Metabolite profiles: implications for fertility and treatment

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Abstract

Childlessness is a life crisis with impaired fertility affecting 1 in 7 couples globally. Since the introduction of in vitro fertilisation in 1978, the treatment of subfertility has evolved substantially to incorporate an advanced set of clinical diagnostic tests and novel stratified care pathways encompassing appropriate and effective treatment strategies. To accompany these technological developments, the continued elucidation of the contribution of the female and male preconceptual phenotype in determining clinical outcomes, and identification and influence of potentially modifiable biological pathways is critical.

In this thesis, I outline the development of an original prospective cohort of 400 women and their male partners undertaking in vitro fertilisation. For each participant, I performed extensive pre-treatment phenotyping, collected meticulous clinical and laboratory data, and undertook prolonged follow-up allowing ongoing pregnancies from both fresh and frozen embryo outcomes to be considered as clinical outcomes. In parallel, I collected biological samples enabling the creation of a unique comprehensive biobank encompassing pre-treatment serum, plasma, DNA, and vaginal swab samples for each participant.

Utilising a high-throughput NMR metabolomics platform, covering a wide range of metabolic pathways including lipoprotein lipids, fatty acids, amino acids, ketone bodies, and glycaemic traits which are highly relevant to cardiometabolic risk and overall health, I assessed the association of 155 metabolites with the ovarian reserve, semen parameters, the correlation between couples and the clinical outcome of treatment as defined by an ongoing pregnancy. I conduct a novel insight into the association of ovarian biomarkers with metabolic profiles conjointly with other recent studies. My results suggest that dyslipidaemia has a limited role in the relationship between ovarian reserve and cardiovascular diseases. Some fatty acids and amino acids may have a role in mediating an effect. My study provides preliminary data on a range of metabolic pathways and their association with semen parameters. I identified several metabolites associated with the odds of total motile sperm count. Higher levels of glycolysis metabolites

and ketone bodies were associated with increased odds of low total motile sperm counts. I have also explored within couples correlations of multiple metabolomic traits and found weak to modest positive correlations for the vast majority of traits that are known to be influenced by assortative mating or shared couple behaviours. This correlation suggests assortative mating and might have some potential weak to modest impact on couples having similar metabolic traits. After assessing the association of metabolite concentrations with ongoing pregnancy, I was the first to report a higher likelihood of ongoing pregnancy after IVF among both women and men with higher pre-conceptual histidine concentrations. For all these findings, confirmation with a larger number of participants to evaluate the potential clinical implications more fully is essential. My aspiration is that this unique cohort and accompanying biobank will be an important resource contributing to and enabling future collaborative and contemporary research efforts to provide novel insights and personalised approaches to fertility care.

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I am dedicating this thesis to my beloved children, who were patient, caring and understanding. They all enlighten my life with their unconditional love and support. When things are not going well, or when I'm stressed, they cheered me up and kept me going. To all my children, thanks for believing in me!

Author's Declaration

The research reported is my own original work which I carried out in collaboration with others as follows:

I drafted and obtained Ethics and Research Development approval, recruited all participants, collected and recorded all data relating to their pretreatment phenotype, the laboratory, clinical and pregnancy outcomes for both their ovarian stimulation cycles and frozen embryo transfer cycles, performed all biological sample collection and preparation. In conjunction with Professor Nelson and Professor Lawlor I developed the initial research questions, arranged the appropriate Material Transfer Agreements to enable the transfer of samples and measurement of the metabolites on the Nightingale Health's platform at the University of Bristol. The statistical analyses of the NMR data were undertaken in conjunction with Dr Amy Taylor and Dr Neil Goulding of the MRC Integrative Epidemiology Unit at the University of Bristol. All clinical and embryological procedures that constitute the components of an IVF cycle were undertaken by the Human Fertilisation and Embryology Authority licensed staff of NHS Greater Glasgow and Clyde.

I have had sole responsibility for the conduct of all aspects of the research presented within this thesis. Professor Scott Nelson and Professor Mary Ann Lumsden have reviewed drafts of this thesis.

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not previously been presented for a higher degree at the University of Glasgow or any other institution.

Karema Al Rashid, January 2021

Abbreviations

ADA	American Diabetes Association
AMH	Anti-Müllerian Hormone
AFC	Antral Follicle Count
ACS	Assisted Conception Service
ART	Assisted Reproductive Technology
ACE	Association of Clinical Embryologists
BCAA	Branch Chain Amino Acids
CRF	Case Report Form
COH	Controlled Ovarian Hyper-stimulation
CRP	C-Reactive Protein
COCs	Cumulus-Oocyte Complexes
DAG	Directed Acyclic Graphs
EDTA	Ethylenediamine Tetra-acetic Acid
FF	Follicular Fluid
FET	Frozen Embryo Transfer
HBP	High Blood Pressure
HDL	High Density Lipoprotein
HOMA	Homeostatic Model Assessment
hCG	human Chorionic Gonadotrophin
HFEA	Human Fertilisation Embryology Authority
ICH	International Council for Harmonisation
IVF	In Vitro Fertilisation
IRT	Inhibitor Removal Technology
ICSI	Intra-Cytoplasmic Sperm Injection
IQR	Inter-Quantile Range

IMCL	Intra-Myo-Cellular Lipids
IUI	Intra-Uterine Insemination
LC-MS	Liquid Chromatography–Mass Spectrometry
LoD	Limit of Detection
LoQ	Limit of Quantitation
LIPO	Lipoprotein
LMWM	Low-Molecular-Weight Metabolites
NHS	National Health Service
NEQAS	National External Quality Assessment Scheme
NEFA	Non-Esterified Fatty Acids
NMR	Nuclear Magnetic Resonance
PIS	Participant Information Sheet
PUFAs	Polyunsaturated Fatty Acids
PGT-M	Pre-implantation Genetic Testing for Monogenic disorders
PGT-SR	Pre-implantation Genetic Testing for Structural Rearrangements
R&D	Research & Development
SCNT	Somatic Cell Nuclear Transfer
SDF	Sperm DNA Fragmentation
SD	Standard Deviation
SNPs	Single Nucleotide Polymorphism
TLS	Time-Lapse System
TMSC	Total Motile Sperm Count
WHO	World Health Organisation

Chapter 1 Introduction

Fertility is a key element of reproductive health and infertility is recognized as a global public health issue by the World Health Organisation (WHO) (Boivin et al., 2007, Macaluso et al., 2010). Infertility is defined by the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and WHO as the 'failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse'(Zegers-Hochschild et al., 2009). The experience of infertility can cause those affected personal distress (Schneider and Forthofer, 2005), significant treatment costs (Bell, 2010) and in some contexts, ostracism and discrimination (Cui, 2010). The significant medical, social and demographic consequences of infertility have led to its widespread awareness.

Infertility may be considered a characteristic of the couple, with female or male factors implicated, or in some cases, both. Some risk factors such as age and lifestyle factors have been the topic of considerable epidemiological research. However, other risk factors including maternal and paternal metabolism have received little investigation, with the majority of research focusing on novel therapies rather than considering assessing and identifying potential baseline determinants of treatment outcomes. This lack of attention has continued despite increasing recognition of pre-treatment maternal and paternal phenotypes as critical factors in determining clinical outcomes. Even in the context of single or heavily sponsored multicentre clinical trials, Biobanks are rarely established, limiting the potential for discovery-based science even when the trial findings are null.

This thesis describes the establishment of a cohort of couples undertaking IVF in a single centre, with an accompanying comprehensive Biobank. Thereby enabling the analytical investigation of the role of the circulating maternal and paternal metabolite profile on both the pre-treatment phenotype but also clinical outcomes, while also establishing a future resource for researchers for discovery or replication focused studies. Each results chapter has its own pertinent introduction and discussion, consequently this overall introduction is used to set the scene of the global issue of infertility, its assessment, management and the

theoretical potential for the baseline phenotype to be a critical determinant of both immediate and long-term outcomes.

1.1 Infertility – a global problem

Infertility or the inability to conceive remains a problem of global proportions. In the third decade of the new millennium, six demographic realities regarding infertility treatment remain salient, and are summarised in Table 1.1, with the first and last particularly relevant to this thesis. The first demographic reality is that millions of people around the globe continue to suffer from infertility. The totality of the worldwide population of infertile couples is extremely difficult to ascertain because of (i) the heterogeneity in the criteria used to define infertility (e.g. 1 versus 2 versus 5 years of trying); (ii) the critical differences between estimates of infertility based on large-scale population surveys versus epidemiological studies of infertility and (iii) whether infertility is defined as being located in 'women', 'couples', 'people' or 'individuals', units of analysis that are often used interchangeably or without precision (Gurunath et al., 2011, Mascarenhas et al., 2012b).

Demographic surveys have provided varying estimates (Boivin et al., 2007, Mascarenhas et al., 2012a), with the most recent providing a global examination of infertility trends based on analysis of 277 reproductive and health surveys from 190 countries and territories during the period of 1990 to 2010 (Mascarenhas et al., 2012a). In this study rather than use the WHO's clinical or epidemiological definitions (i.e. absence of conception after 1 or 2 years of trying, respectively), primary infertility was defined as the 'inability to have any live birth' and secondary infertility as the 'inability to have an additional live birth'. The primary outcome assessed was a live birth over a 5-year exposure period, based on the couple being in a stable union, neither used contraception and that there was a clear desire for a child. Using this demographically based definition of infertility, the study estimated that 48.5 million couples were affected by infertility in 2010 – considerably lower than previous reports (Boivin et al., 2007, Mascarenhas et al., 2012a). However, by reducing the time-frame from 5 to 2 years would be associated with an increase in the total number of infertile couples of 2.5-fold to 121 million (Mascarenhas et al., 2012a). Within the UK it is estimated that 1 in 7 couples are affected by infertility (NICE 2013), with little more than half of women

and men who experienced infertility seeking medical or professional help (Datta et al., 2016, Mills et al., 2011).

Demographic Reality
1. Millions of people around the globe suffer from infertility
2. Women in many low-resource settings continue to suffer from high rates of secondary infertility
3. Africa continues to suffer from inordinately high rates of infertility
4. High rates of infertility coexist with high rates of fertility in Africa—a demographic paradox known as 'barrenness amid plenty'
5. Lack of infertility prevention and treatment services is often justified as a form of population control, particularly in high-fertility settings such as sub-Saharan Africa
6. Those parts of the world with the highest rates of infertility are least likely to offer reliable diagnosis and treatment, including IVF services

Table 1.1 Global infertility: six demographic realities.

Despite the differing estimates of global infertility prevalence, and the lack of information on the total number of infertile men, who contribute substantially to the cases of childlessness, infertility rates are only slowly increasing over the preceding decades as compared to other non-communicable diseases (Mascarenhas et al., 2012b). A Global Burden of Disease survey reported that between 1990 and 2017, the age-standardised prevalence of infertility increased annually by 0·37% in women and by 0·29% in men (Sun et al., 2019). This relatively small increase reflects that global fertility rates have dropped significantly – i.e. fewer people are trying to have children as population growth has slowed (Mascarenhas et al., 2012a). Accompanying this decline in intended family size is the competing / opposing risk factor of an increase in the anticipated age at which couples wish to have a family, with a marked trend for delaying the timing of first birth in both developed and more recently developing countries (Rosero-Bixby et al., 2009). The inevitable consequence of this postponement of parenthood is the potential for impaired fertility and the risk that prolonged deferral of parenthood will rule it out even as a possibility with respect to natural conception or the use of autologous oocytes (Leridon, 2008). It is therefore likely that the prevalence of

infertility will continue to rise albeit slowly, with the concomitant need for increased interventions across society.

The other pertinent demographic reality relates to access to infertility services. The limited access to in vitro fertilisation (IVF) and related ART can be considered as a global reproductive health disparity (Jain, 2006). Those parts of the world with the highest rates of infertility are least likely to have reliable diagnosis and treatment, including IVF services (Adamson et al., 2018). This is particularly true in sub-Saharan Africa (Ombelet and Onofre, 2019), where tubal infertility dominates and despite IVF being originally developed to overcome blocked fallopian tubes, these nations are the least likely to be served by IVF clinics (Giwa-Osagie, 2002). Even within developed countries access to IVF is not equitable for couples with primary infertility, with fertility treatment provision not routinely mandated by all insurance providers or state funders (Bitler and Schmidt, 2012). Furthermore, secondary infertility is largely not addressed by state providers, except in a few countries such as Belgium, where a lifetime approach to reproductive health is taken and reimbursement of six IVF cycles per patient in a lifetime is available to ensure minimal financial burden to the patient (Ombelet et al., 2005). Interventions which could reduce the prevalence of infertility and / or reduce the need for more advanced therapies would be welcome additions to the public health armamentarium in its battle to reduce the global burden of infertility

1.2 Male infertility

The cause of infertility lies solely with the man in 20–30% of cases and a male cause is contributory in a further 20% (Thonneau et al., 1991). A large meta-analysis by Carlsen and colleagues in 1992, initially highlighted concerns regarding declining sperm counts with the suggestion that sperm counts had declined by 50% during a 60-year period (Carlsen et al., 1992). Subsequently, numerous studies have shown similar declines globally (Mishra et al., 2018, Swan et al., 2000), and although some studies have disputed this claim (Ravanos et al., 2018), there is now a large body of evidence that supports a reduction in sperm counts of 50–60% between 1973 and 2011, and an overall increase in the burden of infertility that is attributable to the male (Levine et al., 2017).

The multitude of causes and risk factors contributing to the overall increase on the incidence of male infertility (Krausz, 2011, Unuane et al., 2011), can be principally considered as congenital, acquired, and idiopathic. The primary genetic causes of male infertility are congenital bilateral absence of the vas deferens secondary to cystic fibrosis gene mutations, Kallmann syndrome, chromosomal abnormalities leading to deterioration of testicular function, and Y chromosome microdeletions resulting in isolated spermatogenic defects. Among acquired factors, varicocele is the most common and correctable cause of infertility in men, with a prevalence of 40% (Damsgaard et al., 2016). In addition to this various childhood conditions (e.g., cryptorchidism, postpubertal mumps orchitis, and testicular torsion or trauma) can result in testicular atrophy or decreased semen quality (Lewis et al., 2009). Infections of the male urogenital tract (prostatitis, urethritis, epididymitis, and orchitis) may also be implicated (Salonia et al., 2020), as the prevalence of male urogenital tract infection was reported to be as high as 35% in a study of more than 4000 subfertile men (Henkel et al., 2007). While a cross-sectional study of 1689 men revealed that 20% of men with primary infertility had asymptomatic semen infections (Boeri et al., 2020). It is estimated that ~17% of men with infertility have erectile dysfunction, or premature ejaculation, or both (McCabe et al., 2016). Sexual disorders, including hypoactive sexual desire and an absence of sexual satisfaction (pleasure, positive feeling, and orgasm) are less common but may coexist with other classical causes (Lotti and Maggi, 2018). The psychological effects of sexual dysfunction and / or other pathological causes of male infertility can be overwhelming and when combined may have a substantial detrimental impact on overall fecundity.

In the absence of a demonstrable pathological cause, about 30–50% of male infertility cases are idiopathic. In these seminal oxidative stress may contribute, and abnormal levels as evidenced by increased sperm DNA damage, have been associated with altered semen characteristics (Agarwal et al., 2019b). Environmental or occupational exposure to toxic chemicals and various lifestyle factors (e.g., smoking (Sharma et al., 2016a), alcohol consumption (Ricci et al., 2017b), recreational drug use (Gundersen et al., 2015), obesity (Eisenberg et al., 2014a) and psychological stress (Durairajanayagam, 2018)) are also all potential risk factors for male infertility. The association between obesity and male infertility has been widely investigated as the global prevalence of obesity

continues to rise (Craig et al., 2017). Obesity-induced endocrine alterations that result in peripheral conversion of testosterone to oestrogen have been linked with reduced sperm concentrations (Alshahrani et al., 2016). Among the subsets of obesity, metabolically unhealthy obesity (i.e., with metabolic abnormalities such as diabetes, hypertension, dyslipidaemia, insulin resistance) is known to be a risk factor for erectile dysfunction, and the combination of erectile dysfunction and metabolically healthy obesity (i.e., without evidence of metabolic and cardiovascular disease) in men represents an early marker for future adverse metabolic consequences (Rastrelli et al., 2019).

1.2.1 Assessment of the male

Routine evaluation is recommended for couples who do not conceive naturally after at least 12 months of regular, unprotected sexual intercourse, or after 6 months for couples in which the female partner is older than 35 years. Inevitably evaluation and treatment may be considered prior to these broad time limits based on medical history or physical examination. For the male, initial evaluation is a reproductive history and at least one semen analysis (Salonia et al., 2020). At present laboratory evaluation is still required as home-based testing systems are primarily limited to assessment of sperm concentrations (Yu et al., 2018). The WHO Laboratory Manual for the Examination and Processing of Human Semen and Sperm–Cervical Mucus Interaction has been published since 1980, with the most recent manual released in 2010 (WHO, 2010).

The recommended cut-off values for semen parameters have evolved dramatically over the years (Table 1.2). The lower reference limits in the latest iteration shown in Table 1.2 are derived from the statistical analysis of the semen parameters of 1953 fertile men from around the world (Cooper et al., 2010). However, these reference limits have been criticised for not considering the female factor, high biological variation among individuals, and the absence of data from representative ethnic groups (Patel et al., 2018). Consequently, standard semen analysis has limited accuracy for determining male fertility potential or predicting reproductive success. In fact, interpreting semen analysis using WHO 2010 reference values resulted in samples being considered normal that would have been considered abnormal if using the 1999 manual.

	WHO manual 1st edn (1980)	WHO manual 2nd edn (1987)	WHO manual 3rd edn (1992)	WHO manual 4th edn (1999)	WHO manual 5th edn (2010)
Volume	-	≥2·0 mL	≥2·0 mL	≥2·0 mL	≥1·5 mL
Sperm concentration	20–200 × 10^6/mL	≥20×10^6/mL	≥20×10^6/mL	≥20×10^6/mL	≥15×10^6/mL
Total sperm count	≥40×10^6/mL	≥40×10^6/mL	≥40×10^6/mL	≥40×10^6/mL	≥39×10^6/mL
Sperm motility (% progressive)	≥60%	≥50%	≥50%	≥50%	≥32%
Sperm vitality (%)	ND	≥50%	≥75%	≥75%	≥58%
Sperm morphology (% normal)	≥80·5%†	≥50%	≥30%‡	≥15%§	≥4%

Table 1.2 The evolution of normal values for semen parameters from 1980 to 2010 across the first five editions of the *WHO Laboratory Manual for the Examination and Processing of Human Semen and Sperm-Cervical Mucus Interaction*
Data extracted from WHO manuals. ND=not defined. †Mean of fertile population. ‡Arbitrary value. §Value not defined but strict criteria and in-vitro fertilisation data suggest a 14% cutoff value.

Interpretation of a semen sample report is primarily referent to the individual categories presented in Table 1.2, with an overall judgement made on whether it is normal or not. Consistent with this overall view, analysis of the diagnostic potential of the cut-off values for each single value, suggested that single sperm parameters were of little prognostic value in differentiating men who were fertile from subfertile men, and that a combination of these parameters should be used to predict a man's fertility status (Ombelet et al., 1997). Notably sperm morphology, which is frequently not assessed within the UK - only 5% of UK

laboratories are compliant with the WHO recommendations (Riddell et al., 2005), exhibited the best area under the receiver operator characteristic curve (Ombelet et al., 1997). Semi and fully automated computer assisted sperm analysis systems have limited capacity to analyse sperm morphology, however their wider adoption would potentially ensure stricter adherence to quality control procedures and more accurate quantification of sperm numbers and motility (Mortimer et al., 2015). Unfortunately, at the time of sample collection for this thesis neither morphology nor computer assisted sperm analysis systems were in use at Glasgow Royal Infirmary.

Routine physical examination and hormonal investigation (follicle stimulating hormone and testosterone) are still considered by some to be part of the standard investigation for every male patient with infertility (Ring et al., 2016). Although international societies recommend limiting their use to particular groups of patients, including men with a sperm concentration below 5×10^6/mL or impaired sexual function, or if an endocrinopathy is suspected (EAU Medicine, 2015). However, endocrine testing is not routinely performed in many centres based solely on a reduced sperm count, with the validity of these guidelines for predicting hypogonadism challenged due to their low accuracy (Ventimiglia et al., 2016).

Testing for genetic abnormalities, is mainly targeted based on evidence of defective spermatogenesis, resulting in severe oligozoospermia or azoospermia and largely takes the form of karyotype, Y-microdeletions and CF status. (Lipshultz and Lamb, 2007) Although a wide range of genes have been linked to different male infertility phenotypes targeted panels have yet to be fully developed (Oud et al., 2019), rather the focus is on identifying karyotypic abnormalities which are the most common with a prevalence of 12–15% in azoospermia, 5% in severe oligozoospermia, as compared to less than 1% in men with normal semen analysis (Ravel et al., 2006).

Additional tests to evaluate specific functional aspects of spermatozoa have also been developed to augment baseline semen analysis. The necessity for these came to light after the emergence of in vitro fertilisation and intracytoplasmic sperm injection (ICSI). In conventional in vitro fertilisation, defective sperm–zona

interaction is the main reason for fertilisation failure. However, in the current era of ICSI, hemizona or acrosome function assays are no longer used in clinical practice, because the penetrating capability of sperm is bypassed by direct intracytoplasmic injection of the sperm. Therefore, greater emphasis has been placed on the assessment of sperm chromatin quality using sperm DNA fragmentation testing. Despite a substantial body of work implicating DNA damage in altered sperm function, the absence of strict standardisation and clear threshold values for diagnostic tests (Agarwal et al., 2019a), have deterred its wider application or recommendation by professional bodies (EAU Medicine, 2015). Additional alternative tests such as determination of seminal oxidative stress remain experimental and are not recommended pending validation of their utility in randomised controlled trials (Salonia et al., 2020).

1.2.2 Management of male infertility

The clinical management of male infertility is largely dependent on the cause and whether there is sperm albeit of a reduced concentration or quality in the ejaculate or if there is azoospermia, which can then be classified as pretesticular, testicular, or post-testicular (Agarwal et al., 2020). The overarching themes of the different clinical pathway are to improve sperm number and quality and obtain sufficient sperm to enable natural or assisted reproduction. For many men ICSI is the final step in their clinical journey.

In men with idiopathic infertility, treatment consists of assisted reproductive technology and / or empirical medical therapy, which includes lifestyle improvement and hormonal or non-hormonal therapy. Lifestyle modifications (e.g., weight loss, physical activity, and cessation of smoking) are important non-invasive measures (Salonia et al., 2020), and have been linked to improvements in sperm parameters (Ibañez-Perez et al., 2019, Guthauser et al., 2013, Prentki Santos et al., 2011).

Separate treatment pathways for the different causes of azoospermia exist but all with the primary purpose of ensuring adequate sperm for conception whether this be natural or assisted (Agarwal et al., 2020). Patients with obstructive azoospermia have several options, including epididymal or testicular sperm retrieval (two forms of surgical sperm retrieval) for intracytoplasmic sperm

injection or surgical reconstruction. For men with impaired spermatogenesis as the cause of their non-obstructive azoospermia, although sperm production in non-obstructive azoospermia is often inadequate to reach the ejaculate, the finding of heterogeneous patchy spermatogenesis on testicular biopsy, and demonstrable sperm within the testes in 60% of men with non-obstructive azoospermia, provide the rationale for sperm retrieval in the management of non-obstructive azoospermia (Schlegel, 1999). For those men where sperm retrieval or induction of spermatogenesis is unsuccessful the option of donor sperm insemination or adoption are the only viable options.

1.2.3 Future therapies and challenges for male fertility

The use of ICSI has substantially improved the ability of men with severe male factor infertility, to have their own biological children. Despite the success of this technique, the importance of the paternal contribution and the need to optimise preconceptual sperm function cannot be understated, as ICSI from men with severe oligoasthenoteratozoospermia and non-obstructive azoospermia have substantially poorer clinical outcomes when compared to sperm derived from normozoospermic men (Lee et al., 2009).

Advancements in omics technologies have facilitated improved diagnosis and management of male infertility at genetic, molecular, and cellular levels. Next-generation sequencing technologies, such as disease-targeted sequencing, whole exome and genome sequencing, and epigenetic analysis of sperm, will enable further elucidation of the genetic architecture of male infertility (Thirumavalavan et al., 2019). Discoveries on the role of small RNAs and microRNAs in epigenetic regulations, and their involvement in spermatogenesis and epididymal sperm maturation, will continue to expand current understanding of these processes (Hilz et al., 2016). Metabolic fingerprinting of seminal plasma is another promising area of research, especially in cases of idiopathic male infertility (Jafarzadeh et al., 2015). Analysis of the circulating metabolome referent to sperm function is a similarly another exciting area. However, at present studies using a scalable reproducible platform providing quantitative measures are limited. In the current thesis I detail how I have used one such approach, nuclear magnetic resonance (NMR) spectroscopy, to begin to assess whether the circulating metabolite profile is associated with sperm parameters and clinical outcomes.

1.3 Female infertility

Primary female subfertility refers to women who have never achieved a clinical pregnancy, whereas secondary female subfertility refers to women who have previously achieved a clinical pregnancy who cannot establish a subsequent clinical pregnancy (Zegers-Hochschild et al., 2017). These definitions are clinical definitions that are designed for the early detection and treatment of subfertility, as previously noted alternative definitions of infertility may be used for demographic or epidemiological purposes (Mascarenhas et al., 2012a).

In women several critical steps are involved in achieving a natural conception. In brief follicular development leading to oocyte release from the ovary requires a normal menstrual cycle, which is coordinated by a complex interplay of pituitary-derived and follicle-derived hormones. After an oocyte is released, it enters the fallopian tube where it can be fertilized by spermatozoa (sperm) that have travelled through the female genital tract. The fertilized oocyte develops as it moves through the fallopian tube into the uterine cavity, where the developing embryo implants in the endometrium. In women, subfertility can involve any of these processes, whereas in men as discussed above, subfertility centres around absent or inadequate sperm production.

Common causes of female subfertility include ovulatory dysfunction (such as ovarian insufficiency or polycystic ovary syndrome (PCOS)), damaged or blocked fallopian tubes, endometriosis and uterine fibroids. Ovarian insufficiency is a loss of function of the ovaries that reduces the chance of pregnancy in all women from ~35 years of age but occurs prematurely in some women. Blocked or damaged fallopian tubes can cause subfertility or an increased risk of tubal pregnancy (Shaw et al., 2010). Endometriosis can create an unfavourable pelvic environment (Horne and Saunders, 2019), whereas uterine fibroids can impede tubal transport, cause tubal obstruction or affect embryo implantation, any of which can lead to subfertility in women (Hur et al., 2019). In up to 30% of couples, no identifiable cause of subfertility can be found after completion of a standard investigation protocol, which would include tests of ovulation, tubal patency and semen analysis, all of which would be normal (Ray et al., 2012). These cases are commonly referred to as unexplained subfertility. In this thesis, I retain this term for consistency with the literature but with the proviso that unexplained does not

mean that subfertility has no underlying cause, rather, it means that the cause has not been identified through a conventional diagnostic work-up, however, age which has a critical factor in determining fecundity is not recognised as a diagnostic label for a cause of infertility (ESHRE Group, 2017).

1.3.1 Key risk factors for female subfertility

1.3.1.1 Age

The pre-eminent and best-established factors that affects fertility is female age (Eijkemans et al., 2014)(Figure 1.1). This risk factor is increasingly relevant as women worldwide delay childbearing and choose to have their first child at an older age. The impact of age is such that for women, the likelihood of pregnancy is stable from puberty to approximately 30 years of age, after which it declines in an accelerating rate until menopause, when the chance of pregnancy approaches zero (Broekmans et al., 2009). Concomitant with this reduction in natural fertility is an increase in the risk of miscarriage (Andersen et al., 2000) reflecting a decline in oocyte quality and increase in embryos with aneuploidies.

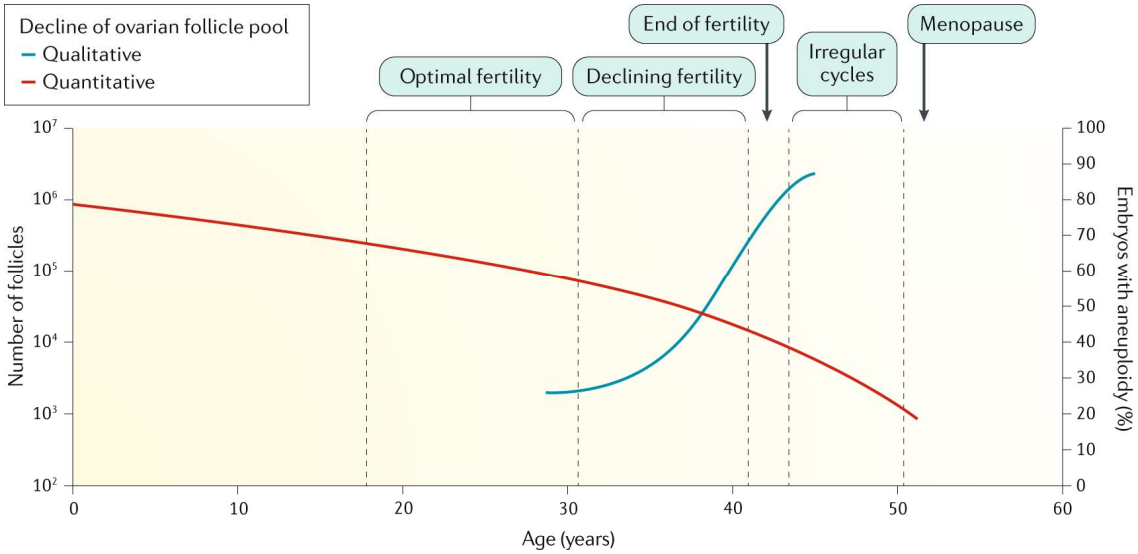


Figure 1.1 Reduction in female natural fertility with increasing age.
The reduction of the ovarian follicle or oocyte pool with increasing age likely dictates the onset of declining fertility, the transition from regular to irregular menstrual cycles and the onset of menopause. Ageing is associated with a logarithmic decline in the number of ovarian follicles and a simultaneous decrease in oocyte quality, the latter of which is reflected here by an increased percentage of embryos with chromosomal abnormalities / aneuploidies. Reproduced from (Farquhar et al., 2019).

As such chronological age is a strong predictor of the chance of pregnancy, in part through its association with the number of follicles present in the ovaries and that it reflects oocyte quality. At birth, girls have ~2 million oocytes in their ovaries, of which more than half are lost before puberty (Baker, 1963). Following the onset of ovulation, a cohort of 40–500 follicles develops each month, of which usually only one (the so-called dominant follicle) releases an oocyte (Hodgen, 1983). The other follicles degenerate and are ultimately lost through the process of follicular atresia. This overall pattern of decline in the number and quality of remaining oocytes with increasing age (Figure 1.1), leads to the age-related decline in female fertility.

The detrimental effect of increased age on oocyte quality and chance of pregnancy is further illustrated by pregnancy outcomes in women who receive donor oocytes, with oocytes from young donors having a substantially higher rate of subsequent pregnancy than oocytes from older donors, irrespective of the age of the recipient (Sunderam et al., 2018). Except for the use of donor oocytes, no other established interventions to prevent the age-related decline in fertility are available. The precise mechanism by which age affects oocyte quality is unknown. One hypothesis is an accumulation of DNA damage in oocytes over time through environmental stressors, for example, by certain food or drug residues or by reactive oxygen species (Harraan, 1955, Armstrong, 1986). Alternatively, a first-in-first-out principle (also known as a production line principle) has been proposed, whereby high-quality oocytes are primarily ovulated early in life, causing lower quality oocytes to be ovulated at later ages. This hypothesis is based on the decrease in the frequency of chiasmata (the physical crossover of homologous chromosomes) in mouse oocytes with increasing maternal age (Polani and Crolla, 1991, Henderson and Edwards, 1968). The reduction in chiasmata frequency could also explain the increased number of aneuploidies in oocytes, embryos and other products of conception in miscarriages in older women (Hassold and Hunt, 2001, van Echten-Arends et al., 2011, Nakagawa and FitzHarris, 2017).

1.3.1.2 Lifestyle factors

In observational studies, lifestyle factors such as smoking, excessive alcohol consumption and caffeine use have been consistently associated with reduced fertility (Hakim et al., 1998). Several additional negative lifestyle factors

significantly associated with current subfertility, including being overweight, unprotected sexual intercourse with multiple partners, stress and use of marijuana have also been identified as possible contributors (Bunting and Boivin, 2010). At present whether occupations involving exposure to reproductive toxins such as nitrosamines and formaldehyde affect fertility continue to be evaluated in prospective studies (Pak et al., 2013). These lifestyle factors are recognised in the NHS Scotland eligibility criteria for IVF and form the foundation for access to NHS funded IVF, with further details provided in Chapter 2 with discussion of the cohort eligibility.

1.3.2 Specific causes of female subfertility, initial evaluation and treatment

A comprehensive fertility assessment is designed to identify the underlying cause of subfertility and establish a prognosis. In the context of the recruited cohort for this thesis, all couples were already proceeding to IVF so only a brief summary of female causes, and diagnostic workup is provided here. In women the causes may be related to ovulatory dysfunction, tubal abnormalities ranging in severity from scarring and mild adhesions (fibrous bands between organs) to complete blockage or absence of the fallopian tubes, endometriosis, defects in the development of the Mullerian duct (which forms the cervix, uterus, fallopian tubes and part of the vagina), or other uterine abnormalities can be caused by fibroids, polyps or adenomyosis (in which endometrial tissue grows within the muscle walls of the uterus). A detailed medical history encompassing menstrual patterns, previous pregnancies, medical and surgical factors, lifestyle and family history is designed to point to these different causes and guide investigations.

Given the extensive range of causes, baseline investigations for subfertility primarily consist of tests to confirm ovulation and tests to identify structural abnormalities. A history of regular menstrual cycles is suggestive of satisfactory ovulation in most women, although a test to confirm ovulation such as a mid-luteal-phase progesterone or urinary LH estimation via self-testing is normally recommended, with the latter having a strong concordance with both ultrasound and serum hormone detected ovulation (Behre et al., 2000). Tubal patency tests are advised for suspected tubal diseases, with commonly performed tests including hysterosalpingography (HSG), hysterosalpingo contrast sonography

(HyCoSy) and diagnostic laparoscopy with chromotubation. Hormonal tests (for example, serum prolactin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels) are advised for suspected ovulatory dysfunction. For suspected uterine abnormalities, two- or three-dimensional ultrasonography is the first-line investigation.

Ovarian reserve tests can be used to identify women with diminished ovarian reserve and may identify women at high risk of compromised ovarian function owing to increased age (>35 years of age), a family history of early menopause, previous gonadotoxic therapy, ovarian surgery or idiopathic declines (La Marca et al., 2010). The currently available tests of the ovarian reserve essentially provide an estimate of ovarian responsiveness to exogenous stimulation i.e., the "functional ovarian reserve" and can be useful for planning the dose of ovarian stimulation in women who intend to use ART. Common tests of ovarian reserve include circulating levels of follicle stimulating hormone (FSH) and Anti-Müllerian Hormone (AMH), and the Antral Follicle Count (AFC).

Increased levels of serum FSH during the early follicular phase of the menstrual cycle are associated with reduced ovarian reserve. AMH is secreted by the granulosa cells of pre-antral and small antral follicles (<8 mm), and AMH concentrations decline with advancing age, mirroring the decline in primordial follicles (Hansen et al., 2011) Pretreatment baseline AMH concentrations are strongly positively correlated with oocyte yields in IVF cycles (Iliodromiti et al., 2015). The AFC is defined as the number of ovarian antral follicles (measuring 2–10 mm in diameter) in the early follicular phase and can be measured using transvaginal ultrasonography. An AFC of ≥20 per ovary when measured using a transvaginal transducer with a frequency of 8.0 MHz, is used to define polycystic ovarian morphology (Teede et al., 2018), whereas an AFC of ≤10 is suggestive of diminished ovarian reserve (Kline et al., 2005), and a very low AFC (3–6 follicles) strongly suggestive of a poor response during IVF (Hendriks et al., 2005). Although both AMH and AFC are good predictors of ovarian response to gonadotropin stimulation during IVF, they are generally poor at predicting pregnancy as they primarily reflect oocyte quantity rather than oocyte quality (Broer et al., 2013).

The initial management of women with subfertility requires explanation of any identified cause of subfertility, the provision of a realistic prognosis (with and without treatment), advice on management options (including non-intervention) and provision of information and support. For the women who have participated in this thesis, expectant management, induction of ovulation and / or intrauterine insemination, surgical interventions had all been unsuccesful, and so their rationale or biological basis is not discussed further, as all participants had moved on to receiving IVF.

1.4 In vitro fertilisation

IVF is a fertility treatment whereby mature oocytes are collected from the ovaries and fertilized by sperm outside the body and the resulting embryo(s) judged to have the best chance of pregnancy are introduced directly into the uterus. IVF is the mainstay of treatment for women with tubal disease and men with poor sperm quality but it is also frequently used for unexplained subfertility, or where other less invasive treatments have been unsuccesful. The success rates of IVF are broadly similar irrespective of the cause of subfertility, with the notable exception that unexplained infertility has been associated with marginally higher success rates (Nelson and Lawlor, 2011). It is however worth noting that reporting and comparing data on the outcomes of IVF is difficult, owing to a lack of a universally agreed way of reporting plus the inherent difficulties in accounting for differences between women (for example, age and length of subfertility) and treatment (for example, whether pre-implantation testing for aneuploidy (PGT-A) was undertaken and how many embryos were transferred). To further highlight this although data from >144,000 embryo transfer cycles in European centres in 2013 (including women with all causes of subfertility) showed a clinical pregnancy rate of 34.5% per embryo transfer, single embryos were transferred in only 31% of women, whereas two embryos were transferred in 56% of women, with the remaining 13% receving three or more (Consortium et al., 2017). In contrast in Australia and New Zealand, 87.7% of cycles transferred one embryo, and the overall clinical pregnancy rate was 33% per embryo transfer (Fitzgerald et al., 2017).

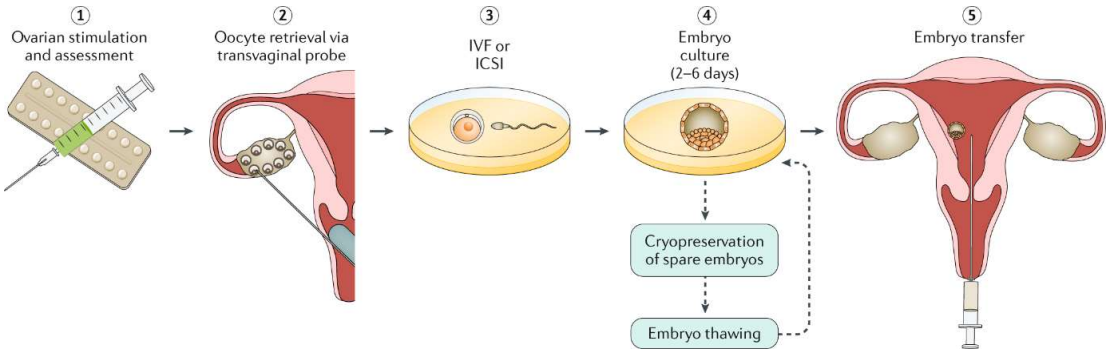


Figure 1.2 Summary of the five key steps in IVF.

The treatment pathway for IVF is complex, comprising many steps and decision points; each step has alternative clinical approaches, some of which are supported by robust evidence, whereas others require more research. In broad principal the five steps are shown in Figure 1.2. These include the administration of gonadotropins to stimulate the growth of ovarian follicles, and gonadotropin-releasing hormone (GnRH) agonists to suppress the natural menstrual cycle and down regulate the pituitary gland (step 1). GnRH antagonists are an alternative to GnRH agonists, as they are not associated with hypo-oestrogenic adverse effects, flare-up or a long down regulation period and can be used at any time during the follicular phase; both regimens have similar live birth rates (Farquhar et al., 2017). During the ovulation stimulation phase, regular monitoring using ultrasonography (with or without blood tests) is undertaken to assess the growth of the ovarian follicles and to avoid ovarian hyperstimulation (Kwan et al., 2014). When the follicles have reached an appropriate size, final maturation of the oocytes is induced (ovulation triggering). Human chorionic gonadotropin (hCG) is commonly used as a trigger but can negatively affect endometrial receptivity (Evans, 2020, Volvosky et al., 2018, Evans & Salamonsen, 2013) and embryo quality and increase rates of ovarian hyperstimulation syndrome (OHSS). An alternative is the agonist trigger, which is given if there is an increased risk of OHSS. Oocytes are collected, usually with a transvaginal ultrasonography probe for guidance, under conscious sedation (step 2). Oocytes and sperm are combined in a Petri dish for IVF, or alternatively, ICSI can be used when sperm concentration and motility are low (step 3). The fertilized oocyte, now called a preimplantation embryo, is cultured for 2–6 days in vitro (step 4). From the pool of available embryos, usually one embryo (although more can be transferred) is selected for transfer (step 5). Supernumerary embryos of good quality are cryopreserved by the process of

vitrification and can be subsequently thawed and transferred one-by-one if pregnancy was not established after the initial fresh embryo transfer or if all embryos were electively cryopreserved or if the couple desires an additional child. Drugs such as progesterone or rarely hCG are administered for luteal support to improve the likelihood of implantation and pregnancy and are usually stopped at 12 weeks of gestation (van der Linden et al., 2011). All of these individual variables were recorded in the preparation of this thesis and associated database, however, in chapter 6 I have focused on the principal outcome of ongoing pregnancy as defined by a fetal heart at 20 weeks gestation from the first embryo transfer whether it be a fresh or frozen embryo transfer cycle.

1.5 Impact of cardiometabolic health on reproductive outcomes in men and women

In men several studies have shown that men with lower quality semen — as indexed by concentration, count, motility — have an increased risk of mortality, chronic disease, and health service use in the future (Jensen et al., 2009, Latif et al., 2017, Eisenberg et al., 2014b), suggesting that semen quality may be an early biomarker of long-term health (Del Giudice et al., 2020). Of the observed associations with future-health and morbidity, the strongest associations are with the full range of cardiometabolic disease including hypertension, hyperlipidaemia, peripheral vascular complications, ischaemic heart disease and diabetes mellitus (Latif et al., 2017, Eisenberg et al., 2016), with little or no association observed with future mental health disorders like depression (Latif et al., 2017, Eisenberg et al., 2016).

The mechanistic link between male factor infertility and future adverse health remains uncertain, although several alternative potentially non-mutually exclusive explanations have been proposed. Firstly as approximately 10% of the male genome is involved in reproduction, it is likely that other nonprocreative processes may also be affected by aberrations in fertility (Matzuk and Lamb, 2008). Secondly, investigators have hypothesized that fetal exposures may lead to both adverse reproductive and somatic health in adulthood, with testicular dysgenesis and testicular cancer being the archetypal example of this (Barker, 1995). A third theory proposes a hormonal explanation. Men with infertility have lower circulating testeosterone levels than fertile men (Andersson et al., 2004).

Because hypogonadism is a risk factor for cardiovascular disease and mortality, such an explanation may link infertility to cardiovascular disease (Kay-Tee and Chir, 2007). Fourthly, although infertility may be a marker of diminished fitness, which may accelerate the development of impaired health in the future, it may also occur as a consequence of current ill health, whether it be clinical or subclinical (Salonia et al., 2009). As such, unrecognized preclinical chronic diseases may diminish male fertility and lead to impaired health later in life. Lastly unmeasured confounding may also contribute as the men who have participated in these studies (irrespective of whether through data linkage or prospective cohorts) have largely been identified through infertility clinics and potentially important covariates have typically not been collected. While there seems to be a consensus that selected risk factors for mortality — cigarette smoking (Sharma et al., 2016b), higher alcohol intake (Ricci et al., 2017a), and obesity or overweight (Sermondade et al., 2013) — are correlated with poorer semen quality, there is a paucity of evidence for many other characteristics, particularly biomarkers such as blood pressure, blood glucose and blood cholesterol, which have only been assessed in a few studies (Batty et al., 2019, Ma et al., 2020). Assessment of the association of metabolite profiles including lipoprotein lipids, fatty acids, amino acids, ketone bodies, and glycemic traits which are already known to be associated with cardiometabolic disease and long-term mortality (Deelen et al., 2019), would be of interest as it may identify a common mediation pathway and contribute further to the etiologic understanding of male factor infertility (Würtz et al., 2017).

In women, adverse pregnancy outcomes have long been recognised as a potential sentinel event for identifying those at increased risk of future cardiovascular disease (Bellamy et al., 2007). Women with history of hypertensive disorders of pregnancy (HDP) have approximately a 2-fold increased risk of cardiovascular disease compared with women with normotensive pregnancies (Brown et al., 2013, Wu et al., 2017). Reproductive risk factors are not however limited to the obstetric period. Common causes of secondary infertility, including polycystic ovary syndrome, premature ovarian insufficiency, endometriosis, and pelvic inflammatory disease, have all been linked to an increased risk of cardiovascular disease (Anderson et al., 2014, Mu et al., 2016, Liou et al., 2013). Also, early age at menarche, early menopause, and use of hormonal contraceptive agents are

associated with an increased risk of cardiovascular disease (Lidegaard et al., 2012).

Adverse pregnancy outcomes and cardiovascular disease share certain common (traditional) risk factors, including hypertension, hyperglycaemia, and obesity. It has been suggested that in women experiencing pre-eclampsia and the range of hypertensive disorders of pregnancy, the attributable excess risk of cardiovascular disease related to these conventional risk factors may be substantial (Haug et al., 2019). One cohort study suggested that blood pressure and body mass index alone were associated with up to 77% of the excess risk, while glucose and lipids were associated with smaller proportions (Haug et al., 2019). Others have previously found that only up to 15% of the risk of coronary heart disease in young women (aged <65) could not be accounted for by traditional risk factors (Khot et al., 2003). That women with hypertensive disorders of pregnancy already had more adiposity, higher blood pressure and glucose levels, and more adverse lipid levels before their first pregnancy (Magnussen et al., 2007), and that their cardiovascular risk factor levels remained higher than women without HDP through age 50 years and beyond (Haug et al., 2018), suggests a theoretical risk trajectory which is quite different from women without reproductive complications. It also suggests that the adverse risk profile precedes any index event or pregnancy and potentially continues to be abnormal thereafter. This divergent adult cardiovascular risk factor trajectory may reflect a combination of genetic, in-utero conditions, and different lifestyle patterns in childhood and adolescence that culminate in breaching a diagnostic threshold and manifesting clinical disease in adult life (Sattar and Greer, 2002).

Despite the wealth of data assessing the key events across a womens life course and there association with cardiovascular disease (Okoth et al., 2020), there has been limited assessment of the need for fertility treatment i.e. subfertility with long term cardiometabolic disease (Park et al., 2015). There is some suggestion that a history of subfertlity identifies a subpopulation at increased risk. Women who experienced some degree of infertility but eventually still conceive, there is evidence of a small increase in cardiovascular disease risk even after accounting for traditional cardiovascular risk factors (Parikh et al., 2012). There is also some weak evidence supporting an association of both miscarriage and recurrent

miscarriage with cardiovascular disease (Ranthe et al., 2013, Pell et al., 2004). However, receiving fertility treatment (ovulation induction, in vitro fertilisation, and intrauterine insemination with drug treatment) has not been associated with increasing the risk of developing a composite cardiovascular disease outcome or stroke as compared to infertile women not receving fertility treatment (Dayan et al., 2017). Collectively this suggests that it is the baseline pretreatment phenotype which is critical, and that the associated cardiometabolic profile may determine both the immediate IVF clinical outcome and risk of pregnancy complications but also the longer term risk of cardiovascular disease.

1.6 Summary

In conclusion, the preceding literature review has provided evidence regarding the magnitude of the problem of infertility, summarised the basic causes, investigations and treatment pathways of the infertile couple and tried to highlight the need for novel quality research regarding the baseline phenotype and its potential impact on male, female and couple outcomes. In addition to the continued efforts to improve established assisted conception techniques and radical new approaches including the use of stem cells to create oocytes or sperm, the creation of synthetic embryos, gene editing, prolonged embryo culture, artificial gametes, and the development of an artificial uterus, three overarching priorities for future research have recently been highlighted (Farquhar et al., 2019). These include addressing the preventable causes of subfertility, providing support and alternatives for individuals with subfertility and encouraging new initiatives to increase the global accessibility, affordability, and acceptability of assisted reproductive techniques. The establishment of a cohort of couples requiring IVF with a detailed and comprehensive biobank will contribute to two of these overall priorities. Similarly, clarification of the role of the metabolic profile in determining male and female fertility and clinical outcomes informs these priority areas, as identification of a baseline metabolic phenotype which is associated with important clinical outcomes may aid development of novel interventions or guide exploration of previously unconsidered biological pathways.

1.7 Overall aim and specific objectives

This thesis details my recruitment and development of a cohort of 400 women and their male partners undertaking IVF, with a detailed and comprehensive database detailing pretreatment, clinical and laboratory information and the establishment of an extensive accompanying Biobank of biological samples including maternal and paternal plasma, serum and DNA and isolated RNA from vaginal swabs suitable for future microbiome analysis. On completion of recruitment, I performed detailed metabolic profiling of both female and male participants using a high-throughput NMR metabolomics platform, covering a wide range of metabolic pathways including lipoprotein lipids, fatty acids, amino acids, ketone bodies, and glycemic traits, which are highly relevant to cardiometabolic risk and overall health. This has enabled me to address four specific objectives:

1. Determine the association of the functional ovarian reserve with the circulating metabolite profile.
2. To determine whether 155 circulating metabolic measures relevant to lifestyle and metabolic health are associated with sperm parameters (as measured by concentration, motility and total motile sperm count (TMSC)).
3. To assess the correlation within couples undergoing fertility treatment of physical, social and behaviour characteristics, and 155 circulating metabolic measures.
4. To establish whether in men and women undergoing IVF if preconceptual circulating metabolites are associated with ongoing pregnancy rates. While live-birth per cycle would be the preferred clinical outcome, this was not feasible due to time restraints and the prolonged follow-up that would be required for those women with multiple frozen embryos. However, it is increasingly recognised that ongoing pregnancy per cycle, is an appropriate surrogate particularly if ongoing pregnancy is defined at later gestations (e.g. 20 weeks as used in this thesis) due to the low fetal loss rates beyond this gestation (Mukherjee et al 2013).

Chapter 2 Material and Methods

2.1 Introduction

This chapter addresses the methods employed in recruiting volunteers for the research, the data collected from the participants and the measurements used to conduct the study. Through this thesis, multiple methodologies have been used and adapted to address the research questions. In broad terms, participants were prospectively recruited, baseline characteristics and medical history recorded, biological samples (blood samples and vaginal swab) collected and then comprehensive ovarian stimulation, embryology and clinical outcomes were gathered for each participant as they progressed through their treatment pathway. A unique comprehensive database was developed to record this information for all participants. Appropriate statistical analyses were developed for each individual research question.

This study was conducted with the ethical approval of Scotland A Research Ethical Committee (REC reference 16/WM/308) and Clinical Research & Development in NHS Greater Glasgow and Clyde (R&D reference GN16OG323). A research passport was granted from the Occupational Health Unit at the University of Glasgow to the principal investigator (KA). A copy of the ethical approval documentation is provided in Appendix I. Patient recruitment commenced in September 2016, with the last particiant recruited in June 2018, with data collection censored in January 2020. This allowed 18 months from recruitment of the last participant for completion of treatment prior to closure of the study.

2.2 Design, Setting and Participants

This was a prospective longitudinal cohort study of 731 participants, comprising of 400 women and 331 male partners, receiving assisted conception at Glasgow Royal Infirmary between September 2016 and January 2020. The study was wholly conducted by the Principal Investigator (KA), with oversight provided by the Chief Investigator (SN).

2.2.1 Setting

Participants were recruited from the assisted conception Unit at Glasgow Royal Infirmary, an NHS Scotland Assisted Conception Service providing quaternary level care to a regional population of 2,810,900 (Wikipedia, 2018). The service is licensed by the Human Fertilisation Embryology Authority (HFEA) for a range of assisted conception treatments including ovulation induction with oral agents and gonadotrophins, Intra-uterine Insemination (IUI), in vitro fertilisation (IVF), intra-cytoplasmic sperm injection (ICSI), frozen embryo transfer (FET), pre-implantation genetic testing for structural rearrangements (PGT-SR), pre-implantation genetic testing for monogenic disorders (PGT-M), pre-implantation genetic testing for aneuploidy (PGT-A) and storage of gametes and embryos. These services are provided under a combination of NHS and private funded treatments with approximately 1100 IVF/ICSI cycles and 500 FETs undertaken per annum.

NHS Scotland eligibility criteria for the provision of assisted conception services and in particular IVF/ICSI are as follows; infertility with an appropriate cause, of any duration, or unexplained infertility of two years for heterosexual couples or unexplained infertility following six cycles of donor insemination. Additional eligibility criteria include; neither partner to have undergone voluntary sterilisation or undertaken reversal of sterilisation. Couples must have been cohabiting in a stable relationship for a minimum of two years. Couples where only one partner has legal parenthood of a child (or a biological child) can access NHS funded treatment as long as all other access criteria are met in full. Same sex couples will not be eligible if they already have a child in the home and both have consented to legal parenthood of that child. There is a responsibility on patients to fulfil the following access criteria in the interests of the welfare of the child and the effectiveness of treatment. The female partner must have a BMI above 18.5 and below 30. Both partners must be non-smoking for at least three months before treatment and couples must continue to be non-smoking during treatment. Both partners must abstain from illegal and abusive substances. Both partners must be Methadone free for at least one year prior to treatment. Neither partner should drink alcohol prior to or during the period of treatment.

For NHS funding purposes one full cycle of IVF includes ovulation induction, egg retrieval, fertilisation, transfer of fresh embryos followed by the freezing of

suitable embryos and the subsequent replacement of these, provided the couple still fulfil the access criteria. If suitable embryos are frozen these should be transferred before the next stimulated treatment cycle. No individual (male or female) can access more than the number of NHS funded IVF treatment cycles supported by NHS Scotland under any circumstances, even if they are in a new relationship.

The number of cycles initiated by the date of the female's 40th birthday is up to three cycles of IVF/ICSI, where there is a reasonable expectation of a live birth. Clinical judgement is required to be applied to determine this, using an assessment of ovarian reserve before the first cycle. If there has been no or a poor response to ovarian stimulation (<3 eggs retrieved) in the first cycle no further IVF/ICSI treatment will be funded. Fresh treatment cycles must be initiated by the date of the female partner's 40th birthday and all subsequent frozen embryo transfers must be completed before the woman's 41st birthday.

For women aged 40 to 42 years old one cycle of treatment may be funded for couples if the female has never previously had IVF treatment, if there is no evidence of poor ovarian reserve and if in the treating clinician's view it is in the patients' interest. Eligible patients must be screened before the female partners 42nd birthday.

For frozen embryo transfers, should circumstances change and couples no longer meet the NHS eligibility criteria self-funding for any future transfers will be required.

For patients who have previously funded treatment NHS funding may be given to those patients who have previously paid for IVF treatment, if in the treating clinician's view, the individual clinical circumstances warrant further treatment.

Patients not meeting these criteria are eligible to self-fund treatment provided the female partner has a BMI ≤35kg/m^2 and the criteria for drug use are met.

2.2.2 Participants

I prospectively recruited 731 participants undergoing IVF/ICSI treatment. The final cohort comprised of 400 females and 331 male partners. The discordance in the number of female and male participants reflected that some female participants were receiving treatment as either single women or as a same-sex couple using donor sperm, the male partner did not accompany the female partner to the clinic, the male partner attended on a different day that conflicted with other patient's recruitment / pre-existing commitments, or in rare occasions, the male partner refused to participate. Detailed inclusion and exclusion criteria are provided below, but in brief all participants were greater than 18 years in age, were using autologous oocytes and were not participating in oocyte or embryo donation. Some couples were female same-sex couples and were using a sperm donor. Of the 399 female participants with complete metabolite data, 271 (67.9%) participants had primary infertility and 127 (31.8%) secondary infertility. The cause of infertility similarly varied, with 211 (52.9%) participants having unexplained infertility, 84 (21.1%) with female factor and 87 (21.8%) with male factor. Although this range of diversity may have contributed to potential heterogeneity between the participants and the associated metabolic profiles, adjusted and unadjusted analyses to assess any potential effects of infertility status.

2.2.3 Eligibility

All women and their partners from all races attending the assisted conception service unit at Glasgow Royal Infirmary for IVF/ICSI treatment were eligible for participation irrespective of whether they were NHS or privately funded. There was no upper age limit. The number of female participants recruited above the age of 40 was 34 (8.5%), reflecting the local clinical population. Patients receiving NHS funded treatment met the NHS Scotland criteria for funding as listed above.

2.2.3.1 Inclusion criteria

Undergoing IVF/ICSI treatment at Glasgow Royal Infirmary.

2.2.3.2 Exclusion criteria

Participants were excluded if ether partner was less than 18 years old or the participant required oocyte or embryo donation for their IVF/ICSI treatment. This

study includes a large number of participants, reflecting a potentially heterogenous clinic population. However, this inclusive approach ensured timely recruitment, a diverse population ensuring generalisability of my results and I undertook appropriate sensitivity, multivariate and univariate analyses to ensure the robustness of my findings.

2.3 Study documentation

All the documents used in the study were developed by the principal investigator (KA). Each participant received three documents. The Participant Information Sheet (PIS) (Appendix II) was the first document to be handed to the prospective participant, as it contained comprehensive details of the study, its purpose, aim and anticipated outcome. In addition, it contained the contact details of the chief investigator and the principal investigator. The second document was the informed consent (Appendix III) which acted as the contract between the principal investigator and the participant. By signing the informed consent, the participatant acknowledged that the study has been explained, they were aware of what was involved and that they consented to participate in in the study. The third document represented the baseline questionnaire (Appendix IV). This document asked the participant to provide the principal investigator with personal information including baseline lifestyle, medical, fertility and family history details. Case Report Form (CRF) (Appendix V) is the document where I the principal investigator (KA) recorded additional medical information for each participant by gaining access to the participant's medical record. All documents were created in two versions; male and female. All documents were approved by the Ethics panel and Research and Development prior to commencement of the study.

2.4 Recruitment process

The study and participant recruitment were designed to align with routine clinical care pathways to minimise disruption to patients and maximise recruitment. The majority of the patients treated within the ACS Unit were in receipt of NHS funding and thus were subject to NHS waiting list for assisted conception prior to commencing treatment. When patients are at the top of the waiting list for NHS treatment, they are routinely provided with three separate consecutive clinic visits prior to the commencement of ovarian stimulation. There are 2-3 weeks

between the first and the second visit. Whereas the third visit will be after 4-7 weeks depending on the patient's menstrual cycle. The principal purpose of these visits is to provide baseline health screening, completion of HFEA consents and blood sampling for blood borne-viruses respectively. This routine care pathway provided a logical sequence of clinics to engage with participants to provide information regarding the study, obtain informed consent and collect additional blood samples when undergoing routine phlebotomy. The participants were not required to attend any additional appointments other than their routine appointments within the ACS Unit. An overview of the routine care pathway, recruitment process and additional study-specific procedures is shown in Figure 2.1.

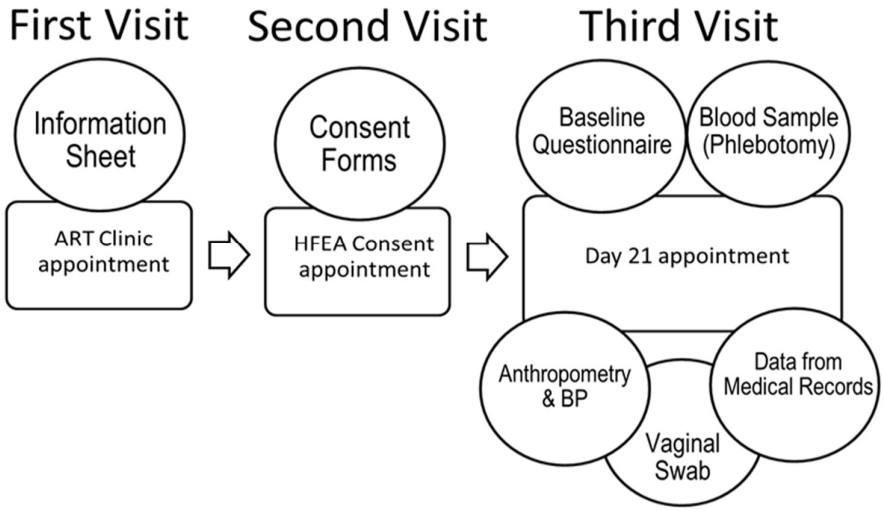


Figure 2.1 Flowchart illustrating the recruitment process
Routine care visits are contained within rectangles and the action by the principal investigator KA at each visit are shown in the circles.

At the first visit, all couples attending the ACS Unit to commence their IVF/ICSI treatment were provided with a Patient Information Sheet. The principal investigator (KA) discussed the study in detail with the potential participants and answered any questions they might have. The principal investigator assured the participants that they had the right to decline participation or withdraw from the study at any time, without any adverse on their clinical care.

At their second visit, prospective participants were asked if they wished to participate, and those who agreed completed and signed the consent form for

participation in the study. This enabled me to collect routine clinical data related to their treatment from their medical notes and perform study specific procedures. After obtaining the consent, both partners were asked to complete the baseline questionnaire, which took approximately 10 minutes to complete. After completing the baseline questionnaire, participant height, weight and blood pressure were measured as detailed below.

At the third and final visit, on approximately day 21 of the menstrual cycle prior to initiating ovarian stimulation, blood was routinely taken from both partners for assessment of blood borne-viruses (Hepatitis B, Hepatitis C and Human Immunodeficiency Virus (HIV)). At this visit, I took an additional aliquot of blood (24mL) for isolation of serum, plasma and DNA. In addition, a high vaginal swab was obtained for DNA extraction and future bacterial taxonomic assessment.

After this final visit there was no further direct interaction between myself and the participant, with all subsequent data collected from routine clinical records. The recruitment process took 21 months to be completed.

2.5 Development of the study-specific database

A comprehensive study-specific database was created by myself in Excel (Microsoft, Redmond, USA) to facilitate detailed data collection for each participant. This database was designed to enable recording of all lifestyle, family and personal medical history characteristics, baseline infertility assessment, ovarian stimulation details, embryological outcomes including time-lapse parameters for transferred embryos and clincial and pregnancy outcomes for both the fresh and frozen embryo transfers. In additon the database incorporated the Nuclear Magnetic Resonance (NMR) testing results. For each participant the final database contained more than 500 individual variables, with subsequent transfer to Stata v15.1 (College Station, Texas, USA) for statistical analyses.

2.5.1 Participant's baseline, personal and family medical history and lifestyle characteristics.

Each participant completed a questionnaire to provide general lifestyle data (Appendix IV). Full details of how these variables were categorised are provided

in the relevant methods of each results chapter. In brief these characteristics included their self-reported ethnicity, their marital status, their occupation, educational attainment, smoking status, alcohol consumption, the frequency and intensity of exercise per week. With respect to their own medical history participants were asked if they were currently receiving any medical care or taking any medication and specifically whether they have taken steroids within the last 2 years. They were also asked if they had suffered a prolonged illness or been hospitalized in the past, or had any major surgery. Participants were specifically questioned regarding a personal history of cardiometabolic conditions including heart attacks, strokes, high blood pressure, elevated cholesterol, diabetes, congenital heart disease, cardiac operations and obesity. Female participants were also asked regarding vaginal symptoms including vaginal discharge and itch, dyspareunia and whether they had ever sought medical advice for the symptoms and if so the diagnosis and treatment provided. Both male and female participants were asked regarding whether their sexual partner had symptoms of irritation burning or discharge and if so the details of this including diagnosis and treatment provided. With respect to family history they were asked whether first-degree relatives had experienced cardiometabolic conditions including heart attacks under the age of 50, strokes under the age of 50, hypertension, elevated cholesterol, diabetes, congenital heart disease, cardiac operations or obesity.

2.5.2 Additional details regarding the participant's medical history

As part of the consent the participant had signed, permission to access the participant's medical record in the NHS database was provided. Additional information regarding any operations, or past medical history of note was recorded.

2.6 Anthropometry and blood pressure

All participants male and female had baseline anthropometric and blood pressure measurements recorded.

2.6.1 Height

A stadiometer (Invicta Plastics Ltd, Leicester, UK) was used to measure the height of the participant with no shoes on, their feet joint at their heels and resting flat. The participant's buttocks and upper part of their body were positioned against a fixed surface and touching the stadiometer. The participants were instructed to straighten their back and take a deep breath in and the height was measured at the end of the exhalation by the PI lowering a moveable headboard on the top of the patient's head by applying a gentle pressure downward to allow hair compression. The measurement was taken to the nearest millimetre.

2.6.2 Weight and body mass index

The body weight of the participants was measured using calibrated scales (Marsden Ltd, Rotherham, UK), approved for medical and research purposes. Patients were weighted wearing lightweight clothing, barefoot, having both feet flat and arms in a lateral resting position. Measurements were taken to the nearest 0.1 kilogram.

Body Mass Index was calculated as weight / height^2 and recorded to one decimal place.

2.6.3 Blood pressure

Resting seated blood pressure was measured using an Omron automated monitor (Omron Healthcare Ltd, Milton Keynes, UK). Patients were asked to sit with their arm resting on a table, relax, not to cross their legs during the procedure and to stop talking. Two blood pressure recordings were taken two minutes apart and the average of the two blood pressure measurements recorded.

2.7 Creation of the Biobank

2.7.1 Blood sample collection

A non-fasting blood sample (total 24mls) was collected at the third visit by myself using venipuncture from an antecubital vein with the use of a vacutainer blood collection set (BD Vacutainer; Push Button Blood Collection Set, BD, New Jersey). Blood samples were collected in collection tubes (BD Vacutainer; Blood Collection Tubes, BD, New Jersey); four 4ml Ethylenediamine Tetra-acetic Acid (EDTA -

purple top) and two 4ml serum blood (yellow top). The collected sample tubes were inverted several times after collection and left on ice, or in the fridge at 4ºC if multiple participants were having venepuncture consecutively before being moved by the PI to the laboratory for sample preparation.

2.7.2 Sample preparation and storage

On transfer to the laboratory the blood samples were centrifuged at 2,000 G-force and 4-6 °C for 10 minutes. After centrifugation, the sample layers could be distinguished. For the EDTA tubes; a layer of clear fluid (the plasma), a layer of red fluid containing most of the red blood cells, and a thin layer in between, the buffy coat which contains most of the white blood cells and platelets (Figure 2.2 below). The plasma was aliquoted into multiple separate tubes each containg at least 350µl and the the separated buffy coat stored separately with each tube labelled with the participant unique ID. Serum samples were similarly aliquoted into labelled tubes. All aliquots were split between two -80 °C freezers and stored at -80 °C in the main laboratory at Level 3, New Lister Building (Glasgow Royal Infirmary) (each participant has 4 plasma aliquots and 2 serum aliquots).

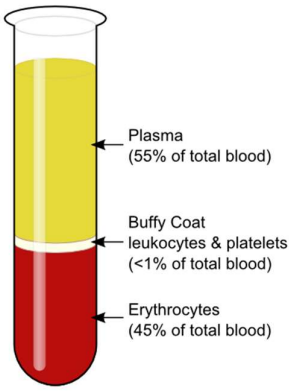


Figure 2.2 Illustration of the different layers of blood after centrifugation.

EDTA samples were centrifuged at 2,000 g for 10 minutes and the plasma and buffy coat layers stored separately.

2.8 Vaginal swab samples

The development of non-culture-dependent DNA sequencing has aided our general understanding of the composition of the microbial communities which inhabit the human body called "the microbiome". This understanding has led to the suggestion

that the balance of microbial species may greatly affect the health of the host, with disturbances in the composition of the bacterial communities, contributing to various disease states including infertility. As part of the development of a comprehensive Biobank we collected a vaginal swab sample from each participant, with isolation of the DNA and storage for future interrogation.

2.8.1 Collection procedure

The vaginal swab was collected using a Regular Nylon Flocked Swab which was individually wrapped to prevent contamination (Thermo Scientific, UK). The swab was taken by the participant herself in the third visit as it was a relatively straightforward procedure with guidance provided by the PI during the process. The PI encouraged the participant to wash her hands before and after the process. The patient was instructed to pull the cap with the attached swab off the tube and not to touch the soft tip or lay the swab down. Then, to hold the swab by the cap with one hand so the swab tip is pointing toward the participant and with the other hand, gently spread the skin outside the vagina to insert the tip of the swab into the vaginal opening pointing the tip toward her lower back with her muscles relaxed. The patient was advised to gently slide the swab no more than two inches into the vagina. If the swab does not slide easily, then the participant was instructed to gently rotate the swab as she pushed and to make sure the swab touched the walls of the vagina so that moisture was absorbed by the swab. Finally, the participant was asked to rotate the swab for 10 to 15 seconds before withdrawing the swab without touching the skin and place it in the tube and secure the cap. Figure 2.3 shows the process of the vaginal swab sample collection. The swabs were appropriately labelled after collection by the participant and stored in cold environment (i.e the clinic refrigerator), before being transferred later that day to the laboratory. 500µl of DNA free treated water was placed into a 2ml microtube and the end of the swab placed into it and agitated for 2 minutes before the swab was cut appropriately to facilitate secure closure and then stored in the assigned -80^{o}C freezer in the laboratory of Level 3 in the New Lister Building, Glasgow Royal Infirmary.

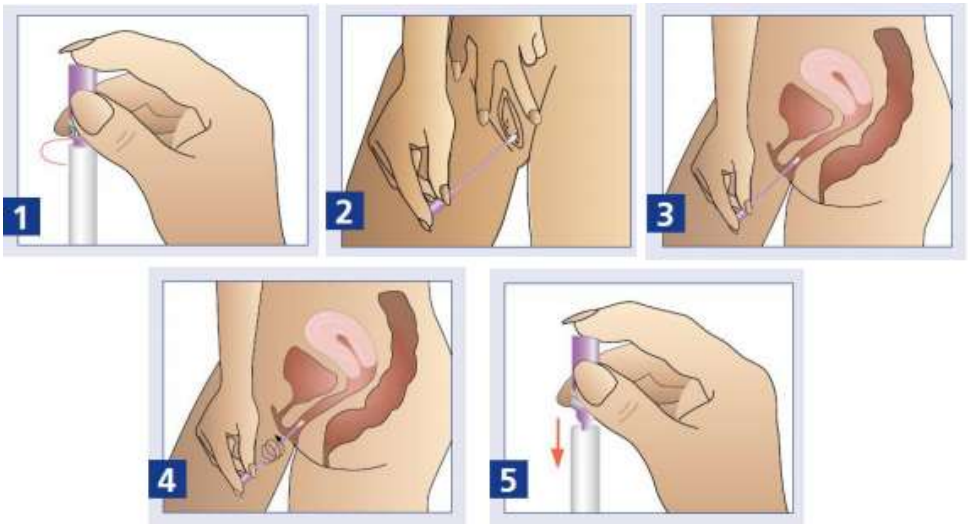


Figure 2.3 Illustration of the steps for vaginal swab self-collection.

Panel 1: Twist the cap to break the swab seal. Panel 2: gently spread the outer skin of the vagina and slowly insert the swab through the vagina. Panel 3: keep inserting the swab for no more than 2 inches approximately. Panel 4: Rotate the swab to collect the sample from the inner wall of the vagina. Panel 5: slowly retrieve the swab and immediately put it again into the cap and reseal it by twisting the swab with caution.

2.8.2 DNA extraction protocol

As there is the potential for DNA degadation with time, it was essential to perform DNA extraction within 6 weeks of sample collection. To ensure uniformity of storage conditions I selected three weeks after the sample was collected as an appropriate time to perform DNA extraction.

Several methods were initially compared for DNA extraction using pilot samples obtained from healthy volunteers. The optimal method based on DNA purity and quantity was the DNeasy PowerSoil kit (Qiagen, Germany), which was performed according to the manufacturers instructions. In brief I added 0.25g of the swab sample to the PowerBead Tubes which contained a proprietary buffer and then gently vortexed to mix. After that, 60µl of solution C1 was added and the tube vortexed briefly (Solution C1 contains SDS and additional disruption agents required for cell lysis). Next, the PI heated the mixture to 70°C for 5 minutes then vortexed it for 3-4 seconds. The PI used the fast preparation methodology with 24 devices treated concurrently for 60 seconds with setting 6, then rested for 2-3 minutes before repeating. After that, the tubes were centrifuged at 10,000g for 2

minutes at room temperature. A supernatant appeared within the tube and this was subsequently transferred to a clean 2ml collection tube. I added 250µl of solution C2 and vortexed for 5 seconds then incubated at 4°C for 5 minutes. After that, the tubes were centrifuged at room temperature for 1 minute at 10,000g. Avoiding the pellet, I transferred up to, but not more than, 600µl of supernatant to a clean 2ml collection tube. Then, a 200µl of solution C3 was added and vortexed briefly before it was incubated at 4°C for 5 minutes. Solution C3 is patented Inhibitor Removal Technology (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris and proteins. The tubes were centrifuged at 10,000g at room temperature for 1 minute. Avoiding the pellet, I transferred up to, but no more than, 750µl of supernatant into a clean 2ml collection tube. Then, 1200µl of solution C4 (a high-concentration salt solution) was added to the supernatant and vortexed for 5 seconds. Approximately 675µl was loaded onto a Spin column and centrifuged at 10,000g for 1 minute at room temperature, with the flow through discarded. This was repeated with an additional 675µl of supernatant added to the Spin column and centrifuged at 10,000g for 1 minute at room temperature. Next, the remaining supernatant loaded onto the Spin column and centrifuged at 10,000 g for 1 minute at room temperature. I then added 500µl of solution C5 (an ethanol based wash solution) and centrifuged the sampel at room temperature for 30 seconds at 10,000g and discarded the flow through, then centrifuged again at room temperature for 1 minute at 10,000g. After that, the Spin column was placed carefully in a clean 2ml collection tube while ensuring there was no splashing of any Solution C5 onto the Spin column. Then, 100µl of solution C6 (sterile elution buffer) was added to the centre of the white filter membrane and centrifuged at room temperature for 30 seconds at 10,000g. The final step was discarding the Spin Filter and by then, the DNA in the tube was ready for any downstream application.

I used a Nanodrop Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) to quantify and assess the purity of DNA extracted from each sample. This device performs full-spectrum UV-Vis absorbance analyses (220-750nm) and is designated for measuring the absorbance of DNA, RNA, microarray labelling dyes, and proteins. The advantages of the Nanodrop Spectrophotometer include the very small amount of the sample needed to be assessed (1µl), no

dilution needed for most samples and no cuvettes or capillaries. Figure 2.4 below shows two examples of DNA extraction from two different vaginal swab samples. Samples that has a 260/280 value greater than 1.6, a 260/230 ratio >2.0 and a concentration greater than 50 ng/uL were deemed acceptable.

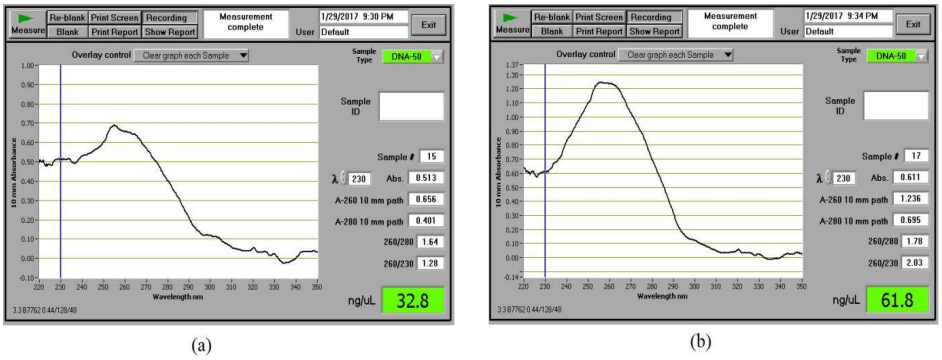


Figure 2.4 Nanodrop Spectrophotometer screenshots assessing purity and quantity of DNA derived from vaginal swabs.

Panel (a): The concentration is 32.8 ng/uL, the 260/280 value is 1.64, and the 260/230 value is 1.28. The 260/280 value, which indicated the contamination relative to other protein, was at low end of the acceptable range. In addition, the 260/230 value of 1.28 indicates potential contamination. The concentration is also low. Overall this sample was not acceptable.

Panel (b): The concentration is 61.8 ng/uL, the 260/280 value 1.78, and the 260/230 value 2.03. This was deemed an acceptable sample and suitable for DNA sequencing.

2.9 Clinical procedures

2.9.1. Clinical investigation

All patients underwent full clinical assessment to diagnose the cause of infertility prior to being placed on the waiting list for treatment. All blood tests (FSH, Oestradiol, LH, Prolactin, Thyroid hormones, Androgens, Progesterone, viral screen) and semen evaluation were performed by Clinical Pathology Accredited/ ISO15189 laboratories within 12 months of treatment starting. Tubal patency testing if undertaken was either by hysterosalpingogram, hysterosonography or laparoscopic hydrotubation as deemed appropriate by the referring clinician.

2.9.2 Anti-Müllerian Hormone Measurement

Anti-Müllerian Hormone serum concentrations were determined when the patient was at the top of the waiting list, (visit 1 of their routine care pathway), to

determine which ovarian stimulation protocol they would receive. AMH was measured on a random day of the menstrual cycle using the Beckman Coulter AMH automated method on a clinically validated immunoassay platform (Access 2, Beckman Coulter, USA). The assay was calibrated and quality controlled using the manufacturer's reagents and is known to have a measuring range of 0.08 – 24ng/ml (0.57 – 171 pmol/l). The limit of quantitation (LoQ) was 0.02 ng/ml (0.014pmo/l), with the 20% CV LoQ 0.08ng/ml (0.57pmol/l). The coefficient of variation between runs for two levels of control ran at <4.4%.

2.9.3. Antral Follicle Count Measurement

Antral Follicle Count was determined on day 2–4 of the menstrual cycle, prior to the patient commencing ovarian stimulation. Transvaginal ultrasound was performed by reproductive endocrinologists or nurses experienced in the measurement of AFC. AFC was defined as the sum of follicles 2–10 mm in diameter in both ovaries (Broekmans *et al.*, 2010)

2.9.4 Down-regulation, stimulation and follicle tracking

Stimulation protocols were based on plasma AMH for treatment naïve patients (Nelson *et al.*, 2009), and for those with previous treatment this was guided by previous ovarian response to gonadotrophin stimulation. The aim for all patients was to optimize the ovarian response and facilitate a fresh embryo transfer while maintaining a low risk of OHSS and minimal treatment burden.

Pituitary Regulation was achieved in one of two ways:
(a) Down regulation with agonist leuprorelin acetate 3.75mg (Prostap, Takeda UK Ltd, UK) was commenced on approximately day 21 of the cycle and topped up if required with buserelin (Suprecur®, supplied by Pharmasure) given at a dose of 0.5mg once a day, and continued throughout FSH administration;

(b) GnRH Antagonist; Cetrorelix acetate (Cetrotide® Merck Serono) was given as an additional injection during the time patients were being given stimulation drugs. Cetrorelix acts directly on the pituitary stopping its production of LH and FSH.

For those patients receiving down-regulation this was confirmed after 14 days by determination of serum oestradiol <50pg/ml or 180pmol/L and a transvaginal ultrasound of the uterus and ovaries (endometrial thickness, ovarian volume, number and size of follicles). If the endometrium was not less than 4mm or a cyst ≥10mm was observed (functional or not), the scan was repeated in one week. If the endometrium continued to be greater than 3mm or a cystic structure was seen on the ovaries (polyps ≥1cm are removed), then the stimulation cycle was delayed pending further investigation.

FSH stimulation (Menopur® Ferring or Gonal F® Serono) was started at a dose that was determined by the serum AMH and response in previous cycles (starting dose of 150IU, with increments of 75IU to a possible maximum of 450IU of FSH).

All patients were scanned by ultrasound on Day 6 or 8 of FSH administration at which time endometrial thickness was determined and the sizes of all ovarian follicles above 10mm were recorded. In addition, the number of follicles less than 10mm were recorded. Daily FSH administration was continued at the same dose, if follicular development was seen, and patients scanned at regular intervals. The daily dose of FSH was adjusted according to the total follicle number and size of the follicular cohort. Patients were only accepted for egg collection when there were three follicles ≥17mm in diameter on ultrasound, or there was no potential for further follicular development in anticipated poor responders. Thirty-six hours prior to oocyte retrieval, 6,500IU of human Chorionic Gonadotrophin (hCG; Ovitrelle® Merck) was administered. Patients stopped Buserelin injections or Cetrotide after the hCG was administered and started taking micronized progesterone (Cyclogest® Abbott) 400mg twice daily the evening of the oocyte retrieval. For those patients at risk of excessive response, the cycle was either cancelled if it was an agonist-controlled cycle or for those on antagonist protocols an agonist trigger was administered (0.5mg Buserelin) and the cycle segmented, with cryopreservation of all embryos.

2.9.5 Oocyte retrieval and sperm preparation

Oocyte retrieval was performed 36 hours after hCG injection under conscious sedation with a double channel 15-gauge needle. All follicles greater than 10mm were drained. Eggs were placed into commercial culture medium from Vitrolife

(G-MOPS, Vitrolife). Eggs were collected into tubes containing gassed G-MOPS and incubated at 6% CO2/ 5%O2/Bal N_2 at 37ºC for up to 4 hours prior to insemination or injection.

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

2.9.6 Insemination, injection and fertilisation

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

Where ICSI was indicated, oocytes were placed into 50ul droplet of cumulase (Origio) for 40 seconds, before being transferred to 50ul droplets of HEPES (QA). After 5 minutes, the cumulus and corona cells were removed by mechanical manipulation. Approximately 4 hours after retrieval, MII oocytes were loaded in individual 4ul droplets of HEPES surrounding a central droplet containing PVP (Origio) and 1ul of sperm. Injections were performed on an Integra Ti micromanipulator (Research Instruments) fitted with Hoffman Modulation Contrast System. Humagen micromanipulation tools were used to immobilise the oocyte with the polar body at the '6 o'clock' position and injection at '3 o'clock' position. Once injected, oocytes were cultured in QA cleavage medium in 4-well culture plates at 6% CO2/ 5% O2/ Bal N2 at 37ºC.

2.9.7 Embryo culture

Approximately 17 ± 1 hours after insemination or injection, oocytes were assessed for fertilisation and normally fertilised embryos were moved to G-TL media (Vitrolife) and placed within the EmbryoScope.

2.10 Time-lapse imaging

Time lapse technology has been introduced into human IVF in recent years, and has shown great potential in improving treatment outcomes, most likely due to the optimised embryo culture conditions via closed incubation and/or improved embryo selection based on morphokinetic information collected via continuous observations. The traditional approach for morphological assessment of human embryos is carried out at a low frequency (mostly 24 hour intervals or even longer) and the timing of these may not be consistent but rather reflect the workflow of the laboratory. The emergence of time-lapse imaging in the human IVF has further offered as opportunity to perform consistent temporal scoring, and more detailed developmental processes throughout the first days of development. All embryos cultured at Glasgow Royal infirmary are cultured within a time-lapse imaging system, EmbryoScope Time-Lapse System (TLS) (FertiliTech (Vetrolife), Denmark). Figure 2.5 illustrates the components of the EmbryoScope TLS.

The key component is the EmbryoScope Incubator which is an integrated benchtop incubator with the capacity of 6 patients holding 12 embryos in each culture dish. The EmbryoScope Incubator ensures a stable incubation environment while automatically taking high-quality images of the embryo development at pre-set intervals (10 minutes). These images are transferred to the EmbryoScope server, which is the second component of the time-lapse system. The EmbryoScope server will receive the images from the incubator and transformed the data into user-friendly formate to be accessed by the user via the EmbryoViewer software and hardware, which is the third and last component of the EmbryoScope TLS. The EmbryoViewer is the tool for embryo analysis and evaluation. The hardware consists of a thin client computer and computer workstation (high-resolution monitor with keyboard and mouse). Figure 2.6 shows some an example of the observed embryo development and how to distinguish between the embryos in each developmental stage.

Figure 2.5 The Embryoscope system.

This is a complete Time-Lapse System, including EmbryoScope Incubator, EmbryoScope Server and EmbryoViewer Software & Hardware. This system has been used in the study for observing the development of the embryos for each participant

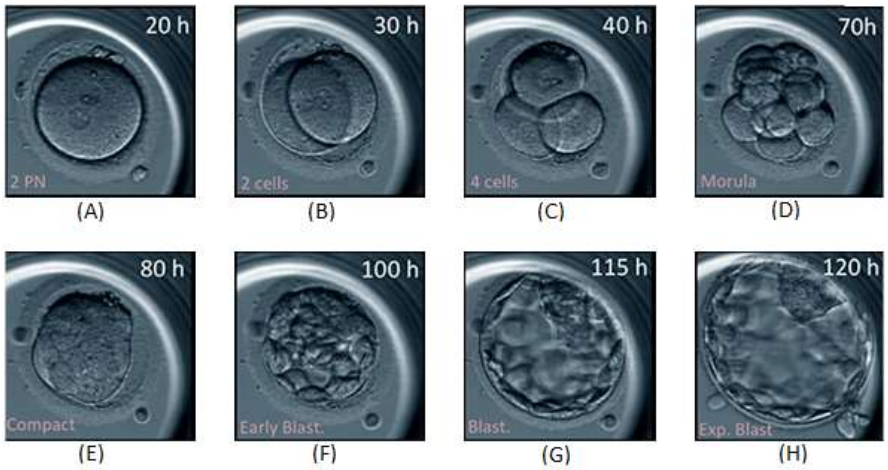


Figure 2.6 Screenshots of an embryo contained within the EmbryoScope.

The panels show the different embryo development stages along with the time the image was taken and the current development stage. A: The 2PN, which refers to two ProNucleus, is the first indication that an egg has become an embryo. As day1 passes, the pronuclei breakdown and cell division begin. B: The embryo has already started to divide. Blastomeres often divided evenly, thus 2 cells. C: The second day almost passes and the embryo still dividing evenly. D: A morula stage contains 10-30 cells. It's the final stage prior to the formation of a fluid-filled cavity, which called blastocoel cavity. E: The embryo continues to grow and develop into a compact ball of cells, about 16 morulas. F: The embryo is progressing fast and continues dividing forming early Blastocyst. G: Blastocyst stage is where the embryo has developed to have two different cell components and a fluid cavity. H: Expand Blastocyst is an advanced development stage of Blastocyst where the blastocoel cavity enlarges and the zona pellucida (the protective shell) is thin.

2.10.1 Embryo culture within the Embryoscope

2.10.1.1 Device setup

The EmbryoScope is turned on at least three hours prior to use to allow temperature and gaseous equilibrium. The device is checked to be grounded through the power connector, gas connections are not leaking, and the gas reservoir is full. The residual pressure of gas cylinders are checked daily, with CO2 or nitrogen cylinders replaced if the pressure is below 40 bar. The back pressure in the connecting tubes should not exceed 1 bar or drop below 0.6 bar.

2.10.1.2 EmbryoSlides preparation and culture

On confirmation of fertilisation the embryos are transferred from each participant are transferred into an EmbryoSlide, which have been allowed to calibrate for 20 hours with media in a general incubator with similar temperature and gas conditions. Each EmbryoSlide contains a large oil reservoir with 12 25µl wells enabling the incubation of 12 individual embryos. Within each well, there is a central depression where the embryo resides, with a diameter of approximately 250 µm.

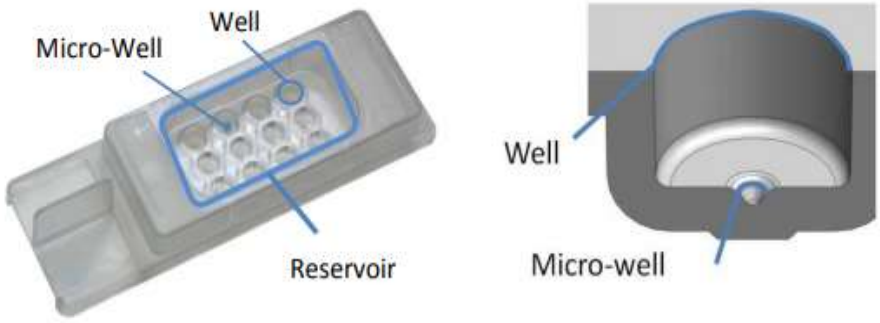


Figure 2.7 The well and micro-well in the EmbryoSlide where the embryos were placed and then transferred into the incubator for observation

Each individual well are numbered at their bottom, allowing tracking of each embryo within the well. As the embryo naturally migrates to he micro-well, the inverted microscope that is built into the Embryoscope can immediately start to acquire images as soon as the Embryoslide is loaded within the system.

2.10.2 Embryo grading

Embryos were principally graded according to the Association of Clinical Embryologists (ACE) national grading schemes with the additional morphokinetic

information taken into account where there was a cohort of embryos available for selection. For cleavage stage embryos the scheme takes into account the cell number, evenness of cells and degree of fragmentation (Table 2.1). The ACS unit primarily focuses on cleavage stage assessment on day 3 (68 ± 1 h) (Figure 2.9), with a 8 cell/4/4 being a top quality embryo.

Criterion	Grade	Description
Blastomere Number		Presented as nc (n= number of cell)
Blastomere size	4	Regular, even division
	3	<20% difference (cell diameter)
	2	20-50% difference
	1	>50% difference
Fragmentation	4	<10% fragmentation by volume
	3	10-20%
	2	20-50%
	1	>50%

Table 2.1 BFS and ACE cleavage stage embryo grading system.

On the morning of day 5 (116 ± 1 h), embryos were subsequently graded according to their degree of expansion status and integrity of the inner cell mass and trophoectoderm (Table 2.2) (ESHRE, 2011).

Expansion score	Expansion status	ICM/TE score	Inner Cell Mass (ICM)	Trophectoderm (TE)
6	Hatched Blastocyst (The blastocyst has evacuated the ZP			
5	Hatched Blastocyst (Trophectoderm has started to herniate through ZP)			
4	Expanded (blastocoel volume larger than the embryo with thinning of ZP)	A	ICM prominent, easily seen, tightly adhered completed cell	Continuous layer of small identical cells
3	Full blastocyst (blastocoel completely fills the embryo)	B	ICM less prominent (cells appear compacted and large in size, loosely adhered)	Fewer cells with gaps, not continuous
2	Blastocyst (blastocoel >50% volume of embryo)	C	Very few cells visible (cells similar to TE)	Fewer small cells with large cells, not continuous
1	Early blastocyst (blastocoel <50% volume of embryo	D	No visible cells or visible cells are degenerate or necrotic	Spares cells, large/flat/degenerate

Table 2.2 Blastocyst grading scheme.

As all embryos were cultured in the Embryoscope, further information regarding their development was determined by scrolling through the time-lapse images and ensuring no abnormal cell divisions occurred. The annotation of key events combined with the D5 KiD score were used to decide which of two similar graded embryos were to be replaced.

2.10.3 Time-lapse parameters for transferred embryos.

For all embryos which were transferred, I recorded the timing of the morphokinetic events outlined as in Figure 2.8 and 2.9 and Table 2.3 in accordance

with international guidelines. The derived morphokinetic variables indicated in Table 2.4 were also recorded.

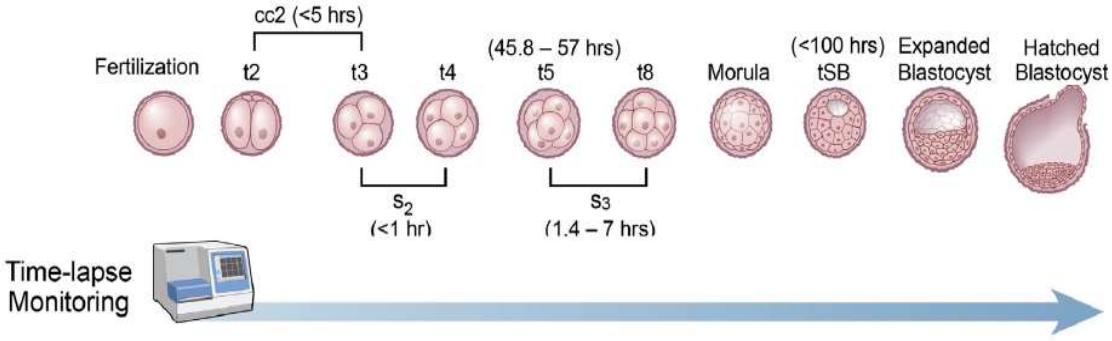


Figure 2.8 Embryo growth and development over time which illustrates all the abbreviations used in the algorithm

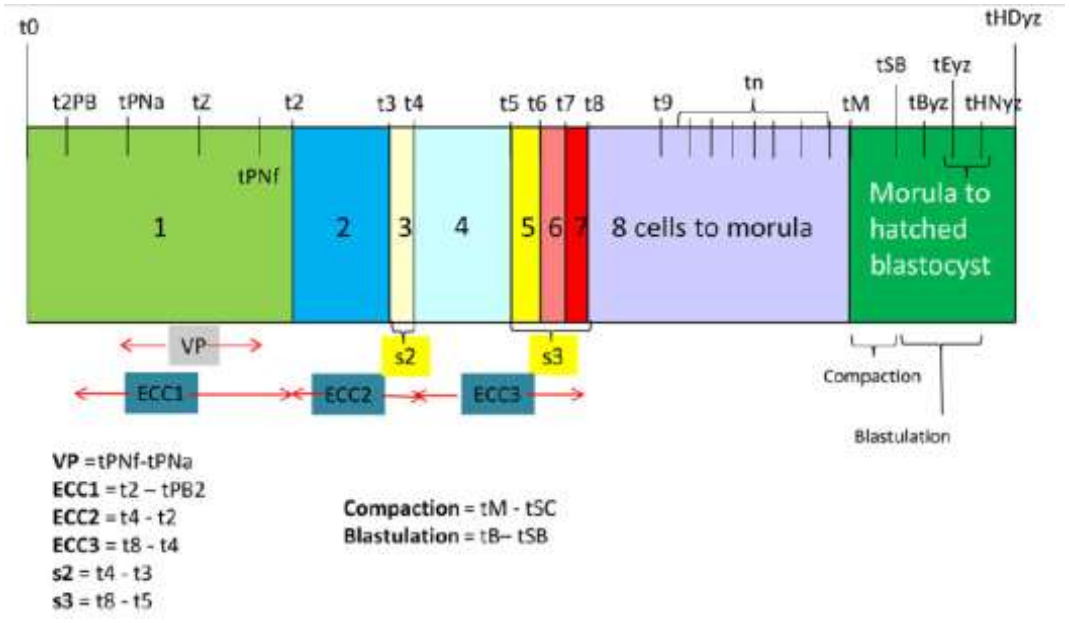


Figure 2.9 Definitions for the dynamic monitoring of human preimplantation embryo development.

Time	Definition of expected event
t0	Time of IVF or mid-time of micro/injection (ICSI/IMSI)
tPB2	The second polar body is completely detached from the oolemma
tPN	Fertilisation status if confirmed
tPNa	Appearance of individual pronuclei ; tPN1a, tPN2a, tPN3a....
tPNf	Time of pronuclei disappearance ; tPN1f, tPN2f....
tZ	Time of PN scoring
t2 to t9	two to nine discrete cells
tSC	First evidence of compaction
tMF/p	End of compaction process (last frame before cavity formation) 'f' corresponds to fully compacted 'p' corresponds to partial compaction
tSB	iniriation of blastulation
tByz	Full blastocyst (last fram before zona starts to thin) 'y' corresponds to morphology of inner cell mass 'z' corresponds to morphology of trophectoderm cell
tEyz	Initiation of expansion; first frame of zona thinning
tHNyz	Herniation; end of expansion phase and initiation of hatching process
tHDyz	Fully hatched blastocyst

Table 2.3 Summary of morphokinetic variables and proposed definitions.

Each timing defines the first time lapse frame in which the expected phenomenon is observed or detected.

Annotation	Calculated duration of events	Dynamic event
VP	tPNf-tPNa	PN dursation
ECC1	t2-tPB2	Duration of first cell cycle
ECC2	t4-t2 cc2a=t3-t2 cc2b=t4-t2	Duration of second embryo cell cycle
ECC3	t8-t2 cc3a=t5-t4 cc3b=t6-t4 cc3c=t7-t4 cc3d=t8-t4	Duration of third embryo cell cycle
S2	t4-t3	Synchronisation of cell divisions
S3	t8-t5	Synchronisation of cleavage pattern
dcom	tMf-tSC (Full compaction) tMp-ttSC (Partial compaction)	Duration of compaction
dB	tB-tSB	Duration blastulation
dexp	tHN-tE	Duration of blastocyst expansion
dcol	tBCend(n)-tBC(n)	Duration of blastocyst collapse: 'n' is number of episodes of collapse and re-expansion
dre-exp	tre-exp end(n)-tre-expi(n)	Duration of re-expansion
dHN	tHN-tHD	Duration of herniation

Table 2.4 Summary of calculated variables of dynamic monitoring of human preimplantation embryo development.

2.11 Embryo selection transfer and cryopreservation

In accordance with the multiple birth minimisation strategy one or two of the top scoring embryos were selected for transfer. The number of embryos transferred reflected the female age, overall embryo quality score and past treatment history. All excess good quality embryos (3Bb or above) were vitrified on day 5 or 6.

2.12 Determination of pregnancy outcome / perinatal data

Positive pregnancy was determined by a positive urinary pregnancy test 10 days after embryo transfer. A clinical pregnancy was defined as presence of a fetal heartbeat at 6 to 8 weeks gestation. An ongoing pregnancy was defined as a fetal heartbeat at 20 weeks gestation. Pregnancy outcome, offspring sex, birthweight, gestational age and mode of delivery were self-reported.

2.13 Detailed metabolic analysis using NMR metabolomics platform

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform [Nightingale Health© (Helsinki, Finland)] at the University of Bristol. The platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, the glycolysis precursors lactate and pyruvate, ketone bodies, and amino acids in absolute concentration units (mostly mmol/l). The NMR-based metabolite quantification is achieved through measurements of three molecular windows from each sample. Two of the spectra (LIPO and LMWM windows) are acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts are conducted at 37^{O}C and 22^{O}C, respectively. Details of this platform have been published previously (Wurtz *et al.*, 2017; Soininen *et al.*, 2009) and it has been widely applied in genetic and observational epidemiological studies (Akbaraly *et al.*, 2018; Wurtz *et al.*, 2014,2015; Deelen *et al.*, 2019; Wang *et al.*, 2016,2018; Fischer *et al.*, 2014; White *et al.*, 2017; Mills *et al.*, 2019).

I transferred the samples to Bristol, under dry ice and the samples were then analysed in accordance with standard operating procedures in two runs. A summary of the process is provided in Figure 2.10 and accompanying figure legend, with a summary of the individual measures provided in Table 2.5.

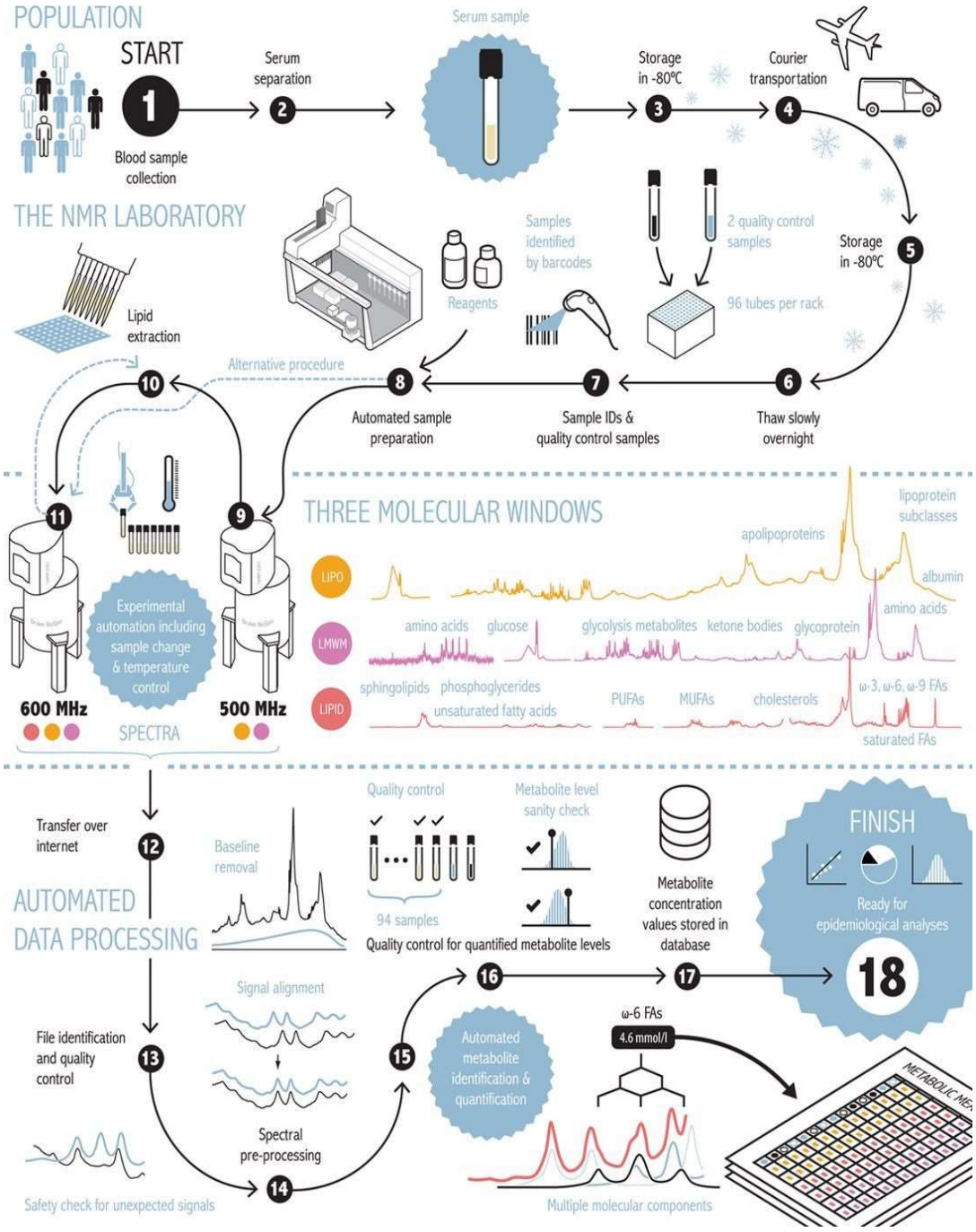


Figure 2.10 NMR spectroscopy complete system with an extended illustration of the process.

The automated high-throughput serum NMR metabolomics platform—process from the population to the individual comprehensive quantitative molecular data. Any routine serum (or ethylenediaminetetraacetic acid plasma) samples can be profiled. For integrity and biological stability, long-term (>1 month) storage must be at −80°C and the samples must stay frozen during the transport. The samples are handled in 96-well plates, every plate containing 2 quality control samples—a

serum mimic and a mixture of 2 low-molecular–weight metabolites. The former is used to monitor the consistency of quantifications, whereas the latter is a technical reference to monitor the performance of the automated liquid handler and the spectrometer. Barcoding is preferred for sample identification. Before the NMR measurements, 260 µL of serum and 260 µL of a sodium phosphate buffer (75 mmol/L Na_2HPO_4 in 80%/20% H_2O/D_2O, pH 7.4; including also 0.08% sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 and 0.04% sodium azide) is carefully mixed and moved to the NMR tubes. All the liquid handling steps for native serum samples are done with a PerkinElmer JANUS Automated Workstation equipped with an 8-tip dispense arm with Varispan. The automated liquid handler can prepare 96 samples in ≈30 minutes. The current NMR metabolomics laboratory setup at the University of Bristol combines a Bruker AVANCE III 500 MHz and Bruker AVANCE III HD 600 MHz spectrometers, both with the SampleJet robotic sample changer. The SampleJet can hold 480 samples (5 positions for NMR tubes in the standard 96-well plate array) in a cooled (+6°C) temperature. The cooled sample changer is a prerequisite when working with biological samples to prevent degradation. The 500 MHz spectrometer is equipped with a selective inverse room temperature probe head, whereas the 600 MHz system has a cryogenically cooled triple resonance probe head (CryoProbe Prodigy TCI). For the native serum samples the Lipoprotein (LIPO) and Low-Molecular-Weight Metabolites (LMWM) data can be automatically collected either with the 500 MHz or the 600 MHz spectrometer. Standardized parameters are used for data acquisitions. The entire time required for the sample handling and measurements is ≈8 and 5 minutes at 500 and 600 MHz, respectively. After these measurements, the same samples go through a standardized lipid extraction procedure based on multiple extraction steps containing saturated sodium chloride solution, methanol, dichloromethane, and deuterochloroform. The extraction procedure is done manually with an Integra Biosciences VIAFLO 96 channel electronic pipette. These lipid extracts are then moved into the NMR tubes and the extracted lipid data are collected in full automation with the 600 MHz instrument using a standard parameter set. The time required for the sample handling and the LIPID measurement is ≈5 minutes. The depicted setup of a 500 MHz and a 600 MHz platform allows ≈80 000 samples to be analysed annually. The initial data processing, including the Fourier transformations to NMR spectra and automated phasing are done using the computers that control the spectrometers; the spectra are then automatically

transferred to a centralized server, which performs various further automated spectral processing steps, including overall signal check for missing/extra peaks, background control, baseline removal and spectral area-specific signal alignments. The spectral information of the actual sample also undergoes various comparisons with the spectra of the 2 quality control samples; the data for which is also followed and compared in a consecutive manner. For those spectral areas that pass all the quality control steps, regression modelling are performed to produce the quantified molecular data. Also, the individual metabolic measures undergo various statistical quality control steps and are also checked against an extensive database of quantitative molecular data. The metabolic measures that pass all quality control steps are stored in the database and are ready for various epidemiological data analysis. FA indicates fatty acid; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

Molecular class	Lipid, lipoprotein or metabolite name	Units*
Extremely large VLDL	Concentration of chylomicrons and extremely large VLDL particles	mol/l
	Total lipids in chylomicrons and extremely large VLDL	mmol/l
	Phospholipids in chylomicrons and extremely large VLDL	mmol/l
	Total cholesterol in chylomicrons and extremely large VLDL	mmol/l
	Free cholesterol in chylomicrons and extremely large VLDL	mmol/l
	Triglycerides in chylomicrons and extremely large VLDL	mmol/l
Very large VLDL	Concentration of very large VLDL particles	mol/l
	Total lipids in very large VLDL	mmol/l
	Phospholipids in very large VLDL	mmol/l
	Total cholesterol in very large VLDL	mmol/l
	Cholesterol esters in very large VLDL	mmol/l
	Free cholesterol in very large VLDL	mmol/l
	Triglycerides in very large VLDL	mmol/l
Large VLDL	Concentration of large VLDL particles	mol/l
	Total lipids in large VLDL	mmol/l
	Phospholipids in large VLDL	mmol/l
	Total cholesterol in large VLDL	mmol/l l
	Cholesterol esters in large VLDL	mmol/l
	Free cholesterol in large VLDL	mmol/l
	Triglycerides in large VLDL	mmol/l
Medium VLDL	Concentration of large VLDL particles	mol/l
	Total lipids in small VLDL	mmol/l
	Phospholipids in small VLDL	mmol/l
	Total cholesterol in small VLDL	mmol/l
	Cholesterol esters in small VLDL	mmol/l
	Free cholesterol in small VLDL	mmol/l
	Triglycerides in small VLDL	mmol/l
Small VLDL	Concentration of very small VLDL particles	mol/l
	Total lipids in very small VLDL	mmol/l
	Phospholipids in very small VLDL	mmol/l
	Total cholesterol in very small VLDL	mmol/l
	Cholesterol esters in very small VLDL	mmol/l
	Free cholesterol in very small VLDL	mmol/l
	Triglycerides in very small VLDL	mmol/l
IDl	Concentration of IDL particles	mol/l
	Total lipids in IDL	mmol/l
	Phospholipids in IDL	mmol/l
	Total cholesterol in IDL	mmol/l
	Cholesterol esters in IDL	mmol/l
	Free cholesterol in IDL	mmol/l
	Triglycerides in IDL	mmol/l

Large LDL	Concentration of large LDL particles	mol/l
	Total lipids in large LDL	mmol/l
	Phospholipids in large LDL	mmol/l
	Total cholesterol in large LDL	mmol/l
	Cholesterol esters in large LDL	mmol/l
	Free cholesterol in large LDL	mmol/l
	Triglycerides in large LDL	mmol/l
Medium LDL	Concentration of medium LDL particles	mol/l
	Total lipids in medium LDL	mmol/l
	Phospholipids in medium LDL	mmol/l
	Total cholesterol in medium LDL	mmol/l
	Cholesterol esters in medium LDL	mmol/l
	Free cholesterol in medium LDL	mmol/l
	Triglycerides in medium LDL	mmol/l
Small LDL	Concentration of small LDL particles	mol/l
	Total lipids in small LDL	mmol/l
	Phospholipids in small LDL	mmol/l
	Total cholesterol in small LDL	mmol/l
	Cholesterol esters in small LDL	mmol/l
	Free cholesterol in small LDL	mmol/l
	Triglycerides in small LDL	mmol/l
Very large HDL	Concentration of very large HDL particles	mol/l
	Total lipids in very large HDL	mmol/l
	Phospholipids in very large HDL	mmol/l
	Total cholesterol in very large HDL	mmol/l
	Cholesterol esters in very large HDL	mmol/l
	Free cholesterol in very large HDL	mmol/l
	Triglycerides in very large HDL	mmol/l
Large HDL	Concentration of large HDL particles	mol/l
	Total lipids in large HDL	mmol/l
	Phospholipids in large HDL	mmol/l
	Total cholesterol in large HDL	mmol/l
	Cholesterol esters in large HDL	mmol/l
	Free cholesterol in large HDL	mmol/l
	Triglycerides in large HDL	mmol/l
Medium HDL	Concentration of medium HDL particles	mol/l
	Total lipids in medium HDL	mmol/l
	Phospholipids in medium HDL	mmol/l
	Total cholesterol in medium HDL	mmol/l
	Cholesterol esters in medium HDL	mmol/l
	Free cholesterol in medium HDL	mmol/l
	Triglycerides in medium HDL	mmol/l
Small HDL	Concentration of small HDL particles	mol/l
	Total lipids in small HDL	mmol/l
	Phospholipids in small HDL	mmol/l
	Total cholesterol in small HDL	mmol/l
	Cholesterol esters in small HDL	mmol/l
	Free cholesterol in small HDL	mmol/l
	Triglycerides in small HDL	mmol/l
Lipoprotein particle size	Mean diameter for VLDL particles	nm
	Mean diameter for LDL particles	nm
	Mean diameter for HDL particles	nm

Cholesterol concentrations	Total cholesterol	mmol/l
	Total cholesterol in VLDL	mmol/l
	Remnant cholesterol (non-HDL and non-LDL cholesterol)	mmol/l
	Total cholesterol in LDL	mmol/l
	Total cholesterol in HDL	mmol/l
	Total cholesterol in HDL2	mmol/l
	Total cholesterol in HDL3	mmol/l
	Esterified cholesterol	mmol/l
	Free cholesterol	mmol/l
Glycerides and phospholipid concentrations (and one ratio)	Total triglycerides	mmol/l
	Triglycerides in VLDL	mmol/l
	Triglycerides in LDL	mmol/l
	Triglycerides in HDL	mmol/l
	Total phosphoglycerides	mmol/l
	Ratio of triglycerides to phosphoglycerides	
	Phosphatydilcholine and other cholines	mmol/l
	Sphingomyelins	mmol/l
	Total cholines	mmol/l
Apolipoprotein concentrations (and one ratio)	Apolipoprotein A-1	g/l
	Apolipoprotein B	g/l
Fatty acid concentrations	Total fatty acids	mmol/l
	Estimated degree of saturation	
	22:6, docosahexaenoic acid	mmol/l
	18:2 linoleic acid	mmol/l
	Omega-3 fatty acids	mmol/l
	Omega-6 fatty acids	mmol/l
	Polyunsaturated fatty acids	mmol/l
	Monounsaturated fatty acids; 16:1, 18:1	mmol/l
	Saturated fatty acids	mmol/l
Fatty acid ratios	Ratio of 22:6, docosahexaenoic acid to total fatty acids	%
	Ratio of 18:2 linoleic acid to total fatty acids	%
	Ratio of omega-3 fatty acids to total fatty acids	%
	Ratio of omega-6 fatty acids to total fatty acids	%
	Ratio of polyunsaturated fatty acids to total fatty acids	%
	Ratio of monounsaturated fatty acids to total fatty acids	%
	Ratio of saturated fatty acids to total fatty acids	%
Glycolysis related metabolite	Glucose	mmol/l
	Lactate	mmol/l
	Pyruvate	mmol/l
	Citrate	mmol/l
	Glycerol	mmol/l
	Alanine	mmol/l

	Glutamine	mmol/l
Amino acid concentrations	Glycine	mmol/l
	Histidine	mmol/l
branched	Isoleucine	mmol/l
branched	Leucine	mmol/l
branched	Valine	mmol/l
aromatic	Phenylalanine	mmol/l
aromatic	Tyrosine	mmol/l
Ketone body concentrations	Acetate	mmol/l
	Acetoacetate	mmol/l
	3-hydroxybutyrate	mmol/l
Fluid balance marker	Albumin	mmol/l
	Creatinine	mmol/l
Inflammation marker	Glycoprotein acetyls, mainly a1-acid glycoprotein	mmol/l

Table 2.5 metabolic measures
*These are the units used for each of the metabolic measures, unless we state that we are presenting results in standard deviation (SD) units.
VLDL: very low density lipoprotein; LDL: low density lipoprotein; IDL: intermediate density lipoprotein; HDL: high density lipoprotein

2.14 BioBank and database creation

I created two excel sheet to organise the participant's appointments and the data collected from them. The first datasheet included the basic identification information of the participant, including their name, CHI number, when they signed the informed consent, their unique alphanumeric ID for the study and any other remarks or notes. The second datasheet acted as the core database for the study as it included all data collected from the participants including (but not limited to); the participant's CHI number, unique alphanumeric ID, personal details (name, address and contact number, daily job, education level and date of birth), anthropometric data (height, weight and BMI), lifestyle (amount of alcohol consumption per week, number of cigarettes smoked per week and frequency of weekly exercise), medical history (routenly taken medication, major operation and any allergies), family medical history (diabetes, cardiometabolic health, blood pressure and obesity), obstetric history (number of previous natural pregnancies, number of natural lifebirth, number of previos ART pregnancies, number of IVF lifebirth), infertility history (duration of infertility and cause and type of the infertility), hormone level (AMH), ultrasound baseline scan, ultrasound last tracking scan, semen parameters before preparation, semen parameters after preparation, treatment plan (type, starting date, number of attempt, treatment

drug, drug protocol, date of stimulation, drug dose, date of triggering, number and date of oocytes collected, number and date of oocytes inseminated, number of oocytes fertilised, total number and date of embryos transferred, number of embryos freezed and treatment cycle remarks), embryo transferred details (includes all the phases of development starting from day 1 until embryo transfer or vitrification), embryo kinetic data and treatment cycle outcome (including the pregnancy outcome and any complications and remarks).

2.15 Statistical analysis

2.15.1 Sample size and power calculation

The ACS performs approximately 1,000 IVF/ICSI cycles for ages 18-44 every year. Considering the historical success rates and age of the treated population the success rate for ongoing pregnancy per cycle was estimated to be 30%. I recruited 400 couples over the duration of recruitment (19 months), to ensure I had 80% power to detect a 0.23 standard deviation difference in baseline measures between women who achieved ongoing pregnancy to those who do not at an alpha of 5%. This equates to roughly 2 mmHg of systolic blood pressure and 0.15 mmol/l of cholesterol (based on general population statistics for adult women). The sample size and power calculation were based on the Avon Longitudinal Study of Parents and Children (ALSPC) (Macdonald-Wallis et al., 2015) where a small (~ 2 mmHg) nadir of systolic blood pressure was the reported difference between pregnant women at 17-18 weeks of gestation and non-pregnant women.

2.15.2 Statistical analysis

All statistical analyses were performed using Stata (version 15.1, StatCorp, Texas, USA) and/or the statistical software package R (version 3.4.2), with additional related specialist packages as required. Full details of the analyses are provided in each of the respective chapters.

Chapter 3 Association of the functional ovarian reserve with serum metabolomic profiling by nuclear magnetic resonance spectroscopy: A cross sectional study of ~400 women.

3.1 Introduction

Female reproductive aging is the result of a gradual decrease in both the quantity and quality of oocytes (Velde & Pearson, 2002). Genetic, environmental and lifestyle factors are all known to contribute to the timing and depletion of the ovarian reserve. Markers of diminished ovarian reserve, such as low AMH and low AFC have been shown to associate with earlier menopause (Broekmans *et al.*, 2004; Dolleman *et al.*, 2013). Several observational studies have investigated the association between these markers of diminished ovarian reserve and cardiovascular risk factors, with several (Bleil *et al.*, 2013, 2017; Kat *et al.*, 2016; Tehrani *et al.*, 2014; Dam *et al.*, 2019) but not all studies (Yarde *et al.*, 2016; Anderson *et al.*, 2013; Ramezani *et al.*, 2016), suggesting that a diminished ovarian reserve may be associated with an unfavourable circulating cardiometabolic risk profile and cardiovascular events. However, these studies have been limited to a restricted number of established cardiovascular risk factors, including evaluation of total and LDL cholesterol (Tehrani *et al.*, 2014), Homeostatic Model Assessment as a surrogate for Insulin Resistance (HOMA-IR) (Park *et al.*, 2010) or have considered these established risk factors together as a composite outcome of cardiometabolic risk (Bleil *et al.*, 2013; Kat *et al.*, 2016).

Detailed metabolic profiling or metabolomics, has been applied successfully to identify novel biomarkers for the development of cardiovascular disease (Akbaraly *et al.*, 2018; Wurtz *et al.*, 2015) and all-cause-mortality (Deelen *et al.*, 2019), with improved prediction as compared to models containing conventional risk factors (Deelen *et al.*, 2019). That serum NMR metabolomics enables reproducible quantification of circulating lipids and abundant metabolites (Wurtz *et al.*, 2017) has facilitated its use in the assessment of the changes in metabolites with adiposity (Wurtz *et al.*, 2014), glycemia (Wurtz *et al.*, 2012), pregnancy (Wang *et al.*, 2016) and menopausal status (Wang *et al.*, 2018).

The aim of the current study was to assess the association of ovarian reserve (as measured by AMH and AFC) with 155 circulating metabolic measures. These measures were profiled by a high-throughput NMR metabolomics platform, covering a wide range of metabolic pathways including lipoprotein lipids, fatty acids, amino acids, ketone bodies, and glycemic traits, which are highly relevant to cardiometabolic risk and overall health.

3.2 Methods

3.2.1 Study Design and Participants

Cross-sectional study of women aged 18 to 45 who presented at Glasgow Royal Infirmary, UK for assessment prior to assisted conception between 1 April 2017 and 31 March 2019. Exclusion criteria were a documented positive pregnancy test at time of presentation, body mass index (BMI) ≥35kg/m^2, and/or requiring oocyte or embryo donation. A total of 400 women were recruited and of these 398 (99%) had complete data on AMH, AFC and at least one NMR metabolite and were included in the analyses presented in this chapter (see chapter 2 section 2.2 for further details).

The study was conducted according to ICH Guideline for good clinical practice, the Declaration of Helsinki and the Convention of the Council of Europe. All women provided written informed consent. The study protocol was approved prior to study initiation by the relevant institutional review boards (see Materials & Methods Chapter for further details).

3.2.2 Study procedures

Demographic, lifestyle, fertility and medical history was obtained by self-reported questionnaire and clinical data by linkage to electronic medical records (further details can be found in chapter 2 section 2.5).

AFC was determined by two- or three-dimensional transvaginal ultrasound (AFC was defined as the total number of antral follicles with a size of 2–10 mm in both ovaries) on menstrual cycle day 2-4. Follicle counts greater than 20 follicles per ovary were classed as ≥20, consistent with the diagnostic threshold for PCOS, and that ovary was not counted further (Teede *et al.*, 2018). Due to the potential for

inter-sonographer variability, sonographers were provided with training and were asked to follow published practical recommendations for accurate transvaginal ultrasound (Broekmans *et al.*, 2010); each sonographer used standard equipment (Acuson Sequoia, Siemens Germany) (further details can be found in chapter 2 section 2.9.3).

Non-fasted blood samples were collected during the same visit that transvaginal ultrasound was performed. AMH was measured using the Beckman Coulter AMH automated method on a clinically validated immunoassay platform (Access 2, Beckman Coulter, USA). The assay was calibrated and quality controlled using the manufacturer's reagents and is known to have a measuring range of 0.08 – 24ng/ml (0.57 – 171 pmol/l). The Limit of Quantitation (LoQ) was 0.02 ng/ml (0.014pmo/l), with the 20% CV LoQ 0.08ng/ml (0.57pmol/l). The coefficient of variation between runs for two levels of control ran at <4.4% (further details can be found in chapter 2 section 2.9.2).

Additional blood samples were taken for NMR analyses and immediately spun and frozen at −80 °C and all NMR assays completed for this study were undertaken within 1 year of storage and with no previous freeze/thaw cycles (further details can be founr in chapter 2 section 2.7).

3.2.3 NMR protocol

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform [Nightingale Health© (Helsinki, Finland)] at the University of Bristol. The platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, the glycolysis precursors lactate and pyruvate, ketone bodies, and amino acids in absolute concentration units (mostly mmol/l). The NMR-based metabolite quantification is achieved through measurements of three molecular windows from each sample. Two of the spectra (LIPO and LMWM windows) are acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts are conducted at 37^{O}C and 22^{O}C, respectively. Details of this platform

have been published previously (Wurtz *et al.*, 2017; Soininen *et al.*, 2009) and it has been widely applied in genetic and observational epidemiological studies (Akbaraly *et al.*, 2018; Wurtz *et al.*, 2014,2015; Deelen *et al.*, 2019; Wang *et al.*, 2016,2018; Fischer *et al.*, 2014; White *et al.*, 2017; Mills *et al.*, 2019). Further details of the platform are provided in Chapter 2 (Materials & Methods Chapter)

3.2.4 Metabolite quantification and quality control

The NMR spectra were analysed for absolute metabolite quantification (molar concentration) in an automated fashion. For each metabolite a ridge regression model was applied for quantification in order to overcome the problems of heavily overlapping spectral data. In the case of the lipoprotein lipid data, quantification models were calibrated using high performance liquid chromatography methods, and individually cross-validated against NMR-independent lipid data. Low-molecular-weight metabolites, as well as lipid extract measures, were quantified as mmol/l based on regression modelling calibrated against a set of manually fitted metabolite measures. The calibration data are quantified based on iterative line-shape fitting analysis using PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland). Absolute quantification cannot be directly established for the lipid extract measures due to experimental variation in the lipid extraction protocol. Therefore, serum extract metabolites are scaled via the total cholesterol as quantified from the native serum LIPO spectrum.

3.2.5 Assessment of potential confounders

In relation to our analyses and control for confounders, we used currently recommended practice of defining confounders a priori (before undertaking analyses) using Directed Acyclic Graphs (DAG) (Pearce & Lawlor, 2016; Williamson *et al.*, 2014). This approach defines confounders as any characteristic that is known to cause variation in the exposure (here ovarian biomarkers AMH and AFC) and outcome (NMR metabolites) or is plausibly a cause of exposure and outcome. Using a priori knowledge and relevant literature, our selected confounders were age, BMI, educational attainment, ethnicity, family history of cardiovascular disease (defined as first degree relative affected) physical activity, alcohol intake, smoking status, duration and cause of infertility whether infertility was primary or secondary (with secondary defined as a woman unable to establish a clinical pregnancy but who has previously been diagnosed with a clinical pregnancy)

(Zegers-Hochschild *et al.*, 2017). These are known to or plausibly influence both ovarian reserve and the NMR metabolites (Mathews *et al.*, 2009). Figure 3.1 shows the DAG for this, our main, confounder-adjusted analyses. Following reviewer comments, we also considered the extent to which PCOS might be part of a confounding path and whether we should also adjust for it. There is evidence ovarian reserve and PCOS may share underlying common causes, including genetic variation and intrauterine exposures. If these, specific genetic variants and exposures are also related to cardiometabolic health, then PCOS may be on a confounding path between ovarian reserve and cardiometabolic health. Three possibilities are considered in Figure 3.1b–d. Whether or not to adjust for PCOS is in part related to the fact that we do not have genetic or intrauterine data on the women included in this study, and so, these would be potential unmeasured confounders. In Figure 3.1b, we assume that there is no causal relationship between PCOS and cardiometabolic health. Whilst observational studies have shown associa-tions of PCOS with type 2 diabetes, and some also show associations with CHD, a recent Mendelian randomisation study has suggested no causal effect of PCOS on type 2 diabetes, coronary heart disease or stroke (Pearce & Lawlor, 2017), making this scenario plausible. In this scenario, PCOS is not on a confounding path, and adjusting for it should not alter the results. Figure 3.1c and Figure 3.1d suggest PCOS does causally influence cardiometabolic health. In Figure 3.1c, PCOS is on a confounding path, and we would want to adjust for it. In Figure 3.1d, it is on a mediating path, and we would not want to adjust for it. Given we cannot be certain which of these scenarios is correct in additional analyses we, repeat all of our main analyses with those with known PCOS removed. We chose to remove them rather than add to a multivariable model because there were only 24 cases.

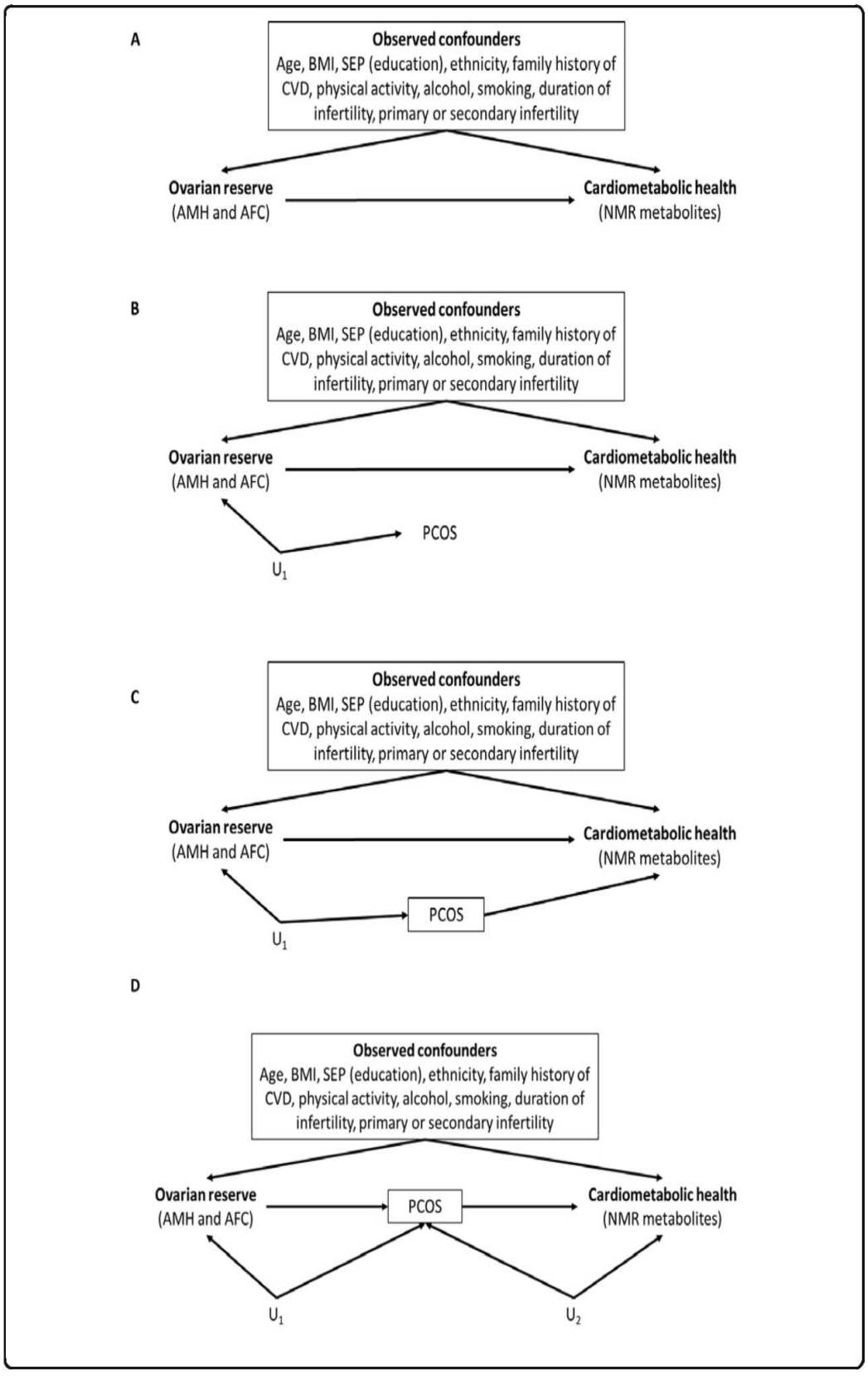


Figure 3.1 Directed acyclic graphs (DAGs) of the association of ovarian reserve with cardiometabolic health.

Our main analyses, in which we a priori considered factors that were known to, or highly plausibly influenced ovarian reserve and cardiometabolic health, as confounders to be controlled for. Figures 3.1b–d shows further consider whether we should or should not control for PCOS. In all of these, we assume that there are underlying unmeasured factors that generate an association between ovarian reserve and PCOS (U_1). For example, it has been suggested that common genetic and/or intrauterine factors affect both of these. In Figure 3.1b we assume that PCOS is not causally related to cardiometabolic health (no arrow from PCOS to cardiometabolic health). Whereas in Figure 3.1c, PCOS does causally influence cardiometabolic health and that it is on a confounding path between ovarian reserve and cardiometabolic health via U_1. Figure 3.1d assumes that PCOS causally influences cardiometabolic health but that it is a mediator between ovarian reserve and cardiometabolic health. Deciding whether we need to adjust for PCOS therefore depends on evidence for a causal link between PCOS and cardiometabolic health and if there is a link evidence as to whether PCOS is likely to be a confounder or a mediator. In relation to point 1, whilst there have been several observational studies showing an association of PCOS with cardiometabolic health, recent Mendelian randomisation studies suggest that PCOS does not causally influence type 2 diabetes, coronary heart disease or stroke (making Figure 3.1b a plausible scenario) (Zhu, 2020). If the scenario depicted in Figure 3.1b is correct, then controlling for PCOS is not necessary, but if done, it should have no impact on the results. It is not uncommon in observational epidemiology to have a risk factor for an outcome and be unsure which is more plausible that it is a confounder or a mediator. If PCOS is on the confounding path between unmeasured factors (U_1) as shown in Figure 3.1c, we would definitely want to adjust for it as this would be the only way to block this confounding (given U_1 variables are unmeasured). However, if PCOS is a mediator (Figure 3.1d), then we would not want to adjust for it. Primarily, this is because we want to know the 'total' potential effect of ovarian reserve on cardiometabolic health and not remove any of that going via mediation. It is also possible that adjusting for a mediator can introduce what is known as collider bias (Pearce & Lawlor, 2017). If there are unmeasured confounders of the mediator (PCOS) and cardiometabolic health (shown by U_2), then adjusting for PCOS could generate spurious associations between ovarian reserve and cardiometabolic health.

Weight and height [used to calculate the body mass index (BMI)] were measured in light clothing and unshod. Weight was measured to the nearest 0.1 kg using Tanita scales; height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Smoking status was categorised as ever versus never (to be considered for state funded assisted conception women had to have not smoked for at least 3 months and this was confirmed by a negative cotinine breath test). All other confounders, including an existing diagnosis of PCOS, were obtained by a questionnaire or from medical notes when the women were originally recruited. Full details of the assessment of these confounders is provided in the result sub-section.

3.2.6 Statistical analysis

All analyses were conducted using R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Characteristics were summarized as n, total range, mean, standard deviation, median, and Inter-Quantile Range (IQR) of 25th and 75th quantiles as appropriate. Multivariable linear regression was used to examine the associations of functional ovarian reserve markers (treated as exposures) with serum metabolic profiles (treated as outcomes). Robust standard errors were estimated for all associations, as some metabolite concentrations had skewed distributions. The metabolic measures were scaled to Standard Deviation (SD) units (by subtracting the mean and dividing by the standard deviation of all women included in the analyses). This scaling allows easy comparison of multiple metabolic measures with different units or with large differences in their concentration distributions. AMH and AFC were also scaled to SD units in the same way as the metabolites. This was done for ease of comparison of results between the two biomarkers. Associations were adjusted for all a priori selected confounders (age, ethnicity, education, family history of cardiovascular disease, BMI, physical activity, alcohol, smoking, duration of infertility and whether the woman had primary or secondary infertility).

3.2.7 Additional analyses

In addition to presenting my main results as the difference in mean metabolite in SD units per SD of AMH or AFC, I have also tabulated the full results (confounder adjusted) in the metabolite, AMH and AFC original units and presented these in an online repository of the associated manuscript (https://bit.ly/2MnlM2v). One

woman was taking lipid-lowering medication, and removal of her from the analyses did not alter any of the findings from the main results. We repeated our main analyses only on those women with a known partner cause of infertility (N = 87 (22% of the cohort). We knew a priori that this sample size would lack statistical power for reliable results but wanted to compare the point estimates in this group to the results of the whole cohort to provide some indication as to whether our results might be driven by the cause of infertility in the women or potentially generalisable to women of reproductive age without infertility. We compared the magnitudes of the results in women who were going to undergo assisted conception because of partner infertility with those of the whole cohort using a scatterplot. As 99% of the women recruited had full data on ovarian reserve and at least one metabolite, with < 1% of these having missing covariable data (see Table 3.1), we did not need to undertake any additional analyses to explore potential biases due to missing data. To explore the departure from linearity where there was evidence of an association, AMH and AFC were split into quarters of their distribution and regression models were run with these quarters as a continuous score and as a 4 level categorical variable (with 3 indicators). A likelihood ratio test was used to compare these two models. Statistical support that the second (3 indicators) model was a better fit of the data would suggest a possible non-linear association.

3.2.8 Accounting for multiple testing

Due to the correlated nature of the metabolic biomarkers, over 95% of the variation in the 155 metabolic biomarkers was explained by 14 principal components. Therefore, multiple testing correction, accounting for 14 independent tests using the Bonferroni method, resulted in $P < 0.0036$ (0.05/14) being denoted as statistically significant.

3.3 Results

Three hundred and ninety-eight women (99% of the 400 recruited) with available AMH and AFC levels and data on at least one metabolite were included in the study. Characteristics of the participants are shown in Table 3.1. Mean (SD) age of the women was 35.5 (4.4) years and the majority (92%) were white European, with over 50% having a university degree, 26% being ever smokers, 68% exercising more than 3-4 times per week, 52% having a family history of cardiovascular

disease and median alcohol consumption being 4 units per week. Mean (SD) BMI was 25 (3) Kg/m^2 and 95% were due to start their first treatment cycle, with 49% having an unknown cause of infertility and 24% a cause related to their partner. Median AMH was 16.1 pmol/l (IQR 8.8, 28.0 pmol/l) and median AFC of 12 (IQR 7, 16). AMH and AFC were positively correlated (Spearman's correlation coefficient = 0.55, p<0.001).

Age (years): Mean, SD, Range	35.5 (4.43) 22-45
Ethnicity: N(%) White European Asian Other	 365 (92%) 28 (7%) 5 (1%)
Highest Education N(%) School leaving exams Undergraduate degree Postgraduate degree	 185 (46%) 139 (35%) 74 (19%)
BMI: Mean, SD, Range	24.7 (3.2) 18.2-32.5
Ever smoked: N(%)	104 (26%)
Alcohol (units per week) : Median, IQR, Range	4 (1,8) 0-27
Physical activity (times per week) N(%) Never Once Twice 3-4 times 5-7 times 7+ times	 12 (3%) 29 (7%) 86 (22%) 239 (60%) 25 (6%) 7 (2%)
Family history of cardiometabolic disease: N(%)	208 (52%)
Gravidity: Median, IQR, Range	0 (0,1) 0-12
Parity: Median, IQR, Range	0 (0,0) 0-4
Duration infertility (years): Median, IQR, Range	3 (2,4) 1-13
Primary infertility: N(%)	271 (68%)
Cause of infertility Unexplained Tubal disorder Endometriosis Ovulatory disorder Male factor/No male partner Other	 203 (51%) 44 (11%) 32 (8%) 24 (6%) 87 (22%) 8 (2%)
First IVF cycle (missing data N=3)	376 (95%)
AMH: Median, IQR, Range	16.1 (8.9,28.0) 1-170.8
Total AFC: Median, IQR, Range	12 (7,16) 0-40

Table 3.1 Baseline characteristics of the study population (N=398)

The unadjusted associations of AMH and AFC with selected confounders are shown in Tables 3.2 and 3.3, respectively. Age was negatively associated with AMH (difference in mean −0.09 SD per 1-year older age, 95% CI −0.11, −0.07) and AFC (−0.06 SD per 1-year older age, 95% CI −0.08, −0.04). An ovulatory cause of infertility compared with an unexplained cause was associated with a higher AMH (difference in mean 0.93 SD, 95% CI 0.48, 1.37) and AFC (0.65, 95% CI 0.20, 1.11). Secondary infertility was strongly negatively associated with AFC (−0.34SD (95% CI −0.55, −0.13), with a weaker negative association with AMH (−0.11 (95%CI −0.32, 0.11)). Family history of cardiometabolic diseases was negatively associated, and non-White European ethnicity positively associated with AMH and AFC. There was no strong statistical evidence of an association between BMI or other potential confounders and either measure of ovarian reserve.

	Difference in mean AMH per unit or category of confounder (SD) (95% CI)	**P-value**
Age (years)	-0.09 (-0.11, -0.07)	<0.001
BMI (kg/m2)	-0.002 (-0.03, 0.03)	0.91
Education High School Undergraduate Postgraduate	 REF 0.21 (-0.01, 0.42) -0.19 (-0.45, 0.08)	 - 0.06 0.17
Physical activity (per category increase)	-0.01 (-0.12, 0.10)	0.85
Ever smoked	0.10 (-0.12, 0.33)	0.32
Alcohol (units per week)	-0.005 (-0.03, 0.01)	0.60
Family History of cardiometabolic diseases (yes vs no)	-0.17 (-0.37, 0.02)	0.09
Ethnicity (Non-white vs White European)	0.33 (-0.02, 0.69)	0.06
Secondary vs primary infertility	-0.11 (-0.32, 0.11)	0.33
Duration of infertility (years)	0.01 (-0.04, 0.06)	0.59
Endometriosis vs unexplained cause	-0.01 (-0.41,0.39)	0.96
Tubal vs unexplained cause	0.24 (-0.10,0.57)	0.16
Ovulatory vs unexplained cause	0.93 (0.48, 1.37)	<0.001
Male factors vs unexplained cause	0.03 (-0.21, 0.28)	0.78

Table 3.2 Associations of possible confounders with AMH (SD) N=398

	Difference in mean AFC per unit or category of confounder (SD) (95% CI)	P-value
Age (years)	-0.06 (-0.08, -0.04)	<0.001
BMI (kg/m2)	-0.01 (-0.04, 0.02)	0.61
Education High School Undergraduate Postgraduate	 REF -0.07 (-0.29, 0.15) -0.17 (-0.45, 0.10)	 - 0.54 0.21
Physical activity (per category increase)	0.004 (-0.11, 0.12)	0.94
Ever smoked	-0.14 (-0.36, 0.08)	0.22
Alcohol (units per week)	-0.02 (-0.04, 0.002)	0.07
Family History of cardiometabolic diseases (yes vs no)	-0.26 (-0.45, -0.06)	0.01
Ethnicity (Non-white vs White European)	0.31 (-0.04, 0.67)	0.09
Secondary vs primary infertility	-0.34 (-0.55, -0.13)	0.001
Duration of infertility (years)	-0.005 (-0.06, 0.04)	0.83
Endometriosis vs unexplained cause	0.01 (-0.39, 0.41)	0.96
Tubal vs unexplained cause	0.14 (-0.20, 0.48)	0.41
Ovulatory vs unexplained cause	0.65 (0.20, 1.11)	0.005
Male factors vs unexplained cause	0.12 (-0.13, 0.38)	0.34

Table 3.3 Associations of possible confounders with Total AFC (SD) N=398

The adjusted (for all potential confounders) associations between AMH and AFC and the respective metabolomics measures is SD units per SD of AMH/AFC are presented in Figures 3.2, 3.3 and 3.4. With the unadjusted associations presented in Figures 3.5, 3.6 and 3.7.

In confounder adjusted analyses AMH levels were positively correlated with measures of components of medium HDL and small HDL and with concentrations of omega-6 fatty acids and polyunsaturated fatty acids (PUFA). AMH was also positively associated with the amino acids isoleucine, leucine and tyrosine, and it was negatively associated with acetate concentrations. Although there were positive associations with lipoprotein subclasses, other cholesterol subtypes, glycerides and apolipoproteins, several of which had point estimates of ~0.1SD or larger differences, these had wide confidence intervals that included the null and did not reach our multiple testing threshold for statistical significance.

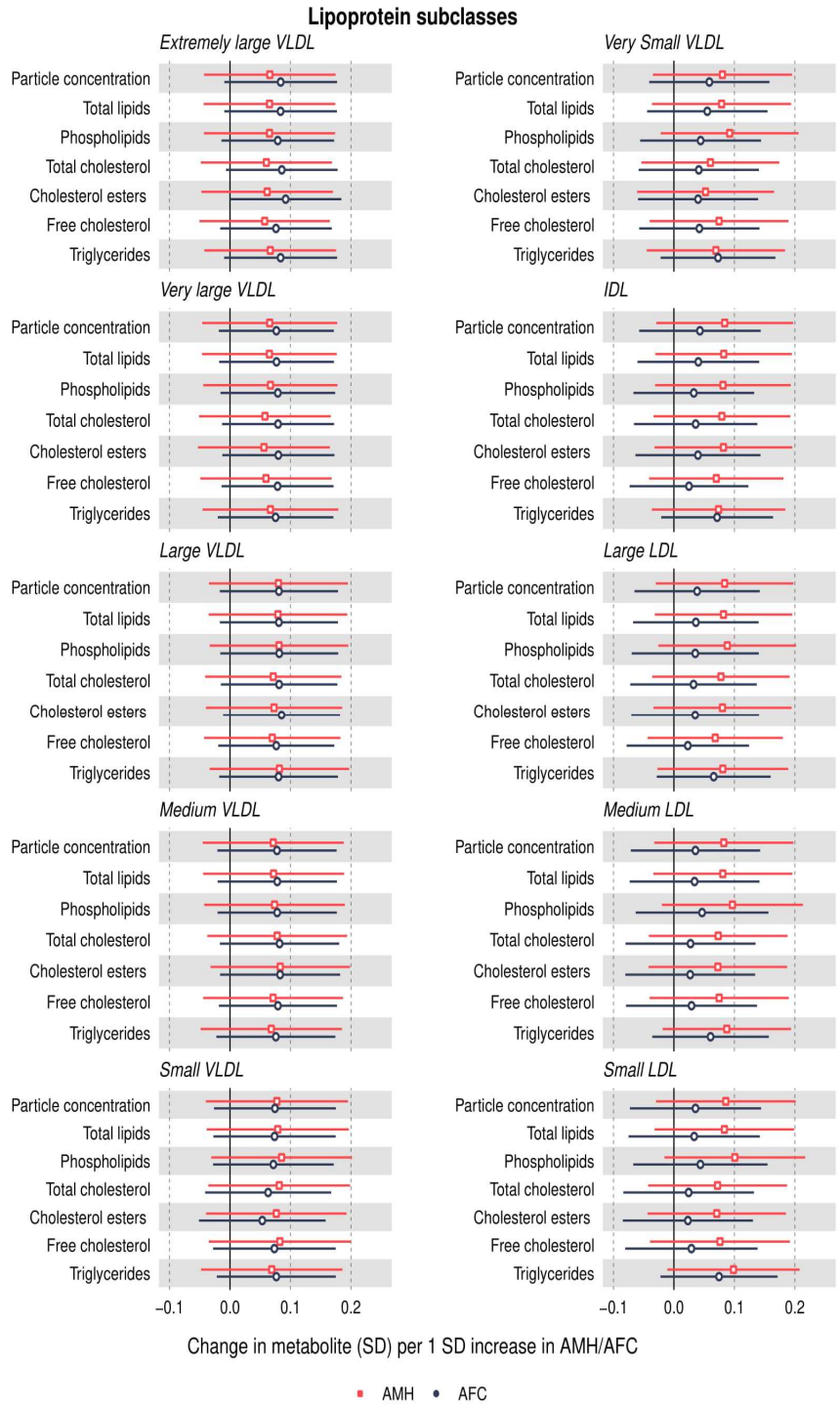


Figure 3.2 Associations of lipoprotein classes with AMH and AFC in women awaiting IVF.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, cause of infertility, primary/secondary infertility.

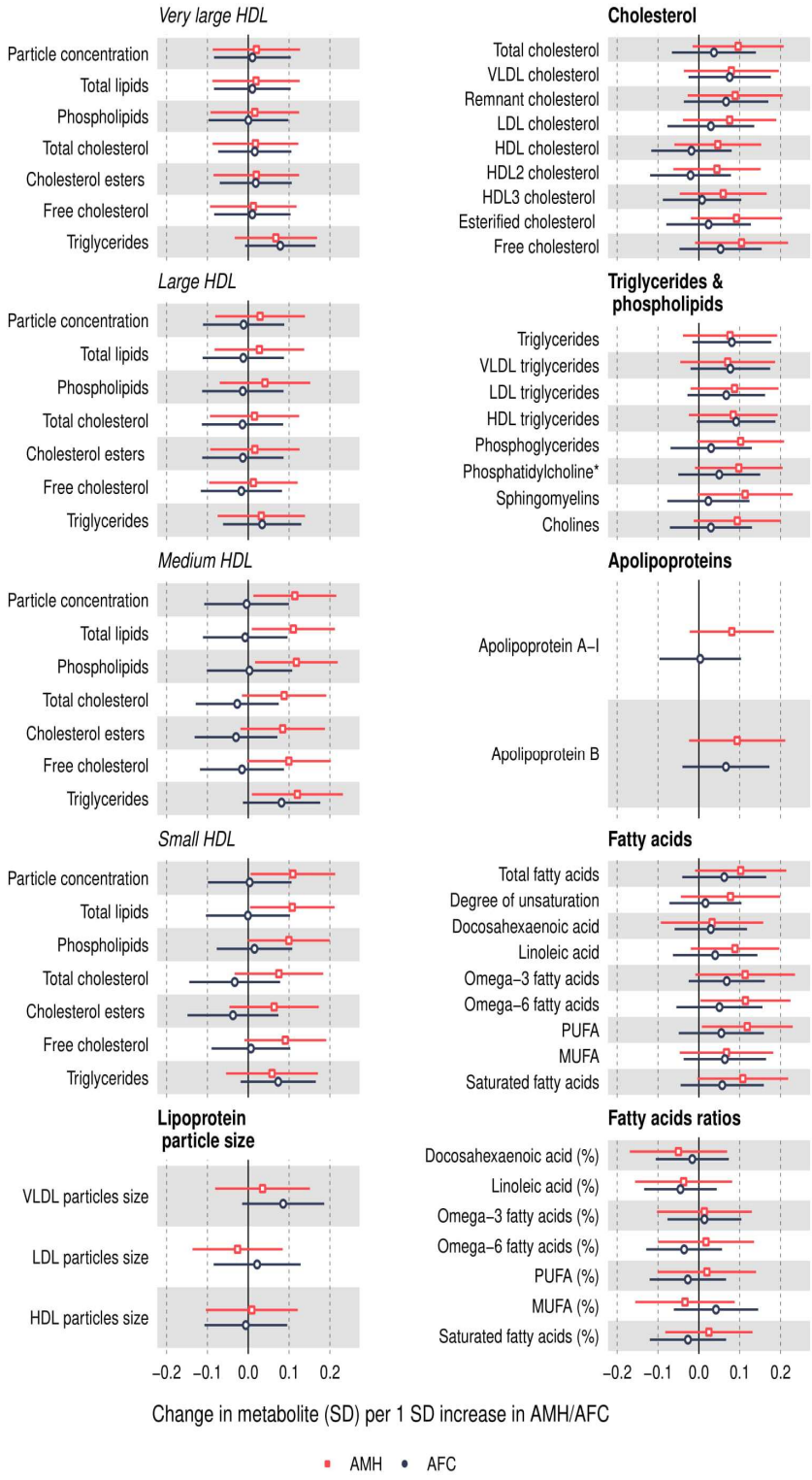


Figure 3.3 Associations of lipoprotein classes and fatty acids with AMH and AFC in women awaiting IVF.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, cause of infertility, primary/secondary infertility.

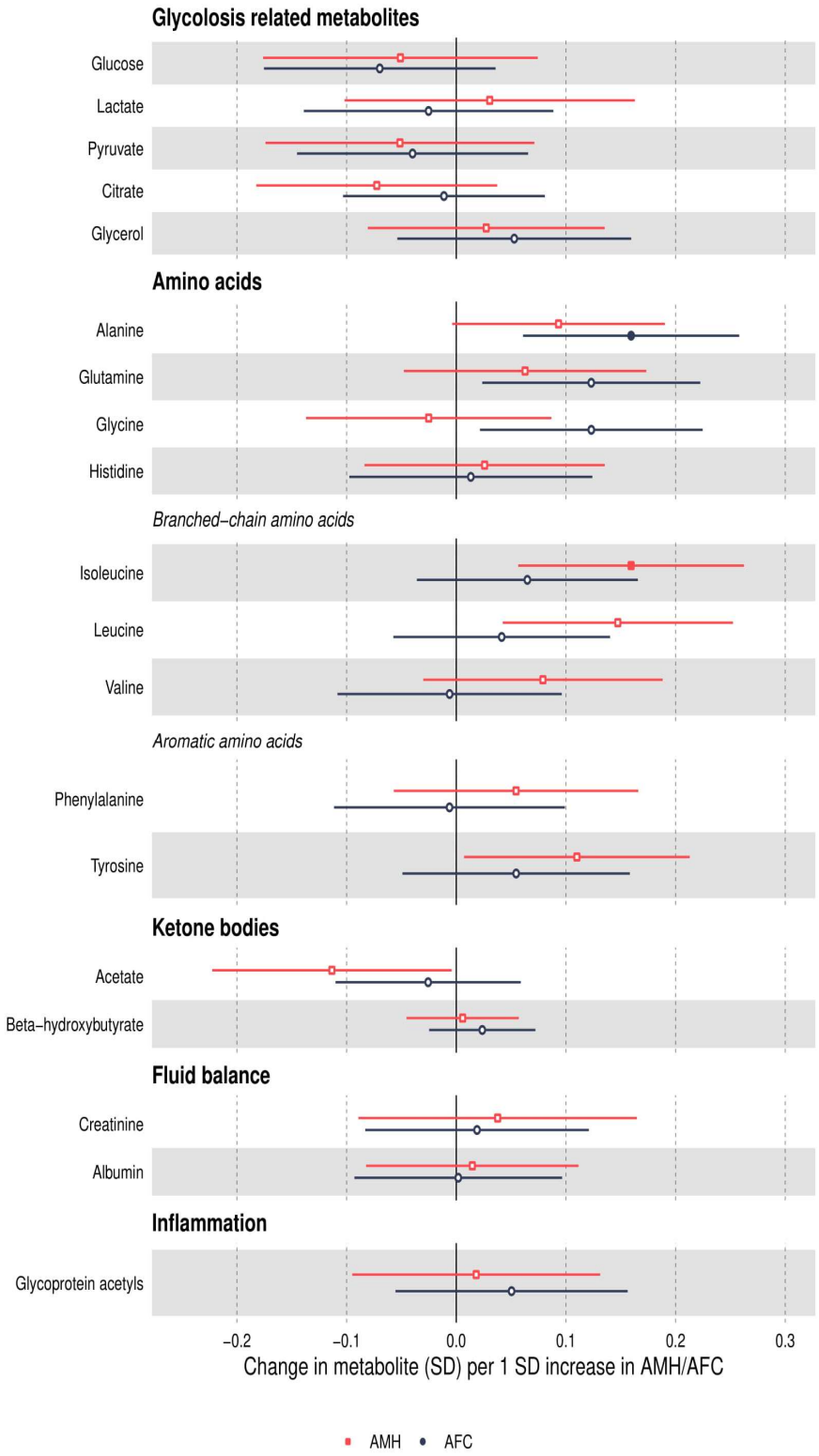


Figure 3.4 Associations of metabolic traits with AMH and AFC in women awaiting IVF.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, cause of infertility, primary/secondary infertility.

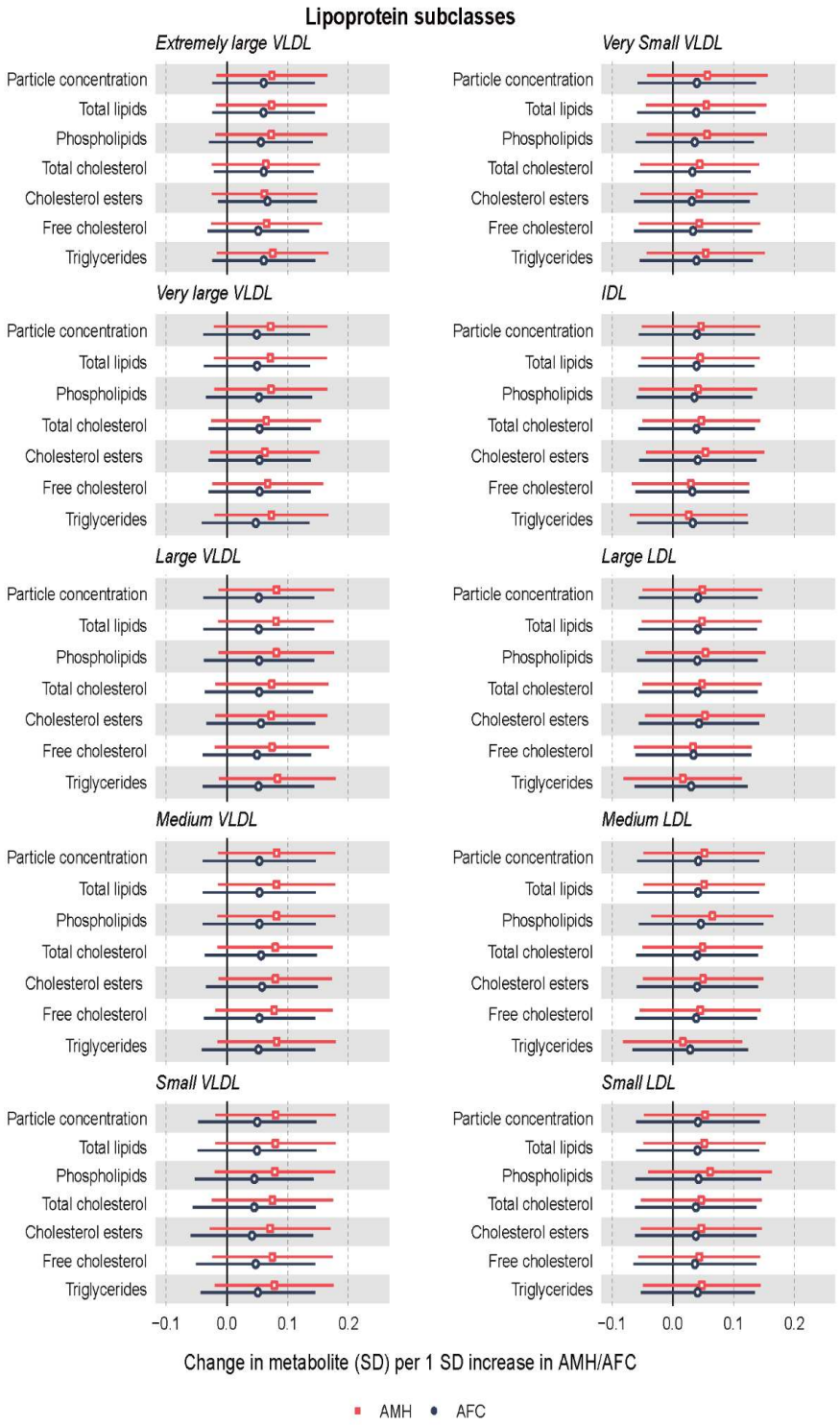


Figure 3.5 Associations of lipoprotein classes with AMH and AFC (unadjusted). Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black).

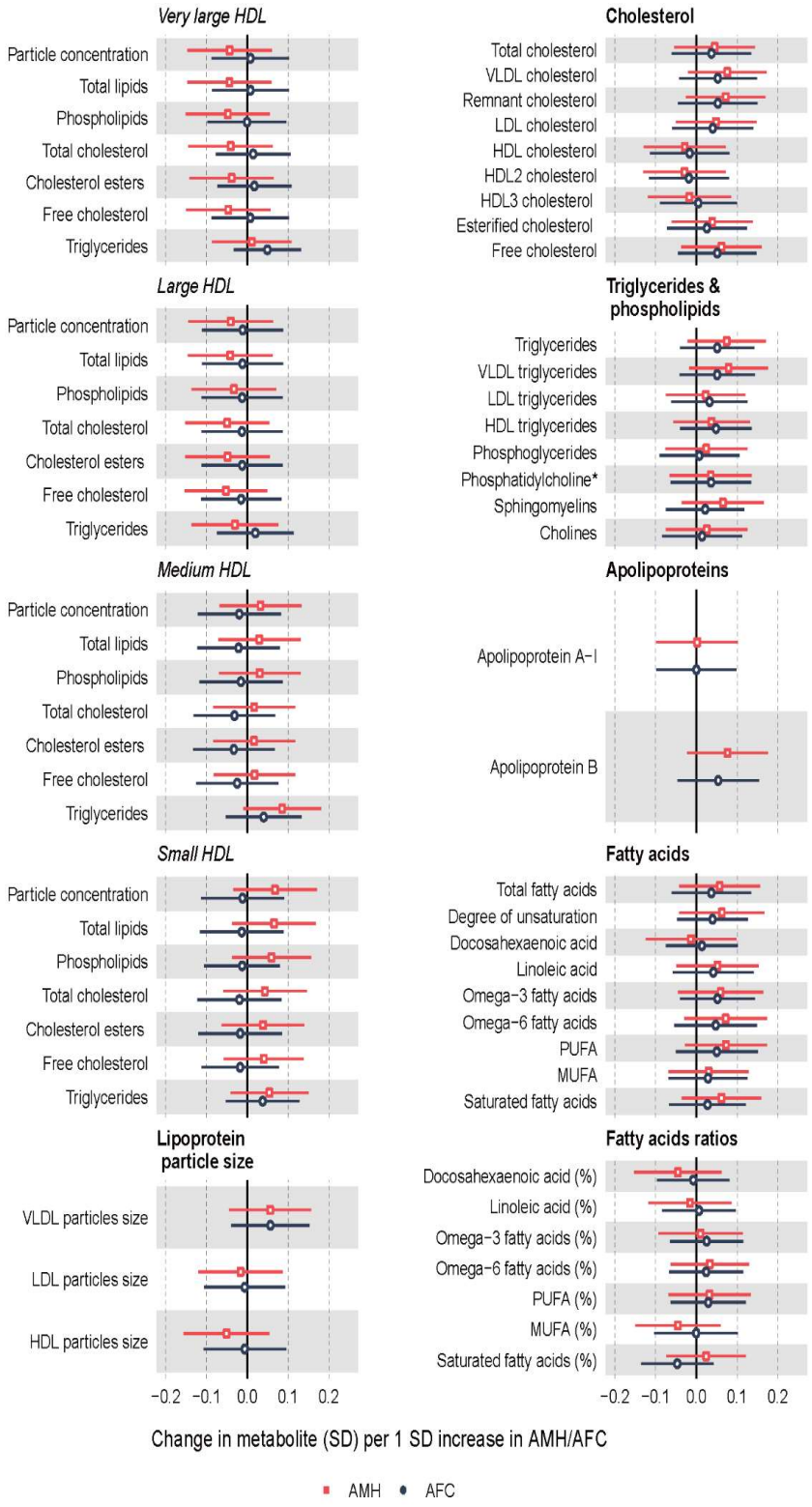


Figure 3.6 Associations of lipoprotein classes and fatty acids with AMH and AFC (unadjusted).
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black).

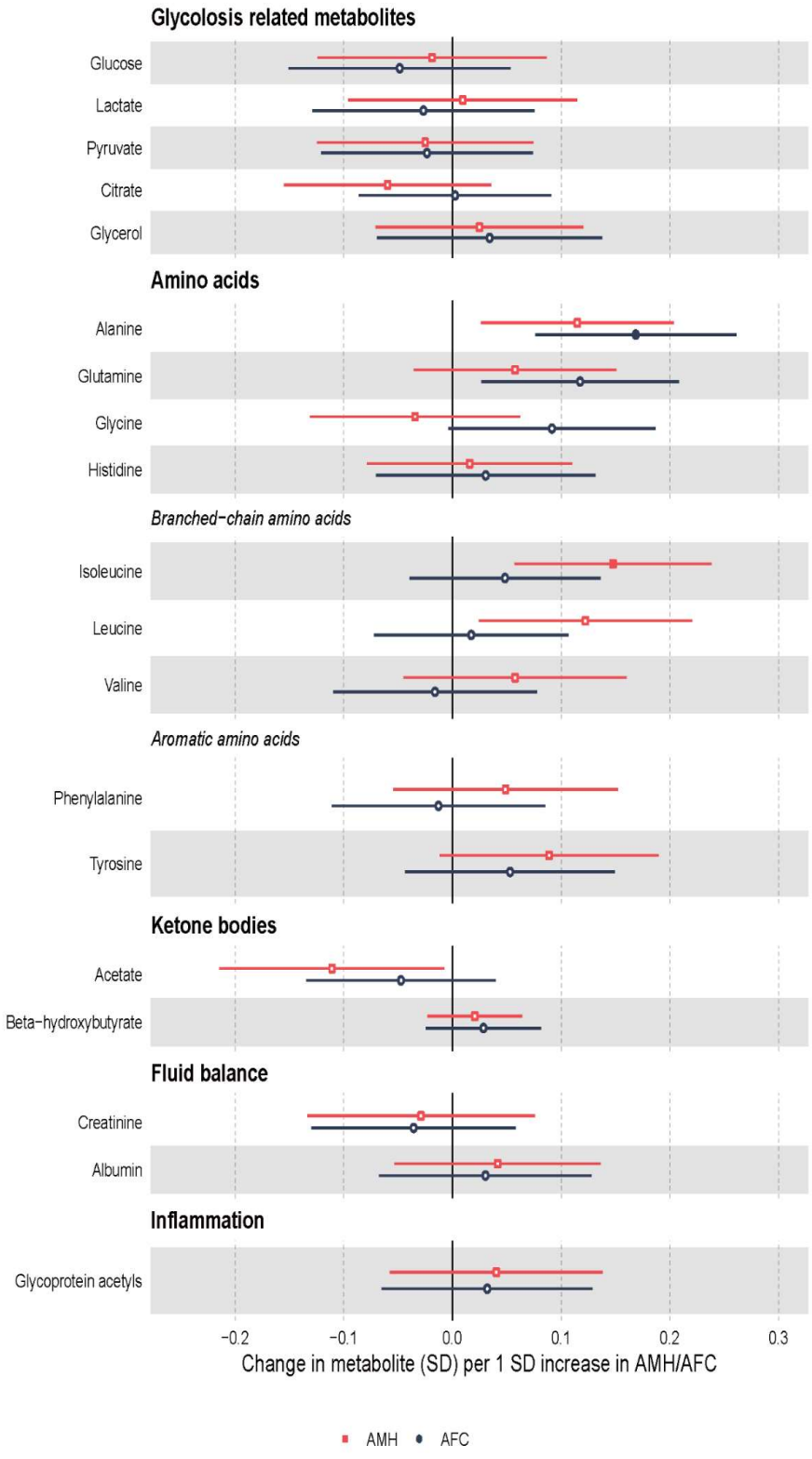


Figure 3.7 Associations of metabolic traits with AMH and AFC (unadjusted). Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black).

Overall, the associations for AFC with the metabolic measures were in the same direction as those observed for AMH, however they were weaker in magnitude, and for many of them close to unity (Figures 3.2, 3.3 and 3.4). The notable exceptions were the positive associations with the amino acids alanine, glutamine and glycine.

There was no strong evidence of departure from linearity for any of the observed associations (all likelihood ratio p-values comparing a model with four categories as 3 indicator variables to the simpler model as a 4 level score ≥ 0.05). When analyses were repeated only in those undergoing assisted conception due to partner infertility (n=87), most of the 155 associations of AMH were stronger than those in the main analyses including all women (Figure 3.8a). This included strengthening of the previously noted associations of AMH with the metabolomic markers. Positive associations were also observed for the various lipid concentrations within the medium and small very-low-density lipoproteins, intermediate-density lipoprotein and low-density lipoproteins, cholesterol, the mono-unsaturated fatty acids, and alanine, with a negative association with glucose emerging; the negative association with acetate attenuated (Figures 3.9, 3.10 and 3.11). In this subgroup some associations of AFC also strengthened (Figure 3.8b), with positive associations with various lipid concentrations across the size range of the very-low-density lipoproteins, triglycerides, apolipoprotein-B, mono-unsaturated fatty acids, and the amino acids alanine and glutamine (Figures 3.12, 3.13 and 3.14). When analyses were compared between the whole cohort and after exclusion of women with PCOS (n = 24), the associations were similar for AMH and AFC with correlations of 0.97 and 0.98, respectively (shown in Figure 3.15).

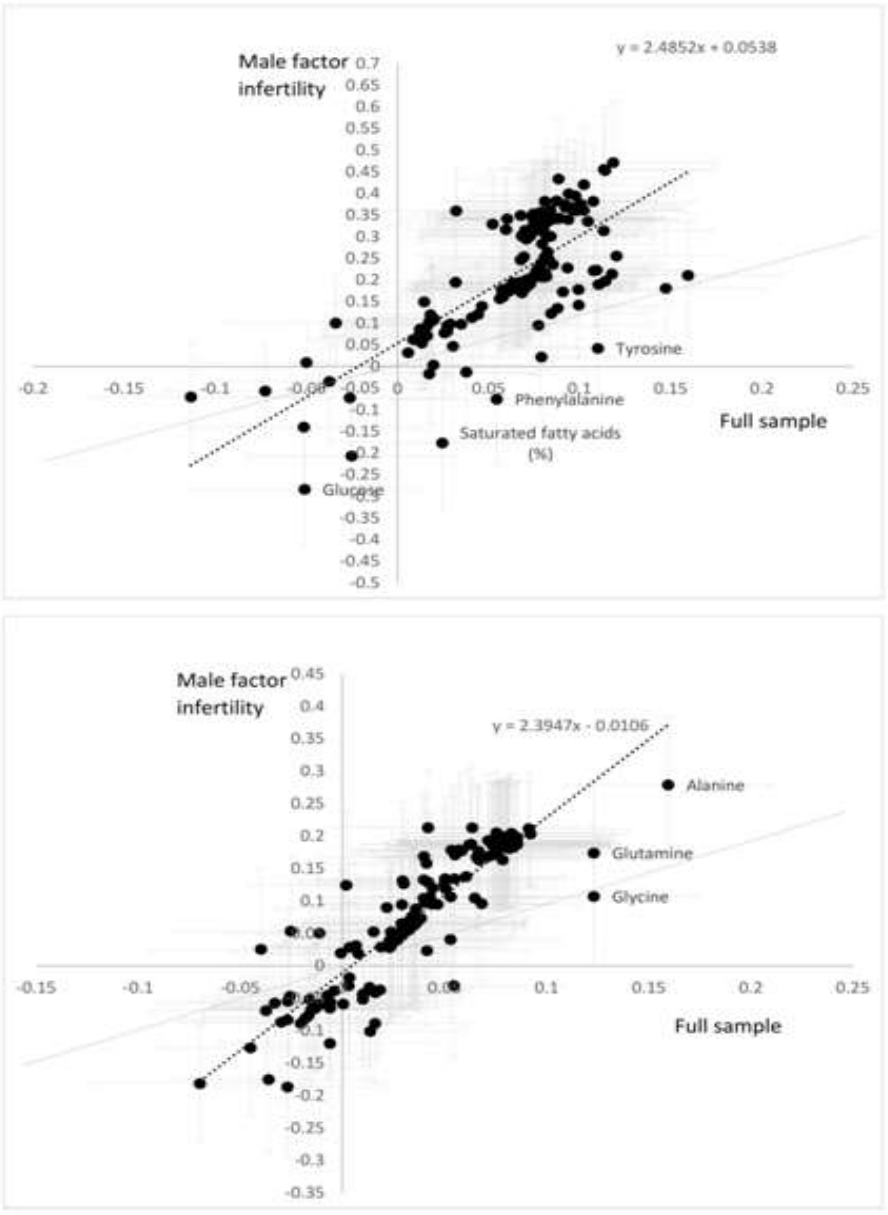


Figure 3.8 Scatterplot of associations between AMH and AFC and metabolites in full sample (N = 398) and sub-sample restricted to females with a reported male partner cause of infertility (N = 87).

Figure 3.8a Top panel shows associations with AMH, Figure 3.8b lower panel shows associations with AFC

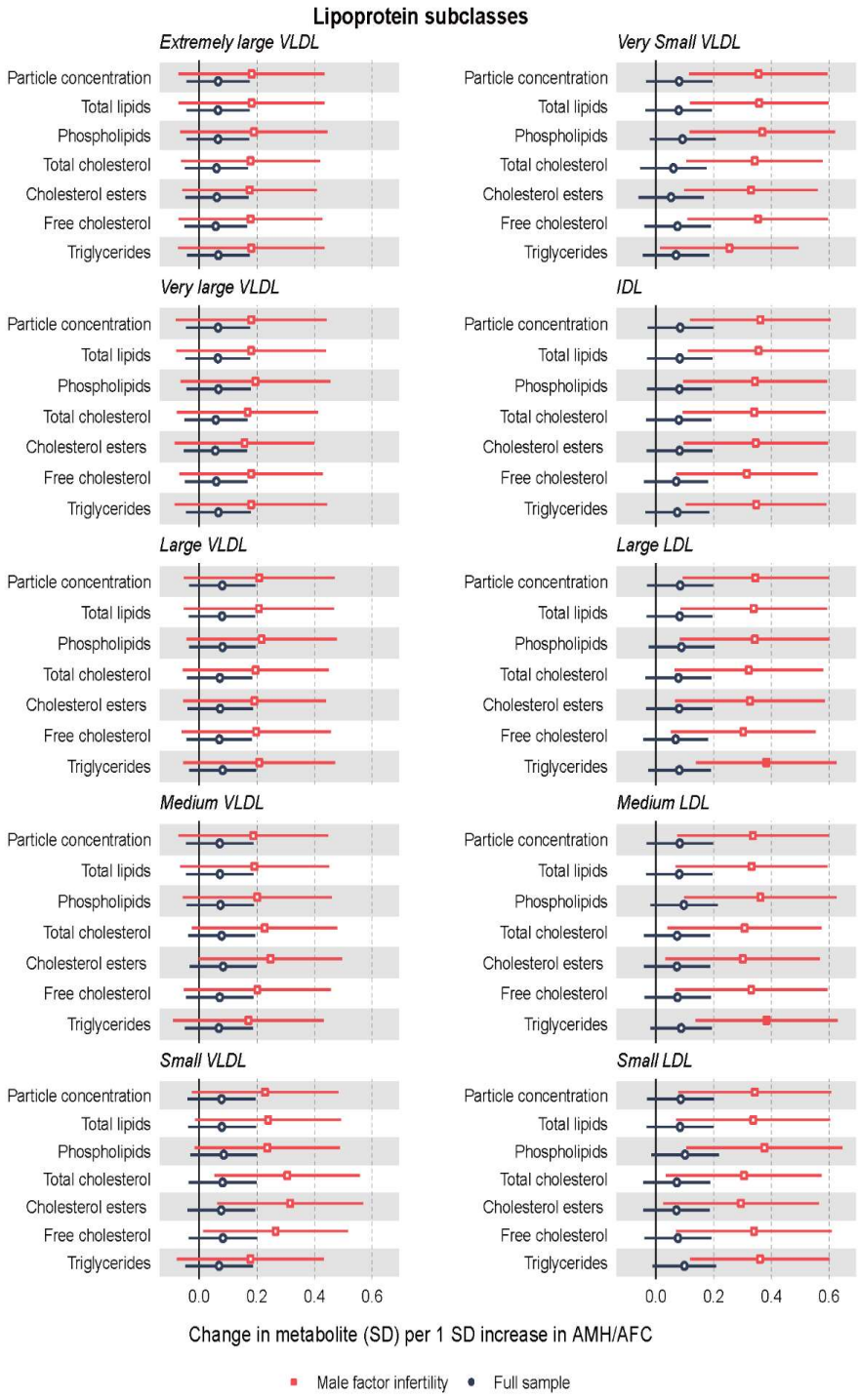


Figure 3.9 Associations of lipoprotein classes with AMH in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

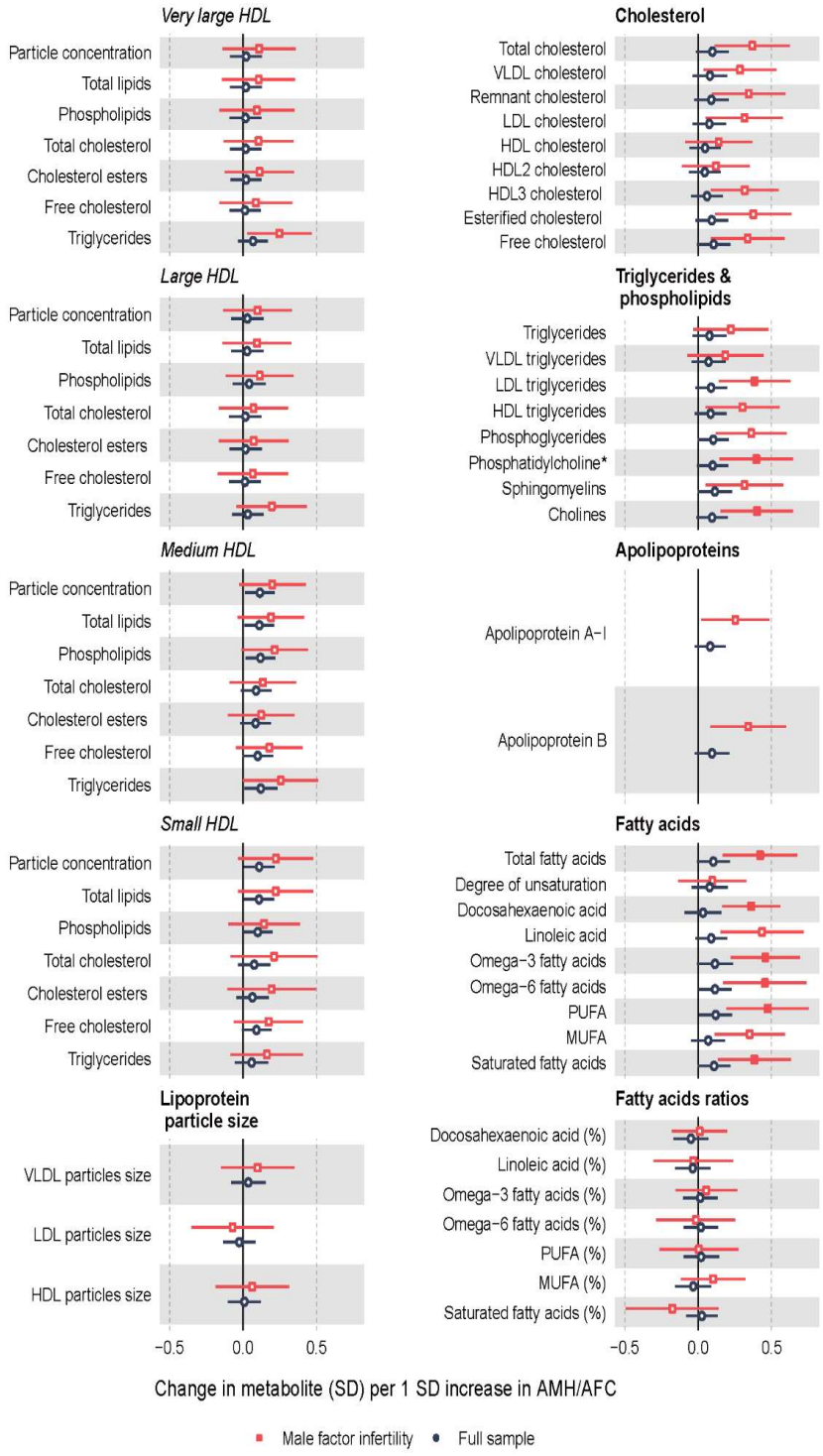


Figure 3.10 Associations of lipoprotein classes and fatty acids with AMH in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

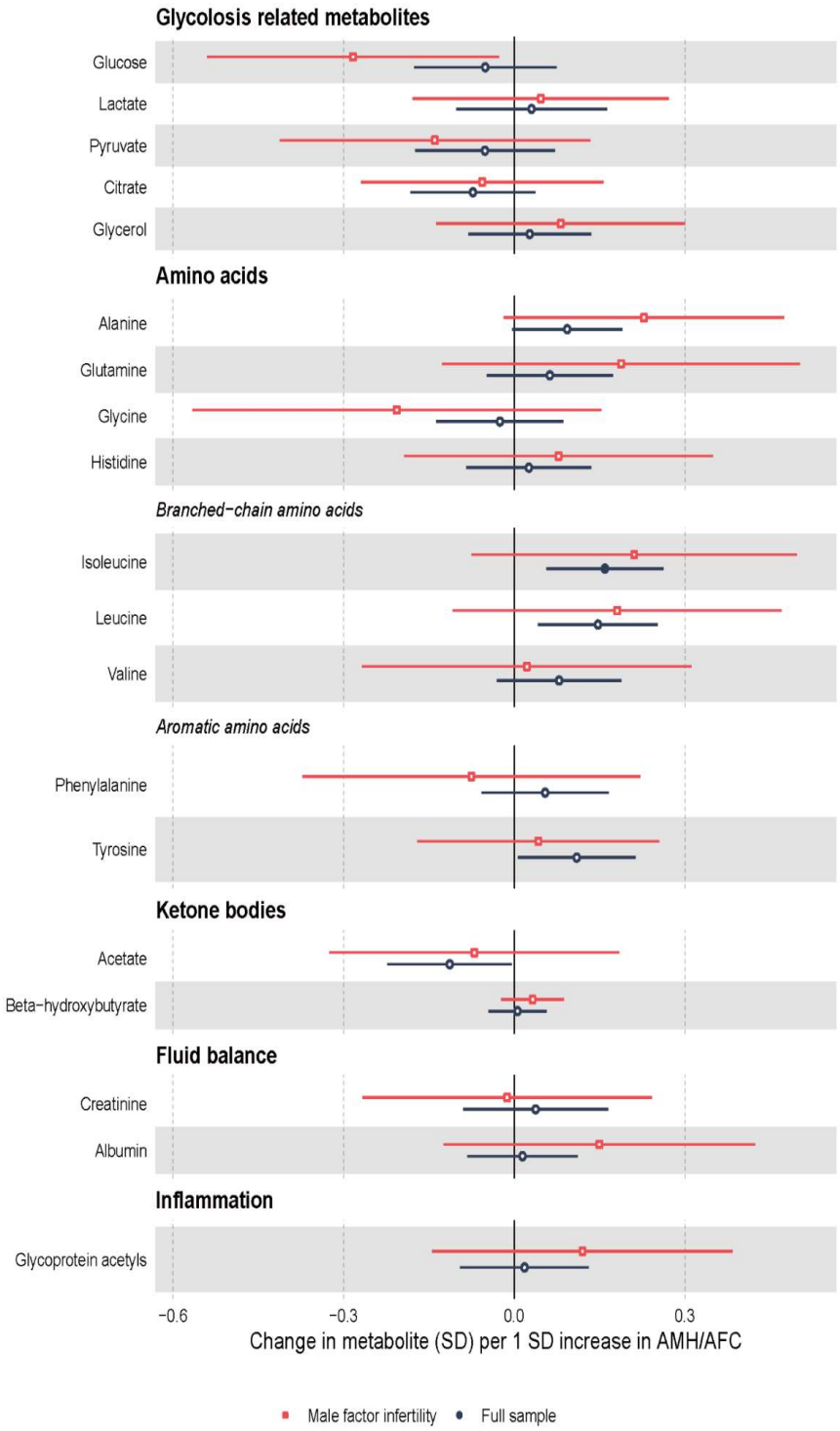


Figure 3.11 Associations of metabolic traits with AMH in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

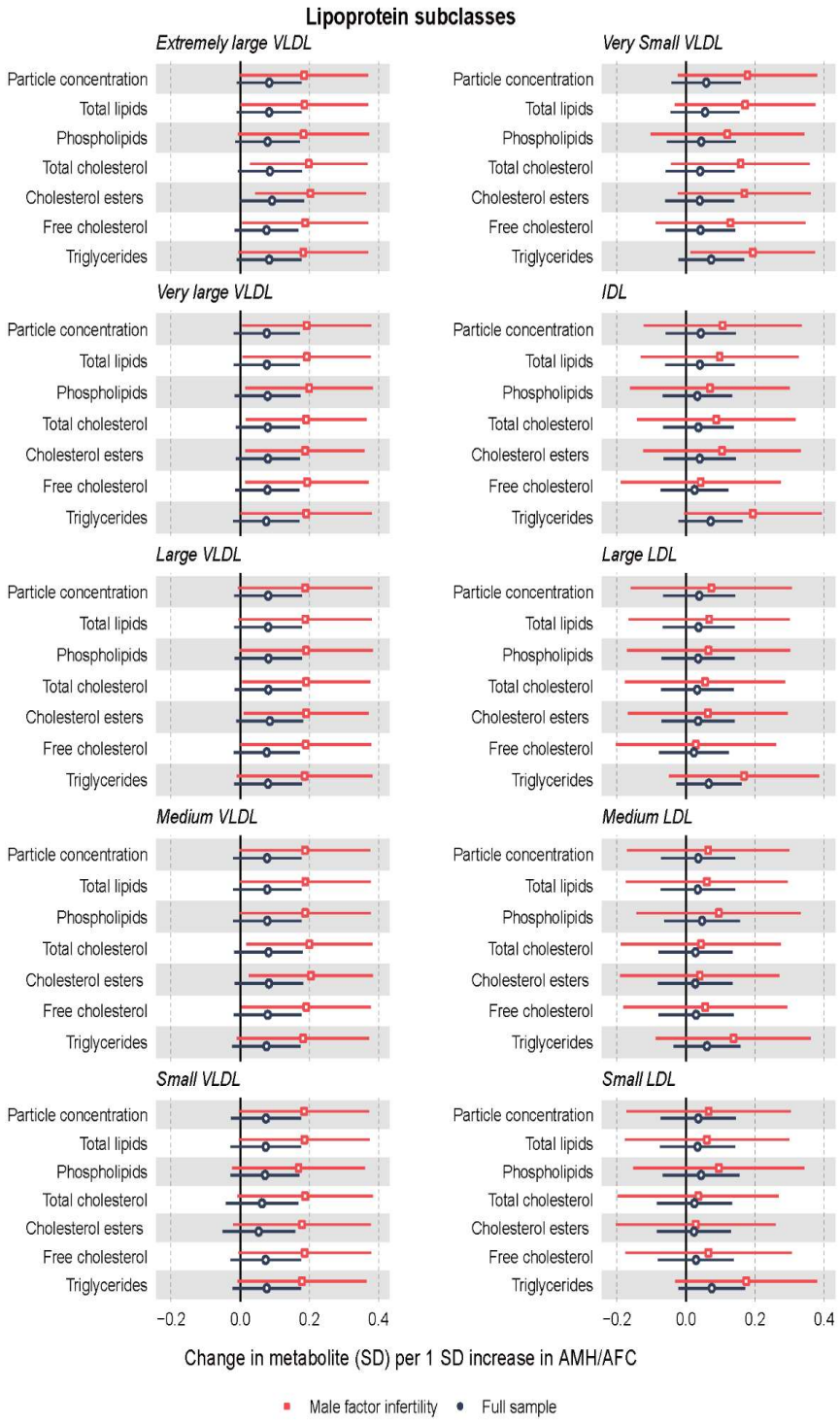


Figure 3.12 Associations of lipoprotein classes with AFC in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

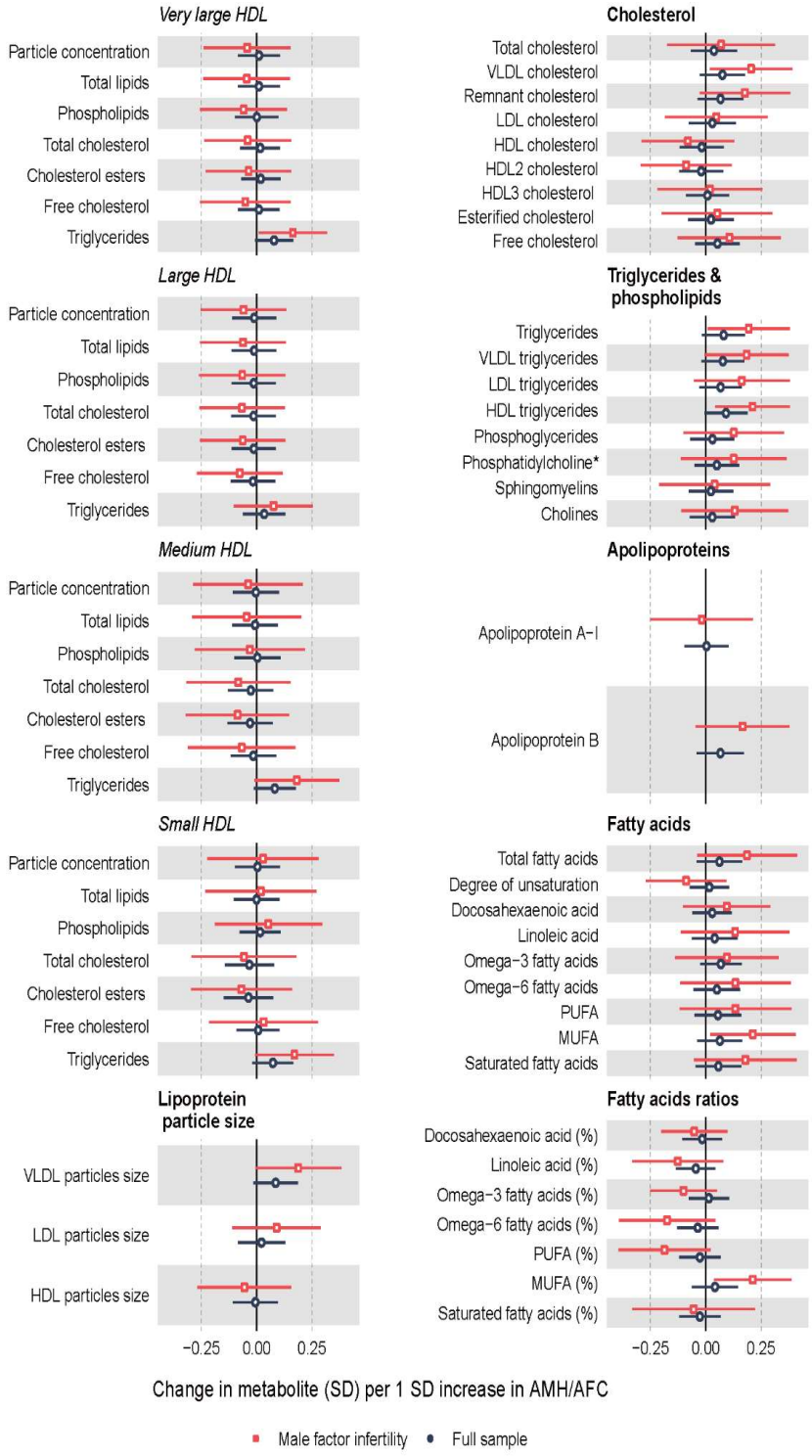


Figure 3.13 Associations of lipoprotein classes and fatty acids with AFC in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

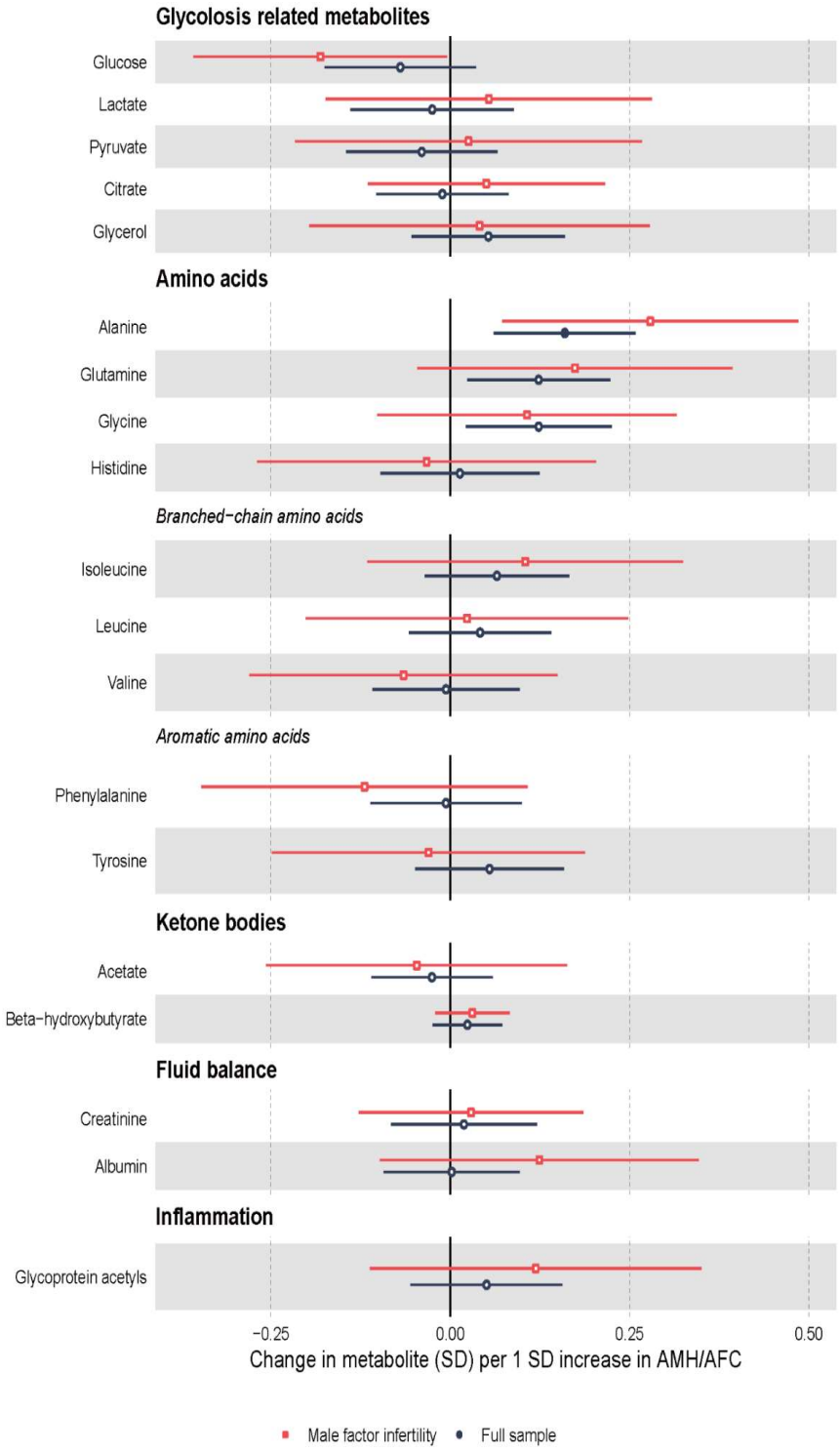


Figure 3.14 Associations of metabolic traits with AFC in women with male factor infertility or no male partner.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for AMH (red) and AFC (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, primary/secondary infertility.

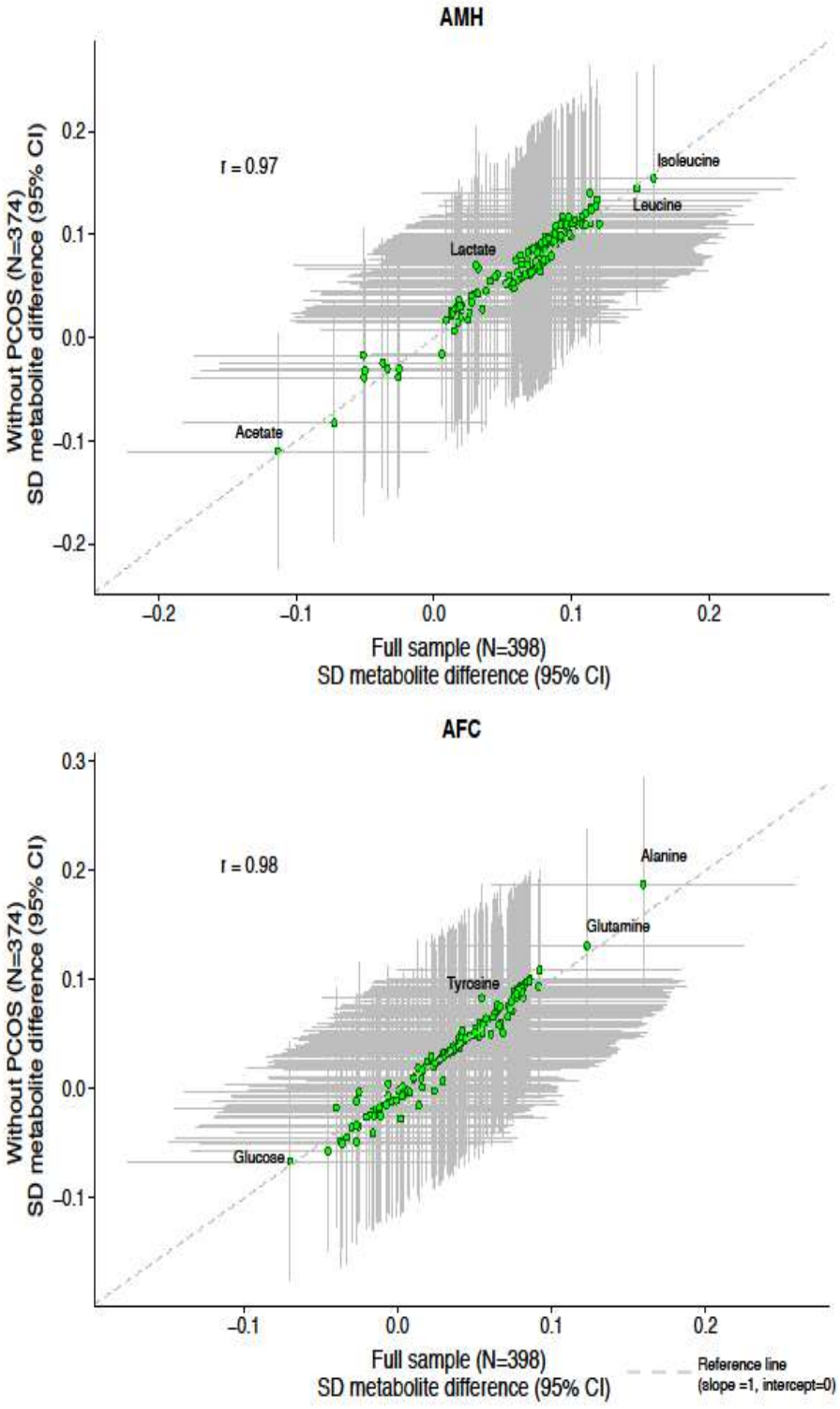


Figure 3.15 Comparison of associations for AMH and AFC for whole cohort and with PCOS cases (n=24) removed.

3.4 Discussion

In this cross-sectional study of women attending a fertility clinic for evaluation I identified several novel associations between the biomarkers of ovarian reserve, as measured by AMH and AFC, and circulating metabolites. Specifically, AMH showed positive associations with the amino acids isoleucine, leucine and tyrosine, Medium HDL and Small HDL, omega-6 fatty acids and polyunsaturated fatty acids, and a negative association with acetate. AFC had directionally consistent associations to those seen for AMH but the magnitudes of association (measured on the same scale as SD difference in metabolite for a 1SD increase in AMH or AFC) were weaker overall. Statistical evidence for positive associations with alanine, glutamine and glycine were observed. In my main analyses, including all women, I did not find strong statistical support for either biomarker being associated with an extensive lipid profile. When I restricted analyses to the subgroup (22%) of women with male partner infertility, observed associations strengthened and we observed positive associations of AMH and AFC with a range of lipids, suggesting that the women infertility phenotypes may mask associations with lipid profiles.

Previous studies have suggested an association of lower ovarian reserve with vascular health (Kat *et al.*, 2017; Appt *et al.*, 2012; Looby *et al.*, 2016). Whether alteration in serum lipids contribute to this association is unclear. One small study (n=50) reported a positive correlation between AMH and total cholesterol and LDL-C, and inverse correlation with HDL-C (Skalba *et al.*, 2011), while a larger study (N=252) of women with PCOS, found a weak positive correlation with HDL-C, which was attenuated when adjusted for BMI (Feldman *et al.*, 2017). In contrast, larger studies of Chinese women did not observe an association between AMH and any of total cholesterol, LDL-C, HDL-C or triglycerides in either women with PCOS (N=304), infertile women (N=1,896) (Cui *et al.*, 2014), or in a general cohort of women (N=6,763) (Cui *et al.*, 2016). This is consistent with our own findings, with limited statistical evidence of an association between AMH and lipoprotein subclasses, lipoprotein particle size, cholesterol, LDL or HDL subtypes or triglycerides, phosphoglycerides, or apolipoproteins when the whole population was considered. Collectively, the evidence to date (including from our study) would support recent observational studies suggesting that the association of AMH with atherosclerosis may be independent of lipid levels (Kat *et al.*, 2017; Appt *et al.*, 2012; Looby *et al.*, 2016). As an alternative mechanism it has been proposed

that AMH has a direct effect on cardiovascular tissue (McLennan & Pankhurst, 2015), making it more prone to injury and atherosclerosis (Kat *et al.*, 2017). Though specific evidence for this is lacking. We did not demonstrate associations between AFC and lipids, which have to our knowledge not been previously explored, further supporting suggestions that alternative mechanisms to dyslipidaemia may underlie the relationships between ovarian reserve and cardiovascular disease. Though as acknowledged below the greater variability in AFC compared with AMH may have attenuated some of these results towards the null.

I report a positive association between AMH and higher circulating levels of PUFA and omega-6 fatty acids. However, a systematic review and meta-analysis found no evidence of either observational associations between dietary intake or measured circulating concentrations omega-6 on cardiovascular diseases or any effect of dietary supplementation with omega-6 in randomised controlled trials (Chowdhury *et al.*, 2014), suggesting that even if the association of AMH with omega-6 is causal this is unlikely to be a mechanism for preventing cardiovascular diseases.

Branch chain amino acids (BCAA), such as isoleucine, leucine, and valine, have been found to positively associated with a number of cardiometabolic risk factors, including adiposity, fasting glucose, insulin resistance, blood pressure, dyslipidemia, and indicators of coronary artery disease, in cross-sectional studies (Tobias *et al.*, 2018). There are also positive associations of BCAA and aromatic amino acids with incident cardiovascular events in several studies large prospective studies (Wurtz *et al.*, 2014; Shah *et al.*, 2010; Magnusson *et al.*, 2013; Ruiz-Canela *et al.*, 2016). However, whether these are causally related is at present unknown. My observed AMH-BCAA association are of interest, but replication of these findings and confirmation of biological plausibility of causality would be required to assess whether the association of AMH with CVD may be mediated to some extent by BCAA.

The same NMR platform has previously been used to identify 14 metabolites associated with all-cause mortality in a meta-analysis of 12 cohorts and 44,168 participants (Deelen *et al.*, 2019), with subgroup analyses of 7603 participants

identifying seven metabolites (XXL-VLDL-L, PUFA, lactate, histidine, leucine, phenylalanine and albumin) inversely associated with cardiovascular mortality and three (glucose, lactate and glycoprotein acetyls) positively associated with cardiovascular mortality (Deelen *et al.*, 2019) . Overall, the observed direction of associations wass consistent with markers of low ovarian reserve relating to these metabolites that have previously been shown to robustly associated with cardiovascular disease mortality. Associations of AMH with PUFA and leucine met our adjusted statistical threshold. These findings, if replicated in larger cohorts, would support the overall concept that a diminished ovarian reserve may be associated with an unfavourable circulating cardiometabolic risk profile (Kat *et al.*, 2016).

This study has several strengths. To my knowledge, I am not aware of any study with a similar or larger sample size with detailed phenotypic and metabolite measurements. All women attended during the early follicular phase for measurement of AMH and AFC. I included all women across the range of ovarian reserve including some women with extremely low and high AMH and AFC. I wrote, and have used, a prespecified analysis plan which incorporated adjustment for a wide range of a priori specified potential confounders. Sub-group analyses in those women who were awaiting assisted conception because of male partner infertility were also assessed, as these women may reflect a general population of women of reproductive age.

I do however acknowledge several limitations. The analyses are cross sectional and therefore could be explained by variation in metabolism (e.g. of amino acids) influencing ovarian reserve rather than the other way around, as we have assumed. Furthermore, I cannot assume that the small number of associations identified are causal. Residual confounding may have resulted from crude questionnaire measurements of physical activity, alcohol intake and family history of cardiovascular disease and the lack of any data on dietary intake. However, I adjusted for the measures we had of alcohol, physical activity and family history of cardiovascular disease, as well as education and BMI, which influence diet and physical activity, or are influenced by it, and thus may have captured key confounding paths. Of note our adjustment for confounders is more extensive than previous studies of the association of AMH with lipids and other cardiovascular risk

factors. Whilst, this is one of the larger studies to explore these associations our results were imprecisely estimated, with wide confidence intervals and we are not aware of any independent study that has measures of ovarian reserve and multiple metabolites (or even the amino acids and fatty acids that we observed associations with) in which to attempt to replicate our findings.

Women were awaiting IVF and related to that were confirmed (through cotinine breath test) non-smokers and were of a relatively restricted BMI range. The women were also largely white European and educated to degree level. This homogeneous relatively healthy population may have resulted in some selection bias and may mean that our results do not generalise to a general population of women of reproductive age or other infertile populations. AFC was measured by several operators, and the known intra- and inter-observer variability of AFC (Iliodromiti *et al.*, 2014) and application of a threshold for counting per ovary, with the highest value for AFC of 40 (range 0-40), as compared to AMH of 171 (range 1-171 pmol/l), may explain why I did not see the same strength of associations of AFC with the various metabolites compared to what I observed with AMH. AMH was measured in a single laboratory on an automated analyser with a wide analytical range and low coefficient of variation.

Analyses were undertaken on non-fasting samples. This was necessary to align with clinical processes for a population who are undergoing assisted conception, where caloric restraint may be detrimental. In collaborations of several studies using this same NMR analysis platform results have not differed notably between studies in which the analyses were undertaken in participants who had been advised to fast and those who had not, including in analyses exploring the associations of these metabolites with cardiovascular diseases (Wurtz *et al.*, 2015).

The NMR platform used in these analyses covers considerably more of the lipidome than conventional clinical chemistry measures (total cholesterol, LDL-C, HDL-C and triglycerides) that have previously been explored in relation to ovarian reserve, and in addition includes fatty acids, amino acids, glycolysis metabolites, ketone bodies and an inflammatory marker. I acknowledge it misses a high proportion of the currently quantifiable metabolites in human serum/plasma, including markers of energy balance, microbiota metabolism, vitamins, co-factors

and xenobiotics, that may be influenced by ovarian reserve. High through put analyses of a wider range of metabolites measured by mass spectrometry is possible in epidemiological studies but considerably more expensive than the NMR platform used here, resulting in their use being frequently restricted to subsamples of cohorts (Sovio *et al.*, 2020).

3.5 Conclusion

This study provides novel insight into the association of the ovarian biomarkers, AMH and AFC, with metabolic profiles. Taken together with other recent studies my results suggest that dyslipidaemia may have a limited role to play in the relationship between ovarian reserve and cardiovascular diseases. The novel associations we find with some fatty acids and amino acids may have a role in mediating any effect of ovarian reserve on cardiometabolic diseases, but these require replication in large prospective studies, and we cannot assume that the small number of associations identified are causal.

Chapter 4 Association of the serum metabolomic profile by nuclear magnetic resonance spectroscopy with sperm parameters: A cross sectional study of 325 men.

4.1 Introduction

Despite increasing concern regarding the significant decline in sperm counts over the last few decades (Levine, et al., 2017), therapeutic options for male infertility are limited (Barratt, et al., 2017). The significant body of data to support the concept that oxidative stress (Aitken, et al., 2014) and also potentially metabolic dysfunction (Sermondade, et al., 2013) are associated with sperm dysfunction, have driven recent intervention trials to primarily focus on preconceptual optimisation of health (Barratt, et al., 2017, Schisterman, et al., 2020, Smits, et al., 2019).

Robust identification of additional modifiable and non-modifiable risk factors for poor semen quality have however had limited success. For example smoking (Li, et al., 2011, Povey, et al., 2012), adiposity (Bieniek, et al., 2016, Macdonald, et al., 2013, Pacey, et al., 2014, Povey, et al., 2012, Sermondade, et al., 2013) and alcohol excess (Marinelli, et al., 2004, Muthusami and Chinnaswamy, 2005, Povey, et al., 2012) have all shown non-consistent associations with the risk of poor semen parameters. Consequently, advice is primarily based on a "good health advice" model, rather than robust evidence for a detrimental effect on semen parameters (Pfeifer, et al., 2017). An alternative approach to identify risk factors for an abnormal semen analysis would be to examine circulating metabolites, which may provide insights into downstream factors as well as more upstream exposures (Soininen, et al., 2009). Detailed metabolic profiling has previously been used to explore the metabolic pathways that may underpin phenotypes, including adiposity (Wurtz, et al., 2014), and has been widely used in epidemiological studies to identify novel risk factors for a variety of reproductive and pregnancy characteristics (Mills, et al., 2019, Taylor, et al., 2019, Wang, et al., 2016, Wang, et al., 2016, Wang, et al., 2016, Wurtz, et al., 2015, Wurtz, et al., 2017). The application of NMR spectroscopy to male infertility has however been limited to the assessment of selected metabolites within the sperm metabolome (Calvert, et al., 2018, Paiva, et al., 2015, Reynolds, et al., 2017) or

seminal fluid (Neto *et al.*, 2020; Morgia *et al.*, 2020; Mehrparvar *et al.*, 202; Mumcu *et al.*, 2020). Alternative metabolomic approaches (liquid chromatography coupled to mass spectrometry (LC-MS) or high resolution mass spectrometry (LC-HRMS) or gas chromatography-mass spectrometry (GC-MS)) have been undertaken on plasma, serum, seminal plasma and urine, but studies have been of varying size (n=30 to 260), primarily used a case-control design with no adjustment for confounders, have variable thresholds for defining cases and have utilised conventional discovery based metabolomic approaches that have not provided absolute quantities (Qiao *et al.*, 2017; Ma *et al.*, 2019; Zhang *et al.*, 2014; Courant *et al.*, 2013).

The aim of the current study was to undertake an exploratory analysis of the association of 155 circulating metabolic measures with sperm parameters. These measures were profiled by a high throughput cost efficient NMR platform, covering a range of metabolic pathways, predominantly a lipidome, including lipoprotein lipids, fatty acids, as well as some amino acids, ketone bodies, and glycaemic traits.

4.2 Methods

4.2.1 Study Design and Participants

Cross-sectional study of men aged 18 to 55 who presented to Glasgow Royal Infirmary, UK between 1 April 2017 and 31 March 2019 for assessment prior to assisted conception. A total of 331 men were recruited and of these 325 (98%) had complete data on semen parameters and at least some NMR metabolites and were included in the analyses presented in this chapter.

The study was conducted according to ICH Guideline for good clinical practice, the Declaration of Helsinki and the Convention of the Council of Europe. All men provided written informed consent. The study protocol was approved prior to study initiation by the relevant institutional review boards (see Materials & Methods Chapter).

4.2.2 Study procedures

Demographic, lifestyle, fertility and medical history was obtained by self-reported questionnaire and clinical data by linkage to electronic medical records. Blood samples were taken for NMR analyses and immediately spun and frozen at −80 °C. All NMR assays completed for this study were undertaken within 1 year of storage and with no previous freeze/thaw cycles.

4.2.3 Semen analysis

The semen sample was provided in the morning of the same day as the blood sample for NMR analyses. Semen analysis was completed utilizing the World Health Organisation guidelines (WHO, 2010). The men were advised to produce a semen sample after 2–3 days of sexual abstinence. The semen sample was collected in the hospital or at home in the morning (instructions to produce sample within one hour of semen analyses appointment which were at 30 minute intervals from 0800 to 1000) of the same day as the blood sample for NMR analyses were taken (with blood samples taken between 1330 and 1600). Giving a range of 3.5 hours to 8 hours between ejaculation and the blood sample for NMR analysis. Semen volume was measured using a graded tube. The concentration was measured in an Improved Neubauer chamber in two replicates and at least 200 spermatozoa counted in each replicate, at a magnification of 200×. Where the difference between the counts was greater than acceptable based on the 95% confidence intervals, the first two values were discarded, and two fresh dilutions of semen prepared and assessed. Motility was scored manually, as percentages of (A) fast forward progressive, (B) slow forward progressive, (C) non-progressive and (D) immotile spermatozoa in 200 spermatozoa in at least five power fields per replicate, according to recommendations (WHO, 2010). The Glasgow Royal Infirmary clinic from which study participants were recruited, collaborates in a nationwide quality control system organized by the UK National External Quality Assessment Scheme (NEQAS). Where sperm motility categories did not total to 100% (N=11), motility was rescaled by dividing by the total percentage and multiplying by 100. For those men where the initial and repeat sample exhibited azoospermia a surgical sperm retrieval was performed, and the sample evaluated prior to cryopreservation.

4.2.4 Total Motile Sperm Count

The men were grouped according to the Total Motile Sperm Count (TMSC), calculated by multiplying the sample volume by the density and the percentage of A and B motility divided by 100%. Three groups were created: men with TMSC > 15 million (M); men with a TMSC of 5 to 15M; and men with TMSC of <5M (including those with azoospermia) (Borges Jr, et al., 2016, Hajder, et al., 2016, Tiegs, et al., 2019) (further details can be founr in chapter 2 section 2.5)

4.2.5 NMR protocol

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform (Nightingale Health© (Helsinki, Finland)) at the University of Bristol. The platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, various glycolysis precursors, ketone bodies, and amino acids in absolute concentration units. The NMR-based metabolite quantification is achieved through measurements of three molecular windows from each serum sample. Two of the spectra (LIPO and LMWM windows) are acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts are conducted at $37^{O}C$ and $22^{O}C$, respectively. Details of this platform have been published previously (Soininen, et al., 2009, Wurtz, et al., 2017) and it has been widely applied in genetic and observational epidemiological studies (Fischer, et al., 2014, Mills, et al., 2019, Wang, et al., 2018, Wang, et al., 2016, White, et al., 2017, Wurtz, et al., 2015). Further details of the platform are provided in the Materials & Methods Chapter (Chapter 2).

4.2.6 Metabolite quantification and quality control

The NMR spectra were analysed for absolute metabolite quantification (molar concentration) in an automated fashion. For each metabolite, a ridge regression model was applied for quantification in order to overcome the problems of heavily overlapping spectral data. In the case of the lipoprotein lipid data, quantification

models were calibrated using high performance liquid chromatography methods, and individually cross-validated against NMR-independent lipid data. Low-molecular-weight metabolites, as well as lipid extract measures, were quantified as mmol/l based on regression modelling calibrated against a set of manually fitted metabolite measures. The calibration data are quantified based on iterative line-shape fitting analysis using PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland). Absolute quantification cannot be directly established for the lipid extract measures due to experimental variation in the lipid extraction protocol. Therefore, serum extract metabolites are scaled via the total cholesterol as quantified from the native serum LIPO spectrum.

4.2.7 Assessment of potential confounders

Age, BMI, educational attainment, ethnicity, family history of cardiovascular disease (defined as first degree relative affected), physical activity, alcohol intake, smoking status, and duration of infertility were considered as potential confounders because they plausibly influence both semen parameters and the NMR metabolites (Eisenberg, et al., 2015). All confounders were obtained by a questionnaire or from medical notes when the men were originally recruited.

4.2.8 Statistical analysis

All analyses were conducted using Stata (Version 15.1) and R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). An analysis plan was written in June 2019. Characteristics were summarized as number and percentage, total range, mean, standard deviation, median, and 25th and 75th quantiles (IQR) as appropriate. Multivariable linear regression was used to examine the associations of serum metabolic profiles (treated as exposures) with % progressive motility and sperm concentration (treated as outcomes). Robust standard errors were estimated for associations with continuous sperm outcomes. The metabolic measures were scaled to standard deviation (SD) units (by subtracting the mean and dividing by the standard deviation of all men included in the analyses). This scaling allows easy comparison of multiple metabolic measures with different units or with large differences in their concentration distributions. Sperm concentration (natural log transformed) and progressive motility were also scaled to SD units in the same way as the metabolites. Multivariable logistic regression was used to examine the associations of the serum

metabolic profiles (treated as exposures) with TMSC. Two analyses were undertaken: (i) exploring the odds of having a low TMSC (<15M) compared to a normal TMSC (i.e. >15M) and (ii) exploring the odds of having a very low TMSC (<5M) compared to a low or normal TMSC (i.e. ≥5M). We adjusted for all a priori selected confounders (age, ethnicity, education, family history of cardiovascular disease, BMI, physical activity, alcohol, smoking, and duration of infertility) in both the multivariable linear and logistic regression analyses.

4.2.9 Additional analyses

In addition to presenting our main results as difference in mean metabolite in SD units per SD of concentration or motility, I also present the full tabulated results (confounder adjusted) in the metabolite, concentration and motility original units in an online repository (https://bit.ly/35aUmDz). I repeated our main analyses on those men with a known male factor cause of infertility (N=66 (20% of the cohort)) and compared these associations to those men with an unknown or known female partner cause of infertility (N=258 (79%))(Figures 4.13 to 4.18). I wished to compare the point estimates in this group to the results of the whole cohort to provide some indication as to whether our results might be driven by the cause of infertility or potentially generalisable to men of reproductive age without infertility. I compared the magnitudes of the results in men who were going to undergo assisted conception because of the couple having unexplained or a known female cause of infertility with those of the whole cohort using a scatterplot. As 99% of the men recruited had full data on semen parameters and at least one metabolite, with <1% of these having missing covariable data (see Table 1) I did not need to undertake any additional analyses to explore potential biases due to missing data.

4.2.10 Accounting for multiple testing

Due to the correlated nature of the metabolic biomarkers, over 95% of the variation in the 155 metabolic biomarkers was explained by 15 principal components. Therefore, multiple testing correction, accounting for 15 independent tests using the Bonferroni method, resulted in $P_{Bonferroni} < 0.0033$ (0.05/15) being denoted as statistically significant.

4.3 Results

Three hundred and twenty-five men (98% of the 331 recruited) with available semen analysis and metabolomics evaluation were included in the study. Characteristics of the participants are shown in Table 1. Mean (SD) age of the men was 37.2 (5.7) years with a mean (SD) BMI of 25.2 (3) Kg/m^2. The majority (92%) were white European, with over 50% having a university degree, 30% being ever smokers, 69% reporting exercising equal to or more than 3-4 times per week, 47% having a family history of cardiovascular disease and median alcohol consumption being 4 units per week. For 54% of the men the cause of them and their partner requiring referral for assisted conception was unexplained and for 26% the cause was related to their female partner; the source of requiring assisted conception was deemed due to sperm / male causes in 20% of the men included in this study. Sample production was by masturbation for 98%, with 6 men requiring surgical sperm retrieval. The median sperm concentration was 35 Million per ml (IQR 15, 69 Million per ml) and median progressive motility was 53% (IQR 42, 67 %) in the whole sample. These differed by cause of infertility, with the median sperm concentration in those with a male cause being 12 Million per ml (IQR 1.4, 23 Million per ml) and the median progressive motility being 32.5% (IQR 15,58) and equivalent values in men where the cause of infertility was unknown or a known cause in their female partner being 42 Million per ml (IQR 23, 84) and 55% (IQR 46, 68).

Age (years): Mean, SD, Range	37.2 (5.7) 25-55
Ethnicity: N(%) White Asian Other	 299 (92%) 19 (6%) 7 (2%)
Ever smoked: N(%)	97 (30%)
Alcohol (units per week) : Median, IQR, Range	4 (2,9) 0-40
BMI: Mean, SD, Range	25.2 (3.0) 19.4-35.1
Highest Education N(%) High School Undergraduate Postgraduate	 147 (45%) 138 (42%) 40 (12%)
Physical activity (times per week) N(%) Never/Once Twice 3-4 times 5-7 times 7+ times	 29 (9%) 73 (22%) 176 (54%) 35 (11%) 12 (4%)
Family history of cardiometabolic disease: N(%)	153 (47%)
Cause of infertility (missing data N=1) Azoospermia Oligozoospermia Female factor Other/Unexplained/Avoidance of genetic disorder	 11 (3%) 55 (17%) 84 (26%) 174 (54%)
Sperm production method Masturbation MESA/TESA/Other	 319 (98%) 6 (2%)
Total motile sperm count < 5 Million 5- 15 Million >15 Million	 45 (14%) 33 (10%) 247 (76%)
% Motile : Median, IQR, Range	53 (42,67) 0-91
Sperm concentration (M per ml): Median, IQR, Range	35 (15,69) 0-230

Table 4.1 Baseline characteristics of the study population (N=325).

The unadjusted associations of sperm concentration, motility and TMSC with confounders are shown in Tables 4.2, 4.3, and 4.4 respectively. With the exception of established male factor infertility having a negative association with sperm concentration, motility and TMSC there was no clear evidence for associations of baseline demographic or lifestyle characteristics, including BMI, with semen parameters.

	Difference in mean concentration per unit or category of confounder (SD) (95% CI)	P-value
Age (years)	0.004 (-0.02, 0.02)	0.71
BMI (kg/m2)	0.02 (-0.01, 0.06)	0.21
Highest Education: High School Undergraduate Postgraduate	 - 0.05 (-0.19, 0.28) 0.17 (-0.18, 0.53)	 - 0.68 0.34
Physical activity (per category increase)	-0.02 (-0.14, 0.10)	0.78
Ever smoked	-0.13 (-0.37, 0.11)	0.29
Alcohol (units per week)	-0.0003 (-0.02, 0.02)	0.97
Family History (Cardiometabolic)	0.12 (-0.10, 0.34)	0.30
Ethnicity (Non-white vs White European)	-0.25 (-0.65, 0.16)	0.23
Cause of infertility (missing data N=1) Female factor Azoospermia Oligozoospermia Other/Unexplained/Avoidance of genetic disorder	 - -1.32 (-1.88, -0.77) -1.25 (-1.56, -0.95) -0.13 (-0.36, 0.10)	 - <0.001 <0.001 0.27

Table 4.2 Associations of demographics/lifestyle factors with sperm concentration (SD).

	Difference in mean motility per unit or category of confounder (SD) (95% CI)	P-value
Age (years)	-0.002 (-0.02, 0.02)	0.88
BMI (kg/m2)	0.001 (-0.03, 0.04)	0.94
Highest Education High School Undergraduate Postgraduate	 - 0.05 (-0.19, 0.28) 0.22 (-0.13, 0.57)	 - 0.70 0.21
Physical activity (per category increase)	-0.11 (-0.23, 0.002)	0.06
Ever smoked	-0.09 (-0.32, 0.15)	0.48
Alcohol (units per week)	-0.004 (-0.02, 0.01)	0.63
Family History (Cardiometabolic)	0.13 (-0.08, 0.35)	0.23
Ethnicity (Non-white vs White European)	-0.22 (-0.62, 0.18)	0.28
Cause of infertility (missing data N=1) Female factor Azoospermia Oligozoospermia Other/Unexplained/Avoidance of genetic disorder	 - -1.36 (-1.94, -0.78) -0.99 (-1.31, -0.67) -0.15 (-0.39, 0.09)	 - <0.001 <0.001 0.23

Table 4.3 Associations of possible confounders with sperm motility (SD) (N=325).

	≥15 million	<15 million	P-value
Age (years): Mean, SD	37.2 (5.8)	37.0 (5.4)	0.70
Ethnicity: N(%) **White** **Non-white**	 229 (93%) 18 (7%)	 70 (90%) 8 (10%)	 0.40
Ever smoked: N(%)	70 (28%)	27 (35%)	0.29
Alcohol (units per week) : Median, IQR	4 (1,9)	4 (2,9)	
BMI: Mean, SD	25.4 (3.0)	24.7 (3.0)	0.06
Highest Education N(%) **School** **Undergraduate** **Postgraduate**	 113 (46%) 102 (41%) 32 (13%)	 34 (44%) 36 (46%) 8 (10%)	 0.69
Physical activity (times per week) N(%) **Never/Once** **Twice** **3-4 times** **5-7 times** **7+ times**	 22 (9%) 57 (23%) 139 (56%) 23 (9%) 6 (2%)	 7 (9%) 16 (21%) 37 (47%) 12 (15%) 6 (8%)	 0.19
Family history of cardiometabolic disease: N(%)	121 (49%)	32 (41%)	0.22
Cause of infertility (missing data N=1) **Azoospermia/Oligozoospermia** **Female factor** **Other/Unexplained/Avoidance of genetic disorder**	 27 (11%) 76 (31%) 143 (58%)	 39 (50%) 8 (10%) 31(40%)	 <0.001

Table 4.4 Association of demographics/lifestyle factors with TMSC (N=325).

The adjusted associations between sperm concentration and progressive motility and the respective metabolomics measures are presented in Figures 4.1, 4.2 and 4.3. With the unadjusted analyses for sperm concentration and progressive motility presented in Figures 4.4, 4.5 and 4.5.

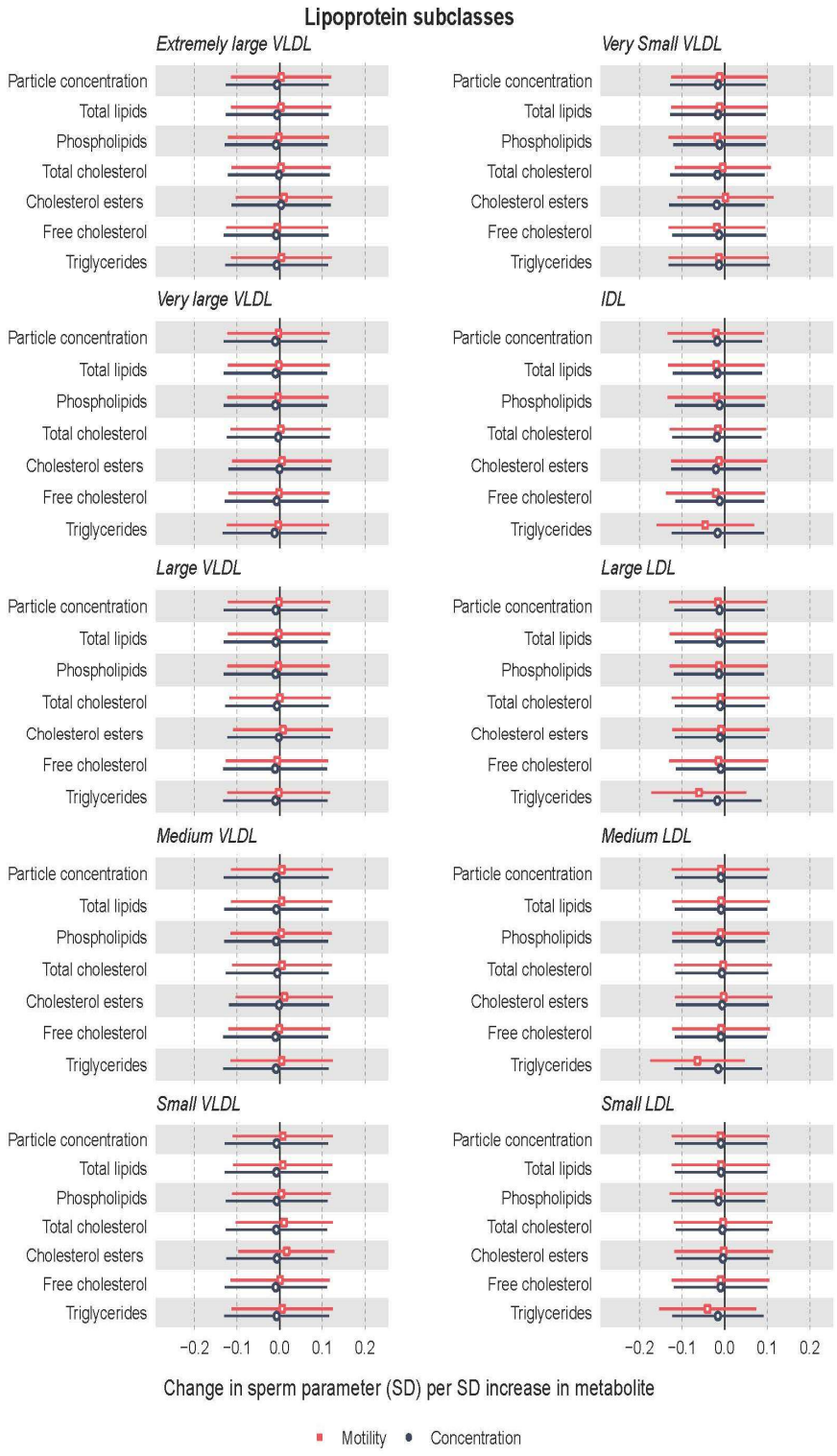


Figure 4.1 Associations of lipoprotein classes with sperm concentration and progressive motility in men undertaking assisted conception.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration (red) and progressive motility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

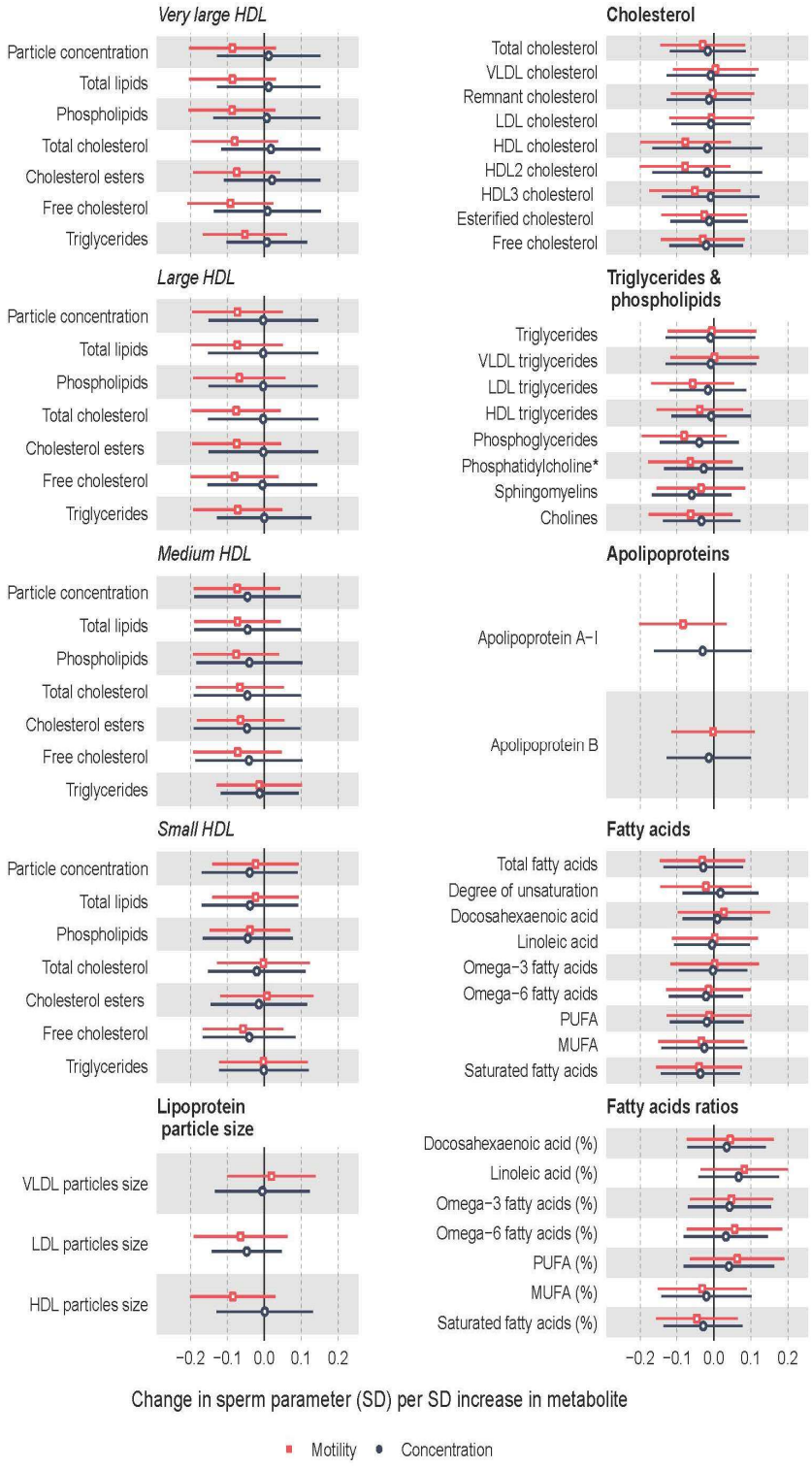


Figure 4.2 Associations of lipoprotein classes and fatty acids with sperm concentration and progressive motility in men undertaking assisted conception.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration (red) and progressive motility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

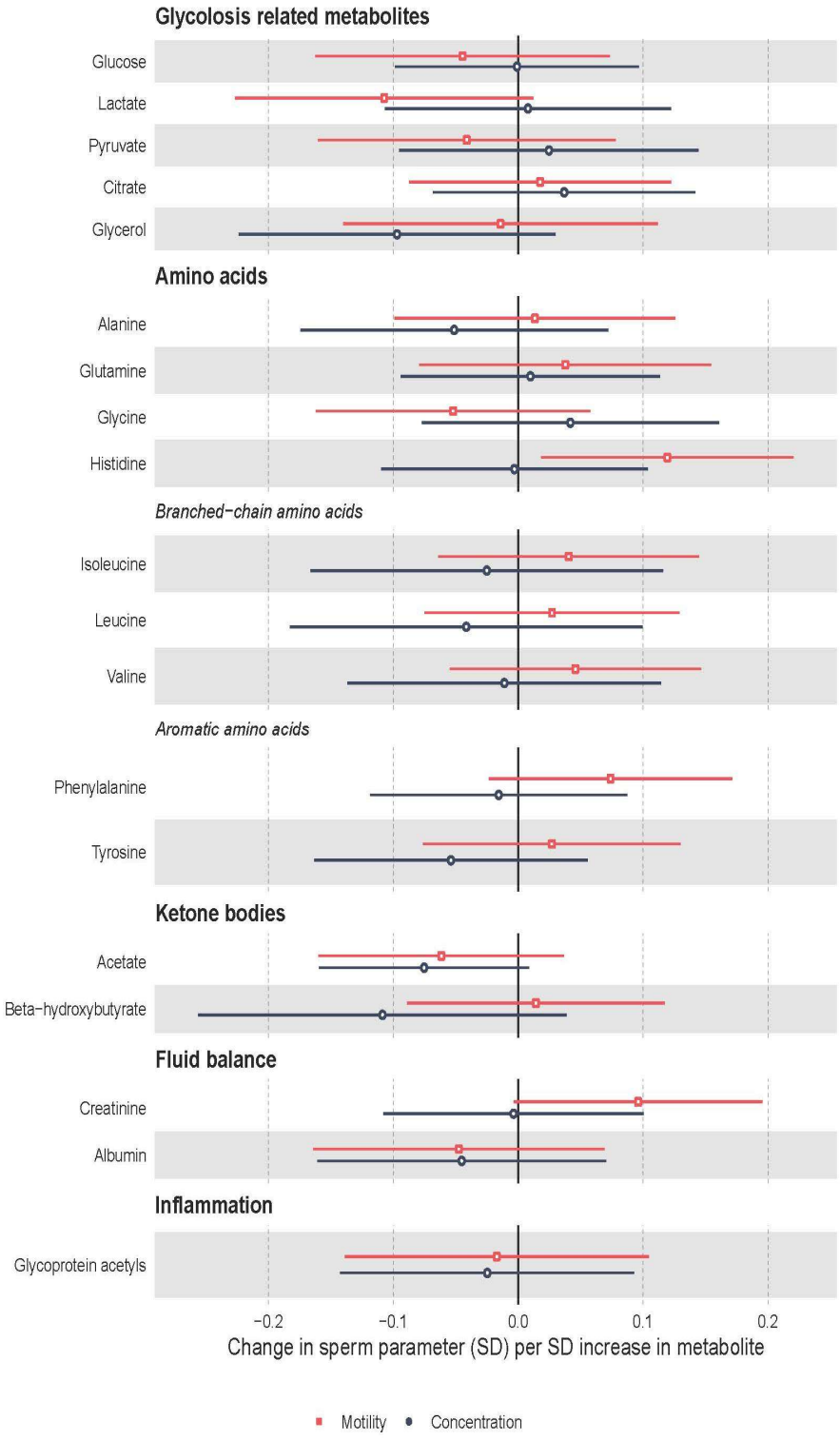


Figure 4.3 Associations of metabolic traits with sperm concentration and progressive motility in men undertaking assisted conception.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration (red) and progressive motility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

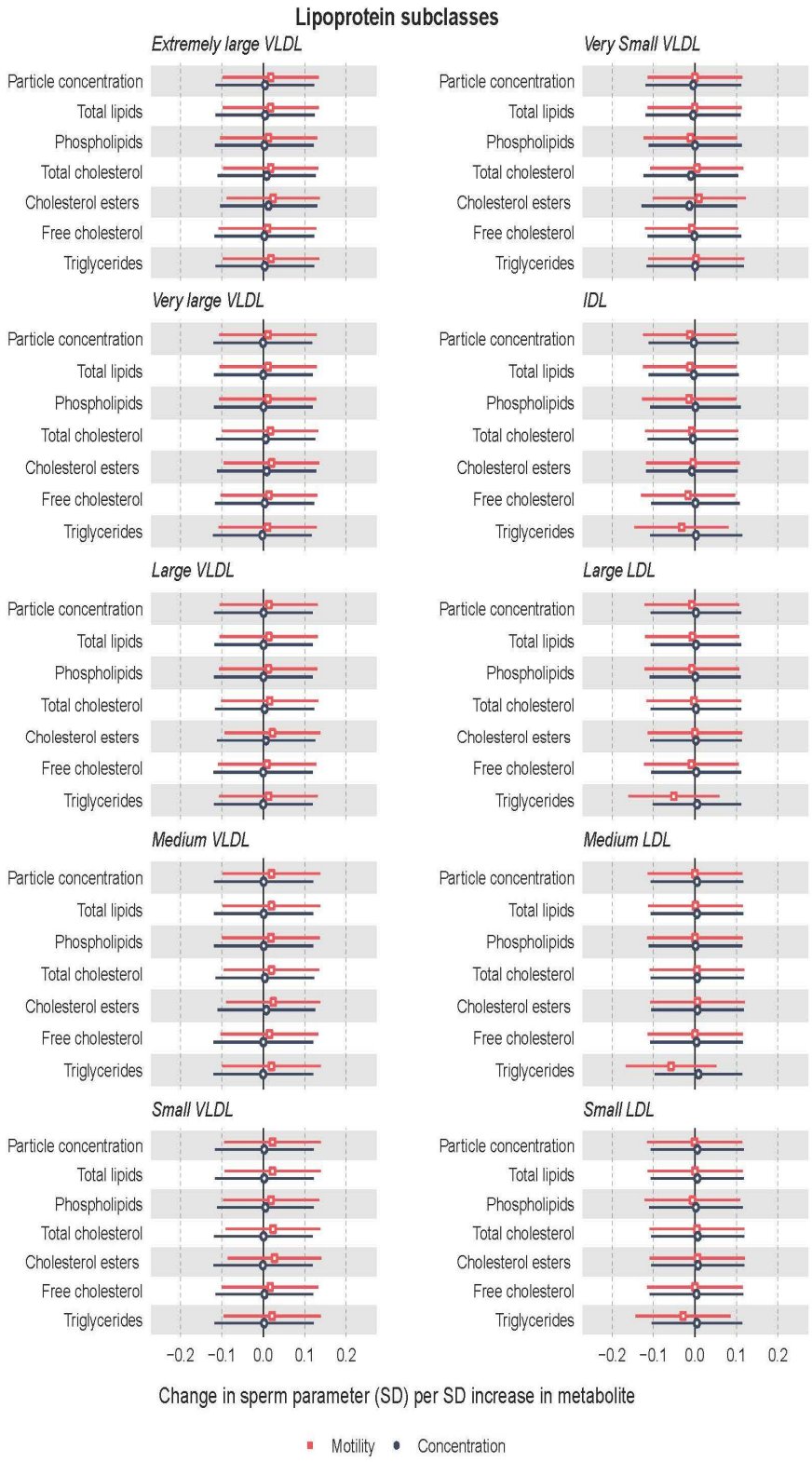


Figure 4.4 Associations of lipoprotein classes with sperm concentration and motility (unadjusted).
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility (red) and concentration (black).

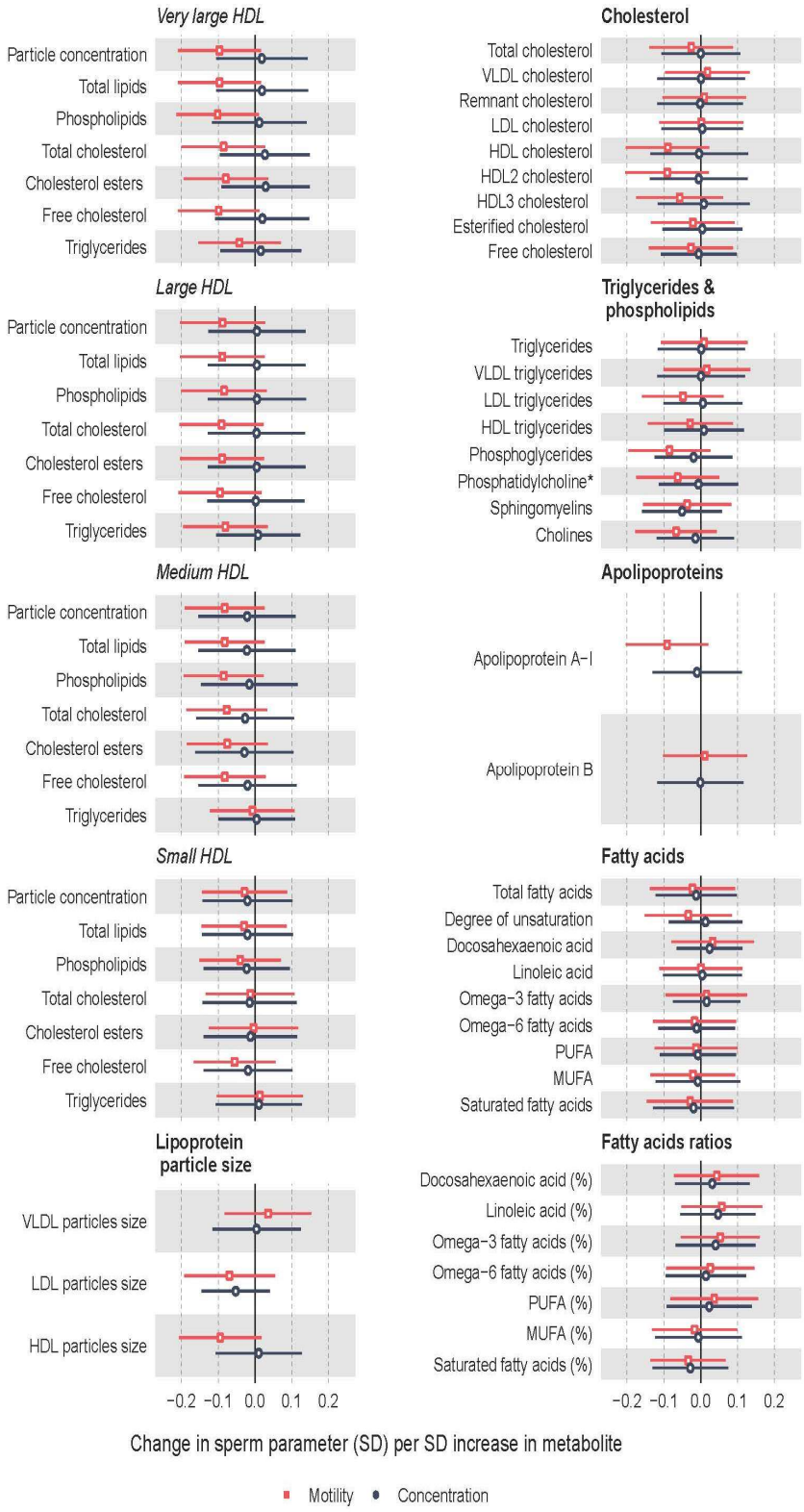


Figure 4.5 Associations of lipoprotein classes and fatty acids with sperm concentration and motility (unadjusted).
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility (red) and concentration (black).

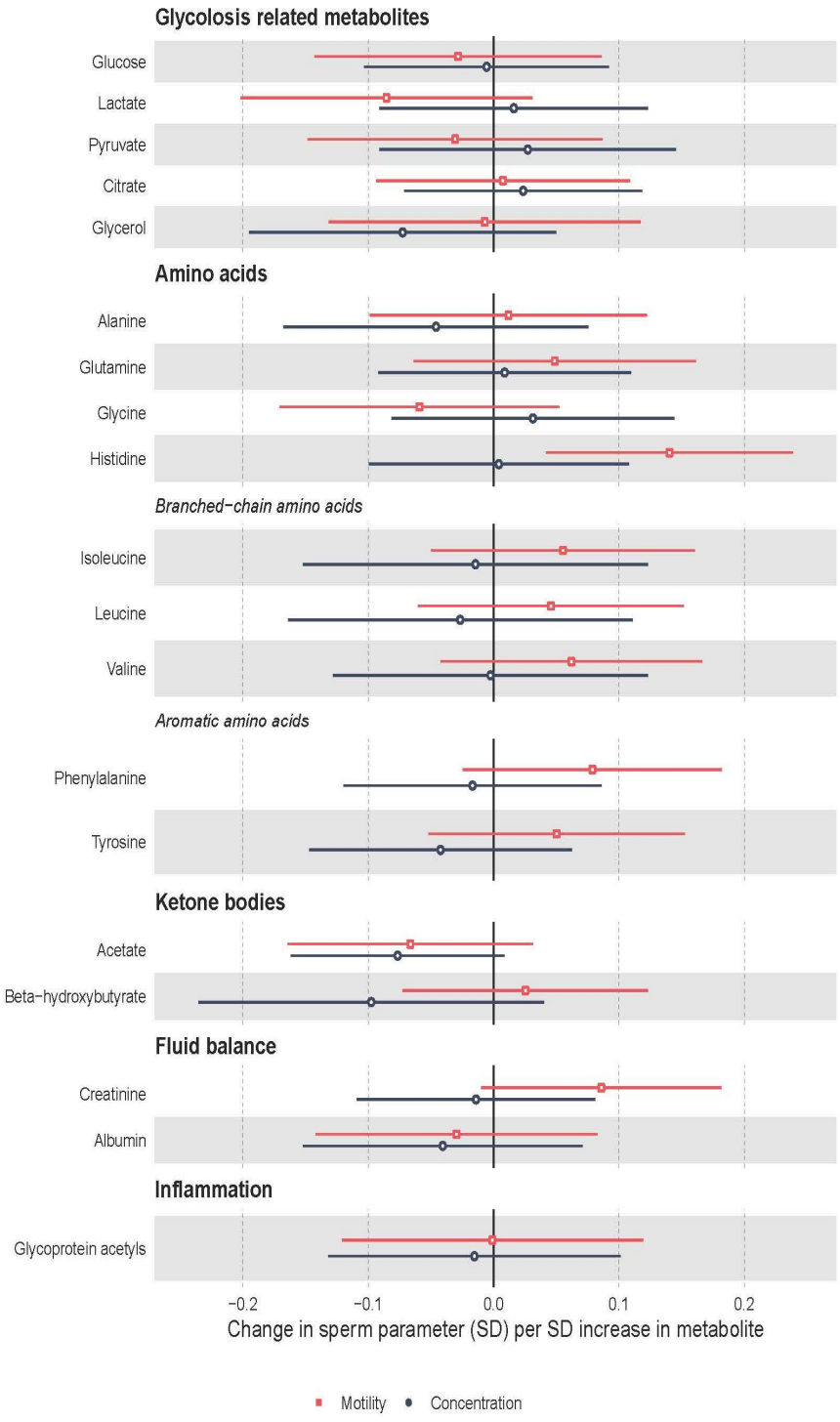


Figure 4.6 Associations of metabolic traits with sperm concentration and motility (unadjusted).
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility (red) and concentration (black).

Histidine was the only metabolite with Bonferroni corrected statistical evidence of an association with motility in the unadjusted analyses and there was no statistical evidence of any metabolites associating with sperm concentration. Whilst all of the associations were imprecisely estimated (i.e. with wide

confidence intervals), most of the point estimates for the lipids were very close to the null, except for very large, large and medium HDL particle concentrations and apolipoprotein A-1 which had inverse associations with point estimates of ~ -0.1SD with sperm motility per 1SD higher lipid. Creatinine also has a point estimate of 0.1SD, showing a positive association with sperm motility, though again with wide confidence intervals and not reaching the Bonferroni corrected statistical significance. In results adjusted for confounders (Figure 4.1, 4.2 and 4.3) the associations showed very little difference to the unadjusted results. The adjusted association for histidine with motility was 0.12 SD per 1 SD higher histidine concentration (95%CI: 0.02, 0.22), though the p-value was slightly higher than the Bonferroni corrected value at 0.02. In contrast, lactate showed a negative association with motility (-0.11 SD in sperm motility (95%CI -0.23, 0.01) per 1 SD increase in lactate concentration) but no association with concentration.

Analyses of the associations of metabolites with odds of a low TMSC compared with a healthy count (Figure 4.7, 4.8 and 4.9) and of odds of a very low TMSC compared to either a low or healthy count (Figure 4.10, 4.11 and 4.12) did not have statistical (Bonferroni corrected) support for any associations, though these analyses have less power than with the continuously measured outcomes and wide confidence intervals. Point estimates for adjusted associations were close to the null for all lipids, lipoproteins and fatty acids. For glucose, lactate, pyruvate, both ketone bodies and glycoprotein acetyls point estimates suggested positive associations with reduced compared to a normal TMSC with odds ratios of ~1.2 and for glycerol a positive association with an odds ratio of ~1.3. There was also a point estimate of 0.8 for creatinine with low versus normal TMSC. When comparing TMSC of <5 million to ≥ 5 million the adjusted associations for most very small VLDL, IDL and large, medium and small LDL concentrations, and some of the cholesterols and triglycerides and phospholipids had point estimates of ~0.8 suggesting potentially important reduced odds of a very low sperm count with higher levels of these lipids but for which we had limited power to obtain precise estimates that excluded the null (Figure 4.10, 4.11 and 4.12). Degree of fatty acid unsaturation had a point estimate OR of ~0.6 in these analyses and some other metabolites were associated with very low TMSC with OR suggestive of a relative increase or decrease in odds of 20% or more.

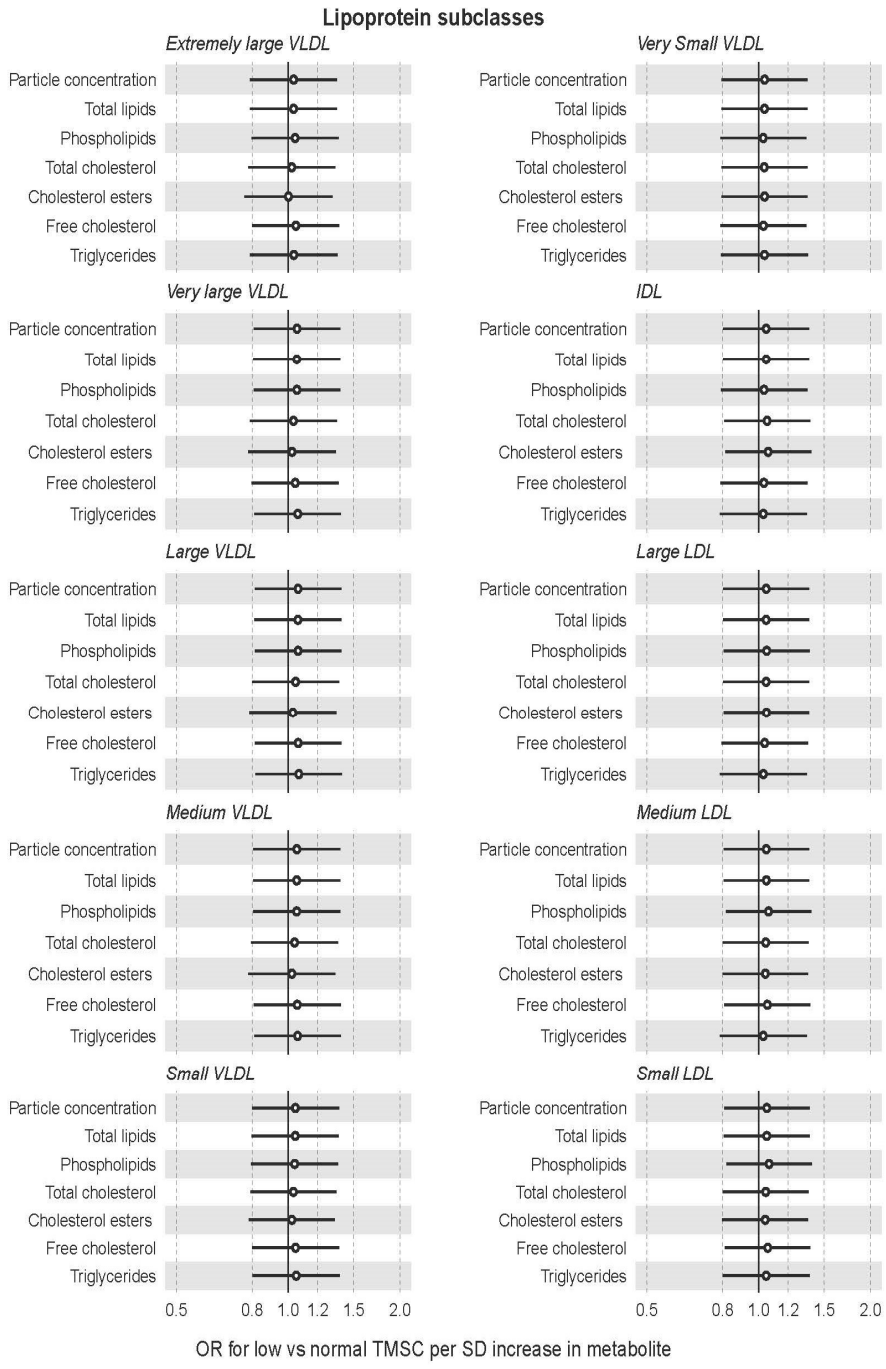


Figure 4.7 Associations of lipoprotein classes with odds of having a TMSC less than 15 Million compared with greater or equal to 15 million.
Odds ratio for having a total motile sperm count less than 15 Million and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

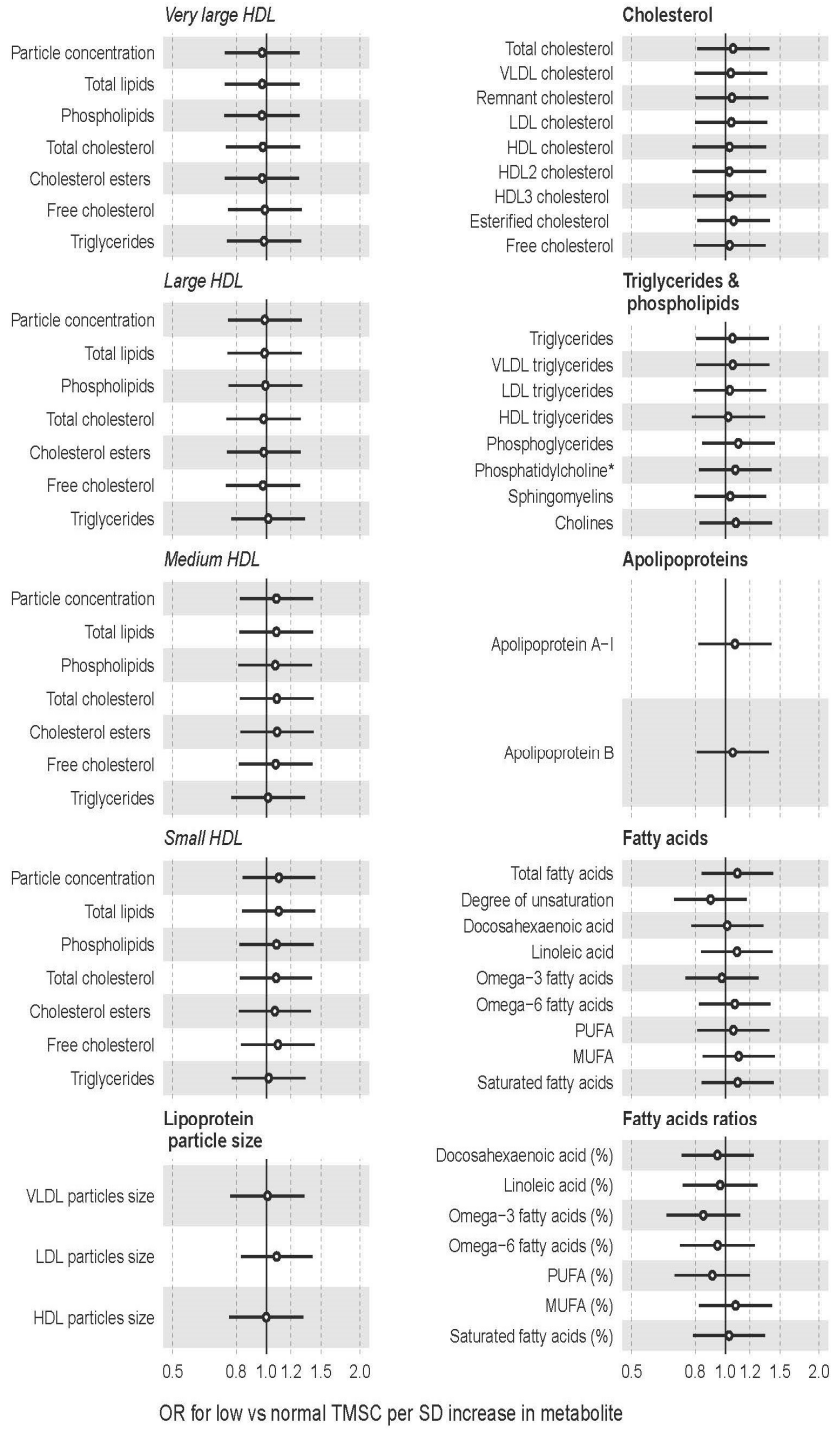


Figure 4.8 Associations of lipoprotein classes and fatty acids with odds of having a TMSC less than 15 Million compared with greater or equal to 15 million.

Odds ratio for having a total motile sperm count less than 15 Million and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

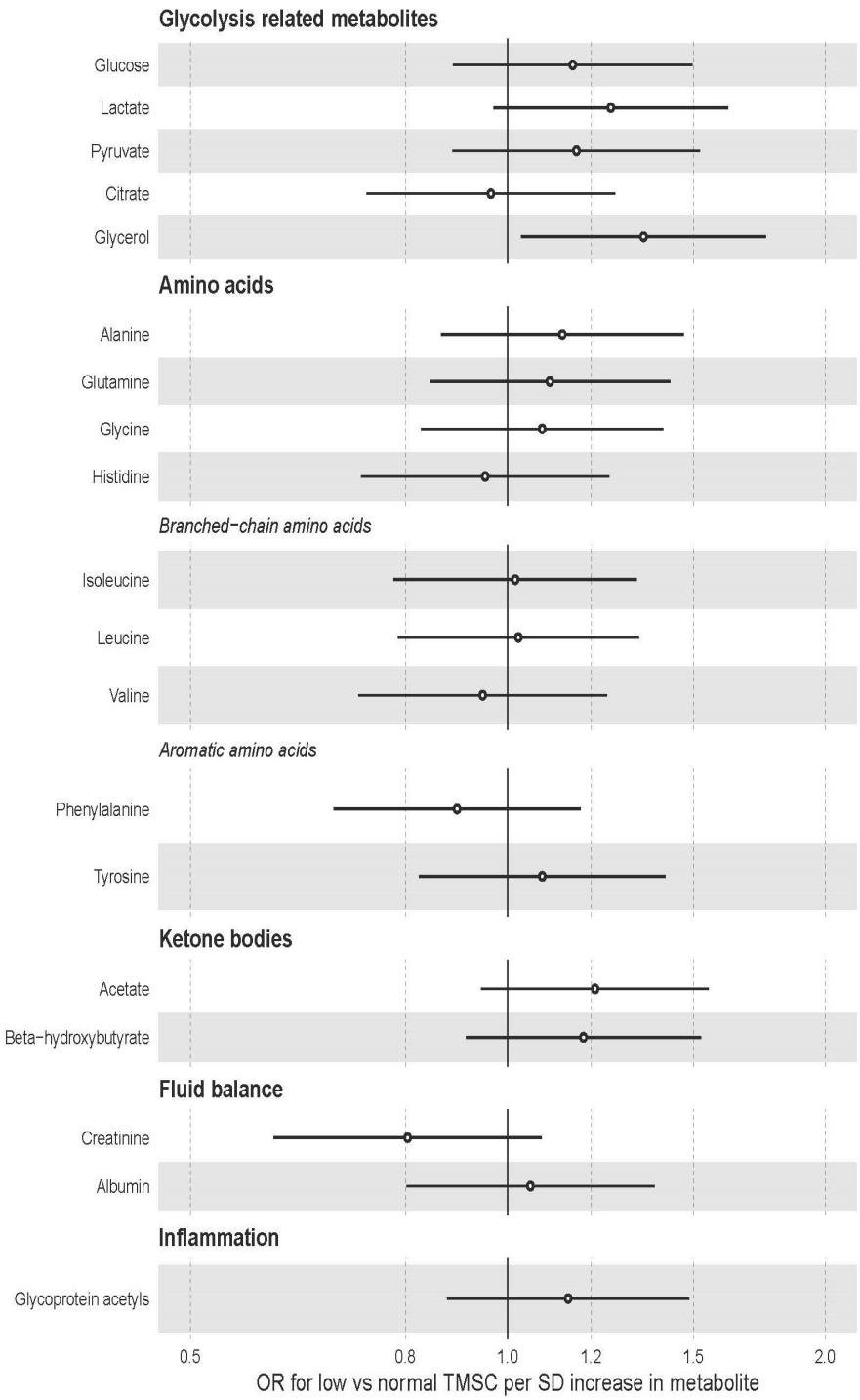


Figure 4.9 Associations of metabolic traits with odds of having a TMSC less than 15 Million compared with greater or equal to 15 million.
Odds ratio for having a total motile sperm count less than 15 Million and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

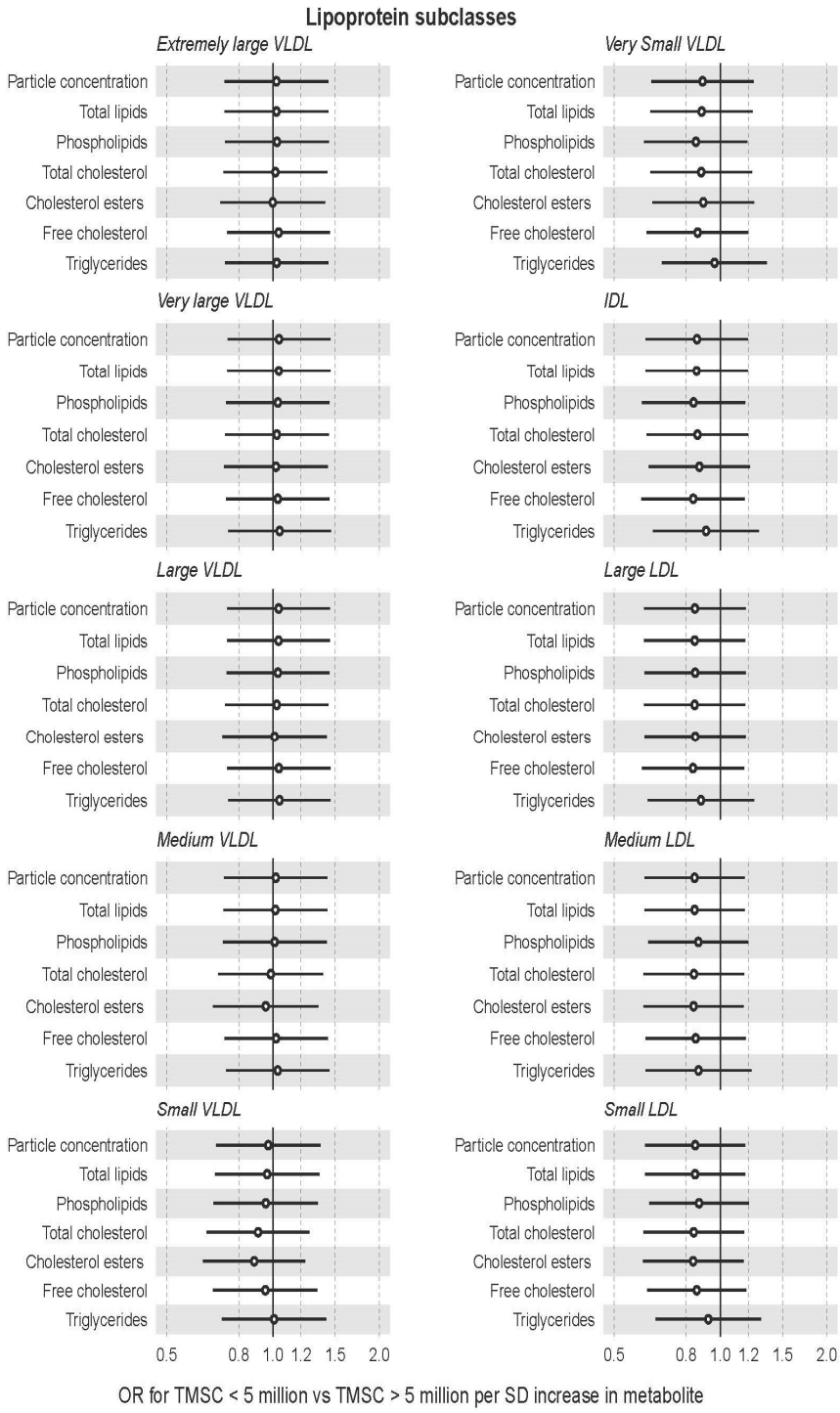


Figure 4.10 Associations of lipoprotein classes with odds of having a TMSC less than 5 Million compared with 5 million or more.
Odds ratio for having a total motile sperm count less than 5 Million compared with 5 million or more and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

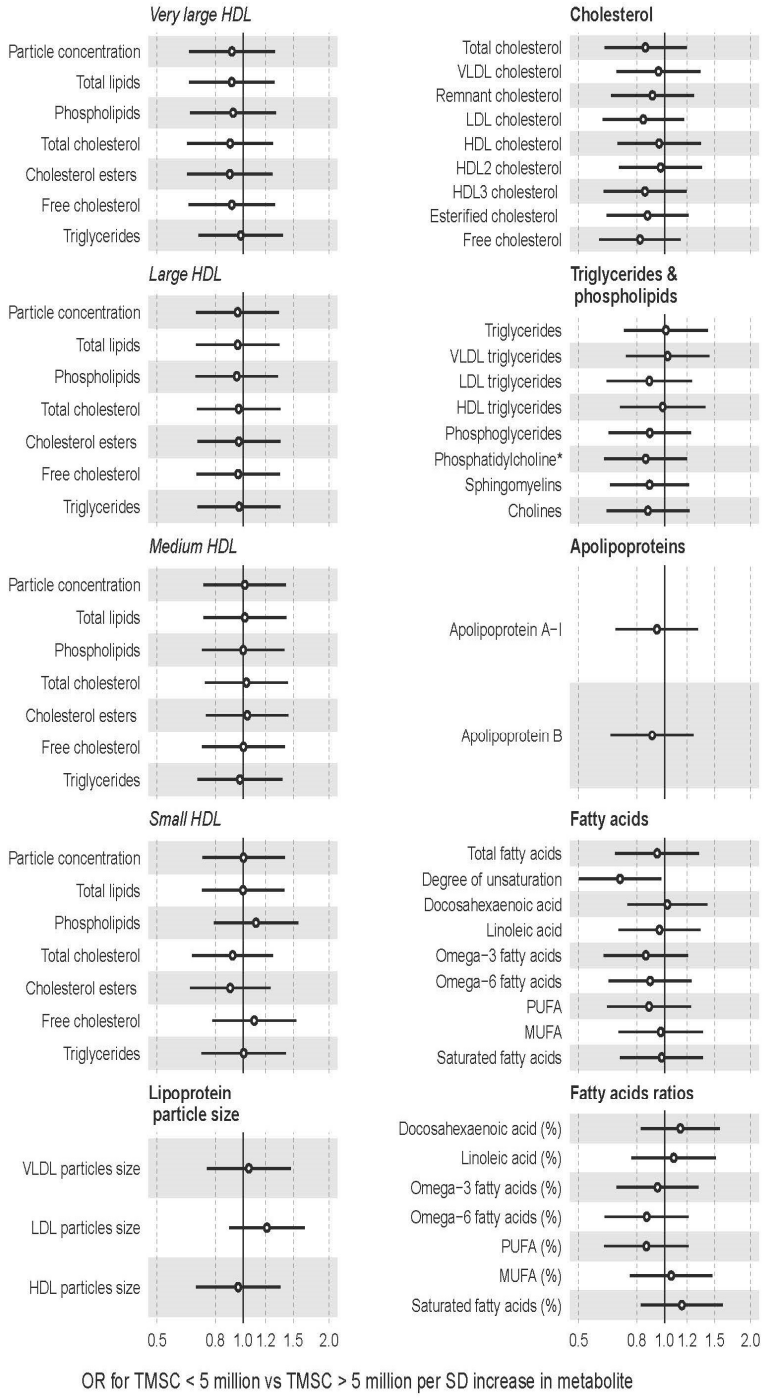


Figure 4.11 Associations of lipoprotein classes and fatty acids with odds of having a TMSC less than 5 Million compared with 5 million or more.
Odds ratio for having a total motile sperm count less than 5 Million compared with 5 million or more and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

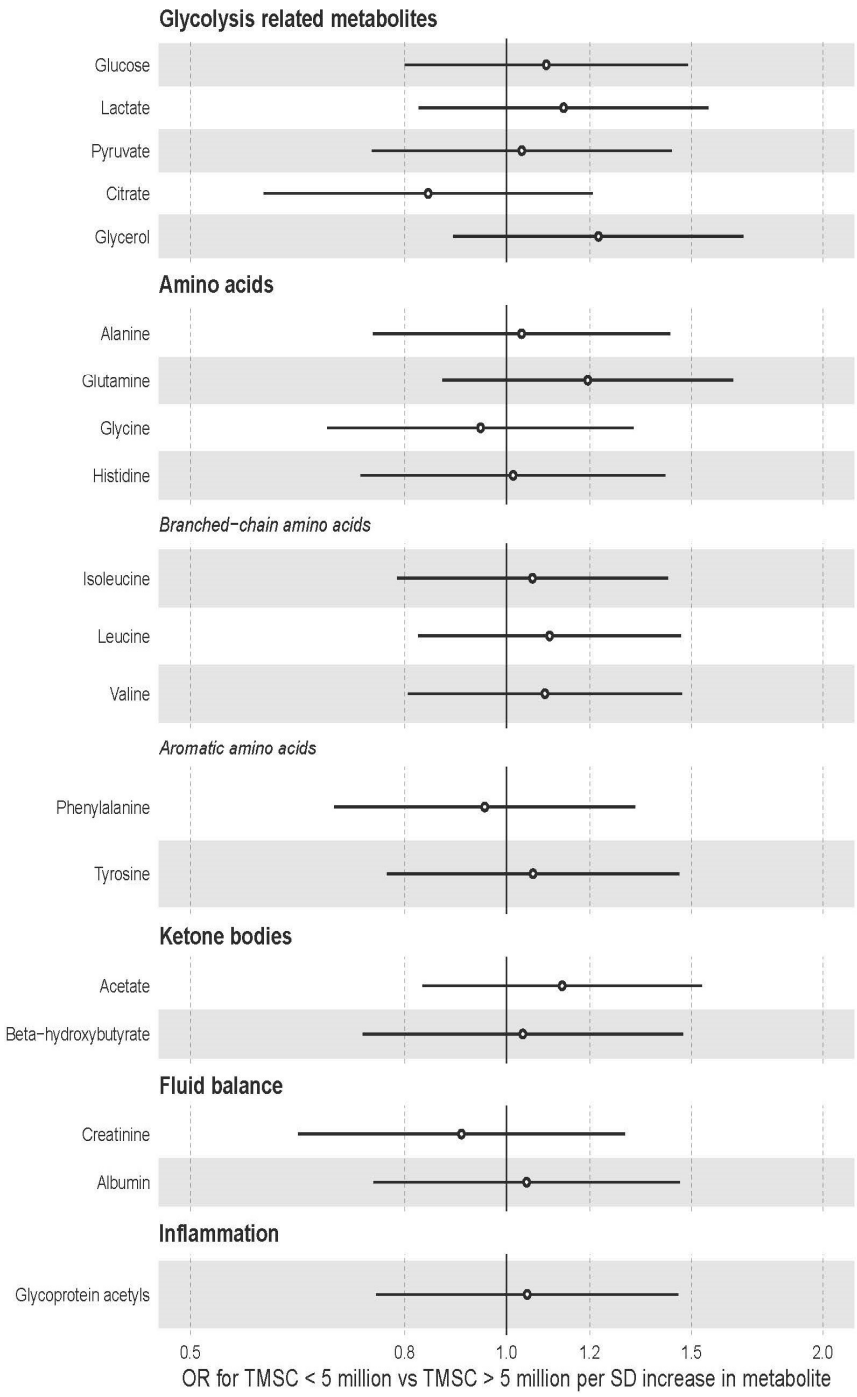


Figure 4.12 Associations of metabolic traits with odds of having a TMSC less than 5 Million compared with 5 million or more.
Odds ratio for having a total motile sperm count less than 5 Million compared with 5 million or more and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

When analyses were repeated only in those undergoing assisted conception for male factor (n=66) as opposed to unexplained or female cause of infertility (n=258), most of the 155 associations were broadly consistent, with a tendency for those with male factor associations to be stronger for both concentration and motility (Figures 4.13 to 4.18). In those with male factor infertility there was evidence of higher phenylalanine associating with higher sperm concentration and motility (0.44 SD (95%CI 0.09, 0.80) per 1 SD for both outcomes). Comparing all point estimates across all 155 metabolites between those with male factor infertility and those with female or unknown causes showed poor consistency between the two groups (Figure 4.19; goodness of fit statistic R^2 = 0.1 for sperm concentration and 0.03 for motility). Indeed, most of the associations with sperm concentration were in the opposite directions between the two groups (slope = -0.92 (95% CI –0.48, 1.36)), including for associations of phenylalanine and creatinine which associated positively with concentration in those with male cause infertility and weakly inversely in those without. For sperm motility many associations were also directionally inconsistent (weak positive slope 0.35 (95% CI 0.004, 0.70)); associations of both phenylalanine and creatinine were positively associated with motility, with a stronger association in those with male cause fertility than those without (Figure 4.19).

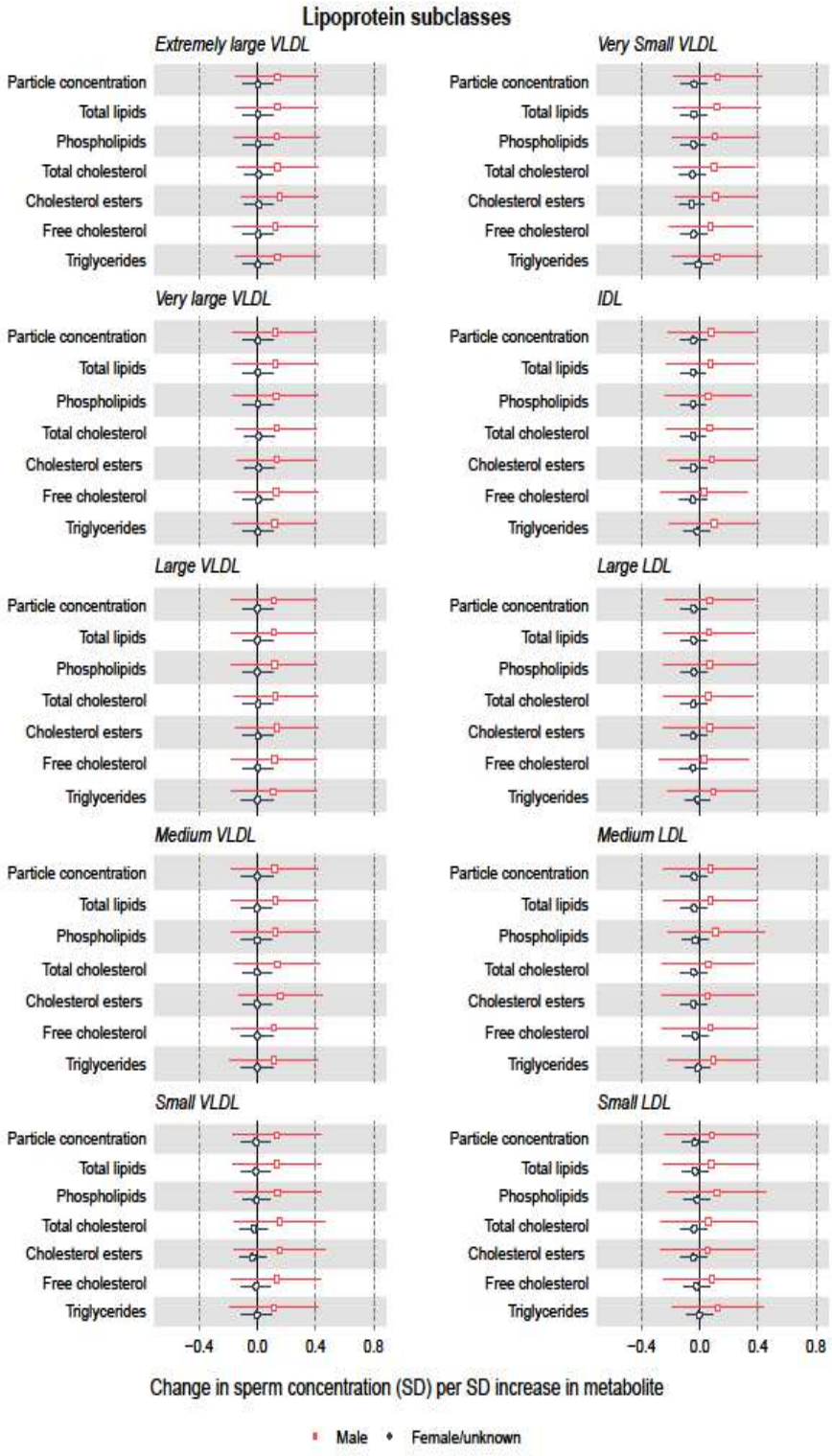


Figure 4.13 Association of lipoprotein classes with sperm concentration by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

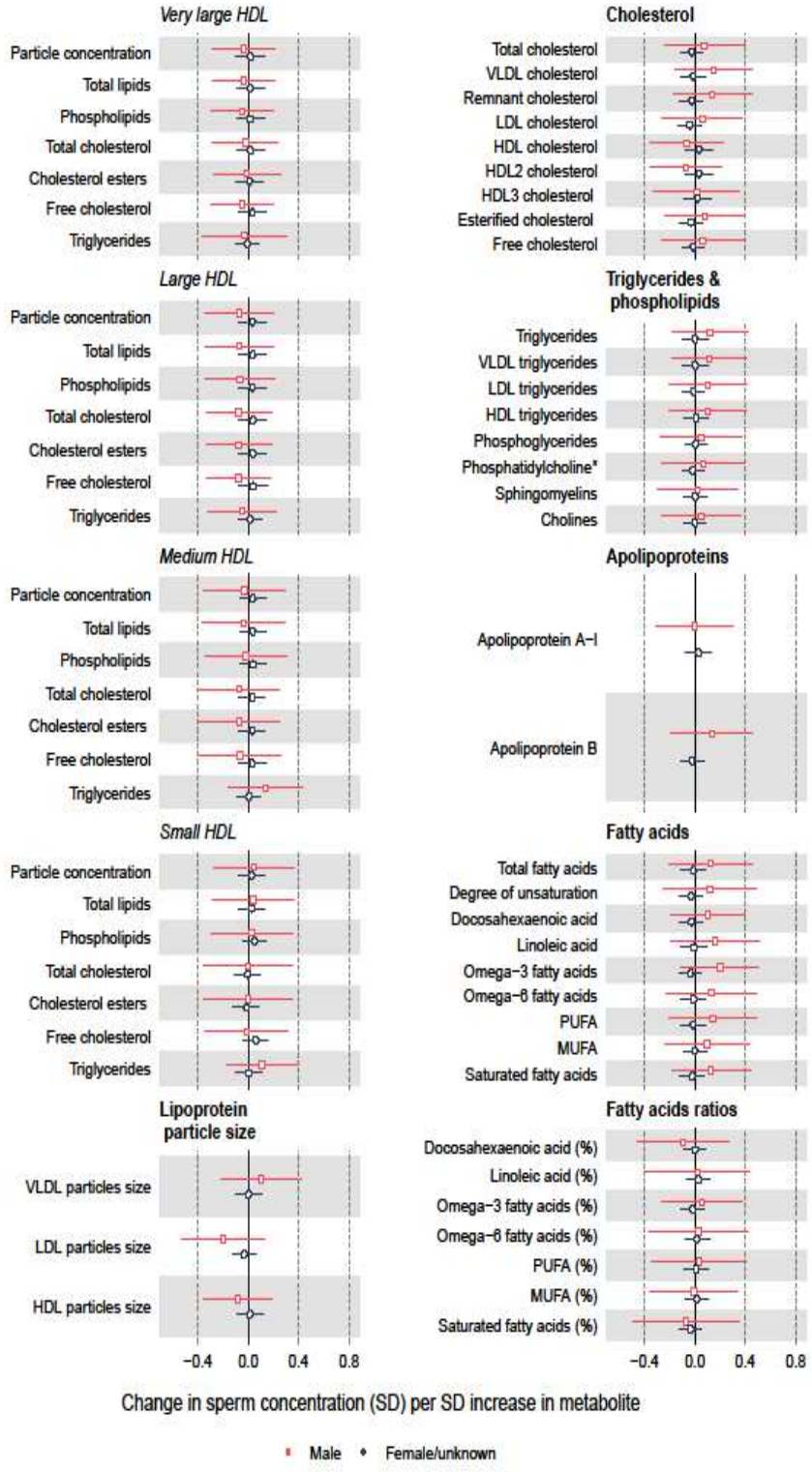


Figure 4.14 Association of lipoprotein classes and fatty acids with sperm concentration by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

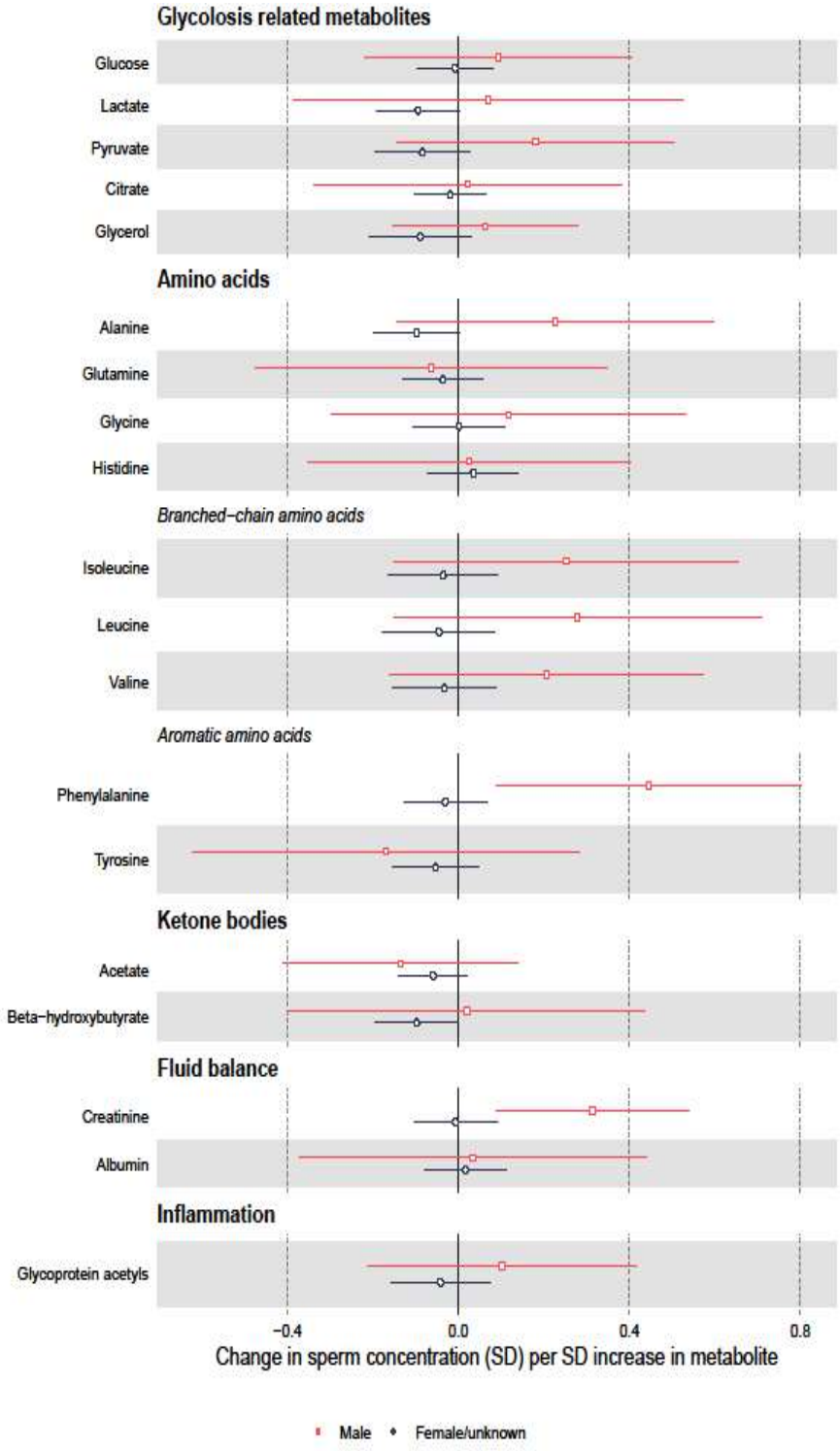


Figure 4.15 Association of metabolic traits with sperm concentration by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

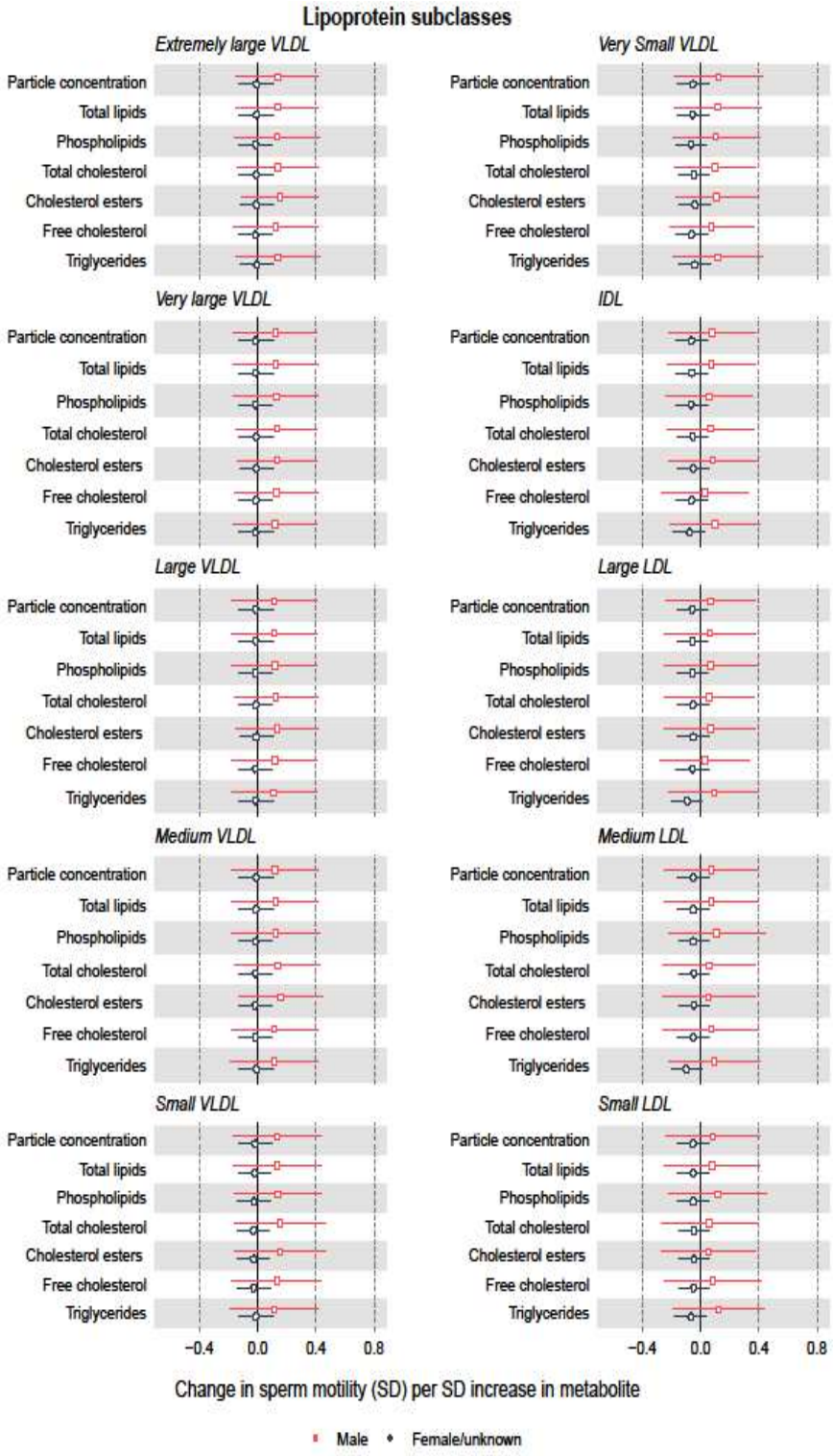


Figure 4.16 Association of lipoprotein classes with sperm motility by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

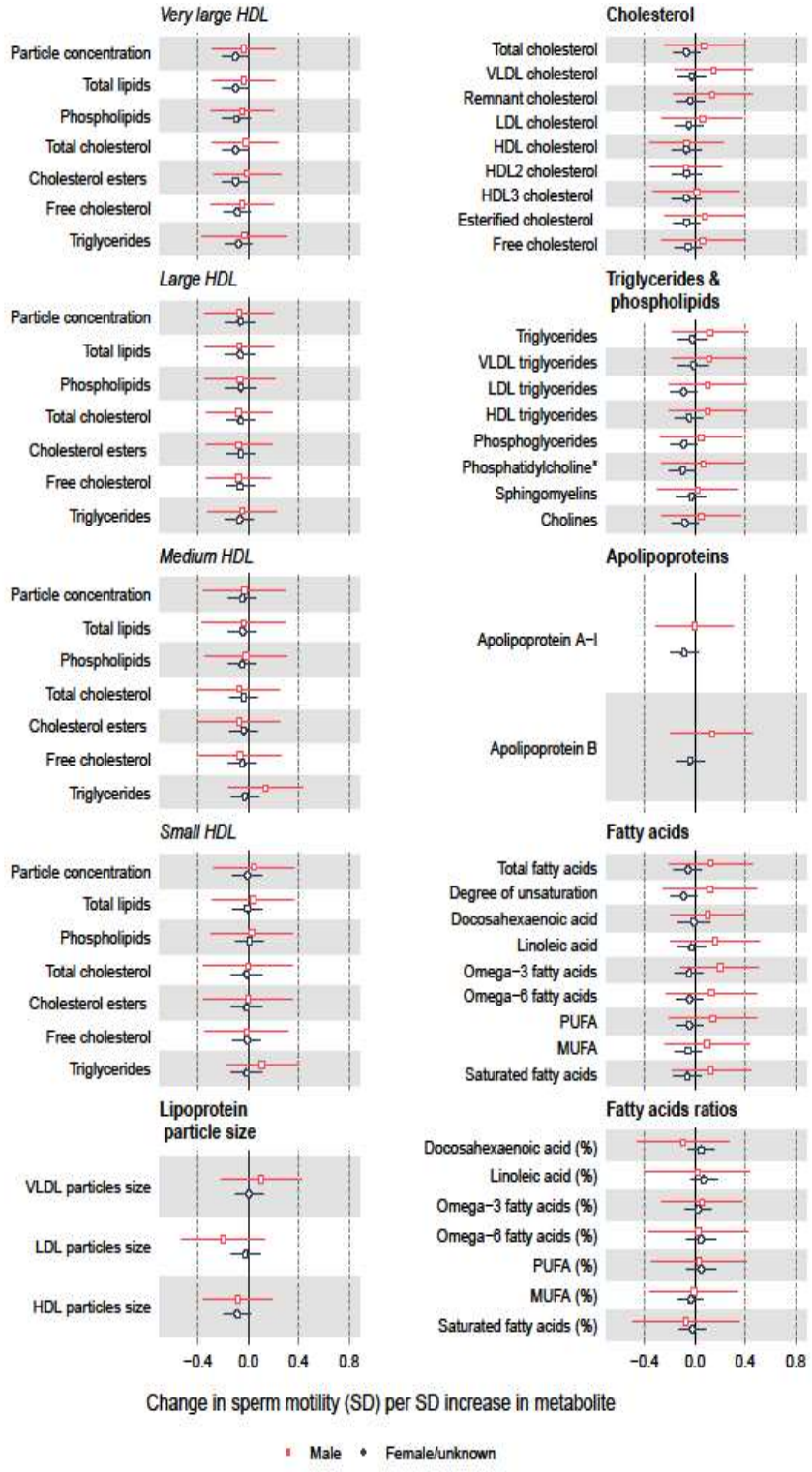


Figure 4.17 Association of lipoprotein classes and fatty acids with sperm motility by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

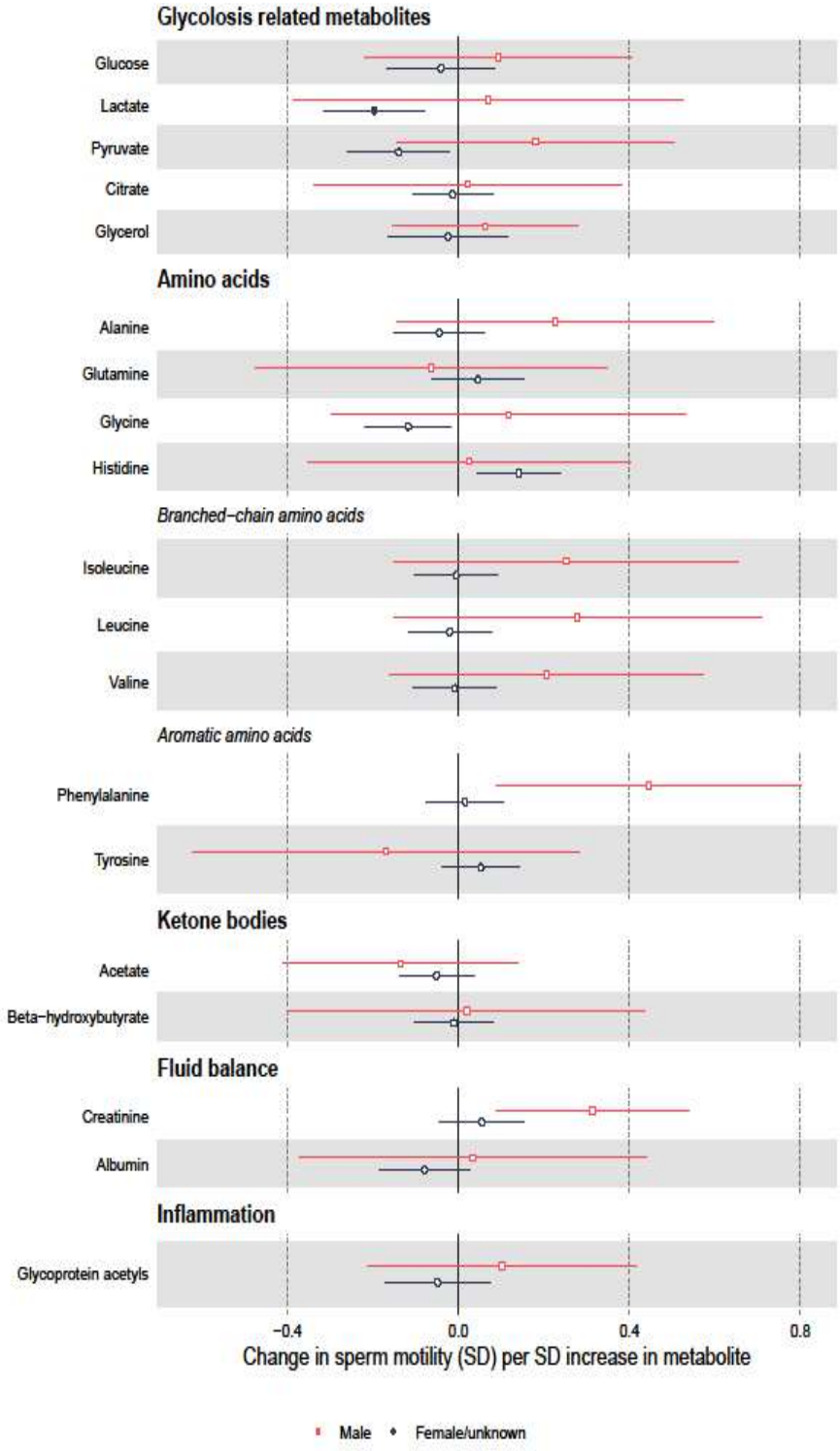
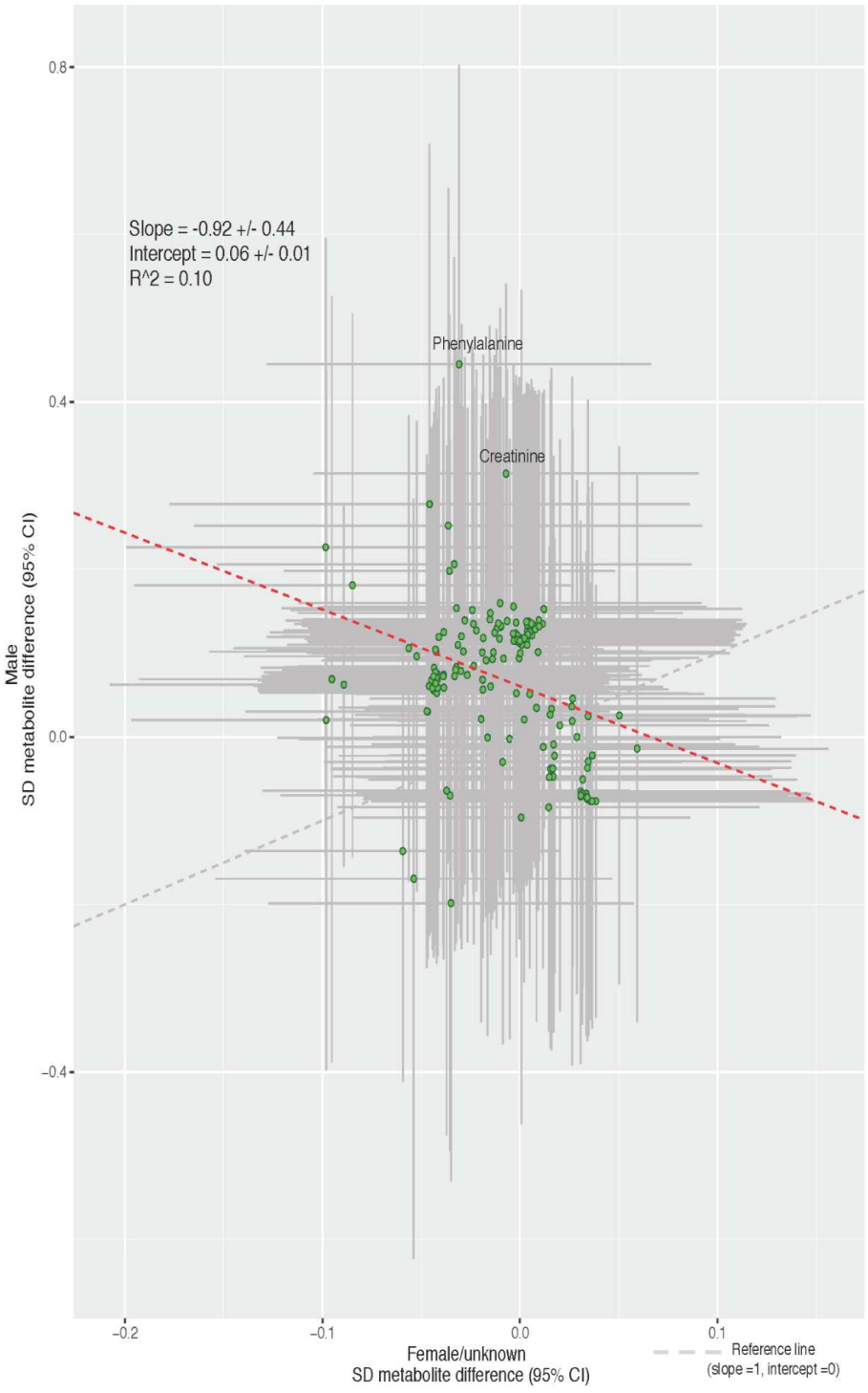


Figure 4.18 Association of metabolic traits with sperm motility by cause of infertility

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.


Slope = -0.92 +/- 0.44
Intercept = 0.06 +/- 0.01
R^2 = 0.10
Phenylalanine
Creatinine
0.8
0.4
0.0
−0.4
−0.2
−0.1
0.0
0.1
Male
SD metabolite difference (95% CI)
Female/unknown
SD metabolite difference (95% CI)
Reference line
(slope =1, intercept =0)

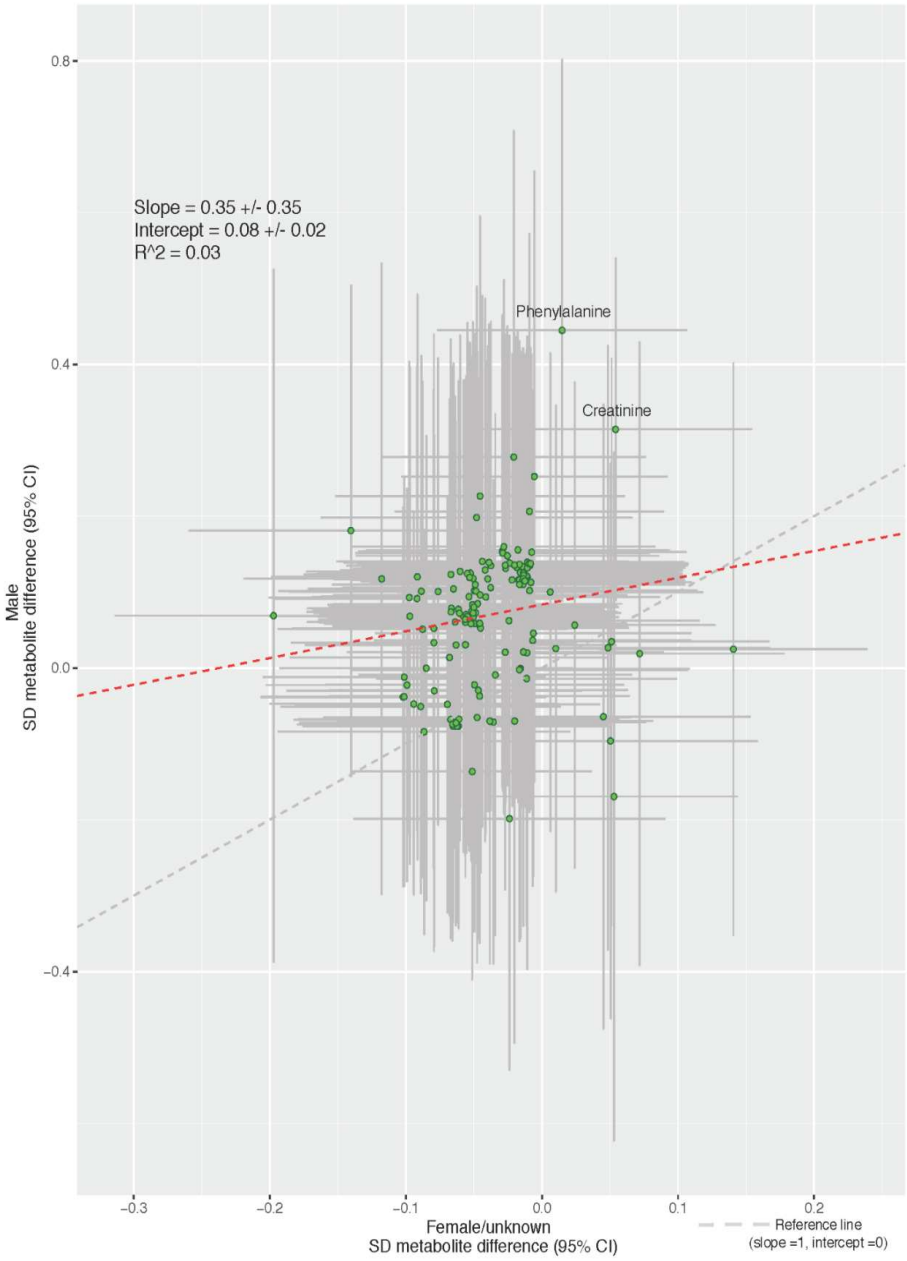


Figure 4.19 Scatterplot of associations between sperm concentration and motility and metabolites in those males with female or unknown cause of infertility (N = 398) and restricted to males with a reported male partner cause of infertility (N = 87).

Figure 4.19a shows associations with concentration by cause of infertility, Figure 4.19b shows associations with motility by cause of infertility. The green dots highlight the individual metabolites, with phenylalanine and creatinine highlighted. The grey dashed line reflects the reference line (slope 1, intercept 0) and the red dashed line is the best line of fit.

4.4 Discussion

In this cross-sectional exploratory study of men attending a fertility clinic for evaluation I did not identify any associations between circulating metabolites and sperm concentration, sperm progressive motility or the risk of a low or very low TMSC on the basis of reaching conventional 5% levels of statistical significance after Bonferroni correction for multiple testing. However, point estimates suggested some potentially clinically important associations. Higher levels of glycolysis metabolites and ketone bodies were associated with increased odds of TMSC <15M compared with ≥15M (odds ratios of ~1.2 to 1.3), and several lipids/lipoprotein concentrations appeared to protect against very low TMSC (<5M compared with ≥5M) with odds ratios of ~0.8 or greater. That this study is unique in exploring these associations highlights the importance of funding to undertake larger studies that could precisely estimate associations and provide more robust evidence of how large any associations are likely to be. Based on a post-hoc power calculation we estimate that a sample size of 3,188 would be required at the Bonferroni corrected p-value used in this study to detect a 20% relative difference in odds (i.e. OR of 0.8 or 1.2) or larger with 80% power.

A potential role for lipids and semen parameters is plausible as cholesterol is the precursor of steroid hormones, including testosterone which is produced in the Leydig cells (Payne and Youngblood, 1995), testicular cholesterol has been shown contribute to normal spermatogenesis (de Neergaard, et al., 2018) and cholesterol-fed rats and rabbits showed reduced spermatid cell numbers, reduced seminiferous tubules' diameters, and smaller Leydig cell nuclear dimensions (Gupta and Dixit, 1988). However, the association of serum lipids and semen parameters has been inconsistent, with some (Ergün, et al., 2007) but not all studies (de Neergaard, et al., 2018, Hagiuda, et al., 2014) finding an inverse association with lipids and sperm concentration and / or motility. The LIFE study (n=501) which quantified 35 different semen parameters, found that total and free cholesterol and phospholipid concentrations were negatively associated with several sperm head morphology defects but not with concentration or motility (Schisterman, et al., 2014). In the largest study to date of healthy individuals (n= 7,920) total cholesterol level was positively correlated with total sperm motility and progressive motility, but there was no association observed for HDL, LDL, Triglyceride or VLDL with concentration or motility (Liu, et al., 2017). A similarly

large study, with a comprehensive evaluation of the lipoprotein spectrum as per the current study combined with the extensive assessment of semen parameters as per the LIFE study would be useful. Further clarification of whether lipoproteins levels in the male reproductive tract are regulated locally as per the rat (Cooper, 1980), and not through passive diffusion from serum would also be useful.

I observed a weak negative association of increasing lactate concentrations with reduced motility. Human sperm have been shown to be capable of metabolising 13C labelled glucose and pyruvate to lactate via lactate dehydrogenase (Hereng *et al.,* 2011), and that lactate oxidation by lactate dehydrogenase isoenzymes has a significant role in energy metabolism during the middle and later stages of spermatogenesis (Goldberg *et al.,* 2010). Lactate has been shown to have an inhibitory effect on sperm motility, in part by acidification of the cytosol (Matsuzaki *et al.,* 2015; Carr *et al.,* 1985), but also it has been suggested that lactate may inhibit the binding site of lactate dehydrogenase (Matsuzaki *et al.,* 2015). It is possible that export of lactate from sperm leads to acidification of the extracellular medium and contributes to a reduction in sperm motility. Confirmation that circulating lactate concentrations may also have a negative association on sperm motility warrants replication given that lactate concentrations increase during acute illness, and other systemic insults but also during strenuous exercise (Andersen *et al.,* 2013).

The impact of supplementation with omega-3 fatty acids on semen parameters has been assessed in two small (n=74 and 138) randomised controlled trials (Akbaraly, et al., 2018). Supplementation with docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) (990 mg/d and 135 mg/d, respectively) for 10 weeks demonstrated no effect on sperm parameters but improved sperm DNA fragmentation (Martínez-Soto, et al., 2016). Although supplementation with higher amounts of DHA + EPA (0.72 g/d and 1.12 g/d, respectively) led to significant improvements in total sperm count and concentration, sperm motility, and morphology (Safarinejad, 2011). However, I was unable to detect any robust association with either omega-3 or omega-6 fatty acids and sperm parameters; for these exposures all point estimates were close to the null (thought the degree of saturation of fatty acids did have a point estimate odds ratio of ~0.8 for TMSC< 5M compared with ≥5M). Similarly, although a variety of amino acids, including

arginine and cysteine are frequently incorporated into anti-oxidant preparations and, have been suggested as a means of improving semen parameters and potentially live-birth rates (Smits, et al., 2019), neither branched chain or aromatic amino acids were associated with semen parameters. Replication the association of histidine with motility would be useful, as it has been suggested that at ejaculation sperm chromatin is critically stabilized by salt bridges in which zinc interchelates between thiols and possibly the imidazole groups of histidine (Björndahl and Kvist, 2009). Low phenylalanine concentrations in seminal plasma have previously been reported for oligospermic males (Gupta, et al., 2011), however whether supplementation is beneficial is unknown.

I did not observe any association with baseline demographics, including adiposity or alcohol intake and semen parameters. Previous larger population studies and meta-analyses have differed in their findings with respect to whether there is or is not an inverse association between adiposity and sperm concentration and motility (Bieniek, et al., 2016, Macdonald, et al., 2013, Pacey, et al., 2014, Povey, et al., 2012, Sermondade, et al., 2013). Whether this reflects that BMI is a relatively crude marker for adiposity, although it is strongly related to other health outcomes including mortality (Global, et al., 2016), or whether metabolic health rather than adiposity per se underlies associations with semen parameter as recently suggested (McPherson and Tremellen, 2020), is unclear. However, the NMR platform used in the current study has previously detected differences for a range of metabolic disorders including adiposity (Wurtz, et al., 2014), suggesting that adiposity or mild deviation of metabolic health may have a limited impact on crude semen parameters such as concentration and motility. My findings are broadly in line with a recent systematic review and meta-analyses, which did not find any consistent associations of adiposity with sperm concentration or motility (Salas-Huetos et al 2020). Although the authors reported an isolated negative association of class III obesity ($\geq$40kg/m^2) with sperm motility, my cohort had few such participants to be able to replicate this association. Similarly, I did not observe a detrimental effect of alcohol on semen parameters, consistent with previous meta-analyses which have suggested that a detrimental effect of alcohol seemed to be limited to daily drinkers, whereas occasional drinkers or low intake as per our participants were apparently similar to never drinkers (Ricci, et al., 2017).

While my findings are of interest, I acknowledge some important limitations, the relatively small sample size, and lack of a replication cohort which is why I present the novel analyses I have undertaken as exploratory. As noted above I am unable to exclude some potentially clinically important associations and have estimated that a sample size of at least 3,188 would be required to obtain more precise and statistically robust results. The cohort comprised of men presenting to a tertiary infertility service, including some men with extremely low sperm counts, those with azoospermia and those with known female factor infertility and although we assessed whether the observed associations differed depending on the cause of infertility, my findings may not be generalisable to healthy individuals. As TMSC reflects the product of sperm concentration and motility, it may reflect a heterogeneous clinic population, particularly as a similar TMSC may be derived from low sperm concentration with high motility or high sperm concentration with low motility. Future studies of men with similarly derived TMSC or clinical phenotypes such as non-obstructive azoospermia would be useful. Furthermore, that men were confirmed (through cotinine breath test) as non-smokers, were of a relatively restricted BMI range and largely white European and educated to degree level may have introduced a selection bias which have attenuated our results towards the null. A single semen sample was assessed by a single trained operator, consistent with recent guidance that the intraclass coefficient of the parameters assessed is adequate to enable a single sample to be used for infertility referral and treatment (Barratt, et al., 2017). I used the TMSC thresholds derived by Tiegs and colleagues (Tiegs *et al.,* 2019), as that study assessed 119,972 semen samples, rather than the 20 million threshold described by others in much smaller studies (Borges *et al.,* 2016; Hajder *at al.,* 2016) I did not assess other aspects of sperm function including sperm DNA fragmentation, however, at present there is no consensus on the optimal assay methodology and its contribution to prognosis. I acknowledge that at present it is unknown whether ejaculation (whether during sex or masturbation) alters blood metabolites levels, and future studies of paired pre and post ejaculation blood samples would be useful to clarify this. I also acknowledge that the metabolic composition of seminal fluid may have influenced sperm function, with several studies of seminal fluid suggesting associations of metabolites with semen parameters (Neto *et al.,* 2020; Murgia *et al.,* 2020; Mehrparvar *et al.,* 2020; Mumcu *et al.,* 2020). Similarly repeat metabolite measures across the window of spermatogenesis may further inform a potential

role of specific metabolites. Lastly, serum metabolites were measured on the day of the semen sample and therefore may not reflect the entire spermatogenic cycle, however, previous analyses of short- and long-term repeat ^{1}H NMR measures (<6 months and >3 years) have shown good intra-class coefficients supporting that they would be representative of exposures of the spermatogenic cycle (Li-Gao *et al.*, 2019).

4.5 Conclusion

This study provides preliminary data on a range of metabolic pathways and their association with semen parameters. While I found most point estimates to be close to the null and few associations that met conventional statistical thresholds after adjustment for multiple testing, I identified several metabolites that associated with odds of TMSC with potentially of important clinical effect sizes (differences in relative odds of 20%) which warrant further exploration and replication in a larger prospective study.

Chapter 5 Spousal concordance of serum metabolomic profiles by nuclear magnetic resonance spectroscopy: A cross sectional study of 326 couples.

5.1 Introduction

Phenotype-based assortative mating is well established in humans for several traits including age (Dribe and Lundh 2009, Sánchez-Andrés and Mesa 1994), height (Silventoinen et al. 2003, Stulp et al. 2017) and other physical characteristics such as skin pigmentation(Baldwin and Damon 1973), eye and hair colour (Sebro et al. 2017). In addition, there are other behavioural and social factors that are correlated between spouse-pairs and are thought to affect mate selection such as educational level (Hur 2003, Sánchez-Andrés and Mesa 1994), occupation (Sánchez-Andrés and Mesa 1994), socio-economic status (Dribe and Lundh 2009), smoking (Di Castelnuovo et al. 2009), alcohol consumption(Grant et al. 2007), language and culture (Nagoshi et al. 1990). For other physical and physiological characteristics such as weight (Hur 2003), body mass index (Silventoinen et al. 2003) and blood pressure (Di Castelnuovo et al. 2009), weak to modest positive correlations are also observed, potentially reflecting both initial assortative mating, and subsequent spousal interaction and convergence through a shared environment and behaviours (Luo 2017).

Over the last decade epidemiological studies have increasingly measured circulating multiple metabolic traits, which collectively provide information on genomic, environmental and lifestyle traits. As physical, social and behavioural assortative mating traits may be associated with these metabolic profiles, correlations between spouses for a range of metabolites may be anticipated. However, despite the long established positive correlations of physical, social, and behavioural characteristics between couples, the assessment of metabolic measures in couples has been limited and primarily focused on conventional cardiovascular risk factors (Di Castelnuovo et al. 2009). For example, for total cholesterol, LDL cholesterol, and triglycerides, the within-spouse correlation coefficients are generally weak with coefficients ranging from 0.05 to 0.10 (Di Castelnuovo et al. 2009), with limited evidence of correlation for glucose and HDL cholesterol (Di Castelnuovo et al. 2009).

Serum nuclear magnetic resonance (NMR) metabolomics enables reproducible quantification of circulating lipids and abundant metabolites (Wurtz et al. 2017) and has been used to assess the differences in metabolites with adiposity (Wurtz et al. 2014), height (Jelenkovic et al. 2013), glycemia (Wurtz et al. 2012) and a range of physiological and pathogenic disease states (Wang et al. 2018, White et al. 2017, Wurtz et al. 2015). Furthermore, detailed metabolic profiling has been applied to assess the heritability and genetic architecture of blood metabolites (Draisma et al. 2013, Hagenbeek et al. 2020, Sirota et al. 2015, Tremblay et al. 2019), that may underlie established physical, social and behavioural assortative mating traits. We could only identify three studies assessing four cohorts for within-spouse metabolite correlations. These included sample sizes of 281, 327, 64 and 6 spousal pairs, respectively, used different metabolite platforms to each other and the one we use here, which covered 120, 110, 51 and 147 metabolic trait measures and were undertaken in extended families of twin studies (Draisma et al. 2013, Sirota et al. 2015, Tremblay et al. 2019). In general, they found weak metabolic trait spousal correlations (mean = 0.08, 0.18, 0.24), with these being weaker than twin correlations.

The aim of the current study was to assess the correlation within couples undergoing fertility treatment of physical, social and behaviour characteristics, and 155 circulating metabolic measures. These measures were profiled by a high throughput cost efficient NMR platform, covering a range of metabolic pathways, predominantly a lipidome, including lipoprotein lipids, fatty acids, as well as some amino acids, ketone bodies, and glycaemic traits. Whilst other studies have explored spousal similarity of the physical, social and behavioural characteristics that we also explore here, it is important that we explore these in this group of couples to help interpretation of metabolite correlations. Specifically, if we see similar correlations in these infertile couples to those seen in general populations it provides some rationale for assuming the results for the metabolites might generalise to a more general population.

5.2 Methods

5.2.1 Study Design and Participants

Cross-sectional study of women aged 18 to 45 and their male partners who presented at Glasgow Royal Infirmary, UK for assessment prior to assisted conception between 1 April 2017 and 31 March 2019. Referral for state-funded assisted conception is limited to those where the female body mass index (BMI) ≤30kg/m^2 and both partners are non-smokers smokers and in a stable relationship defined as cohabiting for ≥2 years, while for self-funding patients female BMI should be <35kg/m^2. Exclusion criteria for study participation were a documented positive pregnancy test at time of presentation and /or requiring gamete or embryo donation. A total of 400 women were recruited, 331 of whom had a male partner who agreed to participate and of those 326 couples (100%) had a blood sample suitable for NMR analyses (further details can be found in chapter 2).

The study was conducted according to ICH Guideline for good clinical practice, the Declaration of Helsinki and the Convention of the Council of Europe. All women provided written informed consent. The study protocol was approved prior to study initiation by the relevant institutional review boards (see Appendix).

5.2.2 Study procedures

Physical, social, behavioural, fertility and medical history was obtained by self-reported questionnaire at the baseline visit or from the medical notes at initial recruitment. Weight and height [used to calculate the body mass index (BMI)] were measured in light clothing and unshod. Weight was measured to the nearest 0.1 kg using Tanita scales; height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Smoking status was categorised as ever versus never (to be considered for state funded assisted conception both women and men had to have not smoked for at least 3 months and this was confirmed by a negative cotinine breath test) (further details can be found in chapter 2 section 2.5).

Non-fasted blood samples were collected during the same baseline visit for NMR analyses and immediately spun and frozen at −80 °C and all NMR assays completed for this study were undertaken within 1 year of storage and with no previous freeze/thaw cycles (further details can be found in chapter 2 section 2.9.3)

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5.2.3 NMR protocol

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform [Nightingale Health© (Helsinki, Finland)] at the University of Bristol. The platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, the glycolysis precursors lactate and pyruvate, ketone bodies, and amino acids in absolute concentration units (mostly mmol/l). The NMR-based metabolite quantification is achieved through measurements of three molecular windows from each sample. Two of the spectra (LIPO and LMWM windows) are acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts are conducted at 37^{O}C and 22^{O}C, respectively. Details of this platform have been published previously (Wurtz *et al.,* 2017; Soininen *et al.,* 2009) and it has been widely applied in genetic and observational epidemiological studies (Wurtz *et al.,* 2014,2015; Wang *et al.,* 2014,2018; White *et al.,* 2018; Fischer *et al.,* 2014; Mills *et al.,* 2019; Deelen *et al,.* 2019; Akbaraly *et al.,*2018). Further details of the platform are provided in Materials and Methods Chapter 2.

5.2.4 Metabolite quantification and quality control

The NMR spectra were analysed for absolute metabolite quantification (molar concentration) in an automated fashion. For each metabolite a ridge regression model was applied for quantification in order to overcome the problems of heavily overlapping spectral data. In the case of the lipoprotein lipid data, quantification models were calibrated using high performance liquid chromatography methods, and individually cross-validated against NMR-independent lipid data. Low-molecular-weight metabolites, as well as lipid extract measures, were quantified as mmol/l based on regression modelling calibrated against a set of manually fitted metabolite measures. The calibration data are quantified based on iterative line-shape fitting analysis using PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland). Absolute quantification cannot be directly established for the lipid extract measures due to experimental variation in the lipid extraction protocol. Therefore, serum extract metabolites are scaled via the total cholesterol as quantified from the native serum LIPO spectrum.

5.2.5 Statistical analysis

All analyses were performed in Stata (version 15.1, StataCorp. 2017 College Station, TX), with figures created in R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Characteristics were summarized as n, total range, mean, standard deviation, median, and 25th and 75th quantiles (IQR) as appropriate. Associations between physical, social and behavioural characteristics within couples were investigated using both Pearson and Spearman's rank correlation (continuous variables) and phi for dichotomized categorical variables(Cohen 2013). In females and males separately, the metabolic measures were scaled to standard deviation (SD) units (by subtracting the mean and dividing by the standard deviation of all women or men respectively included in the analyses). This scaling allows easy comparison of multiple metabolic measures with different units or with large differences in their concentration distributions. The primary analysis was the correlation of the unadjusted metabolite measures. In secondary analyses I assessed whether these were influenced by potentially similar physical, social and behavioural characteristics. This was done by using linear regression, with robust standard errors as some metabolite concentrations had skewed distributions, the sex-specific standard deviation (SD) scores of metabolites on a priori selected characteristics that might result in spousal correlations (age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, ethnicity) and obtaining the residuals from these regression models (i.e. the sex specific metabolic concentrations having removed variation due to the observed characteristics listed above). The correlations between the residuals from these regressions within couples were then calculated using both Pearson and Spearman's rank correlation. Confidence intervals for these correlations were calculated using bootstrapping. To test whether the skewness of the metabolite data was having any impact on correlations, regressions were repeated using quantile regression and results compared. Lastly to assess whether observed physical, social and behavioural characteristics that have been shown to correlate in couples explained any metabolite correlations I compared the covariates adjusted and unadjusted correlation using a scatter plot of these and exploring the linear fit. As women are excluded from infertility treatment if their BMI is greater than 30 kg/m2 and this exclusion (which is not applied to their male

partners) may influence spousal correlations, I reassessed the correlation of spousal BMI after exclusion of the 17 male partners with a BMI > 30 kg/m2.

5.3 Results

Three hundred and twenty-six couples with complete physical, social and behavioural characteristics and NMR data were included in the study. Characteristics of the participants are shown in Table 5.1. Couples exhibited correlations of varying strength for most physical, social and behavioural characteristics including age, height, alcohol consumption, education, smoking status, family history and ethnicity, with correlation coefficients ranging from 0.22 to 0.73 (Table 5.1). There was no evidence of within couple correlation for BMI and weight, where the correlation coefficients were -0.03 (95%CI -0.14, 0.08) and 0.01 (95%CI -0.10, 0.12) respectively (Table 5.1). When we repeated the analyses after excluding couples (n=17) where the male BMI was >30kg/m2, the results were unchanged with a correlation for BMI of -0.02 (95%CI -0.13, 0.09), p=0.75.

The correlation estimates for the unadjusted metabolite measures are shown in Figures 5.1, 5.2 and 5.3, with overall similar results for the Spearman correlation coefficients (Figures 5.4, 5.5 and 5.6). Across the metabolites correlation point estimates were all positive and ranged from very weak to modest, with the median coefficient across all 155 measures being 0.12 (full range 0.01 to 0.37 and interquartile range 0.10 to 0.18). For lipoproteins, the correlation coefficients ranged from 0.11 for very large VLDL and 0.13 for medium VLDL, to 0.12 to 0.21 for very large HDL, large HDL, medium HDL and small HDL. For fatty acids the overall degree of unsaturation was correlated within couples (0.26, 95% CI 0.13, 0.38). Of the individual fatty acids docosahexaenoic acid (0.37, 95%CI 0.22, 0.52) and omega-3-fatty acids (0.32, 95%CI 0.20, 0.43) exhibited modest correlations within couples, with the correlation of docosahexaenoic acid the strongest correlation across all of the NMR measures. The contributions of individual fatty acid classes to total fatty acid concentrations was broadly similar within couples with coefficients ranging from 0.20 to 0.27. For all the glycolysis related metabolites, there was modest evidence of positive correlations within couples with the strongest effects observed for pyruvate (0.32, 95%CI 0.22, 0.43), citrate (0.29, 95%CI 0.14, 0.44) and glycerol (0.26,

95%CI 0.15, 0.38), with the correlation for glucose 0.25 (95%CI 0.08, 0.41). Of the amino acids only, histidine had evidence of modest correlation (0.32 95%CI 0.23, 0.41), with alanine, isoleucine, leucine, valine, phenylalanine, glycine and tyrosine exhibiting weaker positive correlations (0.12 to 0.29) within couples.

	Females	Males	Correlation coefficient (95%CI)	P-value for association between traits in couples*
Age (years)	35.6 (4.4)	37.2 (5.7)	0.61 (0.53, 0.69)	<0.001
Height (cm)	164.2 (6.3)	176.5 (5.1)	0.22 (0.12, 0.32)	<0.001
Weight (kg)	66.7 (9.7)	78.6 (10.2)	0.01 (-0.10, 0.12)	0.86
BMI (kg/m^2)	24.7 (3.24)	25.2 (3.0)	-0.03 (-0.14, 0.08)	0.55
Alcohol (units per week)	4 (1,8)	4 (2,9)	0.62 (0.50, 0.74)	<0.001
Education **School** **Undergraduate** **Postgraduate**	 146 (45%) 115 (35%) 65 (20%)	 147 (45%) 139 (43%) 40 (12%)		<0.001
Smoking **Ever** **Never**	 83 (25%) 243 (75%)	 97 (30%) 229 (70%)	0.47 (0.36, 0.57)	<0.001
Physical activity (times per week) **Never/once** **Twice** **3-4 times** **> 4 times**	 33 (10%) 70 (21%) 196 (60%) 27 (8%)	 29 (9%) 73 (22%) 177 (54%) 47 (14%)		<0.001
Family history of cardiometabolic disease **Yes** **No**	 167 (51%) 159 (49%)	 154 (47%) 172 (53%)	0.39 (0.29, 0.50)	<0.001
Ethnicity **White** **Non-white**	 299 (92%) 27 (8%)	 300 (92%) 26 (8%)	0.73 (0.59 ,0.88)	<0.001

Table 5.1 Demographic/lifestyle characteristics of couples undergoing IVF treatment (N=326)

* From Pearson's correlation/chi square test

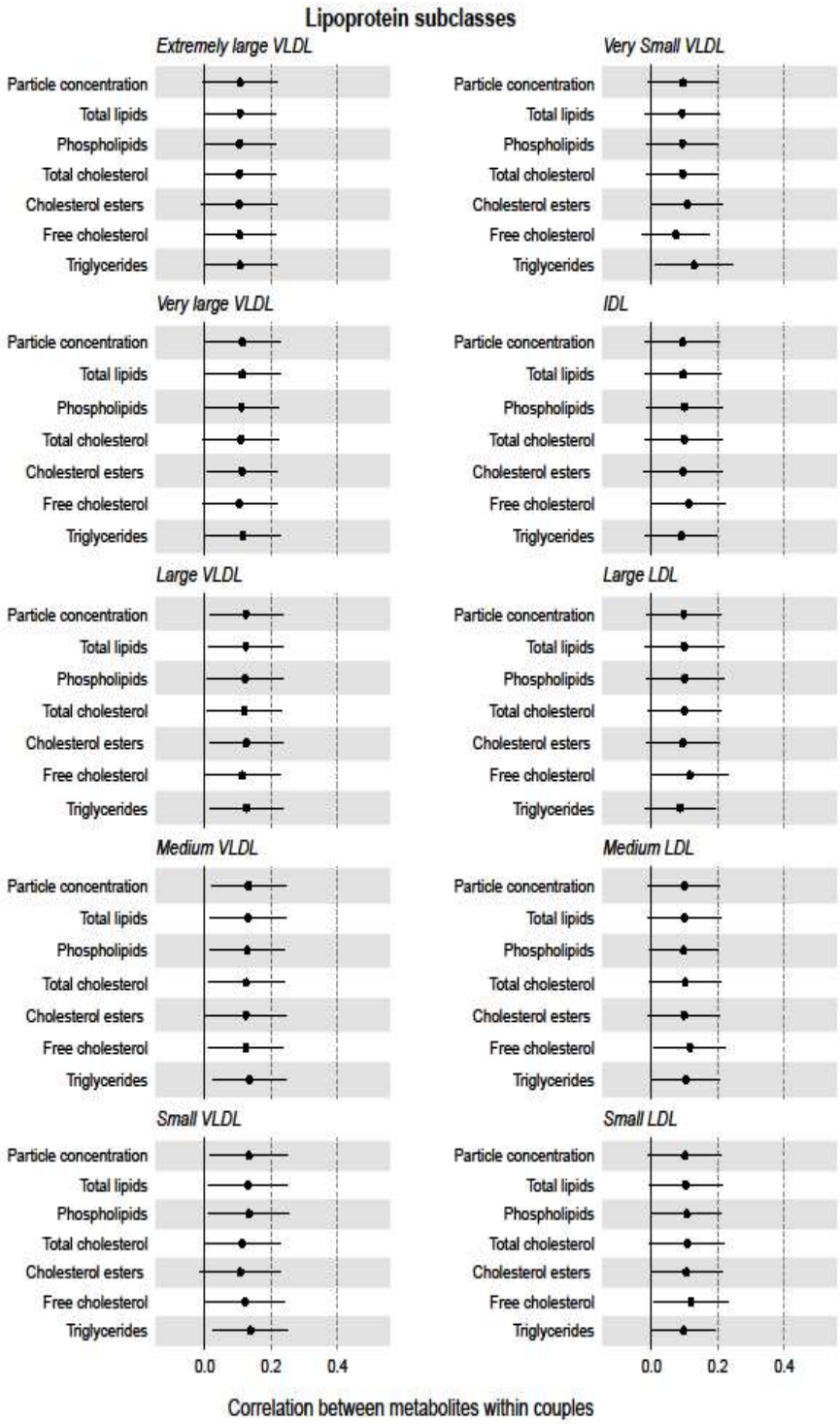

Figure 5.1 Correlations of lipoprotein classes in couples awaiting IVF
Values are Pearson correlation coefficients and 95% confidence intervals

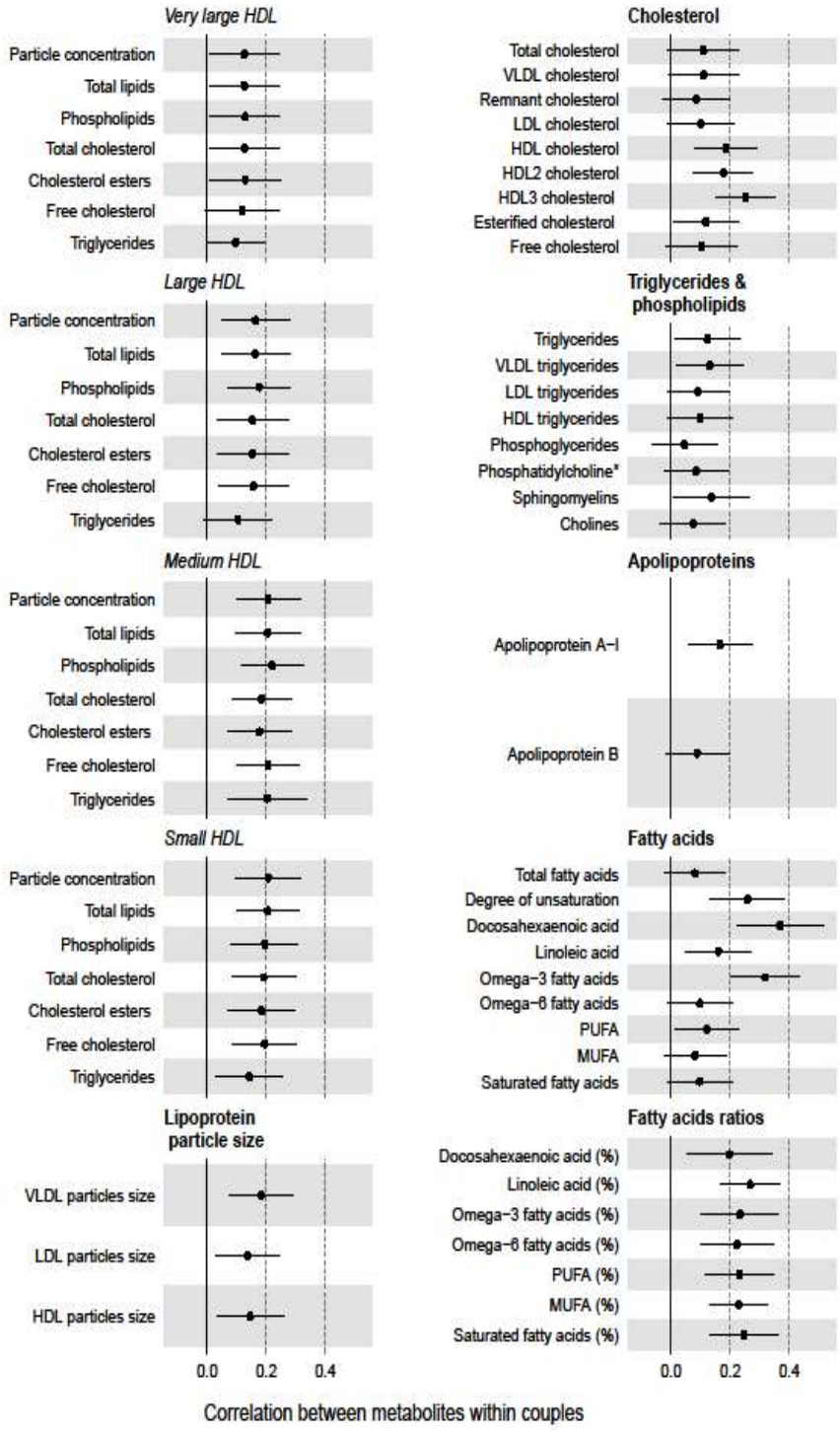

Figure 5.2 Correlations of lipoprotein classes and fatty acids in couples awaiting IVF
Values are Pearson correlation coefficients and 95% confidence intervals

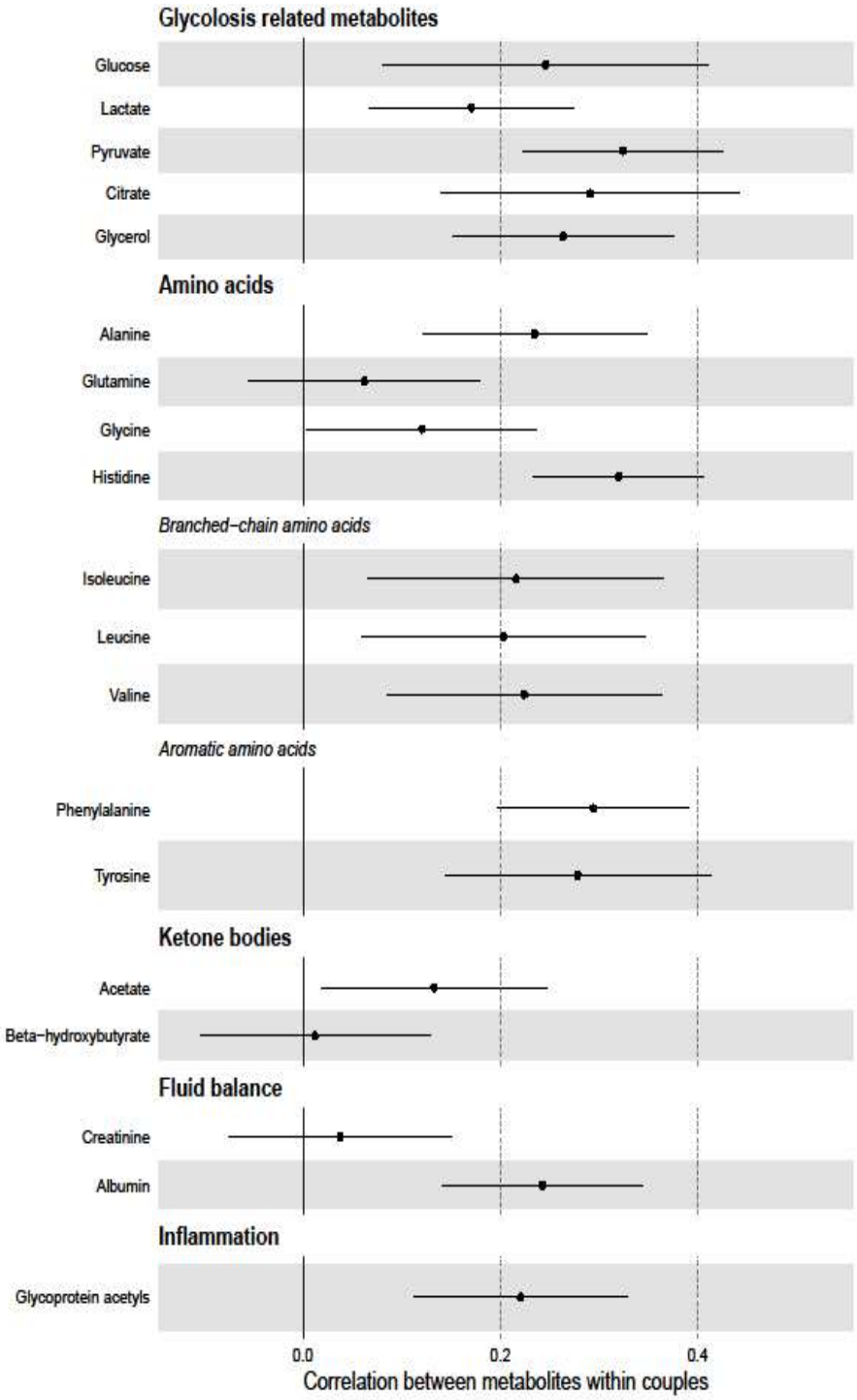

Figure 5.3 Correlations of metabolic traits in couples awaiting IVF
Values are Pearson correlation coefficients and 95% confidence intervals

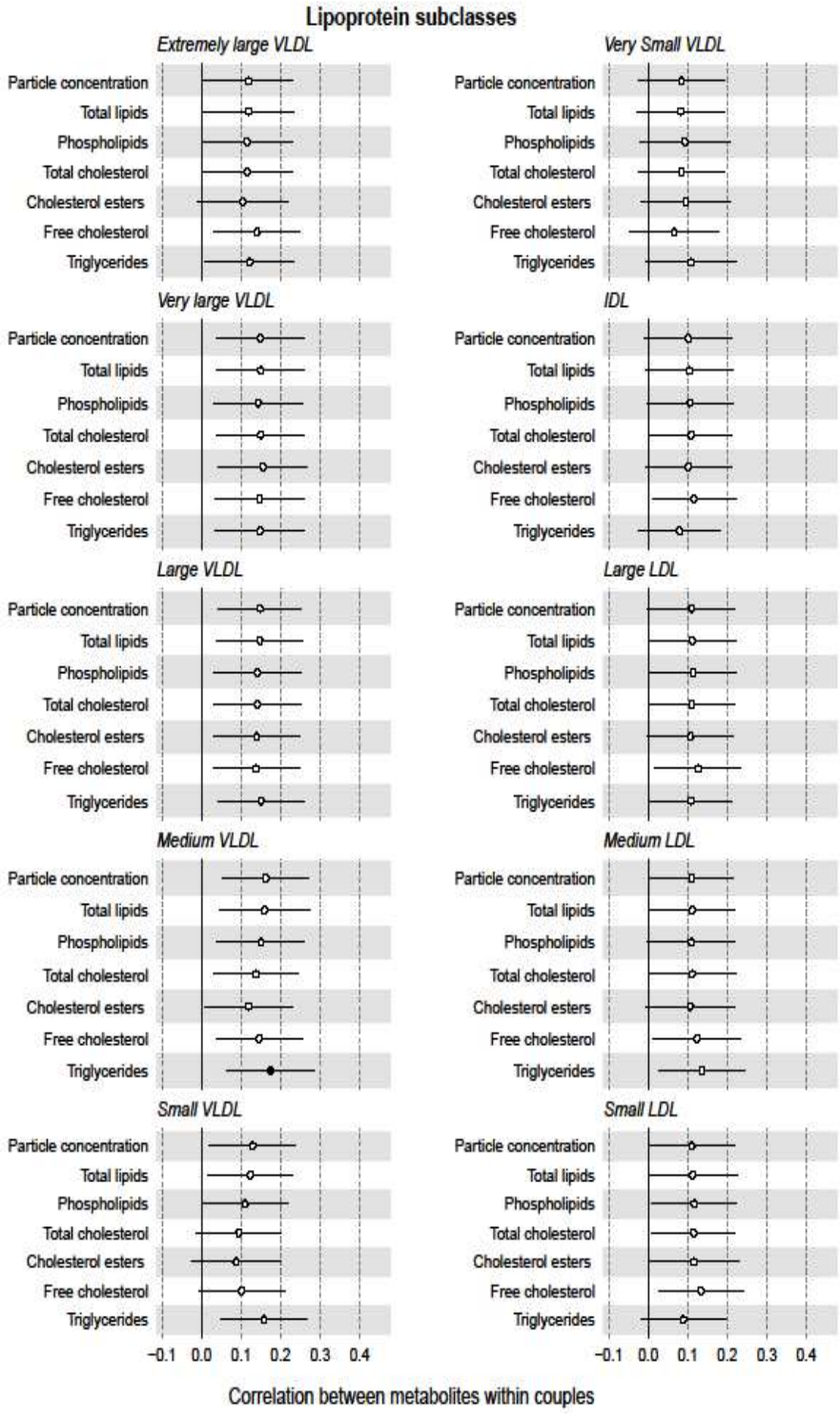

Figure 5.4 Spearman correlations of lipoprotein classes in couples awaiting IVF. Values are Spearman correlation coefficients and 95% confidence intervals

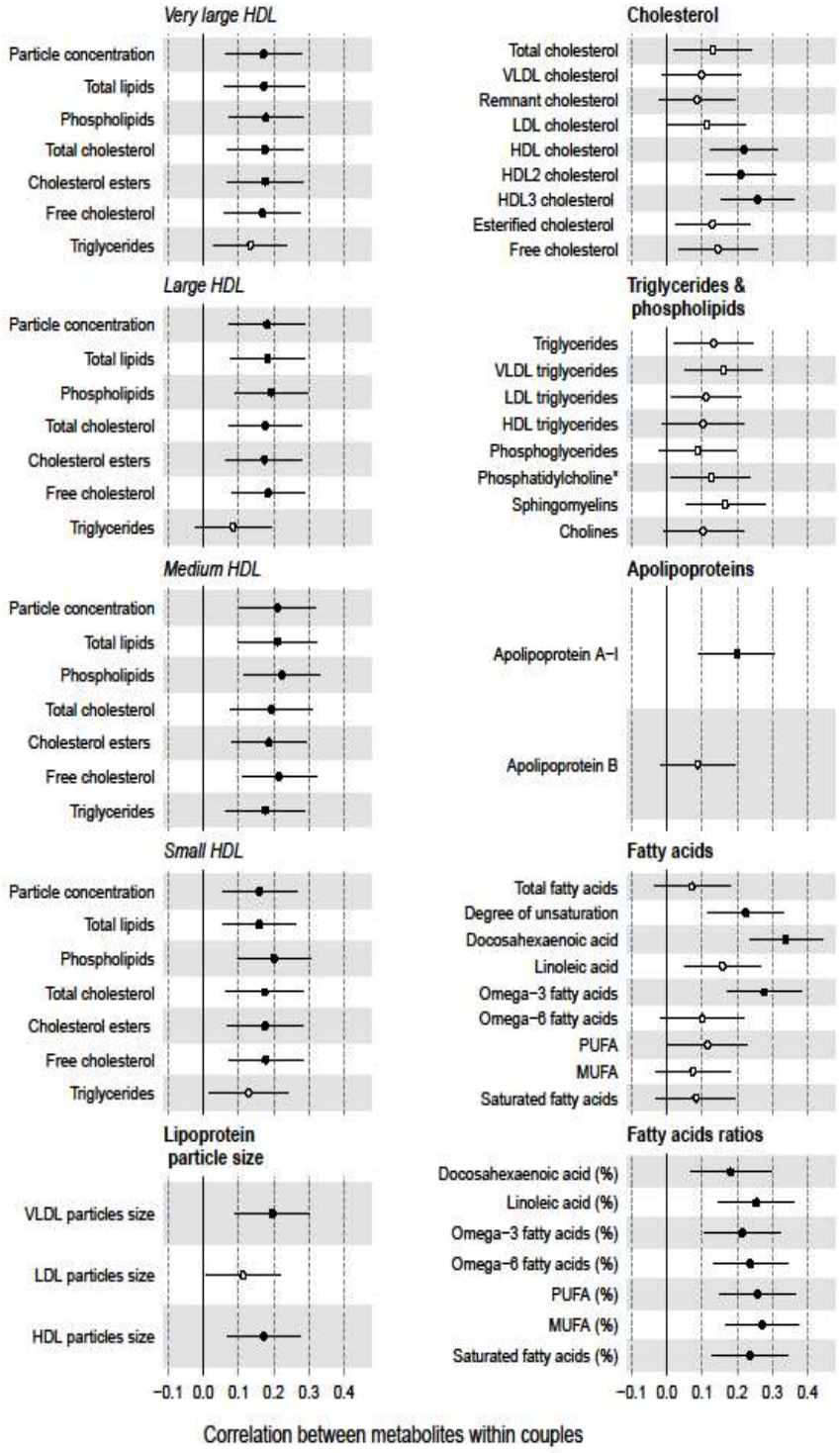

Figure 5.5 Spearman correlations of fatty acids in couples awaiting IVF. Values are Spearman correlation coefficients and 95% confidence intervals

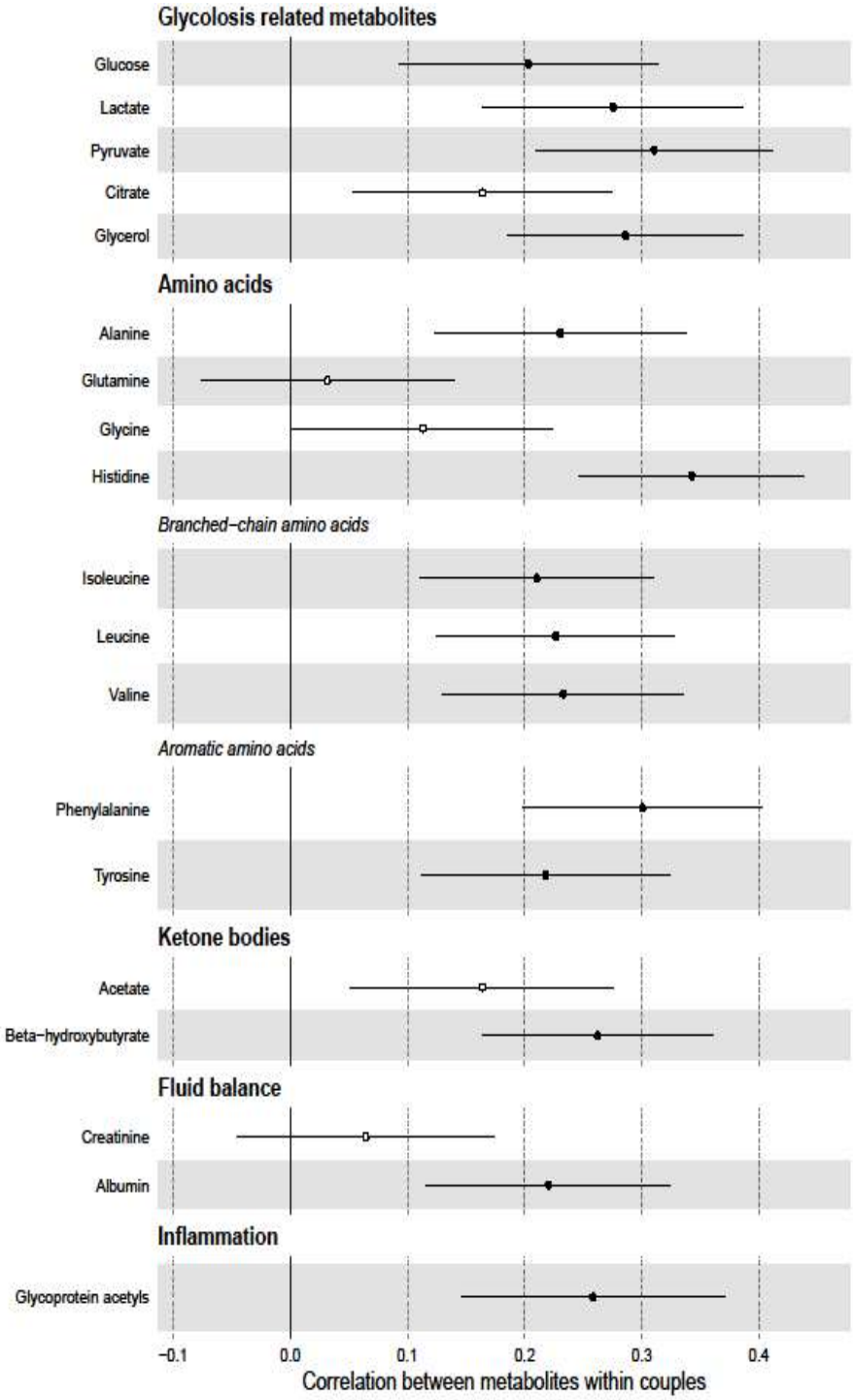

Figure 5.6 Spearman correlations of metabolic traits in couples awaiting IVF. Values are Spearman correlation coefficients and 95% confidence intervals

Correlation estimates within couples were similar in adjusted versus unadjusted analyses (Figure 5.7) for most metabolites (R^2 = 0.96 across all metabolites coefficients)

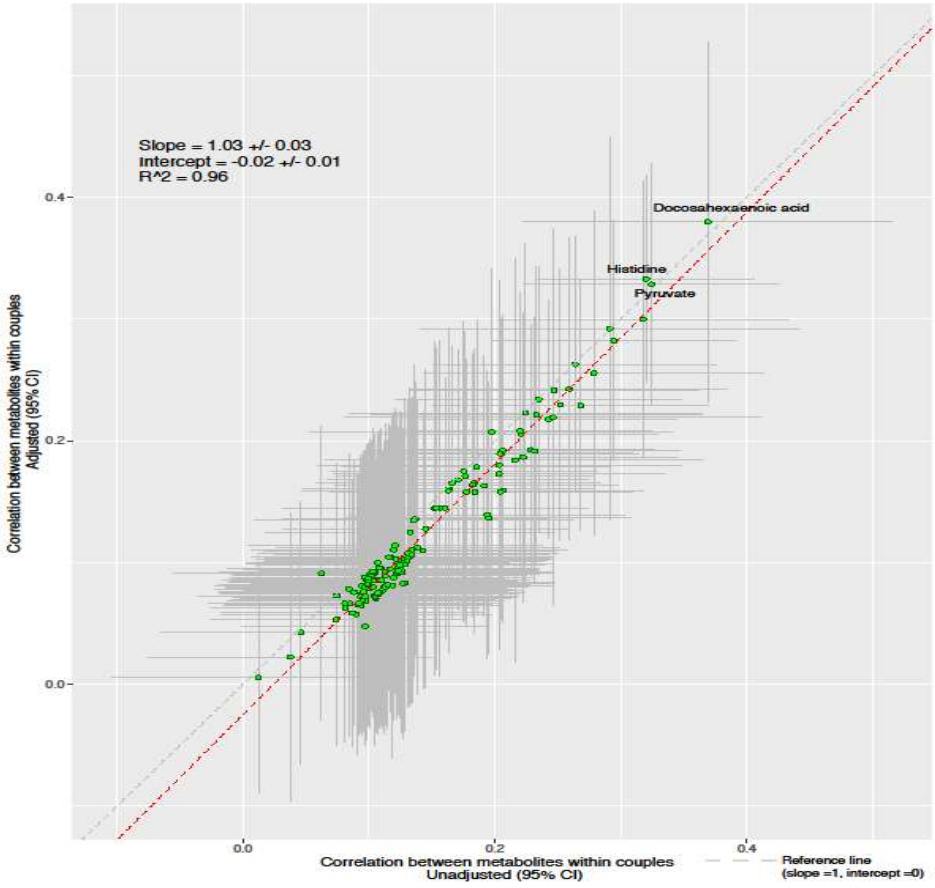

Figure 5.7 Scatterplot of adjusted versus unadjusted within couple correlation estimates across all metabolites.
In adjusted analyses in males and females separately, metabolites (in SD units) were regressed on covariates (age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity) and the residuals of those models use to estimate the adjusted Pearson correlation coefficients within couples. Unadjusted analyses were the Pearson correlation coefficients of the metabolites in SD units.

For adjusted analyses the results were similar when covariates were adjusted by linear or quantile regression (Figure 5.8, 5.9, 5.10 and Figure 5.11, 5.12, 5.13 respectively).

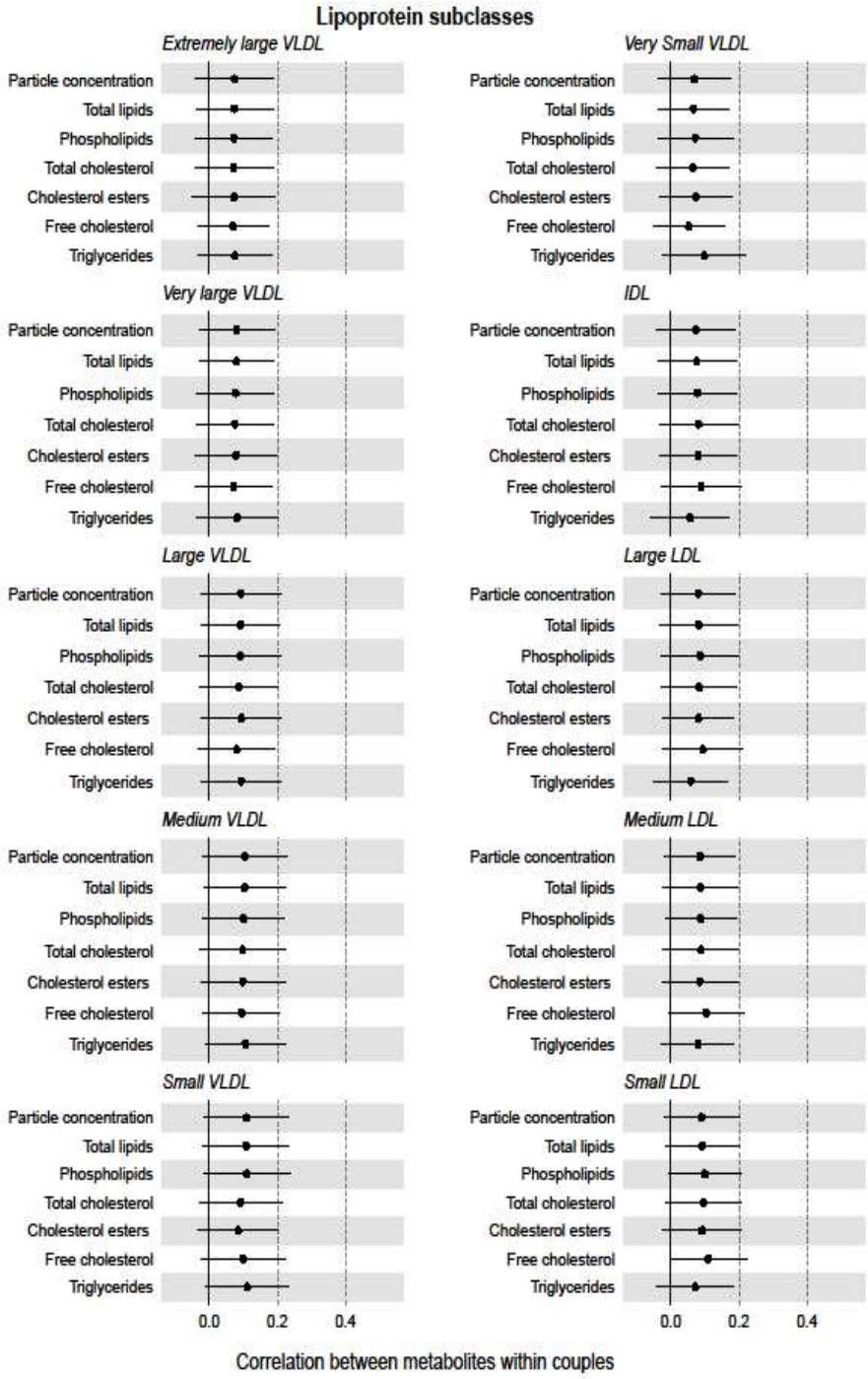

Figure 5.8 Adjusted correlations of lipoprotein classes in couples awaiting IVF. Pearson correlation coefficients and 95%CI of male and female linear regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

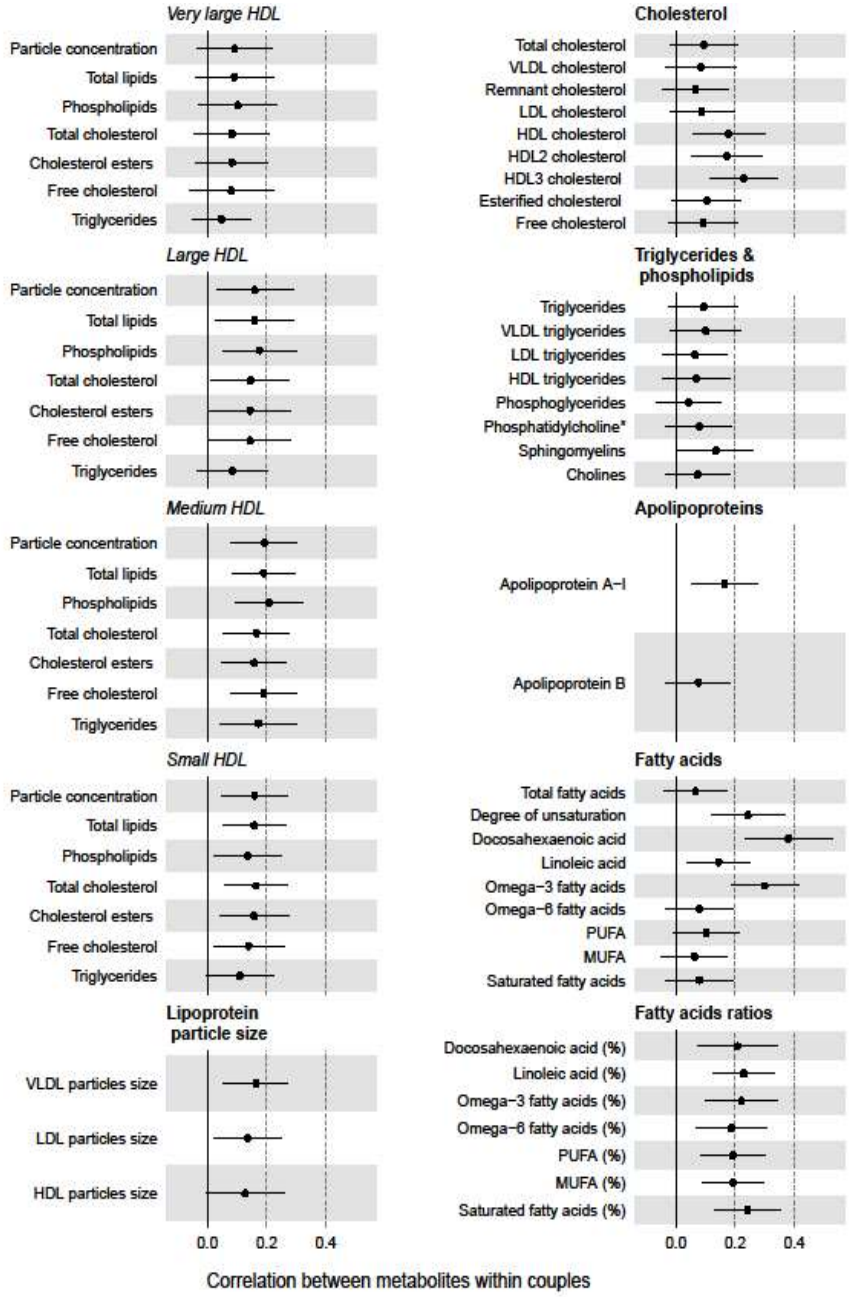

Figure 5.9 Adjusted correlations of lipoprotein classes and fatty acids in couples awaiting IVF.
Pearson correlation coefficients and 95%CI of male and female linear regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

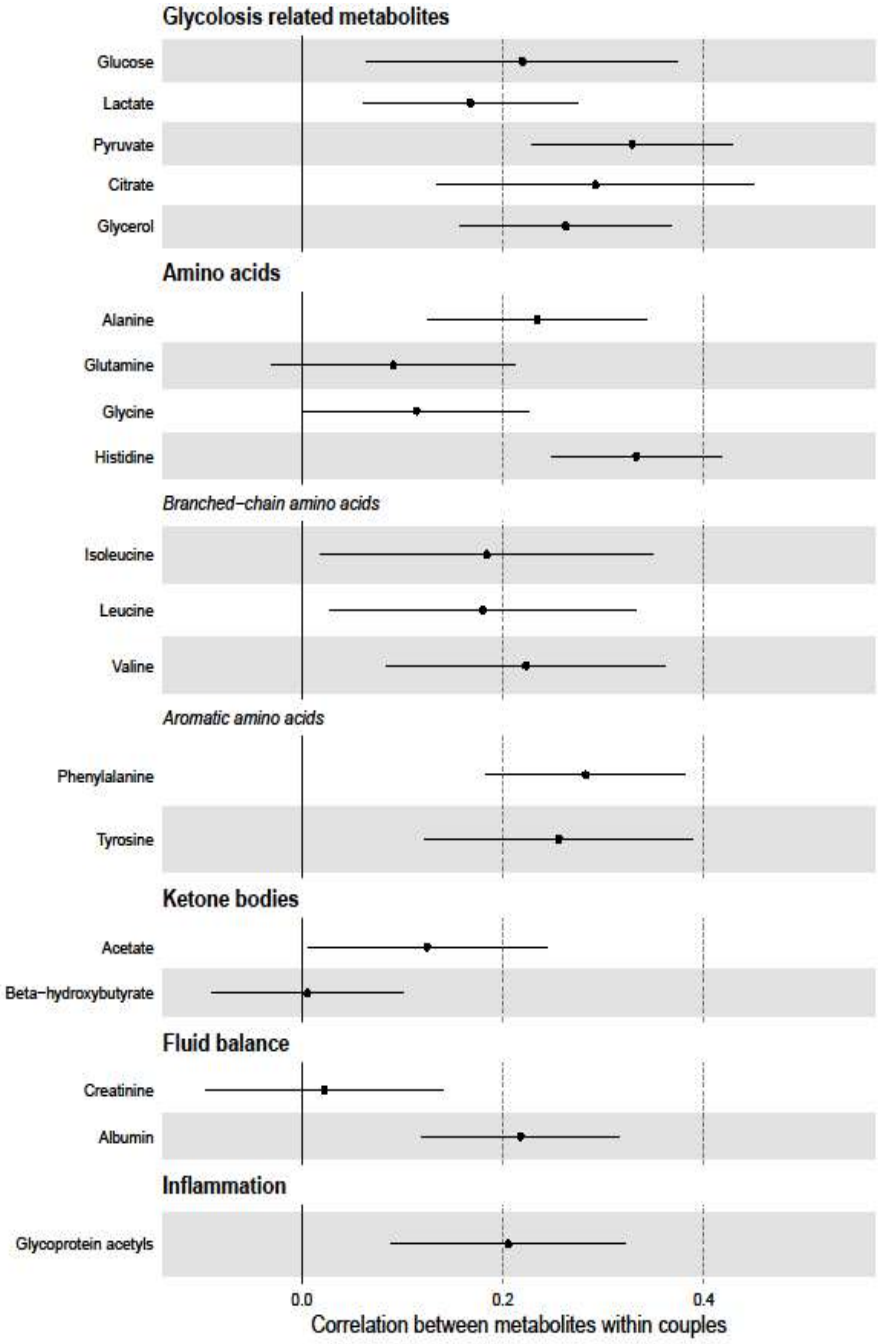

Figure 5.10 Adjusted correlations of metabolic traits in couples awaiting IVF. Pearson correlation coefficients and 95%CI of male and female linear regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

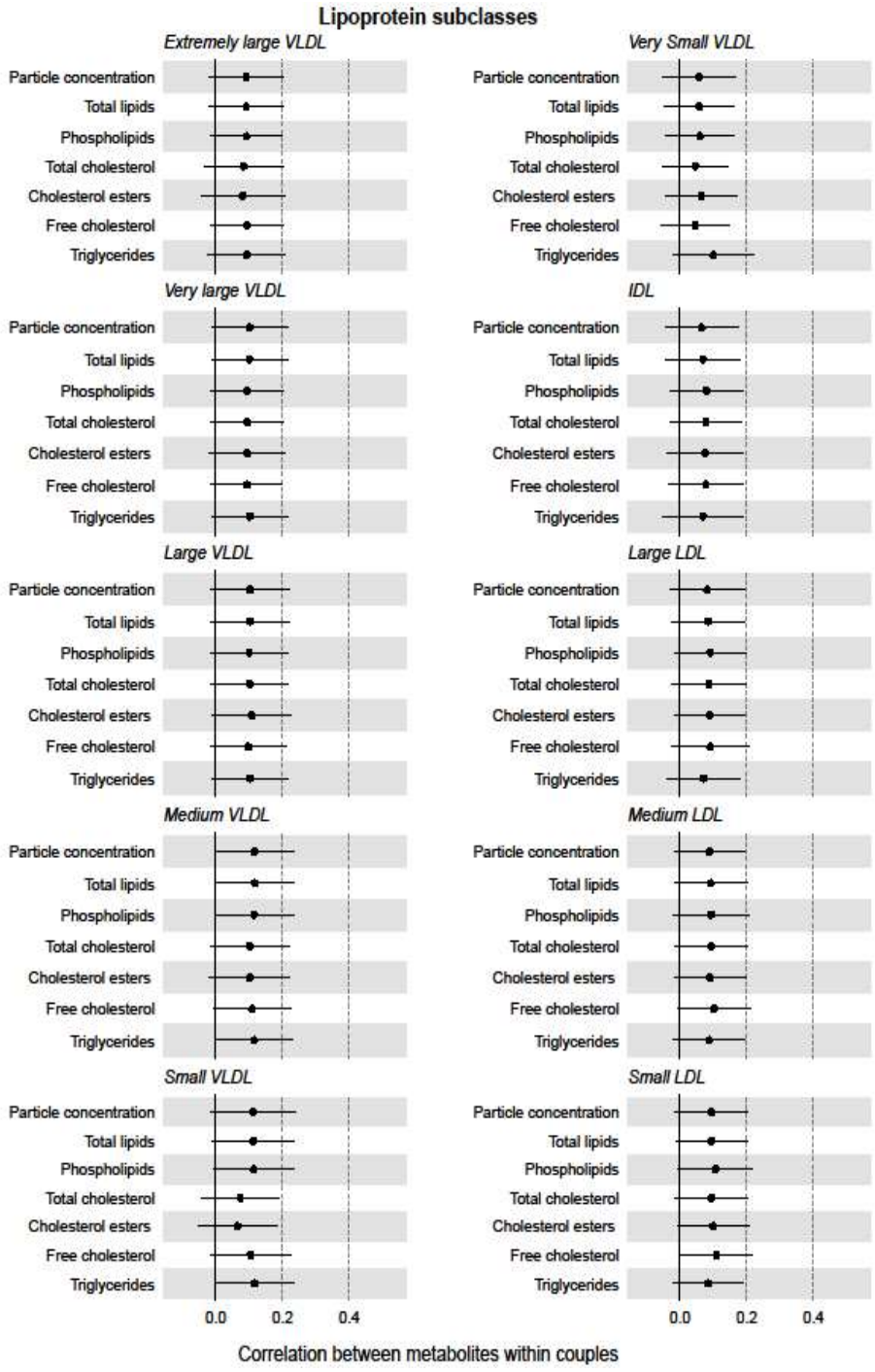

Figure 5.11 Correlations of lipoprotein classes in couples awaiting IVF using quantile regression.

Pearson correlation coefficients and 95%CI of male and female quantile regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

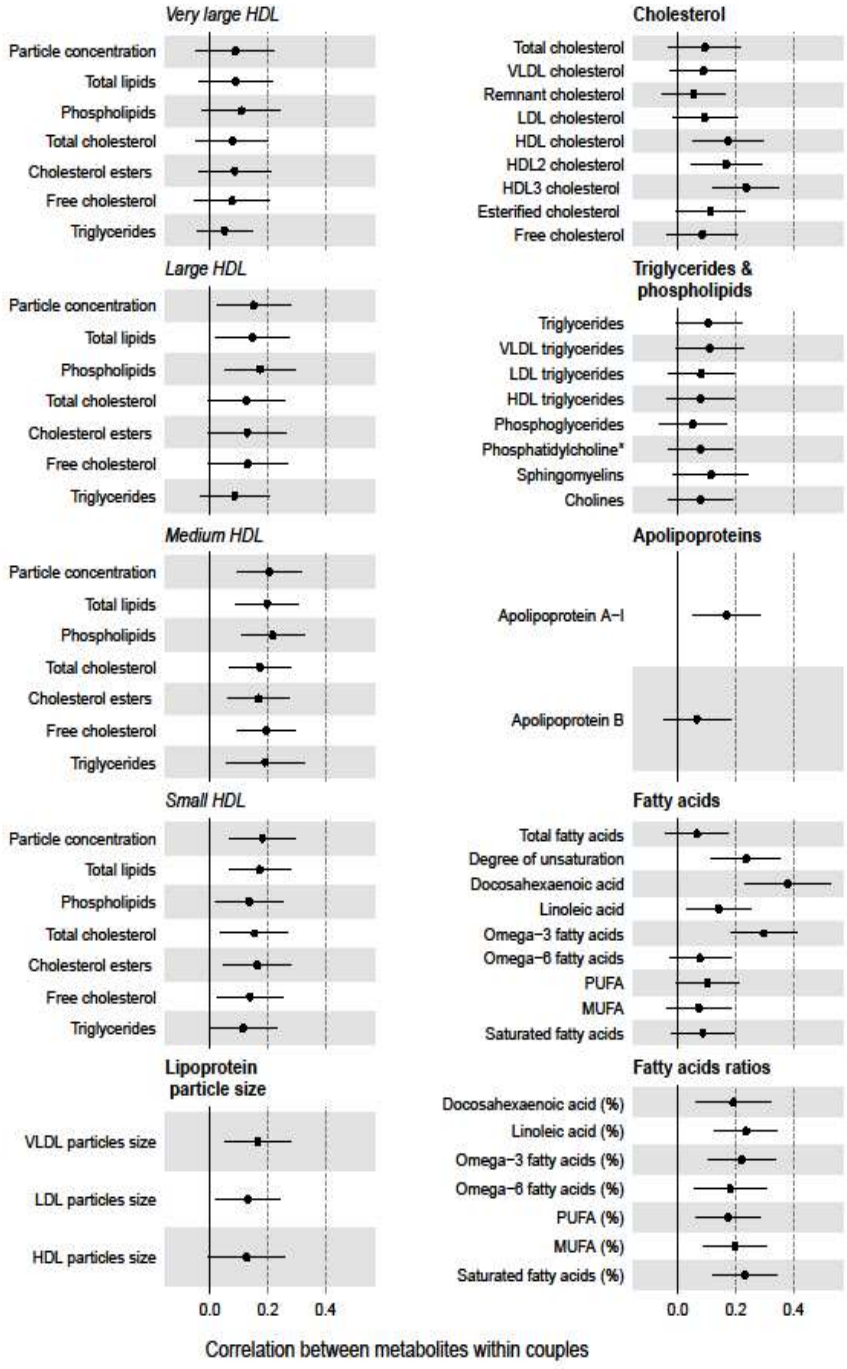

Figure 5.12 Correlations of lipoprotein classes and fatty acids in couples awaiting IVF using quantile regression.
Pearson correlation coefficients and 95%CI of male and female quantile regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

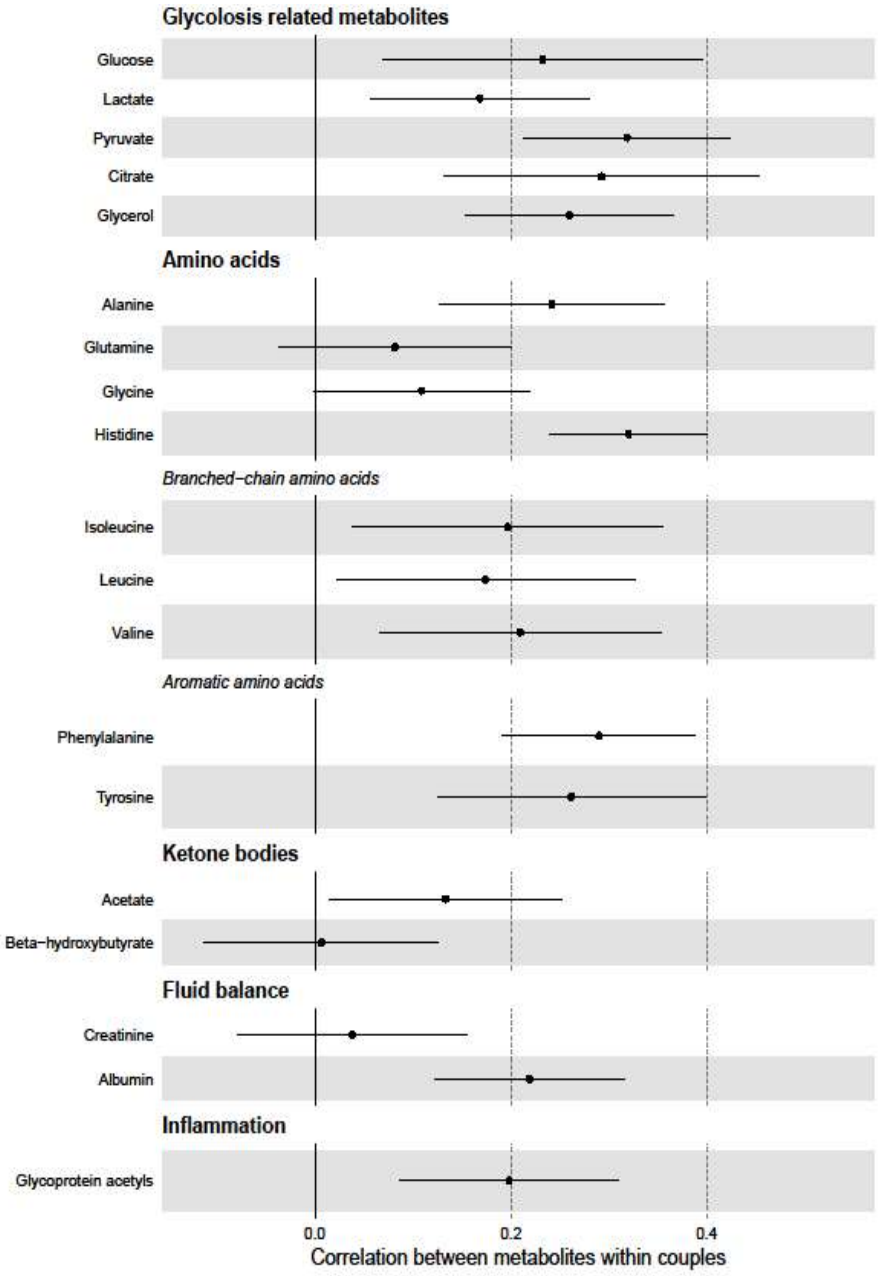

Figure 5.13 Correlations of metabolic traits in couples awaiting IVF using quantile regression.
Pearson correlation coefficients and 95%CI of male and female quantile regression residuals after sex-specific adjustment for age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity.

Figure 5.14 shows the scatter plots of the unadjusted metabolite concentrations (in SD units) for each woman versus her male partner for the four metabolites with a Pearson's correlation of greater than 0.3; Docosahexanoic acid, pyruvate, histidine and omega-3-fatty acids. For all four metabolites the concentrations were mostly concentrated around central values in both women and men but with a spread showing the weak to moderate correlations.

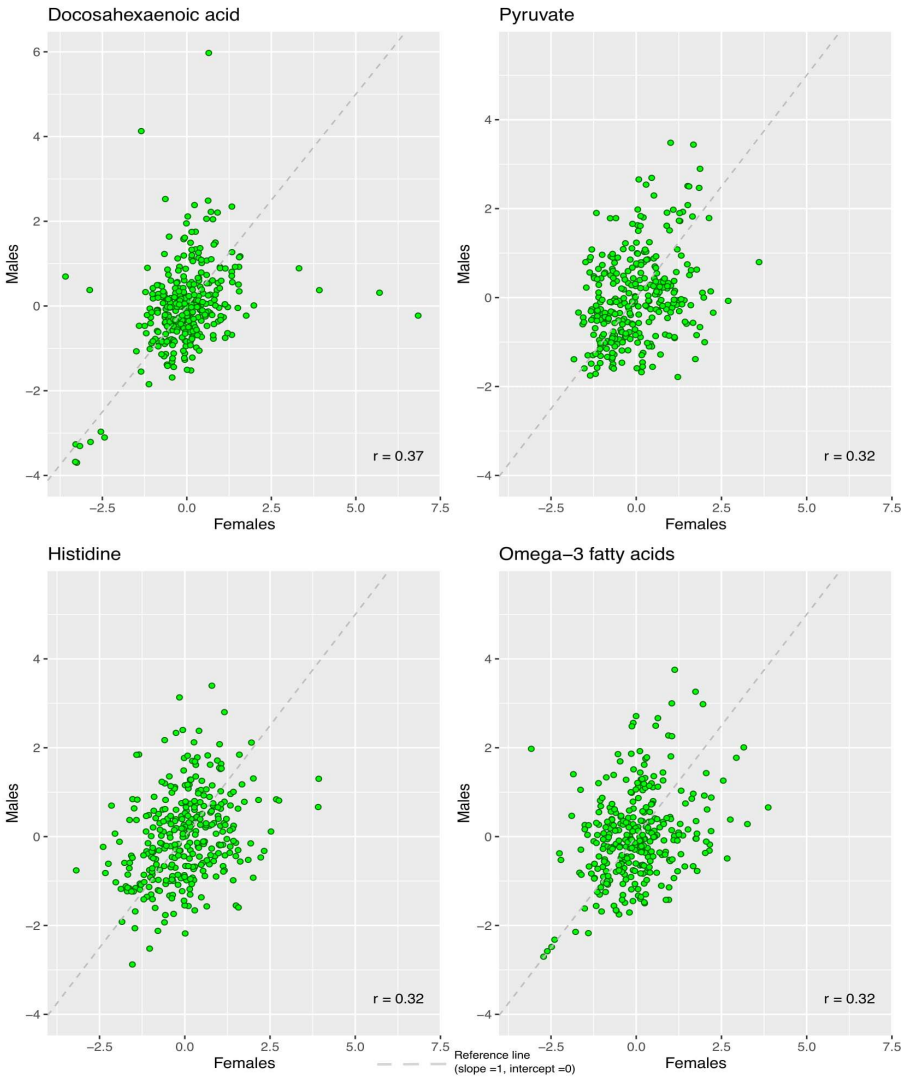

Figure 5.14 Multi-panel scatterplot of individual measures of metabolites in 326 women and their male partners for four selected metabolites with a within couple correlation of greater than 0.3.

Post-hoc analyses

When comparing Pearson with Spearman's correlations I noticed that beta-hydroxybutyrate exhibited different correlation coefficients (Pearson = 0.01 (95%CI -0.10, 0.13) versus Spearman = 0.26 (95% CI 0.16, 0.36))(Figure 5.3 versus Figure 5.6). On further investigation there was an obvious outlier for beta-hydroxybutyrate, with the difference attenuated by removal of the outlier: Spearman (adjusted) = 0.18, Pearson (adjusted) = 0.08. When males with a BMI > 30 kg/m^2 were excluded, there was still no evidence of a correlation of BMI within couples (n = 309, rho=-0.02(-0.13,0.09), p=0.75).

5.4 Discussion

In this cross-sectional study I demonstrate that couples attending for infertility treatment exhibit strong correlations for a range of physical, social and behaviour characteristics and modest to weak correlations for a range of lipids and some other metabolic measures. The similarity in correlations for height, education and ethnicity, with those found in other publications not restricted to couples seeing fertility treatment (Hur 2003, Nagoshi et al. 1990, Sánchez-Andrés and Mesa 1994, Silventoinen et al. 2003, Stulp et al. 2017), suggest that conventional assortative mating is similar in infertile couples as in the general population' couples. That diet is the principal source for several of the metabolites; docosahexaenoic acid, histidine, phenylalanine and omega-3-fatty acids, would suggest that convergence due to a shared environment and active co-participation in daily activities including food consumption facilitates convergence of some metabolites. However, I acknowledge that for the vast majority of the metabolic measures assessed here correlations were weak and in this study I have no information for how long the couples have co-habited, beyond the minimum of two years.

In line with previous research, the present analyses provide strong evidence of concordance and assortative mating for age (Feng and Baker 1994, George et al. 2015, Schwartz and Graf 2009), modest concordance for height (Stulp et al. 2017) and strong concordance for educational levels (Hur 2003, Sánchez-Andrés and Mesa 1994), with strong evidence of endogamy with respect to self-declared ethnicity (Luo 2017). Age is well established as showing the greatest level of couple similarity among all personal characteristics, with spousal age correlations typically ranging from 0.70 to 0.90 (Feng and Baker 1994, George et al. 2015, Luo

2017, Schwartz and Graf 2009). The reasons for our slightly lower estimate (0.61 95% CI 0.53, 0.69) are unclear but may reflect recruitment of participants with known fertility issues, as both maternal and paternal age are known to be independently inversely associated with fecundity, however, the median age gap was similar to that observed in the general population(Ford et al. 2000). The observed modest estimate for height is similar to previous meta-analyses, which have suggested moderate assortative pairing for height across human populations (r =0.23, 95%CI 0.21, 0.23), and that the strength of this assortment appears to be relatively constant over time (Stulp et al. 2017). For ethnicity although endogamy remains the norm in Scotland, it has declined over recent years with similar declines observed in other Western countries (Frimmel et al. 2013, Luo 2017). In contrast to this decline in ethnic endogamy, most studies in line with our own study, have indicated a sustained increase in educational homogamy (George et al. 2015), with moderate partner similarities for potential drivers for education including socioeconomic status, abilities and intelligence all documented (Luo 2017). Despite previous meta-analyses suggesting weak associations (r = 0.10 to 0.15) for BMI, weight and related indices including waist circumference and waist to hip ratios (Di Castelnuovo et al. 2009), we did not observe any correlation, thus, no concordance for BMI between couples. This is likely to reflect our unique population, as even when we restricted the analyses to couples where the male BMI was also ≤30kg/m2 to account for the female BMI treatment eligibility criteria we still did not observe a correlation.

The observed convergence of additional lifestyle factors like alcohol consumption (r = 0.62, 95%CI 0.50, 0.74) emphasizes a strong concordance between couples, with a dominance of consumption of a low number of units compliant with national guidelines (≤14 units per week), may in part reflect that the population were drawn from an infertility clinic where healthy preconceptual lifestyle behaviours may be anticipated. Meta-analyses have previously suggested an overall moderate similarity for alcohol use (r = 0.36)(Reynolds et al. 2006), though levels of similarity observed in different studies have ranged from negligible to high. For exercise, studies have generally reported correlations between 0.15 and 0.30 (Aarnio et al. 1997, PÉRUSSE et al. 1989, Price and Vandenberg 1980) albeit some higher than 0.40 (Jurj et al. 2006, Willemsen et al. 2003). That our observed correlation of smoking status (r= 0.47 95%CI 0.36, 0.57) which shows modest

concordance, is marginally higher than previous estimates reported in a meta-analyses of smoking habits (r = 0.23, 95% CI: 0.12, 0.36) (Di Castelnuovo et al. 2009) may reflect our eligibility criteria, as in Scotland placement on the waiting list for public funding of fertility treatment is dependent on confirmation of non-smoking by cotinine breath testing for both partners.

Limited evidence from twin and family studies of multiple metabolites suggest that the heritability (h2; proportion of phenotypic variance due to genetic factors) of lipids and lipid-like molecules have a mean h2 levels of 47% (range from h2 = 0.11 to h2 0.66), while for organic acids and derivatives the mean is 0.41 (0.14-0.72), essential amino acids 0.42 (0.23-06.4) and non-essential amino acids 0.39 (0.22-0.69)(Draisma et al. 2013, Hagenbeek et al. 2020, Kettunen et al. 2012, Sirota et al. 2015, Tremblay et al. 2019). As direct genetic variation in metabolites profiles would not produce a correlation between couples due to the invisible nature of both genes and metabolites, my observed correlations are likely to be due to through indirect pathways including assortative mating for social and behavioural characteristics. In a systematic review for coronary risk factors, significant but low (upper limit of 95% confidence interval, maximal 0.10) spousal correlations were identified for total and LDL cholesterol and total triglycerides (Di Castelnuovo et al. 2009). These meta-analysis estimates are very similar to those reported here; total cholesterol 0.07 vs 0.11, LDL cholesterol 0.06 vs 0.10 and triglycerides 0.08 vs 0.12, with the detailed NMR breakdown of the lipid subclasses and lipoproteins providing further similar estimates of spousal correlation for lipid metabolism, that illustrates very weak concordance. To date given limited prospective longitudinal studies, inference on whether assortative mating and/or cohabitation and thereby a shared environment underlie these associations has been achieved by using marriage duration as a surrogate for a common environment and potential convergence (Di Castelnuovo et al. 2009). These support initial indirect assortative mating (i.e. on social or behaviour factors that influence metabolism), and that a shared environment may further influence lipid metabolism but to a lesser degree (Di Castelnuovo et al. 2009).

I observed weak to moderate spousal correlations for a range of essential amino acids and omega-3-fatty acids including the subtype docosahexaenoic acid all of which have diet as their principal source (Benevenga and Steele 1984, Horrocks

and Yeo 1999). The sharing of a common household larder and most main meals has been proposed as a mechanism by which couples have similarities in types of food, and nutrient intakes(Bove et al. 2003). Although gender asymmetry in the spousal adoption of health-related dietary changes has been reported, with female partners more likely to adopt male partners changes than vice versa (Savoca and Miller 2001), this may not apply to preconceptual diets where females may have a dominant role in preparation for pregnancy. Consistent with the suggestion that shared diet may have a critical influence, heritability variance estimates for circulating serum levels of histidine, docosohexaenoic acid, phenylalanine have been all lower than those observed for lipids, with environmental factors such as diet having a much greater contribution (Kettunen et al. 2012).

Glucose, the downstream glycolysis product of pyruvate and then the citric acid cycle carbon flux product of citrate, plus lactate and the glyceroneogenesis pathways were all weakly correlated, which elucidates modest concordance in my results. A meta-analyses of six studies, estimated that history of spousal diabetes was a risk factor for diabetes in their partner (effect estimate 1.26 (95% CI 1.08, to 1.45)(Leong et al. 2014). A data mining study of 5,643 couples and 5643 non-couple pairs similarly found strong associations of having diabetes within couples (5.2% both of the couple had diabetes) than non-couples (0.1%)(Wang et al. 2017). Heritability and shared environmental factors are proposed to account for at least half of the variability in normalised fasting glucose (Boehnke et al. 1987), however, my study is unable to delineate their respective contributions to the weak association observed here.

This study adds to the small number (3) of studies we could identify that have previously explored spousal metabolite correlations (Draisma et al. 2013, Sirota et al. 2015, Tremblay et al. 2019). It has a similar sample size to one of those previous studies (Draisma et al. 2013) and examines a similar number of metabolic traits to two of them (Draisma et al. 2013, Tremblay et al. 2019). It adds to those previous studies which were examining correlations in twin studies, where one or both of a spousal pair had been recruited based on being a twin. I do however acknowledge several limitations. Participants were couples awaiting IVF and this homogeneous relatively healthy population may have resulted in some selection

bias and may mean that our results do not generalise to a general population of couples of reproductive age or same-sex populations. Previous population studies have suggested that regardless of sex composition of the partnership, all couples demonstrate substantial within couple similarity in demographics including for age, education, race/ethnicity, work hours, and earnings (Andersson et al. 2006). Determination of metabolite concentrations were undertaken on non-fasting samples, however, collaborations of several studies using this same NMR analysis platform results have not differed notably between studies in which the analyses were undertaken in fasted and non-fasted participants (Wurtz et al. 2015). The analyses are cross-sectional and included couples within a narrow age range. With repeat assessments of couple correlations over time, or with cross-sectional data including couples with a wide age range and number of years of being together, it would be possible to explore the relative contributions of assortative mating and convergence on the weak metabolite correlations we have observed. Previous studies that have tried to explore this using marriage / cohabitation duration as a surrogate have found little evidence of any convergence for physical measures such as BMI or blood pressure, while behaviours such as smoking and alcohol converged during the initial period of a relationship prior to marriage / cohabitation, whereas convergence in physical activity was sustained throughout life (Ask et al. 2012) (Tambs and Moum 1992). The NMR platform used misses a proportion of the currently quantifiable metabolites in human serum/plasma, including markers of microbiota metabolism, vitamins, co-factors, and xenobiotics, that may be influenced by diet and preconceptual supplements. I did not undertake detailed dietary questionnaires, which would allow me to confirm the suggestion that a shared environment and common food would contribute to the observed correlations of metabolites.

The study exhibits a strong to modest concordance between couples in most baseline characteristics, such as age, height, level of education attained, alcohol consumption and smoking status. In addition, there were weak to modest positive correlations for most metabolites, including very weak concordance in lipoproteins, modest concordance in fatty acids, amino acids, and glycolysis related metabolites.

5.5 Conclusion

I have explored within couple correlations of multiple metabolomic traits and find weak to modest positive correlations for the vast majority that are not influenced by adjustment for traits know to be influenced by assortative mating or shared couple behaviours. This suggests assortative mating, for example via genes linked to assortative characteristics such as height and education, might have some potential weak to modest impact on couples having similar metabolic traits. Whilst we acknowledge replication in a general population would be valuable the broadly similar within couple correlations of physical, social, and behavioural traits in these couples provides some evidence that our findings might be generalisable. Longitudinal studies would be valuable to fully explore the relative roles of assortative mating and convergence.

Chapter 6 Preconceptual male and female metabolite profiles are associated with ongoing pregnancy after IVF.

6.1 Introduction

The study of metabolites has become a fast-growing area of interest for research into the causes, prognosis, screening, diagnosis, and treatment of many conditions, including pregnancy (Wang, et al., 2018, White, et al., 2020, White, et al., 2017, Wurtz, et al., 2015, Wurtz, et al., 2017). For infertility, mass spectrometry-based metabolomics have primarily focused on elucidating sperm and oocyte physiology and embryo selection (Gardner, et al., 2015, Nel-Themaat and Nagy, 2011, Sakkas, et al., 2015). Characterisation of preconceptual/pre-treatment circulating metabolites with IVF outcomes has however been limited, but initial small studies suggest that circulating metabolites including lipids and lipoproteins may be associated with embryo quality and clinical pregnancy rates (Jamro, et al., 2019, Onyiaodike, et al., 2018).

There is considerable experimental literature supporting a role for lipids in males and female fertility (Maqdasy, et al., 2013, Miettinen, et al., 2001), as higher high-density lipoproteins (HDL) concentrations have been associated with better oocyte and embryo outcomes as well as improved spermatogenesis (Fujimoto, et al., 2010). However, the concentration of the individual lipoprotein particles may be critical as abnormal serum lipid profiles have been associated with poorer oocyte quality and embryo development (Grummer and Carroll, 1988). Furthermore, elevated free cholesterol has been associated with reduced fecundity in women independent of BMI (Schisterman, et al., 2014), particularly if cholesterol was abnormally elevated (Pugh, et al., 2017). A potential paternal contribution may also exist as male free cholesterol concentrations have been suggested as an independent risk factor for reduced fecundability, irrespective of female lipid levels (Schisterman, et al., 2014).

Improvements in standard analytical procedures including those of high-throughput 1H-nuclear magnetic resonance (NMR) techniques have enabled clinical quantitation of a far wider range of the lipidome than conventional clinical

chemistry measures, while also providing quantitation of fatty acids, amino acids, glycolysis metabolites, ketone bodies and an inflammatory marker (Al Rashid, et al., 2020, Rankin, et al., 2014, Wang, et al., 2018, White, et al., 2020, White, et al., 2017, Wurtz, et al., 2017). I am not aware of any study which has assessed female and male preconceptual metabolite levels, and the role of female and male preconceptual/pre-treatment metabolic profiles with IVF pregnancy outcomes is unknown. The aim of this study was to assess the association between 155 metabolites in females and males undergoing their first cycle of IVF treatment and ongoing pregnancy rates.

6.2 Methodology

6.2.1 Study Design and Participants

Prospective cohort study of women aged 18 to 45 and their male partners who presented at Glasgow Royal Infirmary, UK for assessment prior to assisted conception between 1 April 2017 and 31 March 2019. Exclusion criteria were a documented positive pregnancy test at time of presentation, body mass index (BMI) ≥35kg/m2, and/or requiring gamete or embryo donation. A total of 398 women were recruited and 325 male partners (further details can be found in chapter 2).

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The study was conducted according to ICH Guideline for good clinical practice, the Declaration of Helsinki and the Convention of the Council of Europe. All participants provided written informed consent. The study protocol was approved prior to study initiation by the relevant institutional review boards (see Appendices).

6.2.2 Study procedures

Individual demographic, lifestyle, fertility and medical history data was obtained through self-completed questionnaires and from clinical data by linkage to electronic medical records. Weight and height [used to calculate the body mass index (BMI)] were measured in light clothing and unshod. Weight was measured to the nearest 0.1 kg using Tanita scales; height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Smoking status was categorised as ever versus never (to be considered for state funded assisted conception women had to have

not smoked for at least 3 months and this was confirmed by a negative cotinine breath test). Additional blood samples were taken for NMR analyses, immediately spun and frozen at −80 °C. All NMR assays completed for this study were undertaken within 1 year of sample storage and with no previous freeze/thaw cycles (further details can be found in chapter 2 section 2.7).

Ovarian stimulation was performed in accordance with local treatment protocols according to baseline AMH and AFC measurements. In brief GnRH antagonist or agonist-controlled cycles were used according to the patient phenotype with a variable dose of gonadotrophins administered based on ovarian reserve markers and bodyweight. Ultrasound and biochemical monitoring were performed throughout stimulation with cycles cancelled if there was no evidence of follicular development. Final oocyte maturation was triggered with 6,500IU recombinant hCG when 3 or more follciles were ≥17mm in size. In cases where there was a clinical concern regarding potential OHSS, GnRH agonist triggering (0.5mg Buserelin) with subsequent elective vitrification of blastocysts was undertaken. Oocyte retrieval was performed under sedation 36 hours after the final trigger. All oocytes were fertilised by ICSI, with incubation in a time lapse incubator and selected embryos transferred on day 5, with vitrification of surplus embryos. Progesterone supplementation was commenced on the day after oocyte retrieval. For those having a frozen embryo transfer, a GnRH controlled medicated cycle was used with oestradiol valerate 6mg from day 2 of the cycle and confirmation of adequate endometrial thickness on day 10 prior to commencing vaginal progesterone. The number of embryos transferred was in accordance with the multiple birth minimisation strategy of the Human Fertilisation and Embryology Authority.

Ongoing pregnancy was defined as presence of a fetal heart at 20 weeks gestation. Ongoing pregnancy from the first embryo transfer irrespective of whether it was fresh or frozen was used as the primary outcome.

6.2.3 NMR protocol

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform which applies a single experimental setup, based at the University of Bristol [Nightingale Health© (Helsinki, Finland)]. This

platform allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, various glycolysis precursors, ketone bodies, and amino acids in absolute concentration units.

The NMR-based metabolite quantification was achieved by measuring through three molecular windows from each sample. Two of the spectra (LIPO and LMWM windows) were acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts were conducted at 37OC and 22OC, respectively. Details of this platform have been published previously (Soininen, et al., 2009, Wurtz, et al., 2017) and it has been widely applied in genetic and observational epidemiological studies (Fischer, et al., 2014, Mills, et al., 2019, Wang, et al., 2018, Wang, et al., 2016, White, et al., 2017, Wurtz, et al., 2015). Further details of the platform are provided in Chapter 2 Materials and Methods.

6.2.4 Metabolite quantification and quality control

For each sample, the NMR spectra were analysed for absolute metabolite quantification (molar concentration) in automated fashion. A ridge regression model was applied for quantification of each metabolite in order to overcome the problems of heavily overlapping spectral data. Quantification of lipoprotein lipid data was performed by calibrating against high performance liquid chromatography methods, and then individually cross-validated against NMR-independent lipid data. Low-molecular-weight metabolites, as well as lipid extract measures, were quantified as mmol/l based on regression modelling calibrated against a set of manually fitted metabolite measures. The calibration data was quantified based on iterative line-shape fitting analysis using PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland). Absolute quantification could not be directly established for the lipid extract measures due to experimental variation in the lipid extraction protocol. Therefore, serum extract metabolites have been scaled via the total cholesterol as quantified from the native serum LIPO spectrum.

6.2.5 Assessment of potential confounders

For women and men the principal confounders of age, BMI, educational attainment, ethnicity, family history of cardiometabolic disease (defined as first degree relative affected), physical activity, alcohol intake, smoking status, duration and cause of infertility were considered because they plausibly influenced both NMR metabolites and the chance of ongoing pregnancy (Lintsen, et al., 2005, Matthews, et al., 2009, Mushtaq, et al., 2018, Sermondade, et al., 2019). For women whether this was primary or secondary infertility, with the latter defined as following a previous naturally conceived pregnancy, was also adjusted for as a confounder.

6.2.6 Statistical analysis

Statistical analyses were conducted using R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Characteristics of the participants were summarized as n, total range, mean, standard deviation, median, and 25th and 75th quantiles (IQR) as appropriate. Multivariable logistic regression was used to examine the associations of serum metabolic profiles with ongoing pregnancy separately for males and females. Robust standard errors were estimated for all associations, as some metabolite concentrations had skewed distributions. The metabolic measures were scaled to sex-specific standard deviation (SD) units (by subtracting the mean and dividing by the standard deviation of either all men or women included in the analyses). This scaling allows easy comparison of multiple metabolic measures with different units or with large differences in their concentration distributions. As an additional sensitivity analyses to account for any potential sex-specific differences in metabolite distributions we also assessed the associations in the original metabolite units for both sexes (for example per 1mmol/l increase).

In the main analysis, adjustments were made for all a priori selected confounders (age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility and whether the woman had primary or secondary infertility). I assessed the consistency of the magnitude and direction of the covariate adjusted odds ratios for women and men with ongoing pregnancy using a scatterplot and exploring the linear fit. As I have previously shown weak to moderate spousal

concordance for metabolite profiles (Chapter 5, Al Rashid, et al., 2020), I performed additional analyses to assess the association of metabolite concentrations with ongoing pregnancy after restriction to women (n=322) with a male partner in the study.

6.3 Results

Four hundred women and 326 male partners were initially recruited, with 392 women (98%) and 322 men proceeding to treatment. Baseline characteristics of the female and male participants are shown in Table 6.1. The mean (SD) age of the women was 35.5 (4.4) years, with the majority (92%) White European, with a mean (SD) BMI of 24.7 (3.2) and 68% with primary infertility. Median AMH was 16.1 pmol/l (IQR 8.9, 28.0) and median AFC of 12 (IQR 7, 16). The male partners were of a slightly older mean age 37.2 (5.7) years, and 20% exhibited a known male factor cause of infertility.

	Females (N=398)	Males (N=325)
Age (years): Mean, SD, Range	35.5 (4.43) 22-45	37.2 (5.7) 25-55
BMI (kg/m^2) : Mean, SD, Range	24.7 (3.2) 18.2-32.5	25.2 (3.0) 19.4-35.1
Alcohol (units per week): Median, IQR, Range	4 (1,8) 0-27	4 (2,9) 0-40
Highest Education N(%)		
School	185 (46%)	147 (45%)
Undergraduate	139 (35%)	138 (42%)
Postgraduate	74 (19%)	40 (12%)
Ever smoked: N(%)	104 (26%)	97 (30%)
Physical activity (times per week)		
Never/once	41 (10%)	29 (9%)
Twice	86 (22%)	73 (22%)
3-4 times	239 (60%)	176 (54%)
> 4 times	32 (8%)	47 (15%)
Family history of cardiometabolic disease: N(%)	208 (52%)	153 (47%)
Ethnicity: N(%)		
White European	365 (92%)	299 (92%)
Asian	28 (7%)	19 (6%)
Other	5 (1%)	7 (2%)
Cause of infertility		
Tubal disorder	44 (11%)	
Endometriosis	32 (8%)	
Ovulatory disorder	24 (6%)	
Azoospermia		11 (3%)
Oligozoospermia		55 (17%)
Male factor/No male partner	87 (22%)	
Female factor		84 (26%)
Other/Unexplained	211 (53%)	174 (54%)
Missing data		1 (<0.1%)
Duration infertility (years): Median, IQR, Range	3 (2,4) 1-13	
Primary infertility: N(%)	271 (68%)	-
Gravidity: Median, IQR, Range	0 (0,1) 0-12	-
Parity: Median, IQR, Range	0 (0,0) 0-4	-
AMH (pmol/l): Median, IQR, Range	16.1 (8.9,28.0) 1-170.8	-
Total AFC:	12 (7,16) 0-40	-

Table 6.1 Baseline characteristics of the study population.

The clinical outcomes of the index ovarian stimulation cycle are shown in Table 6.2. An equal weighting of GnRH agonist and antagonist cycles were undertaken with a median duration of 11 (IQR 9, 12) days of stimulation. A small proportion of cycles were either cancelled due to no response or no oocytes retrieved or no

embryos available on day 5 for transfer. The majority (83%) of women had a single blastocyst transferred, with an overall ongoing pregnancy rate of 47.2% per cycle started and a multiple pregnancy rate of 1.1%.

	Clinical outcomes (N=392)
Stimulation protocol: N (%)	
GnRH agonist	193 (49.2%)
GnRH antagonist	198 (50.5%)
Total FSH units (IU): Median, IQR	2588 (1800, 3150)
Duration of stimulation (days): Median, IQR	11 days (9,12)
Number of oocytes retrieved: Median, IQR, Range	8 (6, 11) 0 - 33
Number of MII oocytes: Median, IQR, Range	7 (4, 9) 0 - 28
Number of 2PN embryos: Median, IQR, Range	5 (3, 7) 0 - 23
Number of embryos frozen: Median, IQR, Range	1 (0, 3) 0 - 23
Number of embryos transferred: N (%)	
Single embryo	293 (83.2%)
Double embryo	59 (16.8%)
Clinical outcomes per cycle started: N (%)	
Cycle cancelled for anticipated poor response	12 (3.1%)
No oocytes retrieved	6 (1.5%)
No Embryo available for transfer	23 (5.9%)
Not Pregnant	128 (32.6%)
Biochemical Pregnancy	21 (5.3%)
Miscarriage	16 (4.1%)
Ongoing Pregnancy*	185 (47.2%)
Pregnancy terminated**	1 (0.3%)
Multiple ongoing pregnancy: N (%)	183 (98.9%)
Singleton	
Twins	2 (1.1%)

Table 6.2 Clinical outcomes of the study population.
*An ongoing pregnancy was defined as each pregnancy showing a positive heartbeat at ultrasound after 20 weeks of gestation.
**pregnancy terminated for medical reasons

The adjusted associations of the metabolites for females and males and ongoing pregnancy are presented in Figure 6.1, 6.2 and 6.3 with the unadjusted analyses of male and female metabolites and ongoing pregnancy presented in Figure 6.4, 6.5 and 6.6.

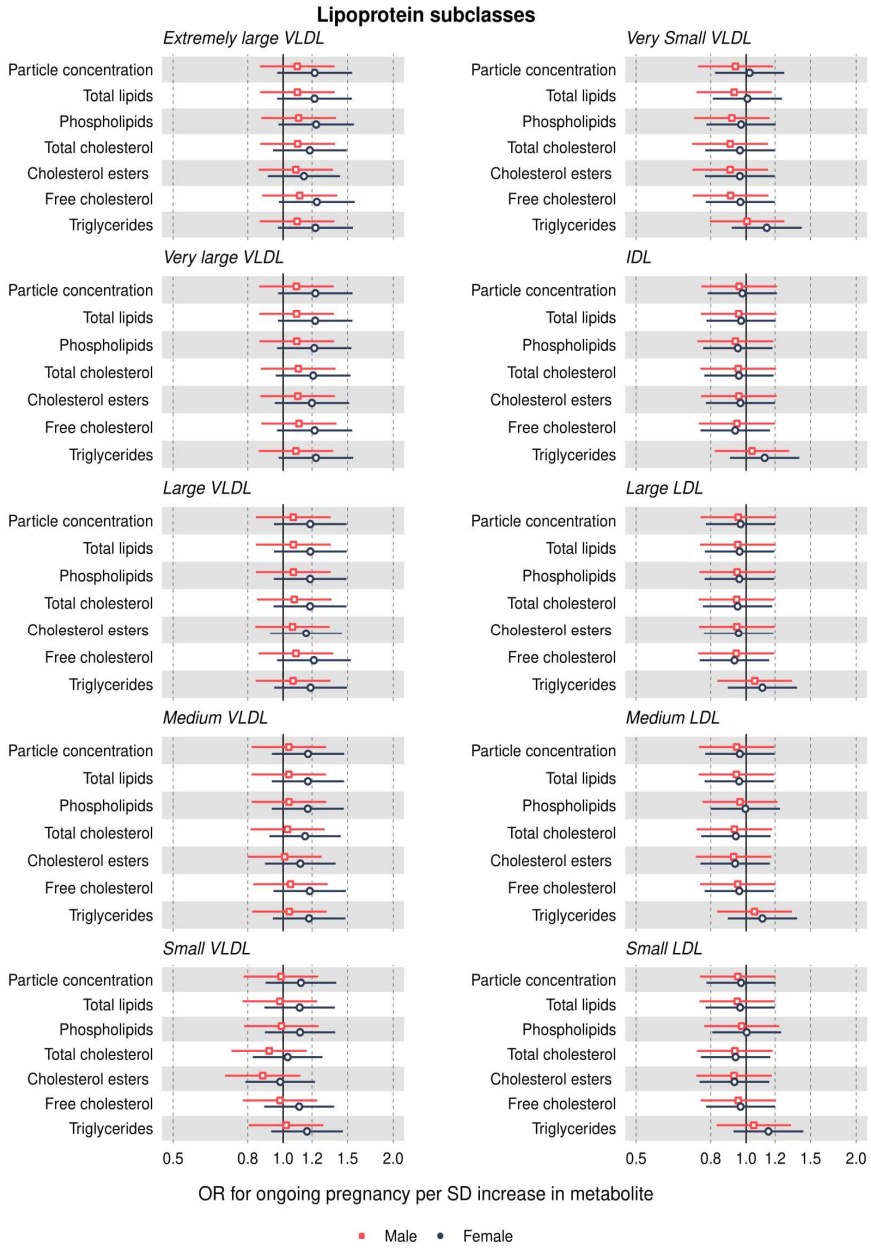

Figure 6.1 Associations of lipoprotein classes with ongoing pregnancy in males and females.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility

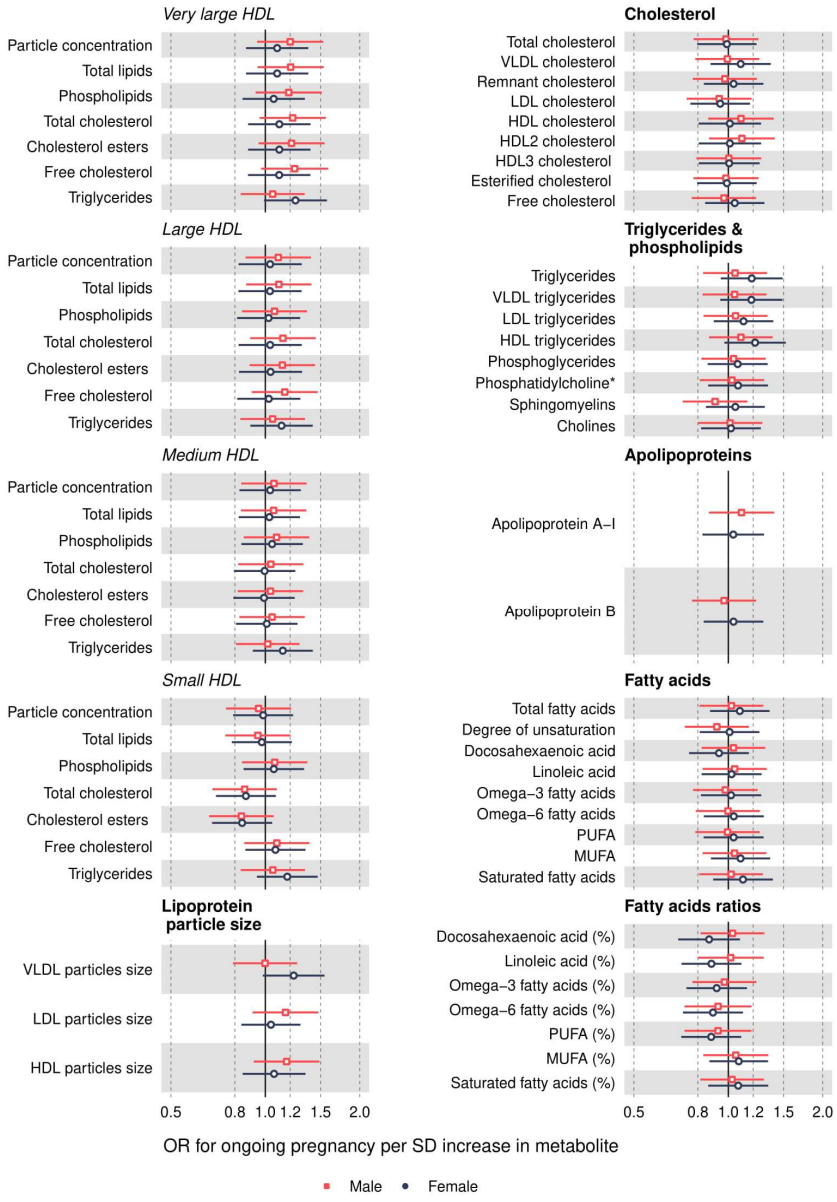

Figure 6.2 Associations of lipoprotein classes and fatty acids with ongoing pregnancy in males and females.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility

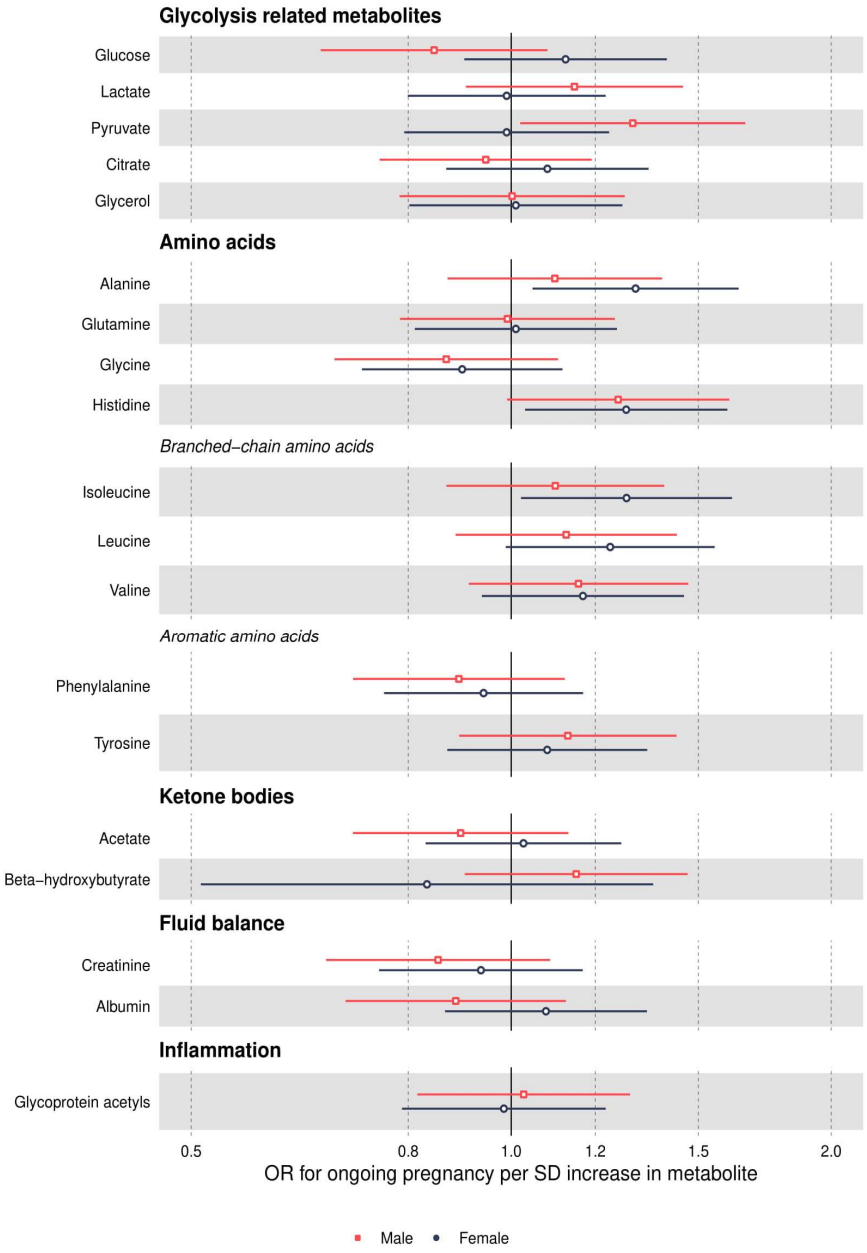

Figure 6.3 Associations of metabolic traits with ongoing pregnancy in males and females.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility

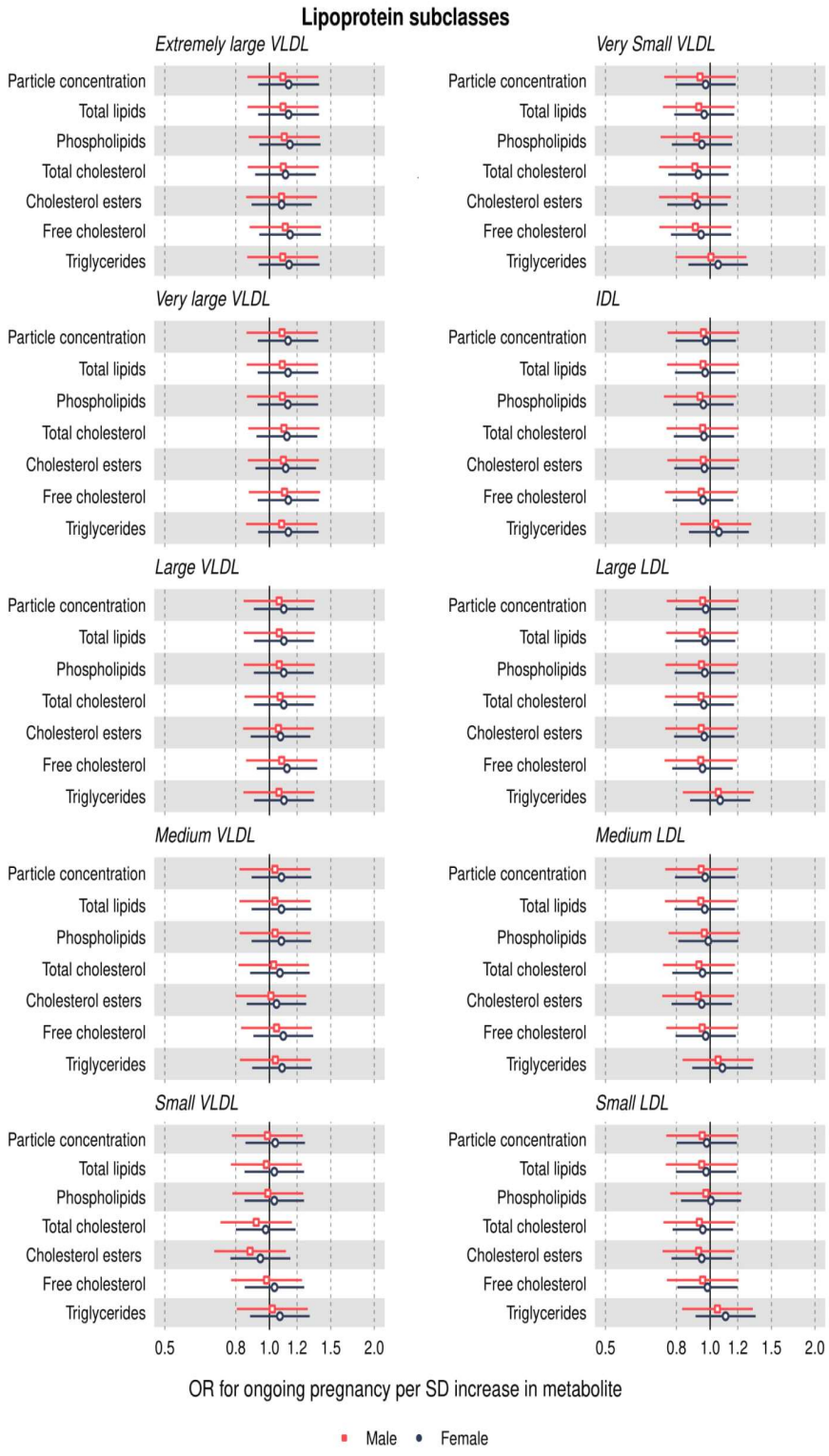

Figure 6.4 Unadjusted associations of lipoprotein classes in male and female with ongoing pregnancy.

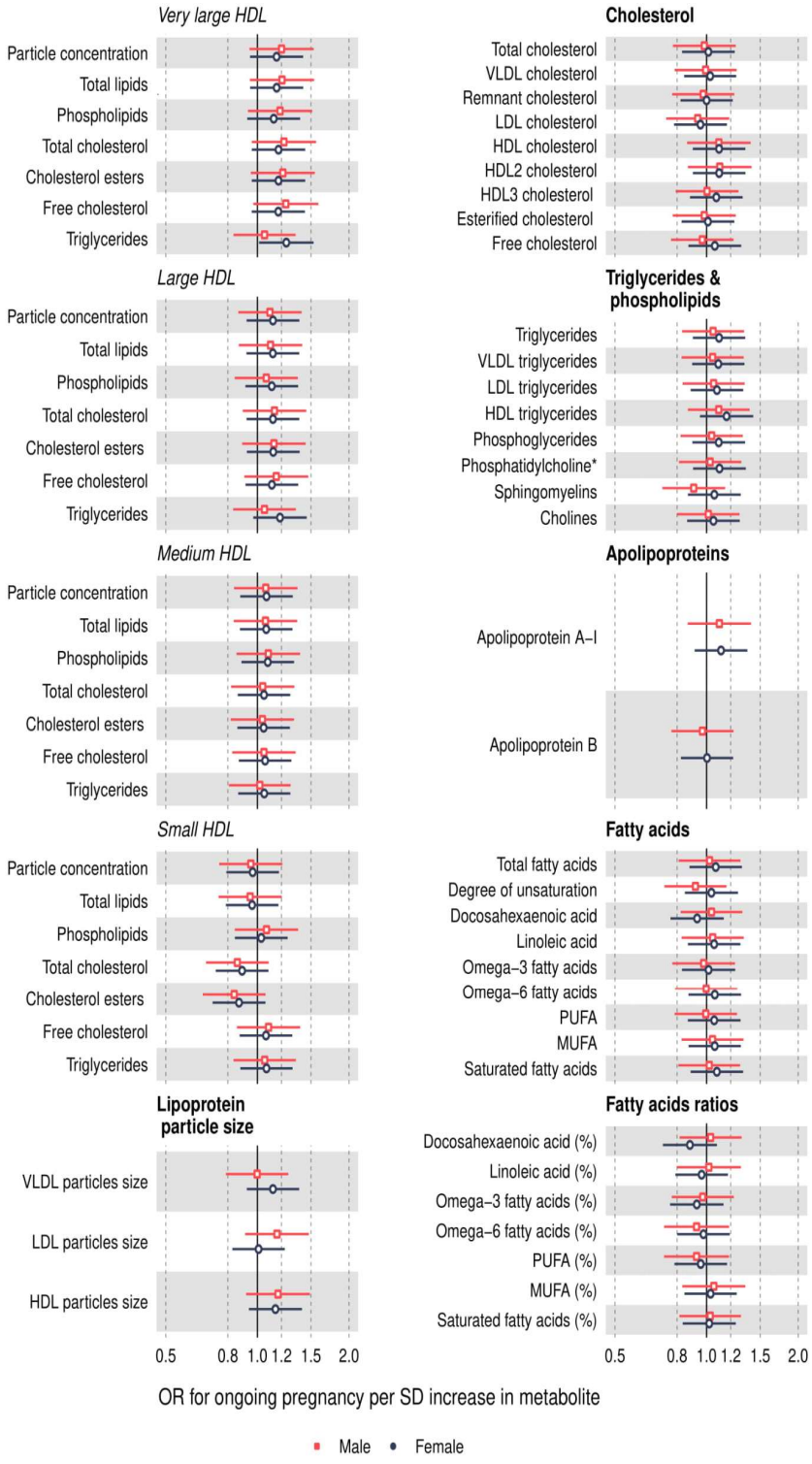

Figure 6.5 Unadjusted associations of lipoprotein classes and fatty acids in male and female with ongoing pregnancy.

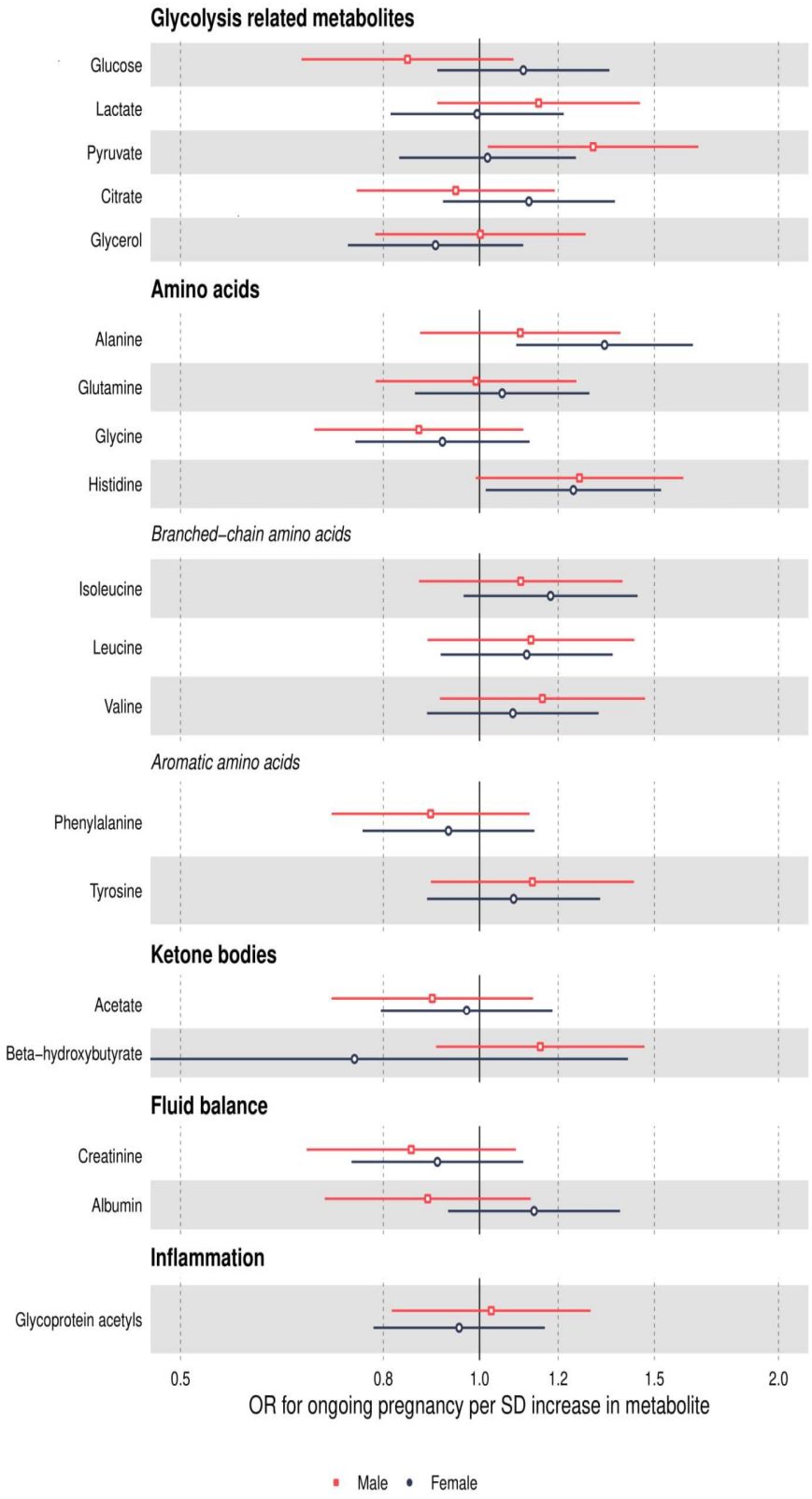

Figure 6.6 Unadjusted associations of metabolite traits in male and female with ongoing pregnancy.

There was evidence of an association between Histidine and ongoing pregnancy in females (OR = 1.28 (95% CI 1.03, 1.60) per one SD increase) and also in males, with similar magnitudes in both (OR = 1.26 (95% CI 0.99, 1.60) per one SD increase). In females, alanine (OR=1.31 (1.05, 1.64)), Isoleucine (OR=1.28 (1.02,1.61)) and Leucine (OR=1.24 (0.99,1.55)) were positively associated with ongoing pregnancy. Pyruvate has a positive association in males and ongoing pregnancy (OR=1.30 (1.02,1.66)). Other metabolic measurements, including lipids, lipoprotein sub-participles and fatty acids were closer to the null in both parents.

Across all metabolite measurements there was very little evidence of consistency (of magnitude and direction) in associations between women and men (R2 = 0.29; Figure 6.7). When I repeated analyses only including women (n=322) with a male partner in the study, results were consistent with the main analysis results (Figures 6.8, 6.9 and 6.10).

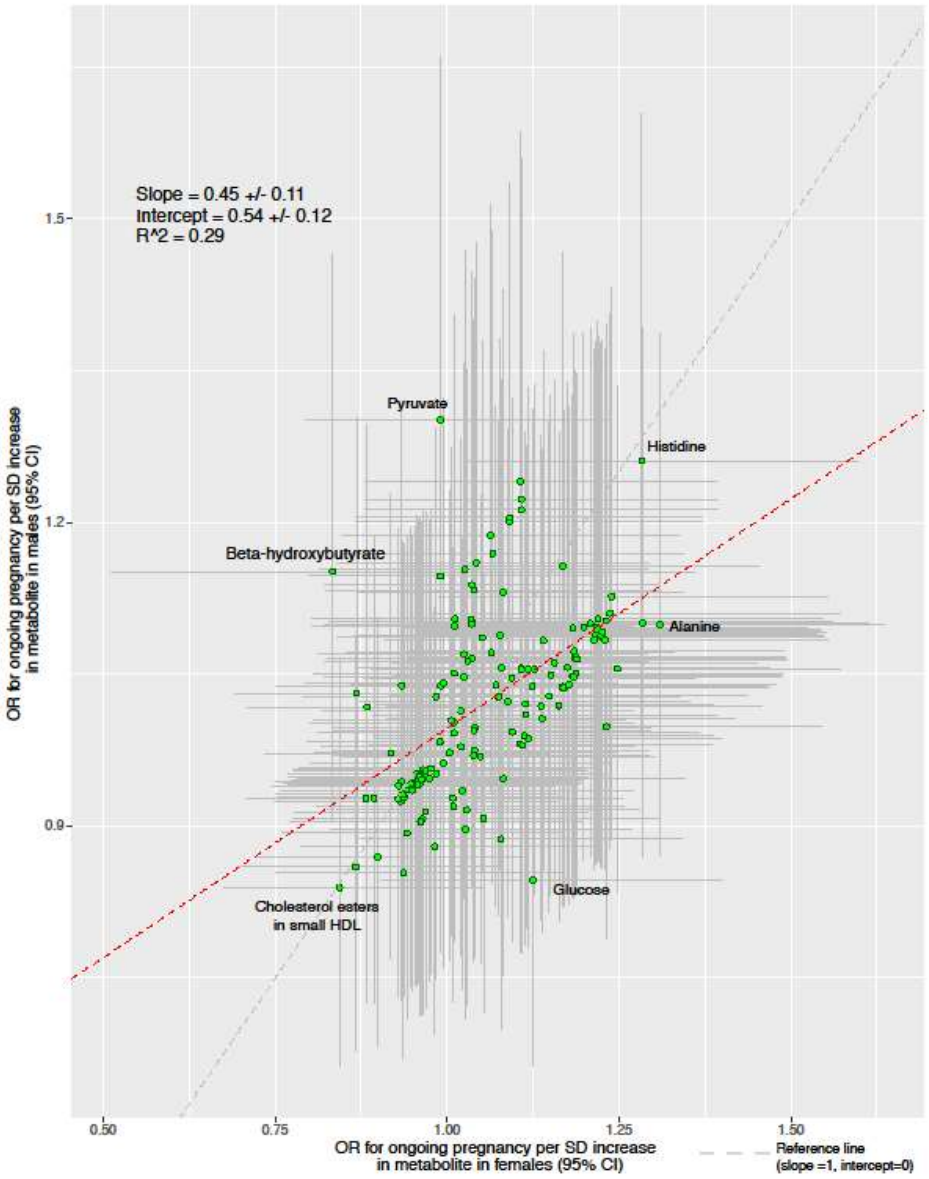

Figure 6.7 Scatterplot of associations of male and female metabolite profiles with ongoing pregnancy rates.

In adjusted analyses in males and females separately, standard deviation (SD) scores of metabolites were regressed on covariates (age, educational attainment, ever smoking, physical activity, family history of cardiometabolic disease, alcohol consumption, BMI, and ethnicity) with ongoing pregnancy rates. The green dots highlight the individual metabolites. The grey dashed line reflects the reference line (slope 1, intercept 0) and the red dashed line is the best line of fit.

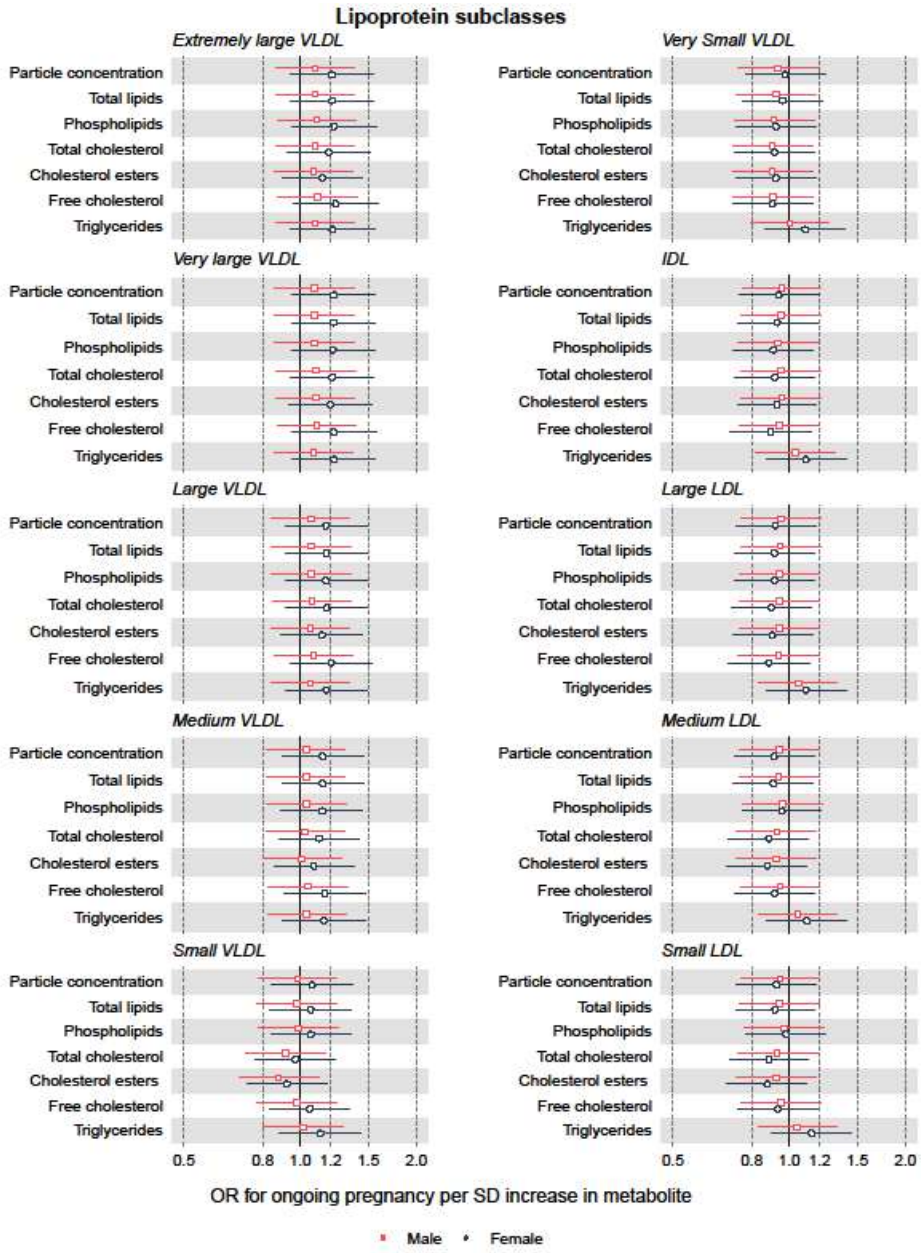

Figure 6.8 Associations of lipoprotein classes with ongoing pregnancy in males and females for the 322 women with male partners in the study.
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility.

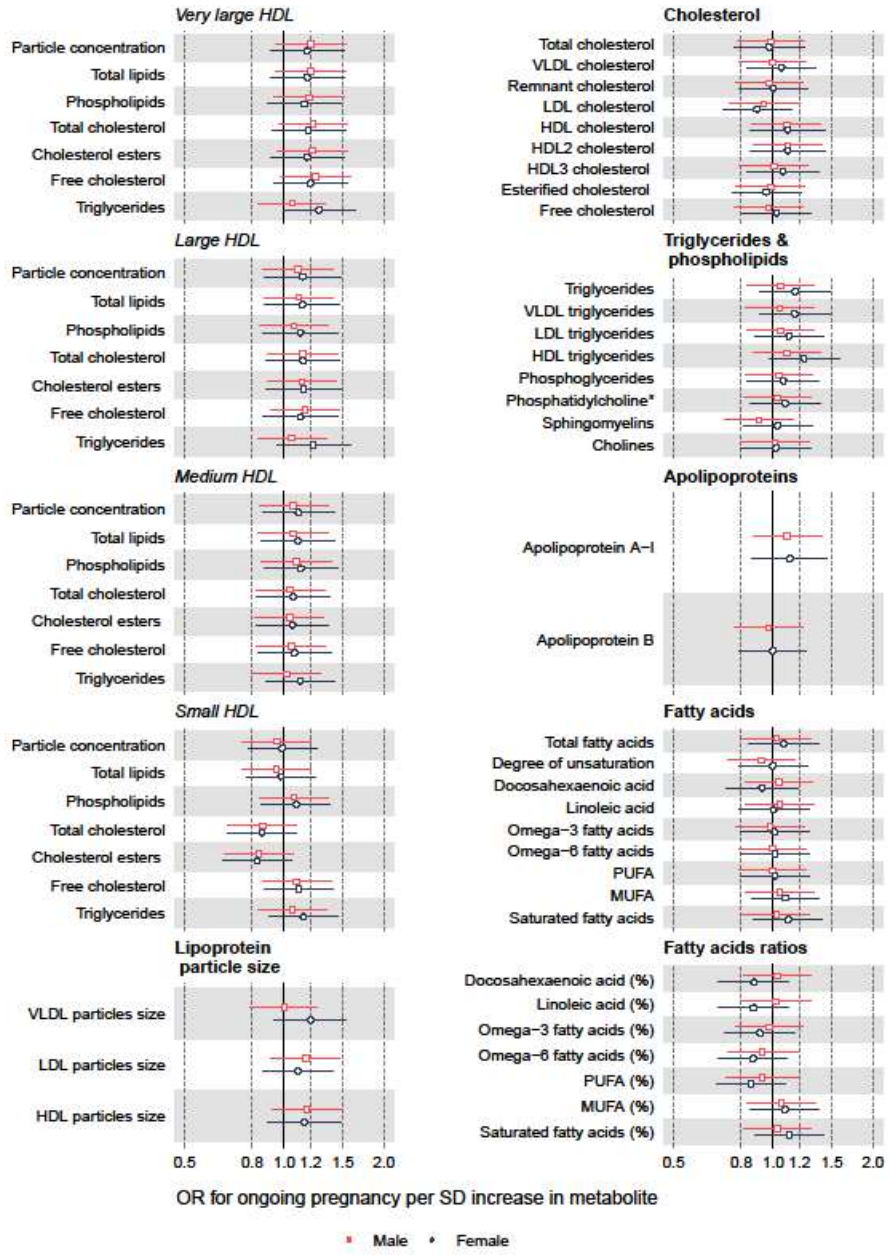

Figure 6.9 Associations of lipoprotein classes and fatty acids with ongoing pregnancy in males and females for the 322 women with male partners in the study.

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility.

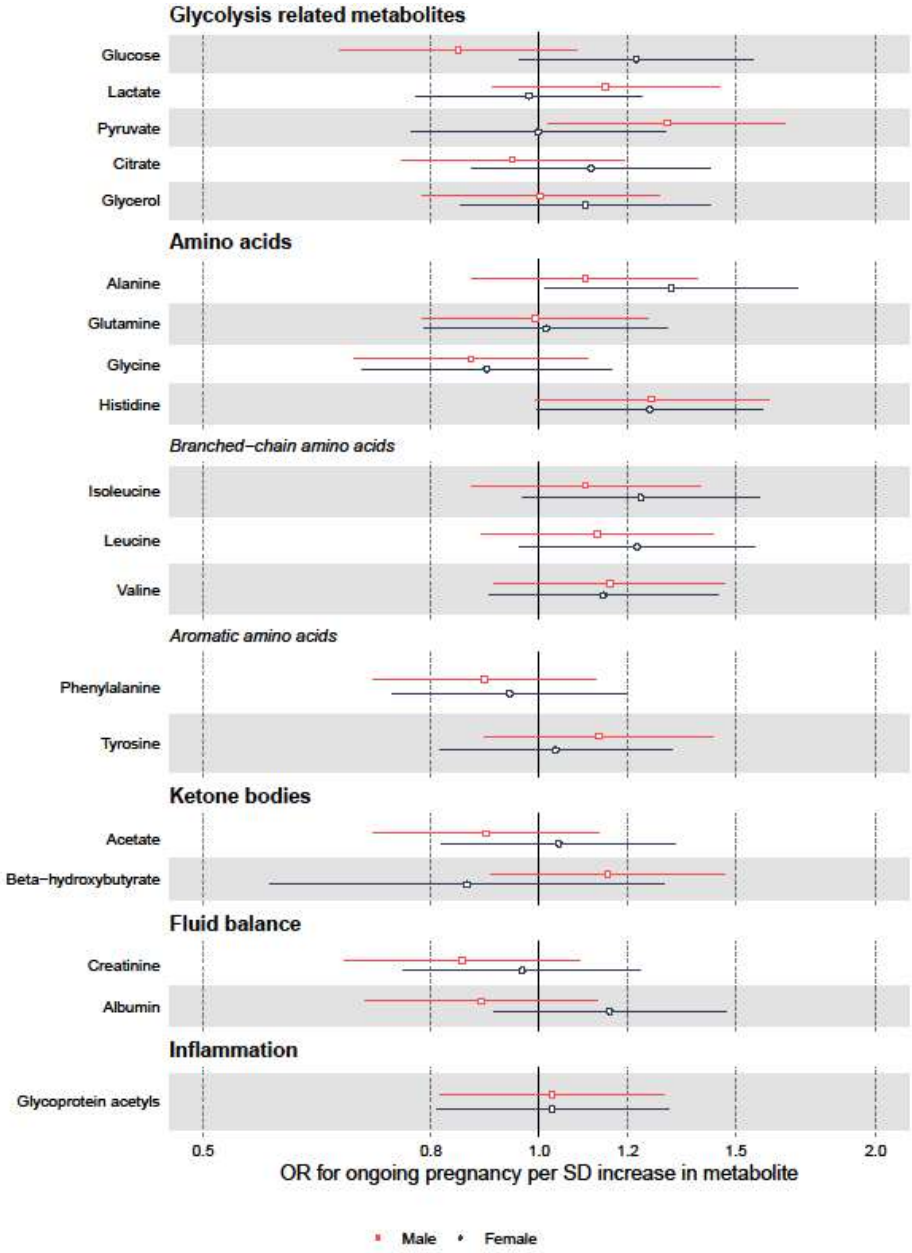

Figure 6.10 Associations of metabolic traits with ongoing pregnancy in males and females for the 322 women with male partners in the study

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for males (red) and females (black). Analyses in females adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of female infertility, duration of infertility, type of infertility (primary vs secondary). Analyses in males adjusted for age, education, alcohol use, physical activity, smoking, BMI, ethnicity, family history of cardiometabolic disease, cause of male infertility, duration of infertility

6.4 Discussion

In this prospective cohort of women and men undergoing their first cycle of IVF treatment, I demonstrate that women and men with higher preconception serum histidine levels were more likely to experience an ongoing pregnancy from IVF than those with lower levels. In addition, I identify positive associations of isoleucine, leucine and alanine in women, and of pyruvate in men which associated with successful IVF. I did not find clear associations with lipids, lipoproteins, fatty acids or other metabolic measurements in either parent and across all metabolites, there was little evidence of consistency between women and men. These results were robust to adjustment for demographic and clinical confounders. To my knowledge, this is the first study assessing detailed metabolic profiling in both men and women with clinical pregnancy outcomes. However, estimates were imprecisely estimated and I lacked statistical power to determine specific differences between women and men (as can be seen in Figure 6.1, 6.2 and 6.3 the confidence intervals overlap between the parents for all associations).

The identification of histidine, an essential alpha amino acid with a functional imidazole group as being associated with ongoing pregnancy is novel. Histidine is a positively charged α-amino acid and contains an α-amino group (which is in the protonated $-NH_3^+$ form under biological conditions), a carboxylic acid group (which is in the deprotonated $-COO^-$ form under biological conditions), and an imidazole side chain (which is partially protonated). The acid-base properties of the imidazole side chain are relevant both to formation of complexes with many metal ions as the it commonly serves as a ligand in metalloproteins and it can also improve the catalytic efficiency of many enzymes due to the rapid proton shuffling.

Histidine is a precursor for several biologically active amines including histamine and carnosine and it can also be converted to ammonia and urocanic acid via the enzyme histidine ammonia lyase, or 3-methylhistidine which serves as a biomarker for skeletal muscle damage via certain methyltransferase enzymes. The imidazole ring is also capable of scavenging reactive oxygen species. It has been reported that low plasma concentrations of histidine are associated with protein-energy wasting, and markers of inflammation, oxidative stress and insulin sensitivity (Mihalik, et al., 2012, Niu, et al., 2012, Watanabe, et al., 2008). With short-term

supplementation in a placebo-controlled randomised controlled trial associated with improved adiposity, insulin resistance, inflammatory oxidative stress markers (Feng, et al., 2013). In both women and men, increasing adiposity is inversely associated with IVF success rates (Mushtaq, et al., 2018, Sermondade, et al., 2019), however, the observed associations persisted after adjustment for BMI suggesting alternative pathways may also contribute. These may include its anti-oxidant properties, as anti-oxidant therapy has been associated with improved clinical outcomes in some but not all trials (Showell, et al., 2017, Smits, et al., 2019).

Alternatively, the observed association may be through the composition of other proteins such as histidine-rich glycoprotein (HRG) which has been extensively documented throughout the female reproductive tract, including in blastocysts (Nordqvist, et al., 2010)Human HRG is a 507-amino acid multidomain protein consisting of 2 N-terminal regions with homology to cystatin-like domains (termed N1 and N2), a central histidine-rich region (HRR) flanked by 2 proline-rich regions (PRR1 and PRR2), and a C-terminal domain. On the basis of the modular architecture of HRG, it has been proposed that HRG may act as an adaptor molecule that interacts with multiple ligands simultaneously through several independent binding sites and that collectively, HRG can potentially regulate numerous biologic processes. Previous small studies have also suggested that single nucleotide polymorphism (SNPs) in HRG that increase HRG circulating levels, may be associated with the chance of successful IVF treatment in both sexes (Lindgren, et al., 2016, Nordqvist, et al., 2011). The biological mechanism underlying this is less clear, as HRG can function as an adaptor molecule and regulate numerous important biologic processes, such as immune complex/necrotic cell/pathogen clearance, cell adhesion, angiogenesis, coagulation, and fibrinolysis (Poon et al., 2011). With respect to its immune function HRG can modulate the formation of immune complexes, FcγR function, exhibits antimicrobial and endotoxin-neutralizing properties, and also facilitate the removal of later ie plasma membrane permeabilised apoptotic and necrotic cells (Poon et al., 2011). Studies utilising HRG-/- mice have shown that HRG is an important factor for efficient clearance of fungal and bacterial infections and may play a minor role in regulating hemostasis.

With respect to angiogenesis, HRG and peptides derived from the histidine-rich region of HRG have been shown to exhibit both pro- and/or anti-angiogenic properties, depending on the experimental systems (Poon et al., 2001). Further assessment of the potential contribution of HRG peptides to endometrial and embryo development demonstrated that HRG enhanced migration and tube formation of human endometrial endothelial cells but inhibited proliferation (Lindgren et al., 2016). vascular endothelial growth factor (VEGF)-induced migration was also inhibited by the HRG proline peptide and both peptides reduced VEGF-induced proliferation. Except for a prolonged time from first cleavage after thawing to development of the morula, no difference in embryo morphokinetics or embryo quality was noted in human embryos cultured in the presence of the HRG proline peptide. Taken together, these results suggest that treatment with a specific HRG peptide might prime the endometrium for implantation and be beneficial for adequate placentation. However, addition of a specific HRG proline peptide to human embryos has no beneficial effects in terms of embryo development (Lindgren et al., 2016). Whether HRG simply functions as a regulator or is a key molecule in either a nonredundant or redundant pathway remains to be determined. Whilst the consistent association in both women and men makes chance less likely, I acknowledge this association needs independent replication and studies that can determine causality.

In previous analyses detailed in Chapter 3, I demonstrated cross-sectional associations of AMH with isoleucine, leucine and tyrosine (Al Rashid et al., 2020), suggesting that these are influenced by or reflect ovarian reserve and the possibility that associations of isoleucine and leucine with successful pregnancy in women in this study may reflect the influence of variation in ovarian reserve (underlying fertility) on IVF pregnancy success. Alanine, which was also associated with pregnancy in this study, and leucine have both previously been demonstrated within follicular fluid (Marianna, et al., 2017), with reduced levels in the sera and follicular fluid of endometriosis patients associated with lower pregnancy rates than controls (Jana, et al., 2013). In a porcine in vitro maturation model alanine and leucine supplementation have also been associated with improved pronuclear development and blastocyst formation, suggesting a female-specific contribution (Hong and Lee, 2007). Concentrations of these and other amino acids have also been observed to be reduced in uterine flushings of pregnancies after somatic cell

nuclear transfer (SCNT) as compared to standard IVF pregnancies (Groebner, et al., 2011), and through this disrupted embryo-maternal interaction in the preimplantation period have been suggested to contribute to the observed developmental abnormalities and lower pregnancy rates observed for SCNT cycles (Zhou, et al., 2014).

Few previous observational studies have examined the association between circulating lipoproteins and clinical IVF outcomes in detail. A previous smaller (N=167) analysis was limited to conventional serum lipid measurements (HDL, LDL and Triglycerides) and suggested that Triglycerides were negatively associated with the chances of a live-birth, however, this association was attenuated to the null when analyses were restricted to non-Asian participants (Jamro, et al., 2019). In contrast, analyses of natural cycle frozen embryo transfers (N=143) suggested that higher triglyceride and the erythrocyte ratio of saturated to unsaturated fatty acids concentrations were both positively associated with the chance of ongoing pregnancy (Onyiaodike, et al., 2018). In the current study with a predominantly non-Asian population, there was also evidence of a weak association of Triglycerides in women and ongoing pregnancy. Endogenous triglycerides are a key energy source during oocyte maturation and preimplantation embryo development (Ferguson and Leese, 2006), with mammalian blastocysts dependent on their own energy stores to drive cell division (Gardner, 1998). However, human embryos that successfully develop to blastocysts have lower triglyceride content, although it is difficult to determine if this is due to less initial storage or greater oxidation rates providing more energy while the blastocyst was being formed (Leary, et al., 2014). A well-stocked oocyte in terms of lipid available for oxidation (i.e. saturated fat) may have an advantage over one that lacks such lipid (Onyiaodike, et al., 2018).

This study has several strengths including the detailed phenotyping and that I assessed both women and men, enabling the consideration of maternal and paternal metabolic profiles on the chances of ongoing pregnancy. That I previously showed in Chapter 5 limited spousal correlation for the serum metabolite measured, further supports their evaluation in both partners. I do however acknowledge several limitations including that I cannot assume that the small number of associations are causal. Whilst this is the largest study to explore these associations the results were imprecisely estimated, with wide confidence

intervals and I am not aware of any independent study that has measures of multiple metabolites and ongoing pregnancy (or even the amino acids that we observed associations with) in which to attempt to replicate our findings. Though I acknowledge the necessity to replicate and hope that my findings will stimulate others to obtain these measures in couples undergoing IVF. The small sample size also limits our ability to robustly test sex differences and the suggestive sex-specific results should be treated with caution until they can be replicated in larger studies. Participants were confirmed (through cotinine breath test) non-smokers and the women were of a relatively restricted BMI range. The participants were also largely white European and educated to degree level. This homogeneous relatively healthy population may have resulted in some selection bias and may mean that the results do not generalise to other infertile populations or other IVF centres with different treatment protocols. I limited analyses to the clinical outcome of the first embryo transfer, to provide a time-limited evaluation of the primary outcome with all participants able to contribute equally. Given the predicted number of pregnancies after completion of all embryo transfers and current point estimates I anticipate their inclusion for those who were initially unsuccessful would have limited impact on the overall conclusions. Live-birth data was not available due to the regional provision of assisted conception services. Analyses were undertaken on non-fasting samples, however, several studies using this same NMR analysis platform results have not differed notably between studies in which the analyses were undertaken in participants who had been advised to fast and those who had not (Maqdasy *et al.*,2013). Lastly, while NMR platform used in these analyses covers considerably more of the lipidome than conventional clinical chemistry measures we acknowledge it misses a high proportion of the currently quantifiable metabolites in human serum/plasma, including markers of energy balance, microbiota metabolism, vitamins, co-factors and xenobiotics, that may influence the chance of pregnancy. High throughput analyses of a wider range of metabolites measured by mass spectrometry are possible but considerably more expensive than the NMR platform used here, resulting in their use being frequently restricted to subsamples of cohorts (Sovio, et al., 2020).

6.5 Conclusion

My findings are the first to report a higher likelihood of ongoing pregnancy after IVF among both women and men with higher preconceptual histidine concentrations, as part of a wider profile that encompasses lipoproteins, a range of amino acids and other metabolites related to overall health. The association of isoleucine and leucine with pregnancy in women may reflect an influence of variation in ovarian reserve influencing pregnancy success in IVF. Although the mechanisms underlying these associations are unclear our results if confirmed have the potential for clinical intervention. Serum metabolites are readily ascertained, and modification of concentrations including that of histidine may be achieved through supplementation. Confirmation of these findings is warranted with a larger number of participants to evaluate the potential clinical implications more fully.

Chapter 7 General discussion

In this thesis, I have outlined the development of a novel cohort of 731 participants (400 women and 331 men) undertaking assisted conception with extensive pre-treatment phenotyping, meticulous clinical and laboratory data and prolonged follow-up allowing ongoing pregnancies from both fresh and frozen embryo outcomes to be considered as clinical outcomes. Each participant has also contributed to the creation of a unique comprehensive biobank encompassing pre-treatment serum, plasma, DNA and vaginal swab samples. I have used this cohort to address a range of novel and clinically important questions, with a particular focus on circulating metabolic measures and the ovarian reserve, semen parameters, the correlation between couples and the clinical outcome of treatment as defined by an ongoing pregnancy. In the creation of this exceptional resource as summarised in Figure 7.1, I anticipate that this cohort through collaboration will continue to make a major contribution to a considerable number of areas of active research and further understanding of how the pre-treatment phenotype may impact on immediate and longer-term outcomes. It is with this aspiration that this final chapter concludes my thesis and suggests potential areas of future research.

7.1 General findings

The study discussed in chapter 3 observed a positive association between AMH and branch chain amino acids, such as isoleucine, leucine and tyrosine, medium HDL and small HDL, omega-6 fatty acids and polyunsaturated fatty acids, and negative association with acetate. AFC showed directionally consistent association but with overall weaker in magnitude. These associations strengthened when I restricted the analysis to a subgroup of women with male factor infertility. In addition, I observed a positive association of AMH and AFC with a range of lipids. Some of the associated metabolites, particularly branched-chain amino acids, have been linked to several cardiometabolic risk factors, including adiposity, fasting glucose, insulin resistance, blood pressure, dyslipidaemia, and indicators of coronary artery

disease (Tobias et al., 2018). My work would support a potential mechanism linking ovarian reserve to cardiometabolic health.

The exploratory study conducted in chapter 4 shows no identification of any associations between the measured metabolites and sperm parameters, such as concentration, progressive motility, or the risk of low or very low total motile sperm count. However, the point estimates indicate some potentially important associations. For instance, higher glycolysis metabolites and ketone bodies were associated with higher odds of total motile sperm count <15M; also, various lipids/lipoprotein concentrations seem to prevent extremely low TMSC. There was also a weak negative association between acetate and sperm motility. Replication of these findings in both larger studies and in smaller less heterogenous sub-populations would be useful.

In chapter 5 I demonstrated that couples show a strong correlation for the range of physical, social, and behavioural characteristics with modest to weak correlations for lipids and a few other metabolic measures which are solely diet derived. This suggests that assortative mating is similar in infertile couples as in the general population, with further convergence through active sharing in the shared environment and diet.

In the prospective cohort study presented in chapter 6, I demonstrate a novel finding that a higher level of preconception serum histidine in both women and men is associated with higher chances of achieving ongoing pregnancy after undergoing IVF than those with lower levels of histidine. I also identify positive associations of leucine, isoleucine and alanine in women and pyruvate in men, associated with IVF success rate. However, I did not observe associations with lipids, lipoproteins, fatty acids or other metabolic measurements in either partner, and there was little evidence of consistency between women and men. To my knowledge, this is the first study to evaluate the detailed metabolic characteristics of men and women with clinical pregnancy outcomes.

An a priori power calculation defined the sample size for the cohort, however, I acknowledge that for several of the outcomes, potentially clinically relevant but modest effect sizes were observed. Replication in larger studies would be useful however, these would require sample sizes ~10 times larger than used here. The

lack of power in IVF cohorts and trials is a well-recognised issue, with small studies of seemingly well-defined clinical phenotypes often not replicating in larger studies. That I chose to include a more heterogeneous population improves the generalisability of my findings, but I appreciate that potential effects in specific sub-populations may be attenuated.

7.2 Ovarian ageing

Ovarian ageing involves the natural decline in both quantity and quality of oocytes in the primordial follicle pool with increasing age. The endpoint of this process is menopause when too few follicles remain to sustain a normal menstrual cycle. The median age of menopause is 51 years, with accelerated ovarian ageing and early menopause (<45 years) occurring in 10% of women, with 1-5% experiencing premature ovarian insufficiency before the age of 40 years (Coulam et al 1985, Shuster et al. 2010). The concept of the ovarian reserve as a biomarker for health has been supported by several studies identifying that the age of natural menopause and especially a young age of natural menopause to be associated with an increased risk of disease or events normally considered to be age-related such as cardiovascular disease (CVD), coronary heart disease, cancer-related mortality and all-cause mortality (Jacobsen et al., 2003; Ossewaarde et al., 2005; Otsuki et al., 2018; Zhu et al., 2019). Similarly, women with a poor response to ovarian stimulation, and a low

Figure 7.1 Summary of IVF cohort, outcomes and associated Biobank samples.

The number of females and males recruited, and the associated biological samples NMR metabolomics has been undertaken, timelapse variables of the embryos, clinical pregnancy outcomes, vaginal swabs processed for microbiome analysis, pretreatment characterisation (full details of forms in Appendix IV), stimulation and laboratory details, maternal and paternal DNA samples (stored as buffy coats for preparation) and plasma and serum aliquots.

oocyte yield had a significantly higher hazard of age-related events including CVD, osteoporosis and early receipt of retirement benefit and an overall higher Charlson comorbidity index (Christensen et al., 2020). Thus, premature depletion of the ovarian reserve, and the onset of either POI or early menopause, may be a marker of accelerated somatic ageing. The concept of the ovarian reserve as a biomarker for health is further supported by data showing that women with late menopause i.e. greater ovarian reserve, seem to have a lower risk of CVD, ischemic heart

disease and a decreased risk of all-cause mortality than women with an average age of menopause (Ossewaarde et al., 2005)

The underlying mechanisms of the increased CVD risk and morbidity associated with premature depletion of the ovarian reserve remain to be clarified, although the timing of the increased disease risk in relation to the menopause has led some to suggest that oestrogen deficiency may be a contributing cause (Rocca et al., 2018). However, consistent with my findings premenopausal women with unexplained very low ovarian reserve (AMH, AFC) have previously been shown to have a more unfavourable CVD risk profile, with bone mass mineral density also reduced, as compared to women of a similar age but with normal ovarian reserve (Pal et al., 2008; Bleil et al., 2013; Cedars, 2013; Verit et al., 2014; de Kat et al., 2016). This would suggest that oestrogen independent processes are also contributing. That I observed in young women associations between ovarian reserve markers and non-lipid metabolites which have previously been robustly been associated with cardiovascular disease mortality (Deelen et al., 2019), would further support that a range of mechanisms that are not purely atherosclerotic may be responsible for the link between ovarian ageing and somatic ageing. This may include shared genetic, metabolic and environmental factors, and clarification of their relative contribution may be feasible through future cohorts incorporating mother-offspring pairs and sibling studies where their respective contribution of genetic and a shared environment can be delineated.

Telomere length has been proposed to act as a biomarker for ageing (Sanders and Newman 2013) and the possible relationship between female fertility and telomere length continues to be debated (Kosebent et al., 2018). DNA methylation (DNAm) patterns have previously also been used to predict chronological age with high accuracy (Horvath S. 2013), with the predicted DNAm age considered a biological measure of ageing. When the estimated DNAm age is greater than the chronological age, the term “age acceleration” is used to describe faster rates of ageing (Horvath, 2013). To date, several studies have found such deviations between the DNAm age and the chronological age in various tissue types and individuals (Horvath et al., 2015a; Horvath, et al., 2015b; Sehl et al., 2017), with accelerated DNAm age in blood associated with all-cause risk of mortality (Marioni et al., 2015; Perna et al., 2016). Since the development of the multi-tissue age

predictor, several more specialized epigenetic clocks have been developed to measure different aspects of the ageing process (Weidner et al., 2014; Giuliani et al., 2016; Levine et al., 2018). Of these DNAm age acceleration in leukocytes has been associated with reproductive life events such as menarche, puberty, and early menopause (Levine et al., 2016; Simpkin et al., 2017; Binder et al., 2018). Given these links, establishing the relationship of DNAm with the ovarian reserve biomarkers (AMH and AFC) would be of interest, with preliminary data from 39 women suggesting that accelerated ageing was associated with a lower AMH (Monseur et al., 2020). In young women (n =175), a poor ovarian response has also been associated with epigenetic age acceleration within WBC samples (Hanson et al., 2020). In contrast, age-related changes were not observed in CC (Hanson et al., 2020), potentially reflecting that epigenetic clocks failed to accurately predict the age of cumulus cells, (Morin et al., 2018). Whether DNAm clocks are associated with oocyte quality or the probability of pregnancy would be worth exploring further and could be evaluated using the leucocyte samples (n= 400 women) stored for DNA preparation within my cohort.

Clarification of whether a low ovarian reserve and/or ART treatment outcomes such as low oocyte yield enables simple and early identification of women at risk for accelerated somatic ageing is urgently required, as this may allow the early onset of preventative initiatives such as lifestyle changes and/or pharmacological interventions. Although suggested interventions may appear relatively generic and include counselling on adopting a healthier lifestyle (e.g. regular physical exercise, avoidance of smoking and importance of maintaining a healthy body weight), that the individual would have been identified through a different mechanism to classical risk factor screening may lead them to consider this "teachable" moment more seriously, thereby allowing potentially greater mitigation of some of the adverse risks associated with premature ageing.

7.3 Male Factor

Male factor infertility is a very common problem, affecting 1 in 10 men of reproductive age (Datta et al., 2016). The majority of cases are idiopathic, with no relevant past medical history or abnormal findings on physical examination, endocrine, genetic and biochemical laboratory testing. There is currently no proven treatment that the man can take, nor be added to his sperm in vitro, to

improve sperm function (Martins da Silva et al., 2017). This large unmet need for a universally available direct treatment for the sub-fertile man (i.e. men with poor sperm) to increase the chance of natural conception and live birth is of critical global importance and prompted the analysis of metabolite profiles and their association with semen parameters. Although I identified several novel associations, the effect sizes were modest and substantially larger study sizes of >3,000 participants would be required to robustly confirm our findings. Future work incorporating alternative and more comprehensive metabolome profiling may identify alternative therapeutic targets, that may be amenable to either dietary or pharmaceutical modification.

Alternative targets that may not be highlighted by metabolite profiling include the potential contribution of reactive oxygen species (ROS) which are normal by-products of cellular metabolism. Small amounts of ROS are required for normal sperm function, but when ROS are in excess or uncontrolled, they become toxic (Aitken et al., 2011), with excessive ROS causing direct damage to DNA (Gharagozloo et al., 2016), as well as to the sperm cell membrane, which impacts on motility and the ability to fertilise an oocyte (Aitken and Curry, 2011). The current thinking is that free radical generation, oxidative DNA damage, DNA fragmentation and cell death lie on a continuum (Aitken et al., 2011). Spermatozoa are particularly sensitive to ROS because they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack (Koppers et al., 2010). Whilst there is universal agreement that OS is an underlying cause and affects a significant proportion (30-70%) of sub-fertile men (Aitken et al., 2017) and there is a rationale for using antioxidant supplements, accurate identification of who would benefit from treatment has been difficult to determine. To date, assays have primarily focused on indirect oxidative stress determination for example sperm DNA damage. Whether alternative blood oxidative stress assays may be useful is at present unknown, but there are several advantages including easy collection and assay execution, relatively low cost and the facility to test many samples in laboratory routine or large studies. An example of this would be Diacron reactive oxygen metabolites test (d-ROM) (Vassalle et al 2008), a simple commercial colourimetric assay, designed to quantify early peroxidation species (reactive oxygen species) rather than their end products. This test is based on the principle that transition metals

can catalyze peroxides in the sample under acidic conditions (pH 4.8), forming alkoxy and peroxy radicals, which subsequently react with an amine, leading to the formation of coloured products that can be measured spectrophotometrically.

Reactions require iron ions; thus, samples must be collected as serum and heparinized plasma, which I have collected. The stability of the reactive oxygen species in stored serum samples results in reliability; with differences between samples that were immediately dosed and aliquots stored for 24 h at 4°C nonsignificant, with serum samples showed a very good degree of stability for up to 1 year of storage at -20 and -80°C (Jansen et al., 2015). Further work could include the measurement of d-ROM within the stored samples and correlation with semen parameters and clinical outcomes to evaluate its potential in the stratification of males who would benefit from anti-oxidant therapy. This may also inform the design of future randomised controlled trials of anti-oxidants, as at present the WHO Expert Synthesis Group (ESG) recently concluded that there was insufficient evidence to support the use of supplemental antioxidant therapies to treat men with abnormal semen parameters and/or male infertility (Barratt et al., 2017). Rather than simply randomising all men with abnormal semen parameters or all men receiving fertility treatment irrespective of their sperm count, identification of a sub-group with elevated ROS may be feasible. This would have the beneficial effect of increasing power and reducing trial size, by avoiding any therapeutic effect being diluted by a wider population where it would be unlikely to be beneficial as their ROS status was normal.

Alternatively, by measuring ROS or metabolites with semen itself rather than blood may be a means as to which identify men which may have relative deficiencies of anti-oxidants or excessive or deficient metabolites that may be amenable to correction. However, although several studies have undertaken metabolomics in seminal fluid (Murgia et al., 2020; Mumcu et al., 2020; Mehrparvar et al., 2020), they have been of limited size, have primarily used select groups and have used single samples, albeit some performing technical repeats, rather than replicating the results on a separate ejaculate sample (Gilany et al., 2017). This approach may also be potentially limited to those with evidence of sperm in the ejaculate rather than non-obstructive azoospermia where there would be limited interaction between sperm and the seminal fluid. Future cohorts

could collect seminal fluid, including repeat samples and used automated semen analyses to help further reduce variability than my single operator approach. However, validation of collection and processing of the semen samples would be critical, to ensure that stored samples are representative of fresh ejaculates with respect to ROS concentrations and metabolomics.

The sperm epigenome is uniquely complex (Immler et al., 2018) and susceptible to environmentally associated modification (Schagdarsurengin & Steger, 2016) in part because there are various points in the development that the epigenome is susceptible to modification, that is, paternal embryonic development, spermatogenesis, and offspring early embryonic development (Gold et al., 2018). At each time point, the internal and external environments appear to have a major influence on how the epigenome is modified (Soubry et al., 2014). For example, sperm DNA methylation patterns have recently been shown to predict paternal age with a high degree of accuracy (Jenkins et al., 2018). There is also evidence to suggest that idiopathic male subfertility and abnormal semen parameters, in particular oligozoospermia, are associated with abnormal spermatozoal DNA methylation of imprinted regions (Åsenius et al., 2020). However, no specific DNA methylation signature of either subfertility or abnormal semen parameters has been convincingly replicated in genome-scale, unbiased analyses and the clinical benefits of findings to date is therefore limited. Preliminary data also suggest that environmental factors, such as smoking, may also alter DNA methylation patterns to an age beyond actual chronologic age (Åsenius et al., 2020). While two small cohorts (n = 67 and n= 294) have assessed the association with adiposity on sperm methylation of imprinted loci in targeted analyses, the results are non-consistent with the larger study finding no associations (Soubry et al., 2016; Potabattula et al., 2019). Overall, despite several studies independently demonstrating associations between spermatozoal DNA methylation and fertility, smoking status, exercise interventions and exposure to some environmental pollutants, none of these studies has consistently identified DNA methylation changes at the same genomic regions (Åsenius et al., 2020). Therefore, although this data is of interest, the drawbacks of analysis of human spermatozoal DNA methylation is similar to other epigenetic studies of human phenotypes and tissues, and include study design, experimental processes and statistical analyses, while some challenges are specific to the nature of sperm as a tissue. A critical issue would be that to generate

robust, reproducible findings, sample sizes need to be sufficiently large to meet the power needs of the study design. As sperm is not routinely collected outside of reproductive medicine settings, multicentre collaborations will likely be required to meet the need for increased sample sizes. Such collaborations should harmonize protocols for phenotyping of participants, processing samples and data analyses to limit batch effects. In that regard, blood is routinely taken from all patients, and clarification of the extent to which peripheral blood DNA methylation reflects semen parameters and clinical outcomes would be useful to determine and could be done using my stored samples, whether as an initial pilot or part of a larger collaboration.

My work with metabolites and sperm highlighted the critical issue; of study size, as the effect sizes that I observed, would be biologically plausible estimates (odds ratios of 1.2 to 1.3), and if confirmed in independent cohorts would be potentially clinically meaningful. It is highly unlikely that interventions that could be applied to the general population are associated with substantially greater effect sizes. In contrast, male reproductive research is unfortunately frequently undertaken in substantially smaller studies with limited evidence or attempts at replication. Similarly randomised controlled trials of interventions have been small, for example in the assessment of supplementary oral antioxidants, 61 different randomised controlled trials assessing 18 different antioxidants have been undertaken (26 RCTs were placebo-controlled), but the overall population contained within all these trials was only 6,264 subfertile men (Smits et al., 2019). Of these only 11 studies (with 15 treatment arms) reported clinical pregnancy rates per couple (Peto OR 2.97, 95% CI 1.91 to 4.63, 786 men), and 7 RCTs reporting live birth rates (Peto odds ratio (OR) 1.79, 95% confidence interval (CI) 1.20 to 2.67, 750 men) (Smits et al., 2019). For the male reproductive field to move forward substantially, greater effort in understanding basic biology, validation and replication of possible therapeutic targets and then large scale RCTs with biologically relevant endpoints such as clinical pregnancy or live-birth are required. Fortunately, global opinion leaders are beginning to campaign for male reproductive health to be placed centre stage in the political and research agenda (De Jonge et al., 2019). Whether a paradigm shift can occur with increasing recognition of the links between reproductive health and somatic health is at present unclear (Capogrosso et al., 2018; Choy et al., 2018; Latif et al., 2018, Jensen et al., 2009), but I hope that my Biobank of male serum, plasma and DNA samples may help inform future work.

7.4 Couple-based Interventions

Infertility clinics are relatively unique in medicine, as they accommodate the needs of couples rather than individuals. Partners support each other during treatment and the emotional upheavals engendered by it. My analyses demonstrating that the couples within my cohort exhibit strong correlations for a range of physical, social and behaviour characteristics is consistent with conventional assortative mating, with infertile couples similar to couples within the general population. A critical difference was the lack of correlation for BMI or weight, as previous meta-analyses have suggested weak correlations between partners with regards to BMI (r = 0.15 across 34,582 couples in 19 studies) and weight (r = 0.11 across 6,765 couples in 9 studies) (Di Castelnuovo et al., 2009). Although this meta-analysis included all age-groups, a study examining 3356 expectant couples attending antenatal clinics found a positive partner correlation for BMI (r = 0.21) (Edvardsson et al., 2013). In that study, a woman's odds of being obese were more than six times higher if their partner was also obese, in comparison with women whose partner was of normal weight (OR 6.2, 95% CI 4.2–9.3). More than one-third (37.8%, P < 0.001) of the couples in the Pregnancy in PCOS II trial (PPCOS II) were concordant for obesity (Polotsky et al., 2015). A Danish population cohort study reported that couples presenting for IVF resembled each other in BMI, though they did not supply supportive data (Petersen et al., 2013). Despite these observations in similar populations to my own, that I did not observe an association may lead to some to question the proposal for couple-based weight-loss interventions in infertile couples (Best et al., 2017). However, as I observed concordance in diet-derived metabolites and physical activity and that the main barriers to exercise reported by women (Banting et al., 2014) is lack of time and fatigue, and that their main physical activity supports are their partners (Banting et al., 2014), this compels me to consider whether couple-based interventions might be more useful than individual interventions.

For weight loss, the few available trials including partners have used a variety of intervention approaches. These have included partner training for social support to increase positive reinforcement (e.g. praise), role modelling healthier eating, setting goals and focusing on problem-solving; also reduction of negative social control including criticism, punishment and nagging (McLean et al., 2003). To identify the social processes most relevant to couples seeking fertility treatment,

it will be necessary to study support but also processes that have received less attention such as social control, companionship, person-to-person contact, and access to resources and material goods (Berkman et al., 2000). Given the inherent strains of infertility and the irritability that accompanies behaviour change, interventions should also promote relationship-strengthening behaviours such as companionship and emotional and physical intimacy. Several RCTs have been recently been developed using a couple-based approach to different aspects of infertility management including optimisation of preconceptual lifestyle advice (PreLiFe-RCT) (Boedt et al., 2019), and the assessment of a web-based educational programme for the treatment of unexplained subfertility (Pleasure&Pregnancy RCT)(Dancet et al., 2019) with their results eagerly awaited.

That I observed weak to moderate spousal correlations for a range of metabolites including some that are purely dietary derived is also consistent with previous reports (Draisma et al. 2013; Sirota et al. 2015; Tremblay et al. 2019). If these and the lipids associated with cardiometabolic disease were amenable to modification and shown to exhibit sustained changes after intervention through maintained behaviour change this could have beneficial effects across the lifespan for both partners and potentially for their offspring. Future intervention studies will, however, need to account for the strong cultural differences in eating, physical activity, and close relationships and require adequately powered studies to also be undertaken in other countries to establish the generalizability of their findings.

7.5 Implantation

Embryo implantation is the highly coordinated process whereby a blastocyst-stage embryo attaches and invades the maternal endometrium before placentation and pregnancy (Dey et al., 2004; Achache and Revel, 2006). This complex multistep process classically comprising three phases: apposition, adhesion and invasion. During apposition, the human blastocyst is usually guided to a specific endometrial location to implant by bidirectional crosstalk mediated by soluble molecules (Cha et al., 2012; Thouas et al., 2015), enabling orientation of the embryonic pole with the inner cell mass (ICM) towards the epithelium (Dey et al., 2004; Cha et al., 2012). It then directly contacts the endometrium during the adhesion phase by establishing interactions between trophectoderm (TE) and endometrial epithelial

cells (EECs). In this phase, communication between blastocyst and endometrium is mainly based on ligand-receptor reactions, but soluble factors are also involved in the dialogue. Finally, in the invasion phase, TE cells undergo differentiation and the embryonic trophoblast becomes invasive and penetrates the epithelium, breaking through the basement membrane into the stroma to reach the maternal vasculature (Schlafke and Enders, 1975; Bentin-Ley et al., 2000). Despite substantial advances in clinical and laboratory techniques, such as new strategies for oocyte stimulation, oocyte/embryo vitrification, selection of euploid embryos, have all contributed to enhanced pregnancy rates, at present, there is limited ability to predict or control the embryo's ability to attach to the maternal endometrium. Greater clarification of the physiological and molecular factors involved in this process is essential for designing effective strategies to correct implantation failure.

To date different omic based approaches have largely focused on unravelling the complexity of the implantation process, with the assessment of endometrial receptivity through analysis of endometrial biopsies or endometrial secretions, while embryo potential has been evaluated by assessment of follicular fluid, cumulus cells, embryo culture media or embryo biopsy derived material. With each sample type having their respective transcriptomic, epigenomic, proteomic and metabolomic signatures evaluated as appropriate. However, despite a large amount of biomarker related data from this wide range of techniques there is still insufficient evidence to highlight the differential regulation of a specific molecule with the clinical outcome of ongoing pregnancy. In my thesis I did not focus on any of these specific tissues, but rather applied metabolomics to peripheral blood samples from the couple to assess whether a common signature that would embrace both maternal and paternal components and therefore also potentially the embryo could be identified, with possibly additional specific molecules from either source.

The identification of histidine as a metabolite which was common to both partners was unexpected, and the mechanism underlying this remains unclear. This has not been identified in any of the omic studies done to date on endometrial, oocyte, or embryo related samples and requires urgent replication. Putative mechanisms may be through its inverse association with inflammation, as evidence of sub-

clinical low-grade inflammation (as determined by C-reactive protein (CRP) concentrations) before implantation has been associated with poorer reproductive outcomes (Brouillet et al., 2020). Determination of CRP levels and other potential systemic markers of inflammation within the cohort as part of further work would be useful, as to date studies evaluating CRP concentrations before IVF have been small (N = 16 to 135). However, there does appear to be a consistent association between increased CRP levels and impaired folliculogenesis (Lorenz et al., 2015), poor embryo development (Haikin Herzberger et al., 2019) and a reduced window of implantation (Macklon et al., 2014) all contributing to reduced pregnancy rates (Levine et al., 2019).

I also identified several amino acids with the maternal circulation that exhibited novel positive associations with ongoing pregnancy, isoleucine, leucine and alanine. Previous studies assessing the metabolome of embryos have similarly identified; alanine, and leucine concentrations within embryo culture media and embryo viability, with a systematic review identifying that these findings had been replicated in at least two studies concerning embryo viability, or clinical pregnancies (Hernández-Vargas eta l., 2020). Determination of whether maternal circulating amino acid concentrations reflect those of the embryo would be useful in future studies as although embryonic culture concentrations were thought to reflect DNA damage and correction (Houghton et al 2004), evidence of correlations between the circulating levels and culture media would suggest alternative mechanisms may also contribute. Equally evaluation as to whether the wider embryo secretome (Seli et al., 2008, Sanchez-Ribas et al., 2012, Nadal-Desbarats et al., 2013, Pudakalakatti et al., 2013) or follicular fluid (Pinero-Sagredo et al., 2010) as determined by NMR, are correlated with maternal circulating metabolite profile similarly determined by NMR would be of interest.

7.6 Alternative Analytical Platforms

At present, the primary analytical technologies used in metabolomics include liquid chromatography coupled with single-stage mass spectrometry (LC-MS) (or tandem mass spectrometry (LC-MS/MS), gas chromatography coupled to mass spectrometry (GC-MS), high or ultrahigh performance liquid chromatography coupled to UV or fluorescent detection (HPLC/UPLC), and nuclear magnetic resonance (NMR) spectroscopy (Ashrafian et al., 2020). Each analytical platform

has its advantages and disadvantages, with the choice of platform primarily depending on the focus of the study as well as on the nature of the samples. While LC-MS and GC-MS methods are increasingly popular, accounting for more than 80% of the published metabolomics studies to date), there is still considerable and growing interest in the use of NMR for metabolomics reflecting its unique advantages. In particular, NMR is non-destructive, nonbiased, easily quantifiable, requires little or no chromatographic separation, sample treatment, or chemical derivatization, and it permits the routine identification of novel compounds. Furthermore, NMR is highly automatable and exceptionally reproducible, making high-throughput, large-scale metabolomics studies much more feasible with NMR spectroscopy than with LC-MS or GC-MS. NMR spectroscopy can be used to assess unique classes of metabolites (especially protein-bound metabolites such as lipoprotein particles), that cannot be done via LC-MS and GC-MS (Dona et al., 2016).

However, NMR also has several disadvantages, with the most significant challenge for NMR being its lack of sensitivity. Compared to LC-MS and GC-MS, NMR spectroscopy is often 10 to 100 times less sensitive. This means that a typical NMR-based metabolomic study usually only returns information on 50–200 identified metabolites with concentrations >1 μM, while a typical LC-MS study can return information on 1000+ identified metabolites with concentrations of >10 to 100 nM (Emwas et al., 2019). It is widely appreciated that there are several thousand measurable or detectable metabolites in human blood, yet only a few of these (the most abundant), can be reliably be detected by NMR. While high-abundance metabolites are almost always physiologically important, low-concentration metabolites are often more important as diagnostic biomarkers. This means that NMR-based metabolomics are often unable to detect these important molecules or cannot be used in diagnostic clinical applications.

In general, NMR spectrometers are also quite expensive compared to mass spectrometers or many other common analytical tools. Moreover, NMR instruments require highly skilled operators as well as substantial laboratory space with nonvibrational floors and isolation from magnetic and radio frequency interference. These factors, in addition to the overriding issue of low sensitivity, have made it challenging for NMR to expand its user base in metabolomics. In

terms of translational clinical applications, the same issues regarding space, cost, and personnel also make it difficult for NMR to replace existing clinical analysers for routine measurements in hospitals or clinics. As a general rule, mass spectrometry has had more success than NMR in the clinical chemistry lab because of its lower costs, its smaller instrumental footprint, and its ability to measure low-concentration metabolite species that are more frequently used in diagnostic assays.

Despite these limitations the advent of near-total automation in the NMR workflow (including sample preparation, sample loading, spectral acquisition, and spectral deconvolution), the ability to quantify absolute levels, comprehensive blood metabolite coverage (155 metabolites), the high-throughput, the robust quality control, the cost-effectiveness and the already extensive use in genetic and epidemiological studies, the ^{1}H-NMR platform was an obvious choice for use in my thesis. Future studies will be able to use my stored samples on alternative metabolomics platforms, potentially in a more targeted sub-group and matched controls, for example, men with severe oligospermia (<5 million sperm per ml), or couples with failed fertilisation, thereby overcoming the potential cost issues while facilitating a discovery-based approach for new biological insights and potential interventions given their wider and untargeted coverage. This approach has been successfully done in other cohorts with detailed phenotyping, for identification of novel biomarkers for prediction of pre-eclampsia (Sovio et al., 2020) and fetal growth restriction (Sovio et al., 2020b).

Several analytical platforms can be used in metabolic profiling. However, the choice of this specific NMR platform reflected that this platform is able to quantify a wide range of measures, with equivalent results to standard clinical chemistry analysers, has been shown to be scalable with widespread use in large epidemiological cohorts including the UK Biobank, the analytes have been shown to relevant cardio-metabolic health outcomes and that is a cost-efficient platform which has been used widely by our long-standing collaborators at the Bristol University where an NMR platform is established as part of a global network.

7.7 The Microbiome

While not addressed in my thesis, there are strong indications that the vaginal and endometrial microbiomes are associated with female reproductive health (Hong

et al., 2020). The invasive techniques required for endometrial samples limited my ability to attain samples on a large scale, with the collection of vaginal samples enabling widespread acceptance by patients, standardisation relative to the menstrual cycle and a large number of self-obtained samples to be collected. That the vaginal microbiome is recognised as playing an important role in protecting the vagina, the first barrier from the external environment to the upper reproductive tract, was also a critical factor in my decision to collect vaginal samples.

In non-pregnant, healthy women the vaginal microbiome is dominated by four *Lactobacillus* species: *L. crispatus*, *L. iners*, *L. jensenii* or *L. gasseri* (Ravel et al., 2011). There appears to be five vaginal microbial community state types (CST); four of them (I, II, III and V) are *Lactobacillus*-dominant and are more commonly found in European and Asian women. CST-IV, more frequent in Hispanic and African-American women, differs due to the increased abundance of strictly anaerobic bacteria (*Gardnerella*, *Ureaplasma*) and reduced presence of *Lactobacillaceae* (Ravel et al., 2011). *Lactobacillus* spp. produce lactic acid that helps to keep the vaginal pH below 4.5 and creates inhospitable conditions for pathogens to grow (Graver and Wade, 2011). In addition to the vaginal microbiota composition exhibiting large ethnic differences it also appears to vary across the life-course depending on physiological events, like menstrual cycle and pregnancy, and external factors, such as sexual activity, hygiene habits and medical treatments (Gajer et al., 2012; Ravel et al., 2011; Zhou et al., 2007).

Several studies suggest that altered vaginal microbiota may be associated with poor IVF and pregnancy outcomes. Specifically, vaginal microbiota which is rich in *Lactobacillus* spp. without bacterial vaginosis, either clinical or subclinical leads to more positive outcomes with ART (Babu et al., 2017; Eckert et al., 2003; Mangot-Bertrand et al., 2013; Moore et al., 2000). The extent to which an unfavourable microbiota is associated with an unfavourable outcome after IVF appears to be strong as Haahr et al. (2016) studied the microbiota of 84 women undergoing IVF, and of the 22 women who had an altered composition, only two women (9%) achieved a clinical pregnancy as compared to 29 women (47%) with a normal microbiota composition. Similarly, in a prospective study of 192 women undertaking IVF, embryo implantation was less successful in women with reduced

Lactobacillus spp. in their vaginal microbiota (5.9% if unfavourable profile) (Koedooder et al., 2019). The dominance of *L. crispatus* was also identified as an important factor in predicting pregnancy, as women who had a favourable profile based on <60% *L. crispatus* had a high chance of pregnancy: more than half of these women (50 out of 95) achieving pregnancy. Using this data and with confirmation by external validation the authors proposed an algorithm with high specificity [97%], but low sensitivity [26%] for not becoming pregnant after IVF (Koedooder et al., 2019). Others have also confirmed the positive association of *L. crispatus* dominant microbiomes with higher live birth rates (Haahr et al 2019, Vergaro et al., 2019). However, as can be seen from these essentially pilot studies, it is at present hard to draw definitive conclusions given the different molecular approaches used, the small sample sizes and the non-standardised collection techniques. The samples collected within my cohort combined with detailed knowledge of potential exogenous factors including smoking, stress, drug exposure and antibiotic exposure, which have all been shown to alter the gut microbiome (Falony et al., 2016), could make a potentially further useful addition to the literature once sequencing techniques and bioinformatic pipelines have been standardised (Koedooder et al 2016).

7.8 Conclusion

In this thesis, I have shown that it is feasible to establish a novel cohort of couples undertaking IVF with extensive and detailed pre-treatment phenotyping with linkage to laboratory and clinical outcomes to address a series of novel interlinked research questions. That my work will also have the legacy of an extensive Biobank encompassing maternal and paternal plasma, serum and DNA and vaginal samples already prepared and suitable for future microbiome analysis, further enhances the utility of the cohort, thereby ensuring that my work will continue to have impact and enable novel insights into the pathophysiology of female and male infertility.

Appendices

Appendix I: The Ethical Approval

Coordinator: Joanne McGarry
Telephone Number: 0141 232 1818
E-Mail: joanne.mcgarry@ggc.scot.nhs.uk
website www.nhsggc.org.uk/r&d

Clinical Research & Development
West Glasgow ACH
Dalnair Street
Glasgow G3 8SJ
Scotland, UK

12/08/2016

Dr Karema Alrashid
New Lister Building,
Level 2, Reproductive & Maternal Medicine,
Glasgow Royal Infirmary
10 Alexander Parade
Glasgow
G31 2ER

NHS GG&C Board Approval

Dear Dr Karema Alrashid

Study Title:	Does cardiometabolic health determine IVF treatment outcome?
Principal Investigator:	Dr Karema Alrashid
GG&C HB site	Glasgow Royal Infirmary
Sponsor	NHS Greater Glasgow and Clyde
R&D reference:	GN16OG323
REC reference:	16/WM/0308
Protocol no: (including version and date)	Version 2.1 13/05/16

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Approval** for the above study.

Conditions of Approval

1. **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004
   a. During the life span of the study GGHB requires the following information relating to this site
      i. Notification of any potential serious breaches.
      ii. Notification of any regulatory inspections.

It is your responsibility to ensure that all staff involved in the study at this site have the appropriate GCP training according to the GGHB GCP policy (www.nhsggc.org.uk/content/default.asp?page=s1411), evidence of such training to be filed in the site file.

2. **For all studies** the following information is required during their lifespan.
   a. Recruitment Numbers on a quarterly basis
   b. Any change of staff named on the original SSI form
   c. Any amendments – Substantial or Non Substantial
   d. Notification of Trial/study end including final recruitment figures
   e. Final Report & Copies of Publications/Abstracts

NHS
Greater Glasgow and Clyde

Please add this approval to your study file as this letter may be subject to audit and monitoring.

Your personal information will be held on a secure national web-based NHS database.

I wish you every success with this research study

Yours sincerely,

Joanne McGarry
Research Co-ordinator

CC: Emma-Jane Gault

NHS
Health Research Authority
West Midlands - Black Country Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

28 June 2016

Professor Scott Nelson
Level 2, Room 2.52
Reproductive & Maternal Medicine
New Lister Building
Glasgow Royal Infirmary
G31 2ER

Dear Professor Nelson

Study title:	**Does cardiometabolic health determine IVF treatment outcome?**
REC reference:	**16/WM/0308**
IRAS project ID:	**202216**

The Proportionate Review Sub-committee of the West Midlands - Black Country Research Ethics Committee reviewed the above application on 27 June 2016.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Miss Georgia Copeland, nrescommittee.westmidlands-blackcountry@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

1. The Participant Information Sheets should be updated as follows;
    a. The use of the word 'your' should be removed from the opening sentence of the first paragraph of each document.
    b. To contain the name of the Chief Investigator.
    c. To contain the contact details for the local PAL service or other independent complaints procedure. (Further guidance is available via; http://www.hra-decisiontools.org.uk/consent/content-sheet-support.html)
    d. The wording 'with your consent' should be added to the section of the Participant Information Sheet titled; 'How long will the study last?'
2. The Informed Consent Forms should be updated to contain the name of the Chief Investigator.

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Summary of discussion at the meeting

Care and protection of research participants; respect for potential and enrolled participants' welfare and dignity

The Sub-Committee commented on the storage of samples at the end of the research project and acknowledged that some participants may not wish for their samples to be stored in a tissue bank. The Sub-Committee agreed it should be made explicit within the Participant Information Sheet that this is optional, and requested the wording 'with your consent' be added to the section of the Participant Information Sheet titled; 'How long will the study last?'

Informed consent process and the adequacy and completeness of participant information

The Sub-Committee reviewed the Participant Information Sheets and Informed Consent Forms, and requested that the name of the Chief Investigator be added to these documents.

The Sub-Committee commented on the opening sentence of the first paragraph of the Participant Information Sheets, and considered that the use of the word 'your' may lead participants to believe that the study is part of the treatment protocol, or that taking part in the study will help with their own problems. The Sub-Committee agreed use of this word should be removed from the sentence.

The Sub-Committee noted that the Participant Information Sheets are without a complaints section, and requested that the documents be updated to contain reference to the local PAL service or a relevant independent complaints procedure.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
IRAS Checklist XML [Checklist_20062016]		20 June 2016
Letter from funder		25 April 2016
Non-validated questionnaire	2.1	13 May 2016
Other [Case Information Note]	2.1	13 May 2016
Other [Participant Information Sheet (Male)]	2.2	19 June 2016
Other [Informed Consent (Female)]	2.2	19 June 2016
Other [Informed Consent (Male)]	2.2	19 June 2016
Other [Participant Information Sheet (Female)]	2.2	19 June 2016
REC Application Form [REC_Form_16062016]		16 June 2016
Research protocol or project proposal	2.1	20 May 2016
Summary CV for Chief Investigator (CI)		20 May 2016
Summary CV for student		20 May 2016
Summary CV for supervisor (student research)		20 May 2016

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

16/WM/0308	**Please quote this number on all correspondence**

Yours sincerely

Reverend Keith Lackenby
Acting Chair

Email: nrescommittee.westmidlands-blackcountry@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers"

Copy to: *Ms EmmaJane Gault*
Mr. Paul Dearie, Glasgow University

Appendix II: Participant Information Sheet

University of Glasgow

NHS Greater Glasgow and Clyde

Participant Information Sheet (Female)

Does Cardiometabolic Health determine IVF/ICSI Treatment Outcome?

You are invited to take part in this research study which is aimed at assessing whether your cardiometabolic health affects the outcome of your In-Vitro Fertilisation (IVF)/Intra-Cytoplasmic Sperm Injection (ICSI) treatment. Before you decide, please take your time and carefully read the following information about the study. It is important that you understand what the study involves and what will be required. Please feel free to discuss it with your friends, family and health care team before deciding. If you have any query or require further information, please do not hesitate to contact us.

Thank you very much for taking the time to read this.

What is the purpose of the study?

We recognise that many different factors from how we stimulate your ovaries to the laboratory methods can affect IVF/ICSI success rates. The impact of maternal and paternal health has been less well studied. This study is being conducted in order to enhance the healthcare provided by Assisted Conception Service (ACS) which will be reflected in the overall success rates of all IVF/ICSI cycles. The purpose of this study is to identify whether both maternal and paternal general health and in particular their cardiometabolic health (BMI, blood pressure, circulating lipids, insulin resistance) influences the outcome of IVF/ICSI.

Why have we been chosen?

You are being asked to take part in this study because you and your partner are undergoing an IVF or intra-cytoplasmic sperm injection treatment which does not require any type of donation (oocyte or embryo), and are aged greater than 18.

Do we have to take part?

It is entirely up to you if you want to participate in this study; however, if you choose to take part, you will be asked to keep this information sheet and to sign a consent form. You are free to withdraw from the study at any time without giving a reason. This will not affect the standard clinical service that you receive.

What do we have to do?

If you agree to participate in this study, we will ask you to sign the consent form firstly. No additional visits will be required. You will be asked to complete a baseline questionnaire

(which contains basic information and straight forward questions about your general health) that will take 10 minutes. We will also measure your height, weight, blood pressure and we will obtain an extra 10mL blood sample which will be taken at the same time as your routine blood tests. This will include serum and a DNA sample which can be stored and analysed in the future. We will also take an additional high vaginal swab in conjunction with the routine two swabs that we take prior to commencing an IVF/ICSI cycle. During the study, you will follow your ordinary clinical treatment protocol and the study does not require you or your partner to adapt any changes to your treatment plan. The researcher will also seek your permission to access your medical notes in order to obtain information about your IVF/ICSI treatment cycle, reproductive and general health history.

How long will the study last?

Your participation in the study last as long as your IVF/ICSI treatment cycle. Samples will be analysed after all patients have been recruited and the generated data published. At the end of the research project all samples will be transferred to the GGC Biobank and made available to other research staff.

Are there any risks for us in taking part in this study?

There are no health risks to participants taking part in this study. All participants will follow their ordinary clinical treatment protocol and the study does not oblige you to adopt any changes to your treatment plan.

What are the benefits?

There are no direct benefits for you. However, the potential results from this study will contribute to science and provide substantial information regarding the impact of maternal and paternal health on IVF/ICSI outcome.

Will our information be kept confidential?

After the participants have signed the consent forms, each participant will be allocated an alphanumeric code which will be unique to each participant and will be used to label all related documents and samples to remain anonymous during the study and laboratory analyses. Names, addresses and contact details (emails, telephone and mobile numbers) will be kept in separate file locker in a locked office within New Lister Building in Glasgow infirmary and will be accessed only by the research staff. Your information may also be looked at by representatives of the study Sponsor, NHS Greater Glasgow and Clyde, to ensure that it is being conducted correctly.

What are the costs for taking part in this study?

There will be no cost for taking part in this study.

University of Glasgow

NHS Greater Glasgow and Clyde

Can the participant withdraw after agreeing to participate?

You will have the right to withdraw at any time during the study with no impact on the care you receive. Please do not hesitate to contact the researcher anytime to discuss any issues related to your participation in the study and inform us in case you decide not to continue.

What about dissemination of the results?

The results of the study will be written up as a Ph.D. thesis, available through the University of Glasgow website as well as in peer-reviewed scientific journals and scientific conference presentations. A summary of the research findings will be made available to the participants on completion of the project or on the publication of any papers,

Who is organising and funding this study?

The principal investigator and associates from ACS are conducting this cohort study as part of a doctoral thesis at the University of Glasgow in coordination with Glasgow Royal Infirmary. The study is being funded by Kuwait Cultural Office.

Who has reviewed this study?

The study procedures have been checked, reviewed and agreed by a National Health Service Research Ethics Committee (NHS REC).

Contact information

For more information, any doubts or questions, please feel free to contact:	
Dr. Karema Alrashid, MBChB, MSc PhD Researcher (Principal Investigator) Mobile : Email: New Lister Building, Glasgow Royal Infirmary 10 Alexandra Parade, Glasgow Second floor Office Number 2.75 Glasgow G31 2ER	Prof. Scott Nelson, MBChB, PhD, MRCOG Muirhead Chair of Reproductive & Maternal Medicine, University of Glasgow (Chief Investigator) Tel: Email: Scott.Nelson@glasgow.ac.uk New Lister Building, Glasgow Royal Infirmary 10 Alexandra Parade, Glasgow Second floor Office Number 2.52 Glasgow G31 2ER

University of Glasgow

NHS Greater Glasgow and Clyde

Participant Information Sheet (Male)

Does Cardiometabolic Health determine IVF/ICSI Treatment Outcome?

You are invited to take part in this research study which is aimed at assessing whether your cardiometabolic health affects the outcome of your In-Vitro Fertilisation (IVF)/Intra-Cytoplasmic Sperm Injection (ICSI) treatment. Before you decide, please take your time and carefully read the following information about the study. It is important that you understand what the study involves and what will be required. Please feel free to discuss it with your friends, family and health care team before deciding. If you have any query or require further information, please do not hesitate to contact us.

Thank you very much for taking the time to read this.

What is the purpose of the study?

We recognise that many different factors from how we stimulate your ovaries to the laboratory methods can affect IVF/ICSI success rates. The impact of maternal and paternal health has been less well studied. This study is being conducted in order to enhance the healthcare provided by Assisted Conception Service (ACS) which will be reflected in the overall success rates of all IVF/ICSI cycles. The purpose of this study is to identify whether both maternal and paternal general health and in particular their cardiometabolic health (BMI, blood pressure, circulating lipids, insulin resistance) influences the outcome of IVF/ICSI.

Why have we been chosen?

You are being asked to take part in this study because you and your partner are undergoing an IVF or ICSI treatment which does not require any type of donation (oocyte or embryo), and are aged greater than 18.

Do we have to take part?

It is entirely up to you if you want to participate in this study; however, if you choose to take part, you will be asked to keep this information sheet and to sign a consent form. You are free to withdraw from the study at any time without giving a reason. This will not affect the standard clinical service that you receive.

What do we have to do?

If you agree to participate in this study, we will ask you to sign the consent form firstly. No additional visits will be required. You will be asked to complete a baseline questionnaire (which contains basic information and straight forward questions about your general health)

that will take 10 minutes. We will also measure your height, weight, blood pressure and we will obtain an extra 10mL blood sample which will be taken at the same time as your routine blood tests. This will include serum and a DNA sample which can be stored and analysed in the future. During the study, you will follow your ordinary clinical treatment protocol and the study does not require you or your partner to adapt any changes to your treatment plan. The researcher will also seek your permission to access your medical notes in order to obtain information about your IVF/ICSI treatment cycle, reproductive and general health history.

How long will the study last?

Your participation in the study last as long as your IVF/ICSI treatment cycle. Samples will be analysed after all patients have been recruited and the generated data published. At the end of the research project all samples will be transferred to the GGC Biobank and made available to other research staff.

Are there any risks for us in taking part in this study?

There are no health risks to participants taking part in this study. All participants will follow their ordinary clinical treatment protocol and the study does not oblige you to adopt any changes to your treatment plan.

What are the benefits?

There are no direct benefits for you. However, the potential results from this study will contribute to science and provide substantial information regarding the impact of maternal and paternal health on IVF/ICSI outcome.

Will our information be kept confidential?

After the participants have signed the consent forms, each participant will be allocated an alphanumeric code which will be unique to each participant and will be used to label all related documents and samples to remain anonymous during the study and laboratory analyses. Names, addresses and contact details (emails, telephone and mobile numbers) will be kept in separate file locker in a locked office within New Lister Building in Glasgow infirmary and will be accessed only by the research staff. Your information may also be looked at by representatives of the study Sponsor, NHS Greater Glasgow and Clyde, to ensure that it is being conducted correctly.

What are the costs for taking part in this study?

There will be no cost for taking part in this study.

University of Glasgow

NHS Greater Glasgow and Clyde

Can the participant withdraw after agreeing to participate?

You will have the right to withdraw at any time during the study with no impact on the care you receive. Please do not hesitate to contact the researcher anytime to discuss any issues related to your participation in the study and inform us in case you decide not to continue.

What about dissemination of the results?

The results of the study will be written up as a Ph.D. thesis, available through the University of Glasgow website as well as in peer-reviewed scientific journals and scientific conference presentations. A summary of the research findings will be made available to the participants on completion of the project or on the publication of any papers,

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Who has reviewed this study?

The study procedures have been checked, reviewed and agreed by a National Health Service Research Ethics Committee (NHS REC).

Contact information

For more information, any doubts or questions, please feel free to contact:	
Dr. Karema Alrashid, MBChB, MSc PhD Researcher (Principal Investigator) Mobile : Email: New Lister Building, Glasgow Royal Infirmary 10 Alexandra Parade, Glasgow Second floor Office Number 2.75 Glasgow G31 2ER	Prof. Scott Nelson, MBChB, PhD, MRCOG Muirhead Chair of Reproductive & Maternal Medicine, University of Glasgow (Chief Investigator) Tel: Email: Scott.Nelson@glasgow.ac.uk New Lister Building, Glasgow Royal Infirmary 10 Alexandra Parade, Glasgow Second floor Office Number 2.52 Glasgow G31 2ER

Appendix III: Informed Consent

University of Glasgow

Participant's ID

NHS Greater Glasgow and Clyde

Informed Consent (Female)

Does Cardiometabolic Health Determine
IVF/ICSI Treatment Outcome?

I, the undersigned, confirm that (please initial as appropriate):

1.	I have read and understood the information about the project, as provided in the Information Sheet dated ____/____/_____ and version _____.	
2.	I have been given the opportunity to ask questions about the project and my participation.	
3.	The procedures regarding confidentiality have been clearly explained (e.g. use of names, pseudonyms, anonymisation of data, etc.) to me.	
4.	The use of the data in research, publications, sharing and archiving has been explained to me.	
5.	I understand that other researchers will have access to this data only if they agree to preserve the confidentiality of the data and if they agree to the terms I have specified in this form.	
6.	I understand that my DNA will be stored for future analysis.	
7.	I understand that data from my medical notes, but only that part relevant to this study, will be looked at by the researchers of this study and possibly by the NHS Greater Glasgow & Clyde R&D office for evaluation purposes. I give permission for these individuals to have access to my medical records.	
8.	I understand I can withdraw at any time without giving reasons and that I will not be penalised for withdrawing nor will I be questioned on why I have withdrawn.	
9.	I voluntarily agree to participate in the project.	
10.	I, along with the Researcher, agree to sign and date this informed consent form.	

Participant:

_____	_____	_____
Name of Participant	Signature	Date

Researcher:

_____	_____	_____
Name of Researcher	Signature	Date

Version 2.2 (19/6/2016)

University of Glasgow

Participant's ID

NHS Greater Glasgow and Clyde

Informed Consent (Male)

Does Cardiometabolic Health Determine IVF/ICSI Treatment Outcome?

I, the undersigned, confirm that (please initial as appropriate):

1.	I have read and understood the information about the project, as provided in the Information Sheet dated ___/___/_____ and version _____.	
2.	I have been given the opportunity to ask questions about the project and my participation.	
3.	The procedures regarding confidentiality have been clearly explained (e.g. use of names, pseudonyms, anonymisation of data, etc.) to me.	
4.	The use of the data in research, publications, sharing and archiving has been explained to me.	
5.	I understand that other researchers will have access to this data only if they agree to preserve the confidentiality of the data and if they agree to the terms I have specified in this form.	
6.	I understand that my DNA will be stored for future analysis.	
7.	I understand that data from my medical notes, but only that part relevant to this study, will be looked at by the researchers of this study and possibly by the NHS Greater Glasgow & Clyde R&D office for evaluation purposes. I give permission for these individuals to have access to my medical records.	
8.	I understand I can withdraw at any time without giving reasons and that I will not be penalised for withdrawing nor will I be questioned on why I have withdrawn.	
9.	I voluntarily agree to participate in the project.	
10.	I, along with the Researcher, agree to sign and date this informed consent form.	

Participant:

_____	_____	_____
Name of Participant	Signature	Date

Researcher:

_____	_____	_____
Name of Researcher	Signature	Date

Version 2.2 (19/6/2016)

Appendix IV: Baseline Questionnaire

University of Glasgow

Participant's ID

NHS Greater Glasgow and Clyde

Baseline Questionnaire (Female)

Does Cardiometabolic Health determine IVF/ICSI Treatment Outcome?

The research team would grateful if you would complete the questionnaire below. Through this brief questionnaire, your answers will be helpful in understanding your general health and wellbeing. It's important to answer all the questions truthfully. We have estimated 10 minutes to complete it. Please feel free to discuss any question. Your responses will only be used for the study purposes, and all the information provided will remain confidential and will be available to the research team only.

Thank you very much for taking the time to complete this.

Ethnicity:

White (Scottish, English, Northern Irish, Welsh)	yes [ ]	no [ ]
Other white ethnic group (Details.....)	yes [ ]	no [ ]
Mixed or multiple ethnic (Details.....)	yes [ ]	no [ ]
Asian/ Asian British (Indian, Pakistani, Chinese)	yes [ ]	no [ ]
Other Asian ethnic group (Details.....)	yes [ ]	no [ ]
Black (Black British, African, Caribbean)	yes [ ]	no [ ]
Other black ethnic group (Details.....)	yes [ ]	no [ ]
Arab	yes [ ]	no [ ]
Other white ethnic group (Details.....)	yes [ ]	no [ ]

Marital status, please tick appropriate:

Married ☐

Single ☐

Separated ☐

Divorced ☐

Widow ☐

Occupation:

Student (If yes details.....)	yes [ ]	no [ ]
Employee (If yes details.....)	yes [ ]	no [ ]
Self-employed (If yes details.....)	yes [ ]	no [ ]
Unemployed (If yes details.....)	yes [ ]	no [ ]

Education attainment:

High school	yes [ ]	no [ ]
Undergraduate degree	yes [ ]	no [ ]
Postgraduate	yes [ ]	no [ ]

University of Glasgow

NHS Greater Glasgow and Clyde

Smoking status:

Smoker (If yes Details.....cigarettes/day)	yes [ ]	no [ ]
Ex-smoker (If yes Details..... cigarettes/day)	yes [ ]	no [ ]
Non-smoker	yes [ ]	no [ ]

Do you (or used to) drink alcohol? yes [ ] no [ ]
(If yes details.....units/week)

How many times per week do you exercise that makes your heart rate increase (more than 15 minutes)?

Once	☐
Twice	☐
3-4 times	☐
5-7 times	☐
More than 7 times	☐
Never	☐

Medical history

Are you presently under medical care or taking any medication types other than your treatment cycle (tablets, medicines or drugs)?	yes [ ]	no [ ]
Are you taking or have you taken steroids in the last two years?	yes [ ]	no [ ]
Have you ever had a prolonged illness or been hospitalised?	yes [ ]	no [ ]
Have you had any major/serious operations or radiation therapy?	yes [ ]	no [ ]
Do you have any allergies?	yes [ ]	no [ ]

Have you ever had any of the following:

Check those to which the answer is yes (leave other blank).

- ☐ Heart attacks
- ☐ Strokes
- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Congenital heart disease (existing at birth but not hereditary)
- ☐ Heart operations
- ☐ Obesity (20 or more pounds overweight)

If YES to any of previous questions, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled). (Use a separate sheet if necessary)

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University of Glasgow

NHS Greater Glasgow and Clyde

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.....

Familial Diseases

Have you or your blood relatives had any of the following (include grandparents, aunts and uncles, but exclude cousins, relatives by marriage and half-relatives)?

Check those to which the answer is yes (leave other blank).

- ☐ Heart attacks under age 50
- ☐ Strokes under age 50
- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Congenital heart disease (existing at birth but not hereditary)
- ☐ Heart operations
- ☐ Obesity (20 or more pounds overweight)

If any of the above chosen, please give details:

.....

.....

.....

.....

Vaginal Complaints

Do you have burning after intercourse?	yes [ ]	no [ ]
Do you have vaginal discharge?	yes [ ]	no [ ]
Do you have vaginal burning?	yes [ ]	no [ ]
Is your vagina itchy?	yes [ ]	no [ ]

If "YES" to any of previous questions, have you seen a physician for those symptoms? yes [ ] no [ ]

If "YES", please give details including the diagnosis and treatment given (if any):

.....

.....

.....

Does your sexual partner have symptoms of irritation, burning or discharge? yes [ ] no [ ]

If "YES", please give details including the diagnosis and treatment given (if any):

.....

.....

.....

Thank you for taking time to complete this questionnaire

University of Glasgow

Participant's ID

NHS Greater Glasgow and Clyde

Baseline Questionnaire (Male)

Does Cardiometabolic Health determine IVF/ICSI Treatment Outcome?

The research team would grateful if you would complete the questionnaire below. Through this brief questionnaire, your answers will be helpful in understanding your general health and wellbeing. It's important to answer all the questions truthfully. We have estimated 10 minutes to complete it. Please feel free to discuss any question. Your responses will only be used for the study purposes, and all the information provided will remain confidential and will be available to the research team only.

Thank you very much for taking the time to complete this.

Ethnicity:

White (Scottish, English, Northern Irish, Welsh)	yes [ ]	no [ ]
Other white ethnic group (Details.....)	yes [ ]	no [ ]
Mixed or multiple ethnic (Details.....)	yes [ ]	no [ ]
Asian/ Asian British (Indian, Pakistani, Chinese)	yes [ ]	no [ ]
Other Asian ethnic group (Details.....)	yes [ ]	no [ ]
Black (Black British, African, Caribbean)	yes [ ]	no [ ]
Other black ethnic group (Details.....)	yes [ ]	no [ ]
Arab	yes [ ]	no [ ]
Other white ethnic group (Details.....)	yes [ ]	no [ ]

Marital status, please tick appropriate:

Married ☐

Single ☐

Separated ☐

Divorced ☐

Widower ☐

Occupation:

Student (If yes details.....)	yes [ ]	no [ ]
Employee (If yes details.....)	yes [ ]	no [ ]
Self-employed (If yes details.....)	yes [ ]	no [ ]
Unemployed (If yes details.....)	yes [ ]	no [ ]

University of Glasgow

NHS Greater Glasgow and Clyde

Education attainment:

High school	yes [ ]	no [ ]
Undergraduate degree	yes [ ]	no [ ]
Postgraduate	yes [ ]	no [ ]

Smoking status:

Smoker (If yes Details.....cigarettes/day)	yes [ ]	no [ ]
Ex-smoker (If yes Details..... cigarettes/day)	yes [ ]	no [ ]
Non-smoker	yes [ ]	no [ ]

Do you (or used to) drink alcohol? yes [ ] no [ ]
(If yes details.....units/week)

How many times per week do you exercise that makes your heart rate increase (more than 15 minutes)?

Once	☐
Twice	☐
3-4 times	☐
5-7 times	☐
More than 7 times	☐
Never	☐

Medical history

Are you presently under medical care or taking any medication types other than your treatment cycle (tablets, medicines or drugs)?	yes [ ]	no [ ]
Are you taking or have you taken steroids in the last two years?	yes [ ]	no [ ]
Have you ever had a prolonged illness or been hospitalised?	yes [ ]	no [ ]
Have you had any major/serious operations or radiation therapy?	yes [ ]	no [ ]
Do you have any allergies?	yes [ ]	no [ ]

Have you ever had any of the following:

Check those to which the answer is yes (leave other blank).

- ☐ Heart attacks
- ☐ Strokes
- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Congenital heart disease (existing at birth but not hereditary)
- ☐ Heart operations
- ☐ Obesity (20 or more pounds overweight)

University of Glasgow

NHS Greater Glasgow and Clyde

If YES to any of previous questions, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled). (Use a separate sheet if necessary)

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Familial Diseases

Have you or your blood relatives had any of the following (include grandparents, aunts and uncles, but exclude cousins, relatives by marriage and half-relatives)?

Check those to which the answer is yes (leave other blank).

- ☐ Heart attacks under age 50
- ☐ Strokes under age 50
- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Congenital heart disease (existing at birth but not hereditary)
- ☐ Heart operations
- ☐ Obesity (20 or more pounds overweight)

If any of the above chosen, please give details:

.....

.....

.....

.....

Thank you for taking time to complete this questionnaire

Appendix V: Case Report Form

University of Glasgow

Participant's ID

NHS Greater Glasgow and Clyde

Case Report Form

Does Cardiometabolic Health Determine IVF/ICSI Treatment Outcome

Patient's personal details

Female Information	**Male Information**
Fore names:	Fore names: .....
Surname:	Surname: .....
DOB: / /	DOB: / /
Address:..... ....Post Code:	Address:..... ....Post Code: .....
Contact number:	Contact number: .....
CHI Number.....	CHI Number.....

Reproductive History

Female

Parity (Gravida, Para): .....

Vaginal discharges (colour, odour, vulval irritation, previous sexually transmitted disease)

.....
.....

Diagnosis of previous pregnancies (if any)

.....
.....

Cause of infertility

.....
.....
.....

Duration of infertility

.....
.....

Obstetric history (natural pregnancies, ART pregnancies, natural live birth, ART life birth)

.....
.....
.....

Previous IVF/ICSI treatment details (type of cycle, dose, number of oocytes retrieved, number of embryos transferred, outcome)

.....
.....
.....
.....

Details of baseline scan

Date of the scan: / /

Endom thickness: .....

IFC (Right): .....

IFC (Left): .....

Last tracking scan

Date of the scan: / /

Endom thickness:.....

TFC (Right): .....

Maximum size (Right): .....

Maximum size (Left): .....

IFC (Right): .....

IFC (Left): .....

TFC(Left) :.....

Remarks

.....
.....
.....

Male

History of fatherhood

.....
.....

History of any sexual problems (coital frequency, any erectile or ejaculatory problems)

.....
.....
.....

Remarks

.....
.....
.....

Female

Anthropometrics data

Height: .....cm Weight: .....kg

Blood Pressure: .....

Any medication intake

.....
.....
.....

Any allergies

.....
.....

Family history of genetic abnormalities

.....
.....
.....

History of medical surgeries

.....
.....
.....

Any disabilities

.....
.....

Cardiometabolic health markers

Hypertension:

.....

Diabetes and Insulin resistance: .....

Glucose: .....

Lipid profile:

- Triglycerides: .....
- Total cholesterol: .....
- High-density lipoprotein cholesterol [HDL-C]: .....
- Low-density lipoprotein cholesterol [LDL-C]: .....

Inflammatory conditions (CRP): .....

Remarks

.....
.....
.....

Male

Anthropometrics data

Height: .....cm Weight: .....kg

Blood Pressure: .....

Any medication intake

.....
.....
.....

Any allergies

.....
.....

Family history of genetic abnormalities

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History of medical surgeries

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.....

Any disabilities

.....
.....

Cardiometabolic health markers

Hypertension: .....

Diabetes and Insulin resistance: .....

Glucose: .....

Lipid profile:

- Triglycerides: .....
- Total cholesterol: .....
- High-density lipoprotein cholesterol [HDL-C]: .....
- Low-density lipoprotein cholesterol [LDL-C]: .....

Inflammatory conditions (CRP): .....

Remarks

.....
.....
.....

IVF/ICSI Treatment Cycle

Female/ male

Date of attending ACS clinic: / /

Date of last menstrual cycle: / /

Cycle number:.....

Follow-up ultrasound scan comments:

.....
.....
.....

Details of protocol used (stimulation drug type and brand, long/short protocol...etc.)

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.....
.....

Details of oocyte development (dates and progression of scanning)

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.....
.....

Date of Oocyte triggering use: / /

Type of Oocyte triggering used

.....
.....

Date of Oocytes retrieval: / /

Details of Oocytes retrieved (quality and number)

.....
.....
.....

Semen analysis details:

- Production date: / /
- Production method: .....
- Ejaculation problems: .....
- Semen volume (mL): .....
- Sperm concentration: .....
- Motile concentration: .....
- Total number of sperm (10^6 per ejaculation): .....
- Sperm concentration (10^6 per mL): .....
- Consistency (thickness) of the semen: .....
- Progressive sperm motility (PR, %): .....
- Sperm morphology (normal forms, %): .....

Semen analysis after preparation details:

- Production date: / /
- Production method: .....
- Ejaculation problems: .....
- Semen volume (mL): .....
- Sperm concentration: .....
- Motile concentration: .....
- Total number of sperm (10^6 per ejaculation): .....
- Sperm concentration (10^6 per mL): .....
- Consistency (thickness) of the semen: .....
- Progressive sperm motility (PR, %): .....

Sperm morphology (normal forms, %): .....

Fertilisation method used

.....

Insemination date: / /

Number of oocytes injected/fertilised: .....

Embryo development Morphologically

Presence of pronuclear:

.....

.....

Evidence of multinucleation:

.....

.....

Day 3:

.....

.....

Day 5:

.....

.....

Key kenetic parameters for embryo development

Presence of 2 pronuclears (2PN):.....
Fading of 2 pronuclears (2PNf):..... 1st Synchronise division S_1(t_2-2PNf):.....
Time to 2 cells (t_2):..... 2nd Cycle CC_2 (t_3-t_2):.....
Time to 3 cells (t_3):..... 3rd Cycle CC_3 (t_5-t_3):.....

Time to 4 cells (t_4):..... 2nd Synchronise devision $S_2(t_4- t_3)$:.....
Time to 5 cells (t_5):..... 3rd Synchronise devision $S_3(t_8- t_5)$:.....
Time to 8 cells (t_8):.....
Time to 9 cells (t_9):.....
Time to Morula (tMor):.....
Time to Start Blastocyst (tSB):.....
Time to Blastocyst (tBL):.....

IVF/ICSI cycle

Number of embryos transferred

.....
.....

Embryo transfer date: / /

Outcome:

- HCG test:
  - Date: / /
  - Positive ☐
  - Negative ☐
- Gestational sac:
  - Date: / /
- Fetal heart beat:
  - Date: / /
  - Positive ☐ Number of heart beat:.....
  - Negative ☐
- Live birth:
  - Week born: /40
  - Baby weight: .....
- Miscarriage (details): .....

  .....

  .....
- Abortion (details): .....

List of References

Aarnio, M., Winter, T., Kujala, U. & Kaprio, J. 1997. Familial aggregation of leisure-time physical activity-a three generation study. *International Journal of Sports Medicine,* 18**,** 549-556.

Achache, H. & Revel, A. 2006. Endometrial receptivity markers, the journey to successful embryo implantation. *Human Reproduction Update,* 12**,** 731-746.

Adamson, G., Zegers-Hochschild, F., Dyer, S., Chambers, G., Ishihara, O., Mansour, R., Banker, M. & De Mouzon, J. Icmart world report 2014. Human Reproduction, 2018. London: Oxford University Press, 65-65.

Agarwal, A., Majzoub, A., Parekh, N. & Henkel, R. 2020. A schematic overview of the current status of male infertility practice. *World J Mens Health,* 38**,** 308-322.

Agarwal, A., Panner Selvam, M. K., Baskaran, S. & Cho, C.-L. 2019. Sperm DNA damage and its impact on male reproductive health: A critical review for clinicians, reproductive professionals and researchers. *Expert Review of Molecular Diagnostics,* 19**,** 443-457.

Agarwal, A., Parekh, N., Panner Selvam, M. K., Henkel, R., Shah, R., Homa, S. T., Ramasamy, R., Ko, E., Tremellen, K. & Esteves, S. 2019. Male oxidative stress infertility (mosi): Proposed terminology and clinical practice guidelines for management of idiopathic male infertility. *The World Journal of Men's Health,* 37**,** 296-312.

Aitken, R. J. & Curry, B. J. 2011. Redox regulation of human sperm function: From the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxidants & redox signaling,* 14**,** 367-381.

Aitken, R. J., Smith, T. B., Jobling, M. S., Baker, M. A. & De Iuliis, G. N. 2014. Oxidative stress and male reproductive health. *Asian journal of andrology,* 16**,** 31.

Akbaraly, T., Würtz, P., Singh-Manoux, A., Shipley, M. J., Haapakoski, R., Lehto, M., Desrumaux, C., Kähönen, M., Lehtimäki, T. & Mikkilä, V. 2018. Association of circulating metabolites with healthy diet and risk of cardiovascular disease: Analysis of two cohort studies. *Scientific Reports,* 8**,** 1-14.

Akbaraly, T., Wurtz, P., Singh-Manoux, A., Shipley, M. J., Haapakoski, R., Lehto, M., Desrumaux, C., Kahonen, M., Lehtimaki, T., Mikkila, V., Hingorani, A., Humphries, S. E., Kangas, A. J., Soininen, P., Raitakari, O., Ala-Korpela, M. & Kivimaki, M. 2018. Association of circulating metabolites with healthy diet and risk of cardiovascular disease: Analysis of two cohort studies. *Scientific Reports,* 8**,** 8620.

Al Rashid, K., Goulding, N., Taylor, A., Lumsden, M. A., Lawlor, D. A. & Nelson, S. M. 2020. Spousal associations of serum metabolomic profiles by nuclear magnetic resonance spectroscopy: A cross sectional study of 326 couples. . *Submitted*.

Al Rashid, K., Taylor, A., Lumsden, M. A., Goulding, N., Lawlor, D. A. & Nelson, S. M. 2020. Association of the functional ovarian reserve with serum metabolomic profiling by nuclear magnetic resonance spectroscopy: A cross-sectional study of ~ 400 women. *BMC Medicine,* 18**,** 247.

Al Rashid, K., Taylor, A., Lumsden, M. A., Goulding, N., Lawlor, D. A. & Nelson, S. M. 2020. Association of the serum metabolomic profile by nuclear magnetic resonance spectroscopy with sperm parameters: A cross-sectional study of 325 men. *F&S Science,* 1**,** 142-160.Alshahrani, S., Ahmed, A. F., Gabr, A., Abalhassan, M. & Ahmad, G. 2016. The impact of body mass index on semen parameters in infertile men. *Andrologia,* 48**,** 1125-1129.

Andersen, A.-M. N., Wohlfahrt, J., Christens, P., Olsen, J. & Melbye, M. 2000. Maternal age and fetal loss: Population based register linkage study. *BMJ,* 320**,** 1708-1712.

Andersen, L. W., Mackenhauer, J., Roberts, J. C., Berg, K. M., Cocchi, M. N. & Donnino, M. W. Etiology and therapeutic approach to elevated lactate levels. Mayo Clinic Proceedings, 2013. Elsevier, 1127-1140.

Anderson, E. L., Fraser, A., Mcnally, W., Sattar, N., Lashen, H., Fleming, R., Nelson, S. M. & Lawlor, D. A. 2013. Anti-müllerian hormone is not associated with cardiometabolic risk factors in adolescent females. *PLoS One,* 8**,** e64510.

Anderson, S. A., Barry, J. A. & Hardiman, P. J. 2014. Risk of coronary heart disease and risk of stroke in women with polycystic ovary syndrome: A systematic review and meta-analysis. *International Journal of Cardiology,* 176**,** 486-487.

Andersson, A.-M., Jørgensen, N., Frydelund-Larsen, L., Rajpert-De Meyts, E. & Skakkebaek, N. 2004. Impaired leydig cell function in infertile men: A study of 357 idiopathic infertile men and 318 proven fertile controls. *The Journal of Clinical Endocrinology & Metabolism,* 89**,** 3161-3167.

Andersson, G., Noack, T., Seierstad, A. & Weedon-Fekjaer, H. 2006. The demographics of same-sex marriages in norway and sweden. *Demography,* 43**,** 79-98.

Appt, S. E., Chen, H., Clarkson, T. B. & Kaplan, J. R. 2012. Premenopausal antimullerian hormone concentration is associated with subsequent atherosclerosis. *Menopause* 19**,** 1353.

Armstrong, D. T. 1986. Environmental stress and ovarian function. *Biology of Reproduction,* 34**,** 29-39.

Åsenius, F., Danson, A. F. & Marzi, S. J. 2020. DNA methylation in human sperm: A systematic review. *Human Reproduction Update,* 26**,** 841-873.

Ashrafian, H. S., V Glen, R Ebbels, T Blaise, B.J Kalra, D Kultima, K Spjuth, O, Tenori, L. S., R Kale, N Haug, K Schober, D Rocca-Serra, P O'donovan, C Steinbeck, C & Cano, I. D. A., P Cascante, M 2020. Metabolomics - the stethoscope for the 21st century. *Medical Principles and Practice*.

Ask, H., Rognmo, K., Torvik, F. A., Røysamb, E. & Tambs, K. 2012. Non-random mating and convergence over time for alcohol consumption, smoking, and exercise: The nord-trøndelag health study. *Behavior Genetics,* 42**,** 354-365.

Babu, G., Singaravelu, B. G., Srikumar, R. & Reddy, S. V. 2017. Comparative study on the vaginal flora and incidence of asymptomatic vaginosis among healthy women and in women with infertility problems of reproductive age. *Journal of Clinical and Diagnostic Research: JCDR,* 11**,** DC18.

Baker, T. 1963. A quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society of London. Series B. Biological Sciences,* 158**,** 417-433.

Baldwin, J. C. & Damon, A. 1973. Some genetic traits in solomon island populations. V. Assortative mating, with special reference to skin color. *American Journal of Physical Anthropology,* 39**,** 195-201.

Banting, L. K., Gibson-Helm, M., Polman, R., Teede, H. J. & Stepto, N. K. 2014. Physical activity and mental health in women with polycystic ovary syndrome. *BMC women's health,* 14**,** 51.

Barker, D. J. 1995. Fetal origins of coronary heart disease. *BMJ,* 311**,** 171-174.

Barratt, C. L., Björndahl, L., De Jonge, C. J., Lamb, D. J., Osorio Martini, F., Mclachlan, R., Oates, R. D., Van Der Poel, S., St John, B. & Sigman, M. 2017. The diagnosis of male infertility: An analysis of the evidence to support the development of global who guidance—challenges and future research opportunities. *Human Reproduction Update,* 23**,** 660-680.

Batty, G. D., Mortensen, L. H. & Shipley, M. J. 2019. Semen quality and risk factors for mortality. *Epidemiology,* 30**,** e19-e21.

Behre, H. M., Kuhlage, J., Gaßner, C., Sonntag, B., Schem, C., Schneider, H. P. & Nieschlag, E. 2000. Prediction of ovulation by urinary hormone measurements with the home use clearplan® fertility monitor: Comparison with transvaginal ultrasound scans and serum hormone measurements. *Human Reproduction,* 15**,** 2478-2482.

Bell, A. V. 2010. Beyond (financial) accessibility: Inequalities within the medicalisation of infertility. *Sociology of Health & Illness,* 32**,** 631-646.

Bellamy, L., Casas, J.-P., Hingorani, A. D. & Williams, D. J. 2007. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: Systematic review and meta-analysis. *BMJ,* 335**,** 974.

Benevenga, N. & Steele, R. 1984. Adverse effects of excessive consumption of amino acids. *Annual review of nutrition,* 4**,** 157-181.

Bentin-Ley, U., Horn, T., Sjogren, A., Sorensen, S., Falck Larsen, J. & Hamberger, L. 2000. Ultrastructure of human blastocyst-endometrial interactions in vitro. *Journal of Reproduction and Fertility,* 120**,** 337-350.

Berkman, L. F., Glass, T., Brissette, I. & Seeman, T. E. 2000. From social integration to health: Durkheim in the new millennium. *Social Science & Medicine,* 51**,** 843-857.

Best, D., Avenell, A., Bhattacharya, S. & Stadler, G. 2017. New debate: Is it time for infertility weight-loss programmes to be couple-based? *Human Reproduction,* 32**,** 2359-2365.

Bieniek, J. M., Kashanian, J. A., Deibert, C. M., Grober, E. D., Lo, K. C., Brannigan, R. E., Sandlow, J. I. & Jarvi, K. A. 2016. Influence of increasing body mass index on semen and reproductive hormonal parameters in a multi-institutional cohort of subfertile men. *Fertility and Sterility,* 106**,** 1070-1075.

Binder, A. M., Corvalan, C., Mericq, V., Pereira, A., Santos, J. L., Horvath, S.,

Shepherd, J. & Michels, K. B. 2018. Faster ticking rate of the epigenetic clock is associated with faster pubertal development in girls. *Epigenetics,* 13**,** 85-94.

Bitler, M. P. & Schmidt, L. 2012. Utilization of infertility treatments: The effects of insurance mandates. *Demography,* 49**,** 125-149.

Björndahl, L. & Kvist, U. 2009. Human sperm chromatin stabilization: A proposed model including zinc bridges. *MHR: Basic science of reproductive medicine,* 16**,** 23-29.

Bleil, M., Adler, N., Gregorich, S., Sternfeld, B., Rosen, M. & Cedars, M. 2012. Does accelerated reproductive aging underlie premenopausal risk for cardiovascular disease? *Fertility and Sterility,* 98**,** S43-S44.

Bleil, M. E., Gregorich, S. E., Mcconnell, D., Rosen, M. P. & Cedars, M. I. 2013. Does accelerated reproductive aging underlie premenopausal risk for cardiovascular disease? . *Menopause,* 20**,** 1139.

Boedt, T., Dancet, E., Fong, S. L., Peeraer, K., De Neubourg, D., Pelckmans, S., Van De Vijver, A., Seghers, J., Van Der Gucht, K. & Van Calster, B. 2019. Effectiveness of a mobile preconception lifestyle programme in couples undergoing in vitro fertilisation (ivf): The protocol for the prelife randomised controlled trial (prelife-rct). *BMJ open,* 9**,** e029665.

Boehnke, M., Moll, P. P., Kottke, B. A. & Weidman, W. H. 1987. Partitioning the variability of fasting plasma glucose levels in pedigrees: Genetic and environmental factors. *American Journal of Epidemiology,* 125**,** 679-689.

Boeri, L., Pederzoli, F., Capogrosso, P., Abbate, C., Alfano, M., Mancini, N., Clementi, M., Montanari, E., Montorsi, F. & Salonia, A. 2020. Semen infections in men with primary infertility in the real-life setting. *Fertility and Sterility*.

Boivin, J., Bunting, L., Collins, J. A. & Nygren, K. G. 2007. International estimates of infertility prevalence and treatment-seeking: Potential need and demand for infertility medical care. *Human Reproduction,* 22**,** 1506-1512.

Borges Jr, E., Setti, A., Braga, D., Figueira, R. & Iaconelli Jr, A. 2016. Total motile sperm count has a superior predictive value over the who 2010 cut-off values for the outcomes of intracytoplasmic sperm injection cycles. *Andrology,* 4**,** 880-886.

Bove, C. F., Sobal, J. & Rauschenbach, B. S. 2003. Food choices among newly

married couples: Convergence, conflict, individualism, and projects. *Appetite,* 40**,** 25-41.

Broekmans, F., Soules, M. & Fauser, B. 2009. Ovarian aging: Mechanisms and clinical consequences. *Endocrine Reviews,* 30**,** 465-493.

Broekmans, F. J., De Ziegler, D., Howles, C. M., Gougeon, A., Trew, G. & Olivennes, F. 2010. The antral follicle count: Practical recommendations for better standardization. *Fertility and Sterility,* 94**,** 1044-1051.

Broekmans, F. J., Faddy, M. J., Scheffer, G. & Te Velde, E. R. 2004. Antral follicle counts are related to age at natural fertility loss and age at menopause. *Menopause,* 11**,** 607-614.

Broer, S. L., Van Disseldorp, J., Broeze, K. A., Dolleman, M., Opmeer, B. C., Bossuyt, P., Eijkemans, M. J., Mol, B.-W. J., Broekmans, F. J. & Group, I. S. 2013. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Human Reproduction Update,* 19**,** 26-36.

Brouillet, S., Boursier, G., Anav, M., Du Boulet De La Boissière, B., Gala, A., Ferrieres-Hoa, A., Touitou, I. & Hamamah, S. 2020. C-reactive protein and art outcomes: A systematic review. *Human Reproduction Update*.Brown, M. C., Best, K. E., Pearce, M. S., Waugh, J., Robson, S. C. & Bell, R. 2013. Cardiovascular disease risk in women with pre-eclampsia: Systematic review and meta-analysis. *European Journal of Epidemiology,* 28**,** 1-19.

Bunting, L. & Boivin, J. 2010. Development and preliminary validation of the fertility status awareness tool: Fertistat. *Human Reproduction,* 25**,** 1722-1733.

Calvert, S., Reynolds, S., Paley, M., Walters, S. & Pacey, A. 2019. Probing human sperm metabolism using 13c-magnetic resonance spectroscopy. *MHR: Basic science of reproductive medicine,* 25**,** 30-41.

Capogrosso, P., Ventimiglia, E., Boeri, L., Cazzaniga, W., Chierigo, F., Montorsi, F. & Salonia, A. 2018. Male infertility as a proxy of the overall male health status. *Minerva Urologica e Nefrologica,* 70**,** 286-299.

Carlsen, E., Giwercman, A., Keiding, N. & Skakkebæk, N. E. 1992. Evidence for decreasing quality of semen during past 50 years. *BMJ,* 305**,** 609-613.

Carr, D. W., Usselman, M. C. & Acott, T. S. 1985. Effects of ph, lactate, and viscoelastic drag on sperm motility: A species comparison. *Biology of Reproduction,* 33**,** 588-595.

Catteau-Jonard, S., Jamin, S. P., Leclerc, A., Gonzalès, J., Dewailly, D. & Di Clemente, N. 2008. Anti-mullerian hormone, its receptor, fsh receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism,* 93**,** 4456-4461.

Cedars, M. I. Biomarkers of ovarian reserve—do they predict somatic aging? Seminars in Reproductive Medicine, 2013. Thieme Medical Publishers, 443-451.

Cha, J., Sun, X. & Dey, S. K. 2012. Mechanisms of implantation: Strategies for successful pregnancy. *Nature Medicine,* 18**,** 1754-1767.

Chowdhury, R., Warnakula, S., Kunutsor, S., Crowe, F., Ward, H. A., Johnson, L., Franco, O. H., Butterworth, A. S., Forouhi, N. G. & Thompson, S. G. 2014. Association of dietary, circulating, and supplement fatty acids with coronary risk: A systematic review and meta-analysis. *Annals of internal medicine,* 160**,** 398-406.

Choy, J. T. & Eisenberg, M. L. 2018. Male infertility as a window to health. *Fertility and Sterility,* 110**,** 810-814.

Christensen, M. W., Kesmodel, U., Christensen, K., Kirkegaard, K. & Ingerslev, H. 2020. Early ovarian ageing: Is a low number of oocytes harvested in young women associated with an earlier and increased risk of age-related diseases? *Human Reproduction,* 35**,** 2375-2390.

Ciborowski, M., Lipska, A., Godzien, J., Ferrarini, A., Korsak, J., Radziwon, P., Tomasiak, M. & Barbas, C. 2012. Combination of lc–ms-and gc–ms-based metabolomics to study the effect of ozonated autohemotherapy on human blood. *Journal of Proteome Research,* 11**,** 6231-6241.

Cnop, M., Hannaert, J. C., Hoorens, A., Eizirik, D. L. & Pipeleers, D. G. 2001. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes,* 50**,** 1771-1777.

Cohen, J. 2013. *Statistical power analysis for the behavioral sciences*, Academic press.

Consortium, E. I.-M., Reproduction, E. S. O. H., Embryology, Calhaz-Jorge, C., De Geyter, C., Kupka, M., De Mouzon, J., Erb, K., Mocanu, E., Motrenko, T. & Scaravelli, G. 2017. Assisted reproductive technology in europe, 2013: Results generated from european registers by eshre. *Human Reproduction,* 32**,** 1957-1973.

Cook, C. L., Siow, Y., Brenner, A. G. & Fallat, M. E. 2002. Relationship between serum müllerian-inhibiting substance and other reproductive hormones in untreated women with polycystic ovary syndrome and normal women. *Fertility and Sterility,* 77**,** 141-146.

Cooper, G. S. & Sandler, D. P. 1998. Age at natural menopause and mortality. *Annals of epidemiology,* 8**,** 229-235.

Cooper, T. 1980. The general importance of proteins and other factors in the transfer of steroids into the rat epididymis. *International Journal of Andrology,* 3**,** 333-348.

Cooper, T. G., Noonan, E., Von Eckardstein, S., Auger, J., Baker, H., Behre, H. M., Haugen, T. B., Kruger, T., Wang, C. & Mbizvo, M. T. 2010. World health organization reference values for human semen characteristics. *Human Reproduction Update,* 16**,** 231-245.

Coulam, C. B., Adamson, S. C. & Annegers, J. F. 1986. Incidence of premature ovarian failure. *Obstetrics & Gynecology,* 67**,** 604-606.

Courant, F. D. R., Antignac, J.-P., Monteau, F. & Le Bizec, B. 2013. Metabolomics as a potential new approach for investigating human reproductive disorders. *Journal of Proteome Research,* 12**,** 2914-2920.

Craig, J. R., Jenkins, T. G., Carrell, D. T. & Hotaling, J. M. 2017. Obesity, male infertility, and the sperm epigenome. *Fertility and Sterility,* 107**,** 848-859.

Cui, L., Qin, Y., Gao, X., Lu, J., Geng, L., Ding, L., Qu, Z., Zhang, X. & Chen, Z.-J. 2016. Antimüllerian hormone: Correlation with age and androgenic and metabolic factors in women from birth to postmenopause. *Fertility and Sterility,* 105**,** 481-485. e1.

Cui, W. 2010. Mother or nothing: The agony of infertility. *World Health Organization. Bulletin of the World Health Organization,* 88**,** 881.

Cui, Y., Shi, Y., Cui, L., Han, T., Gao, X. & Chen, Z.-J. 2014. Age-specific serum antimüllerian hormone levels in women with and without polycystic ovary syndrome. *Fertility and Sterility,* 102**,** 230-236. e2.

Dam, V., Van Der Schouw, Y. T., Onland-Moret, N. C., Groenwold, R. H., Peters, S. A., Burgess, S., Wood, A. M., Chirlaque, M.-D., Moons, K. G. & Oliver-Williams, C. 2019. Association of menopausal characteristics and risk of coronary heart disease: A pan-european case–cohort analysis. *International Journal of Epidemiology,* 48**,** 1275-1285.

Damsgaard, J., Joensen, U. N., Carlsen, E., Erenpreiss, J., Jensen, M. B., Matulevicius, V., Zilaitiene, B., Olesen, I. A., Perheentupa, A. & Punab, M. 2016. Varicocele is associated with impaired semen quality and reproductive hormone levels: A study of 7035 healthy young men from six european countries. *European Urology,* 70**,** 1019-1029.

Dancet, E. A., D'hooghe, T. M., Dreischor, F., Van Wely, M., Laan, E. T., Lambalk, C. B., Repping, S. & Custers, I. M. 2019. The 'pleasure&pregnancy'web-based interactive educational programme versus expectant management in the treatment of unexplained subfertility: Protocol for a randomised controlled trial. *BMJ open,* 9**,** e025845.

Das, M., Djahanbakhch, O., Hacihanefioglu, B., Saridogan, E., Ikram, M., Ghali, L., Raveendran, M. & Storey, A. 2008. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism,* 93**,** 881-887.

Datta, J., Palmer, M., Tanton, C., Gibson, L., Jones, K., Macdowall, W., Glasier, A., Sonnenberg, P., Field, N. & Mercer, C. 2016. Prevalence of infertility and help seeking among 15 000 women and men. *Human Reproduction,* 31**,** 2108-2118.

Datta, J., Palmer, M. J., Tanton, C., Gibson, L. J., Jones, K. G., Macdowall, W., Glasier, A., Sonnenberg, P., Field, N., Mercer, C. H., Johnson, A. M. & Wellings, K. 2016. Prevalence of infertility and help seeking among 15 000 women and men. *Human Reproduction,* 31**,** 2108-2118.

Dayan, N., Filion, K. B., Okano, M., Kilmartin, C., Reinblatt, S., Landry, T., Basso, O. & Udell, J. A. 2017. Cardiovascular risk following fertility therapy: Systematic review and meta-analysis. *Journal of the American College of Cardiology,* 70**,** 1203-1213.

De Kat, A., Verschuren, W., Eijkemans, M., Van Der Schouw, Y. & Broekmans, F. 2016. The association of low ovarian reserve with cardiovascular disease risk: A cross-sectional population-based study. *Human Reproduction,* 31**,** 1866-1874.

De Kat, A. C., Verschuren, W. M., Eijkemans, M. J., Broekmans, F. J. & Van Der Schouw, Y. T. 2017. Anti-müllerian hormone trajectories are associated with cardiovascular disease in women: Results from the doetinchem cohort study. *Circulation,* 135**,** 556-565.

De Neergaard, R., Nielsen, J., Jørgensen, A., Toft, B., Goetze, J. & Jørgensen, N. 2018. Positive association between cholesterol in human seminal plasma and sperm counts: Results from a cross-sectional cohort study and immunohistochemical investigations. *Andrology,* 6**,** 817-828.

Deelen, J., Kettunen, J., Fischer, K., Van Der Spek, A., Trompet, S., Kastenmüller, G., Boyd, A., Zierer, J., Van Den Akker, E. B., Ala-Korpela, M., Amin, N., Demirkan, A., Ghanbari, M., Van Heemst, D., Ikram, M. A., Van Klinken, J. B., Mooijaart, S. P., Peters, A., Salomaa, V., Sattar, N., Spector, T. D., Tiemeier, H., Verhoeven, A., Waldenberger, M., Würtz, P., Davey Smith, G., Metspalu, A., Perola, M., Menni, C., Geleijnse, J. M., Drenos, F., Beekman, M., Jukema, J. W., Van Duijn, C. M. & Slagboom, P. E. 2019. A metabolic profile of all-cause mortality risk identified in an observational study of 44,168 individuals. *Nat Commun,* 10**,** 3346.

Del Giudice, F., Kasman, A. M., Ferro, M., Sciarra, A., De Berardinis, E., Belladelli, F., Salonia, A. & Eisenberg, M. L. 2020. Clinical correlation among male infertility and overall male health: A systematic review of the literature. *Investigative and Clinical Urology,* 61**,** 355-371.

Dey, S., Lim, H., Das, S. K., Reese, J., Paria, B., Daikoku, T. & Wang, H. 2004. Molecular cues to implantation. *Endocrine Reviews,* 25**,** 341-373.

Di Angelantonio, E., Bhupathiraju, S. N., Wormser, D., Gao, P., Kaptoge, S., De Gonzalez, A. B., Cairns, B. J., Huxley, R., Jackson, C. L. & Joshy, G. 2016. Body-mass index and all-cause mortality: Individual-participant-data meta-analysis of 239 prospective studies in four continents. *The Lancet,* 388**,** 776-786.

Di Castelnuovo, A., Quacquaruccio, G., Donati, M. B., De Gaetano, G. & Iacoviello, L. 2009. Spousal concordance for major coronary risk factors: A systematic review and meta-analysis. *American Journal of Epidemiology,* 169**,** 1-8.

Dolleman, M., Faddy, M., Van Disseldorp, J., Van Der Schouw, Y., Messow, C., Leader, B., Peeters, P., Mcconnachie, A., Nelson, S. & Broekmans, F. 2013. The relationship between anti-müllerian hormone in women receiving fertility assessments and age at menopause in subfertile women: Evidence from large population studies. *The Journal of Clinical Endocrinology & Metabolism,* 98**,** 1946-1953.

Dona, A. C., Kyriakides, M., Scott, F., Shephard, E. A., Varshavi, D., Veselkov, K.

& Everett, J. R. 2016. A guide to the identification of metabolites in nmr-based metabonomics/metabolomics experiments. *Computational and Structural Biotechnology Journal,* 14**,** 135-153.

Draisma, H. H., Beekman, M., Pool, R., Van Ommen, G.-J. B., Vaarhorst, A. A., De Craen, A. J., Willemsen, G., Slagboom, P. E. & Boomsma, D. I. 2013. Familial resemblance for serum metabolite concentrations. *Twin Research and Human Genetics,* 16**,** 948-961.

Dribe, M. & Lundh, C. 2009. Status homogamy in the preindustrial marriage market: Partner selection according to age, social origin, and place of birth in nineteenth-century rural sweden. *Journal of Family History,* 34**,** 387-406.

Durairajanayagam, D. 2018. Lifestyle causes of male infertility. *Arab Journal of Urology,* 16**,** 10-20.

Durlinger, A. L., Gruijters, M. J., Kramer, P., Karels, B., Ingraham, H. A., Nachtigal, M. W., Uilenbroek, J. T. J., Grootegoed, J. A. & Themmen, A. P. 2002. Anti-mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology,* 143**,** 1076-1084.

Eckert, L. O., Moore, D. E., Patton, D. L., Agnew, K. J. & Eschenbach, D. A. 2003. Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following in-vitro fertilization. *Infectious diseases in obstetrics and gynecology,* 11**,** 11-17.

Edvardsson, K., Lindkvist, M., Eurenius, E., Mogren, I., Small, R. & Ivarsson, A. 2013. A population-based study of overweight and obesity in expectant parents: Socio-demographic patterns and within-couple associations. *BMC Public Health,* 13**,** 923.

Eijkemans, M. J., Van Poppel, F., Habbema, D. F., Smith, K. R., Leridon, H. & Te Velde, E. R. 2014. Too old to have children? Lessons from natural fertility populations. *Human Reproduction,* 29**,** 1304-1312.

Eisenberg, M. L., Kim, S., Chen, Z., Sundaram, R., Schisterman, E. F. & Buck Louis, G. M. 2014. The relationship between male bmi and waist circumference on semen quality: Data from the life study. *Human Reproduction,* 29**,** 193-200.

Eisenberg, M. L., Li, S., Behr, B., Cullen, M. R., Galusha, D., Lamb, D. J. & Lipshultz, L. I. 2014. Semen quality, infertility and mortality in the USA. *Human Reproduction,* 29**,** 1567-1574.

Eisenberg, M. L., Li, S., Behr, B., Pera, R. R. & Cullen, M. R. 2015. Relationship between semen production and medical comorbidity. *Fertility and Sterility,* 103**,** 66-71.

Eisenberg, M. L., Li, S., Cullen, M. R. & Baker, L. C. 2016. Increased risk of incident chronic medical conditions in infertile men: Analysis of united states claims data. *Fertility and Sterility,* 105**,** 629-636.

Emwas, A.-H., Roy, R., Mckay, R. T., Tenori, L., Saccenti, E., Gowda, G., Raftery, D., Alahmari, F., Jaremko, L. & Jaremko, M. 2019. Nmr spectroscopy for metabolomics research. *Metabolites,* 9**,** 123.

Engel, K. M., Baumann, S., Rolle-Kampczyk, U., Schiller, J., Von Bergen, M. & Grunewald, S. 2019. Metabolomic profiling reveals correlations between spermiogram parameters and the metabolites present in human spermatozoa and seminal plasma. *PLoS One,* 14**,** e0211679.

Ergün, A., Köse, S., Aydos, K., Ata, A. & Avci, A. 2007. Correlation of seminal parameters with serum lipid profile and sex hormones. *Archives of andrology,* 53**,** 21-23.

Evans, J. (2020). *100 Years of Human Chorionic Gonadotropin: Reviews and New Perspectives (Chapter 3.2 - The role of hCG in endometrial receptivity and embryo implantation)*. Amesterdam, 153-166.

Evans, J. and Salamonsen, Louis. (2013). Too much of a good thing? Experimental evidence suggests prolonged exposure to hCG is detrimental to endometrial receptivity. *human reproduction*.

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., Kurilshikov, A., Bonder, M. J., Valles-Colomer, M. & Vandeputte, D. 2016. Population-level analysis of gut microbiome variation. *Science,* 352**,** 560-564.

Fanchin, R., Schonäuer, L. M., Righini, C., Frydman, N., Frydman, R. & Taieb, J. 2003. Serum anti-müllerian hormone dynamics during controlled ovarian hyperstimulation. *Human Reproduction,* 18**,** 328-332.

Farquhar, C., Marjoribanks, J., Brown, J., Fauser, B. C., Lethaby, A., Mourad, S., Rebar, R., Showell, M. & Van Der Poel, S. 2017. Management of ovarian stimulation for ivf: Narrative review of evidence provided for world health organization guidance. *Reproductive Biomedicine Online,* 35**,** 3-16.

Farquhar, C. M., Bhattacharya, S., Repping, S., Mastenbroek, S., Kamath, M. S.,

Marjoribanks, J. & Boivin, J. 2019. Female subfertility. *Nature Reviews Disease Primers,* 5, 7.

Fedorcsák, P., Dale, P. O., Storeng, R., Ertzeid, G., Bjercke, S., Oldereid, N., Omland, A. K., Åbyholm, T. & Tanbo, T. 2004. Impact of overweight and underweight on assisted reproduction treatment. *Human Reproduction,* 19, 2523-2528.

Feldman, R. A., O'neill, K., Butts, S. F. & Dokras, A. 2017. Antimüllerian hormone levels and cardiometabolic risk in young women with polycystic ovary syndrome. *Fertility and Sterility,* 107, 276-281.

Feng, D. & Baker, L. 1994. Spouse similarity in attitudes, personality, and psychological well-being. *Behavior Genetics,* 24, 357-364.

Feng, R., Niu, Y., Sun, X., Li, Q., Zhao, C., Wang, C., Guo, F., Sun, C. & Li, Y. 2013. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: A randomised controlled trial. *Diabetologia,* 56, 985-994.

Feng, R. N., Niu, Y. C., Sun, X. W., Li, Q., Zhao, C., Wang, C., Guo, F. C., Sun, C. H. & Li, Y. 2013. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: A randomised controlled trial. *Diabetologia,* 56, 985-994.

Ferguson, E. M. & Leese, H. J. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Molecular reproduction and development,* 73, 1195-1201.

Ferramosca, A. & Zara, V. 2014. Bioenergetics of mammalian sperm capacitation. *BioMed research international,* 2014, 902953-902953.

Fischer, K., Kettunen, J., Wurtz, P., Haller, T., Havulinna, A. S., Kangas, A. J., Soininen, P., Esko, T., Tammesoo, M. L., Magi, R., Smit, S., Palotie, A., Ripatti, S., Salomaa, V., Ala-Korpela, M., Perola, M. & Metspalu, A. 2014. Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: An observational study of 17,345 persons. *PLoS Med,* 11, e1001606.

Fitzgerald, O., Harris, K., Paul, R. C. & Chambers, G. M. 2017. Assisted reproductive technology in australia and new zealand 2015. *Sydney: National Perinatal Epidemiology and Statistics Unit, the University of New South Wales Sydney*.

Ford, W., North, K., Taylor, H., Farrow, A., Hull, M. & Golding, J. 2000.

Increasing paternal age is associated with delayed conception in a large population of fertile couples: Evidence for declining fecundity in older men. *Human Reproduction,* 15**,** 1703-1708.

Frimmel, W., Halla, M. & Winter-Ebmer, R. 2013. Assortative mating and divorce: Evidence from austrian register data. *Journal of the Royal Statistical Society: Series A (Statistics in Society),* 176**,** 907-929.

Fujimoto, V. Y., Kane, J. P., Ishida, B. Y., Bloom, M. S. & Browne, R. W. 2010. High-density lipoprotein metabolism and the human embryo. *Human Reproduction Update,* 16**,** 20-38.

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M., Zhong, X., Koenig, S. S., Fu, L., Ma, Z. S. & Zhou, X. 2012. Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine,* 4**,** 132ra52-132ra52.

Gardner, D. K. 1998. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology,* 49**,** 83-102.

Gardner, D. K., Meseguer, M., Rubio, C. & Treff, N. R. 2015. Diagnosis of human preimplantation embryo viability. *Human Reproduction Update,* 21**,** 727-747.

George, D., Luo, S., Webb, J., Pugh, J., Martinez, A. & Foulston, J. 2015. Couple similarity on stimulus characteristics and marital satisfaction. *Personality and Individual Differences,* 86**,** 126-131.

Gharagozloo, P., Gutiérrez-Adán, A., Champroux, A., Noblanc, A., Kocer, A., Calle, A., Pérez-Cerezales, S., Pericuesta, E., Polhemus, A. & Moazamian, A. 2016. A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: Promising preclinical evidence from animal models. *Human Reproduction,* 31**,** 252-262.

Giuliani, C., Cilli, E., Bacalini, M. G., Pirazzini, C., Sazzini, M., Gruppioni, G., Franceschi, C., Garagnani, P. & Luiselli, D. 2016. Inferring chronological age from DNA methylation patterns of human teeth. *American Journal of Physical Anthropology,* 159**,** 585-595.

Giwa-Osagie, O. F. 2002. Art in developing countries with particular reference to sub-saharan africa. *Current practices and controversies in assisted reproduction***,** 22.

Gold, H. B., Jung, Y. H. & Corces, V. G. 2018. Not just heads and tails: The

complexity of the sperm epigenome. *Journal of Biological Chemistry,* 293**,** 13815-13820.

Goldberg, E., Eddy, E. M., Duan, C. & Odet, F. 2010. Ldhc: The ultimate testis-specific gene. *Journal of Andrology,* 31**,** 86-94.

Grant, J. D., Heath, A. C., Bucholz, K. K., Madden, P. A., Agrawal, A., Statham, D. J. & Martin, N. G. 2007. Spousal concordance for alcohol dependence: Evidence for assortative mating or spousal interaction effects? *Alcoholism: Clinical and Experimental Research,* 31**,** 717-728.

Graver, M. A. & Wade, J. J. 2011. The role of acidification in the inhibition of neisseria gonorrhoeae by vaginal lactobacilli during anaerobic growth. *Annals of Clinical Microbiology and Antimicrobials,* 10**,** 1-5.

Groebner, A. E., Zakhartchenko, V., Bauersachs, S., Rubio-Aliaga, I., Daniel, H., Büttner, M., Reichenbach, H. D., Meyer, H. H., Wolf, E. & Ulbrich, S. E. 2011. Reduced amino acids in the bovine uterine lumen of cloned versus in vitro fertilized pregnancies prior to implantation. *Cellular Reprogramming (Formerly" Cloning and Stem Cells"),* 13**,** 403-410.

Group, E. C. W. 2017. A prognosis-based approach to infertility: Understanding the role of time. *Human Reproduction,* 32**,** 1556-1559.

Grummer, R. & Carroll, D. 1988. A review of lipoprotein cholesterol metabolism: Importance to ovarian function. *Journal of Animal Science,* 66**,** 3160-3173.

Gundersen, T. D., Jørgensen, N., Andersson, A.-M., Bang, A. K., Nordkap, L., Skakkebæk, N. E., Priskorn, L., Juul, A. & Jensen, T. K. 2015. Association between use of marijuana and male reproductive hormones and semen quality: A study among 1,215 healthy young men. *American Journal of Epidemiology,* 182**,** 473-481.

Gupta, A., Mahdi, A. A., Ahmad, M. K., Shukla, K. K., Jaiswer, S. P. & Shankhwar, S. N. 2011. 1h nmr spectroscopic studies on human seminal plasma: A probative discriminant function analysis classification model. *Journal of Pharmaceutical and Biomedical Analysis,* 54**,** 106-113.

Gupta, R. & Dixit, V. 1988. Effect of dietary cholesterol on spermatogenesis. *Zeitschrift für Ernährungswissenschaft,* 27**,** 236-243.

Gurunath, S., Pandian, Z., Anderson, R. A. & Bhattacharya, S. 2011. Defining infertility—a systematic review of prevalence studies. *Human Reproduction Update,* 17**,** 575-588.

Guthauser, B., Boitrelle, F., Plat, A., Thiercelin, N. & Vialard, F. 2013. Chronic excessive alcohol consumption and male fertility: A case report on reversible azoospermia and a literature review. *Alcohol and Alcoholism,* 49**,** 42-44.

Haahr, T., Jensen, J., Thomsen, L., Duus, L., Rygaard, K. & Humaidan, P. 2016. Abnormal vaginal microbiota may be associated with poor reproductive outcomes: A prospective study in ivf patients. *Human Reproduction,* 31**,** 795-803.

Haahr, T., Jensen, J. S. & Humaidan, P. 2019. Vaginal microbiota and ivf outcomes: Poor diagnosis results in flawed conclusions. *Reproductive Biomedicine Online,* 39**,** 178.

Hagenbeek, F. A., Pool, R., Van Dongen, J., Draisma, H. H., Hottenga, J. J., Willemsen, G., Abdellaoui, A., Fedko, I. O., Den Braber, A. & Visser, P. J. 2020. Heritability estimates for 361 blood metabolites across 40 genome-wide association studies. *Nature Communications,* 11**,** 1-11.

Hagenbeek, F. A., Pool, R., Van Dongen, J., Draisma, H. H. M., Jan Hottenga, J., Willemsen, G., Abdellaoui, A., Fedko, I. O., Den Braber, A., Visser, P. J., De Geus, E. J. C. N., Willems Van Dijk, K., Verhoeven, A., Suchiman, H. E., Beekman, M., Slagboom, P. E., Van Duijn, C. M., Barkey wolf, J. J. H., Cats, D., Amin, N., Beulens, J. W., Van Der Bom, J. A., Bomer, N., Demirkan, A., Van Hilten, J. A., Meessen, J. M. T. A., Moed, M. H., Fu, J., Onderwater, G. L. J., Rutters, F., So-Osman, C., Van Der Flier, W. M., Van Der Heijden, A. a. W. A., Van Der Spek, A., Asselbergs, F. W., Boersma, E., Elders, P. M., Geleijnse, J. M., Ikram, M. A., Kloppenburg, M., Meulenbelt, I., Mooijaart, S. P., Nelissen, R. G. H. H., Netea, M. G., Penninx, B. W. J. H., Stehouwer, C. D. A., Teunissen, C. E., Terwindt, G. M., 'T Hart, L. M., Van Den Maagdenberg, A. M. J. M., Van Der Harst, P., Van Der Horst, I. C. C., Van Der Kallen, C. J. H., Van Greevenbroek, M. M. J., Van Spil, W. E., Wijmenga, C., Zwinderman, A. H., Zhernikova, A., Jukema, J. W., Mei, H., Slofstra, M., Swertz, M., Van Den Akker, E. B., Deelen, J., Reinders, M. J. T., Harms, A. C., Hankemeier, T., Bartels, M., Nivard, M. G., Boomsma, D. I. & Consortium, B. M. 2020. Heritability estimates for 361 blood metabolites across 40 genome-wide association studies. *Nature Communications,* 11**,** 39.

Hagiuda, J., Ishikawa, H., Furuuchi, T., Hanawa, Y. & Marumo, K. 2014.

Relationship between dyslipidaemia and semen quality and serum sex hormone levels: An infertility study of 167 japanese patients. *Andrologia,* 46, 131-135.

Haikin Herzberger, E., Miller, N., Ghetler, Y., Tamir Yaniv, R., Neumark, E., Shulman, A. & Wiser, A. 2019. A prospective study of c-reactive protein in patients with obesity during ivf. *Human Fertility*, 1-6.

Hajder, M., Hajder, E. & Husic, A. 2016. The effects of total motile sperm count on spontaneous pregnancy rate and pregnancy after iui treatment in couples with male factor and unexplained infertility. *Medical archives,* 70, 39.

Hakim, R. B., Gray, R. H. & Zacur, H. 1998. Alcohol and caffeine consumption and decreased fertility. *Fertility and Sterility,* 70, 632-637.

Hansen, K. R., Hodnett, G. M., Knowlton, N. & Craig, L. B. 2011. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertility and Sterility,* 95, 170-5.

Hanson, B. M., Tao, X., Zhan, Y., Jenkins, T. G., Morin, S. J., Scott, R. T. & Seli, E. U. 2020. Young women with poor ovarian response exhibit epigenetic age acceleration based on evaluation of white blood cells using a DNA methylation-derived age prediction model. *Human Reproduction,* 35, 2579-2588.

Hassold, T. & Hunt, P. 2001. To err (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews Genetics,* 2, 280-291.

Haug, E. B., Horn, J., Markovitz, A. R., Fraser, A., Klykken, B., Dalen, H., Vatten, L. J., Romundstad, P. R., Rich-Edwards, J. W. & Åsvold, B. O. 2019. Association of conventional cardiovascular risk factors with cardiovascular disease after hypertensive disorders of pregnancy: Analysis of the nord-trøndelag health study. *JAMA Cardiology,* 4, 628-635.

Haug, E. B., Horn, J., Markovitz, A. R., Fraser, A., Vatten, L. J., Macdonald-Wallis, C., Tilling, K., Romundstad, P. R., Rich-Edwards, J. W. & Aasvold, B. O. 2018. Life course trajectories of cardiovascular risk factors in women with and without hypertensive disorders in first pregnancy: The hunt study in norway. *Journal of the American Heart Association,* 7, e009250.

Henderson, S. & Edwards, R. 1968. Chiasma frequency and maternal age in mammals. *Nature,* 218, 22-28.

Hendriks, D. J., Mol, B.-W. J., Bancsi, L. F., Te Velde, E. R. & Broekmans, F. J. 2005. Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: A meta-analysis and comparison with basal follicle-stimulating hormone level. *Fertility and Sterility,* 83**,** 291-301.

Henkel, R., Maaß, G., Jung, A., Haidl, G., Schill, W. B. & Schuppe, H. C. 2007. Age-related changes in seminal polymorphonuclear elastase in men with asymptomatic inflammation of the genital tract. *Asian Journal of Andrology,* 9**,** 299-304.

Hereng, T., Elgstøen, K., Cederkvist, F., Eide, L., Jahnsen, T., Skålhegg, B. & Rosendal, K. 2011. Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. *Human Reproduction,* 26**,** 3249-3263.

Hernández-Vargas, P., Muñoz, M. & Domínguez, F. 2020. Identifying biomarkers for predicting successful embryo implantation: Applying single to multi-omics to improve reproductive outcomes. *Human Reproduction Update,* 26**,** 264-301.

Hilz, S., Modzelewski, A. J., Cohen, P. E. & Grimson, A. 2016. The roles of microrna*s* and sirnas in mammalian spermatogenesis. *Development,* 143**,** 3061-3073.

Hippisley-Cox, J., Coupland, C., Pringle, M., Crown, N. & Hammersley, V. 2002. Married couples' risk of same disease: Cross sectional study. *BMJ,* 325**,** 636.

Hodgen, G. D. 1983. The dominant ovarian follicle. *Fertility and Sterility,* 39**,** 54-73.Hong, J. & Lee, E. 2007. Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. *Theriogenology,* 68**,** 728-735.

Hong, X., Ma, J., Yin, J., Fang, S., Geng, J., Zhao, H., Zhu, M., Ye, M., Zhu, X. & Xuan, Y. 2020. The association between vaginal microbiota and female infertility: A systematic review and meta-analysis. *Archives of Gynecology and Obstetrics*, 1-10.

Horne, A. W. & Saunders, P. T. K. 2019. Snapshot: Endometriosis. *Cell,* 179**,** 1677-1677.e1.

Horrocks, L. A. & Yeo, Y. K. 1999. Health benefits of docosahexaenoic acid

(dha). *Pharmacological research,* 40**,** 211-225.

Horvath, S. 2013. DNA methylation age of human tissues and cell types. *Genome Biology,* 14**,** 3156.

Horvath, S., Mah, V., Lu, A. T., Woo, J. S., Choi, O.-W., Jasinska, A. J., Riancho, J. A., Tung, S., Coles, N. S. & Braun, J. 2015. The cerebellum ages slowly according to the epigenetic clock. *Aging (Albany NY),* 7**,** 294.

Horvath, S., Pirazzini, C., Bacalini, M. G., Gentilini, D., Di Blasio, A. M., Delledonne, M., Mari, D., Arosio, B., Monti, D. & Passarino, G. 2015. Decreased epigenetic age of pbmcs from italian semi-supercentenarians and their offspring. *Aging (Albany NY),* 7**,** 1159.

Houghton, F. D., Hawkhead, J. A., Humpherson, P. G., Hogg, J. E., Balen, A. H., Rutherford, A. J. & Leese, H. J. 2002. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction,* 17**,** 999-1005.

Hur, C., Rehmer, J., Flyckt, R. & Falcone, T. 2019. Uterine factor infertility: A clinical review. *Clin Obstet Gynecol,* 62**,** 257-270.

Hur, Y.-M. 2003. Assortative mating for personality traits, educational level, religious affiliation, height, weight, and body mass index in parents of a korean twin sample. *Twin Research and Human Genetics,* 6**,** 467-470.

Hur, Y. M. 2003. Assortive mating for personaltiy traits, educational level, religious affiliation, height, weight, adn body mass index in parents of korean twin sample. *Twin Research and Human Genetics,* 6**,** 467-70.

Ibañez-Perez, J., Santos-Zorrozua, B., Lopez-Lopez, E., Matorras, R. & Garcia-Orad, A. 2019. An update on the implication of physical activity on semen quality: A systematic review and meta-analysis. *Archives of Gynecology and Obstetrics,* 299**,** 901-921.

Iliodromiti, S., Anderson, R. A. & Nelson, S. M. 2015. Technical and performance characteristics of anti-müllerian hormone and antral follicle count as biomarkers of ovarian response. *Human Reproduction Update,* 21**,** 698-710.

Immler, S. 2018. The sperm factor: Paternal impact beyond genes. *Heredity,* 121**,** 239-247.

Jacobsen, B. K., Heuch, I. & Kvåle, G. 2003. Age at natural menopause and all-cause mortality: A 37-year follow-up of 19,731 norwegian women. *American Journal of Epidemiology,* 157**,** 923-929.

Jafarzadeh, N., Mani-Varnosfaderani, A., Minai-Tehrani, A., Savadi-Shiraz, E., Sadeghi, M. R. & Gilany, K. 2015. Metabolomics fingerprinting of seminal plasma from unexplained infertile men: A need for novel diagnostic biomarkers. *Molecular Reproduction and Development,* 82**,** 150-150.

Jain, T. 2006. Socioeconomic and racial disparities among infertility patients seeking care. *Fertility and Sterility,* 85**,** 876-881.

Jamro, E. L., Bloom, M. S., Browne, R. W., Kim, K., Greenwood, E. A. & Fujimoto, V. Y. 2019. Preconception serum lipids and lipophilic micronutrient levels are associated with live birth rates after ivf. *Reproductive Biomedicine Online,* 39**,** 665-673.

Jana, S. K., Dutta, M., Joshi, M., Srivastava, S., Chakravarty, B. & Chaudhury, K. 2013. 1h nmr based targeted metabolite profiling for understanding the complex relationship connecting oxidative stress with endometriosis. *BioMed Research International,* 2013.

Jansen, E., Beekhof, P., Viezeliene, D., Muzakova, V. & Skalicky, J. 2015. Long-term stability of cancer biomarkers in human serum: Biomarkers of oxidative stress and redox status, homocysteine, crp and the enzymes alt and ggt. *Biomarkers in Medicine,* 9**,** 425-432.

Jelenkovic, A., Bogl, L. H., Rose, R. J., Kangas, A. J., Soininen, P., Ala-Korpela, M., Kaprio, J. & Silventoinen, K. 2013. Association of height and pubertal timing with lipoprotein subclass profile: Exploring the role of genetic and environmental effects. *Am J Hum Biol,* 25**,** 465-72.

Jelenkovic, A., Bogl, L. H., Rose, R. J., Kangas, A. J., Soininen, P., Ala-Korpela, M., Kaprio, J. & Silventoinen, K. 2013. Association of height and pubertal timing with lipoprotein subclass profile: Exploring the role of genetic and environmental effects. *American Journal of Human Biology,* 25**,** 465-472.

Jenkins, T. G., Aston, K. I., Cairns, B., Smith, A. & Carrell, D. T. 2018. Paternal germ line aging: DNA methylation age prediction from human sperm. *BMC Genomics,* 19**,** 763.

Jensen, T. K., Jacobsen, R., Christensen, K., Nielsen, N. C. & Bostofte, E. 2009. Good semen quality and life expectancy: A cohort study of 43,277 men. *American Journal of Epidemiology,* 170**,** 559-565.

Jurj, A. L., Wen, W., Li, H.-L., Zheng, W., Yang, G., Xiang, Y.-B., Gao, Y.-T. & Shu, X.-O. 2006. Spousal correlations for lifestyle factors and selected diseases in chinese couples. *Annals of epidemiology,* 16**,** 285-291.

Kay-Tee, K. & Chir, M. 2007. Endogenous testosterone and mortality due to all causes, cardiovascular disease, and cancer in men. *Am Heart Association,* 116, 2694-701.

Kettunen, J., Tukiainen, T., Sarin, A.-P., Ortega-Alonso, A., Tikkanen, E., Lyytikäinen, L.-P., Kangas, A. J., Soininen, P., Würtz, P. & Silander, K. 2012. Genome-wide association study identifies multiple loci influencing human serum metabolite levels. *Nature Genetics,* 44, 269-276.

Khot, U. N., Khot, M. B., Bajzer, C. T., Sapp, S. K., Ohman, E. M., Brener, S. J., Ellis, S. G., Lincoff, A. M. & Topol, E. J. 2003. Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA,* 290, 898-904.

Kline, J., Kinney, A., Kelly, A., Reuss, M. & Levin, B. 2005. Predictors of antral follicle count during the reproductive years. *Human Reproduction,* 20, 2179-2189.

Knuiman, M. W., Divitini, M. L., Bartholomew, H. C. & Welborn, T. A. 1996. Spouse correlations in cardiovascular risk factors and the effect of marriage duration. *American Journal of Epidemiology,* 143, 48-53.

Koedooder, R., Mackens, S., Budding, A., Fares, D., Blockeel, C., Laven, J. & Schoenmakers, S. 2018. Identification and evaluation of the microbiome in the female and male reproductive tracts. *Human Reproduction Update,* 25, 298-325.

Koedooder, R., Singer, M., Schoenmakers, S., Savelkoul, P. H., Morré, S. A., De Jonge, J. D., Poort, L., Cuypers, W.-J. S., Beckers, N. & Broekmans, F. 2019. The vaginal microbiome as a predictor for outcome of in vitro fertilization with or without intracytoplasmic sperm injection: A prospective study. *Human Reproduction,* 34, 1042-1054.

Koppers, A. J., Garg, M. L. & Aitken, R. J. 2010. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radical Biology and Medicine,* 48, 112-119.

Krausz, C. 2011. Male infertility: Pathogenesis and clinical diagnosis. *Best Practice & Research Clinical Endocrinology & Metabolism,* 25, 271-285.

Kwan, I., Bhattacharya, S., Kang, A. & Woolner, A. 2014. Monitoring of stimulated cycles in assisted reproduction (ivf and icsi). *Cochrane Database of Systematic Reviews*.

La Marca, A., Sighinolfi, G., Radi, D., Argento, C., Baraldi, E., Artenisio, A. C.,

Stabile, G. & Volpe, A. 2010. Anti-müllerian hormone (amh) as a predictive marker in assisted reproductive technology (art). *Human Reproduction Update,* 16**,** 113-130.

Latif, T., Kold Jensen, T., Mehlsen, J., Holmboe, S. A., Brinth, L., Pors, K., Skouby, S. O., Jørgensen, N. & Lindahl-Jacobsen, R. 2017. Semen quality as a predictor of subsequent morbidity: A danish cohort study of 4,712 men with long-term follow-up. *American Journal of Epidemiology,* 186**,** 910-917.

Leary, C., Leese, H. J. & Sturmey, R. G. 2015. Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. *Human Reproduction,* 30**,** 122-132.

Lee, S. H., Song, H., Park, Y. S., Koong, M. K., Song, I. O. & Jun, J. H. 2009. Poor sperm quality affects clinical outcomes of intracytoplasmic sperm injection in fresh and subsequent frozen-thawed cycles: Potential paternal effects on pregnancy outcomes. *Fertility and Sterility,* 91**,** 798-804.

Leong, A., Rahme, E. & Dasgupta, K. 2014. Spousal diabetes as a diabetes risk factor: A systematic review and meta-analysis. *BMC Medicine,* 12**,** 12.

Leridon, H. 2008. A new estimate of permanent sterility by age: Sterility defined as the inability to conceive. *Population Studies,* 62**,** 15-24.

Leroy, J., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., De Kruif, A., Genicot, G. & Van Soom, A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction,* 130**,** 485-495.

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R. & Swan, S. H. 2017. Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update,* 23**,** 646-659.

Levine, L. D., Holland, T. L., Kim, K., Sjaarda, L. A., Mumford, S. L. & Schisterman, E. F. 2019. The role of aspirin and inflammation on reproduction: The eager trial. *Canadian Journal of Physiology and Pharmacology,* 97**,** 187-192.

Levine, M. E., Lu, A. T., Chen, B. H., Hernandez, D. G., Singleton, A. B., Ferrucci, L., Bandinelli, S., Salfati, E., Manson, J. E. & Quach, A. 2016. Menopause accelerates biological aging. *Proceedings of the National*

Academy of Sciences, 113**,** 9327-9332.

Levine, M. E., Lu, A. T., Quach, A., Chen, B. H., Assimes, T. L., Bandinelli, S., Hou, L., Baccarelli, A. A., Stewart, J. D. & Li, Y. 2018. An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany NY),* 10**,** 573.

Lewis, J. M., Kaplan, W. E., Caroppo, E., Zirkin, B. R., Papadopoulos, V., Hardy, M. P., Alukal, J. P., Lamb, D. J., Niederberger, C. S. & Makhlouf, A. A. 2009. Anatomy and embryology of the male reproductive tract and gonadal development. *Infertility in the Male. 4th ed. New York*.

Li, S., Rosenberg, L., Wise, L. A., Boggs, D. A., Lavalley, M. & Palmer, J. R. 2013. Age at natural menopause in relation to all-cause and cause-specific mortality in a follow-up study of us black women. *Maturitas,* 75**,** 246-252.

Li, Y., Lin, H., Li, Y. & Cao, J. 2011. Association between socio-psycho-behavioral factors and male semen quality: Systematic review and meta-analyses. *Fertility and Sterility,* 95**,** 116-123.

Lidegaard, Ø., Løkkegaard, E., Jensen, A., Skovlund, C. W. & Keiding, N. 2012. Thrombotic stroke and myocardial infarction with hormonal contraception. *N Engl J Med,* 366**,** 2257-2266.

Li-Gao, R., Hughes, D. A., Le Cessie, S., De Mutsert, R., Den Heijer, M., Rosendaal, F. R., Willems Van Dijk, K., Timpson, N. J. & Mook-Kanamori, D. O. 2019. Assessment of reproducibility and biological variability of fasting and postprandial plasma metabolite concentrations using 1h nmr spectroscopy. *PLoS One,* 14**,** e0218549.

Lindgren, K. E., Nordqvist, S., Kårehed, K., Sundström-Poromaa, I. & Åkerud, H. 2016. The effect of a specific histidine-rich glycoprotein polymorphism on male infertility and semen parameters. *Reproductive Biomedicine Online,* 33**,** 180-188.

Lintsen, A., Pasker-De Jong, P., De Boer, E., Burger, C., Jansen, C., Braat, D. & Van Leeuwen, F. 2005. Effects of subfertility cause, smoking and body weight on the success rate of ivf. *Human Reproduction,* 20**,** 1867-1875.

Lintsen, A. M. E., Pasker-De Jong, P. C. M., De Boer, E. J., Burger, C. W., Jansen, C. a. M., Braat, D. D. M., Van Leeuwen, F. E. & On Behalf of The, O. P. G. 2005. Effects of subfertility cause, smoking and body weight on the success rate of ivf. *Human Reproduction,* 20**,** 1867-1875.

Liou, T.-H., Wu, C.-W., Hao, W.-R., Hsu, M.-I., Liu, J.-C. & Lin, H.-W. 2013. Risk

of myocardial infarction in women with pelvic inflammatory disease. *International Journal of Cardiology,* 167**,** 416-420.

Lipshultz, L. I. & Lamb, D. J. 2007. Risk of transmission of genetic diseases by assisted reproduction. *Nature clinical practice Urology,* 4**,** 460-461.

Liu, C.-Y., Chou, Y.-C., Lin, S.-H., Wu, S.-T., Cha, T.-L., Chen, H.-I. & Tsao, C.-W. 2017. Serum lipid profiles are associated with semen quality. *Asian journal of andrology,* 19**,** 633.

Looby, S. E., Fitch, K. V., Srinivasa, S., Lo, J., Rafferty, D., Martin, A., Currier, J. C., Grinspoon, S. & Zanni, M. V. 2016. Reduced ovarian reserve relates to monocyte activation and subclinical coronary atherosclerotic plaque in women with hiv. *AIDS (London, England),* 30**,** 383.

Lorenz, T. K., Worthman, C. M. & Vitzthum, V. J. 2015. Links among inflammation, sexual activity and ovulation: Evolutionary trade-offs and clinical implications. *Evolution, Medicine, and Public Health,* 2015**,** 304-324.

Lotti, F. & Maggi, M. 2018. Sexual dysfunction and male infertility. *Nature Reviews Urology,* 15**,** 287-307.

Luo, S. 2017. Assortative mating and couple similarity: Patterns, mechanisms, and consequences. *Social and Personality Psychology Compass,* 11**,** e12337.

Ma, J. X., Wang, B., Li, H. S., Jiang, X. J., Yu, J., Ding, C. F. & Chen, W. Q. 2020. Association between obesity-associated markers and semen quality parameters and serum reproductive hormones in chinese infertile men. *Reprod Biol Endocrinol,* 18**,** 95.

Ma, P., Zhang, Z., Zhou, X., Luo, J., Lu, H. & Wang, Y. 2019. Characterizing semen abnormality male infertility using non-targeted blood plasma metabolomics. *PLoS One,* 14**,** e0219179.

Macaluso, M., Wright-Schnapp, T. J., Chandra, A., Johnson, R., Satterwhite, C. L., Pulver, A., Berman, S. M., Wang, R. Y., Farr, S. L. & Pollack, L. A. 2010. A public health focus on infertility prevention, detection, and management. *Fertility and Sterility,* 93**,** 16. e1-16. e10.

Macdonald, A., Stewart, A. & Farquhar, C. 2013. Body mass index in relation to semen quality and reproductive hormones in new zealand men: A cross-sectional study in fertility clinics. *Human Reproduction,* 28**,** 3178-3187.

Macdonald-Wallis, C., Silverwood, R. J., Fraser, A., Nelson, S. M., Tilling, K.,

Lawlor, D. A., Bianca, d. L. 2015. Gestational-age-specific reference ranges for blood pressure in pregnancy findings from a prospective cohort. *Journal of Hypertension, 33*(1), 96-105

Macklon, N. S. & Brosens, J. J. 2014. The human endometrium as a sensor of embryo quality. *Biology of Reproduction,* 91**,** 98, 1-8.

Magnussen, E. B., Vatten, L. J., Lund-Nilsen, T. I., Salvesen, K. Å., Smith, G. D. & Romundstad, P. R. 2007. Prepregnancy cardiovascular risk factors as predictors of pre-eclampsia: Population based cohort study. *BMJ,* 335**,** 978.

Magnusson, M., Lewis, G. D., Ericson, U., Orho-Melander, M., Hedblad, B., Engström, G., Östling, G., Clish, C., Wang, T. J. & Gerszten, R. E. 2013. A diabetes-predictive amino acid score and future cardiovascular disease. *European Heart Journal,* 34**,** 1982-1989.

Maheshwari, A., Stofberg, L. & Bhattacharya, S. 2007. Effect of overweight and obesity on assisted reproductive technology—a systematic review. *Human Reproduction Update,* 13**,** 433-444.

Mangot-Bertrand, J., Fenollar, F., Bretelle, F., Gamerre, M., Raoult, D. & Courbiere, B. 2013. Molecular diagnosis of bacterial vaginosis: Impact on ivf outcome. *European Journal of Clinical Microbiology & Infectious Diseases,* 32**,** 535-541.

Maqdasy, S., Baptissart, M., Vega, A., Baron, S., Lobaccaro, J.-M. A. & Volle, D. H. 2013. Cholesterol and male fertility: What about orphans and adopted? *Molecular and cellular endocrinology,* 368**,** 30-46.

Marianna, S., Alessia, P., Susan, C., Francesca, C., Angela, S., Francesca, C., Antonella, N., Patrizia, I., Nicola, C. & Emilio, C. 2017. Metabolomic profiling and biochemical evaluation of the follicular fluid of endometriosis patients. *Molecular BioSystems,* 13**,** 1213-1222.

Marinelli, D., Gaspari, L., Pedotti, P. & Taioli, E. 2004. Mini-review of studies on the effect of smoking and drinking habits on semen parameters. *International Journal of Hygiene and Environmental Health,* 207**,** 185-192.

Marioni, R. E., Shah, S., Mcrae, A. F., Chen, B. H., Colicino, E., Harris, S. E., Gibson, J., Henders, A. K., Redmond, P. & Cox, S. R. 2015. DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biology,* 16**,** 1-12.

Martínez-Soto, J. C., Domingo, J. C., Cordobilla, B., Nicolás, M., Fernández, L., Albero, P., Gadea, J. & Landeras, J. 2016. Dietary supplementation with docosahexaenoic acid (dha) improves seminal antioxidant status and decreases sperm DNA fragmentation. *Systems biology in reproductive medicine,* 62**,** 387-395.

Martins Da Silva, S. J., Brown, S. G., Sutton, K., King, L. V., Ruso, H., Gray, D. W., Wyatt, P. G., Kelly, M. C., Barratt, C. L. & Hope, A. G. 2017. Drug discovery for male subfertility using high-throughput screening: A new approach to an unsolved problem. *Human Reproduction,* 32**,** 974-984.

Mascarenhas, M., Flaxman, S. & Boerma, T. 2012. National, regional, and global trends in infertility prevalence since 1990: A systematic analysis of 277 health surveys. . *PLoS Med,* 9**,** e1001356.

Mascarenhas, M. N., Cheung, H., Mathers, C. D. & Stevens, G. A. 2012. Measuring infertility in populations: Constructing a standard definition for use with demographic and reproductive health surveys. *Population Health Metrics,* 10**,** 17.

Matsuzaki, M., Mizushima, S., Hiyama, G., Hirohashi, N., Shiba, K., Inaba, K., Suzuki, T., Dohra, H., Ohnishi, T. & Sato, Y. 2015. Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. *Scientific Reports,* 5**,** 17643.

Matthews, K. A., Crawford, S. L., Chae, C. U., Everson-Rose, S. A., Sowers, M. F., Sternfeld, B. & Sutton-Tyrrell, K. 2009. Are changes in cardiovascular disease risk factors in midlife women due to chronological aging or to the menopausal transition? *Journal of the American College of Cardiology,* 54**,** 2366-2373.

Matzuk, M. M. & Lamb, D. J. 2008. The biology of infertility: Research advances and clinical challenges. *Nature Medicine,* 14**,** 1197-1213.

Mccabe, M. P., Sharlip, I. D., Lewis, R., Atalla, E., Balon, R., Fisher, A. D., Laumann, E., Lee, S. W. & Segraves, R. T. 2016. Incidence and prevalence of sexual dysfunction in women and men: A consensus statement from the fourth international consultation on sexual medicine 2015. *The Journal of Ssexual Medicine,* 13**,** 144-152.

Mclean, N., Griffin, S., Toney, K. & Hardeman, W. 2003. Family involvement in weight control, weight maintenance and weight-loss interventions: A systematic review of randomised trials. *International Journal of Obesity,*

27, 987-1005.

Mclennan, I. S. & Pankhurst, M. W. 2015. Anti-mullerian hormone is a gonadal cytokine with two circulating forms and cryptic actions. *Journal of Endocrinology,* 226, R45-R57.Mcpherson, N. O. & Tremellen, K. 2020. Increased bmi 'alone'does not negatively influence sperm function-a retrospective analysis of men attending fertility treatment with corresponding liver function results. *Obesity Research & Clinical Practice*.

Medicine, A. S. I. R. & Embryology, E. S. I. G. O. 2011. The istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting†. *Human Reproduction,* 26, 1270-1283.

Medicine, P. C. O. T. a. S. F. R. 2015. Diagnostic evaluation of the infertile male: A committee opinion. *Fertility and Sterility,* 103, e18-e25.

Mehrparvar, B., Chashmniam, S., Nobakht, F., Amini, M., Javidi, A., Minai-Tehrani, A., Arjmand, B. & Gilany, K. 2020. Metabolic profiling of seminal plasma from teratozoospermia patients. *Journal of Pharmaceutical and Biomedical Analysis,* 178, 112903.

Miettinen, H. E., Rayburn, H. & Krieger, M. 2001. Abnormal lipoprotein metabolism and reversible female infertility in hdl receptor (sr-bi)–deficient mice. *The Journal of Clinical Investigation,* 108, 1717-1722.

Mihalik, S. J., Michaliszyn, S. F., De Las Heras, J., Bacha, F., Lee, S., Chace, D. H., Dejesus, V. R., Vockley, J. & Arslanian, S. A. 2012. Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: Evidence for enhanced mitochondrial oxidation. *Diabetes Care,* 35, 605-611.

Mikkelsen, A. L. & Lindenberg, S. 2001. Morphology of in-vitro matured oocytes: Impact on fertility potential and embryo quality. *Human Reproduction,* 16, 1714-1718.

Mills, H. L., Patel, N., White, S. L., Pasupathy, D., Briley, A. L., Ferreira, D. L. S., Seed, P. T., Nelson, S. M., Sattar, N. & Tilling, K. 2019. The effect of a lifestyle intervention in obese pregnant women on gestational metabolic profiles: Findings from the uk pregnancies better eating and activity trial (upbeat) randomised controlled trial. *BMC Medicine,* 17, 15.

Mills, H. L., Patel, N., White, S. L., Pasupathy, D., Briley, A. L., Santos Ferreira, D. L., Seed, P. T., Nelson, S. M., Sattar, N., Tilling, K., Poston, L. & Lawlor, D. A. 2019. The effect of a lifestyle intervention in obese

pregnant women on gestational metabolic profiles: Findings from the uk pregnancies better eating and activity trial (upbeat) randomised controlled trial. *BMC Medicine,* 17**,** 15.

Mills, M., Rindfuss, R. R., Mcdonald, P. & Te Velde, E. 2011. Why do people postpone parenthood? Reasons and social policy incentives. *Human Reproduction Update,* 17**,** 848-860.

Mishra, P., Negi, M. P. S., Srivastava, M., Singh, K. & Rajender, S. 2018. Decline in seminal quality in indian men over the last 37 years. *Reproductive Biology and Endocrinology,* 16**,** 103.

Monseur, B., Murugappan, G., Bentley, J., Teng, N. & Westphal, L. 2020. Epigenetic clock measuring age acceleration via DNA methylation levels in blood is associated with decreased oocyte yield. *Journal of Assisted Reproduction and Genetics***,** 1-7.

Moore, D. E., Soules, M. R., Klein, N. A., Fujimoto, V. Y., Agnew, K. J. & Eschenbach, D. A. 2000. Bacteria in the transfer catheter tip influence the live-birth rate after in vitro fertilization. *Fertility and Sterility,* 74**,** 1118-1124.

Morin, S. J., Tao, X., Marin, D., Zhan, Y., Landis, J., Bedard, J., Scott Jr, R. T. & Seli, E. 2018. DNA methylation-based age prediction and telomere length in white blood cells and cumulus cells of infertile women with normal or poor response to ovarian stimulation. *Aging (Albany NY),* 10**,** 3761.

Mortimer, S. T., Van Der Horst, G. & Mortimer, D. 2015. The future of computer-aided sperm analysis. *Asian Journal of Andrology,* 17**,** 545.

Mu, F., Rich-Edwards, J., Rimm, E. B., Spiegelman, D. & Missmer, S. A. 2016. Endometriosis and risk of coronary heart disease. *Circulation: Cardiovascular Quality and Outcomes,* 9**,** 257-264.

Mukherjee S., Edwards D. R. V., Baird D. D., Savitz D. A., Hartmann K. E. (2013). Risk of Miscarriage Among Black Women and White Women in a US Prospective Cohort Study. *American Journal of Epidemiology, 177*(11), 1271–1278.

Mumcu, A., Karaer, A., Dogan, B. & Tuncay, G. 2020. Metabolomics analysis of seminal plasma in patients with idiopathic oligoasthenoteratozoospermia using high-resolution nmr spectroscopy. *Andrology,* 8**,** 450-456.

Murgia, F., Corda, V., Serrenti, M., Usai, V., Santoru, M. L., Hurt, K. J., Passaretti, M., Monni, M. C., Atzori, L. & Monni, G. 2020. Seminal fluid

metabolomic markers of oligozoospermic infertility in humans. *Metabolites,* 10**,** 64.

Mushtaq, R., Pundir, J., Achilli, C., Naji, O., Khalaf, Y. & El-Toukhy, T. 2018. Effect of male body mass index on assisted reproduction treatment outcome: An updated systematic review and meta-analysis. *Reproductive Biomedicine Online,* 36**,** 459-471.

Muthusami, K. & Chinnaswamy, P. 2005. Effect of chronic alcoholism on male fertility hormones and semen quality. *Fertility and Sterility,* 84**,** 919-924.

Nadal-Desbarats, L., Veau, S., Blasco, H., Emond, P., Royere, D., Andres, C. R. & Guérif, F. 2013. Is nmr metabolic profiling of spent embryo culture media useful to assist in vitro human embryo selection? *Magnetic Resonance Materials in Physics, Biology and Medicine,* 26**,** 193-202.

Nagoshi, C. T., Johnson, R. C. & Danko, G. P. 1990. Assortative mating for cultural identification as indicated by language use. *Behavior Genetics,* 20**,** 23-31.

Nakagawa, S. & Fitzharris, G. 2017. Intrinsically defective microtubule dynamics contribute to age-related chromosome segregation errors in mouse oocyte meiosis-i. *Current Biology,* 27**,** 1040-1047.

Nardo, L. G., Yates, A. P., Roberts, S. A., Pemberton, P. & Laing, I. 2009. The relationships between amh, androgens, insulin resistance and basal ovarian follicular status in non-obese subfertile women with and without polycystic ovary syndrome. *Human Reproduction,* 24**,** 2917-2923.

Nelson, S. M. & Lawlor, D. A. 2011. Predicting live birth, preterm delivery, and low birth weight in infants born from in vitro fertilisation: A prospective study of 144,018 treatment cycles. *PLoS Med,* 8**,** e1000386-e1000386.

Nelson, S. M., Yates, R. W., Lyall, H., Jamieson, M., Traynor, I., Gaudoin, M., Mitchell, P., Ambrose, P. & Fleming, R. 2009. Anti-müllerian hormone-based approach to controlled ovarian stimulation for assisted conception. *Human Reproduction,* 24**,** 867-875.

Nel-Themaat, L. & Nagy, Z. P. 2011. A review of the promises and pitfalls of oocyte and embryo metabolomics. *Placenta,* 32**,** S257-S263.

Neto, F. T. L., Marques, R. A., De Freitas Cavalcanti Filho, A., Araujo, L. C. N., Lima, S. V. C., Pinto, L. & Silva, R. O. 2020. 1h nmr-based metabonomics for infertility diagnosis in men with varicocele. *Journal of Assisted Reproduction and Genetics,* 37**,** 2233-2247.

Niu, Y.-C., Feng, R.-N., Hou, Y., Li, K., Kang, Z., Wang, J., Sun, C.-H. & Li, Y. 2012. Histidine and arginine are associated with inflammation and oxidative stress in obese women. *British Journal of Nutrition,* 108**,** 57-61.

Nordqvist, S., Kårehed, K., Hambiliki, F., Wånggren, K., Stavreus-Evers, A. & Åkerud, H. 2010. The presence of histidine-rich glycoprotein in the female reproductive tract and in embryos. *Reproductive Sciences,* 17**,** 941-947.

Nordqvist, S., Kårehed, K., Stavreus-Evers, A. & Åkerud, H. 2011. Histidine-rich glycoprotein polymorphism and pregnancy outcome: A pilot study. *Reproductive Biomedicine Online,* 23**,** 213-219.

Okoth, K., Chandan, J. S., Marshall, T., Thangaratinam, S., Thomas, G. N., Nirantharakumar, K. & Adderley, N. J. 2020. Association between the reproductive health of young women and cardiovascular disease in later life: Umbrella review. *BMJ,* 371**,** m3502.

Ombelet, W., Bosmans, E., Janssen, M., Cox, A., Vlasselaer, J., Gyselaers, W., Vandeput, H., Gielen, J., Pollet, H. & Maes, M. 1997. Semen parameters in a fertile versus subfertile population: A need for change in the interpretation of semen testing. *Human Reproduction,* 12**,** 987-993.

Ombelet, W., Cooke, I., Dyer, S., Serour, G. & Devroey, P. 2008. Infertility and the provision of infertility medical services in developing countries. *Human Reproduction Update,* 14**,** 605-621.

Ombelet, W., De Sutter, P., Van Der Elst, J. & Martens, G. 2005. Multiple gestation and infertility treatment: Registration, reflection and reaction—the belgian project. *Human Reproduction Update,* 11**,** 3-14.

Ombelet, W. & Onofre, J. 2019. Ivf in africa: What is it all about? *Facts, views & vision in ObGyn,* 11**,** 65-76.

Onyiaodike, C. C., Murray, H., Zhang, R., Meyer, B., Jordan, F., Brown, E. A., Nibbs, R. J. B., Lyall, H., Sattar, N., Nelson, S. & Freeman, D. 2018. Pre-conception maternal erythrocyte saturated to unsaturated fatty acid ratio predicts pregnancy after natural cycle frozen embryo transfer. *Scientific Reports,* 8.

Onyiaodike, C. C., Murray, H. M., Zhang, R., Meyer, B. J., Jordan, F., Brown, E. A., Nibbs, R. J., Lyall, H., Sattar, N. & Nelson, S. M. 2018. Pre-conception maternal erythrocyte saturated to unsaturated fatty acid ratio predicts pregnancy after natural cycle frozen embryo transfer. *Scientific Reports,* 8**,** 1-8.

Onyiaodike, C. C., Murray, H. M., Zhang, R., Meyer, B. J., Jordan, F., Brown, E. A., Nibbs, R. J. B., Lyall, H., Sattar, N., Nelson, S. M. & Freeman, D. J. 2018. Pre-conception maternal erythrocyte saturated to unsaturated fatty acid ratio predicts pregnancy after natural cycle frozen embryo transfer. *Scientific Reports,* 8**,** 1216.

Ossewaarde, M. E., Bots, M. L., Verbeek, A. L., Peeters, P. H., Van Der Graaf, Y., Grobbee, D. E. & Van Der Schouw, Y. T. 2005. Age at menopause, cause-specific mortality and total life expectancy. *Epidemiology***,** 556-562.

Otsuki, S., Saito, E., Sawada, N., Abe, S. K., Hidaka, A., Yamaji, T., Shimazu, T., Goto, A., Iwasaki, M. & Iso, H. 2018. Female reproductive factors and risk of all-cause and cause-specific mortality among women: The japan public health center–based prospective study (jphc study). *Annals of epidemiology,* 28**,** 597-604. e6.

Oud, M. S., Volozonoka, L., Smits, R. M., Vissers, L. E. L. M., Ramos, L. & Veltman, J. A. 2019. A systematic review and standardized clinical validity assessment of male infertility genes. *Human Reproduction,* 34**,** 932-941.

Pacey, A., Povey, A., Clyma, J.-A., Mcnamee, R., Moore, H., Baillie, H., Cherry, N. & Chaps-Uk, P. C. O. 2014. Modifiable and non-modifiable risk factors for poor sperm morphology. *Human Reproduction,* 29**,** 1629-1636.

Paiva, C., Amaral, A., Rodriguez, M., Canyellas, N., Correig, X., Ballesca, J., Ramalho-Santos, J. & Oliva, R. 2015. Identification of endogenous metabolites in human sperm cells using proton nuclear magnetic resonance (1h-nmr) spectroscopy and gas chromatography-mass spectrometry (gc-ms). *Andrology,* 3**,** 496-505.

Pak, V. M., Powers, M. & Liu, J. 2013. Occupational chemical exposures among cosmetologists: Risk of reproductive disorders. *Workplace health & safety,* 61**,** 522-528.

Pal, L., Bevilacqua, K., Zeitlian, G., Shu, J. & Santoro, N. 2008. Implications of diminished ovarian reserve (dor) extend well beyond reproductive concerns. *Menopause,* 15**,** 1086-1094.

Parikh, N. I., Cnattingius, S., Mittleman, M. A., Ludvigsson, J. F. & Ingelsson, E. 2012. Subfertility and risk of later life maternal cardiovascular disease. *Human Reproduction,* 27**,** 568-575.

Park, H. T., Cho, G. J., Ahn, K. H., Shin, J. H., Kim, Y. T., Hur, J. Y., Kim, S. H.,

Lee, K. W. & Kim, T. 2010. Association of insulin resistance with anti-mullerian hormone levels in women without polycystic ovary syndrome (pcos). *Clinical Endocrinology,* 72**,** 26-31.

Park, K., Wei, J., Minissian, M., Merz, C. N. B. & Pepine, C. J. 2015. Adverse pregnancy conditions, infertility, and future cardiovascular risk: Implications for mother and child. *Cardiovascular Drugs and Therapy,* 29**,** 391-401.

Patel, A. S., Leong, J. Y. & Ramasamy, R. 2018. Prediction of male infertility by the world health organization laboratory manual for assessment of semen analysis: A systematic review. *Arab Journal of Urology,* 16**,** 96-102.

Payne, A. H. & Youngblood, G. L. 1995. Regulation of expression of steroidogenic enzymes in leydig cells. *Biology of Reproduction,* 52**,** 217-225.

Pearce, N. & Lawlor, D. A. 2016. Causal inference—so much more than statistics. *International Journal of Epidemiology,* 45**,** 1895-1903.

Pell, J. P., Smith, G. C. & Walsh, D. 2004. Pregnancy complications and subsequent maternal cerebrovascular events: A retrospective cohort study of 119,668 births. *American Journal of Epidemiology,* 159**,** 336-342.

Pellatt, L., Hanna, L., Brincat, M., Galea, R., Brain, H., Whitehead, S. & Mason, H. 2007. Granulosa cell production of anti-mullerian hormone is increased in polycystic ovaries. *The Journal of Clinical Endocrinology & Metabolism,* 92**,** 240-245.

Perna, L., Zhang, Y., Mons, U., Holleczek, B., Saum, K.-U. & Brenner, H. 2016. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a german case cohort. *Clinical Epigenetics,* 8**,** 64.

Pérusse, L., Tremblay, A., Leblanc, C. & Bouchard, C. 1989. Genetic and environmental influences on level of habitual physical activity and exercise participation. *American Journal of Epidemiology,* 129**,** 1012-1022.

Petersen, G. L., Schmidt, L., Pinborg, A. & Kamper-Jørgensen, M. 2013. The influence of female and male body mass index on live births after assisted reproductive technology treatment: A nationwide register-based cohort study. *Fertility and Sterility,* 99**,** 1654-1662.

Pfeifer, S., Butts, S., Fossum, G., Gracia, C., La Barbera, A., Mersereau, J., Odem, R., Paulson, R., Penzias, A. & Pisarska, M. 2017. Optimizing natural fertility: A committee opinion. *Fertility and Sterility,* 107**,** 52-58.

Pigny, P., Merlen, E., Robert, Y., Cortet-Rudelli, C., Decanter, C., Jonard, S. & Dewailly, D. 2003. Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: Relationship to the ovarian follicle excess and to the follicular arrest. *The Journal of Clinical Endocrinology & Metabolism,* 88**,** 5957-5962.

Piñero-Sagredo, E., Nunes, S., De Los Santos, M. J., Celda, B. & Esteve, V. 2010. Nmr metabolic profile of human follicular fluid. *NMR in Biomedicine,* 23**,** 485-495.

Polani, P. E. & Crolla, J. A. 1991. A test of the production line hypothesis of mammalian oogenesis. *Human Genetics,* 88**,** 64-70.

Polotsky, A. J., Allshouse, A. A., Casson, P. R., Coutifaris, C., Diamond, M. P., Christman, G. M., Schlaff, W. D., Alvero, R., Trussell, J. & Krawetz, S. A. 2015. Impact of male and female weight, smoking, and intercourse frequency on live birth in women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism,* 100**,** 2405-2412.

Poon I. K. H., Patel K. K., Davis D. S., Parish C. R., Hulett M. D. (2011). Histidine-rich glycoprotein: the Swiss Army knife of mammalian plasma. *Blood, 117*(7).

Potabattula, R., Dittrich, M., Schorsch, M., Hahn, T., Haaf, T. & El Hajj, N. 2019. Male obesity effects on sperm and next-generation cord blood DNA methylation. *PLoS One,* 14**,** e0218615.

Povey, A., Clyma, J.-A., Mcnamee, R., Moore, H., Baillie, H., Pacey, A., Cherry, N. & Chaps-Uk, P. C. O. 2012. Modifiable and non-modifiable risk factors for poor semen quality: A case-referent study. *Human Reproduction,* 27**,** 2799-2806.

Prentki Santos, E., López-Costa, S., Chenlo, P., Pugliese, M. N., Curi, S., Ariagno, J., Repetto, H., Sardi, M., Palaoro, L. & Mendeluk, G. 2011. Impact of spontaneous smoking cessation on sperm quality: Case report. *Andrologia,* 43**,** 431-435.

Price, R. A. & Vandenberg, S. G. 1980. Spouse similarity in american and swedish couples. *Behavior Genetics,* 10**,** 59-71.

Pudakalakatti, S. M., Uppangala, S., D'souza, F., Kalthur, G., Kumar, P., Adiga, S. K. & Atreya, H. S. 2013. Nmr studies of preimplantation embryo metabolism in human assisted reproductive techniques: A new biomarker for assessment of embryo implantation potential. *NMR in Biomedicine,* 26**,**

20-27.

Pugh, S. J., Schisterman, E. F., Browne, R. W., Lynch, A. M., Mumford, S. L., Perkins, N. J., Silver, R., Sjaarda, L., Stanford, J. B. & Wactawski-Wende, J. 2017. Preconception maternal lipoprotein levels in relation to fecundability. *Human Reproduction,* 32**,** 1055-1063.

Pugh, S. J., Schisterman, E. F., Browne, R. W., Lynch, A. M., Mumford, S. L., Perkins, N. J., Silver, R., Sjaarda, L., Stanford, J. B., Wactawski-Wende, J., Wilcox, B. & Grantz, K. L. 2017. Preconception maternal lipoprotein levels in relation to fecundability. *Human Reproduction,* 32**,** 1055-1063.

Qiao, S., Wu, W., Chen, M., Tang, Q., Xia, Y., Jia, W. & Wang, X. 2017. Seminal plasma metabolomics approach for the diagnosis of unexplained male infertility. *PLoS One,* 12**,** e0181115.

Ramezani Tehrani, F., Montazeri, S., Khalili, D., Cheraghi, L., Broekmans, F., Momenan, A., De Kat, A. & Azizi, F. 2016. Age-specific anti-müllerian hormone and electrocardiographic silent coronary artery disease. *Climacteric,* 19**,** 344-348.

Rankin, N. J., Preiss, D., Welsh, P., Burgess, K. E., Nelson, S. M., Lawlor, D. A. & Sattar, N. 2014. The emergence of proton nuclear magnetic resonance metabolomics in the cardiovascular arena as viewed from a clinical perspective. *Atherosclerosis,* 237**,** 287-300.

Ranthe, M. F., Andersen, E. a. W., Wohlfahrt, J., Bundgaard, H., Melbye, M. & Boyd, H. A. 2013. Pregnancy loss and later risk of atherosclerotic disease. *Circulation,* 127**,** 1775-1782.

Rastrelli, G., Lotti, F., Reisman, Y., Sforza, A., Maggi, M. & Corona, G. 2019. Metabolically healthy and unhealthy obesity in erectile dysfunction and male infertility. *Expert review of endocrinology & metabolism,* 14**,** 321-334.

Ravanos, K., Petousis, S., Margioula-Siarkou, C., Papatheodorou, A., Panagiotidis, Y., Prapas, N. & Prapas, Y. 2018. Declining sperm counts… or rather not? A mini review. *Obstetrical & gynecological survey,* 73**,** 595-605.

Ravel, C., Berthaut, I., Bresson, J. L., Siffroi, J. P. & Cecos, A. T. G. C. O. T. F. F. O. 2006. Prevalence of chromosomal abnormalities in phenotypically normal and fertile adult males: Large-scale survey of over 10 000 sperm donor karyotypes. *Human Reproduction,* 21**,** 1484-1489.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., Mcculle, S. L., Karlebach, S., Gorle, R., Russell, J. & Tacket, C. O. 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences,* 108**,** 4680-4687.

Ray, A., Shah, A., Gudi, A. & Homburg, R. 2012. Unexplained infertility: An update and review of practice. *Reproductive Biomedicine Online,* 24**,** 591-602.

Reynolds, C. A., Barlow, T. & Pedersen, N. L. 2006. Alcohol, tobacco and caffeine use: Spouse similarity processes. *Behavior Genetics,* 36**,** 201.

Reynolds, S., Calvert, S., Paley, M. & Pacey, A. 2017. 1h magnetic resonance spectroscopy of live human sperm. *MHR: Basic science of reproductive medicine,* 23**,** 441-451.

Ricci, E., Al Beitawi, S., Cipriani, S., Candiani, M., Chiaffarino, F., Viganò, P., Noli, S. & Parazzini, F. 2017. Semen quality and alcohol intake: A systematic review and meta-analysis. *Reproductive BioMedicine Online,* 34**,** 38-47.

Riddell, D., Pacey, A. & Whittington, K. 2005. Lack of compliance by uk andrology laboratories with world health organization recommendations for sperm morphology assessment. *Human Reproduction,* 20**,** 3441-3445.

Ring, J. D., Lwin, A. A. & Köhler, T. S. 2016. Current medical management of endocrine-related male infertility. *Asian Journal of Andrology,* 18**,** 357.

Rocca, W. A., Gazzuola Rocca, L., Smith, C. Y., Grossardt, B. R., Faubion, S. S., Shuster, L. T., Kirkland, J. L., Lebrasseur, N. K., Schafer, M. J. & Mielke, M. M. 2018. Loss of ovarian hormones and accelerated somatic and mental aging. *Physiology,* 33**,** 374-383.

Rosero-Bixby, L., Castro-Martín, T. & Martín-García, T. 2009. Is latin america starting to retreat from early and universal childbearing? *Demographic Research,* 20**,** 169-194.

Ruiz-Canela, M., Toledo, E., Clish, C. B., Hruby, A., Liang, L., Salas-Salvadó, J., Razquin, C., Corella, D., Estruch, R. & Ros, E. 2016. Plasma branched-chain amino acids and incident cardiovascular disease in the predimed trial. *Clinical Chemistry,* 62**,** 582-592.

Rydengård V., Shannon O., Lundqvist K., Kacprzyk L., Chalupka A., Olsson A. K., Mörgelin M., Jahnen-Dechent W., Malmsten M., Schmidtchen A. (2088). Histidine-Rich Glycoprotein Protects from Systemic Candida Infection.

PLOS Pathogens, 4, 8.

Safarinejad, M. 2011. Effect of omega-3 polyunsaturated fatty acid supplementation on semen profile and enzymatic anti-oxidant capacity of seminal plasma in infertile men with idiopathic oligoasthenoteratospermia: A double-blind, placebo-controlled, randomised study. *Andrologia,* 43**,** 38-47.

Sakkas, D., Ramalingam, M., Garrido, N. & Barratt, C. L. 2015. Sperm selection in natural conception: What can we learn from mother nature to improve assisted reproduction outcomes? *Human Reproduction Update,* 21**,** 711-726.

Salas-Huetos A., Maghsoumi-Norouzabad L., James E. R., Carrell D. T., Aston K. I., Jenkins T. G., Becerra-Tomás N., Javid A. Z., Abed R., Torres P. J., Luque E. M., RamírezN. D., Martini A. C., Salas-Salvadó J. (2020). Male adiposity, sperm parameters and reproductive hormones:An updated systematic review and collaborative meta-analysis. *Obesity Reviews*.

Salonia, A., Bettocchi, C., Carvalho, J., Corona, G., Jones, T. & Kadioglu, A. 2020. Eau guidelines on sexual and reproductive health. *European Association of Urology Guidelines*.

Salonia, A., Matloob, R., Gallina, A., Abdollah, F., Sacca, A., Briganti, A., Suardi, N., Colombo, R., Rocchini, L. & Guazzoni, G. 2009. Are infertile men less healthy than fertile men? Results of a prospective case-control survey. *European Urology,* 56**,** 1025-1032.

Sánchez-Andrés, A. & Mesa, M. S. 1994. Assortative mating in a spanish population: Effects of social factors and cohabitation time. *J Biosoc Sci,* 26**,** 441-50.

Sánchez-Ribas, I., Riqueros, M., Vime, P., Puchades-Carrasco, L., Jönsson, T., Pineda-Lucena, A., Ballesteros, A., Domínguez, F. & Simón, C. 2012. Differential metabolic profiling of non–pure trisomy 21 human preimplantation embryos. *Fertility and Sterility,* 98**,** 1157-1164. e2.

Sattar, N. & Greer, I. A. 2002. Pregnancy complications and maternal cardiovascular risk: Opportunities for intervention and screening? *BMJ,* 325**,** 157-60.

Savoca, M. & Miller, C. 2001. Food selection and eating patterns: Themes found among people with type 2 diabetes mellitus. *Journal of Nutrition Education,* 33**,** 224-233.

Scarr, S. & Mccartney, K. 1983. How people make their own environments: A theory of genotype→ environment effects. *Child development*, 424-435.

Schagdarsurengin, U. & Steger, K. 2016. Epigenetics in male reproduction: Effect of paternal diet on sperm quality and offspring health. *Nature Reviews Urology,* 13, 584.

Schisterman, E. F., Mumford, S. L., Browne, R. W., Barr, D. B., Chen, Z. & Louis, G. M. B. 2014. Lipid concentrations and couple fecundity: The life study. *The Journal of Clinical Endocrinology & Metabolism,* 99, 2786-2794.

Schisterman, E. F., Mumford, S. L., Chen, Z., Browne, R. W., Boyd Barr, D., Kim, S. & Buck Louis, G. 2014. Lipid concentrations and semen quality: The life study. *Andrology,* 2, 408-415.

Schisterman, E. F., Sjaarda, L. A., Clemons, T., Carrell, D. T., Perkins, N. J., Johnstone, E., Lamb, D., Chaney, K., Van Voorhis, B. J. & Ryan, G. 2020. Effect of folic acid and zinc supplementation in men on semen quality and live birth among couples undergoing infertility treatment: A randomized clinical trial. *JAMA,* 323, 35-48.

Schlafke, S. & Enders, A. C. 1975. Cellular basis of interaction between trophoblast and uterus at implantation. *Biology of Reproduction,* 12, 41-65.

Schlegel, P. N. 1999. Testicular sperm extraction: Microdissection improves sperm yield with minimal tissue excision. *Human Reproduction,* 14, 131-135.

Schneider, M. G. & Forthofer, M. S. 2005. Associations of psychosocial factors with the stress of infertility treatment. *Health & social work,* 30, 183-191.

Schwartz, C. & Graf, N. 2009. Assortative matching among same-sex and different-sex couples in the united states, 1990-2000. *Demographic Research,* 21, 843-878.

Schwartz, C. R. & Graf, N. L. 2009. Assortative matching among same-sex and different-sex couples in the united states, 1990–2000. *Demographic research,* 21, 843.

Sebro, R., Peloso, G. M., Dupuis, J. & Risch, N. J. 2017. Structured mating: Patterns and implications. *PLoS Genetics,* 13, e1006655.

Sehl, M. E., Henry, J. E., Storniolo, A. M., Ganz, P. A. & Horvath, S. 2017. DNA methylation age is elevated in breast tissue of healthy women. *Breast Cancer Research and Treatment,* 164, 209-219.

Seli, E., Botros, L., Sakkas, D. & Burns, D. H. 2008. Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertility and Sterility,* 90**,** 2183-2189.

Sermondade, N., Faure, C., Fezeu, L., Shayeb, A., Bonde, J. P., Jensen, T. K., Van Wely, M., Cao, J., Martini, A. C. & Eskandar, M. 2013. Bmi in relation to sperm count: An updated systematic review and collaborative meta-analysis. *Human Reproduction Update,* 19**,** 221-231.

Sermondade, N., Faure, C., Fezeu, L., Shayeb, A. G., Bonde, J. P., Jensen, T. K., Van Wely, M., Cao, J., Martini, A. C., Eskandar, M., Chavarro, J. E., Koloszar, S., Twigt, J. M., Ramlau-Hansen, C. H., Borges, E., Jr., Lotti, F., Steegers-Theunissen, R. P., Zorn, B., Polotsky, A. J., La Vignera, S., Eskenazi, B., Tremellen, K., Magnusdottir, E. V., Fejes, I., Hercberg, S., Lévy, R. & Czernichow, S. 2013. Bmi in relation to sperm count: An updated systematic review and collaborative meta-analysis. *Human Reproduction Update,* 19**,** 221-31.

Sermondade, N., Huberlant, S., Bourhis-Lefebvre, V., Arbo, E., Gallot, V., Colombani, M. & Fréour, T. 2019. Female obesity is negatively associated with live birth rate following ivf: A systematic review and meta-analysis. *Human Reproduction Update,* 25**,** 439-451.

Shah, S. H., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Crosslin, D. R., Haynes, C., Dungan, J., Newby, L. K., Hauser, E. R. & Ginsburg, G. S. 2010. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. *Circulation: Cardiovascular Genetics,* 3**,** 207-214.

Shannon O., Rydengård V., Schmidtchen A., Mörgelin M., Alm P., Sørensen O. E., Björck L. (2010). Histidine-rich glycoprotein promotes bacterial entrapment in clots and decreases mortality in a mouse model of sepsis. *Blood, 116*(13), 2365-2372.

Sharma, R., Harlev, A., Agarwal, A. & Esteves, S. C. 2016. Cigarette smoking and semen quality: A new meta-analysis examining the effect of the 2010 world health organization laboratory methods for the examination of human semen. *European Urology,* 70**,** 635-645.

Shaw, J. L., Dey, S. K., Critchley, H. O. & Horne, A. W. 2010. Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction*

Update, 16**,** 432-44.

Showell, M. G., Mackenzie-Proctor, R., Jordan, V. & Hart, R. J. 2017. Antioxidants for female subfertility. *Cochrane Database of Systematic Reviews,* 7**,** Cd007807.

Shuster, L. T., Rhodes, D. J., Gostout, B. S., Grossardt, B. R. & Rocca, W. A. 2010. Premature menopause or early menopause: Long-term health consequences. *Maturitas,* 65**,** 161-166.

Silventoinen, K., Kaprio, J., Lahelma, E., Viken, R. J. & Rose, R. J. 2003. Assortative mating by body height and bmi: Finnish twins and their spouses. *American Journal of Human Biology,* 15**,** 620-627.

Simpkin, A. J., Howe, L. D., Tilling, K., Gaunt, T. R., Lyttleton, O., Mcardle, W. L., Ring, S. M., Horvath, S., Smith, G. D. & Relton, C. L. 2017. The epigenetic clock and physical development during childhood and adolescence: Longitudinal analysis from a uk birth cohort. *International Journal of Epidemiology,* 46**,** 549-558.

Sirota, M., Willemsen, G., Sundar, P., Pitts, S. J., Potluri, S., Prifti, E., Kennedy, S., Ehrlich, S. D., Neuteboom, J. & Kluft, C. 2015. Effect of genome and environment on metabolic and inflammatory profiles. *PLoS One,* 10**,** e0120898.

Skalba, P., Cygal, A., Madej, P., Dabkowska-Huc, A. & Sikora, J. 2011. Is the plasma anti-müllerian hormone (amh) level associated with body weight and metabolic, and hormonal disturbances in women with and without polycystic ovary syndrome? *European Journal of Obstetrics, Gynecology, and Reproductive Biology,* 158**,** 254-259.

Smits, R. M., Mackenzie-Proctor, R., Yazdani, A., Stankiewicz, M. T., Jordan, V. & Showell, M. G. 2019. Antioxidants for male subfertility. *Cochrane Database of Systematic Reviews,* 3**,** Cd007411.

Smits, R. M., Mackenzie-Proctor, R., Yazdani, A., Stankiewicz, M. T., Jordan, V. & Showell, M. G. 2019. Antioxidants for male subfertility. *Cochrane Database of Systematic Reviews*.

Snowdon, D. A., Kane, R. L., Beeson, W. L., Burke, G. L., Sprafka, J. M., Potter, J., Iso, H., Jacobs Jr, D. R. & Phillips, R. L. 1989. Is early natural menopause a biologic marker of health and aging? *American Journal of Public Health,* 79**,** 709-714.

Soininen, P., Kangas, A. J., Wurtz, P., Tukiainen, T., Tynkkynen, T.,

Laatikainen, R., Jarvelin, M. R., Kahonen, M., Lehtimaki, T., Viikari, J., Raitakari, O. T., Savolainen, M. J. & Ala-Korpela, M. 2009. High-throughput serum nmr metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst,* 134**,** 1781-5.

Soininen, P., Kangas, A. J., Würtz, P., Tukiainen, T., Tynkkynen, T., Laatikainen, R., Järvelin, M.-R., Kähönen, M., Lehtimäki, T. & Viikari, J. 2009. High-throughput serum nmr metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst,* 134**,** 1781-1785.

Soubry, A., Guo, L., Huang, Z., Hoyo, C., Romanus, S., Price, T. & Murphy, S. K. 2016. Obesity-related DNA methylation at imprinted genes in human sperm: Results from the tieger study. *Clinical Epigenetics,* 8**,** 51.

Soubry, A., Schildkraut, J. M., Murtha, A., Wang, F., Huang, Z., Bernal, A., Kurtzberg, J., Jirtle, R. L., Murphy, S. K. & Hoyo, C. 2013. Paternal obesity is associated with igf2hypomethylation in newborns: Results from a newborn epigenetics study (nest) cohort. *BMC Medicine,* 11**,** 29.

Sovio, U., Goulding, N., Mcbride, N., Cook, E., Gaccioli, F., Charnock-Jones, D. S., Lawlor, D. A. & Smith, G. C. 2020. A maternal serum metabolite ratio predicts fetal growth restriction at term. *Nature Medicine,* 26**,** 348-353.

Sovio, U., Mcbride, N., Wood, A. M., Masconi, K. L., Cook, E., Gaccioli, F., Charnock-Jones, D. S., Lawlor, D. A. & Smith, G. C. 2020. 4-hydroxyglutamate is a novel predictor of pre-eclampsia. *International Journal of Epidemiology,* 49**,** 301-311.

Stulp, G., Simons, M. J., Grasman, S. & Pollet, T. V. 2017. Assortative mating for human height: A meta-analysis. *Am J Hum Biol,* 29.

Sun, H., Gong, T.-T., Jiang, Y.-T., Zhang, S., Zhao, Y.-H. & Wu, Q.-J. 2019. Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: Results from a global burden of disease study, 2017. *Aging (Albany NY),* 11**,** 10952.Sunderam, S., Kissin, D. M., Crawford, S. B., Folger, S. G., Boulet, S. L., Warner, L. & Barfield, W. D. 2018. Assisted reproductive technology surveillance—united states, 2015. *MMWR Surveillance Summaries,* 67**,** 1.

Swan, S. H., Elkin, E. P. & Fenster, L. 2000. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. *Environmental Health Perspectives,* 108**,** 961-966.

Tambs, K. & Moum, T. 1992. No large convergence during marriage for health,

lifestyle, and personality in a large sample of norwegian spouses. *Journal of Marriage and the Family*, 957-971.

Taylor, K., L Santos Ferreira, D., West, J., Yang, T., Caputo, M. & A Lawlor, D. 2019. Differences in pregnancy metabolic profiles and their determinants between white european and south asian women: Findings from the born in bradford cohort. *Metabolites,* 9, 190.

Te Velde, E. R. & Pearson, P. L. 2002. The variability of female reproductive ageing. *Human Reproduction Update,* 8, 141-154.

Teede, H. J., Misso, M. L., Costello, M. F., Dokras, A., Laven, J., Moran, L., Piltonen, T. & Norman, R. J. 2018. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. *Human Reproduction,* 33, 1602-1618.

Tehrani, F. R., Erfani, H., Cheraghi, L., Tohidi, M. & Azizi, F. 2014. Lipid profiles and ovarian reserve status: A longitudinal study. *Human Reproduction,* 29, 2522-2529.

Thirumavalavan, N., Gabrielsen, J. S. & Lamb, D. J. 2019. Where are we going with gene screening for male infertility? *Fertility and Sterility,* 111, 842-850.

Thonneau, P., Marchand, S., Tallec, A., Ferial, M.-L., Ducot, B., Lansac, J., Lopes, P., Tabaste, J.-M. & Spira, A. 1991. Incidence and main causes of infertility in a resident population (1 850 000) of three french regions (1988–1989). *Human Reproduction,* 6, 811-816.

Thouas, G. A., Dominguez, F., Green, M. P., Vilella, F., Simon, C. & Gardner, D. K. 2015. Soluble ligands and their receptors in human embryo development and implantation. *Endocrine Reviews,* 36, 92-130.

Tiegs, A. W., Landis, J., Garrido, N., Scott Jr, R. T. & Hotaling, J. M. 2019. Total motile sperm count trend over time: Evaluation of semen analyses from 119,972 men from subfertile couples. *Urology,* 132, 109-116.

Tobias, D. K., Mora, S., Verma, S. & Lawler, P. R. 2018. Altered branched chain amino acid metabolism: Towards a unifying cardiometabolic hypothesis. *Current Opinion in Cardiology,* 33, 558.

Tremblay, B. L., Guénard, F., Lamarche, B., Pérusse, L. & Vohl, M.-C. 2019. Familial resemblances in human plasma metabolites are attributable to both genetic and common environmental effects. *Nutrition Research,* 61,

22-30.

Tsuchida-Straeten N., Ensslen S., Schäfer C., Wöltje M., Denecke B., Moser M., Gräber S., Wakabayashi S., Koide T., Jahnen-Dechent W. (2005). Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG). *j Thromb Haemost, 3*(5), 865-872.

Unuane, D., Tournaye, H., Velkeniers, B. & Poppe, K. 2011. Endocrine disorders & female infertility. *Best Practice & Research Clinical Endocrinology & Metabolism,* 25**,** 861-873.

Van Der Linden, M., Buckingham, K., Farquhar, C., Kremer, J. A. & Metwally, M. 2011. Luteal phase support for assisted reproduction cycles. *Cochrane Database of Systematic Reviews*.

Van Echten-Arends, J., Mastenbroek, S., Sikkema-Raddatz, B., Korevaar, J. C., Heineman, M. J., Van Der Veen, F. & Repping, S. 2011. Chromosomal mosaicism in human preimplantation embryos: A systematic review. *Human Reproduction Update,* 17**,** 620-627.

Van Hoeck, V., Sturmey, R. G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H. J., Bols, P. E. & Leroy, J. L. 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLoS One,* 6**,** e23183.

Vassalle, C. 2008. An easy and reliable automated method to estimate oxidative stress in the clinical setting. *Advanced protocols in oxidative stress i.* Springer.

Ventimiglia, E., Capogrosso, P., Boeri, L., Ippolito, S., Scano, R., Moschini, M., Gandaglia, G., Papaleo, E., Montorsi, F. & Salonia, A. 2016. Validation of the american society for reproductive medicine guidelines/recommendations in white european men presenting for couple's infertility. *Fertility and Sterility,* 106**,** 1076-1082. e1.

Vergaro, P., Tiscornia, G., Barragán, M., García, D., Rodriguez, A., Santaló, J. & Vassena, R. 2019. Vaginal microbiota profile at the time of embryo transfer does not affect live birth rate in ivf cycles with donated oocytes. *Reproductive Biomedicine Online,* 38**,** 883-891.

Verit, F. F., Keskin, S., Omer, B., Yalcinkaya, S. & Sakar, N. 2014. Is there any relationship between cardiovascular risk markers and young women with diminished ovarian reserve? *Gynecological Endocrinology,* 30**,** 697-700.Wang, J. Y., Liu, C. S., Lung, C. H., Yang, Y. T. & Lin, M. H. 2017.

Investigating spousal concordance of diabetes through statistical analysis and data mining. *PLoS One,* 12**,** e0183413.

Wang, J.-Y., Liu, C.-S., Lung, C.-H., Yang, Y.-T. & Lin, M.-H. 2017. Investigating spousal concordance of diabetes through statistical analysis and data mining. *PLoS One,* 12**,** e0183413.

Wang, Q., Ferreira, D. L. S., Nelson, S. M., Sattar, N., Ala-Korpela, M. & Lawlor, D. A. 2018. Metabolic characterization of menopause: Cross-sectional and longitudinal evidence. *BMC Medicine,* 16**,** 1-12.

Wang, Q., Wurtz, P., Auro, K., Makinen, V. P., Kangas, A. J., Soininen, P., Tiainen, M., Tynkkynen, T., Jokelainen, J., Santalahti, K., Salmi, M., Blankenberg, S., Zeller, T., Viikari, J., Kahonen, M., Lehtimaki, T., Salomaa, V., Perola, M., Jalkanen, S., Jarvelin, M. R., Raitakari, O. T., Kettunen, J., Lawlor, D. A. & Ala-Korpela, M. 2016. Metabolic profiling of pregnancy: Cross-sectional and longitudinal evidence. *BMC Medicine,* 14**,** 205.

Wang, Q., Würtz, P., Auro, K., Morin-Papunen, L., Kangas, A. J., Soininen, P., Tiainen, M., Tynkkynen, T., Joensuu, A. & Havulinna, A. S. 2016. Effects of hormonal contraception on systemic metabolism: Cross-sectional and longitudinal evidence. *International Journal of Epidemiology,* 45**,** 1445-1457.

Watanabe, M., Suliman, M. E., Qureshi, A. R., Garcia-Lopez, E., Bárány, P., Heimbürger, O., Stenvinkel, P. & Lindholm, B. 2008. Consequences of low plasma histidine in chronic kidney disease patients: Associations with inflammation, oxidative stress, and mortality. *The American journal of clinical nutrition,* 87**,** 1860-1866.

Weidner, C. I., Lin, Q., Koch, C. M., Eisele, L., Beier, F., Ziegler, P., Bauerschlag, D. O., Jöckel, K.-H., Erbel, R. & Mühleisen, T. W. 2014. Aging of blood can be tracked by DNA methylation changes at just three cpg sites. *Genome Biology,* 15**,** R24.

White, S. L., Begum, S., Vieira, M. C., Seed, P., Lawlor, D. L., Sattar, N., Nelson, S. M., Welsh, P., Pasupathy, D. & Poston, L. 2020. Metabolic phenotyping by treatment modality in obese women with gestational diabetes suggests diverse pathophysiology: An exploratory study. *PLoS One,* 15**,** e0230658.

White, S. L., Pasupathy, D., Sattar, N., Nelson, S. M., Lawlor, D. A., Briley, A.

L., Seed, P. T., Welsh, P. & Poston, L. 2017. Metabolic profiling of gestational diabetes in obese women during pregnancy. *Diabetologia,* 60**,** 1903-1912.

Who. 2010. *Who laboratory manual for the examination and processing of human semen* [Online]. Available: https://www.who.int/publications/i/item/9789241547789 [Accessed November 15 2020].

Wikipedia. 2018. *List of scottish council areas by population* [Online]. Available: https://en.wikipedia.org/wiki/List_of_Scottish_council_areas_by_population [Accessed November 15 2020].

Willemsen, G., Vink, J. M. & Boomsma, D. I. 2003. Assortative mating may explain spouses' risk of same disease. *BMJ,* 326**,** 396.

Williamson, E. J., Aitken, Z., Lawrie, J., Dharmage, S. C., Burgess, J. A. & Forbes, A. B. 2014. Introduction to causal diagrams for confounder selection. *Respirology,* 19**,** 303-311.

Wu, P., Haththotuwa, R., Kwok, C. S., Babu, A., Kotronias, R. A., Rushton, C., Zaman, A., Fryer, A. A., Kadam, U. & Chew-Graham, C. A. 2017. Preeclampsia and future cardiovascular health: A systematic review and meta-analysis. *Circulation: Cardiovascular Quality and Outcomes,* 10**,** e003497.

Wurtz, P., Havulinna, A. S., Soininen, P., Tynkkynen, T., Prieto-Merino, D., Tillin, T., Ghorbani, A., Artati, A., Wang, Q., Tiainen, M., Kangas, A. J., Kettunen, J., Kaikkonen, J., Mikkila, V., Jula, A., Kahonen, M., Lehtimaki, T., Lawlor, D. A., Gaunt, T. R., Hughes, A. D., Sattar, N., Illig, T., Adamski, J., Wang, T. J., Perola, M., Ripatti, S., Vasan, R. S., Raitakari, O. T., Gerszten, R. E., Casas, J. P., Chaturvedi, N., Ala-Korpela, M. & Salomaa, V. 2015. Metabolite profiling and cardiovascular event risk: A prospective study of 3 population-based cohorts. *Circulation,* 131**,** 774-85.

Würtz, P., Kangas, A. J., Soininen, P., Lawlor, D. A., Davey Smith, G. & Ala-Korpela, M. 2017. Quantitative serum nuclear magnetic resonance metabolomics in large-scale epidemiology: A primer on-omic technologies. *American Journal of Epidemiology,* 186**,** 1084-1096.

Wurtz, P., Tiainen, M., Makinen, V. P., Kangas, A. J., Soininen, P., Saltevo, J., Keinanen-Kiukaanniemi, S., Mantyselka, P., Lehtimaki, T., Laakso, M., Jula, A., Kahonen, M., Vanhala, M. & Ala-Korpela, M. 2012. Circulating

metabolite predictors of glycemia in middle-aged men and women. *Diabetes Care,* 35, 1749-56.

Wurtz, P., Wang, Q., Kangas, A. J., Richmond, R. C., Skarp, J., Tiainen, M., Tynkkynen, T., Soininen, P., Havulinna, A. S., Kaakinen, M., Viikari, J. S., Savolainen, M. J., Kahonen, M., Lehtimaki, T., Mannisto, S., Blankenberg, S., Zeller, T., Laitinen, J., Pouta, A., Mantyselka, P., Vanhala, M., Elliott, P., Pietilainen, K. H., Ripatti, S., Salomaa, V., Raitakari, O. T., Jarvelin, M. R., Smith, G. D. & Ala-Korpela, M. 2014. Metabolic signatures of adiposity in young adults: Mendelian randomization analysis and effects of weight change. *PLoS Med,* 11, e1001765.

Xu, X., Jones, M. & Mishra, G. D. 2020. Age at natural menopause and development of chronic conditions and multimorbidity: Results from an australian prospective cohort. *Human Reproduction,* 35, 203-211.

Yarde, F., Spiering, W., Franx, A., Visseren, F., Eijkemans, M., De Valk, H., Broekmans, F., Group, O. S., Joosten, C. A. & Klaassen, I. P. 2016. Association between vascular health and ovarian ageing in type 1 diabetes mellitus. *Human Reproduction,* 31, 1354-1362.

Yu, S., Rubin, M., Geevarughese, S., Pino, J. S., Rodriguez, H. F. & Asghar, W. 2018. Emerging technologies for home-based semen analysis. *Andrology,* 6, 10-19.

Zegers-Hochschild, F., Adamson, G., De Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E. & Van Der Poel, S. 2009. Technology icfmar, organization wh. The international committee for monitoring assisted reproductive technology (icmart) and the world health organization (who) revised glossary on art terminology, 2009. *Fertility and Sterility,* 92, 1520-4.

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., De Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L. & Cooke, I. D. 2017. The international glossary on infertility and fertility care, 2017. *Human Reproduction,* 32, 1786-1801.

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., De Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L., Cooke, I. D., Simpson, J. L. & Van Der Poel, S. 2017. The international glossary on infertility and fertility care, 2017. *Fertility and Sterility,* 108, 393-406.

Zhang, J., Huang, Z., Chen, M., Xia, Y., Martin, F. L., Hang, W. & Shen, H. 2014.

Urinary metabolome identifies signatures of oligozoospermic infertile men. *Fertility and Sterility,* 102**,** 44-53. e12.

Zhou, W., Gosch, G., Guerra, T., Broek, D., Wu, G., Walker, S. & Polejaeva, I. 2014. Amino acid profiles in first trimester amniotic fluids of healthy bovine cloned pregnancies are similar to those of ivf pregnancies, but not nonviable cloned pregnancies. *Theriogenology,* 81**,** 225-229.

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. C., Hansmann, M. A., Joyce, P., Foster, J. A. & Forney, L. J. 2007. Differences in the composition of vaginal microbial communities found in healthy caucasian and black women. *The ISME journal,* 1**,** 121-133.

Zhu, D., Chung, H.-F., Dobson, A. J., Pandeya, N., Giles, G. G., Bruinsma, F., Brunner, E. J., Kuh, D., Hardy, R. & Avis, N. E. 2019. Age at natural menopause and risk of incident cardiovascular disease: A pooled analysis of individual patient data. *The Lancet Public Health,* 4**,** e553-e564.

Zhu, T. & Goodarzi, M. O. 2020. Sun-016 mendelian randomization reveals that polycystic ovary syndrome is not a causal risk factor for type 2 diabetes, coronary heart disease, or stroke. *Journal of the Endocrine Society,* 4**,** SUN-016.