MOLECULAR AND PHYSICAL INTERACTIONS OF HUMAN SPERM WITH FEMALE TRACT SECRETIONS

by

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ABSTRACT

To achieve fertilisation, human sperm have to navigate and interact with the female reproductive tract (FRT) on molecular and mechanical levels. The current knowledge of some aspects of both types of interactions are limited and they were examined in this research.

Proteomic analysis of crude and depleted human follicular fluid (hFF) by three proteomic approaches identified 479 hFF-proteins of which 22% were novel. A table of hFF-proteins, compiled from twenty-four hFF proteomic studies, resulted in 1586 hFF proteins; a resource for folliculogenesis and discovery of hFF biomarkers.

A comparative proteomic study of media-capacitated human sperm versus capacitated sperm in the presence of hFF revealed certain hFF proteins were acquired by sperm during capacitation. Comparative metabolomics revealed some elevated metabolites in the sperm capacitation-media following 6-hour incubation compared to 1-hour swim-up, which may have relevance to sperm energy metabolism and potentially to sperm signalling mechanisms in the FRT causing remodelling for fertilisation and preparation for implantation.

Sperm micro-particle image-velocimetry revealed an average fluid velocity around the motile sperm of $\leq$ 25- 45 μm/s and $\leq$ 20-35 μm/s in low and high viscosity media respectively. The averaged fluid vorticity manifested a trail of spatially confined mixing of the fluid surroundings motile sperm. To the best of our knowledge, this work is the first to conduct a comparative proteomic analysis of human sperm versus sperm-hFF interaction and to perform metabolomics analysis of human sperm capacitation media at two time points. The novel perspective of mechanical aspects of sperm motility by studying fluid

velocity and vorticity around motile sperm adds a new approach to the study of sperm motility and chemotaxis.

In conclusion, sperm-FRT interactions involve complex molecular and physical interactions and regulatory events. Further research of these interactions may enhance our understanding of potential applications for improved assisted reproductive techniques' outcome and possible diagnostic approaches of infertility.

Dedications

To the memory of my father, I miss you every second of my life

To the memory of my brothers Mustafa and Abdullah, I hope you are in heaven

To my devoted mother, I love you so much

To my sister Abier, you taught me the love of books

To all my sisters, Eman, Hana, Nada, Nisreen and Raja, I love you all so much

To my brothers, Ahmed and Osama, I hope you all the best in your life

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LIST OF ABBREVIATIONS

µPIV:	micro-particle image velocimetry
cP	centipoise
1cP	1cP = 0.001Pa.s
2D-PAGE:	2 dimensional Polyacrylamide Gel Electrophoresis
ART	Assisted Reproductive Techniques
BSA	Bovine serum albumin
CASA	computer assisted semen analysis
COC	cumulus oocyte complex
DTT	Dithiothreitol
EDTA	Ethylene diaminetetra acetic acid
ESI	electro spray ionization
ESI-QTOF	electrospray ionization-quadrupole time of flight mass spectrometry
FC	fold change
FDR	False discovery rate
FF	follicular fluid
fps	Frame per second
FRT	Female reproductive tract
GC–MS	gas chromatography–mass spectrometry
GCs	granulosa cells
HAPs	High Abundance Proteins
hFF	Human follicular fluid
HMD	Human Metabolome Database
HPLC	high performance liquid chromatography
HVM	High viscosity media
i TRAQ	Isobaric tags for relative and absolute quantitation
IEF	Iso Electric Focusing
LAPs	Low abundance proteins
LPC	lysophosphatidylcholine
LVM	low viscosity media

MALDI-TOF	Matrix-assisted laser desorption/ ionization-Time of Flight
MC	methylcellulose
MS	Mass spectrometry
NMR	nuclear magnetic resonance
Pa.s	Pascal.second
PS beads	Polystyrene beads
PBS	Phosphate buffered solution
ROI	Region of interest
rnd proteins	random sequence proteins
SCX	Strong cation exchange chromatography
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sEBSS	supplemented Earle's balance salt solution
SELDI-TOF	Surface-enhanced laser desorption/ionization- Time of Flight
SP	seminal plasma
SPE	Solid phase extraction
TP1	1-hour swim-up samples (sperm capacitation media at time point at1h)
TP7	7-hour samples (sperm capacitation media at time point after 7-hour capacitation)
UPLC–MS	Ultra-performance liquid chromatography–mass spectrometry

Chapter 1

GENERAL INTRODUCTION

1.1The Sperm cell

1.1.1 Spermatogenesis:

Spermatogenesis is a distinct biological process by which undifferentiated spermatogonia develop, over a period of several weeks, into mature sperm cells with a haploid number of chromosomes (Hermo et al., 2010). This process occurs in the testis within the seminiferous epithelium, which is composed of Sertoli cells and several germ cells types. Spermatogenesis begins at puberty and continues throughout adult life with approximately 200 million sperm per ejaculate in human. Four consecutive stages are involved in spermatogenesis with the whole cycle taking around 64-74 days to finally produce mature sperm (**figure 1-1**) (Heller and Clermont, 1963, Heller and Clermont, 1964). The first phase of spermatogenesis involves spermatogonial proliferation through several mitotic divisions resulting in two diploid cells (spermatogonia and primary spermatocytes). The primary spermatocyte undergoes the second phase of cellular division through two rounds of meiosis; from primary spermatocyte to two secondary spermatocytes, then to four haploid round spermatids. The third phase of spermatogenesis is the spermiogenesis in which the spherical spermatids transform by differentiation of a head and tail structure and reduction of cytoplasm to produce the mature spermatozoa, which is followed by the final stage of spermatogenesis (spermiation), marked by the sperm release into the tubule lumen (McLachlan et al., 2002, Amann, 2008). These stages are interdependent; nevertheless, each stage has distinctive regulatory microenvironment contributed by the secretions of the neighbouring Sertoli cells, Leydig cells and the surrounding vascular network in endocrine and paracrine patterns (Amann, 2008). Testosterone, growth factors and neuroendocrine compounds secreted by Sertoli and Leydig cells determine the intrinsic regulatory mechanism of spermatogenesis (Middendorff et al., 1997) while follicle stimulating

hormone (FSH) secreted by the anterior pituitary gland and gonadotropin releasing hormone (GnRH) secreted by the hypothalamus determine the extrinsic regulation of spermatogenesis (Holstein et al., 2003). FSH stimulates Sertoli cells whereas GnRH induces luteinizing hormone (LH) release from the hypophysis which in turn stimulates Leydig cells to secrete testosterone (Holstein et al., 2003). Additionally, feedback mechanisms are manifested by inhibin secreted via Sertoli cells and testosterone levels released from Leydig cells to regulate the hypophysis-GnRH secretory activity (Holstein et al., 2003).

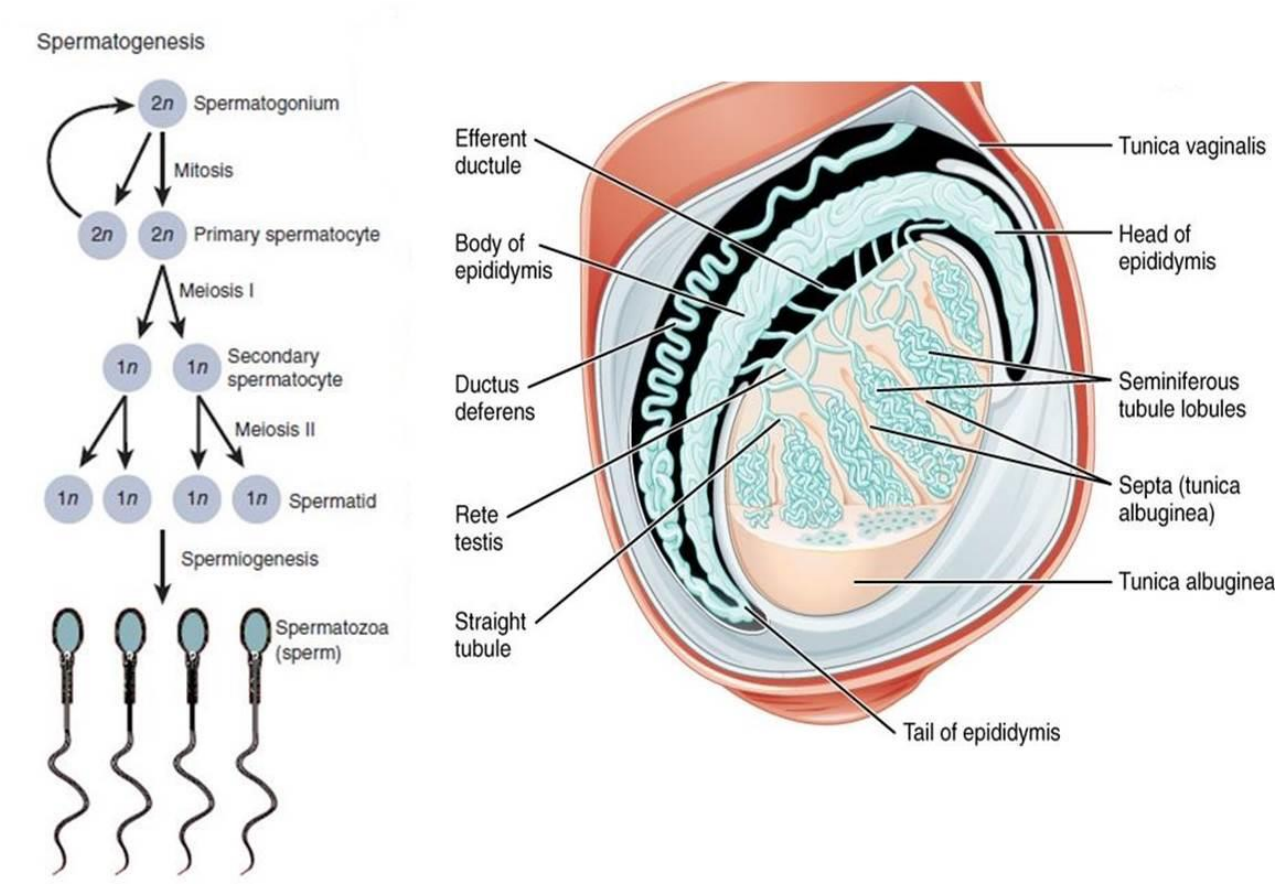


Figure 1-1 Stages of spermatogenesis and illustration of sagittal view of the seminiferous tubule and epididymis (adapted from OpenStax College, Anatomy & Physiology book available at http://cnx.org/content/col11496/latest/ also available on public domains).

1.1.2 Sperm cell structure:

Mature sperm are compact, highly differentiated and compartmentalised cells with a haploid number of chromosomes. All mammalian sperm share a common structure of a head and tail (flagellum) (Peter and Gaurishankar, 2006) (**figure 1-2**). The sperm head is approximately 4-5µm long and is composed of a membrane-enclosed nucleus and acrosome (Mortimer, 1997); the nucleus is highly condensed due to the partial replacement of histones with protamines during spermatogenesis (Wouters-Tyrou et al., 1998). This nuclear hyper-condensation is important for sperm head hydrodynamic shape and compactness which enables sperm motility and oocyte penetration (Brewer et al., 2002). Nonetheless, this highly packed DNA within the sperm nucleus means sperm are not capable of transcription and translation (Luconi et al., 2004). This inability to synthesise new proteins results in sperm reliance on post-translational modifications of already existing proteins to achieve functional competence and fertilisation (Naz and Rajesh, 2004). These modifications are evident within the epididymis (see section 1.1.3) and during capacitation (section 1.4.1).

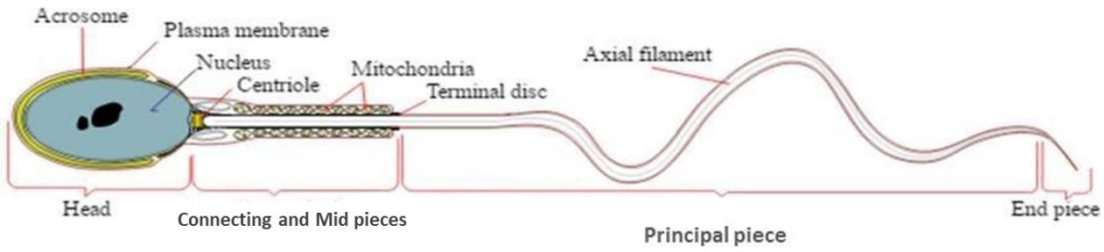


Figure 1-2 human sperm cell structure with two main components: head and tail. The tail is subdivided into four regions: conncting piece, mid piece, principal piece and end piece (adapted from https://www.boundless.com/ physiology/textbooks), figure is available on public domains.

The sperm acrosome is a secretory vesicle packed with a complex combination of lytic enzymes, including hyaluronidase and acrosin which are released upon acrosome exocytosis (Yanagimachi, 1994b). The acrosomal cap is formed from the Golgi apparatue during early spermiogenesis and covers the anterior two thirds of the sperm nucleus (Yoshinaga and Toshimori, 2003), **figure 1-3**.

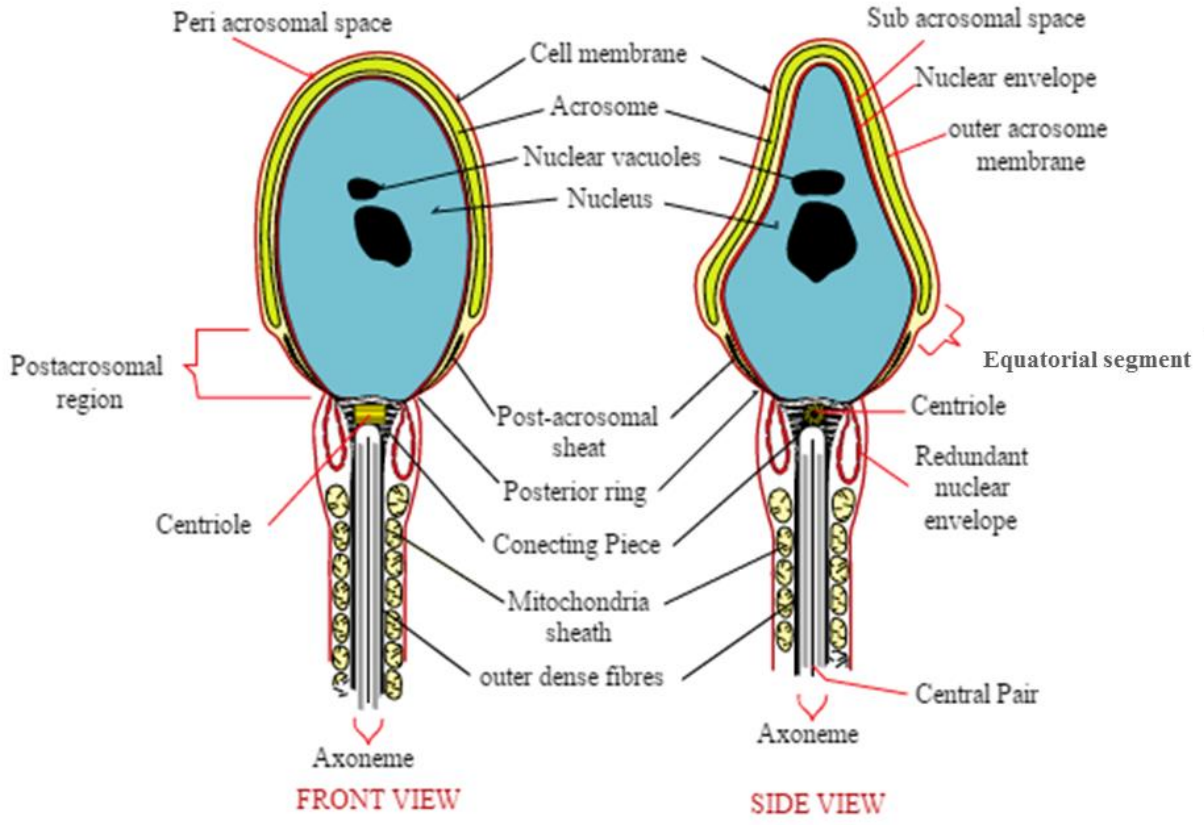


Figure 1-3 sperm head is composed of an acrosome and nucleus, the connecting piece contains the centriole while the midpiece mainly contains mitochondrial sheath (adapted from https://www.boundless.com/ physiology/ textbooks, also available on public domians)

Acrosome abnormalities, such as acrosome hypoplasia in which the acrosome is small and detached and chromatin condensation is diminished, are associated with male infertility (Chemes and Alvarez Sedo, 2012). Total globozoospermia, where the semen contains only round-headed sperm with absent or malformed acrosomes is a further example of acrosomal deficiency resulting in male infertility (Dam et al., 2007). Increased levels of DNA damage and perturbed chromatin compaction have also been observed in the globozoospermic sperm (Dam et al., 2007).

The sperm tail (flagellum) is generally divided into four regions (**figure 1-4**); the connecting piece, which links the tail with head region; the mid-piece, which links the connecting piece with the annulus which marks the end of the mid-piece and the beginning of the principal piece and finally the end piece (Mortimer, 1997, Fawcett, 1975). Each of the four regions has some unique characteristics, for example, the midpiece exclusively contains the mitochondria essential for adenosine triphosphate (ATP) production required for sperm flagellar motility while the principal piece exclusively contains the fibrous sheath (FS) (Turner, 2006).

The ultrastructure of the sperm tail is critical for its function (Turner, 2006). The axoneme is the motor part and is arranged in 9 + 2 microtubule doublets extending throughout the length of the four tail regions and it has inner and outer dynein arms which extend from each of the outer nine doublets (Turner, 2006). These dynein arms are believed to be responsible for the generation of the propulsive force of the sperm flagellum (Fawcett, 1975, Clermont et al., 1990, Turner, 2006). In the mid-piece, the presence of nine outer dense fibres (ODFs) surrounding each of the nine outer axonemal microtubules enclosed in a mitochondrial sheath is characteristic of this region (Turner, 2006). The principal piece has seven ODFs as two of the nine ODFs are replaced by two longitudinal columns of FS

in this region and both ODFs and FS taper and terminate at the end of the principal piece (Turner, 2006). The end piece has only the axoneme surrounded by plasma membrane and marks terminal section of the sperm flagellum (Turner, 2006).

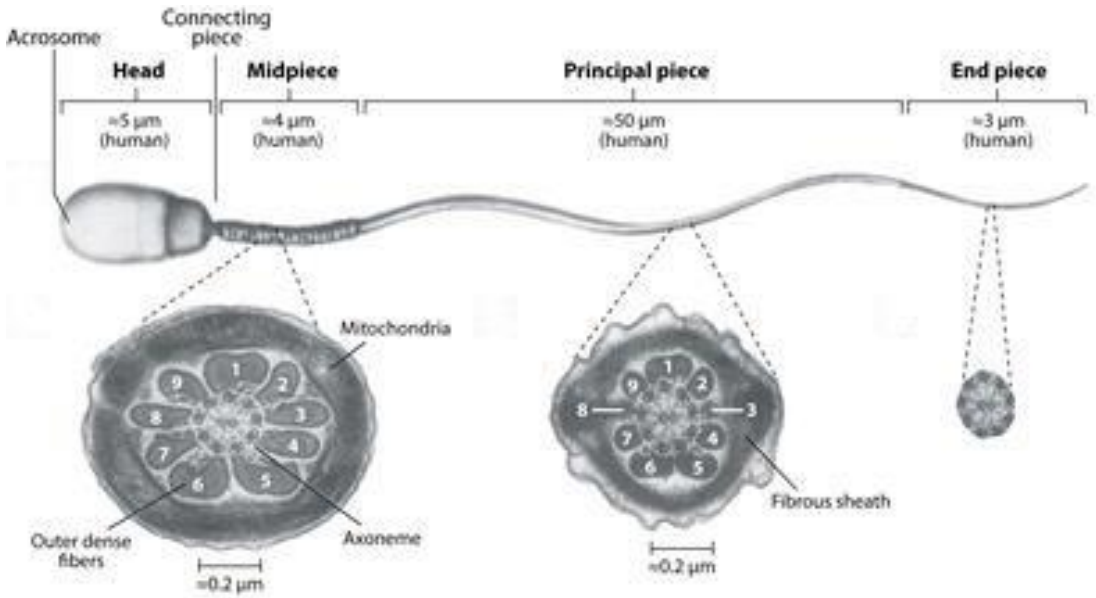


Figure 1-4 Diagram representing human sperm and ultrastructure of the flagellum. The flagellum is subdivided into four regions: conncting piece, mid piece, principal piece and end piece (from Gaffney et al, 2011, no permission is required from Annual Reviews).

1.1.3 Sperm maturation in the Epididymis:

From the testis to the deferent duct, sperm cells are exposed to a distinct environment created by very restrictive exchange that is isolated from the rest of the body by a blood-testis barrier (Dacheux and Dacheux, 2014). Both Sertoli cells within the testis and epididymal epithelium play major roles in the formation of this unique milieu which sperm are exposed to prior to ejaculation (Dacheux and Dacheux, 2014). However, testicular sperm are unable to fertilise oocytes (Cornwall, 2009) and as they transit through the epididymis, sperm acquire progressive motility and the ability to capacitate in a series of

sequential events termed maturation which culminates with the sperm's capability for fertilisation (Cooper, 1995, Cooper, 2007). The epididymis is a long convoluted tubule, several metres in length, which is divided into distinct segments that each have a unique microenvironment (Cornwall and von Horsten, 2007). These epididymal segments (namely caput, corpus and cauda) synthesise and secrete proteins in a segment-defined manner (Cornwall, 2009). The transit time of sperm through the epididymis varies from a few days to three weeks with an average of 11 days in humans (Cooper, 2007).

The maturing sperm acquire some epididymal proteins which are vital for functional modulation (Cooper, 1995). Simultaneously, the epididymal milieu's composition also undergoes consecutive changes throughout the epididymis length in parallel to the sperm maturation process (Dacheux and Dacheux, 2014). Extensive research has been devoted to understand the interplay between both sperm maturation within the epididymis and the changes in epididymal environment composition and the role of these changes in sperm fertilisation in several species (reviewed in Dacheux and Dacheux (2014)).

During their transit through the epididymis, sperm are exposed to changes in the composition of the epididymal fluid caused by water reabsorption, leading to increased sperm concentration paralleled to remarkable changes in the epididymal fluid ionic composition (Da Silva et al., 2006). Furthermore, there are resultant increases in the luminal fluid's protein concentration which reaches an average of 20-30 mg/ml in the distal parts of the epididymis (Fouchécourt et al., 2000, Belleannée et al., 2011, Dacheux et al., 2012). Modifications of epididymal protein and ionic compositions have been linked to sperm maturation events such as cytoplasmic droplet migration, initiation of flagellar beating and ability to bind to the zona pellucida in several mammalian species (Dacheux and Dacheux, 2014). The roles of epididymal proteins in the modification of the

sperm surface and their contribution to sperm survival and function have been demonstrated in several studies (reviewed in Dacheux and Dacheux (2014)). Sperm acquire low molecular weight compounds from epididymal secretions during the maturation process (Cooper, 2007). The sperm interaction with epididymal proteins is critically important as the sperm are unable to actively synthesise new proteins as discussed earlier (Cornwall, 2009). However, the exact biochemical and molecular changes which are involved in the sperm maturation within the epididymis remain elusive (Cornwall, 2009).

Several studies have aimed to identify the epididymal proteins' composition and to understand the specific roles of the identified proteins in the sperm maturation process (Turner et al., 1979, Brooks, 1981, Dacheux and Dacheux, 2014). Despite considerable progress in proteomics technology in recent years, the complete epididymal protein profile is still proving technically challenging (Dacheux and Dacheux, 2014). This is attributed to the immense variation in protein concentrations seen in epididymal fluid which exceeds 12 orders of magnitude in comparison with the 4-6 orders of magnitude detectable by the most sensitive mass spectrometers (Dacheux and Dacheux, 2014). Currently, hundreds of the epididymal proteins have been identified (Dacheux and Dacheux, 2014). Albumin, clusterin, lactoferrin, α1-antitrypsin, extracellular matrix protein, transferrin, actin binding protein, prostaglandin D2 synthase (PTGDS) and Niemann-Pick disease type C2 (NPC2) protein represent approximately 77% of the total human epididymal proteins (for the full list of the identified human epididymal proteins and their proportions see (Dacheux et al., 2006). However, the molecular functions of these proteins in sperm maturation is still not fully characterised (Cornwall, 2009). One of the important epididymal, non-sperm expressed proteins acquired by sperm during their epididymal transit is the cysteine-rich

secretory protein 1 (CRISP1) (Cohen et al., 2000). CRISP1 has been shown to work as a decapacitation factor to maintain sperm in a quiescent state within the epididymis (Koppers et al., 2011).

1.1.4 Semen

In humans, ejaculated semen comprises of sperm and seminal plasma (SP). The SP is a complex fluid mixture which determines the chemical properties of the ejaculate and is crucial for sperm survival (Juyena and Stelletta, 2012). Seminal plasma is a mixture of constituents secreted by a number of glands (Owen and Katz, 2005). First are the secretions of the Cowpers (bulbourethral) and Littre glands which contribute about 5% of the ejaculate while the prostate forms the second part which is around 15-30% of the ejaculated semen (Owen and Katz, 2005). The seminal vesicle contributes the bulk of the semen while the ampulla and epididymis contribute small amounts (Owen and Katz, 2005). Notably, these constituents are not entirely mixed and therefore, the first part of the ejaculated semen is not completely homogenous (Owen and Katz, 2005). Within the ejaculate, sperm form only around 1-5% of the total semen volume (Mortimer, 1994). The roles of SP have been studied extensively in different species (Juyena and Stelletta, 2012). Some components within SP have been shown to contribute to fundamental events in sperm function, to provide optimum nutrition and to prevent premature capacitation through capacitation inhibitors (Desnoyers and Manjunath, 1992, Villemure et al., 2003, Juyena and Stelletta, 2012). For example, prostasomes, which are sub-micron, membrane-bound secretory granules secreted by the prostate epithelium and form a fraction of the seminal fluid (Burden et al., 2006, Ronquist and Brody, 1985), have been shown to undergo fusion with sperm resulting in direct transfer of the prostasomes' proteins to the sperm (Ronquist and Nilsson, 2004). The newly acquired prostasomes' proteins contribute

to sperm Ca^{2+} signalling mechanisms (Park et al., 2011) and are important for sperm survival and motility in the female reproductive tract (FRT) (Ronquist and Nilsson, 2004). Prostasomes and their functions in sperm are extensively reviewed in (Ronquist, 2012) and for a review of seminal plasma properties see Owen and Katz (2005) and Juyena and Stelletta (2012).

The response of the FRT to semen exposure and its effect on the success of natural fertility and ART has been the core of numerous studies in several species. Immunological, inflammatory and molecular changes have been observed following the deposition of semen into the FRT (Muller et al., 2006) in several species. Both sperm (Pandya and Cohen, 1985, Thompson et al., 1992) and seminal fluid (Sharkey et al., 2007) have been suggested to elicit signalling response in human FRT, mainly in the form of leucocytic infiltration of the vagina and cervix. Exposure of the FRT to semen has also been shown to provoke an inflammatory response manifested by a remarkable infiltration of macrophages, T cells and dendritic cells and increased cytokines release (Schuberth et al., 2008); in pig (O'Leary et al., 2006), in sow (Rodriguez-Martinez et al., 2010) in mouse (Robertson et al., 1996) and in humans (Sharkey et al., 2007).

It has also been suggested that prior contact of the FRT to semen may enhance endometrial receptivity and improve pre-implantation embryo development (Katila, 2012, Tremellen et al., 2000). This is attributed to enhanced immune tolerance of the FRT prompted by the previous exposure to seminal fluid which represents the paternal alloantigens (Robertson et al., 2009, Katila, 2012). Furthermore, greater risk of failed implantation, spontaneous miscarriage and pre-eclampsia have been linked to lack of prior exposure to semen in women in their first pregnancy or who conceived via IVF or in those using barrier method

of contraception such as condoms (Klonoff-Cohen et al., 1989, Robillard et al., 1994, Bellinge et al., 1986, Tremellen et al., 2000).

In mouse, transforming growth factor-b (TGF-B) from seminal fluid has been identified as a significant component to maintain maternal tolerance to pregnancy and implantation (Robertson et al., 2006a, Robertson et al., 2009) through regulation of endometrial epithelium and stimulation of certain pro-inflammatory cytokines such as Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Tremellen et al., 1998). Similarly, TGF-B from human seminal fluid has been suggested to play a role in cervical immune response to infection (Sharkey et al., 2012).

The evidence supporting significant role of seminal fluid in improving pregnancy rate in several other species is still elusive (Hansen, 2011). For instance, there was no impact of seminal vesicle surgical excision on fertility rates in boars (Davies et al., 1975) and no difference was observed in pregnancy rates in pigs (Flowers and Alhusen, 1992) and mare (Newcombe and Cuervo-Arango, 2011) whether the pregnancy followed natural mating or was induced by artificial insemination in which only a fraction of the seminal fluid is introduced to the FRT (Hansen, 2011). In contrast, removal of the seminal vesicle resulted in decreased implantation rate and poor embryo development in mice (Robertson, 2014, Robertson et al., 1996). Obesity, abnormal metabolic parameters and decreased glucose tolerance were also manifested in the male mice offspring in another study which highlighted the implications of FRT exposure to seminal fluid on the offspring health (Robertson et al., 2006b).

1.2The female reproductive tract (FRT):

1.2.1 Menstrual cycle:

From the onset of menarche, the female reproductive tract (FRT) undergoes hormonal and developmental changes and the menstrual cycle commences to provide female gametes for fertilisation. Menstruation occurs in response to coordinated interactions between hypothalamic, anterior pituitary and ovarian hormones (Johnson and Everitt, 2000). The gonadotropins secretion by the anterior pituitary manifests a pulsatile pattern with pulse frequency and amplitude fluctuating according to the phase of menstrual cycle with LH reaching a maximum prior to onset of ovulation (**figure 1-5**). The median duration of menstrual cycle is 28 days divided into two phases: follicular or proliferative phase and luteal or secretory phase. The follicular phase starts from the first day of menstrual bleeding until the onset of ovulation (days 1-14) and is dominated by the hormone oestrogen. During this phase, follicle stimulating hormone (FSH) is secreted by the anterior pituitary leading to the development of ovarian follicles and LH receptors formation on the surface of follicular granulosa cells (Johnson and Everitt, 2000). The growing follicles produce large amounts of oestrogen which results in thinning of cervical mucus, proliferation and increased vascularisation of uterine endometrium and increased FSH receptors on the granulosa cells (GCs) (Johnson and Everitt, 2000). Oestrogen also provides feedback regulation to the hypothalamic-pituitary axis which regulates the secretions of gonadotropin-releasing hormone (GnRH) from the hypothalamus and the LH and FSH by the anterior pituitary (Johnson and Everitt, 2000). The sharp rise in oestrogen secretion around day 10-14 causes positive feedback to the hypothalamic-pituitary axis which results in the onset of LH surge, around mid-cycle, followed 36 hours later by ovulation (Johnson and Everitt, 2000). Exposure to 200 pg/ml of oestrogen for

approximately 50 hours has been shown to induce hypothalamic secretion of GnRH with subsequent increase of LH and FSH from the anterior pituitary gland (Young and Jaffe, 1976).

The resultant LH surge triggers resumption of meiosis in the oocyte and the expulsion of the first polar body 10-12 hours following the peak of the LH surge (Johnson and Everitt, 2000). The mechanisms by which the LH surge induces the release of the oocyte from the mature follicle is not fully clear and is suggested to involve LH-induced rise in the follicular contents of collagenase, plasminogen activator and prostaglandins (LeMaire et al., 1973). These factors are suggested to contribute to the increased fluid pressure within the follicle (Diaz-Infante Jr et al., 1974, Koos and Clark, 1982) and digestion of the follicular wall and rupture of the follicle releasing the cumulus oocyte complex (COC) (Beers et al., 1975, Downs and Longo, 1983). The next phase of the menstrual cycle is the luteal phase (day 14-28) which marks the formation of corpus luteum from the follicular remains that contributes predominantly to the secretion of progesterone. If oocyte fertilisation does not occur, the corpus luteum degenerates leaving scar tissue known as corpus albicans and resulting in progesterone withdrawal and onset of menstrual bleeding. The decline in progesterone level relieves negative feedback on the anterior pituitary inducing secretion of FSH and a new cycle commences.

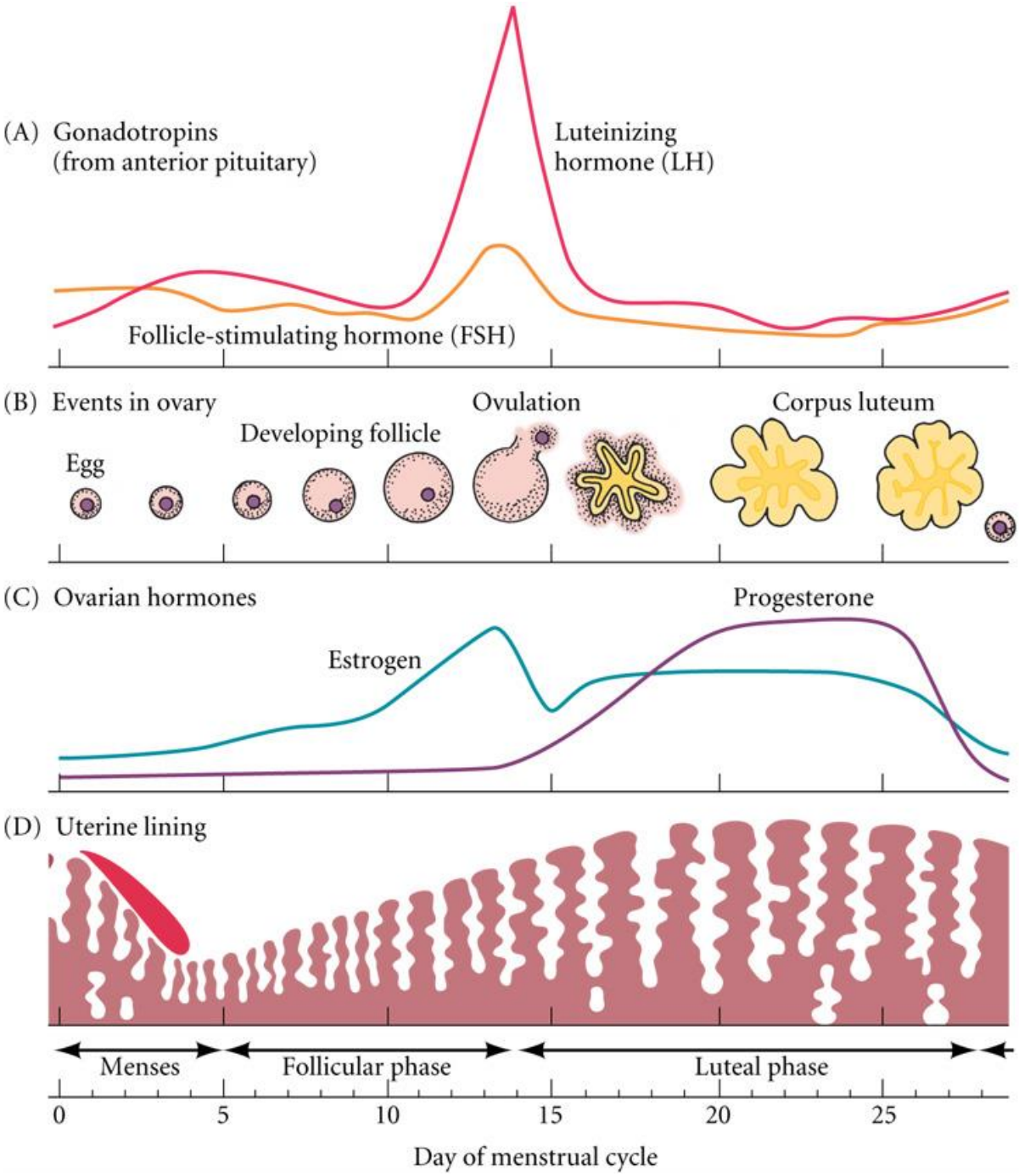


Figure 1-5 The human female reproductive cycle showing changes in ovarian hormones and uterine lining (from https://www.britannica.com/science/menstrual-cycle) also available on public domains.

1.2.2 Oogenesis:

The general accepted concept regarding the reserve of primary oocytes is that females have a finite number of follicles/oocytes which is established before birth and declines with female age until the permanent cessation of menstruation at menopause (Rodrigues et al., 2008). However, some recent studies have opposed this concept and suggested the presence of de novo synthesis of oocytes similar to the spermatogenesis that operates in human adult testis (Zou et al., 2009, White et al., 2012). The presence of postnatally functional stem cells at different stages of development in the mammalian ovaries including in humans has raised the question of formation of oogonia during adulthood (Virant-Klun, 2015). A recent review has critically presented the supporting and the contradicting views of postnatal oogenesis in humans (Virant-Klun, 2015).

Oogenesis is an episodic process which commences in the ovaries during the fetal period of life followed by a stage of arrest which continues until puberty (Johnson and Everitt, 2000).

The primordial germ cells (PGCs), with high rates of mitosis, divide into oogonia (premeiotic germ cells) which reach approximately 6 million cells at 20 weeks of gestation after which the rate of mitosis diminish progressively parallel to an increased rate of atresia which continues throughout the rest of the female life (Oktem and Oktay, 2008). Oogonia divide in a number of mitotic cycles to progress to the early stage of meiosis I forming diploid primary oocytes (Oktem and Oktay, 2008; Virant-Klun, 2015). At this stage, the primary oocytes become arrested at the prophase I of the first meiosis (diplotene stage) and their nucleus is known as the germinal vesicle (GV). The GV oocytes become enclosed within the primordial follicle (see section 1.2.3). This stage of arrested meiosis is vital for the oocyte development and storage (Voronina and Wessel, 2003). All the

primary oocytes are formed by the end of the fetal period and only ~1 million are available at birth (Oktem & Oktay, 2008). The synthesis of mRNA and rRNA by the primary oocytes continues despite being in meiotic arrest and this process is crucial for the generation of proteins required for oocyte maturation and development following fertilisation (Johnson and Everitt, 2000).

Due to continuing atresia, by puberty, only 3000-4000 primary oocytes are still available and recruitment of around 15-20 takes place during each menstrual cycle with one secondary oocyte being released at ovulation (Oktem and Oktay, 2008). During maturation and prior to ovulation, the primary oocytes resume meiosis and complete meiosis I and undergo an asymmetric division into two daughter cells: a secondary oocyte with haploid number of chromosomes and first polar body (Johnson and Everitt, 2000). The secondary oocytes proceed to the second meiotic division and undergo a second arrest at the metaphase II until the time of fertilisation when the completion of meiosis is activated (Oktem and Oktay, 2008).

1.2.3 Folliculogenesis:

The ovarian follicles are the functional units of the ovary and they undergo growth and development to achieve the ovulatory stage of a mature oocyte in a highly complex process termed folliculogenesis (Oktem and Oktay, 2008). Folliculogenesis involves an array of events which include proliferation and morphological changes of GCs, steroidogenesis, gonadotrophin receptor expression, oocyte maturation and ends with ovulation (Oktem and Oktay, 2008).

The earliest stage of follicular development commences at week 7 of embryo development in humans and involves the formation of primordial follicles which are dormant follicles; each contains a primary oocyte encapsulated in a layer of squamous pre-GCs and a basal

membrane (Oktem and Oktay, 2008). The residence of the primary oocyte in the quiescent state within the primordial follicle may extend for decades maintained by a complex inhibitory signalling array of events (reviewed in McLaughlin and McIver (2009)). The primordial follicles represent the ovarian reserve and they undergo transition into primary follicles which are recruited into a pool of growing follicles during each menstrual cycle (Oktem and Oktay, 2008). The recruitment of primordial to primary follicles commences at puberty and continues until menopause (Oktem and Oktay, 2008). During the transition from primordial to primary follicles, several morphological changes occur including increase in the oocyte diameter and its encapsulation in zona pellucida (ZP) (Rankin et al., 1996). The pre-antral stage of follicle is characterised by proliferation of GCs into a multi-layered structure surrounding the enlarging oocyte along with the formation of basal lamina and theca interna cell layer from the condensed stroma cells (Rankin, et al., 1996) with a follicle diameter reaching 40-120 μm ((Knight and Glister, 2006, Oktem and Oktay, 2008).

Factors contributing to trigger the transition from the quiescent primordial to the growing primary follicles are not fully characterised (Oktem and Oktay, 2008). The lack of FSH receptors on the primordial follicles suggested that the transition process is gonadotropin-independent (Oktay et al., 1997, Oktem and Oktay, 2008). Locally produced members of the transforming growth factor-beta family (TGF-β), bone morphogenetic proteins BMP4 and BMP7 from stromal and theca cells (Nilsson and Skinner, 2003, Lee et al., 2001) have been shown along with growth differentiation factor 9 (GDF9) (Vitt et al., 2000) and FIGalpha (Soyal et al., 2000) to be crucial factors for the formation of primary follicle. Factors contributing to the transition of primordial to primary follicle are reviewed in Oktem and Urman (2010). The regulation of primordial follicle quiescence/activation

involves interactions with somatic cells and oocytes (Kim, 2012). Several factors such as antimullerian hormone (AMH), Foxo3a and Foxl2 are involved in maintaining primordial follicle quiescence and survival and prevention of premature activation and depletion of primordial follicles reserve (reviewed by Kim (2012)).

In the antral stage of follicle development, the secondary follicle is formed over a prolonged period of several months and is characterised by extensive proliferation of the GCs forming numerous layers surrounding the oocyte. GCs undergo differentiation into two distinct cell types: mural GCs which form the follicle wall and responsible for steroidogenesis; and cumulus cell mass surrounding the oocyte (Chian et al., 2004, Huang and Wells, 2010). The size of the oocyte and its surrounding follicle increases progressively along with increased vascularisation and formation of fluid-filled space with follicular fluid (FF) accumulation. At this stage, the GCs become responsive to FSH which is along with members of the (TGF-β) family, GDF9, BMP15, BMP6 and activin promote granulosa cell proliferation and positive regulation of follicle growth (Oktem and Urman, 2010). Bidirectional signalling communications between the oocyte and its surrounding GCs and between the granulosa and theca cells in the presence of growth factors are some of the suggested critical events involved in triggering follicle growth and development (Oktay et al., 2000, Eppig, 2001, Skinner, 2005).

The regulation of follicle growth and development and the mechanisms involved in the cyclic recruitment of follicles and selection of a dominant (tertiary) follicle is a complex multifactorial process (Oktem and Umran, 2010). This process involves a complex array of events in which both local (paracrine) and endocrine factors are involved (Oktem and Urman, 2010). Both LH and FSH play principal roles in the endocrine modulation of follicle development with more prominent role of high FSH (and oestradiol) in the

formation of the dominant follicle (Danforth, 1995, Salha et al., 1998). One of the suggested mechanisms implicated in the dominant follicle selection is the differentially expressed FSH sensitivity in the growing cohort of follicles which is regulated by members of the TGF-β family, activin, inhibin and anti-mullerian hormone (AMH) (Oktem and Oktay, 2008). While the majority of follicles undergo atresia, the selected dominant follicle undergoes further development and expansion with increased human follicular fluid (hFF) accumulation (Johnson, 2007). It has also been observed that low concentrations of follicular FSH and oestradiol are associated with follicle atresia (Salha et al., 1998). Local factors within the ovarian follicle such as growth factors, steroids, cytokines and inhibin/activin are also suggested to be involved in the modulation of follicle growth and atresia via modification of FSH and LH response during folliculogenesis (Erickson and Danforth, 1995).

At ovulation, the cumulus-oocyte complex (COC) is released along with hFF (**figure 1-6**) (Johnson, 2007). In some studies ovulation is described as an inflammatory process as the majority of hFF proteins detected are acute phase proteins which belong to the complement cascade category (Angelucci et al., 2006). However, ovulation is also described in other studies as a hormone-induced injury as many of hFF proteins belong to angiogenesis and blood coagulation categories (Jarkovska et al., 2010) and tissue restoration (Murdoch et al., 2010).

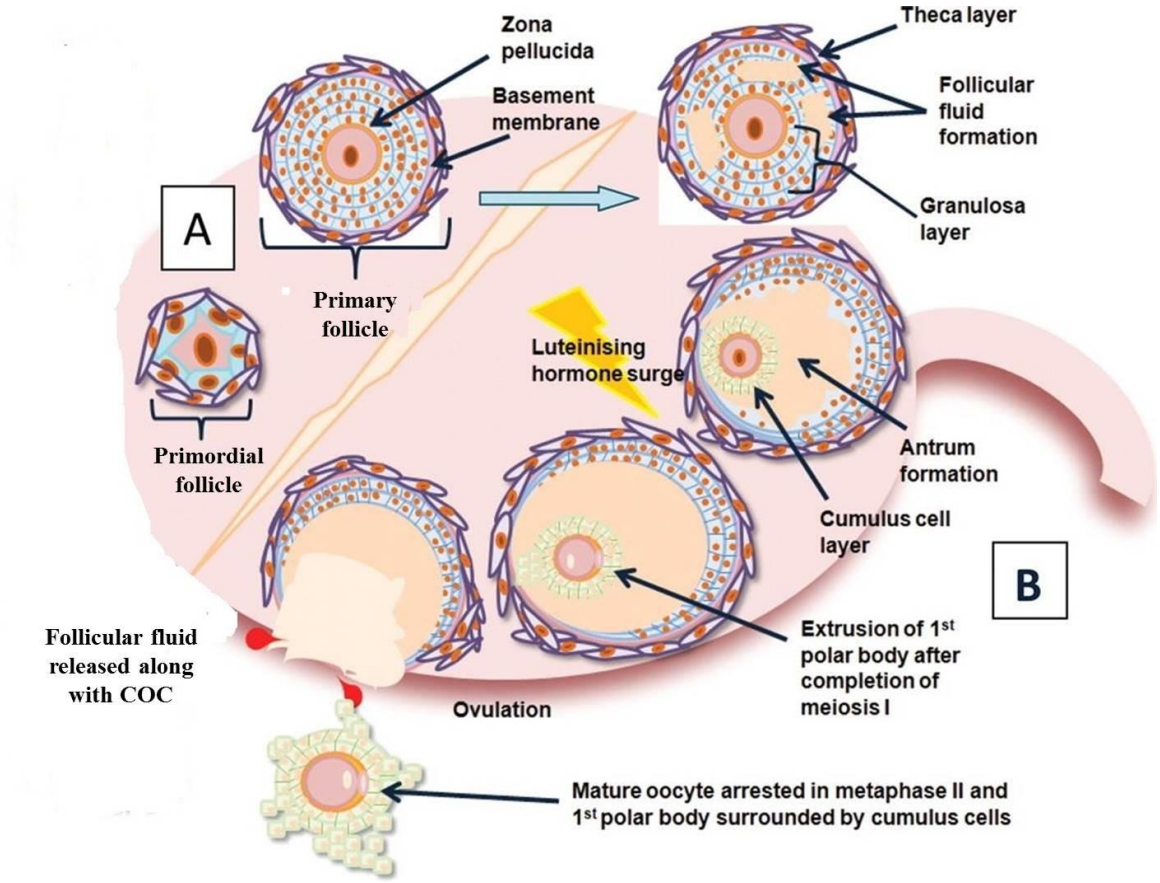


Figure 1-6 Stages of follicular development and ovulation. A- Preantral stage of folliculogenesis, B- Antral stage of folliculogenesis, accumulation of hFF and ovulation (from Huang and Wells, 2010, with permission).

The oocyte, zona pellucida and cumulus cells:

The oocyte is the female gamete which is involved in fertilisation and is considered one of the largest cells in the body (Johnson and Everitt, 2000). In humans, the diameter of the oocyte prior to ovulation is around 100 µm (Mehlmann, 2005). At ovulation, the secondary oocyte is surrounded by zona pellucida (ZP) and cumulus mass forming the COC) which is released into the peritoneal cavity and transferred to the ampullary region of the oviduct where fertilisation by sperm may take place.

All mammalian oocytes are encapsulates in the ZP which is a 7-20μm thick matrix of highly glycosylated proteins formed during oogenesis (Talbot, 1985).

In humans, ZP is composed of four glycoproteins ZP1, ZP2, ZP3 and ZP4 (Lefievre et al., 2004). The roles of the ZP glycoproteins have been studied extensively in murine species which only possess three ZP proteins, unlike humans (Lefievre et al., 2004). In mouse, ZP1 has been shown to provide structural integrity to the ZP matrix (Wassarman, 1999, Wassarman, 1988), ZP2 and ZP3 are both sperm receptors with ZP3 being the primary sperm ligand and inducer of AR (Bleil and Wassarman, 1983) while ZP2 is the secondary sperm ligand which binds to AR-sperm and contributes to polyspermy prevention (Bleil and Wassarman, 1980). In human, ZP2 has been shown as the sperm-oocyte binding ligand and the site for gamete recognition and prevention of polyspermy (Baibakov et al., 2012).

Several functions have been attributed to ZP including species-specific binding of sperm, prevention of polyspermy (Yanagimachi, 1994) and protection of the embryo prior to implantation as it has been shown to surround embryos until shortly before implantation (Wasserman, 2008; Florman et al, 2006). ZP is also considered as the principal physiological inducer of AR following sperm-ZP binding (Kopf and Gerton, 1991) although, as will be discussed later, other more recent studies indicate the onset of AR prior to the encounter of both gametes (reviewed in Gervasi and Visconti (2016)).

The cumulus oophorous is approximately 700 μm in size and comprises two layers of cells; the cumulus cell mass which is the outer layer rich in hyaluronic acid and the corona radiata cells which form the inner layer adjacent to and penetrating through the ZP to contact the oocyte. The cumulus cells contribute significantly to the development of competent oocytes via bi-directional communication established by gap junctions (Huang

and Wells, 2010). Both of these cell layers contribute to steroid hormones and protein secretions of the COC (Laufer et al., 1984, Osman et al., 1989, Tesarik et al., 1988) which are rich in progesterone and other chemotactic substances (De Jonge, 2005). Progesterone is secreted in the concentration of 5–10µM by the oocyte and the cumulus mass during ovulation (Osman et al, 1989) and the level of progesterone in the pre-ovulatory hFF is approximately 22 µM (Anderson, 1991). Progesterone is highly available at the vicinity of the ovulated egg and may be encountered by sperm prior to fertilisation (Florman et al, 2008).

In addition to their secretory functions, Ca^{2+} signal propagation through their gap junctions with the oocyte is another important feature of the corona radiata cells (Motta et al., 1994, Mattioli and Barboni, 2000). Phagocytosis has been also suggested as a selective mechanism operated by the cumulus and corona cells to remove excessive and abnormal sperm cells from the site of fertilisation (Nottola et al., 1998).

Human follicular fluid (hFF):

Human follicular fluid (hFF) is a straw coloured, slightly viscous fluid (Edwards, 1974) with an average volume of 2.7 ml in mature follicles (Simonetti et al., 1985). hFF within the follicle is separated from the circulation by blood-follicle barrier which is determined by cellular and physiological properties (Edwards, 1974). The generally accepted theory about hFF formation suggests that secretions from granulosa cells along with diffused substances from blood-follicle barrier are the principal source of the hFF contents (Fortune, 1994, Spitzer et al, 1996, Anahory et al, 2002, Jarkovska et al, 2010). The blood-follicle barrier is suggested to work as a selective molecular sieve only permeable to substances of molecular weight up to 500 kDa (Cran et al., 1976). The high permeability of the basement membrane of the GCs in growing follicles enables the transmission of

molecules between the follicular cells without active transport being involved (Edwards, 1974).

Some of the suggested physical functions of hFF are to facilitate the escape of the oocyte from the follicle and to provide a medium for oocyte transport and protection during ovulation (Edwards, 1974). At ovulation, the movement of the fimbria near the ovary where the oocyte is released creates an environment for the ciliary beat to gently sweep the oocyte into the ampulla (Edwards, 1974). Although not clearly elucidated, hFF is suggested to modulate the oviductal environment to enable oocyte transport to the ampulla (Edwards, 1974). Some of the suggested mechanisms include hFF enhancement of tubal fimbria contractility via its prostaglandin content (Sterin-Speziale et al., 1978) and increases in ciliary beat frequency in the oviduct (Lyons et al., 2006). Both mechanisms are suggested to assist oocyte pick-up and facilitate gametes transport through the oviduct contributing an increased chances of pregnancy (Lyons et al, 2006).

Despite the significance of the formation of follicular antrum and synthesis of FF in mammalian reproduction, only few studies have considered this mechanism and elaborated on its implications (Clarke et al., 2006). One of the few proposed theories which explains the mechanism of FF accumulation states that large molecular weight molecules contained within the follicules are osmotically active and thereby create an osmotic gradient which attracts fluid from the highly vascular stroma to accumulate and form an antrum (Clarke et al., 2006).

Most studies considered the assessment of follicular expansion by ultrasound as a satisfactory tool to evaluate follicular maturity when conducting *in vivo* studies, while relying on granulosa cells proliferation when carrying out *in vitro* research (Rodgers and Irving-Rodgers, 2010). Interestingly, the rate of follicular antrum expansion and not the

thickness of granulosa cells is the sign for the maturation of a dominant follicle for ovulation (Clarke et al., 2006). Nonetheless, Rodgers and Irving-Rodgers (2010) demonstrated that not only should the expansion of the antrum be considered but also follicular growth which incorporates several aspects; oocyte growth, follicular cell proliferation and antral enlargement and all should be considered when evaluating follicle maturity. The size of the mature Graafian follicle seem to vary widely among species, ranging from few millimetres in mouse to up to 5cm in horse (Edwards, 1974). The rate of FF accumulation and the size of resultant follicles seem to also be correlated to the species type (Rodgers and Irving-Rodgers, 2010). Smaller amount of fluid accumulates within the follicles of smaller animals such as rats and mice, while larger species have larger follicles with greater amount of FF within their antrum (Rodgers and Irving-Rodgers, 2010). The protein and steroid hormone contents of FF have also been shown to differ according to the stage of follicular development (Edwards, 1974). The biochemical composition of FF has been related to the developmental potential of oocyte in several studies (Hartshorne, 1989, Nayudu et al., 1989, Salha et al., 1998). FF composition is highly complicated and **table 1-1** summarises the contents of FF.

Table 1-1 Summary of Follicular fluid contents:

FF main components groups	Examples	References
1. Hormones: **1.1 Steroid hormones**	Progesterone, Oestrogens (17beta-estradiol) and androgen (testosterone)	Uehara et al., 1985 Tesarik and Mendoza, 1997
1.2 Gonadotrophins :	FSH, LH, human chorionic gonadotropin (hCG)	Suchanek et al., 1988, Ellsworth et al., 1984 , Cha et al., 1986
1.3 Growth hormone		Tarlatzis et al., 1993
1.4 Corticoids	Cortisol, cortisone	Keay et al., 2002
1.5 prolactin		Lindner et al., 1988
2-growth factors of the Transforming Growth Factor-beta (TGF-beta) superfamily	Inhibin and Activin Anti-mullerian hormone, Bone morphogenetic protein-15	Lau et al., 1999, Ebner et al., 2006, Wu et al., 2007
3-Other growth factors and interleukins	Insulin-like growth factors (IGF), Amphiregulin	Wang et al., 2006, Inoue et al., 2009, Bili et al., 1998
4-Reactive Oxygen Species (ROS) and antioxidant factors	enzymes: superoxide dismutase (SOD) and selenium-dependent glutathione peroxidase (SeGPx) Vascular Endothelial Growth Factor (VEGF), vitamin C, E	Sabatini et al., 1999, Attaran et al., 2000, Lee et al., 2000, Oyawoye et al., 2003, Monteleone et al., 2008, Prieto et al., 2012
5-Proteins, peptides and amino acids	Albumin, fibronectin, clusterin	Nandedkar et al., 1992 Schweigert et al., 2006 Sinclair et al., 2008
6-Sugars	Hyaluronan, Myo-inositol, Glucose, fructose, Lactic acid, Protein bound hexose and fucose.	Józwik et al., 2007, Saito et al., 2000, Anifandis et al., 2010, Chiu et al., 2002
7-Lipids	Cholesterol, Triglycerides, Non esterified fatty acids (NEFA), vitamin D	Chang et al., 1976, Sinclair et al., 2008, Anifandis et al., 2010
8-anti-apoptotic factors	Tumor Necrosis Factor (TNF), soluble FAS (sFas) and its ligand sFas-L	Lee et al., 2000, Malamitsi - Puchner et al., 2004
9-Prostanoids	Prostaglandin F2alpha, PGE2	Jeremy et al., 1987
10-Ions	sodium, potassium, chloride, magnesium	Knudsen et al., 1979 Nandi et al., 2007,

hFF protein contents versus plasma proteins:

Remarkable similarity has been observed between hFF and blood plasma/serum proteins (Anderson et al, 1976). The increased follicular permeability during maturation especially in the pre-ovulatory follicle allows more proteins from the serum to cross the blood-follicular barrier (Gerard et al., 2002, Gosden et al., 1988, Bianchi et al., 2013). Some studies have run comparative proteomic analysis of paired hFF and blood serum samples from the same patients to differentiate proteins which are specific to the follicle from common non-specific proteins from the blood (Schweigert et al., 2006; Angelucci et al., 2006; Jarkovska et al., 2010). The protein concentration in hFF ranges from 35 to 52 mg/ml (Ambekar et al, 2013). **Table 1-2** shows a comparison of certain High Abundance Proteins (HAPs) in hFF with blood serum (adapted from Johnson (1973) in Edwards (1974).

Table 1-2 A comparison of certain HAPs contents in hFF with blood serum (adapted from Johnson, 1973, Edwards, 1974)

Protein name	hFF (mg%)	Human serum (mg%)
Albumin	**2560**	**4400**
Alpha1 glycoproteins	**45**	**90**
IgG	**580**	**1300**
Beta1-A globulin	**45**	**100**
Transferrin	**149**	**320**
Haptoglobin	**65**	**160**
IgA	**75**	**210**
IgM	**17**	**140**
Alpha 2-macroglobulin	**28**	**240**

1.3 Sperm journey toward the oocyte:

Exposure of sperm to the FRT different environments is critical for sperm to acquire the necessary equipment to accomplish their journey in the FRT and to achieve its principal goal to fertilise oocyte. In the FRT, sperm travel a relatively long journey and encounter several anatomical and biochemical obstacles to arrive at the fertilisation site (Druart, 2012). This section highlights the stages of the sperm journey in the FRT.

1.3.1 Sperm transport through the vagina and cervix:

Following their deposition in the vagina, human sperm escape this acidic and immuno-hostile environment by swimming directly toward the cervix (Sobrero and Macleod, 1962). In other species the the site of semen deposition varies due to anatomical differences, for instance, while straight deposition of the ejaculate into the uterine cavity occurs in pigs (Hunter, 1981, Roberts, 1986), rodents deposit sperm into the vagina from which sperm immediately pass through the cervix to the uterine cavity (Zamboni, 1972, Bedford and Yanagimachi, 1992).

In humans, cervical mucus is described as a physiological selective barrier which prevents the passage of sperm with abnormal morphology and poor motility into the uterine cavity (Hanson and Overstreet, 1981, Barros et al., 1984, Katz et al., 1997, Suarez and Pacey, 2006). Additionally, the priming influence of the cervical mucus on sperm capacitation is well recognised in the literature (Lambert et al., 1985, Overstreet and VandeVoort, 1989). The high viscosity of the cervical mucus may serve as a guide to allow sperm to swim in a straight path during their journey toward the uterus (Katz et al., 1978, Suarez and Pacey, 2006). The viscosity of the cervical mucus varies with the stage of menstrual cycle; thin watery mucus formation is induced by oestrogen while thick hostile mucus is formed in response to high progesterone (Wolf et al., 1978). Furthermore, cervical mucus facilitates

the elimination of seminal components which maintain sperm plasma membrane stability and thus initiates sperm capacitation (De Jonge, 2005). An immune response has been recognised in the cervical mucus in the form of immunoglobulins A, G and leukocytes especially neutrophils which aim to protect the cervix from microbial invasion without damaging the sperm (Suarez and Pacey, 2006). However, the presence of antibodies in the cervical secretion which identify antigens on the sperm surface (anti-sperm antibodies) may cause infertility (reviewed in Suarez and Pacey, 2006).

1.3.2 Sperm transport through the uterus and uterotubal junction:

The viscosity of uterine fluid is lower than that of cervical mucus (Eytan and Elad, 1999). Uterine contractions have been suggested to enable sperm transport to the oviduct in domestic animals with less contribution from sperm motility (Troedsson et al., 2001, Katila, 2001). Dead and immotile sperm have been recovered from some mammalian oviducts (Overstreet and Tom, 1982, Katila, 2001) which may indicate that mechanisms other than sperm motility may contribute to sperm transport in the FRT (Suarez and Pacey, 2006). SP may also enhance uterine contractions (Katila, 2001) containing oxytocin (Watson et al., 1999), prostaglandins (Harper, 1988, Katila, 2001) and oestrogen; the later stimulates prostaglandin F-2α production which enhances uterine contractions (Claus, 1990, Suarez and Pacey, 2006) and contributes to sperm transport. SP is also involved in the protection against the defensive uterine environment (Troedsson, 2001, Katila, 2001) by inhibition of complement cascade and suppression of uterine polymorphonuclear leukocyte (PMN) chemotaxis (Rozeboom et al., 1999). Nonetheless, the uterine inflammatory response and phagocytosis in response to sperm deposition has been described as a physiological process aiming to eliminate the damaged and excessive sperm from the uterine cavity in mares (Troedsson et al., 2001, Troedsson et al., 2005).

Only a few thousands of sperm migrate through the uterotubal junctions (UTJ) to reach the oviduct where a combination of thermotaxis and chemotaxis may guide sperm to reach the oocyte (Eisenbach and Tur-Kaspa, 1999, Bahat et al., 2003). In the UTJ, passage of sperm may be hindered by anatomical and physiological challenges such as the narrowness and tortuosity of the UTJ which may also contain thick mucus (Suarez and Pacey, 2006). Further elimination of some seminal plasma components from the surface of sperm takes place during sperm passage through the UTJ (Polge, 1978, Einarsson et al., 1980, Hunter, 2008). Furthermore, not only normal morphology and motility are required for sperm to successfully pass through the UTJ but also possession of normal genes (Suarez and Pacey, 2006) which encode: fertilin β or Adam2 (Cho et al., 1998) calmegin (Ikawa et al., 1997, Ikawa et al., 2001) and testis-specific angiotensin converting enzyme (ACE) (Krege et al., 1995, Hagaman et al., 1998). These observations were obtained following experimentations on null mutant mice for the genes encoding these proteins (fertilin β, calmegin and ACE) as sperm from these mice were unable to cross the UTJ and also incapable of ZP-binding (Suarez and Pacey, 2006). More recently, further sperm factors have been identified to be essential for sperm passage through UTJ (Druart, 2012) which include: Adam1a or fertilin alpha (Nishimura et al., 2004), Adam3 or cyritestin (Yamaguchi et al., 2009), PGAP1 (Post-GPI Attachment to proteins 1) (Ueda et al., 2007), Calreticulin3 or calsperin (Ikawa et al., 2011) and PDILT (Tokuhiro et al., 2012). Therefore, evidence has been established for the significance of certain sperm surface proteins for successful passage through the UTJ (Suarez and Pacey, 2006, Druart, 2012).

1.3.3 Sperm transport along the oviduct

Despite the fact that the ejaculate may contain millions of sperm, only hundreds to thousands actually arrive at the oviduct of which only one sperm may succeed in fertilising

the oocyte (Motta et al., 1995, De Jonge, 2005, Suarez and Pacey, 2006). The influences of oviductal epithelium and fluid on sperm have been investigated in several species (Scott, 2000, Reeve et al., 2005). In humans, both the oviductal fluid and epithelium lining have been areas of interest in reproductive research particularly their influence on sperm fertilising capacity (Hunter, 2008; Zumoffen et al., 2010). However, due to ethical and practical challenges, the limited number of studies on human tubal fluid have been conducted with conditioned media prepared from cells and tissue explants of the human oviductal epithelium (Lippes et al., 1972, Zumoffen et al., 2010).

The mammalian oviduct functions as a sperm reservoir in animals (Suarez et al., 1991a, Lefebvre et al., 1995, Suarez, 1998, Reeve et al., 2005) and in the maintenance of motility, viability and fertilising capability of sperm (Smith and Yanagimachi, 1990, Pollard et al., 1991, Smith and Nothnick, 1997, Reeve et al., 2005). The oviductal environment has been described as an indispensable site for sperm modulation prior to fertilisation (Holt et al., 2006). Oviductal secretions are suggested to influence the rate of sperm capacitation and acrosome reaction (Zumoffen et al., 2010) and to ensure synchronisation of sperm capacitation with the onset of ovulation (Reeve et al., 2005). Broadening the sperm capacitation window to prolong sperm survival is suggested to be one of the mechanisms by which oviductal fluid retain sperm fertilising capacity (Cohen-Dayag et al., 1995, Eisenbach, 1999a, Holt et al., 2006, Rodriguez-Martinez, 2007, Hunter, 2008, Zumoffen et al., 2010, Hunter and Rodriguez-Martinez, 2004). Oviductal fluid has been suggested to increase sperm plasma membrane stability and therefore contribute to the reduction of sperm capacitation and AR (Morales et al., 1996, Zumoffen et al., 2010).

Assessment of tyrosine phosphorylation revealed diminished levels of tyrosine phosphorylation for sperm incubated in medium conditioned with oviductal secretions

(Zumoffen et al., 2010). Inhibition of the cAMP/PKA pathway and/or enhancement of dephosphorylation processes are suggested as mechanisms involved in the reduced sperm tyrosine phosphorylation observed in this study (Zumoffen, 2010). Yao et al. (1999) indicated that sperm AR induced by Ca^{2+} ionophore was suppressed in sperm incubated with media conditioned by oviductal cells. Selection of the most competent sperm which may arrive at the fertilisation site also presumably occurs within the oviductal environment (Cohen-Dayag et al., 1995, Zumoffen et al., 2010).

Sperm oviductal epithelium binding and the existence of a sperm reservoir in human are still controversial and has been described as non-essential for successful achievement of fertilisation in mammals (Hunter, 2008). This was shown when direct insemination into the ampullary region of the oviduct resulted in fertilisation and live birth (Yaniz et al., 2002, Hunter, 1998, Hunter, 2008). In contrast, some studies on the effect of endometriosis on the sperm-endosalpingeal epithelial interaction suggested that infertility in patients with endometriosis could be due to the damaged sperm-oviductal interaction (Reeve et al., 2005). The mechanism of sperm release from the oviductal reservoir in mammals is suggested to be facilitated by several mechanisms (Reeve et al., 2005). For example, hyperactivated sperm motility (Demott and Suarez, 1992, Pacey et al., 1995) thermo-tactic (Bahat et al., 2003) and chemotactic (Eisenbach, 1999b) factors within the ipsilateral oviduct and the diminished sperm-epithelium bonds as sperm undergo capacitation (Lefebvre and Suarez, 1996, Fazeli et al., 1999). Thus, some studies suggested that a normally functioning sperm plasma membrane which responds efficiently to the several molecular signalling in the surrounding environment is critical for sperm release from the oviductal reservoir in mammals (De Jonge, 2005).

1.4 Regulation of sperm function

1.4.1 Sperm capacitation:

Sperm have been shown to acquire fertilising capacity only following their deposition in the FRT (Chang, 1951, Austin, 1951). In the FRT, sperm undergo sequential, highly complex physiological and biochemical modifications and membrane remodelling, collectively termed capacitation, which equip sperm with their fertilising capability (de Lamirande et al., 1997, Visconti et al., 2011). Capacitation involves acquisition of hyperactivated motility through capacitation induced-signalling in the tail region (Eddy et al., 2003) while preparing sperm for acrosome reaction (AR) through capacitation induced-signalling in the head region (Mayorga et al., 2007). Additionally, it has been shown that only capacitated sperm may undergo a chemotactic response to a progesterone gradient *in vitro* (Uñates et al., 2014). In the FRT, capacitation is believed to mainly occur in the oviduct through sperm interaction with components from oviductal, FF and cumulus secretions (Quintero et al., 2005, Suarez, 2008b). The contribution of such components in the modulation of sperm capacitation and preparation for fertilisation has been explored in several studies (Martinez-Leon et al., 2015, Harrison and Miller, 2000, Quintero et al., 2005).

The molecular aspects of capacitation are not yet fully elucidated and whether the different signalling events are interdependent or coordinated is not known (Gervasi and Visconti, 2016). Nevertheless, the linear pathway description of molecular signalling in capacitation is disputed and more independent, compartmentalised and multi-factorial mechanisms are suggested (Gervasi and Visconti, 2016). Several studies have revealed a series of changes during sperm capacitation involving cholesterol efflux from the plasma membrane (Davis, 1982), loss of membrane stability (Wolf et al., 1986), an increase in pH (Fraser, 1995) and

alteration in ion concentration mainly of calcium (Ca^{2+}) (Fraser and McDermott, 1992) and bicarbonate (HCO_3^-) (Leclerc et al., 1996). Other two important aspects of sperm capacitation are activation of the cyclic adenosine monophosphate/protein kinase A (cAMP/ PKA) pathway (Harrison, 2004) and increased tyrosine phosphorylation (Leclerc et al., 1997, Visconti et al., 1995a).

The crucial roles of some of the above mentioned factors, namely HCO_3^-, Ca^{2+} and albumin have been challenged in some studies which suggested cell vitality, intracellular pH and high availability of ATP are sufficient for sperm to undergo capacitation and tyrosine phosphorylation (Baker et al., 2004, Aitken et al., 1998b, Ecroyd et al., 2004, Aitken and Nixon, 2013).

A sequence of changes in sperm membrane lipid composition takes place during capacitation (McNutt and Killian, 1991). Loss of cholesterol from the sperm surface is described as the initial stage of capacitation as cholesterol is considered a potent de-capacitation factor which maintains sperm plasma membrane stability in the epididymis prior to ejaculation (Davis et al., 1979, Davis and Bilayer, 1980, Aitken and Nixon, 2013). Cholesterol transfer from the sperm membrane is enhanced by the presence of albumin and high density lipoproteins (HDLs) which act as acceptor molecules for cholesterol (Aitken and Nixon, 2013). Cholesterol peroxidation and oxysterol formation induced by oxidative stress have also been shown to play a significant role in facilitating cholesterol efflux and increased membrane fluidity during capacitation (Brouwers et al., 2011). Additionally, an ATP-binding cassette (ABC A17), a member of cholesterol transport family, has been suggested to promote the transport of cholesterol to albumin (Morales et al., 2012). Both oviductal and follicular fluids are rich sources of albumin and HDLs which have been

shown to have greater affinity to binding cholesterol than serum albumin (Gangwar and Atreja, 2015).

In addition to albumin, HCO_3^- has been indicated as an important ion in sperm capacitation involved in lipid peroxidation, formation of reactive oxygen species (ROS) by the sperm (Ecroyd et al., 2003, Leclerc et al., 1996, Boerke et al., 2013) and enhancement of albumin affinity for cholesterol (Flesch et al., 2001). The presence of high levels of HCO_3^- also results in the stimulation of sperm-specific pH-dependent soluble adenylyl cyclase (sAC) (Gangwar and Atreja, 2015). Activation of sAC increases levels of cAMP which is involved in the activation of cAMP-dependent PKAs and in tyrosine phosphorylation which are events critical for capacitation (Flesch et al., 2001, Harrison and Miller, 2000, Gadella and Harrison, 2002). HCO_3^- is also involved in PKA-dependent alterations in the sperm plasma membrane lipid architecture through cholesterol redistribution and phospholipid scrambling via activation of a phospholipid scramblase enzyme which further enhances albumin-mediated cholesterol efflux and subsequent capacitation (Flesch et al., 2001, Gadella and Harrison, 2002).

The biphasic role of Ca^{2+} in capacitation:

Another vital ion involved in capacitation is Ca^{2+} (Ruknudin and Silver, 1990) which has been shown to have both stimulatory and inhibitory effects on enzymatic pathways linked to tyrosine phosphorylation and capacitation (Navarrete et al., 2015). Controversial results have been obtained in independent studies regarding the effects of Ca^{2+} in the regulation of tyrosine phosphorylation (Baker et al., 2004). An increase in Ca^{2+} concentration has been shown to enhance tyrosine phosphorylation through positively modulating sAC activity (Jaiswal and Conti, 2003) which activate cAMP/PKA pathway (Hess et al., 2005). Conversely, Ca^{2+} interaction with calmodulin (CaM), a Ca^{2+} modulating protein, results in

activation of Ca^{2+} /CaM-dependent enzymes, for instance phosphodiesterase 1 (PDE1), which negatively regulate cAMP-dependent pathways and supress tyrosine phosphorylation (Navarrete et al., 2015). **Figure 1-7** summarises the signalling pathways during capacitation.

Tyrosine phosphorylation in sperm capacitation:

An increase in tyrosine phosphorylation has been observed during sperm capacitation in all species studied to date (Aitken and Nixon, 2013) and is considered a marker of sperm capacitation (Visconti et al., 1995a, Ficarro et al., 2003). Tyrosine phosphorylation is an important post-translational modification event, which is particularly relevant to the transcriptionally and translationally inactive sperm cells, and is involved in the regulation of capacitation, hyperactivation and acrosome exocytosis (Jagan Mohanarao and Atreja, 2011). Characterisation of proteins involved in tyrosine phosphorylation is believed to be critical in unravelling molecular principles of signal transduction during capacitation (Naz and Rajesh, 2004). Some phosphoproteins have been revealed as targets for tyrosine phosphorylation during human sperm capacitation including a number of A kinase anchoring proteins (AKAP3, AKAP4, AKAP82) (Mandal et al., 1999, Vizel et al., 2015, Carrera et al., 1996) and the Ca^{2+}-binding and tyrosine phosphorylation-regulated protein (CABYR) (Naaby-Hansen et al., 2002).

cAMP, a key second messenger in many biological systems, has a distinctive signalling pathway in sperm cells through the activation of PKA which is involved in the regulation of tyrosine phosphorylation in several species (Naz and Rajesh, 2004). The compartmentalisation of PKA in sperm cells enables the activation of specific functions via binding to the relevant AKAPs subunits (Naz and Rajesh, 2004). PKA activation is

important in sperm capacitation and its associated hyperactivated motility (Baker et al., 2009).

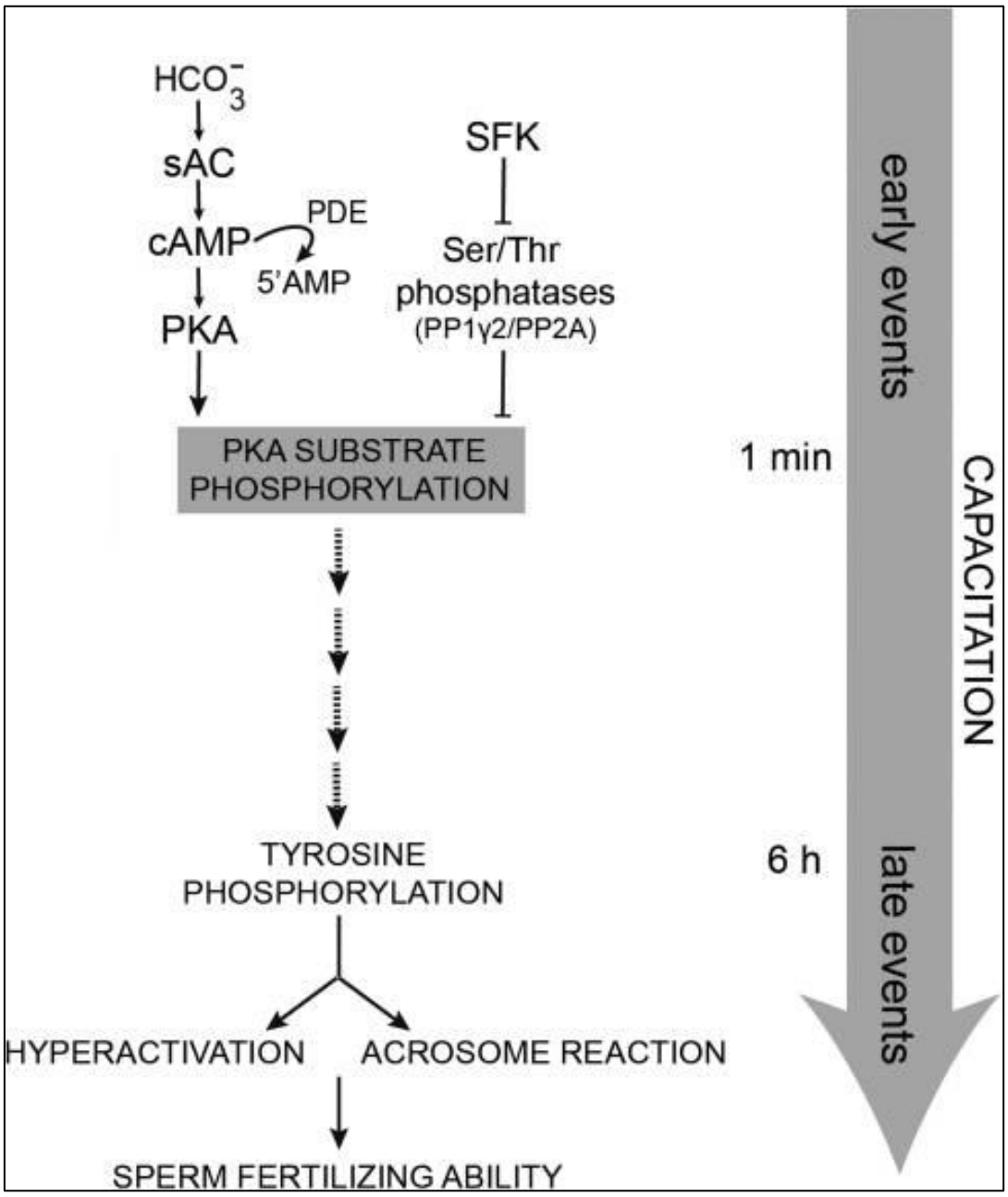


Figure 1-7 Signalling pathways in human sperm capacitation. Two pathways are essential for sperm function. The first is the HCO^{-3} dependent activation of PKA by sAC/cAMP and the second involves the down-regulation of Ser/Thr phosphatases. Human capacitation involve both early (1 minute) and late events (at least 6 h) to achieve functional capacitation state, SFK stands for Src family kinase. Figure from (Battistone et al., 2013) with permission.

The mechanism of regulation of tyrosine phosphorylation in sperm during capacitation via cAMP/PKA pathway occurs via a unique cascade of signal transduction events (White and Aitken, 1989, Aitken et al., 1998a). Increased or decreased levels of intracellular cAMP production have been shown to have equivalent effects on the levels of tyrosine phosphorylation of the cell (Visconti et al., 1995b, Rivlin et al., 2004, Baker et al., 2004, Baker et al., 2006). Factors such as ROS, Ca^{2+} levels and HCO_3^- have been shown to regulate the cAMP-dependent tyrosine phosphorylation events in sperm via stimulation of sAC (Aitken et al., 1995, Baker et al., 2004, Visconti et al., 1995b). The mechanism of regulation of cAMP-mediated activation of PKA is complicated and involves both direct and indirect pathways (Baker et al., 2006, Aitken and Nixon, 2013). Direct activation of protein kinases, increased availability of cAMP and inhibition of tyrosine phosphatases action along with pH buffering are some of the suggested mechanisms involved (Leclerc et al., 1996, Baker et al., 2006, Aitken et al., 1998b). For recent reviews of sperm capacitation, see Gervasi and Visconti (2016) and Aitken and Nixon (2013).

The role of de-capacitation factors in regulation of capacitation:

Although not fully elucidated, the mechanism of *in vivo* capacitation regulation is suggested to involve acquisition and/or loss of certain ligands, some of which become absorbed on or integrated into the sperm surface in a receptor-mediated mechanism (Breitbart and Etkovitz, 2011, Aitken and Nixon, 2013). Several de-capacitation factors, which sperm acquire from the epididymal secretions and seminal fluid, have been shown to regulate capacitation in its early stages (Aitken and Nixon, 2013). For example, glycodelin S from seminal plasma retains the ejaculated sperm in un-capacitated state (Yeung et al., 2006) while cholesterol associated with the sperm membrane (Davis and Bilayer, 1980) and phosphatidylethanolamine binding protein 1 (Nixon et al., 2006) have

been suggested to prolong sperm survival via prevention of premature capacitation and subsequent sperm apoptosis (Fraser, 1998, Gibbons et al., 2005). Dissociation of such de-capacitation factors from sperm membrane may initiate the onset of capacitation (Aitken and Nixon, 2013).

1.4.2 Post-translational modifications (PTM) of sperm proteins :

Post-translational modifications (PTM) is defined as any variations between the original polypeptide sequence produced following translation and the final structure of a fully functional protein (Samanta et al., 2016) which may also result in alteration of protein mass and characteristics (Baker, 2016). Currently, a list of 307 types of PTM has been generated in the database of UniProt (http://www.uniprot.org/docs/ptmlist) (Baker, 2016). Several types of PTM, such as phosphorylation, glycosylation, nitrosylation, ubiquitination, acetylation and methylation, have been detected in sperm following epididymal maturation and capacitation and have been linked to sperm acquisition of functional competence (Baker, 2016, Cornwall, 2009). Phosphorylation is one of the most significant PTM occurring in sperm (Baker, 2016) with tyrosine phosphorylation of several proteins during capacitation as a prominent example. During epididymal transit, one of the most important proteins which undergo phosphorylation is IZUMO 1, the sperm- oocyte fusion receptor (Inoue et al., 2005), which has manifested seven phosphorylation sites in mature sperm (Baker et al., 2012). Glycosylation is another significant example of PTM in sperm with various glycoproteins being identified as sperm-associated epididymal proteins such as CD59 and fertilin (Baker, 2016). Glycosylation of sperm proteins is also suggested as a possible mechanism by which sperm escape the immune recognition and damage by the FRT (Clark et al., 1997; Baker,

2016). S.nitrosylation has also been shown as an important PTM in sperm with 240 proteins being identified to undergo nitrosylation (Lefievre et al., 2007). Sperm cells rely considerably on PTM of existing proteins and proteins acquisition and/or loss to achieve maturation in the epididymis and functional competence in the FRT (Baker, 2016).

1.4.3 Sperm motility

Two distinct categories of sperm motility are described in the literature as crucial for fertilisation; the progressive active motility in the fresh ejaculate and the hyperactivated motility mainly in the ampulla (site of fertilisation) (**figure 1-8**) (Suarez et al., 1991b, Suarez and Ho, 2003) . Both types are essential in mammalian fertility for the sperm to migrate toward the oocyte, disconnect from the oviductal epithelium and to penetrate the ZP (Suarez, 2008b). As described previously, testicular sperm are immotile and only acquire motility after the transit period in the epididymis where sperm undergo maturation steps (section1.1.3).

The unique structure of the sperm flagellum and the modulations sperm undergo during capacitation enable sperm to display both motility patterns according to the surrounding environment and signalling pathways (Turner, 2006). Progressive motility with low amplitude and symmetrical flagellar waves drives the sperm in more or less a straight line (Turner, 2006; Suarez, 2008) and is suggested to enable sperm to navigate the FRT (Turner, 2006). The second type of motility, hyperactivation, is mainly observed at the site of fertilisation (ampulla) and is considered as an event accompanying capacitation with major tyrosine phosphorylation of sperm tail proteins as an important feature (Suarez et al, 2008; Baker et al, 2006). Hyperactivation is described as a pattern of sperm motility in which sperm cells exhibit high amplitude of lateral head displacement associated with low linearity and whiplash flagellar motion (de Lamirande et al., 1997, Kulin et al., 1994,

Yanagimachi, 1970, Suarez and Ho, 2003). The significance of sperm hyperactivation is linked to sperm release from oviductal epithelium in animals and sperm ability to penetrate ZP via the propulsive forces of sperm tail (Suarez and Ho, 2003, Suarez, 2008b, Visconti et al., 2011). In human, hyperactivation is suggested to enable sperm motility through the viscous oviductal fluid and to penetrate ZP and cumulus matrix surrounding the oocyte (Pacey et al., 1995, Suarez, 2008a). Physiological inducers of *in vivo* hyperactivation are not fully elucidated, however, several *in vitro* studies have shown that hyperactivation can be triggered by hFF (Yao et al, 2000), progesterone (Sueldo et al., 1993) and cumulus cells surrounding the oocyte (Fetterolf et al., 1994).

Regulation of sperm motility and hyperactivation involves a number of signalling pathways (Yanagimachi, 1994b, Suarez and Ho, 2003, Turner, 2006). The regulation of sperm motility is highly complex as Ca^{2+} signalling, cAMP/PKA signalling pathway and tyrosine phosphorylation of flagellar proteins are some of the suggested mechanisms (Ho and Suarez, 2001, Darszon et al., 2006). In brief, activation of cAMP production via sAC in the presence of Ca^{2+} and HCO_3^- will result in activation of PKA (Chen et al., 2000, Xie et al., 2006, Marquez and Suarez, 2008). PKA activation will initiate a signalling pathway to enhance tyrosine phosphorylation of flagellar proteins associated with hyperactivation (Si and Okuno, 1999, Suarez, 2008a).

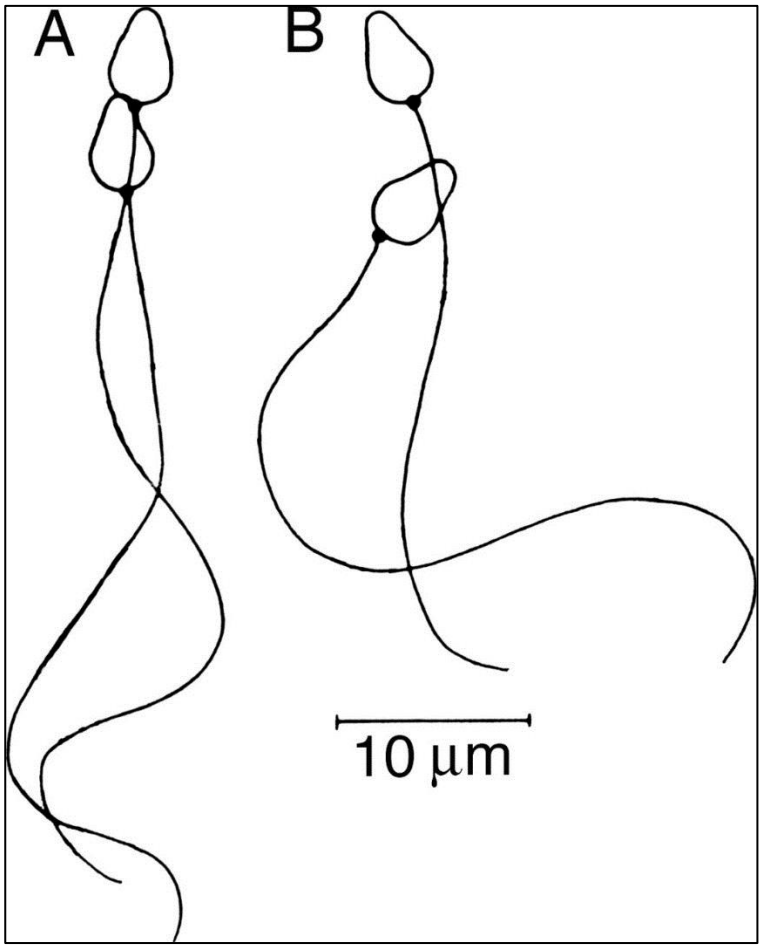


Figure 1-8 Human sperm flagellar bending patterns in A- progressive motility and B- hyperactivated motility (figure from Suarez et al (2008a) with permission, adapted from Morales et al. (1988) .

1.4.4 Sperm guidance mechanisms in the FRT

Several factors have been suggested to guide sperm in their lengthy journey toward the oocyte (**figure 1-9**); chemotaxis (Spehr et al., 2003, Eisenbach, 2004) and thermotaxis (Bahat et al., 2003) have been suggested to provide guidance in the immediate vicinity of the oocyte. Rheotaxis has been suggested to guide sperm on longer distance scales (Miki and Clapham, 2013).

The concept of chemotaxis in mammalian sperm and its correlation to fertilisation is relatively novel and only proposed approximately twenty years ago (Villanueva-Diaz et

al., 1990, Ralt et al., 1991, Eisenbach and Tur-Kaspa, 1999, Eisenbach and Giojalas, 2006). Sperm chemotaxis can be defined as the change of the direction exhibited by motile sperm exposed to a chemical gradient, such as human follicular fluid (hFF) (Ralt et al., 1994, Fabro et al., 2002). The directional swimming of the sperm in the FRT toward the ipsilateral site where the oocyte resides in the oviduct is attributed partially to the chemo-attractant effect of hFF and mainly progesterone (Serrano et al., 2001). Chemotaxis has been suggested as a selective measure for high competence sperm to approach the COC at the appropriate time and in the proper condition (only capacitated sperm) (Ralt et al., 1991). Some studies suggested that only good quality and capacitated sperm manifest a chemotactic response to hFF (Tacconis et al., 2001, Fabro et al., 2002). The chemotactic response of sperm to hFF was only observed in normozoospermic samples while poor sperm samples with oligoasthenozoospermia or asthenoteratozoospermia failed to manifest chemotactic responses to FF even following prolonged incubation for more than 6 hours with neither chemotactic response nor hyperactivation observed (Tacconis et al., 2001).

The ability of sperm to orient their motility to a temperature gradient is termed thermotaxis and is suggested to direct sperm toward the ampulla of the ovulating oviduct where the temperature is 2°C greater than in the isthmus as described for rabbit (Bahat et al., 2003). Rheotaxis is another phenomenon which has been observed in some marine species and is defined as the orientation response of motile cells and microorganisms to the alterations in a gradient of fluid flow (Kantsler et al., 2014, Miki and Clapham, 2013). Rheotaxis has been reported for sperm long time ago as indicated by Miki and Clapham (2013) and was described as positive rheotaxis for cells which have the tendency to direct their motility against the fluid flow (Miki and Clapham, 2013).

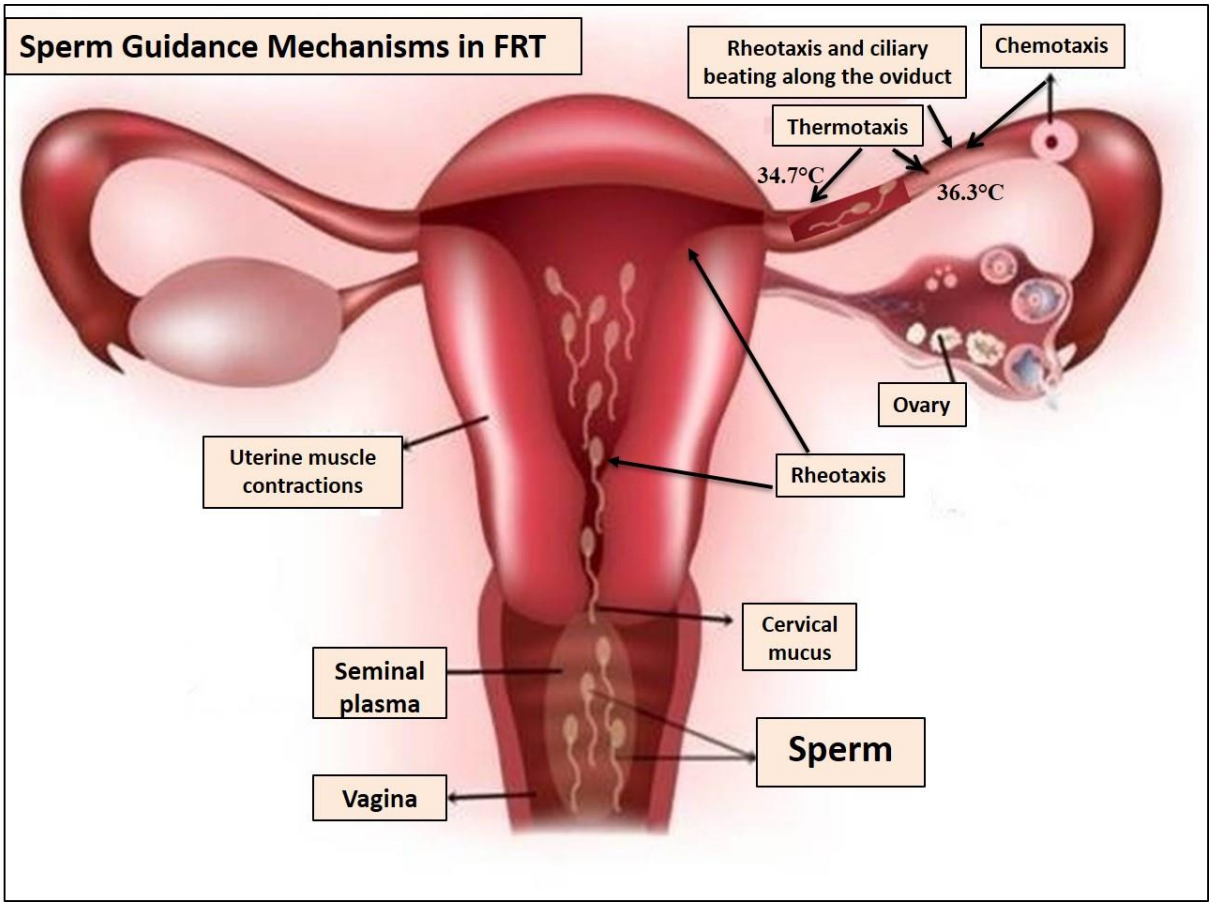


Figure 1-9 Sperm guidance mechanisms in the mammalian FRT namely chemotaxis, thermotaxis, rheotaxis, ciliary beating and muscular contractions. Thermoatxis was described for rabbit (Bahat et al., 2005).

1.4.5 Acrosome reaction (AR):

The process of AR involves the fusion of the outer acrosomal membrane at various points with the overlying plasma membrane (**figure 1-10**) (Harper et al., 2008). This membrane fusion results in the dispersal of the acrosomal contents and exposure of sites on the inner acrosomal membrane that are required for gamete fusion (Patrat et al., 2000, Harper et al., 2008). AR is an indispensable pre-requisite for fertilisation and is considered as an essential modification step to provide sperm with competence for oocyte fusion and interaction (Florman et al., 2008).

Sperm capacitation is an essential pre-requisite to enable AR as similar signalling transduction mechanisms activated during capacitation are required to initiate AR (de Lamirande et al., 1997, Saling and Storey, 1979). Therefore, some studies use the ability of sperm to undergo AR as a marker of the occurrence of capacitation (Baldi et al., 2000, Leclerc et al., 1996). While the biochemical and physiological changes occurring during capacitation are reversible (Bedu-Addo et al., 2005), AR is an irreversible process and therefore the onset of AR has to be coordinated spatially and temporally to coincide with the presence of oocytes (Florman et al., 2008).

Although not fully elucidated, factors contributing to *in vivo* AR induction are suggested to involve receptor-mediated mechanism (Daniel et al., 2010, Florman et al., 2008). ZP2 have been shown to mediate sperm-ZP binding and human sperm-oocyte recognition (Baibakov et al., 2012). AR has been also triggered *in vitro* by sperm exposure to the oviductal fluid and/or FF both containing progesterone and nitric oxide and described as priming factors for AR (Serrano and Garcia-Suarez, 2001, Machado-Oliveira et al., 2008). Sperm traversing through the cumulus cell layer or incubated with COC have also been shown to undergo AR (Tesarik et al., 1988, Carrell et al., 1993, De Jonge et al., 1988). The principal AR priming factor introduced by these conditions is suggested to be the steroid hormone progesterone (Tesarik et al., 1988, Carrell et al., 1993, De Jonge et al., 1988). Progesterone has been shown to trigger AR through the release of calcium influx by the direct activation of the cation channel CatSper (Lishko et al., 2011, Strunker et al., 2011).

Various other components of the FRT secretions have also been shown to induce AR *in vitro* such as prostaglandins (Joyce et al., 1987), atrial natriuretic peptide (ANP) (Rotem et al., 1998) and EGF (Lax et al., 1994, Daniel et al., 2010). Although not yet confirmed,

some studies suggested that such ligands may also have a role in *in vivo* AR induced either by interaction with other FRT ligands or ZP via synergistic actions (Roldan et al., 1994, Daniel et al., 2010).

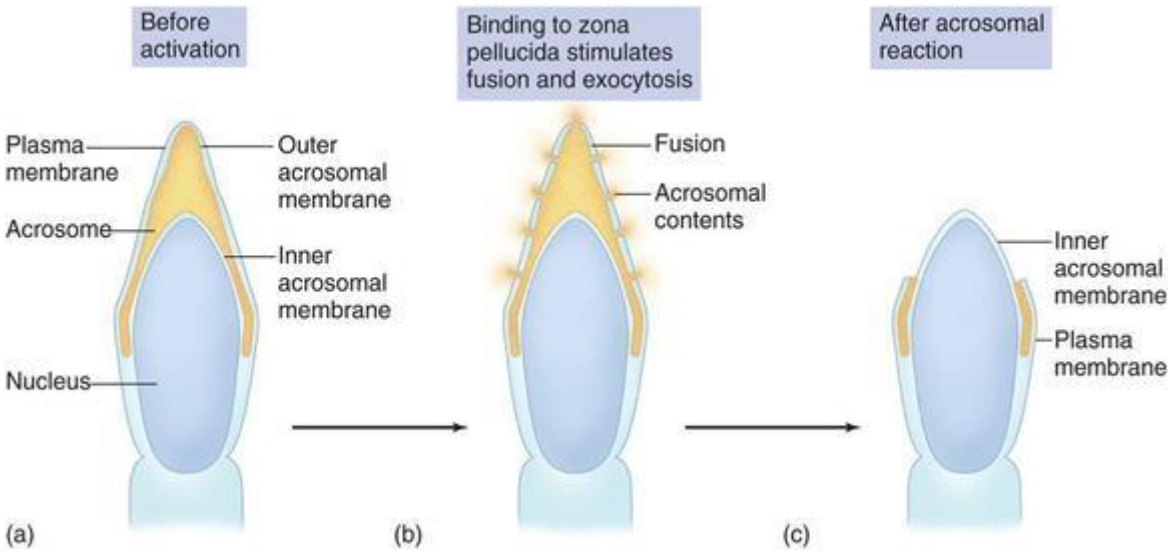


Figure 1-10 Stages of acrosome reaction in human sperm; fusion of the outer acrosomal membrane with the plasma membrane and dispersal of acrosomal contents (from http://humanphysiology2011.wikispaces.com/15.%20Reproductive%20Physiology), also available on public domains.

Similar to capacitation, AR timing and place within the FRT are important for sperm to achieve fertilisation of the oocyte (Florman et al., 2008). Sperm undergoing premature onset of AR have been shown to be incapable of passing through the cumulus oophorous surrounding the oocyte (Cummins and Yanagimachi, 1986). This premature or spontaneous AR has been suggested in some studies as a selection mechanism by which FRT eliminate sperm with abnormal morphology prior to fertilisation (Breitbart and Etkovitz, 2011).

AR is considered as a key for fertilisation (Florman, et al., 2008) and this is attributed partially to the actions of the lytic enzymes released which, along with hyperactivated sperm motility, are suggested to facilitate sperm penetration through the zona layer (Talbot, 1985). Another suggested mechanism for AR role in fertilisation is the change of the sperm's apical head shape from blunt to tapered following AR which further enables sperm penetration through physical thrust (Bedford, 1998).

Some recent studies have debated whether only acrosome-intact sperm are capable of binding to the ZP (Gervasi and Visconti, 2016) as acrosome-reacted sperm have been shown to fertilise ZP-enclosed oocytes (Inoue et al., 2011). Another study applied real-time tracking of fluorescent protein (142 EGFP, a mutant of Green Fluorescent Protein) developed by Nakanishi et al (1999) to track the sperm acrosome region revealed that mouse sperm that had lost their acrosome in advance of arriving at the ZP were still able to fertilise the oocyte (Jin et al., 2011). These observations and others suggest that AR may commence before the sperm encounter the ZP layer of the oocyte (Gervasi and Visconti, 2016). Two more recent studies have also shown that sperm in the isthmus region of the oviduct are still acrosome-intact while those in the ampullary region are acrosome-reacted (La Spina et al., 2016, Muro et al., 2016). These results have suggested that acrosome-reacted and not acrosome-intact sperm are required for sperm-ZP interaction (Inoue et al. 2011) and that sperm-ZP binding is not essential for fertilisation (Okabe, 2013; Gervasi and Visconti, 2016).

Further work is required to resolve these contradictory results linking AR and sperm-ZP binding and whether an intact acrosome is indispensable for sperm-ZP binding as shown in numerous previous studies (Gervasi and Visconti, 2016). One suggested explanation could

be the discrepancy between sperm-ZP binding used in *in vitro* assays and the equivalent *in vivo* events necessary for fertilisation (Gervasi and Visconti, 2016).

Regardless of the timing of AR, sperm-oocyte fusion has been shown to only occur between acrosome-reacted sperm and oocyte as the AR is necessary for exposure of sperm receptors on the inner acrosomal membrane that are required for gamete fusion (Patrat et al., 2000, Harper et al., 2008, Gervasi and Visconti, 2016). Izumo1 is a sperm-specific protein located on the anterior acrosome, which has been shown by gene deletion in mice to be an indispensable protein for gamete fusion (Sutovsky, 2009, Inoue et al., 2005). Izumo 1 has been shown to interact with Juno, from the oocyte and this interaction facilitates gamete fusion (Okabe, 2013, Bianchi et al., 2014). The re-localisation of Izumo1 during AR is dependent on the presence of testis-specific kinase (TSSK6) with Tssk6-null mice demonstrating failed sperm-oocyte fusion (Sosnik et al., 2009). The translocation of Izumo1 within the sperm head during AR and proper final location of this protein are both suggested crucial for successful gamete fusion (Gervasi and Visconti, 2016, Sosnik et al., 2009, Miranda et al., 2009).

1.5 FF and sperm function and kinetics:

FF has been shown to play an important role in the process of fertilisation by provision of a favourable environment for sperm capacitation, hyperactivation, chemotaxis and acrosome reaction (Hong et al., 1993). Progesterone, a major steroid hormone in hFF, is suggested to be the main component involved in these aspects of sperm function (Uhler et al, 1992, Eisenbach and Giojalas, 2006, Roldan et al, 1994). The micro-molar levels of progesterone have been shown to induce AR (Osman et al., 1989). Other studies investigated the effects of progesterone when the exposure is presented in a gradient; the sperm responses were mainly observed in hyperactivation and chemotaxis (Harper and

Publicover, 2005, Harper et al., 2004, Teves et al., 2006). This section highlights the effects of FF on sperm regulation with emphasis on the role of hFF proteins. FF is used to refer to follicular fluid from any species while hFF is specific for human follicular fluid.

1.5.1 FF and sperm capacitation:

Since capacitation is a critical prerequisite for fertilisation (Yanagimachi, 1994a); appropriate timing of capacitation to coincide with ovulation is also essential (Hunter and Rodriguez-Martinez, 2004). Sperm capacitation has been described to occur in a rapid response to hFF exposure which is relevant for sperm-FF contact *in vivo* within the oviduct which occurs prior to the onset of fertilisation (Yao et al., 2000). The rate of sperm capacitation on exposure to hFF has been shown as dose dependent (Yao et al., 2000). Different hFF concentrations (for example 10%, 20%, 50% and 75%) have demonstrated a range of sperm capacitation with no response at 1% hFF and the highest response at 75% (Yao et al., 2000). In bovine FF, Therien et al. (2005) purified and investigated two glycosaminoglycans, the first was heparan sulphate which was demonstrated to have high potency to induce bovine sperm capacitation. The second was chondroitin sulphate B which has similar binding capacity to heparan sulphate but less affinity to induce capacitation (Therien et al., 2005).

1.5.2 FF and sperm motility:

Several studies have demonstrated that FF induces progressive sperm motility in *in vitro* studies in both animal and human sperm (Calvo et al., 1989, Falcone et al., 1991, Getpook and Wirotkarun, 2007, Revelli et al., 1992). FF has also been shown to stimulate sperm hyperactivation (Mbizvo et al., 1990, Kulin et al., 1994) and this stimulatory effect has been attributed to the steroid hormones in hFF, mainly progesterone (Uhler et al., 1992, Kulin et al., 1994). The addition of activated charcoal to hFF media, which eliminate the

hFF steroid constituents, has been shown to inactivate the hFF ability to induce sperm hyperactivation and support the concept that progesterone is the main hyperactivation inducer in hFF (Calogero et al., 2000, Jaiswal et al., 1999).

The effects of FF on sperm kinetics in the previously published data were contradictory with earlier studies indicated inhibitory effects (Mortimer and Camenzind, 1989) whereas more recent ones showed stimulatory effects of FF on sperm motility parameters (Yao et al., 1999, Hong et al., 1993, Getpook and Wirotkarun, 2007). Nonetheless, these inconsistent observations in the previous literature may have resulted from several factors such as varied levels of FF concentrations and whether the sperm were capacitated or not (Yao e al., 1999).

The effect of different concentrations of FF and duration of action (of up to 48 hours) on sperm motility was also investigated by numerous studies (Kulin et al., 1994, Fabbri et al., 1998, Mendoza and Tesarik, 1990, Getpook and Wirotkarun, 2007). Most of these studies indicated the ability of FF to enhance sperm motility at various concentrations (20, 30, 50, 80 and 100%) and to preserve this action for prolonged periods of time of up to 12 hours (Mendoza and Tesarik, 1990, Chao et al., 1991, Fabbri et al., 1998, Getpook and Wirotkarun, 2007). However, the best suggested hFF concentration is 20-50% and period of incubation of a maximum of 12 hours after which sperm motility starts to decline (Getpook and Wirtkarun, 2007). Further to these conditions of incubation, Kulin et al., (1994) suggested that FF can only exert this stimulatory effect on sperm motility following a pre-incubation period in capacitating media (Kulin et al., 1994). However, vast majority of other studies has demonstrated that no requirement for sperm pre-incubation in order to be stimulated by FF (Getpook and Wirtkarun, 2007). The activating influence of FF on sperm motility was suggested to diminish following FF heating to 100°C for a period of 30

minutes (Chao et al., 1991). A heat unstable substance in the hFF was, therefore, suggested as one of the important sperm motility's stimulator in hFF (Chao et al., 1991). The chemical structure of the proposed substance was described as highly similar to apolipoprotein H with an identical sequence of the N- terminal amino acid (Chao et al., 1991).

In addition to studying the effects of hFF on normozoospermic freshly collected semen samples, some studies have considered the effect of hFF on post thawed cryopreserved and oligozoospermic semen samples (Briton-Jones et al., 2001). Since sperm cryopreservation is shown to reduce sperm motility and to affect fertilising capacity (Briton-Jones et al., 2001), optimisation of assisted reproduction results following thawing demands the design of appropriate incubation media to improve post-thaw sperm motility (Briton-Jones et al., 2001). Aiming to enhance motility in cryopreserved oligozoospermic sperm , Briton-Jones et al. (2001) incubated the thawed sperm from good and poor quality semen samples in FF and compared the result with the incubation in media containing platelet activating factor (PAF) and another with human tubal fluid (Briton-Jones et al., 2001). This study demonstrated that, while hFF led to an increase in progressively motile sperm in normozoospermic samples, no such a stimulatory influence was detected in oligozoospermic cryopreserved sperm (Briton-Jones et al., 2001). Nonetheless, further studies on the effect of hFF on post-thawed cryopreserved sperm with multiple concentrations of hFF and for variable durations may show more promising outcomes in enhancing sperm motility following cryopreservation (Briton-Jones et al., 2001).

1.5.3 FF and sperm chemotaxis

FF has been demonstrated to attract sperm in *in vitro* study media in humans (Tacconis et al., 2001, Ralt et al., 1994), murine species (Giojalas and Rovasio, 1998, Oliveira et al.,

1999), rabbit (Fabro et al., 2002) and pig (Serrano et al., 2001). This chemotactic activity of FF has been mainly linked to progesterone (Villanueva-Diaz et al., 1995) and/or ANP (Anderson et al., 1995). However, few studies have suggested that the chemotactic effect of progesterone is only observed when progesterone is bound to a certain protein in FF (Cohen-Dayag et al., 1995, Ralt et al., 1994, Serrano et al., 2001). Some studies suggested that progesterone is not the only chemo-attractant agent in FF and proposed the presence of other active chemo-attractant components in hFF (Jaiswal et al., 1999). This study indicated that progesterone action on sperm is predominantly manifested as hyperactivation which results in sperm accumulation and trapping and not genuine chemotaxis (Jaiswal et al., 1999). This study applied strict criteria to define chemotaxis by the alteration in sperm direction of swimming toward the origin of the chemo-attractant described as directed turning behaviour of the sperm (Jaiswal et al., 1999). The authors also examined the effect of hFF in the presence of progesterone and following its removal by the treatment of hFF by dextran coated charcoal (Jaiswal et al., 1999). This study showed that similar chemotactic figures represented by sperm trajectory toward the hFF were obtained prior and following charcoal addition to the hFF which suggested that progesterone is not the only major chemo-attractant in hFF (Jaiswal et al., 1999). Some other studies suggested a non-hydrophobic low molecular weight protein of approximately 8.6 kDa which is homologous to apolipoprotein B2 to be responsible for this chemotactic effect of hFF (Ralt et al., 1994, cohen-Dayag et al., 1995, Serrano et al., 2001).

1.5.4 FF and sperm acrosome reaction:

FF has been shown to act as a biological promoter of AR in IVF media and was recognised as a potent AR mediator in several studies (Suarez et al., 1986, Calvo et al., 1989, Revelli et al., 1992, Tesarik, 1985, Morales et al., 1992, Siegel et al., 1990). The duration

following which FF induces sperm AR and the rate of induction varied in the literature (Siegel et al., 1990, Yao et al., 2000). AR following 1-6 hours of sperm incubation with FF has been observed (Gearon et al., 1994, Yao et al., 2000). The rate of AR induction also ranged from 5% to up to 45% in some studies (Mortimer and Camenzind, 1989, Suarez et al., 1986, Siegel et al., 1990). This diversity of FF influence on AR may be attributed to the variations among different laboratories in sperm preparation, period of incubation in FF and the procedures applied to detect sperm AR (Siegel et al., 1990).

Comparison of peritoneal and follicular fluids effects on sperm AR in human revealed that hFF has higher AR inducing capacity due to its considerably greater concentrations of both steroid hormones and proteins compared to peritoneal fluid (Munuce et al., 2006). Most studies indicated that steroid hormone progesterone is the principal component in hFF which induces AR (Morales et al., 1992, Saaranen et al., 1993). However, there has been a developing body of evidence to indicate the presence of other contents in the hFF participating in the induction of sperm AR (Fabbri et al.. 1998, Morales et al.. 1992). For example; proteins and peptides may play a part in the induction of sperm AR (Fabbri et al.. 1998, Morales et al.. 1992). Morales et al. (1992) showed that hFF samples treated with charcoal and dextran to reduce their progesterone contents by 1000 time could only restore 88% of their original detected ability to induce AR when progesterone was re-added to the media (Morales et al., 1992). It has also been demonstrated that albumin is highly abundant in hFF with approximately 70-80% of the overall protein contents (Munuce et al., 2004) and suggested that albumin may act as a cholesterol sink to facilitate cholesterol efflux and enhance membrane fluidity and thereby contribute in sperm capacitation followed by AR (De Jonge, 2005, de Lamirande et al., 1997).

Burrello et al (2004) indicated that AR is hFF dose dependent and thay examined the role of gamma aminobutyric acid (GABA) receptors in inducing AR by sperm incubation in hFF at different concentrations. Nonetheless, the authors stated that GABA receptors binding may not be the only mechanism by which hFF stimulates AR in humans as GABA receptors antagonists could not totally inhibit the AR induced by hFF (Burrello et al., 2004). Their study did not also identify which components in the hFF may interact with the GABA receptors to induce AR.

Another study suggested that sperm AR triggered by hFF is a multicomponent effect and progesterone alone is insufficient to induce AR (Fehl et al., 1995). Some observations in this study suggested the requirement for progesterone to be bound to another component to be able to induce sperm AR (Fehl et al., 1995). The observed low rate of AR in sperm incubated in hFF which had proteins eliminated by the addition of non-specific protease enzyme (Fehl et al., 1995). Further to this work, some studies have shown that the substance which expected to be bound to progesterone has a molecular weight in the range of 37-53 kDa (Fehl et al., 1995) while other authors indicated a range of 45-50 kDa (Siiteri et al., 1988, Suarez et al., 1986, Miska et al., 1994). Therefore, evidence indicating the requirement for certain proteins in the FF for the progesterone to possess its AR stimulating capacity requires further investigations (Fehl et al., 1995). Additionally, the effect of corticosteroid-binding globulin (CBG) antibodies on the hFF ability to stimulate sperm AR was examined and revealed diminished rate of sperm AR (Miska et al., 1994). CBG-Progesterone complex was suggested to be the AR triggering substance in hFF (Miska et al., 1994). hFF treated with proteases enzymes was shown to be biologically inactive even following addition of progesterone which may demonstrate the importance of the progesterone-binding protein (Calogero et al., 2000).

1.5.5 FF and sperm quality:

The observed stimulatory effects of FF on sperm motility was suggested to be linked to improved sperm quality following incubation in FF (Bahmanpour et al., 2012). The presence of antioxidant factors in FF such as superoxide dismutase and glutathione peroxidase have been suggested to inhibit ROS formation and minimize sperm DNA damage (Bahmanpour et al., 2012). Improvement of sperm functionality and reduction of DNA damage following sperm incubation in FF have been suggested to enhance fertilisation and improve pregnancy rate in ART (Fabro et al., 2002, Bahmanpour et al., 2012). A study examining the influence of hFF on the chromatin content of sperm in *in vitro* culture media suggested that hFF can be used as incubation media to enhance sperm quality and reduce DNA damage (Bahmanpour et al., 2012). The study indicated that prolonged incubation period in hFF of up to 180 minutes enhanced more histones to be replaced with protamines and decreased abnormal chromatin in the incubated sperm cells (Bahmanpour et al., 2012). However, these results were not statistically significant and further validation of these observations in future studies is recommended (Bahmanpour et al., 2012).

1.6 Techniques:

Several techniques have been utilised in the current research work and this section highlights the basic principles of these techniques. Further details are provided in the relevant chapters.

1.6.1 Solid Phase Extraction (SPE):

Solid phase extraction (SPE) is a valuable technique for proteins fractionation and peptides purification, which allows retention and elution of analytes from complex samples,

elimination of interfering compounds, and sample concentration (Martínez-Maqueda et al., 2013). SPE is a rapid and selective sample preparation technique which enhances peptides detection for the mass spectrometry analysis (Martínez-Maqueda et al., 2013). Numerous commercial sorbents (for example: C18, C8, C2, and ion exchange bonded materials, among others) are available to enhance the flexibility of SPE according to the range of physico-chemical properties of the analytes of interest (Martínez-Maqueda et al., 2013). Sample preparation has a significant effect on protein identification and peptide separation and their subsequent analysis. In this work, hFF will be prepared using SPE by Discovery Carbon 18% spin columns (DSC18 SCs) from Suppleco (chapter 2, section 2.4.1). DSC18 SCs are polymerically bonded trifunctional C18 silica sorbent that have high binding capacity due to their great carbon loading (18% C) (Martínez-Maqueda et al., 2013). In the SPE technique, the DSC18 spin columns extract protein elements from their liquid condition into a resin or sorbent (a stationary phase) which allows the extraction of wide ranges of analytes from the sample with great sample recovery (Martínez-Maqueda et al., 2013). The high binding capacity of the column enables maximum amount of the analytes to be retained from the sample and minimises sample loss (Martínez-Maqueda et al., 2013). The carbon loading uniformity of the DSC18 SCs ensure high reproducibility of the obtained results and enables effective and quick extraction, isolation and purification of peptides from biological samples (Martínez-Maqueda et al., 2013).

According to the manufacturer's instructions, the DSC18 SC is first conditioned and equilibrated to activate the sorbent and permit maximum interaction and retention of the target sample, which is then loaded to the column. The column is then washed using a strong solvent to remove any contaminants which are not bound to the sorbent. Finally, the DSC18 SC is eluted to release the purified target sample analytes (the purified hFF

peptides in this research) from the sorbent, **figure 1-11**, for further analysis by high performance liquid chromatography mass spectrometry (HPLC-MS), see also chapter 2, section 2.4.1 for further detail.

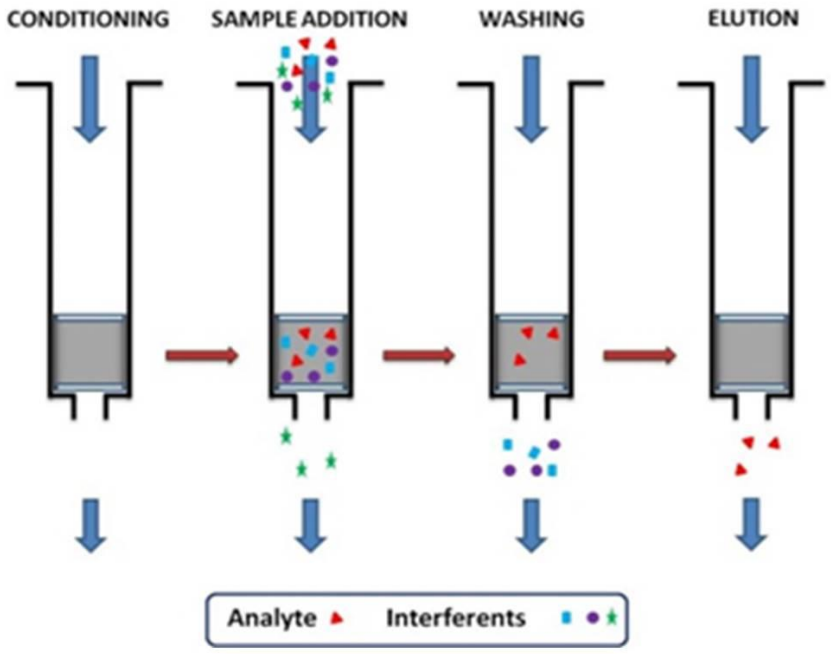


Figure 1-11 Solid phase extraction by DSC18 spin columns, four main steps are performed to achieve sample peptides purification as shown in the figure: column conditioning, sample addition, washing to remove interferents (contaminants) and elution of the target sample peptides (figure from DSC18 manual, also available on public domains).

1.6.2 High Abundance Proteins Depletion (HAPs depletion):

In follicular fluid, high abundance proteins such as albumin and immunoglobulins occupy approximately 85-90% of the total protein constituents in humans (Angelucci et al., 2006;

Hanrieder et al., 2008; Bayasula et al., 2013) and in the follicular fluid of other species such as porcine and equine (Fahiminiya et al., 2011a). The presence of HAPs may hinder the detection of low abundance proteins (LAPs) which are defined as being in the concentration range of < 100 ng/ml (Millioni et al., 2011). LAPs are considered more follicle-specific as they may be produced locally within the follicle itself and may be valuable for understanding follicle growth and development (Jarkovska et al., 2010).

Several types of depletion formats are available such as columns, cartridges, micro-columns or spin columns based on ion exchange, affinity ligands, antibodies or combination of all of these techniques, provided by companies such as Agilent, GenWay, Biotech, BioRad, Sigma–Aldrich and Amersham Biosciences (reviewed in Ahmed (2009)).

In this research, hFF will be prepared by HAPs depletion using immunoglobulin yolk spin columns (IgY-12 SCs) (chapter 2, section 2.4.2) to overcome the high complexity of hFF protein composition and to enhance low and medium abundance proteins identification (Jarkovska et al., 2010, Ambekar et al., 2013). The IgY-12 SCs are designed on the basis of avian antibody-antigen interactions; the column contains 12 avian polyclonal immunoglobulin yolk (IgY) antibodies against 12 of the most high abundance proteins in a biological sample covalently coupled to microbeads to pack the column (Ahmed, 2009). The HAPs, which are targeted for depletion by the IgY-12 SCs according to manufacturers' specifications, are: albumin, immunoglobulin G (IgG), IgM, IgA, transferrin, fibrinogen, alpha2-macroglobulin, alpha1-antitrypsin, haptoglobin, alpha1-acidic glycoprotein and apolipoproteins A-I and A-II, **figure 1-12**.

HAPs depletion by IgY-12 SCs involves a selective immune-depletion which provides very specific binding and clean capture compared to mammalian antibodies to provide an enriched pool of LAPs (Corrigan et al, 2011, Qian et al, 2008).

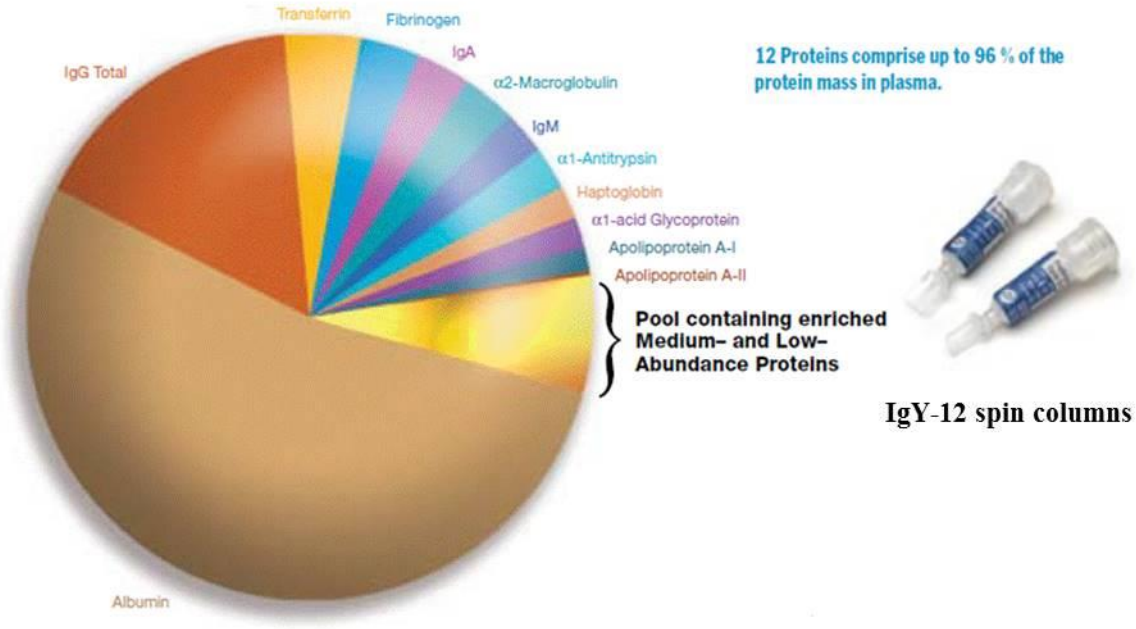


Figure 1-12 The twelve high abundance proteins (HAPs) targeted by IgY-12 SCs in plasma which are similar to that in hFF and the pool of enriched medium and low abundance proteins obtained following depletion. The IgY-12 SCs are also shown (both figures from Beckman coulter IgY-12 manual at http://www.protocol online.org/ also available on public domains).

The IgY-12 SCs kit contains two 1.2 ml IgY-12 spin columns and three buffers (dilution buffer, stripping buffer and neutralisation buffer) to prepare the column (see appendix 1 for buffers' recipes). The IgY-12 SCs can be regenerated for reusability by eluting and stripping off the bound proteins in 1× stripping buffer and immediately neutralising the

column in the 1 × neutralisation buffer and then re-suspending the columns' beads in the dilution buffer to store the column at 2-8°C for future use. For full details of the IgY SCs regeneration procedure see Beckman Coulter, proteomelab IgY-12 SCs manual at http://www.protocol online.org. The reusability of the IgY-12 SCs following their regeneration makes their application cost effective and relatively inexpensive. Another technique utilised in this research to enhance the protein yield was filtration and concentration of the depleted hFF samples which is highlighted in chapter 2, section 2.4.3.2.

1.6.3 One dimensional Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Pre-separation of proteins by one- or two-dimensional gel electrophoresis (1D SDS-PAGE and 2D SDS-PAGE) are the workhorse in the majority of proteomic studies (Shevchenko et al., 2007). SDS-PAGE is a powerful most widely used electrophoretic method for the analysis of complex proteins mixtures that separates proteins according to their molecular weights (Berkelman and Stenstedt, 1998). Proteins identification using gel electrophoresis provides significant advantages compared to gel-free techniques such as the in depth analysis of a wide dynamic range of proteins in a safe to handle matrix (Shevchenko et al, 2007).

The principle of the technique is that charged molecules migrate through the gel matrix upon application of a constant electric field (Wright et al, 2012). The presence of SDS, an ionic denaturing detergent, in both the prepared protein samples and the running buffer masks the charge of the sample proteins and results in a constant net negative charge per unit mass leading to proteins migration governed by molecular weights (Wright et al, 2012). Additionally, SDS, assisted by heat, enables partial unfolding of the protein

molecules, disruption of hydrogen bonds and blocks hydrophobic interaction (Wright et al, 2012). Additionally, a reducing agent such as dithiothreitol (DTT) is added to enhance the cleavage of the proteins' disulphide bonds and unfold any tertiary or quaternary protein structures (Wright et al, 2012).

A pre-stained protein standard of known proteins masses (known as molecular weight marker or protein ladder) is run in the same gel alongside the protein samples to allow determination of the mass of the sample proteins, **figure 1-13**. Protein samples are loaded into the gel wells and the proteins start to migrate through the gel matrix once the electric current is applied creating lanes or bands of proteins (Wright et al, 2012). Protein bands differ in their migration patterns according to the SDS-PAGE buffer system utilised, **figure 1-13**. Each buffer system has a slightly different pH which affects the charge of a protein and its binding capacity for SDS and therefore their migration (Wright et al, 2012). The gels are then stained, to visualise the separated proteins, using dyes such as Coomassie Brilliant blue, zinc imidazol, and silver nitrate (Wright et al, 2012).

The pore size of the acrylamide matrix works as a sieve, allowing larger proteins to migrate slower while smaller proteins propagate much faster. Low-percentage gels are used to resolve large proteins, and high-percentage gels are used to resolve small proteins (Wright et al., 2012) (see chapter 3, section 3.1.1 for further detail of SDS-PAGE application in proteomics studies). In this research work, 10% NuPAGE gel with MOPs buffer system will be utilized. This selection is based on our preliminary data obtained following the experimentations on hFF proteins and sperm + hFF protein interactions which showed clear protein bands using this selection. For convenience, reproducibility and high quality, a precast NuPAGE gel will be utilised in this research work.

Protein	Approximate Molecular Weights (kDa)			
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS
Myosin	250	210	188	191
Phosphorylase	148	105	98	97
BSA	98	78	62	64
Glutamic Dehydrogenase	64	55	49	51
Alcohol Dehydrogenase	50	45	38	39
Carbonic Anhydrase	36	34	28	28
Myoglobin Red	22	17	17	19
Lysozyme	16	16	14	14
Aprotinin	6	7	6	n/a
Insulin, B Chain	4	4	3	n/a

Figure 1-13 The range of proteins molecular weights migration in NuPAGE Bis-Ris gels 4-12% according to the buffer system utilised in running the gels. BSA: bovine serum albumin, MOPS: (3-(N-Morpholino) propanesulfonic acid), MES: 2-(N-morpholino) ethanesulfonic acid(figure from www.invitrogen.com, available on public domains).

1.6.4 Mass spectrometry:

Mass spectrometry (MS) is the analytical technique of choice for proteins identification and quantitation in proteomic studies, which offers accuracy, sensitivity and flexibility in biological research (Ho et al, 2003). MS instruments are generally composed of the

following main components: **i-** an ionisation source into which the molecules of interest are introduced to acquire positive or negative charges, **ii-** mass analyser which allows the travel of the ionised molecules according to their mass/charge (m/z) ratio, **iii-** detector which detects the generated signals from the ionised molecules and delivers them to the forth component of MS **iv-** a computer system which displays the signals as a mass spectrum according to the m/z ratio recorded (Ho et al, 2003), **figure 1-14**.

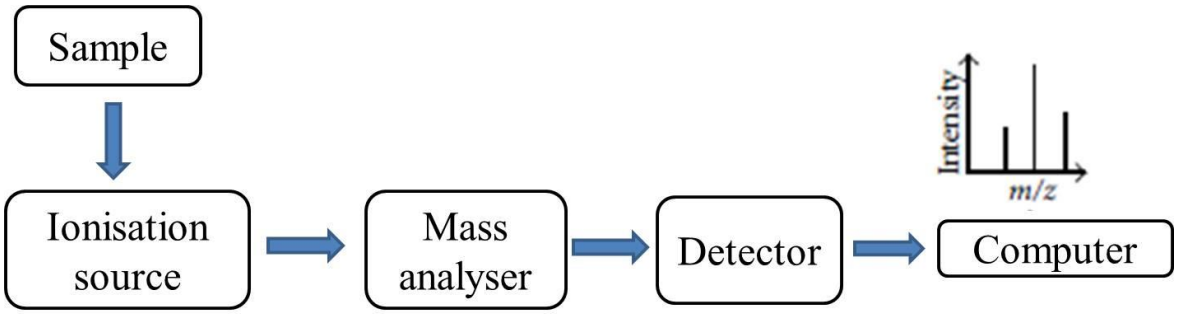


Figure 1-14 Basic components of mass spectrometry instruments; prepared samples are introduced into the ionisation source where molecules are charged into positive and negative charges and then analysed in the mass analyser according to their m/z ratio, the detector detects signals from the ionised molecules and delivers them to the computer system where the signals are interpreted to a mass spectrum.

Several types of MS are available, for example Electro Spray Ionisation – Time of Flight MS (ESI -TOF MS), Matrix-assisted laser desorption/ ionization-Time of Flight (MALDI-TOF MS) among others, based on different technical principles. Coupled with high performance liquid chromatograph (HPLC) for molecular fractionation prior to MS

analysis, HPLC-MS has become a very powerful technique capable of analysing at femto-mole quantities in micro-litre sample volumes of a complex biological sample (Ho et al., 2003).

In this research work, ESI -TOF MS will be utilised for proteomic analysis (chapter 2, section 2.4.4 and chapter 3, sections 3.1.1 and 3.4.5) and Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) for metabolomics analysis (chapter 4, section 4.3.3).

1.6.5 Micro-Particle Image Velocimetry (µPIV):

The principles of the technique of micro-particle image velocimetry (µPIV) are highlighted in detail in chapter 5, sections 5.1.1 and 5.3.3.

1.7Research aims:

To achieve fertilisation, human sperm have to navigate and interact with the female reproductive tract (FRT) on molecular and mechanical levels. This research intends to investigate some molecular and mechanical aspects of sperm interactions with the FRT secretions, mainly follicular fluid and high viscosity fluids such as cervical mucus (represented in this study by 1% methylcellulose). Limited knowledge is available about the role of hFF proteins in sperm regulation and whether sperm acquire any hFF proteins. The study of the human sperm metabolome under *in vitro* conditions is a novel approach in this research, which may enable characterisation of the sperm metabolic substrates for better understanding of sperm energy metabolism. The lack of knowledge regarding the

effects of sperm on their surrounding fluids in terms of mixing and perturbation were the main motivations for the sperm micro-particle image velocimetry study in this work.

The aims of the current research work are:

- To identify proteins present in the human follicular fluid, which is one of the fluid milieu sperm are exposed to just prior to fertilisation. A combination of proteomic techniques will be utilised along with compiling the human follicular fluid protein data from the existent literature.
- To examine if sperm may acquire any hFF proteins and the possible roles of the acquired hFF proteins, utilising proteomic approaches.
- To investigate and identify sperm metabolites released and/or consumed during capacitation, which is another aspect of molecular interaction of sperm with the FRT, using metabolomics techniques.
- To examine some physical aspects of sperm motility represented by the effect of motile sperm on their surrounding fluid's instantaneous velocity and vorticity in low and high viscosity media using a μ-particle image velocimetry technique.

Finally, a global discussion and conclusions are included with suggestions for future research. Appendices are also included which contain buffers and recipes, list of hFF proteins identified, list of proteins identified in sperm and hFF proteomic interactions, list of metabolites identified in sperm metabolomics and codes for fluid vorticity analysis in MATLAB.

Chapter 2

IDENTIFICATION OF NOVEL HUMAN FOLLICULAR FLUID PROTEINS BY PROTEOMIC TECHNIQUES

2.0 Abstract

Human follicular fluid (hFF) is the *in-vivo* microenvironment for oocytes to develop and mature. The composition of hFF is highly complicated with a high variety of steroid hormones and proteins. The knowledge of hFF protein constituents and their roles in folliculogenesis and reproductive biology is still limited. Aims: to perform an in-depth analysis of hFF proteins and to classify the identified proteins according to their biological functions and cellular localisation. Methods: a combination of proteomic techniques were utilised; solid phase extraction (SPE), proteins fractionation and peptide purification by DSC 18 spin columns followed by HPLC/MS was used as a first proteomic approach, High Abundance Proteins (HAPs) depletion followed by SDS-PAGE/MS as a second approach and HAPs depletion followed by filtration and concentration by Amicon Ultra centrifugal filter units and SDS-PAGE/MS as the third approach. Results: 212, 431 and 81 proteins were detected by the first, second and third approaches respectively with 180 proteins detected in both crude and depleted hFF samples. A total of 479 proteins were identified of which 103 (22%) were novel hFF proteins newly added to the repertoire and 376 proteins confirm those previously detected hFF proteins. Classification of the identified hFF proteins according to their molecular function in this study indicated that catalytic activity was the most common category with 38% followed by binding function with 29%. This study will contribute to the knowledge of hFF protein composition by increasing the data of the repertoire present. The study also compiled available hFF protein data from 24 published studies into a hFF protein table. The extensive hFF proteins table complied in this study comprising 1586 hFF proteins is an important resource for further research into folliculogenesis, discovery of biomarkers in hFF, study of fertilisation environment and potential improvements in assisted reproductive techniques (ART).

2.1 Introduction:

Human follicular fluid (hFF) is the *in vivo* microenvironment in which oocytes develop and mature (Fortune, 1994). During folliculogenesis, hFF is formed by secretions from the GCSs of the growing follicle and diffused exudate from the blood-follicle barrier (Fortune, 1994, Edwards, 1974). The hFF composition is recognised as highly complex with a variety of chemical substances ranging from steroid hormones, inorganic salts, muco-polysaccharides to a diversity of peptides and proteins (Spitzer et al, 1996) important for oocyte development and maturation (Emiliozzi et al., 1996, De Jonge, 2005). hFF has been the core of several studies which aimed to evaluate the roles of hFF steroid hormones, particularly progesterone, in sperm regulation and fertilisation (De Jonge et al., 1993, Harper et al., 2004, Revelli et al., 2009). hFF also denotes a distinctive and an enriched pool of biologically significant markers in terms of proteins and peptides (Kolialexi et al., 2007). Although the high dynamic range of hFF proteins renders its proteomics' study challenging (Bianchi et al., 2013), advances in proteomic approaches in hFF analysis have been evolving progressively and enabled identification of hundreds of hFF proteins up to date (Ambekar et al, 2014; Zamah et al, 2015). **Table 2-1** lists the recent proteomic studies of hFF proteome and techniques utilised.

Some of the hFF proteomic studies considered the levels of expression of certain hFF proteins as a biomarker in reproductive pathologies such as recurrent spontaneous miscarriage (Kim et al., 2006), endometriosis (Lo Turco et al., 2010, Lo Turco et al., 2013, Cunha-Filho et al., 2003) and polycystic ovarian syndrome (PCOS) (Moos et al., 2009, Ambekar et al., 2014).

Table 2-1 List of proteomic studies of hFF protein since 1996 to date, abbreviations are included in the list of abbreviations

Techniques utilised	Number of proteins identified	Reference
iTRAQ labelling and strong cation-exchange chromatography + LC-MS/MS analysis	**770**	(Ambekar et al., 2014)
SDS-PAGE, OFF GEL and SCX-based separation followed by LC–MS/MS	**480**	(Ambekar et al., 2013)
2D-PAGE + MALDI MS	**13**	(Anahory et al., 2002)
2D-PAGE + MALDI-TOF-MS	**69**	(Angelucci et al., 2006)
LTQ Orbitrap + (LC-MS/MS).	**503**	(Bayasula et al., 2013) (excluded from the hFF table)
2D-PAGE+IEF +MALDI-TOF/TOF MS	**43**	(Bianchi et al, 2013)
2D-PAGE LC –tandem MS	**11**	(Estes et al., 2009)
IEF, nano-LC MALDI TOF /TOF MS	**69**	(Hanrieder et al., 2008)
IEF, MALDI TOF MS/MS	**73**	(Hanrieder et al., 2009)
2D-PAGE + MALDI-TOF-TOF MS	**6**	(Hashemitabar et al., 2014)
2D-PAGE+ LC + MALDI MS	**15**	(Jarkovska et al., 2010)
In solution digestion + Nano-LC-QTOF	**73**	(Kushnir et al., 2012)
2D-PAGE + MALDI-TOF MS + nano-LC MS/MS	**5**	(Kim et al., 2006)
2D-PAGE +MALDI MS	**10**	(Lee et al., 2005)
Protein digestion + MALDI-TOF MS	**18**	(Liu et al., 2007)
SELDI-TOF-MS + cation exchange protein chip	**4**	(Li et al., 2008) (excluded from the hFF table)
Multidimensional Protein Identification Technology (MudPIT)	**170**	(Lo Turco et al., 2010)
2D-PAGE + ESI-QTOF MS	**21**	(Lo Turco et al., 2013)
Immunoprecipitation + SULPHATESELDI- TOF-MS-based ProteinChip System	**4**	(Schweigert et al., 2006)
Multiplexed protein array and iTRAQ	**30**	(Severino et al., 2013)
2D-PAGE MALDI-TOF MS	**37**	(Sim and Lee, 2008)
2D-PAGE	**7**	(Spitzer et al., 1996)
pre-fractionation of proteolytic peptides and nanoflow reversed-phase LC-MS/MS	**246**	(Twigt et al., 2012)
MALDI-TOF MS	**11**	(Wu et al., 2015)
In solution protein digestion + Q-TOF MS	**535**	(Regiani et al., 2015)
HPLC-MS	**742**	(Zamah et al., 2015)
Total hFF proteins	**1586 proteins**	

Identification of potential biomarkers for oocyte quality, pregnancy outcome and IVF success in hFF has also been considered in several studies (Michael et al., 1993, Kolialexi et al., 2007, Michael and Papageorghiou, 2008, Kushnir et al., 2012). For example, the level of 11 β- hydroxyl steroid dehydrogenase in hFF has been recommended as a feasible predictor of IVF success with lower levels of 11 βHSD increasing the chances of successful IVF (Michael et al., 1993). Additionally, levels of sex hormone binding globulin (SHBG), corticosteroid binding globulins (CBG) (Andersen, 1990) and some cytokines such as IL8 and IL18 (Sarapik et al., 2012) in hFF have been recommended as predictors of IVF success. hFF has also been investigated as a source for proteins supplementation in IVF to replace the previously commonly used fetal cord serum and maternal serum (Chi et al., 1998). Furthermore, hFF has been shown as a valuable source of proteins in *in vitro* maturation media of immature oocytes (Cha et al., 1991, Jee et al., 2008) and for supplementation of embryo culture media to enhance embryo cleavage during IVF (Davoodi et al., 2005, Hemmings et al., 1994). It has also been shown that hFF stimulates several sperm functions (Getpook and Wirotkarun, 2007, Yao et al., 2000, Mbizvo et al., 1990, Ralt et al., 1994) (section 1.5, chapter 1). The possible beneficial effects of hFF on implantation prior to intracytoplasmic sperm injection (ICSI) have been investigated by flushing the uterine cavity with hFF prior to embryo transfer to enhance implantation, however, no significant improvement in implantation rates was detected in this study (Hashish et al., 2014). Protein profiling of hFF has also been suggested as a fundamental approach for understanding ovarian folliculogenesis and as an indicator of follicular developmental stage (Liu et al., 2007, Spitzer et al., 1996, Bianchi et al., 2013). For example, the biochemical profile of FF has been shown to vary between large and small follicles in the same species which suggests links between the FF composition,

follicle size and the stage of oocyte maturation (Thomas et al., 2000, Revelli et al., 2009). In animals, some studies were performed to determine the relationship between protein concentration, FF volume and follicle size and some of their results were conflicting (Nandi et al., 2007). Studies on bovine FF (Andersen et al., 1976, Brantmeier et al., 1987) and ovine FF (Nandi et al., 2007) indicated similar or increased total protein concentration in small and large follicles. In human, comparison of hFF protein contents from mature and immature follicles revealed remarkable differences in the pattern and amount of proteins of these follicles and was suggested as a potential tool to identify the stage of follicular maturation (Spitzer et al., 1996).

Metabolic changes in hFF chemical constituents have also been observed in obese patients seeking fertility treatment with dramatic elevation in C-reactive protein concentration in their hFF samples (Robker et al., 2009). hFF from patients with advanced maternal age and diminished ovarian reserve also showed alteration in the metabolic profile of hFF which was attributed to changes in follicular cell activity (mainly GCs) (Pacella et al., 2012). Pacella et al (2015) indicated that changes in the carbohydrate and hormonal contents of hFF were statistically significant while those in ions and proteins were not. These studies applied metabolomics techniques which are novel approach aims to analyse the total molecular metabolites in a biological fluid (Revelli et al, 2009, Pacella et al, 2012).

The various applications of hff protein contents, as shown in the above mentioned studies, provide impetus for extensive hFF proteomic analysis and exploration (Hanrieder et al., 2008). The high complexity of the different processes involved in oocyte maturation (Picton et al., 1998) may also indicate the necessity for the whole hFF proteome profile to predict the oocyte quality for ART instead of relying on single biomarkers (Twigt et al,

2012). The lack of the complete hFF protein profile and the currently limited understanding of the diverse range of functions of its proteins contents render further research into hFF proteins indispensable (Kushnir et al., 2012). This chapter presents hFF proteomic study using a combination of proteomic techniques and also introduces a hFF protein table compiled from 24 hFF proteomic studies. A table with the potential hFF biomarkers in several applications has also been constructed in this study.

2.2 Objectives:

- To identify novel hFF proteins and to compile the existing hFF protein data from the literature in hFF protein table.
- To describe the functional classifications of the identified hFF proteins and any potential implications in reproduction.
- To use the hFF protein identified in this study and the compiled hFF protein table in the comparison and data analysis of the sperm + hFF proteomic interaction which is introduced in chapter 3 of this thesis.

2.3 Materials and methodology:

2.3.1 Materials:

Table 2-2 Materials

Product name	Company	Product code
96 Well flat bottom plate	Corning Incorporated, New York, USA	3595
Acetonitrile	Millipore, Watford, UK.	UN1648
Amicon Ultra 0.5ml-30K centrifugal filters units	Millipore, Watford, UK.	UFC503096
Ammonium bicarbonate	Sigma, Dorset, UK	09830-1KG
Bicinchoninic Acid protein assay kit	Sigma, Dorset, UK.	B9643
Bovine serum albumin	Millipore, Watford, UK.	82-002-4
Dithiothreitol (DTT)	Melford Laboratories , Suffolk, UK	MB1015
DSC18 spin columns	Sigma, Dorset, UK	52602-U
Ethylene diamine tetra-acetic acid (EDTA)	Sigma, Dorset, UK	E5134EDTA
Formic acid	Sigma(Fluka analytical), Dorset, UK	14265-50ML
IgY-12 spin column kit	Beckman Coulter, High Wycombe, UK	A24618
Iodoacetamide	BioRad laboratory, Hertfordshire, UK	1632109
NuPAGE Novex (10% Bis-Tris gel)	Invitrogen, Paisley, UK	NP0302Box
Sodium deoxycholate	Sigma, Dorset, UK	30970
Sodium dodecyl sulphate (SDS)	Sigma, Dorset, UK	L6026-250G
Tris (hydroxymethyl) amino-methane	Sigma, Dorset, UK	252859
Trypsin (sequencing grade, modified, porcine)	Promega, Southampton, UK	V511A
Trypsin Resuspension Buffer	Promega, Southampton, UK	V542A
Urea	Sigma, Dorset, UK	U5378-5KG

2.3.2 hFF collection:

Follicular fluid was obtained under Local Research Ethical Approval from South Birmingham 2003/239. Follicular fluid was obtained from women undergoing fertility treatment (IVF or ICSI) at the Assisted Conception Unit (HFEA Centre: 0119), Birmingham Women's Hospital following informed consent in agreement with the HFEA (Human Fertilization and Embryology Authority) Code of Practice (Ethical approval number 2003/239) (Frettsome, 2011). Patients received hormonal treatment to stimulate multiple follicles development prior to oocytes retrieval; briefly, down regulation of patient's cycle was achieved using Buserelin acetate (a GnRH agonist) (Sanofi-Aventis, France) followed by long protocol stimulation using urinary follicle stimulating hormone (FSH) (Frettsome, 2011). The follicles were aspirated under trans-vaginal ultrasound guided puncture to collect COCs and follicular fluid. A flushing media containing heparinised Hartman's solution was used in some cases to aspirate the follicle (Frettsome, 2011). Only clear hFF with no blood or flushing media contamination and with follicle size ranging from 16-18 mm and containing an oocyte were included in the study. Centrifugation of the collected hFF was carried out at 3500g, for 30 minutes at room temperature to eliminate any cellular debris (Frettsome, 2011). hFF from three donors were pooled together and then divided into small aliquots of 50µl which kept in the freezer at -80°C until use.

Donor codes: 119ECF11, 119ECF25 (patient with male factor infertility) and 119ECF04 (patient with unexplained infertility), all three female donors were healthy and had successful cycles and positive pregnancy outcomes.

2.3.3 BCA assay for hFF protein concentration estimation:

The concentration of the total proteins in the pooled sample of hFF was estimated by the BCA (Bicinchoninic acid assay). Briefly, 200 µl of the working solution (bicinchoninic acid and copper (II) sulphate, 50:1 respectively) was added to 25µl diluted hFF samples (diluted at a concentration range of 1:5, 1:10, 1:20). Similarly, 200 µl of the working solution was added to a bovine serum albumin (BSA) standard (ranging from 0 µg/ml to 1000 µg/ml). All the preparations were added to a flat bottomed 96 well plate and left to incubate for 30 minutes at 37°C. The optical density values were measured in a plate spectrophotometer at 540 nm wavelength. The protein concentration was determined by comparison to BSA standard curve that was constructed in parallel.

2.4 hFF preparations

Three proteomic approaches were used and the details of each approach are shown in the sections below as follows:

2.4.1 1st Approach: Solid Phase Extraction/High Performance Liquid Chromatography/Mass Spectrometry (SPE/HPLC/MS):

For abbreviation, this approach is referred to as SPE/HPLC/MS. In this approach, hFF was prepared using Discovery DSC18 column from Suppleco (see section 1.6.1 for further detail of the technique principles).

hFF was prepared prior to loading into the DSC18 columns as follow:

30 µl of hFF were added to 470 µl of 50mM ammonium bicarbonate. 500 µl of 2% sodium deoxycholate prepared in 10mM DTT/100mM ammonium bicarbonate was added to the hFF preparation (to end up with 1% sodium deoxycholate/5mM DTT/50mM ammonium bicarbonate) and incubated for 60 minutes at 65°C. The sample was left to cool down

before adding 20 µl of 1M iodoacetamide/50mM ammonium bicarbonate to the prepared hFF sample and left in the dark at room temperature for 20 minutes. Then, 150 µl of trypsin on ice was added to the sample and left overnight on a shaker at 37°C. Next, 125 µl of 100% formic acid was added to the 1250 µl prepared hFF sample to achieve a final concentration of 10% formic acid. The preparation was left for 5-7 minutes and then centrifuged at 2000g for 15 minutes. Only the supernatant fluid (without disturbing the pellet which was discarded) was collected to be loaded into the equilibrated DSC 18 columns.

The DSC 18 column was prepared prior to loading the hFF sample as follow: the DSC 18 column was conditioned by adding 1ml of 1% formic acid in acetonitrile and allowed to dribble slowly under the column under gravity into a Falcon tube and discarded (all the next additions were left in a similar way, under gravity effect). The column was then equilibrated by adding 1ml of 1% formic acid in high purity water and left to dribble and the flow through was discarded.

Then, the prepared hFF sample was loaded into the DSC 18 column and left to dribble and collected into a Falcon tube. When the dribbling was completed, 1ml of 1% formic acid was added to wash the DSC18 column, to remove any contaminants, and left to dribble into the same Falcon tube. Another 1ml of a mixture of 75% of 1% formic acid in acetonitrile and 25% of 1% formic acid in distilled water was added to elute the DSC 18 column and release the target analyte of hFF and the dribbling fluid was collected in a clean Falcon tube to be analysed. The collected hFF sample was divided into two parts (50 µl and 950 µl); both were dried completely under vacuum in the Speedvac. The 50µl sample was used to confirm presence of peptides by MS while the second part of 950µl was kept for further analysis. When the presence of peptides in the 50µl sample was

confirmed, further processing of the 950µl was pursued as follows: 110µl of mixed HPLC solvent A was added to the dried 950µl sample, vortexed and sonicated for 10 minutes. The sample was then centrifuged at 2000g for 5 minutes and the supernatant was collected before immediately transferring the sample into the HPLC machine. The yield from the HPLC was collected every 3 minutes into clean 0.65 µl Eppendorf tubes. The collected samples were dried completely in Speedvac at 45°C for around 120 minutes and finally they were re-suspended in 20µl of 1% formic acid and kept frozen until further analysis by the MS. **Figure 2-1** summarises the methodology for the SPE/HPLC/MS approach.

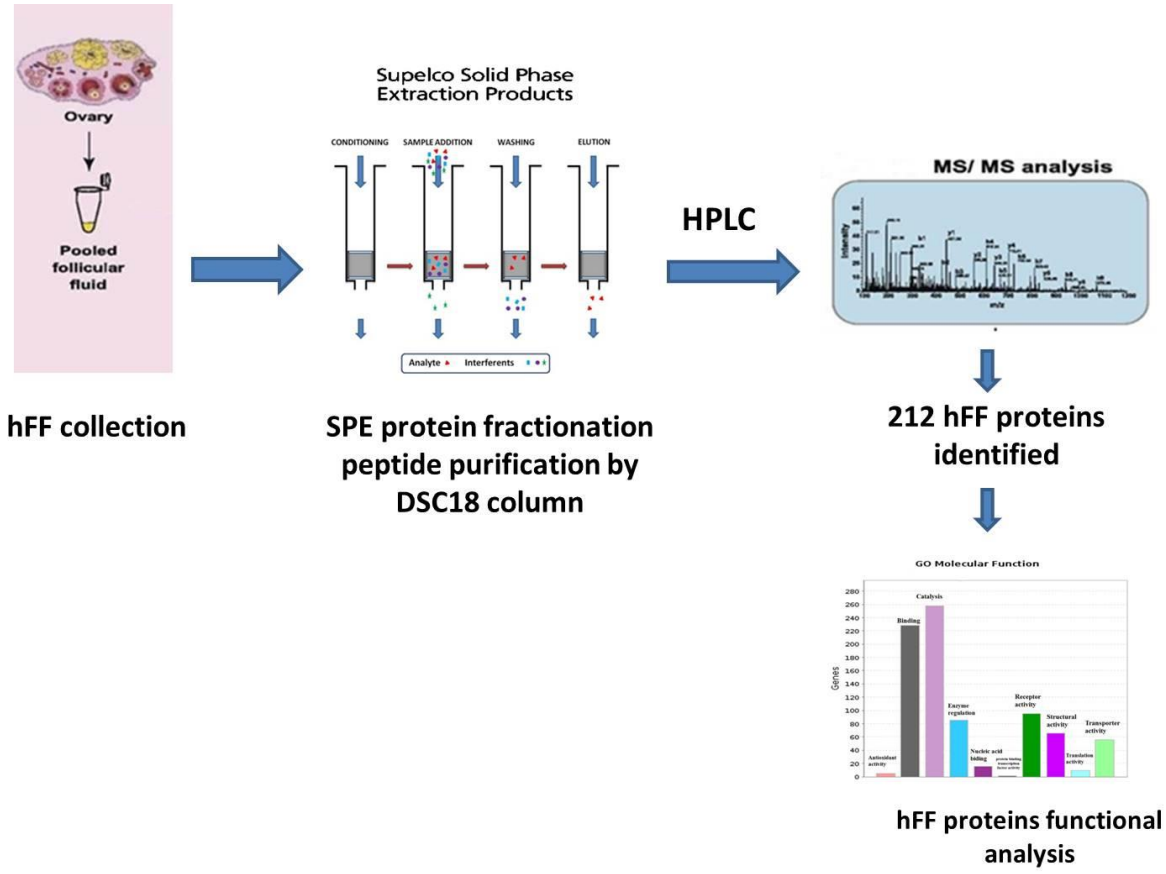


Figure 2-1 Summary of the 1st approach methodology, Solid Phase Extraction/High Performance Liquid Chromatography/Mass Spectrometry (SPE /HPLC/MS).

2.4.2 2nd Approach : High Abundance Proteins (HAPs) depletion/Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis/Mass Spectrometry

For abbreviation, this approach is referred to as HAPs/SDS-PAGE/MS.

2.4.2.1 hFF (HAPs) depletion prior to SDS-PAGE:

In this approach hFF sample was first depleted from the 12 most highly abundant proteins using IgY-12 spin columns (IgY-12 SCs) (see section 1.6.2 for further detail). The procedure of HAPs depletion was performed according to the manufacturer's instructions. Briefly, the IgY-12 SCs were centrifuged at 2000g for 30 seconds to obtain dried beads. 65µl of hFF was diluted in 1× Dilution buffer (provided with the IgY-12 SCs kit) to a final volume of 1300 µl. The diluted hFF sample was divided into two tubes, 650 µl each and each was added to one of the IgY-12 SCs and mixed on an end to end rotator for 15 minutes to assure complete blending of the IgY-12 SCs' beads with the hFF samples. The two IgY-12 SCs were then centrifuged for 30 seconds at 2000g and the flow-through hFF samples were collected. The hFF samples were then prepared for running the SDS-PAGE as shown below. The IgY-12 SCs can be regenerated for reusability by eluting and stripping off the bound proteins (see section 1.6.2, chapter 1).

2.4.2.2 SDS -PAGE:

The same SDS-PAGE protocol was used in the preparation of both the depleted hFF samples (section 2.4.2) and the depleted and filtered/concentrated hFF samples (section 2.4.3). Briefly, samples were divided into 20 µl aliquots and 5µl of 5 × SDS-PAGE sample buffer (0.05% (w/v) bromophenol blue, 0.25 M DTT, 50% (v/v) glycerol, 5% (w/v) SDS and 0.225 M Tris-Cl, pH 6.8) was added to each of the aliquots, heated at 100°C for 5 minutes and centrifuged at 500g for another 5 minutes immediately before loading the samples into SDS-PAGE gel along with 5 µl a pre-stained protein standard.

Proteins were resolved by electrophoresis on a precast 10% NuPAGE Bis-Tris gel for 60 minutes at 200 V voltages in MOPS buffer (1 × MOPS prepared by diluting 25 ml of 20 × MOPS in high purity water up to a total volume of 500 ml). The gels were stained overnight with Coomassie Brilliant Blue stain on a shaker at room temperature. Each whole protein lane was then carefully cut into approximately 23 pieces 1mm each according to the stained protein bands. Positive pressure hood, sterile scalpels and clean (dust free) Eppendorf tubes were used throughout the experiments to minimise keratin contamination of the gels. The sliced protein bands were further treated according to standard in-gel protein digestion protocol for MS analysis as follows:

2.4.2.3 In-gel protein digestion and MS:

In- gel protein digestion protocol of Coomassie Brilliant Blue stained gels (Shevchenko et al., 2007) was applied, with minor modifications, to digest proteins in the gels and prepare them for MS analysis as follows: (all the in-gel protein digestion steps were carried out inside a positive pressure hood as mentioned earlier and all chemical preparations were freshly made on the day of the experiment).

De-staining : On a clean glass plate and inside a positive pressure hood the whole Coomassie stained gel lanes were sliced by a sterile scalpel. Each lane was sliced into around 23 small pieces (around 1mm each) which preferably coincide with the stained protein bands in the gel. 500µl of 50% Acetonitrile/50mM Ammonium bicarbonate was added to each of the Eppendorf tubes containing one gel slice and left to incubate for 20-45 minutes with vigorous shaking on a shaker at room temperature. Then the supernatant was aspirated by a suction machine and discarded.

Reduction and alkylation: 250µl of 50mM DTT in 10% Acetonitrile/50mM Ammonium bicarbonate was added to the Eppendorf tubes containing the gel slices and incubated for

60 minutes at 54°C. The supernatant fluid was aspirated by suction machine and 250µl of 100mM Iodoacetamide in 10% Acetonitrile/50mM Ammonium bicarbonate was added to each tube and incubated in the dark at room temperature for 30 minutes. The supernatant fluid was then discarded and 500µl of 10% Acetonitrile/50mM ammonium bicarbonate was added and the tubes were left on a rotator for 15-30 minutes. The tubes were dried in the Speedvac for 120 minutes or until the gel pieces were completely dry.

In-gel protein digestion: sequencing grade modified trypsin was prepared according to the manufacturer's specifications and 20-40µl of the prepared trypsin was added to the dried gel pieces and left overnight at 37°C on a shaker. All trypsin additions were carried out quickly on ice.

Protein extraction: On the following day, 150µl 1% formic acid in 50% acetonitrile was added to each of the tubes containing gel slices and incubated for 60 minutes on a shaker. Then the supernatant fluid was transferred to new Eppendorf tubes and the pipette tips were left inside the new tubes (to minimise peptide loss). Next, 150µl 1% formic acid in distilled water was added to each gel slice in the original tubes and left for 60 minutes on a shaker and the supernatant fluid from each tube was then transferred into the same new tubes again. This step was performed twice and the collected supernatant fluids were then dried completely in the Speedvac for 120-180 minutes. Finally, 20µl of 1% formic acid was added to each tube and the samples were ready for MS analysis (kept frozen at -20°C until the MS analysis was performed). **Figure 2-2** summarises the methodology for the HAPs/SDS-PAGE/MS approach.

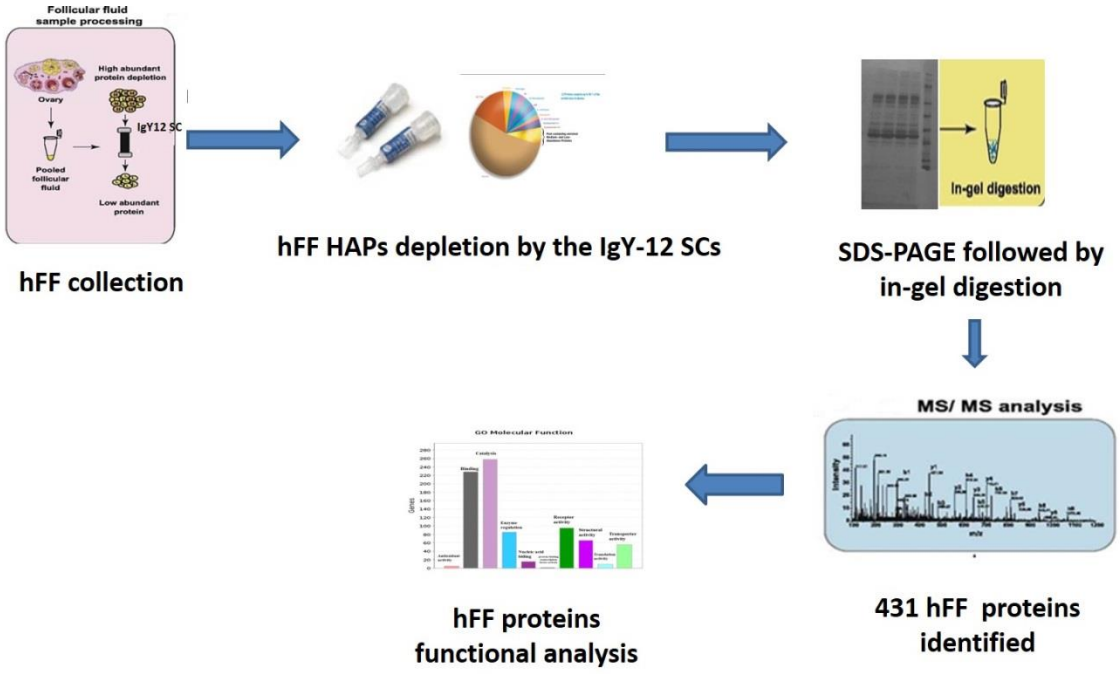


Figure 2-2 Summary of the 2nd approach methodology, High Abundance Protein depletion/ Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis/Mass Spectrometry (HAPs/SDS-PAGE/MS).

2.4.3 3rd Approach: High Abundance Proteins (HAPs) depletion/ filtration and concentration/SDS-PAGE/MS (HAPs/FC/SDS-PAGE/MS)

For abbreviation, this approach is referred to as HAPs/FC/SDS-PAGE/MS.

2.4.3.1 hFF (HAPs) depletion prior to SDS-PAGE:

See section 2.4.2.1

2.4.3.2 hFF concentration and filtration following the HAPs depletion:

To further enhance the yield of hFF proteins detection, a step of filtration and concentration by Amicon Ultra 0.5ml centrifugal filter units (with pore size 30 kDa molecular weight cut off) was introduced following the depletion technique in this approach. According to the manufacturer's specifications, these centrifugal filter units have a concentration factor of 15-20 times. The depleted hFF sample was processed

further to obtain depleted concentrated hFF sample as follows: 300µl of the depleted hFF sample was loaded into each Amicon Ultra 0.5ml centrifugal filter unit following addition of 8M urea and 1% SDS to the depleted hFF sample. The filter units were then centrifuged for 15minutes and the fluid in the filter device of the centrifugal units was retrieved for running on SDS-PAGE. **Figure 2-3** summarises the methodology for the HAPs/FC/SDS-PAGE/MS approach.

2.4.3.3 SDS-PAGE:

See section 2.4.2.2

2.4.3.4 In-gel digestion:

See section 2.4.2.3

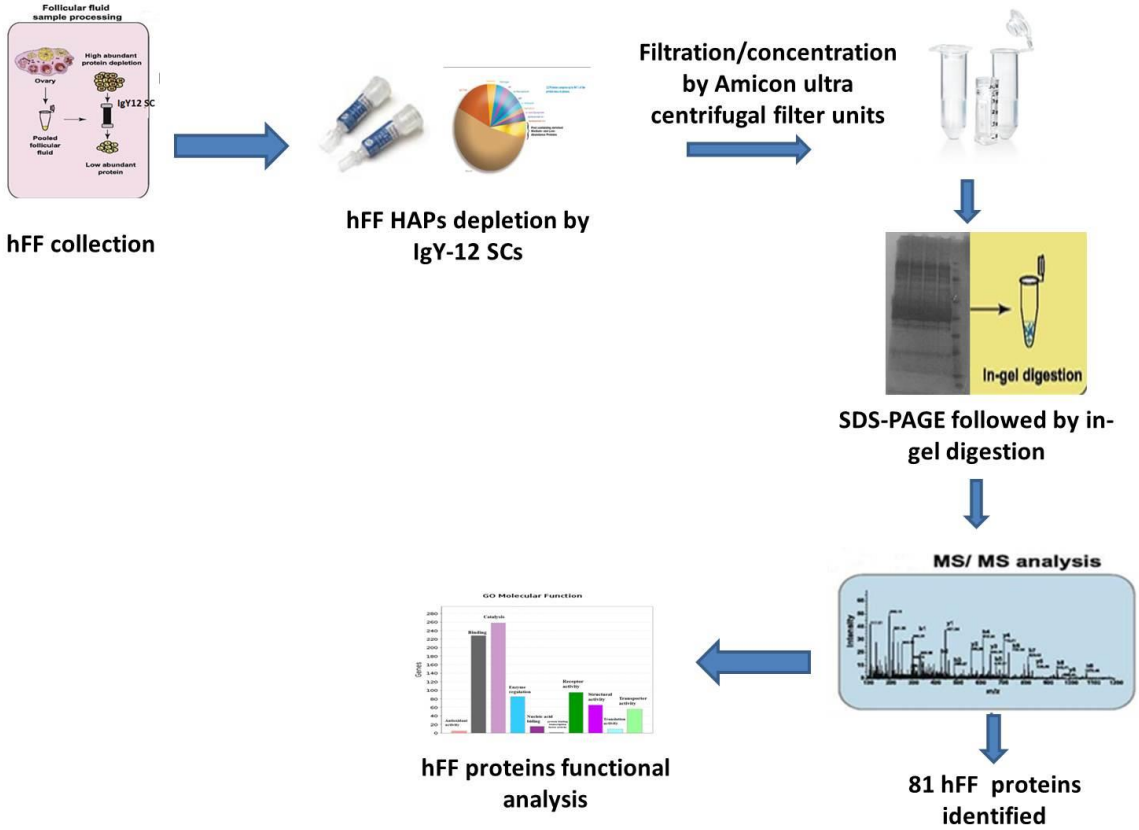


Figure 2-3 Summary of the methodology in the 3rd approach, High Abundance Proteins depletion/ Filtration concentration/Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis/Mass Spectrometry (HAPs/FC/SDS-PAGE/MS).

2.4.4 Mass spectrometry analysis:

This work was completed by Dr. Ashley Martin and Dr Douglas Ward, University of Birmingham. Briefly, the obtained tryptic peptides from the individual protein bands from the three hFF proteomic approaches were subjected to an on-line, direct infusion into an Electro Spray Ionisation interface – Time of Flight MS (ESI -TOF MS) that has MS/MS capability. The peptides were sprayed into a MicroTof II MS via a nano ESI interface following separation on a Dionex Ultimate 3000 HPLC system. The HPLC column was an Acclaim C18 Pep Map (25 cm long, 75 µm internal diameter, 3µm particle size) (Frettsome, 2011). An online nanospray source fitted with a metal needle with a 10 µm tip connected the HPLC column to a Bruker ETD Amazon mass spectrometer (Frettsome, 2011). The drying gas set to 6.5 l/min at 180°C and 1600 V was applied to the end plate (Frettsome, 2011).

An MS survey scan from 350 to 1600 m/z was accomplished and the selection of the five most abundant ions in each MS spectrum was performed (Frettsome, 2011). An exclusion list was applied following each two cycles of fragmentation. The data search was performed by the Mascot search engine (version 3.0) and applying 10 ppm (part per million) mass accuracy rate for both the parent and fragment ions. The search was performed by using SwissProt sequence of database for human. Trypsin as a protease, 0.5 Da for minimum mass accuracy for the parent and fragment ions and carbaminomethylation of cysteines as a fixed modification were selected. Protein identifications were filtered using both a 1% false discovery rate (FDR) (99% probability) and a requirement of a minimum 2 or more peptides using Proteinscape 2.1 software (Frettsome, 2011).

2.4.5 hFF proteins functional analysis:

The identified hFF proteins in this study were classified according to molecular functions, biological processes and cellular localisation. The functional classification was achieved using PANTHER (Protein ANalysis THrough Evolutionary Relationship) Classification System Version 10 at http://www.pantherdb.org/(accessed in July 2015). PANTHER is part of the Gene Ontology Reference Genome Project and is a comprehensive free online knowledgebase of protein pathways which was designed to classify proteins in order to facilitate high-throughput analysis (Mi et al., 2013, Thomas et al., 2006). The following terms are used in the PANTHER classification system: molecular function which is the function of a protein at a biochemical level e.g. a protein kinase; biological process: the function of a protein in the context of a larger network of proteins that interact to achieve a process at the level of the cell or organism, e.g.metabolic process.

2.5 hFF compiled protein table from the literature:

2.5.1 Methodology for the hFF compiled protein data:

Following an extensive literature search in Medline via the PubMed search platform using the following search terms: (“human follicular fluid” OR (“Follicular fluid”[All Fields] AND “proteins”[All Fields]]) OR “human follicular fluid proteomics”[All Fields] OR (“follicular fluid proteins ”[All Fields] AND “identification”[All Fields]) OR “follicle ”[All Fields]) AND (“fluid”[Subheading] OR (“biological”[All Fields] AND “markers”[All Fields]) OR “biological markers”[All Fields] OR “biomarker”[All Fields]) and by selecting (Human) for species. Studies retrieved from the PubMed search were assessed for eligibility using the title and abstract or where necessary the full text. Twenty-six studies were available online from 1996 up to July 2015 on hFF proteomic

studies published in English. A list of the techniques applied in each of the 26 identified studies and the number of proteins identified per study are illustrated in **table 2-1** and **figure 2-4**. All studies included in the analysis were on human follicular fluid. Twenty-four studies were included in the hFF ptotein table while two studies were excluded (one study only the abstract was available in English and the article and protein data were only available in Chinese (Li et al, 2008). The second study (Bayasula et al, 2013) was excluded since most of the FF proteins described in the hFF protein list were of none *homo sapien* origin, including yeast and mouse proteins. None of the studies were excluded on the basis of the proteomic techniques used which is unlike a compiled study of human sperm proteome (Amaral, et al, 2014a) in which the authors excluded studies using MALDI-TOF/ MS as the technique is described as outdated. Nonetheless, inclusion of potentially false positive hFF proteins was minimised by applying the following selection criteria for protein identification in the included published studies according to: identification of at least two peptides per protein and a false discovery rate (FDR) < 5% or an FDR < 1%. In studies when the hFF protein data retrieved by SDS-PAGE, only protein data from direct amino acid sequencing of proteins were included. The inclusion criteria for each of the studies included are highlighted in appendix 3.

All the proteins identified in the included 24 hFF studies were loaded into UniProt knowledge base (the Universal Protein Resource, http://www.uniprot.org/uploadlists) which is one of the major databases for protein information containing an exhaustive and accurate annotation for proteins along with their sequences. All proteins were annotated using Swiss-Prot accession number even when the original data was in other forms of protein ID (for example: GI accession number or in some cases ENSEMBL proteins). This was performed to achieve homogeneity of the data in terms of protein names and accession

numbers and also to avoid proteins redundancy. For example, in three studies (Kushnir et al, 2012; Ambekar et al, 2013, Ambekar et al, 2014) which used GI numbers for protein data annotation, two or more GI numbers have only one UniProt accession number. Thus a study referring to 770 proteins identification (Ambekar et al., 2014), actually had 404 proteins when the protein data was loaded into UniProt knowledge base (UniProtKB) and annotated using UniProt accession numbers. Some of the listed proteins in the study by Ambekar et al (2014) and Regiani et al (2015) were either fragments or deleted proteins and their GI numbers have been merged into a single UniProt accession number.

2.5.2 Results and discussions of the compiled hFF protein data from the literature:

By compiling the protein data from the available articles, a list of 1586 proteins was generated along with the references to the studies identified them. This table only contains proteins with UniProt accession numbers; any proteins with accession number that has been merged into another protein's accession number or proteins with deleted or obsolete accession numbers were not included in the compiled hFF protein table. Obsolete or deleted proteins are considered as redundant proteins. In proteomic, a redundant protein is defined as the one in which all or nearly all protein sequences are highly similar or identical to an existing protein from the same species (as defined in UniProt knowledge base, www.uniprot.org). The UniProtKB further reduces proteins redundancy by merging all proteins sequences which are encoded by the same gene into a single UniProt entry record and are shown as merged proteins in the UniProt database. In this compiled study, some proteins were found to be merged into their corresponding proteins following their annotation with a UniProt accession number.

In the compiled hFF protein data from the literature, around one third of the proteins were reported more than once, which may indicate the high biological variation in the proteins identified by the different proteomic techniques in terms of protein depletion, fractionation and analysis. This also shows the importance of compiling hFF proteins to include up to date hFF proteins reported in the literature. This work may provide a resource for further hFF proteomic research. This observation is similar to the work done in plasma proteome where combining data for the Plasma Proteome's Reference Database from different plasma proteomic studies which applied different sample preparation techniques resulted in the identification of considerably diverse sets of proteins (Farrah et al., 2011). The total number of hFF proteins identified also varied from previous publications. Ambekar et al, 2013 reported that 789 hFF proteins are found in hFF proteomic data while Zamah et al (2015) indicated that hFF contains 982 proteins up to the date of their publication. In this work, 1586 hFF proteins were compiled out of 24 published studies from 1996 to 2015.

The list of compiled hFF proteins was searched in PANTHER classification system http://pantherdb.org/ to describe the biological processes involved for hFF protiens, **figure 2-5.**

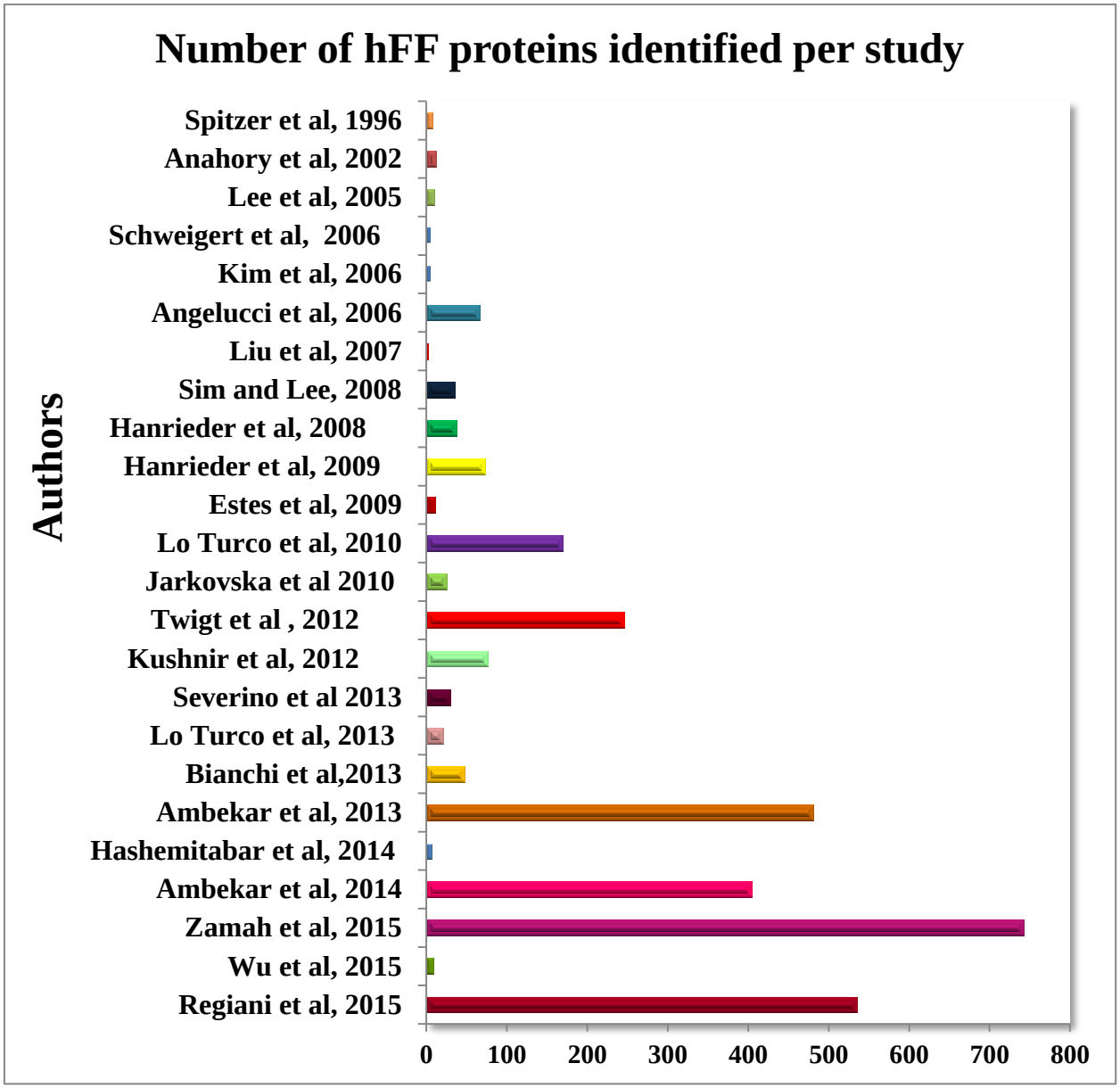


Figure 2-4 The number of hFF proteins identified in each of the 24 published studies included in the compiled hFF protein data. See **table 2-1** for the detail of techniques utilised in each study.

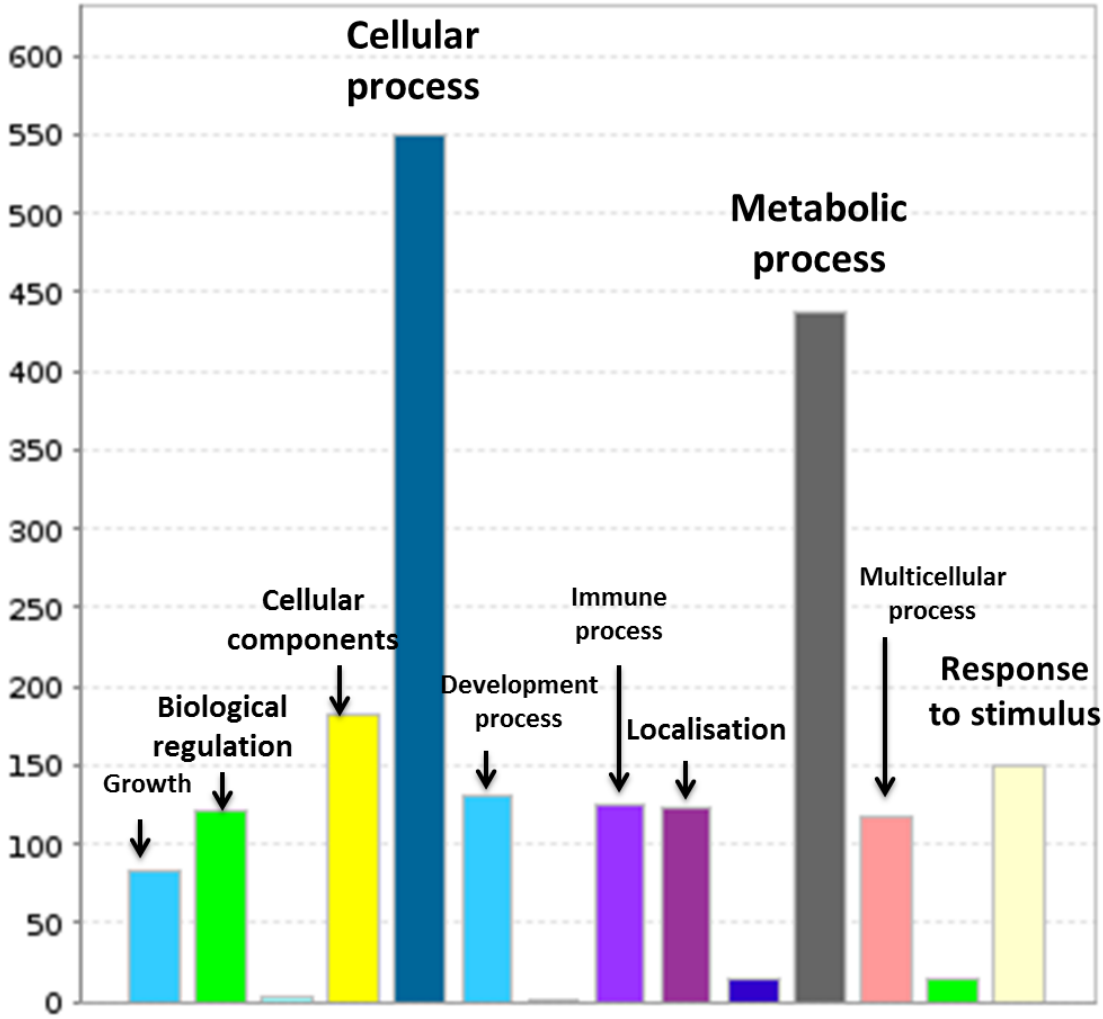


Figure 2-5 Biological processes involved in the compiled hFF proteins according to PANTHER classification system. Cellular process and metabolic process were among the highest categories.

2.6 Results of hFF proteomics:

The hFF proteins identified in the three approaches (n = 3) were compared and compiled in one table for the total number of distinct hFF proteins detected in this study. The original data obtained was 346, 660 and 102 proteins from the 1st, 2nd and 3rd approaches respectively. Random sequence proteins (rnd proteins) were deleted from the data at 1% FDR. A total of 479 hFF proteins were included in the final analysis; 212 proteins from

the 1st approach (SPE/HPLC/MS), 431 proteins from the 2nd approach (HAPs/SDS-PAGE/MS) and 81 proteins from the 3rd approach (HAPs/FC/SDS-PAGE/MS). The 1st approach using a crude non-depleted hFF sample enabled the detection of 32 proteins which were not detected in the other two approaches following HAPs depletion. Alternatively, 267 proteins out of the total 479 were only identified following the HAPs depletion in the 2nd and 3rd approaches (154 and 17 proteins respectively with 4 proteins detected in both). 180 proteins were identified in both crude and depleted hFF samples in this study. Venn diagrams **figure 2-6** and **2-7** illustrate the number of proteins detected in each approach and numbers of proteins in the crude and depleted hFF samples respectively.

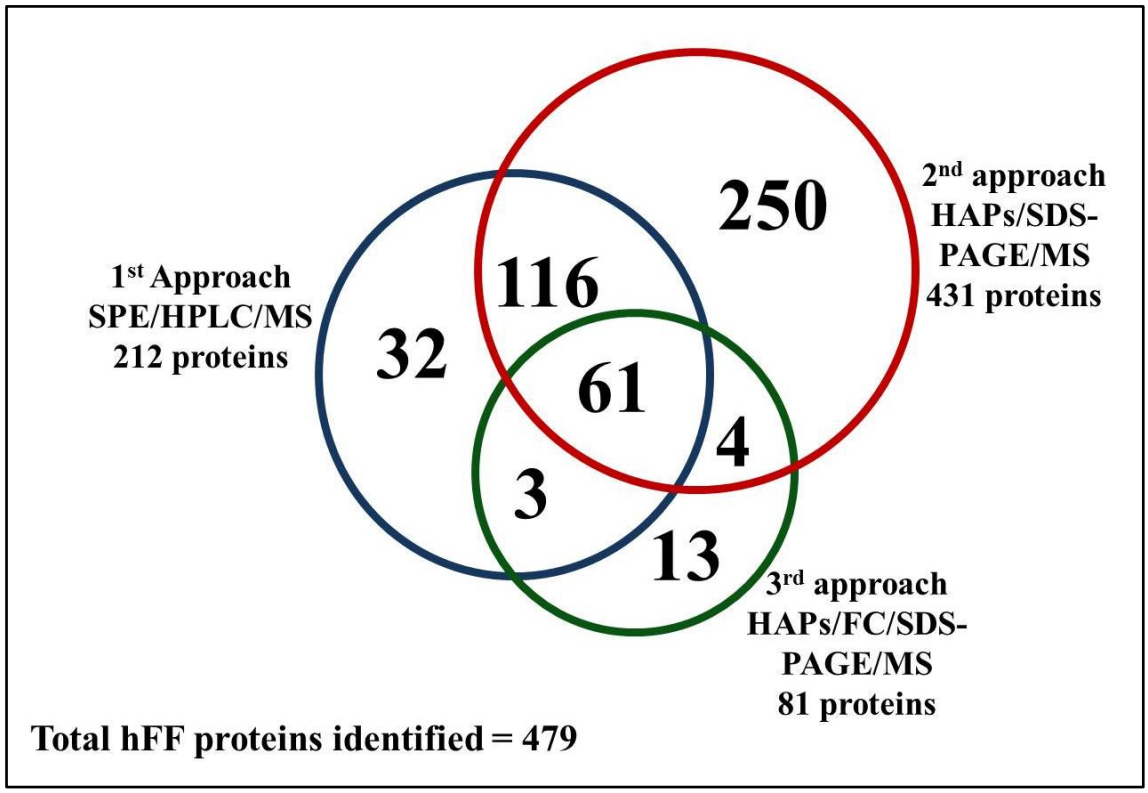


Figure 2-6 Venn diagram of the results of the three approaches compiled together with the number of proteins identified in each approach. Some proteins were only detected the crude or the HAPs depleted hFF.

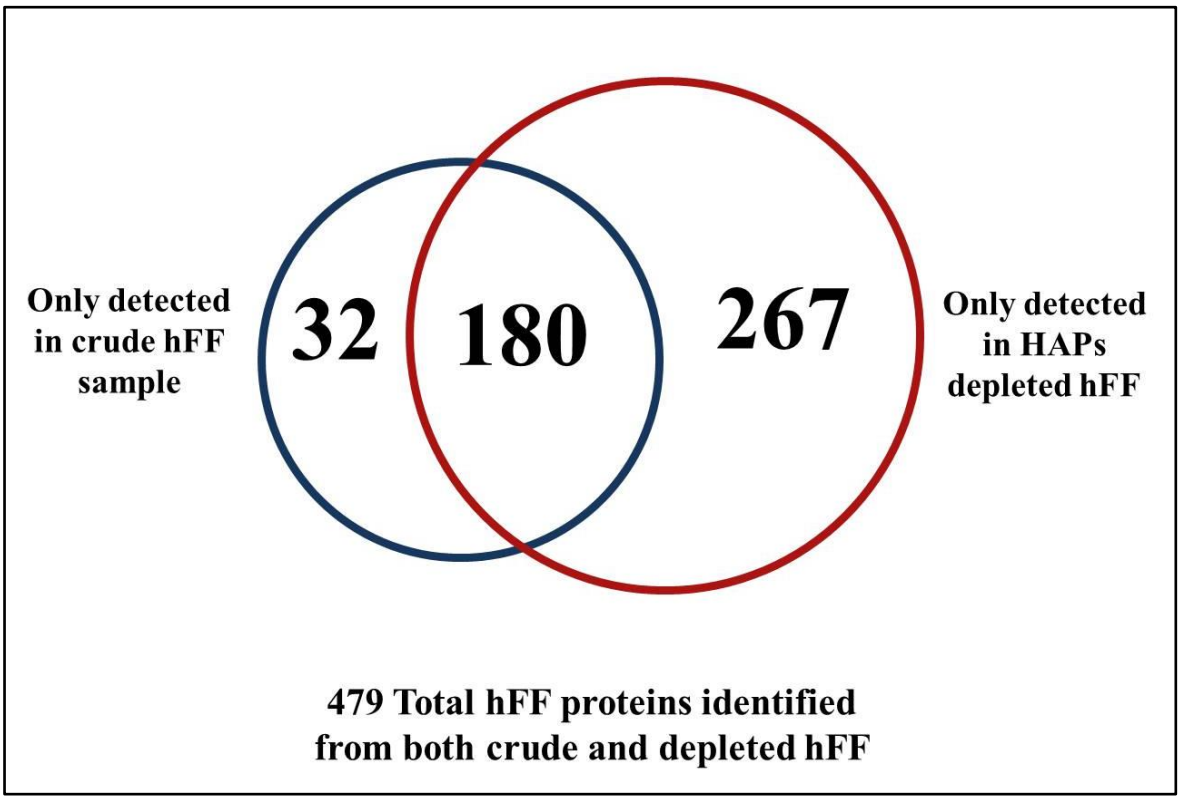


Figure 2-7 Venn diagram comparing number of proteins detected in the crude and HAPs depleted hFF in this study, 180 proteins were detected in both samples while 32 proteins were only detected only in the crude hFF and 267 proteins only detected in the HAPs depleted hFF sample.

The list of the compiled proteins detected in this study was loaded into UniProt knowledge base (http://www.uniprot.org/uploadlists/) to retrieve UniProt accession numbers for each protein. The annotation of the identified proteins with UniProt accession numbers enabled the comparison of our data with the data in the literature(an exhaustive hFF protein table from 24 hFF proteomic published studies was compiled in this work, section 2.5.1).

When the hFF protein data was compared with the hFF data compiled from the literature, 103 (approximately 22%) out of the total detected proteins were novel hFF proteins while

376 proteins (approximately 78%) have been previously reported in the hFF proteomic literature (**figure 2-8**).

Previous detection of each of the hFF proteins in this study was verified by comparison of UniProt accession numbers of the identified hFF proteins with the list of the compiled hFF proteins from the published studies. The list of the hFF proteins detected in this study are included in appendix 2.

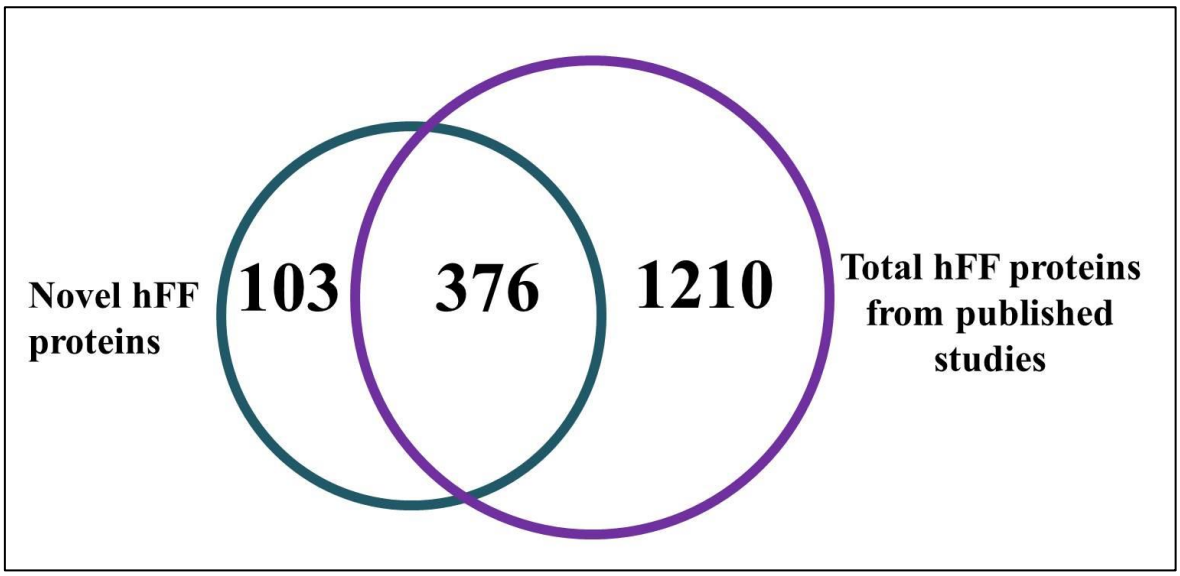


Figure 2-8 Venn diagram showing the number of hFF proteins identified in this study compared with the number of hFF proteins from the hFF proteomic literature, 103 novel hFF proteins were detected in our study.

Functional analysis of hFF proteins identified

Based on the PANTHER Classification System, 38 % of the identified hFF proteins in this study belong to the enzyme catalytic function and 29 % belong to the binding category followed by 24 % with receptor activity. Other hFF functional categories included enzyme regulation, transport activity, structural and antioxidant activities (**figure 2-9**). However,

74 proteins out of the 479 proteins loaded into PANTHER Classification System were not mapped into any specific function (unknown function).

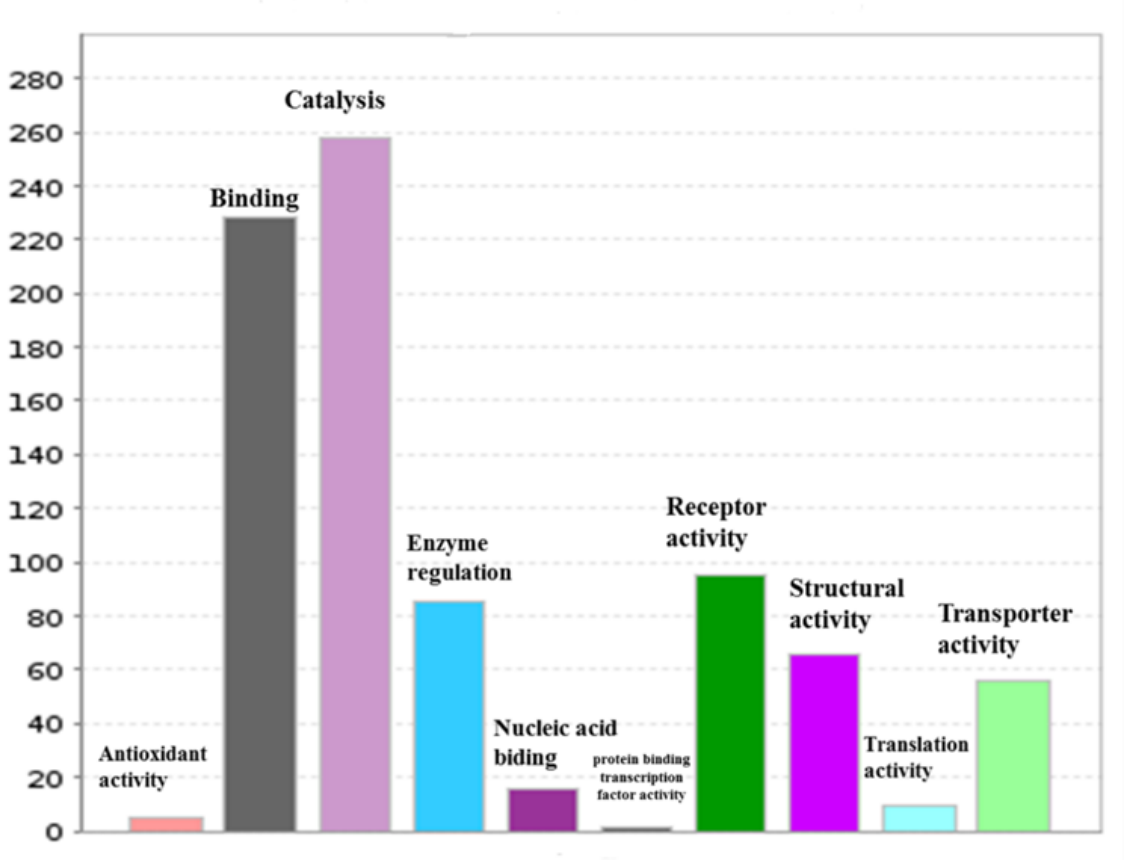


Figure 2-9 hFF protein classification according to molecular functions in PANTHER classification system; catalysis and binding were among the highest categories identified in this study followed by receptor activity and enzyme regulation.

In terms of protein classification by biological process, the majority of the hFF proteins were shown to belong to the metabolic process category (75%) followed by cellular process and bioloigical regulation (**figure 2-10**). Response to stimulus, developmental process and immune system were the next categories of hFF proteins. Most proteins were involved in more than two types of biological processes.

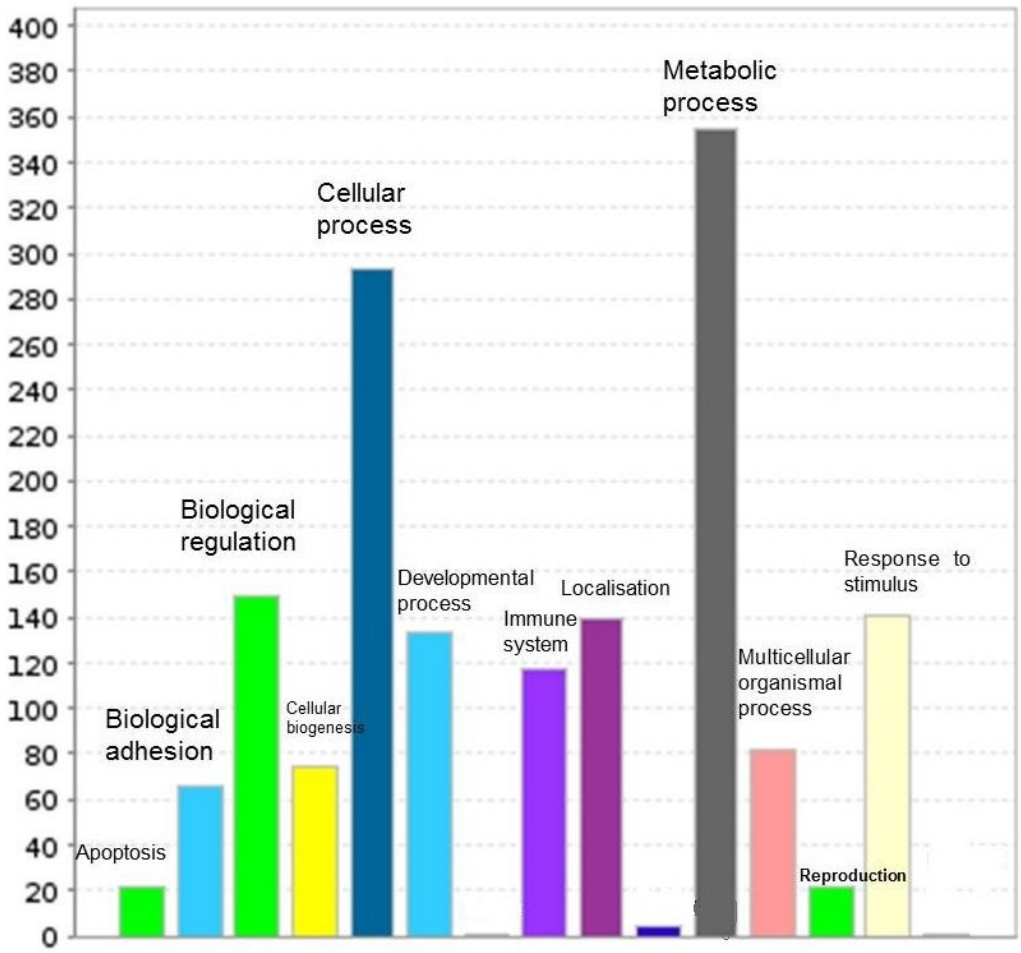


Figure 2-10 hFF protein classification according to biological process in PANTHER classification system; metabolic processes and cellular processes were among the highest categories followed by biological regulation

On the basis of cellular localisation, the majority of the identified hFF proteins in this study were extracellular proteins followed by cell part (**figure 2-11**). The next localisations of the identified hFF proteins were in organelles, extracelluar matrix and macromolucular complex (which is defined in PANTHER Classification System as a stable assembly of two or more constituents (or macromolecules), for example, proteins, nucleic acids, carbohydrates or lipids to function together).

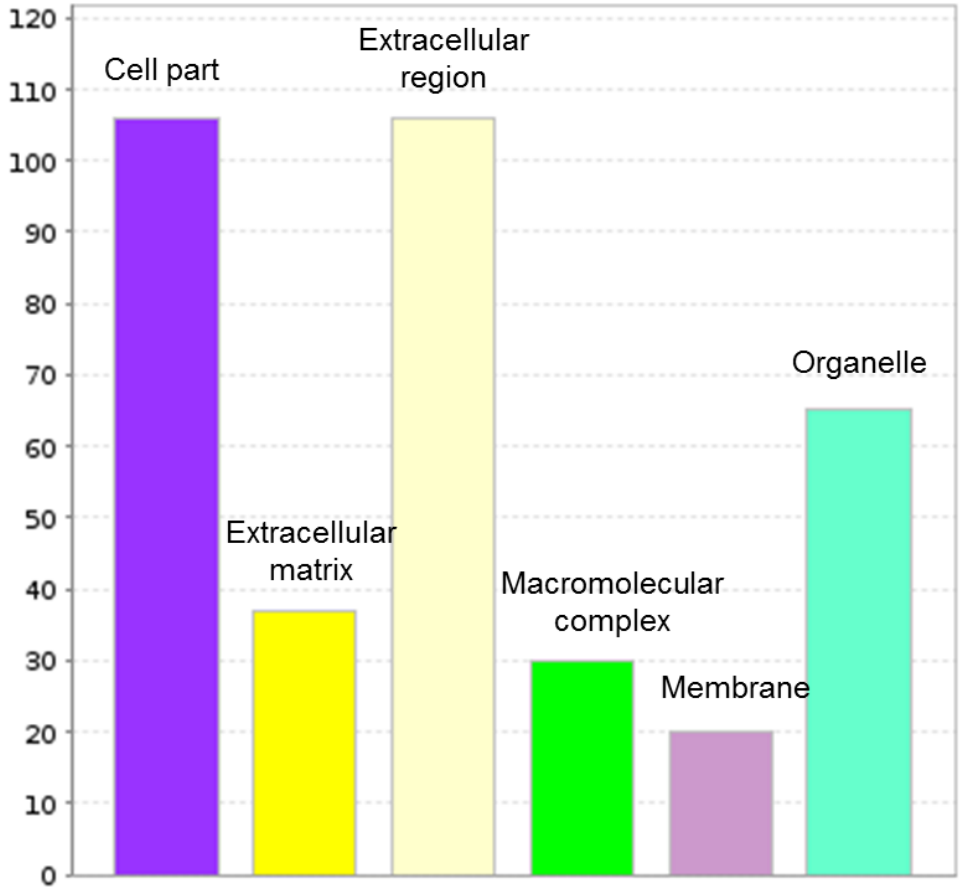


Figure 2-11 hFF protein classification according to cellular localisation in PANTHER classification system, extracellular region and cell part were among the highest categories followed by organelle and extracellular matrix

Novel hFF proteins:

One hundred and three novel hFF proteins have been detected in the current study. Some of the novel hFF proteins identified in this study are members of protein families with the other members being identified in previous hFF proteomic studies. For example, three types of annexins have been detected in the hFF in this study, one of them was annexin A1,a novel hFF protein. In the literature, annexins A2, A5, A6 (Zamah, et al, 2015), annexin A4 (Angelucci et al, 2006) and annexin A10 (Ambekar et al, 2014) have been detected in the hFF. Annexins are protein family with different biological functions

(Takagi et al., 2002). Annexin A1 is a calcium-dependent phospholipid binding protein which is involved in several cellular functions mainly anti-inflammatory response and apoptotic functions (Flower and Rothwell, 1994). Annexin A1 has been shown to be expressed in syncytiotrophoblast of the placenta (Sun et al., 1996) and to be elevated in plasma of women with pre-eclampsia (Perucci et al., 2015). Further functions of this protein in hFF may require further research.

Four enzymes which belong to the protein disulphide isomerases (PDI) family have been detected in this study which includes PDIA1, PDIA3, PDIA4 and PDIA6 members. PDIA1 (Zamah, et al, 2015) and PDIA3 (Angelucci et al, 2006; Zamah et al, 2015) have already been identified in hFF while PDIA4 and PDIA6 were detected for the first time in this study. This makes 4 members of the PDIs family identified in hFF. PDIs enzymes are involved in the catalysis of thiol/disulphide reactions (Wilkinson and Gilbert, 2004) and participate in several cellular functions (Turano et al., 2002).

Up to date 15 SERPINs (SERpin Peptidase INhibitors) have been identified in hFF (Ambekar et al., 2013, Bayasula et al., 2013, Kushnir et al., 2012) namely SERPIN A1, A3, A4, A5, A6, A7, A8, A10, C1, D1, E1, E2, F1, F2, G1. Two more SERPINs have been identified in this study namely SERPIN B5 and B6 (serpin peptidase inhibitor clade B, member 5 and member 6) making 17 SERPINs in hFF. The functions of SERPINs range from coagulation, fibrinolysis and cellular differentiation and they have been suggested to be vital for ovarian follicle maturity and ovulation (Ambekar et al., 2013).

Interestingly, some of the published studies have listed keratins as components of hFF proteome (Ambekar et al, 2013), however, in the current study they were considered as a potential contamination from hair and dust and were only included in the final analysis when they were found in the hFF protein data from the literature.

There are clearly many other proteins some of which may have key roles in fertility; however, it is out of the scope of this work to include all of them. The functional analysis of the hFF protein data may highlight the general function of the identified hFF proteins and their localisation.

2.7 Discussion

This study identified a total of 479 distinct proteins adding 103 novel proteins to the hFF repertoire and confirming 376 proteins of the previously reported hFF proteins in the literature (**figure 2-8**). hFF protein profiling was achieved by a combination of proteomics approaches in an attempt to overcome the challenges of the high dynamic range of hFF proteins (Bianchi et al, 2013) which is defined as the variation in the abundance between the high abundance detectable proteins and the lowest abundance detectable proteins in a biological samples (Brewis and Gadella, 2010). In proteomic studies, it is a well documented challenge to detect the lower abundance proteins, which generally have high biological functional relevance (Brewis and Gadella, 2010).

Discussion of the methodolgical choices, strengths and limitations:

The application of a combination of proteomic techniques to study hFF is highly valuable to enable the detection of large number of variety of proteins (Twigt et al, 2012) and also to overcome the limitation of one technique by the advantages of the others.

In the first approach, a technique of protein fractionation and peptide purification using SPE of crude hFF samples by DSC 18 spin columns was used. One of the advantages of the use of crude hFF in this approach was to have an overall view of the hFF with a minimum sample manipulation and to maintain the physiological and biochemical nature of the hFF (Bianchi et al, 2013). SPE has the advantages of being a rapid and selective sample preparation technique which enhances peptides detection for the MS analysis

(Martínez-Maqueda et al., 2013). Protein fractionation of highly complex biological samples is suggested as a vital procedure to reduce sample complexity prior to subsequent proteomic analysis (Josic and Clifton, 2007). One of the reasons to apply the SPE technique in this study was to tackle the challenge of high complexity of the hFF protein composition. Coupled with HPLC/MS, the SPE using DSC18 SC enabled the detection of 212 proteins in a crude none depleted sample of hFF of which 30 proteins were novel hFF proteins.

Although advantageous, the use of crude none depleted hFF may create challenges in the detection of Lower Abundance Proteins (LAPs) (Fahiminiya et al., 2011a). Research into hFF proteomics is mostly interested in the identification of candidate biomarkers which are generally LAPs rather than the HAPs which are considered as common non-specific proteins (Fahiminiya et al., 2011), (see also section 1.6.2 for further detail).

Since HAPs are usually described as housekeeping proteins (Gorg et al., 2004) and their presence may mask the detection of LAPs on SDS-PAGE (Fahiminiya et al., 2011a), the second and third approaches in this study applied HAPs depletion of the hFF prior to SDS-PAGE/MS. The technique of HAPs depletion has been recommended for hFF proteomics mainly to overcome the high complexity of hFF protein composition (Jarkovska et al., 2010, Ambekar et al., 2013). Some studies have applied the technique of HAPs depletion successfully on human FF (Jarkovska et al., 2010, Ambekar et al., 2013, Zamah et al., 2015) and animals such as mare FF (Fahiminiya et al., 2011b) to enhance low and medium abundance proteins identification. In this study, a total of 447 proteins were detected following HAPs depletion of hFF samples in two sets of experiments (2^{nd} and 3^{rd} approaches). 267 hFF proteins out of the total proteins identified were only detected in the

depleted hFF samples and were not detected in the crude hFF sample prepared by the SPE approach. The 2nd and 3rd approaches with HAPs depletion enabled the detection of 107 novel hFF proteins (of which 15 proteins were also detected in the crude hFF sample).

However, similar to other techniques, HAPs depletion strategy has some drawbacks. Although the HAPs depletion technique is designed to target 12 of the mostly abundant proteins in a biological sample (the targeted 12 HAPs are listed in section 1.6.2), the depletion process could be non-specific (Zhou et al., 2004). For instance, some studies indicated that the process of proteins elimination during HAPs depletion carries the risk of loss of potential candidate proteins which were bound to the depleted proteins and also eliminated none specifically during the HAPs depletion (Jacobs et al., 2005, Bayasula et al,, 2013). This would be supported by our observations where SPE technique using the crude none depleted hFF has detected a series of proteins (32 proteins) not identified in the HAPs depleted samples. The most likely cause being that these proteins were also depleted, non-specifically, during sample treatment. This is described in the literature as the sponge phenomenon (Zhou et al., 2004) in which albumin and other HAPs may bind other fragments of LAPs and lead to some LAPs loss during the depletion process.

However, the IgY-12 SCs used in this study, have been shown as selective and a robust technique for biological fluids analysis (Corrigan et al., 2011, Qian et al., 2008). The high specificity of the column is attributed to the avian nature of the polyclonal IgY antibodies of the column which provides very specific binding and clean capture compared to mammalian antibodies (Corrigan et al, 2011, Qian et al, 2008). It has also been observed that only very few non-specific proteins loss occur following the use of the IgY-12 SCs (Carrigan et al, 2011).

In the current study, out of the 12 HAPs targeted by the IgY-12 SCs five were removed completely (not detected following depletion), while seven proteins were still detectable following the depletion cycle. For example, albumin, apolipoprotein AI and II were still detected in the depleted hFF samples in this study although they were targeted by the depletion column. This observation is similar to work by others, for example (Jarkovska et al, 2010, Ambekar et al, 2013). In some studies which applied the HAPs depletion on hFF, application of more than one depletion cycle was performed to ensure complete depletion of HAPs (Ambekar et al., 2013). Only one depletion cycle was performed in the current study in both the 2nd and 3rd approaches. However, further enhancement of the depletion technique was achieved in the third approach by the application of Amicon Ultra centrifugal filter units for concentrating the depleted hFF samples and to increase LAPs detection. The use of the centrifugal filter units enabled the concentration of hFF sample by approximately 15-20 times (that of the previously depleted hFF) to run on SDS-PAGE. 81 proteins were detected following the use of the third approach of which 13 proteins were only detected in the depleted concentrated hFF and 4 of them were novel hFF proteins. However, a recognised issue with the use of the filtration/concentration technique is the risk of increased sample loss due to increased samples manipulation (Jacobs et al., 2005). This was prominent when only 81 proteins were detected in the HAPs depleted/concentrated sample compared to 431 in the depleted sample without concentration/filtration.

In this study, 180 proteins were identified in both depleted and none depleted hFF samples. However, 32 proteins out of the total 479 were only detected in the crude none depleted hff sample which may indicate that some of these proteins were removed, non-specifically, by the depletion technique as highlighted earlier. Additionally, the role of certain proteins can

be overlooked when considering only the depleted hFF samples, for example albumin, which is one of the HAPs, has been shown to play a role in oocyte protection against oxidative damage (Otsuki et al., 2012). Conversely, 267 proteins out of the total 479 hFF detected in this study were only detected in the depleted hFF samples following HAPs depletion which may also indicate the advantages of the application of HAPs depletion in hFF proteomic studies along with other techniques for enhanced coverage of the protein range in hFF.

Both depleted and depleted concentrated hFF samples from the 2nd and 3rd approaches were run on SDS-PAGE for proteins separation. SDS-PAGE along with MS are considered the workhorse in proteomic studies particularly in investigating highly complicated body fluids such as hFF (Gorg et al., 2004, Bianchi et al., 2013). The application of MS in hFF proteomic studies has resulted in enormous advancements in the detection of proteins in hFF and their potential importance for reproduction (Hanrieder et al, 2008). Specific peptide peak patterns detected by MALDI-TOF MS have been described as potential biomarkers for follicular development and maturity (Liu et al., 2007).

The application of SDS-PAGE in hFF proteomic analysis was described for the first time by the pioneering work of Spitzer et al (1996). Advances in proteomic approaches of hFF analysis have been evolving progressively since then and some disadvantages have been linked to the use of SDS-PAGE (Baker et al, 2007). For example, although the gel lanes are sliced into approximately 1mm pieces, yet each slice may contain up to 40 proteins leading potentially to difficulty in identifying all the proteins present (Baker et al., 2007). Another issue is the risk of contamination with keratin from dust and hair. In this study,

contamination was minimised by following certain precautions such as preparation of the samples in positive pressure hoods, use of gloves, clean tubes and high purity chemicals to avoid possible contamination of the gels while running hFF samples.

Discussion of functional hFF proteins analysis:

The functional classification of the hFF proteins differed from one study to another in the literature according to the range of proteins identified in the study data. For example, some studies have described acute phase proteins as the major category in hFF proteins and defined ovulation as an inflammatory process (Anahory et al., 2002; Lee et al., 2005; Angelucci et al., 2006; Hanrieder et al., 2008). In contrast, other later studies manifested that other functions such as complement cascade, blood coagulation and angiogenesis are also involved in hFF protein functions (Jarkovska et al, 2010; Bianchi et al, 2013) adding another definition to ovulation as a hormone-induced injury (Murdoch et al., 2010) because of the involved coagulation and tissue restoration. In the current study, majority of hFF protein were metabolic proteins with catalytic enzyme activity, binding proteins followed by receptor activity are the major categories.

With regards to cellular localisation analysis of hFF in this study, the majority of the identified hFF proteins were extracellular followed by proteins from cell parts. These findings are similar to the classification results obtained by Ambekar et al (2013) of hFF proteins using HPRD (Human Protein Reference Database). The presence of proteins from the cell part and organelle specific proteins (intracellular proteins) has been suggested as a result of cellular apoptosis and epithelial shedding during follicular growth (Ambekar et al., 2013, Markstrom et al., 2002).

Limitations of the study around number of samples analysed:

In addition to the above discussed limitations, such as non-specific loss of LAPs using HAPs depletion, there were some limitations in this study in terms of number of pooled hFF samples. The hFF proteomic analysis in this study was performed on a pooled hFF sample from three healthy donors with positive pregnancy outcomes and comparable follicle maturity (follicle diameter 16-18 mm) only seeking fertility treatment for male factor infertility and unexplained infertility. Most proteomic studies utilise pooled samples of hFF from at least two or more donors to increase hFF protein coverage (Twigt et al, 2012). In this study, the hFF sample was pooled from three donors which again may result in limited analysis of biological variations. Since hFF may be considered as a by-product of IVF (Bianchi et al, 2013), future studies may consider pooling hFF samples from larger numbers of patient, or analysing many samples individually to ensure enriched proteomic analysis of biological variation (Ambekar et al, 2014).

In the current study, hFF was obtained following controlled ovarian stimulation. A study which examined the variation in hFF protein profile from 6 women with natural cycles versus 6 women undergoing ovarian stimulation suggested increased immune and inflammatory response with eight proteins being differentially expressed in the ovarian stimulation group (Wu et al, 2015). However, the authors indicated that the results of their study are preliminary and large-scale studies are required to reveal any differences in the protein profile from women with natural cycle versus patients with induced cycles (Wu et al, 2015).

hFF biomarker detection and reproductive biology applications:

It is beyond the scope of this work to list all the potential biomarkers in the hFF proteins detected up to date, however, this section highlights some of the important aspects of hFF

protein applications in reproduction. One of the advantages of the application of hFF as a source of biomarkers is its easy collection in a non-invasive way as it is described as a by-product during the IVF procedure (Bianchi et al., 2013). The criteria of an ideal biomarker are non-invasive collection (Liu et al., 2007, Kovac et al., 2013), sensitivity, specificity and identification of disorders at an initial stage when diagnosis and appropriate management are more advantageous for patients (Kovac et al., 2013, Liu et al., 2007). Biomarkers for diagnosis and prognosis of ovarian hyperstimulation syndrome especially in its severe form have been suggested in hFF (Moos et al., 2009, Jarkovska et al., 2011).

As a result of the growing trend toward single embryo transfer, the identification of the highest quality oocyte for selection in ART has become highly critical (Bianchi et al., 2013). Some correlation between hFF contents of several substances and oocyte maturity and folliculogenseis have been indicated in the literature (Kawano et al., 2004, Ocal et al., 2004, Lee et al., 2005, Wu et al., 2007). Nonetheless, the role of several proteins in the characterization of follicle growth and oocyte quality is still elusive (Revelli etal., 2009). **Table 2-3** lists some of the hFF biomarkers described in the literature and compiled in this study. For a review of the suggested FF biomarkers for oocyte quality see Revelli et al. (2009).

Table 2-3 hFF biomarkers described in the literature

Applications	Potential hFF biomarkers	References
PCOS and pre-eclampsia	Apo lipoprotein A IV	(Kim et al., 2013)
oocyte maturity and folliculogenseis	Macrophage inflammatory protein-3alpha, inhibins A and B, follistatin, activin, transforming growth factor beta family and bone morphogenetic protein 15 (BMP15)	(Kawano, 2004, Ocal, 2004, Lee, 2005, Wu, 2007)
IVF success	Haptoglobin alpha, apolipoprotein H, dihydrolipoyl dehydrogenase, lyzozyme C, fibrinogen alpha-chain, and immunoglobulin heavy chain V-III (region BRO), antithrombin, vitamin D–binding protein, and complement C3 11 β- hydroxyl steroid dehydrogenase	(Estes, et al, 2009) (Michael et al., 1993)
Recurrent spontaneous miscarriage	Fibrinogen γ	(Kim et al., 2006)
Predictor of IVF outcome	Sex hormone binding globulin (SHBG), corticosteroid binding globulins (CBG) Cytokines such as IL8 and IL18	(Andersen, 1990) (Sarapik et al., 2012)

Despite the above mentioned advantages of hFF as a source of biomarkers for oocyte quality and other reproductive biology applications, hFF is obtained as a by-product of ART (Bianchi et al, 2013). In future research, the identification of good biomarkers in hFF may demand further studies to attempt detecting signs of the identified biomarkers in other body fluids such as blood or urine. This may enable the application of the diagnostic biomarkers for women with natural, none stimulated ovulatory cycle.

Concluding remarks:

This study adds further novel hFF proteins to the repertoire and confirms some of the previously described hFF proteins. The compiled hFF protein data from the literature in this study is a resource for future reference of the currently available hFF protein data. Enhanced understanding of protein components of hFF is vital for further research into biomarkers for oocyte quality, understanding of the fertilisation environment and potential improvements in ART.

Chapter 3

COMPARATIVE PROTEOMIC STUDY OF SPERM VERSUS SPERM AND HUMAN FOLLICULAR FLUID INTERACTIONS

3.0 Abstract:

Sperm are transcriptionally and translationally inactive and they rely markedly on proteins acquisition/loss and post-translational modification to achieve functional competence. hFF has been shown to provide a favourable media for sperm longevity, capacitation, and acrosome reaction. Aim: to perform a comparative proteomic analysis and identify any hFF proteins which may bind to sperm and may be linked to sperm acquisition of fertilisation capability. Method: sperm cells were prepared by Percoll gradient centrifugation and sperm were either left to capacitate in standard ART media alone (control sperm samples) or capacitated in the media with hFF for 90-120 minutes to allow for capacitation and any protein binding to occur. Both control sample and sperm + hFF preparations were processed using two approaches, total binding and membrane-associated protein approach. In both approaches, two cycles of washing were performed for both sample types (to remove any unbound hFF fractions in sperm + hFF samples). In the total binding approach, the pellet part for both samples (representing total binding fractions in sperm + hFF samples) were run on SDS-PAGE and analysed by MS. In the membrane-associated approach, control and sperm + hFF samples were further incubated in 2 M NaCL and Tris-base pH 10.6 for 40-60 minutes and centrifuged for 5 minutes. The supernatant part (representing the sperm + hFF membrane-associated proteins) was run on SDS-PAGE and analysed by MS. Results: comparative data analysis of control versus sperm + hFF samples from both approaches revealed 24 hFF proteins in the sperm + hFF samples. Functional analysis revealed that sperm-bound hFF proteins have lipid transport and antioxidant activities which may suggest their role in cholesterol efflux during capacitation and sperm protection against oxidative damage. Future studies are recommended to illustrate the roles of the sperm-bound hFF proteins in fertilisation.

3.1 Introduction:

Sperm have been shown to only acquire motility and fertilising potential following their maturation in the epididymis (Cornwall, 2014) and capacitation in the FRT (Chang, 1951, Austin, 1951). Following these distinctive maturational processes (chapter 1, sections 1.1.3 and 1.4.1), testicular sperm, which are immotile and unable to fertilise oocytes acquire their fertilisation potential (Cornwall, 2009). Remarkably, both processes of sperm maturation in the epididymis and capacitation in FRT occur despite the lack of de novo protein synthesis in sperm cells (Baker, 2016). Sperm cells are transcriptionally and translationally inactive (Miller et al., 2010, Oliva and Castillo, 2011), however, their protein profile undergoes dynamic changes which are highly attributed to PTM and proteins loss/acquisition (Samanta et al, 2016).

During the epididymal transit, cysteine-rich secretory protein1 (CRISP1) is one of the important proteins secreted by the epididymal epithelium (Cameo and Blaquier, 1976) and acquired by the sperm (Kohane et al., 1980, Cohen et al., 2000). CRISP1 has been suggested as a decapacitation factor when loosely associated with the sperm surface (Roberts et al., 2003, Cohen et al., 2000, Kohane et al., 1980) whereas the strongly bound CRISP1 has been linked to sperm-ZP binding and sperm-oocyte fusion (Ernesto et al., 2015).

In their journey toward the ampulla, sperm are exposed to several variable environments including FRT's epithelial lining and different types of fluid and secretions (Zhu et al., 1994, De Jonge, 2005, Suarez and Pacey, 2006, Lambert et al., 1985). However, the knowledge of molecular events facilitating sperm's acquirement of fertilisation capability in FRT is still limited (Barratt et al., 2011). Several studies have indicated the contribution of proteins in the oviductal microenvironment in the modulation of sperm function

(Munuce et al., 2009, Zumoffen et al., 2015, Zumoffen et al., 2010). Sperm incubated with human oviductal-tissue conditioned medium with high protein contents, mainly lactoferrin, were shown to inhibit sperm capacitation (Zumoffen et al, 2010, Zumoffen et al, 2015). The authors suggested that this effect of oviductal proteins may broaden the capacitation window and thereby extend the lifespan of sperm in the oviduct (Zumoffen et al, 2015). In ram, some oviductal proteins including heat shock protein 8 (HSPA8) and soluble apical plasma membrane proteins (sAPM) extracted from the oviductal epithelial cells have been shown to enhance sperm survival for up to 48 hours (Lloyd et al., 2009).

hFF is also considered as a rich source of steroid hormones mainly progesterone, proteins and other factors such as atrial natriuretic peptide (Revelli et al., 2009) important for sperm function and fertilisation capability (Emiliozzi et al., 1996, De Jonge, 2005). hFF is produced during folliculogenesis by GCs of the developing ovarian follicle (Fortune, 1994). At ovulation, hFF is released along with the COC, hence hFF may travel along the oviduct and become available at the ampulla for the sperm to be exposed to on their way towards the COC (De Jonge, 2005, Hansen et al., 1991). This spread of hFF to the site of fertilisation and therefore its potential role in guiding sperm through chemotaxis (Tacconis et al., 2001) and or modulating fertilisation environment is of interest in understanding the effects of hFF on sperm functions. hFF is also a potential culture media for preservation of sperm vitality and motility (Hamamah et al., 1995, Getpook and Wirotkarun, 2007). Follicular fluid has been shown to stimulate sperm capacitation (Yao et al., 2000), hyperactivation (Mbizvo et al., 1990, Kulin et al., 1994), sperm progressive motility (Falcone et al., 1991, Getpook and Wirotkarun, 2007, Hong et al., 1993, Revelli et al., 2009), chemotaxis (Tacconis et al., 2001, Ralt et al., 1994) and acrosome reaction (Burrello et al., 2004) in several species.

Limited knowledge is available about the exact mechanism by which hFF enhances sperm function apart from the effects of progesterone (Jaiswal et al., 1999). Progesterone is one of the prominent components of both hFF and cumulus secretions (Laufer et al., 1984, Chian et al., 1999, De Jonge, 2005) and it has been shown to induce sperm chemotaxis (Villanueva-Diaz et al., 1995, Wang et al., 2001) and AR (Morales et al., 1992, Saaranen et al., 1993).

Nonetheless, not only progesterone and other steroid hormones but also proteins from hFF have been shown to affect sperm function and fertilisation (Tesarik et al., 1990, Yao et al., 2000). For instance, the high abundance of albumin, lysophosphatidylcholine (LPC) and platelet activating factor in hFF has been suggested to contribute to hFF capacitating effect by enhancing sperm membrane cholesterol efflux (Lepage et al., 1993). In other studies, sperm capacitation induced by hFF was attributed to a lipid transfer mechanism (Ravnik et al., 1992, Muller and Ravnik, 1995) involving a lipid transfer protein (LTP1) also known as cholesteryl ester transfer protein which was purified from hFF (Ravnik et al., 1992). Glycosaminoglycans, which are proteoglycans formed of protein core and covalently bound repeated disaccharide units, have also shown to affect sperm motility and fertilisation capability (Hamamah et al, 1995).

Some studies focused on four glycodelins, which are a group of glycoproteins that have a similar protein core but different glycosylation sites, and considered their effects on sperm (Yeung et al., 2006). Glycodelins have been shown to affect sperm function in different ways (**figure 3-1**) (Yeung et al., 2006). During early pregnancy, Glycodelin-A (GdA) is also suggested to protect the fetus against maternal immune response by the suppression of the cytotoxic activity of natural killer cells, which represent 70 % of the uterine leukocyte (Seppala et al., 2007).

Sperm receptors for glycodelin F (GdF) from hFF have been described as a low affinity receptor of fucosyltransferase-5 on sperm surface which binds both GdF and GdA (Chiu et al., 2003b, Chiu et al., 2007, Yeung et al., 2009). Determination of other hFF proteins that bind to sperm similar to GdF may enhance our understanding of sperm regulation induced by hFF. The identification of the hFF candidate proteins acquired by sperm may also enable the design of media to enhance sperm function and longevity.

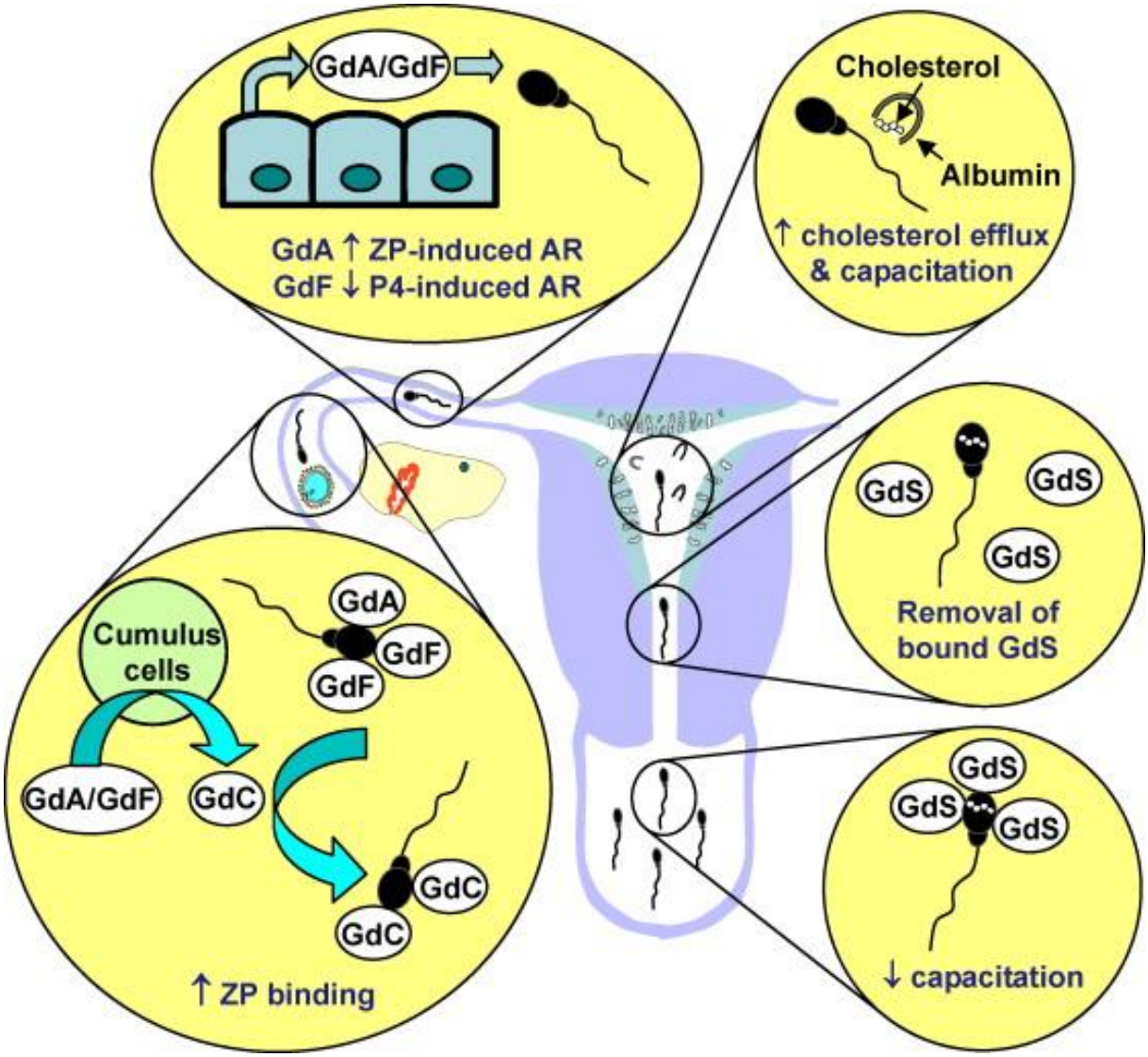


Figure 3-1 Diagrammatic representation of the roles of glycodelins in reproduction (from Yeung et al, 2009, with permission). Glycodelin S (GdS) in seminal plasma works as a de-capacitation factor which retains the ejaculated sperm in non-capacitated condition by inhibiting albumin-induced cholesterol efflux (Yeung et al., 2006; Uchida et al, 2013). Following semen deposition in the FRT, cervical mucus removes the GdS from the sperm surface and permits capacitation by enhancing cholesterol efflux (Yeung et al., 2006). GdA, found in endometrial fluid (Li et al, 1993) and GdF (from hFF) bind to sperm and GdF suppresses early onset AR induced by P4 (progesterone) and inhibits sperm zona binding while GdC, from the cumulus cells, stimulates sperm-zona (ZP) binding (Chiu et al., 2003a, Chiu et al., 2007, Yeung et al., 2006, Yeung et al., 2009, Chiu et al., 2005, Chiu et al., 2003b).

3.1.1 Human sperm and hFF proteomics:

Proteomics is defined as the systematic analysis of the whole proteins present in a cell, tissue or organism under specific conditions and at a particular time (Domon and Aebersold, 2006, Cox and Mann, 2007). The term proteome combines both words PROTEin and genOME and was introduced by Wilkins et al. (1996) as a new concept for analysis of the entire proteins expressed by a genome. Proteins form major structural and functional components of the cells and their study may provide better understanding of molecular pathways (Wilkins et al, 1996). Human follicular fluid proteome has been studied extensively in the literature and was highlighted in chapter 2. Human sperm proteome has also gained great interest in recent years with thousands of sperm proteins being identified in several studies (Baker et al., 2007, Azpiazu et al., 2014, Castillo et al., 2014, Amaral et al., 2014b). The accessibility, easy purification and lack of transcriptional and translational activity of the human sperm cells, as mentioned earlier, ensure the high suitability of these cells for proteomic studies (Oliva et al., 2009, Amaral et al., 2014a). The compiled human sperm proteome from 30 proteomic studies produced a list of 6198 unique proteins with highlights of possible metabolic pathways involved (Amaral et al, 2014a). Several approaches have been applied to study sperm proteomic such as the whole cell proteomic analysis (Baker et al., 2007, Wang et al., 2013, de Mateo et al., 2007) and the subcellular proteome analysis of sperm cell fractions. For example, sperm head, tail (Amaral et al., 2013, Baker et al., 2013), nucleus (de Mateo et al., 2011) and fibrous sheath (Kim et al, 2007) have been analysed to improve throughput of sperm proteins identification (de Mateo et al, 2011; Amaral et al, 2014a).

Prior to sperm proteomic analysis, an initial step of sperm samples purification from seminal plasm, leukocytes and round cells is important and is usually achieved using

swim-up or Percoll density gradient centrifugation (de Mateo et al., 2013, Amaral et al., 2014a). Following protein extraction, two different techniques are generally applied in human sperm proteomic analysis using mass spectrometry (de Mateo et al, 2013). The first technique involves sperm protein separation using either one or two dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE, 2D-PAGE) with subsequent in-gel proteins digestion and MS analysis using either LC-MS/MS or MALDI-MS of the generated peptides (Paiva, 2015, Amaral et al., 2014a, de Mateo et al., 2013). This approach has been shown to enhance the quality of the protein identification due to reduced complexity of the protein mixture as a result of the prior separation (Baker et al., 2007, Amaral et al., 2013, Wang et al., 2013). The high efficiency of the combined techniques of LC and MS along with enhanced throughput by prior protein separation and digestion resulted in thousands of human sperm proteins being identified in several studies using this approach (Baker et al., 2007, Baker et al, 2013, Amaral et al., 2013; Wang et al., 2013). In the second proteomic strategy, sperm proteins are directly digested into peptides with no prior separation and then the generated peptides are either separated by LC or isoelectric focusing (IEF) followed by MS analysis (de Mateo et al, 2013; Amaral et al, 2014a). The peptide/protein identification for both proteomic techniques is then achieved using a software search of peptide/protein databases (Amaral et al, 2014a).

Amaral et al (2014a) indicated that 2D-PAGE was the classic approach to separate human sperm proteins and resulted in the detection of numerous proteins (< 200) in several studies (Pixton et al., 2004, Chu et al., 2006, Martinez-Heredia et al., 2006). Recently, studies that applied LC-MS/MS for peptide detection of digested proteins with prior separation using 1D SDS-PAGE have resulted in the identification of a higher throughput of up to 400-1000 proteins (Amaral et al, 2013, Wang et al, 2013, Amaral et al, 2014a).

Nonetheless, protein digestion into peptides prior to LC-MS/MS results in inability to identify possible PTM of the detected proteins, which is considered as a drawback of this technique (Amaral et al, 2014a). **Figure 3-2** summarises the different flow options of sperm proteomic strategies.

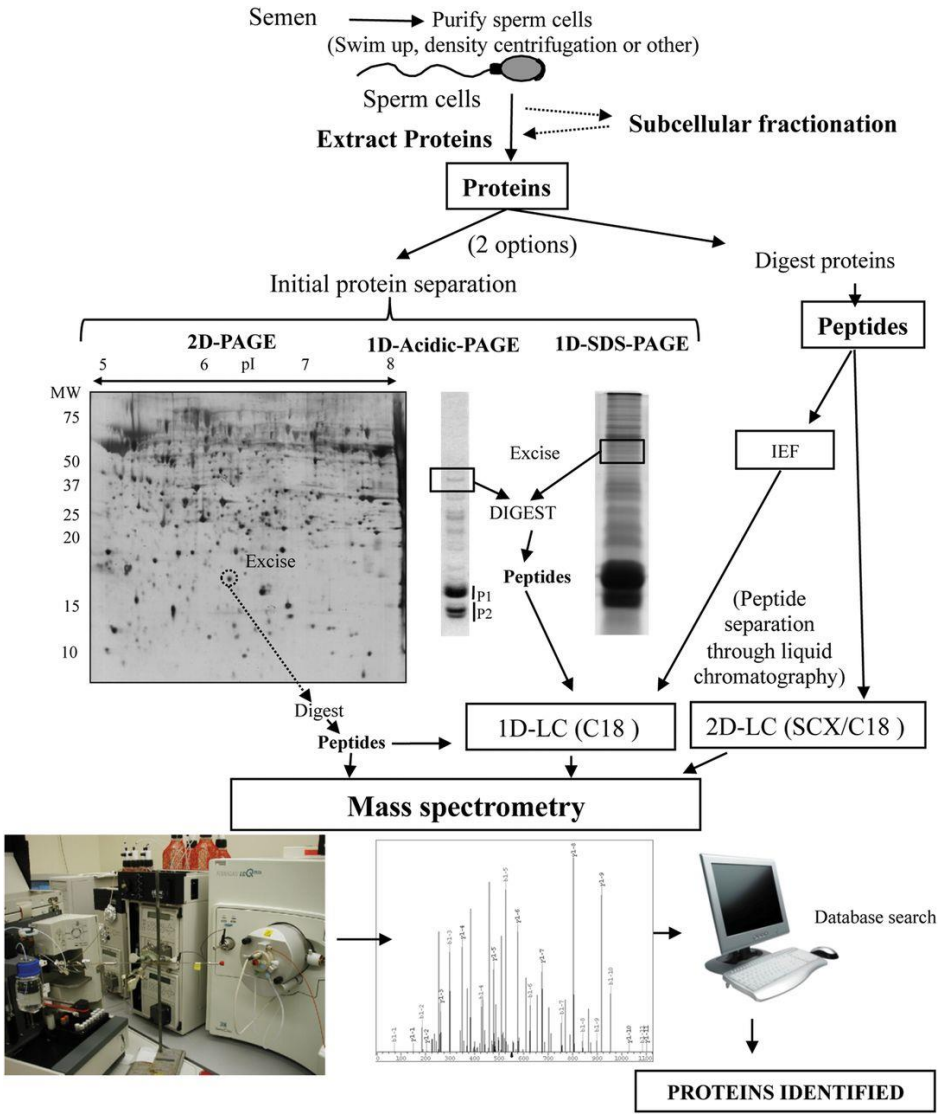


Figure 3-2 The sequential steps in different human sperm proteomic approaches starting with sperm purification using swim-up or Percoll gradient centrifugation to the final analysis of peptides using mass spectrometry (from Amaral et al, 2014a, with permission).

3.2 Objectives

To conduct a comparative proteomic analysis of sperm capacitated in capacitation media alone versus sperm capacitated in the presence hFF to identify any putative hFF which may have bound to sperm. The analysis also aims to conduct functional classification of the putative hFF proteins and investigate their possible role in sperm regulation.

3.3 Methods:

3.3.1 Materials:

Table 3-1

Product name	**Company**	**Product code**
Acetonitrile	Millipore, Watford, UK.	UN1648
Ammonium bicarbonate	Sigma, Dorset, UK	09830-1KG
Bovine serum Albumin (BSA)	Millipore, Watford, UK.	82-002-4
Dithiothreitol (DTT)	Melford Laboratories, Suffolk, UK	MB1015
Ethylene-diamine tetra-acetic acid (EDTA)	Sigma, Dorset, UK	E5134EDTA
Formic acid	Sigma, Dorset, UK	14265-50ML
Iodoacetamide	BioRad laboratory, Hertfordshire, UK	1632109
20 × MOPS SDS Running Buffer (3-(N-Morpholino) propanesulfonic acid)	Sigma, Dorset, UK.	M1254
NuPAGE Novex (10% Bis-Tris gel)	Invitrogen, Paisley, UK	NP0302Box
Percoll	Sigma, Dorset, UK.	P1644
Phpsphate Buffered Saline (PBS)	Invitrogen, Paisley, UK	14040083
Seeblue plus 2 pre-stained standard	Invitrogen, Paisley, UK	LC5925
Sodium chloride (NaCl)	Sigma, Dorset, UK.	S9888
Sodium dodecyl sulfate (SDS)	Sigma, Dorset, UK	L6026-250G
supplemented Earle's balanced Salt Solution (sEBSS)	Geneflow, Fradley, UK	06-2010-03-1B
Tris-(hydroxymethyl)aminomethane	Sigma, Dorset, UK	252859
Trypsin (sequencing grade, modified, porcine)	Promega, Southampton, UK	V511A
Urea	Sigma, Dorset, UK	U5378-5KG

3.3.2 Sperm preparation:

Semen samples were obtained by masturbation from healthy research donors at Birmingham Women's Hospital (Human Fertilisation and Embryology Authority Centre 0119; HRA NRES Ethical Approval for the Centre for Human Reproductive Science Reproductive Tissue Bank: 13/EM/0272; within the bank approval this project was reference: Application Project 5) following 2-5 days of sexual abstinence. Informed consent was obtained from all the donors and all procedures were performed in accordance to HFEA Code of Practice. Semen samples were allowed to liquefy for 30 minutes and then sperm cells were prepared by Percoll density gradient centrifugation. 100 % isotonic Percoll (285-300 mOsm/kg) solution was prepared from the original Percoll by the addition of 10 × M medium (1.37 M NaCl, 25 mM KCl, 200 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), 100 mM glucose). Two different Percoll fractions (80% and 40%) were made isotonic by the addition of 1 × M medium (137 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 10 mM glucose) (Lefievre et al., 2007). A density gradient of Percoll was prepared by under-layering 1 ml of 40 % Percoll with 1 ml 80 % Percoll and a top layer of 1 ml liquefied semen was added in a 15 ml Falcon tube and centrifuged for 20 minutes at 500 g at room temperature. The supernatant fluid was discarded and pellet part was washed with 5 ml of Phosphate Buffered Saline (PBS), centrifuged for 5 minutes at 500 g. Sperm concentration was determined by Neubauer haemocytometer and at least 400 cells were scored. Semen samples for this study were obtained from three different donors.

3.3.3 Follicular fluid collection:

See section 2.3.2 for hFF sample collection.

3.4 Methodology:

In this study, two approaches were used as follows:

3.4.1 Sperm + hFF total protein binding approach:

Following sperm preparation by Percoll gradient centrifugation, the total count of the sperm cells was adjusted to 1×10^6 cells using sEBSS + 0.3% BSA. Two different preparations were performed: control sperm samples in which sperm cells were capacitated in sEBSS + 0.3% BSA alone, total volume 50 µl and sperm + hFF samples in which sperm cells were incubated in the presence of hFF (30 % final hFF concentrations), total volume 50 µl, (n= 3).

The samples were incubated with the lids open for 90-120 minutes at 37°C, 6% CO_2 to allow sperm capacitation and for any sperm and hFF protein binding to occur. The samples were then centrifuged at 500 g for 5 minutes at room temperature and the supernatant fluid was discarded to remove any excess hFF and only minimum volume of the pellet was left in each tube (around 20 µl). The pellets were washed twice using 500 µl PBS by centrifugation at 500 g for 5 minutes to remove any unbound excess hFF. The supernatant was discarded and minimum volume of the pellet was left in each tube (around 20 µl) to run on SDS-PAGE (section 3.4.3) and then in-gel protein digestion (section 3.4.4) was carried out on the protein bands. **Figure 3-3** summarises the methodology for the sperm + hFF total binding approach.

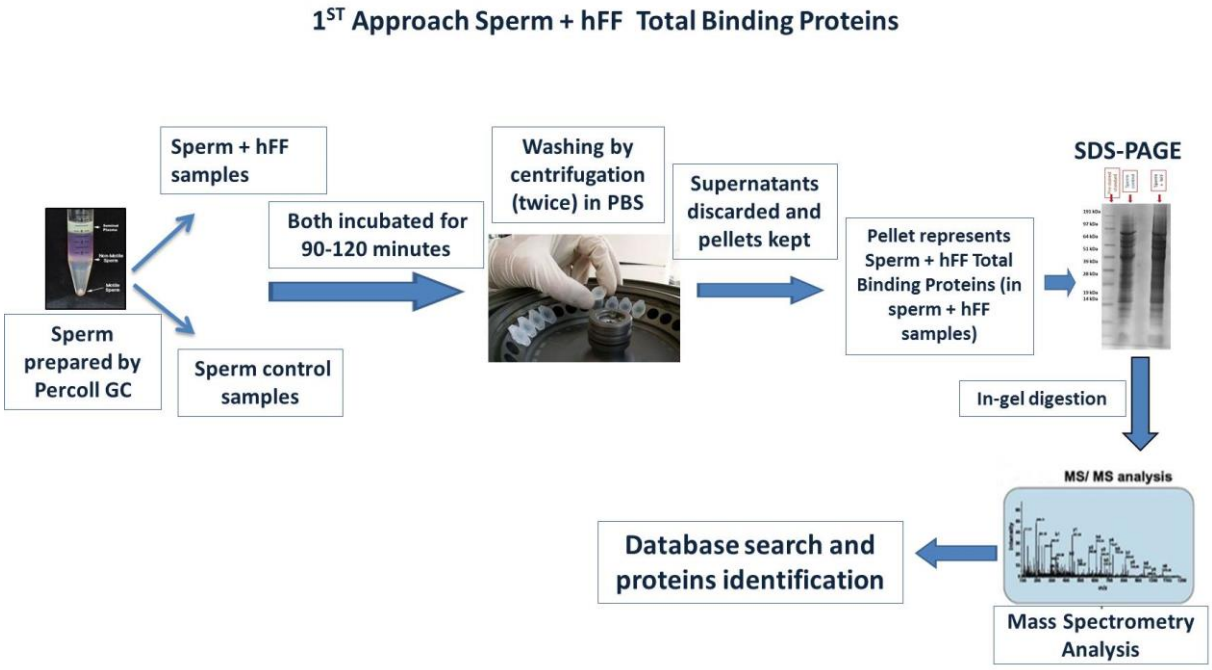


Figure 3-3 Summary of the methodology of the sperm + hFF total binding approach, sperm cells were prepared by Percoll gradient centrifugation (Percoll GC), two sets of samples prepared with and without hFF, both samples were incubated and washed prior to SDS-PAGE using only the pellet parts, SDS-PAGE/MS was then performed

3.4.2 Sperm + hFF membrane-associated proteins approach:

Similar to the total binding proteins, following sperm preparation by Percoll gradient centrifugation sperm cells total count was adjusted to 1×10^6 cell using sEBSS media + 0.3% BSA and sperm were either incubated in sEBSS containing 0.3% BSA alone as sperm control samples or in hFF (30% final concentration). Samples were incubated with the lids open for 90-120 minutes at 37°C, 6% CO_2 to capacitate and to allow time for any sperm and hFF protein binding. The samples were then centrifuged at 500 g for 5 minutes at 24°C, the supernatant fluid was discarded and only minimum volume of the pellet (around 20 µl) was left in each tube. Two cycles of washing in 500 µl PBS by centrifugation at 500 g for 5 minutes were performed for all samples. The supernatant fluid was discarded and minimum volume of the pellet was left in each tube (around 20 µl). To

achieve detection of the sperm + hFF membrane-associated protein fractions, different chemical conditions were first tested to select the appropriate method to carry on solubilisation of sperm membrane proteins along with any bound hFF.

One of the following chemical preparations was used on one pair of samples (control sperm alone and sperm + hFF) as follows:

- First preparations (EDTA): 5mM EDTA, 150mM NaCl , 20mM Tris-base pH7.5
- Second preparations (high pH):150mM NaCl, 20mM Tris-base pH 10.6
- Third preparations (high molarity salt) : 500mM NaCl, 20mM Tris-base pH7.5
- Forth preparations (heat): 150mM NaCl, 20mMTris-base pH7.5 and kept at 65° C in the block heater.

25 µl of one of these preparations was added to one pair of samples (both sperm control samples and sperm + hFF samples). The samples were then left for 40-60 minutes in the incubator at 37°C, 6% CO_2 except for the heat preparation which was kept at 65°C in the block heater for 40-60 minutes. Each tube was then centrifuged at 500 g for 5 minutes at room temperature and the supernatant fluid (which represent the sperm + hFF membrane-associated proteins in sperm + hFF samples) from each tube was collected and run on SDS-PAGE and the protein bands were compared from the different preparation techniques. The high pH (second preparation) and the high molarity NaCl (third preparation) showed darker protein bands on the SDS-PAGE compared to the other preparations. Therefore, the next step was to carry out a salt concentration curve experiment to determine the appropriate NaCl molarity and pH to use for the detection of sperm + hFF membrane-associated proteins. In the NaCl concentration curve experiment, samples preparations followed the same protocol above until the stage where the different chemical preparations were used. In this experiment, three different molarities of NaCl

prepared with two different pHs (Tris-base pH 7.5 and pH 10.6) were used to determine the appropriate salt molarity and pH for the membrane-associated approach. The NaCl molarities and pH used were as follow:

- 750 mM NaCl Tris-base pH 7.5
- 750 mM NaCl Tris-base pH 10.6
- 1M NaCl Tris-base pH 7.5
- 1M NaCl Tris-base pH 10.6
- 2M NaCl Tris-base pH 7.5
- 2M NaCl Tris-base pH 10.6

25 µl of one of these preparations was added to one pair of samples (both sperm control samples and sperm + hFF samples). The samples were then left for 40-60 minutes in the incubator at 37°C, 6% CO_2. Each tube was then centrifuged at 500 g for 5 minutes at room temperature and the supernatant fluid (which represent the sperm + hFF membrane-associated proteins in sperm + hFF samples) from each tube was collected and run on SDS-PAGE and the protein bands were compared from the different preparation techniques. From this experiment, the 2M NaCl in Tris-base pH10.6 preparation produced the darkest protein bands in the SDS-PAGE (**figure 3-4**) and thus this preparation was used in the sperm + hFF membrane-associated proteins experiment throughout the rest of the experiments (n=4).

The supernatant fluids obtained in these experiments were run on SDS-PAGE and then in-gel protein digestion was carried out on the protein bands. **Figure 3-5** summarises the methodology for the membrane-associated proteins approach.

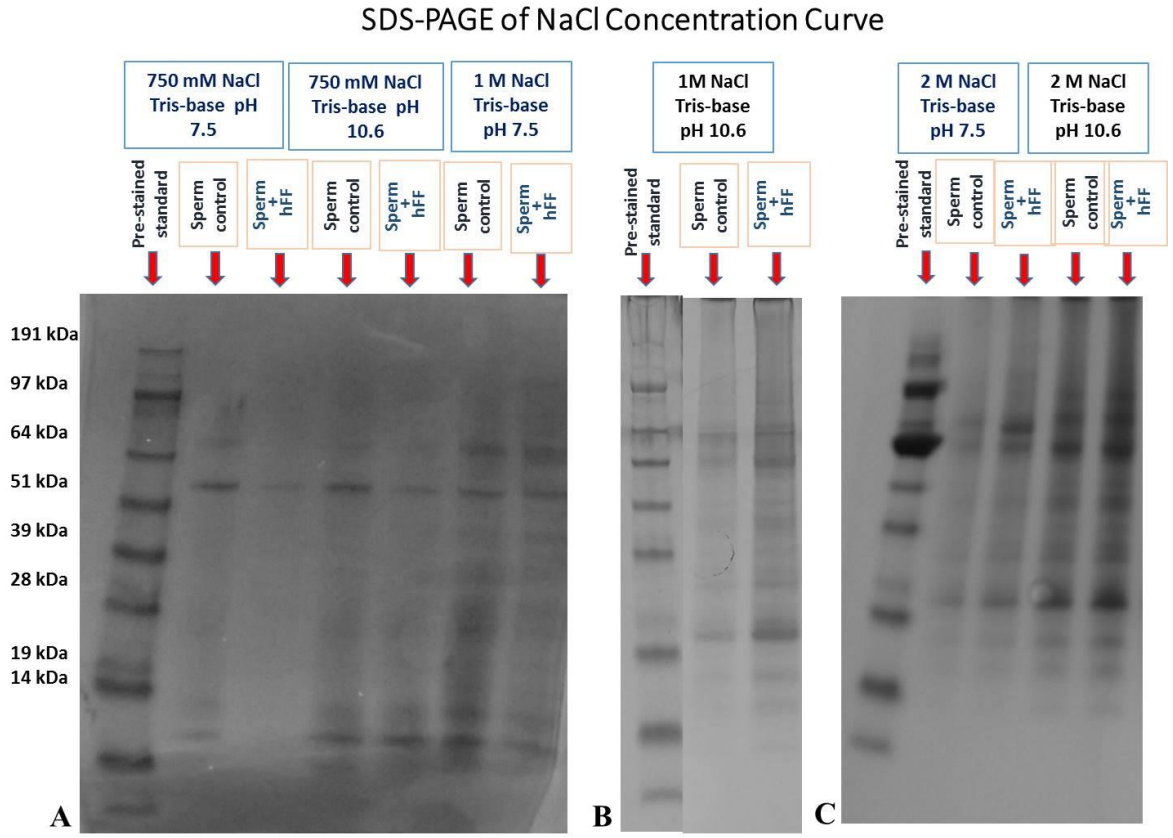


Figure 3-4 NaCl concentration curve for membrane-associated approach. NaCl molarity and Tris-base pH in these experiments were as follow: **A-** 750 mM NaCl/Tris-base pH 7.5, 750 mM NaCl/Tris-base pH 10.6 and 1M NaCl/Tris-base pH 7.5 (samples were prepared from the same ejaculate from one donor). **B-** 1 M NaCl/Tris-base pH 10.6, and **C-** 2 M NaCl/Tris-base pH 7.5, 2 M NaCl/Tris-base pH 10.6 (the two gels in **B** and **C** were prepared from two ejaculates from the same donor) .

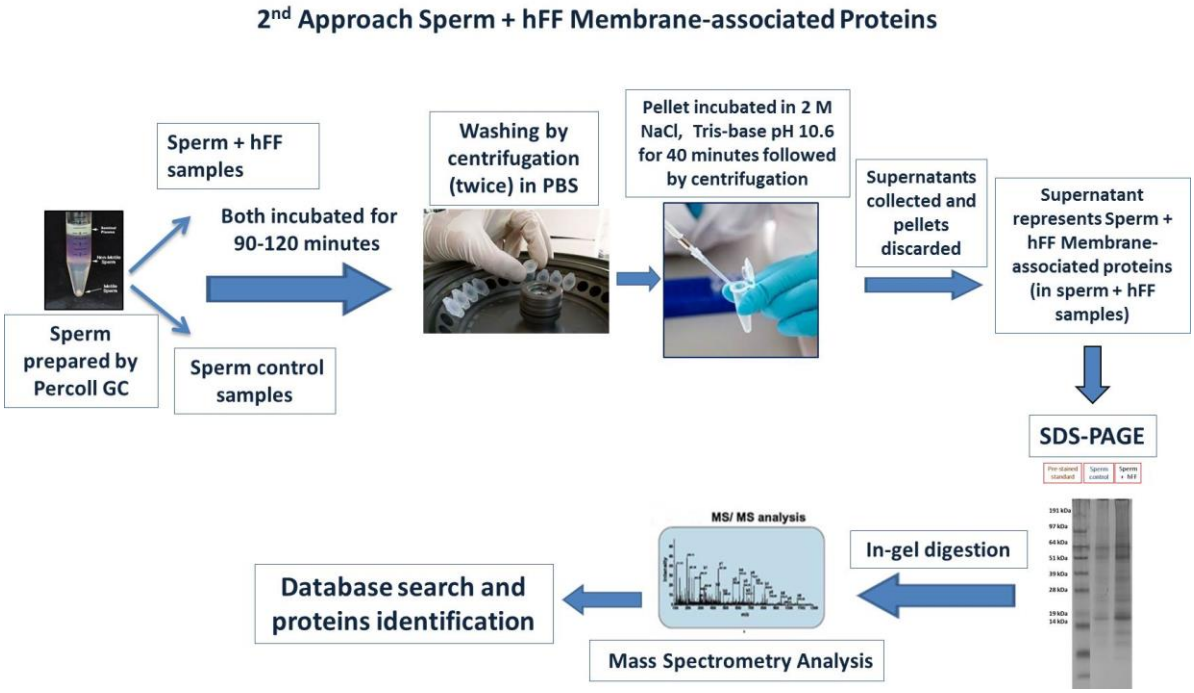


Figure 3-5 Summary of the methodology of sperm + hFF membrane-associated proteins; sperm cells were prepared by Percoll gradient centrifugation (Percoll GC), two sets of samples prepared with and without hFF, both samples were incubated, washed and the pellet parts were further incubated in 2M NaCl prior to SDS-PAGE using only the supernatant parts, SDS-PAGE/MS was then performed.

3.4.3 SDS -PAGE:

The same SDS-PAGE protocol was used for both total binding and membrane-associated approaches. For details of SDS-PAGE methodology, see section 2.4.2.2. **Figure 3-6** shows the SDS-PAGE for both approaches.

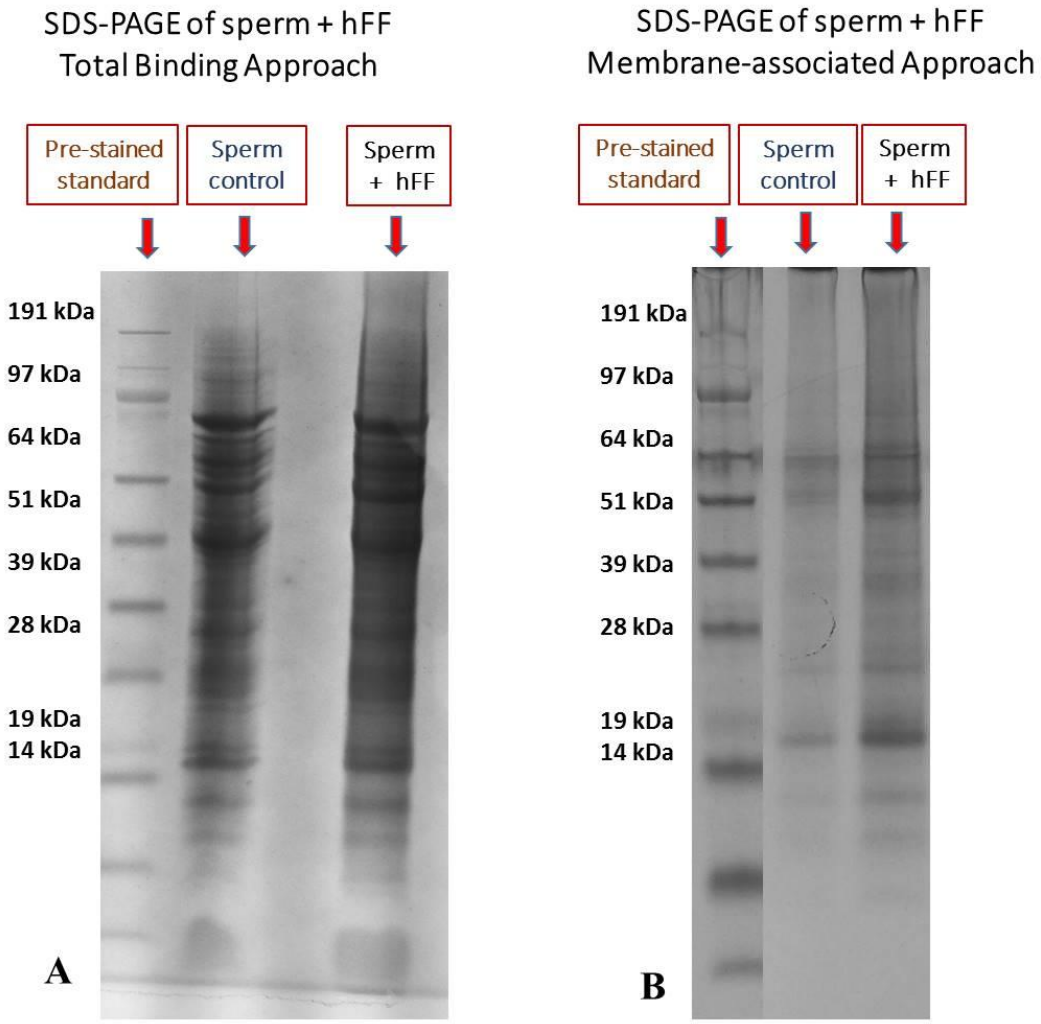


Figure 3-6 SDS-PAGE of samples in both approaches (**A-** Total binding approach and **B-** membrane-associated approach). Sperm and sperm + hFF samples were run along with a prestained standard in a precast 10% gel, gels were stained with Coomassie Brilliant blue.

3.4.4 In-gel protein digestion:

See section 2.4.2.3.

3.4.5 Mass spectrometry analysis:

This work was completed by Dr Ashley Martin and Dr Douglas Ward, University of Birmingham. An on-line, direct infusion into an ESI (ElectroSpray Ionisation) interface –

TOF (Time of Flight) mass spectrometry that has MS/MS capability was utilised in the data analysis. Fro more details of MS analysis, see section 2.4.4.

3.4.6 Acrosome reaction assay

3.4.6.1 Materials and methods

Table 3-2 Materials

Product name	**Company**	**Product code**
Progesterone (4-pregnene-3,20-dione)	Sigma, Dorset, UK.	P0130
poly-D-Lysine	Millipore, Watford, UK	A-003-E
Pisum sativum (pea) agglutinin FITC labelled (FITC-PSA) powder	Sigma, Dorset, UK.	L0770
sEBSS	Geneflow, Fradley, UK	06-2010-03-18
Fluoromount	BDH Merck ,Poole, Dorset, UK	360982B
Dimethyl sulfoxide (DMSO)	Life Technology (Thermo Fisher Scientific), Rockford, USA	D12345
Phosphate buffered saline (PBS)	Invitrogen, Paisley, UK	14040083
Calcium Ionophore/ A23187	Sigma, Dorset, UK.	C5149
Methanol	Fisher Scientific, Loughborough, UK.	M/3950/17
Bovine Serum Albumin (BSA)	Millipore, Watford, UK	82-002-4
Sodium citrate	Sigma, Dorset, UK.	S1804
Fructose	Sigma, Dorset, UK.	F0127
5 mL Falcon round-bottom polystyrene tube	Becton Dickinson Labware, New Jersey, USA.	2054

3.4.6.2 Sperm preparation and capacitation:

Semen samples were obtained in a similar procedure to section 3.3.2. Sperm cells were prepared by swim-up (Mortimer, 1994). Briefly, 1ml of sEBSS + 0.3 % BSA was under-layered with 400 µl of liquefied semen in 5ml Falcon round-bottom tubes and left to incubate for 1 hour at an angle of 45° at 37° C, 6% CO_2. The top 700 µl containing motile sperm fraction was gently harvested using sterile transfer pipette and pooled into 15 ml Falcon tube. Sperm concentration in the collected fraction was assessed by a Neubauer hemocytometer and the final concentration adjusted to 6 $\times 10^6$ sperm/ml using sEBSS + 0.3% BSA. The sperm samples were divided into aliquots of 100 µl and left to capacitate for 4-6 hours at 37°C, 6% CO_2, (n = 6).

Following sperm capacitation, sperm aliquots were stimulated either by addition of progesterone (final concentration of 13.5 µM), 10 µM ionomycin /A23187 as a positive control, hFF (30 % final concentration) and DMSO (1:1000) as a negative control. The different preparations were left to incubate for 1 hour then centrifuged at 300g for 5minutes. The supernatants were discarded and sperm were re-suspended and incubated in 500 µl of hypo-osmotic swelling medium (HOS) (0.74% sodium citrate, 1.35 % fructose in distilled water) for 45 minutes. The sperm preparations were then centrifuged at 300g for 5 minutes and re-suspended in a minimum volume of the HOS medium (approximately 50µl). The different sperm preparations were then smeared on duplicate slides (25 µl of each sperm preparation on each slide) pre-coated with 0.001% poly-D-lysine solution and left to air dry. The slides were then immersed in 100 % methanol for 1 minute to permeabilize the cells and slides were dried on a hot plate. 100 µl of 50 µg/ml FITC-PSA in PBS was laid on each slide and left to incubate for 45 minutes in a moist chamber at

37°C. The slides were then rinsed with PBS and left to air dry before they were mounted with fluoromount and left overnight in the dark to prevent fade.

Sperm were visualised using a 100 × oil immersion lens and assessment of acrosomal status was performed using a fluorescence microscopy (Cross et al., 1988). Only viable sperm with curly tails were scored with 200 cells being scored per treatment (100 sperm per slide). Sperm cells were scored according to the criteria described by Mendoza et al. (1992).

The Mean of acrosomal status for each treatment was obtained from the duplicates of each treatment scored. AR percentage data was converted to an arcsine values before testing for statistical significance between treatment groups using paired T tests and statistical significance was set at P value < 0.05. All calculations and statistical analyses were accomplished by the application of Microsoft Excel 2010 software.

The percentages of AR induction for hFF and progesterone treatments were also normalised to both the positive control (10 μM ionmoycin) and spontaneous AR (negative control, DMSO) and expressed as percentage stimulation of AR according to the formula:

$$\%\ \text{Stimulation of AR} = \left[\frac{\text{treatment AR\%} - \text{spontaneous AR\%}}{\text{Positive AR\%} - \text{spontaneous AR\%}}\right] \times 100$$

3.5 Results

3.5.1 Sperm + hFF total binding approach:

In one of the experiments of the sperm + hFF total binding approach the MS analysis did not detect any peptides which may have resulted from contamination during samples preparation. The results of the two successful experiments were analysed according to the sample type (sperm + hFF or sperm control) as follow:

Sperm +hFF samples: Twenty-one hFF proteins were identified in sperm + hFF samples this approach, the details of the data analysis are summarised in **figure 3-7**. The protein lists from sperm + hFF samples were compiled and loaded into UniProt knowledge base to obtain UniProt accession numbers. Proteins were then compared with the sperm protein data in a recent systematic review compiles sperm proteins from 30 sperm proteomic studies with 6198 sperm proteins (Amaral et al., 2014a) and also protein data from the sperm control samples in this study. Following the comparison, 31proteins were not sperm proteins and they were compared with the hFF protein data (a table with all the hFF proteins described in the published studies up to date was produced in this study, see chapter 2). Twenty-one hFF proteins were identified in the sperm + hFF samples. Sperm do not previously possess these proteins as they were found neither in sperm control nor in Amaral et al (2014a) compiled sperm data.

The total number of proteins identified in the sperm + hFF samples was 1060 proteins. 1030 were sperm proteins, found in the sperm compiled protein data Amaral et al (2014a) and/or in the sperm control samples. Fourteen proteins out of the 1030 were novel sperm proteins, detected in the sperm control samples but not in Amaral et al (2014a) sperm data. There was another group of proteins which was considered as unknown source proteins that were only found in sperm + hFF samples but not in the sperm control samples. These proteins were neither found in sperm compiled data (Amaral et al, 2014a) nor in hFF data. These proteins were detected for the first time in this study and therefore they may belong to either the novel sperm or the novel hFF proteins. Nine proteins belong to this category which may add to the sperm + hFF total binding if they are novel hFF proteins. **Figures 3-7** and **3-8** illustrate the data analysis for the sperm +hFF samples in the total binding approach.

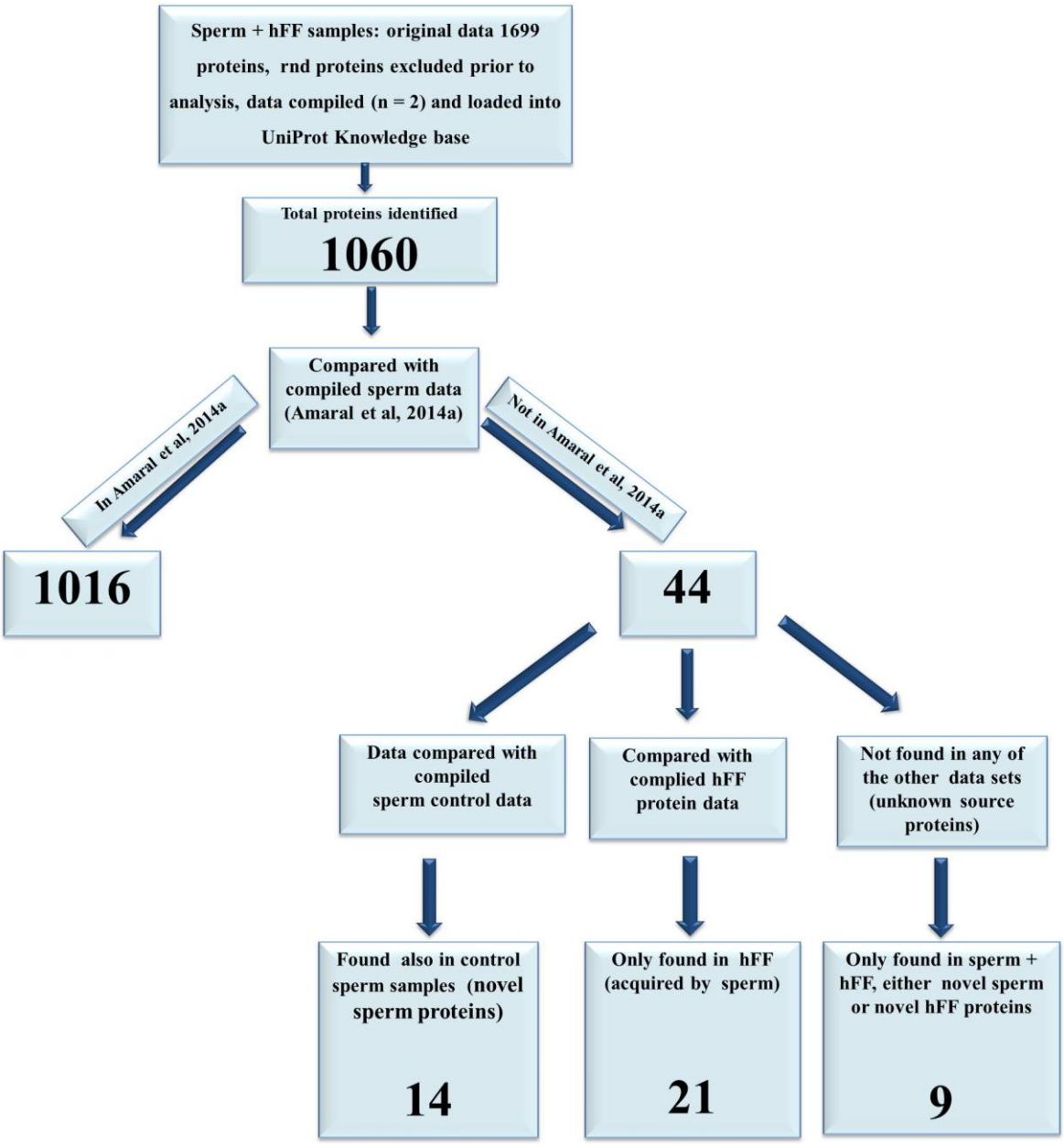


Figure 3-7 Summary of the data analysis of the of sperm + hFF samples in the total binding approach. Protein data were compared with sperm data from Amaral et al, 2014a to recognise novel sperm proteins. Data were also compared with hFF compiled data from the literature to identify hFF acquired by sperm.

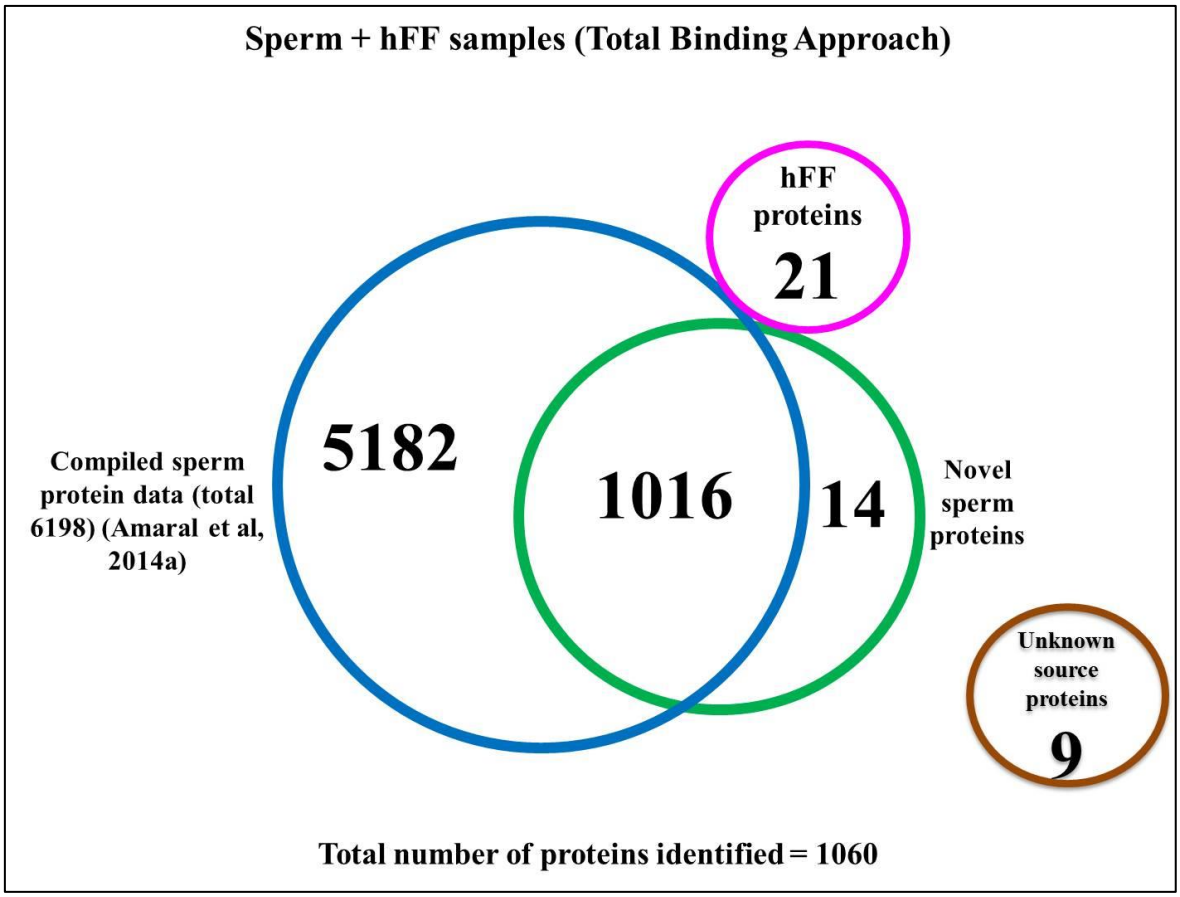


Figure 3-8 Venn diagram for the results of sperm + hFF samples in the total binding approach, 21 hFF proteins were identified in the sperm + hFF samples and 14 novel sperm proteins in this approach.

Sperm control samples data analysis: The sperm control protein data were compiled from the two experiments performed and a total of 1303 proteins were identified. The compiled list of the sperm control proteins was loaded into UniProt knowledgebase to obtain the UniProt accession numbers. The list was compared with sperm protein data in Amaral et al (2014a) to distinguish any novel sperm proteins, which were detected for the first time in this study. Fifty-three proteins were not previously described in Amaral et al (2014a) and were considered as novel sperm proteins. Fourteen out of the 53 novel sperm proteins were detected in both sperm control and sperm +hFF samples in this approach.

Figures 3-9 and **3-10** show a summary of the data analysis and a Venn diagram of sperm control protein results.

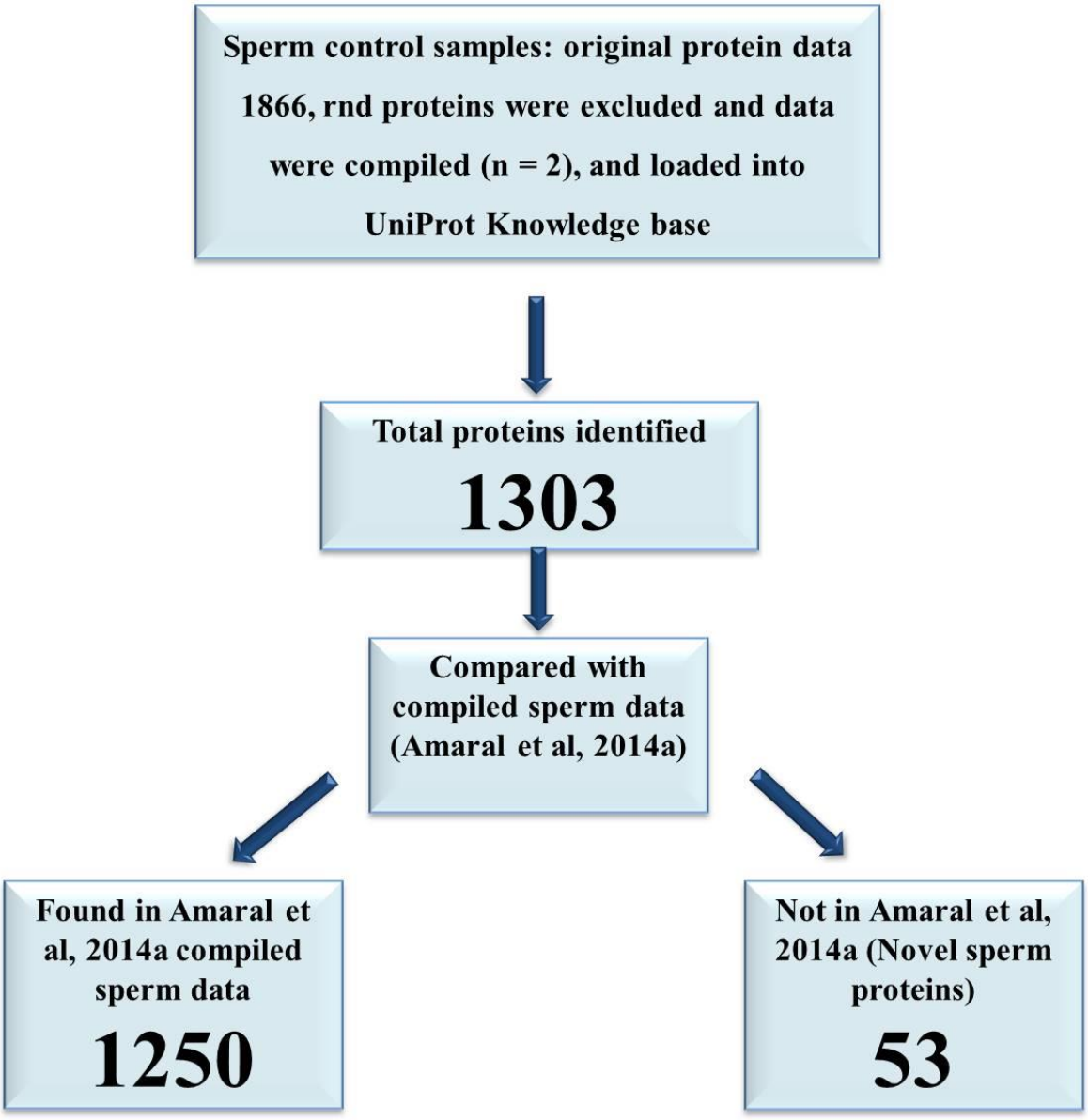


Figure 3-9 Summary of the data analysis of sperm control samples in the total binding approach. Protein data were compared with sperm data from Amaral et al, 2014a to recognise novel sperm proteins.

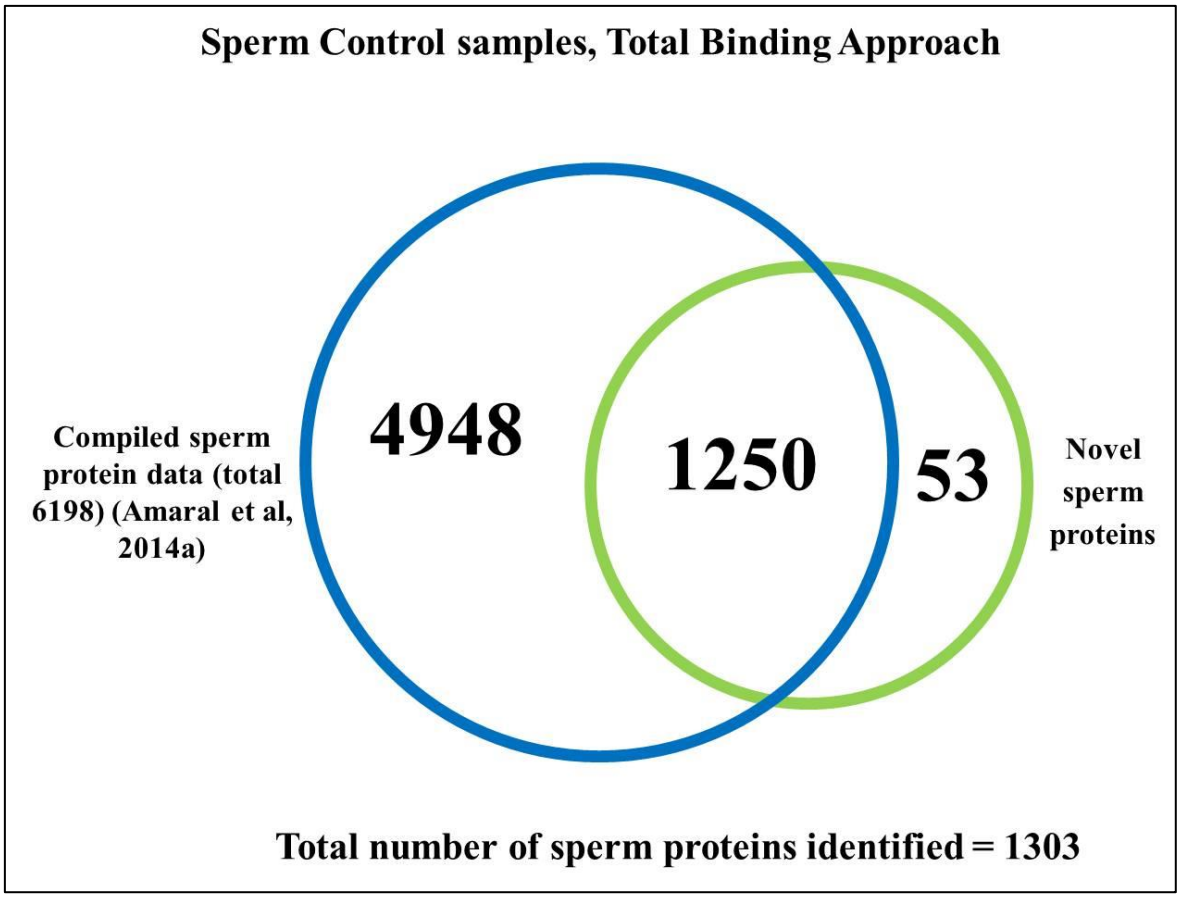


Figure 3-10 Venn diagram of the results of sperm control protein data analysis in the total binding approach, 53 novel sperm proteins were identified in this approach

3.5.2 Sperm + hFF membrane-associated proteins approach:

Five hFF proteins were identified as sperm membrane-associated proteins. The details of the analysis are summarised below.

Sperm + hFF samples data analysis: similar to the total binding approach data analysis, the protein data from the sperm + hFF membrane-associated approach were analysed to determine which hFF proteins were bound to the sperm. Proteins from the sperm + hFF samples were compiled and a total of 278 proteins identified. The protein data were loaded into UniProt knowledge base to determine the UniProt accession number for each protein.

The data were compared with sperm data (Amaral et al, 2014a) and sperm control samples in the membrane-associated approach. Two hundred and seventy proteins were sperm protein out of which eight were novel sperm proteins. Eight proteins were not found in the sperm data and they were compared with the hFF protein data in the literature. Five proteins were only found in hFF data and these proteins were considered as hFF protein acquired by the sperm following sperm incubation with hFF. Three proteins were unknown source proteins, detected only in the sperm + hFF samples (either novel sperm or novel hFF proteins). **Figures 3-11 and 3-12** summarise the analysis and results of the sperm + hFF membrane-associated proteins approach.

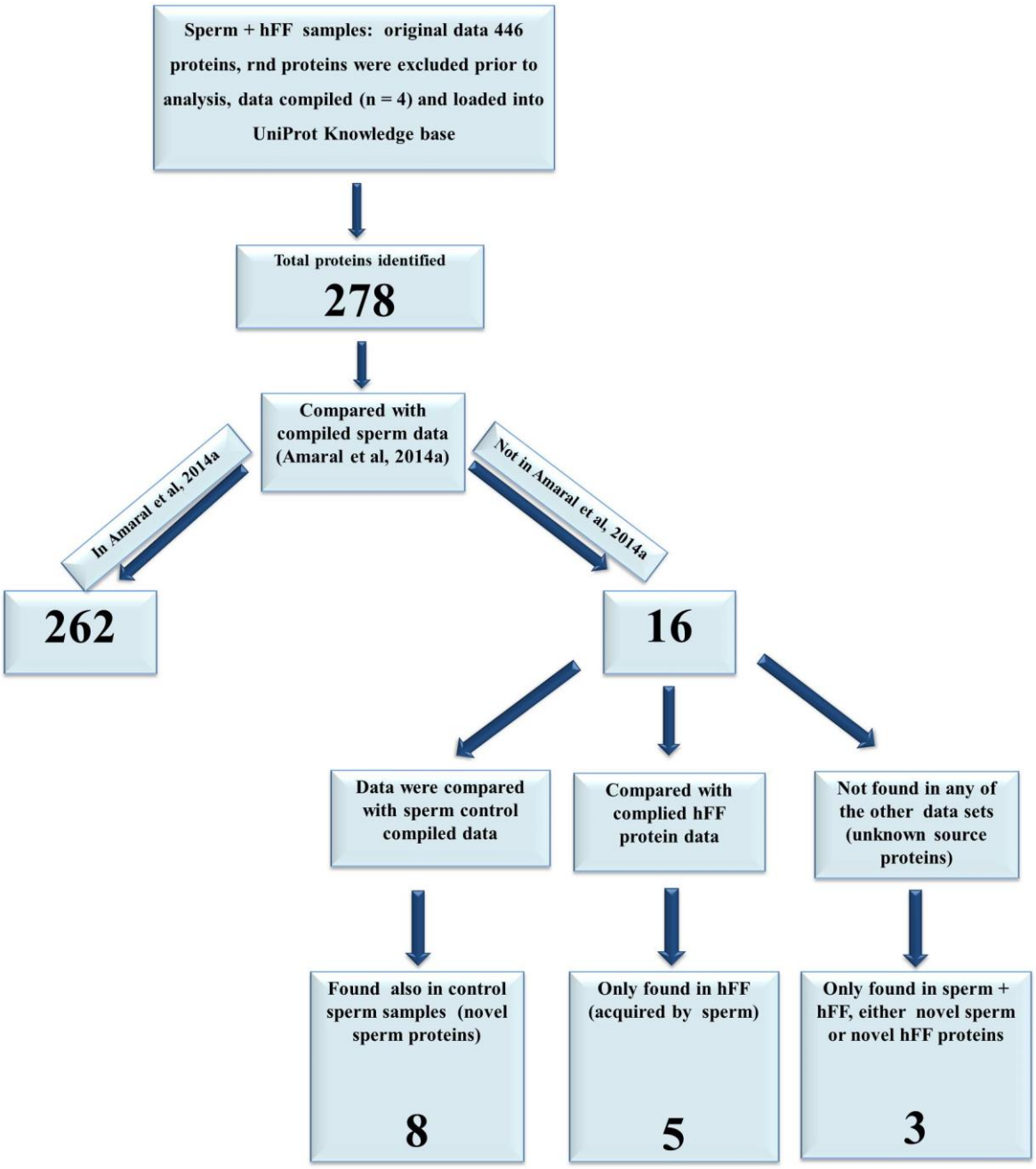


Figure 3-11 Summary of the data analysis of sperm + hFF samples in the membrane-associated approach. Protein data were compared with sperm data from Amaral et al, 2014a to recognise novel sperm proteins. Data were also compared with hFF compiled data from the literature to identify hFF acquired by sperm.

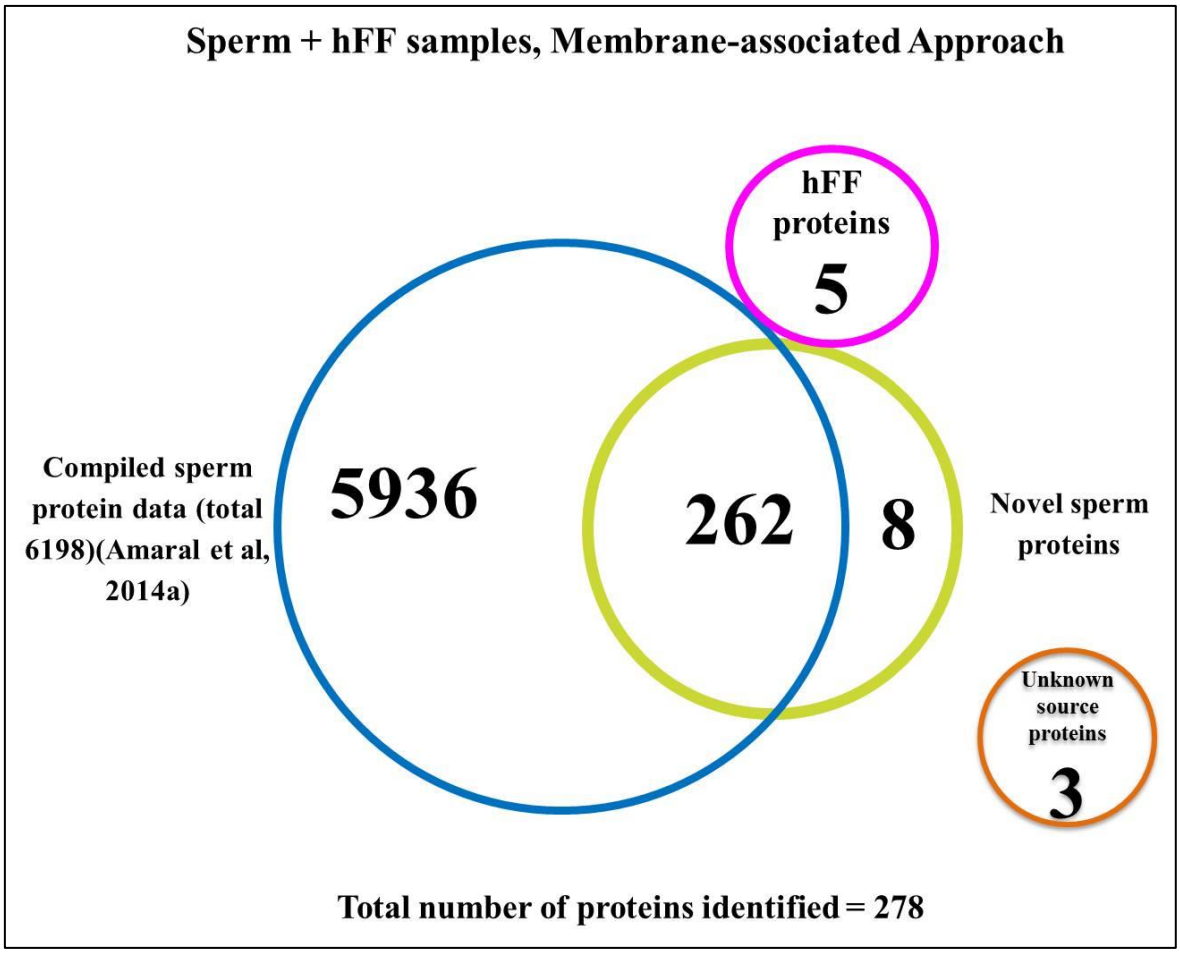


Figure 3-12 Venn diagram of the results of the sperm +hFF membrane-associated approach. Five hFF proteins identified in the sperm + hFF samples, 8 novel sperm proteins and 3 unknown source proteins.

Sperm control data analysis: protein data from the sperm control samples were compiled and compared with sperm data (Amaral, et al, 2014a) to distinguish any novel proteins which were detected for the first time in this study. A total of 212 proteins were detected in the sperm control samples with 13 proteins not found in Amaral et al (2014a) sperm data (novel sperm proteins). Seven out of the 13 novel sperm proteins were also found in the sperm + hFF samples. **Figures 3-13** and **3-14** summarise the results of the membrane-associated proteins approach.

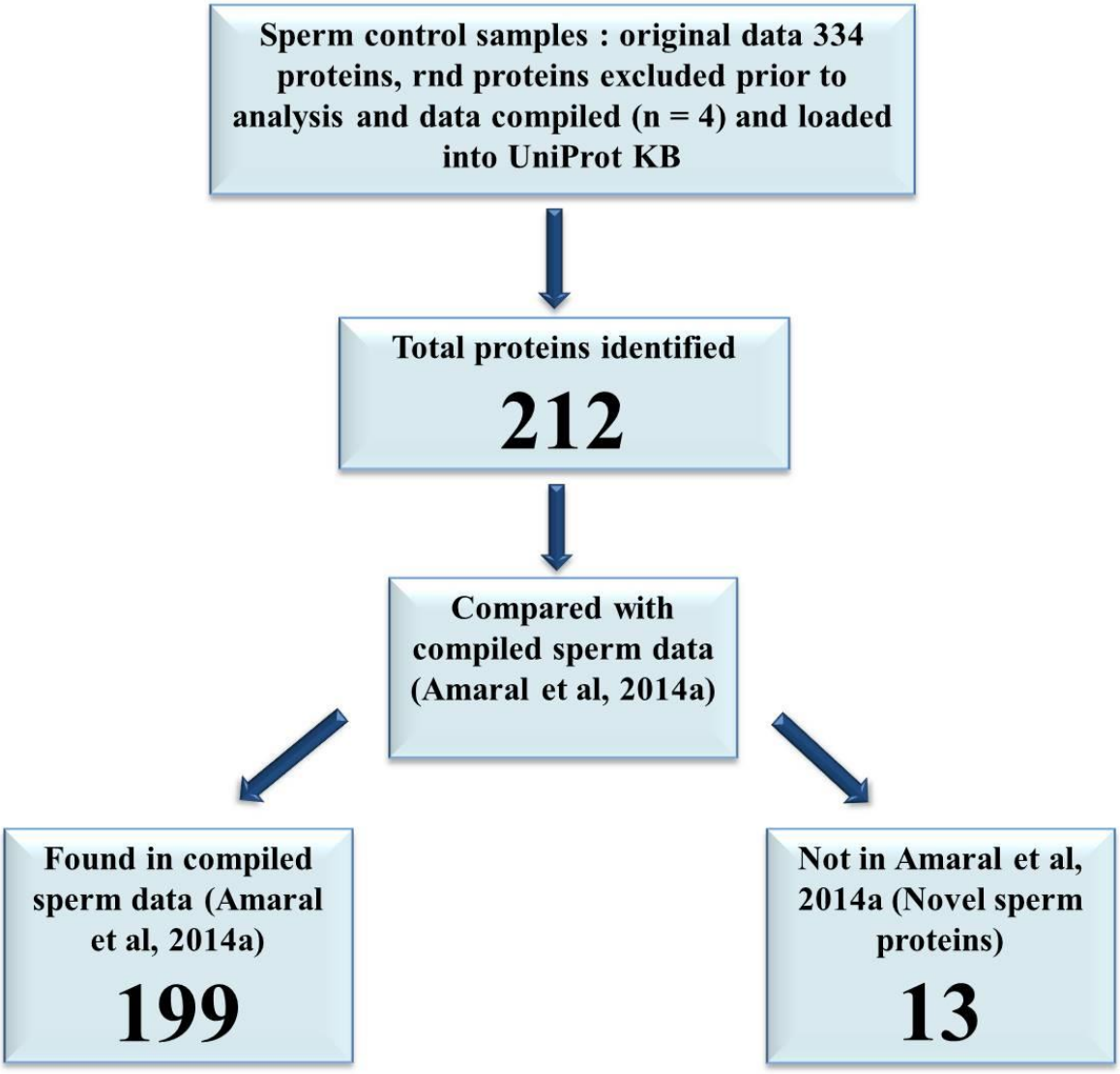


Figure 3-13 Summary of the data analysis of sperm control samples in the membrane-associated approach. Protein data were compared with sperm data from Amaral et al, 2014a to recognise novel sperm proteins, 13 proteins were novel sperm proteins.

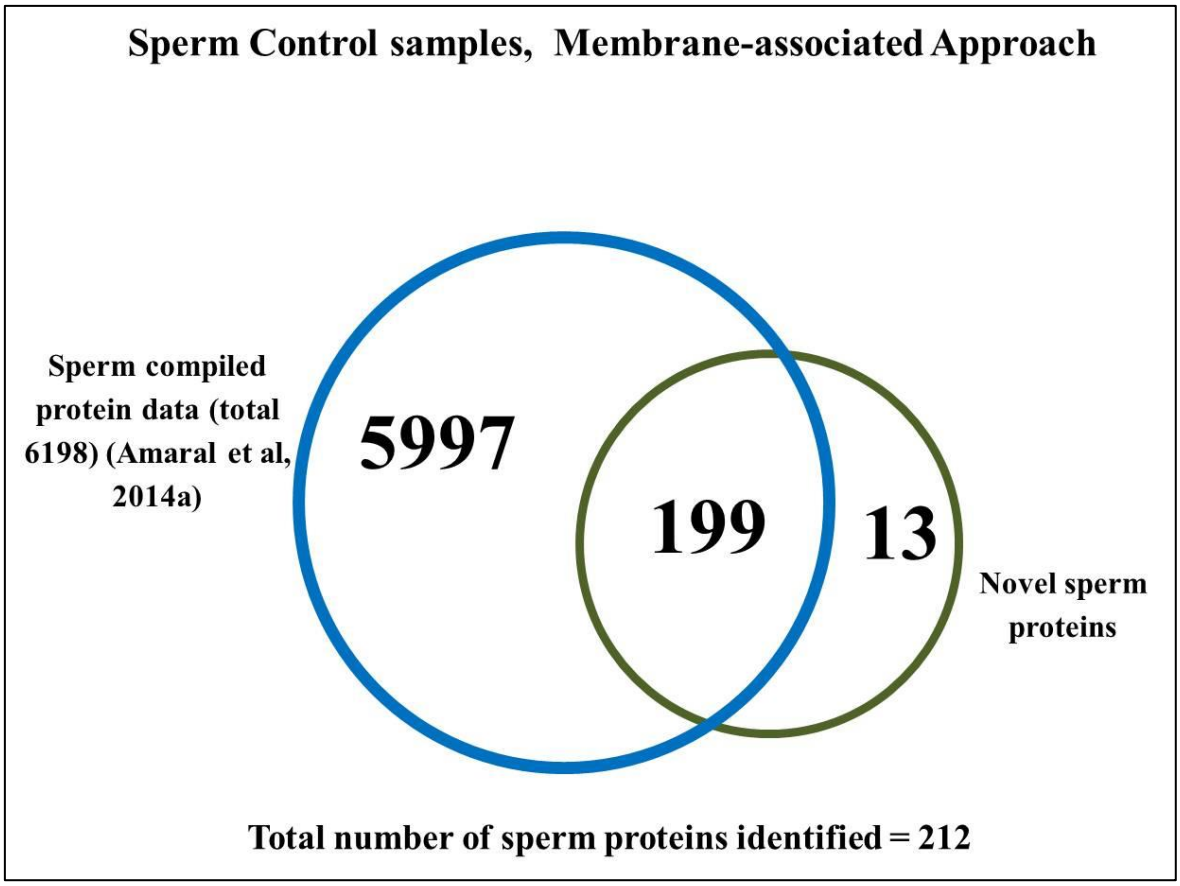


Figure 3-14 Venn diagram of the protein results of sperm control samples in the membrane-associated approach, 13 novel sperm proteins were identified.

3.5.3 Compiled results from both approaches:

Twenty-four hFF proteins (never previously detected in sperm) were detected in the sperm + hFF samples from both approaches. Two out of the 24 hFF proteins (vitronectin and alpha-1-antichymotrypsin) were detcted in both the total binding and the membrane-assoicated approaches. The total number of proteins identified in both approaches (total binding and membrane-associated) was 1530 of which 953 proteins (62%) were identified in two or more samples and/or independent experiments and 241 proteins were detected in both approaches. Out of the 1530 proetins, 1428 were described in sperm compiled data (Amaral et al, 2014a) while 103 proteins were not found in Amaral et al (2014a) sperm

data. Out of the 103 proteins, 63 proteins were novel sperm proteins found in sperm control samples. Eleven proteins from sperm + hFF samples were neither found in sperm control nor in hFF data from the literature and were considered as unknown source proteins (either novel sperm or novel hFF proteins). Some proteins were common between sperm and hFF data from the literature (not shown in the diagram). Four proteins were keratin and excluded from the final protein compiled data list leaving 1526 proteins included. **Figures 3-15** and **3-16** illustrate these data.

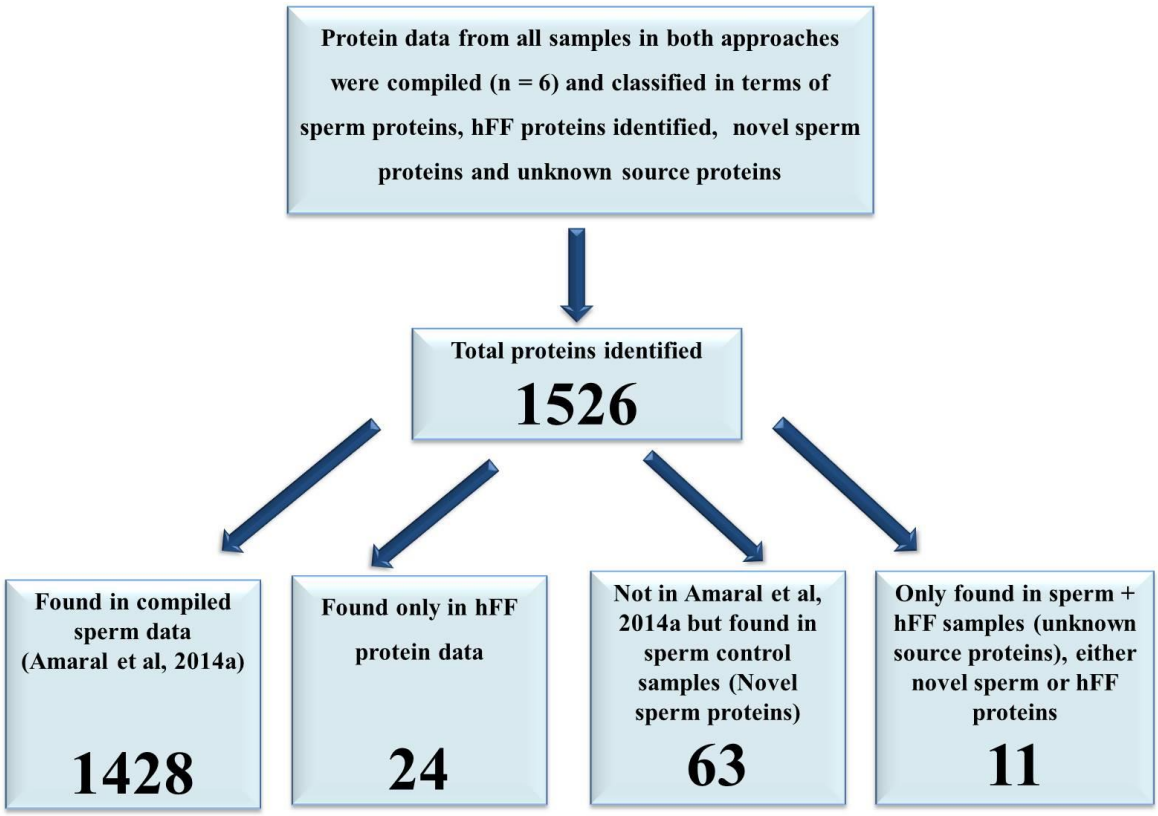


Figure 3-15 Summary of the data analysis of the compiled results from both approaches. The total number of proteins identified in this study was 1530 proteins, Keratin proteins (four) were excluded from the final list.

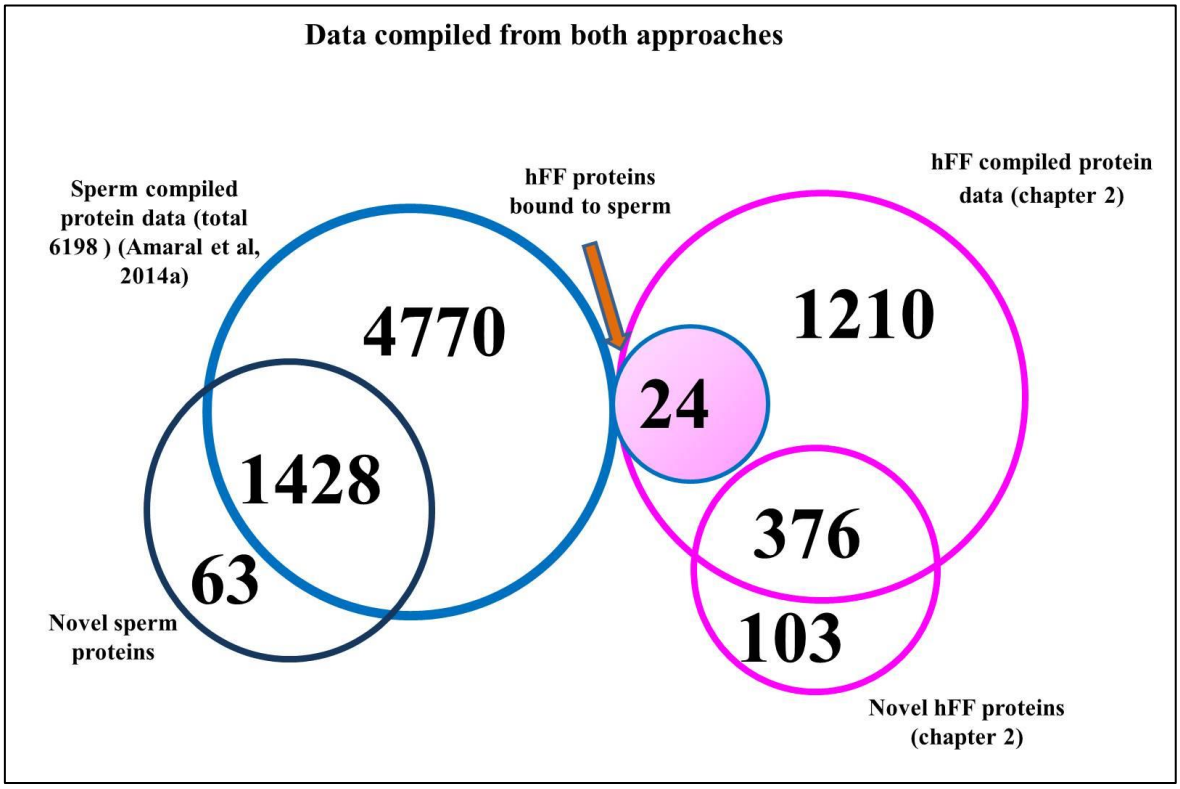


Figure 3-16 Data compiled from both approaches, total binding and membrane-associated (both sperm control and sperm + hFF samples). hFF compiled proteins data were obtained from chapter two. The total number of proteins identified in this study was 1530 proteins, 11 proteins were unknown source proteins (either novel sperm or novel hFF) not shown in this Venn diagram, four other proteins were keratins and excluded from the final list.

Novel sperm proteins:

In addition to identification of hFF sperm-bound proteins, novel sperm proteins were identified in this study. A total of 63 proteins from both approaches were detected in sperm control samples with some being detected also in sperm + hFF samples.

3.5.4 hFF proteins identified in sperm + hFF binding fraction:

Twenty-four hFF were identified in the sperm + hFF samples in this study compiled from both approaches (**table 3-3**). The lsit of identified proteins (**table 3-3**) were searched for functional classification and localisation and any relevance to sperm fertilisation.

Table 3-3 hFF proteins detected in sperm + hFF samples compiled from both approaches and their known function according to PANTHER Classification system

UniProt accession number	Protein name	Approach	Known function	References in hFF proteomic studies
P04004	Vitronectin	Total binding and Membrane-associated	Adhesion	Hanrieder et al, 2008; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Bianchi et al, 2013; Zamah et al, 2015
P01011	Alpha-1-antichymotrypsin	Total binding and Membrane-associated	serine protease inhibitor	Angelucci et al, 2006; Hanrieder et al, 2008;Hanrieder et al, 2009; Jarkovska et al, 2010; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Zamah et al, 2015
P01860	Ig gamma-3 chain C region	Total binding	Immune response	Hanrieder et al, 2009; Twigt et al, 2012
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	Total binding	Immune response	Hanrieder et al, 2008; Twigt et al, 2012; Ambekar et al, 2013; Zamah et al, 2015
P02679	Fibrinogen gamma chain	Total binding	Signalling	Anahory et al, 2002; Angelucci et al, 2006; Kim et al, 2006; Hanrieder et al, 2008; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Severino et al, 2013; Bianchi et al, 2013; Zamah et al, 2015; Wu et al, 2015
P00738	Haptoglobin	Total binding	Antioxidant	Spitzer et al, 1996; Anahory et al, 2002; ngelucci et al, 2006; Hanrieder et al, 2008; Schweigert et al, 2006; Estes et al, 2009; Sim and Lee; 2008; Kushnir et al, 2012; Twigt et al, 2012;Ambekar et al, 2013;Bianchi et al, 2013; Ambekar et al, 2014; Severino et al, 2013; Zamah aet al, 2015; Wu et al, 2015
P01042	Kininogen-1	Total binding	Catalysis	Hanrieder et al, 2008; Hanrieder et al, 2009; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Bianchi et al, 2013; Severino et al, 2013;Ambekar et al, 2014;Hashemitabar et al, 2014; Zamah et al, 2015
P13671	Complement component C6	Total binding	Immune response	Twigt et al, 2012; Ambekar et al, 2013; Aseverino et al, 2013; Zamah et al, 2015
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	Total binding	Immune response	Hanrieder et al, 2008; Hanrieder et al, 2009; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; ;Ambekar et al, 2014; Zamah et al, 2015
Q14624	Inter-alpha-trypsin inhibitor	Total binding	Immune response	Hanrieder et al, 2008; Hanrieder et al, 2009; Sim and Lee, 2008; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Bianchi et al, 2013; Severino et al, 2013;Ambekar et al, 2014; Zamah et al, 2015

	heavy chain H4			
P01766	Ig heavy chain V-III region BRO	Total binding	Immune response	Hanrieder et al, 2008; Hanrieder et al, 2009; Estes et al, 2009; Twigt et al, 2012; Zamah et al, 2015
P04217	Alpha-1B-glycoprotein	Total binding	catalysis	Hanrieder et al,2008; Hanrieder et al, 2009; Kushnir et al, 2012; Twigt et al, 2012; Bianchi et al, 2013; Ambekar et al, 2013;Ambekar et al,2014; Zamah et al, 2015
P01625	Ig kappa chain V-IV region Len	Total binding	Immune response	Twigt et al, 2012
P01765	Ig heavy chain V-III region TIL	Total binding	Immune response	Hanrieder et al, 2008; Hanrieder et al, 2009
O14791	Apolipoprotein L1	Total binding	Lipid transport	Twigt et al, 2012; Ambekar et al, 2013; Zamah et al, 2015
O95445	Apolipoprotein M	Total binding	Lipid transport	Ambekar et al, 2013; Bianchi et al,2013; Zamah et al, 2015
P01597	Ig kappa chain V-I region DEE	Total binding	Immune response	Twigt et al, 2012
P02671	Fibrinogen alpha chain	Total binding	Signalling molecule	Angelucci et al, 2006; Hanrieder et al, 2008; Hanrieder et al, 2009; Estes et al, 2009; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013; Severino et al, 2013; Zamah et al, 2015
P02765	Alpha-2-HS-glycoprotein	Total binding	cysteine protease inhibitor	Angelucci et al, 2006; Hanrieder et al, 2008; Hanrieder et al, 2009; ; Ambekar et al, 2013; Bianchi et al, 2013; Ambekar et al,2014; Zamah et al, 2015
P04433	Ig kappa chain V-III region VG (Fragment)	Total binding	Immune response	Hanrieder et al, 2009; Twigt et al, 2012
P80748	Ig lambda chain V-III region LOI	Total binding	Immune response	Twigt et al, 2012
P02749	Beta-2-glycoprotein 1	Membrane-associated	Binding	Angelucci et al, 2006; Estes et al, 2009; Twigt et al, 2012; Bianchi et al, 2013; Ambekar et al, 2013; Ambekar et al, 2014; Zamah et al, 2015
P06703	S100-A6	Membrane-associated	Calcium signalling	hFF data from hFF proteomic study (chapter 2)
P05155	Plasma protease C1 inhibitor	Membrane-associated	Protease inhibitor	Hanrieder et al, 2008; Kushnir et al, 2012; Twigt et al, 2012; Ambekar et al, 2013;Zamah et al, 2015

Functional classification of the hFF proteins bound to sperm:

The list of the 24 hFF proteins **(table 3-3)** identified in the sperm + hFF samples were analysed in terms of molecular function and biological processes, using PANTHER Classification System Version 10.0 (section 2.4.5) at http://www.pantherdb.org/ accessed in July 2015, to recognize their functions and their possible role in sperm modulation. The molecular functions and protein class of the 24 hFF proteins are illustrated in **figures 3-17** and **3-18**.

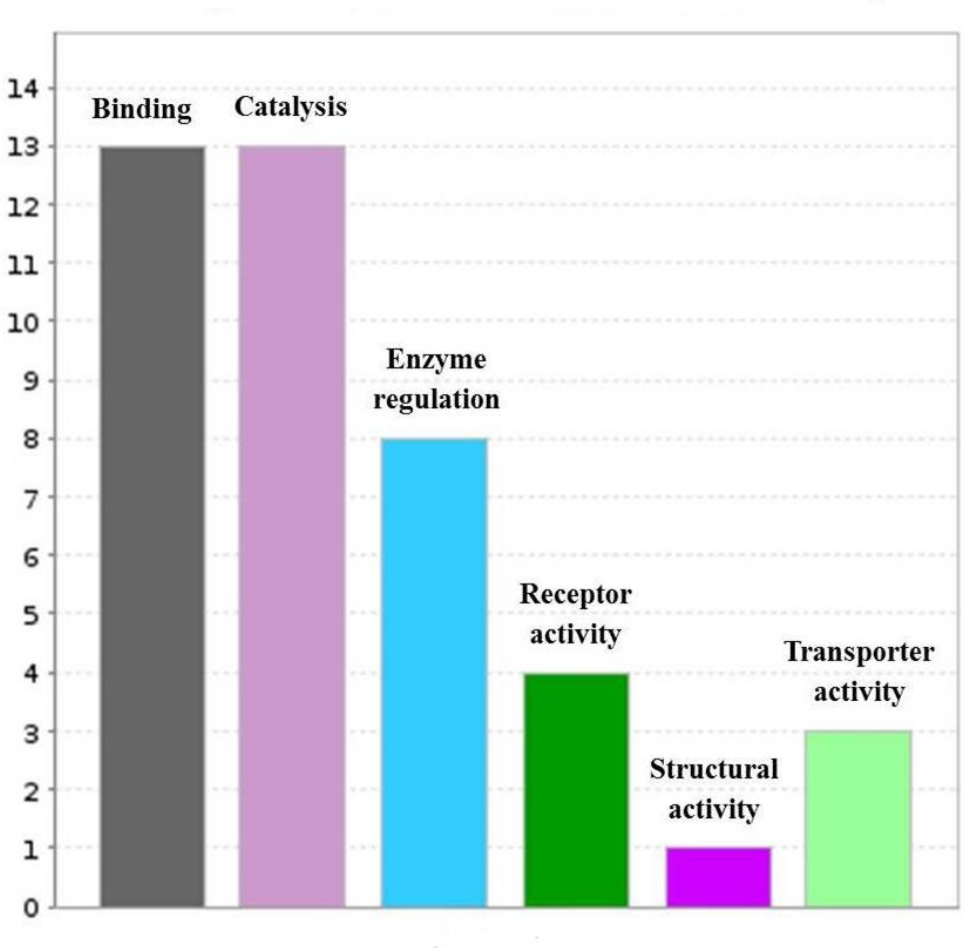


Figure 3-17 Molecular functions of the hFF proteins bound to sperm using PANTHER classification system; binding and catalysis were among the the highest categories followed by the enzyme regulation and receptor activity

Binding and enzyme catalysis were among the highest protein categories along with enzyme regulation and receptor activity. Some proteins were mapped to more than one molecular function. For example, alpha-2-macroglobulin was mapped into the enzyme catalytic category and receptor activity. However, seven proteins had no PANTHER hit in the functional classification with no functions described. These proteins were immunoglobulins heavy chain proteins with different regions. Further details of the functional analysis of the hFF proteins bound to sperm are included in section 3.6.

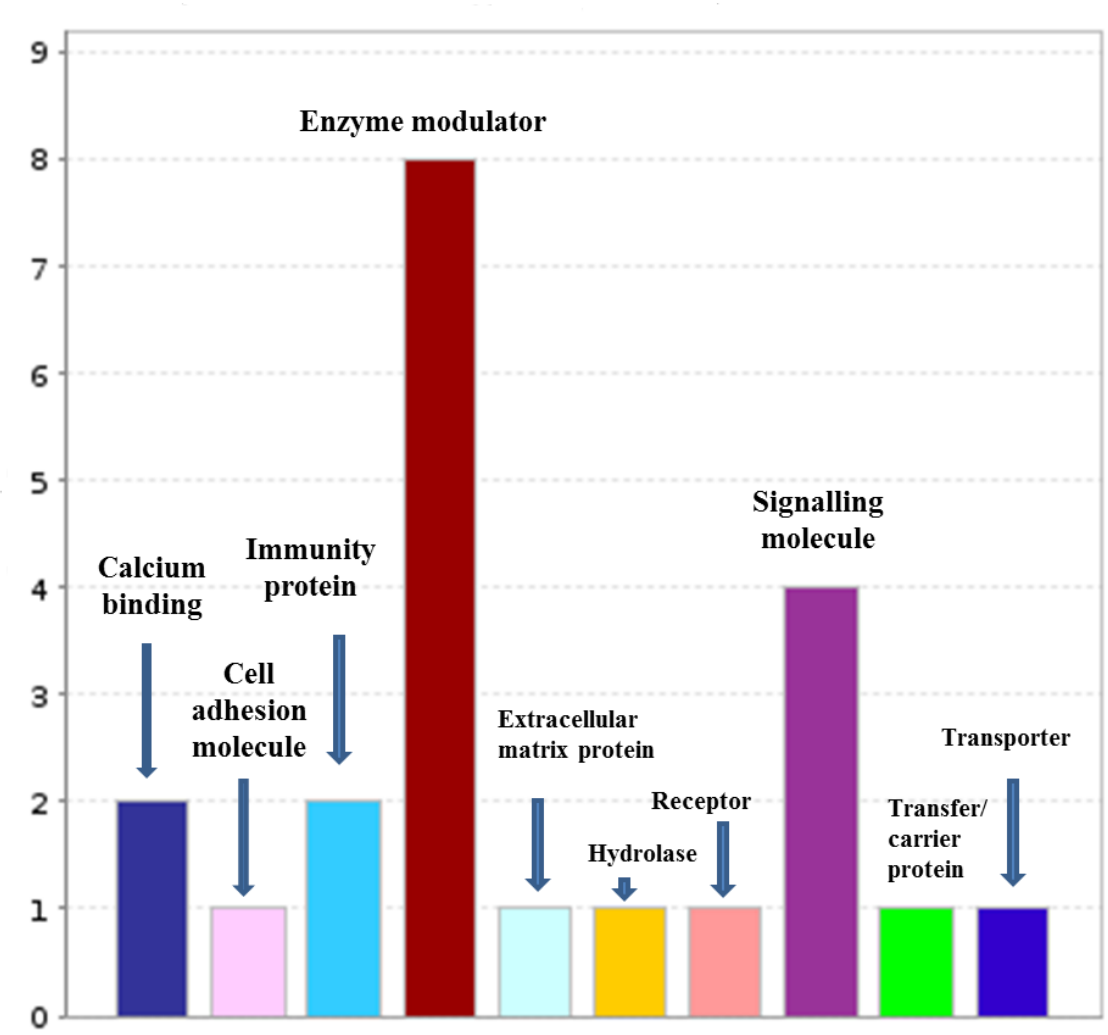


Figure 3-18 Functional analysis for protein class of the hFF proteins acquired by sperm using PANTHER classification system. Enzyme modulators and signalling molecules were among the highest categories.

3.5.5 Acrosome reaction results:

Across this replicates (n = 6), 30% hFF induced statistically significant AR when compared to the negative control (DMSO) with p value < 0.0002. AR triggered by 30 % hFF was not significantly different from 13.5 µM progesterone (p. value < 0.03), **figure 3-19**.

AR was also evaluated using the AR stimulation formula (section 3.4.6.2) which enables the analysis of AR based on excluded spontaneous AR in the sperm population (represented by the negative control DMSO) and manifests maximum stimulation of AR levels observed in sperm exposed to the positive control ionomycin/calcium ionophore, (Correia, 2011), **table 3-4.**

Table 3-4 % stimulation of acrosome reaction in the different treatments

Treatments	**% of Stimulation of acrosome reaction**						
	Exp1	**Exp2**	**Exp3**	**Exp4**	**Exp5**	**Exp6**	**Mean ± SEM**
Ionomycin	**100**	**100**	**100**	**100**	**100**	**100**	**100 ± 0**
hFF	**23**	**17.4**	**14.6**	**21.4**	**15.7**	**26.3**	**19.7 ± 1.9**
Progesterone	**30**	**23.5**	**17**	**22**	**17.5**	**28**	**23 ± 2. 2**
DMSO	**0**	**0**	**0**	**0**	**0**	**0**	**0 ± 0**

.

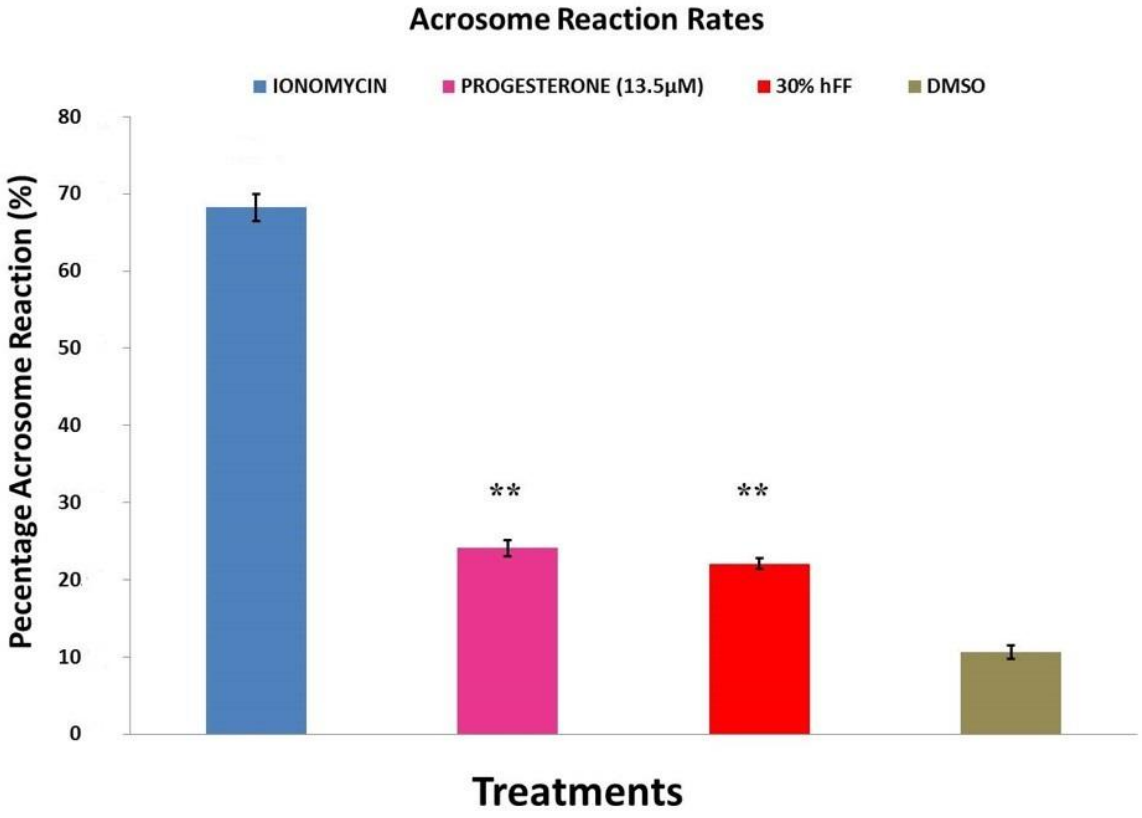


Figure 3-19 Acrosome reaction analysis data (n = 6) plotted as mean ± SEM. Sperm cells were treated with ionomycin (positive control), DMSO (negative control), progesterone and hFF. * indicates a significant difference between control and treatments (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.0001$).

3.6 hFF proteins identified in the sperm + hFF samples:

3.6.1 Functional relevance: The hFF proteins detected in both the sperm + hFF total binding and membrane-associated approaches, which were possibly acquired by sperm following incubation, were searched in PubMed at http://www.ncbi.nlm.nih.gov/pubmed with the key words: sperm AND/OR fertilisation AND/OR reproduction, to construct possible links to sperm regulation mechanism. Some of the hFF proteins were selected according to their relevance to sperm and reproduction in the searched literature and a summary for the identified hFF protein function is highlighted in this section.

Immunoglobulins: six out of the 24 hFF proteins detected in the sperm + hFF samples were immunoglobulins. In a study investigating the effect of heavy and light chains of human IgG, the authors suggested that the purified preparation of IgG induced sperm acrosome reaction (Saragüeta et al., 1994). This effect was only examined by anti-human lgG antibody in the study and was not supported by any further studies in the literature .

Vitronectin is a glycoprotein with ability to interact with several molecules including glycosaminoglycans and proteoglycans enabling it to contribute to numerous biological functions such as cell adhesion and cell surface proteolysis (Gibson et al., 1999, Bronson et al., 2000, Thys et al., 2012). In human, low concentrations of vitronectin (< 100 nM) supplementation was suggested to enhance sperm-egg adherence, while at higher concentrations, vitronectin was suggested to reduce sperm-egg adhesion (Fusi et al., 1996). In cattle, addition of high concentrations (more than 500nM) of exogenous vitronectin to the IVF culture media has been shown to inhibit sperm-oocyte interaction and thus suggesting that vitronectin could be a ligand in the bovine sperm-oocyte recognition process (Thys et al., 2012, Tanghe et al., 2004). Whether vitronectin from hFF which has bound the sperm following sperm + hFF incubation in this study possesses similar effects to the exogenously added vitronectin in terms of enhancing sperm-egg adhesion and interaction described in the above indicated studies is interesting for further research. The presence of vitronectin as an intrinsic sperm protein has been claimed in some studies which applied monoclonal anti-vitronectin antibody on semen sample from one donor (Bronson et al., 2000). The same research group has suggested enhanced expression of vitronectin on sperm surface following capacitation and vitronectin release following acrosome reaction (Fusi et al., 1996, Fusi et al., 1992, Fusi et al., 1994). Another study has described vitronectin to be localised to the equatorial area of the sperm head (Jeremias and

Witkin, 1996) and to have correlation with sperm' ability to acrosome react (Fusi et al., 1992). Nevertheless, none of the recent studies analysing sperm proteins via mass spectrometry have detected vitronectin in sperm cells (vitronectin was searched in a systematic review of compiled 6198 sperm proteins from 30 human sperm proteomic studies (Amaral et al., 2014a). In the current study, vitronectin was only detected in two independent experiments in sperm + hFF samples and not in any sperm control data from the two approaches applied.

Vitronection described on sperm surface following capacitation and acrosome reaction (Fusi et al., 1996) might have been acquired from exogenous sources and become bound to sperm during the incubation period. Additionally, the Velcro effect of vitronectin on sperm-oocyte adhesion (Fusi et al, 1996) was only observed when vitronectin was detected on the sperm surface following capacitation. The role of hFF vitronectin in sperm functions may require further research by the application of techniques such as recombinant proteins to identify the exact role of this protein in sperm functions.

Kininogen-1 is the precursor protein for both high and low molecular weights kininogens and is part of the kallikrein-kinin system which has been suggested to be involved in human sperm motility regulation (Schill, 1975). Kininogens and the other three components of the kallikrein-kinin system namely kallikreins, kinins and kininases have been detected in the human male seminal secretions and have been shown to enhance sperm metabolism and motility (Schill and Miska, 1992). However, kininogen-1 has not been described in the compiled sperm proteome by Amaral et al (2014a). The mechanism by which the components of the kallikrein- kinin system affect the sperm is not clearly understood, however, some studies advocated the presence of certain sperm membrane bradykinin subtype B receptors for the kinins' effects on sperm (Schill and Miska, 1992).

Furthermore, enhancement of sperm penetration to cervical mucus was shown following sperm incubation in media with of kininogen, kallikrein and acrosin and also suggested the likelihood of some traces of kininogen anchored to the sperm surface allowing release of kinin (Schill et al., 1979). In these studies, porcine pancreatic kallikerins were used to investigate their effects on cervical mucus penetration. In the current study, kininogen-1 from hFF was found in sperm + hFF sampes and may have similar effects to the pancreatic-derived kininogen, however, further research is necessary to confirm this suggestion.

S100 A6 is a member of the S100 protein family which are involved in calcium signalling and phosphorylation (Schafer and Heizmann, 1996, Donato, 2001) and in regulation of cell motility, growth, and survival (Gross et al., 2013). S100 A6 is a calcium binding protein with the main function in calcium signalling by the interaction with other proteins (Nedjadi et al., 2009). S100 A6 is also involved indirectly in the reorganization of the actin cytoskeleton and in cell motility (Nedjadi et al., 2009). Until now, the exact physiological function of S100A6 is unknown (Leśniak et al., 2005), however, it has been suggested that S100A6 may contribute to cellular motility (Gross et al., 2013), cytoskeleton rearrangement (Breen and Tang, 2003), cell proliferation and exocytosis (Timmons et al., 1993). The link between S100A6 and cell motility was shown mainly in cancer cells such as pancreatic cancer and osteosarcoma through S100A6 interaction with Annexin A2 (Nedjadi et al., 2009). Depleted levels of S100 A6 show loss of cell migration and invasion of pancreatic cancer cells (Nedjadi et al., 2009). However, the specific regulation mechanism by which S100A6 affects cell motility is still elusive (Gross et al., 2013). No further articles exploring the effect of S100A6 on cellular motility in none cancer cells such as sperm were found in the literature. Although the invasive nature of malignant cells

and their migration are distinct from motility in sperm cells and its ability to penetrae the egg; the role of the bound S100A6 on sperm surface requires further study.

Plasma protease C1 inhibitor: also known as C1 esterase inhibitor and SERPING1 with UniProt accession number P05155 (IC1_HUMAN) and its short name is C1-Inh which will be used in this study. C1-Inh is a protease inhibitor which participates in several physiological functions such as complement cascade, fibrinolysis and coagulation (Davis, 1988) and it belongs to the SERPIN superfamily. As indicated earlier, C1-Inh was not among the 6198 proteins listed in Amaral, et al (2014a) compiled sperm data and was not detected in sperm control samples and only detected in sperm + hFF in this study and in hFF protein data . However, the identification of a C1- Inh-Like molecule on the sperm surface mainly the head and midpiece regions was claimed in a study using polyclonal antibody (Jiang et al., 1997). The authors assumed that C1- Inh-like was synthesised in the testis or within the sperm itself and it became attached to the sperm surface (Jiang et al., 1997). Prevention of sperm lysis in the presence of C1- Inh-Like on the sperm surface was suggested to be part of the complement regulation mechanism played by this protein (Jiang et al., 1997). Since sperm and extra-embryonic membranes are allogenic to the maternal cells, the involvement of complement system regulation in fertilisation is believed to play significant role for successful fertilisation and prevention of complement-mediated damage to the sperm and embryonic tissues (Vanderpuye et al., 1992). This observation was illustrated in the case of infertile patients with C1-Inh-Like deficiency in semen (Jiang et al., 1996) who have sperm antibodies which bind C1 complement component and results in complement-mediated destruction of sperm (Jiang et al., 1997). C1-Inh-Like on the sperm surface was also suggested to play a role in regulation of sperm motility and that antibodies against this protein inhibit motile sperm (Jiang et al., 1997).

Nonetheless, neither C1- Inh nor its C1- Inh-Like homologue has been identified in the sperm in any of the 30 sperm proteomic studies included in Amaral et al, 2014a sperm proteome. This could open the door for speculations on the role of this protein which is present in hFF and may bind to the sperm surface following their incubation with hFF as investigated in this study. hFF displays complement activity (D'Cruz et al., 1990) and the presence of C1- Inh in both hFF and in the sperm + hFF samples may signify its role in the protection of sperm against sperm damage caused by complement cascade activation. Such speculations require further research to elucidate possible roles of C1- Inh in sperm fertilisation.

3.6.2 Future work:

Although the current knowledge of the function of the acquired hFF proteins may not indicate any direct effects on sperm regulation and fertilising capability; further research into their roles is essential. Techniques, such as recombinant proteins or combinations of purified forms of these specific hFF proteins acquired by sperm, and studying their effect on sperm parameters may increase our understanding of their role in sperm fertilisation. Investigation of the effects of specific antibodies to the acquired hFF proteins (for example, monoclonal anti-vitronectin antibodies (Bronson et al., 2000)) on sperm regulation may enable better understanding of the role of the acquired hFF proteins in capacitation and AR. Subcellular fractionation of the sperm samples following their incubation with hFF may be an alternative approach to enable localisation of the site of hFF proteins binding e.g. on the head or tail regions. Validation of the detected hFF in sperm + hFF samples by western blot may also enable further confirmation of the identity of the acquired hFF proteins. The application of these techniques, however, was out of the scope of this study.

3.7 hFF and sperm acrosome reaction data:

In the current study, among six replicates, 30% hFF induced AR in approximately 20% subpopulation of sperm following capacitation of 4-6 hours. The ability of hFF to induce AR in a subpopulation of sperm has been documented in several studies (Tesarik, 1985, Calvo et al., 1989, Yao et al., 2000). The rate of AR in response to hFF has been shown as dose-dependent (Burrello et al., 2004). For example, the rate of AR in response to 25% - 50% concentrations of hFF varied from 11% to up to 59% of sperm (Siegel et al., 1990, Tesarik, 1985, Yao et al., 2000). This variation in the rate of AR among these studies may have resulted from differences in the experimental design such as the duration of incubation with the treatments, sperm pre-incubation for capacitation and the difference in the hFF dose applied (Yao et al, 2000).

In the current study, capacitated sperm were incubated in hFF for 1 hour. The duration after which hFF induces AR varied widely in the literature ranging from 15 minutes to up to 24 hours of extended exposure in some studies (Pilikian and Mimouni, 1988, Stock and Fraser, 1989, Yao et al., 2000). While Yudin et al. (1988) stated that hFF stimulated AR in 40% of the sperm population within 180 seconds, Gearon et al. (1994) claimed that a minimum of 6 hours is required to induce sperm acrosomal exocytosis in response to hFF. Most other studies agree on one hour duration of incubation in order for hFF to induce AR (Tesarik, 1985; Calvo et al, 1989; Siegel, 1990; De Jonge et al, 1993; Yao et al, 2000).

3.8 Discussion:

To our knowledge, this is the first study to conduct a comparative proteomic analysis of sperm capacitated in capacitation media alone versus sperm capacitated in the capacitation media in the presence of hFF. Protein identification was achieved using MS analysis. Twenty-four hFF proteins were detected in sperm + hFF samples following two proteomic

approaches and these hFF proteins may have been acquired by the sperm following sperm incubation with hFF.

In the total binding approach, 1488 proteins were detected and they were mostly soluble sperm proteins with only 81 (approximately 5%) sperm membrane proteins when compared to sperm membrane proteins from Gu et al (2011) and 22 hFF proteins were detected. Membrane proteins are the site where sperm interaction with their surrounding environments in both the male and female reproductive tracts is believed to take place (Naaby-Hansen et al., 1997). To have better access to the sperm membrane proteins along with any bound hFF proteins and to investigate sperm surface protein remodelling following exposure to hFF, the membrane-associated approach was designed. The first set of experiments in the membrane-associated approach was performed to determine which chemical conditions are appropriate to disrupt sperm membrane and enable separation of any membrane-associated hFF proteins using chemical elution. Elution is a process in which some substances are extracted from another using washing with chemical solvents. 2M NaCl/Tris-base pH 10.6 preparation was selected for the membrane-associated approach following SDS-PAGE salt concentration curve. Preparations containing high salt concentrations has been shown to disrupt electrostatic interactions between the integral and peripheral membrane proteins resulting in the release of the peripheral fraction of membrane proteins (Gadella, 2009). However, out of the total 328 proteins detected in the membrane-associated approach from both sperm control and sperm + hFF samples, 52 (approximately 15%) were sperm membrane proteins when compared to sperm membrane proteins from Gu et al (2011) and five hFF proteins were detected.

The challenge of detecting sperm membrane proteins along with any bound hFF proteins may be attributed to the nature of the membrane proteins in general. It is well documented

that membrane protein solubilisation is a major challenge in proteomic studies with no well established approach to achieve their purification from intracellular protein contamination (Kalipatnapu and Chattopadhyay, 2005, Jamshad et al., 2011). In proteomics, it is a well-recognised difficulty to solubilise and extract all proteins from cells, particularly highly hydrophobic proteins using standard techniques (Brewis and Gadella, 2010). Despite the significance of sperm membrane proteomic study in understanding the initial stages of fertilisation, the best choice of techniques for solubilisations of sperm membrane proteins is still lacking (reviewed in Brewis and Gadella (2010) and Nowicka-Bauer and Kurpisz (2013). Several techniques have been applied in sperm membrane proteomic studies such as nitrogen cavitation, ultra-sonication and biotinylation (Gadella, 2009, Brewis and Gadella, 2010). However, low purity of the detected sperm membrane proteins with more intracellular proteins being detected are still problematic in these techniques (Brewis and Gadella, 2010).

Discussion of the study's strengths, limitations and methodological choices,:

While 62 % of the proteins detected in this study were detected more than once, 38% sperm proteins were only detected once in one experiment and/or approach. This may have resulted from physiological reasons such as the generally observed heterogeneity in sperm samples (Holt and Van Look, 2004) with intra (between ejaculates from the same sperm donor) and inter-variabilities (between ejaculates from different donors) being observed in sperm proteomic studies (Amaral et al, 2014a). Additionally, sperm capacitation has been shown to only occur in a subpopulation of sperm and does not occur simultaneously in all sperm cells and may not occur in some sperm and this may affect the protein profile among the same population of sperm (Amaral et al, 2014a). Since only a subpopulation of sperm may undergo capacitataion at a given time (Amaral et al, 2014a) it may be

reasonable to expect only this subpopulation to acquire some hFF proteins during the sperm surface membrane remodelling in the current study.

Low abundance of certain hFF is another possible explanation for missing detection of potential hFF proteins bound to sperm. As highlighted in chapter 2, low abundance follicle-specific hFF proteins are generally of high relevance to the functional aspects of hFF proteins in reproduction (Jarkovska et al, 2010). Whether the detection of the identified hFF proteins in the sperm + hFF samples was due to their binding to the sperm or their abundance in hFF requires further research. Low number of replicates in this study is another limitation. Six experiments (two in the total binding and four in the membrane-associated approaches) were performed in this work. Future studies may apply larger number of semen samples in the study of sperm + hFF interactions to achieve better representation of biological variation.

Discussion of the methodological choice:

Sperm purification, in this study, was performed using Percoll density gradient centrifugation which has been shown to effectively eliminate seminal plasma and none sperm cells such as leukocytes (Gandini et al., 1999, Wang et al., 2013). Out of the 30 sperm proteomic studies compiled by Amaral et al (2014a), 21 studies have utilised density gradient centrifugation for sperm purification (see **table 3-5** for studies' references). It has also been shown that Percoll density gradient centrifugation produced superior reproducibility with less variation between gels prepared from same semen donors on different occasions compared to swim-up technique (Pixton et al, 2004). This is suggested to possibly result from elimination of seminal plasma sperm-adhered proteins by density gradient centrifugation that are not removed by swim-up (Pixton et al, 2004).

In the current study, sperm + hFF were incubated for 90-120 minutes to allow for sperm capacitation and any hFF binding to take place. It has been suggested that sperm cells may have a rapid response, within one hour, to the hFF exposure since this exposure occurs in the oviduct shortly prior to fertilisation (Yao et al, 2000). Yao et al (2000) has manifested that sperm capacitation following incubation with hFF occurs within one hour as evaluated by CTC stain for cells undergoing AR as an indicator of capacitation.

Previous work in our group has examined the effects of human cumulus cells and hFF on sperm modulation and their relationship to pregnancy outcomes (Frettsome, 2012). Preliminary data from our group has shown that sperm incubated with hFF manifested darker protein bands on 10% SDS gel compared to sperm control samples (with no hFF) which may indicate that sperm acquire some of the hFF proteins.

Protein separation in this study was achieved using 1D-SDS-PAGE which is considered superior to 2D-PAGE in terms of number of protein identification and also its compatibility with the use of charged detergents. The use of 2D-PAGE in sperm membrane proteomic is not highly recommended because of the limited compatibility of this technique with the use of charged detergents to solubilise membrane proteins (Brewis and Gadella, 2010). Additionally, large and highly charged proteins are difficult to resolve in 2D-PAGE (Rabilloud, 2009). In a comparative study of capacitated versus ejaculated sperm using 2D-PAGE, Secciani et al. (2009) identified 58 sperm proteins of which only one was a membrane protein.

hFF proteins and sperm modulation: Sperm cells are transcriptionally and translationally inactive and they may rely on other sources of factors and proteins to achieve functional competence (Miller et al., 2010, Oliva and Castillo, 2011). In this study, sperm are shown to acquire hFF proteins which sperm never have and the putative

hFF proteins may induce modulation of sperm function. Although most of the published studies support the concept of hFF steroid hormones, specifically progesterone, to be responsible for the observed effects of hFF on sperm function (Morales et al., 1992, Fabbri et al., 1998). Nonetheless, there is a growing body of evidence to indicate the significance of proteins in hFF to participate in the induction of sperm functions (Hamdi et al., 2010). Apolipoproteins and high-density lipoproteins (HDL) in hFF have been suggested to play a role in sperm hyperactivation (Hamdi et al, 2010). Some of the other suggested mechanisms by which hFF may modulate sperm and induce capacitation include lipid transfer and enhancement of cholesterol efflux which may increase sperm cell membrane fluidity (Ravnik et al., 1992, Muller and Ravnik, 1995, Lepage et al., 1993, Hamamah et al., 1995). Follicular and oviductal fluids are rich in albumin and lipoproteins which have been shown to induce cholesterol efflux important for sperm capacitation (Ehrenwald et al., 1990, Visconti et al., 1999). In the current study, two of the hFF proteins (apolipoprotein L1 and complement component C6) which were found in the sperm + hFF samples belong to the transport activity which is defined in the PANTHER classification system as the ability of the protein to facilitate the directed movement of lipids into, out of or within a cell, or between cells. Lipid transport activity of proteins have been shown to play a role in facilitating sperm capacitation by enhancing cholesterol efflux (Hamdi et al, 2010). The role of these two hFF proteins may require further research to establish their effects on sperm modulation for capacitation. Additionally, the antioxidant properties of hFF has been described in the literature (Huang et al., 2014) and in this study one of the hFF sperm-bound proteins was haptoglobin which is known for its antioxidant characteristics (Jelena et al., 2013).

In the current study, comparison of protein profiles from sperm incubated in capacitating media alone (sperm control samples) and sperm incubated in the capacitation media along with hFF (sperm + hFF samples) was performed. This comparative proteomic analysis may reveal the global protein modification of sperm proteins following capacitation in the presence of hFF. It has been suggested that comparative proteomics analysis, in which sperm protein profiles from two sets of samples are compared, may enable the identification of sperm proteins with functional relevance (Aitken and Baker, 2008). Such comparative proteomic studies have been applied to compare sperm protein profiles from capacitated versus ejaculated sperm (Secciani et al, 2009) or fertile donors against infertile patients (Rajeev and Reddy, 2004, Pixton et al., 2004). The study of changes in sperm protein profile in response to exposure to body's milieus has also been recommended (Gadella, 2009). In the current study, the aim of the comparative proteomic analysis of sperm cells was to study sperm protein profile following exposure to hFF proteins and to identify any hFF in the sperm +hFF samples. Twenty-four hFF proteins were detected in the sperm + hFF samples following the concomitant incubation which suggest that, sperm may have uniquely acquired these proteins. The relevance of the putative hFF proteins to sperm fertilisation potential requires further investigation as illustrated in section 3.6. Some proteins were common proteins found in both sperm data and in hFF data in the literature and were detected in sperm + hFF samples in this study. The origin of these proteins in this study was not ascertained and they were not considered in the binding fraction of hFF. However, the potential of sperm cells to replace or add proteins they already have with some from the hFF proteins that common to both, require further research. During their transiet in the epididymis, sperm acquire some proteins which are similar to the testicular proteins which sperm already possess (Cooper and Young, 2006).

Similarly, we may suggest, from this study, that sperm may acquire hFF proteins which sperm already have.

Concluding remarks:

In summary, this study is the first to our knowledge to present comparative proteomic profile of sperm incubated in capacitating media alone versus sperm incubated in capacitation media with hFF. Identification of candidate hFF proteins may enhance our understanding of their role in sperm fertilisation. Supplementation of IVF media with the candidate hFF proteins will also be a promising approach prior to IVF to enhance sperm function and fertilisation. Alternatively, the knowledge of candidate hFF proteins which influence sperm fertilisation may enable the development of diagnostic tools of male infertility or design of male contraceptive.

Table 3-5 Methods of sperm purification for proteomic analysis in the literature, data taken from Amaral et al, 2014a, total number of studies 29

Technique of sperm cells purification	Number of studies utilised the technique	References
Density gradient centrifugation (Percoll or Pure Sperm)	**19**	Ficarro et al, 2003, Pixon et al, 2004, Martínez-Heredia et al., 2006, Lefievre et al, 2007, de Mateo et al, 2007, Li et al, 2007, Baker et al, 2007, Zhao et al, 2007, Martinez-Heredia et al, 2008, Chan et al, 2009, Liao et al, 2009; Frapsauce et al, 2009, Siva et al, 2010, Nixon et al, 2011; Redgrove et al, 2011, Wang et al. 2013, Amaral et al, 2013, Baker et al, 2013, Vigodner et al, 2013
Swim-up	**3**	Gu et al, 2011, Xu et al, 2012, Secciani et al, 2009
Both Percoll fractionation and/or swim-up	**2**	Naaby-Hansen et al, 2010, Naaby-Hansen and Herr, 2010
Washing and/or isolation of sperm subcellular compartments	**5**	Johnston et al, 2005, de Mateo et al, 2011, Kim et al , 2007, Parte et al, 2012

Chapter 4

SPERM METABOLOMICS

4.0 Abstract

Metabolomics enables the identification of low molecular weight exogenous and endogenous molecules involved in metabolism such as amino acids and peptides contained within a biological sample. Sperm metabolomics may provide understanding of sperm energy metabolism, biomarkers of infertility and insight into possible signalling mechanisms induced by metabolites released by sperm into the female reproductive tract (FRT). However, current knowledge of the human sperm metabolome is still very limited. The current study is the first to our knowledge to conduct a comparative metabolomic analysis of released sperm metabolites following one hour of swim-up and 6 hours of capacitation. Method: sperm cells were prepared by swim-up and adjusted to 6×10^6 sperm/ml. Three sample sets were prepared. Media alone controls which had not been exposed to sperm; TP1 in which sperm had 1 hour of swim-up and TP7 samples, where sperm has a further 6 hours capacitation time. All samples were centrifuged and the supernatant filtered through 0.22µm sterile filter before being frozen for subsequent metabolomics analysis. Reults: ninety-four statistically significant metabolites ($p < 0.05$) were detected in two sets of data (n=18) (the p-value was not adjusted for multiple calculations). The fold change of the metabolites concentrations (TP1 metabolite concentration/TP7 metabolite concentration) are reported. Fatty acids and oxidised fatty acids had statistically different concentrations between TP1 and TP7 samples. The results of this study indicate that sperm consume and release metabolites from/into the capacitation media. Further research for fertility diagnostics and into the effects of these metabolites on FRT signalling is warranted.

4.1 Introduction:

The omics fields, namely, genomics, transcriptomics, proteomics, and metabolomics are now the main techniques to study complex biological samples (Courant et al., 2013). Metabolomics is one of the comprehensive approaches, which enables the study of metabolites (low molecular weight exogenous and endogenous molecules involved in metabolism) such as amino acids and peptides in a biological sample (Courant et al., 2013, Nicholson et al., 2002). Discovery of diseases' biomarkers, understanding the biological pathways, drug development and investigation of disease pathology are some of the substantial applications of metabolomics (Zhang et al., 2012). The significance of metabolomics emerges from the fact that metabolites are the main building blocks involved in the regulation of the metabolic processes in human body which also provide valuable insights into the human metabolic phenotype (Dunn et al., 2011).

Metabolomics is a promising novel approach in scientific research, but the field is still in its infancy in terms of the available human metabolome databases compared to the human genome (Patti et al., 2012, Zhang et al., 2012). Hence, not all the currently identified metabolites are recorded in the available metabolomics databases which results in incomplete understanding of cellular metabolism (Baker, 2011, Kind et al., 2009). Currently, the human metabolome (defined as the complete quantitative identification of metabolites in a biological system) (Oliver et al., 1998) is estimated to have more than 40,000 metabolites (Wishart et al., 2013) with around seventy human-specific metabolic pathways involved (Ma et al., 2007). The high complexity of the human metabolome with massive diversity of metabolites including amino acids, peptides and nucleotides creates another challenge in metabolomics studies (Zhang et al., 2012). Nevertheless, considerable progress has been achieved in the metabolomics studies by the application of

advanced techniques that enabled better access to the human metabolome (Dunn et al., 2015). Several advanced techniques such as nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–mass spectrometry (GC–MS) and ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) have been applied in metabolomics with the latter two (GC–MS and UPLC–MS) being superior in accessing the whole metabolome (Dunn et al., 2015, Kell and Goodacre, 2014). This is attributed to the sample preparation in which the chromatographic separation of the thousands of human metabolites precedes the mass spectrometry analysis and increases the metabolome coverage of these techniques (Kell and Goodacre, 2014, Thiele et al., 2013, Dunn et al., 2015).

In metabolomics research, two approaches are currently applied which are targeted and untargeted metabolomics workflows with more recently a semi-targeted approach being recognised as an intermediate technique (Dunn et al., 2011). The untargeted approach is a comprehensive metabolic profiling of a whole range of metabolites in a biological sample which provides a wide range of metabolomics data important in generating hypothesis and expanding knowledge in certain aspects in the metabolic network (Dunn et al, 2011). By contrast, the targeted metabolomics approach starts with a specific hypothesis and aims to answer particular questions in which certain metabolites linked to specific pathways are quantified in the samples (Dudley et al., 2010). In the current study, an untargeted approach was utilised to accomplish a global overview of sperm metabolites during capacitation.

It is noteworthy that the metabolomics software applied in metabolite detection only provides p-value and fold change (FC) associated with changes in the concentrations of certain metabolites between classes of samples (Patti et al., 2012). To proceed further with

metabolite identification, the metabolite databases are searched using the accurate mass of the metabolite(s) of interest (Patti et al., 2012). Nonetheless, further confirmation of the metabolite identity is required as the database match only indicates a putative metabolite and comparison of the metabolites of interest's retention times and MS/MS data with that of a model compound may confirm the identity of the metabolite of interest (Patti et al, 2012).

In many cases a search may not return any matches due to the incomplete and evolving nature of current metabolite databases and therefore, complete identification of the entire metabolite profile in a sample is still challenging (Patti et al, 2012). Similar to other evolving technologies, metabolomics faces some challenges such as the incomplete identification of both the active metabolic pathways and the chemical structure of many of the detected metabolites (Deepinder et al., 2007, Hollywood et al., 2006). Additionally, the multivariate and complex nature of metabolomics data is another difficulty that necessitates special validations of the generated data prior to their upload into the databases (Deepinder et al., 2007). Both genetic and environmental factors are involved in the regulation of metabolism and any differences in these components among studied individuals may result in huge perturbations in the metabolomics data acquired (Johnson and Gonzalez, 2012). For example, differences in the individuals' diet, life style, stress patterns and gender have to be considered when designing metabolomics studies (Johnson and Gonzalez, 2012).

4.1.1 Metabolomics in reproductive biology:

Metabolomics studies have already made significant contributions in reproductive biology such as identification of serum metabolites linked to ovarian malignancy where leading to the discovery of six serum metabolites considered as potential biomarkers for early

diagnosis of the disease (Chen et al., 2011). The application of metabolomic techniques in semen profiling has the advantage of being a non-invasive and potentially cost effective strategy for semen quality assessment (Deepinder et al., 2007). Recently, some studies have identified certain metabolites such as superoxide anion (Iuliis et al., 2006) which is linked to defective sperm function and alanine, glycerophosphocholine, citrate, tyrosine and phenylalanine from seminal fluid as potential biomarkers for male infertility (Gupta et al., 2011b) amongst others (Courant et al, 2013). For a review of male infertility biomarkers in metabolomics see Lee and Foo (2014) and Bieniek et al., (2016).

Several other promising applications of metabolomics in reproductive biology may include testing of endometrial receptivity and fetal monitoring by analysis of samples from the endometrium and amniotic fluid respectively (Deepinder et al., 2007, Chu, 2016). One of the metabolomics studies on testicular tissue biopsies also indicated significant differences in the tissue concentration of the metabolites phosphocholine and taurine between testis with normal spermatogenesis (NS) and those with Sertoli-cell only syndrome (SCO) (Aaronson et al., 2010). This study suggested the potential of using this definable distinctive metabolic signature to differentiate between testis with NS and SCO and to diagnose non- obstructive azoospermia in a non-invasive way (Aaronson et al., 2010).

Previous work in our group has shown that sperm trigger calcium-signalling in oviductal tissue and that whatever induces this signal is a diffusible messenger released by sperm during capacitation (Connolly, 2012). The application of metabolomic and proteomic studies to investigate human sperm-FRT interactions has not been much considered in published research to date. The molecular basis of sperm-oviductal communication(s) are still poorly characterised in human (Huang et al., 2015) compared to other mammals in which a carbohydrate moiety on the surface of the oviduct has been characterised and

shown to bind a molecule on the sperm plasma membrane overlying the acrosome (Suarez, 2001, Reeve et al., 2003).

4.1.2 Sperm metabolomics:

Knowledge of the sperm metabolome is still limited as only a few studies in this relatively new field have applied metabolomic techniques to study sperm (Egea et al., 2014). Compared to more than thirty studies which have considered the human sperm proteome and have identified over 6000 proteins (Amaral et al, 2014a); sperm metabolomics is still in its infancy. Only one previous study has investigated the human sperm metabolome from sperm extract and identified 69 metabolites (Paiva et al, 2015). Metabolomic techniques were applied to determine sperm DNA damage caused by oxidative stress (Sanchez et al., 2012) and to compare oxidative DNA damage in semen and blood samples from fertile and infertile subjects (Guz et al., 2013). Metabolomic analyses of human seminal plasma from infertile patients receiving traditional Indian herbal medicines revealed twenty-three different metabolites (Gupta et al., 2011a, Gupta et al., 2013). Metabolomic studies of sperm occurred in rhesus monkey (Lin et al., 2009, Hung et al., 2009), boar (Marin et al., 2003) and goat (Patel et al., 1999) with only twenty metabolites being detected in total (Paiva et al., 2015).

Sperm cells have a high energy requirement to fuel their motility and function (Amaral et al., 2013). Following over 60 years of research into sperm energy metabolism since Mann (1946) identified that fructose rather than glucose is the carbohydrate substrate in seminal plasma, the mechanism of energy production for sperm motility has still not been fully characterised (Storey, 2008, Amaral et al., 2013). Two principal metabolic pathways are described: glycolysis which mainly occurs in the principal piece and oxidative phosphorylation in mitochondria (Storey et al, 2008, Amaral et al, 2013). However, the

principal energy sources of sperm motility under *in vivo* conditions in the FRT where sperm have been shown to survive for up to 120 hours (Gould et al., 1984) are yet to be elucidated (Amara et al, 2013).

Sperm energy metabolism:

Energy metabolism is a vital feature for sperm motility and signalling pathways (Miki, 2007, Goodson et al., 2012) which involves energy production and formation of essential substances for cell survival (Amaral et al, 2014a). Both glycolysis and oxidative phosphorylation are involved in ATP production by the sperm cells (Visconti, 2012). It has been shown that sperm may require different metabolic substrates and metabolic pathways during their different modification processes in the FRT (Miki, 2007). For example, capacitation has been shown to involve mainly glycolysis for ATP production in the presence of pyruvate (Hereng et al., 2011) while glucose and mannose are required for sperm hyperactivation and tyrosine phosphorylation in mouse (Goodson et al, 2012). In the absence of any metabolic substrates, the motility of sperm cells have been shown to decline as the ATP levels rapidly decrease and the cells are incapable to undergo hyperactivation (Goodson et al, 2012; Visconti, 2012). However, human sperm have been shown to survive in a culture medium of PBS for prolonged period of more than one week without any external energy substrates (Amaral et al., 2011) which may suggest the ability of sperm to utilise endogenous energy substrates for their energy requirement (Amaral et al, 2013).

4.2 Objectives:

To use an untargeted metabolomics approach to examine metabolites released and/or consumed by sperm following immediate swim-up preparation (TP1 samples) and following 6-hour incubation in capacitation media (TP7 samples) (to mimic the *in vivo* FRT physiology). A comparative metabolomics analysis of capacitation media alone, used as a control, compared to both TP1 and TP7 samples was conducted.

4.3 Methods

4.3.1 Materials:

Table 4-1

Product name	**Company**	**Product code**
Bovine Serum Albumin (BSA)	Millipore, Watford, UK.	82-002-4
Disposable sterile filter unit, membrane pore size 0.22µm	Millipore, Watford, UK.	SLGP033RS
Supplemented Earle's Balanced Salt Solution (sEBSS)	Geneflow, Fradley, UK	06-2010-03-1B
10 ml sterile syringes	Becton Dickinson Labware, New Jersey, USA.	302188
5ml Falcon round bottom tubes	Becton Dickinson, Labware, New Jersey, USA.	352054
15 ml Falcon tubes	Becton Dickinson Labware, New Jersey, USA.	352095

Table 4-2 Components of suplemented Earls Balanced Salt Solution (sEBSS)

Components	Molecular weight	Concentrations	
		g/L	mM
Sodium phosphate (NaH2PO4·2H2O)	156.01	0.158	1.01
Potassium chloride (KCl)	74.55	0.4	5.37
Magnesium sulphate (MgSO4·7H2O)	246.48	0.2	0.81
Dextrose (C6H12O6)	180.16	1	5.55
Sodium pyruvate (C3H3NaO3)	110.05	0.3	2.73
dl-lactic acid (C3H6O3)	112.06	4.68	41.76
Calcium chloride (CaCl2·2H2O)	147.02	0.264	1.80
Sodium bicarbonate (NaHCO3)	84.01	2.2	26.19
Sodium chloride (NaCl)	58.44	6.8	116.36

sEBSS : 285-295 mOsm, pH 7.15-7.45, produced by Geneflow, Fradely, UK. 0.3% BSA was added before use for final capacitating formulation (sEBSS + 0.3 % BSA).

4.3.2 Sperm preparations:

All semen samples were obtained in accordance with HFEA Code of Practice in this project (see section 3.3.2 for semen collection procedure). Semen samples were allowed to liquefy for approximately 30 minutes and were prepared by swim-up (Mortimer, 1994). Briefly, 1ml of sEBSS + 0.3 % BSA was under-layered with 400 µl of liquefied semen in 5ml Falcon round-bottom tubes and left to incubate for 1 hour at an angle of 45° at 37 °C, 6% CO_2. The top 700 µl containing motile sperm fraction was gently harvested using sterile transfer pipette and pooled into 15 ml polystyrene Falcon tube. Sperm concentration in the collected fraction was assessed by a Neubauer hemocytometer and the final concentration adjusted to 6×10^6 sperm/ml using sEBSS + 0.3% BSA. Three different sets of samples were then prepared as follows: media alone controls (sEBSS + 0.3% BSA) which had not been exposed to sperm; TP1 in which sperm had 1 hour of swim-up and TP7 samples, where sperm has a further 6 hours capacitation time at 37 °C, 6% CO_2. All samples were centrifuged for 5 minutes at 500g and the supernatants filtered through 0.22 µm pore size sterile filter to remove any sperm before being frozen for subsequent metabolomics analysis. **Figure 4.1** summarises the methodology of samples preparations. Nine different sperm donors were used in the samples preparations (n = 18). For the purpose of metabolomics data analysis, the samples from the first 13 experiments (n = 13) were sent for metabolomics analysis as a first set of data. The second set of data (n = 5) were sent for metabolomics analysis a few months later. The results from both data sets were compiled and analysed as shown in the results section. The chemical contents of the capacitation media (sEBSS + 0.3 % BSA) are included in **table 4-2** in the material section 4.2.1.

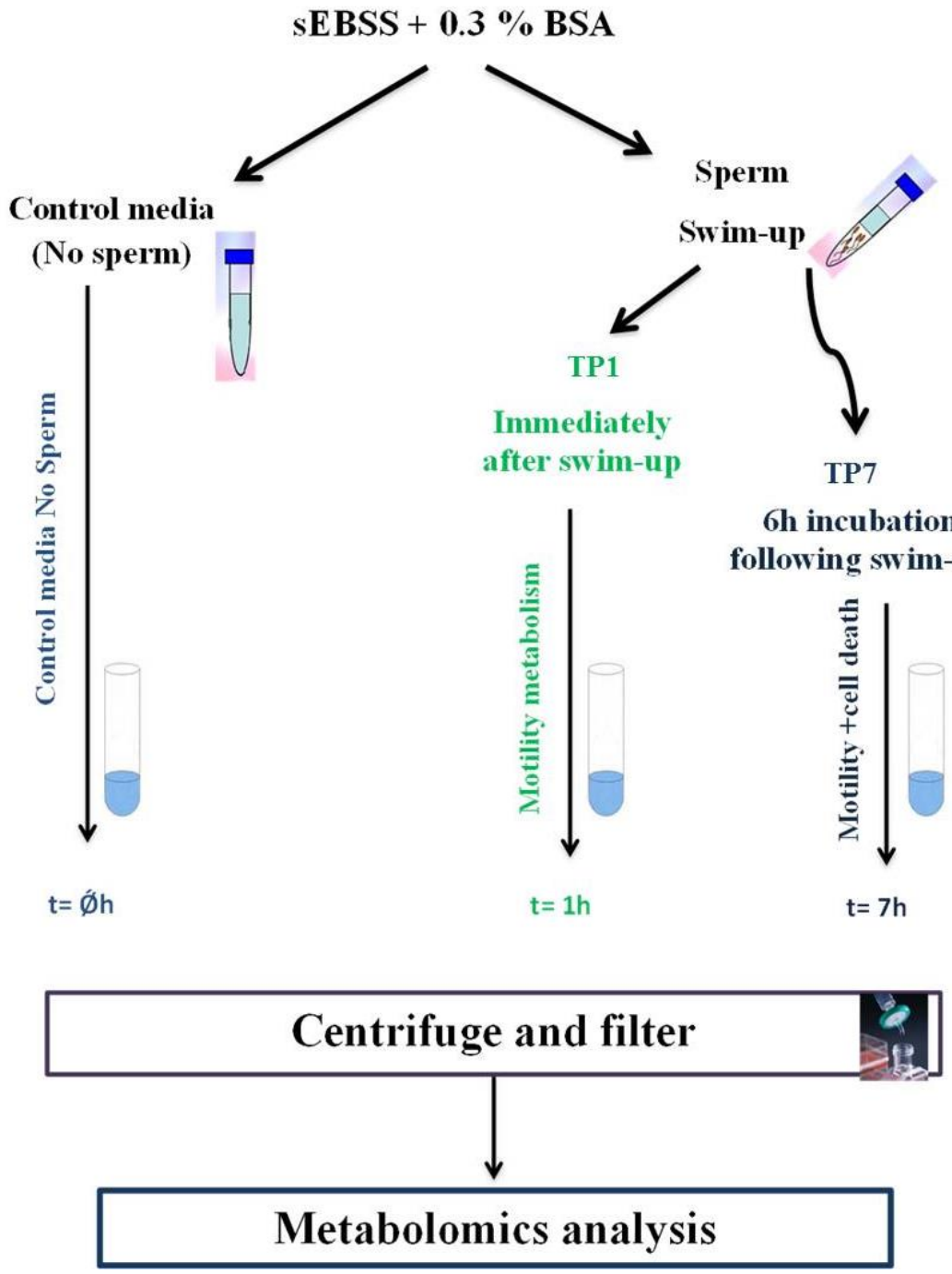


Figure 4-1 Summary of the methodology of sperm sample preparations for metabolomics analysis. Three sets of samples were prepared: Control media (no sperm), TP1: 1 hour swim-up and TP7: 6 hours capacitation following 1 hour swim-up

4.3.3 Metabolic profiling of samples:

The metabolomics analysis was performed by Dr Warwick Dunn in Phenome Centre Birmingham at the University of Birmingham using untargeted metabolomics approach.

Metabolomics analysis was performed using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) followed by statistical analysis to identify statistically significant metabolites and fold changes of the detected metabolites and to compare the three sets of samples in terms of metabolic profiles.

Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS):

Samples were thawed on ice for approximately 60 minutes and de-proteinised by addition of 600µl of methanol (pre-chilled to -20°C for 24h; HPLC grade, Sigma-Aldrich Chromasolv) to 200µl of cell media followed by vortexing (15 seconds), centrifugation (15-minutes, 13500 xg, 3°C) and drying of 600µl of the supernatant (Thermo Scientific Savant SpeedVac Concentrator SPD111V). A single pooled quality control (QC) sample was prepared by combining 120µl aliquots of each biological sample followed by vortex mixing for one minute. 200µl aliquots were prepared in an identical process as described above. All samples were analysed applying Ultra Performance Liquid Chromatography (Ultimate3000RS; Thermo Scientific, Hemel Hempstead, UK) interfaced to an electrospray mass spectrometry (Q Exactive Focus, Thermo Scientific, Hemel Hempstead, UK). Samples were reconstituted in 100µl 80/20 methanol/water and analysed with QC samples analysed ten times at the start of the batch (for system equilibration and MS/MS data acquisition), followed by injection after every 5th sample and finally two QC samples were analysed at the end of the analytical batch. Samples were randomised across a single analytical batch for analysis. The mass spectrometer was tuned and calibrated applying standard procedures and solutions as defined by Thermo Scientific. UPLC separations

were performed applying a Hypersil Gold C18 reversed phase column (100 x 2.1 mm, 1.9 mm) at a flow rate of 400 mL.min-1, temperature of 40°C and with two solvents: solvent A (HPLC grade water +0.1% formic acid) and solvent B (HPLC grade methanol +0.1% formic acid). A gradient elution was performed as follows: hold 100% A 0–1.5 min, 100% A–100% B 1.5–6 min curve 3, hold 100% B 6–12 min, 100% B–100% A 12–13 min curve 3, hold 100% A 13–15 min. Injection volume was 5µL. UPLC eluent was introduced directly into the electrospray mass spectrometer with source conditions as follows: spray voltage -4.0 kV (ESI-) and +4.5 kV (ESI+), sheath gas 30 arbitrary units, aux gas 15 arbitrary units, capillary voltage 35 V, tube lens voltage -100 V (ESI-) and +90 V (ESI+), capillary temperature 280°C, ESI heater temperature 300°C. Data were acquired in ion mode switching in the m/z range 100–1000 at a mass resolution of 35 000 (FWHM defined at m/z 200), with a scan speed of 0.4 s and an AGC setting of 1 x 106.

Data pre-processing:

Data were processed applying XCMS as described previously (Dunn et al., 2008). All metabolites reporting a relative standard deviation (RSD) > 20% and which were detected in <70% of QC samples from injection nine onwards were removed. Metabolites were annotated applying PUTMEDID_LCMS (Brown et al., 2011). All metabolites were annotated according to level 2 (putatively annotated metabolites) as defined by the Metabolomics Standards Initiative (Sumner et al., 2007). Statistically significant metabolites were grouped into classes related to chemical structure or metabolic function similarity.

Univariate and multivariate analysis:

Processed data was analysed using paired Mann-Whitney test for statistical significance. Application of a critical p-value of <0.05 without the application of FDR correction was

performed. The p-value was not adjusted for multiple calculations in these data as the number of samples was low in each class and corrections do not work well with small numbers of samples (Dr Dunn, personal communication). Calculation of the fold changes was performed using the equation: median peak area at TP1 hour /median peak area at TP7.

4.4 Results:

The preliminary results of the current study have revealed differences in the metabolomics level between capacitated (TP7 samples) and sperm immediately prepared following swim-up (TP1 samples) across the 18 replicates. A total of 203 metabolites have been detected in the sperm incubation media from two sets of experiments (1st set n = 13, 2nd set n = 5). There were certain classes of metabolites which were statistically significant (p-values of ≤ 0.05) and showed the same relative change (upregulated or downregulated) throughout the study samples namely, fatty acids, oxidised fatty acids and peptides. Multiply charged metabolites (most likely peptides/proteins) also showed the same statistically significant changes in both sets of data, however, the exact identity of these metabolites is yet to be identified. Amino acid metabolism, glycerophospholipids, arginine and proline metabolism, sterol and steroid metabolism and sulphur metabolism were among the other classes of metabolites identified in this study. Arginine and proline metabolism and amino acid metabolism show a major change in the level only in one set of data (second set of experiments, n = 5).

For the purpose of data analysis, four classes of samples were analysed; quality samples (QC) applied for quality control; media or control sample (sEBSS + 0.3 %BSA); TP1 is the 1 hour samples following swim-up and TP7 is the 7-hour samples (6-hour capacitation following swim-up). The concentration of the metabolites in the TP1 and TP7 samples

were compared using paired Mann-Whitney test with statistical significance at a p.value ≤ 0.05 (appendix 5). For all the metabolites which manifested a statistically significant differences between TP1 and TP7 samples in the 2nd set of data, a boxplot analysis for all the sample classes analysed (QC, media, TP1 and TP7) was performed. The boxplot analysis demonstrated whether the identified metabolites have higher median concentration in the TP1 samples (immediately following swim-up) or in the TP7 samples (following 6 hours of capacitation) or in the original media (control samples with no sperm). From the boxplot analysis, 46 (16 known identity and 30 multiply charged most likely peptide/proteins) metabolites showed higher median concentration in the TP1 samples compared to both the control media and TP7 samples. 41 Metabolites (38 known identity and 3 multiply charged most likely peptide/proteins) had higher median concentrations in the TP7 samples compared to both the control media and TP1 samples. Seven metabolites showed higher median concentration in the media (control, no sperm) compared to TP1 and TP7. Details of the metabolites identity and the boxplot figures are shown in **table 4-3** and **figures 4-2, 4-3, 4-4, 4-5**, **4-6, 4-7, 4-8 4-9, 4-10, 4-11, 4-12 and 4-13**. These results may indicate that sperm release or secrete certain metabolites while consuming others. This is manifested by the higher concentration of certain metabolites following the 7-hours incubation period of sperm (TP7) which may indicate that sperm have released these metabolites into the media. Forty-one metabolites demonstrated higher median concentration in the TP7 samples with a p.value ≤ 0.05. In contrast, some metabolites were found in lower concentrations in the TP7 samples compared to the TP1 samples which may indicate that these metabolites have been consumed by the sperm. Forty-six metabolites with a p.value ≤ 0.05 belong to this category. Another set of

metabolites were highest in the control media with no sperm which may suggest they have been consumed by sperm in the swim-up (TP1) samples and in the TP7 samples.

Table 4-3 Details of number of metabolites and their classes in each sample type

Sample type	Number of metabolites	Metabolite class
TP1	**31**	Possible protein and peptide
	6	Fatty acids and oxidised fatty acids
	4	Peptides
	2	Glycerophospholipids
	1	hormones
	1	Vitamin D metabolism
	1	others
Total	**46**	
TP7	**9**	Fatty acids and oxidised fatty acids
	7	Mixed class
	5	Other class
	4	Amino acid metabolism
	3	Glycerophospholipids
	3	Arginine and proline metabolism
	3	Possible protein and peptides
	2	Sterol and steroid metabolism
	2	Carbohydrate
	1	Sulphur metabolism
	1	Peptides
	1	Acyl carnitines
Total	**41**	
Media	**2**	Sulphur metabolism
	2	Others
	1	Sterol and steroid metabolism
	1	Peptides
	1	Mixed class
Total	**7**	

Figure 4-2 Metabolites with the median concentration highest in the TP1 samples (one hour swim-up) compared to the TP7 samples (7 h-hour capacitation) and media (control).

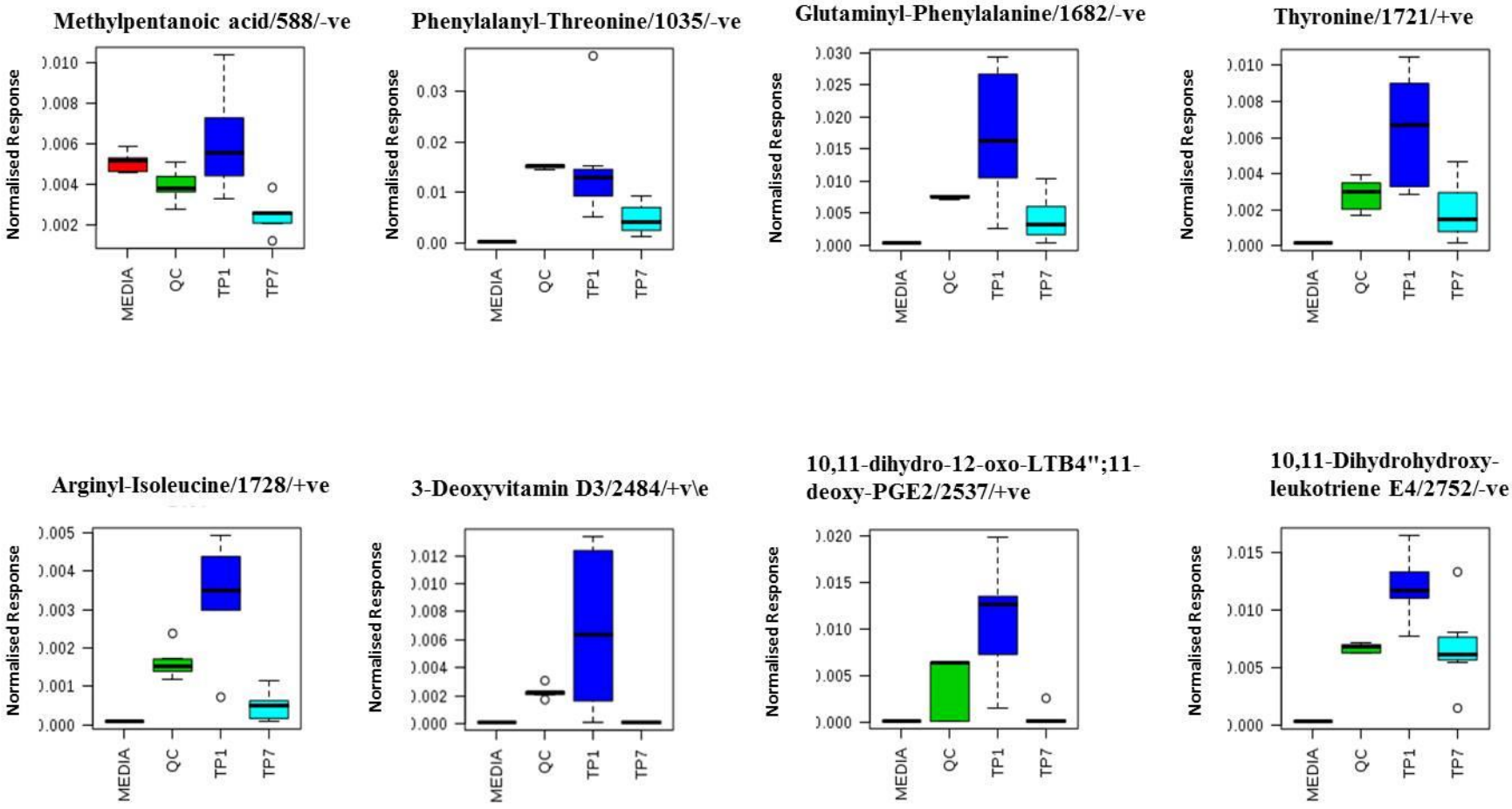


Figure 4-3 Metabolites with the median concentration is highest in the TP1 samples (one hour swim-up) compared to the TP7 samples (7 h hour capacitation) and media (control)

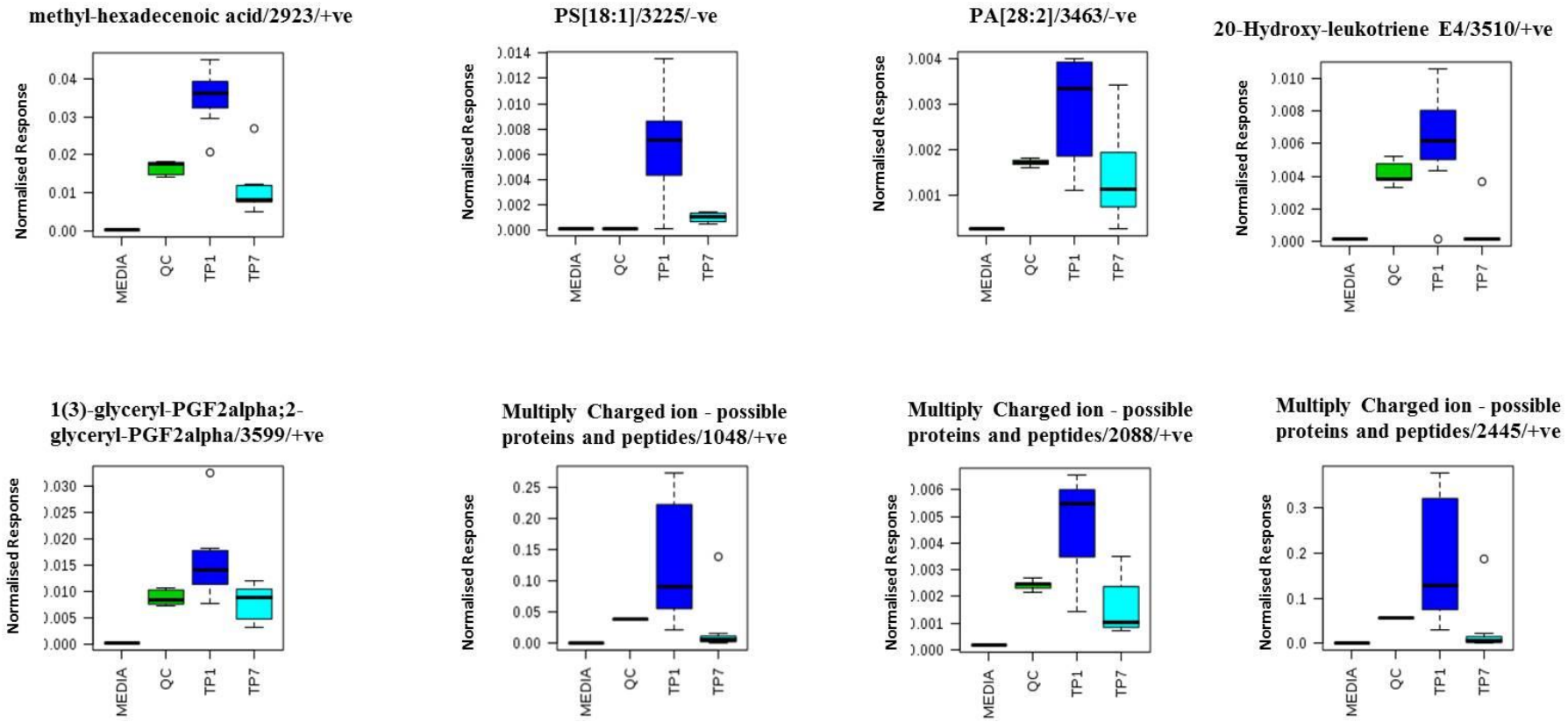
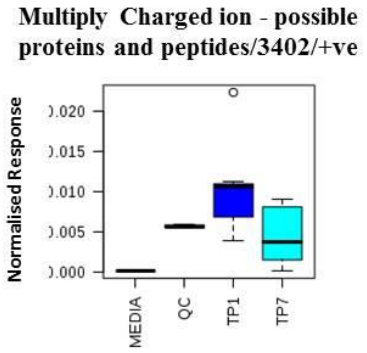
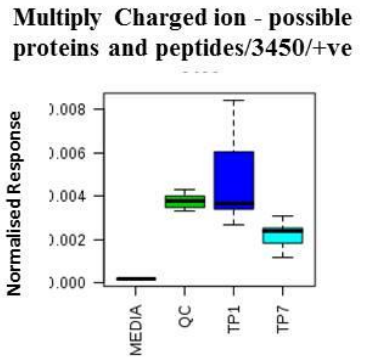
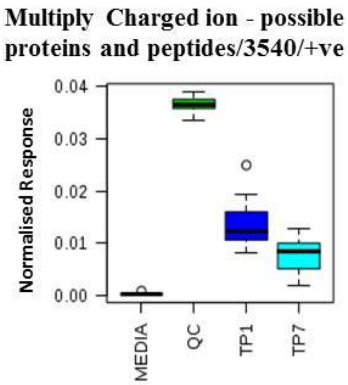
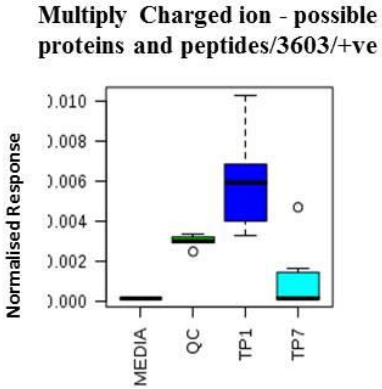


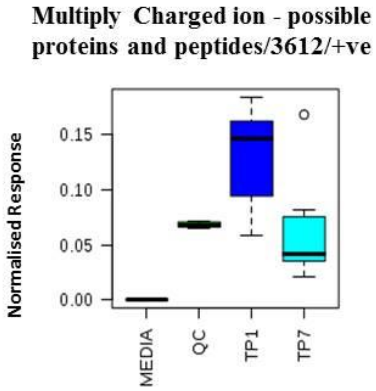
Figure 4-4 Metabolites with the median concentration is highest in the TP1 samples (one hour swim-up) compared to the TP7 samples (7 h-hour capacitation) and media (control)

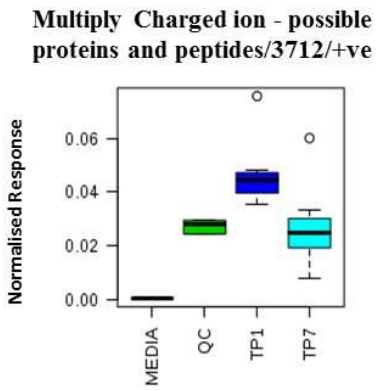


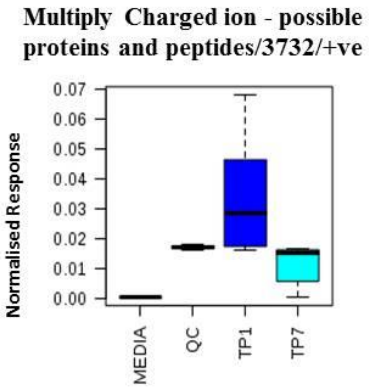












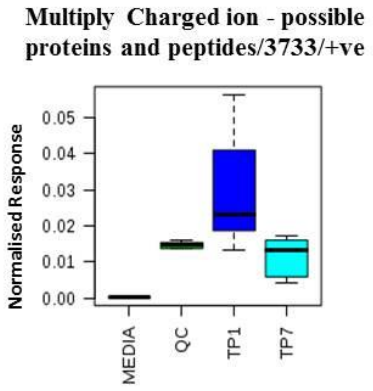


Figure 4-5 Metabolites with the median concentration is highest in the TP1 (one hour swim-up) compared to the TP7 samples (7 h-hour capacitation) and media (control)

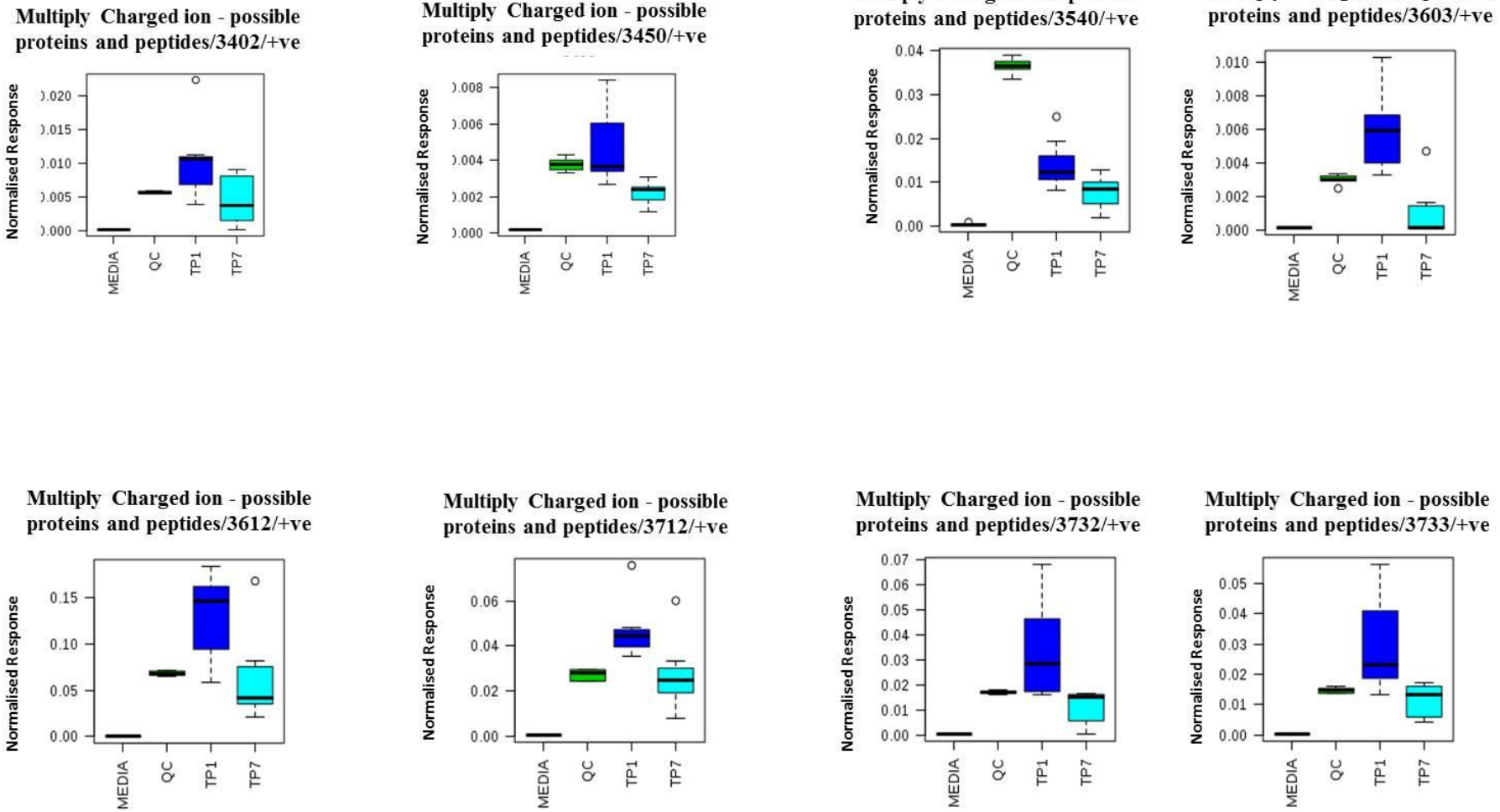


Figure 4-6 Metabolites with the median concentration is highest in the TP1 samples (one hour swim-up) compared to the TP7 samples (7 h-hour capacitation) and media (control)

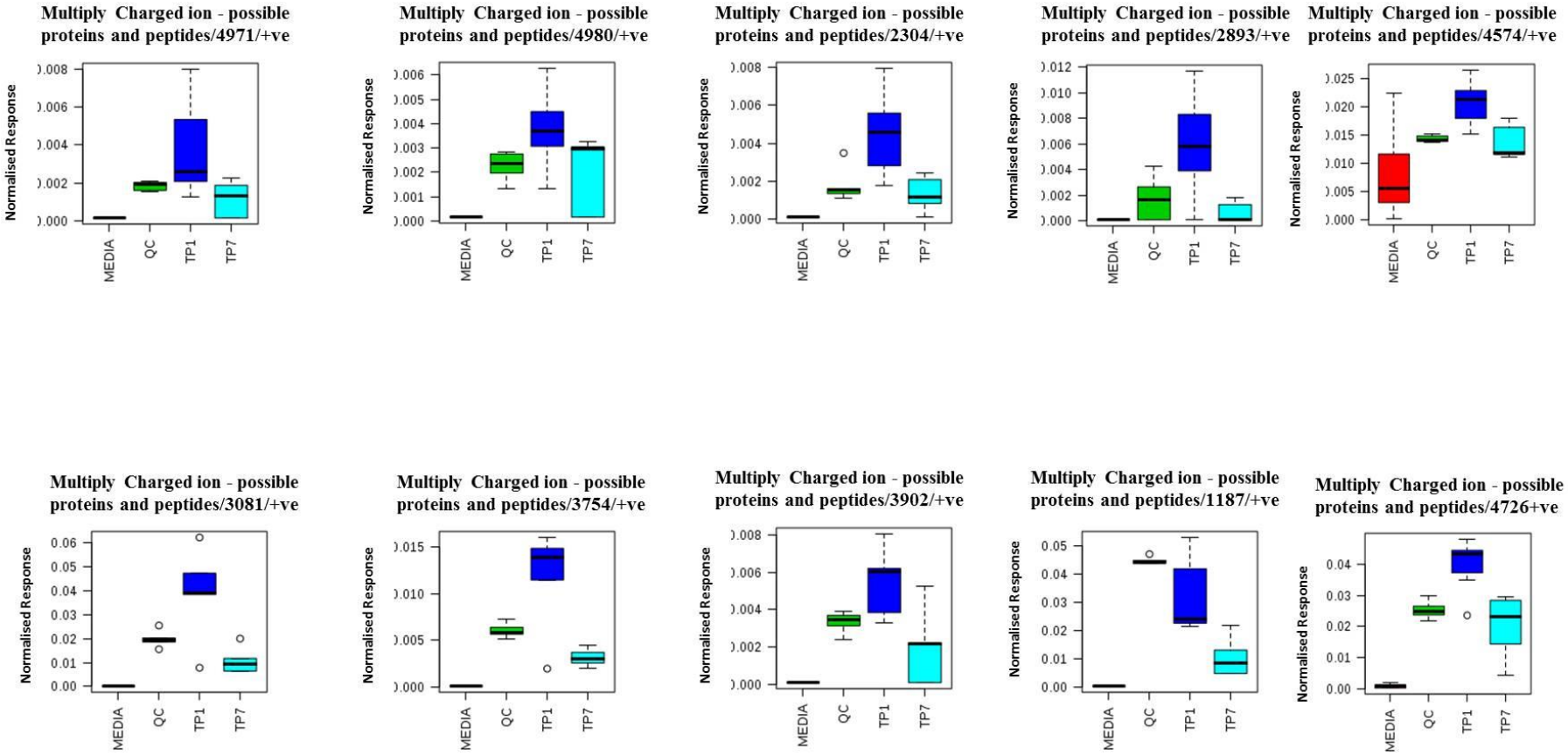


Figure 4-7 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one hour swim-up) samples and control media

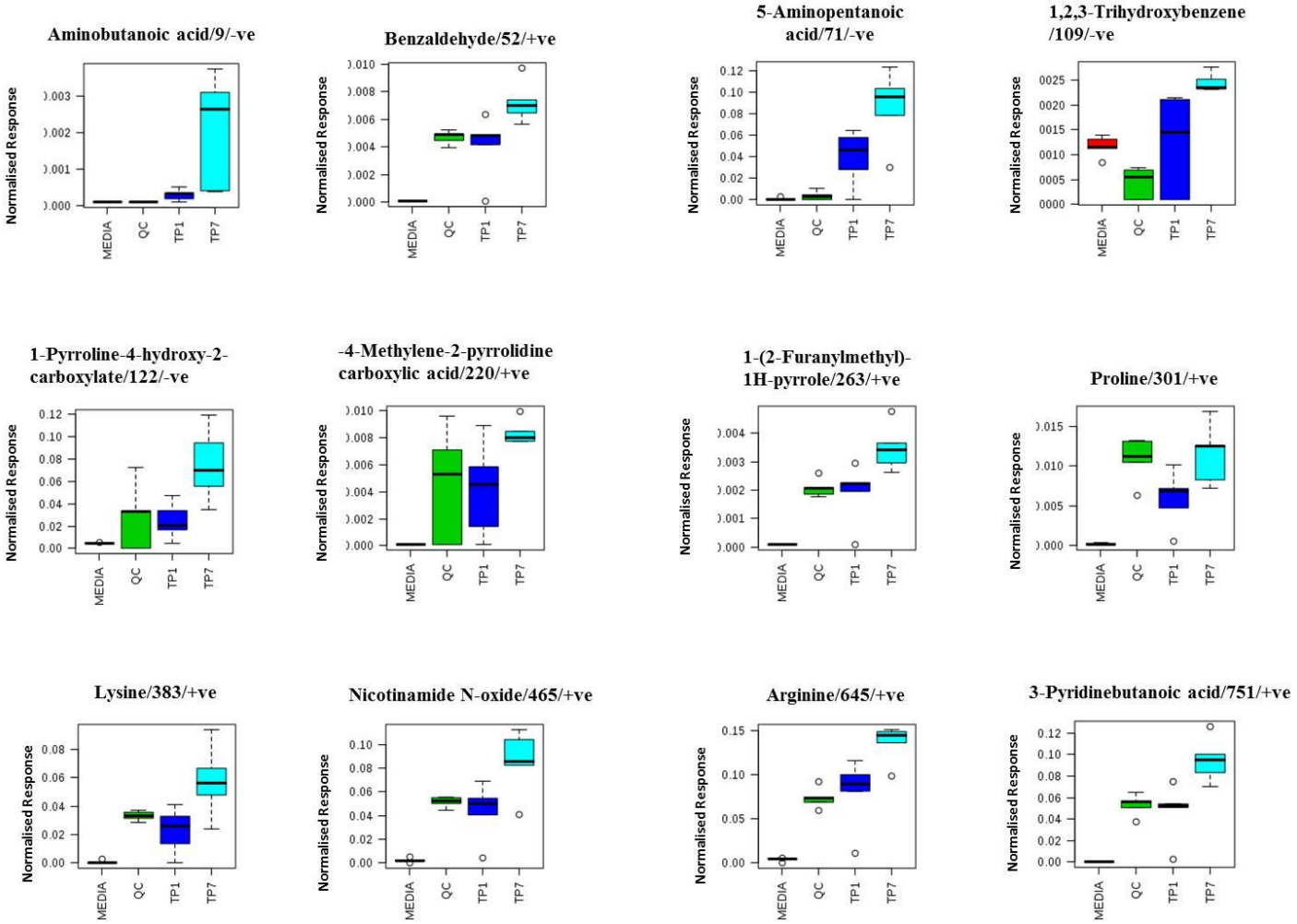


Figure 4-8 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media

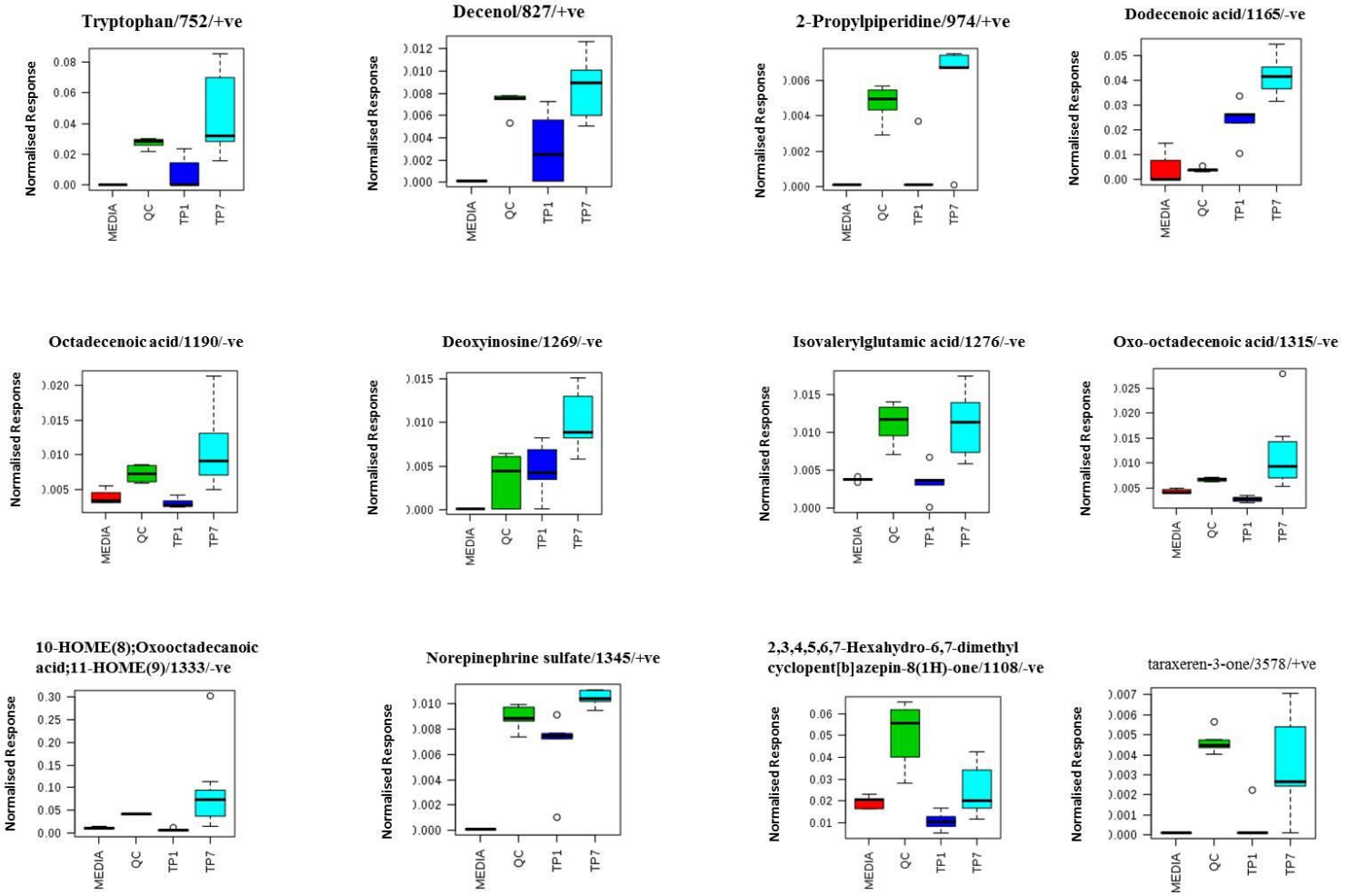


Figure 4-9 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media

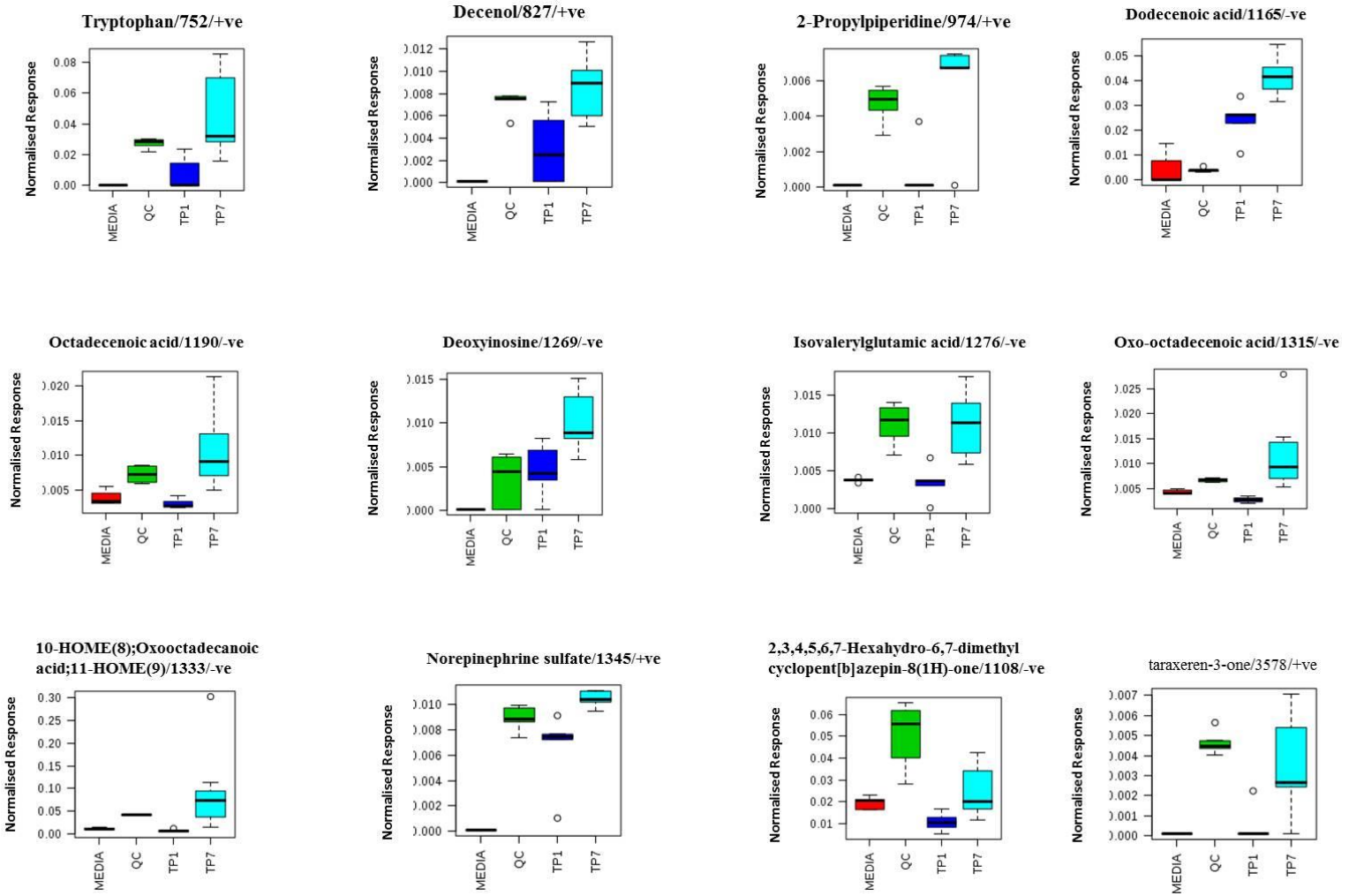


Figure 4-10 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media

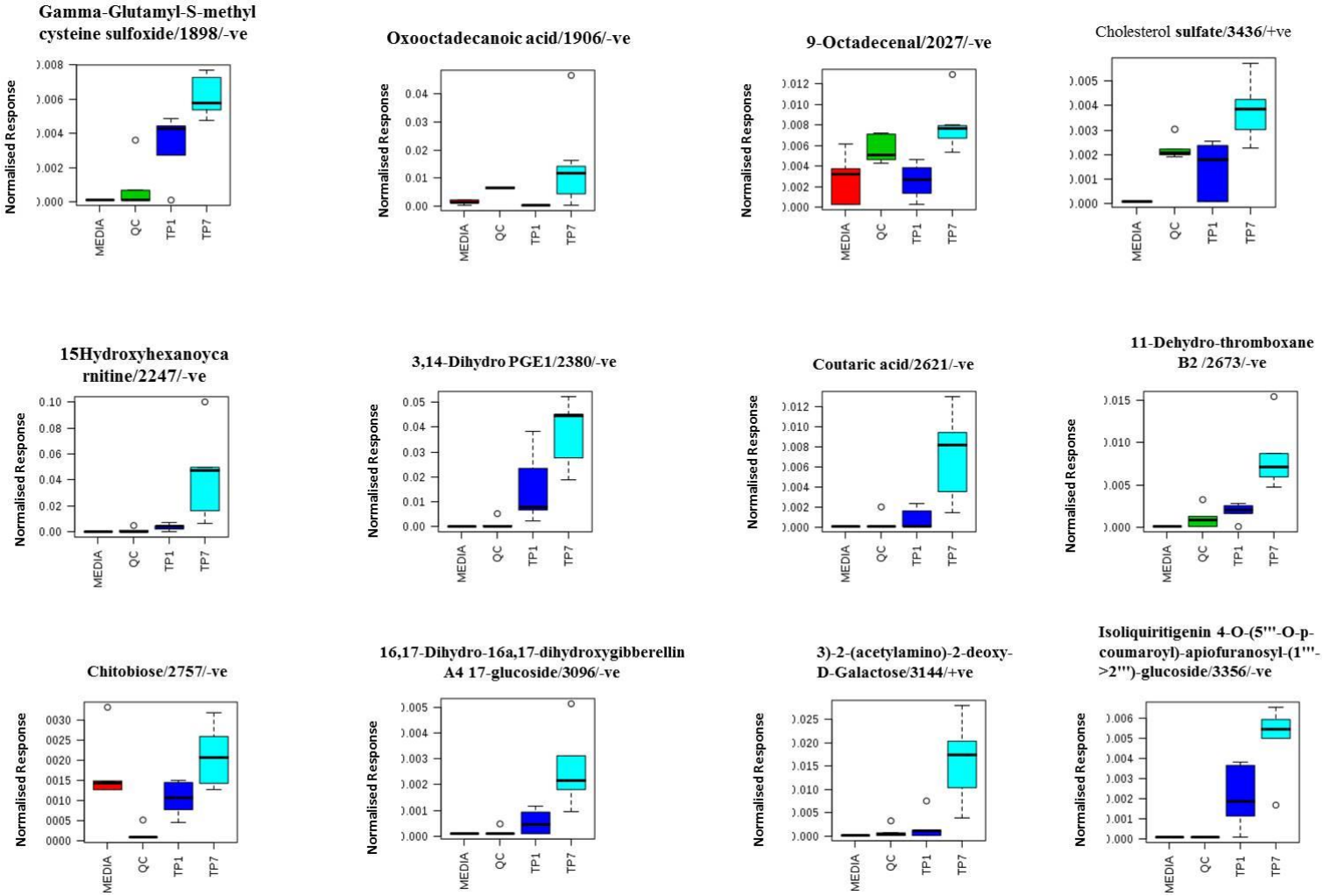


Figure 4-11 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media

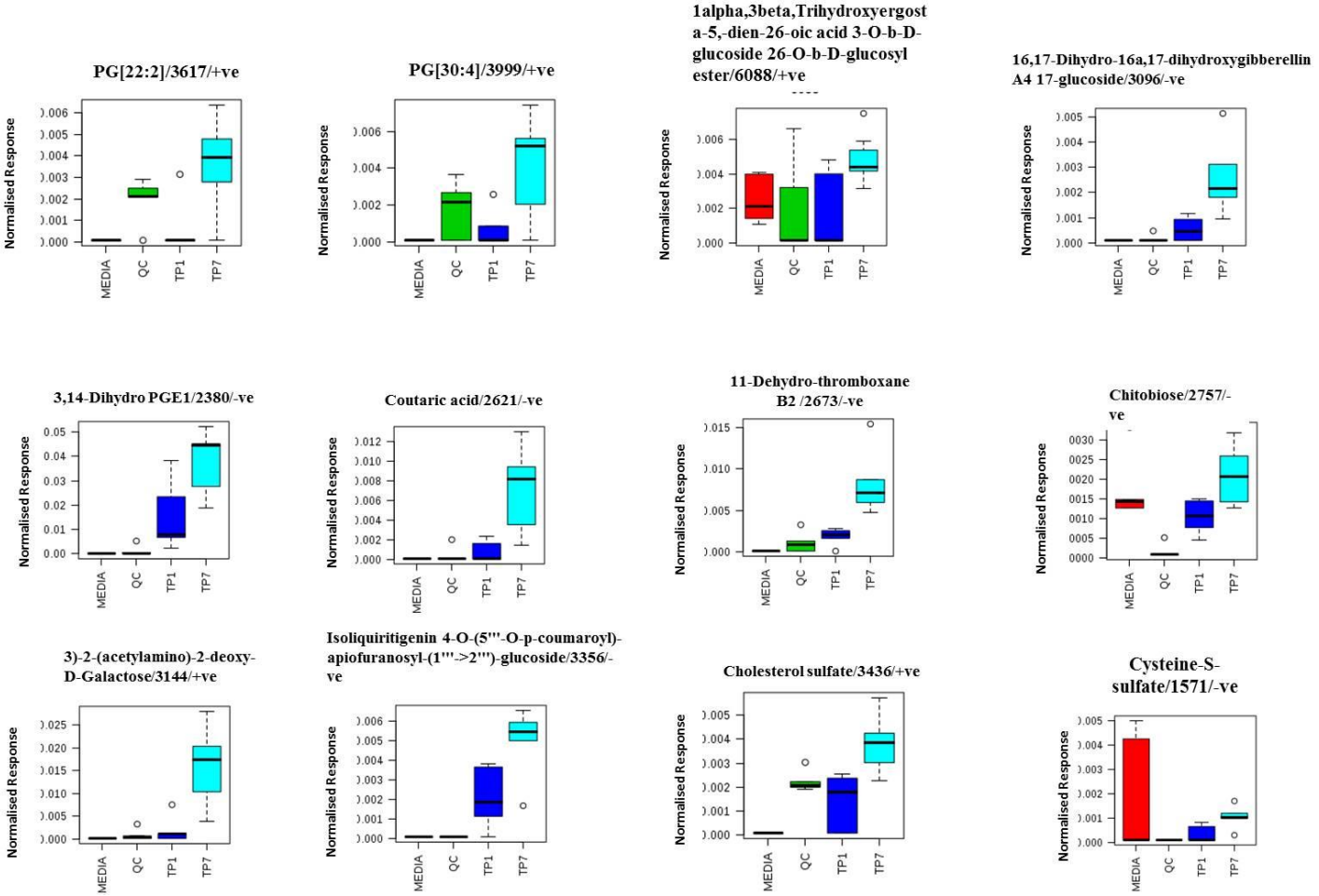


Figure 4-12 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media

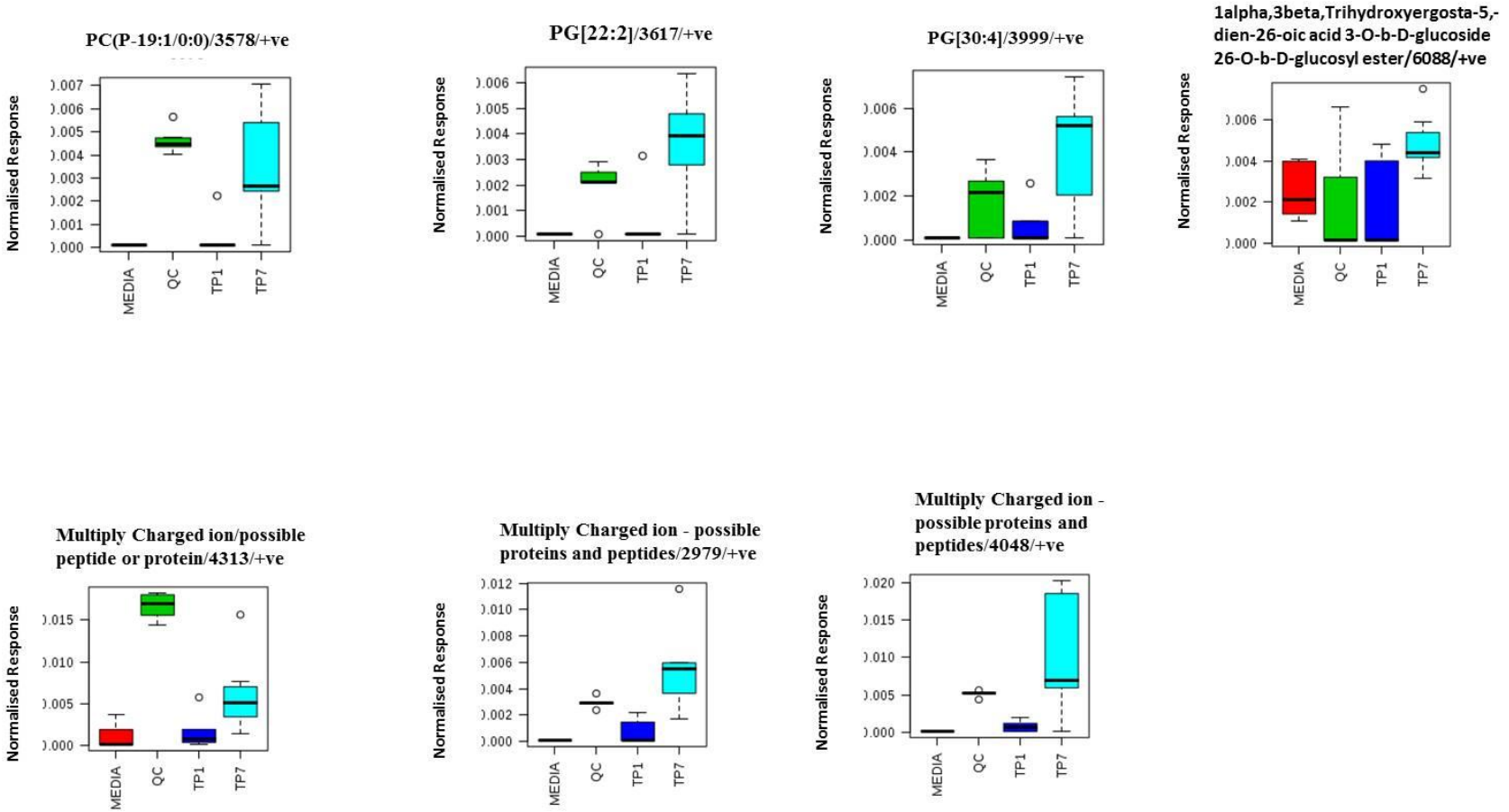
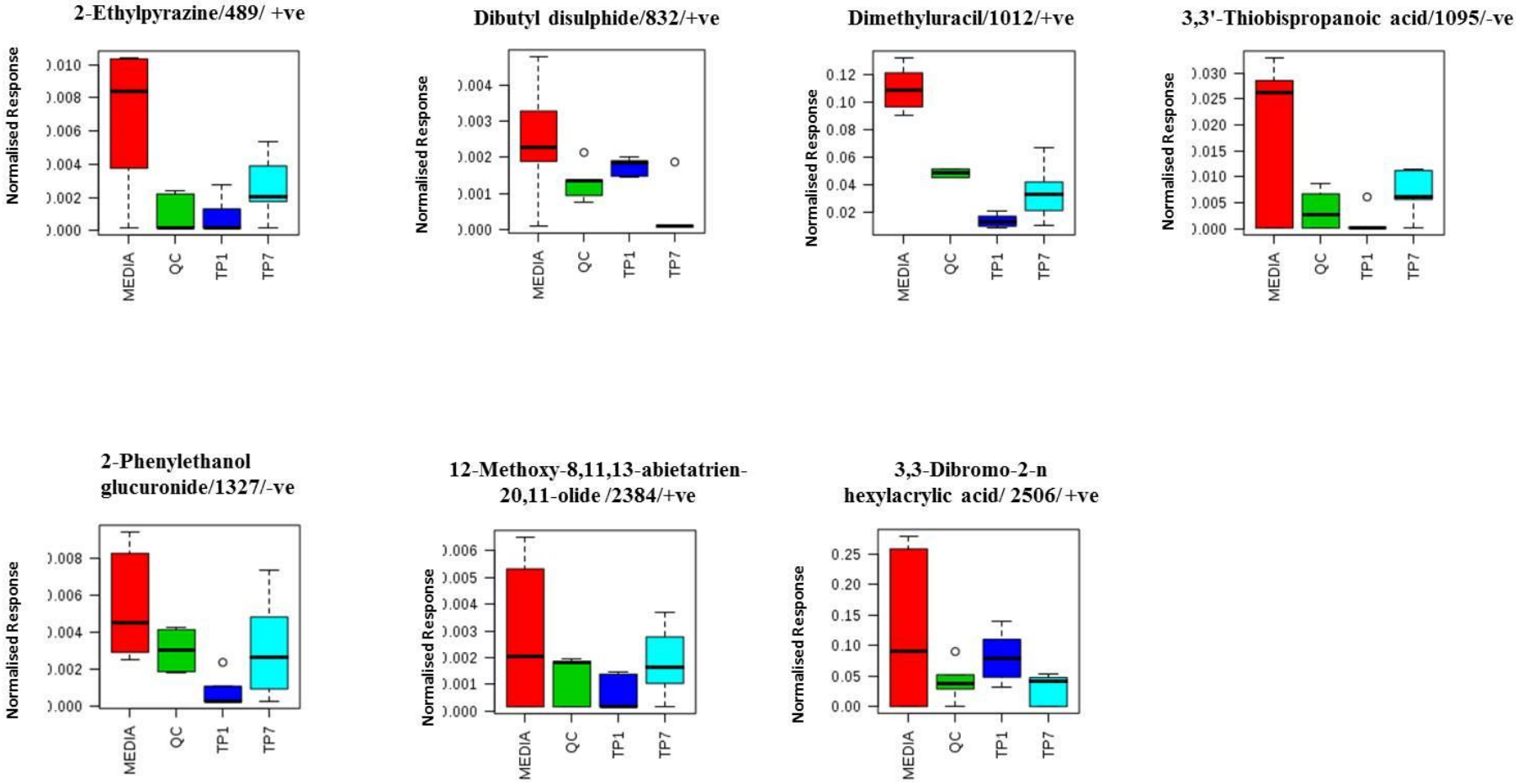


Figure 4-13 Metabolites with the median concentration is highest in the TP7 (7-hour capacitation) samples compared to the TP1 (one-hour swim-up) samples and control media



4.5 Discussion:

To the best of our knowledge, this is the first human sperm metabolomics study conducted to investigate the metabolite changes during sperm capacitation. While more than 30 studies have considered the human sperm proteome and have identified thousands of sperm proteins as mentioned earlier (Amaral et al, 2014a), only one previous study has investigated the human sperm metabolome (Paiva et al, 2015).

Variation in the metabolites detected in both data sets:

In the current study, both data sample sets were prepared and analysed in an identical methodology and the only reason to separate them was that the 1st data set was prepared in January 2016 while the 2nd was in July 2016. However, none of the metabolites was detected in both sets of data. This could be attributed to the high intra- and inter-individual variabilities, which have been generally observed in previous metabolomics studies of human samples (Courant et al, 2013). Additionally, physiological reasons such as the generally observed heterogeneity in semen samples with inter- and intra-variability being well documented (Holt and Van Look, 2004; Secciani et al, 2009) may be the reason for the differences in metabolites detected in the two data sets. The fact that no results were duplicated across the sets would seem to imply that a wide range of pathways and therefore metabolites are available within a sperm, only further work would reveal whether certain ones occur in specific individuals or phenotypes. Further replicates would presumably provide a more comprehensive and coherent dataset.

Human metabolomes are highly diverse compared to the genome and proteome due to the effects of the environmental factors such as diet which highly contribute to the metabolome and make the characterisation of the human metabolomes challenging (Wishart et al, 2013). It may be that this has also influenced our data, though this would

imply diet having a long-term effect on sperm cell metabolism after their removal from the body fluids.

The comparison of the metabolites detected in the current study with those detected in Paiva et al (2015) did not also show any similar metabolites. This could be attributed to the difference in the sample type (human sperm extract in Paiva et al, (2015) study of endogenous sperm metabolites versus exogenous metabolites in human sperm incubation media in the current study). The differences in the techniques applied (NMR and GC-MS versus UPLC-MS) in both studies may also be another reason for the dissimilarity of the metabolites detected.

Eighteen replicates (5 and 13 replicates in the 1st and 2nd data sets) were analysed in the current study with 203 metabolites were detected. This could be due to the considerably smaller number of metabolites when compared to the number of proteins in the cells (Kouskoumvekaki and Panagiotou, 2011; Wishart et al., 2013). One previous study on metabolomics of human sperm extract revealed 69 sperm metabolites out of 15 sperm samples (n =9) with only four metabolites detected by both techniques (proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and gas chromatography coupled to mass spectrometry (GC-MS) applied in the study (Paiva et al, 2015).

Discussion of the methodological choice:

The choice of using untargeted metabolomics approach in this study aims to obtain a global view of the metabolites released/consumed in sperm capacitation media at the two time points since this is the first study, to our knowledge, to investigate human sperm metabolomics. Mass spectrometry has been shown to have higher sensitivity than NMR

spectroscopy with sub-nanomolar concentrations of metabolites for the MS compared to sub-millimolar concentrations detected by NMR spectroscopy (McRae et al., 2012).

The study design in terms of capacitation media utilised and the incubation time of 6 hours were selected to best mimic physiological conditions in the FRT. In this study, sperm cells were prepared by swim-up to enable selection of the best motile fraction of sperm, representative of those that may accomplish their journey in the FRT. The metabolites detected in higher concentration in the TP1 samples, therefore, may represent the metabolites released by sperm during active motility and homeostatic energy metabolism, the lack of their detection later may imply that they can also be consumed by sperm at other stages of their metabolic cycle. Metabolites detected in higher concentration in the TP7 samples may additionally represent those released by sperm following capacitation and cell death. Metabolites which had highest concentration in the control media may represent substrates that have been consumed by sperm following swim-up and 6 hour capacitation. Future research may help confirm the identity of metabolites detected in the three cases.

The background chemical noise was not a problem for the detection of metabolites or the annotation of metabolites. This is untargeted metabolomics where the analysis never provides a name to all detected metabolites and have a global view of the contents analysed. Some of the expected metabolites such as ATP were not detected in this study. ATP is an intracellular metabolite and is not detected in extracellular samples as cells do not secrete ATP. As we were studying the sperm capacitation media, ATP was not detected.

Relevance of the detected sperm metabolites to future applications:

In understanding sperm energy metabolism: Research into sperm metabolomics is significant in unravelling the uncertainty surrounding sperm energy metabolism and understanding the vital biological process sperm undergo to achieve fertilisation (Paiva et al, 2015). The study of the human sperm metabolome under *in vitro* conditions may also enable characterisation of the metabolic substrates consumed or released by sperm for better understanding of sperm energy metabolism. This study is the first, to our knowledge, to conduct a comparative metabolomics study of human sperm metabolites detected in sperm capacitation media following 1h swim-up versus 6 hours capacitation.

Knowledge of the human sperm metabolome is still limited as only a few studies in this relatively new field have applied metabolomics techniques to study human sperm (Egea et al., 2014). The identified human sperm metabolites in this study may have relevance to future studies investigating human sperm energy metabolism during capacitation and identification of capacitation biomarkers. In the current study, 203 human sperm metabolites were detected in the capacitation media following 1-hour swim-up and 6-hours capacitation. The relevance of these detected metabolites to sperm metabolism is still elusive. In the current study, fatty acids and oxidised fatty acids were among the metabolites which show statistically significant fold changes (up and down regulations) in the concentrations between TP1 and TP7 samples. The role of the identified fatty acids and oxidised fatty acids in sperm energy metabolism for energy production during motility and capacitation, therefore, deserve further research. Fatty acid oxidation of endogenous substrates is a form of metabolic pathway which has been suggested to operate in human sperm in the lack of exogenous energy substrates (Amaral et al, 2013). Fatty acid

oxidation is involved in the modulation and transfer of fatty acids and acyl groups into mitochondria for beta-oxidation (Paiva et al, 2015). Lipid metabolism has been suggested as an important mechanism for sperm energy production (Amaral et al, 2014a; Amaral et al, 2014b). Incubation of human sperm with etomoxir, an inhibitor of fatty acid oxidation, resulted in a dramatic decline in sperm motility which indicated the significance of fatty acid oxidation for human sperm energy production (Amaral et a, 2013). Some studies reported a correlation between lower levels of certain sperm metabolites, mainly L-carnitine, in semen and male infertility (Matalliotakis et al., 2000; Li et al., 2007). Future studies may identify the significance of other sperm metabolites to male reproductive health.

In the female reproductive tract signalling: a significant application of sperm metabolomics is to study sperm signalling in the FRT. The importance of sperm-oviduct interaction cannot be undermined as numerous studies have shown the beneficial effects on sperm following co-incubation with oviductal epithelial cells such as enhanced motility (Yao et al., 2000, Yeung et al., 1994) and prolonged viability (Morales et al., 1996).

Key for my findings is that human sperm have recently been shown to induce calcium signalling in uterine and oviductal cells, but importantly this response could also be induced by culture media conditioned by their presence - indicating a possible role for sperm metabolites in this signalling (Connolly, 2012). It is quite possible that the signals observed in Connolly (2012) would cause sperm-induced changes in endometrial proteome. In turn these would go on to influence implantation or future child health as per the observations in murine species in response to seminal plasma (Robertson, 2014, Schjenken and Robertson, 2015). A next stage for this research would be to examine identified metabolites for their signalling effects in the female tract cells as utilised by

Connolly (2012). If a metabolite(s) was identified which caused similar tract signalling a randomised clinical trial of infusion of the sperm metabolite(s) to the uterus and evaluation of implantation rates and pregnancy success rates may enable better understanding of the role of sperm metabolites in human reproduction and open up an exciting new therapy. This would have particular significance as the Robertson's data may suggest it would improve the health of children being born not just a treatment success rate (Robertson et al, 2015).

In the study of implantation and changes in the oviduct secretory proteomic profile:

The metabolites detected in the sperm capacitation media in this study may have significant relevance to future studies into implantation and changes in the oviduct secretory proteome. The newly recognised potential for sperm to influence the female tract in implantation and ongoing child health, stems from mouse evidence reviewed in Schjenken and Robertson (2015). Removal of the seminal vesicle resulted in decreased implantation rate and poor embryo development in mice (Robertson, 2014, Robertson et al., 1996). Obesity, abnormal metabolic parameters and decreased glucose tolerance were also manifested in the male mice offspring in this study which highlighted the implications of FRT exposure to seminal fluid on the offspring health (Robertson et al., 2006b). In humans, seminal plasma does not reach the uterine cavity so if the murine-type signalling was to occur in humans it would actually be based upon a sperm-induced signal caused by released metabolites.

Sperm have also been shown to induce changes in the oviduct secretory proteomic profile in some species such as pigs (Georgiou et al., 2005) and to enhance prostaglandin secretion in bovine oviduct (Kodithuwakku et al., 2007). The significance of the activation of specific sperm signalling cascade within the oviduct, which results in

modulation of its gene expression and protein secretions, has also been linked to prolonged fertilisation span and sperm survival (Murray and Smith, 1997, Yeste et al., 2014, Kolle, 2015). Sperm ability to activate a particular transduction pathway following their binding to oviductal epithelial cells has been suggested to cause changes in certain heat shock proteins gene expression in pigs (Yeste et al., 2014). The changes in these specific heat shock protein (namely clusterin CLU, heat shock proteins: HSP90AA1, HSPA5, and HSPA8) have been proposed to play role in modulation of sperm viability within the oviduct (Lloyd et al., 2009, Elliott et al., 2009, Yeste et al., 2014). Another study by Georgiou et al. (2007) demonstrated that sperm may induce alteration of expression of up to twenty proteins in the FRT, mainly the oviduct, including fibrinogen and complement C3.

Limitations of the study:

As discussed earlier, some limitations were inevitable in terms of limited number of replicates and variation and heterogeneity in semen samples. Additionally, sperm samples were prepared by swim up to obtain the best motile fraction of sperm which may achieve fertilisation *in vivo*. The assessment of efficiency of sperm purification from contaminants such as leukocytes and epithelial cells was not performed. Paiva et al, (2015) recommended checking the prepared sperm samples with phase contrast microscopy to ensure complete purity of the analysed samples prior to metabolomics analysis, none were observed when cell counts were performed in this study, but that does not preclude the presence of contaminants at a low level.

Additionally, assessment of the percentage of live and dead sperm could be relevant to sperm apoptosis, to consider whether the detected metabolites in the TP7 samples are partly because of cell death. In the current study, sperm metabolomics of capacitated versus uncapacitated human sperm has shown statistically significant pattern of metabolites which may also be a novel approach for population capacitation assessment. However, the TP1 samples in this study (considered as uncapacitated sperm) were timed after one hour of swim-up during which some sperm cells may have already undergone protein phosphorylation (Secciani et al, 2009). Further studies are certainly required to identify more human sperm metabolites and to relate the sperm metabolomics data to sperm functionality and fertilisation competence.

Metabolomics versus proteomics sperm studies:

Compared to more than thirty studies which have considered the human sperm proteome and have identified over 6000 proteins (Amaral et al, 2014a) as highlighted earlier; sperm metabolomics is still in its infancy. Comparison of the proteomic profile of capacitated versus ejaculated sperm and fertile versus infertile subjects have revealed different protemic feature between the two compared sperm populations (Rajeev and Reddy, 2004; Pixton et al, 2004; Secciani et al, 2009). Similarly, comparative metabolomics studies of different sperm populations, for example from fertile versus infertile subjects, in future studies may provide insights of the underlying pathologies in patients with unexplained infertility. Another interesting application of sperm metabolomics is to link to sperm proteomic data by exploring the relevance of certain metabolic pathways such as lipid metabolism to the proteins with metabolic function (Amara et al, 2014a, Paiva et al, 2015). The fact that sperm metabolomics are generally performed in *in vitro* setting may highlight

the challenge in accurately identifying the contribution of the different metabolic pathways in sperm metabolism in the FRT (Amaral, et al, 2014a). Combining sperm proteomic data with sperm metabolomics data may enable better understanding of *in vivo* sperm metabolomics pathway by the identification of sperm proteins/enzymes which are actively involved in these pathways (Amaral et al, 2014a).

Concluding remarks: In summary, this study presents, for the first time, a comparative metabolomics study of human sperm metabolites detected in sperm capacitation media following 1h swim-up versus 6 hours capacitation. Fatty acids and oxidised fatty acids were among the statistically significant metabolic class detected in this study. The detected sperm metabolites are of relevance for understanding sperm energy metabolism during capacitation and for future study of signalling in FRT.

Chapter 5

SPERM MICRO-PARTICLE IMAGE VELOCIMETRY

(Sperm μPIV)

5.0 Abstract

To achieve fertilisation, sperm have to navigate the complex architecture of the FRT by actively propelling through the different viscous milieus they face during this journey. Motility is, therefore, one of the most significant aspects of sperm functional competence for natural fertilisation and is a target for enhancing animal breeding. The fluid dynamics of sperm motility is a subject of evolving significance for understanding the sperm journey toward the oocyte. Biological and molecular aspects of progressive sperm motility have gained much attention compared to the mechanical aspects. The effects of the motile sperm on the surrounding fluid in terms of fluid mixing which could be relevant to biological mechanisms such as chemotaxis has not been previously considered. A micro-particle image velocimetry (μPIV) using polystyrene beads as tracing micro-particles in 10 μm chambers have been applied in this study to investigate fluid velocity and vorticity around motile sperm in both low and high viscosity media. Data analysis was achieved using computing programming language MATLAB and its applications. Fluid velocity around motile sperm was estimated in the range of 25-45 μm/s in low viscosity media (LVM) and 20-35 μm/s in high viscosity media (HVM). The preliminary results of this study indicated that minimum fluid disturbance is observed around the motile sperm with localised fluid vorticity averaged as a trail for sperm motility path. The negligible fluid mixing exerted by the motile sperm may be relevant to sperm chemotaxis with minimum disruption to chemical gradients created in the vicinity of the egg. This work may provide an initial platform for future studies aiming to explore sperm- fluid interaction in terms of fluid mixing in *in vitro* setting and for chemotactic studies to investigate sperm motility from fluid mixing perspectives.

5.1 Introduction:

Fertilisation requires sperm cells to traverse the FRT and to arrive at and penetrate the oocyte vestments (Suarez and Ho, 2003, Suarez et al., 1991b). The FRT represents a challenging environment to the sperm to swim through. The complex geometry of the FRT from the narrow cervical crypts to the confined tortuous UTJ and oviduct represent mechanical challenges to the sperm (Katz et al, 1989, Suarez and Pacey, 2006). The high viscosity of the cervical, uterine and oviductal fluids as well as the narrow opposed surfaces in these passages may also hinder sperm migration (Katz et al., 1989). Not surprisingly, only a tiny fraction of sperm out of the hundreds of millions deposited into the vagina may accomplish their journey and arrive at the ampulla (Williams et al., 1993, Scott et al., 2000).

Progressive motility has been shown as an essential functional element of ejaculated sperm to navigate the FRT and achieve fertilisation (Mortimer and Mortimer, 2013, Mortimer, 1997). The prediction of functional competence of the sperm during semen analysis is also suggested to be more reliant on sperm progressive motility than on sperm concentration in a semen sample (Macleod and Gold, 1951, Mortimer and Mortimer, 2013). Progressive motility has been shown as an interactive process between both sperm cells and the surrounding environment with physical and chemical characteristics of the FRT playing major roles in this process (Katz et al, 1989). The biophysical activities of the FRT including uterine peristaltic movements, ciliary beating and fluid currents within the tract may contribute to the sperm transport (Kunz et al., 1996, Katz et al., 1989). It has also been suggested that the fluid flow and ciliary beating within the oviduct may enable reorientation of sperm trapped in the cul de-sacs of the oviductal epithelium and contribute to maintain luminal patency of the oviduct (Katz et al, 1989). Alternatively, sperm

deposition in the FRT may enhance uterine peristalsis via seminal fluid's contents of oxytocin and prostaglandins (Katila, 2001, Watson et al., 1999, Harper, 1988).

The unique ultrastructural characteristics of the sperm flagellum with dynein, the motor molecules in the axoneme, being the main inducer of the sliding movement are vital for sperm motility (Lindemann et al., 1992). Sperm motility is shown to be driven by a combination of factors including the forces created by the passive flagellar structures and the sliding movements initiated by dynein molecules (Gaffney et al, 2011). The mechanisms activating the coordinated activity of the dynein arms are still elusive; however, it has been shown that these arms act by ATP-ase enzyme converting chemical energy into mechanical one (Lindemann, 2009). The sperm flagellum waveform (curvature) is suggested to result from a balance between internal sliding forces generated by dynein microtubules, the elastic rigidity of the flagellum and the viscous fluid's response to the moving flagellum (Teran et al., 2010, Smith et al., 2009b, Brokaw, 1966). The sperm flagellar bending has been shown to confine to the distal flagellum in HVM in both human and bull sperm (Katz et al., 1989, Ishijima et al., 1986, Rikmenspoel, 1984).

Several studies have considered characterisation of molecular aspects of sperm motility (Yanagimachi, 1994a, Carlson et al., 2007, Pereira et al., 2015, Luconi et al., 2005). Nonetheless, mechanical aspects of sperm motility have gained less attention in the literature. Studies investigating the impact of the motile human sperm on the fluid surrounding them in terms of mixing and disturbance are also scarce. Additionally, Smith et al (2009b) and Eamer et al. (2015) indicated that most of the sperm motility studies have applied low viscosity media which is in contrast to the *in vivo* conditions where sperm encounter high viscosity FRT fluids such as cervical mucus (Suarez and Pacey, 2006). The first study to consider modelling sperm motility in a viscoelastic fluid was in 1998 by

Fulford et al. Recently, advances in high speed live-cell imaging and computational analysis have provided great opportunities to study sperm motility in the view of fluid dynamics (Gaffney et al, 2011). Multidisciplinary studies are in the heart of sperm motility research with collaborations between mathematicians and biologists have resulted in pioneering studies (Gaffney et al, 2011, Smith et al, 2009b). The application of high speed imaging technology and fluid mechanics in studies of sperm motility may enhance the understanding flagellar propulsive movements by allowing access to better through-put image capture for sperm flagellar wave characterisation (Smith et al, 2009b; Gaffney et al, 2011). Both theory and experimentation in such studies play major roles in understanding sperm swimming behaviour (Fulford et al, 1998, Gillies et al, 2009, Friedrich et al, 2010, Gaffney et al, 2011).

Some studies have investigated the changes in the sperm swimming patterns and beat frequency in response to alterations in fluid viscosity and encountering boundaries (Smith et al., 2009a, Smith et al., 2009b). The application of high viscosity media (HVM) in *in vitro* studies of sperm motility and guidance is crucial to enable understanding of the sperm migration behaviour in the FRT where high viscosity fluids and chemotaxis may play role in sperm fertilisation capability (Smith et al, 2009b). Furthermore, the mechanism of sperm interaction with boundaries has potential implications in understanding certain aspects of sperm journey in the FRT (Gaffney et al, 2011, Smith et al, 2009a) since the close proximity between the opposing epithelial surfaces and the motile sperm (Katz et al, 1989). Sperm have the tendency to swim near surfaces (Woolley, 2003, Rothschild, 1963) but the impact of surrounding boundaries on sperm flagellar beat pattern is still elusive (Smith et al, 2009a). Katz et al (1989) indicated that when the distance between the sperm and the surrounding boundaries is less than the

sperm's length, the fluid movement caused by the flagellar beating is constrained by the boundary (Woolley, 2003, Katz et al., 1989, Katz et al., 1975). The increased energy resulted from the boundary constrain causes the sperm flagellum to thrust harder to accomplish similar local fluid motion obtained without this constrain (Katz et al, 1989, Katz et al, 1975). Additionally, the boundary effect results in increased passive resistance to the sperm head subsequent to the increased hydrodynamic drag on the head and thus suggested to counter any possible increase in flagellar thrust (Katz et al, 1989). Nevertheless, the mechanism by which increased viscosity may enhance the efficiency of a three- dimensional swimmer like sperm is still uncharacterised (Katz et al, 1989; Teran et al, 2009). The tendency of swimming flagellated objects in close vicinty to beat in synchrony was observed more than 60 years ago (Taylor, 1951). However, the relevance of this observation to human sperm transport and fertilisation has not been explored extensively (Smith et al, 2009a). Flagellar synchronisation has been observed in bull sperm (Woolley et al, 2009), micro-organism such as between the two flagellae of the Chlamydomonas (Polin et al., 2009) and some types of bacteria (Hsu and Dillon, 2009). The synchronisation of the two flagellae of Chlamydomonas has been suggested to be crucial for chemotaxis (Berg and Brown, 1972, Polin et al., 2009). Understanding of sperm-fluid dynamics and its applications may provide answers to such unresolved questions in reproductive biology (Gaffney et al, 2011). The application of sperm-fluid dynamic studies has also been implicated in the design of sperm-sorting micro-devices for the selection of competent sperm based on sperm motility (Seo et al., 2007, Shao et al., 2007, Gaffney et al., 2011) and also in advancing the currently available CASA machines (Computer Assisted Semen Analysis) (Smith et al, 2009a). For a comprehensive review of sperm –fluid dynamic see Gaffney et al (2011).

5.1.1 Micro-resolution particle image velocimetry (μPIV):

In this study, a technique of micro-resolution particle image velocimetry (μPIV) was applied to investigate both fluid velocity and fluid vorticity created by the motile sperm in low and HVM in a micro-chamber. μPIV is a technique used in microfluidic studies for measurements of fluid motion in a spatially determined way with the length scales in the range of 10^{-4} - 10^{-7} m (100 nm to 100 μm) (Wereley and Meinhart, 2010).

The flow tracing micro-particles added to the fluid enable the fluid motion to be observed and traced as the successively captured images of the moving particles are compared (Wereley and Meinhart, 2010). In μPIV, each pair of the flow-tracing particles' images is captured sequentially with specific time interval denoted as Δt. The image pairs are divided into interrogation areas (**figure 5-1**) which are cross correlated to identify the local displacement of the tracing particles Δx.

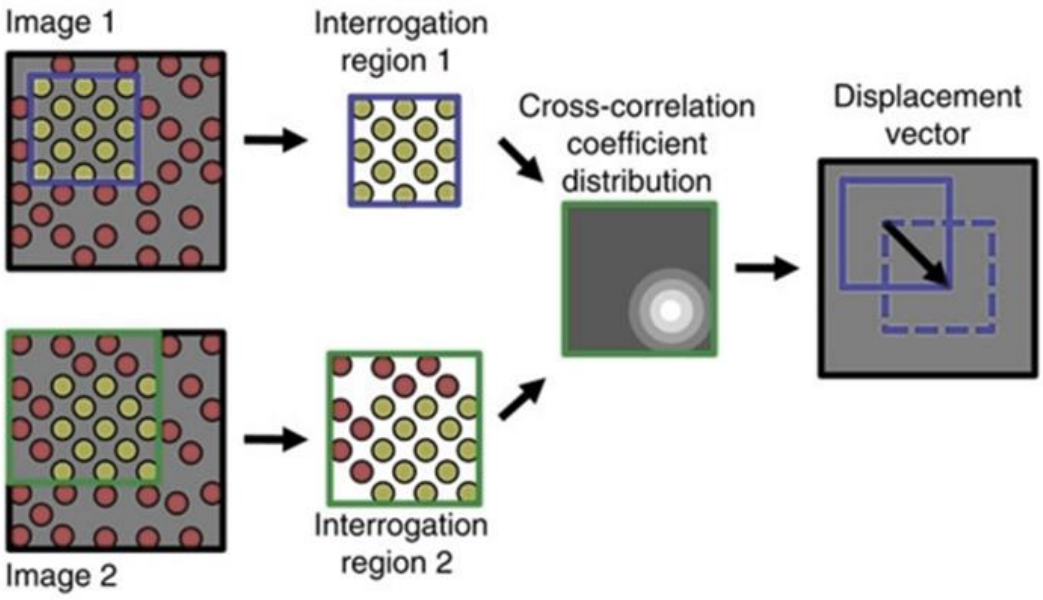


Figure 5-1 Selection of interrogation regions for PIV analysis; two interrogation areas are selected and cross correlation coefficient is calculated to determine vector displacement per image analysed.

The fluid velocity (**u**) is then calculated by the equation $\mathbf{u} = \Delta\mathbf{x} / \Delta t$. Thus, the tracing micro-particles local motion is used to infer the velocity of the fluid of interest within the microchannel used in the experiment (Wereley and Meinhart, 2010).

µPIV, or in some articles, µPTV (µParticle Tracking Velocimetry) has several applications such as study of blood flow velocity in the microcirculation (Ravnic et al., 2006), measurement of intra-aneurysmal flow in cerebrovascular aneurysm (Liou et al., 2004) and evaluation of aqueous flow in the eye following laser iriditomy (Yamamoto et al., 2006). For data analysis, MATLAB (MATrix LABoratory, MATLAB R2015b, Mathworks, Natick, Massachusetts) a powerful data analysis and visualisation tool which enables image processing and interpretation (Thielicke and Stamhuis, 2014) was used in this study. PIVlab-GUI (Particle Image Velocimetry lab_ Graphical User Interface) which is software applies some of the built-in MATLAB functions (Thielicke and Stamhuis, 2014) was applied to provide quantitative imaging of fluid flow and fluid velocity magnitude around sperm .

Sperm are considered neutrally buoyant (have an average density equivalent to the surrounding fluid allowing them to neither sink nor rise) with gravity being the only force affecting the flow and this force becomes negligible on microscopic scales (Montenegro-Johnson, 2013). The Reynold number for motile sperm is also very low, approximately 10^{-3}, and therefore inertia (the tendency to remain unchanged) is considered negligible (Gaffney et al, 2011) and the small length scales described for the µPIV become applicable. Reynolds number is the ratio between inertial forces and viscosity (Or et al., 2011). At low Reynold numbers, the fluid flow is usually laminar and viscosity forces dominate compared to high Reynold numbers where the flow is turbulent and inertial forces dominate (Videler, 1993).

The environment within the FRT is described to encompass complex non-Newtonian fluids (Fauci and Dillon, 2006) which signify that the properties of the fluid flow cannot be described by a constant viscosity value since the fluid viscosity alters in response to shear forces applied to them (Winet et al., 1984).

In this study, fluid motion was evaluated in the form of fluid vorticity, which is a microscopic measure of local rotation at any point in the fluid while circulation is the macroscopic measure of fluid rotation (Liu et al., 2015). Vorticity quantifies rotational motion of the fluid. The study of fluid vorticity may provide insights to the influence of sperm on fluid mixing and pattern and extent of this mixing and its relevance to chemotaxis. Study of fluid velocity around sperm in the view of fluid vorticity is a new perspective for sperm motility studies and also for chemotaxis.

Both fluid flow and viscosity are suggested to play a role in sperm swimming pattern within the FRT and ability of sperm to explore larger surface area of the oviduct (Kantsler et al., 2014). An upstream spiralling motion has been described for both human and bull sperm and has been suggested to enhance the likelihood of sperm encountering the oocyte (Kantsler et al., 2014). Several other guidance mechanisms such as chemotaxis, thermotaxis and rheotaxis have been suggested in the FRT to guide sperm to encounter the oocyte (chapter1, section 1.5.4). While in *in vitro* assays of chemotaxis the linear gradient of the chemotactic attractant can be maintained, it has been suggested that in *in vivo* conditions the maintenance of such gradient could be challenging where the oviduct is exposed to muscular contractions and ciliary beating (Fauci and Dillon, 2006). Limited knowledge is available regarding mechanical mixing induced by motile human sperm of their surrounding fluid environment which could be interesting for chemotaxis studies.

5.2 Objectives:

The aims of this study were to investigate the fluid velocity and vorticity in the vicinity of the motile sperm in both low (LVM) and high viscosity media (HVM) to evaluate the range of fluid perturbation caused by the motile sperm.

5.3 Methods:

5.3.1 Material

Table 5-1

Product name	**Company**	**Product code**
Bovine serum albumin (BSA)	Millipore, Watford, UK.	82-002-4
CellVision Counting Chamber 10µm manual counting chamber	Mitrone Healthcare Ltd (CellVision) , Dublin, UK	CV1010- 4cv
Cristaseal	Hawksley, Sussex, UK	01503-00
5ml Falcon round bottom tubes	Becton Dickinson, UK	352054
Methylcellulose 4000	Sigma, Dorset, UK	M0512
Poly D lysine solution	Sigma, Dorset, UK.	P8920
Polystyrene beads (PS beads) 0.50 µm mean diameter , 10% solids	Bangs laboratory, Stratech Scientific limited, Suffolk, UK	PS03N
Percoll	Sigma, Dorset, UK.	P1644
supplemented Earle's balanced Salt Solution (sEBSS) without phenol red, containing energy substrates 5 mM glucose, 2.5 mM Na pyruvate and 19 mM Na lactate	Geneflow, Fradley, UK	06-2010-03-1B
TWEEN-20	Sigma, Dorset, UK.	P1379

5.3.2 Methodology:

The tracer micro-particles used in this study were the polystyrene beads and they will be referred to as PS beads throughout the study. The study was conducted in LVM and HVM as follow:

5.3.2.1 Free motile sperm (single and ≤ 2-10 sperm in the field of view) in low viscosity media (LVM):

a- PS Beads preparation in LVM

The LVM was prepared using supplemented Earle's Balanced Salt Solution (sEBSS) + 0.3% BSA and the coating of the PS beads was achieved by simple adsorption. The PS beads were prepared in the LVM according to the following protocol:

The PS beads were coated with BSA a day before the experiment as follow: 50µl PS beads was mixed with 950µl sEBSS and centrifuged at 10,000g for 2 minutes and the supernatant fluid was discarded and this step was repeated three times. The pellet was then re-suspended in 950µl of the coating solution (sEBSS + 5% BSA + 0.02% Tween). The preparation was vortexed and left in a cold room at 6°C on a shaker overnight to facilitate the mixing and coating process which reduce beads sticking to each other and to the surface of chamber walls. On the day of the experiment, the preparation of the coated beads was centrifuged at 10,000g for 2 minutes, the supernatant was discarded and the pellet was re-suspended in 950µl of the washing solution (sEBSS + 0.02% Tween). The washing step was performed three times by centrifugation at 10,000g for 2 minutes and discarding the supernatant and re-suspending in the washing solution each time. The washing steps before and after PS beads coating aimed to reduce the toxicity of the PS beads on sperm. Tween-20 as a surfactant also reduces the beads hydrophobicity. Finally, the PS beads were re-suspended in the final suspension solution (sEBSS + 0.3 % BSA).

All the preparation solutions (coating, washing and final suspensions) were prepared a day before the experiment. The PS beads solution was vortexed between washes as the PS beads tend to form a pellet which is trapped and do not mix with the added washing solutions. **Figure 5-2** shows an image of sperm in LVM.

The LVM was prepared using sEBSS which has a viscosity similar to water at 20˚C of approximately 10^{-3} Pa.s (Smith et al, 2009b). Following the addition of the PS beads, the viscosity of the LVM was calculated using Einstein's formula as follows:

$\eta\hat{} \approx \eta (1 + 2.5 \varphi)$

Where ($\eta\hat{}$) is the viscosity of the LVM containing PS beads, (η)is the viscosity of the sEBSS prior to the addition of the PS beads (10^{-3} Pa.s) and φ is the volume fraction of the PS beads in the preparation (The final concentration of the PS beads in the preparation was 0.005% (v/v) since the original Ps beads preparation contains 10 % solids).

$\eta\hat{} \approx \eta (1 + 2.5 \varphi) =$

$\eta\hat{} \approx 10^{-3} \times (1 + 2.5 \times 0.005) \approx 1.0125 \times 10^{-3}$ Pa.s (final viscosity of the LVM containing PS beads).

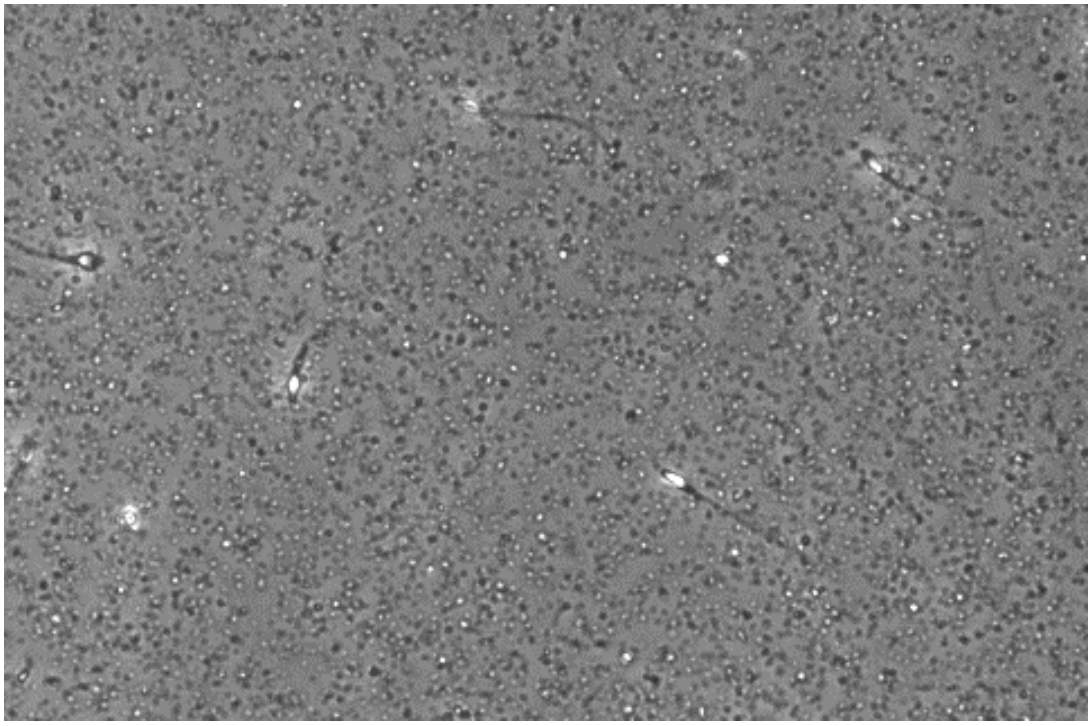


Figure 5-2 Image of sperm cells ≤ 2-10 in low viscosity media (LVM), PS beads' diameter is 0.5µm) viewed in a 10 micron chamber under 40 × magnification lens.

b- Sperm preparation:

All semen samples were obtained in accordance with HFEA Code of Practice in this project (see section 3.3.2 for semen collection procedure). The semen samples were left to liquefy for 30 minutes and the sperm cells were prepared by swim-up in sEBSS + 0.3 % BSA (section 3.4.7.2). Sperm concentration was adjusted by dilution in sEBSS + 0.3% BSA to obtain only ≤ 2-10 sperm cells per field of view. The addition of BSA to the PS beads coating preparation and sperm solution may prevent the adherence of the PS beads and sperm cells to the chamber walls (Weibel et al., 2005).

All the semen samples used in this study (both in LVM and HVM) were delivered to the laboratory within one hour of ejaculation. Imaging and data analysis were performed on sperm from 15 donors. CASA machine (Hamilton-Thorne IVOS Version 10.9, Beverly) in

Birmingham women's Hospital was used to evaluate the primary base-line for the sperm samples motility prior to subsequent experimentations.

c- Chamber preparation:

The capacity of each loading zone in the 10 µm chamber (**figure 5-3**) is only 2 µl.

1 µl of the prepared LVM (containing PS beads) was injected into one of the chamber's loading zones immediately following PS beads preparation. 2-5 µl of sperm preparation was loaded at the open end of the chamber's loading zone which has the beads loaded in. The sperm were left to swim into the chamber's loading zone thereafter aided by capillary action and the excess solution was wiped off and the chamber loading zone was sealed with cristaseal.

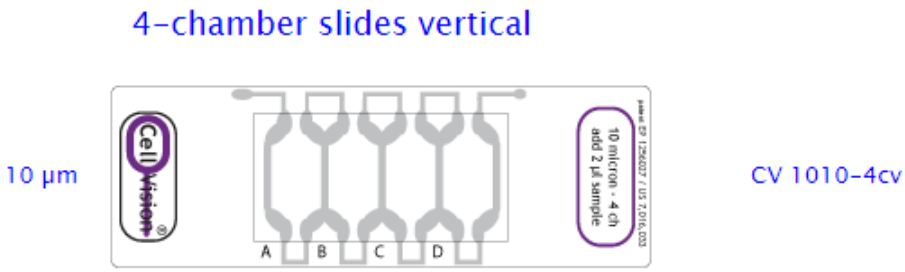


Figure 5-3 10 µm vertical depth chamber used in this study; A, B, C, D are the loading zones with loading capacity of 1-2 µl.

d- Imaging

Sperm cells were imaged using an inverted Olympus (Nikon TE200) microscope with positive phase contrast lens (40×) connected to a high-speed video camera (FASTCAM SA3, Photron), pixel size 17 µm. The imaging data was streamed directly to a Dell PC workstation. The microscope stage was kept at 37 °C by a LINKAM C0102 stage heater.

The recording of the movies was performed at a frame rate of 500fps (frame per second). The field of view was adjusted either to the full 1024×1024 pixel resolution and then the images were cropped using image J software or a region of interest were selected during the PIV data analysis. In some movies, the resolution was adjusted to only include region of interest (ROI) for imaging sperm and to have longer time recording. Imaging and data analysis were performed on sperm from at least 15 donors; data presented in the results section were taken from eight representative sperm donors.

5.3.2.2 Free motile sperm (≤ 2-5 sperm in the field of view) in high viscosity media (HVM):

a- PS beads preparation in HVM

The HVM was prepared by dissolving methylcellulose (MC) powder in sEBSS + 0.3% BSA to obtain 1% MC which has a viscosity of approximately 0.14 Pa.s which comparable to midcycle mucus (Smith et al, 2009b). Following the addition of the PS beads, the viscosity of the HVM (1% MC + PS beads) was calculated using Einstein's formula as follows:

$\eta\hat{} \approx \eta\,(1 + 2.5\,\varphi)$

Where ($\eta\hat{}$) is the viscosity of the HVM containing PS beads, (η) is the viscosity of the 1 % MC prior to the addition of the PS beads (0.14 Pa.s) and φ is the volume fraction of the PS beads in the preparation (The final concentration of the PS beads was 0.005% (v/v)).

$\eta\hat{} \approx \eta\,(1 + 2.5\,\varphi) =$

$\eta\hat{} \approx 0.14 \times (1 + 2.5 \times 0.005) \approx$ 0.14175 Pa.s (final viscosity of the HVM + PS beads).

The HVM was prepared in two different ways to overcome the problem of beads clustering following the addition of the methylcellulose powder to the sEBSS media and Ps beads. In the first method, the PS beads were prepared and coated (according to the PS

beads preparation protocol in section (a) in 5.3.2.1 for LVM preparation). On the day before the experiment, 0.01 g of MC powder was added to 1ml of the final solution of sEBSS + 0.3 % BSA containing suspended PS beads, mixed vigorously and left on a roller overnight in the cold room to mix homogenously. However, this preparation resulted in the PS beads forming clumps after the addition of the MC powder (**figure 5-4**).

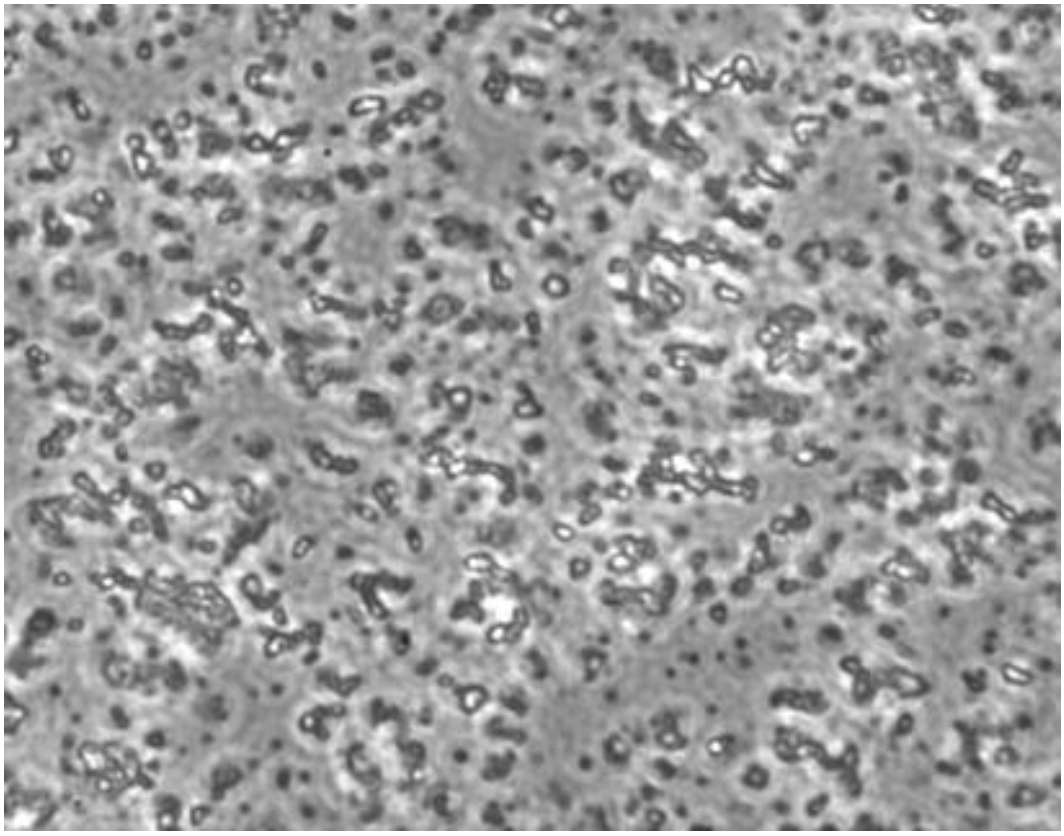


Figure 5-4 Clumps formation following the addition of the methylcellulose powder to the PS beads preparation to obtain high viscosity media of 1 % methylcellulose + PS beads, this preparation was not utilised in the study.

To overcome this problem, the PS beads were prepared in 2% MC and then adjusted to 1 % MC as follows: 0.2g MC powder was dissolved in 10 ml of sEBSS + 0.3 % BSA and left overnight in the cold room at 6˚C to mix on a roller. The PS beads were prepared according to the protocol (section (a) in 5.3.2.1) and left overnight to coat. On the next

day, equal volumes of PS beads solution and the 2% MC preparation were mixed to achieve the desired 1% MC viscosity + PS beads. The new preparation was left on a roller overnight in the cold room to ensure homogenous 1 % MC with PS beads (**figure 5-5**). The prepared HVM (PS beads in 1% MC) were viewed under 100 × magnification to visualise whether the PS beads are moving within the preparation. The HVM was also viewed on a slide with coverslip to confirm the beads were moving along with the fluid flow. Some degree of PS beads cluster formation was unavoidable; however, the second preparation technique reduced the PS clusters in the MC media and was used throughout the rest of this study in the HVM experiments.

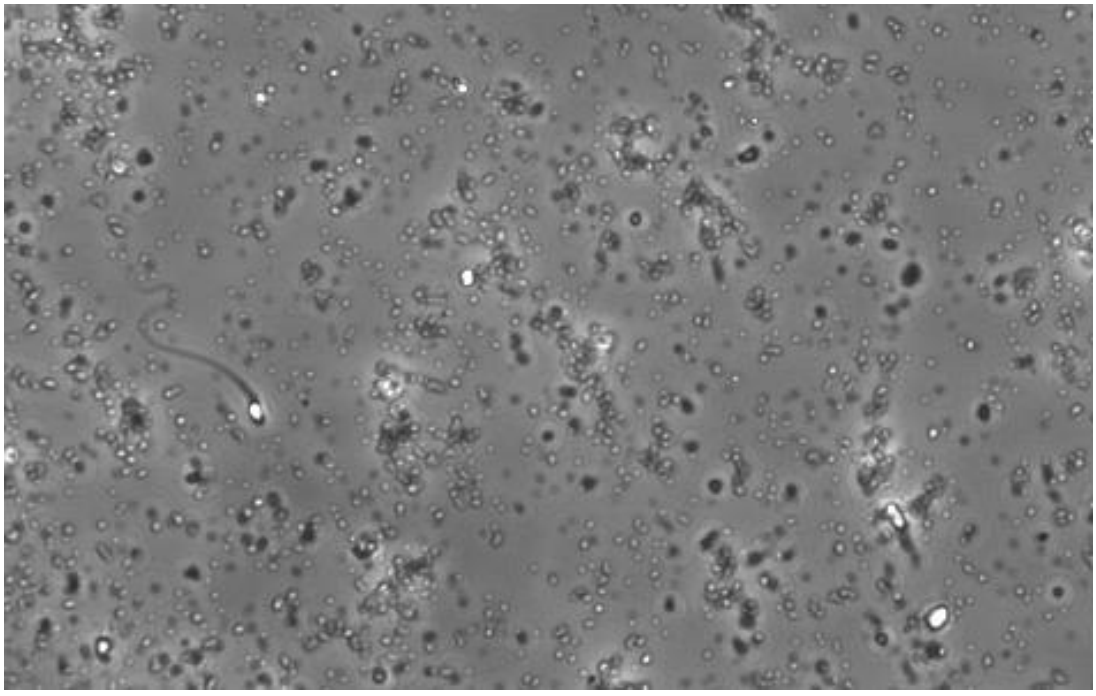


Figure 5-5 Improved HVM preparations with minimum clumps formation following the addition of PS beads solution to the 2% MC (methylcellulose) to achieve final concentration of 1% MC containing PS beads

b- Sperm preparation:

Sperm cells were prepared by swim-up into 1% MC prepared in sEBSS + 0.3% BSA + PS beads preparation (as above). Several aliquots of 300 µl of liquefied semen was under-layered beneath 700 µl of HVM (1% methylcellulose + PS beads) in 5 ml round bottom Falcon tubes and were incubated at 37°C and 6% CO_2 for one hour. The motile fraction of the sperm were collected from the top 400 µl of the medium and transferred to a new tube.

c- Chamber preparation:

2µl of sperm prepared by swim up in HVM was injected into the loading zone of the chamber and any residue was left for sperm to swim into the loading zone of the chamber aided by capillary action. To avoid drying of the media containing sperm, the chamber preparation were left in a moist box in the incubator at 37˚C and 6% CO_2 and the number of sperm swimming into the chamber was checked after 5-10 minutes of loading. The open end of the chamber's loading zone was sealed by cristaseal after any excess fluid was wiped dry.

d- Imaging

Similar to imaging technique in LVM, (section 5.3.2.1, part d). Imaging and data analysis was performed on sperm from 4 donors; data presented in the results section were taken from two representative donors.

5.3.3 PIV data analysis:

Representative movies were selected from LVM (8 movies) and HVM (4 movies) and were converted into image sequence using movie to image sequence code in MTALB and images were cropped or rotated using Image J software. The sequenced images were loaded into PIVlab_GUI for analysis of fluid velocity and vorticity around motile sperm. **Figure 5-6** illustrates the steps followed for the PIV data analysis.

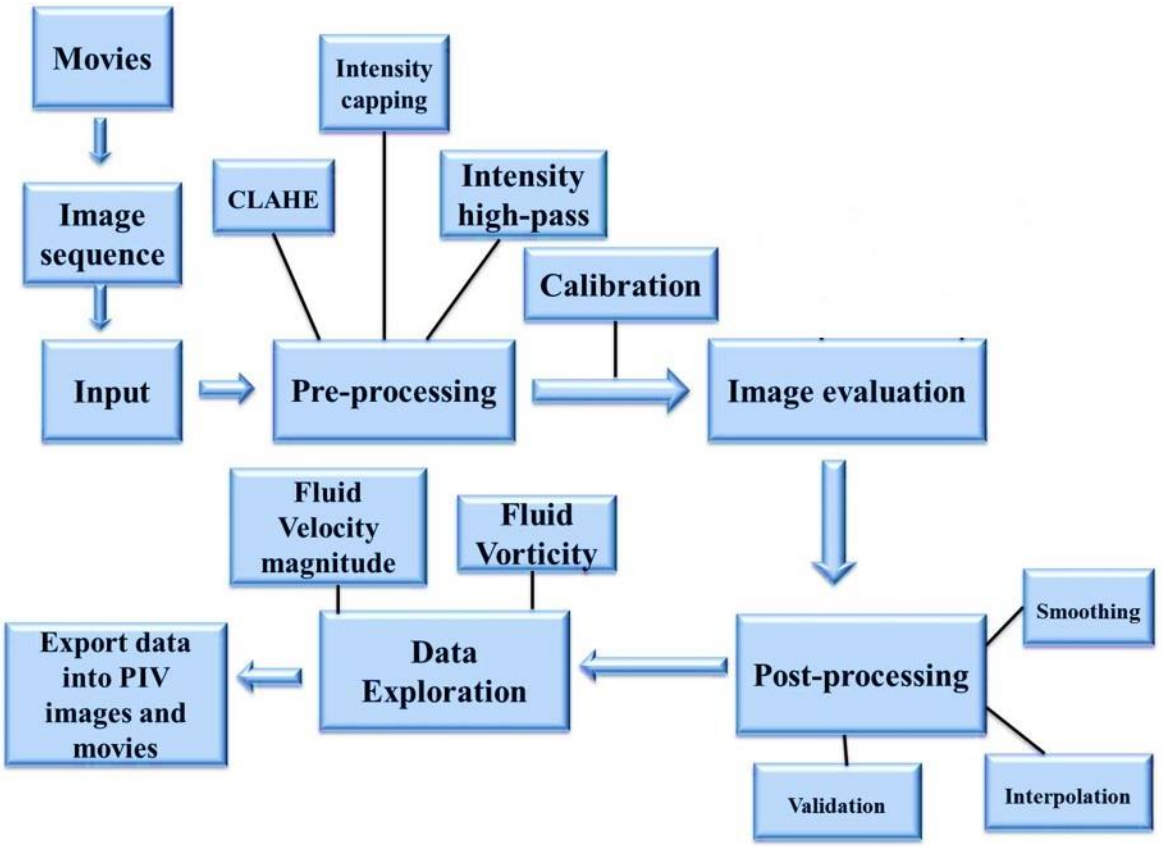


Figure 5-6 PIV analysis workflow for fluid velocity and vorticity analysis. Recorded sperm movies were sequenced into images and the quality of the retrieved images was enhanced using pre and post-processing tools available in MATLAB built-in function.

As shown in **figure 5-6**, each analysis step involves selection of data smoothing tools. For example, in the pre-processing step three filters can be enabled to smooth the loaded images (**figure 5-7** and **5-8**). CLAHE (Contrast limited adaptive histogram equalisation) improves the readability of the image data by equalisation of light intensities on the different regions of the image (Thielicke and Stamhuis, 2014). The intensity high-pass filter allows the removal of background noise caused by inhomogeneous illumination of the images by eliminating any low frequency information (Shavit et al, 2007; Thielicke and Stamhuis, 2014). While intensity capping filter prevents the high brightness in some areas of the image to cause bias in the analysis by selecting an upper limit of the greyscale

intensity which is applied as a threshold to adjust the image brightness (Shavit et al, 2007; Thielicke and Stamhuis, 2014). These pre-processing tools were used to enhance the image quality and were applied to all the frames in each movie analysed in this study.

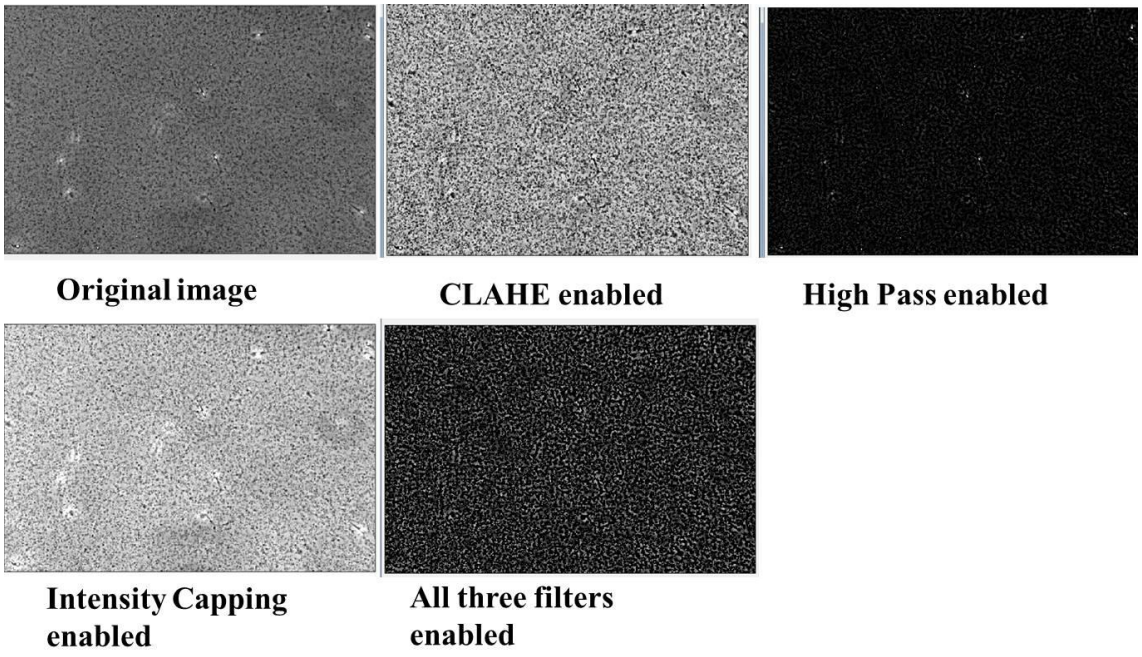


Figure 5-7 Sperm in LVM, original image before and after pre-processing filters enabled. CLAHE (Contrast limited adaptive histogram equalisation) enables equalisation of light intensities on the different regions of the image. The intensity high-pass filter allows the removal of background noise while the intensity capping filter enables selection of a threshold to adjust the image brightness.

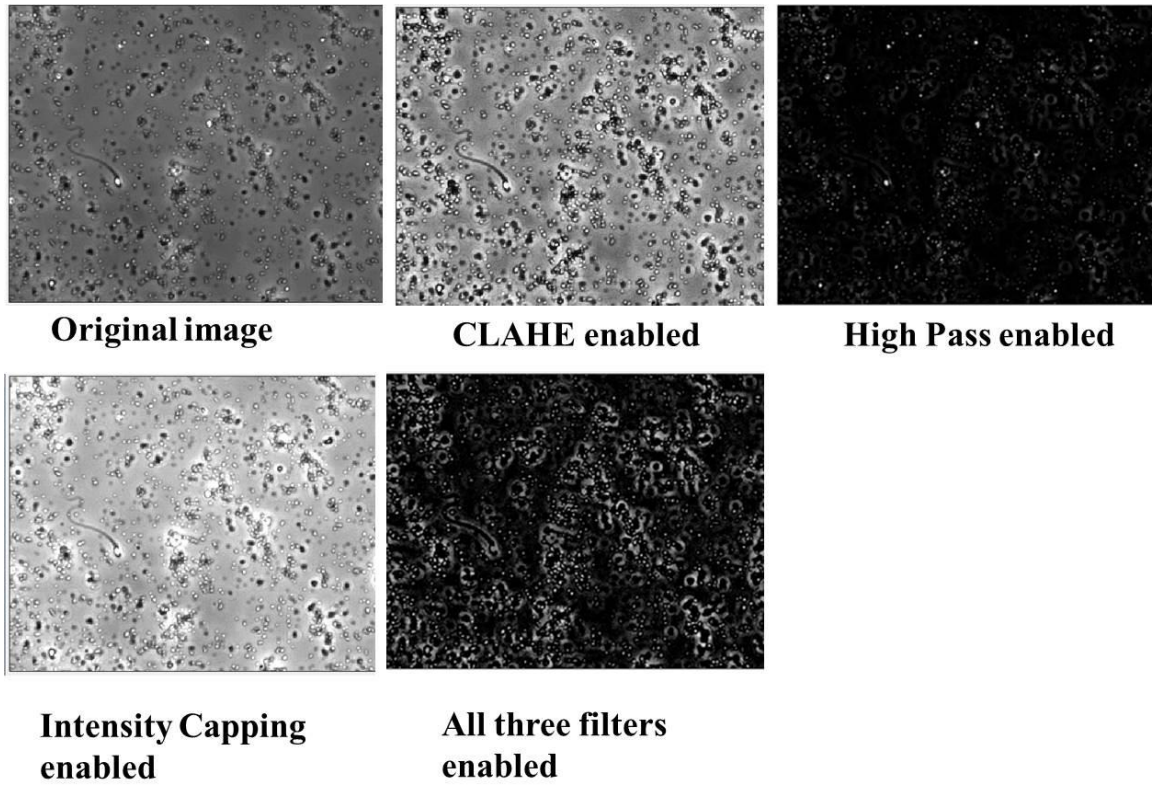
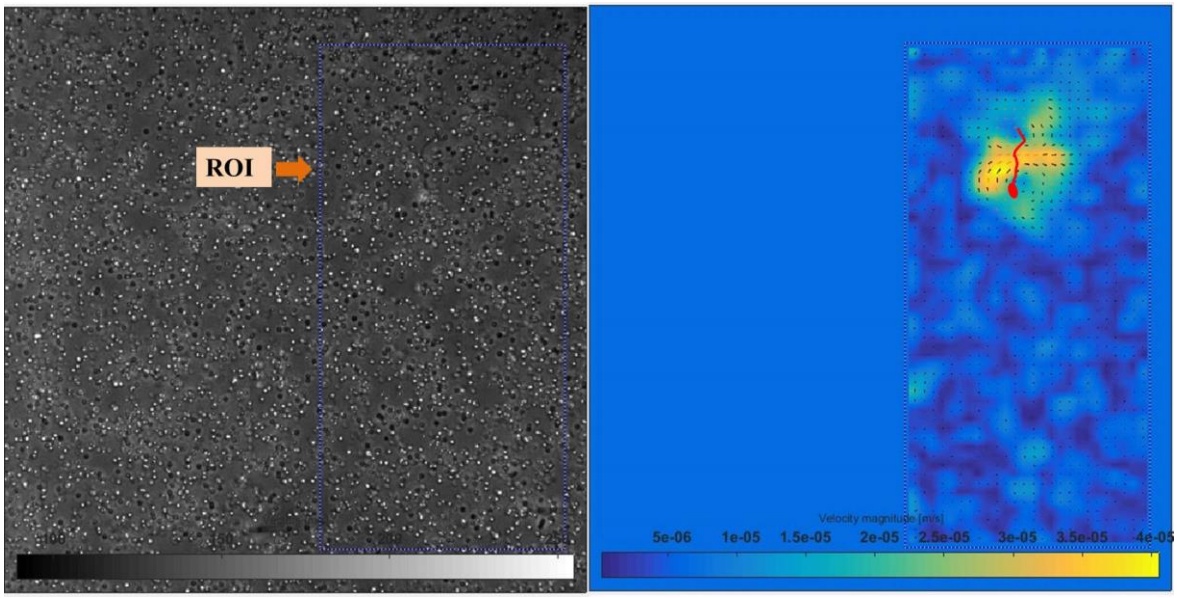


Figure 5-8 Sperm in HVM (high viscosity media), original image before and after pre-processing filters enabled (the function of the filters are illustrated in **figure 5-7** in LVM)

The PIV setting also enables selection of region of interest (ROI) from the full field of view (**figure 5-9**). The selection of ROI allows the analysis of one area (for example around single sperm) within the field of view.

The calibration filters allow selection of a reference distance and real time. For calibration of real time and real distance in this study, 0.002 second and 0.000425mm (0.425µm) were used respectively. These measurements were based on the frame rate (500fps, 1frame takes 0.002second) and the pixel size for the camera and lens magnification (17 microns (pixel size) / 40 × magnification = 0.425µm). The calibration step enables the velocity magnitude to be evaluated in m/second instead of pixel/ frame prior to the calibration. The analysis setting was saved and was used consistently among all the movies analysed in this study.



A **B**

Figure 5-9 **A-** Selection of region of interest (ROI) from the full field of view in the original movie and **B-** the analysis of the velocity magnitude based on the selected ROI. Sperm position is indicated on the velocity plot by illustration taken from the matching frame in the original movie.

The command Fast Fourier Transform (FFT) enables cross correlation of the interrogation areas of each image pair to select the most probable particle movement in the analysed interrogation areas (Thielicke and Stamhuis, 2014). For further details on FFT please refer to Thielicke and Stamhuis (2014).

In this study, FFT was used with 3 passes using an interrogation area of 64 × 64 pixels in pass1, 32 × 32 pixels in pass 2 and 16 × 16 pixels in the pass 3 (each pass with 50 % overlap between the neighbouring interrogation areas), **figure 5-10**.

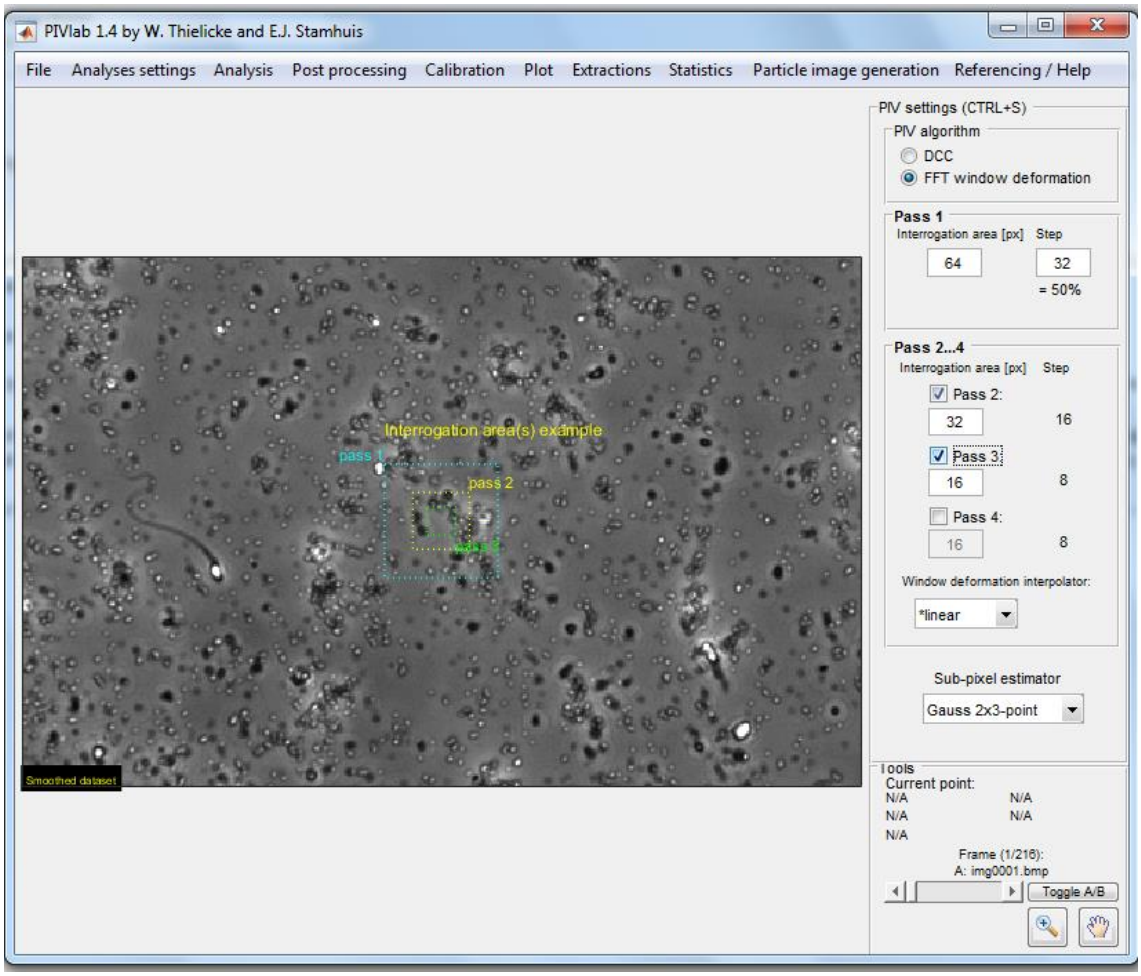


Figure 5-10 Fast Fourier Transform (FFT) with three passes and interrogation areas selected, pass 1 at 64 × 64 pixels, pass 2 at 32× 32 pixels and pass 3 at 16× 16 pixels (each pass with 50 % overlap between the neighbouring interrogation areas). The image was taken from sperm in HVM movie 2.

The post processing filters aim to validate the data by the selection of velocity limits from the scatter plot which excludes any outliers from the analysis. The interpolating missing data command enables replacement of missing vectors (the outliers which were removed in the validation step) by taking the nearest value to include in the data analysis and applying this to all the frames (Thielicke and Stamhuis, 2014).

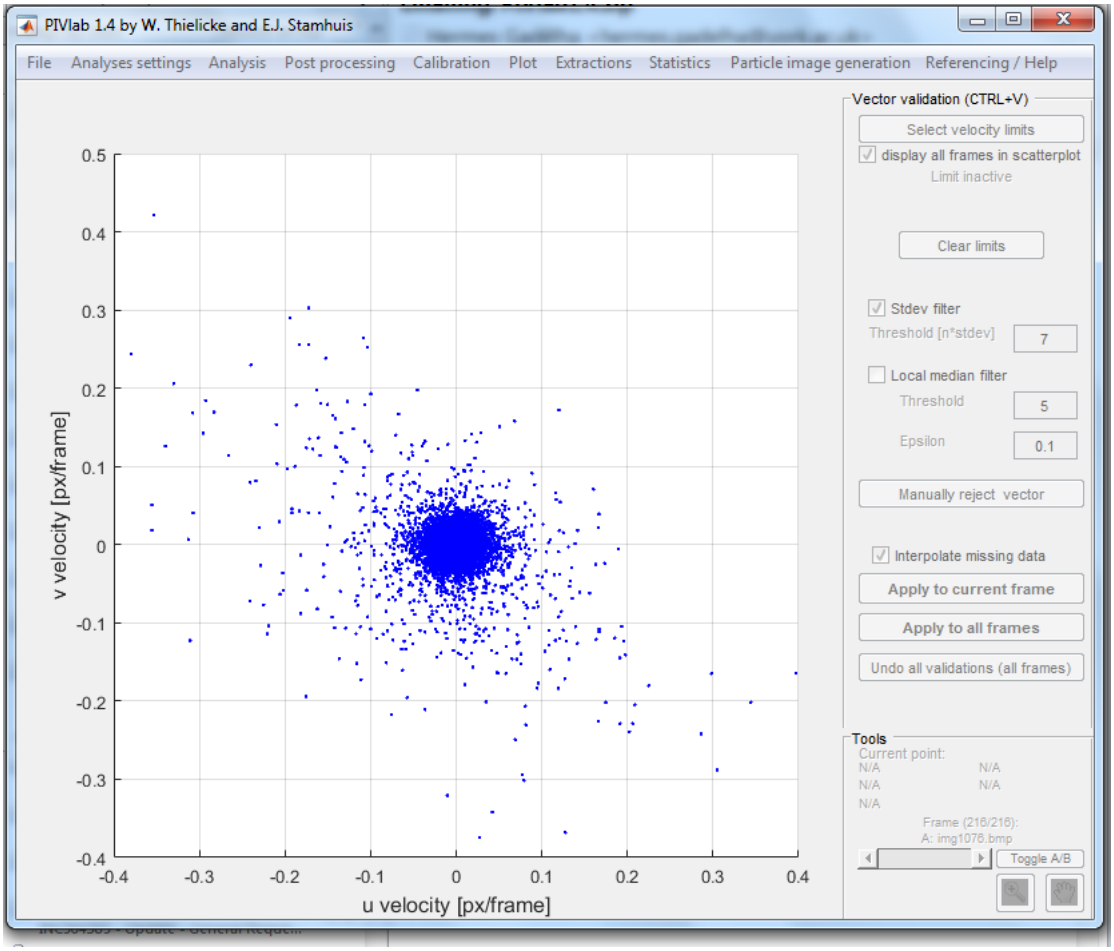


Figure 5-11 An example of a scatter plot to select velocity limit and exclude any outliers which may affect the average value in the data analysis (velocity magnitude is shown in pixel/frame, prior to calibration).

Following fluid velocity analysis, fluid vorticity was calculated using MATLAB built-in function. For averaging the fluid vorticity around the motile sperm, a MATALAB code, created by Dr Hermes Gadelha, was used to estimate the average fluid vorticity along the sperm motility path across the image frames in each movie. The colourmap in the software can be adjusted to enhance the final view of the averaged fluid vorticity aroun the motile sperm (**figure 5-15** and **5-16**, section 5.4).

5.4 Results:

The results of this study were divided according to the viscosity of the media used in the experiments, either low (LVM) or high viscosity media (HVM) as follows:

5.4.1 Results in LVM:

5.4.1.1 Fluid velocity around motile sperm:

Fourteen movies in LVM from ten donors were analysed and fluid velocity magnitude around motile sperm was evaluated in each movie. The data presented here are taken from eight representative samples. The fluid velocity around motile sperm in LVM was evaluated in the range of 25-45 µm/s across the repliacates. The colour codes in MATLAB software indicate the range of fluid velocity in the field of view and by comparing the original movie with the velocity magnitude analysis the area adjacent to the motile sperm can be determined (**figure 5-12 and 5-13**). The fluid velocity in areas distant from sperm in these movies manifested lower range of ≤ 5 µm/s. To further confirm this observation, control areas where no sperm is present in the field of view were analysed to determine the fluid velocity in the absence of sperm. The fluid velocity in the control area was in the reange of 0.5-4 µm/s (**figure 5-14**).

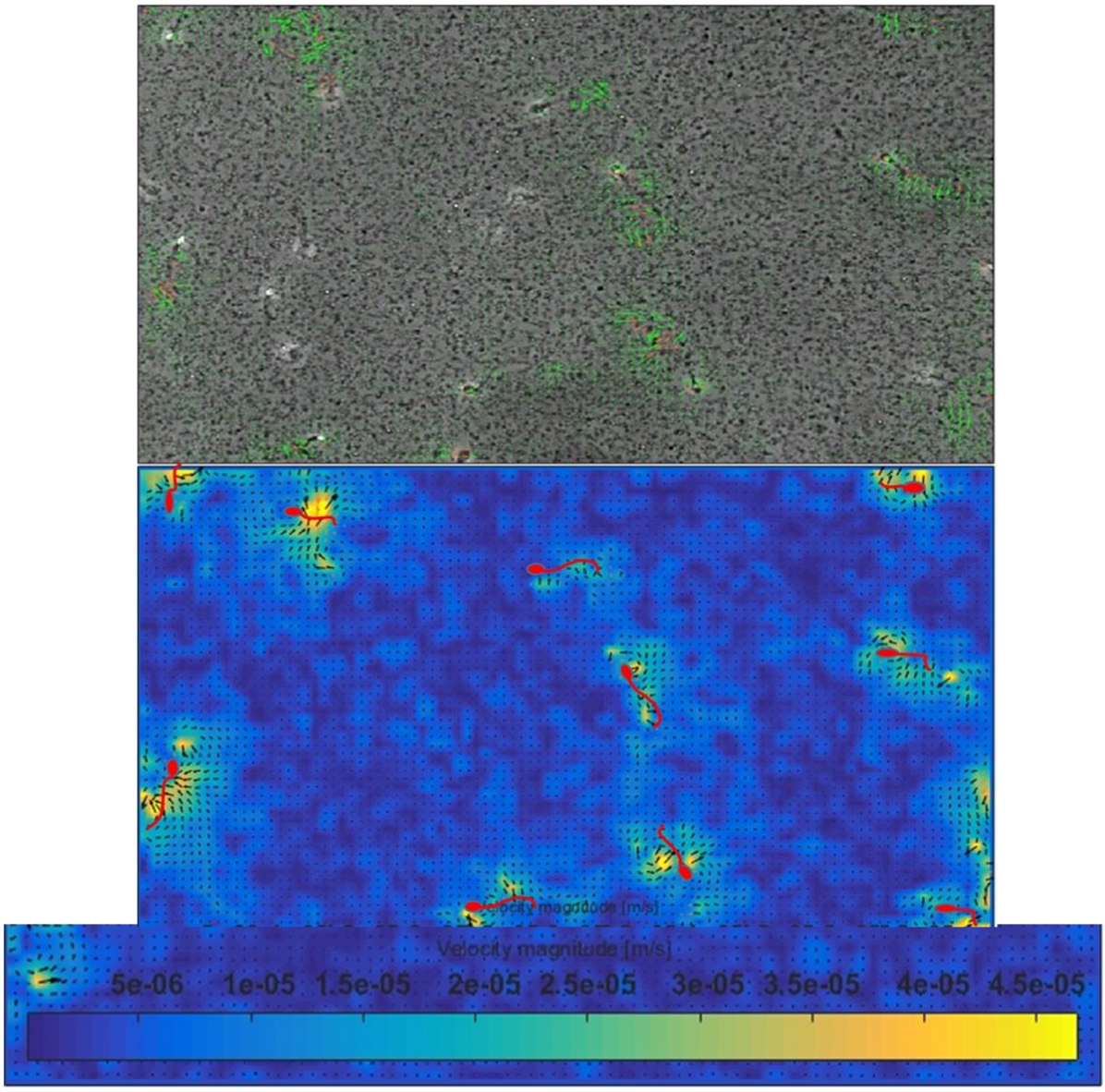


Figure 5-12 Fluid velocity around motile sperm coded in yellow colour shows a maximum velocity magnitude 4.5×10^{-5} m/s (45 µm/s). Minimum velocity was 5×10^{-6} m/s (5 µm/s) in areas distant from the motile sperm coded in blue colour. The range of fluid velocity near a motile sperm in this movie was 30-45 µm/s. Sperm position is indicated on the velocity plot by illustration taken from the matching frames in the original movies. Data taken from LV8: low viscosity 8th movie with ≤ 2-10 motile sperm in the field of view.

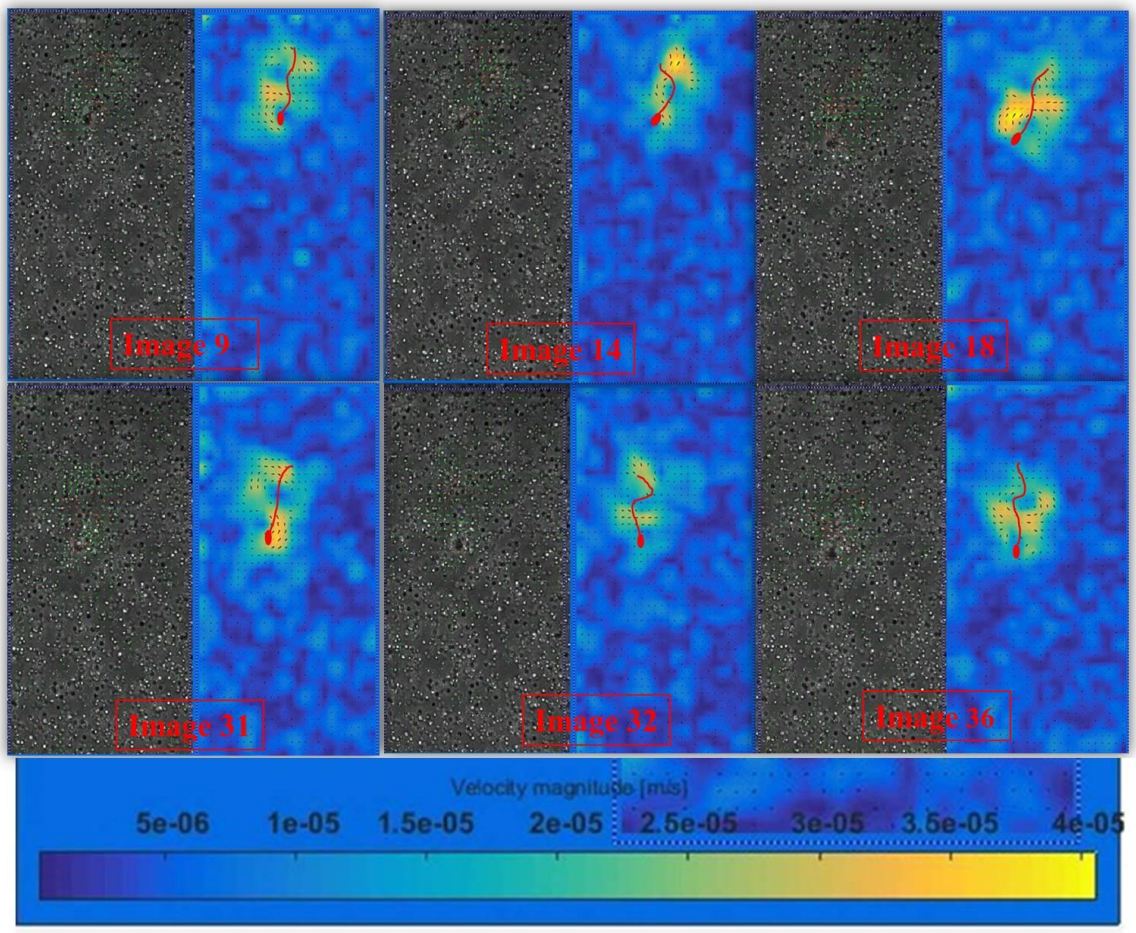


Figure 5-13 Fluid velocity magnitude around single motile sperm in LVM at different frame points. Sperm position is indicated on the velocity plot by illustration taken from the matching frames in the original movie. Fluid velocity magnitude near motile sperm (yellow coded areas) in this movie ranged from 2.5×10^{-5} m/s – 4×10^{-5} m/s (25-40µm/s)

.

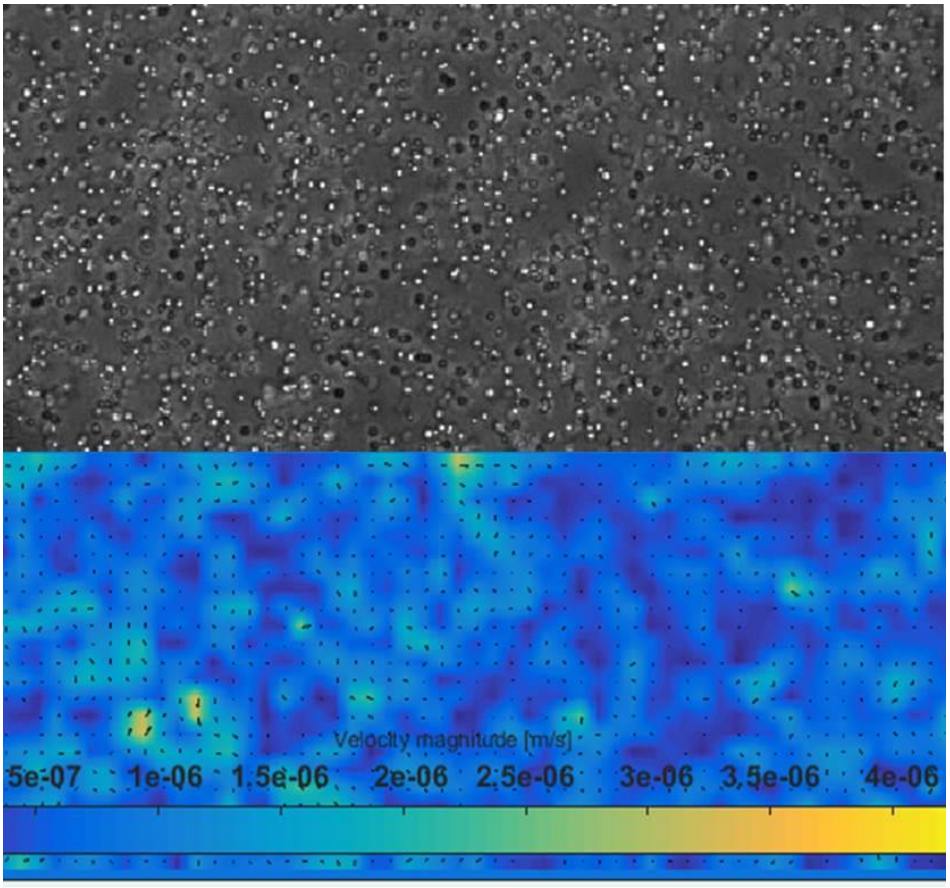


Figure 5-14 Control area with no sperm in the field of view in LVM, the maximum velocity magnitude coded in yellow was 4×10^{-6} m/s (4 µm/s) and the minimum was 0.5 10^{-6} m/s (0.5 µm/s)

5.4.1.2 Fluid vorticity around motile sperm :

By averaging the fluid vorticity over the frame range in the analysed movies, a trail was demonstrated (**figure 5-16** and **5-17**). Using vorticity codes in MATLAB (created for this work by Dr Hermes Gadelha), a trail was visualised along the sperm swimming path in the LVM which decays rapidly in time once the sperm has moved past. In movies where the whole field of view was imaged and analysed with single sperm in the field of view, the vorticity trail was only observed in areas where sperm have passed (along the sperm swimming path, **figure 5-19**) while areas with no sperm have no trail (trail LV1, **figure 5-16**). In movies with single sperm in the field of view and where a region of interest (ROI)

was selected, the trail was also observed to confine to the sperm path (trail LV2, **figure 5-16**).

Interestingly, in movies with only two sperm in the field of view, two trails were observed (trails LV3 and LV4, **figure 5-15** and **5-16**). In movies with ≤ 2-10 sperm in the field of view, several trails can be observed (trails LV5 and LV6, **figure 5-17**). To further confirm this observation (time-averaged fluid vorticity around motile sperm produce a trail), control area with no sperm was analysed to identify the average fluid vorticity in the absence of sperm. The time-averaged fluid vorticity in the selected control area did not show any trails (**figure 5-18**).

In one of the movies (LV7), a single motile sperm in the field of the view was adhered to the slide and the average fluid vorticity around this sperm was confined to the vicinity of the sperm and the plotted trail was circumscribed area around the adhered sperm (trail LV7, **figure 5-17**). In another movie where ≤ 2-10 sperm were in the field of view with one sperm adhered to the slide, the averaged fluid vorticity around the freely motile sperm showed trails while the averaged fluid vorticity around the adhered sperm showed circumscribed area similar to the one observed in the LV7 movie (trail LV8, **figure 5-17**). The MATLAB codes for fluid vorticity calculation are included in appendix 6. The colour code represents the averaged vorticity for the movie sequence. To clearly visualise the formed trails in each data analysis, the colourmap bar was adjusted accordingly and therefore the colour scales change from one data set to another as seen in **figures 5-15** and **5-16**.

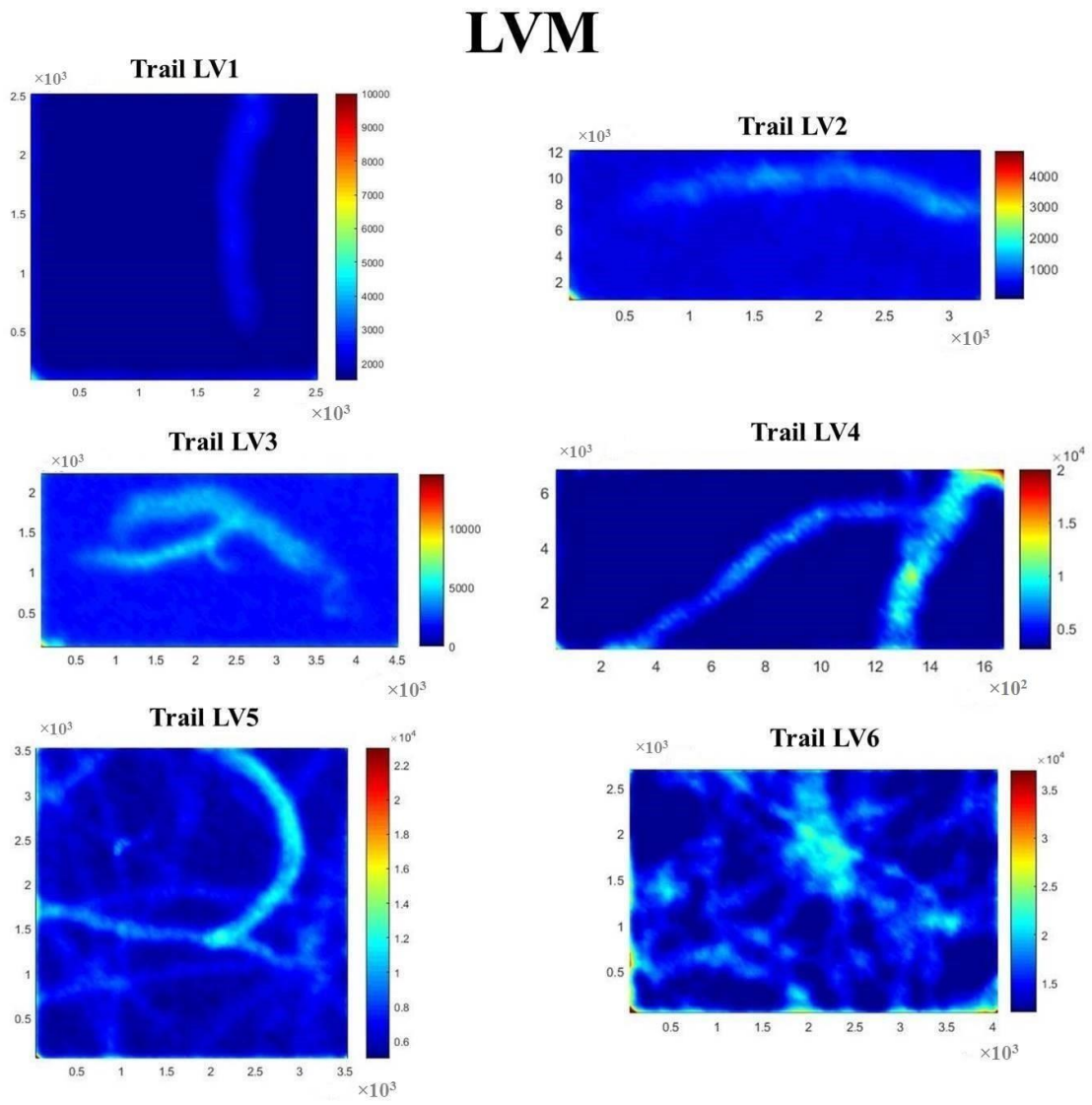


Figure 5-15 The time-averaged fluid vorticity near motile sperm manifested as sperm trails along the sperm swimming paths. Trail LV1 is for a single sperm analysed in a full field of view, trail LV2 is for a single sperm analysed in a ROI, trails LV3 and LV4 for two sperm analysed in ROI and trails LV5 and LV6 are for ≤ 2-10 sperm in the full field of view, colour codes represent fluid vorticity around motile sperm measured in inverse seconds S^{-1} .

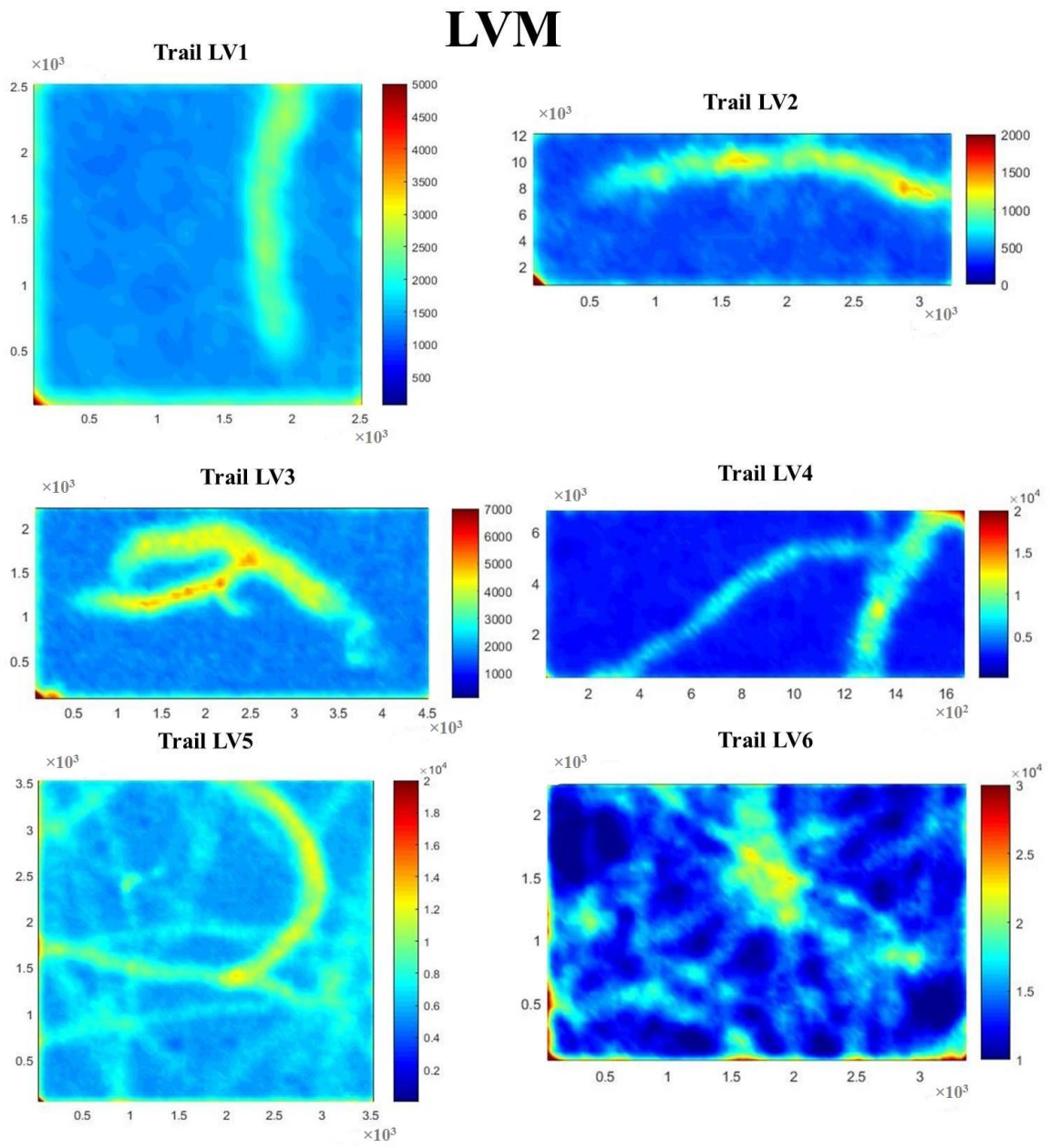


Figure 5-16 The same time-averaged fluid vorticity near motile sperm in **figure 5-15** to show how adjusting the colormap bar in each data set will enhance the visualisation of the averaged vorticity trails analysed.

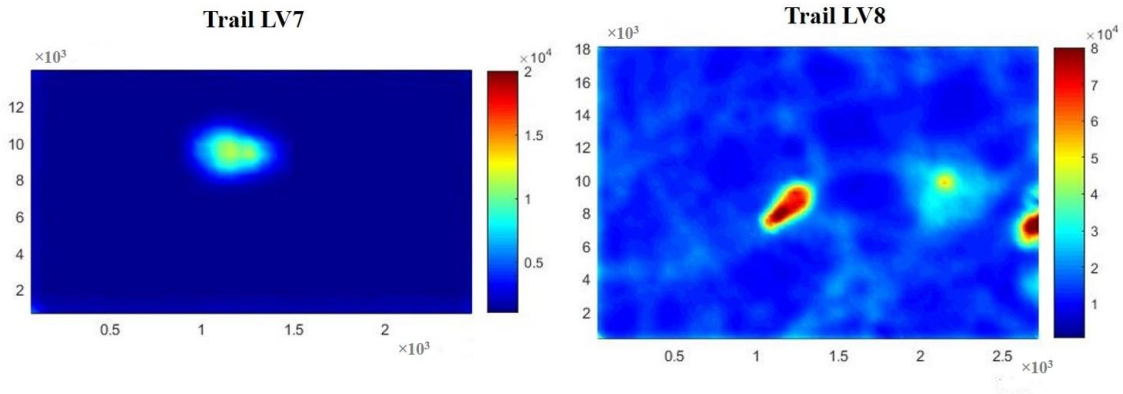


Figure 5-17 Time-averaged fluid vorticity for a single sperm adhered to the surface of the chamber's wall. LV7: averaged vorticity trail manifested as a circumscribed area around the adhered sperm in LVM with single sperm in the field of view. LV8: averaged fluid vorticity around ≤ 2-10 sperm in the field of view with one sperm adhered to the chamber's inner wall. The adhered sperm showed a circumscribed area for the averaged fluid vorticity similar to the one observed in LV7 while the freely motile sperm showed trails.

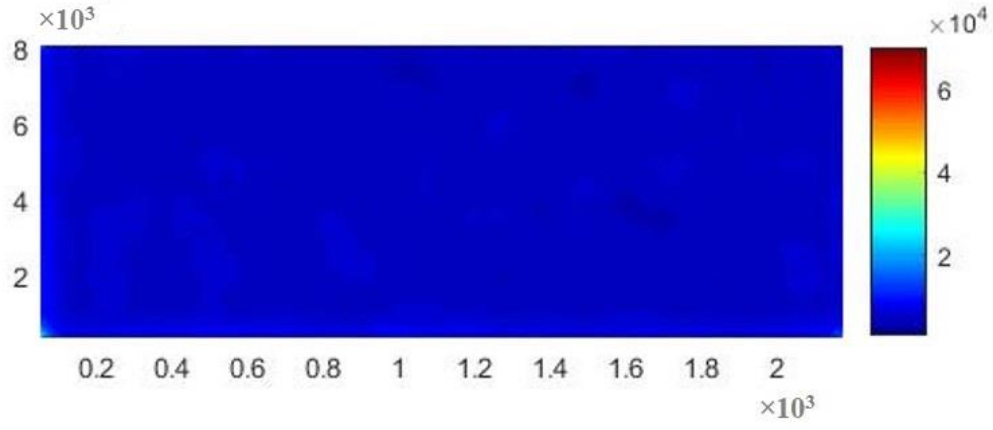


Figure 5-18 Averaged fluid vorticity for control area with no sperm in the field of view in LVM, , no trail was manifested.

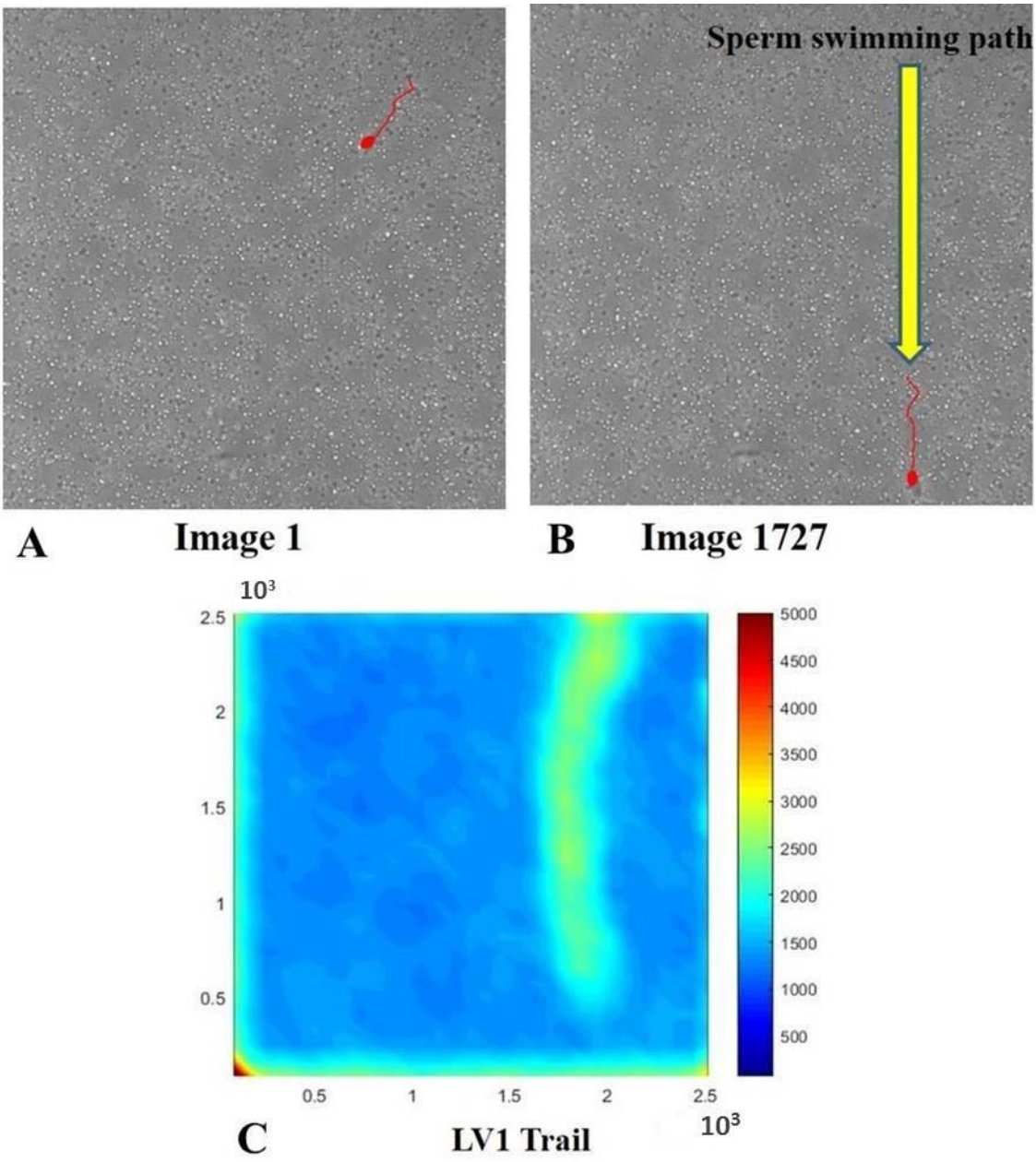


Figure 5-19 Sperm-motility path of a single sperm **A-** Image 1, the first image in the original movie with sperm position highlighted in red, **B-** Image 1727, the last image in the image sequence with sperm position highlighted in red. **C-** The averaged fluid vorticity analysis over the whole frame range of image sequence; a fluid vorticity trail can be visualised along the sperm motility path (images were taken from movie LV1 in LVM).

Calculation of Brownian error :

In the current study the effect of the Brownian error was substantially minimised by the application of the cross correlation analysis of multiple interrogation areas in the image pairs to identify the local displacement of the PS beads as shown in section 5.3.3. However, the realtive error caused by Browinian motion is a recommended consideration in studies using microparticle tracer particles to trace velocity of >10 µm/s (Santiago et al., 1998). Brownian error (εB) results from Brownian motion, a random thermal noise observed for the PS beads as they vibrate randomly (Santiago et al., 1998) even in the absence of any motile sperm (observed in the control areas with no sperm).

To calculate εB in the data the following equation from Santiago et al (1998) and Wereley and Meinhart (2010) was used :

$$\varepsilon B = \frac{1}{u}\sqrt{\frac{2D}{\Delta t}}$$

Where **u** is the local fluid velocity (calculated in this study 25-45µm/s); Δt is the time interval (calibrated at 0.002 second) and **D** is the diffusivity of the PS beads given by the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi \mu d_p}$$

where kB is Boltzmann's constant (1.38×10^{-23} J/k)

T is the absolute fluid temperature (37 + 273 = 310 Kelvin (all experiments in the study were conducted on a stage heater at 37˚C), π = 3.14

µ is the viscosity of the fluid (the viscosity of the LVM with PS was calculated as 1.0125 $\times 10^{-3}$ Pa.s) and d_p is the diameter of the PS beads which is 0.5×10^{-6} m (0.5µm). The

calculated Brownian error by the above equation for a maximum fluid velocity of 45μm/s in LVM was = 2.1 % which is the relative error due to Browinian motion.

Calculation of the Peclet number and diffusion coefficient for chemoattractants:

The relevanace of the study of fluid vorticity and velocity around motile sperm may be significant for understanding sperm chemotaxis i*n vivo* and for studies of chemical gradient assays *in vitro*. To evaluate the relative importance of the sperm-driven fluid flow and diffusion in the current study, the Peclet number (a dimensionless ratio to illustrate relative significance of convection to diffusion (Huysmans and Dassargues, 2005) was calculated for some physiological ligands.

Peclet number = velocity × length scale/diffusion coefficient

The diffusion coefficient for progesterone is 6.88×10^{-6} cm^2/s (Seki et al., 2003) and the diffusion coefficient for bourgeonal (a sperm chemoattractant (Spehr et al, 2003) is 5.7×10^{-6} cm^2/s (Correia , 2011) (both in aqueous solution). The lengthscale assumed in the model is the length of the oviduct (approximately 10 cm) and fluid velocity of 45 μm/s in LVM:

Peclet number for progesterone = 45×10^{-4} cm/s × 10 cm /6.88×10^{-6} cm^2/s = 6.54×10^3

Peclet number for bourgeonal = 45×10^{-4} cm/s × 10 cm /5.7×10^{-6} cm^2/s = 7.89×10^3

5.4.2 Results in HVM

Fluid velocity magnitude near motile sperm in HVM was evaluated in the range of 20-35 µm/s (yellow coded areas around motile sperm), **figures 5-19** and **5-20**.

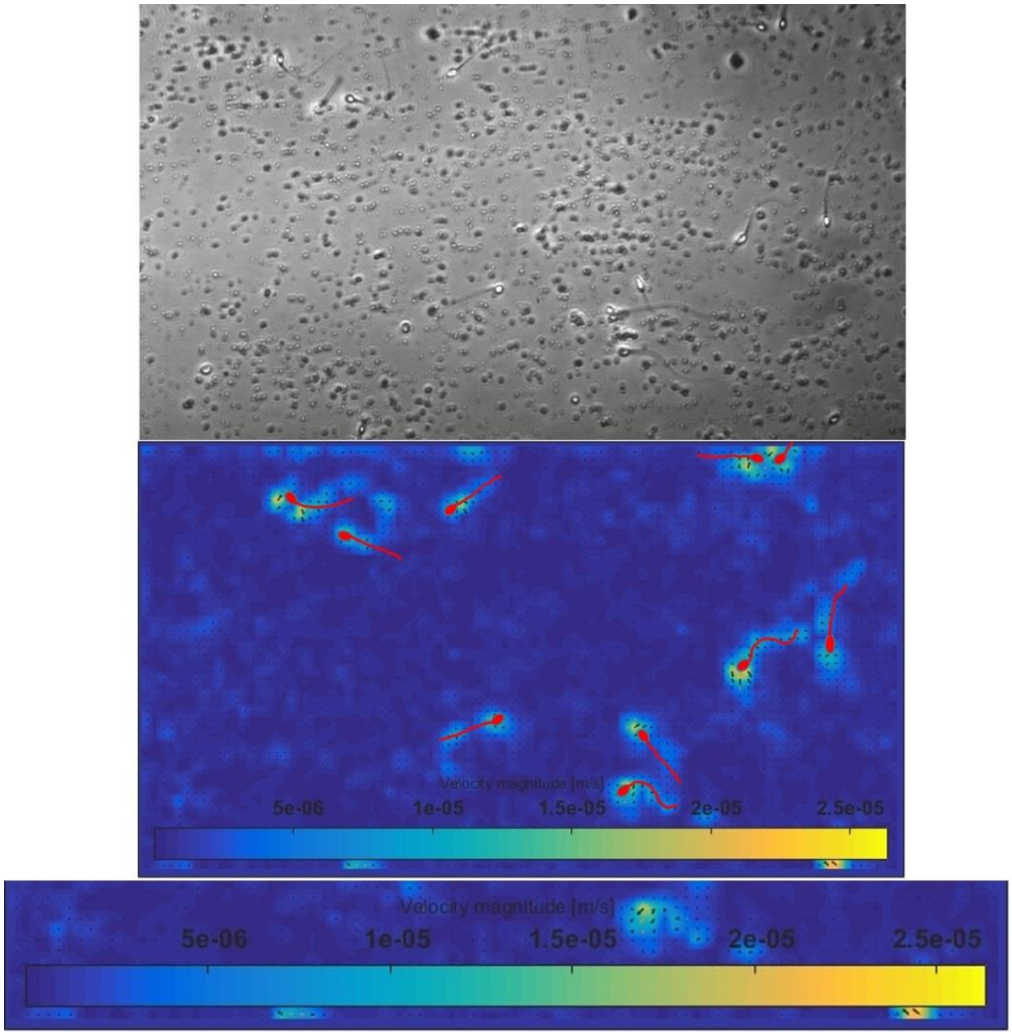


Figure 5-20 Fluid velocity magnitude around motile sperm coded in yellow colour shows a maximum velocity magnitude of **2.5×10^{-5} m/s** (**25 µm/s**). Minimum velocity magnitude was < **5×10^{-6} m/s (5 µm/s)** in areas distant from sperm, coded in blue colour. The range of fluid velocity near a motile sperm in this movie was **15-25 µm/s**. Sperm position is indicated on the velocity plot by illustration taken from the matching frames in the original movies. Data taken from HV4 movie with ≤ 2-10 sperm in the field of view.

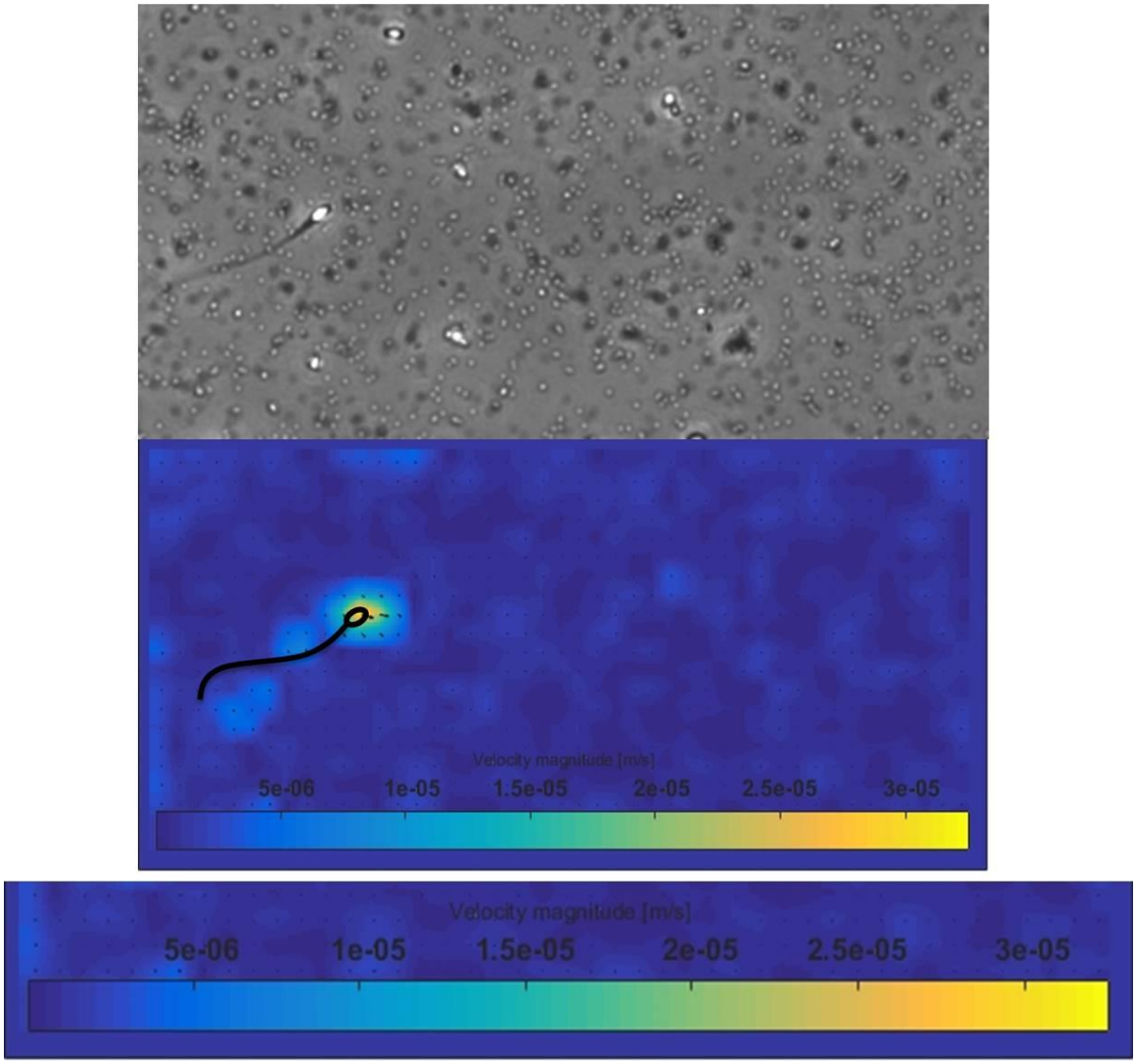


Figure 5-21 Sperm velocity magnitude in HVM in the range of **20-30 µm/s** in this movie. Sperm position is indicated on the velocity plot by illustration taken from the matching frames in the original movies, data taken from movie HV2, ROI.

Similar to the LVM, the range of the fluid velocity magnitude in HVM was highest near the motile sperm and minimum in areas with no sperm (≤ 5 µm/s).

The analysis of averaged fluid vorticity in HVM also demonstrated a trail created for the sperm swimming path in the fluid (**figure 5-21**). The Browninan error was estimated for HVM (using the same equations in section 4.5.1 and changing fluid viscosity µ to 0.14175 Pa.s, the estimated Brownian error was = 21.8 %.

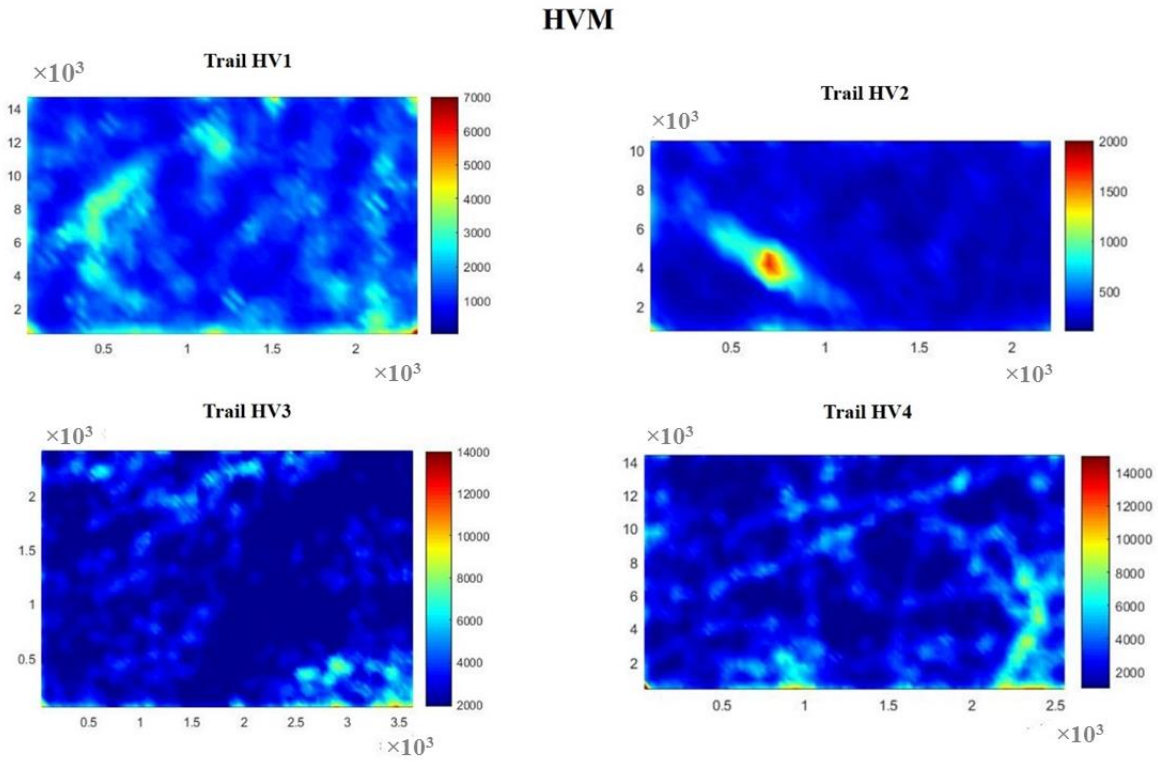


Figure 5-22 Averaged-fluid vorticity around motile sperm analysed in HVM; trails HV1, HV3 and HV4 analysed with ≤ 2-10 sperm in the field of view while HV2 was analysed with a single sperm in the field of view

Similar to averaged vorticity in LVM, single sperm produced one localised trail of vorticity (trail HV2, **figure 5-22**) and sperm of ≤ 2-10 showed multiple vorticity trails (trails HV1, HV3 and HV4, **figure 5-22**). To clearly visualise the formed trails in each data analysis, the colourmap bar was adjusted accordingly and the colour bar scales change from one data set to another as seen in **figure 5-22**.

Peclet number for sperm chemoattractant is also relevant in HVM, however, the diffusion coefficients of progesterone and burgeonal was only available in aqueous solution and not viscous ones.

5.5 Discussion:

This study is the first to our knowledge to examine human sperm motility from this perspective of fluid vorticity and mixing using PS beads as tracing micro-particles. The current study investigated fluid velocity and vorticity around motile sperm imaged in a 10 μm chamber in low and HVM. This study's preliminary results indicated that sperm cells move with minimal mixing of their fluid surrounding and. The observed fluid motion or 'mixing' around the motile sperm was localised spatially to the vicinity of the sperm body and when averaged across the sperm path a trail was observed.

Both free single sperm and a small population of sperm (≤ 2-10 in the field of view) were imaged and fluid vorticity around their trajectory was investigated. Studies of single-cell biomechanics has been shown as a valuable approach for exploring the cell's response to modulation in the surrounding environment (Kohles et al., 2007, Nève et al., 2008). Although the results of the study are preliminary, this work may serve as a platform for future studies employing high frame rate imaging to explore how sperm affect the fluid environment around them *in vitro*. The first observation in this study was that, sperm do not cause major disturbance in the fluid environment around their swimming trajectory. The fluid mixing is also observed to be restricted to the sperm immediate vicinity as illustrated by the "trail" of fluid vorticity. The averaged vorticity trails formed by the motile sperm observed in the current study may imply the locality of the fluid mixing exerted by the motile sperm. These observations could be significant for reproductive phenomena such as chemotaxis, as sperm moving with minimum mixing may maintain any established chemical gradients. In some marine species, it has been observed that the fertilisation success is largely linked to the relationship between chemical signals and fluid flow, which regulates the sperm-egg encounter rate (Zimmer and Riffell, 2011).

The significance of the identified fluid "trail" formed by the human sperm in the current study may link to maintenance of minimum disruption to established chemotactic gradients traversed by sperm. Further inferences may require additional studies.

No previous studies have explored fluid mixing caused by human sperm. PIV studies of fluid mixing have been applied in some marine species to explore biogenic mixing, a phenomenon described for motile macroscopic organisms to enhance nutrients and other materials transport in the environment, for example in oceans (Kurtuldu et al., 2011, Katija and Dabiri, 2009). On microscopic level, local mixing has been described for flagellated microorganisms such as chlamydomonas for optimum nutrient uptake and transport (Tam and Hosoi, 2011, Short et al., 2006).

The limited understanding of the mechanical impact of motile human sperm on the fluid milieus within the FRT and scarcity of sperm models looking at the potential roles of this mechanism were the main motivations for this study. Fluid mixing or stirring could be relevant for chemotactic response which has been observed for human sperm (Spehr et al., 2003, Eisenbach and Giojalas, 2006). Sperm chemotaxis in the vicinity of the COC has been suggested as an important guide mechanism to direct sperm to encounter the oocyte (Cohen-Dayag et al, 1995, Fabro et al., 2002; Eisenbach, 2004). The local sperm mixing action on the fluid environment observed in this study with no large scale disturbance could be significant for sperm swimming through, for example hFF or progesterone hormone, on their way to the oocyte.

The calculation of the Peclet number of both progesterone and burgeonal showed both sperm chemoattractant have high Peclet numbers (> 1-2) which suggests that mixing at a distance of 10cm in LVM is negligible. However, since chemotaxis is shown to be relevant in the vicinity of the COC (Eisenbach, 2004, Eisenbach and Giojalas, 2006), the

calculation requires estimated distance from the COC when sperm chemotaxis is operating which is not available in the literature and the diffusion coefficient of both chemoattractant should be in viscosity comparable to oviductal fluid.

The study of fluid mixing may also be relevant to investigate the influence of motile sperm on each other in terms of navigating their way through the same fluid gradients. The minimum mixing of the fluid environment by the vanguard sperm (the sperm in the frontline population), may maintain any chemical gradient for the following sperm to swim through in their journey toward the fertilisation site. For example, progesterone, which has been shown as an important chemoattractant factor for sperm (Serrano et al., 2001, Teves et al, 2006) may exist in a gradient concentration in the fluid environment in the oviduct (released from COC and present in hFF (Uñates et al., 2014). This negligible mixing of the environment observed by the low vorticity and confined trails evaluated in this study may indicate that there is minimum perturbation of the fluid during sperm journey which commences with large numbers of sperm and may end with only few near the fertilisation site (Williams et al, 1993). The averaged fluid vorticity over the image sequenced movies demonstrated a trail which may indicate the localised effect of motile sperm on the surrounding fluid which also disappears near instantaneously once the sperm has moved on.

The study of sperm motility from the point of view of fluid vorticity may be vital for research into chemotaxis and other aspects of fluid flow and rheotaxis. *In vivo*, sperm motility is modulated by several factors such as boundary constraints, the viscoelastic properties of the FRT secretions and their flow and the epithelial lining of the tract (Katz et al., 1989). Therefore, fluid mechanics is a relevant aspect to study sperm motility behaviour and this approach has been applied in several studies in reproductive biology

(Smith et al, 2009a, Gaffney et al, 2011, Hyun et al, 2012, Miki and Clapham, 2013, Eamer et al, 2015). Such studies require interdisciplinary teamwork and application of techniques from both reproductive biology and mathematics. The high complexity of the techniques applied and the requirement of mathematical data processing in such studies are sometimes challenging.

In addition to investigating fluid vorticity, velocity magnitude around human sperm was also considered in both LVM and HVM in this study. Fluid velocity around motile sperm was evaluated to be 25- 45 μm/s in LVM and 20-35 μm/s in HVM. The lower average values in the HVM could be attributed to the reduced PS bead motion with the fluid flow after certain periods particularly in the HVM as illustrated below.

The solid boundary created by the 10 μm chamber surfaces are also known to constrain the fluid motion generated by the sperm swimming near the chamber surfaces (Barratt et al., 2011). This boundary effect is complicated with several aspects including the tendency of sperm cells to swim near surfaces (Smith et al., 2009a) and also to the restrained flagellum active thrust due to constrained sperm head movement caused by increased passive resistance (Zhu et al., 1994, De Jonge, 2005).

Methylcellulose, which is an inert, non-toxic synthetic viscoelastic fluid medium, was used in this study to mimic the high viscosity fluid encountered by the sperm in the FRT. 1% methylcellulose preparation has been shown as a good analogue to peri-ovulatory cervical mucus ((Smith et al., 2009b, Ivic et al., 2002). However, the procedure of mixing the PS beads with the methylcellulose for HVM preparation was problematic as the PS beads tend to stick to each other and form clumps which did not follow the fluid flow faithfully in some settings. The hydrophobic characteristic of the polystyrene beads causes non-specific binding due to hydrophobic interactions or the charge-based interactions (Kim et al.,

2004). The PS beads were moving along with the fluid flow, however, some degree of beads clumping was unavoidable, but was minimised (as shown in the methodology section 5.3.2.2, part a). Coating the PS beads with BSA was based on simple adsorption which prevents non-specific binding and enables minimising the beads sticking to each other and prevents adherence of the beads to the surfaces of the chamber (Weibel et al., 2005). The successive washing steps of the PS beads also aimed to reduce any toxicity for the sperm cells.The lack of evident movement of the PS beads in the HVM under the 40 × lens could be anticipated due to the increased viscosity which causes the thermal movement to be damped. However, under higher magnification, the PS beads were confirmed to be moving with the fluid flow.

Discussion of the methodological choice, strengths and limitations of the study:

In this study, both low and high viscosity media were utilised to study fluid velocity and vorticity around motile sperm. The study of sperm motility in HVM is highly relevant to *in vivo* sperm modulations and natural conception (Smith et al, 2009b). In fluid dynamic studies of sperm, the use of HVM setting is recommended (Smith et al., 2009b, Zimmer and Riffell, 2011) since sperm in the FRT encounter highly viscous fluid environments (Katz et al, 1989; Suarez and Pacey, 2006). The viscosity of human FRT fluids is estimated in the range of 1.7 Pa.s (1700 centipoise) and above (Hyun et al., 2012, Tam et al., 1980). Nonetheless, study of sperm motility in LVM is also vital to understand sperm motility modulations *in vitro* and probably in FF at ovulation (Smith et al, 2009b).

The size of the used PS beads in this study was 0.5 µm which is both sufficiently small to follow the flow and yet can be visualised under microscope. It has been suggested that the smaller the beads compared to the scale of the flow the better the beads follow the fluid

flow in a reliable manner (Sharp and Adrian, 2005). On the other hand, the beads have to be sufficiently large to be visualised and imaged under microscope and also reduce errors caused by Brownian motion (Wereley and Meinhart, 2010). It has been suggested that Brownian error is a significant consideration for particles with 50 to 500 nm diameter with velocity flow of approximately 1×10^{-3} m/s (Wereley and Meinhart, 2010). In the current study the Brownian error was calculated as 2.1%, however, as discussed earlier, this figure was not considered in the data analysis based on the validation steps taken during data analysis using FFT correlation.

One of the limitations of the study is the tendency of the number of moving PS beads to decrease with time even in the LVM, therefore, immediate imaging was aimed after each chamber preparation in both types of media as well as using a new chamber with PS beads preparation vortexed immediately before loading the chamber. The addition of the PS beads to the media also increased the viscosity of both LVM and HVM as seen in the calculations of media viscosity following Ps beads addition in the methodology section which also reduced the beads movement.

In addition to the reduced movements of the PS beads after some time of the chamber preparation, the background noise caused by the Brownian motion of the PS beads was another consideration during data analysis. The impact of the Brownian motion was minimised by both obtaining the average of numerous particles in each interrogation area and averaging of multiple calculations of the velocity magnitude (Thielicke and Stamhuis, 2014). Three passes using an interrogation area of 64 × 64 pixels in pass 1, 32 × 32 pixels in pass 2 and 16 × 16 pixels in the pass 3 (each pass with 50% overlap between the neighbouring interrogation areas) were applied, (see figures **5-1** and **5-10**). Additionally, the velocity magnitude was plotted as an average over the whole frame range in each

movie and the fluid velocity magnitude presented was an average over eight movies in LVM and 4 movies in HVM. In PIV analysis, the correlation analysis is important since identification of the exact particle displacement for the many thousands of PS beads between frames is impossible (Thielicke and Stamhuis, 2014). In this study, correlation was performed using Fast-Fourier Transform (FFT) algorithms.

During the analysis process, several filters were used to smooth and validate the data at every step of the analysis using the software (as shown in the **figures 5-6, 5-7, 5-8, 5-9, 5-10 and 5-11**). The data smoothing filters were used to minimise the background noise caused by Brwonian motion and to ensure data validation. These filters enabled the acquisition of applicable results for the fluid velocity and fluid vorticity near the motile sperm in the PIV analysis. The advantage of using fluid vorticity along with the velocity analysis is that fluid vorticity is not affected by the time and distance calibration from one movie to another as vorticity expresses local rotation of the fluid. In future studies, the μPIV technique may be further validated on larger scales in other studies to use on models for calculations of fluid vorticity around sperm.

In addition to viscosity, the velocity of the sperm has been shown to be highly temperature dependent (Esfandiari et al., 2002, Appell and Evans, 1977, Milligan et al., 1978). In this study, the stage heater was maintained at 37°C throughout the experiments included in the data analysis to ensure optimum physiological conditions for sperm motility. Nonetheless, some studies have suggested a decrease in the viscosity of the methylcelluose media when temperature is increased (Hyun et al., 2012). In this study, MC was prepared at room temperature with estimated viscosity 0.14 Pa.s at 20°C.

The flagellar beat frequency in human sperm has been linked to the viscosity of the media surrounding the swimming sperm and has been estimated as 10-20 Hz (Smith et al. 2009b). Fluid viscosity has also been suggested to cause pivotal effects on sperm motility and flagellar waveform (Hyun et al, 2012, Smith et al, 2009b, Eamer et al, 2015). A study investigating the effects of high viscosity media on sperm motility's energetics suggested that fluid viscosity influences the mechanical aspects of sperm motility and not the molecular pathways involved in its energetics (Hyun et al., 2012). Increased viscosity is associated with increased frictional forces and suggested to decrease sperm curvilinear velocity (Hyun et al, 2012) and decrease flagellar wave amplitude (Eamer et al, 2015), however, in this study, these parameters have not been examined. Other studies looking at the effect of viscoelastic fluids on two dimensional sheet swimmers indicated that the response of the viscous fluid may enhance the velocity of the swimmer in a mechanism not fully understood (Montenegro-Johnson et al., 2013, Teran et al., 2010).

While sperm are shown in this study to cause spatially confined mixing or stirring of the fluid around them, the mechanical influence of the fluid has also been suggested in other studies to guide steering of sperm on large-scale distances (Miki and Clapham, 2013). Therfore, while sperm are stirring the fluid, the fluid is steering the sperm to guide them in their journey toward the oocyte. This study's preliminary results indicated that sperm cells move with minimal mixing of their fluid surrounding which was spatially localised to the vicinity of the sperm body.

Future research and recommendations

In the current study, fluid vorticity around freely motile sperm with progressive motility were investigated. In prospective studies, it is worth considering fluid vorticity around

sperm with hyperactivation, which manifest vigorous asymmetric flagellar beating (Suarez and Ho, 2003). Comparison of the fluid mixing patterns caused by both types of motility (progressive and hyperactivated) may enable elucidation of the different roles of both type of motility in fertilisation (for example in chemotaxis). A study modelling tethered sperm has investigated the role of hyperactivated motility in sperm escape from the oviduct epithelial surfaces (Curtis et al., 2012). The study revealed that hyperactivated sperm may exert stronger force which enables sperm to be released from the oviductal epithelium (Curtis et al., 2012).

Modelling of tethered sperm in HVM may represent similar setting to sperm bound to the oviductal epithelium reservoir which has been described for mammalian sperm (Suarez et al., 1991a, Reeve et al., 2005). The formation of the functional sperm reservoir is suggested to be facilitated by the high viscosity of the oviductal fluid which counteracts the progressive sperm motility and assists sperm attachment and adhesion to the epithelial lining of the oviduct (Hunter, 2008). Nonetheless, modelling of sperm may require the consideration of numerous factors involved in sperm motility modulation including fluid flow created by ciliated epithelia, boundaries effect, muscular contractions along with rheotaxis and non-Newtonian fluids (Winet et al., 1984). It is, therefore, challenging to model sperm *in vitro* and apply the results to *in vivo* sperm behaviour. Studies of sperm fluid dynamics may eventually enable the design of microfluidic device for sperm selection for IVF according to sperm velocity (Eamer et al, 2015).

Another aspect which has not been studied extensively is the hydrodynamic interaction of a population of human sperm in terms of synchronisation (Woolley et al., 2009) and altruism (Pizzari and Foster, 2008). In wood mice sperm, the apical hook on the head has been shown to become hooked to the neighbouring sperm flagellum or apical hook in the

fertilisation media forming clusters of hundreds of sperm (Moore et al, 2002, Fauci and Dillon, 2006, Pizzari and Foster, 2008). This phenomenon is described as "sperm trains", which is suggested to enhance the wood mice sperm velocity (Moore et al, 2002, Pizzari and Foster, 2008). Although human sperm lack hooks, the possibility of other mechanisms of cooperation is still possible and requires further research. Future studies considering human sperm synchronisation may investigate fluid vorticity created by synchronised sperm and their effect on sperm chemotaxis.

This study presented a new perspective of mechanical aspects of human sperm motility and indicated the importance of multidisciplinary research in understanding sperm fertilisation.

Chapter 6

GENERAL DISCUSSION AND RECOMMENDATIONS

General discussion

The sperm journey through the female reproductive tract (FRT) is not merely a passive passage, but is an interactive process in which sperm cells interact with the FRT's epithelial lining and secretions (Yeung et al., 2009, Suarez, 2016). The research presented has investigated certain aspects of sperm-FRT interactions including at both the molecular and physical/mechanical levels, **figure 6-1**.

Sperm modulation by the environmental milieu of the FRT during capacitation, motility and oocyte interaction are differing aspects of molecular sperm-FRT interaction. Sperm cells may also interact in the reverse direction with the FRT by releasing certain substances or metabolites that may elicit chemical signalling relevant to fertilisation (Connolly, 2012) or later events. The FRT of a number of non-primate mammals has been shown to respond to the presence of sperm by the release of complex combination of proteins and hormones, which play roles in sperm regulation and preparation for fertilisation (Suarez, 2016). Finally, physical modulation of the sperm's migration may occur for numerous reasons including: orientation along the complex geometry of the FRT; sperm response to the fluid flow induced by the ciliary beat; and changes in the viscoelastic secretions in which sperm migrate through the tract (Suarez, 2016).

Characterisation of hFF proteome was the first stage of this research. hFF is a highly complex body fluid surrounding the oocyte, through which a fertilising sperm will pass and which proceeds ahead of the ovulated oocyte through the female tract.

A combination of proteomic approaches were applied in order to tackle the challenge of the high dynamic range of hFF proteins and to achieve better detection of low abundance proteins. This study applied solid phase extraction, high abundance proteins depletion and filtration/concentration of the depleted hFF coupled with high performance liquid

chromatography/mass spectrometry for thorough proteomic analysis. A total of 479 proteins were identified compiled from both crude and depleted hFF samples with 103 novel hFF proteins added to the repertoire. Most recent hFF proteomic studies have applied a combination of proteomic techniques (Hanrieder et al., 2008; Hanrieder et al., 2009; Jarkovska et al, 2010; Bianchi et al, 2013, Ambekar et al., 2014; Zamah et al., 2015) to achieve better hFF proteins identification. The results of this study in terms of number of identified proteins is similar to other recent hFF studies which identified a range of 240 to up to 770 proteins in the hFF with at least one third novel proteins identified (see **table 2.1**, chapter 2 for further details). This may highlight the high variety of hFF proteins and the significance of the application of a combination of proteomic techniques to study hFF proteins (Twigt et al, 2012).

Some of the novel hFF proteins identified in this study are members of protein families with the other members being identified in previous hFF proteomic studies. For example, annexin A1 from the annexins family was identified for the first time in this study while annexins A2, A4, A5, A6 and A10 have been previously detected in hFF (Zamah, et al, 2015; Angelucci et al, 2006; Ambekar et al, 2014). This observation may indicate the significance of compiling hFF protein data from several hFF studies and to work toward the complete hFF proteome for better understanding of the functions of these hFF proteins in reproduction (Zamah, et al, 2015). Therefore, along with the protein data identified in this study, hFF proteins from 24 published studies were also compiled. The compiled hFF proteome from the literature resulted in 1586 hFF proteins presented in a table with their UniProt accession numbers and references of the studies detected them. This hFF protein table provides a resource for future research into folliculogenesis, discovery of biomarkers

for oocyte quality, study of fertilisation environment and potential improvements in IVF media used in assisted reproductive techniques.

The study of the hFF proteome is not only relevant for biomarkers of oocyte quality but also to sperm as sperm is exposed to hFF in the ampulla just prior to fertilisation. The relevance of this exposure is seen in different aspects of sperm functions (as summarised in chapter 1, section1.5)

Brewis and Gadella (2010), suggested that prior proteomic study of the fluid milieus sperm encounter in male and female reproductive tracts may be beneficial in evaluating their influence on sperm surface proteins since proteins and other factors from these milieus may induce alteration of sperm surface protein composition and organisation (Brewis and Gadella, 2010). In the current work this strategy has been followed, hFF proteomics being characterised prior to examination of sperm-hFF proteomic interaction.

A comparative proteomic study of sperm incubated in capacitation media alone and sperm incubated in capacitation media along with hFF was conducted to investigate whether sperm may acquire hFF proteins. Sperm are transcriptionally and translationally inactive and therefore may rely on other sources of proteins to achieve maturation and fertilisation (Miller et al., 2010, Oliva and Castillo, 2011) or to survive for long periods of time. In this study, sperm were incubated with and without hFF in capacitation media and two analytical approaches were applied. The total binding protein approach allowed the analysis of the total sperm + hFF protein sample preparation with high number of soluble sperm proteins being identified and therefore sperm + hFF membrane-associated approach was designed aiming to identify sperm loosely-associated surface membrane proteins along with newly bound hFF proteins.

During the comparative protein data analysis, the compiled hFF proteome table (generated from 24 hFF proteomic studies in the literature and hFF data in this work) was used to compare protein data from the sperm + hFF samples to identify any hFF protein in the data set. Twenty-four hFF proteins, never previously identified in sperm, were found in the sperm + hFF samples. The results of this study, therefore, suggest that sperm may acquire proteins from hFF and their putative effects warrant further study into their role in sperm functions. The identified hFF proteins in the sperm + hFF samples may also be relevant for future studies interested in purifying relevant hFF proteins to prepare media either for IVF or increasing the longevity of sperm. The possible roles of the putative hFF proteins identified in this study on sperm may involve complement regulation mechanism to protect sperm against damage caused by the immune response in the FRT, protection of sperm from oxidative damage by the antioxidant properties of some of these hFF proteins and enhancement of cholesterol efflux and sperm membrane fluidity important for capacitation. In addition to the detection of the hFF proteins in the sperm + hFF proteomic study, 63 novel sperm proteins were also identified in this study that add new proteins to the characterised human sperm proteome.

The next objective of this work was to conduct a comparative metabolomics study to investigate the human sperm metabolites released during sperm capacitation of 6 hours and those present prior to sperm capacitation (1h swim-up). An untargeted metabolomics technique using UPLC-MS analysis was applied to characterise, for the first time, the human sperm metabolites in two preparations. The choice of using untargeted metabolomics approach was to obtain a global view of the metabolites released/consumed in sperm capacitation media since this is the first study, to our knowledge, to investigate human sperm metabolomics. The capacitation media utilised and the incubation time of 6

hours were selected to best mimic physiological conditions in the FRT. Two hundred and three metabolites were detected in this study with fatty acids, oxidised fatty acids and peptides being among the main groups of metabolites identified. Sperm releasing certain metabolites while consuming others during *in vitro* capacitation may be relevant to sperm capacitation in the FRT. Although the results of the current sperm metabolomics study are preliminary, it shows validity and promise for its use in further research exploring sperm metabolism. The detected sperm metabolites are also relevant to research into sperm signalling in the FRT. Previous work in our group has shown that sperm trigger calcium-signalling in oviductal tissue and that whatever induces this signal is a diffusible messenger released by sperm during capacitation (Connolly, 2012). The current work has identified some of the metabolites, which are produced by the sperm into their surrounding media during capacitation, a key next stage would be to examine the effect of these metabolites upon the female tract at relevant concentrations to discover whether any signals are induced. The significance of sperm signalling observed in Connolly (2012) to changes in the endometrial proteome and preparation for implantation may require further research. In murine species, it has been shown that signalling induced by seminal plasma may influence implantation and future child health (Robertson, 2014, Schjenken and Robertson, 2015).

The next aspect that was investigated in this work was fluid velocity and vorticity around human sperm in both LVM and HVM. The physical aspects of human sperm motility are relatively overlooked with most studies focusing on the molecular and biochemical characterisation of sperm motility (Smith et al, 2009b). Sperm migration in the FRT may introduce mixing of the fluid in the tract, which may have relevance in terms of studying chemotaxis and other aspects of chemical and mechanical sperm-FRT interactions. The

currently available knowledge of whether sperm cause any perturbation or mixing of the fluids they swim through is almost unknown and was the main motivation for this study. This work was the first to our knowledge to investigate the effects of human sperm motility from the perspective of fluid velocity and vorticity in LVM and HVM. In collaboration with mathematicians, the application of µPIV using MATLAB and PIVlab_GUI programes enabled the study of fluid velocity and vorticity around motile sperm. In this study, sperm were shown to cause minimum perturbation to the fluid surrounding them while migrating in a confined space represented by a 10-micron chamber. A trail was demonstrated, for the first time in this study, by averaging the fluid vorticity around the motile sperm along their migration path using MATLAB codes developed for this work. The sperm trails may signify the locality of the sperm mixing effect on the fluid surrounding them. This observation may be relevant to sperm chemotaxis and to sperm's ability to only cause minimum mixing of any established chemotactic gradient *in vivo* in the vicinity of the COC. Fluid velocity around motile sperm was also investigated and the average fluid velocity was 25-45 µm/s in the LVM and 20-35 µm/s in the HVM.

In conclusion, the study of sperm and FRT interactions needs to involve both physical and molecular aspects to illustrate the different impacts of these factors on sperm fertilisation capability (as also suggested by Suarez, 2016).

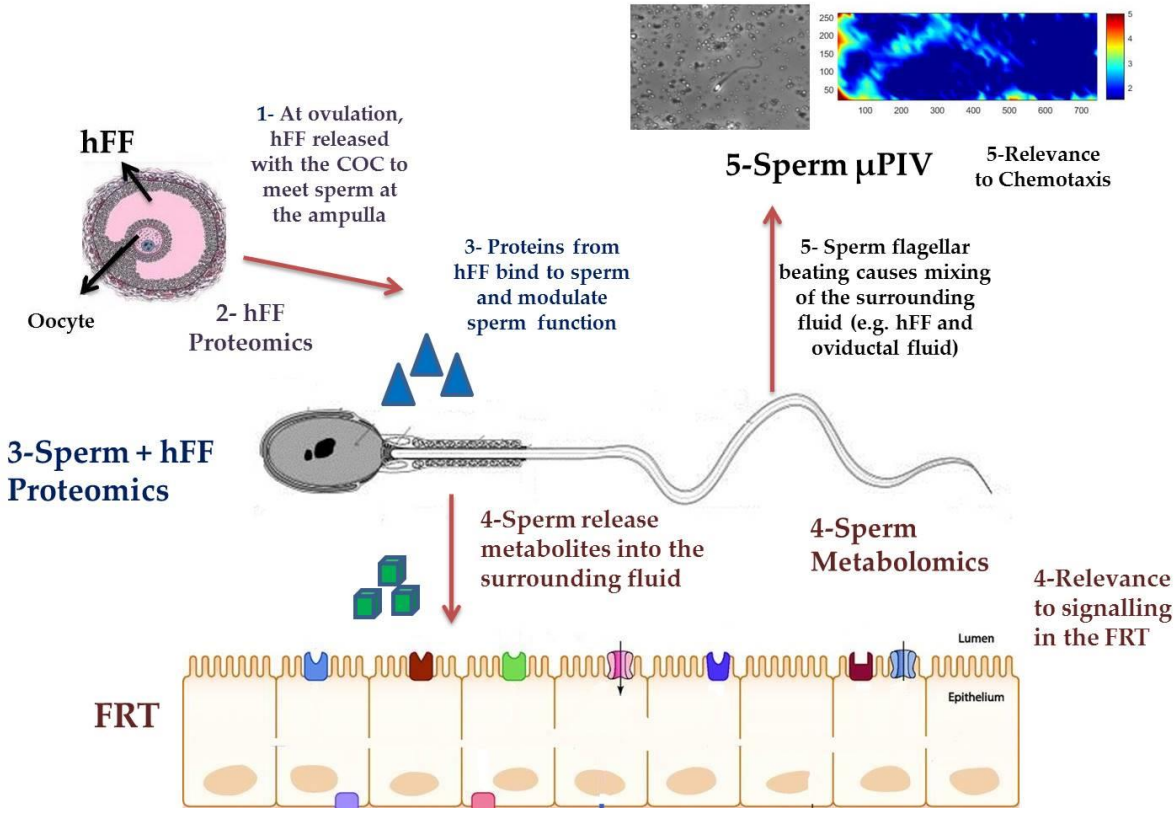


Figure 6-1 Some of the different aspects of sperm and FRT interactions. **1-** hFF is released along with the COC at ovulation **2-** hFF proteomic analysis was conducted in this work and identified 479 proteins **3-** sperm + hFF proteomic interactions with hFF proteins acquired by sperm and their relevance to sperm modulation **4-** Sperm metabolomics and the relevance of sperm metabolites to FRT signalling **5-** Sperm µPIV and its relevance to chemotaxis

Future work:

Several studies have considered hFF proteins, however, the ideal hFF proteomic profile which may enable prediction of best oocyte quality and IVF outcome is yet to be characterised (Benkhalifa et al., 2015). The high dynamic protein range and the presence of HAPs which may hinder the detection of follicle-specific proteins in hFF may limit protein biomarkers detection (Bianchi et al, 2013). Additionally, another challenge in the study of hFF proteomic is to progress from lists of detected proteins to a more advanced understanding of molecular function (Twigt et al, 2012). Future studies exploring hFF

proteomics may take advantage of the growing field of metabolomics and combine hFF proteo-metabolomics analysis.

Recently, coupled proteomic and metabolomics analysis of human sperm extract revealed interesting relationship between the proteomic profile of sperm and their metabolites in normozoospermic and asthenozoospermic samples (Paiva et al, 2015). A similar approach could be applied in hFF analysis, hFF metabolomics profiling as has already been suggested as a promising future approach in clinical practice for the evaluation of oocyte developmental potential prior to IVF (McRae et al., 2012, Xia et al., 2014). Coupled with advanced proteomic techniques, hFF metabolomics analysis may enable the detection of the best oocyte quality for IVF (McRae et al, 2012).

The majority of hFF studies support the concept that steroid hormones in hFF, mainly progesterone, are responsible for the observed effects of hFF on sperm function (Morales et al., 1992, Fabbri et al., 1998). This work has revealed that some hFF proteins may bind to sperm following incubation and therefore we suggest that these proteins may have relevance to the different aspects of sperm functions including acquisition of fertilisation competence and longer sperm survival. Future work investigating the effects of, for example, purified hFF proteins in the presence versus in the absence of progesterone on sperm functions may reveal new insights of the role of hFF proteins in sperm fertilisation. The role of hFF protein in sperm fertilisation may also be relevant to the discovery of new targets for male contraception. In this study, sperm incubated with hFF was investigated in the view of whole sperm proteome and membrane-associated proteome. Future studies may apply subcellular proteomics to study sperm and hFF interactions on the level of, for example, the sperm acrosome, head and flagellum. While such studies may be technically challenging and time-consuming (Brewis and Gadella, 2010; Amaral et al, 2014a), they

may enable localisation of any binding sites of hFF to sperm and provide more insights of the functional roles of the bound hFF proteins in sperm fertilisation capability. The subcellular proteomic approach may also enable the identification of low abundance hFF proteins which bound to sperm and may not be detected in whole sperm proteomic analysis. A further advantage of subcellular sperm proteomic is the reduced sample complexity and better detection of low abundance sperm proteins (de Mateo et al., 2011; Amaral et al., 2013).

In this work, comparison of sperm metabolites from normozoospermic samples following 1h swim-up and 6h capacitation was conducted. Future studies may consider differences between human sperm metabolome in fertile and infertile subjects which may enable the discovery of certain male infertility biomarkers. Currently, only one study has applied comparative analysis of sperm metabolomics in normozoospermic and asthenozoospermic samples with initial results indicating an overall lower concentration of the identified metabolites in the asthenozoospermic samples (Paiva et al, 2015).

Study of the effect of different environmental factors such as smoking, diet and physical exercise on the pattern of human sperm metabolomics may also provide insights into the effects of the different environmental factors on male fertility.

The examination of the signalling effect of the sperm metabolites identified in this work on the female tract cells would be the next stage of this research. As described by Connolly (2012), a signalling response was elicited in an oviductal tissue extract following its co-incubation with sperm. If a metabolite(s) was identified which caused similar tract signalling, a randomised clinical trial of infusion of the sperm metabolite(s) to the uterus and evaluation of implantation rates and pregnancy success rates may enable better understanding of the role of sperm metabolites in human reproduction and open up an

exciting new therapy. This would have particular significance as the Robertson's group data(Robertson, 2007, Robertson et al., 2006a, Schjenken and Robertson, 2015) may suggest it would improve the health of children being born not just a treatment success rate.

The mechanical aspects of sperm motility in terms of perturbing chemical gradients such as hFF, progesterone or other relevant fluid in the FRT has gained little attention in the literature. Future studies exploring the fluid vorticity and the effects of motile sperm on an established gradient of, for example, hFF or progesterone are therefore recommended. Sperm stirring and the role of sperm population in causing mechanical changes in the FRT fluid and the impact of such type of hydrodynamic interaction on fertilisation rates have not been explored in human sperm. Future studies applying μPIV to explore the relationship between fluid flow, sperm chemotaxis and rate of fertilisation may be relevant to *in vivo* conception. The rate of fertilisation success in some marine species has been highly linked to sperm-oocyte encounter regulated by fluid flow and chemotaxis (Zimmer and Riffell, 2011). In humans, sperm chemotaxis to hFF has been correlated to oocyte fertilise-ability (Ralt et al, 1999). Nonetheless, the roles of fluid flow and sperm mechanical motility on sperm- oocyte encounter and fertilisation rates have not been thoroughly investigated.

Development of virtual sperm models which respond to the signalling and variation of the FRT's ambivalent microenvironments in comparable way to the *in vivo* sperm is the ultimate goal of multidisciplinary research in reproduction and mathematics (Gaffney et al, 2011). In this work, a 10-micron glass chamber was used to investigate sperm velocity and fluid vorticity. For future studies, it may worth applying micro-channels with elastic walls, which may mimic the microenvironment in the FRT to better model sperm in *in*

vitro studies. An elastic silicon-based polydimethylsiloxane has been used recently to create micro-channels to study sperm swimming behaviour near surfaces (Denissenko et al., 2012). The elasticity of such chambers may provide better resemblance to the *in vivo* epithelial surface in the FRT (Suarez, 2016). Another microfluidic model has been developed to simulate the fluid flow and the micro-crypts of the FRT to study the effects of the FRT biophysical characteristics, mainly fluid flow and microgrooves on sperm migration (Tung et al., 2014, Tung et al., 2015). The advancement in understanding of sperm fluid dynamics may enable the design of sperm-sorting micro-devices for the selection of competent sperm based on sperm motility (Seo et al., 2007, Shao et al., 2007, Gaffney et al., 2011).

To the best of our knowledge, this work is the first to conduct a comparative proteomic analysis of human sperm versus sperm-hFF interaction and to perform a metabolomics analysis of human sperm's capacitation media at two time points. As knowledge of sperm regulation in the female tract advances, possible new approaches for male infertility diagnosis based on sperm metabolic profile and/or the pattern of sperm proteomic interactions with the FRT secretions may be developed. Development of sperm media enriched with hFF proteins relevant to sperm survival and regulation may also enhance sperm fertilisation capability. Prior exposure of FRT to certain sperm metabolites may be a new approach to enhance implantation and pregnancy outcome. The novel perspective of mechanical aspects of sperm motility by studying fluid velocity and vorticity around motile sperm may add also a new approach to the study of sperm motility and chemotaxis. The improved understanding mechanical aspects of sperm motility may also enable the design of microfluidic devices for sperm selection based on motility.

APPENDICES

Appendix 1:

Buffers and recipes:

- SDS sample buffer (5 ×): Bromophenol blue (0.25%), DTT (dithiothreitol; 0.5 M), Glycerol (50%), SDS (sodium dodecyl sulphate; 10%), Tris-Cl (0.25 M, pH 6.8).
- 20× MOPS buffer (3-(N-morpholino) propanesulfonic acid) for running the gel (50mM MOPS, 50mM Tris base, 1mM EDTA, 400ml of distilled water. 25 ml of 20 × MOPS in distilled water up to a total volume 500 ml to obtain 1 × MOPS buffer
- Coomassie Brilliant Blue stain: 1 g of Coomassie Brilliant Blue (Bio-Rad) in 1 liter of the following solution: 50% Methanol, 10% Glacial acetic acid, 40% H_2O
- Phosphate Buffered Saline (PBS) :137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
- 10 × M medium (1.37 M NaCl, 25 mMKCl, 200 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), 100 mM glucose)
- HPLC solvent A: 1ml of trifluoroacetic acid (TFA) in 1L of distilled water, mixed thoroughly in a glass cylinder and then kept in the HPLC reservoir bottle.
- Buffers provided with the IgY12 SCs are : 1 × Dilution buffer: 1 × Tris Buffered Saline: 10 mM Tris-HCl, pH 7.4, 150 mM sodium chloride), 1× stripping buffer (0.1 M Glycine-HCl, pH 2.5) and 1× neutralization buffer (0.1 M Tris-HCl, pH 8.0)

Appendix2: (chapter 2)

List of hFF proteins identified in the hFF proteomic study:

Protein	Peptides	Score	**Protein**	Peptide	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score
P01024	113	6895.6	P01023	18	917.4	P0CG05	10	709.6	P08238	5	205.9	Q96IY4	5	160.2
P02768	106	6892.4	P05155	18	1163.2	P07360	9	649.4	P01764	6	254.8	P16070	5	270.7
P98160	73	4176.2	P01876	18	1102.3	P12111	9	458.3	P22105	6	256.5	P04207	5	163.3
P0C0L5	64	4032.7	P03952	18	1030.6	P0C0L4	9	510.5	P43251	6	367.6	P80362	5	279.5
P02787	62	4532.1	P01011	17	1088.6	P05452	9	495.8	P47972	6	300.5	P04206	5	477.6
P01009	45	2976.5)	P02675	17	956.9	P05154	9	474.9	P05543	6	467.2	P18065	4	178.2
P08603	43	2301.3	P01859	17	969.4	P68032	9	364.5	P00742	6	232.0	O95445	4	173.0
P00734	37	2420.3	P01860	17	987.9	P11047	8	558.7	P08263	6	236.1	P13645	4	131.1
P06727	34	1764.4	P10909	16	1023.8	P02750	8	360.8	P15309	6	271.1	P08195	4	167.0
P02774	34	1963.2	P02760	16	1107.1	P01834	8	1006.1	Q16610	6	243.4	P63104	4	144.2
Q14624	32	1936.2	P01861	16	920.0	P04196	8	451.8	P01622	6	330.8	P19320	4	157.3
P01042	30	2054.9	P05156	15	888.5	P29622	8	337.8)	Q03591	5	229.5	P08779	4	138.1
P02790	29	1607.6	P01019	15	842.4	P07225	8	320.5	P35527	5	216.5	P32119	4	140.8
P02647	28	1655.6	P13671	15	749.1	Q9NZP8	7	307.1	P07737	5	276.8	P30101	4	225.0
P01008	28	1811.5	P02748	14	756.1	P04075	7	280.3	P36980	5	179.7	P01871	4	267.0
P02751	28	1713.5	P02765	14	1204.8	P02649	7	352.5	P27169	5	248.1	P01619	4	286.6
P19823	27	1713.5	P05090	14	627.8	P04004	7	434.9	P09871	5	354.7	P01765	4	267.0
P00747	26	1479.6	P36955	14	802.6	P05546	7	381.3	P01620	5	427.4	P01766	4	311.0
P19827	25	1728.5	P07357	13	833.5	P04278	7	327.4)	P80748	5	210.5	P06310	4	189.0
P06681	24	1172.4	P00738	13	762.8	P01768	7	282.4	P01597	5	346.4	P19652	4	128.6
P06396	24	1296.9	P01877	13	801.0	P04003	7	316.6	P01625	5	417.6	P01880	4	125.4
P43652	23	1099.8	P07358	13	750.6	P55268	7	238.5	Q04756	5	221.2)	P01594	4	257.3

Protein	Peptides	Score	**Protein**	Peptide	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score
P00751	22	1258.9	P02749	12	831.8	P62937	7	322.2	O75882	5	294.2	P01610	4	198.5
P04114	22	1108.9	P00748	12	587.3	P07093	7	352.6	P33151	5	221.0	P01762	4	228.4
P00450	21	1372.0	P08697	12	662.2	P00736	7	337.2	Q96KN2	5	302.6	P01767	4	202.7
P01857	21	1346.8	P51884	12	580.6	P11142	7	296.4	Q96PD5	5	344.5	P01781	4	254.9
Q06033	21	1296.0	P02679	11	549.8	P06733	7	364.4	P04275	5	156.2	P04406	4	148.4
P01031	20	901.3	P60709	11	476.9	P22792	7	440.1	P13796	5	149.4	P15169	4	171.0
P02671	20	901.5	P02766	10	740.2	P18135	7	338.8	P23142	5	316.8	P17936	4	173.5
P00739	20	1110.2	P02753	10	626.2	P02652	6	218.2	P80108	5	252.8	P26927	4	172.8
P10643	19	1062.2	P35858	10	537.2	Q9UGM5	6	319.9	Q14520	5	232.3	P07996	4	250.3
P04217	19	1252.2	P13611	10	469.0	P04264	6	283.2	P07900	5	221.6	P06858	4	161.9
Q9UK55	4	210.2	P81605	3	130.3	P07355	3	224.9	P09382	2	78.4	P55290	2	49.0
P03951	4	264.4	P01034	3	244.4	P12277	3	131.0	P13667	2	135.0	P25311	2	79.7
P00740	4	169.7	P02746	3	155.2	Q6UVK1	3	192.9	P16144	2	141.8	P02747	2	78.6
P15311	4	119.7	Q15113	3	83.3	P07237	3	169.4	P26439	2	61.5	Q9BXR6	2	53.9
Q12805	4	183.0	P27918	3	78.9	P13598	3	122.5	P30520	2	139.6	P39059	2	79.4
P14625	4	213.9	Q15166	3	139.4	P13639	3	122.8	P35221	2	46.9	P59665	2	97.3 (
O60664	4	172.5	P02533	3	71.2	P14314	3	108.6	P37837	2	98.6	P14174	2	80.1
P07602	4	220.4	O43866	3	173.8	P30041	3	93.6	P43490	2	149.6	Q9Y5Y7	2	107.8
P14618	4	273.3	O75874	3	109.5	P35241	3	86.0	P48741	2	77.3	P02655	2	124.8
P19338	4	224.0	P80188	3	117.6	P37802	3	115.2	P49321	2	47.9	O00533	2	75.8
P22314	4	308.0	P22692	3	152.5	Q02818	3	91.7	P55060	2	90.2	P08123	2	64.3
P55072	4	171.3	P35908	3	216.4	P04259	3	131.6	P61221	2	78.7	P13591	2	53.0
Q16658	4	219.5	P08253	3	99.6	P35579	3	172.8	P63241	2	96.6	P13647	2	115.2
P04430	4	171.5	P10599	3	121.0	P01596	3	227.0	P67936	2	66.4	P16035	2	73.9
P34932	4	142.4	P48668	3	113.8	P01599	3	227.0	Q02952	2	62.3	P26022	2	80.8
Q15366	4	116.3	Q99584	3	129.5	P01604	3	138.9	Q14103	2	83.0	P41222	2	75.4

Protein	Peptides	Score	**Protein**	Peptide	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score
Q99715	4	179.5	Q04695	3	71.3	P01609	3	259.0	Q14574	2	70.3	P48740	2	46.2
P01593	4	292.2	P31151	3	158.2	P06316	3	125.9	Q15084	2	103.2	Q15262	2	109.0
O00391	3	165.8	P60174	3	157.7	P31948	3	144.1	Q15149	2	70.4	Q76LX8	2	88.5
P25391	3	142.0	P09211	3	105.1	P01608	3	179.1	Q92598	2	74.4	Q8NBP7	2	100.8
Q06830	3	126.3	P35542	3	89.2	P01613	3	191.9	Q92973	2	57.8	P08476	2	48.5
Q14126	3	150.4	Q08380	3	161.0	P01614	3	230.9	Q9NQ38	2	71.9	P49908	2	49.0
Q6EMK4	3	115.6	P01617	3	169.7	P01701	3	69.2	Q9Y653	2	99.6	Q13103	2	76.2
P04180	3	166.7	O75636	3	87.1	P01714	3	185.8	P01623	2	150.3	P00338	2	72.8
P20742	3	158.6	P08185	3	193.6	Q9NPH3	2	70.4	P23083	2	108.1	P00441	2	106.8
O14791	3	95.6	P01598	3	251.5	P04434	2	77.8	P83593	2	64.2	P00915	2	63.9
P02786	3	162.2	P01602	3	220.3	Q96NH3	2	58.9	P01601	2	84.1	Q6UXB8	2	96.5
P00746	3	92.9	P01779	3	203.2	P04083	2	114.2	P01611	2	133.5	Q9HCB6	2	53.3
P00558	3	117.9	P98066	3	150.6	P05455	2	66.6	P01763	2	105.0	P00918	2	99.2
P05067	3	167.9	P55058	3	157.0	P09104	2	109.7	P13489	2	89.6)	P31025	2	144.2
P02788	2	63.6	P09172	2	64.1	P00488	2	105.9	P18206	2	89.1	Q13630	1	46.4
P04433	2	95.8	P12259	2	117.7	P23284	2	122.6	P22626	2	119.7	Q14257	1	40.8
P02763	2	79.8	Q99435	2	62.2	Q8IWY4	2	140.0	P36952	1	92.4	Q15392	1	67.7
P02656	2	134.1	Q13822	2	75.9	P68871	2	113.8	P39023	1	60.5	Q15404	1	77.3
P01700	2	106.5	O43707	2	49.8	P30153	2	112.0 (	P39687	1	87.1	Q15750	1	63.7
P01773	2	65.0	P05997	2	73.7	P52565	2	145.4	P49006	1	61.4	Q16401	1	47.6
P01769	2	86.2	P08758	2	110.4	P54578	2	106.1	P49411	1	72.9 (	Q16787	1	69.9
P05160	2	93.3	P12814	2	81.2	P83110	2	68.0	P49588	1	70.4	Q86UP2	1	65.4
P11021	2	83.1	P16112	2	67.8	Q02809	2	114.4	P49756	1	72.4	Q8WY91	1	55.8
P35443	2	69.1	P19022	2	130.4	Q5SZK8	2	57.0	P50502	1	68.8	Q8WZA0	1	48.2
P49747	2	120.4	P23528	2	87.4	Q9BTY2	2	130.8	P17706	1	55.8	Q9BY32	1	65.2
Q99969	2	59.7	P52209	2	58.1	Q9Y4L1	2	54.3	P17987	1	88.9	Q9H0U4	1	72.4

Protein	Peptides	Score	**Protein**	Peptide	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score	**Protein**	Peptides	Score
P08571	2	116.4	Q9Y5C1	2	101.7	P04070	2	60.4	Q9H8L6	1	106.4	Q9H0X4	1	50.4
P09455	2	68.7	P01033	2	150.7	P07942	2	86.9	P15814	1	87.2	Q9H4M9	1	72.0
P01591	2	115.2	P01344	2	57.3	P09238	2	64.6	Q9NZT1	1	36.5	Q9NUQ9	1	78.2
Q7Z7G0	1	65.4	P07108	1	41.7	O00232	1	83.8	P52907	1	49.9	Q9NY33	1	44.4
P19883	1	52.3	P08519	1	47.4	O00267	1	44.4	P58546	1	58.9	Q9NYQ8	1	44.0
O00339	1	50.6	P10451	1	64.6	O00469	1	45.5	P61970	1	65.4	Q9UMS4	1	47.3
P05019	1	52.9	P12004	1	53.1	O14950	1	79.3	Q09328	1	78.8	Q9UQE7	1	52.9
P35237	1	105.9	P14209	1	45.1	O75937	1	49.5	Q13561	1	57.3	Q9HAV7	1	55.2
Q01995	1	51.5	P16422	1	117.6	O76003	1	45.1	Q14195	1	53.6	P01699	1	30.9
Q13421	1	44.4	P02461	1	61.4	O76096	1	41.6	P80723	1	63.5	P01777	1	79.5
Q13838	1	43.3	P07339	1	64.4	O95716	1	57.6	O75144	1	52.1)	P27482	1	53.1
Q16543	1	71.8	Q9BWP8	1	79.7	P02730	1	47.7	Q12841	1	60.6	Q9UGM3	1	29.4)
Q99733	1	90.5	P02462	1	79.1	P02776	1	41.3	P02775	1	40.8	P04208	1	88.0
Q99985	1	45.7	P0CG47	1	57.8	P04746	1	51.0	P07307	1	44.5	P01825	1	61.4
Q9H173	1	62.7	P0DJI8	1	60.8	P05787	1	61.0	P18669	1	76.4	Q15582	1	71.6
O43790	1	74.3	P01717	1	79.3	P14151	1	56.3	P25787	1	68.4	P22352	1	61.3
P21741	1	67.5	O94985	1	43.7	P05111	1	81.7	P29401	1	50.0	P18428	1	99.6
Q8IVF4	1	66.0	O95479	1	55.0	P02654	1	43.8	P30044	1	44.9	P06276	1	63.8
P01742	1	47.8	P40925	1	67.1	P01621	1	79.2	P30530	1	44.8	P27348	1	98.0
P01833	1	82.6	Q13790	1	47.3	P05109	1	38.5	P08670	1	68.8	P69905	1	41.1
A5YKK6	1	56.2	Q9Y490	1	64.2	P61981	1	43.7	P09622	1	47.4	P07195	1	52.2
O00170	1	45.4	P06702	1	28.7	P61626	1	79.1	P43121	1	56.9			

Appenidix 3 (chapter 2)

The inclusion criteria for hFF protein data for the hFF proteins table

Authors	Criteria for protein, peptide identifications
Ambekar et al, 2013	False discovery rate (FDR) was calculated using peptide validator node of the Proteome Discoverer where, FDR is calculated based on decoy database search. Peptide spectral matches which qualified for 1% FDR were considered for drawing protein inference from the peptide data
Ambekar et al, 2104	Precursor and fragment mass tolerance were set to 20 ppm and 0.1 Da, respectively. Peptide identifications were filtered with 1% false discovery rate threshold at the peptide level. Differentially abundant proteins were identified with 1.5-fold change as the cut off value.
Anahory et al, 2002	The maximum number of missed cleavage sites was set to 1. Peptide coverage of the full-length protein exceeded 15% confirming the protein to be RBP. The RBP spot identified by MALDI-MS was also confirmed by co-migration of RBP purified on IEF followed by SDSPAGE (data not shown).
Angelucci et al, 2006	Protein identification was confirmed by peptide mass finger printing analysis. Score is −10 Log(P), where P is the probability that the observed match is a random event, it is based on NCBInr database using the MASCOT searching program as MALDI-TOF data. Significance evaluated with Mann-Whitney test, P<0.0001. A spot was considered as such only if it was detected in three out of five replicates for all patients and defined "common spot" (i.e., always present and in the same position on all 25 follicular fluid gels). Relative spot volumes were determined by modelling the optical density in individual spot segments using a two-dimensional Gaussian analysis. Spot identification was carried out by gel comparison with the human plasma 2-DE reference map available on-line
Bianchi et al, 2013	Protein identification was carried out by peptide mass fingerprinting and by peptide sequencing on an Ultraflex III MALDI-TOF/TOF mass spectrometer equipped with a 200 Hz smartbeam™ I laser. Tandem MS was also performed on a nanoscale LC-ESI/MS-MS system using a Micro-HPLC Pump Phoenix 40 (Thermo Finnigan, San Jose, CA) and a LCQ DECA IT mass spectrometer (Thermo).
Estes et al, 2009	The presence of spots A and B was confirmed in five out of the eight additional pairs in the similar pattern identified in the original two pairs (spots present in a total of seven out of ten pairs). Subsequently, because of the large number of gels analyzed (16 samples), PDQuest program was used to maximize comparison of the additional gels. The purpose of the PDQuest analysis program is to identify differences that may not otherwise be noted. An independent evaluation of the raw spot density data of the five spots identified by PDQuest and the two spots identified visually (data extracted manually from PDQuest by using the 3 3 mm average area density of each spot in the subsequent 16 2D PAGE images) confirmed the significant differences between the success and failure groups (P<.05).Quantitative Validation by ELISA: The total antithrombin (AT) level in follicular fluid was quantified by using direct ELISA with the purified polycolonal antibody against antithrombin.
Hanrieder et al, 2008	Proteins were found and identified by integrated mascot database batch search of all MS/MS in UniProt KB. All matches are identified significantly. Identified proteins are considered as a positive match on at least a 99% significance level (p<0.01) corresponding to a significance threshold in score of 28. At least one matching peptide for each identified protein must fulfil significance criteria
Hanrieder et al, 2009	The significance threshold was set to a minimum of 95% (p≤0.05). The reproducibility of protein and peptide identification was evaluated by analysis of three consecutive CE-MALDI runs of one sIEF fraction.

Hashemitabar et al, 2014	Only those spots that were present on all three replicate gels were quantified and enrolled for statistical analysis. The spots with significant differences in expression levels ($p < 0.05$) between the two groups were considered up- or down-regulated.
Jarckovska et al, 2010	For PMF database searching, peak lists in XML data format were created using flex Analysis 3.0 program with SNAP peak detection algorithm. No smoothing was applied and maximal number of assigned peaks was set to 50. After peak labelling, all known contaminant signals were removed. No restrictions on protein molecular weight and pI value were applied. Proteins with MOWSE score over the threshold 56 calculated for the used settings were considered as identified. If the score was lower or only slightly higher than the threshold value, the identity of protein candidate was confirmed by MS/MS analysis.
Kim et al, 2006	for declaring a protein hit, protein score > 13, peptide score > 10, and SPI (%) >70 were applied throughout the data analysis procedures. All the proteins identified based on at least two peptides assignment
Kushnir et al, 2012	Criteria used for acceptance of peptide assignments for proteins identified based on single peptide were: (a) score for the peptides match greater than 10; (b) error in mass accuracy of the parent ion within 20 ppm (for over 80% of the peptides error was within 10 ppm); (c) good alignment of predicted vs observed ion series; (d) number of unmatched ions in mass spectra less than 50% of the expected number of ions; (e) forward score greater than the reverse score; (f) well-defined chromatographic peaks at the retention time where the mass spectrum was acquired.
L Turco et al, 2013	considering at least two unique proteotypic peptides
Lee et al, 2005	Using ExPASy PeptIdent with an error tolerance of 100 ppm, information on the peptide mass fingerprinting data was searched against databases of relevant species.
Liu et al, 2007	To validate the search results, Western blot analysis on apolipoprotein A-I and Collagen type IV was performed. In these cases, the expression changes of the selected proteins further confirmed the direct analysis of tryptic digests by MALDI-TOF MS (Data were normalized by using the external standard signals (B) (means±SD, n=10). * value significantly differs from the control ($P<0.05$), ** value significantly differs from the control ($P<0.01$).
Lo Turco et al, 2010	Only proteins in attendance scores and confidence higher than 50% and 99%, respectively, were considered in order to accept these database searches, and when the same protein was identified for different MS/MS fragment ions, those presenting the highest score were considered for comparisons and data presentation
Regiani et al, 2015	Each pool in this study was analyzed in quadruplicate and only proteins present in at least two replicates were included in the study. The minimal fragment ion matches per protein was 7 and the minimal peptide matches per protein was 1.
Schweigert et al, 2006	To surely identify proteins, an immunoassay was applied. The different antibodies (haptoglobin, prealbumin and transferrin were coupled on the chip surface. Mass resolution (defined as $m/\Delta m$) is routinely in the range of 300–400, and mass accuracy was within 0.1%. Peaks with amplitudes at least five times greater than the average background noise level were considered.
Severino et al, 2013	Protein and peptide scores were set up to maintain the false positive peptide ratio below 5%
Sim and Lee, 2008	We searched for identical known protein sequences from NCBInr and EST databases using the MASCOT search program (www.matrixscience.com) and BLAST in order to identify the proteins.
Spitzer et al, 1996:	Amino acid sequences were compared with the PIR protein sequence database (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711, USA). Position numbers were assigned

	to correspond with the position of amino acid residues in the protein as given in the database.
Twigt et al, 2012	Scaffold (version Scaffold_2_06_02, Proteome Software,Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least one identified peptides (resulting in a 0.1% protein false discovery rate). Protein probabilities were assigned by the Protein Prophet algorithm.
Wu et al, 2015	Proteins matching more than four peptides and with a MSCOT score >64 were considered significant ($P < 0.05$). Proteins with a minimal 1.5-fold change between groups and $P < 0.05$ were considered significantly differentially expressed.
Zamah et al, 2015	Proteins detected with 5% local FDR were reported. To determine significant changes in protein abundances log-transformed iTRAQ ratios were analyzed using Student's t-test and the threshold for differential abundance was $p < 0.05$

Appendix 4 (chapter 3)

List of the proteins identified in the sperm + hFF proteomic interactions

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q5JQC9	80	4318.1	P04259	22	1441.4	Q8N1F7	16	657.8	P04075	16	867.3
O75969	51	2573.2	P11142	22	1087	Q9Y4L1	16	548.8	P13639	16	506
P07900	47	2297.8	P14625	21	833.2	P40926	16	948.4	P21266	16	849
P54652	44	2236.7	P26640	21	764.7	P40939	16	689.4	Q5THR3	16	531
P02788	44	2170.5	P60174	21	1116.2	Q13618	16	646.1	Q6BCY4	16	652.1
Q5BJF6	42	1922.1	O14556	20	880.1	P10323	15	589.7	O75952	11	429.8
Q5VU65	41	1643.4	O75694	20	830	P13647	15	560	O95757	11	490.9
Q96JB1	40	1379.2	P33121	20	741	P36969	15	573.6	P01857	11	739.3
Q01813	37	1681	P49327	20	649.3	P49368	15	519.5	P04843	11	387.5
Q9UFH2	37	1218.6	P55072	20	831.8	P60709	15	744.2	P06733	11	395
P08238	32	1436.2	Q02383	20	840.1	P68363	15	677.7	P07237	11	342.9
P11021	29	1388.2	Q9BVA1	20	942.5	Q16698	15	752.1	P46459	11	416.4
P04264	28	1449.6	P07437	19	976.8	Q8NCR6	15	612.5	P55786	11	442.9
P07864	27	1213.9	P08779	19	762.4	Q9Y277	15	927.9	P10809	11	454.3
P14618	27	1130.9	P35527	19	921.9	O60309	14	521.7	Q8WUD1	11	432.1
P13645	26	1247.7	P49221	19	848.4	P30101	14	546.1	Q9BZX4	11	607.6
Q9BZW7	26	1107.6	Q13748	19	910.8	P61019	14	724.6	Q9NY65	11	502.8
A6NMS7	25	1119.8	Q99798	19	721.8	Q13509	14	719.6	Q96RQ9	11	373.5
P35908	25	1173.5	P02538	18	728.5	Q6NUT2	14	488.3	Q9UKU0	11	353.4
P02768	24	1059	P25705	18	756.6	Q8WXX0	14	530.2	Q14568	11	555.1
P07205	24	999.4	P48668	18	724.7	Q8TC71	13	440.8	Q14990	11	597.1
P34931	24	1276.2	Q04695	18	1052.2	Q96A08	13	798.2	P17987	12	425.8

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q00610	24	1002.2	Q8NEB7	18	894.4	Q99832	13	423.8	P36873	12	459
Q14997	24	840.8	Q8TEM1	18	651.5	Q9BXF9	13	479	P45880	12	637.9
P04279	23	990.1	Q92621	18	555.4	Q9BYZ2	13	511.5	P62805	12	731
P48741	23	1142.2	P02533	17	643.5	Q9HAT0	13	680.3	P63261	12	513.8
P19367	23	930.5	Q14203	17	583.4	P04350	13	792.2	Q96QE4	12	560.9
P29144	23	761.4	Q71U36	17	791.6	P04406	13	588.3	Q13733	13	493.5
P68371	23	1158.3	Q86VP6	17	679.7	P08237	13	472.4	Q13939	13	635.5
Q9H0B3	23	790.2	Q96PU9	17	615.1	P25786	13	514.7	P05141	12	473.2
P47897	13	460.7	P12235	11	470.5	P84243	9	335.2	P53396	9	331.4
P50991	13	541	P35579	11	425.5	Q13162	9	349.9	P02743	9	368.9
Q9BS86	12	609.8	O00410	10	384.9	Q15008	9	271.8	P05109	9	414.8
Q9BUN1	12	742.7	O75976	10	334.1	Q16531	9	276.7	P0C0S8	9	546.3
Q9H0C2	12	490.1	P00918	10	438.6	Q4G0X9	9	331.1	P12277	9	403.1
Q9NTJ4	12	439.4	P02787	10	337	Q6J272	9	304	P12821	9	299
Q9NXE4	12	411.8	P06576	10	570.5	Q92820	9	372.3	P22695	9	335.6
Q9Y6C9	12	490.3	P15309	10	356.1	P13073	9	288.8	P25788	9	338.4
P07814	12	395.7	P24752	10	457.4	P17066	9	423.3	Q9UJT2	9	290.5
P15104	12	486.5	P29218	10	393.8	O43847	8	299.8	P31040	9	284
P17174	12	420.8	Q5SRE5	10	345	P00558	8	305.9	P22492	9	315
Q8TC56	11	357.2	Q6UWU2	10	395.7	P00747	8	228.3	A8MYP8	8	277.7
P10909	11	517.1	Q7L266	10	592	Q8TBY8	8	264.6	O14645	8	254
P00338	9	416.3	Q99623	8	323.5	Q8IVF4	8	204.5	Q53H82	9	352.9
Q5JST6	9	297.3	Q9NSE4	8	247.9	Q9HBV2	8	448.9	P08758	8	318.9
Q5JX69	9	320.9	P62140	8	288.1	Q9BTM1	8	493.2	P13646	8	374.1
Q9UIA9	9	309.6	P68366	8	455	Q9BVM2	8	263.8	P13667	8	275.2

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q96KX2	9	306.7	Q14697	8	242.7	Q99613	8	220.6	P13861	8	275.5
Q9H1X1	9	351.9	Q3LXA3	8	304.3	P50990	8	382.8	P27348	8	291.9
P25789	9	430.7	Q5VTE0	8	342.6	P38646	9	358.8	P28074	8	289.8
P26641	9	416	Q6ZR08	8	253.1	P35232	8	318.9	P30041	8	296.1
Q00325	8	387	Q5JVL4	7	266.8	Q9HDC9	8	265.2	P30044	8	296.9
Q02978	8	349.6	Q8NBX0	7	270.4	P57105	8	276.8	P36542	8	311.2
Q04837	8	364.8	Q15046	7	210.3	P60900	8	361.8	P61106	8	262.9
Q8TEX9	8	256.1	P48643	8	224.1	P41250	8	224.3	Q13576	7	237.5
Q93009	8	253	Q9C0G6	8	278.3	P47756	8	282.2	Q7Z745	7	244.4
Q96BH3	8	295.5	Q9Y230	8	283.4	O00743	7	202.9	P61006	7	219.1
P12273	7	279.2	P01860	7	268	O75390	7	257.6	P62258	7	295
P15144	7	259.9	P01861	7	456.6	O75746	7	246.9	P63104	7	337.3
P15586	7	239	P06899	7	359.5	P01024	7	209.9	P68104	7	337.7
P22061	7	257	P07195	7	275.5	Q93077	7	309.5	Q16891	7	220.3
P22314	7	190.6	P07954	7	274.6	Q96KK5	7	325.5	Q1ZYL8	7	244.6
P43155	7	256.6	P10515	7	268.7	Q96P26	7	233.4	Q562R1	7	249.7
P49748	7	288.4	P11177	7	311	Q7Z3B4	7	293.2	P68400	6	213.5
P54136	7	246.8	Q96RL7	7	221.2	P78406	6	170	Q8IXA5	6	283.1
Q6UW49	7	324.5	Q99447	7	287	P98160	6	196	Q8IYK2	6	180.7
Q75WM6	7	290.1	Q99880	7	375.8	Q00796	6	266.1	Q8N427	6	172.1
Q5T9G4	7	207.8	P21912	6	216.2	O14818	6	204.7	Q02218	6	217.3
Q9UBX3	7	265	P22748	6	206.7	O43242	6	211.8	Q06210	6	245.8
Q9UI46	7	222.4	P25685	6	223.1	O43772	6	183	Q12931	6	279.3
Q9UJS0	7	274.9	P28066	6	229.2	O75874	6	158.1	Q13200	6	206.7
Q9UL16	7	228.9	P28331	6	241.5	O96011	6	183.2	Q14974	6	222.7
Q9NTM9	7	256	P31937	6	231.5	P06702	6	239.4	Q58FF6	6	246.8

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q7Z4H3	7	237.5	P31948	6	192.1	P06727	6	329.2	Q6NXT2	6	213.7
P68871	6	188.6	P34932	6	209.2	P06744	6	284.7	Q6X784	6	222.3
Q86VQ3	6	220	P53621	6	163.7	P07737	6	315.7	Q15631	5	221.5
Q9Y262	6	182.1	P54577	6	149.8	P08727	6	278.6	Q15907	5	140.1
P60842	6	219.6	P55060	6	169.8	P0C5Z0	6	350.5	Q5CZC0	5	139.6
P61247	6	189.8	P55084	6	197.6	P13796	6	283.2	Q5JX71	5	187.1
Q92523	6	187.2	P25787	5	283.9	O94905	5	152.1	Q96GK7	5	160.3
Q92526	6	214.3	P13798	6	194.5	Q5T4S7	5	151.4	Q96PF2	5	143.1
Q92598	6	264.4	P16152	6	253.1	Q6P2I3	5	184.1	Q96QH8	5	316.2
Q96JQ2	6	216.3	Q9NS25	6	409.6	Q6URK8	5	157.3	Q99536	5	160
Q96M32	6	203.4	Q9NSD9	6	176	Q6ZN84	5	164.6	Q9BS26	5	167.4
Q96M98	6	190.3	Q9NQT6	6	286	Q6ZQR2	5	185.1	Q9BXA6	5	195.7
Q96NG3	6	249.1	P62987	6	304.1	Q86X76	5	184	Q96DC9	5	232
Q96QV6	6	286.3	A0AVT1	6	198.7	Q8IUE6	5	227.9	Q96FW1	5	132
Q9C099	5	156.9	P20618	5	179.7	Q8IZ16	5	217.4	Q8TAA3	5	214.2
Q9H0K4	5	161.8	P20674	5	198.4	Q9P0L0	5	189.9	Q8TCU4	5	206.7
Q9H4B8	5	218.6	P21796	5	240.4	Q9P225	5	131.1	Q8TDQ7	5	156.5
Q9H4Y5	5	168.7	P23526	5	142.1	Q9UFE4	5	134.2	Q8WW24	5	200.2
Q9NY33	5	257.8	P23634	5	190.4	Q9UIF3	5	139.3	Q8WZ59	5	201.5
P26373	5	234.4	P25325	5	237.7	Q9Y371	5	168.8	Q01469	4	182.3
P28838	5	147.1	O95861	5	182.4	Q9Y5B8	5	180.2	Q07020	4	137.5
P30086	5	238.5	P00568	5	196.5	P99999	5	201.2	Q0VFZ6	4	136.6
P37837	5	218.8	P01876	5	163.7	Q00059	5	188.9	Q14410	4	109.9
P38117	5	124.5	P02751	5	128.3	Q12907	5	177.1	P18669	5	173.8
P40925	5	186.2	P04004	5	167.1	Q13011	5	209.4	P19827	5	178.2
P42126	5	168.6	P05388	5	137	Q15257	5	126.6	P22612	4	93.2

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P46926	5	176.4	P08574	5	179.1	Q15506	5	310.4	P23284	4	102
P55145	5	196.4	P0C7P4	5	167.2	P63244	4	172.3	Q9P0J0	4	148.5
P60660	5	178.6	P11310	5	133.8	P67812	4	127.8	Q9P2R7	4	123.6
P61313	5	169.2	P11766	5	169.7	Q9BTE6	4	132.6	Q9UFN0	4	131.4
P61604	5	199.3	P12429	5	229.8	Q9BTW9	4	104.1	Q9UJ70	4	135.6
P61981	5	202.7	P14314	5	227.8	Q9C002	4	116.9	Q9UM54	4	119.7
P62158	5	233.4	P15374	5	163.3	Q9GZS0	4	173.6	Q9UNN5	4	150.7
P62241	5	144	P15531	5	186.5	Q15435	4	123.2	Q9Y265	4	157.6
P62249	5	177.9	P16219	5	120	Q15785	4	113.2	Q9Y4P3	4	125.4
P78371	5	171.3	P16403	5	155.3	Q15836	4	141.6	Q9Y570	4	186.6
P23368	4	127.5	P62269	5	199.9	Q16836	4	130.2	Q9Y6A4	4	136.9
P23396	4	148.6	A2RUT3	4	208.3	Q4G0P3	4	94.4	P62888	4	168.1
P26436	4	142.3	A6NDG6	4	158.9	Q53EV4	4	142.9	P62906	4	95
P27487	4	132	O00231	4	117	Q5T2S8	4	125.3	P62701	4	117
P29401	4	125.2	O00468	4	142.4	Q6UW15	4	169.6	Q9H0J4	4	148
P29803	4	151.6	O15173	4	99.2	Q6UX06	4	184.7	Q9HB07	4	188.2
P30040	4	124.7	O75602	4	94.4	Q6ZU69	4	107	Q9HC21	4	208
P30046	4	148.6	O75947	4	128.2	Q6ZUB1	4	122.4	Q9NQ86	4	146.1
P33176	4	140.1	P00450	4	169.2	Q8IYT1	4	179.5	Q9NUJ1	4	212.4
P35268	4	154.9	P00492	4	221.4	Q8N158	4	141	Q9NY87	4	263.2
P35580	4	116.6	P00738	4	124.2	Q8N1V2	4	140	Q9NYU2	4	166
P36404	4	142.7	P01834	4	326.5	Q8N4E7	4	151.3	Q8WZ42	4	126.5
P39656	4	163.1	P01859	4	180.5	Q8N6M8	4	174.1	Q92928	4	145.7
P40227	4	115.5	P02679	4	128.5	Q8NA47	4	143.4	Q96HS1	4	140.3

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P42765	4	119.5	P04792	4	166.5	Q8NFW8	4	122.5	Q96JN2	4	129.1
P43490	4	121.5	P05023	4	118.4	P62826	4	169.2	Q96KX0	4	170.8
P46379	4	107.7	P0C0L5	4	139.1	P18754	4	124.8	Q96MR6	4	126.1
P49821	4	91.7	P0C0S5	4	170.6	P19012	4	136.4	Q99426	4	153.2
P49913	4	193.6	P10644	4	128.3	Q8WWU5	4	130.5	Q99497	4	135.1
P50502	4	134	P10768	4	139.8	Q8WXX5	4	169.2	Q99714	4	121.6
P51665	4	131.5	P11169	4	208.6	P56556	4	94.8	P59910	4	162.5
P51854	4	154.4	P12236	4	147.5	Q8TB22	4	122.5	Q99878	4	223
P52292	4	117.3	P13489	4	120.1	Q8TC29	4	142.3	P19013	4	128.4
P53007	4	141.1	P13637	4	162.6	Q8TC94	4	145	P20338	4	126.8
P54727	4	141.4	P13804	4	103.4	P02675	3	89.7	P27797	3	91.9
Q8TD57	4	127	P14550	4	127.5	P02763	3	102.9	P28070	3	85.8
P17612	4	116.3	A4FU69	3	81.5	P04083	3	113.7	P29508	3	80.9
P18124	4	152.3	A5D8V7	3	113.1	P05155	3	160	P29692	3	183.9
Q8TDY3	4	180.2	O00232	3	63.8	P05164	3	128.2	P30042	3	85.9
P62330	3	120.5	O00291	3	125.6	P05783	3	106.8	P30084	3	88.5
P62333	3	134.4	O00299	3	107.8	P06396	3	96.3	P32119	3	98.1
P62854	3	112.4	O00519	3	93.9	P06865	3	108.9	P32189	3	118.7
P63167	3	124.8	O14744	3	77.4	P07288	3	117.3	P35998	3	67.7
Q02878	3	82.9	O15230	3	88.3	P07339	3	98.6	P38567	3	87.1
Q06830	3	118.6	O43175	3	79.2	P08134	3	118.2	P38606	3	79.9
Q13508	3	77.2	O43236	3	108.4	P08603	3	73.8	P40616	3	104.9
Q13617	3	77.7	O43488	3	106.9	P08865	3	89.8	P41252	3	73.4
Q13642	3	167.3	O43592	3	81.1	P09622	3	141.7	P42785	3	85.4

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q14139	3	92.8	O43617	3	76.8	P0C0L4	3	82.2	P43304	3	104.7
Q14409	3	127.9	O43822	3	152.1	P0CG04, P0CG05, P0CG06	3	180.3	P43487	3	97.9
Q15005	3	82.8	O60733	3	74.6	P10253	3	90.4	P46781	3	93
Q15370	3	145.6	P60673	3	113.1	P10412	3	74.1	P47895	3	88.9
O75340	3	71.6	P61018	3	92.8	P11047	3	93.9	P47929	3	168.7
O75477	3	125.6	P61160	3	94.2	P12814	3	82.1	P48047	3	118.6
O75521	3	75.9	P61163	3	130.6	P14555	3	115.8	P49189	3	82.4
O75531	3	111.4	P61204	3	69.5	P14649	3	148.4	P49589	3	115.5
O75569	3	99.6	P62195	3	85.2	P14854	3	125.6	P49755	3	88.5
O75882	3	98.4	P62244	3	147.7	P14927	3	78.6	P50213	3	124.6
O95202	3	109.2	P62263	3	91.2	P15259	3	101.2	P50914	3	114.1
O95292	3	138.8	Q8N0W7	3	154.2	P15529	3	89.4	P51148	3	105.5
O95336	3	88.2	Q8N335	3	80.4	P18077	3	115	P51157	3	93.5
P00387	3	95.1	Q8N4L4	3	84.9	P20155	3	92.9	P52209	3	86.8
P00505	3	131.8	Q8N5Q1	3	97.4	P23528	3	82.6	Q9BQA1	3	92.7
P01009	3	216.5	Q8NA82	3	86.8	P24539	3	86.6	Q9Y282	3	99.9
P01023	3	81.9	Q8NDM7	3	165.1	Q9UKF2	3	146.7	Q9Y615	3	84
P02647	3	182.2	Q8NEP4	3	116.6	Q9UKR5	3	104.8	Q9NR28	3	108.8
P02649	3	77.6	Q8NFH5	3	120.4	Q9UKU7	3	122.1	Q9NSB4	3	121.2
P24666	3	110.4	Q8TC12	3	132.6	Q9UNZ2	3	126	Q9NVJ2	3	98.1
P25398	3	118.9	Q8TC44	3	97	Q96FJ2	3	141.3	Q9H0R4	3	114.1
P26440	3	83	Q8TDZ2	3	127.5	Q96HR9	3	73.8	Q9H1K1	3	91.7
P26639	3	75.4	Q9BT09	3	92.8	Q96M34	3	84.4	Q8IYX7	3	87.5
P27169	3	82.8	Q9BT78	3	107.6	Q96M63	3	112.3	Q8IZP2	3	134.1

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P53597	3	121.9	Q9BTX1	3	110.2	Q96SB4	3	76.6	Q9BX68	3	222.4
P54709	3	118.3	Q9BU02	3	85.2	Q9H503	3	136.6	P05387	2	102.3
P54920	3	94.8	Q9BWH2	3	81.8	Q9HAE3	3	108.8	P06753	2	93.1
P56385	3	106.6	Q16775	3	77.2	Q99567	3	76.6	P07108	2	63.4
P59665	3	105.9	Q16854	3	93.9	Q99766	3	107.2	P07355	2	55.7
P60228	3	82	Q53FA7	3	110.6	Q9BYD9	3	112.9	P08133	2	101
Q5JRX3	3	71.8	Q5BN46	3	107.2	Q9H0I3	3	102.1	P08174	2	46.1
Q5T749	3	85.9	Q8WUM4	3	106	Q9UGP8	3	91.6	P08670	2	92.5
Q5TA50	3	77.1	Q8WWB3	3	73.6	Q9UHG3	3	76.6	P09211	2	84.4
Q5TZJ5	3	72	Q8WYR4	3	69.5	Q8IWZ5	3	118.9	P09382	2	57.7
Q5VYK3	3	86.4	Q92930	3	92.4	Q99436	3	122.7	P09669	2	61.3
Q63HN1	3	77.4	Q969U7	3	87	O75190	3	146.9	P0C874	2	56.8
Q68CQ1	3	115.6	Q96A26	3	80.5	Q9P2D7	2	66.5	P10606	2	61
Q6IA69	3	145	Q96C74	3	111	Q9UBQ7	2	48.3	P11216	2	55.9
Q6JEL2	3	93.4	Q9NZ45	3	77.4	Q9UBS4	2	99.5	P11279	2	55.3
Q6P4A8	3	129.2	Q9UBF2	3	113.1	O95168	2	49.5	P11908	2	73.3
Q71UI9	3	117.8	Q9UBX1	3	87.8	O95573	2	86.5	P13671	2	50.8
Q7Z4W1	3	76.6	A4D1T9	2	74.7	O95825	2	52.4	P13716	2	66.3
Q7Z794	3	122.4	A6NCJ1	2	62.4	O95831	2	61.2	P14174	2	71.3
O15296	2	62	A6NFA0	2	46.9	O95881	2	67.7	P14406	2	54.3
O15372	2	47.7	A6NFE3	2	68.4	O95994	2	43.3	P15121	2	96.6
O15393	2	52.6	A6NJV1	2	62.3	O96000	2	48.2	P15289	2	55.1
O43298	2	86.8	A6NKQ3	2	74.7	O96008	2	77.6	P15311	2	52.8
O43324	2	59.5	Q96PK2	2	42.5	P00390	2	49.6	P15735	2	92.2
O43678	2	47.8	B1ANS9	2	50.5	P00403	2	68.1	P15924	2	50.5
O43684	2	53.2	O00159	2	71.3	P00441	2	99.3	P16401	2	62.6

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
O43708	2	70.3	O00487	2	70.3	P01011	2	108.5	P36578	2	46.2
O43813	2	78	O14520	2	110.7	P01040	2	106	P36776	2	115.7
O43920	2	52.2	O14618	2	55	P01042	2	68.5	P38405	2	53.8
O60809	2	53.1	O14746	2	65.4	P01623	2	190.8	P39019	2	69.2
O75367	2	53.6	O14829	2	65.5	P01766	2	68.2	P40429	2	50.4
Q9UHL4	2	54.6	O14910	2	58	P02753	2	136.7	P40763	2	54.4
Q9UHP6	2	62.7	O14950	2	69.7	P02774	2	57.8	P41218	2	86.8
Q9UHR6	2	68.7	O14980	2	53.8	P04179	2	43.8	P41222	2	81.9
Q9UII2	2	51.7	O15068	2	43	P04217	2	65.6	P42766	2	63
Q9ULC5	2	81.9	P25311	2	111.7	P04554	2	73.8	P43034	2	53.6
Q9UM00	2	53.8	P26885	2	67.2	P31930	2	98	P43358	2	48.9
Q9UM07	2	49.2	P27105	2	130.7	P31939	2	53.9	P43897	2	53.3
Q9UNM6	2	50.7	P27635	2	55.6	P31946	2	58.9	P98095	2	83
Q9UNX3	2	58.4	P27824	2	79.4	P31947	2	73.5	Q00169	2	55.4
Q9UPN7	2	49.7	P28072	2	123.7	P32969	2	60.8	Q01518	2	62.2
Q9UQ80	2	78.5	P30038	2	60.2	P34896	2	66.5	Q02127	2	54.7
Q9Y234	2	74.7	P30048	2	52.9	P34913	2	54.3	Q04760	2	55.9
Q9Y266	2	62.8	P30153	2	57	P35237	2	61.2	Q05469	2	58.5
Q9Y2B4	2	60.6	P31025	2	146.1	P35658	2	52.2	Q06323	2	58.7
Q9Y303	2	61.8	P31150	2	70.3	P35813	2	65.5	Q15366	2	54
Q9Y376	2	55.1	P50402	2	60.1	P36405	2	75.7	Q15369	2	46.6
Q9Y3A5	2	61.8	P51149	2	52.8	P56134	2	109.5	Q16181	2	67.8
Q9Y3B3	2	113.4	P52272	2	43.1	P56180	2	46.1	Q16222	2	57
Q9Y4W6	2	80.8	P52565	2	59.6	P56192	2	50.7	Q16401	2	48
O75396	2	51.7	P52566	2	49	P56730	2	51.8	Q16563	2	101.1
O75438	2	56.1	P52907	2	47.1	P56851	2	53.4	Q16566	2	56.6

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
O75951	2	75.9	P53602	2	49.6	P57678	2	64.1	Q16718	2	54.6
O76003	2	52.6	P53680	2	78.4	P58340	2	77.5	Q8IV63	2	67.3
O94788	2	68.1	P54107	2	90.9	P59282	2	122.1	Q8IWZ6	2	55.1
O94808	2	82.3	P55884	2	64.1	P60510	2	62	Q8IXS2	2	58.2
P16435	2	73.8	Q08722	2	53.5	P60866	2	66.8	Q8IXY8	2	63.7
P16562	2	92.9	Q13057	2	67.4	P61009	2	85.8	Q8IY82	2	45.9
P17050	2	77.9	Q13404	2	107.2	Q14156	2	63.7	Q8IYP2	2	48.5
P18085	2	67.4	Q13561	2	46.2	Q14165	2	104	Q8IYX1	2	52.1
P19652	2	93.8	Q13564	2	54.9	Q14204	2	43.4	Q96L21	2	48.9
P19784	2	45.4	Q13765	2	95.3	Q14507	2	44.8	Q96LM6	2	48.4
P19823	2	68.4	Q14019	2	46.7	Q14624	2	47.3	Q96M29	2	51.4
P20142	2	65.6	Q14093	2	42.1	Q15102	2	70.7	Q96MA6	2	46.3
P20810	2	56.5	Q14152	2	58.3	Q15181	2	62.9	Q96S97	2	52.7
P22732	2	66.9	Q5W111	2	76.4	Q15363	2	54.6	Q99417	2	67.5
P23786	2	91.3	Q5XKE5	2	53.5	Q7Z5L4	2	54.1	Q99460	2	68
P24534	2	81.8	Q66GS9	2	43.6	Q7Z6W1	2	56.2	Q99523	2	66.7
P61353	2	61.1	Q68DN1	2	69.3	Q86SX6	2	53.7	Q99598	2	48.8
P61962	2	77.9	Q6DD88	2	53.6	Q86WT1	2	42	Q99963	2	62.3
P62191	2	106.3	Q6IAN0	2	60.2	Q86Y39	2	44.1	Q9BRA2	2	50.7
P62280	2	56.3	Q6ICG8	2	71.8	Q8N6Q1	2	57.6	Q9BRY0	2	72.9
P62312	2	85.5	Q6NXR0	2	61.9	Q8N7U6	2	64	Q9NR31	2	75.4
P62424	2	66.4	Q6P1M0	2	61.3	Q8N865	2	60	Q9NZC3	2	58.5
P62491	2	56.8	Q6P656	2	55.8	Q8N9V2	2	70.7	Q9Y581	2	75.1
P62714	2	95.9	Q6TDU7	2	73.1	Q8WW22	2	60.4	Q9Y5Z4	2	48.5
P62829	2	47.9	Q8TBQ9	2	57.9	Q969H8	2	47.8	P45954	2	73.4

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P62753	2	119.5	Q6UWP2	2	66	Q8WXQ8	2	53	Q9Y697	2	55.1
P62851	2	81.6	Q8TC36	2	73.9	Q969V4	2	63.2	P45974	2	122.9
P62917	2	78.8	Q8TC99	2	67.5	Q96D96	2	64.8	P46776	2	86.3
P62937	2	62.4	Q8TCS8	2	81.8	Q96E40	2	70.6	P46777	2	79.8
P63173	2	55.3	Q8TDM5	2	130.1	Q96GG9	2	57.8	P47985	2	52.7
P63208	2	61.4	Q8WUK0	2	95.4	Q96HA9	2	66.9	P48729	2	78.4
P67870	2	63.1	Q8WUW1	2	69.2	Q96IU4	2	90.1	P49411	2	86.6
P78362	2	54.5	Q8WVP5	2	93.2	Q96IX5	2	76.2	P49588	2	48.7
P78395	2	49.5	Q9NRW1	2	54.3	Q96L03	2	47.9	P49591	2	75.7
P83731	2	76.5	Q9NSB2	2	53.8	Q9NRG9	2	80.4	P49720	2	88.1
P84098	2	60.4	Q9NTJ5	2	59.3	Q9NRN7	2	63	P49721	2	75
Q6UWQ5	2	61.2	Q9NU02	2	45	Q9H845	2	120.7	P49773	2	58.6
Q6UXV1	2	76.8	Q9NVH1	2	65.9	P07478	1	36	Q5FVE4	2	44.6
Q6UXV4, Q9BUR5	2	67.8	Q8IYX3	2	98.9	P07738	1	26.2	Q5GAN3	2	78.1
Q6V702	2	70.1	Q8IZ96	2	60.1	P07741	1	34.9	Q5JU67	2	56.6
Q6ZU64	2	59.2	Q8IZS6	2	55.7	P07910	1	44.8	Q5RI15	2	77.1
Q6ZUB0	2	80.2	Q8N0U8	2	47.4	P08559	1	39	Q5SRN2	2	63.5
Q6ZVS7	2	58.6	Q8N3X6	2	63.6	P09417	1	25.7	Q5SVJ3	2	52.8
Q7L2H7	2	69.7	Q8N6G2	2	60.4	P09496	1	40.4	Q5T655	2	62.2
Q7Z4W2	2	60.9	Q9BU61	2	81.4	P09936	1	49.8	Q5T871	2	43.2
Q8NCW5	2	57.7	Q9BVK6	2	61.4	P0C7M6	1	56	Q5TCS8	2	63.3
Q8NE09	2	79.9	Q9BWD1	2	47.8	P62979	1	55.7	Q2M243	2	45.2
Q8NE22	2	85	Q2TAA8	2	51	P10599	1	69.5	Q9BXW7	2	118.8
Q8NE62	2	50.6	Q3ZCQ8	2	67	P12830	1	29.3	Q9C093	2	56.2

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q8NEG0	2	63.5	Q495T6	2	47.5	P13987	1	35	Q9GZZ8	2	83.9
Q8NEH6	2	85.9	Q499Z3	2	77.4	P14621	1	32	Q9H061	2	76
Q8NFH3	2	74.4	Q53FT3	2	54.8	A1A4V9	1	31.4	Q9BWS9	2	43
Q8NHS0	2	104.2	Q53QW1	2	50	A1A519	1	27.9	O60664	1	54.1
Q8NHU2	2	52.1	Q5D862	2	78.5	A4QMS7	1	49	O60763	1	43
Q9HAQ2	2	52.9	Q9H095	2	69.2	A6NFR6	1	29.5	O60884	1	35.2
Q9HC35	2	59.1	Q9H299	2	80.6	A7E2U8	1	48.5	O60888	1	44.8
Q9NPJ3	2	82.9	Q9H2U2	2	86.3	A8MV24	1	42.7	O75223	1	26.5
Q9NQ48	2	64.3	Q9H361	2	61.8	A8MYZ5	1	28.4	O75251	1	26.2
Q9NQP4	2	57.6	O14782	1	33.1	B3GLJ2	1	63.7	O75306	1	29.6
P14735	1	48.6	P17980	1	36.9	P81605	1	38.8	Q02413	1	25
P14868	1	24.9	P18440	1	27	P83881	1	24.9	Q02543	1	30.4
P15291	1	28.1	P20151	1	31.2	Q00577	1	38.6	Q07021	1	51.5
P15880	1	27.4	P21399	1	30.3	Q00765	1	52	Q07955	1	39
P16444	1	28.5	P22033	1	34	Q00839	1	34.2	Q12798	1	33
P16949	1	37.4	P78368	1	25.1	Q13144	1	43.9	Q12904	1	28.9
P17152	1	31.2	P80748	1	33.9	Q13155	1	52	Q13107	1	37.1
O96015	1	26.8	O14787	1	33.4	O00154	1	45.8	O75323	1	26.2
P00846	1	33.6	O14791	1	51	O00161	1	28.3	O75348	1	45.1
P01008	1	40	O14880	1	52	O00170	1	26	O75369	1	26
P01033	1	25.7	O14949	1	28.9	O00303	1	51.4	O75380	1	38.9
P01597	1	51.1	O15054	1	37.5	O00330	1	44.5	O75425	1	42.9
P01616	1	35.2	O15084	1	38.8	O00483	1	37.9	O75487	1	31.7
P01619	1	46.3	O15371	1	55.4	O14494	1	31.2	O75489	1	38.7
P01625	1	32.4	O15511	1	49.7	O14521	1	32.6	O75503	1	25
P01709	1	34	O43247	1	35.8	O14523	1	30.6	O75608	1	31.9

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P01764	1	42.4	O43396	1	50.8	O14561	1	25.4	O75676	1	32.3
P01765	1	42.4	O43414	1	26	O14579	1	27.4	O75928	1	27.1
P01777	1	71.8	O43676	1	37.4	O14684	1	28	O75935	1	28.9
P02511	1	54.1	O43716	1	25.4	O14737	1	47	O75964	1	34.7
P02671	1	45.7	O43759	1	27.6	O95445	1	34.6	O94979	1	44.1
P02749	1	53.7	O43765	1	26.2	O95456	1	52.1	O95816	1	44.3
Q92506	1	34.8	Q96IY4	1	35.2	Q5T681	1	36.7	Q13630	1	46.5
Q92530	1	48.5	Q96LK8	1	44.1	Q5TZF3	1	43.1	Q9Y285	1	34.6
Q92542	1	32.7	Q96M91	1	35.5	Q5VTH2	1	30.6	Q9UHV9	1	29.7
Q92597	1	48.2	Q9NY47	1	25.4	Q5VTT2	1	33.3	Q13347	1	27.4
Q92696	1	25.7	Q9NZL4	1	32.9	Q5VVP1	1	36	P33316	1	38.1
Q93100	1	36.2	Q9P1Z9	1	33.9	Q5VZ72	1	33.8	P46821	1	26.7
Q969L2	1	26.8	Q9P2J5	1	29.6	Q5W041	1	43.1	O95865	1	31.9
Q96A05	1	33.4	Q9P2M1	1	56.9	Q9UI09	1	27.5	O95870	1	26.3
Q96AB3	1	38	Q9P2X0	1	30.9	Q9UIC8	1	30.3	O95989	1	32
Q96CN7	1	45.2	Q9UBN7	1	26	Q9UJ68	1	44.2	O95995	1	31.8
Q96CT7	1	35.1	Q9UDW1	1	34	Q9UJW0	1	36.9	P62266	1	30.1
Q96DB2	1	37.5	Q9UGM3	1	82.6	Q9UKF5	1	25.6	P62277	1	42.3
Q96DB5	1	36.4	Q9UGV2	1	26.2	Q9UMX2	1	54.5	P62306	1	37.7
Q96EY8	1	49.8	O43805	1	47	O95473	1	55.5	P62310	1	40.8
Q96FC7	1	26.9	O43808	1	29.6	O95749	1	28.4	P62316	1	32.9
Q96FZ7	1	68.1	O43924	1	59.4	P56381	1	31.9	P62341	1	32.7
Q96IV0	1	29.6	O60220	1	41.6	P56537	1	26.1	P62495	1	41.5
P02765	1	31	O60271	1	26.1	P56597	1	51.1	P62750	1	44.6
P02766	1	26.8	O95159	1	27.6	P57088	1	52.7	P62837	1	32.8

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
P02790	1	48.7	O95169	1	41.3	P58546	1	39.9	P62857	1	72.9
P04066	1	41.6	O95373	1	25.5	P60981	1	33.4	P62910	1	34.9
P04196	1	38.8	O95433	1	28.6	P61026	1	31.8	P62913	1	44.2
P04433	1	39.3	P51659	1	24.8	P61088	1	41.9	P62942	1	47.6
P05496	1	31	P51692	1	55.8	P61289	1	36.7	P63172	1	28.2
P06703	1	27.6	P52198	1	58.2	P61626	1	58.6	P68402	1	48.2
P07203	1	35.6	P52788	1	28.4	P61769	1	29.8	P78318	1	32.2
P07311	1	31.3	P52948	1	40	P61803	1	46	Q8N7F7	1	41.1
P48163	1	28.4	P53367	1	27.2	P61923	1	49.3	Q8N9A8	1	48.4
P48449	1	27.4	P53618	1	34.4	P61966	1	34.9	Q8NA61	1	41.6
P48454	1	32.3	P54578	1	67.6	P61970	1	31.7	Q8NCM2	1	43.5
P48507	1	26.6	P54819	1	28.5	P62081	1	26.3	Q8NCS7	1	29.1
P48556	1	34.5	P55036	1	69.4	Q8IW45	1	29	Q8ND76	1	48.6
P48637	1	25.2	P55064	1	27.1	Q8IWG1	1	30.3	Q8NDH3	1	26.2
P49223	1	34.4	P55809	1	55.2	Q8IY31	1	45.5	Q8NG35	1	28.9
P49585	1	33.8	Q6UY27	1	28.6	Q8IYF3	1	56	Q8NHX4	1	37
P49840	1	62.8	Q6YN16	1	32.3	Q8IYM1	1	30	Q8TAP6	1	26.9
P49841	1	25.2	Q6ZNM6	1	42.3	Q8N104	1	54.2	Q8TAQ9	1	28.9
P50395	1	34.9	Q6ZQQ2	1	25	Q8N1D5	1	29	Q8TBP0	1	28.4
P51571	1	44.6	Q6ZST8	1	28.2	Q8N443	1	32.3	Q8TCD5	1	27.2
Q5XX13	1	43.8	Q6ZVC0	1	39.3	Q8N5M9	1	29.2	Q8TF05	1	41.2
Q68G75	1	35.4	Q76KD6	1	26.1	Q8N688	1	30.4	Q8TF09	1	70.5
Q6DKK2	1	33.9	Q7RTN6	1	26.7	Q8N6K0	1	36.7	Q8TF72	1	28.5
Q6EEV6	1	73.2	Q7Z3U7	1	24.9	Q8N6Q3	1	31.3	Q8WUD6	1	35.1
Q6GMV3	1	37.3	Q7Z4T9	1	27.1	Q8N7B9	1	29.9	Q8WW14	1	39.2

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q6IPR1	1	24.9	Q7Z7G8	1	26.1	Q9BUP3	1	36.6	Q8WW32	1	35.6
Q6NVY1	1	26.8	Q7Z7H3	1	33.2	Q9BVL2	1	33.1	Q8WXA2	1	52.1
Q6NXE6	1	33.9	Q7Z7H5	1	35.6	Q9BVM4	1	59.7	Q9HD34	1	41.8
Q6P047	1	26.6	Q86U86	1	34.6	Q9BW83	1	27.2	Q9NPE6	1	34.4
Q6P1Q0	1	25.1	Q86WA6	1	32.2	Q9BWX1	1	26.2	Q9NQ60	1	38.2
Q6P9G0	1	30	Q86Y82	1	37.8	Q9BY14	1	42.4	Q9NQ88	1	41.3
Q6PEW0	1	38.8	Q86YW0	1	28.8	Q9H0T7	1	34.1	Q9NQC3	1	28.4
Q6PEY2	1	35.6	Q99733	1	28.5	Q9H0W9	1	29.6	Q9NQE9	1	36.2
Q6PF18	1	39.6	Q99932	1	42.7	Q9H1E5	1	37.5	Q9NQW7	1	25.8
Q6PI48	1	41.8	Q99988	1	25.4	Q9H3G5	1	34.2	Q9NRG7	1	70.3
Q6SA08	1	28.6	Q9BPX5	1	31.6	Q9H4A4	1	38.4	Q9NS69	1	33.4
Q6UW68	1	29.6	Q9BRX2	1	50.9	Q9H4K1	1	35.5	Q9NUQ2	1	26.2
Q96MT7	1	34.6	Q9BSF0	1	25.9	Q9H579	1	27.5	Q16595	1	39.9
Q96RM1	1	33.8	Q9GZT8	1	30.6	Q9H7C9	1	33.3	Q16851	1	26.4
Q96RT7	1	40.2	Q9H069	1	27.7	Q9H9E3	1	52.2	Q16864	1	58.1
Q96RY7	1	25.9	Q9H0D6	1	29.3	Q9HC38	1	71.6	Q17RC7	1	29.4
Q96S96	1	49.5	Q9H0E2	1	28.7	Q9HC84	1	44.9	Q3B820	1	61.1
Q99643	1	33.2	Q9H0I9	1	35.2	Q9HCN8	1	30	Q3KQZ1	1	27.7
Q9BT73	1	25.3	Q9Y2Z0	1	37.5	Q13724	1	32.2	Q4G0N4	1	27.4
Q9BUD6	1	31.3	Q9Y315	1	37	Q13838	1	26.1	Q5HYI8	1	32.3
Q9BZ72	1	25	Q9Y512	1	32	Q14257	1	38.5	Q5JWF8	1	25.6
Q9BYC2	1	39.3	Q9Y333	1	42.6	Q14166	1	32.1	Q5J5C9	1	29.2
Q9BZH6	1	39.3	Q9NVV0	1	43.3	Q14435	1	32.8	Q5SQS8	1	26.9

Protein	Peptides	Score	**Protein**	Peptide	score	**Protein**	Peptide	Score	**Protein**	Peptide	Score
Q9BZV1	1	42.1	Q9NWV4	1	27.2	Q149M9	1	25.6	Q5T0N1	1	28.9
Q9C0B2	1	34.7	Q9UHY7	1	32.9	Q15084	1	70.1	Q5T1B0	1	32.4
Q9Y547	1	42.4	P22234	1	25.9	Q15185	1	29.3	Q5T5A4	1	27.4
Q9Y586	1	28.4	P23381	1	40.7	Q15365	1	36.8	P35052	1	27.2
Q9Y5Y2	1	29.9	P24821	1	28.4	Q15388	1	31.7	Q9UNK0	1	35.7
Q9Y6I9	1	30	P26447	1	33.1	Q15645	1	31.4	P43686	1	38.1
Q9Y6M0	1	30.4	P30049	1	59.6	Q15742	1	27.5	P45877	1	26.2
P35080	1	28.8	P30050	1	32.6	Q16186	1	31.3	P46782	1	54.9
P35270	1	73.2	P30085	1	28.6	Q16568	1	40	P31153	1	39.2
P35606	1	32.5	P30740	1	35.9	P46977	1	46.5	P31949	1	37.8
P35754	1	34.6	P31151	1	43.8	P47914	1	35.1	P37198	1	31.4
P36957	1	54.3	P40121	1	47						

Appendix 5 (chapter 4)

List of the metabolites identified in the sperm metabolomics study:

1- Metabolites higher in the TP1 samples

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
588	0.021584	0.9759	183.0646185	255.077586	Methylpentanoic acid;Caproic acid	Fatty acids and oxidised fatty acids	negative
1035	0.035158	0.99901	265.1194304	114.885498	Phenylalanyl-Threonine	Peptides	negative
1048	0.027021	0.99865	234.8004888	135.053322	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
1187	0.0013843	0.51021	245.6462623	257.383779	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
1457	0.030621	0.99865	267.1330763	115.19577	Phenylalanyl-Threonine	Peptides	positive
1469	0.029119	0.99865	268.1366039	114.523758	Phenylalanyl-Threonine	Peptides	positive
1682	0.00703	0.99901	338.1356017	162.22971	Glutaminyl-Phenylalanine;Phenylalanyl-Gamma-glutamate	Peptides	negative
1691	0.0079209	0.99901	339.1387588	162.54696	Glutaminyl-Phenylalanine;Phenylalanyl-Gamma-glutamate	Peptides	negative
1721	0.0086368	0.99865	291.1343325	63.913953	Thyronine	Hormones	positive
1728	0.037948	0.93227	289.2066532	104.588253	Arginyl-Isoleucine;Arginyl-Leucine	Peptides	positive
2088	0.0041405	0.87782	321.9179841	263.844756	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
2304	0.022531	0.93227	358.3741645	63.71853	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
2445	0.024818	0.99865	351.6980238	133.96035	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
2480	0.0046204	0.88272	354.6834161	270.943686	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
2484	0.005529	0.93227	386.3797216	225.127602	3-Deoxyvitamin D3	Vitamin D metabolism	positive
2516	0.015997	0.99865	357.8359618	182.646258	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
2537	0.010531	0.93227	395.1956368	237.543084	10,11-dihydro-12-oxo-LTB4";11-deoxy-PGE2;11-HpETE;"11,12-DiHETE";"11H-14,15-EETA";11HPETE;12HPETE;12Leukotriene B4;"14,15-DiHETE";"14,15-Dihydroxy-8(17),13(16)-labdadien-19-oic acid";"14,15-HxA3 ";"14,15-HxB3 ";15-epi-PGA1;"15H-11,12-EETA";15HPETE;"17,18-DiHETE";5-HPETE;"5,12-DiHETE";"5,15-DiHETE";5Hydroperoxyeicosatetraenoic acid;6-trans-12-epi-Leukotriene B4;6-trans-Leukotriene B4;"6,7-dihydro-5-oxo-12-epi-LTB4";8-iso-PGA1;"8,15-DiHETE";"8,9-DiHETE";8HPETE;9-HpETE;HpETE;Leukotriene B4;Prostaglandin A1;Prostaglandin B1	Fatty acids and oxidised fatty acids	positive
2575	0.016202	0.99865	363.5077124	97.088226	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
2752	0.0086724	0.99901	478.2256405	290.591064	10,11-Dihydrohydroxy-leukotriene E4	Fatty acids and oxidised fatty acids	negative
2814	0.00023337	0.23407	384.6878227	286.063743	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
2893	0.029376	0.93227	447.716048	64.117023	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
2923	8.51E-05	0.23407	395.1933549	281.223576	methyl-hexadecenoic acid;Heptadecenoic acid	Fatty acids and oxidised fatty acids	positive
3059	0.023352	0.99865	409.5531826	273.804912	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
3081	0.015335	0.93227	482.7239441	224.530227	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
3165	0.046435	0.99865	419.2239033	329.877678	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
3211	0.044981	0.99865	424.1909035	66.400026	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
3225	0.034585	0.9759	590.273408	237.65019	PS[18:1];;PS[20:4]	Glycerophospholipids	Negative
3325	0.023855	0.99865	436.1976457	62.781819	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
3337	0.042989	0.99865	437.7129274	90.449511	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
3356	0.035481	0.9759	712.2213762	140.085261	Isoliquiritigenin 4-O-(5'''-O-p-coumaroyl)-apiofuranosyl-(1'''->2''')-glucoside;Licorice glycoside B;Licorice glycoside D1;Licorice glycoside D2	Other class	Negative
3402	0.049125	0.99865	443.554656	267.34929	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3450	0.0076587	0.99865	448.4564397	257.254476	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3463	0.04037	0.99901	713.3204854	272.530044	PA[28:2]	Glycerophospholipids	Negative
3510	0.0018643	0.55761	456.2422176	286.002705	20-Hydroxy-leukotriene E4;20-hydroxy-LTE4;Lipoxin E4	Fatty acids and oxidised fatty acids	Positive
3540	0.030947	0.99865	460.2389423	283.194828	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3599	0.030629	0.99865	467.2400769	275.054916	1(3)-glyceryl-PGF2alpha;2-glyceryl-PGF2alpha;;"4,5-epoxyHDHA";;14-F2-dihomo-IsoP;15-methylPGF2alpha methyl ester;"16,16-dimethyl-PGE1";"16,16-dimethyl-PGF2beta";17-F2-dihomo-IsoP;"1a,1b-dihomo-PGE1";"1a,1b-dihomo-PGF2alpha";20-ethyl PGF2alpha;7-F2-dihomo-IsoP	Fatty acids and oxidised fatty acids	Positive
3603	0.0010891	0.51021	467.5706048	273.406038	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3612	0.030094	0.99865	468.8948025	272.435157	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3712	0.033981	0.99865	480.2403847	290.608452	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3732	0.012331	0.99865	482.720317	172.808772	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3733	0.011481	0.99865	482.7204151	115.928622	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
3754	0.010539	0.93227	643.6290981	225.460407	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
3902	0.024119	0.93227	688.7547238	250.125924	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
4574	0.0034705	0.81904	592.7948106	277.852761	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
4726	0.0016813	0.55761	613.8240498	274.291953	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
4971	0.017709	0.99865	643.2905047	115.2348	Multiply Charged ion - possible peptide or protein	Possible protein and	Positive

						peptide	
4980	0.0378	0.99865	644.0980283	286.062792	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive
5378	0.018065	0.99865	702.8383263	272.282946	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	Positive

2- Metabolites higher in the TP7 samples

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
9	0.036447	0.9759	102.0561245	84.281442	Aminobutanoic acid;Aminoisobutanoic acid;Dimethylglycine	Other class	negative
52	0.033867	0.93227	107.0492223	141.868272	Benzaldehyde	Fatty acids and oxidised fatty acids	positive
71	0.043134	0.9759	116.0718273	56.486883	5-Aminopentanoic acid;Betaine;Norvaline	Amino acid metabolism	negative
109	0.026818	0.9759	125.0246933	237.375054	1,2,3-Trihydroxybenzene;"1,3,5-Trihydroxybenzene";4-Hydroxy-2H-pyran-3-carboxaldehyde;Methyl 2-furoate	Mixed class	negative
122	0.01562	0.9759	128.0354654	52.7772594	1-Pyrroline-4-hydroxy-2-carboxylate;N-Acryloylglycine;Pyroglutamic acid;Pyrrolidonecarboxylic acid;Furoic acid	Arginine and proline metabolism	negative
220	0.031304	0.93227	128.0706205	214.923684	(▒)-4-Methylene-2-pyrrolidinecarboxylic acid;"2,3,4,5-Tetrahydropiperidine-2-carboxylate";D-1-Piperideine-2-carboxylic acid;;"1,3-Benzenediol";"2,4-Hexadienedial";Muconic dialdehyde;Pyrocatechol	Mixed class	positive
263	0.030506	0.93227	132.0541475	141.604116	1-(2-Furanylmethyl)-1H-pyrrole	Other class	positive
301	0.04311	0.93227	138.0527504	51.807444	Proline	Arginine and proline metabolism	positive
383	0.031405	0.93227	147.1129879	44.4615126	Lysine	Amino acid metabolism	positive
465	0.034795	0.93227	156.0770912	46.4663982	Nicotinamide N-oxide;Urocanic acid	Mixed class	positive
645	0.024376	0.93227	175.1188903	47.3949654	Arginine	Arginine and proline metabolism	positive
751	0.012425	0.93227	188.0683809	141.817647	3-Pyridinebutanoic acid;Norsalsolinol	Mixed class	positive

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
752	0.025975	0.93227	188.0709048	241.02711	Tryptophan	Amino acid metabolism	positive
827	0.026436	0.93227	195.115485	62.931615	Decenol	Fatty acids and oxidised fatty acids	positive
974	0.015242	0.93227	212.1047932	68.125701	2-Propylpiperidine	Other class	positive
1108	0.046479	0.9759	236.083055	270.957468	2,3,4,5,6,7-Hexahydro-6,7-dimethylcyclopent[b]azepin-8(1H)-one;"3,4-Dimethyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one";"trans-4,5-Dimethyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one	Other class	negative
1165	0.01096	0.9759	241.118974	258.181632	Dodecenoic acid;Methyl-undecenoate	Fatty acids and oxidised fatty acids	negative
1190	0.0040069	0.99901	281.2486169	577.774284	Octadecenoic acid;methyl-heptadecenoic acid	Fatty acids and oxidised fatty acids	negative
1269	0.034021	0.9759	251.0797958	259.758522	Deoxyinosine;Dethiobiotin	Mixed class	negative
1276	0.011314	0.9759	252.0859138	247.56129	Isovalerylglutamic acid;Suberylglycine	Amino acid metabolism	negative
1315	0.0090482	0.99901	295.2276752	496.389285	Oxo-octadecenoic acid;Hydroxy-octadecadienoic acid;12-OxoOME;"12,13-EpOME";"12,13-EpOME(9)";6-HODE;9-HODE;"9,10-EpOME(12)";Epoxyoctadecenoic acid	Fatty acids and oxidised fatty acids	negative
1333	0.040588	0.99901	297.2432106	507.39738	10-HOME(8);Oxooctadecanoic acid;11-HOME(9);12-HOME;13-HpOME;17-HOME;19-HOME;5-HOME(2);;8-HOME(9);9-HOME;9-HOME(12)	Fatty acids and oxidised fatty acids	negative
1339	0.039973	0.99901	298.2465666	507.385632	10-HOME(8);Oxooctadecanoic acid;11-HOME(9);12-HOME;13-HpOME;17-HOME;19-HOME;5-HOME(2);;8-HOME(9);9-HOME;9-HOME(12)	Fatty acids and oxidised fatty acids	negative
1345	0.024823	0.93227	250.0388463	142.672257	Norepinephrine sulfate	Sterol and steroid metabolism	positive
1571	0.033151	0.9759	283.9293679	113.475189	Cysteine-S-sulfate	Sulphur metabolism	negative

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
1898	0.024197	0.9759	317.0211759	82.773975	gamma-Glutamyl-S-methylcysteine sulfoxide;Cysteinyl-Hydroxyproline	Peptides	negative
1906	0.045118	0.99901	365.2306628	506.671614	10-HOME(8);Oxooctadecanoic acid;11-HOME(9);12-HOME;13-HpOME;17-HOME;19-HOME;5-HOME(2);;8-HOME(9);9-HOME;9-HOME(12)	Fatty acids and oxidised fatty acids	negative
2027	0.00068443	0.41887	381.1720615	576.789336	9-Octadecenal	Fatty acids and oxidised fatty acids	negative
2037	0.0069045	0.99901	382.1755899	576.762948	9-Octadecenal	Fatty acids and oxidised fatty acids	negative
2247	0.03945	0.9759	358.1262286	76.049436	Hydroxyhexanoycarnitine	Acyl carnitines	negative
2380	0.041963	0.9759	377.2294313	301.21443	13,14-Dihydro PGE1;"13,14-Dihydro PGF2a";"13,14-dihydro-15-keto-PGF1alpha";"13,14-dihydro-PGF2alpha";PGF1beta;Prostaglandin F1a	Fatty acids and oxidised fatty acids	negative
2621	0.017857	0.9759	416.1792654	69.228369	Coutaric acid	Other class	negative
2673	0.0091069	0.9759	425.1721083	272.675535	11-Dehydro-thromboxane B2;11-dehydro-TXB2;19-Hydroxy-PGE2;"20-COOH-10,11-dihydro-LTB4";20-dihydroxyleukotriene B4;20-hydroxy-PGD2;20-Hydroxy-PGE2;"5,12-diHPETE";"5,15-diHPETE";5(6)-Epoxy Prostaglandin E1;6-Ketoprostaglandin E1;"6,15-Diketo,13,14-dihydro-PGF1a";"8,15-diHPETE";D17, 6-keto PGF1a";diHPETE;hydroperoxy-PGD2;hydroperoxy-PGE2;Prostaglandin G2;16,17-epoxy-DHA";"16,DHA-epoxide	Fatty acids and oxidised fatty acids	negative
2757	0.033887	0.9759	442.166732	645.41436	Chitobiose	Carbohydrates	negative
3096	0.023398	0.9759	527.211275	186.498876	16,17-Dihydro-16a,17-dihydroxygibberellin A4 17-glucoside;;"3)-2-(acetylamino)-1,5-anhydro-2-deoxy-D-arabino-Hex-1-enitol";"4)-2-(acetylamino)-1,5-anhydro-2-deoxy-D-arabino-Hex-1-enitol	Mixed class	negative
3144	0.01282	0.9759	545.222018	82.201905	3)-2-(acetylamino)-2-deoxy-D-Galactose	Carbohydrates	negative
3436	0.014969	0.93227	557.2904566	238.479102	Cholesterol sulfate;;"26,27-Dihomo-1alpha-hydroxy-24-epivitamin D2";"26,27-Dihomo-1alpha-hydroxyvitamin D2";28-Hydroxy-14-taraxeren-3-one;32-oxolanosterol;3beta-3-Hydroxy-18-lupen-21-one;3beta-Hydroxy-22(30)-hopen-29-al;"4,4-Dimethyl-14a-formyl-5a-cholesta-8,24-dien-3b-ol	Sterol and steroid metabolism	positive
3578	0.048024	0.93227	594.3073006	245.573466	PC(P-19:1/0:0)	Glycerophospholipids	positive
3617	0.044844	0.93227	609.312446	248.819763	PG[22:2]	Glycerophospholipids	positive
3999	0.045369	0.93227	725.3757941	260.206179	PG[30:4];PA[32:4]	Glycerophospholipids	positive

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
4313	0.034015	0.99865	561.8434171	275.787015	Multiply Charged ion - possible peptide or protein	Possible protein and peptide	positive
6088	0.012633	0.99865	807.4122655	282.513804	1alpha,3beta,Trihydroxyergosta-5,-dien-26-oic acid 3-O-b-D-glucoside 26-O-b-D-glucosyl ester";;PA[39:7];Octaprenyl diphosphate;PG[29:0]	Mixed class	positive
4048	0.040556	0.93227	749.7087436	258.129228	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive
2979	0.02015	0.93227	464.2461608	237.615198	Multiply Charged ion - possible proteins and peptides	Possibly proteins or peptides	positive

3- Metabolites higher in the control media

IDX	p.value	FDR	mz	rt	Metabolites	Metabolite Class	ION MODE
489	0.042884	0.99865	177.0641043	385.675848	2-Ethylpyrazine	Other class	positive
832	0.0084477	0.93227	196.1196554	732.23673	Dibutyl disulfide	Sulphur metabolism	positive
1012	0.024578	0.99865	231.0344358	154.954944	Dimethyluracil;Imidazolepropionic acid;Methylimidazoleacetic acid	Mixed class	positive
1095	0.049493	0.9759	234.9805272	53.8880178	3,3'-Thiobispropanoic acid	Sulphur metabolism	negative
1327	0.049503	0.99901	297.0973778	316.389606	2-Phenylethanol glucuronide;;Asparaginyl-Lysine;Lysyl-Asparagine	Peptides	negative
2384	0.04777	0.99865	346.2359678	552.538209	12-Methoxy-8,11,13-abietatrien-20,11-olide;"9,11alpha-epoxypregn-4-ene-3,20-dione	Sterol and steroid metabolism	positive
2506	0.011861	0.99865	356.9082142	808.62708	3,3-Dibromo-2-n-hexylacrylic acid	Other class	positive

Appendix 6 (chapter 5)

MTALAB codes for vorticity analysis :

```
for j=1:456
    clear u v vort x y
    load(sprintf('PIVlab_%04d.mat',j));

    u1(:,:,j) = u;
    v1(:,:,j) = v;
     w(:,:,j) = vort;
     V(:,:,j) = sqrt(u.^2 + v.^2);
    x1(:,:,j) = x;
    y1(:,:,j) = y;

end

%%
figure;
for j=1:456

  pcolor(x1(:,:,j),y1(:,:,j),w(:,:,j)); shading interp
 % pcolor(x1(:,:,j),y1(:,:,j),V(:,:,j)); shading interp
hold on;
quiver(x1(:,:,j),y1(:,:,j),u1(:,:,j),v1(:,:,j),'b')
hold off
axis equal tight
colormap jet
title(sprintf('%04d',j))
colorbar

%set(gca, 'CLim', [Vmin, 20*10^-6]);
set(gca, 'CLim', [-80, 80]);
    F(j) = getframe;
end

%%

for j=1:456

  Vmean(j) = mean(mean(V(:,:,j)));
  Vstd(j) = std(std(V(:,:,j)));

  wmean(j) = mean(mean(abs(w(:,:,j))));
  wstd(j) = std(std(w(:,:,j)));

end

%%
figure;
subplot(1,2,1);plot(Vmean)
subplot(1,2,2);plot(Vstd)

figure
subplot(1,2,1);plot(wmean)
```

```
subplot(1,2,2);plot(wstd)

figure;
plotyy(1:456,Vmean,1: 456,wmean)

%% Fourier Transform
figure;
Fs = 100;                    % Sampling frequency
T = 1/Fs;                     % Sampling period
L = 456;                     % Length of signal
t = (0:L-1)*T;                % Time vector

f = Fs*(0:(L/2))/L;
Y = fft(Vmean);
P2 = abs(Y/L);
P1 = P2(1:L/2+1);
P1(2:end-1) = 2*P1(2:end-1);

plot(f,P1)
title('Single-Sided Amplitude Spectrum of S(t)')
xlabel('f (Hz)')
ylabel('|P1(f)|')

%%
wsum=0;
Vsum=0;
for j=1:456

  wsum = wsum + abs(w(:,:,j));
  Vsum = Vsum + V(:,:,j);
end
%%
figure
 pcolor(x1(:,:,j),y1(:,:,j),wsum); shading interp

axis equal tight
colormap jet

colorbar

set(gca, 'CLim', [0, 2000]);
```

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