Ovarian follicular fluid reflects the clinical condition and oocyte cumulus homeostasis

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Abstract

Infertility is a worldwide problem that is often overlooked. Although assisted reproductive technology has been developed over decades, many women still suffer from infertility. More knowledge is needed to understand ovarian homeostasis to optimise fertility treatment. This study aimed to explore the relationship of lipids and glucose levels in blood and follicular fluid, and compare these substrates among women with normal or abnormal metabolic condition. It also sought to measure lipid content within human oocytes as well as the expression of endoplasmic reticulum stress marker genes in cumulus cells, and their relationship with metabolic substances, obesity and IVF outcome.

The blood, follicular fluid, cumulus cells and unfertilised oocytes from 88 women, who underwent IVF in FertilitySA from February 2011 to August 2011, were collected and analysed for glucose, lipids and endoplasmic reticulum stress markers.

Follicular glucose, insulin, high density lipoprotein cholesterol (HDL-C) and majority of polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) levels correlated with the serum levels (r= 0.16-0.23). Insulin was associated with the BMI, waist circumference, metabolic syndrome and many fatty acids, but not follicular glucose. However, the immaturity rate of the retrieved oocytes correlated with the follicular glucose and total fatty

acids (r = 0.19-0.26, p <0.04). Variability of the unfertilised oocyte morphology correlated with follicular glucose, and the immaturity rate also differed among the metabolic syndrome group.

An increase of follicular 18:3 n-3 (alpha-linolenic) and decrease of 20:3 n-3 (eicosatrienoic acid; ETA) existed in women with a waist circumference of more than 80 cm. The follicular 20:5 n-3 (eicosapentaenoic acid; EPA) percentage correlated with fertilisation and cleavage rate (r = -0.32, p = 0.003 and r = -0.35, p = 0.001). The follicular low density lipoprotein cholesterol (LDL-C) and HDL-C related to follicular fatty acids. Higher levels of serum LDL-C (2.31 ± 0.65 and 1.98 ± 0.61mmol/L, p 0.02) and follicular fatty acid (0.26 ± 0.09 and 0.22 ± 0.05 mmol/L, p = 0.03) were found in non-pregnant women.

There was a wide variability of ER stress expression in cumulus cells among women in this study. There was no obvious correlation between ER stress markers and other measurements. The unfertilised oocyte BODIPY fluorescence intensity had high variability among women and individuals. However, the unfertilised oocyte lipid content correlated with the serum LDL-C level.

Substances with a good follicular-serum relationship may be transported directly from blood to the follicle. The discorrelation might be affected by intrafollicular metabolism. Insulin may be involved in follicular lipid metabolism because it correlated with many follicular fatty acids and cholesterols. The equilibrium between follicular fatty acids involving insulin modulation may affect oocyte quality.

Overall, this study found there were correlations between serum and follicular lipids, follicular insulin and cholesterol with follicular fatty acids and the importance of serum LDL-C and follicular omega-3 fatty acids. Serum insulin and LDL-C screening might be another tool for predicting the follicular lipid dysequilibrium and poor IVF outcome. The unfertilised oocyte may be a useful tool for a study on oocyte quality. A larger study is needed to recruit more women of different ages and BMI for a stronger correlation between follicular insulin, glucose and lipid metabolism, and ER stress markers.

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Chapter 1 Literature review

1.1 The effects of being overweight and obese on reproduction

1.1.1 Overweight and obesity

Obesity is a major problem throughout the world. It is often classified by the body mass index (BMI) category, which is body weight in kilogram divided by height in metres squared. People who have BMI 25-29.9 kg/m^2 are defined as overweight and BMI 30 kg/m^2 or more are classified as obese (1). In 2000, 52% of Australian women had a BMI more than 25 kg/m^2 and nearly half of them were obese (2). The number of overweight women is predicted to grow to a similar proportion of the USA and the UK (3). Obesity is also classified into two types; peripheral obesity and the central obesity. Central or visceral obesity is diagnosed by the waist circumference or waist/hip circumference measurement and is a better predictor of diabetes mellitus risk than BMI. Although, the waist circumference is the best surrogate marker of visceral fat accumulation, it still includes the abdominal subcutaneous fat, not only the visceral fat unlike that measured from CT-scan (4, 5). Central obesity has a high impact on health. The mesenteric fat has higher lipogenic activity than subcutaneous fat and it releases free fatty acids to the portal circulation. The excess of free fatty acids causes liver lipid synthesis enhancement leading to insulin resistance, hyperlipidaemia and hypertension. The effect of visceral fat on deregulation of adipocytokine production and secretion induces a higher risk of diabetes mellitus, dyslipidaemia, hypertension and cardiovascular disease (6).

Obesity relates to many reproductive health problems including polycystic ovary syndrome (PCOS) which affects about 50% of obese women (7, 8). It mainly increases the chance of abnormal menstruation, anovulation, subfertility and pregnancy loss (9-11).

1.1.1.1 Female Infertility and Obesity

Infertility is a hidden problem worldwide. The prevalence in Australia is similar to the UK, at around 10-17% (12, 13). Ninety percent of anovulatory infertility in women is caused by PCOS and both PCOS and obesity decrease the chances of conception and the success rate of assisted reproductive techniques (9, 14). Infertility also impacts the psychology and quality of life, especially in women. The childless infertile woman has an increased risk of anxiety disorders, personal, marital and social distress (15-18). Half of the women who undergo fertility treatment rate infertility as their most stressful experience and the severity of anxiety and depression are comparable to women with cancer or coronary heart disease (19, 20).

The fecundity of overweight or obese women is lower than normal weight women with the reduction in pregnancy rate of at least 8% in overweight and 18% in obese patients (21-24). The effect of obesity on spontaneous pregnancy is clinically and epidemiologically obvious when the BMI is more than 29 kg/m^2 (25). The two major biochemical substances that are associated with obesity related anovulation leading to infertility are insulin and leptin. Obese patients, especially those who are centrally obese have an increased risk of insulin

resistance and hypersecretion of insulin, which inhibits hepatic Sex-Hormone Binding Globulin (SHBG) and Insulin-like Growth Factor Binding Protein (IGFBP) synthesis. The decrease of SHBG leads to a higher level of free sex steroid hormone, while the increase of Insulin-like Growth Factor (IGF), produced mainly from the liver also stimulates ovarian steroidogenesis and both of them cause abnormal gonadotrophin secretion which affects normal folliculogenesis and ovulation (9, 10, 26, 27). Moreover, obese patients experience an increase in serum leptin, which is the protein secreted by adipocytes and can inhibit folliculogenesis, dysregulate gonadotrophin releasing hormone (GnRH) and alter ovarian steroidogenesis (9). About 50% of obese women of reproductive age also have PCOS (7, 8) and both the obese and PCOS patients have similar insulin and hyperandrogenism pathophysiology (10, 28).

There is no absolute consensus based on evidence about the effect of obesity on infertility treatment. Obesity may prolong the time to pregnancy during ovulation induction, increases the gonadotrophin dose required, decreases the number of mature follicles and increases cycle cancellation rate (29, 30). The impairment of sex steroid hormone regulation may lead to longer periods of follicular stimulation, more chance of follicular asynchrony, cycle cancellation and lower live birth rates among obese patients who undergo In Vitro Fertilisation (IVF) compared to normal weight infertile patients (9). Every 1 unit increase of BMI decreases the probability of pregnancy following IVF by 2.2- 4.3% (31). The intracytoplasmic

injection (ICSI) fertilisation rate does not seem to be affected but the implantation rate of obese patients are less than normal weight patients (32). The obesity effect is still debated in regards to oocyte retrieval number, quality and maturity, fertilisation, embryo quality and pregnancy rate, but the outcome tends to be worse in obese patients, especially, morbid obesity (23, 29, 33-37). PCOS, which is included in some obesity studies may also affect the pregnancy rate (38). Overall, the obese patient might have oocyte, hormone, metabolic and endometrial dysfunction. The degree of dysfunction needs to be identified to optimise their fertility.

Pregnancies resulting from IVF do not depend solely on the number of oocytes; good quality oocytes and/ or endometrial function are the key factors for successful IVF outcome (39-42). Oocyte factors may be affected by obesity because clinical pregnancy rate is lower in obese patients, but obese patients who use donor oocyte do not have poor clinical pregnancy rates (43). However, evidence has shown that primiparous obese women have a higher rate of early miscarriage than normal weight women (44). Although the implantation rate in donor cycles of obese recipient is still debated, the chance of miscarriage is higher than in a normal weight recipient. Obese recipients have a 4-fold higher miscarriage risk compared to normal weight recipients, leading to lower live birth rates. (45-49). Moreover, obese women also have aberrant gene expression regulation during the window of implantation (50).

There are few studies examining the effect of fat distribution on outcomes in infertile patients. A study in patients undergoing intrauterine insemination showed that every 0.1 unit increase of waist/hip circumference ratio relates to a 30% decrease in the probability of conception per cycle (HR 0.71) (51). Likewise, a study of IVF patients found that a waist-hip ratio more than 0.8 associates with a lower chance of pregnancy (52). Weight-loss programs reveal a higher pregnancy rate in patients who reduce their waist circumference (53). Fertility, hormones, ovulation, live birth rate and psychometric measurement all improve after weight loss (54-57). Weight loss provides many benefits for the obese patient, but, changing eating behavior and maintenance of ideal weight is difficult and hard to achieve (58, 59). Many of the infertile patients attend infertility clinics at an age more than 30 and may not have much time to wait until they can lose weight because age itself is the major factor of declining fertility (60-63).

1.1.1.2 Effect on male factor

Infertility is a couple problem and the male contribution needs to be assessed simultanously with female infertility. Studies have found that couples tend to have similar age, weight, BMI, lean mass and fat mass, indicating an obese woman may have an obese male partner (22, 64, 65) and there is a higher risk of being subfecund if both are obese (66). So, the effect of obesity on IVF outcome may arise from both male and female obesity. Male

obesity increases the risk of sleep apneoa, abnormal sexual function, hormone alteration, increased scrotal temperature and impaired semen parameters (67, 68). Male BMI has an inverse correlation with androgen hormones, but the effect on semen parameters is still inconclusive (67, 69-71). However, morbid obesity has a detrimental effect on the sperm count and sperm DNA damage (72). Fatty acids affect sperm function; the amount of polyunsaturated fatty acids and docosahexaenoic acid (DHA) have a positive correlation with sperm concentration and motility, while monounsaturated, oleic and total saturated fatty acids have an inverse correlation (73-76). An overweight male partner tends to produce with his partner decreased blastocyst development, lower clinical pregnancy rate and live birth when compared to normal weight males. This effect is obvious in IVF cycles, but less so with ICSI (77, 78). The effect of male obesity also has been shown by weight loss, as, this can restore fertility and normal hormone profiles (68).

1.1.1.3 Anovulation and PCOS

Under the Rotterdam criteria, polycystic ovary syndrome (PCOS) is diagnosed by two of three criteria which are hyperandrogenism, polycystic ovaries on ultrasound and chronic anovulation (79). The prevalence of PCOS is 6-17% of reproductive- aged women (80, 81). PCOS patients commonly present with menstrual problems, hyperandrogenism and subfertiliy (82). This syndrome is associated with infertility, obesity, dyslipidaemia, metabolic syndrome,

impaired glucose tolerance, diabetes mellitus, cardiovascular disease and endometrial hyperplasia (7). The risk is different among subgroups of PCOS, for instance, obese PCOS patients have more chance of anovulation and insulin resistance than lean PCOS patients (83). However, the risk, prevention and specific treatment for each PCOS subgroup is still unclear because of limited knowledge of causes and pathophysiology of PCOS.

PCOS patients can be diagnosed by three major classifications, namely the National Institute of Health (NIH) 1990, the Rotterdam 2003, and the Androgen Excess and PCOS Society (AES) 2006 descriptions. Among these three criteria, Rotterdam weighs the importance of hyperandrogenism equal to anovulation and polycystic ovaries from ultrasound pictures, while the AES pays more attention to hyperandrogenism and the NIH is strict on both hyperandrogenism and anovulation (8). Indeed, the prevalence of PCOS is highest when using the Rotterdam criteria, followed by the AES. NIH and AES criteria classed PCOS patients have a greater severity of clinical manifestation when compared to patients who are diagnosed by the Rotterdam's criteria (84-86). The application of criteria depends on the purpose of the studies. The NIH and AES classifications are commonly used in hormonal, metabolic and cardiovascular issues; meanwhile, the Rotterdam is more appropriate for early detection strategies and infertility issues (8). PCOS clinical manifestation is variable because of the heterogeneity of PCOS (79). There are 10 phenotypes of the Rotterdam PCOS, 9 in AES and 6 in the NIH as shown in Table 1.1 (8). Although each PCOS phenotype is quite similar, in

some circumstances, such as obesity and insulin resistance, the pathophysiology, metabolic risk and appropriate treatment for lean and obese PCOS is different (81, 83, 87, 88). Obese PCOS patients have more severe phenotypes in both metabolism and reproduction because hyperinsulinaemia can affect ovarian steroidogenesis, decrease SHBG level and increase free androgen hormone (9, 81, 88-90). Moreover, adipose tissue can store lipid soluble steroids, such as androgens and has steroidogenic activity in its own right converting more androgen precursors to active androgens such as androstenedione and testosterone. (91).

Table 1.1 Phenotypes of PCOS patients according to different criteria
(Adapted from Azziz R, 2009 (8))

Features	Phenotypes									
Hyperandrogenaemia	+	+	+	+	-	-	+	-	+	-
Hirsutism	+	+	-	-	+	+	+	+	-	-
Oligo-anovulation	+	+	+	+	+	+	-	-	-	+
Polycystic ovaries	+	-	+	-	+	-	+	+	+	+
NIH criteria	/	/	/	/	/	/				
Rotterdam criteria	/	/	/	/	/	/	/	/	/	/
AES criteria	/	/	/	/	/	/	/	/	/	

+ is the present of each feature

/ is the diagnosis by individual criteria

1.1.2 Glucose and lipid in overweight-obese patients

The imbalance of food intake and activity causes excess fat storage, affecting both glucose and lipid metabolism. Fat accumulation, especially in the visceral fat causes insulin resistance, hyperglycaemia and diabetes mellitus. Meanwhile, it can also induce cholesterol production and free fatty acids synthesis (92). Glucose and lipid metabolism have a close association, likewise, the serum triglyceride (TG) and high density lipoprotein cholesterol (HDL-C) level relate to the insulin level and can be a predictor for insulin resistance (93). The homeostatic model assessment (HOMA) correlates with TG level even in a normal glucose tolerance patient and patients who have high triglyceride and low HDL-C have a higher chance to have high level of C-reactive protein (CRP), blood pressure, glucose, insulin either currently or within a three years follow up period (94-96).

Insulin is a major peptide hormone produced from beta cells of the pancreas. It plays a significant role in carbohydrate, fat and protein metabolism (97). The gold standard for insulin sensitivity testing is the hyperinsulinaemic-euglycaemic clamp but, it is time-consuming, labour intensive, invasive and not suitable for a practical setting. Many studies have tried to look for a simpler test of insulin resistance, such as fasting insulin, glucose-insulin ratio and HOMA, which is calculated by fasting insulin multiplied by fasting glucose and divided by 22.5. This test has a good relationship with the gold standard tests and cardiovascular and diabetes mellitus risk. The cut off value for Caucasians is more than 3.8

(98-101). Insulin potentiates steroidogenic response to gonadotrophins by increasing the LH receptor or increases pituitary sensitivity to GnRH. This hormone also inhibits hepatic IGPBP-1 and SHBG production leading to the increase of IGF and free steroid hormone (97).

Lipids are an important energy supply, with the excess lipid intake stored in adipose tissue. Fatty acids are stored in white adipose tissue in triacylglycerol form before they are released as free fatty acids entering the blood circulation for energy production. Obese patients tend to have high serum free fatty acids (FFAs), especially saturated fatty acids. The prevalence of increased fatty acids levels is higher among obese patients who have higher upper body fat distribution. Elevated serum FFA levels often lead to insulin resistance and hepatic inflammation. This relates to the increase of diabetes mellitus, hypertension and dyslipidaemia risks (102-104). FFAs, especially, long chain FFAs can promote insulin secretion through insulin resistance and inhibit carbohydrate oxidation, glycogen synthesis and intracellular glucose transportation (102). PCOS patients tend to have higher FFA levels when compared to non- PCOS patients and this is obviously significant in those who have high serum fasting insulin levels and truncal obesity (105-107). This may be explained by the increase of upper body fat distribution in PCOS patients, especially, obese PCOS (108-111). Moreover, the amount of visceral fat in PCOS patients also correlates with insulin resistance, hypertriglyceridemia and SHBG (108, 112).

There are 2 major groups of fatty acid in human serum, the saturated and unsaturated. The predominant saturated fatty acids are myristic (14:0), palmitic (16:0), stearic (18:0) and arachidic (20:0). The unsaturated fats are either monounsaturated (MUFA) or polyunsaturated (PUFA). The PUFA group includes omega-9 (n-9), omega-6 (n-6)and omega-3 (n-3) subgroups. Humans cannot synthesize the n-6 or n-3 PUFA, and these two fatty acid subgroups must be obtained from diet or synthesized by gut micro-organisms (113). Major saturated fatty acids in human diet are palmitic, stearic, myristic and lauric acid (114). Fatty acids from food are absorbed and transformed to the membrane lipid, stored in triglyceride form or oxidized for energy (113). Elevation of total fatty acid levels induce insulin resistance by three mechanisms, namely inhibition of insulin stimulated glucose uptake, inhibition of glycogen synthesis and inhibition of insulin stimulated glucose oxidation. Moreover, fatty acids cause hepatic insulin resistance leading to an increased rate of endogenous glucose production. Eighty percent of obese patients have insulin resistance only because they can compensate for free fatty acid mediated insulin resistance by increased insulin secretion, but, those who cannot compensate may develop type II diabetes mellitus (115). The increase of polyunsaturated intake may partially protect the body from insulin resistance because the polyunsaturated fatty acid levels relate with the increase of resting metabolic rate that may lead to lower fat accumulation (113, 116). Studies in rat have shown that n-3 PUFA consumption improves insulin receptor function (113). The data from epidemiological studies

suggest that an increase in saturated fatty acid intake is associated with hyperinsulinaemia, while monounsaturated and polyunsaturated fatty acid intake decreases the risk of insulin resistance (116, 117).

Recently, obesity has been associated with chronic inflammatory disease. The key inflammatory mediators related to the lipids are n-6 eicosanoids, PGE2 and leukotrieneB4 (LTB4), which are derived from n-6 PUFA. Eicosapentaenoic acid (EPA) is one of the n-3 family. EPA competes using the same enzyme as arachidonic acid, transforming itself to PGE3 and LTB5 which have weaker inflammatory activity when compared to PGE2 and LTB4 that are derived from arachidonic acid. Eicosatrienoic acid (ETA) is derived from n-9 fatty acid also competes for the enzyme, but not as strongly as EPA (118). These n-3 fatty acids can suppress TNF-alpha and IL-1beta production. A high intake of n-3 fatty acid associates with the reduction of plasma TNF and CRP and this effect is enhanced when people have high serum n-6 supplemented with n-3 fatty acid (119). The high serum n-6/n-3 ratio relates to the increase of TNF-alpha and IL-6, significant proinflammatory cellular cytokines in human (120).

Besides the n-6 and n-3 proportion that may be beneficial on lipid and glucose metabolism, consumption of high monounsaturated fatty acid (MUFA) sources like olive oil can decrease low density lipoprotein cholesterol (LDL-C), increase HDL-C and may decrease blood pressure (121). The benefit of fatty acid diets has also been shown in Australian studies where people who decreased their saturated fatty acid intake lowered

their total cholesterol while the increase of n-3 fatty acid in the diet can slightly increased HDL-C and decreased TG (122). Replacing dietary saturated fatty acid with unsaturated fatty acid decreases LDL-C, VLDL and TG levels. Higher PUFA n-6 and n-3 intake increases HDL and decreases TG and LDL (116, 123), while n-3 supplements also decrease the risk of cardiovascular disease (124, 125).

Each 2% increase of trans-unsaturated fat intake associates with a greater risk of ovulatory infertility with relative risk from 1.73 to 2.31 (126). Infertile women have lower plasma eicosapentaenoic acid and erythrocyte docosahexaenoic acid levels than fertile women (127). While there is no difference in serum total fatty acids, palmitic acid, stearic, oleic, linoleic and myristic acid among IVF patients who succeed or who do not get pregnant (128), higher levels of alpha-linolenic acid in women undergoing IVF were associated with decreases in the pregnancy rate (128).

1.2. The ovarian follicle

1.2.1 The oocyte

1.2.1.1 Oocyte development

The oocyte is the largest cell in female mammals. Mammalian oocytes form in early fetal life and remain suspended at the diplotene stage of the first meiotic prophase until the commencement of follicular recruitment during puberty. The oocyte increases in volume, replicates and redistributes cytoplasmic organelles and protein, accumulates water, ions, lipid vesicles, glycogen granules, multivesicular and crystalline bodies. Oocyte meiosis finishes to produce a haploid gamete when ovulation occurs and extrusion of the 1st polar body into the perivitelline space and second meiotic division takes place at metaphase II (129, 130). The oocyte surface is covered with the acellular glycoprotein membrane comprising the zona pellucida with a width around 15 um (131).

The immature oocyte has fewer microvilli than mature oocyte and smaller lipid droplets as during the oocyte maturation process, the droplets gradually merge and increase in size. The mature oocyte also contains less rough endoplasmic reticulum, a lower volume of Golgi complex , but an increase in mitochondria, accumulation of ribosomes and volume of smooth endoplasmic reticulum (SER) (132, 133). Normal oocyte development needs the follicular cells surrounding the egg for physical, nutrient and metabolic precursor support as well as for the production of growth factors and hormones (131). The growing follicle and

granulosa cells which surround the oocyte have bidirectional communication and the essential substances are transfered via the zona pellucida and gap junctions (131). In morphologically abnormal oocytes, there is an increase in the presence of degenerate structures, as well as granulosa cell and cumulus cell apoptosis (133).

The most abundant organelles in human oocytes are the smooth endoplasmic reticulum (SER) and mitochondria (134-136). Unfertilised oocytes have a decrease of mitochondrial gene expression, increased numbers of deleted DNA and accumulation of rearranged mitochondrial DNA (137, 138). The number of mitochondria in unfertilised oocytes are fewer than in mature human oocytes, pronuclear zygotes and arrested embryos (139).

In general, there is a small amount of free lipid droplets in the resting oocyte, but, the number of lipid droplets increases when atresia is initiated and are located mainly in multivesicular bodies of the perinuclear area. Sign of atresia within the resting oocyte are dilatation of SER and Golgi complexes, irregular mitochondria, loss of mitochondria-SER association, ruptured mitochondrial membrane and extensive vacuolarization. The mitochondrial dysfunction might lead to oxygen radical overproduction in the resting oocyte (136). The process of environmental injury starts from an increase in cell membrane permeability, followed by the destruction of the cytoplasmic structure and nuclear degeneration. Women of advanced age tend to have more ooplasmic vacuoles, mitochondrial

matrix, dilated SER and Golgi complexes that may affect the quality of the oocyte (140).

1.2.1.2 Oocyte morphology

Under the light microscope, normal mature human oocytes show a round clear zona pellucida, small perivitelline space, single unfragmented 1st polar bodies and a pale, moderately granular cytoplasm. Ninety three percent of patients undergoing assisted reproductive technologies have at least 1 abnormal oocyte (141). Many of the oocytes are dysmorphic, with cytoplasmic abnormalities, large perivitelline spaces, dark zona pellucida and fragmented polar bodies (142-144). Cytoplasmic granularity is the most common abnormal defect. One-quarter of oocytes have double anomalies, 5.9% have triple anomalies and 2.9% have abnormal shapes (141-143, 145).

The oocyte cytoplasmic dysmorphism or multiple morphology abnormalities impacts on blastocyst development. Some studies found that dysmorphism is not associated with higher risks of aneuploidy, fertilisation rate and cleavage rate (146). However, further studies revealed that the timing of dysmorphism occurring is important. The oocyte dysmorphism that occurs in early meiotic maturation has 50% chance of aneuploidy and this oocyte may have fertilisation incompetence, but the oocyte dysmorphism which occurs after metaphase stage has equal chance of aneuploidy to normal cytoplasmic oocyte at 15% (144, 147).

One quarter of unfertilised oocytes have abnormal chromosomes. Non-dysjunction is the most common aneuploidy defect, but aneuploidy of unfertilised oocytes is not related with the female age (147). However, there is a lot of inter-patient variation in the prevalence of hypohaploidy and chromosome abnormalities. Chromosomal fragmentation is not different between the unfertilised oocyte after intracytoplasmic sperm injection (ICSI) or standard in vitro fertilisation (IVF) (147-149).

Most oocytes with normal morphology have a good implantation rate, but an abnormal polar body, large perivitelline space, centrally located granular area, granular cytoplasm and vacuole are associated with a poor implantation or pregnancy rate (142, 143, 150-152). However, the effect of oocyte dysmorphism on fertilisation rate and embryo quality is still debated (141-143, 150, 151, 153, 154). Patients who have more than one abnormality, especially a centrally located granular area, abnormal polar body or vacuoles have a higher chance of having lower embryo quality and clinical pregnancy rate. These abnormalities also have a weak correlation with female age, basal FSH and weak negative correlation with the number of oocyte retrieved (141). Although, the percentage of mature and immature oocytes is similar in obese and non-obese patients, there are more granular cytoplasm oocytes in overweight-obese patients when compared to normal weight patients (32).

Studies have shown that an abnormal polar body with or without perivitelline space enlargement are found more often in women aged more than 35 years (151). Oocytes with a

good shape 1st polar body and undergoing ICSI have higher fertilisation rates, embryo development, implantation and pregnancy rate, compared to oocytes with fragmented 1st polar body which develop more fragmented embryos and fewer blastocysts, while the cytoplasmic abnormalities may not play a role in this effect (145, 151, 155-157). However, other groups found that the presence of a fragmented polar body does not affect fertilisation rate, embryo quality, pregnancy and implantation rate (158, 159). The effect on pregnancy outcome may depend on the variation in timing of meiotic maturation because a normal post mature polar body may be degenerate naturally (160).

Besides the cytoplasmic abnormality that may relate to poor IVF outcome, 0.3% of the overall oocytes are giant oocytes (20% larger than normal size). Unfertilised giant oocytes are diploid (FISH), while fertilised giant oocytes have normal cleavage and blastocyst development (161). Eighteen percent of ICSI patients also have at least one ovoid oocyte and the ovoid oocyte has a different cleavage pattern and delayed preimplantation development (162).

The zona pellucida is an acellular area composed of several glycoproteins and carbohydrates. It is the protector of the oocyte and also connects the oocyte with cumulus cells by gap junctions. After fertilisation, the zona changes its chemical structure to prevent poly-spermic fertilisation and protects the early embryo during transportation through the fallopian tube. Zona pellucida defects might be the cause of fertilisation failure (163, 164).

Zona pellucida thickness variation (ZPTV) is calculated by measuring four areas of zona pellucida at 3,6,9 and 12 o'clock. Then, minus the maximal zona pellucida thickness with mean zona pellucida thickness and divided by mean zona pellucida thickness and 100%. High values of ZPTV correlate with good clinical pregnancy rates and high grade embryos (163, 165). Although the embryo score can predict pregnancy rates, the ZPTV enhances the prediction accuracy in poor quality embryos (166). The unfertilised oocyte has significantly thicker ZPT when compared to fertilised and polyspermic oocyte (mean 18.9, 16.4 and 15.1 um) (167).

Overall, the studies about oocyte abnormal morphology that affect the IVF outcome are shown in Table 1.2

Table 1.2 Abnormal morphology of oocytes related to poor IVF outcome

Abnormal Morphology	Fertilisation rate	Cleavage quality	Blastocyst quality	Aneuploidy	Implantation rate	Pregnancy Rate	Cryosurvival Rate
Granular cytoplasm	Lower (141, 151) Not affected (143, 144, 146, 150) Early meiotic abnormalities affected(147)	Lower (151) Not affected (146)	Lower (146)	Not affected (146) Early meiotic abnormalities affected (144, 147)	Lower (150) Not affected (142)	Not affected (142) Lower (143, 152)	Lower (134)
Centrally located granular area	Not affected (146, 152)	Not affected (146, 152) Lower (141)	Lower (146)	Not affected (146)	Lower (150)	Lower (152)	Lower (134)
Vacuole	Not affected (146) Lower (141)	Not affected (146)	Lower (146)	Not affected (146) Early meiotic abnormalities affected (144)	Lower (150)		Lower (134)
Cytoplasmic inclusion	Not affected (143, 146)	Not affected (146)	Lower (146)	Not affected (146)		Lower (143)	
Irregular cytoplasm	Not affected (146)	Not affected (146)	Lower (146)	Not affected (146) Early meiotic abnormalities affected (144)			Lower (134)

Table 1.2 (cont.) The abnormal morphology of oocytes related to poor IVF outcome

Abnormal Morphology	Fertilisation rate	Cleavage quality	Blastocyst quality	Aneuploidy	Implantation rate	Pregnancy Rate	Cryosurvival Rate
Giant oocyte		Not affected (161)	Not affected (161)				
Ovoid oocyte		Lower (162)	Delayed development (162)				
Large perivitelline space	Lower (141, 151) Not affected (142, 146)	Lower (151) Not affected (146)	Lower (146)	Not affected (146)	Lower (150) Not affected (142)	Not affected (142)	
1st polar body	Lower (141, 151) Not affected (146, 158, 159)	Lower (145, 151, 155-157) Not affected (146, 158, 159)	Lower (145, 146, 155-157)	Not affected (146)	Lower (145, 150, 155-157) Not affected (158, 159)	Lower (145, 155-157) Not affected (158, 159)	
ZPTV &ZPT	Lower (166, 167)	Lower (163, 165, 166)				Lower (163, 165)	

ZPT = Zona pellucida thickness

ZPTV = Zona pellucida thickness variation

1.2.1.3 Oocyte lipid profile

Energy substrates used by the oocyte's mitochondria can be derived exogenously and endogenously. The mitochondria and SER associate with lipid droplets, concerning that the lipid is the most important endogenous source for energy production together with glycogen (129).

Fatty acids are an important energy source for mouse oocyte maturation and early embryo development (168). The oocyte lipid droplets (BODIPY stain) are small and diffuse in preovulatory mouse and porcine cumulus-oocyte complexes, before increasing and relocating centrally after maturation (133, 169). In general, there is a small amount of free lipid droplet in the resting oocyte, but lipid droplet number increases with atresia and droplets locate mainly in multivesicular bodies in the perinuclear area (136). The immature bovine and porcine oocyte contains more triglyceride than mature oocyte and 2-cell embryo (170, 171). Thus, the oocyte lipid should be an important substrates for oocyte maturation and an atresia predictor.

There is more lipid in the unstimulated porcine oocyte than in the cow, sheep and mouse, respectively. In immature porcine oocytes, the major lipid component is triglyceride, followed by cholesterol and phospholipid. The predominant fatty acids are palmitic and oleic acids, while the percentage of polyunsaturated fatty acid is only 16% (172). The proportion of saturated fatty acid is more than monounsaturated and polyunsaturated fatty acid. (171, 173, 174). In bovine immature and mature oocytes, palmitic acid is the most abundant fatty acids at 33-35%, followed by oleic acid and

stearic acid. In vitro matured oocytes contain a lower proportion of linoleic, arachidonic acid, triglyceride and total cholesterol when compared to immature oocytes. This assumes maturation of the bovine oocyte needs fatty acids metabolism (175), which has been confirmed by two studies showing that during maturation, the oxygen consumption is quite steady until the blastocyst stage, when there is a marked increase of oxygen consumption (171). At the same time, the fatty acid oxidation rate is constant in mouse unfertilised oocytes and early cleavage stage, but, increases after the 8- cell stage. The simultaneous change of oxygen consumption and the fatty acid oxidation rate during oocyte maturation and embryo development may assume that fatty acids are the important substrates for these two processes. Moreover, Research in bovine oocytes has revealed that there is a decrease in the capacity for immature bovine oocyte to develop to the blastocyst stage after exposure to methypalmoxinate (MP) which is a mitochondria carnitine palmitoyltransferase inhibitor, leading to blockage of fatty acids oxidation. MP also inhibits oxygen consumption of immature and mature bovine oocytes. This research group also found that the oocyte can develop without exogenous energy substrate, so, the source of energy should be from the intracellular lipid (176).

The adipose tissue of Americans is composed of 27% saturated, 52% monounsaturated and 20% polyunsaturated fatty acids. In the saturated fatty acid group, the majority is palmitic acid (20%), while the stearic acid only represents 4%. Oleic acid is still the most abundant monounsaturate at 10% of total fatty acids. The proportion of fatty acids in adipose tissue vary among ethnic groups that have different food preferences

(177). The fatty acid proportion in oocytes is different from the adipose tissue, with the majority of fatty acids in unfertilised human oocytes, being saturated fatty acids (79.2%); the percentage of monounsaturated fatty acid is 14.3% and polyunsaturated fatty acid is only 6.5%. The most abundant saturated fatty acids are stearic acid and palmitic acid (38.6% and 32.7%), while oleic acid is the most abundant monounsaturated fatty acid at 9.8% (178).

The difference in oocyte lipid amount in each species (pig> cow> sheep and mouse) may relate to the time to attachment of the embryo as the pig embryo has a longer time to attach to the uterus when compared to mouse and human embryos that may need less endogenous energy reserve (129). The pig has a higher percentage of abdominal fat than cow and sheep (179). The amount of oocyte lipid may resemble the amount of abdominal fat. However, the body fat of humans is hard to compare with animals. The human body fat distribution assessed by dual energy x-ray absorptiometry (DXA) shows that the trunk fat distribution of overweight patients may be quite similar to the cow, while the percentage of the trunk fat of normal weight people is similar to the sheep (179, 180). Thus, the lipid content in the human oocyte may be different among overweight and normal weight patients, especially, people who have truncal obesity (181).

Induced obesity in the mouse also leads to an alteration of mitochondrial distribution in the oocyte, increased mitochondrial potential, mitochondrial DNA content and biogenesis and reactive oxygen species (ROS), while glutathione is decreased showing the oxidative stress and fewer blastocysts than lean mice. The obese mouse has

more apoptotic ovarian follicles, smaller and fewer mature oocytes and smaller fetuses (182).

These findings may explain the effect of obesity on infertility, but, there has been no study in the oocyte of obese woman. My thesis opens the possibility of examining this area.

1.2.2 Follicular glucose and lipid

The ovary is an organ that has two major roles, namely the production of oocytes and steroidogenesis. The granulosa cell and cumulus cell are the cells that surround the oocytes from the pre-antral stage and bathe the oocyte with follicular fluid which is secreted and filtered from the plasma by the follicular epithelium (183, 184). The number of granulosa cells and the follicular fluid volume increase along with the developing oocyte and contribute to folliculogenesis (185, 186). The follicular fluid contains glucose, pyruvate, lactate, lipid, amino acids, albumin, protein, glycoprotein, peptide, steroid hormones, cytokines, growth factors, and electrolytes (183, 184). Sodium, potassium and chloride levels resemble those in plasma , but the pH is more acidic than plasma (187). Normally, the primordial follicle develops to be primary, secondary, pre-antral, small antral and large antral follicles before ovulation (185).

The cumulus oocyte complex needs fatty acids, amino acids, electrolytes, purine, pyrimidines and metabolites for oocyte maturation. Glucose is an essential energy substrate for oocyte maturation, with too high or low concentrations affecting oocyte

maturation. However, the oocyte itself has poor capacity for glucose metabolism. It can directly use pyruvate while other substrates are metabolized by the follicular cell and supplied to the oocyte through paracrine regulation (188-191). The glucose transporter of the cumulus cells is insulin sensitive and relies on insulin and insulin-like growth factor, not only the glucose concentration (190). Lactate is the end product of the glycolysis pathway (glucose utilization), especially in anaerobic glycolysis, which is essential for energy production in the growing follicle (192).

Follicular glucose concentration has a negative correlation with follicular size and lactate concentration. During oocyte maturation, the follicular glucose levels decrease to concentrations lower than plasma levels, while the lactate production increases. This supports the anaerobic glycolysis mechanism (under follicle stimulating hormone; FSH control). (183, 193-195). Under high insulin stimulation, the granulosa cell of PCOS patients cannot utilize glucose well, via glycolysis, as shown by less lactate production when compared to the control group (192).

There is detectable insulin in follicular fluid: the level does not correlate with follicular oestradiol or androgen, but it does correlate with progesterone and serum insulin (97, 196). Follicular insulin levels correlate with BMI, serum insulin and serum glucose (196). It may transude from the blood circulation and stimulate both granulosa and theca cells promoting ovarian steroidogenesis. Excess or diminished levels of glucose can affect in vitro oocyte maturation in animals (190, 197). In obese and infertile women, follicular glucose, insulin, lactate, triglyceride and C-reactive protein levels are higher than

in non-obese women, while the sex hormone binding globulin levels are lower (198). The insulin receptor mRNA expression in granulosa cells also increases in PCOS patients (196).

Bovine cumulus oocyte complexes (COCs) have constant phosphofructokinase glucose-6- phosphate dehydrogenase (PFK) activity during maturation. However, lipase activity decreases significantly over this time. PFK and glucose-6 –phosphate dehydrogenase (G-6PDH) are enzymes involved in glycolysis. They are more abundant in cumulus cells than in the oocyte, but the amount of lipase is more than G-6PDH and PFK in the denuded oocyte (199).

The oocyte might need lipid metabolism for normal development. HDL-C levels in serum are double those in follicular fluid and there is little LDL-C in follicular fluid. However, human granulosa cells need LDL-C and HDL-C for progesterone synthesis. Cholesterol may be provided from three sources, namely intracellular synthesis from acetate, mobilization from intracellulary stored cholesterol ester and extracellular lipoprotein uptake from follicular fluid (200-203). The lack of LDL-receptor or scavenger receptor class B type I (SR-BI), which is the physiologically relevant lipoprotein receptor which mediates uptake of cholesteryl esters from the lipoprotein core leads to the decrease of progesterone secretion (201). The SR-BI mechanisms are still not clear, but it is required for oocyte development and also correlates with the number of oocytes retrieved following hormone stimulation and fertilised (204, 205). Although obese patients have a higher follicular triglyceride level without differences in LDL-C and HDL-C level

when compared to non-obese infertile groups (198), no study has been performed on PCOS patients, which have a high prevalence of dyslipidaemia (206, 207). If lipid and cholesterol levels in follicular fluid of PCOS patients mimic blood levels, this may affect pregnancy rate since the level of HDL-C has negative correlation with embryo fragmentation (208).

The predominant fatty acids in follicular fluid are oleic, palmitic, linoleic and stearic acids. Although it is similar to the fatty acid component in serum, the correlation between follicular and specific serum fatty acid levels is quite low (r= 0.25-0.31) (209). Women who have high levels of follicular fatty acids tend to have poorer COCs morphology (209). A small study has shown that overweight women with higher serum palmitic and linoleic acid levels have no significant differences in follicular fatty acids levels when compared to normal weight women (209). However, The high level of follicular fatty acids relates to poor oocyte and embryo quality in cattle as well as granulosa cells survival and humans (210, 211). Mouse COCs cultured with palmitic acid results in the induction of endoplasmic reticulum (ER) stress, leading to a decrease in oocyte mitochondrial membrane potential, impaired secretion of cumulus matrix protein, altered mitochondrial activity and poor development competence (212). Murine blastocysts exposed to high palmitic acid have altered embryonic metabolism and growth (213). So, the change of follicular fatty acids equilibrium may contribute to the infertility of obesity.

1.2.3 Follicular cell stress and apoptosis

The cumulus cell (the specialized granulosa cell which attached the oocyte) has a close interaction with the oocyte. Normal oocyte development needs the follicular cells surrounding the egg for physical, nutrient and metabolic precursor support as well as producing growth factors and hormones (131). Studies in mammals found that the oocyte regulates granulosa and cumulus cell differentiation, proliferation, steroidogenesis, deposition of extracellular matrix, tPA (tissue-type plasminogen activator) production and expansion by secreting soluble paracrine growth factor that promotes growth and regulate energy metabolism and sterol biosynthesis of the cumulus cell (131, 214). On the other hand, the cumulus cell also regulates oocyte development. There are trans-zonal cytoplasmic projections forming cumulus-oocyte complexes that transfer small molecules like ions, metabolites and amino acids through gap junctions (186). The cumulus cell and oocyte both nurture each other by bidirectional communication (215-217). While the oocyte is not able to take up L-alanine or synthesize cholesterol from acetate and has poor glucose metabolism, the cumulus cell can metabolize amino acid, cholesterol and glucose which are essential for oocyte development and function (216). Cumulus cell apoptosis is higher in germinal vesicle or metaphase I oocytes when compared to metaphase II oocytes. Apoptosis is also found more in nonfertilised oocytes (165, 215). In abnormal morphologic oocytes, degenerate structures, granulosa cell and cumulus cell apoptosis are increased (133). The cumulus cell may help resumption of meiosis, decrease oxidative stress and influence embryonic metabolism (215, 218). The gene

expression of cumulus cells relate to the oocyte maturation, fertilisation, chromosomal abnormalities, oocyte and embryo competence and pregnancy (215, 216, 219-221). Cumulus cell co-culture and embryo transfer can also improve implantation and pregnancy rate (222).

In the mouse, high serum glucose increases oxidative stress and disrupts calcium homeostasis that may lead to cell apoptosis (223). Additional studies in mice showed that obesity is associated with ER stress, mitochondrial dysfunction and granulosa cell apoptosis (181, 211, 224). The diabetic mouse has more fragmented and aggregated distribution of mitochondria in cumulus cells. The disruption of mitrochondrial function leads to lower ATP levels that may cause oocyte incompetence and poor pregnancy outcomes (225). This is similar to lipotoxicity that has been described in other cells, which follows the increase of intracellular lipid from lipid overload or synthesized from excess glucose, leading to ER stress and cell apoptosis (226-228). Mice fed a high fat diet showed higher levels of lipids in the oocyte and increases in granulosa cell ER stress marker genes expression (ATF4 and GRP78) but not CHOP10 and Hsp70 (181). This might lead to higher granulosa cell apoptotic index in high fat fed mice (181). PCOS patients have a lower fertilisation rate when compared to non- PCOS patients (229). This may be caused by the apoptosis of cumulus cells that affect fertilisation (165). However, the granulosa cell apoptosis theory in the polycystic ovary is still inconclusive (230, 231). The cumulus cell stress marker and unfertilised oocyte composition may be the clues for our knowledge on obesity and PCOS oocyte quality.

Overall, there have been many studies in obesity and PCOS but there are still some data which are important for the pathophysiology of the disease that have not been collected to date as shown in Table 1.3.

Table 1.3 Summary of data about substances and cell stress markers in serum and follicular fluid of PCOS and obese patients

Substances	Sample	PCOS		Non-PCOS
		General PCOS	Obese compared with non-obese	Non-obese compared with Obese
Glucose	Blood	Increase in PCOS patients (207)	Increase in obese PCOS (232)	Increase in obese patients (1)
	Follicular fluid	Increase in PCOS patients (233)	**No data**	Increase in obese patients (198)
	Relationship	No data exploring the correlation between follicular glucose, lipid metabolism and cell stress		
Insulin	Blood	Increase in PCOS patients (85, 234)	Increase in obese PCOS (9, 89, 232)	Increase in obese patients (9, 10)
	Follicular fluid	The increasing level correlate with BMI, but not different from non-PCOS (196, 233, 234)		Increase in obese patients (198)
	Relationship	No data exploring the correlation between follicular insulin, lipid metabolism and cell stress		
Lipids	Blood	Increase in TG and LDL, decrease HDL in PCOS patients (85)	Increase TG, decrease HDL in obese PCOS patients (235)	Increase TG and LDL, decrease HDL in obese patients (1)
	Follicular fluid	**No data** in PCOS patients		Increase TG in obese patients (198)
	Relationship	**No data** in PCOS patients		

Substances	Sample	PCOS		Non-PCOS
		General PCOS	Obese compared with non-obese	Non-obese compared with obese
Fatty acids	Blood	Increase in PCOS patients, no composition detail (106, 107)	**No data**	Increase in obese patients, no composition detail (236)
	Follicular fluid	**No data** in PCOS patients		**No data** in obese patients
	Relationship	**No data** exploring the correlation between follicular fatty acids, lipid-glucose metabolism and cell stress		
ER-stress marker	Granulosa cell	**No data** in PCOS patients		Obese patients increase ATF4 expression (181)
Apoptosis	Granulosa cell	Inconclusive data (230, 231)	**No data**	Increase apoptosis (related to oxLDL receptor) (237)

1.3 Hypothesis and objectives

1.3.1 Hypothesis: Alterations in ovarian follicular fluid reflect the clinical condition and oocyte cumulus homeostasis

1.3.2 Objectives:

1. To explore the relationship between lipids and glucose levels in blood and follicular fluid in infertile women.
2. To compare and contrast these substances among women with normal and abnormal metabolic condition.
3. To measure lipid content within human oocytes and the relationship with metabolic substances, obesity and IVF outcome.
4. To measure expression of endoplasmic reticulum stress marker genes in cumulus cells and relate these with metabolic substances, obesity and IVF outcome.

Chapter 2 General methods and demographic data

2.1 Methods

This study was approved by the Human Research Ethics Committee (HREC) of the University of Adelaide (H-217-2010), Southern Adelaide Health Service/Flinders University HREC and the St Andrew's Hospital Ethics Committee (No 54).

2.1.2 Patients

Infertile patients who intended to undergo in-vitro fertilisation (IVF) at Fertility SA, South Australia between February 2011 and August 2011 were given information about this study to enable written informed consent. After they signed the consent form, data about infertility treatment was collected. Patients underwent general examination, body weight, height and waist circumference measurements. They started an oocyte stimulation protocol with recombinant FSH and ovulation was blocked with a GnRH antagonist (Cetrotide® ; Merck Serono or Orgalutran®; MSD) or GnRH analogue (Synarel®; Merck Serono or Lucrin®; MSD)(238). When at least three follicles reached 16 mm diameter, recombinant human chorionic gonadotropin (Ovidrel®) 250 ug was injected and oocyte recovery performed 36 hours later. Fasting blood and clear follicular fluid samples were collected on the day of oocyte aspiration in the operation theatre. The details about stimulation protocol, fertilisation

rate, embryo quality and pregnancy rate was recorded.

2.1.3 Blood samples

Blood was taken from each patient. After spinning at 3,000 rpm for 10 minutes, the serum and plasma samples were stored at -20 ^{0} C for 2 weeks before transfered to The University of Adelaide and stored at -80 ^{0} C. The plasma glucose, serum triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were assessed using the Roche Hitachi 912 Chemistry Analyzer. Fatty acids were analysed by gas chromatography mass spectrometry-based metabolomics (Waite campus, The University of Adelaide).

The Hitachi analysis for glucose was done using the Roche® UV test by the hexokinase method which catalyses the phosphorylation of glucose to glucose-6-phosphate by ATP. Then, oxidized glucose-6-phosphate is converted in the presence of NADP to gluconate-6-phosphate. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and can be measured photometrically. The measuring range is 0.11-41.6 mmol/L.

Insulin was analysed by the Millipor Ultra sensitive human insulin RIA kit(Linco Research, St. Charles, MO Cat.#HI-11K). All samples were assayed in duplicate in 2 assays.

The coefficient of variation of the duplicates was less than 10%. The low and high QC values were within the manufacturer's limits. The lowest detectable concentration was 0.4 uU/ml.

The TG analysis was done with the Roche® enzymatic colorimetric test using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacted with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidise to form a red dye. The measuring range is 0.05-11.3 mmol/L.

The analysis for LDL-C and HDL-C were done by the Roche® homogeneous enzymatic colorimetric assay using cholesterol esterase to break down cholesterol quantitatively into free cholesterol and fatty acids. Then, the cholesterol is oxidised in the presence of oxygen by the cholesterol oxidase to Δ^4cholestenone and hydrogen peroxide and reacts the hydrogen peroxide with 4-aminoantipyrine and HSDA to form a purple-blue dye. The colour intensity of this dye then directly proportional to the cholesterol concentration and is measured photometrically. The measuring range is 0.08-14.2 mmol/L for LDL-C and 0.08-3.10 mmol/L for HDL-C.

The total fatty acid was measured by the Wako® method, converting the non-esterified fatty acid to Acyl-CoA, AMP and pyrophosphoric acid by the action of Acyl-CoA synthetise under coexistence with coenzyme A and adenosine-5'-triphosphate disodium salt. Then, the

Acyl-CoA was oxidized and yields 2,3-trans-enoyl-CoA and hydrogen peroxide by the action of Acyl-CoA oxidase. Finally, the hydrogen peroxide formed yields a blue purple pigment by quantitative oxidation condensation with MEHA and 4-aminoan-tipyrine. The non-esterified fatty acids concentration was obtained by measuring absorbance of the blue purple color. The measuring range is 0.05- 2 mmol/L.

The quality control of glucose, TG and cholesterol tests were done by the Precinorm U (PNU®) and Precipath U (PPV®) kit with acceptable target values. The coefficient of variation was less than 2%. The quality control of total fatty acid was done by the Biorad® QCS1 and QCS2 kit.

The fatty acid gas chromatography mass spectrometry-based metabolomics analysis was done by extracting the fatty acid from the sample by AR methanol (Analytical reagent) and heptadecanoic acid in 9:1 choloroform:methanol solution. Then, the sample was placed on a thin layer chromatography plate. The free fatty acid band including silica was scraped to methylate with 1% H_2SO_4 in methanol. The gas chromatography method started by separating the fatty acid and measuring in a Hewlett-Packard 6890 gas chromatograph (CG) equipped with a 50 metre capillary column (0.32 mm internal diameter SGE, Victoria) coated with 70% cyanopropyl polysilphenylene-siloxane (BPX-70) (0.25 um film thickness) which was fitted with a flame ionization detector. Helium was the carrier gas and the split-ratio was 20:1. The injector temperature was set at 250 ^{o}C and the detector temperature at 300 ^{o}C. The initial

oven temperature was 140 °C and programmed to rise to 220 °C at 5 °C per minute. Fatty acids in percentage were identified based on the retention time of standards obtained from Nucheck Prep Inc. (Elysian, MN) using the Chemstation software. The standard control was run for quality control with 463 mixture from Nuchek®

Forty two fatty acids were identified in percentage as named in table 2.1

Table 2.1 Fatty acid equivalent names in the study

Molecular name	Scientific name	Common name
14:0	Tetradecanoic acid	Myristic acid
15:0	Pentadecanoic acid	-
dma16:0	Dimethyl acetyl Hexadecanoic acid	-
16:0	Hexadecanoic acid	Palmitic acid
dma18:0	Dimethyl acetyl Octadecanoic acid	-
18:0	Octadecanoic acid	Stearic acid
20:0	Eicosanoic acid	Arachidic acid
22:0	Docosanoic acid	Behenic acid
24:0	Tetracosanoic acid	Lignoceric acid
Trans 16:1	Transpalmitelaidic acid	-
Trans 18:1 n-9	9-trans-octadecenoic acid	-
Trans 18:1n-7	Δ7 transvaccenic acid	-
14:1	Tetradecenoic acid	Myristoleic acid
15:1	Pentadecenoic acid	-
16:1 (undifferentiated)	Hexadecenoic acid	Palmitoleic acid
16:1n-9	Δ9 palmitelaidic acid	-
16:1n-7	9-hexadecenoic acid	Elaidic acid
17:1	Heptadecenoic acid	-
18:1 (undifferentiated)	Octadecenoic acid	Oleic acid
18:1 n-9	9-octadenoic acid	-
18:1n-7	11-octadecenoic acid	Vaccenic acid
19:1	Nonadecanoic acid	-
20:1 n-11	Δ11 eicosanoic acid	-
20:1n-9	11-eicosenoic acid	Eicosenoic acid
22:1 (undifferentiated)	Docosenoic acid	Erucic acid
22:1n-9	13-docosenoic acid	-
24:1n-9	15-tetracosanoic acid	Nervonic acid
18:2 (undifferentiated)	Octadecadienoic acid	Linoleic acid
18:2 n-9		-
20:2n-9	Eicosadienoaic acid	-
9,11 18:2 cLA		-
18:2 n-6	9,12-octadecadienoic acid	-
18:3 (undifferentiated)	Octadecatrienoic acid	Linolenic acid
18:3n-6	6,9,12-octadecatrienoic acid	Gamma-linolenic acid (GLA)
20:2n-6	11,14-Eicosadienoic acid	Eicosadienoic acid
20:3n-6	8,11,14-eicosatrienoic acid	Homo-gamma-linolenic acid (DGLA)

Molecular name	Scientific name	Common name
20:4 (undifferentiated)	Eicosatetraenoic acid	Arachidonic acid
20:4n-6	5,8,11,14-eicosatetraenoic acid	Docosatetraenoic acid
22:2 n-6	13,16-docosadienoic acid	Docosadienoic acid
22:4n-6	7,10,13,16-docosatetraenoic acid	Docosatetraenoic acid
22:5n-6	4,7,10,13,16-Docosapentaenoic acid	Clupanodonic acid
16:2 (undifferentiated)	Hexadecadienoic acid	-
16:2 n-3	Palmitoleatic acid	-
18:3n-3	9,12,15-octadecatrienoic acid	α-linolenic acid (ALA)
18:4 (undifferentiated)	Octadecatetraenoic acid	Parinaric acid
18:4 n-3	6,9,12,15-octadecatetraenoic acid	Stearidonic acid
20:3n-3	11,14,17-eicosatrienoic acid	Eicosatrienoic acid (ETA)
20:5n-3	5,8,11,14,17-eicosapentaenoic acid	Eicosapentaenoic acid (EPA)
22:5 n-3	7,10,13,16,19-docosapentaenoic acid	Docosapentaenoic acid (DPA)
22:6n-3	4,7,10,13,16,19-docosahexaenoic acid	Docosahexaenoic acid (DHA)

2.1.4 The follicular fluid

The follicular fluid was collected by transvaginal ultrasound guided aspiration. After the oocyte was separated, the blood-free follicular fluid from each patient was collected separately. The fluid was spun at 3,000 rpm for 10 minutes and granulosa cells removed, then stored at -20 ^{0} C for 2 weeks before transfered to The University of Adelaide and stored at -80 ^{0} C. The follicular fluid was thawed for insulin, glucose, TG, LDL-C, HDL-C and fatty acids analysis using the same methods as for plasma and serum measurement. All methods were validated for the fluid examined.

2.1.5 The cumulus cells

Cumulus cells were collected after physically denuding the cumulus cell from the oocyte for intracytoplasmic sperm injection (ICSI). The cumulus oocyte complex was denuded with 75 IU/ml hyaluronidase and manual pippeting for 30 seconds. The cells from each patient were pooled and stored at -20 ^{0} C for 2 weeks before being transfered to The University of Adelaide and stored at -80 ^{0} C until analysed. The detail of cumulus cells analysis is in Chapter 5.

2.1.6 The unfertilsed oocyte

After 20 hours insemination, any unfertilised oocytes were collected and fixed with 4% paraformaldehyde. After 4- 6 hours, oocytes were transfered into phosphate buffer saline (PBS) with 1 mg/ml of polyvinylpyrolidone (PVP) and stored at 4 ^{0}C until used. The details of unfertilsed oocyte staining is in Chapter 5.

2.1.6 Terms and definition

Weight delineation is classified by the body mass index (BMI) category, which is calculated as the body weight in kilogram divided by height in metre squared. People who have BMI 25-29.9 kg/m^{2} are defined as overweight. Those with a BMI 30 kg/m^{2} or more are classified as obese (1). A patient was diagnosed as polycystic ovarian syndrome (PCOS) if

they had 2 of the 3 Rotterdam 2003 criteria, which are hyperandrogenism, polycystic ovary and chronic anovulation (79). Hyperglycaemia was diagnosed when plasma glucose was equal or higher than 5.6 mmol/L (239, 240). The degree of insulin resistance was diagnosed using the homeostatic model assessment (HOMA), which is calculated by fasting insulin multiplied by fasting glucose and divided by 22.5, (hyperinsulinaemia was diagnosed when HOMA is 3.8 or greater (98-101)). Hypertriglyceridaemia was diagnosed if the serum triglyceride level was higher or equal to 1.7 mmol/L (240). LDL-C was abnormal at 2.58 mmol/L or higher and low HDL-C cut off value was below 1.29 mmol/L (240). Metabolic syndrome was diagnosed according to both the International Diabetes Federation (IDF) and the National Cholesterol Education Program Adult treatment Panel III (NCEP) criteria. IDF criteria are the central obesity (BMI > 30 kg/m2 or waist circumference > 80 cm) plus two of the four criteria, which are triglyceride ≥ 1.7 mmol/L, HDL-C < 1.29 mmol/L, blood pressure ≥ 130/85 mmHg and fasting glucose ≥ 5.6 mmol/L. NCEP metabolic syndrome was diagnosed when women had 3 of the 4 criteria, which are waist circumference > 88 cm, triglyceride ≥ 1.7 mmol/L, HDL-C < 1.29 mmol/L, BP ≥ 130/85 mmHg and fasting glucose ≥ 6.1 mmol/L(240).

A mature oocyte is that which a first polar body in the perivitelline space is visualised under the light microscope on the day of collection. An oocyte that does not have a first polar body was classified as immature.

The fertilisation rate is defined as the number of oocytes that have 2 pronuclei after

insemination for 18 hours divided by overall oocyte collection. The cleavage rate was defined as the number of cleavage embryos in the second or third day after oocyte collection divided by the number of overall oocytes. Biochemical pregnancy was defined when the serum beta-hCG was detectable above 25 u/l in the second week after embryo transfer. Clinical pregnancy was defined when the fetal heart beat was seen on ultrasound after embryo transfer for 4- 6 weeks.

2.1.7 Sample size calculation

The sample size was calculated by correlation formula for testing the hypothesis that the follicular lipids level correlates with the serum level.

$$n = \left(\frac{Z_\alpha + Z_\beta \sqrt{1-r^2}}{r} \right)^2 + 2$$

Z_α = 1.96 (α = type I error = 5%)

Z_β = 0.84 (β= type II error = 20%)

r = correlation between follicular fatty acid and serum fatty acid as done previously (209) = 0.30

N = 86.7

The number of patients needed was at least 87 to test the significance of correlation at 0.30 or more.

2.1.8 Statistical analyses

Statistical analyses were done using the SPSS program (PASW statistics 18). Spearman's correlation was used to explore correlations between each measure. To explore the difference of continuous data between two individual groups, the distribution test was done, then, the t-test was used for the normal distribution data and the nonparametric t-test was selected for the variables which do not have normal distribution. The comparison between more than 2 individual groups was done by ANOVA or Kruskal's Wallis test based on the distribution.

2.2 Demograhic data

From 18th February to 26th August 2011, 90 patients were recruited into this study. There were 88 women who had clear follicular fluid and/or unfertilsed oocytes for the study. The mean age of the women undergoing IVF was 34.2 ± 4.6 years. Their partners' mean age was 36.8 ± 6.6 years. Fifty three percent of the participating couples were suffering from primary infertility and 47.1% from secondary infertility. The mean infertile period was 26.8 ± 17.4 months. Thirty one percent of these 88 couples had an unexplained cause of infertility, 37.9% had predominant male infertility. Anovulation and inactive endometriosis causes were at 6.9%. Nine percent of overall couples had both male and anovulation problems. There was only 1 leiomyoma and 1 tubal case.

Forty three women had normal weight, 24 women were overweight and 17 were obese. There were 12 PCOS patients in this study. Four of them were overweight, 4 were obese and 4 had normal weight.

Seventy percent of the women underwent IVF by the FSH/GnRH antagonist stimulation protocol and 28.7% by the FSH-GnRH agonist down regulation protocol. Only 1 patient used the flare up protocol. There was no statistical significantly differences for demographic parameters among the antagonist and down regulation protocol. The standard IVF procedure was done in 32 couples and intracytoplasmic sperm injection (ICSI) was done in 49 couples. Six couples decided to do both IVF and ICSI on half of their oocytes. The mean number of retrieved oocyte was 10.4 ± 5.8. The immaturity rate was 6.4 ± 11.6 percent. The fertilisation, cleavage and blastocyst rate per cycle were 56.2 ± 25.0, 55.1 ± 26.4 and 31.3 ± 20.8 percent, respectively. Seventy nine percent of the couples transfered 1 fresh embryo, 13.8% transfered 2 embryos, only 1 patient transfered 3 embryos and 5 couples did not have a transfer. The overall biochemical pregnancy rate in this study was 51.2% and the clinical pregnancy rate was at 41.0%.

Chapter3 Effect of BMI, body fat distribution and metabolic disturbance on follicular metabolism

3.1 Introduction

There are few studies on the effect of fat distribution on infertile women (51-53), and no absolute consensus based on evidence about obesity affecting infertility. However, the obese patient might have oocyte, hormone, metabolic and endometrium dysfunction that affects fertility (9, 23, 29, 30, 32-37). PCOS, which is included in some obesity studies, may also affect the pregnancy rate (38).

Obese women have a higher level of follicular triglyceride and insulin with minor differences in LDL-C and HDL-C levels when compared to non-obese women (198). A small study found that there were no significant differences in major follicular fatty acid levels between obese and normal weight women (209). The follicular environment may not rely solely on the body mass index. Thus, women with specific metabolic abnormalities, such as PCOS, insulin resistance and metabolic syndrome have a high risk of diabetes mellitus, dyslipidaemia and cardiovascular disease, which might have some abnormal follicular glucose-lipid metabolism (240). The aim of this chapter is to explore the differences of follicular metabolites among women with normal or abnormal metabolic condition.

3.2 Methods

General methods have been described in Chapter 2. Eighty four women were classified as normal weight, overweight or obese as well as put into PCOS groups according to the criteria mentioned in Chapter 2. The diagnosis of hyperglycaemia, dyslipidaemia, hyperinsulinaemia and metabolic syndrome is defined in detail in Chapter 2.

Statistical analyses

Statistical analysis was carried out using the SPSS program, as described in Chapter 2

3.3 Results

Eighty four women participated in the study. Forty three of them had normal weight, 24 were overweight and 17 were obese. The women who were obese and those with high waist circumference had high follicular insulin levels. Significant differences among BMI and waist circumference classified subgroups are shown in Table 3.1 and 3.2.

Table 3.1 Comparison between normal weight, overweight and obese subgroups

Variables	Normal weight n = 43 (Mean ± SD)	Overweight n=24 (Mean ± SD)	Obese n=17 (Mean ± SD)	P- value
Waist circumference (cm)	81.98 ± 7.91	95.62 ± 9.16	114.06 ± 10.77	<0.001
Waist/Hip ratio	0.83 ± 0.09	0.87 ± 0.06	0.89 ± 0.06	0.02
Serum glucose (mmol/L)	4.82 ± 0.38	5.16 ± 0.54	5.37 ± 0.73	0.001
Serum TG (mmol/L)	1.15 ± 0.48	1.27 ± 0.36	1.66 ± 0.92	0.04
Serum HDL (mmol/L)	1.53 ± 0.31	1.39 ± 0.28	1.28 ± 0.24	0.007
Serum Insulin (uU/ml)	8.15 ± 4.48	12.81 ± 6.07	21.49 ± 8.42	<0.001
Serum 20:2 n-6% (docosadienoic acid)	0.22 ± 0.13	0.19 ± 0.11	0.16 ± 0.11	0.03
Follicular insulin (uU/ml)	5.47 ± 3.15	8.68 ± 5.37	18.69 ± 11.30	<0.001

Table 3.2 Comparison between women with a normal waist circumference (n=18), 80-88 cm (n=20), and the group with more than 88 cm (n=44)

Variables	Waist circumference (cm)			P- value
	<80 (Mean ± SD)	80-88 (Mean ± SD)	> 88 (Mean ± SD)	
Serum Glucose (mmol/L)	4.72 ± 0.28	4.76 ± 0.42	5.30 ± 0.56	<0.001
Serum HDL-C (mmol/L)	1.57 ± 0.28	1.57 ± 0.36	1.32 ± 0.24	0.001
Serum Insulin (uU/ml)	7.19 ± 1.81	8.70 ± 6.14	16.03 ± 8.12	<0.001
Follicular Glucose (mM)	2.61 ± 0.72	2.64 ± 0.68	3.22 ± 0.99	0.01
Follicular Insulin (uU/ml)	5.57 ± 3.98	6.40 ± 3.60	11.90 ± 9.20	0.003
Follicular Triglyceride (mM)	0.28 ± 0.49	0.64 ± 0.97	0.58 ± 1.55	0.009
Follicular 20:0 % (arachidic acid)	0.06 ± 0.11	0.02 ± 0.06	0.11 ± 0.16	0.04
Follicular F20:1 n-11% (Δ11 eicosanoic acid)	0.03 ± 0.09	0.13 ± 0.19	0.02 ± 0.09	0.003
Follicular 18:3 n-3 (ALA)%	0.36 ± 0.18	0.32 ± 0.21	0.44 ± 0.23	0.04
Follicular 20:3 n-3(ETA)%	0.74 ± 0.30	0.64 ± 0.36	0.46 ± 0.29	0.004
Follicular 20:4 n-6% (docosatetraenoic acid)	0.70 ± 0.34	0.65 ± 0.27	0.94 ± 0.56	0.02

The waist circumference had a strong correlation (r =0.85, p < 0.0001) with BMI. BMI and waist circumference affected the value of many substances in serum and follicular fluid, especially the serum and follicular insulin that had the strongest correlation with both BMI and waist circumference, as shown in Table 3.3 and 3.4.

Table 3.3 Serum analytes and significant correlation with BMI and waist circumference among 84 women

Measurement	BMI		Waist circumference	
	r	p	R	p
Plasma glucose	0.46	<0.0001	0.52	<0.0001
Serum Insulin	0.68	<0.0001	0.71	<0.0001
Serum TG	0.29	0.008	0.28	0.01
Serum HDL-C	- 0.37	0.0006	-0.40	0.0002
Serum LDL-C	-	NS	-	NS
Serum total fatty acid	-	NS	-	NS

Table 3.4 Significant correlation of follicular substances with BMI and waist circumference among 84 women

Measurement	BMI		Waist circumference	
	r	p	R	p
Follicular glucose	0.26	0.02	0.31	0.006
Follicular insulin	0.53	<0.0001	0.49	<0.0001
Follicular TG	0.29	0.008	0.25	0.03
Follicular HDL -C	0.24	0.03	-	NS
Follicular LDL-C	-	NS	-	NS
Follicular total fatty acids	-	NS	-	NS

The results in Table 3.3 and 3.4 show that BMI and waist circumference have similar correlations among insulin and metabolites. Thus, for subsequent analyses of specific fatty acids, these are presented in terms of BMI.

Among the fatty acids, only the percentage of follicular 20:3 n-3 (eicosatrienoic acid; ETA) had a negative correlation with waist circumference ($r = -0.34$, $p = 0.002$) and BMI, as shown in Figure 3.1. Graphs of the relationship between serum and follicular specific fatty acid – BMI are shown in Appendix 2.

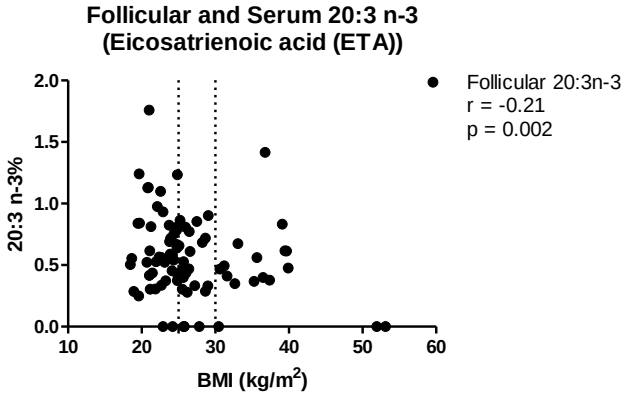


Figure 3.1 The follicular eicosatrienoic acid correlation with BMI.

There were 12 PCOS women in this study; with 4 classified as obese, 4 were overweight and 4 were normal weight. There was no difference of serum or follicular substances between the PCOS and non-PCOS group.

The women with hyperglycaemia (n = 13) had higher BMI (32.0 ± 7.7 vs controls 25.8 ± 5.9 kg/m^2 , p = 0.002), waist circumference (107.9 ± 15.4 and 89.3 ± 13.5 cm, p=<0.001), serum insulin (19.4 ± 8.4 and 10.9 ± 6.9 uU/ml, p=<0.001), serum total fatty acid (0.57 ± 0.16 and 0.46 ± 0.16 mmol/L, p=0.02), percentage of serum 18:1n-7 (vaccenic acid) (1.89 ± 0.47 and 1.62 ± 0.46, p = 0.02) and percentage of serum trans 16:1 (transpalmitelaidic acid) (0.09 ± 0.06 and 0.05 ± 0.07, p= 0.03); and lower serum HDL-C (1.26 ± 0.19 and 1.47 ± 0.31 mmol/L, p =0.02), serum 20:3n-3 (ETA) (0.20 ± 0.08 and 0.29 ± 0.17 p= 0.04) and serum 22:6n-3 (DHA) percentages (0.66 ± 0.41 and 0.94 ± 0.63, p = 0.05). There were no differences in follicular components when compared to women with normal levels of plasma glucose (n=71).

When compared to women with an HOMA of less than 3.8, those with a greater score (more than 3.8, n=21) had higher levels of serum triglyceride (1.74 ± 0.78 and 1.14 ± 0.43 mmol/L, p = 0.002) and serum trans16:1 (transpalmitelaidic acid) percentages (0.09 ± 0.06 and 0.05 ± 0.07, p =0.008); and lower levels of serum HDL-C (1.28 ± 0.20 and 1.49 ± 0.31 mmol/L, p =0.001), serum 17:1 (heptadecenoic acid) (0.17 ± 0.22 and 0.20 ± 0.21, 0.04), serum 20:2 n-6 (eicosadienoic acid) (0.17 ± 0.10 and 0.21 ± 0.13, p= 0.03) and serum 20:4n-

6 (docosatetraenoic acid) percentages (0.96 ± 0.85 and 1.06 ± 0.64, p=0.05). They also had higher levels of follicular insulin and LDL-C, but lower levels of follicular triglyceride, as shown in Table 3.5.

Table 3.5 Significant comparison between women with a HOMA score < 3.8 (n=63) and those who had more than 3.8 (n=21)

Variables	Low score (63) (Mean ± SD)	High score (21) (Mean ± SD)	p-value
Follicular triglyceride (mmol/L)	0.58 ± 1.426	0.37 ± 0.405	0.04
Follicular LDL-C (mmol/L)	0.01 ± 0.286	0.02 ± 0.044	0.02
Follicular insulin (uU/ml)	6.11 ± 3.828	17.00 ± 10.56	<0.001

The women with hypertriglyceridaemia (n=15) had a higher level of serum insulin than patients who had normal serum triglyceride (18.4 ± 10.42 and 10.8 ± 6.39 uU/ml, p = 0.02). They also had lower follicular 22:5 n-6 (clupanodonicate) percentages (0.06 ± 0.09 and 0.14 ± 0.11 , p =0.02). The women with a low level of HDL-C had a higher level of serum insulin (15.0 ± 8.5 and 11.0 ± 7.2 uU/ml, p = 0.03) and glucose (5.31 ± 0.63 and 4.91 ± 0.48 mmol/L , p = 0.002), when compared to those who had high HDL- C. Follicular glucose was significant in women who had low HDL-C, as shown in Table 3.6. There was a lower percentage of follicular saturated fat in hypercholesterolemic women. The fatty acid percentage of serum and follicular fluid in women with high levels of LDL-C is shown in Table 3.7.

Table 3.6 Significant differences of follicular substances between women with normal serum HDL-C level (n=25) and those with less than 1.29 mmol/L (n=59)

Variables	Normal level (59) (Mean ± SD)	Low level (25) (Mean ± SD)	p-value
Follicular glucose (mmol/L)	2.78 ± 0.83	3.39 ± 0.92	0.004
Follicular 24:0 (lignoceric acid)%	0.01 ± 0.04	0.05 ± 0.10	0.05
Follicular 22:2 n-6 (docosadienoate)%	0.10 ± 0.13	0.04 ± 0.08	0.03

Table 3.7 Significant difference of fatty acid percentage between women with normal serum LDL-C level (n=67) and those with more than 2.56 mmol/L (n=17)

Variables	Normal level (67) (Mean ± SD)	High level (17) (Mean ± SD)	p-value
Serum Total n-6%	13.71 ± 5.67	11.26 ± 5.25	0.04
Serum 18:2 n-6 % (9,12-octadecadienoic acid)	11.82 ± 5.11	9.31 ± 3.99	0.02
Serum 18:1 n-9% (9-octadenoic acid)	38.92 ± 10.63	41.91 ± 9.74	0.02
Serum 16:2 n-3% (Palmitoleatic acid)	0.09 ± 0.08	0.13 ± 0.10	0.06
Follicular 16:0 (palmitic acid)%	32.15 ± 5.2	28.15 ± 3.8	0.004
Follicular 18:0 (stearic acid)%	16.10 ± 3.76	13.75 ± 2.25	0.002
Follicular total saturated%	50.08 ± 8.73	43.68 ± 6.27	0.006
Follicular monounsaturated%	31.89 ± 8.06	37.43 ± 9.80	0.02
Follicular total n-9%	27.84 ± 7.69	33.56 ± 9.67	0.01

There were significant differences in serum and follicular insulin among women who had metabolic syndrome. The follicular glucose level was also high in women who had metabolic syndrome, according to the NCEP criteria, but, not the IDF criteria. The level of insulin was increased according to the length of metabolic syndrome criteria, as shown in Table 3.8 and 3.9.

Table 3.8 Significant comparison between women with none of the NCEP metabolic syndrome criteria (n=29), 1 criterion (n=25), 2 criteria (n=20) and 3-4 criteria (n=10) group

Measurement	Number of NCEP criteria				
	0 (Mean ± SD)	**1** (Mean ± SD)	**2** (Mean ± SD)	**3-4** (Mean ± SD)	**p- value**
Serum Insulin (uU/ml)	6.97 ± 1.98	11.61 ± 7.03	16.24 ± 7.88	20.63 ± 8.74	<0.001
Follicular Insulin (uU/ml)	5.82 ± 3.66	6.62 ± 4.33	12.63 ± 10.87	15.38 ± 9.38	0.006
Follicular Glucose (mmol/L)	2.49 ± 0.71	3.02 ± 0.70	3.33 ± 0.93	3.37 ± 1.31	0.02
Follicular 18:3 n-3 % (ALA)	0.30 ± 0.18	0.43 ± 0.21	0.43 ± 0.23	0.46 ± 0.26	0.04
Serum trans 16:1% (transpalmitelaidiate)	0.06 ± 0.07	0.43 ± 0.07	0.06 ± 0.06	0.11 ± 0.06	0.05

Table 3.9 Significant difference of substances between women with none or some of the IDF metabolic syndrome criteria (n=15), 1 IDF criterion (n=35), 2 criteria (n=22) and 3-4 criteria (n=12) group

Measurement	Number of IDF criteria				
	0 (Mean ± SD)	**1** (Mean ± SD)	**2** (Mean ± SD)	**3-4** (Mean ± SD)	**P-value**
Serum Insulin (uU/ml)	6.69 ± 1.30	9.71 ± 5.14	16.15 ± 8.87	18.99 ± 8.87	<0.001
Follicular Insulin (uU/ml)	5.68 ± 3.99	6.51 ± 4.14	11.57 ± 10.65	14.44 ± 8.93	0.003

3.4 Summary of the results

1. BMI was strongly correlated with waist circumference.
2. Follicular insulin levels had the strongest correlation with BMI and waist circumference.
3. There were increases of follicular alpha-linolenic acid (ALA) and a decrease of follicular eicosatrienoic acid (ETA) in women with a waist circumference greater than 80 cm.
4. Follicular insulin levels were increased among metabolic syndrome groups.
5. Women with a high level of serum LDL-C had a different follicular fatty acid profile compared to those who had a normal LDL-C level.

3.5 Discussion

As expected, the waist circumference had a strong correlation with the BMI. However, women with a high waist circumference tended to have more deviations in follicular fatty acid levels. The alpha-linolenic acid (ALA) and eicosatrienoic acid (ETA) were decreased among women with a waist circumference of more than 80 cm. ETA is derived from omega-9 fatty acid and it competes for the use of the same enzyme for changing arachidonic acid into strong inflammatory mediators, but with less competing ability than eicosapentaenoic acid

(EPA) (118). The low level of follicular ETA in women who had a waist circumference of more than 80 cm may be involved with the inflammatory process in the follicular environment (241). ALA is a short chain omega 3 fatty acid, with higher levels of serum alpha-linolenic acid in women undergoing IVF decreasing the chance of pregnancy (128). Thus, women with a waist circumference more than 88 cm may have serum-follicular ALA disturbances, which lead to a poor chance of pregnancy. The correlation among serum and follicular ALA is shown in Chapter 4.

The NCEP classified metabolic syndrome group had more significant differences in follicular glucose and fatty acid when compared to the metabolic syndrome subgroup, which was classified by the IDF with weaker criteria (lower waist circumference and serum glucose diagnostic value). This means that BMI- and IDF-classifications of metabolic syndrome might be a good screening tool for classifying women into the high metabolic risk group, which may contain follicular metabolic disturbance. However, waist circumference and NCEP- classified metabolic syndrome associate better with follicular lipid and glucose disturbances. Although there have been few studies on waist circumference affecting infertility or IVF outcome (51, 52), weight loss programs have resulted in a higher pregnancy rate in women who show a significant decrease in the circumference of their waist (53).

The number of obese, PCOS and metabolic syndrome women in this study may be too small to examine significant differences between groups. However insulin, which early

indicate metabolic disturbances, shows a good correlation with BMI and waist circumference. Metabolic syndrome combines three metabolic risks. Therefore, the significance of metabolic disturbance involves more than assessing individual serum glucose or lipids levels. There were no differences in the levels of follicular lipids among the metabolic syndrome groups, except for ALA, but the insulin may still be different amongst them. Thus, women who had metabolic syndrome may have follicular disequilibrium caused by the effect of insulin. Although there is no study on the pregnancy rate of women who have metabolic syndrome, the prevalence of metabolic syndrome is high in PCOS women with clomiphene citrate (CC) resistance, when compared to CC responders and fertile PCOS (242). In this study, women with metabolic syndrome also had a high percentage of follicular ALA, which may affect the chance of pregnancy (128). Besides the ETA, there was no correlation in BMI and waist circumference with specific fatty acids. However, women with hypercholesterolemia had different proportions of follicular fatty acid when compared to those who were normolipidaemic, but this was not found in females who were hyperinsulinaemic or hyperglycaemic. Saturated fat is the most abundant fatty acid in follicular fluid, and high follicular saturated fat can impair mouse oocyte maturation (243). However, moderate levels of fatty acids are also needed for oocyte maturation (168). The percentage of saturated fat was lower among the hypercholesterolemic group. Thus, hypercholesterolemia may be another major factor in disturbing the follicular equilibrium.

In this chapter, there were some differences in follicular substances, especially concerning insulin among different metabolic groups. More data on the follicular-serum relationship between glucose and lipids are discussed in the next Chapter.

Chapter 4 Relationship between serum and follicular glucose and lipids

4.1 Introduction

Follicular fluid is secreted and filtered from plasma by follicular epithelium (183, 184), and it bathes the oocyte and follicular granulosa cells. It also contains many essential substances and plays an important role in folliculogenesis (183-186).

As mentioned in the literature review, glucose and lipids are essential energy substrates for oocyte maturation (188-191, 200-203).

It is possible that disturbances in the follicular glucose and lipid equilibrium affects oocyte quality, embryo development and hormone production (188-191, 200-203, 212). An understanding of the relationship between glucose and lipid levels in serum and follicular fluid might provide some insight into how follicular glucose and lipid metabolism that may affect fertility. The aim of this chapter was to explore the relationship of lipids and glucose levels in blood and follicular fluid.

4.2 Assay methods

The general methods have been written in Chapter 2. The follicular fluid and serum from all 84 women including those who had metabolic disturbances and those who had not were thawed and analysed for insulin, glucose, triglyceride, low-density lipoprotein (LDL-C), high-density lipoprotein cholesterol (HDL-C) and fatty acids.

Statistical analysis was performed as described in Chapter 2.

4.3 Results

Correlation analyses were carried out on 84 women for both serum and follicular fluid. Follicular metabolic substances were significantly different from blood level, as shown in Table 4.1. There were higher levels of metabolic substances in the serum. Follicular insulin had the strongest correlation with serum levels, when compared to other measurements. The serum-follicular relationship of lipids and glucose substances are shown in Figure 4.1.

Table 4.1 Mean concentrations of metabolic substances in serum and follicular fluid of 84 women

Substance	**Serum** (Mean ± SD)	**Follicular fluid** (Mean ± SD)	**p-value**
Insulin (uU/ml)	12.18 ± 7.77	8.87 ± 7.71	<0.001
Glucose (mmol/L)	5.03 ± 0.55	2.95 ± 0.89	<0.001
TG (mmol/L)	1.29 ± 0.59	0.51 ± 1.23	<0.001
LDL-C (mmol/L)	2.18 ± 0.66	0.01 ± 0.03	<0.001
HDL-C (mmol/L)	1.44 ± 0.30	0.62 ± 0.18	<0.001
Total fatty acid (mmol/L)	0.47 ± 0.17	0.24 ± 0.08	<0.001

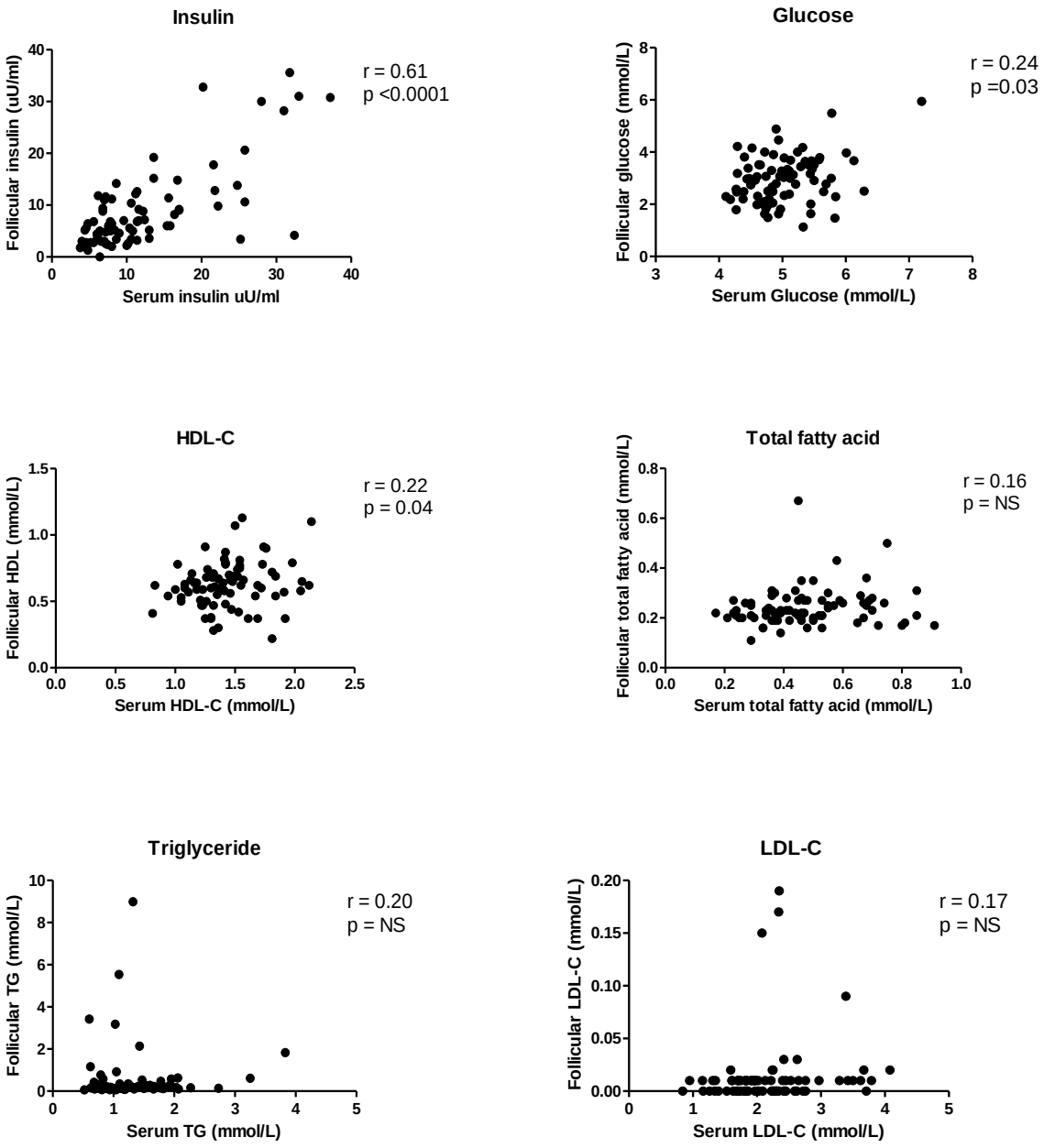
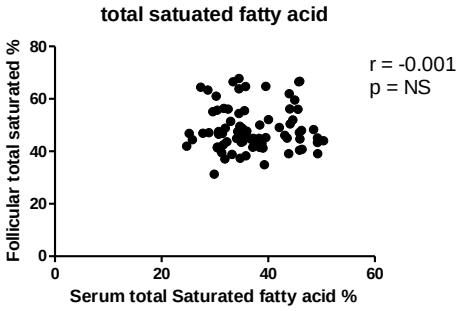


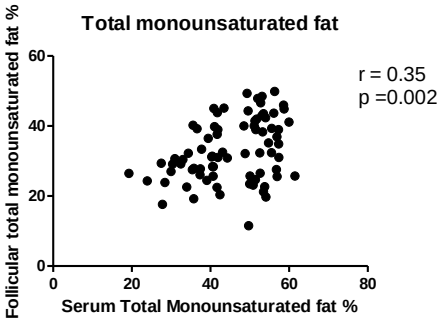
Figure 4.1 Correlation between serum and follicular substances among 84 women

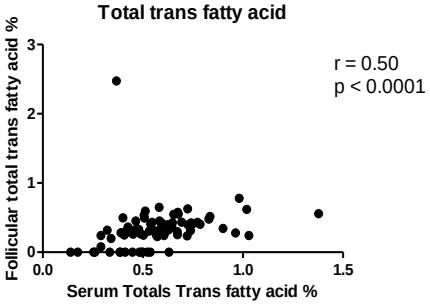
The percentage of total fatty acids in follicular fluid was different from the serum proportion, except for the total n-7 group, which had a similar percentage in both serum and follicular fluid. The percentage of total saturated fatty acid was higher in the follicular fluid. Meanwhile, the monounsaturated and total n-9 fatty acid percentages were higher in serum, as shown in Table 4.2. However, there were moderate to strong correlations among serum and follicular fatty acid classes, except for the total saturated group, as shown in Figure 4.2.

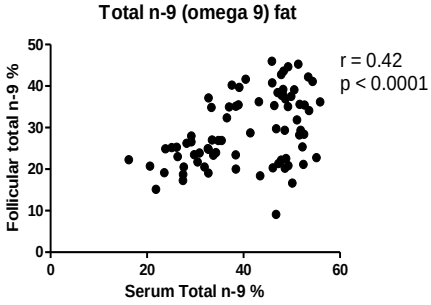
Table 4.2 Mean percentage of each total fatty acid class in serum and follicular fluid of 84 women

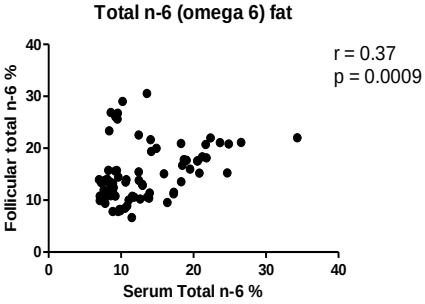
Fatty acid (%)	**Serum (%)** (Mean ± SD)	**Follicular fluid (%)** (Mean ± SD)	**p-value**
Total saturated fatty acid	36.95 ± 6.54	48.78 ± 8.65	0.000
Total monounsaturated fatty acid	45.36 ± 9.72	33.01 ± 8.67	0.000
Total trans fatty acid	0.59 ± 0.23	0.37 ± 0.57	0.000
Total n-9 fatty acid (omega 9)	40.52 ± 10.05	29.00 ± 8.39	0.000
Total n-6 fatty acid (omega 6)	13.20 ± 5.64	14.84 ± 5.45	0.01
Total n-3 fatty acid (omega 3)	3.32 ± 1.46	2.29 ± 0.81	0.000
Total n-7 fatty acid (omega 7)	4.63 ± 1.84	3.93 ± 1.28	NS

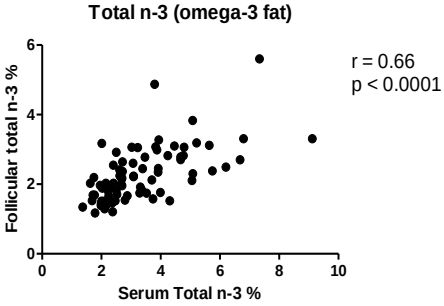












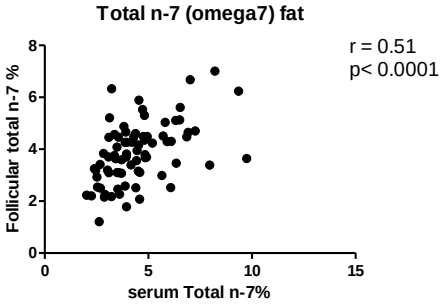


Figure 4.2 Correlation between serum and follicular fatty acids

The predominant fatty acid in both serum and follicular fluid was 16:0 (palmitic acid), 18:1 n-9 (9-octadenoic acid), 18:1 n-6 (9,12-octadecadienoic acid) and 18:0 (stearic acid). The percentage of specific fatty acid in serum was significantly different from the follicular percentage. The percentage of 16:0 (palmitic acid) and 18:0 (stearic acid) were higher in follicular fluid, as shown in Table 4.3.

Table 4.3 Mean percentage of fatty acid in serum and follicular fluid

Fatty acid	**Serum (%)** (Mean ± SD)	**Follicular fluid (%)** (Mean ± SD)	**p-value**
14:0 (Myristic acid)	1.40 ± 0.50	1.36 ± 0.26	NS
15:0 (Pentadecanoic acid)	0.25 ± 0.08	0.25 ± 0.10	NS
dma16:0 (Dimethyl acetyl hexadecanoate)	0.15 ± 0.44	0.02 ± 0.08	0.009
16:0 (Palmitic acid)	24.57 ± 4.01	31.34 ± 5.18	<0.001
dma18:0 (Dimethyl acetyl octadecanoiate)	0.06 ± 0.18	0	0.007
18:0 (Stearic acid)	10.40 ± 2.86	15.63 ± 3.62	<0.001
20:0 (Arachidic acid)	0.03 ± 0.07	0.08 ± 0.14	NS
22:0 (Behenic acid)	0.04 ± 0.13	0.08 ± 0.27	NS
24:0 (Lignoceric acid)	0.04 ± 0.07	0.02 ± 0.07	0.002
Trans 16:1 (Transpalmitelaidic acid)	0.06 ± 0.07	0	<0.001
Trans 18:1 n-9 (9-octadenoic acid)	0.26 ± 0.17	0.23 ± 0.55	<0.001
Trans 18:1n-7 (Δ7 transvaccenic acid)	0.26 ± 0.19	0.14 ± 0.12	<0.001
14:1 (Myristoleic acid)	0.22 ± 0.13	0.21 ± 0.33	0.01
15:1 (Pentadecenoic acid)	0.03 ± 0.06	0.94 ± 0.38	NS
16:1n-9 (Δ9 palmitelaidic acid)	0.35 ± 0.20	0.24 ± 0.10	<0.001
16:1n-7 (Elaidic acid)	2.97 ± 1.41	1.94 ± 0.66	<0.001
17:1 (Heptadecenoic acid)	0.19 ± 0.21	0.26 ± 0.53	<0.001
18:1 n-9 (9-octadenoic acid)	39.55 ± 10.46	27.36 ± 8.91	<0.001
18:1n-7 (Vaccenic acid)	1.66 ± 0.47	1.99 ± 0.81	0.001
19:1 (Nonadecanoic acid)	0.02 ± 0.05	0	0.02

Table 4.3 (cont.) Mean percentage of fatty acid in serum and follicular fluid

Fatty acid	Serum (%) (Mean ± SD)	Follicular fluid (%) (Mean ± SD)	p-value
20:1 n-11 (Δ11 eicosanoic acid)	0.06 ± 0.10	0.05 ± 0.12	0.01
20:1n-9 (Eicosenoic acid)	0.20 ± 0.12	0.43 ± 0.22	<0.001
22:1n-9 (13-docosenoic acid)	0.04 ± 0.09	0.17 ± 0.24	0.001
24:1n-9 (Nervonic acid)	0.06 ± 0.05	0.23 ± 0.09	<0.001
18:2 n-9	0.04 ± 0.08	0.03 ± 0.13	0.002
20:2n-9 (Eicosadienoaic acid)	0.28 ± 0.41	0.55 ± 0.59	0.001
9,11 18:2 cLA	0.25 ± 0.19	0.13 ± 0.13	<0.001
18:1 n-6 (9,12-octadecadienoate)	11.30 ± 4.98	12.24 ± 4.93	NS
18:3n-6 (Gamma-linolenic acid; GLA)	0.17 ± 0.20	0.15 ± 0.16	NS
20:2n-6 (Eicosadienoic acid)	0.20 ± 0.12	0.52 ± 0.24	<0.001
20:3n-6 (Homo-gamma-linolenic acid; DGLA)	0.27 ± 0.24	0.32 ± 0.13	<0.001
20:4n-6 (Docosatetraenoic acid)	1.03 ± 0.70	0.82 ± 0.47	0.04
22:2 n-6 (Docosadienoic acid)	0	0.09 ± 0.12	<0.001
22:4n-6 (Docosatetraenoic acid)	0.15 ± 0.07	0.58 ± 0.28	<0.001
22:5n-6 (Clupanodonicate)	0.08 ± 0.07	0.12 ± 0.11	0.008
16:2 n-3 (Palmitoleatic acid)	0.10 ± 0.08	0.03 ± 0.06	<0.001
18:3n-3 (α-linolenic acid; ALA)	1.45 ± 0.89	0.39 ± 0.21	<0.001
18:4 n-3 (Parinaric acid)	0.02 ± 0.06	0	NS
20:3n-3 (Eicosatrienoic acid; ETA)	0.28 ± 0.17	0.57 ± 0.33	<0.001
20:5n-3 (Eicosapentaenoic acid; EPA)	0.28 ± 0.25	0.12 ± 0.16	<0.001
22:5n-3 (Docosapentaenoate;DPA)	0.31 ± 0.19	0.33 ± 0.18	NS
22:6n-3 (Docosahexaenoic acid;DHA)	0.90 ± 0.61	0.77 ± 0.38	NS

There was a moderate to strong correlation between the level of follicular 14:0 (myristic acid), 20:4 n-6 (docosatetraenoic acid), 22:6 n-3 [docosahexaenoic acid (DHA)] and 22:5 n-3 [docosapentaenoic acid (DPA)] and serum levels, as shown in Table 4.4.

Table 4.4 Correlation between serum and follicular fatty acids

Substance (%)	r	p	Substance (%)	r	p
Saturated fat----------			**Monounsaturated fat--**		
14:0 (Myristic acid)	0.65	<0.0001	**15:1 (Pentadecenoic acid)**	-0.10	NS
15:0 (Pentadecanoic acid)	0.36	0.001	**17:1 (Heptadecenoic acid)**	-0.08	NS
dma 16:0 (Dimethyl acetyl Hexadecanoate)	0.32	0.004	**16:1 n-9 (Δ9 palmitelaidic acid)**	0.50	<0.0001
16:0 (Palmitic acid)	0.02	NS	**16:1n-7 (Elaidic acid)**	0.51	<0.0001
dma18:0 (Stearic acid)	0.08	NS	**18:1 n-9 (9-octadenoic acid)**	0.46	<0.0001
18:0 (Stearic acid)	-0.02	NS	**18:1 n-7 (Vaccenic acid)**	0.52	<0.0001
20:0 (Arachidic acid)	0.22	NS	**19:1 (Nonadecanoic acid)**	0.36	0.001
22:0 (Behenic acid)	0.02	NS	**20:1 n-11 (Δ11 eicosanoic acid)**	-0.01	NS
24:0 (Lignoceric acid)	-0.09	NS	**20:1 n-9 (Eicosenoic acid)**	0.33	0.003
Trans-fat----------			**22:1 n-9 13-docosenoic acid)**	0.52	<0.0001
Trans16:1 (Transpalmitelaidate)	0.19	NS	**24:1 n-9 (Nervonic acid)**	-0.02	NS
Trans 18:1 n-9 (9-octadenoic acid)	0.12	NS	**18:1 n-6 (9,12-octadecadienoiate)**	0.33	0.003
Trans 18:2	-0.04	NS			

*F = follicular substance, S = serum substance, - = negative correlation

Table 4.4 (cont.) Correlation between serum and follicular fatty acids

Substance (%)	r	p	Substance (%)	r	p
Polyunsaturated fat----					
18:2 n-9	0.20	NS	**22:4 n-6 (Docosatetraenoic acid)**	0.37	0.0008
20:2 n-9 (Eicosadienoaic acid)	0.20	NS	**22:5 n-6 (Clupanodonicate)**	0.16	NS
9,11 18:2cLA	0.55	<0.0001	**16:2 n-3 (Palmitoleatic acid)**	0.14	NS
18:3 n-6 (GLA)	0.45	<0.0001	**18:3 n-3 (ALA) (alpha-linolenic acid)**	0.50	<0.0001
20:2 n-6 (Eicosadienoic acid)	0.51	<0.0001	**18:4 n-3 (Parinaric acid)**	-0.04	NS
20:3 n-6 (DGLA)	0.47	<0.0001	**20:3 n-3 (ETA)**	-0.01	NS
20:4 n-6 (Docosatetraenoic acid)	0.61	0.004	**20:5 n-3 (EPA)**	0.58	<0.0001
22:2 n-6 (Docosadienoic acid)	-0.09	NS	**22:5 n-3 (DPA)**	0.60	<0.0001
			22:6 n-3 (DHA)	0.61	<0.0001

There was no detectable levels of 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 11:1, 12:1, 13:1, 18:1 n-12 or 20:3 n-9 in either follicular fluid or serum.

There were many correlations between follicular fluid and serum substances. Follicular glucose levels did not correlate highly with any fatty acids. However, the follicular insulin had weak to moderate correlation among metabolic substances, as shown in Table 4.5- 4.7.

Table 4.5 Significant correlation of follicular glucose with other substances

Substance (mmol/L)	r	P
Serum HDL-C	-0.28	0.01
Serum insulin	0.25	0.03
Follicular total fatty acid	0.24	0.03

Table 4.6 Significant correlation of follicular insulin with other substances

Substance (mmol/L)	r	p	Substance (mmol/L)	R	p
Serum TG	0.26	0.02	**Follicular TG**	0.28	0.01
Serum Glucose	0.37	0.0008	**Follicular LDL-C**	0.28	0.0
Serum HDL	-0.35	0.001	**Follicular HDL-C**	0.22	0.05
			Follicular total fatty acid	0.40	0.0002

Table 4.7 Significant correlation of follicular insulin with follicular fatty acids

Substance (%)	r	p	Substance (%)	R	p
S-20:2 n-6 (Eicosadienoic acid)	-0.30	0.008	**Ftotal n-3 (Omega-3)**	-0.31	0.005
			F16:0 (Palmitic acid)	-0.29	0.008
S-16:2 n3 (Palmitoleatic acid)	0.23	0.004	**F17:1 (Heptadecenoic acid)**	-0.33	0.002
			F16:1n-7 (Elaidic acid)	0.35	0.001
			F20:3n-3 (ETA) (Eicosatrienoic acid)	-0.36	0.001

***F = follicular substance, S = serum , - = negative correlation**

The follicular cholesterol and triglyceride levels were related to many substances, as shown in Table 4.8-4.13.

Table 4.8 Significant correlation of follicular triglyceride with other substances

Substance (mmol/L)	r	p	Substance (mmol/L)	R	p
Serum Glucose	0.27	0.02	**Follicular insulin**	0.28	0.01
Serum HDL	-0.23	0.04	**Follicular total fatty acid**	0.32	0.003
Serum Insulin	0.30	0.006			

Follicular triglyceride levels had a weak, negative correlation with follicular heptadecenoic acid (17:1) percentage (r = -0.30, p = 0.005), and serum total omega 7 (r = -0.32, p = 0.005), elaidic acid (r = -0.30, p = 0.007), vaccenic acid (r= -0.35, p = 0.001), 13-docosenoic acid (r= -0.38, p = 0.0007) and eicosadienoic acid (r = -0.43, P< 0.0001) percentages.

Table 4.9 Significant correlation of follicular LDL-C with other substances

Substance (mmol/L)	r	P
Follicular HDL-C	0.34	0.001
Follicular insulin	0.28	0.01
Follicular total fatty acid	0.42	<0.0001

Table 4.10 Significant correlation of follicular LDL-C with other substances

Substance (%)	r	P	Substance (%)	r	p
S-total monounsaturated	0.34	0.002	**Ftotal saturated**	-0.38	0.0004
S-total n-9	0.32	0.005	**Ftotal monounsaturated**	0.30	0.005
S-total n-6	-0.40	0.003	**Ftotal n-9**	0.31	0.005
S-15:1 (Pentadecenoic acid)	0.34	0.002	**F16:0 (Palmitic acid)**	-0.40	0.0002
S-18:1n-9 (9-octadenoic acid)	0.31	0.006	**F18:0 (Stearic acid)**	-0.30	0.006
S-18:2n-6	-0.38	0.0007	**F17:1 (Heptadecenoic acid)**	-0.34	0.002
S-20:3n-6 (DGLA)	-0.31	0.0006	**F18:1n-9 (9-octadenoic acid)**	0.32	0.004
			F20:2n-9 (Eicosadienoaic acid)	-0.34	0.001

*F = follicular substance, S = serum substance, - = negative correlation

There was little follicular LDL-C fluid in women. Thirty nine women had no detectable LDL-C levels in their follicular fluid, while 34 had 0.01 mmol/L and 11 were in the range of 0.02 to 0.19 mmol/L. Thus, the sample size for testing and confirming significance of these correlations was not as strong as that for other substances. However, there were some differences among women with and without detectable follicular LDL-C, as shown in Table 4.11.

Table 4.11 Significant differences between women with and without detectable follicular LDL-C

Fatty acid	Detectable follicular LDL-C (Mean ± SD) (n =45)	Undetectable follicular LDL-C (Mean ± SD) (n=39)	p-value
S- total monounsaturated%	48.45 ± 7.78	41.46 ± 10.97	0.006
S- total n-3%	2.98 ± 1.16	3.80 ± 1.74	0.02
S- dma 16:0 %	0.04 ± 0.17	0.30 ± 0.63	0.03
S- 22:0 (Behenic acid)%	0.02 ± 0.10	0.08 ± 0.17	0.02
S- 15:1 (Pentadecenoic acid)%	0.04 ± 0.05	0.01 ± 0.04	0.003
S- 18:1 n-9 (9-octadenoic acid) %	42.75 ± 8.63	35.97 ± 11.69	0.01
S- 24:1 n-9 (Nervonic acid)%	0.07 ± 0.05	0.05 ± 0.06	0.01
S- total n-9%	43.63 ± 8.28	37.00 ± 11.24	0.01
S- 18:1 n-6 (9,12-octadecadienoic acid)%	9.47 ± 3.35	13.61 ± 5.87	0.001
S- 20:3 n-6% acid (DGLA)	0.21 ± 0.11	0.35 ± 0.33	0.03
S-20:4 n-6 (Docosatetraenoic acid)%	0.86 ± 0.40	1.27 ± 0.93	0.05
S- 22:4 n-6% (Docosatetraenoic acid)	0.13 ± 0.07	0.17 ± 0.08	0.04
S- total n-6%	11.04 ± 3.74	15.89 ± 6.63	0.000
S- 16:2 n-3 (Palmitoleatic acid)%	0.12 ± 0.08	0.06 ± 0.07	0.001
S- 18:3 n-3% (α-linolenic acid (ALA))	1.21 ± 0.64	1.77 ± 1.10	0.02
F- HDL-C (mmol/L)	0.67 ± 0.19	0.56 ± 0.14	0.004
F- insulin (uU/ml)	10.16 ± 7.94	7.30 ± 7.22	0.02
F- total fatty acid (mmol/L)	0.26 ± 0.09	0.22 ± 0.03	0.001
F- total saturated%	46.62 ± 8.43	51.28 ± 8.32	0.05
F-total monounsaturated%	35.35 ± 8.99	30.32 ± 7.53	0.02
F- total n-3%	2.11 ± 0.81	2.49 ± 0.78	0.005
F- 16:0 (Palmitic acid)%	29.99 ± 5.08	32.90 ± 4.89	0.002
F- 18:0 (Stearic acid)%	14.88 ± 3.44	16.48 ± 3.68	0.03
F- 17:1 (Heptadecenoic acid)%	0.16 ± 0.98	0.38 ± 0.76	0.006
F- 18:1 n-9 (9-octadenoic acid)%	29.91 ± 9.24	24.41 ± 7.60	0.01
F- 20:2 n-9 (Eicosadienoaic acid)%	0.36 ± 0.42	0.76 ± 0.68	0.003
F- total n-9%	31.32 ± 8.87	26.32 ± 7.00	0.02
F- 20:3 n-6 (DGLA) %	0.30 ± 0.12	0.34 ± 0.14	0.03
F- 20:3 n-3 (Eicosatrienoic acid (ETA))%	0.50 ± 0.30	0.66 ± 0.34	0.02

***F = follicular substance, S = serum substance

The follicular HDL-C also correlated with many substances, as shown in Table 4.12-4.13

Table 4.12 Significant correlation of follicular HDL-C with other substances

Substance (mmol/L)	r	p	Substance (mmol/L)	R	p
S-Insulin	0.24	0.03	**Finsulin**	-0.35	0.001
			FLDL-C	0.35	0.001
			Ftotal fatty acid	0.42	<0.0001

*F = follicular substance, S = serum substance, - = negative correlation

Table 4.13 Significant correlation of follicular HDL-C with fatty acids

Substance (%)	r	p	Substance (%)	R	p
S-15:1 Pentadecenoic acid)	0.30	0.008	**Ftotal saturated**	-0.32	0.003
			Ftotal n-9	0.30	0.006
			F16:0 (Palmitic acid)	-0.35	0.001
			F17:1 (Heptadecenoic acid)	-0.28	0.009
			F18:1 n-9 (9-octadenoic acid)	0.30	0.006

*F = follicular substance, S = serum substance, - = negative correlation

There were few correlations between the serum substances and other follicular fatty acids. The serum HDL-C had a weak correlation with follicular 24:0 (lignoceric acid) (r = -0.30, p = 0.006). The serum insulin had a weak correlation with follicular 20:4n-6 (docosatetraenoic acid) (r=0.29, p = 0.008) and 20:3n-6 (homo-gamma-linolenic acid ;DGLA)

(r= -0.30, p = 0.006). The serum LDL-C had a weak correlations to some follicular fatty acids, as shown in Table 4.15.

Table 4.15 Significant correlation of serum LDL-C with other substances

Substance (%)	r	P	Substance (%)	R	P
Ftotal saturated	-0.33	0.002	**F16:2 n-3 (Palmitoleatic acid)**	0.32	0.004
F16:0 (Palmitic acid)	-0.35	0.001			
F18:0 (Stearic acid)	-0.29	0.008			

*F = follicular substance, S = serum substance, - = negative correlation

4.4 Summary of the results

1. The serum levels of glucose, insulin and lipids were higher than follicular levels.
2. The follicular glucose, insulin and HDL-C levels were correlated with the serum level, but not with TG, LDL-C and total fatty acid.
3. The proportion of fatty acid in follicular fluid correlated with the serum percentage, except for saturated fatty acid.
4. The serum insulin had the strongest correlation with the follicular level.
5. The most abundant fatty acids were different to the predominance of those in serum. The most abundant fatty acids in follicular fluid was saturated, monounsaturated and omega-9 fatty acids.

The predominant fatty acids in serum was monounsaturated, omega-9 and saturated fatty acids.

6. Follicular insulin correlated with follicular lipids, including palmitic acid, ETA and total omega3.
7. Follicular LDL-C and HDL-C, and serum LDL-C levels correlated with many follicular fatty acids.

4.5 Discussion

Follicular fluid contains many essential substances. Although it is filtered from plasma, the composition is not exactly the same as the serum component. This difference may depend on follicular epithelium secretion and intra follicular metabolism (183, 184).

The predominant fatty acids in both serum and follicular fluid are 16:0 (palmitic acid), 18:1 n-9 (9-octadenoic acid), 18:1 n-6 (9, 12-octadecadienoic acid) and 18:0 (stearic acid). The correlation between serum and follicular fluid levels among major fatty acids, such as 16:0 (palmitic acid) and 18:0 (stearic acid), was non-existent and only weak in this and a previous study, respectively (209). The proportion of follicular saturated fatty acids was higher in follicular fluid than in the serum. Meanwhile, the proportion of monounsaturated and n-9 fatty acids was higher in serum.

The majority of polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA), especially the omega-6, omega-3, omega-9 and omega-7 subgroups, had a moderate correlation between serum and follicular fluid. The omega-6 and omega-3 fatty acids might be transported directly from serum because humans cannot synthesize omega-6 or omega-3 PUFA, as these two fatty acid subgroups must be obtained from diet or synthesized by intestine micro-organisms (113). The majority of follicular omega-7 and omega-9 fatty acids might be transported from the serum as well. There has been no evidence about the synthesis of fatty acids in the ovarian follicle, but there have been signs of follicular fatty acid utilization (168). The percentage of saturated fatty acids, which are the most abundant fatty acids in follicular fluid, might be affected by intrafollicular metabolism.

As mentioned in the literature review, excess dietary fat is associated with insulin resistance, inflammation, high level of serum LDL-C, TG and blood pressure (116, 117, 119-122, 124, 125). There has been no study on the benefit of PUFA and MUFA on fertility, but the moderate correlation between serum and follicular fluid percentage found in this study might lead to further examination on whether these two groups of fatty acid are involved in the cause and treatment of infertility.

The proportion of fatty acids in the follicular environment might affect reproductive function. The literature suggests that high levels of follicular fatty acids also relate to poor oocyte and embryo development in animals, as well as decreased human granulosa cell

survival (210-213). A high omega-3 dietary feed for cows and ewes leads to a lower omega-6: omega-3 ratio in both plasma and follicular fluid. The mature oocytes, follicular progesterone and cleavage rate are increased among high omega-3 diet fed cattle (244, 245). Thus, the strong correlation between the serum and follicular total omega-3 fatty acid (r= 0.66) found in this study might lead to the serum total omega-3 being a predictor of follicular total omega-3, which that may affect fertility. Moreover, we also found the moderate serum-follicular correlation of the alpha-linolenic acid, the omega 3 fatty acid that affect the chance of pregnancy (128). There have been studies revealing that diet and lifestyle associates with the infertility (126). A further study on omega-3 rich diets may have role in the infertility treatment in the future.

Insulin is a pancreatic hormone that has many important roles in metabolic pathways in the human body. Serum insulin levels are associated with fatty acids, as they have a close metabolic relationship as mentioned in literature review (246, 247). The result from this study has shown a moderate- strong correlation between serum and follicular insulin, the same as in previously published data (196). Follicular insulin may transudate from the blood circulation. It stimulates both granulosa and theca cells promoting ovarian steroidogenesis. The glucose transporter of the cumulus cell is insulin sensitive (190), In this study, follicular insulin did not correlate with the follicular glucose, which is the essential energy substrate for cumulus cell metabolism and oocyte maturation. Thus, follicular glucose utilization may not rely solely on

insulin modulation. However, the high level of serum insulin might lead to the high level of follicular insulin and may involve follicular lipid metabolism because insulin correlated with many particular fatty acids. There was weak negative correlation between insulin and the majority of follicular saturated fatty acid, but, there was weak positive correlation with total monounsaturated fat. This correlation is different to the metabolism in serum, when the saturated fat can induce hyperinsulinaemia and insulin resistance (116, 117). The equilibrium between the follicular saturated fatty acid and other fatty acids affected by insulin modulation may affect the oocyte quality. Moreover, follicular insulin also had a negative correlation with the ETA, supporting the significance of this fatty acid with the waist circumference in chapter 3 that may be involved in the inflammatory process in the follicular environment (241)

The human granulosa cell needs LDL-C and HDL-C for progesterone synthesis. (200-203). The lack of LDL-receptor might affect the number of oocyte retrieved and fertilsed oocyte (204, 205). The level of follicular HDL-C has a negative correlation with embryo fragmentation (208). This study found that the follicular LDL-C and HDL-C also had a relationship with follicular fatty acids. These two cholesterols may have a role in follicular fatty acid transportation. The very low level of LDL-C in human follicular fluid may affect the interpretation of correlations and the importance of this cholesterol in follicular lipid and glucose metabolism. However, the negative correlation between the follicular saturated fat and the follicular HDL-C, LDL-C and insulin might refer to the unique follicular glucose and

lipid metabolism. The detrimental effect of the cholesterol and insulin on fertility may be explained by the relationship with the follicular fatty acid equilibrium.

The weak point of this study might be the number of patients. As mentioned in Chapter 2, there was not much variability among participating women. Thus, a larger study may be needed to recruit more women in different ages and BMIs for better comparison of follicular insulin, glucose and lipid metabolism.

Chapter 5 Oocyte lipid, cumulus cell ER stress and embryo and pregnancy outcome

5.1 Introduction

The cumulus cell is a specialized granulosa cell attached to the oocyte, with which it has a close anatomical and biological interaction. Normal oocyte development requires cumulus cells surrounding the egg for physical, nutrient and metabolic precursor support as well as production of growth factors and hormone (131). The cumulus cell and oocyte both nurture each other by bidirectional communication (215-217) (Chapter 1).

Studies have shown that obesity is associated with endoplasmic reticulum (ER) stress, mitochondrial dysfunction and granulosa cell apoptosis (181, 211, 224). Furthermore, obese mice also have higher lipid concentrations within the oocyte, altered mitochondrial function, oxidative stress and decreased blastocysts development rate (181, 182) (Chapter 1).

The aim of this chapter is to explore whether there are relationships between serum or follicular metabolic measures and ER stress markers in cumulus cells, and whether these affect embryo development or pregnancy outcome.

5.2 Methods

General methods, terms and definition are covered in Chapter 2.

The cumulus cells were collected, as mentioned in Chapter 2, before being thawed and the RNA isolated by the RNeasy Micro Kit (Qiagen). The RNA concentration and purity were determined by using the Nanodrop ND-1000 spectrophotometer (Biolab). The specimen was reverse transcribed by random primers and superscript III reverse transcriptase (Invitrogen). The C-DNA was triplicated by real-time PCR using SYBR* Green PCR Master Mix (Applied Biosystems) and a Rotor-Gene 6000 (Corbett) real-time rotary analyser. L19 was used as the housekeeping gene. Finally, the Real-time RT-PCR data were analysed by the $2^{-\Delta\Delta_{CT}}$ method.

Unfertilised oocytes were collected and stored as described in Chapter 2. Before staining, the oocytes were washed in PBS plus 1 mg/ml of polyvinylpyrrolidone (PVP) for 5 minutes 3 times. Then, they were placed in staining solution, composed of 1 µg/ml of neutral lipid stain BODIPY 493/503 (Invitrogen), 7.5 µg/ml of nuclear stain HOESCHT (Invitrogen) and 1 ml of PBS plus PVP. Each oocyte was washed and incubated individually in 1 ml of staining solution. After incubating for 1 hour at 4 ^{0}C, the oocytes were washed in PBS plus PVP for 5 minutes 2 times and mounted on coverslips.

The BODIPY 493/503 (4,4 difluoro- 1,3,5,7,8-pentamethyl-4-bora-3a, 4a-diaza-s-indacene) stain is an intrinsically lipophilic amine-reactive green fluorescent dye. It has

maximum fluorescence excitation at 500-650 nm and emission at 510- 665 nm, with a narrow spectral band width. This dye is equal to, or more specific than Nile red dye (Invitrogen) for cellular neutral lipid droplets (169).

Hoechst is a bisbenzimidazole derivative and cell permeable nucleic acid blue florescent dye that is sensitive enough for detecting >3ng of DNA.

Twenty oocytes were stained each time. Two mouse oocytes were stained with the same protocol to be a standardized control.

Images of each oocyte were captured using a Leica SP5 spectral scanning confocal microscope, using the same magnification and settings throughout the experiments. The plane with the largest diameter of oocyte was captured, as in the mid plane of the oocyte. The average lipid content was determined by Analysis LS professional software (Olympus^{R}). A square region of interest was placed over the area of the oocyte, as shown in Figure 5.1. The average lipid content was determined by the average intensity of the fluorescence pixel dot in the boxed area of each oocyte.

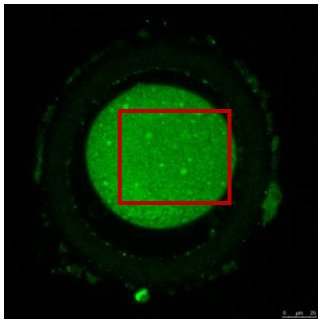


Figure 5.1 The boxed area determining the average of the fluorescence pixel dot.

The morphology of the unfertilised oocytes were scored from the image under the normal light source of a confocal microscope. A normal oocyte has a round clear zona pellucida, small perivitelline space, single unfragmented 1st polar body and a pale, moderately granular cytoplasm without irregular cytoplasm (32, 144). The morphology was classified into three categories:

Mild abnormality; abnormal shape of the zona pellucida, irregular polar body or irregular cytoplasm surface.

Moderate abnormality; particle in the pervitelline space, fragmented polar body, giant polar body, abnormal cytoplasm shape, granular found in the cytoplasm, small vacuole, or small or large oocyte (defined by being 20% larger or smaller than the average size).

Severe abnormality; distorted perivitelline space, divided polar body, large vacuole found in the cytoplasm or shrunk cytoplasm.

The zona pellucida thickness is difficult to assess. However, no abnormal thickness was seen clearly.

Every oocyte was scored as follows:

0 = normal morphology.

1 = good (irregular cytoplasm surface or abnormal shape of zona pellucida).

2 = fair (oocyte with moderate abnormality or two mild abnormalities).

3 = poor (oocyte with severe abnormality or more than one moderate abnormality).

Normal, good and fair oocytes were included in the lipid study (32, 144). Poor oocytes were excluded from further lipid stain analysis. If the patient had more than one normal-fair oocyte, the representative fluorescence data were taken from the average of all of them. The photos representing oocyte morphology are shown in Figure 5.2.

Morphologic variability score was based on the similarity of each unfertilised oocyte from individual patients. If all of the oocytes had the same score, the variability would be 0%. If one out of three had a different score, the variability would be 33.33%, and if each oocyte was different, the variability would be 100%.

The percentage of good unfertilised oocytes is the number of oocytes with a 0-2 score divided by the total number of unfertilised oocytes of each patient, multiplied by 100.

Statistical analyses

The statistical analysis was performed, as in Chapter 2. The comparison between oocyte maturity and morphological effect on fluorescence intensity was carried out by the linear mix effect model.

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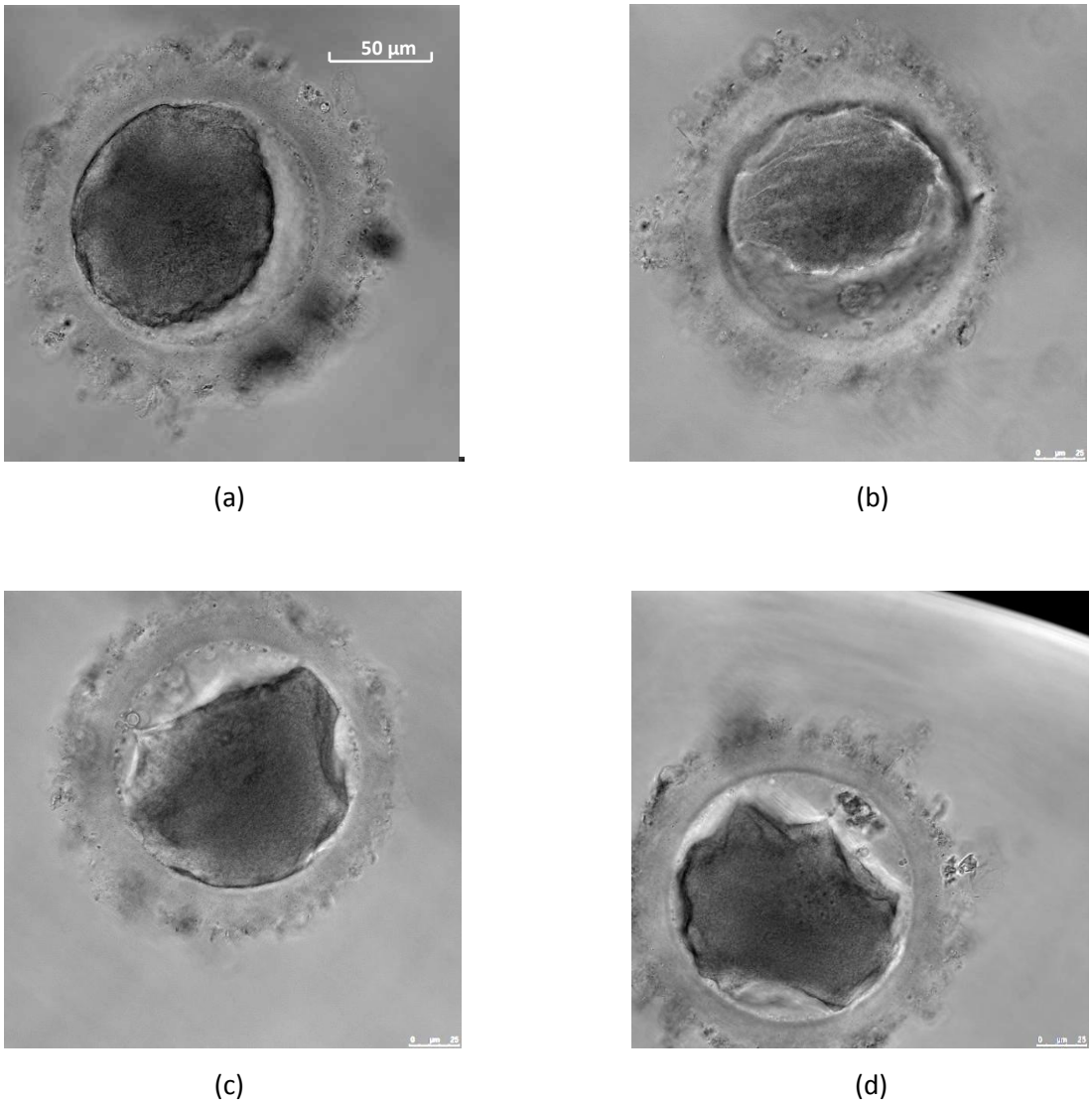


Figure 5.2 The morphology of oocytes from the same patient

(a) is a good oocyte (score=1) with irregular cytoplasm surface

(b) is a fair oocyte (score=2) with abnormally shaped cytoplasm

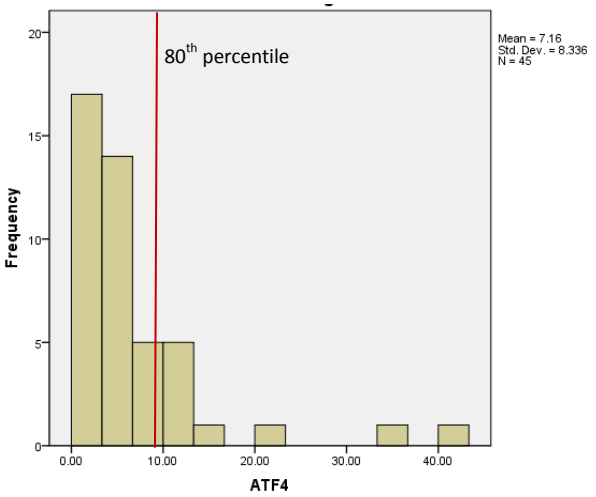
(c) is a fair oocyte (score=2) with abnormally shaped cytoplasm

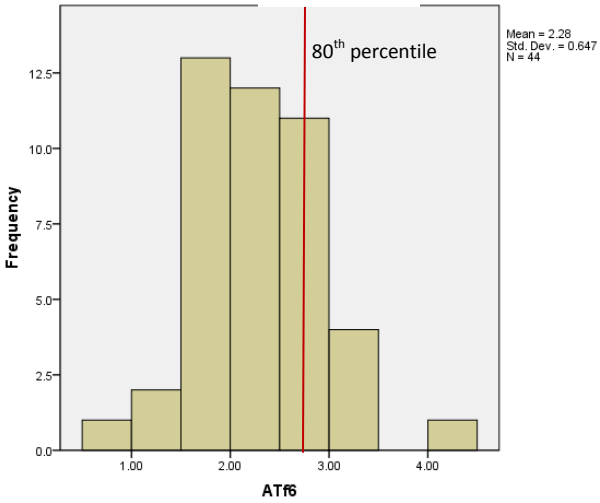
(d) is a poor oocyte (score=3) with abnormally shaped cytoplasm and fragmented polar body

The (b), (c) and (d) oocytes were included in the fluorescence study, and the morphologic variability of this patient was 50%.

5.3 Results

Forty five women had cumulus cells analysed for ER stress marker genes (ATF4, ATF6, GRP78) that were distributed, as shown in Figure 5.3. The ATF6 was distributed normally, but the ATF4 and GRP78 were not. Thus, a high ER stress expression was defined as a value at the 80^{th} percentile or above.





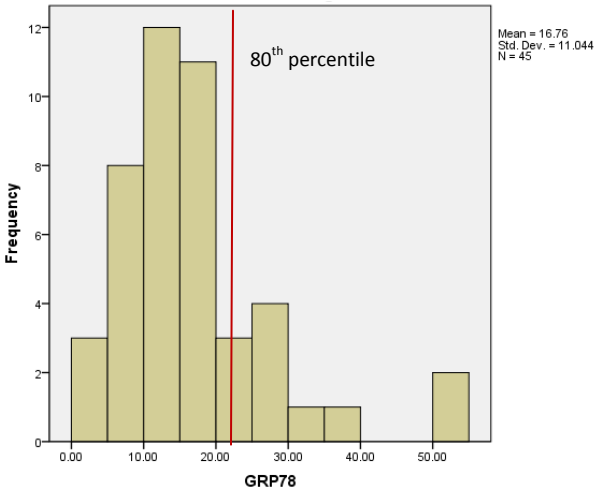


Figure 5.3 The distribution of ER stress marker gene expression in cumulus cells.

The relative expression of the each ER stress marker is shown in the x-axis.

The relationship between each of the ER stress markers was examined as well as correlations with the metabolic markers in serum and follicular fluid. There was a strong correlation between ATF4 and GRP78 expression ($r = 0.59$, $p < 0.001$).

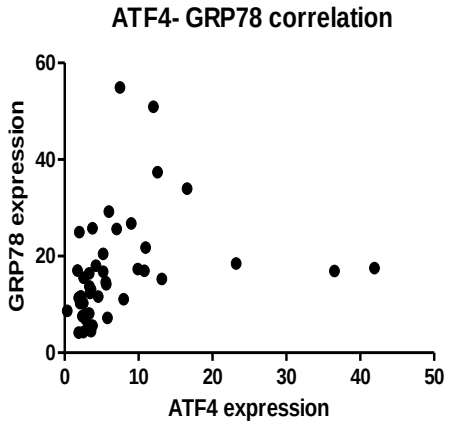


Figure 5.4 The correlation between ATF4 and GRP78 expression among 45 women.

GRP78 expression correlated with BMI only ($r = -0.30$, $p = 0.02$). ATF4 expression did not correlate highly with any variables, while the ATF6 expression significantly correlated with only serum 18:3 n-6 (GLA) ($r = 0.39$, $p = 0.006$).

When women were classified into the high and low expression of ER stress markers, there were no differences in any variables among the ATF4 group. The high GRP78 and ATF6 expression group showed some differences in fatty acid percentage. The follicular eicosapentaenoic acid (EPA) was lower in the high GRP78 expression group than in the lower one, as shown in Table 5.1 and 5.2, which show the significant differences between two expression groups.

Table 5.1 Significant comparisons between women with less and more than the 80th percentile of GRP78 expression

Variables	<80th percentile (36) (Mean ± SD)	≥ 80th percentile (9) (Mean ± SD)	p-value
S-total n-3%	3.84 ± 1.85	2.57 ± 0.45	0.01
S- 18:3 n-6 (ALA)%	0.22 ± 0.21	0.08 ± 0.11	0.04
S-20:5 n-3 (EPA) %	0.33 ± 0.31	0.12 ± 0.06	0.04
Follicular 17:1%	0.18 ± 0.13	0.23 ± 0.07	0.04
F-20:5 n-3 (EPA) %	0.16 ± 0.18	0.04 ± 0.07	0.04
Good unfertilised oocyte %	94.6 ± 9.2	100 ± 0.00	0.03

*F = follicular substance, S= serum substance

Table 5.2 Significant comparisons between women with less and more than the 80th percentile of ATF6 expression

Variables (%)	<80th percentile (36) (Mean ± SD)	≥ 80th percentile (9) (Mean ± SD)	p-value
S- total saturated	37.52 ± 7.08	32.28 ± 5.77	0.02
S- total trans-fatty acid	0.54 ± 0.18	0.81 ± 0.34	0.04
S- total n-7	4.26 ± 1.62	5.99 ± 2.23	0.01
S- total n-6	12.31 ± 5.00	18.97 ± 7.63	0.005
S- total n-3	3.18 ± 1.32	5.06 ± 2.33	0.01
S-dma16:0	0.15 ± 0.50	0.06 ± 0.05	0.004
S-18:0 (Stearic acid)	10.88 ± 3.03	7.23 ± 2.55	0.02
S- 20:0 (Arachidic acid)	0.03 ± 0.07	0.06 ± 0.04	0.005
S- 16:1 n-7 (Elaidic acid)	2.69 ± 1.22	3.88 ± 1.72	0.03
S- 18:1n-7 (Vaccenic acid)	1.57 ± 0.44	2.10 ± 0.58	0.007
S- 16: 1n-9 (Δ9 palmitelaidic acid)	0.33 ± 0.18	0.66 ± 0.28	0.006
S-trans 18:1 n-9 (9-octadenoic acid)	0.06 ± 0.06	0.10 ± 0.06	0.04
S-24:1 n-9 (Nervonic acid)	0.07 ± 0.05	0.03 ± 0.02	0.03
S-16:2 n-3 (Palmitoleatic acid)	0.12 ± 0.09	0.03 ± 0.05	0.007
S- 18:2 n-6 (9,12-octadecadienoic acid)	10.54 ± 4.34	16.74 ± 7.38	0.007
S- 18:2 n-9	0.03 ± 0.06	0.14 ± 0.10	0.001
S- 9,11 18:2 cLA	0.21 ± 0.16	0.41 ± 0.23	0.01
S- 18:3 n-6 (GLA)	0.15 ± 0.15	0.35 ± 0.29	0.002
S-18:3 n-3 (ALA)	1.40 ± 0.83	2.47 ± 1.44	0.009
S-20:3 n-3 (ETA)	0.30 ± 0.18	0.19 ± 0.08	0.03
S- 18:4 n-3 (Parinaric acid)	0.003 ± 0.02	0.08 ± 0.13	0.001
S-22:5 n-3 (DPA)	0.29 ± 0.16	0.54 ± 0.29	0.01
F- total n-3	2.10 ± 0.64	3.14 ± 1.21	0.009
F-20:1 n-9 (Eicosenoic acid)	0.39 ± 0.21	0.63 ± 0.17	0.007
F- 20:2 n-9 (Eicosadienoaic acid)	0.45 ± 0.50	0.96 ± 0.52	0.02
F-20:2 n-6 (11,14-Eicosadienoic acid)	0.48 ± 0.19	0.77 ± 0.30	0.02

*F = follicular substance, S= serum substance

There were differences in the expression of ER stress markers among women who had metabolic problems. The women with high levels of serum triglyceride ($\geq$ 1.7 mmol/L ; n=10) had a lower ATF4 expression when compared to those who had normal triglyceride (n = 35) (6.14 ± 10.76 and 7.45 ± 7.67, p = 0.05). The women with detectable follicular LDL-C (n=22) had a lower ATF6 expression than those who had undetectable LDL-C (n =19) (2.04 ± 0.40 and 2.39 ± 0.68, p=0.05). Expression among the PCOS group had a wide standard deviation, as shown in Table 5.3. There were no other clinical data that correlated with ER stress markers.

Table 5.3 ER stress markers in PCOS and non-PCOS women

Variables	Non-PCOS (n=37) (Mean ± SD)	PCOS (n=8) (Mean ± SD)	P-value
ATF4	8.04 ± 8.95	3.11 ± 1.44	0.03
ATF6	2.33 ± 0.66	2.03 ± 0.55	0.25
GRP78	18.30 ± 11.45	9.65 ± 4.70	0.04

From 88 women who participated in this study, 67 donated their unfertilised oocytes, of which 17 had both mature and immature oocytes, 44 had only mature ones and 6 had only immature ones.

For the lipid study, 55 women had normal to fair unfertilised oocytes and 23 normal to fair immature ones. Thirty nine women had more than one unfertilised oocyte. The

morphology of oocytes from the same patient was not always identical. The mean percentage of morphologic variability was 70.4%. Only 5.3% of these 39 women had the same morphology score in every oocyte. Fifty three percent had a different morphology score in each oocyte. Ten percent had 50% variability, 7.9% had 33.3% and 25% variability and 5.3% had 40% variability.

Among 39 women with more than one unfertilised oocyte, the degree of variability in the morphology score correlated with follicular glucose, as shown in Table 5.4. The follicular glucose might associate with simultaneous development of the oocyte.

Table 5.4 Significant correlations of the degree of morphologic variability in unfertilised oocytes with other measurements

Substance	r	p
Serum HDL-C	-0.30	0.03
Serum LDL-C	-0.31	0.03
Follicular glucose	0.34	0.02

Among the unfertilised oocytes (n=61), 49.2% of the women had good morphology in every unfertilised oocyte, 14.8% had good morphology in half of their unfertilised oocyte pool, 8.2% had good unfertilised oocytes in one-fourth of their pool and 11.5% had no good unfertilised oocytes. To study whether the number of poor unfertilised oocytes affected any variables, the percentage of good unfertilised oocytes was identified by the number of oocytes with a score of 0-2 divided by the total number of unfertilised oocytes from each patient, multiplied by 100.

Women with all good unfertilised oocytes had a lower serum saturated fat percentage, when compared to those with at least one abnormally morphologic oocyte, as shown in Table 5.5.

Table 5.5 Significant comparisons between women with all good unfertilised oocytes and those with at least one abnormally morphologic oocyte

Variables (%)	100% Good unfertilised oocyte (n= 30) (Mean ± SD)	Less than 100% good unfertilised oocyte (n=31) (Mean ± SD)	p-value
S-total saturated fat	35.25 ± 6.45	39.36 ± 5.85	0.02
S-16:0 (Palmitic acid)	23.51 ± 3.93	26.19 ± 3.64	0.01
S-18:0 (Stearic acid)	0.0 ± 0.01	0.10 ± 0.23	0.02
S-24:1 n-9 (Nervonic acid)	0.05 ± 0.05	0.08 ± 0.07	0.05
S-20:3 n-3 (ETA)	0.25 ± 0.16	0.33 ± 0.19	0.03
S-20:5 n-3 (EPA)	0.23 ± 0.27	0.34 ± 0.25	0.02
S-22:6 n-3 (DHA)	0.74 ± 0.56	1.02 ± 0.64	0.02
F-20:5 n-3 (EPA)	0.09 ± 0.17	0.15 ± 0.16	0.04

*F = follicular substance, S= serum substance

Seventy seven percent of participating women had good polar body morphology in every one of their unfertilised oocytes. Eighteen percent had good polar body in 50-83% of them and only 5% had none.

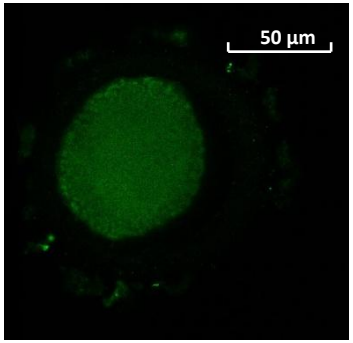
There were differences in follicular alpha-linolenic and omega-7 fatty acid percentage among women with a good polar body in every unfertilised oocyte, and also in patients who had at least one abnormal polar body in their unfertilised oocyte pool, as shown in Table 5.6.

Table 5.6 Significant comparisons between women with good polar body in every one of their unfertilised oocytes and those who had abnormal polar body in some of their oocytes

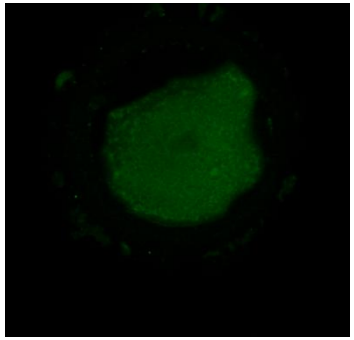
Variables (%)	100% Good polar body unfertilised oocyte (n= 45) (Mean ± SD)	Less than 100% good polar body unfertilised oocyte (n=15) (Mean ± SD)	p-value
S- HDL-C (mmol/L)	55.29 ± 13.39	61.36 ± 8.41	0.03
S-total n-7	4.53 ± 1.94	5.81 ± 2.29	0.05
S-14:0 (Myristic acid)	1.38 ± 0.50	1.65 ± 0.57	0.04
S-15:0 (Pentadecanoic acid)	0.23 ± 0.08	0.29 ± 0.09	0.03
S-trans 18:1 n-7 (Δ7 transvaccenic acid)	0.22 ± 0.19	0.33 ± 0.17	0.02
S-16:1 n-7 (Elaidic acid)	2.91 ± 1.49	3.87 ± 1.75	0.05
S-18:1 n-7 (Vaccenic acid)	1.62 ± 0.50	1.94 ± 0.57	0.05
S-20:1 n-11 (Δ11 eicosanoic acid)	0.07 ± 0.11	0.00 ± 0.00	0.01
S-22:1 n-9 (13-docosenoate)	0.03 ± 0.07	0.11 ± 0.14	0.02
S-20:2 n-6 (11,14-Eicosadienoic acid)	0.17 ± 0.09	0.28 ± 0.18	0.02
S-16:2 n-3 (Palmitoleatate)	0.10 ± 0.08	0.04 ± 0.06	0.02
F-total n-3	2.16 ± 0.90	2.54 ± 0.89	0.05
F-total n-7	3.75 ± 1.35	4.64 ± 1.38	0.04
F-dma 16:0 (Palmitic acid)	0.02 ± 0.07	0.06 ± 0.10	0.05
F-18:0 (Stearic acid)	15.97 ± 3.62	14.17 ± 3.64	0.05
F-trans 18:1 n-9 (9-octadenoic acid)	0.24 ± 0.69	0.36 ± 0.61	0.01
F-18:1 n-7 (Vaccenic acid)	1.93 ± 0.98	2.33 ± 0.79	0.04
F-20:2 n-6 (11,14-Eicosadienoic acid)	0.47 ± 0.23	0.63 ± 0.22	0.03
F-18:3 n-6 (GLA)	0.12 ± 0.16	0.23 ± 0.15	0.02
F-18:3 n-3 (ALA)	0.37 ± 0.22	0.53 ± 0.27	0.05
F-22:6 n-3 (DHA)	0.68 ± 0.39	0.88 ± 0.32	0.02
Number of oocyte retrieved	10.33 ± 5.34	14.87 ± 6.39	0.02

*F = follicular substance, S= serum substance

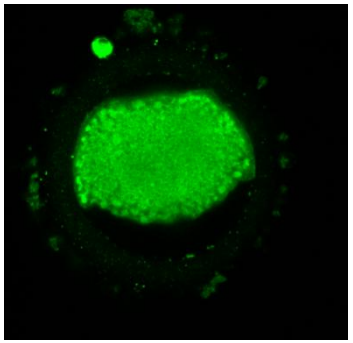
Morphology of oocytes and their maturity did not affect fluorescence intensity of BODIPY 493/506 ($p = 0.42$ and 0.32). However, oocytes with a poor score were excluded from the lipid statistic analysis. The mean fluorescence difference in every oocyte from the same patient was 49.1 ± 41.9 (4.8- 180.1). Pictures of oocyte BODIPY staining is shown in Figure 5.5. There was wide variability among each individual oocyte from the same patient. The fluorescence intensity of each mature oocyte, according to the BMI, is shown in Figure 5.6 and the mean fluorescence of the mature and immature oocytes of each patient is shown in Figure 5.7 and 5.8.



(a) Fluorescence intensity = 65.9

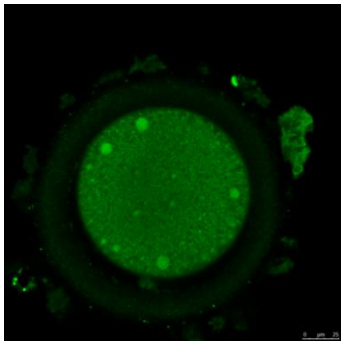


(b) Fluorescence intensity = 53.5

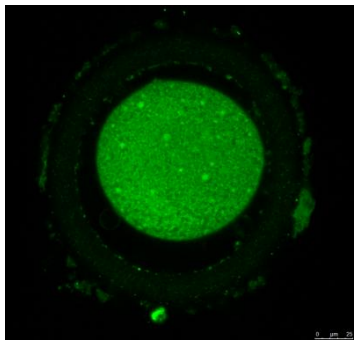


(c) Fluorescence intensity = 164.2

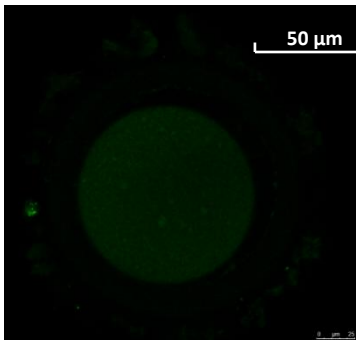
A



(d) Fluorescence intensity = 86.4



(e) Fluorescence intensity = 132.6



(f) Fluorescence intensity = 26.2

B

Figure 5.5 Picture of individual BODIPY staining of a good normal oocyte from the same patient:

(a),(b) and(c) are an individual oocyte from the same patient (A) and (d), (e), (f) are that from another patient (B).

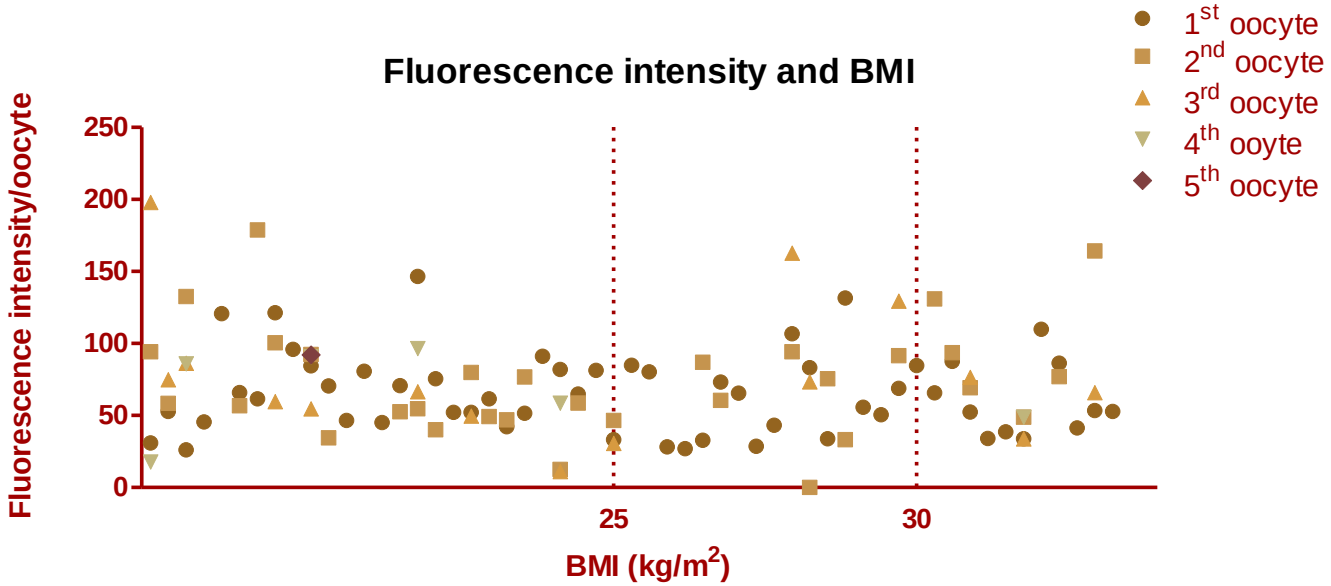


Figure 5.6 Fluorescence intensity of individual mature oocytes from 55 women according to the BMI.

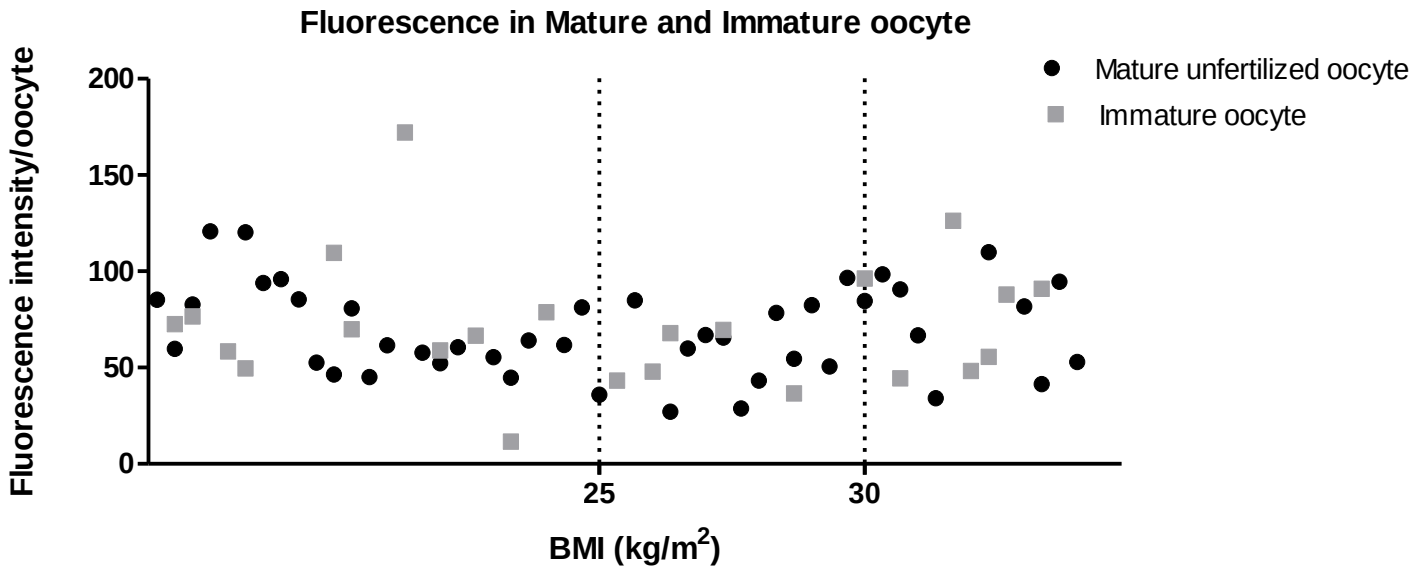


Figure 5.7 Mean fluorescence intensity of mature and immature oocytes according to the BMI.

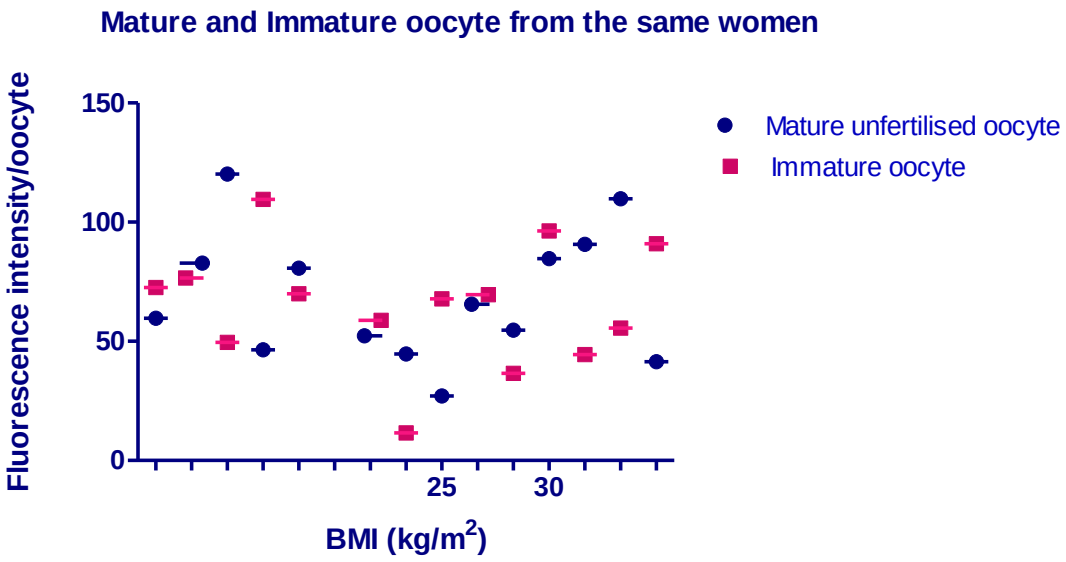


Figure 5.8 Mean fluorescence intensity of mature and immature oocytes from women with both.

There was wide variability of lipid stain among the women, and no significant difference of oocyte fluorescence intensity among the BMI group, as shown in Figure 5.9.

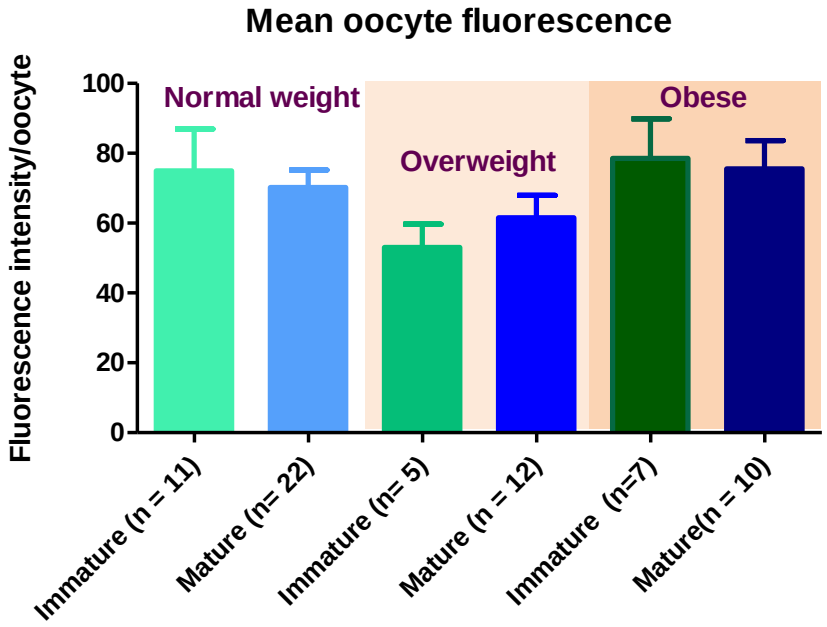


Figure 5.9 The mean fluorescence intensity of the mature and immature oocytes in each BMI subgroup.

Although there was much variability in the lipid stain, the lipid content in mature oocytes (n=55) correlated with morphologic variability (r= -0.46, p=0.005). Lipid content in immature oocytes (n =23) had a significant correlation with serum LDL-C (r=0.45, p = 0.03) and serum lignoceric acid (24:0) percentage (r = -0.56, p = 0.005).

Women with a high level of serum LDL-C (n=10) had lower mature oocyte lipids when compared to those who had normal serum LDL-C (n=35) (54.6 ± 19.3 and 72.5 ± 23.4, p = 0.03).

Clinical data of 88 women showed that the immaturity rate correlated with follicular glucose (r= 0.26, p = 0.009) and follicular total fatty acid (r= 0.19, p = 0.04).

Women who had NCEP (1) metabolic syndrome had higher immaturity rates of the oocyte.

	Number of NCEP criteria				
	0 (n=29)	**1 (n=25)**	**2 (n=20)**	**3-4 (n=10)**	**P-value**
Immaturity rate	3.82 ± 7.03	2.59 ± 4.44	9.91 ± 10.61	16.55 ± 25.01	0.03

The fertilisation rate had a negative correlation with the follicular 20: 5 n-3 (EPA) (r = -0.32, p = 0.003) percentage as well as the cleavage rate (r = -0.35, p= 0.001). However, this fatty acid did not correlate with the blastocyst rate, as in Table 5.8. The blastocyst rate did not correlate with anything.

Morphology of unfertilised oocytes affects the fertilisation and cleavage rate, as shown in Table 5.7.

Table 5.7 Comparison between women with all good unfertilised oocytes and those who had some abnormally morphologic unfertilised oocytes

Variables (%)	100% Good unfertilised oocyte (n= 30) (Mean ± SD)	Less than 100% good unfertilised oocyte (n=31) (Mean ± SD)	p-value
Fertilisation rate	59.68 ± 20.61	45.83 ± 24.84	0.03
Cleavage rate/cycle	59.68 ± 20.61	43.95 ± 25.83	0.01

Women became pregnant from a fresh cycle that had lower serum LDL-C and follicular total fatty acid, as shown in Table 5.8. Nevertheless, there were age differences (32.6 ± 4.3 and 35.4 ± 4.5 , p = 0.005) and serum 15:1 (pentadecenoic acid) % differences (0.01 ± 0.02 and 0.04 ± 0.06, p = 0.006) among clinical pregnancy (n=34) and non-clinical pregnancy groups (n=49).

Table 5.8 Comparison between biochemically pregnant women and women who were non-pregnant from the fresh cycle

Variables	Pregnant patient (n=43) (Mean ± SD)	Non-pregnant patient(n =41) (Mean ± SD)	p-value
Fertilisation rate(%)	62.28 ± 22.00	49.49 ± 26.78	0.02
Serum LDL (mmol/L)	1.98 ± 0.61	2.31 ± 0.65	0.02
Follicular total fatty acid (mmol/L)	0.22 ± 0.05	0.26 ± 0.09	0.03
Good polar body unfertilised oocyte %	94.53 ± 18.89	80.77 ± 29.94	0.01

By Chi-square test analysis, the number of pregnancies in women with a high level of serum LDL-C (n=17) was less than that for the normal level group (n= 67) (biochemical

pregnancy; 11.6% and 30%, and clinical pregnancy; 8.8% and 29.2%). The number of biochemical pregnancies in the PCOS (n=10) group was higher than that of the non-PCOS (n=32) (83.3% and 45.1%, p =0.01) group, as well as the clinical pregnancy (66.7% and 35.2%), p = 0.04) group. However, there was no difference in serum LDL-C, follicular total fatty acid or good polar body percentage among the PCOS subgroup. The oocyte lipid was no different among any of the metabolic or pregnant groups.

The women with 100% good oocytes in their unfertilised oocyte pool (n=30) had a greater number of pregnancies (70.4% and 45.2%, p =0.05) as well as a 100% good polar body oocyte pool (66.7% and 26.7%, p = 0.007).

5.4 Summary of the results

1. There was much variability in the expression of ER stress marker in cumulus cells.
2. The ATF6 expression was correlated well with the GRP78 expression.
3. There was much variability in oocyte morphology among patients.
4. There was much variability in BODIPY 493/506 fluorescence intensity among each patient.
5. Variability of unfertilised oocyte morphology correlated with follicular glucose.
6. Good polar body morphology correlated with the number of oocytes retrieved.

7. The immaturity rate correlated with follicular glucose, total fatty acid and differences in the metabolic syndrome group.
8. The follicular 20:5 n-3 (EPA) percentage had a negative correlation with good morphology of the unfertilised oocyte, fertilisation and cleavage rates.
9. There were differences in follicular n-7 and n-3 percentages among women who had 100% good polar body in their unfertilised oocyte pool and those who had not.
10. There were more pregnancies in women who had lower serum LDL-C and follicular total fatty acid levels.
11. The high LDL-C group had lower mature oocyte lipids, but higher immature lipids than normal LDL-C women.

5.5 Discussion

There appears to be more cumulus cell apoptosis in immature and abnormal oocytes (165, 215). Much cumulus cell gene expression relates to oocyte maturation, fertilisation, chromosomal abnormalities, oocyte and embryo competence and pregnancy (215, 216, 219-221). ATF4 expression had a good correlation with GRP78 expression because they are in the same pathway of ER stress (248). Studies involving ER stress markers and lipids have shown that mice fed on high fat diets showed higher levels of lipids in the oocyte and increased granulosa cell ER stress marker gene expression, which included ATF4 and GRP78 (181).

This might lead to a higher granulosa cell apoptotic index in mice fed on a high fat diet (181). Exposure to human lipid rich follicular fluid also increases the lipid content and ER stress marker in mouse oocytes (243). No strong correlation existed between the ER stress marker and other measurements in this study: the expression was low among the PCOS group and hypertriglyceridaemic women. Evidence of granulosa cell apoptosis in PCOS is still inconclusive (165, 230, 231). The human cumulus cells in this study came from the cumulus cell pool of individual patients, who could provide good or poor oocytes that may cause high variation. Thus, more samples may be needed to solve the expression of wide variability between each patient, and confirm the significance of lipids on human cumulus ER stress markers.

Abnormal morphology of oocytes might relate to lower fertilisation, cleavage, blastocyst, aneuploidy, implantation and cryosurvival rate (42, 134, 141-144, 146, 150, 151). Among women who had more than one unfertilised oocyte, only 5.3% of them had the same morphology score in every one. The degree of variability and immaturity rate correlated with follicular glucose. Thus, follicular glucose should play a role in simultaneous oocyte maturation in the stimulated cycle. This result is supported by animal studies that show the effect of glucose on oocyte maturation (190, 197). Follicular glucose levels that increase in obese women may associate with the quality of oocyte (198). Fatty acids are the important energy source for mouse oocyte maturation and early embryo development (133, 168-171).

This study also revealed a weak correlation between follicular total fatty acid and immaturity. Women with 100% well formed polar bodies in their oocyte pool had a mean of 10.3 retrieved oocytes, which were fewer than those from women with polar body defects in their unfertilised oocyte pool. The high number of stimulated oocytes might affect nuclear maturation as well (249). The results from this study support the importance of glucose and fatty acid as well as the benefit of mild ovarian stimulation on oocyte maturity and quality.

A study of ideal oocyte quality should be carried out in the overall oocyte pool, and not only in unfertilised oocytes. However, in this study, the percentage of good morphology in unfertilised oocytes is associated with fertilisation, cleavage and biochemical pregnancy rate. Thus, an unfertilised oocyte study may be a useful tool in representing overall oocyte quality. There was no significant correlation among blastocyst rates and any variables. Blastocyst development depends on natural selection and laboratory quality. A poor cleavage embryo cannot develop successfully into a blastocyst. The effect of metabolic disturbance and oocyte morphology in this study may affect fertilisation and cleavage rates, but it is not strong enough to affect the blastocyst that develops from the best oocyte in the pool. Clinical pregnancy rates did not correlate with the lipid profile, and this may be hard to interpret in relation to the effect of lipids and glucose because of complications affecting endometrial quality (250, 251). A further study on frozen embryo transfer cycles might conclude a better overall effect of lipids and glucose on IVF outcome.

Despite the morphologic variability among each patient, the serum saturated 20:5 n-3 (EPA) and follicular 20:5 n-3 (EPA) percentages were high in women with poor unfertilised oocyte. The 20:5 n-3 (EPA) also had a negative correlation with fertilisation and cleavage rate. Eicosapentaenoic acid (EPA) is one of the n-3 families. EPA competes using the same enzyme as arachidonic acid by transforming itself into PGE3 and LTB5, which have weaker inflammatory activity when compared to PGE2 and LTB4 derived from arachidonic acid (118). Thus, the inflammatory process may play a role in oocyte quality and fertilisation as well. The omega3, omega7 fat as well as alpha-linolenic acid (ALA) percentages were high in women with poor form polar body in their unfertilised oocyte. These two fatty acid groups had a moderate correlation between serum and follicular measurements ($r= 0.58$, $p < 0.0001$ and 0.51, $p < 0.0001$; Chapter 4). The ALA level also associates with the waist circumference (Chapter 3) and chance of pregnancy (128). Thus, the detection in serum might be predictive of oocyte quality and maturation.

The importance of fatty acids and cholesterol was shown in the pregnancy data, when high levels of serum LDL-C and follicular fatty acid were found in non-pregnant women. The serum LDL-C relates with many serum and follicular fatty acids, as shown in Chapter 3 and 4. Serum LDL-C screening might be another tool for predicting follicular lipid dysequilibrium and poor IVF outcome as well as the importance of metabolic syndrome that relates to the immaturity rate.

In this human study, variability of fluorescence intensity among women and individuals was presented. However, the unfertilised oocyte lipid may correlate with the serum LDL-C. Quality of the unfertilised oocyte itself might not reveal a strong correlation with the clinical outcome, when compared to good fertilised oocytes because the lipid droplets also increase in the atretic resting oocyte (136). More oocyte pools might be needed to confirm the effect of oocyte lipids in human unfertilised oocytes, as well as optimising the resolution of the confocal microscope setting in order to produce a better resolution in pictures of the human unfertilised oocyte. The human oocyte might need 3 captures of the oocyte planes to evaluate the mean fluorescence intensity because the human oocyte is bigger than a mouse oocyte.

Chapter 6 Overall discussion and conclusion

6.1 Introduction

Follicular fluid contains many substances essential for ovulation and oocyte health. Although the fluid is filtered from plasma, the composition is not exactly the same as the serum component. Differences in the follicular composition also depend on the follicular epithelium and intra follicular metabolism (183, 184). Glucose and lipids are essential energy substrates for oocyte maturation (171, 175, 176, 188-191), and excess or diminished glucose concentrations, as well as high levels of follicular fatty acids affect oocyte maturation and quality (210-212). Thus, this study revealed the follicular glucose and lipid relationship with a systemic metabolic status and clinical outcome.

6.2 Effect of systemic metabolic disturbance on follicular metabolism

This study found that follicular insulin was associated with BMI (r= 0.53), waist circumference (r= 0.49) and metabolic syndrome (Chapter 3). Insulin that also correlated with follicular lipids (Chapter 4) and specific fatty acids in a follicular environment might affect reproductive function. The literature suggests that murine blastocysts exposed to palmitic acid also exhibits altered embryonic metabolism and growth (213). A high omega-3 dietary

feed for cows and ewes leads to a lower omega-6: omega-3 ratio in both plasma and follicular fluid. Mature oocytes, follicular progesterone and cleavage rate are increased among high omega-3 diet fed cattle (244, 245). This study found a low follicular alpha-linolenic acid (ALA) percentage in women with high waist circumference (Chapter 3). The ALA also had a moderate correlation between serum and follicular levels (r = 0.50). Thus, a high serum ALA that associates with a poorer chance of pregnancy, as found in a previous study, may associate with follicular ALA and significant waist circumference, which affects fertility (128).

Eicosatrienoic acid (ETA) had the negative correlation with waist circumference (r = -0.34). This fatty acid is derived from omega-9 fatty acid, and competes for enzyme that changes arachidonic acid into strong inflammatory mediators (118). However, eicosapentaenoic acid (EPA) is another fat in the omega-3 group that had negative correlations with fertilisation and cleavage rate. This finding may relate to obesity and a chronic inflammatory relationship that might affect follicular metabolism (241).

Few follicular fatty acids correlated with BMI (Chapter 3). However, a lower percentage of follicular saturated fatty acid existed in women with high serum LDL-C (43.68 ± 6.27 and 50.08 ± 8.73%) (Chapter 3). Women with hyperlipidaemia had different proportions of follicular fatty acids, when compared to normolipidaemic females, but this was not found in hyperinsulinaemic or hyperglycaemic women (Chapter 3). The importance of cholesterol was clearly shown in pregnancy data, when higher levels of serum LDL-C were found in non-

pregnant women (2.31 ± 0.65 and 1.98 ± 0.61 mmol/L) (Chapter 5). Higher follicular fatty acid levels also were found in non-pregnant women (0.26 ± 0.09 and 0.22 ± 0.05 mmol/L) (Chapter 5).

6.3 Relationship between serum and follicular glucose and lipids

As in Chapter 4, this study found that the serum levels of glucose, insulin and lipids were different from follicular levels. It is known that follicular insulin correlates with serum insulin (196). This study revealed more knowledge of the relationship between glucose and lipids and follicular-serum when it found that follicular glucose, insulin and HDL-C levels correlated with the serum level (r= 0.24, 0.61 and 0.22, respectively), but not with TG, LDL-C and total fatty acids. The majority of polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) also had a moderate correlation between serum and follicular fluid (r= 0.37- 0.66) (Chapter 4). In general, the levels of metabolic substances in serum were higher than follicular levels (Chapter4). Follicular substances might be transported mainly from serum. However, the percentage of saturated fatty acids, which are the most abundant fatty acids in follicular fluid, did not correlate with the serum value. This might be affected by intrafollicular metabolism, as it is known that oocyte maturation needs fatty acids and glucose metabolism (168).

Insulin correlated with many follicular fatty acids and cholesterols (Chapter4), and has the strongest follicular-serum relationship. Thus, the high level of serum insulin might lead to a high level of follicular insulin, and may be involved in follicular lipid metabolism. Follicular insulin also had a negative correlation with ETA, which decreased in women with high waist circumference. However, the comparison between hyperinsulinaemic and normoinsulinaemic women did not show a difference of follicular fatty acids between groups (Table3.5, Chapter 3). Further investigations may be needed to understand more about follicular insulin-fatty acid relationships.

This study found that follicular LDL-C and HDL-C also correlated with many follicular fatty acids (Chapter4), and these two cholesterols should play a role in follicular fatty acid transportation, because human granulosa cells need LDL-C and HDL-C for progesterone synthesis. (200-203). The lack of LDL-receptor affects the number of retrieved and fertilised oocytes (204, 205). Furthermore, the level of follicular HDL-C has a negative correlation with embryo fragmentation (208). The negative correlation between follicular saturated fat and follicular HDL-C, LDL-C and insulin (r=-0.32, -0.38 and - 0.33, respectively) might refer to the unique follicular glucose and lipid metabolism that is different from general body metabolism (116, 117, 122, 123). Saturated fat is needed for normal oocyte metabolism, but in very high levels that might affect this complicated process (168, 213).

6.4 Endoplasmic stress marker and the oocyte study

The result in Chapter 5 showed the importance of glucose and fatty acids on oocyte development, as the rate of retrieving immature oocytes was correlated with follicular glucose (r = 0.26) and total fatty acid (r= 0.19). The morphologic variability of the unfertilised oocyte correlated with follicular glucose (r= 0.34). The immaturity rate also increased among women who met more of the metabolic syndrome criteria (Chapter 5). Thus, follicular glucose and fatty acids should be involved in oocytes development.

There were differences in follicular omega-7 and omega-3 percentages between women who had a good polar body in 100% of their unfertilised oocyte pool and compared with those who had oocytes with poor polar bodies (Chapter 5). In addition, the follicular 20:5 n-3 (eicosapentaenoic acid; EPA) percentage associated with good morphology of the unfertilised oocyte, fertilisation and cleavage rate (r = -0.29, -0.32 and -0.35, respectively) (Chapter 5). The EPA competes using the same enzyme as arachidonic acid by transforming itself into PGE3 and LTB5, which have weaker inflammatory activity when compared to PGE2 and LTB4 derived from arachidonic acid. (118). This inflammation also may play a role in oocyte quality.

There is more cumulus cell apoptosis in immature and abnormal oocytes (165, 215). A lot of cumulus cell gene expression relates to oocyte maturation, fertilisation, chromosomal abnormalities, oocyte and embryo competence and pregnancy (215, 216, 219-221). Mice fed

high fat diets showed higher levels of lipids in the oocyte and increased granulosa cell ER stress marker gene expression, ATF4 and GRP78 (181). Mouse oocytes exposed to human lipid rich follicular fluid also had higher lipid content and increase induction of ER stress markers (243). However, ER stress marker gene expression between the women was variable widely in this study and there was no obvious correlation between ER stress markers and other measurements.

Abnormal morphology of oocytes might relate to lower fertilisation, cleavage, blastocyst, aneuploidy, implantation and cryosurvival rate (42, 134, 141-144, 146, 150, 151). Women with a 100% well formed polar body had a mean of 10.33 retrieved oocytes, which were fewer than those in women who had a polar body defect in their unfertilised oocyte pool (Chapter 5). Thus, the high number of stimulated oocytes also might affect nuclear maturation (249).

In this human study, there was variable fluorescence intensity among women and individuals. Nevertheless, the unfertilised oocyte lipid correlated with serum LDL-C (r=0.45) (Chapter 5). Quality of the unfertilised oocyte itself might not reveal strong correlation with clinical outcome, when compared to a good fertilised oocyte, because the lipid droplet also increases in the atretic resting oocyte (136). However, when compared to women with normal serum LDL-C, hypercholesterolemic women had a lower lipid in mature oocytes (54.6 ± 19.3 and 72.5 ± 23.4), but higher one in immature oocytes (r= 0.45) (Chapter 5).

6.5 Study limitation and future research

The weak point of this study might be the number of participants (Chapter 2, descriptive data). There was not much variability among the women involved. Thus, a larger study is needed to recruit more women of different ages and BMIs for a stronger correlation between follicular insulin, glucose and lipid metabolism, and ER stress markers. More oocyte pools also are needed to confirm the effect of oocyte lipids in human unfertilised oocytes, as well as optimising the resolution of a confocal microscope setting and technique for producing better resolution in pictures of human unfertilised oocytes.

Further study on omega-3 fat metabolism and diet may play a role in future infertility treatment. A frozen embryo transfer study also might conclude a better overall effect of lipids and glucose on IVF outcome.

6.6 Conclusion

Overall, this study found the correlation of serum and follicular lipids, relationships between follicular insulin and cholesterol with follicular fatty acids and the importance of serum LDL-C and omega-3 fatty acids. The unfertilised oocyte may be a useful tool for

studying oocyte quality and serum insulin, and LDL-C screening might be another method for predicting follicular lipid dysequilibrium and poor IVF outcome.

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Appendix1

Information and consent forms

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Phone 08 8232 0577
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Ground Floor
120 Hutt Street, Adelaide

Our Doctors
also consult from
- Calvary Central Districts Hospital
- North Adelaide
- Burnside
Regional Centres

Specialists
Dr Alex Hubczenko
Dr Louise Hull
Professor Robert Norman
A/Professor Ossie Petrucco
Dr Jodie Semmler
Dr Michelle Wellman

Participant Information Sheet for the Research Study:

Understanding the basis of female infertility through analysis of ovarian cells

Why is this research being done?

Thank you for reading this Information Sheet. This study is being done to gain a better understanding of the causes of female infertility. The knowledge gained will assist us in developing new strategies to improve treatments for women with infertility.

Who can take part?

Any woman planning to undergo oocyte aspiration (egg collection) as part of their IVF/ICSI cycle will be invited to participate.

What will happen in this research study?

This study does not involve any investigational drugs or procedures, or any alterations to your normal treatment. During your IVF/ICSI cycle you will have an egg retrieval in theatre. As a normal part of this procedure lots of extra ovary cells and fluid come out with the egg and are usually discarded. This study will involve simply collecting these cells and fluids that are normally discarded. Your egg recovery will proceed along normal grounds. In the laboratory any of the excess, non-egg cells that are attached to the egg and normally thrown away will also be retained. Your eggs will be fertilised by the methods previously agreed upon with your doctor. The next day all eggs will be assessed for fertilisation by the embryologist. Those eggs that are fertilised will be managed in the usual manner for our clinic. Those eggs that have not fertilised and would normally be thrown away would also be retained. These eggs are unfertilisable and will not be used to create embryos, but will be used for studies looking at the structure of the egg and its proteins under a microscope. The ovary cells and fluids will be used to measure various metabolites and hormones or for analysis of genes associated with cell metabolism. While you are under anaesthetic, a 10ml blood sample will also be taken by the anesthetist and this will be analysed in an identical manner and compared to your

ovary fluid. Information about your infertility diagnosis and the outcome of your IVF/ICSI treatment will also be recorded. Your samples may be stored for up to 5 years in a laboratory at the University of Adelaide but they will be labelled with your patient number only, and can only be linked to your personal records by your clinical provider. They may be assessed in a variety of ways during this time but only ever for studies which have been approved by a Human Research Ethics Committee.

How do I participate in this study?

If you choose to participate a consent form (attached) must be signed by you, as well as your doctor or a nurse at your IVF clinic. The consent form must be signed before you attend theatre for egg collection. Your own doctor will continue to oversee all aspects of your treatment. Your excess materials will be collected for the current IVF cycle only.

Are there any benefits?

The study will be of no benefit to you, however it will enable the acquisition of new knowledge about how the ovary works and whether alterations detected in the blood or ovary cells may be contributing to infertility in some women. Ultimately this new information will help improve treatments and pregnancy rates in women suffering from infertility.
There is a very small chance that some commercial value may arise from this study; if that would happen you would not be offered a share in the profits.

Are there any risks to me or my embryos if I take part in the study?

There will be no additional risks or discomforts to you, other than those that would normally be experienced during an IVF/ICSI cycle and which will have been explained to you by your doctor. Your eggs and embryos will not be affected by the study because only discarded cells and fluids will be collected and analysed.

What if I don’t want to take part?

Taking part in this research study is your decision, and you may wish to discuss the matter with a relative, friend, your doctor, nurse or counselor. It is important that you understand that your participation is voluntary, and if you do not wish to take part you are under no obligation to

do so. If you decide to take part but later change your mind, you are free to withdraw your consent and this should be communicated verbally or in writing to the medical, nursing, laboratory or counseling staff at Fertility SA. If you withdraw your consent, all materials you already contributed to the study will be destroyed and any information relating to you will be deleted from study records. Your decision to take part, not to take part, or to withdraw, will not affect your routine medical treatment or your relationship with those treating you or your relationship with Fertility SA.

Will information about me remain confidential?

Your privacy is important to us and normal Fertility SA patient confidentiality procedures will be followed at all times. Only your Fertility SA Patient Number will be used on data storage forms. All identifying information will be removed from your samples before they are analysed. The coded information will be released if required for monitoring the proper conduct and reporting of this study. When the analysis of the samples is complete, all participants' results will be pooled together and published in a medical journal. Your involvement in the study will in no way be identifiable, so that confidentiality will maintained at all times.

What if I have questions about the study?

If you have any questions regarding this study, you are welcome to contact your clinic or doctor or the Principal Investigator, Professor Robert J Norman FRANZCOG, on (08) 8232 0577 or (08) 8303 8166. You can talk to Professor Norman at any time about questions and concerns you may have about this study. This study has been approved by the St Andrew's Hospital Ethics Committee. You can get information about the conduct of the study and the rights of research participants from the Executive Secretariat of the St Andrew's Hospital Ethics Committee, on 8408 2139.

ST ANDREW'S HOSPITAL CONSENT FORM FOR RESEARCH PARTICIPANTS

STUDY NAME:
Understanding the basis of female infertility through analysis of ovarian cells

PRINCIPAL INVESTIGATORS:

Professor Robert J Norman FRANZCOG

Fertility SA, 345 Carrington Street, Adelaide 5000

School of Paediatrics and Reproductive Health, University of Adelaide

Dr Rebecca L Robker

School of Paediatrics and Reproductive Health, University of Adelaide

I understand that the study involves collecting my ovary cells and fluids and blood samples that are normally discarded at the end of my IVF/ICSI cycle.

The following extra tissues may be retained for research:

blood sample	☐ Yes	☐ No
ovary fluid	☐ Yes	☐ No
ovary cells	☐ Yes	☐ No
unfertilised eggs	☐ Yes	☐ No

In addition,

- I have understood and am satisfied with the explanations that I have been given and hereby consent to my participation.
- I understand that the results of these studies may be published, but my identity will be kept confidential.
- I understand that the procedure may not be of any benefit to myself, and that I may withdraw my consent at any stage without affecting my rights or the responsibilities of the investigator in any respect, or my medical care, both now and in the future
- I understand that representatives from the Hospital Ethics Committee or Government Regulatory Authorities may need to access my medical record for information related to the study for the purpose of audit. I authorise access to my medical record for this purpose.
- I declare that I am over the age of 18 years.
- I have had the opportunity to discuss taking part in this study with a family member or friend.

Name: _____

Signed: _____

Dated: _____

Witness: _____

I certify that I have explained the study to the patient/volunteer and consider that he/she understands what is involved.

Signed:_____Dated:_____

(Investigator)

Appendix 2

The serum and follicular fatty acid-BMI relationship.

The dotted lines in the figure are the mark of 25 and 30 kg/m^2, which are the cut off BMI for the overweight and obese diagnosis

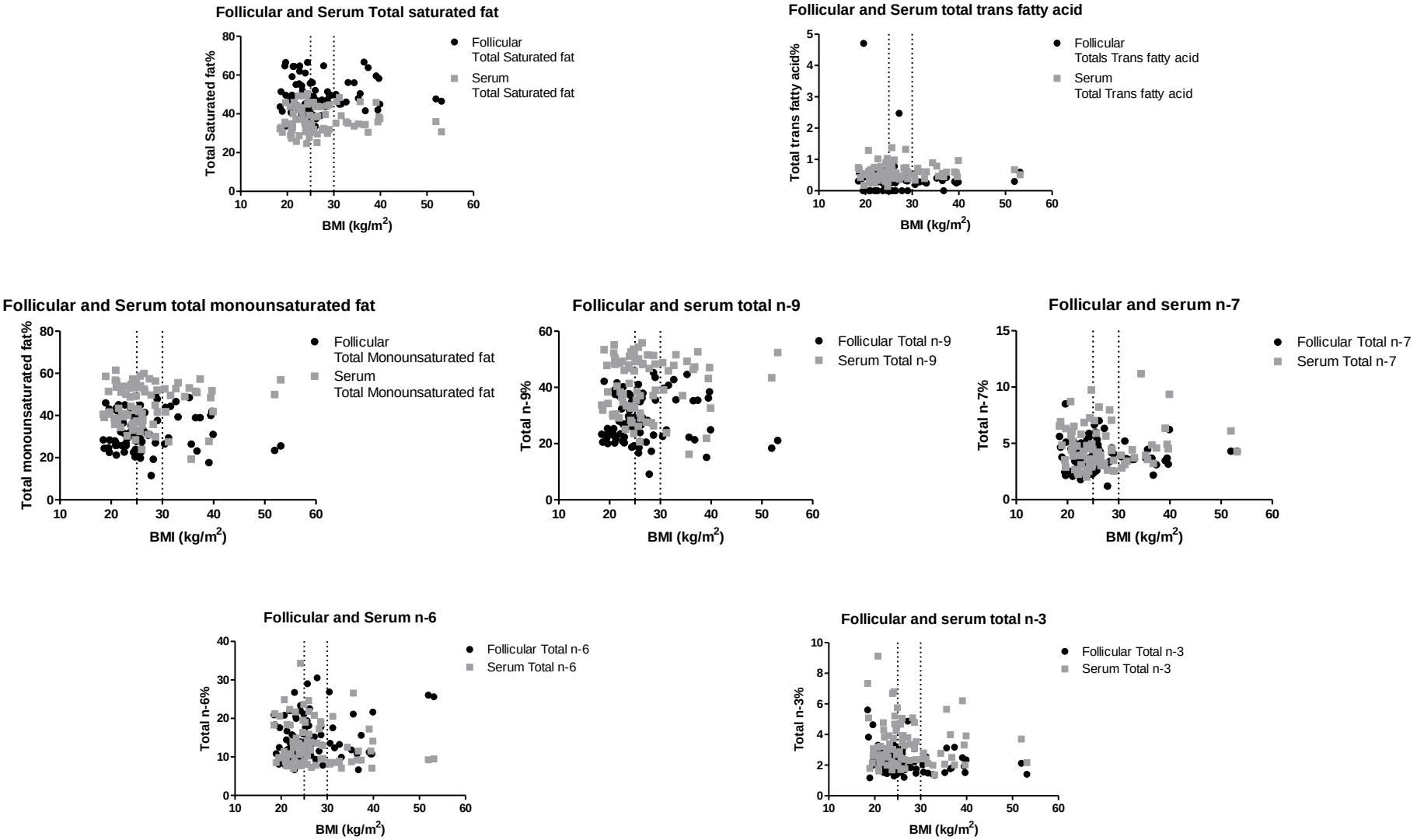

Figure A2.1 The distribution of Follicular and Serum total fatty acid among individual patient according to the BMI

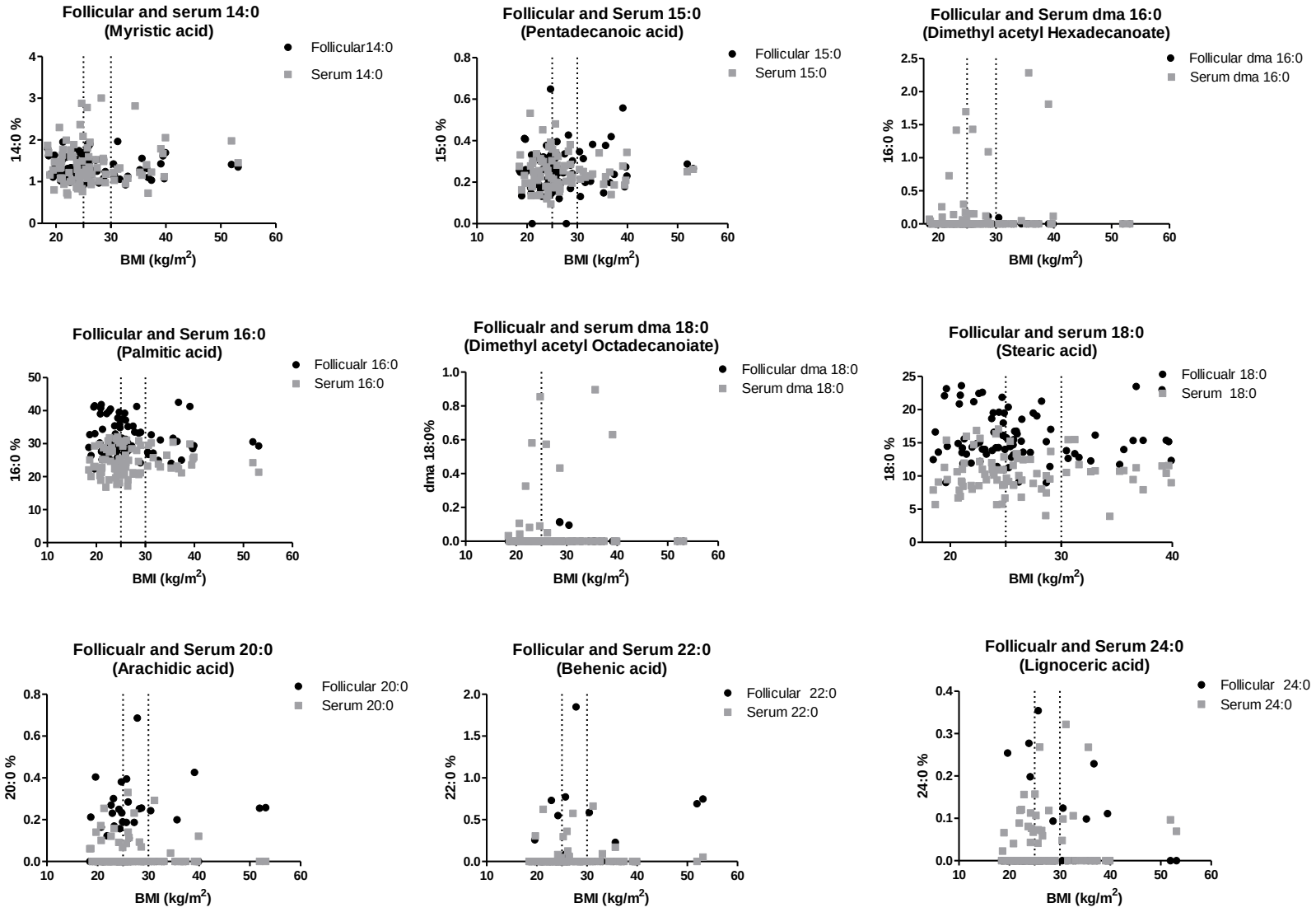

Figure A2.2 The distribution of Follicular and Serum saturated fatty acid among individual patient according to the BMI

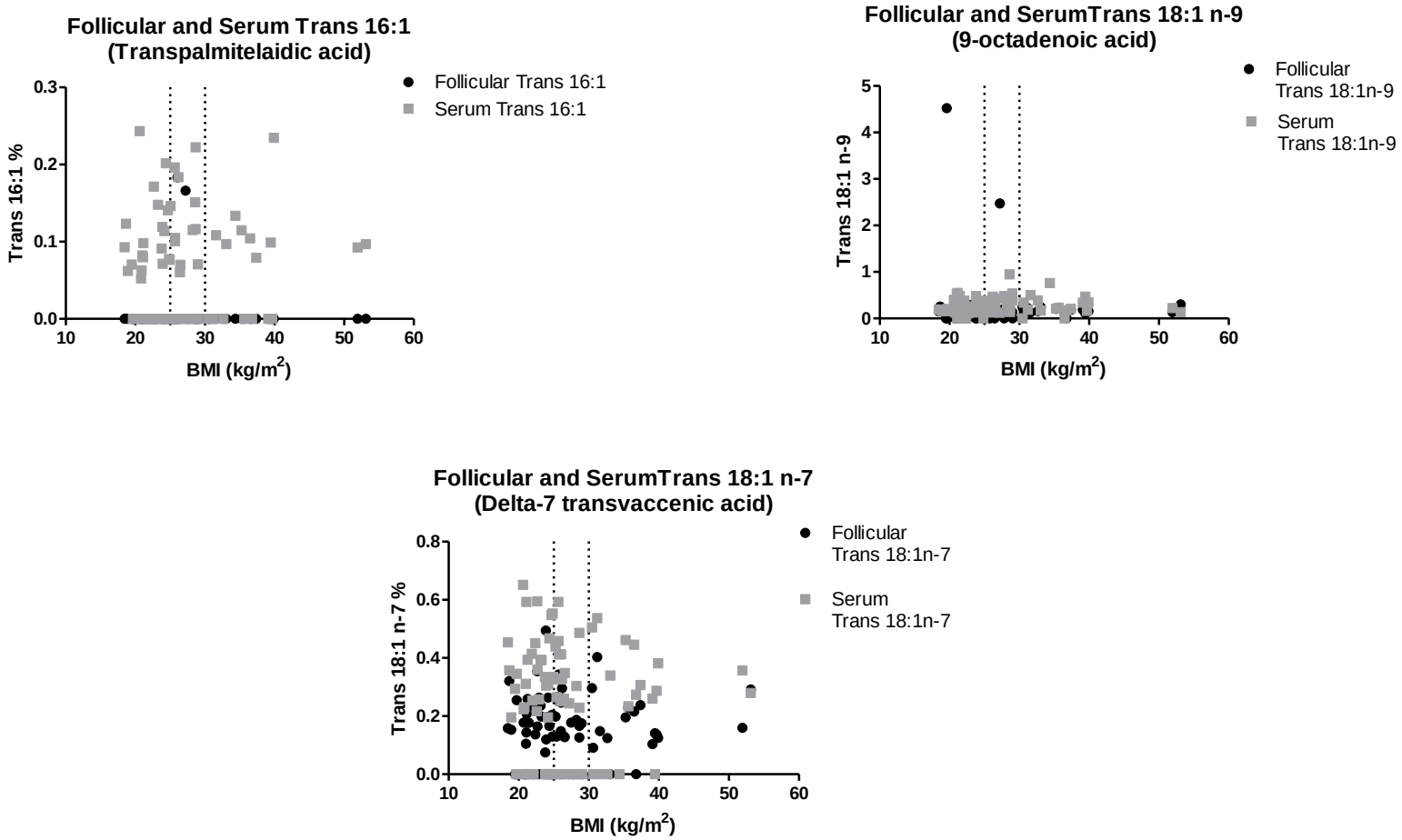

Figure A2.3 The distribution of Follicular and Serum trans- fatty acid among individual patient according to the BMI

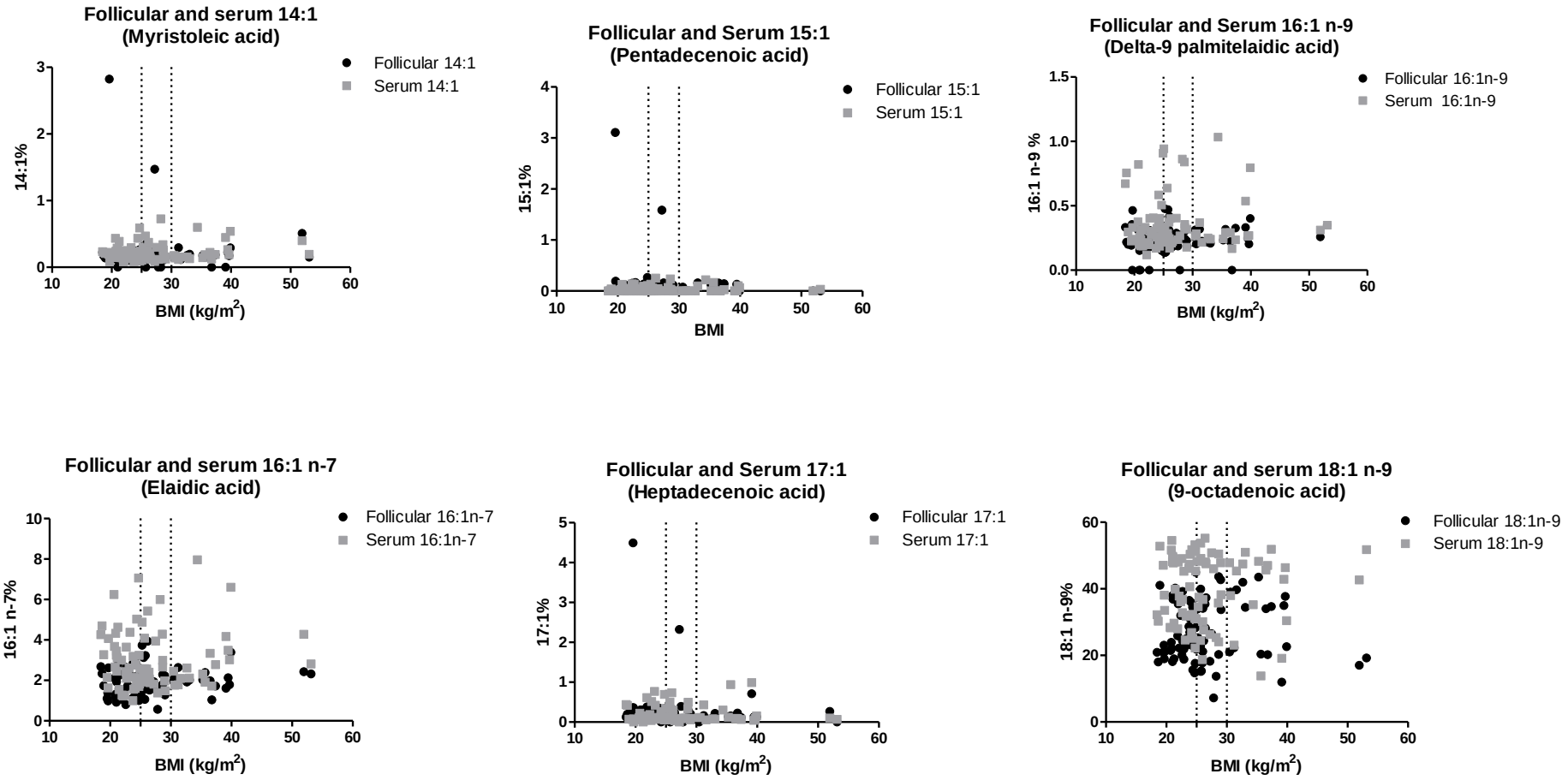

Figure A2.4 The distribution of Follicular and Serum Monounsaturated fatty acid among individual patient according to the BMI

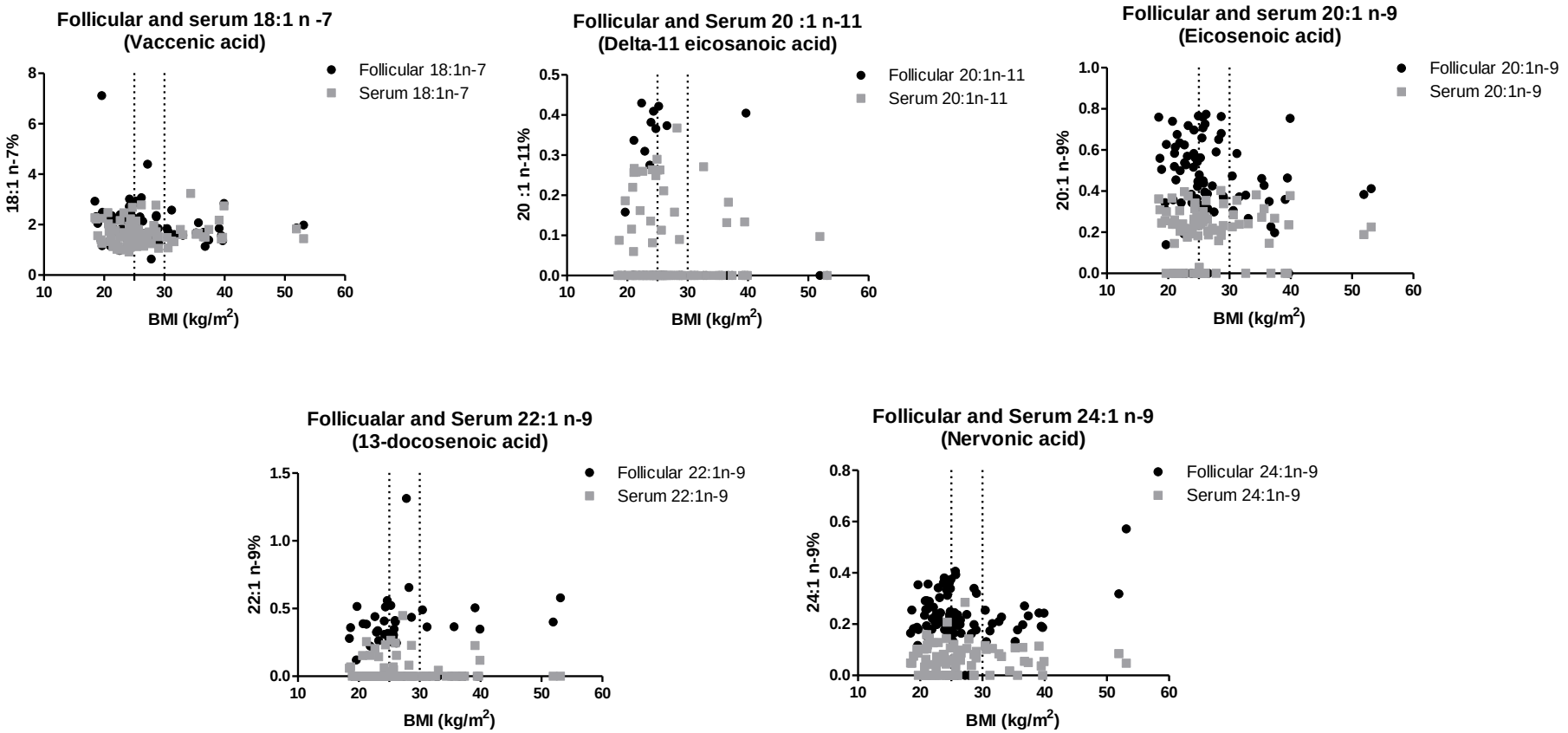

Figure A2.4 (cont.) The distribution of Follicular and Serum Monounsaturated fatty acid among individual patient according to the BMI

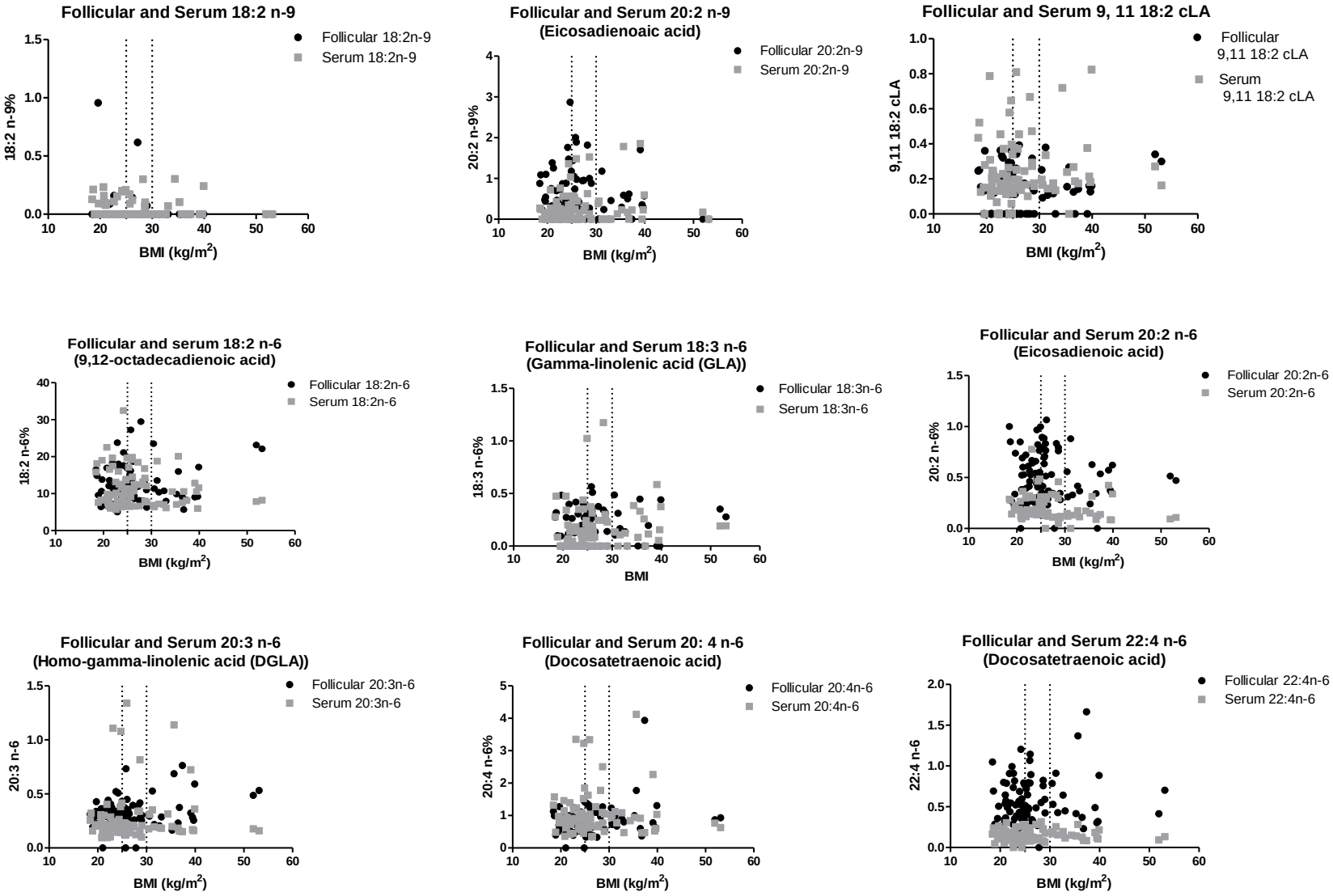

Figure A2.5 The distribution of Follicular and Serum polyunsaturated fatty acid among individual patient according to the BMI

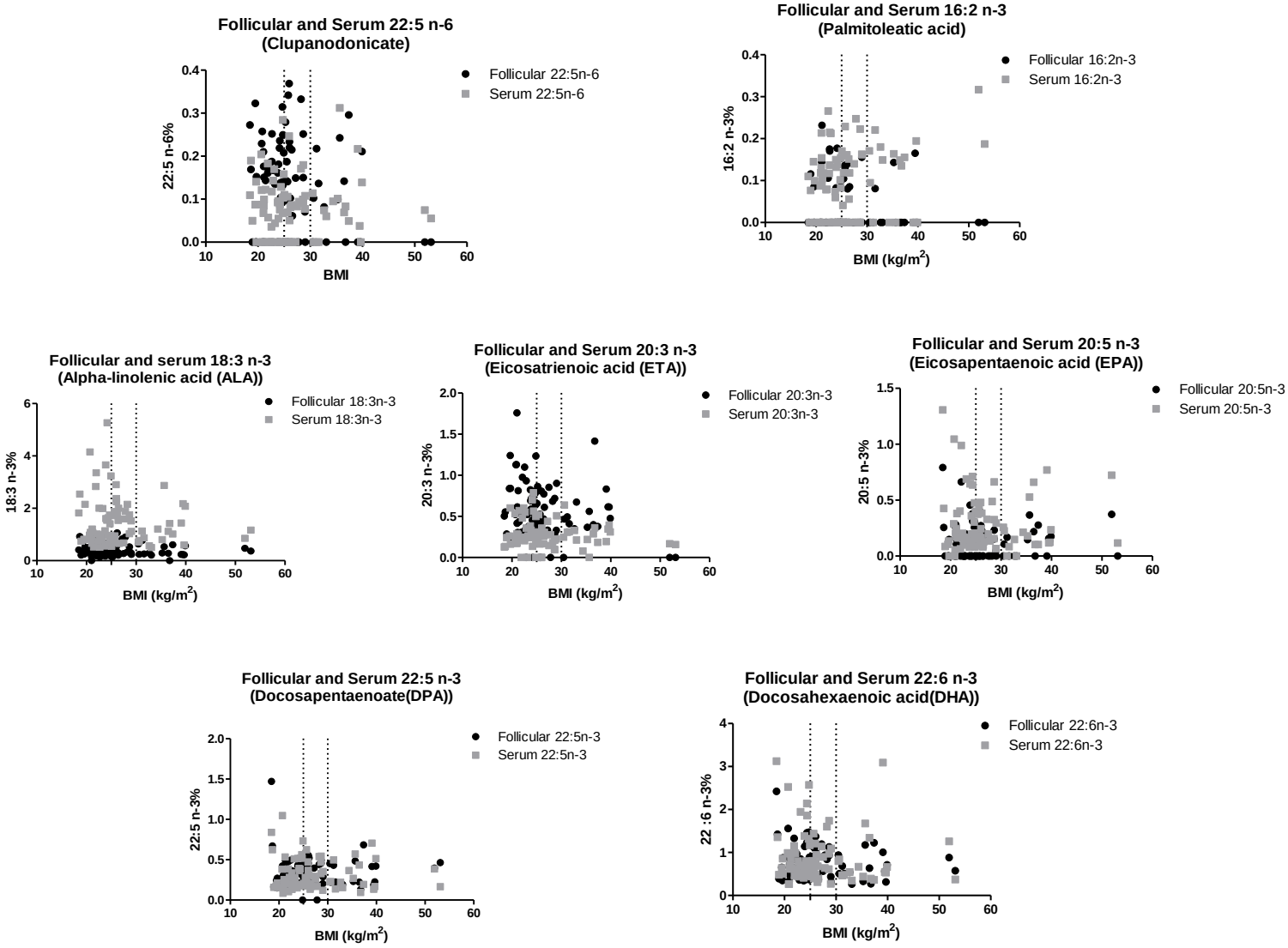

Figure A2.5 (cont.) The distribution of Follicular and Serum polyunsaturated fatty acid among individual patient according to the BMI

Appendix 3

The number of women who had undetectable levels of particular fatty acids among overall 84 women

Fatty acid	Serum (N)	Follicular fluid (N)
dma 16:0	66	76
dma 18:0	73	82
22:0 (Behenic acid)	71	75
trans 16:1	58	83
trans 18:2	80	83
19:1 (Nonadecanoic acid)	77	84
20:1 n-11 (Δ11 eicosanoate)	58	74
22:1 n-11	75	80
18:2 n-9	62	77
10,12 18:2 cLA	83	85
18:4 n-3 (Parinaric acid)	78	83