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Unit of Human Development and Health

Periconceptional Nutritional and Environmental Determinants of Human Embryo Health

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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PERICONCEPTIONAL NUTRITIONAL AND ENVIRONMENTAL DETERMINANTS OF HUMAN EMBRYO HEALTH

Alexandra Jayne Kermack

Introduction

Whilst undergoing artificial reproductive treatments, physicians and patients try a wide variety of approaches to improve the periconceptional environment of the developing gametes and embryos and hence the chances of success. Recently, a 'Mediterranean' diet, high in vegetable oils and fish, has been reported to increase pregnancy rates by up to 40%. In addition to parental dietary changes, keeping the embryos in a more stable environment (observing them via time lapse technology rather than removing them from their incubator to grade them) may improve their quality. However, up to now, few prospective randomized controlled trials have investigated the impact of periconceptional dietary interventions or the stability of the embryos' culture environment on fertility outcomes.

Methods

Two trials have been carried out to examine these hypotheses:

1. The PREPARE trial: A randomised double blinded controlled trial of 111 couples examining the effect of a six week dietary intervention of omega-3 fatty acids and vitamin D on morphokinetic markers of embryo quality.
2. The PROMOTE trial: A prospective and randomised trial comparing embryo development and metabolic activity of embryos cultured in the MINC (standard benchtop) versus the EmbryoScope (time lapse) incubators.

Results

There was a statistically significant increases in Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) in erythrocytes and in vitamin D in blood serum following the dietary intervention (all $p<0.001$). The development of embryos generated from the couples in the study group to the four cell stage (CC4) was accelerated compared to the control group ($p<0.001$). These embryos also demonstrated a significantly shortened S3 (meaning more synchronous cell division from the five to eight cell stage) ($p=0.031$). The fatty acid composition of follicular fluid was altered by the dietary intervention and correlations were made with the embryo quality. The short intervention did not have an effect on sperm quality.

Culturing embryos in the EmbryoScope significantly increased the number of blastocysts formed on day 5 (159 EmbryoScope versus 133 MINC; $P = 0.015$) and altered the carbohydrate utilisation and amino acid consumption and production of those embryos.

Conclusions

Improving a couple's preconceptional diet and providing a more stable environment for embryo culture improves markers of embryo quality. Further work is needed to examine whether this improvement causes an increase in pregnancy rates and to establish dosing and the longevity of a dietary intervention before clinical recommendations can be made.

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DECLARATION OF AUTHORSHIP

I, Alexandra Jayne Kermack declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Periconceptional Nutritional and Environmental Determinants of Human Embryo Health

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

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Signed: .....

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Definitions and Abbreviations

2PN	2 pronuclei embryo
AAP	American Academy of Paediatrics
ACOG	American College of Obstetrics and Gynaecology
ACTH	Adrenocorticotropin hormone
AFC	Antral follicle count
Ala	Alanine
ALA	Alpha-linoleic acid
AMH	Anti-müllerian hormone
ANOVA	Analysis of variance
Arg	Arginine
ART	Assisted Reproductive Technology
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
AZF	Azoospermic factor
BMI	Body mass index
BRC	Biomedical Research Centre
cAMP	Cyclic adenosine monophosphate
CBAVD	Congenital bilateral absence of the vas deferens
CC2	Time taken for the embryo to develop from two to three cells
CC3	Time taken for the embryo to develop from three to five cells
CC4	Time taken for the embryo to develop from five to nine cells
CEMACH	Confidential enquiry into maternal and child health
CRF	Case report form

CRH	Corticotropin releasing hormone
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EDTA	Ethylenediamine tetra-acetic acid
EmbryoScope	Validated time lapse incubator produced by Unisense, Fertilitech, Denmark
EPA	Eicosapentaenoic acid
ERGO	Ethics and Research Governance Online
ESHRE	European Society of Human Reproduction and Embryology
ETA	Eicosatetraenoic acid
ICMART	International Committee for Monitoring Assisted Reproductive Technology
ISRCTN	International Standard Randomised Controlled Trial Number
FA	Fatty acid
FAMEs	Fatty acid methyl esters
FID	Flame ionising detector
FSH	Follicle stimulating hormone
GC	Gas chromatography
GCP	Good Clinical Practice
GLA	Gamma-linoleic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GnRH	Gonadotropin releasing hormone
hCG	Human Chorionic Gonadotrophin
HFEA	Human Fertilisation and Embryology Authority

His	Histidine
HPLC	High pressure liquid chromatography
HSG	Hysterosalpingography
HyCoSy	Hysterosalpingo contrast sonography
ICM	Inner cell mass
IgG	Immunoglobulin G
Ile	Isoleucine
Infertility	A disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse
IRAS	Integrated Research Approval System
IUI	Intrauterine insemination
IVF	*In vitro* fertilisation
IVF-ICSI	*In vitro* fertilisation – intra-cytoplasmic sperm injection
KIDScore D3	Known Implantation Data Score of the embryo on day 3
KIDScore D5	Known Implantation Data Score of the embryo on day 5
LA	Linoleic acid
LDH	Lactate dehydrogenase
Leu	Leucine
LH	Luteinizing hormone
Lys	Lysine
MAIS	Mild androgen insensitivity syndrome
MAPK	Mitogen activated protein kinase pathway
MAR	Mixed antiglobulin reaction
Met	Methionine
MINC	Standard benchtop incubator produced by Cook Medical, Indiana, USA
MPF	M-phase promoting factor

MRC	Medical Research Council
MRI	Magnetic resonance imaging
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
OHSS	Ovarian hyperstimulation syndrome
OPA	o-Phthaldialdehyde
P-5-P	Pyridoxal-5-Phosphate
PBS	Phosphate buffered saline
PCA	Phosphino carboxylic acid
PCOS	Polycystic ovarian syndrome
PCT	Postcoital test
Phe	Phenylalanine
PID	Pelvic inflammatory disease
PREPARE	PREconception dietary suPplements in Assisted REproduction
PUFAs	Polyunsaturated fatty acids
R+D	Research and Development
RBC	Red blood cells
RCOG	Royal College of Obstetrics and Gynaecology
ROS	Reactive oxygen species
S2	Synchronicity of the cell cycle of the embryo between three and four cells
S3	Synchronicity of the cell cycle of the embryo between five and eight cells
Ser	Serine
SHBG	Sex hormone binding globulin

SLE	Supported liquid extraction
SPSS	Statistical Package for the Social Science
SST	Serum separator tubes
t2	Time at which the embryo contains two cells
t3	Time at which the embryo contains three cells
t4	Time at which the embryo contains four cells
t5	Time at which the embryo contains five cells
t6	Time at which the embryo contains six cells
t7	Time at which the embryo contains seven cells
t8	Time at which the embryo contains eight cells
t9	Time at which the embryo contains nine or more cells
tB	Time at which the embryo forms a blastocyst
TCA	Tricarboxylic acid
TE	Trophectoderm
tEB	Time at which blastocyst expansion occurs
Thr	Threonine
TLS	Time lapse system
tHB	Time at which the blastocyst begins hatching
tM	Time at which the embryo becomes a morula
tPN	Time at the appearance of the pronuclei in the embryo
tPNf	Time at the disappearance of the pronuclei in the embryo
Trp	Tryptophan
tSB	Time at which the embryo starts to form a blastocyst
Tyr	Tyrosine
Val	Valine
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Overview

Despite advancing technology in assisted reproductive techniques (ART), success rates over the last two decades have remained static. This places a high emotional, physical and financial burden on couples undergoing fertility treatment. Therefore, patients and their healthcare team are willing to try different methods, prior to treatment and in the periconceptional period, in order to try and improve outcomes. These include patient centred approaches such as acupuncture and use of nutritional supplements, and laboratory based advances, for example improvements in culture techniques and methods of assessing embryo quality.

There is a multi-million pound nutraceutical industry marketing food products and supplements to couples trying to become pregnant. Observational cohort studies have suggested links between preconception diet and fertility treatment outcomes (Vujkovic et al., 2010, Toledo et al., 2011). For example, a prospective observational study demonstrated an association between dietary intake of polyunsaturated fatty acids and improved embryo morphology (Hammiche et al., 2011). Data are also now emerging indicating that dietary vitamin D intake may be related to embryo and fetal development (Mnallah, 2017). Such observations from non-intervention studies have increased the uptake of omega 3 fatty acid (FA) and vitamin D supplements. However, up to now, few prospective randomized controlled trials have investigated the impact of periconceptional dietary interventions on fertility outcomes.

Nourishing the embryo in a stable environment has also been shown to be important. Time-lapse incubators allow embryos to be observed without removing them from the culture chamber. However, much of the research into their benefits has investigated both the more stable culture environment and the enhanced methods of embryo selection combined and their subsequent effect on pregnancy rates (Meseguer et al., 2012, Kahraman et al., 2012, Rubio et al., 2014). Studies separating these two factors, to understand the impact of the more stable culture environment alone remain scarce. Furthermore, our group has previously demonstrated that the metabolic activity measured by amino acid uptake is inversely correlated to embryo viability prior to embryo compaction and cavitation (Houghton et al., 2002). The use of pyruvate and glucose utilisation rates has also been proposed as a method of assessing embryo quality (Conaghan et al., 1993, Gardner et al., 2011). However, data from randomised controlled trials demonstrating clinical efficacy and the impact of timelapse incubators on embryo quality and metabolic activity are limited.

This thesis uses two randomised controlled trials to examine the effect of omega 3 fatty acids (FAs) and vitamin D supplementation and a more stable culture environment on the developing embryo.

1.2 Infertility

Infertility is defined by the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organisation (WHO) as 'a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse' (Zegers-Hochschild et al., 2009). Clinicians subdivide infertility into primary (diminished fertility throughout the reproductive years) and secondary (a failure to conceive after one or more successful pregnancies) (Mascarenhas et al., 2012). It is estimated that approximately nine per cent of couples worldwide suffer from infertility (Boivin et al., 2007). In the developed world, epidemiological studies have found this figure to vary between 3.5% in Australia (Webb and Holman, 1992) and 31.8% in Germany (Küppers-Chinnow and Karmaus, 1997). However, it should be noted that there is great variation in the definitions used to assess prevalence of infertility (Gurunath et al., 2011).

The National Institute for Health and Care Excellence (NICE) states that over 80% of couples in the general population will conceive within 1 year when having regular unprotected vaginal intercourse if the woman is aged under 40 years. Of those who do not conceive in the first year, a further 50% will conceive in the second year (cumulative pregnancy rate of 90%). NICE recommends that any woman who has been having unprotected intercourse and not conceived within one year of trying, in the absence of any known fertility problems, should be referred with her partner for clinical assessment and further investigations. Earlier referral should be made if the woman is aged over 36 years or if there is a known clinical cause for the infertility or a predisposing factor in the patient's history (NICE, 2013).

1.2.1 Causes and investigations of infertility

In most couples the causes of infertility are multifactorial but to ease assessment and decisions in treatment, they may be grouped into female factors (approximately 50%), male factors (20 – 26%) or unexplained factors (25 - 30%) (Evers, 2002).

Female factors can be further subdivided into disorders of ovulation, tuboperitoneal factors and cervical factors (see Figure 1.1).

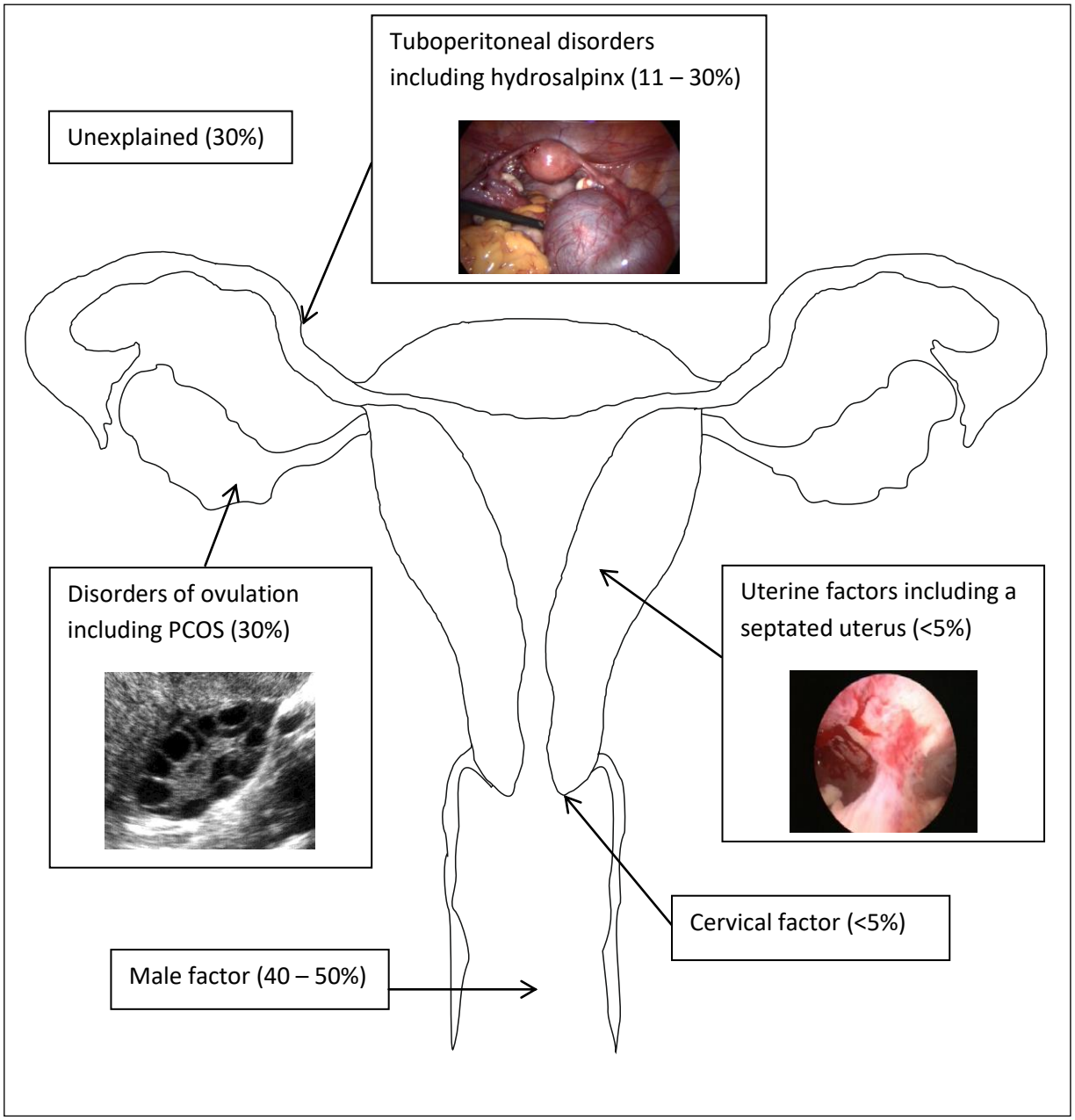


Figure 1.1: Illustration of the causes of infertility

1.2.1.1 Disorders of ovulation

Ovulation can be assessed using mid-luteal phase serum progesterone levels, with values of greater than 30 nmol/L indicating ovulation has taken place; by endometrial biopsy, the presence of secretory-phase endometrium is taken as evidence of ovulation; and by using ultrasonography to identify follicular growth. More than 95% of women who have regular menstrual cycles are shown to be ovulating using serum progesterone levels or endometrial biopsy results (Rosenfeld and Garcia, 1976). However, it should be noted that the only true evidence that ovulation is taking place is pregnancy.

The WHO subdivides women with ovulatory disorders into three distinct groups (Rowe, 1997):

Group I: hypothalamic pituitary failure (hypothalamic amenorrhoea and hypogonadotrophic hypogonadism).

Group II: hypothalamic-pituitary-ovarian dysfunction (predominantly polycystic ovarian syndrome).

Group III: ovarian failure.

1.2.1.1.1 Group I ovulatory disorders

Group I is caused by a lack of gonadotropin-releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) which usually stimulate the ovaries to produce and release an oocyte. Hypogonadotropic hypogonadism may be primary or secondary. Primary is Kallmann's syndrome, a genetic condition usually caused by the KAL-1 mutation, which leads to an impairment of the hypothalamus to produce and release GnRH. Secondary or acquired hypogonadotropic hypogonadism is more common and may be caused by damage to the pituitary gland such as in Sheehan's syndrome (very rare) or pituitary tumours e.g. microadenomas (prolactin secreting tumours). Other causes include haemochromatosis and high doses or long term use of steroids or opioids.

Functional hypothalamic dysfunction (which accounts for a third of cases of secondary amenorrhoea (Medicine, 2006)) may be triggered by excessive weight loss such as in anorexia nervosa, exercise, or stress. The pathophysiology of this is complex and poorly understood; the GnRH pulsatile secretion (that is impeded in this condition) is controlled by numerous neuropeptides, neurotransmitters and neurosteroids. These include corticotropin releasing

hormone (CRH), beta-endorphin, leptin, Kisspeptin, neuropeptide Y, and ghrelin (Meczekalski et al., 2014).

Studies have demonstrated elevated levels of CRH (Kaye et al., 1987) and/or cortisol (Brundu et al., 2006) in the cerebrospinal fluid of patients with anorexia nervosa. CRH stimulates the pituitary to release adrenocorticotropin hormone (ACTH) and other hormones such as beta-endorphin (Rivier et al., 1982). This results in an increase in glucocorticosteroids which inhibit GnRH and gonadotrophin release. Furthermore, there may also be an alteration in the corticosteroid receptors expressed in the hippocampus, with increased mineralocorticoid as opposed to glucocorticoid receptors in times of chronic stress (Sapolsky et al., 1984); this may also alter the hypothalamic GnRH response to hypoestrogenism (Whirledge and Cidlowski, 2010).

Leptin is a peptide hormone produced by adipocytes (and therefore women with anorexia nervosa produce low amounts) and it is well recognised that a deficiency results in hypogonadtrophic hypogonadism in humans (Clement et al., 1998). However, GnRH neurons lack receptors to leptin and therefore it has been hypothesised that Kisspeptin and its G protein-coupled receptor, GPR54 may be an intermediary between leptin and GnRH function (Smith et al., 2006). Exogenous Kisspeptin administered to women with functional hypothalamic dysfunction has been shown to stimulate gonadotropin release (Sills and Walsh, 2008).

Neuropeptide Y stimulates appetite and neurons expressing this are in close proximity to Kisspeptin neurons in the arcuate nucleus of the hypothalamus (Backholer et al., 2010). Neuropeptide Y stimulates GnRH release under certain conditions (normal levels of circulating sex hormones); however, an inhibitory effect is observed in hypoestrogenism (i.e. women with hypothalamic dysfunction) (Kalra and Crowley, 1992).

Ghrelin also stimulates appetite but suppresses fat utilisation, and therefore levels are increased in women with anorexia nervosa (Ogiso et al., 2011). Gherelin inhibits the hypothalamic-pituitary-gonadal axis and prolongs amenorrhoea even once leptin has returned to normal following weight gain (Schneider and Warren, 2006).

1.2.1.1.2 Group II ovulatory disorders

Research has demonstrated that approximately 30% of women who present with secondary amenorrhoea have normal levels of FSH and LH (Baird et al., 1977). Group II ovulatory disorders are normogonadotrophic anovulatory syndromes presenting with a lack of menses or more

commonly irregularities in the menstrual cycle. Most (91%) of these women meet the criteria for polycystic ovarian syndrome (PCOS) (i.e. morphological ovarian characteristics on ultrasound plus a clinical feature) (Broekmans et al., 2006). In these women, follicular development and hence oestrogen production occur but arrest prior to full maturation. The unopposed oestrogen production (because of the lack of corpus luteum producing progesterone) leads to a hyperplastic endometrium.

The pathophysiology of PCOS is thought to be primarily due to the thickened thecal layer within polycystic ovaries that results in excessive androgen secretion from the thecal cells either under basal conditions or in response to LH (Gilling-Smith et al., 1994). The hyperandogenism and excess follicles result in an increase in anti-müllerian hormone (AMH) which in turn inhibit the FSH induced action of aromatase in the granulosa cells and therefore oestradiol synthesis which is required for follicular maturation (Jonard and Dewailly, 2004). This is further exacerbated by hyperinsulinaemia which causes premature advancement of granulosa cell differentiation (Jonard and Dewailly, 2004), increases the 17α hydroxylase activity within the thecal cells (Rosenfield et al., 1990) and decreases the hepatic production of sex hormone binding globulin (SHBG) therefore increasing free testosterone (Plymate et al., 1990).

PCOS is a familial condition which strongly supports the role of genetic factors in the development of the condition. However, differences in phenotypic features between and within families demonstrate the importance of environmental factors. A number of genes have been implicated as contributing to PCOS, including 7β-hydroxysteroid-dehydrogenase type 6, but the genetics of PCOS are complex and involve mutations leading to alterations in the molecular structures of gonadotrophins, their receptors and the enzymes involved in steroidogenesis (Prapas et al., 2009). In addition, exposure to hyperandrogenism and hyperinsulinaemia in utero is thought to impair oestrogen and progesterone inhibition of GnRH, resulting in the increased pulse frequency and hence PCOS (Blank et al., 2007). It appears probable that the increase in GnRH pulsatility is intrinsic rather than as a result of the reduction in serum progesterone (which follows ovulation) (Daniels and Berga, 1997). Factors such as obesity and a sedentary lifestyle also play a role by leading to metabolic dysfunction; increased glucose intolerance and hyperinsulinaemia (Després and Lemieux, 2006).

1.2.1.1.3 Group III ovulatory disorders

Group III (ovarian failure or insufficiency) can be congenital or acquired. Turner's syndrome, a congenital cause, may result in a failure of ovarian development and reduced number of

primordial follicles before birth leading to primary amenorrhoea and raised LH and FSH (hypergonadotrophic hypogonadism). Acquired causes include damage to ovarian tissue from endometriosis, ovarian surgery and chemo or radiotherapy.

1.2.1.2 Tuboperitoneal factors

It is known that the cause of infertility is tuboperitoneal disease in between 11 and 30 percent of couples (Evers, 2002). The commonest cause of tubal damage is pelvic inflammatory disease (PID) caused by *Chlamydia trachomatis* infection; other causes include endometriosis and adhesions following pelvic inflammation or surgery (for example in appendicitis or peritonitis).

Tubal patency is evaluated using one of three methods; hysterosalpingography (HSG), hysterosalpingo contrast sonography (HyCoSy), or laparoscopy with dye insufflation. There are advantages and disadvantages to each of these investigations and these should be considered before deciding on the best test for the patient. In recent years serological studies for *Chlamydia trachomatis* as raised Immunoglobulin G (IgG) antibody titres are a consistent finding in women with infertility associated with tubal occlusion; however, more studies are required to establish the utility of this as a screening tool.

1.2.1.3 Uterine factors

Anatomical abnormalities of the uterus can also be implicated as a cause for infertility (and early pregnancy loss); these include congenital abnormalities (unicornuate, bicornuate or septated), abnormalities following diethylstilbestrol exposure in utero (T-shaped), uterine fibroids or polyps, adenomyosis and Ashermann's syndrome. They can be investigated by HSG, by filling the cavity with saline during a transvaginal ultrasound or by hysteroscopy. Magnetic resonance imaging (MRI) may also be used to clarify the abnormality further, but it may not be necessary if a clear diagnosis has already been achieved.

1.2.1.4 Cervical factors

Cervical factors include both abnormal cervical anatomy and cervical hostility (the existence of which is debated by fertility experts).

Cervical stenosis is the most common anatomical abnormality associated with infertility, it may be congenital or, more commonly, acquired. Acquired causes include diathermy excision of cervical lesions or other cervical surgical procedure such as a cone biopsy for cervical

intraepithelial neoplasia, radiotherapy, infection or endometriosis. This is normally discovered whilst trying to pass a dilator through the cervical canal during another procedure e.g. a HyCoSy.

Cervical hostility can be investigated using a postcoital test (PCT); however the application of this test has been intensely debated. A positive result in this test implies that well timed intercourse has taken place with receptive cervical mucus and adequate semen quality. However, a review of this assessment was critical; it was concluded that the test lacked the ability to determine fertility status and the method for performing the test was not standardized (Griffith and Grimes, 1990). Due to these reasons, the use of the PCT is largely historical and it is no longer routinely offered as part of fertility investigations.

1.2.1.5 Male factor infertility

Male factor infertility is primarily investigated by semen analysis. Semen is collected in a sterile container following mastubation after a period of abstinence of between 3 and 5 days. In 2010, the WHO published new semen analysis reference values. These criteria state that the ejaculated volume should be greater than 1.5 ml with a sperm concentration of greater than 15 million per ml, a progressive motility of 32% and normal morphology of 4% (Cooper et al., 2010). Figure 1.2 demonstrates some of the abnormal morphology that may be observed in spermatozoa.

The cause of a decreased sperm quality is unknown in approximately 50% of cases; causes may be pre-testicular, testicular or post-testicular and should be investigated thoroughly. Hypogonadotrophic hypogonadism may be secondary to congenital genetic anomalies such as Kallmann syndrome (failure to produce GnRH) or mutations in genes involved in the hypothalamic-pituitary-gonadal axis (for example mutations of G-protein coupled receptor GPR54 and its ligand Kisspeptin) (Huhtaniemi and Alevizaki, 2007). Other causes of hypogonadotrophic hypogonadism may be acquired and include pituitary adenomas and meningiomas or anabolic steroid abuse (Silveira and Latronico, 2013). Other pre-testicular causes of male factor infertility are structural and include erectile dysfunction and retrograde ejaculation. Retrograde ejaculation is ejaculation into the bladder and may be found in patients with diabetes, MS and spinal cord injury (Jefferys et al., 2011).

Testicular dysfunction can also be subdivided into congenital and acquired pathologies. Genetic anomalies such as Klinefelter syndrome (karyotype XXY), Y chromosome microdeletions (azoospermic factor (AZF) or *gr/gr* deletions), and mild androgen insensitivity syndrome ((MAIS) or mutations of the androgen receptor gene) (Krausz, 2011) may result in decreased sperm

production. Congenital structural defects include anorchia (lack of testes) or cryptorchidism (testes not descended into the scrotal sac) (Lambert et al., 2010). However, more commonly the cause is acquired secondary to testicular trauma, orchitis, a varicocoele or chemo or radiotherapy.

Congenital absence of the vas deferens (either as a feature of cystic fibrosis or congenital bilateral absence of the vas deferens (CBAVD)) is a post testicular cause of male infertility (Mercier et al., 1995). Others include infection and inflammation of the epididymis or accessory glands (seminal vesicles, prostate gland and the bulbourethral glands). In addition to obstructive structural damage caused by infection, leukocytes increase the reactive oxygen species which has a negative effect on sperm motility and results in the spermatozoa having a reduced ability to fertilise an oocyte (Krausz et al., 1994).

Secondary investigations should be based on the semen analysis and clinical suspicion of the pathophysiology; they may include serum FSH, LH, testosterone and SHBG, genetic testing, urethral swab, microbiological examination of semen and urine and a scrotal ultrasound.

However, these semen analysis parameters do not address sperm function and they have limited associations with artificial reproductive techniques (ART) outcomes. In recent years, sperm deoxyribonucleic acid (DNA) damage testing is thought to be a useful adjunct (Lewis et al., 2013). It has been closely associated with numerous indicators of reproductive health, including fertilisation (Simon et al., 2014), implantation, spontaneous abortion and childhood diseases (Simon et al., 2013). However, there remain controversies; a systematic review by Li et al. demonstrated no correlation between sperm DNA damage and fertilisation rates but did demonstrate a decrease in clinical pregnancy rate following IVF (Li et al., 2006). Furthermore, 8% of men with normal semen parameters have been shown to have sperm DNA damage (Zini et al., 2001) and it is not yet known what level of sperm DNA damage can be regarded as normal and how best to treat abnormal results (Schulte et al., 2010).

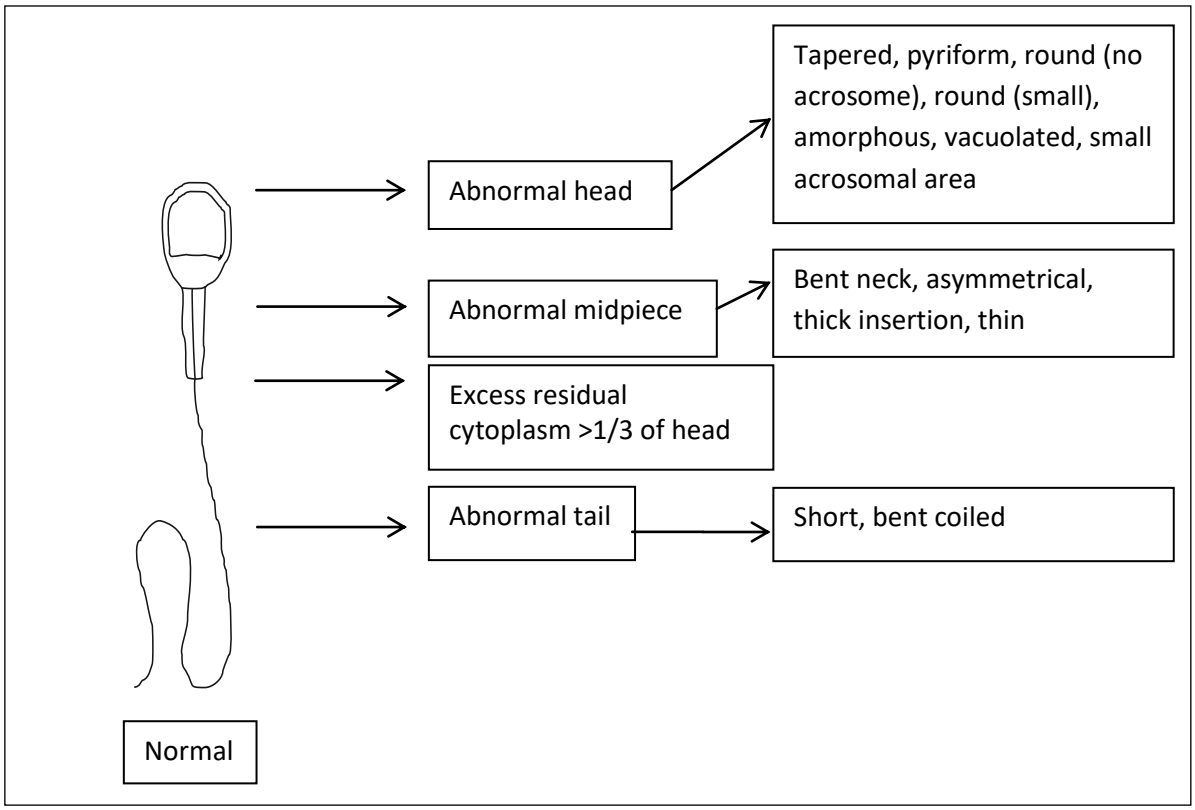


Figure 1.2: Sperm morphology and types of abnormality

1.2.1.6 Unexplained infertility

Despite thorough investigation, in approximately one third of couples no cause for their infertility is found; this figure has decreased as more diagnostic tests have become available. In addition, the chance of pregnancy decreases as the duration of a patient's unexplained infertility increases; to 40% after three years and then 20% after five years (Collins and Rowe, 1989). Interestingly, a trial which carried out fertility investigations on couples with normal fertility found that in two thirds of them at least one of the tests was found to be abnormal. This implies that abnormal fertility results cannot always be substantiated as the cause of the couple's difficulties conceiving. The extent to which unexplained infertility is contributed to by modifiable lifestyle factors such as suboptimal nutrition, increased alcohol or caffeine intake or stress is not known. Further research into this area is required so that accurate preconception advice can be given to all couples.

1.2.2 Treatment of infertility

The treatment of infertility depends on the cause and the number of couples seeking reproductive assistance has increased greatly over the last twenty years as treatments become more acceptable and accessible. This is due in part to an increase in sexual transmitted infections such as *Chlamydia trachomatis* causing tubal occlusion and a shift in social pressures meaning that women are often older when they decide to start a family. The Office for National Statistics in the UK showed that the number of live births in women over 40 years more than quadrupled between 1981 (6,860) and 2015 (29,241) (Statistics, 2016). The association between increasing maternal age and decreasing conception rates is well established. Without recourse to ART, a female aged 30 years has a 75% chance of conceiving within one year, and by age 40 years this has decreased to 44% (Leridon, 2004).

There are a number of different methods used to attempt to overcome infertility. Disorders of ovulation can be treated with pulsatile GnRH with or without a human chorionic gonadotrophin (hCG) trigger (for group I) or anti-oestrogens such as Clomiphene Citrate, a selective oestrogen receptor modulator, or Letrozole, an aromatase inhibitor (for group II).

In addition, for women with PCOS, weight loss and ovarian diathermy or Metformin may also be recommended. A recent study examining whether a delay in fertility treatment (Clomiphene Citrate) would be beneficial for patients with PCOS whilst they underwent a weight loss programme demonstrated that there was an improved chance of ovulation and live birth rate in those who postponed in order to carry out the lifestyle modifications (Legro et al., 2016). Interestingly, in 2015 there was a cohort study that demonstrated an inverse association between PCOS and eating a Mediterranean style diet; it is thought that this demonstrates an improvement in eating patterns once a woman is diagnosed with PCOS but recommends that further investigation is needed to examine specific dietary modifications that may be useful to this group of women (Moran et al., 2015).

Women with tubal occlusions are often ovulating and therefore ovulation induction is not required. The treatments for this specific group are either open microsurgery to unblock the tubes or *in vitro* fertilisation (IVF) (with or without a salpingectomy) in order to overcome the problem. The risk of ectopic pregnancy in women undergoing this type of surgery is high (although comparable with women undergoing IVF as a treatment for tubal occlusion) (Schippert et al., 2012) and clinicians should have a low threshold for investigation in the early stages of pregnancy. Surgery and laparoscopic excision is a treatment option for patients with

endometriosis and it is thought to improve both rates of natural conception and IVF outcomes (Abbott et al., 2003). Uterine factors may also be treated with surgery including polypectomies and/or myomectomies as appropriate (Varasteh et al., 1999).

Cervical factors and unexplained infertility can be treated using intrauterine insemination (IUI), it is considered less physically and emotionally demanding than IVF and in the UK has an 11% pregnancy rate per cycle in women aged under 35 years (HFEA, 2008). Success rates are higher if ovarian stimulation is used in conjunction with IUI (rising to 13%) and in couples with a short duration of unexplained infertility, the male of whom has a high percentage of progressively motile spermatozoa (Tomlinson et al., 1996). IUI may also be used in cases of mild or moderate endometriosis or following oral treatment methods in disorders of ovulation such as PCOS.

The next line of treatment for all couples experiencing infertility or for those in whom the cause is male factor, is IVF or *in vitro* fertilisation with intra-cytoplasmic sperm injection (IVF-ICSI), respectively. IVF may also be used as a first treatment approach in women who are older and therefore have less time to try less invasive treatments. According to the Human Fertilisation and Embryology Authority (HFEA), in 1991, 6,146 women in the UK underwent IVF (HFEA, 2008), and by 2014 this had increased to 51,258 (with a total of 67,708 cycles) (HFEA, 2008). Parallel with this rising demand, the field has seen significant developments in hormone treatments and laboratory techniques, including embryo assessment, and while this has impacted on outcomes, ongoing pregnancy rates per started cycle of treatment remain at approximately 25% (Dobson, 2002).

An important component of the IVF process is ovarian stimulation, the goal of which is to facilitate the ongoing development of multiple follicles and allow the retrieval of multiple mature oocytes. Traditionally, ovarian stimulation is performed using a long protocol which involves initial pituitary suppression using a GnRH agonist leading to reduced endogenous FSH and LH and hence inhibition of ovarian steroidogenesis, follicle production and premature ovulation. The GnRH agonist is usually started in the woman's mid luteal phase (day 21) of her cycle, there is an initial increase in the production of FSH and LH by the pituitary once the GnRH agonist is administered. Starting it later in the cycle decreases the chance of functional follicular cysts forming and hence decreases the chance of the IVF cycle being delayed or cancelled. Once down regulation has been achieved, daily exogenous FSH is administered usually until 3 or more follicles are greater than 18 mm in diameter; hCG is then administered and 36 hours later oocyte retrieval occurs. This method is expensive and women often suffer with significant side effects

such as headaches, hot flushes, depression and night sweats. However, it is still commonly used in women who have multiple cysts prior to starting IVF or those suffering with endometriosis.

It is becoming more common for clinics to offer a milder stimulation regime to patients in order to minimise side effects, reduce the risk of ovarian hyperstimulation syndrome (OHSS) and reduce costs. In mild stimulation IVF, the woman's endogenous FSH is not suppressed but utilised with lower doses of exogenous FSH to encourage multiple dominant follicle formation. GnRH antagonists are started on approximately day 6 of stimulation in order to prevent an LH surge and premature ovulation. HCG is administered 36 hours before oocyte retrieval. Studies have demonstrated that fewer oocytes are retrieved using the milder stimulation regime; however improved implantation rates are achieved with fewer embryos and therefore concern about the number of oocytes retrieved using this protocol may be unjustified (Verberg et al., 2009).

In addition to using either the long protocol or the shorter milder protocol, different drugs can be used for ovarian stimulation. The majority of women are prescribed recombinant FSH, but in cases where previous response to this has been poor or in women with hypogonadotropic hypogonadism, human menopausal gonadotrophin, which contains urinary derived FSH and LH activity may be used. Different ovulation triggers can also be used, depending on the number of follicles and levels of oestradiol. In the majority of cases, hCG is used, however in cases where the woman is at risk from OHSS (i.e. more than 18 follicles greater than 14 mm diameter at the time the trigger is prescribed and/or an oestradiol level of greater than 18000 pmol/L) then a GnRH agonist can, in certain circumstances, be used as the trigger. GnRh agonists cause the pituitary to release an FSH and LH surge, which allows oocyte maturation but since the duration of the surge is much shorter than that mimicked by hCG, it is associated with a reduction in the risk of OHSS compared with hCG triggering. If a GnRH agonist is used as a trigger then all embryos may be cryopreserved; this is because, although the risk of OHSS is decreased, it is not completely negated and pregnancy could cause the syndrome to worsen.

1.2.2.1 The developing embryo and markers to predict viability

As previously stated, patients are willing to try a wide variety of approaches to improve their chances of success in IVF; however, one of the great challenges is the difficulty in determining an embryo's viability. Since the invention of ART, scientists have investigated a number of methods to try to determine which embryo is most likely to implant and result in a live birth. These approaches have included examining the embryos morphologically at specified time points,

looking at morphokinetic markers of individual embryos and analysing an embryo's uptake of key nutrients.

1.2.2.1.1 Morphological and morphokinetic markers

The most frequently used method of evaluating human embryos in IVF is by ocular assessment of their morphology. This is performed by the embryology team at various time points during embryo culture. Since the development of the EmbryoScope, morphokinetic markers have been used to try to predict embryo viability. The EmbryoScope allows the development of an embryo to be observed throughout culture as opposed to it being graded at specified time points, thus giving the embryology team more information to make a decision about which embryo to transfer.

In IVF, the spermatozoa must undergo capacitiation, which allows them to bind to and penetrate the oocyte. This process occurs at 37°C and takes approximately 6 hours in humans. During capacitation, fluctuations of calcium and bicarbonate lead to the activation of intracellular signalling pathways which result in protein tyrosine phosphorylation (this change in the proteins leads to either activation or inactivation). There is removal of the seminal proteins from the surface of the spermatozoon, alteration of glycoproteins on the sperm plasma membrane and an efflux of cholesterol resulting in an increase in the membrane fluidity. Reactive oxygen species (ROS) such as hydrogen peroxide and nitric oxide are involved in the regulation of protein tyrosine phosphorylation and therefore capacitation. Lower concentration of ROS may activate cyclic adenosine monophosphate (cAMP) pathways, within one of the cell signalling pathways, leading to increased protein tyrosine phosphorylation. However, higher levels of ROS may bind with molecules such as nicotinamide adenine dinucleotide (NADH) (Rivlin et al., 2004) and lactate dehydrogenase (LDH) (Duan and Goldberg, 2003) which are known to promote capacitation. Furthermore, high levels of ROS may lead to peroxidation of the polyunsaturated fatty acids (PUFAs) in the cell membrane leading to a loss of cell membrane integrity and fluidity and therefore cell dysfunction (Aitken et al., 1993). Docosahexaenoic acid (DHA) is found in high levels in spermatozoa and seminal plasma and is known to play an important role in the regulation of the fluidity of the cell surface membrane (Connor et al., 1998) and as an antioxidant (Martinez-Soto et al., 2016). Following capacitation, the spermatozoon has hyperactive mobility and zona pellucida receptors to enable oocyte binding (Naz and Rajesh, 2004).

Once the capacitated spermatozoon has bound with the zona pellucida, the acrosome reaction can occur. In humans, this is a relatively rapid process taking between 2 and 15 minutes. The acrosome is produced in the Golgi system of the spermatid; a number of small proacrosomal vesicles are produced by fusion with several smaller vesicles, one grows in size and migrates towards the nucleus forming the acrosome. Research in mice has demonstrated that DHA is essential for the proacrosomal vesicle fusion and therefore the acrosome reaction (Roqueta-Rivera et al., 2011). Attachment to the zona pellucida results in activation of calcium channels (O'Toole et al., 2000) and a release of intracellular calcium (Florman et al., 1998), which activates cAMP and phosphokinase A pathways. Vitamin D has been shown to be important in this process due to its role in calcium homeostasis (Krasznai et al., 2006). The acrosomal membrane then fuses with the sperm head and releases lysins, under the regulation of Protein C inhibitor (Laurell et al., 1992). The main lysin is proacrosin, which is quickly converted into its active form β-acrosin which lyses the zona pellucida; the cell membrane of the spermatozoon and the oocyte are then able to fuse. Once sperm penetration has occurred, the zona pellucida undergoes modification to change it into a protective outer layer for the developing embryo.

A process similar to sperm capacitation must occur in the oocyte to ensure it is developmentally mature for successful fertilisation to occur. Following the mid-cycle LH surge or hCG administration in IVF, the oocyte resumes meiosis and progresses to metaphase II. A decrease in cAMP results in the activation of M-phase promoting factor (MPF) (Jones, 2004), comprised of cyclin-dependent kinase 1 and cyclin B1. Homologous chromosomes converge on the spindle equator and extrusion of the first polar body occurs. Following this the transition from meiosis I to meiosis II occurs under the regulation of c-mos activity and the mitogen activated protein kinase pathway (MAPK) (Verlhac et al., 2000). This leads to migration of the spindle to the cortex and hence preparation for an asymmetric cell division. The second meiotic division is then arrested at metaphase II, but the mechanism causing this is poorly understood (Jones, 2004).

Following these processes, fusion between the postacrosomal region of the sperm head plasma membrane and the oocyte membrane occur. This happens in two steps: Firstly, adhesion molecules bring the membranes in close apposition and secondly the membranes fuse (Anifandis et al., 2014). A number of molecules have been identified as having a crucial role in this process including fertilin α, fertilin β and cyritestin. Once this fusion has occurred, the spermatozoon's tail stops beating immediately and the sperm is drawn into the oocyte by elongation. A massive influx of sodium ions enter the oocyte causing depolarisation, this

alteration prevents polyspermy in those initial moments (Georgadaki et al., 2016). Subsequently, calcium is released from the endoplasmic reticulum which propagates the cortical reaction; the release of cortical granules from the oocyte to establish a permanent barrier to further sperm entry and polyspermy (Georgadaki et al., 2016). Interestingly, calcium release from the endoplasmic reticulum is dependent on the quality and quantity of mitochondria. Work in mice has demonstrated an decrease in the distribution of mitochondria in the outer region of the oocyte in mice fed a diet enriched with omega 3 FAs (Wakefield et al., 2008). Furthermore, this was associated with decrease oocyte competence and fertilisation rates, possibly due to a decreased or inadequate cortical reaction, as well as decreased energy production in this area (Wakefield et al., 2008). Finally meiosis resumes and there is extrusion of the second polar body.

Following this process, the two pronuclei appear; these contain the genetic material from the spermatozoa and the oocyte. They migrate towards one another and following the disappearance of their membranes, the chromosomes within them combine to form the single zygote nucleus. The embryo is assessed between 16 and 18 hours following fertilisation; abnormal fertilisation is characterised by more or less than two pronuclei and these embryos can be discarded at this point.

The zygote then undergoes mitotic divisions to two cells, four cells and eight cells at approximately 27.9, 40.7 and 59.1 hours post fertilisation (Herrero et al., 2013). This mitosis does not contain growth stages like cell division in adult cells but simply a synthesis or S phase (during which DNA replication occurs), followed by mitosis (the separation of the chromosomes) and then cytokinesis (separation of the cell components). This means that the resulting cells (or blastomeres) have a reduced cytoplasmic volume with each cell division. However, the cell membrane requirements increase rapidly with the dividing embryo; as the embryo divides to from one to four cells there is a 74% increase in membrane surface area (Pratt and George, 1989). This requires the uptake of omega-3 FAs which are an essential part of the cell membrane. Furthermore, the embryo requires energy and utilises both pyruvate and carbohydrate (glucose) to produce adenosine triphosphate (ATP). However, β oxidation of FAs is also an important energy source providing significantly more ATP per molecule than glucose (Sturmey et al., 2009a).

Embryologists grade these early embryos by recording the number of cells present and then scoring (between one and four) depending on the amount of fragmentation, degree of regularity in the size of the blastomeres and the presence or absence of multinucleated cells. By day 2

post fertilisation, it is expected that the embryo has at least two cells, preferably 4 and by day 3, the embryo should contain at least six cells, preferably eight. Embryos with a higher cell number, minimal fragmentation, similar sized blastomeres and no multinucleation have a higher chance of implantation. The morphokinetic markers at this stage that have been proposed as important in the embryo's development include the appearance and disappearance of the pronuclei, time to two, three, four, five, six, seven, eight and nine cells (t2 – t9 respectively). Cell cycle times between two and three cells, three and five cells and five and nine cells (CC2, CC3 and CC4) and the synchronicity of the cell cycles between three and four cells and five and eight cells (S2 and S3) (see Figure 1.3) can also be used in conjunction with the KIDScore D3 (Known Implantation Data Score) algorithm to predict viability and the likelihood of implantation.

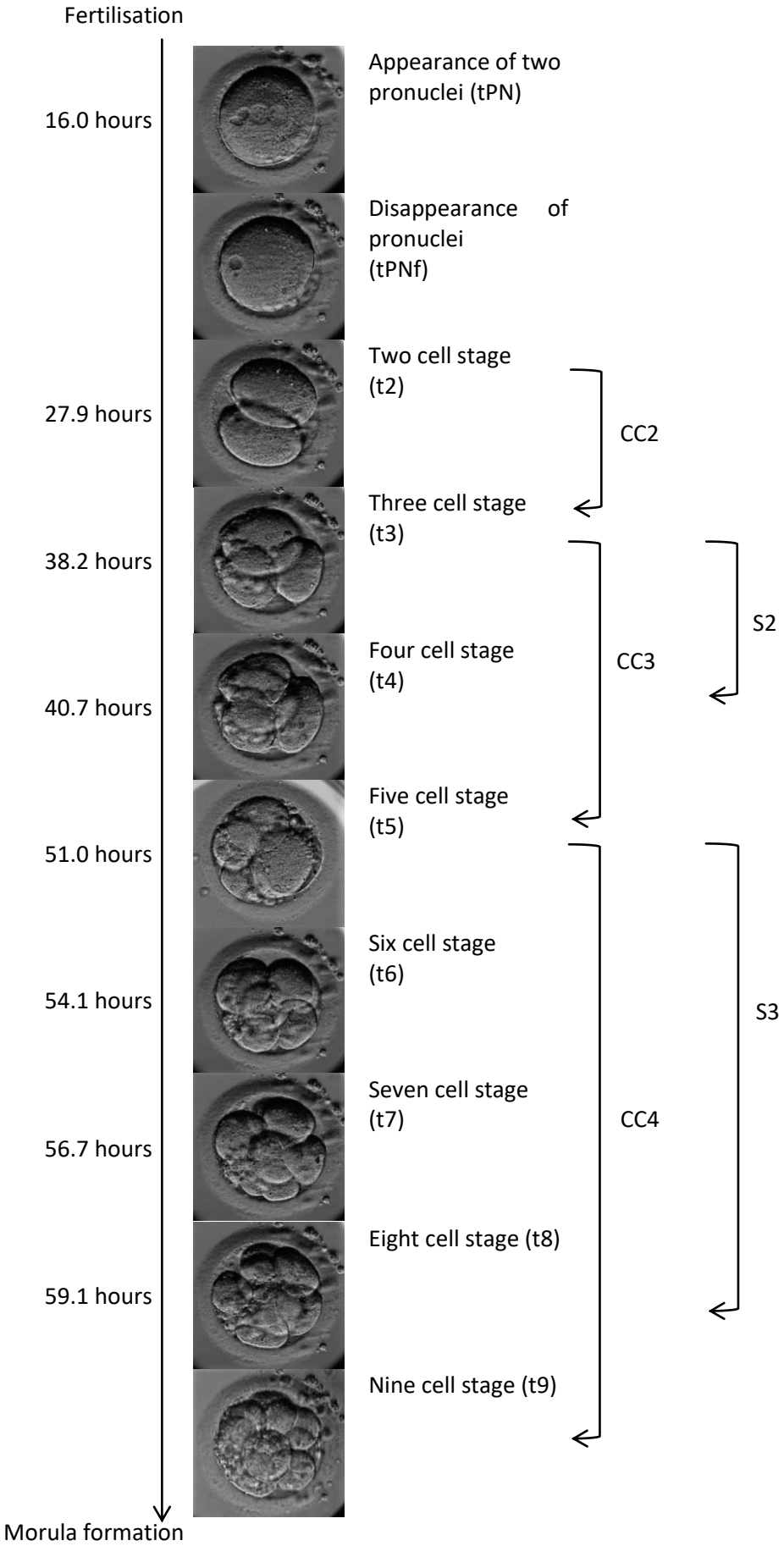


Figure 1.3: The development of the embryo from the point of fertilisation of the oocyte to morula formation (timings taken from averages from the general population (Herrero et al., 2013)) (images taken from PREPARE patient 157).

Once the embryo reaches the eight or sixteen cell stage, it starts to compact within the zona pellucida; tight junctions form between the external blastomeres resulting in a sealed sphere. Between the internal blastomeres gap junctions form allowing the movement of molecules and ions between the cells. There is evidence from other tissues that increased DHA content in the cell membrane may increase the gap junction capacity (Champeil-Potokar et al., 2007) which is essential for embryo development; cells with poor gap junction communication with the embryo are excluded (Hardy et al., 1996). At approximately 86.6 hours post-fertilisation, the embryo contains in the region of 30 cells and is termed a morula. The external cells will give rise to the trophectoderm and the internal cells to the inner cell mass once the blastocyst is formed.

The outer blastomeres start to express sodium transporters; the movement of sodium then creates an osmotic effect and causes a blastocoel cavity to form. By day 5 (120 hours post-fertilisation, on average 104.1 hours) the human embryo should be at the blastocyst stage of development. A human blastocyst consists of between 80 and 160 cells and comprises of an inner cell mass (ICM) (approximately 30% of cells) and a trophectoderm (TE). Finally, the blastocyst starts to hatch out of the zona pellucida. The most common method of scoring blastocysts was developed by David Gardner and William Schoolcraft in 1999; blastocysts are scored according to their stage of development, the quality of the trophectoderm and the quality of the inner cell mass.

The morphokinetic markers measured at this stage of development include time to the morula stage (tM), the start of blastocyst formation (tSB), the point at which the blastocyst is formed (tB), blastocyst expansion (tEB) and blastocyst hatching (tHB). Day 5 algorithms, such as the KIDScore D5 (as yet unpublished), may also be used to assess the likelihood of blastocyst implantation.

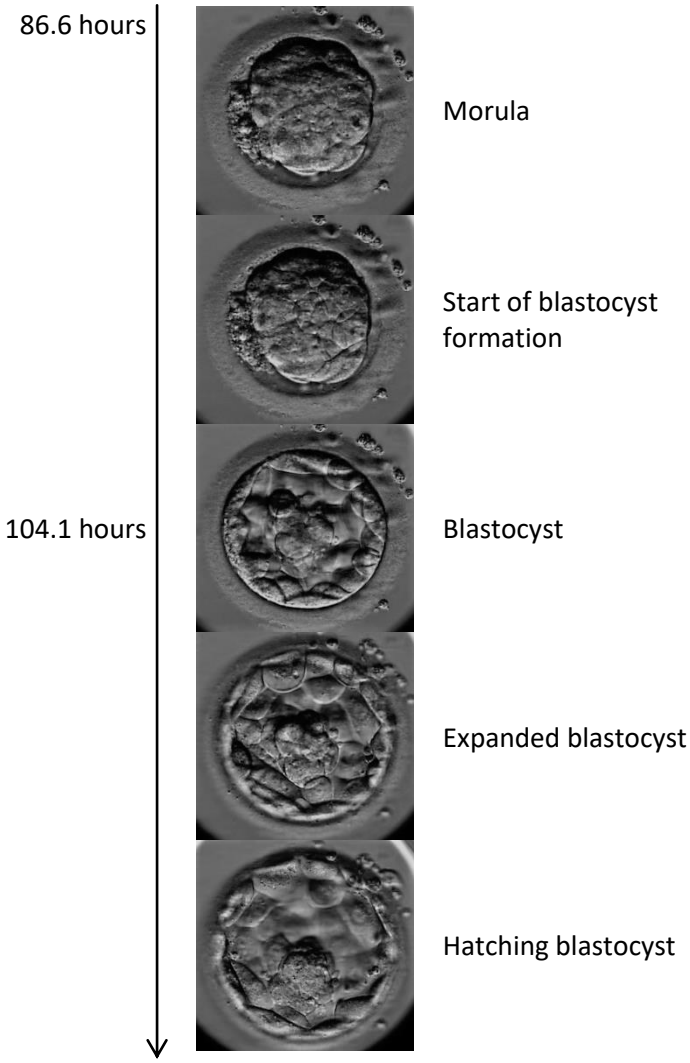


Figure 1.4: The development of the embryo from a morula to a hatching blastocyst

(timings taken from averages from the general population (Herrero et al., 2013)) (images taken from PREPARE patient 157).

These morphokinetic markers have been linked to a number of endpoints (see Table 1.1).

Table 1.1: Evidence for morphokinetic markers of embryo quality

Acronym		Evidence
tPNf	Time of pronuclei fade	Live birth (Azzarello et al., 2012)
t2	Time to two cells	Development to a blastocyst (Wong et al., 2010), chance of implantation (Meseguer et al., 2011)
t3	Time to three cells	Development to a blastocyst (Milewski et al., 2016), chance of implantation (Meseguer et al., 2011)
t4	Time to four cells	Development to a blastocyst (Cruz et al., 2012, Milewski et al., 2016), chance of implantation (Freour et al., 2013, Meseguer et al., 2011), clinical pregnancy (Milewski et al., 2016)
t5	Time to five cells	Development to a blastocyst (Cruz et al., 2012, Milewski et al., 2016), chance of implantation (Meseguer et al., 2011)
t6	Time to six cells	Development to a blastocyst (Milewski et al., 2016), clinical pregnancy (Milewski et al., 2016)
t7	Time to seven cells	Development to a blastocyst (Milewski et al., 2016, Dal Canto et al., 2012)
t8	Time to eight cells	Development to a blastocyst (Milewski et al., 2016, Dal Canto et al., 2012)
t9	Time to nine cells	Development to a blastocyst (Milewski et al., 2016), chance of implantation (Milewski et al., 2016), clinical pregnancy (Milewski et al., 2016)
CC2	Second cell cycle (t3-t2)	Development to a blastocyst (Milewski et al., 2016), chance of implantation (Meseguer et al., 2011, Wong et al., 2010)
CC3	Third cell cycle (t5-t3)	Development to a blastocyst (Milewski et al., 2016), clinical pregnancy (Milewski et al., 2016)
CC4	Fourth cell cycle (t9-t5)	Development to a blastocyst (Milewski et al., 2016), chance of implantation (Milewski et al., 2016), clinical pregnancy (Milewski et al., 2016)
S2	Synchrony of second cell cycle (t4-t3)	Development to a blastocyst (Cruz et al., 2012, Hashimoto et al., 2012), chance of implantation (Meseguer et al., 2011, Freour et al., 2013, Wong et al., 2010)
S3	Synchrony of third cell cycle (t8-t5)	Development to a blastocyst (Hashimoto et al., 2012, Milewski et al., 2016)
tM	Time to morula	Development to a blastocyst (Cruz et al., 2012)
tSB	Time to start of blastocyst formation	Chance of implantation (Campbell et al., 2013)
tB	Time to blastocyst	Chance of implantation (Campbell et al., 2013)
tEB	Time to an expanded blastocyst	
tHB	Time to a hatching blastocyst	

Despite the use of morphology and morphokinetic markers to assess whether an embryo will successfully implant and produce a healthy liveborn offspring, scientists are continually looking for methods to improve this assessment. One of these methods is to examine the embryo's metabolic activity by measuring the uptake of nutrients by the embryo from the culture media.

1.2.2.1.2 Embryo metabolic activity

A recent review article has examined the methods that can be used for examining embryo metabolic activity (Thompson et al., 2016). These are either indirect, examining the spent media from cultured embryos, or direct, examining intracellular activity (however, the invasive nature means the latter is not currently acceptable for use in human embryos that are to be transferred) (Thompson et al., 2016). A number of substrates and products have been suggested over the last twenty years as potentially useful at predicting embryo viability, with varying degrees of success, including amino acids, pyruvate, lactate and glucose. It should be noted that the concentrations of these different substrates required by the embryo alter as the embryo develops, hence the widespread use of sequential media (G1 changing to G2 on day 3) in IVF. The amino acids required by the embryo change; the early cleavage stage embryo requires non-essential amino acids for protein metabolism, whereas once the embryo contains more than eight cells essential amino acids become vital. Pyruvate is the main energy source for the cleavage stage embryo and glucose is found in very minimal levels in G1 media (enough to meet the requirements of the sperm during insemination), whereas to support the embryo developing from the 8-cell size to blastocyst, glucose metabolism is required and therefore in G2 media glucose is found in higher concentration with lower levels of pyruvate.

It has been shown that the amino acid metabolism of human preimplantation embryos cultured *in vitro* correlates inversely with their viability. In other words, metabolically 'quiet' embryos which turn over amino acids at a lower rate are developmentally higher quality than those with a higher amino acid turnover (Houghton et al., 2002, Stokes et al., 2007). Interestingly, amino acid profiling is also able to distinguish between developmentally competent embryos of the highest morphological grade (Stokes et al., 2007). Furthermore, alanine production on days 2 and 3 post insemination was highest in embryos which did not form a blastocyst (Houghton et al., 2002). Evidence from animal studies suggests that a high amino acid turnover positively correlates with DNA damage (Sturmey et al., 2009b) highlighting the benefit of a quiet metabolism. Conversely, a study in Southampton has demonstrated that increased total amino acid turnover at the compaction and cavitation stage of formation was associated with increased assembly of junctional proteins (Eckert et al., 2007). This may appear contradictory to the

previous research but it is important to recognise that the quiet embryo hypothesis examined amino acid turnover in cleavage stage embryos whereas Eckert et al. analysed the embryos at a later stage of development. The analysis of embryo metabolism through amino acid profiling has been proposed as a novel, functional means of assessing embryo viability and selection for transfer (Brison et al., 2004). Interestingly, research has also demonstrated that the depletion or production of amino acids is significantly affected by whether the embryo is predominantly euploid or aneuploid and the sex of the offspring (Picton et al., 2010).

There is some evidence that pyruvate metabolism may be used to predict embryo viability; uptake was higher in embryos which formed a blastocyst compared to those which arrested at the cleavage stage and uptake was lower in embryos which failed to implant compared to those which did on both day 2 and day 3. However, the uptake was so variable that it was concluded that this was not a good method to improve the assessment of morphologically similar embryos (Conaghan et al., 1993). More recent research has led to similar conclusions despite the fact that on day 2 pyruvate uptake was significantly higher in the embryos of women who became pregnant compared to those who did not. Pyruvate metabolism, once again, did not correlate with embryo morphology and therefore could not be used as a tool to aid embryo selection (Devreker et al., 2000).

Glucose metabolism has also shown promise as a useful assessment tool particularly following compaction of the embryo. Embryos which resulted in a pregnancy consumed significantly higher amounts of glucose on days 4 and 5 post insemination compared to those which did not and this was independent of the morphological grade of the embryos. In addition, the sex of the offspring also affected glucose consumption, with females consuming 28% more glucose on day 4 than males (Gardner et al., 2011).

Measuring the metabolic status of embryos is not currently used clinically to aid the selection of embryos for transfer. However, in the future, the ability to determine intracellular metabolic activity (for example using endogenous fluorophores) may provide the additional information required and combined with morphokinetic parameter may improve outcomes.

1.2.2.1.3 Culturing embryos in the lab

In addition to selecting the best embryo for transfer and thus increasing the chance of success, a large amount of research has taken place in order to optimise the culture conditions for these early embryos. As stated previously, sequential culture media are used to ensure that the appropriate nutrients (lactate, pyruvate, glucose and amino acids) for each stage of

development are available to the growing embryo. Embryos are cultured at 37°C which, interestingly, produces a better blastocyst formation rate than if the embryos are cultured at the more physiological temperature of 36°C (Hong et al., 2014). They are also cultured in 5% O_2 (Waldenstrom et al., 2009, Meintjes et al., 2009) and 6% CO_2 (as part of a bicarbonate/CO_2 buffer system, to allow the maintenance of a physiological pH (between 7.2 and 7.4)). In addition to these carefully controlled parameters, even air pollutants within the IVF laboratory, especially NO_2, have been shown to have an effect on the developing embryo (Legro et al., 2010). It is believed that keeping the embryos in as stable an environment as possible throughout their *in vitro* culture should improve outcomes. Due to this, single step medium has been developed, although there is limited evidence to date about whether it improves outcomes (Sfontouris et al., 2016). Furthermore, the use of an EmbryoScope or other time lapse incubator allows the embryos to be observed without removing them from the culture chamber. Analysing the morphological parameters and nutrient uptake of embryos cultured in different environments may provide a tool in assessing the quality of these conditions.

1.3 Preconception care

It is now becoming clear that a further major determinant of fecundity and success of IVF is the patient's preconceptional health and lifestyle and in contrast to age, these factors may be amenable to intervention. However, little is known about which diet or nutritional supplements may cause the greatest benefit in this group.

1.3.1 History of preconception care

Preconception care is not a new concept. Indeed references to improving women's health prior to pregnancy can be found in the Old Testament (Judges 13 verse 4), where it states "Now therefore beware, I pray thee, and drink not wine nor strong drink, and eat not any unclean thing". However, in modern times little attention was given to preconceptual health until the 1980s. It was then that it became increasingly apparent that conventional models of prenatal care made limited impact on the incidence of congenital abnormalities. Moreover, it became understood that pregnancy represents an unique window of opportunity for health promotion because this is a time when women are most amenable to changing unhealthy habits in order to improve the health of their unborn child (Bille and Andersen, 2009). Leaders in health care began to encourage redefinition of the perinatal prevention paradigm in the USA (Freda et al., 2006a). In 1983, the American College of Obstetrics and Gynaecology (ACOG) and the American

Academy of Paediatrics (AAP) published guidelines for perinatal care and within this was advice regarding the need for preconception care in order that parents should have optimal physical health and be emotionally prepared for parenthood (Freda et al., 2006b). In 1985 Moos and Cefalo developed the preconceptional healthcare appraisal (Moos, 1989) to aid healthcare professionals to efficiently assess health prior to conception and therefore target advice. However, for many decades, the advice given was too little, too late. In 2004 the "Preconception Health and Health Care Initiative" (Waggoner, 2012) published in the USA advised that all women of childbearing age should be counselled at all health encounters regarding appropriate medical care and optimising health in order to improve pregnancy outcomes. This inclusive approach was considered necessary as a large proportion of pregnancies are unplanned (Lakha and Glasier, 2006) and few women seek medical advice prior to conceiving (Mazza et al., 2013). In the UK, preconception advice has been specifically encouraged for 'high risk' groups including those with a body mass index (BMI) of greater than 30 kg/m^2 (RCOG, 2010), those from low income families (NICE, 2008) and those with diabetes (CEMACH, 2007). Preconception care is considered of particular value in these high risk groups because waiting until their first antenatal appointment means that major developmental milestones have already passed and lifestyle modifications at this point will have less impact on outcomes. For example, women taking antiepileptic medication should be on high dose folic acid prior to conception as folic acid is essential for neural tube development which occurs between day 22 and day 26 after fertilisation.

Prenatal complications often have their origins before conception. Women with a BMI greater than 35 kg/m^2 have been reported to have a stillbirth rate that is twice that of the general population (8.6 versus 3.9 per 1000 singleton births respectively) (RCOG, 2010). Detrimental effects are not only to the baby, as obese women have an increased risk of venous thromboembolism, gestational diabetes and pregnancy induced hypertension. These women are also 1.5 times more likely to undergo a caesarean section and four times more likely to have a postpartum haemorrhage than the general population (RCOG, 2010). It is clear that efforts to reduce a high BMI before conception are likely to be rewarded by reductions in such risks.

In contrast to those caring for pregnant women, those providing fertility treatment are far better placed to offer preconceptional lifestyle advice in order to improve that patient's chance of conception, the health of the couple and that of the not yet conceived child. In addition to this, giving these couples information about how they can improve their fecundity, empowers them through their fertility journey, enabling them to take some responsibility for the outcome of the

treatments that they are undergoing. Van der Zee et al. have examined the idea of preconception advice from an ethical point of view, stating that responsible fatherhood begins before conception and that healthcare professionals should involve fathers in lifestyle modifications prior to conception in order to boost their health and therefore the health of their family (van der Zee et al., 2013).

In addition to this, physicians and healthcare professionals working in reproductive medicine should view not only the couple undergoing the treatment as their patients but also the embryo. In this context, work in epidemiological studies have demonstrated a relationship between in utero conditions and adult chronic disease such as coronary heart disease (Barker et al., 1993), stroke, type 2 diabetes and osteoporosis (Gluckman et al., 2008).

Despite this growing body of evidence demonstrating how vulnerable the early embryo is to its environment and the need to optimise maternal health prior to conception, the impact of interventions on outcomes remains unclear. In a recent Cochrane review summarising the preconception advice that should be given to people who suffer with infertility (Anderson et al., 2010), the lack of randomised control trials examining the effectiveness of lifestyle modifications in this particular group of people was highlighted.

1.3.2 The importance of diet in the preconception period

Women planning a pregnancy do not in general appear to alter their nutrient intake (Crozier et al., 2009). In contrast, there is evidence that they continue to eat an inadequate diet rich in saturated fats and with levels of a number of essential micronutrients that are below the recommended daily intake, when compared to women who are not trying to conceive (de Weerd et al., 2003). This is despite a growing body of evidence including large prospective cohort studies which have demonstrated the impact of female and male preconceptional nutritional status on fertility, perinatal and long term health of the offspring (Inskip et al., 2006). More recently, research has shown that variations in preconceptional diet may impact on IVF outcomes. A 'Mediterranean' diet high in vegetable oils, fish, vegetables and legumes and low in carbohydrate-rich snacks was positively associated with red blood cell folate and vitamin B6 in blood and follicular fluid and with a 40% increase in the probability of pregnancy (Vujkovic et al., 2010). The findings of a subsequent case control study by another research group also suggested that fertility outcomes were improved in couples with a Mediterranean diet (Toledo et al., 2011). Furthermore, a Dutch study reported that male and female patients undergoing IVF/ICSI who described eating a Mediterranean–style diet in the week prior to treatment appeared to

increase their chance of an ongoing pregnancy by 65% (Twigt et al., 2012). Interestingly, this change in diet may not need to be for a prolonged duration. Fleming's group in Southampton have demonstrated that dietary manipulation in the rodent for just three days prior to implantation can have a profound effect on in-uteri growth trajectories and even behaviour development (Fleming et al., 2011). These data provide strong support for the sensitivity of the peri-implantation embryo to programming in response to periconceptional nutritional environment.

1.3.3 Diet affects the intrauterine environment

Research related to the Developmental Origins of Disease theory has demonstrated a link between maternal diet in early pregnancy and the development of chronic diseases, for example cardiovascular disease in later life (Barker, 2007). It is also known that BMI (independent from pathology linked to obesity such as polycystic ovarian syndrome (PCOS)) (van der Steeg et al., 2008) and age (Leridon, 2004) affect fertility although the potential mechanisms involved are still being investigated.

Research has demonstrated that what a woman eats affects the amino acid concentration within her intrauterine environment and hence, presumably, the nutritional composition within the reproductive tract during preimplantation embryo development (Kermack, 2015). A diet identified by a validated scoring system as being less healthy was associated with higher concentrations of asparagine, histidine, serine, glutamine, valine, phenylalanine, isoleucine and leucine in the uterine fluid compared with a healthier diet, defined as one with a higher intake of fresh vegetables, fruit, whole-grain products and fish and a low intake of red and processed meat and high fat dairy products. These findings were comparable to what has previously been observed in mice fed a low protein diet, where reductions in the branched chain amino acids were also observed (Eckert et al., 2012). This suggested that, like the mouse, the nutritional environment of human uterine fluid is sensitive to diet. These data offer the potential to facilitate the production of embryo culture media containing physiologically relevant concentrations of amino acids based on those found in uterine fluid, and perhaps also to guide preconception dietary interventions to optimise the intrauterine environment. Further research is needed to examine what dietary interventions may alter the intrauterine environment and whether these in turn will improve outcomes for patients undergoing fertility treatments.

1.3.4 Nutritional supplements in couples undergoing ART

In order to try to improve their chances of success some couples undergoing ART request, or self medicate with, a range of adjuvant therapies including acupuncture and increasingly, nutritional supplements. A multi million pound nutraceutical industry has grown to meet both this demand and that of women trying to conceive spontaneously. In a recent study of women about to embark on IVF in a centre in the UK, 55% reported taking some kind of multivitamin preparation containing folate and a further 16% folic acid only (Kermack and Macklon, 2014). While this suggests considerable enthusiasm for the use of multivitamins prior to IVF, only a proportion were following the established advice to take folate supplements prior to conceiving, in order to reduce the chance of having an offspring with a neural tube defect (MRC, 1991). This finding is consistent with previous research which revealed that around one third of women starting IVF are not taking folic acid supplements (Boxmeer et al., 2009). On the other hand, around half may be taking expensive multivitamins unnecessarily. Given this rapidly expanding industry fuelled by strong and prevalent marketing, the scientific evidence supporting the routine use of vitamin supplements warrants scrutiny.

1.3.4.1 Folate

The place of folate supplementation in preconceptional preparation is well established and current advice is to supplement a healthy diet with 400 micrograms of folic acid daily (unless there is a clinical indication for a higher dose). More than twenty years ago, a double blinded randomised controlled trial demonstrated its efficacy in reducing the chance of neural tube defects (MRC, 1991). In recent years, its role in modulating fertility and embryo health has become apparent and has led to interest in folic acid as a supplement for those trying to conceive by IVF. Folic acid supplementation increases plasma folate levels and reduces the concentration of plasma homocysteine. These alterations have also been demonstrated in follicular fluid and hence supplementation can be shown to influence the nutritional environment of the maturing oocyte (Boxmeer et al., 2008). Indeed, an improvement in embryo quality and a three-fold increase in the chance of a biochemical pregnancy following IVF was demonstrated in a cohort study of women regularly taking a folic acid supplement prior to conception, defined as women with a serum folate concentration of greater than 22.5 nmol/L (Boxmeer et al., 2009). However, a more recent study reported that despite altering plasma concentrations, folic acid supplementation did not increase the chance of achieving a pregnancy during fertility treatment in women with unexplained infertility (Murto et al., 2014).

Folic acid supplementation is often recommended to males as a means of improving sperm quality. This is supported by a retrospective study examining diet in relation to semen volume and sperm quality (concentration, motility and morphology), which demonstrated the latter to be associated with higher intakes of folate (the naturally occurring form of folic acid), carbohydrate, fibre, vitamin C and lycopene and lower intakes of protein and fat (Mendiola et al., 2010). However, the results from prospective trials of supplementary folic acid in this context are equivocal. An improvement in both spermatozoa number and motility and a decrease in round cell numbers was observed in males supplemented with a high dose of 15 mg per day for a three month period (Bentivoglio et al., 1993). In contrast, other studies have shown either no impact on sperm count (Landau et al., 1978) or improvement only when zinc was co-supplemented (Wong et al., 2002).

1.3.4.2 B vitamins

A study from Anquing in China reported that women with higher levels of vitamin B6 had an increased chance of conception (HR=1.4) when compared to women with a deficiency (Ronnenberg et al., 2007). In addition, a Dutch study examining a Mediterranean diet in couples undergoing IVF demonstrated an increase in plasma vitamin B6 levels and in those with high adherence to the diet, a 40% increase in the probability of pregnancy (Vujkovic et al., 2010). However, there has been little prospective research examining the effect of supplementing individual B vitamins, including thiamine, riboflavin, niacin, vitamin B6 and vitamin B12 on IVF outcomes.

However, the B vitamin Cobalamin, has been shown to be of importance in spermatogenesis, as low levels of serum and seminal fluid Cobalamin correlated with decreased sperm concentrations (Boxmeer et al., 2007). However, prospective randomised studies confirming the clinical efficacy of vitamin B supplementation in IVF are still awaited.

1.3.4.3 Antioxidants: Vitamins C and E

Antioxidants are widely reported to offer a broad range of health benefits and the field of infertility has been quick to enthuse about their possible benefits. A number of studies of their use in IVF have been performed, and the efficacy of antioxidants to improve fertility outcomes in subfertile women has been subject to systematic review (Showell et al., 2013). This showed no evidence that antioxidants cause a significant increase in clinical pregnancy rates or live birth rates when compared to placebo or no treatment. Vitamin E supplementation in women undergoing ovarian induction has been reported to improve endometrial thickness but this was

not associated with increased implantation or pregnancy rates (Cicek et al., 2012). However, a recent study in women aged over 35 years indicated that use of vitamin E supplements or a high total vitamin E in the diet (as assessed by a 110 item food frequency questionnaire) were associated with a decreased time to pregnancy rate in women undergoing infertility treatment (Ruder et al., 2014).

With regard to vitamin C, a randomised controlled trial comparing three doses of vitamin C to placebo for 14 days following oocyte retrieval also showed no improvement in clinical pregnancy rates, concluding that there was no obvious benefit to taking vitamin C supplements in the luteal phase during infertility treatments (Griesinger et al., 2002). When vitamin C supplementation was provided earlier in treatment, during the period of ovarian stimulation, no statistically significant impact on pregnancy rates was observed (Crha et al., 2003). However, more recent research has shown in women with a BMI of less than 25 kg/m^2 and aged under 35 years old, vitamin C supplementation was associated with a shorter time to pregnancy when undergoing ART (Ruder et al., 2014). In addition, in those women with a BMI of greater than 25 kg/m^2, β carotene supplementation decreased time to pregnancy.

Vitamin C and E supplementation have also been reported to influence parameters of male fertility. Studies have shown that vitamins C and E (Greco et al., 2005, Schmid et al., 2012) decrease DNA damage in spermatozoa. These vitamins have also been shown to improve the total motile sperm count: vitamin C in a young healthy male population (Minguez-Alarcon et al., 2012) and vitamin E in those with a decreased motility (Suleiman et al., 1996). The former study also demonstrated the importance of β carotene supplementation on the same parameter.

There has been no research carried out into the benefits of supplementation with resveratrol in humans undergoing fertility treatment; however, recent work in mice demonstrated a fertility sparing effect to counteract age associated infertility (Liu et al., 2013). This was a proof of concept experiment and further work is needed in clinical trials before the advice can be given to patients undergoing fertility treatment.

1.3.4.4 Zinc and Selenium

Zinc and selenium have been suggested to be of benefit for both male and female reproductive health. A study examining the selenium levels in follicular fluid demonstrated that they were significantly lower in women with unexplained infertility when compared to those with tubal occlusion or male factor infertility (Paszkowski et al., 1995). There is limited research examining the importance of sufficient zinc intake in women trying to conceive; a descriptive study has

described longstanding infertility noticed in women with coeliac disease and normal menstrual cycles who also have serum zinc deficiency (Jameson, 1976). However, following this study further work has shown no correlation between plasma zinc levels and pregnancy rates (Soltan and Jenkins, 1983) and zinc levels in the follicular fluid in women undergoing IVF and chance of oocyte fertilisation (Ng et al., 1987). Men undergoing fertility treatment are also often advised to take zinc supplements and whilst zinc concentrations in the seminal plasma appear to be correlated to sperm concentration and motility, excessively high concentrations have been linked to poor motility in patients with asthenozoospermia (Fuse et al., 1999). However, prospective randomized trials examining the supplementation of Zinc and Selenium and their effect on ART outcomes are still needed.

1.3.4.5 Vitamin D

The prevalence of vitamin D insufficiency among certain populations appears to be high, and is currently a focus of attention among health policy makers. A recent study in North West London demonstrated a surprisingly high vitamin D deficiency rate in women of child bearing age with 35% being classed as clinically deficient and a further 45% demonstrating insufficient levels (McAree et al., 2013). However the significance of Vitamin D insufficiency for IVF outcomes remains unclear. A recent prospective cross sectional study in which serum Vitamin D levels were measured during cycle preparation for IVF treatment in 335 women reported significantly reduced pregnancy rates in those with a level below <20 ng/mL, while those with the highest serum vitamin D levels had the greatest chance of conceiving (Paffoni et al., 2014). Low levels of vitamin D both in plasma (Polyzos et al., 2014) and follicular fluid (Ozkan et al., 2010) have also been shown to be associated with a reduced chance of achieving a clinical pregnancy after IVF, and a correlation between serum and follicular fluid vitamin D concentrations has been demonstrated (Anifandis et al., 2010). However, a recent publication demonstrated that an increased concentration of vitamin D in the follicular fluid was associated with a poorer quality oocyte from that follicle (Ciepiela et al., 2018). Vitamin D deficiency has also been shown to negatively impact on spermatozoa motility, progressive motility and morphology (Blomberg Jensen et al., 2011). It is known to be essential for flagellar beating and the acrosome reaction due to its regulation of calcium homeostasis (Krasznai et al., 2006). However, its effects are again controversial and one large study has reported that low Vitamin D is not a risk factor for poor semen quality in healthy males, but this study was limited to morphological assessment of quality (Ramlau-Hansen et al., 2011).

There is emerging evidence that vitamin D deficiency may impact on endometrial receptivity. In a study of outcomes after oocyte donation treatment, pregnancy rates in vitamin D depleted oocyte donor recipients, assessed by measuring serum levels using radioimmunoassay, were significantly decreased (37%) when compared to vitamin D repleted recipients (78%) (Rudick et al., 2014). However, the picture is not wholly consistent as increased vitamin D levels in follicular fluid have been associated with poorer embryo quality and a reduced chance of conceiving from IVF treatment (Anifandis et al., 2010).

Taken together, current interest in vitamin D supplementation as a potential modulator of fertility in those found to be deficient seems justified. However, prospective studies of supplementation have yet to show any clear benefit, raising the possibility that Vitamin D deficiency may simply be a marker of some other health issue impacting on fertility, rather than a direct cause.

1.3.4.6 Omega-3 fatty acids

Public awareness of the putative benefits of omega-3 FAs is high as many food packages proclaim their high content of these fatty acids, including alpha linoleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fatty acids are required by the developing embryo as a source of energy (Sturmey et al., 2009a) and for the rapidly increasing cell membrane (Pratt and George, 1989). There is also some evidence that increased levels of omega-3 FAs in the cell membrane may increase the gap junction capacity of the morula, hence improving embryo quality (Champeil-Potokar et al., 2007). Furthermore, they are a precursor for eicosanoids which are essential signalling molecules that regulate cell growth (Zhou and Nilsson, 2001). In the context of IVF, cohort studies have suggested that increased levels of DHA and EPA are associated with improved embryo morphology but a reduction in the number of follicles produced following ovarian stimulation (Hammiche et al., 2011). Furthermore, a study in overweight and obese women demonstrated that those who became pregnant following IVF had higher omega-6 and linoleic acid dietary intake (and a trend to increased omega-3 intake) compared to those women who did not achieve a pregnancy (Moran et al., 2016). Omega-3 FA supplementation (4 g EPA and DHA daily for one month) has also been shown to reduce serum FSH levels in women with a normal BMI, but not in obese women (Al-Safi et al., 2016). This could potentially imply that omega-3 FA supplementation may extend a woman's reproductive lifespan; however, further research is needed to examine the effect on women with a poor ovarian reserve.

Omega-3 FA supplements may also have a positive effect on male fertility, potentially affecting the concentration, motility and morphology of the spermatozoa. PUFAs are important in male fertility because:

1. DHA is found in high concentrations in the tail and due to its large number of double bonds promotes sperm motility (Connor et al., 1998).

2. Both acrosome reaction (secretion of enzymes to allow the sperm to penetrate the zona pellucida) and sperm-oocyte fusion (the release of the sperm nucleus into the oocyte cytoplasm) are dependent on the membrane structure of the spermatozoa, which crucially contains a high proportion PUFAs, particularly DHA.

3. They work as antioxidants, potentially reducing the percentage of spermatozoa suffering from DNA damage (Martinez-Soto et al., 2016).

A double blinded, randomised controlled trial strengthened the case for omega-3 FA supplementation in men with oligoasthenoteratospermia. A dose of 1.86 g per day of DHA and EPA or placebo was given to the men for a 32 week period; total sperm count and sperm concentration were improved in the treatment group (Safarinejad, 2011). Two other smaller studies have also been carried out, also supplementing DHA but for shorter time periods, 12 weeks (Conquer et al., 2000) and 24 weeks (Comhaire et al., 2000). The first showed no difference in the sperm parameters, the latter showed an improvement in sperm concentration in oligozoospermic males, as well as an increase in the acrosome reaction and reduced levels of reactive oxygen species. Another study demonstrated an increase of 1.9% of spermatozoa with normal morphology when men with higher intake levels were compared to those with lower consumption (Attaman et al., 2012). In addition, research has shown that walnuts, a plant source of alpha-linolenic acid (ALA), increased the serum fatty acid profile and also the vitality, motility and morphology of the participants' sperm (Robbins et al., 2012). However, there is some controversy around the benefit of supplementation. Conquer et al. demonstrated similar serum levels of DHA between asthenozoospermic and normospermic men suggesting similar dietary intake despite significantly lower levels of DHA in the seminal plasma and the spermatozoa of the asthenozoospermic men. The authors concluded that this difference could potentially be explained by a difference in the metabolism of DHA within the testes of the asthenozoospermic men as opposed to differences in dietary intake (Conquer et al., 1999).

The importance of DHA has been well recognised, with one study demonstrating it makes up more than 60% of the PUFAs found in the phospholipids within the head and tail of the

spermatozoa (Zalata et al., 1998) and a further study showing preferential accumulation within the spermatozoa with levels 6.2 times higher than in the seminal plasma (Zerbinati et al., 2016). Increased levels of DHA in the spermatozoa and seminal plasma have also been correlated with less loss of motility following sperm cryopreservation (Martinez-Soto et al., 2013). Additional work has also had similar findings with seminal fluid DHA levels positively correlating, and stearic acid levels negatively correlating, with sperm motility (Zerbinati et al., 2016).

Taken together, this literature points to possible benefits of a diet high in omega-3 FAs, perhaps together with Vitamin D; these might be considered to be important components of what is popularly referred to as the "Mediterranean diet'. In a Dutch cohort study, those reporting taking a more Mediterranean than Dutch diet were found to have significantly higher chance of conceiving after IVF (Vujkovic et al., 2010).

1.3.4.7 Multivitamin preparations

While over half of women embarking on fertility treatments in one UK clinic reported taking a multivitamin preparation regularly (Kermack and Macklon 2014), to date, only two randomised controlled trials have examined their efficacy at improving outcomes in subfertile women. Compared with folic acid supplementation alone, the use of a proprietary multivitamin was associated with an increase in conception rates and a decreased chance of miscarriage in women undergoing ovulation induction treatment (Agrawal et al., 2012). The second study also demonstrated an increase in pregnancy rates by 16% between the group taking the nutritional supplement containing chasteberry, green tea, L-arginine, vitamins (including folate) and minerals when compared with the placebo group (Westphal et al., 2006). However, these studies were small and therefore subject to chance effects. Despite this, the outcomes from these studies have been used for high profile marketing purposes by the manufacturers.

In the absence of additional data from prospective studies, a degree of caution is indicated as the doses of individual components of multivitamin preparations may exceed daily requirements, and excessive vitamin exposure has been associated with a number of detrimental effects (Ko and Sabanegh, 2012). Indeed it has been advocated that using over the counter multivitamins in male infertility should be avoided as often the nutrients can be obtained through a balanced diet (Ko and Sabanegh, 2012). Further research in the form of well designed randomised controlled trials are needed in this area to allow clinicians to advise patients on the most appropriate diet and/or nutritional supplements to improve their chances of conception either naturally or whilst undergoing fertility treatment.

1.4 Aim

The aim of this thesis is to investigate how the periconceptional environment affects the developing embryo. This will be done by determining whether a specific dietary intervention (mimicking the Mediterranean diet) instituted six weeks prior to IVF and/or the stability of the environment an embryo is cultured in improve embryo quality.

1.5 Hypothesis

The hypothesis for this thesis is that the periconceptional environment in which an embryo develops impacts on its health. This work was divided into two distinct studies:

- The PREPARE trial: It was hypothesized that a Mediterranean diet containing high levels of omega-3 fatty acids and vitamin D would improve the environment for the developing oocytes and spermatozoa and hence result in better quality sperm and embryos.
- The PROMOTE trial: It was hypothesized that embryos cultured in the more stable environment of the EmbryoScope were more likely to form a blastocyst (and be of better quality) than those cultured in the routine 'MINC' benchtop incubator.

1.6 Personal contribution

Due to the multifaceted nature of this work, both the PREPARE trial and the PROMOTE trial were projects on which many people worked.

1.6.1 Contribution to the PREPARE trial

My contribution to the PREPARE trial was:

- Finalising the protocol and preparation of the manuscript describing the protocol for publication.
- Preparation of all trial documentation including the patient information sheet, consent forms and case report forms (CRFs).
- Completion of the IRAS form, attendance at the ethics committee meeting and correspondence with the committee for any amendments.

- Completion of the paperwork for Research and Development (R+D) team approval and ERGO approval.
- Registration with the ISRCTN registry.
- Recruitment of participants.
- Purchasing, anonymizing and distributing oils and spreads and distributing drinks.
- Conducting research appointments and completing CRF.
- Venepuncture.
- Preparation for storage of follicular fluid, semen, seminal plasma and sperm.
- Data entry into SPSS.
- Statistical analysis.
- Presentation at national and international meetings (ESHRE 2017 and Fertility 2018) and preparation of manuscripts.

Contribution of other team members to the trial:

- Randomisation of participants.
- Processing and analysis of blood samples including fatty acid analysis, vitamin D, B6, B12, and folate.
- Fatty acid analysis of follicular fluid, seminal fluid and sperm.
- Sperm DNA damage analysis.
- Oocyte retrieval.
- Analysis of embryo morphology and annotation of morphokinetic markers.
- Semi structured interviews to ascertain couples' experience, including transcribing interviews and analyzing data.

1.6.2 Contribution to the PROMOTE trial

My contribution to the PROMOTE trial:

- Successfully applying for grant funding from the NIHR BRC (Nutrition) Commercial and Enterprise fund.
- Writing the protocol.
- Writing patient information leaflet, consent forms and preparing the CRF.
- Completion of the IRAS form, attendance at the ethics committee meeting and correspondence with the committee for any amendments.
- Completion of the paperwork for Research and Development (R+D) team approval and ERGO approval.
- Registration with the ISRCTN registry.
- Recruitment of patients and training of the research team in the recruitment of patients.
- Storage of spent media.
- Analysis of glucose, pyruvate, lactate and amino acids in spent media.
- Data entry into SPSS.
- Statistical analysis.
- Presentation at a national meeting (Fertility 2018) and preparation of manuscripts.

Contributions of other team members to the trial:

- Recruitment of some couples.
- Analysis of morphology of embryos.
- Analysis of glucose, pyruvate, lactate and amino acids in some media.
- Presentation at an international conference (ESHRE 2017).

Chapter 2: Methods: The “PREPARE” trial.

2.1 Study Objective

Although retrospective studies have suggested a link between preconception diet and human fecundity, several questions still remain, for example which nutrients are beneficial and how long they need to be taken prior to conception. This prospective, randomized, controlled trial (the PREPARE trial (PREconception dietary suPplements in Assisted REproduction)) aimed to address the questions of the effect of a diet rich in marine omega-3 FAs and Vitamin D on a developing embryo and the intrauterine environment.

2.2 Hypothesis

It was hypothesised that a diet rich in marine omega-3 FAs and Vitamin D would be beneficial to a developing embryo and thus improve morphokinetic markers used to predict embryo viability and implantation.

2.3 Ethics

This study was designed using the guidelines for good clinical practice (GCP) as well as the Declaration of Helsinki 1964 as revised and recognised by governing laws and EU Directives. Full ethical approval (13/SC/0544) was granted from South Central (Oxford A) Research Ethics Committee (NRES) via the Integrated Research Application System (IRAS). In accordance with the GCP guidelines, written informed consent prior to randomization was mandatory.

2.4 Sample size and power considerations

In a previous retrospective study from our group, a diet rich in the omega-3 FAs was shown to be associated with improved embryo morphology when assessed on day 3 post fertilisation (Hammiche et al., 2011). Using a published scoring system between 1 (highest quality) and 5 (lowest quality), embryos generated in women with a daily mean total intake of omega-3 FAs in the 4 weeks prior to IVF greater than 1.7 g scored a mean of 0.6 (SE 0.26) points higher than those embryos derived from women reporting an intake of <1 g per day. Recently, morphokinetic analysis of human embryos has shown the duration of key development phases to be correlated to standard conventional morphological criteria, but more highly predictive of implantation potential (Meseguer et al., 2011, Kirkegaard et al., 2012). One of the most sensitive predictors of implantation has been shown to be CC2. Embryos with a CC2 shorter than 11.9

hours (pooled SD 2.25 hours, but not normally distributed) have been reported to have an implantation rate of 35% compared to 28% when this key developmental step took longer than 11.9 hours. Moreover, in a study correlating morphokinetic parameters with static morphology scoring, a CC2 less than 11.9 hours was shown to correlate with an overall increase in embryo score of 0.5 points on a 5 point scale (Meseguer et al., 2011). This is similar in magnitude to the impact on embryo morphology reported to be achieved by exposure to a high omega 3 FA diet as described above (Hammiche et al., 2011). The primary end point of this study was therefore the difference in mean CC2 score of embryos generated by IVF after exposure of the couple to the intervention versus control diet. In order to detect a minimum absolute difference of 12% (1.4 hours) in mean CC2, or effect size of 0.670, with power ≥ 80% at $p < 0.05$ a non-parametric comparison (Wilcoxon test) indicated a requirement of 46 couples per group in the analysis.

To allow for drop outs and failure to produce sufficient viable embryos, a further 20% were recruited. This required the randomization of a minimum of 55 couples per group (110 in total).

2.5 Study population and recruitment

Women participating in the PREPARE trial were between the ages of 18 and 41 years, with a body mass index (BMI) between 18 and 32 kg/m^2; they could not have had more than two previous unsuccessful cycles of IVF or IVF-ICSI. Their partners had to be using their own sperm and be prepared to provide a fresh sample prior to the dietary intervention and on the day of oocyte retrieval. Participants had to provide written informed consent to participate in the study.

Exclusion criteria were: any medical contraindication to IVF or IVF-ICSI treatment or to the specific dietary intervention, previously diagnosed diabetes, taking prescribed medication or herbal remedies apart from simple painkillers, and eating oily fish (as defined by the UK Food Standards Agency) more than once a week.

Eligible patients were informed about the PREPARE study during information evenings or at their initial consultation with the medical team. A minimum reflection period of 3 days was offered. Eligible patients who wished to participate were randomized after providing written consent. If an eligible patient declined participation, basic characteristics were obtained to identify any selection bias.

2.6 Study design

The PREPARE trial was a single centre, randomized controlled trial, powered to demonstrate a difference in mean duration of the second cell cycle (CC2) of embryos generated by IVF after exposure of the couple to the intervention versus control. The intervention group received a six week diet of olive oil for cooking, an olive oil based spread, and a daily supplement drink enriched with Vitamin D (10 micrograms daily) and the marine omega-3 FAs eicosapentaenoic acid (EPA) (800 milligrams daily) and docosahexaenoic acid (DHA) (1200 milligrams daily) versus the control diet of sunflower seed oil for cooking, a sunflower oil based spread, and a daily supplement drink without vitamin D, EPA or DHA.

Following inclusion into the study but prior to randomisation, consenting participants were invited to complete a preconception questionnaire and the short Southampton Food Frequency Questionnaire (FFQ) in order to characterise their lifestyle and diet prior to entry into the study (Crozier et al., 2010). At this time, samples of blood were obtained from the women in order to provide baseline data of the nutritional content prior to the intervention. The male participants were asked to provide a semen sample which was analysed for concentration, motility and morphology (Cooper et al., 2010).

After collecting the baseline data, participating couples were randomised to one of the two intervention groups and were provided with a 6 week supply of the respective intervention components of drinks, oil and spread in unmarked containers. Permuted block randomization was used with blocks of varying size and allocation concealment, stratification was performed at randomisation for planned mode of fertilisation: IVF or IVF-ICSI. The trial was double blinded; neither the couples nor the research or clinical teams knew which arm of the study a couple had been assigned. Unblinding was only performed once all couples had completed the dietary intervention and the annotation of all embryos had been performed. Compliance with the diet was monitored by weekly communication (either phone calls or email) by the research team; this was recorded in the case report form (CRF).

The women embarking on the study underwent ovarian stimulation according to standard protocol of the Complete Fertility Centre, provided no abnormality was seen on their baseline scan on day 2 of their cycle. Oocyte retrieval was performed 36 hours following the single dose of human chorionic gonadotrophin (hCG) or gonadotrophin releasing hormone agonist (GnRH agonist), which promote oocyte maturation. At the point of oocyte retrieval, further blood was taken from the female participants and the follicular fluid and cumulus cells that are routinely

removed from the oocyte prior to insemination were snap frozen. A further semen sample from the males was analysed prior to insemination.

The embryos were cultured in Vitrolife IVF medium in a validated time lapse incubator (EmbryoScope, Unisense, FertiliTech, Denmark) in 5% CO_2, 5% O_2 and at 37°C. During the incubation, twenty one plane focal images were generated every hour and analysed according to morphological and morphokinetic markers (Chamayou et al., 2013).

2.7 Assessing the couples' diet and lifestyle

The couples' diets were assessed using the Southampton Women's Survey short food frequency questionnaire. This comprised of asking each individual about the frequency that they consumed 20 food items (see Table 2.1). The short food frequency questionnaire was derived by extracting the 20 foods that best characterized a prudent dietary pattern from a 100 item, interviewer administered, food frequency questionnaire used during the Southampton Women's Survey (Crozier et al., 2010). In order to calculate the prudent diet score, the frequency that the participant consumed the food item was calculated in the units provided in Table 2.1. The mean in Table 2.1 was subtracted from the score and then this was divided by the SD. This number was then multiplied by the positive (healthier) or negative (less healthy) coefficient of the food item, the twenty scores were then added together to give the prudent diet score.

Table 2.1: Twenty items found in short food frequency questionnaire with mean SD and coefficient

Food	Mean	SD	Coefficient	Units
Peppers	1.061397	1.4904	0.1974	Frequency per week
Tomatoes	2.62901	2.431786	0.1928	Frequency per week
Vegetable dishes	0.864513	1.631979	0.1898	Frequency per week
Courgettes, marrows and leeks	0.5266601	0.97666	0.1875	Frequency per week
Green salad	2.439183	2.343691	0.1673	Frequency per week
Wholemeal bread	6.181649	8.616229	0.1640	Slices per week
Onions	2.587543	2.237814	0.1590	Frequency per week
Vegetarian foods	0.6203704	1.596571	0.1547	Frequency per week
Pasta	1.872092	1.579819	0.1502	Frequency per week
Spinach	0.2208598	0.6283572	0.1423	Frequency per week
Full-fat liquid milk	0.1290752	0.3354734	-0.1420	Pints per day
Beef	0.5680372	0.7974675	-0.1430	Frequency per week
Crisps and savoury snacks	3.090659	3.341807	-0.1461	Frequency per week
Yorkshire puddings and savoury pancakes	0.3032224	0.5110254	-0.1486	Frequency per week
White bread	12.43189	11.8666	-0.1611	Slices per week
Sugar	2.560397	5.050369	-0.1665	Teaspoons per day
Gravy	1.342487	1.515248	-0.1825	Frequency per week
Sausages	0.6230788	0.8352866	-0.1874	Frequency per week
Meat pies	0.3688938	0.6061124	-0.1905	Frequency per week
Chips and roast potatoes	1.460222	1.595467	-0.2087	Portions* per week

*Portion defined as a large baking potato, new potato counts as $1/6^{th}$ of an old/baking potato

2.8 Blood sampling and processing

2.8.1 Collecting the samples

Bloods were taken from female and male participants following informed consent and as per hospital protocol, prior to randomisation and on the day of egg collection. After locating an appropriate vein in the antecubital fossa of the participant, the overlying skin was cleaned using an alcohol wipe (70% isopropyl alcohol), and a tourniquet applied to the upper arm. A closed vacutainer system was then used for venesection, this method was employed in order to minimise the risk of haemolysis. If possible, blood was collected in the following tubes and order: one 6 ml tube containing Lithium Heparin (green top), two 5 ml serum separator tubes (SSTs, yellow top) and two 6 ml tubes containing ethylenediamine tetra-acetic acid (EDTA, lavender top). The tubes were labelled using the participant's study ID, initials, date of birth and the date and time of collection. Samples were transported to be processed in the Wellcome Trust Clinical Research Facility laboratory at ambient temperature, except for the EDTA tubes

which were transferred on ice and protected from light. All samples were moved to the laboratory as quickly as possible and always within one hour of collection.

2.8.2 Processing the bloods for storage

2.8.2.1 Lithium Heparin blood tube

Upon arrival at the Wellcome Trust Clinical Research Facility laboratory, the tube was inverted several times and centrifuged at 1000 g for 15 minutes at 20°C. The plasma was then transferred using a plastic pipette into labelled aliquot tubes (0.5 ml per aliquot). The red cell pellet was then pipetted into a 15 ml falcon tube and this was filled to 15 ml with room temperature PBS. The tube was gently inverted to wash the cells and then centrifuged at 400 g for 10 minutes at room temperature. The PBS was discarded and the wash step was repeated. The red cell pellet was then transferred into labelled 0.5 ml aliquots. All aliquots were stored at -80°C until analysis.

2.8.2.2 SST blood tubes

The sample within the SST blood tubes was mixed by inversion and then centrifuged at 1500 – 2000 g for between 10 and 15 minutes at 20°C. The serum was removed and pipetted into labelled tubes, these were then stored at -80°C until analysis.

2.8.2.3 EDTA blood tubes

These tubes were kept protected from light and on ice until processing could be undertaken, this was in order to prevent degradation of Pyridoxine (vitamin B6) and hence an inaccurate result. Upon arrival at the laboratory, the samples were mixed by inversion and centrifuged at 1500 g and 4°C for 10 minutes. The plasma was pipetted into 0.5 ml labelled aliquots. The remainder contained a concentrated leukocyte band (or buffy coat), this was removed and discarded. Phosphate buffered saline (PBS) was added to the tube to replace the removed plasma (same volume) in order to wash the red cells. The sample was mixed using inversion and centrifuged at 4°C and 1500 g for 10 minutes. The supernatant was removed and discarded, the same volume of PBS was added again and the wash repeated. Once washed, the red cells were pipetted into 0.5mls labelled aliquots and stored at -80°C until analysis.

2.8.3 Blood sample analysis

2.8.3.1 Fatty acid analysis

The fatty acid content of the red blood cells was measured in Professor Calder's laboratory and by his team, using gas chromatography. This allowed the separation of nineteen fatty acids including; Myristic acid (14:0); Palmitic acid (16:0); Palmitoleic acid (16:1n-7); Stearic acid (18:0); Oleic acid (18:1n-9); Vaccenic acid (18:1n-7); Linoleic acid (LA) (18:2n-6); Gamma-linolenic acid (GLA) (18:3n-6); Alpha-Linolenic acid (ALA) (18:3n-3); Arachidic acid (20:0); Gondoic acid (20:1n-9); Eicosadienoic acid (20:2n-6); Dihomo-gamma-linolenic acid (20:3n-6); Arachidonic acid (20:4n-6); Behenic acid (22:0); Eicosatetraenoic acid (ETA) (20:4n-3); Eicosapentaenoic acid (EPA) (20:5n-3); Docosapentaenoic acid (DPA) (22:5n-3); and Docosahexaenoic acid (DHA) (22:6n-3).

The red blood cell samples were removed from the storage freezer and thawed. The red cell lipids were then extracted from the sample by Folch extraction. Each sample (0.8 ml) was placed into labelled test tubes and 5 ml of chloroform/methanol (2:1) was added to the samples. The specimens were vortexed in order to cause homogenisation. Sodium chloride solution (1 ml of 1M) was then added and the samples were vortexed again and then centrifuged at 1000 g for 10 minutes, this caused separation of the chloroform/methanol and aqueous layers (with a protein plug in between) and partitioning of the lipids into the organic (chloroform/methanol) phase. The lipid layer was then removed from the base of the test tubes using a pipette and placed into clean test tubes. The samples were placed onto a drying plate, heated to 40°C and nitrogen was blown through the solutions in order to remove the chloroform/methanol.

The fatty acids within the lipid extracts were then methylated in order to produce fatty acid methyl esters (FAMEs); to do this a methyl group is added to the carboxylic acid end of the fatty acid by reaction with a methyl donor (methanol) in the presence of a sulfuric acid catalyst. The methylation process is done in order to lower the boiling point of the fatty acids and allows increased separation of the fatty acids during gas chromatography (GC) at more moderate temperatures. Toluene (0.5 ml) was added to each sample in order to dissolve any nonpolar lipids. 1 ml 2% sulfuric acid in methanol was then added to the samples and they were vortexed. They were then heated to 50°C for two hours in order to allow methylation to occur.

After two hours, the samples were removed from the heat plate and 1 ml of neutralising agent (potassium hydrogen carbonate and potassium carbonate) was added to each tube, followed by 1 ml of hexane. The samples were vortexed and then centrifuged at 250 g for 2 minutes. The toluene and hexane formed the top layer of the samples following centrifugation and this was

removed using a pipette and placed into labelled test tubes. The samples were then placed on the drying plate, heated to 40°C and nitrogen was blown through them in order to remove the toluene and hexane. Hexane (75 µl) was added to the test tubes and the samples were vortexed, the sample was then transferred by pipette to the GC vials. A further 75 µl was added to the test tube and the process repeated.

The samples are then placed on to the GC in order to separate and quanitify the FAMEs. Each sample (1 µl) was rapidly heated to between 250°C and 300°C in the injection port and carried into the column by hydrogen. The fused silica capillary column has a lower initial temperature than the injection port and so the FAMEs condense (however the hexane, in which the FAMEs are dissolved, is very volatile and so does not condense). The column is then heated incrementally, separating out the FAMEs as their boiling point is reached. The temperature at which each FAME becomes volatile is dependent on the carbon chain length (longer chain length FAMEs have increased boiling point) and the number and position of the double bonds (more double bonds in a FAME decreases the boiling point). In turn, each FAME then reaches the flame ionising detector (FID) at the end of the column. The hydrogen flame causes combustion of the FAME and this generates an ion current that is proportional to the amount of FAME in the sample. A chromatogram is produced and each FAME produces a peak, the area of which allows the proportion of that fatty acid (as a percentage of total fatty acids) to be calculated.

2.8.3.2 Vitamin D

Serum vitamin D concentrations were analysed in Laboratory Medicine, Department of Pathology at University Hospital Southampton NHS Trust, using serum aliquots taken from the SST tubes, by Liquid Chromatography/Tandem Mass Spectrometry. 25-OH-Vitamin D2 and D3 were freed from their binding proteins by mixing 150 µl of serum with 150 µl of propan-2-ol and water (50:50 mix), containing an internal standard. The internal standard and 25-OH-Vitamin D2 and D3 were then extracted using supported liquid extraction (SLE) technology. This works by adding the serum to a plate containing a support material, the serum forms a layer of immobilised aqueous droplets over the surface of the material. Analyte elution then occurs by adding a water immiscible, organic extraction solvent (heptane, 750 µl followed by a second 750 µl 5 minutes later) and pressure. This causes the release of the analytes whilst other components such as proteins and phospholipids remain dissolved in the aqueous droplets on the material. The clean organic solvent containing the analyte is collected, dried and reconstituted into a mobile phase using 70 µl of 2 mM Ammonium Formate (aqueous) with 0.1% formic acid and 30 µl of 2 mM Ammonium Formate (99% Methanol and 1% aqueous) with 0.1% formic acid.

The extracts were then analysed by liquid chromatography (allowing the separation of the 25-OH-Vitamin D2 and D3) followed by triple quadrupole mass spectrometry. Liquid chromatography works by separating the analytes on a chromatography column. The column contains a stationary phase (containing long alkyl chains containing 8 to 18 carbons which absorb analytes that are soluble in organic solvents). The sample is injected and then absorbed onto the stationary phase, desorbed and then driven further down the column where the process recurs. The chemical properties of the analyte are what causes time differences in the absorption and desorption and hence separation. Once separation has occurred, the analytes are introduced to the mass spectrometer through an electrospray ionisation source. A triple quadrupole mass spectrometer consists of two mass analysers with a linear accelerator in between, resulting in three quadrupoles or stages. The first (Q1) filters the sample according to the mass to charge ratio of the ions it contains. Q1 contains four rods; two rods have a positive DC (direct current with the charge always flowing in one direction) voltage applied and the other two a negative DC voltage, all four rods have an AC (alternating current) radio frequency voltage applied to them. The voltages change; as they change and the ions are accelerated between the rods only certain ions with a specific mass to charge ratio (resonant ions) are able to move through them, the remainder collide with the rods and lose their charge; these are then removed by a vacuum pump. The resonant ions then move into the second quadrupole (Q2). Q2 is a non-mass resolving radio-frequency only quadrupole, this means that no DC voltage is applied so that all ions are able to move through this stage. Within Q2, the ions are forced to collide with a neutral gas in order to fragment the ions; this is known as collision induced dissociation. These fragments then accelerate towards the third quadrupole (Q3), where once again they are filtered according to their mass to charge ratio, using the same method as in Q1. The 25-OH-Vitamin D2 and D3 then reach the detector and can be measured.

2.8.3.3 Folate

Serum folate was analysed in Laboratory Medicine, Department of Pathology at University Hospital Southampton NHS Trust. Blood taken in the SST tube and the Beckman Coulter DXI analyser was used. Serum was analysed using the Access Folate assay, a competitive binding receptor assay.

Sodium ascorbate with hydrochloric acid was added to the serum to release folate from its binding proteins. Folic acid-alkaline phosphatase conjugate was then added and the sample was left to incubate for 10 minutes at 36.5°C. Milk folate binding protein, paramagnetic particles coated with goat anti-mouse IgG/anti folate binding protein monoclonal antibody complexes

and potassium phosphate were added to the solution. The solution was then incubated for a further 15 minutes at 36.5°C. Folate in the sample competed with the folic acid-alkaline conjugate for binding sites on the milk folate binding protein. A wash was then used to remove unbound material, this was performed three times. Lumi-Phos 530 (Lumigen PPD) was added to the reaction, this is an alkaline phosphatase substrate. On reaction with the folic acid-alkaline phosphatase conjugate, Lumi-Phos 530 emits light. Therefore, the less folate in the sample, the more folic-acid-alkaline phosphatase conjugate and hence the more light emitted (measured by a luminometer). The precise amount of folate was calculated using a previously stored, multi-point calibration curve.

2.8.3.4 Vitamin B12

Vitamin B12 was measured in serum prepared from blood taken in the SST tube, using a competitive immunoenzymatic assay on the Beckman Coulter DXI analyser, in Laboratory Medicine, Department of Pathology at University Hospital Southampton NHS Trust.

Vitamin B12 was released from its binding proteins and converted to cyanocobalamin by adding alkaline potassium cyanide and dithiothreitol to the serum. Intrinsic factor ALP was then added and bound to the cyanocobalamin. Paramagnetic particles coated with goat anti-mouse IgG/mouse monoclonal anti-intrinsic factor were added. The intrinsic factor ALP that was already bound to the cyanocobalamin could not bind to the anti-intrinsic factor, the remaining intrinsic factor ALP did. A wash was then performed to remove materials not bound to the paramagnetic particles. The chemilimunescent substrate Lumi-Phos 530 was added to the reaction and the emitted light measured. The light produced was inversely proportional to the level of vitamin B12 and concentrations were calculated using a multi-point calibration curve.

2.8.3.5 Vitamin B6

In order to assess the Vitamin B6 status of the patients, Pyridoxal-5-Phosphate (P-5-P) was measured. This is the metabolically active coenzyme form of vitamin B6 and is the main circulating form released from the liver. This analysis was performed by Christiaan Gelauf in the WISH laboratory at SCBR, University Hospital Southampton NHS Foundation Trust.

Aliquots of plasma processed from the EDTA blood tubes were thawed with a P-5-P standard. Plasma (400 µl) was added to semicarbazide hydrochloride/glycine reagent (32 µl) and the solution was vortexed. The tubes were placed at room temperature and left for 20 minutes; this

allowed a reaction with the P-5-P to form a fluorescent semicarbizone derivative catalysed by the glycine.

Following incubation, 32 µl of phosphino carboxylic acid (PCA) was added to each tube in order to remove proteins within the sample. Each tube was vortexed and then left at 4°C for between 5 and 10 minutes. Following this the tubes were centrifuged at 10°C and 10000 g for 10 minutes.

Whilst the solution was being centrifuged, 25 µl of 25% sodium hydroxide (NaOH) was added to labelled amber chromatography vials, these vials were used to protect the sample from light. Following centrifugation, 250 µl of the supernatant was pipetted into each labelled chromatography vial and the vial was vortexed. The NaOH was added to neutralise the sample.

Standards containing a known concentration of vitamin B6 were prepared in the same way. Samples were stored at 4°C in the dark until analysis.

The HPLC (high pressure liquid chromatography) buffer was prepared by dissolving 8.8 g of disodium hydrogen orthophosphate and 400 mg of disodium EDTA in 700 ml of distilled water. HPLC grade methanol (200 ml) was then added and the pH of the solution was adjusted to 7.3 by using a drop wise method to add concentrated phosphoric acid. The solution was made up to a 1L using distilled water and the whole solution was filtered through 0.45 µm filter paper prior to use.

The Beckman System Gold HPLC was used for analysis of the samples. The samples were injected and pumped through the liquid mobile phase (buffer) into the column or stationary phase. The different compounds within the sample were then separated according to their polarity and eluted from the column at different time points. The fluorescence at that time point was measured using a fluorimeter (PerkinElmer, UK) and gave a chromatogram for each sample. The area of the peak known to be P-5-P was measured and compared to the known standards in order to ascertain the concentration of P-5-P or vitamin B6 in the sample.

2.9 Semen sampling and processing

2.9.1 Collecting the sample

The male participant was asked to abstain from ejaculation for at least two days and a maximum of seven days prior to providing a sample for the trial. The sample was produced by

masturbation into a clean container. The sample was then placed in the laboratory to undergo analysis; the private room in which the male participant produced his sample was near the laboratory in order to avoid fluctuations in temperature during this time.

2.9.2 Semen analysis

Prior to processing for storage, semen analysis was performed by an andrologist on all samples produced by the participants.

The sample was placed in the warming oven, at 37°C, for a minimum of 15 minutes before the analysis was begun. Once the pot was removed, it was gently swirled to assess liquefaction. If the sample had not liquefied at this point then it was returned to the warming oven for a further 60 minutes; after this a final check was performed and it was recorded whether or not the sample had liquefied. Volume and viscosity were then assessed; the semen sample was drawn into a serological pipette and the volume measured and recorded. While drawing the semen into the serological pipette, the viscosity was also observed. If threads of more than 2 cm were observed then the sample was found to be viscous, whereas if the sample was expelled from the pipette in droplets then the sample was documented as not viscous. The sample was considered normal if it liquefied within one hour and was not viscous.

The next variables to be examined were sperm concentration and motility. Seven microliters of the sample were pipetted into the chamber on a Leja slide, ensuring the chamber was completely filled. The Leja slide was then placed in the warming oven for 60 seconds. The slide was then examined under the microscope (using an objective magnification of x20). The distribution of the sperm was checked to ensure whether it was uniform or not as this would have implications for the results. Using the aid of a counter, the number of motile sperm in a 30 square area was counted and recorded. This process was repeated, in the same area for non-progressive motile, and non-motile sperm and then for all three extents of motility in a further two areas. At this point, if more than 200 sperm in total had been counted then the concentration was determined, if not then further areas were counted until this target was reached. The concentration was calculated using the equation:

$$\frac{\text{Total number of sperm counted}}{\text{Number of areas assessed}} = \text{Concentration of sperm (million per ml)}$$

The percentages of motile sperm and progressively motile sperm were then calculated. Motile sperm are sperm that are moving in any direction, whereas progressively motile sperm are those which are predominantly moving in a straight line.

If fewer than 5 sperm were seen in the 30 square area then the number of areas assessed was increased to 10 (i.e. 300 squares) in order to allow an accurate concentration to be calculated.

Over 15 million spermatozoa per ml of ejaculate was considered normal with a progressive motility of at least 32%.

In samples in which the sperm concentration was assessed to be greater than 5 million/ml, sperm morphology was also examined. 2.5 µl of sample was pipetted onto the centre of the stained area of a Testsimplets slide. A coverslip was placed over the sample and gently pressed down so that the sample covered the area beneath it. The slide was examined under oil after a minimum of 15 minutes and a maximum of 4 hours using a 100x objective. A minimum of 200 sperm were evaluated and scored as either "normal" or "abnormal". For a spermatozoon to be considered as "normal" then its head and tail had to both be normal. According to the *WHO Laboratory Manual for the Examination and Processing of Human Semen*, the head had to be smooth and oval with a well defined acrosomal area (comprising 40-70% of the head area). The acrosomal area had to contain no large vacuoles and not more than two smaller vacuoles. The tail needed to be approximately 45 µm long (ten times the length of the head). The tail could be looped back but there could not be a sharp angle as that would have suggested a flagellar break. All borderline forms were considered abnormal and the sample was considered normal if 4% of more of the sperm were found to be normal.

Aggregation and agglutination or "clumping" was also assessed. The percentage of sperm that were sticking to one another and forming immotile clumps was recorded and a percentage of greater than 30% was considered abnormal.

Finally, following this initial analysis, the percentage of anti-sperm antibodies was assessed using the mixed antiglobulin reaction (MAR) test. 5 µl of latex beads were pipetted onto a labelled microscope slide, followed by 5 µl of antiserum and finally 5 µl of the semen sample. The sample was then thoroughly mixed, the coverglass applied and the slide was left for 5 minutes before assessment. The slide was examined using 20x objective, the percentage of motile sperm with beads bound was recorded (having evaluated at least 200 free swimming sperm). The significance of the presence of antisperm antibody is not fully understood and therefore in accordance with WHO criteria a value of greater than 50% was considered abnormal.

2.9.3 Processing the semen for storage

Following the semen analysis, the semen were prepared for storage. Prior to the dietary intervention, the whole sample was processed and stored; however the sample produced on the day of egg retrieval (post dietary intervention) was primarily used for treatment and any remaining sample was stored for later analysis.

A density gradient to separate the seminal fluid and sperm was created by placing 1 ml of Nidacon PureSperm®100 90% solution in the bottom of a conical centrifuge tube. A layer of 1 ml of Nicadon PureSperm®100 45% solution was then pipetted gently onto the top, taking care not to disturb the interface between the two layers. 2 ml of seminal plasma (if available or the whole sample if the volume or remaining volume was smaller) was carefully placed onto the density gradient, using a pipette and again taking care not to disturb the interface. The sample was centrifuged at 400 g for 20 minutes. Following this, the top layer of seminal plasma was removed from the sample and divided into 1 ml aliquots, which were pipetted into labelled cryovials and placed into the -80°C freezer. The supernatant was then removed from the sample and discarded. 10 ml of PBS was added to the conical centrifuge tube, which now contained only the sperm pellet at its base. The tube was then centrifuged again at 400 g for 5 minutes. This was performed in order to wash the spermatozoa. The supernatant was then removed and discarded and the sperm pellet was placed into a labelled cryovial and stored in the -80°C freezer to await analysis.

In 58 of the couples, semen was also stored for DNA fragmentation analysis. Semen was left to liquefy for at least 30 minutes following ejaculation, between 100 µl and 200 µl was then placed into a labelled aliquot tube. The aliquot was plunged into liquid nitrogen (-196°C) to snap freeze and then stored at -80°C until analysis.

2.9.4 Semen, sperm and seminal fluid analysis

2.9.4.1 Sperm and seminal fluid fatty acid analysis

The fatty acid analysis of the seminal fluid and sperm was carried out using the same techniques as for the red blood cell analysis (see section 2.7.3.1).

2.9.4.2 Sperm DNA damage

Sperm DNA fragmentation was analysed by Professor Lewis's team at Lewis Testing Facility in association with Queen's University Belfast. Sperm DNA damage was assessed using the SpermComet assay, a second generation sperm DNA test which allows the percentage of DNA damage in each sperm that is analysed to be measured. A collaboration was formed with Professor Lewis. This assay was used as it only requires approximately 5000 sperm and therefore was suitable for the assessment of the small volumes of sample left over following clinical use. Furthermore, it provided a quantifiable result appropriate for statistical analysis (Lewis, 2013).

2.10 Follicular fluid and sampling and analysis

The collection of follicular fluid is described in chapter 4 and its fatty acid content was analysed as described in section 2.7.3.1.

2.11 Embryo morphology and morphokinetic markers analysis

As previously described in these methods, the embryos were cultured in a validated time-lapse incubator and during the incubation, twenty one plane focal images were generated every hour.

2.11.1 Morphology assessment of embryos

Morphological assessment of the embryos was made by the Complete Fertility embryology team contemporaneously, in order to allow them to decide which embryo was most suitable for transfer and which should be cryopreserved or discarded. The embryos were analysed on day 1 to assess whether normal fertilisation had taken place, this was done by ensuring that two pronuclei were present (one containing female and one male DNA). Embryos with fewer or greater than two pronuclei were discarded. This initial period of incubation was carried out in MINC benchtop incubators; once it was ascertained that the embryo had fertilised normally it was placed onto an EmbryoScope dish in 25 µl of media and cultured in the EmbryoScope.

A further morphological assessment of the embryos was made on day 3. At this point, the number of cells was assessed and the embryo was given a grade between 1 and 4 depending on

whether the cells were even in size, the amount of fragmentation of the cells and whether any multinucleation was seen (see Table 2.2).

Table 2.2: Grading of day 3 embryos

Grade of embryo	Amount of fragmentation		Cell size even?		Multinucleation present?
1	<5%		Yes		No
2	6 – 20%	And/or	No		No
3	20 – 50%		No	And/or	Yes
4	>50%		No		Yes

An 8 cell embryo with 20% fragmentation was therefore recorded as $8c^2$ on the embryology record sheet.

In order to assess the morphological grade of the embryos on day 3, each embryo was given a score according to its grade, the scoring system was as follows (see Table 2.3):

Table 2.3: Method of converting morphological grades on day 3 into scores.

Morphological embryo grade	Score
$7c^1 – 10c^1$	1
$7c^2 – 10c^2$	2
$6c^1$ or $6c^2$ or $>10c^1$ or $>10c^2$	3
$<5c^1$ or $<5c^2$ or $1c^3 – 14c^3$	4

The next morphological assessment of the embryos was carried out on day 5 using Gardner's blastocyst grading system. The number related to the blastocyst development stage, the first letter to the ICM quality and the second letter to the TE quality (see Table 2.4, Table 2.5 and Table 2.6).

Table 2.4: Scoring system for blastocyst expansion

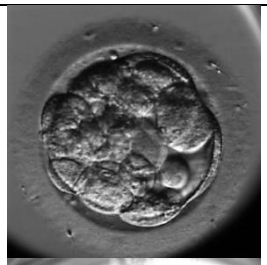
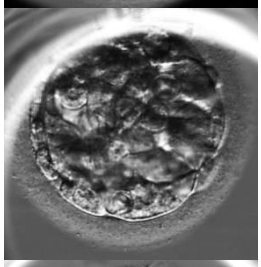
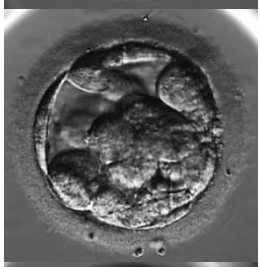
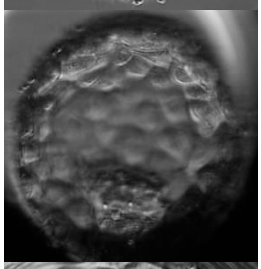
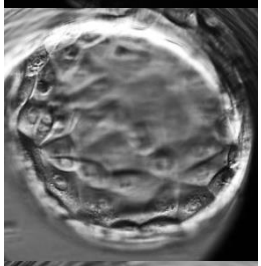
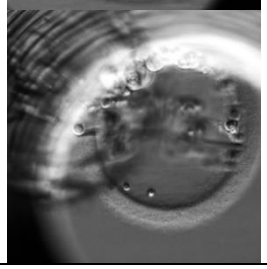
Grade of blastocyst expansion	Description	Example
1	Blastocoel (cavity forming within the mass of cells) is less than half the volume of the embryo	
2	Blastocoel is more than half the volume of the embryo	
3	Fully formed blastocyst (cavity filling the embryo)	
4	Blastocyst expanding in size causing thinning of the zona pellucida	
5	Blastocyst has started hatching out of the zona	
6	Blastocyst has completed hatching	

Table 2.5: Scoring system for inner cell mass at blastocyst stage (ICM)

Grade of ICM	
A	Large number of tightly packed cells
B	Smaller number of loosely grouped cells
C	Very few cells
(D	Absent or degenerating ICM – not in Gardner's grading but used here)

Table 2.6: Scoring system for trophectoderm at blastocyst stage (TE)

Grade of TE	
a	Large number of cells, forming a cohesive layer
b	Smaller number of cells, forming a loose epithelium
c	Very few large cells
(d	Absent or degenerating TE – not in Gardner's grading but used here)

In order to assess the blastocyst morphology, the grades were converted using the following scoring system (see Table 2.7).

Table 2.7: Method of converting morphological grades of ICM and TE on day 5 into scores.

Morphological blastocyst grade of ICM and TE	Score
AA	1
AB or BA	2
BB	3
BC or CB	4
CC or CD or DC or DD	5
1-- or 2--	6

On day 5, a top quality blastocyst would be expected to have an expansion grade of 3, 4 or 5 with a score of A/a or B/b for both the ICM and the TE. Embryos with these scores were selected for transfer or were cryopreserved. Embryos were also assessed using the same grading system on day 6. At this point, they were expected to have an expansion grade of between 4 and 6 and a score of A/a or B/b for the ICM and the TE, blastocysts achieving these results were cryopreserved.

Day 5 and 6 embryos could be graded if they had not yet made the blastocyst stage) (see Table 2.8).

Table 2.8: Alternative grading for embryos on day 5 or 6 that have not yet reached the blastocyst stage

Grade		Score
M	Morula	
CM	Cavitating morula (showing first stages of cavitation)	7
Nec	Necrotic – degenerating embryo	
Arr	Arrested – cleavage stage embryo which has not yet compacted or cavitated.	8

2.11.2 Morphokinetic assessment of embryos

The embryos were annotated retrospectively, but prior to the trial being unblinded, by the embryology team at Complete Fertility, Southampton. The time points that were marked were tPNf (the time the pronuclei fade) t2, t3, t4, t5, t6, t7, t8 and t9 (the time at which the embryo reaches the two cell, three cell, four cell, five cell, six cell, seven cell, eight cell and nine cell stage). These then allowed the calculations of the second, third and fourth cell cycle lengths (CC2, CC3 and CC4) and the synchrony of the second cell cycle and the third cell cycle (S2 and S3). Furthermore, the time points were noted when the embryo formed a morula (tM), started blastocyst formation (tSB), formed a blastocyst (tB), formed an expanded blastocyst (tEB) and a hatching blastocyst (tHB). If the embryo died then the time point at which this occurred was also noted (tDead). These time points were then used to calculate the day 3 (see Figure 2.1) (Petersen et al., 2016) and day 5 known implantation data scores (KIDScores). The day 5 KIDScores were calculated by Professor Markus Montag as the algorithm has not yet been published and therefore is not in the public domain.

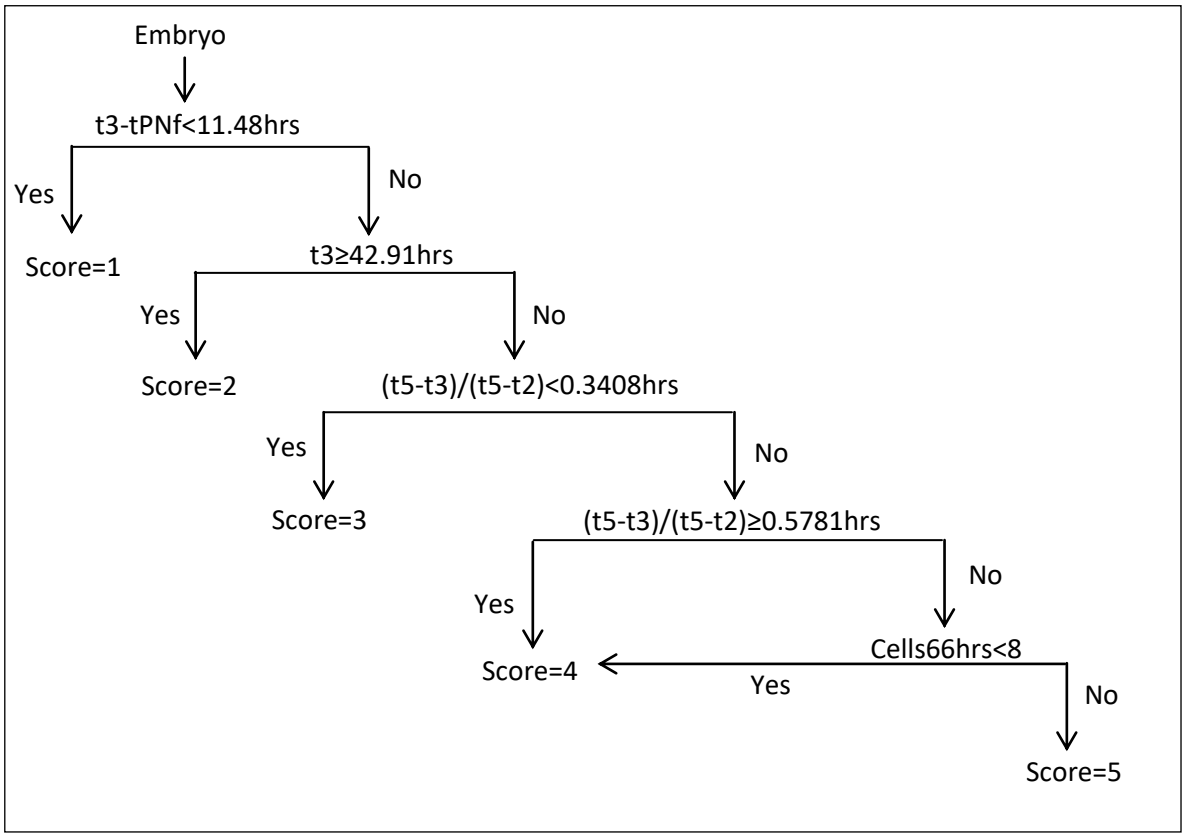


Figure 2.1: Algorithm for the calculation of KIDScore on day 3

2.12 Primary and secondary endpoints

The primary endpoint was the mean period in hours for the cleaved embryos from participants in each arm of the study to develop from the 2 cell to the 3 cell stage (CC2). Secondary endpoints included using the EmbryoScope to examine embryo quality, including S2 (synchrony during second set of cleavage divisions), t5 (time between fertilisation and the five cell embryo formation) (Meseguer et al., 2011, Hashimoto et al., 2012), CC3 (duration of the third cell cycle) (Hashimoto et al., 2012), and the disappearance of the pronuclei (tPNf) (Azzarello et al., 2012). The KIDScores on day 3 and day 5 were analysed as a secondary endpoint as these incorporated a number of morphokinetic markers of embryo quality (see section 2.11.2). The metabolomics of the embryos cultured including pyruvate and glucose consumption, lactate production and amino acid turnover was examined in the spent media. Nutritional markers, including the concentration of fatty acids, Vitamins D, B12, and B6, folate, were measured in blood pre and post intervention in both male and female participants. In male participants, changes in semen quality pre and post dietary intervention was recorded according to standard parameters,

changes in seminal fluid and sperm fatty acid profiles were analysed and changes in sperm DNA fragmentation.

2.13 Data collection

Data collection was performed using a CRF. Data relating to clinical treatment outcomes, including embryo quality measurements was acquired from the Complete Fertility Centre's electronic data collection system (IDEAS). Questionnaire responses were input electronically into the CRF. Additional data relating to laboratory assays was obtained directly from the laboratory's database or the respective external laboratories and entered into the CRF. This minimised the risk of transcription errors; however, all data was verified by double data entry and cleaned prior to analysis.

2.14 Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Results are recorded as mean ± standard deviation unless otherwise stated. Differences between the sociodemographic characteristics of participants in the two groups were analysed using ANOVA; characteristics that were not normally distributed and were scalar were adjusted by log transforming and then included in the ANOVA analysis.

ANOVA was also used to compare the levels of fatty acids, vitamin D, B12, B6 and folate in the blood of the participants in the two groups. Results that were not normally distributed were log transformed and then included in the analysis.

Other statistical methods have been described in their relevant chapters.

Chapter 3: Results: The effect of a six week dietary supplement of omega-3 fatty acids and vitamin D on *in vitro* embryo development (PREPARE TRIAL)

3.1 Introduction

A growing body of evidence from prospective cohort observational studies indicates that the peri-conceptional nutritional status of both men and women impacts on early fetal development, perinatal and long term health of the offspring, and fertility (Inskip et al., 2006). However, despite these studies and the rapidly expanding nutraceutical industry targeting women during the preconception and pregnancy periods, there is limited and often confusing evidence about which diet or micronutrient supplements couples planning, or in the early stages of a pregnancy, might consider using. Furthermore, women planning a pregnancy rarely alter their nutritional intake (Crozier et al., 2009); rather they tend to continue their habitual diet, even when inadequate in terms of saturated fat and essential micronutrient content (de Weerd et al., 2003). This may reflect uncertainty as to the optimal dietary intervention, or the perceived burden of a prolonged dietary change, particularly for those couples who require fertility treatment.

In recent years, a growing number of observational studies have indicated that variations in preconceptional diet may impact fertility, embryo quality and hence fecundity in couples undergoing IVF. Particular interest has been afforded to the possible benefits of a Mediterranean diet. Important components of the Mediterranean diet are considered to include olive oil, omega-3 FAs from seafood and vitamin D. The intake of the omega-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is low in many pregnant women (Miles et al., 2011, Sioen et al., 2017). Cohort studies have suggested that increased dietary intake of DHA and EPA is associated with improved embryo morphology (Hammiche et al., 2011) and omega-3 FA supplements been reported to have a positive effect on men's fertility, potentially affecting the concentration, motility and morphology of the spermatozoa (Safarinejad, 2011, Attaman et al., 2012).

A prospective observational study reported that a 'Mediterranean' diet high in vegetable oils, fish, vegetables and legumes and low in carbohydrate-rich snacks was positively associated with red blood cell folate and vitamin B6 in blood and follicular fluid and with a 40% reported increase in the probability of achieving a pregnancy (Vujkovic et al., 2010). A subsequent case control study also suggested that fertility outcomes were improved in couples adhering to a Mediterranean diet (Toledo et al., 2011), and a further study of men and women undergoing IVF/ICSI reported a 65% increase in the chance of achieving an ongoing pregnancy in those who described eating a Mediterranean–style diet in the week prior to treatment (Twigt et al., 2012). More recently, an observational study reported that higher serum omega 3 fatty acids (FAs) in

women undergoing IVF were associated with an increased chance of clinical pregnancy and live birth (Chiu et al., 2018).

Vitamin D has also been implicated as a key factor in fertilisation, affecting sperm-egg binding and the activity of acrosine which digests the zona pellucida (Dabrowski et al., 2015), and serum levels have been associated with pregnancy rates after IVF (Mnallah, 2017).

The use of morphokinetic markers to assess embryo quality provides a more standardised and objective measurement than morphology alone (Sundvall et al., 2013) and there is a growing body of evidence that demonstrates strong correlations between morphokinetic markers and the embryo's ability to develop into a blastocyst (Wong et al., 2010, Dal Canto et al., 2012, Milewski et al., 2016), chance of implantation (Campbell et al., 2013, Freour et al., 2013, Meseguer et al., 2011) and likelihood of resulting in a clinical pregnancy (Milewski et al., 2016) and live birth (Azzarello et al., 2012). Critics have postulated that morphological evaluation of embryos alone is subjective and may not provide the best method to predict chromosome status of a blastocyst (Alfarawati et al., 2011) or improve the probability of implantation (Stokes et al., 2007). However, concerns have also been raised about time-lapse parameters, including variation in timings due to confounding factors such as insemination method (Cruz et al., 2013), the type of gonadotrophins used for stimulation (Muñoz et al., 2012) and age (Campbell et al., 2014). These methods are the only non-invasive approaches to assess embryo quality; however, studies examining correlations between the two remain sparse.

While a growing body of literature points to beneficial effects of the key components of the Mediterranean diet on fertility and IVF outcomes, there remains a lack of randomized, blinded controlled trials. Moreover, the duration of such a preconceptional dietary intervention necessary to achieve measurable effects on morphological markers of pre-implantation embryo development is not known. While it has been proposed that the intervention should be of sufficient duration to cover a three month period of in-vivo gamete maturation (van Dijk et al., 2017), it has been shown in rodent studies that a very short dietary manipulation of just three days prior to implantation can have a profound effect on embryo development (Fleming, et al., 2011), suggesting that a shorter diet manipulation prior to IVF might provide significant benefit without requiring excessive delay to starting treatment. The PREconception dietary suPplements in Assisted REproduction (PREPARE) trial (Kermack et al., 2014) was a prospective, double blinded randomized controlled trial investigating whether a drink high in omega-3 FAs (both EPA and DHA) and providing vitamin D taken by both the man and the woman for 6 weeks prior to conception by IVF improves embryo quality. Performing a trial such as this in couples undergoing

assisted reproductive technology (ART) provides the opportunity to examine the effect of the dietary intervention on early embryo development.

There is limited research into the acceptability and feasibility of clinical trials in IVF but it is recognised that women undergoing ART are a vulnerable group of individuals (Haimes et al., 2012). Examining the couples' experience is therefore an important part of the research process to inform future research protocols and to protect this group of patients.

3.1.1 Aim

The aims of this chapter are to:

- Assess the compliance of couples undertaking a dietary supplements trial prior to IVF and understand their experiences of taking part.
- To determine correlations between morphological and morphokinetic markers of embryo quality.
- Investigate the changes on the fatty acid composition of erythrocytes and serum vitamin D levels in participants of the PREPARE trial.
- Examine the effect of the six week dietary intervention on morphological and morphokinetic markers of embryo quality.

3.1.2 Hypotheses

Our hypothesis is that a diet rich in marine omega-3 FAs and Vitamin D will be beneficial to a developing embryo and thus improve morphokinetic markers used to predict embryo viability and implantation.

3.2 Methods

3.2.1 PREPARE trial

The PREPARE trial was carried out as described in Chapter 2: Methods: The "PREPARE" trial. The demographic details of the women were collected and their diet and lifestyle assessed (see section 2.7).

3.2.2 Blood sample analysis

Blood samples were collected from the women and men and analysed as described in Chapter 2.8.3.

3.2.3 Assessment of embryo quality

Embryos were analysed using morphology on day 3 and 5 and morphokinetic markers to assess their quality (see Chapter 2.11).

3.2.4 Pregnancy outcomes

Whether or not the patient had a positive pregnancy test following embryo transfer (9 days after a day 5 embryo transfer or 11 days after a day 3 embryo transfer) was recorded.

Patients were scanned at approximately 7 weeks gestation, to ensure that the pregnancy was intrauterine and a fetal heart beat could be seen.

At birth, gestation duration, birthweight and sex of the infants who delivered after 24 weeks gestation were recorded.

3.2.5 Statistical analysis

Results are reported as mean ± standard deviation unless otherwise stated. Differences between the sociodemographic characteristics of participants in the two groups were analysed using ANOVA; characteristics that were not normally distributed and were scalar were adjusted by log transforming and then included in the ANOVA.

ANOVA was also used to compare the levels of fatty acids and vitamin D in the blood of the participants in the two groups. Results that were not normally distributed were log transformed and then included in the analysis.

A mixed effect model was used to analyse the effect of the intervention on the morphokinetic markers of embryo quality. A random effect was fitted for each couple and a fixed effect for treatment and the methodology (i.e. IVF or IVF-ICSI) used to inseminate the embryo. Treatment effects are summarised by the regression coefficient and its standard error. The intra-class correlation coefficient measures the within to between couple variation.

3.2.6 Assessment of the trial experience

Ten of the couples participating in the PREPARE trial underwent a semi-structured interview, performed by a psychologist (not previously involved in the trial). Couples were questioned on their motivation for participating in the trial, the acceptability of the six week dietary intervention, their willingness to delay their IVF for a longer period in order to extend the trial, their experiences of taking part with their partner and the support of the research team. The interviews were recorded, transcribed and then cleaned. A thematic analysis was performed (by the psychology team) and themes were developed using Braun and Clarke's (2006) six phase procedure for thematic analysis.

3.3 Results

3.3.1 Who was eligible to participate in the PREPARE trial and who was recruited?

One hundred and eleven couples were recruited to the PREPARE trial between February 2014 and November 2015. During this time, 372 couples were screened for eligibility, given the patient information leaflet and talked to about the trial (see figure 3.1).

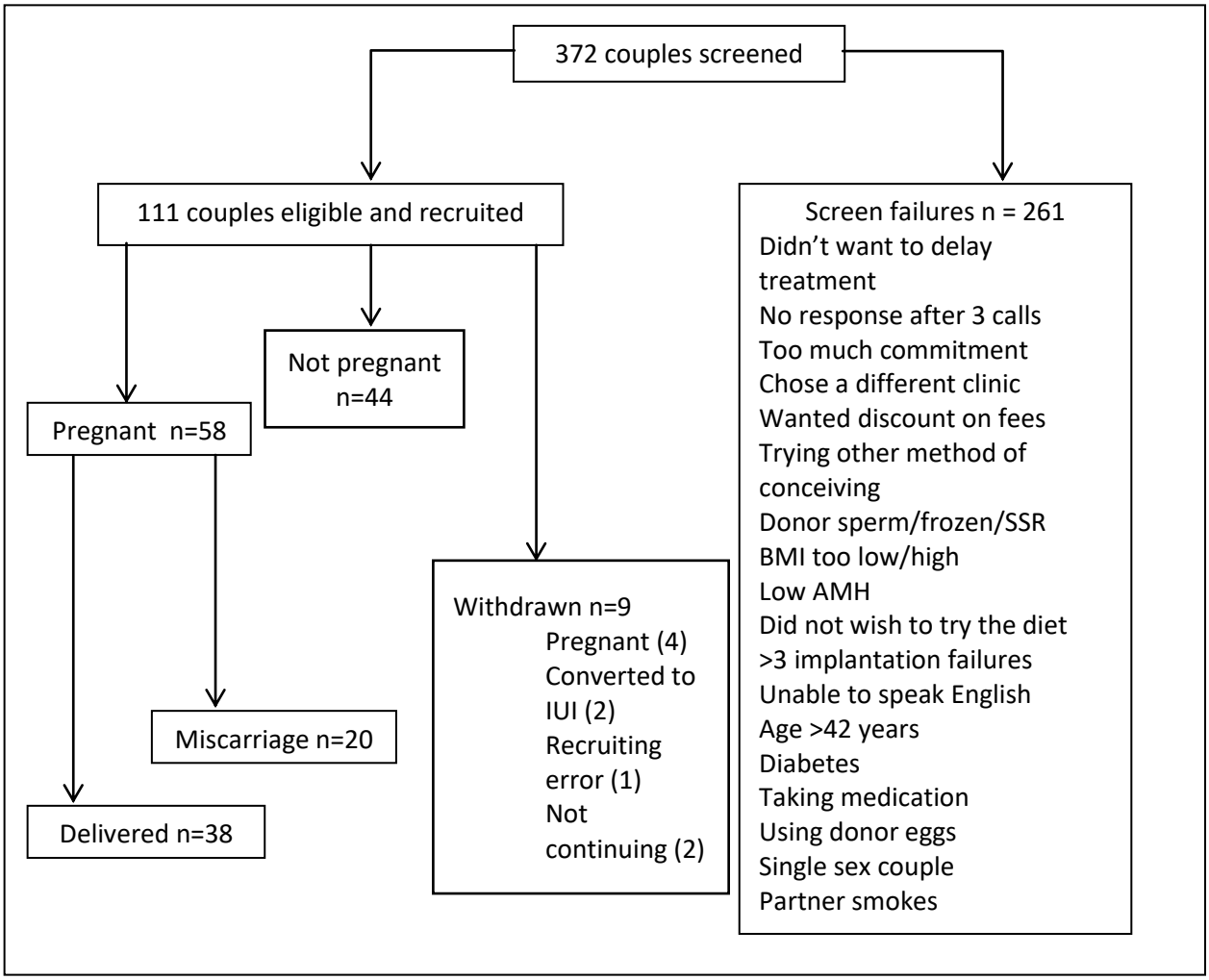


Figure 3.1: Consort diagram for the PREPARE trial

Two hundred and sixty one couples were not eligible or did not want to participate in the study for a variety of reasons, most commonly because they did not want to delay treatment or they felt that participating in the trial would be too much commitment (when combined with the already great time pressure of an IVF cycle).

3.3.2 What were the basic characteristics of couples participating in the PREPARE trial?

Ninety one per cent of the women and 93.7% of the men described themselves as having a white Caucasian ethnicity. Other ethnicities identified in the women included Indian (3.6%), Chinese (1.8%), Bangladeshi (0.9%), Black Caribbean (0.9%) and other (1.8%). A similar pattern was seen in the male participants with Indian (1.8%), Chinese (1.8%), Bangladeshi (0.9%), Black Caribbean (0.9%) and other (0.9%). It should be noted that in the 2011 Census, 86% of the population of Southampton and 93% of the population of Hampshire described themselves as white Caucasian; thus the ethnicity of subjects recruited in PREPARE is similar to that of the population from which they come. The average age of the participants in the trial was 33.4 years ± 4.2 for the women and 36.0 years ± 5.5 for the men. Other demographic details including the age the participants were when they left full time education, BMI, prudent diet score, quantity of alcohol and caffeine consumed and number of hours of exercise per week are described in Tables 3.1 and 3.2. There was no difference in the general characteristics between those randomised to the treatment group when compared to the placebo group (see Table 3.1 and Table 3.2).

Table 3.1: Characteristics of females participating in the PREPARE trial

	Mean ± standard deviation	Range	Mean ± standard deviation within treatment group	Mean ± standard deviation within placebo group	p value
Age (years)	33.4 ± 4.2	22.2 – 41.5	33.3 ± 4.1	33.4 ± 4.3	0.895
Age left full time Education (years)	20.1 ± 3.6	15.0 – 32.0	20.2 ± 4.0	20.1 ± 3.2	0.851
BMI (kg/m^2)	24.7 ± 3.5	17.7 – 33.5	24.3 ± 3.1	25.0 ± 3.9	0.294
Prudent diet score	0.04 ± 1.02	-2.84 – 5.03	0.02 ± 0.9	0.05 ± 1.1	0.903
Amount of alcohol per week (units)	3.8 ± 5.0	0.0 – 30.0	5.9 ± 6.1	5.0 ± 3.9	0.451
Amount of caffeine per day (mg)	111.8 ± 144.9	0.0 – 756.0	131.5 ± 162.0	132.4 ± 138.9	0.975
Number of hours exercise per week (hours)	3.7 ± 3.8	0.0 – 25.0	3.7 ± 3.4	3.7 ± 4.3	0.957

Table 3.2: Characteristics of males participating in the PREPARE trial

	Mean ± standard deviation	Range	Mean ± standard deviation within treatment group	Mean ± standard deviation within placebo group	p value
Age (years)	36.0 ±5.5	24.4 – 51.5	35.6 ± 5.8	36.4 ± 5.3	0.442
Age left full time education (years)	19.5 ± 4.0	14.0 – 32.0	18.8 ± 3.9	20.3 ± 4.1	0.042
BMI (kg/m^2)	27.0 ± 4.0	19.4 – 43.1	26.8 ± 4.0	27.2 ± 4.0	0.571
Prudent diet score	-0.05 ± 1.0	-3.71 – 3.54	0.01 ± 0.9	-0.11 ± 1.1	0.523
Amount of alcohol per week (units)	7.7 ± 7.4	0.0 – 35.0	9.7 ± 6.5	8.6 ± 7.9	0.466
Amount of caffeine per day (mg)	202.2 ± 170.9	0.0 – 648.0	228.1 ± 187.0	203.0 ± 146.0	0.451
Number of hours exercise per week (hours)	5.4 ± 5.4	0.0 – 40.0	5.2 ± 4.8	5.5 ± 6.0	0.775

Participants had a wide variety of occupations (Table 3.3). These were classified as professional, managerial or technical, skilled (either non manual or manual), partly skilled and unskilled. Approximately equal numbers of women had professional (30.6%) and managerial or technical (31.5%) occupations. The majority of male participants were in managerial or technical roles (45.9%), followed by skilled (25.2%) and then professional (18.9%). Less than 5% of women and 2% of men were in unskilled occupations (see Table 3.3).

Table 3.3: Percentages of classifications of occupations of participants in the study.

	Females (n=111)		Males (n=111)	
	Number	**Percentage**	**Number**	**Percentage**
Professional	34	30.6	21	18.9
Managerial and technical	35	31.5	51	45.9
Skilled (non-manual or manual)	18	16.2	28	25.2
Partly skilled	19	17.1	9	8.1
Unskilled	5	4.5	2	1.8

The mean age for leaving full time education was 20.1 years in the women and 19.5 years in the men. Forty one per cent of the women had achieved a degree and 3.6% a HND, with the highest level of academic attainment being A-levels for 18 of the women (16.2%) and GCSEs or equivalents (grade A to C) for 18 (16.2%). Eight per cent (9/111) of the women had no qualifications and no GCSEs at grade C or above. The remaining 14% classified their educational attainment as "other", in the majority of cases this was a higher degree including a Masters or a PhD. Less of the men participating in the trial had attained a degree (34.2%) or a HND (5.4%), a third had completed A-levels as their highest educational achievement and 9.9% GCSEs or equivalent (grades A to C). Eleven per cent (12/111) of the men had no qualifications or GCSEs grade C or above.

The general health of the participants was assessed by analysing their BMI, prudent diet score, alcohol intake, caffeine intake, whether they had felt stressed in the last month and the amount and type of exercise undertaken. Participants were also asked about their use of dietary supplements.

The mean BMI was 24.7 kg/m^2 ± 3.5 in the women and 27.0 kg/m^2 ± 4.0 in the men. The mean prudent diet score was 0.04 ± 1.02 and -0.05 ± 1.00 in the women and men respectively, where a positive score represents a healthier diet and a negative score an unhealthy one. Twenty seven of the women participating in the trial (24.3%) were taking only a folic acid supplement at the point of recruitment; a further 5.4% (6/111) were taking folic acid and additional supplements such as Omega 3 or Vitamin C and/or Vitamin D. Fifty two women (46.8%) were taking a multivitamin and six per cent (7/111) were taking an Omega 3 supplement. It should be noted that all Omega 3 supplements were reviewed and considered low dose (less than 200 mg of EPA and DHA) and therefore the participants were deemed eligible to participate in the trial. Interestingly, 22.5% of women were not taking any supplements at all (see Figure 3.2).

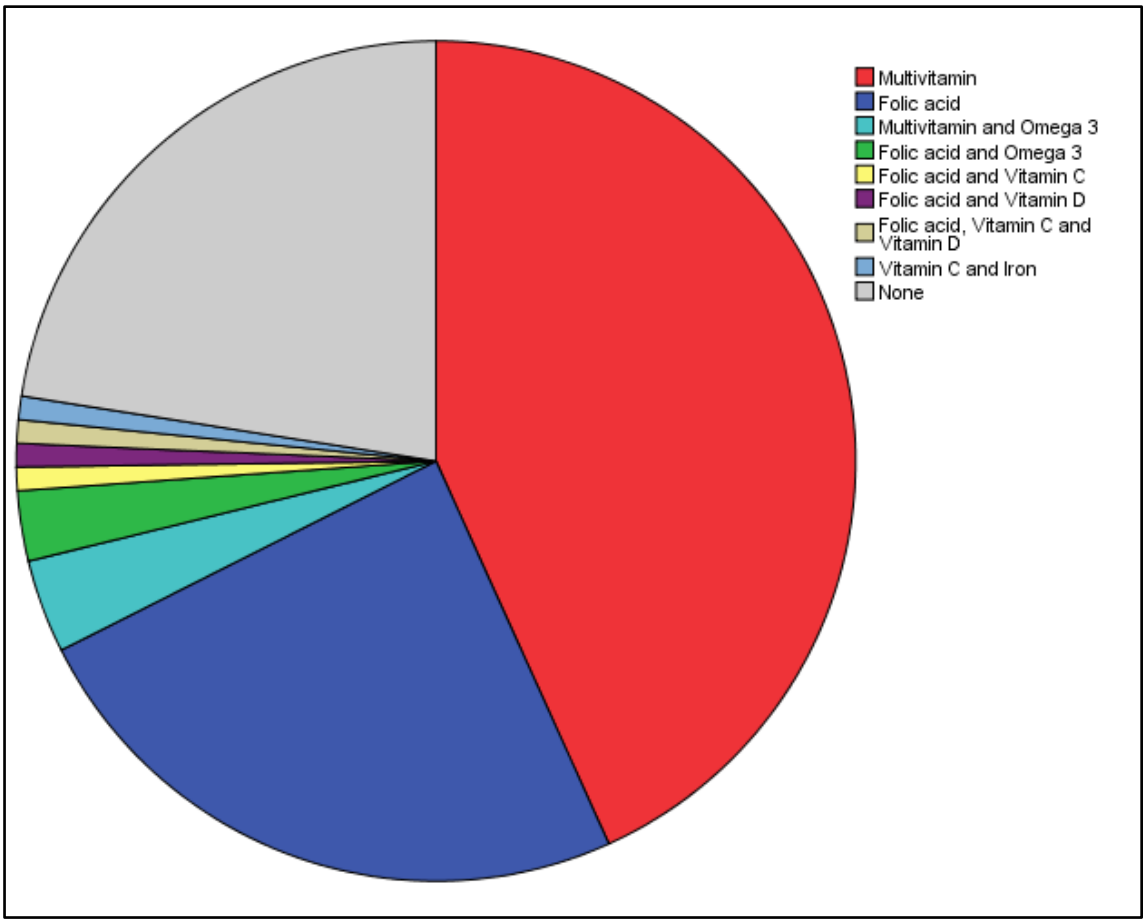


Figure 3.2: Diagram showing the proportion of recruited women taking dietary supplements.

A larger proportion of the male participants were not taking any dietary supplements (55.0%). The majority of those taking supplements were taking multivitamins (64.0%) or multivitamins with Omega 3 (14.0%); protein shakes (2%); Omega 3 and protein shakes (2%); Omega 3 and Zinc (4%); or protein shakes and Zinc (2%). Two participants were taking only protein shakes, one

just Omega 3 and one only Zinc. One participant was taking a combination of protein shakes, Omega 3 and Zinc. One participant was also taking homeopathic supplements (see Figure 3.3).

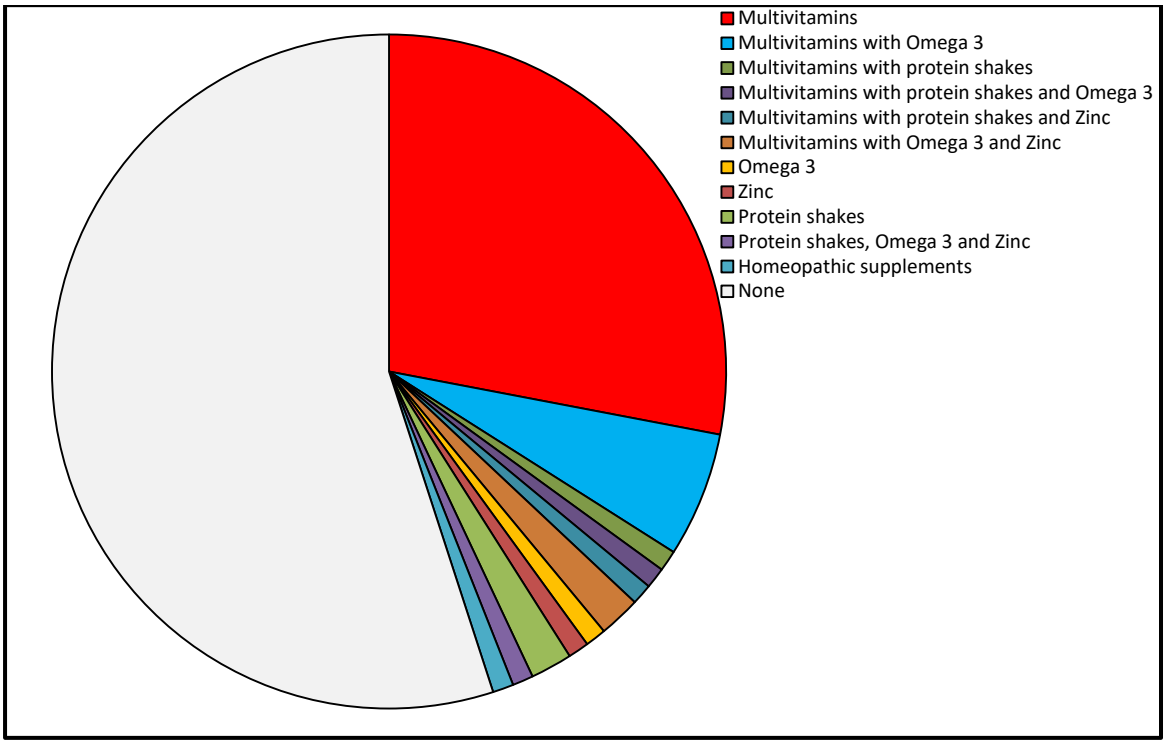


Figure 3.3: Diagram showing the proportion of recruited men taking dietary supplements.

Almost seventy per cent of women participating in the study had drunk alcohol in the last three months. The majority of these (57.6%) drank alcohol on a maximum of two days per week, with only six participants (5.4%) reporting drinking alcohol on four or more days in a week. A small proportion (4.5%) of women described drinking more than the recommended 14 units of alcohol per week, with most of them (83.7%) drinking less than seven units per week. Eighty three per cent of male participants reported drinking alcohol; 73.8% on three or less days during a week and 81% stated they drank less than the recommended 14 units per week. The majority of participants stated they regularly consumed caffeine at the initial questionnaire: 84.7% of women and 93.7% of men. The majority of women drank caffeine on a daily basis (51.4%) but only 4.5% drank over 500 mg per day (thought to be the threshold at which fertility starts to be affected (Bolumar et al., 1997)). The male participants drank more caffeine on average, with 59.5% drinking caffeine daily and 9.0% stating they drunk over 500 mg per day. Thirty one per cent of women and 24.3% of men reported feeling under an unusually high amount of stress in the last month; the most common reasons for which were work related pressures and the toll of the fertility treatment.

The majority of women stated that they exercised between one and two (33.3%) or three and six (31.5%) times per week, with only 7.2% claiming they never did any physical activity and 10.8% claiming they performed exercise on a daily basis. On average, they exercised 3.7 hours per week ± 3.9. The women predominantly performed cardiovascular exercise including running, walking and aerobics. The results were similar in the male participants with 31.5% stating they exercised between one and two times per week and 37.8% between three and six. A smaller proportion (3.6%) claimed that they never performed any and 16.2% were carrying out physical exercise on a daily basis. On average, the men were carrying out 50% more exercise than their partners with an average of 5.3 hours per week ± 5.4. A high proportion of men also reported cardiovascular exercise as their physical activity of choice but in addition 22.5% reported carrying out strength training, for example lifting weights.

Participants were also asked about the amount of time they spent outdoors and about their use of sun cream. Interestingly, there was no statistical difference in the total number of hours spent outside in the winter months when compared to the summer months in either the female or male participants (p=0.277). However, this may be due to the majority of participants' jobs being inside; when hours spent outside on the weekend were analysed then a statistical difference was seen (p=0.035) with an average of 5.1 hours ± 3.1 spent outside in the Summer months (May to August) compared to 4.8 hours ± 2.6 in Spring (March and April) or Autumn (September and October) and 3.6 hours ± 2.0 in Winter (November to February). In addition, the number of days that sun cream was used by the participants in the various seasons was also statistically significant (p=0.001).

Following the initial assessment, the couples were provided with drinks, their compliance was checked weekly by phone or email until the day of egg collection.

3.3.3 Compliance

3.3.3.1 What was the compliance to the trial and what factors affected it?

The mean number of days between recruitment (supplying the drinks) and oocyte retrieval was 67.1 ± 29.3. The minimum number of days was 36 (5 weeks and 1 day) and the maximum was 174 (24 weeks and 6 days). Over 62% of the women and 50% of the men were 100% compliant. In women, there was a negative correlation (-0.425) between the number of days since the drinks were supplied and compliance (p<0.001) (see Figure 3.4). This was also true when looking at compliance in the male subjects although the correlation was less marked (-0.224, p=0.022).

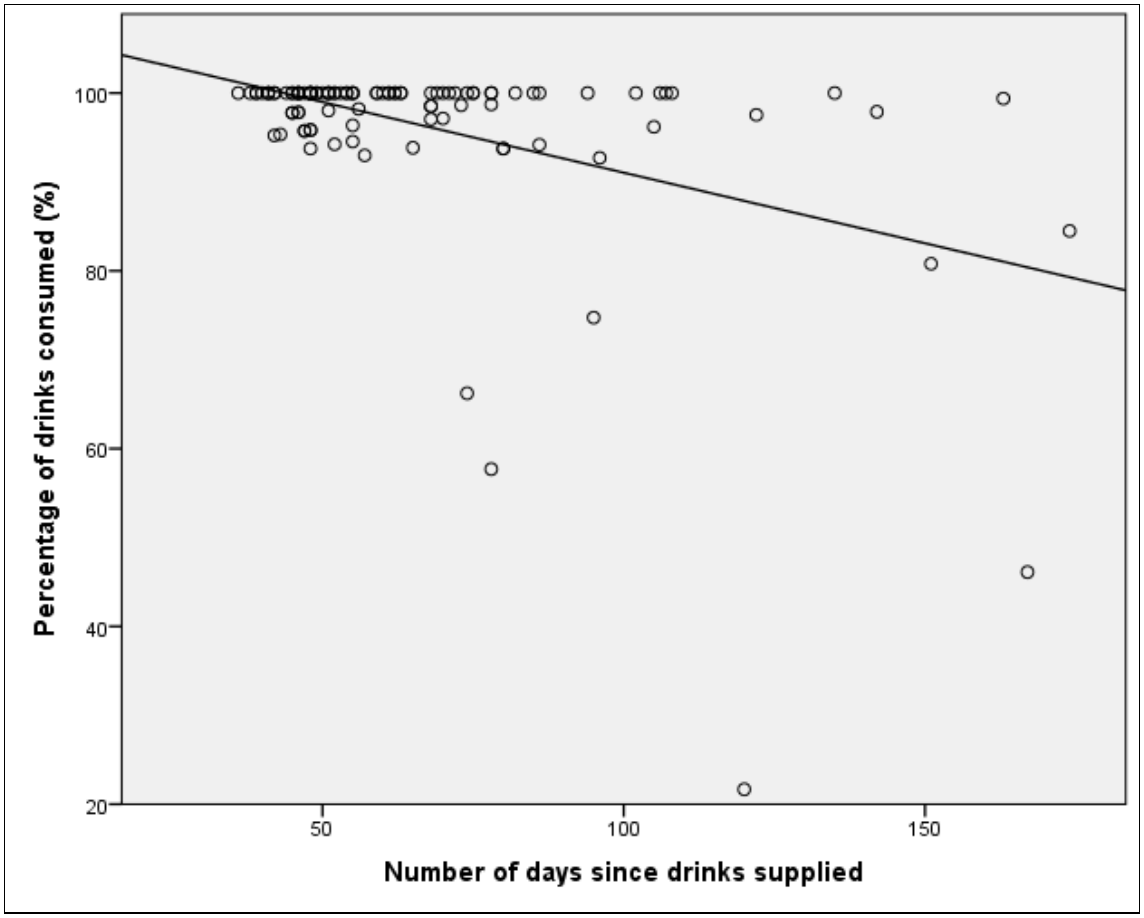


Figure 3.4: Graph demonstrating the correlation between the number of days a woman was required to take the drinks and compliance

There was no statistical difference seen for compliance between the treatment group and the placebo group in either women (p=0.799) or men (p=0.089). The mean compliance was 96.3% ± 10.9 for women and 94.5% ± 13.9 for men. Interestingly, using a Spearman's rho test, the compliance of the male and female participants within the same couple was positively correlated (0.629, p<0.001).

Couples were also asked about their oil and spread usage; the majority of women had not used any alternative oil (67.3%) or spread (64.4%) to that which had been provided to them for the duration of the trial. These figures were similar in the male participants (66.3% and 69.2% respectively). Out of the women who used alternative oil during the trial, 29.4% (10/34) used the substitute monthly, 55.9% (19/34) weekly and 14.7% (5/34) daily. The most common replacement, in 47% of cases, was olive oil or virgin olive oil (16/34) but other alternatives included vegetable oil (8.8%), sunflower oil (8.8%), coconut oil (5.9%) and an oil spray (frylight) (11.8%). In six cases (17.6%) women did not know what substitutions they had used as it had been whilst living outside of their normal environment e.g. on holiday or a business trip. The results were the same for the male participants, although one extra person worked away from

home during the week (7/35) and therefore used an unknown alternative on these days. Similar results were also seen when examining the use of alternative spreads; 27.0% (10/37) of women used their substitution monthly, 51.4% weekly (19/37) and 21.6% daily (8/37). Substitute spreads used included butter (37.8%), olive oil spread (16.2%), vegetable oil spread (8.1%), sunflower spread (8.1%) and a dairy free spread (2.7%). Ten women (27.0%) could not identify the spreads they had used as the substitutions had been used when they were not in their home environment. Fewer of the male participants used alternative spreads (32/104) and those that did used them less frequently; 31.3% monthly, 46.9% weekly and 21.9% daily. The most commonly used substitute, as with the female participants, was butter (28.1%). Other replacements included olive oil spread (18.8%), sunflower spread (12.5%) and vegetable oil spread (6.3%). Notably, in the vast majority of cases both the male and female in the same couple either did not substitute or used the same replacement products of oil (95.2%) and spread (89.4%).

In order to assess the compliance further it was possible to analyse changes in the patients' blood composition including the proportions of different fatty acids and the concentration of Vitamin D.

3.3.4 Analysis of blood samples

3.3.4.1 What was the fatty acid composition of the erythrocytes pre and post the dietary intervention?

The proportion of 19 different fatty acids in the red blood cells (RBC) were measured; these were Myristic acid, Palmitic acid, Palmitoleic acid, Stearic acid, Oleic acid, Vaccenic acid, Linoleic acid (LA), gamma-Linolenic acid (GLA), α-Linolenic acid (ALA), Arachidic acid, Gondoic acid, Eicosadienoic acid, Dihomo-gamma-linolenic acid, Arachidonic acid, Behenic acid, Eicosatetraenoic acid (ETA), Eicosapentaenoic acid (EPA), Docosapentaenoic acid (DPA) and Docosahexaenoic acid (DHA).

Fatty acid data that were not normally distributed were Myristic acid, Palmitoleic acid, Stearic acid, gamma-Linolenic acid (GLA), α-Linolenic acid (ALA), Arachidic acid, Eicosatetraenoic acid (ETA), and Eicosapentaenoic acid (EPA); these were log10 transformed and then analysed. Using a one way ANOVA, no statistically significant difference was seen in the percentages of different fatty acids between the treatment and placebo groups in either the female or the male participants prior to intervention (see Table 3.4).

No statistically significant difference was seen between the percentages of EPA or DHA in the red blood cells of the 12 male volunteers who took omega 3 supplements prior to the start of the trial and those who did not. Interestingly, there was a slight increase in the levels of Stearic acid in the group who were not taking the supplements (15.42% ± 0.94 compared to 14.68% ± 0.99, p=0.009). However, in the female cohort, the EPA levels were significantly higher in the seven participants who took omega 3 supplements (1.24% ± 1.45 compared to 0.94% ± 1.34). No other differences were noted and by chance those who took omega 3 supplements were evenly distributed between the treatment group (9/19) and the placebo group (10/19).

Table 3.4: Mean % of fatty acids in red blood cells for female and male participants prior to the intervention.
*** means the data has been log transformed to use a parametric test and then raised to the power 10 to calculate the mean.**

	Female participants			Male participants		
	Mean ± standard deviation % of fatty acids		p value	Mean ± standard deviation % of fatty acids		p value
	Treatment (n=55)	Placebo (n=55)		Treatment (n=55)	Placebo (n=55)	
Myristic acid	0.39* ± 1.29	0.38* ± 1.26	0.816	0.38* ± 1.29	0.38* ± 1.34	0.837
Palmitic acid	24.57 ± 0.98	24.56 ± 0.99	0.961	24.80 ± 1.02	24.59 ± 0.97	0.283
Palmitoleic acid	0.55* ± 1.31	0.57* ± 1.32	0.588	0.56* ± 1.37	0.55* ± 1.37	0.685
Stearic acid	15.36* ± 1.07	15.31* ± 1.06	0.763	15.26* ± 1.07	15.35* ± 1.06	0.634
Oleic acid	16.27 ± 0.88	16.51 ± 1.21	0.222	16.81 ± 0.97	16.90 ± 0.94	0.629
Vaccenic acid	1.32 ± 0.18	1.32 ± 0.17	0.885	1.27 ± 0.15	1.23 ± 0.13	0.148
Linoleic acid	12.46 ± 1.45	12.62 ± 1.44	0.549	12.68 ± 1.65	12.96 ± 1.42	0.349
gamma-Linolenic acid	0.09* ± 1.42	0.09* ± 1.49	0.627	0.09* ± 1.43	0.10* ± 1.49	0.829
α-Linolenic acid	0.24* ± 1.30	0.24* ± 1.31	0.827	0.24* ± 1.43	0.24* ± 1.39	0.800
Arachidic acid	0.11* ± 1.26	0.11* ± 1.27	0.659	0.12* ± 1.19	0.11* ± 1.23	0.792
Gondoic acid	0.39 ± 0.06	0.38 ± 0.06	0.604	0.38 ± 0.06	0.38 ± 0.06	0.987
Eicosadienoic acid	0.30 ± 0.05	0.31 ± 0.05	0.741	0.29 ± 0.05	0.28 ± 0.05	0.273
Dihomo-gamma-linolenic acid	1.83 ± 0.28	1.93 ± 0.38	0.091	2.04 ± 0.45	2.03 ± 0.41	0.958
Arachidonic acid	16.40 ± 1.39	16.10 ± 1.33	0.264	15.81 ± 1.54	15.93 ± 1.37	0.667
Behenic acid	0.19 ± 0.05	0.19 ± 0.05	0.817	0.19 ± 0.05	0.19 ± 0.05	0.896
Eicosatetraenoic acid	0.19* ± 1.52	0.19* ± 1.55	0.928	0.20* ± 1.59	0.18* ± 1.54	0.427
Eicosapentaenoic acid	0.97* ± 1.36	0.96* ± 1.35	0.731	0.94* ± 1.36	0.91* ± 1.36	0.638
Docosapentaenoic acid	3.07 ± 0.43	3.06 ± 0.34	0.818	3.14 ± 0.55	3.03 ± 0.38	0.236
Docosahexaenoic acid	5.16 ± 0.98	5.03 ± 1.10	0.517	4.66 ± 1.09	4.51 ± 1.14	0.493

There was however a statistically significant difference between the female and male participants for oleic acid (p=0.001), vaccenic acid (p<0.001), eicosadienoic acid (p=0.019), dihomo-gamma-linolenic acid (p=0.003), arachidonic acid (p=0.046), docosahexaenoic acid (p=0.001).

Following the dietary intervention, a statistically significant difference was seen in the proportions of various FAs between those who were consuming the treatment drinks and those who were having the placebo (see Table 3.5). The percentages of stearic acid, linoleic acid, gamma-linolenic acid, eicosadienoic acid, dihomo-gamma-linolenic acid, arachidonic acid and behenic acid were lower in women in the treatment group than in the placebo group. Vaccenic acid, gondoic acid, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid proportions were higher in the treatment group (see Table 3.5). The same FAs were upregulated and downregulated in a similar pattern in the males except for eicosadienoic acid, behenic acid and docosapentaenoic acid (see Table 3.5). Furthermore, the percentages of stearic acid, oleic acid, linoleic acid, gamma-linolenic acid, dihomo-gamma-linolenic acid, and arachidonic acid decreased in women in the treatment group. Conversely, vaccenic acid, gondoic acid, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid proportions increased in the treatment group. The same FAs were upregulated and downregulated in a similar pattern in the males except for stearic acid and docosapentaenoic acid (see Table 3.6).

Table 3.5: Mean % of fatty acids in red blood cells for female and male participants following the intervention.
*** means the data has been log transformed to use a parametric test and then raised to the power 10 to calculate the mean.**

	Female participants			Male participants		
	Mean ± standard deviation % of fatty acids		p value	Mean ± standard deviation % of fatty acids		p value
	Treatment (n=49)	Placebo (n=53)		Treatment (n=50)	Placebo (n=53)	
Myristic acid	0.36* ± 1.28	0.39* ± 1.28	0.161	0.38* ± 1.31	0.40* ± 1.30	0.303
Palmitic acid	24.51 ± 0.96	24.73 ± 0.85	0.240	24.54 ± 1.20	24.60 ± 0.98	0.783
Palmitoleic acid	0.54* ± 1.32	0.56* ± 1.30	0.577	0.58* ± 1.33	0.54* ± 1.37	0.269
Stearic acid	14.93* ± 1.07	15.55* ± 1.07	0.002	14.98* ± 1.09	15.60* ± 1.06	0.007
Oleic acid	15.98 ± 0.89	16.06 ± 1.27	0.684	16.55 ± 0.94	16.71 ± 1.12	0.425
Vaccenic acid	1.42 ± 0.14	1.33 ± 0.16	0.004	1.30 ± 0.15	1.20 ± 0.13	<0.001
Linoleic acid	11.13 ± 1.47	12.37 ± 1.52	<0.001	12.04 ± 1.82	12.83 ± 1.39	0.015
gamma-Linolenic acid	0.06* ± 1.62	0.07* ± 1.45	0.005	0.08* ± 1.46	0.09* ± 1.45	0.037
α-Linolenic acid	0.23* ± 1.39	0.24* ± 1.36	0.340	0.25* ± 1.35	0.24* ± 1.39	0.752
Arachidic acid	0.11* ± 1.22	0.12* ± 1.27	0.059	0.11* ± 1.25	0.11* ± 1.23	0.483
Gondoic acid	0.52 ± 0.08	0.39 ± 0.06	<0.001	0.47 ± 0.08	0.37 ± 0.05	<0.001
Eicosadienoic acid	0.29 ± 0.05	0.33 ± 0.05	0.001	0.28 ± 0.05	0.30 ± 0.06	0.066
Dihomo-gamma-linolenic acid	1.40 ± 0.23	1.99 ± 0.41	<0.001	1.68 ± 0.38	2.04 ± 0.41	<0.001
Arachidonic acid	13.76 ± 1.33	16.41 ± 1.21	<0.001	13.59 ± 1.21	15.87 ± 1.57	<0.001
Behenic acid	0.19 ± 0.04	0.21 ± 0.05	0.019	0.20 ± 0.05	0.21 ± 0.05	0.278
Eicosatetraenoic acid	0.19* ± 1.50	0.20* ± 1.58	0.578	0.20* ± 1.58	0.11* ± 1.70	0.947
Eicosapentaenoic acid	2.95* ± 1.23	0.86* ± 1.38	<0.001	2.38* ± 1.29	0.91* ± 1.37	<0.001
Docosapentaenoic acid	3.30 ± 0.36	2.98 ± 0.31	<0.001	3.13 ± 0.37	3.03 ± 0.41	0.204
Docosahexaenoic acid	7.96 ± 1.04	5.05 ± 1.16	<0.001	7.07 ± 1.12	4.57 ± 1.17	<0.001

Table 3.6:Difference in fatty acid percent before and after the study according to sex and intervention group.

Fatty acid	Women				Men			
	Study (n=53)		Control (n=48)		Study (n=53)		Control (n=49)	
	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value
Myristic*	0.92 (0.85, 1.00)	0.06	1.00 (0.92, 1.08)	0.93	0.98 (0.90, 1.07)	0.72	1.05 (.096, 1.14)	0.33
Palmitic	-0.07 (-0.33, 0.20)	0.61	0.14 (-0.11, 0.39)	0.26	-0.28 (0.61, 0.06)	0.10	0.09 (-0.23, 0.41)	0.57
Palmitoleic*	0.97 (0.90, 1.05)	0.50	0.97 (0.90, 1.05)	0.51	1.02 (0.93, 1.12)	0.64	1.00 (0.92, 1.08)	0.92
Stearic*	**0.97 (0.95, 1.00)**	**0.02**	1.01 (0.99, 1.04)	0.22	0.98 (0.96, 1.01)	0.20	1.01 (0.99, 1.03)	0.23
Oleic	**-0.28 (-0.47, 0.09)**	**0.004**	**-0.48 (-0.69, -0.26)**	**<0.001**	**-0.24 (-0.49, 0.00)**	**0.05**	-0.18 (-0.41, 0.06)	0.14
Vaccenic	**0.08 (0.05, 0.12)**	**<0.001**	0.00 (-0.03, 0.03)	0.90	**0.03 (0.00, 0.06)**	**0.04**	-0.02 (-0.05, 0.00)	0.09
Linoleic	**-1.38 (-1.71, -1.05)**	**<0.001**	-0.16 (-0.46, 0.14)	0.30	**-0.67 (-1.05, -0.29)**	**0.001**	-0.15 (-0.55, 0.25)	0.47
gamma-Linolenic*	**0.66 (0.57, 0.76)**	**<0.001**	**0.74 (0.66, 1.19)**	**<0.001**	**0.86 (0.77, 0.96)**	**0.007**	0.99 (0.87, 1.11)	0.81
α-Linolenic*	0.94 (0.85, 1.03)	0.19	0.99 (0.90, 1.09)	0.87	1.03 (0.93, 1.14)	0.54	1.03 (0.92, 1.16)	0.55
Arachidic*	1.02 (0.95, 1.11)	0.53	1.08 (0.99, 1.83)	0.08	1.02 (0.95, 1.11)	0.54	1.05 (0.97, 1.13)	0.23
Gondoic	**0.13 (0.11, 0.16)**	**<0.001**	0.01 (-0.01, 0.03)	0.21	**0.09 (0.07, 0.11)**	**<0.001**	-0.01 (-0.02, 0.01)	0.42
Eicosadienoic	-0.01 (-0.02, 0.00)	0.19	**0.02 (0.00, 0.03)**	**0.01**	-0.01 (-0.02, 0.00)	0.07	**0.02 (0.01, 0.04)**	**0.001**
Dihomo-γ-Linolenic	**-0.43 (-0.48, -0.38)**	**<0.001**	0.06 (-0.01, 0.12)	0.08	**-0.36 (-0.44, -0.28)**	**<0.001**	0.01 (-0.05, 0.06)	0.84
Arachidonic	**-2.55 (-2.83, -2.27)**	**<0.001**	0.27 (0.04, 0.58)	0.08	**-2.17 (-2.54, -1.80)**	**<0.001**	-0.10 (-0.46, 0.27)	0.60
Behenic	-0.01 (-0.02, 0.01)	0.53	0.02 (0.00, 0.04)	0.06	0.01 (-0.01, 0.03)	0.188	**0.02 (0.01, 0.04)**	**0.01**
Eicosatetraenoic*	1.03 (0.87, 1.21)	0.74	1.06 (0.88, 1.28)	0.53	1.02 (0.85, 1.23)	0.79	1.13 (0.94, 1.36)	0.18
Eicosapentaenoic*	**2.30 (2.80, 3.21)**	**<0.001**	**0.82 (0.87, 0.97)**	**0.002**	**2.51 (2.30, 2.74)**	**<0.001**	0.99 (0.93, 1.05)	0.73
Docosapentaenoic	**0.21 (0.10, 0.31)**	**<0.001**	-0.07 (-0.14, 0.00)	0.06	-0.03 (-0.13, 0.07)	0.54	0.00 (-0.09, 0.09)	0.97
Docosahexaenoic	**2.80 (2.57, 3.03)**	**<0.001**	0.06 (-0.12, 0.23)	0.52	**2.42 (2.12, 2.71)**	**<0.001**	0.08 (-0.07, 0.23)	0.31

*** distribution skewed, so analysed using logarithms to produce a ratio rather than a simple difference. Difference is calculated as "after – before" intervention, so a positive difference or a ratio greater than 1.0 signify an increase over time.**

Amongst women, EPA levels increased 3.27 times more in the study group than in the control group (95% CI 3.00 to 3.55) and DHA levels increased by 2.74% more (2.47 to 3.02). The corresponding figures for men were 2.54 (2.29 to 2.81) and 2.34 (2.02 to 2.66). These differences were all statistically significant at $p<0.0001$. The male participants in the treatment group showed a smaller percentage change in their combined DHA and EPA levels than their female counterparts. This could be explained by the lower mean compliance of the male participants. At oocyte retrieval, the percentage of EPA in the red blood cells of the male participants correlated with their compliance levels ($r_s=0.326$, $p=0.017$). This correlation was not seen with the DHA percentages ($r=0.259$, $p=0.061$) in the males or with either EPA ($r_s=0.056$, $p=0.688$) or the DHA ($r=-0.119$, $p=0.397$) percentages and compliance in the female volunteers.

3.3.4.2 What were the Vitamin D levels in the trial pre and post the dietary intervention?

Blood serum collected from the male participants prior to the dietary intervention (n=109) and analysed for vitamin D levels including 25-Hydroxyvitamin D2 (25(OH)D2), 25(OH)D3 and total 25(OH) vitamin D showed mean levels of 0.51 nmol/L ± 1.79, 67.28 nmol/L ± 27.43 and 67.80 nmol/L ±27.43 respectively. Almost 12% (13/109) of the male patients had a total vitamin D level of less than 30 nmol/L (regarded as deficient), with a further 14.7% (16/109) with levels between 30 and 50 nmol/L (borderline deficiency). The season of recruitment ($p<0.001$) and the average number of hours spent outside ($p=0.001$) both affected the level of vitamin D; men who were recruited in the summer (77.75 nmol/L ± 25.01) had higher mean total levels compared to those recruited in winter (49.07 nmol/L ± 22.51). Men who spent more time outside also had higher mean levels of total vitamin D (see Figure 3.5).

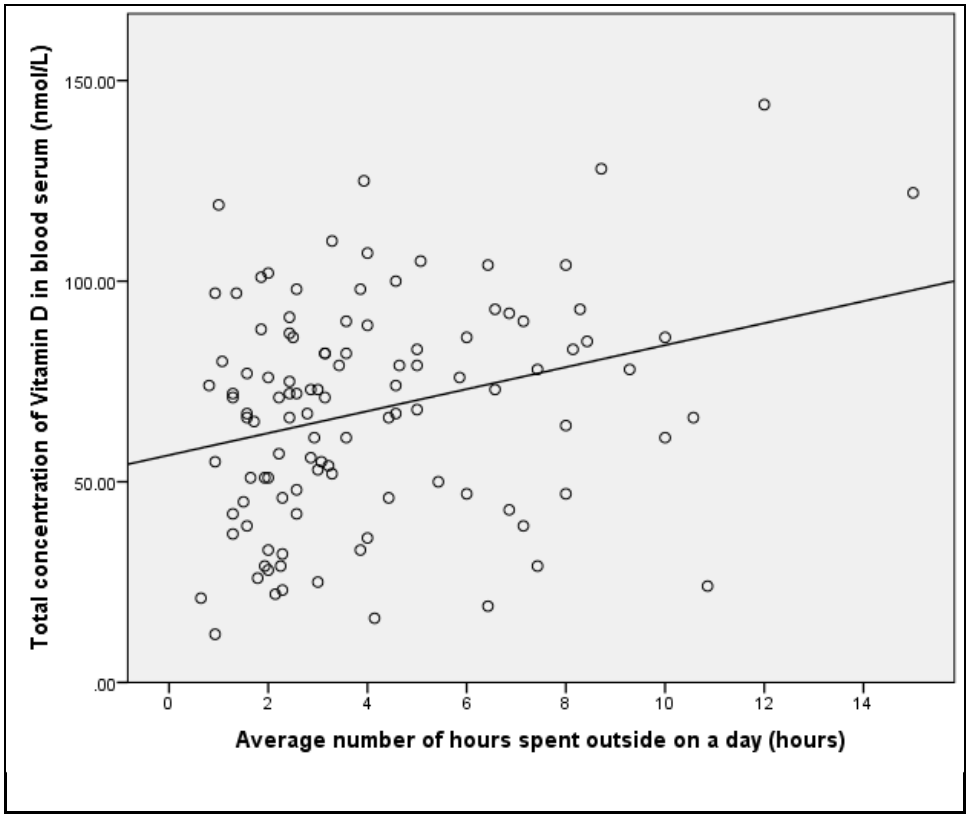


Figure 3.5: Correlation between hours per day spent outside and total vitamin D levels for male participants

Only 4.5% (5/110) of the female participants had a total vitamin D blood serum level of less than 30 nmol/L while a further 18.2% (20/11) had borderline deficient levels. The mean total vitamin D blood serum level for female volunteers was 72.97 nmol/L ± 27.06, comprised of 72.41 nmol/L ± 27.44 of 25(OH)D3 and 0.56 nmol/L ± 2.56 of 25(OH)D2. As with the male volunteers, the total levels of vitamin D in the blood serum were statistically different depending on the season the female patient was recruited in (p=0.001). Mean levels were measured at 49.47 nmol/L ± 18.42 for those recruited in the winter compared to 82.13 nmol/L ± 26.06 for those recruited in summer. However, surprisingly, in the female participants, the average number of hours spent outside did not correlate significantly with the vitamin D levels, although there was a strong trend (p=0.072).

Prior to the start of the trial, only two female participants took vitamin D supplements exclusively and therefore no statistically significant differences were seen between those who used these supplements and those who did not. However, when examining those who used a multivitamin (the vast majority of which contain vitamin D) or exclusive vitamin D supplements, a difference was seen in the female participants; total vitamin D levels were 78.01 nmol/L ± 1.38 in those that took supplements compared to 61.60 nmol/L ± 1.60 in those who did not

(p=0.0004). This difference was not seen in the male participants who did and did not take multivitamins (p=0.197).

Following the dietary intervention, the group who received the treatment drinks had significantly higher levels of total vitamin D in their blood serum when compared to the placebo group (see Table 3.7).

Table 3.7: Serum Vitamin D levels in female and male participants following the dietary intervention.
*** means the data has been log transformed to use a parametric test and then raised to the power 10 to calculate the mean.**

	Female participants			Male participants		
	Mean ± standard deviation		p value	Mean ± standard deviation)		p value
	Treatment (n=50)	Placebo (n=49)		Treatment (n=51)	Placebo (n=53)	
25(OH)D2 (nmol/L)	1.10* ± 1.47	1.24* ± 1.79	0.243	1.03* ± 1.28	1.78* ± 2.30	<0.001
25(OH)D3 (nmol/L)	154.35* ± 1.56	67.64* ± 1.52	<0.001	137.15* ± 1.55	64.54* ± 1.50	<0.001
Total vitamin D (nmol/L)	154.63* ± 1.56	68.50* ± 1.51	<0.001	137.31* ± 1.54	66.57* ± 1.49	<0.001

The total vitamin D levels at visit 4 in the male participants (r_s=0.351, p=0.010) correlated with their compliance to the drinks, this was not, however, the case for the female participants (r_s=0.240, p=0.094), possibly due to the high number of females who were 100% compliant. In addition, there was a greater percentage increase in the total vitamin D levels in the winter (347.15% ± 262.63) when compared to the summer months (149.09% ± 227.85, p=0.021) in the treatment group, however there was no statistical difference in the absolute concentration changes (111.62 nmol/L ± 52.59 compared to 81.80 nmol/L ± 63.93, p=0.276).

3.3.4.3 What were the folate, vitamin B12 and vitamin B6 levels in the participants pre and post the dietary intervention?

At recruitment, the levels of folate (11.82 ng/ml ± 1.66 compared to 6.42 ng/ml ± 1.59, p<0.001), vitamin B12 (355.63 ng/L ± 1.40 compared to 243.56 ng/L ± 1.48, p<0.001) and vitamin B6 (147.50 nmol/L ± 2.11 compared to 68.77 nmol/L ± 1.75, p<0.001) were higher in the male volunteers who utilised multivitamins compared to those who did not. As was expected, following the intervention, there was no statistical difference between the mean levels of these vitamins in the treatment group and the placebo group (p=0.239, 0.211, 0.428 respectively).

Over three quarters (79/104) of the female participants were taking folic acid, a multivitamin or a combination of the two at recruitment. At recruitment, there was a statistically significant difference ($p<0.001$) in the plasma folate levels between those who were taking supplements (14.30 ng/ml ± 1.63) compared to those who were not (7.16 ng/ml ± 1.63). As folic acid was found in all the drinks (treatment and placebo groups), no difference was seen between the two groups, the mean plasma folate level at the time of oocyte retrieval in the female volunteers was 12.74 ng/ml ± 1.70. In addition, there was no significant difference seen ($p=0.513$) between the mean (log transformed) folate levels prior and after the intervention, this is likely to be due to the high proportion of women who were using folate containing supplements prior to the trial. There was no statistical difference in the female volunteers in the vitamin B12 and B6 levels either before and after the intervention ($p=0.645$ and 0.072) or between the treatment groups following the intervention ($p=0.066$ and 0.824).

3.3.4.4 Did the pre-intervention blood results correlate with the prudent diet score of the participants?

No difference was seen when the prudent diet score at the time of recruitment was compared to the score on the day of oocyte retrieval in either the male ($p=0.483$) or the female ($p=0.583$) volunteers. The prudent diet score in the male participants positively correlated with pre-intervention blood levels of DHA ($r=0.218$, $p=0.022$), folate ($r_s=0.270$, $p=0.005$), total vitamin D ($r_s=0.238$, $p=0.013$) and vitamin B6 ($r_s=0.218$, $p=0.022$). Blood levels of EPA ($p=0.191$) and vitamin B12 ($p=0.741$) showed no correlation with the male's prudent diet score. Similar correlations are seen with the female diet prior to the intervention; EPA ($r_s=0.282$, $p=0.003$), DHA ($r=0.377$, $p<0.001$), folate ($r_s=0.283$, $p=0.003$) and vitamin B6 ($r_s=0.311$, $p=0.001$) were all positively correlated with the prudent diet score. No statistically significant correlations were seen with vitamin B12 ($p=0.064$) and vitamin D ($p=0.082$).

3.3.5 Analysis of IVF cycle

3.3.5.1 What was the IVF regime followed by women participating in the trial?

The vast majority (90.4%) of women underwent the short antagonist stimulation regime, with 10 women using a long protocol; by chance these women were all in the treatment group. Further to this, 6 of the women using the antagonist regime (3 in the treatment group and 3 in the placebo group) and 3 of the women who were on the long protocol used Menopur® (human

menopausal gonadotrophin) as their stimulation drug whilst the remainder used Gonal F® (recombinant follitropin alfa) (see Table 3.8).

Table 3.8: The different IVF protocols, stimulation drugs and doses used in the two groups in the PREPARE trial

	Stimulation drug	N		Mean dose (I.U.)		p value
		Treatment	Placebo	Treatment	Placebo	
Long Protocol	Gonal F	7	0	225.00 ± 55.90	0	
	Menopur	3	0	350.00 ±0	0	
Antagonist short protocol	Gonal F	40	48	219.06 ±66.87	205.71 ±50.57	0.289
	Menopur	3	3	275.00 ±43.30	316.67 ±80.36	0.473
TOTAL		53	51			

All the women who used the long protocol were triggered with Ovitrelle (hCG), as would be expected and 87.1% (81/93) of those who followed the short antagonist protocol; the remainder of women (12/93) were at increased risk of OHSS and these were triggered with Buserelin (GnRH). Of the women who were triggered by Buserelin, 8 were in the placebo group and 4 were in the treatment group, this difference was not statistically significant (p=0.152). Two patients did not undergo oocyte retrieval as they were not stimulated sufficiently (i.e. they only had one follicle) and therefore they were converted to an IUI cycle; both of these women were in the placebo group.

3.3.5.2 Did the dietary intervention affect the number of follicles, oocytes, embryos or blastocysts?

The mean antral follicle count (AFC) at the female participant's baseline scan was 14.06 ± 6.70. There was no difference between those consuming drinks containing the high levels of omega-3 and vitamin D and those consuming placebo (p=0.540). The mean endometrial thickness at this scan was 4.10 mm ± 1.50 and again there was no difference between the women in the two groups (p=0.386). There was no statistical difference between the AFC or endometrial thickness at baseline scan in women who underwent the long protocol (11.90 follicles ± 2.42 and 3.90 mm ± 1.05) compared to those who used the short antagonist protocol (14.29 follicles ± 6.97 and 4.12 mm ± 1.54, p=0.286 and 0.6663).

Following stimulation, there was no difference in the number of follicles greater than 14 mm prior to an ovulation trigger being administered between the two treatment groups (this remained true when patients who had undergone the long protocol were excluded) (see Table 3.9). There was also no correlation between the RBC DHA and EPA levels and the number of follicles (p= 0.576 and p=0.828, respectively). A difference was however seen in the percentage of eggs fertilised with a higher fertilisation rate seen in the placebo group (73.23% ± 23.10) compared to the treatment group (63.24% ± 22.45) (p=0.029), although there was no difference between absolute numbers fertilised (the number of 2PN embryos).

There was no difference between the number of blastocysts formed and the number of blastocysts suitable for cryopreservation in the two treatment groups (see Table 3.9). In addition, there was no significant difference between the two groups in the proportion of embryos that developed to blastocyst stage (p=0.674) and the proportion of blastocysts suitable for cryopreservation (p=0.476).

Table 3.9: The number of embryos and blastocysts in the treatment group compared to the placebo group

	Treatment (median and IQR) n=53	**Placebo (median and IQR) n=49**	**p value**
Number of follicles greater than 14mm prior to trigger	8.00 (4.00 – 11.75)	8.00 (6.00 – 13.00)	0.280
Number of oocytes retrieved	10.00 (6.00 – 15.75)	11.00 (7.50 – 18.50)	0.500
Number of 2PN embryos	6.00 (2.00 – 9.00)	6.00 (3.50 – 12.00)	0.299
Number of blastocysts formed	4.00 (0.00 – 6.00)	3.00 (0.00 – 9.00)	0.619
Number of blastocysts suitable for cryopreservation	3.00 (0.00 – 4.00)	2.00 (0.00 – 5.00)	0.823

3.3.6 Analysis of embryo quality

3.3.6.1 Are there correlations between the morphology and the morphokinetic markers of the embryos?

Correlations were observed between the embryo grade on day 3 (see Table 2.2) and the time points to pronuclei fade, 2 cells, 4 cells, 5 cells, 6 cells, 7 cells and 8 cells; the third and fourth cell stages; and the synchronicity of these cell cycles. These correlations were also seen with the day

3 embryo scores (see Table 2.3). Surprisingly, no correlations were seen with either the grade or the score on day 3 and the length of the second cell cycle (CC2) (see Table 3.10).

Both the morphological grade and the converted score of the day 3 embryos correlated with the KIDScore on day 3 (R_s=-0.410, p<0.001 and R_s=-0.469, p<0.001, respectively), with morphologically better embryos having a higher KIDScoreD3 (i.e. improved implantation potential).

Correlations were also detected between the quality of the ICM (see Table 2.5) and TE (see

Table **2.6**) on day 5 with the time the embryo took to form a morula and the time taken to form a blastocyst (see Table 3.11).

Furthermore, as the score on day 5 (both with only embryos that formed a blastocyst (see Table 2.7) and all embryos including those that had not yet formed a blastocyst (see Table 2.8)) increased (i.e. the blastocyst or embryo quality was worse) so did the time that the embryo took to form a morula or blastocyst (see Table 3.12).

Table 3.10: Spearman Rho correlations between embryo grade and score on day 3 and morphokinetic markers of embryo quality

	n	Correlation with embryo grade on day 3 (R_s)	p-value	Correlation with embryo score on day 3 (R_s)	p-value
tPNf	738	0.137	<0.001	0.169	<0.001
t2	734	0.226	<0.001	0.262	<0.001
t3	722	0.068	0.069	0.118	0.001
t4	711	0.213	<0.001	0.257	<0.001
t5	703	0.144	<0.001	0.226	<0.001
t6	680	0.232	<0.001	0.287	<0.001
t7	657	0.294	<0.001	0.347	<0.001
t8	607	0.301	<0.001	0.340	<0.001
CC2	722	-0.005	0.891	0.048	0.200
CC3	703	0.208	<0.001	0.291	<0.001
CC4	578	0.146	<0.001	0.151	<0.001
S2	711	0.278	<0.001	0.292	<0.001
S3	607	0.373	<0.001	0.400	<0.001

Table 3.11: Correlation of grade of ICM and TE with morphokinetic markers of embryo quality

	n	Correlation with grade of ICM on day 5 (R)	p-value	Correlation with grade of TE on day 5 (R)	p-value
tM	391	0.306	<0.001	0.274	<0.001
tB	386	0.448	<0.001	0.433	<0.001

Table 3.12: Correlation of the stage of blastocyst or embryo on day 5 with morphokinetic markers of embryo quality

	n	Correlation with blastocyst score on day 5 (R)	p-value	Correlation with the embryo score on day 5 (including embryos that did not form a blastocyst) (R)	p-value
tM	391	0.352	<0.001	0.658	<0.001
tB	386	0.508	<0.001	0.771	<0.001

3.3.6.2 Does the dietary intervention have an effect on the embryo morphology?

Seven hundred and fifty four embryos were analysed, with the day 3 score recorded in 745 cases. There was no significant difference observed between the number of cells; the amount of fragmentation, blastomere eveness and multinucleation (i.e. the embryo grade); or the embryo score (see Table 2.3) on day 3 between the embryos in the intervention group and those in the placebo group (see Table 3.13).

Four hundred and one embryos had formed a blastocyst on day 5 (120 hours). No statistical difference was seen between the stage of blastocyst expansion (see Table 2.4), the ICM or trophectoderm grades (see Table 2.5 and Table 2.6) or the blastocyst score (as allocated based on the stage of expansion and the ICM and TE grading, see Table 2.7) (see Table 3.14).

There was also no difference when all embryos were included (see Table 2.8) with a mean embryo score of 5.07 ± 2.27 in the treatment group and 5.23 ± 2.16 in the placebo group (p=0.058), it should be noted that a higher number is lower quality in the scoring system.

Table 3.13: Mixed model analysing day 3 markers of morphology

Morphological marker	Treatment n=353 Mean ± SD	Placebo n=392 Mean ± SD	Mixed Effects Model		
			Treatment Effect	95% CI	p-value
Number of cells	7.40 ± 2.24	7.18 ± 2.30	0.32	-0.25 – 0.88	0.267
Grade of embryo	1.84 ± 0.76	1.90 ± 0.86	-0.15	-0.36 – 0.05	0.146
Day 3 embryo score	2.31 ± 1.13	2.39 ± 1.20	-0.18	-0.46 – 0.11	0.221

Table 3.14: Mixed model analysing day 5 markers of morphology

Morphological marker	Treatment (n=190) Mean ± SD	Placebo (n=211) Mean ± SD	Mixed Effects Model		
			Treatment Effect	95% CI	p-value
Stage of blastocyst expansion	3.73 ± 0.84	3.56 ± 0.73	0.18	-0.03 – 0.38	0.087
ICM grade	2.13 ± 0.83	2.26 ± 0.76	-0.13	-0.32 – 0.06	0.183
TE grade	2.19 ± 0.75	2.35 ± 0.74	-0.16	-0.33 – 0.02	0.073
Blastocyst score	3.26 ± 1.28	3.56 ±1.28	-0.26	-0.87 – 0.35	0.393

3.3.6.3 Does the dietary intervention have an effect on the morphokinetic markers of embryo quality?

Seven hundred and fifty embryos were analysed (356 in the study group and 394 in the control group). Of these, 742 embryos cleaved to the two cell stage (351/356, 98.6% vs. 391/394, 99.2%, p=0.392), 719 cleaved to the four cell stage (344/356, 96.6% vs. 375/394, 95.2%, p=0.319) and 610 to the eight cell stage (295/356, 82.9% vs. 315/394, 79.9%, p=0.306). Furthermore, 487 embryos formed a blastocyst (231/356, 64.9% vs. 256/394, 65.0%, p=0.980). Table 3.15 gives median and quartile values of the morphokinetic markers for the study and control groups. The markers were expressed in standardised form and the treatment effects are shown in Table 3.15 and Figure 3.6.

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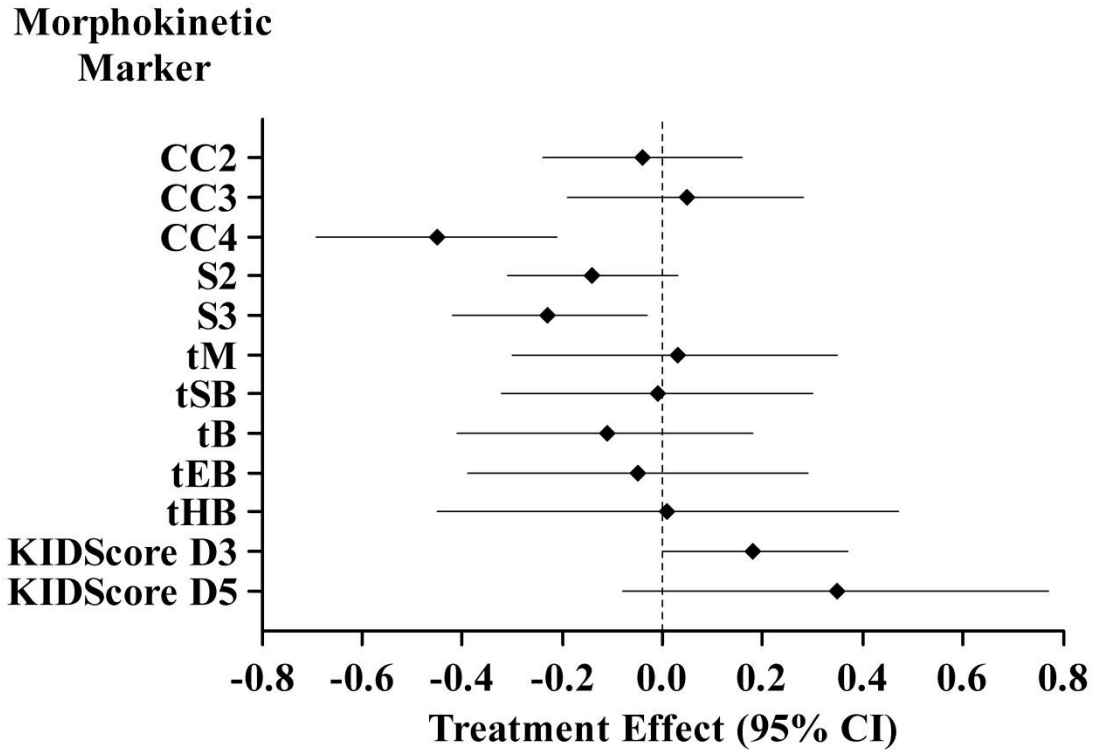

Figure 3.6: Effect of the dietary intervention on morphokinetic markers of embryo quality. A negative treatment effect in CC2, CC3, CC4, S2, S3, tM, tSB, tB, tEB and tHB indicates a shorter length of time and hence improved morphokinetic marker. Increased KIDScores indicate an increased chance of implantation. A significantly beneficial effect of the intervention diet was seen in CC4 ($p<0.001$), S3 ($p=0.02$) and the KIDScoreD3 ($p=0.05$).

Table 3.15: A mixed model analysis of the morphokinetic markers of embryo quality according to intervention group.
LQ = lower quartile, UQ = upper quartile, Treatment Effect set as "Intervention – Control" with markers in standardised (z-score) form;
CI = confidence interval; ICC = intra-class correlation coefficient.

Morphokinetic marker	Study intervention group			Control intervention group			Mixed model analysis			
	n	median	LQ, UQ	n	median	LQ, UQ	Treatment Effect	95% CI	p-value	ICC
CC2	349	11.51	10.51, 12.34	381	11.51	10.67, 12.51	-0.04	-0.24, 0.16	0.71	0.10
CC3	338	13.50	11.96, 15.02	368	13.01	11.51, 15.34	0.05	-0.19, 0.28	0.70	0.18
CC4	**283**	**19.34**	**16.79, 23.07**	**298**	**21.94**	**18.51, 26.48**	**-0.45**	**-0.69, -0.21**	**<0.001**	**0.15**
S2	344	0.50	0.17, 1.33	375	0.67	0.33, 1.80	-0.14	-0.31, 0.03	0.11	0.05
S3	**295**	**4.67**	**2.17, 14.34**	**315**	**5.84**	**2.50, 18.67**	**-0.23**	**-0.42, -0.03**	**0.02**	**0.06**
tM	298	94.89	88.04, 102.00	313	94.52	88.23, 100.98	0.03	-0.30, 0.35	0.87	0.38
tSB	282	100.91	93.61, 108.87	298	100.04	94.55, 107.15	-0.01	-0.32, 0.30	0.95	0.34
tB	231	109.29	102.25, 118.98	256	110.72	104.06, 118.37	-0.11	-0.41, 0.18	0.45	0.26
tEB	185	116.83	109.95, 129.87	192	118.12	112.06, 127.27	-0.05	-0.39, 0.29	0.77	0.31
tHB	98	119.41	112.19, 132.85	78	123.49	114.81, 131.89	0.01	-0.45, 0.47	0.96	0.47
KIDScore D3	**340**	**4**	**3, 5**	**376**	**4**	**2, 5**	**0.18**	**0.00, 0.37**	**0.05**	**0.17**
KIDScore D5	323	2	1, 5	355	2	1, 4	0.35	-0.08, 0.77	0.11	0.21

CC2 times were 0.04 standard deviations shorter in the study group (95% CI -0.16 to 0.24, p=0.71) than the control group. There were, however, statistically significant reductions in CC4 and S3 times and an increase in KIDScore on day 3.

No difference between groups was observed in the time it took the embryos to form a morula (tM), start blastocyst formation (tSB), form a blastocyst (tB), or form an expanded blastocyst (tEB) and a hatching blastocyst (tHB) (see Table 3.15

Table 3.15).

3.3.6.4 Did the dietary intervention affect the timing or number of embryos transferred or pregnancy rates?

There was no difference in the proportion of cycles that resulted in all the embryos being cryopreserved (usually due to the risk of OHSS in the female partner) between the treatment (10/53 cycles, 18.9%) and placebo (9/49 cycles, 18.4%) groups (p=0.948). In two cases in the treatment group, no embryo was transferred or cryopreserved. No difference was observed between the day of embryo transfer in the treatment group and the placebo group (p=0.402). 78% of cases that had a fresh embryo transfer had one embryo transferred, in the remaining 18 cases (that underwent a two embryo transfer), 12 were in the placebo group and 6 in the treatment group (however this was not statistically significant (p=0.096)).

No difference was observed in the pregnancy rate between the two groups (p=0.956) (see Table 3.16).

Table 3.16: Pregnancy rates in the treatment and placebo groups

	Treatment group	Placebo group	Total
Pregnant	30	28	58
Not pregnant	23	21	44
Total	53	49	102

There was also no difference in the live birth rates with 73% (22/30) of those achieving a pregnancy in the treatment group having a live birth and 57% (16/28) in the placebo group (p=0.195).

3.3.6.5 Did the dietary intervention affect the neonatal outcomes of the infants?

Eight of the liveborn babies were born preterm (before 37 weeks gestation), three in the placebo group and five in the treatment group (p=0.767). There was no statistical difference in the birthweights (3143 grams ± 708 in the placebo group compared to 3447 grams ± 663 in the treatment group, p=0.071) or birthweights adjusted for gestation duration (3193 grams ± 471 in the placebo group compared to 3466 grams ± 411 in the treatment group, p=0.082) of the infants born at term, although a trend towards bigger babies was seen in the treatment group. None of the babies in either group were growth restricted (less than 2.5 kg at term).

3.3.7 Analysis of the participants' experience

3.3.7.1 What was the experience of couples participating in the PREPARE trial?

Ten of the couples who participated in the trial underwent dyadic semi-structured interviews to ascertain their experience of the trial.

The couples that were interviewed demonstrated a good understanding of the trial and the blinding. The biggest reason participants gave for wanting to participate in the PREPARE trial was the possible chance that it would increase their success rates:

> *I think for me, my desire was that potentially it could help. So I think the whole way through I had that in the back of my mind. So you know that was probably [a] driving factor for me* (Couple 026, female participant).

Many of them also viewed the trial altruistically, believing that even if it did not benefit them directly it may help others in the future. Furthermore, they viewed the addition of the use of the EmbryoScope as a motivating factor in recruitment and retention to the trial:

> *"Yeah, I think we had a lot of questions that the microscope might have answered, so yes I think we would have done it anyway but it was a definite yes when we knew about that"* (Couple 019, female participant).

Couples experienced no major difficulties in following the dietary regime and establishing a routine for taking the drinks, this was reflected in the high compliance observed (see chapter 3.3.3):

> "*We just had them on the side. I had one every morning and I tend to go to work a bit earlier than A so I left his on the side and he would have it when he got up. So we just made it a bit of a routine of it really, having it in the morning."* (Couple 021, female participant).

Overall, couples demonstrated a high willingness and behavioural intention to do what it took in order to conceive.

The length of the trial and the delay in treatment starting were discussed. They perceived that the six weeks was a short length of time in a long process and that the benefits of participating in the trial outweighed this. The couples were also asked how long they would have been prepared to delay their treatment for; some couples were willing to delay for up to three or six months, with one couple even contemplating taking part in a similar trial for up to a year. However, they wanted a stronger evidence base to warrant them delaying their treatment for longer:

> *"Would we have delayed it? Probably because I think the thought that it could help was an underlying factor for me, so I think if I knew that by doing it for three months that it would really increase our chances then I probably would have said ok that's fine."* (Couple 026, female participant).

Conversely, other couples felt that the six week delay was the maximum that they were willing to undergo. Older couples also acknowledged that by delaying their treatment for more than three months for a trial, they may be decreasing their chances of success due to the effect of age on fertility.

The support that the couples provided by the PREPARE trial team was received positively, with the team being described as *"helpful"*, *"encouraging"*, *"enthusiastic"*, *"professional"* and *"honest"*. Couples also felt that participating as a couple was beneficial as they felt more of a team, and the male participants felt they played a more active role in the treatment.

Overall, the interviews demonstrated a highly motivated group of individuals with a willingness to do whatever was necessary to conceive. The trial was viewed as a positive addition to their treatment by the participants and many would have participated even if the trial length had been longer.

3.4 Discussion

To my knowledge, this is the first randomized controlled trial using IVF as a model to study the impact of a relatively brief dietary intervention on pre-implantation embryo development. The dietary intervention did not reveal a significant impact on CC2, but other markers considered to be equally valid, indicated a positive impact on embryo development. Specifically, CC4 and S3 were shortened in embryos derived from couples who had taken a 6 week intervention of high dose of omega 3 FAs and the recommended vitamin D intake along with olive oil. The observed impact of the dietary intervention on CC4 and S3, as opposed to the earlier morphokinetic markers (including CC2), may simply reflect a cumulative amplification of the effect over time. A shortened CC4 has been shown to be positively predictive of continuing development to the blastocyst stage, and of achieving a clinical pregnancy (Milewski et al., 2016). An improvement in the day 3 KIDScore was also observed, demonstrating an overall improvement in the morphokinetic markers of embryo quality. Interestingly, a difference in time to blastocyst formation was not noted which might imply that blastocysts with a slower CC4 have fewer cells in the trophectoderm and inner cell mass; this area needs further research but was beyond the scope of this trial.

There was no difference in the number of follicles after ovarian stimulation between the two groups and the number of follicles did not correlate with the RBC levels of EPA and/or DHA; this did not agree with a previous study which demonstrated an inverse relationship of number of follicles with dietary intake of omega 3 FAs (Hammiche et al., 2011). This study found no difference in the embryo morphology or the number of blastocysts formed. Again, this does not agree with the research by Hammiche et al. which found that embryo morphology was directly proportional to dietary intake of ALA and DHA. Interestingly, this paper also demonstrated a decrease in serum oestradiol levels in patients whose diets reportedly contained higher levels of omega 3 and hypothesised that the omega 3 FAs may affect the granulosa cells' response to gonadotrophins.

This study also demonstrated the feasibility of recruiting couples who are trying to conceive to randomization controlled nutritional intervention trials. Compliance was high and biochemical markers of omega-3 FA status and vitamin D in blood indicated significant enrichment in the both men and women in the study group. The semi-structured interviews combined with the compliance demonstrated that the couples were able to follow the six week dietary intervention with ease, however there were differing opinions on delaying the treatment for a longer period of time. Interestingly, another piece of qualitative research demonstrated that males

undergoing the IVF process were less keen to try low-technology approaches to enhance their fertility than their female counterparts (Throsby and Gill, 2004). This trial did demonstrate a marginally lower compliance level for the men rather than the women, although there was a high correlation between the compliance of the man and woman from the same couple, indicating a joint commitment to the study.

Randomisation was shown to be successful with no statistical differences found in the sociodemographic details between the two groups. There was also no difference in the prudent diet scores of either the female or male volunteers between the two groups. The RBC folate levels of the volunteers correlated with their prudent diet score with a similar positive coefficient to that which was seen in the original paper from which the 20 item food frequency questionnaire was extrapolated (Crozier et al., 2010). The proportion of participants taking multivitamins or other supplements were found to be similar to levels found in previous studies which examined patients from the same clinic (Kermack and Macklon, 2014).

As was expected, the total 25 (OH) vitamin D levels varied with the season of recruitment and for the male volunteers correlated with the amount of time spent outside, this is in agreement with recent research examining the seasonality aspect of serum vitamin D levels (Bonelli et al., 2016). Interestingly, our mean 25 (OH) vitamin D levels were found to be substantially greater than those who participated in the MAVIDOS trial, a double blinded, randomised controlled trial examining vitamin D levels and supplementation on women from 14 weeks of pregnancy (based in Southampton, Oxford and Sheffield) (Cooper et al., 2016). This may be due to vitamin D depletion during the first trimester of pregnancy or because a higher proportion of the PREPARE women were taking supplementation for planned pregnancies prior to the trial. Cooper et al. also demonstrated seasonal changes and found that women who conceived in the summer or autumn months had higher levels of 25 OH vitamin D at 14 weeks gestation, our data concurred with this.

A number of limitations of this trial should however be noted. The study was not powered to demonstrate any impact on pregnancy rates, so no conclusions regarding the impact of the diet on clinical outcomes can be drawn. The duration of the intervention was six weeks, which might have limited the effect on embryo development as it was shorter than the reported duration of oocyte and sperm maturation, considered to take around 3 months (Gougeon, 1986) and 72 days (Clermont, 1972) respectively. However, the duration of the intervention was supported by studies in mice that have demonstrated a remarkable impact of very short term preconceptional dietary changes in embryo and offspring development (Fleming et al., 2011). Moreover, RBC

levels of omega 3 FAs and serum vitamin D were increased by six weeks of dietary supplements, showing that the trial intervention was efficacious in altering the in-vivo nutritional milieu.

The FA profile of the women prior to the intervention was similar to that reported in a previous fish oil supplementation study in pregnancy (Dunstan et al., 2004). The fish oil supplementation in pregnancy study gave the female participants 1.1 g EPA and 2.2 g DHA and therefore similar alterations in the percentages of measured fatty acids were noted. In both studies, percentages of linoleic acid, dihomo-gamma-linoleic acid and arachidonic acid were decreased in the treatment group when compared to the placebo group and percentages of EPA and DHA were increased. Interestingly, in Dunstan et al.'s study, no changes were noted in the levels of decosapentaenoic acid in the women in the treatment group (which mimicked the changes in our male volunteers). There was also no difference seen between stearic acid and gondoic acid in their study (unlike PREPARE which saw a decrease and increase, respectively). This suggests that the RBC fatty acid profile of the studied women studied is representative of the wider female population of this age. However, it should be noted that the study population was predominantly white Caucasian and care should be taken when extrapolating the evidence to those from other ethnic backgrounds.

Despite these limitations, this interventional study addresses the need for randomized interventional studies to confirm or refute the proposed benefits of a Mediterranean diet in the preconception period and shows that even a short intervention can impact on markers of embryo quality. It should be noted however that most over-the-counter omega 3 supplements provide lower doses than those provided in this trial, and the evidence base for their effect remains weak.

In conclusion, the present study demonstrated that levels of omega-3 FAs and vitamin D in the blood can be enhanced by a six week dietary intervention and that a short period of dietary intervention has a significant and demonstrable impact on embryo quality. Omega 3 FA and Vitamin D supplementation should be recommended for couples undergoing artificial reproductive treatments, and these data also support their use in heterosexual couples trying to conceive naturally. Further intervention studies are to examine the effect of longer duration of intervention, to investigate the impact of preconceptional dietary interventions on clinical outcomes and their mechanism of action.

Chapter 4: Results: Effect of lifestyle and diet on female fertility

4.1 Introduction

Approximately one third of subfertility is unexplained and there are a number of hypotheses, currently being investigated. This study begins to question whether it is possible that in a proportion of cases of unexplained subfertility, suboptimal nutritional milieu in the follicular fluid surrounding the maturing oocyte may result in poorer oocyte and therefore embryo quality. Previous studies have examined whether alteration in the follicular fluid could be affected by lifestyle. Increased levels of stearic acid (a saturated fatty acid) have been observed in the follicular fluid of women with a higher BMI (Mirabi et al., 2017) and decreased antioxidant enzyme levels have been demonstrated in older women (Carbone et al., 2003). Furthermore, conditions such as Polycystic Ovarian Syndrome (PCOS) have been shown to alter the fatty acid profile of human follicular fluid; with an increase in palmitoleic acid and oleic acid observed in obese PCOS patients compared to non-obese PCOS patients and controls (Niu et al., 2014). However, the effect of diet and other lifestyle factors such as alcohol intake on the follicle environment have not been extensively investigated.

The importance of lipid metabolism in the process of oocyte maturation is becoming increasingly recognised. Fatty acids are required as an energy source (utilised by β oxidation following the LH surge) in order to support oocyte maturation and early embryo development. Animal studies show that inhibition of β oxidation decreases oocyte quality and subsequent blastocyst formation (Ferguson and Leese, 2006, Dunning et al., 2010). In addition to their use as an energy source, fatty acids are required for the increasing cell surface membrane of the rapidly dividing early embryo (division from 1 to 4 cells results in a 1.74 increase in surface area (Pratt and George, 1989)), and play a vital role in the process of embryo cavitation (Watson, 1992, Berger and Wood, 2004). Haggarty et al. demonstrated that embryos that developed beyond the four cell stage contained higher levels of linoleic and oleic acids and lower levels of saturated fatty acids compared to those that did not (Haggarty et al., 2006). Linoleic acid is an essential fatty acid and must come from the diet. Despite the possible consequences on the early stages of development, the effect of varying fatty acid profiles on embryo quality remains unclear.

Bovine studies have demonstrated that it is not simply the presence of fatty acids in general that is important but the type of fatty acid plays a role; a differing fatty acid composition was observed in oocytes with varying cytoplasmic quality (Kim et al., 2001). Palmitic and stearic acids have been shown to have a negative effect on oocyte maturation, fertilisation, cleavage rate and blastocyst formation (Leroy et al., 2005), possibly due to their effects on amino acid

metabolism (Van Hoeck et al., 2011) and nuclear fragmentation (Sturmey et al., 2009b). However, oleic acid has been shown to counteract the negative effects of palmitic and stearic acid and improve oocyte quality (Aardema et al., 2011).

In humans, linoleic acid levels in follicular fluid were shown to be positively correlated with oocyte fertilisation rates as opposed to arachidonic acid which showed a negative correlation. In the same study the ratio of omega 6 FAs:omega 3 FAs was lower in the follicular fluid of women who achieved a pregnancy following artificial reproductive techniques (ART) (Shaaker et al., 2012). Conversely, one study examining the fatty acid content of human follicular fluid demonstrated that follicular fluid containing increased levels of oleic, palmitic, linoleic and stearic acids resulted in a lower percentage of cumulus oocyte complexes with favourable morphology (Jungheim et al., 2011). In addition, these fatty acids have been shown to be higher in the follicular fluid of women who did not achieve a pregnancy following IVF-ICSI (Mirabi et al., 2017), possibly due to the disruption of oocyte development to the MII stage and cumulus cell expansion (Marei et al., 2010) or the decreased ability of these oocytes to form pronuclei after IVF-ICSI (Ciepiela et al., 2015).

Previous studies have demonstrated that dietary supplementation may alter the nutritional milieu of human follicular fluid (Özkaya et al., 2011). The ability to alter the fatty acid content of follicular fluid has been demonstrated in bovine studies (Childs et al., 2008) but to date, no randomised controlled trials have been conducted in humans.

In addition, to altering the follicular fluid content, previous studies have indicated that fatty acids may modulate folliculogenesis by altering steroid secretion of theca and granulosa cells. Cows supplemented with poly unsaturated fatty acids (PUFAs) reveal increased numbers of follicles (Beam and Butler, 1997) and these were larger (Bilby et al., 2006). However, in a human observational study, higher intakes of the omega-3 PUFAs, DHA and EPA, were associated with a reduction in the number of follicles following ovarian stimulation (Hammiche et al., 2011). Taken together, there remains a need for further investigation of the effects of lifestyle factors on human follicular fluid and the examination of how an altered follicular fluid fatty acid profile affects oocyte and embryo development.

4.1.1 Aims

The aims of this chapter are to:

- Identify the fatty acid profile of human follicular fluid.

- Examine correlations between the levels of specific fatty acids found in erythrocytes compared to follicular fluid.
- Investigate the effect of demographic characteristics and lifestyle on women's follicular fluid fatty acid profile.
- Study the effect of the PREPARE dietary intervention on the fatty acid profile of follicular fluid and on folliculogenesis, oocyte maturation and oocyte fertilisation.
- Examine the effects of the fatty acid profile on morphokinetic markers of embryo quality.

4.1.2 Hypotheses

It is hypothesised that it is possible to alter the fatty acid content of human follicular fluid by dietary supplementation. Moreover, increasing the omega 3 FAs content of follicular fluid may decrease follicle number but enhance oocyte maturation and quality reflected in improved markers of early embryo development.

4.2 Methods

4.2.1 PREPARE trial

The PREPARE trial was carried out as described in Chapter 2: Methods: The "PREPARE" trial. The demographic details of the women were collected and their diet and lifestyle assessed (see section 2.7).

4.2.2 Blood sample analysis

Blood samples were collected from the women and analysed as described in Chapter 2.8.3.

4.2.3 Follicular fluid collection

Follicular fluid was collected during the oocyte retrieval. Once the oocytes had been removed, the first 5 ml collected was placed into a conical centrifuge tube and centrifuged at 400 g for 5 minutes. The supernatant was then removed by pipette and placed into 1 ml aliquots (three or four per patient). These were stored in the -80°C freezer to await analysis.

4.2.4 Follicular fluid analysis

Fatty acid analysis was performed on the follicular fluid. Total lipid was extracted into chloroform: methanol (2:1, vol/vol). The lipid extract was heated to 50°C for 2 hours with 2% methanol in sulphuric acid to produce fatty acid methyl esters (FAMEs). FAMEs were separated and identified by gas chromatography performed according to conditions described elsewhere (Fisk et al., 2014). FAMEs were identified by comparison of run times with those of authentic standards. Fatty acid concentrations are expressed as % of the total fatty acids present in order to maintain consistency with the other tissues measured (i.e. erythrocytes).

4.2.5 Assessment of embryo quality

Embryos were analysed using morphology on day 3 and 5 and morphokinetic markers to assess their quality (see Chapter 2.11)

4.2.6 Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Results are reported as mean ± standard deviation unless otherwise stated. A p value of less than 0.05 was considered significant.

The effect of the lifestyle factors on fatty acid profile in follicular fluid in the placebo group was determined using Pearson's correlation for normally distributed, continuous data and Spearman's rho for non-normally distributed data. Correlations were also examined between the proportions of the specific FAs in the erythrocytes and follicular fluid. Comparisons were made between the proportions of the PUFAs in the follicular fluid between the placebo and treatment groups using ANOVA (any non-normally distributed data was log transformed prior to the analysis).

ANOVA was used to analyse the difference between the follicle count, percentage of oocytes fertilised and percentage of 2PN embryos forming a blastocyst between the placebo group and the treatment group in the PREPARE trial.

In order to ascertain relationships between morphokinetic markers of embryo quality and the fatty acid profile of the follicular fluid, the two groups were analysed separately and a Pearson's correlation or a Spearman's rho correlation was performed as appropriate.

4.3 Results

4.3.1 What is the fatty acid composition of human follicular fluid?

21 fatty acids were identified in the follicular fluid of 49 women in the placebo arm of the PREPARE trial in order to ascertain the baseline follicular fluid profile. Palmitic acid was found in the highest levels (28.50% ± 1.33), followed by linoleic acid (23.12% ± 2.86), oleic acid (17.36% ± 1.93) and stearic acid (12.01% ± 0.91) (see Figure 4.1).

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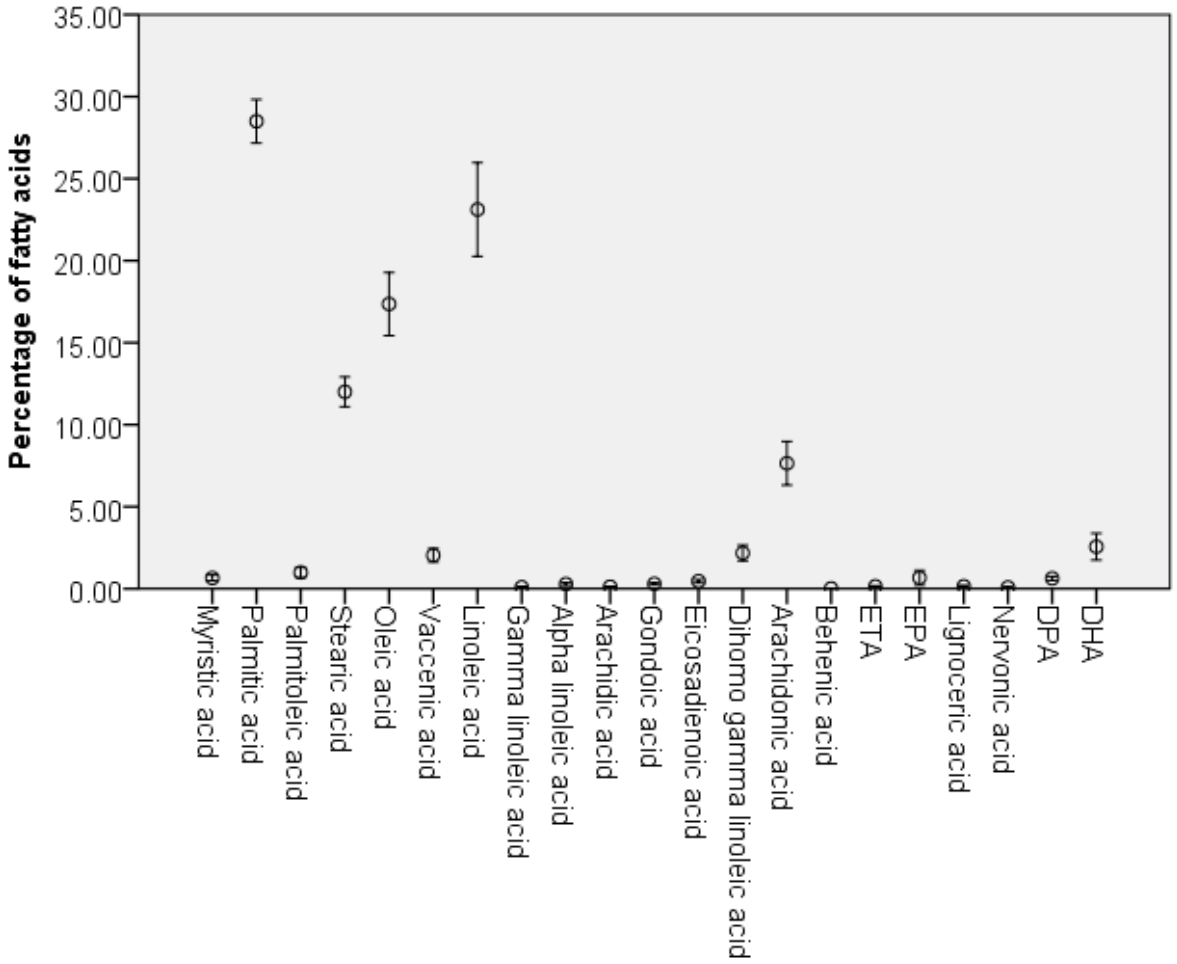

Figure 4.1: Fatty acid profile of women in the placebo arm of the PREPARE trial.

No differences were observed between women in whom the primary cause of the subfertility was a female factor or unexplained and those with primarily male factor subfertility, although the number of women with PCOS in the placebo group was small.

4.3.2 Do the fatty acids measured in the follicular fluid of a woman correlate with those in her erythrocytes?

In the women in the placebo group, positive correlations were observed between the following fatty acids in the erythrocytes and follicular fluid; myristic acid (R_s=0.307, p=0.036), palmitic acid (R=0.464, p=0.001), palmitoleic acid (R_s=0.468, p=0.001), stearic acid (R_s=0.377, p=0.009), oleic acid (R_s=0.298, p=0.042), vaccenic acid (R_s=0.619, p<0.001), linoleic acid (R=0.785, p<0.001), dihomo gamma linoleic acid (Rs=0.544, p<0.001), alpha linoleic acid (Rs=0.326, p=0.025), eicosadienoic acid (R=0.395, p=0.006), dihomo gamma linoleic acid (R=0.565, p<0.001), arachidonic acid (R=0.685, p<0.001), EPA (R_s=0.406, p=0.005) and DHA (R_s=0.538, p<0.001).

4.3.3 Do lifestyle factors affect the fatty acid composition of human follicular fluid (analysis of placebo group)?

An increase in prudent diet score (and hence a healthier diet) was associated with a decrease in the percentage of arachidonic acid measured in the follicular fluid (R=-0.367, p=0.010) (see Figure 4.2).

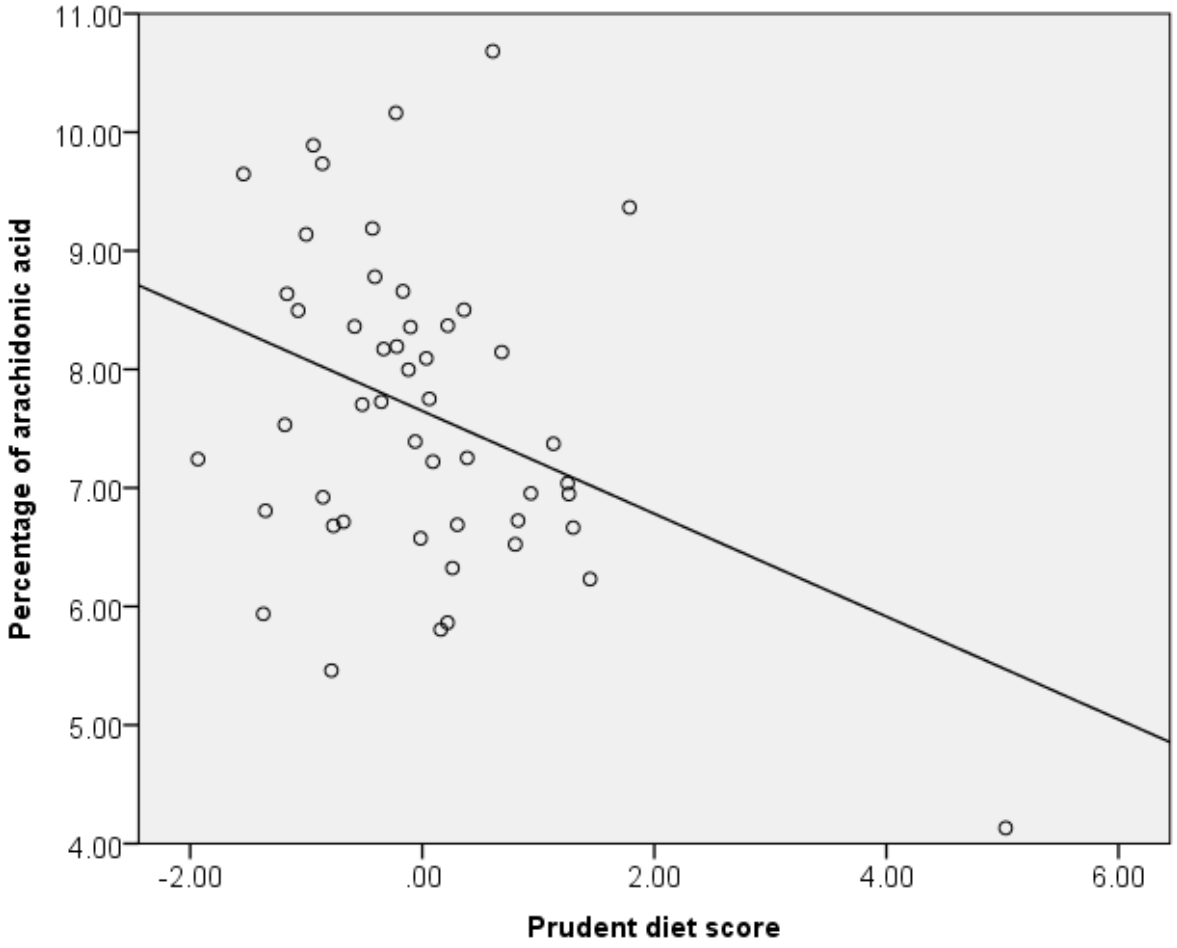

Figure 4.2: Correlation between prudent diet score and percentage of arachidonic acid in follicular fluid
O represents woman's prudent diet score and correlating percentage of arachidonic acid, --- represents linear regression (R^2=-0.134, p=0.010)

BMI was correlated with follicular fluid palmitoleic acid (R_s=0.286, p=0.047), vaccenic acid (R_s=-0.315, p=0.028), gamma linoleic acid (R_s=0.571, p<0.001), dihomo-gamma linoleic acid (R=0.441, p=0.002) and ETA (R=0.418, p=0.003). A negative correlation was observed between alcohol intake and palmitic acid (R=-0.313, p=0.029).

Furthermore, 7 of the fatty acids demonstrated a correlation with the woman's age; negatively with palmitoleic acid (R_s=-0.315, p=0.028); oleic acid (R_s=-0.355, p=0.012), dihomo-gamma linoleic acid (R=-0.297, p=0.038), lignoceric acid (R_s=-0.312, p=0.029); and positively with alpha linoleic acid (R_s=0.298, p=0.038), EPA (R=0.496, p<0.001) and DHA (R_s=0.484, p<0.001).

No correlations were observed between lifestyle factors and stearic acid, linoleic acid, arachidic acid, gondoic acid, eicosadienoic acid, behenic acid, nervonic acid and DPA.

4.3.4 Does the PREPARE dietary intervention alter the fatty acid composition of the participants' follicular fluid?

Significant differences were observed in the percentages of linoleic acid (p<0.001), gamma linoleic acid (p<0.001), arachidic acid (p=0.001), gondoic acid (p<0.001), eicosadienoic acid (p<0.001), dihomo gamma linoleic acid (p<0.001), arachidonic acid (p<0.001), ETA (p=0.034), EPA (p<0.001), lignoceric acid (p<0.001), DPA (p<0.001) and DHA (p<0.001) between the treatment group and the placebo group (see Table 4.1).

Table 4.1: Mean percentage of fatty acids in follicular fluid following the intervention. * data were log transformed to use a parametric test and then raised to the power 10 to calculate the mean.

	Mean ± standard deviation % of fatty acids		p value
	Treatment (n=53)	Placebo (n=49)	
Myristic acid	0.61 ± 0.12	0.66 ± 0.18	0.084
Palmitic acid	28.61 ± 1.40	28.50 ± 1.33	0.697
Palmitoleic acid	0.94* ± 1.35	0.95* ± 1.24	0.893
Stearic acid	11.65 ± 0.91	12.01 ± 0.91	0.055
Oleic acid	16.85* ± 1.10	17.26* ± 1.11	0.227
Vaccenic acid	2.08* ± 1.13	2.00* ± 1.21	0.251
Linoleic acid	20.89 ± 2.43	23.12 ± 2.86	<0.001
gamma-Linolenic acid	0.05* ± 1.53	0.08* ± 1.36	<0.001
α-Linolenic acid	0.26* ± 1.40	0.27* ± 1.36	0.493
Arachidic acid	0.09* ± 1.40	0.11* ± 1.31	0.001
Gondoic acid	0.41* ± 1.19	0.29* ± 1.24	<0.001
Eicosadienoic acid	0.38 ± 0.09	0.46 ± 0.12	<0.001
Dihomo-gamma-linolenic acid	1.39 ± 0.35	2.18 ± 0.48	<0.001
Arachidonic acid	6.55 ± 1.22	7.65 ± 1.35	<0.001
Behenic acid	0.01 ± 0.00	0.01 ± 0.00	0.715
Eicosatetraenoic acid	0.16 ± 0.03	0.15 ± 0.03	0.034
Eicosapentaenoic acid	2.66* ± 1.32	0.58* ± 1.65	<0.001
Lignoceric acid	0.08* ± 1.43	0.14* ± 1.47	<0.001
Nervonic acid	0.07* ± 1.35	0.07* ± 1.31	0.962
Docosapentaenoic acid	0.87 ± 0.17	0.63 ± 0.12	<0.001
Docosahexaenoic acid	5.05* ± 1.17	2.45* ± 1.35	<0.001

4.3.5 Does the PREPARE dietary intervention affect folliculogenesis, oocyte number and maturation or the rate of fertilisation?

There was no difference between the two group in the AFC (antral follicle count) at the baseline scan (p=0.759), the number of follicles greater than 14 mm prior to ovulation trigger (p=0.386) or the number of follicles at oocyte retrieval (p=0.739). Furthermore, no differences were

observed between the treatment group and the placebo group in the number of eggs collected at oocyte retrieval (p=0.540) or the percentage of these that were mature (p=0.810).

A difference was however seen in the percentage of eggs fertilised with a higher fertilisation rate seen in the placebo group (73.23% ± 23.10) compared to the treatment group (63.24% ± 22.45) (p=0.029), although there was no difference between absolute numbers fertilised (8.04 ± 5.84 in the placebo group vs. 6.72 ± 5.33 in the treatment group (p=0.234)). No difference was seen between the percentage of 2 pronuclei (2PN) embryos that formed a blastocyst (p=0.593) or in the number of blastocysts produced per couple in each group (p=0.356). The mean number of blastocysts in the placebo group was 5.08 ± 5.28 and in the treatment group was 4.21 ± 4.22 (p=0.356).

4.3.6 Do the fatty acids measured in follicular fluid of a woman correlate with her embryo quality?

394 embryos from 49 women were analysed in the placebo group. Women with an increased percentage of stearic acid in their follicular fluid had embryos with delayed pronuclei fade (R=0.145, p=0.004); time to the two cell stage (R=0.227, p<0.001), the four cell stage (R=0.151, p=0.003), the eight cell stage (R=0.134, p=0.017) and time to blastocyst formation (R=0.228, p<0.001). A higher percentage of linoleic acid also appeared to prolong the time it took the embryo's pronuclei to fade (R=0.176, p<0.001), the division into two cells (R=0.133, p=0.009) and for blastocyst formation to occur (R=0.281, P<0.001). Conversely, increased percentages of palmitic acid are associated with decreased time to the two cell stage (R=-0.116, p=0.022), time to the four cell stage (R=-0.124, p=0.016) and time to blastocyst formation (R=-0.213, p=0.001). Oleic acid also decreased the time to blastocyst formation (R_s=-0.215, p<0.001), as did DHA (R_s=-0.138, p=0.027) (see Figure 4.3).

356 embryos from 53 women were analysed in the treatment group. Interestingly, no correlations were observed between stearic, linoleic and palmitic acids and the morphokinetic markers of embryo quality. A quicker time to pronuclei fade (R_s=-0.206, p<0.001) and the two cell stage (R_s=-0.131, p=0.014) was seen with increased linoleic acid. Furthermore, higher proportions of DHA in the follicular fluid shortened the time to pronuclei fade (R_s=-0.248, p<0.001, two cells (R_s=-0.196, p<0.001), four cells (R_s=-0.253, p<0.001) and eight cells (R_s=-0.155, p=0.008) but not to blastocyst formation (p=0.303) (see Figure 4.3).

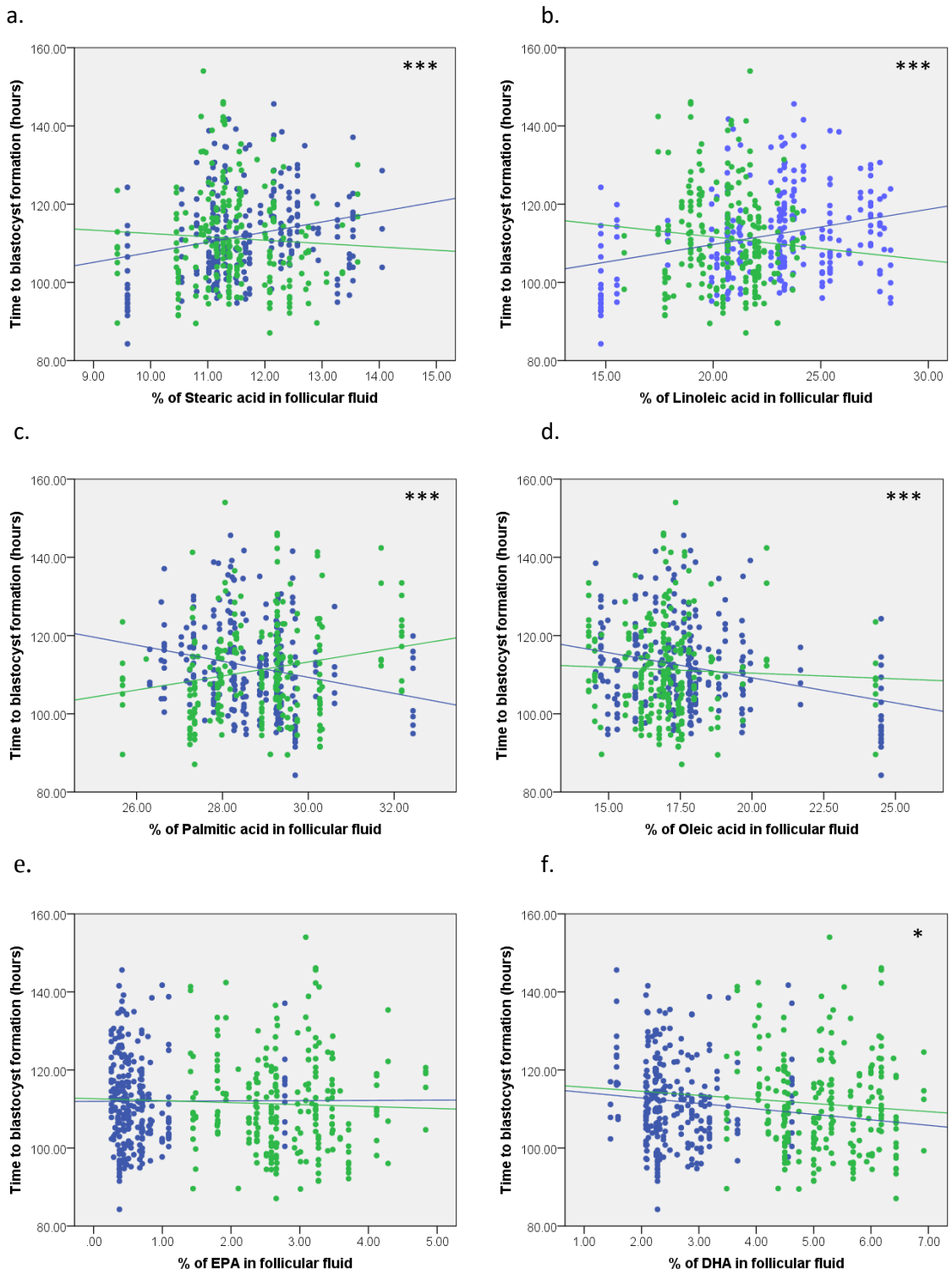

Figure 4.3: The correlation between time to blastocyst formation and percentage of a. stearic acid, b. linoleic acid, c. palmitic acid, d. oleic acid, e. EPA and f. DHA in the placebo group (blue) and the treatment group (green).
Line represents linear regression in the placebo group (blue) and the treatment group (green).
***p value <0.05, **p value<0.01, ***p value<0.001 in placebo group.**
#p value<0.05, ##p value<0.01, ###p value<0.001 in treatment group.

4.4 Discussion

The fatty acids found in the highest percentages in human follicular fluid were palmitic, stearic, oleic and linoleic acids, in agreement with previous findings (Dunning et al., 2014, Leroy et al., 2005). In addition, 14 of the fatty acids measured showed correlations between the levels found in the erythrocytes and in follicular fluid. Such correlations have also been demonstrated by Hauschka et al. (Hauschka et al., 2009).

Although the study did not demonstrate any differences in the fatty acid profile in relation to the cause of subfertility, the vast majority of the included patients had either unexplained or male factor subfertility. Further investigation is needed into whether the fatty acid content in follicular fluid is altered by diseases such as PCOS and endometriosis, as other studies have indicated that this may be the case (Niu et al., 2014). The current study did demonstrate a relationship between follicular fluid FAs and lifestyle which is perhaps unsurprising given the effect of lifestyle on fatty acids found in erythrocytes (Genio, 2015) and the relationships between the fatty acid profiles in the two tissues.

The findings of this research are the first to demonstrate in humans that a short dietary intervention of around six weeks can significantly alter the fatty acid composition of human follicular fluid. A decrease in the proportion of linoleic acid found in the follicular fluid was observed in the treatment group. Linoleic acid is an omega 6 PUFA and animal studies have suggested that increased linoleic acid inhibits cumulus cell expansion and development of the mature oocyte (Marei et al., 2010). Furthermore, in human studies associations have been made with higher levels of cumulus oocyte complexes with unfavourable morphology (Jungheim et al., 2011) and decreased success following IVF-ICSI (Mirabi et al., 2017). A decrease in arachidonic acid in the treatment group was also observed. A recently published study, utilising a "one follicle – one retrieved oocyte – one resulting embryo" investigational model, demonstrated that increased arachidonic acid and linoleic acid and their derivatives resulted in oocytes that did not develop two pronuclei and degenerated following ICSI (Ciepiela et al., 2015). In addition to the decrease in linoleic acid and arachidonic acid, a statistically significant increase in the proportions of EPA and DHA were observed in the treatment group. This alteration in the follicular fluid content has been associated with improved folliculogenesis and IVF performance in bovine studies (Moallem et al., 2013).

While this study did not demonstrate an impact of the dietary intervention on the number of follicles or mature oocytes, a decrease in fertilisation rate was observed in the treatment group.

It is difficult to explain this finding , but very high levels of DHA added during *in vitro* maturation of oocytes has been reported to diminish their quality due to altering the lipid and steroid metabolism of the cumulus cells (Oseikria et al., 2016). Future work should examine the effects of varying doses of the omega 3 FAs on the follicular fluid and oocyte maturation. Despite the difference in fertilisation rate, no difference was observed in blastocyst number relieving some anxiety of possible detrimental effects of the dietary supplement. Furthermore, these outcomes were not the primary outcome of the study and therefore the study was not powered accordingly. Future work should be carried out to examine these specific outcomes.

Despite no difference being seen in markers of folliculogenesis, correlations were observed between the follicular fluid fatty acids and the morphokinetic markers of embryo quality, especially the time to blastocyst formation. Higher levels of stearic acid, linoleic acid and palmitic acid appeared to be detrimental and slow the time to blastocyst formation whereas higher levels of oleic acid and DHA were protective. A similar pattern of detrimental and beneficial fatty acids has been demonstrated in animal studies (Leroy et al., 2005, Aardema et al., 2011). However, additional research is required to try to understand the mechanism of action and the possible implications of this.

A limitation of this study was the inability to examine the fatty acid content of the oocytes themselves, as previous research, in cattle has demonstrated selective uptake of saturated fatty acids by the cumulus-oocyte complex as opposed to PUFAs supplemented in the diet (Fouladi-Nashta et al., 2009, Adamiak et al., 2006). However, cumulus cells from a proportion of women who undertook the PREPARE trial have been stored and it would be interesting to assess the fatty acid profile of these to ascertain whether it is similar to that of the follicular fluid and/or manipulated by the diet.

In conclusion, dietary supplementation can alter the fatty acid profile of human follicular fluid and this may have an effect on morphokinetic markers of embryo quality. However, more research is needed to investigate the implications of these findings and to examine other lifestyle factors that may have an impact.

Chapter 5: Results: Effect of lifestyle and diet on male fertility

5.1 Introduction

Infertility is estimated to affect nine per cent of the world's population (Boivin et al., 2007). Male factors are thought to be the primary cause in approximately a quarter of cases (Evers, 2002) and this proportion is likely to increase if the reported significant decline in semen quality over the last 50 years is accurate (Carlsen et al., 1992). Indeed it has been proposed that this decline in sperm count may, in fact, be due to improvement in semen analyses and in the standardisation of techniques and training as opposed to a true decline (Pacey, 2013). Nevertheless, a number of demographic and lifestyle factors have been shown to impact on semen quality. These include age (Kumar et al., 2017); education level (Parn et al., 2015); body mass index (BMI) (Belloc et al., 2014, Hammoud et al., 2008, Tsao et al., 2015); diet (Kermack and Macklon, 2013, Oostingh et al., 2017, Salas-Huetos et al., 2017); alcohol intake (La Vignera et al., 2013, Povey et al., 2012); caffeine intake (Figa-Talamanca et al., 1996) and exercise levels (Gaskins et al., 2014).

Sperm and seminal fluid contain a high amount of the omega-3 fatty acid docosahexaenoic acid (DHA), which makes up more than 60% of the fatty acids found in the phospholipids within the head and tail of human spermatozoa (Zalata et al., 1998). DHA appears to accumulate preferentially within the spermatozoa, which has higher levels than measured in the seminal plasma (Zerbinati et al., 2016). Seminal plasma DHA levels positively correlate with sperm motility (Zerbinati et al., 2016), and higher levels of DHA in the spermatozoa and seminal plasma have been associated with better maintenance of sperm motility following sperm cryopreservation (Martinez-Soto et al., 2013). The mechanisms of action causing the alteration in the spermatozoa motility by DHA are related to increases in the fluidity of the cell membrane. This is caused by the large number of double bonds in DHA, which appear to be necessary to permit the fluidity required for the motility of the sperm tails (Connor et al., 1998). DHA in the seminal plasma works as an antioxidant, by suppressing the action of reactive oxygen species and hence decreasing oxidative stress, resulting in improved sperm function (Safarinejad, 2011) and reduced DNA damage (Aitken et al., 2010).

These studies point to a likely therapeutic benefit from omega-3 fatty acid supplementation; a double blind, randomised controlled trial in men with oligoasthenoteratospermia demonstrated an improvement in total sperm count and sperm concentration with a daily 1.86 g dose of DHA for a 32 week period (Safarinejad, 2011). However, there remains uncertainty in relation to the benefits of supplementation; previous work concluded a difference in the metabolism of DHA

within the testes of asthenozoospermic compared to normospermic men was responsible for the observed different DHA contents, as opposed to a variance in their dietary intake (Conquer et al., 1999).

Vitamin D deficiency has also been shown to negatively impact on spermatozoa morphology, motility and progressive motility (Blomberg Jensen et al., 2011). There is high expression of vitamin D receptors (VDR) in the testis, epididymis, seminal vesicle and spermatozoa which suggests that vitamin D is important in spermatogenesis, however the mechanism of action remains unclear (Fu et al., 2017). Vitamin D is essential for calcium homoeostasis and a 2-3 fold higher calcium concentration has been observed in epididymal fluid compared to serum (Blomberg Jensen et al., 2010). A calcium influx across the cell membrane is essential to trigger flagellar beating (and hence motility of the spermatozoa) and the acrosome reaction precipitating fertilisation (Krasznai et al., 2006). Furthermore, research has demonstrated a link between vitamin D levels and serum testosterone (an important regulator of spermatogenesis), both in mice (Bouillon et al., 2008) and in humans (Pilz et al., 2011).

Although much of the previous work is based on observational studies, it does point to possible benefits on sperm quality of a diet high in omega-3 fatty acids and vitamin D. There is a need for randomised controlled trials of dietary interventions testing the effects of diets or dietary components on sperm quality. This study is part of the PREPARE trial (Kermack et al., 2014) which demonstrated that this short term dietary intervention had a positive impact on embryo quality; this work has allowed exploration into one of the potential mechanisms behind the improvement seen.

5.1.1 Aims

The aim of this chapter is to:

- Examine the lifestyle factors that affect sperm quality.
- Explore the relationships between FA composition of erythrocytes, seminal plasma and spermatozoa.
- Investigate whether a short dietary intervention (containing omega-3 PUFAs and Vitamin D) providing omega-3 fatty acids, vitamin D and olive oil can alter DHA and EPA in the seminal plasma and spermatozoa.
- Analyse the effect of the dietary intervention on semen analysis parameters and sperm DNA fragmentation.

5.1.2 Hypothesis

Sperm quality is affected by a number of lifestyle factors and a short dietary intervention (of omega 3 PUFAs and Vitamin D) will increase the levels of DHA and EPA in seminal plasma and spermatozoa. Improved semen parameters will be seen with increased levels of DHA and EPA.

5.2 Methods

5.2.1 PREPARE trial

The PREPARE trial was carried out as described in Chapter 2: Methods: The "PREPARE" trial. The demographic details of the men were collected and their diet and lifestyle assessed (see section 2.7).

5.2.2 Blood sample analysis

Blood samples were collected from the men and analysed as described in Chapter 2.8.3.

5.2.3 Semen analysis

Male participants were asked to produce a semen sample on the day of recruitment and on the day of their partner's oocyte retrieval. Semen analysis was performed by a blinded andrologist on all samples. Sample liquefaction and viscosity; the volume of semen produced; sperm concentration; percentage of motile and progressively motile sperm; and percentage of sperm with normal morphology were recorded. The sample was considered normal if at least 1.5 ml of semen was produced; it liquefied within one hour and was not viscous; and it had a least 15 million spermatozoa per ml of ejaculate with a progressive motility of at least 32% and a normal morphology of at least 4% (see Chapter 2.9.2).

Following analysis and, on the day of oocyte retrieval, clinical use, the remaining semen was prepared for storage. Semen (2 ml or the maximum volume available if less) was placed on a density gradient (created using 1 ml of Nidacon PureSperm®100 90% solution overlaid with a 1 ml of Nicadon PureSperm®100 45% solution) and centrifuged at 400 g for 20 minutes to separate the seminal plasma from the sperm. The supernatant (seminal plasma) was removed and stored in 1 ml aliquots at -80°C. The sperm pellet was washed with phosphate-buffered saline (PBS) and centrifuged at 400 g for a further 5 minutes, this sperm pellet was collected and stored at -80°C prior to analysis (see 2.9.3).

For DNA fragmentation analysis, semen was left to liquefy for at least 30 minutes following ejaculation. Between 100 µl and 200 µl was then placed into a tube and plunged into liquid nitrogen to snap freeze and then stored at -80°C until analysis. Sperm DNA fragmentation was assessed using the SpermComet assay, a second generation sperm DNA test (Lewis et al., 2013) (see Chapter 2.9.4.2). This test allows the analysis of the amount of DNA damage per sperm and

not simply its presence or absence; providing greater specificity and sensitivity than previous methods.

5.2.4 Fatty acid analysis

Fatty acid analysis was performed on seminal plasma and sperm. In all cases, total lipid was extracted into chloroform: methanol (2:1, vol/vol). The lipid extract was heated to 50°C for 2 hours with 2% methanol in sulphuric acid to produce fatty acid methyl esters (FAMEs). FAMEs were separated and identified by gas chromatography performed according to conditions described elsewhere (Fisk et al., 2014). FAMEs were identified by comparison of run times with those of authentic standards. Fatty acid concentrations are expressed as % of the total fatty acids present (see Chapter 2.8.3.1). Analysis on the sperm was only performed if the sperm concentration was greater than 15 million per ml and there was enough semen remaining following clinical use on the day of oocyte retrieval.

5.2.5 Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Results are reported as mean ± standard deviation unless otherwise stated. Differences between the sociodemographic characteristics of participants in the two groups were analysed using ANOVA; characteristics that were not normally distributed and were scalar were adjusted by log transforming and then included in the ANOVA analysis. Fatty acid measurements were log transformed and then a t test was used to examine the percentage change. A p value of less than 0.05 was considered significant. The effect of the demographic and lifestyle factors on the semen analysis parameters prior to the intervention was determined using Pearson's correlation for continuous data and ANOVA for grouped data; the effect was examined for the entire cohort and for those undergoing IVF or IVF-ICSI separately. Comparisons were made between the proportions of the PUFAs in the erythrocytes, seminal plasma and sperm and the serum levels of Vitamin D using ANOVA (any non-normally distributed data was log transformed prior to the analysis). This method of analysis was also used to compare the semen analysis parameters in the two groups. Correlations were then examined between these parameters and the proportions of DHA and EPA in the erythrocytes, seminal plasma and sperm. For normally distributed data Pearson's correlation was used, while for non-normally distributed data Spearman's Rho was used.

5.3 Results

5.3.1 Does the males' demographic data and lifestyle have an effect on their semen analysis?

Prior to the intervention, demographic and lifestyle factors potentially affecting the semen analysis parameters were assessed. A higher sperm concentration was observed in male participants older than 35 years compared to those younger than 35 years (the division was made at this age as this allowed for similar numbers in each group). Caffeine intake also appeared to have an effect on some sperm quality parameters: a decrease in the percentage of sperm with normal morphology was observed as the amount of caffeine consumed increased. No effect on the semen analysis parameters was detected when examining the participants' BMI, alcohol intake or amount of exercise per week (see Table 5.1). None of the men smoked cigarettes as this is a stipulation of the IVF unit, they did also not use any illicit substances and therefore these lifestyle choices could not be analysed.

Table 5.1: Effect of demographic and lifestyle factors on semen analysis parameters at study entry.
All values are mean ± standard deviation. Analysis performed using ANOVA.Φ General Certificate of Secondary Education (UK examinations taken age 16 years)
Ψ General Certificate of Education Advanced level (UK examinations taken age 18 years)

		Sample (n)	Semen volume (ml)	Sperm concentration (M/ml)	Total motility (%)	Progressive motility (%)	Morphology (% normal forms)	Samples (n)	Sperm DNA fragmentation(%)
Age (years)	**<35**	50	3.66 ±1.61	**46.0 ±31.60**	52.92 ±10.68	41.85 ±10.53	6.77 ±3.68	23	29.14 ±11.41
	>35	58	3.36 ±1.51	**50.93 ±34.32**	53.33 ±9.45	43.37 ±9.20	8.81 ±4.12	30	28.83 ±7.66
	p value		0.279	**0.032**	0.225	0.100	0.871		0.292
Educational attainment	**None**	3	2.80 ±0.53	19.67 ±7.23	35.67 ±22.03	22.67 ±20.40	6.67 ±3.06	2	23.15 ±14.82
	GCSEsϕ or equivalent	19	3.35 ±1.32	52.50 ±34.31	50.17 ±16.06	39.06 ±17.56	9.89 ±5.57	9	26.42 ±5.123
	A-levelsψ or equivalent	35	3.73 ±1.74	29.98 ±28.18	48.68 ±11.75	37.18 ±12.33	7.44 ±3.51	19	34.93 ±11.09
	University degree or above	51	3.13 ±1.36	37.53 ±30.64	49.78 ±14.22	38.96 ±15.28	8.91 ±4.92	23	32.65 ±9.62
	p value		0.287	0.062	0.394	0.318	0.295		0.108
BMI (kg/m^2)	**18 – 25 (normal)**	34	3.27 ±1.47	35.30 ±39.68	47.42 ±12.58	36.27 ±14.08	8.42 ±4.55	17	35.79 ±8.83
	25 – 30 (overweight)	50	3.41 ±1.62	38.40 ±26.04	50.16 ±15.85	40.22 ±15.81	8.48 ±4.05	26	30.18 ±10.30
	>30 (obese)	24	3.35 ±1.24	37.07 ±27.28	49.17 ±11.84	35.39 ±14.56	9.15 ±6.25	10	30.57 ±10.73
	p value		0.917	0.906	0.688	0.331	0.844		0.179
Prudent diet score	**<-0.5 (very unhealthy)**	36	3.16 ±1.33	31.94 ±24.25	50.19 ±14.73	38.50 ±14.65	8.03 ±4.04	17	32.46 ±11.22
	-0.5 – 0 (unhealthy	28	3.40 ±1.65	34.10 ±26.78	47.04 ±15.38	36.79 ±17.70	9.46 ±5.85	12	31.19 ±11.12
	0 – 0.5 (healthy)	19	3.58 ±1.49	35.43 ±32.56	48.84 ±14.40	36.32 ±15.97	8.94 ±4.28	9	33.01 ±5.98
	>0.5 (very healthy	23	3.41 ±1.56	50.41 ±40.33	50.09 ±11.09	39.83 ±11.30	8.14 ±4.35	15	31.70 ±10.70
	p value		0.770	0.132	0.818	0.852	0.662		0.977
Alcohol intake	**None**	0	-	-	-	-	-	-	-
	1 – 14 units per week (recommended)	73	3.26 ±1.51	36.61 ±30.70	47.56 ±15.31	36.83 ±15.96	8.77 ±5.03	36	32.60 ±10.03
	>14 units per week	35	3.55 ±1.42	38.29 ±31.70	52.35 ±10.21	40.29 ±12.54	8.27 ±3.88	17	30.90 ±10.41
	p value		0.349	0.794	0.100	0.269	0.632		0.573
Caffeine intake	**<100 mg/day (low)**	27	3.87 ±1.63	36.04 ±20.44	50.50 ±11.47	40.69 ±11.42	**10.52 ±5.29**	11	30.14 ±9.73
	100 – 500 mg/day (moderate)	63	3.11 ±1.39	38.75 ±35.91	48.05 ±14.78	36.00 ±16.04	**8.23 ±4.35**	31	32.82 ±9.89
	>500 mg/day (excessive)	18	3.44 ±1.44	33.22 ±24.94	50.67 ±15.01	40.67 ±15.42	**6.94 ±3.99**	11	31.81 ±11.60
	p value		0.080	0.786	0.663	0.287	**0.034**		0.755
Amount of exercise	**<150 minutes per week (inadequate)**	31	3.23 ±1.27	34.02 ±24.29	49.19 ±14.68	38.94 ±16.00	8.96 ±5.83	15	33.53 ±7.66
	150 – 300 minutes per week (adequate)	40	3.61 ±1.62	40.66 ±33.76	49.08 ±14.57	38.51 ±14.98	8.91 ±4.19	17	30.08 ±9.62
	>300 minutes per week (elevated)	37	3.19 ±1.50	36.03 ±33.08	49.03 ±14.01	36.47 ±14.38	7.97 ±4.16	21	32.59 ±12.00
	p value		0.407	0.651	0.999	0.767	0.642		0.607

When correlations were analysed separately in men whose sperm was of sufficient quality to undergo conventional IVF and those requiring IVF-ICSI, a relationship between BMI and progressive sperm motility was observed (r=-0.282, p=0.034) in the IVF group. In addition, a trend towards less motile sperm (r=-0.248, p=0.063) and a lower concentration of sperm (r=-0.221, p=0.098) was seen in men with a higher BMI within this subset. Furthermore, there were trends towards a higher sperm concentration with a more prudent diet (r=0.222, p=0.097) and towards more motile sperm with increased caffeine intake (r_s=0.258, p=0.062) in those undergoing IVF but not in those having IVF-ICSI. In addition, those who were undergoing IVF-ICSI showed a trend towards less DNA sperm fragmentation in those men who consumed less alcohol (r_s=-0.480, p=0.0620) (see Table 5.2).

Table 5.2: Correlation between demographic data for patients and semen analysis parameters.

Pearson's correlation used to demonstrate the relationships with age, BMI and prudent diet score. Spearman's correlation was used to examine the relationships with alcohol and caffeine intake and amount of exercise.

		Patients undergoing IVF						Patients undergoing IVF-ICSI					
		Semen volume (ml)	Sperm concentration (M/ml)	Total motility (%)	Progressive motility (%)	Morphology (% normal forms)	Sperm DNA fragmentation(%)	Semen volume (ml)	Sperm concentration (M/ml)	Total motility (%)	Progressive motility (%)	Morphology (% normal forms)	Sperm DNA fragmentation(%)
Age (years)	**R**	-0.197	-0.044	-0.019	0.001	-0.067	0.028	-0.152	0.243	0.045	0.142	0.012	0.124
	p	0.141	0.745	0.890	0.993	0.621	0.875	0.331	0.126	0.780	0.376	0.952	0.602
BMI (kg/m^2)	**R**	0.102	-0.221	-0.248	**-0.282**	0.099	-0.169	-0.008	0.003	0.117	0.067	0.077	-0.357
	p	0.452	0.098	0.063	**0.034**	0.462	0.348	0.959	0.986	0.467	0.675	0.691	0.123
Prudent diet score	**R**	0.066	0.222	0.139	0.174	-0.012	-0.117	0.214	-0.024	-0.067	-0.082	-0.047	0.350
	p	0.624	0.097	0.302	0.196	0.932	0.516	0.169	0.882	0.675	0.611	0.810	0.131
Alcohol intake	**R**	-0.051	0.049	-0.028	-0.059	-0.022	-0.051	0.112	-0.056	0.231	0.106	-0.175	-0.480
	P	0.726	0.735	0.846	0.684	0.879	0.795	0.521	0.753	0.189	0.551	0.392	0.060
Caffeine intake	**R**	-0.049	0.101	0.258	0.190	-0.164	0.021	-0.295	-0.014	-0.120	-0.221	-0.169	-0.033
	p	0.726	0.474	0.062	0.174	0.240	0.911	0.068	0.935	0.478	0.188	0.418	0.896
Amount of exercise	**R**	-0.132	0.108	0.164	0.128	-0.020	-0.237	0.219	0.233	-0.181	-0.278	-0.012	0.123
	p	0.329	0.425	0.223	0.341	0.885	0.184	0.158	0.142	0.257	0.078	0.949	0.606

5.3.2 Are there correlations between the percentages of DHA and EPA in erythrocytes, seminal plasma and sperm and the semen analysis?

Prior to the intervention, no correlations were observed between the percentages of DHA and EPA in the erythrocytes and the percentages of these FAs found in the seminal plasma or sperm. There was also no correlation between the DHA and EPA in the seminal plasma and the sperm. Surprsingly, there was a negative correlation between the percentage of DHA in the sperm and the percentage of EPA (R_S=-0.487, p<0.001).

Furthermore, correlations were observed between the semen parameters and the percentages of DHA in the seminal plasma and the sperm. The percentage of DHA in the seminal plasma positively correlated with sperm concentration (r=0.230, p=0.022). Furthermore, increasing levels of DHA within the sperm were associated with increased sperm concentration (r_s=0.700, p<0.001); increased sperm motility (r_s=0.408, p=0.002); increased progressive motility (r_s=0.528, p<0.001); increased percentage of sperm with normal morphology (r_s=0.442, p=0.001); and with decreased sperm DNA fragmentation (r_s=-0.523, p=0.006) (see Table 5.3). EPA in the erythrocytes, seminal plasma and sperm and serum vitamin D concentration did not correlate with any semen analysis parameters.

Table 5.3: Correlation between DHA in erythrocytes, seminal plasma and sperm and semen analysis parameters prior to the dietary intervention.

Pearson's correlation was used to demonstrate the relationships with DHA in the erythrocytes and the seminal plasma. Spearman's correlation was used to examine the relationships with DHA in the sperm.

Correlation	DHA in the erythrocytes (%)	DHA in the seminal plasma (%)	DHA in the sperm (%)
Concentration of sperm (million per mL)	0.063	**0.230**	**0.700**
p value	0.533	**0.022**	**<0.001**
Sperm motility (%)	-0.097	0.077	**0.408**
p value	0.339	0.445	**0.002**
Sperm progressive motility (%)	-0.043	0.091	**0.528**
p value	0.676	0.370	**<0.001**
Sperm with normal morphology (%)	0.037	-0.097	**0.442**
p value	0.731	0.371	**0.001**
Sperm DNA fragmentation (%)	-0.161	0.048	**-0.523**
p value	0.248	0.732	**0.006**

5.3.3 What effect did the dietary intervention have on erythrocyte percentages of EPA and DHA and serum concentrations of Vitamin D?

As reported previously, an increase in erythrocyte EPA and DHA and serum vitamin D was observed in the treatment arm of the trial (see **Error! Reference source not found.** and Table 3.7).

5.3.4 What effect did the dietary intervention have on percentages of DHA and EPA in seminal plasma and sperm?

After the dietary intervention, the percentage of EPA measured in the sperm was higher in the treatment compared to the placebo group (0.08 % ± 1.57 vs 0.06 % ± 1.57, p=0.007) as was EPA measured in the seminal plasma (0.12 % ± 1.82 vs. 0.09 % ± 2.05, p=0.032). However no difference was seen in the percentage of DHA in sperm (15.43 % ± 2.64 vs. 13.85 % ± 1.89, p=0.379) or seminal plasma (8.99 % ± 3.84 vs. 9.24 % ± 4.45, p=0.764) (see Table 5.4).

Table 5.4: DHA and EPA at study entry and following the dietary intervention in the treatment and placebo groups.

*** data were log transformed for analysis and then raised to the power 10 to calculate the mean.**

	Treatment group		p value	Placebo group		p value	p value comparing post intervention levels
	Prior to intervention	Post intervention		Prior to intervention	Post intervention		
N	53	51		55	51		
% DHA in erythrocytes	4.69 ± 1.10	7.11 ± 1.12	<0.001	4.52 ± 1.15	4.57 ± 1.18	0.834	<0.001
% DHA in seminal plasma	8.78 ± 3.78	8.99 ± 3.84	0.779	9.11 ± 3.96	9.24 ± 4.45	0.878	0.764
N	18	18		21	21		
% DHA in sperm	16.54* ± 2.14	15.43* ± 2.64	0.189	18.08* ± 1.95	13.85* ± 1.89	0.049	0.679
N	53	51		55	51		
% EPA in erythrocytes	0.93* ± 1.36	2.38* ± 1.30	<0.001	0.91* ± 1.36	0.91* ± 1.34	0.965	<0.001
% EPA in seminal plasma	0.10* ± 1.84	0.12* ± 1.82	0.111	0.08* ± 1.80	0.09* ± 2.05	0.796	0.032
N	18	18		21	21		
% EPA in sperm	0.06* ± 1.67	0.08* ± 1.57	0.528	0.05* ± 1.67	0.06* ± 1.57	0.233	0.007

There was a correlation between the percentage of EPA in erythrocytes and sperm (r_s=0.421, p=0.008) however, no other correlations between EPA and DHA in the erythrocytes, seminal fluid and sperm were observed.

5.3.5 What effect did the dietary intervention have on the semen analysis parameters?

There was no effect of the intervention on any of the semen parameters (see

Figure **5.1**); this remained true for both the men undergoing conventional IVF and those utilising IVF-ICSI.

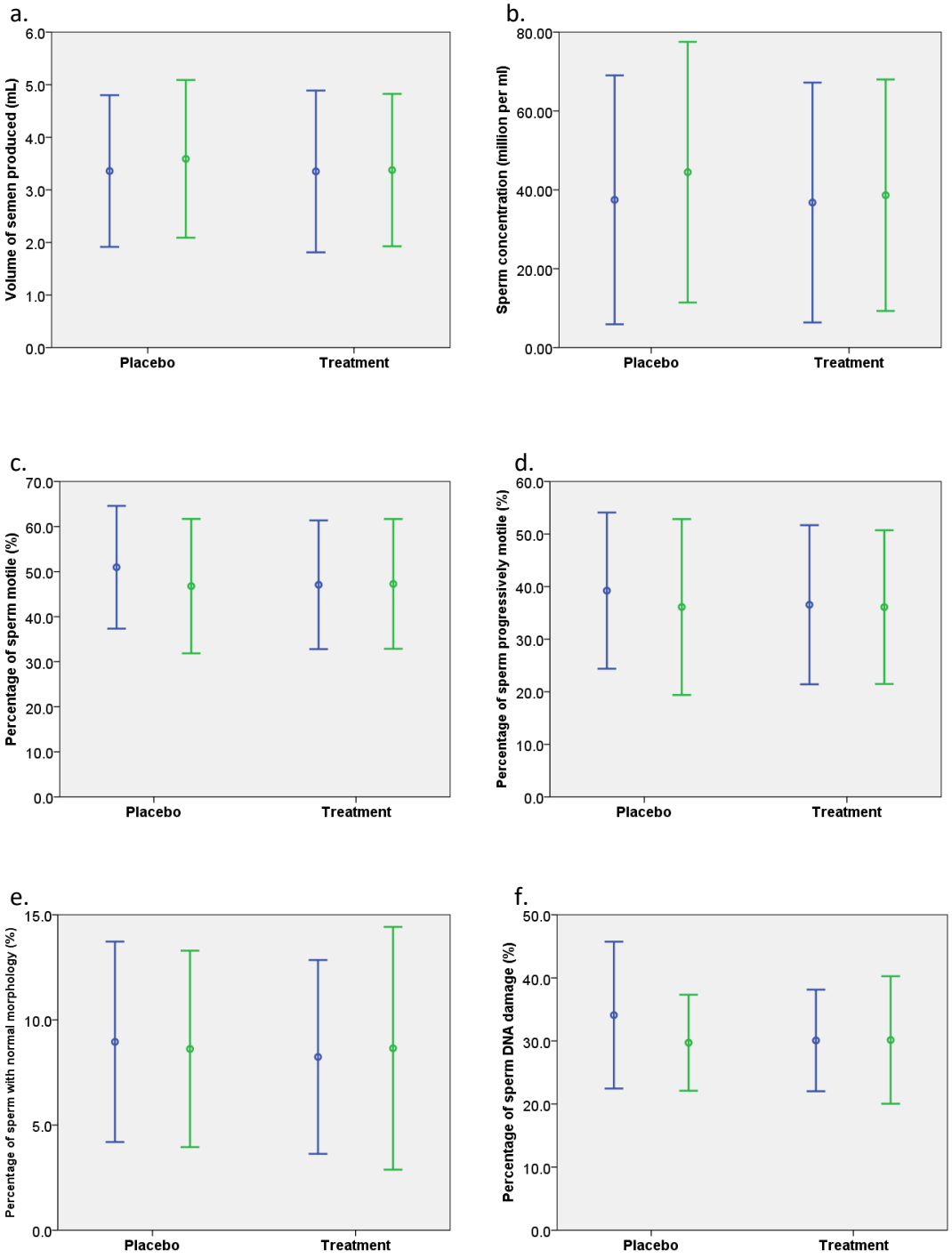

Figure 5.1: Supplementation with Omega 3 fatty acids for six weeks does not affect the semen analysis parameters or percentage of sperm DNA fragmentation.

Effect of dietary intervention (pre intervention= blue, post intervention=green) on (a) volume of semen, (b) concentration of sperm, (c) sperm motility, (d) sperm progressive motility, (e) sperm morphology and (f) sperm DNA fragmentation. Values are mean ± standard deviation.

5.4 Discussion

The current trial showed that, although a dietary intervention providing 2 g of EPA plus DHA per day (1.2 g DHA daily) significantly increased the content of both EPA and DHA in erythrocytes, there was no increase in EPA or DHA in seminal plasma or in DHA in sperm or improvement in semen quality parameters. Seminal plasma and sperm DHA content was positively related to better sperm quality and morphology suggesting that increasing DHA content of sperm through nutritional strategies might still improve sperm quality. The lack of effect on DHA content of seminal plasma and sperm may be because the time period of intervention was too short to allow for DHA incorporation into newly developing sperm. An alternative explanation may be that the DHA content of sperm is determined more by metabolic processes in the testes than by dietary intake or blood levels of DHA. The latter explanation is supported by the lack of correlation found between the DHA in blood, seminal plasma and sperm in this research. Further studies should be undertaken with longer time periods of intervention in order to examine and distinguish these possibilities.

The DHA content of seminal plasma and sperm reported here is in accordance with values published by others (Safarinejad et al., 2010, Safarinejad, 2011). The findings from our study also agree with work that has demonstrated that increased age in subfertile males is associated with increased sperm concentration (Brahem et al., 2011). However, this finding should be interpreted with caution because often partners within a couple are of a similar age, and as a couple becomes older the difficulty in conceiving is more likely a female factor rather than male hence accounting for a perceived improved sperm concentration in older males. In this trial, there was a strong association between the age of the man and his female partner ($r=0.571$, $p<0.001$). In couples seeking IVF, if the women is aged over 35 years then it may be presumed that the diagnosis is more likely to be due to poor ovarian reserve rather than a male factor.

When examining the demographic features of the participants, the greatest correlation of modifiable lifestyle factors (BMI, prudent diet score and caffeine) with sperm quality indicators was seen in men undergoing IVF, this is possibly because those requiring ICSI may have had additional pathologies which could not be overcome by simply making healthier lifestyle choices. The relationship between BMI and sperm quality is inconsistent. No differences were seen in the sperm parameters among men with differing BMIs, which concurs with other research findings indicating there is no significant link between BMI and sperm parameters (Eskandar et al., 2012, Povey et al., 2012). However, when men with sperm quality suitable to be used for IVF were examined, a negative impact of BMI was observed. This has also been demonstrated by previous

studies which have shown a detrimental effect of increasing BMI on sperm parameters including decreased semen volume (Belloc et al., 2014), lower sperm concentration (Tsao et al., 2015) and proportionally fewer progressively motile sperm (Hammoud et al., 2008).

A previous study demonstrated that men with a more prudent diet had an increased percentage of progressively motile sperm when compared to those with a less prudent diet (Gaskins et al., 2012). Our results did not demonstrate this, but did show a possible positive correlation between prudent diet and sperm concentration, a relationship also noted in recent research (Oostingh et al., 2017). However, in this recent study, the correlation was more pronounced in men with a poor sperm count, whereas the current study demonstrated this relationship in those with a normal sperm count.

No correlation was seen between the men's alcohol intake and the sperm parameters. This concurred with a large case referent study which demonstrated no significant association (Povey et al., 2012). However, the majority of participants had a weekly alcohol intake within recommended limits which could account for the fact that no difference was seen; research in which a decrease in semen sample size and sperm concentration was observed was in men with alcohol dependence syndrome (i.e. not those with low to moderate intake) (Kucheria et al., 1985). Furthermore, an older study performed on a cohort of 258 men attending a fertility clinic showed similar results to ours with regards to both the levels of alcohol intake and the lack of correlation with sperm parameters (Dunphy et al., 1991). The effect of increased caffeine intake on sperm quality is contentious (Ricci et al., 2017); however, this study supports findings of increased caffeine intake being linked with increased abnormal sperm morphology (Figa-Talamanca et al., 1996). Our study also showed a possible improvement in sperm motility (in IVF patients) with increased caffeine exposure. A direct effect of caffeine on sperm motility in a laboratory model has been demonstrated (Moussa, 1983) but to our knowledge this has not been reported in a human study. However, when considering these findings it is important to recognise that both alcohol and caffeine intake were assessed by questionnaire, potentially resulting in recall bias.

Recently, papers have been published examining the relationship between sperm parameters and exercise (Maleki and Tartibian, 2017, Rosety et al., 2017). Our findings did not agree with those of these studies: increasing amount of exercise improves sperm parameters. However, the previous studies examined men with a poor semen analysis to begin with or those who were obese prior to starting the exercise regime, so comparisons between studies should be made with caution.

We did demonstrate a correlation between sperm concentration, sperm motility and progressive sperm motility and the proportion of DHA in the sperm, which concurs with data published showing that men with asthenozoospermia, oligozoospermia and oligoasthenozoospermia had lower concentrations of DHA in their sperm than those with normozoospermia (Aksoy et al., 2006, Safarinejad et al., 2010).

The lack of difference in the sperm parameters between the two treatment groups and the fact that there was no increase in the seminal plasma and sperm DHA when dietary intake of DHA and erythyrocyte levels of DHA both increased implies that either the seminal plasma and sperm is a protected environment (although this is not in keeping with the increase in sperm EPA) or that the dietary intervention was not taken for long enough to enrich sperm with DHA. A further trial should be undertaken to see if a 12 to 24 week intervention with DHA improves sperm quality outcomes.

Our study has several strengths. First, retention of participants was high (102 out of 111 participants completed the trial). Secondly, compliance in both groups was high. Thirdly, we obtained a large and broad amount of demographic information about the participants. Fourthly, sperm parameters were measured according to WHO guidelines. Finally, we measured omega-3 fatty acids in erythrocytes, a recognised indicator of dietary intake (Harris and Von Schacky, 2004, von Schacky, 2011) and of concentrations in some tissues (Harris et al., 2004). However, there are also some limitations. First, the duration of the dietary intervention may have been too short to affect DHA incorporation into sperm. Secondly, the participants were heterogeneous with regard to the quality of their sperm. Thirdly, sperm fatty acid composition was assessed in only a sub-set of participants.

In conclusion, this study confirmed that DHA is fundamental to semen quality but demonstrated that the DHA content of seminal plasma and sperm could not be altered with a relatively short dietary intervention providing DHA. Further work is required to see if increasing the length of dietary intervention will increase the DHA content of semen; this should be aimed at men who are diagnosed with male factor infertility as a strategy to improve the chances of their partner becoming pregnant.

Chapter 6: A prospective randomised trial comparing embryo development (morphological and metabolic) in the standard versus the Embryoscope incubator. (PROMOTE trial)

6.1 Introduction

Embryos are cultured *in vitro* in strictly controlled conditions that have been shown to be vital to embryo quality and blastocyst formation rates. Key conditions include a temperature of 37°C (Hong et al., 2014) and a premixed triple gas mix containing 89% nitrogen, 5% O_2 (Waldenstrom et al., 2009, Meintjes et al., 2009) and 6% CO_2. The latter varies marginally due to the altitude of the clinic and acts via a bicarbonate/CO_2 buffer system to allow the maintenance of pH between 7.2 and 7.4. While incubator conditions are crucial to successful development, appropriate selection of the most viable embryos for transfer to the uterus is also important. The assessment of embryos in human IVF has traditionally been performed using morphological grading systems at the cleavage stage (day 2 and/or day 3 after fertilisation) and following blastocyst formation (day 5 and/or day 6). However, in order to make this assessment, embryos have needed to be removed from the incubator, thus disrupting the strictly controlled conditions. It has been shown that decreasing the number of times embryos are exposed to the environment outside of an incubator improves blastocyst formation rate (Zhang et al., 2010). Furthermore, fluctuations in embryo temperature (Wang et al., 2002), oxygen tension (Gomes Sobrinho et al., 2011) and pH (Swain, 2010) have been demonstrated to decrease embryo quality.

The Embryoscope is a time-lapse system (TLS), which allows embryos to be monitored without removing them from their culture chamber, and allows dynamic markers of embryo development to be assessed (morphokinetic parameters). There is some evidence to suggest that embryos cultured in a time-lapse incubator may offer higher success rates than those cultured in traditional benchtop incubators. However, most studies comparing time-lapse to traditional bench top incubator have not been designed to discern the relative contributions of the more stable environment offered, or the additional information provided to aid embryo selection for transfer (Meseguer et al., 2012, Kahraman et al., 2012, Rubio et al., 2014). One previous study did separate these confounding factors and aimed to examine the effect of the more stable environment on embryo development by using standard morphology for embryo selection. No differences were observed. However, embryos in this trial were transferred on day 2, as opposed to day 3 or on day 5, following blastocyst formation (Park et al., 2015). A Cochrane review in 2015 stated that “there were insufficient differences in live birth or clinical pregnancy to choose between time lapse systems (TLS) and conventional incubators” and called for further data to investigate the environment (Armstrong et al., 2015). Following this call for more research, a number of papers have been published comparing live birth rates (Mascarenhas et al., 2018, Barrie et al., 2017), including demonstrating a higher mean

birthweight resulting from embryos cultured in a TLS (Mascarenhas et al., 2018). However, a more recent Cochrane review stated that the evidence continued to be poor and demonstrated no difference in live birth rate that could be attributed solely to the more stable culture environment (i.e. when time lapse incubators were compared with standard incubators but all embryos were assessed using conventional morphological assessment) (Armstrong et al., 2018).

Despite the advancing laboratory techniques, success rates in IVF have remained static, this may reflect the limitations of morphological and morphokinetic markers to discern embryo functional quality. There has therefore been growing interest in the use of metabolic markers as indicators of quality (Thompson et al., 2016). The metabolic profile of an embryo varies greatly prior to and after compaction. In the pre-compaction stage of embryo development, the embryo is relatively quiescent and predominantly utilises pyruvate as its preferred energy source (Gott et al., 1990). Pyruvate is used by mitochondria in the tricarboxylic acid (TCA) cycle in order to produce ATP. Pyruvate uptake has been shown to be approximately 28 pmol/embryo/hour on day 2.5, rising to 40 pmol/embryo/hour on day 4.5 (Hardy et al., 1989). Although attempts have been made to assess pyruvate utilisation as an early predictor of embryo quality (Conaghan et al., 1993, Turner et al., 1995), success has been limited, probably due to the low metabolic activity of the cleavage stage embryo. However, one publication demonstrated that on day 4 uptake was significantly higher in embryos that went on to form a blastocyst than those which arrested at prior to compaction (Hardy et al., 1989).

Conversely, following compaction, glucose is consumed preferentially by the embryo, leading to glycolysis being the main method of energy production (Gott et al., 1990). Hardy et al. demonstrated that the glucose consumption was approximately 8 pmol/embryo/hour on day 2.5, 14 pmol/embryo/hour on day 4.5 and 24 pmol/embryo/hour on day 5 (Hardy et al., 1989). Multiple studies have demonstrated a positive relationship between glucose metabolism and blastocyst formation (Hardy et al., 1989, Gardner et al., 2001, Gott et al., 1990) and pregnancy and live birth rate (Gardner et al., 2011).

It has been shown that the amino acid metabolism of human precompaction embryos cultured *in vitro* correlates inversely with their viability. Thus, the analysis of embryo metabolism through amino acid profiling has been proposed as a novel, functional means of assessing embryo viability and selection for transfer (Brison et al., 2004). Metabolically 'quiet' embryos which turnover amino acids at a lower rate are developmentally higher quality than those with a higher amino acid turnover (Houghton et al., 2002; Stokes et al., 2007). Interestingly, amino acid profiling is also able to distinguish between developmentally competent embryos of the highest

morphological grade (Stokes et al., 2007). Evidence from animal studies suggests that a high amino acid turnover at the cleavage stage of development positively correlates with DNA damage (Sturmey et al., 2009) highlighting the benefit of a quiet metabolism. More recently, research in Southampton has examined amino acid turnover during compaction and cavitation, demonstrating embryos with a greater turnover had improved assembly of Zo-1α+ and occludin required for the assembly of trophectoderm junctional complexes (Eckert et al., 2007). Increased amino acid turnover may therefore be associated with improved embryo quality at the time of blastocyst formation.

Most of the previous studies examined only the metabolic profile of embryos that had been inseminated using IVF-ICSI (Brison et al., 2004). This is because cumulus cells are metabolically active and are not removed prior to IVF (as opposed to IVF-ICSI) (Gardner et al., 1996); their presence therefore should be considered prior to analysis of the metabolic profile of an embryo.

Due to difficulties in measuring the substrates and inconsistent results, metabolomic markers have not entered clinical practice for assessing embryo viability. However, measuring the metabolic status of embryos by analyzing nutrient uptake from culture media may provide a tool for assessing the quality of culture conditions.

6.1.1 Aims

The aims of this chapter are to:

- Investigate whether the more stable culture environment provided by the Embryoscope has an effect on early embryo development including morphology and blastocyst formation rate.
- Assess carbohydrate utilisation and amino acid production and consumption in human embryos cultured in G1+ Vitrolife sequential media (days 1 to 3) and G2+ Vitrolife sequential media (days 3 to 5).
- Ascertain differences in the carbohydrate and amino acid utilisation between embryos following insemination by IVF and IVF-ICSI, due to the presence of cumulus cells in those fertilized by IVF.
- Assess whether carbohydrate utilisation and amino acid utilisation and production correlate with embryo morphology and blastocyst formation.

- Examine whether embryos cultured in the Embryoscope have an altered rate of carbohydrate (glucose and pyruvate) utilisation compared to those cultured in the MINC benchtop incubator on day 3 and day 5.
- Determine any differences in amino acid utilisation and production in the EmbryoScope (TLS) and MINC benchtop incubator (standard) on day 3 and day 5.

6.1.2 Hypotheses

This study was designed to test the following hypotheses:

1. That a more stable culture environment provided by the EmbryoScope will improve embryo morphology and blastocyst formation rates compared to the MINC benchtop incubator, and

2. That embryos cultured in the time-lapse system demonstrate an altered metabolic profile compared to the standard incubator; with increased glucose utilisation at the blastocyst stage, decreased amino acid consumption and production at the cleavage stage of development and then increased amino acid consumption and production during compaction and cavitation.

6.2 Methods

6.2.1 Ethical Approval

Ethical approval was sought and received from the South Central - Berkshire Research Ethics Committee (NRES) via the Integrated Research Application System (IRAS) (Ethics number: 14/SC/1260). The project was conducted in accordance with the recommendations for physicians involved in research on human participants adopted by the 18th World Medical Assembly, Helsinki 1964 as revised and recognised by governing laws and EU Directives; and the principles of GCP and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations 2004, as amended in 2006 and any subsequent amendments.

6.2.2 Study population and recruitment

The study was a single centre trial and couples were recruited from Complete Fertility Centre, situated in Princess Anne Hospital, Southampton. They were eligible to take part if the female was aged under 42 years, with an antral follicle count (AFC) of greater than 12 or an anti-müllerian hormone (AMH) of greater than 10 pmol/L. They needed to be having IVF or IVF-ICSI for a standard medical reason. Couples who met the inclusion criteria were approached whilst attending for their baseline scan (on day 2 of their menstrual cycle) and given an information leaflet. A member of the research team then contacted them by telephone to discuss the study further and answer any questions. At a future scan appointment or on the day of egg retrieval, written consent was obtained by the study doctor or nurse (this had to be no less than 1 day after the subjects had been fully informed about the study).

It should be noted that subjects were excluded after the oocytes were checked for fertilisation if fewer than six 2 pronuclei (2PN) embryos were available for culture.

6.2.3 Study design

The participants' notes were accessed in order to record basic demographic details and clinical history. According to standard clinical procedures, if no abnormality was seen on the baseline scan, women commenced ovarian stimulation with daily injections of FSH, and then from stimulation day 5 commenced additional daily injections with a GnRH antagonist to prevent premature ovulation. The patients underwent regular ultrasound monitoring and then were

given a further hormone injection when the criterion for scheduling egg retrieval had been met (at least 3 follicles of 17 mm or more). The patient attended the clinic for egg retrieval 36 hours later. The eggs were then fertilised according to standard clinical procedure. Following fertilisation, if 6 or more 2PN embryos resulted (embryos which had fertilised normally) then they were divided into two groups. Each group was randomly selected to undergo culture in either the EmbryoScope or in a MINC incubator (see Figure 6.1).

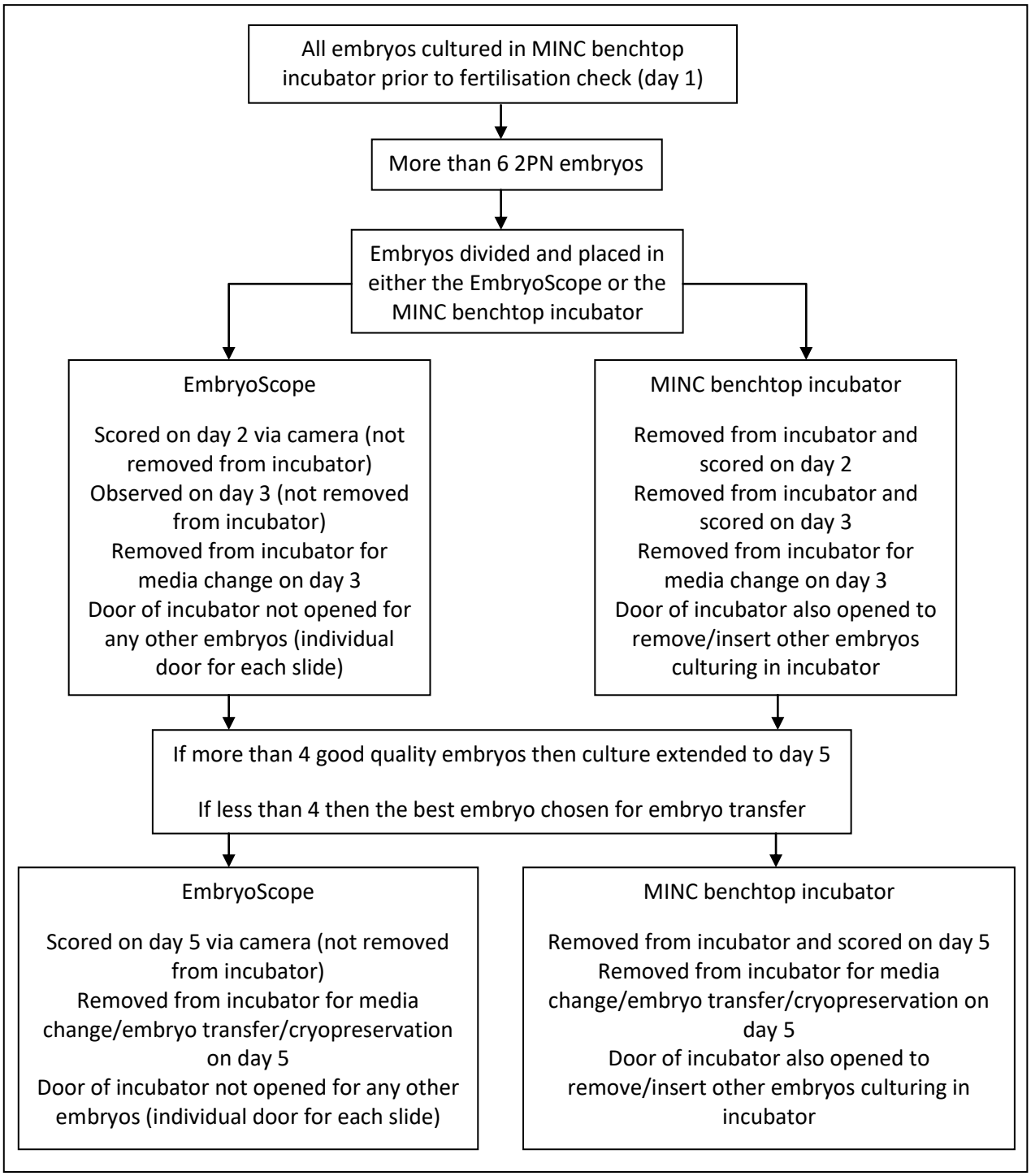

Figure 6.1: Pathway for embryos in the PROMOTE trial

The quality of the embryos was scored on day 3 using morphological parameters in order to accurately compare the two groups and in accordance with the grading system previously described (in section 2.10). The embryologists performing the grading were not blinded to the incubator in which the embryos were cultured.

If fewer than 4 embryos in total were considered viable, one or two were selected for transfer on day 3, in line with routine clinical procedure. The remaining embryos were kept in culture and reassessed on day 5, using the Gardner's scoring system (see section 2.10). When 4 or more embryos were considered viable on day 3, culture was extended to day 5 and selection for transfer was made on day 5. Remaining high quality embryos were cryopreserved.

6.2.4 Collecting and storing embryo media

All embryos were cultured in 25 µl drops of media in either the EmbryoScope or the MINC benchtop incubator. They were cultured in Vitrolife IVF sequential media; GIVF+, G1+ and G2+; GIVF+ media during egg collection and insemination (day 0), G1+ media post ICSI culture and for all embryos from day 1 to day 3 and then G2+ media until day 5. If the embryos were not transferred, cryopreserved or discarded on day 5 then the media was changed to fresh G2+ media at this point (day 5-6 media was not collected for analysis). All media were overlaid with Ovoil, which helped to maintain a stable temperature, osmolality and pH (Sifer et al.). GIVF+ media was not collected. The remaining spent media (G1+ and G2+) for each embryo and control media for each patient was transferred from the culture dish (on day 3 and day 5) to a labelled Eppendorf and then stored at -80°C until analysis.

6.2.5 Pyruvate analysis

The following fluorometric method was used to measure pyruvate concentration in the spent media.

An EPPS (4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid) buffer was made by dissolving 2.52 g EPPS (Sigma, UK) in 150 ml of distilled water. The pH of the buffer was altered to 8 using a 1 M solution of NaOH (Fisher Scientific, UK). Once the pH was correct, distilled water was added to make the total volume up to 200 ml. 10 mg of penicillin G (Sigma, UK) and 10 mg of streptomycin (Sigma, UK) were then added. The solution was mixed well and stored until needed.

A 5 mM NADH Disodium salt solution was made; 17.74 mg NADH Disodium salt (Sigma, UK) was added to 5 ml water.

The pyruvate cocktail was then made by combining 14 ml EPPS buffer with 0.15 ml 5 mM NADH and 0.2 ml LDH (Sigma, UK). This was mixed well and stored at -20°C until required for use (but for a maximum of 2 months).

Pyruvate standards were made using 27.5 mg of 99% sodium pyruvate (Sigma, UK) and 50 ml of distilled water resulting in a 5 mM solution, this was further diluted to give concentrations of 0.025, 0.05, 0.1, 0.2, 0.4 and 0.5 mM in order to produce the standard curve.

A 96 well plate (Greiner, UK) was used and 45 µl of pyruvate cocktail was pipetted into each well. 5 µl of the standard, control (media placed in the incubator without an embryo) or the sample were then placed in each well (each one was run in triplicate) and the samples were mixed well. Media needed to be diluted in a ratio of two parts media to three parts distilled water (in order to ensure the measured concentrations of pyruvate were on the standard curve). Once all wells were filled the plate was covered in foil and left for three minutes to allow the reaction to occur:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

The plate was placed on the plate reader (FLUOstar OPTIMA, BMG Labtech, Germany) and the fluorescence of each well was measured using an excitation wavelength (Ex) of 340 nm and an emission wavelength (Em) of 459 nm. A standard curve of fluorescence versus concentration was then produced and the pyruvate concentration within each media sample calculated (being sure to adjust for the dilution of the day 3 media). The pyruvate consumption could then be calculated using the following equation:

$$\text{Pyruvate consumption (pmol/embryo/hour)} = \frac{\text{Pyruvate conc. in control} - \text{Pyruvate conc. in spent medium}}{\text{Number of hours the embryo was cultured in the medium}}$$

For day 3 media, the pyruvate consumption per cell per hour was also calculated.

6.2.6 Glucose analysis

The concentration of glucose in the spent media was measured using the following fluorimetric technique. First, EPPS buffer was prepared (see section 6.2.5). A solution containing 15 ml EPPS buffer, 2 ml 5 mM dithiothreitol (Sigma, UK), 3 ml 37 mM magnesium sulphate (Sigma, UK), 1 ml

10 mM ATP (Sigma, UK), 3 ml 10 mM NADP (Sigma, UK) and 1 ml Hexokinase/Glucose-6-phosphate dehydrogenase (Sigma, UK) was produced.

Glucose standards were made by diluting 1 mM concentrate to 0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM.

Day 3 and 5 media were diluted to ensure concentrations fitted onto the standard curve. Media was diluted in a ratio of 1 part media to 2 parts HPLC grade water (1 in 3).

A 96 well plate (Greiner, UK) was used and 45 µl of glucose cocktail was pipetted into each well. The standards, controls (medium placed in incubator without an embryo) and diluted medium (5 µl) were added and the test was performed in triplicate. The plate was left to stand for 10 minutes to allow the reactions to occur:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-Phosphate} + \text{ADP} + H^+$$

$$\text{Glucose-6-Phosphate} + 2NADP^+ + H_2O \longrightarrow \text{Ribose-5-phsphate} + 2NADPH + 2H^+ + CO_2$$

The fluorescence was measured using an Ex of 340 nm and an Em of 459 nm. The concentration was calculated from the standard curve and then the glucose consumption per embryo per hour (pmol/embryo/hour) and for day 3 media glucose consumption per cell per hour was calculated.

6.2.7 Amino acid analysis

The concentration of amino acids in embryo media was analysed using reverse phase high pressure liquid chromatography (HPLC) (Agilent 1100). This method allowed the separation and analysis of 18 amino acids; including essential amino acids (histidine (His), glutamine (Gln), arginine (Arg), threonine (Thr), tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Iso), leucine (Leu), and lysine (Lys)) and non-essential amino acids (aspartic acid (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glycine (Gly), and alanine (Ala)). This method did not allow the measurement of proline and cysteine.

A working o-Phthaldialdehyde (OPA) reagent (Sigma, UK) was made in the fume cupboard by adding 10 µl β2-mercaptoethanol (Sigma, UK) to 5 ml of OPA. The solution was mixed and then 1.5 ml aliquots were pipetted into 2 ml standard brown glass HPLC vials. These were then stored at -20°C until use (for a maximum of 10 weeks).

A stock of 83 mM Sodium Acetate Solution was made up by placing 56.5 g of sodium acetate trihydrate (Fisher Scientific, UK) and 4.5 L of HPLC grade water (Fisher Scientific, UK) into a 5 L

volumetric flask. The pH was adjusted to 5.9 by adding glacial acetic acid (Fisher Scientific, UK), 700 µl was added initially then 200 µl and then dropwise until the desired pH was achieved. The solution was then made up to 5 L by adding more HPLC grade water. This was stored at room temperature until use (for a maximum of 6 months). Using this solution the HPLC buffers were then made up; buffer C (comprising of 50% HPLC grade methanol (Fisher Scientific, UK) and 50% HPLC grade water), followed by buffer B (containing 800 ml HPLC grade methanol and 200 ml 83 mM sodium acetate pH5.9) and buffer A (containing 800 ml 83 mM sodium acetate pH5.9, 200 ml HPLC grade methanol and 15 ml tetrahydrofuran (Fisher Scientific, UK). The buffers were made in this order, they were filtered through a 0.45 µm cellulose nitrate membrane filter Fisher Scientific, UK) and stored in the dark at room temperature prior to use (for a maximum of 3 months).

Control medium or spent medium (2 µL) was mixed with 23 µl of HPLC grade water and placed into a clear HPLC vial (Sigma, UK) in order to produce the injectable sample. Standards (50 µM) were prepared. Three standards were run at the start of each day to ensure that the HPLC machine, OPA or buffers were allowing a good standard of chromatography to be produced. A standard was also run after every three media samples.

The sample was injected into the mobile phase and combined with OPA, this allowed a reaction between the OPA and the primary amines within the amino acids to produce a fluorescent derivative. The buffers moved the sample through the polar mobile phase, altering this phase as the quantities of individual buffers changed; this started to separate the amino acids according to their polarity and charge. The sample then reached the stationary phase or column (Gemini 3u C18 Column, Phenomenex 00b-4439-e0, USA). In reverse phase HPLC, the stationary phase is non polar and hydrophobic; the polar amino acids therefore moved more quickly through the stationary phase and were detected sooner by the fluorescence detector. The non-polar amino acids were absorbed and desorbed and therefore detected later. The retention time was the time at which each amino acid left the column, this allowed the identification of each amino acid from the chromatogram. The quantity of each amino acid was calculated by comparing the area under the peak in the standard chromatography of a particular amino acid with the peak of that amino acid within the sample. The amino acid utilisation and production was calculated by subtracting the concentration of individual amino acids in the spent media from the concentration in the control media (i.e. media that had been kept in a well that did not contain an embryo but was in the same culture dish as the spent media and developing embryos). The utilisation or production rate per embyo per hour was then calculated.

6.2.8 Sample size and power considerations

Anecdotal evidence has suggested that use of the EmbryoScope is associated with a marked increase in pregnancy rates. Since blastocyst formation rates are key to clinical outcome, this parameter was selected as the primary outcome measure. In order to show, with 80% power at a p value of <0.05, an increase in blastocyst formation rate from 30% observed in our centre using MINC benchtop incubators to 45% proposed for the EmbryoScope, 176 embryos were required in each arm. In a previous study examining metabolic parameters, embryos generated by 53 patients were sufficient to discern an amino acid metabolic profile of embryo viability in spent medium (Brison et al., 2004). In order to generate sufficient embryos to address both main research questions, 80 couples were to be recruited. This number was anticipated to be sufficient to generate at least 180 embryos for analysis in each arm, even when 25% of subjects did not generate 6 or more 2PN embryos, necessitating removal from the study.

6.2.9 Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Results are reported as mean ± standard deviation unless otherwise stated. A p value of less than 0.05 was considered significant.

A Chi squared test was used to compare the numbers of embryos that formed blastocysts, were suitable for cryopreservation and were transferred or cryopreserved from each type of incubator. This test was also used to compare pregnancy rates.

The mean morphological scores of the embryos on day 3 and day 5 from each type of incubator were compared using a student's t-test.

Two way ANOVA was used to compare the carbohydrate metabolism and amino acid consumption and production of the embryos comparing those inseminated by IVF and by IVF-ICSI, the quality of the embryo and the differing culture environments. Non-normally distributed data was log transformed prior to analysis.

6.3 Results

6.3.1 What were the basic characteristics of the participants of the PROMOTE trial?

Between January 2015 and November 2016, 81 couples undergoing either IVF or IVF-ICSI at Complete Fertility Centre, Southampton were recruited to the PROMOTE trial. Of these 81, 51 couples met the criteria for their embryos to be randomised (i.e. they had more than six 2PN embryos) (see Figure 5.1). These 51 couples produced a total of 585 embryos, 289 of which were cultured in the EmbryoScope and the remaining 296 in the MINC benchtop incubator.

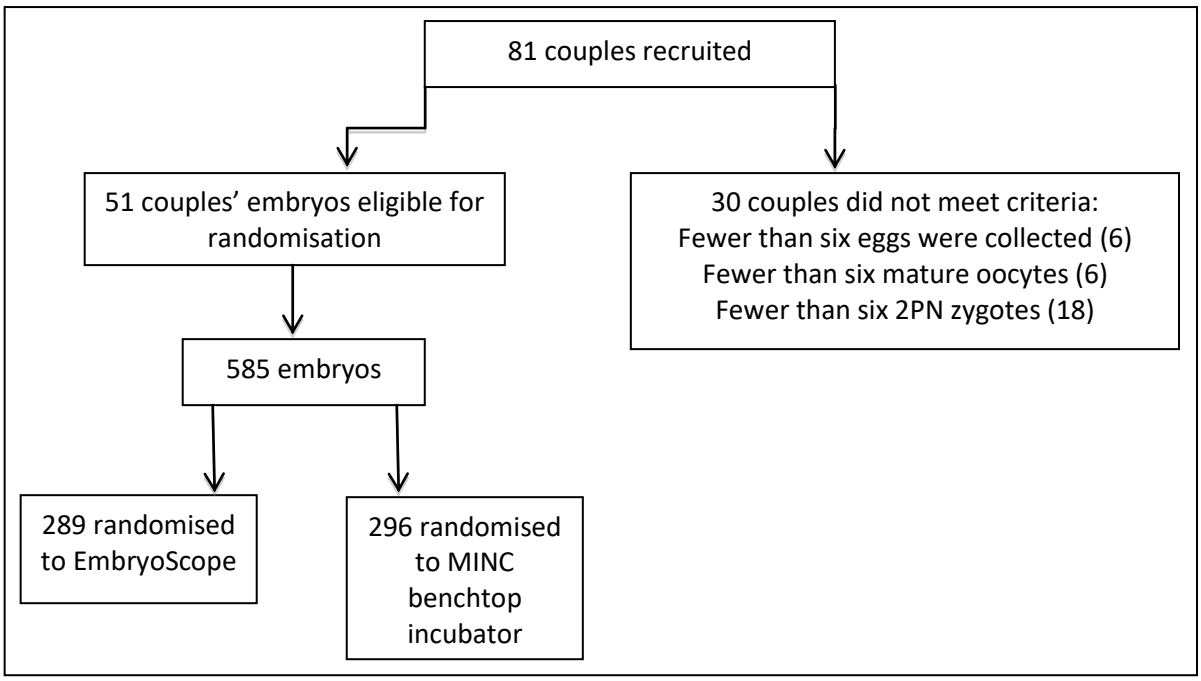

Figure 6.2: Consort diagram for recruitment to the PROMOTE trial

The average age of the women recruited to the trial was 32.6 years ± 3.8. If the women whose embryos were not randomised were excluded then the average age was 32.4 years ± 4.0. The reasons for requiring ART reflected those reported in the general population undergoing IVF, with 22.2% suffering from female infertility (PCOS 12/81 and tubal factors 6/81); 35.8% male infertility (29/81); 28.4% unexplained (23/81); and in 11 of the 81 cases there was more than one cause recorded. The majority of couples who were withdrawn from the trial were those in whom male factor was the cause of their infertility (60%), followed by unexplained (16.7%).

The Antral Follicle Count (AFC) was recorded at the baseline scan, the median score was 18 with an interquartile range of ± 8 follicles. The minimum AFC recorded was 4 and the maximum was

52. The majority of women recruited (86.4%) were triggered with human chorionic gonadotrophin (hCG) and the remainder with GnRH. Women who were triggered with GnRH were those with a higher AFC at baseline and a serum oestradiol of greater than 18000 pmol/L and hence at risk of ovarian hyperstimulation syndrome (OHSS). The number of eggs collected on the day of oocyte retrieval varied between 1 and 42 with a mean value of 15.1. Excluding the six couples with less than six oocytes, the mean number of mature oocytes collected was 14.6 with a range of between 2 and 42. After excluding the further six patients who did not meet the criteria (i.e. had less than six mature oocytes), the mean number of 2PN zygotes was 9.5 ±5.5 and a range of between 0 and 27. Eighteen couples produced fewer than 6 2PN embryos and were therefore excluded from the trial at this point. The embryos from the remaining 51 couples were randomised to the EmbryoScope or the MINC benchtop incubator.

6.3.2 Were there differences in the blastocyst formation rate and embryo quality between the two incubators?

The primary endpoint for the study was the percentage of normally fertilized oocytes forming a blastocyst on day 5 (see Table 6.1). The percentage of embryos cultured in the EmbryoScope that developed into blastocysts on day 5 (55%) was significantly higher than those allocated to the MINC incubator (45%) (p=0.015). Furthermore, if the total number of blastocysts was examined (i.e. blastocysts formed on day 5 or day 6) then a statistically significant difference was also seen (p=0.025); with more blastocysts formed in the EmbryoScope (192/289; 66%) than in the MINC benchtop incubator (170/296; 57%).

The number of embryos suitable for cryopreservation on day 5, defined as a blastocyst which scored A or B for the quality of the ICM and TE on the Gardner's score, was analysed in each incubator. Following culture in the EmbryoScope 90 out of 289 embryos were deemed suitable for cryopreservation versus 69 out of 296 embryos in the MINC benchtop incubator (p=0.033).

Table 6.1: Number of blastocysts and number of embryos suitable for cryopreservation in the EmbryoScope versus the MINC benchtop incubator.

	EmbryoScope (n=289)		MINC (n=296)		p-value
	Yes	**No**	**Yes**	**No**	
Did the embryo form a blastocyst on day 5?	159	130	133	163	0.015
Did the embryo form a blastocyst (day 5 or day 6)?	192	97	170	126	0.025
Were the embryos suitable for cryopreservation on day 5?	90	199	69	227	0.033
Were the embryos suitable for cryopreservation (on day 5 or day 6)?	99	190	80	216	0.058

The average morphological score on day 3 (see Table 2.3) was examined to ascertain whether it was greatest in the embryos cultured in the EmbryoScope when compared to the MINC incubator; there was no statistical difference between the two groups (p=0.635). The mean score in the EmbryoScope was 2.55 ± 1.2 and in the MINC incubator was 2.45 ± 1.2.

The mean score of blastocysts on day 5 was 3.04 ± 1.3 in the EmbryoScope and 3.15 ± 1.3 in the MINC benchtop incubator; this was not statistically significant (p=0.519). The same was true on day 6 (p=0.155) with a mean score of 4.26 ± 1.1 in the EmbryoScope and 4.34 ± 0.9 in the MINC.

Culturing embryos within the EmbryoScope did not significantly increase the blastocyst utilisation rate (61% in the EmbryoScope versus 59% in the MINC, p=0.231), the number of blastocysts frozen (51% in the EmbryoScope versus 47% in the MINC (p=0.392)) or transferred (9% in the EmbryoScope versus 8% in the MINC (p=0.558)) (see Table 6.2).

Table 6.2: Number of blastocysts used, cryopreserved and transferred in the EmbryoScope versus the MINC benchtop incubator.

	EmbryoScope (n=192)		MINC (n=170)		p-value
	Yes	**No**	**Yes**	**No**	
Was the blastocyst used (either for transfer or for cryopreservation)?	117	75	100	70	0.231
Was the blastocyst cryopreserved?	99	93	80	90	0.392
Was the blastocyst transferred?	18	174	13	157	0.558

Furthermore there was no significant difference in pregnancy rate per embryo transfer (71% EmbryoScope versus 73% MINC, p=1.00) or clinical pregnancy rate (59% EmbryoScope versus 53% MINC, p=1.00).

6.3.3 Assessment of the embryos' metabolic profile on day 3

The mean number of hours that the embryos were cultured for in G1 media was 48.48 ± 0.81. The metabolic profile of the individual embryos was assessed including carbohydrate utilisation (glucose and pyruvate) and amino acids' consumption and production.

6.3.3.1 What was the mean carbohydrate utilisation in G1+ media (Vitrolife IVF sequential media for days 1 to 3)?

A control drop of medium was placed into the EmbryoScope and the MINC benchtop incubator for each set of embryos studied (i.e. the embryos from one couple). On day 3, the mean ± SD measured glucose concentration was 0.55 mM/L ± 0.10 in the EmbryoScope and 0.55 mM/L ± 0.11 in the MINC. The pyruvate concentration in the media was measured as 0.22 mM/L ± 0.09 in the EmbryoScope and 0.23 mM/L ± 0.08 in the MINC. These control concentrations were then used to calculate the utilisation of the substrates.

As shown in Figure 6.3, the mean glucose consumption (including all embryos) between day 1 and day 3 was 53.81 pmol/embryo/hour ± 58.07; and mean pyruvate consumption was 37.79 pmol/embryo/hour ± 55.38.

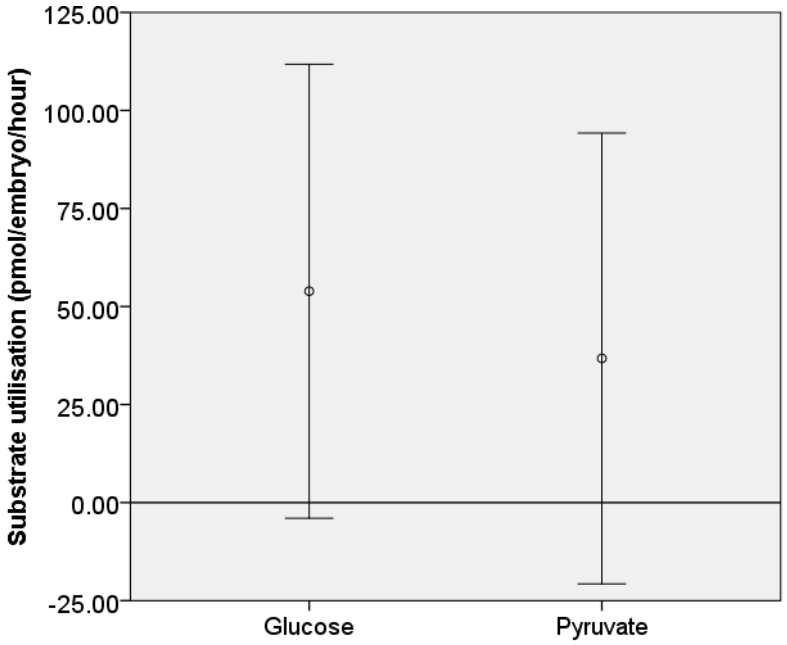

Figure 6.3: Carbohydrate utilisation per embryo per hour in the spent media between day 1 and day 3 (positive=substrate utilisation, negative=substrate production).
Values are mean percentage ± standard deviation.

A negative correlation was observed between the utilisation of glucose and the utilisation of pyruvate in the embryos (R=-0.125, p=0.017).

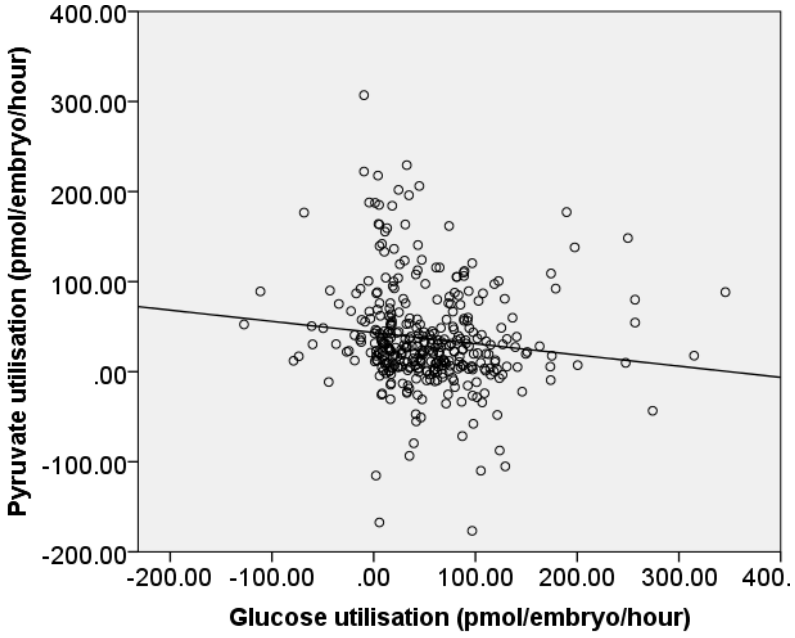

Figure 6.4: Correlation between glucose utilisation and pyruvate utilisation in G1+ media (between day 1 and day 3 of embryo culture).
(Positive=substrate utilisation, negative=substrate production).
Circles represent individual embryo's utilisation. Line represents linear regression.
***p value <0.05**

6.3.3.2 Were there differences in carbohydrate utilisation in G1+ media between embryos inseminated by IVF and those inseminated by IVF-ICSI?

No differences were observed in carbohydrate utilisation between those embryos fertilised by IVF and those fertilised by IVF-ICSI. However, when the number of cells per embryo was taken into account, a difference was seen in the utilisation of glucose between the two fertilisation methods; increased glucose consumption was seen in the IVF group compared to the IVF-ICSI group (see Table 6.3).

Table 6.3: Mean glucose and pyruvate consumption (pmol/cell/hour) in G1+ media in embryos inseminated by IVF and those inseminated by IVF-ICSI

	IVF group			IVF-ICSI group			
	n	**Mean consumption (pmol/cell/hour)**	**Standard deviation**	**n**	**Mean consumption (pmol/cell/hour)**	**Standard deviation**	**p value**
Glucose	231	9.40	12.43	140	6.81	10.52	0.040
Pyruvate	264	6.07	9.23	197	5.29	8.46	0.352

Due to these differences, data from the two fertilisation methods were analysed separately.

6.3.3.3 Did embryo quality have an effect on carbohydrate consumption in G1 media?

A blastocyst was formed from 62% (362/585) of the embryos.

In the IVF group, 65% (241/371) formed a blastocyst. In this group, those that formed a blastocyst consumed less glucose per cell per hour than those that did not develop into a blastocyst (6.85 pmol/cell/hour ± 7.60 vs. 14.40 pmol/cell/hour ± 17.60 ($p<0.001$)). No difference was seen in the pyruvate utilisation.

Furthermore, increased glucose and pyruvate utilisation was demonstrated in poorer quality embryos compared to higher quality embryos (scored according to Table 2.3) ($p<0.001$ and 0.023, respectively) (see Figure 6.5).

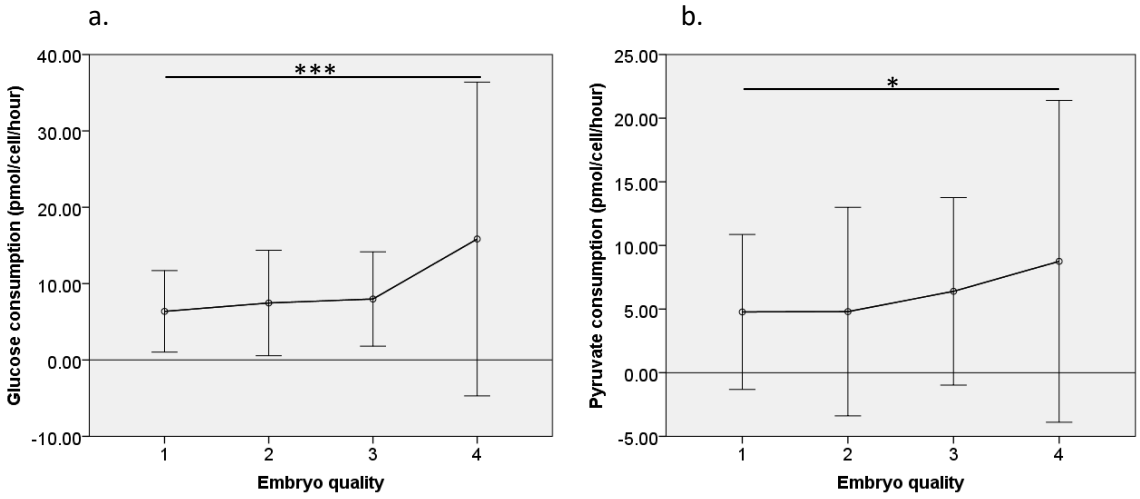

Figure 6.5: Graphs depicting a. glucose utilisation and b. pyruvate utilisation according to the quality of the embryo. (n=79 in group 1, 80 in group 2, 29 in group 3 and 75 in group 4). Values are mean utilisation (pmol/cell/hour) ± standard deviation.
***p value <0.05, **p value<0.01, ***p value<0.001**

A blastocyst was produced from 57% (121/214) of the embryos fertilised using IVF-ICSI; no statistically significant differences were seen in the carbohydrate utilisation of those that formed blastocysts and those that did not. However, a trend towards higher pyruvate utilisation (p=0.053) in embryos that failed to form a blastocyst were observed. Furthermore, higher quality embryos (scoring a 1) consumed less pyruvate than lower quality embryos (scoring a 4) (3.88 pmol/cell/hour ± 4.22 vs. 8.05 pmol/cell/hour ± 10.81 (p=0.011)).

6.3.3.4 Was there a difference in carbohydrate consumption in G1 media between the two types of incubator?

Measurement of the carbohydrate utilisation in embryos produced both by IVF and IVF-ICSI demonstrated differences between the usage of glucose and pyruvate in the two different types of incubators (see Figure 6.5**Figure 6.6**). Embryos inseminated by IVF and by IVF-ICSI, cultured in the EmbryoScope showed decreased glucose utilisation (p=0.060, p=0.014 respectively). However, increased pyruvate utilisation was demonstrated in embryos inseminated by IVF and cultured in the EmbryoScope (p=0.035) and decreased pyruvate utilisation in embryos inseminated by IVF-ICSI and cultured in the EmbryoScope (p=0.029).

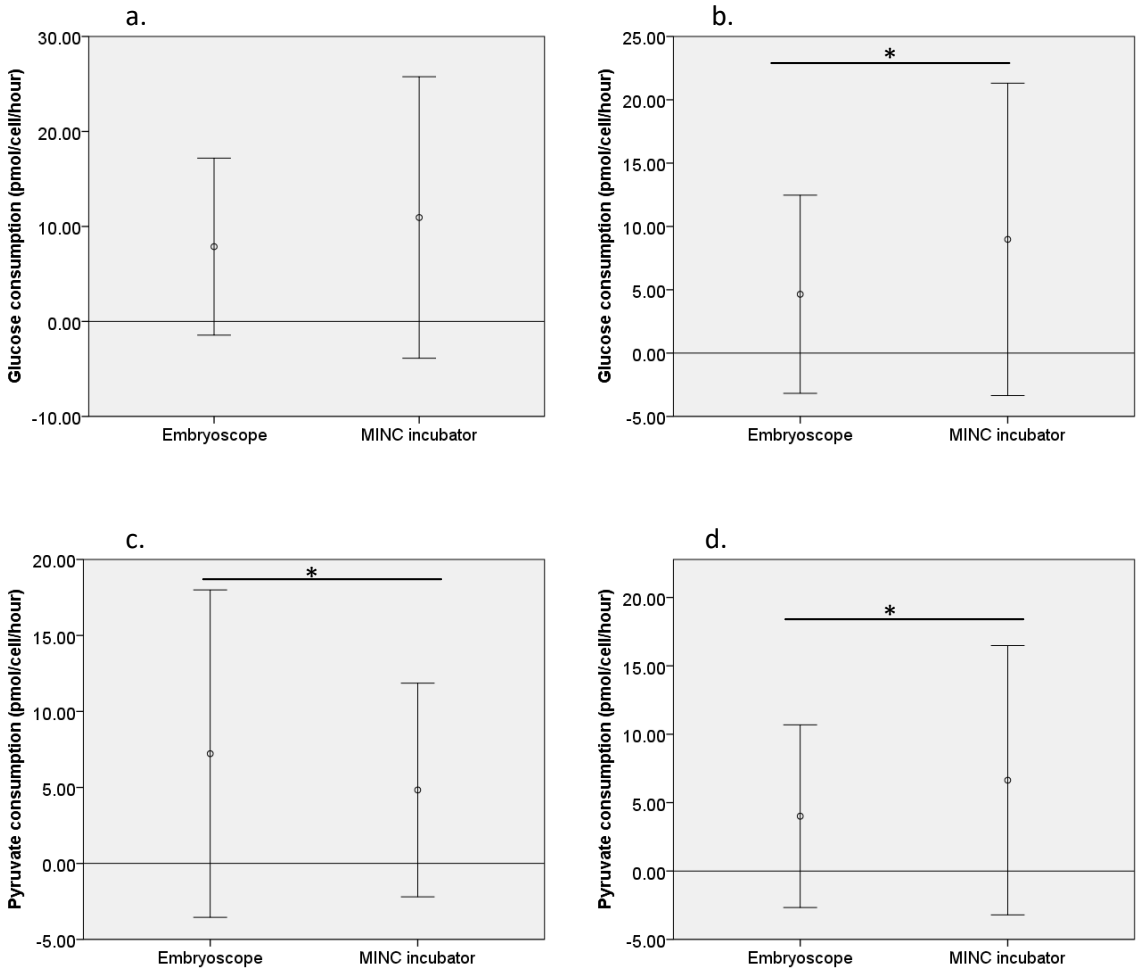

Figure 6.6: Differences between carbohydrate consumption between embryos cultured in the EmbryoScope and MINC incubator, examining a. glucose consumption in embryos inseminated by IVF (n=116 in EmbryoScope, n=115 in MINC), b. glucose consumption in embryos inseminated by IVF-ICSI (n=70 in EmbryoScope, n=70 in MINC), c. pyruvate consumption in embryos inseminated by IVF (n=137 in EmbryoScope, n=127 in MINC), d. pyruvate consumption in embryos inseminated by IVF-ICSI (n=101 in EmbryoScope, n=96 in MINC).
Values are mean utilisation (pmol/cell/hour) ± standard deviation.
***p value <0.05**

6.3.4 What was the mean amino acid concentration in G1+ media?

The amino acid concentrations in control droplets of G1+ media (medium placed in the incubators at the same time as the embryos but that did not contain an embryo) were measured for each couple. The mean total concentration of amino acids measured was 737.62 µM ± 54.71 in the EmbryoScope and 725.69 µM ± 59.41 in the MINC benchtop incubator. The mean concentrations of amino acids found in the control drops of media are found in Table 6.4.

Table 6.4: Mean concentration ± standard deviation of amino acids measured in control G1 media drops in the EmbryoScope and the MINC incubator (μM)

		Control media in EmbryoScope		Control media in MINC benchtop incubator	
		Mean concentration	Standard deviation	Mean concentration	Standard deviation
Aspartic acid	Asp	93.28	14.36	92.58	10.69
Glutamic acid	Glu	81.35	16.97	81.07	14.33
Asparagine	Asn	65.72	10.64	65.28	7.80
Histidine	His	0.00	0.00	0.00	0.00
Serine	Ser	81.63	9.45	79.71	8.30
Glutamine	Gln	107.51	23.55	111.59	36.26
Arginine	Arg	1.83	0.20	1.76	0.117
Glycine	Gly	77.90	12.27	74.66	22.30
Threonine	Thr	0.00	0.00	0.00	0.00
Alanine	Ala	214.44	34.63	204.94	52.08
Tyrosine	Tyr	0.00	0.00	0.00	0.00
Methionine	Met	2.04	0.30	2.20	0.27
Valine	Val	0.00	0.00	0.00	0.00
Tryptophan	Trp	0.00	0.00	0.00	0.00
Phenylalanine	Phe	0.00	0.00	0.00	0.00
Isoleucine	Ile	1.62	0.64	1.70	0.41
Leucine	Leu	1.85	0.29	1.90	0.41
Lysine	Lys	8.44	1.97	8.28	1.80

The concentrations of the following amino acids were log transformed on day 3 prior to the analysis as they were not normally distributed: His, Arg, Thr, Tyr, Met, Val, Phe, Ile, Leu.

6.3.4.1 What was the effect of the insemination method on amino acids' consumption and production?

The embryos were divided into those that were fertilised using conventional IVF and those fertilised using IVF-ICSI as it is recognised that cumulus cells are metabolically active (and chemically stripped prior to ICSI) therefore may alter the metabolic profile of the embryos on day 3. As can be seen in Figure 6.7, a difference was noted in the consumption or production of Asp (p=0.004), Asn (p=0.008), His (p=0.015), Gly (p=0.017), Met (p=0.018) and Phe (p=0.026).

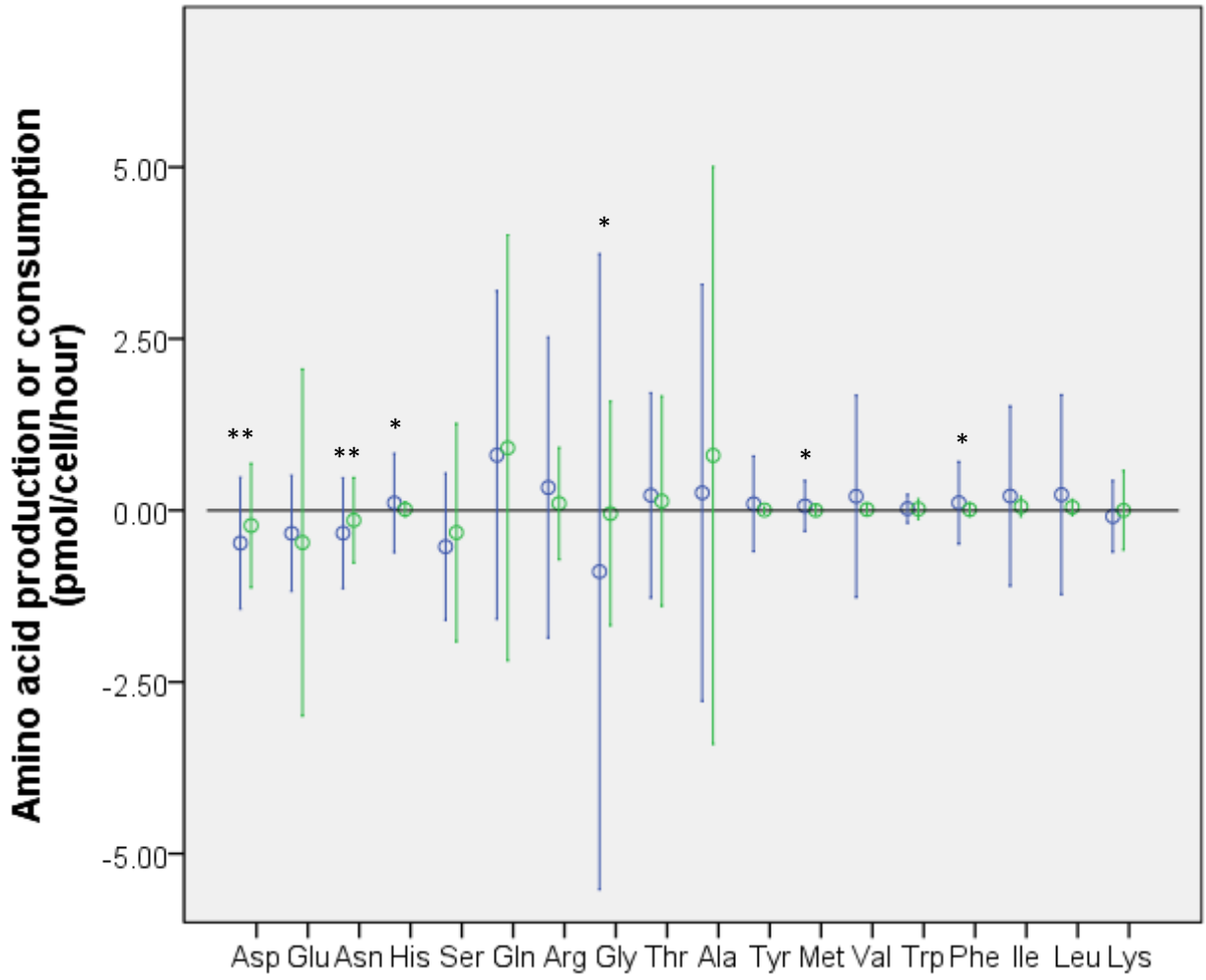

Figure 6.7: Consumption and production of individual amino acids in G1+ media according to method of fertilisation (blue=IVF (n=258), green=ICSI (n=186))
Positive values indicate production and negative values indicate consumption.
Values are mean utilisation (pmol/cell/hour) ± standard deviation.
***p value <0.05, **p value<0.01**

Increased usage of Asp was noted in the IVF group compared to the ICSI group (0.48 pmol/cell/hour ± 0.96 compared to 0.22 pmol/cell/hour ± 0.91). Furthermore, Asn (0.33 pmol/cell/hour ± 0.80 compared to 0.14 pmol/cell/hour ± 0.62) and Gly (0.89 pmol/cell/hour ± 4.63 compared to 0.05 pmol/cell/hour ± 1.63) demonstrated increased utilisation in the IVF group. Increased production of His was seen in the IVF group 0.11 pmol/cell/hour ± 0.72 vs. 0.02 pmol/cell/hour ± 0.11. The was also true of Met (production of 0.07 pmol/cell/hour ± 0.37 in embryos inseminated by IVF compared to 0.001 pmol/cell/hour ± 0.07 in the IVF-ICSI group) and Phe (production of 0.11 pmol/cell/hour ± 0.60 in the IVF group compared to 0.01 pmol/cell/hour ± 0.10). Due to these observed differences, the two groups were analysed separately.

6.3.4.2 What relationship was observed between the quality of the embryos inseminated by IVF and amino acids' consumption and production on day 3?

The embryos were scored according to the methods previously described (see table 2.3).

When all embryos were observed, a statistically significant difference was observed in the consumption of His (p=0.001). Higher quality embryos (scoring a 1 or a 2) produced less His when compared to lower quality embryos. Embryos that scored 1 (n=79) produced 0.07 pmol/cell/hour ± 0.48 compared to those who scored 4 (n=78) which produced 0.14 pmol/cell/hour ± 1.07. A trend was also seen in the utilisation of Ser (p=0.052) with increased utilisation in lower quality embryos).

Interestingly when only embryos that went on to develop a blastocyst were analysed, the production of Gln and Ala was decreased in higher quality embryos. Gln was produced at a rate of 0.73 pmol/cell/hour ± 1.48 in high quality embryos (n=70) and 2.71 pmol/cell/hour ± 2.93 in poorer quality embryos (n=23) ($p<0.001$), Ala was produced at a rate of 0.40 pmol/cell/hour ± 1.82 in high quality embryos compared to 2.15 pmol/cell/hour ± 3.18 in poorer quality embryos (p=0.002).

6.3.4.3 What relationship was observed between the quality of the embryos inseminated by IVF-ICSI and amino acid consumption or production on day 3?

In embryos inseminated by IVF-ICSI, no statistically significant differences were observed between the individual amino acids' consumption or production when the embryos were divided into groups according to their quality.

6.3.4.4 Was there a difference in the amino acid production or consumption of embryos cultured in the EmbryoScope compared to those cultured in the MINC benchtop incubator?

When examining embryos inseminated by IVF, no differences were observed in the production or consumption of amino acids between embryos cultured in the EmbryoScope compared to those cultured in the MINC benchtop incubator.

However, a difference in Ala was shown in embryos inseminated using IVF-ICSI, with those in the EmbryoScope producing greater quantities (1.49 pmol/cell/hour ± 4.46) compared to those

cultured in the MINC (0.11 pmol/cell/hour ± 3.83) (p=0.025). No other differences were observed.

6.3.4.5 Overview of day 3 metabolomics

Table 6.5: Overview of metabolic differences between embryos inseminated using different methods, of differing quality and cultured in different incubators on day 3.

NS depicts no significant results.

	Different insemination methods	Embryo quality		Type of incubator	
		IVF	IVF-ICSI	IVF	IVF-ICSI
Glucose consumption	Increased consumption in IVF embryos	Decreased consumption in higher quality embryos	NS	NS	Decreased consumption in the EmbyoScope
Pyruvate consumption	NS	Decreased consumption in higher quality embryos	Decreased consumption in higher quality embryos	Increased consumption in EmbryoScope	Decreased consumption in the EmbryoScope
Amino acids' consumption and production	Increased utilisation of Asp, Asn and Gly and increased utilisation of His, Met and Phe in IVF embryos	Decreased His utilisation, decreased Gln and Ala production in higher quality embryos	NS	NS	Increased Ala production in EmbryoScope

6.3.5 Assessment of the embryos' metabolic profile on day 5

6.3.5.1 What was the mean carbohydrate utilisation in G2+ media?

The controls were analysed to ascertain the concentration of glucose, pyruvate and lactate in the G2+ media. The mean glucose concentration was measured to be 2.66 mM/L ± 0.20 in the EmbryoScope and 2.80 mM/L ± 0.18 in the MINC benchtop incubator. The mean pyruvate concentration in the G2+ media was 0.17 mM/L ± 0.07 in the EmbryoScope and 0.17 mM/L ± 0.11 in the MINC benchtop incubator.

The mean glucose consumption in the G2+ media was 36.75 pmol/embryo/hour ± 99.80 and pyruvate utilisation was 35.42 pmol/embryo/hour ± 30.31 (see Figure 6.8).

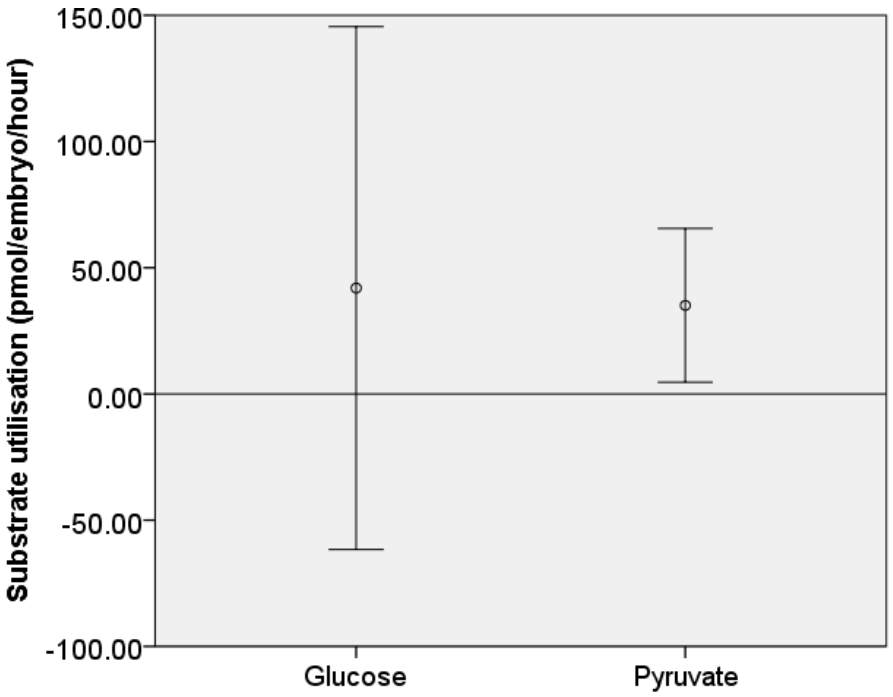

Figure 6.8: Carbohydrate utilisation per embryo per hour in the spent media between day 3 and day 5 (positive=substrate utilisation, negative=substrate production).
Values are mean percentage ± standard deviation.

6.3.5.2 Was there a difference in carbohydrate utilisation in the G2+ media between embryos inseminated by IVF and those inseminated IVF–ICSI?

As opposed to the G1 media, where differences were observed in the glucose usage, in the G2+ media increased pyruvate usage in the embryos fertilised by IVF-ICSI was seen (39.33 pmol/embryo/hour ± 29.31 compared to 32.53 pmol/embryo/hour ± 30.78 (p=0.036)). As a difference was observed, the IVF and the IVF-ICSI fertilised embryos were analysed separately.

6.3.5.3 What is the relationship between embryo quality and carbohydrate consumption in G2 media?

In the IVF group, when embryos that formed a blastocyst on day 5 were examined, a statistically significant difference was detected in glucose utilisation between embryos suitable for cryopreservation (high quality: 58.94 pmol/embryo/hour ± 86.21) and those not suitable (low

quality: 25.79 pmol/embryo/hour ± 105.70 (p=0.018)). No difference in pyruvate utilisation was seen between the high quality and low quality embryos (p=0.717).

No statistically significant differences was seen when the embryos were further subdivided using the day 5 scoring system (see Table 2.7) however; the trends appeared to be maintained.

In the IVF-ICSI group, no differences were observed between the blastocysts suitable for cryopreservation and those that were not in either the glucose utilisation (p=0.354) or the pyruvate utilisation (p=0.474). Again, no differences were observed when the day 5 scoring system was used to further subdivide the embryos.

6.3.5.4 Was there a difference in carbohydrate consumption in G2+ media between embryos cultured in the two types of incubator?

In the embryos inseminated using conventional IVF, a trend towards increased glucose consumption in the EmbryoScope compared to the MINC benchtop incubator was observed (p=0.059). However, no difference was seen between the pyruvate utilisation in the two types of incubator. However, the increased blastocyst formation rate in the EmbryoScope produced a bias; and when only embryos that formed a blastocyst were analysed, no differences in any of the metabolites were observed.

Interestingly, in the IVF-ICSI group, increased pyruvate consumption was observed in the embryos cultured in the EmbryoScope (44.18 pmol/embryo/hour ± 30.98 vs. 33.25 pmol/embryo/hour ± 26.04 (p=0.022). When only embryos that formed a blastocyst were analysed, the difference in pyruvate utilisation between those embryos cultured in the EmbryoScope compared to those cultured in the MINC benchtop incubator was maintained (p=0.043).

6.3.5.5 What was the mean amino acids' concentrations measured in G2+ media?

The mean total concentration of amino acids measured in the G2+ media controls was 2039.69 µM ± 288.88 in the EmbryoScope and 2023.34 µM ± 137.03 in the MINC benchtop incubator. The mean concentrations of amino acids found in the control drops of media are found in Table 6.6.

Table 6.6: Mean concentration ± standard deviation of amino acids measured in control G2 media drops in the EmbryoScope and the MINC incubator (μM)

	Control media in EmbryoScope		Control media in MINC benchtop incubator	
	Mean concentration	**Standard deviation**	**Mean concentration**	**Standard deviation**
Asp	96.70	11.88	93.83	9.76
Glu	80.39	16.60	80.52	9.03
Asn	68.90	12.22	68.58	10.78
His	74.52	15.87	78.54	5.86
Ser	85.56	12.84	84.27	11.60
Gln	103.19	36.39	99.68	37.22
Arg	247.96	39.88	245.01	27.96
Gly	89.47	19.27	88.62	16.32
Thr	150.14	30.92	159.37	19.49
Ala	210.64	47.27	196.44	46.13
Tyr	71.52	14.31	73.84	7.17
Met	37.53	8.41	41.14	4.79
Val	145.16	28.33	146.80	24.98
Trp	22.73	6.20	23.44	4.02
Phe	74.66	14.75	77.72	10.75
Ile	140.76	27.60	150.74	17.86
Leu	146.89	28.37	156.49	14.98
Lys	192.96	45.59	194.72	48.12

When examining the day 5 data; Ser, Gln, Arg were not normally distributed and therefore were log transformed.

6.3.5.6 Was there a difference in the amino acids' consumption and production of embryos inseminated by IVF and those inseminated by IVF–ICSI?

Interestingly, there was still a statistically significant difference observed in six of the amino acids between embryos produced using conventional IVF and those produced by IVF-ICSI (see Table 6.7).

Table 6.7: Comparison of the amino acids' consumption or production of embryos inseminated by IVF compared to those inseminated by IVF-ICSI
Negative means consumption, positive means production.
***means the data has been log transformed to use a parametric test and then raised to the power 10 to calculate the mean.**

	Embryos inseminated by IVF (n=279)		Embryos inseminated by IVF-ICSI (n=171)		
	Mean amino acid utilisation or production (pmol/embryo/hour)	**Standard deviation**	**Mean amino acid utilisation or production (pmol/embryo/hour)**	**Standard deviation**	**p-value**
Asp	-2.14	4.67	-1.56	5.92	0.251
Glu	-0.47	4.01	-0.82	6.83	0.491
Asn	**-2.26**	**4.13**	**-0.68**	**5.51**	**0.001**
His	-1.93	4.81	-2.61	10.70	0.360
Ser*	-1.76	20.50	-1.19	8.63	0.729
Gln*	57.96	49.72	57.03	49.25	0.848
Arg*	-3.86	14.95	1.09	86.95	0.353
Gly	-2.36	12.51	-2.22	8.73	0.897
Thr	-0.29	10.19	-2.51	13.82	0.051
Ala	54.72	53.72	53.91	55.06	0.877
Tyr	**0.10**	**5.02**	**-1.24**	**6.39**	**0.014**
Met	0.10	3.00	-0.40	3.45	0.110
Val	0.40	11.54	-1.45	13.88	0.127
Trp	-0.21	1.92	-0.01	2.63	0.335
Phe	**0.33**	**5.60**	**-1.29**	**7.56**	**0.010**
Ile	**-0.13**	**10.51**	**-2.58**	**12.39**	**0.025**
Leu	**-0.58**	**10.11**	**-3.67**	**13.96**	**0.007**
Lys	**-1.06**	**19.92**	**-9.60**	**31.71**	**<0.001**

Therefore, due to these findings, the two groups were analysed separately.

6.3.5.7 Was there a relationship between the embryo quality and the amino acids' consumption and production on day 5?

In the IVF group, lower production of Ala was demonstrated in embryos that formed a blastocyst compared to those that did not (47.01 pmol/embryo/hour ± 42.30 vs. 71.46 pmol/embryo/hour

± 70.00, p<0.001). A decreased production of Gln was also observed (50.83 pmol/embryo/hour ± 39.25 in those that formed a blastocyst compared to 73.44 pmol/embryo/hour ± 64.67 in those that did not, (p=0.014)).

When, only embryos that formed a blastocyst were examined, a statistically significant increase in Ala (p=0.017) production was also observed in embryos not suitable for cryopreservation, compared to those that were suitable.

Interestingly, when these blastocysts were further subdivided on day 5 (see Table 2.7), differences were observed in Asp (p=0.038), Asn (p=0.034), His (p=0.014), Ser (p=0.024) Gln (p=0.013), Ala (p=0.025). With the lowest quality embryos consuming and producing the greatest quantities of amino acids (see

Figure 6.9).

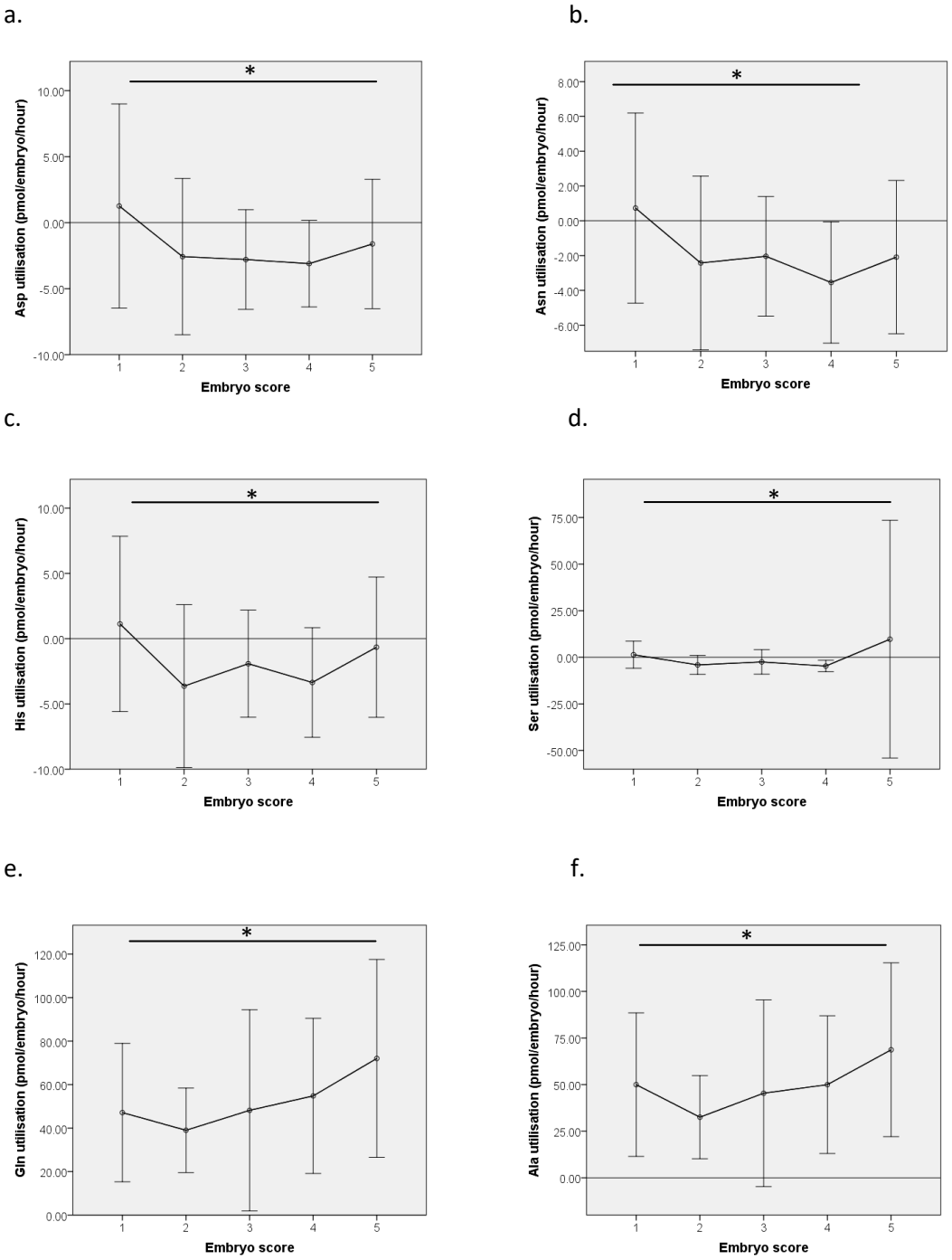

Figure 6.9: Mean consumption and production of individual amino acids a. Asp, b. Asn, c. His, d. Ser, e. Gln and f. Ala on day 5 according to embryo score (n in group 1 = 16, group 2 = 28, group 3 = 45, group 4 = 20 and group 5 = 24) (in those inseminated by IVF)
Values are mean ± standard deviation.
Negative=consumption, positive=production
***p value <0.05**

Interestingly, in the IVF-ICSI group, the same amino acids (Gln and Ala) demonstrated a statistically significant difference between those that formed a blastocyst and those that did not.

Embryos that formed a blastocyst produced less Gln (46.58 pmol/embryo/hour ± 42.63) than those that arrested prior to this stage (71.06 pmol/embryo/hour ± 54.15, p=0.002). A reduction in production was also noted in Ala between these groups (44.88 pmol/embryo/hour ± 48.59 vs. 66.03 pmol/embryo/hour ± 60.97, p=0.013). Furthermore, in the IVF-ICSI group, no differences were seen in the amino acid metabolic profile between those embryos suitable for cryopreservation and those that were not. In addition, when the embryos were further subdivided into the 5 categories, no statistically significant differences were seen.

6.3.5.8 Was there a difference in the amino acid consumption and production between embryos cultured in the EmbryoScope and those cultured in the MINC benchtop incubator?

Blastocysts produced by IVF and cultured in the EmbryoScope appear to have an altered amino acid utilisation profile compared to those cultured in the MINC. Increased utilisation of His ($p<0.001$), Arg ($p=0.017$), Gly ($p=0.029$), Thr ($p<0.001$), Tyr ($p<0.001$), Met ($p=0.005$), Val ($p<0.001$), Trp ($p<0.001$), Phe ($p<0.001$), Ile ($p<0.001$), Leu ($p<0.001$), Lys ($p<0.001$) and decreased production of Gln ($p<0.001$) and Ala ($p<0.001$) was observed in the EmbryoScope (see Figure 6.10).

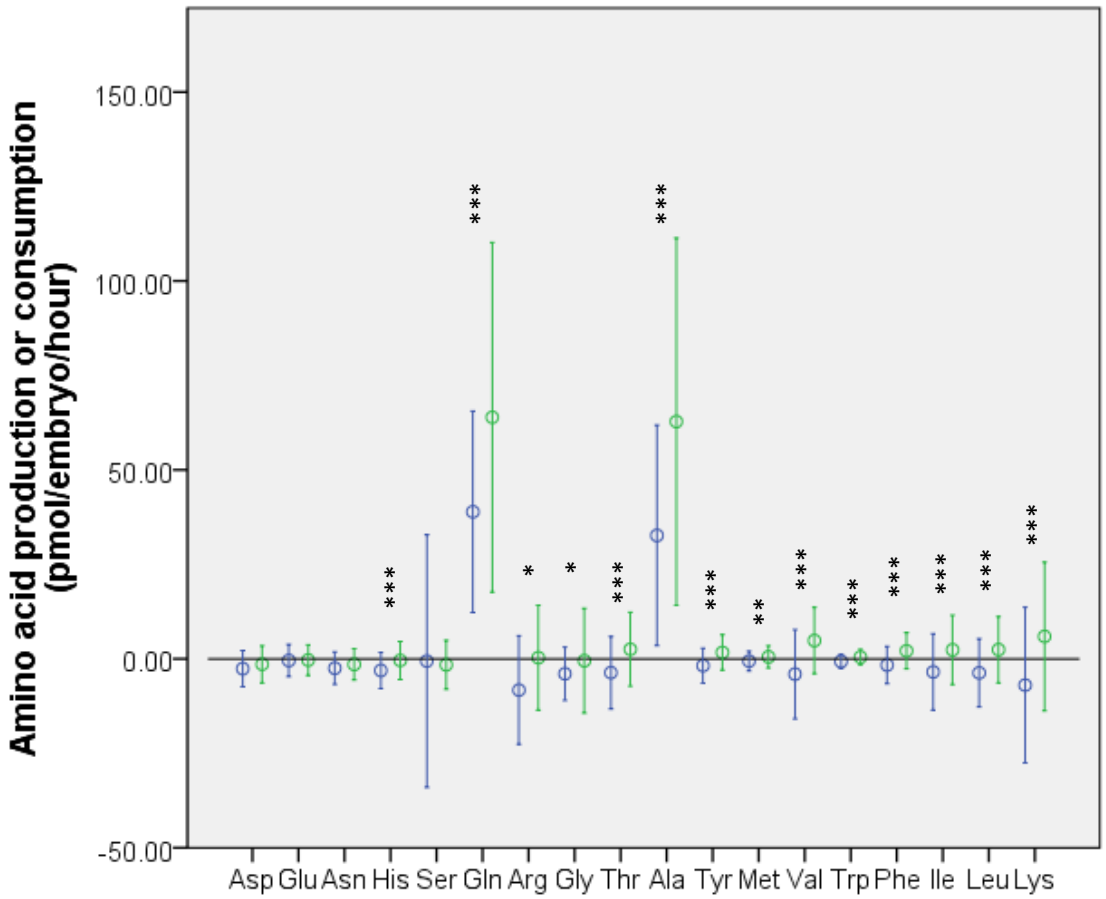

Figure 6.10: Consumption and production of individual amino acids in G2+ media in embryos inseminated by IVF according to the type of incubator (blue=EmbryoScope (n=125), green=MINC (n=116))
Positive values indicate production and negative values indicate consumption.
Values are mean utilisation (pmol/embryo/hour) ± standard deviation.
***p value <0.05, **p value<0.01, ***p value<0.001**

In the IVF-ICSI group, blastocysts cultured in the Embryoscope demonstrated a difference in consumption and production of Thr (p=0.004), Ala (p=0.009), Tyr (p<0.001), Val (p<0.001), Trp (p=0.027), Ile (p=0.009) and Lys (p=0.003) when compared to those cultured in the MINC benchtop incubator (see Figure 6.11).

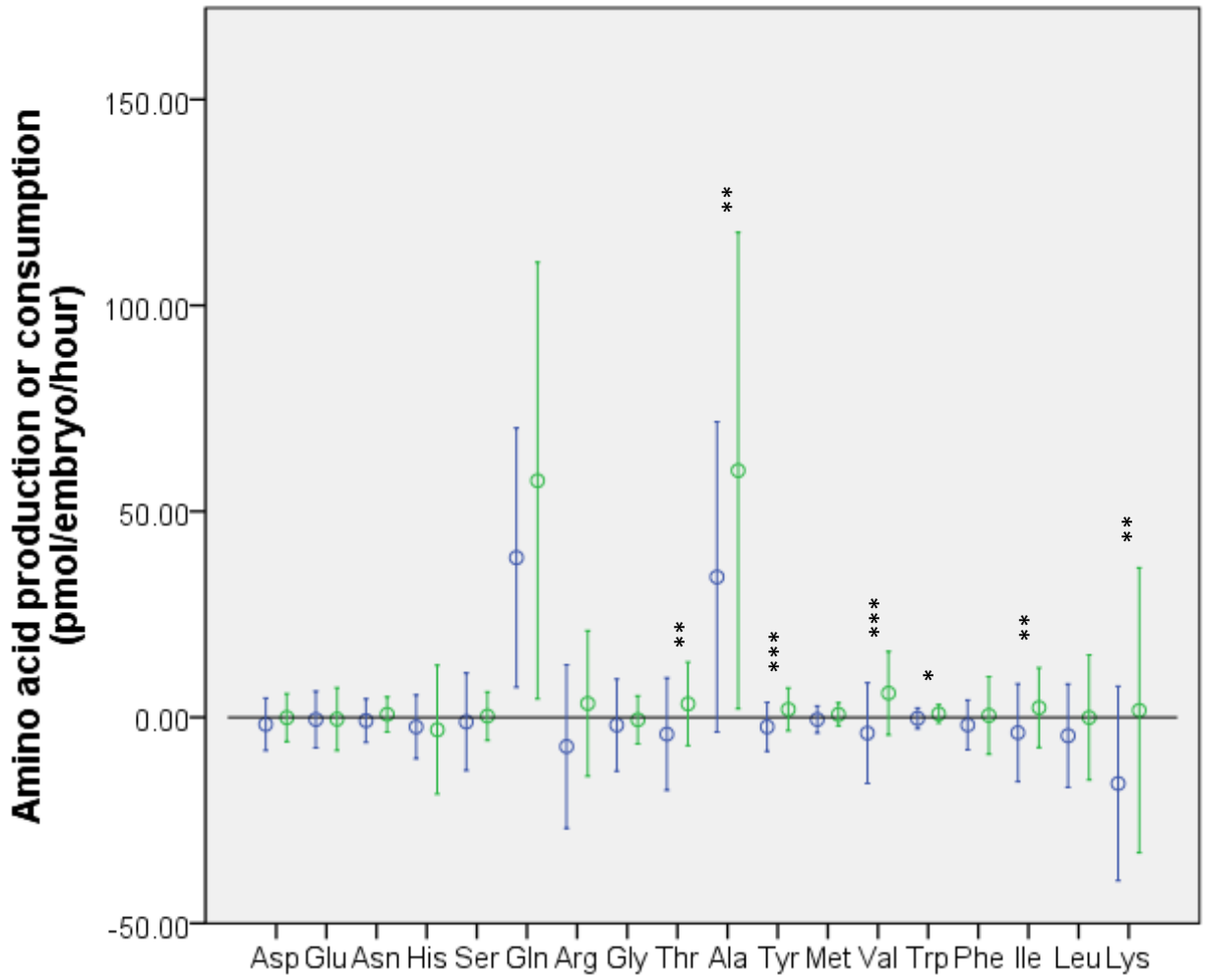

Figure 6.11: Consumption and production of individual amino acids in G2+ media in embryos inseminated by IVF-ICSI according to the type of incubator (blue=EmbryoScope (n=67), green=MINC (n=54))
Positive values indicate production and negative values indicate consumption.
Values are mean utilisation (pmol/embryo/hour) ± standard deviation.
***p value <0.05, **p value<0.01, ***p value<0.001**

6.3.5.9 Overview of day 5 metabolomics

Table 6.8: Overview of metabolic differences between embryos inseminated using different methods, of differing quality and cultured in different incubators on day 5.

NS depicts no significant results.

	Different insemination methods	**Embryo quality**		**Type of incubator**	
		IVF	**IVF-ICSI**	**IVF**	**IVF-ICSI**
Glucose consumption	NS	Increased consumption in higher quality embryos	NS	NS	NS
Pyruvate consumption	Decreased in embryo inseminated by IVF	NS	NS	NS	Increased consumption in the EmbryoScope
Amino acids' consumption and production	Increased Asn and decreased Tyr, Phe, Ile, Leu and Lys utilisation in embryos inseminated by IVF	Decreased Asp, Asn and His utilisation and decreased Ser, Gln and Ala production in higher quality embryos	Decreased Gln and Ala production in higher quality embryos	Increased utilisation of His, Arg, Gly, Thr, Tyr, Met, Val, Trp, Phe, Ile, Leu, Lys and decreased production of Gln and Ala in the EmbryoScope	Increased utilisation of Thr, Tyr, Val, Trp, Ile and Lys, decreased production of Ala in the EmbryoScope

6.4 Discussion

This study demonstrates that culturing embryos in a more stable environment improves blastocyst formation rate and this is the first study to examine how this alters blastocyst carbohydrate and amino acid utilisation. Culturing an embryo in a time lapse incubator increases the chance that it will form a blastocyst on day 5 or day 6. Although incubation of embryos in the EmbryoScope increased the number and quality of blastocysts formed, this was not reflected in an impact on pregnancy rates. While this may be due to insufficient statistical power, it should be noted that both incubators provided good outcomes. These findings concur with those from a recent publication demonstrating improved 8 cell development rate, blastocyst development rate and implantation rates in embryos who underwent uninterrupted culture (Alhelou et al., 2018).

The carbohydrate and amino acid concentrations measured in the control drops of G1+ media are similar to those found in the human fallopian tube (Lane and Gardner, 2007) and to those previously described (Gardner and Lane, 1999). The measurements in the G2+ media are also similar to those previously published (Gardner and Lane, 1999) demonstrating a good technique in measuring the substrates.

This work demonstrated different metabolic profiles between embryos inseminated by IVF and those inseminated by IVF-ICSI. The increased glucose utilisation in the G1 media in the IVF group may be due to the increased carbohydrate requirement of the cumulus cells (which are removed prior to IVF-ICSI).

To my knowledge, these data are the first to demonstrate differences in carbohydrate utilisation between embryos cultured in a stable environment (i.e. a timelapse incubator) as opposed to a disturbed one. This is likely to be due to the effect of increased oxidative stress and fluctuations in pH and temperature on the embryo. Variances were observed on day 3 despite the embryo disturbance until this point being minimal. However, on day 5 increased glucose uptake by embryos inseminated by IVF and increase pyruvate consumption by embryos inseminated by IVF-ICSI was observed in embryos cultured in the EmbryoScope. Both of these metabolic markers have been previously correlated with improved embryo quality (Gott et al., 1990).

These data demonstrate that in the early stages of development (day 1 to day 3) both pyruvate and glucose play an important role as energy sources. This has been previously demonstrated in mouse models; in which the development to the two cell stage had an absolute requirement for pyruvate (Biggers et al., 1967), whilst glucose became a more important carbohydrate source

after the 4 cell stage (Brinster and Thomson, 1966). One previous study (Conaghan et al., 1993) has demonstrated a positive relationship between pyruvate utilisation in the early embryo and embryo quality, the current study did not find this relationship. However, it should be noted that in the Conaghan et al. study (Conaghan et al., 1993), the embryos were cultured in very different conditions, including 20% O_2 and therefore comparisons cannot easily be drawn. Other studies (Gott et al., 1990, Gardner et al., 2001) have examined pyruvate metabolism and demonstrated improved embryo quality with an increased pyruvate uptake, however these studies have analysed the metabolic profile of the embryo on day 4, which the PROMOTE trial was unable to do.

After day 3 (approximately the 8 cell stage), glucose becomes the energy source of choice for the embryo; however, in the PROMOTE trial, 43.8% of the embryos had less than 8 cells when the media was changed on day 3 which may account for the high pyruvate utilisation in the G2 media. On day 5, a positive correlation between glucose consumption and embryo quality, in the IVF group, was observed which agrees with previous work (Gott et al., 1990, Gardner et al., 2011).

Previous research examining the amino acid turnover in the cleavage stage embryo has demonstrated that a "quiet" embryo i.e. one which took up and produced lower quantities of amino acids was more likely to develop into a blastocyst (Houghton et al., 2002). However, during the formation of a blastocyst, amino acids are essential for the production of the trophectoderm junctional complexes. Research from Southampton has demonstrated that embryos with a greater amino acid turnover (at the time of compaction and cavitation) have improved assembly of these junctional complexes (Eckert et al., 2007). Furthermore, in murine studies of post compaction embryos, those cultured at 20% O_2 (and therefore under increased oxidative stress) demonstrated a decreased consumption of asparagine, glutamate, tryptophan, lysine, threonine, tyrosine, methionine, valine, isoleucine, leucine and phenyalanine compared to those cultured at 5% O_2 (Wale and Gardner, 2012). The current data comparing the amino acid metabolic profiles of embryos cultured in the EmbryoScope and the MINC benchtop incubator had remarkably similar results. Interestingly, a statistically significant increase in glutamine and alanine production was also observed in embryos cultured in the MINC benchtop incubator and in embryos that were of a poorer quality; studies have demonstrated an increased transamination of these amino acids by blastocysts when they are placed under increased stress (Wale and Gardner, 2013).

The division of embryos rather than patients meant that potential genetic variation and maternal confounding factors were controlled. However, ideally the embryologists would have been blinded to the type of incubator that the embryos had been cultured in whilst they were analysing them (this was one of the main criticisms of the 2018 Cochrane review). Furthermore, the embryos cultured in the EmbryoScope were disturbed when slides were removed to change the media. With the development of a new single stage medium, the importance of a stable environment with no changes in temperature or oxygen and carbon dioxide levels can be investigated further as the embryos would not need to be removed from the Embryoscope until transfer or cryopreservation occurred. A further limitation was that whilst medium was refreshed in the EmbryoScope, embryos in the MINC were transferred to new dishes. The assessment of the metabolite usage was only performed at two time points, when media was being changed, and assumed that the nutrient uptake was the same throughout the 48 hour time period which has been demonstrated to not be true in both human (Hardy et al., 1989) and mouse models (Biggers et al., 1967). The study was also not powered to determine a difference in clinical outcome parameters.

In conclusion, the PROMOTE trial demonstrated that less interruptions to an embryo's culture environment improves blastocyst formation rates and the metabolic profile of the developing embryo including increased consumption of carbohydrates and decreased production of Gln and Ala. Increased utilisation of glucose was shown to correlate with improved embryo quality, as was the decreased utilisation and production of certain amino acids, strengthening the case for using metabolic markers as predictors of embryo viability.

Chapter 7: Discussion

7.1 Overview

This thesis demonstrates the importance of the periconceptional period on early embryo development and manipulation of this by altering the diet of a couple to include higher levels of Omega 3 FAs or by improving the stability of the environment the embryo is cultured in. The work presented is the first double blinded randomised controlled trial examining the effect supplementing omega-3 FAs and Vitamin D on morphokinetic markers of embryo quality. Furthermore, the PROMOTE trial is the first trial to examine the effect on carbohydrate utilisation and amino acid consumption of the early embryo and production of a stable culture environment.

Despite the relatively short length of time the dietary intervention was taken for, a positive impact on morphokinetic markers of embryo quality, specifically CC4 and S3, was observed. When the protocol for the PREPARE trial was written, the most predictive measure of blastocyst formation (Cruz et al., 2012) and subsequent chance of implantation (Meseguer et al., 2011) was thought to be CC2 and therefore this was chosen as the primary endpoint of the trial. The use of morphokinetic markers is a rapidly evolving field and other markers such as CC3 and CC4 have emerged as being predictive of clinical pregnancy (Milewski et al., 2016).

Furthermore, a number of algorithms have been developed, including the KIDScore on day 3 (Petersen et al., 2016) and day 5, which take into account a number of different morphokinetic parameters to give a score which corresponds to the likelihood of implantation. These scores may offer a more robust method of assessing embryo viability than taking one morphokinetic marker in isolation and therefore were also analysed. A number of algorithms have been published, but many of them are based on data from one clinic or a small group of clinics and therefore are not generally applicable due to variations in laboratory conditions and protocols between clinics (Kirkegaard et al., 2014). In addition, morphokinetic time ranges predicting implantation vary between clinics (Freour et al., 2015). The KIDScore, including morphological and morphokinetic markers, was developed based on a large, heterogeneous data set and has maintained its predictive value following testing on subsets of patients using various fertilisation methods and culture conditions (Kovacs, 2016). These data demonstrate an improvement in the day 3 KIDScore of embryos from couples in the treatment arm of the trial. Nevertheless, new algorithms are being developed and video recordings were made of each embryo in order to have the possibility of studying any more discerning parameters that might emerge in the future. It would also be interesting to develop an algorithm applicable to the patients and embryos at

Complete Fertility and analyse the effect of the dietary intervention using this potentially more specific approach.

There are several potential mechanisms of action by which increased levels of omega-3 FAs and vitamin D may improve embryo quality. Fatty acids are metabolised by β oxidation to produce ATP and therefore provide a potentially important energy source for the developing embryo (Sturmey et al., 2009a), providing significantly more ATP per molecule compared to glucose. A 5-fold increase in β-oxidation is observed at the time of blastocyst formation (Flynn and Hillman, 1980) and increasing this with dietary L-carnitine improves embryo development (Dunning et al., 2011). Omega-3 FAs are a precursor to eicosanoids, essential signalling molecules that regulate cell growth (Zhou and Nilsson, 2001). Furthermore, they are needed for the rapidly increasing cell membrane requirement of the dividing early embryo. Previous research has demonstrated that poorly developing embryos contained increased levels of saturated fatty acids and low unsaturated fatty acids, mimicking the pattern observed in oocytes that failed to fertilise (Matorras et al., 1998).

Fatty acids are also essential for the developing gametes. They are β oxidised in mitochondria following the LH surge and pharmacological inhibition of this impairs oocyte maturation (Dunning et al., 2010, Dunning et al., 2014). As a secondary outcome of the PREPARE trial, the fatty acid composition of the follicular fluid was analysed. This work demonstrated that the nutritional milieu of the follicular fluid could be altered by diet and correlations were observed between embryo quality and oleic, palmitic and docosahexaenoic acids (improved quality) and stearic and linoleic acids (decreased quality). However, we did not observe the expected increase in oocyte maturation number. This is possibly due to a lack of power as numbers of immature oocytes collected were low in both arms of the trial.

Interestingly, in contrast to these results and the previous observational studies in humans, which have demonstrated improved oocyte maturation and embryo quality with increased omega-3 FA intake, research in mice has shown the opposite. Reactive oxygen species production due to increased lipid peroxidation was increased and intracellular calcium homeostasis altered in mice fed diets enriched with omega-3 FAs resulting in a reduced proportion of cleaved zygotes (Wakefield et al., 2008). This study also demonstrated decreased fertilisation rates, possibly due to an alteration in the distribution of active mitochondria and mitochondrial calcium levels in the outer region of the oocyte thus impacting on the energy production in this area (Wakefield et al., 2008). This study did observe a decrease in fertilisation rates in the treatment arm of the trial but no difference was observed in fertilisation number or

in blastocyst formation rate or number. It would be interesting to ascertain whether our dietary intervention also alters the mitochondria distribution in human oocytes, but this would be difficult to due to the invasive nature of mitochondrial analysis (Chen et al., 2012).

The vitamin D status within the follicular fluid was not measured within this trial. Furthermore, the relationship between vitamin D and oocyte competence remains unclear. A recent publication has demonstrated that an increased concentration of vitamin D in the follicular fluid was negatively correlated with the quality of the oocyte from that follicle (Ciepiela et al., 2018). Conversely, a recent meta-analysis demonstrated that Vitamin D deficiency was associated with lower live birth rate but not with lower clinical pregnancy rate in women undergoing ART (Lv et al., 2016). It may be possible to examine Vitamin D levels in follicular fluid in future work and look at correlations with markers of embryo quality. A limitation of our trial compared to that conducted by Ciepela et al. is that the follicular fluid was not collecting in a manner that allowed it to be paired with the retrieved oocytes and resulting embryos and therefore such an extensive analysis of local follicular fluid levels will not be possible.

Despite the recognised importance of DHA in spermatogenesis, no effect was seen of our dietary intervention on the semen analysis parameters. This is likely to be because the dietary intervention was for too short a period for the DHA to be incorporated into the newly developing sperm. Previous research has demonstrated a benefit when supplementation of omega-3 FAs was for a more prolonged period of time (for example 12 weeks (Robbins et al., 2012) or 10 weeks (Martinez-Soto et al., 2016)). Surprisingly, a more recent study has shown an effect on sperm motility with a daily dose of 1 g (similar to our dose) after 1 month, but the effect was most evident in the asthenozoospermic patients (Gonzalez-Ravina et al., 2018). However in the PREPARE study, no difference was seen on the proportion of DHA in the sperm between the two groups. Further work is therefore needed to ascertain the length of time DHA takes to be incorporated into sperm, and therefore to have an effect on motility (a result of the large number of double bonds in DHA (Connor et al., 1998)). Furthermore, unlike the follicular fluid, no increase was seen in the DHA found in the seminal plasma. This is could be due to the relative higher proportion in the seminal plasma implying it is actively transported and determined by other processes in the male reproductive organs than by diet or levels in the blood.

Vitamin D levels were not measured in either the seminal plasma or the sperm as this was not part of the original protocol and funding. In view of the fact that no difference was seen in the omega 3 PUFAs, it would be interesting to see if this was also true of vitamin D levels implying a

tightly controlled nutritional milieu and content of sperm required for normal motility and the acrosome reaction.

In addition to a dietary intervention for six weeks prior to IVF improving embryo quality, the stability of the conditions an embryo was cultured in also had an impact. The data demonstrated that culturing the embryos in a more stable environment improved blastocyst formation rates; however we did not see any impact on pregnancy rates. Over recent years, these findings have been contradicted (Wu et al., 2016) and more recently agreed with (Alhelou et al., 2018). This study also demonstrated a change in nutrient uptake by embryos cultured in the different incubators. Taking into account the increased blastocyst formation rate, embryos cultured in the EmbryoScope had increased pyruvate consumption on day 5 and increased utilisation of His, Arg, Gly, Tyr, Met, Val, Trp, Phe, Ile, Leu and Lys and decreased production of Gln, Thr and Ala. Pyruvate consumption has been shown to correlate with increased blastocyst formation rate (Hardy et al., 1989). However, other research was of the opinion it is a poor and unreliable marker (Conaghan et al., 1993, Turner et al., 1995). Conversely, the pattern of consumption and production observed in the EmbryoScope compared to the MINC benchtop incubator mimicked mouse embryos cultured at 5% O_2 compared to 20% O_2 (and therefore under increased oxidative stress) (Wale and Gardner, 2012). These data give further evidence to compel IVF laboratories to make the length of time an embryo is exposed to a change in environment an important goal.

As this objective has already been recognised and following the invention of time lapse incubators (thus eradicating the need for embryos to be removed from their incubator for morphological assessment), a new single stage medium has been developed. As it has been demonstrated that this medium does not need to be "refreshed" on day 3 (Rambhia and Desai, 2014), the embryos do not need to be removed from the EmbryoScope until transfer or crypreservation. It would be interesting to examine whether the effects on the metabolic profile, particularly the amino acids' consumption and production, were amplified by this advancement. This would be a very difficult study to perform as the amino acid utilisation would need to be measured after the 120 hour period and would vary considerably within this time period.

7.2 Strengths

The results of this trial are reliable due to the high number of embryos analysed. The recruitment to both trials was successful and the sample sizes required by the power

calculations were achieved. This is likely to be because these are a highly motivated group of individuals who have often been trying to conceive for many years and are keen to improve their chances of success in any way possible; this has been previously demonstrated as a "willingness to do anything" in order to improve the likelihood of achieving a pregnancy (Mahon and Cotter, 2014). Interestingly, another piece of qualitative research demonstrated that males undergoing the IVF process were less keen to try low-technology approaches to enhance their fertility than their female counterparts (Throsby and Gill, 2004). This trial did show marginally lower compliance level for the men compared to the women (still greater than 90%), although there was a high correlation between the compliance of the man and woman from the same couple, indicating a joint commitment to the study.

Randomisation to the PREPARE trial was successful with no difference being demonstrated between the sociodemographic characteristics of the participants in the two groups. Blinding was also successful and unblinding was only performed once all participants had completed the trial and all embryos had been annotated and morphokinetic markers calculated. The division of the embryos in the PROMOTE trial rather than the patients meant that potential genetic variation and maternal confounding factors were controlled for, however there were limitations as it was not possible to blind the embryologists to the style of incubator.

Conducting the PREPARE trial in an IVF unit (instead of comparing fecundity in the general population) allowed a unique opportunity to examine the effect of the dietary intervention on the first few days of embryo development and start to postulate on possible mechanisms of action, as opposed to assessing the benefit to clinical outcome.

The methodology of assessing the participants' diet was shown to be robust as the RBC folate correlated with the prudent diet score with a similar positive coefficient to that which was seen in the original paper from which the 20 item food frequency questionnaire was extrapolated (Crozier et al., 2010). Furthermore, the methods used to measure fatty acids in the erythrocytes, follicular fluid, seminal plasma and sperm have been previously trialled and validated (Fisk et al., 2014). Furthermore, the data from this study was similar to measurements previously made (Browning et al., 2012, Dunning et al., 2014, Safarinejad et al., 2010). In addition, the methods used to measure glucose, pyruvate and amino acids have been previously used (Kermack, 2015, Houghton et al., 1996) and the measurements in the control media were similar to those published (Gardner and Lane, 1999).

7.3 Limitations

Both trials were single centre studies and although the demographic details of the participants were typical of both the fertility unit and Southampton (2011 Census), care should be taken when extrapolating the results to a wider audience. The majority of the participants were white Caucasian and therefore may have a different diet and lifestyle compared to other ethnic groups. It would be interesting to repeat the trial in a different clinic in another area with a different population to compare the results.

An exclusion criterion of the PREPARE trial was the consumption of more than two portions of oily fish per week and the assumption was made that couples eating less than this would be deficient. Research has demonstrated that dietary reports are inaccurate and correlations between the amount of oily fish people report to eat and the erythrocyte content of DHA and EPA are unreliable (Browning et al., 2012). Furthermore, dietary oily fish intake in the UK is low and infrequent (SACN, 2004) and the DHA and EPA proportions measured in the erythrocytes in our trial prior to the intervention were similar to those previously reported (Browning et al., 2012). This was not the case for vitamin D. Our mean 25 (OH) vitamin D levels were found to be substantially greater than those of women who participated in the MAVIDOS trial; a double blinded, randomised controlled trial examining vitamin D levels and supplementation on women from 14 weeks of pregnancy (based in Southampton, Oxford and Sheffield) (Cooper et al., 2016). There are two possible explanations for this; the first is that participants in the MAVIDOS trial may have become vitamin D deplete during the first trimester of pregnancy. The second and more likely explanation is that the women participating in PREPARE had been taking vitamin supplements, including vitamin D, for a prolonged period prior to starting the trial. Hence, a surprisingly small percentage of women (less than 5%) were deplete prior to starting the trial, compared to cited levels of between 35 and 45% (McAree et al., 2013). The lack of deficiency may mean it is possible that the full effect of the vitamin D supplementation was not elicited. Analysing this subset of the participants was considered, but was not possible as the number was low.

A criticism of the PREPARE trial could be the use of morphokinetic markers as a surrogate marker of live birth rate, instead of using the latter itself as the primary endpoint of the trial. Whilst it is acknowledged that a double blinded randomised controlled trial to examine the effect on clinical outcomes of our dietary intervention would be fascinating, the PREPARE trial was primarily designed to test the hypothesis derived from previous observational studies (Hammiche et al., 2011, Vujkovic et al., 2010) that even short-term changes in preconceptional

maternal diet can affect human embryo development. In designing this project focusing on diet and the embryo, an important research gap that had developed between observational studies reporting associations between diet and embryo development and the interventional studies necessary to confirm or refute those observations has been satisfied. Furthermore, given the challenges of carrying out an appropriately powered study with an endpoint of live birth, pending such a trial being undertaken, this data provides value to couples undergoing ART treatment and wishing to take an evidence based approach to their dietary supplementation.

A similar criticism could also be made of the PROMOTE trial as again this used blastocyst formation as the primary end point rather than live birth rate as requested by the 2015 Cochrane review (Armstrong et al., 2015). However, the design of the study allowed the effect of the culture time on embryo development to be assessed. The division of embryos rather than patients meant that potential genetic variation and parental confounding factors were controlled. Furthermore, by assessing all embryos according to morphological parameters (rather than morphokinetic markers), it allowed for the important distinction between the improvement in pregnancy rates in time lapse incubators (Meseguer et al., 2012) secondary to enhanced methods of embryo selection or due to the increased stability of culture conditions to be ascertained.

Another limitation of the PROMOTE trial was the inability to blind the embryologists to the type of incubator; this was because of differences in the designs of the dish used in each type of incubator. Furthermore, there were differences in the protocols with embryos in the EmbryoScope having their media "refreshed" on day 3 (i.e. G1+ media removed from the dish and G2+ media added) whereas embryos cultured in the MINC benchtop incubator were moved into a different dish on day 3 (containing the G2+ media). Despite this difference in the protocols for media changes the mean concentrations of glucose, pyruvate and individual amino acids taken from the controls from the two incubators were very similar. Furthermore, to try and negate this difference, controls from each couple were placed into each incubator at the same time as their embryos and then used to ascertain the substrate utilisation or production.

7.4 Further work

In the future, I plan to apply for a NIHR Clinical Lectureship. This will allow me time to further develop my research interests and career.

To take forward my work on the PREPARE trial I would like to:

- Examine the fatty acid composition of the cumulus cells to ascertain if the diet had an effect.
- Given the changes in the follicular fluid observed, analyse whether this short dietary intervention also altered the fatty acid composition of uterine fluid.
- Investigate the effect of different causes of subfertility on the follicular fluid fatty acid profile of women.
- Look at the effect of the fatty acid profile on the distribution of mitochondria in the oocyte to explore further possible mechanisms of action to explain the difference in fertilisation rate observed.
- Consider altering the length of the intervention (to 12 weeks) to observe whether an improvement in semen analysis parameters would then be seen.
- Run a large multicenter trial looking at pregnancy rates as the primary outcome.

Future work within the PROMOTE trial should include:

- Analysis of the lactate production of embryos cultured in the two incubators to ascertain whether this correlates with carbohydrate utilisation.
- As a difference in blastocyst formation rate has been demonstrated in a number of studies, perform an appropriately powered study to examine the effect of the more stable culture environment on live birth rate.
- With the invention and use of algorithms including morphological and morphokinetic markers of quality for embryo selection, assess the possible addition of carbohydrate utilisation and/or amino acids' consumption or production to improve the effect of the algorithm application on live birth rates.

7.5 Implications for the future

This thesis has produced some interesting and exciting findings that demonstrate that not only what a woman consumes during a pregnancy is important to fetal outcome but also the couples' intake prior to conception may have an effect. Furthermore, these data show that a relatively short period of dietary supplementation alters that the fatty acid profile of erythrocytes and follicular fluid and has a demonstrable impact on embryo quality. These beneficial results will

add to the currently small body of evidence that advises couples what supplements they should be taking prior to IVF. However, more double blinded randomised controlled trials looking at clinical outcomes are required before definite recommendations can be made.

In addition, to the importance of the couples' lifestyle prior to IVF, improvements can be made in the incubation of embryos in the laboratory to increase IVF outcomes. By limiting the amount of time an embryo is removed from an incubator, by using time lapse incubators for embryo assessment, blastocyst formation rates improve. The carbohydrate utilisation and amino acids' consumption and production of the early embryos in the two types of incubator strengthen these findings. The thesis demonstrates that the environment of an early embryo is of paramount importance and couples and healthcare professionals should strive to enhance this to obtain the best possible fertility outcomes.

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Appendix A

Reproductive Health Research Team
Southampton General Hospital
Tremona Road
Southampton
Hampshire
SO16 6YD

Alexandra Kermack
Academic Clinical Fellow
Direct tel: +44 (0)23 81206044
Email: Alexandra.Kermack@uhs.nhs.uk

Date: 20/01/2014

Project Title: PREPARE: A double-blind randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment

Ethics Approval Number: 13/SC/0544

Dear Sir and Madam

As I am sure you are aware, there is a lot of information on the internet and in the press regarding vitamins and minerals that it may be beneficial for you to take while trying to conceive. Unfortunately, the evidence to support these claims is often sparse. Here at the Complete Fertility Centre, we are conducting a research project into the supplementation of micronutrients taken before you conceive, and would like to invite you to participate in our project. I have enclosed an information leaflet which details the study. This study is being completed as part of the educational requirements for a PhD.

It should be noted that your participation in this study is entirely voluntary and you may withdraw from it at any time. In addition, whether you decide to participate in this study or not, we recommend that all women take Folic Acid while trying to conceive.

Please do not hesitate to contact me if you would like any further information regarding this study.

Kind regards,

Alexandra Kermack

Academic Clinical Fellow

Direct tel: +44 (0)23 81206856

Email: Alexandra.Kermack@uhs.nhs.uk

Follow up: As part of your follow up, you may be contacted by one of the research team by either phone or email.
You will be monitored throughout your pregnancy by routine ultrasound appointments. Your baby's birthand placental weight will be recorded and we will ask to take samples from the placenta. You may also be asked if you would be happy for your baby to undergo a bone density scan as part of the research. Further information will be given about this following the delivery of your baby and you will be asked to sign a separate consent form.
Samples and data that have been provided may be stored for use in future ethically approved studies.

What are the possible disadvantages and risks in taking part?

You will be asked to stick to the healthy diet supplement program to which you have been randomised. Additional samples of fluid and tissue will be taken, the risks of which are negligible.

What are the possible benefits of taking part?

We will provide you with a food hamper that may improve your health. We will also carry out an endometrial scratch (taking a sample from the lining of the womb) which is thought to increase the chances of success during IVF. We will use an Embryoscope to culture and closely monitor the growing embryos and if an embryo is transferred then we will give you a copy of this film.

Will my taking part in this study be kept confidential?

All information collected about you will be kept strictly confidential. Your GP and obstetric team will be informed of your participation.

What will happen to the results of the research?

These findings will be presented at scientific meetings, nationally and internationally, published in medical journals, and possibly in the local and national press. You will not be identified in these reports or publications in any way.

What if there is a problem?

If you have a concern about any aspect of this study, please ask to speak to Dr Alexandra Kermack, who will do her best to answer any questions you have (Alexandra.kermack@uhs.nhs.uk).
You can also speak to Mr Nick Brook, a Consultant in Reproductive Medicine, who is independent from the study (Nick.Brook@uhs.nhs.uk).

What if I want to complain?

If you wish to complain formally, you can do this through the NHS Complaints Procedure (via Southampton General Hospital).

Who has reviewed the study?

This study has been reviewed and approved by South Central Oxford A Research Ethics Committee (Ethics No.: 13/SC/0544). It has also been peer reviewed by Professor Robert Norman at the University of Adelaide, Australia.

Who is organizing and funding the research?

This study is funded by the NIHR Biomedical Research Centre (BRC) Southampton and the Complete Fertility Unit, and organized by the BRC, Complete Fertility Unit and University of Southampton.

Contact for further information

For further information please contact Professor Nick Macklon (Chief Investigator) or Dr Alexandra Kermack at the University of Southampton on Alexandra.Kermack@uhs.nhs.uk.

Version 4.0 16/06/2014

University Hospital Southampton NHS
NHS Foundation Trust
UNIVERSITY OF Southampton

You are invited to take part in

Prepare

A double-blind randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment.

If you are interested in taking part, please contact us at the earliest opportunity on:

Telephone: 02381206856

Email: Alexandra.Kermack@uhs.nhs.uk

You are being invited to take part in a trial of dietary supplementation in women and men undergoing IVF treatment. There is a lot of information in the press regarding the vitamins that women should be taking while trying to conceive, however the scientific evidence behind these claims is weak. The University of Southampton is conducting exploratory research to investigate this further. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with anyone you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?

This is an exploratory research project to examine whether or not increased levels of certain micronutrients in a couple's diet improves embryo quality, and if so, how.

Why might I be approached?

You are planning to undergo IVF treatment at Complete Fertility Centre, Southampton.

Am I eligible to take part?

You are eligible to take part if:

- You have not had more than two unsuccessful cycles of IVF.
- For female partners, you are aged 41 or under and have a body mass index (BMI) of between 20 and 32kg/m^2.
- You are not known to have diabetes, HIV, or Hepatitis B or C.
- You have been non-smokers for the last six months.
- You do not eat oily fish more than once a week.
- You are not taking any high dose vitamin supplements – please ask one of the team if you would like clarification on this.
- You are not taking any prescribed medications other than simple painkillers.
- You are not currently participating in any other trials.

Do I have to take part?

You decide whether or not to take part. If you do take part you are still free to withdraw at any time and without giving a reason. This will not affect the treatment or standard of care you receive.

What will happen to me if I take part?

4 weeks prior to starting your IVF treatment: Both you and your partner will be asked to complete a preconception and food frequency questionnaire in order to assess your current lifestyle and diet. You will be asked to give samples of blood, urine, fluid from the womb and the lining of the womb, which will be taken with your consent. We will also request a semen sample from your partner.

You will then be randomised to either of two healthy diet supplement programs (to simulate diets shown in previous studies to potentially be of benefit). You will be given a food hamper containing smoothie drinks (one each per day), oils and spreads which you should use to compliment your normal diet for the next four weeks. You will be asked to keep a diary to record use of the hamper.

Day 2 of cycle: You will come to Complete Fertility for a baseline scan and be asked to return with your used straws. You will meet with a member of the research team who will look at your diary and count the returned straws. They will then give you another hamper, which should last 2 weeks.

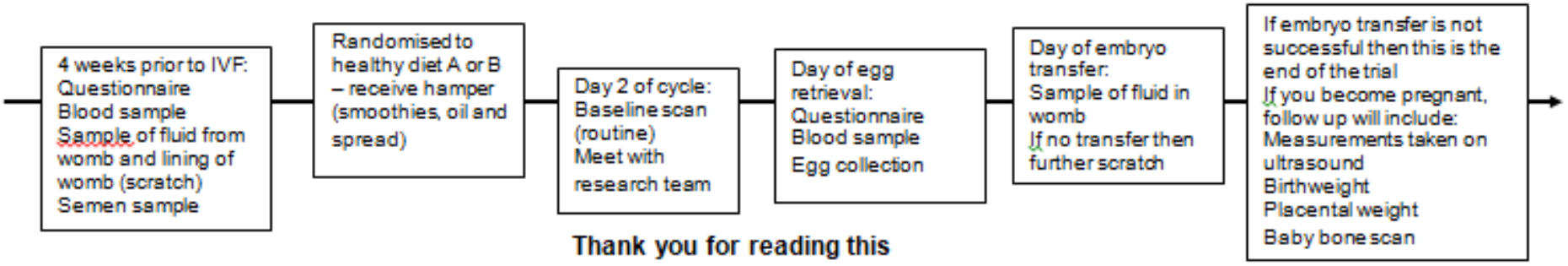
Day of egg retrieval: You and your partner will both be asked to complete a further questionnaire to assess any changes in your lifestyle and diet.

You will be asked to give further blood and urine samples and will then proceed with your egg retrieval. The process by which egg collection occurs involves taking the fluid and cells from around the egg; instead of being discarded these waste tissues will be kept for analysis. The partner's semen will be analysed prior to insemination.

The embryos will be monitored and filmed using an Embryoscope.

Day of embryo transfer: The fluid in the womb will be removed prior to the embryo transfer and kept for analysis. If embryo transfer is not carried out for clinical reasons, then we will request a sample of the fluid in the womb and the lining of the womb. This is optional and will be taken only with your additional consent.

Fold out for trial outline:



Thank you for reading this

complete FERTILITY CENTRE SOUTHAMPTON

University Hospital Southampton NHS NHS Foundation Trust

UNIVERSITY OF Southampton

Prepare

A double-blind randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment

Ethics Approval Number: 13/SC/0544

Initial Consent form - Male

PREPARE serial no:

Please initial each box:

1) I confirm that I have read and understand the information sheet dated 16/06/2014 (version 3.0) for the above study and have had the opportunity to ask questions. ☐

2) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

3) I agree to information being gathered from my hospital notes, to give blood and semen samples and to these being stored for analysis. Gametes collected will be rendered non-viable prior to storage. ☐

4) I understand samples will be analysed for metabolic, immunological and genetic studies and that they will not be used to diagnose any individual genetic conditions. ☐

5) I agree to have my embryos' development monitored and recorded by an ~~embryoscope~~. ☐

6) I agree for my General Practitioner to be informed of my participation in this study. ☐

7) I agree to being contacted by the research team about participation in future aspects of this research. ☐

8) I agree that the Health Authority and my GP may disclose my new contact details to the researchers if I move house. ☐

9) I agree to take part in the study and use the study foods and drink that are provided to me. ☐

.....	.....	.....
Name of participant giving consent	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature

complete FERTILITY CENTRE SOUTHAMPTON

University Hospital Southampton NHS NHS Foundation Trust

UNIVERSITY OF Southampton

Prepare

A double-blind randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment

Ethics Approval Number: 13/SC/0544

Initial Consent form - Female

PREPARE serial no:

Please **initial** each box:

1)	I confirm that I have read and understand the information sheet dated 13/11/2013 (version 2.0) for the above study and have had the opportunity to ask questions.	☐
2)	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.	☐
3)	I agree to information being gathered from my hospital notes and to have blood, urine, uterine fluid (fluid from inside the womb) and endometrium (lining of the womb) samples taken and stored for later analysis.	☐
4)	During egg retrieval, I agree to have a sample of follicular fluid (fluid around the egg) and the cumulus cells (cells around the egg) stored and analysed as part of the research project. These will be held securely and disposed of once the study is complete.	☐
5)	I understand samples will be analysed for metabolic, immunological and genetic studies and that they will not be used to diagnose any individual genetic conditions.	☐
6)	I understand that my embryos may be incubated and monitored by an Embryoscope if sufficient equipment is available.	☐
7)	I understand that I may be invited to undergo additional ultrasound scans in pregnancy to monitor development.	☐
8)	I agree for my General Practitioner and obstetric team to be informed of my participation in this study.	☐
9)	I agree to being contacted by the research team about participation in future aspects of this research.	☐
10)	I agree that the Health Authority and my GP may disclose my new contact details to the researchers if I move house.	☐
11)	I agree to take part in the study and use the study foods and drink that are provided to me.	☐

I

.....	.....	.....
Name of participant giving consent	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature

PRECONCEPTION AND DIET QUESTIONNAIRE

PREPARE serial no: ☐☐☐☐ **Date:** ☐☐.☐☐.☐☐☐☐

Gender: Male ☐ Female ☐

Visit: 1 ☐ 4 ☐

Date of birth: ☐☐.☐☐.☐☐☐☐

Ethnicity: ☐☐

1. White
2. Black Caribbean
3. Black African
4. Black Other
5. Indian
6. Pakistani
7. Bangladeshi
8. Chinese
9. Other Asian Group
10. Other _____

Your Body Composition

1. Height ☐☐.☐☐ metres
2. Weight ☐☐☐.☐☐ kgs
3. BMI ☐☐.☐☐ kgs/m^2

Your lifestyle

1. What is your occupation? _____
2. How old were you when you left full time education? ☐☐ years
   (don't round up; count current age if still studying)

3. Have you passed any exams or do you have any formal qualifications? ☐
    1. None
    2. CSE/ School cert/ GCSE grade D or lower/ NVQ1/ Foundation GNVQ
    3. O levels/ Matric/ GCSE grade A,B,C/ RSA secretarial/ NVQ2/Intermediate GNVQ
    4. A levels/ City & Guilds/ EM(G)/ ONC/ NNEB/ BTech (day release)/ NVQ3/ Advanced GNVQ/OND/HNC
    5. HND/ RGN/ Teaching Cert/ NVQ4
    6. Degree/ NVQ5
    7. Other _____

4. How often do you exercise? ☐
    1. Never
    2. Once a month
    3. Once every two weeks
    4. 1-2 times per week
    5. 3-6 times per week
    6. Daily

    ii. How much exercise do you do? ☐☐.☐☐ hours per week

    iii. What type of exercise? _____

5. In the last month, on average how much time have you spent outside on a day during the week?

    ☐☐ hrs ☐☐ mins

6. In the last month, on average how much time have you spend outside on a day during the weekend?

    ☐☐ hrs ☐☐ mins

7. In the last month, how many days have you used suncream? ☐☐ days

8. Do you feel that you have been under an unusually high amount of stress within the last month?

    Yes ☐ No ☐

    a. If yes:

        i. Why? _____

9. Do you smoke? Yes ☐ No ☐ Never ☐

    a. If no:

        i. When did you stop? ☐☐.☐☐.☐☐☐☐

    b. If yes:

i. How many cigarettes in an average day? ☐☐☐ cigarettes per day

10. In the last three months, have you drunk alcohol? ☐ Yes ☐ No

a. If yes:

i. How many days a week do you drink alcohol? ☐

ii. How many units per week? ☐☐☐ units per week

Document which alcoholic drinks the participant drinks per week and quantities.

11. In the last three months, have you consumed caffeine? ☐ Yes ☐ No

a. If yes:

i. How many days a week do you consume caffeine? ☐

ii. How much per day? ☐☐☐ mg per day

Document which caffeinated drinks the participant drinks per day and quantities.

Your Eating Habits

1. Over the past month how often have you eaten these foods? **Tick one box** on each line for every item.

	Over the past month	**Never**	**Once a month**	**Once every 2 weeks**	**1-2 times per week**	**3-6 times per week**	**Once a day**	**More than once a day**
1.	Roast potatoes and chips							
2.	Peppers and watercress							
3.	Tomatoes							
4.	Meat pies							

1. Over the past month how often have you eaten these foods? **Tick one box** on each line for every item.

	Over the past month	**Never**	**Once a month**	**Once every 2 weeks**	**1-2 times per week**	**3-6 times per week**	**Once a day**	**More than once a day**
5.	Vegetable dishes							
6.	Courgettes, marrow and leeks							
7.	Sausages and sausage rolls							
8.	Gravy							
9.	Green salad							
10.	Wholemeal bread							
11.	White bread							
12.	Onion							
13.	Vegetarian food							
14.	Pasta							
15.	Yorkshire pudding & savoury pancakes							
16.	Crisps & savoury snacks							
17.	Beef							
18.	Spinach							
19.	Fresh fruit							
20.	Approximately how many teaspoons of **sugar** do you add each day to breakfast cereals, tea and coffee, etc.?							☐☐ Teaspoons
21.	How much **full-fat milk** on average do you use per day in your drinks, add to breakfast cereals, etc.?							☐☐.☐ Pints

Your Dietary Supplements

1. During the past month have you taken any pills, tonics or tablets to supplement your diet? (e.g. vitamins, minerals, iron tablets, folic acid, fish oils etc.)

Yes ☐ No ☐

a. If yes:
   i. Please state which? (for number per day, record number of tablets/capsules/teaspoons per day, as appropriate)

Supplement	Number per day?	How many days in the last 30?
	☐☐	☐☐
	☐☐	☐☐
	☐☐	☐☐
	☐☐	☐☐
	☐☐	☐☐

PROMOTE

Reproductive Health Research Team
Southampton General Hospital
Tremona Road
Southampton
Hampshire
SO16 6YD

Alexandra Kermack
Academic Clinical Fellow
Direct tel: +44 (0)23 81206856
Email: Alexandra.Kermack@uhs.nhs.uk

Date: 27/08/2014

Project Title: PROMOTE: A Prospective Randomised trial cOMparing embryO development in the standard versus the Embryoscope incubator

Ethics Approval Number: 14/SC/1260

Dear Sir and Madam

As I am sure you are aware, there is a lot of information on the internet and in the press regarding the best method of growing embryos for patients undergoing IVF or IVF-ICSI. Here at the Complete Fertility Centre, we are conducting a research project examining which of two different types of incubators are better at supporting the needs of a developing embryo. If you agree to participate in the project, half of your embryos will be put into a benchtop "MINC" incubator and the other half into an Embryoscope. They will be monitored, as usual, by our highly skilled team of embryologists. The best embryo or embryos for transfer will be selected on the basis of our standard validated criteria in order to give you the highest chance of pregnancy. The embryo(s) for transfer may therefore come from either of the incubators. With your consent, any other healthy embryos will be frozen according to our standard procedures. In addition, we will examine the fluid drop that your embryos were grown in to enable us to examine their nutrient use. We plan to do this because there is evidence to suggest that embryos that use fewer nutrients are in fact healthier and this will help us to decide which incubator provides a more favourable environment.

I have enclosed an information leaflet which details the study. This study is being completed as part of the educational requirements for a PhD.

It should be noted that your participation in this study is entirely voluntary and you may withdraw from it at any time.

Please do not hesitate to contact me if you would like any further information regarding this study.

Kind regards,

Alexandra Kermack

Academic Clinical Fellow

Direct tel: +44 (0)23 81206856

Email: Alexandra.Kermack@uhs.nhs.uk

complete FERTILITY University of Southampton University Hospital Southampton NHS NHS Foundation Trust

I

PROMOTE

Introduction

You are being invited to take part in a trial of two different types of incubators in order for us to understand whether one or the other increases the quality of embryos available for transfer. The Embryoscope is a new type of incubator which may improve IVF outcomes, but this has not yet been demonstrated. We wish to compare this incubator with our standard incubator. Both have been shown to be safe and effective in clinical practice. Before you decide whether you wish to take part in the study, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with anyone you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

This is a research project to examine whether there is any difference between the growth and development of embryos cultured in two different incubators; the standard benchtop 'MINC' incubator and an Embryoscope.

Why might I be approached?

You are planning to undergo IVF treatment at Complete Fertility Centre, Southampton.

Am I eligible to take part?

You are eligible to take part if:

- You are female and aged 42 or under.
- You have a Follicle Count of greater than 12.

Do I have to take part?

You decide whether or not to take part. If you do take part you are still free to withdraw at any time and without giving a reason. This will not affect the treatment or standard of care you receive. It is important that you decide as a couple whether or not you wish to participate in the research as both of you will be required to sign a consent form.

What will happen to me if I decide to take part?

Day of egg retrieval: You and your partner will both be asked to give consent to participating in the study. If you have more than 6 healthy fertilised eggs, then half of the total number will be randomised to the benchtop incubator and the other half to the Embryoscope. The embryos will be monitored closely as per usual clinical practice by the highly experienced embryology team.

Day 3 and day 5 of culture: Each embryo is cultured in its own nutrient fluid drop which is routinely changed on day 3 and 5, instead of being discarded it will be kept for analysis. We will analyse the fluid drop to assess the amount of particular nutrients used by the embryo as there is evidence that embryos that use less nutrients are healthier (it is not possible to do this before embryo transfer and therefore we will not have this information when choosing the best embryo to transfer).

Day of embryo transfer: The best embryo or embryos for transfer will be selected on the basis of our standard validated criteria in order to give you the highest chance of pregnancy. The embryo(s) for transfer may therefore come from either of the incubators. With your consent, any other healthy embryos will be frozen according to our standard procedures.

What are the possible disadvantages and risks in taking part?

There are not thought to be any disadvantages to taking part in the study, as both incubators have been shown to be safe and effective in clinical practice.

What are the possible benefits of taking part?

Your embryos will be monitored in two clinically proven incubators. If an embryo is transferred from the Embryoscope then we will give you a copy of a film recording its development in the incubator. Culture in the Embryoscope will be free of charge.

Will my taking part in this study be kept confidential?

All information collected about you will be kept strictly confidential. With your consent, your GP and obstetric team will be informed of your participation.

What will happen to the results of the research?

The findings will be presented at scientific meetings, nationally and internationally, published in medical journals, and possibly in the local and national press. You will not be identified in these reports or publications in any way.

What if there is a problem?

If you have a concern about any aspect of this study, please ask to speak to Dr Alexandra Kermack, who will do her best to answer any questions you have (Alexandra.kermack@uhs.nhs.uk).
You can also speak to Mr Nick Brook, a Consultant in Reproductive Medicine, who is independent from the study (Nick.Brook@uhs.nhs.uk).

What if I want to complain?

If you wish to complain formally, you can do this through the NHS Complaints Procedure (via Southampton General Hospital).

Who has reviewed the study?

This study has been reviewed and approved by South Central - Berkshire Ethics Committee (Ethics No.: 14/SC/1260). It has also been peer reviewed by Professor Marcus Montag from the University Women's Hospital Heidelberg, Germany and Mrs Emma Adolfsson from Goteborg Universitet, Sweden.

Who is organising and funding the research?

This study is jointly funded by the NIHR Southampton BRC Commercial & Enterprise Incubator Fund and Complete Fertility Unit. The Embryoscope has been provided by Unisense, however they are playing no part in funding the research. The study is being led by Professor Nick Macklon, from the University of Southampton.

Contact for further information.

For further information please contact Dr Alexandra Kermack at the University of Southampton on 02381206856 or Alexandra.Kermack@uhs.nhs.uk.

complete FERTILITY CENTRE SOUTHAMPTON

University Hospital Southampton NHS NHS Foundation Trust

UNIVERSITY OF Southampton

PROMOTE

A Prospective Randomised trial cOMparing embryO developmenT in the standard versus Embryoscope incubator

Ethics Approval Number: 14/SC/1260

Consent form - Male

PROMOTE serial no:

Please initial each box:

1) I confirm that I have read and understand the information sheet dated 20/10/2014 (version 2.0) for the above study and have had the opportunity to ask questions. ☐

2) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

3) I agree to information being gathered from my hospital notes for the purposes of this study. ☐

4) I understand that if our cycle produces 6 or more embryos, half will be incubated in the MINC incubator and the other half in the Embryoscope. I understand that they will be closely monitored by a member of the embryology team and that the best quality embryo or embryos will be chosen for transfer, according to the standard criteria applied by the IVF laboratory. ☐

5) I understand that the research team will store and analyse the culture media from all embryos. ☐

6) I agree for my General Practitioner to be informed of my participation in this study. ☐

7) I agree that the Health Authority and my GP may disclose my new contact details to the researchers if I move house. ☐

8) I agree to being contacted by the research team about participation in future aspects of this research. ☐

.....	.....	.....
Name of participant giving consent	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature

complete FERTILITY CENTRE SOUTHAMPTON

University Hospital Southampton NHS NHS Foundation Trust

UNIVERSITY OF Southampton

PROMOTE

A Prospective Randomised trial cOMparing embryO developmenT in the standard versus Embryoscope incubator

Ethics Approval Number: 14/SC/1260

Consent form - Female

PROMOTE serial no:

Please **initial** each box:

1) I confirm that I have read and understand the information sheet dated 20/10/2014 (version 2.0) for the above study and have had the opportunity to ask questions. ☐

2) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

3) I agree to information being gathered from my hospital notes for the purposes of this study. ☐

4) I understand that if our cycle produces 6 or more embryos, half will be incubated in the MINC incubator and the other half in the Embryoscope. I understand that they will be closely monitored by a member of the embryology team and that the best quality embryo or embryos will be chosen for transfer, according to the standard criteria applied by the IVF laboratory. ☐

5) I understand that the research team will store and analyse the culture media from all embryos. ☐

6) I agree for my General Practitioner and obstetric team to be informed of my participation in this study. ☐

7) I agree that the Health Authority and my GP may disclose my new contact details to the researchers if I move house. ☐

8) I agree to being contacted by the research team about participation in future aspects of this research. ☐

.....	.....	.....
Name of participant giving consent	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature

Appendix B

PEER REVIEWED PUBLICATIONS

Kermack AJ, Van Rijn BB, Houghton FD, Calder PC, Cameron IT, Macklon NS. The 'Developmental Origins' Hypothesis: relevance to the obstetrician and gynecologist. J Dev Orig Health Dis. 2015 Oct;6(5):415-24.

Kermack AJ, Macklon NS. Nutritional supplementation and artificial reproductive technique (ART) outcomes. Reprod Fertil Dev. 2015 May;27(4):677-83.

Kermack AJ, Finn-Sell S, Cheong YC, Brook N, Eckert JJ, Macklon NS, Houghton FD. Amino acid composition of human uterine fluid: association with age, lifestyle and gynaecological pathology. Hum Reprod. 2015 Apr;30(4):917-24.

Kermack AJ, Calder PC, Houghton FD, Godfrey KM, Macklon NS. A randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment (PREPARE trial). BMC Womens Health. 2014 Nov 18;14:130.

Kermack AJ and Macklon N. Preconception care and fertility. Minerva Ginecol. 2013 Jun;65(3):253-69.

PRESENTATIONS TO LEARNED SOCIETIES

A Kermack, P Lowen, S Wellstead, M Montag, F Houghton, P Calder, N Macklon. PREPARE trial: A randomised double blinded controlled trial of a preconception Omega 3 and Vitamin D rich dietary supplement in couples undergoing assisted reproduction treatment. Fertility Conference 2018 3rd – 5th January 2018. (oral)

A Kermack, P Lowen, S Wellstead, P Calder, F Houghton, N Macklon. A prospective randomised trial comparing embryo development in the MINC incubator versus the EmbryoScope incubator. Fertility Conference 2018 3rd – 5th January 2018. (oral)

A Kermack, T Yoo, K Donnelly, S Wellstead, H Fisk, F Houghton, S Lewis, N Macklon, P Calder. The effect of a six week dietary supplement of Omega-3 fatty acids on sperm quality: A double blinded randomised controlled trial. (poster)

A Kermack, P Lowen, S Wellstead, M Montag, F Houghton, P Calder, N Macklon. Does Omega 3 and Vitamin D Supplementation in the Six Weeks Prior to *In Vitro* Fertilisation Improve Embryo Quality? SRI MRC-CRH Symposium 16th-18th August 2017. (poster)

P Tucker, **A Kermack,** S Wellstead, P Calder, F Houghton, N Macklon. A Prospective and Randomised Trial Comparing Embryo Development in the MINC Incubator Versus the EmbryoScope Incubator. European Society of Human Reproduction and Embryology 2nd July – 5th July 2017. (poster)

A Kermack, P Lowen, S Wellstead, M Montag, F Houghton, P Calder, N Macklon. PREPARE trial: A randomised double blinded controlled trial of a preconception Omega 3 and Vitamin D rich dietary supplement in couples undergoing assisted reproduction treatment. European Society of Human Reproduction and Embryology 2nd July – 5th July 2017. (poster talk)

A Kermack, P Lowen, S Wellstead, P Calder, F Houghton, N Macklon. A Prospective Randomised Trial Comparing Embryo Development in the MINC Incubator Versus the EmbryoScope

Incubator. University of Southampton Postgraduate Medicine Conference 14th-15th June 2017. (poster)

A Kermack, P Lowen, S Wellstead, H Fisk, M Montag, F Houghton, P Calder, N Macklon. PREPARE trial: A randomised double blinded controlled trial of a preconception Omega 3 and Vitamin D rich dietary supplement in couples undergoing assisted reproduction treatment. University of Southampton Postgraduate Medicine Conference 14th-15th June 2017. (oral)

A Kermack, P Kirkham, W Lawrence, F Houghton, P Calder, NS Macklon, M Barker. Participating in a trial of dietary supplements six weeks prior to IVF: The patients' perspective. Annual Academic Meeting at RCOG 2nd-3rd March 2017. (poster)

A Kermack, Y Cheong, N Brook, N Macklon, F Houghton. Investigation of the amino acid composition of human uterine fluid: effect of age, body composition and diet. Institute for Life Sciences Conference 17th September 2014. (poster)

A Kermack, Y Cheong, N Brook, N Macklon, F Houghton. Effect of maternal diet on the amino acid composition of human uterine fluid**.** World Congress of Reproductive Biology 2nd-4th September 2014. (poster)

A Kermack, S Finn, Y Cheong, N Brook, J Eckert, N Macklon, F Houghton. Analysis of the amino acid content of human uterine fluid and the effect of female gynaecological pathology, age, body composition and diet. European Society of Human Reproduction and Embryology 30th June – 2nd July 2014. (oral)

A Kermack, N Macklon. Vitamin supplement usage in 400 women embarking on in vitro fertilisation treatment. British Fertility Society conference 8th-9th January 2014. (poster)

BOOK CHAPTERS

A Kermack, B Ng, N Macklon. Preconception Lifestyle Modification of Decidua to Decrease Early Pregnancy Loss: Feeding the Endometrium in the book How to Improve Preconception Health to Maximise IVF Success (edited by Robert Norman and Gab Kovacs).

PRIZES

"Rising star" Junior Doctors Award at University Hospital Southampton NHS Foundation Trust (2018)

Highly commended for poster presentation at Southampton University Postgraduate Medicine Conference (2017)

BMC Womens Health. 2014 Nov 18;14:130. doi: 10.1186/1472-6874-14-130.

A randomised controlled trial of a preconceptional dietary intervention in women undergoing IVF treatment (PREPARE trial).

Kermack AJ[1], Calder PC, Houghton FD, Godfrey KM, Macklon NS.

Author information

Abstract

BACKGROUND: In vitro fertilisation (IVF) treatment provides an opportunity to study early developmental responses to periconceptional dietary interventions. Retrospective studies have suggested links between preconception diet and fertility, and more recently, a "Mediterranean" diet has been reported to increase pregnancy rates by up to 40%. In addition, a prospective study examining increased intake of omega-3 polyunsaturated fats demonstrated a quickened rate of embryo development after IVF. However, up to now, few prospective randomised controlled trials have investigated the impact of periconceptional dietary interventions on fertility outcomes.

METHODS AND DESIGN: The study is a randomised controlled trial of a dietary intervention consisting of olive oil for cooking, an olive oil based spread, and a daily supplement drink enriched with Vitamin D (10 microgram daily) and marine omega-3 fatty acids (2 g daily) for 6 weeks preconception versus a control diet of sunflower seed oil for cooking, a sunflower oil based spread, and a daily supplement drink without added Vitamin D or marine omega-3 fatty acids. Couples undergoing IVF will be randomised to either the intervention or control group (55 in each arm). The primary endpoint is embryo developmental competency in vitro, measured by validated morphokinetic markers. Secondary outcomes will include the effect of the dietary intervention on the nutritional content of the intrauterine environment.

DISCUSSION: This approach will enable rigorous examination of the impact of the dietary intervention on early embryo development, together with the influence of the peri-implantation intra-uterine nutritional environment.

TRIAL REGISTRATION: ISRCTN50956936.

PMID: 25407227 PMCID: PMC4289275 DOI: 10.1186/1472-6874-14-130

[Indexed for MEDLINE] Free PMC Article

Hum Reprod. 2015 Apr;30(4):917-24. doi: 10.1093/humrep/dev008. Epub 2015 Feb 18.

Amino acid composition of human uterine fluid: association with age, lifestyle and gynaecological pathology.

Kermack AJ[1], Finn-Sell S[2], Cheong YC[3], Brook N[4], Eckert JJ[2], Macklon NS[5], Houghton FD[6].

Author information

Abstract

STUDY QUESTION: Do the amino acid levels of human uterine fluid vary with age, BMI, phase of menstrual cycle, benign pathology or diet?

SUMMARY ANSWER: The levels of 18 amino acids in human uterine fluid were shown to be affected only by maternal diet.

WHAT IS KNOWN ALREADY: Murine, bovine and ovine uterine amino acid content has been reported, but no reliable data on the human exist. Murine studies have demonstrated that the intrauterine periconceptional nutritional environment is affected by maternal diet.

STUDY DESIGN, SIZE, DURATION: Uterine secretions were aspirated from 56 women aged 18-45 years. The women were recruited preoperatively from gynaecological theatre operating schedules or hysterosalpingo-contrast-sonography (HyCoSy) lists. A proportion of these women had proven fertility; however, the majority were being investigated for subfertility. The BMI, gynaecological history and dietary pattern of these women were also assessed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Reverse phase high performance liquid chromatography was used to analyse the concentrations of 18 amino acids within the uterine fluid and blood serum. The results were analysed against the women's stage of cycle, age, BMI and diet.

MAIN RESULTS AND THE ROLE OF CHANCE: The profile of 18 amino acids in uterine fluid was described. In total, human uterine fluid was observed to contain an amino acid concentration of 3.54 mM (interquartile range: 2.27-6.24 mM). The relative concentrations of 18 amino acids were not significantly altered by age, BMI, cycle phase or the presence of specific benign gynaecological pathologies. However, a diet identified by a validated scoring system as being less healthy was associated with higher concentrations of asparagine ($P = 0.018$), histidine ($P = 0.011$), serine ($P = 0.033$), glutamine ($P = 0.049$), valine ($P = 0.025$), phenylalanine ($P = 0.019$), isoleucine ($P = 0.025$) and leucine ($P = 0.043$) in the uterine fluid compared with a healthier diet, defined as one with a higher intake of fresh vegetables, fruit, whole-grain products and fish and a low intake of red and processed meat and high fat dairy products. There were no significant correlations between serum amino acid concentrations and those in the uterine fluid.

LIMITATIONS, REASONS FOR CAUTION: Our results enabled us to detect the effect of diet on the concentrations of amino acids in human uterine fluid; however, the study may not have had sufficient numbers to detect mild effects of BMI or age.

WIDER IMPLICATIONS OF THE FINDINGS: These findings increase our understanding of the nutritional environment encountered by the preimplantation embryo, and indicate how periconceptional diet may alter this. Given the importance of early embryo environment for programming of development and future health, this information may aid in the development of nutritional interventions aimed at optimizing the preimplantation phase of human embryo development in vivo.

STUDY FUNDING/COMPETING INTERESTS: This work was funded by the NIHR, the Medical Research Council (G0701153) and the University of Southampton and was supported by the NIHR BRC in Nutrition and Southampton University NHS Foundation Trust. The authors declare no conflicts of interest.

KEYWORDS: BMI; amino acids; diet; human uterine fluid; menstrual cycle

PMID: 25697730 PMCID: PMC4359399 DOI: 10.1093/humrep/dev008

Minerva Ginecol. 2013 Jun;65(3):253-69.

Preconception care and fertility.

Kermack AJ[1], Macklon N.

Author information

Abstract

Preconceptional health has been shown to be an important determinant of fertility, fecundity and perinatal outcomes. In recent years the impact of periconceptional factors on developmental programming, and the health of the resultant child have become increasingly clear. Since fertility specialists care for couples during this critical phase, they have a unique opportunity to collaborate with the couple to optimise preconceptional health and thus fertility and pregnancy outcomes. In this review article, the current evidence available for the importance of preconceptional health care is considered, specific lifestyle and dietary interventions are described and the care of the medically complicated patient prior to fertility treatment is discussed. Finally strategies for overcoming challenges in implementing preconceptional care into the fertility clinic are addressed.

PMID: 23689168

[Indexed for MEDLINE]

Reprod Fertil Dev. 2015 May;27(4):677-83. doi: 10.1071/RD14304.

Nutritional supplementation and artificial reproductive technique (ART) outcomes.

Kermack AJ[1], Macklon NS[2].

Author information

Abstract

Approximately one in six couples suffer from subfertility, and many seek treatment with artificial reproductive technologies (ART). Despite improvements in laboratory techniques and ovarian stimulation, ongoing pregnancy rates per cycle remain at ~25%. Couples wanting to improve their chances may turn to adjuvant therapies, such as nutritional supplements. There is growing evidence that periconceptional nutritional status is a key determinant of fertility and long-term health of the offspring, and a lucrative market has developed to meet the demand based on these benefits. However, the practice of routine dietary supplementation before and during IVF treatment has not been subject to well-powered prospective randomised trials. In this article, the potential roles of specific nutritional supplements in the context of improving IVF outcomes are reviewed and an assessment is made of the evidence base supporting their clinical use in this context. Finally, current research needs in the field are outlined.

PMID: 25846211 DOI: 10.1071/RD14304

[Indexed for MEDLINE]

J Dev Orig Health Dis. 2015 Oct;6(5):415-24. doi: 10.1017/S2040174415001324.

The 'Developmental Origins' Hypothesis: relevance to the obstetrician and gynecologist.

Kermack AJ[1], Van Rijn BB[2], Houghton FD[1], Calder PC[2], Cameron IT[2], Macklon NS[2].

Author information

Abstract

The recognition of 'fetal origins of adult disease' has placed new responsibilities on the obstetrician, as antenatal care is no longer simply about ensuring good perinatal outcomes, but also needs to plan for optimal long-term health for mother and baby. Recently, it has become clear that the intrauterine environment has a broad and long-lasting impact, influencing fetal and childhood growth and development as well as future cardiovascular health, non-communicable disease risk and fertility. This article looks specifically at the importance of the developmental origins of ovarian reserve and ageing, the role of the placenta and maternal nutrition before and during pregnancy. It also reviews recent insights in developmental medicine of relevance to the obstetrician, and outlines emerging evidence supporting a proactive clinical approach to optimizing periconceptional as well as antenatal care aimed to protect newborns against long-term disease susceptibility.

KEYWORDS: developmental origins; ovary; placenta; pregnancy

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