Investigating the effects of neuropeptides on human sperm function and fertility

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List of Abbreviations

Full	Abbreviation
4’,6-diamidino-2-phenylindole	DAPI
5'—C—phosphate—G—3'	CpG
5-methylcytosine	5mC
Adenosine triphosphate	ATP
Adrenocorticotropic hormone	ACTH
Ammonium persulfate	APS
Analysis of variance	ANOVA
Androgen binding protein	ABP
Anti-Müllerian hormone	AMH
Aquaporin	AQP
Assisted reproductive technology	ART
Atrial natriuretic peptide	ANP
Autism spectrum disorder	ASD
Bicinchonic acid assay	BCA
Body mass index	BMI
Bovine serum albumin	BSA
Carbonyl cyanide-4 trifluoromethoxy pheylhydrazone	FCCP
Cholecytokinin	CCK
c-Jun N-terminal kinases	JNK
Cocaine and amphetamine regulated transcript	CART
Colony stimulating factor-1	CSF
Complimentary deoxyribonucleic acid	cDNA
Computer assisted sperm/semen analysis	CASA
Confidence interval	CI
Corticotropin-releasing hormone	CRH
Curvilinear velocity	VCL
Cyclic adenosine monophosphate	cAMP
delta-9-tetrahydrocannabinol	THC
Deoxyribonulceic acid	DNA
Desmopressin	dDAVP
Diacylcycerol	DAG
Dimethyl sulfoxide	DMSO
Discs-Large Associated Protein 2	DLGAP2
Distilled water	dH_2O
DNA methyltransferase	DNMT
Dulbecco’s modified eagle’s medium	DMEM
Dulbecco's phosphate buffered saline	DPBS
Electrochemiluminescence	ECL
Endoplasmic reticulum	ER
Enzyme linked immunosorbent assay	ELISA
Ethylenediaminetetraacetic acid	EDTA
Extracellular acidification rate	ECAR
Extracellular signal related kinases	ERK

Fluorescein isothiocyanate	FITC
Foetal bovine serum	FBS
Follicle stimulating hormone	FSH
Galanin-like peptide	GALP
Genome-wide association study	GWAS
Glucagon-like peptide	GLP
Gonadotropin releasing hormone	GnRH
G-protein coupled receptors	GPCRs
Growth hormone-releasing hormone	GHRH
Guanine neucleotide exchange factors	GEFs
Guanosine diphosphate	GDP
Guanosine triphosphate	GTP
Hemonkinin-1	HK1
Horseradish peroxidase	HRP
Hydrochloric acid	HCL
Hypothalamic – neurohypophysial system	HNS
Hypothalamic – pituitary – adrenal	HPA
Hypothalamic – pituitary – gonadal	HPG
Hypothalamic – pituitary – thyroid	HPT
Immunoglobulin G	IgG
In vitro fertilisation	IVF
Intracytoplasmic sperm injection	ICSI
Linearity	LIN
Luteinising hormone	LH
Melanin-concentrating hormone	MCH
Melanocyte-stimulating hormone	MSH
Neurokinin A	NKA
Neurokinin B	NKB
Neuropeptide Y	NPY
Oxidative phosphorylation	OXPHOS
Oxygen consumption rate	OCR
Oxytocin	OXT
Oxytocin receptor	OXTR
Pancreatic polypeptide Y	PPY
Paraformaldehyde	PFA
Paraventricular nucleus	PVN
Peptide YY	PYY
Phosphate buffered saline	PBS
Phosphatidylinositol bisphosphate	PIP_2
Phosphatidylinositol triphosphate	PIP_3
Phosphatidylinositol-3-kinsase	Pl3K
Phospholipase C	PLC
Phospholipase C Zeta	PLCζ
Phospholipase Cβ	PLCβ
Pisum sativum agglutinin	PSA
Pituitary adenylate cyclase-activating peptide	PACAP

Polycystic ovarian syndrome	PCOS
Polymerase chain reaction	PCR
Prolactin-releasing peptide	PrRP
Protein kinase A	PKA
Protein kinase B	PKB
Protein kinase C	PKC
Protein kinase G	PKG
Quantitative polymerase chain reaction	qPCR
Radioimmuno assay	RIA
Radioimmunoprecipitation assay	RIPA
Reactive oxygen species	ROS
Repeated measures	RM
Reverse transcription polymerase chain reaction	RT-PCR
Ribonucleic acid	RNA
S-adenosylmethionine	SAM
Scanning electron microscopy	SEM
Semen Analysis with Machine Intelligence	SAMi
Sodium chloride	NaCl
Sodium dodecyl sulphate	SDS
Square root	SQRT
Standard deviation	SD
Standard error of the mean	SEM
Straight-line velocity	VSL
Suprachiasmatic nuclei	SCN
Supraoptic nucleus	SON
ten-eleven translocation methylcytosine dioxygenase	TET
Tetramethylethylenediamine	TEMED
Thyroid-stimulating hormone	TSH
Thyrotropin-releasing hormone	TRH
Thyroxine	T4
Transforming growth factor-beta	TGF
Triiodothyronine	T3
Tris Borate EDTA	TBE
Tris buffered saline	TBS
Tumour necrosis factor α	TNFα
United Kingdom	UK
United States of America	USA
Variance inflation factor	VIF
Vasoactive intestinal peptide	VIP
Vasopressin	AVP
Vasopressin receptor 1a	AVPR1a
Vasopressin receptor 1b	AVPR1b
Vasopressin receptor 2	AVPR2
World Health Organisation	WHO
Zona pellucida sperm-binding protein 3	ZP3

Acknowledgements

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Ada - it is all for you, noodle. With love.

Abstract

An ever-growing body of research has reported decreasing trends in male fertility globally. In particular a recent meta-regression analysis demonstrated a significant decline in sperm count occurring over the past 40 years warranting further investigation. This study sought to investigate the role of the neuropeptides, oxytocin and vasopressin on sperm function and fertility.

Neuropeptides have major functions in reproduction that are well documented. They are essential for normal function in the hypothalamic-pituitary-gonadal (HPG) axis, influencing the activity of the reproductive endocrine system, fecundity and sexual behaviour. Despite the involvement of neuropeptides in reproduction, little is known about their effects on sperm function and within the follicular fluid, an essential microenvironment where the oocyte matures.

This study used *in vitro* assays to elucidate concentrations of neuropeptides in follicular fluid and semen, investigate their effects on sperm function and to investigate oxytocin receptor methylation in sperm. Regression analyses were used to investigate the relationship between the neuropeptides and the clinical outcomes in the patient cohorts of men and women undergoing assisted reproductive technology (ART).

This study demonstrated that the neuropeptides vasopressin and oxytocin have a role in fertility. It is the first to discover the vasopressin receptor 2 on the acrosome region of human sperm and demonstrate a role for vasopressin in sperm motility, hyperactivation, calcium response, mitochondrial respiration and glycolysis. In the clinical cohorts of men and women undergoing *in vitro* fertilisation (IVF), increasing concentrations of oxytocin in follicular fluid was found to be negatively associated with fertilisation of oocytes in women and increasing concentrations of oxytocin in semen was negatively associated with sperm count and concentration in men. In sperm, increases in the oxytocin receptor methylation at CpG sites 924 and 934 were negatively associated with sperm concentration. The data presented in this thesis is highly suggestive of a novel role for vasopressin and oxytocin in sperm function, spermatogenesis, fertilisation and potentially indicating epigenetic effects for the oxytocin receptor and warrants further investigation.

Chapter 1: Introduction

1.0 Introduction

1.1 Reproduction

Animal reproduction involves cellular, physiological, endocrine and behavioural functions in the production of offspring to propagate their genes into the next generation. In mammals, successful reproduction is controlled by a complex interplay of hypothalamic-pituitary-gonadal (HPG) axis, reproductive organs, and the neuroendocrine system. Human reproduction is regulated via the hypothalamic-pituitary-gonadal axis. In brief, gonadotropin-releasing hormone (GnRH) is secreted; this triggers luteinizing hormone (LH) and follicle stimulating hormone (FSH) to be released and increases production of steroid hormones by the male and female gonads which in turn act upon the hypothalamus and pituitary in a feedback loop (Ebot et al., 2018).

1.2 Male reproduction

The hypothalamic-pituitary-testicular axis is responsible for all aspects of male reproduction. Sperm are the male gamete, a highly specialised cell, produced via the process of spermatogenesis, which occurs in the testes and takes approximately 70 days to complete. Spermatogenesis begins at puberty and continues over the course of the male's life (Bronson, 2011).

1.2.1 Sperm structure

Sperm have a highly specialised structure, which is vital for their function of delivering the paternal genome to the oocyte (Suarez and Pacey, 2006). Sperm are comprised of three main sections; the head, midpiece and tail (Figure 2) (Pacey and Williams, 2018). The head contains the paternal genome and the acrosome, which contains the essential enzymes such as hyaluronidase and acrosin that digest the hyaluronic acid in the corona radiata and involved the penetration of the zona pellucida respectively, thus allowing fertilisation to occur (Osman et al., 1989; Yoshinaga and Toshimori, 2003). The midpiece primarily contains the neck of the sperm and mitochondria for adenosine triphosphate (ATP)

production (Piomboni et al., 2012). The neck of the sperm consists of two centrioles; proximal and distal, the proximal centriole is also deposited during fertilisation as the oocyte does not contain centrioles and the distal centriole is the beginning of the axial filament comprised of a 9+2 arrangement of the microtubules, mitochondria are wrapped around the axial filament in approximately 10-14 spirals. The axial filament continues into the sperm tail and is only surrounded by cytoplasm and cell membrane, the tail propels the sperm towards the oocyte using an elliptical cone whipping motion (Figure 1, Figure 3) (Sutovsky and Manandhar, 2006).

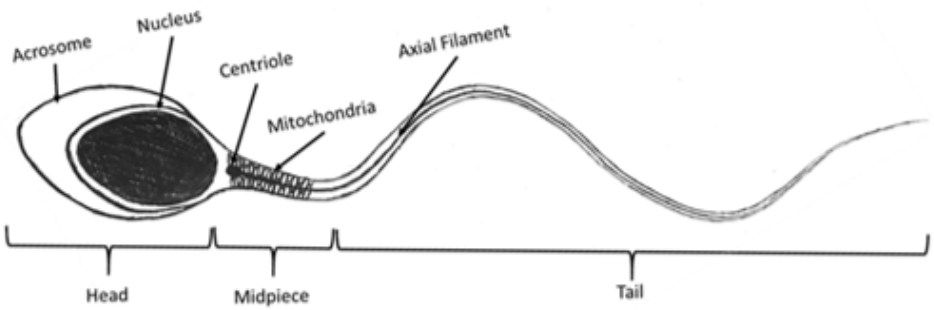

Figure 2. The anatomy of the human sperm. Comprised of 3 sections; head, midpiece and tail. The head contains the haploid paternal DNA in the nucleus and the acrosome. The midpiece or 'neck' joins the head to the tail and consists of the centriole and mitochondria which provide energy for motility and are enveloped around the axial filament which protrudes through the entire tail. Adapted from Pacey and Williams, 2018.

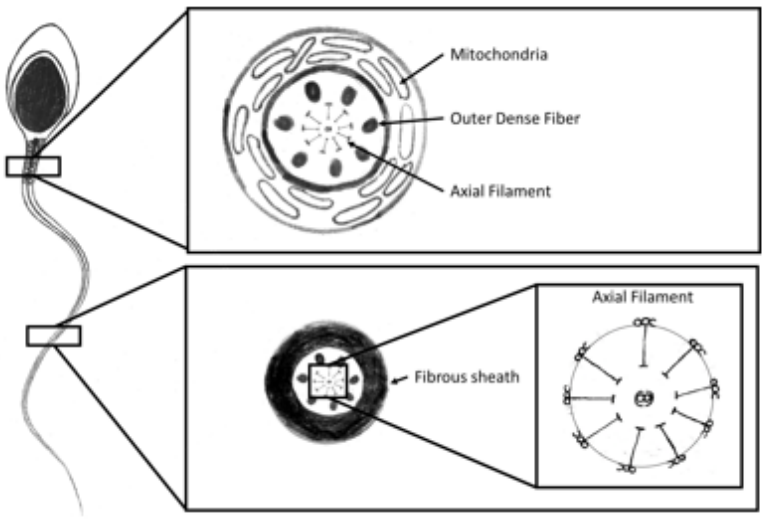

Figure 1. Cross sections of the midpiece and tail of human sperm. The axial filament that runs throughout the midpiece and the tail is comprised of a 9+2 doublet arrangement of the microtubules, this is surrounded by a fibrous sheath, in the midpiece mitochondria are wrapped around the axial filament to provide energy for motility. Adapted from Pacey and Williams, 2018.

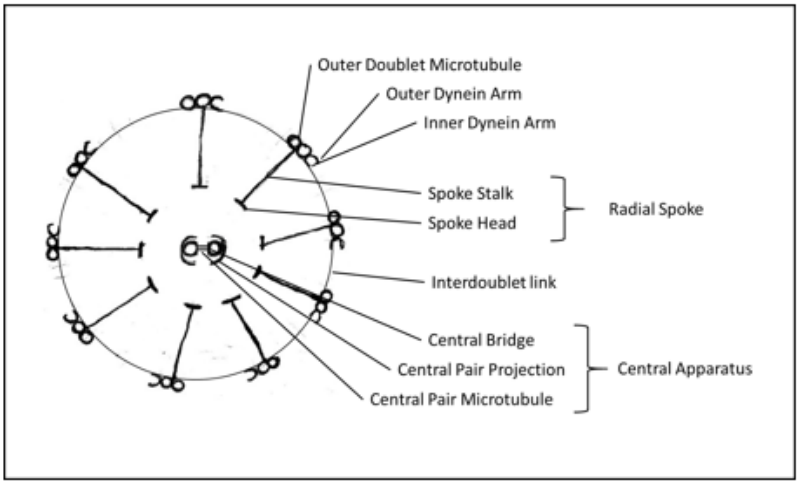

Figure 3. The 9 + 2 doublet arrangement of the microtubules in the axial filament. Motility is due to the action of the dynein arms against the microtubule doublets, resulting in a rhythmic longitudinal motion between the tubules which propels the sperm. Adapted from Pacey and Williams, 2018.

1.2.2 Spermatogenesis and maturation

In brief, the preoptic nucleus and the arcuate nucleus of the hypothalamus secrete GnRH. GnRH travels via the hypopheaseal portal system and binds to the gonadotrophs in the anterior pituitary. The gonadotrophs release FSH and LH into the blood stream. LH binds to its receptor on the Leydig cells (cells surrounding the seminiferous tubules) triggering a signalling cascade which produces specific enzymes which catalyse the conversion of cholesterol to testosterone, known as steroidogenesis (Bronson, 2011). The enzymes involved in the steroidogenesis of testosterone are catagorized primarily into two classes; cytochrome P450 heme-containing proteins and hydroxysteroid dehydrogenases (Payne and Hales, 2004). Testosterone is secreted into the blood stream and at high concentrations, testosterone will exert a negative feedback on the hypothalamus and the anterior pituitary inhibiting LH and FSH production. In the testes the seminiferous tubules are composed of Sertoli cells, which are responsible for spermatogenesis. FSH binds to its receptor on the Sertoli cells and triggers the production of androgen binding protein (ABP) which is involved in the homeostasis of testosterone in the lumen of the seminiferous tubules where testosterone is secreted (Holdcraft and Braun, 2004). Spermatogenesis relies on both ABP and Testosterone which are regulated through LH and FSH (Ebot et al., 2018). The Sertoli cells are linked together via tight junctions and adherens junctions – these form the blood-testes barrier to ensure that the lumen of the seminiferous tubules are an immune privileged site. This is vital as spermatogenesis begins at puberty – after the immune system is developed, without this the sperm cells would be recognised as foreign

bodies and destroyed. The seminiferous tubules consist of 3 main compartments, the basal compartment, adluminal compartment and the lumen. The male gamete stem cell known as the spermatogonia, is found in the basal compartment of the seminiferous tubules (Figure 4) (Ehmcke et al., 2006). The spermatogonia (2n) replicates via mitosis to produce 2 diploid daughter cells which are either type A or type B (Phillips et al., 2010). Type A cells remain as spermatogonia to continue this initial mitosis process to maintain the reservoir of stem cells. The type B cells release chemicals that open the tight junctions to allow them to pass from the basal compartment to the adluminal compartment and continue the process of spermatogenesis. Once in the adluminal compartment the type B cells are known as a primary spermatocyte. The primary spermatocyte undergoes meiosis I resulting in 2 haploid secondary spermatocytes. The secondary spermatocytes undergo meiosis II resulting in 4 haploid cells known as spermatids (round haploid cells) (Wistuba et al., 2007). The spermatids undergo spermiogenesis, the cells become flagellated, develop an acrosome and the nucleus condenses to produce the specialised sperm cells (Figure 5). The Sertoli cells support spermiogenesis and also maintain sperm production equilibrium. If the Sertoli cells detect a high concentration of sperm in the seminiferous tubules, they secrete inhibin, which exerts a negative feedback on the hypothalamus and anterior pituitary inhibiting FSH and LH production and therefore spermatogenesis (Holdcraft and Braun, 2004). At this stage the sperm are immotile, in order to gain the ability to be motile they must undergo additional maturation processes in the epididymis (Moore, 1998). The epididymis is a coiled tube comprised of three sections, the *caput* (head), *corpus* (body) and *cauda* (tail), which is between the testes and vas deferens. It is responsible for the continued maturation, storage and transportation of the sperm cells. For sperm to undergo the essential maturation processes in the epididymis it takes around two weeks (Figure 6) (Coward and Wells, 2013). The epididymis is very complex, the microenvironment of the epididymis is highly concentrated with proteins that interact with the sperm as they pass through . These proteins remodel the appearance of the nucleus and acrosome as well as changes in overall cholesterol and phospholipids through epididymal transport (Jones et al., 2007; Olson et al., 2002). These modifications are essential for fertilisation as prior to passing through the epididymis the sperm do not have the capacity to be progressively motile (Cornwall, 2009).

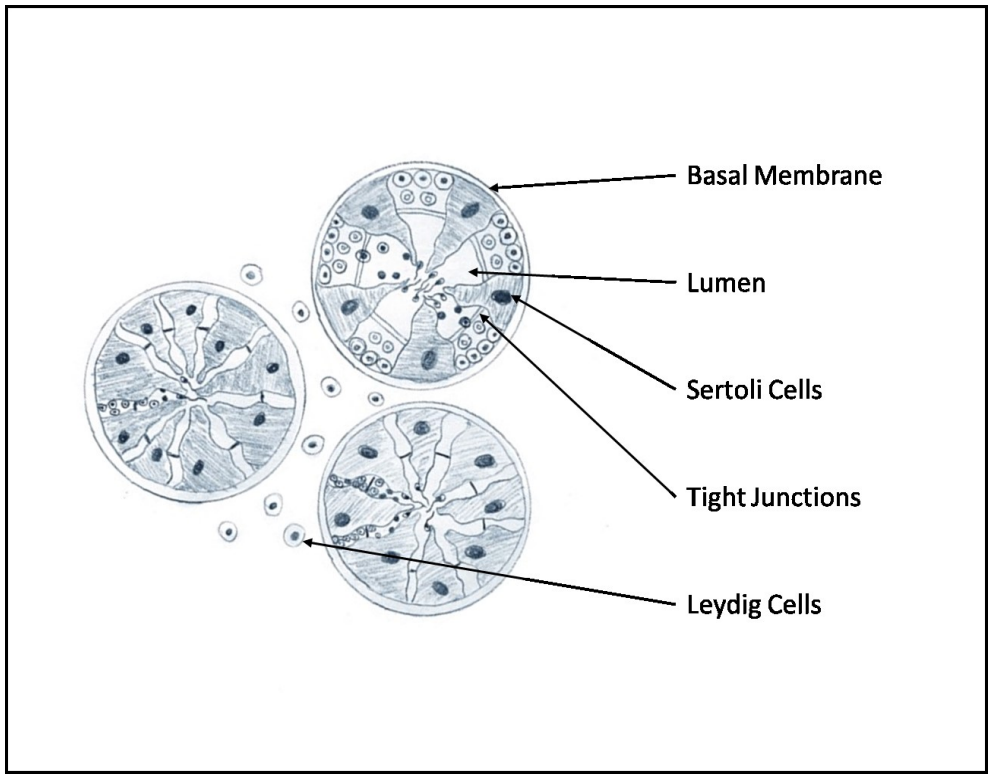

Figure 4. The seminiferous tubules. In the testes, the seminiferous tubules are the site of spermatogenesis, the tight junctions form the blood-testes barrier, the Leydig cells and Sertoli cells maintain spermatogenesis through feedback mechanisms within the HPG axis.

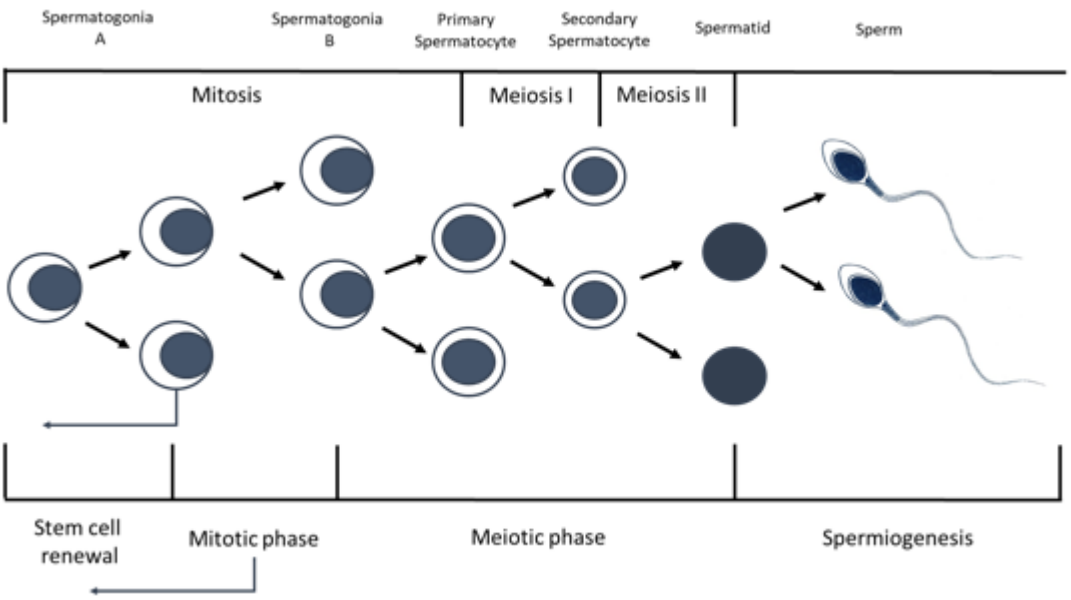

Figure 5. Spermatogenesis. The process of differentiation resulting in sperm. Initially the spermatogonia either self-renew or differentiate into Type B. The Type B cells lead to primary spermatocytes which further differentiate into secondary spermatocytes then the round spermatid. The process of differentiation from round spermatid to sperm is known as spermiogenesis where cells undergo specialised morphological changes.

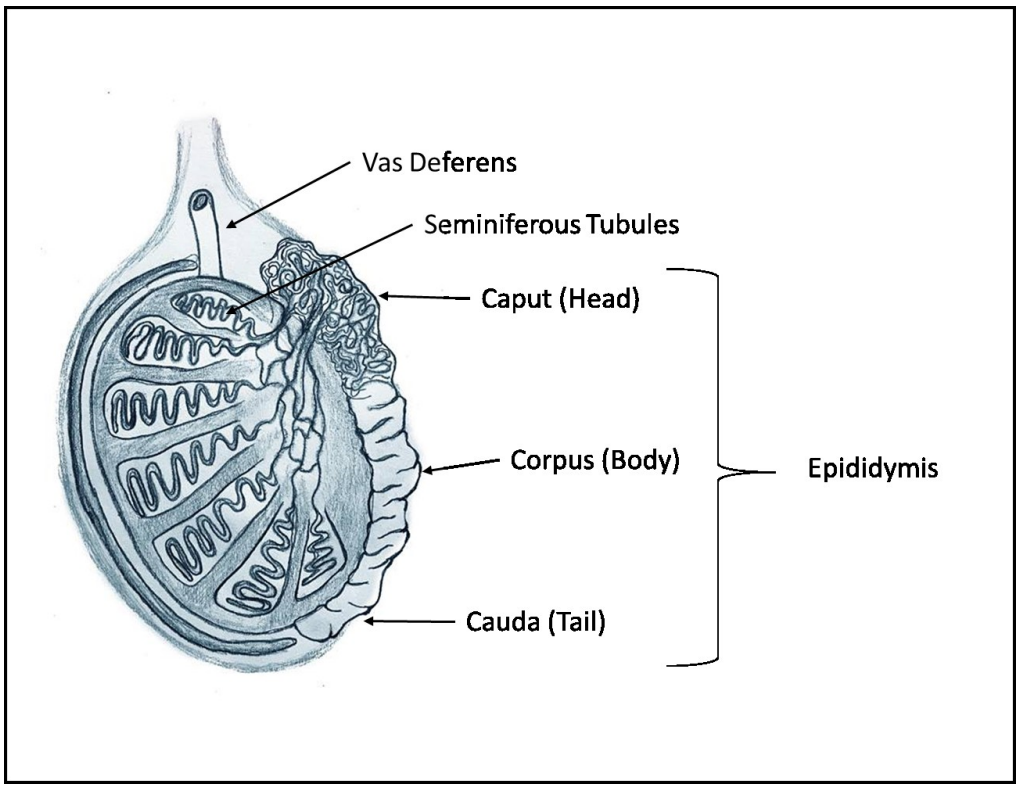

Figure 6. Testicle. Comprised of the seminiferous tubules which are the site of spermatogenesis, the epididymis where sperm are further matured, acquire motility and are stored prior to ejaculation and the vas deferens, a muscular tube which carries sperm to the ejaculatory ducts.

1.2.3 Sperm function, capacitation and the acrosome reaction

In the epididymis capacitation is prevented via the secretion of epididymal proteins such as Crisp-1, β-glucuronidase and others (Gwatkin et al., 1974; Roberts et al., 2003). The final maturation steps for human sperm occur in the female reproductive tract. Sperm must undergo capacitation – a process of hyperactivation, which is an essential perquisite for sperm to be able to undergo the acrosome reaction, and therefore essential for fertilisation. Capacitation is due to a calcium ion influx through the sperm cell membrane, increases in cyclic AMP (cAMP), high bicarbonate concentration and soluble adenylate cyclase in the seminal fluid. The cAMP activates protein kinase A (PKA), PKA phosphorylates serine and threonine residues on many target proteins (Yoshida et al., 2008). During capacitation sperm also undergo essential membrane stripping, altering the cholesterol content and removing non-essential proteins leaving only modified specific glycoproteins, increasing membrane fluidity in preparation for the sperm to undergo acrosome reaction upon binding to the oocyte.

1.2.4 Fertilisation

The journey of the sperm is a complicated process in humans and begins with the sperm deposited directly in the female reproductive tract. The sperm then has to make its way to the ampulla, region where the oocyte awaits (Carroll, 2018a).

After the sperm have passed through the cervix and through the uterotubal junction, entering the Fallopian tube, it must then bypass the cumulous layer (the cells surrounding the oocyte), which is rich in hyaluronic acid (Suarez and Pacey, 2006). The capacitated sperm release hyaluronidase, which breaks the hyaluronic acid connections between the cells of the cumulous cells, permitting the entrance of the sperm to reach the oocyte (Salicioni et al., 2007). Sperm then binds to zona pellucida receptors, the sperm-binding protein 3 (ZP3). Calcium influx triggers the acrosome to fuse and release its contents such as acrosin and proteases, which facilitate the passage through the zona pellucida. This process is known as acrosome reaction.

After sperm penetrate the zona pellucida, it enters the perivitelline space where it binds to an oocyte-specific receptor, folate receptor-4 (Folr4) also known as Juno via the sperm-specific Izumo1 (Bianchi et al., 2014; Inoue, 2017; Inoue et al., 2005).

Following sperm-oocyte binding – the sperm's contents enter the ooplasm, which include the paternal genome, the centrioles and various coding and non-coding RNAs (Carroll, 2018a). The fertilising sperm also activated the oocyte in order to exit its meiotic arrest. This sperm activation is achieved by eliciting a series of intracellular calcium oscillations (Jones, 2005). These calcium oscillations originate from the point of sperm fusion and propagate the oocytes in a wave-like manner (Carroll et al., 2003). In mammals, these calcium transients are initiated by the activity of a sperm-specific phospho lipase C (PLC ζ), also referred to as the sperm factor (Saunders et al., 2002). These sperm-triggered calcium oscillations are essential for the activation of the anaphase promoting factor, which leads to the degradation of factors such as cdk1:cyclinB that enables the exit of meiotic arrest (Jones, 2005). The male pronucleus and female pronucleus then fuse and form the zygote (Sutovsky, 2009).

Any perturbation of spermatogenesis through disruption of the HPG axis or testicular function can result in infertility, which may require assisted reproductive technology to increase the changes of parenting a biological child.

1.3 Male factor infertility, treatment and assisted reproductive technology

Male factor infertility affects approximately half of couples with infertility, either in combination with female factor or as the sole cause (Keihani et al., 2019). Recent studies demonstrated a significant 50-60% decline in sperm count over the past 40 years (Levine et al., 2017). There are many causes of male factor infertility such as; infections (including sexually transmitted infections, testicular damage/torsion, varicocele, maldescent testicles, sexual and ejaculatory dysfunction, endocrinopathies, obstruction and most commonly can be idiopathic (Rowe et al., 2000).

Male factor infertility is primarily diagnosed via semen analysis, where a semen sample is assessed against the World Health Organisation's (2010) reference ranges for semen parameters. Any sample that falls below the recommended levels is considered abnormal, the World Health Organisation's (2010) lower reference ranges for semen parameters are as follows; semen volume < 1.5 mL, total motility < 40%, progressive motility < 32%, total sperm count < 39 x 10^6 cells, sperm concentration 15 x 10^6/mL, morphology < 4% normal

forms. Alterations to these semen parameters can be low sperm concentration (oligozoospermia), low sperm motility (asthenozoospermia), total absence of sperm in the sample (azoospermia), low semen volume and abnormal morphological forms (World Health Organization, 2010a).

Infertility can be treated through assisted reproductive technology (ART). ART comprise of methods of gamete extraction, *in vitro* fertilisation, embryo culture and transfer. Briefly, sperm is separated from seminal plasma via methods including 'swim-up' or density gradient separation, oocytes are collected usually via ultrasound-guided transvaginal oocyte retrieval. Fertilisation is then achieved through insemination (adding sperm at a determined concentration to the oocytes in culture media), or via injecting a single sperm in to the ooplasm (IntraCytoplasmic Sperm Injection – ICSI). Fertilised embryos are then cultured and monitored for up to 5 /6 days before been selected for embryo transfer (Carroll, 2018b).

One important element of ART is to retrieve a good number of quality oocytes. This is achieved by ovarian stimulation. The stimulation protocols directly regulate follicular development and therefore number of oocytes that are obtained, many other factors that may influence each woman's response to the stimulation. Genetic factors such as the FSH receptor genotype and the oestrogen receptor genotype and genetic factors affecting metabolism of the selective oestrogen receptor modulator, clomifene citrate, have been shown to have an effect on the response to treatment (Georgiou et al., 1997; Greb et al., 2005; Rostami-Hodjegan et al., 2004). Antral follicle count has been demonstrated as a predictor of how effective gonadotrophin stimulation will be on ovarian response to treatment (Hendriks et al., 2005). Furthermore there are several other important predictors of cycle outcome such as; anti-Müllerian hormone (AMH), day 3 FSH concentration and inhibin B concentration (Keck et al., 2005).

1.4 Biomarkers for infertility

Assessment of infertility for both male and female patients include physical, cellular and biochemical analysis to increase diagnostic accuracy. However, approximately 15 - 30% of couples will be diagnosed with unexplained infertility (Practice Committee of the American Society for Reproductive Medicine, 2006). The development of additional diagnostic tools will potentially reveal more concerning the causation of infertility. A biomarker in

reproduction could be also be used to differentiate subgroups with potentially different causes of infertility and predict outcome (Bonassi et al., 2001).

The neuroendocrine system is integrated with all aspects of reproductive function and dysfunction and offers potential avenues for biomarkers in reproduction.

1.5 The neuroendocrine system

The neuroendocrine system refers to the interaction between the nervous system and the endocrine system in maintaining homeostasis and a variety of responses to the organism's surroundings. The neuroendocrine system is comprised of the hypothalamus, the pituitary and endocrine organs, these interact with each other to produce physiological responses to environmental stimuli (Melmed, 2016).

The neuroendocrine system can be separated into 3 axis; the hypothalamic-pituitary-adrenal axis (HPA), the hypothalamic-pituitary-thyroid axis (HPT), the hypothalamic-pituitary-gonadal axis (HPG) and the hypothalamic – neurohypophysial system (HNS).

1.5.1 Hypothalamic – pituitary – adrenal (HPA) axis

The HPA axis is primarily involved in stress response though also regulates further processes such as immune response, mood, digestion, energy expenditure/storage and sexuality. The neuropeptides primarily involved in regulation of the HPA axis are vasopressin (AVP) and corticotropin-releasing hormone (CRH). AVP and CRH stimulate the release of adrenocorticotropic hormone (ACTH) which regulates the adrenal cortex and releases glucocorticoid hormones such as cortisol. The glucocorticoid hormones regulate the hypothalamus and pituitary through negative feedback and surpress further release of CRH/ACTH (Smith and Vale, 2006).

1.5.2 Hypothalamic – pituitary – thyroid (HPT) axis

The HPT axis regulates metabolism. In response to low circulating levels of the thyroid hormones Triiodothyronine (T3) and Thyroxine (T4) the hypothalamus releases thyrotropin-releasing hormone (TRH), which stimulates the anterior pituitary to release thyroid-stimulating hormone (TSH), this in turn stimulates the release of thyroid hormone, T3 and T4, release from the thyroid. T3 and T4 have a negative feedback regulatory effect

on the hypothalamus and pituitary. The majority of T3 is mainly produced in the periphery via organs and tissues such as; the liver, adipose tissue, glia and skeletal muscle by deiodination of the T4 that is circulating. This deiodination processes is regulated via many hormones such as TSH and vasopressin (Fliers et al., 2014).

1.5.3 Hypothalamic – pituitary – gonadal (HPG) axis

The HPG axis is comprised of the hypothalamus, pituitary gland and gonads and refers to their interactions as a system. The HPG axis regulates reproduction, development, sexual dimorphism, aging and has a role in the immune system. In brief, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) which stimulates the anterior pituitary to secrete luteinising hormone (LH) and follicle stimulating hormone (FSH). LH and FSH stimulate the gonads to produce their sex specific hormones, testosterone for males, oestrogen and progesterone for females (Ebot et al., 2018).

GnRH is a small neuropeptide synthesised in the arcuate nucleus of the hypothalamus. The activity of GnRH is low throughout childhood and during puberty becomes active and is secreted in a pulsatile manner which is essential for sexual maturation and healthy reproductive function. GnRH is secreted into the hypophysial portal where it is transported to the anterior pituitary. In the anterior pituitary, GnRH binds to its receptor which is a G-protein coupled receptor of the Gq subtype triggering the PLC, calcium, PKC signalling cascade, this stimulated the gonadotropes to release lutieinizing hormone and follicle-stimulating hormone into the periphery (Strauss III and Barbieri, 2013). Luteinising hormone is responsible for supporting the production of androgens and precursors of oestradiol in the theca cells of the ovary, ovulation and the formation of the corpus luteum in females and regulates the production of testosterone from the Leydig cells in males. Follicle stimulating hormone works synergistically with luteinising hormone to regulate the reproductive system, it stimulates follicular growth through the stimulation of the granulosa cells in females, FSH stimulates the Sertoli cells to maintain spermatogenesis and to induce inhibin B secretion in males, FSH also matures primordial germ cells in both sexes (Mac E Hadley, 1996).

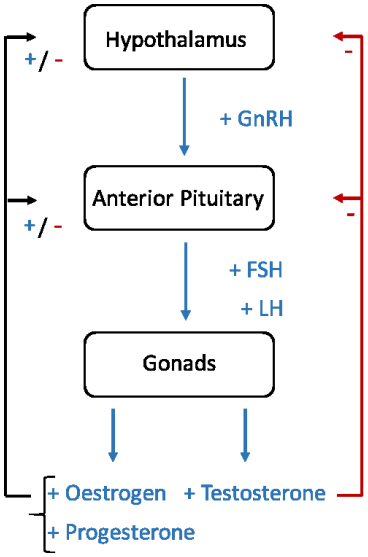

Figure 7. Simplified schematic representation of the hypothalamic-pituitary-gonadal axis in humans. The positive (blue lines) and negative (red lines) feedback loops are demonstrated between the hormones and the glands which secrete them.

1.6 An overview of hormones and their receptors

There are two main types of hormones, steroid hormones and peptide hormones. Steroid hormones are lipid soluble and therefore their receptors are intracellular, either intracytosolic or intranuclear. These steroid receptors are bound with a heat shock protein which is displaced when the steroid hormone binds to its receptor, the receptor then binds to its target, for example a gene sequence and activates a hormone response element triggering protein synthesis or mitosis. Peptide hormones are water-soluble but not lipid soluble, they cannot pass through the lipid bilayer of the cell and therefore need a receptor on the cell surface membrane, they function through secondary messenger systems via G-protein coupled receptors (Melmed et al., 2015).

G-protein coupled receptors (GPCRs) are a protein complex comprised of seven transmembrane α-helicases, an extracellular N-terminus and an intracellular C-terminus (Holmes et al., 2003). These are heterotrimeric and are bound to 3 subunits, *alpha* (α), *beta* (β) and *gamma* (γ) (Hurowitz et al., 2000).

The Gs receptor in its inactive state exists as a trimer with guanosine diphosphate (GDP) bound to the α-subunit. Once the receptor is bound to by its corresponding ligand this causes a conformational change, GDP is displaced and guanosine triphosphate (GTP) binds (active state) (Iismaa et al., 1995). There are 4 main subfamilies of GPCRs; Gs (stimulatory), Gq, Gi (inhibitory), $G_{12,13}$ (involved in regulation of RhoGTPase family). The signalling pathway depends on the receptor type. GPCRs of the Gs subtype stimulate the effector protein adenylate cyclase, which in turn stimulates an increase in cyclic adenosine monophosphate (cAMP) via the hydrolysis of adenosine triphosphate (ATP), this stimulates protein kinase A (PKA) activity, PKA phosphorylates target proteins. GPCRs of the Gq subtype stimulate the effector protein phospholipase Cβ (PLCβ), PLCβ hydrolyses phosphatidylinositol bisphosphate (PIP_2) to diacylcycerol (DAG) and inositol triphosphate (IP_3) – IP_3 increases intracellular calcium (Ca^{2+}) and DAG acts as a secondary messenger which activates protein kinase C (PKC) which phosphorylates target proteins. The Gi subfamily main function is through the inhibition of adenylate cyclase, thus inhibiting cAMP production and PKA activity (Alberts et al., 2002). The Gi stimulates phosphatidylinositol-3-kinsase (Pl3K) which catalyses the phosphorylation of PIP_2 to make phosphatidylinositol triphosphate (PIP_3) which primarily activates protein kinase B (PKB), it also inhibits

adenylate cyclase therefore downregulating cAMP. $G_{12,13}$ activates some guanine neucleotide exchange factors (GEFs) in the Rho family of GTPases, these are members of the RAS family of monomeric GTP-binding proteins. GEFs activate Rho family of GTPases via the catalysis of removing GDP in exchange for GTP (Rossman et al., 2005). These go on to stimulate further effector proteins that are involved in regulation of microtubule cytoskeleton, organelle development, gene expression and various other important cell functions (Marks et al., 2017) (Figure 8).

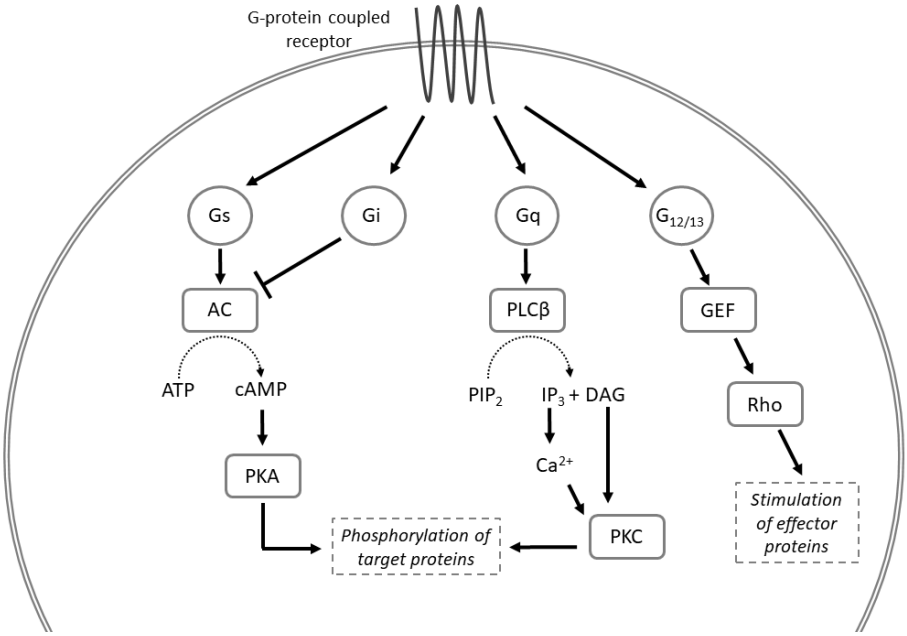

Figure 8. G-protein coupled receptor pathways. Simplified representation of the signalling cascades of Gs, Gi, Gq and G12/13 receptors.

1.7 Neuropeptides

Neuropeptides are a class of short chain polypeptides, which function as modulators in neuronal activity in the brain and as hormones in the periphery. Neuropeptides function through the neuroendocrine system, which is essential for homeostasis; it regulates growth and development, fluid balance, stress response, behaviour, mood, hunger/satiety, thirst, mood and reproduction (Melmed et al., 2015).

1.7.1 Neuropeptides in reproduction

The neuroendocrine system is intertwined, each axis is not an entirely separate entity. The neuropeptides impact each other and are essential for reproduction, the HPG axis regulates reproduction and the HPT and HPA axis can also modulate the HPG axis, this is evident in pathologies of both these axes. Thyroid disorders are implicated in both male and female infertility, dysfunction causes abnormalities in spermatogenesis, disturbances of the menstrual cycle and an increased risk of miscarriage (Trokoudes et al., 2006). Adrenal disorders affect female and male fertility, these disorders affect GnRH secretion severely affecting spermatogenesis and ovulation resulting in conditions such as oligozoospermia and aspermia, anovulation and premature ovarian failure (McNally, 1987; Unuane et al., 2011). Further, the HPA axis regulates stress response, with emotional stress implicated as a factor in infertility. Catecholamine hormones interact with the HPG axis, resulting in infertility, which results in further emotional stress for couples trying to conceive (Schenker et al., 1992).

The neuropeptides can broadly be separated into 8 groups; neurohypophyseal hormones, hypophysiotropic hormones, endogenous opioids, melanocortins, tachykinins, vasoactive peptides, appetite regulating peptides and miscellaneous (Melmed et al., 2015). The neurohypophyseal hormones consist of oxytocin and vasopressin. Oxytocin has a well-established role in parturition, lactation, maternal-infant bonding, and social bonding whereas the primary role of vasopressin is in osmotic homeostasis. Vasopressin is less understood in terms of reproduction (Akerlund, 2004; Wathes, 1984). Currently vasopressin is thought to be involved in maternal and social behaviour, it has been implicated in maternal depression and negative clinical outcomes in women undergoing ART (Murgatroyd et al., 2004; Rotzinger et al., 2010; Smeenk et al., 2005; Thiering et al., 1993). In the male, vasopressin is involved in contractility of the male reproductive tract and potentially may impact sperm function (Kwon et al., 2013). The hypophysiotropic hormones are hormones that stimulate other endocrine glands, these include direct regulators of the neuroendocrine axis, and all are involved in regulating the HPG axis either directly or indirectly (i.e through regulation of prolactin) (Dittrich et al., 2011; Gallego et al., 2005; Garcia et al., 2007; Hugues et al., 1991). The endogenous opioid peptides primary role is in modulating nociception. Some endogenous opioid peptides have been measured

in reproductive fluids and has some function in modulating sperm concentration, motility, acrosome reaction (Foresta et al., 1986; Sastry et al., 1991; Subirán et al., 2012). These endogenous opioid peptides have also been shown control GnRH and progesterone secretion and are involved in oocyte maturation (Facchinetti et al., 1986; Lehman et al., 2010). The melanocortin peptides are melanocyte-stimulating hormone (MSH) and corticotropin (ACTH). MSH regulates melanin secretion and is implicated in appetite suppression and sexual arousal, it has been found in the ovaries and shown to increase LH and FSH secretion (Backholer et al., 2009; Reid et al., 1981; Shaha et al., 1984). ACTH is a key mediator of the stress response and has differing effects depending on exposure length, acute exposure has a stimulatory effect on reproduction, increasing LH, FSH, sexual behaviour and ovulation, however chronic exposure inhibits LH, ovulation, follicular maturation with negative impacts on pregnancy (Brann and Mahesh, 1991). The tachykinins are a group of peptides that have a variety of effects such as regulating blood pressure, immune system, nociception and neurokinin B directly modulates GnRH and LH secretion (Latronico, 2009; Navarro, 2013; Rance et al., 2010). Tachykinins have been shown to regulate sperm motility, ovarian function and the HPG axis through effects on GnRH and LH (Blasco et al., 2020; Pennefather et al., 2006; Roman et al., 2012).

The vasoactive peptides are regulators of blood pressure and are found in both male and female reproductive tissues (Herr et al., 2013; Müller et al., 2004). Vasoactive peptides have been shown to regulate the ovulatory cycle, steroidogenesis, spermatogenesis and may affect sperm function and fertilisation (Ivanova et al., 2003; Kim et al., 1997; Klipper et al., 2004; Leung and Sernia, 2003; Speth et al., 1999; Tsai et al., 2005). Appetite regulating peptides are primarily involved in regulating energy balance, some have been identified in the reproductive tissues and fluids of both male and females, this group includes neuropeptide Y (NPY) which has been established as a key regulator of reproduction through modulating GnRH and LH secretion and steroidogenesis (Allen et al., 2011; Crown et al., 2007; Hanson and Dallman, 1995). Other appetite regulating peptides are involved in modulating GnRH and LH also, they are implicated in sexual behaviour, uterine function and erection and ejaculation (Cheung et al., 1996; Giacobini and Wray, 2007; Gottsch et al., 2004; Kozyrev et al., 2012). Within the miscellaneous neuropeptides there are known essential regulators of the HPG such as prolactin and kisspeptin which directly modulate GnRH and important in healthy reproductive function (Bachelot and Binart, 2007; Roa et al., 2011). Other neuropeptides implicated in the regulation of GnRH

are; Cocaine and amphetamine regulated transcript (CART), Melanin-concentrating hormone (MCH) and Neuromedin S which have a variety of other roles in reward, stress and the circadian rhythm (Chiocchio et al., 2001; Folger et al., 2013; True et al., 2013; Vigo et al., 2007; Yang et al., 2009). Orexin, Prolactin-releasing peptide (PrRP) and Neuromedin U modulate LH secretion (Backholer et al., 2009; Barreiro et al., 2004; Quan et al., 2003; Seal et al., 2000). Neurotensin, Vasoactive intestinal peptide (VIP) and PACAP have more local actions within the gonads (Benjamin Davoren and Hsueh, 1985; Fahrenkrug, 2001; Goodnough et al., 1979; Hiradate et al., 2014; Reglodi et al., 2012).

It is clear that neuropeptides play an integral role in all aspects of mammalian reproduction and several studies have demonstrated the role of neuropeptides in sperm function and fertility. Table 1 summarises the neuropeptides involved or associated with reproduction and fertility in both human and animal studies. The interaction between various neuropeptides and the HPG axis is outlined in Figure 9.

Table 1. Overview of neuropeptides, their general function and current evidence for roles in reproduction.

Neuropeptide	General function	Expression in reproductive tracts or tissues	Effect on fertility	Reference
Neurohypophyseal hormones				
Vasopressin	Water homeostasis/blood pressure/social behaviour/maternal behaviour/penile erection	Yes in both male and female	Implicated in negative clinical outcomes in women undergoing ART, involved in contractility of male reproductive tract, may impact sperm function. Implicated in maternal depression.	(Kwon et al., 2013; Murgatroyd et al., 2004; Rotzinger et al., 2010; Smeenk et al., 2005; Thiering et al., 1993)
Oxytocin	Parturition/sexual reproduction/social bonding	Yes in both male and female	Essential for many aspects of reproduction	(Akerlund, 2004; Assinder et al., 2000a; Carmichael et al., 1987; Carter, 1992; Filippi et al., 2002; Kumaresan et al., 1974)
Hypophysiotropic hormones				
Thyrotropin-releasing hormone (TRH)	Regulates HPT axis and stimulates release of prolactin	Yes - receptors on oocytes also	Affects prolactin, GnRH and sex steroids, pathology in TRH can lead to infertility	(Aghajanova et al., 2009; Dittrich et al., 2011; Williams, 2011)
Growth hormone-releasing hormone (GHRH)	Regulates release of growth hormone	Yes receptors	Can increase sensitivity of ovaries to gonadotropin stimulation	(Gallego et al., 2005; Hugues et al., 1991; Magon et al., 2011)
Ghrelin	Increases appetite, involved in reward pathway and sleep/wake cycle	Yes receptors	Modulates HPG axis through inhibition of LH	(Garcia et al., 2007; Lorenzi et al., 2009; Tena-Sempere, 2007)

Gonadotropin-releasing hormone (GnRH)	Regulates reproduction	Yes receptors	Essential for all aspects of reproduction	(Clayton and Catt, 1981; Herbison, 2018)
Corticotropin-releasing hormone (CRH)	Regulates stress response	Yes	Suggested to regulate timing of parturition and inhibitory effects on HPG axis, inhibition of GnRH, LH and steroid hormones	(Kalantaridou et al., 2010; OLSTER and FERIN, 1987)
Somatostatin	Inhibits growth hormone, inhibits thyroid stimulating hormone and release of prolactin and insulin	Yes in semen and male reproductive tissue	Inhibits GnRH	(Bhattarai et al., 2010; Pekary et al., 1984; SASAKI and YOSHINAGA, 1989)
Cortistatin	Induces slow wave sleep, binds to all somatostatin receptors	Unknown	Inhibits prolactin and corticotropin, affects growth hormone	(Ibáñez-Costa et al., 2017)
Endogenous opioid peptides				
B-endorphin	Reduces stress, maintains homeostasis, associated with pain, hunger, thrill, reward and sexual behaviour	Yes	May have a role in oocyte maturation, involved in acrosome reaction, may modulate sperm concentration	(El-Haggar et al., 2006; Jaschke et al., 2018; Urizar-Arenaza et al., 2016)
Enkephalin	Modulates nociception	Yes in sperm, semen and follicular fluid	Increases sperm motility, increases progesterone secretion by granulosa cells	(Facchinetti et al., 1986; Foresta et al., 1986; Sastry et al., 1991; Subirán et al., 2012)
Dynorphin	Modulates nociception	Unknown	Modulates GnRH and LH secretion, inhibitory effects on both via progesterone	(Lehman et al., 2010)
Nociceptin	Modulates nociception	Unknown	Inhibits uterine contractions in pregnancy, stimulation of receptor decreased female fertility	(Enright et al., 2012; Klukovits et al., 2010)
Melanocortin peptides				
Melanocyte-stimulating hormone (MSH)	Increases melanin secretion, implicated in appetite suppression and sexual arousal	Yes in ovaries	Increase LH and FSH secretion	(Backholer et al., 2009; Reid et al., 1981; Shaha et al., 1984)
Corticotropin (ACTH)	Regulates stress response	Unknown	Acute exposure: stimulates LH and FSH, facilitates sexual behaviour and ovulation. Chronic exposure: Inhibits LH,	(Brann and Mahesh, 1991;

			follicle development, ovulation, sexual maturation and negatively impacts pregnancy.	Dobson and Smith, 2000; Li and Wagner, 1983)
Tachykinins (neurokinins)				
Substance P	Regulates vasodilation, inflammation and nociception	Yes in semen, sperm, male reproductive tract and ovaries	Increases sperm motility, may modulate ovarian function	(Barad et al., 1988; Sastry et al., 1991)
Neurokinin A (NKA) (a.k.a. Substance K)	Involved in pain and inflammatory response	Unknown	Unknown	
Neurokinin B (NKB)	Modulates GnRH and LH secretion	Receptors in female reproductive system and in sperm	Modulates GnRH and LH secretion	(Blasco et al., 2020; Latronico, 2009; Navarro, 2013; Rance et al., 2010; Roman et al., 2012)
Hemokinin-1 (HK1)	Involved in immune system	Yes in sperm and receptor in myometrium	Increases sperm motility, uterine stimulant	(Blasco et al., 2020; Pennefather et al., 2006; Pinto et al., 2010; Ravina et al., 2007)
Neuropeptide K	May regulate sensation and may be involved in hypotension		Inhibits sexual behaviour, inhibit LH	(Debeljuk and Lasaga, 1999; Dornan et al., 1993; Tatemoto et al., 1985)
Vasoactive peptides				
Angiotensin II	Regulates vasoconstriction, stimulates release of aldosterone	Yes in both	May have a role in ovulation, spermatogenesis, sperm transport, capacitation, acrosome reaction and fertilisation	(Herr et al., 2013; Leung and Sernia, 2003; Speth et al., 1999)
Atrial natriuretic peptide (ANP)	Modulates extracellular fluid volume, decreases blood pressure	Yes, female reproductive tissues and receptors in male reproductive tissues	Inhibits follicular development, ovulation and steroidogenesis. May be involved in spermatogenesis.	(Ivanova et al., 2003; Kim et al., 1997; Müller et al., 2004; Tsai et al., 2005)

Endothelin	Vasoconstrictor	Yes	Regulates corpus luteum, involved in gamete transport	(Kedzierski and Yanagisawa, 2001; Klipper et al., 2004; Rosselli et al., 1994)
Appetite regulating peptides				
Galanin	Regulates appetite, implicated in nociception, sleep/wake cycle, blood pressure, mood	Yes in both	May contract uterus, involved in LH surge	(Cheung et al., 1996; Lerner et al., 2008; Stjernquist et al., 1988)
Galanin-like peptide (GALP)	Regulates appetite, roles in sexual behaviour, stress and inflammation	Unknown	Involved in LH surge, increases testosterone production, involved in modulating sexual behaviour	(Cheung et al., 1996; Gottsch et al., 2004; Gundlach, 2002; Kauffman et al., 2005; Krasnow et al., 2003)
Glucagon-like peptide (GLP)	Implicated in obesity and hypoglycemia	Unknown	May stimulate LH, may improve fertility in obese and PCOS females, may decrease pulsitivity of testosterone	(Beak et al., 1998; Holst, 2007; Jeibmann et al., 2005; Jensterle et al., 2019)
Cholecystokinin (CCK)	Aids digestion of fats and proteins and involved in appetite suppression	Unknown	May be implicated in reproductive behaviour, may inhibit GnRH, involved in GnRH neuron migration during embryo development	(Giacobini and Wray, 2007; Micevych et al., 2002; Wierman et al., 2011)
Gastrin releasing peptide	Stimulates gastrin in the stomach	Yes in female reproductive tissues	Implicated in erection and ejaculation, may be involved in uterine function	(Budipitojo et al., 2004; Kozyrev et al., 2012; Moghimzadeh et al., 1983; H. Sakamoto et al., 2008; Tamura et al., 2017; Whitley et al., 1996)

Neuropeptide Y (NPY)	Feeding behaviour/response to blood pressure/regulation of appetite/immune response	Yes in both	Modulates GnRH, LH secretion and gonadal steroids	(Allen et al., 2011; Crown et al., 2007; Fitzgerald et al., 2003; Hanson and Dallman, 1995; Kanzaki et al., 1996; Körner et al., 2011; Mcshane et al., 1992; Terado et al., 2006; Zhu et al., 2008)
Peptide YY (PYY)	Feeding behaviour, appetite regulation, related to NPY and binds to the same Y receptors, preferentially Y2	NPY family - binds to NPY receptors	Implicated in modulation of GnRH	(Lin et al., 2007; Murphy and Bloom, 2006; Woods and D'Alessio, 2008)
Pancreatic polypeptide (PPY)	Feeding behaviour, appetite regulation, binds to the Y receptors, preferentially Y4 and Y5	NPY family - binds to NPY receptors	Y4 and Y5 receptors implicated in inhibiting reproductive function via GnRH suppression	(Lin et al., 2007; Murphy and Bloom, 2006; Sainsbury et al., 2002; Toufexis et al., 2002)
Miscellaneous				
Cocaine and amphetamine regulated transcript (CART)	Involved in reward, stress and inhibits appetite	Yes in follicles	Role in follicular devlopment, may facilitate effect of leptin on GnRH, may stimulate GnRH neurons	(Folger et al., 2013; Rondini et al., 2004; True et al., 2013)
Orexin	Regulates wakefulness, arousal and appetite	Yes in uterus, ovaries and male gonads	May be involved in stimulating LH via being activated by melanocortins, may increase testosterone secretion, may suppress genes in Sertoli cells	(Backholer et al., 2009; Barreiro et al., 2005, 2004; Nitkiewicz et al., 2012)
Melanin-concentrating hormone (MCH)	Regulates sleep/wake cycle, energy balance, mood and behaviour	Unknown	Regulation of LH in the presence of oestrogen, regulation of GnRH in relation to energy balance	(Chiocchio et al., 2001; Naufahu et al., 2013; Wu et al., 2009)

Prolactin-releasing peptide (PrRP)	Regulates prolactin	Expressed in both male and female reproductive tracts.	Increases oxytocin (and vasopressin in females), modulates prolactin, LH, FSH and testosterone.	(Fujii et al., 1999; Maruyama et al., 1999; Seal et al., 2000)
Kisspeptins (Metastin)	Activates GnRH, directly involved in modulating reproduction and puberty	Yes	Regulates GnRH secretion, timing of puberty, mediation of sex steroids, fertility, modulates sperm function, modulates ovarian function	(Hu et al., 2018; Pinto et al., 2012; Roa et al., 2011)
Neuromedin U	Contraction of smooth muscle cells (in uterus also), regulates nociception, bone growth and appetite	Yes	Regulates follicugenesis, modulates LH, stimulates uterine contractions	(Brighton et al., 2004; Minamino et al., 1985; Quan et al., 2003)
Neuromedin S	Regulates circadian rhythm, appetite and the release of vasopressin, oxytocin and LH	Yes in testis	Regulates GnRH, LH and oestrogen, stimulates oxytocin release, may have a role in spermatogenesis	(Ma et al., 2017; Sakamoto et al., 2007; T. Sakamoto et al., 2008; Vigo et al., 2007; Yang et al., 2009)
Neurotensin	Regulates LH, prolactin and interactions within dopaminergic system	Yes in female reproductive tissues	Stimulates uterine contraction, modulates sperm capacitation and acrosome reaction	(Carraway and Leeman, 1973; Hiradate et al., 2014; Reinecke, 1987; Rodríguez et al., 2010; Umezu et al., 2016)
Vasoactive intestinal peptide (VIP)	Increases contractility in heart, vasodialator, increases vaginal lubrication	Yes in female reproductive tissues	Regulates oocyte transport, sexual arousal in males and females, regulates steroidgenesis in females and males, promotes vaginal lubrication	(Benjamin Davoren and Hsueh, 1985; Fahrenkrug, 2001; Goodnough et al., 1979; Graf et al., 1995; Kasson et al., 1986; Levin, 1991; Palle et al., 1989)
Pituitary adenylate cyclase-activating polypeptide (PACAP)	Binds to VIP receptors, stimulates gastric cells	Yes in female reproductive tissues	Regulates ovarian function	(Reglodi et al., 2012; Scaldaferri et al., 1996;

				Steenstrup et al., 1995; Winters and Moore Jr, 2011)
Prolactin	Growth regulator/Lactation/receptor involved in embryo implantation	Yes in both	Essential for corpus luteum function, development of mammary glands, maternal behaviour, implicated in modulating LH, GnRH and some role in the prostate	(Bachelot and Binart, 2007; Heller and Jacobs, 1978)

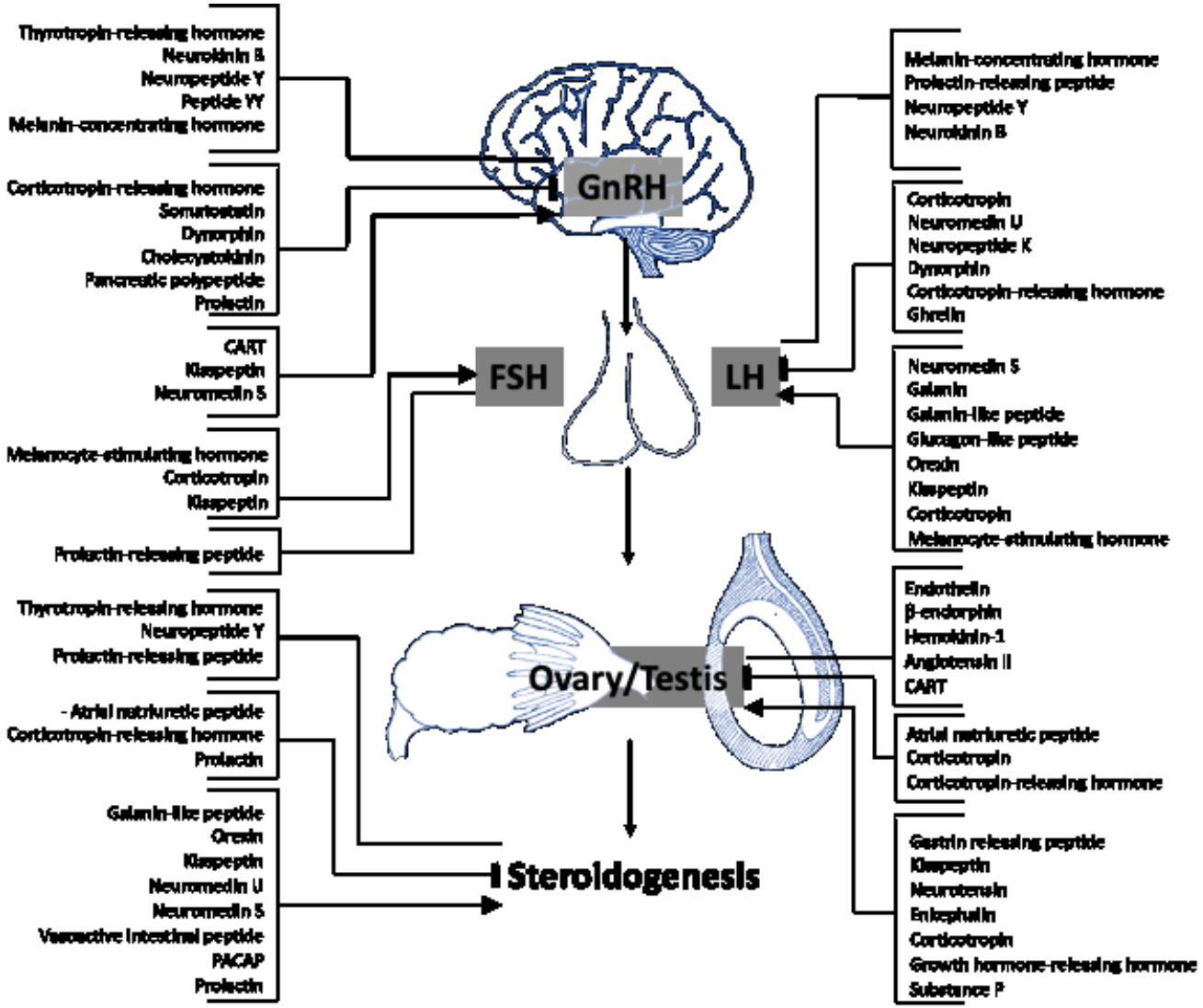

Figure 9. The known impacts of neuropeptides on the HPG axis. An arrow represents a stimulatory effect, a line with a blunted end represents an inhibitory effect and a line alone represents an effect with either unknown or complex consequences.

1.7.2 Neuropeptides effects on sperm

Although the role of neuropeptides in reproduction is evident as outlined in studies presented in Table 1, their role in sperm function is less explored. In the present study a number of neuropeptides, which had been investigated in semen or plasma comparatively in the samples of fertile and infertile males were further investigated through short meta-analysis.

Infertility is defined as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" by the WHO (Zegers-Hochschild et al., 2009). The WHO also has an epidemiological definition where it is specified that "women of reproductive age, at risk of becoming pregnant, who report trying unsuccessfully for a pregnancy for 2 or more years" and other definitions use a 5 year time bracket (Cavallini and Beretta, 2015). Male infertility is rarely included as an individual disorder apart from and even so involves a panel of examinations of the semen and reproductive tracts in order to diagnose infertility or subfertility (World Health Organization, 2010a). With this lack of consensus and limitation in the definition of male infertility caution is necessary when interpreting the following data as other research groups may use variations in definitions.

A meta-analysis is a statistical procedure that integrates the results of several independent studies (Haidich, 2010) and in this study a review of the literature for neuropeptides available for meta-analysis were leptin, prolactin, β-Endorphin and ghrelin.

Leptin is a neuropeptide which inhibits appetite and regulates energy balance through its impacts on the arcuate nucleus (Ahima et al., 2000). Prolactin is the regulator of lactation and growth and is essential in many aspects of reproduction (Bachelot and Binart, 2007; Heller and Jacobs, 1978). β-Endorphin is an endogenous opioid primarily involved in nociception (Hartwig, 1991). Ghrelin can be considered as an opposite to leptin, it stimulates appetite and increases food intake (Wren et al., 2001). Currently, there is no consensus in regard to whether these neuropeptides have a positive or negative effect on human sperm.

To compile, analyse and present the available data Review Manager (RevMan 5.3) was used. RevMan 5.3 is software developed by Cochrane, which can be used to support systematic reviews and meta-analysis. The data in the meta-analysis in this study were presented as forest plots (Haidich, 2010). There are two important factors in forest plots,

the heterogeneity which determines the variation that is not due to chance between the included studies which assesses their suitability to be assessed together where a non-significant *p*-value indicates no heterogeneity. The I^2 statistic is representative of total variability where above 50% is high heterogeneity, 25-50% is moderate and below 25% there is little to no heterogeneity. The *p*-value of the overall effect determines the significance of all the included studies.

In the studies included in the meta-analysis the neuropeptides were all measured in either the serum or the semen of both fertile and infertile men.

The concentration of the neuropeptides for fertile men were compared to infertile men across multiple studies. The meta-analysis showed that leptin concentration is significantly lower in normozoospermic males, β-Endorphin concentration was significantly higher in normozoospermic males, ghrelin concentration was significantly lower in normozoospermic males, however, the variability in the studies investigating ghrelin was extreme and therefore the validity of the meta-analysis is questionable. Studies investigating prolactin concentration were highly variable and therefore overall prolactin concentration showed no significant difference between fertile and infertile males.

Due to the limited nature of the studies available, low heterogeneity was not able to be ensured in the meta-analyses. The overall paucity of data available in the literature regarding the role of neuropeptides and sperm function warrants further research.

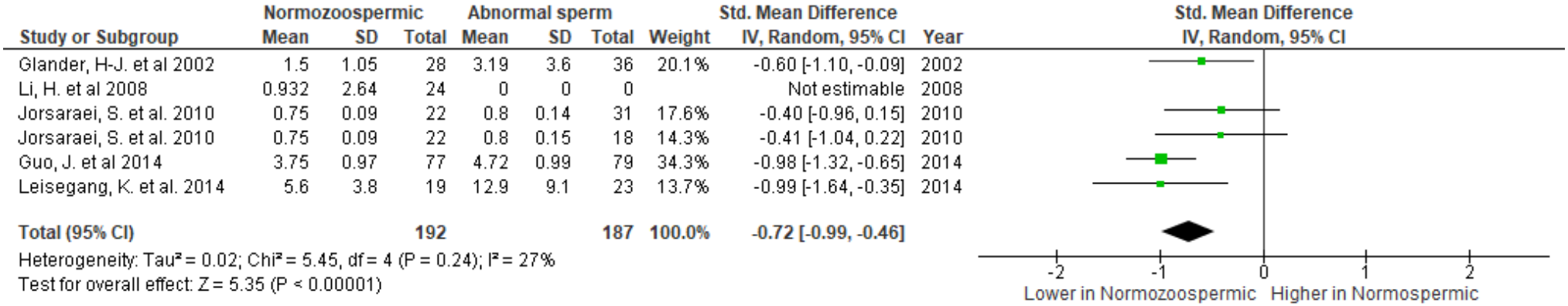

Figure 10. Meta-analysis for 5 studies investigating leptin (ng/mL) concentration and semen parameters.

Table 2. Characteristics and overview of studies included in Leptin meta-analysis.

Study	Author	Country	N	Method	Summary	Positive/Negative effect on fertility	
Leptin exists in tubuli seminiferi and in seminal plasma	(Glander et al., 2002)	Germany	64	RIA	No relationship between leptin concentrations and semen parameters.	None	*converted to SD
Effect of leptin on motility, capacitation and acrosome reaction of human spermatozoa	(Li et al., 2009)	China	24	ELISA	No significant effect on sperm motility, capacitation or acrosome reaction.	None	*converted to SD
The leptin concentrations in seminal plasma of men and its relationship to semen parameters	(Jorsaraei et al., 2010)	Iran	40	ELISA	Significant negative correlation between semen leptin concentration and sperm motility.	Negative	
Sperm motility inversely correlates with seminal leptin levels in idiopathic asthenozoospermia	(Guo et al., 2014)	China	156	ELISA	Significant negative correlation between semen leptin concentration and sperm motility and serum testosterone.	Negative	
Obesity is associated with increased seminal insulin and leptin alongside reduced fertility parameters in a controlled male cohort	(Leisegang et al., 2014)	South Africa	42	ELISA	No correlation between semen leptin concentration and sperm motility or vitality.	None	

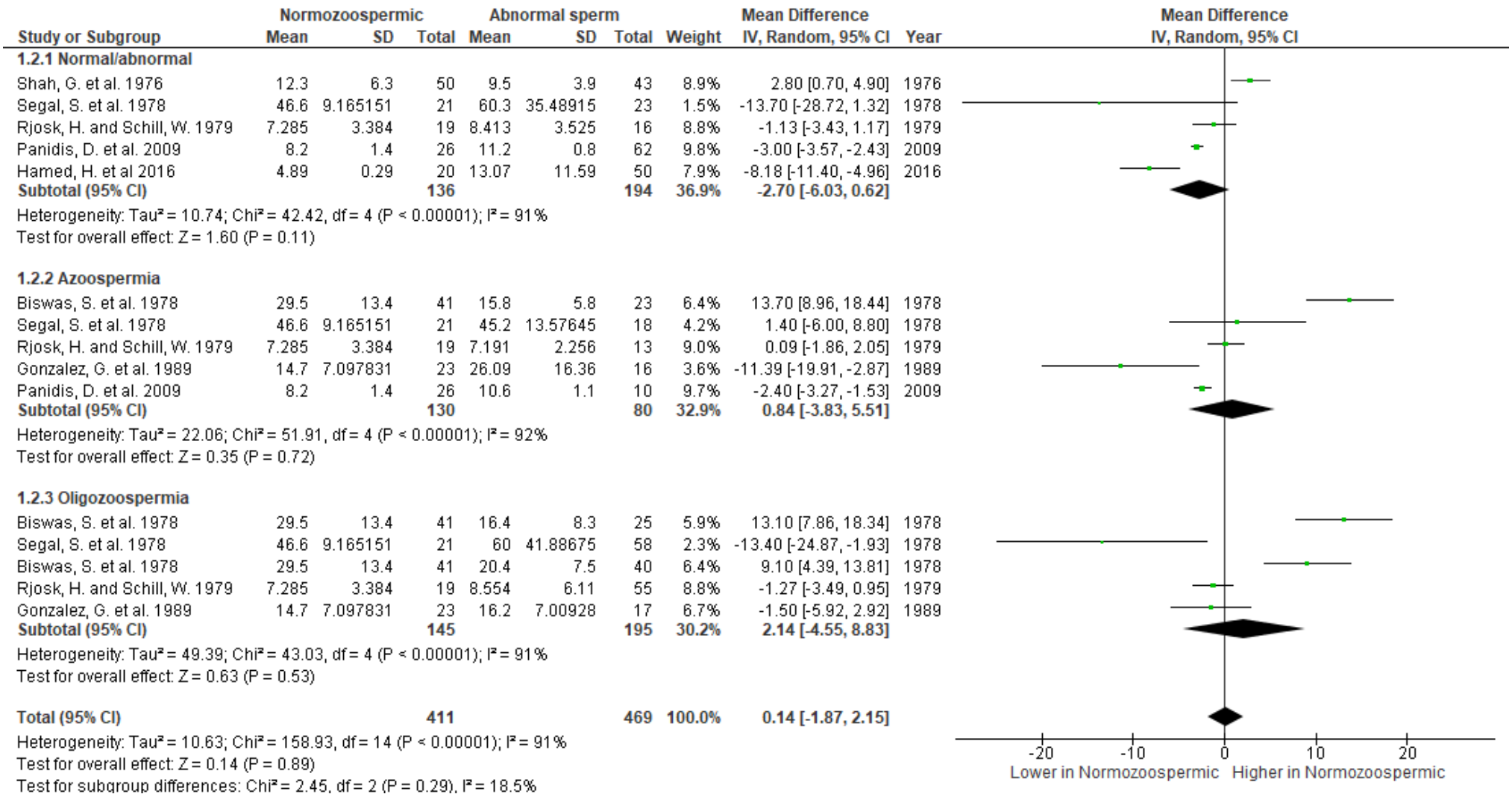

Figure 11. Meta-analysis for 7 studies investigating prolactin (ng/mL) concentration and semen parameters.

Table 3. Characteristics and overview of studies included in Prolactin meta-analysis.

Study	Author	Country	N	Method	Summary	Positive/Negative effect on fertility	
Correlations between seminal radioimmunoreactive prolactin, sperm count, and sperm motility in prevasectomy and infertility clinic patients	(Smith et al., 1979)	USA	93	RIA	Strong positive correlation between semen prolactin and sperm motility and sperm count in all patients.	Positive	*converted to SD
Serum prolactin in male infertility	(Rjosk and SCHILL, 1979)	Germany	103	RIA	No correlation between serum prolactin and sperm motility or concentration. No significant difference in serum prolactin was found between normozoospermia, asthenozoospermia, oligozoospermia and azoospermia was found.	None	*converted to ng/mL *serum measurements used
Seminal prolactin and its relationship to sperm motility in men	(Gonzales et al., 1989)	Peru	56	RIA	Seminal prolactin levels positively correlated with motility and negatively with sperm concentration. Serum prolactin was higher in azoospermia.	Unknown	*converted to SD
Prolactin in seminal plasma of infertile men	(Segal et al., 1978)	Israel	120	RIA	No relationship found between sperm count, motility and serum or seminal prolactin levels. Excessively high serum and seminal plasma prolactin levels found in a few of the men in the infertile groups. Prolactin in seminal plasma	None	*converted to SD

					was 2 to 3 times higher than in serum.		
Fructose and hormone levels in semen: their correlations with sperm counts and motility	(Biswas et al., 1978)	UK	129	RIA	Significantly higher levels of prolactin was found in seminal plasma of normozoospermic compared to sub/infertile groups. Prolactin was found to have a significantly positive correlation with sperm concentration but not motility.	Positive	
The effect of ghrelin On sex hormones in infertiled men	(Hamed et al., 2016)	Iraq	70	ELISA serum measurements	Significantly higher serum prolactin in infertile patients.	Negative	*serum measurements
Serum and seminal plasma ghrelin levels in men with normospermia and dyspermia	(Panidis et al., 2008)	Greece	98	Immunochemiluminescence	No difference found between fertile and infertile males serum levels of prolactin.	None	*serum measurements

Table 4. Characteristics and overview of studies included in β-Endorphin meta-analysis.

Study or Subgroup	Normozoospermic Mean	SD	Total	Abnormal sperm Mean	SD	Total	Weight	Mean Difference IV, Fixed, 95% CI
Davidson, A. et al 1989	290	175	25	335	315	49	0.4%	-45.00 [-156.74, 66.74]
El-Haggar, S. et al 2006	87.23	29.55	20	20.36	13.39	20	25.3%	66.87 [52.65, 81.09]
El-Haggar, S. et al 2006	87.23	29.55	20	23.13	4.7	20	29.7%	64.10 [50.99, 77.21]
El-Haggar, S. et al 2006	87.23	29.55	20	51.3	27.37	20	16.4%	35.93 [18.28, 53.58]
El-Haggar, S. et al 2006	87.23	29.55	20	51.88	9.47	20	27.6%	35.35 [21.75, 48.95]
Singer, R. et al 1983	278.6	151.0348	12	191.1	139.1941091	31	0.5%	87.50 [-11.01, 186.01]
Total (95% CI)			**117**			**160**	**100.0%**	**51.91 [44.76, 59.06]**

Heterogeneity: Chi² = 19.81, df = 5 (P = 0.001); I² = 75%
Test for overall effect: Z = 14.23 (P < 0.00001)

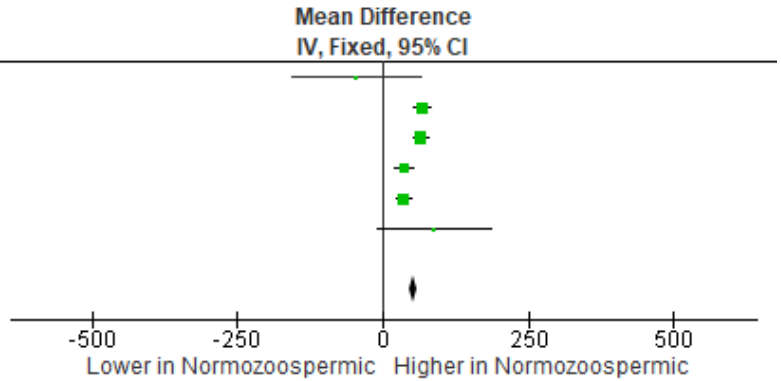

Figure 12. Meta-analysis for 3 studies investigating β-Endorphin (pg/mL) concentration and semen parameters.

Study	Author	Country	N	Method	Summary	Positive/Negative effect on fertility	
β-Endorphin in normozoospermic and pathologic human semen	(Singer et al., 1985)	Israel	43	RIA	Significantly higher β-Endorphin in normozoospermic patients. Semen levels were significantly higher than serum levels.	Positive	*converted to SD
Presence of immunoreactive β-Endorphin and calcitonin in human seminal plasma, and their relation to sperm physiology	(Davidson et al., 1989)	USA	74	RIA	No significant difference in seminal plasma β-endorphin levels in fertile and infertile males. β-endorphin levels had no relationship with semen parameters.	None	*converted to SD
Beta-endorphin in serum and seminal plasma in infertile men	(El-Haggar et al., 2006)	Egypt	100	ELISA	Semen levels had positive correlation with sperm concentration	Positive	*converted to mean and SD from range and percentiles

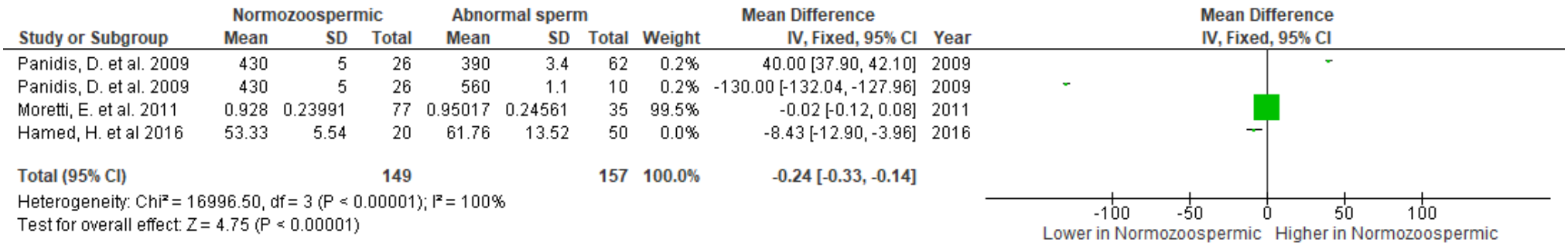

Figure 13. Meta-analysis for 3 studies investigating ghrelin (ng/mL) concentration and semen parameters.

Table 5. Characteristics and overview of studies included in Ghrelin meta-analysis.

Study	Author	Country	N	Method	Summary	Positive/Negative effect on fertility	
The effect of ghrelin On sex hormones in infertiled men	(Hamed et al., 2016)	Iraq	70	ELISA serum measurements	Significantly lower ghrelin in serum of control participants.	Negative	
Serum and seminal plasma ghrelin levels in men with normospermia and dyspermia	(Panidis et al., 2008)	Greece	98	ELISA	No difference found between fertile and infertile males semen levels of ghrelin, negatively correlated with semen volume.	None	*converted to ng/mL
Detection of obestatin in seminal plasma and its relationship with ghrelin and semen parameters	(Moretti et al., 2011)	Italy	112	RIA	Higher levels of ghrelin in semen than in serum.	None	*converted to ng/mL

Table 6 vasopressin, oxytocin and their effects on fertility

Study	Author	Year	Species	Country	Neuropeptide	N	Method	Summary	Positive/Negative effect on fertility
Vasopressin effectively supresses male fertility	Kwon, W. et al	2013	Mouse	Korea	Vasopressin	6 repeats	In vitro assay	Vasopressin has a dose dependant decrease on mouse sperm motility	Negative
Fertility in female chickens as affected by the injection of oxytocin and arginine vasopressin near the time of insemination	Hughes, B. and Parker, J.	1971	Chicken	USA	Vasopressin/Oxytocin	15 Chickens	Intravenous injection	Intravenous injections of vasopressin given immediately after insemination significantly reduced fertility. Intravenous injections of oxytocin significantly reduced fertility immediately after insemination and 30 minutes post insemination.	Negative
Oxytocin and vasopressin stimulate anion secretion by human and porcine vas deferens epithelia	Hagedorn, T. et al	2007	in vitro human/porcine cell culture	USA	Vasopressin/Oxytocin	3-5 repeats	Cell culture and RT-PCR	Oxytocin and vasopressin modulate ion transport across vas deferens epithelia by independent mechanisms. VP and OXT therefore have the potential to acutely change the environment in which sperm are exposed to.	Unknown
The in vitro effects of oxytocin and vasopressin on spontaneous contractility of the mouse cauda epididymis	Hib, J.	1974	Mouse	Uruguay	Vasopressin/Oxytocin	20 mice	ex vivo	OXT and VP caused an increase in the frequency and amplitude of contractions of the mouse cauda epididymis. VP caused an incremental rise in the baseline tension.	Unknown
Effect of vasopressin on fertility of male rats	Ratnasooriya, W. and Jayakody, J.	2004	Rat	Sri Lanka	Vasopressin	18 rats	Vaso-epididymal injection	Daily injections of vasopressin into the cauda epididymis of rats for 7 consecutive days resulted in oligospermic ejaculates. Sexual behaviour was unaffected.	Negative

Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa	Kwon, W. et al	2015	Boar	Korea	Proteomics analysis	3 repeats	Proteomics , 2-DE and MS	20 proteins showed differential expression levels in small and large litter size groups, 19 of which showed decreased expression in large litter size. Glutathione S-transferase Mu3 and glutathione peroxidase 4 were related to the glutathione metabolic pathway and vasopressin receptor 2 was linked to vasopressin receptor 2/STAT and these were differentially expressed in small and large litter sizes.	Negative
Urinary vasopressin in male infertility	Puri, S. and Puri, V.	1984	Human	India	Vasopressin	10 men	Extracted using zinc ferrocyanide absorption	A highly statistically significant negative correlation was observed between urinary vasopressin concentration and sperm motility and sperm count.	Negative
Some effects of vasopressin on sexual behaviour and seminal characteristic in intact and castrated rabbits	Kihlstrom, J. and Agmo, A.	1973	Rabbit	Sweden	Vasopressin	Unknown	Intravenous injection	Rabbits were intravenously injected with different doses of vasopressin, a low dose had no effect on behaviour or semen parameters. Doses of 40 or 200 mu. Significantly increased semen volume and sperm content in the ejaculates.	Positive
[Patent] Method of reducing mammalian fertility and drugs therefor	Cheesman, D.	1976	Rats	USA	Vasotocin			0.5-5 ug vasotocin per kilogram of body weight was suggested to suppress the preovulatory phase in females therefore suppressing ovulation and fertility. Suggested as a method to reduce fertility in males via reducing LH secretion by using vasotocin.	Negative

Variations in plasma concentrations of vasoprssin during the menstrual cycle	Forsling, M. et al	1980	Human	Sweden	Vasopressin	8 women	RIA	Plasma concentrations of vasopressin significantly increased on day 16-18 of the menstrual cycle in a cohort of health women	Unknown
Arginine vasopressin and oxytocin in the porcine corpus luteum	Choy, V. and Watkins, W.	1988	Pig	New Zealand	Vasopressin/Oxytocin	176 corpus lutea from 18 sows	Acetic acid extraction, HPLC and RIA	Mid cycle corpus lutea showed presence of arginine vasopressin like material but not lysine vasopressin like material when investigated with HPLC. Oxytocin content was unchanged during luteal phase but declined at the end of the cycle. AVP remained unchanged during luteal phase but significantly increased towards the end of the cycle.	Unknown
[Review] Effects of psychological stress on male fertility	Nargund, V.	2015	Human	UK	Vasopressin/Multiple	None - review		Vasopressin is released with corticotropin releasing hormone under psychological stress, triggering signalling pathways that negatively affect fertility and spermatogenesis in males	Negative
Vasopressin: another pregnancy protein in human seminal plasma	Brotherton, J.	1990	Human	Germany	Vasopressin	20 semen, 19 amniotic fluid	Ethanol/nitrogen extraction & RIA	Vasopressin was found in seminal plasma of human males at similar concentrations to plasma levels. Vasopressin was found in trace amounts in half of the samples of amniotic fluid.	Unknown
A comparison of plasma vasopressin and oxytocin concentrations during the the oesterous cycle of the ewe	Wathes, D. et al	1991	Sheep	UK	Vasopressin/Oxytocin	5 ewes	Petroleum ether extraction and RIA	Oxytocin concentrations increased during the early luteal phase and reached plateau then declined either preceding or at ovulation. Vasopressin showed significant dependence on the day of the cycle with concentrations lowest at oestrus and minor peaks on day 4 and 8-9.	None

Vasopressin concentrations were significantly higher in the morning than in the afternoon and would rise again in the evening. Minor variations postulated to have no effect on reproduction at the concentrations found.

There are several studies in the literature highlighting the role of vasopressin and oxytocin in mammalian reproduction, including many aspects of human reproduction as summarised in Table 6. However, as integral as these neuropeptides are in reproductive physiology, there are still many gaps in the knowledge. The research presented in this thesis set out to further explore the role of vasopressin and oxytocin has in human reproduction and fertility, and in particular sperm function.

Overall aim:

The aim of this research was to investigate how vasopressin and oxytocin function as in relation to sperm physiology and fertility.

To identify any associations between oxytocin, vasopressin and TNFα in follicular fluid and clinical outcomes, and to investigate any correlation with seminal plasma vasopressin and oxytocin with clinical outcomes. To establish presence of the vasopressin receptors in human sperm and explore the effect of vasopressin through treatments with agonists *in vitro*. To identify the role of methylation status in oxytocin receptor CpG sites 924 and 934 in human sperm function in clinical sperm samples.

Chapter 2: Methods and Materials

2.0 Methods and materials

2.1 List of materials, equipment and software used

Reagents	Supplier	Catalogue #
[Arg8] Vasopressin acetate salt	Sigma	V9879
[deamino-Cys1, D-Arg8]-Vasopressin acetate salt hydrate	Sigma	V1005-1MG
10 ml, premixed 4x Laemmli protein sample buffer for SDS-PAGE	Bio-Rad	1610747
2-Mercaptoethanol	Sigma	M7154
5x DNA Loading Buffer Blue	Bioline	BIO-37045
8-bromo-cAMP	Abcam	ab141448
Acrylamide/Bis 30% solution	Bio-Rad	161-0158
Agarose	Fisher scientific	M-12198
Agarose, low gelling temperature	Sigma-Aldrich Co. Ltd	A9045-25G
Alexa fluor® 488 F(ab') 2 goat anti-mouse IgG, IgM (H+L)	Thermo Fisher Scientific	A-10684
Alexa fluor® 488 goat anti-rabbit IgG (H+L)	Invitrogen	A-11008
AllPrep DNA/RNA Mini Kit	Qiagen	80204
AmershamTM Protran® Western blotting membranes, nitrocellulose	AmershamTM	GE10600001
Ammonium persulphate	Sigma	A3678
Anti-alpha Tubulin antibody	Abcam	ab7750
Anti-Aquaporin 2 antibody	abcam	ab62628
Anti-AVPR V2 antibody	Abcam	Ab188748
Anti-goat HRP	Sigma	A5420
Anti-oxytocin receptor	Abcam	ab87312
Anti-oxytocin receptor	Abcam	ab115664
Anti-Phosphotyrosine antibody	Abcam	ab10321
Anti-prolactin receptor	Abcam	ab2773
Aquaporin 2 Polyclonal antibody	Invitrogen	PA5-78809
Aquaporin 2 Polyclonal antibody	Invitrogen	PA5-22865
Aquaporin 2 Polyclonal Antibody	Thermo Fisher Scientific	PA5-22865
Arg8-Vasopressin ELISA Kit	Abcam	ab205928
AVPR1A antibody (7HCLC), ABfinityTM Rabbit Oligoclonal	ThermoFisher Scientific	711640
Benzoanase Nuclease	Sigma	E1014-5KU
Bovine serum albumin	Sigma	A2153
Bromophenol blue	Sigma	B8026
Calcium Ionophore A23187	Sigma	C7522 - 5MG
CELLview™ Cell Culture Dish, one compartment	Greiner Bio-One Ltd	627 861
Chloroform	Sigma	C7559
DAPI	Sigma	D9542

deamino-Cys1, D-Arg8]-Vasopressin acetate salt hydrate	Sigma	V1005-5MG
Dimethyl Sulfoxide	Fisher Scientific	67-68-5
DPBS 1X	Corning	21-031-CVR
Dulbecco's Modified Eagle's Medium - high glucose	Sigma	D1145
EBSS CUSTOM MADE MEDIA,100ml	GENEFLOW LTD	K1-0522
Epitect Bisulfite Kit (48)	Qiagen	59104
Ethanol	Sigma	51976
EZ-ECL	Biological industries	20-500-120
Falcon™ 15mL Conical Centrifuge Tubes	Fisher scien	10263041
Fluo-4, AM, cell permeant	thermofisher	F14217
Foetal bovine serum	Sigma	F0804
Gibco™ Fetal Bovine Serum	Fisher Scientific UK	11550356
glass square coverslip	Fisherbrand™	12363138
Glycerol	Sigma	G5516
Glycine	Sigma	G8898
Goat anti-rabbit HRP	Bio-Rad	170-6515
GoTaq® 2-step RT-qPCR system	Promega	A6010
GoTaq® Probe 2-Step RT-qPCR System	Promega	A6110
Halt protease and phosphotase inhibitor cocktail	Fisher Scientific	16085973
Hydrochloric acid	Sigma	H1758
HyperLadderTM 25bp	Bioline	BIO-33057
HyperLadderTM 50bp	Bioline	BIO-33054
ImmEdge Hydrophobic Barrier Pen (PAP pen)	Vector	H-4000
Invitrogen™ SYBR™ Gold Nucleic Acid Gel Stain	Fisher Scientific UK	10358492
ISOLATE II Genomic DNA Kit	Bioline	BIO-52066
Isopropanol	Sigma	I9516
Lectin from Pisum sativum	Sigma	L0770-2MG
L-Glutamine	Sigma	G5792
MagicMarkTM XP western protein standard	Life technologies	LC5603
Marvel dried milk powder	Sainsbury's	2051857
Methanol	Sigma	494437
Microplates for Fluorescence-based Assays	Thermo Scientific	M33089
Monoclonal Anti-β-Tubulin antibody produced in mouse	Sigma	T4026
M-PER™ Mammalian Protein Extraction Reagent	Fisher Scientific UK	11885095
MyTaqTM DNA Polymerase	Bioline	BIO-21105
MyTaq™ DNA Polymerase	Bioline	BIO-21105
N,N,N',N'-Tetramethylethylenediamine	Sigma	T9281
Normal goat serum	Vector	S-1000
Nuclease free water (10 x 50 mL)	thermofisher	AM9906
OPC 31260 hydrochloride	Sigma	O1266-10MG
Oxytocin acetate salt hydrate	Sigma	O6379-1MG
Oxytocin ELISA Kit	Abcam	ab133050
Penicillin-Streptomycin	Sigma	P4333
Phosphate Buffered Saline	Oxoid	BR0014G
Phospho(Ser/Thr) PKA substrate antibody	Cell signalling	9621S
Phosphotase inhibitor cocktail set III	Millipore	524627
Pierce® BCA protein assay kit	Pierce	23225
Pluronic® F-127	Sigma	P2443
Poly-D-Lysine solution, 1.0 mg/mL	sigma	A-003-E
Ponceau S	Sigma	P3504

Precision plus proteinTM standards	Bio-Rad	161-0376
Precision plus standard dual colour	Bio-Rad	1610374
Precut 7 x 8.4 cm Immun-Blot PVDF Membrane	Bio-Rad	1620174
Primers	Invitrogen	A15612
Protease inhibitor cocktail	Sigma	P8340
Proteinase K (10 ml)	Qiagen	19133
Puresperm	BioTipp	PBS-100
Puresperm 100	BioTipp	PS 100-100
PyroMark PCR Kit (200)	Qiagen	978703
RIPA Lysis buffer	Fisher Scientific	10230544
RQ1 RNase-Free DNase	Promega	M6101
Sodium bicarbonate	Sigma	S3817
Sodium chloride	Sigma	S3014
Sodium deoxycholate	Sigma	S1827
Sodium dodecyl sulphate	Sigma	L3771
Sperm freezing medium	Origio	10670010A
Sperm preparation medium with Phenol Red	Origio	10705060A
SR144528	Sigma	SML1899-5MG
SuperFrost™ Microscope Slides	Fisher Scientific	12372098
SupraSperm® System	Origio	10922060A
Taq DNA Polymerase (250 U)	Qiagen	201203
TGX FastCastTM Acrylamide kit, 10%	Bio-Rad	161-0173
TNFα ELISA Kit	Sigma	RAB0476
Tris base	Fisher scientific	BP152-1
TritonTM X-100	Sigma	T8787
Triton™ X-100 (Electrophoresis),	Fisher Scientific	9002-93-1
TRIzol® Reagent	Ambion life technologies	15596018
Trypsin	Sigma	T2600000
Tween® 20	Sigma	P9416
Vectashield® mounting medium for fluorescence with DAPI	Vector	H-1200

Equipment/Materials	Supplier
0.2mL PCR Tube, Flat Cap	Star Labs
3-16PK centrifuge	Sigma
Autoflow Direct Heat CO2 Incubator – Cell culture incubator	NUAIRE
Axio Imager Z1 – Fluorescence microscope	Zeiss
Biological Safety Cabinets – Class II safety cabinet	NUAIRE
CASA counting chamber 20 micron	Cell Vision
Cell culture plates and flasks	Nunc by Fisher Scientific
ChemiDocTM Touch Imaging System – Western blot and agarose gel imaging system	Bio-Rad
Confocal	Leica
DFC 365 FX – Fluorescent microscope	Leica
Dri-Block® DB-2D – Heat block	Techne
Eppendorf thermal cycler	Eppendorf
Eppendorf tubes	Starlabs
Falcon Tubes	Sarstedt
Glassware	Thermofisher Scientific
Microplates	Grenia
Mini-PROTEAN® Comb, 10 well	Bio-Rad
Mini-PROTEAN® Tetra System	Bio-Rad
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-Rad
Mini-Rocker Shaker	Grant-Bio
NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer	Thermofisher Scientific
Olympus CX41 – Phase contrast microscope (CASA)	Olympus
Pipette tips	Starlabs
Pipettes	Gilson
Plasticware	Sarstedt/Fisher Scientific
PowerPac Basic	Bio-Rad
PowerPacTM Basic Power Supply - power supply for gel tanks and transfer system	Bio-Rad
PyroMark Q24 System	Qiagen
Sample collection pots	Sterilin UK
Specimen pots	BioTipp
Sub-Cell® GT Cell – Agarose gel electrophoresis tank	Bio-Rad
Superfrost® Plus Glass Slides	Thermo-Fisher
SureCycler 8800 – Thermal cycler	Agilent Technologies
SYNERGY HT Microplate Reader	BioTek®
Thermo-Shaker	Peqlab
Tomy Capsulefuge PMC-860 - Micro centrifuge	ProLabMas
Trans-Blot® SD Semi-Dry Transfer System – Western blot transfer system	Bio-Rad
Vortexer	SLS Lab Basics
Whatman® gel blotting papers	Whatman®

Software

AxioVision version 4.8.2.0
Bio-Rad Image Lab™ software
Gen 5 version 2.05.5 BioTek®
GraphPad Prism7
IBM SPSS Statistics 25
ImageJ 1.52i
Leica LAS X Suite microscope imaging software
Matlab R2018a
MetaMorph®
Microsoft® Excel® 2016 version MSO (16.0.4738.1000)
Microsoft® PowerPoint 2016 version MSO (16.0.4738.1000)
Microsoft® Word 2016 version MSO (16.0.4738.1000)
RevMan 5.3
SAMi® Pro Creative Diagnostics 1.0
Wave 2.6 Software Agilent
ZEN 2 Blue Edition

2.2 Sperm sample procurement, assessment and storage

Semen samples were produced by masturbation after 2-5 days of abstinence from healthy males aged 18+. Donors were provided with a participant information sheet, which contained details of the study, two copies of the consent form, one for their own reference and a medical screening questionnaire upon initial consent (see appendix). Full faculty ethical approval (EthOS Reference Number: 0381; REC approval: Reference number: 12/SC/0649, Study number: SE1617126). Semen samples were either produced on site in a designated room or at the participant's home. Samples produced from home had to be brought to the laboratory within an hour of production.

Semen samples were assessed in accordance with the WHO laboratory manual for the examination and processing of human semen 5th edition (2010) guidelines. Semen was liquefied at 37°C for 30 minutes in a direct heat 5% CO_2 incubator. This was followed by measurement of semen volume, sperm concentration and sperm motility.

Sperm motility, kinematics and concentration were measured and analysed using SAMi (Procreative Diagnostics) computer assisted sperm analysis (CASA) system, where 5 µl of the semen was pipetted onto a counting chamber slide (Cell Vision 20 micron depth) (Figure 14).

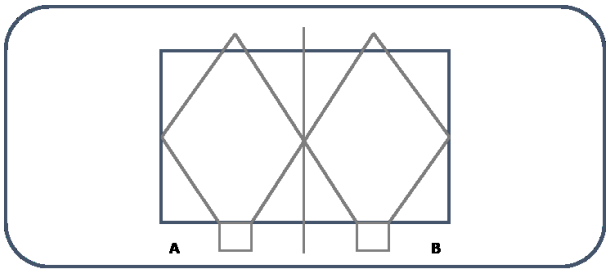

Figure 14. Schematic of CASA slide.

To measure sperm motility - the slide was places onto a 37°C heated stage on the microscope (Olympus CX41), the SAMi software records 60 frame one-second videos of the sample and categorises the selected sperm into 4 grades for motility. A – fast progressive (faster than 25 microns per second and linear movement), B – slow progressive (slower than 25 microns per second and linear movement), C – non-progressive (twitching, moving in very small circles), D – immotile (no movement at all). SAMi provides these grade outputs as a percentage of the sample and the number of cells, also concentration in million/ml and velocity in microns/second.

Sperm kinematics were assessed using the coordinates of the paths generated in the CASA, a MATLAB script was used to assess the straight line path (VSL µm/s), the curvilinear path (VCL µm/s) and the linearity of the sperm path (LIN %) LIN = VSL/VCL x100.

Sperm concentration was also measured via haemocytometer. A wet preparation (10 µL semen on a glass slide with a 24 mm x 24 mm coverslip) and a 40X objective were used to initially count sperm in the field of view. Once sperm in the field of view were counted this was used to decide which dilution was appropriate for an accurate calculation of sperm concentration using the following.

Sperm per 400x field	Dilution	Semen (µL)	Diluent (µL)
2-15	1:2	12.5	12.5
16-100	1:5	12.5	50
> 100	1:20	12.5	237.5

The dilutions were made in duplicate using 4% formaldehyde. Using a standard haemocytometer 10 µL of each dilution were placed in each chamber (Figure 15).

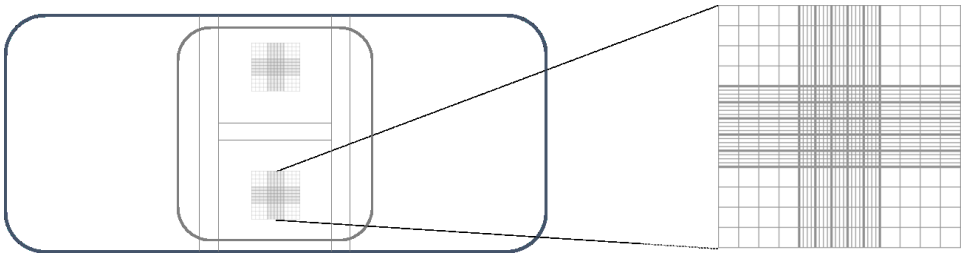

Figure 15. Schematic Haemocytometer.

Full row of the grid were counted until over 200 sperm were counted. This was done for each chamber, if the difference between counts was greater than 10% the chambers were recounted, if the difference was still greater than 10% the dilutions were remade and recounted. The concentration was calculated as follows.

Dilution	Correction factor
1:2	÷ 10
1:5	÷ 4

1:20	÷ 1	

Concentration = $\left(\frac{total\ number\ of\ sperm\ counted}{total\ number\ of\ rows\ counted}\right)$ ÷ correction factor

Clinical samples of semen (80) and follicular fluid (52) were obtained from consenting patients undergoing assisted conception treatment at the Department of Reproductive Medicine, St Mary's Hospital Manchester. All samples were pseudonymised prior to collection.Follicular fluid samples were separated into 500 µL aliquots upon acquisition, snap frozen in liquid nitrogen and stored at -80°C. All semen samples were processed via density gradient, used in *in vitro* assays and/or RNA, DNA or protein was extracted depending on the assay.

2.3 Sperm isolation – density gradient

Density centrifugation was used to select a population, motile and mature sperm with intact DNA for motility experiments (Le Lannou and Blanchard, 1988; Sakkas et al., 2000). Sperm cells were separated from seminal plasma post liquefaction. The density gradient (PureSperm® 100 Nidacon) was initially diluted using the 100% stock and PureSperm® Buffer to create 80% and 40% solutions. The density gradient was prepared by carefully layering 2 mL of 80% phase followed by 2 mL of a 40% phase and 1 mL of semen into a 15 mL falcon tube and centrifuged at 300 x g for 20 minutes (Figure 16). The supernatant was discarded and the pellet was resuspended in 3 mL of PureSperm® Buffer and centrifuged at 300 x g for 5 minutes, the supernatant was discarded, this step was repeated. The pellet was resuspended in 1 mL of PureSperm® Buffer and the washed sample was assessed by CASA.

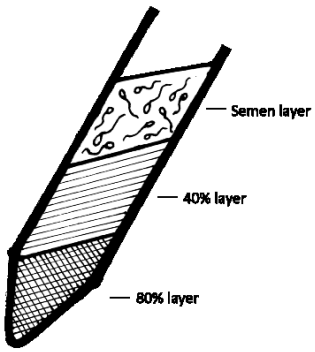

Figure 16. Falcon tube containing 80%, 40% density gradient layers and semen layer.

2.4 Cell culture and in vitro assays

All cell lines used were cultured using Class II safety cabinets and direct heat 5% CO_2 incubators at 37°C. All somatic cells were cultured using standard cell culture media: Dulbecco's modified eagle's medium (DMEM), 10% foetal bovine serum (FBS) with 1% L-glutamine and 1% penicillin-streptomycin. Standard cell culture technique was used to grow cells in either 6 well plates, T25 flasks or T75 flasks. Any *in vitro* assays for somatic cell lines were performed using standard cell culture media (Jones, 1998). Somatic cells were frozen using 10% dimethyl sulfoxide (DMSO) in standard cell culture media in 2 mL cryovials, placed in Mr. Frosty freezing container at -80°C overnight before transferring to liquid nitrogen for long-term storage.

For *in vitro* assays sperm were incubated using a sperm specific buffered Earle's balanced salt solution.

For incubation with neuropeptides various concentrations of neuropeptide agonists (10 pM, 10 nM, 10 µM or appropriate volume of vehicle for control) were added to 10 million sperm in 1 mL of media and incubated for 60 minutes in a 37°C 5% CO_2 direct heat incubator. Motility was measured via CASA post incubation and samples were used for protein extraction directly after for use in western blotting.

2.4.1 Capacitation

Sperm were incubated for 3 hours in Earle's balanced salt solution with 3% bovine serum albumin (BSA) in a 37°C direct heat 5% CO_2 incubator as previously described (Castillo et al., 2019).

2.5 Immunocytochemistry

For sperm, the sample (10 µL) was smeared onto a glass slide using the feathering technique and left to air dry. To fix the sample the slide was immersed in ice-cold methanol or 4% formaldehyde for 10 minutes and air dried. The slide was either used directly for immunocytochemistry or stored at -20°C until required.

Slides that were used from storage in -20°C were left to thaw at room temperature and placed in a humidity chamber for prior to staining. The perimeter or necessary section of the slide was drawn around using a hydrophobic PAP pen and were rehydrated in phosphate buffered saline (PBS) for 10 minutes. Slides were permeabilised in 0.1% PBS-Tween (v/v) for a further 10 minutes. Blocking buffer (PBS-Tween 0.1% with 10% goat serum) was added to the slides and slides were then replaced into the humidity chamber and covered with the lid and incubated for 1 hour at room temperature. The anti-vasopressin receptor 2 primary antibody (Anti-AVPR V2 - ab188748) was diluted (1:500) in blocking buffer and 200 µl was added to the slides or 200 µl of blocking buffer alone for no primary control. Slides were incubated at 4°C overnight. This was followed by washing the slides three times in 0.1% PBS-Tween for five minutes. Slides were then incubated with Alexa fluor® 488 goat anti-rabbit IgG secondary antibody (Invitrogen, A-11008) at a 1:500 dilution in PBS-Tween for 1 hour at room temperature. Slides were washed three times with PBS-Tween. VECTASHIELD® mounting medium with DAPI was added to each slide then covered with coverslips and sealed with an enamel sealant (Kricka, 2001). Appropriate controls were used to ensure there was no cross reaction with antibodies. Slides were incubated for 30 minutes at room temperature prior to imaging with a fluorescent microscope (Zeiss Axio Imager Z1/Leica DFC 365 FX).

2.6 Protein extraction and quantification (sperm, somatic cells)

Radioimmunoprecipitation assay (RIPA) (50 mM Tris base adjusted to pH 8 (0.607g), 150 mM sodium chloride (NaCl) (0.292g), 0.5% Sodium deoxycholate (0.5g), 0.1% sodium dodecyl sulphate (SDS) (0.1g), 1% Triton X-100 in 100 mL distilled water (dH_2O) lysis buffer was used for protein extraction from somatic cells (Six and Kasel, 1978). Suspension cells were pelleted prior to addition of lysis buffer, for adherent cells lysis buffer was added directly to flask or plate. Per 10 million cells, 1 mL of RIPA with 1:100 (v/v) protease inhibitor cocktail and 1:100 (v/v) phosphotase inhibitor cocktail was used. Cells were resuspended several times to ensure homogenisation and vortexed for 5 minutes. Protein samples were stored in -20°C.

Sperm protein extraction was performed using a modified protocol adapted from Lefievre et al., (2002). The sperm pellet following density gradient centrifugation was resuspended in 1 mL PBS, centrifuged at 300 x g for five minutes and the supernatant discarded, this was

repeated. The sperm pellet was resuspended in 300 µL solubilisation buffer (2% sodium dodecyl sulphate, 10% glycerol, 1.4% dithiothreitol, 62.5 mM Tris-HCL, pH 6.8 with phosphatase and protease inhibitor cocktail at 1:100). The lysate was vortexed for five minutes until the pellet was thoroughly solubilised. Lysate was boiled using a dry heat block at 100°C for 5 minutes. The sperm lysate was centrifuged at 15000 x g for 15 minutes at 4°C. The supernatant (protein) was transferred to a fresh Eppendorf tube and the pellet was discarded. The lysate was diluted with 100% ethanol at 1:9 (lysate : ethanol) ratio and incubated at -80°C for 2 hours minimum. Post incubation the lysate was centrifuged at 15000 x g for 30 minutes at 4°C. The ethanol was removed via decanting and the pellet was left to dry for 5 – 10 minutes at room temperature. Once all the ethanol had evaporated the protein pellet was resuspended in 2% SDS and stored at -20°C for use in downstream applications.

All proteins were quantified using the bicinchonic acid assay (BCA) (Pierce™ BCA protein assay kit), which is a colourmetric assay that determines total protein concentration via comparison of colour changes in the known standards and the unknown samples. Protein standards were made using the manufacturers recommendations. Two reagents were provided in the kit, reagent A was mixed with reagent B at 50:1 (v/v) (A:B), known as the working reagent. In a flat bottom 96 well plate 10 µL of standard or sample was pipetted into each well and 200 µL of the working reagent was added to each well containing standard or sample. The 96 well plate was incubated at 37°C for 30 minutes and then measured using a microplate reader at 562 nm absorbance. A standard curve was plotted using Microsoft® Excel software and equation from curve was used to quantify unknown proteins using $y=mx+c$.

2.7 Dot blot

Protein samples were loaded onto a nitrocellulose membrane by pipetting 2 µL as evenly spaced “spots” and left to air dry for 15 minutes. The membrane was blocked using 5% BSA in TBS-Tween (Tris buffered saline with Tween; 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% tween) (5% bovine serum albumin in 0.1% TBS-T v/v) for 1 hour at room temperature under agitation. The blocking buffer was discarded, and Anti-Aquaporin 2 antibody (ab62628) was added to the membrane, diluted in blocking buffer (1:1000). The membrane was incubated at 4°C overnight under constant agitation.

The following day the primary antibody was removed, and the membrane was washed three times in 0.1% TBS-Tween for 5 minutes. The horseradish peroxidase (HRP) conjugated secondary antibody was diluted in blocking buffer at 1:1000 and was added to the membrane. The membrane was incubated at room temperature for 1 hour under agitation.

The solution was discarded, and the membrane was washed three times in 0.1% TBS-Tween for five minutes. Electrochemiluminescence (ECL) solution was used to develop the membrane, ECL solution A and B were mixed at 1:1 to create the working solution. The membrane was placed on the imaging tray with the protein side up. ECL was added to the membrane, enough to cover the surface and left to develop at room temperature for 1 minute (Stott, 2000). The ECL was drained off the membrane prior to imaging with chemiluminescence on the ChemiDoc™ Touch Gel and Western Blot Imaging System.

2.8 Western blotting

Gel casting glass plates (1 mm) were assembled on the casting stand according to manufacturer's instruction. The Bio-Rad TGX™ FastCast™ Acrylamide Kit was used to make 10% acrylamide gels for SDS-PAGE. For two 10% acrylamide gels 3 ml of Resolver A, 3 mL resolver B, 30 µL ammonium persulfate (APS), 3 µL tetramethylethylenediamine (TEMED) were combined in a falcon tube. In a separate falcon tube 1 mL of Stacker A, 1 mL of stacker B, 10 µL APS and 2 µL TEMED were combined. The resolver solution was pipetted into the glass plates until around 1 cm below the top of the plate. The stacker solution was pipetted on top of the resolver solution in the glass plates until it reached the rim and a 10 well comb was inserted. The gels were left to polymerise for 30 minutes prior to electrophoresis.

When the gels had polymerised they were removed from the casting stand and placed into a gel tank. The gel tank was filled with electrophoresis buffer until the marker on the tank (25 mM tris, 190 mM glycine, 0.1% SDS) and the well combs removed. The protein samples were diluted in 4X laemmli buffer (8% SDS, 40% glycerol, 0.008% bromophenol blue, 0.25 M Tris-HCL, pH 6.8) with 5% β-mercaptoethanol. The protein samples were denatured at 100°C for five minutes using a dry heat block. The standard concentration of protein loaded was 25 µg per well unless otherwise stated as well as a protein ladder. A power pack was

connected to the gel tank and ran at 120V until the dye front reached the bottom of the gel.

Once the gel had finished running the glass plates were opened and the stacking gel was removed and discarded. A container with transfer buffer (25 mM tris, 190 mM glycine, 20% methanol) was used to soak 8 pieces of blotting paper per gel, and the nitrocellulose membrane for 5 minutes. A 'sandwich' was assembled on the semi-dry transfer system, 4 pieces of blotting paper, the nitrocellulose membrane, the acrylamide gel and finally another 4 pieces of blotting paper. Each time a layer was added a roller was used to remove any air bubbles. The lid was carefully placed on top and the system was connected to the power pack and ran at 10V(400 mA) for 1 hour.

When the transfer was complete the membrane was washed for 1 minute in Tris buffered saline with Tween (TBS-Tween) (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% tween) and the rest of the 'sandwich' was discarded. The membrane was incubated for 1 hour at room temperature in blocking buffer (5% bovine serum albumin in 0.1% TBS-T v/v) under agitation. The blocking buffer was discarded and the primary Anti-Aquaporin 2 antibody (ab62628) was added to the membrane, which was diluted in blocking buffer at 1:1000. The membrane was incubated at 4°C overnight under constant agitation.

The following day the primary antibody was removed and the membrane was washed three times in 0.1% TBS-Tween for 5 minutes. Horseradish peroxidase (HRP) conjugated secondary antibody was diluted in blocking buffer at 1:1000 unless and added to the membrane. The membrane was incubated at room temperature for 1 hour under agitation.

The secondary antibody was discarded and the membrane was washed three times in 0.1% TBS-Tween for five minutes. Electrochemiluminescence (ECL) solution was used to develop the membrane, ECL solution A and B were mixed at 1:1 to create the working solution. The membrane was placed on the imaging tray with the protein side up. ECL was added to the membrane, enough to cover the surface and left to develop at room temperature for 1 minutes. The ECL was drained off the membrane prior to imaging with chemiluminescence on the ChemiDoc^{TM} Touch Gel and Western Blot Imaging System.

Membranes were washed three times with 0.1% TBS-Tween for five minutes and incubated with an appropriate loading control antibody using the same process as described above (Mahmood and Yang, 2012).

2.9 DNA extraction

For sperm DNA extraction the Isolate II Genomic DNA Kit (Bioline) was used with a few modifications to the manufacturer's protocol. Per each extraction 20 million sperm cells were used. The pellet was resuspended in 360 µL Lysis Buffer GL, 50 µL Proteinase K solution (1 mg/mL), 3.8 µL RNase A (10 mg/mL) and 50 µL DTT (5mM) and incubated at 56 °C under agitation for 3 hours. The sample was vortexed and 400 µL of Lysis Buffer G3 was added, vortexed vigorously and incubated at 70 °C for 10 minutes under agitation. The sample was vortexed and 420 µL of absolute ethanol was added and vortexed vigorously. An Isolate II Genomic DNA spin column was placed into a 2 mL collection tube. A maximum of 500 µL of the sample was loaded into the spin column, centrifuged for 1 minute at 11000 x g and flow through was discarded. This step was repeated until the full volume of sample had passed through the spin column then 500 µL of Wash Buffer GW1 was added to the spin column and centrifuged for 1 minute at 11000 x g. The flow through was discarded and 600 µL of Wash Buffer GW2 was added to the spin column and centrifuged for 1 minute at 11000 x g. The flow through was discarded and the spin column was centrifuged for 1 minute at 11000 x g then left to air dry for 1 minute at room temperature to remove any residual ethanol. The spin column was placed in a 1.5 mL microcentrifuge tube and 100 µL of pre-heated Elution Buffer G (70 °C) was added onto the centre of the silica membrane. The spin column was incubated at room temperature for 1 minute then centrifuged for 1 minute at 11000 x g. The DNA concentration and purity of the extract was determined using NanoDrop. All DNA was stored at -20°C.

2.9.1 Bisulphite conversion of DNA

The Epitect® Bisulphite kit (Qiagen) was used for all bisulphite conversion of DNA. The buffers in the kit were prepared to manufacturer's instructions prior to use. The DNA to be converted was thawed and 250 ng was used in each reaction (volume variable), the Bisulphite Mix was reconstituted using 800 µL per each aliquot and vortexed thoroughly. Each bisulphite reaction was prepared in 0.2 mL microcentrifuge tubes as shown in Table 7.

Table 7. Bisulphite reaction components.

Component	Volume (per reaction)
DNA solution (250 ng)	Dependant on concentration
RNase-free water	Dependant on volume of DNA (use water to make up to 20 µL)
Bisulphite mix	85 µL
DNA protect buffer	35 µL

The tubes were mixed thoroughly until the solution had turned from blue to green which signified correct pH and adequate mixing. The tubes were then placed in the thermal cycler (Agilent) and incubated with the settings in Table 8.

Table 8. Thermal cycler conditions for bisulphite conversion.

	Temperature °C	Time
Denaturation	95°C	5 minutes
Incubation	60°C	25 minutes
Denaturation	95°C	5 minutes
Incubation	60°C	85 minutes
Denaturation	95°C	5 minutes
Incubation	60°C	175 minutes
Hold	20°C	-

Once the conversion was complete the reactions were transferred to 1.5 mL Eppendorf tubes and 560 µL of fresh buffer BL containing 10 µg/mL carrier RNA was added to each reaction and vortexed. Each reaction was then transferred to an EpiTect spin column and collection tube and centrifuged for 1 minute at maximum speed. The flow through was discarded and 500 µL buffer BW was added to each spin column and centrifuged for 1

minute at maximum speed. The flow through was discarded and 500 µL buffer BD was added to each spin column and incubated for 15 minutes at room temperature. After incubation the tubes were centrifuged for 1 minute at maximum speed. The flow through was discarded and 500 µL buffer BW was added to each reaction and centrifuged at maximum speed for 1 minute. The flow through was discarded and the previous step was repeated once more. The spin columns were placed in new collection tubes and centrifuged for 1 minute at maximum speed. The spin columns were then placed in 1.5 mL Eppendorf and incubated for 5 minutes at 56°C with the lids open. The spin columns were placed in new 1.5 mL Eppendorf tubes and 20 µL buffer EB was added to the centre of the membrane in each spin column. The spin columns were centrifuged at 15000 x g and converted DNA was stored in -20°C until use.

2.10 PCR

All primers used were designed using the Primer3 Plus software by NCBI for use with BLAST. Not all primers were able to span an exon-exon junction therefore; RNA was treated with RQ1 RNase-free DNase (Promega) prior to reverse transcription. The digestion reactions were set up in 200 µL microcentrifuge tubes using 1 unit of RQ1 RNase-free DNase per microgram of RNA, for less than 1 microgram of RNA 1 unit of DNase was also used. The reaction was to the manufacturers recommendations as follows:

RNA (in water or TE buffer)	1-8 µg
RQ1 RNase-free DNase 10X reaction buffer	1 µL
RQ1 RNase-free DNase	1 u per µg of RNA
Nuclease-free water to a final volume of	10 µL

The reactions were incubated at 37°C for 30 minutes. Following this 1 µL of RQ1 DNase stop solution was added to each reaction and incubated at 65°C for 10 minutes using a dry heat block. The DNase treated RNA was then used directly for reverse transcription. Any treated RNA that was not used was stored at -80°C.

Reverse transcription was performed using the GoTaq® 2-step RT-qPCR system (Promega). The following reactions were combined on ice using manufacturer's recommendations:

RNA template (up to 5 µg per reaction)	1 – 5 µL
Oligo(dT)$_{15}$ Primer (0.5 µg/µl)	1 µL
Random Primers (0.5 µg/µl)	1 µL
Nuclease-free water	To a final volume of 10 µL

Using Agilent's SureCycler 8800 thermal cycler the reactions were incubated at 70°C for 5 minutes, then at 4°C for 5 minutes and briefly centrifuged to return contents to the bottom of the tubes. A reverse transcription mix was prepared per reaction to manufacturer's recommendations:

Nuclease-free water	1.5 µL
GoScript™ 5X reaction buffer	4 µL
$MgCl_2$	2 µL
PCR nucleotide mix	1 µL
Recombinant RNasin® ribonuclease inhibitor	0.5 µL
GoScript™ reverse transcriptase	1 µL
Final volume	10 µL

The 10 µL of reverse transcription reaction mix was added to each 10 µL RNA reaction tube from the previous step to a final volume of 20 µL. To synthesise the cDNA Agilent's SureCycler 8800 thermal cycler was used, the reactions were incubated at 25°C for 5 minutes, 42°C for 1 hour, and finally 70°C for 15 minutes. All cDNA was either used immediately for PCR or stored at -20°C until required.

All custom primers and cDNA were checked and optimised via endpoint PCR prior to use in quantitative PCR (qPCR) using the HotStarTaq® PCR kit (Qiagen).

Using manufacturer's recommendations, the reactions were set up as follows (per 1 reaction):

10X PCR buffer	2 µL
dNTP mix (10 mM)	0.4 µL
Primer Mix (forward and reverse)	4 µL
HotStarTaq DNA polymerase	0.1 µL
Template (either water or cDNA)	< 1 µg/100 µL reaction (usually 1 µL)
Nuclease-free water	To a final volume of 20 µL

The cycle was performed using Agilent's SureCycler 8800 thermal cycler. The reactions were incubated at 95°C for 15 minutes then denatured at 94°C for 1 minute, annealed on a gradient of 55°C - 65°C for 1 minute and extended 72°C for 1 minute, the previous 3 steps were cycled 30 times (unless otherwise stated), after the reactions were incubated at 72°C for 10 minutes. PCR product was used immediately for agarose gel electrophoresis or stored at -20°C for later.

All agarose gels were 2% agarose (w/v) in 1X Tris Borate EDTA (TBE) buffer (0.89 M tris base, 0.89 M boric acid, 0.02 M EDTA). The agarose/TBE mixture was heated in a microwave for approximately 1-2 minutes until the agarose had dissolved fully. Midori green (GENEFLOW) was used for the DNA intercalating agent and was added to the agarose gel prior to casting. The agarose gel mixture was poured into the gel casts (comprised of a cassette and a comb) while still hot and was left at room temperature for 30 minutes to solidify. Once the gels had solidified, the gel combs were carefully removed. The gels were placed into a horizontal electrophoresis tank and covered with 1X TBE buffer. The appropriate volume of 5X DNA loading buffer blue (Bioline) was added to each PCR product reaction. The samples were loaded on the gel alongside Hyperladder™ 25bp or 50bp depending on the expected amplicon size of the primers and the gel was ran until the dye front reached the end of the gel, usually at 80V for 1 hour. The gels were imaged using "GelGreen" setting on ChemiDoc™ Touch Gel and Western Blot Imaging System.

2.11 Pyrosequencing

All pyrosequencing primers were designed using PyroMark Assay Design software. The region of interest was chosen using UCSC Genome Browser and input into the PyroMark Assay Design software which designed appropriate primers.

PCR was performed using the relevant bisulphite converted DNA (conversion protocol: methods 2.10.1) and relevant PCR product was used downstream in the pyrosequencing.

Prior to beginning the heat block was set to 85°C and the cartridge to be used was washed with warm water by pushing it through each segment.

The troughs in the PyroMark Q24 vacuum work station were filled with approximately 50 mL of the appropriate solutions; 1 – 70% ethanol, 2 – Denaturation solution (0.2 M Sodium hydroxide), 3 – Wash buffer (10 mM Tris-Acetate, pH 7.6), 4 – Distilled water, 5 – Distilled water (Figure 17).

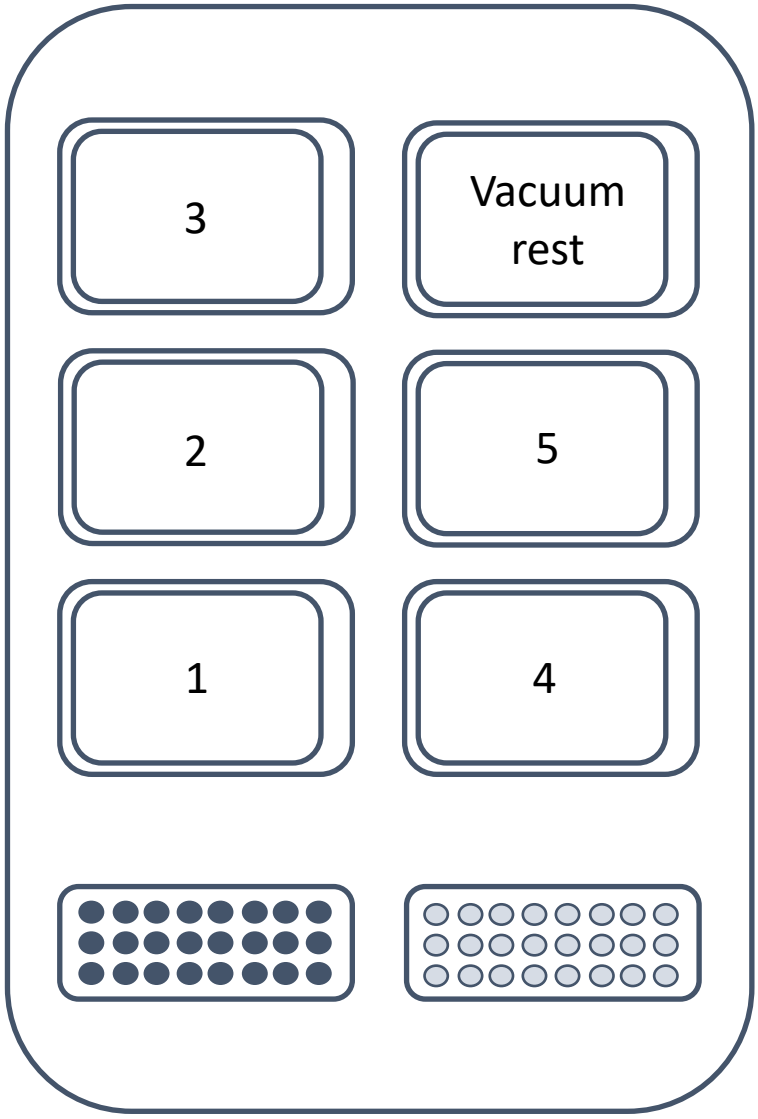

Figure 17. Schematic of the PyroMark Q24 Vacuum Workstation.

The reagents were left to equilibrate to room temperature prior to use. A master mix was made as demonstrated in Table 9 and 70 µL was aliquoted per well in the plate.

Table 9. Pyrosequencing master mix.

	Per reaction
Sepharose beads	1 µL
Binding buffer	40 µL
Nuclease free water	29 µL

The PCR product (10 µL) was added to the master mix to a total of 80 µL. The plate was sealed and placed onto a plate shaker for a minimum of 10 minutes. The sequencing primer was diluted to 0.3 µM (2 µL of 100 µM primer stock in 648 µL annealing buffer) and vortexed thoroughly. The primer solution was aliquoted (25 µL) into each well of the PyroMark Q24 Plate and this was then placed in the correct slot in the Workstation (below trough number 4). Once the 10 minutes shaking were complete the handheld vacuum was turned on and used to aspirate the samples from the plate for approximately 15 seconds until there was no liquid left, immediately the vacuum was placed in the 70% ethanol (Trough 1, Figure 17) and left to aspirate for 5 seconds, then moved to the denaturation buffer (Trough 2, Figure 17) for a further 5 seconds, then finally to the wash buffer (Trough 3, Figure 17) for 10 seconds. While still on the vacuum was inverted to remove surplus wash buffer. To deposit the samples the still turned on vacuum was carefully held above the PyroMark Q24 plate in position, then turned off before being placed into the PyroMark Q24 plate. The vacuum was shaken from side to side for 10 seconds to allow the samples to deposit into the wells and placed in trough 4 (Figure 17) to wash. The PyroMark Q24 plate was heated to 80 degrees for 2 minutes and loaded immediately into the pyrosequencer. The cartridge was loaded with the appropriate amount of sequencing reagents which is calculated by the PyroMark Q24 Advanced software during set up, then inserted into the pyrosequencer, the appropriate sequence was loaded and ran.

2.12 Acrosome reaction assay

Prior to staining all sperm were obtained and separated from the seminal plasma using density gradient centrifugation as described in sections 2.3 and used at a concentration of 10 million/mL. A slide of pre-treated sperm (10 µL) was prepared using the feathering method per sample. Sperm were capacitated using a modified Earle's balanced salt solution media and 3% (w/v) bovine serum albumin (BSA) for 3 hours at 37°C in a direct heat 5% CO_2 incubator. Once capacitated the sperm were further treated with the following; a vehicle (DMSO), 10 µM progesterone, 10 µM calcium ionophore A23187, 10 µM vasopressin, 10 µM desmopressin for 1 hour. The treated sperm were washed with modified Earle's balanced salt solution media three times prior to further use to remove traces of BSA.

2.12.1 FITC PSA

Slides of all the treatments were made in duplicate using the feathering method and air-dried or sperm were fixed in 4% paraformaldehyde (PFA), smeared and left to air dry. Slides that were air dried alone were fixed using ice-cold methanol for 10 minutes. The slides were stained in a humidity chamber, the fluorescein isothiocyanate-pisum sativum agglutinin (FITC-PSA) stain was used at a 2 µM final concentration in PBS. The FITC-PSA stain was added to the slides, the slides were covered with the lid and incubated at 4°C for 1 hour (World Health Organization, 2010a). Slides were washed under gently running dH_2O for 5 minutes each to ensure adequate removal of unbound stain. The slides were mounted with 1 drop of VECTASHIELD® with 4',6-diamidino-2-phenylindole (DAPI) mounting medium and covered with a glass coverslip. The perimeter of the coverslip and slide were sealed using an enamel sealant and incubated for a further 30 minutes before imaging with a fluorescent microscope (Zeiss Axio Imager Z1/Leica DFC 365 FX). Sperm that showed a complete staining of the acrosome were classed as not acrosome reacted, sperm that showed staining as linear across the middle of the head were classed as acrosome reacted. Images were analysed post acquisitionally.

2.12.2 Scanning electron microscopy (SEM)

Silicon wafers were cut into 1 cm x 1 cm and placed into the wells of a 48 well plate. The wafers were coated in poly-d-lysine (1 mg/mL) for 10 minutes, the poly-d-lysine was aspirated and the wafers were washed with dH_2O three times, covered and left to dry in a heated drying cabinet. The samples were placed onto the silicon wafers (50 µL) and left to adhere for 10 minutes. The supernatant was aspirated and discarded and the wafers were washed with PBS three times for 5 minutes. The samples were fixed onto the wafers in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The wafers were then washed 3 times with dH_2O before being dehydrated in an ethanol gradient (20%, 40%, 60%, 80% and 100%) for 30 minutes at each dilution, twice at 100%. The wafers were dried overnight in a vacuum-assisted desiccator and then mounted onto 12.5 mm diameter aluminium SEM specimen stubs (Agar Scientific) using 12 mm carbon tabs (Agar Scientific). The samples were sputter coated using gold and imaged via scanning electron microscopy using Supra 40VP and SmartSEM software (Carl Zeiss Ltd, Germany).

2.13 Calcium assay

Calcium assays were performed on consenting donors' sperm. Prior to assay all sperm were obtained and separated from the seminal plasma using density gradient centrifugation as described in sections 2.3 and used at a concentration of 10 million/mL. The calcium indicator Fluo-4 AM (ex/em of Ca^2 bound form: 494/506) was used to measure calcium in sperm following the addition of agonists. Post density gradient and washing the sperm were incubated in a calcium free media (Dulbecco's PBS, no calcium, no magnesium) with 0.2% (w/v) pluronic F-127 and 4 µM Fluo-4 AM for 45 minutes in the dark at 37°C in a direct heat 5% CO_2 incubator. The sperm were centrifuged at 300G for 5 minutes and the supernatant was discarded, they were resuspended in a modified Earle's balanced salt solution and the centrifugation step was repeated. Sperm were resuspended in a modified Earle's balanced salt solution at 10 million/mL. Sperm calcium changes were measured either via microplate and live cell imaging.

2.13.1 Microplate fluorescence assay

The fluo-4 AM loaded sperm were aliquoted (100 µL) into a microplate or fluorescence-based assays, 96-well (ThermoFisher scientific) which was precoated with poly-D-lysine (Sigma-Aldrich). BioTek® SYNERGY HT Microplate Reader was used to measure changes in sperm calcium at 488 nm using a kinetic assay sequence over the period of 10 minutes and 45 seconds (unless otherwise stated). The background fluorescence was measured over 45 seconds prior to the addition of agonists or a vehicle control of DMSO (10 µM calcium ionophore A23187, 10 µM vasopressin, 10 µM desmopressin (dDAVP) and 10 µM oxytocin) all were performed in duplicate.

2.13.2 Live cell calcium imaging

Live cell calcium imaging was performed in collaboration with the University of Birmingham. Sperm samples were acquired from consented males at Birmingham Women's Hospital (HFEA Centre 0119), under NRES REC Reference: 18/EM/0223. Semen samples were liquefied at 37°C for 30 minutes in a direct heat 5% CO_2 incubator and sperm were separated from seminal plasma using the swim-up technique as described in the WHO

2010 manual for semen analysis, in brief, 1 mL of semen was placed beneath 2 mL of supplemented earle's balanced salt solution with 3% BSA and incubated at an angle of approximately 45° at 37°C for 30 minutes in a direct heat 5% CO_2 incubator, the layer of media was then removed and this fraction contained the sperm to be used downstream. Sperm were diluted to 6 million/mL and capacitated for 6 hours prior to experiment. Sperm were loaded with calcium indicator as follows; 150 µL sperm with 10 µM calcium green 1AM (Thermofisher, C3011MP) for 30 minutes in 37°C in a direct heat 5% CO_2 incubator. The calcium indicator loaded sperm were placed in a RC-20 imaging chamber (Warner instruments) on a glass cover slip treated with 0.1% poly-L-lysine and incubated for a further 30 minutes in 37°C in a direct heat 5% CO_2 incubator in order to adhere to the coverslip. The sperm were imaged in time-lapse with the image taken every 3 seconds for a duration of 8 minutes in total, initial 4 minutes were a control period with just media flow over the cells, the following 2 minutes were treatment with either 10 µM vasopressin or 10 µM dDAVP, the final 2 minutes were treatment with 500 nM progesterone. A peristaltic pump was used to perfuse treatments across the surface of the coverslip in the chamber and another removed the treatments simultaneously. MetaMorph® software was used for analysis of time-lapse data, the region of interest were drawn around each sperm cell and the average fluorescence intensity values per frame were exported to excel for statistical analysis.

2.14 Enzyme linked immunosorbent assay (ELISA)

ELISA were performed to measure the levels of vasopressin, oxytocin and TNFα in human samples following the manufactures protocols.

To measure vasopressin levels in semen and follicular fluid, the Arg^8-Vasopressin ELISA Kit (ab205928) was used. All reagents and standards were prepared as instructed in the assay protocol. Briefly; standard of vasopressin were prepared from stock solution providing a rage from 4.1 pg/mL to 1,000 pg/mL in to Assay Buffer. Before use, all material and reagents were equilibrated to room temperature. Semen and follicular fluid were diluted in 1:3 in Assay Buffer. All samples and standards and controls were assayed in duplicate. The plate was set up as per manufacture's protocol, with 100 µL of Assay Buffer added to the control wells (non-specific binging and blank) - 100 µL of the standards were added to the appropriately labelled wells, 100 µL samples were added to the appropriate wells and 50

µL Vasopressin Biotin Conjugate was added to each well (except the blank wells). This was followed by adding Vasopressin polyclonal rabbit Antibody to all the wells (except the non-specific binding and blank). The plate was then tapped gently to mix, sealed and incubated at 4°C for 18-24 hours.

The plate was washed with wash solution (400 µL) X 3 times. After removing all the wash solution, 200 µL of SA-HRP was added to each well (except the blank wells). The plate was sealed and incubated at room temperature on a plate shaker for 30 minutes (~500 rpm), after which, it was washed as above. After incubation a stop solution was added to every well and the absorbance (optical density) was measured immediately at 405 nm, using BioTek® SYNERGY HT Microplate Reader.

For measurements of oxytocin in semen and follicular fluid Oxytocin ELISA Kit (ab133050) was used using the manufactures protocol. Standard of oxytocin were prepared from stock solution providing a rage from 15.6 pg/mL to 1,000 pg/mL in to diluent. As above, all reagents and materials were equilibrated at room temperature. Samples of semen and follicular fluid were diluted 1:3 in assay buffer before use.

The assay procedure was carried out as per protocol instructions, and similar the vasopressin ELISA. All standards and samples were assayed in duplicate. The Oxytocin-alkaline phosphatase conjugate and Oxytocin antibody provided by the kit were used.

As per the vasopressin ELISA - the absorbance (optical density) was measured immediately at 405 nm, using BioTek® SYNERGY HT Microplate Reader.

For measurements of TNFα in follicular fluid, the Human Tumor Necrosis Factor α ELISA Kit (SIGMA -RAB0476) was used. The assay was preformed using the manufacture's protocol. Standards were prepared from stock solution providing a rage from 24.58 pg/mL to 6,000 pg/mL in to Assay Diluent. 100 µL of each standard and sample was added into appropriate wells. The plate was covered and incubated incubate for 2.5 hours at room temperature at 4 °C with gentle shaking. After discarding the solutions, each well was washed 4x with Wash Solution. 100 µL of 1x prepared Biotinylated Detection Antibody was then added to each well and incubated for 1 hour at room temperature with gentle shaking, after which the solution was discharged and the wells washed with Wash Solution. 100 µL of prepared HRP-Streptavidin was added solution to each well and incubated for 45 minutes at room temperature with gentle shaking. After discarding the solution and washing as above 100

µL of ELISA Colorimetric TMB Reagent was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. This was followed by adding 50 µL of Stop Solution to each well and read at 450 nm immediately using BioTek® SYNERGY HT Microplate Reader.

2.15 Seahorse XFp metabolic analysis

The cell mito stress test starter kit (Agilent 103708-100) along with the seahorse XFp FluxPak (Agilent 103022-100) were used to determine sperm oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The OCR and ECAR allow for the determination of further parameters of mitochondrial and non-mitochondrial respiration through the use of three compounds to modulate the function of the electron transport chain.

Oligomycin which inhibits the complex V (ATP synthase), the inhibition of complex V decreases the OCR levels and is related to ATP production within the cell. Carbonyl cyanide-4 trifluoromethoxy pheylhydrazone (FCCP), an uncoupling agent which functions via the collapse of the proton gradient across the mitochondrial matrix and the intermembrane space thus interferes with the membrane potential of the mitochondria allowing electrons to pass freely and fully restores the electron transport chain, this stimulates an increase in OCR which is used to calculate spare respiratory capacity that is indicative of how able the cell is to respond to increased demand for energy or stress. Rotenone and antimycin A (rot/AA) which are complex I and complex III inhibitors respectively these entirely inhibit mitochondrial respiration which allows for the determination of non-mitochondrial dependant respiration, the respiratory processes that are not performed by mitochondria (Figure 18).

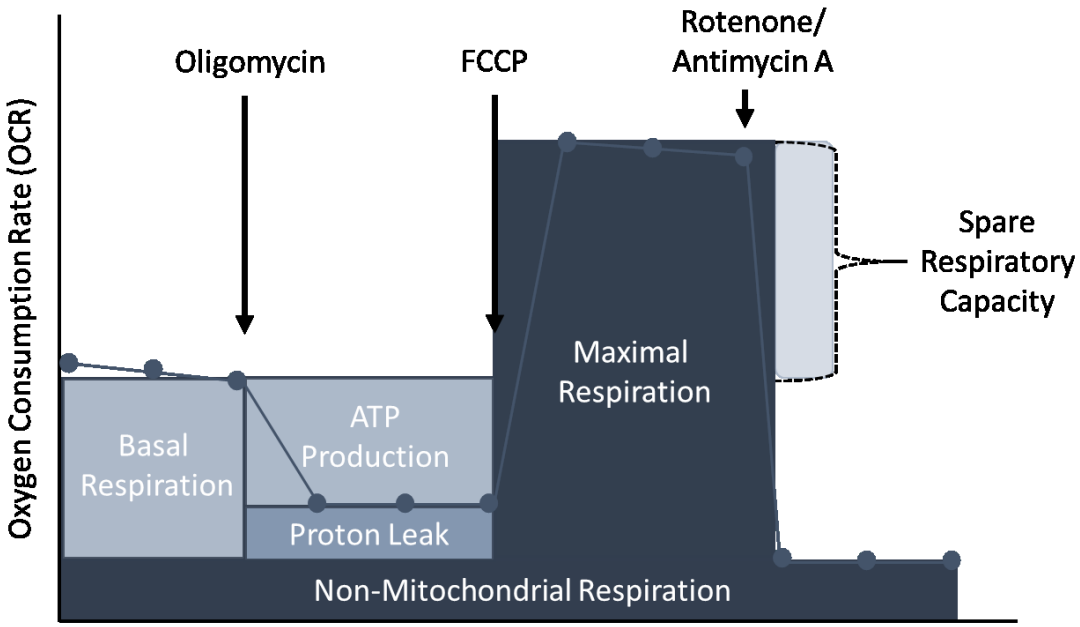

Figure 18. Seahorse schematic. Demonstration of parameter calculations from oxygen consumption rate results. *Adapted from Agilent.*

Sperm were separated from the seminal plasma and washed as per section 2.3. The mini plate was pre-coated with poly-d-lysine prior to use. Sperm were used at a concentration of 200,000 cells per well. The assay was optimised using the titrations recommended by the user handbook, the optimal working concentrations of each compound were determined and were as follows; oligomycin – 1.5 µM, FCCP – 1 µM and rotenone/antimycin A – 0.5 µM.

The day prior to running the cell mito stress test assay there were several preparation steps; the Agilent Seahorse XFp Analyzer was turned on and left overnight to warm up as a minimum of 5 hours is required, the miniplates to be used were coated with poly-d-lysine (0.1 mg/mL) and the sensor cartridge was hydrated in the Agilent Seahorse XF calibrant solution overnight in a non-CO_2 incubator at 37°C.

On the day of the cell mito stress test the assay medium was prepared as follows; DMEM (without phenol red) was supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose. As all reagents were purchased from Agilent and the recommended concentrations were used the pH of the medium was already correct at 7.4. The assay medium was warmed up in a 37°C waterbath prior to use. After the sperm were washed they were resuspended in the seahorse assay medium at 20 million/mL, 100 µL of sperm suspension was added to each well of the miniplate and each well was topped up with 80 µL of assay medium to a final volume of 180 µL per well. The miniplate loaded with sperm

(except for two wells that were used for background adjustments) was degassed in a non-CO_2 incubator at 37°C for 1 hour prior to running the assay.

During the degassing step of the miniplate the stock compounds were prepared with assay medium as follows; oligomycin in 280 µL of assay medium (45 µM), FCCP 288 µL of assay medium (50 µM), Rotenone/Antimycin A in 216 µL of assay medium (25 µM). These stock solutions were used to make 300 µL of the appropriate working solutions as follows; oligomycin stock 100 µL in 200 µL of assay medium (15 µM), FCCP stock 60 µL in 240 µL assay medium (10 µM), rotenone/antimycin A stock 60 µL in 240 µL assay medium. The test compounds vasopressin and dDAVP were prepared to a stock of 100 µM in assay medium, a vehicle control (DMSO) proportional to that used to dilute compounds was also prepared. The modified assay was used therefore 25 µL of each compound was loaded into each port of the sensor cartridge as follows; port A – test compound, port B – oligomycin, port C – FCCP and port D – rotenone/antimycin A. Care was taken not to introduce air bubbles into the ports while loading. The modified 4-port assay was selected on the XFp analyzer and the sensor cartridge was placed into the instrument for calibration. Once calibrated the degassed miniplate containing sperm was loaded into the XFp analyser and the assay was left to run.

Once the assay was over the miniplate was further utilised for normalisation to the protein content of the sperm that had adhered. All assay medium was aspirated and discarded and the sperm protein was extracted as described 2.6 and the protein was interpolated using a BCA assay as described in 2.6.

Data was analysed using the Seahorse Wave software v2.6 and Microsoft Excel 2016.

2.16 Statistical analysis and software used

Microsoft word 2016, Microsoft Excel 2016, Microsoft PowerPoint 2016, Image J, SAMi, Matlab, script was designed by Joshua Brothers. All statistical analysis was performed using IBM SPSS version 25. Seahorse Wave desktop v2.6. Statistical modelling was performed after descriptive analysis of outcome variables. The number of oocytes and number fertilised were count data which were skewed and therefore negative binomial logistic regressions were performed to analyse any relationship between the variables and concentration of neuropeptide concentrations measured. The choice between freeze all

embryos and embryo transfer and clinical pregnancy were both binary variables and therefore binary logistic regressions were used to investigate any relationship between the variables and neuropeptides measured. Semen parameters were all transformed for use in linear regressions with the neuropeptides measured and the oxytocin receptor methylation. *In vitro* assays with vasopressin and dDAVP were statistically analysed using either one way or repeated measures analysis of variance.

Chapter 3 – Investigating neuropeptide levels in follicular fluid and seminal plasma, and clinical outcomes

3.0 Investigating neuropeptide levels in follicular fluid and seminal plasma and clinical outcomes

3.1 Introduction

Infertility affects approximately 1 in 7 couples in the United Kingdom and is defined as the inability to conceive within 12 months of unprotected regular intercourse (World Health Organization, 2009). There are multiple causes to both male and female infertility, and several risk factors, such as; age, BMI, smoking, alcohol, stress and environmental factors such as occupational hazards (Tomova and Carroll, 2018). Although stress is a contributing factor to infertility, infertility itself has also been shown to have detrimental effects on mental health via psychological stress with studies highlighting the negative impact this can on fertility treatment itself (Boivin and Schmidt, 2005; Peterson et al., 2007).

Stress is mediated primarily via the HPA axis, once activated, the paraventricular nucleus in the hypothalamus releases corticotropin-releasing hormone and vasopressin. This stimulates the release of adrenocorticotropic hormone, which induces the production and release of cortisol, adrenal androgens and aldosterone into the bloodstream. The HPA axis functions through a negative feedback where cortisol inhibits further release of corticotropin-releasing hormone and adrenocorticotropic hormone (Stephens and Wand, 2012). Glucocorticoids, such as cortisol, have been previously shown to impact the HPG axis via the direct inhibition of GnRH and indirectly through negatively impacting the Kisspeptin (KISS1) neurons (Takumi et al., 2012; Ubuka et al., 2009). Gluticocorticoids have been shown to have an effect on ovarian function, including oocytes, granulosa cells and luteal cells (Joseph and Whirledge, 2017; Zhang et al., 2016).

3.1.1 Ovulation and oogenesis

Ovulation and oogenesis are regulated by the HPG axis. In the hypothalamus, the pre-optic nucleus and the arcuate nucleus produce GnRH, this stimulates the gonadotropes in the anterior pituitary. Once stimulated the gonadotropes secrete LH and FSH from the anterior pituitary into the bloodstream, which stimulate the ovarian steroidogenesis (Gilloteaux and Coey, 2018).

Ovulation is split into three phases; follicular phase (days 1-14), ovulatory phase (days 14-15), luteal phase (days 15-28).

3.1.1.1 Follicular phase

At birth, the female has all the oogonia (diploid stem cells) required for her reproductive life. During prepubescent childhood oogonia are converted to primordial follicles, which are diploid and arrested in prophase of meiosis 1. Once the female reaches puberty, localised androgens stimulate the primordial follicles into primary follicles. Surrounding the primary follicles is a single layer of granulosa cells. FSH stimulates the conversion of the primary follicle to the early secondary follicle, the granulosa cells proliferate to several layers and secrete an acidophilic glycoprotein layer that becomes the zona pellucida encapsulating the oocyte (Raven, 2013). LH binds to the LH receptor on the thecal cells that lie adjacent to the granulosa cell layer in the follicle. The binding of LH to its receptor initiate an intracellular cascade converting cholesterol into androgens. The granulosa cells become responsive to FSH stimulates the conversion of these androgens into oestrogen using aromatase enzymes. In the late secondary follicle (diploid) LH continues to stimulate the thecal cells to produce androgens and FSH continues to stimulate the granulosa cells to proliferate and secrete small volumes of follicular fluid rich in hyaluronic acid. FSH and LH continue to stimulate the late secondary follicle that matures further to the Graffian follicle (secondary oocyte, haploid, arrested in metaphase II). The volume of follicular fluid increases forming the antrum. Oestrogen has a negative feedback mechanism, high levels in the blood inhibit the hypothalamus from secreting GnRH and the pituitary from secreting FSH and LH. There are two oestrogen peaks in the follicular phase, mid follicular phase it has inhibitory effect, while at the end of follicular phase oestrogen causes a surge of GnRH from the hypothalamus and a surge of LH from the anterior pituitary. The inhibin B from

the Graffian follicle inhibits the anterior pituitary from releasing FSH (Gilloteaux and Coey, 2018).

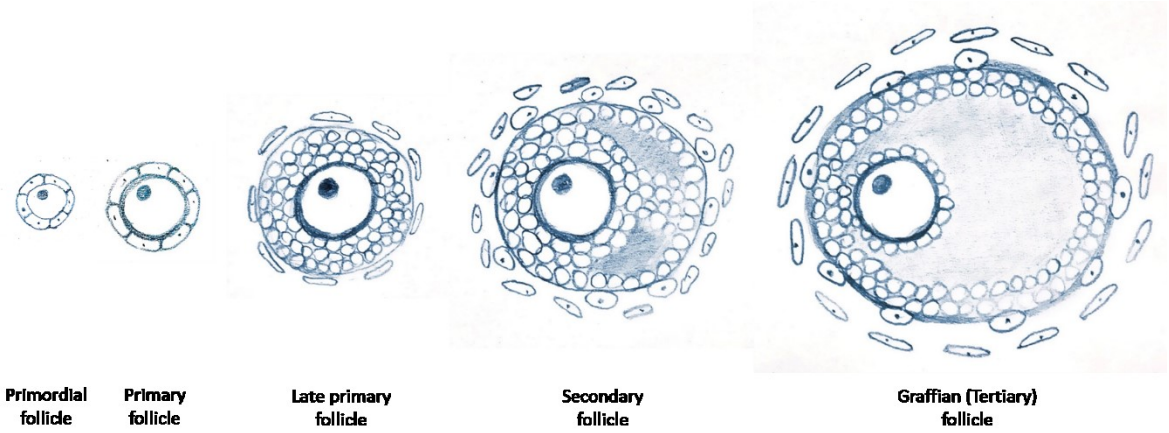

Figure 19. Follicular development in oogenesis.

3.1.1.2 Ovulatory phase

Around day 14 of the menstrual cycle and a day after the LH surge, the follicle bulges from the ovary, the theca externa cells tighten around the Graffian follicle, proteases cause degradation of the membrane and pressure from the follicular fluid causes the follicle wall to rupture. The oocyte-cumulous complex is expelled with the follicular fluid into the abdominal cavity and is captured by the fimbriae cilia cells of the fallopian tube (Gilloteaux and Coey, 2018).

3.1.1.3 Luteal phase

Post ovulation (days 15 – 28) the remainder of the follicle fills with blood and is invaded by connective cells; LH stimulates the ruptured follicle to become the corpus luteum and stimulates the production of progesterone. If no fertilisation occurs within 12 hours of ovulation the oocyte-cumulous complex dies in the fallopian tube and the corpus luteum undergoes degradation becomes fibrous known as the corpus albicans, where local macrophages slowly remove this leaving scar tissue. If fertilisation does occur the corpus luteum provides hormonal support to the embryo and is degraded into a corpus albicans post-pregnancy.

During follicular maturation, follicular fluid is produced during the formation of the follicular antrum. Follicular fluid forms a substantial portion of the volume (can be above 95% in human) of the follicle at maturity (Graafian follicle) and provides an essential

microenvironment for the oocyte development and maturation (Rodgers et al., 2001). The composition of the follicular fluid is affected by the secretory process of the theca interna cells (responsible for androgen and progesterone secretion) and the granulosa cell layer (responsible for oestradiol secretion) (Ambekar et al., 2013; Edwards, 1974). It is has been suggested that follicular fluid is produced by the granulosa cells, which produce hyaluronan and versican (a large chondroitin sulfate proteoglycan) generating an osmotic gradient, which accumulates fluid from the thecal vasculature (Cavender and Murdoch, 1988). Some molecules may cross-link for retention in the follicular fluid the aquaporins in the granulosa cells may be involved in further transport of water into the follicle (Gosden et al., 1988; Rodgers and Irving-Rodgers, 2010; Skowronski et al., 2009).

Follicular fluid is aspirated from the follicle during oocyte retrieval as a routine procedure in Assisted Reproductive Technology (ART), and is readily available as an optimal source of non-invasive biochemical predictors of oocyte quality (Piñero-Sagredo et al., 2010; Yell, 2018). The constituents of follicular fluid are complex and attempts to identify them through proteomics and metabolomics have revealed an extensive list including; hormones, sugars, growth factors of the transforming growth factor-beta (TGF beta) superfamily, further growth factors and interleukins, proteins/peptides and amino acids, prostinoids, reactive oxygen species (ROS), anti-apoptotic factors and many more (Ambekar et al., 2013; Zamah et al., 2015). There have been no clear established prediction marker or combination of markers in follicular fluid for the best oocytes to be utilized in ART (Revelli et al., 2009). Follicular fluid has also been found to have chemoattractant properties towards sperm and triggers the acrosome reaction *in vitro,* implying a role in fertilisation (Suarez et al., 1986; Wang et al., 2001).

Follicular fluid also contains neuropeptides and a small number of studies have explored the levels the vasopressin and oxytocin. Schaffer et al (1984) found the presence of oxytocin and vasopressin in human follicular fluid using radioimmuno assays and showed the levels of both have been found to be 30-fold greater than levels in serum (Schaeffer et al., 1984). Such high levels are suggestive of a paracrine role of these hormones in the regulation of ovarian or fallopian tube functions. However, there is a lack of investigation into the effects of oxytocin and vasopressin in the human follicular fluid and clinical outcomes.

In ART, controlled ovarian stimulation protocols are used to obtain multiple mature oocytes from a treatment cycle and thus increasing chances of pregnancy (Pacchiarotti et al., 2016). The two stimulation protocols used in this cohort of patient were the antagonist protocol and the long down regulation. The long down-regulation stimulation protocol uses a GnRH agonist which is started in the mid-luteal phase to suppress the production of endogenous FSH and to inhibit dominant follicle selection thus allowing for multiple follicles to develop simultaneously, once suppression is confirmed via ultrasound and serum oestradiol concentration (10-14 days of agonist treatment) then gonadotrophin is administered. The antagonist protocol uses a GnRH antagonist post gonadotrophin administration has begun to prevent endogenous ovulation via the immediate suppression of endogenous gonadotrophins and allowing for multiple follicles to develop (Al-Inany and Aboulghar, 2002). These both use a gonadotrophin to stimulate the ovary and trigger growth of multiple dominant follicles. Both protocols end with a final oocyte maturation trigger of human chorionic gonadotrophin (hCG) and oocytes are collected 3 days after (Tsampras and Fitzgerald, 2018).

hCG is also know to stimulate the production of Tumor necrosis factor α (TNFα) in cultured granulosa cells (in combination with colony stimulating factor-1 (CSF)). It has been proposed that hCG may act by inducing CSF receptors with the CSF then responsible for the TNFα increase (Zolti et al., 1990).

TNFα is a prominent cytokine involved with the acute phase reaction in systemic inflammatory response. TNFα is a pyrogen, able to induce apoptosis and has an intense response in the cell stress related JNK pathway. It stimulates the HPA axis and increases corticotropin-releasing hormone, and is associated with multiple human diseases such as cancer, Alzheimer's and depression (Dowlati et al., 2010; Liu, 2003; Locksley et al., 2001; Swardfager et al., 2010).

TNFα is synthesised throughout the female reproductive tract and studies have detected TNFα in the follicular fluid of several species including, humans (Hunt, 1993). Oocytes, granulosa cells, thecal cells, luteal cells, endothelial cells, and macrophages are sources of TNFα. Follicular development, ovulation, and luteal regression are the processes regulating expression of ovarian TNFα. In small developing follicles, TNFα suppresses the responsiveness of the ovary to gonadotropins, whereas in preovulatory follicles, TNF stimulates steroidogenesis (Davis et al., 2003).

TNF-α influences ovarian steriodogenesis, by modulating receptor function of gonadotropins and by modifying ovarian folliculogenesis (Adashi et al., 1989). TNFα has also been shown to be significantly higher with corresponding lower concentrations of oestradiol in the follicular fluid of women with immunological factor infertility when compared women with tubal factor infertility. In this study the women with immunological factor infertility had lower rates of fertilisation (Cianci et al., 1996). Furthermore, a recent study has demonstrated that vasopressin modulates the effects of TNFα in human aortic endothelial cells, acting through OXTR. In this manner, vasopressin may have anti-inflammatory effects (Yang et al., 2019). In porcine corpus luteum oxytocin stimulates oestradiol production and inhibits progesterone, TNFα was shown to have inhibitory effects on both progesterone and oestradiol and TNFα in the presence of oxytocin persisted to have inhibitory effects on both (Pitzel et al., 1993).

The present study sought to investigate any relationship between TNFα, vasopressin and oxytocin in follicular fluid in women undergoing ART.

3.1.2 Seminal fluid

Seminal fluid is the fluid that is mixed with sperm in the ejaculate and is composed of fluids produced in the seminal vesicles, bulbourethral glands and the prostate. Seminal vesicles produce 60-70% of seminal fluid, containing fructose for energy for sperm and other constituents including prostaglandins and coagulase. The bulbourethral glands secretions primarily enter the urethra during arousal as lubrication for the penis for entry into the vagina. The prostate gland secretions contributes approximately 30% of the ejaculate volume. Seminal fluid is comprised of prostaglandins, amino acids, potassium, fructose, citric acid, enzymes, phosphorylcholine, zinc and amino acids (Sulaiman and Coey, 2018).

Prostate gland accounts for around 30% of seminal fluid, provides citrate (energy source), fribrinolysin, prostate specific antigen.

The main function of seminal fluid is to facilitate transportation of the sperm from the testis to the female reproductive tract, and buffer the sperm from the acidic conditions of the vagina. However, seminal plasma may have other functions It has been implicated in the health of offspring in mouse studies, where it was demonstrated that the absence of seminal fluid at conception caused the offspring to have increased fat accumulation, hypertension and metabolism alterations. It is suggested that seminal fluid regulates the

female reproductive tract environment and the inflammatory events involved to ensure optimal support for a developing embryo (Bromfield, 2014).

In addition to several components present in semen – there are a number of neuropeptides that may have functional roles in sperm activity. Oxytocin and vasopressin are synthesised in the hypothalamus and secreted from the posterior pituitary. Furthermore, the receptors for both neuropeptide are expressed throughout the male and female reproductive tracts. Oxytocin's involvement in reproduction is well documented and is commonly associated with sperm transfer, pregnancy, birth, lactation, maternal behaviour and bonding (Anthony W Norman and Helen L Henry, 2015). Vasopressin regulates water/salt balance and blood pressure, but is also involved in human social behaviour, maternal aggression and stress response through stimulating the release of adrenocorticotropic hormone (ACTH) which in turn stimulates the release of cortisol (Caldwell and Young III, 2006; Gibbs, 1986; Robertson, 1977). Vasopressin has been associated with depression in both humans and animal models and maternal depression has been implicated in negative clinical outcomes in women undergoing fertility treatments (Murgatroyd et al., 2004; Rotzinger et al., 2010; Smeenk et al., 2005; Thiering et al., 1993). Vasopressin has been previously detected in human semen at levels similar to those in plasma (~1.84 pg/mL) (Brotherton, 1990). Oxytocin has also been detected in human semen (~1.28 – 1.72 pg/mL). However, no statistically significant correlation was detected between oxytocin concentration and sperm morphology, motility and count. These early studies were carried out in the 1990's and very few subsequent studies have been conducted, in particular the relationship of seminal plasma levels of vasopressin and oxytocin semen parameters and clinical outcomes (Goverde et al., 1998). In 2015, a study investigated males with and without varicocele and found higher concentrations of oxytocin (61.1 – 85.5 pg/mL) and negative correlations with sperm count and motility, and positive correlation with abnormal sperm morphology. This suggests that oxytocin may have a negative impact on sperm quality (Mostafa et al., 2015).

Hypothesis:

Clinical outcomes in females will be associated with levels of vasopressin, oxytocin and TNFα in follicular fluid in women undergoing ART and semen parameters in males will be associated with levels of vasopressin and oxytocin in seminal plasma of men undergoing ART.

Aims: The overall aim of this research is to explore any association between oxytocin, vasopressin and TNFα in follicular fluid and clinical outcomes, and to investigate any correlation with seminal plasma vasopressin and oxytocin with clinical outcomes. These aims will be achieved via the following objectives:

1. Measure oxytocin, vasopressin and TNFα levels in human follicular fluid samples from women undergoing fertility treatments both *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) using enzyme-linked immunosorbent assay (ELISA), and investigate any associations between the levels of both neuropeptides, TNFα and the clinical outcomes, biometrics, and stimulation treatment.
2. Measure the levels of oxytocin and vasopressin in seminal plasma and investigate any association of sperm parameters and clinical outcomes.

3.2 Methods

All samples used were obtained from consenting donors undergoing ART at Saint Mary's Hospital, Manchester, United Kingdom.

This work was approved under local faculty ethical approval (EthOS Reference Number: 038) and REC approval [Reference number: 12/SC/0649] in collaboration with Department of Reproductive Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust.

Caution must be observed with any small clinical study, the limitations of studying any outcome from IVF are the very many confounders. These include but are not limited to: female age/egg quality, all laboratory processes, training and ability of laboratory staff, ability of clinician at embryo transfer, stimulation protocol and all male factors have a bearing on outcome. Male factors are only a small component (Tomlinson et al., 2013).

3.2.1 Follicular fluid

Follicular fluid samples (n=52) were obtained from consented patients undergoing assisted conception treatment at St Mary's Hospital, Manchester. The samples were measured for TNFα, oxytocin and vasopressin using ELISAs. The parameters available for modelling were as follows; levels of TNFα (pg/mL), oxytocin (pg/mL) and vasopressin (pg/mL) present in the follicular fluid, age, treatment undergone (referred to as IVF/ICSI), the stimulation protocol, the gonadotrophin drug used, the gonadotrophin dose, the number of oocytes collected during egg collection after stimulation treatment (hereafter referred to as "number of oocytes"), the number of oocytes that were mixed with the partner sperm (hereafter referred to as "number mixed with sperm"), the number of oocytes that normally fertilised after mixing with sperm (hereafter referred to as "number fertilised"), the number of embryos replaced into the female (here after referred to as "number replaced"), the day of embryo transfer, the number of embryos frozen and the cycle outcome (freeze all, pregnant, not pregnant).

The data obtained were modelled in IBM SPSS Statistics version 25. Unadjusted and fully adjusted regression models were created for each outcome variable separately in order to obtain meaningful analysis. To reduce over fitting, the regression models, all outcome

variables, predictor variables and confounders were chosen *a priori* and each predictor was modelled with each outcome separately. All relevant confounders were included whether they were statistically significant or not. However, sample size was a limitation and further investigation would increase the validity of the regression models (Table 10).

Table 10. Outcome variables, predictor variables and covariates used in the modelling follicular fluid data.

Outcome Variables	Predictor Variables	Confounders/Covariates
Number of oocytes	TNFα (pg/mL)	Age
Number fertilised	Oxytocin (pg/mL)	IVF or ICSI
Freeze all or embryo transfer	Vasopressin (pg/mL)	Stimulation protocol
Pregnant or not pregnant		Gonadotrophin used
		Gonadotrophin dose
		Number replaced

3.2.2 Semen

Semen samples (n = 80) were obtained from men undergoing assisted conception treatment at St Mary's Hospital, Manchester. The samples were measured for oxytocin and vasopressin using ELISAs (section 2.14). The parameters available for modelling were as follows; progressive motility, total motility, concentration, count, volume, oxytocin concentration, vasopressin concentration, age or normal/abnormal semen parameters using WHO reference ranges.

The data obtained were modelled in IBM SPSS Statistics version 25. The data was explored using descriptive statistics and appropriate further tests were chosen based on the distribution of the outcome variables. Initial relationships between semen parameters and predictor variables were investigated using partial correlations. Regression models were created for each outcome variable separately in order to obtain meaningful analysis. The semen parameters were appropriately transformed where possible in order to perform univariate regressions. To reduce over fitting, the regression models, all outcome variables, predictor variables and confounders were chosen *a priori* and each predictor was modelled with each outcome separately. All relevant confounders were included whether they were

statistically significant or not. However, the models were limited due to the only confounder available to use was age (Table 11).

Table 11. Outcome variables, predictor variables and covariates used in the modelling of seminal plasm data.

Outcome Variables	Predictor Variables	Confounders/Covariates
Progressive motility (%) Total motility (%) Concentration (million/mL) Count (million) Volume (mL) Normal/Abnormal semen parameters	Oxytocin (pg/mL) Vasopressin (pg/mL)	Age

3.3 Results

3.3.1 Follicular fluid concentrations of neuropeptides, TNFα and their relationships with clinical parameters

3.3.1.1 Follicular fluid data descriptives

Standard descriptive analysis was used to explore the data and identify the correct type of model to use for each outcome variable. In order to model the data binary logistic regressions were used for the binary outcomes (attempt pregnant or freeze all and pregnant or not pregnant) and after investigating the distribution of oocytes obtained and the number of oocytes fertilised both of which were positively skewed and therefore a negative binomial regression was used for both of these outcomes. Reference categories for binary variables were chosen as either what is expected as 'normal' (i.e. not pregnant as the women are already not pregnant) or if there was no expected normal then whichever variable dominated the data was the reference category (i.e. for stimulation protocol antagonist was the reference category as long down regulation consisted of only 5 patients).

Overall concentrations of TNFα and oxytocin in follicular fluid showed a large variation whereas vasopressin concentrations in follicular fluid appeared to be tightly regulated (Table 12).

Table 12. Descriptive statistics for predictor variables in the full cohort.

	N	Mean	Median	Std Dev	InterQ Range	Min	Max
TNFα pg/mL	28	177.78	46.39	394.71	119.72	0.36	1783.15
Oxytocin pg/mL	36	694.21	559.23	452.18	242.06	285.26	2442.17
Vasopressin pg/mL	36	3079.87	3081.97	7.97	10.86	3055.56	3091.08

The descriptive statistics for each binary outcome variable demonstrated the differences in means, median, standard deviation, interquartile range (InterQ Range), min and max values for all the predictor variables (Table 13, Table 14, Table 15).

Table 13. Descriptive statistics for TNFα concentration in follicular fluid and all binary variables.

	TNFa pg/mL						
	N	Mean	Median	Std Dev	InterQ Range	Min	Max
IVF	13	304.95	52.71	556.28	297.31	0.36	1783.15
ICSI	15	67.56	32.31	84.76	78.35	1.83	311.98
Long down regulation (Stimulation protocol)	3	429.39	52.71	675.66	-	26.04	1209.42
Antagonist (Stimulation protocol)	25	147.58	46.02	358.27	114.87	0.36	1783.15
Menopur (gonadotrophin drug used)	16	229.79	27.51	508.32	139.02	0.36	1783.15
Bemfola (gonadotrophin drug used)	12	108.42	52.53	144.47	86.03	4.38	486.16
Freeze all embryos	8	64.91	50.85	45.05	80.37	14.64	142.51
Embryo transfer	19	171.00	28.99	409.95	152.15	0.36	1783.15
Not pregnant	8	295.83	54.01	610.58	271.48	0.36	1783.15
Pregnant	11	80.22	28.99	141.48	39.60	4.38	486.16

Table 14. Descriptive statistics for oxytocin concentration in follicular fluid and all binary variables.

	Oxytocin pg/mL						
	N	Mean	Median	Std Dev	InterQ Range	Min	Max
IVF	16	711.63	605.66	504.57	291.60	285.26	2442.17
ICSI	20	680.28	544.05	418.57	221.91	357.42	2264.92
Long down regulation (Stimulation protocol)	4	536.79	559.23	167.39	305.80	311.85	716.84
Antagonist (Stimulation protocol)	32	713.89	571.95	473.86	250.30	285.26	2442.17
Menopur (gonadotrophin drug used)	24	586.42	559.23	198.20	249.97	285.26	1196.40
Bemfola (gonadotrophin drug used)	12	909.80	625.31	701.71	546.09	398.03	2442.17
Freeze all embryos	12	844.63	576.95	727.80	435.83	285.26	2442.17
Embryo transfer	23	614.75	555.11	203.68	225.25	357.42	1196.40
Not pregnant	9	596.23	588.78	135.72	232.79	357.42	753.46
Pregnant	14	626.65	544.05	241.82	230.51	398.03	1196.40

Table 15. Descriptive statistics for vasopressin concentration in follicular fluid and all binary variables.

	Vasopressin pg/mL						
	N	Mean	Median	Std Dev	InterQ Range	Min	Max
IVF	16	3079.62	3080.62	6.85	10.57	3064.60	3091.08
ICSI	20	3080.08	3082.55	8.93	11.53	3055.56	3090.70
Long down regulation (Stimulation protocol)	4	3078.16	3078.17	7.14	13.43	3070.75	3085.54
Antagonist (Stimulation protocol)	32	3080.09	3081.97	8.14	9.53	3055.56	3091.08
Menopur (gonadotrophin drug used)	24	3080.76	3081.39	6.11	10.72	3070.55	3091.08
Bemfola (gonadotrophin drug used)	12	3078.09	3083.03	10.89	18.26	3055.56	3088.79
Freeze all embryos	12	3082.82	3083.61	4.68	6.13	3074.09	3091.08
Embryo transfer	23	3078.21	3077.99	9.05	11.84	3055.56	3090.70
Not pregnant	9	3079.81	3077.61	5.69	9.98	3073.50	3089.37
Pregnant	14	3077.18	3080.32	10.76	16.52	3055.56	3090.70

The number of oocytes and the number of oocytes fertilised were both positively skewed and therefore it was necessary to use a negative binomial regression for these count data (Figure 20, Figure 21).

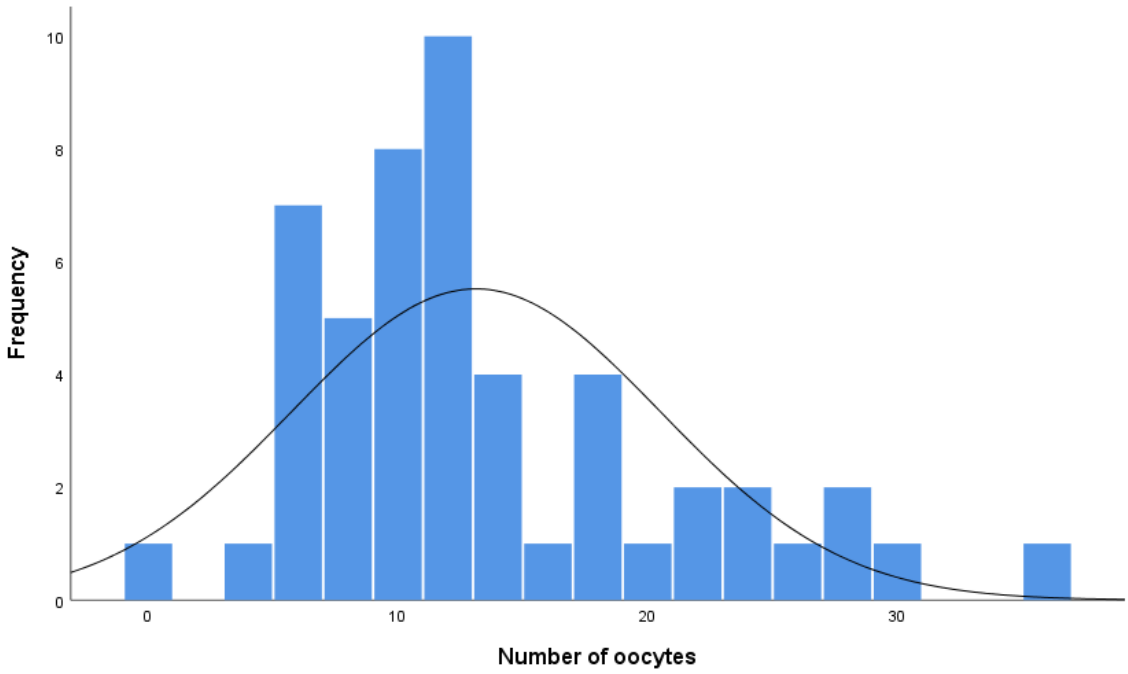

Figure 20. Frequency distribution of the number of oocytes obtained. The distribution demonstrates a positive skew of the data.

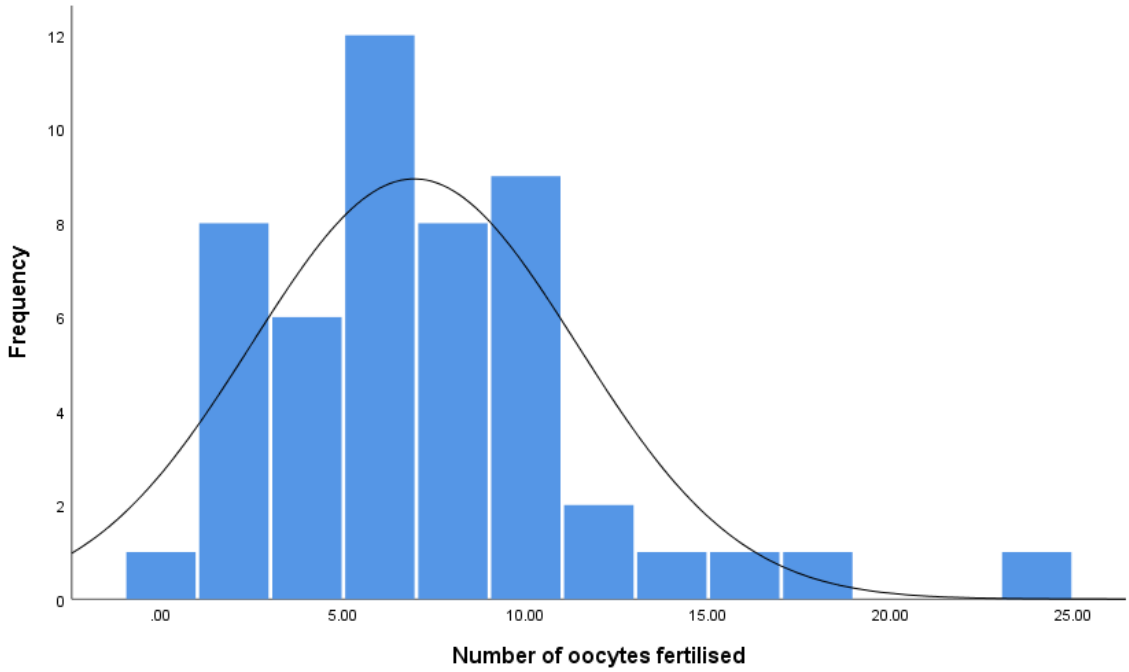

Figure 21. Frequency distribution of the number of oocytes fertilised. The distribution demonstrates a positive skew of the data.

The Pearson correlation was used to investigate any collinearity between the predictor variables in order to ensure their effects on the outcome variables are unique to each predictor variable. No statistically significant correlation was found between any of the predictor variables (Table 16).

Table 16. Pearson's correlation matrix for predictor variables.

		TNFα	Oxytocin	Vasopressin
TNFα	Pearson Correlation	1	0.037	-0.096
	p-value		*0.859*	*0.641*
Oxytocin	Pearson Correlation	0.037	1	0.016
	p-value	*0.859*		*0.928*
Vasopressin	Pearson Correlation	-0.096	0.016	1
	p-value	*0.641*	*0.928*	

The model chosen for number of oocytes was a negative binomial logistic regression. The model chosen for number fertilised was a negative binomial logistic regression with number mixed with sperm as the offset. The model chosen for the cycle outcome was a positive binomial logistic regression.

3.3.1.2 Follicular fluid regression model results

In order to investigate any association between vasopressin, oxytocin and TNFα and the outcome variables regression modelling was used.

The data was modelled per 10 pg/mL increase of TNFα, vasopressin and oxytocin.

Rate ratio, also referred to as Exp (B), is representative of the probability that an event will occur if all other factors in the model remain constant. The unadjusted models represent the individual predictor variables overall main effects on the outcome variable, the adjusted models represent the effects that each predictor variable has on the outcome when all of them are taken into consideration. Any warnings were taken into consideration when finalising the fully adjusted model, in some cases the stimulation protocol was not possible to include as there was only an N of 5 in the long down regulation protocol in the overall cohort, thus causing interference with the step-halving of the regression, both the warning and the variable, which has caused this to occur are marked with **. A mean centred interaction variable was created for the gonadotrophin used and the dose of gonadotrophin used, as these two variables are directly linked it was important to establish whether their interaction significantly impacted upon the regression models, this variable was only included where statistically relevant.

3.3.1.2.1 Follicular fluid - number of oocytes negative binomial logistic regression

A negative binomial logistic regression was used to investigate any relationship between the predictor variables and the number of oocytes obtained. The interaction variable was not significant in the unadjusted model and therefore not included in subsequent regression models. A warning was issued (marked with **) in association with the stimulation protocol variable in the unadjusted model (Table 17)[1] In the unadjusted model only the dose of gonadotrophin drug used was found to be significant showing that with a 10 unit increase in dose there was a probability that the number of oocytes obtained would decrease by a factor of 0.974 (Table 17). The stimulation protocol did not cause any issues in the fully adjusted models (Table 18, Table 19, Table 20).

Table 17. Unadjusted negative binomial logistic regression for number of oocytes including all variables.

Unadjusted model				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.928	0.996	0.922	1.077
Long down regulation (Stimulation protocol)**	0.177	0.508	0.19	1.358
Antagonist (Stimulation protocol)**	.	1	.	.
Menopur (gonadotrophin drug used)	0.393	0.772	0.426	1.399
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0.03	0.974	0.951	0.997
Interaction variable	0.059	0.997	0.994	1
Vasopressin (per 10 pg increase)	0.664	1.097	0.723	1.664
Oxytocin (per 10 pg increase)	0.437	1.003	0.995	1.01
TNFα (per 10 pg increase)	0.356	0.995	0.986	1.005

The fully adjusted negative binomial logistic regression model including all available confounders showed no significant association between vasopressin concentration in follicular fluid and number of oocytes obtained (Table 18).

Table 18. Fully adjusted negative binomial logistic regression for vasopressin concentration in follicular fluid and number of oocytes.

Fully adjusted model vasopressin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.841	1.011	0.911	1.121
Long down regulation (Stimulation protocol)	0.446	0.636	0.198	2.038
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.972	1.016	0.426	2.421
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0.294	0.981	0.948	1.016
Vasopressin (per 10 pg increase)	0.813	1.06	0.655	1.714

The fully adjusted negative binomial logistic regression model including all available confounders showed no significant association between oxytocin concentration in follicular fluid and number of oocytes obtained (Table 19).

Table 19. Fully adjusted negative binomial logistic regression for oxytocin concentration in follicular fluid and number of oocytes.

Fully adjusted model oxytocin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.92	1.005	0.91	1.111
Long down regulation (Stimulation protocol)	0.428	0.629	0.2	1.976
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.85	1.088	0.454	2.611
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0.256	0.981	0.949	1.014
Oxytocin (per 10 pg increase)	0.708	1.002	0.993	1.01

The fully adjusted negative binomial logistic regression model including all available confounders showed no significant association between TNFα concentration in follicular fluid and number of oocytes obtained (Table 20).

Table 20. Fully adjusted negative binomial logistic regression for TNFα concentration in follicular fluid and number of oocytes.

Fully adjusted model TNFα				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.904	0.992	0.877	1.123
Long down regulation (Stimulation protocol)	0.472	0.605	0.154	2.38
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.922	1.055	0.365	3.052
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0.123	0.97	0.934	1.008
TNFα (per 10 pg increase)	0.213	0.993	0.983	1.004

3.3.1.2.2 Follicular fluid: number of oocytes fertilised negative binomial logistic regression

A negative binomial logistic regression was used to investigate any relationship between the predictor variables and the number of oocytes fertilised. All models were offset with the number of oocytes mixed with sperm to adjust for the potential number of oocytes fertilised. In the unadjusted model the following variables showed significant associations with the number of oocytes fertilised; choosing ICSI over IVF showed an extremely significant increase in association with fertilisation, the long-down regulation stimulation protocol showed an extremely significant increase in association with fertilisation, Menopur over Bemfola as the gonadotrophin used showed a statistically significant increase in association with fertilisation, with every 10 unit increase in the gonadotrophin drug used showed a statistically significant increase in association with fertilisation, the interaction variable was highly significant therefore included in all fully adjusted models and oxytocin concentration in follicular fluid was statistically significant and showed decrease in number of eggs fertilised per 10 pg increase in concentration in follicular fluid by a factor of 0.95. No statistically significant association was found between number of oocytes fertilised and age, vasopressin concentration in follicular fluid or TNFα concentration in follicular fluid (Table 21).

Table 21. Unadjusted negative binomial logistic regression for number of oocytes fertilised including all variables

	Unadjusted model			
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.223	1.064	0.963	1.176
ICSI	0	16.582	7.354	37.389
IVF	.	1	.	.
Long down regulation (Stimulation protocol)	0.014	7.701	1.499	39.568
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.004	3.671	1.513	8.904
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0	1.134	1.097	1.171
Interaction variable	0	1.015	1.011	1.019
Vasopressin (per 10 pg increase)	0.804	1.105	0.504	2.423
Oxytocin (per 10 pg increase)	0	0.95	0.941	0.959
TNFα (per 10 pg increase)	0.075	0.989	0.977	1.001

The fully adjusted negative binomial logistic regression model including all available confounders showed no significant association between vasopressin concentration in follicular fluid and number of oocytes fertilised (Table 22).

Table 22. Fully adjusted negative binomial logistic regression for vasopressin concentration in follicular fluid and number of oocytes fertilised.

Fully adjusted model vasopressin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0	0.773	0.693	0.861
ICSI	0	635.327	168.296	2398.399
IVF	.	1	.	.
Long down regulation (Stimulation protocol)	0.099	0.195	0.028	1.357
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.201	2.288	0.643	8.141
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0	1.273	1.12	1.446
Interaction variable	0	0.968	0.952	0.985
Vasopressin (per 10 pg increase)	0.256	0.669	0.335	1.338

The fully adjusted negative binomial logistic regression model including all available confounders showed a significant negative association between oxytocin concentration in follicular fluid per 10 pg increase and a 3% decrease in number of oocytes fertilised (Table 23).

Table 23. Fully adjusted negative binomial logistic regression for oxytocin concentration in follicular fluid and number of oocytes fertilised.

Fully adjusted model oxytocin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.026	0.884	0.792	0.985
ICSI	0	277.326	69.728	1102.996
IVF	.	1	.	.
Long down regulation (Stimulation protocol)	0.343	0.401	0.06	2.655
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.56	1.456	0.411	5.155
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0	1.222	1.107	1.349
Interaction variable	0	0.974	0.961	0.988
Oxytocin (per 10 pg increase)	0	0.968	0.957	0.98

The fully adjusted negative binomial logistic regression model including all available confounders showed no significant association between TNFα concentration in follicular fluid and number of oocytes fertilised (Table 24).

Table 24. Fully adjusted negative binomial logistic regression for TNFα concentration in follicular fluid and number of oocytes fertilised.

Fully adjusted model TNFα				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.138	0.897	0.778	1.035
ICSI	0	18.389	3.96	85.404
IVF	.	1	.	.
Long down regulation (Stimulation protocol)	0.73	1.426	0.191	10.669
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.689	0.721	0.145	3.581
Bemfola (gonadotrophin drug used)	.	1	.	.
Gonadotrophin drug dose (per 10 units)	0.19	1.094	0.957	1.25
Interaction variable	0.843	0.998	0.983	1.015
TNFα (per 10 pg increase)	0.255	1.012	0.991	1.033

3.3.1.2.3 Follicular fluid binary logistic regression embryo transfer or freeze all embryos

A binary logistic regression was used to investigate any relationship between the predictor variables and whether embryos were transferred or the embryos were frozen. The interaction variable was not significant in the unadjusted model and therefore not included in subsequent regression models. A warning was issued (marked with **) in association with the stimulation protocol variable, the gonadotrophin used variable and the gonadotrophin drug dose variable in the unadjusted model (Table 25)[Error! Bookmark not defined.]. Despite the issues in the unadjusted model these variables were not problematic in the fully adjusted models and therefore remained included. The only significant variable in the unadjusted model was the gonadotrophin drug dose, which caused a statistically significant 6% decrease in likelihood that embryos would be frozen per 10 unit dose increase (Table 25).

Table 25. Unadjusted binary logistic regression for embryo transfer or freeze all embryos including all variables.

Unadjusted model				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.769	1.024	0.876	1.196
ICSI	0.663	1.222	0.496	3.014
IVF	.	1.000	.	.
Long down regulation (Stimulation protocol)**	0.755	0.689	0.066	7.192
Antagonist (Stimulation protocol)	.	1.000	.	.
Menopur (gonadotrophin drug used)**	0.880	0.909	0.265	3.118
Bemfola (gonadotrophin drug used)	.	1.000	.	.
Gonadotrophin drug dose (per 10 units)**	0.044	0.943	0.890	0.998
Interaction variable	0.116	0.994	0.988	1.001
Vasopressin (per 10 pg increase)	0.118	2.456	0.795	7.584
Oxytocin (per 10 pg increase)	0.199	1.011	0.994	1.029
TNFα (per 10 pg increase)	0.550	0.980	0.916	1.048

The fully adjusted binary logistic regression model including all available confounders showed no significant association between vasopressin concentration in follicular fluid and whether embryos were transferred or if the embryos were frozen (Table 26).

Table 26. Fully adjusted binary logistic regression for vasopressin and embryo transfer or freeze all embryos.

Full adjusted model vasopressin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.058	1.280	0.992	1.652
ICSI	0.027	0.095	0.012	0.764
IVF	.	1.000	.	.
Long down regulation (Stimulation protocol)	0.915	1.260	0.018	89.263
Antagonist (Stimulation protocol)	.	1.000	.	.
Menopur (gonadotrophin drug used)	0.669	1.581	0.194	12.922
Bemfola (gonadotrophin drug used)	.	1.000	.	.
Gonadotrophin drug dose (per 10 units)	0.239	0.937	0.841	1.044
Vasopressin (per 10 pg increase)	0.084	3.787	0.837	17.133

The fully adjusted binary logistic regression model including all available confounders showed no significant association between oxytocin concentration in follicular fluid and whether embryos were transferred or if the embryos were frozen (Table 27).

Table 27. Fully adjusted binary logistic regression for oxytocin and embryo transfer or freeze all embryos.

Full adjusted model oxytocin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.176	1.177	0.929	1.490
ICSI	0.038	0.134	0.020	0.897
IVF	.	1.000	.	.
Long down regulation (Stimulation protocol)	0.288	3.169	0.377	26.615
Antagonist (Stimulation protocol)	.	1.000	.	.
Menopur (gonadotrophin drug used)	0.097	0.926	0.845	1.014
Bemfola (gonadotrophin drug used)	0.994	1.013	0.043	23.608
Gonadotrophin drug dose (per 10 units)	.	1.000	.	.
Oxytocin (per 10 pg increase)	0.360	1.010	0.989	1.030

The fully adjusted binary logistic regression model including all available confounders showed no significant association between TNFα concentration in follicular fluid and whether embryos were transferred or if the embryos were frozen (Table 28).

Table 28. Fully adjusted binary logistic regression for TNFα and embryo transfer or freeze all embryos.

Full adjusted model TNFα				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.626	0.887	0.547	1.438
ICSI	0.067	0.054	0.002	1.228
IVF	.	1.000	.	.
Long down regulation (Stimulation protocol)	0.604	0.359	0.007	17.245
Antagonist (Stimulation protocol)	.	1.000	.	.
Menopur (gonadotrophin drug used)	0.033	0.832	0.703	0.985
Bemfola (gonadotrophin drug used)	0.639	4.611	0.008	2713.452
Gonadotrophin drug dose (per 10 units)	.	1.000	.	.
TNFα (per 10 pg increase)	0.260	0.934	0.829	1.052

3.3.1.2.4 Follicular fluid binary logistic regression pregnant or not pregnant

A binary logistic regression was used to investigate any relationship between the predictor variables and whether pregnancy was achieved or not. The interaction variable was not significant in the unadjusted model and therefore not included in subsequent regression models. In the unadjusted model none of the variables showed any statistically significant relationship with whether pregnancy was achieved or not (Table 29). The number of embryos replaced was included as this has been long established in literature to directly affect the pregnancy rate therefore this was accounted for statistically (Edwards and Steptoe, 1983; Wramsby et al., 1987).

Table 29. Unadjusted binary logistic regression for pregnancy achieved or not pregnant including all variables.

Unadjusted model				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.706	0.967	0.811	1.152
ICSI	0.966	1.032	0.247	4.303
IVF	.	1	.	.
Long down regulation (Stimulation protocol)	0.773	1.444	0.118	17.671
Antagonist (Stimulation protocol)	.	1	.	.
Menopur (gonadotrophin drug used)	0.494	0.6	0.139	2.595
Bemfola (gonadotrophin drug used)	.	1	.	.
Number embryos replaced	0.881	0.909	0.261	3.163
Gonadotrophin drug dose (per 10 units)	0.437	0.977	0.923	1.035
Interaction variable	0.571	0.998	0.991	1.005
Vasopressin (per 10 pg increase)	0.492	0.704	0.259	1.913
Oxytocin (per 10 pg increase)	0.722	1.008	0.965	1.053
TNFα (per 10 pg increase)	0.37	0.982	0.944	1.022

The fully adjusted binary logistic regression model including all available confounders showed no significant association between vasopressin concentration in follicular fluid and whether pregnancy was achieved or not (Table 30).

Table 30. Fully adjusted binary logistic regression for vasopressin and pregnancy achieved or not pregnant.

Full adjusted model Vasopressin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.832	0.974	0.760	1.247
ICSI	0.779	1.321	0.189	9.254
IVF	.	1.000	.	.
Number embryos replaced	0.865	0.857	0.145	5.077
Menopur (gonadotrophin drug used)	0.433	0.431	0.053	3.535
Bemfola (gonadotrophin drug used)	.	1.000	.	.
Gonadotrophin drug dose (per 10 units)	0.954	0.998	0.922	1.080
Long down regulation (Stimulation protocol)	0.747	0.545	0.014	21.709
Antagonist (Stimulation protocol)	.	1.000	.	.
Vasopressin (per 10 pg increase)	0.514	0.663	0.193	2.277

The fully adjusted binary logistic regression model including all available confounders showed no significant association between oxytocin concentration in follicular fluid and whether pregnancy was achieved or not (Table 31).

Table 31. Fully adjusted binary logistic regression for oxytocin and pregnancy achieved or not pregnant.

Full adjusted model Oxytocin				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.804	0.965	0.728	1.279
ICSI	0.797	1.297	0.178	9.419
IVF	.	1.000	.	.
Number embryos replaced	0.731	0.726	0.117	4.503
Menopur (gonadotrophin drug used)	0.350	0.359	0.042	3.074
Bemfola (gonadotrophin drug used)	.	1.000	.	.
Gonadotrophin drug dose (per 10 units)	0.955	1.002	0.926	1.084
Long down regulation (Stimulation protocol)	0.830	0.674	0.019	24.440
Antagonist (Stimulation protocol)	.	1.000	.	.
Oxytocin (per 10 pg increase)	0.626	1.014	0.960	1.070

The stimulation protocol was unable to be fitted into the fully adjusted binary logistic regression for TNFα, this was down to the long down regulation group only having 5 patients in total and caused issues with accuracy of the model. The fully adjusted binary logistic regression model including all available confounders showed no significant

association between TNFα concentration in follicular fluid and whether pregnancy was achieved or not (Table 32).

Table 32. Fully adjusted binary logistic regression for TNFα and pregnancy achieved or not pregnant.

Full Adjusted model TNFα				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.466	0.866	0.589	1.274
ICSI	0.335	0.232	0.012	4.511
IVF	.	1.000	.	.
Number embryos replaced	0.217	0.179	0.012	2.741
Menopur (gonadotrophin drug used)	0.416	0.294	0.015	5.593
Bemfola (gonadotrophin drug used)	.	1.000	.	.
Gonadotrophin drug dose (per 10 units)	0.998	1.000	0.880	1.135
TNFα (per 10 pg increase)	0.395	0.959	0.872	1.055

3.3.2 Semen concentrations of neuropeptides and clinical parameters

3.3.2.1 Semen data descriptives

The Shapiro-Wilk test for normality was used to initially investigate whether the continuous outcome variables (count, concentration, volume, progressive motility and total motility) were normally distributed. As all the variables were statistically significant this indicated they were not normally distributed and were further investigated using frequency histograms (Table 33).

Table 33. Shapiro-Wilk tests for normality for the continuous outcome variables.

	Shapiro-Wilk Statistic	df	Sig.
Count	0.822	73	0
Concentration	0.92	73	0
Progressive Motility	0.933	73	0.001
Total Motility	0.905	73	0

The frequency histograms of the semen parameters show the distribution of the continuous outcome variables. Count, concentration and volume all show abnormal distribution with positive skew, whereas progressive motility and total motility show abnormal distribution with negative skew. The data was either square root transformed to correct the positive skew or log gamma transformed to correct the negative skew prior to use in linear regressions (Figure 22).

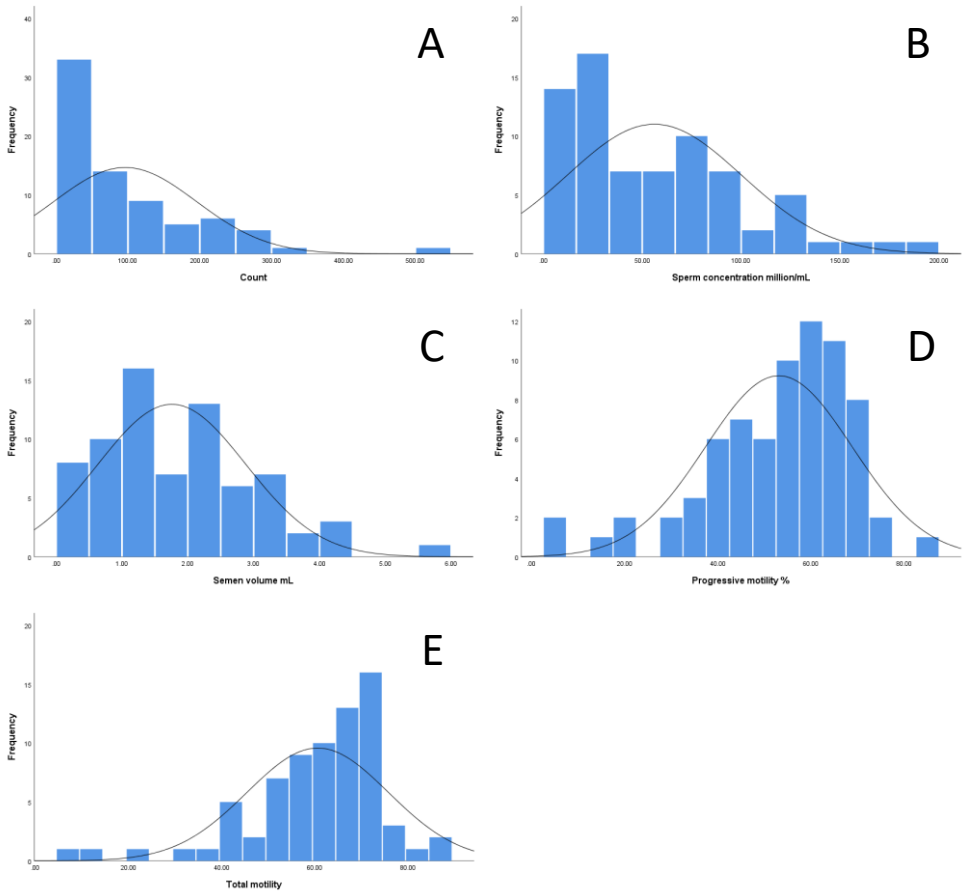

Figure 22. Distribution histograms of semen parameters. A – sperm count (million), B – sperm concentration (million/mL), C – semen volume (mL), D – progressive motility (%), E – total motility (%).

The table of descriptives for the binary variable of normal or abnormal semen parameters, defined by using WHO reference ranges, show the n, mean, median, standard deviation (Std Dev), interquartile range (interQ range), minimum and maximum concentration of vasopressin or oxytocin in semen per group (Table 34).

Table 34. Descriptives for normal or abnormal semen parameters and oxytocin or vasopressin concentration in seminal plasma.

		N	*Mean*	*Median*	*Std Dev*	*InterQ Range*	*Min*	*Max*
Oxytocin	**Normal**	28	952.07	843.76	532.94	337.63	496.55	2963.38
	Abnormal	37	1074.96	911.53	573.88	372.05	513.99	3100.24
Vasopressin	**Normal**	29	1085.32	1016.76	477.63	762.18	143.35	2019.99
	Abnormal	40	1204.80	1124.73	551.64	719.03	192.86	2827.24

The Pearson correlation was used to investigate any collinearity between the predictor variables in order to ensure their effects on the outcome variables are unique to each predictor variable. No statistically significant correlation was found between any of the predictor variables (Table 35).

Table 35. Pearson's correlation matrix for predictor variables.

	Vasopressin pg/mL		Oxytocin pg/mL	
	r	*p*-value	r	*p*-value
Vasopressin pg/mL	1		0.122	0.327
Oxytocin pg/mL	0.122	0.327	1	

3.3.2.2 Semen parameters partial correlations

Partial correlations were performed on the appropriately transformed outcome variables using age as a confounding factor. The r value represents the direction and strength of the correlation. The only statistically significant correlation was between oxytocin concentration in semen and sperm count (r = -0.311, p = 0.022). There were no further statistically significant correlations between oxytocin concentration in semen and concentration, volume, progressive motility, total motility or normal/abnormal semen parameters. There were no statistically significant correlations between concentrations of vasopressin in semen and any of the semen parameters investigated (Table 36).

Table 36. Values for partial correlation between vasopressin and oxytocin concentration in semen and transformed semen parameters.

	Vasopressin pg/mL		Oxytocin pg/mL	
	r	*p*-value	r	*p*-value
Concentration (SQRT Transformed)	0.095	0.496	-0.18	0.192
Volume (SQRT Transformed)	-0.219	0.111	-0.201	0.145
Count (SQRT Transformed)	-0.047	0.738	-0.311	0.022
Progressive motility (Log gamma Transformed)	0.018	0.895	-0.091	0.512
Total motility (Log gamma Transformed)	-0.008	0.952	-0.064	0.648
Normal or abnormal	0.109	0.432	0.218	0.114

3.3.2.3 Semen parameters univariate regressions

In order to thoroughly investigate any potential relationship between the concentrations of oxytocin and vasopressin in semen and semen parameters univariate regressions were used. The only confounder available was age. Variables were transformed appropriately either using square root transformation to correct for the positive skew or log gamma transformations to correct for negative skew and be normally distributed prior to use in univariate regressions. Standardised β values are the relative change in the outcome variables (semen parameters) due to the change in the predictor variables (oxytocin/vasopressin concentration in semen).

Oxytocin concentration in semen had a statistically significant negative relationship with sperm count and sperm concentration but no statistically significant relationship with

volume, progressive motility or total motility in both the unadjusted and fully adjusted models (Table 37, Table 38).

Table 37. Unadjusted univariate regression values for transformed semen parameters and oxytocin concentration in semen.

	Unadjusted oxytocin (pg/mL)			
			CI (95%)	
	p-value	Standardised β	Lower	Upper
SQRT count	0.006	-0.332	-0.004	-0.001
SQRT concentration	0.027	-0.268	-0.003	0
SQRT volume	0.089	-0.203	0	0
Log gamma progressive motility	0.164	-0.167	-0.041	0.007
Log gamma total motility	0.162	-0.168	-0.041	0.007

Table 38. Fully adjusted univariate regression values for transformed semen parameters and oxytocin concentration in semen.

	Fully adjusted oxytocin (pg/mL)			
			CI (95%)	
	p-value	Standardised β	Lower	Upper
SQRT count	0.004	-0.373	-0.005	-0.001
SQRT concentration	0.024	-0.293	-0.003	0
SQRT volume	0.069	-0.229	0	0
Log gamma progressive motility	0.103	-0.207	-0.048	0.005
Log gamma total motility	0.107	-0.204	-0.048	0.005

Vasopressin concentration in semen had no statistically significant relationship with any of the semen parameters in both the unadjusted and fully adjusted models (Table 39, Table 40).

Table 39. Unadjusted univariate regression values for transformed semen parameters and vasopressin concentration in semen.

	Unadjusted vasopressin (pg/mL)			
			CI (95%)	
	p-value	Standardised β	Lower	Upper
SQRT count	0.599	-0.066	-0.003	0.002
SQRT concentration	0.686	0.051	-0.001	0.002
SQRT volume	0.122	-0.188	0	0
Log gamma progressive motility	0.771	-0.035	-0.031	0.023
Log gamma total motility	0.6	-0.064	-0.034	0.02

Table 40. Fully adjusted univariate regression values for transformed semen parameters and vasopressin concentration in semen.

	Fully adjusted vasopressin (pg/mL)			
			CI (95%)	
	p-value	Standardised β	Lower	Upper
SQRT count	0.477	-0.072	-0.324	0.154
SQRT concentration	0.821	0.03	-0.001	0.002
SQRT volume	0.206	-0.161	0	0
Log gamma progressive motility	0.962	-0.006	-0.028	0.026
Log gamma total motility	0.462	0.094	-1.69	3.68

3.3.2.4 Normal or abnormal semen parameters binary logistic regression

The data was modelled per 10 pg/mL increase of vasopressin and oxytocin. Rate ratio also referred to as Exp (B) is representative of the probability that an event will occur if all other factors in the model remain constant. The unadjusted models represent the individual predictor variables overall main effects on the outcome variable, the adjusted models represent the effects that each predictor variable has on the outcome when all of them are taken into consideration.

In order to investigate whether there is any relationship between normal or abnormal semen parameters a binary logistic regression was used (assessed as normal if all WHO reference ranges were met and abnormal is any of the WHO reference ranges were not met).

In the unadjusted binary logistic regression neither oxytocin concentration nor vasopressin concentration in semen had any statistically significant relationship with normal or abnormal semen parameters (Table 41).

Table 41. Unadjusted binary logistic regression for normal or abnormal semen parameters and oxytocin concentration and vasopressin concentration in semen.

	Unadjusted model normal/abnormal			
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.66	0.977	0.882	1.083
Vasopressin (per 10 pg increase)	0.348	1.005	0.995	1.014
Oxytocin (per 10 pg increase)	0.384	1.004	0.995	1.014

Oxytocin concentration in semen had no statistically significant relationship with normal or abnormal semen parameters (Table 42).

Table 42. Fully adjusted binary logistic regression for normal or abnormal semen parameters and oxytocin concentration in semen.

	Full adjusted model oxytocin			
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.556	0.966	0.862	1.083
Oxytocin (per 10 pg increase)	0.151	1.011	0.996	1.025

Vasopressin concentration in semen had no statistically significant relationship with normal or abnormal semen parameters (Table 43).

Table 43. Fully adjusted binary logistic regression for normal or abnormal semen parameters and vasopressin concentration in semen

	Full adjusted model vasopressin			
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Age	0.677	0.978	0.882	1.085
Vasopressin (per 10 pg increase)	0.353	1.005	0.995	1.014

3.4 Discussion

The concentrations of vasopressin, oxytocin and TNFα were measured in follicular fluid and vasopressin, oxytocin was measured in semen samples obtained from consenting patients undergoing ART treatment. The associations between these molecules and several clinical outcomes for both males and females undergoing ART were investigated using a variety of regression models.

The follicular fluid concentrations of TNFα (0.36 – 1783.15 pg/mL) and oxytocin (285.26 – 2442.17 pg/mL) ranged greatly whereas vasopressin (3055.56 – 3091.08 pg/mL) concentrations were very consistent, this may be suggestive that vasopressin is tightly regulated.

TNFα has been previously detected in human follicular fluid at similar levels to the lower range found in this study 0.36 pg/mL to 7.8 pg/mL and was positively associated with poor quality oocytes and found that it may be involved in stimulating prostaglandin production and the proliferation of follicular cells (Lee et al., 2000; Wang et al., 1992). However, there has been reported that TNFα is not associated with success of IVF (Mendoza et al., 2002). These levels previously found are similar to lower boundary in the present study, which is the first study to demonstrate a high variability in TNFα concentrations in follicular fluid, where no associations were found between TNFα concentrations and the clinical outcomes measured; number of oocytes, number fertilised, the choice between freeze all and embryo transfer and clinical pregnancy. TNFα has been demonstrated to interact with other cytokines such as; the proinflammatory interleukins 1, 6 and 8 as well as colony-stimulating factors (CSFs), interferons (IFNs) and transforming growth factor beta (TGF-β). TNFα is a known stimulator of many cytokines and in combination may have differing effects; either synergistic or antagonistic (Neta et al., 1992). The cytokines are implicated in many aspects of female reproduction from regulating ovulation to potential effects on pregnancy outcomes (Bedaiwy et al., 2007; Büscher et al., 1999; Gaafar et al., 2014; Mendoza et al., 2002). Ideally, measurements of TNFα would coincide with measurements of the cytokines it interacts with to gain insight into the role it may play in female reproduction and the mechanisms involved in this.

Previously, vasopressin has been shown to modulate the effect of TNFα within human aortic endothelial cells through the oxytocin receptor, where vasopressin caused shedding of the tumour necrosis factor receptor 1 (TNFR1) through a calcium influx into the cells. However, no relationship was found between the levels of vasopressin, oxytocin or TNFα within the human follicular fluid, the interaction may have been a unique observation within human aortic endothelial cells, which so far have only been found to express the oxytocin receptor and not any vasopressin receptors, oxytocin receptor has an affinity for vasopressin due to the similarity of the nonapeptides at high enough concentrations (Thibonnier et al., 1999; Yang et al., 2019).

In previous studies, oxytocin was found in human follicular fluid from the pre-ovulatory follicles at concentrations of 12.85 pg/mL at higher levels than in immature follicles (5.54 pg/mL) (Tjugum et al., 1986). In tissue extracts of human ovaries acquired through surgeries, oxytocin was found at 1.4 - 60 ng/g in the preovulatory follicles. It was suggested that oxytocin may function as a luteolytic agent (degradation of the corpus luteum where pregnancy does not occur) (Khan-Dawood and Dawood, 1983). Further studies showed a concentration of 299 pg/mL of oxytocin in human follicular fluid, 30 times greater than the plasma concentrations (Schaeffer et al., 1984). The oxytocin concentrations detected in this study were similar to those reported in previous studies at the lower boundary and ranged to far greater than previously reported.

Vasopressin has previously been detected at 131 pg/mL in human follicular fluid, 30 times higher than in plasma (Schaeffer et al., 1984). Other studies have disputed that vasopressin was found at levels either lower or equal to plasma levels within human follicular fluid at a mean concentration of 5.8 – 9.6 pg/mL (Verges et al., 1986). The vasopressin levels found in this study are far greater than those reported in previous literature, however the studies investigating vasopressin levels in human follicular fluid are few in number and nearly 30 years ago. The consistent levels of vasopressin throughout all follicular fluid samples may be related to the regulation of aquaporin-2 and the osmotic gradient in the follicular antrum. Vasopressin regulates the water channels aquaporin-2 and aquaporin-3 which allow influx of water into the cell and out of the cell respectively (Knepper, 1997). Many of the aquaporins have previously been demonstrated to be present in the uterus and fallopian tubes of humans (Hildenbrand et al., 2006; Mints et al., 2007; Mobasheri et al., 2005). Aquaporin-2 mRNA in granulosa cells has been found to be 35-fold higher in the

early ovulatory phase than the pre-ovulatory phase, with aquaporin-2 mRNA levels remaining upregulated during ovulation (Thoroddsen et al., 2011).

In follicular fluid, while controlling for all available confounding factors, the only statistically significant result was a negative association between oxytocin and number of oocytes fertilised (RR – 0.968 [0.957, 0.98], *p* – 0.000). Oxytocin was not associated with the number of oocytes obtained, whether embryos were transferred or frozen, or pregnancy outcome. TNFα and vasopressin were not associated with any of the outcomes analysed.

Most previous studies have investigated the effects of oxytocin and vasopressin within animal studies or tissue extracts of ovaries of women undergoing surgery. In cow and sheep models the oxytocin concentration in the periphery and the ovary are concordant with activity of the corpus luteum throughout the oestrous cycle, there is an increase in the early luteal phase and a decline at luteolysis. Maximum levels of circulating oxytocin and vasopressin have been found at ovulation. Within cow, sheep and goat studies oxytocin was shown decline post ovulation and to be lower in pregnancy. Both vasopressin and oxytocin have been implicated in influencing secretion of gonadotropin (Wathes, 1984).

The follicular fluid used in this study is pooled from multiple follicles within the same woman, there may have been immature and mature follicles aspirated and there have been previous studies detecting differences in the constituents in the follicular fluids of immature and mature follicles (Spitzer et al., 1996; Tjugum et al., 1986).

In semen, while controlling for all available confounding factors, it was found that oxytocin was negatively associated with sperm count (standardised β: - 0.332 [-0.004, -0.001], *p* – 0.004) and concentration (standardised β: -0.293 [-0.003, 0], *p* – 0.000). Oxytocin was not associated with volume, progressive motility, total motility or overall normal/abnormal semen parameters. Semen vasopressin concentration was not associated with any of the outcomes analysed. Previous studies has mainly investigated levels of vasopressin in the testes or male reproductive tract within animal models, demonstrating potential involvement in motility and contractions of the epididymis, vas deferens and seminiferous tubules (Wathes, 1984).

Previously, plasma levels of oxytocin was found to have no correlation with sperm motility, count and morphology (Ogawa et al., 1980). In more recent studies, plasma oxytocin had been correlated with negative impacts on sperm (reduced motility, count and increased abnormal morphology) and a lower concentration of oxytocin was found in fertile men than men with idiopathic oligozoospermia, idiopathic asthenozoospermia and obstructive azoospermia which may suggest that an increase in oxytocin negatively impacts human sperm (Lui et al., 2010; Mostafa et al., 2015). Oxytocin in the testis potentially has an autocrine/paracrine role on steroid metabolism, previously shown to have a role in the contractility of seminiferous tubules (Ivell et al., 1997). Any modulation in steroid metabolism or contractility of the seminiferous tubules may have further implications in spermatogenesis and male fertility.

3.5 Conclusion

In this study only oxytocin concentration demonstrated any significance and was found to have a significant negative association with number of oocytes fertilised, sperm count and sperm concentration in men and women undergoing ART. Oxytocin was not associated with the number of oocytes obtained, whether embryos were transferred or frozen, pregnancy outcome, volume, progressive motility, total motility or overall normal/abnormal semen parameters. Neither TNFα nor vasopressin had any statistically significant association with any of the clinical parameters and outcomes.

Chapter 4 – The role of vasopressin in human sperm function

4.0 Vasopressin and Human sperm

4.1 Introduction

Arginine Vasopressin (AVP) (in humans) or vasopressin is a small neuropeptide (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2), which is nine amino acids in length differing by only two from oxytocin (OXT). Vasopressin is synthesised by the supraoptic nucleus (SON), and also in the paraventricular nucleus (PVN) in the hypothalamus of the brain and is stored and secreted by the posterior pituitary (Garrahy and Thompson, 2019).

The primary function of vasopressin in humans is as an antidiuretic and is key to salt/water balance and regulating blood pressure and there is evidence that vasopressin plays a role in animal and human sexual behaviour, maternal aggression, pair bonding and social behaviour (Knafo et al., 2008). The general role of vasopressin/oxytocin family of peptides and its analogues in sexual behaviours is conserved through phylogeny (Reaume and Sokolowski, 2011).

Vasopressin is a derivative from the preprohormone prepropressophysin (also comprised of a copeptin and neurophysin II), the preprohormone is cleaved into the prohormone in the Golgi apparatus and is then stored in secretory vesicles until necessary (Acher et al., 2002; Cuzzo and Lappin, 2019). The prohormone is cleaved into vasopressin before reaching the posterior pituitary where it is released from. Vasopressin is the primary regulator for osmotic homeostasis within the body, in a hyperosmotic state the osmoreceptors within the neurons in the hypothalamus respond swiftly to even slight blood osmolarity changes and secrete vasopressin. Vasopressin then acts in the kidney by binding to the principle cells within the nephron and triggering a cell signalling cascade

through the vasopressin receptor 2, increasing cyclic AMP, activating protein kinase A which phosphorylated target proteins resulting in the translocation of aquaporin-2 channels to the cell surface membrane allowing the passive movement of water into the cell due to the osmotic gradient within the cell (high intracellular concentration of salt and urea) (Boone and Deen, 2008; Davies, 1972).

In hypovolemia (reduced fluid volume of the blood) the baroreceptors are stimulated and trigger the vagus nerve to stimulate the release of vasopressin which promotes the kidneys to reabsorb water and at high enough concentrations vasopressin will also stimulate vasoconstriction via the vasopressin receptor 1a in vascular smooth muscle, stimulating the phospholipase C signalling cascade, produces IP3 and DAG, causing a release of calcium from intracellular stores, DAG and calcium stimulate PKC and this phosphorylates target proteins. Vasopressin can also be stimulated by pain, nicotine, nausea, hypoglycaemia and angiotensin II and is inhibited by alcohol (Boone and Deen, 2008; Schrier and Bichet, 1981).

Vasopressin receptors are found throughout the human male and female reproductive tracts. Vasopressin has 3 different receptors, vasopressin receptor 1a (AVPR1a) (Gq), vasopressin receptor 1b (AVPR1b) (Gq) and vasopressin receptor 2 (AVPR2) (Gs) as described in Figure 8.

The AVPR1a receptor is primarily localised in brain, liver, kidney and throughout peripheral vasculature, this receptor is responsible for vasoconstriction, platelet aggregation and release of factor VIII as well as gluconeogenesis in the liver (Tiwari and Ecelbarger, 2018). Study in humans found correlation between receptor expression and extra-pair mating in females but not males (Zietsch et al., 2015). Cerebrospinal fluid vasopressin levels in humans correlated with a history of aggression (Coccaro et al., 1998).

AVPR1b is also involved in homeostasis in the regulation of blood osmolality, mediates corticotropin secretion and is localised primarily to the anterior pituitary (Stewart et al., 2008). The AVPR1b receptor has been shown to be involved in behaviour in animal studies; a mouse knockout model showed reduced aggression and the use of a AVPR1b specific agonist in hamsters also showed reduced aggression (Blanchard et al., 2005; Wersinger et al., 2002). In humans there is also evidence for AVPR1bs involvement in behaviour, a single nucleotide polymorphism of this receptor has been implicated in depression within humans (Van West et al., 2004).

AVPR2 is primarily responsible for free water reabsorption in the renal medullar via translocation of AQP2 channels to the cell surface membrane, AQP2 allows for water influx into the cell and therefore AVPR2 is responsible for concentrating the urine, disfunction in this receptor or AQP2 causes nephrogenic diabetes insipidus (Bichet, 2006). In vascular endothelium AVPR2 stimulation causes release of von Willebrand factor and factor VIII (Jackson, 2018).

In multiple species including humans, vasopressin has been found to be present in the suprachiasmatic nuclei (SCN), which is responsible for control of circadian rhythms, and a daily rhythm of vasopressin synthesis and neurones expressing vasopressin in the SCN are present in these multiple species (Kalsbeek et al., 2010). This suggests that vasopressin is important in regulating the HPG axis as the circadian rhythm is involved in many aspects of reproduction such as regulating pulsatile activity of LH, vasopressin has been shown to have a direct stimulatory effect on the LH surge (Palm et al., 2001).

The vasopressin receptor 1a/oxytocin receptor antagonist (atosiban) has been demonstrated to improve pregnancy rates in IVF patients with recurrent failures, this is suggested to be due to inhibiting contractile activity in the uterus post embryo transfer (Pierzynski, 2011).

Vasopressin has been detected in seminal plasma at various levels, between those similar to plasma and in the previous chapter at higher levels (Brotherton, 1990). Vasopressin has been found to stimulate contractile activity in the seminiferous tubules (Harris and Nicholson, 1998) and it's receptors have been found to be expressed in testis (Assinder et al., 2000b; Kasson et al., 1985). Studies in several mammals demonstrated an influence in male reproduction; an increase in seminal volume (rabbit), a high urine concentration of vasopressin was correlated with a decrease in sperm count and decreased motility (human) and decreased motility in mouse sperm (Kihlström and Ågmo, 1974; Puri and Puri, 1985; Śliwa, 1994). In a 2013 study, the vasopressin receptor 2 was found in mouse sperm and incubation with dDAVP was found to inhibit sperm motility, increase calcium concentration, decrease intracellular pH and decrease PKA phosphorylation (Kwon et al., 2012). The effects of vasopressin on human sperm have not been effectively elucidated to date.

Hypothesis (experimental): Sperm function is modulated by vasopressin and its downstream signalling cascade.

Aim:

- To investigate the presence of vasopressin receptors on human sperm.
- To investigate the effect of vasopressin on human sperm through treatments with agonists in a set of *in vitro* experiments.

To investigate the role of vasopressin in human sperm function the following objectives will be performed:

- Investigate presence of vasopressin receptors on human sperm using immunocytochemistry.
- Incubate human sperm with vasopressin and dDAVP *in vitro* and assess sperm motility and kinematics using CASA and MATLAB.
- Investigate regulation of phosphotyrosine (and pPKA) after vasopressin and dDAVP treatment.
- Explore the presence of AQP2 channels on sperm via immunocytochemistry and western blot.
- Investigate a vasopressin-induced calcium response in sperm using a Fluorescence Assays and live cell imaging.
- Investigate the role of vasopressin and dDAVP on the acrosome reaction using FITC-PSA and SEM

4.2 Methods

Consenting donors (18 +) were recruited under faculty approved ethics (Faculty Ethics approval: SE1617126). Semen samples were separated from seminal plasma as described in section 2.3. Immunolocalisation of the vasopressin receptor 2 was investigated with immunofluorescent staining (section 2.5), motility and sperm kinematics were measured using CASA (section 2.2). To analyse any calcium response in sperm treated with vasopressin was measured via fluorescence microplate reader and live cell imaging, acrosome reaction was investigated by both FITC-PSA immunofluorescent staining and scanning electron microscopy, oxygen consumption rate and extracellular acidification rate were measured using Seahorse analysis, the presence of AQP2 channels was investigated using dot blots and western blot analysis (see methods, section 2.8). Anti-Phosphotyrosine antibody [PY20] (Abcam, ab10321) was used to investigate capacitation in sperm treated with vasopressin and dDAVP through western blot analysis (methods, section 2.8)

4.3 Results

4.3.1 Immunolocalisation of vasopressin receptor 2 on human sperm

Human sperm were separated from seminal plasma, fixed and examined by immunofluorescence using anti-AVPR2 antibody (see Methods section 2.5). Sperm had intense AVPR2 labelling on the acrosomal region (Figure 23, A (insert)). There was no unspecific labelling (Figure 23, B and C).

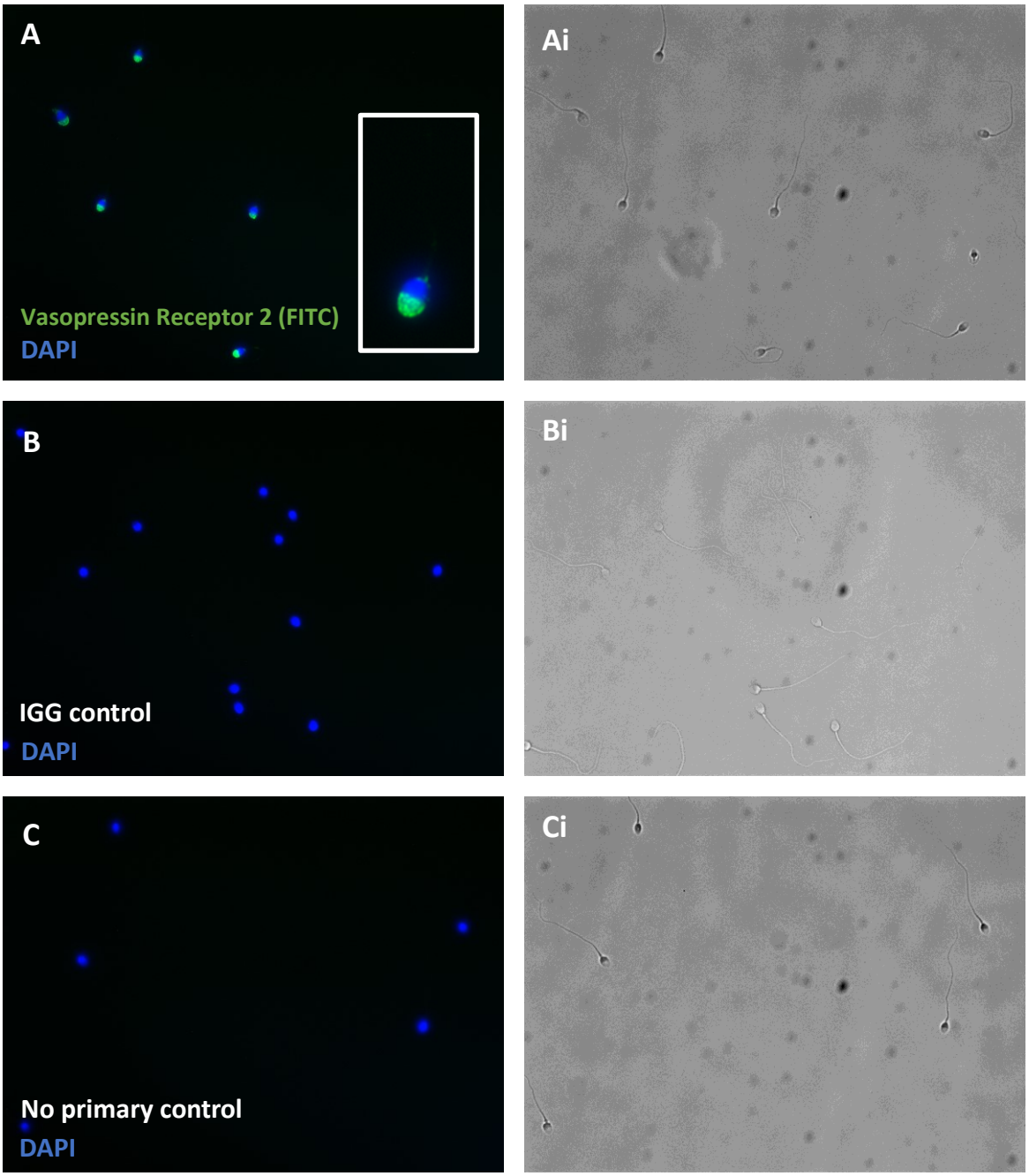

Figure 23. Immunolocalisation of the vasopressin receptor 2 in human sperm cells. Vasopressin receptor 2 (green) localised on the acrosome of human sperm (A, inset). IGG control on human sperm (B). No primary control on human sperm (C). Nuclear labelling with DAPI in all (blue). Phase contrast micrographs of each image (Ai, Bi, Ci). Representative of n = 10. 630 x.

4.3.2 Sperm motility and kinematics

Sperm motility and kinematics are important parameters in determining function *in vitro*. The motility and way that sperm swim are indicative of normal function, sperm that are immotile or unable to swim linearly will be unlikely to fertilise an oocyte (Wallach et al., 1992).

Human sperm were isolated from the seminal plasma using density gradient centrifugation. Sperm were resuspended to 10 million/mL and incubated with serial dilutions of vasopressin and dDAVP for 1 hour (10 pM, 10 nM and 10 µM). Sperm motility was assessed using CASA. Sperm kinematics were assessed using the coordinates of the paths generated in the CASA, a MATLAB script was used to assess the straight line path (VSL µm/s), the curvilinear path (VCL µm/s) and the linearity of the sperm path (LIN %) LIN = VSL/VCL x100.

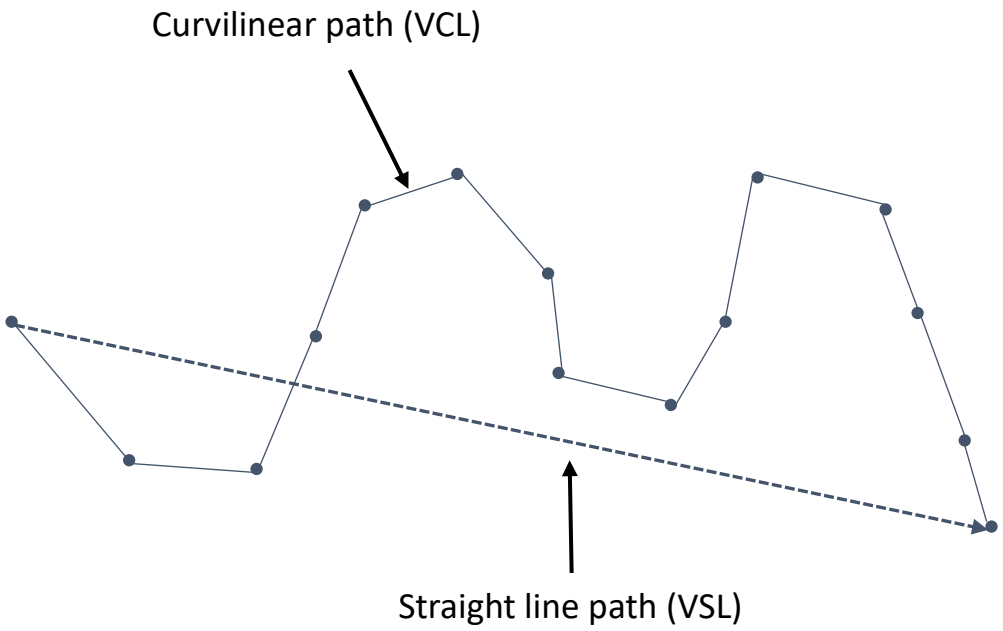

Figure 24. Terminology for variables measured in CASA. Adapted from WHO (2010)

4.3.2.1 Sperm motility

Sperm motility is an important part of semen analysis and is sperm motility is essential for reproduction (Donnelly et al., 1998). Progressive sperm motility is used as an indicator for sperm health and below 32% progressively motile sperm in a sample is considered abnormal (asthenozoospermia). In order to calculate progressive motility grade A and grade B sperm were combined (World Health Organization, 2010a). To eliminate the

baseline variation between participants prior to statistical analysis, the data were normalised to each participant's baseline (vehicle control). The distribution of the motility data was analysed using the Shapiro-Wilk test in GraphPad Prism and was found to be normally distributed and therefore was analysed using a one-way ANOVA and Dunnett's test as a post-hoc analysis for multiple comparisons.

Overall differences between groups was observed at F (7, 52) = 2.34, p = 0.037. The post-hoc analysis showed a statistically significant increase in progressive motility in the 10 µM vasopressin treatment group at p = 0.019 when compared to vehicle control. No other treatment groups showed statistically significant differences in progressive motility when compared to the vehicle control (Figure 25, Table 44).

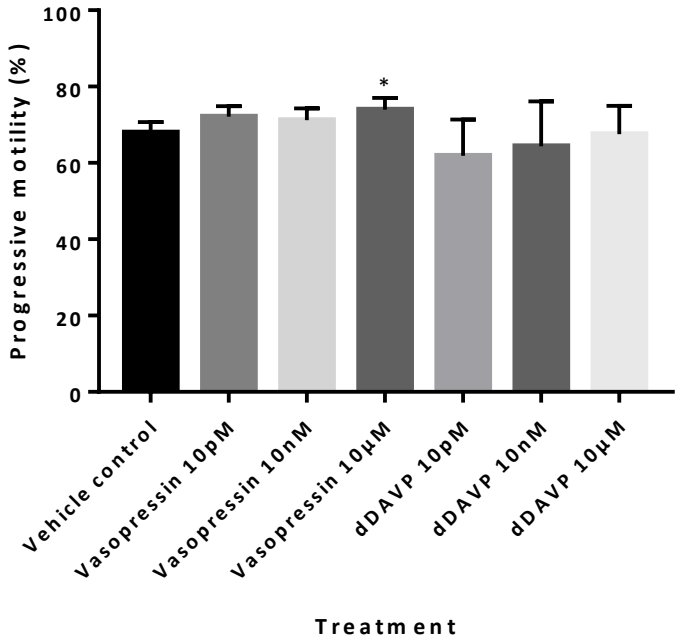

Figure 25. Percentage difference progressive motility after 1 hour incubation with serial dilutions of vasopressin and dDAVP. Each participants data was normalised to their control and analysed using a two tailed T-Test. Statistically significant differences were found at 10 pM, 10 nM and 10 µM dDAVP. * indicates $p < 0.05$. n = 10.

Table 44. Delta progressive motility following 1 hour incubation with vasopressin or dDAVP. One-Way ANOVA for overall differences and Dunnett's multiple comparisons test for analysis of differences between treatment groups compared to vehicle control.

One-Way ANOVA progressive motility	
F (DFn, DFd)	**P value**
F (7, 52) = 2.34	P=0.0373

Dunnett's multiple comparisons test	**95% CI of diff.**	**Adjusted P Value**
Vasopressin 10pM	-0.1883 to 0.05127	0.4814
Vasopressin 10nM	-0.182 to 0.05757	0.5813
Vasopressin 10µM	-0.2369 to -0.01517	0.0194
dDAVP 10pM	-0.2921 to 0.2733	0.9999
dDAVP 10nM	-0.3674 to 0.198	0.9416
dDAVP 10µM	-0.2946 to 0.019	0.1064

4.3.2.2 Sperm linearity

Sperm linearity is the measure of the linearity of the curvilinear path as a ratio between the straight line velocity and curvilinear velocity (VSL/VCL x 100). Linearity is a kinematic assessment. To investigate if VP and dDAVP modulates sperm kinematics further, human sperm linearity was calculated from the sperm track coordinates provided from the CASA (SAMi software) using Matlab script. The data were normalised to each individual participants baseline (vehicle control) prior to any statistical analysis to control for individual baseline variation (delta linearity). Normality was investigated using the Shapiro-Wilk test in Graph-Pad Prism. Linearity was found to be not normally distributed and therefore the non-parametric Kruskal-Wallis analysis was used with Dunn's multiple comparison test for post-hoc analysis of differences between treatment groups. Overall significance of differences between groups was found at <0.0001. Statistically significant differences were found in the 10 nM vasopressin, 10 µM vasopressin and 10 µM dDAVP treatment conditions when compared to the control at p = 0.000 for the three treatment conditions. The 10 nM and 10 µM vasopressin treatments increased sperm linearity whereas 10 µM dDAVP reduced sperm linearity. No other treatment condition was found to be statistically significant. N = 3 (Figure 26, Table 45).

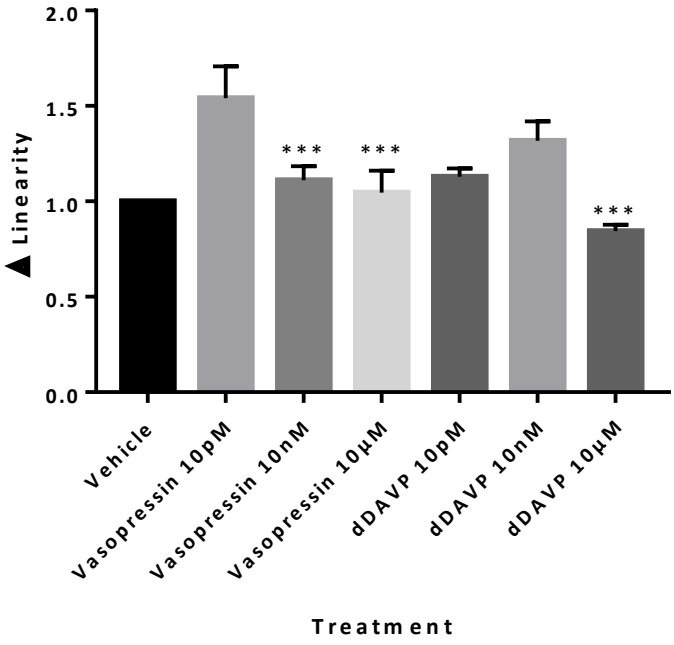

Figure 26. Sperm linearity after 1 hour incubation with serial dilutions of vasopressin and dDAVP. Analysed using Kruskal-Wallis test and Dunn's multiple comparisons test. Statistically significant differences found at 10 nM, 10 µM vasopressin and 10 µM dDAVP., *** indicates p < 0.001. n = 3.

Table 45. Delta linearity following 1 hour incubation with vasopressin or dDAVP. Kruskal-Wallis test for overall differences and Dunn's multiple comparisons test for analysis of differences between treatment groups compared to vehicle control.

Kruskal-Wallis test Linearity		
P value	<0.0001	
Dunn's multiple comparisons test	**Mean rank diff.**	**Adjusted P Value**
Vasopressin 10pM	-46.76	>0.9999
Vasopressin 10nM	168.8	0.0002
Vasopressin 10µM	299.9	<0.0001
dDAVP 10pM	45.14	>0.9999
dDAVP 10nM	6.943	>0.9999
dDAVP 10µM	358.7	<0.0001

4.3.2.3 Sperm curvilinear velocity (VCL)

Human sperm curvilinear velocity (VCL) was calculated from the sperm track coordinates provided from the CASA (SAMi software) using Matlab script. The data were normalised to each individual participants baseline (vehicle control) prior to any statistical analysis to control for individual baseline variation (delta VCL). Normality was investigated using the Shapiro-Wilk test in Graph-Pad Prism. VCL was found to be not normally distributed and therefore the non-parametric Kruskal-Wallis analysis was used with Dunn's multiple comparison test for post-hoc analysis of differences between treatment groups. Overall significance of differences between groups was found at <0.0001. Statistically significant differences were found in the 10 pM, 10 nM, 10 µM vasopressin and 10 pM, 10 nM dDAVP treatment conditions when compared to the control at p = 0.000 for the five treatment conditions. The vasopressin and dDAVP treatments increased sperm VCL. The 10 µM dDAVP treatment was not found to be statistically significant. N = 3. (Figure 27, Table 46).

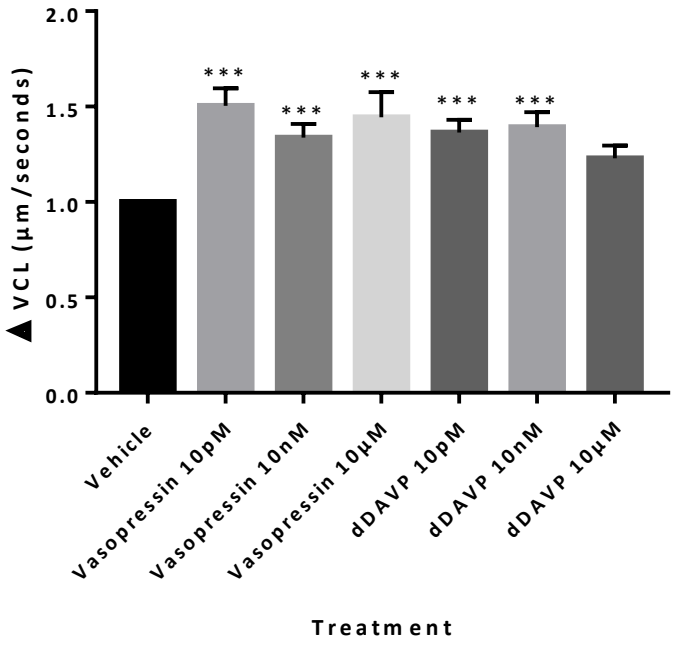

Figure 27. Sperm VCL after 1 hour incubation with serial dilutions of vasopressin and dDAVP. Analysed using Kruskal-Wallis test and Dunn's multiple comparisons test. Statistically significant differences found at 10pM, 10 nM, 10 µM vasopressin and 10 pM, 10 nM dDAVP., *** indicates $p < 0.001$. n = 3.

Table 46. Delta VCL following 1 hour incubation with vasopressin or dDAVP. Kruskal-Wallis test for overall differences and Dunn's multiple comparisons test for analysis of differences between treatment groups compared to vehicle control.

Kruskal-Wallis test VCL		
P value	<0.0001	
Dunn's multiple comparisons test	**Mean rank diff.**	**Adjusted P Value**
Vasopressin 10pM	-320.8	<0.0001
Vasopressin 10nM	-192.1	<0.0001
Vasopressin 10µM	-259.5	<0.0001
dDAVP 10pM	-260.9	<0.0001
dDAVP 10nM	-255.5	<0.0001
dDAVP 10µM	-87.97	0.1936

4.3.2.4 Sperm straight line velocity (VSL)

Human sperm straight line velocity (VSL) was calculated from the sperm track coordinates provided from the CASA (SAMi software) using Matlab script. The data were normalised to each individual participants baseline (vehicle control) prior to any statistical analysis to control for individual baseline variation (delta VSL). Normality was investigated using the Shapiro-Wilk test in Graph-Pad Prism. VSL was found to be not normally distributed and therefore the non-parametric Kruskal-Wallis analysis was used with Dunn's multiple comparison test for post-hoc analysis of differences between treatment groups. Overall significance of differences between groups was found at <0.0001. Statistically significant differences were found in the 10 pM, 10 nM, 10 µM vasopressin and 10 µM dDAVP treatment conditions when compared to the control at p = 0.000 for the four treatment conditions. The 10 pM vasopressin treatment increased sperm VSL and the 10 nM and 10 µM vasopressin and 10 µM dDAVP treatments decreased sperm VSL. No other treatment was found to be statistically significant. N = 3. (Figure 28, Table 47).

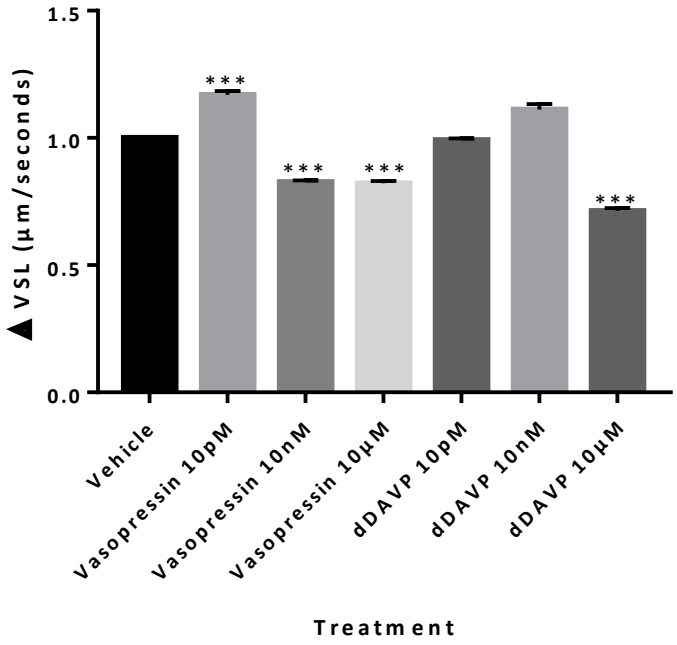

Figure 28. Sperm VSL after 1 hour incubation with serial dilutions of vasopressin and dDAVP. Analysed using Kruskal-Wallis test and Dunn's multiple comparisons test. Statistically significant differences found at 10 pM, 10 nM, 10 µM vasopressin and 10 µM dDAVP., *** indicates p < 0.001. n = 3

Table 47. Delta VSL following 1 hour incubation with vasopressin or dDAVP. Kruskal-Wallis test for overall differences and Dunn's multiple comparisons test for analysis of differences between treatment groups compared to vehicle control.

Kruskal-Wallis test VSL	
P value	<0.0001

Dunn's multiple comparisons test	Mean rank diff.	Adjusted P Value
Vasopressin 10pM	-255.6	<0.0001
Vasopressin 10nM	543.2	<0.0001
Vasopressin 10µM	538.6	<0.0001
dDAVP 10pM	99.82	0.0949
dDAVP 10nM	37	>0.9999
dDAVP 10µM	697.7	<0.0001

4.3.3 Intracellular calcium measurements

4.3.3.1 Microplate fluorescence assay

Capacitation and acrosome reaction are prerequisite maturation steps that are essential for fertilisation to be possible. Capacitation and acrosome reaction are mediated via PKA and protein tyrosine phosphorylation, which is dependent on the influx and efflux of calcium in the cell (Puga Molina et al., 2018). Calcium assays are an effective measure of capacitation and acrosome reaction in sperm from the cell as specific ligands may trigger these signalling cascades indicating a role in either capacitation, acrosome reaction or both (Parodi, 2014).

Human sperm were isolated from the seminal plasma. Sperm were loaded with Fluo-4 AM (4µM) and were resuspended to a concentration of 10 million/mL and loaded into the wells of a 96 well plate for fluorescence coated with poly-D-lysine. F0 (starting background fluorescence) was read for 45 seconds then the individual treatments were added to the relative wells and read for a further 10 minutes. Sperm were treated with 10 µM ionophore, 3 µM progesterone, 10 µM, 10 nM and 10 pM vasopressin, 10 µM, 10 nM and 10 pM dDAVP, 10 µM oxytocin* and DMSO as the vehicle control. Each well was normalised to its baseline (F0) prior to statistical analysis. (Figure 29)

[*Although the oxytocin receptor was not localised on human sperm (data not included) – it can elicit a intracellular response via the vasopressin receptor (Song and Albers, 2018)]

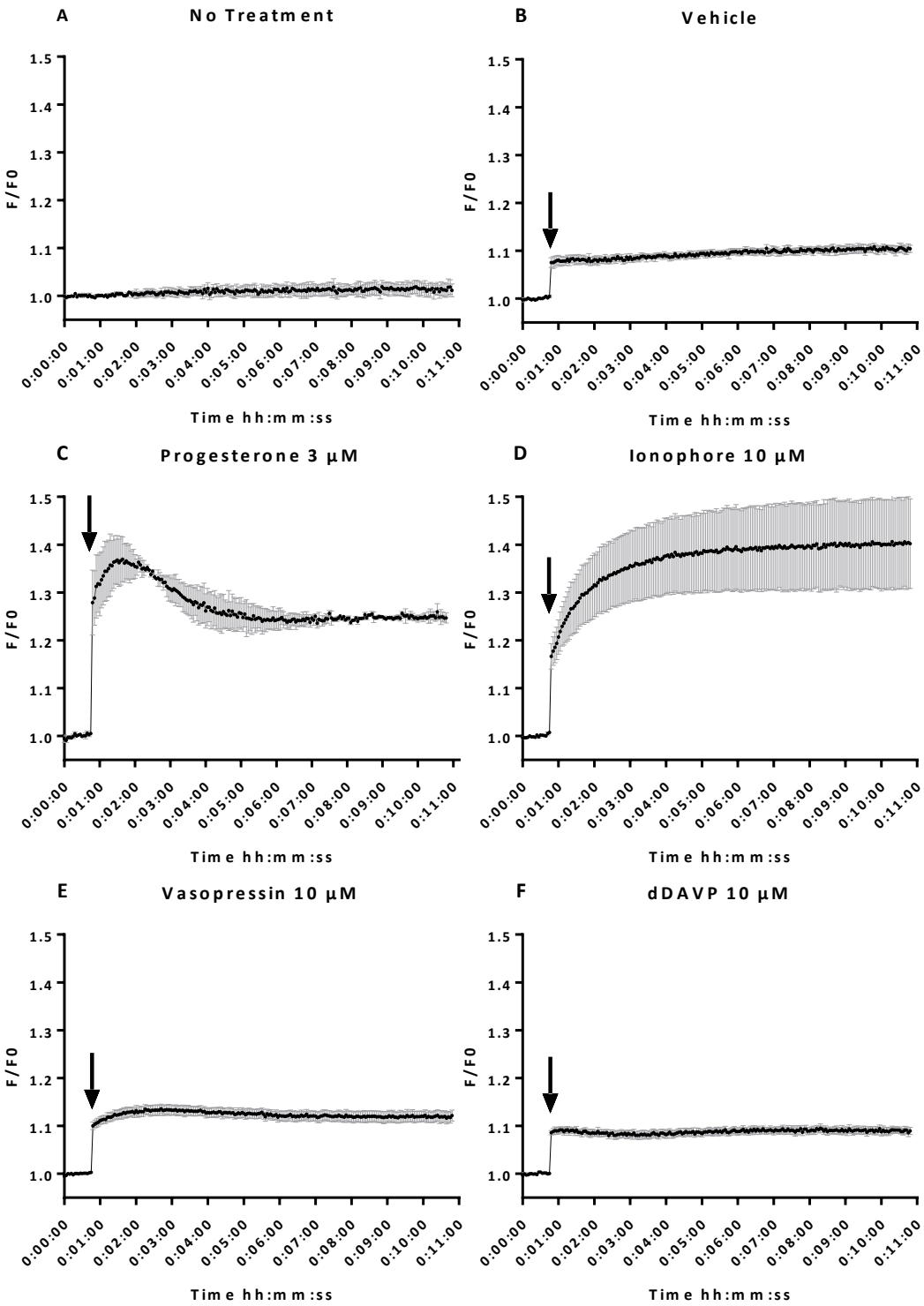

Figure 29. Average calcium traces over 10 minutes and 45 seconds with injection of compounds. Sperm cells loaded with calcium indicator Fluo4-AM. A – no treatment, B – Vehicle control (DMSO), C – progesterone 3 µM, D – calcium ionophore A23187 10 µM, E – vasopressin 10 µM, F – dDAVP 10 µM. Arrow indicates time of injection (45 seconds). N = 4.

Differences between treatments were assessed using a one-way ANOVA the overall test was statistically significant at $p < 0.000$. The Dunnett's test for multiple comparison was used to analyse differences between each treatment compared to the vehicle control.

Statistically significant differences in calcium response were observed when compared to the vehicle control in the 10 pM vasopressin ($p = 0.0001$), 10 nM vasopressin ($p = 0.0403$), 10 µM vasopressin ($p = 0.0001$), 10 pM dDAVP ($p = 0.0001$), 10 µM ionophore ($p = 0.0001$), 3 µM progesterone ($p = 0.0001$). N = 4. (Figure 30, Table 48).

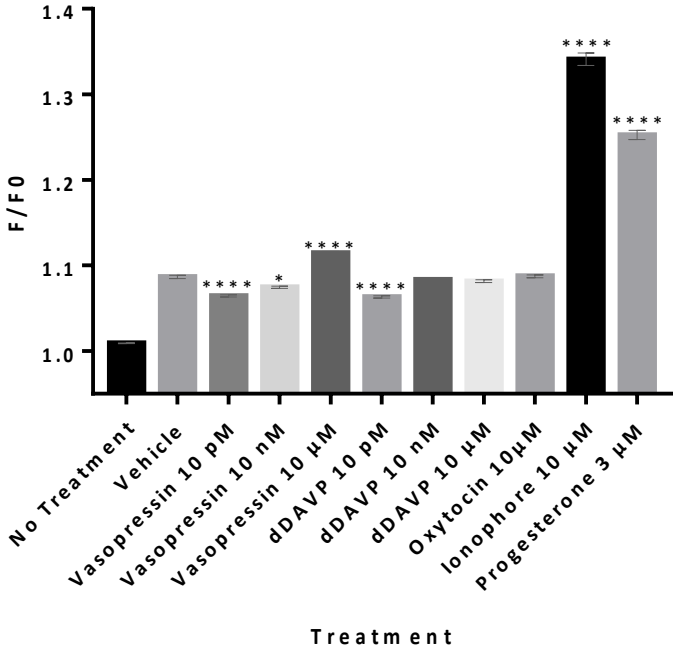

Figure 30. Differences between calcium traces of sperm treated with vasopressin, dDAVP, oxytocin, ionophore and progesterone. Significance determined via One-Way ANOVA and Dunnett's multiple comparisons test. **** indicates $p < 0.0001$, * indicates $p < 0.05$. N = 4.

Table 48. Significance of calcium response in sperm treated with vasopressin, dDAVP, oxytocin, ionophore and progesterone. One-Way ANOVA for overall differences and Dunnett's multiple comparisons test to investigate differences between the treatments and vehicle control. N = 4.

One-Way ANOVA			
F (DFn, DFd)		F (10, 2376) = 961.2	
P value		P<0.0001	
Dunnett's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Adjusted *P* Value**
Vasopressin 10 pM	0.02243	0.01055 to 0.03431	0.0001
Vasopressin 10 nM	0.01222	0.000341 to 0.0241	0.0403
Vasopressin 10 µM	-0.02752	-0.0394 to -0.01564	0.0001
dDAVP 10 pM	0.02357	0.01169 to 0.03545	0.0001
dDAVP 10 nM	0.003275	-0.008605 to 0.01515	0.9887
dDAVP 10 µM	0.005186	-0.006693 to 0.01707	0.8281
Oxytocin 10µM	-0.00067	-0.01255 to 0.01121	0.9998
Ionophore 10 µM	-0.2543	-0.2662 to -0.2424	0.0001
Progesterone 3 µM	-0.1658	-0.1777 to -0.1539	0.0001

4.3.3.2 Live cell calcium imaging

Single cell calcium imaging was carried out to establish spatiotemporal patterns of calcium signalling after treatment vasopressin (10 µM) and dDAVP (10 µM) Progesterone (500nM) was used as a positive control (Figure 33) as per methods section 2.13.2.

Out of the 175 cells analysed for responses to vasopressin treatment 85.7% of them responded to progesterone, any unresponsive cells were not further assessed. Of the 150 cells that responded to progesterone 10.7% of them responded to the vasopressin treatment (Figure 31).

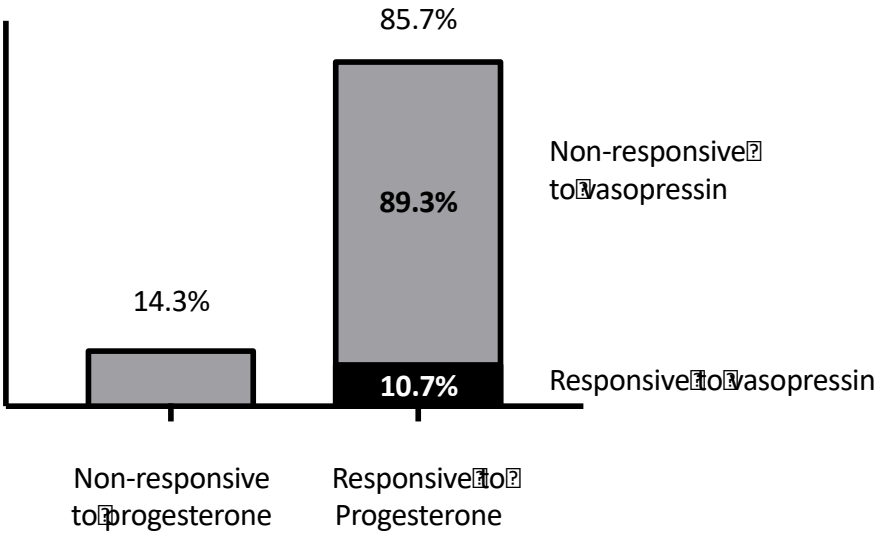

Figure 31. Proportion of cells responsive to the positive control progesterone and of those the proportion responsive to vasopressin treatment.

Out of the 492 cells analysed for responses to dDAVP treatment 77% of them responded to progesterone, any unresponsive cells were not further assessed. Of the 379 cells that responded to progesterone 19.5% of them responded to the dDAVP treatment (Figure 32).

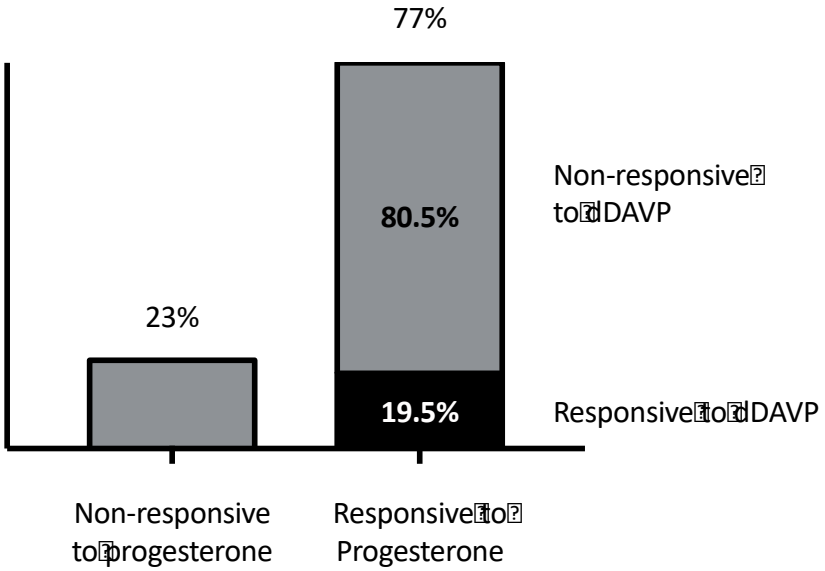

Figure 32 Proportion of cells responsive to the positive control progesterone and of those the proportion responsive to dDAVP treatment.

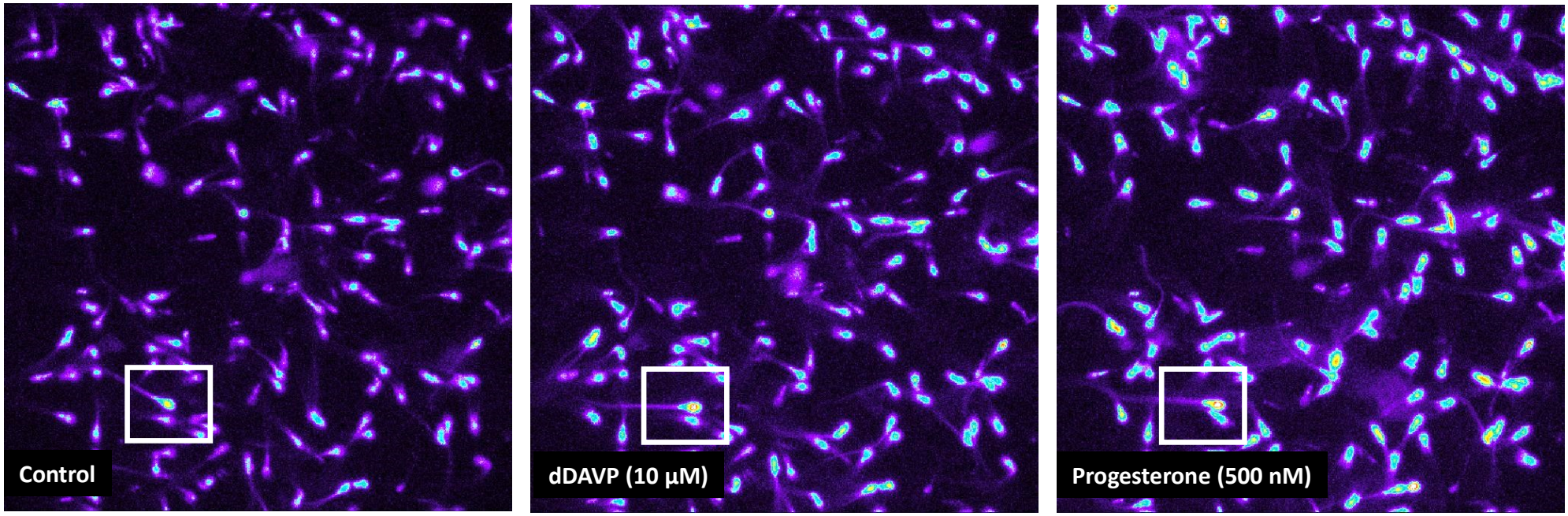

Figure 33. Fluorescent micrograph of sperm loaded with calcium green demonstrating response to dDAVP and progesterone treatment.

Sperm cells loaded with calcium green 1AM and perfused with vasopressin showed a mild transient response. At 6 minutes the sperm were perfused with progesterone, which induced a larger calcium response (Figure 34).

The first 4 minutes of the trace was baseline calcium levels.

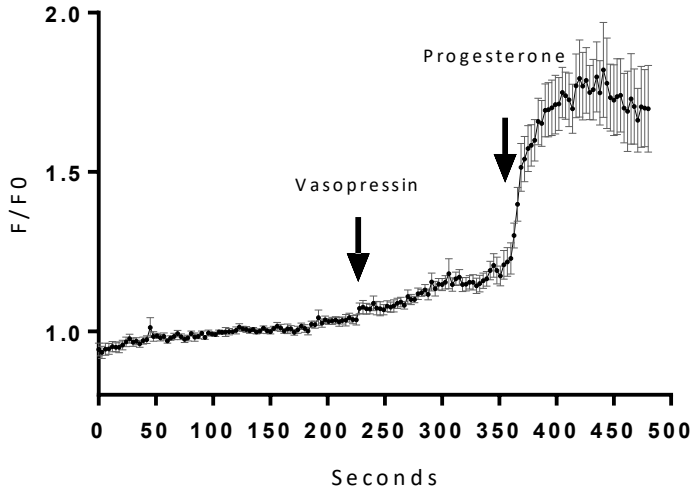

Figure 34. Average calcium traces of live sperm cells which responded to vasopressin over 8 minutes. Sperm cells loaded with calcium indicator calcium green. Arrow indicates time of perfusion of vasopressin (4 minutes) and progesterone (6 minutes). N = 3.

The cells that responded to vasopressin were then statistically analysed using a repeated measures ANOVA. The overall differences were statistically significant at $p < 0.0001$, the Tukey's multiple comparisons test was used for post-hoc analysis of differences between treatment periods and statistically significant differences were found between all treatment groups (control, vasopressin 10 μM and progesterone 500 nM) at adjusted $p < 0.0001$ (Figure 35, Table 49).

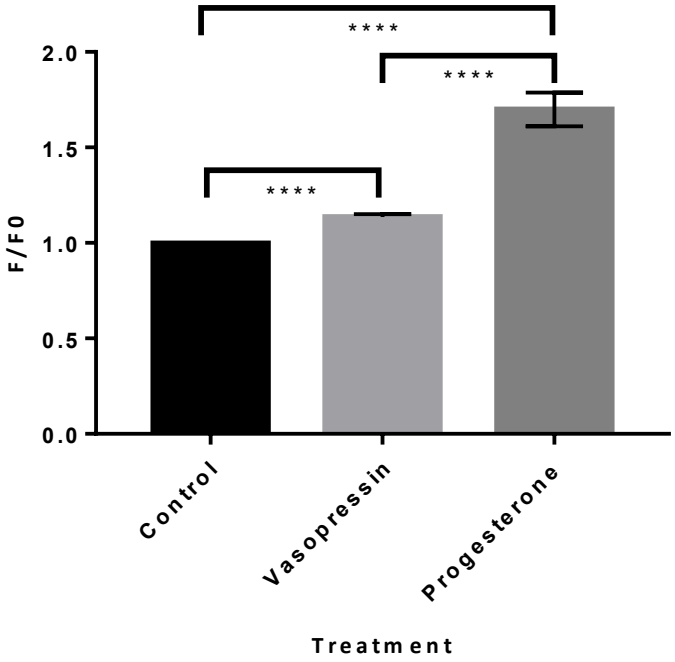

Figure 35. Differences in calcium response between control period, vasopressin and progesterone perfusion in live sperm which responded to vasopressin treatment. N = 3.

Table 49. Repeated measures ANOVA for overall differences in treatments with vasopressin and progesterone and Tukey's multiple comparisons test for differences between treatments.

Repeated Measures ANOVA			
F (DFn, DFd)		F (1.043, 15.65) = 55.12	
***P* value**		P<0.0001	
Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Adjusted *P* Value**
Control vs. Vasopressin	-0.1384	-0.1786 to -0.09829	<0.0001
Control vs. Progesterone	-0.702	-0.932 to -0.4721	<0.0001
Vasopressin vs. Progesterone	-0.5636	-0.7805 to -0.3466	<0.0001

Sperm cells perfused with dDAVP elicited a calcium peak, which was followed by a progesterone-induced response after 6 minutes (Figure 34, Figure 36).

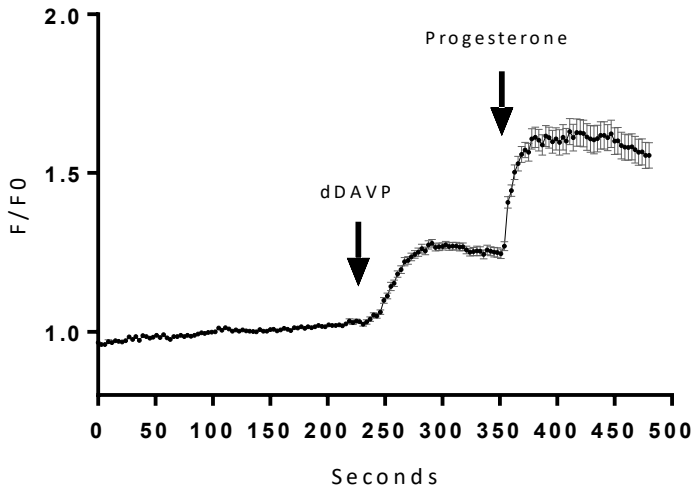

Figure 36. Average calcium traces of live sperm cells which responded to dDAVP over 8 minutes . Sperm cells loaded with calcium indicator calcium green. Arrow indicates time of perfusion of dDAVP (4 minutes) and progesterone (6 minutes). N = 3.

The cells that responded to dDAVP were then statistically analysed using a repeated measures ANOVA. The overall differences between treatment and controls were significant at $p < 0.000$, the Tukey's multiple comparisons test was used for post-hoc analysis of differences between treatment periods and significant differences were found between all treatment groups (control, dDAVP 10 μM and progesterone 500 nM) at adjusted $p < 0.000$ (Figure 37, Table 50).

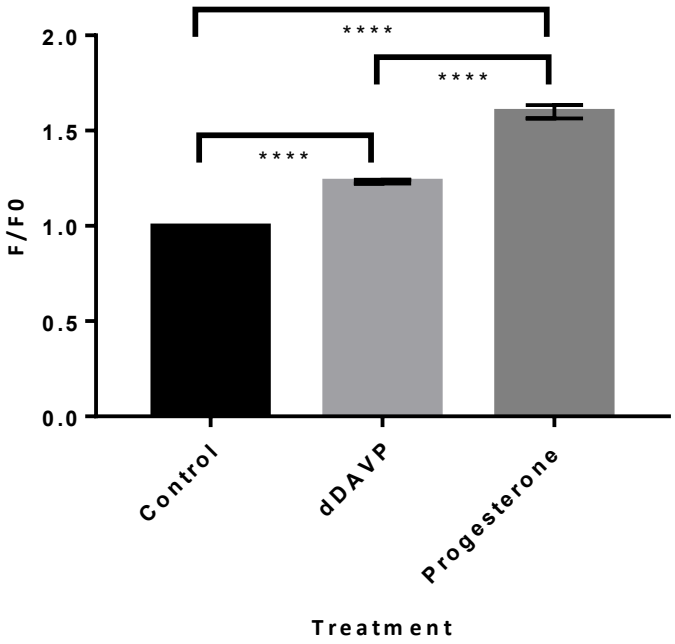

Figure 37. Differences in calcium response between control period, dDAVP and progesterone perfusion in live sperm which responded to vasopressin treatment. N = 3.

Table 50. Repeated measures ANOVA for overall differences in treatments with dDAVP and progesterone and Tukey's multiple comparisons test for differences between treatments.

Repeated Measures ANOVA			
F (DFn, DFd)		F (1.115, 81.36) = 220	
***P* value**		P<0.0001	
Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Adjusted *P* Value**
Control vs. dDAVP	-0.2343	-0.257 to -0.2115	<0.0001
Control vs. Progesterone	-0.6003	-0.6836 to -0.517	<0.0001
dDAVP vs. Progesterone	-0.366	-0.4487 to -0.2834	<0.0001

4.3.4 Phospho-tyrosine western blots

Protein tyrosine phosphorylation is involved in capacitation and results in the hyperactivation and acrosome reaction of sperm (Naz and Rajesh, 2004). To examine how vasopressin may regulate sperm capacitation - protein tyrosine phosphorylation was investigated using western blot using anti-phosphotyrosine antibody on human sperm samples treated with serial dilutions of vasopressin and dDAVP. The samples were normalised to β-tubulin and checked for normal distribution using Shapiro-Wilk test in GraphPad Prism. Relative intensity was analysed using a repeated measures One-Way ANOVA and Dunnett's multiple comparisons test and for non-parametric the Friedman's test and Dunn's multiple comparisons test. No treatment was found to be statistically significant. N = 3. (**Figure *38***, **Figure *39***, **Table *51***, **Table *52***, **Table *53***, **Table *54***).

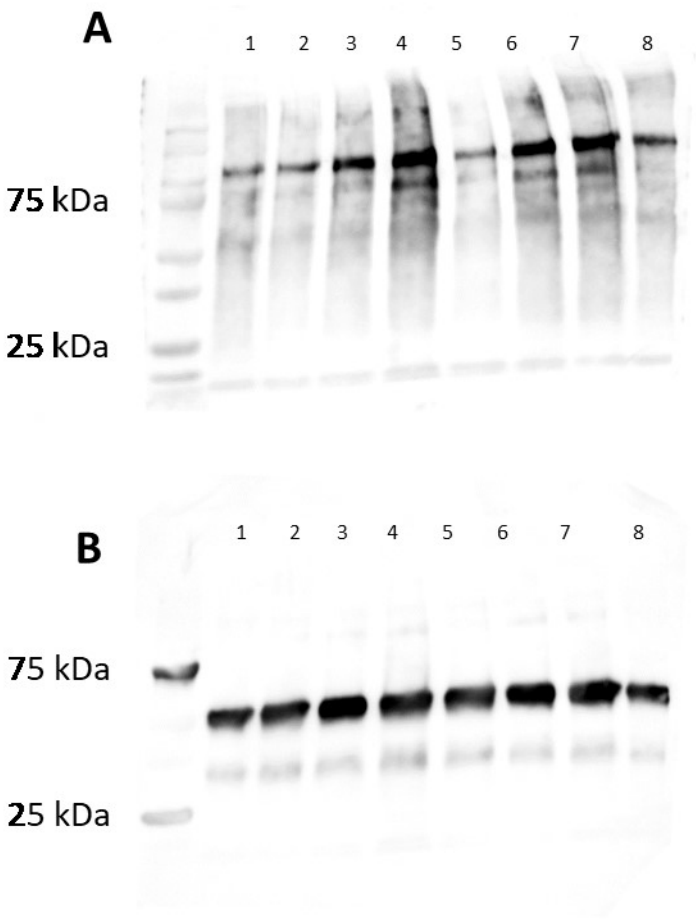

Figure 38. Western blots to detect phospho-tyrosine in protein lysates from sperm treated with vasopressin and dDAVP. A – phospho protein tyrosine. B – β-tubulin. Lane 1 – no treatment, lane 2 – vehicle control, lane 3 – vasopressin 10 pM, lane 4 – vasopressin 10 nM, lane 5 – vasopressin 10 µM, lane 6 – dDAVP 10 pM, lane 7 – dDAVP 10 nM, lane 8 – dDAVP 10 µM. Representative of n = 3.

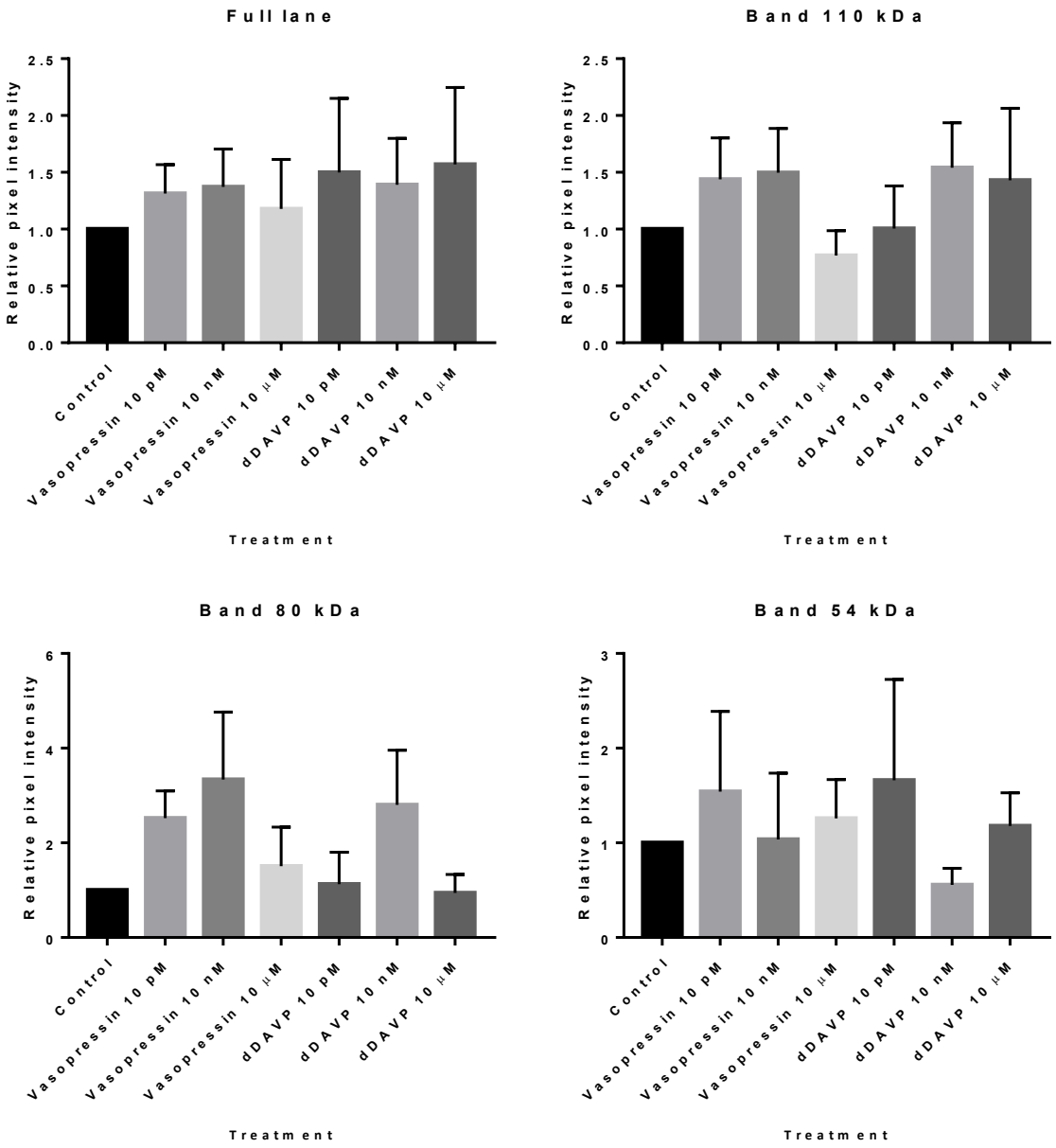

Figure 39. Relative pixel intensity of western blots of sperm treated with vasopressin and dDAVP. Relative pixel intensity of the full lane, band 1 – 110 kDa, band 2 – 80 kDa and band 3 – 54 kDa. N = 3.

Table 51. Repeated measures One-Way ANOVA for overall differences in all treatments for the full lane and Dunnett's multiple comparisons test for differences between treatments and vehicle control.

Full lane RM one-way ANOVA	
F (DFn, DFd)	**P value**
F (1.652, 3.304) = 0.2696	P=0.7429

Dunnett's multiple comparisons test		
	95% CI of diff.	**Adjusted P Value**
Vasopressin 10 pM	-2.115 to 1.486	0.7041
Vasopressin 10 nM	-2.742 to 1.997	0.7589
Vasopressin 10 µM	-3.282 to 2.928	0.9916
dDAVP 10 pM	-5.143 to 4.146	0.908
dDAVP 10 nM	-3.295 to 2.513	0.8302
dDAVP 10 µM	-5.401 to 4.262	0.8797

Table 52. Repeated measures One-Way ANOVA for overall differences in all treatments for band 1 and Dunnett's multiple comparisons test for differences between treatments and vehicle control.

Band 1 RM one-way ANOVA	
F (DFn, DFd)	**P value**
F (1.395, 2.79) = 1.23	P=0.3847

Dunnett's multiple comparisons test		
	95% CI of diff.	**Adjusted P Value**
Vasopressin 10 pM	-3.035 to 2.157	0.7213
Vasopressin 10 nM	-3.277 to 2.285	0.6921
Vasopressin 10 µM	-1.329 to 1.793	0.7861
dDAVP 10 pM	-2.671 to 2.66	0.9999
dDAVP 10 nM	-3.364 to 2.282	0.6518
dDAVP 10 µM	-4.956 to 4.097	0.9378

Table 53. Friedman's test for overall differences in all treatments for band 2 and Dunn's multiple comparisons test for differences between treatments and vehicle control.

Band 2 Friedman test	
P value	0.0929

Dunn's multiple comparisons test		
	Rank sum diff.	**Adjusted P Value**
Vasopressin 10 pM	-9	0.5338
Vasopressin 10 nM	-11	0.2258
Vasopressin 10 µM	-3	>0.9999
dDAVP 10 pM	2	>0.9999
dDAVP 10 nM	-7	>0.9999
dDAVP 10 µM	0	>0.9999

Table 54. Friedman's test for overall differences in all treatments for band 3 and Dunn's multiple comparisons test for differences between treatments and vehicle control.

Band 3 Friedman test	
P value	0.8438

Dunn's multiple comparisons test		
	Rank sum diff.	**Adjusted P Value**
Vasopressin 10 pM	0	>0.9999
Vasopressin 10 nM	5	>0.9999
Vasopressin 10 µM	2	>0.9999
dDAVP 10 pM	0	>0.9999
dDAVP 10 nM	6	>0.9999
dDAVP 10 µM	1	>0.9999

4.3.5 Acrosome reaction

The acrosome is a structure on the anterior part of the head of the sperm containing essential enzymes believed to facilitate the penetrance of the oocyte zona pellucida. In order to release these enzymes the sperm must undergo the exocytotic process of the acrosome reaction. Impairments in the ability for sperm to undergo acrosome reaction, or premature acrosome reaction are indicators of sperm function (Fénichel et al., 1991).

In order to assess the acrosomal status of human sperm FITC-PSA was used. Human sperm were capacitated and treated with either 10 µM vasopressin or 10 µM Ionophore with DMSO as a vehicle control. The acrosomal status was statistically analysed using an independent t-test. Statistically significant differences were observed in the vasopressin treatment at $p = 0.006$ when compared to the vehicle control.

Previous studies have reported the acrosome reaction assay scoring in to three classes of lectin labelling (Glenn et al., 2007). In the present study acrosome reaction was investigated using FITC-PSA and 3 classifications of acrosomal status was observed; intact, partially reacted and fully reacted Figure 40.

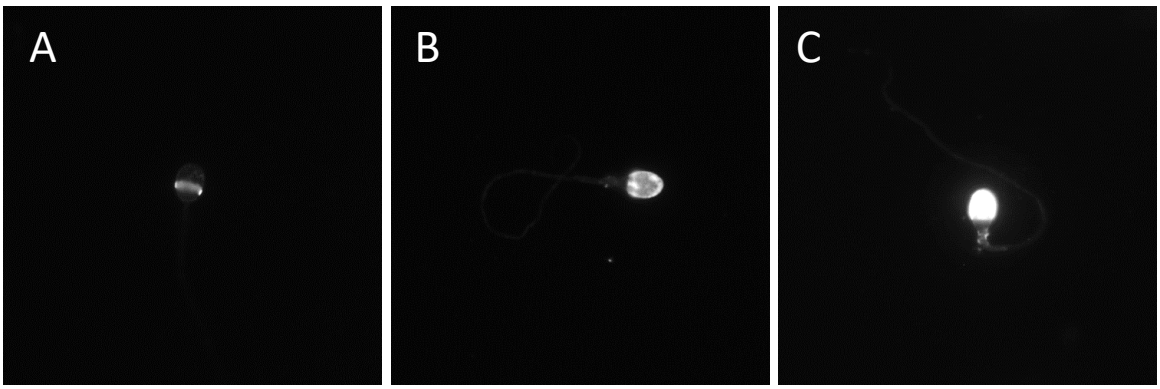

Figure 40. Representative images of FITC-PSA stained sperm. A – acrosome reacted, B – partially reacted, C – acrosome intact. 630 X magnification.

Vasopressin showed an inhibitory effect on the acrosome reaction, however the ionophore treatment, which was used as a positive control did not induce acrosome reaction (Figure 41). N = 5.

Due to inconsistencies with the FITC-PSA assay, it was decided to further explore the acrosome reaction after treatment with vasopressin using different fixative protocols and scanning electron microscopy.

Sperm treated with vasopressin and controls were prepared for SEM. The same test sampled were prepared in different fixatives for FITC-PSA. The FITC-PSA method was unreliable regardless of fixation methods or incubation times, the data showed extreme differences from true acrosomal status as is observable in the same sample in SEM. The SEM data showed that the ionophore did induce acrosome reaction whereas vasopressin and dDAVP mildly inhibited the acrosome reaction in this sample (Figure 42, Table 55). N = 1.

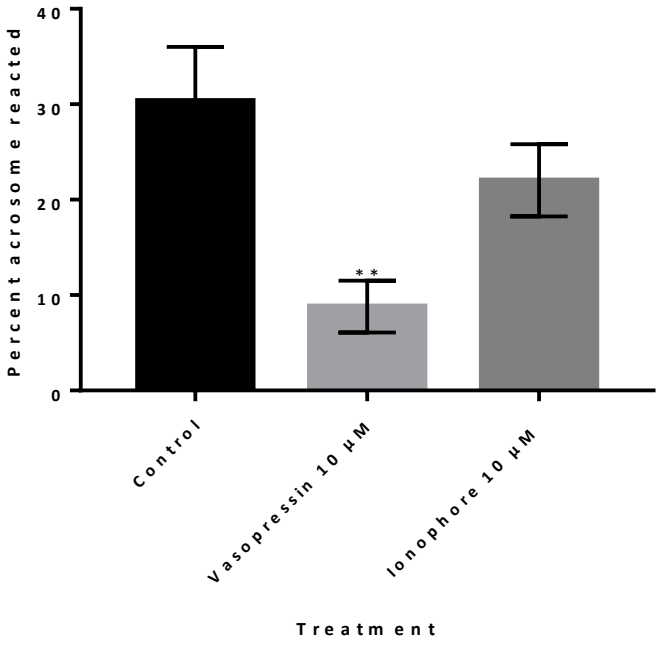

Figure 41. Percent acrosome reacted human sperm with vasopressin and ionophore. Human sperm incubated with 10 µM vasopressin and 10 µM Ionophore stained with FITC-PSA. A statistically significant difference was observed in vasopressin treatment * indicates $p < 0.05$, ** indicates $p < 0.01$. N = 5.

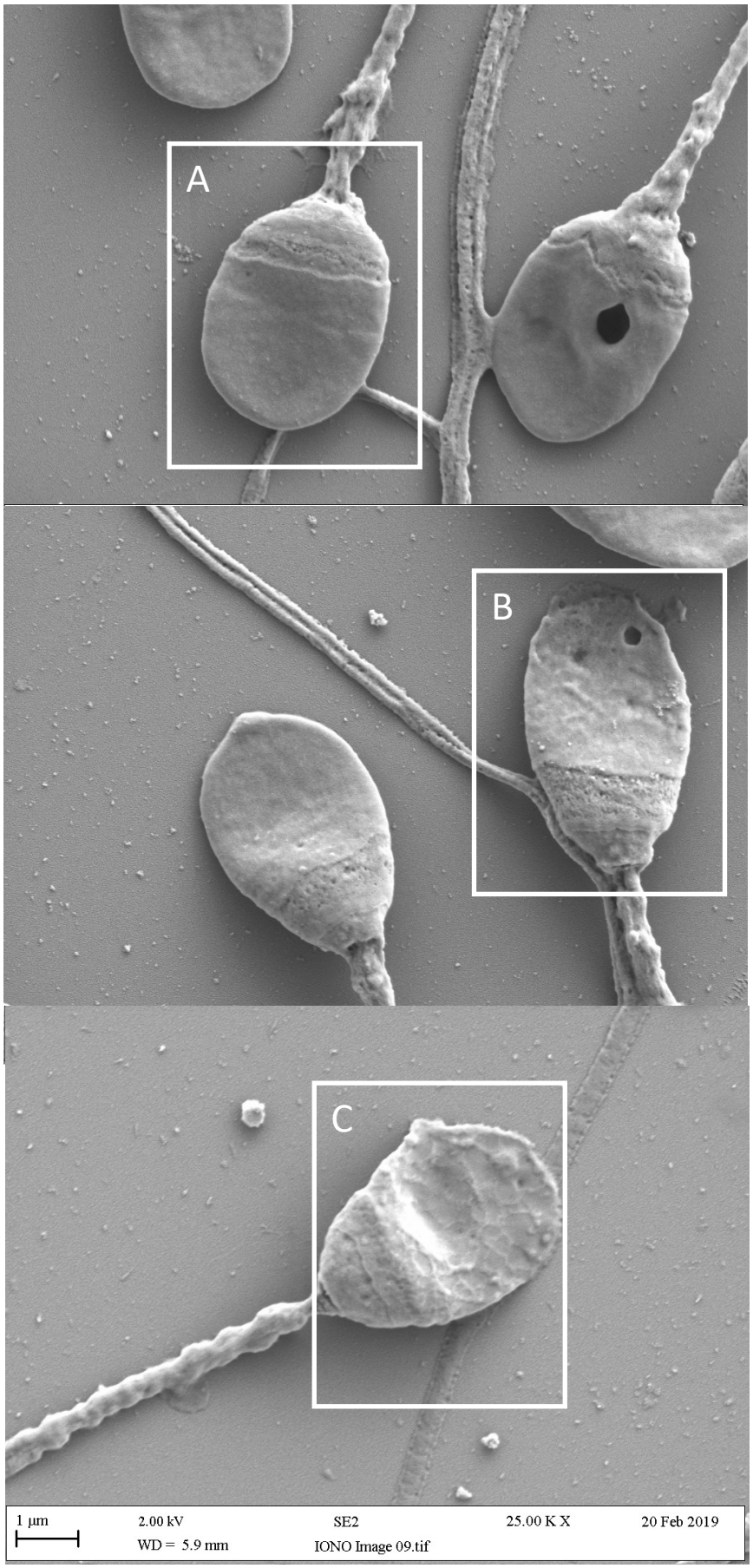

Figure 42. Examples of SEM images of sperm. A – acrosome intact, B – partially reacted, C – acrosome reacted. 25000 X magnification.

Table 55. Percentages of acrosomal status in human sperm. Vasopressin 10 µM, dDAVP 10 µM ionophore 10 µM and DMSO as vehicle control used as treatments. FITC-PSA method of detecting acrosomal status, either air-dried or fixed in PFA prior to staining and SEM data for acrosomal status in the same sample. N = 1.

%	No Treatment	Vehicle	Vasopressin 10 µM	dDAVP 10 µM	Ionophore 10 µM
FITC-PSA PFA					
Intact	22	11.3	9.2	18	16.3
Partial	67.7	76.1	63.2	67.4	74.5
Reacted	10.2	12.6	27.6	14.6	9.2
FITC-PSA Air dry					
Intact	2.4	1.6	2	0.3	1.7
Partial	29.2	26.6	12	22.9	13.3
Reacted	68.4	71.8	86	76.8	85
SEM					
Intact	47.8	41.2	45.7	51.9	4
Partial	47.8	57.6	48.5	45.5	46.8
Reacted	4.4	1.8	6.2	2.6	49.2

4.3.6 Seahorse cell mito stress test

The Seahorse cell mito stress test is a metabolomic assay that can be used to assess mitochondrial function in response to various components. The Seahorse cell mito stress test is used to measure oxygen consumption rate and extracellular acidification rate allowing for the determination of multiple parameters of mitochondrial function using modulators of the electron transport chain. Figure 43 demonstrates the real time changes in OCR following injections of treatment (control/vasopressin/dDAVP), oligomycin, FCCP and rotenone/antimycin A.

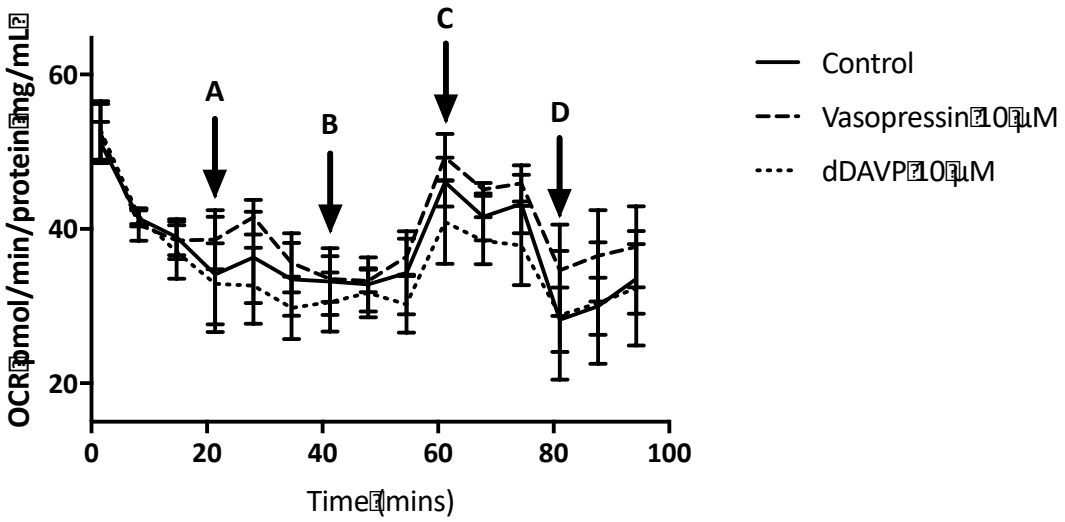

Figure 43. Oxygen consumption rate of sperm over time. Oxygen consumption rate was normalised to protein concentration per well and each individual participant were normalised to their baseline. A – first injection of either vehicle control (DMSO), vasopressin 10 µM or dDAVP 10 µM. B – second injection of oligomycin. C – third injection of FCCP. D – fourth injection of rotenone/antimycin A. N = 3.

Statistical analysis was performed in GraphPad Prism. The data was tested for normality using the Shapiro-Wilk test for normality, showing all OCR data normally distributed. A one-way repeated measures ANOVA was used to investigate differences between the treatment groups. The F statistic represents the ratio between two variances; the between-group variance and the within-group variance, this represents whether the variability between the means of the groups are higher than random chance or not, the higher the F the greater the null hypothesis can be rejected. The one-way repeated measures ANOVAs showed statistically significant differences were observed in the OCR rates between the

treatment groups and the control overall and at treatment, FCCP and rotenone/antimycin A injections (Table 56).

Table 56. Significance (*p*-values) of repeated measures one-way ANOVA tests for OCR between treatment groups and vehicle control within injection groups determined.

	Repeated measures ANOVA	
	F (DFn, DFd)	**P value**
Overall	F (1.636, 22.91) = 25.85	P<0.0001
Baseline	F (1.493, 2.985) = 0.000	P>0.9999
Treatment	F (1.87, 3.739) = 27.04	P=0.0061
Oligomycin	F (1.063, 2.125) = 6.934	P=0.1124
FCCP	F (1.092, 2.184) = 100.5	P=0.0072
Rot/Anti A	F (1.02, 2.04) = 66.59	P=0.0139

Dunnett's multiple comparisons test was used to investigate differences between groups and correct for multiple comparisons where statistically significant differences were observed compared to the control between overall vasopressin and dDAVP OCR. Statistically significant differences were found at the FCCP injection in the vasopressin and dDAVP OCR compared to control. At the rotenone/antimycin A injection vasopressin OCR was significantly different from control (Table 57).

Table 57. Significance (p value) of differences in OCR between treatment groups and control within each injection using Dunnett's test to correct for multiple comparisons.

	Dunnett's multiple comparisons test			
	Adjusted P Value	**95% CI**	**Adjusted P Value**	**95% CI**
	Vasopressin		**dDAVP**	
Overall	0.0009	-4.237 to -1.263	0.0054	0.6443 to 3.4
Baseline	0.9999	-3.346 to 3.346	0.9999	-5.512 to 5.512
Treatment	0.0795	-8.981 to 1.112	0.1103	-1.543 to 7.271
Oligomycin	0.3459	-4.065 to 2.136	0.1447	-2.091 to 7.315
FCCP	0.0108	-4.556 to -1.706	0.0385	0.5817 to 8.535
Rot/Anti A	0.0283	-9.967 to -1.473	0.9876	-3.039 to 3.189

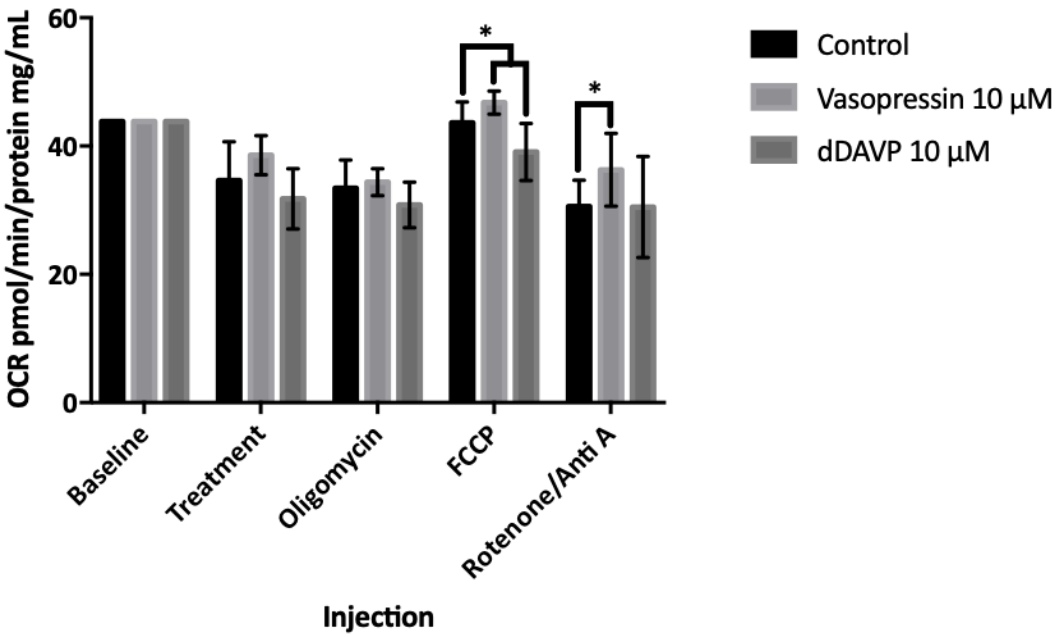

Figure 44. Average oxygen consumption rate at each injection period for control, vasopressin and dDAVP conditions. * is representative of $p = < 0.05$. N = 3.

The data was tested for normality using the Shapiro-Wilk test for normality and all ECAR data was normally distributed. A one-way repeated measures ANOVA was used to investigate differences between the treatment groups. The one-way repeated measures ANOVAs showed statistically significant differences were observed in the ECAR rates between the treatment groups and the control overall and at treatment, oligomycin, FCCP and rotenone/antimycin A injections (Table 58).

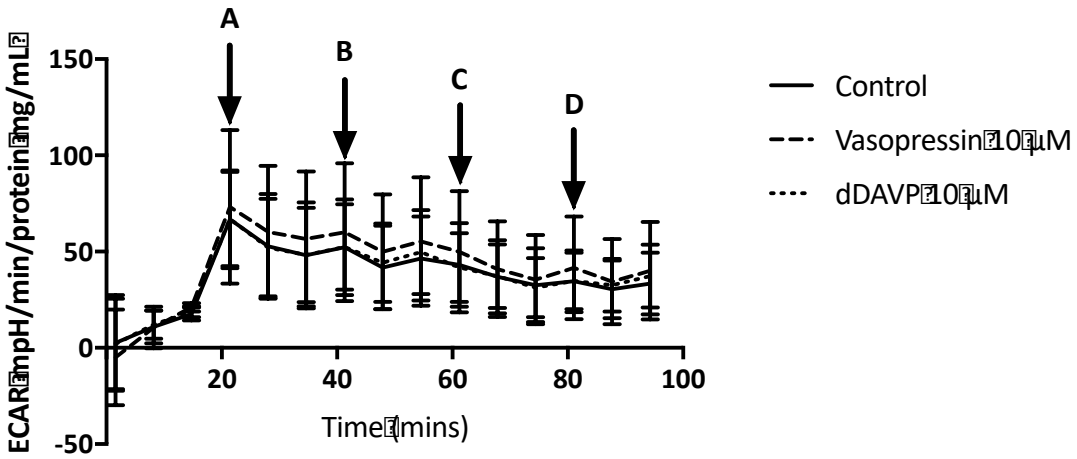

Figure 45. Extracellular acidification rate of sperm over time. Extracellular acidification rate was normalised to protein concentration per well and each individual participant were normalised to their baseline. A – first injection of either vehicle control (DMSO), vasopressin 10 µM or dDAVP 10 µM. B – second injection of oligomycin. C – third injection of FCCP. D – fourth injection of rotenone/antimycin A. N = 3.

Table 58. Significance (*p*-values) of repeated measures one-way ANOVA tests for ECAR between treatment groups and vehicle control within injection groups determined.

	Repeated measures ANOVA	
	F (DFn, DFd)	**P value**
Overall	F (1.224, 17.14) = 16.6	P=0.0004
Baseline	F (1.007, 2.014) = 0.3546	P=0.6129
Treatment	F (1.222, 2.443) = 132.5	P=0.0034
Oligomycin	F (1.02, 2.039) = 76.85	P=0.0120
FCCP	F (1.195, 2.391) = 13.99	P=0.0485
Rot/Anti A	F (1.455, 2.91) = 11.7	P=0.0421

Dunnett's multiple comparisons test was used to investigate differences between groups and correct for multiple comparisons where statistically significant differences were observed compared to the control between overall vasopressin ECAR. Statistically significant differences were found at the oligomycin injection in the vasopressin ECAR compared to control. Statistically significant differences were found at the FCCP injection in the vasopressin ECAR compared to control. At the rotenone/antimycin A injection vasopressin ECAR was significantly different from control (Table 59).

Table 59. Significance (p value) of differences in ECAR between treatment groups and control within each injection using Dunnett's test to correct for multiple comparisons.

	Dunnett's multiple comparisons test			
	Adjusted P Value	95% CI	Adjusted P Value	95% CI
	Vasopressin		dDAVP	
Overall	0.001	-7.66 to -2.219	0.1763	-1.762 to 0.2976
Baseline	0.8777	-16.15 to 19.01	0.577	-3.671 to 2.494
Treatment	0.0097	-10.71 to -4.263	0.8144	-1.227 to 1.514
Oligomycin	0.0013	-9.637 to -6.988	0.2488	-7.127 to 3.091
FCCP	0.0924	-10.73 to 1.765	0.3538	-1.981 to 3.724
Rot/Anti A	0.0395	-11.01 to -0.684	0.2962	-7.987 to 3.85

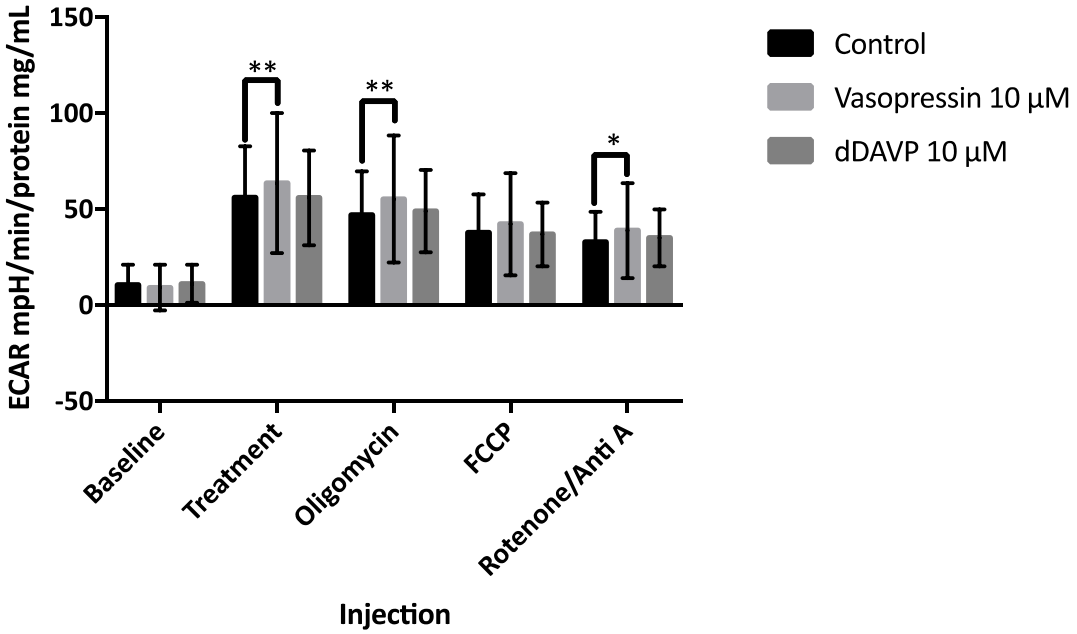

Figure 46. Average extracellular acidification rate of sperm per injection. Extracellular acidification rate was normalised to protein concentration per well and each individual participant were normalised to their baseline. Base – readings prior to injection. Treatment – first injection of either vehicle control (DMSO), vasopressin 10 µM or dDAVP 10 µM. Oligo – second injection of oligomycin. FCCP – third injection of FCCP. Rot – fourth injection of rotenone/antimycin A. * is representative of $p = < 0.05$, ** is representative of $p = < 0.01$. N=3.

4.3.7 Aquaporin-2 and human sperm

The vasopressin receptor 2 is found in renal cells where it is essential for water homeostasis. Vasopressin binds to the vasopressin receptor 2 and triggers an increase in intracellular cAMP which results in phosphorylation of aquaporin-2 (AQP2) channels which results in their translocation to the cell surface membrane. Aquaporin-2 allows water influx into the cell driven via the osmotic gradient (Boone and Deen, 2008). It was postulated that as the vasopressin receptor 2 is present on the acrosome of human sperm that the presence of AQP2 channels may indicate a mechanistic role in the acrosome reaction.

4.3.7.1 Dot blot investigating the presence of AQP2 channels

The dot blot is a simple method to determine presence of a target protein of interest in a sample of interest (section 2.7). HeLa and HK2 cells were used as positive controls, sperm protein was probed for aquaporin-2 and then protein content was confirmed with β-tubulin. Aquaporin-2 was found in sperm total protein as well as in HeLa and HK2 total protein (Figure 47).

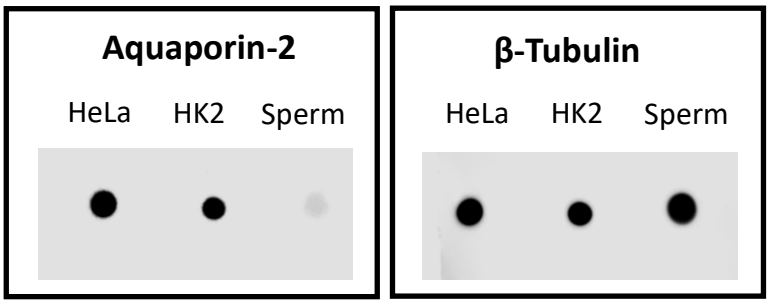

Figure 47. Dot blot investigating HeLa, HK2 and sperm protein for the presence aquaporin-2 and β-Tubulin. Representative of n = 3.

4.3.7.2 Western blots investigating the presence of AQP2 channels

As the presence of aquaporin-2 was detected via dot blot it was further investigated via western blotting. HK2 (aka human proximal tubular cells) total protein was used as a positive control (Bouley et al., 2009). Sperm were either untreated, treated with 10 µM vasopressin, or capacitated as described in methods (2.4.1). Bands present at the glycosylated form of aquaporin-2 at around 45-50 kDa for HK2, sperm, sperm treated with 10 µM vasopressin and capacitated sperm. The non- glycosylated form of aquaporin-2 was detected in sperm, sperm treated with 10 µM vasopressin and capacitated sperm but not in the HK2 sample. B-tubulin was present in all samples and used as a loading control (Figure 48).

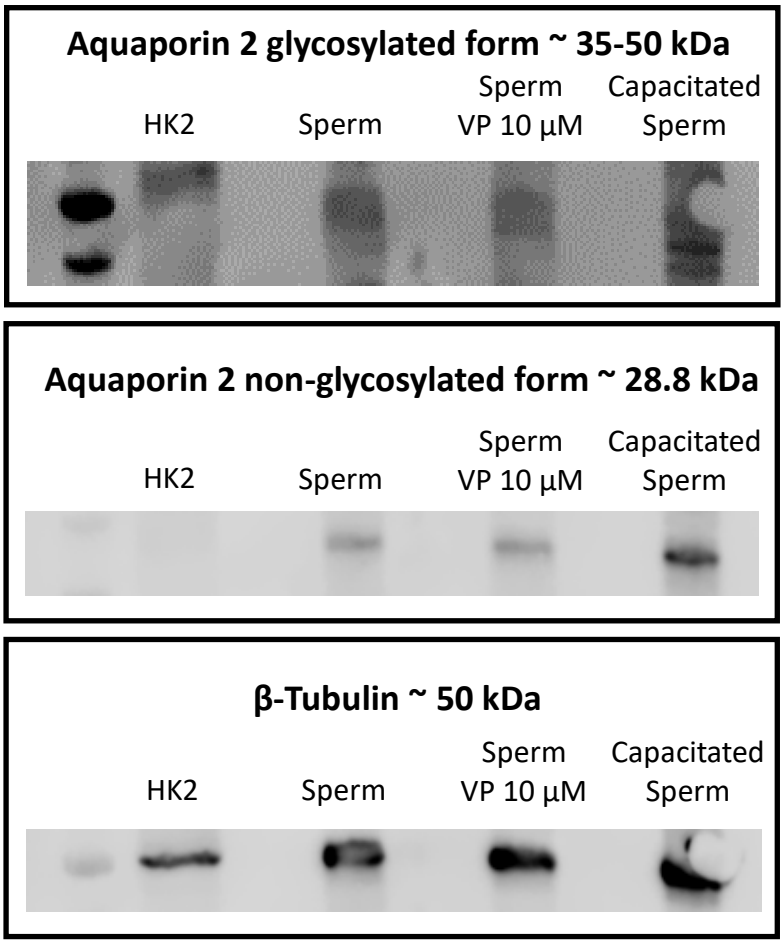

Figure 48. Western blot investigating HK2, sperm, sperm treated 10 µM vasopressin, capacitated sperm protein for aquaporin-2 and β-Tubulin. Representative of n = 1-3.

4.4 Discussion

Any modulator of sperm function can be considered for use as a fertility treatment or as a contraceptive. This is the first study to investigate the effects of vasopressin in human sperm *in vitro* and the first to detect the vasopressin receptor 2 by immunocytochemistry on the acrosome region of human sperm.

Sperm motility is an important factor in male reproduction where low sperm motility or asthenozoospermia (less than 32% progressively motile sperm) and can account for around 40-50% of male factor infertility. Absolute asthenozoospermia (100% immotile sperm in sample) affects around 1 in 5000 men (Ortega et al., 2011). In the present study, sperm progressive motility was significantly increased at 10 μM vasopressin when compared to the vehicle control.

Sperm kinematics can be described as the pattern of movement that sperm undertake, although motility is the predominantly used measure for sperm quality, kinematics allows for important insight into the sperm behaviour such as patterns of hyperactivation and the trajectory that the sperm swim in (Valverde et al., 2019). Though it has been noted that kinematics results are dependent on a number of factors in the system used; the depth and type of counting chamber, the frame-rate recorded and the field of observation therefore it is important to note that all readings were performed on the SAMi CASA system at 60 frames per second in a 20 μm deep counting chamber (Bompart et al., 2018; Nöthling and Dos Santos, 2012; Wilson-Leedy and Ingermann, 2007). The kinematics measured in this study were linearity (how linear the curvilinear path of the sperm is VSL/VCL), curvilinear velocity (VCL) μm/s (the velocity of the sperm head along its actual path averaged over time) and straight-line velocity (VSL) μm/s (the velocity of the sperm head along the straight line from the first position to its end point averaged over time) (World Health Organization, 2010b). The 10 nM and 10 μM vasopressin treatments significantly increased sperm linearity whereas 10 μM dDAVP significantly reduced sperm linearity compared to the vehicle control. The curvilinear velocity was significantly increased by the 10 pM, 10 nM, 10 μM vasopressin and 10 pM, 10 nM dDAVP treatment conditions when compared to the control. The sperm straight-line velocity was significantly increased by the 10 pM vasopressin treatment and was significantly decreased by the 10 nM and 10 μM vasopressin and 10 μM dDAVP treatments. Sperm kinematics have previously been associated with assessing the behaviours of hyperactivated human sperm, where VCL is

increased this indicated hyperactivated patterns, VSL and linearity was decreased in hyperactivated sperm (Mortimer and Mortimer, 1990). Previously, it has been found that human epididymal sperm with higher VCL have higher fertilisation rates, as VCL suggests hyperactivation this can be considered a measure of capacitation and fertilising potential (Davis et al., 1991; Katz et al., 1989).

In the present study it was found that vasopressin may influence sperm fertilisation potential by increasing VCL significantly from 10 pM to 10 µM treatments, and at the 10 µM vasopressin treatment sperm progressive motility also significantly increased, this may indicate that the sperm are hyperactivated by vasopressin. As the AVPR2 has been localised to the acrosome of human sperm and no other vasopressin receptors have been found in human sperm to date, this would suggest that vasopressin functions through this receptor to modulate the sperm motility and behaviour. When the vasopressin receptor 2 specific agonist (dDAVP) was used there was no significant differences in motility however the VCL was still modulated at the 10 pM and 10 nM dDAVP treatment conditions, this may be down to the small sample size used.

Capacitation and acrosome reaction are prerequisite steps which modify the sperm in order for fertilisation to occur, these processes are mediated via PKA and protein tyrosine phosphorylation, which are dependent on the influx and efflux of calcium in the cell (Puga Molina et al., 2018). Calcium assays in sperm may be considered a method of measuring capacitation and acrosome reaction as specific ligands can trigger these signalling cascades indicating a role in either capacitation, acrosome reaction or both (Parodi, 2014). In this study calcium response was measured via two methods; a fluorescence plate-reader assay measuring global calcium response and single cell live calcium imaging, which allowed for investigation into the individual cells' response.

The fluorescence plate reader assays demonstrated responses in almost all of the treatment conditions. Those of statistical significance in comparison with the vehicle control following a one-way ANOVA and Dunnett's test for post-hoc analysis were as follows; 10 pM vasopressin, 10 nM vasopressin, 10 µM vasopressin, 10 pM dDAVP, 10 µM ionophore, 3 µM progesterone. All of which were significant to $p = 0.0001$ except the 10 nM vasopressin treatment group which was significant to $p = 0.0403$. The 10 pM vasopressin, 10 nM vasopressin and 10 pM dDAVP inhibited the calcium response compared to the vehicle control (however there was still a greater response than the no

treatment controls). The 10 µM vasopressin, the ionophore and progesterone treatments all caused a significantly increased calcium response. This indicates that high concentrations vasopressin elicits increased calcium response in sperm, which may lead to hyperactivation and increased capacitation status.

Human sperm are a heterogeneous population of cells, where spontaneous capacitation and hyperactivation can occur in some cells (Ickowicz et al., 2012). This differential response to treatment and control conditions was demonstrated using the live cell calcium imaging, where individual sperm responded differently to treatments. For those cells that did not respond to the positive control of progesterone, they were omitted for further analysis. Only a certain portion of the populations treated and analysed responded to the vasopressin and dDAVP treatment (10-20%). Of the sperm that responded, the response was significantly different from the vehicle control period and the positive control of progesterone to $p = < 0.0001$ (repeated measures ANOVA, Tukey's test for multiple comparisons). Although progesterone response can be near ubiquitous in humans, it has been previously noted that there are subpopulations that do not respond, the same can be true for all molecules, individual sperm may have differential expression of surface proteins (Aitken and McLaughlin, 2007).

Vasopressin receptor 2 was localised to the acrosome region of human sperm, therefore it was postulated that it may be involved as a potential modulator of the acrosome reaction. Acrosome reaction of human sperm following treatment with vasopressin and ionophore (positive control) post capacitation was measured primarily by FITC-PSA. Although the level of acrosome reacted sperm was significantly decreased in the vasopressin group the ionophore did not induce a significantly higher rate of acrosome reaction. These inconsistencies lead to further investigation by using different fixatives for the FITC-PSA (Figure 40) staining and scanning electron microscopy (Figure 42) to visualise true acrosomal status. The SEM demonstrated that vasopressin had a 3 fold increase in acrosome reacted sperm when compared to vehicle control (6.2 compared to 1.8), the ionophore showed the expected increase in acrosome reacted sperm (Cummins et al., 1991) The same sample had been used for FITC-PSA staining with both standard air-dry/methanol fixation and PFA fixation which showed inconsistent results especially when compared to the SEM. It can be suggested that vasopressin may induce acrosome reaction in sperm compared to vehicle control. The assessment for the acrosome status using SEM

was carried out on a single sample. Therefore, to confirm the acrosome status with SEM, further assays are required.

The majority of studies investigating the role of vasopressin and male reproduction involve sperm transport through the epididymis and vas deferens and primarily carried out in animal models (Assinder et al., 2000b; Nicholson et al., 1999). Except the Kwon et al (2013) mouse study there are no studies investigating the effects that exposing sperm to vasopressin and dDAVP may have. A previous study in humans investigated the relationship between urinary vasopressin and the sperm of infertile males, they found a negative correlation with vasopressin levels and sperm count and motility. However, this study only had a cohort of 10 males, no control group and investigated using correlations alone with no corrections for confounding factors which is important when discussing relationships in biosciences in order to avoid Simpson's paradox; a phenomenon where a trend may occur across groups which is then reversed when considering all factors (Blyth, 1972; Puri and Puri, 1985).

Kwon et al (2013) demonstrated that vasopressin had an inhibitory effect on sperm function by decreasing motility, calcium response, acrosome reaction, decreased PKA and protein tyrosine phosphorylation, decreased fertilisation and embryo development rate in the mouse. The data presented in this chapter demonstrates that activation of the vasopressin 2 receptor on sperm, increased sperm motility - which is in contrast with the findings in the mouse (Kwon et al, 2013). The differences in the response to vasopressin treatment in mouse and human are unsurprising. Mouse sperm differ in many ways to human sperm and are not considered suitable sentinel for human fertility (Neuber and Powers, 2000). Furthermore, the vasopressin receptor 2 is differentially localised in mouse and human sperm. In humans, AVPR2 was localised to the acrosome region of the head of the sperm whereas in mouse sperm from the caput AVPR2 was solely detected in the midpiece and in sperm from the cauda AVPR2 was localised in both the midpiece and acrosome region (Kwon et al., 2012).

Mature sperm do not have an endoplasmic reticulum for calcium storage, there is evidence to suggest that sperm have two stores of intracellular calcium; in the acrosome and in the neck/midpiece. Mitochondria can serve as a calcium store, within the matrix space mitochondria are able to accumulate calcium predominantly through the mitochondrial calcium uniporter and calcium uptake occurs via a negative membrane potential in the

matrix (Costello et al., 2009a). Release of calcium stores from the mitochondria is possible through a sodium/calcium exchanger (Bernardi, 1999). Sperm is able to mobilise the stores and modulate the interactions between the calcium stores of the neck/midpiece and the CatSper channels and thus regulates motility and induces hyperactivation. In the acrosome, the calcium stores have been found to be involved in the exocytosis of the acrosome and in calcium free media the mobilisation of these stores alone can trigger acrosome reaction (Costello et al., 2009a). In the micrograph of the sperm treated with vasopressin (Figure 22) appears that the calcium stores in the neck/midpiece are being mobilised to the flagella indicating a possible role in hyperactivation and motility.

Mitochondria are essential for the motility and hyperactivation of sperm, it has been previously found that vasopressin increase the mitochondrial calcium concentration and elicit activation of essential enzymes for mitochondrial oxidative metabolism, the mitochondrial oxidative metabolism has also been implicated in water uptake into cells and mitochondrial swelling within a rat liver model (Assimacopoulos-Jeannet et al., 1986; Lehninger and Neubert, 1961). In more recent studies using male rats, it was found that vasopressin inhibited mitochondrial respiration and metabolism linked to complex I, II and III in kidney mitochondria, however this was in a haemorrhagic shock model where vasopressin is shown to preserve mitochondrial function and to prevent acute kidney injury (Sims et al., 2017). There is limited information on the direct effects of vasopressin on mitochondrial function within current literature (Bordt et al., 2019a). An inhibition of mitochondrial respiration negatively impacts the ATP production and therefore motility in mice (Tourmente et al., 2015). The Seahorse cell mito stress test was used to measure oxygen consumption rate and extracellular acidification rate in sperm following injections of vasopressin, dDAVP, vehicle control and modulators of the electron transport chain. Oxygen consumption rate is considered a direct measurement of oxidative phosphorylation (OXPHOS) and extracellular acidification rate is considered a direct measure of glycolysis through the excretion of lactic acid (Li and Graham, 2012; Wu et al., 2007). Post injection with the uncoupling agent FCCP vasopressin significantly increased oxygen consumption rate when compared to vehicle control whereas dDAVP significantly decreased OCR compared to the vehicle control. After the complex I and III inhibitors rotenone and antimycin A (total inhibition of mitochondrial respiration) were injected the vasopressin treated sperm showed a significantly increased OCR compared to vehicle control. Changes in mitochondrial function can affect motility, hyperactivation and calcium response as

mitochondria act as a calcium store in the neck/midpiece (Costello et al., 2009a). The extracellular acidification rate (ECAR) is due to both anaerobic glycolysis and CO_2 production during respiration however the source of ECAR is dependent on multiple factors such as cell type and media, and therefore without the proper controls through measurements in glucose free media, additions of glucose to measure glycolysis and the addition of 2-deoxyglucose to inhibit glycolysis and determine non-glycolytic extracellular acidification it is not possible to entirely determine the rate of glycolysis driven extracellular acidification (Mookerjee et al., 2015; TeSlaa and Teitell, 2014). In the present study it was found that vasopressin significantly increased extracellular acidification rate at the injection of vasopressin, oligomycin and rotenone/antimycin A. As both the OCR and ECAR are linked similar trends can be expected, the significantly increased ECAR at treatment and oligomycin injection may indicate that vasopressin increases glycolysis and not oxidative phosphorylation initially and then increases the maximal respiration potential at FCCP injection. At the rotenone/antimycin A vasopressin significantly increased OCR and ECAR which both indicate an increase in non-mitochondrial linked respiration through glycolysis.

The activation of vasopressin receptor 2 results to the Gs cell signalling cascade that ultimately leads to aquaporin-2 channels being translocated to the cell surface membrane. It was postulated that as vasopressin is located on the acrosome of human sperm that it may be involved in the acrosome reaction through AQP2 channels to aid the exocytosis of the membrane. Initially, sperm were probed for the presence of AQP2 through dot blot and it was detected at low levels. Sperm were treated with vasopressin or capacitated and further probed via western blots where bands at both the glycosylated and non-glycosylated isoforms of AQP2 were detected, glycosylation is vital for AQP2s function at the cell surface membrane as a water channel and the non-glycosylated form may be indicative of stored AQP2 (Hendriks et al., 2004). However, sperm do not have an endoplasmic reticulum or Golgi apparatus where N-linked glycosylation typically occurs therefore any glycosylation is likely to happen within the epididymis (Taylor and Drickamer, 2011; Tulsiani et al., 1993) Vasopressin is known to result in the phosphorylation of sites on AQP2 that are associated with trafficking to the cell surface membrane which could be phosphorylated via PKA (protein kinase A), PKC (protein kinase C), PKG (protein kinase G), ERK (extracellular signal related kinases) and casein kinase II (Brown et al., 2008).

4.5 Conclusion

The vasopressin receptor 2 was immunolocalised to the acrosome of human sperm. Vasopressin treatment of human sperm significantly modulates motility, kinematics, calcium response, mitochondrial function and glycolysis indicating a potential role in hyperactivation and capacitation. Vasopressin may also play a role in the acrosome reaction through the translocation of AQP2 channels to the cell surface membrane however, further work is needed to investigate this hypothesis. Any modulator of sperm function has a potential role as a contraceptive or a fertility aid, as vasopressin is also present in both follicular fluid and semen as demonstrated in chapter 3 it is important to understand its role in reproduction.

Chapter 5: The methylation of the oxytocin receptor and its role in human sperm

5.0 Chapter 5: Methylation

5.1 Introduction

The cause of male factor infertility can have a genetic origin (Krausz and Riera-Escamilla, 2018). In addition to changes to DNA sequence, gene expression is controlled by changes in DNA and / or chromatin structure (Nevin and Carroll, 2015), which leads to epigenetic transmission found to affect the offspring of males with infertility (O'Brien et al., 2010).

Significant alterations in in sperm DNA methylation patterns between fertile and infertile males has led to the notion that idiopathic male infertility may be associated with methylation (Hammoud et al., 2010).

Epigenetics was coined by Conrad Waddington in 1953 initially as a term to describe events leading to the development of a fully mature organism from a zygote as a result of the interactions between genes, their products and the phenotype (Felsenfeld, 2014; Waddington, 1953). Epigenetics is now defined as ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Dupont et al., 2009). DNA methylation and acetylation can act as on/off switches for gene expression. Methylation is the addition of a methyl group (CH_3) by DNA methyltransferases (DNMT) to a cytosine residue using the methyl donor s-adenosylmethionine (SAM) resulting in 5-methylcytosine (5mC). This predominantly occurs in a cytosine-guanine sequence aka CpG sequence on the DNA, the methylation of CpG sites is primarily associate with gene silencing (Figure 49). There is also histone modification via the addition of acetyl, methyl and other groups to the histone tail, acetylation is associated with transcription activation, methylation with transcription repression (Bannister and Kouzarides, 2011).

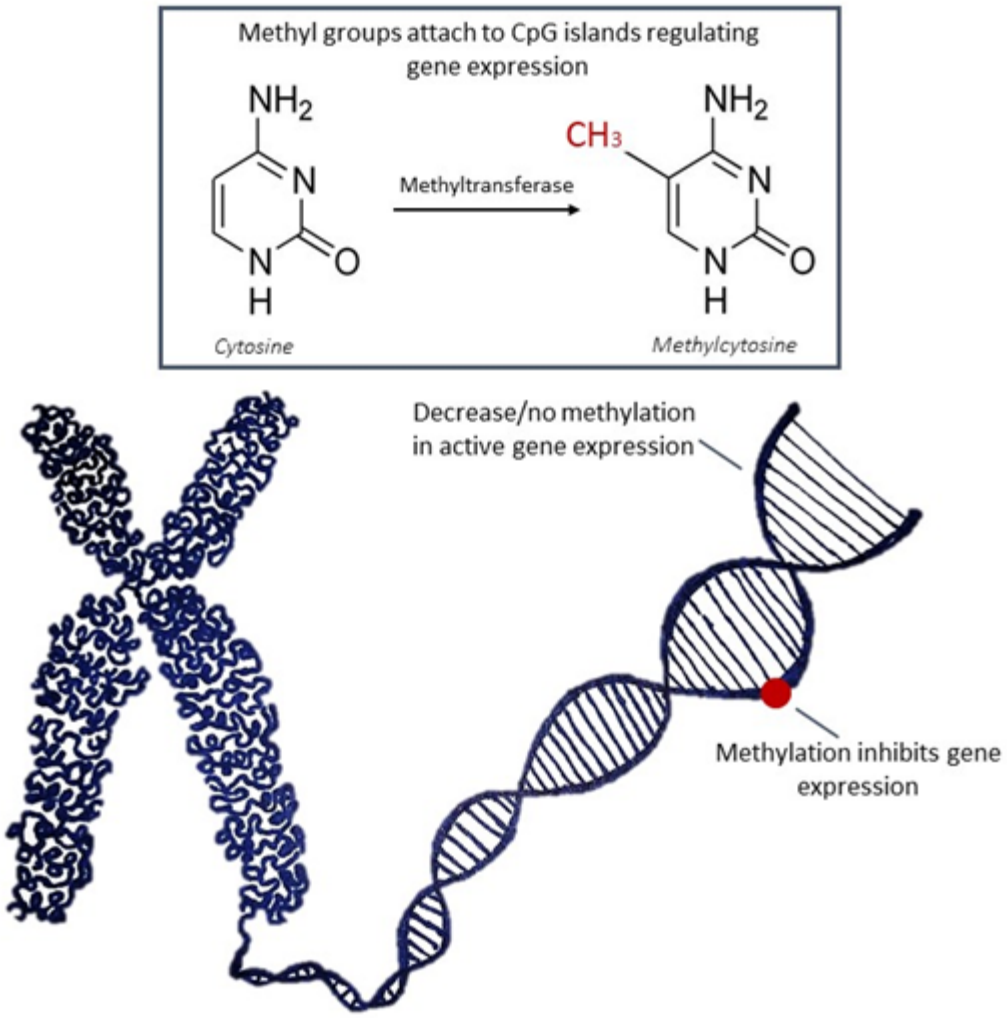

Figure 49. DNA methylation

During embryonic development epigenetic methylation is established. The egg and sperm are methylated with the maternal and paternal methylation patterns prior to fertilisation (Nevin and Carroll, 2015). At fertilisation, the paternal genome undergoes genome wide demethylation and remains demethylated for multiple divisions. The maternal genome undergoes a more gradual passive demethylation, this demethylation is regulated by ten-eleven translocation methylcytosine dioxygenase (TET) enzymes. However some genes are omitted from the global demethylation and the parental methylation pattern is preserved - these are known as imprinted genes (Santos et al., 2002; Smallwood and Kelsey, 2012). At blastocyst stage the embryo undergoes *de novo* DNA methylation via DNMT3a and DNMT3b in order to differentiate cells into various cell types, DNMT1 maintains the DNA methylation state during cell division (Figure 50).

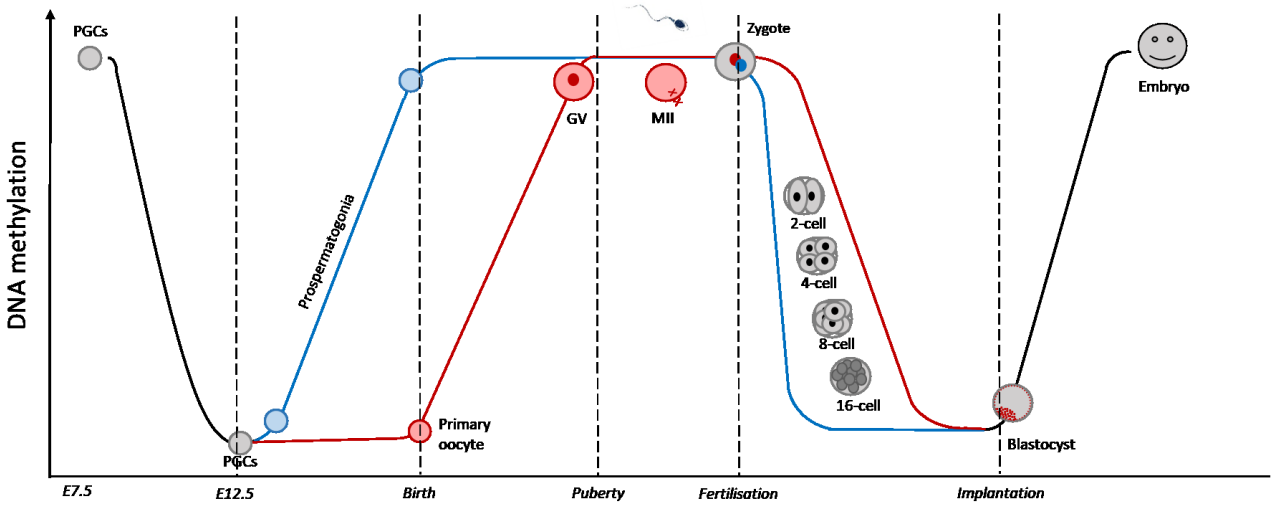

Figure 50. DNA methylation changes during development. Primordial germ cells (PGCs) are globally demethylated. Post sex-determination, new DNA methylation occurs in the male and female germ cell precursor cells. In the male (blue line) all de novo methylation is established prior to birth. In the female (red line), de novo methylation is established post-birth during follicular growth. Upon fertilisation the paternal DNA is rapidly, actively demethylated and the maternal DNA is passively demethylated during replication. *Adapted from Smallwood and Kelsey, 2012.*

DNA methylation is important in a variety of biological processes such as aging, X-chromosome inactivation, transgenerational inheritance/genomic imprinting, carcinogenesis and is vital for differentiation of cells (Heo et al., 2017; Meissner et al., 2008). Primordial germ cells are globally demethylated of the genomic imprints between E7.5 and E12.5, then spermatogenesis initiates, the primordial germ cells become spermatogonia which replicate via a couple of rounds mitosis to become primary spermatocytes. The primary spermatocytes divide via meiosis to develop into round spermatids which then undergo the process of spermiogenesis where several molecular and morphological changes occur resulting in mature sperm and during which time the genome is remethylated (Kishigami et al., 2006). This has led to the concept that it may be likely that sperm abnormalities could be linked to aberrant methylation of the DNA during spermatogenesis (Nevin and Carroll, 2015).

Previous research has identified significant differences in sperm methylation patterns between fertile and infertile men and found these methylation patterns to have predictive potential of embryo quality (Aston et al., 2015). As well as global methylation patters, several imprinted genes have also been found to be significantly altered in the sperm of infertile men compared to fertile men (Hammoud et al., 2010). Elevated methylation in sperm has also been associated with poor semen parameters (Houshdaran et al., 2007).

Genome-wide association study (GWAS) study identified 2752 CpG sites that have aberrant methylation patters in the sperm of men with idiopathic infertility when compared with fertile men, the CpG sites showing aberrant methylation were associated with sites that are methylated in sperm compared to somatic cells (Urdinguio et al., 2015).

Aberrant methylation in the promoter of oestrogen receptor genes in the endometrium have been implicated in the development and progression of endometriosis (Xue et al., 2007). Hypermethylation of the progesterone receptor gene via notch signalling in the uterus has been shown to lead to infertility in mouse studies (Su et al., 2016). However, there is extremely little investigating the effects that receptor gene methylation may have on semen parameters in current literature.

Oxytocin and the oxytocin receptor is expressed throughout male reproductive tracts in humans and a number of animals, there is evidence to suggest that oxytocin may regulate local steroidogenesis and has a role in sperm transport (Einspanier and Ivell, 1997; Frayne and Nicholson, 1998a; Whittington et al., 2001). The oxytocin receptor is a g-protein coupled receptor (specifically the Gq subtype which activates the PLC cascade resulting in phosphorylation of target proteins and an influx of calcium) and is essential in labour, lactation and maternal behaviours as well as in male ejaculation (Bales and Perkeybile, 2012; Caldwell and Young III, 2006; Ogawa et al., 1980).

Increased OXTR methylation has been associated with social deficits in autism spectrum disorder (ASD), callous-unemotional traits in young people, anorexia, emotional regulation issues, mood disorders, poor facial and emotional recognition. Low OXTR methylation has been associated with depression, stress, social anxiety and autism in children (Hollander et al., 2007; Kim et al., 2014; Maud et al., 2018; Reiner et al., 2015).

[*The initial aim of this study was to explore the methylation status of the vasopressin receptor 2 and the oxytocin receptor. However, due to technical issues and time constraints this study focused on the oxytocin receptor CpG sites 924 and 934*].

Hypothesis: Semen parameters will be associated with methylation percentage in the oxytocin receptor CpG sites 924 and 934 in the sperm of men undergoing ART.

To investigate the role of methylation status in oxytocin receptor CpG sites 924 and 934 in human sperm function the following objectives will be performed:

1. Perform pyrosequencing on DNA from sperm samples derived from patients undergoing ART.
2. Explore associations between methylation status of CpG sites 924 and 934 and sperm parameters

5.2 Methods

Semen samples (n = 94) were obtained from men undergoing assisted conception treatment at St Mary's Hospital, Manchester (see section 2, methods). The parameters available for modelling were as follows; progressive motility, concentration, count, volume, oxytocin receptor CpG 924 methylation, oxytocin receptor CpG 934 methylation, age, BMI, smoker status, alcohol units per week and normal/abnormal semen parameters using WHO reference ranges to classify patients as normozoospermic or abnormal if any of the criteria were not met.

To investigate oxytocin receptor methylation primers were designed for CpG island 1 at CpG site 924 and CpG site 934, 500 ng of DNA was isolated from sperm samples and bisulphite converted using a Bisulfite Conversion Kit (Qiagen) as described in (2.9.1) and the percentage methylation at the CpG sites of interest was investigated using pyrosequencing (**Error! Reference source not found.**).

The primers used to amplify CpG sites 924 and 934 of the oxytocin receptor are described in Table 60.

Table 60. Sequences used for pyrosequencing of CpG island in human oxytocin receptor.

Primer	Id	Sequence	Nt	Tm, ºC	%GC
PCR	F1	GGGGGGAGTTAATTTTAGGTT	21	58.7	42.9
PCR	R1	CTCAATCCCCAAAAATCACTTTACAATCT	29	59.0	34.5
Sequencing	S1	TTTTGTTTTTGGAGGAG	17	44.0	35.3

The data obtained were modelled in IBM SPSS Statistics version 25. Regression models were created for each outcome variable separately in order to obtain meaningful analysis. To try to reduce over fitting the regression models all outcome variables, predictor variables and confounders were chosen *a priori* and each predictor was modelled with each outcome separately, all relevant confounders were included whether they were statistically significant or not (Table 61).

Table 61. Outcome variables, predictor variables and covariates used in the modelling.

Outcome Variables	Predictor variables	Confounders/Covariates
Count	OXTR CpG 924	Male age
Concentration	OXTR CpG 934	Male BMI
Volume		Smoker status
Progressive motility		Alcohol units per week
Normal/Abnormal semen parameters		

5.3 Results

5.3.1 Descriptive statistics

The Shapiro-Wilk test for normality was used to initially investigate whether the continuous outcome variables (count, concentration, volume and progressive motility) were normally distributed. As all the variables were statistically significant this indicated they were not normally distributed and were further investigated using frequency histograms (Table 62).

Table 62. Shapiro-Wilk tests for normality for the continuous outcome variables.

	Shapiro-Wilk	
	Statistic	**Sig.**
Concentration	0.8	0
Volume	0.942	0
Total sperm count	0.886	0
Progressive motility	0.962	0.009

The table of descriptives for the binary variable of normal or abnormal semen parameters – defined by using WHO reference ranges, show the n, mean, median, standard deviation (Std Dev), interquartile range (interQ range), minimum and maximum percentage of oxytocin receptor methylation for both CpG sites per group (Table 63).

Table 63. Descriptives for normal or abnormal semen parameters and oxytocin receptor methylation at CpG 924 and 934 in sperm.

	Descriptives - percentage methylated for each CpG site						
	N	***Mean***	***Median***	***Std Dev***	***InterQ Range***	***Min***	***Max***
OXTR CpG 924							
Normal	59	5.86	5.00	2.63	3.00	3.00	18.00
Abnormal	22	6.91	6.00	3.79	2.63	3.00	18.00
OXTR CpG 934							
Normal	59	5.08	4.00	2.48	2.00	3.00	18.00
Abnormal	22	6.52	6.00	2.69	3.25	2.00	13.00

The frequency histograms of the semen parameters show the distribution of the continuous outcome variables. Concentration, count and volume all show abnormal distribution with positive skew, whereas progressive motility shows abnormal distribution with negative skew. Concentration was Log10 transformed to correct the positive skew, count and volume were both square root transformed to correct the positive skew and progressive motility was log gamma transformed to correct the negative skew prior to use in linear regressions (Figure 51).

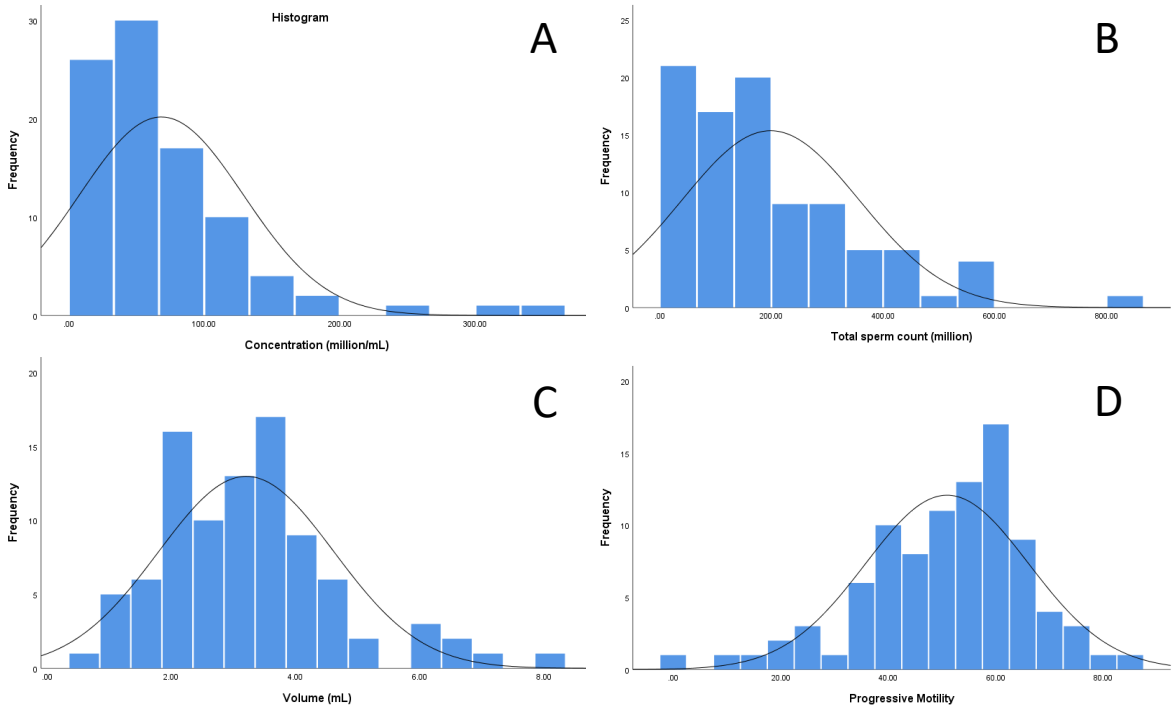

Figure 51. Distribution histograms of semen parameters. A – sperm count (million), B – sperm concentration (million/mL), C – semen volume (mL), D – progressive motility (%).

The Pearson correlation was used to investigate any collinearity between the predictor variables in order to ensure their effects on the outcome variables are unique to each predictor variable. Both oxytocin receptor CpG sites were significantly correlated and therefore were subsequently regressed separately in order to investigate the individual CpG sites relationship with the outcome variables (Table 64).

Table 64. Pearson correlation matrix for predictor variables.

	OXTR CpG 934		**OXTR CpG 924**	
	r	***p*-value**	**r**	***p*-value**
OXTR CpG 934	1.00	-	.848**	0.00
OXTR CpG 924	.848**	0.00	1.00	-

5.3.2 Independent t-tests and semen parameters

To initially investigate any differences the semen parameters were coded as binary variables using the WHO reference ranges, and independent t-tests were performed for each outcome variable. Statistically significant differences were observed in the oxytocin receptor (OXTR) methylation percentage at CpG 924 and low sperm concentration (p = 0.029), where low concentration had a significantly higher methylation at OXTR CpG 924 when compared to those with normal concentration. No statistically significant differences were found for count, volume, progressive motility or normal/abnormal parameters for OXTR CpG 924 (Figure 52, Table 65). Statistically significant differences were observed in the OXTR CpG 934 methylation for concentration, count and normal/abnormal semen parameters (p = 0.000, 0.001, 0.026 respectively), where low concentration, low count and abnormal semen parameters had significantly higher methylation at OXTR CpG 934 when compared to those with normal concentration, normal count and normal semen parameters. No statistically significant differences were observed for OXTR CpG 934 with volume or progressive motility (Figure 53, Table 65).

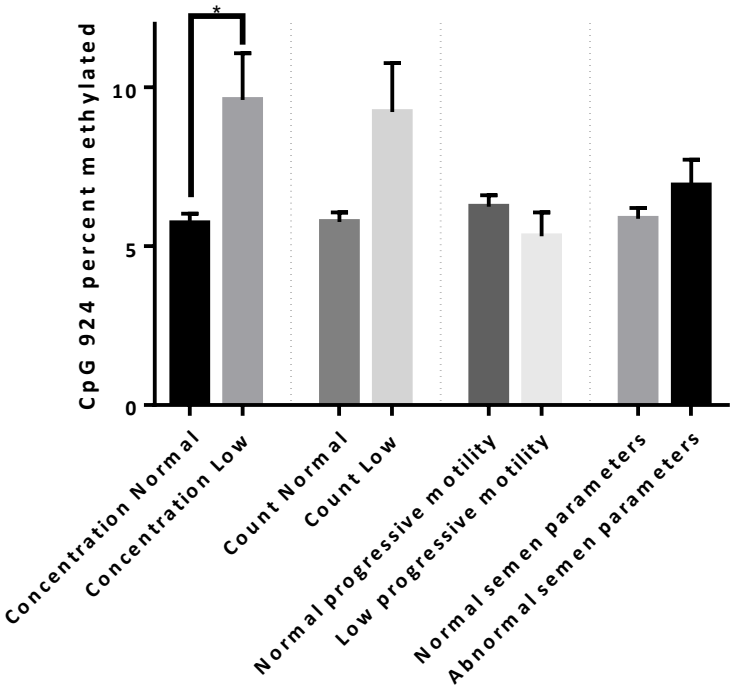

Figure 52. Mean percentage DNA methylation at oxytocin receptor CpG 924. Semen parameters classed using WHO (2010) reference ranges, mean percentage methylation for each group shown. Statistically significant differences found in methylation of CpG site 924 of the oxytocin receptor with independent t- tests for sperm concentration. * represents $p < 0.05$. N = 81.

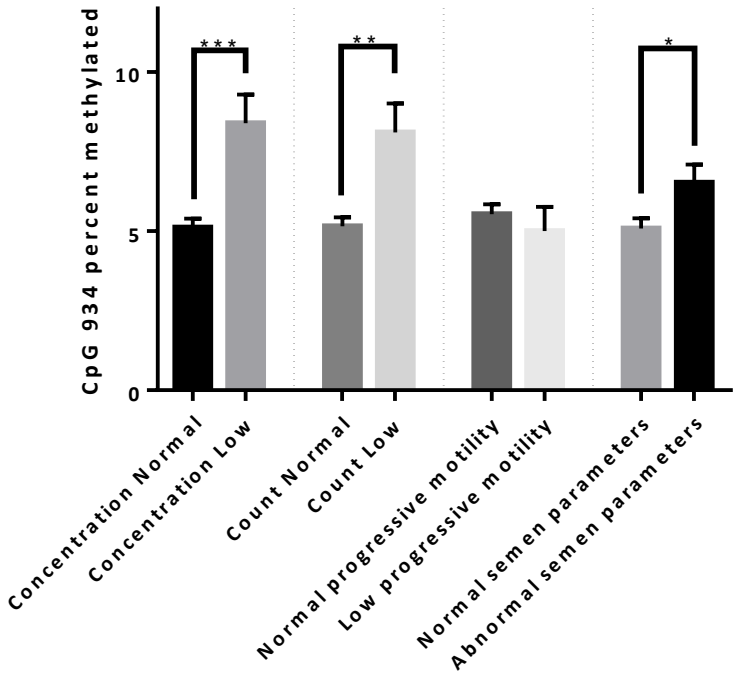

Figure 53. Mean percentage DNA methylation at oxytocin receptor CPG 934. Semen parameters classed using WHO (2010) reference ranges, mean percentage methylation for each group shown. Statistically significant differences found in methylation of CpG site 934 of the oxytocin receptor using independent t-tests for sperm concentration, count and normal/abnormal overall semen parameters. *** represents $p < 0.001$, ** represents $p < 0.01$, * represents $p < 0.05$. N = 81.

Table 65. Independent t-test *p*-values for OXTR CpG 924 and 934. Mean and standard error shown for each group and outcome variable.

		OXTR CpG 924			OXTR CpG 934		
		Mean	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**
Concentration	**Normal**	5.72	0.30		5.11	0.28	
	Low	9.61	1.46	0.029	8.39	0.90	0.000
Count	**Normal**	5.76	0.30		5.15	0.28	
	Low	9.22	1.54	0.057	8.11	0.90	0.001
Progressive Motility	**Normal**	6.24	0.36		5.53	0.31	
	Low	5.31	0.75	0.410	5.00	0.76	0.590
Normal/Abnormal parameters	**Normal**	5.86	0.34		5.08	0.32	
	Abnormal	6.91	0.81	0.165	6.52	0.57	0.026

5.3.3 Partial correlations with oxytocin receptor methylation and semen parameters

To further investigate any potential relationship between each OXTR CpG island methylation and semen parameters partial correlations were performed using both the binary outcome variables and the transformed continuous outcome variables. Both partial correlations were controlled using the confounding variables; age, BMI, smoker status and alcohol consumption.

The partial correlation with the binary outcome variables using the WHO reference ranges were coded as 0 for normal and 1 as low/abnormal, therefore any positive correlation is indicative of a negative relationship. The correlations showed that there was a statistically significant negative correlation between OXTR CpG 934 and sperm count, concentration and normal semen parameters. There was not statistically significant correlation with OXTR CpG 934 and volume and motility. There was a statistically significant negative correlation between OXTR CpG 924 and sperm count and concentration, there was no statistically significant correlation between OXTR CpG 924 and volume, motility or normal semen parameters (Table 66).

Table 66. Partial correlations for OXTR CpG 924 and 934 and binary outcome variables.

	OXTR CpG 934		OXTR CpG 924	
	r	***p*-value**	**r**	***p*-value**
Count	0.341	0.003	0.324	0.005
Concentration	0.367	0.001	0.371	0.001
Motility	-0.02	0.866	-0.069	0.558
Normal/Abnormal	0.234	0.045	0.116	0.327

Partial correlations with the transformed continuous outcome variables showed a statistically significant negative correlation between OXTR CpG 934 and concentration but no statistically significant correlations with count, volume or progressive motility. Statistically significant negative correlations were found between OXTR CpG 924 and concentration and volume, no statistically significant correlation was found with count or progressive motility (Table 67).

Table 67. Partial correlation for OXTR CpG 924 and 934 and transformed continuous outcome variables.

	OXTR CpG 934		OXTR CpG 924	
	r	***p*-value**	**r**	***p*-value**
Log 10 concentration	-0.294	0.012	-0.281	0.017
Square root count	-0.097	0.415	-0.036	0.762
Square root Volume	0.152	0.203	0.252	0.033
Log gamma progressive motility	-0.05	0.674	-0.009	0.938

5.3.4 Univariate regressions semen parameters

To investigate any relationship between OXTR CpG 924 and 934 methylation and semen parameters (count, concentration, volume and motility) univariate regressions were used. In order to perform univariate regressions the outcome variables must be normally distributed, this was achieved via transformation prior to regressing. The outcome variables were individually modelled with each CpG site methylation separately due to collinearity issues, modelling separately allowed for investigation of each sites individual effect. Confounders included in each model were age, BMI, smoker status and alcohol consumed per week. Standardised β values are the relative change in the outcome variables (semen parameters) due to the change in the predictor variables (CpG site 924 and CpG 934 methylation). The unadjusted models show the individual effects of each predictor variables or confounders on the outcome variables. The fully adjusted models show the effects of the predictor variables have on the outcome variables when the confounders are adjusted for.

Multicollinearity for confounders within the models was investigated using tolerance and variance inflation factor (VIF) during regression analysis. A VIF value between 1 and 10 is acceptable and indicates no multicollinearity issues within the model. All the variables within all the regressions were acceptable.

5.3.4.1 Univariate regressions for sperm concentration and CpG 924 and 934 methylation

The unadjusted univariate regression showed that both CpG site 924 and CpG site 934 had a statistically significant negative association with sperm concentration. None of the confounding factors (age, BMI, smoker status and alcohol consumption) showed a significant relationship with sperm concentration individually (Table 68).

Table 68. Unadjusted univariate regression values for sperm concentration.

Unadjusted univariate regression log 10 concentration				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.021	0.846	-0.016	0.013
Male BMI	-0.049	0.648	-0.026	0.017
Smoker	-0.045	0.673	-0.189	0.123
Alcohol (units per week)	0.171	0.102	-0.002	0.017
OXTR CpG 924	-0.303	0.006	-0.063	-0.011
OXTR CpG 934	-0.306	0.006	-0.073	-0.013

The fully adjusted univariate regression for CpG site 924 methylation and sperm concentration demonstrated that the CpG 924 methylation had a statistically significant negative association with sperm concentration. Alcohol consumption showed a statistically significant positive association with sperm concentration in the model. No other variables showed any significant association with concentration (Table 69).

Table 69. Fully adjusted univariate regression values for sperm concentration and CpG 924 methylation.

Fully adjusted univariate regression log 10 concentration CpG 924				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.05	0.663	-0.019	0.012
Male BMI	-0.081	0.471	-0.031	0.014
Smoker	-0.08	0.467	-0.216	0.1
Alcohol (units per week)	0.305	0.01	0.003	0.025
OXTR CpG 924	-0.27	0.016	-0.059	-0.006

The fully adjusted univariate regression for CpG site 934 methylation and sperm concentration demonstrated that the CpG 934 methylation had a statistically significant negative association with sperm concentration. Alcohol consumption showed a statistically

significant positive association with sperm concentration in the model. No other variables showed any significant association with concentration (Table 70).

Table 70. Fully adjusted univariate regression values for sperm concentration and CpG 934 methylation.

Fully adjusted univariate regression log 10 concentration CpG 934				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.061	0.592	-0.019	0.011
Male BMI	-0.087	0.44	-0.031	0.014
Smoker	-0.041	0.715	-0.189	0.131
Alcohol (units per week)	0.328	0.006	0.004	0.026
OXTR CpG 934	-0.288	0.012	-0.072	-0.009

5.3.4.2 Univariate regressions for sperm count and CpG 924 and 934 methylation

The unadjusted univariate regression showed no statistically significant association between both CpG site 924 and CpG site 934 and sperm count. None of the confounding factors (age, BMI, smoker status and alcohol consumption) showed a significant relationship with sperm count individually (Table 71).

Table 71. Unadjusted univariate regression values for sperm count.

Unadjusted univariate regression square root count				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	0.018	0.863	-0.19	0.226
Male BMI	-0.119	0.266	-0.505	0.141
Smoker	-0.036	0.736	-2.703	1.917
Alcohol (units per week)	0.151	0.152	-0.038	0.241
OXTR CpG 924	-0.082	0.468	-0.558	0.259
OXTR CpG 934	-0.154	0.169	-0.793	0.141

The fully adjusted univariate regression for CpG site 924 methylation and sperm count demonstrated that the CpG 924 methylation had no association with sperm count. Alcohol consumption showed a statistically significant positive association with sperm count in the model. No other variables showed any significant association with sperm count (Table 72).

Table 72. Fully adjusted univariate regression values for sperm count and CpG 924 methylation.

Fully adjusted univariate regression square root count CpG 924				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.02	0.865	-0.257	0.217
Male BMI	-0.15	0.201	-0.588	0.126
Smoker	-0.06	0.598	-3.13	1.815
Alcohol (units per week)	0.285	0.018	0.035	0.364
OXTR CpG 924	-0.04	0.722	-0.49	0.341

The fully adjusted univariate regression for CpG site 934 methylation and sperm count demonstrated that the CpG 934 methylation had no association with sperm count. Alcohol consumption showed a statistically significant positive association with sperm count in the model. No other variables showed any significant association with sperm count (Table 73).

Table 73. Fully adjusted univariate regression values for sperm count and CpG 934 methylation.

Fully adjusted univariate regression square root count CpG 934				
			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.025	0.829	-0.262	0.21
Male BMI	-0.143	0.219	-0.573	0.134
Smoker	-0.041	0.725	-2.937	2.053
Alcohol (units per week)	0.297	0.014	0.043	0.372
OXTR CpG 934	-0.119	0.305	-0.743	0.236

5.3.4.4 Univariate regressions for sperm progressive motility and CpG 924 and 934 methylation

The unadjusted univariate regression model showed no association between CpG site 924 or CpG site 934 methylation and progressive motility. The confounding factors showed no significant association with progressive motility (Table 74).

Table 74. Unadjusted univariate regression values for progressive motility.

Unadjusted univariate regression log gamma progressive motility			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.089	0.398	-2.9	1.162
Male BMI	0.032	0.768	-2.717	3.667
Smoker	0.103	0.326	-11.31	33.668
Alcohol (units per week)	-0.052	0.624	-1.721	1.037
OXTR CpG 924	0.036	0.748	-3.44	4.767
OXTR CpG 934	0.022	0.845	-4.264	5.199

The fully adjusted univariate regression for sperm progressive motility and CpG site 924 showed no significant associations between CpG 924 methylation and sperm progressive motility. The confounding factors showed no associations with sperm progressive motility (Table 75).

Table 75. Fully adjusted univariate regression values for progressive motility and CpG 924 methylation.

Fully adjusted univariate regression log gamma progressive motility CpG 924			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.112	0.361	-3.64	1.343
Male BMI	0.118	0.33	-1.919	5.639
Smoker	0.124	0.295	-12.27	39.858
Alcohol (units per week)	-0.072	0.557	-2.243	1.219
OXTR CpG 924	-0.007	0.954	-4.462	4.212

The fully adjusted univariate regression for sperm progressive motility and CpG site 934 showed no significant associations between CpG 934 methylation and sperm progressive motility. The confounding factors showed no associations with sperm progressive motility (Table 76).

Table 76. Fully adjusted univariate regression values for progressive motility and CpG 934 methylation.

Fully adjusted univariate regression log gamma progressive motility CpG 934			95% CI	
	Standardised β	*p*-value	Lower	Upper
Male age (years)	-0.114	0.352	-3.664	1.321
Male BMI	0.122	0.314	-1.853	5.689
Smoker	0.131	0.276	-11.88	40.95
Alcohol (units per week)	-0.068	0.584	-2.22	1.259
OXTR CpG 934	-0.041	0.734	-6.016	4.258

5.3.5 Normal or abnormal semen parameters binary logistic regression

Rate ratio also referred to as Exp (B) is representative of the probability that an event will occur if all other factors in the model remain constant. The unadjusted models represent the individual predictor variables overall main effects on the outcome variable, the adjusted models represent the effects that each predictor variable has on the outcome when all of them are taken into consideration.

In order to investigate whether there is any relationship between normal or abnormal semen parameters a binary logistic regression was used (assessed as normal if all WHO reference ranges were met and abnormal is any of the WHO reference ranges were not met).

A warning was issued (marked with **) in association with the alcohol consumed per week in the unadjusted model[1]. This warning was issued for the fully adjusted model for OXTR CpG 924 and therefore alcohol consumed per week had to be removed as a confounder from this model.

The unadjusted binary logistic regression showed no statistically significant relationship between normal or abnormal semen parameters and any of the other variables (Table 77).

Table 77. Unadjusted binary logistic regression for normal/abnormal semen parameters.

Unadjusted binary logistic regression				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Ex-Smoker	0.386	0.664	0.263	1.676
Non-Smoker	.	1	.	.
Male BMI	0.089	1.118	0.983	1.272
Male age (years)	0.092	1.074	0.988	1.167
Alcohol consumed per week**	0.999	1	0.946	1.057
OXTR CpG 924	0.176	1.111	0.954	1.295
OXTR CpG 934	0.041	1.223	1.008	1.483

[1] *The maximum number of step-halvings was reached but the log-likelihood value cannot be further improved. Output for the last iteration is displayed. The GENLIN procedure continues despite the above warning(s). Subsequent results shown are based on the last iteration. Validity of the model fit is uncertain*

The fully adjusted binary logistic regression showed no statistically significant relationship between OXTR CpG 924 and normal/abnormal semen parameters (Table 78).

Table 78. Fully adjusted binary logistic regression for OXTR CpG 924 methylation and normal/abnormal semen parameters.

Adjusted binary logistic regression for OXTR CpG 924				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Ex-Smoker	0.317	0.568	0.188	1.72
Non-Smoker	.	1	.	.
Male BMI	0.221	1.099	0.945	1.279
Male age (years)	0.066	1.098	0.994	1.214
OXTR CpG 924	0.402	1.076	0.907	1.277

The fully adjusted binary logistic regression showed no statistically significant relationship between OXTR CpG 934 and normal/abnormal semen parameters (Table 79).

Table 79. Fully adjusted binary logistic regression for OXTR CpG 934 methylation and normal/abnormal semen parameters.

Adjusted binary logistic regression for OXTR CpG 934				
			95% CI	
	Sig.	Exp(B)	Lower	Upper
Ex-Smoker	0.297	0.549	0.178	1.696
Non-Smoker	.	1	.	.
Male BMI	0.143	1.125	0.961	1.317
Male age (years)	0.034	1.12	1.009	1.244
Alcohol consumed per week	0.17	0.947	0.877	1.023
OXTR CpG 934	0.345	1.089	0.913	1.298

5.4 Discussion

This study investigated the percentage methylation of the oxytocin receptor. This was measured at 2 CpG sites (924 and 934) via pyrosequencing in the sperm of patients undergoing ART at St Mary's Hospital, Manchester. The relationship between percentage oxytocin receptor methylation and clinical parameters was investigated through a variety of statistical analyses.

Oxytocin has a well-established involvement in reproduction in particular in the female; lactation, birth and maternal-infant bonding (Fuchs et al., 1982; Marlin et al., 2015; Nickerson et al., 1954). Oxytocin is involved in the human sexual response for both males and females with increased levels at orgasm and involvement in arousal, sociosexual interactions and sexual satiety (Carmichael et al., 1987; Carter, 1992).

Oxytocin and its receptor has been implicated in spermatogenesis. Within the seminiferous tubules there are discontinuous layers of fibroblast/smooth muscle cell-like cells known as peritubular myoid cells (Hermo et al., 1977). The peritubular myoid cells support the seminiferous tubules via the contractile propulsion of fluid containing immotile sperm to the rete testes, this contractile function is modulated via angiotensin II and oxytocin (Schell et al., 2010; Welter et al., 2014). The human leydig cells have been shown to produce oxytocin and act upon the peritubular myoid cells increasing contractility in the seminiferous tubules, it has been proposed that oxytocin may also have an autocrine role in spermatogenesis via the stimulation of steroidogenesis (Frayne and Nicholson, 1998a; Thackare et al., 2006). Previously in mice, oxytocin treatments *in vitro* and *in vivo* have been found to increase proliferation of germ cells (spermatocytes and spermatids) in the testis in a dose dependant manner (Anjum et al., 2018).

Aberrant DNA methylation has been demonstrated to have an effect on male fertility. Significant differences in sperm DNA methylation patterns are found between fertile and infertile men and it is thought that the methylation patterns may have a predictive factor in embryo quality in IVF. DNA methylation is important in all aspects of male fertility such as; spermatogenesis, male organ and sexual development and sexual behaviour (Aston et al., 2015; Cisneros, 2004).

Methylation levels at the OXTR CpG sites 924 and 934 have primarily been associated with modulating social behaviour. Previous studies showed increased methylation at OXTR CpG 924 and 934 in autism and the increased methylation resulted in a 20% decrease in OXTR expression in males (Tops et al., 2019). Increased methylation at OXTR CpG site 934 has been associated with decreased social perception, decreased emotional regulation and increased callous-unemotional traits (Dadds et al., 2014a; Puglia et al., 2015). However, this is the first study investigating oxytocin receptor methylation in male infertility, any modulations in the methylation status of the oxytocin receptor can result in altered expression and function. A previous study investigated oxytocin receptor levels in the lymphocytes of fertile and infertile males and demonstrated a significant increase in the monomer form of oxytocin receptor in the asthenozoospermia and oligozoospermia group when compared to normozoospermic controls and obstructive azoospermia group (Lui et al., 2010).

Both of the oxytocin receptor CpG sites measured were significantly correlated and therefore were regressed separately to elucidate the individual relationships each CpG site had with the semen parameters.

Initial differences between oxytocin receptor methylation at both sites were investigated using independent t-tests. The CpG site 924 was found to have a statistically significantly higher percentage methylation in patients with low concentration when compared to those with normal concentration. The CpG site 924 did not show any other statistically significant differences with the other parameters. The CpG site 934 was found to have statistically significantly higher percentage methylation in patients with low concentration, low count and abnormal overall semen parameters when compared to those with normal concentration, normal count and normal semen parameters. The CpG site 934 did not show any further statistically significant differences with the other semen parameters.

Further investigations with partial correlations accounting for confounding factors (BMI, age, alcohol consumption and smoking status) were performed on both the binary variables and transformed continuous variables for semen parameters. The partial correlations demonstrated that CpG site 924 and 934 had statistically significant negative impacts on sperm count and concentration, and CpG site 934 also had a statistically significant correlation with overall abnormal semen parameters (i.e. categorised as normozoospermic or not). For the transformed continuous variables CpG 934 showed a

statistically significant negative correlation with sperm concentration, CpG 924 showed a statistically significant negative correlation with sperm concentration. No other outcomes were significantly correlated with the CpG sites 924 and 934.

The fully adjusted univariate regressions demonstrated a statistically significant negative association between sperm concentration and both CpG 924 (Standardised β: -0.27 [-0.059, - 0.006], p = 0.016) and CpG 934 (Standardised β: -0.288 [-0.072, -0.009], p = 0.012).

The fully adjusted binary logistic regression showed a no statistically significant associations between overall normal/abnormal semen parameters and CpG 924 or CpG 934.

The overall outcome of the analyses demonstrates the novel negative association between oxytocin receptor methylation levels at CpG sites 924 and 934 with sperm concentration. Although the oxytocin receptor has not been localised on mature sperm to date previous research has localised it throughout the male reproductive tract (Einspanier and Ivell, 1997; Frayne and Nicholson, 1998b). The oxytocin receptor may be expressed in spermatids/spermatocytes or primordial germ cells and is worthy of further investigation as oxytocin has been demonstrated in previous studies to be involved in spermatogenesis and steroidogenesis as well as sperm transport through the male reproductive system and local production by the leydig cells any modulation in the receptor function through methylation may have negative implications for male fertility (Anjum et al., 2018; Frayne and Nicholson, 1998a; Thackare et al., 2006).

There are further implications in transgenerational epigenetics. The primordial germ cells develop while the foetus is *in utero*, the maternal environment during pregnancy can have an effect on the future health of the developing foetus and there is further evidence to suggest that parental experiences can be transmitted through both male and female germlines which can have multigenerational impacts (Szyf, 2015). Furthermore, paternal exposures to lifestyle and/or environmental toxicants can alter the sperm epigenome, with transgenerational effects. A recent study demonstrated that exposure of cannabis can have adverse neurodevelopmental outcomes in offspring. Schrott et al, (2019) provided data demonstrating widespread DNA methylation changes in human and rat sperm upon exposure to delta-9-tetrahydrocannabinol (THC). This study reported that the Discs-Large Associated Protein 2 (DLGAP2), which is involved in synapse organization, neuronal signaling, and strongly implicated in autism, exhibited significant hypomethylation at 17 CpG sites in human sperm. In the same study, adult male rats exposed to THC, showed

differential DNA methylation at Dlgap2 in sperm, together with the nucleus accumbens of rats whose fathers were exposed to THC prior to conception. (Schrott et al., 2020).

These data and the findings in the present study indicate that the sperm epigenome and alterations in DNA methylation may impact the health of the offspring, especially where receptors are not present in the mature sperm.

5.5 Conclusion

The methylation of oxytocin receptor DNA CpG sites 924 and 934 are significantly negatively associated with sperm concentration which may be indicative of a further role for the oxytocin receptor in spermatogenesis than currently present in literature. Furthermore, alterations in the sperm DNA methylation can be used as indicators for long-term health risks in the offspring.

Chapter 6: Overall discussion

6.0 Discussion

An increasing trend in male fertility has been reported demonstrating a significant decline in the sperm count. Levine et al (2017) reported this decline in sperm count occurring over the past 40 years. These data warrant further research to elucidate the causes of this decline. This study sought to investigate the role of the neuropeptides, oxytocin and vasopressin on sperm function and fertility.

The role of neuropeptides in reproduction is well documented with major functions in the HPG axis, influencing the activity of the reproductive endocrine system, fecundity and sexual behaviour (see Table 1). Despite the involvement of neuropeptides in reproduction, little is known about their effects on sperm function and within the follicular fluid, an essential microenvironment where the oocyte matures.

In the follicular fluid of women undergoing assisted reproduction the concentrations of TNFα, oxytocin and vasopressin were detected. The concentrations of TNFα and oxytocin were largely variable in the cohort at 0.36 – 1783.15 pg/mL and 285.26 – 2442.17 pg/mL respectively. The concentration of vasopressin was highly consistent at 3055.56 – 3091.08 pg/mL.

In published literature, TNFα concentration in human follicular fluid has been found at similar levels to the lower range found in this study and has been associated with poor quality oocytes (Lee et al., 2000; Wang et al., 1992). Other studies have demonstrated that TNFα concentration in human follicular fluid is not associated with successful IVF (Mendoza et al., 2002). The present study did not find any relationship between TNFα concentrations and the clinical outcomes investigated; number of oocytes, number fertilised, the choice between freeze all and embryo transfer and clinical pregnancy. However, it is the first to demonstrate a large variability of TNFα concentration in human follicular fluid and previous literature demonstrated a potential impact upon oocyte quality which was not a measured clinical outcome in this study. It has been previously demonstrated that TNFα interacts with other cytokines in a synergistic or antagonistic manner resulting in differing effects (Neta et al., 1992). Cytokines are involved in various aspects of female reproduction such as

regulating ovulation and potential involvement in pregnancy outcomes (Bedaiwy et al., 2007; Büscher et al., 1999; Gaafar et al., 2014; Mendoza et al., 2002). Measuring the concentrations of the cytokines that interact with TNFα in combination with the concentration of TNFα would better demonstrate its role in female reproduction and potentially the mechanistic process through which it functions.

Oxytocin has a well-established involvement in reproduction in particular in the female; lactation, birth and maternal-infant bonding and is also involved in the human sexual response in both sexes including; arousal, sociosexual interactions and sexual satiety (Carmichael et al., 1987; Carter, 1992; Fuchs et al., 1982; Marlin et al., 2015; Nickerson et al., 1954).

Previously oxytocin and vasopressin have been detected in human follicular fluid at concentrations ranging from 5.54 – 299 pg/mL and 5.8 – 131 pg/mL respectively (Khan-Dawood and Dawood, 1983; Schaeffer et al., 1984; Tjugum et al., 1986; Verges et al., 1986). The higher range of previous studies was similar to the lower range of the oxytocin concentrations found in human follicular fluid by this study and is the first to report a large variation in oxytocin concentration. The oxytocin concentration in human follicular fluid was found to have a significantly negative association with the number of oocytes fertilised (RR – 0.968 [0.957, 0.98], p – 0.000) using regression analysis. In the present study, vasopressin was detected at very high concentrations in human follicular fluid and concentrations were consistent across the patient cohort. Vasopressin regulates the water channels aquaporin-2 and aquaporin-3, which allow influx of water into the cell and out of the cell respectively (Knepper, 1997). The high and consistent concentrations of vasopressin in human follicular fluid may play a role in maintaining the osmotic gradient in the follicular antrum during follicular maturation. This idea may be supported in literature, the mRNA of aquaporin-2 in granulosa cells has been found to be upregulated throughout ovulation (Thoroddsen et al., 2011). The high concentrations of vasopressin may also be due to multiple follicle aspirates from stimulated women, which is something worth further investigation. The vasopressin concentration in human follicular fluid was not found to have a relationship with any of the clinical outcomes measured in this study.

This is the first study to report concentrations of TNFα, oxytocin and vasopressin in human follicular fluid at these levels. Although a relationship between vasopressin and TNFα has been demonstrated in previous studies where vasopressin functions through the oxytocin

receptor to result in the shedding of tumour necrosis factor receptor 1, this may be unique to human aortic endothelial cells as no relationship between oxytocin, vasopressin and TNFα was detected in this study (Yang et al., 2019). Previously, the effects of oxytocin and vasopressin in female fertility have primarily been investigated through animal studies or ovarian tissue extracts from women undergoing surgery. These studies demonstrated a potential role for oxytocin in the oestrous cycle and luteolysis and both oxytocin and vasopressin may have a role in influencing the secretion of gonadotropin (Wathes, 1984).

In the semen of men undergoing ART concentrations of vasopressin and oxytocin were detected at 143.35 - 2827.24 pg/mL and 496.55 - 3100.24 pg/mL respectively. The levels of vasopressin and oxytocin were modelled statistically to elucidate any relationship with semen parameters. Vasopressin concentration in semen did not show any relationship with any of the semen parameters. However, the data presented in this study demonstrate that there may be a role for oxytocin in regulating sperm count and concentration. In semen, an increased concentration was negatively associated with sperm count (standardised β: -0.332 [-0.004, -0.001], *p* – 0.004) and concentration (standardised β: -0.293 [-0.003, 0], *p* – 0.000) and not found to be associated with volume, progressive motility, total motility or overall normal/abnormal semen parameters, the relationship between oxytocin concentration and sperm count/concentration may be indicative of a direct role in spermatogenesis. This study is in concordance with some previous findings. One study found that plasma levels of oxytocin were not correlated with sperm motility, count or morphology. However, other more recent studies have found plasma levels of oxytocin to be negatively correlated with lower sperm motility, lower sperm count and lower normal morphology with higher concentrations found in infertile men (Lui et al., 2010; Mostafa et al., 2015; Ogawa et al., 1980). Another study performed a small single blind experiment where acute oxytocin administration prior to sperm collection increased sperm release in human oligozoospermic patients. It was postulated that oxytocin has a physiological role in sperm transport and epididymal motility (Filippi et al., 2002). It is clear oxytocin may play a role in spermatogenesis and possibly sperm function and fertilisation rate. However, there is no consensus in the published literature.

The data presented in this research is the first to localise the vasopressin receptor 2 has on the acrosome of human sperm. Furthermore, this is the first study to investigate the any effects vasopressin may have in human sperm function *in vitro*. Vasopressin was found to

modulate several aspects of sperm function. During *in vitro* motility and kinematic assays it was found that vasopressin significantly increased human sperm progressive motility at 10 µM, significantly increased linearity at 10 nM and 10 µM, increased VCL at all concentrations used (10 pM – 10 µM), increased VSL at 10 pM and decreased VSL at 10 nM and 10 µM. The vasopressin receptor 2 agonist dDAVP was found to decrease sperm linearity at 10 µM, increased VCL at 10 pM and 10 nM, decreased VSL at 10 µM. Although VCL and VSL are related, they can be considered as a measure of cell vigour and straight line progression respectively, VCL is considered to be directly related to hyperactivation and VSL is considered to be related to non-hyperactivated sperm (Cancel et al., 2000; Mortimer and Mortimer, 1990). The calcium measurements found in both the microplate fluorescence assay and the live cell imaging at 10 µM vasopressin significantly increased calcium response in human sperm and in the live cell imaging calcium response was also significantly increased with 10 µM dDAVP. It has been well established that calcium response plays an essential role in regulating motility and particularly, hyperactivation of sperm (Costello et al., 2009a; Lishko et al., 2011; Publicover et al., 2007; Strünker et al., 2011). Although hyperactivation is considered a visual and behavioural measure of capacitation, it has been debated that both processes may be independent of each other in human sperm, where hyperactivation specifically refers to the pattern of movement controlled by the flagella and capacitation refers to the membrane changes which alter the location of calcium binding sites in the plasma membrane of the sperm head (de Lamirande and Cagnon, 1993; Mortimer et al., 1998). The capacitation, hyperactivation and acrosome reaction of sperm are prerequisites to fertilisation (De Lamirande et al., 1997; Wang et al., 1993). Assessment of the acrosome reaction using FITC-PSA produced inconsistent results. Therefore, scanning electron microscopy was used to investigate the acrosome reaction in further depth. It was found that vasopressin increased the amount of acrosome reacted sperm by approximately 3-fold compared to the vehicle control. The results of this study suggest that vasopressin increases hyperactivation of sperm *in vitro*, vasopressin may have a role in capacitation and acrosome reaction also.

The mitochondria essential in human sperm motility, hyperactivation and calcium response as the mitochondria can act as a calcium store in the neck/midpiece and an inhibition of mitochondrial respiration has been shown to negatively impact ATP production and sperm motility in mouse studies (Costello et al., 2009b; Kasai et al., 2002; Tourmente et al., 2015). Vasopressin has been found to increase mitochondrial calcium concentration, activate

essential enzymes for oxidative metabolism which has been demonstrated to be involved in water uptake into the cells in rat liver models (Assimacopoulos-Jeannet et al., 1986; Lehninger and Neubert, 1961). In rat haemorrhagic shock models vasopressin was found to prevent acute kidney damage via the inhibition of mitochondrial respiration linked to complex I, II and III however, there is little known in published literature about the direct effects of vasopressin on mitochondrial function (Bordt et al., 2019b; Sims et al., 2017). Using the Seahorse cell mito stress test to measure human sperm mitochondrial oxygen consumption rate and extracellular acidification rate following injections of vasopressin, dDAVP, vehicle control and modulators of the electron transport chain, it was found that vasopressin treatment significantly increased oxygen consumption rate after FCCP (uncoupling agent) and rotenone/antimycin A (total inhibitors of mitochondrial respiration) injections. The dDAVP treatment significantly decreased oxygen consumption rate following FCCP injection. The extracellular acidification rate was significantly increased in vasopressin treatment following injection of the treatment, oligomycin and rotenone/antimycin A. The oxygen consumption rate is considered a direct measurement of oxidative phosphorylation (OXPHOS) and extracellular acidification rate is considered a direct measure of glycolysis through the excretion of lactic acid (Li and Graham, 2012; Wu et al., 2007). However, extracellular acidification rate can be due to both anaerobic glycolysis and CO_2 production through respiration, both of which are reliant on several factors including cell type and media used, further tests would be necessary to determine the non-glycolytic extracellular acidification and glycolysis driven extracellular acidification (Mookerjee et al., 2015; TeSlaa and Teitell, 2014). The findings in the present study demonstrate that vasopressin may increase glycolysis rather than oxidative phosphorylation, whilst increasing maximal respiration potential following the uncoupling at the FCCP injection and the increase in both oxygen consumption and extracellular acidification rate following the rotenone/antimycin A injection indicates an increase in non-mitochondrial linked respiration through glycolysis. There is a large body of research suggesting that glycolysis is the primary source of ATP production required for motility and alterations in this would impact sperm motility and hyperactivation (du Plessis et al., 2015; Lardy and Phillips, 1941; Mukai and Okuno, 2004).

Overall, the data would indicate that vasopressin stimulates an increase in human sperm hyperactivation through activation of key pathways such as glycolysis and the calcium response. Vasopressin may also have a role to play in modulating human sperm motility

and capacitation. Hyperactivation, capacitation and motility are all essential to male human fertility.

Aberrant DNA methylation has been demonstrated to have an effect on male fertility with significant differences in sperm DNA methylation patterns found between fertile and infertile men. DNA methylation is important in all aspects of male fertility such as; spermatogenesis, male organ and sexual development and sexual behaviour (Aston et al., 2015; Cisneros, 2004). Oxytocin and the oxytocin receptor have previously been implicated in spermatogenesis, steroidogenesis, the healthy contractile function in the seminiferous tubules (Frayne and Nicholson, 1998a; Schell et al., 2010; Thackare et al., 2006; Welter et al., 2014). The oxytocin receptor CpG sites 924 and 934 have been associated with modulating social behaviours, autism, mood and personality traits (Dadds et al., 2014b; Puglia et al., 2015; Tops et al., 2019). This study investigated the methylation levels in the oxytocin receptor at CpG sites 924 and 934 in men undergoing ART and is the first to find a relationship between oxytocin receptor methylation and male fertility. There was a significant negative association between sperm concentration and both CpG 924 (Standardised β: -0.27 [-0.059, - 0.006], p = 0.016) and CpG 934 (Standardised β: -0.288 [-0.072, -0.009], p = 0.012). Although presently the oxytocin receptor has not been localised on mature sperm it is found throughout the male reproductive tract, the receptor may be expressed in immature sperm cells (spermatid/spermatocytes/primordial germ cells) and would warrant further investigation due to the findings in this study and in previous studies implicating oxytocin receptor in spermatogenesis (Einspanier and Ivell, 1997; Frayne and Nicholson, 1998b).

During *in utero* development of the foetus, the maternal environment and paternal exposures to lifestyle and environmental toxicants can affect the future health of the offspring via alterations in the epigenome, which may have multigenerational impacts (Szyf, 2015). For instance, paternal exposure to cannabis prior to conception has been demonstrated to have adverse neurodevelopmental effects on the offspring through the methylation of discs-large associated protein 2(Schrott et al., 2020).

The findings in the present study demonstrate that the oxytocin receptor methylation may be important in spermatogenesis and alongside previous research may implicate the oxytocin receptor methylation in the health of the offspring, especially where CpG sites 924 and 934 are associated with mood disorders and autism.

The presence of the neuropeptides oxytocin and vasopressin in the semen and follicular fluid and the association between oxytocin and lower fertilisation rate, sperm concentration and sperm count, the presence of the vasopressin receptor 2 on human sperm and vasopressin's positive effects on sperm *in vitro* and the negative association between oxytocin receptor methylation and sperm concentration indicate important roles for these neuropeptides in human fertility. The neuropeptides oxytocin and vasopressin are very similar, differing by only 2 amino acids, this results in the cross reactivity between the neuropeptides and both their receptors (Song and Albers, 2018). However, the data from this study indicate that oxytocin and vasopressin have different effects on human fertility. Vasopressin has been shown to stimulate sperm hyperactivation, glycolysis and calcium response and may potentially influence sperm motility and capacitation, these processes are vital for male fertility. However, oxytocin is associated with negative impacts on fertility, it is negatively associated with fertilisation and sperm count and concentration.

In many studies, such as the present one, it is important to consider confounding factors that can influence the outcomes. The confounders included in the regression analyses were all chosen *a priori* and included due to their established biological relevance to the outcomes measured.

It is well established within the literature that increased age has negative implications for fertility, both maternal and paternal advancing age is associated with poor fertility, complications in pregnancy and adverse health outcomes in offspring. In males the testosterone levels decrease with age resulting in hypogonadism, semen parameters decrease potentially as soon as 35 years, after 40 years males show an increased amount of sperm DNA damage and decreased motility and vitality. In the female fertility, declines significantly over the age of 36, both the ability to get pregnant and maintain the pregnancy are negatively impacted, there is an increased risk of aneuploidy, spontaneous abortion and chromosomal abnormalities (Sharma et al., 2013). In the models used in this study, age was found to be significantly relevant when modelling fertilisation of oocytes and in the methylation cohort. Age was found to have a negative association with fertilisation in women undergoing IVF which is in concordance with current literature and found to have a paradoxical positive association with overall normal semen parameters (classified as normozoospermic using the WHO reference ranges for semen parameters).

Whether IVF or ICSI was used was an important confounding factor as it with ICSI the sperm are directly injected into the oocyte, fertilisation rate has been investigated with sibling oocyte split treatment studies and in some has been shown to have an effect on outcome where IVF has a higher rate of total failed fertilisation rates than ICSI, however, in other studies no statistically significant effect was found (Fan et al., 2012; Lee et al., 2017; Plachot et al., 2002; Staessen et al., 1999). In the follicular fluid cohort it was found that using ICSI over IVF significantly increased fertilisation of oocytes and significantly decreased the choice to freeze all embryos.

Body mass index (BMI) is considered a parameter which affects clinical outcomes. Obesity (a BMI greater than or equal to 30) is associated with several diseases as well as hypogonadism and other impacts on the endocrine systems therefore affecting both male and female fertility (Brewer and Balen, 2010; Phillips and Tanphaichitr, 2010; Reece, 2008). In women, obesity is associated with poor response to ART treatments leading to lower fertilisation rates and lower live birth rates. Obesity in women is also associated with increases in miscarriage, gestational diabetes, larger foetuses and subsequent delivery complications (Homan et al., 2007; Pandey et al., 2010). In men, obesity is associated with reduced circulating testosterone levels and poor sperm concentration, motility and morphology (Cabler et al., 2010; MacDonald et al., 2009). Women who are clinically underweight (BMI less than 18.5) have an increased risk of infertility, this is suggested to be due to an increase in FSH levels, a short luteal phase leading to secondary amenorrhoea due to reduced leptin levels (Frisch, 1987; Grodstein et al., 1994a). Low BMI is also associated with reduced clinical pregnancy rates and increased miscarriage rates in comparison to women of a healthy BMI, they have higher rates of premature labour and a reduced birth weight (Davies, 2006; Han et al., 2010). In this study BMI was only available to be included for the models of oxytocin receptor methylation in sperm and was not found to be statistically significant in any of the models of sperm parameters.

Smoking tobacco has repeatedly been demonstrated to have a severe negative health consequences and is thought to be a predominant contributor to alterations in CpG methylation (Breitling, 2013). The WHO state that there are no levels of exposure that are considered safe (World Health Organization and Tobacco Free Initiative (World Health Organization), 2007). In reproduction, both male and female fertility is impacted by cigarette smoking as well as second hand smoke exposure, increased DNA damage to

sperm, detrimental effects on semen parameters, it increases the risk of early menopause, higher risk of tubal factor, reduced oocyte retrieval, higher incidence of diploid and triploid oocytes, poor conception rates, reduced IVF/ICSI rates, doubled ectopic pregnancy risk, increased rate of miscarriage by 80% (Evans et al., 1981; Potts et al., 1999; Tomova and Carroll, 2018). In this study smoker status was only available to be included for the models of oxytocin receptor methylation in sperm and was not found to be statistically significant in any of the models of sperm parameters, this was included as either ex-smoker or non-smoker, patients undergoing ART are required to quit smoking prior to treatment and therefore the exposure of the ex-smokers to tobacco smoke may be vastly different.

Alcohol consumption has been well established in the literature to be associated with many negative health outcomes, in fertility, it is associated with severe pregnancy complications, poor development of the foetus, increases in female factor infertility due to ovulatory factors or endometriosis, increased association with miscarriage (Grodstein et al., 1994b; Henriksen et al., 2004). In men, chronic alcohol use is associated with an increase in FSH, oestrogen and LH, decreases in progesterone and testosterone and significant negative impacts on sperm count, morphology, motility and decreased seminal volume (Muthusami and Chinnaswamy, 2005). In this study, regression analysis demonstrated that alcohol consumption had a positive association with sperm concentration and count.

6.1 Overall conclusion

This study demonstrated the neuropeptides vasopressin and oxytocin have a role in fertility and sperm function. It is the first to locate the vasopressin receptor 2 on the acrosome region of human sperm and demonstrate a role in sperm motility, kinematics, calcium response, mitochondrial respiration and glycolysis. In the clinical cohorts of men and women undergoing IVF where increasing concentrations of oxytocin in follicular fluid was found to be negatively associated with fertilisation of oocytes in women and increasing concentrations of oxytocin in semen was negatively associated with sperm count and concentration in men. In sperm, increases in the oxytocin receptor methylation at CpG sites 924 and 934 were negatively associated with sperm concentration. These data indicate a novel role for vasopressin and oxytocin in sperm function, spermatogenesis, fertilisation and potentially indicating transgenerational implications for the oxytocin receptor.

6.2 Limitations

Research using human samples and clinical cohorts come with limitations and there are some limitations recognised in this study.

One of the main limitations of this study is the available confounding factors (as discussed above). In order to robustly model the relationship between predictor variables and outcome variables it would be ideal to have a high enough number of patients and all confounders relevant to each outcome. When investigating the neuropeptide levels in semen the only confounding variable available was age whereas other factors influence semen parameters such as BMI, smoke exposure and alcohol consumption. In the follicular fluid cohort there were only 5 patients overall who had the long-down regulation stimulation protocol which caused difficulty in the regression models. The type of infertility, male/female/type of either, was not known and therefore not accounted for in the regression models. All patients were from the same clinic and there may be a difference in the semen parameters of the patients who chose to participate in the research compared to those who chose not to.

There are always limitations with live cell *in vitro* assays as the controlled and simple microenvironment used to investigate direct effects of compounds on cells is rarely representative of the complex *in vivo* environment. For any research using human sperm – it is important to obtain a fresh sample on the day of experimentation and from a healthy donor. For the *in vitro* assays, participants were recruited from the general population. Although participants were available, due to the nature of our recruitment process and ethical approval procedures only a limited number of participants could be recruited. Therefore, a smaller sample size results in less power and higher variation. Furthermore, the Seahorse extracellular flux analysis required further optimisation, as this was the first study to use this system with human sperm to date.

DNA methylation analysis of human sperm requires primer design targeting regions of interest. The methylation analysis intended to include vasopressin receptor 2 however, all primers designed did not amplify the region of interest and required further design optimisation. However, due to time constraints, optimisation and further primer design was not possible.

6.3 Future work

This is the first study to localise vasopressin receptor 2 on human sperm and although a functional role of vasopressin in sperm was presented – this research has raised a number of future research possibilities.

Further investigation of the aquaporin receptor in sperm function, in particular acrosome integrity and the acrosome reaction is worth pursuing. As the FITC-PSA assays were inconclusive and could not be repeated – the SEM was utilised. More SEM work to evaluate the acrosome status after vasopressin treatment would be useful. Moreover, localisation of the aquaporin receptor and its translocation with vasopressin treatment would reveal a novel functional role of vasopressin and aquaporin receptor and sperm function.

Further investigations into the *in vivo* effects of vasopressin could be investigated through a cohort of healthy males, males on vasopressin receptor 2 antagonists (e.g. tolvaptan) and males on vasopressin receptor 2 agonists (desmopressin a.k.a dDAVP) through analysis of the semen parameters in these cohorts. However, it may be difficult to discern direct effects of the vasopressin receptor 2 agonists/antagonists as patients may likely be on further medications, a large enough patient cohort would be required to be able to statistically account for additional medications.

In the present study vasopressin was demonstrated to modulate sperm motility and increase calcium concentrations in sperm. In mouse, vasopressin was shown to negatively affect sperm function (Kwon et al., 2013b), however the present study has shown the opposite. These data suggest a novel function of vasopressin on sperm, which may be to influence sperm function in the female reproductive tract. This warrants further investigation.

Investigate vasopressin receptor 2 methylation to elucidate any relationship between AVPR2, which is expressed in mature sperm, and semen parameters. As

As cell and molecular research progresses, the "-omics" are becoming increasingly informative. Ideally, a further study would be conducted with a cohort of fertile and infertile males. Proteomics, metabolomics and NextGen sequencing performed on the sperm samples to elucidate differences between the fertile and infertile males. This would provide further insight into neuropeptides within sperm and highlight further neuropeptides of interest to investigate.

Proteomics on the follicular fluid of the female cohort would provide further insight into the constituents and their effects on the number of oocytes, number fertilised and pregnancy rate. The data from proteomics could also be mined for differences leading to investigations into potential biomarkers for reproductive success.

6.4 Research outputs

Ana-Maria Tomova & Michael Carroll. *Lifestyle and Environmental Impacts on Fertility*. Clinical Reproductive Science. Wiley-Blackwell 2018

Ana-Maria Tomova, Jessica Hughes, Helen Hunter, Daniel Brison, Jason Ashworth, Chris Murgatroyd, Michael Carroll. *The role of Vasopressin in human sperm function.* Fertility 2021 [abstract / poster presentation]

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Appendices

Appendix

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

MEMORANDUM

TO	Michael Carroll
FROM	Karen Hartley
DATE	26th June 2017
DATE OF EXPIRY:	30th June 2020
SUBJECT	Application for Ethical Approval **(SE1617126)**

On the 26th June 2017 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (**SE1617126**) entitled "Human Sperm in vitro toxicity study". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Professor Tristan McKay) and the Research Degrees Administrator. Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence. Please notify Professor Tristan McKay of any issues relating to this.

If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the MMU Request for Amendment form (found on the Graduate School website) and submit it to the Administrator.

Regards

Karen Hartley
Research Administrator
All Saints North

Page **1** of **1**

Medical Screening Questionnaire **Participant code:**

It is important that the investigators are aware of any health conditions before participation in this research study. This information will be kept strictly confidential.

Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication? **YES/NO**

Are you currently attending your GP? **YES/NO**

Have you ever suffered from a cardiovascular problem? **YES/NO**

i*.e. high blood pressure, anaemia, heart attack etc*

Have you ever suffered from a neurological disorder? **YES/NO**

i.e. epilepsy, convulsions etc

Have you ever suffered from an endocrine disorder? **YES/NO**

i.e. diabetes etc

Have you ever suffered from a chronic gastrointestinal disorder? **YES/NO**

i.e. Crohn's disease, irritable bowel syndrome etc

Have you ever suffered from a skin disorder? **YES/NO**

i.e. eczema etc

Do you suffer from any allergies? **YES/NO**

i.e. any medications, foods etc

Do you smoke cigeretes **YES/NO**

If yes, how many per day? _____

Have you had a vasectomy or any urological surrey? **YES/NO**

i.e. testicular surgery

Have you had Mumps? **YES/NO**

Have you had any testicular injuries / torsions? **YES/NO**

Do you knowingly have, or had a Sexually Transmitted Infection? **YES/NO**

If you have answered "yes" to any of these questions, please provide details below:

Participant informed consent. ID code ……

Name: ……

Date of Birth: ……

Project title:

In vitro toxicity studies.

Principal Investigator:

Dr Michael Carroll

Investigator/Collaborators:

Dr Oliver Sutcliffe, Senior Lecturer, MMU

Dr Stéphane Berneau, PDRA, MMU

Ethics approval number: SE1617126

I have read and understood the information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.

Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason. I understand that my sperm will not be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos. I understand that my semen may be also used for teaching purposes. I also understand that no personal identifying information will be attached to any data derived from this sample and all data presented or published will be anonymised.

My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the School of Healthcare Sciences, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

- I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis (*please circle*) **YES NO**

- I give my consent for samples to be donated to MMU for use in future scientific / medical research and /or teaching purposes. I understand that my sample will not be used for any reproductive activities
- (*please circle*) YES NO

Date

Signed ....

Date

Witnessed

Name (Print)..... .....

SE1617126 v.1

Study Procedures

The following is a brief description of the procedures and techniques that will be employed during this study.

Semen procurement:

Semen will be produced by masturbation in to sterile containers provided. On occasions where participants cannot provide a specimen from home they will be asked to produce a sample on site in a dedicated, secured room. The specimen container will be placed in the plastic bag with the completed 'semen procurement form'.

Semen analysis:

Semen analysis is carried out within 30 minutes of specimen production. Volume, pH and other physical characteristics are noted. Sperm motility and concentration is measured and a sample of semen is smeared on to a glass slide for fixing and morphological analysis.

Sperm toxicity assays:

Sperm will be exposed to various compounds, after which measurements of sperm vitality and function will be measured, such as vitality, motility, morphology and DNA damage.

Cells and / or DNA will be stored for future analysis after collection.

SE1617126 v.1

Principle Investigator:

Dr Michael Carroll (michael.carroll@mmu.ac.uk)

0161 247 1231

Participant information sheet

Title of Study: *In Vitro Toxicity study*

Study Background

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm are produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. One major factor resulting in male infertility are exposures to chemicals (environmental chemicals, medicines, drugs etc).

This study will utilise in vitro assays to measure any potential sperm damaging effects of various chemicals.

This information will offer more insight to potential causes of male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.

The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. There will be no genetic testing. The samples will be stored at -80°C for further analysis.

Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes. No personal identifying information will be attached to any data derived from this sample and all data presented or published will be anonymised.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm. Recompense is not given for this study.

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

Become A Volunteer

We are looking for men to participate (regularly or depending on your availabilities) in various studies looking at human sperm biology and at Manchester Metropolitan University.

This is an opportunity to play an important role in Reproductive Biology research and to further our understanding on sperm quality (sperm count, how they move and how they look), which may help couples suffering from fertility issues.

Who can participate? Men, age 18+

For further information and to take part in this study, please see contact details below

Manchester Metropolitan University

Contact: **Dr Michael Carroll**
Senior Lecturer, Reproductive Science
Manchester Metropolitan University
Dr Stéphane Berneau
Research Associate, Reproductive Science
Tel: 0161 247 3366
Email: s.berneau@mmu.ac.uk

26/06/2018

Project Title: The Role of Neuropeptides and Sperm Function

EthOS Reference Number: 0381

Ethical Opinion

Dear Ana-Maria Tomova,

The above application was reviewed by the Science and Engineering Research Ethics and Governance Committee and on the 26/06/2018, was given a favourable ethical opinion. The approval is in place until 01/09/2020 and is based on the documentation submitted with your application.

Conditions of favourable ethical opinion

The Science and Engineering Research Ethics and Governance Committee favourable ethical opinion is based on the following conditions

Adherence to Manchester Metropolitan University's Policies and procedures

This ethical approval is conditional on adherence to Manchester Metropolitan University's Policies, Procedures, guidance and Standard Operating procedures. These can be found on the Manchester Metropolitan University Research Ethics and Governance webpages.

Amendments

If you wish to make a change to this approved application, you will be required to submit an amendment. Please visit the Manchester Metropolitan University Research Ethics and Governance webpages or contact your Faculty research officer for advice around how to do this.

We wish you every success with your project.

Science and Engineering Research Ethics and Governance Committee

Participant information sheet

Title of Study: *Investigating the role of neuropeptides and their effects on sperm function.*

Study Background

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm are produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. Neuropeptides are small proteins that function both as neurotransmitters in the brain and as hormones in the body. Neuropeptides role in reproduction are very well documented however, very little is currently known about their role in sperm function.

We will investigate the presence of neuropeptide receptors on sperm and the effects that neuropeptides may have on sperm function. This information may offer potential therapeutic options to improve male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.

The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.

Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes.

You may also be asked to provide a blood sample (optional). The blood will be taken by an experienced phlebotomist and is a quick and relatively painless procedure. However, there may be some bruising. Blood will be prepared and stored at -80°C until required. These samples will be used for biochemical and molecular analysis.

*Please note that this is **not** a diagnostic test and you will **not** be informed of the quality of your semen/sperm.*

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

A slight risk of fainting might also occur during/after blood collection via venepuncture due to fasting or a low blood pressure. The procedure may cause alight discomfort or pain, and some bruising. Post care and advice will be provided.

How do I withdraw from the study?

To withdraw simply state that you wish to do so in an email to Principle Investigator (michael.carroll@mmu.ac.uk) and all information/samples/data will be destroyed. You do not need to provide a reason for withdrawal from this study. You have the right to withdraw at any point in time.

Participant informed consent. ID code ……

Name: ……

Date of Birth: ……

Ethics approval number:

Project title:

Investigating the role of neuropeptides and their effect on sperm function.

Principal Investigator:

Dr Michael Carroll

Investigator/Collaborators:

Ana-Maria Tomova

Dr Stephane Berneau

Dr Chris Murgatroyd

Dr Jason Ashworth

Prof. Daniel Brison

I have read and understood the participant information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.

Any questions I have about the study, or my participation in it, have been answered to my satisfaction.

I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason and I understand how to do this.

I understand that my sperm will **not** be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos.

I understand that my semen may be also used for teaching purposes at Manchester Metropolitan University.

I understand that my participation in this study will be confidential unless I choose to break the confidentiality myself.

I understand that my sample will be anonymised for all processing purposes.

My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of Faculty of Science and Engineering, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

- I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis (*please circle*) **YES NO**

- I give my consent for blood collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis (*please circle*) **YES NO**

Signed _____ Date _____

Name (Print) _____

Witnessed _____ Date _____

Name (Print) _____