Investigating the regulation of male infertility

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Declaration of originality and contributors

Declaration of originality: The work of this thesis is my own and all collaborations and assistance is reported below. Contributors are within the Department of Metabolism, Digestion and Reproduction, Imperial College London unless stated otherwise.

Chapter 2: The ethics of this study was designed by Dr Channa N Jayasena. I led on this study and recruited all the participants and performed all seminal reactive oxygen species testing. The seminal microbiome analysis was carried out by Dr David Macintyre's team and the DNA fragmentation testing using the TUNEL assay was performed by Ms Dalia Khalifa, Dr Emad Sindi and Professor Ralf Henkel. The semen analysis was performed by the Andrology Department, Hammersmith Hospital, Imperial College Healthcare NHS trust.

Chapter 3: I led on this study with the supervision of Dr Channa N Jayasena and Professor Suks Minhas. Dr Daniel Foran assisted with the screening of the studies and Dr Giovanni Corona (Endocrinology Unit, Medical Department, Azienda Usl Bologna Maggiore-Bellaria Hospital, Italy) assisted with the statistical analysis for the meta-analysis. Professor Andrea Salonia (Department of Experimental Oncology/Unit of Urology, URI, IRCCS Ospedale San Raffaele, Italy), Professor Nikolaos Sofikitis (Department of Urology, Ioannina University School of Medicine, Greece), Professor Aleksander Giwercman (Department of Translational Medicine, Lund University, Sweden), Professor Csilla Krausz (Department of Experimental and Clinical Biomedical Sciences, University Hospital of Careggi, University of Florence, Italy) and Mr Tet Yap (Department of Urology, Guy's and St Thomas' Hospital, UK) helped preparation of the manuscript which has been published in Human Reproduction Update. (https://doi.org/10.1093/humupd/dmac016)

Chapter 4: The ethics of this study was designed by Dr Channa N Jayasena and Dr Emad Sindi. I co-led this study with Dr Emad Sindi. I recruited participants in the study and took the blood samples. The genetic sequencing and analysis was performed in collaboration with Dr Emad Sindi and Dr Anu Sironen (University College London, Great Ormond Street, UK)

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Abstract

Infertility is defined as the inability to become pregnant following one year of practicing regular and unprotected sexual intercourse and is estimated to affect 72.4 million people globally. Within the U.K., infertility has been estimated to affect 14% of couples. In 50% of cases of infertility the cause will be attributable to poor sperm quality and the main treatment is assisted reproductive technologies (ART) such as in-vitro fertilisation (IVF). However, ART is resource limited and IVF has an estimated success rate of 29%. Thus, there is an urgent need to improve our understanding of the pathophysiological mechanisms that underpin male infertility to help develop more cost-effective therapies. Recent studies have highlighted the effects of oxidative stress (including seminal reactive oxygen species (ROS) and sperm DNA fragmentation (SDF)) in sperm dysfunction. Furthermore, there is limited data showing differences in seminal microbiome in infertile compared to fertile men. However, it is unclear from the literature how oxidative stress and the seminal microbiome correlate with semen analysis. This is pertinent given that semen analysis is the gold standard investigation for diagnosing male infertility.

Non obstructive azoospermia (NOA) is the absence of sperm in the ejaculate due to impaired spermatogenesis. The only method for men with NOA to conceive biological children is through sperm retrieval surgery combined with ART. However, the success rate of testicular sperm extraction in men with NOA is only 50%. In half of all cases of NOA the cause is unknown but there is emerging data showing that genetic mutations may be contributory. This knowledge is helpful in both patient counselling and clinical management. For example, men with NOA who have either Azoospermia factor A or B gene deletions have a lower likelihood of testicular tissue containing sperm and should be counselled against sperm retrieval surgery. There is emerging data showing that in some cases of idiopathic NOA, a genetic mutation may be causal. Further genetic studies are needed to help improve our understanding of the aetiology of NOA and this may help identify future therapeutic targets for men with infertility.

Spermatogenesis is stimulated by gonadotropins and intratesticular testosterone. Some clinicians have trialed hormone stimulation therapy to improve sperm retrieval rates in men with NOA. However, no study has critically evaluated the literature regarding the effects of hormone stimulation therapy in improving sperm retrieval rates and also the potential adverse events.

This thesis includes the first study investigating how oxidative stress markers and seminal microbiome differ in different cohorts of male infertility and fertile controls. Furthermore, I performed the first meta-analysis investigating the effects of hormone stimulation therapy on surgical sperm retrieval rates in men with NOA. I have also investigated for novel genetic mutations in a cohort of infertile men with idiopathic NOA. Collectively, the results from this thesis will improve our understanding on the aetiological factors, pathophysiological mechanisms and management of male infertility.

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Table of contents

List of Figures

List of tables

Abbreviations

ABG	Androgen binding globulin
AD	Autosomal dominant
AHH	Acquired hypergonadotropic hypogonadism
AIs	Aromatase inhibitors
AO	Acridine Orange Assay
AR	Autosomal recessive
ART	Assisted reproductive technologies
AUC	Area Under the Curve
BMI	Body mass index
BDI	Beck depression inventory
CPM	Counted photons per minute
CESD	The centre for epidemiological studies depression scale
CFTR	cystic fibrosis transmembrane conductance regulator gene
CHH	Congenital hypergonadotropic hypogonadism
cHH	Compensated hypergonadotropic hypogonadism
CI	Confidence Intervals
COPD	Chronic obstructive pulmonary disease
CTESE	Conventional testicular sperm extraction surgery
DFI	Sperm DNA fragmentation index
DMSO	Dimethyl Sulfoxide
DN	Deletion
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
E_2	Oestradiol
FDR	False discovery rate
FR	Fertilisation rate
FSH	Follicle stimulating hormone
GPx	Glutathione peroxidase
GnRH	Gonadotrophin Releasing hormone
GU	Genitourinary tract infection
hCG	Human chorionic gonadotropin
HFEA	Human Fertilisation and Embryology Authority
HH	Hypergonadotropic hypogonadism
hMG	Human menopausal gonadotropin
HPG	Hypothalamic-pituitary-gonadal axis
HPV	Human papillomavirus
HSROC	Hierarchical Summary Receiver Operating Characteristic

HST	Hormone stimulation therapy
IGV	Integrative Genome Viewer
insertion/deletions	Indels
I.M	intramuscular injection
ICRF	Imperial College Research Facility
ICSI	Intracytoplasmic sperm injection
ISNT	In situ nickel translation
IUI	In uterine insemination
IVF	In vitro fertilisation
KNDy	Kisspeptin-dynorphin-neurokinin B complex
LBR	Live birth rate
LH	luteinising hormone
LEfSe	Linear discriminant analysis effects size
LoF	Loss of function
mMol/L	Millimole per litre
MA	Maturation arrest
MAGI	Male accessory gland infection
MDA	Malondialdehyde
MS	Missense mutation
MR	Miscarriage rate
mTESE	Micro testicular sperm extraction
NBT	Nitroblue tetrazolium
NG	Normogonadotropic eugonadism,
NGH	Normogonadotropic hypogonadism
NGS	Next generation sequencing
nmol/L	Nanomole per litre
NOA	Non obstructive azoospermia
NR	Not reported
OA	Obstructive azoospermia
OR	Odds Ratio
PBS	phosphate buffer solution
PCR	Polymerase chain reaction
PR	Pregnancy rate
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-analyses
RCTs	Randomised controlled trials
RLU	Relative light units
ROS	Reactive oxygen species
rpm	Revolutions per minute
RR	Risk Ratio
rRNA	Ribosomal ribonucleic acid
S.C	Subcutaneous injection
SCOS	Sertoli cell only syndrome
SD	Standard deviation
SERMs	Selective oestrogen receptor modulators
SDF	Sperm DNA fragmentation
SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay

SNVs	Splice site-disrupting nucleotide variants
SSR	Surgical sperm retrieval
SOD	Superoxide dismutase
T	Testosterone
TAC	Total antioxidant capacity
T:E	Testosterone oestrogen ratio
TESE	Testicular sperm extraction
TEX 11	Testis expressed 11 gene
TOS	Total oxidant status
TUNEL	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling
UKNEQAS	United Kingdom National External Quality Assessment Service
UV	Ultraviolet
WES	Whole exome sequencing
WHO	World Health Organisation
XLR	X-linked recessive

Chapter 1:

General introduction

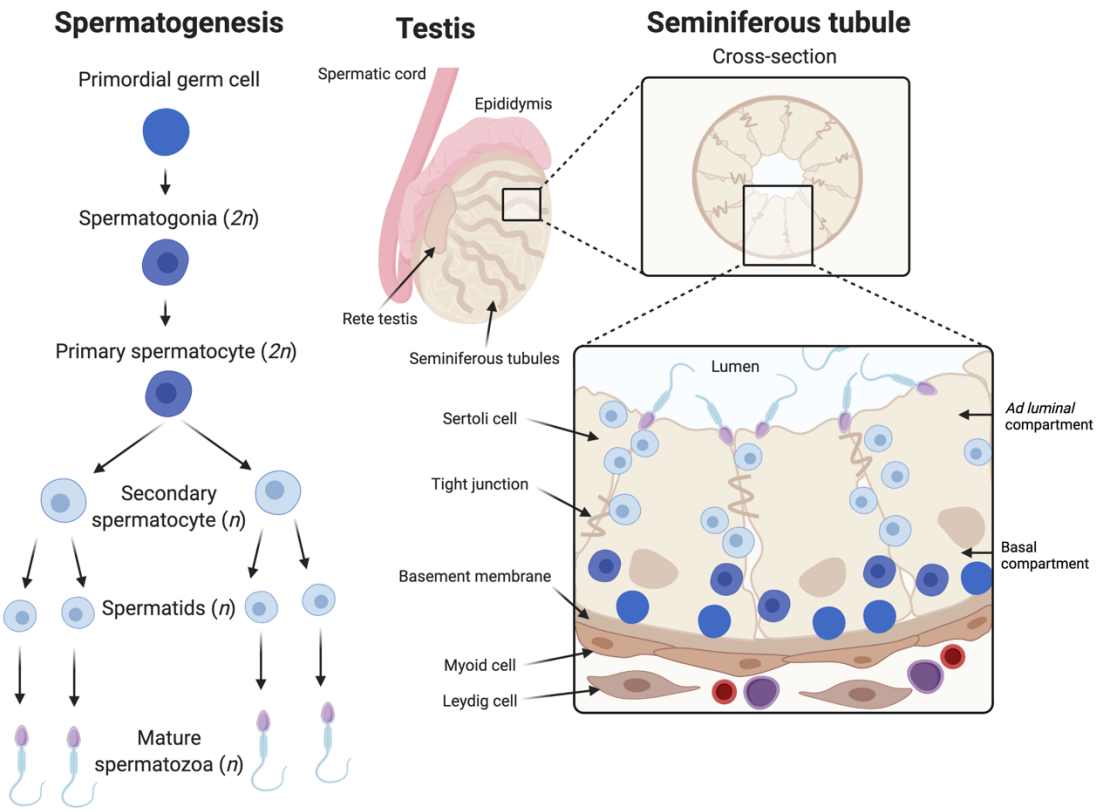
1.1 An overview of spermatogenesis

Spermatogenesis describes the differentiation of primordial germ cells into spermatozoa, and this occurs in the seminiferous tubules of the testes of adult men. The average spermatogenesis cycle takes 74 days in humans (1,2) and studies have reported that men aged 20-50 years old produce between 150-275 million spermatozoa every day (3,4).

The testicular parenchyma consist of 200-300 lobules and each individual lobule contains one to three seminiferous tubules (4). The seminiferous tubules contain a basement membrane and Sertoli and germ cells and a peritubular tissue layer comprising of myoid cells, adventitial cells and collagen matrix encloses each seminiferous tubule (4). Leydig cells are located between seminiferous tubules and blood vessels (4) (Figure 1.1).

Figure 1.1: Diagram of the spermatogenesis cycle.

Spermatogonia migrate from the *ad luminal* compartment to the basal area of the seminiferous tubule and undergo mitosis to become primary spermatocytes and subsequently two stages of meiosis to become 4 haploid spermatids.



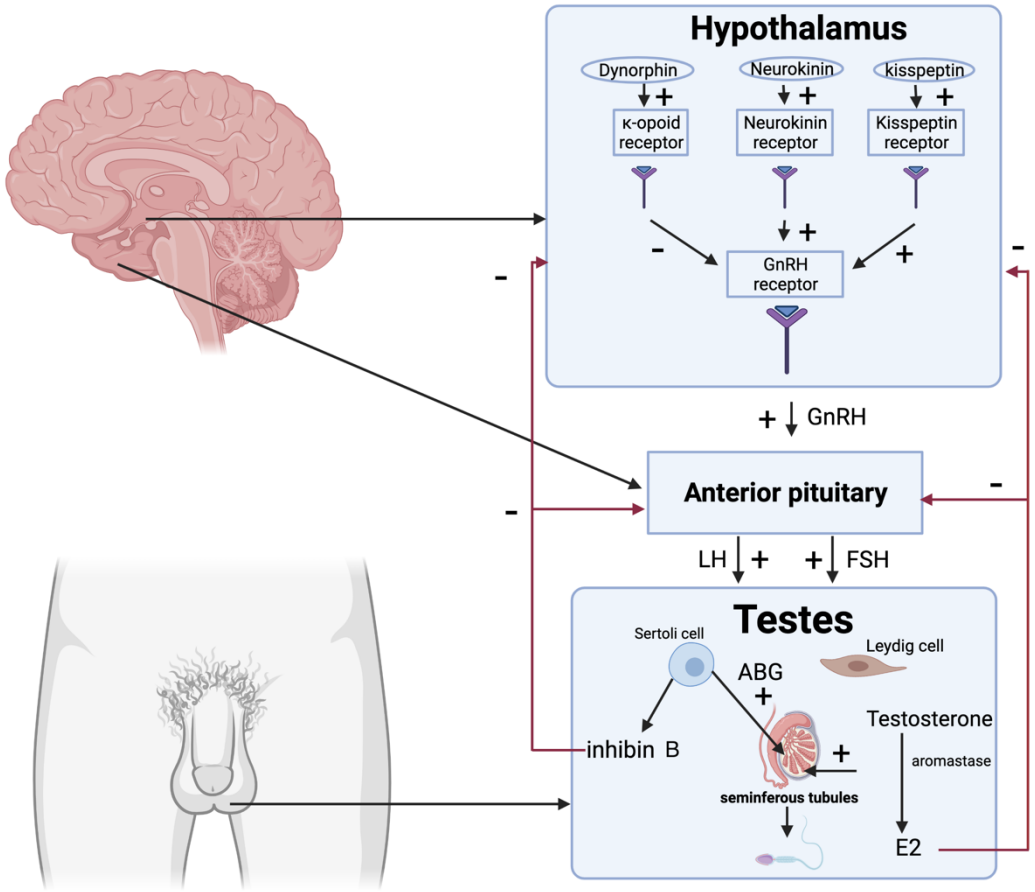
1.1.1 The regulation of spermatogenesis

The hypothalamic-pituitary-gonadal (HPG) axis regulates spermatogenesis (5) (Figure 1.2). Spermatogenesis commences at the onset of puberty (6,7) with a sustained increase in Gonadotrophin Releasing hormone (GnRH) production from the GnRH pulse generator located in the hypothalamus. The GnRH pulse generator is regulated by both active factors (e.g. kisspeptin), inhibitory factors (e.g. dynorphin) and other modulatory neuropeptides (such as neurokinin B) (7,8). The Kisspeptin-dynorphin-neurokinin B (KNDy) neurones stimulate GnRH neurones to synthesise the GnRH (8,9). The GnRH is transmitted through the hypophyseal portal circulation to the anterior pituitary gland, where it stimulates the gonadotrophs to secrete pulses of luteinising hormone (LH) and follicle stimulating hormone (FSH) every 1-2 hours (8,10). The LH stimulates Leydig cells to produce and secrete testosterone, which promotes cell maturation (11). Both FSH and testosterone promote Sertoli and germ cell maturation (10–12). The enzyme aromatase is located in the testes and converts testosterone into oestradiol, which stimulates a negative feedback on the HPG axis and reduces both the GnRH and gonadotropin secretion (10). Sertoli cells produce Inhibin B which downregulates the pituitary production of FSH through a negative feedback (10,13).

Figure 1.2: Diagram of hypothalamic- pituitary- gonadal axis.

+ = stimulation, - = inhibition, GnRH = Gonadotropin releasing hormone, E2 = Oestradiol, ABG = Androgen binding globulin

The Kisspeptin-dynorphin-neurokinin B complex (KNDy) stimulates the Gonadotropin regulating hormone receptor to release Gonadotrophin Regulating hormone (GnRH) which regulates the anterior pituitary gland to synthesise luteinising hormone (LH) and follicle stimulating hormone (FSH). LH and FSH stimulate Sertoli and Leydig cells, respectively. Leydig cells synthesis testosterone (T) which regulates spermatogenesis, and this is bound to androgen binding globulin (ABG). Sertoli cells synthesises ABG and also inhibin B which negatively regulates the HPG axis. Testosterone is downgraded to oestradiol (E2) by aromatase and E2 negatively regulates the HPG axis.



1.1.2 The stages of spermatogenesis

Spermatogenesis is broadly categorised into three stages: 1) Spermatogoniogenesis, 2) Maturation of spermatocytes and 3) Spermiogenesis.

1) Spermatogoniogenesis

Located within the basal aspect of the germinal epithelium are three types of spermatogonia (Type A (pale and dark) and Type B) (6). Type A spermatogonia are classified according to the colour of their nuclei; pale nuclei stem cells do not undergo mitosis whilst dark nuclei stem cells undergo mitosis to produce Type B spermatogonia (6). Type B spermatogonia undergo mitosis to produce primary spermatocytes (6). Spermatogoniogenesis is facilitated by local factors released from the Sertoli cells; glial cell-derived neurotrophic factor induces spermatogonia stem cell renewal whist retinoic acid promotes differentiation (4).

2) Spermatocyte Maturation

Primary spermatocytes migrate across the blood-testicular barrier from the basal to the *ad luminal* compartment of the seminiferous tubules (4,6). Primary spermatocytes undergo one cycle of meiosis to form 2 haploid secondary spermatocytes. Following this a further meiosis cycle occurs to produce four haploid spermatids (6). During this transition, spermatocytes undergo immunological development and the most mature forms (spermatids) are present in the *ad luminal* compartment whilst the most immature (primary spermatocytes) are located at the basal area (14) (see Figure 1).

3) Spermiogenesis

Spermiogenesis describes the processes (condensation of the nuclear chromatin, development of the acrosome cap and creation of the flagellum structures) facilitating the differentiation of spermatids into spermatozoa (6). Spermiogenesis is dependent on intratesticular testosterone and both human and animal studies have observed that androgen suppression results in a retention of spermatids and failure of spermiogenesis (15,16). The resultant sperm are released into the lumen of the seminiferous tubule through the coordination of the Sertoli cell's intermediate filament and cytoplasmic tubules (6). The myoid cells contract to mobilise spermatozoa to the rete testis and through the efferent ducts to the epididymis (6). Within the epididymis further spermatozoa maturation

occurs including the loss of the cytoplasmic droplet. The failure of these developmental mechanisms can result in abnormal sperm motility or morphology (17,18). Developed sperm are stored in the epididymis until ejaculation occurs.

1.2 The definition, epidemiology, and classification of infertility

1.2.1 The classification of infertility

Infertility is defined as the inability of a heterosexual couple to become pregnant following one year of practicing regular and unprotected sexual intercourse (19) and is estimated to affect 72.4 million people globally with 40.5 million seeking medical care (20). Within the U.K., infertility has been estimated to affect 14% of couples (21).

The Global Burden Of Disease study (22) assessed trends in infertility amongst 195 countries in the time period 1950-2017. This study measured both age-specific-fertility rate (the annual number of live birth rates to women of a specified age group per 1000 women in that age group) and the total-fertility rate (the average number of children a woman would bear if she survived through the end of the reproductive age span (10-54 years) and experienced at each age a particular set of age specific fertility rates observed in the year of interest). The authors reported a decrease in the total fertility rates from 4.7 live births to 2.4 (49.4%). Moreover, the age-specific-fertility rate of mothers aged 10-19 had declined from 37 live births to 22 live births.

In addition to this, there is data showing that semen parameters have declined with time. A meta-analysis (23) that included 42,935 men, demonstrated that sperm counts have decreased by 0.70million/ml/year within the period of 1981-2013 (95% CI -0.72 to -0.69; $p<0.001$). However, this study has been criticised because of the large variation in semen analysis practices between the laboratories included and a large non response rate in the overall data (24).

It is unclear why infertility rates are rising but it has been postulated that changes in societal and behavioural practices may be contributing. There are more women entering higher education and working and as a consequence choosing to conceive at older ages (25,26). It has been reported that increasing female age is a risk factor for infertility (from age 32 years onwards but most pronounced above the age of 37 years) (27,28).

There is data showing that household and environmental chemicals (termed endocrine disrupting chemicals) can cause both sperm and sexual function abnormalities (29,30). Phthaltes are chemicals present in many consumer products (e.g. shampoos) and have been associated with decreased sperm production in several murine studies (31). Nassan et al. investigated the effects of di-butyl

phthalate coating on mesalamine medications in 73 men. The authors reported that di-butyl phthalate coating was negatively associated with total sperm motility ($p<0.007$). Bisphenol-A is a chemical present in many plastics (such as food storage containers) and Li et al. investigated the association between urine bisphenol-A concentrations and sexual function in a cohort of 427 men (32). The authors observed that increasing urine bisphenol-A was associated with erectile dysfunction ($p<0.001$) and a lower sexual desire ($p<0.01$). The current literature on endocrine disrupting chemicals is limited because of issues related to the measurement of single exposures given the ubiquitous nature of endocrine disrupting chemicals. Furthermore, there is a paucity of human studies (33).

In addition to this, there is data showing that obesity and diabetes can adversely affect sperm parameters. Jensen et al. (34) performed a cross sectional study investigating the relationship between body mass index (BMI) and sperm parameters and observed that a raised BMI (>25kg/m^2) compared to BMI within the normal range (20-25kg/m^2) was associated with a decreased sperm concentration (39million/ml vs 46million/ml) and total sperm count (116 million vs 138 million). The authors also reported that the serum testosterone was lower in those with a high BMI suggesting that hypogonadism might have been contributing to the observed differences. There are human studies showing that type 1 diabetes is associated with a decrease in sperm motility and normal morphology but not sperm count (35,36). Moreover, murine studies have reported that diabetes is associated with an increase in seminal oxidative stress (37). Thus, the rising levels of obesity and diabetes (38,39) may be contributing to the increasing rates of infertility.

Infertility can be classified by the underlying cause. An abnormality of the semen analysis, according to World Health Organisation (WHO) reference ranges, is described as male factor infertility (Table 1.1) (40). Female factor infertility is related to ovulatory or fallopian tubal abnormalities (Table 1.2).

Table 1.1- Table summarising the nomenclature for semen analysis abnormalities according to World Health Organisation (WHO) reference ranges.

Nomenclature	WHO definition	Description
Azoospermia	No spermatozoa are detected in the sediment of a centrifuged sample	Absence of sperm in the ejaculate.
Oligospermia	< 15 million sperm per millilitre (x 10^6 mL^{-1}) detected in the semen	Decreased sperm concentration compared to reference ranges.
Asthenospermia	< 32% of sperm detected are progressively motile	Decreased sperm motility compared to reference ranges.
Teratospermia	< 4% of sperm present have a normal morphology.	Decreased normal sperm morphology compared to reference ranges.

Reference values and definitions derived from WHO laboratory manual for the Examination and processing of human semen. Fifth edition. (40)

Table 1.2- Table summarising the common causes of female infertility

Abnormality	Classification	Pathology	Conditions
Ovulatory dysfunction*	Type I	Hypogonadotropic Hypogonadism	Kallman syndrome, functional hypothalamic amenorrhea.
	Type II	Dysfunction of the Hypothalamic-pituitary-ovarian axis.	Polycystic ovary syndrome, congenital adrenal hyperplasia.
	Type III	Hypergonadotropic hypogonadism	Primary ovarian insufficiency.
Tubal abnormalities	Unilateral or Bilateral	Surgical trauma, compression from a mass, internal obstruction	Iatrogenic injury, ectopic pregnancy, pelvic mass.

**WHO classification for ovulatory dysfunction* (41)

Azoospermia is the absence of sperm in the ejaculate and occurs in 1% of the male population and 10-20% of patients presenting with infertility (42). Azoospermia is classified as obstructive (OA) if there is an obstruction to the reproductive ductal system or non-obstructive (NOA) when there is an impairment of spermatogenesis (43). NOA results from either a pathology of the testicle itself (e.g., mumps orchitis) or insufficient hormone stimulation to the testicle (such as hypogonadotropic hypogonadism). NOA men cannot conceive naturally and require sperm retrieval surgery and assisted reproductive technologies (ART) to father biological children.

The histological classification of NOA is defined by the extent of spermatogenesis cycle dysfunction (44). Hypospermatogenesis describes the presence of mature spermatozoa but at a reduced total number. Maturation arrest is characterised by incomplete sperm maturation and further classified into early and late if the disruption has occurred at the primary or secondary spermatocyte stage respectively. Sertoli cell only syndrome is defined by the absence of all germ cells. Hypospermatogenesis is associated with the highest surgical sperm retrieval rate and Sertoli cell only syndrome the lowest (45,46) but mixed histological patterns in the same testicle are common (47).

Unexplained infertility is defined as infertility where the man has normal semen parameters and his female partner has normal ovulation and fallopian tube patency (48). Unexplained infertility has been estimated to affect 15-30% of couples (48,49). There is no definitive method for assessment of ovulation however it is widely accepted that a regular menstrual history (Eumenorrhea) is a highly accurate marker of ovulation. Other markers of ovulation include luteal phase elevations of serum progesterone, and the appearance of a dominant follicle with subsequent visualisation of a corpus luteum during ultrasound of the ovaries. Tubal patency can be assessed by hysterosalpingography or laparoscopic inspection.

1.2.2 Epidemiology of Infertility

A WHO study (50) reported that male factor infertility was the single cause of infertility in 20% of couples whilst in 27% of couples the aetiology was related to both male and female factors. The authors also observed that in 15% of couples no cause could be identified. Therefore, abnormalities in sperm function contribute to a significant proportion of infertility cases and in many couples the cause is unclear.

1.3 Aetiology of male infertility

Male infertility is categorised according to the anatomical location of the pathology (pre-testicular, testicular and post testicular). The genetic causes of male infertility will be discussed separately (see 1.4 The genetic causes of male infertility)

1.3.1 Pre-Testicular

The pretesticular causes of male infertility are caused by secondary hypogonadism, due to insufficient gonadotrophin stimulation of the testicles with decreased testosterone production and reduced spermatogenesis. Secondary hypogonadism arises from a pathology of the hypothalamus or pituitary gland and is categorised by the age of onset into congenital and acquired hypogonadotropic hypogonadism.

Congenital hypogonadotropic hypogonadism (CHH) is a clinically heterogenous disorder but is characterised by the presence of pubertal developmental abnormalities in the absence of any hypothalamic or pituitary gland anatomical abnormalities (51). Kallman syndrome is CHH with anosmia or hyposmia and contributes to two thirds of the cases of CHH (52).

Acquired hypogonadotropic hypogonadism (AHH) is due to a functional or structural abnormality of the hypo-pituitary-gonadal axis. The causes of AHH include drugs (exogenous testosterone and anabolic steroids (53)), tumours of the pituitary gland (51), infiltrative disorders (e.g. sarcoidosis) (51), and systemic diseases such as diabetes (35,54) and obesity (55–57).

1.3.2 Testicular

The testicular causes of male infertility arise from pathology of the testicle (Table 1.3) and may be associated with primary hypogonadism (low testosterone and normal or increased gonadotrophin levels).

Table 1.3- Table summarising causes of testicular failure related infertility

Cause	Examples
Infection	Mumps virus (58)
Trauma	Testicular trauma (59)
Drugs	Chemotherapy (60)
Congenital abnormalities	Cryptorchidism (61)
Genetic	Klinefelter's syndrome (62)
Systemic diseases	Diabetes (35)
Iatrogenic	Testicular damage post hernia surgery (63)

1.3.3 Post testicular

The post testicular causes of male infertility are due to a blockage in the conduit of the sperm rather than any abnormalities of spermatogenesis. The main causes of post testicular infertility are the absence of the vas deferens (either congenital (associated with Cystic Fibrosis) or iatrogenic (such as post vasectomy)) and epididymitis (commonly associated with genital tract infection).

1.4: Genetic disorders

The genetic conditions associated with impaired sperm function and male infertility are described in Table 1.4. Johnson et al. performed a pooled analysis of 11 studies containing 9766 infertile men with either azoospermia or oligospermia and observed a 5.8% incidence rate of chromosomal abnormalities (64). Vincent et al. (65) performed a retrospective analysis of data pertaining to 2651 infertile men at a single institution. The authors subclassified oligospermia by the sperm concentration into mild (>10-20 million x 10^6/ml), moderate (5-10 million x 10^6/ml) and severe (<5 million x 10^6/ml) and reported that NOA men had the highest incidence of chromosomal abnormalities (16.7%) followed by severe (9.7%), moderate (4.3%) and mild (0.5%) oligospermia. The above studies highlight that genetic conditions are common in infertile men and the frequency of genetic abnormalities is related to the severity of sperm dysfunction (66). The discovery of Y chromosome microdeletions has revolutionised the management of NOA. Three regions were identified on the long arm of the Y chromosome (Yq11), that are associated with failure of spermatogenesis if absent (AZFa, AZFb, and AZFc) (67). These microdeletions were observed in 10-20% NOA men (68,69) and provided prognostic information on surgical sperm retrieval success. Consequently, men with AZFa, AZFb or AZFb+c microdeletions have a poor likelihood of spermatogenesis and thus are typically counselled against sperm retrieval surgery. However, men with the AZFc microdeletion have a high chance of successful sperm extraction (69) and couples may choose to only select female embryos for implantation to prevent transmission of this genetic disorder into any conceived child. This highlights that genetic testing can help diagnose the cause for infertility and shape the management of individuals.

Both the American Urology Association (70) and European Association of Urology (66) recommend karyotype and Y microdeletion testing in patients with azoospermia and Cystic fibrosis transmembrane conductance regulator gene (CFTR) testing in those suspected of having absence vas deferens.

Table 1.4: Table summarising common genetic conditions associated with infertility

Condition	Genetic abnormality	Infertility sequalae
Cystic Fibrosis	Cystic fibrosis transmembrane conductance regulator gene (long arm of chromosome 7	Associated with the congenital bilateral absence of vas deferens resulting in obstructive azoospermia
Klinefelter syndrome	Extra X chromosome (XXY) or mosaicism 47XXY/ 46 XY	Associated with loss of testicular tissue and seminiferous tubules fibrosis resulting in testicular atrophy and non-obstructive azoospermia.
Primary ciliary dyskinesia	Autosomal recessive disorder (30 genes reported to be causative-linked to abnormalities in dynein protein)	Immotile sperm
XX male syndrome	Y chromosome mosaicism, X linked mutations of the differentiation pathway sequences, translocation of Y chromosome sequences onto the X chromosome or autosome.	Testicular atrophy and non-obstructive azoospermia

The need for further genetic studies

There is increasing data showing that genetic mutations are contributing to male infertility (71,72) The emergence of next generation sequencing (NGS) has allowed for quicker and cheaper genetic testing compared to traditional sanger sequencing and a recent systematic review demonstrated that NGS has identified variants of 28 genes related to male infertility (72).

Whole exome sequencing (WES) identifies all the genetic variants in the exome (and protein) coding region of the genome. There have been 2,000 genes reported to contribute to spermatogenesis (73) and at least 50 candidate genes that have an association with NOA (74). The most commonly identified gene causing meiotic arrest is Testis Expressed 11 (TEX11*)* and this has been observed to be present in approximately 15% of men with NOA (75). Yang et al. studied 246 men with idiopathic NOA and detected 3 different genetic sequence variants in the TEX 11 gene (76). The contemporary literature have reported that karyotype abnormalities (most commonly Klinefelter syndrome) and Y chromosome microdeletions are present in 17% and 2-10% of cases NOA, respectively (71).

Kasak et al. (71) performed a literature search for monogenetic causes in NOA using the Human Phenotype Ontology, Online Mendelian Inheritance in Man and PubMed databases. The authors reported 10 causative genes for NOA that have been validated by two independent studies and 16 candidate genes that have been only reported in a single study (Table 1.5).

Table 1.5 Table summarising the literature on monogenetic causes for non obstructive azoospermia.

MS = missense mutation; LoF = loss of function; DN= deletion; AR = Autosomal recessive; AD = Autosomal dominant; XLR = X-linked recessive; SCOS = Sertoli cell only syndrome; MA = Maturation arrest

Adapted table from Kasak et al.(71)

Gene	Gene function	Validated by two independent studies	NOA Histology	Extra testicular phenotypes	Genetic inheritance	mRNA expression	Variant type	Mouse model
FANCM	DNA repair	Yes (77) (78)	SCOS	Breast, prostate, ovarian neoplasia and premature ovarian failure.	AR	Testis	LoF	Yes
MEI1	Chromosome synapsis	Yes (79) (80)	MA	Hydatidiform mole	AR	Testis	MS, LoF	Yes
MEIOB	DNA double strand break repair	Yes (81) (82)	MA	Premature ovarian failure.	AR	Testis	MS, LoF	yes
STAG3	DNA double strand break repair	Yes (83) (84)	MA	Premature ovarian failure	AR	Testis	MS, LoF	Yes
TEX11	Chromosome synapsis	Yes (85) (86) (75)	MA	NR	XLR	Pancreas testis	MS, LoF	yes
TEX14	Meiotic intercellular bridges development	Yes (74) (81)	MA, SCOS	NR	AR	Testis	MS, LoF	Yes
TEX15	DNA double strand break repair	Yes (87) (88)	MA	NR	AR	Endometrium, smooth muscle, testis	LoF	Yes
NR5A1	Sex determination	Yes (89)	SCOS, MA	46,XY and 46XX syndrome;	AD	Adrenal gland, ovary, spleen	MS, LoF	Yes

	transcription factor	(90) (91)		adrenocortical insufficiency and premature ovarian failure				
SETX	DNA & RNA processing	Yes (92) (93)	MA	Amyotrophic lateral sclerosis; ataxia with oculomotor apraxia type 2 and premature ovarian failure	AR	All tissues	MS, LoF	Yes
WT1	Transcription factor	Yes (94) (95) (96)	SCOS, MA	Wilms tumour; nephrotic syndrome; mesothelioma; Meacham syndrome; Frasier syndrome; Denys-Drash syndrome and premature ovarian failure	AD	Endometrium, fallopian tube, smooth muscle	MS	yes
CCDC155	Homologue pairing in meiosis	No (96)	MA	NR	AR	Testis	MS	Yes
DMC1	DNA double strand break repair	No (97)	MA	Premature ovarian failure	AR	Testis	MS	Yes
MCM8	DNA double strand break repair	No (98)	unknown	Premature ovarian failure	AR	Testis	LoF	Yes
NANOS2	Spermatogonia stem cell preservation	No 74)	SCOS	NR	AR	Testis	MS	Yes
PLK4	Centriole duplication	No (99)	SCOS	Autosomal recessive microcephaly and chorioretinopathy	AD	Testis	DN	Partial
RNF212	Meiotic recombination	No (83)	MA	Recombination rate	AR	Mixed	LoF	Yes
SPINK2	Acrosin Inhibitor	No (100)	Post meiotic block	NR	AR	Epididymis	LoF	Yes

SPO11	Initiation of DNA double strand break	No (74)	MA	NR	AR	Testis	MS	Yes
SYCE1	Chromosome synapsis	No (101)	MA	Premature ovarian failure	AR	Testis	LoF	Yes
TAF4B	Transcriptional coactivator	No (102)	Unknown	NR	AR	Mixed	LoF	Yes
TDRD9	Suppression of transposable elements	No (103)	MA	NR	AR	Testis, parathyroid	LoF	Yes
WNK3	Electrolyte homeostasis	No (74)	SCOS	NR	XLR	Epididymis and testis	MS	No
ZMYND15	Supressor of transcription	No (102)	MA	NR	AR	Parathyroid, testis	LoF	Yes
FANCA	Crosslink repair	No (104)	SCOS	Fanconi Anaemia and premature ovarian failure	AR	Mixed	MS DN	Yes
TDRD7	Translation regulation	No (105)	MA	Cataract	AR	All tissues; highest in testis and eyes	LoF	yes
XRCC2	DNA double strand break repair	No (106)	MA	Fanconi anaemia	AR	Mixed	MS	Yes

Understanding potential genetic causes for NOA is clinically relevant because it would not only help with patient counselling but could also direct health care provision by stratifying patients with spermatogenesis present (e.g., Y microdeletions) and thus those who would benefit from sperm retrieval surgery

1.5 Idiopathic male infertility

Although there are several recognised causes of impaired sperm function including infection, drug use and testicular torsion, in 50% of male infertility cases the cause is unknown (107). Moreover, there is increasing data to suggest that there may be sperm dysfunction even in men with apparently normal sperm parameters by WHO criteria (108). There have been several postulated causes for unexplained infertility including scrotal hyperthermia, testicular hypoxia, vascular factors and oxidative stress mechanisms.

1) Scrotal hyperthermia

Humans have intra-scrotal testes because optimal testicular function occurs at temperatures lower (physiological temperatures for the scrotum are 32-35°C) than the core body temperature (109). Lue et al. (110) studied the semen parameters and testosterone levels in 3 adult cynomolgus monkeys who had their scrotum submerged in a water bath (43°C) for 30 minutes once daily for 6 consecutive days. The authors reported that two of the monkeys developed azoospermia and one oligospermia and this was associated with a decrease in testosterone in two of the monkeys. The authors performed testicular biopsies and reported an increase in germ cell apoptosis in all three monkeys and a full recovery of spermatogenesis was observed 12 weeks post water bath. Mieusset et al. measured the scrotal temperature of 150 infertile and 37 fertile men and reported that the mean scrotal temperature was significantly higher in the interfile cohort (111). Hjollund et al. measured semen samples and scrotal skin temperature (using a 24-hour continuous portable device) in 99 healthy men. The authors observed a negative association between median scrotal temperature and sperm concentration ($p<0.01$). The definitive mechanisms for which scrotal hyperthermia impairs testicular function is unclear but has been postulated to be due to testicular heat stress causing increased reactive oxygen species and oxidative stress and germ cell apoptosis. However, most of the studies on heat stress and testicular function have been performed in animals and few studies have investigated clinical outcomes such as pregnancy and live birth rates. Whilst there is data showing that hot sauna and occupational exposure to radiant heat (e.g. bakers and welders) may adversely affect sperm parameters (109), these risk factors are not applicable to many men with unexplained infertility.

2) Testicular Hypoxia

There is limited data showing that high altitude may affect sperm function. Verrati et al. performed semen analyses on 6 mountain trekkers and reported that following high altitude climbing there was a significant decrease in sperm count ($p=0.004$) and increase in abnormal sperm morphology ($p=0.0067$). The authors reported a recovery of pre-hypoxic levels in all participants within 6 months. Okumura et al. (112) measured semen parameters in 3 men climbing 7821m above sea level. The authors also reported a decline in sperm count and this was associated with a decrease in testosterone levels. Several studies have also investigated whether certain medical conditions associated with hypoxia may impact testicular and sperm function. Semple et al. studied testosterone levels in men with pulmonary fibrosis (113) and chronic obstructive pulmonary disease (COPD) (114) and observed a significant correlation between a decrease in testosterone levels and arterial hypoxia. This suggests that hypoxia causes hypogonadism although LH and FSH are not reported and therefore it is unclear whether this is due to primary or secondary hypogonadism. Torres et al. (115) used a murine model to investigate the impact of obstructive sleep apnoea (OSA) on fertility. The authors compared mice subjected to periodic hypoxia (20 seconds of 5% oxygen followed by 40 seconds of room air oxygen for 6 hours per day) to mice breathing room air oxygen only. The authors reported a decrease in sperm motility ($p<0.04$), antioxidant enzymes (Glutathione peroxidase 1 and superoxide dismutase 1) ($p<0.05$) and number of pregnant females per mating ($p=0.04$) in the intermittent hypoxia cohort compared to the normoxic control group. There has not been any study in the literature comparing sperm parameters and live birth rates in men with and without OSA and therefore it is unclear whether OSA effects male fertility in humans. Although the above studies highlight that hypoxia may affect male fertility, the data is limited by small cohort sizes and a lack of randomised controlled studies. Moreover, risk factors such as high-altitude climbing are uncommon in the male infertility population.

3) Vascular Factors

A varicocele is an abnormal dilation of the venous pampiniform plexus within the scrotum (116) and has been reported to be present in 15% of the general population and 35-40% of men presenting with infertility (117). Several meta-analyses have reported that varicocele repair can improve sperm parameters (118–120). A Cochrane review reported that varicocele repair in couples diagnosed with

unexplained infertility increased spontaneous pregnancy rates (odds ratio 1.47, p=0.03) and this was most pronounced when analysis was limited to men with clinical varicoceles and abnormal sperm parameters (odds ratio 2.39, p=0.03). However, the authors observed that no study reported live birth outcomes and the literature was of low-quality evidence. In contrast, Kim et al. performed a meta-analysis investigating the relationship between varicocele repair and pregnancy rate in unexplained infertility and observed no statistically significant association (p =0.16) (121) . However, when the authors limited analysis to infertile men with clinically palpable varicoceles and abnormal semen analysis, there was a significant increase in pregnancy rates associated with varicocele repair (odds ratio 4.15, p<0.001). The pathophysiological mechanism by which a varicocele causes impaired sperm function is unclear but has been postulated to be a result of scrotal hyperthermia and oxidative stress. Agarwal et al. (122) performed a meta-analysis investigating the relationship between oxidative stress and varicoceles and reported that the presence of a varicocele conferred to a significantly higher reactive oxygen species level (p<0.0001) and lower total antioxidant level (p<0.00001). However, this study was limited because it only included 4 studies of which none were randomised controlled trials and the overall cohort was 118 patients. Several human and animal studies have reported that the presence of a varicocele can increase intratesticular temperatures and thus the presence of a varicocele may cause impair sperm parameters through scrotal hyperthermia (123). Lee et al. (124) measured the expression of hypoxia-induced-factor-1-α (a surrogate marker for tissue hypoxia) in men with varicoceles compared to controls. The authors reported that the presence of a clinical varicocele was associated with a 7 times higher concentration of hypoxia-induced-factor-1-α than controls. However, the study was limited because of its small cohort size of 8 patients. Although varicoceles appear to impair sperm parameters, it is unclear why only a small proportion of men with varicoceles develop infertility (125). Moreover, the current literature suggests that only clinically palpable varicoceles adversely affect sperm parameters, and this is usually identified in the diagnostic work up of an infertile man. Therefore, it is unlikely that varicoceles contribute significantly to infertility in men with unexplained infertility.

4) Oxidative stress

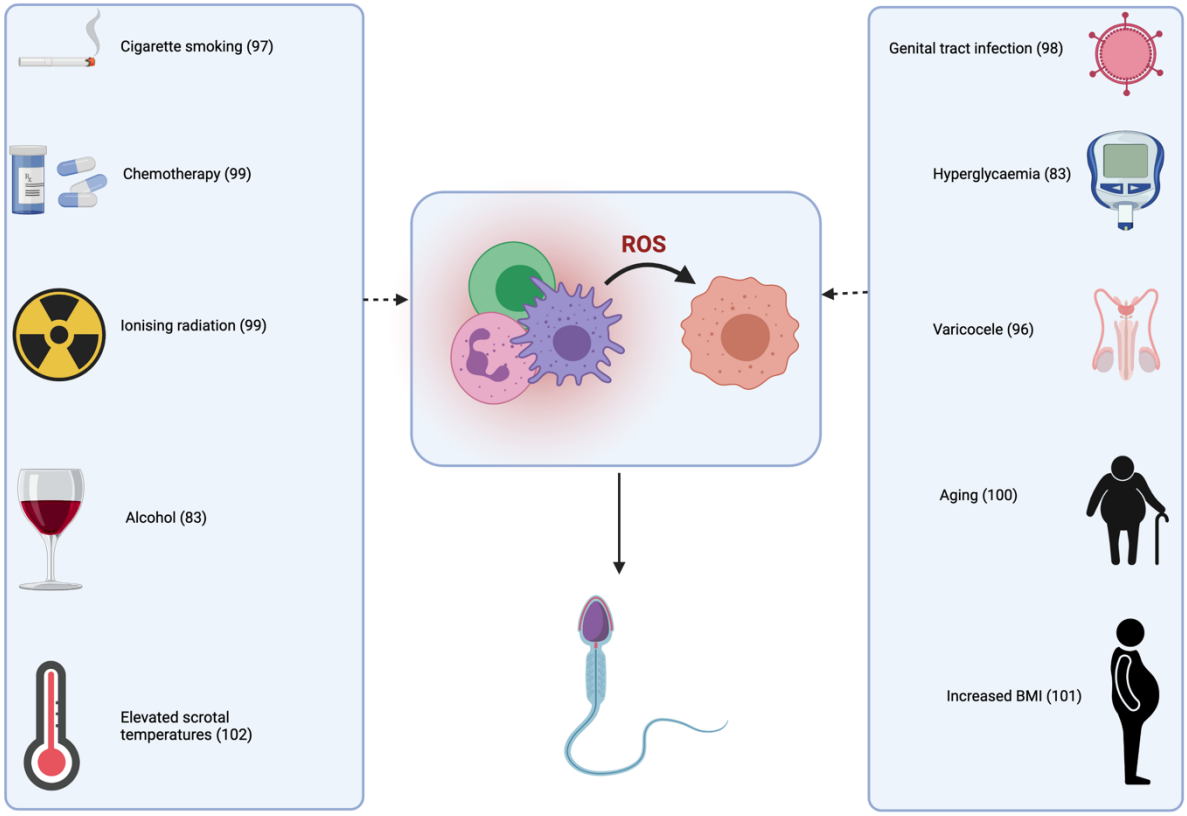
Oxidative stress can be due to scrotal hyperthermia, testicular hypoxia, varicoceles, and idiopathic mechanisms. Seminal reactive oxygen species (ROS) are free radical derivates of oxygen that are

required for both physiological and pathological processes within the human body (126). Seminal ROS is necessary for the maturation of sperm (including the capacitation stage) (127) but supraphysiological levels of ROS generate oxidative stress which causes sperm DNA fragmentation (SDF) and also disruption to the plasma membrane of sperm (128,129). These changes are more consequential because sperm lack the necessary enzymes to repair oxidative stress damage (130,131) and the consequences are asthenozoospermia (132,133), oligospermia (134) and teratozoospermia (135) .

Seminal ROS levels are determined by the balance of ROS production and antioxidant concentration. However, there is data to suggest that supraphysiological concentrations of seminal ROS are predominantly due to increased ROS production rather than decreased antioxidants (136,137). Seminal ROS is mainly produced by polymorphonuclear leucocytes (128) and an increased level of seminal leucocytes can be attributed to infection or inflammation. Although, the WHO define leukocytospermia as $\geq 1 \times 10^6$ leukocytes/mL in the ejaculate (40) there is data showing that levels below this threshold can still cause oxidative stress (138). Sharma et al. studied the oxidative stress levels (measuring ROS via a chemiluminescence assay) in 271 infertile men and 28 healthy controls (138). The authors reported that the seminal ROS was significantly higher in leucocyte levels of 0.4-0.8 x 10^6 compared to zero leucocytes and that there was no minimum white blood cell count in the semen which was associated with oxidative stress. This highlights that the current WHO reference ranges may be inaccurate at discriminating what level of seminal leucocytes causes sperm impairment. The other causes of an abnormal seminal ROS concentration are illustrated in Figure 1.3.

Figure 1.3: Diagram showing reported causes of increased seminal reactive oxygen species

ROS = reactive oxygen species, BMI = body mass index



(126,139–144)

SDF can be caused by oxidative stress (145), defective packaging of sperm chromatin (146) and apoptosis mechanisms (48,147). The adverse effects of SDF include abnormal sperm, epididymal and embryo development (148,149). The causes for a raised SDF are similar to those of seminal ROS (48) and include the presence of varicocele (150,151), male genital tract infections (152), aging (153), increased body mass index (154), cigarette smoking (140,155), chemotherapy (156,157) and ionising radiation (157).

The need for further understanding of the causes of male infertility

Although oxidative stress provides a mechanism for sperm dysfunction, in many cases there will not be an obvious cause. This is clinically relevant because identifying the cause of the oxidative stress may provide a potential reversible target for optimising sperm function. Moreover, further understanding of the pathophysiological mechanisms that underpin male infertility will allow disease and treatment stratification and could help identify which couples would benefit from ART from those who would likely conceive naturally. This has implications for public health provision, as only 12% of clinical commissioning groups in the NHS offer the recommended 3 cycles of in vitro fertilization (IVF); instead choosing to prioritise other areas of health and disease (158).

Another aspect to patient care which is often not addressed clinically is the psychological distress associated with the diagnosis of infertility. Domar et al. (159) compared the mental health of infertile and fertile women using the Beck depression inventory (BDI) and the centre for epidemiological studies depression scale (CESD). The authors reported higher BDI (36.7 vs 18.4, $p=0.025$) and CSED (25.8 vs 13.2, $p=0.086$) scores in infertile women compared to fertile controls. The absence of an identifiable pathology can also cause further patient distress. Although there are no specific studies analysing unexplained infertility, Rhodes et al. (160) investigated patients with chronic back pain and reported those with an undiagnosed pathology felt distressed at the uncertainty and also that those who went on to be diagnosed with organic pathology felt a sense of relief and validation. This highlights that understanding the pathophysiological mechanisms or aetiological factors to male infertility may be beneficial in patient counselling and also in aiding patients mental health.

1.6 The role of microbes in male infertility.

1.6.1 Microbial infections

Genitourinary (GU) infections have been reported to be the cause of male infertility in 6.9% of cases (50). GU infections can be of bacterial, viral, and fugal aetiology (Table 1.6).

Table 1.6: Table summarising recognised microbial infections causing impaired sperm parameters.

Categories	Types of microbes	Clinical manifestation	Impact on male infertility
Bacterial	Chlamydia trachomatis Neisseria gonorrhea	Painful, swollen epididymis/testicle or Urethral discharge but can be asymptomatic.(161)	Orchitis causing germ cell atrophy. Peritubular fibrosis causing an epididymal stricture resulting in obstructive azoospermia. (161)
Viral	Mumps	Parotid and testicular swelling	Orchitis causing germ cell atrophy.(58)
Fungal	Candida albicans	Infection of the glans penis.	Candida albicans produces soluble factors that reduce sperm motility.(162)

Both infection and inflammation of the urethra, prostate, testicle, and epididymis can result in abnormalities in spermatogenesis and the WHO developed the nomenclature male accessory gland infections (MAGI) as an umbrella term to describe infectious and inflammatory processes occurring in the seminal pathways.

Table 1.7- Table summarising the World Health Organisation classification for male accessory gland infection (MAGI)

Categories	Description	Diagnostic Features
A	Clinical History	Urinary tract infection, sexually transmitted infection, epididymitis.
	Clinical Examination	Thickened epididymis, thickened spermatic cord, abnormal digital rectal examination.
B	Testing of urine post prostatic massage	>10 leukocytes/high-poer microscopic field(magnification x400), presence of chlamydia trachomatis
C	Testing of ejaculate	Peroxidase-positive leukocytes $>10^6$/ml, culture with significant growth of pathogens, presence of chlamydia trachomatis, increased inflammatory markers, reactive oxygen species or abnormal biochemical parameters in the seminal plasma.

The WHO diagnosis of a MAGI (Table 1.7) requires the presence of an abnormality in sperm count, motility, or morphology (according to WHO reference ranges) combined with either 2 positive findings in categories A-C or two positive findings from category C. This diagnostic classification system has been criticised because a prerequisite for the diagnosis of a MAGI is abnormal semen parameters and also the presence of symptoms is heavily weighted on the diagnostic criteria when many patients with infection and infertility can be asymptomatic. Ricci et al. (163) performed semen analyses and culturing on 285 infertile men at a single institution. The authors reported that 29.1% (83/285) of the semen samples were positive for a pathogen and the presence of a positive semen culture was negatively associated with both total (p=0.012) and progressive sperm motility (p=0.0098). Sutton et al. reported that the prevalence of chlamydia trachomatis was 2.48% in a cohort of 1252 American male college cadets and 93.6% of the those infected were asymptomatic (164).

Currently the main methods of testing for infection are bacterial culture and polymerase chain reaction (PCR) testing and there are several limitations to these laboratory diagnostic investigations. The culture method typically requires a high bacterial load and some bacteria may require specific conditions or medium that prevents successful culture testing (165). The PCR method necessitates a *priori* knowledge of the organisms suspected and the clinical sample is only tested for these organisms and therefore may not detect other organisms (166). NGS analysis detects the bacteria present in specimens by identifying the sequence and alignment of the 16S ribosomal ribonucleic acid (rRNA) genes of bacteria (165). This technique is highly accurate at detecting all the bacteria present within a sample because it tests for the genome of bacteria rather than rely on bacterial growth on media (165). Therefore, the NGS of semen could provide a greater insight into the microbial causes of male infertility.

1.6.2 The seminal microbiome

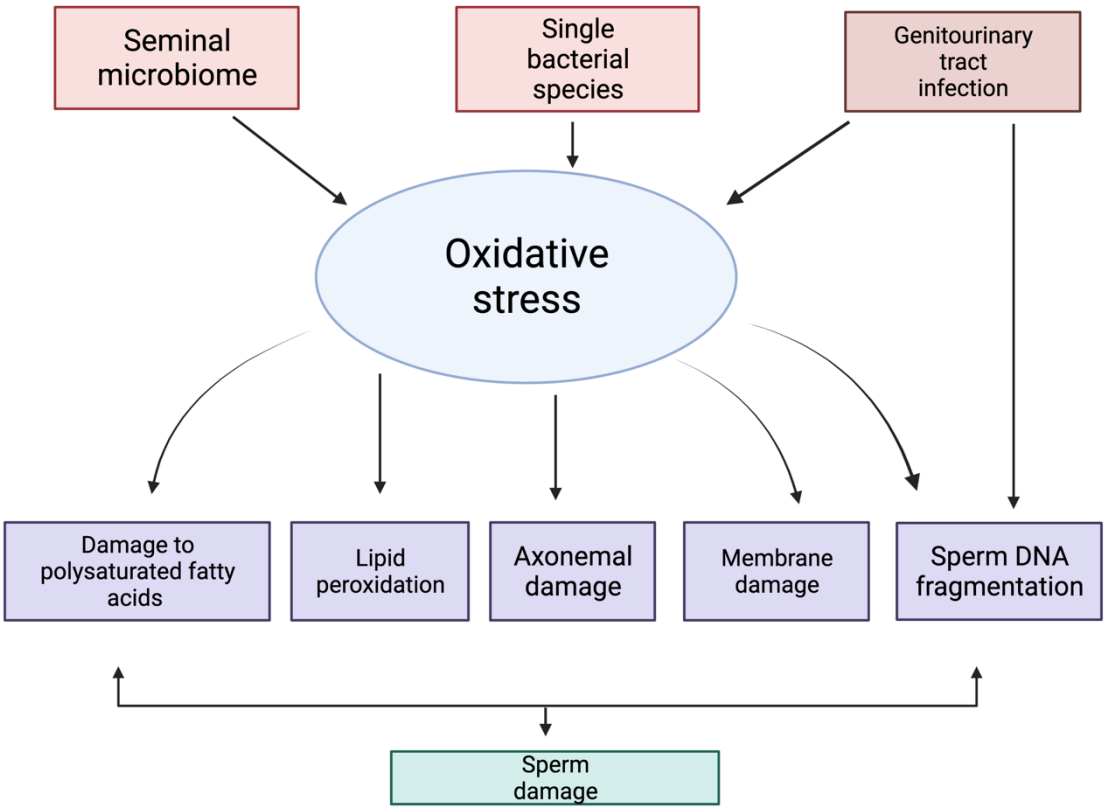
As discussed, in a significant proportion of infertile men the cause will be unknown. Whilst SDF and seminal ROS have been postulated to be potential mechanisms for male infertility, it is unclear in many cases what is the aetiological cause. Technological advancements such as NGS have provided a new insight into the role of bacteria in human health and disease and it has become apparent that the number of individual bacteria within the body are equivalent or more than human cells (167). Moreover, these bacteria work in a symbiotic relationship with human processes to regulate

physiology and homeostasis. The microbiome is the catalogue of all the genes of the microbial organisms together with the host. The urogenital tract has been reported to make up 9% of the total human microbiome (168). It has been postulated that certain bacterial species or asymptomatic bacterial infections may contribute to male infertility (169) and this has been supported by studies showing that the seminal bacteria may adversely affect sperm function (170,171). Our understanding is evolving with improvements in technology and increasing data suggests that semen is not sterile (172) (see Table 1.6).

Given that the main producer of seminal ROS is leucocytes it is theoretically plausible that asymptomatic infections or the seminal microbiome may be causative in idiopathic male infertility (see Figure 1.4)

Figure 1.4: Purported pathophysiological mechanism for which seminal microbiome causes male infertility

Genitourinary tract infection (GU), bacterial species and seminal microbiome can cause both oxidative stress and sperm DNA fragmentation. Oxidative stress can result in damage to polysaturated fatty acids in sperm cell membranes resulting in lipid peroxidation, which results in axonemal(the collective term for the cytoskeletal elements within the tail that produce motility and include microtubules, dyneins and regulatory structures(173)) damage, reduced sperm viability and an increase in sperm morphology malformations(134). Collectively, these abnormalities result in deterioration in sperm motility, sperm protein damage, lipid peroxidation, membrane damage and DNA fragmentation(134). Oxidative stress damages the chromatin present in the nucleus of sperm resulting in DNA fragmentation(134). Oxidative stress triggers apoptotic pathways (including the release of capsases), which results in apoptosis of sperm leading to decreased sperm concentration(134).



However, the current literature regarding seminal microbiome has several limitations (Table 1.8). Several studies use normal and abnormal semen analysis as a proxy for fertile and infertile status respectively. Most studies have small cohort sizes, and no study has compared seminal microbiome with SDF. A single study has correlated seminal ROS with microbiome but this study only examined 37 men (174). Given that the seminal microbiome may be a potential cause for unexplained infertility further research is needed as this will not only help with patient counselling but could also be a target for future therapies.

Table 1.8: Table summarising the current literature investigating male infertility and seminal microbiome

*Only testicular tissue analysed

** patients underwent rectal swab, seminal and urine microbiome

NOA = non obstructive azoospermia, OA = obstructive azoospermia

Study	Comparator groups	Population	Results
Hou et al(2013)(175)	58 Infertile men vs 19 sperm donors	China	Diverse kinds of bacteria were present in the human semen, there were no significant differences between sperm donors and infertility patients. The presence of Anaerococcus might be a biomarker for low sperm quality.
SL Weng et al (2014)(176)	Normal Semen analysis (n=36) vs Abnormal Semen analysis (n=60)	Taiwan	The proportion of Lactobacillus and Gardnerella was significantly higher in the normal samples, while that of Prevotella was significantly higher in the low-quality samples.
Chen et al (2018)(177)	NOA (n=6) vs OA (n=6) vs Fertile Control (n=5)	China	NOA/OA had greater numbers of Bacteroidetes and Firmicutes phyla, whereas the number of Proteobacteria and Actinobacteria was decreased compared with the Control group.
Baud et al (2019)(178)	Normal semen analysis (n=26) vs Abnormal semen analysis (n=68)	Switzerland	Differences in overall microbiota composition did not correlate with semen analysis parameters.
Alfano et al (2018)(179)*	5 men with idiopathic NOA and negative surgical sperm retrieval, 5 men with idiopathic	Italy	Normozoospermic cohort- predominant phyla included *Actinobacteria*, *Bacteroidetes*, *Firmicutes Proteobacteria* In idiopathic NOA with positive surgical sperm retrieval there were increased bacterial DNA with decreased *Bacteroidetes* and *Proteobacteria*.

	NOA and positive surgical sperm retrieval and 5 normozoospermia men undergoing orchidectomy		In idiopathic NOA with negative surgical sperm retrieval there was a decrease in *Firmicutes* and *Clostridia* and an absence of *Peptoniphilus asaccharolyticus* but an increased level of *Actinobacteria*.
Monterio et al (2018)(180)	Normal semen parameters (n=29) vs infertile men with abnormal semen parameters (n=89)	Portugal	In infertile men with hyperviscositity there was increase in Proteobacteria and decreased firmicutes (although this was not statistically significant). Lactobacillus was low in all species but highest proportion in control group (0.6%) and lowest in hyperviscosity infertile male cohort (>0.1%).
Mandar et al (2015)(181)	23 couples with male infertility	Estonia	Concordance between vaginal and seminal microbiomes. In vaginal samples, lactobacilli were the most predominant species. Proteobacteria was in a higher concentration in leukocytospermic men.
Amato et al (2019)(182)	23 couples with idiopathic infertility undergoing IUI treatment. Males had normal semen parameters compared with previous databases of healthy participants.	Italy	Vaginal swabs had a decrease in Lactobacillaceae and an increase in Bifidobacteriaceae compared to healthy controls (not statistically significant). There were no significant differences between idiopathic infertility and controls.

Yang et al (2020)(183)	74 patients with abnormal semen parameters and 58 healthy controls	China	Composition of seminal microbiome significantly different in cohort of men with abnormal semen parameters (*Lactobacillus*, *Bacteroides*, *Delftia*, *Sneathia*, *Enhydrobacter*, *Anaerococcus*, *Mycoplana*, *Finegoldia*, *Stenotrophomonas*, *Methylobacterium*, *Coprobacillus*, *Aerococcus*, *Atopobium*, *Chryseobacterium*, *Kocuria*, *Megasphaera*, *Ralstonia*, *Achromobacter*, *Erwinia*, *Ureaplasma*, and *Filifactor*, and species of *Prevotella copri*, *Saccharopolyspora hirsuta*, *Kocuria palustris*, *Prevotella nigrescens*, *Porphyromonas endodontalis*, *Lactobacillus coleohominis*, *Bacteroides barnesiae*, and *Lactobacillus iners)* compared to controls (*Pelomonas*, *Propionibacterium*, *Bosea genosp*, *Bosea*, *Afipia*, *Sphingomonas*, *Vogesella*, *Brevibacillus*, *Xylanimicrobium*, *Flexispira*, *Pedomicrobium*, *Phyllobacterium*, *Aquimonas*, *Dietzia*, *Sediminibacterium*, *Mycobacterium*, and *Eikenella*, and species of *Brevibacterium aureum*, *Propionibacterium acnes*, *Corynebacterium simulans*, *Eubacterium dolichum*, and *Bacillus thermoamylovorans)*
Lundy et al (2020)(174)	25 primary idiopathic male infertility and 12 men with proven fertility**	America	Infertile men harboured increased seminal α-diversity and distinct β-diversity, increased seminal *Aerococcus,* and decreased rectal *Anaerococcus*. *Prevotella* abundance was inversely associated with sperm concentration, and *Pseudomonas* was directly associated with total motile sperm count.

1.7 Current diagnostic tests in male infertility

1.7.1 Semen analysis

The most widely used investigation for male infertility is semen analysis. Semen analysis involves a sample of ejaculate being inspected microscopically to assess sperm count, motility and morphology. Most practice follows the guidelines and recommendations set out by the WHO (40).

Semen analysis provides a quick representation of sperm production and sperm motility and gives an estimation of the number of "normal" appearing sperm according to the Kruger's strict criteria (184). The main strength of semen analysis is that indicates whether there is a male factor contributing to the couple's infertility. It also highlights whether a surgical sperm retrieval procedure is required i.e., in cases of azoospermia. However, semen analysis also has several limitations:

1) Reference ranges

The WHO reference ranges are derived from studies reviewing fertile men and men of unknown fertility. Subsequently, whether they are a suitable threshold to define infertility is questionable. Moreover, the studies for which the reference ranges were derived from did not have an equal geographical distribution and 90% of the study population were from the Northern Hemisphere (185). This may affect the applicability of the data.

2) Inter-subject variability

Several studies (186–189) have demonstrated that both infertile and fertile men show a significant variation in semen parameters with each ejaculate. Keel et al. (190) analysed 5 consecutive semen analyses for infertile men and noted a coefficient of variation of 54.2% and 34.4% in sperm count and motility respectively. Moreover, Oshio et al. (191) demonstrated that within healthy volunteers semen analysis would change over the course of monthly semen samples with the sperm concentration varying by 4.8+/-4.3 fold (1.5 to 17.2 fold). This highlights that one semen analysis is insufficient in the assessment of fertility.

3) Errors

Semen analysis is subject to errors related to the examination process. As the vast majority of semen samples are heterogenous, insufficient mixing can result in an unrepresentative sample. The WHO guidelines recommend the use of a haemocytometer to calculate sperm concentration. However, variations in manufacturing standards, age and the quality of chambers may alter the sperm concentration results (192).

4) Intra-operator variability

There is limited data showing (193,194) intra-operator variability in semen analysis. Jorgensen et al. (195) compared semen analyses between different andrological teams using 26 different semen specimens. The authors reported that the analysis of variance between andrological teams showed a statistically significant difference in sperm concentration ($F=4.47$, $p=0.0061$) and semen volume ($F=10.16$, $p=0.0001$) interpretation between different teams. Moreover, both sperm morphology and sperm motility evaluation had very poor inter-laboratory consistency although the authors do not report any statistical analysis. Auger et al. (196) compared sperm concentration and motility assessments between different andrologists and observed no significant difference in sperm concentration interpretation but a significant difference in sperm motility evaluation ($p<0.05$ using a random model effect). Thedford et al. (193) compared the sperm morphology assessments of three andrologists for 35 semen samples. The authors observed that the median value among the participants were significantly different ($p=0.021$).

5) Clinical application

Another criticism of semen analysis is that it has been demonstrated to have limited correlation to fertility. Guzick et al.(108) compared the semen parameters between 765 infertile men and 696 fertile men (defined as a man who's partner was either pregnant or who had conceived within the previous two years) and noted an extensive overlap between the subfertile and fertile ranges. The mean and median sperm concentration, motility and morphology were all within the WHO reference ranges for fertility for both fertile and infertile cohorts.

Boeri et al. (197) compared the sperm parameters of 1957 infertile men with 103 fertile controls. The authors reported that 12.1% and 40.8% of the infertile and fertile cohorts respectively had normal semen analysis according to WHO reference ranges.

Leushuis et al. (198) used a cox multivariable regression model to assess the discriminatory capacity of semen analysis to predict natural conception. The authors reported that the predictive power was poor for both a single semen analysis (receiver operator characteristics (ROC) curve (area under the curve (AUC) 0.56 (95% confidence interval (CI)):0,51-0.61))) and the average of two semen analyses (ROC (AUC 0.53(95% CI 0.46-0.56)))

Men with infertility and normal semen analysis without evidence of subfertility in their partners might be a fruitful target for more detailed investigation of other potentially impacting factors.

As previously discussed, there is emerging research into the role of oxidative stress in male infertility and this has spurred newer molecular tests.

1.7.2 Seminal reactive oxygen species

There are several techniques to measure seminal ROS and these are broadly classified into direct (recording the oxidation level) and indirect (recording the products of oxidative stress e.g. lipid peroxidation) tests. Table 1.9 demonstrates the different techniques used to measure ROS.

Table 1.9: Table summarising the different techniques to measure reactive oxygen species

Adapted from review by Agarwal A and Majzoub A(199)

Direct tests	Indirect tests
Electron spin resonance	Myeloperoxidase
Nitroblue tetrazolium activity	Oxidation Redox potential
Flow cytometry	Lipid peroxidation concentrations
Chemiluminescence	Isoprostane
Xylenol orange-based assay	Chemokines
The reduction of cytochrome C	Total antioxidant capacity

Table 1.10: Table of studies investigating the association between seminal reactive oxygen species and male infertility.

ROS (reactive oxygen species); Cpm (counted photons per minute); µMol/L (micromoles per litre); mMol/L(millimole per litre); nmol/L(nanomole per litre); RLU/s (relative light units/sec); NBT (nitroblue tetrazolium); MDA (malondialdehyde); TAC (Total antioxidant capacity); TOS (Total oxidant status); superoxide dismutase (SOD) and glutathione peroxidase (GPx).

Study	Methods	ROS Measurement	Results
Venkatesh et al (200)	33 Infertile men compared to 18 fertile men	Chemiluminescence assay	Significantly higher seminal ROS in infertile compared to fertile group (24.90 vs 0.167 10^6cpm, p<0.0001)
Subramnian et al.(201)	87 Infertile men compared to 23 fertile men.	ROS measured :Thiobarbituric acid reactive species assay (lipid peroxidation) TAC (measured using the 2-diphenyl-2-picrylhydrazyl free radical assay)	Significantly higher Seminal ROS (18.6 vs 6.1nmol/ml, p<0.05) and lower TAC (62.7 vs 83.05%, p<0.05) in the infertile cohort compared to the fertile group.
Riaz et al.(202)	20 fertile and 20 infertile men	Total oxidant (using the Xylenol orange-based assay) and TAC (Fenton's reaction)	Significantly higher TOS (7.42 vs 5.10 µMol/L, p<0.05) and significantly lower TAC (1.29 vs 1.41 mMol/L, p<0.05) in the infertile cohort compared to the fertile cohort.
Agarwal et al.(203)	318 infertile men and 28 fertile men	Chemiluminescence assay	Seminal ROS was significantly higher in the infertile cohort compared to the fertile cohort

			(267.5 vs 64.8 RLU/s, p<0.001).
Fingerova et al.(204)	91 infertile men and 34 fertile controls	Chemiluminescence assay	Seminal ROS levels were significantly higher in infertile men with abnormal sperm parameters compared to fertile controls (1.1 vs 0.26 RLU x 10^3, p<0.005) Infertile men with normal semen parameters did not have a significant difference compared to the fertile cohort (0.31 vs 0.26 RLU x 10^3, p<0.52).
Sharma et al.(205)	28 infertile men and 12 fertile controls	Chemiluminescence	A significant increase in seminal ROS (2.29 vs 1.39, p=0.01) and a significant decrease in TAC (1650 vs 1051.98, p=0.0003) in the infertile group compared to the fertile group.
Ammar et al.(206)	19 fertile men (control) 27 infertile men with asthenoteratozoospermia and 32 teratozoospermia.	NBT and MDA.	Seminal ROS was significantly higher in the infertile cohort compared to the fertile cohort. NBT (%) -AT vs control (71.92 vs 37.21, p<0.001)

			-TT vs control (69.55 vs 37.21 p=0.001) MDA (nmol/L) AT vs control (1.095 vs 0.56, p=0.007) -TT vs control (0.84 vs 0.56 p=0.001)
Agarwal et al.(135)	79 Teratozoospermia infertile men and 28 fertile men	Chemiluminescence	Significantly higher seminal ROS in teratozoospermic patients compared to fertile control (145.4 vs 58.8 RLU)/sec/106, p<0.05)
Dorostghoal et al.(207)	112 infertile men and 105 fertile men	Lipid peroxidation measuring MDA Antioxidant enzyme activity assessed by activity of SOD and GPx	Significantly higher levels of MDA and significantly lower levels of GPx and SOD were present in the infertile compared to fertile cohort (p<0.001).
Shemshaki et al.(208)	100 infertile men and 50 fertile men	Chemiluminescence	The infertile men cohort had a significantly higher seminal ROS compared to the fertile cohort (145 vs 71.4, p<0.5)

Table 1.10 highlights that the majority of studies (utilising a variety of seminal ROS measurement techniques) support a negative association between seminal ROS levels and male fertility. Seminal ROS levels have been used as a marker to predict the success of ART such as IVF but the single meta-analysis examining this relationship only contained 3 studies with an overall cohort of 122 patients (209). The authors reported a significant association between a high ROS and low oocyte

fertilisation rate. This study was limited because it did not provide data regarding the association between ROS and pregnancy or live birth rates.

Limitations of Reactive oxygen species measurement

Although there are several studies that have correlated abnormal semen parameters such as asthenospermia with elevated levels of seminal ROS there are reports that have observed no such association (210). Moreover, there is conflicting evidence on the role of ROS in predicting IVF outcomes (211,212). Agarwal et al. (209) meta-analysis reported a significant association between high ROS and low oocyte fertilisation rate. However, this study was limited as it did not account for male or female factor infertility (137). Moreover the same meta-analysis combined different ROS measurement techniques and the end point was oocyte fertilisation rate rather than pregnancy or live birth rates (137). Consequently, at present there is still controversy regarding the use of ROS in the clinical assessment of infertility.

1.7.3 Sperm DNA fragmentation rate

SDF can be measured either directly (recording the extent of DNA damage) or indirectly (though susceptibility of DNA to protein denaturation) (213). Table 1.11 demonstrates the different techniques to measure SDF.

Table 1.11: Table summarising the different methods of measuring sperm DNA fragmentation

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling); ISNT(in situ nickel translation); SCD (sperm chromatin dispersion); SCSA(sperm chromatin structure assay)

Adapted from Tharakan et al.(48)

Direct	Indirect
TUNEL assay- measures sperm DNA damage through the attachment of deoxyuridine triphosphate to single- and double-strand DNA breaks using terminal deoxynucleotidyl transferase(214).	Comet assay- uses electrophoresis to separate damaged DNA(both single and double stranded) which migrate to form a comets tail whilst the stable double stranded DNA makes up the comets head. (215)
ISNT assay quantifies biotinylated dUTP that attaches to single-strand DNA breaks through DNA polymerase.(216)	SCD assay allows for acid denaturation and removal of nuclear proteins. Consequently, stable DNA (but not fragmented DNA) will produce a halo of dispersed DNA loops.
	SCSA assay measures the degree of DNA denaturation is determined by measuring the changes in colour of acridine orange in the DNA, from green fluorescence to red fluorescence, after heat or acid treatment(217)

There are limitations to each individual SDF test. Both the Tunnel and SCD assay have been observed to have a high intra-assay and interlaboratory variation (218) and a standardised protocol for the comet assay have not been developed (219). The SCSA necessitates a high concentration of sperm and therefore may not be applicable to patients with severe oligospermia and the ISNT assay only detects single stranded DNA breaks (219).

Clinical implications

1) Fertility status

Santi et al. (220) performed a meta-analysis to investigate the ability of the COMET, SCD, SCSA and TUNEL assays to discriminate fertile and infertile men. The authors included 28 studies and observed a significantly higher SDF in infertile compared to fertile men in all the individual assays except the

COMET assay. The authors pooled the data from all the individual assays and reported that overall SDF was significantly higher in the infertile compared to fertile cohorts ($p<0.001$). However, the meta-analysis included data where normozoospermic men and healthy donors were considered fertile rather than those with proven childbearing status.

Cui et al.(221) pooled data from 8 studies to investigate the use of TUNEL, SCD, SCSA and COMET assays. The authors reported an overall (inclusive of the SDF assays) pooled sensitivity of 0.80 and pooled specificity of 0.85 for SDF to diagnose infertility. Furthermore, the TUNEL assay was observed to be the most accurate assay with a sensitivity and specificity of 0.77 and 0.91, respectively. A limitation to the study was that there was significant heterogeneity between the studies.

2) Natural Conception

Zini et al. (222) meta-analysis of three studies, comprising of 616 couples, suggested that a high SDF (using the SCSA test) was negatively associated with the ability to conceive naturally (Odds Ratio of 7.01 (95% Confidence Interval, 3.68– 13.36)).

3) Assisted Reproductive Technologies

The has been several studies investigating the role of SDF in predicting the success of ART. Table 1.12 highlights all of the meta-analyses investigating this topic of research.

Table 1.12 Table summarising the current meta-analyses investigating the association between sperm DNA fragmentation and ART outcomes.

Sperm DNA fragmentation (SDF); Sperm DNA fragmentation index(DFI); IVF(in vitro fertilisation); IUI(in uterine insemination); ICSI (intracytoplasmic sperm injection); PR (pregnancy rate); LBR (live birth rate); MR (miscarriage rate); AO (Acridine Orange Assay); SCSA (Sperm Chromatin Structure Assay); SCD (Sperm Chromatin Dispersion); TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling); OR (Odds Ratio); RR (Risk Ratio); CI (Confidence Intervals); HSROC (Hierarchical Summary Receiver Operating Characteristic); AUC (Area Under the Curve).

Adapted from Tharakan et al. (48)

Name	**ART methods**	**DNA fragmentation Assay**	**Result**
Evenson et al. (2006**)** (223)	IUI and ICSI	COMET, SCD, SCSA and TUNEL assay	For IUI: -DFI<30% associated with a higher chance PR (P=0.0001) and LBR (P=0.03). For ICSI: DFI<30% was not associated with higher chance of achieving pregnancy/delivery (P=0.06)
Collins et al. (2008) (224)	IVF and ICSI cycles	SCSA and TUNEL assay	Abnormal SDF was negatively associated with PR for IVF and ICSI (P=0.045)
Robinson et al. (2012) (225)	IVF, IUI, ICSI and natural conception	AO, COMET, SCSA and TUNEL assay.	MR was significantly associated with a high SDF compared with a low SDF (P<0.00001).
Zini et al. (2013) (222)	IVF and ICSI	SCSA and TUNEL assay	High SDF is associated with lower IVF PR (P<0.05) but no association with ICSI PR (P=0.65)

			For both ICSI and IVF a high SDF associated with pregnancy loss (P<0.0001).
Zhao et al. (2014) (226)	IVF and ICSI	AO, COMET, SCSA and TUNEL assay	High SDF negatively associated with PR for IVF (*P*=0.008) but not ICSI (P=0.65). MR significantly associated with high sperm DNA damage (P<0.0001)
Osman et al. (2015) (227)	IVF and ICSI	COMET, SCSA and TUNEL assay	ICSI and IVF studies: Significant association between low SDF and LBR (*P*=0.0005). This trend persisted when each assay was investigated individually; IVF (*P*=0.01) and ICSI (*P*=0.04).
Li et al. (2016) (228)	IVF and ICSI	SCSA and TUNEL assay	TUNEL assay: Abnormal SDF was negatively associated with PR with IVF (*P*=0.0006) and ICSI (*P*=0.09) SCSA assay: Abnormal SDF was not associated with PR with IVF (P=0.19) or ICSI (*P*=0.38)
Cissen et al. (2016) (229)	IVF and ICSI	COMET, SCD, SCSA, and TUNEL assay	Both the SCSA and SCD assay had a poor predictive accuracy for pregnancy with both IVF and ICSI cycles.

			Both the TUNEL and COMET assay had a fair predictive accuracy for pregnancy for both IVF and ICSI cycles. For the TUNEL assay the HSROC curve sensitivity was 0.84 (95% CI: 0.75-0.90), specificity 0.24 (95% CI:0.11-0.44) and AUC 0.71 (95% CI:0.66-0.74). For the COMET assay the HSROC curve sensitivity was 0.79 (95% CI:0.61-0.90), specificity 0.60 (95% CI 0.48-0.71) and AUC 0.73 (95% CI:0.19-0.97).
Simon et al. (2017) (230)	IVF and ICSI	COMET, SCD, SCSA and TUNEL assay	For ICSI and IVF studies combined an abnormal SDF was negatively associated with PR (*P*≤0.0001) For IVF studies only: OR was 1.15 [95% CI:1.05–1.27 (*P*=0.0033)] For ICSI studies only: OR was 0.89 [95% CI:0.80–0.99 (*P*=0.0254)] Studies which mixed IVF and ICSI: OR: 2.00 [95% CI:1.66–2.41 (*P*<0.0001)]
Deng et al. (2019) (231)	IVF and ICSI	SCD, SCSA, COMET and TUNEL assay	The LBR was not significantly associated with SDF. The PR was lower in the high DFI group than in the low DFI group (risk ratio = 0.85, P<0.01).

			Subgroup analyses demonstrated that this trend was in the IVF subgroup but not the ICSI cohort.
Chen et al. (2019) (232)	IUI	SCD and SCSA assay	SCSA and SCD data combined: high SDF was associated with decreased PR(RR: 0.34[95% CI:0.22–0.52 (P<0.001)]) When analysed by assay a significant association was present for the SCSA but not SCD.
Sugihara et al. (2019) (233)	IUI	SCSA assay	A Low SDF was associated with a higher PR (RR: 3.30 [95% CI: 1.16- 9.39])

Table 1.12 illustrates that there is no consensus regarding the use of SDF in predicting ART success. This can be partly explained by the significant heterogenicity of current data both in terms of methods (i.e., type of SDF test) but also ART treatments (IVF alone or in combination with ICSI).

Limitations to sperm DNA fragmentation tests

Most reviews analysing SDF tests are limited because of the heterogenicity of the data. There are several techniques to investigate SDF which all have inter-assay variability and are yet to be standardised. Moreover, the study populations vary greatly with some including male factor only whilst others include female factor or combined gender issues. The timing of the SDF test was not always uniform and confounding factors such as female age and number of oocytes are not corrected for. Within this context, the use of testing SDF in the assessment of male infertility remains experimental.

1.7.4 Need to further investigate the pathogenesis of unexplained infertility

Currently, both seminal ROS and SDF testing are not routinely performed in the assessment of male infertility and this is because both tests are costly, subject to inter-assay and inter-laboratory variability and require specialist expertise and equipment (234). More evidence of clinical utility is required before recommending their routine use in clinical practice. More studies are required to identify

whether men in unexplained infertility couples have molecular abnormalities such as high seminal ROS or SDF. Given that unexplained infertility is characterised by normal semen parameters, accessibility to further diagnostic tests may allow clinicians to discriminate those with sperm dysfunction from those who are fertile. There are also limitations in our understanding of female infertility; there are no tests that accurately predict oocyte function or the likelihood of successful implantation (49). Moreover, there is no current clinical investigation that tests for bidirectional tubal motility which is needed for embryo transportation (49). Therefore, although this thesis will focus on potential male factors for unexplained infertility, it is worth noting that undiagnosed female factors may also be contributing to unexplained infertility.

1.8 Current management of male infertility

There are no pharmacological treatments available to treat male infertility and conservative, medical, radiological and surgical treatments are aimed at optimising semen parameters or retrieving sperm for use in ART.

1.8.1. Smoking

Smoking was reported to have a negative association with sperm count and morphology in a recent meta-analysis comprising of 16 studies (235). Another meta-analysis comprising of 57 cross sectional studies with a cohort size of 2542 men observed that smoking was associated with abnormalities in sperm count, morphology, motility and semen volume (236). There is little evidence regarding the impact of cessation of smoking on semen parameters or fertility other than one case report (237).

1.8.2 Antioxidants

A Cochrane review (238) investigated the use of antioxidants for male infertility. The report consisted of 48 randomised controlled trials comprising of 4179 men. Only 4 trials reported the impact of antioxidant therapy on natural conception and live birth rates. These studies noted an increased live birth rate with antioxidant use compared with placebo or no treatment (OR 4.21, 95% CI 2.08 to 8.51, $p<0.0001$). However, the studies were of low-quality evidence and three of the studies did not reveal their methods of randomisation or allocation concealment.

In the same Cochrane review (238), two studies reported on live birth rates in the context of ART. These studies collectively showed an increase in live birth rates with antioxidant use compared with placebo (OR 3.61, 95% CI 1.27 to 10.29, $p = 0.02$). Of interest, the individual studies reviewing antioxidants in the ART cohorts, did not themselves demonstrate an increase in clinical pregnancy rate versus placebo (OR 2.64, 95% CI 0.94 to 7.41, $p = 0.07$).

The Cochrane review (238) also reported that there was inconsistent evidence on the impact of antioxidants on semen parameters.

In summary, the current evidence on antioxidants use is of low-quality. This may have limited the clinical use of both SDF and seminal ROS testing because there is no established treatment for an abnormal level of oxidative stress.

1.8.3 Medical

The medical management of male infertility largely consists of hormonal stimulation therapy. The rationale for using this approach is that it may increase intra testicular testosterone and improve or restore spermatogenesis. Hormone stimulation therapy has been shown to be highly effective in hypogonadotropic hypogonadism (5,239) but for other indications, such as idiopathic infertility, data on its efficacy is scarce and inconsistent (5).

1.8.4 Radiological and surgical

The use of varicocele embolisation or surgery in the context of infertility is contentious. A Cochrane review (240) highlighted that varicocele treatment does not improve pregnancy rates. However, this meta-analysis has been criticised for including patients with sub-clinical varicoceles and also patients with normal semen parameters. A further Cochrane review suggested a benefit to varicocele treatment in infertile men with regards to pregnancy rate (OR:1.47 (95% CI 1.05 to 2.05) (241).

1.8.5 Surgery

Men with NOA require surgical sperm retrieval to obtain sperm for ART. Advancements in surgery have improved sperm retrieval rates but even in the most favourable histological subtype (Hypospermatogenesis) the success rates range from 73% to 100% (242). In the least favourable histological subtype (Sertoli cell syndrome) the sperm retrieval rates have been estimated to be 22.5-41% (242).

1.8.6 Assisted reproductive technologies

IVF is the most utilised treatment for couples presenting with infertility. It has been estimated that about 2–4% of births in developed countries are the result of ART (243). Furthermore, the use ART is increasing by 4% per year (244) and the annual NHS budget for IVF alone is £250 million (245).

However, the success rates of IVF have been estimated to be 29% for women aged less than 35 years with declining rates with increasing age groups (245).

In summary, there is a shortage of evidence-based therapies for male infertility and this may be related to a lack of understanding regarding the pathological mechanisms that underpin the disease. Furthermore, given the financial burden of IVF and questionable “success” rate, newer knowledge and further treatments are needed.

1.9 Hormone therapies in non obstructive azoospermia

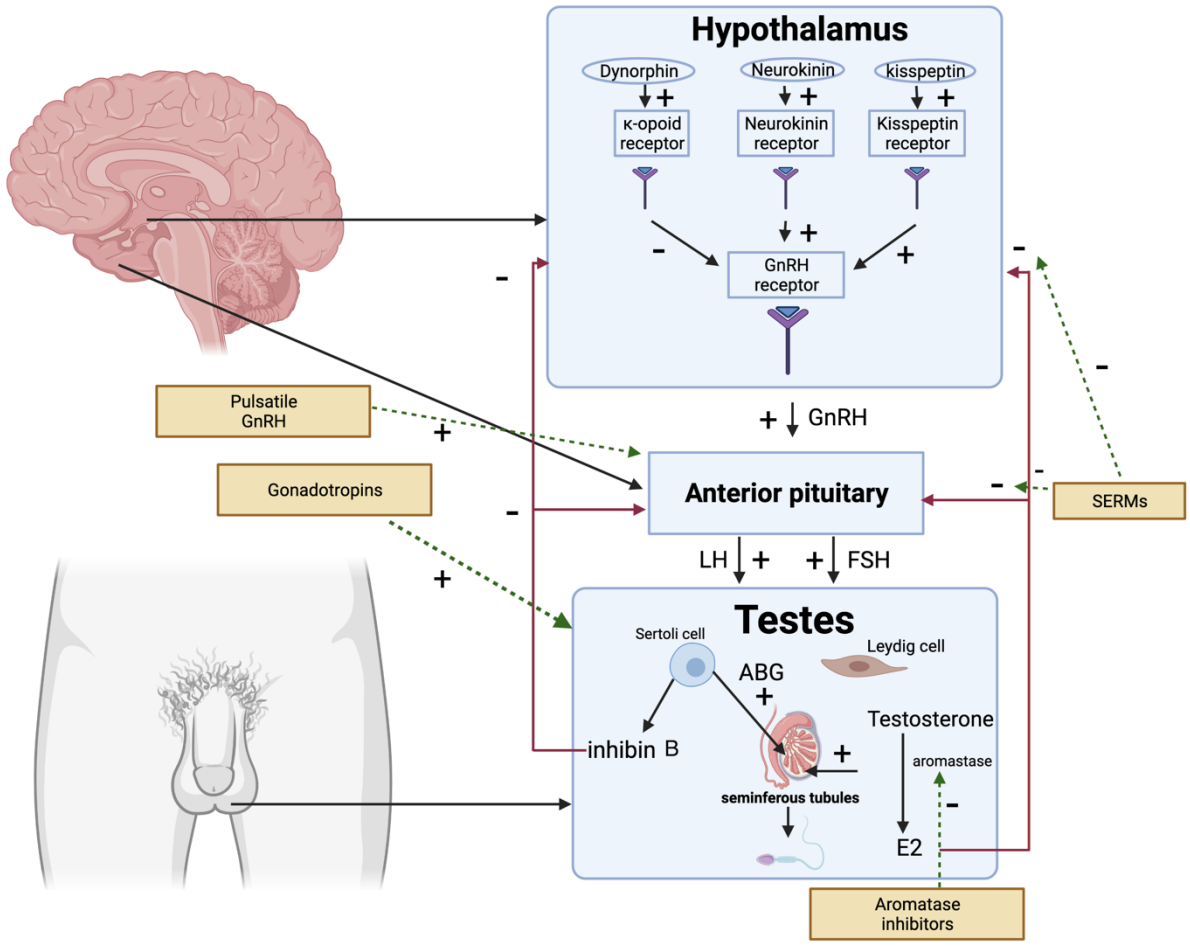
The diagnosis of azoospermia was previously associated with sterility, but the development of testicular sperm extraction surgery combined with ART has allowed men with NOA to father biological children. Conventional testicular sperm extraction surgery (cTESE) involves random, wedge biopsies of the testicle, whilst the micro testicular sperm extraction technique (mTESE) utilises an operative microscope to target larger and more opaque seminiferous tubules (which are more likely to contain sperm) (246). There is data showing that mTESE has a higher surgical sperm retrieval and lower tissue excision rate than cTESE (246,247). However, the surgical sperm retrieval rate in men with NOA is approximately 40-60% (248,249) and there are no established pharmacological therapies to optimise surgical outcomes. The failure of testicular extraction surgery to yield sperm can result in negative psychological sequalae especially in couples where sperm donation is not permissible due to cultural or religious views. Within this context, hormone stimulation therapy has been utilised empirically as an adjuvant therapy to optimise spermatogenesis prior to testicular extraction surgery. A survey of American urologists demonstrated that 65% of respondents use hormone therapy to treat idiopathic male infertility (250). The rationale for hormone therapy in eugonadal (normal hormone status) or hypergonadotropic (raised gonadotrophin levels) men is to stimulate an increase in intra-testicular testosterone (ITT), which is needed for sperm maturation (251). ITT has been reported to be 100 times higher than serum testosterone (252) but the only method for measuring ITT is through testicular aspiration which is invasive. However, within the literature there is no optimal serum testosterone or ITT level that is associated with increased surgical sperm retrieval and similarly the most optimal hormone agent regimen is unclear. There have been no meta-analyses assessing hormone stimulation therapy as an adjunctive therapy for testicular sperm extraction surgery.

The most common hormone stimulation therapies used in clinical practice are gonadotrophins, selective oestrogen receptor modulators (SERMs) and aromatase inhibitors. Gonadotropins include human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG). hCG and hMG are agonists of LH and FSH receptors on Leydig and Sertoli cells respectively and increase testosterone production (11,253–255). SERMs, (e.g. Clomiphene Citrate) inhibit the oestrogen receptors of the pituitary gland and hypothalamus resulting in upregulation of the HPG axis and

increased gonadotropin levels (5,256). Aromatase inhibitors (e.g. Anastrozole) inhibit the aromatase enzyme, which converts testosterone into oestrogen, and thus increase testosterone levels (254,257).

Figure 1.5: Hypothalamic- Pituitary- Gonadal Axis and Hormone stimulation Therapies

Key: + = stimulation, - = inhibition, GnRH = Gonadotropin releasing hormone, T = Testosterone, E2 = Oestradiol, ABG = Androgen binding globulin; SERMs = Selective Oestrogen Receptor Modulators.



There is data suggesting that hormone stimulation therapy can cause side effects including loss of libido, cutaneous rash and venous thromboembolism. The European Association of Urology do not advocate hormone stimulation therapy prior to testicular sperm extraction surgery in men with idiopathic NOA (258). Thus, a meta-analysis of the available evidence is urgently needed to ascertain the benefits and risks of hormone stimulation therapy as an adjunctive therapy to surgical sperm retrieval.

1.10 Rationale, hypothesis and aims of the thesis

1.10.1 Rationale

1) The only method to treat unexplained infertility is through ART. An understanding of the causes of unexplained infertility may help patient counselling, disease and fertility outcome stratification and the formulation of future therapies.

2) Hormone stimulation therapy is being utilised empirically to improve surgical sperm retrieval rates in NOA. An understanding of the efficacy and side effects of this therapy can rationalise its future clinical use to improve the quality and safety of treatment for couples with NOA.

3) In 80% of cases of NOA the cause will be unknown. Identifying a genetic cause for idiopathic NOA may help patient counselling, disease and fertility outcome stratification and formulation of future therapies.

1.10.2 Hypotheses

1) Men in couples diagnosed with unexplained infertility have abnormalities in their seminal ROS, SDF and seminal microbiome compared to fertile controls.

2) Hormone stimulation therapy improves surgical sperm retrieval rates in men with NOA.

3) Some men with idiopathic NOA have underlying novel, monogenic loss of function variants in genes implicated in testicular failure.

1.10.3 Aims

1) To compare the seminal microbiome, SDF and seminal ROS in men with male factor infertility, unexplained infertility and of proven fertility.

2) A systematic review and meta-analysis to investigate whether hormone stimulation therapy improves sperm retrieval outcomes in men with NOA.

3) To identify novel genetic variants in infertile men with idiopathic NOA.

Chapter 2:

Investigating the Seminal reactive oxygen species, sperm DNA fragmentation and seminal microbiome in male infertility

2.1 Introduction

In 30% of infertile couples the man will have a normal semen analysis and their partner will have normal ovulation and fallopian tubal patency (termed ‘unexplained infertility’). The mainstay of treatment for couples diagnosed with unexplained infertility is ART and The Human Fertilisation and Embryology Authority (HFEA) reported that the most common indication for ART in the U.K within the period 2015-2016 was male factor infertility (37% of recorded reasons), followed by unexplained infertility (32%) (259). Guzick et al. pooled data from 45 studies and observed that in unexplained infertility, the pregnancy rate for expectant management was 1.3-4.1% and for intrauterine insemination (IUI) and IVF were 3.8% and 20.7%, respectively (260). The European Society for Human Reproduction and Embryology performed a multicentre, randomised controlled trial investigating the fertility outcomes of couples with unexplained infertility and reported that the pregnancy rate per cycle for IUI was 27.4% and for IVF was 25.7%. Therefore, the current literature highlights a low pregnancy rate for couples diagnosed with unexplained infertility treated with ART. This is pertinent given that IVF has a risk of ectopic pregnancy (1.5-2.1%) (261), ovarian hyperstimulation syndrome (20-30%) (262) and multiple pregnancies (6%) (263). Moreover, Baram et al. (264) investigated the psychological ramifications of IVF in a cohort of 86 couples and observed the presence of depression in 66% of women and 40% of men following the failure of an IVF cycle. In addition to this, Bahadur et al. analysed the HFEA data for ART during 2016 and estimated that the cost burden (cycles and additional maternal and neonatal costs) of IVF to the NHS was £455-653 million whilst IUI was £6.18-8.24 million (265). Therefore, the current management paradigm for unexplained infertility needs to be addressed because it is costly and confers to a low pregnancy rate.

The diagnosis of unexplained infertility is reliant on semen analysis with normalcy defined by WHO reference ranges (40). However, there is data showing that semen analysis has several limitations and is a poor discriminator between infertile and fertile men (chapter 1.7.1). Guzick et al.(108) compared the semen parameters between 765 infertile men and 696 fertile men (defined as a man who’s partner was either pregnant or who had conceived within the previous two years) and noted an

extensive overlap between the subfertile and fertile ranges. Within this context, there has been interest in novel diagnostic markers, including seminal reactive oxygen species (ROS) and sperm DNA fragmentation (SDF) as more accurate discriminators of fertility status (see chapter 1.7). Studies have reported that both seminal ROS (Table 1.8) and SDF (Table 1.10) can accurately discriminate between fertile and infertile men. However, the role of seminal ROS and SDF in the management of unexplained infertility is unclear.

Most of the literature on seminal ROS is related to male factor infertility with a scarcity of data on unexplained infertility. Saleh et al. (266) observed no significant differences in seminal ROS (using a chemiluminescence assay) in men with unexplained infertility compared to fertile controls (p=0.56). However, two studies (267,268) measuring levels of intracellular hydrogen peroxide and superoxide anion reported significantly higher levels of seminal ROS in the unexplained infertility cohort compared to the fertile group.

There have been several studies that have reported a significantly higher SDF in unexplained infertility compared to fertile controls (266,267,269). However, none of these studies compared unexplained infertility to male factor infertility. Indeed, most of the current data investigating unexplained infertility is with reference to fertile controls and it may be beneficial to compare men diagnosed with unexplained infertility with both male factor infertility and fertile controls; this type of analysis would provide insight into how closely related these conditions are and would help guide future diagnostics and management.

Furthermore, there is some data showing that antioxidant therapy may improve sperm parameters (270) and ART outcomes (271). Therefore, should men diagnosed with unexplained infertility have abnormal levels of oxidative stress (SDF and or seminal ROS) they may benefit from antioxidants.

There is increasing data investigating the role of seminal microbiome in male infertility (Table 1.6). However, only one study has investigated the seminal microbiome in men with unexplained infertility and this did not find any statistically significant differences between the infertile and control cohort. This study was limited because it only included 23 men and it made comparisons with a historical control group of healthy men rather than those with male factor infertility or a fertile cohort. Moreover, this study only included Caucasian men. Within this context, a study investigating seminal microbiome

differences in male factor, unexplained infertility and fertile controls was performed to investigate the pathophysiological mechanisms that underpin male infertility.

2.2. Hypothesis and aims

2.2.1 Hypotheses

1) Infertile men with male factor infertility or unexplained infertility will have a significantly different seminal ROS, SDF and seminal microbiome compared to fertile controls.

2) Seminal ROS and SDF levels will be negatively associated with sperm concentration, sperm morphology and sperm motility

2.2.2 Aims

To determine the following:

1) Seminal ROS, SDF and seminal microbiome in males with male factor infertility, unexplained infertility and of proven fertility status.

2) The associations between seminal ROS, SDF and semen analysis.

2.3 Methods

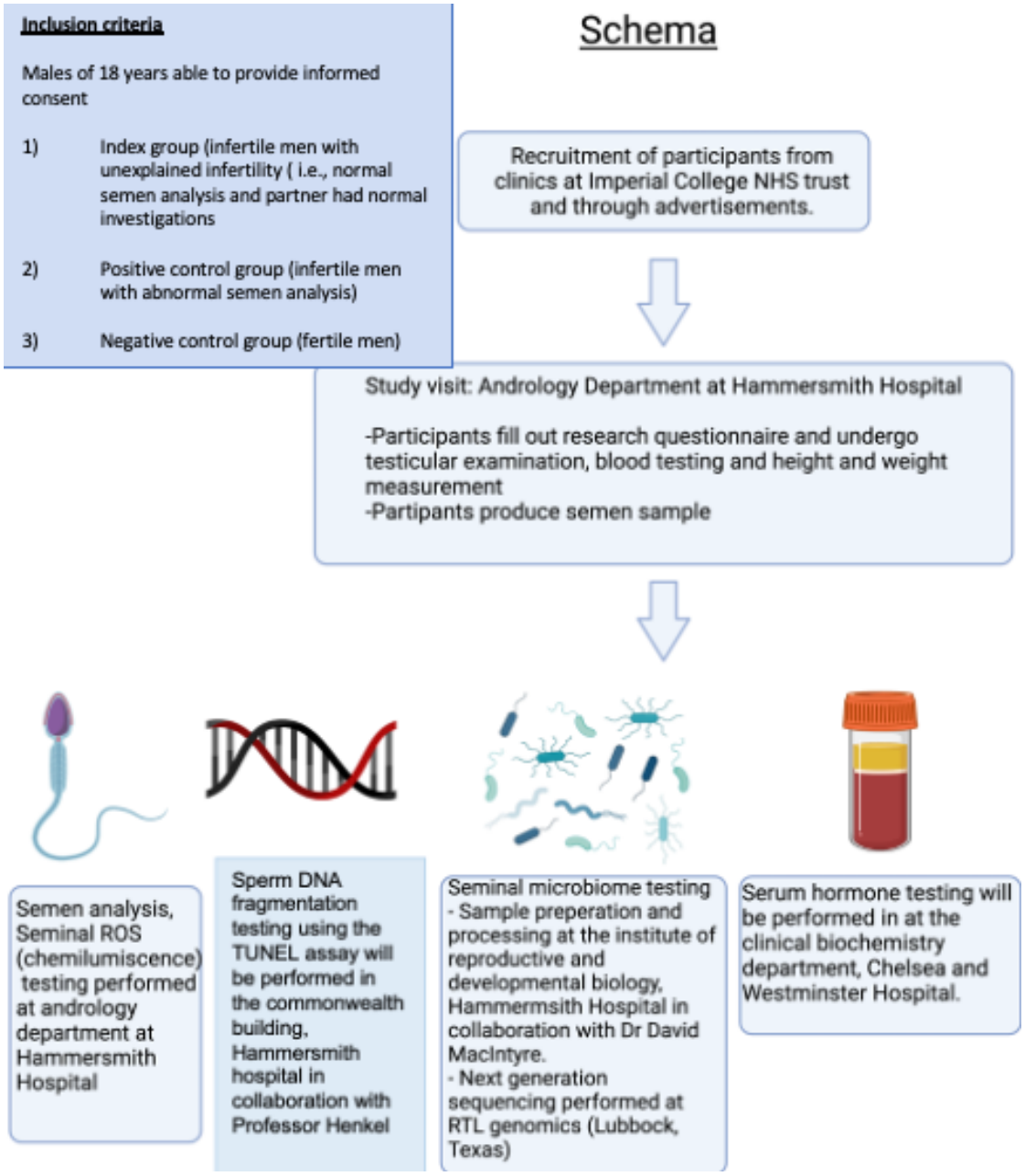
2.3.1 Study design

Ethical approval was granted by the West London & GTAC Human Ethics Research Committee (registration number: 14/LO/1038). Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

This was a prospective paired cohort study comparing semen analysis, SDF, seminal ROS, and microbiome between males with male factor infertility, unexplained infertility and proven fertility status (Figure 2.1).

Figure 2.1: Figure showing the study design and protocol for the study.

TUNEL = Terminal deoxy Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling; ROS = reactive oxygen species



2.3.2 Subjects

Subjects were recruited from infertility clinics at Imperial College Healthcare Trust and poster advertisements within the same institution. Infertility was defined as the inability of a heterosexual couple to become pregnant following one year of practicing regular and unprotected sexual intercourse (19) and male factor infertility was defined as an infertile man with one or more abnormalities of their semen analysis (according to WHO reference ranges(40)). Unexplained infertility was defined as an infertile couple where the man had a normal semen analysis (according to WHO reference ranges(40)) and their female partner has normal ovulation (diagnosed clinically by a regular menstrual periods) and tubal patency (confirmed by hysterosalpingography or laparoscopic visualisation). The fertile cohort was defined as any man who was able to conceive through natural conception. No power calculation was performed as this was a proof-of-concept study but the sample size of the cohorts were comparable to other studies investigating seminal microbiome (Chapter 1,Table 1.8)

Participants were evaluated in the Imperial College Research Facility (ICRF) for a single visit and asked to complete a questionnaire regarding their clinical and reproductive history (see 1.1 questionnaire in appendix). Participants also underwent height and weight measurement and testicular examination. Participants produced their semen sample in an allocated private room within the Andrology Department, Hammersmith Hospital. Participants must have completed a period of abstinence of 2-7 days prior to their study visit and all cleaned their glans penis with Clinell (GAMA healthcare, Hertfordshire, U.K) antibacterial wipe prior to masturbation. Samples were collected in a Sterilin (Sterilin Limited, Gwent U.K) 60ml specimen pot.

2.3.2.1 Comparison of serum luteinising hormone, follicle stimulating hormone and testosterone between men with unexplained infertility, male factor infertility and proven fertility status.

Blood was sampled from each participant between the time-period 09:00-12:00 using an aseptic technique with venipuncture. Samples were collected in plain serum Vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA) and allowed to clot prior to centrifugation (using a Hettich EBA 20

machine (Hettich International, Tuttlingen, Germany) for 10 minutes at 3000rpm). Serum was separated and stored at -20°C until analysis.
Serum samples were tested for luteinising hormone (LH), follicle stimulating hormone (FSH) and testosterone levels in the clinical biochemistry department at Chelsea and Westminster Hospital using an automated immunoassay platform with the United Kingdom National External Quality Assessment Service (UKNEQAS) accreditation.

2.3.2.2 Comparison of seminal semen analysis between men with unexplained infertility, male factor infertility and proven fertility status.

All semen samples underwent investigation at the Andrology Department, Hammersmith Hospital, which has UKNEQAS accreditation to perform semen analysis. The specimen was incubated (at temperature of 36+/-1°C) for between 15-60 minutes to allow for liquefaction. Semen analysis was performed according to WHO guidelines (40) and sperm morphology was assessed using Kruger strict criteria. Seminal leucocyte esterase was tested using Siemens (Siemens, Dublin, Ireland) multistix 8SG reagent strips and leucocytes were diagnosed within the following range: 0μL, 15 μL, 70μL, 125μL and 500μL. A level of between 70-500μL was considered positive.

2.3.2.3 Comparison of seminal ROS between men with unexplained infertility, male factor infertility and proven fertility status.

Seminal ROS was measured with a chemiluminescence assay using a luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) solution. The luminometer (GloMax; Promega Corporation; Madison, WI, USA) measures the light emissions when luminol is oxidized in the presence of ROS in the semen (272). The luminol stock solution was prepared every 3 months (validated optimal duration period) and stored in an aluminum foil-covered falcon tube at room temperature in the dark (272).

Prior to seminal ROS testing a working solution (consisting of 50μl luminol stock solution mixed with 950μl Dimethyl Sulfoxide (DMSO)) was prepared, and internal quality control procedures (measuring negative and positive controls) took place. The negative control solution comprised of 400μl of

phosphate-buffered saline (PBS) and 100μl of luminol working solution and this was aliquoted into a 1.5mL eppendorf and tested in the luminometer. The positive control solution contained a mixture of 395μl of PBS, 5μl of 30% hydrogen peroxide and 100μl of luminol working solution and this was aliquoted into a 1.5mL eppendorf and tested in the luminometer. The luminometer expresses readings in relative light units (RLU) at 1-minute intervals for 10 minutes and a mean value is calculated (RLU/sec). The validated negative and positive control value readings to provide accurate seminal ROS measurements are <120RLU/sec and >100,000 RLU/sec, respectively (272). The semen sample must be tested within twenty minutes of ejaculation as data has shown that seminal ROS declines significantly following this time-point (272). An aliquot of 400μL neat semen is pipetted into a 1.5mL eppendorf and mixed with 100μL luminol working solution and then inserted into the luminometer. The luminometer will express 10 readings at 1-minute intervals and a mean value is calculated, the negative control value is then subtracted to provide the seminal ROS value. The normal reference range for seminal ROS is <3.8 RLU/sec/million sperm (272).

2.3.2.4 Comparison of Sperm DNA fragmentation between men with unexplained infertility, male factor infertility and proven fertility status.

A 400 μl of the semen sample was aliquoted into a 15ml falcon tube and stored at -20ºC until analysis. The TUNEL (Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling) assay detects sperm DNA damage through the attachment of dUTP to single- and double-strand DNA breaks using terminal deoxynucleotidyl transferase (214).
The DNA fragmentation assay protocol was a validated methodology from the Cleaveland Clinic. 6 μL of the sample was aliquoted onto a fixed cell chamber for manual evaluation of sperm concentration. A 5mls of the sample was aliquoted into four test tubes (2 patient negative samples and 2 test samples) and each sample spun at 1700rpm for 7 minutes. The seminal plasma was removed from each sample.

For the spermatozoa positive control samples, a diluted hydrogen peroxide solution (1:15 dilution of Hydrogen Peroxide 30%)) was prepared. Spermatozoa was suspended in 1ml of the diluted hydrogen peroxide solution and heated at 50°C for one hour.

The solution was centrifuged for 7 minutes at 1600rpm, the supernatant removed and replaced with 1ml of PBS. This process was repeated a further three times.

The positive and negative controls and the sample were centrifuged for 7 minutes at 1600rpm and the supernatant removed. A 1ml of a 3.7% paraformaldehyde solution was then added to the controls and the samples and all were centrifuged for 4 minutes at 2500rpm. The paraformaldehyde was then removed and 1ml of PBS added. The subsequent samples underwent a second centrifuge for 4 minutes at 2500rpm and a second wash with PBS. The supernatant was removed from the samples and replaced with a 1ml of ice-cold ethanol (70%) and the samples were stored at 4ºC for 15-30 minutes.

A staining solution was prepared (10.00 µL reaction buffer, 0.75 µL terminal deoxynucleotidyl transferase enzyme, 8.00 µL of fluorescein-12-dUTP and 32.25 µL distilled water). The cell pellet was suspended in 50 µL of the staining solution and the sperm was incubated in the staining solution for 60 minutes at 37˚C. Following this, 1.0 mL of rinse buffer was added to each tube and each tube centrifuged at 1600rpm for 7 minutes. The supernatant was discarded from each tube and this process was repeated. The cell pellet was resuspended in 0.5 mL of the propidium/RNase staining buffer and the cells were incubated in the dark for 30 minutes at room temperature.

Both controls and samples were then evaluated by flow cytometry. A minimum of 10,000 events were examined for each measurement at a flow rate of 100 events on a flow cytometer. The spermatozoa obtained in the plots were gated using a forward-angle light scatter and side angle light scatter dot plot to exclude debris, aggregates and other cells. The normal reference range for SDF is <17% TUNEL positive sperm.

2.3.2.5 Comparison of seminal microbiome between men with unexplained infertility, male factor infertility and proven fertility status.

The seminal microbiome analysis was performed by David Macintyre and his team (Institute of Reproductive and Developmental Biology, Imperial College London). A 600µl of the semen sample was aliquoted into an Ultraviolet (UV) irradiated 2 ml eppendorf tube (STARLAB, Milton Keynes, U.K) and stored at -80ºC until analysis. All equipment and non-biological reagents utilised underwent UV irradiation to minimise the risk of bacterial contamination. A 200µl sample of the semen underwent

enzymatic lysis using a combination of lyzosyme, mutanolysin (sigma-aldrich), lysostaphin (sigma-Aldrich) and TE50 buffer through a validated method previously described (27). The sample then underwent a mechanical disruption phase with glass beads oscillation at 25Hz for one minute (Tissue Lyser, Qiagen, Germany). The subsequent lysate was processed using QIAamp DNA mini Kit (Qiagen, U.K) according to the manufacture's protocol. The amplification of the V1 and V2 hypervariable regions of 16S rRNA gene amplicons was achieved using mixed primers 28F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1 with 388R reverse primers. The metataxonomic profiling was conducted via the Illumina MiSeq platform (Illumina, United States of America). The primer sequences were trimmed using Cutadapt (273) and read quality was checked using FastQC (274). The amplicon sequence variant counts per sample were calculated using the Qiime2 pipeline. The Divisive Amplicon Denoising Algorithm software (28) and STIRRUPS reference database (29) were used to categorise sequences into taxonomical species.

The Microbiome analysist software (275) was used to analyse 16S rRNA abundance data.

2.3.2.6 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Macintosh (Version 27.0. 2020. Armonk, New York). Shapiro-Wilk tests were conducted to assess the distribution of the data. The variables which were parametric were sperm motility, semen volume, sperm DNA fragmentation and BMI, whilst the non-parametric variables were sperm concentration, sperm morphology, seminal leucocyte esterase, seminal ROS age and seminal microbiome. The equality of variance of the data was assessed using Levene (both parametric and non-parametric) tests. All variables except sperm motility and seminal leucocyte esterase contained homoscedastic data. Subsequently, the Kruskall wallis test was performed for the variables of sperm concentration, sperm morphology, seminal ROS, seminal leucocyte esterase, age and seminal microbiome. The ANOVA test was performed on semen volume, BMI and sperm DNA fragmentation and the Welch ANOVA for sperm motility. The post hoc analysis for the non parametric variables (sperm concentration, seminal ROS, age and sperm morphology) was performed using the Man-Whitney U test. The sperm motility had unequality of

variance and was therefore assessed using the post hoc Games-Howell test. The variables of semen volume and BMI were assessed with the Hochberg's GT2 test because they were parameteric, of equal variance but contained different sample sizes. The correlation between sperm parameters and seminal ROS was assessed using Kendall's Tau-B correlation test because the dataset was not linear. Parametric data was expressed as mean values (standard deviation) whilst non parametric data was presented as median values (interquartile range).

2.3.3 Results

Two participants were excluded from the study because their semen analysis showed azoospermia and for five participants seminal ROS testing could not be performed owing to insufficient semen volume. In eighteen participants, SDF testing could not be performed owing to insufficient semen volume. In three participants, sperm morphology was not assessed due to measurement difficulties related to a low sperm concentration. Table 2.1 displays the clinical characteristics of the three cohorts studied and the male factor group had the most participants (n = 43), followed by the unexplained infertility (n =31) and fertile control groups (n=24). There was a significantly higher age in the fertile cohort compared to the male factor infertility (40.00 years vs 34.50 years, $p<0.05$) and unexplained fertility cohorts (40.00 years vs 33.50 years, $p<0.05$). Moreover, there was a significantly higher BMI in the fertile group compared to the male factor infertility (27.43 vs 24.96, $p<0.05$) and the unexplained infertility (27.43 vs 24.70, $p<0.05$) groups.

Table 2.1 Clinical characteristics of participants.

Data for age expressed as mean (standard deviation) and body mass index (BMI) presented as median (interquartile range)

= statistically significant ($p<0.05$) when unexplained infertility cohort compared to fertile cohort

* = statistically significant ($p<0.05$) when male factor cohort compared to fertile cohort

	Male Factor	Unexplained	Fertile
Sample size	43 (44%)	31(32%)	24(24%)
Age	34.50 (4.00)*	33.50 (5.00)#	40.00(18.00)*#
BMI	24.96 (3.12)*	24.70 (3.08)#	27.43 (3.66)*#
White British	25(58.1%)	18(58.1%)	9(37.5%)
White Irish	1(2.3%)	1(3.2%)	1(4.2%)
White Other	4(9.3%)	3(9.7%)	3(12.5%)
Indian	7(16.3%)	7(22.6%)	5(20.8%)
Bangladesh	2(4.7%)	1(3.2%)	1(4.2%)
African			2(8.3%)
Caribbean	1(2.3%)		
Black Other		1(3.2%)	
Chinese			1(4.2%)
Other	3(7.0%)		2(8.3%)
Smoker (%)	2(4.7%)	1(3%)	3(12.5%)
Previous STI	2(4.7%)	2(6.5%)	2(8.3%)
Mumps	8(18.6%)	0(0%)	1(4.2%)
Cryptorchidism	6(14.0%)	1(3%)	0(0%)
Varicocele	10(23.3%)	4(12.9%)	0(0%)

2.3.3.1 Semen analysis, seminal ROS and sperm DNA fragmentation.

Comparisons were made between the three study cohorts with regards to the sperm (sperm concentration, sperm motility, sperm morphology, sperm DNA fragmentation) and seminal (semen volume, seminal ROS and seminal leucocyte esterase) parameters (Table 2.2).

Table 2.2: Table showing the seminal and sperm characteristics.

* = Kruskal- Wallis test

= ANOVA test

† = Welch ANOVA test

ROS = reactive oxygen species; RLU = relative light units; sec = seconds

Parametric data (sperm motility, semen volume, sperm DNA fragmentation and BMI) expressed as mean values (standard deviation).

Non-parametric (sperm concentration, sperm morphology, seminal leucocyte esterase, seminal ROS and age) expressed as median values (interquartile range).

Parameter	Male factor	Unexplained	Fertile	p-value
Sperm concentration ($x10^6$/ml)	16.10 (41.20)	82.80 (76.80)	79.00 (112.95)	<0.001*
Sperm motility (%)	43.79 (18.53)	57.45 (7.29)	53.08 (12.89)	<0.001†
Sperm morphology (%)	1.00 (2.00)	2.00 (3.00)	3.00 (3.00)	0.001*
Semen Volume (ml)	3.67 (1.24)	3.35 (1.19)	3.20 (1.43)	0.310#
Seminal ROS (RLU/sec/sperm million)	2.60 (13.33)	0.70 (2.37)	0.99 (3.89)	0.002*
Seminal leucocyte esterase (μL)	70.00(55.00)	70.00(110.00)	97.50(336.25)	0.164*
Sperm DNA fragmentation (%)	24.42(3.37)	19.55(1.99)	17.22(2.63)	0.183#

We observed a significantly lower sperm concentration (Figure 2.2a) in the male factor infertility cohort compared to the unexplained infertility ($p<0.01$) and fertile ($p<0.05$) groups. However, there was no significant difference between the unexplained infertility and fertile cohorts. Similarly, both

sperm motility (Figure 2.2b) and sperm morphology (Figure 2.2c) were significantly lower in the male factor infertility group compared to the unexplained infertility and fertile groups, but no differences were observed between the unexplained infertility and fertile cohorts in these two variables. There was a significantly higher seminal ROS (Figure 2.2d) level in the male factor infertility group compared to the fertile group ($p<0.05$). However, no significant differences were observed between the unexplained infertility and fertile cohorts. Our analysis demonstrated a significantly higher seminal ROS in the male factor infertility group compared to the unexplained infertility group ($p<0.05$).

Figure 2.2a: Box and whisker plot displaying sperm concentration for each different cohort.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.001, Man-Whitney U test p<0.05 (#) between male factor infertility and fertile control; p<0.001 (##) between male factor infertility and unexplained infertility cohort.

↑ = outlier outside the scale, with the adjacent figure representing the sperm concentration level and the figure within the brackets the participant identification code.

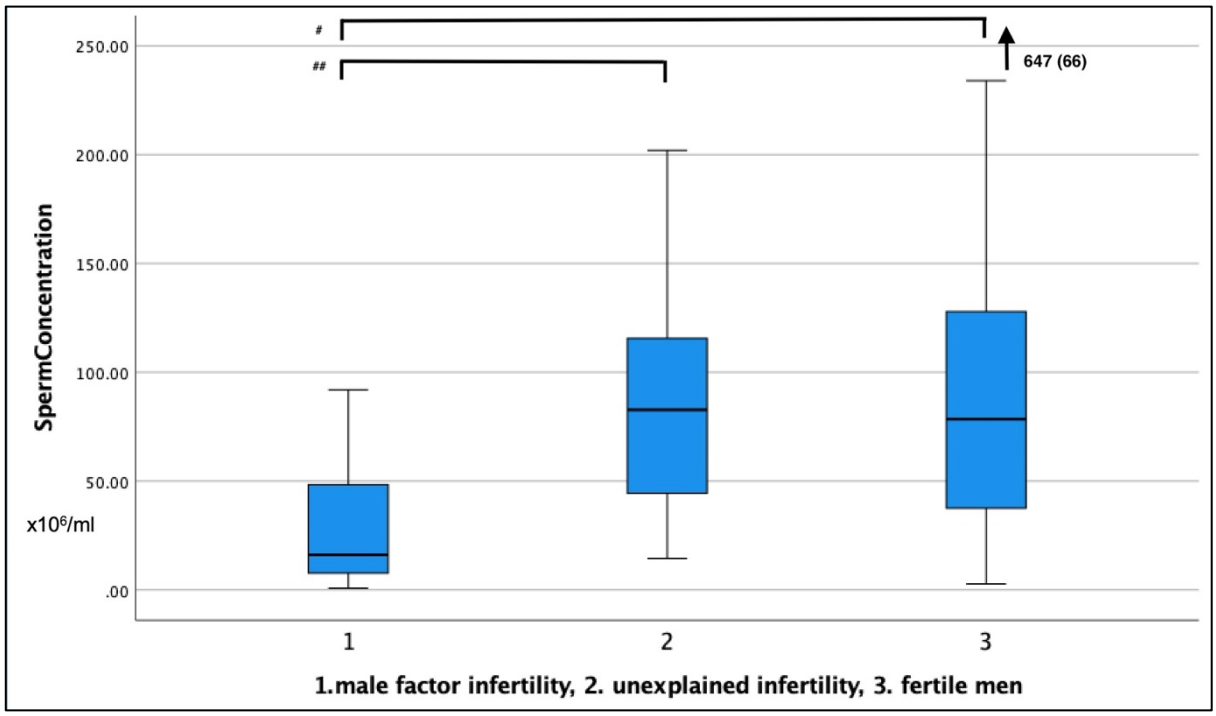


Figure 2.2b: Box and whisker plot displaying sperm motility for each different cohort.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Welch ANOVA: <0.001. Games-Howell test: p<0.05 (#) between male factor infertility and fertile control; p<0.001 (##) between male factor infertility and unexplained infertility cohort.

* = extreme outlier (defined as >3 box lengths from the upper hinge), with the adjacent figure representing the participant identification code.

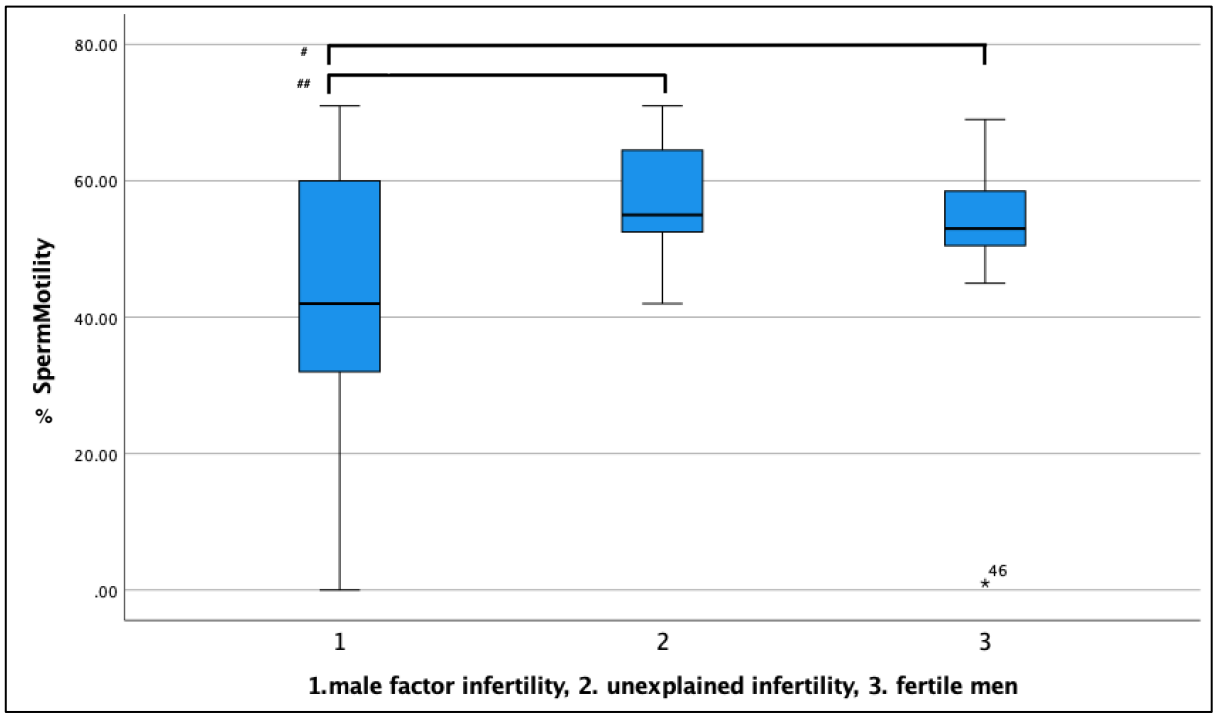


Figure 2.2c: Box and whisker plot displaying sperm morphology for each different cohort.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.001, Man-Whitney U test p<0.05 (#) between male factor infertility and fertile control; p<0.001 (##) between male factor infertility and unexplained infertility cohort. ° = low outlier (defined as >1.5 box lengths from the lower upper hinge), with the adjacent figure representing the participant identification code.

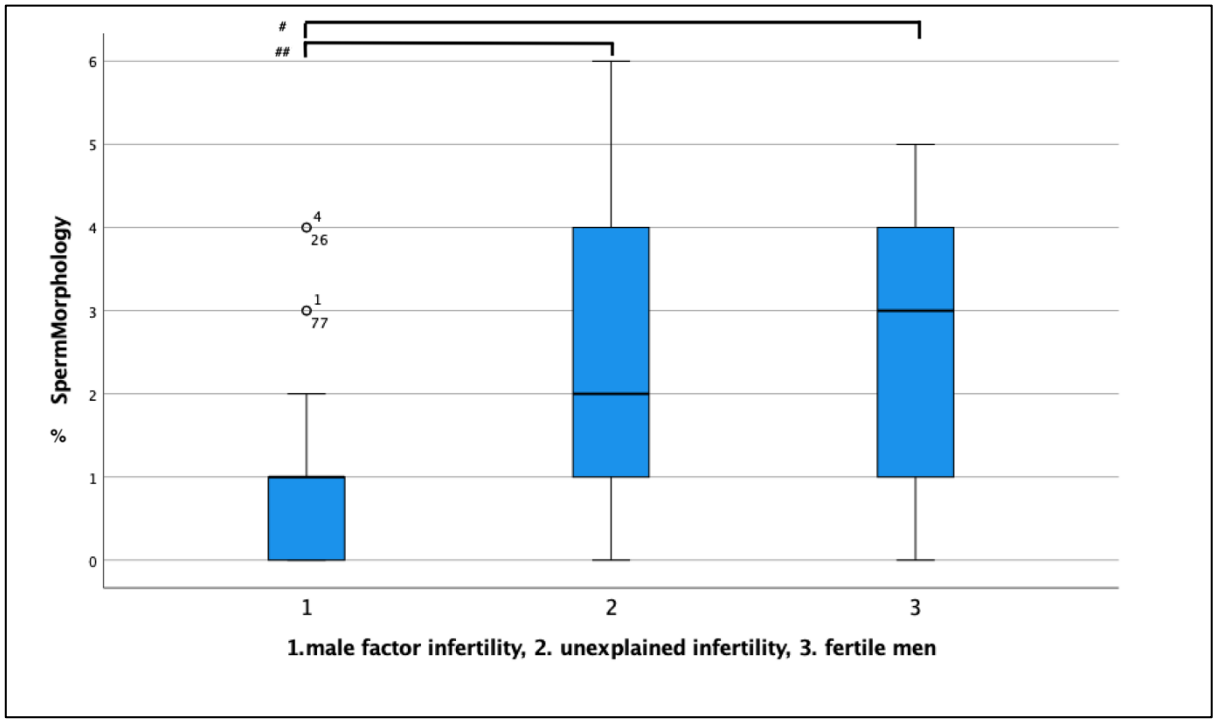
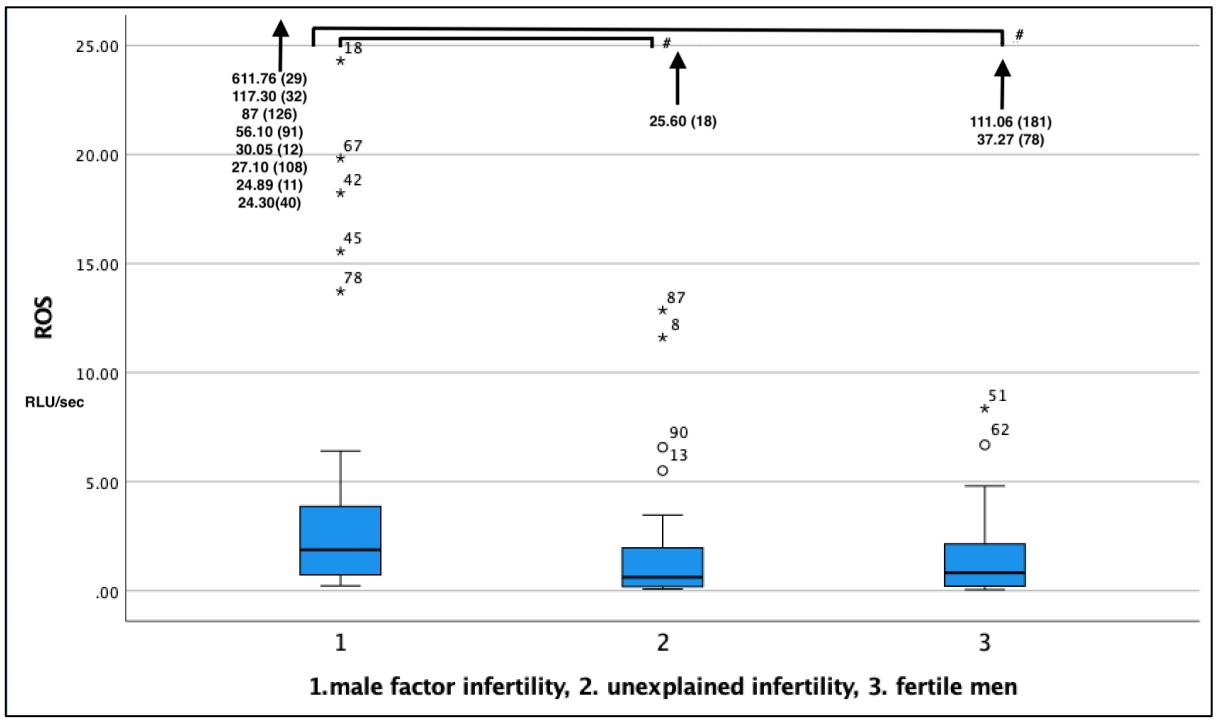


Figure 2.2d: Box and whisker plot displaying seminal reactive oxygen species (ROS) for each different cohort.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.001, Man-Whitney U test p<0.05 (#) between male factor infertility and fertile control; ° = low outlier (defined as >1.5 box lengths from the lower upper hinge), * = extreme outlier (defined as >3 box lengths from the upper hinge) with the adjacent figure representing the participant identification code. ↑ = outlier outside the scale, with the adjacent figure representing the seminal ROS level and the figure within the brackets the participant identification code. RLU = relative light units; sec = seconds.



2.3.3.2 Correlation between sperm parameters and seminal ROS

To assess the association between seminal ROS and sperm and semen parameters, correlation studies were performed. The data was non-linear and non-parametric and therefore Kendall's tau-b correlation studies were conducted. Sperm concentration (Figure 2.3a, -0.488, $p<0.001$), Sperm motility (Figure 2.3c, -0.181, $p=0.001$) and Sperm morphology (Figure 2.3d, -0.34, $p<0.001$) were all significantly negatively correlated with seminal ROS. The seminal leucocyte esterase was significantly positively correlated (Figure 2.3e, 0.198, $p= 0.012$). The seminal volume (Figure 2.3b) was not significantly correlated with seminal ROS ($p = 0.936$)

Figure 2.3a: Scatter plot displaying association between sperm concentration and seminal reactive oxygen species (ROS).

RLU = relative light units; sec = seconds

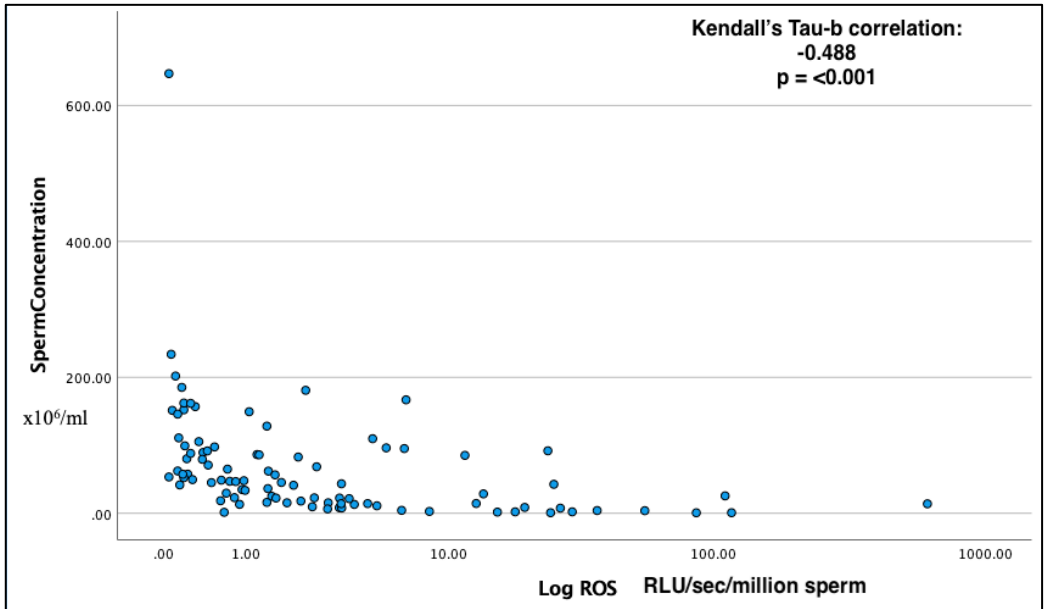


Figure 2.3b: Scatter plot displaying association between seminal volume and seminal reactive oxygen species (ROS).

RLU = relative light units; sec = seconds

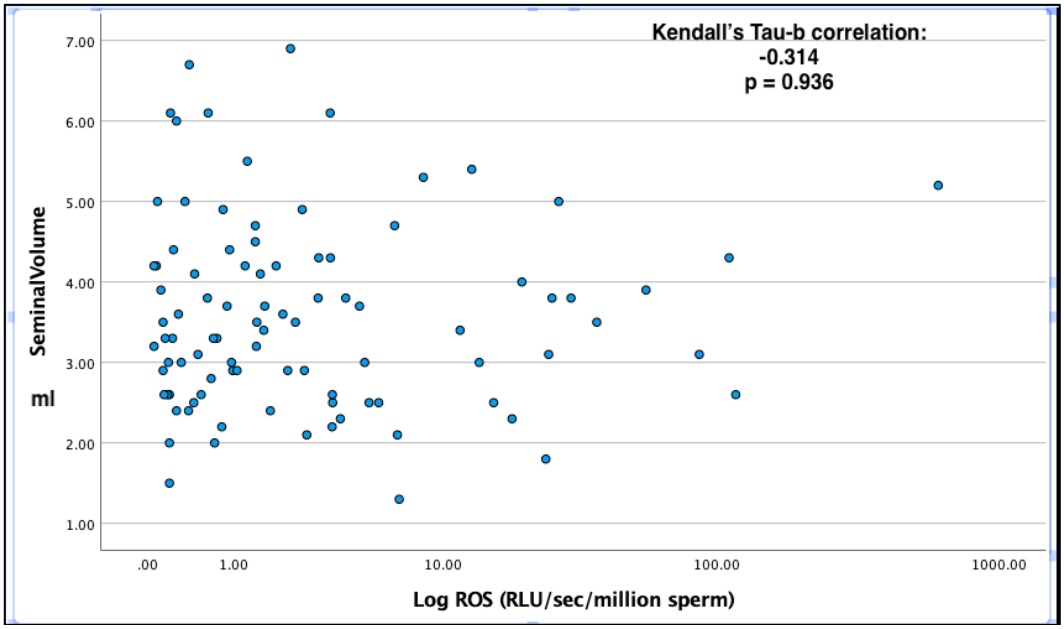


Figure 2.3c: Scatter plot displaying association between sperm motility and seminal reactive oxygen species (ROS).

RLU = relative light units; sec = seconds

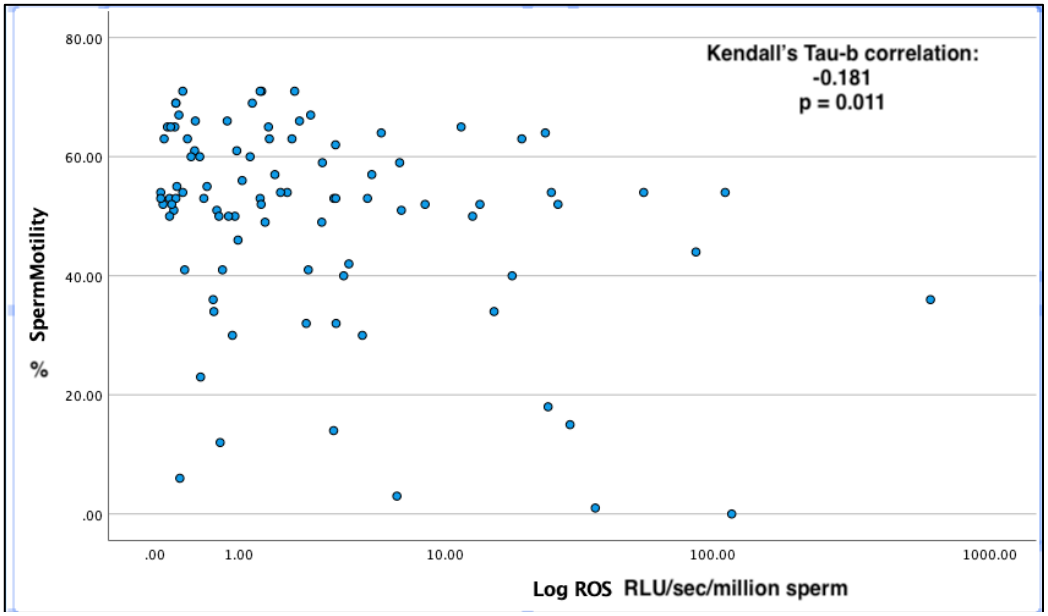


Figure 2.3d: Scatter plot displaying association between sperm morphology and seminal reactive oxygen species (ROS).

RLU = relative light units; sec = seconds

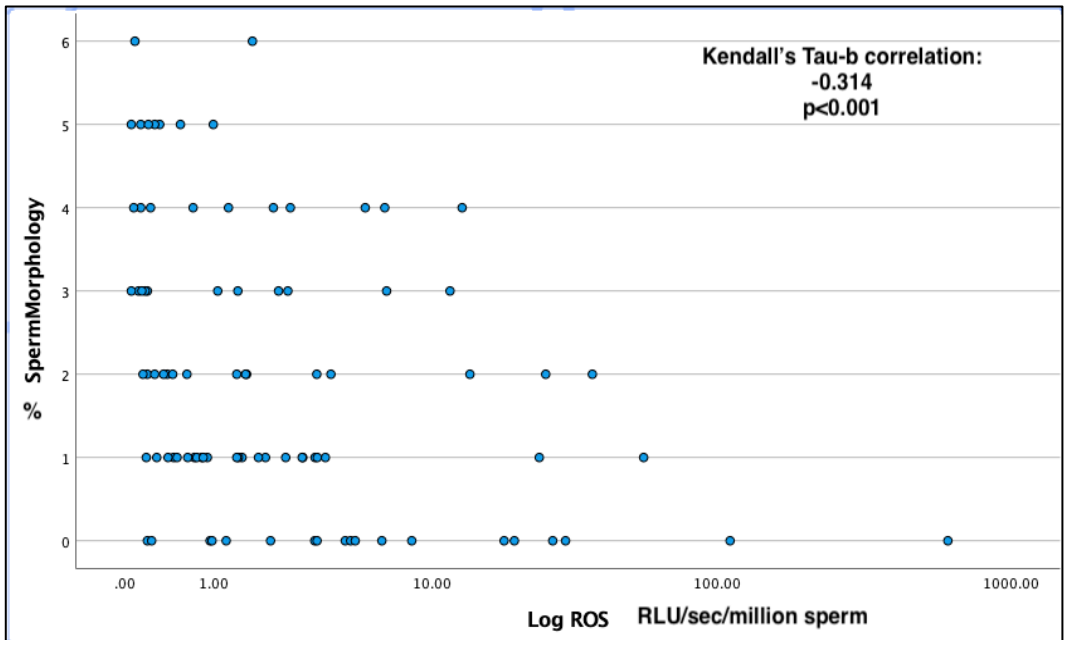
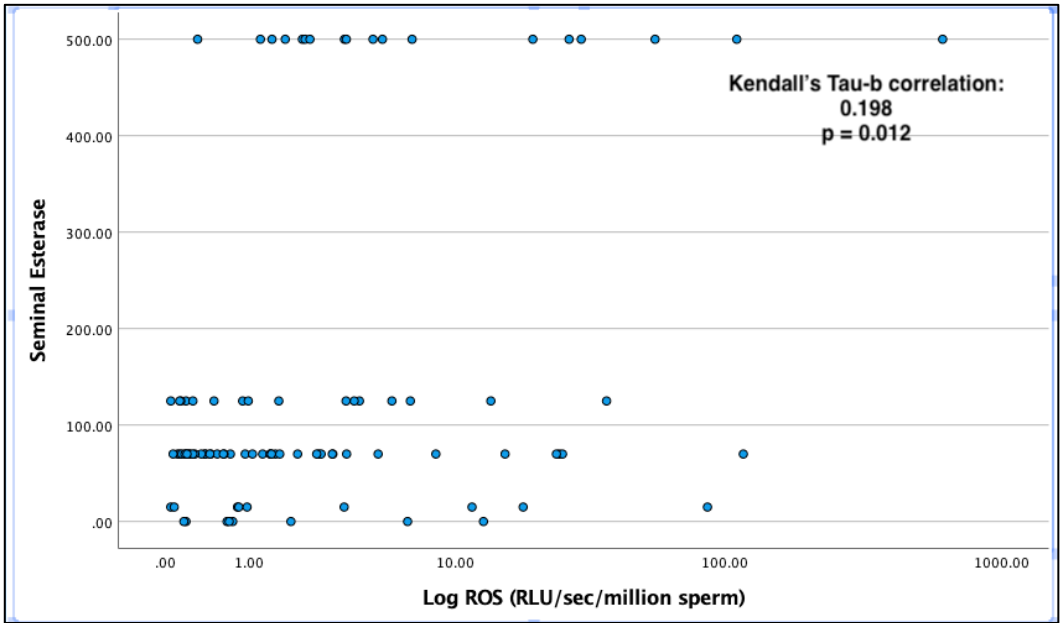


Figure 2.3e: Scatter plot displaying correlation between seminal leucocyte esterase and seminal reactive oxygen species (ROS).

RLU = relative light units; sec = seconds



2.3.3.3 Comparison of hormone profiles

Owing to the COVID-19 pandemic laboratory restrictions on staff personnel and working hours, we do not have the results of the hormone profile tests for the end of the study.

2.3.3.4 Relationship between seminal ROS and semen analysis

A comparison of seminal and sperm parameters between men with normal (<3.8 RLU/sec/million sperm) seminal ROS and abnormal (>3.8 RLU/sec/million sperm) seminal ROS was performed. The variables of sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase were observed to be non-parametric and seminal volume was detected to be parametric. The equality of variance testing demonstrated that sperm concentration, seminal volume, sperm motility and sperm morphology contained homoscedastic data, but seminal leucocyte esterase consisted of heteroscedastic data. Subsequently, Kruskal-Wallis testing was performed for the variables of sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase and ANOVA testing for seminal volume (Table 2.3).

Table 2.3: Table showing sperm and seminal characteristics in men with normal and abnormal seminal reactive oxygen species (ROS).

* = Kruskal- Wallis test

= ANOVA test

RLU = relative light units

Non-parametric data (sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase) expressed as median values (interquartile range).

Parametric data (seminal volume) expressed as mean values (standard deviation).

	Normal Seminal ROS (<3.8 RLU/sec/million sperm) N= 67	Abnormal Seminal ROS (>3.8 RLU/sec/million sperm) N = 26	P-value
Semen volume (ml)	3.63 (1.21)	3.45 (1.10)	0.499#
Sperm concentration (10^6/ml)	53.50 (74.60)	12.10 (50.70)	<0.001*
Sperm motility (%)	54.00 (13.00)	51.50 (21.75)	0.015*
Sperm morphology (%)	2.00 (2.00)	0.50 (2.00)	0.014*
Seminal leucocyte esterase (μL)	70.00 (55.00)	125.00 (430.00)	0.080*
Sperm DNA fragmentation (%)	15.55 (19.80)	21.30 (24.00)	0.194*

We observed significant differences between sperm concentration (Figure 2.4a, p<0.001), sperm motility (Figure 2.4b, p=0.015) and sperm morphology (Figure 2.4c, p=0.014) in men with a normal seminal ROS level compared to an abnormal seminal ROS level. However, both seminal leucocyte esterase and semen volume showed no significant differences between men with normal and abnormal seminal ROS levels.

Figure 2.4a: Box and whisker plot displaying sperm concentration for normal and abnormal seminal reactive oxygen species (ROS) cohorts.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.001(##) between abnormal seminal ROS and normal seminal ROS levels. ° = low outlier (defined as >1.5 box lengths from the lower upper hinge), * = extreme outlier (defined as >3 box lengths from the upper hinge) with the adjacent figure representing the participant identification code.

↑ = outlier outside the scale, with the adjacent figure representing the sperm concentration level and the figure within the brackets the participant identification code.

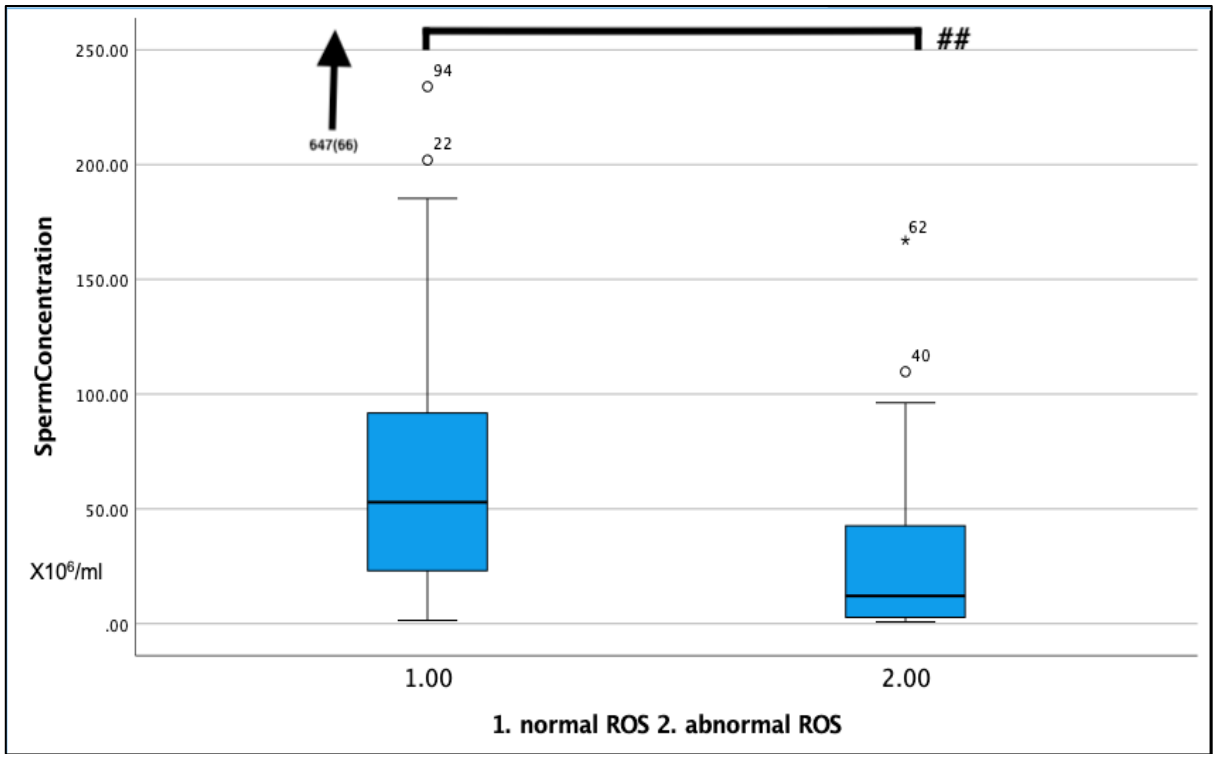


Figure 2.4b: Box and whisker plot displaying sperm motility for normal and abnormal seminal reactive oxygen species (ROS) cohorts.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.05 (#) between abnormal seminal ROS and normal seminal ROS levels. ° = low outlier (defined as >1.5 box lengths from the lower upper hinge), * = extreme outlier (defined as >3 box lengths from the upper hinge) with the adjacent figure representing the participant identification code.

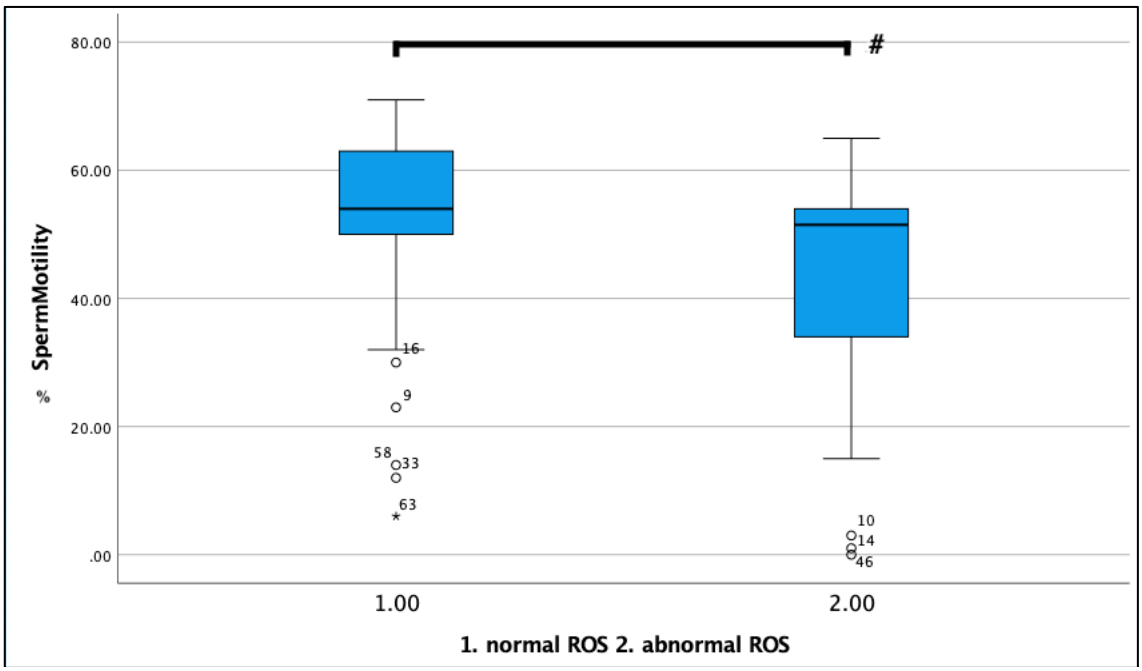
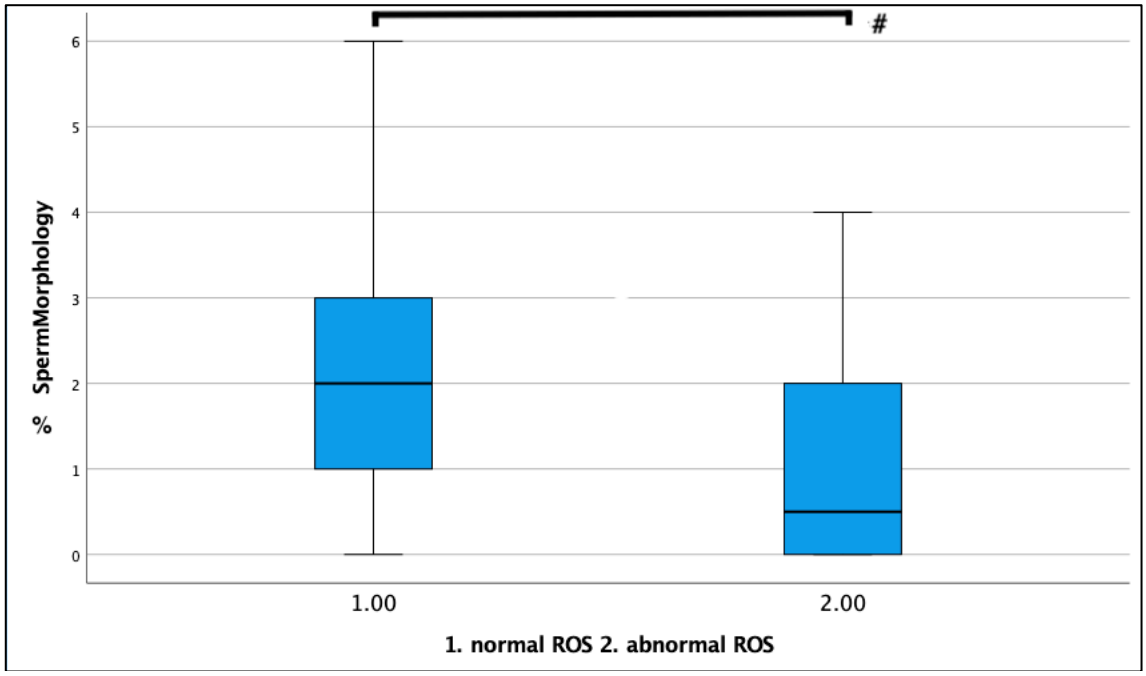


Figure 2.4c: Box and whisker plot displaying sperm morphology for normal and abnormal seminal reactive oxygen species (ROS) cohorts.

The box covers the middle 50% of data values, between the upper and lower quartile. The central line represents the median. Whiskers extent to the maximum and minimum values. Kruskal-Walis: <0.05 (#) between abnormal seminal ROS and normal seminal ROS levels.



There is data suggesting an association between BMI, infection, and seminal ROS (chapter 1, figure 1.3) and thus further sub analysis was conducted on these variables. Although smoking and the presence of a varicocele has also been postulated to be causes of high seminal ROS, the data contained low frequencies of these variables and therefore no statistical analysis was performed.

2.3.3.5 Relationship between a normal and abnormal seminal leucocytes esterase and seminal ROS

Participants results were further stratified by the manufacture's (SIEMENS, Dublin, Ireland: Multistix 8G) recommended thresholds of a normal (<70μL) and abnormal (≥70μL) seminal leucocyte esterase level. The statistical analysis demonstrated that the data was non-parametric and of equality of variance and therefore Kruskal-Wallis testing was performed. We did not observe a significant

difference in seminal ROS levels between those with an abnormal compared to normal seminal leucocyte esterase level (p= 0.616)

2.3.3.6 Relationship between a normal, overweight, and obese BMI and seminal ROS

Participants results were further stratified by the NHS thresholds of a normal (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese (≥30 kg/m^2) BMI level. The statistical analysis demonstrated that the data was non-parametric and of equality of variance and therefore Kruskal-Wallis testing was performed. We did not observe a significant difference in seminal ROS levels (p=0.197) using the omnibus test and therefore post Hoc analysis was not performed.

2.3.3.7 Relationship between sperm DNA fragmentation and semen analysis

There were no significant differences in sperm DNA fragmentation between the unexplained infertility, male factor fertility and fertile cohorts (p=0.183).

A comparison of seminal and sperm parameters between men with normal (<17%) sperm DNA fragmentation and abnormal (>17%) sperm DNA fragmentation was performed. The variables of sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase were observed to be non-parametric and seminal volume was detected to be parametric. The equality of variance testing demonstrated that sperm concentration, seminal volume, sperm motility, sperm morphology, seminal leucocyte esterase and seminal volume contained homoscedastic data. Subsequently, Kruskal-Wallis testing was performed for the variables of sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase and ANOVA testing for seminal volume (Table 2.4).

Table 2.4: Table showing sperm and seminal characteristics in men with normal and abnormal sperm DNA fragmentation.

* = Kruskal- Wallis test

= ANOVA test

Non parametric data (sperm concentration, sperm motility, sperm morphology and seminal leucocyte esterase) expressed as median values (interquartile range).

Parametric data (seminal volume) expressed as mean values (standard deviation).

	Normal sperm DNA fragmentation (<17%) N= 41	Abnormal sperm DNA fragmentation (>17%) N = 39	P-value
Semen volume (ml)	3.43 (1.07)	3.74 (1.31)	0.349#
Sperm concentration (10^6/ml)	62.20 (76.60)	45.40 (75.35)	0.141*
Sperm motility (%)	53.50 (12.75)	55.00 (14.00)	0.736*
Sperm morphology (%)	2.00 (2.00)	2.00 (3.00)	0.314*
Seminal leucocyte esterase (μL)	70.00 (55.00)	70.00 (55.00)	0.661*
Seminal ROS (RLU/sec/million sperm)	1.03 (3.03)	3.03 (3.46)	0.976*

There were no significant differences in sperm concentration, sperm motility, sperm morphology, seminal leucocyte esterase and seminal ROS in those with normal DNA fragmentation compared to those with abnormal DNA fragmentation (Table 2.4).

There is data suggesting an association between BMI (154), infection (152), and SDF and thus further sub analysis was conducted on these variables. Although smoking and the presence of a varicocele

has also been postulated to be causes of high SDF, the data contained low frequencies of these variables and therefore no statistical analysis was performed.

2.3.3.8 Relationship between a normal and abnormal seminal leucocytes esterase and sperm DNA fragmentation

Participants results were further stratified by the manufacture's recommended thresholds of a normal (<70μL) and abnormal (≥70μL) seminal leucocyte esterase level. The statistical analysis demonstrated that the data was non-parametric and of equality of variance and therefore Kruskal-Wallis testing was performed. We did not observe a significant difference in sperm DNA fragmentation levels between those with an abnormal compared to normal seminal leucocyte esterase level (p= 0.657)

2.3.3.9 Relationship between a normal, overweight, and obese BMI and sperm DNA fragmentation
Participants results were further stratified by the NHS thresholds of a normal (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese (≥30 kg/m^2) BMI level. The statistical analysis demonstrated that the data was parametric and of equality of variance and therefore ANOVA testing was performed. We did not observe a significant difference in sperm DNA fragmentation levels (p=0.119) using the omnibus test and therefore post Hoc analysis was not performed.

2.3.4. Seminal microbiome in male factor, unexplained infertility and fertile controls
The analysis of the microbiome samples showed a total read count of 2707512 and the average count per samples was 26807 (maximum per sample was 59677 and minimum per sample 1101). The most abundant bacterial genera overall were *Streptococcus* (18%) followed by *Prevotella* (14%). Streptococcus was also the most abundant bacterial genera in the male factor infertility (24%) and fertile (18%) cohorts and *Prevotella* (16%) in the unexplained infertility group
The most abundant bacterial species overall was *Finegoldia Magna* (9%) followed by *Streptococcus Oralis* (7%). *Streptococcus Anginosus* was the abundant bacterial species in the male factor cohort (10%) whilst *Lactobacillus Iners* (11%) was the most abundant in the unexplained infertility group. The most abundant bacterial species in the fertile cohort was *Finegoldia Magna* (10%).

Figure 2.5: Colour coded stack chart demonstrating the relative abundances of genera in each different cohort

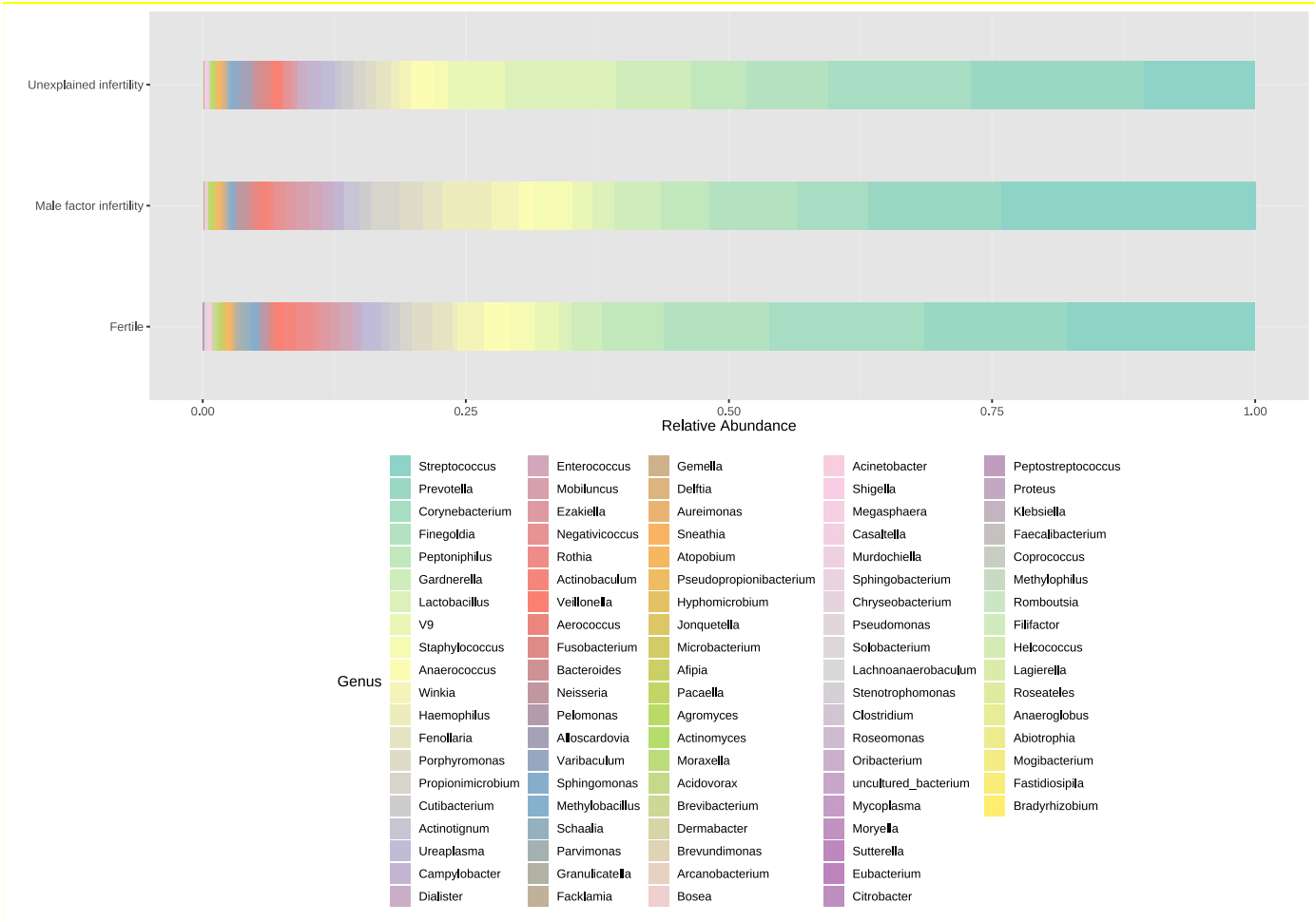


Figure 2.6: Colour coded stack chart demonstrating the relative abundances of species in each different cohort

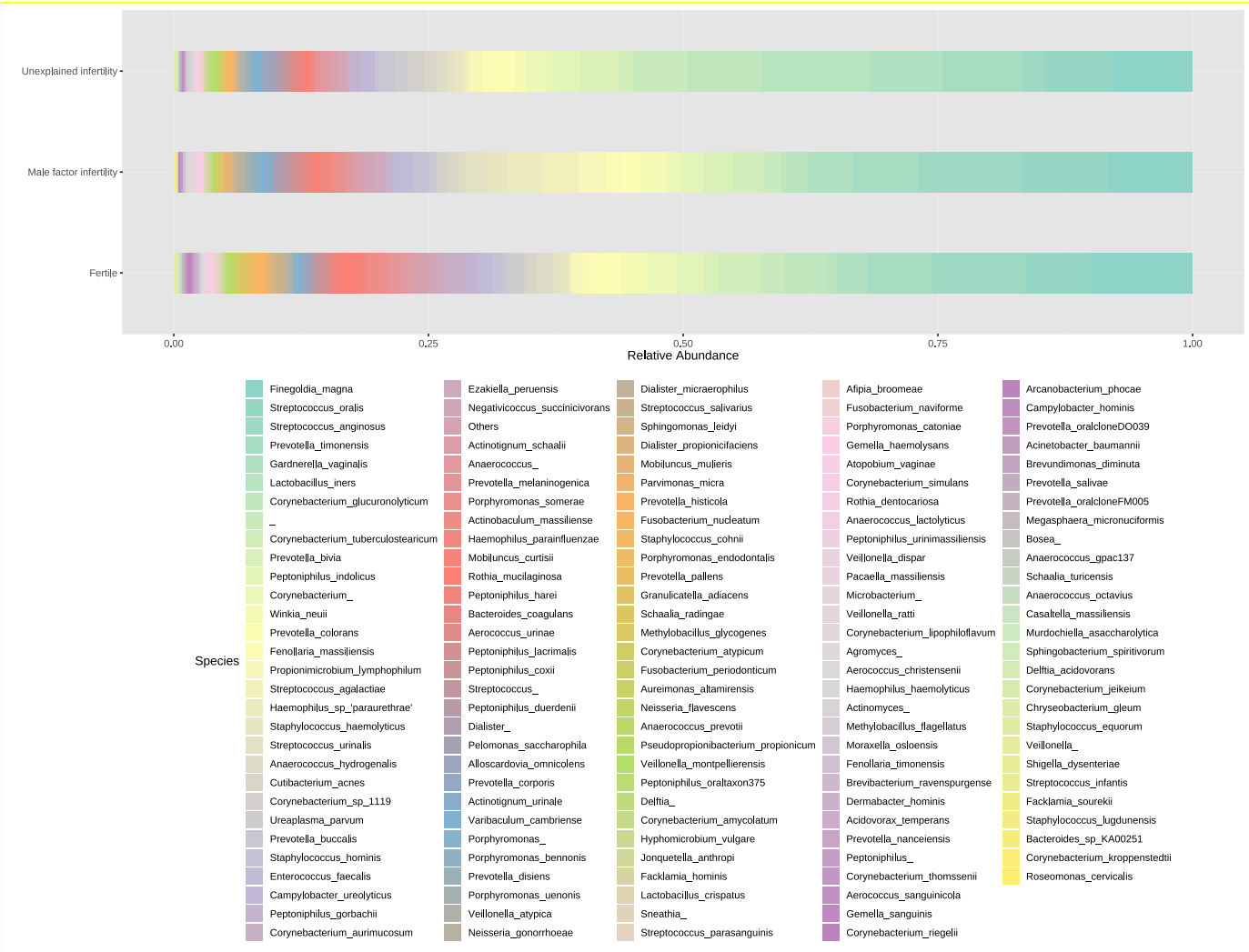
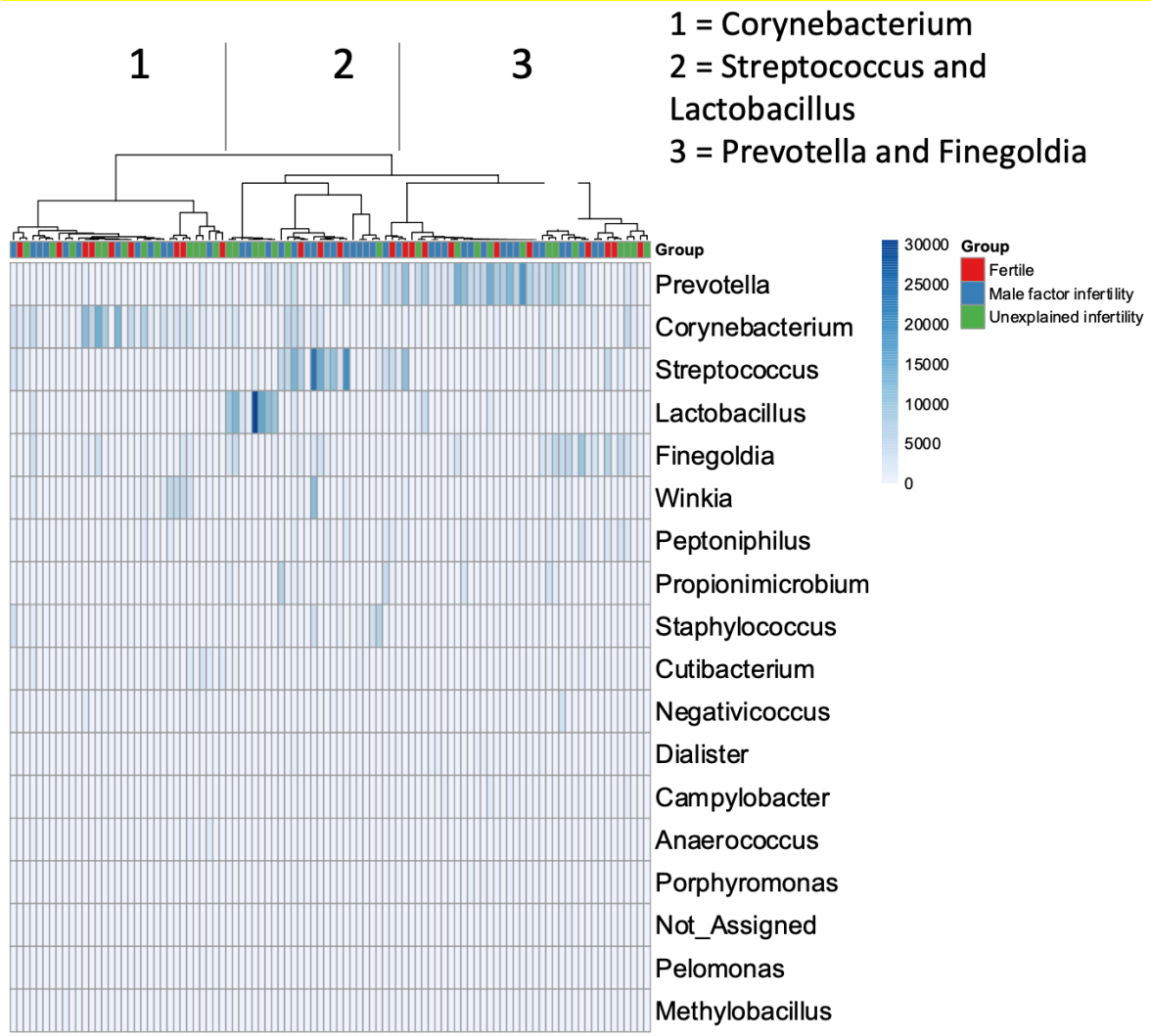


Figure 2.7: Hierarchical clustering of semen microbial communities and heat map demonstrating the proportions of bacterial genera.



Linear discriminant analysis effects size (LEfSe) analysis (276) of the relative abundances of the bacterial profiles in the dataset demonstrated three major microbial signature cohorts : 1) *Corynebacterium*, 2) *Streptococcus* and *Lactobacillus* and 3) *Prevotella* and *Finegoldia* (Figure 2.5). A comparison of these microbiome signatures between the three studied cohorts was performed by Fishers exact test and showed no significant difference ($p > 0.05$).

The data was non-parametric and Kruskal Wallis test was performed to determine whether there were any significant differences between the unexplained infertility, male factor infertility and fertile cohorts in terms of microbiome at the Species (Table 2.5) and Genera (Table 2.6) level. Any significant association underwent further analysis by the Benjamin-Hochberg procedure to determine whether the association withstood the false discovery rate (i.e. the expected proportion of falsely rejected null hypotheses among all of the rejected null hypotheses (277))

Table 2.5: Table showing univariate analysis of seminal species

P value calculated using Kruskal Wallis analysis.

FDR (false discovery rate) calculated using Benjamin-Hochberg procedure

	P value	FDR
iners	0.032576	0.78183
glucuronolyticum	0.11133	0.9905
anginosus	0.1932	0.9905
ureolyticus	0.20076	0.9905
sp_1119	0.2351	0.9905
uenonis	0.29823	0.9905
hominis	0.3101	0.9905
coxii	0.38809	0.9905
tuberculostearicum	0.41586	0.9905
micraerophilus	0.43625	0.9905
lymphophilum	0.49403	0.9905
bivia	0.51384	0.9905
neuii	0.59907	0.9905
magna	0.64082	0.9905
glycogenes	0.70216	0.9905
acnes	0.80143	0.9905
haemolyticus	0.8177	0.9905
indolicus	0.83337	0.9905
saccharophila	0.83778	0.9905
timonensis	0.91287	0.9905
succinicivorans	0.93029	0.9905
colorans	0.96092	0.9905
hydrogenalis	0.9905	0.9905

Table 2.6: Table showing univariate analysis of seminal species

P value calculated using Kruskal Wallis analysis.

FDR (false discovery rate) calculated using Benjamin-Hochberg procedure

	Pvalues	FDR
Lactobacillus	0.032576	0.58637
Streptococcus	0.1932	0.9905
Campylobacter	0.20076	0.9905
Porphyromonas	0.29823	0.9905
Corynebacterium	0.36683	0.9905
Dialister	0.38469	0.9905
Propionimicrobium	0.49403	0.9905
Staphylococcus	0.53882	0.9905
Winkia	0.59907	0.9905
Finegoldia	0.64082	0.9905
Methylobacillus	0.70216	0.9905
Prevotella	0.76262	0.9905
Cutibacterium	0.80143	0.9905
Pelomonas	0.83778	0.9905
Negativicoccus	0.93029	0.9905
Peptoniphilus	0.95764	0.9905
Anaerococcus	0.9905	0.9905

Although the *Iners* species and *Lactobacillis* genus were observed to be significantly different between the three cohorts, neither association withstood the false discovery rate.

The Simpson diversity index was compared between the groups. The data was parametric and ANOVA analysis demonstrated no significant differences (p =0.14). The bacterial richness was also compared between the three cohorts. The data was parametric and ANOVA analysis demonstrated no significant differences (p=0.38)

Due to the COVID-19 pandemic laboratory restrictions on staff personnel and working hours, we do not have the analysis of potential correlations between the seminal microbiome and sperm and seminal parameters for the end of the study.

2.4 Discussion

In 30-50% of infertile men, the WHO semen analysis will be normal, and no underlying cause can be found. Current guidelines do not recommend the routine use of seminal ROS or SDF testing in the diagnostic pathway of male infertility (258). This is the first study evaluating seminal ROS, SDF, seminal microbiome and WHO semen analysis in the cohorts of male factor infertility, unexplained infertility and fertile controls. We report that multiple sperm parameters (concentration, motility, morphology) negatively correlated with seminal ROS. Moreover, there was a significant difference in sperm concentration, sperm motility, sperm morphology and seminal ROS levels between the male factor and the unexplained infertility and fertile cohorts. There was no significant difference in these parameters between the unexplained infertility and fertile groups.

Our data highlights an association between seminal ROS and abnormal sperm parameters and supports that oxidative stress may be contributory to the development of male factor infertility. Our study findings suggest that either seminal ROS and SDF are not contributing to the pathophysiological mechanisms of unexplained infertility or that men diagnosed with unexplained infertility may be fertile with the problems of fecundity arising from potentially undiagnosed female factors. This suggests that antioxidants would not be suitable for men diagnosed with unexplained infertility. Furthermore, the study findings suggest that both SDF and seminal ROS are likely to be normal if semen analysis is within the normal WHO reference range. The current definition of unexplained infertility is limited because it only necessitates bilateral tubal patency in the female partner; however, bidirectional tubal motility is also needed for embryo transportation (49). However, there are no current clinical tests to assess tubal motility and also there are no investigations in use that measure oocyte function or the likelihood of successful implantation (49).

We did not observe any significant differences in SDF between male factor infertility, unexplained infertility and fertile controls. Moreover, our data did not show any significant differences in sperm concentration, sperm motility, sperm morphology, seminal leucocyte esterase and seminal ROS in those with normal SDF compared to those with abnormal SDF. This is inconsistent with the current literature and there are reports of a significant increase in SDF in male factor infertility compared to fertile controls with the TUNEL assay (220) and other SDF assays (221). Our results may have been

affected by the limitations of the TUNEL assay and our methodology. The TUNEL assay requires the terminal deoxynucleotidyl transferase (TdT) enzyme to attach to the deoxynucleotides of the 3'-hydroxyl terminus of DNA breaks. However, should the protamine bound chromatin be resistant to nucleases, then TdT would not attach to the deoxynucleotides and hence the estimation of DNA fragmentation may be lower (278). We did not process our semen prior to freezing and subsequently there is a risk of dead cells in the samples (278). The presence of dead cells in the semen can increase the risk of DNA fragmentation and therefore confound our results. Similarly, sperm preparation can affect the results of the TUNEL assay and explain the discrepancies in our results compared to contemporary literature (278). Moreover, other variables such as light exposure and temperature could have altered our results and are other potentially confounding factors.

Our data suggested that sperm has a diverse microbiome, and this is consistent with the current literature. We observed that the most prevalent genus was *Streptococcus*. Previous studies have reported streptococcus in the seminal microbiome but have observed different bacteria to be more abundant in semen samples including *Lactobacillus* (176,181), *Prevotella* (178), *Enterococcus* (180) and *Tissierellacea* (182). Two studies have observed a high prevalence of *Streptococcus* in the seminal microbiome. Tuominen et al. investigated the seminal microbiome in 31 fertile healthy men and reported a high abundance of *Streptococcus* in men with human papillomavirus (HPV) (279). Moreover, Liu et al. investigated the seminal microbiome of 49 men who have sex with men and observed a high abundance of *Streptococcus* (280). We did not investigate the HPV status or sexual practices of our participants and therefore cannot confirm whether these factors may have affected our results. There is also data (281) showing that *Streptococcus* is a common bacteria in the distal urethra and coronal sulcus. Therefore, it is plausible that our samples may have been contaminated by skin or urethral bacteria. With regards to skin contamination, we asked all participants to wash their hands and clean the penile glans with an alcohol wipe prior to masturbation. However, given the intimate nature of semen production we were not able to confirm that all participants did this or did this effectively despite our instructions. There would be no pragmatic method of avoiding urethral contamination of semen.

We observed a higher prevalence of *Prevotella* in the unexplained infertility cohort. This is in contrast with two other studies that have reported an association between seminal *Prevotella* and abnormal

semen analyses (174,176). *Prevotella* is has been reported to be an oral and vaginal flora and it would be interesting to identify whether this was present in the vaginal microbiome of the participants' partners (176). It is important to acknowledge that intercourse has the potential to cause transmission of bacteria between sexual partners and the effects of some species may be unique to one partner or influential to both. Indeed, there is data (282) showing that sexual intercourse significantly alters vaginal microbiota and it is plausible that this association could be bidirectional (175). A major limitation of this study, and most of the current seminal microbiome literature, is the absence of data investigating the male and female partner concurrently.

Our study did not show a significant difference in seminal microbiome between the three cohorts which is supported by a study by Hou et al (175) which did not observe a significant difference between fertile and infertile men and a study by Amato et al (182) which did not report any significant difference in the seminal microbiome between unexplained infertile men and fertile controls. Our results could have been affected by limitations in our study methodology. We sequenced the V1 and V2 regions of 16S RNA using mixed primers. Although these primers anneal to most known bacterial 16S rNA genes, it has been observed that they do not detect all taxa (175). The efficiency of annealing sequence targets is variable, which can affect the number of bacteria detected and it would be interesting to sequence other regions to determine whether different microbiome signatures are detected (175).

No previous study has investigated the association between semen parameters, SDF, seminal microbiome and seminal ROS in a wide spectrum of infertility disorders. The generation of ROS in spermatozoa occurs via the Nicotinamide adenine dinucleotide dependent oxido-reductase reaction at the mitochondrial level and or the Nicotinamide adenine dinucleotide phosphate metabolism at the level of the sperm plasma membrane (283). Seminal ROS is predominantly produced from neutrophils, macrophages and immature spermatozoa. Seminal ROS are necessary for both sperm maturation and the acrosome reaction (284) but at supraphysiological levels can impair fertility by generating oxidative stress which stimulates germ cell apoptosis (134) and damages sperm plasma membrane thereby causing SDF (128,133). The resulting sequalae include impaired sperm motility (133) and a reduced sperm count.

We observed a significant negative correlation between sperm concentration, sperm motility and sperm morphology with seminal ROS. The seminal leucocyte esterase was significantly positively correlated with seminal ROS.

We also observed that an abnormal seminal ROS (>3.8 RLU/s/10^6) was negatively associated with sperm concentration ($p<0.001$), sperm motility ($p=0.015$) and sperm morphology ($p=0.014$) compared to a normal seminal ROS (<3.8 RLU/s/10^6). There have been similar results reported in other studies (203,285) but there is also data reporting no significant association between sperm motility and the levels of seminal ROS production (210). There was no significant association between an abnormal seminal ROS and ejaculate volume. This is possibly because ejaculate volume is related to secretions from the prostate, seminal vesicles and testis and epididymis and hence more affected by structural blockages rather than oxidative stress.

Further, sub- analysis confirmed that there was also no significant association between an abnormal (≥70μL) and normal (<70μL) seminal leucocyte esterase and seminal ROS ($p<0.252$). Moreover, there was no significant differences between normal, overweight and obese BMI levels and seminal ROS. A raised BMI (143) and infection/inflammation (141) have been reported to be associated with abnormal seminal ROS levels but this was not supported by our study findings; this could be due to the sample sizes of our study and we did not perform sub-analysis on the variables smoking (n=7) and varicocele (n=14) because the frequencies in the data set were low.

It is important to recognise the limitations to this study including its sample size. The study recruitment was restricted by the influence of the COVID-19 pandemic and hence the unequal cohort sizes. We did not perform any power calculation as this was a proof-of-concept study. Furthermore, there was a significantly higher age and BMI in the fertile cohort compared to the infertility cohorts. Moreover, there is heterogeneity in personal performing semen analysis testing which could lead to inter-laboratory variability.

Several methods of seminal ROS measurement are available. We used the chemiluminescence assay, which is a direct measure of seminal ROS but it is limited because it is time consuming and requires specialist equipment (234). Furthermore the chemiluminescence assay can be affected by variables such as semen age and temperature (234). It would be interesting to evaluate these study results with other seminal ROS assays such as the oxidation-reduction potential which has been

reported to be less time consuming and requiring less expertise than the chemiluminescence assay (234).

We performed the TUNEL assay which has been reported to have a high intra-assay and interlaboratory variability (218) and it would be useful to identify how other SDF assays correlate with these study findings.

Although efforts were made to restrict contaminants in the semen sample both in terms of processing (sterile equipment and containers, participants cleaned their prepuce with a sterile wipe prior to semen production) and analysis (exclusion of commonly recognised bacterial contaminates) this could also be a limitation to the seminal microbiome measurement. However, given the intimate and private nature of masturbation and the costs, invasiveness, and risks (including a general anaesthetic) of electroejaculation it is difficult to produce semen in a completely aseptic technique.

Another limitation to our study was we were unable to investigate any correlations between seminal microbiome and semen analysis, SDF and seminal ROS. The study was affected by COVID-19 laboratory restrictions on staff personnel and working hours. Moreover, we were unable to process our hormone profile samples due to similar circumstances. However, we did report the differences in seminal microbiome between our three cohorts which was a main aim of the study.

2.5 Conclusion

In summary, we report the first study comparing seminal ROS, semen parameters, SDF and seminal microbiome in different infertile populations and fertile controls. We report significant associations in seminal ROS and sperm concentration, morphology and motility parameters between male factor infertility compared to unexplained infertility or fertile controls. We did not observe any significant associations with regards to SDF or seminal microbiome between the different infertile populations or fertile controls. This suggests that pathophysiological mechanisms other than the seminal microbiome may be contributing to the observed elevation of seminal ROS in male factor infertility.

Chapter 3:

Investigating the effects of hormonal therapy on surgical sperm retrieval rates in men with non-obstructive azoospermia: A Systematic Review and Meta-analysis

3.1 Introduction

Non obstructive Azoospermia (NOA) is the absence of sperm in the ejaculate because of abnormalities of spermatogenesis (43). NOA has been reported to be present in 1% of the male population and 10-20% of men presenting with infertility (42,286). It has been observed that hypogonadism is present in approximately 50% of all NOA patients (286–288). The practice of hormonal stimulation in NOA patients with hypogonadotropic hypogonadism (i.e., central or secondary hypogonadism) is well established, but this represents a small minority (2-3%) of infertile men (289–293). However, the effects of hormone therapy in NOA men with hypergonadotropic hypogonadism (i.e., primary hypogonadism) or normal gonadotrophins is unclear. The rationale for the use of hormone therapy in men with normal or raised gonadotrophins is to increase intra-testicular testosterone (ITT) levels, which are necessary for sperm maturation (251). The measurement of ITT requires testicular aspiration, which is invasive and serum testosterone has been reported to be an inaccurate surrogate marker with studies reporting differences ranging from 100 to 1000 times (294). Therefore, hormone therapy has been utilised empirically on the basis that optimisation of ITT levels may stimulate spermatogenesis and therefore increase the probability of successful surgical sperm retrieval (SSR). However, there is no recognised optimal serum testosterone or ITT level for spermatogenesis, and the most suitable pharmacological regimen for hormonal therapy remains unclear. Furthermore, there are no meta-analyses assessing the risk or benefits of this treatment in this population of men with NOA.

There is murine data showing that a supraphysiological follicle stimulation hormone (FSH) level may compensate for a low testosterone and stimulate spermatogenesis through a testosterone independent pathway (295). This provides a rationale for using hormone therapy in NOA men with normal hormone status but these findings have not been validated in human studies and there are studies showing that FSH is not predictive of testicular sperm extraction (TESE) success (296,297). Therefore, further understanding is needed to discern the role of hormone stimulation as an adjunctive therapy for SSR.

The common hormone stimulation therapies used in clinical practice are gonadotrophins, selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (AIs). Gonadotropins, such as

human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG) act as agonists of LH and FSH receptors on Leydig and Sertoli cells, respectively, stimulating testosterone production and spermatogenesis (11,253–255). SERMs (e.g., Clomiphene Citrate) inhibit the oestrogen receptors that mediate negative feedback of the hypothamo-pituitary-gonadal (HPG) axis and thus upregulate the HPG axis leading to increased gonadotrophins (5,256). AIs (such as Anastrozole) inhibit the aromatase enzyme, found in both adipose and gonadal tissue. Aromatase normally converts testosterone into oestrogen and thus inhibition of the aromatase enzyme increases testosterone levels and reduces oestrogen levels, thereby reducing negative feedback on the pituitary and hypothalamus and upregulating the HPG axis (254,257).

The SSR rates for NOA patients is approximately 40-60% and have remained static over the last decade (248,249,286). Therefore, hormonal therapy has been hypothesized as a useful adjunctive therapy to improve fertility outcomes (i.e., SSR rates and production of sperm into the ejaculate). However, this needs to be assessed in the context of potential adverse events and the potential time delays associated with adjuvant treatment in this setting. This is especially pertinent given that increasing female age is associated with poorer assisted reproductive techniques (ART) outcomes (298). The European Association of Urology guidelines on Male Sexual and Reproductive Health state that the role of hormone stimulation therapy prior to SSR in men with idiopathic NOA is limited and is currently not recommended in routine clinical practice (66). Despite this, a survey reported that 64.9% of urologists prescribe hormone therapy to treat idiopathic male infertility, with Clomiphene Citrate the most commonly prescribed drug (250).

3.1.1 Hypothesis and aims

Hypothesis

The use of hormone stimulation will increase SSR rates in NOA men undergoing testicular sperm extraction surgery.

Aims

(i) To compare the SSR rates of NOA men treated with hormone therapy compared to those given placebo or no treatment.

(ii) To determine if baseline hormone status (hypergonadotropic vs. normogonadotropic NOA men) alters the effects of hormone therapy on NOA men undergoing SSR.

3.2 Methods

This systematic review and meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) guidelines (286). See Appendix 2: Prisma 2020 checklist). The study was registered in the international prospective register of systematic reviews (PROSPERO, ID CRD42019145226, “Does hormonal stimulation improve sperm retrieval rates in men with non-obstructive azoospermia?”, https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=145226.)

3.2.1 Literature search

A literature search was conducted with the Medline, Embase, Web of Science and Clinicaltrials.gov databases (286). The literature search included studies published between 01/01/1946 to 17/09/2020. The search terms used were: azoospermia, selective oestrogen receptor modulators, tamoxifen, clomiphene, gonadotropins, gonadotropin releasing hormone, aromatase inhibitors, anastrozole, letrozole, testolactone, chorionic gonadotropin, human chorionic gonadotropin, menotropins, human menopausal gonadotropin, sperm retrieval, testicular sperm extraction, microdissection testicular sperm extraction, testicular sperm aspiration, and the corresponding abbreviations (286).

3.2.2 Inclusion and exclusion criteria

All case series, case-control studies and randomised controlled trials (RCTs) were eligible for selection, but authors needed to confirm that the participants had NOA and report the hormone status of the participants and both the type and duration of hormone treatment (286). There were no age restrictions and abstracts and full text studies were reviewed (286). However, animal studies and non-English language studies were excluded. Where there were multiple publications with overlapping cohorts, only the most recent study was included unless specified otherwise (286). The systematic review had no restrictions on study design but the meta-analysis only included data from controlled studies (286).

3.2.3 Study selection

The screening of abstracts and full-text articles were conducted by two independent reviewers (Dr Tharu Tharakan and Dr Daniel Foran) and any differences in opinions were discussed and consensus

achieved by a third reviewer (Dr Channa Jayasena). Where outcome measures were absent from the full-text article, the authors of the study were contacted to provide the data (286).

3.2.4 Outcomes and quality assessment of included studies

There are no reference LH, FSH or testosterone levels to attain optimal spermatogenesis in men with either primary hypogonadism or normal hormone status (286). We investigated the differences in serum testosterone, FSH, and LH between the different types of therapies. We accepted the mean or median cohort testosterone values as a representation of the overall cohort hormone status and a successful SSR was defined as a single spermatozoon or more (286). Conventional TESE (cTESE) was defined as single or multiple wedge biopsies of testicular tissue (286). Microdissection TESE (mTESE) was defined as TESE under operative magnification as described by Schlegel (246,286). Hormone status was defined according to the reference ranges utilised in each individual study or the authors descriptions of the hormone status (e.g., normal hormone profiles) (286). In cases of ambiguity, the authors were contacted for clarification and in the absence of a response, a FSH level of ≥12 mUI/ml and an LH ≥10mUI/ml was used to define hypergonadotropic hypogonadism as these were the most common (mode) upper limit thresholds used in all of the included studies (286). Similarly, hypogonadism was defined as a serum testosterone level <8.8 nmol/l as this was the average (mean) lowest reference threshold for hypogonadism in the included studies (286). If a single gonadotrophin was raised than this was categorised as hypergonadotropic. In addition to this men with a raised FSH or LH and a normal testosterone level were classified as compensated hypergonadotropic hypogonadism (cHH) (286).

Where applicable, we measured baseline hormone parameters, type and duration of hormone agent, type of surgery, surgical sperm retrieval rates, sperm production in the ejaculate and adverse events.

The risk of bias was evaluated using the ROBINS-1 tool (299) (See Appendix 3: ROBINS-1 tool) for the non-RCTs (300–304) studied in the meta-analysis (286). Two reviewers (Dr Tharu Tharakan and Dr Daniel Foran) performed independent assessments of the risk of bias with differences of opinions being resolved by a third reviewer (Professor Suks Minhas).

3.2.5 Meta-analysis and statistical analysis

We investigated whether hormone stimulation therapy (irrespective of class) improved SSR rates and whether this was affected by baseline hormone status (hypergonadotropic vs. normgonadotropic NOA men).

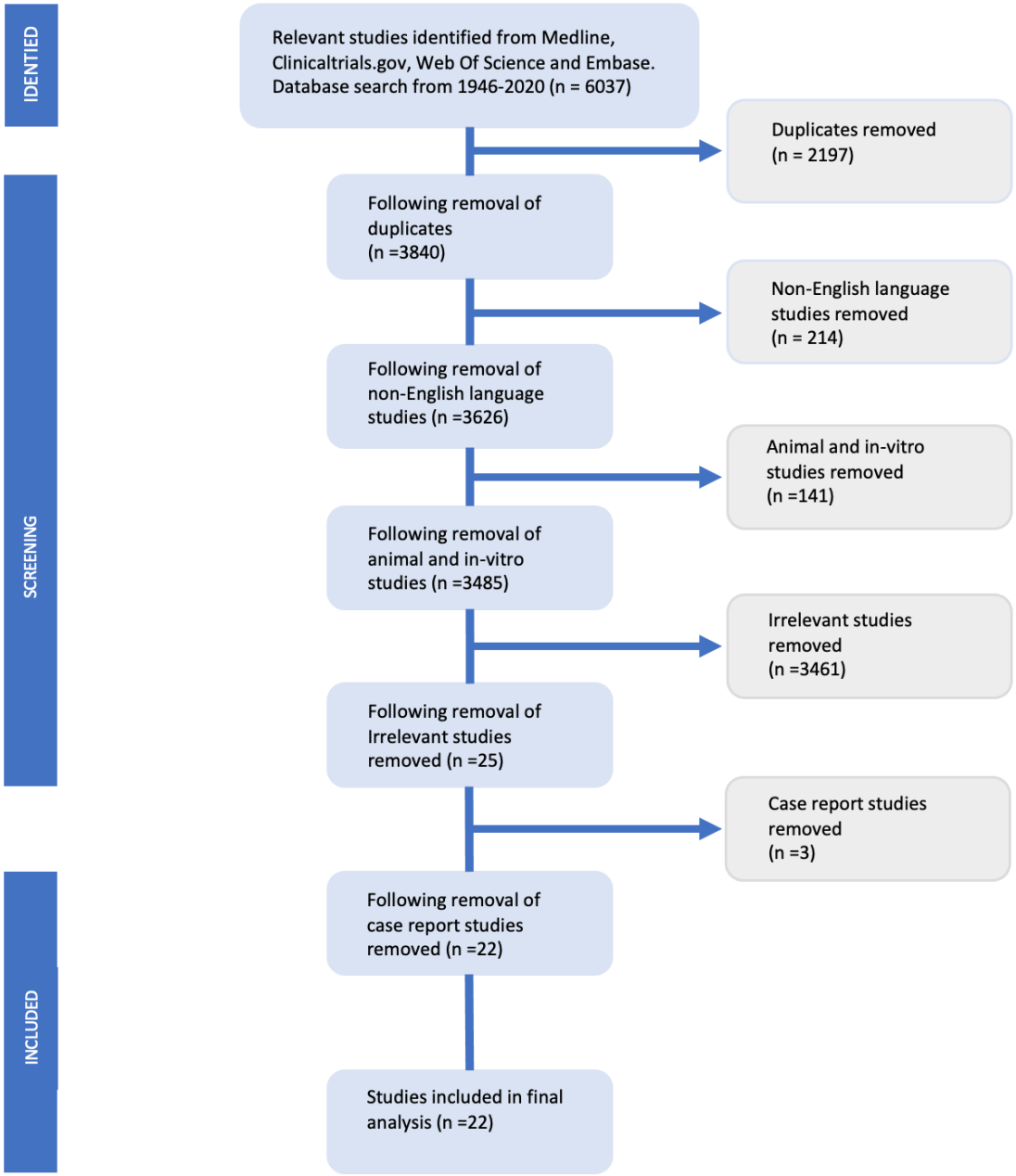
The heterogeneity in SSR was assessed using I^2 statistics. Even when low heterogeneity was detected, a random-effect model was applied because the validity of tests of heterogeneity can be limited with a small number of component studies (286). A funnel plot and the Begg adjusted rank correlation test was utilised to estimate possible publication or disclosure bias (286,305); however, undetected bias may still be present, because these tests have low statistical power when the number of trials is small (286). Overall SSR is expressed as mean percentage (95% confidence interval) (286). All data were calculated using the Comprehensive Meta-analysis Version 2, Biostat (Englewood, NJ, USA).

3.3 Results

3.3.1 Evidence synthesis

Figure 1 shows the PRISMA flow-chart of the studies. 3840 studies were screened and 22 studies included (Table 1) of which 10 were case-control studies, 11 were case series and one was a RCT.

Figure 3.1: PRISMA Flow-chart



For the purposes of the systematic review, we subdivided the different classes of hormonal treatments:

3.3.2 Gonadotropin Therapies

Primary surgical sperm retrieval surgery

Cocci et al. (302) treated 25 normogonadotropic, eugonadal NOA patients with hCG for 3 months prior to a primary mTESE. They observed that 20% of the treated group produced sperm in their ejaculate (mean concentration 0.9 million/ml), compared to zero in a quasi-control group represented by a retrospective cohort of patients who had not received any hormonal therapy ($p<0.05$). The authors also reported a significant improvement in SSR rates (40% vs. 28%; $p<0.05$) associated with hormonal therapy.

In contrast, two other studies using hCG (301) and FSH (300) in NOA patients with normal hormone status reported no overall differences in SSR rate compared to patients that received no hormone therapy. However, when stratifying for histology there was a significant increase in SSR rates in men treated with FSH who had focal spermatogenesis/hypospermatogenesis on histology when compared to the control group ($p<0.05$).

Sen et al. (306) treated twelve normogonadotropic, hypogonadal NOA men with hCG for 3 months prior to a primary mTESE. The sperm retrieval outcomes were compared with 12 hypergonadotropic NOA men who proceeded straight to mTESE without any hormonal therapy. The authors reported that 3/12 (25%) of the intervention group produced sperm into their ejaculate compared to 0/12 of the control group. Moreover, 6/9 (66.6%) of the intervention group had a successful SSR compared to 3/12 (33.3%) of the control group. Overall, the sperm retrieval rate (both surgical and production in the ejaculate) was significantly higher in the cohort receiving gonadotrophin therapy compared to the control group (75% vs. 33.3%, $p<0.05$).

Secondary or salvage surgical sperm retrieval surgery

Hu et al. (303) investigated the use of Goserelin treatment followed by hCG and hMG in cHH NOA patients who had a prior failed cTESE. In the treatment group, one patient (4%) produced sperm in his ejaculate (concentration 1.42 x 10^6/ml) and two patients (8%) had a successful SSR on salvage

cTESE. This coincided with a decline in circulating FSH, LH ($p<0.001$) and an increase in total testosterone ($p<0.05$). In the control arm (n=10) that received no hormonal therapy there were no significant changes noted in gonadotrophin or testosterone levels and no subject produced sperm in the ejaculate or had a successful SSR.

Amer et al. (307) studied the use of testosterone and gonadotrophin treatment in men with NOA and hypergonadotropic hypogonadism who had a previously negative mTESE. Twenty participants received 4 months of testosterone ethanoate and 3 months of hCG and recombinant FSH prior to a secondary mTESE. The control group received no hormone therapy and proceeded directly to surgery. There was a higher SSR rate in the intervention group compared to the control group but this was not statistically significant (10% vs. 0%, $p=0.072$).

Shiraishi et al. (248) treated NOA men with cHH who had a previously failed mTESE. The intervention group consisted of 28 men who were given hCG with the addition of recombinant FSH if their gonadotrophin levels declined. The control group included 20 men who did not tolerate this treatment, but the side effects were not reported. In patients receiving hormone therapy there was a significant increase in testosterone levels and reduction in LH and FSH compared to baseline measurements ($p<0.0001$). Furthermore, sperm were obtained at the second mTESE from 6 men who had received hormonal therapy (21%), compared to zero in the control group ($p<0.05$). Success at the second mTESE was more likely if histology at the primary mTESE demonstrated hypospermatogenesis ($p<0.05$). Of those undergoing hormone therapy, three men (11%) exhibited symptoms of acne, and two (7%) gynecomastia.

There have been two case series reporting the use of hCG and FSH in NOA men undergoing a secondary SSR; the SSR rate was 9% and 22% in those who had cHH (308) or normal hormone status (309), respectively.

No pregnancy, fertilisation or live birth rates were reported in any of the aforementioned studies.

Kobori et al. (310) treated 26 NOA patients that had a failed SSR with FSH therapy. This cohort included both cHH and normogonadotopic, eugonadal men and all patients had a histological diagnosis of late maturation arrest. Following hormone therapy, 5/26 (19.2%) men subsequently produced sperm in their ejaculate (concentration <1million/ml), with two resultant pregnancies and one live birth.

Summary of studies investigating the use of gonadotrophin therapy

The literature investigating the use of gonadotrophin therapy in NOA men with normogonadotropic or hypergonadotropic hormone status is of low-quality evidence. There were no RCTs, and the data largely consisted of case series with 7 case control studies. There was a significant heterogenicity within the literature with regards to the patient cohorts (first mTESE vs. secondary mTESE), treatment regimens (FSH, FSH + hCG, Goserelin followed by hMG and hCG and testosterone ethanoate, hCG and recombinant FSH) and treatment durations (range 3-10 months). Furthermore, there was a variability in inclusion criteria with some studies including men with chromosomal anomalies or Y microdeletions and other studies excluding participants with genetic abnormalities. Moreover, some studies did not report post treatment hormone values.

Adverse events were not recorded in 5 studies but in 3 studies (300,301,311) no side effects were observed. Two studies (248,308) reported acne and gynaecomastia as a result of gonadotrophin therapy. Hu et al. (303) observed that 40% of participants developed androgenic deprivation side effects (i.e., loss of libido, erectile dysfunction and asthenia) whilst on Goserelin therapy, but these symptoms resolved with subsequent hCG treatment.

Overall, the available studies suggest that gonadotrophin therapy can improve sperm retrieval rates (both surgical and sperm production into the ejaculate). Moreover, there is data suggesting that the histological subtype may be important in stratifying patients that would benefit from treatment with those with late maturation arrest and hypospermatogenesis having higher SSR rates.

3.3.3 Aromatase inhibitors

There has been no study investigating the role of AIs in secondary SSR and the majority of trials have investigated the impact of this drug class on sperm production in the ejaculate. Cavallini et al. (312) performed the only RCT in the literature. The authors investigated the use of Letrozole on a mixed cohort of NOA and oligospermia patients with a testosterone oestradiol ratio (T:E) of less than 10. The NOA patients in this study had normal hormone status and all of those who received hormone therapy (6/6) produced sperm in their ejaculate compared to zero (0/5) in the control group that received a placebo. The serum LH, FSH and testosterone all significantly increased ($p<0.01$) with Letrozole whilst serum oestradiol (E_2) and T:E decreased ($p<0.01$). However, 4/22 (18%) patients dropped out of the treatment arm due to side effects (i.e., loss of libido, hair loss and cutaneous rash). Cavallini et al. (313) also reported the only case series specifically evaluating the use of AIs in a cohort of NOA patients. The authors trialled 3 months of Letrozole in 4 NOA patients with normal hormone parameters. All patients produced sperm in their ejaculate and this was associated with a significant increase in gonadotropin and testosterone levels, and reduced E_2 levels ($p<0.05$). However, all patients complained of a loss of libido and a cutaneous rash was noted in two patients and the feeling of nervousness in one.

Saylam et al. (314) treated a mixed cohort of infertile men (17 NOA, 10 oligospermia) with Letrozole for a mean treatment duration of 6.59 months. The NOA cohort had hypergonadotropic hypogonadism and 4 patients (24%) produced sperm in their ejaculate. This coincided with a significant increase in serum testosterone ($p=0.001$) and T:E ($P=0.001$) and a significant decrease in E_2 levels ($p=0.001$).

In contrast, Pavlovich et al. (315) observed that the use of Testolactone in 12 NOA patients with hypergonadotropic hypogonadism resulted in no patients producing sperm in their ejaculate. This study contained a mixed cohort of oligospermic and NOA patients and the use of Testolactone was associated with an increase in T:E ($p<0.01$) and testosterone ($p <0.01$) and a reduction in E_2 levels ($p <0.01$). Testolactone use was also associated with an asymptomatic deterioration in liver function tests in 18% of participants, which improved on monitoring and resolved on discontinuation of the drug.

Shoshany et al. (316) treated 86 infertile men (28 NOA, and 58 with other abnormal semen parameters) with hypergonadotropic hypogonadism and a T:E <10 with Anastrazole. The use of Anastrazole was associated with a significant decrease in E_2 levels and significant increase in LH, FSH, testosterone and T:E levels. None of the NOA patients produced sperm in the ejaculate and 11 elected to undergo mTESE of which 8 (73%) were successful. Anastrozole treatment was discontinued in 8 patients (9.3%). The side effects of Anastrozole included joint and tendon pain, limb swelling, decreased libido, irritability, depression, bilateral breast tenderness, ocular pruritus, ocular pain, dry mouth and a paradoxical increase in E_2 levels.

Summary of studies investigating the use of aromatase inhibitors

The majority of studies investigating AIs were case series with no case control studies and one RCT. In the RCT, letrozole converted all of the azoospermic men to oligospermia. However, this study and the majority of other studies contained mixed cohorts of oligospermic and azoospermic men and no further stratification was performed and as such we could not assess the impact of these pharmacological agents on the hormone levels of NOA men. There was heterogenicity within the literature with regards to the treatment regimens (Testolactone, Letrazole and Anastrazole) and treatment durations (4-6 months). Furthermore, whilst 3 studies excluded patients with genetic abnormalities, 1 study included NOA men with chromosomal abnormalities. Adverse events were reported in all of the studies and there was a drop-out rate of 18% in the one RCT (312) and 9.3% in a further case series (316). The main side effects reported were loss of libido (3/5 studies) (312,313,316) and cutaneous rash (2/5 studies) (312,313). Given the above limitations, the use of AIs cannot be advocated especially given the high number of studies which reported adverse events (66).

3.3.4 Selective oestrogen receptor modulators

We identified three studies investigating the use of SERMs in men with NOA but two were excluded from analysis (Hussein et al. (317) and Moein et al. (318)) because the study cohorts included NOA patients with hypogonadotropic hypogonadism.

Kumar et al. (319) reported a case series of 45 NOA patients with cHH or normal hormone status. These patients underwent either hCG or Clomiphene Citrate therapy for a minimum of 6 months. No patient produced sperm in their ejaculate. The authors did not report any changes in hormone levels or side effects profiles.

Given the paucity of studies investigating the use of SERMs in NOA men with hypergonadotropic hypogonadism or normal hormone status, we cannot advocate this drug class in routine clinical practice (66).

3.3.5 Combined hormonal therapy

Several studies have employed more than one hormone drug class in treating NOA men.

Hussein et al. (304) investigated the use of Clomiphene Citrate and other hormonal agents in a cohort of 612 NOA patients with normal gonadotropin levels. All patients were initially commenced on Clomiphene Citrate 50mg on alternate days with the aim of reaching a testosterone level in the range of 600-800 ng/dl. At 2 weeks, the patients serum hormones were re-evaluated and the patients regrouped according to their response, with treatments including Clomiphene Citrate, hCG and hMG. All patients treated with hormone therapy reached the target testosterone range of 600-800 ng/dl. Overall, 54 (10.9%) patients produced sperm in their ejaculate, with a mean sperm concentration (standard deviation) of 2.3 (4.1) million/ml. No patients in the control cohort (who received no hormone therapy) produced sperm in the ejaculate. The proportion of men who underwent a successful SSR with mTESE following hormone therapy was 57.0% compared to 33.6% in the control group ($p<0.001$). Subgroup analysis revealed no significant differences in age, testicular volume, initial serum total testosterone, or FSH in patients who produced sperm in their ejaculate or who had successful SSR compared to those who remained azoospermic or who had a negative SSR.

Sujenthiran et al. (320) studied the use of hormone therapy in hypergonadotropic hypogonadal patients with Klinefelter syndrome prior to mTESE. The intervention group received either Clomiphene Citrate or hCG and FSH for 6 months, whilst the control group proceeded directly to surgery. The authors reported a higher SSR rate in those receiving hormone therapy (40% vs 13%) but no statistical significance testing was reported. Moreover, it is not clear whether the two cohorts were matched in terms of testicular histopathology.

Majzoub et al. (321) investigated the use of hormonal therapy in hypergonadotropic men with Klinefelter syndrome undergoing mTESE. The study included 3 cohorts: 10 men receiving Anastrazole; 6 men receiving Clomiphene Citrate and hCG; and 4 men who received no hormone therapy (control group). After the therapy, the E_2 level was significantly lower in the Anastrozole cohort compared to the Clomiphene Citrate and hCG group, whilst the FSH, LH and T:E were significantly higher. Overall, the SSR rate was higher in those patients who received hormonal therapy compared with the control group (37.5% versus 0%).

Reifsnyder et al. (288) reported a retrospective analysis on the use of different hormonal therapy regimens in a cohort of NOA men with hypergonadotropic hypogonadism. The authors defined hypogonadism as a testosterone level less than 300 ng/dl. Patients were treated for a minimum of 2-3 months with a variety of hormone agents including Anastrazole ± hCG, Testolactone ± hCG, Clomiphene Citrate and hCG. This study also included 38 NOA men who were on unknown hormone therapies. The results of mTESE were compared with a group of NOA patients with a testosterone <300 ng/dl who received no hormonal therapy. There was no significant difference in SSR rates and pregnancy or live birth rates between patients who received hormonal therapy (any) and those who did not. The authors defined a "response" to hormone therapy as a testosterone level ≥250 ng/dl as this was shown in a previous study to be associated with a higher SRR rate (322); there were no significant differences in the aforementioned parameters between patients who had responded to hormonal treatment and those who had not. However, this study is limited because it included patients who were on unknown hormone therapies.

Song et al. (323) reported the use of testosterone undecanoate and Tamoxifen in a mixed cohort of oligospermic and NOA patients with normal hormone status. Following two months of treatment, all 4 NOA patients produced sperm in their ejaculate. The authors reported that the use of hormone therapy was associated with a significant increase in LH and FSH ($p<0.01$), but not testosterone levels. However, these hormone changes were derived from a mixed cohort of azoospermic and oligospermic patients. Moreover, the NOA cohort size was too small to provide any significant recommendations.

Summary of studies employing multiple hormone therapies

The evidence in the aforementioned studies is conflicting. Reifsnyder et al. (288) observed that neither baseline testosterone level nor response to hormonal therapy affected overall sperm retrieval, clinical pregnancy or live birth rates. However, studies by Hussein et al. (304) and Majzoub et al. (321) reported improvements in sperm retrieval (both surgical and production into the ejaculate) following hormone stimulation therapy. This may be a reflection of the different choice of drugs, treatment durations and protocols with Reifsynder's cohort aiming for a testosterone level of 250 ng/dl whilst Hussein and Majzoub targeted a testosterone within the range of 600-800 ng/dl. Furthermore,

there were differences in hormone status and inclusion criteria (presence of chromosomal abnormalities and FSH levels). The only adverse event reported was a paradoxical decline of testosterone in 3% of patients treated with Clomiphene Citrate. Most studies pooled the data for different hormone treatments and therefore it is difficult to determine the specific effects of individual hormone agents.

Table 3.1: Eligible studies for the systematic review and meta-analysis

Adapted from a meta-analysis published by Tharakan et al. (286)

SD = Standard Deviation, LH = Luteinising hormone, FSH =follicle stimulating hormone, T:E = Testosterone Oestrogen ratio, hCG = human chorionic gonadotropin, hMG = human menopausal gonadotropin, I.M = intramuscular injection, S.C = subcutaneous injection, SSR = surgical sperm retrieval, FR = Fertilisation rate, PR = Pregnancy rate, LBR = Live birth rate, FR = Fertilisation rate, mTESE= micro testicular sperm extraction, cTESE = conventional testicular sperm extraction, NG = normogonadotrophic eugonadism, NGH – normogonadotrophic hypogonadism, cHH = Compensated hypergonadotrophic hypogonadism. HH = hypergonadotrophic hypogonadism, NR = Not reported

Study	**Methods**	**Population**	**Genetics**	**Mean Age (SD) (*Range) *in years* **=median**	**Intervention**	**Type of Surgery**	**Hormone changes**	**Sperm in ejaculate/ Surgical Sperm Retrieval**	**Pregnancy Live birth rate**	**Adverse Events**
Kumar et al(319) (1990)	Case Series	50 NG and cHH NOA men 29 Oligospermic men	Chromosomal abnormalities excluded	31 (4.7)	2000 units hCG, twice a week for 6 months Or Clomiphene Citrate (25 days per month of 50mg for 6months)	N/A	NR	No sperm produced into the ejaculate in NOA men.	N/A	NR
Pavlovich et al(315) (2001)	Case series	43 HH NOA + 20 oligospermic men No differentiation between oligospermic	Chromosomal abnormalities included	37 (*31-43)	Testolactone (orally) 50mg twice daily for a mean duration of 5 months. If oestradiol still high after one month then Testolactone (orally) increased to 100mg twice daily.	N/A	Increases in mean serum Testosterone (*p<0.01*) and T:E (*p<0.01*) Decrease in mean serum oestradiol (*p<0.01*).	Only 12 NOA men underwent semen analysis and none produced sperm in their ejaculate.	NR	8 patients (18%) developed asymptomatic liver function test derangement, which resolved on cessation of treatment.

		and NOA men.			Mean treatment duration: 5 months					
Aydos et al(300) (2003)	Case control	NG NOA (n=174) Intervention (n=63) Control (n=45)	Chromosomal abnormalities included	29 (*21-39)	75 IU FSH I.M. three times a week for 3 months Control group: no treatment	Primary cTESE	Significant increase in FSH in intervention group compared to controls (*p<0.001*)	No significant difference in SSR outcomes between intervention and control groups. However, significant increase in SSR was associated with focal spermatogenesis & hypospermatogenesis cohorts (P<0.05). Intervention group successful SSR: 40/63 (63.5%) Control group successful SSR: 15/45 (33.3%)	NR	No adverse effects observed
Selman et al(309) (2005)	Case series	NG NOA (n=49)	Chromosomal abnormalities excluded	(*32-41)	75 IU rFSH alternate days for 2 months 150 IU rFSH alternate days for 4 months At 4th month addition of hCG 2000 IU twice weekly for 2 months	Secondary cTESE	NR	No sperm produced in the ejaculate cTESE successful SSR: 11/49 (22.4%)	PR: 3 LBR: 3	NR
Saylam et al(314) (2011)	Case series	17 HH NOA + 10 oligospermia men (all T:E<10) No differentiation between oligospermic and NOA men.	NR	34.92 (6.66) (*26-49)	Letrozole 2.5 mg, orally, once daily for ≥6 months. Mean treatment duration: 6.59 ± 0.88 months	N/A	Significant increase in Testosterone and T:E and decrease in Oestrogen (*p=0.001*). No significant differences in FSH and LH.	In 4/17 (23.5%) patients, sperm returned to ejaculate.	NR	Two patients (7%) had mild headaches
Cavallini et	Case series	NG NOA (N=4)	Chromosomal	37.25 (*29-44)	Letrozole 2.5mg, orally, once daily for 6 months	N/A	Significant Increases in Testosterone, FSH and, LH (*p<0.05*).	In 4/4 (100%) patients, sperm returned to the ejaculate.	NR	Loss of libido

al(313) (2011)			abnormalities excluded				Oestrogen significantly decreased (*p<0.01*)			Cutaneous rash and anxiety
Shiraishi et al(248) (2012)	Case control	cHH NOA (n=48) Intervention (n=28) Control (n=20)	Chromosomal abnormalities excluded	34 (5.7)	5000 IU of hCG three times a week for 4-5 months (n =13) 5000 IU of hCG thre times a week for 5 months + 150 IU FSH three times a week for 2 months (n= 15) Control group: no treatment	Secondary mTESE	In hCG only cohort: Significant increase in Testosterone (*p<0.01*) and significant decrease in LH **(***p<0.05*) compared to baseline, but not FSH. In hCG and FSH cohort: significant increase in Testosterone (*p<0.0001*), and significant decreases in LH and FSH (*p<0.0001*) compared to baseline.	Successful SSR was significantly higher in intervention group compatred to controls (p<0.05). Intervention group successful SSR: 6/28 (21.4%) Control group successful SSR: 0/20 (0%) Hypospermatogenesis, was associated with higher successful SSR (p<0.05)	NR	Three patients (11%) showed symptoms of acne, and two (7%) of gynecomastia
Song et al(323) (2012)	Case Series	4 NG NOA + 8 oligospermic men No differentiation between NOA and other men.	Chromosomal abnormalities excluded	(*25-39)	Testosterone undecanoate 40mg twice daily and Tamoxifen Citrate 10mg twice daily for 4 months.	N/A	Significant increases in FSH and LH (*p<0.01*).	In 4/4 (100%) NOA patients, sperm had returned to the ejaculate by 2 months.	NR	NR
Hussein A et al(304) (2012)	Case control	612 NGH NOA	NR	26.7 (4.9)	Different treatment modalities depending on initial response to Clomiphene Citrate. Aiming for serum testosterone 600-800ng/dl. Intervention group:	Primary mTESE	All groups reached target testosterone level. FSH increased in all groups.	Rate of return of sperm to the ejaculate: Intervention group 1: 41/372 (11.0%) Intervention group 2: 7/62 (11.3%) Intervention group 3: 4/46 (8.7%) Intervention group 4: 2/16 (12.5%) Control group: 0/116 (0%)	NR	16 patients (3%) experienced a paradoxical decrease in testosterone level on Clomiphene citrate.

					-Group 1 (n=372) Clomiphene Citrate (for 6.4+/- 2 months) -Group 2 (n=62) Clomiphene Citrate and hCG (for 4.1+/-2.4 months) -Group 3 (n=46) hMG + hCG (for 4.2+/-1.1 months) -Group 4 (n=16) hMG + hCG (for 4.2+/-1.1 months) Control group(n=116): no treatment			Successful SSR: Intervention group 1: 191/331 (57.7%) Intervention group 2: 31/55 (56.3%) Intervention group 3: 22/42 (52.4%) Intervention group 4: 8/14 (57.1%) Control group: 39/116 (33.6%) Overall sperm retrieval rate (conversion rate and SSR) was significant in intervention groups 1 (P< 0.001), 2 (P< 0.001), and 4 (< 0.05) compared to control group.		
Reifsnyder et al(288) (2012)	Case control	348 HH NOA men Intervention group: treated with hormone therapy prior to mTESE(n=307) Control group: no hormone therapy but mTESE(n=41)	Exclusion of Azoospermia Factor Gene a, b and c Y microdeletion. Included some chromosomal abnormalities ie. Klinefelter syndrome	35	Regimes unspecified: Anastrozole (n= 180) Anastrozole + hCG (n=29) Clomiphene Citrate (n=66) Testolactone (n=14) Testolactone + hCG (n=12) hCG (n=9) Other combinations/unknown (n=38) **INCLUDED cohort with unknown or combinations** Treatment duration: minimum 2-3 months prior to surgery	Primary mTESE	Significantly increased FSH in intervention group compared to controls (*p=0.02*).	No significant difference in successful SSR between intervention group and controls (*p=0.31*). Intervention group successful SSR: 157/307 (51.1%) Control group successful SSR: 25/41 (61.0%) No significant difference in successful SSR between those who had responded to hormone therapy in the intervention group (resultant testosterone >250ng/dl) compared to those who had not (p=0.97).	No significant difference in, PR and LBR	NR
Effesoy et al(311) (2013)	Cohort study (no control)	NG NOA (n=11)	NR	31.09 (4.52)	100-150 IU FSH two - three times a week Mean treatment duration (7.45+/- 4.5 months)	Primary mTESE	Significant increase in FSH (*p=0.004*).	2/11 (18.1%) patients produced sperm in ejaculate (p=0.323) mTESE successful SSR: 2/11 (18.1%)	NR	No adverse events observed

Cavallini et al(312) (2013)	Randomised controlled trial	Intervention (n=6 HH NOA + 16 cryptospermia) Control (n=5 HH NOA + 19 cryptospermia) No differentiation between oligospermic and NOA men.	Chromosomal abnormalities excluded	Intervention group: 44 (*37-52) Control group: 45 (*38-53)	Letrozole 2.5 mg, orally, once daily for 6 months Placebo for control	NA	Significant increases in Testosterone, FSH and LH in intervention group at 3 and 6 months ($p<0.01$).	Rate of return of sperm to the ejaculate: Intervention group: 6/6 (100%) Control group: 0/5 (0%)	PR: 0	4 patients (18%) dropped out of treatment group due to side effects including loss of libido, loss of hair and cutaneous rash.
Kobori et al(310) (2014)	Case Series	HH, cHH and NG NOA (n=26) Only reported data for the five patients who produced sperm in their ejaculate	Chromosomal abnormalities excluded	34.6 (*29-38)	75 IU FSH twice a week for the first 3 months FSH was increased to 150 IU twice a week subsequently. Mean duration to produce sperm into ejaculate: 4.4 months	N/A	NR	5/26 (19.2%) patients produced sperm in the ejaculate (<1million/ml).	PR: 2 LBR: 1	NR
Shiraishi et al(308) (2016)	Case series	cHH NOA (n=21)	Chromosomal abnormalities excluded	32.2 (3.1) (*29-36)	5000 IU of hCG three times a week for 4 months 150 IU FSH three times a week for 3 months Overall duration: 4 months	Secondary mTESE	Testosterone and Oestrogen significantly increased and FHS and LH significantly decreased compared to baseline ($p<0.01$).	mTESE successful.SSR: 2/21 (9.5%) Successful SSR associated with Hypospermatogenesis and late maturation arrest ($p<0.01$).	PR: 1 LBR: 1	Three patients (14%) developed acne
Gul U et al(301) (2016)	Case control	NG NOA (n=83)	Chromosomal	34 (5.7)	hCG 2500 IU twice a week S.C. for 10-14 weeks	Primary cTESE (and if	NR	Successful SSR was not significantly different between the	No significant	No adverse events observed

		Intervention (n=34) Control (n=49)	abnormalities excluded		Control group: no treatment	this failed then mTESE)		intervention and control cohorts (p=0.338) Intervention group successful SSR: 17/34 (50%) Control group successful SSR: 28/49 (57.1%)	difference in FR, PR and LBR	
Majzoub et al(321) (2016)	Case Control	16 HH NOA patients	All subjects had non mosaic Klinefelter syndrome Exclusion of Azoospermia Factor Gene a, b and c Y microdeletions.	32.9	Intervention group: -Group A1 (n=10): Anastrozole 1mg, orally once daily for 6 months. -Group A2 (n=6): Clomiphene Citrate 25mg, orally, once daily and hCG 5000 IU once weekly. No duration of treatment specified. Control group (n=4): no treatment	Primary mTESE	Statistically significant increase in Testosterone in intervention group compared to controls (*p=0.01*), but no difference in FSH and LH.	The successful SSR was higher in those who received hormone therapy compared to controls. Intervention group successful SSR: 6/16 (37.5%) Control group successful SSR: 0/4 (0%)	PR: 3 LBR: 3	NR
Shoshany et al(316) (2017)	Case series	28 HH NOA + 58 men (normal and abnormal semen parameters) No differentiation between NOA and other men.	Chromosomal abnormalities excluded	**37 (*32–41)	Anastrazole 1mg, orally, once daily for 4 months	Primary mTESE	At 3 weeks there were significant increases in LH, FSH, Testosterone, and T:E (*p<.0001*). Oestrogen significantly decreased (*p<.0001*).	No return of sperm to ejaculate in any NOA patient. 11 patients underwent mTESE. 8/11 (72.7%) had successful mTESE. 17/28 did not undergo surgery.	NR	Anastrozole treatment was discontinued in 8 patients (9.3%) due to side effects including joint pain, lower limb swelling, low libido, depression, mastalgia, ocular pruritus and pain and dry mouth.

Cocci et al(302) (2018)	Case Control	NG NOA (n=50) Intervention (n= 25) Control (n=25)	NR	35.5 (4.3)	150 IU FSH, S.C. three times a week for 3 months. Control group (retrospective cohort): no treatment	Primary cTESE	NR	Significant increase in conversion rate in treatment group (20%) compared to controls (0%) (p= <0.05). Significant increase in successful SSR in treatment group (24%) compared to controls (8%) (p=<0.05). Rate of return of sperm to the ejaculate: Intervention group: 5/25 (20%) Control group: 0/25 (0%) Successful SSR via cTESE: Intervention group successful SSR: 6/25 (24%) Control group successful SSR: 2/25 (8%)	Significantly increased FR and PR in treated group compared to control (P=<0.05)	NR
Hu X et al(303) (2018)	Case control	cHH NOA (n=35) Intervention (n=25) Control (n=10)	Chromosomal abnormalities excluded	Intervention group: 25.8 (3.4) Control group: 26.6 (3.3)	3.6 mg Goserelin S.C. once every four weeks for a total of 6 months. 2000 IU hCG I.M. Once/week for 5 months. 150 IU hMG I.M. twice a week for 4 months Control group (men who did not tolerate the treatment): no treatment.	Secondary mTESE	Significant increase in total Testosterone (*p<0.05*) and significant decreases in FSH and LH (*p<0.001*) in intervention group.	Rate of return of sperm to the ejaculate: Intervention group: 1/25 (4%) - (Sperm concentration was 1.42 x10^6/ml and the total sperm count was 3.98 x10^6) Control group: 0/10 (0%) Successful SSR via mTESE: Intervention group successful SSR: 1/25 (4%) Control group successful SSR: 0/25 (0%)	NR	10 patients (40%) developed symptoms of androgen deprivation (e.g erectile dysfunction) on Goserelin, which resolved with hCG therapy. 10 patients did not tolerate treatment

Sen et al(306) 2020	Case Control	NGH and HH NOA (n=24) Intervention (NGH) (n=12) Control (HH) (n=12)	NR	Intervention group: 36.58 (2.01) Control group: 41 (2.37)	250 mcg recombinant HCG once/week for 6 months. Control group: no treatment	Primary mTESE	In intervention group serum Testosterone increased from 8.03(+/-0.97) to 15.66(+/-2.20).	Rate of sperm to the ejaculate: Intervention group: 3/12 (25%) Control group:0/12 Successful SSR via mTESE: Intervention group successful SSR: 6/12 (66.6%) Control group successful SSR: 4/12 (33.3%) p<0.05	NR	NR
Amer et al(307) 2020	Case control	HH NOA (n=40) Intervention (n=20) Control (n=20)	NR	Intervention group: 36.2 (4.3) Control group: 35.9 (5.4)	250mg testosterone enanthate once/week for 1 month, Subsequently, 5000 IU hCG I.M. once/week, 150 IU I.M. purified urinary FSH 3x/week and 250mg testosterone enanthate once/week for 3 months.	Secondary mTESE	NR	Successful SSR via mTESE: Intervention group Successful SRR: 2/20(10%) Control group successful SSR : 0/20 (0%) (p =0.072)	NR	NR
Sujenthiran et al(320)	Case series	HH NOA (n =23) Intervention (n=15) Control (n=8)	All Klinefelter syndrome patients	**33(IQR 30-34)	Intervention group received: Clomiphene Citrate or hCG and FSH. Control group: no treatment	NR	NR	Successful SSR via mTESE: Intervention group successful SRR: 6/15(40%) Control group successful SSR: 1/8(13%)	In the intervention group: PR: 4/15(27%) LBR:3/15 (20%)	NR

3.4 Meta-analysis

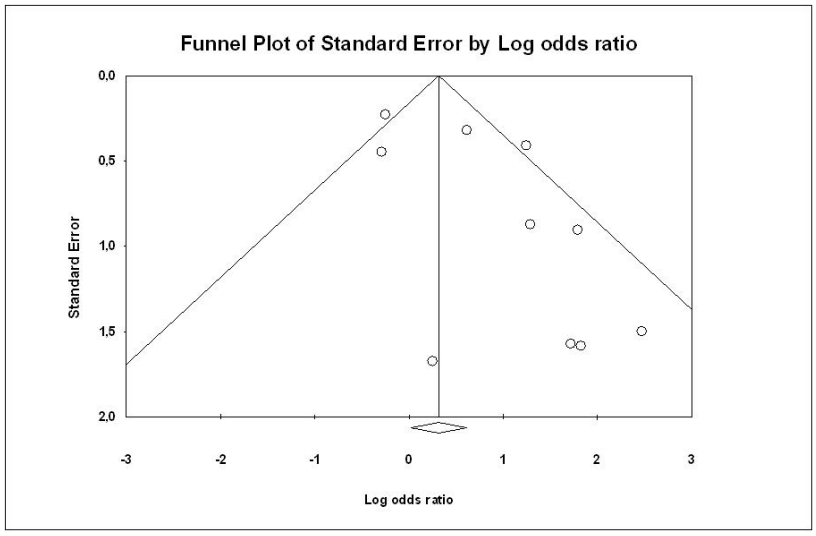
For the meta-analysis, only controlled studies were included. Owing to the limited number of studies, data were aggregated for all hormone classes and no analysis was performed on the individual drug agents. Of the retrieved texts, 10 studies were included in the meta-analysis (Table 1) (286). Among them, 5 studies (248,288,303,307,321) included hypergonadotropic subjects and 5 (300–302,304,306) included normogonadotropic men (Table 1). The characteristics of the retrieved studies are reported in Table 1. The meta-analysis included 985 patients with a mean (±SD) age of 31.9±4.2 years and a mean follow-up of 17.2±9.4 weeks (286). The treatment modalities differed among the studies (Table 1).

The I^2 in trials assessing overall SSR was 58.2 ($p<0.01$). A funnel plot and Begg adjusted rank correlation test (Kendall's τ: 0.00 p=1.00) suggested no publication bias (Figure 3.2).

Figure 3.2 – Funnel plot and Begg Adjusted rank correlation test

Adapted from a meta-analysis published by Tharakan et al. (286)

A funnel plot of standard error of sperm retrieval rate by Mantel–Haenszel log odds ratio.

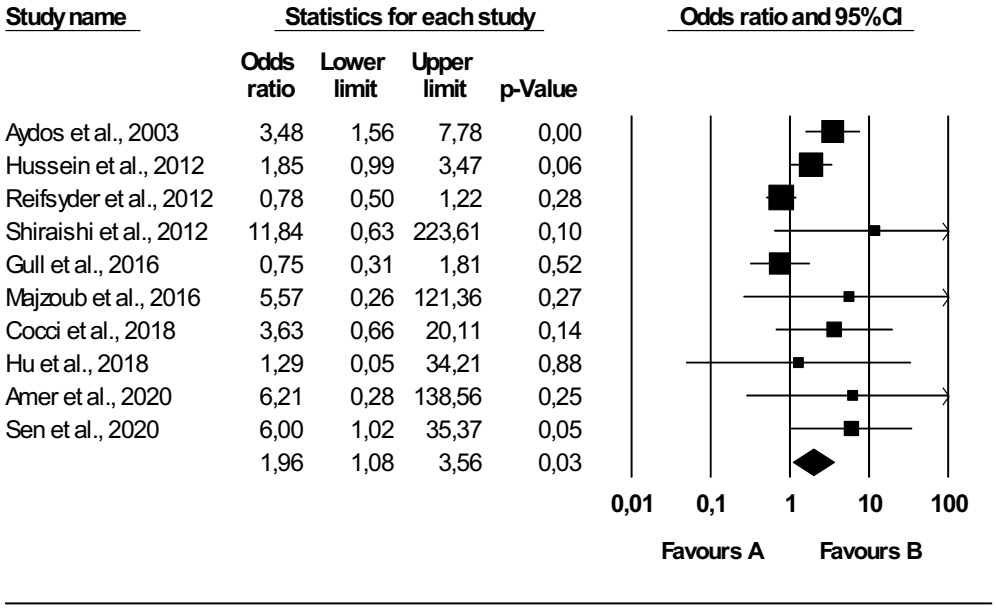


Overall, a higher SSR in subjects pre-treated with hormonal therapy was observed (OR 1.96, 95% CI:1.08-3.56, p=0.03) (Figure 3.3).

Figure 3.3: Effect of hormone therapy on surgical sperm retrieval rate.

Adapted from a meta-analysis published by Tharakan et al. (286)

A Forrest plot demonstrating the individual and cumulative odds ratios for SSR. A = no sperm retrieval, B = sperm retrieval.

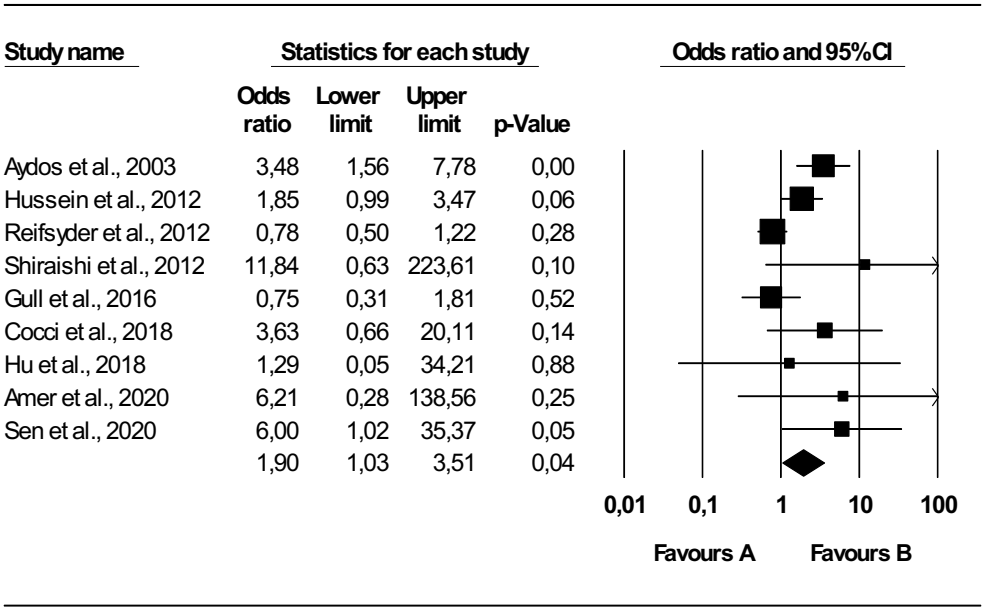


Sensitivity analysis, excluding one study enrolling only patients with Klinefelter syndrome (321), confirmed the previous observation that hormone therapy was associated with a higher SSR (OR 1.90, 95% CI:1.03-3.51, p=0.04) (Figure 3.4).

Figure 3.4: Effect of hormone therapy on surgical sperm retrieval rate (excluding a study (Majzoub et al. (321)) **that included only patients with Klinefelter syndrome).**

Adapted from a meta-analysis published by Tharakan et al. (286)

A Forrest plot demonstrating the individual and cumulative odds ratios for SSR. A = no sperm retrieval, B = sperm retrieval.

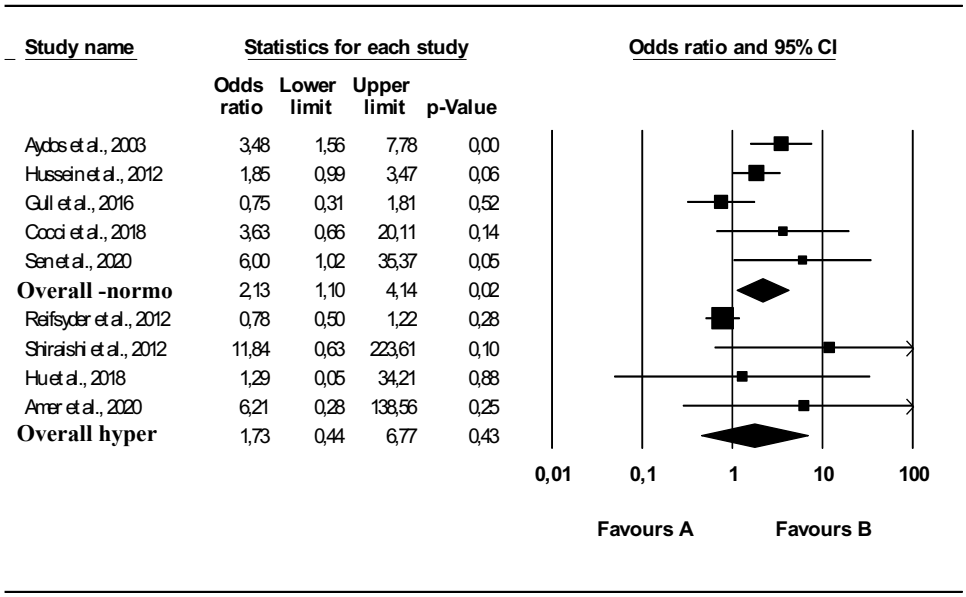


Further subgroup analysis of baseline hormone status demonstrated a significant improvement in normogonadotropic men (OR 2.13, 95% CI: 1.10-4.14, p=0.02) but not in hypergonadotropic patients (OR 1.73, 95% CI: 0.44-6.77, p=0.43) (Figure 3.5).

Figure 3.5: Effect of baseline hormone status (normogonadotropic (normo) vs. hypergonadotropic (hyper)) on surgical sperm retrieval rates.

Adapted from a meta-analysis published by Tharakan et al. (286)

A Forrest plot demonstrating the individual and cumulative odds ratios for SSR. A = no sperm retrieval, B = sperm retrieval.



Finally, when the only study not published as a full text (Sen et al., 2020) was excluded, there was a non-statistically significant trend towards a higher SSR in the normogonadotropic group compared to the hypergonadotropic cohort (SSR=1.9[0.95;3.78]; p=0.07).

3.5 Risk of bias

Key

Bias	
Unclear	
Low risk of bias	
Moderate risk of bias	
Serious risk of bias	
Critical risk of bias	

Table 3.2: Risk of bias for non-randomised controlled trials

Adapted from a meta-analysis published by Tharakan et al. (286)

Study name	Confounding	Patient selection	Interventions classification	Deviations form intended interventions	Missing data	Measurement outcomes	Selection of reported result	Outcome
Aydos (2013)								
Coccil (2018)								
Gul (2016)								
Hu (2018)								
Hussein (2013)								
Shiraishi (2012)								
Reifsynder (2012)								
Majzoub (2016)								
Sen et al (2020)								
Amer et al (2020)								

Summary

Overall, the methodological quality of the non-randomised trials had either moderate or serious risk of bias.

3.6 Discussion

This is the first meta-analysis (286) to investigate the use of hormone stimulation therapy in men with NOA and hypergonadotropic hypogonadism or eugonadism.

There are no established therapies to treat NOA in men with primary hypogonadism and successful surgical sperm retrieval rates have been reported to be only 40-60% (248). Hence, hormone therapies have been utilised empirically to increase the chances of sperm retrieval. However, there are no large-scale, randomised controlled trials to support the use of hormone stimulation therapy for this indication. There is a theoretical basis (252) to the use of hormone therapy in this context because intratesticular testosterone is needed for spermatogenesis and there is data showing that hormone therapy can increase intratesticular testosterone (294). However, it is unclear what level of intratesticular testosterone is required to improve sperm retrieval and the measurement of intratesticular testosterone necessitates testicular aspiration which is an invasive procedure. Furthermore, serum testosterone has been observed to poorly correlate with intratesticular testosterone levels (252). There is murine data suggesting that FSH stimulation may increase spermatogenesis through a testosterone independent pathway (295) and this provides an alternative justification to the use of hormone stimulation therapy. Thus, hormone therapy has been rationalised in clinical practice by theoretical plausibility and because of a lack of alternative treatments to optimise sperm retrieval.

This study has shown that the current literature investigating hormone therapy in men with NOA and hypergonadotropic hypogonadism or eugonadism is of low quality evidence. There is only one randomised controlled trial, and many studies are case series. There is heterogeneity in study design with a wide variability in drug regimens, participant cohorts and surgical procedures. Furthermore, many studies do no report post treatment hormone profiles or adverse events outcomes. We also observed that the studies included in the meta-analysis were of moderate to serious risk of bias. Within these limitations, we observed that hormone therapy significantly improved surgical sperm retrieval but this was only in men with NOA and normal gonadotropin levels. It is unclear why the benefits of hormone therapy are limited to eugonadal men, but it may be that primary hypogonadism

reflects a more severe form of NOA with irreversible damage and thus refractory to hormone stimulation. However, there are no studies within the literature to validate this theory.

There is data suggesting that FSH receptor gene polymorphisms may affect endocrinological and reproductive outcomes (286). Selice et al. treated 70 oligospermic men with recombinant FSH for three months and observed significant improvements in sperm concentration, total motility and normal morphology in only those who were homozygote Ala307-Ser680/ Ala307-Ser680 or heterozygote Thr307-Asn680/ Ala307-Ser680 common allelic variants (324).

Lindgren et al. studied the hormone profiles and genotypes of 313 Swedish men and reported that men homozygous for the Thr307Thr/Asn680Asn single nucleotide polymorphism combination had a significantly lower FSH and total testosterone level then carriers of other FSH receptor variants (325).

The above studies suggest that polymorphisms in the FSH receptor gene may account for differential responses to hormone stimulation therapy, and hence may be contributory to our study findings. However, further studies are needed to investigate FSH receptor gene polymorphisms in NOA.

It is important to acknowledge the limitations of this study. Most of the data were not randomised or prospective and hence the observations of this analysis should be treated with caution because of the low-quality evidence.

It is recognised that surgical sperm retrieval outcomes can be influenced by the type of surgery (326), the experience of the surgeon (327), the embryological sperm extraction processes (328) and testicular histopathological subtype (242). Unfortunately, many of the studies did not report on the above variables and hence our analysis did not correct for any of these confounding factors. However, it is important to acknowledge that it is common for NOA patients to have mixed histopathological patterns within the same testicle (47).

Due to the shortage of controlled studies within the literature, no sub-analysis was performed on the individual hormone classes and therefore it is unclear what is the optimal hormone regimen. There is also a lack of information regarding the costs of different hormone therapies and hence no cost-effective analysis could be performed. Too few studies reported clinical outcomes such as pregnancy and live birth rates and this should be a focus of future research as it is important to understand how hormone therapy affects ART outcomes. Furthermore, future studies should investigate the presence

of hypogonadal symptoms in infertile men. This data could help rationalise hormone stimulation therapy for men with NOA based on infertility and symptomatic hypogonadism.

3.7 Conclusions

Our study findings suggest that hormone stimulation therapy may increase surgical sperm retrieval rates in NOA men with normal hormone status. However, the current literature is of low quality evidence with a moderate or severe risk of bias. Furthermore, it is unclear regarding the optimal hormone regimen or potential side effects. Therefore, currently we can only recommend the use of hormone therapy in men with NOA within a clinical trial setting and not in routine clinical practice.

Chapter 4:

Investigating novel genetic variants in idiopathic non obstructive azoospermia

4.1 Introduction

Non-obstructive azoospermia (NOA) is the absence of sperm in the ejaculate due to an impairment of spermatogenesis and has been reported to affect 1% of all men (329). NOA represents the most severe form of male infertility and men with NOA can only father biological children through testicular sperm extraction surgery (TESE) coupled with assisted reproductive technologies (ART). However, a recent meta-analysis reported that the success rate of testicular sperm extraction from men with NOA is approximately 50% (249).

NOA is classified into histological subtypes based on the degree of spermatogenesis cycle dysfunction (see Chapter 1.1.2) (44). The histological classification of NOA has been correlated with the likelihood of surgical sperm retrieval with the subtype hypogospermatogenesis associated with the highest (73-100%) success and Sertoli cell only syndrome the lowest (23-41%) (242). However, it is recognised that mixed histological patterns within the same testis are common (47). Historically, there was no clinical or biochemical variable that could predict sperm retrieval surgery success, but the discovery of Y chromosome microdeletions has allowed disease stratification of NOA patients. Three regions were identified on the long arm of the Y chromosome (Yq11), that are associated with failure of spermatogenesis if absent (AZFa, AZFb, and AZFc) (67) and these microdeletions have been reported to occur in 10-20% of NOA men (68,69) and provide prognostic information on surgical sperm retrieval success. Hopps et al. (69) performed both TESE and testicular biopsies on 78 men with AZF deletions. The authors reported that those with AZFa, AZFb, AZFb+c deletions were azoospermic and no sperm was found with TESE or biopsy. However, an isolated AZFc deletion conferred to a successful sperm retrieval rate in 75% (9/12) by TESE and 45% (9/20) on biopsy (56% overall). Consequently, both the American Urology Association (70) and European Association of Urology (66) recommend Y microdeletion testing in all men presenting with NOA and advocate performing surgical sperm retrieval in those with an AZFc deletion only. This highlights that identifying a genetic mutation in NOA can help shape patient management and be important in patient counselling. This is pertinent given that sperm retrieval surgery has limited availability and requires sub-specialist expertise. Moreover, sperm retrieval surgery can be costly and has risks including hypogonadism, testicular atrophy and those pertaining to anaesthesia.

Topuz et al. investigated the levels of depression and anxiety (using the Beck depression and anxiety inventory, respectively) in a cohort of 40 men with NOA. The authors reported that 65% of the patients suffered from idiopathic NOA and in the overall cohort 27.5% had mild depression and 10% moderate depression. Moreover, 2.5% of patients suffered from mild anxiety. An understanding of the aetiology of NOA may help patient counselling and provide knowledge that could potentially ameliorate the psychological implications related to the diagnosis of unexplained NOA.

Surgical sperm retrieval rates in patients with NOA have remained static in the last 10 years. Historically, the only method of sperm retrieval surgery was a wedge biopsy of the testicle (cTESE). However, Schlegal et al. reported the micro-testicular sperm extraction (mTESE) technique in 1999 (246) and this procedure utilised an operative microscope to target larger and more opaque seminiferous tubules that are more likely to contain sperm. Bernie et al. (242) performed a meta-analysis of 15 studies comparing surgical sperm retrieval techniques and reported that mTESE conferred to a higher surgical sperm retrieval rate compared to the cTESE (42.9-63% vs 16.7-45%). However, apart from the development of mTESE, there have been no recent advancements to improve surgical sperm retrieval success. It is also worth noting that a recent meta-analysis (249), comprising of 117 studies reported no significant difference in cTESE and mTESE surgical sperm retrieval rates. In approximately 50% of cases of male infertility the cause is unknown (330) and in only 25% of cases of NOA can a genetic cause be identified (331). Thus, it could be argued that not understanding the pathophysiological mechanisms that underpin NOA has limited research into novel treatments.
Within this context, identifying genetic abnormalities in patients diagnosed with idiopathic NOA may confer prognostic information and could potentially stratify patients into those with spermatogenesis and of whom would likely to benefit from surgery from those who would not. This would help with health provision management and avoid the risks associated with surgery. Moreover, a further understanding of the aetiology of NOA may help identify future therapeutic targets for men with infertility.

The emergence of next generation sequencing has allowed quicker and cheaper genetic testing compared to traditional sanger sequencing and our understanding of the genetic causes of NOA is increasing (Chapter 1.5, Table 1.5). Imperial College Healthcare Trust is the regional andrology

service for Northwest London. Consequently, the department is referred a large, ethnically diverse population and this provides an opportunity to screen patients with idiopathic NOA for novel genetic variants.

4.2. Hypothesis and aims

4.2.1 Hypothesis

1) Men with idiopathic NOA have genetic variants

4.2.2 Aim

To determine the following:

1) Novel genetic variants in men with idiopathic NOA.

4.3 Methods

4.3.1 Study design

Ethical approval was granted by the West London & GTAC Human Ethics Research Committee (registration number: 14/LO/1038). Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

4.3.2 Subjects

Subjects were recruited from infertility clinics at Imperial College Healthcare Trust. Non obstructive Azoospermia was defined as no spermatozoa detected in the sediment of a centrifuged sample of semen (40)). Non obstructive azoospermia was based on a histological diagnosis from testicular biopsy samples. Idiopathic NOA was defined as NOA without any recognised cause (Table 4.1)

Table 4.1 demonstrates causes of non obstructive azoospermia

Causes of NOA	Examples
Infection	Mumps
Genetic	Klienfelter syndrome, Y chromosome deletions
Iatrogenic	Chemotherapy, Radiotherapy
Hormone dysfunction	Hypogonadotrophic Hypogonadism

Participants were evaluated in the Imperial College Research Facility (ICRF) for a single visit to complete a questionnaire regarding their clinical and reproductive history (see appendix 1: study questionnaire), undergo height and weight measurement and testicular examination, and provide blood samples.

The inclusion and exclusion criteria for the study are listed in Table 4.2.

Table 4.2 Table demonstrating the inclusion and exclusion criteria for the study.

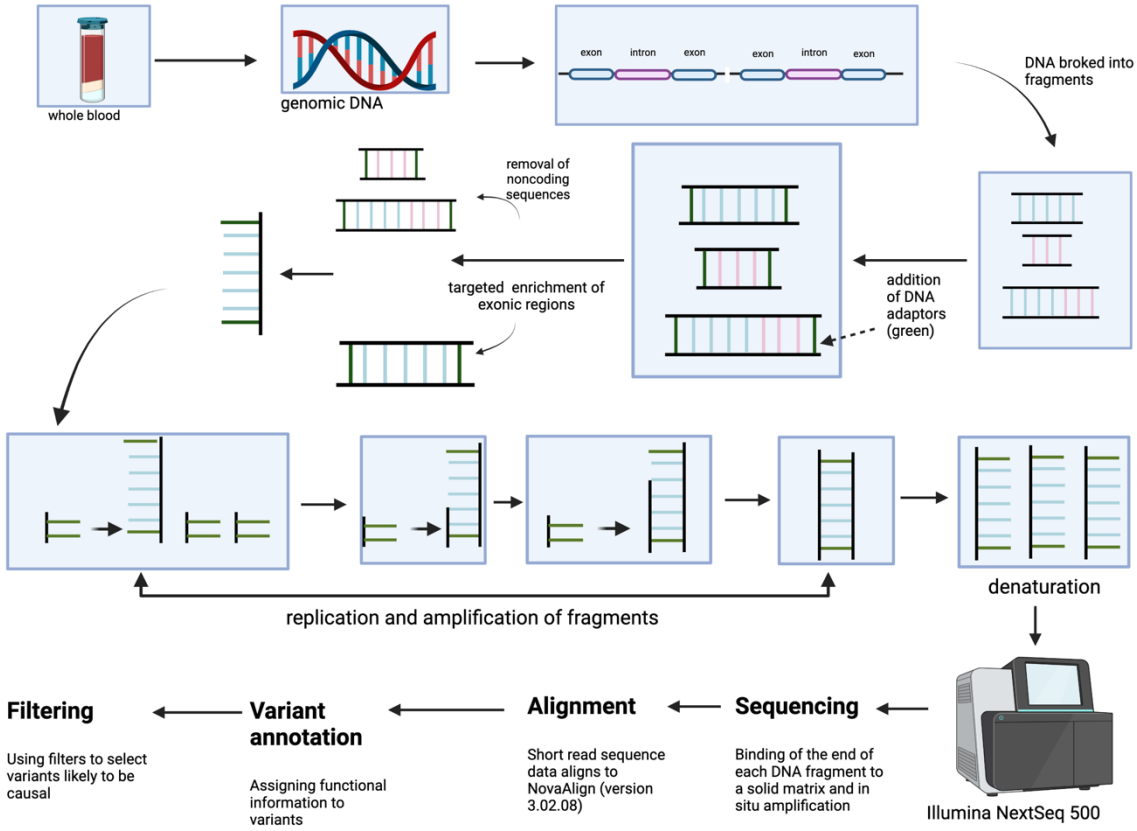
Inclusion criteria	Exclusion Criteria
Non obstructive azoospermia	Any history of: - Mumps - Radiotherapy - Chemotherapy - Steroid or testosterone use - Testicular torsion - Cryptorchidism - Testicular trauma
Capacity to consent	Hypogonadotrophic hypogonadism
	Any recognised genetic disorder (e.g. Klinefelter syndrome, Y chromosome microdeletion)

4.3.3 Study methodology

This was a prospective cohort study investigating the whole exome sequences of men with idiopathic NOA. Figure 4.1 describes the methodology and genetic analysis of the study samples.

The genetic sequencing and analysis was performed in collaboration with Dr Emad Sindi and Dr Anu Sironen (University College London, Great Ormond Street, UK)

Figure 4.1 Summary of the methodology of whole exome genome sequencing



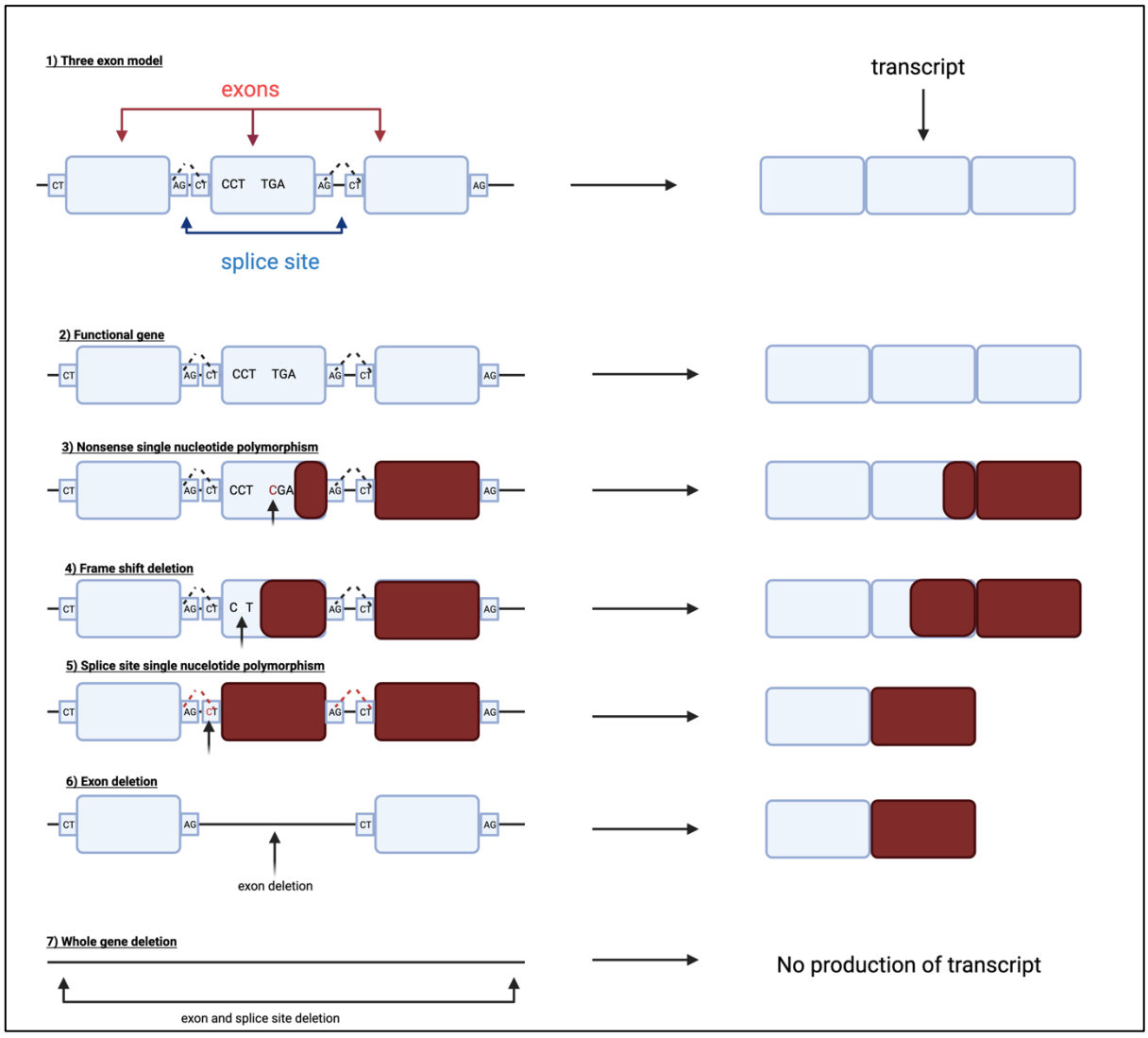
Blood was sampled from each participant with venipuncture. Samples were collected in plain whole blood Vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA) and were stored in -20°C until analysis. The genomic DNA was extracted from the blood using the QIAAmp DNA Blood Mini kit (QIAQEN, Maryland, USA). DNA was enzymatically fragmented and adaptor ligands bonded to each end of individual fragments. Target enrichment and exon capture was performed using Cell3 Target (Nonacus, Birmingham, U.K); fragments were amplified and then ligated to biotin-labelled probes and streptavidin coated beads. This hybridization target enrichment process captures exonic sequences and non exonic sequences are washed away. The subsequent exome library underwent pair end sequencing on the Illumina NextSeq 500 high-throughput sequencer with a mean exome coverage of 60 times. This sequencing uses parallel sequencing techniques to generate millions of reads and generates raw data in the FASTQ format. The data was analysed using the Phenopolis bioinformatics platform (332). The short-read sequence data was aligned with the NovaAlign (version 3.02.08, Novocraft Technologies) reference genome and variants and insertion/deletions (indels) were called

according to GATK (version 3.5.0, Broad Institute) best practices (joint variant calling followed by variant quality score recalibration). The variant annotation and filtering were done using SVS software (Golden Helix, Montana, U.S) and UCSC RefSeq Genes 63 was used for annotation and GenomAD exome for allele frequencies. The variants were filtered to include those with an allele frequency <0.001 because a rare variant is more likely to have a functional effect that one found frequently (333). Loss of function variants are those variants likely to correlate with complete loss of function of the gene transcript and include frameshift insertions/deletions, splice site-disrupting nucleotide variants (SNVs), introduction of stop codon (nonsense) and exon and whole gene deletions (Figure 4.2).

Figure 4.2 demonstrates the different types of loss of function variants

Adapted from MacArthur et al. (334)

Figure 4.2 uses a three-exon model to demonstrate how a nonsense single nucleotide polymorphism, frame shift deletion, splice site single nucleotide polymorphism, exon deletion and whole gene deletion (as indicated by the black vertical arrow on the left side of the figure) can affect gene transcription (right side of the figure). The crimson boxes represent loss of protein coding function.



Data has recently shown that loss of function variants are contributory to all known severe haploinsufficient human disease genes (335).

Missense variants are those that change an amino acid and can be subclassified into silent (the protein remains the same despite the change of an amino acid), conservative mutation (where the replacement amino acid shares biochemical properties with the original amino acid e.g. charge) and non-

conservative mutation (where the replacement amino acid has different biochemical properties to the original amino acid).

We filtered missense and nonsense variants, and all candidate variants were visualised with the Integrative Genome Viewer (IGV). Further filtering was performed based on whether the gene was on a database of known NOA genes based on mouse data (83) and whether the gene was expressed in the testicles using the Gene Expression Omnibus profiles (https://www.ncbi.nlm.nih.gov/geoprofiles).

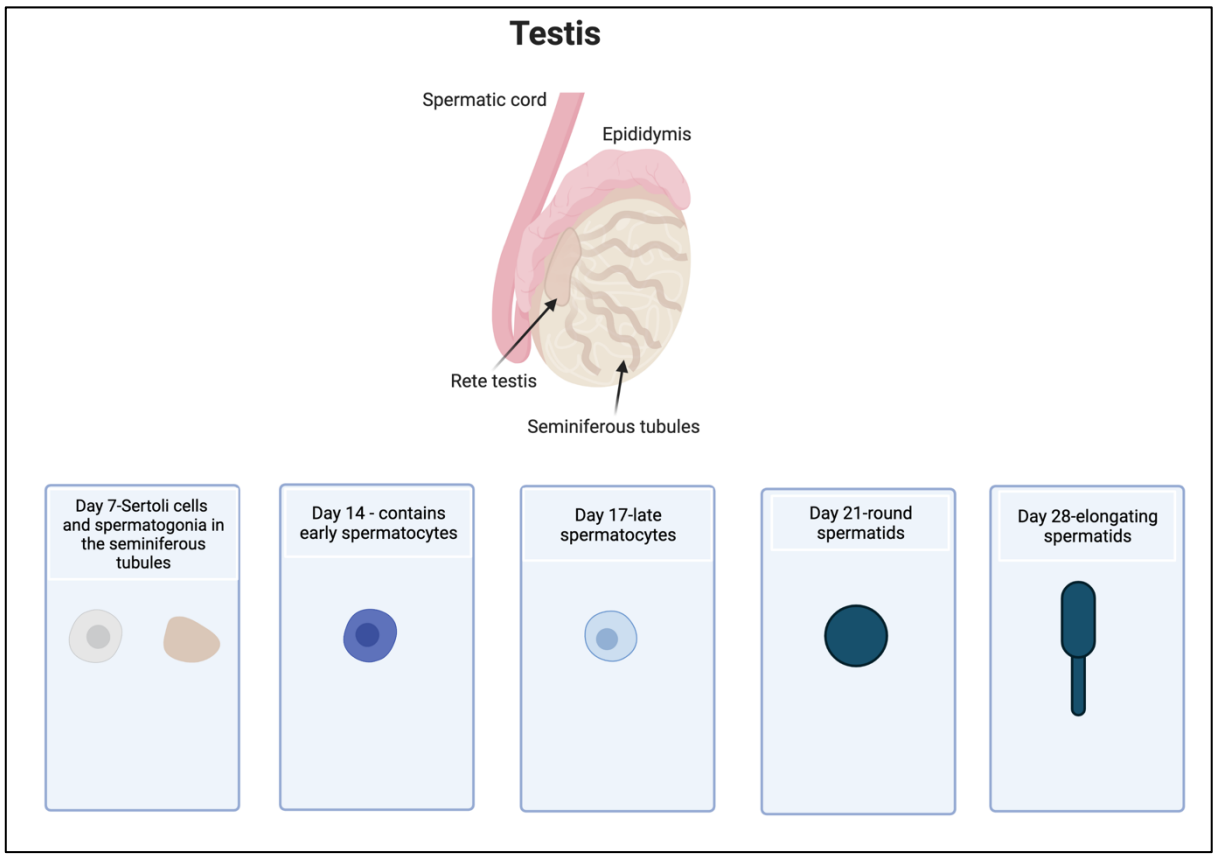
Identified genetic variants that fit the above criteria underwent the following analysis:

a) Sanger sequencing – It is still widely accepted that next generation sequencing results need to be validated by the current gold standard of sanger sequencing, therefore we performed sanger sequencing on any identified missense or nonsense variant that had an allele frequency of <0.0001 and which was associated with NOA in the current literature. Gene specific primers for any variants were designed using NCBI Primer-BLAST and genomic sequence amplification was performed using standard polymerase chain reaction conditions and annealing at the primer melting temperature.

b) The Enselbl reference human transcript annotation (version 103, (336)) was utilised to generate RNA sequences for genes, and changes caused by the identified variants for NOA transcripts and protein sequences translated with the Expasy translate tool (337). The pairwise sequence alignment tool (EMBOSS Needle sequence comparison (https://www.ebi.ac.uk/Tools/psa/emboss_needle/)) was used for protein sequence comparison between reference and NOA sequences. The 2D protein domains were predicted using InterProScan and 3D structures using Phyre2 tools (338). Multiple protein sequence alignment was done using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

c) Expression analysis: A comparison of RNA sequence data was made using contemporary literature from both human (339) and murine studies (340). Fagerberg et al. (339) investigated the RNA transcript expression data for 27 human tissues. Laiho et al. (340) investigated the gene expression data at 5 distinct points during the first wave of spermatogenesis in the mouse (post natal day 7, 14, 17, 21 and 28); Figure 4.3 describes the different cell content of the testes during these time points.

Figure 4.3 demonstrates the different cell contents of the mouse testes during different time points



4.4 Results

4.4.1. Study participants

17 participants fit the recruitment criteria (Table 4.2). The study cohort had a median age of 35 years, body mass index of 25.40 and included a variety of ethnicities (Table 4.3).

Table 4.3 Table demonstrating clinical characteristics of participants.

Data for age and body mass index (BMI) expressed as median (interquartile range).

	Male Factor
Sample size	17
Age	35.00 (7.00)
BMI	25.40 (4.60)
White British	1(5.88%)
White other	1(5.88%)
Indian	2(11.8%)
Pakistani	4 (23.53%)
Bangladesh	2(11.8%)
African	1(5.88%)
Other	5 (29.4%)

4.4.2. Candidate genes

This filtering pipeline resulted in 3 candidate variants in 3 genes within two unrelated participants (AZ001 and AZ031). All variants were loss of function with an allele frequency of <0.001 (Table 4.4).

Table 4.4 demonstrates the novel candidate variants identified in the study

*Allelle frequencies reported in gnomAD exomes and genomes

LoF (loss of function)

Gene	Position	Variant	Sequence ontology	Effect	Reference SNP identity	Allele frequency*	Patient ID
MEIOB	16:1889331	ATCA/-	Frameshift variant	LoF	766633975	0.0000715882	AZ031
FADH1	16:1889331	ATCA/-	3' Prime UTR variant	LoF	766633975	0.0000715882	AZ031
FKBP6	7:72745658	A/T	Splice acceptor variant	LoF	Not present on reference SNP identity database	Not present GenomeAD database	AZ001

There was a 4 base pair deletion (ATCA/-, chr16:1889331) present in both FAHD1 (fumarylacetoacetate hydrolase domain containing 1) and MEIOB (meiosis specific with OB-fold) genes but the FAHD1 variant was a 3' Prime UTR variant, whilst the MEIOB variant was a frameshift variant. The identified FKBP6 variant is a splice site mutation at the acceptor splice site chr7:72745658 (A>T) for exon 5.

4.4.3. Candidate genes expression

The FAHD1 gene has a broad expression pattern and has a role in mitochondrial function (341) and FKBP6 and MEIOB genes are testis specific and have been reported to have a role in meiosis (342,343). Therefore, the genes of FKBPS and MEIOB are more likely than FAHD1 to be causal mutations for NOA. This is supported by data investigating the expression pattern of these genes during the first wave of mouse spermatogenesis (340), which showed high expression during meiosis (postnatal day, PND 14-17) for FKBP6 and MEIOB, but not for FADH1 (Figure 4.4).

Figure 4.4 demonstrates the expression pattern of genes MEIOB, FKBP6 and FAHD1 in different organs.

RPKM (Reads per kilo base per million mapped reads); FAHD1 (fumarylacetoacetate hydrolase domain containing 1) and MEIOB (meiosis specific with OB-fold).

FAHD1 is broadly expressed in several organs whilst MEIOB and FKBP6 is predominantly expressed in the testis.

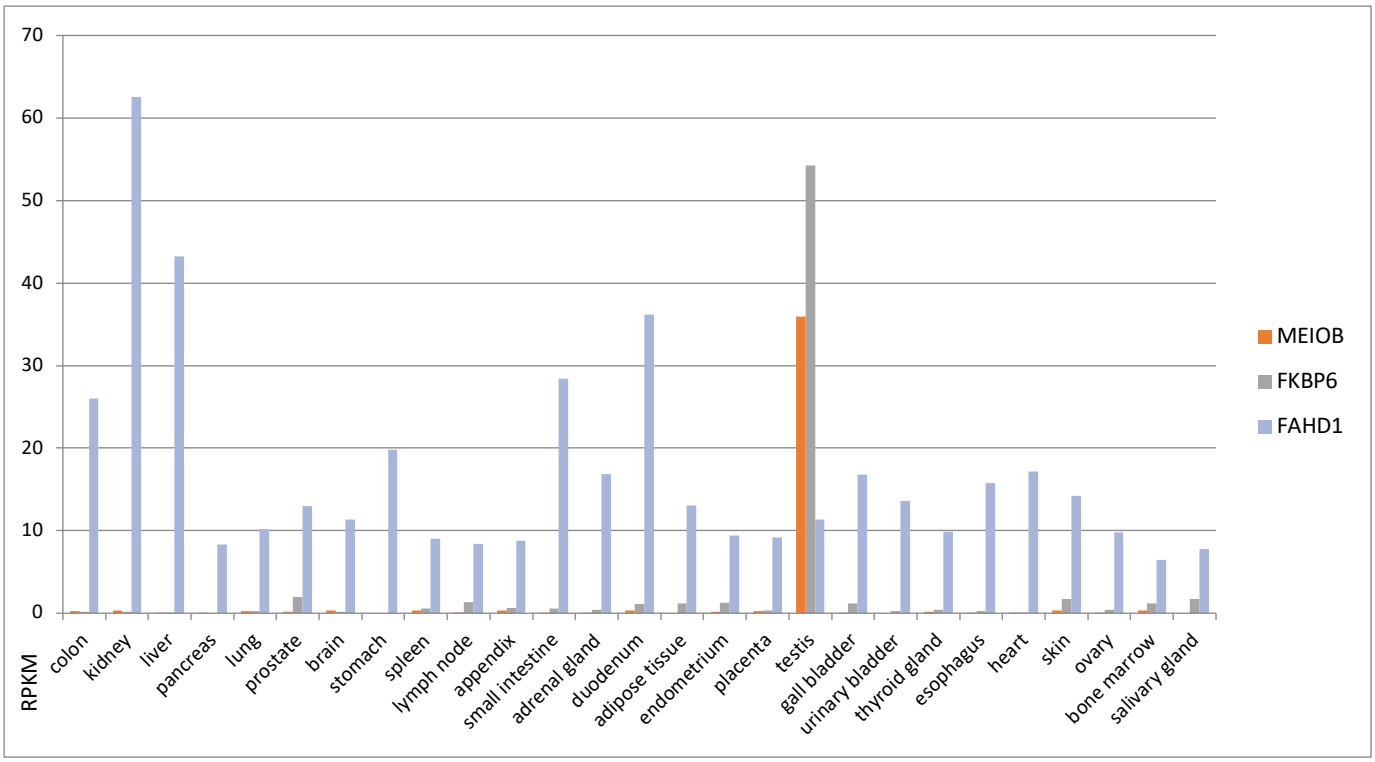
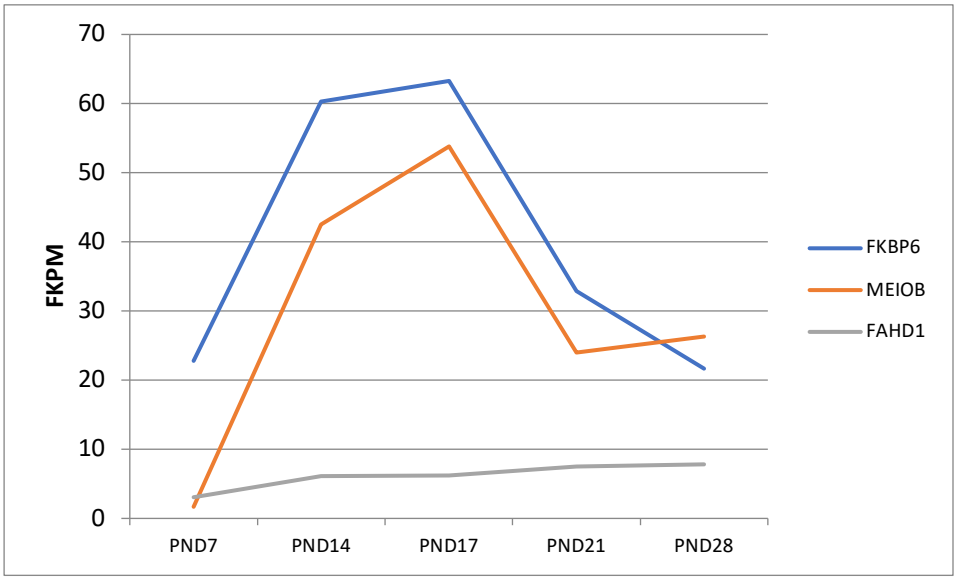


Figure 4.5 expression pattern of genes MEIOB, FKBP6 and FAHD1 during the first wave of spermatogenesis in the mouse according to data by Laiho et al.(340)

FKPM (Fragments Per Kilobase of transcript per Million mapped reads); FAHD1 (fumarylacetoacetate hydrolase domain containing 1) and MEIOB (meiosis specific with OB-fold). PND (post natal day)

Figure 4.5 demonstrates a high expression pattern during meiosis (postnatal day, PND 14-17) for FKBP6 and MEIOB but not FAHD1 genes during the first wave of mouse spermatogenesis.



The histological analysis of the seminiferous tubule cross sections of both patients showed spermatogenic arrest at meiosis, which is consistent with the known function of FKBP6 and MEIOB. Therefore, based on expression pattern, murine study data and the histology of the affected patients, FKPP6 and MEIOB (but not FADH1) are likely to be causal candidate genes for NOA.

4.4.4 Sanger sequencing of candidate genes

Sanger sequencing confirmed the presence of a homozygous alternative allele in participant AZ001 for FKBP6 (Figure 4.6a) and in AZ031 for MEIOB (Figure 4.6b).

Figure 4.6a Sanger sequencing of FKBP6 gene variant.

Figure 4.6a demonstrates the sanger sequencing of participant AZ001 and shows the A/T change at exon 5 acceptor splice site in FKBP6.

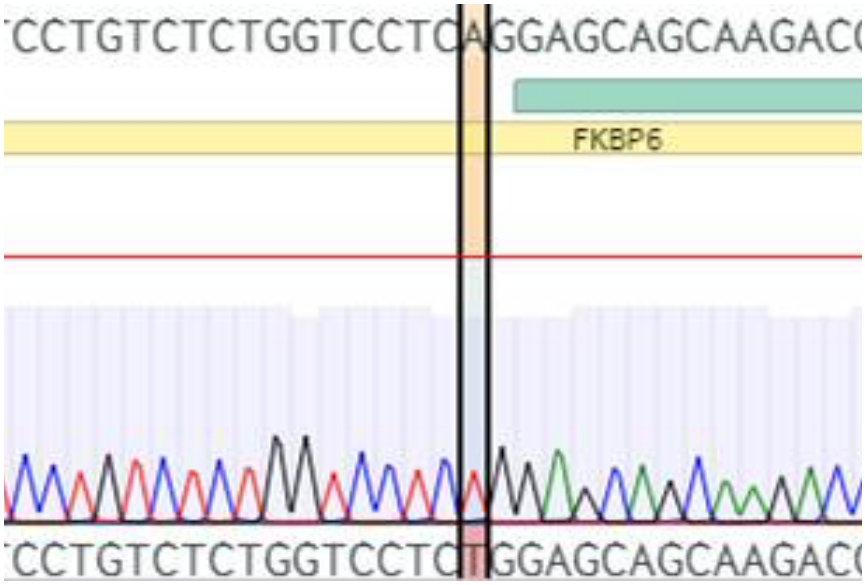
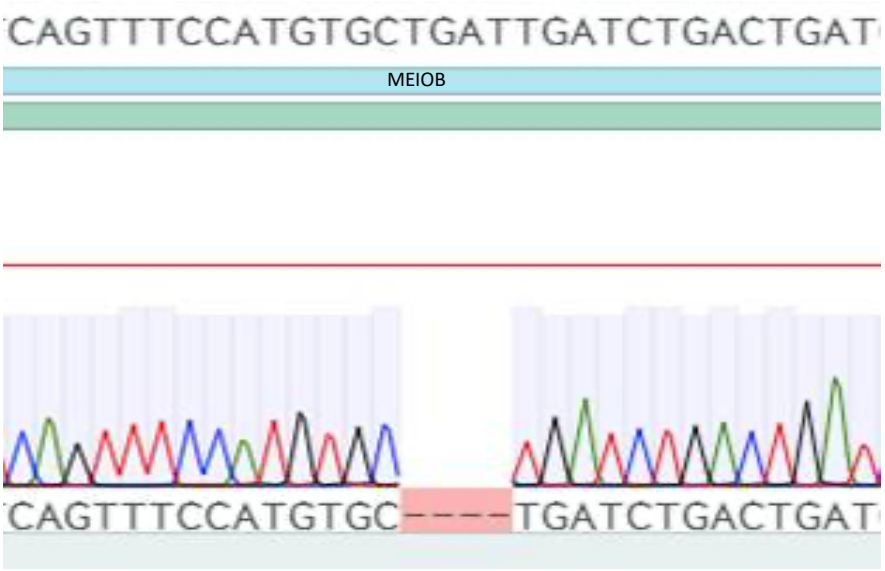


Figure 4.6b Sanger sequencing of MEIOB gene variant.

Figure 4.6b demonstrates the sanger sequencing of participant AZ031 and the four base pair deletion in MEIOB.



2.3.3.3 Protein sequences

The genomic sequence between exons 4-6 (chr7:73,330,150- 73,340,832) in FKBP6 was used for splice site prediction with Alternative Splice Site Predictor (ASSP, http://wangcomputing.com/assp/).

Figure 4.7a Predicted effect of FKBP6 variant on protein sequence

FKBP6 (reference protein sequence); NOA (FKBP6 variant protein sequence found in NOA patient)

Figure 4.7a compares the protein sequence in the FKBP6 variant (NOA) in the NOA participant with the addition of alternative splice site within FKBP6 exon 5 compared to the reference sequence (FKBP6).

```
FKBP6              1 MGGSALNQGVLEGDDAPGQSLYERLSQRMLDISGDRGVLKDVIREGAGDL     50
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
NOA                1 MGGSALNQGVLEGDDAPGQSLYERLSQRMLDISGDRGVLKDVIREGAGDL     50

FKBP6             51 VAPDASVLVKYSGYLEHMDRPFDSNYFRKTPRLMKLGEDITLWGMELGLL    100
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
NOA               51 VAPDASVLVKYSGYLEHMDRPFDSNYFRKTPRLMKLGEDITLWGMELGLL    100

FKBP6            101 SMRRGELARFLFKPNYAYGTLGCPPLIPPNTTVLFEIELLDFLDCAESDK    150
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
NOA              101 SMRRGELARFLFKPNYAYGTLGCPPLIPPNTTVLFEIELLDFLDCAESDK    150

FKBP6            151 FCALSAEQQDQFPLQKVLKVAATEREFGNYLFRQNRFYDAKVRYKRALLL    200
                     ||||||                          |||||||||||||||||
NOA              151 FCALSA----------------------------NRFYDAKVRYKRALLL    172

FKBP6            201 LRRRSAPPEEQHLVEAAKLPVLLNLSFTYLKLDRPTIALCYGEQALIIDQ    250
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
NOA              173 LRRRSAPPEEQHLVEAAKLPVLLNLSFTYLKLDRPTIALCYGEQALIIDQ    222

FKBP6            251 KNAKALFRCGQACLLLTEYQKARDFLVRAQKEQPFNHDINNELKKLASCY    300
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
NOA              223 KNAKALFRCGQACLLLTEYQKARDFLVRAQKEQPFNHDINNELKKLASCY    272

FKBP6            301 RDYVDKEKEMWHRMFAPCGDGSTAGES    327
                     ||||||||||||||||||||||||||
NOA              273 RDYVDKEKEMWHRMFAPCGDGSTAGES    299
```

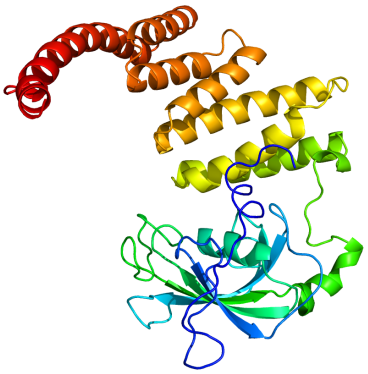
For the FKBP6 variant an alternative acceptor splice site within exon 5 was predicted at position chr7:73331655. The presence of this splice site shortens the exon 5 by 84bp to 36bp resulting in a reduction of 28 amino acids (amino acids 157-184, Figure 4.7a) affecting the tetratricopeptide repeat (TPR, 171-286aa), which form scaffolds to facilitate protein-protein interactions. The absence of this domain is likely to result in misfolding of FKBP6 and impact the interaction with other proteins. Figure 4.7b is a 3D prediction of both the NOA FKBP6 and reference protein structure and this demonstrates a misfolding of the mutated FKBP6.

Figure 4.7b: Predicted effects of FKBP6 variant on 3D structure of FKBP6

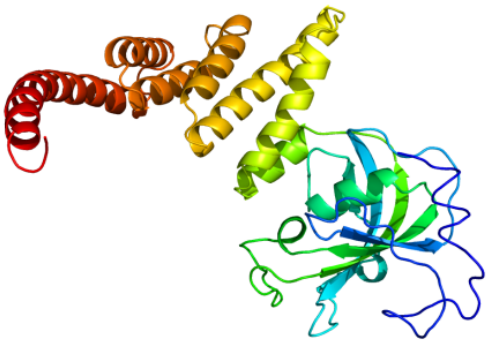
FKBPS (reference 3D structure), FKBPS NOA (3D structure of FKBP6 variant in NOA participant)

Figure 4.7b demonstrates the misfolding of the FKBP6 variant found in the non-obstructive azoospermia patient (FKBP6 NOA) compared to the reference FKBP6 structure (FKBP6).

FKBP6



FKBP6 NOA



In the MEIOB gene variant the premature stop codon causes depletion of amino acids 381-471 in the protein sequence (Figure 4.8a).

Figure 4.8a Predicted effect of MEIOB variant on protein sequence

MEIOB (reference protein sequence); NOA (MEIOB variant protein sequence found in NOA patient)

Figure 4.8a compares the protein sequence in the MEIOB variant (NOA) in the NOA participant with the addition of the premature stop codon created by MEIOB frameshift variant compared to the reference sequence (MEIOB).

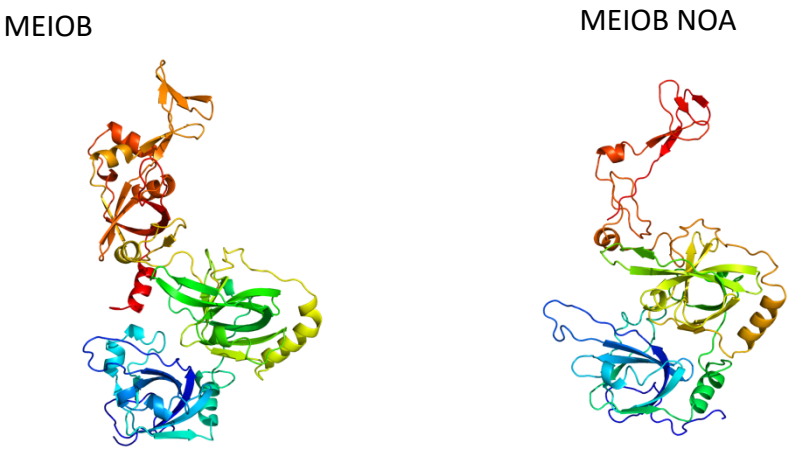
```
MEIOB           251 SKTIITTNPDIPEANILLNFIRENKETNVLDDEIDSYFKESINLSTIVDV    300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
NOA             251 SKTIITTNPDIPEANILLNFIRENKETNVLDDEIDSYFKESINLSTIVDV    300

MEIOB           301 YTVEQLKGKALKNEGKADPSYGILYAYISTLNIDDETTKVVRNRCSSCGY    350
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
NOA             301 YTVEQLKGKALKNEGKADPSYGILYAYISTLNIDDETTKVVRNRCSSCGY    350

MEIOB           351 IVNEASNMCTTCNKNSLDFKSVFLSFHVLIDLTDHTGTLHSCSLTGSVAE    400
                    ||||||||||||||||||||||||||||||
NOA             351 IVNEASNMCTTCNKNSLDFKSVFLSFHVLI--------------------    380

MEIOB           401 ETLGCTVHEFLAMTDEQKTALKWQFLLERSKIYLKFVLSHRARSGLKISV    450

NOA             381 ------------------------------------------------    380

MEIOB           451 LSCKLADPTEASRNLSGQKHV    471

NOA             381 ----------------------    380
```

This protein region contains a conserved DNA binding domain and C-terminal truncating mutations have also been reported previously in Israeli patients (81). The premature stop codon often initiates nonsense mediated decay of the transcript resulting in no protein or a shortened NOA isoform with misfolding of the MEIOB (Figure 4.8b).

Figure 4.8b: Predicted effects of MEIOB variant on 3D structure of MEIOB

MEIOB (reference 3D structure), MEIOB NOA (3D structure of MEIOB variant in NOA participant)

Figure 4.8b demonstrates the misfolding of the MEIOB variant found in the non-obstructive azoospermia patient (MEIOB NOA) compared to the reference MEIOB structure (MEIOB).



Both depleted protein sequences are highly conserved across mammalian species indicating an evolutionary important role in sperm production (Figure 4.9a and 4.9b)

Figure 4.9a Conservation of protein sequences of FKBP6 for other species

```
Sus         ALSVDQQDQFPLEKVLKVAATEREFGNYLFRQNRFYD   37
Mus         ALSAEQQEQFPLQKVLKVAATEREFGNYLFRQNRFCD   37
Equus       ALSAEQQDQFPLQKVLKVAATEREFGNYLFRQHRFYD   37
Homo        ALSAEQQDQFPLQKVLKVAATEREFGNYLFRQNRFYD   37
Canis       ALSAEQQDQFPLQKVLKVAATEREFGNYLFRQNRFYD   37
Bos         ALSAEQQSQFPLQKVLKVAATEREFGNYLFRQNRFYD   37
            ***.:**.****:******************:** *
```

Figure 4.9b Conservation of protein sequences of MEIOB for other species

```
Mus         DLTDHTGTLHSCSLSGSIAEETLGCTINEFLTMTSEQKTKLKWQLLLERSKIYLKLILSH     60
Sus         DLSDHTGTLHFCSLTGSVAEETLGCTVNEFLAMTDEQRTALKWQLLLERTKIYLKVSLLH     60
Homo        DLTDHTGTLHSCSLTGSVAEETLGCTVHEFLAMTDEQKTALKWQFLLERSKIYLKFVLSH     60
Equus       DLTDHTGTLHSCSLTGSVAEETLGCTVNEFLAMTDAQKTALKWQFLLERSKIYLKFFLSH     60
Canis       DLTDHTGTLHACSLTGGVAEETLGCTVNEFLAMTDEQKTALKWQFLLERSKIYLKFFLSH     60
            **:******* ***:*.:********::***:**. *:* ****:****:*****. * *

Mus         RARGGLKVTILSCKLADPTEASRNLARQGH-     90
Sus         RARARLRMSVLSCKLADPVEASRSLSG----     87
Homo        RARSGLKISVLSCKLADPTEASRNLSGQKHV     91
Equus       RARGGLRISVLSCKLADPIEASRNLSGQRNI     91
Canis       RARGGLRISVLSCKLADPIEASRNLSGGGNI     91
            ***. *:::::******** ****.*:
```

4.5 Discussion

There are no therapeutic options for NOA other than surgical sperm retrieval of which the success rate is only 47% (249). NOA represents the pathology in 10-20% of patients presenting with infertility (42) and in the vast majority of cases the cause will be unknown. This represents a huge gap in our understanding of infertility and further knowledge is needed to help counsel patients but also to rationalise and develop future treatments.

In this study, whole exome sequencing and bioinformatics analysis was performed to identify novel causative variants for NOA. The allele frequency of the MEIOB variant is low (0.0000715882) and no homozygous variants are present in the gnomAD database. Contemporary literature has reported variants in MEIOB as the cause for male (NOA) and female (primary ovarian insufficiency) infertility. Three disease causing variants have been reported (81,82,344) and two of the variants cause C-terminal truncation in accordance with the findings of this study. It has been shown that the truncation of MEIOB C-terminus disrupts the interaction with SPATA22 (345–347). Both the proteins MEIOB and SPATA22 are required to induce DNA double-strand breaks and successful recombination of chromosomes (342,346) providing a theoretical plausibility that a MEIOB variant could be the cause of NOA.

This is the first report of a potential FKBP6 causative variant in an NOA patient and the identified FKBP6 variant is not present in the gnomAD database. FKBP6 appears to be a male specific protein required for sex-specific synaptonemal complex maintenance. Crackower et al. (343) reported that the targeted inactivation of FKBP6 results in abnormal pairing and misalignments between homologous chromosomes, nonhomologous partner switches, and autosynapsis of X chromosome cores in meiotic spermatocytes resulting in azoospermic mice. Miyamoto et al. (348) reported a hetereozygous FKBPS polymorphism (premature stop codon) in exon 3 (245C→G) in an analysis of 19 idiopathic NOA patients but the authors also noted that the same heterozygous sequence was present in 10/30 of a fertile control group.This is the first study to identify the presence of a homozygous FKBP6 variant in a NOA patient.

A further review of the literature highlighted that both depleted protein sequences are highly conserved across other mammalian species indicating an evolutionary importance. This supports that the identified variants are likely to be causal in NOA.

Unfortunately, tissue samples for RNA or protein level studies were not available but predicted consequences of the identified variants strongly support their causality.

However, it is important to recognise the limitations to this study. We recruited only 17 participants. This was because idiopathic NOA is uncommon and also our recruitment was limited by the emergence of the COVID-19 pandemic both in terms of participant interest and laboratory practice restrictions. Future research should consider multiple centres to increase the potential recruitment catchment area. Indeed, Professor Frank Tuttlemann (University of Munster) and Professor Joris Veltmann (University of Newcastle) have formed a consortium investigating genetic variants in male infertility and these collaborations increase the likelihood of recruiting larger sample cohorts.

I performed whole exome sequencing (WES) which is inferior to whole genome sequencing (WGS) because it may not detect copy number or non coding variants (349). However, most heritable diseases are caused by alterations in protein-coding regions of the genome and WES is cheaper and quicker to perform (350). There is also data showing that WES is comparable to WGS in clinical and research applications (350). Therefore, whilst WES may not be as comprehensive as WGS it was suitable for the purposes of this study.

Causality can be proven with parent-offspring genetic trio data or knockout gene studies. Parent-offspring genetic trio data involves comparing the exome sequences of the NOA patient with that of their parents; if the variant was present in one or both parents then this would increase the likelihood that the genetic variant is causal. However, in my study the parents of the participants with novel variants were unavailable (deceased or abroad) and I was therefore unable to confirm causality using this approach. Another method to prove causality is through knockout gene studies in animals. I was unable to perform functional studies due to time restrictions, but my study has highlighted two candidate variants that can be tested in future studies. However, given the strict bioinformatics pipeline criteria (rare, homozygous, loss of function variants expressed in genes associated with NOA and expressed in the testis) there is a high likelihood that these gene variants are causal. We are planning further functional studies using targeted gene knockout in drosphila flies.

4.6 Conclusion

We have observed two novel loss of function variants in a small cohort of idiopathic NOA men. A homozygous splice acceptor variant in the FKBP6 gene and a frameshift variant in the MEIOB gene. The candidate genes are associated with azoospermia and highly expressed in the testes suggesting causality. However, future knockout studies are required to confirm causality.

Chapter 5:

General Discussion

The advent of assisted reproductive technologies (ART) such as in vitro fertilisation (IVF) has undoubtedly allowed many infertile couples to conceive children. However, most ART function by bypassing issues related to male sperm. Indeed, the main indication for ART in the U.K was recently reported to be male factor infertility (259). The widespread use of ART may have reduced commercial interest into setting up studies investigating the cause for male infertility. However, the success rate of IVF is 29.6% (265) and given the limited resources within the NHS the availability of ART is declining (263). Within this context, further understanding of the pathophysiological mechanisms that underpin male infertility is needed.

Unexplained infertility represents 30% of all infertile couples (48,49) and the only diagnostic criteria for men is a normal semen analysis (according to WHO reference ranges) even though there is data showing that the WHO reference ranges for semen analysis are a poor discriminator between infertile and fertile populations (108). There is increasing data showing that oxidative stress may contribute to sperm dysfunction and that sperm DNA fragmentation (SDF) and reactive oxygen species (ROS) levels may be raised in infertile men compared to fertile controls (48). Currently, both seminal ROS and SDF testing are not routinely performed in the assessment of male infertility and this is because both tests are costly, subject to inter-assay and inter-laboratory variability and require specialist expertise and equipment (351). Therefore, more evidence regarding clinical utility is needed before recommending their use in clinical practice. Moreover, data is needed to determine whether men diagnosed with unexplained infertility have molecular abnormalities such as high seminal ROS or SDF. This is of clinical importance because the majority of men with unexplained infertility proceed to ART; but if they were found to have abnormal oxidative stress they could be investigated and treated for potential reversible causes such as varicocele, hyperglycaemia, alcohol excess, cigarette smoking, raised BMI and genital tract infections (126,139–144). Moreover, these men may be candidates for antioxidant therapy as there is data showing that antioxidant therapy may improve sperm parameters (270) and ART outcomes (271). Therefore, I sought to identify whether there was an increase in oxidative stress in men diagnosed with unexplained infertility in comparison with male factor and fertile controls and this was measured by SDF and seminal ROS.

I also investigated a potential cause for male infertility and studied the differences in seminal microbiome between infertile and fertile men. Although there are many recognised causes of sperm dysfunction including infection, drug use and trauma, in approximately 50% of male infertility cases

the cause is unknown (107). There is data reporting the importance of the microbiome in gastrointestinal tract disorders, and treatments such as faecal microbiota transplantation have been successfully used to treat clostridia difficile infection (352). The emergence of next generation sequencing has provided novel data and an informative insight into the function of bacteria in human health and disease (167) and the urogenital tract has been observed to contribute 9% of the total human microbiome (168). It has been postulated that certain bacterial specifies or communities of different bacteria may contribute to male infertility (169) and this has been supported by data reporting a negative association between specific seminal bacteria and sperm function (170,171). However, there is a paucity of studies related to the seminal microbiome. We performed a proof-of-concept study to investigate whether the seminal microbiome was significantly different between male factor, unexplained infertility and fertile controls. Our aims were to investigate whether the seminal microbiome may be a potential aetiological factor for male infertility. Moreover, we sought to add to the current understanding of the pathophysiological mechanisms of male infertility and provide a focus for future studies. I performed the first study to investigate differences in seminal microbiome between male factor, unexplained infertility and fertile cohorts.

I observed no differences in seminal ROS, SDF and seminal microbiome between unexplained infertility and fertile controls. Moreover, there was no significant difference in seminal microbiome and SDF between any of the three cohorts. The only significant difference identified was an increased seminal ROS in the male factor infertility cohort compared to the unexplained infertility or fertile controls. The current literature has shown that male factor infertility has a higher seminal ROS level compared to fertile controls (135,200,201) but in discordance with other studies, we did not observe a significant difference in SDF (353,354). Moreover, in contrast to other studies we did not observe any elevation in SDF in the unexplained infertility cohort compared to fertile controls (266,269,355). The current literature is conflicted (266–268) as to whether seminal ROS is elevated in unexplained infertile men compared to fertile controls and I did not observe any significant difference. Collectively this suggests that infertile men in unexplained infertility couples are either: (1) fertile with the issues of fecundity related to undiagnosed female factors, (2) That seminal microbiome, SDF and seminal ROS are unable to discriminate differences between unexplained infertile men and fertile controls or (3) Our findings were biased due to limitations of our study. A potential limitation to the study was that the

sample sizes were too small to detect a statistically significant difference in variables. Our recruitment was severely affected by the COVID-19 pandemic both in terms of participant interest and laboratory restrictions on staff personnel and working hours. However, this was a proof-of-concept study and therefore no power and sample size estimation calculations were performed. We observed a significant difference between the male factor and unexplained infertility cohort.
Another limitation to the study was that age and BMI were significantly higher in the fertile cohort and this may have masked subtle differences between the unexplained infertility and fertile cohorts. Moreover, I was unable to collect data on fertility outcomes and therefore it is unclear how these novel markers were associated with clinical outcomes such as pregnancy rate and live birth rate. Prior to the end of the study, I was unable to investigate any correlations between seminal microbiome and semen analysis, or SDF and seminal ROS due to the aforementioned COVID-19 restrictions. Moreover, I was unable to process any hormone profiles due to similar reasons. This analysis may be useful in determining whether seminal microbiome or hormone profiles can affect oxidative stress and should be a direction of future research. Moreover, future studies should attempt to have larger matched cohorts with clinical outcomes such as pregnancy and live birth rate. My study results suggest that unexplained infertility may be more similar to fertile controls (as evidenced by semen parameters and seminal ROS,) than male factor infertility and thus may not benefit from andrological assessment or empirical therapies aimed at improving sperm function (such as antioxidants). Furthermore, my study results suggest that infertile men with normal semen analysis should not undergo further testing with oxidative stress markers as they are likely to be normal.

Non obstructive azoospermia (NOA) represents the most severe form of male infertility and men with this disorder require testicular sperm extraction coupled with ART to father children. However, a recent meta-analysis reported that the success rate of finding sperm in testicular sperm extraction procedures is only 47% (249). Clinicians have empirically trialled hormone stimulation therapy (HST) to increase the success rates of testicular sperm extraction but there is a wide variety in terms of HST agents and protocols and it is unclear from the literature the risks and benefits of such an approach. Indeed, the current European Association of Urology guidelines on Male Sexual and Reproductive Health do not recommend the use of HST prior to surgical sperm retrieval in men with idiopathic NOA because of limited data (66). However, a survey of American urologists reported that 64.9% of

participants prescribed hormone therapy to treat idiopathic male infertility, with Clomiphene Citrate the most commonly prescribed drug for both general and fertility trained urologists (250). Within this context, there was an urgent need for a critical appraisal of the evidence supporting HST as a neo-adjuvant therapy for surgical sperm retrieval procedures. I performed the first meta-analysis investigating the effects of HST on surgical sperm retrieval rates in NOA and observed that HST increased the sperm retrieval rate in normogonadotropic but not hypergonadotropic NOA men. This suggests that HST might have a role in optimising sperm retrieval rates in a specific cohort but should not be used for all procedures. However, one major observation from the study was the low-quality evidence currently available and there are no randomised controlled trials investigating the use of HST to optimise surgical sperm retrieval in contemporary literature. This highlights that HST cannot be advocated until prospective randomised controlled trials have occurred. A major strength of this study is that it has provided preliminary data to justify the development of a large, multicentre randomised controlled trial that could potentially provide high level evidence to rationalise future treatments. This trial should investigate the use of gonadotropin therapies in men with normal gonadotropin levels with NOA. The meta-analysis and systemic review suggested that this type of hormone therapy had the least side effects and increased surgical sperm retrieval rate in normogonadotropic men. This trial should include patient reported outcome measure data to assess patients' opinions, compliance issues and also mental health status. This would improve our current understanding of patients' ideas, concerns and expectations and also provide a patient perspective on the therapy and disease. Moreover, the trial should investigate for potential exploratory mechanisms and can include assessment of sperm DNA fragmentation, seminal ROS, hormone levels and radiological investigations including testicular ultrasound. This would potentially provide mechanistic data. Moreover, the trial should include important clinical outcomes such as pregnancy and live birth rates.

The meta-analysis provided a critical overview of the current HST literature (including limitations), and this should help inform both clinicians and patients.

In approximately 50% of cases of male infertility the cause is unknown (330) and in only 25% of cases of NOA can a genetic cause be identified (331). Thus, a greater appreciation of the pathophysiological mechanisms that underpin NOA may help patient counselling and also the development of future

therapies. The discovery of Y chromosome microdeletions has allowed disease stratification of NOA patients and the presence of an AZFa or AZFb deletion is associated with a low sperm retrieval rate (67). Indeed, patients with AZFa or AZFb deletions are counselled against testicular sperm extraction surgery. The development of next generation sequencing has made it cheaper and quicker to perform genome wide association studies to identify variants that causally effect a phenotype (356). I performed a study analysing genetic variants in a small cohort of idiopathic NOA patients and observed two novel genetic variants. I used a strict bioinformatics pipeline (rare, loss of function, homozygous variants expressed in the testis and in genes associated with NOA) to increase the likelihood of finding genuine candidate genetic variants. One challenge with genome wide association studies is the ability to confirm a variant is causal and exclude environmental factors (356). Causality can be proven with parent-offspring genetic trio data and if the identified variant is present in one or both parents then this would increase the likelihood that the genetic variant is causal. Another method of proving causality is by performing knock out studies. We were unable to prove causality as both participant's parents were unavailable and were unable to perform knock-out gene studies due to time restrictions. However, my study has highlighted two novel candidate gene variants and these findings are currently being utilised to apply for funding to perform knockout studies on mice and the drosophila fly.

Another limitation to my study was that it only included 17 men. This is because idiopathic non obstructive azoospermia is uncommon and also due to the COVID-19 pandemic restrictions affecting both participant interest and also laboratory practice. It is worth noting that Professor Frank Tuttlemann (University of Munster) and Professor Joris Veltmann (University of Newcastle) have recently formed a consortium investigating genetic variants in male infertility and future studies might need to involve other centres and closer collaboration between institutions.

In summary, I have performed the first study to compare seminal ROS, SDF and seminal microbiome in different cohorts of infertility. I did not observe any significant differences in seminal microbiome between the different cohorts and no significant differences in seminal ROS or SDF between unexplained infertility compared to fertile controls. This suggests that the seminal microbiome is not a contributor to male infertility and there is no differences in oxidative stress between unexplained fertility and fertile controls. Thus, based on my data, antioxidants should not be used in unexplained

infertility and infertile men with normal semen analysis should not undergo further testing for oxidative stress. I also performed the first meta-analysis to investigate the effects of HST on surgical sperm retrieval rates in NOA and have identified that HST is only effective in men with normal hormone profiles. I have identified two novel variants in idiopathic NOA men and this data will help a targeted genetic approach in future knock out studies.

Overall, my work has added to our current understanding of the aetiology and management of male infertility and can provide the preliminary data for larger, higher-powered studies.

Appendix

1. Study questionnaire

QUESTIONNAIRE 1

Participant study code _____ Date completed _____

Weight kg Height cm Testicular Volume mls

Please answer the following questions, providing as much information as possible. This information will be kept strictly confidential by the research team.

1. What is your ethnicity? Please tick the most appropriate category.

White British	_____
White Irish	_____
White Other	_____
Mixed White-Caribbean	_____
Mixed White-African	_____
Mixed White-Asian	_____
Other Mixed	_____
Indian	_____
Pakistani	_____
Bangladeshi	_____
Chinese	_____
Caribbean	_____
African	_____
Other Black	_____
Other	_____

1. Have you smoked regularly in the last year? **YES/ NO**
   If yes, how many years have you smoked? _____**years**

What do you smoke? (please circle) **CIGARETTES TOBACCO CIGARS OTHER**_____

How many times do you smoke per day _____

2. How many units of alcohol do you drink in a typical week? (1 pint of beer is 3 units, 1 large glass of wine is 3 units, a single measure of spirits is 1 unit)
_____**units**

3. Does any male family member (father, brother, uncle) have a history of infertility? **YES / NO.** If yes, please give details
_____

4. Does any female family member (mother, sister, aunt) have a history of infertility? **YES / NO.** If yes, please give details
_____

5. Are you on any regular medications? **YES / NO**
If Yes, please list any medications you currently take, and for how long you have taken them:

---

6. Have you ever taken any medications for infertility (e.g. clomiphene, tamoxifen)? Please give details and dates of any treatment:

_____
_____

_____
_____

7. How old were you when puberty started (e.g. voice deepening, body hair growth)
_____**years**

8. Have you experienced any of the following illnesses:

Injury or operation to your testicles	**YES / NO**
Undescended testicles	**YES / NO**
Groin problem (such as a hernia)	**YES / NO**
Problems with having erections	**YES / NO**
Mumps	**YES / NO**
Diabetes	**YES / NO**
Heart Problems	**YES / NO**

Kidney problems	**YES / NO**
Liver problems	**YES / NO**
Skin problems	**YES / NO**
Cancer, lymphoma or leukaemia	**YES / NO**

TB (tuberculosis)	**YES / NO**
Hepatitis	**YES / NO**
Sexually transmitted illness (e.g. chlamydia, gonorrhoea, HIV)	**YES / NO**
Problems smelling things	**YES / NO**

Please details and dates of any illnesses you have experienced (please give as much info as possible).

_____
_____

_____
_____

9. Have you ever taken any of the following medications:

Chemotherapy	**YES / NO**
Steroid tablets (such as prednisolone, dexamethasone)	**YES / NO**
Immunosuppressant medications	**YES / NO**

10. Have you taken any recreational drugs within the last 5 years? (this is strictly confidential)

Cannabis	**YES / NO**
Cocaine	**YES / NO**
Heroine	**YES / NO**

Any other recreational drug _____
Please give further details and dates of any recreational drug use:

_____
_____

11. Do you currently have a partner? **YES / NO.** If no, there are no further questions to answer.

    a. What your partner's age? _____**years**
    b. Does your partner have regular periods? **YES / NO**

c. Does your partner have any condition affecting her fertility **YES / NO.** Please give details below:

_____

_____

_____

d. On average, how many times <u>per month</u> do you and your partner have intercourse? Please circle one option?

Less than once **1-3 times** **4-6 times** **7-10 times** **over 10 times**

12. Has your partner ever had any of the following treatments:
    e. Clomiphene citrate (clomid) **YES / NO**
    f. IVF (in vitro fertilisation) **YES / NO**
    g. IUI (intrauterine insemination) **YES / NO**
    h. Any other fertility treatment. _____

    Please give further details and dates of any fertility treatment:

_____

_____

_____

13. Have you ever had any children? **YES / NO**
    If you do have children, please give their age(s) _____

14. Have you ever had a miscarriage or stillbirth? **YES / NO**.
    If yes, please tell us when this happened:

2. **PRISMA CHECKLIST** (357) (available at https://www.prisma-statement.org/documents/PRISMA_2020_checklist.pdf)

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	
Study characteristics	17	Cite each included study and present its characteristics.	
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	
	23b	Discuss any limitations of the evidence included in the review.	
	23c	Discuss any limitations of the review processes used.	
	23d	Discuss implications of the results for practice, policy, and future research.	
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	
Competing interests	26	Declare any competing interests of review authors.	
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

For more information, visit: http://www.prisma-statement.org/

3. **ROBINS 1-Tool** (299) (available at https://www.riskofbias.info/welcome/home)

The Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool

(version for cohort-type studies)

Developed by: Jonathan AC Sterne, Miguel A Hernán, Barnaby C Reeves, Jelena Savović, Nancy D Berkman, Meera Viswanathan, David Henry, Douglas G Altman, Mohammed T Ansari, Isabelle Boutron, James Carpenter, An-Wen Chan, Rachel Churchill, Asbjørn Hróbjartsson, Jamie Kirkham, Peter Jüni, Yoon Loke, Terri Pigott, Craig Ramsay, Deborah Regidor, Hannah Rothstein, Lakhbir Sandhu, Pasqualina Santaguida, Holger J Schünemann, Beverly Shea, Ian Shrier, Peter Tugwell, Lucy Turner, Jeffrey C Valentine, Hugh Waddington, Elizabeth Waters, Penny Whiting and Julian PT Higgins

Version 1 August 2016

ROBINS-I tool (Stage I): At protocol stage

Specify the review question

Participants	
Experimental intervention	
Comparator	
Outcomes	

List the confounding domains relevant to all or most studies

List co-interventions that could be different between intervention groups and that could impact on outcomes

ROBINS-I tool (Stage II): For each study

Specify a target randomized trial specific to the study

Design	Individually randomized / Cluster randomized / Matched (e.g. cross-over)
Participants	
Experimental intervention	
Comparator	

Is your aim for this study…?

- ☐ to assess the effect of *assignment to* intervention
- ☐ to assess the effect of *starting and adhering to* intervention

Specify the outcome

Specify which outcome is being assessed for risk of bias (typically from among those earmarked for the Summary of Findings table). Specify whether this is a proposed benefit or harm of intervention.

Specify the numerical result being assessed

In case of multiple alternative analyses being presented, specify the numeric result (e.g. RR = 1.52 (95% CI 0.83 to 2.77) and/or a reference (e.g. to a table, figure or paragraph) that uniquely defines the result being assessed.

Preliminary consideration of confounders

Complete a row for each important confounding domain (i) listed in the review protocol; and (ii) relevant to the setting of this particular study, or which the study authors identified as potentially important.

"Important" confounding domains are those for which, in the context of this study, adjustment is expected to lead to a clinically important change in the estimated effect of the intervention. "Validity" refers to whether the confounding variable or variables fully measure the domain, while "reliability" refers to the precision of the measurement (more measurement error means less reliability).

(i) Confounding domains listed in the review protocol				
Confounding domain	Measured variable(s)	Is there evidence that controlling for this variable was unnecessary?*	Is the confounding domain measured validly and reliably by this variable (or these variables)?	OPTIONAL: Is failure to adjust for this variable (alone) expected to favour the experimental intervention or the comparator?
			Yes / No / No information	Favour experimental / Favour comparator / No information

(ii) Additional confounding domains relevant to the setting of this particular study, or which the study authors identified as important				
Confounding domain	Measured variable(s)	Is there evidence that controlling for this variable was unnecessary?*	Is the confounding domain measured validly and reliably by this variable (or these variables)?	OPTIONAL: Is failure to adjust for this variable (alone) expected to favour the experimental intervention or the comparator?
			Yes / No / No information	Favour experimental / Favour comparator / No information

* In the context of a particular study, variables can be demonstrated not to be confounders and so not included in the analysis: (a) if they are not predictive of the outcome; (b) if they are not predictive of intervention; or (c) because adjustment makes no or minimal difference to the estimated effect of the primary parameter. Note that "no statistically significant association" is not the same as "not predictive".

Preliminary consideration of co-interventions

Complete a row for each important co-intervention (i) listed in the review protocol; and (ii) relevant to the setting of this particular study, or which the study authors identified as important.

"Important" co-interventions are those for which, in the context of this study, adjustment is expected to lead to a clinically important change in the estimated effect of the intervention.

(i) Co-interventions listed in the review protocol		
Co-intervention	Is there evidence that controlling for this co-intervention was unnecessary (e.g. because it was not administered)?	Is presence of this co-intervention likely to favour outcomes in the experimental intervention or the comparator
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information

(ii) Additional co-interventions relevant to the setting of this particular study, or which the study authors identified as important		
Co-intervention	Is there evidence that controlling for this co-intervention was unnecessary (e.g. because it was not administered)?	Is presence of this co-intervention likely to favour outcomes in the experimental intervention or the comparator
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information

Risk of bias assessment (cohort-type studies)

Responses underlined in green are potential markers for low risk of bias, and responses in red are potential markers for a risk of bias. Where questions relate only to sign posts to other questions, no formatting is used.

Bias domain	Signalling questions	Elaboration	Response options
Bias due to confounding	1.1 Is there potential for confounding of the effect of intervention in this study? **If N/PN to 1.1:** the study can be considered to be at low risk of bias due to confounding and no further signalling questions need be considered	In rare situations, such as when studying harms that are very unlikely to be related to factors that influence treatment decisions, no confounding is expected and the study can be considered to be at low risk of bias due to confounding, equivalent to a fully randomized trial. There is no NI (No information) option for this signalling question.	Y / PY / PN / N
	If Y/PY to 1.1: determine whether there is a need to assess time-varying confounding:		
	1.2. Was the analysis based on splitting participants' follow up time according to intervention received? **If N/PN**, answer questions relating to baseline confounding (1.4 to 1.6) **If Y/PY**, proceed to question 1.3.	If participants could switch between intervention groups then associations between intervention and outcome may be biased by time-varying confounding. This occurs when prognostic factors influence switches between intended interventions.	NA / Y / PY / PN / N / NI
	1.3. Were intervention discontinuations or switches likely to be related to factors that are prognostic for the outcome? **If N/PN**, answer questions relating to baseline confounding (1.4 to 1.6) **If Y/PY**, answer questions relating to both baseline and time-varying confounding (1.7 and 1.8)	If intervention switches are unrelated to the outcome, for example when the outcome is an unexpected harm, then time-varying confounding will not be present and only control for baseline confounding is required.	NA / Y / PY / PN / N / NI
	Questions relating to baseline confounding only		
	1.4. Did the authors use an appropriate analysis method that controlled for all the important confounding domains?	Appropriate methods to control for measured confounders include stratification, regression, matching, standardization, and inverse probability weighting. They may control for individual variables or for the estimated propensity score. Inverse probability weighting is based on a function of the propensity score. Each method depends on the assumption that there is no unmeasured or residual confounding.	NA / Y / PY / PN / N / NI

	1.5. **If Y/PY to 1.4**: Were confounding domains that were controlled for measured validly and reliably by the variables available in this study?	Appropriate control of confounding requires that the variables adjusted for are valid and reliable measures of the confounding domains. For some topics, a list of valid and reliable measures of confounding domains will be specified in the review protocol but for others such a list may not be available. Study authors may cite references to support the use of a particular measure. If authors control for confounding variables with no indication of their validity or reliability pay attention to the subjectivity of the measure. Subjective measures (e.g. based on self-report) may have lower validity and reliability than objective measures such as lab findings.	NA / Y / PY / PN / N / NI
	1.6. Did the authors control for any post-intervention variables that could have been affected by the intervention?	Controlling for post-intervention variables that are affected by intervention is not appropriate. Controlling for mediating variables estimates the direct effect of intervention and may introduce bias. Controlling for common effects of intervention and outcome introduces bias.	NA / Y / PY / PN / N / NI
	Questions relating to baseline and time-varying confounding		
	1.7. Did the authors use an appropriate analysis method that adjusted for all the important confounding domains and for time-varying confounding?	Adjustment for time-varying confounding is necessary to estimate the effect of starting and adhering to intervention, in both randomized trials and NRSI. Appropriate methods include those based on inverse probability weighting. Standard regression models that include time-updated confounders may be problematic if time-varying confounding is present.	NA / Y / PY / PN / N / NI
	1.8. **If Y/PY to 1.7**: Were confounding domains that were adjusted for measured validly and reliably by the variables available in this study?	See 1.5 above.	NA / Y / PY / PN / N / NI
	Risk of bias judgement	See Table 1.	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to confounding?	Can the true effect estimate be predicted to be greater or less than the estimated effect in the study because one or more of the important confounding domains was not controlled for? Answering this question will be based on expert knowledge and results in other studies and therefore can only be completed after all of the studies in the body of evidence have been reviewed. Consider the potential effect of each of the unmeasured domains and whether all important confounding domains not controlled for in the analysis would be likely to change the estimate in the same direction, or if one important confounding domain that was not controlled for in the analysis is likely to have a dominant impact.	Favours experimental / Favours comparator / Unpredictable

Bias in selection of participants into the study	2.1. Was selection of participants into the study (or into the analysis) based on participant characteristics observed after the start of intervention? **If N/PN to 2.1:** go to 2.4 2.2. **If Y/PY to 2.1**: Were the post-intervention variables that influenced selection likely to be associated with intervention? 2.3 **If Y/PY to 2.2**: Were the post-intervention variables that influenced selection likely to be influenced by the outcome or a cause of the outcome?	This domain is concerned only with selection into the study based on participant characteristics observed *after* the start of intervention. Selection based on characteristics observed *before* the start of intervention can be addressed by controlling for imbalances between experimental intervention and comparator groups in baseline characteristics that are prognostic for the outcome (baseline confounding). Selection bias occurs when selection is related to an effect of either intervention or a cause of intervention **and** an effect of either the outcome or a cause of the outcome. Therefore, the result is at risk of selection bias if selection into the study is related to both the intervention and the outcome.	Y / PY / PN / N / NI NA / Y / PY / PN / N / NI NA / Y / PY / PN / N / NI
	2.4. Do start of follow-up and start of intervention coincide for most participants?	If participants are not followed from the start of the intervention then a period of follow up has been excluded, and individuals who experienced the outcome soon after intervention will be missing from analyses. This problem may occur when prevalent, rather than new (incident), users of the intervention are included in analyses.	Y / PY / PN / N / NI
	2.5. **If Y/PY to 2.2 and 2.3, or N/PN to 2.4**: Were adjustment techniques used that are likely to correct for the presence of selection biases?	It is in principle possible to correct for selection biases, for example by using inverse probability weights to create a pseudo-population in which the selection bias has been removed, or by modelling the distributions of the missing participants or follow up times and outcome events and including them using missing data methodology. However such methods are rarely used and the answer to this question will usually be "No".	NA / Y / PY / PN / N / NI
	Risk of bias judgement	See Table 1.	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to selection of participants into the study?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in classification of interventions	3.1 Were intervention groups clearly defined?	A pre-requisite for an appropriate comparison of interventions is that the interventions are well defined. Ambiguity in the definition may lead to bias in the classification of participants. For individual-level interventions, criteria for considering individuals to have received each intervention should be clear and explicit, covering issues such as type, setting, dose, frequency, intensity and/or timing of intervention. For population-level interventions (e.g. measures to control air pollution), the question relates to whether the population is clearly defined, and the answer is likely to be 'Yes'.	Y / PY / PN / N / NI
	3.2 Was the information used to define intervention groups recorded at the start of the intervention?	In general, if information about interventions received is available from sources that could not have been affected by subsequent outcomes, then differential misclassification of intervention status is unlikely. Collection of the information at the time of the intervention makes it easier to avoid such misclassification. For population-level interventions (e.g. measures to control air pollution), the answer to this question is likely to be 'Yes'.	Y / PY / PN / N / NI
	3.3 Could classification of intervention status have been affected by knowledge of the outcome or risk of the outcome?	Collection of the information at the time of the intervention may not be sufficient to avoid bias. The way in which the data are collected for the purposes of the NRSI should also avoid misclassification.	Y / PY / PN / N / NI
	Risk of bias judgement	See Table 1.	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to measurement of outcomes or interventions?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias due to deviations from intended interventions	**If your aim for this study is to assess the effect of assignment to intervention, answer questions 4.1 and 4.2**		
	4.1. Were there deviations from the intended intervention beyond what would be expected in usual practice?	Deviations that happen in usual practice following the intervention (for example, cessation of a drug intervention because of acute toxicity) are part of the intended intervention and therefore do not lead to bias in the effect of assignment to intervention. Deviations may arise due to expectations of a difference between intervention and comparator (for example because participants feel unlucky to have been assigned to the comparator group and therefore seek the active intervention, or components of it, or other interventions). Such deviations are not part of usual practice, so may lead to biased effect estimates. However these are not expected in observational studies of individuals in routine care.	Y / PY / PN / N / NI
	4.2. **If Y/PY to 4.1**: Were these deviations from intended intervention unbalanced between groups *and* likely to have affected the outcome?	Deviations from intended interventions that do not reflect usual practice will be important if they affect the outcome, but not otherwise. Furthermore, bias will arise only if there is imbalance in the deviations across the two groups.	NA / Y / PY / PN / N / NI
	If your aim for this study is to assess the effect of starting and adhering to intervention, answer questions 4.3 to 4.6		
	4.3. Were important co-interventions balanced across intervention groups?	Risk of bias will be higher if unplanned co-interventions were implemented in a way that would bias the estimated effect of intervention. Co-interventions will be important if they affect the outcome, but not otherwise. Bias will arise only if there is imbalance in such co-interventions between the intervention groups. Consider the co-interventions, including any pre-specified co-interventions, that are likely to affect the outcome and to have been administered in this study. Consider whether these co-interventions are balanced between intervention groups.	Y / PY / PN / N / NI
	4.4. Was the intervention implemented successfully for most participants?	Risk of bias will be higher if the intervention was not implemented as intended by, for example, the health care professionals delivering care during the trial. Consider whether implementation of the intervention was successful for most participants.	Y / PY / PN / N / NI
	4.5. Did study participants adhere to the assigned intervention regimen?	Risk of bias will be higher if participants did not adhere to the intervention as intended. Lack of adherence includes imperfect compliance, cessation of intervention, crossovers to the comparator intervention and switches to another active intervention. Consider available information on the proportion of study participants who continued with their assigned	Y / PY / PN / N / NI

		intervention throughout follow up, and answer 'No' or 'Probably No' if this proportion is high enough to raise concerns. Answer 'Yes' for studies of interventions that are administered once, so that imperfect adherence is not possible. We distinguish between analyses where follow-up time after interventions switches (including cessation of intervention) is assigned to (1) the new intervention or (2) the original intervention. (1) is addressed under time-varying confounding, and should not be considered further here.	
	4.6. **If N/PN to 4.3, 4.4 or 4.5**: Was an appropriate analysis used to estimate the effect of starting and adhering to the intervention?	It is possible to conduct an analysis that corrects for some types of deviation from the intended intervention. Examples of appropriate analysis strategies include inverse probability weighting or instrumental variable estimation. It is possible that a paper reports such an analysis without reporting information on the deviations from intended intervention, but it would be hard to judge such an analysis to be appropriate in the absence of such information. Specialist advice may be needed to assess studies that used these approaches. If everyone in one group received a co-intervention, adjustments cannot be made to overcome this.	NA / Y / PY / PN / N / NI
	Risk of bias judgement	See Table 2	
	Optional: What is the predicted direction of bias due to deviations from the intended interventions?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	

Bias due to missing data	5.1 Were outcome data available for all, or nearly all, participants?	"Nearly all" should be interpreted as "enough to be confident of the findings", and a suitable proportion depends on the context. In some situations, availability of data from 95% (or possibly 90%) of the participants may be sufficient, providing that events of interest are reasonably common in both intervention groups. One aspect of this is that review authors would ideally try and locate an analysis plan for the study.	Y / PY / PN / N / NI
	5.2 Were participants excluded due to missing data on intervention status?	Missing intervention status may be a problem. This requires that the *intended* study sample is clear, which it may not be in practice.	Y / PY / PN / N / NI
	5.3 Were participants excluded due to missing data on other variables needed for the analysis?	This question relates particularly to participants excluded from the analysis because of missing information on confounders that were controlled for in the analysis.	Y / PY / PN / N / NI
	5.4 **If PN/N to 5.1, or Y/PY to 5.2 or 5.3**: Are the proportion of participants and reasons for missing data similar across interventions?	This aims to elicit whether either (i) differential proportion of missing observations or (ii) differences in reasons for missing observations could substantially impact on our ability to answer the question being addressed. "Similar" includes some minor degree of discrepancy across intervention groups as expected by chance.	NA / Y / PY / PN / N / NI
	5.5 **If PN/N to 5.1, or Y/PY to 5.2 or 5.3**: Is there evidence that results were robust to the presence of missing data?	Evidence for robustness may come from how missing data were handled in the analysis and whether sensitivity analyses were performed by the investigators, or occasionally from additional analyses performed by the systematic reviewers. It is important to assess whether assumptions employed in analyses are clear and plausible. Both content knowledge and statistical expertise will often be required for this. For instance, use of a statistical method such as multiple imputation does not guarantee an appropriate answer. Review authors should seek naïve (complete-case) analyses for comparison, and clear differences between complete-case and multiple imputation-based findings should lead to careful assessment of the validity of the methods used.	NA / Y / PY / PN / N / NI
	Risk of bias judgement	See Table 2	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to missing data?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in measurement of outcomes	6.1 Could the outcome measure have been influenced by knowledge of the intervention received?	Some outcome measures involve negligible assessor judgment, e.g. all-cause mortality or non-repeatable automated laboratory assessments. Risk of bias due to measurement of these outcomes would be expected to be low.	Y / PY / PN / N / NI
	6.2 Were outcome assessors aware of the intervention received by study participants?	If outcome assessors were blinded to intervention status, the answer to this question would be 'No'. In other situations, outcome assessors may be unaware of the interventions being received by participants despite there being no active blinding by the study investigators; the answer this question would then also be 'No'. In studies where participants report their outcomes themselves, for example in a questionnaire, the outcome assessor is the study participant. In an observational study, the answer to this question will usually be 'Yes' when the participants report their outcomes themselves.	Y / PY / PN / N / NI
	6.3 Were the methods of outcome assessment comparable across intervention groups?	Comparable assessment methods (i.e. data collection) would involve the same outcome detection methods and thresholds, same time point, same definition, and same measurements.	Y / PY / PN / N / NI
	6.4 Were any systematic errors in measurement of the outcome related to intervention received?	This question refers to differential misclassification of outcomes. Systematic errors in measuring the outcome, if present, could cause bias if they are related to intervention or to a confounder of the intervention-outcome relationship. This will usually be due either to outcome assessors being aware of the intervention received or to non-comparability of outcome assessment methods, but there are examples of differential misclassification arising despite these controls being in place.	Y / PY / PN / N / NI
	Risk of bias judgement	See Table 2	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to measurement of outcomes?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in selection of the reported result	Is the reported effect estimate likely to be selected, on the basis of the results, from... 7.1. ... multiple outcome *measurements* within the outcome domain?	For a specified outcome domain, it is possible to generate multiple effect estimates for different measurements. If multiple measurements were made, but only one or a subset is reported, there is a risk of selective reporting on the basis of results.	Y / PY / PN / N / NI
	7.2 ... multiple *analyses* of the intervention-outcome relationship?	Because of the limitations of using data from non-randomized studies for analyses of effectiveness (need to control confounding, substantial missing data, etc), analysts may implement different analytic methods to address these limitations. Examples include unadjusted and adjusted models; use of final value vs change from baseline vs analysis of covariance; different transformations of variables; a continuously scaled outcome converted to categorical data with different cut-points; different sets of covariates used for adjustment; and different analytic strategies for dealing with missing data. Application of such methods generates multiple estimates of the effect of the intervention versus the comparator on the outcome. If the analyst does not pre-specify the methods to be applied, and multiple estimates are generated but only one or a subset is reported, there is a risk of selective reporting on the basis of results.	Y / PY / PN / N / NI
	7.3 ... different *subgroups*?	Particularly with large cohorts often available from routine data sources, it is possible to generate multiple effect estimates for different subgroups or simply to omit varying proportions of the original cohort. If multiple estimates are generated but only one or a subset is reported, there is a risk of selective reporting on the basis of results.	Y / PY / PN / N / NI
	Risk of bias judgement	See Table 2	Low / Moderate / Serious / Critical / NI
	Optional: What is the predicted direction of bias due to selection of the reported result?	If the likely direction of bias can be predicted, it is helpful to state this. The direction might be characterized either as being towards (or away from) the null, or as being in favour of one of the interventions.	Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Overall bias	**Risk of bias judgement**	See Table 3.	Low / Moderate / Serious / Critical / NI
	Optional: What is the overall predicted direction of bias for this outcome?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

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