

This chapter has shown that fewer oocytes are recovered from OWOB women compared to normal weight women. Furthermore, although at first glance the competence of these oocytes may not appear to be compromised – with a similar proportion of metaphase II, fertilising and competent cleaving stage embryos, at the later stages of embryo development differences in competence become apparent. There were three key observations; firstly oocytes from overweight and obese women are significantly smaller than those collected from women with a BMI considered to be in the healthy range. Secondly these smaller oocytes from OWOB women were less likely to reach the blastocyst stage, but those that did so, showed accelerated preimplantation development, and thirdly, the subsequent blastocysts contained fewer cells, notably in the trophectoderm. Overall, the finding that fewer oocytes from overweight and obese women were competent to reach the blastocyst once fertilized may contribute to the lower success rates of fertility treatment reported in overweight and obese women (Bellver et al., 2010; Shah et al., 2011; Chavarro et al., 2012; Moragianni et al., 2012).

4.4.2 Lower oocyte numbers and size

The finding of fewer oocytes from OWOB women compared to normal weight women is supported by 8 other studies, although a further 9 found no difference (Tamer et al 2009). Similar contradictory data have been obtained from animal models and clinical data (Purcell & Moley 2011). However, in contrast to the clinical findings of Spandorfer et al., (2004) and Dokras et al., (2006) found no association between obesity and the proportion of MII oocytes.

A measurable proportion of oocyte growth has been shown to occur in the final hours of oocyte development as observed in human oocytes matured *in vitro* (Cavilla et al., 2008). It is possible that oocytes from OWOB women are not reaching full maturation and growth due to a blunted response to gonadotrophins as a result of increased apoptosis of granulosa cells, such as been observed in a murine model of diabetes (Chang et al., 2005). Furthermore, decreased granulosa cell gap junction communication and connexion expression has been observed in diabetic mice (Ratchford et al., 2008). As obesity is characterised by hyperglycemia and insulin resistance (characteristics of diabetes), such models are highly relevant and may be

applicable to human oocytes of OWOB women. In this regard, the observation by Lucifero et al., (2004), that imprinting of certain genes in mice has been shown to occur in the late growth phase, is also of potential significance when considering disruption in the final growth stages of the oocyte.

Routinely only a small percentage of oocytes fail to complete meiotic maturation after stimulation (estimated to be 20%, from clinic data) and therefore as a measure of oocyte quality the %MII oocytes may not be particularly informative. Discrepancies amongst published study findings may arise due to differences in sample sizes. It is therefore proposed that data obtained from the measurements of oocyte diameter provide a better measure of oocyte quality and developmental potential. This is supported by the finding that smaller oocytes had a significantly reduced ability to undergo embryo cleavage and form blastocysts. This finding was in agreement with those of Otoi et al., (1997), who reported lower rates of blastocyst development for bovine oocytes with a reduced diameter, suggesting that the diameter at which oocytes achieve meiotic competence, maybe below that at which they attain full developmental competence.

Mean MII oocyte diameter was shown to be significantly lower for OWOB women compared to normal weight women. Marquard et al., (2011) reported similar findings, but did not track the characteristics of each oocyte after fertilisation. Likewise, previous observations of human oocyte dimensions have mainly focused on determining minimum threshold diameters at the time of collection to complete maturation (Cavilla et al., 2008; Durinzi et al., 1995). This is the first study to track individual oocyte size to embryo developmental competence and quantify the relationship with female BMI. The data demonstrate clearly that the physiological processes that occur within the confines of the ovarian follicular environment of OWOB women have measurable effects on oocyte size. The implications of smaller oocyte diameter may have lasting ramifications, especially in view of the finding that smaller oocytes in mouse were associated with abnormal fetal development and decreased size (Jungheim et al., 2010).

It is possible that the smaller oocytes generated from OWOB had not have completed their full maturation, as cleavage competence is known to be acquired progressively during both nuclear and cytoplasmic oocyte maturation. In data obtained using the bovine model, approximately 20% of oocytes, displayed asynchronous nuclear and cytoplasmic maturation. However, the diameter of the cytoplasmically mature cohort of

oocytes varied significantly, an observation that may relate to differences in content of triglyceride and/or proteins.

4.4.3 Lipid content

The amount and composition of triglyceride in human oocytes and embryos has not previously been studied and current assays are invasive, rendering oocytes non-viable and thus halting further observations of development. Disappointingly, there was no correlation between TG content and relative weight calculated from the continuous gradient method, indicating that this method for recording density may lack the required sensitivity to act as a surrogate marker of TG.

In domestic animals triglyceride content is accrued during oogenesis and the amount has been shown to be linked to female diet, (reviewed by Mckeegan & Sturmey, 2012). It is possible that aberrant triglyceride accumulation may occur in gametes produced in a high lipid environment and hinder ongoing development. Exposure to saturated fatty acid during development affects the balance of lipid storage in bovine oocytes and developmental competence (Aardema et al., 2011). Furthermore, poorer embryo development has been reported for bovine embryos produced in a hyperlipidaemic environment (Leroy et al., 2010), and excess accumulation of cytoplasmic lipid droplets in embryos in the presence of elevated fatty acids; notable since this affects embryo cryotolerance (Abe et al., 2002). This may provide a possible explanation for the lower cryosurvival and success rates reported in chapter 3 for blastocysts from OWOB women undergoing frozen embryo transfer cycles. This is explored in later chapters of this thesis.

Oocytes from obese mice have been shown to have higher lipid content (Wu et al., 2010), which has been linked to impaired mitochondrial activity (Igosheva et al., 2010) and evidence of endoplasmic reticulum stress (Wu et al., 2010, 2011; Yang et al., 2012). The follicular fluid obtained from obese women contains higher levels of triglyceride and free fatty acids (Robker et al., 2009, Yang et al., 2012). When mouse cumulus-oocyte complexes (COC) are cultured in this fluid, maturation is impaired (Robker et al., 2011) and higher rates of granulosa cell apoptosis are observed (Yang et al., 2012). Such evidence suggests that the obese follicular environment is lipotoxic and COC exposure may elicit a stress response, as lipid accumulates intracellularly and causes damage to organelles. In mitochondria this may manifest as abnormal morphology

(Luzzo et al., 2012), abnormal distribution, increased ROS production and abnormal metabolism (Igosheva et al., 2010).

In the endoplasmic reticulum, stress pathways may be induced and in very simple terms this results in a cohort of genes which slow protein translation and up-regulate protein folding chaperones being activated. As a consequence of this the expression of transcription factors including ATF4, ATF6 is increased and supporting this, is the observation that ATF4 expression is increased in the granulosa cells of obese women (Wu et al., 2010). The treatment of obese mice with an ER stress inhibitor before ovulation has been shown to increase mitochondrial DNA and restore oocyte quality (Wu et al., 2015), providing further suggestive evidence of the cascade of cellular defects aberrant lipid exposure may initiate.

An alternative explanation for the poorer oocyte and subsequent embryo development could be that oocytes from OWOB women are exposed to higher intrafollicular glucose concentrations (Robker et al.,2009) since it has been reported that oocytes from mice with chemically induced hyperglycemia undergo precocious resumption of meiosis (Kim et al., 2007), whilst a number of these oocytes fail to progress through to MII (Chang et al., 2005). There is also evidence that the transcription of cell cycle associated genes is altered in animal models of metabolic syndrome (Ma et al., 2012), leading to poor quality smaller MII oocytes. In addition these authors reported altered expression of enzymes associated with gycolysis, which, together with the data of Chi et al., (2000) who found decreased insulin stimulated glucose uptake and of Chang et al., (2005) who reported structural and functional abnormalities in the mitochondria of oocytes from diabetic mice, suggests these oocytes may be metabolically compromised.

4.4.4 Embryo development

Previous comparison studies of embryo quality in normal verses OWOB embryo cohorts have produced conflicting results (as discussed earlier) and the differences in developmental rates observed in this study could provide an explanation for this. The advanced mitotic pace of development at the cleavage stage in the OWOB embryo cohort could have resulted in these embryos being graded as equivalent in quality to those of normal weight women, if a traditional grading system based on combined cell number and morphology had been used.

Although fewer embryos from OWOB women reached the blastocyst, those that did so developed at a faster rate. Specifically, embryos from overweight and obese women reached the morula stage of development on average 17 h earlier than comparable embryos from women of a healthy weight. Following precocious precompaction blastocysts were formed earlier in overweight and obese women, although the duration of cavitation once the morula stage had been reached did not differ. The reasons behind this precocious development are unclear, particularly given the recent report by Bellver et al., (2013), that embryos from overweight and obese patients had similar timings in cell division to embryos from women of normal weight. An important distinction between the work reported here and that of Bellver et al., (2013) relates to the length of time that embryos were observed; Bellver et al.,(2013) reported findings for 72 h post-fertilization and stopped short of looking beyond day 3 and therefore did not observe differences in development rates. The authors did concede that obesity may play an important role in the later stages of embryo development. The data presented here is the first to demonstrate differences in developmental timing between embryos from OWOB patients that only became apparent after 68 h post-insemination.

Interestingly, in non-obese diabetic mice Moley et al., (1991), reported retarded embryo progression throughout all development stages and that insulin was shown to correct this. Progression through the cell cycle is likely to be influenced by appropriate metabolism and cell cycle checkpoint control. Obesity has previously been associated with a greater prevalence of spindle anomalies and non-aligned chromosomes (Machtinger et al., 2012). This could lead to retarded or uncontrolled advanced development, it is thus likely that a viable blastocyst is one, which has shown development within optimum time ranges at each stage of development – neither too fast nor too slow.

The recorded embryo diameter at a fixed time point (such as movement to extended culture) will likely be related to developmental pace; as blastocysts develop they expand. The embryo size at 116 hpi provides a measure of developmental advancement, which may be less subjective than morphological assessments. At this time point; more embryos from the OWOB cohort remained at the cleavage stage, however those capable of further development did so at an advanced pace, thus assessment of mean diameter per patient maybe misleading. When the analysis was focused to compare equivalent

stage embryos, the blastocysts of OWOB women showed a tendency to be smaller than those generated from normal weight women (trend identified p=0.07).

A larger blastocyst diameter on day 5 indicates advanced blastocyst expansion, which has been shown to be predictive of implantation potential (Shapiro et al., 2007). However, since embryos of OWOB women do not reach an equivalent size of those from normal weight prior to hatching, full expansion is impaired in these embryos.

One possible explanation for reduced expansion capability of embryos from OWOB women, is that impaired energy metabolism in these embryos (as proposed in rodent models described earlier) may result in them being unable to produce sufficient ATP to drive the Na+/K+ ATPase (sodium pump) and protein synthesis, resulting in lower rates of expansion and possibly accounting for the differences in contractile and hatching patterns recorded for the blastocysts from OWOB women. The frequent interruption of expansion due to repeated contractions, as observed in blastocysts generated from OWOB women may disturb the hatching process. The level of pulsatile movements that would be expected as part of normal development is yet to be defined, however viable embryos are likely to be those showing a uniform pattern of contractions (Kirkegaard et al., 2012).

Female BMI was an independent predictor of cell count and OWOB women had significantly lower cell counts. Lower cell counts and increased rates of apoptosis have been reported for blastocysts recovered from diabetic mice (Pampfer et al., 1990; Chi et al., 2000) and those generated from bovine oocytes matured in a hyper-lipid environment (Van Hoeck et al., 2011). Given that blastocysts from OWOB women had fewer cells in the trophectoderm lineage, from which the cytotrophoblast and syncytiotrophoblast will form this might suggest that at implantation, there are fewer chorionicprogenitor cells. This in turn may feasibly have an impact on the size and invasive properties of the trophoblast and subsequent placenta.

4.5 Conclusion

The data presented here provide strong evidence that oocyte developmental competence is compromised in OWOB women. Possible mechanisms and consequences are

summarised in Fig 4.15. The reduced diameters for oocytes from OWOB women are theorised to relate to the nutrient enriched follicular environment.

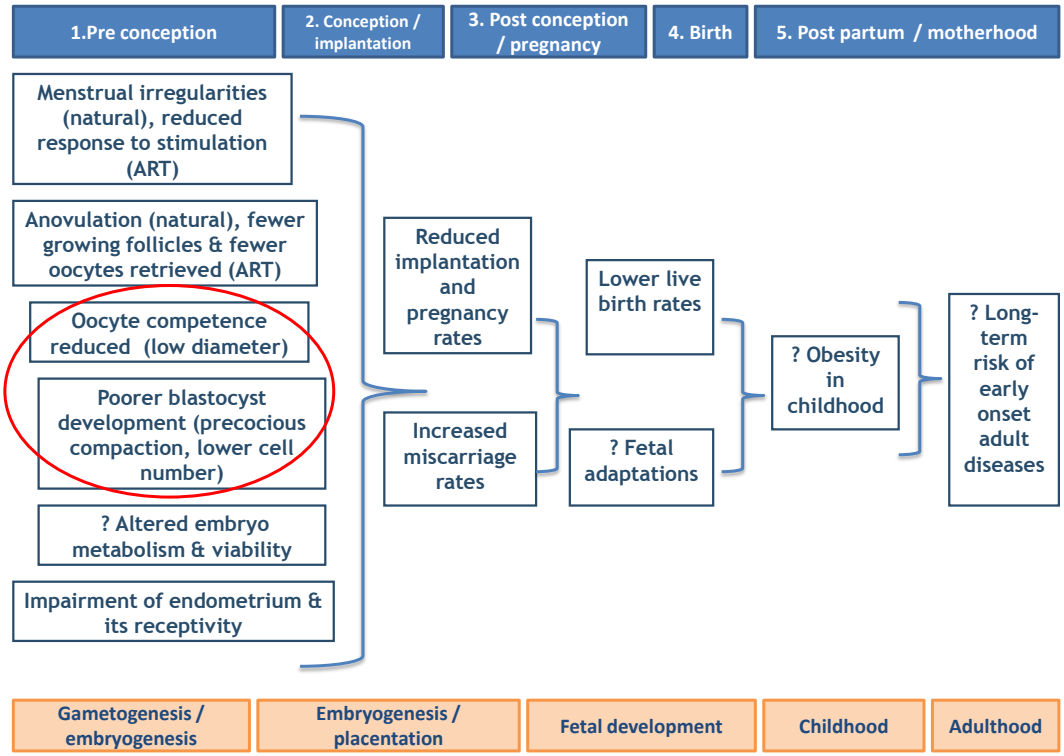


Figure 4.15 Hypothetical impact of maternal obesity- developing picture.

Chapter 5 : Human embryo metabolism

5.1 Introduction

One consequence of the high plane of nutrition of obese women could be nutritional enrichment of the periconceptional environments. Strong evidence of such enrichment in the human has been provided by Robker et al., (2009) who reported significant positive correlations between BMI and the ovarian follicular content of glucose, lactate, triglycerides, insulin and C-reactive protein. It is hypothesised that periconceptional enrichment promotes an aberrant embryo metabolic phenotype which may compromise embryo health and lead to the reduced fertility and increased miscarriage seen in OWOB patients. In addition, there is concern that nutritional stress on the embryo can have major consequences for fetal and neonatal health (Leese et al., 1998) and long term health implications for the offspring.

The evidence supporting the role of metabolic analysis of substrate utilisation as a marker of embryo health is compelling. Human embryos with a pyruvate consumption distributed in the mid to lower range are reported to be most likely to give rise to a pregnancy following embryo transfer (Turner et al., 1994), while Lopes et al., (2007) found that oxygen consumption in the mid range predicted the viability of cattle embryos post-transfer. Most notably, researchers have shown that lower depletion and appearance of amino acids is predictive of the capacity of human embryos to develop to the blastocyst stage in vitro (Houghton et al., 2002) or give rise to a pregnancy following transfer in clinical IVF (Brison et al., 2004).

Metabolic activity has been correlated with molecular damage (Sturmey et al., 2009); embryos with lower levels of damage have characteristically low metabolic activity. This observation underpins the 'quiet embryo hypothesis' (Leese 2002; Sturmey et al 2009) which proposed that viable embryos have less molecular and cellular damage than those which arrest and a reduced need to take up nutrients for repair processes. Leese et al., (2007) extrapolated these findings to animal models; especially domestic ruminants, which indicated that a high level of feeding prior to and during conception leads to adverse developmental outcomes; observations which have been linked to the metabolism of the early embryo.

It is generally accepted that mammalian preimplantation embryo metabolism follows a set pattern, as discussed in Chapter 1. The extent to which the accepted pattern of

uptake and release of these substrates by the developing embryo is linked to maternal obesity has not been explored. Furthermore, the role of endogenous lipid as a source of energy during human embryo development is also unknown.

Examination of the utilisation of exogenous and endogenous metabolites by OWOB and normal weight women may help to answer questions surrounding how the preimplantation embryo processes and adapts to environmental cues. Very little is known about the lipid changes in abundance and composition, which may occur during preimplantation development. Changes, which have previously been observed in animal models are believed to be related to the environment (Kim et al., 2001), as well as the diet of the animal (Wu et al., 2010). It is thus feasible that maternal diet may influence lipid accumulation in the oocyte and subsequent embryo metabolism and development. In light of this proposition it is prudent to address the role of beta-oxidation of TG in embryos from normal and OWOB women. Furthermore, better understanding of metabolic adaptation by early embryos of OWOB women might also provide an explanation for the lack of consensus in the literature linking obesity and embryo quality (Carrell et al., 2001, Metwally et al., 2007, Bellver et al., 2010, 2013). It is possible that phenotypic differences are subtle or do not become immediately apparent, rather leading to more long-term changes in the phenotype of derivative cells. Major findings in animal models in recent years include (1) increased blood pressure (Watkins et al., 2007, Sinclair et al., 2007) (2) increased anxiety related behaviour (Ecker et al., 2004) (3) increased obesity risk (Watkins et al., 2008) (4) decreased insulin sensitivity (Sinclair et al., 2007) (5) altered immune function (Sinclair et al., 2007).

This is the first study to investigate the metabolism of embryos derived from overweight and obese women and those with a BMI in the normal range. The patterns linking single metabolic markers with individual embryo development and viability have been recorded and related to the maternal environment from which they originated.

Aims and objectives

a) To discover if human embryo metabolism is associated with maternal obesity; specifically if an increased maternal BMI is related to an aberrant embryo metabolic profile indicative of reduced viability (i.e. reduced ability to form a blastocyst *in vitro*).

b) To assess if embryo metabolism provides an objective biomarker of human embryo health, measured using a range of quantitative, biochemical methods to examine the relationship with maternal BMI and IVF treatment cycle outcome.
c) To discover if the triglyceride content of embryos generated from OWOB women differs from that of embryos from normal weight women.
d) To discover if triglyceride content of cleavage stage arrested embryos differs to that of those capable of forming blastocysts.

5.2 Materials and Methods

5.2.1 Patient recruitment

IVF patients were consented and recruited to the study as described in chapter 2. A total of 29 consecutive patients presenting for IVF at the Hull IVF Unit donated a total of 150 embryos with full informed consent. Female and male BMI were recorded at the down-regulation appointment and at the commencement of treatment. Embryos that originated from patients classified as OWOB (BMI > 25 kg/m2) were compared with embryos derived from women of normal BMI (18.5–24.9 kg/m2). The study groups being discussed in this chapter are highlighted below (circled in red).

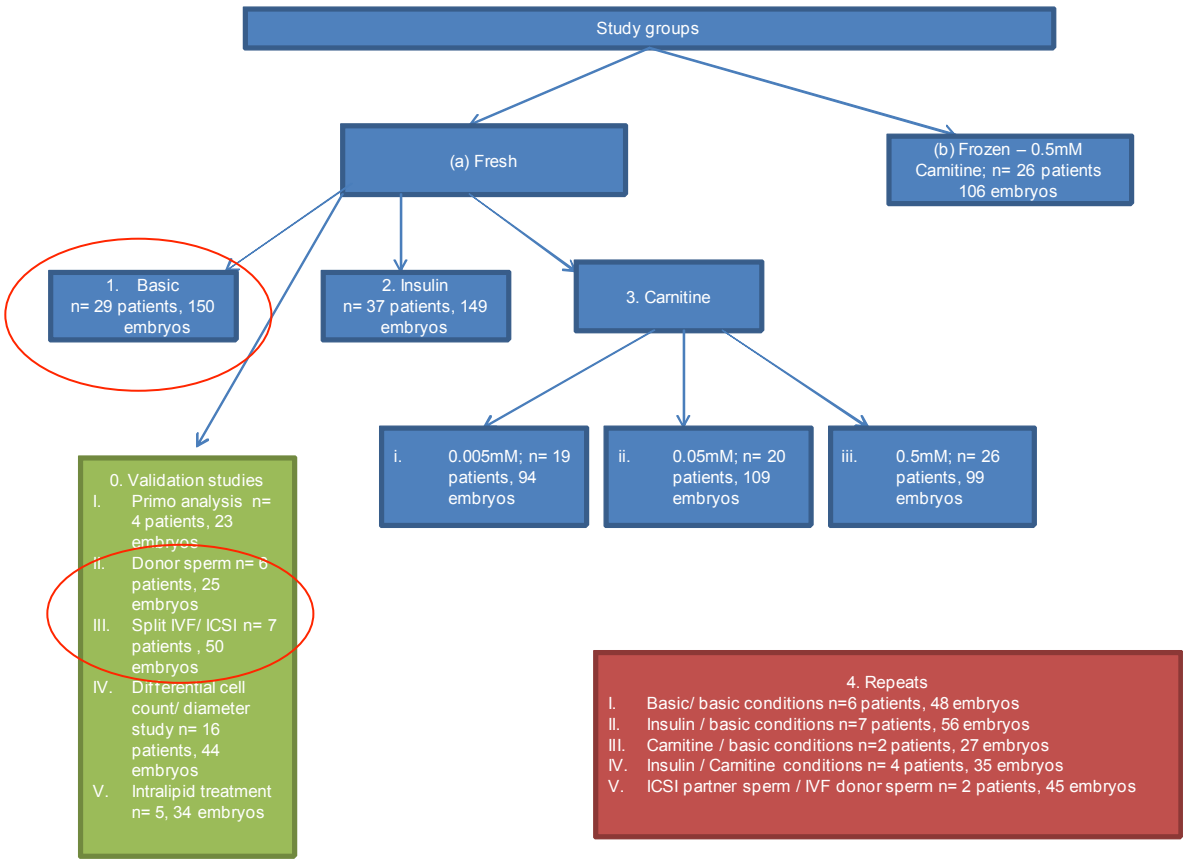


Figure 5.1 Source of study group-

from prospective data, collected from women contributing to the primary research project from 2010 to 2015.

5.2.2 Experimental methods

The experimental methods used to generate metabolic profiles are mainly non-invasive and provide quantitative markers of embryo health.

Surplus embryos donated to research had their development stage recorded before being placed individually into 4 ml drops of Earle's balanced salt solution, supplemented with 1mM glucose, 0.47mM pyruvate, 5mM Lactate, a physiological mixture of amino acids (Houghton et al., 2002; all obtained from Sigma-Aldrich Chemical, Poole, UK) and 0.5% (v/v) QA Serum Protein Substitute. Embryos were cultured under Sage Oil at 37^0Cin 6% CO_2 for 24 hours, alongside embryo-free control drops. Embryos were subsequently moved to fresh culture droplets and developmental observations made, as stated in Chapter 2. All embryos donated for research were profiled and embryos representative of all development stages were cultured and their development monitored. Observations were ended on Day 9. After incubation, the spent culture medium was immediately frozen at -80^0C for later analysis.

Metabolic COnsumption/RElese (CORE) profiles (Guerif et al., 2013) were determined by measuring the depletion and appearance of glucose, pyruvate, lactate and 18 amino acids, according to established techniques, that may be applied to individual oocytes and embryos. The exception being, triglyceride measurement which is determined by a coupled colorimetric assay on samples pooled in groups of 2–5 embryos at equivalent development stages for each patient. Full details of each method are provided in Chapter 2. Results were recorded according to stage reached at the end of the period of culture.

5.2.3 Statistical Analysis

The information from the metabolic profiling of spare embryos was compared for the two study groups and correlated to study endpoints: embryo cleavage development and blastocyst formation. ANOVA was used to assess intra and inter subject variability in combination with linear regression analysis to assess the predictive accuracy of metabolic profile on blastocyst development rate. The best embryos were transferred or cryopreserved as part of patient treatment but the cycle outcome of sibling embryos (which were not assayed) has been incorporated into the analysis.

Full details of the statistical analysis are included in Chapter 2. However additional tests/ calculations conducted included;

- Kolmogorov-Smirnov test for normality was used in the first instance, as mean and variance of CORE profiles was not known and had to be estimated from the data 5-9 data – this allowed a judgement to be made about whether the values attained on each day of development could have come from a normal distribution.
- The 'glycolytic index' of individual embryos was determined based on the assumption that for each molecule of glucose consumed, 2 molecules of lactate are produced.

5.2.4 The study sub-groupings

In the main study group a total of 37 embryos from 7 OWOB women and 113 embryos from 22 normal weight women were assayed for CORE profile. The end point for the study is blastocyst development; 18 embryos had developed to form blastocysts (48.6%) by day 9 in the OWOB group compared to 37 (30.3%) from the normal weight group. There were no differences in patient age and cycle number, but significant differences in follicle and oocyte numbers and male BMI have been appropriately controlled for in the multivariate statistical analyses. It should be noted that if male BMI is used as the denominator, a significantly higher proportion of cases involve ICSI in the OWOB group and this is explored in further detail in the supplementary sub-grouping analysis. The details are provided below.

CORE Research project group
n= 29 consecutive IVF & ICSI patients

	Normal weight	OWOB	p value
Female age (years)	32.86 (1.03)	33.86 (1.75)	>0.05
Male age (years)	35.18 (1.01)	38.86 (1.50)	>0.05
Cycle number	1.23 (0.09)	1.14 (0.14)	>0.05
Female BMI (kg/m^2)	21.76 (1.96)	28.64 (2.01)	<0.001
Male BMI (kg/m2)	25.10 (0.58)	28.11 (1.40)	<0.05
Av. Follicles	14.27 (1.20)	11.86 (2.09)	>0.05
Av. Oocytes	10.45 (0.77)	9.14 (1.30)	>0.05
No. patients IVF / ICSI	15 / 9	3 / 4	>0.05

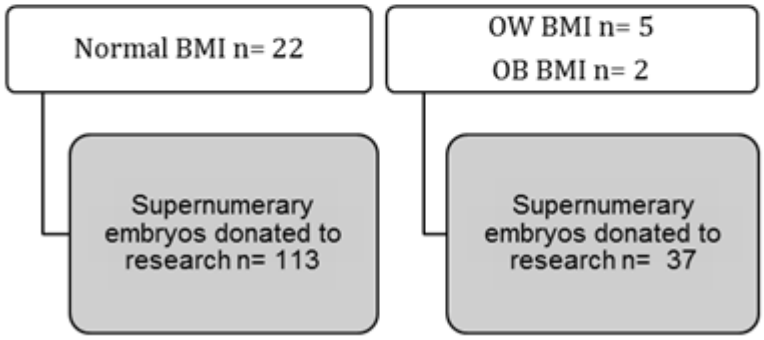


Figure 5.2 Schematic diagram of the study group.

Patient numbers and demographics are indicated (values are ± standard error of the mean). Shaded boxes indicate embryos included in the analysis.

To explore the paternal influence, data from a single male sperm donor that had been used to fertilize oocytes from six women, all of whom had a different BMI was evaluated. With the male factor controlled in this way, the results of the multivariate analysis can be determined.

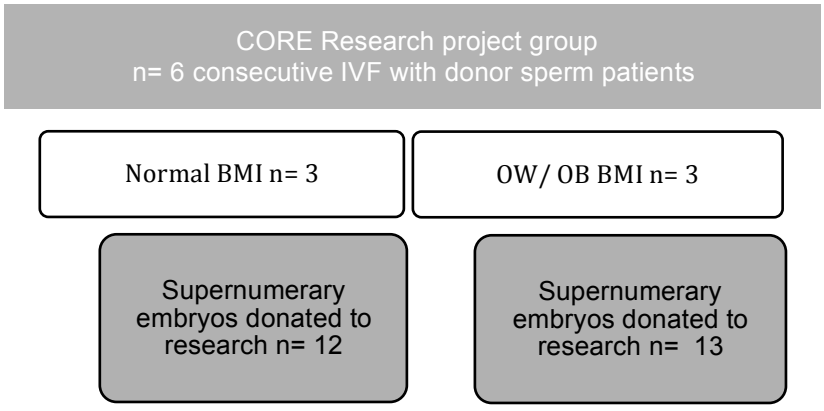


Figure 5.3 Supplementary sub-study groupings for analysis to determine the influence of male factor using a single male sperm donor.

Given the trend for increased use of ICSI in couples in which the male BMI was raised, the influence of insemination method was reviewed. 17 (45.9%) of embryos in the OWOB group of women were fertilised by ICSI, compared to 42 (34.4%) in the normal weight group. The specific demographic differences are displayed below and male semen parameters were significantly poorer in the group receiving ICSI treatment.

	ICSI (n=12)	**IVF (n=17)**	***p*-value**
Female age years	32.91 (1.44)	34.67 (0.99)	0.31
Female BMI kg2	23.65 (0.94)	22.89 (0.99)	0.6
Anti-Mullerian hormone pmol/l	20.89 (3.75)	20.66 (4.32)	0.97
Mean no. oocytes	9.91 (0.83)	9.6 (0.98)	0.82
Male age years	38.73 (2.12)	36.33 (1.26)	0.31
Male BMI kg2	24.42 (0.86)	26.77 (0.86)	0.07
Sperm concentration M/ml	18.42 (5.11)	77.04 (14.93)	<0.01
Sperm motility %	40.91 (4.37)	67.0 (2.97)	<0.001
Sperm morphology %	3.73 (0.45)	7.20 (0.34)	<0.001
Mean no. fertilised oocytes	6.0 (0.38)	7.0 (0.94)	0.39
Mean no. top grade embryos	3.0 (0.56)	3.73 (0.71)	0.73
Mean no. blastocysts	1.64 (0.39)	1.67 (0.37)	0.96
Clinical pregnancy rate %	27.27	66.67	0.11
Implantation rate %	25	57.89	0.14

Figure 5.4 Supplementary sub-study groupings for analysis to determine the influence of insemination method.

Patient demographics are displayed (values are ± standard error of the mean).

5.3 Results

5.3.1 CORE Glucose, lactate, pyruvate and embryo viability

Embryo viability was determined in terms of its developmental stage progression or arrest throughout the study period. Some embryos displayed low viability; undergoing early arrest, conversely others progressed to the hatched blastocyst stage. Embryos reached blastocysts, of differing expansion status/ grade on different days in culture. Mean glucose consumption fluctuated from 136.3pmol/emb/hr on day 5 to 110.9pmol/emb/hr on day 9; lactate production fell from 143.8 to 110.7pmol/emb/hr and pyruvate consumption remained relatively stable between 45.8 and 34.8pmol/emb/hr during the 5 days of culture analysis. The distribution of glucose, lactate and pyruvate metabolism was normal for each day of development, as determined by a one-sample, 2-tail Kolmogorov-Smimov test $p>0.05$ and depicted in Figure 5.5.

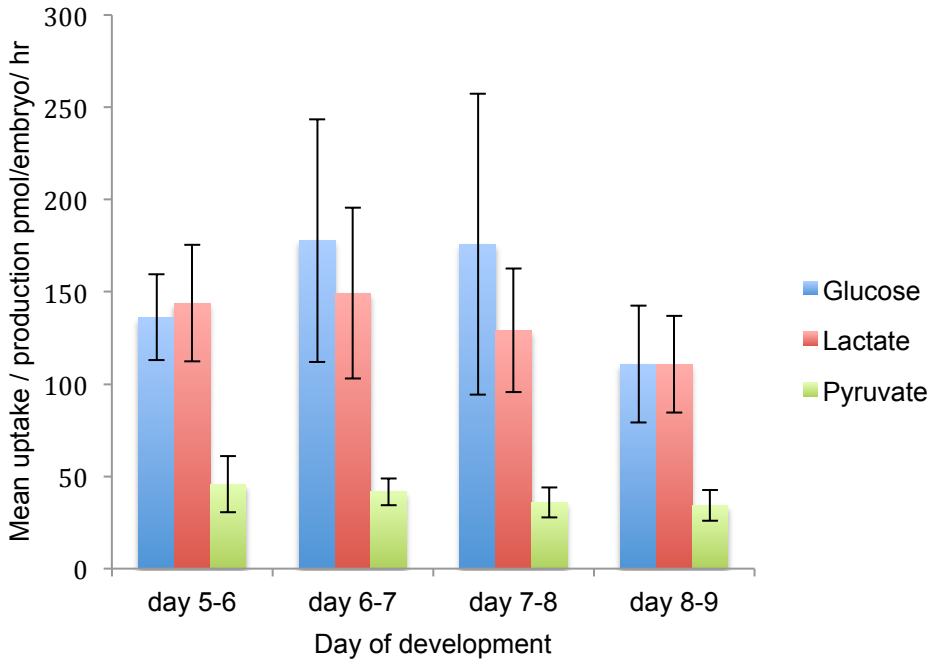


Figure 5.5 Mean embryo metabolism of substrates over 24hr time periods, during 5 days of culture.

In total, measurements were obtained from all 150 embryos derived from 29 patients. The specific breakdown of measurements attained were; n=102 measurements from embryos on day 5-6, n=97 day 6-7, n=87 measurements day 7-8, n=76 day 8 to 9. A minimum 2 sample points were obtained for each embryo. Bars show the glucose and pyruvate consumption and the release of lactate± 95% confidence interval (CI).

The rate of glycolysis did not significantly differ according to day of development, ranging from 52.8% on day 5-6, to 42% day 6-7, 36.7% day 7-8 and 49.9% day 8-9. The mean glucose consumption for a blastocyst which formed on day 6 was 103.02mol/hour, whereas an embryo reaching this stage on day 7 had a mean consumption of 75.89mol/hour at blastulation, this difference was not statistically significant (p=0.42 Figure 5.6). The majority of embryos capable of forming blastocysts, did so by day 6.

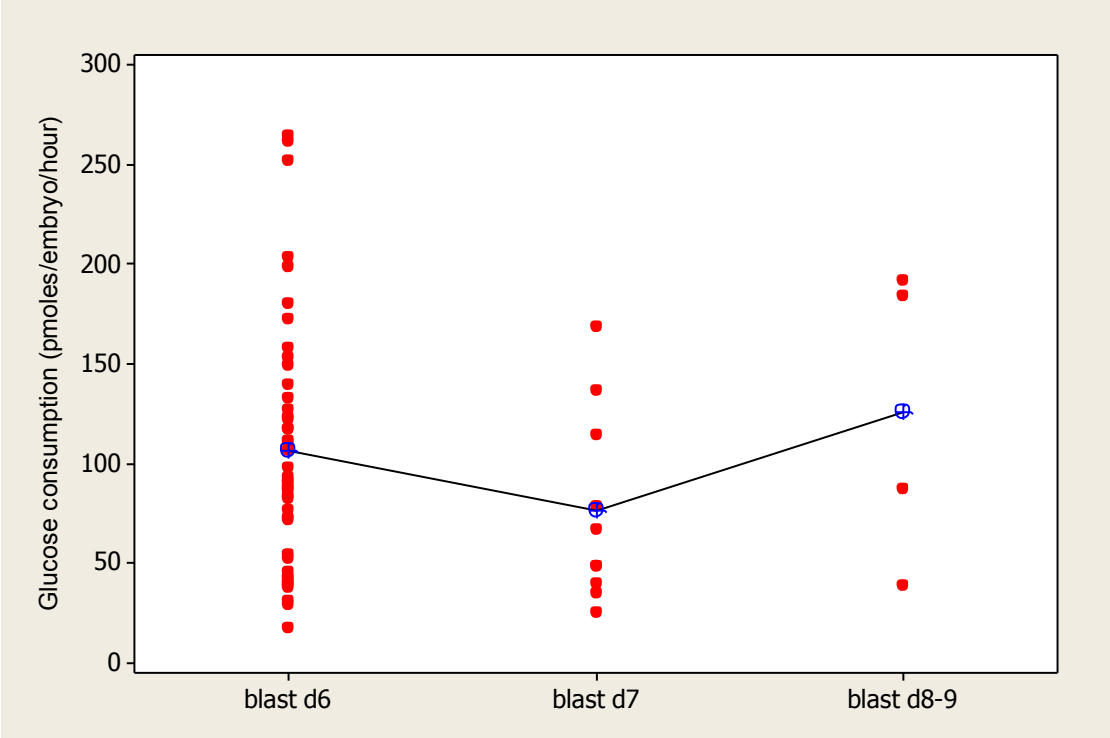


Figure 5.6 Glucose consumption for embryos that formed a blastocyst on day 6, 7 or day 8-9.

Individual plots and mean values depicted. Observations were recorded, at static time points i.e. when culture medium was refreshed, every 24 hours of culture.

The consumption of glucose was found to be significantly different for embryos which had arrested (no change within 48 hours) compared to those continuing to divide / develop on each day of development (ANOVA p=0.026), shown in Figure 5.7.

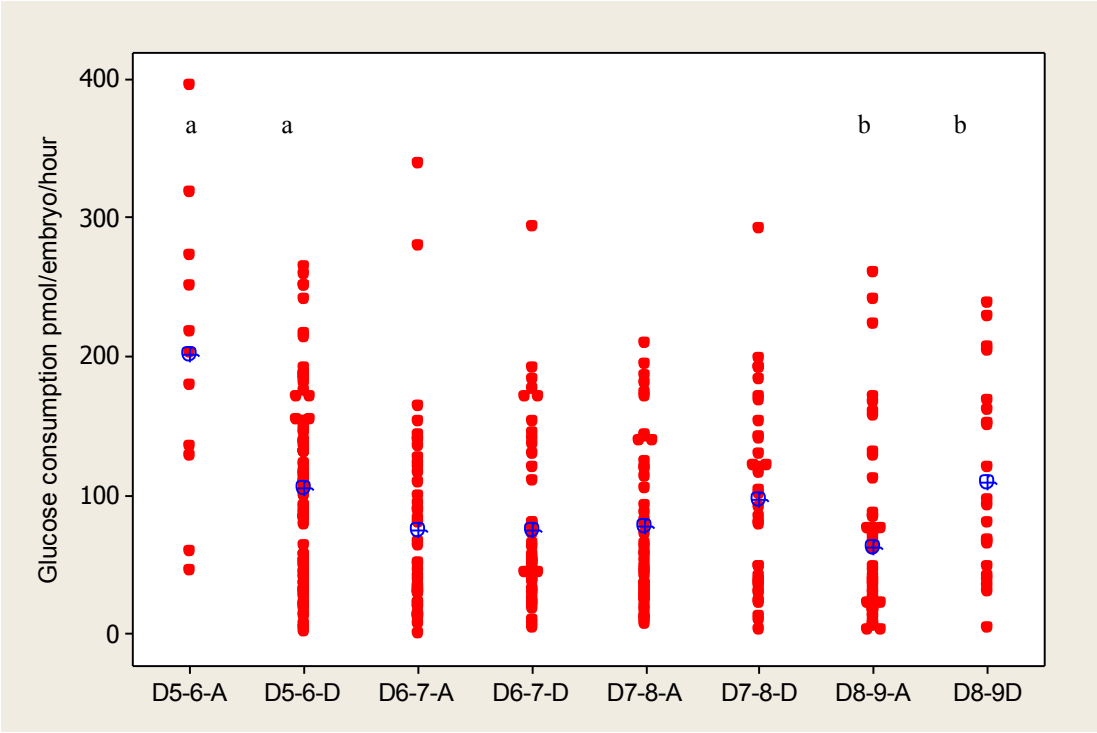


Figure 5.7 Glucose consumption for embryos that arrested (A) and those that continued to develop (D) on each day of extended culture .

Individual plots and mean values depicted. Values with the same superscript were significantly different ($p<0.05$)

Initially embryos arresting between day 5 and 6 consumed significantly more glucose than those continuing to divide (mean consumption 201 verses 105.5 pmoles/embryo/hour respectively). There were no significant differences between those developing and those arresting on days 6 to 8, however, those continuing to develop between days 8-9 consumed significantly more glucose than those arresting (109.8 compared to 63.4 pmoles/embryo/hour).

The results displayed a wide range, although variation was reduced when values were evaluated according to stage of development attained by the end of the study period. Table5.1 shows the mean values for glucose and pyruvate depletion and appearance of lactate over day 5 to 9 for embryos that arrested at each development stage. The distribution of glucose consumption was significantly different across the categories ($p=0.034$), although lactate production ($p=0.713$) and pyruvate consumption ($p=0.095$) do not significantly differ in accordance with stage of arrest.

Table 5.1 CORE profile and stage of development.

The mean glucose uptake, lactate production and pyruvate uptake from embryos (n=150) cultured between day 5 and 9, expressed as pmol/embryo/hr. The values are shown for each stage of development ±SEM, ANOVA was used to assess for differences based on the stage of development. Significant differences were recorded for glucose based on stage of development (p=0.034) Values with the same superscript were significantly different (p<0.05).

	Mean (±SEM) consumption /production in pmol/embryo /hr		
Stage attained prior to arrest	Glucose (pmol/emb/hr)	Lactate (pmol/emb/hr)	Pyruvate (pmol/emb/hr)
Cleavage cell stage (n=43)	74.96 (9.05)[b]	114.79 (15.42)	32.50 (4.18)
Morula (n=48)	99.11 (11.87)	105.00 (10.97)	32.57 (3.01)
Unexpanded blastocyst (n=11)	68.56 (8.09)[a]	77.16 (14.77)	19.48 (2.95)
Expanded blastocyst (n=22)	109.61 (14.82)	109.32 (15.87)	34.53 (5.11)
Hatched blastocyst (n=25)	116.05 (12.94)[a,b]	112.99 (16.07)	36.75 (3.76)

Embryos that arrested at the cleavage and morula stage consumed 87.7pmol/emb/hr of glucose compared to 111.5pmol/emb/hr for those embryos at the equivalent stage that continued to develop. Notably, blastocysts that failed to expand fully had the lowest rates of metabolic activity. Glucose consumption in those embryos that arrested at the unexpanded stage was 68.56pmol/emb/hr, compared to 113.3pmol/emb/hr for blastocysts that continued to develop. In addition, a significantly higher proportion of the arresting blastocysts were generated from OWOB women (p=0.003).

The development stage that an embryo had reached on day 3 (prior to donation to research- information from clinical notes) did not correlate with glucose consumption on day 5-9. Embryos which displayed a slow rate of cell progression on day 3 (<6 cells) or a rapid rate of progression (>8 cells) did not differ in their rate of glucose consumption on days 5-9 compared to those progressing at a more optimal rate of development (6-8 cells day 3- see Appendix A3). Cell number on day 3 was however, related to blastocyst development on day 5-9. The rate of blastocyst development was 18.9% from embryos with less than 6 cells, compared to 45.1% for those with more than 6 cells on day 3 (7/37 and 51/113 respectively, p<0.01). Similarly, lactate and pyruvate metabolism on day 5-9 was comparable for embryos which had reached the 6

cell stage by day 3 and for those developing at a slower rate (<6 cells on day 3 – see Appendix A3).

Glucose consumption was significantly different at different stages of blastocyst development expansion (early unexpanded to hatched i.e. stage 1-6, as shown in Table 5.1). There was however, no significant relationship between blastocyst grade (aa-ec) on day 9 and average glucose consumption from day 5-9. Kruskal-Wallis test demonstrated that the distribution of glucose was the same across categories of grade (p>0.05).

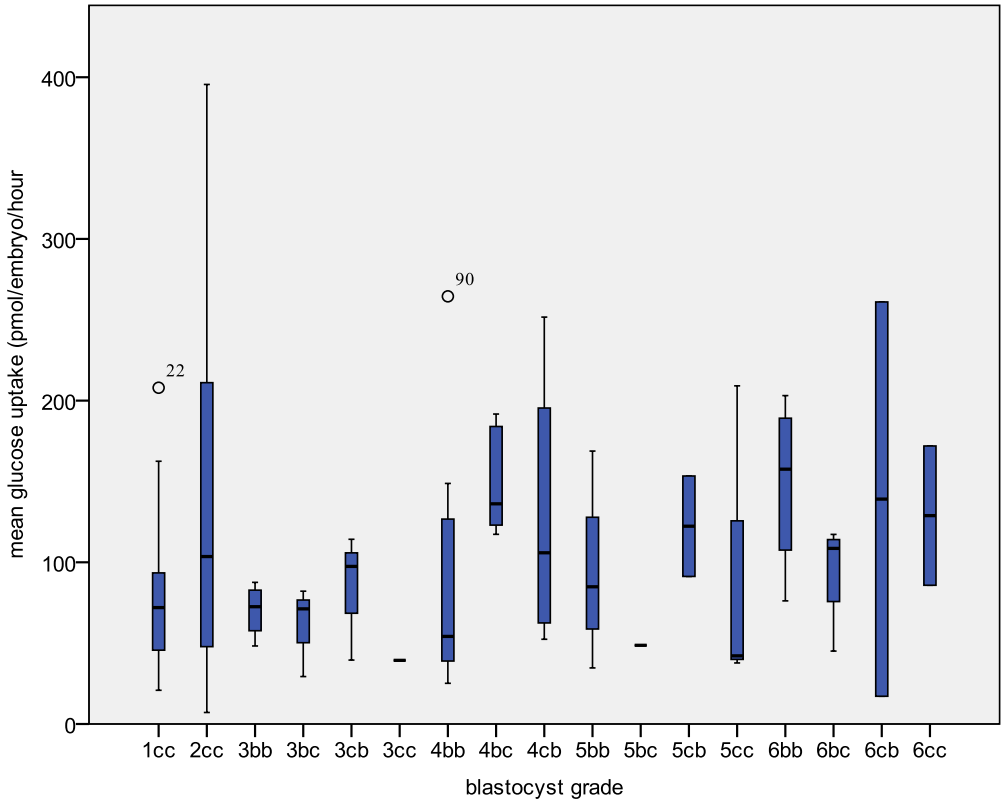


Figure 5.8 Mean glucose consumption from day 5 to 9 and blastocyst development expansion grade (1-6) and ICM (a-e) and TE grade (a-c).

Overall the results indicate that glucose consumption differs between viable developing embryos and those undergoing arrest. Those undergoing early arrest displayed above average consumption, whereas those arresting at a later stage of development had a below average consumption. The relationship between CORE profile and blastocyst development would thus indicate an optimum mid-high range of metabolism indicative of developmental viability.

5.3.2 The influence of female BMI on CORE Glucose, lactate, pyruvate embryo viability profile

The glucose and pyruvate depletion and lactate production by embryos derived from OWOB women was compared to those in the normal BMI range (Figure 5.9). Glucose uptake was significantly lower in embryos generated from OWOB women (p=0.001). The values for lactate and pyruvate did not significantly differ (p=0.259 and 0.318 respectively).

Regression analysis showed a significant negative relationship between glucose uptake and increasing BMI (ß-0.283, t-3.395, p0.001), a weak negative relationship with lactate (ß-0.097, t-1.134, p0.259) and pyruvate uptake was not significant (ß-0.082, t-1.003, p0.318).

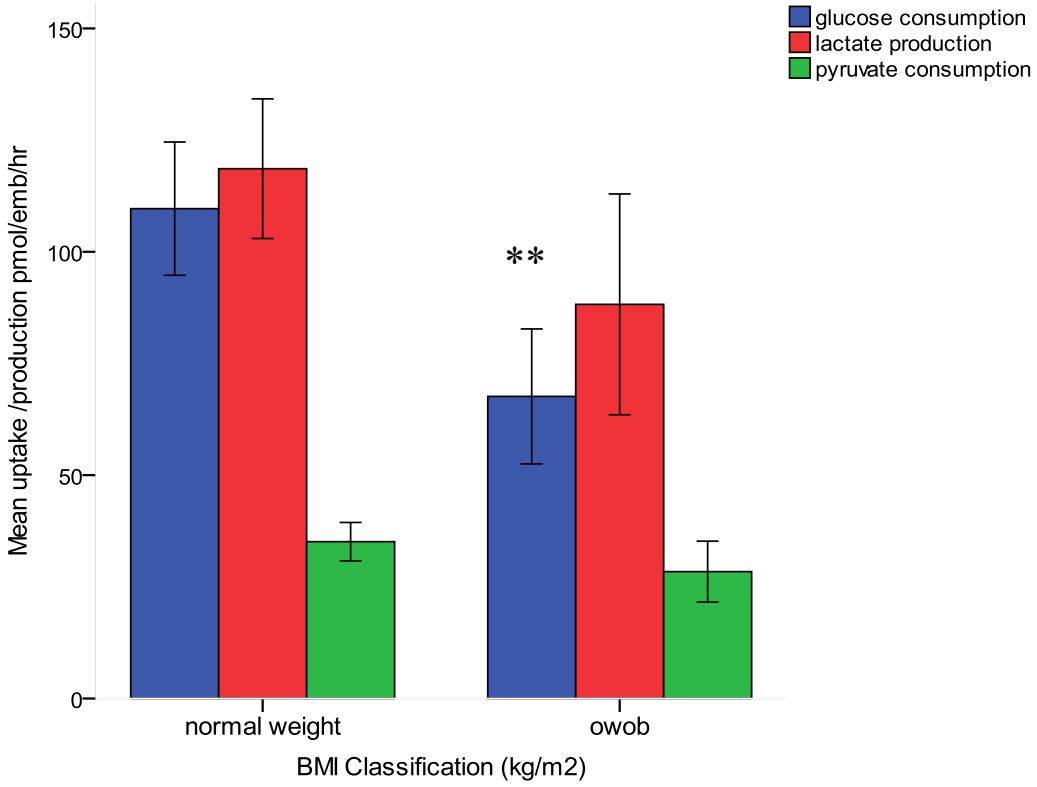


Figure 5.9 Mean embryo metabolism of substrates from day 5 to 9, according to BMI classification of female donor.

37 embryos from 7 OWOB women and 113 embryos from 22 normal weight women. Error bars represent 95% CI, ** P<0.01.

The difference in glucose consumption amongst blastocysts that were able to expand and /or hatch was significantly different in normal (n=33) and OWOB (n=14). Lower rates of glucose uptake were evident for OWOB 87.22pmol/embryo/hr, compared to 132.16pmol/embryo/hr for embryos generated from women of normal weight (p=0.03). ANOVA revealed significant differences in glucose lactate and pyruvate metabolism for normal and OWOB women at each of the different stages of development (p<0.05).

Table 5.2 CORE profile, stage of development and female BMI.

Mean glucose uptake, lactate production and pyruvate uptake from embryos (n=150) cultured between day 5-9, expressed as pmol/embryo/hr, expressed as values for each stage of development ±SD. ANOVA was used to assess for differences amongst embryos generated from OWOB and normal weight women at different stages of development arrest.

Mean (± SD) consumption / production in pmol/emb/hr	BMI	Cleavage cell stage	Morula	Unexpanded	Expanded	Hatched
Glucose	norm	78.18 (59.67)	126.58 (88.8)	80.00 (33.1)	145.79 (61.9)	117.46 (48.6)
	owob	64.40 (60.4)	49.18 (31.9) ***	64.13 (25.3)	46.38 (13.0) ***	114.50 (80.9)
Lactate	norm	104.94 (85.5)	135.16 (75.9)	76.33 (52.6)	142.21 (68.6)	98.69 (54.2)
	owob	147.60 (141.9)	50.17 (34.7) ***	77.50 (51.2)	51.50 (43.9) ***	128.67 (101.9)
Pyruvate	norm	31.39 (18.9)	37.65 (22.6)	23.00 (17.0)	41.71 (27.0)	34.54 (14.5)
	owob	36.50 (47.1)	23.29 (13.4)**	17.86 (4.7)	22.13 (9.9) **	39.42 (22.8)

*p<0.05, ** <0.01 ***<0.001

Significant differences in consumption of glucose and pyruvate and release of lactate, were recorded between embryos from normal and OWOB women at the morula stage and expanded blastocyst stage (p<0.05). Embryos from OWOB women that failed to progress beyond the morula stage consumed significantly lower amounts of glucose and pyruvate and produced less lactate. Embryos from OWOB that failed to hatch also consumed significantly lower amounts of glucose, and pyruvate and produced less lactate, than those arresting at the equivalent stage from normal weight donors.

Results were again analysed according to CORE profile on each day of extended culture, but in this instance, results were sub-classified according to female BMI. Distribution patterns did differ among normal and OWOB populations (Kolmogorov-Smirnov 2-sample 2-tail test p<0.05) and were reflective of developmental activity (see appendix A3). Glucose consumption was significantly lower in embryos generated from OWOB women from day 6 to 9 (p<0.01), however, lactate production was significantly

different between day 5-7 (p<0.05) and pyruvate consumption was significantly lower between day 6-7 only (p<0.05), compared to values from normal weight controls. Between day 5 and 6 approximately 8% of embryos arrested, by day 7 the number arrested accounted for 38% of embryos and by day 9 of culture over 75% of all embryos had arrested their development. A similar proportion of embryos generated from normal and OWOB women had hatched by day 9 (18%). Proportions are represented graphically in Figure 5.10.

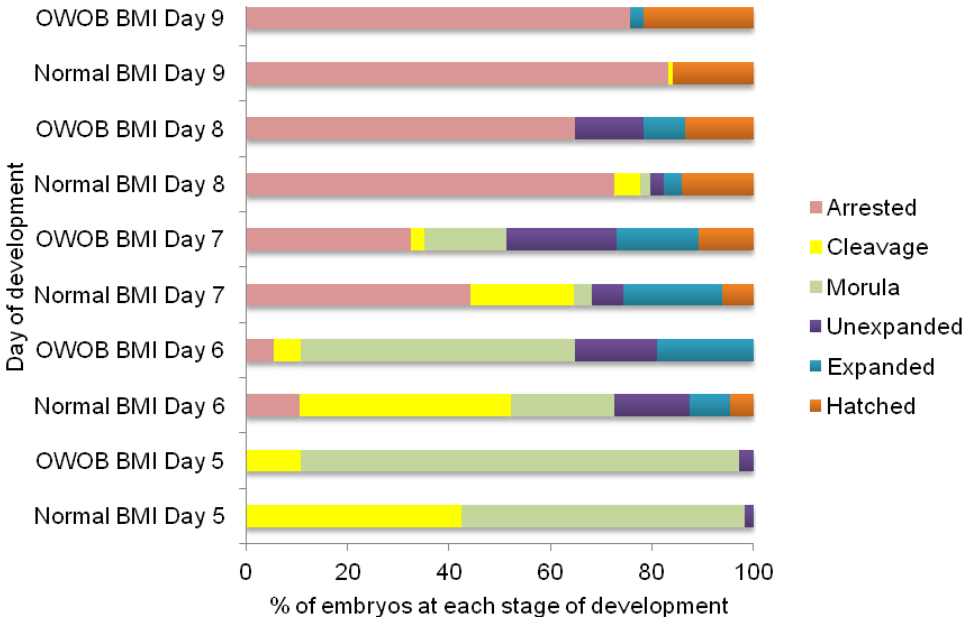


Figure 5.10 Percentage of embryos at each developmental stage at the start of culture on each day of extended culture.

The consumption of glucose was significantly different in both BMI groups for embryos which had arrested compared to those continuing to divide on each day of development (ANOVA p=0.026).

It is clear from the univariate analyses that embryos from women with a BMI in excess of 25 kg/m2 consumed significantly less glucose than embryos from women of a healthy weight at equivalent stages of development (p< 0.001), whilst there were no significant changes in pyruvate uptake and lactate formation (Figure 5.9). This pattern was consistent for each developmental stage (Table 5.3). The reduced consumption of glucose occurred without a compensatory increase in pyruvate uptake, or of glycolytic

activity as determined by lactate formation. In a multivariate analysis, developmental stage and female BMI were significant predictors of glucose uptake ($p<0.05$) and independent of male BMI, age, cause of infertility, embryo grade and day each stage was attained (see Appendix).

The specific details of the regression analysis revealed that the relationship between glucose consumption on day 5-9 and female BMI (female BMI β-0.353, t-4.049, $p<0.001$) was independent of cleavage cell stage embryo developmental kinetics (day 2 β-0.84, t-0.944, day 3 β0.116, t1.304). Average cell counts (±SEM) were 3.19±0.11 and 3.16±0.18 on day 2 and 6.47±0.15 and 6.73±0.27 on day 3 of development for normal and OWOB women respectively. Male BMI was not a significant predictive variable. Similarly the distribution of female BMI was comparable across categories of blastocysts grade.

Overall the results for this section show that glucose consumption is significantly lower in embryos generated from OWOB women compared to normal weight ($p<0.001$). In those embryos which did not undergo early stage arrest and remained viable (reached the next development stage within 48 hours), glucose consumption was significantly lower ($p<0.03$).

Glucose, lactate and pyruvate did not show a correlation with pregnancy outcome of the sibling transferred embryo, however considerable intra-patient variability was observed. This variability was reduced when the analysis was limited to include only developing sibling embryos, however no significant correlation with pregnancy was evident. Despite this, a meaningful analysis of BMI, glucose, lactate and pyruvate metabolism and pregnancy outcome is not possible with the current sample size. However, using logistic regression a predictive model $R^2$0.45, was built (see Appendix). Whilst in general embryos generated from OWOB women had significantly lower rates of glucose consumption, the glucose uptake measured for sibling blastocysts from OWOB women who attained a pregnancy (n=10) did not differ significantly to that of normal weight women (n=15) who established a pregnancy (107.02 verses 129.8pmol/embryo/hr). This suggests that uptake in the mid-range is optimal for viability (i.e. too high a consumption of glucose is also correlated with lower development / implantation potential).

5. 3.3 Embryo amino acid turnover and viability

Previous studies have shown that increased overall amino acid turnover is indicative of poor embryo quality in terms of implantation potential (Brison et al., 2004) and DNA damage (Sturmey et al., 2009), based on findings from cleavage stage embryos. The results presented here distinguish the pattern of uptake and release of amino acids at later stages of development.

It was observed that the net rates of depletion or appearance of amino acids varied between cohorts of embryos; dependent on the stage of development attained (Figure 5.11). Embryos which arrested at the earlier stages of development had a higher turnover of amino acids; specifically asparagine, aspartate, glutamate and alanine production was greater (p<0.05), compared to those that formed blastocysts. Those embryos that developed beyond the unexpanded blastocyst stage produced significantly fewer amino acids, but consumption of methionine was significantly higher in developing blastocysts, as was arginine (p<0.01) and histidine production was high in expanded and hatched blastocysts (p<0.01).

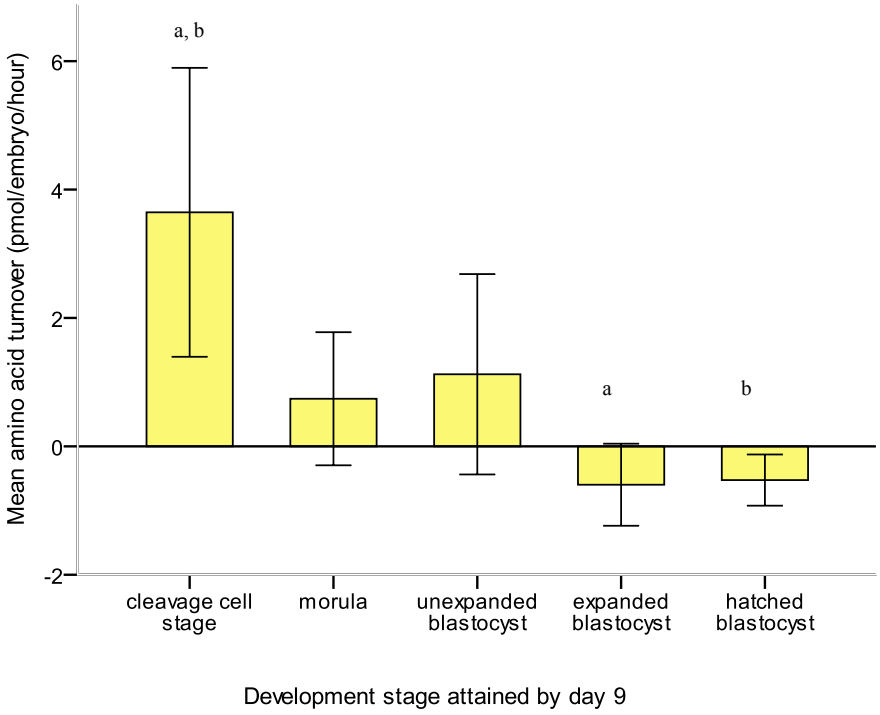


Figure 5.11 The net rates of depletion or appearance of amino acids and stage of development.

Significant differences were evident between those failing to form blastocysts and those blastocysts that failed to expand, as analysed by ANOVA p= 0.048. Error bars represent 95% CI, values with the same superscript were significantly different (p<0.05).

In terms of pregnancy outcome, the turnover of amino acids for all embryos from women achieving pregnancies was lower than in the non-pregnant group and approaching significance (p=0.057; Figure 5.12). When the analysis was limited to compare only developing blastocysts, this difference was less evident, however significant differences were observed in the production of asparagine (p=0.016) and glutamine (p=0.036), which were lower in the pregnant group; similarly the uptake of arginine (p=0.026) was lower.

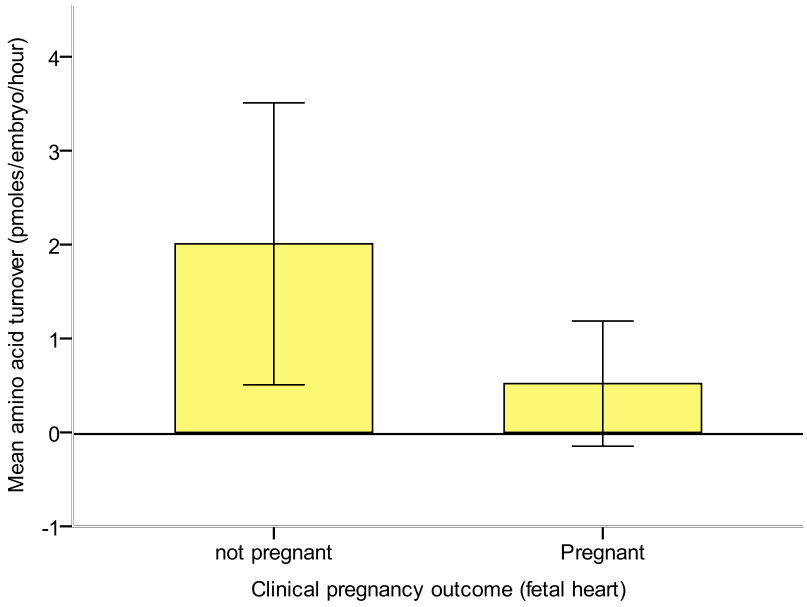


Figure 5.12 Amino acid turn over and pregnancy outcome.

Average sums of amino acid production and depletion, expressed in pmoles/embryo/hour for day 5 to 9 of culture. Error bars represent 95% CI. Results are recorded according pregnancy outcome of sibling embryos (82 embryos from 15 women who achieved a pregnancy and 68 from 29 who did not). Lower production and depletion of amino acids was evident for sibling embryos that gave rise to a pregnancy however this did not reach significance (p=0.057). Significant differences in the individual pattern of uptake and production of amino acids were evident.

5. 3.4 Embryo amino acid turnover and female BMI

When analysed according to BMI classification the difference in the sum of amino acids did not reach significance (p=0.057). However, glutamate (p=0.006), aspartate (p=0.001), asparagine (p=0.011) and tryptophan (p=0.047) appeared in higher concentrations in the medium of embryos from OWOB compared to embryos from those of normal weight. Furthermore, OWOB embryos depleted significantly more

serine (p=0.004) and glutamine (p=0.012) whereas isoleucine was less depleted by embryos from OWOB (p=0.031).

Overall, embryos from OWOB women had a more active turnover of amino acids, amino acid. Figure 5.13 shows the appearance and depletion of specific amino acids according to BMI classification, specifically, for those embryos that developed to blastocyst. There were no significant differences in the sum appearance/ disappearance rates. Only methionine was significantly more depleted from the media by embryos from OWOB women (p<0.05). This was confirmed after principal component analysis.

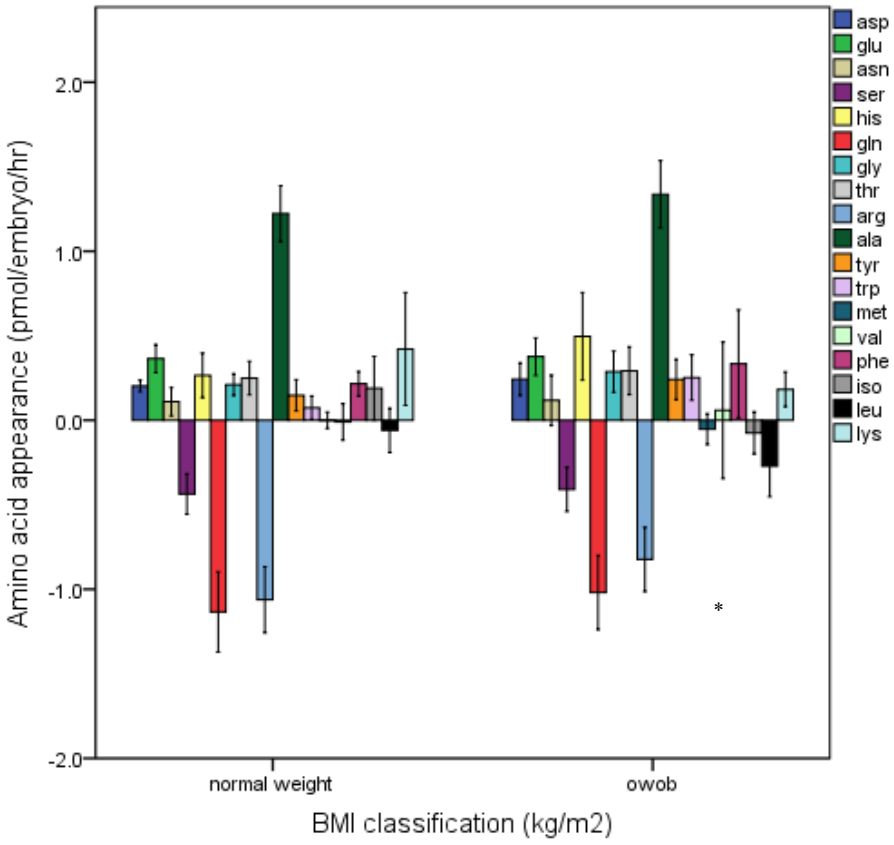


Figure 5.13 Overview of individual amino acid turnover.

Amino acid depletion and appearance by blastocysts of equivalent stage for OWOB (n=20) and normal weight women (n=27). There are no significant differences in the sum of uptake and production, however significant differences are apparent for methionine (p=0.037). Error bars represent 95% CI, *p<0.05

The data from the 18 amino acids was reduced by principal component (PC) analysis (the suitability of the data for this type of analysis was confirmed; standards met =

KMO 0.77, Bartlett's test 0.000). It is likely that differences in the consumption and release of individual amino acids maybe related, therefore prior to further multivariate testing, the variance for each of the 18 individual amino acids was used to create a smaller set of linear combinations. The data were transformed with 5 epigenvectors, accounting for 69% of the total variance. A three-component solution explains 54.9% of the data and is presented in Figure 5.14. Component 1 (PC1) accounted for 36% of the variance.

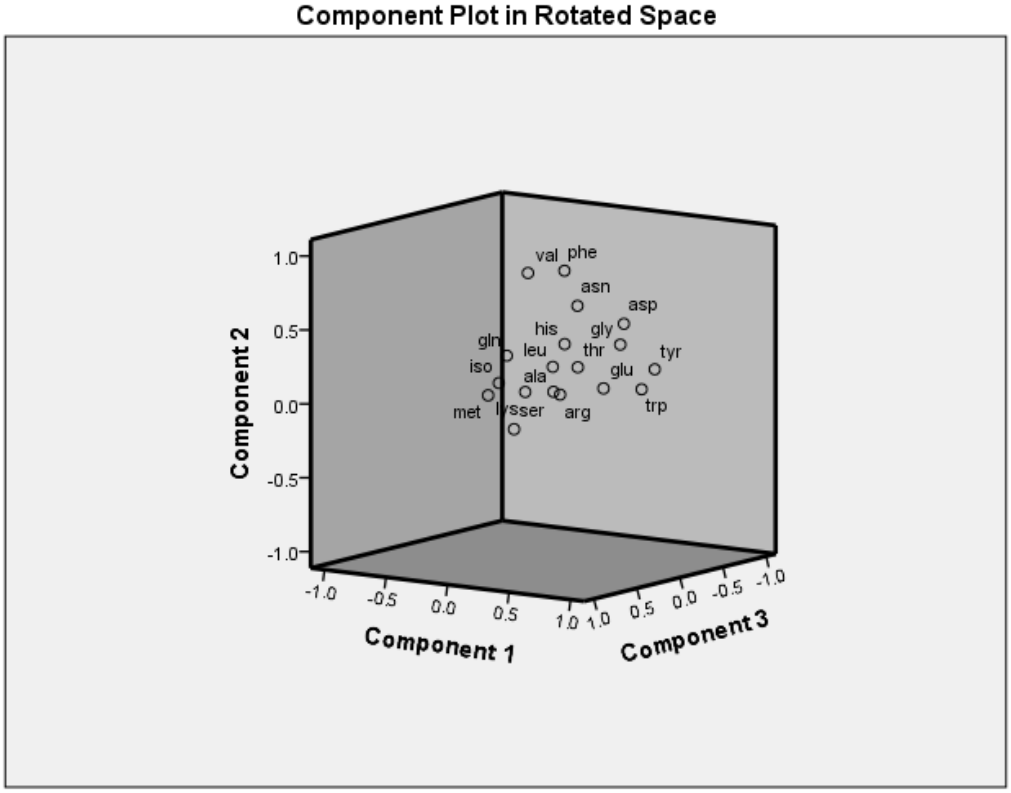


Figure 5.14 PC plot of individual amino acid CORE.

PC component plot in rotated space- rotation converged in 11 iterations (PC1; trp, glu, asp, gly, thr, gln,tyr, ser, asn, his, lys)

The majority of the amino acids included in PC1 are neutral and polar and appear to belong to system N transporters. After the PC adjustment for covariation, a permutated-based adjustment of p-values was undertaken – only methionine depletion significantly differed (p<0.05) between developing blastocysts generated from OWOB women and those with a BMI <24.9Kg/m^2.

5. 3.5 Embryo triglyceride content, viability and female BMI

Figure 5.15 shows that human embryos that arrested contained significantly more triglycerides than those that completed development (11.32 versus 6.7ng; $p<0.001$).

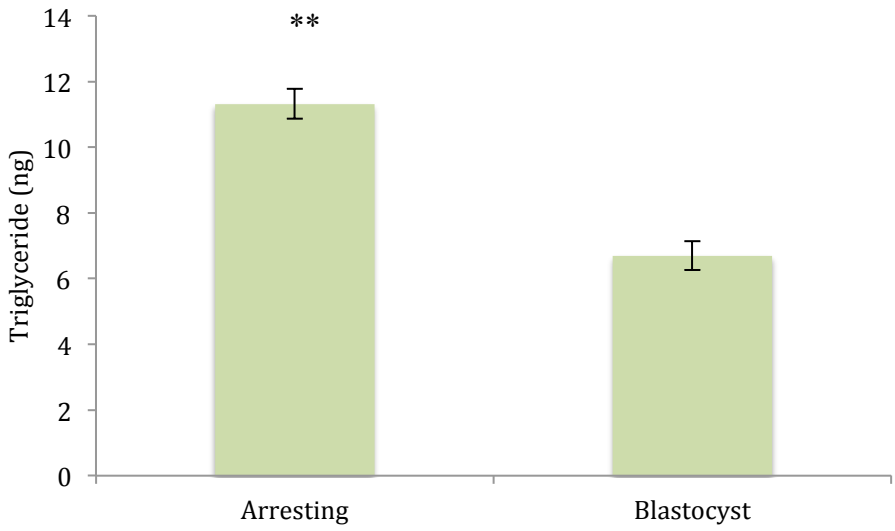


Figure 5.15 Triglyceride content of embryos arresting prior to blastocyst formation and for those capable of forming blastocysts.

95% CI, **$p<0.01$

In light of this finding, results were compared for the pooled samples (groups of 2 to 5 embryos at equivalent stages for each patient) from each patient that achieved a pregnancy from a transferred sibling embryo and those failing to achieve a clinical pregnancy. The data showed a trend for lower triglyceride composition in sibling embryos of those that successfully implanted ($p=0.079$).

In addition, it was observed that Day 9 blastocysts from women with a BMI ≥ 25 kg/m2 contained significantly more triglycerides than comparable embryos from women with a BMI< 24.9 kg/m2 (Figure 5.16; t= 4.11, $p< 0.001$).

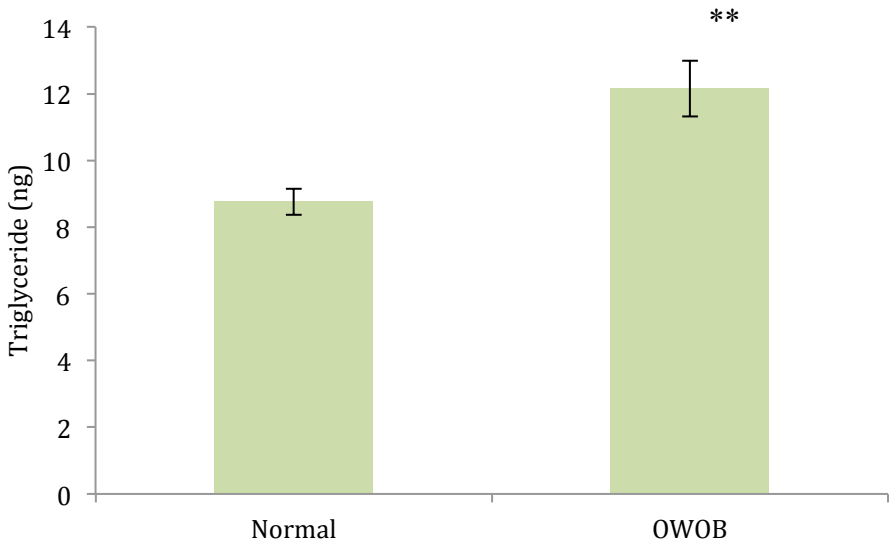


Figure 5.16 Triglyceride content of embryos attained from normal and OWOB women.

95% CI, $^{**}p<0.01$

5.3.6 The maternal verses paternal influence on metabolic profile

To investigate the influence of male BMI, the data have been analysed according to male BMI classification. When stratified in this manner 81 embryos originated from OWOB males and 69 from those of normal weight.

The data in Table 5.3 show that there was no significant effect with male BMI and CORE profile.

Table 5.3 CORE profile and male BMI.

There were no significant differences in the mean glucose, lactate and pyruvate metabolism between days 5 and 9 of culture, for embryos grouped according to the male partner BMI.

	Male	Mean (± SD) consumption / production in pmol/emb/hr	*p*-value
Glucose	norm	101.57 (59.69)	0.279
	owob	89.50 (75.29)	
Lactate	norm	107.88 (70.60)	0.987
	owob	107.66 (89.95)	
Pyruvate	norm	34.94 (19.55)	0.301
	owob	31.16 (24.52)	

A regression analysis, mixed linear model (with female BMI as a fixed effect) showed a weak negative relationship between glucose uptake and increasing male BMI (β-.060, t-.678, p0.499) and weak relationship with lactate (β.070, t.778, p0.438) and pyruvate (β.035, t.407, p0.685). This supports the earlier model findings using female BMI as the primary denominator.

Obesity was more prevalent in the male partner. 45 embryos were generated from discordant male female weight pairings in 40 cases this was due to the pairing of an OWOB male with a normal weight female. ANOVA revealed significant differences in glucose consumption amongst the 4 pairing possibilities i.e. both partners normal weight (n=55 embryos), both OWOB (n=50), male OWOB and normal weight female (n=40), female OWOB and normal weight male (n=5) ($p<0.05$, Figure 5.17).

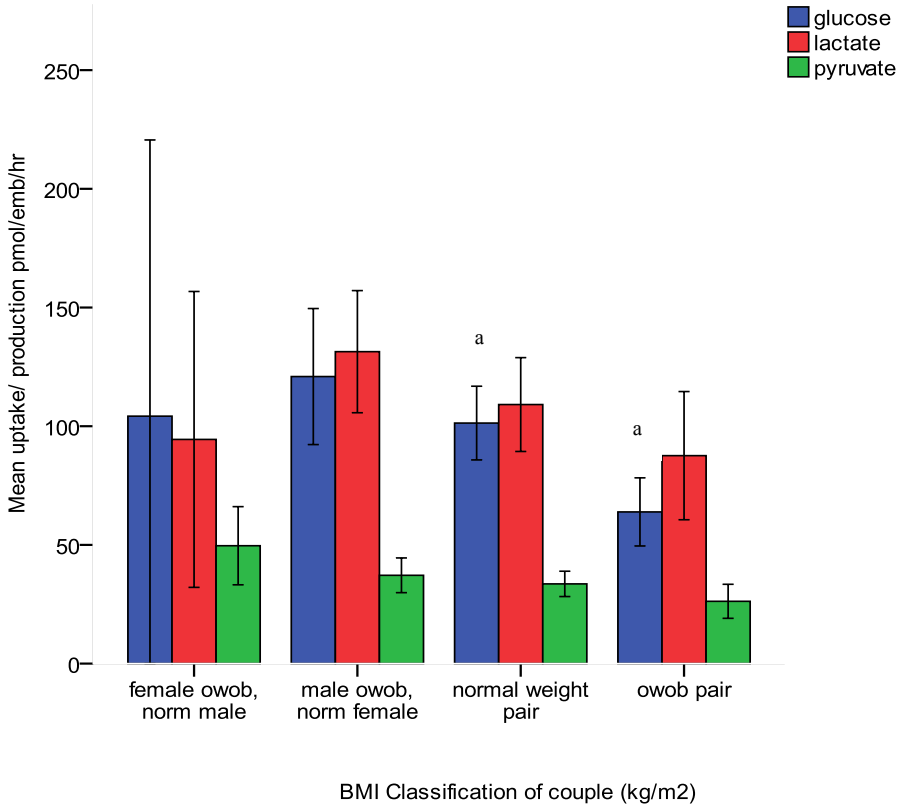


Figure 5.17 Mean core profile day 5-9, according parental BMI classification.

105 embryos from weight matched couples and 45 from weight discordant couples. Error bars represent 95% CI, values with the same superscript are significantly different ($p<0.05$).

Glucose consumption was significantly lower in embryos generated from OWOB pairings. Higher lactate production was linked to male BMI- each unit increase in male

BMI equates to an increase in production of 0.070 pmol/emb/hr, compared to a drop of 0.095 pmol/emb/hr for each unit increase in female BMI. In contrast, increasing female BMI has less of an impact on pyruvate consumption than increasing male BMI. The values for lactate and pyruvate are not significant. The standard deviation is high due to the low sample size (n=5) in the OWOB female and normal weight male.

The strongest evidence that the metabolic results attained had primarily a maternal origin came from the analysis of data using donor sperm. In the course of the study, fortuitously a single male sperm donor had been used to fertilize oocytes from six women, all of whom had a different BMI. With the male factor controlled in this way, it was possible to confirm the results of the multivariate analysis, which suggested that the differences in embryo glucose consumption were independent of male BMI (Figure 5.18).

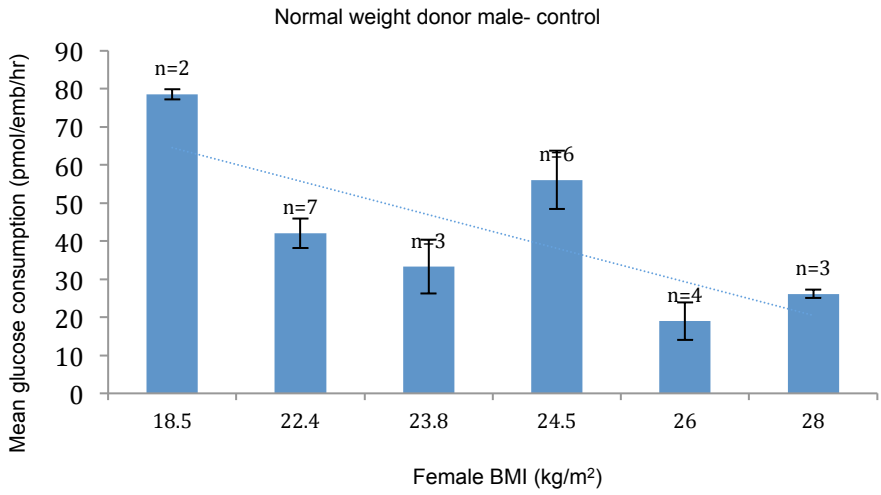


Figure 5.18 Glucose consumption in embryos generated from a single male donor.

The glucose consumption of blastocysts is inversely correlated to female BMI (R^2-0.61; p<0.001) and relates to maternal BMI with little paternal influence. The oocytes in this figure were all fertilized by the same sperm donor, yet reduced glucose consumption was apparent in embryos from OWOB women (12 embryos from 3 women of a normal BMI and 13embryos from 3 OWOB women; see Figure 5.3 for details. Data are expressed as mean ±SEM.

It was noted that despite no significant differences in the proportion of ICSI or IVF treatments performed in the groupings based on female BMI, if male BMI is used as the denominator, a significantly higher proportion of cases involve ICSI in the OWOB male group although not significant if assessed as a continuous variable p=0.07; Figure 5.4. As previous studies had demonstrated, *in vitro* protocols may alter developmental

progress and change gene expression and metabolic profiles (Bell et al., 2008). It would be prudent to evaluate this in further detail.

Table 5.4 shows that uptake of glucose and pyruvate and production of lactate did not differ according to the method of insemination, Values are based on average concentrations from day 5 to 9 (p>0.05).

Table 5.4 ICSI and IVF embryos mean glucose and pyruvate uptake and lactate production between days 5 and 9 of culture, expressed as pmol/hr/embryo(±SEM).

CORE pmol/emb/hr	Glucose	Lactate	Pyruvate
ICSI	83.11 (7.88)	107.70 (10.77)	31.17 (3.48)
IVF	101.60 (7.84)	107.78 (8.74)	33.63 (2.1)

Data were also analysed according to amino acid turnover, Figure 5.19 shows that ICSI embryos produced significantly more amino acids than IVF generated embryos (p=0.04). The specific amino acids which were produced in higher quantities from ICSI embryos were; aspartate (p=0.016), asparagine (p=0.04), histidine (p=0.021), threonine (p=0.009). The consumption of leucine was significantly lower (p= 0.04) in ICSI embryos.

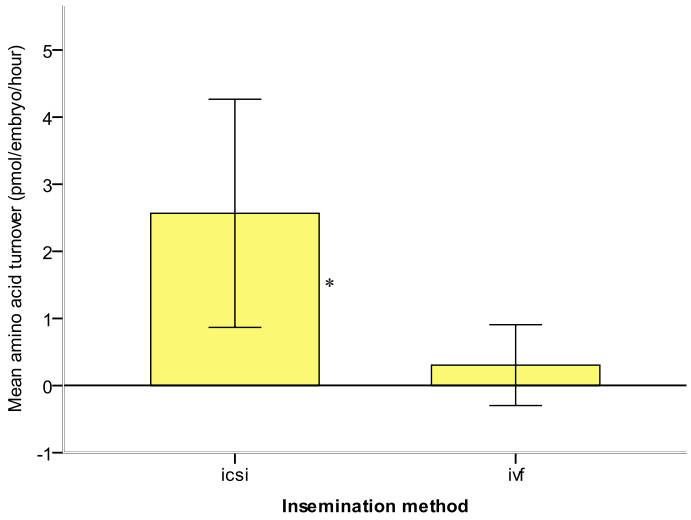


Figure 5.19 Average sums of amino acid production and depletion, according to treatment type.

Data expressed in pmoles/embryo/hour for day 5 to 9 of culture. Significant lower production and depletion of amino acids was evident for IVF embryos, compared to ICSI. Error bars represent 95% CI, *p<0.05.

To explore the possible impact the injection procedure could have on subsequent embryo metabolism and to ascertain if this was a patient specific effect and perhaps reflective of other covariate factors, data were reviewed from an additional subgroup of patients (identified throughout the course of the research study- see figure 5.1 for details), in whom semen parameters had been borderline normal. These patients received a split IVF / ICSI treatment cycle; i.e. half of their oocytes were fertilised by IVF and half by ICSI (n=50 embryos). Individual and collective differences in amino acids were not apparent for sibling embryos created by either technique (Figure 5.20). Interestingly, amino acid turnover was higher for IVF embryos patients-who had borderline suboptimal semen parameters compared to embryos generated via IVF patients, who had normal semen parameters (0.30 verses 0.83 p/mol/embryo/hr respectively) and conversely amino acid turnover was lower for ICSI embryos from those with borderline suboptimal semen evaluation compared to abnormal semen evaluation.

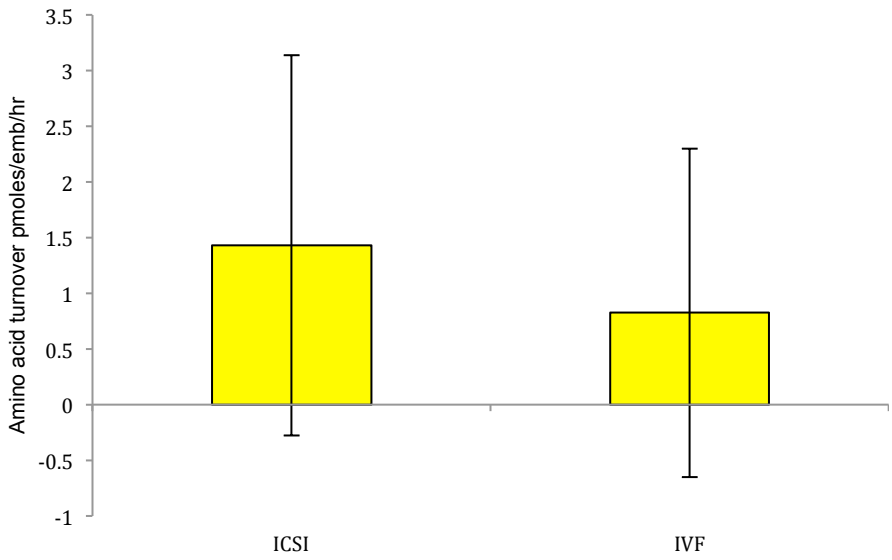


Figure 5.20 Amino acid turn over for sibling embryos, split treatment cycles.

Average sums of amino acid production and depletion, day5-9; comparable for sibling embryos (p=0.39).

Furthermore, blastocyst development rates were comparable (p=0.11), as were rates of uptake and production of substrates from the culture medium. There were no significant difference in glucose (IVF 69.31 versus ICSI 54.45 pmol/embryo/hr p=0.47), lactate (p=0.75) and pyruvate (p=0.93) for IVF and ICSI embryos. Similarly there were no significant differences in the triglyceride content of ICSI and IVF sibling embryos.

5.3.7 Results overview and live birth data

Numerous variables have been reviewed in the course of this chapter. The key findings and how they maybe pieced together will be reviewed in the discussion; however clear observations from this study have been as follows;

1. Glucose consumption is significantly lower in embryos generated from OWOB women compared to those of normal weight.
2. The triglyceride content of embryos from OWOB women was significantly higher than that of normal weight women.
3. Glucose consumption was higher in embryos capable of forming developmentally viable expanded / hatched blastocysts and triglyceride levels were significantly lower amongst blastocysts compared to those arresting at earlier stages of development.

To relate these three dimensions of data a bubble chart has been plotted (Figure 5.21). Blastocyst development ranged from 0-100% amongst cohorts of embryos and complete failure of any embryos within the cohort to reach the blastocyst stage was more common among OWOB patients. However, in cohorts homogenous for development; blastocysts of OWOB consumed less glucose and had higher triglyceride levels. Those from normal weight women forming blastocysts had mid-high glucose consumption and mid-low triglyceride levels.

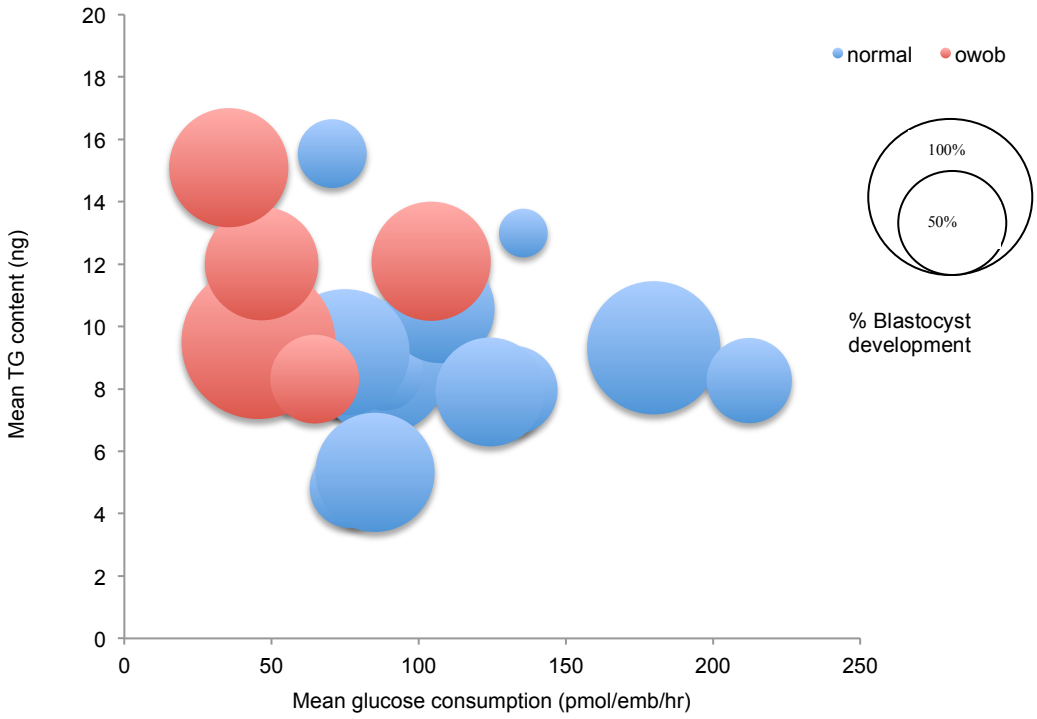


Figure 5.21 Comparison of significant entities influenced by female BMI.

Comparisons plotted in terms of their relative positions with respect to each numeric axis (mean glucose consumption, mean triglyceride content) and size (% blastocyst formation rate) for cohorts of embryos from the 7 OWOB women and 22 normal weight women

In chapter 4 it was established that blastocysts from OWOB women had significantly lower cell counts than those from normal weight women. CORE values were established for these embryos from day 5-7 prior to staining (see Figure 5.1 for study group details). This analysis was undertaken to attempt to clarify if the lower glucose consumption could relate to lower cell numbers. Figure 5.22 and analysis via multiple regression, general linear modelling (R^2 0.08) shows this does not appear to be the case for either total cell count (F 1.053, p=0.56) or covariates; inner cell mass (t-0.85, p=0.4) and trophectoderm (t1.67, p=0.11). BMI remains a significant predictor of glucose (p=0.03) and cell counts (p=0.01); confirmation via Mann Whitney U test that distribution of results for both of these parameters is different in the OWOB population compared to that of the normal weight grouping.

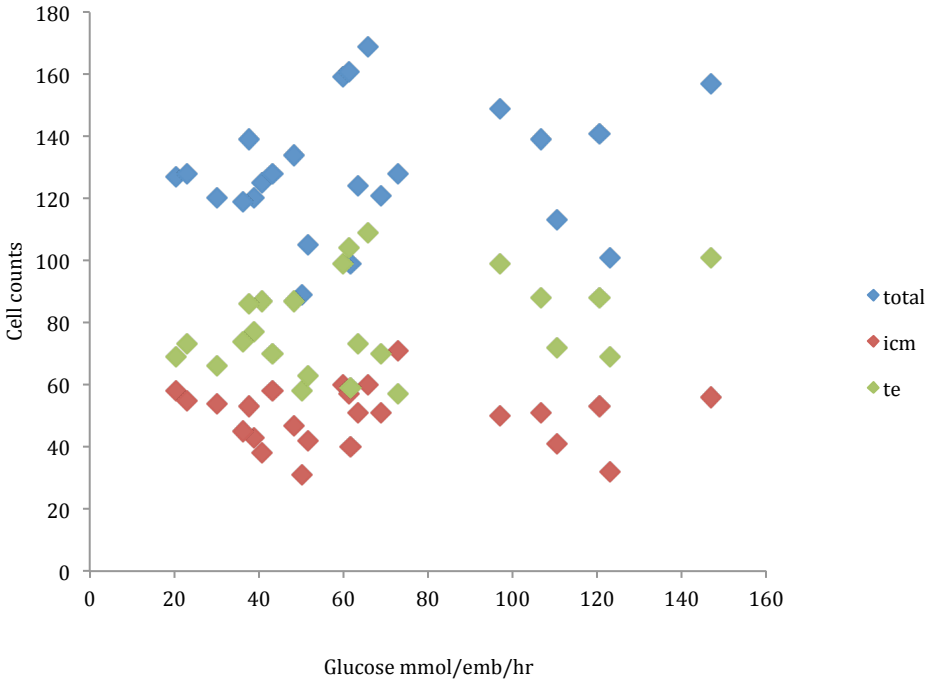


Figure 5.22 Blastocyst cell count and glucose consumption.

Blastocyst cell count (total, or differential) is not a predictor of glucose consumption (R^2 0.08, p>0.05). Data reviewed from CORE profile of 44 embryos from 16 patients.

The data reviewed in chapter 3 implied that clinical pregnancy rates tended to be lower and rates of miscarriage higher among OWOB women compared to those of normal BMI. This did however result in significantly lower live birth rates and data reviewed for live birth rates after DZT and ovum donation implied that fetal growth and development may be influenced by both genetics and the environment. The results from this chapter add to this;

- Clinical pregnancy rates were not significantly different among groupings; 50% (11/22) in the normal weight group and 57% (4/7) in the OWOB group.
- Correlations between metabolic biomarkers and establishment of clinical pregnancy were poor, but as a measure of viability metabolic biomarkers provided information about developmental potential.
- The metabolic effects were mediated via maternal BMI as appose to male BMI and were not significantly influenced by in-vitro handling techniques/ environment.

To determine if metabolic differences observed at the embryonic level were affecting clinical outcome at a later stage, such as fetal growth, live birth data for the two groups of women were reviewed and are displayed in Figure 5.23. Babies born to OWOB

women were significantly heavier (p=0.04) than those born to women of normal weight; mean (±SEM) of 3656.3 (173.9) compared to 3309 (75.8) grams respectively. There were no significant differences in number of weeks gestation (p=0.28) or proportion of male to female infants. The majority of babies born were however, considered appropriate weight for gestational age, with the exception of one large for gestational age baby born to an OWOB mother.

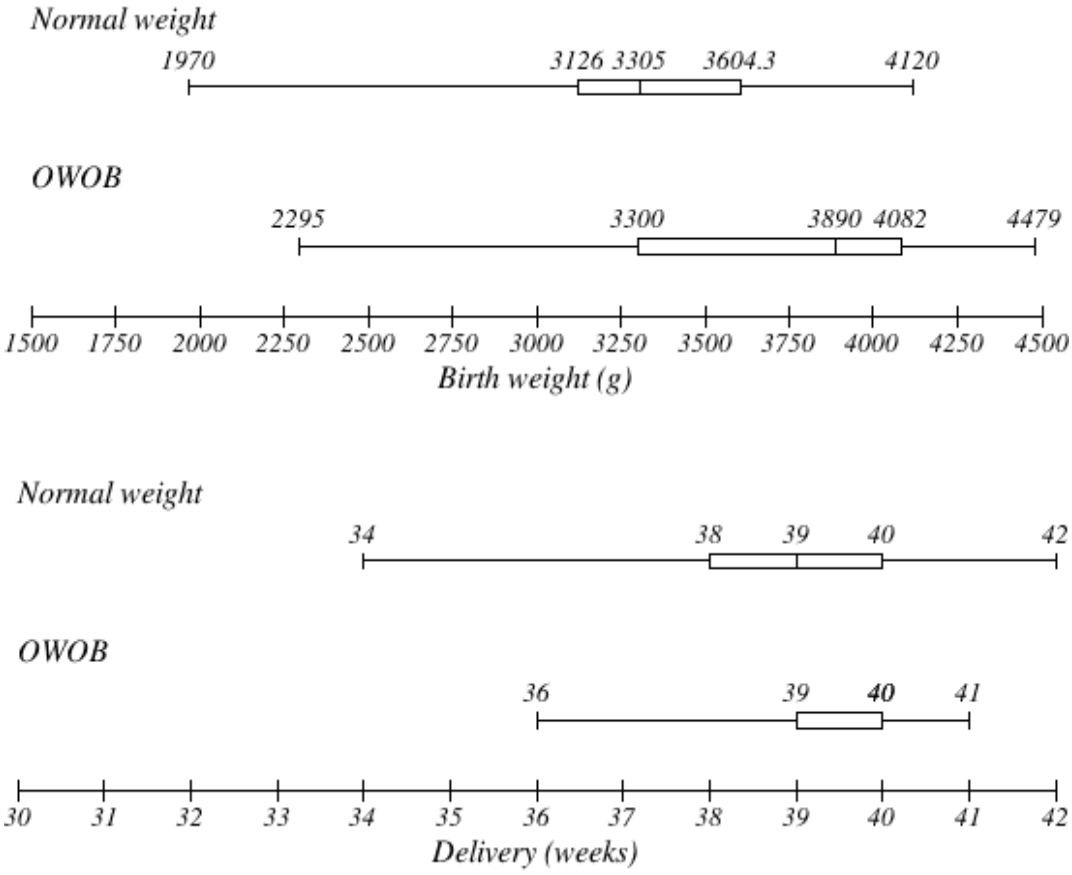


Figure 5.23 Birth outcomes of infants born to normal and OWOB women.

Babies were significantly heavier for stage from OWOB mothers.

To discover potential associations between birth weights and metabolic profile of non-transferred sibling embryos, plots of (a) birth weight and sibling glucose consumption (b) birth weight and sibling triglyceride content, were constructed and compared with those of (c) maternal BMI for those achieving a live birth and sibling embryo glucose consumption and (f) triglyceride content. Data is displayed in Figure 5.24.

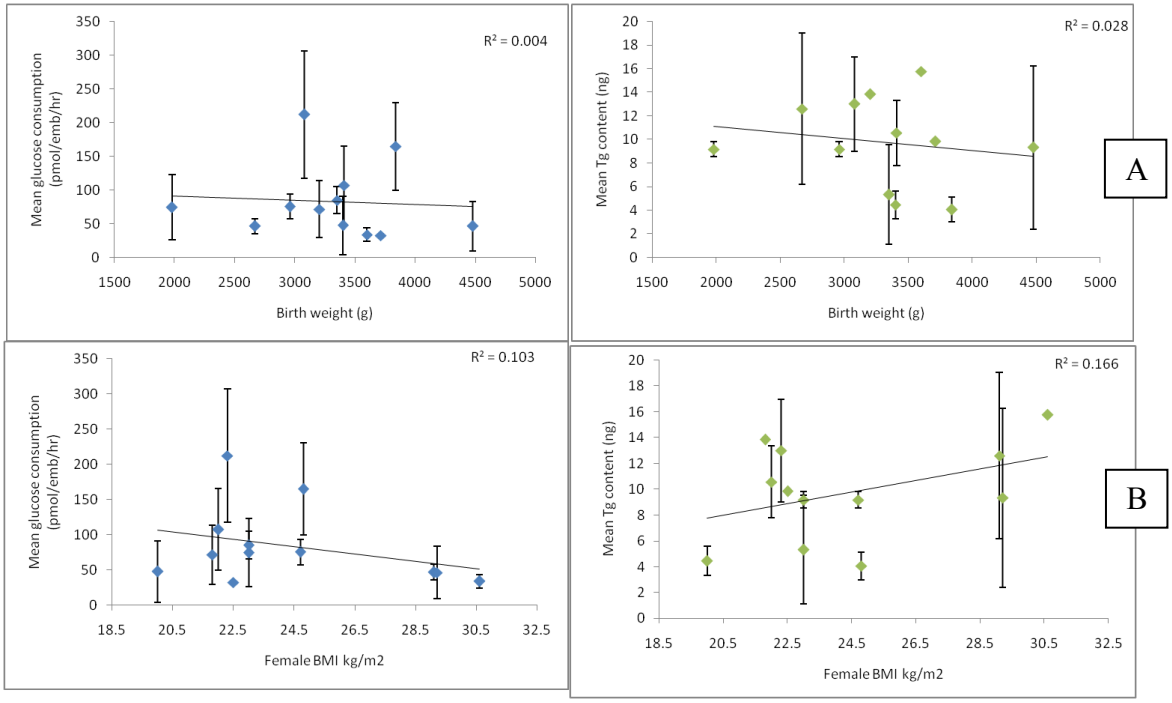


Figure 5.24 Data displayed for women achieving a live birth outcome. Metabolic data for sibling embryos was plotted against (A) birth weight and (B) female BMI.

No significant relationships were established when the patient was used as the denominator. The error bars depict the intra patient variability. The majority of the trends do follow those established as significant amongst individual embryos. The broad-spectrum findings were that;

- Increasing maternal BMI was associated with lower glucose consumption at the pre-implantation stage and higher birth weight infants.
- Increasing maternal BMI was associated with higher measured triglyceride content at the pre-implantation stage, although contrary to this and somewhat unexpectedly, lower sibling triglyceride levels were associated with higher birth weight infants.

5.4 Discussion

5.4.1 Summary of key findings

The data presented here provide strong evidence to support the hypothesis that embryo viability is compromised in OWOB women. The most striking findings were that embryos from OWOB women showed significant metabolic abnormalities, with diminished glucose consumption, altered profile of amino acid metabolism and an increased endogenous triglyceride content. The data provide a link between maternal nutrition, the periconceptional environment, oocyte and preimplantation developmental competence and embryo metabolism, which could have long-term health implications for the offspring.

5.4.2 Metabolic biomarkers, as a measure of embryo viability

As a measure of embryo viability the metabolic biomarkers measured in the course of this study provided strong predictors about an embryo's developmental potential. As outlined in previous studies the association between metabolic activity and viability appears to be independent of embryo morphology (Brison et al., 2004; Gardner et al., 2011). Blastocyst formation, expansion and hatching were observed as key developmental milestones and the attainment of such status rather than the point at which this occurred (day 6, 7 or 8-9), that most influenced metabolic viability. Equally, the stage at which an embryo arrests was closely correlated with its prior metabolism. Early arresting embryos, still at the cleavage stage, displayed a high CORE profile and high triglyceride levels; those arresting at later stages displayed lower uptake of metabolites, as observed for those capable of forming blastocysts, but not competent to undergo expansion. The results for glucose, pyruvate consumption and lactate production from embryos on days 5-9 of development were widely distributed, though were suggestive of an optimum mid-range of activity, in agreement with the findings in embryos, 24 hours following fertilisation, reported by Turner et al., (1994).

Throughout development, there appear to be specific upper and lower optimum values for metabolite consumption and release that define metabolic normality, and as indicated here; embryos displaying metabolic activity outside these ranges may have impaired developmental potential. A 'mid-range' of metabolic activity is suggestive of optimum viability (Gardner et al., 2011; Guerif et al., 2013). Metabolic quiescence seems to be responsible for developmental arrest and delayed implantation (Nilsson et

al., 1982). It has been postulated that too little energy production may result in reduced synthesis of macromolecules or on the contrary, that reduced demand for energy maybe as a result of decreased macromolecule synthesis (Weitlauf & Nieder, 1984). Similarly high metabolic activity has been associated with embryonic arrest, cellular stress and DNA damage, as embryos need to consume higher quantities of substrates, presumably in order to carry out repair processes, compared with those that continue development (Sturmey et al., 2009). Thus, cleavage stage embryos that exhibit a 'quiet' metabolism (Leese, 2002; Leese et al., 2007, 2008), are believed to be more viable. Later in preimplantation development, it has been suggested that, as the capacity to metabolise glucose increases significantly between the morula and blastocyst stages (Devreker et al.,2000), embryos that double their glucose consumption are more likely to form blastocysts than those where glucose consumption remains unchanged (Gardner et al., 2011).

5.4.3 Glucose consumption

In broad terms, the data reported in this chapter on consumption of glucose by single human blastocysts are consistent with those previously reported (Hardy et al., 1989, Gardner et al., 2011). However, blastocysts from OWOB patients consumed significantly less glucose than equivalent embryos from women with a normal BMI. A diminished capacity to metabolize glucose maybe profound since a deviation from what appears to be an evolutionarily conserved metabolic phenotype of increased glucose consumption (Smith & Sturmey, 2013) during blastocyst formation, suggests some degree of metabolic remodelling in the blastocysts derived from oocytes collected form OWOB women. It is proposed that this relates back to the follicular conditions to which oocytes from OWOB women are exposed to prior to fertilisation. Robker et al.,(2009) have shown that the follicle of overweight women is enriched for glucose while Moley et al.,(1998) reported in a mouse model, that elevated follicular glucose leads to alterations in the expression of glycolytic enzymes and the appearance of functional defects in mitochondria.

Embryos from OWOB women consumed significantly less glucose than embryos from women of a healthy weight at all equivalent stages of development, most notably at the points of compaction and expansion. The reduced consumption of glucose occurred without a compensatory increase in pyruvate uptake, or lactate formation and this

pattern appears to mirror data from animal models of nutritional enrichment (Van Hoeck et al., 2011).

A ratio of glucose consumed to lactate produced of 1:2 implies that all the glucose may be accountable by lactate appearance via glycolysis. In the present study the ratio of lactate production was lower than expected in both groups such that glycolytic activity did not significantly differ for normal and OWOB groupings (mean-60.5%). This is supported by findings of Robker et al., 2009 and Binder et al., 2011. As the rate of glycolysis was unchanged, the lower utilisation of glucose by OWOB may be as a consequence of reduced uptake, possibly due to reduced expression of GLUT transporters. Differences between GLUT-1 expression and glucose uptake have been reported in bovine embryos and positively correlated to glucose uptake (Garcia-Herreros et al., 2012), however this has not previously been studied in relation to maternal BMI.

A lower oxidative utilisation of glucose, due to reduced capacity for oxidative phosphorylation is one possibility for the lower rates of glucose consumption among embryos originated from OWOB women – but glycolysis was the same in both groups (60%). One explanation for this might be a reliance on fatty acids to support ATP synthesis. Randle et al.,(1963) proposed a mechanism by which fatty acids may inhibit glycolysis. From the oocyte studies in chapter 4, it was suggested that the oocytes from OWOB, produced in a hyperlipid environment, have higher amounts of triglyceride, this was proven to be the case here in embryos. The fatty acid composition of high and low quality oocytes is reported to differ, lower quality oocytes containing more saturated fatty acids (Aardema et al., 2011), the metabolism of saturated fatty acids being linked to higher rates of developmental arrest. A consequence of the higher fatty acid composition could be higher levels of beta-oxidation. According to Randle's hypothesis this leads to increased levels of acetyl co A and citrate. Accumulation of citrate inhibits phosphofructokinase, leading to accumulation of glucose-6-phosphate, decreased hexokinase activity and ultimately less glucose metabolism and in turn, uptake (mechanism reviewed by Hue et al., 2009). In support of this theory are the findings of Van Hoeck et al (2011), who reported that bovine oocytes matured in medium containing high concentrations of non-esterified fatty acids, had lower glucose consumption than those cultured under control conditions.

The proposition of increased fatty acid oxidation is also in-line with the reports of mitochondrial dysfunction in oocytes from insulin resistant rodent models (discussed in chapter 4; work of Igosheva et al., 2010; Wang et al., 2009). Compromised mitochondrial function and substrate transition are likely to impair the viability of embryos generated from OWOB. Figure 5.25, illustrates how these proposals may be linked.

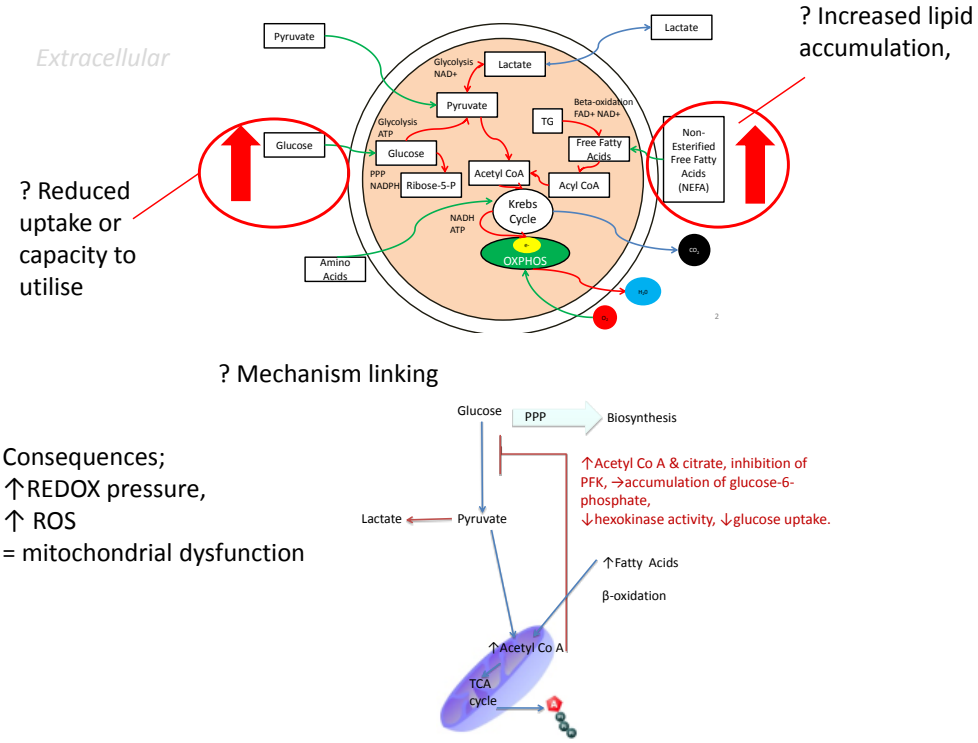


Figure 5.25 Possible mechanism linking lower glucose consumption and higher triglyceride content of embryos from OWOB women (Figure adapted from McKeegan, 2015 and Smith & Sturmey 2013).

5.4.4 Utilisation of endogenous triglyceride stores

This is the first quantitative measure of the triglyceride content in human blastocysts, which was significantly lower than that observed in the domestic species (Ferguson and Leese, 1999; Sturmey & Leese, 2003; Sturmey et al., 2009). Total endogenous triglyceride concentrations were lower in embryos that successfully developed to the blastocyst stage. Moreover blastocysts derived from oocytes of overweight and obese patients contained significantly elevated levels of endogenous triglyceride. It is unlikely that de novo synthesis of fatty acid occurs in the embryo, although this cannot be discounted; it is more likely that oocytes present in the lipid-rich follicles of overweight and obese women accumulate triglycerides from the surrounding environment as reported by Aardema et al. (2011) and Ferguson and Leese (1999) for domestic species.

Since all embryos were cultured in equivalent conditions *in vitro*, the apparent retention of triglyceride observed in embryos originating from OWOB women most likely originates from the period of oocyte development.

Depletion of endogenous lipid stores are evident during maturation; triglyceride stores are reduced in bovine and porcine oocytes (Ferguson & Leese, 1999; Sturmey & Leese, 2003) and lipase activityis increased (Cetica et al., 2002), likewise it is reported that beta-oxidation increases in cumulus-oocyte-complexes, (Dunning et al., 2010). Activity then remains constant, until the blastocyst stage, when there is a 5-fold increase in beta-oxidation (Hillman & Flynn, 1980). When beta-oxidation is inhibited, oocyte developmental competence is reduced (Ferguson & Leese, 2006; Sturmey et al., 2006; Dunning et al., 2010), as is blastocyst development (Hewitson et al., 1996; Ferguson and Leese, 2006; Dunning et al., 2010). This bovine/porcine data support the theory that endogenous lipid stores are being utilized during development and suggests that triglyceride is being broken down into acetyl units by beta-oxidation and utilised as a source of energy.

It can be implied from the animal data and from the hitherto overlooked aspect of human embryo metabolism of endogenous energy stores that oocyte triglyceride levels are reflective of the prior environmental conditions and an optimum level of accumulation is required for viability. Exposure to high levels of lipid in the follicle may result in lipotoxicity (Wu et al., 2011); studies in mice show that dietary induced obesity results in embryos with a higher lipid content than normal weight controls and evidence of endoplasmic reticulum stress, mitochondrial disfunction and apoptosis. The exposure of bovine oocytes to a high-fat environment during final maturation reduces embryo viability post fertilisation, changes the expression of key metabolic genes and modifies metabolic activity in the resulting blastocysts (Van Hoeck et al., 2011). Increased lipid accumulation has been shown in the embryos of high yielding diary cows (Leroy et al., 2005) and also in the embryos of diabetic rats (Sinner et al., 2003). In each model energy and lipid metabolism is disturbed, indicating a mechanism by which obesity may impact on embryo viability.

5.4.5 Amino acid turnover

The measurement of turnover of a variety of amino acids may provide information that can be combined to form an index that can serve as a measure of embryo viability

(Brison et al., 2004). Accordingly, here it has been shown that measurements of amino acid metabolism have provided a more convincing association between blastocyst development and pregnancy outcome than those of glucose and pyruvate. Houghton et al., (2002) reported that blastocyst formation could be predicted by alanine, arginine, glutamine, methionine and asparagine turnover between days 2-3, while Brison et al 2004 reported that pregnancy could be predicted by asparagine, glycine and leucine measured between days 1-2. Similar profiles of amino acid depletion/ appearance have been reported in the present study and shown that blastocyst development and pregnancy outcome could be predicted by differences in the turnover of asparagine, glutamate and alanine.

The embryos of overweight and obese women consumed and produced a number of amino acids in increased quantities, compared with counterparts from women of a normal BMI, further pointing to a degree of alteration of metabolic regulation. Glutamate production was significantly higher in embryos produced from OWOB compared to those of normal weight, consistent with the results for poorer blastocyst development and lower pregnancy rates across the cohort. Increased glutamate may be produced under stress conditions, as shown by Picton et al., 2010. The increase may be an attempt to neutralise increased ammonia produced by these embryos as shown in the mouse (Orsi & Leese 2004). In addition aspartate production and serine and glutamine depletion were greater, whilst less isoleucine was depleted by embryos from OWOB compared to normal weight women. The increased appearance of aspartate and glutamate in embryos from OWOB women might be indicative of a disrupted malate-aspartate shuttle, which is involved in regulating glucose metabolism in mouse blastocysts (Mitchell et al., 2009), and has a further function in regulating the REDOX status of the cytosol.

The present study also sought to identify differences between expanding and hatching human blastocysts and those blastocysts that arrest at the unexpanded stage. Histidine production was higher in developing blastocysts and may reflect a possible role in signalling to the uterus to aid implantation (Zhao et al., 2000). Arginine consumption was high in arrested embryos, while consumption fell in those forming unexpanded blastocysts, before rising again in expanded blastocysts, at which point it may play an important role in signalling via the nitric oxide pathway and in the establishment of a pregnancy (Sengupta et al., 2005). Methionine consumption was significantly higher in

those blastocysts capable of expanding, and this difference was exaggerated in blastocysts from OWOB women. This is potentially significantly since methionine is involved in the metabolic regulation of nucleotide synthesis (Gilbody et al., 2007) and DNA methylation (Grillo & Colombatto, 2008); these processes are likely to be important up to the stage of blastocyst expansion which coincides with the end of DNA demethylation and loss of histone modifications and the onset of methylation (Feng et al., 2010).

The general rise in amino acid turnover prior to arrest may be indicative of lower embryo viability; an elevation in metabolic activity has been correlated with molecular damage (Sturmey et al 2009a). At the other end of the spectrum, an inadequate metabolism of amino acids leads to a delay in trophectoderm development through an mammalian target of rapamycin (mTOR)-dependent pathway (Martin & Sutherland, 2001). Given that a reduction in TE cells was observed in the embryos from OWOB patients, and altered amino acid metabolism, it is tempting to speculate that there is some degree of disruption to the mTOR signalling in these blastocysts.

5.4.6 The influence of paternal factors on embryo metabolism

It is assumed that the origin of the combined metabolic alterations observed in this study can be linked to the environment within the ovary. A caveat to this assumption is that the male partners of the women in the OWOB category also had a significantly higher BMI. There is good evidence that male obesity can indeed impact on fertility and embryo viability (Bakos et al., 2011). Notably, earlier in this thesis lower rates of blastocyst formation on day 5 of development were noted among weight-discordant couples attending for fertility treatment in whom only the male had a BMI>25kg/m^2. However, the impact of male BMI on embryo metabolic markers was negligible. Thus, there was no significant difference in CORE values when data from embryos were stratified according to male BMI and secondly the results of the statistical model suggest that embryo metabolism was independent of male BMI. It was fortuitous that a cohort of six patients who received donor semen from a single donor were identified, allowing a significant negative correlation to be revealed between mean glucose consumption of blastocysts and female BMI, when the male contribution was controlled for.

The role of the paternal influence raised an additional question about the use of ICSI, with a disproportionate number of ICSI cases being performed in instances when the male partner was classified as OWOB (although not significantly different if female BMI is used as the denominator). ICSI adds two possible confounding variables; firstly reduced sperm quality is associated with increased risk of sperm DNA damage (Dupont et al., 2013) and secondly in vitro handling techniques may compromise embryo viability, resulting in epigenetic changes and/or metabolic deregulation (Thompson et al., 2002). Whilst, there we no differences in carbohydrate metabolism or triglyceride levels in embryos generated via either technique amino acid metabolism was significantly higher in the ICSI group compared to the IVF group. When attention was turned to sibling oocyte studies, no significant differences were observed in metabolic phenotype of embryos generated through either IVF or ICSI. Hence, the resolution to this quandary would appear to be that the prior observed differences related more to semen quality than the ICSI procedure per-se. Given then that in the current study, the role of micromanipulation technique has been excluded and all other embryos culture conditions were equivalent, it can be concluded that the origins of the altered amino acid metabolism in human blastocysts from overweight women can be traced back to conditions in the ovary.

5.4.7 Embryo metabolism and pregnancy outcome

Despite the differences in substrate utilisation between normal and OWOB women the relationship with pregnancy outcome was not clear. The data comparing metabolic parameters to the pregnancy outcome of the sibling transferred embryos highlighted the differences in developmental potential apparent in a cohort of embryos; a potential weakness of using the woman as the 'experimental unit' as opposed to individual embryos. This assumes that the intra-follicular conditions were comparable in the ovaries of a given patient. However, even in follicles of comparable size, the degree of vascularization, oxygenation and level of nutrients vary at the time of ovum retrieval (reviewed by Van Blerkom, 2000). The more subtle differences in metabolic regulation and developmental competence of individual embryos could be attributed to these differences and further studies are required on the origin of intra-follicular influences.

5.5 Conclusion

Studies on the consequences of maternal obesity have largely focused on clinical complications for the mother during pregnancy and on offspring health, short- and long-term. Owing to the complexities in working with human embryos and scarcity of material, much research on the impact of obesity on early development has been carried out in experimental animals (Luzzo et al., 2012; Van Hoeck et al., 2011; Moley et al., 1991,). Such data suggest that the early embryo is especially sensitive to nutritional and environmental challenges during the periconceptional period. Recent research efforts have begun to characterize the 're-programming' that occurs at this time, and the consequences for future development. Deviations in fetal growth rates and incidence of fetal abnormalities permit some inferences to be made about the impact of early embryonic adaptations to metabolic adversity. Here it was reported that babies born to OWOB women were significantly larger for gestational age than those born to women of normal weight. Weak associations were evident between birth weight and level of metabolic deregulation, again highlighting the complexities of using the woman as the 'experimental unit' rather than individual embryos. What can however be deduced is that maternal overweight or obesity acts via the ovary to alter the phenotype of the oocyte. These alterations persist in the zygote and manifest as a disrupted metabolism at the blastocyst stage with the potential to compromise subsequent embryonic and fetal growth. The consequences of such phenotypic modifications is unclear, but could impact on the longterm development of non-communicable diseases, including cancer (Walker et al., 2012) cardiovascular disease and diabetes, the aetiology of which are considered to have a developmental component (Hanson et al., 2011).

The data generated in this chapter adds to the developing picture (Figure 5.26, below) of accumulating evidence of an association between obesity and poorer pregnancy outcomes. In chapter 3, lower oocyte recovery rates and live birth rates for OWOB women were demonstrated and chapter 4 described phenotypic differences in oocytes and resulting blastocysts. Here very clear differences in embryo metabolism are recorded for embryos generated from OWOB women compared to normal women. Even allowing for the observed differences in blastocyst formation (i.e. proportion arresting / rate of progression) and subsequent reduced allocation of cells to each lineage, these metabolic differences were evident and independently related to female BMI.

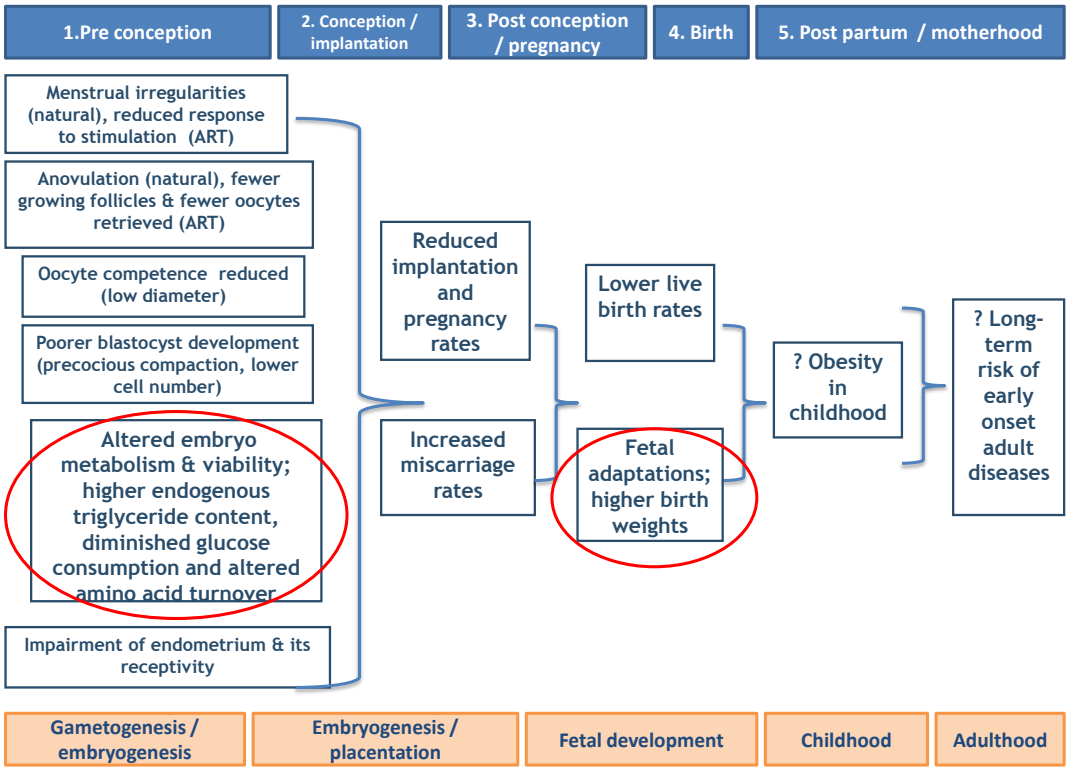


Figure 5.26 Hypothetical impact of maternal obesity- developing picture.

The molecular mechanism by which intra-follicular conditions modify the oocyte and subsequent embryo metabolism is still unclear, but it is considered highly significant that bovine oocytes exposed to fatty acids at concentrations found in human ovarian follicles (Robker et al., 2009; Valckx et al., 2012) display reduced glucose consumption in the subsequent blastocysts (Van Hoeck et al., 2011) as in the present study. Blastocyst formation is clearly dependent on the utilisation of glucose and the observation of decreased utilisation of glucose at each stage of development for embryos from OWOB women is consistent with this. It may be possible to stimulate increased glucose uptake and/or metabolism, by manipulating the *in vitro* environment and this will be reviewed in the following chapter.

Chapter 6 : Modulating the metabolism of oocytes and embryos.

6.1 Introduction

In Chapter 5 it was reported that embryos generated from OWOB women display significant differences in the utilisation of substrates from the culture medium compared to normal weight controls. In Chapter 2 it was reported that the general pattern of substrate utilization and viability is largely comparable among a cohort of developing sibling embryos belonging to a couple and it was further reported in Chapter 3 that the pattern of nutrient utilization is comparable in subsequent cycles of treatment in the same couples. Weight loss may be one way in which the nutrient enriched follicular environment may be modulated- however as already discussed this is a long term commitment and the benefits may be offset by advanced maternal age. A alternative approach is to investigate the possibility of driving embryo metabolic uptake / substrate utilization to correct the observed aberrant patterns of metabolism in embryos generated from OWOB women *in vitro* using tailored specific culture conditions to meet the optimal needs for development.

Adding different compounds to the culture medium may lead the embryo to utilize a particular substrate and / or metabolic pathway. Given the observed findings for lower glucose consumption and higher triglyceride levels in embryos from OWOB women it would seem prudent to attempt to correct this pattern of metabolism; the effects of adding insulin and l-carnitine (LC) will be considered in further detail in this chapter.

6.1.1 Glucose manipulation

Rodent studies have shown that hyperglycemia may compromise blastocyst development, by causing down regulation of glucose transporters leading to abnormal metabolism and apoptosis (Moley et al., 1998, Chi et al., 2000).

Purcell et al., (2009) provided a succinct account of glucose uptake by preimplantation embryos through a large family of glucose transporters, the expression of which is related to the glucose concentration in the environment (summarised in Table 6.1). Glucose can be actively taken up by sodium coupled active glucose transporters (SGLTs) or through facilitative glucose transporters (GLUTs). Most uptake is facilitative (Gardner & Leese 1988), mediated by GLUT family of transporters, of which there are 14 characterised members (Zhao et al., 2007).

Table 6.1 GLUT detected transporter expression in the preimplantation embryo.

Transporter	Expression
GLUT1	mRNA has been detected between the oocyte and blastocyst stage in mouse (Hogan et al., 1991; Morita et al., 1992; Pantaleon et al., 2001), bovine (Lequarre et al., 1997) and human embryos (Dan Goor et al., 1997).
GLUT2	Located on the basolateral membrane side and present from the 8-cell onwards in mice (Hogan et al., 1991)
GLUT3	Expression evident from compaction onwards in mice (Purcell et al., 2009)
GLUT4	Hogan et al., (1991) reported that it is not expressed at any stage in mouse embryos, whereas others have detected expression from the 8-cell in both mouse and human embryos (Dan Goor et al., 1997) and in bovine blastocysts (Navarete Santos et al., 2000). Under insulin stimulation GLUT4 is rapidly translocated from an intracellular location to the cell surface plasma membrane, thus rapidly increasing glucose transport (Gonzalez et al., 2006).
GLUT8	This transporter has been shown to account for insulin responsive transport in the mouse blastocyst (Carayannopoulos et al., 2000), although these findings have not been reproduced in other studies (Tonack et al., 2009).

Insulin signals through the insulin receptor (IR) and additionally through binding with lower affinity to the structurally similar, insulin-like growth factor-I (IGF-1) receptor. The IR and IGF-1 receptor have both been detected in the preimplantation embryos of many species, including; murine (Rappolee et al., 1992) bovine (Schultz et al., 1993) and human (Lighten et al., 1997). IR gene expression can be detected from 8-cell onwards, when the embryo begins glycoltyic metabolism (Harvey & Kayne 1991) and IGF-1 expression has been localised to the surface of the trophectoderm (Heyner et al., 1989). It is thought that insulin binding to the IR regulates glucose uptake in the embryo via the PI3K/ Akt pathway, resulting in GLUT4 trafficking and transport activity, as is the case in adult cells (Figure 6.1).

At the systemic/ whole body level, insulin resistance can arise due to chronic environmental exposure to hyperglycemia, but as it is still to be conclusively shown that

human preimplantation embryos express GLUT4, the action of insulin and any possible mode of insulin resistance in the preimplantation embryo are speculative. Immunohistochemical studies have shown that insulin is internalized by preimplantation mouse embryos (Hayner et al., 1989), although increased glucose uptake in response to insulin maybe acting through the IGF-1 receptor and not the IR (Pantaleon & Kaye 1996). The IGF-1 receptor is present in granulosa cells in human oocytes (Zhou et al., 1993), insulin and IGF-1 may act through this receptor to affect cell proliferation and metabolism via altered gene expression (Figure 6.1).

An alternative explanation for insulin's beneficial effects on embryo development is through its action as a growth factor. In support of this, insulin has been reported to have anabolic and proliferative effects on the early mouse embryo and its addition to the culture medium, increased cell numbers and morphological development (Gardner & Kaye, 1991). Furthermore insulin was shown to stimulate amino acid transport and protein synthesis (Figure 6.1), via insulin receptors present on cells of the ICM and TE (Harvey & Kaye, 1990).

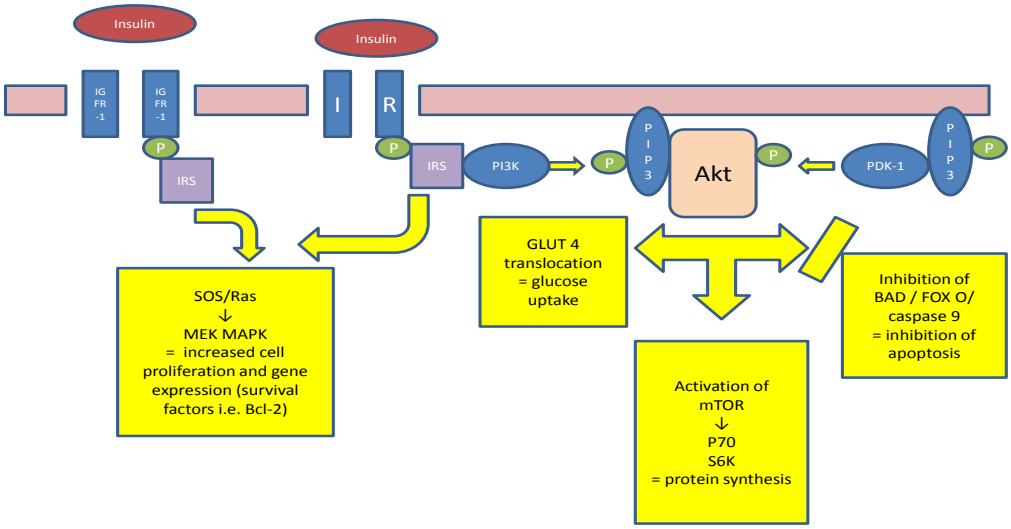


Figure 6.1 Schematic of insulin signalling-

through insulin receptor (IR) and insulin-like growth factor-I (IGF-1) receptor and its possible regulation of glucose homeostasis, gene expression, cellular proliferation, survival and protein synthesis. In the case of glucose regulation it is possible that if human pre-implantation embryos do express GLUT 4 insulin regulates this expression by binding to its receptor, a tyrosine kinase, which phoshorylates (P) IRSs, which is then able to bind PI3K, which phoshorylates PIP2 to PIP3 which can be bound to PDK-1 and Akt. Active Akt can initiate translocation of GLUT 4.

6.1.2 Lipid manipulation

The triglyceride (TG) content of embryos was shown in the previous section, to be significantly higher in embryos generated from OWOB compared to normal weight controls. Strikingly, embryos arresting at the cleavage stages of development had a higher triglyceride content than those arresting at the blastocyst stage. These findings imply two things; firstly that endogenous stores of lipid are influenced by maternal environment and secondly that the embryo may use endogenous stores as an energy source during preimplantation development. If this is indeed the case it was thought that endogenous stores of lipids could be manipulated by stimulating β-oxidation during *in vitro* culture using l-carnitine (LC), which might improve embryo viability.

It is thought that endogenous lipid stores accumulate during oocyte development and are subsequently modified during the final stages of oocyte maturation (Ferguson & Leese 1999). When bovine zygotes were cultured in medium lacking exogenous substrates, but supplemented with LC, the rates of blastocyst formation were higher than those of the control group (Sutton-McDowall et al., 2012). Whilst the endogenous lipid stores of bovine embryos are considerably higher than those reported earlier in the human (Chapter 5), the reduced triglyceride content that was observed in those embryos which formed blastocysts is suggestive of a vital role for β-oxidation of TG during embryo development providing a target for manipulation.

Endogenous long-chain fatty acids are metabolised in the matrix of mitochondria. LC is essential for normal mitochondrial activity; usage affects ATP levels, which in turn influences an oocyte's developmental capacity (Stojkovic et al., 2001). In the zygote, LC increases the transport of fatty acids into mitochondria and thus increases β-oxidation of fatty acids into acetyl-CoA molecules, for metabolism in the TCA cycle and ETC to produce ATP (Van & Wanders, 2002). The initial transport of cytosolic fatty acids into the mitochondria, is the rate limiting step and this is catalyzed by carnitine palmitoyltransferase-IB (CPTIB). This enzyme transesterifes fatty acids in the form of fatty acyl-CoA to LC. Fatty acyl-carnitine then passes through the outer mitochondrial membrane, before being transported into the matrix by carnitine-acylcarnitine translocase (CACT). Subsequently, CPTII then transesterifies the fatty acids to mitochondrial CoA and LC is released and recycled by CACT back to the cytosol. In the mitochondrial matrix, β-oxidation results in the conversion of acyl-CoA

to acetyl-CoA, which can then be metabolised further in the TCA cycle and oxidative phosphorylation to generate ATP.

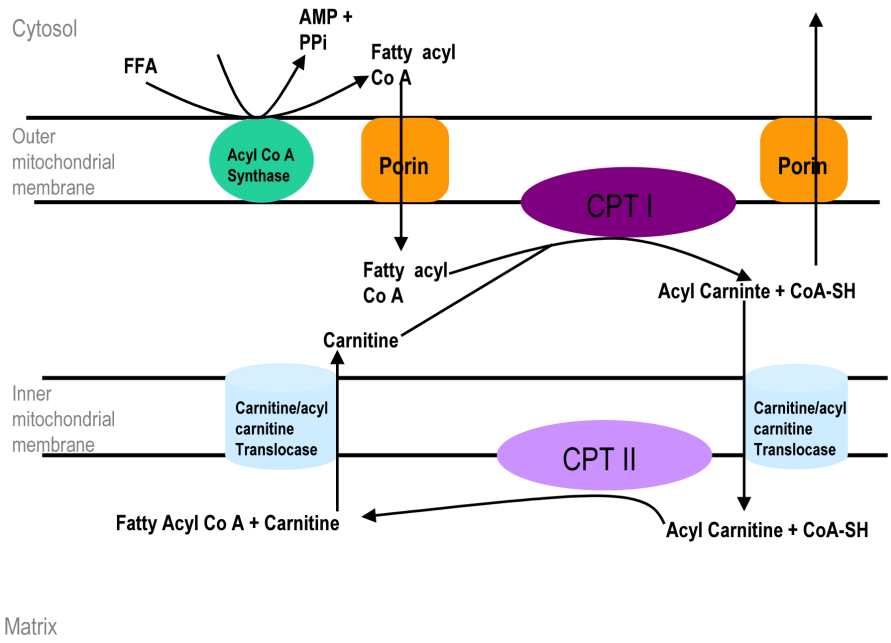


Figure 6.2 Action of LC-

Free fatty acids (FFA) linked to coenzyme A at the outer mitochondrial membrane are activated. Fatty acyl-CoA then binds to LC, catalysed by CPTBI and is translocated across the inner mitochondrial membrane for β-oxidation. (Figure, reproduced with permission of R. Sturmey, 2004)

LC is the biologically active stereoisomer and in humans, 75% of LC is obtained from the diet. However, LC may also be synthesised from the amino acids lysine and methionine, in the kidney, liver and brain (reviewed by Flanagan et al., 2010). It is not clear if embryos have the capacity to synthesis carnitine, or if they must acquire it from the follicular / oviductal fluid. LC is present at a comparable concentration of approximately 29µmol/l, in serum and follicular fluid of women undergoing IVF treatment (Valckx et al., 2012). The presence of LC in the follicular environment provides justification for investigating its role in modulating embryo development. In this chapter, LC was adding it to culture medium at a range of concentrations from 0.005mM to 0.5Mm to determine if human embryos take up LC from the medium and what effect this has on embryo viability.

Despite a number of studies into the effect of LC supplementation, in murine (Pillich et al., 2005; Abdelrazik et al., 2009), bovine (Phongnimitr et al., 2012; Sutton-McDowall et al., 2012) and porcine species (Somfai et al., 2011; Wu et al., 2011), the impact of LC

supplementation on overall embryo metabolism has yet to be addressed, particularly in reference to female obesity.

6.1.3 Lipid content and manipulation of frozen, thawed embryos

Embryo cryopreservation is an integral part of ART. The post thaw survival and viability may differ to those of fresh embryos and few studies have sought to investigate, beyond comparisons of pregnancy, live birth rates and neonatal outcomes.

Cryopreservation may cause cellular damage, affecting membrane permeability and cytoskeletal structure. In this study all donated embryos had been slow-frozen and therefore findings have been related to studies using this method. Slow freezing has been associated with altered gene expression profiles (Tachataki et al., 2003), compared to fresh embryos; this was speculated to relate to the level of cellular trauma. Similarly, the uptake of pyruvate from day 3 embryos has been shown to be lower from slow frozen embryos with poorer post-thaw viability (Balabon et al., 2008), as has the oxygen consumption (Kaidi et al., 2001), when compared to those displaying better post thaw morphology. In one of the few studies to examine amino acid turn-over in human embryos, Stokes et al (2007) conducted their analysis on embryos which had previously been cryopreserved and it was noted that the thawed embryos had a similar amino acid metabolism to those of fresh, however other investigators have reported that the findings may be related to the period of recovery. Fang et al., (2013), demonstrated that the amino acid turnover in the first half an hour post thawing was significantly lower than that reported in the following hour, before returning to pre-freeze levels, suggesting that there is a recovery period. It has been speculated that the disruption of the cytoplasmic microstructure may influence metabolism. The level of disruption may be influenced by the intracellular lipid content of the embryo and lipid composition of membranes.

The membrane lipid composition affects a cell's behaviour after temperature changes, with phospholipids being the most abundant lipids in eukaryotic cell membranes. Adding linoleic acid to bovine embryo culture medium has been shown to enhance cryosurvival of embryos (Pereira et al., 2007), whereas supplementation with palmitic acid has a negative effect on embryo cryosurvial (Shehab-El-Deen et al., 2009). Cytoplasmic lipid accumulation will also affect post thaw survival, as demonstrated in bovine embryos (George et al., 2008) and environmental conditions can cause changes

to lipid metabolism (Abe et al., 2003, Dinnyes et al., 2009, Barcelo-Fimbres et al., 2007, Orsi et al., 2004). Large amounts of intracellular lipids can compromise embryo quality through impaired mitochondrial function (Abe et al., 2002), as demonstrated in bovine embryos cultured using serum free or serum containing media. A greater content of intracellular lipids increases their sensitivity to oxidative stress and may lead to increased free radical generation and increased cell death.

It is widely reported that lipid accumulation can aggravate cryo-injury in porcine and bovine- but these species have a higher embryo lipid content than the human. In chapter 3 lower rates of cryosurvival were reported for blastocysts from OWOB women compared to normal women which were speculated to be due to differences in triglyceride content. In this chapter this concept was tested.

Sufficient ATP production is required for embryo development and cryopreservation has been reported to damage mitochondria leading to ATP losses (Manipalviratn et al., 2011, Zhao et al., 2011). Lipid droplets are a rich source of energy and LC is reported to enhance lipid metabolism and thus ATP production, with this in mind LC was added to the culture medium to evaluate the effects on post- thaw metabolism in embryos from normal and OWOB women.

6.1.4 Intralipid therapy

In the course of this study, it came to light that a small number of the participants, who had had a previous implantation failure received intralipid infusions (20%-100ml). Intralipid is generally administered between days 4 and 9 of ovarian stimulation, and again around the time of embryo transfer, with a 3rd dose being administered immediately after a positive pregnancy test result. Intravenous infusion with intralipid, which is a soybean oil based source of essential free fatty acids; linolenic and linoleic acid, purified egg phospholipids and glycerol has been promoted by a number of clinicians for use as an immunotherapy aimed at improving IVF pregnancy rates (Shreeve & Sadek, 2012; Coulam et al., 2009, Ndukwe et al., 2011). Intralipid has no known long-term effects and reports of adverse reactions are rare – however, substantial data sets are lacking and the mechanisms by which intralipid may modulate the immune system are unclear. It has been postulated that patients with unexpected failed implantation or recurrent miscarriage may have abnormal maternal Natural killer (NK) cell levels and functional cytotoxic activity against the embryo (Kwak-Kim & Gilman-

Sachs, 2008; Matsubayashi et al., 2001). Intralipid reportedly has NK cell suppressive properties (Coulam et al., 2009) and it is hypothesized that this will reduce immune mediated damage to the embryo (Bansal et al., 2012). It is not known if such administration alters the fatty acid profile of the follicular fluid and as such the possible effects on embryo metabolism have thus far been overlooked. In a number of cases embryo metabolic data from the patients previous failed treatment cycle were also available– thus permitting an informative, albeit an under-power assessment of this treatment intervention for both normal and OWOB women. Furthermore, as obesity is also a chronic inflammatory condition and intralipids reportedly also have inhibitory effects on pro-inflamatory factors, such as Th1 cytokines (Granato et al., 2000), the effects of intralipid administration have been compared in normal and OWOB women.

Aims and objectives

The aim of this chapter was to examine the effect of supplementation of the culture medium with insulin and LC on human embryo metabolism and development.

The specific objectives were;

a) To examine the effects of modulating the *in vitro* culture environment by studying the effect of addition of insulin on embryo development and metabolism.
b) To discover if the addition of LC to the culture medium altered the embryo development rate of frozen thawed embryos.
c) To discover if the addition of LC to the culture medium altered embryo metabolism and utilisation of triglyceride.
d) To review data from patients receiving intralipid therapy and consider a potential relationship with embryo metabolism and female BMI.

6.2 Materials and methods

6.2.1 Patient recruitment

IVF patients were consented and recruited to the study as described in chapter 2. A total of 101 consecutive patients presenting for IVF at the Hull IVF Unit donated a total of 451 embryos with full informed consent and a further 25 patients donated 105 frozen embryos no longer required for treatment. Female and male BMI were recorded at the down-regulation appointment and at the commencement of treatment. Embryos that originated from patients classified as OWOB (BMI > 25 kg/m2) were compared with embryos derived from women of normal BMI (18.5–24.9 kg/m2). The study groups being discussed in this chapter are highlighted below.

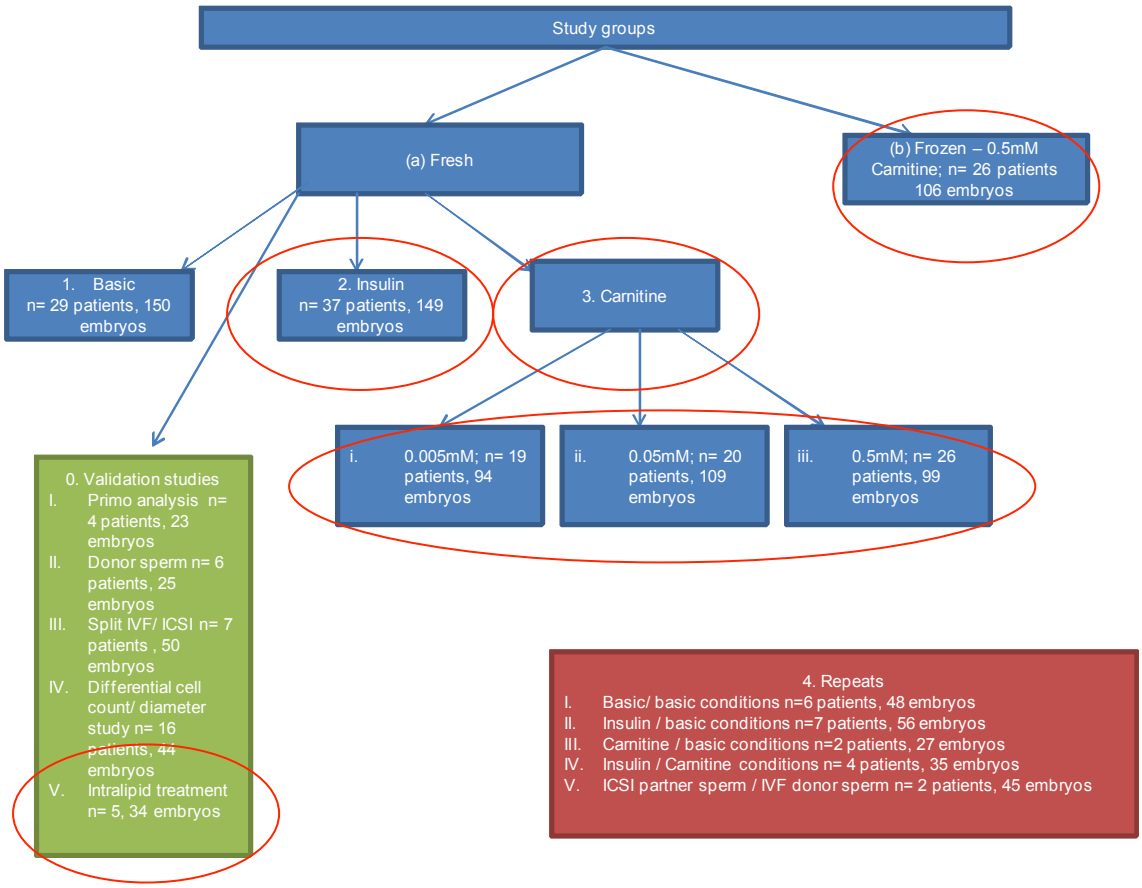


Figure 6.3 Source of study group- from prospective data, collected from women contributing to the primary research project from 2010 to 2015.

6.2.2 Experimental methods

The experimental methods used to generate metabolic profiles were mainly 'non-invasive' and provide quantitative markers of embryo health.

As described in chapters 2 and 5 surplus embryos donated to research had their development stage recorded before being placed individually into 4 ml culture drops. The following protocol differences were implemented;

1. Embryos were cultured under Sage Oil at 37^0C in 6% CO_2 and 5% O_2 for 24 hours, alongside embryo-free control drops. This differs to chapter 5 and due to an improvement to research equipment facilities means that embryos were cultured in-vitro under the same low oxygen conditions, whilst in both clinical and research facilities.
2. Observations were ended on day 7, rather than day 9 – to permit increased consistency in analysis and observations and on the basis of findings discussed in chapter 5.
3. Insulin was added to the culture medium and embryos donated (n= 149) from each patient (n=37) in this portion of the study, were randomly assigned to 'test' and 'standard' media droplets. (Working solution details are provided below).
4. L-C was added to the culture medium at three different concentrations (0.005mM, 0.05mM and 0.5mM) and embryos donated (n= 302) from each patient (n=65) in this portion of the study, were randomly assigned to 'test' and 'standard' media droplets (see Figure 6.3 and 6.4 for further details). (Working solution details are provided below).
5. Frozen embryos that had been donated to research, were thawed using a commercial slow thaw protocol (Origio) and placed in to individual culture droplets. LC was added to the culture medium (0.5mM) and embryos donated (n= 105) from each patient (n=26) in this portion of the study, were randomly assigned to 'test' and 'standard' media droplets. Pre-freeze development was recorded, as was post thaw development / survival. Development was recorded throughout the extended culture, until day 7.

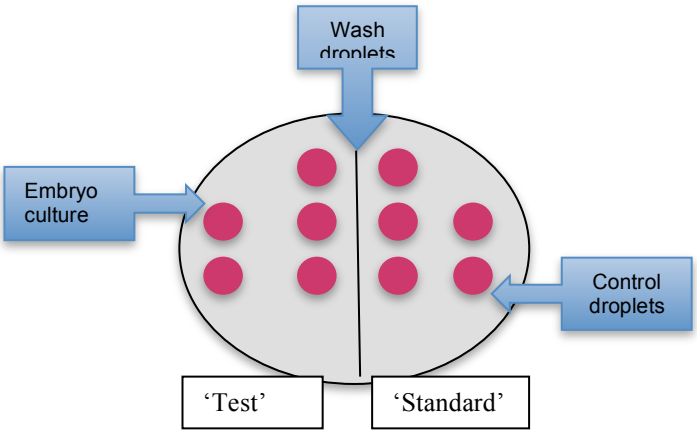


Figure 6.4 Micro-droplet culture set up.

Embryos from each individual patient were randomly divided among 'test' (i.e. addition of Insulin or LC, dependent on study protocol) and 'standard' research culture medium.

As stated previously, culture droplets were refreshed every 24 hours and developmental observations made each day up to termination of culture. After incubation, the spent culture medium was immediately frozen at -80^{0}C for later analysis. Metabolic CORE profiles were determined for glucose, pyruvate, lactate and 18 amino acids, and TG content was measured, all as previously described. Results were recorded according to stage reached at the end of the period of culture. Full details of each method are provided in Chapter 2, however the following protocol deviations were applied;

1. Embryos included in the insulin assessment protocol (test and standard) were placed into RNA later, for future research use and in view of this, no TG analyses have been performed on these embryos.
2. Embryos included in the LC assessment protocol were assayed for carnitine consumption (details given below) and therefore amino acid CORE was not measured.

6.2.3 Addition of Insulin to the culture medium

A stock solution of insulin was prepared using sterile water and was serially diluted and added to culture medium to create a working solution of 0.5ng/ml.

Table 6.2 Insulin supplementation protocol.

Final concentration 0.5ng/ml

A) Stock solution	**mg / 1ml sterile water**
Insulin	5 (5mg/ml)

B) Working solution	**µl embryo culture medium**
5mg/ml Insulin	1000 (5µl/ml)
5µl/ml Insulin	1000 (5ng/ml)
5ng/ml Insulin	10 (0.5ng/ml)

6.2.4 Culture of embryos in the presence of L-Carnitine

A 500m/mol stock solution of L-Carnitine (161.199g/mol, molecular weight) was prepared using sterile water and diluted in culture medium to create a working solution from 5mmol/l, from which dilutions ranging from 0.5 to 0.05m/mol were made.

Table 6.3 LC supplementation protocol-to create 3 concentrations; 0.5, 0.05 and 0.005m/mol

A) Stock solution (500m/mol)	**g / 10ml sterile water**
L-Carnitine	0.806

B) Working solution	**µl/ 1ml embryo culture medium**
500m/mol L-Carnitine	10 (5m/mol)*
*5m/mol L-Carnitine	100 (0.5m/mol)
*5m/mol L-Carnitine	10 (0.05m/mol)
*5m/mol L-Carnitine	1 (0.005m/mol)

Embryos cultured in the presence and absence of LC were assayed for CORE glucose, pyruvate and lactate and at the end of the period of culture the triglyceride content of embryos cultured in LC supplemented and standard media droplets was determined as described in chapter 2. Additionally, spent culture medium was assayed to determine the consumption of LC using a scaled down version of the LC assay kit supplied by Abcam, UK.

The assay was used to measure free LC in the culture medium. In the course of the assay reaction an acetyl group is transferred from CoA to carnitine to form acetylcarnitine.

L-Carnitine + acetyl CoA –*Carnitine acyltransferace*→acetylcarnitine + CoA

The free CoA is further processed and leads to the oxidation of the OxiRed probe to give fluorescence (Ex/Em 535nm 587nm) and absorbance at (570nm).

A six point standard curve was established before each assay (r2>0.9), using LC standards in the range of 0- 1 m/mol- (0. 0.2, 0.4, 0.6, 0.8, 1.0 m/mol-1). Standards were prepared by diluting 1m/mol LC stock with assay buffer. Standards were prepared in triplicate.

The reaction mix was prepared for each assay and consisted of assay buffer, carnitine converting enzyme, development mix and probe. Reactions were carried out in 96 well assay plates. 2.5µl of reaction mix was added to each well and the fluorescence was recorded. 2.5µl of standard (or sample, once a suitable standard curve had been established) was added to each well and after 30 minutes incubation the florescence was recorded.

The amount of LC was calculated by relating changes in fluorescence to those given by the standards. The readings were corrected for background readings and blank culture droplets were also assayed to account for dilution effects during culture.

6.2.5 Statistical Analysis

The information from the metabolic profiling of spare embryos was compared for normal and OWOB patient groups and correlated to study endpoints: embryo cleavage development and blastocyst formation. ANOVA was used to assess intra and inter subject variability in combination with linear regression analysis to assess the predictive accuracy of metabolic profile on blastocyst development rate. Kruskal-Wallis one-way analysis of variance was used, when normal sample distributions were not evident. Full details of the statistical analysis are included in Chapter 2. However additional tests/ calculations conducted included;

- The TG content of developing and early arresting embryos was compared using a 2 Sample t-test or Mann Whitney U test, if assumptions not met.
- Blastocyst rates in the presence of (a) insulin or (b) LC are expressed as a percentage of the total number of embryos cultured and the data compared using a oneway ANOVA with Fisher's or Tukey Test *post-hoc*.
- Kruskal-Walis (2-sided, adjusted for tiers), with Bonferroni correction was used to rank patient BMI and development stage attained. It was also used to compare embryo development according to LC concentration in culture medium.
- CORE profiles of embryos grown in the presence of (a) insulin or (b) LC were compared by oneway ANOVA with Fisher's / Tukey *post-hoc*. General linear modeling was used to account for variations at different CORE levels in stage of development, between (a) BMI sub-groupings and (b) 'test' and 'standard' culture medium.
- Binominal logistic regression was used to predict blastocyst formation (1, 0), using the enter method coded predictor variables included; BMI (<24.9 =1 and ≥ 25kg/m2 = 0) and culture medium (standard =1 and test =0). Cox and Snell R^2 and Nagelkerke R^2 were used to assess the goodness-of-fit and sensitivity / specificity. Wald test was used to determine the significance of independent variables and discover which variables added significantly to the model and predicted blastocyst formation.

Statistical differences at the 5% level were assumed to be significant.

6.2.6 The study sub-groupings

In the insulin study group a total of 76 embryos from 16 OWOB women and 73 embryos from 21 normal weight women were assayed for CORE profile. The end point for the study was blastocyst development; of the supernumerary embryos donated to research 50 embryos had developed to form blastocysts (65.8%) by day 7 in the OWOB group compared to 26 (55.3%) from the normal weight group. There were no differences in patient age and cycle number, but significant differences in male BMI have been appropriately controlled for in the multivariate statistical analyses. The details from the clinical treatment cycles are provided below, including embryo development of all embryos (transferred, frozen, donated to research).

Insulin n=37 consecutive IVF & ICSI patients			
(± SEM)	Normal weight	OWOB	*p* value
Female age (years)	31.86 (0.96)	30.75 (1.04)	>0.05
Male age (years)	37.68 (1.74)	36.64 (1.33)	>0.05
Cycle number	1.43 (0.13)	1.56 (0.18)	>0.05
Female BMI (kg/m2)	22.41 (0.40)	27.54 (0.39)	<0.001
Male BMI (kg/m2)	24.76 (0.29)	25.78 (0.35)	<0.05
Av. Oocytes	10.38 (0.93)	9.38 (0.94)	>0.05
Av. Embryos	6.05 (0.43)	6.25 (0.81)	>0.05
Blastocyst development (%)	32.48 (5.81)	28.84 (7.79)	>0.05

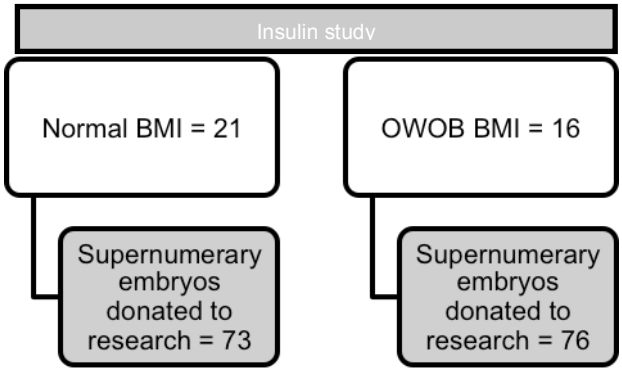


Figure 6.5a Schematic diagram of the study group, indicating patient numbers and demographics (values are±SEM). Shaded boxes indicate embryos included in the analysis.

In the LC study group a total of 134 embryos from 32 OWOB women and 168 embryos from 33 normal weight women were assayed for CORE profile. Significant differences in male BMI have again been controlled for in the analysis and details below refer to the clinical treatment cycle data, further details of supernumerary embryo development are included in the results section.

L-Carnitine n=65 consecutive IVF & ICSI patients			
(± SEM)	Normal weight	OWOB	*p* value
Female age (years)	33.67 (0.64)	31.56 (0.88)	0.06
Male age (years)	35.87 (0.96)	35.76 (1.21)	>0.05
Cycle number	1.55 (0.16)	1.56 (0.16)	>0.05
Female BMI (kg/m2)	22.15 (0.28)	27.68 (0.36)	<0.001
Male BMI (kg/m2)	24.64 (0.23)	26.14 (0.26)	<0.01
Av. Oocytes	11.23 (0.86)	9.72 (0.58)	>0.05
Av. Embryos	8.06 (0.68)	6.81 (0.50)	>0.05
Blastocyst development (%)	44.64 (4.71)	38.85 (5.29)	>0.05

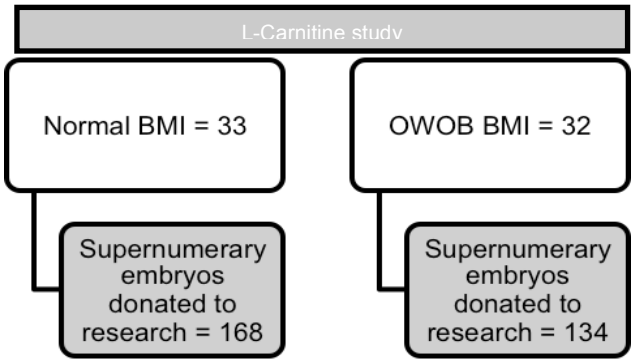


Figure 6.5b Schematic diagram of the study group, indicating patient numbers and demographics (values are ±SEM). Shaded boxes indicate embryos included in the analysis.

In the frozen embryo study group a total of 27 embryos from 5 OWOB women and 79 embryos from 21 normal weight women were assayed for CORE profile. Significant differences in male BMI have again been controlled for in the analysis and details below refer to the clinical treatment cycle data – from the fresh treatment cycle embryos originated from, further details of supernumerary embryo development are included in the results section.

Frozen cycles (details at time of freeze) n=26 consecutive IVF & ICSI patients			
(± SEM)	Normal weight	OWOB	*p* value
Female age (years)	31.33 (1.01)	31.60 (2.54)	>0.05
Male age (years)	35.00 (1.45)	38.67 (1.76)	>0.05
Cycle number	1.62 (0.26)	1.20 (0.20)	>0.05
Female BMI (kg/m2)	22.50 (0.39)	29.22 (1.55)	<0.001
Male BMI (kg/m2)	24.10 (0.36)	26.80 (1.32)	<0.01
Av. Oocytes	12.62 (1.05)	12.40 (1.17)	>0.05
Av. Embryos	9.38 (0.83)	10.40 (1.44)	>0.05
Blastocyst development (%)	57.9 (6.67)	45.56 (22.79)	>0.05

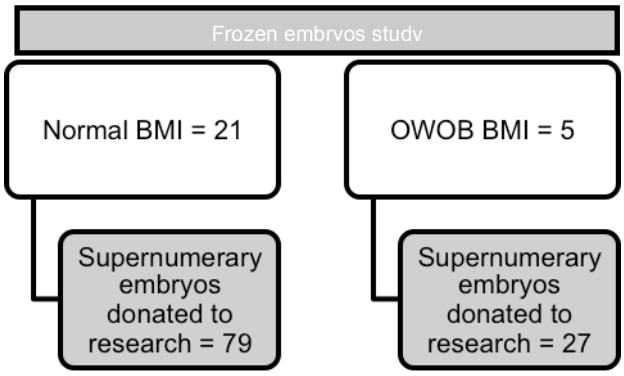


Figure 6.5c Schematic diagram of the study group, indicating patient numbers and demographics (values are ±SEM). Shaded boxes indicate embryos included in the analysis.

In the course of this study it was discovered that a subset of 5 patients had been given intralipid therapy as part of their clinical treatment plan; two of whom were of normal weight and three OWOB. A total of 34 embryos (17 from each category) were donated to research post therapy and subject to assessment.

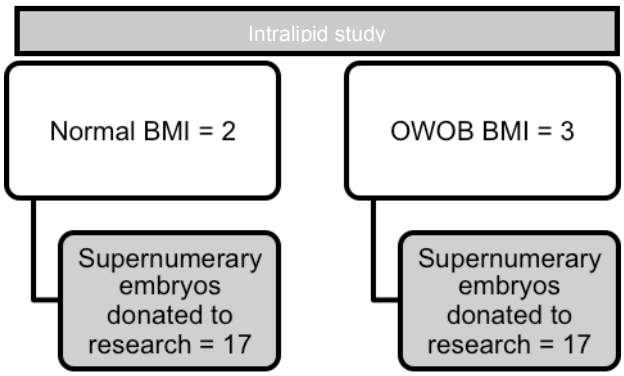


Figure 6.5d Schematic diagram of the Intralipid sub-study group, indicating patient numbers. Shaded boxes indicate embryos included in the analysis.

Figure 6.5 **Overview of study groups (patient and embryo details)**

6.3 Results

6.3.1The addition of insulin to the embryo culture medium

Embryos from OWOB had a significantly lower glucose consumption (69.89pmol/emb/hr versus 96.70pmol/emb/hr) and no significant difference in lactate compared with embryos generated from normal weight women; consistent with the data in Chapter 5. Interestingly, significantly higher pyruvate consumption was evident for OWOB (38.12pmol/emb/hr) compared to embryos from normal weight women (30.56pmol/emb/hr) – a finding not consistent with that reported in Chapter 5. It is assumed that an equal number of embryos had been cultured under experimental 'test' (insulin) and 'standard' conditions in both BMI groupings (confirmed by one sample binominal test p=0.51). However, the proportion of embryos reaching the blastocyst stage donated by normal weight women was significantly lower at 55.3% (26/73) than the 65.8% (50/76) of embryos from OWOB women ($p<0.01$) reaching blastocyst formation by the end of the culture period. There was a trend for increased blastocyst formation when embryos were cultured in medium supplemented with insulin, particularly in the OWOB grouping (58.3% versus 72.3% BD for control and insulin experimental groups respectively).

Table 6.4 CORE profiles for normal (n=21) and OWOB women (n=16) in insulin/ control groups.

Glucose consumption was significantly lower in embryos from OWOB women (n=76) compared to those from normal weight women (n=73), conversely pyruvate consumption was significantly higher and there were no apparent differences in lactate production.

Mean values (±SEM)	*Normal weight*	*OWOB*	*F*	*p-value*
Glucose consumption (pmol/emb/hr)	96.7 (6.4)	69.89 (3.1)	5.6	<0.01
Lactate production (pmol/emb/hr)	92.78 (4.3)	102.08 (4.6)	1.3	n/s
Pyruvate consumption (pmol/emb/hr)	30.56 (1.9)	38.12 (1.8)	2.9	<0.01

There were significant differences in both glucose and pyruvate consumption at different stages of development. However when the data were further sub-classified to

include female BMI, significant differences remained apparent for glucose only (glucose f =2.6 p<0.01, pyruvate f=1.5 p=0.08).

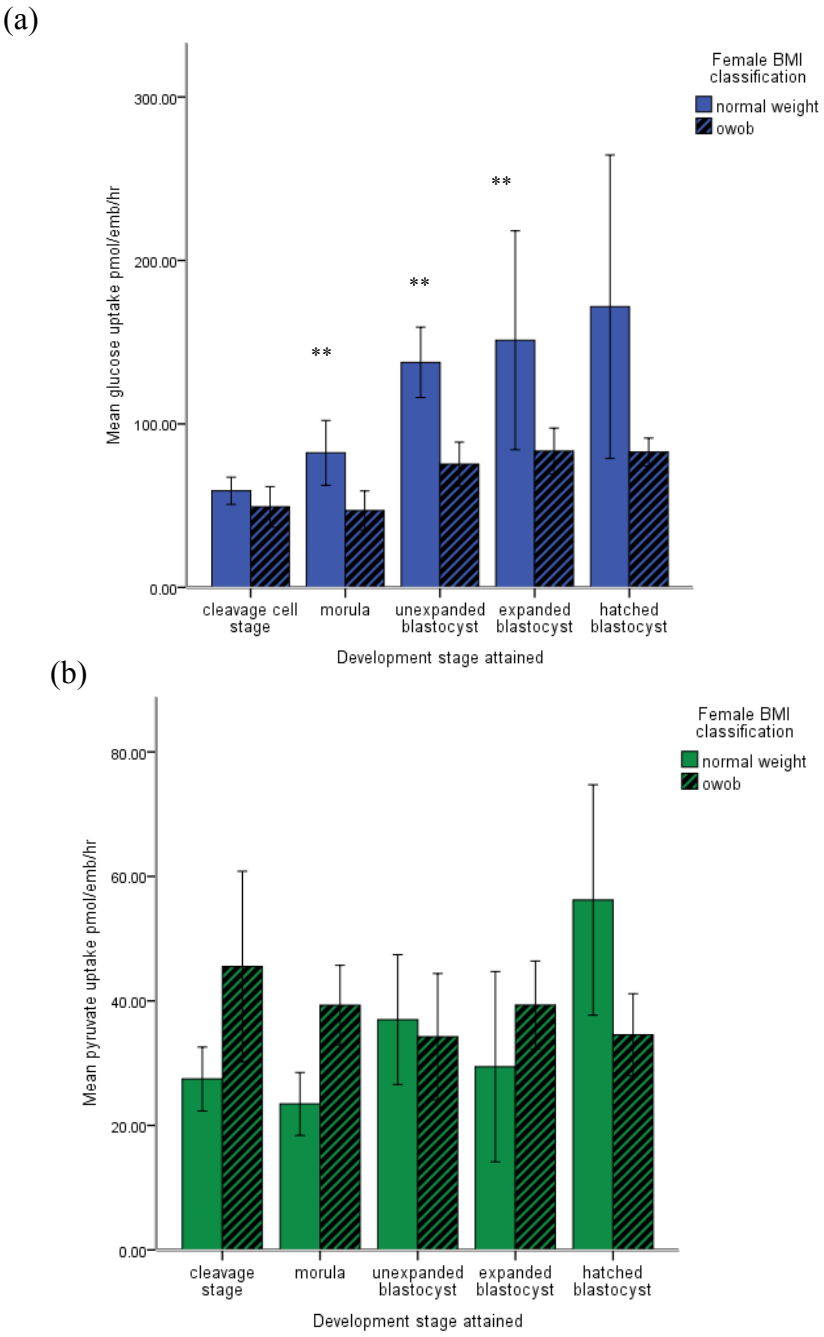


Figure 6.6 Glucose and pyruvate consumption according to stage of embryo development.

Glucose consumption at different developmental stages was shown to be significantly altered (** p<0.01) when the data were stratified into normal and OWOB groupings (a); conversely differences in pyruvate consumption became less apparent after separation into BMI groupings (b).

The addition of insulin (0.5ng/ml) to the culture medium resulted in no significant differences in glucose (p=0.71), lactate (p=0.48) or pyruvate consumption/ release (p=0.3), as determined by independent samples Mann-Whitney U Test and depicted in Figure 6.7.

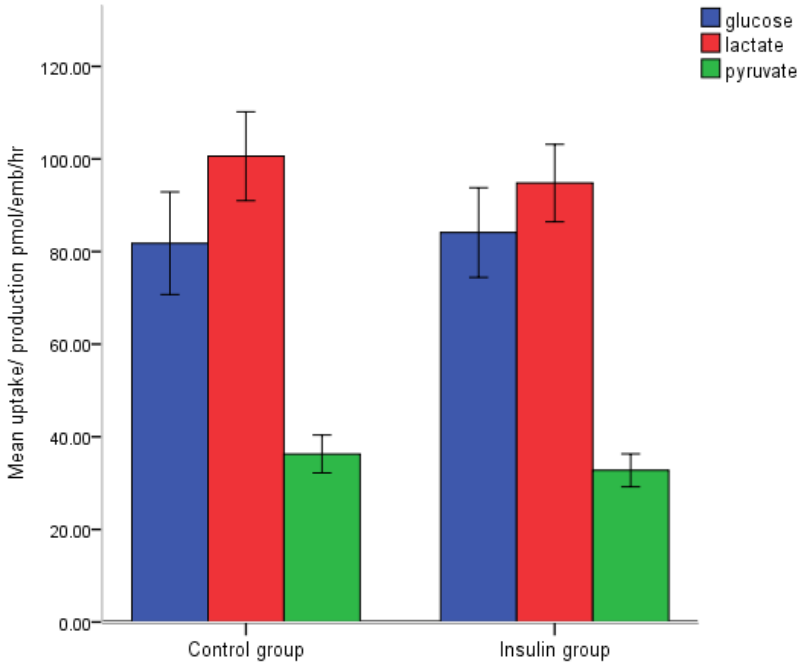


Figure 6.7 The effect of insulin supplementation on mean glucose, lactate and pyruvate metabolism by embryos (n=149) over days 5-7 of development.

In both the normal and OWOB data sets there were no significant differences in glucose, lactate or pyruvate consumption/ release when embryos were cultured in medium supplemented with 0.5ng/ml insulin (Figure 6.8).

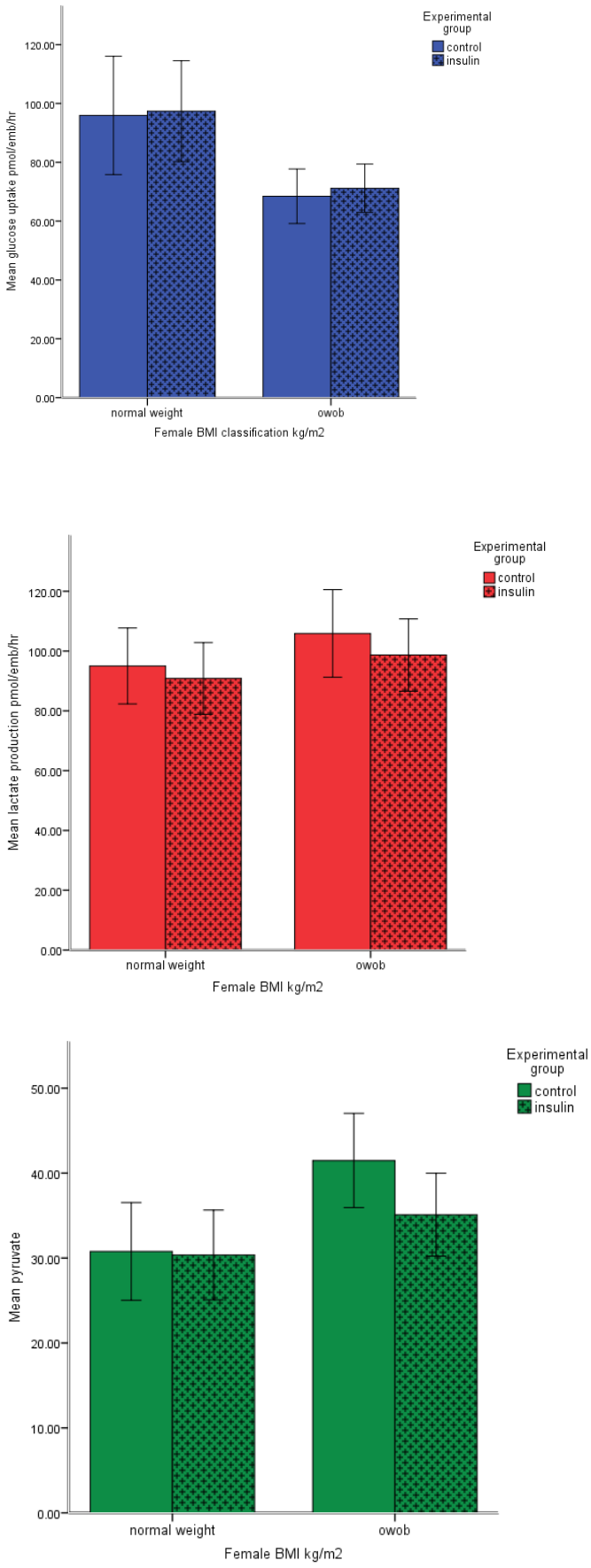


Figure 6.8 The effect of insulin supplementation on embryo metabolism in both the normal and OWOB groupings.

There was considerable variation amongst sibling embryo cohorts, with insulin addition resulting in both positive and negative effects in patients with differing BMIs, as illustrated here for both glucose and pyruvate (the two substrates found to be consumed in significantly different quantities in women of a BMI<24.9kg/m^2 and those with a BMI >25kg/m^2 in this dataset, as shown in Table 6.3). When the results were analysed as a continuous variable, rather than a categorical grouping; a weak negative relationship was evident for glucose and a positive trend for pyruvate metabolism and increasing female BMI was found.

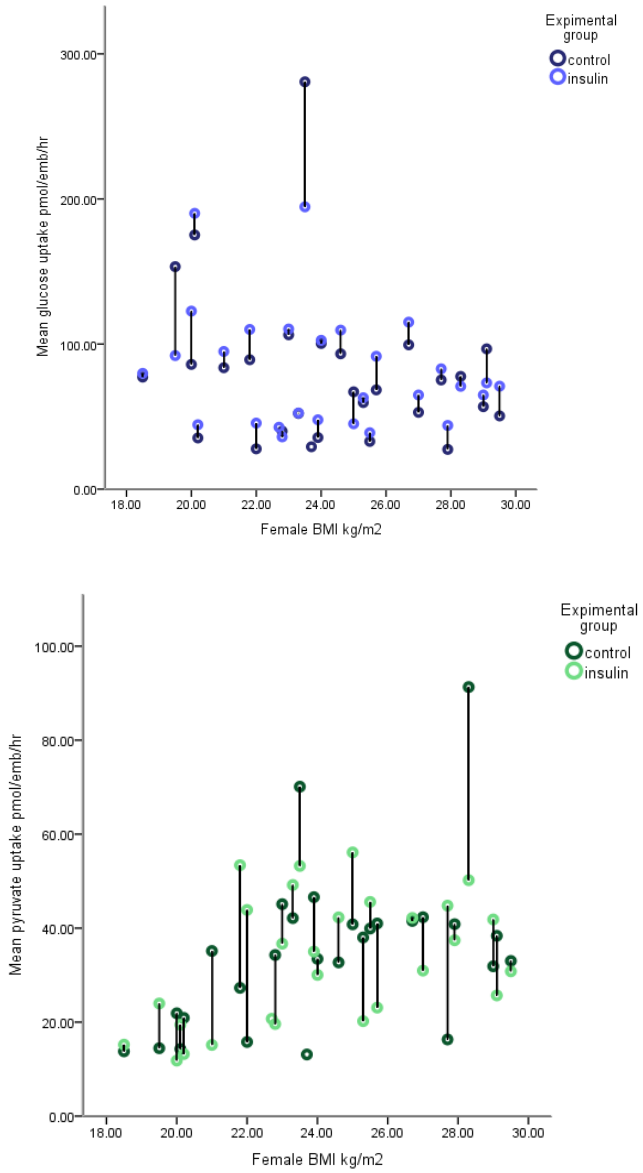


Figure 6.9 Intra-patient variability in substrate metabolism –according to embryo culture conditions (control and supplemented with insulin).

Mean values are depicted for sibling embryos for each patient (connected line) and related to female BMI.

When the data were classified into stage of development, the addition of insulin was associated with significant differences in pyruvate consumption between those embryos forming hatched blastocysts and those arresting at the cleavage stage of development (f=0.26 p=0.045 ANOVA; Figure 6.10). The addition of insulin to the culture medium had no significant effect on CORE glucose (f=0.84 p=0.5) or lactate (f=0.25 p=0.9) at each developmental stage.

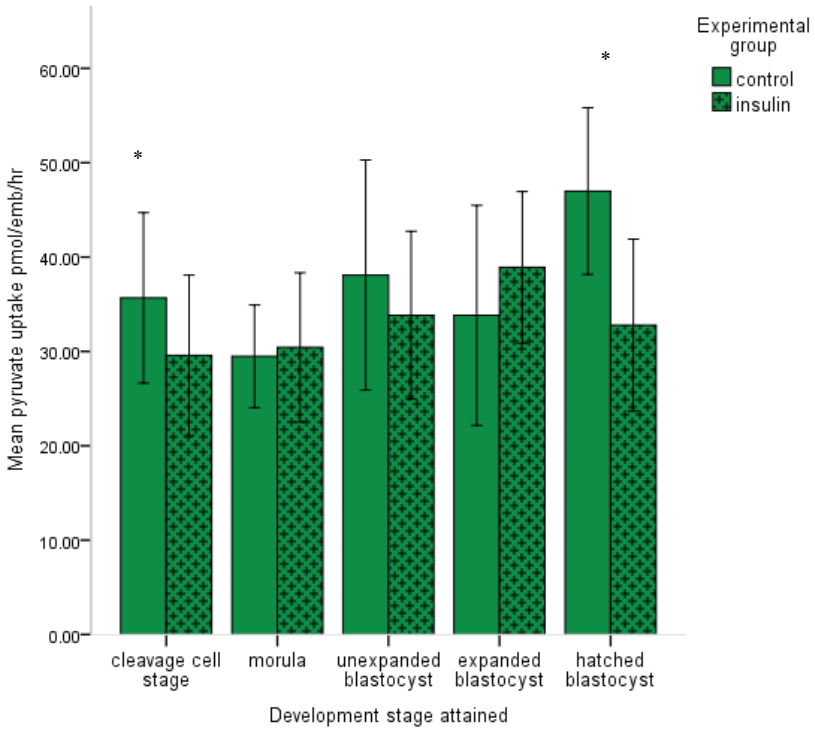


Figure 6.10 Insulin and control culture conditions and pyruvate consumption.

The addition of insulin to culture medium and pattern of uptake at each stage of development for embryos cultured from day 5-9 compared to that of sibling embryos cultured under standard conditions; significantly reduced pyruvate consumption was recorded for embryos arrested at both the cleavage and hatched blastocyst stage (* p<0.05).

Further general linear modelling was conducted to test the between subject effects of female BMI, development stage and the addition / omission of insulin on CORE glucose, lactate and pyruvate metabolism. Manipulation of culture conditions did not result in a change in CORE glucose (p=0.63), lactate (p=0.77) or pyruvate (p=0.74) when other variables were accounted for (including male BMI).

The influence of insulin supplementation has on amino acid turnover was subsequently assessed according to general turnover and individual amino acid CORE values. Data were stratified to review the effects in embryos that had originated from normal and OWOB women Significant differences were apparent in the amino acid turnover of embryos from women of normal BMI classification and those classified as OWOB (f=121.8 p<0.01); the cohort of embryos from normal weight women produced more amino acids than those attained from OWOB women, which depleted amino acids in higher quantities from the medium. The addition of insulin resulted in no significant difference in turnover (f=0.001 p=0.98). This was also the case when general linear modelling was used to assess the combined effects of insulin and BMI (f= 0.73 p=0.77). The data depicted in Figure 6.11 show the large confidence interval for embryos that originated from OWOB women and were cultured under control conditions, suggesting the precision associated with this measurement is low; this is suggestive of an inadequate sample size or that the data generated from CORE amino acid turnover are too variable to give a precise estimate.

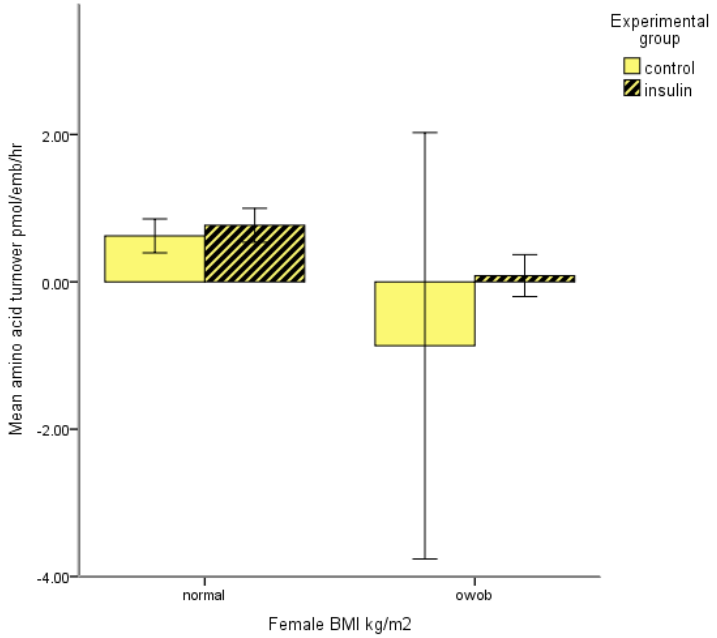


Figure 6.11 Insulin and control culture conditions and amino acid turnover.

Average sums of amino acid production and depletion, expressed in pmoles/embryo/hour for day 5 to 7 of culture. Error bars represent 95% CI. Results are recorded according culture conditions of sibling embryos; 39 embryos cultured in medium with insulin added and 34 in standard culture medium from 21 women with a normal BMI. 40 embryos in insulin supplemented medium, 36 embryos in standard medium from 16 OWOB women. The 95% CI are indicated.

Differences in amino acid turnover were apparent for different developmental stages (f=253.0 p<0.01). The differences were not attributed to culture conditions (f=0.13 p=0.97; Figure 6.12), rather significant differences in BMI were likely to be the associated contributory factor (f=231.18, p<0.01).

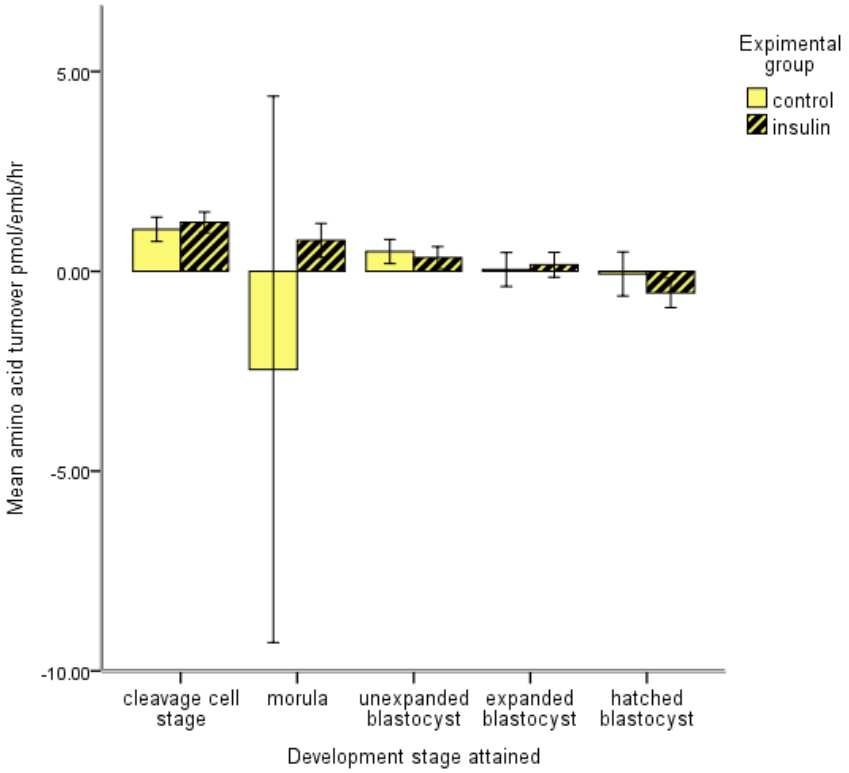


Figure 6.12 Amino acid turnover according to stages of development/ arrest on day 7. Results grouped according to embryo culture conditions.

Collectively significant differences in CORE values were evident between those arresting prior to blastocyst formation and developing onwards (ANOVA p= 0.01). The addition of insulin to the culture medium did not result in significant differences in turnover at each stage of development. Error bars represent 95% CI.

Significant differences in the individual pattern of uptake and production of amino acids were evident, with stage and BMI (asn, arg, trp, met, val, phe, iso; p<0.05) and culture conditions (met and phe; p<0.05 Figure 6.13), as determined by univariate analysis.

A general linear model was then used to test the significance of differences in individual amino acid consumption and release for embryos cultured with/ without the addition of insulin. Independent of stage and female BMI, significant differences were apparent in methionine (p=0.03), phe (p=0.03) and iso (p=0.03)

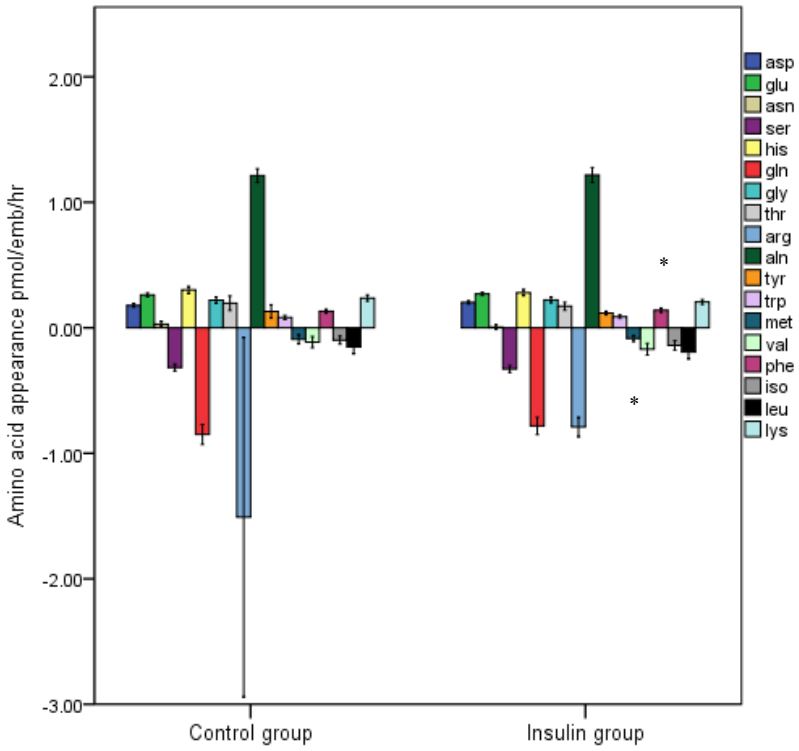


Figure 6.13 Amino acid depletion and appearance by embryos culture with and without the addition of insulin to the culture medium.

There were significant differences (*p<0.05) in methionine depletion and phenylalanine production in the cohorts. Error bars represent 95% CI.

Finally, to relate the findings discussed so far in this insulin study group to the designated study end point – blastocyst development rate, a logistic regression was performed (Table 6.5). The effects of female BMI, glucose, lactate and pyruvate consumption/release and amino acid turnover and experimental group (control / insulin) were evaluated on the likelihood that embryos would reach the blastocyst stage. The logistic regression model explained 59.5% (Nagelkerke R^2) of the variance in blastocyst formation/ development and correctly classified 81.9% of cases. From the results it was evident that female BMI (p = 0.000) and glucose consumption (p = 0.000) added significantly to the model/prediction, but lactate, pyruvate, amino acid turnover and experimental group (control/ insulin) did not add significantly to the model.

Interestingly, embryos from normal weight women were 0.04 times less likely to form blastocysts than those from OWOB females but increasing glucose consumption was associated with an increased likelihood of blastocyst development.

Table 6.5 Binominal logistic regression;

was used to evaluate the statistical significance for each of the independent variables to predict blastocyst development. Glucose and female BMI were shown to be significant. (***p<0.001) The addition of insulin to the culture medium did not influence the probability of blastocyst formation.

Binominal Log regression	Odds Ratio (95% C.I)
OWOB (0), Normal (1)	0.04 (0.01-0.14)***
Glucose uptake	1.06 (1.03-1.08)***
Lactate production	0.99 (0.99-1.01)
Pyruvate uptake	1.00 (0.97-1.03)
Sum amino acid turnover	0.970 (0.89-1.06)
Insulin(0) / standard (1)	0.53 (0.21-1.31)

Considerable intra-patient variability was observed. This variability was reduced when the analysis was limited to include only developing sibling embryos, however as was reported in the previous chapter no significant correlation with pregnancy was evident with the reviewed predictor variables. The clinical pregnancy rate for normal weight women in this study group was 66.7% (14/21) and 37.5% (6/16), the associated respective live birth rates were 52.4% (11/21) and 31.3% (5/16).

As reported in chapter 5, babies born to OWOB women were significantly (p<0.05) heavier (3859.8g) compared to those born to normal weight women (3361.82g).

6.3.2 The addition of LC (0.5, 0.05, 0.005mMol) to the embryo culture medium

Over the course of days 5-7 in extended culture, supernumerary embryos generated from OWOB women consumed significantly lower quantities of glucose than those originating from women of normal weight (74.07 pmol/emb/hr versus 106.51 pmol/emb/hr p< 0.01), with no compensatory adjustments in lactate or pyruvate metabolism (Table 6.6). Notably the comparable rates of uptake of pyruvate are distinct to those reported earlier in this chapter, after insulin had been added to the medium, where upon pyrurate uptake was shown to be higher in embryos from OWOB women compared to normal weight women.

Another finding analogous with data reported in Chapter 5, was that the mean triglyceride content per embryo was significantly higher in embryos from the OWOB group (8.14ng/embryo) compared to the normal weight group (4.94 ng/embryo $p<0.01$).

Table 6.6 CORE profiles for normal (n=33) and OWOB women (n=32) in study group.

Glucose consumption was significantly lower in embryos from OWOB women (n=168) compared to those from normal weight women (n134), and triglyceride content significantly higher. There were no apparent differences in pyruvate, LC consumption or lactate production amongst the groupings

Mean values (±SEM)	*Normal weight (n=168)*	*OWOB (n=134)*	*p-value*
Glucose consumption (pmol/emb/hr)	106.51 (4.4)	69.89 (3.1)	<0.01
Lactate production (pmol/emb/hr)	129.72 (5.5)	121.17 (5.7)	n/s
Pyruvate consumption (pmol/emb/hr)	28.92 (1.3)	28.0.3 (1.5)	n/s
LC consumption (pmol/emb/hr)	20.06 (3.6)	13.06 (2.3)	n/s
Triglyceride content (ng/embryo)	4.94 (0.3)	8.13 (0.4)	<0.01

In this data set, overall blastocyst development rates were statistically comparable at 46.4%(78/168) and 38.8% (52/134) for normal and OWOB categories respectively (p=0.24); although distribution of sample data was not evenly spread between those reaching the unexpanded and hatched stages of development. As such Kruskal- Wallis test was used to evaluate the distribution of female BMI across stage of development reached and significant differences were defined (independent samples Kruskal-Wallis Test H17.4, p=0.02). Furthermore, the distribution of glucose consumption was significantly different across cleavage to hatched blastocyst development stage (H81.1, $p<0.001$).

At each development stage it was observed that glucose consumption increased with developmental progression, this differed to the pattern reported in chapter 5 but mirrors

that reported in the preceding subgroup analysis of embryos cultured with the addition of insulin. Furthermore, at each development stage consumption was lower in the OWOB category – this was more striking at the later development stages, as shown in Figure 6.14 – significant differences in pairings were determined by Bonferroni Dunn's test at the hatched, expanded and cleavage stages (p<0.05).

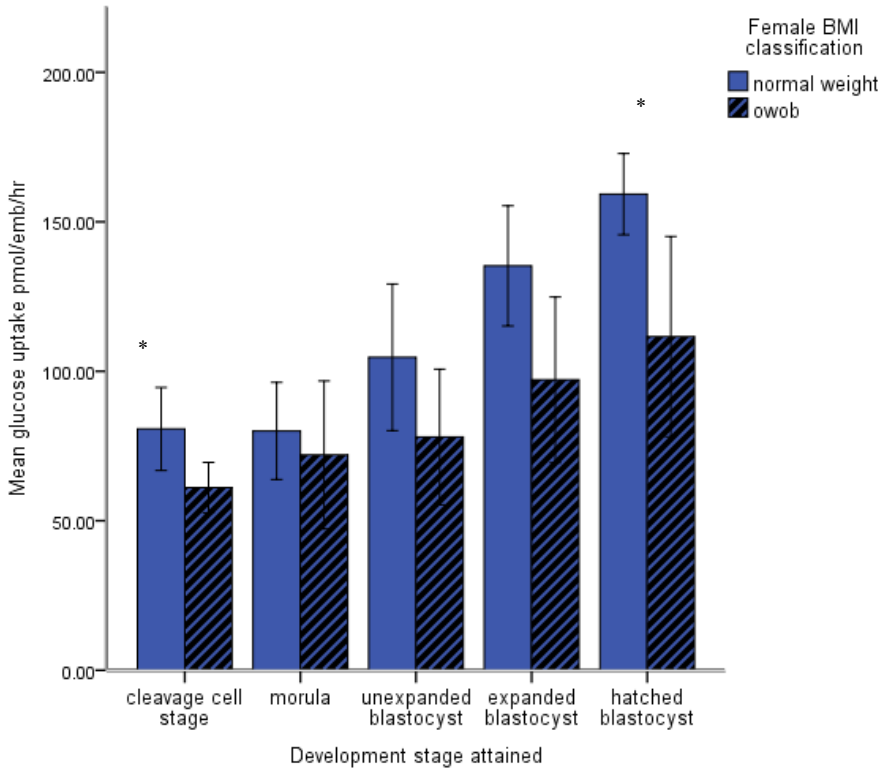


Figure 6.14 Glucose consumption at each developmental stage.

Uptake was shown to be significantly different (* p<0.05) when data was stratified into normal and OWOB groupings.

It was established via a general linear model analysis that in women of both normal and OWOB category of BMI, the addition of LC to the culture medium did not significantly affect the rates of blastocyst development, glucose consumption or lactate production. However, pyruvate consumption was shown to be significantly different amongst cohorts of embryos cultured with and without LC (p=0.02; univariate analysis) – consumption was highest when embryos were cultured in medium with 0.05mMol LC (Figure 6.15).

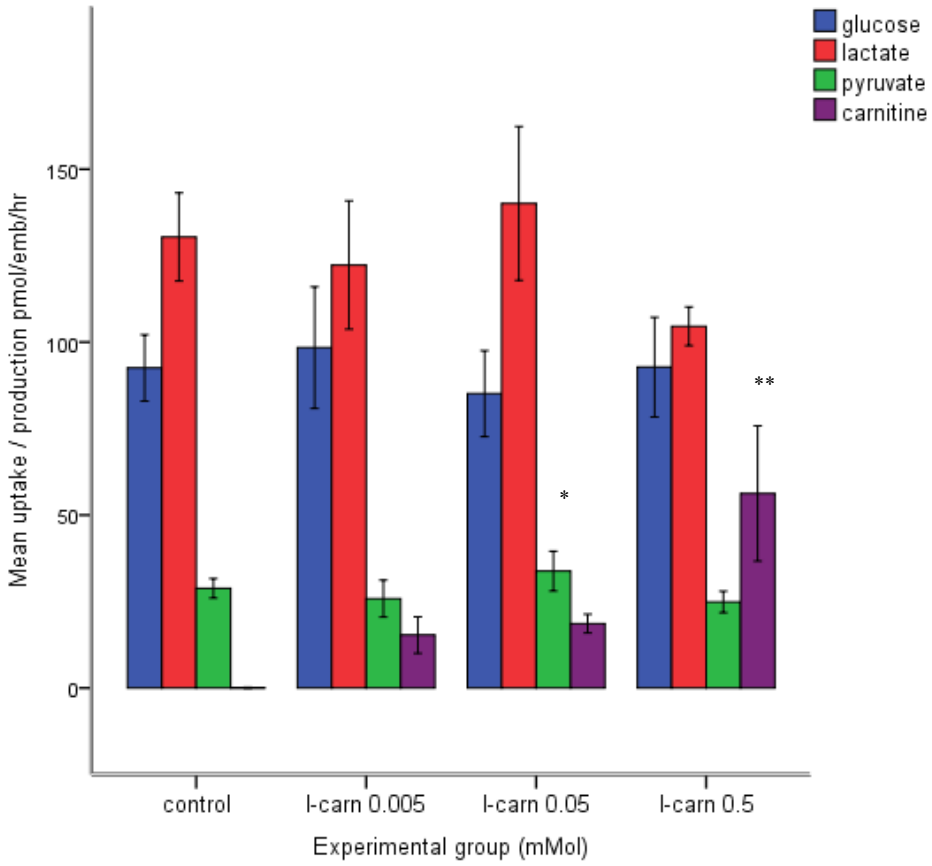


Figure 6.15 The effect of LC supplementation (0.005, 0.05, 0.5mMol)-

on mean glucose, lactate and pyruvate (*p<0.05) metabolism and the uptake of LC (**p<0.01) from supplemented medium by embryos over days 5-7 of development (n=302).

LC uptake was significantly different among the cohorts of embryos cultured in LC free medium and supplemented medium (p<0.01) – uptake was shown to be related to the concentration in the medium (Figure 6.15). LC uptake per se did not significantly differ in embryos originating from normal and OWOB women, as shown in Table 6.5, however highest rates of uptake were reported in embryos cultured in 0.5mM LC that originated from normal weight women, as shown in Figure 6.16. Furthermore LC uptake was related to stage of development (p<0.001), higher rates in hatched blastocysts originating from normal weight women.

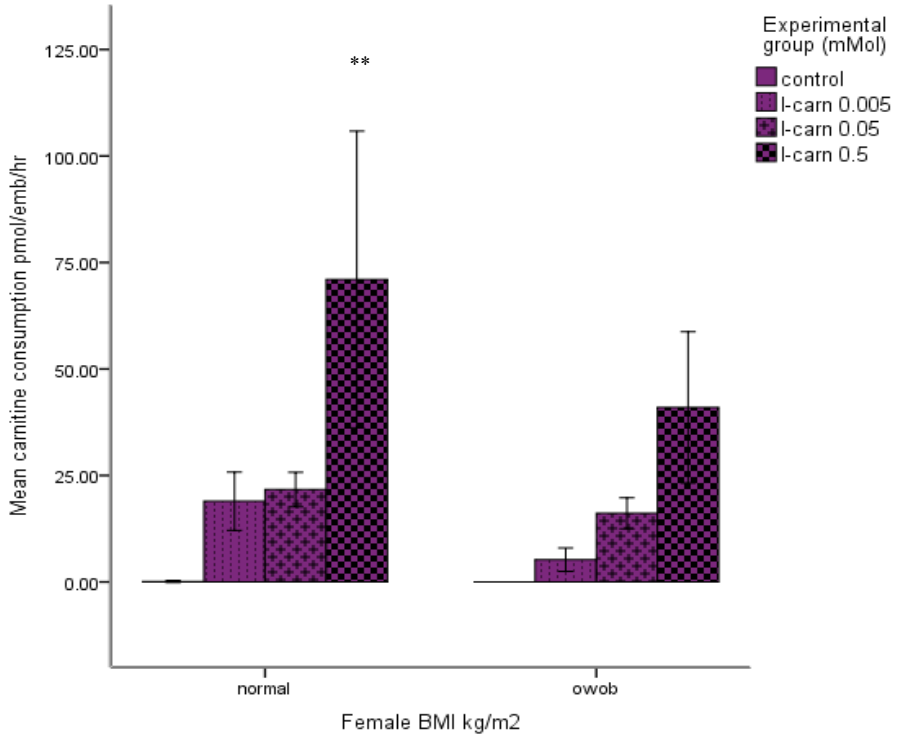


Figure 6.16 The uptake of LC by embryos from normal and OWOB women-

cultured in LC supplemented medium at concentrations from 0 to 0.5mM (**p<0.01).

LC supplementation also resulted in significant differences in the triglyceride content of cohorts of sibling embryos (p<0.01) – this was apparent at different concentrations of supplementation and differing effects were observed in cohorts of embryos originating from normal and OWOB weight women (p=0.002). There was no significant association between stage of development, female BMI and LC supplementation on the triglyceride content of embryos (p=0.92). The finding that triglyceride content, although significantly different in embryos originating from women with a BMI above and below 25kg/m^2(p<0.01), was not significantly lower in those forming blastocysts compared to those arresting prior to compaction, was not expected and is not consistent with any of the previous results reported in this thesis. Generally, triglyceride levels were lower in this study population than in earlier sections of this thesis. Increasing the concentration LC supplementation, was associated with reduced triglyceride levels in embryos from OWOB (p<0.01) and in normal weight women a similar pattern was observed, except at the highest concentration when triglyceride levels were shown to significantly higher than those reported for sibling embryos cultured in un-supplemented medium, at equivalent development stages.

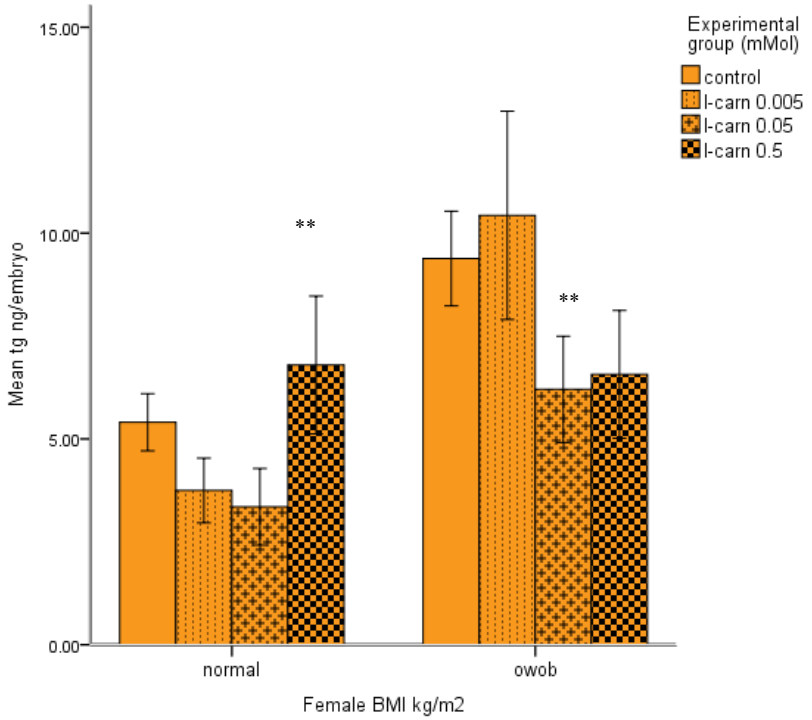


Figure 6.17 LC supplementation and embryo triglyceride content.

The effect of LC supplementation (0.005, 0.05, 0.5mMol) on embryo triglyceride content in normal and OWOB women (**p<0.01).

The data were broken down further to discover the impact on CORE glucose, lactate and pyruvate profiles of supplementation of culture medium with LC on those embryos generated from both normal and OWOB women. Significant differences were apparent for glucose uptake (p=0.021) in embryos from normal and OWOB women. In normal weight women there was a general trend for reduced glucose consumption with increasing concentration of LC in the culture medium, the exception being at 0.5mM supplementation, when glucose uptake was shown to be higher and was akin to that of embryos cultured under control conditions (Figure 6.18 A). Conversely in OWOB women LC supplementation was associated with increased glucose uptake. Differences in pyruvate consumption reported between cohorts of embryos cultured under control and 0.05mM LC were independent of female BMI, with similar uptake patterns evident in embryos originating from normal and OWOB women (Figure 6.18 C).

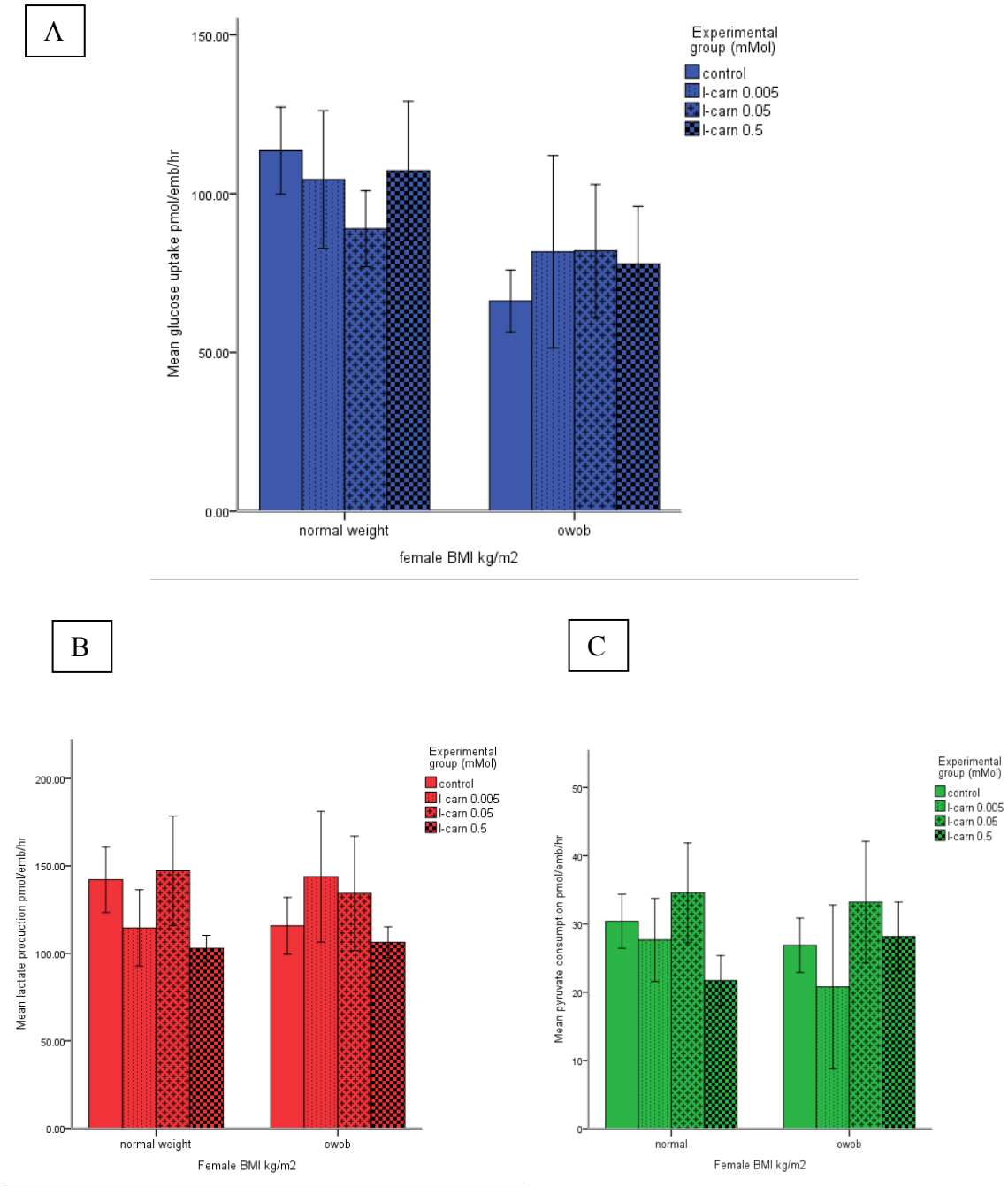


Figure 6.18 The effect of LC supplementation (0.005, 0.05, 0.5mMol) on CORE of (A) glucose, (B) lactate and (C) pyruvate in both the normal and OWOB groupings.

The addition of LC to the culture medium; increased LC uptake, altered pyruvate uptake and decreased triglyceride levels. However, increasing BMI has been associated with lower glucose utilisation and higher triglyceride levels, both of which have been

associated with lower rates of blastocyst formation. The addition of LC to the culture medium had differing effects on the pattern of substrate utilisation for embryos at different stages of development, the inclusion of these 3 variables (female BMI, embryo stage of development and LC supplementation) in a multivariate analysis was used to establish that significant between subject effects were apparent, only for glucose (p=0.008) and LC uptake (p=0.02). Only LC uptake correlated with its concentration in the medium and a general dose dependent response in uptake was evident; uptake was lowest in control medium and highest in that supplemented with the top concentration of LC. This pattern was evident for women irrespective of BMI (Figure 6.19 A), this was not the case with glucose (Figure 6.19 B).

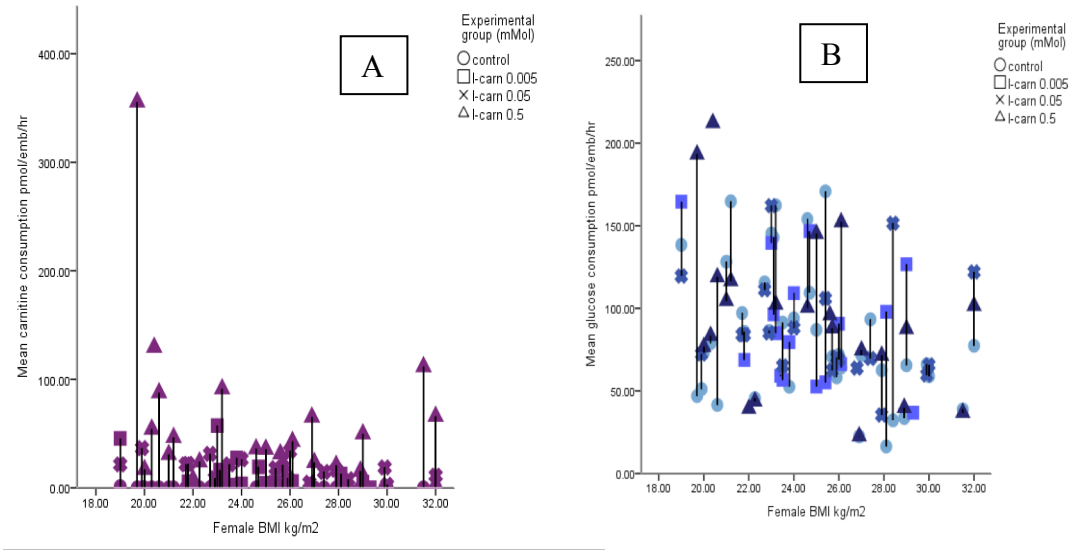


Figure 6.19 Intra-patient variability in uptake of (A) LC and (B) glucose, according to embryo culture conditions (control and supplemented with LC at 0.005, 0.05, and 0.5mM concentration).

Mean values are depicted for sibling embryos for each patient (connected line) and related to female BMI.

Finally, to relate the findings discussed so far in this LC study group, to the designated study end point – blastocyst development rate a logistic regression was performed (Table 6.7). The effects of female BMI, glucose, lactate and pyruvate consumption/release and triglyceride content and experimental group (control / l-carnitine 0.005-0.5mMol) on the likelihood that embryos would reach the blastocyst stage were evaluated. The logistic regression model explained 40% (Nagelkerke R^2) of the variance in blastocyst formation/ development and correctly classified 56.8% of cases.

Unexpectedly, embryos from normal weight women were 0.3 times less likely to form blastocysts than those from OWOB females (a similar finding was reported from the insulin data model) but increasing glucose consumption was associated with an increased likelihood of blastocyst development. Surprisingly LC supplementation did not influence blastocyst formation rates but increased uptake was associated with lower rates of blastocyst formation and in this instance higher triglyceride content was associated with higher rates of blastocyst formation. This would indicate that an optimum mid-range of values is associated with higher rates of blastocyst formation.

Table 6.7 Binominal logistic regression;

was used to evaluate the statistical significance for each of the independent variables to predict blastocyst development. Glucose and female BMI were shown to be significant (***p<0.001, **p<0.01). The addition of l-carnitine to the culture medium did not influence the probability of blastocyst formation.

Binominal Log regression	Odds Ratio (95% C.I)
OWOB (0), Normal (1)	0.37 (0.19-0.69)**
Glucose uptake	1.02 (1.01-1.02)***
Lactate production	1.00 (0.99-1.00)
Pyruvate uptake	1.00 (0.99-1.01)
triglyceride content	1.02 (1.01-1.03)**
l-carnitine uptake	0.77 (0.69-0.84)
l-carnitine (0), Control (1)	1.72 (0.87-3.4)

From the figures presented it was apparent that at 0.05mM LC supplementation resulted in more optimized values of CORE metabolites in OWOB women. At this concentration triglyceride levels were lowest and glucose consumption was highest. At the highest concentrations of culture medium supplementation, LC uptake was also highest and resulted in pronounced disturbances in the patterns of substrate utilisation in normal weight women; notably triglyceride was no longer depleted.

In terms of the clinical outcomes associated with transferred sibling embryos, the clinical pregnancy rate for normal weight women in this sub-study group were 48.5% (16/33) and 15.6% (5/32), the associated respective live birth rates were 9/16 deliveries, plus 4 ongoing pregnancies in the normal weight group and 0 delivered, with 4 ongoing in the OWOB group. The clinical outcomes for this sub-study group are poorer than

those reported in the previous sections, although no significant differences are evident in CORE viability markers between this study population and that of chapter 5.

6.3.3 The addition of LC to the embryo culture medium of frozen and thawed embryos

A total of 21 women of normal weight and 5 OWOB women donated their embryos that had been cryopreserved to research. From these, 79 embryos from normal weight women and 27 embryos from OWOB were thawed and sibling embryos were divided between culture in standard and 0.5mM supplemented culture medium. The addition of LC to the culture medium did not significantly affect the rates of blastocyst development, akin to findings reported in the fresh embryo category. However, the overall rates of blastocyst development post-cryopreservation were significantly lower in the OWOB grouping (4/27 14.8%) compared to the normal weight group (47/79 59.5%) $p<0.001$, as observed in Chapter 3.

In frozen treatment cycles, the metabolism of substrates; glucose, lactate and pyruvate and the triglyceride content of embryos was significantly different in embryos generated from different BMI groupings (assuming an equal split between control and LC supplemented culture).

Table 6.8 CORE profiles for normal (n=21) and OWOB women (n=5) in LC/ control groups.

CORE of glucose, lactate and pyruvate were shown to be significantly lower in embryos from OWOB and triglyceride content was significantly higher in embryos from OWOB women (n=27) compared to those from normal weight women (n=79). There were no apparent differences in LC consumption.

Mean values (±SEM)	*Normal weight*	*OWOB*	*p-value*
Glucose consumption (pmol/emb/hr)	85.12 (7.4)	28.74 (3.9)	<0.01
Lactate production (pmol/emb/hr)	100.41 (6.1)	61.52 (7.4)	<0.01
Pyruvate consumption (pmol/emb/hr)	31.17 (2.5)	14.14 (2.1)	<0.01
LC consumption (pmol/emb/hr)	16.96 (3.1)	20.54 (4.8)	n/s
Triglyceride content (ng/emb)	6.34 (0.4)	13.69 (0.6)	<0.01

When the data are classified according to the culture conditions to which embryos were exposed, supplementation with LC resulted in a trend for higher lactate production (99.6 pmol/emb/hr) than control conditions (80.9 pmol/emb/hr p=0.06). No differences were evident in glucose or pyruvate consumption or indeed triglyceride content of embryos; 7.9ng and 8.9ng/embryo for the respective groupings. When the culture medium was supplemented with LC it was taken up in significant quantities (33.9pmol/emb/hr) (Figure 6.20). The CORE of substrates were lower than those of embryos originating from fresh embryos in the previous section, furthermore in fresh cycles the production of lactate tended to be lower in embryos exposed to culture medium supplemented with LC, compared to those cultured under standard conditions.

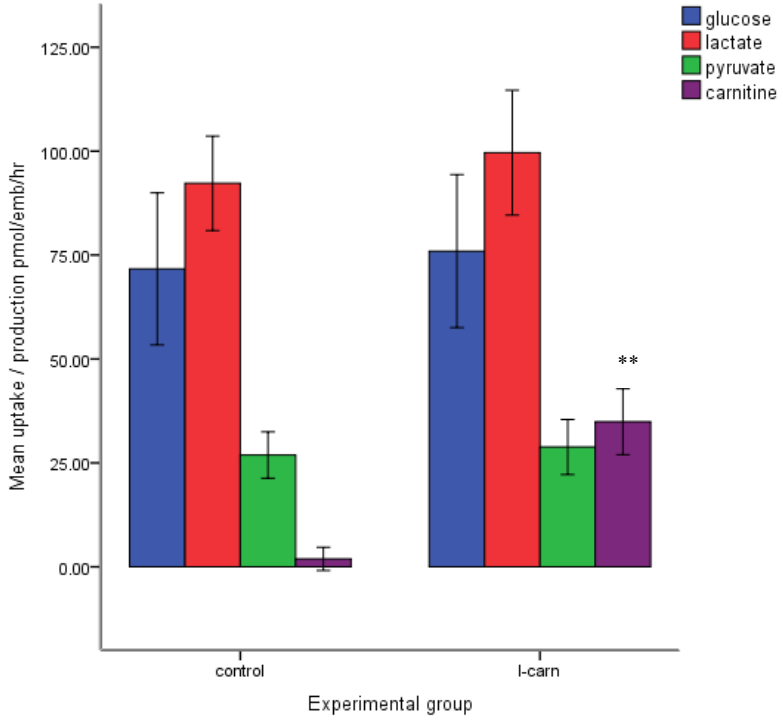


Figure 6.20 The effect of LC supplementation on CORE profile of frozen thawed embryos.

The effect of LC supplementation (0.5mMol) on mean glucose, lactate and pyruvate metabolism and the measured uptake of LC (**p<0.01) from supplemented medium by embryos over days 5-7 of development (n=106).

The addition of LC did not influence substrate utilisation when the data were stratified into groupings according to female BMI (kg/m2). Unlike the findings reported for fresh embryos at 0.5mM supplementation, no significant differences were evident in triglyceride content of embryos from normal weight women cultured in standard

medium (6.64ng/embryo) and those that were culture in supplemented medium (6.06ng/embryo).

A binominal logistic regression model was constructed for normal and OWOB women using experimental group as the dependent variable (control -0, LC -1) and glucose, lactate, pyruvate, triglyceride as covariates. Embryos were no more/ less likely to have a higher metabolism of substrates based upon the culture medium from which they had originated. There was a trend for higher lactate release from embryos of normal weight women cultured in medium supplemented with l-carnitine (B 0.02, p =0.08), as depicted in Figure 6.21, this was not evident in the OWOB group.

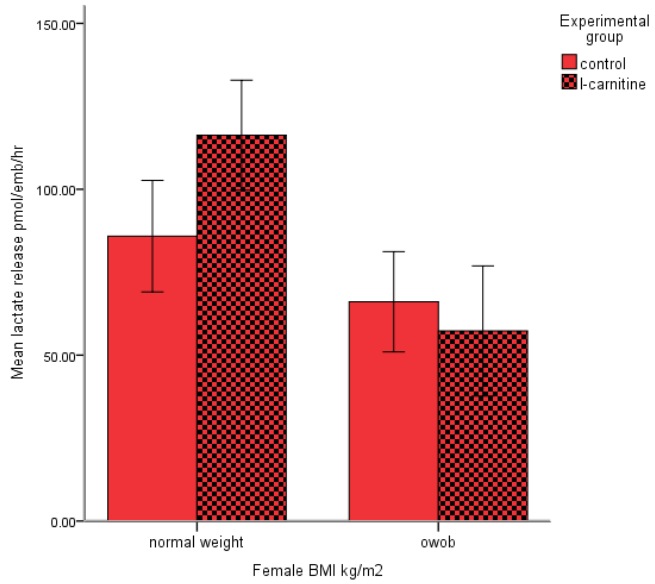


Figure 6.21 The effect of LC supplementation (0.5mMol) on lactate production in both the normal and OWOB groupings (frozen/ thawed embryos).

The clinical pregnancy rates reported from the transfer of sibling embryos in the corresponding fresh treatment cycles were obtained. In the normal weight group 15/21 (71.4%) of the cycles had resulted in a clinical pregnancy and all had resulted in a live birth. In the OWOB group, a clinical pregnancy had been established in 3/5 (60%) of the cycles and again all resulted in a live birth. The birth weights of offspring born to normal and OWOB women were comparable 3175.27g compared to 3175.0g. The high clinical pregnancy rates and comparable birth weights suggest that the embryos that had been transferred in these cycles in both normal and OWOB were of comparably high implantation potential. The sibling cryopreserved embryos from OWOB women did not

appear to exhibit profiles consistent with high viability – hence the influence of cryopreservation on metabolic biomarkers was assessed in the next section.

6.3.4 The influence of cryopreservation per se on CORE profiles

A comparison of two study populations was conducted; those having a fresh treatment cycle (n=26 patients, 99 embryos) and those having a frozen treatment cycle (n=26 patients, 106 embryos). A covariate to this analysis was the fact was that within each treatment group a proportion of embryos were cultured under control conditions and a proportion in 0.5mMol LC. Likewise, a proportion would have been generated from normal weight and an assumed equivalent number from OWOB women. Independent samples Mann-Whitney U Test was used to confirm these assumptions.

The uptake of glucose was significantly lower in embryos that had been cryopreserved compared to those from fresh treatment cycles ($p<0.01$; Figure 6.22) and this in spite of the higher rates of blastocyst formation in embryos belonging to this frozen cohort.

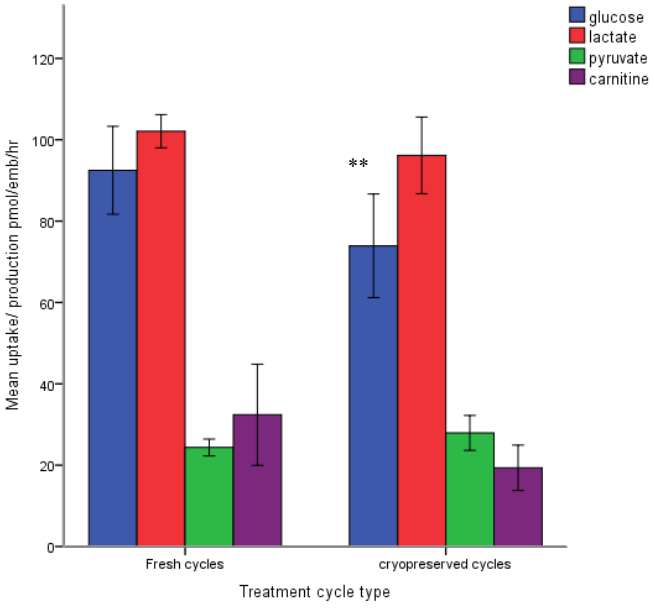


Figure 6.22 Comparison of CORE profile of fresh and frozen embryos.

The effect of cryopreservation on mean glucose (**$p<0.01$), lactate and pyruvate metabolism and the measured uptake of LC from (0.5Mm LC) supplemented medium by embryos over days 5-7 of development.

A binominal logistic regression model was constructed to identify significant differences among the populations of embryos that had been assayed from fresh cycles and frozen cycles (Table 6.9). There were no significant differences in BMI amongst the groups, glucose consumption was significantly different, as were pregnancy rates and rates of blastocyst formation.

Table 6.9 Binominal logistic regression;

was used to evaluate the statistical differences among cohorts of embryos from fresh and frozen cycles., $^{**}p<0.01$, $^{***}p<0.001$.

Binominal Log regression	Odds Ratio (95% C.I)
OWOB (0), Normal (1)	1.14 (0.94-1.38)
Glucose uptake	1.02 (1.01-1.04)**
Lactate production	1.01 (0.99-1.03)
Pyruvate uptake	0.96 (0.92-1.01)
l-carnitine uptake	0.63 (0.00-3.69)
Tg content	0.84 (0.71-1.01)
Clinical pregnancy	11.51 (3.26-40.58)***
Blastocyst formation	0.12 (0.22-0.61)**

Embryos from frozen cycles display different patterns of substrate utilisation to those of fresh cycles.

6.3.5 The influence of Intralipid treatment

In the course of this study it was discovered that a subset of 5 patients had been given intralipid therapy as part of their clinical treatment plan; two of whom were of normal weight and three OWOB. A total of 34 embryos (17 from each category) were donated to research post therapy and subject to assessment.

It was determined that despite following the general reported trend in previous sections glucose concentrations in embryos from OWOB women (75.78 pmol/emb/hr) were not significantly lower than those from normal weight women (107.15 pmol/emb/hr $p=0.06$). Pyruvate consumption was comparable, as was LC consumption, however there was a trend for higher LC consumption in embryos from OWOB (16.86 pmol/emb/hr) compared to embryos from normal weight women (7.42 pmol/emb/hr; $p=0.12$). In contrast to the previous findings, blastocyst development and the

triglyceride content of embryos were comparable between the BMI groupings (10/17 forming blastocysts in each category, with 3.32 and 3.25 ng/emb triglyceride for normal and OWOB respectively).

Lactate production from embryos of normal weight women who had been given intralipid therapy was significantly higher (184.22 pmol/emb/hr) than that from the embryos of OWOB women (101.98 pmol/emb/hr) given the therapy (p<0.001; Figure 6.23). In the normal weight grouping this is notably higher than the lactate values reported in the preceding subsections of this chapter, in which mean production has ranged from 92 to 129pmol/emb/hr for normal weight women and 61 to 121 pmol/emb/hr for OWOB women.

All 5 of the women treated had undergone at least one previous failed cycle of treatment, despite attaining a 'top grade' (≥3Bb) blastocyst for transfer. After treatment with intralipid 3 of the 5 women achieved a clinical pregnancy.

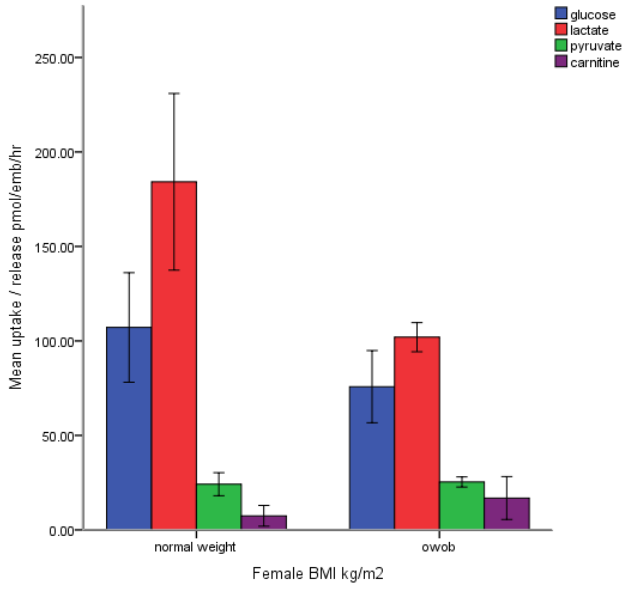


Figure 6.23 Substrate metabolism of embryos generated from women who had received intralipid therapy (n=34 total). Embryos from normal weight women (n=17) display significantly higher lactate production than those form OWOB (n=17) (p<0.001).

It was discovered that 3 of the 5 patients (1 normal weight and 2 OWOB) had previously donated embryos to research in their prior treatment cycles, thus permitting comparisons to be drawn between cycles pre and post intervention. In the repeat cycles

(plus intralipid) it was noted that embryos consumed significantly lower amounts of pyruvate and the mean triglyceride content of embryos at the end of the culture period was significantly lower compared to that of the embryos generated from their initial treatment cycle.

Table 6.10 Patient control cycle (1) and intralipid treatment cycle (2) comparisons

(n=3 women); Post intralipid therapy pyruvate consumption was significantly lower than in the previous cycle of treatment and the triglyceride content of embryos was significantly lower in this cohort (n=14 embryos) compared to their counterparts in the previous control treatment cycle (n=17 embryos).

Mean values (±SEM)	*Cycle 1 – control, minus intralipid*	*Cycle 2 –plus intralipid*	*p-value*
Blastocyst development (%)	7/17	7/14	n/s
Glucose consumption (pmol/emb/hr)	92.88 (9.8)	78.47 (10.8)	n/s
Lactate production (pmol/emb/hr)	98.76 (14.1)	93.48 (6.1)	n/s
Pyruvate consumption (pmol/emb/hr)	39.26 (5.2)	23.21 (0.9)	<0.01
Triglyceride content (ng/emb)	9.5 (1.45)	2.6 (0.47)	<0.001
Clinical pregnancy (%)	0/3	2/3	n/s

The inter-cycle differences were only significant in the cohort of embryos (n=21) generated from the two OWOB women (triglyceride $p<0.001$, pyruvate $p=0.05$), although the cohort of embryos from the normal weight woman (n=10), did mirror these trends.

6.4 Discussion

6.4.1 Summary of the key findings

The experiments presented in this chapter have explored the inclusion of culture media supplements; insulin and LC, selected as promising candidates to not only improve embryo development *in vitro*, but to improve embryo viability, as assessed by metabolic activity.

The embryos donated to research were at different stages of development; cleavage to compaction or very early poor quality blastocysts not suitable for use in clinical treatment. This presented a window of opportunity, where the pluripotency of embryos previously exposed to perturbing conditions might be improved (Campbell et al., 2013). The findings demonstrated that embryos from women with a BMI >25kg/m^2 responded in a different manner to those from normal weight women. Overall glucose consumption was not significantly altered by the addition of insulin to the culture medium. Insulin may however, improve blastocyst development/ cell survival, particularly for embryos from OWOB women. LC was taken up in significant quantities by embryos and triglyceride levels were significantly different as a result. An optimum mid-range for both LC and triglyceride was apparent.

6.4.2 Insulin; blastocyst development and glucose consumption

In this study the addition of insulin to *in vitro* culture did not alter glucose utilisation in embryos originating from normal and OWOB women. Many of the reported adverse effects associated with obesity and hyperglycemia are postulated to originate in the ovary and impaired maternal insulin secretion pre-conception can have detrimental effects on embryo development that persist even after removal from the maternal environment.

There are a number of possible explanations why the addition of insulin to the culture media had no effect on glucose consumption in either group this; firstly, at the concentration added (0.5ng/ml), it could have been too low to have an effect. This has been speculated as a cause of differing responses among different species (Matsui et al., 1997). Secondly; the effect could be dependent upon the stage of development of the embryo prior to insulin exposure i.e. embryos introduced at the cleavage stage of development may not have expressed insulin sensitive GLUT transporters, the

expression of which remains a matter of controversy in human embryos (Purcell et al., 2009).

6.4.3 Insulin; effects in normal and OWOB women

The addition of insulin to the culture medium resulted in; (1) a trend for increased blastocyst formation, (2) significantly lower pyruvate consumption at both the cleavage stage and the hatched blastocyst stage of development and (3) an altered pattern of amino acid utilisation, notably; methionine depletion and phenylalanine production. These findings were not independent of female BMI and considerably higher rates of blastocyst formation were reported from the OWOB group than expected on the basis of past results. Likewise, pyruvate consumption by embryos from OWOB women was higher than expected and the past low rates of substrate utilisation, observed in both cohorts at the unexpanded stage of development were not replicated in these experiments.

One interpretation of these novel findings is that insulin supplementation may have supported the development of embryos from OWOB women that would have otherwise undergone developmental arrest. The rates of blastocyst development were comparable among normal and OWOB women, and there was a trend for higher blastocyst rates from embryos of OWOB women cultured in supplemented medium compared to their siblings cultured in standard media. Insulin, possibly acting through the IGF-1 receptor may support development in this cohort by decreasing apoptosis and increasing cell proliferation. Insulin may promote cell survival by inhibiting ROS generation and has been shown to be antioxidative by inducing Bcl-2 and Bcl-XL via the IGF-1 receptor (Herrler et al., 1998).

The promoted development supported by insulin, may in part explain why substrate utilisation by the OWOB unexpanded blastocyst was not suppressed, as reported in chapter 5. The variability in rate of uptake was considerably less, suggesting that those embryos that had reached this stage on day 7 may still have been capable of further development, as opposed to the majority having simply arrested due to an inability to meet the energy demands of blastocyst expansion. Blastocyst formation is a period of rapid growth requiring increased glucose uptake.

The caveat to this is that the stepwise progressive increase in glucose uptake was also observed in embryos cultured under standard conditions and glucose consumption was not significantly different among sibling embryos that had not been exposed to insulin. It is thus possible that the reduced period of extended culture from day 9 to day 7 of development resulted in a more synchronous development patterns. A further point to consider is that GLUT1 and GLUT4 transcription are reported to be influenced by other environmental conditions, including oxygen, which is reported to induce their expression (Behrooz et al., 1997) and this may explain the different pattern in glucose metabolism at each stage of development reported for this study population, compared to that in chapter 5. An upgrade to research laboratory facilities meant that embryos in this study had been cultured under low oxygen throughout their development (i.e. in both the clinical and research setting).

It had been speculated in Chapter 5, that a co-regulatory cycle exists in the embryo, as in the whole body between glucose and fatty acids (Randle et al., 1963) and the inference was that the energy needs of embryos from OWOB women were met by enhanced β-oxidation of fatty acids. However, and in contrast to data from Chapter 5, it was reported here that the embryos of OWOB women cultured in standard culture medium may have compensated for their diminished uptake of glucose, by using pyruvate as a substrate for ATP generation, via complete oxidation in the TCA cycle, as there was no difference in lactate production. Nevertheless, as two molecules of pyruvate are produced from the glycolysis of one molecule of glucose, the increased uptake of pyruvate may not have been sufficient to compensate completely for lower glucose uptake.

An excess of pyruvate can impair development (Dumollard et al., 2007) and an optimal range of pyruvate consumption has been described in cleavage stage embryos correlating with blastocyst development (Guerif et al., 2013). Pyruvate consumption was highest in embryos of OWOB that arrested at the cleavage stage. At the cleavage stages pyruvate is the preferred substrate of mouse embryos (Leese & Barton, 1984) and glucose uptake is low partly due to limited expression of GLUT1 (Pantaleon et al., 1997). Insulin may increase expression of GLUT transporters, resulting in the premature stimulation of glycolysis glucose being favoured over pyruvate and may explain the significantly lower rates of pyruvate consumption in embryos arrested at this stage after

insulin supplementation. This supports the notion of an optimum mid-range for pyruvate consumption.

In the whole body system, high insulin stimulates the storage of lipids, by stimulating phosphodiesterase-3B, which degrades cyclic AMP, preventing activation of protein kinase A and suppresses the activity of hormone-sensitive lipase in adipose tissue. At the cellular level, *in vitro* cultured embryos of OWOB women, surprisingly reduced their pyruvate uptake after the addition of insulin, assumingly preferentially utilising endogenous lipid to provide Acetyl-CoA for the TCA cycle. Given that this was not seen in sibling embryos cultured in standard medium, it would suggest that the embryos of OWOB women are insulin sensitive; but in both normal and OWOB insulin does not affect glucose transport in the same way. Insulin may act via IGF-1 receptor (Pantaleon & Kaye, 1996) to increase cellular proliferation and metabolic demands for increased growth are met by β-oxidation of fatty acids, in embryos of OWOB women. In support of this conjecture, a higher proportion of embryos from OWOB women cultured in insulin supplemented medium survived to reach the hatched blastocyst stage and moreover pyruvate consumption was significantly lower than those cultured in standard medium.

The altered amino acid turnover observed after insulin supplementation, suggests that these too may be used as energy substrates, entering the TCA cycle at various points. Insulin may facilitate amino acid transport and Koo et al., (1997) demonstrated that insulin and amino acids act synergistically and enhance blastocoel formation in porcine embryos. Increased endogenous protein synthesis has been reported following short term exposure to insulin (Dunglison & Kaye, 1993). Amino acid turnover was shown to be influenced by the addition of insulin and differences were reported in methionine depletion and phenylalanine production, although these were not independent of female BMI. This disrupted methionine metabolism is consistent with the findings reported in Chapter 5 and is important, as such disruption could lead to altered methylation of DNA and expression of developmentally important genes (Ikeda et al., 2012).

6.4.4 Insulin *in vitro* summary

The key message of this section is that, the beneficial effects of insulin on blastocyst development reported for the *in vitro* development of bovine (Matsui et al., 1995), rabbit (Grundker et al., 1996), mouse (Kaye & Gardner, 1999) and here for human

embryos are not necessarily associated with glucose uptake. Insulin had little impact on glucose uptake in human embryos, which may in part suggest that reduced transport by GLUT transporters is unlikely to account for the lower rates of uptake among embryos from OWOB women compared to normal weight women. It is therefore more probable that the utilization of glucose is lower in embryos from OWOB women as alternative substrates are being used.

6.4.5 LC effects on carbohydrate metabolism in normal and OWOB women

In chapter 5 it was shown that human embryos have an endogenous store of triglyceride, which may be affected by maternal diet. This is also consistent with findings reported in the bovine (Leroy et al., 2014). In this chapter LC was used to manipulate the metabolism of these endogenous triglyceride by promoting fatty acid β-oxidation. The addition of LC to the culture medium influenced embryo development and the metabolism of other substrates; furthermore it was possible to resolve differences between embryos from normal and OWOB women. The effects observed were specific to the concentration of LC used to supplement the culture medium.

A general finding from this study sub-population was that glucose consumption was significantly lower in embryos from OWOB women compared to those of normal weight. Concomitantly, glucose consumption was shown to have increased with stage of development reached by individual embryos and this corroborates earlier findings from the other sub-population studied in this chapter (insulin data) and is contrary to the findings reported in chapter 5. This would suggest that rather than media supplements promoting survival and reducing the rates of arrest prior to blastocyst expansion, the change in study conditions to low oxygen culture and 7 days observation, rather than 9 may have been the principle drivers for this difference (see chapter 7 for further analysis).

Addition of LC to the culture media at concentrations ranging from 0 to 0.5mM, led to altered responses in the cohorts of embryos from both normal and OWOB women. LC supplementation influenced carbohydrate metabolism, but the stage of development that an embryo had reached prior to being placed into supplemented medium is likely to complicate any conclusions. This may be particularly important, as CPTIB expression, although confirmed in mouse blastocysts, was not expressed at the 8-cell stages in mice (Dunning et al., 2010). At a concentration of 0.05mM LC supplementation resulted in

significantly higher rates of uptake of pyruvate, however in a multivariate analysis including stage of embryo development and female BMI, glucose consumption was shown to be influenced by LC supplementation. A divergent pattern of utilisation was observed in OWOB women; glucose consumption tended to be marginally higher at higher concentrations of LC. In embryos from normal weight women, glucose consumption was lower at higher LC concentration, with the exception of 0.5mM LC, when consumption was equivalent to embryos cultured in un-supplemented media. One explanation for this observation is that LC may enable embryos from normal weight women to utilise fatty acids instead of glucose, until a threshold concentration is reached at which point alternative metabolic pathways must take over.

Provision of LC has been shown to improved glucose tolerance in insulin resistant rat models (Cave et al., 2008). On the basis of this, the observed obesity-induced lower glucose uptake by blastocysts maybe responsive to metabolic correction if LC was used to deplete triglyceride stores. It would appear that at the concentrations added this was insufficient to elicit a significantly altered response (across all stages of development) or, perhaps as other studies have added LC during oocyte maturation (Sutton-McDowall et al 2012) it was perhaps added too late. As embryo development progresses, the embryo may become less responsive to modulation with LC.

6.4.6 LC consumption and triglyceride levels

A further general observation was that triglyceride levels were significantly higher in embryos generated from OWOB women compared to those of normal weight. Theoretically LC supplementation should act to facilitate increased fatty acid β-oxidation and has been reported to improve embryo developmental competence in mammalian species (Downs et al., 2009, Dunning et al., 2012, Sutton-McDowall et al., 2012). In the present study the addition of LC did not appear to influence rates of blastocyst development, and in a logistic regression model, LC consumption was actually correlated with lower rates of blastocyst development. Furthermore, in contrast to previous findings higher levels of triglyceride were associated with blastocyst formation.

Interestingly the amount of LC consumed and not the amount supplemented, was the significant factor in the model. In general, increasing the concentration of LC in the culture medium resulted in uniformly higher rates of uptake by embryos of normal and

OWOB women, but at the highest concentration 0.5mM a sharp increase in uptake was observed in embryos from women of BMI <25kg/m^2. A corresponding drop in triglyceride levels with LC uptake was demonstrated in embryos from OWOB women. However, in the embryos from women of normal weight, supplementation at the higher concentration of 0.5mMol LC led to a significant increase/ retention of triglyceride. This pronounced difference may be due to higher rates of cleavage stage arrest, possibly implying that at this concentration LC becomes toxic for embryos.

The response in uptake of LC and ability to utilize it for β-oxidation of endogenous lipid stores is also likely to be determined by the stage of development, as during development positional changes in lipid droplets are known to occur. In mice oocytes an initial centrally located lipid droplet becomes larger and droplets are peripherally distributed as development progresses (Wood et al., 2008). Similarly, in porcine co-localised mitochondria and lipid droplets tended to migrate to the periphery of the cell, leading the authors to suggest that lipids form functional units with organelles (Sturmey et al., 2006). In the oocyte, LC has been shown to reduce the amount of lipid droplets and change their position from the cortex to the centre (Chankitisakul et al., 2013),whilst the movement of lipids and associated organelles away from the membrane, may preserve their integrity during freezing it may not be beneficial for immediate development. The reason lipid/mitochondrial functional organelles are hypothesized to move to the periphery is in response to oxygen availability for metabolism (Sturmey et al., 2006).

6.4.7 LC consumption and frozen thawed embryos

Embryos that had been cryopreserved displayed the hallmarks of those with low viability; lower pregnancy rates from sibling embryo transfers and glucose consumption significantly lower than that of fresh controls, and indeed, far lower than that reported in other study groupings in this thesis. This is however at odds with the higher rates of blastocyst formation from frozen embryos compared to the control group. Blastocysts developed from frozen embryos have a lower cell count, as prior cryo-injury and cell loss results in their formation from a lower number of progenitor cells and consequently the lower glucose consumption is reflective of the lower cell count. This may also, in part explain the exceptionally low glucose consumption reported for cryopreserved embryos from OWOB women (28.7pmol/emb/hr), as this is consistent with findings in

chapter 4 that blastocyst cell numbers were lower for OWOB and the report of lower cryosurvival in chapter 3, compared to blastocysts from normal weight women.

Embryos from OWOB women were the most vulnerable post-thaw, with lactate and pyruvate consumption also significantly lower than that of embryos from women <25kg/m^2. However, LC supplementation appeared to offer little benefit. There were no significant differences in triglyceride levels of embryos cultured in control or LC supplemented conditions, furthermore post-thaw developmental potential did not appear to have been influenced by the supplementation. Conceivably, the burden of large amounts of intracellular lipids had already compromised the post-thaw embryo quality of OWOB women, owing to impaired mitochondrial function, increased sensitivity to oxidative stress leading to increased free radical generation and increased cell death (Abe et al., 2002).

In this study sub-group LC was added at the higher concentration of 0.5mM and rather than appearing to have a negative influence on embryo development in women <25kg/m^2, as observed in the fresh grouping, there appeared to have been some marginal benefits. This is perhaps owing to the antiapoptotic action/ antioxidant actions of LC on this cohort of embryos, that were not as metabolically active and therefore uptake of LC was considerably less than in fresh embryos exposed to this concentration.

6.4.8 LC *in vitro* summary

These data show, for the first time, that human embryos are capable of taking up LC in significant quantities from the culture medium, in an almost dose dependent manner. In embryos donated from fresh treatment cycles, supplementation with LC up to a concentration of 0.05mM it led to lower triglyceride levels, implying β-oxidation had been promoted.

It appeared that there are optimum levels of LC consumption that correspond with embryo viability, particularly for cohorts of embryos from women of above and below 25kg/m^2. The impact of LC supplementation on CORE profile of metabolites and blastocyst development appeared to be dependent on(1) the size of the initial store of triglyceride (2) the stage of development attained prior to supplementation and (3) the level of prior oxidative stress / cellular damage.

From the study utilising different concentrations of LC it was apparent that at 0.05mM LC supplementation resulted in more optimized values of CORE metabolites in OWOB women. At this concentration triglyceride levels were lowest and glucose consumption was highest. At the highest concentrations of culture medium supplementation, LC uptake was also highest and resulted in pronounced disturbances in the patterns of substrate utilisation in normal weight women; notably triglyceride was no longer depleted.

6.4.9 Intralipid studies

In the course of this study, it came to light that a small number of the participants, who had, had a previous, unexpected implantation failure received intralipid infusions (20% -100ml). In a number of cases embryo metabolic data from the patient's previous failed treatment cycle was also available and reviewed for both normal and OWOB women.

The data presented provides some insight in to the effects of intralipid therapy on human embryo metabolism, which previous studies have overlooked. It is generally accepted that the chemical profile of the follicular fluid reflects that of the plasma (Rogers & Irvine-Rogers 2010), with measures of lipid components of follicular fluid being directly correlated to plasma concentrations (Valckx et al., 2012) and being notably elevated in the follicular fluid of overweight women (Robker et al., 2009). It is not known if the intralipid infusion that patients received during the mid-stimulation phase of follicular recruitment/ maturation was sufficient to alter the fatty acid profile of the follicular fluid and for how long the effect may have lasted. Intralipid likely contains additional lipids to linolenic and linoleic acid, namely palmitic acid, steric acid and oleic acid. In the bovine addition of palmitic or steric acid to *in vitro* culture during oocyte maturation had a negative effect on meiotic maturation, fertilization and embryo development (Leroy et al., 2005). Van Hoeck et al (2011) demonstrated that exposure of maturing bovine oocytes to elevated NEFA reduced oocyte developmental capacity, embryo viability and metabolism.

These initial findings reported here appeared to suggest that intralipid altered embryo metabolism. It was observed that after exposure to intralipid embryos significantly increased their production of lactate; this was particularly evident in the cohort of embryos from the women of normal weight. This is indicative of high rates of glycolysis, which is favored under times of oxidative stress and may minimize the

production of reactive oxygen species (Kobayahi & Suda 2012), it is also advantageous during periods of rapid cell division (Warburg 1956). In addition increased lactate production may aid implantation- possibly by promoting endometrial tissue breakdown and providing carbon and NADPH (catabolism of glutamine into lactate) to facilitate biosynthesis (Gardner et al., 2015).

Studies reporting on the relationship between the metabolic profile of follicular fluid and oocyte developmental competence have reported that lower granulosa cell lactate production is associated with reduced oocyte developmental competence and pregnancy rates (Wallace et al., 2012, Rice et al., 2005). However, it may also be speculated that excess lactate production is indicative of a 'stress response', suggestive of compromised mitochondrial function – pyruvate could be being shunted away from oxidation in the mitochondria and reduced oxidative metabolism may lead to chronic reduction in ATP levels, associated with implantation failure (Van Blerkom et al., 1995).

A surprising finding was that intralipid resulted in comparable concentrations of triglyceride in embryos from OWOB women, and normal weight women. Data from animal models have shown that, when cultured in the presence of high fat, oocytes and embryos increase their intracellular triglyceride (reviewed by McKeegan & Sturmey 2012). At first glance it may therefore be assumed that the comparable triglyceride content of embryos from women of differing BMI could be explained on the basis that oocytes developing in the follicles of OWOB women are already saturated with triglyceride, whereas the embryos from normal weight women are capable of accumulating additional lipid. This does not appear to be the case; values from normal weight women are comparable to those reported earlier, but values from embryos of OWOB women are lower than expected and are significantly lower in the same patient in cycle 2 (after intralipid) compared to values attained in cycle 1 (without intralipid).

This pilot data would seem to suggest that an embryo's endogenous lipid stores and their rate of utilization maybe inducible by transient changes in the ratio and supply of fatty acid in the follicular fluid bathing the maturing oocyte. If we are to assume that intralipid infusion increases the lipid content of follicular fluid, the reported decrease in triglyceride content of embryos from OWOB is contra to the findings in animal studies and the previously inferred results of this thesis. One possible explanation is that the intralipid infusions are sufficient to alter the availability of individual fatty acids (i.e. the

ratio of unsaturated to saturated fatty acids). Previous studies have shown that developing embryos have higher concentrations of linoleic acid and lower saturates (Haggarty et al., 2006, Matorras et al., 1998). Intralipid, which is high in linoleic acid, may encourage compositional changes– favoring the accumulation of the more abundant unsaturated fatty acids, over saturated fatty acids. As the rate of uptake has previously been reported to reflect the rate of cellular utilization (Haggarty et al., 2002), this differential uptake may in turn encourage selective channeling of unsaturated fatty acids towards oxidation and energy production.

It is important not to read too much into these findings, as the results are based on low patient numbers. This does however pose a promising future area for research.

6.5 Conclusion

The work presented in this chapter has built upon established findings from earlier in this thesis and has sought to identify ways in which aberrant patterns of metabolism in embryos from OWOB women may be corrected.

Throughout this chapter findings of lower glucose consumption and higher triglyceride levels have been confirmed in embryos derived from OWOB women compared to those of normal weight. For the most part, lower glucose has not been accounted for by a compensatory change in the rate of pyruvate uptake or a change in the rate of glycolysis, as indicated by lactate production. Embryos have clearly been shown to utilise LC, implying that embryos from OWOB women may have an increased reliance upon β-oxidation of fatty acids to derive ATP.

Multiple regression models have confirmed that in each sub-study population (insulin treatment, LC treatment, cryopreservation group) that increased glucose consumption was correlated with increased likelihood of blastocyst formation. Nonetheless, blastocyst rates were shown in two of the sub groups, to be higher in the OWOB cohort compared to the normal weight grouping. The stratified data analysis did however confirm that at equivalent stages of development embryos from women <25kg/m^2 were more viable than those of OWOB women.

The addition of insulin to the culture medium had little effect on the utilisation of glucose in either population of embryos from women of above and below 25kg/m^2. Insulin did increase rates of blastocyst formation in the OWOB group and may act as a growth factor to influence embryo development. The stage of development the embryo has attained prior to incorporation of insulin into the culture media maybe important as it is possible that differing effects will be observed pre and post compaction. Insulin is not routinely added to culture media and further research is required before considering this possibility, since the consumption of key amino acids was shown to be influenced by its addition to the culture medium.

Human embryos were clearly shown to take up LC and consumption increased in parallel with concentration. The addition of LC did lead to changes in triglyceride content, carbohydrate utilisation and blastocyst development which were related to female BMI. These consequential differences in women of normal and OWOB BMI were possibly related to the size of the initial store of triglyceride.

LC may influence the metabolic pattern in the embryo, with fatty acid oxidation being promoted at the expense of other pathways. It would appear that there are optimum levels of LC consumption that correspond with embryo viability, particularly for cohorts of embryos from women of above and below 25kg/m^2. LC present in the follicular fluid is unlikely to be sufficient to support the increased demands of oocytes that have developed under conditions of raised glucose and free fatty acids. Oocytes recovered from OWOB women might, therefore benefit from LC supplementation but further work would obviously be required.

Chapter 7 : Overall Conclusions

The primary hypothesis of this thesis was that maternal nutrition in the periconceptional period can have a significant impact on oocyte developmental competence and embryo developmental viability. From the review of clinical data and primary research data clear evidence has been presented to suggest that the oocytes and embryos from OWOB women are compromised developmentally and metabolically.

In this study, putative markers of embryo competence have been identified and a consistent pattern of uptake linked to development stage defined. Specifically, uptake of exogenous substrates was shown to increase with development, in particular glucose consumption was almost twice as high in embryos that had formed blastocysts during extended culture (day 5-9), compared to their slower growing counterparts and this was shown to be the case for embryos from normal and OWOB women. This increase in glucose consumption at the blastocyst stage is consistent with that described in prior accounts in the mouse (Lane et al., 1996) and human (Gardner et al., 2011) and is indicative of embryo health. The work presented here builds upon earlier work that has primarily focussed on cleavage stage human embryos which has sought to use embryo metabolism as a means of selecting embryos with the highest implantation potential (Conaghan et al., 1993; Turner et al., 1994; Houghton et al., 2002; Brison et al., 2004; Gardner et al., 2011). The data presented here are suggestive of a metabolism that is matched to stage and specific for embryos from normal and OWOB women.

Phenotypic responses to a nutritionally enriched maternal environment were evident at; the intracellular biochemical level (CORE of substrates), during cellular development (progression to blastocyst) and ultimately at the whole body level (birth weights), with the potential for long-term consequences.

7.1 Clinical implications of OWOB BMI on ART outcome

If the data from the Hull IVF Unit is assumed to reflect that in the UK, there is compelling evidence that a significant proportion of women attempting to conceive are overweight or obese. Furthermore, the reproductive outcomes for these women are poorer than those of normal weight, in both natural (antenal and subfertility clinic data) and assisted conception cycles (including fresh and frozen cycles, Hull IVF Unit).

The poorer outcomes are primarily mediated through the maternal component, as maternal nutrition in the periconceptional period appears to have an appreciable impact on ovarian responsiveness, resulting in significantly fewer oocytes in OWOB compared to normal weight women. The chances of a live birth were significantly lower for OWOB, compared to normal weight women, furthermore birth weights were significantly higher for babies born to OWOB women compared to those of normal weight women (Figure 7.1). This was not surprising, as maternal supply of macro and micronutrients is known to affect fetal growth (Kulie et al., 2011) and it is becoming increasingly apparent that the developing fetus will make, adaptations, some of which may be irreversible, to its physiology and metabolism (de Boo et al., 2006) in order to survive. However, such adaptations are a source of serious concern, since according to the early origins programming hypothesis developed by Barker et al (1990), size at birth is related to the risk of developing disease later in life.

Potentially more concerning still, are the data presented in this thesis indicating that such adaptations may begin prior to conception and during oocyte development leaving a lasting legacy on the growth and development of the offspring. The nutritionally enriched follicular environment in which oocytes, of OWOB women develop, can result in metabolic adaptations in the embryos, some of which may be inconsistent with further development (figure 7.2) and likelihood of pregnancy (7.1), with others not apparent until after birth. These notions of developmental plasticity and the perceived vulnerability of the preimplantation embryo to its environment are consistent with data from animal models. For example, Eckert & Fleming (2011) compiled a comprehensive review of studies linking maternal diet with a catalogue of conditions in the offspring including; increased blood pressure, obesity risk and decreased insulin sensitivity.

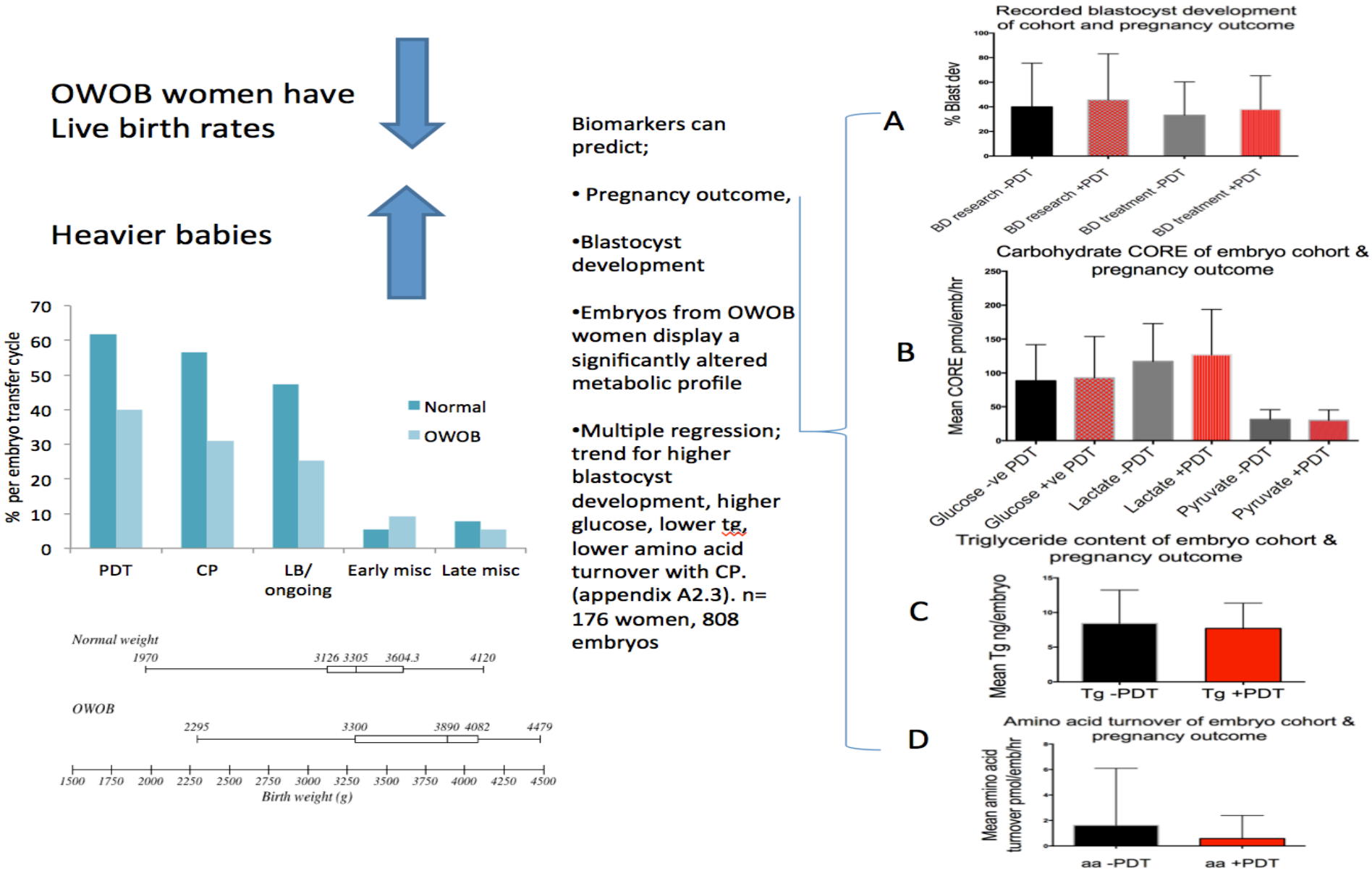


Figure 7.1 An overview of the influence of female BMI (kg/m^2) on clinical outcome (PDT, CP, LBR, misc, birth weights $p<0.05$) and ability of biomarkers to predict pregnancy outcome ($p>0.05$).

7.2 Role of biomarkers to predict clinical pregnancy and blastocyst formation

The levels and nature of metabolites consumed and/or produced characterise an embryos metabolic phenotype, and from this it is possible predict its developmental viability. Viability *in vitro* maybe assessed according to the ability to form a blastocyst in extended culture and the ability of transferred sibling embryos to implant and give rise to a viable pregnancy. Figures 7.1 and 7.2 provide a summary of all data reviewed as part of this thesis from all 176 women, 808 embryos and whilst (a) clinical pregnancy rates were significantly poorer for OWOB women, and (b) putative biomarkers significantly correlated with female BMI; biomarkers were not directly correlated with clinical pregnancy achieved by sibling embryos. Sibling embryos displayed a range of CORE substrate values and although the general pattern was unique to each patient (as evidenced in chapter 2; method validation and chapter 3; repeat cycles), substrate utilisation bore closer correlation to stage of development attained and ability to form a blastocyst (Figure 7.2).

The ability to form a blastocyst could be predicted by the glucose depletion, pyruvate depletion, triglyceride content and amino acid turnover (figure 7.2), each of which has been shown to be correlated with female BMI (in chapters 5 and 6). However, as an independent predictor, when all other confounding variables were considered, female BMI did not predict blastocyst formation per se. Glucose consumption remained a significant predictor variable (appendix A2.3), a finding that was replicated in each subgroup analysis (evidenced in chapter 5 and 6).

It can be concluded, that glucose consumption was generally higher in post-compaction embryos capable of forming developmentally viable expanded / hatched blastocysts and triglyceride levels were significantly lower amongst such blastocysts than those arresting at earlier stages of development. When these three covariates (glucose, triglyceride and development) were combined it became apparent that a mid range was optimum for highest viability.

This idea of an optimum mid range of consumption was first established in Chapter 5, where it was shown that those embryos which underwent early stage arrest on day 5 (start of research culture) had consumed more substrates than those at an equivalent stage on day 5 that continued to develop. This indicates that at the cleavage stages,

consumption in the low to mid-range is more compatible with ongoing development. This is consistent with the data of Turner et al., (1994) and Conaghan et al., (1993), which was reinterpreted here to demonstrate additionally, that lower variability in uptake at this stage of development also implies that the cohort of embryos are dividing synchronously and have an increased implantation potential (established from the data review in chapter 2). Conversely embryos arresting post-compaction were shown in chapter 5 to be consuming below average amounts of substrates, an observation which may reflect insufficient ATP generation to support development. Embryos consuming mid to higher quantities of substrates post-compaction were shown throughout this thesis to be significantly more likely to continued development and achieved blastocyst formation, expansion and hatching. These results are allied with those reported for day 4 human embryos (Gardner et al., 2011). Conjointly, it can thus be argued from this data that metabolism should be appropriate for stage of development.

Alignment of metabolic activity with stage of development seems to be of equivalent importance to temporal pattern of development, in that a blastocyst formed on day 6 may be equally as viable as one which forms on day 7, 8 or 9 providing energy homeostasis is achieved. The influence of the substrate combinations (glucose: fatty acids) that had been utilised to meet the energetic demands has no doubt contributed to the differences in blastocyst expansion rates and pregnancy outcomes.

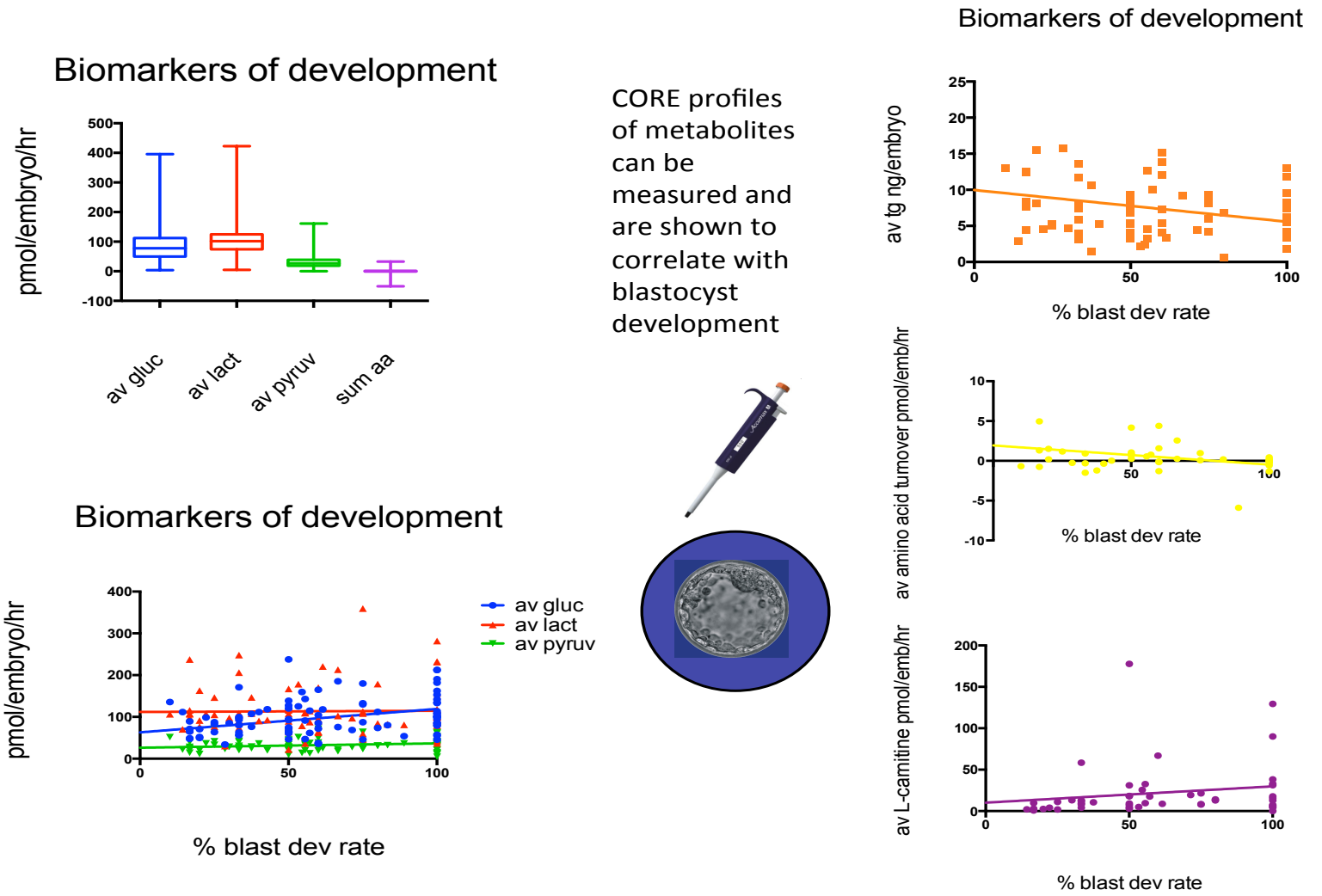


Figure 7.2 Biomarkers indicative of embryo viability; glucose (p<0.0001), pyruvate (p<0.05), triglyceride (p<0.0001) amino acid turnover (p<0.05).

Multiple regression; glucose and triglyceride independent predictors of blastocyst formation (p<0.05), female BMI showed a trend but was not a significant predictor of blastocyst formation. In a multiple regression of factors influencing CORE profile female BMI is a significant independent predictor variable; n= 176 women, 808 embryos.

7.3 Implications of female OWOB BMI on oocyte and embryo viability

The negative impact of obesity on oocyte and embryo morphokinetics was demonstrated in chapter 4; obesity was shown to reduce oocyte size, overall oocyte developmental competence, the subsequent ability of embryos to form blastocysts and affect the kinetics of those capable of compacting. Blastocyst quality was impaired further by reduced blastocyst diameter and cell numbers in relationship to female BMI (summarised in Figure 7.3).

These findings were proposed to relate back to the nutrient enriched environment from which the oocytes were generated, in particular the hyperglycemia and hyperinsulinaemia that were reported in the follicular fluid composition of OWOB women (Robker et al., 2009). The metabolic support provided by bidirectional communication between oocyte cells and cumulus cells is essential for oocyte developmental competence (Sutton-McDowall et al., 2010) and it is likely that suboptimal conditions during oocyte development and maturation may leave a lasting legacy post fertilisation, the effects of which became more apparent as development progressed. In support of this theory, studies in animal models have shown that oocytes exposed to hyperglycemia may undergo precocious maturation (Sutton-McDowall et al., 2010), have dysfunctional AMPK activity (Ratchford et al., 2007), disrupted mitochondrial activity (Wang et al., 2009) and increased follicular apoptosis (Chang et al., 2005).

In chapter 5, it was indeed shown that the embryos derived from oocytes from OWOB women had a higher endogenous triglyceride content, diminished glucose consumption and altered amino acid turnover; markers of a reduced potential to give a pregnancy following embryo transfer. Crucially, it was shown that these observed metabolic differences were independent of male BMI and other confounding variables (appendix A2.3 and chapter 5). Analogous findings have again been reported in animal studies; in the bovine, oocytes exposed to fatty acids at concentrations found in human ovarian follicles (Robker et al., 2009; Valckx et al., 2012) display reduced glucose consumption in the subsequent blastocysts (Van Hoeck et al., 2011) as in the present study.

Blastocyst formation is clearly dependent on the utilisation of glucose and the observation of decreased utilisation of glucose at each stage of development for

embryos from OWOB women was a consistent and striking feature of this thesis. In each study group glucose consumption increased from the cleavage stage to the blastocyst stage. Differences in amino acid turnover were also apparent for different developmental stages. In particular embryos from overweight women consumed significantly more methionine, known to play an important role in the metabolic regulation of nucleotide synthesis and methylation (Grillo and Colombatto, 2008).

The triglyceride data suggested that the amount and composition of triglyceride in the oocyte and embryo can be modified by the environment in which they develop. Embryos from OWOB women contained significantly higher quantities of triglyceride than those of normal weight women. One explanation for this is that triglyceride is accrued during the maturation of the oocyte in lipid rich follicular fluid, as evident in the ovaries of obese women (Robker et al., 2009). Whilst fatty acid oxidation of lipid stores provides a source of ATP essential for oocyte maturation and can be used to support cell proliferation and embryo development processes, a large accumulation of lipid, could impair mitochondrial function and embryo development. The role of lipotoxicity has been widely explored in murine models (reviewed by Wu et al., 2015; Grindler and Moley 2013). The premise of these studies is that lipotoxicity leads to endoplasmic reticulum stress, mitochondrial dysfunction and apoptosis, partly mediated by increased oxidative stress and generation of reactive oxygen species.

The data from this thesis and from animal studies indicate that embryos from overweight and obese women express a different metabolic phenotype to those women with a desirable weight; most notably lower glucose consumption and higher triglyceride content (summarised in Figure 7.3 – for the full data set). This underlines the importance of attaining an optimum pre-pregnancy BMI for the chances of successful live birth.

7.4 *In vitro* manipulation of metabolism; implications for viability

Optimal lipid metabolism, combined with carbohydrate metabolism is essential for development and in chapter 6 ways in which aberrant patterns of metabolism in embryos from OWOB women may be corrected, were sought to be identified. It was established through the supplementation of culture medium with insulin and L-Carntine

(LC) that development and metabolism may be manipulated both independently and in concert (Figure 7.3).

Insulin did not appear to influence glucose consumption, although it did modulate developmental progression and specific amino acid utilisation. On the other hand embryos were shown to utilise LC from the media to modulate their energetic reservoirs and use of exogenous substrates to derive energy. There were optimum levels of LC consumption that correspond with embryo viability, particularly for cohorts of embryos from women of above and below 25kg/m^2. The differing responses of embryos from normal and OWOB women to LC supplementation were suggestive of a distinction in the reliance upon β-oxidation of fatty acids to derive ATP. It is possible that LC derived from the follicular fluid is insufficient to support the increased demands of oocytes/ embryos of OWOB women that have developed under conditions of raised glucose and free fatty acids, the addition of LC to *in vitro* embryo culture medium maybe highly beneficial in this scenario. Chronic over-nutrition is thought to cause 'metabolic gridlock', an increase in mitochondrial membrane potential, accumulation of electrons and intra-mitochondrial acetyl-CoA (Muoio 2014). Once placed into *in vitro* culture medium containing physiological concentrations of glucose, amino acids and serum substitute, the addition of LC to culture medium could act to drive oxidation of triglyceride stores and direct acetyl-CoA through the TCA cycle. If added at an appropriate concentration LC could thus realign metabolic coordination and fuel signalling pathways.

The ability to provide *in vitro* conditions that foster an appropriate metabolic pattern of substrate CORE may improve short term embryo viability and protect against inappropriate adaptations at this vulnerable period of development and programming in the preimplantation embryo. Evidence has been presented here to suggest that it may be possible to manipulate the *in vitro* conditions to realign the embryo metabolism and developmental fate to favour a normal healthy development. However, the complexity of the obese condition means that it is difficult to determine if adaptive or pre conditioning factors influence on the embryo phenotype and metabolic response and thus when intervention steps would be most appropriately applied. Nonetheless the data do suggest a promising future avenue of research.

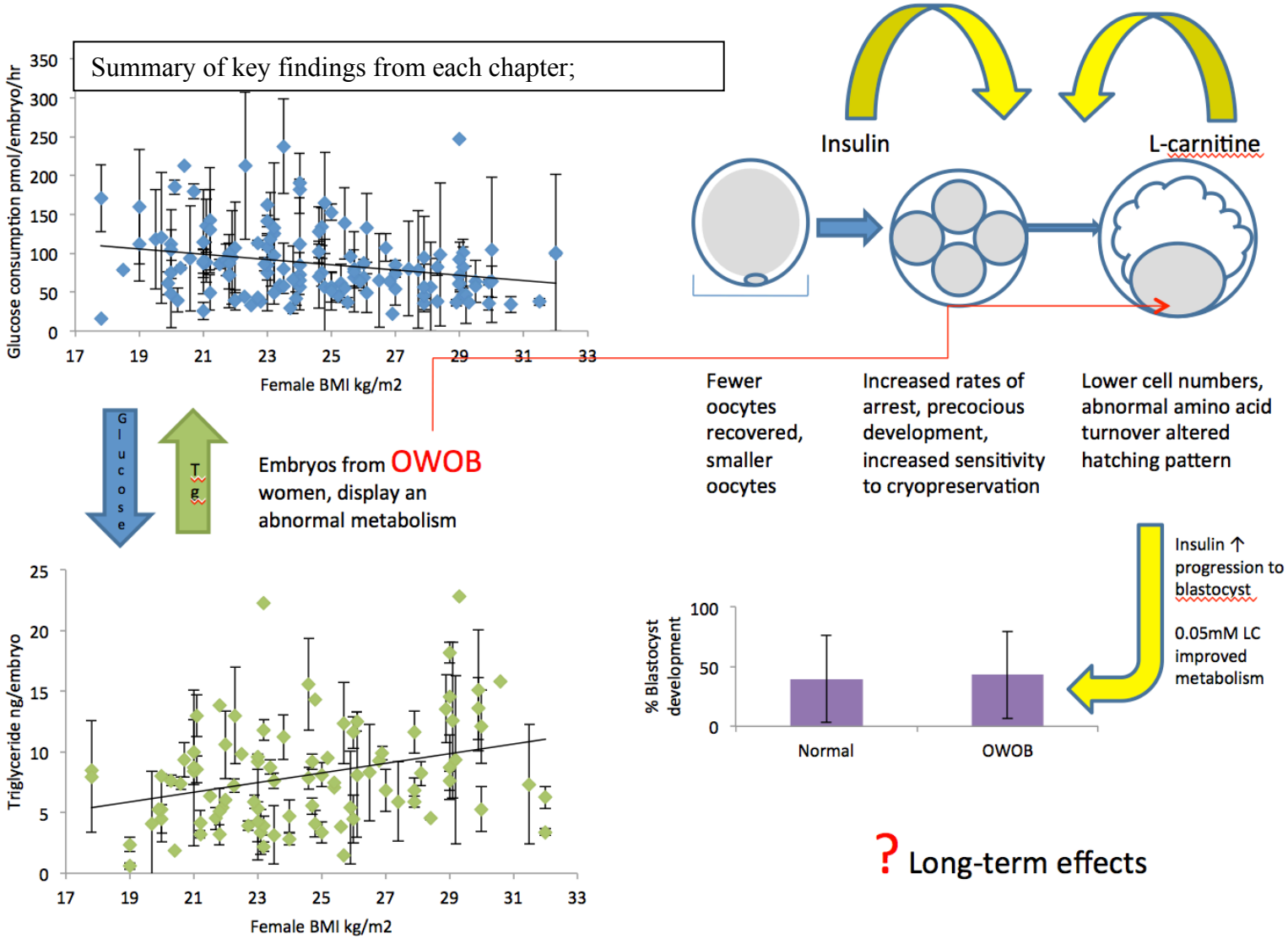


Figure 7.3 Overview of data showing clearly the influence of female BMI kg/m^2 on oocyte and embryo phenotype; lower glucose consumption& higher tiglyceride content; p<0.001; n= 176 women, 808 embryos.

7.5 Caveats; words of caution and confounding variables

Much of what we currently know about the interplay between environmental stimuli and preimplantation embryonic metabolic response has previously been derived from animal studies. However, as Leese (2012) points out, in a succinct review of 40 years research on metabolism of the preimplantation embryo, considerable caution is required when extrapolating such data to human embryos, since mice and other rodents are believed to be particularly vulnerable to dietary modification. This data from human embryos is therefore particularly valuable.

Research using human embryos is quite rightly restricted by ethical constraints and therefore advancements in knowledge about human embryo physiology have not progressed in line with those of other mammalian species. In spite of this, the non-invasive methods applied in this study to determine the relationship between human nutrition and metabolic health have highlighted many parallel findings with those reported in animal models such as the bovine (Leroy et al., 2010, Van Hoeck et al., 2011) and mouse (reviewed by Grindler & Moley 2013).

The scarcity of human embryos for research and the often poor quality of those that are donated (being unsuitable for fresh embryo transfer or cryopreservation) was a concern in this study. Within a cohort of embryos from the same patient there is always a spread of developmental accession and morphological quality, and the merits of drawing conclusions from the poorest embryos of the group maybe contested. Furthermore extrapolating data from embryos placed in extended culture from day 5-9 may be questioned. Clearly at this point, embryos of higher viability would have been expected to have reached the blastocyst stage on the morning of day 5. Be that as it may, this study has shown that those embryos which subsequently develop to reach the blastocysts, albeit at a slower rate of progression exhibit a higher rate of nutrient uptake, indicative of their sustained viability.

A further concern is that the *in vitro* situation is obviously not representative of that *in vivo* and the virtue of drawing conclusions from non-transferred sibling embryos could be questioned. A potential dichotomy exists between the metabolic phenotype of *in vitro* compared to *in vivo* preimplantation embryos, as demonstrated by Gardner et al., (1996). A broad assumption has been made in this study that once placed into *in vitro*

culture, oocytes / embryos retain their native characteristics. This assumption is justified on the basis that culture conditions were identical for embryos of normal and OWOB women, embryos were supplied with complete medium, supplemented with synthetic serum substitute and there is no reason to suspect that one group would be more subject to culture-induced stress than the other. It was shown in chapters 5 and 6 that embryos appeared to be predisposed to utilise exogenous substrates in a particular manner.

Questions remain about whether embryo developmental and metabolic observations are an artefact of the subtleties of *in vitro* conditions; once replaced into the uterine environment would such adaptations be reversed, persist, or indeed be exacerbated? For instance improvements to the metabolic normality of substrate utilisation by the addition of l-carnitine or promotion of blastocyst development by the addition of insulin may well be lost as soon as embryos are returned to the physiological environment. Alternatively it maybe that intervention at a critical 'road-block/ misdirection' can divert the embryo back on to a course of normal development and clearly further research is required on this topic.

A further potentially contentious issue is that of the 'experimental unit' and if this should be regarded as the patient or the individual embryos. This study has been powered on the basis of embryo numbers rather than patient numbers. The observed differences may indeed be patient specific, although ANOVA and variance decomposition were used to assess the variance within and between patients of comparable BMI and cycle outcome. The data comparing metabolic parameters to the pregnancy outcome of the sibling transferred embryos highlights the differences in developmental potential apparent in a cohort of embryos; this is a potential weakness of using the woman as the 'experimental unit' as opposed to individual embryos. This assumes that the intra-follicular conditions were comparable in the ovaries of a patient. However, in a given patient, even in follicles of comparable size, the degree of vascularization, oxygenation and level of nutrients have been shown to vary at the time of ovum retrieval (reviewed by Van Blerkom, 2000). The more subtle differences in metabolic regulation and developmental competence of individual embryos could be attributed to these differences and further studies are required on the origin of intra-follicular influences.

These studies were conducted over the course of 5 years and throughout that period, clinical necessity meant that a number of protocols were changed. In addition, differences arose in the demographics of the study groups. For the most part these variables have been accounted for in the statistical analyses presented in each chapter and have not been found to be of significance. Each is briefly mentioned below to provide further context for the findings of this thesis and also to highlight the cautions and limitations that must be applied when interpreting this body of evidence.

1. **IVF and ICSI** – the influence of the insemination method was reviewed in chapter 5 and a homogenous spread of IVF/ ICSI cases was evident in each study grouping. However as a predictor of blastocyst formation for transfer and cryopreservation, ICSI embryos did form blastocysts at a lower rate, implying that underlying sperm quality does influence developmental progression – as reported in chapter 3. Whether this is solely related to underlying sperm quality or is underpinned with a possible link with male BMI is not clear.
2. **Male BMI** – the most cogent data for the observed embryo metabolic effects reported here being mediated via maternal component as appose to a paternal influence were provided in chapter 5, with the analysis of data from a single male donor and treatment of a range of women of differing BMI. Attempts have been made to control for the impact of male obesity, nonetheless, the detrimental effects of adiposity on fertility are not likely to be limited to the female (as evidenced in animal models; Fullston et al., 2015), even if more evident, based on the chosen bio-markers of viability utilised in this study.
3. **Effects of obesity rather than being overweight –** Changes to health authority funding criteria have meant that obese women cannot access NHS funding (privately funded women are treated up to a BMI of 35kg/m^2) and therefore the majority of the women in this study were overweight rather than obese. The limitations of female BMI as a measure of adipose tissue were discussed in the introduction and the amount of food consumed and composition of the diet will no doubt also have contributed to the variation in output from women of similar BMI.
4. **Female and male age** –this was not found to be significantly different between BMI category groupings in any of the subgroups.
5. **Influence of agonists verses antagonist -** Prior to August 2013 the majority of patients had received an agonist protocol of down regulation; post August 2013,antagonist cycles were most frequently utilized. Antagonist cycles led to

significantly fewer oocytes per patient, higher rates of blastocyst formation and lower implantation rates in treatment cycles, despite no differences in patient demographics including female age, AMH levels and crucially no differences in female BMI. Of those embryos donated to research, blastocyst rates were significantly higher and accordingly the antagonist group of embryos had lower triglyceride content on day 7 of development (7.41ng compared to 9.96ng for the agonist group, $p<0.01$). Glucose, pyruvate and amino-acid metabolism did not differ among the groupings, however lactate production was significantly higher in the antagonist group compared to the agonist group (122.97 &100.42 pmol/embryo/hr respectively $p=0.01$). The excess lactate production may be effective as it may allow faster incorporation of carbon into biomass, which in turn facilitates rapid cell division (Vander Heiden et al., 2009). This may explain the higher rates of blastocyst development, however it could be speculated that, precocious development may be detrimental for implantation, hence the lower pregnancy rates in fresh cycles.

6. **Influence of culture medium -** Generally speaking there are three types of culture regimes employed in ART; (1) non-renewal single medium (2) renewal single medium and (3) sequential media. In the course of this study the clinical protocol employed for the most part was a sequential system (Sage^{TM}), however from June 2014 a single step non-renewal protocol was utilised (Vitrolife^{TM}). Whilst all embryos are cultured in the same medium throughout the research phase of this study, it is well documented that the prior environment of the embryo can affect subsequent metabolism. Metabolic adaption by embryos in culture has previously been reviewed (Leese 1995). The majority of data was collected from embryos that had been cultured previously in sequential medium, however 23 patients (84 embryos) were donated after the change in clinical protocols. There were no significant differences in the patient groupings for female BMI, furthermore blastocyst development rates and CORE profiles were not significantly different. The exception was pyruvate consumption which was borderline significantly ($p=0.05$) different at 25.5 and 32.1pmol/embryo/hr for embryos cultured in Vitrolife and Sage respectively.
7. **Culture to day 7 or day 9**–After the completion of the first phase of this study (Chapter 5), extended culture was arrested at day 7 rather than day 9. The data presented from chapter 5 indicated that 38% of embryos had arrested on day 7, whereas on day 9 the proportion was 75%. The change in profile of uptake at

each stage of development to a continual ‘step-up’ may reflect the increased proportion of embryos still actively developing at the end of culture.

8. **Influence of low oxygen culture** Throughout the clinical phase of treatment embryos were cultured in a low oxygen environment, however once donated to research the embryos were cultured under atmospheric oxygen conditions for the first phase of this study (29 patients, 150 embryos- Chapter 5 data). From July 2012 all culture was performed under 5% O_2 (147 patients, 658 embryos- chapter 6 data). A lower oxygen environment is thought to be more physiological, associated with lower production of ROS and beneficial for embryo development (Wale & Gardner, 2015). Studies have shown that embryos cultured at 5-7% O_2, were more likely to develop to the blastocyst stage than those generated under atmospheric O_2 conditions (Thompson et al 1990, Li & Foote 1993, Dumoulin et al 1999), however here, no significant differences were reported in blastocyst rates (35.5% atmospheric and 44.4% low O_2).

   Incubation at reduced oxygen tension has additionally been reported to cause an increase in mammalian embryo oxygen uptake and pyruvate oxidation; metabolic activity which is suggested to correlates more closely with that of in vivo recovered embryos (Hooper et al., 1995). No significant differences were observed in the present study, in the metabolism pyruvate or other substrates under atmospheric and low oxygen (29.9 and 30.7pmol/embryo/hr respectively). ROS can affect epigenetic markers, thus culture at atmospheric O_2 may have altered expression patterns of genes, including AQP8, CAMK1 and TGFB1 (Rinaudo et al., 2006), genes involved in cell growth and maintenance. This may also have contributed to the altered pattern of substrate utilisation observed in chapter 5 i.e. the low metabolic activity of blastocysts that failed to expand.

   Importantly, women of differing BMI were equally stratified among the groupings, implying this would not have distorted the analysis. What is interesting though is that mean glucose uptake was lower in embryos of OWOB women cultured under atmospheric O_2 than in embryos from OWOB cultured under low O_2 (53.9 and 69.9 pmol/embryo/hr), whereas in normal weight women consumption was approximately the same under both sets of conditions. From this, it could be argued that oocytes from obese women were already

suffering the deleterious effects of oxidative stress and were more vulnerable to assault by ROS. Findings in mice support this idea; Igosheva et al., (2010), reported that a high fat diet could lead to mitochondrial damage and determined that oocytes from such mice displayed an increase in the inner mitochondrial membrane potential, oxidised redox state and higher ROS production than controls. Equally though, oocytes/ embryos from OWOB women would have continued to have been compromised to a greater degree than those of normal weight women under low O_2 conditions. This is proffered on the basis that under low oxygen, hypoxia-inducible factor 1α (HIF1α) forms a heterodimer with HIF1β and regulates the expression of genes involved in glucose metabolism, inducing a shift towards glycolysis (Rafalski et al., 2012). Conversely, on the basis of data presented in this thesis, embryos from OWOB women appear to have a reduced capacity for anaerobic glycolysis and are seemingly predisposed to favour ATP generation via oxidative mechanisms, including β-oxidation of fatty acids. This strategy of OWOB embryos will again result in increased generation of ROS and would also potentially place any uterine transferred embryos at a disadvantage when encountering the low oxygen levels reported *in vivo* (Fischer & Bavister 1993) at the time of implantation.

7.6 Final remarks and future avenues for research

The evidence for compromised oocyte and embryo viability in OWOB women is compelling. Obesity related qualitative phenotypic changes observed in this study have included; significantly smaller oocytes, higher rates of developmental arrest post-fertilisation and aberrant cell-cycle kinetics, resulting in lower ICM and TE cell counts. Quantitative differences in substrate uptake and production were recorded at cleavage to hatched blastocyst stages of development. The substrate CORE profile was established as an important marker of embryo health. Being OWOB significantly alters the metabolism of exogenous substrates, in particular glucose and amino acids and the storage and utilisation of endogenous lipid stores.

In this thesis it has been reported for the first time that human embryos contain a significant store of lipid and that this store can be modified by the environment, akin to

findings in the bovine (Ferguson et al., 2006, Del Collado et al., 2015). Similarly, the amount of triglyceride was lower in embryos assessed at the blastocyst stage, compared to those at the cleavage stage, consistent with findings from cultured porcine embryos (Romek et al., 2010). This finding was suggestive of a role for lipids during embryo development; implying lipid was modified as a result of metabolic processes. Additionally, an altered lipid metabolism in embryos from OWOB women was denoted, with the increased supply and oxidation of fatty acids speculated to have resulted in the accumulation of acetyl-CoA and inhibition of glycolysis (Randle 1998), evidenced by the paired lower rates of glucose uptake observed in these embryos.

The findings on amino acid metabolism suggested that differing rates of CORE of specific amino acids resulted in altered amino acid ratios between embryos of normal and OWOB women. Amino acids interact with the metabolism of carbohydrates in the TCA cycle and in the background of a high fat diet may act synergistically to promote insulin resistance (Newgard, 2012). Of particular note, metabolism of methionine was significantly altered in embryos of OWOB women and after insulin supplementation of the culture medium. Methionine plays a particularly important role in methylation/ imprinting processes and aberrant uptake, as observed in embryos from women with a raised BMI, during a particularly sensitive period of development coinciding with genome activation and establishment of epigenetic marks.

Ample evidence has been presented to support the notion that appropriate development from oocyte maturation through to the hatched blastocyst stage relies upon the provision of conditions that foster a ‘fitting’ metabolism. The nutritional stress of hyperglycemia and lipotoxicity are two components which could lead to cellular damage including altered mitochondria structure, function and copy number, endoplasmic stress responses, inflammation and the generation of reactive oxygen species and longer term undesirable ‘programming events’ in embryos of OWOB women. Chason et al (2011), proposed that the epigenome may serve as a link between the environment and the genome, hence the maternal environment could thus impact upon embryo metabolism and development. Figure 7.4 shows a summary of some of some of the pathways that may be altered by over nutrition, leading to altered gene expression and metabolism. Studies of gene expression are a highly desirable and efforts to test the relationship between maternal BMI and epigenetic marks, metabolism and developmental competence would be an additional future research aim.

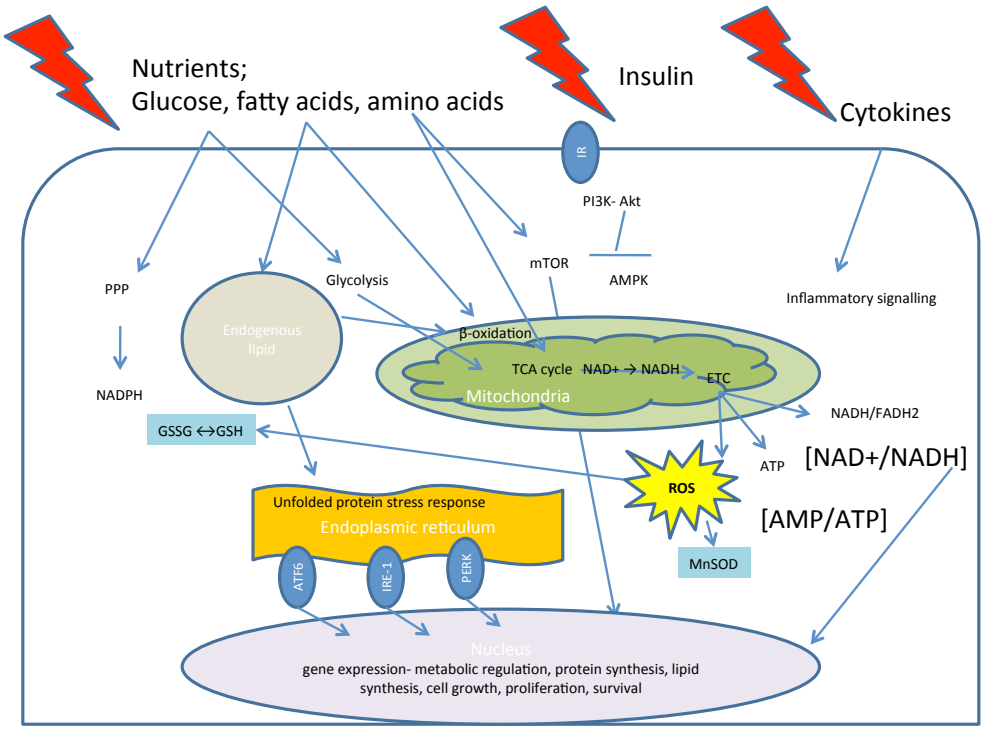


Figure 7.4 Summary diagram linking nutrient uptake to altered gene expression;

Increased intracellular lipid may induce endoplasmic reticulum stress (unfolded protein response, altered gene expression). Oxidative stress and modulation of REDOX circuits occurs as a result of reduced glucose uptake (and use in glycolysis and PPP), increased β-oxidation of FFA, which requires TCA and ETC leading to ROS generation. mTOR is activated by amino acids and insulin and influences gene expression and AMPK interacts with other pathways acting as a fuel sensor.

The preimplantation stage of embryo development is considered to be highly sensitive to environmental perturbation (Steegers-Theunissen et al., 2013) and at no other time point could environmental manipulation / intervention have such an impact on the long-term health and development of an individual. An improved understanding of the interactions between lipid, carbohydrate and amino acid metabolism could potentially lead to the development of specific culture media adapted to improve the metabolic interplay of substrates and embryo health, particularly in embryos from women of high BMI.

To achieve this aim, future work could be directed towards determining the metabolic cost of a process; determining cellular rates of CO_2 production, relative to O_2

consumption would provide a respiratory quotient (RQ), which would be an indicator of glucose oxidation or fat oxidation- if high or low respectively (Muoio, 2014). The energetic efficiency measured alongside nutrient uptake and related to embryo development, would potential provide a more holistic view. Leese (2012) theorised that higher quality embryos will exert reduced effort and suggested this may be determined by measuring oxygen uptake. One proposal would be to apply similar methodology to that used in bovine to measure oxygen consumption in individual embryos (Lopes et al., 2007) and this could be expanded upon with further engineering to incorporate time-lapse analysis.

Efforts need to be made to educate and inform women about the sensitivity of the period prior to conception when the oocyte is maturing in the follicle and the impact that maternal diet and nutritional health may have on embryonic development. A very clear message can be derived from this and animal studies that the maternal environment influences embryonic development in the short term and sets a future trajectory for growth, with the scope to exert a lasting legacy beyond embryonic and fetal life.

On the basis of these data, it is proposed that nutritional enrichment of the periconceptional environment, as apparent in obese women, has a significant and lasting impact on the embryo. Embryos produced in a periconceptionally enriched environment have a compromised metabolism which may reduce embryo health and lead to the reduced fertility; associated with increased miscarriage seen in OVOB patients. In addition, there is concern that nutritional stress on the embryo could have long term health implications for the offspring.

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Appendix

Appendix 1; Culture media preparation, supplies and metabolic profile example data (Chapter 2 Methods)

A1.1 Culture media preparation (Chapter 2 Methods)

Culture media was prepared using sterilised glassware and plasticware. Briefly; new glassware was soaked for 24 hours in 2% hydrochloric acid to remove impurities, rinsed three times in double distilled water (ddH_2O), and dried in a drying oven at 80^oC for 90 minutes before being was wrapped in aluminium foil and sterilised at 160^oC for 4 h. Used glassware was rinsed with tap water and placed in 2% Decon 90 detergent, diluted with ddH_2O, for 1-14 days, before further rinsing with ddH_2O, drying and sterilising, as described above. Plasticware; pipette tips and boxes were autoclaved prior to use and placed in a drying oven at 80^oC to dry. Eppendorf tubes were autoclaved in glass beakers, covered loosely with aluminium foil. Disposable plasticware, including Pasteur pipettes, Falcon tubes and dishes and Nunc flasks were purchased sterile and disposed of after use. All purchased plasticware was suitable for *in vitro* culture of embryos as assessed bythe mouse embryo assay toxicity tested and CE marked.

All media preparation was undertaken in a Class II laminar flow hood. The desired weights of chemicals (all chemicals from Sigma UK unless otherwise; see appendix for full list) were dispensed onto siliconised weighing paper/ weighing boats. The chemicals were then added to a sterile volumetric flask of the required volume fitted with a sterile glass funnel. Water For Injection (Fresenius UK) was used to prepare all media. Chemicals were dissolved by gentle mixing in water in flasks covered with Parafilm. Media stock solutions were prepared of Earle's balanced salt solution (EBBS), buffer, antibiotics, carbohydrates and amino acids (Table 2.3 A1-4) and used to prepare a working solution (Table 2.3 B). All media and stocks were filter-sterilised through 0.22µm Millipore syringe filters, checked for correct osmolarity and stored for 6 weeks at 4^oC.

The osmometer (Osmomat 030, Gonotech GmbH, Germany) was calibrated to 0 mOsm / kg with 100µl of distilled water and to 300 mOsm / kg with 100µl of a commercial

standard (MOD108 Camlab, Cambridge). 100µl of medium were taken to test its osmolarity before adding synthetic serum substitute (SSS) (Sage) SSA. If the osmolarity of the medium was outside of the desired range 288±4mOsm/kg the medium re-made.

Media stock solutions

(1) EBBSx 10 dilution

Component	**g / 100ml sterile water**
CaCl2 (anhydrid)	0.2
KCl	0.4
$MgSO_4.7H_2O$	0.2
NaCl	6.435
NaH2PO4.H2O	0.14
Phenol red	0.01

(2) Buffer

Component	**g / 20ml sterile water**
NaHCO3	0.42

(3) Antibiotics and carbohydrate (CHO)

Component	**g / 10ml sterile water**
Antibiotics	0.06 penicillin + 0.05 streptomycin
Pyruvate	0.0517
Glucose	0.09
Glutamine	0.0146

(4) Amino acids

Component	g / 200ml sterile water	Final conc in working media mMol
Arginine.HCl	0.4214	0.20
Cysteine HCl	0.0315	0.02
Histidine	0.0838	0.04
Isoleucine	0.0262	0.02
Leucine	0.0787	0.06
Lysine	0.1096	0.06
Methionine	0.0298	0.02
Phenylalanine	0.033	0.02
Threonine	0.0596	0.05
Tryptophan	0.0408	0.02
Tyrosine	0.0725	0.04
Valine	0.0351	0.03
Alanine	0.098	0.11
Asparagine	0.0751	0.05
Aspartate	0.0399	0.03
Glutamate	0.1471	0.10
Glycine	0.03	0.04
Proline	0.023	0.02
Serine	0.042	0.04
D-amino butyric acid	0.0516	0.05

Table A1 1.1 Media stock solutions

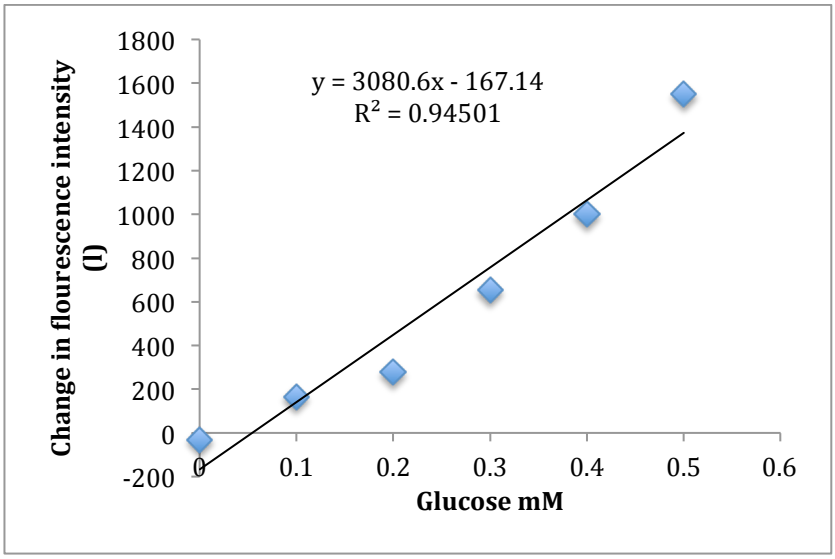
Suppliers;

Chemical	Supplier	Cat No.
ATP	Sigma-Aldrich Company Ltd The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK	A6419
BSA	Sigma-Aldrich	A6003
Calcium Chloride Dihydrate (CaCl2.2H2O)	Sigma-Aldrich	C7902
Decon 90 Detergent	Scientific Laboratory Supplies Ltd (SLS) Wilford Industrial Estate, Ruddington Lane,Wilford, Nottingham NG11 7EP, UK	CLE1020
Di-Sodium Hydrogen Orthophosphate Dihydrate (Na2HPO4.2H2O)	Sigma-Aldrich	71638
Dithiothreitol	Sigma-Aldrich	S1277
Ethanol (Mol.Bio grade)	Sigma-Aldrich	51976-500ML-F
EPPS	Sigma-Aldrich	E9502
FBS	Sigma-Aldrich	
Glucose	Sigma-Aldrich	G6152
Glucose standard, 5.0mmol/L, 30ml	Analox Instruments, Unit 22, Acton Park Estate, The Vale, London, W3 7QE, UK	GMRD-010
Glycine	Sigma-Aldrich	
Hydrazine sulphate	Sigma-Aldrich	
Hexokinase/ G6PDH	Sigma-Aldrich	127-825
Lactate standard, 5.0mmol/L, 25ml	Analox	GMRD-079
L-Carnitine inner salt	Sigma-Aldrich	*C0158*
LDH		
L-Glutamine	Sigma-Aldrich	G8540
Magnesium Sulphate	Sigma-Aldrich	M/1050
Magnesium Sulphate Heptahydrate (MgCl2.7H2O)	Sigma-Aldrich	M2643
NADP, NADH, NAD+ free acid	Roche	128 040
PBS Tablets	Sigma-Aldrich	P4417-100TAB
Penicillin/Streptomycin	Sigma-Aldrich	P0781
Phenol Red Solution	Sigma-Aldrich	P0290
Phosphate Buffered Saline	Sigma-Aldrich	P4417-100TAB
Potassium Chloride (KCl)	Sigma-Aldrich	P5405
Potassium Di-Hydrogen Orthophosphate (KH2PO4)	Sigma-Aldrich	P5655
Pyruvate reagent kit, 30 tests, incl. std	Analox	GMRD-140
Sodium Bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S4772
Sodium Chloride (NaCl)	Sigma-Aldrich	S5886
Sodium Di-Hydrogen Orthophosphate	Sigma-Aldrich	71500
Sodium hydroxide	Fisher Scientific UK Ltd, Bishop, Meadow Road, Loughborough, LE11 5RG, UK	S4920/60
Tris base	Fisher Scientific	
Water for injection	Fresenius Kabi Cestrian Court, Eastgate Way, Manor Park, Runcorn, Cheshire, WA7 1NT, UK	

Table A1 1.2 List of suppliers

A1.2 Example standard curve/ HPLC report – (Chapter 2 Methods)

A1.2.1Glucose



A1.2.2 Amino acids – Example report

Signal 1: FLD1 A, Ex=330, Em=450

Peak #	RetTime [min]	Type	Width [min]	Area LU *s	Height [LU]	Area %
1	2.319	VV	0.1380	44.03182	4.33293	1.6790
2	2.772	VV	0.2398	181.55096	9.70713	6.9227
3	4.918	VV	0.3143	25.61895	1.00632	0.9769
4	16.293	VV	0.8382	94.47572	1.36420	3.6025
5	16.418	VV	0.6310	71.68983	1.36274	2.7336
6	23.674	BV	0.9176	171.91766	2.28253	6.5554
7	24.737	VV	0.4163	46.99915	1.43634	1.7921
8	26.174	VV	0.9243	125.27701	1.71342	4.7769
9	27.728	VV	0.3312	30.02840	1.18093	1.1450
10	28.503	VV	0.2859	26.48116	1.23969	1.0098
11	29.438	VV	0.5896	68.86217	1.45884	2.6258
12	29.707	VV	0.2485	25.96749	1.33544	0.9902
13	30.582	VV	0.3032	29.07711	1.34936	1.1087
14	30.870	VV	0.4775	50.79910	1.36462	1.9370
15	33.170	VV	0.5494	56.21832	1.39713	2.1437
16	33.726	VV	0.4360	69.01418	2.16933	2.6316
17	34.872	VV	0.4572	59.84532	1.71313	2.2820
18	35.527	VV	0.4190	46.32800	1.46347	1.7665
19	36.371	VV	0.2617	31.29744	1.62317	1.1934
20	36.671	VV	0.3154	44.35283	1.93170	1.6912
21	37.069	VV	0.3320	58.81784	2.41231	2.2428
22	37.829	VV	0.3477	46.50774	1.78173	1.7734
23	38.477	VV	0.4723	84.24747	2.31412	3.2124
24	38.855	VV	0.2319	29.53278	1.72671	1.1261
25	39.263	VV	0.3309	45.46843	1.84381	1.7338
26	40.065	VV	0.5519	100.91917	2.33432	3.8482
27	40.577	VV	0.3128	39.62582	1.67477	1.5110
28	40.847	VV	0.2076	26.83591	1.64470	1.0233
29	41.527	VV	0.3523	46.51374	1.67396	1.7736
30	42.473	VV	0.5541	156.33829	4.21128	5.9613
31	42.755	VV	0.2588	100.69277	5.39532	3.8395
32	43.473	VV	0.6531	123.86609	2.54026	4.7231
33	44.464	VV	0.4131	63.94494	2.01668	2.4383
34	45.142	VV	0.3586	70.38461	2.60347	2.6838
35	45.554	VV	0.5426	72.05612	1.60021	2.7476
36	46.606	VV	0.3316	36.22549	1.49920	1.3813
37	47.117	VV	0.3413	38.41654	1.44075	1.4649
38	47.868	VV	0.3567	41.48450	1.45387	1.5818
39	48.141	VV	0.3778	45.35782	1.47656	1.7295
40	48.503	VV	0.2297	25.71348	1.42671	0.9805
41	50.990	VBA	0.6344	69.75315	1.47916	2.6598
Totals :				2622.53530	85.98233	

Figure A1 1.1 Example standard curve and amino acid report

Appendix 2; Statistical model example data (Chapter 5 and 6)

A2.1 Chapter 5

A2.1.1. Predictive of glucose consumption (section 5.3.2);

Forwards, stepwise regression linear model;Target variable glucose. Predictor variables included in analysis; development stage and grade day 3, blastocyst development (i.e. stage day 9), day of formation, blastocyst grade, female BMI, male BMI, female age, male age, cause of infertility.

Effects with significance <0.05 = female BMI (fbmi), blastocyst development stage by day 9 of research culture (bdrs)

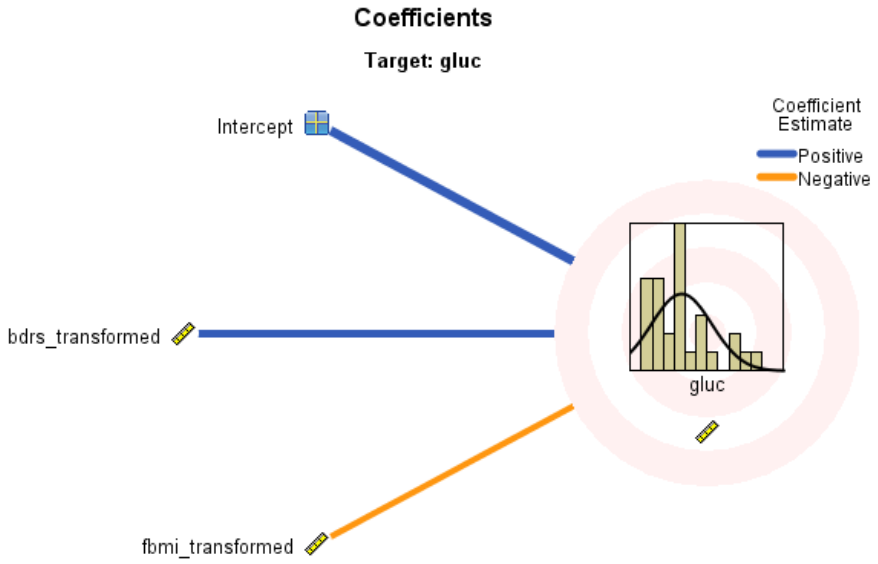


Figure A2 2.1 Forwards, stepwise regression linear model; significant predictive factors of embryo glucose consumption

A2.1.2 General linear Model – Female BMI (section 5.3.2);

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Glucose	170886.133[a]	10	17088.61	4.292	.000
	Lactate	65916.454[b]	10	6591.645	.959	.482
	Pyruvate	3778.887[c]	10	377.889	.721	.704
Intercept	Glucose	207583.559	1	207583.5	52.139	.000
	Lactate	96143.903	1	96143.90	13.981	.000
	Pyruvate	1598.955	1	1598.955	3.051	.083
fbmi	Glucose	99772.378	1	99772.37	25.060	.000
	Lactate	26740.687	1	26740.68	3.889	.051
	Pyruvate	25.188	1	25.188	.048	.827
ivficsi	Glucose	411.212	1	411.212	.103	.748
	Lactate	1985.210	1	1985.210	.289	.592
	Pyruvate	647.367	1	647.367	1.235	.268
stage	Glucose	66918.948	4	16729.73	4.202	.003
	Lactate	13531.320	4	3382.830	.492	.742
	Pyruvate	1773.455	4	443.364	.846	.498
ivficsi * stage	Glucose	38072.044	4	9518.011	2.391	.054
	Lactate	28701.890	4	7175.473	1.043	.387
	Pyruvate	941.309	4	235.327	.449	.773
Error	Glucose	545446.294	137	3981.360		
	Lactate	942081.986	137	6876.511		
	Pyruvate	71807.777	137	524.144		
Total	Glucose	2033051.889	148			
	Lactate	2718985.167	148			
	Pyruvate	233501.687	148			
Corrected Total	Glucose	716332.427	147			
	Lactate	1007998.440	147			
	Pyruvate	75586.664	147			

a. R Squared = .239 (Adjusted R Squared = .183)

b. R Squared = .065 (Adjusted R Squared = -.003)

c. R Squared = .050 (Adjusted R Squared = -.019)

Table A2 2.1 General linear model CORE profile and female BMI.

A2.1.3. CORE profile and pregnancy (section 5.3.2);

Logistic regression

Model Summary

Step	-2 Log likelihood	R^2
1	15.322[a]	0.45

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.forEXP(B)	
								Lower	Upper
Step 1[a]	Female age	.118	.320	.137	1	.711	1.126	.602	2.106
	Female bmi	.742	.605	1.503	1	.220	2.101	.641	6.883
	Male bmi	.506	.730	.480	1	.488	1.658	.397	6.936
	Cause of infert(1)	-1.925	3.008	.409	1	.522	.146	.000	53.031
	Embryo gradeday3	1.895	1.446	1.717	1	.190	6.652	.391	113.222
	Male age	.101	.285	.124	1	.725	1.106	.632	1.935
	Stage of dev day 5	-.152	.156	.939	1	.333	.859	.632	1.168
	Blast dev rate day 9	-.019	.044	.189	1	.664	.981	.900	1.070
	glucose	-.006	.038	.027	1	.871	.994	.923	1.070
	lactate	.003	.015	.035	1	.852	1.003	.974	1.032
	pyruvate	-.256	.175	2.123	1	.145	.774	.549	1.092
	Constant	-30.677	30.178	1.033	1	.309	.000		

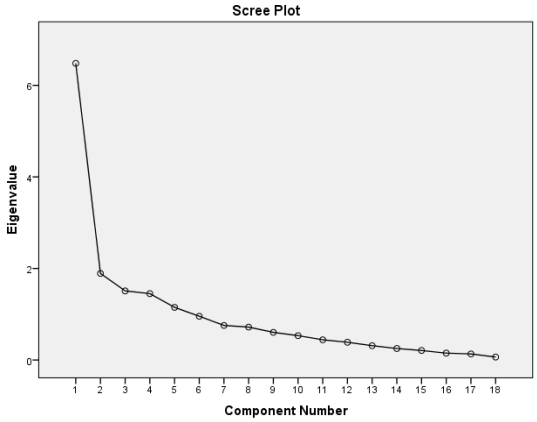
a. Variable(s) entered on step 1: fage, fbmi, mbmi, tx, gradeday3, mage, bdtx, bdrs, gluc, lact, pyruv.

Table A2 2.2 General linear model CORE profile and pregnancy outcome.

A2.1.4. Amino acid Principle component Analysis (section 5.3.4)

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.768
Bartlett's Test of Sphericity	Approx. Chi-Square	1547.372
	Df	153
	Sig.	.000



Rotated Component Matrix[a]

	Component				
	1	2	3	4	5
trp	.758				
glu	.721		.329		
asp	.690	.570			
gly	.680	.433			
thr	.663	.345	.545		
gln	-.604		-.439		
tyr	.552		-.506		
ser	-.498	-.324	-.372		
val		.880			
phe		.877			
asn	.383	.675		.301	
his	.379	.437			-.335
met			.705		
iso			.687		
lys	.304		.643		-.336
ala				.842	
arg				-.733	
leu					.821

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 11 iterations.

Table A2 2.3 Amino acid PC analysis.

A2.1.5 General linear model – Male BMI (section 5.3.7)

Tests of Between-Subjects Effects[d]

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	glucose	1837248.689	10	183724.869	1.695	.088
	lactate	1148043.444	10	114804.344	.678	.743
	pyruvate	97775.384[c]	10	9777.538	.754	.672
Intercept	glucose	1323404.093	1	1323404.093	12.210	.001
	lactate	66345.500	1	66345.500	.392	.532
	pyruvate	18868.313	1	18868.313	1.456	.230
mbmi	glucose	330407.581	1	330407.581	3.048	.083
	lactate	127973.785	1	127973.785	.756	.386
	pyruvate	2405.344	1	2405.344	.186	.667
stage	glucose	720204.315	4	180051.079	1.661	.163
	lactate	527519.309	4	131879.827	.779	.541
	pyruvate	49376.529	4	12344.132	.952	.436
ivficsi	glucose	55567.463	1	55567.463	.513	.475
	lactate	2841.939	1	2841.939	.017	.897
	pyruvate	6579.531	1	6579.531	.508	.477
stage * ivficsi	glucose	492244.854	4	123061.214	1.135	.343
	lactate	386638.454	4	96659.613	.571	.684
	pyruvate	24791.589	4	6197.897	.478	.752
Error	glucose	14849286.81	137	108388.955		
	lactate	23201993.67	137	169357.618		
	pyruvate	1775613.049	137	12960.679		
Total	glucose	45854115.69	148			
	lactate	63307417.15	148			
	pyruvate	5611448.111	148			
Corrected Total	glucose	16686535.50	147			
	lactate	24350037.12	147			
	pyruvate	1873388.433	147			

Table A2 2.4 General linear model CORE profile and male BMI.

A2.2Chapter 6

A2.2.1 Insulin data – general linear model glucose, lactate, pyruvate

Tests of Between-Subjects Effects

Source	Dependen	Type III Sum	df	Mean	F	Sig
Corrected Model	glucose	273425.170[a]	91	3004.672	7.830	.000
	lactate	142155.575[b]	91	1562.149	1.119	.326
	pyruvate	32719.162[c]	91	359.551	2.662	.000
Intercept	glucose	761406.024	1	761406.0	1984.239	.000
	lactate	917422.809	1	917422.8	657.408	.000
	pyruvate	112547.246	1	112547.2	833.201	.000
fbmi	glucose	166729.135	28	5954.612	15.518	.000
	lactate	48326.081	28	1725.931	1.237	.245
	pyruvate	15405.612	28	550.200	4.073	.000
exp	glucose	38.257	1	38.257	.100	.753
	lactate	458.439	1	458.439	.329	.569
	pyruvate	430.618	1	430.618	3.188	.080
stage	glucose	31485.411	4	7871.353	20.513	.000
	lactate	8856.715	4	2214.179	1.587	.190
	pyruvate	1979.958	4	494.990	3.664	.010
fbmi * exp	glucose	4010.175	19	211.062	.550	.925
	lactate	28033.139	19	1475.428	1.057	.416
	pyruvate	3005.192	19	158.168	1.171	.313
fbmi * stage	glucose	25882.075	25	1035.283	2.698	.001
	lactate	31118.450	25	1244.738	.892	.613
	pyruvate	5291.535	25	211.661	1.567	.082
exp * stage	glucose	1295.434	4	323.859	.844	.503
	lactate	1414.291	4	353.573	.253	.906
	pyruvate	1409.265	4	352.316	2.608	.045
fbmi * exp * stage	glucose	676.588	3	225.529	.588	.626
	lactate	1562.506	3	520.835	.373	.773
	pyruvate	170.312	3	56.771	.420	.739
Error	glucose	21872.439	57	383.727		
	lactate	79544.351	57	1395.515		
	pyruvate	7699.452	57	135.078		
Total	glucose	1322328.483	149			
	lactate	1638812.783	149			
	pyruvate	216904.412	149			
Corrected Total	glucose	295297.609	148			
	lactate	221699.926	148			
	pyruvate	40418.613	148			

a. R Squared = .926 (Adjusted R Squared = .808) b. R Squared = .641 (Adjusted R Squared = .068) c. R Squared = .810 (Adjusted R Squared = .505)

Table A2 2.5 General linear model CORE profile and experimental group (control and standard).

A2.2.1 Insulin data – general linear model amino acids (highlighted model utilized)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
fbmi * exp * stage	asp	.002	3	.001	.333	.801
	glu	.002	3	.001	.181	.909
	asn	.008	3	.003	1.134	.343
	ser	.004	3	.001	.152	.928
	his	.013	3	.004	.608	.612
	gln	.148	3	.049	.730	.539
	gly	.000	3	7.629E-005	.010	.999
	thr	.044	3	.015	.227	.877
	arg	.063	3	.021	.305	.822
	aln	.135	3	.045	1.836	.151
	tyr	.002	3	.001	.022	.995
	trp	.000	3	.000	.165	.919
	met	.016	3	.005	3.365	.025
	val	.009	3	.003	.696	.559
	phe	.009	3	.003	3.310	.026
	iso	.021	3	.007	3.088	.034
	leu	.014	3	.005	.483	.696
	lys	.005	3	.002	.483	.696
	sumaa	1.393	3	.464	1.554	.210

Table A2 2.6 General linear model CORE profile and experimental group (control and standard) – individual amino acids.

A2.2.2 Insulin data – Insulin data – Binominal Log Regression

The explained variation in the dependent variable based on the model ranges from 44.6% to 59.5%, based on the Cox & Snell *R2* or Nagelkerke*R2* methods, respectively.

Categorical Variables Codings

		Frequency	Parameter coding
			(1)
exp	control	70	1.000
	insulin	79	.000
fbmiclass	n	73	1.000
	o	76	.000

Coding used control = 1
Normal weight = 1

Classification Table[a]

		Observed	Predicted		
			Blast		Percentage Correct
			0	1	
Step 1	blast	0	60	13	82.2
		1	14	62	81.6
	Overall Percentage				81.9

a. The cut value is .500

Good sensitivity and specificity

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.forEXP(B)	
								Lower	Upper
Step 1[a]	fbmiclass(1)	-3.141	.615	26.073	1	.000	.043	.013	.144
	glucose	.056	.010	30.855	1	.000	1.058	1.037	1.079
	lactate	-.002	.006	.082	1	.775	.998	.987	1.010
	pyruvate	.000	.015	.000	1	.991	1.000	.972	1.030
	sumaa	-.031	.044	.475	1	.490	.970	.889	1.058
	exp(1)	-.645	.469	1.896	1	.169	.525	.209	1.314
	Constant	-2.506	.917	7.462	1	.006	.082		

a. Variable(s) entered on step 1: fbmiclass, glucose, lactate, pyruvate, sumaa, exp.

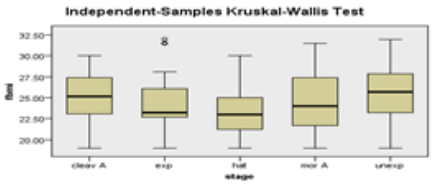
Table A2 2.7 Binominal regression model CORE profile and experimental group (control and standard).

The Wald test ("Wald" column) is used to determine statistical significance for each of the independent variables. The statistical significance of the test is found in the "Sig." column. From these results it is evident female BMI (p = .000) and glucose (p = .000) added significantly to the model/prediction, but lactate, pyruvate, experimental group (control/ insulin) did not add significantly to the model. The probability of an event occurring based on a one unit change in an independent variable when all other independent variables are kept constant can be predicted; the odds of blastocyst formation ("1" category) are0.04 times greater for normal weight as opposed to OWOB women.

A logistic regression was performed to ascertain the effects of female BMI, glucose, lactate and pyruvate consumption/release and amino acid turnover and experimental group (control/insulin) on the likelihood that embryos would reach the blastocyst stage.The logistic regression model explained 59.5% (Nagelkerke R2) of the variance in blastocyst formation/development and correctly classified 81.9% of cases. Embryos from normal weight women were0.04 times more likely to form blastocysts than those from OWOB females and increasing glucose consumption was associated with an increased likelihood of blastocyst development.

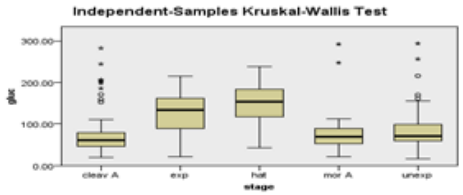
A2.2.3 Carnitine data; BMI, stage, CORE profile and LC concentration

Distribution of BMI across categories of stage and distribution of glucose across stage of blastocyst development- significantly different.



Total N	301
Test Statistic	17.391
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.002

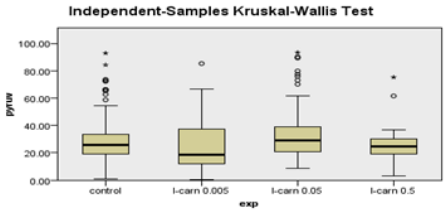
1. The test statistic is adjusted for ties.



Total N	301
Test Statistic	81.115
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

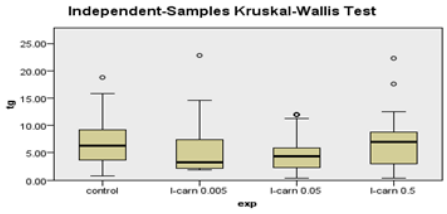
1. The test statistic is adjusted for ties.

The distribution of pyruvate consumption and TG content of embryos is significantly different for different concentrations of carnitine.



Total N	301
Test Statistic	8.141
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.043

1. The test statistic is adjusted for ties.



Total N	301
Test Statistic	18.401
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

Figure A2 2.1 Data variance/ distribution and experimental group (control and standard).

A2.2.4 L-Carnitine data – general linear model glucose, lactate, pyruvate, TG (see highlighted model in red)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
	gluc	20083.146	3	6694.382	3.293	.021
	lact	9248.585	3	3082.862	.665	.574
fbmiclass *	pyruv	1752.404	3	584.135	1.915	.128
exp	carn	9935.749	3	3311.916	4.156	.007
	tg	167.365	3	55.788	5.046	.002
	blast	.000	3	.000	.000	1.000
stage * exp	gluc	58132.112	12	4844.343	2.383	.006
	lact	60306.988	12	5025.582	1.084	.374
	pyruv	3951.386	12	329.282	1.079	.378
	carn	32208.703	12	2684.059	3.368	.000
	tg	64.129	12	5.344	.483	.924
	blast	.000	12	.000	.000	1.000
	gluc	56562.077	12	4713.506	2.319	.008
	lact	36312.340	12	3026.028	.653	.796
fbmiclass *	pyruv	4392.868	12	366.072	1.200	.283
stage * exp	carn	19382.678	12	1615.223	2.027	.022
	tg	63.977	12	5.331	.482	.924
	blast	.000	12	.000	.000	1.000

Table A2 2.8 General linear model CORE profile and experimental group (control and standard).

A2.3 Chapter 7

A2.3.1 Predictors of pregnancy

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 0	Constant	.143	.379	.143	1	.706	1.154

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	.000[a]	.749	1.000

Variables in the Equation

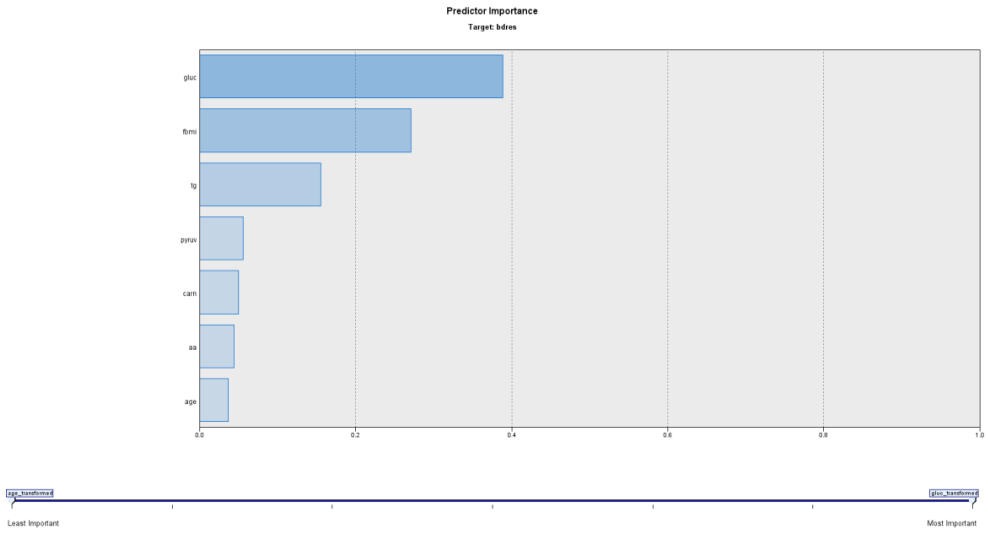
		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.forEXP(B)	
								Lower	Upper
Step 1[a]	age	-3.340	5365.428	.000	1	1.000	.035	.000	.
	fbmi	58.193	19495.813	.000	1	.998	187459415944239530000000000.000	.000	.
	mbmi	27.963	2923.188	.000	1	.992	1393749344680.319	.000	.
	bdtx	-1.353	1335.971	.000	1	.999	.258	.000	.
	bdres	-2.699	235.221	.000	1	.991	.067	.000	1.117E+199
	gluc	1.782	866.893	.000	1	.998	5.942	.000	.
	lact	.374	617.221	.000	1	1.000	1.454	.000	.
	pyruv	-8.367	722.002	.000	1	.991	.000	.000	.
	aa	-9.787	3665.741	.000	1	.998	.000	.000	.
	tg	-30.492	2681.677	.000	1	.991	.000	.000	.
	cycle	119.729	26211.080	.000	1	.996	9.944E+051	.000	.
	tx(1)	-103.062	25791.241	.000	1	.997	.000	.000	.
	Constant	-1459.076	602219.858	.000	1	.998	.000		

a. Variable(s) entered on step 1: age, fbmi, mbmi, bdtx, bdres, gluc, lact, pyruv, aa, tg, cycle, tx.

Table A2 2.9 General linear model- predictors of pregnancy.

A2.3.2 Predictors of blastocyst development

Forwards, stepwise regression linear model;Target variable blastocyst formation. Predictor variables included in analysis; female age, male age, female BMI, male BMI, cause of infertility, cycle number, treatment, culture media, oxygen, down regulation, smoking, embryo grade day 3, glucose, lactate, pyruvate, amino acid, triglyceride, l-carnitine.



Blastocyst formation – treatment – different predictor variable highlighted (N.B. – blastocysts frozen and not donated to research).

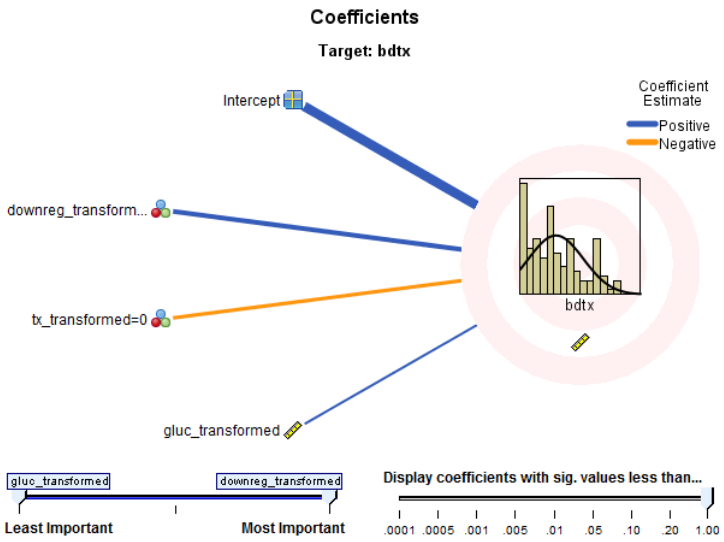


Figure A2 2.2 Forwards, stepwise regression linear model; significant predictive factors of blastocyst development.

Appendix 3 Supplementary figures;

A3.1 Chapter 5

A3.1.1. Relationship between day 3 development and CORE profile day 5-9

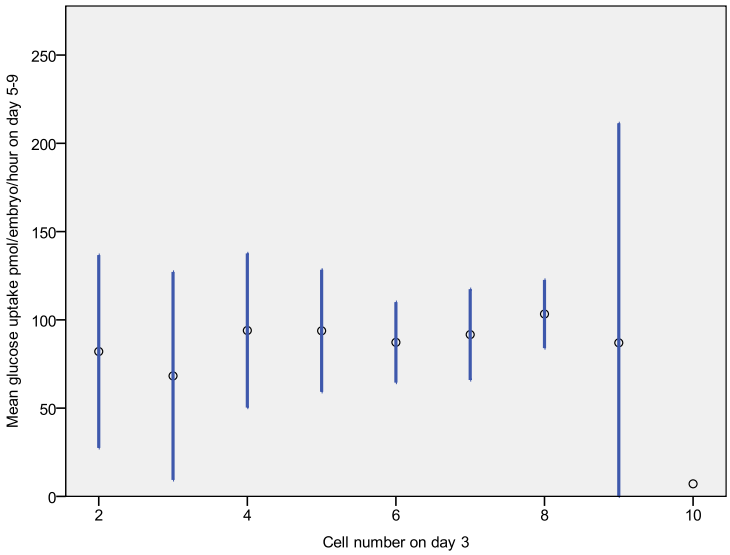


Figure A3 3.1 Mean embryo metabolism of glucose from day 5 to 9, according to developmental cell stage attained on day 3

Day 3 development (<6 cells n=37 and ≥6 cells n=112)			
	d3	Mean (± SEM) consumption / production in pmol/ember/hour	*P*
Glucose	≥6	95.53 (6.45)	0.716
	< 6	90.49 (12.21)	
lactate	≥6	101.49 (7.02)	0.174
	< 6	126.68 (16.86)	
pyruvate	≥ 6	33.86 (2.33)	0.173
	< 6	29.08 (2.59)	

Table A3 3.1 - CORE embryo metabolism of substrates, day 5-9, according to developmental cell stage attained on day 3.

A3.1.1. Relationship between BMI and CORE profile and day of development

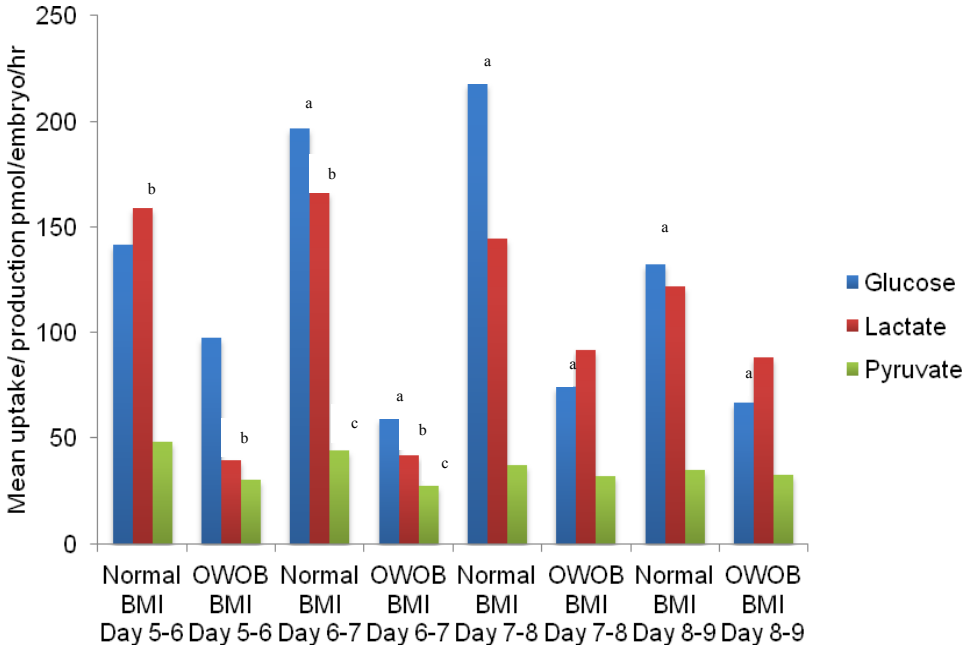


Figure A3 3.2 Mean embryo metabolism of substrates over 24 hour time periods, during 5 days of extended culture. Values with the same superscript are significantly different ($p<0.05$).