Proteomic biomarkers of azoospermia in seminal plasma and testicular interstitial fluid

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I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Raouda Sgaier

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Summary:

Azoospermia, which manifests as the absence of sperm in the semen, is a prevalent form of male infertility, affecting 10-15% of infertile men. Assisted reproductive techniques (ARTs) represent an excellent therapeutic option for azoospermic men, allowing infertile couples to achieve pregnancy. Microsurgical TEsticular Sperm Extraction (micro-TESE) is a surgical technique that allows for the extraction of spermatozoa from any active spermatogenesis pockets in the testis, even in patients with severe testicular failure. The extracted spermatozoa can subsequently be used in ICSI or IVF procedures. Men with normal or quantitatively diminished spermatogenesis (obstructive azoospermia or hypospermatogenesis diagnosis), have better chances for the success of sperm extraction, whereas spermatogenic arrest (SA) or the total loss of germ cells, known as Sertoli cell only syndrome (SCO) means the retrieval of any sperm viable for fertilization is unlikely, lowering the clinical benefit of such procedures. Current clinical tests used for the differential diagnosis of azoospermia are insufficient without a histological examination of testicular tissue. Testicular biopsy, or alternatively an exploratory TESE, remains the golden standard in the diagnosis of idiopathic male infertility.

Novel biomarkers that can accurately predict the outcome of sperm extraction, and that are measurable in semen or in blood plasma would represent an ideal, non-invasive alternative. Deep analysis of seminal plasma, the acellular fraction of semen, and of testicular interstitial fluid (TIF), the biological fluid surrounding seminiferous tubules with access to the bloodstream, can also offer new insights into the etiology of azoospermia and the cellular processes involved in testicular function.

In the first part of this study, we investigated the seminal plasma proteome using label-free mass spectrometry. The comparison of the proteomic composition of seminal plasma of fertile men, used as a control, to that of men diagnosed with obstructive azoospermia (OA), mixed atrophy (MA) and SCO, uncovered a number of proteins of significantly different abundances between the control group and each of the three azoospermia groups (MA, SCO, OA). Based on their relative abundance in seminal plasma, tissue specificity, expression in germ cells, and potential role in spermatogenesis, a selection of these proteins were considered as candidate markers of sperm retrieval. Confirmation experiments (antibody-based imaging, immunoblotting) showed the expression in the testis and abundance in seminal plasma of Lactate dehydrogenase C (LDHC) and Heat shock protein family A (Hsp70) member 2

(HSPA2) are markedly decreased in testicular failure. On the other hand, ELSPBP1 was significantly reduced in OA seminal plasma but shows similarly high levels in MA, SCO, and fertile men. In the second part, we explored the normal TIF proteome via high-resolution proteomic analysis of TIF samples from men diagnosed with obstructive azoospermia and quantitively and qualitatively normal spermatogenesis, which resulted in the identification of more than 25000 proteins, many of which localize specifically to the seminiferous epithelium and are enriched in post-meiotic germ cells. These results suggest that proteins specific to adluminal germ cells can bypass the blood-testis barrier and deposit into the interstitial compartment and potentially into the general circulation. By comparing the TIF proteome in fertile men to that in men diagnosed with MA and SCO using label-free and isobaric labelling mass spectrometry analysis, we uncovered a number of differentially expressed proteins in testicular failure, including LDHC, HSPA2 and Transketolase like 1 (TKTL1). These findings offer new insights into the underlying molecular mechanisms of male infertility and can serve as a starting point for the development of seminal plasma and blood immunoassays for the differential diagnosis of azoospermia and prediction of sperm retrieval outcomes.

Zusammenfassung:

Azoospermie, die sich durch die Abwesenheit von Spermien im Samen Ejakulat manifestiert, ist eine weit verbreitete Form der männlichen Infertilität, von der 10-15% der unfruchtbaren Männer betroffen sind. Assistierte Reproduktionstechniken (ARTs) stellen eine ausgezeichnete therapeutische Option für azoosperme Männer dar, die unfruchtbaren Paaren eine Schwangerschaft ermöglichen können. Die mikrochirurgische Testikuläre SpermienExtraktion (Mikro-TESE) ist eine chirurgische Technik, die die Extraktion von Spermien aus allen aktiven Bereichen, die noch Spermatogenese zeigen, im Hoden ermöglicht, auch bei Patienten mit einem schweren Hodenschaden. Die extrahierten Spermien können anschließend in ICSI- oder IVF-Verfahren verwendet werden. Männer mit normaler oder nur quantitativ verminderter Spermatogenese (Diagnose: obstruktive Azoospermie- oder Hypospermatogenese) haben bessere Chancen auf eine erfolgreiche Spermienextraktion, während bei den Spermatogenesearresten (SA) oder dem totalen Verlust von Keimzellen, bekannt als Sertoli-Cell-Only-Syndrom (SCO), die Gewinnung von befruchtungsfähigen Spermien unwahrscheinlich ist, was den klinischen Nutzen solcher Verfahren verringert. Derzeitige klinische Tests zur Differentialdiagnose der Azoospermie sind ohne histologische Untersuchung des Hodengewebes unzureichend. Die Hodenbiopsie oder alternativ eine explorative TESE bleiben der Goldstandard in der Diagnose der idiopathischen männlichen Infertilität. Neuartige Biomarker, die das Ergebnis der Spermienextraktion genau vorhersagen könnten und die im Ejakulat oder im Blutplasma messbar wären, wären eine ideale, nicht-invasive Alternative. Eine eingehende Analyse des Seminalplasmas, der zellfreien Fraktion des Ejakulates, und der interstitiellen Hodenflüssigkeit (TIF), die die Samenkanälchen mit umgibt und Zugang zum Blutkreislauf hat, kann neue Einblicke in die Ätiologie der Azoospermie und die zellulären Prozesse bieten, die an der Funktion der Hoden beteiligt sind.

Im ersten Teil dieser Studie haben wir das Proteom des Seminalplasmas mittels markierungsfreier Massenspektrometrie untersucht. Der Vergleich der proteomischen Zusammensetzung des Seminalplasmas von fertilen Männern (Kontrolle) und infertilen Männern mit Diagnose einer obstruktive Azoospermie (OA), bunten Atrophie (mixed atrophy, MA) oder SCO, zeigte eine Reihe von signifikant unterschiedlich häufigen Proteinen zwischen Kontrollgruppe und jeder der drei Azoospermie-Gruppen (MA, SCO, OA). Aufgrund ihrer relativen Häufigkeit im Seminalplasma, der Gewebespezifität, der Expression in Keimzellen

und ihrer potentiellen Rolle bei der Spermatogenese wurde eine Auswahl dieser Proteine als mögliche Marker für eine erfolgreiche Spermiengewinnung in Betracht gezogen. Weiterführende Untersuchungen (antikörperbasierte Bildgebung, Immunblotting) zeigten, dass die testikuläre Expression des Gens/Proteins Lactate dehydrogenase C (LDHC) und Heat shock protein family A (Hsp70) member 2 (HSPA2) im Seminalplasma bei Hodenfehlfunktionen qualitativ und quantitativ deutlich verringert sind. Auf der anderen Seite war das Gen/Protein ELSPBP1 im Seminalplasma bei OA signifikant reduziert, zeigte jedoch ähnlich hohe Werte bei MA, SCO und fertilen Männern.

Im zweiten Teil untersuchten wir das normale TIF-Proteom mittels hochauflösender proteomischer Analyse von TIF-Proben von Männern mit diagnostizierter OA und intakter Spermatogenese; dies ermöglichte die Identifizierung von mehr als 25.000 Proteinen, von denen viele spezifisch im Keimepithel exprimiert werden und dort vor allem in postmeiotischen Keimzellen angereichert sind. Diese Ergebnisse legten nahe, dass Proteine, die für adluminale Keimzellen spezifisch sind, die Blut-Hoden-Schranke umgehen und im interstitiellen Kompartiment und möglicherweise im allgemeinen Kreislauf auftreten können. Durch den Vergleich des TIF-Proteoms bei fertilen Männern mit dem von Männern mit diagnostizierter MA und SCO mittels markierungsfreier und isobarer Markierungs-Massenspektrometrie-Analyse, entdeckten wir eine Reihe unterschiedlich exprimierter Proteine bei testikulärer Fehlfunktionen, darunter LDHC, HSPA2 und Transketolase like 1 (TKTL1). Diese Ergebnisse bieten neue Einblicke in mögliche molekulare Mechanismen der männlichen Unfruchtbarkeit und können als Ausgangspunkt für die Entwicklung von Seminalplasma- und Blut-Immunoassays für die Differentialdiagnose der Azoospermie und die Vorhersage von Erfolgsaussichten bei der TESE dienen.

MONASH University

Proteomic biomarkers of azoospermia in seminal plasma and testicular interstitial fluid

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A thesis submitted for the degree of *Doctor of Philosophy*

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Abstract:

Assisted reproductive techniques (ARTs) represent an excellent therapeutic option for men diagnosed with azoospermia (the absence of sperm in the semen). Microsurgical TEsticular Sperm Extraction (micro-TESE) is a surgical technique that allows for the extraction of spermatozoa from any active spermatogenesis pockets in the testis, even in patients with severe testicular failure. Men with normal or quantitatively diminished spermatogenesis (obstructive azoospermia or hypospermatogenesis diagnosis), have better chances for the success of sperm extraction, whereas germ cell aplasia, known as Sertoli cell only syndrome (SCO) means the retrieval of any sperm viable for fertilization is unlikely. Current clinical tests used for the differential diagnosis of azoospermia are insufficient without a histological examination of testicular tissue obtained via testicular biopsy or exploratory TESE. New diagnosis biomarkers that can accurately predict the outcome of sperm extraction, and that are measurable in semen or in blood plasma would represent an ideal, non-invasive alternative. Deep analysis of seminal plasma, the acellular fraction of semen, and of testicular interstitial fluid (TIF), the biological fluid surrounding seminiferous tubules with access to the bloodstream, can lead to the discovery of such biomarkers and offer new insights into the cellular processes involved in testicular function.

In the first part of this study, we investigated the proteomic composition of seminal plasma of fertile men, in comparison to that of men diagnosed with obstructive azoospermia (OA), mixed atrophy (MA) and SCO, uncovered several proteins of significantly different abundances between the control group and each of the three azoospermia groups (MA, SCO, OA). Based on their relative abundance in seminal plasma, tissue specificity, expression in germ cells, and potential role in spermatogenesis, a selection of these proteins was considered as candidate markers of sperm retrieval. Confirmation experiments (antibody-based imaging, immunoblotting) showed the expression in the testis and abundance in seminal plasma of Lactate dehydrogenase C (LDHC) and Heat shock protein family A (Hsp70) member 2 (HSPA2) are markedly decreased in testicular failure. In the second part, we explored the normal TIF proteome via high-resolution proteomic analysis of TIF samples from men diagnosed with obstructive azoospermia /normal spermatogenesis, which resulted in the identification of a large number of post-meiotic germ cell-enriched proteins. These results suggest that proteins specific to adluminal germ cells can bypass the blood-testis barrier and deposit into the interstitial compartment and potentially into the general circulation. By comparing the TIF proteome in fertile men to that in men diagnosed with MA and SCO using label-free and isobaric labelling mass spectrometry analysis, we uncovered a number of differentially expressed proteins in testicular failure, including LDHC, HSPA2 and Transketolase like 1 (TKTL1). These findings offer new insights into the underlying molecular mechanisms of male infertility and can serve as a starting point for the development of seminal plasma and blood immunoassays for the differential diagnosis of azoospermia and prediction of sperm retrieval outcomes.

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Contents :

Abbreviations:

ART Assisted reproductive techniques

BMI Body Mass Index

BTB Blood testes barrier

DNA Deoxyribonucleic acid

FOXA3 Forkhead box A3

FSH Follicle stimulating hormone

GnRH Gonadotropin releasing hormone

Hsp Heat shock protein

ICSI Intracytoplasmic sperm injection

IgG Immunoglobulin G

IVF In vitro fertilization

LC-MS Liquid chromatography – mass spectrometry

LH Luteinizing hormone

RNA Ribonucleic acid

TESE Testicular sperm extraction

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Introduction:

The term "infertility" refers to a failure to conceive despite frequent, unprotected intercourse for at least twelve months (WHO, 2018). It is estimated that 10-15% of couples in industrialized countries suffer from infertility (Gnoth et al. 2005, Boivin et al. 2007). Male infertility is a contributing factor in about 50% of cases (Agarwal et al. 2016). Men can experience infertility due to many factors, including psychological issues, hormonal imbalances, and diseases that have a direct or indirect impact on their ability to produce or deliver sperm (Baker, 1986). Male infertility is often caused by abnormal sperm production or function, which can occur due to maldescended testes, health conditions such as diabetes, genetic defects, or sexually transmitted infections such as gonorrhoea and chlamydia. Various problems can prevent correct sperm delivery, including premature ejaculation, genetic issues like cystic fibrosis, and physical blockages of the vasal ducts. Other factors likely to influence male fertility include exposure to certain types of pesticides, pollutants, alcohol abuse, anabolic steroids, tobacco smoking, and exposure to radiation (Huang et al. 2019). Long-term use of certain medical drugs and frequent exposure to high temperatures in saunas and hot tubs can negatively affect sperm production (Miyamoto et al., 2011, Olayemi, 2010, Durairajanayagam, 2018).

Azoospermia is a condition in which the ejaculate contains no spermatozoa (Mazzilli et al., 2000), and it affects between 5% and 15% of infertile men (WHO, 2018). There are three types of azoospermia based on etiology: pre-testicular, obstructive (OA) and non-obstructive (NOA). The underlying cause of pre-testicular azoospermia is often a pituitary or hypothalamic abnormality. FSH/LH deficiency is the hallmark of hypogonadotropic hypogonadism caused by disorders of the pituitary gland or hypothalamus. This condition can be congenital (Kallman's syndrome), acquired (tumors, hyperprolactinemia), or due to a perturbation of hypothalamic or hypophyseal functions caused by the use of drugs or anabolic steroids. As many as 40% of patients with azoospermia suffer from OA. OA can be congenital, but it can also emerge as a result of reproductive tract surgeries, urogenital infections, as well as infections affecting the epididymis, vas deferens, and ejaculatory tracts. 'Non-obstructive azoospermia' refers to a variety of subtypes of testicular failure, including hypospermatogenesis (where all types of developing sperm cells are present, but in reduced numbers), maturation arrest at a particular point in sperm development, mixed atrophy (MA), and Sertoli cell-only syndrome (SCO) (Nieschlag et al., 2010). MA is a common testicular phenotype in infertile men,

described as the co-presence of Sertoli cell only-tubules and seminiferous tubules containing germ cells with varying degrees of germinal hypoplasia (Nistal and González-Peramato, 2016). SCO on the other hand is defined by the complete absence of germ cells and is frequently associated with elevated LH and FSH levels coupled with microorchidism (abnormally small testes) (Aziz et al. 2014). In addition to congenital conditions (e.g. cryptorchidism, Y chromosome microdeletions), NOA can also be caused by orchitis (i.e. infection of the testis), testicular torsion, tumours, or chemotherapy. Assisted reproduction techniques (ARTs), which have seen notable progress in the last 30 years, allow many couples incapable of achieving natural pregnancy to become parents. Kocourkova et al. (2014) reported that 1.5% of births in Germany in 2009 were a result of ARTs, mainly In Vitro Fertilization (IVF) and IntraCytoplasmic Sperm Injection (ICSI), with levels as high as 5% in other European regions. In 2017, 1.9% of all infants born in the United States of America were conceived through ARTs (CDC, 2020) and approximately 4.72% of all Australian babies born in 2018 were a result of ART treatment (UNSW, 2018). As male factor infertility is nearly as common as female factor infertility (Mascarenhas et al., 2012), assisted reproduction techniques often rely on retrieving viable sperm through TESE (Testicular Sperm Extraction) or m-TESE (microsurgical Testicular Sperm Extraction), especially in azoospermia cases. Clinical parameters cannot be used to accurately predict the presence of sperm in the testes in men with azoospermia, and the outcome of these procedures is unpredictable, especially in men with NOA. Presently, there is no non-invasive method to differentiate between the various types of testicular insufficiency and obstruction, so a testicular biopsy will be required for about 60% of all men with azoospermia (Jarvi *et al.*, 2013). Identifying proteomic markers in the blood or seminal plasma that could differentiate between different types of azoospermia, such as SCO and MA, would provide a non-invasive tool to determine the suitability of sperm extraction as a treatment option.

Translational research is focused on defining disease-specific defective pathways and using that knowledge to develop improved diagnostic methods and treatment modalities. However, basic research in the field of human infertility has been slow to translate into clinical applications (Agarwal et al. 2014; Agarwal et al. 2016, Agarwal et al. 2021). Although evidence from animal models has improved our understanding of the biological mechanisms underlying mammalian infertility, further research is needed on human infertility. Large-scale mapping of the genomes, transcriptomes, proteomes and metabolomes of the various components of the human reproductive system can shed light on the pathways underpinning reproductive disorders

and subsequently lead to the discovery of diagnostics biomarkers and therapeutic targets. With the rapid development of proteomics over the past two decades, large-scale characterization of all the proteins in cells, tissues, and biological fluids has become feasible. Clinical proteomics has been gaining popularity in recent years due to the emergence of new technologies and software that aims to generate protein profiles that can help 'predict, diagnose, and monitor' human diseases and help identify pathological markers (Verrills 2006). Protein profiles linked to high reproductive potential, determined reproductive disorders, and idiopathic infertility present a wealth of information that can help in making more accurate predictions of fertility status and the development of more sensitive diagnosis tests (Seli et al. 2007). The recent development of mass spectrometry as well as orthogonal protein and peptide separation techniques, has greatly enhanced the speed, dynamic range, sensitivity, and reproducibility of proteomic analyses (Bantscheff et al. 2012). The last two decades have seen a surge in bottom-up proteomics studies, in which proteins are broken down into peptides, that are then separated by ultra-high pressure liquid chromatography (UPLC), followed by tandem mass spectrometry analysis (MS/MS) in which the peptides are sequenced and matched against an in-silico database for protein identification (Zhang et al. 2013).

During spermatogenesis, diploid spermatogonia in the testes differentiate into haploid spermatozoa that supply genetic material for the development of totipotent embryos that can successfully differentiate into healthy foetuses. The spermatogenic process is highly dependent on the temporal and spatial regulation of over 2300 genes (Carrell et al. 2016). Reproductive biology research has made extensive use of proteomic strategies. Proteomics studies generate vast amounts of data, which scientists can decode more easily with the help of bioinformatics (Zhu et al. 2013). Proteomic-based studies coupled with advanced bioinformatics methods can assist in elucidating the biology of spermatogenesis and facilitate the discovery of potential biomarkers for the diagnosis and management of male infertility. Protein expression can be monitored in tissues, urine, blood, and other body fluids for the diagnosis of pathological conditions. In molecular medicine, the discovery of biomarkers is heavily reliant on proteomics. The comparison of affected and healthy biological samples provides insights into the pathophysiology of the disease.

Seminal plasma is the lightly alkaline acellular fraction of semen composed of secretions from all the various tissues of the male reproductive system (seminal vesicles, prostate, vasa, epididymis, and testis). The seminal plasma serves a variety of functions related to the nutrition and transport of sperm as well as containing thousands of tissue-specific proteins in

relatively high abundances, presenting a great prospect for the identification of biomarkers for male reproductive system disorders (Drabovich et al., 2014). Testicular interstitial fluid (TIF) is the fluid bathing the interstitial compartment and surrounding the seminiferous tubules in the testis, with access to the general circulation. Besides proteins produced by interstitial cell types (Leydig cells, testicular macrophages), TIF also contains proteins secreted by the seminiferous epithelium, including germ cell-specific proteins (O'Donnell et al. 2021a). Given its proximity to the site of spermatogenesis, the proteomic content of TIF is very likely to be reflective of the male reproductive potential. The proteomic analysis of seminal plasma and TIF of azoospermic men can offer new insights into the aetiology of azoospermia and the cellular processes involved in testicular function. The comparison of protein expression level in seminal plasma and TIF between fertile men and men diagnosed with three prevalent forms of azoospermia: OA, MA, and SCO can allow for the discovery of biomarkers of differential diagnosis.

Literature review

I. Human male reproductive function: overview

I.1. Anatomy and functional organization of the male reproductive tract:

The male reproductive system (MRS) is comprised of a group of internal and external organs, which perform two main functions: production and delivery of sperm to the female reproductive tract, and secretion of sex hormones (Nieschlag et al. 2010). External structures of the MRS include the penis, testes and epididymides, while the vas deferens, prostate, seminal vesicles, bulbourethral glands, and ejaculatory ducts are situated inside the pelvic cavity (Roberts and Pryor, 1997). The different organs of the MRS are responsible for different aspects of male reproductive function: production, storage and transport of the male gametes, secretion of the components of the seminal fluid, the production of androgens and other hormones (estrogen, inhibin), and ejaculation (O'Connor and De Krester, 2004, Bronson, 2011, Hess and Cooke, 2018). All of these functions are under hormonal regulation, primarily by pituitary-derived follicle-stimulating hormone (FSH), and luteinizing hormone (LH) that are stimulated in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus, and testosterone produced by the testicular Leydig cells upon stimulation by LH (Roberts and Pryor, 1997).

I.1.1. Structure and function of the human testis:

The testis is the male gonad in animals. The adult human male usually contains a pair of testes that lie in the scrotum, with the left one usually positioned slightly lower than the one on the right (Nieschlag et al. 2010). The testis is covered by three layers of scrotal tissue, including the serous tunica vaginalis, the fibrous tunica albuginea, and the tunica vasculosa, comprised of a network of blood vessels (Ravindranath et al. 2003, Figure 1). The testis contains coiled tubular structures, called seminiferous tubules, separated by an interstitial compartment (Figure 2). Seminiferous tubules, which represent 60-80% of the total testicular volume are the site of the sperm production process, referred to as spermatogenesis. whereas the interstitial compartment is where steroidogenesis and androgen secretion occur (Nieschlag et al. 2010). The testis is therefore crucial for the development and maintenance of many male physiological functions, regulated by gonadotropic hormones (FSH and LH), as well as hormones produced locally, including androgens and estrogens (Fietz and Bergmann, 2017). The testis connects to the epididymis via the rete testis and the efferent ducts (Ravindranath et al. 2003)

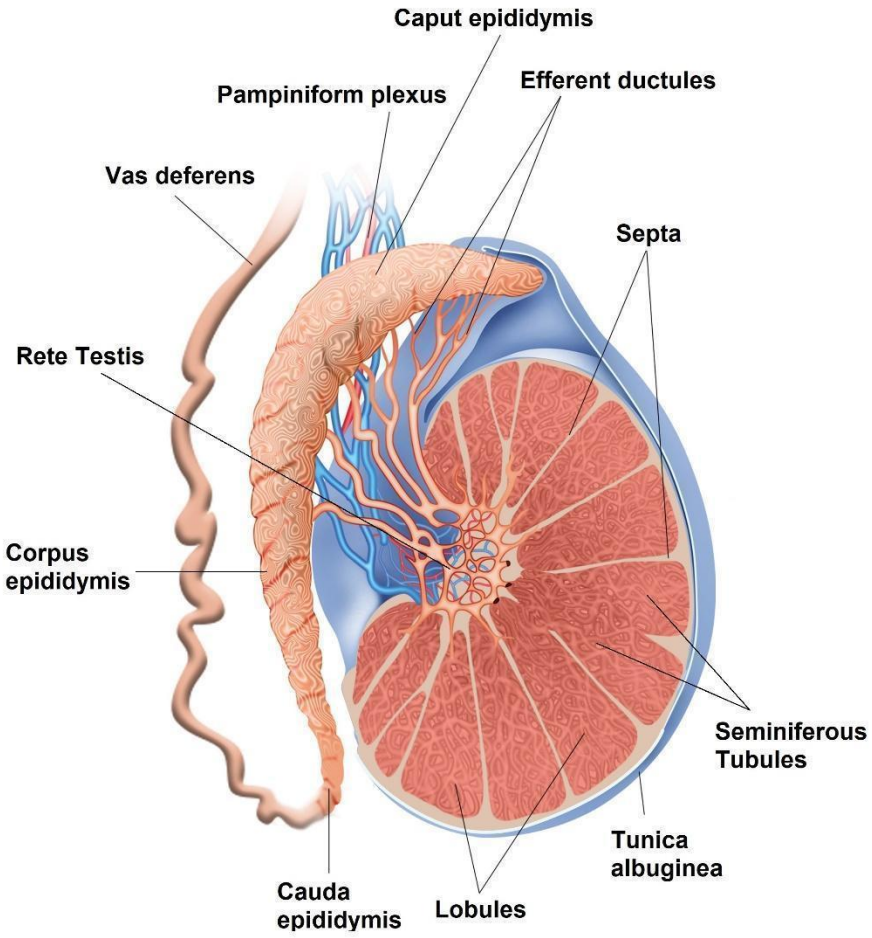


Figure 1: Anatomical diagram of the human testis; The testis is composed of the tightly coiled seminiferous tubules where spermatogenesis takes place and is covered by a fibrous membranous layer called "tunica albuginea". The pampiniform plexus is a convoluted network of veins which in addition to the transportation of deoxygenated blood from the testis, work to cool the adjacent arteries via heat exchange. Seminiferous tubules contained within the lobules converge into the rete testis, where sperm is mixed and transported to the efferent ductules and then to the epididymis. The figure was recreated from Utiger (2018) and *Anatomy and Physiology* (Rice University, 2013; ISBN-10: 1-947172-04-2, Chapter 27.1) using Adobe Illustrator (version 25.0)

a. Seminiferous tubules:

The human testis is composed of roughly 600 seminiferous tubules, organized into 250-300 lobules, contained by fibrous septa. Each testicular lobule holds one, two, or three tightly coiled seminiferous tubules and each tubule is about 1 m in length. Spermatogenesis occurs in the seminiferous epithelium, composed of germ cells (GCs), Sertoli cells (SCs). Peritubular

cells (PTCs) and layers of extracellular matrix form a lamina propria around the tubule (Weinbauer et al. 2010).

- ***Peritubular cells:***

Peritubular cells are partially differentiated smooth muscle cells (myofibroblasts) that possess the capacity to contract using an actin-myosin complex. (Holstein et al. 1996; Schell et al. 2008). Under the influence of hormones and hormone-like active compounds (oxytocin, androgens, prostaglandins), PTCs contract, propelling sperm that are released into the seminiferous tubule lumen towards the rete testis. Additionally, PTCs secrete several connective tissue markers and extracellular matrix proteins (vimentin, collagen, fibronectin, and growth factors) (Schell et al. 2008). The expansion and widening of the layer of collagen that connects PTCs can result in fibrosis of the tubular wall and major disruption of the spermatogenic process (Schlatt, 1999). The integrity of the PTCs and lamina propria is essential for normal spermatogenesis, as is the action of androgen on these cells (Raleigh et al. 2004, Welsh et al. 2009).

- ***Sertoli cells:***

Sertoli cells are tall columnar cells that partially or completely surround germ cells (Rodriguez-Sosa and Dobrinski, 2009). They represent the main supportive structure of the seminiferous epithelium as they rest on the base of the tubule and extend towards its lumen. Sertoli cells are characterized by an irregularly shaped large nucleus and an abundance of co-localized mitochondria and smooth endoplasmic reticulum (Oliveira and Alves, 2015). Sertoli cells are involved in the synthesis and secretion of many regulatory factors and nutrients, such as proteins, cytokines, and growth factors, that are vital to ensure germ cell development and survival (Russell and Griswold, 1993, Weinbauer et al. 2010). Generally, 4 main roles are attributed to SCs: production of germ cell regulatory factors, structural and nutritional support of germ cells, phagocytosis of residual bodies and dead germ cells, and establishment of the blood-testis barrier, providing an immune-privileged micro-environment for the development and differentiation of haploid germ cells (Pöllänen and Cooper, 1994, Dube et al. 2007, Amann et al. 2008).

- ***Sertoli cells, role in spermatogenesis:***

Sertoli cells play a crucial role in spermatogenesis, via direct contact with germ cells and by controlling the environment within the seminiferous tubules (Griswold, 1998, França et al. 2016). Sertoli cells form tight junctional complexes that separate the seminiferous epithelium into two distinct compartments (McGuinness and Griswold, 1994, Stanton, 2016), thereby creating a functional niche that supports SSCs' capacity for replication and self-renewal (De

Rooij, 2015, O'Donnell et al. 2021b). Alterations in gene expression in SCs disrupt this unique microenvironment and could be the underlying cause for certain forms of male infertility (e.g., in Sertoli cell only syndrome (SCO)) (Paduch et al. 2019). FSH and testosterone act on receptors in Sertoli cells to regulate the production of molecular mediators and growth factors necessary for germ cell development and survival (Hogarth and Griswold, 2010, Oatley and Brinster, 2012). Sertoli cells also phagocyte residual bodies and dead germ cells (Baum et al. 2005, Nakagawa et al. 2005) and regulate other aspects of testicular function, including the development and maintenance of the population of steroidogenic adult Leydig cells (Rebourcet et al. 2014, Ye et al. 2017), promoting tissue vascularization (Li et al. 2013) and supporting the function of peritubular myoid cells (Rebourcet et al. 2019). Furthermore, the number of Sertoli cells is a potential determinant factor of the number of meiotic and post-meiotic germ cells and overall spermatogenic output (Rebourcet et al. 2017).

- ***Germ cells:***

Germ cells are the only human cells capable of meiosis, through which they develop into haploid gametes that carry genetic and epigenetic information to the descendants (Neto et al. 2016). Male germ cells are highly ordered within the seminiferous tubules, with less differentiated cells being situated at the basal compartment and advancing towards the lumen as they develop and mature (Clermont, 1963, Heller and Clermont, 1964). Spermatogonia, the most immature germ cells of the testis, are situated on the basal membrane of the tubuli, followed by primary spermatocytes, secondary spermatocytes, and spermatids (Clermont, 1963, de Rooij and Russel, 2000). Human spermatogonial cells fall into four different types: A_{long}, A_{dark}, A_{pale}, and Type B. Dark type A spermatogonia are characterised by a uniformly dark ovoid nucleus with finely granular chromatin surrounding a pale vacuole-shaped area in the middle. Pale Type A spermatogonia have pale stained ovoid nuclei (Schulze, 1979). Type B spermatogonia exhibit large clumps of heterochromatin underneath the nuclear membrane (Sharma and Agarwal, 2011, Griswold, 2016). Spermatogonia become committed to meiosis, a long process in which DNA is replicated to produce two homologous chromosomes consisting each of two sister chromatids, and chromosome recombination occurs to ensure the genetic diversity of each gamete. Meiosis proceeds with two reductive divisions in quick succession, producing haploid (1N) round spermatids. These round spermatids then enter a long period of differentiation (without further division) whereby they develop from simplified round cells to an elongated spermatid with a tightly packed nucleus and an extensive flagellum which will eventually propel the sperm through the female reproductive tract (de Rooij and Russel, 2000, Tanaka et al. 2007, Weinbauer et al. 2010).

b. Interstitial compartment:

Between the seminiferous tubules is the interstitial compartment. This consists of fluid and loose connective tissue that occupies the inter-tubular space (Russell et al. 1990). It contains Leydig cells, lymphocytes, testicular macrophages, and blood vessels (Nieschlag et al. 2010).

- ***Leydig cells:***

Human Leydig cells have an epithelioid shape and a high concentration of organelles involved in steroidogenesis (smooth ER, mitochondria, cytoplasmic lipid inclusions) (Kerr, 1988). Leydig cells are stimulated by luteinizing hormone (LH) to convert cholesterol into testosterone, and to a lower extent androstenedione and dehydroepiandrosterone (Wang et al 2017). Another notable metabolite produced by LCs is insulin-like factor 3 (INSL3), which has an established role in foetal testicular descent as well as germ cell survival in the adult testis (Kawamura et al. 2004, Anand-Ivell et al. 2006). Leydig cells play a role in the regulation of the immune response by interacting with interstitial immune cells present in the interstitial compartment (Hutson, 2006).

- ***Steroidogenesis in Leydig cells:***

Leydig cells are the main site of androgen production in the male. Steroid biosynthesis in Leydig cells consists of a series of enzymatic reactions that convert precursor cholesterol molecules into Testosterone, and to a lesser extent DHEA, androstenediol and androstenedione (Aghazadeh et al. 2015). Cholesterol is mobilised from the plasma membrane or intracellular reserves (lipid droplets) to the mitochondria, where it is converted by Cytochrome P450 family 11 subfamily A member 1 (CYP11A1) into pregnenolone. Pregnenolone transits from the mitochondria to the smooth endoplasmic reticulum where it is converted to progesterone by 3β-Hydroxysteroid dehydrogenase (3β-HSD). Progesterone is then transformed via a two-step hydroxylation catalysed by Cytochrome P450 family 17 subfamily A member 1 (CYP17A1) to Androstenedione, a low potency androgen that is reduced to Testosterone by the testis-specific to 17β-Hydroxysteroid dehydrogenase (17β-HSD) (Miller and Auchus, 2010, Li and Zirkin, 2018). Testosterone production is not only vital for sustaining spermatogenesis (O'Donnell et al. 2006), but also required for the development of secondary sexual characteristics muscle and bone density maintenance, and overall health and well-being in men (Cattabiani et al. 2012, Lang et al. 2012, Morris and Channer, 2012). It has been postulated that age-related declines in LH/testosterone ratios could lead to reduced androgen bioactivity which is linked with various co-morbidities in ageing men (Zitzmann et al. 2006, Nieschlag, 2020). Low serum testosterone

levels are also observed in younger men, particularly those diagnosed with idiopathic infertility (Huhtaniemi & Forti, 2011, Fraietta et al. 2013).

- ***Testicular macrophages:***

The interstitium contains a variety of immune cells, the majority of which are resident testicular macrophages (Hedger, 2002). Testicular macrophages play a crucial role in maintaining normal testis homeostasis (Hedger, 2011) and have been shown to directly interact with and influence the proliferation and function of Leydig cells (reviewed in Mossadegh-Keller and Sieweke, 2018). Through the secretion of Reactive Oxygen Species (ROS), prostaglandins, and pro-inflammatory cytokines, testicular macrophages stimulate steroidogenesis in Leydig cells (Hutson, 2006). Leydig cells in culture exposed to testicular macrophage conditioned medium show significantly increased testosterone production, whereas conditioned macrophage medium does not show the same effect (Yee and Hutson, 1985). Molecular factors secreted by testicular macrophages have been shown to support steroidogenesis in rodent models (Cohen et al. 1997, Nes et al. 2000). (csfmop/csfmop) mutant mice lacking testicular macrophages exhibit decreased production of viable sperm and infertility (Cohen et al. 1996). Testicular macrophages are also thought to be involved in the maintenance of the spermatogonial niche and the differentiation of spermatogonial stem cells, possibly through the retinoic acid and Colony stimulating factor 1 (CSF1) pathways (Oatley et al. 2009, DeFalco et al. 2015). Macrophages accumulate on the surface of seminiferous tubules in regions that contain a high concentration of undifferentiated spermatogonia, promoting their differentiation, through the expression of enzymes and genes involved in retinoic acid synthesis (Hutson, 1994, DeFalco et al. 2015). Moreover, testicular macrophages play a crucial role in preserving the immune privilege in the testis by maintaining an immunosuppressive environment to protect the highly immunogenic post-meiotic germ cells (Hedger, 2002, Bhushan and Meinhardt, 2017). Interstitial macrophages produce high levels of anti-inflammatory cytokines IL-10 and TGF-b (Xue et al. 2014) and have attenuated responses to inflammatory stimulation with LPS, INFg or IL-4 compared to peritoneal macrophages, characterized by lower production of pro-inflammatory cytokines (O'Bryan et al. 2005, Winnall et al. 2011).

c. Blood-testis barrier:

Immune privileged tissues are characterized by the active suppression of the immune response against exogenous and endogenous antigens (Meinhardt and Hedger, 2011). Often, a barrier that largely restricts the access of immune cells to the immune-privileged micro-environment is present but does not completely prevent inflammatory responses from occurring

(Meinhardt and Hedger, 2011). Advanced germ cells start developing at puberty, long after the maturation of the immune system and the establishment of self-tolerance. Many proteins expressed only in spermatocytes and spermatids, therefore, constitute novel antigens. The Blood-Testis-Barrier (BTB) prevents the entry of immune cells into the seminiferous tubules and is composed of cellular tight-junctions formed between somatic Sertoli cells, separating the luminal compartment where antigenic haploid germ cells from the interstitial vasculature and testicular macrophages, lymphocytes, dendritic cells, and T cells present in the interstitium (Hedger, 1997, Hedger, 2002). Beyond the physical sequestration of post-meiotic germ cells, a combination of central tolerance and local immunomodulatory mechanisms contribute to the immunosuppression necessary for the success of the spermatogenic process (Meinhardt and Hedger, 2011, Tung et al. 2017).

I.1.2. Epididymis:

Seminiferous tubules connect to the rete testis via a straight section known as the tubuli seminiferi recti. The network of rete testis tubules leads into the efferent ducts, which in turn converge to form a single highly coiled duct, the epididymis (Hemeida et al. 1978). The epididymis is divided into four gross anatomical sections: the initial segment, head (caput), body (corpus), and tail (cauda) (Robaire et al. 2006). Other than its function as a sperm reservoir, the epididymis is involved in sperm concentration and transport, and immune protection of the male gamete (Sullivan and Mieusset, 2016). The epididymis plays an essential role in sperm maturation, a process during which spermatozoa acquire forward mobility and fertilization capacities (Cooper and Yeung, 2006).

- ***Maturation of the sperm in the epididymal tract:***

Sperm maturation is a complex process that occurs in the caput and corpus epididymis (proximal epididymis) (Cooper and Yeung, 2001). For the male gamete to acquire the capacity to fertilize the egg, it must transit through the epididymis for a certain period first (Robaire et al. 2006). In order to ensure an efficient encounter with the female gamete, several biochemical changes take place in the sperm surface, including changes in the membrane lipid composition (Rejraji et al. 2006); modifications to lectin-binding properties (Hermo et al. 1992); remodelling of plasma membranes structures (Girouard et al. 2011); modifications to surface glycoproteins (Tulsiani, 2006) and surface antigen re-localization (Belmonte et al. 2000).

I.1.3. Accessory glands:

Male accessory glands include the ductus deferens ampulla, seminal vesicles, the prostate, and the bulbourethral glands (Mann and Lutwak-Mann, 1951). These glands secrete several

factors crucial for sperm viability, such as alpha-glucosidase, fructose, prostaglandins, and citric acid. Accessory gland secretions combined represent up to 90% of the contents of the seminal fluid, which serves as a vehicle for sperm during ejaculation (Marconi et al. 2009). The vas deferens brings sperm from the testis to the seminal vesicles, which in turn connect to the prostate. Sperm and seminal vesicle secretions, pass through the prostatic urethra, before being ejaculated through into the ejaculatory ducts, prompted by the contractions of the smooth muscle of the vas deferens and seminal vesicles (Amis and Lang, 1994) (Spring-Mills and Bush, 1982).

a. Seminal vesicles:

The seminal vesicles are a pair of tubular glands that produce several of the major components of the seminal fluid, important for the normal function and survival of sperm (McKay et al. 2020). They are located between the bladder fundus and the rectum (Zaidi et al. 2018). Inside, the seminal vesicles are composed of mucosa lined by a pseudostratified columnar epithelium, where seminal metabolites are produced (e.g., fructose, proteins, enzymes, vitamin C) which make up to 70% of the total volume of the seminal fluid (McKay et al. 2020).

b. Prostate:

The human prostate is a walnut-sized steroid-sensitive gland, situated below the urinary bladder with part of the urethra, referred to as the prostatic urethra, passing through it (Skinner, 2018, Aaron et al. 2016). The prostate gland can be divided into three histological compartments: the non-glandular fibromuscular stroma that surrounds the organ and two glandular regions termed peripheral and central zones which contain a complex ductal system (Amis and Lang, 1994). The latter constitutes most of the base of the prostate and surrounds the ejaculatory ducts (Aaron et al. 2016).

c. Cowper glands/ Bulbourethral glands:

The Bulbourethral glands are a pair of pea-shaped exocrine glands located near to the bulb of the penis and connected to the membranous urethra (Testa-Riva et al. 1994). The main function of these glands is to produce an alkaline lubricating mucus secretion (known as pre-ejaculate), that is added to the final volume of the semen to aid in the passage of the liquid through the spongy urethra during ejaculation (Weinbauer et al. 2010).

I.2. Spermatogenesis:

Spermatogenesis, ultimately resulting in sperm production, is the main function of the testis. It occurs in the seminiferous tubules of the testis, where spermatogonial cells divide and

mature into highly specialized male gametes. This process involves a coordinated interaction between endocrine hormones, growth, and paracrine factors, as well as epigenetic modifiers and gene and protein expression programs (de Kretser et al. 1998; O'Donnell et al. 2017). It requires the cooperation of multiple testicular cell types, including Sertoli cells and Leydig cells, by producing testosterone and expressing many important genes and paracrine factors (Cheng et al. 2010). Spermatogenesis consists of four major processes: spermatogonial proliferation, meiosis, spermiogenesis, and spermiation.

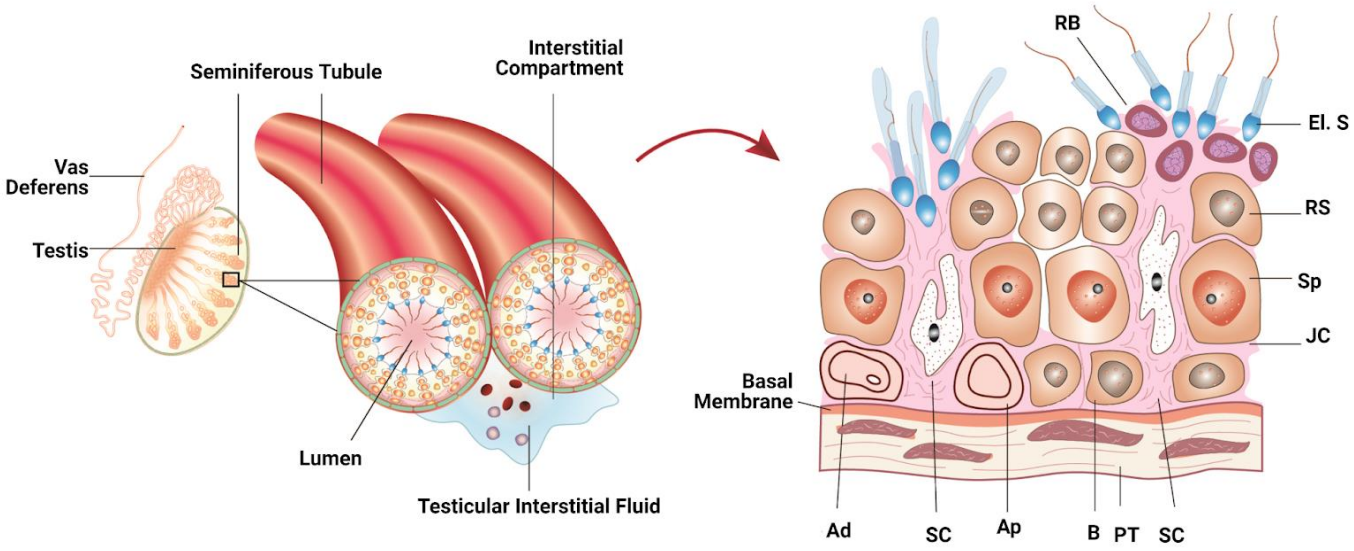


Figure 2: Schematic illustration of the cellular structure of seminiferous tubules. The tubular well is composed of 3-6 layers of peritubular myoid cells (PT). Sertoli cells (SC) extend from the basal membrane to the seminiferous lumen and form cell-cell junctions (JC) with germ cells that divide and differentiate into highly specialized spermatozoa. Reserves of spermatogonial stem cells (SSCs) are maintained via asymmetric division that results in one progenitor cell which advances through the different stages of spermatogenesis and one new stem cell. Ad = A-dark-type spermatogonia (stem cells), Ap = A pale-type spermatogonia; B = B-type spermatogonia, Sp = spermatocyte, RS: round/ early spermatid, El. S= elongated spermatid), RB= residual body. Modified from Weinbauer et al. 2010

I.2.1. Spermatogonial proliferation:

Progenitor A dark spermatogonia are part of the spermatogonial sperm cells (SSCs) pool. They divide rarely, producing progenitor A pale spermatogonia, which can transform into spermatogonial cells that are committed to differentiation (A diff) (Weinbauer et al. 2010). A diff cells divide once per cycle, generating B type spermatogonia, which exhibit a higher level of differentiation (Amann, 2008).

I.2.2. Meiosis:

The meiotic phase covers the progression from diploid primary spermatocytes to haploid spermatids and involves chromosome pairing and chromatid exchange (Griswold, 2016). Type B spermatogonia divide into preleptotene spermatocytes, signalling the beginning of meiosis. Prophase I of meiosis occurs over several weeks and is characterized by DNA replication (to create games with 4C, 2N genetic material) and chromosome condensation, giving a filamentous appearance to the nucleus of leptotene spermatocytes (David et al. 2016). At the zygotene stage, the homologous chromosomes move into a linear arrangement and form synaptonemal complexes, initiating chromosomal crossover which takes place during the pachytene stage (Sharma and Agarwal, 2011). This exchange of genetic material by homologous recombination occurs during the pachytene stage; this involves the induction of DNA double strand breaks, the swapping of genetic material between the homologous chromosomes, followed by DNA repair. Chromosome pairs start separating partially during the diplotene phase and migrate towards opposite poles of the cell during Anaphase I. After the first meiotic division, secondary spermatocytes contain half the genetic material (1N, 2C). The second and final meiotic division quickly follows the first to produce haploid round spermatids. Thus, one primary spermatocyte ultimately produces four haploid spermatids (Weinabauer et al. 2010, Neto et al. 2016).

I.2.3. Spermiogenesis and spermiation:

After the second meiotic division, round, haploid spermatids enter spermiogenesis, where they no longer divide but are gradually transformed into the highly specialised elongated spermatid. This process involves nuclear chromatin condensation: the remodelling of the spermatid's DNA into a tightly coiled structure, and the replacement of histones with arginine- and cysteine-rich protamine during spermatid elongation (Goldberg and Zirkin, 2018). Spermiogenesis also involves the development of the acrosome and the flagellum. The acrosome, located on the surface of the sperm head, contains hydrolytic enzymes necessary to successfully penetrate the zona pellucida of the female egg during fertilization (Goldberg and Zirkin, 2018). The final process of spermiogenesis and consequently spermatogenesis is spermiation, where the most of the round spermatid's cytoplasm is condensed into the so-called residual body, and is removed and phagocytosed by the Sertoli cell as the elongated spermatid is released by the Sertoli cell into the lumen. Spermiation is triggered by signalling within Sertoli cells and is highly vulnerable to disruptions by endocrine disturbances and toxicant exposure (O'Donnell et al. 2011). After sperm are released from the seminiferous tubules, they

are swept along the luman towards the rete testis, propelled within the seminiferous tubule fluid facilitated by contractions of the peritubular myoid cells. As these spermatids transit through the rete testis, efferent ductules and enter the epididymis, they are not motile nor able to fertilize the oocyte (Neto et al. 2016).

I.2.4. Regulation of spermatogenesis:

Spermatogenesis is regulated via a number of distinct factors that include endocrine and autocrine hormones, growth and paracrine factors, gene and protein expression programs, as well as epigenetic modifiers (de Kretser et al. 1998; O'Donnell et al. 2017). Many of these molecular factors are part of the male hypothalamic-pituitary-gonadal axis which regulates androgen synthesis and spermatogenesis through multiple levels of modulation and positive/ negative feedback (Corradi et al. 2016). Gonadotropin releasing hormone (GnRH) is secreted in discrete pulses from the hypothalamus, and binds to GnRH receptors in pituitary gonadotropes, thereby stimulating the secretion of LH and FSH into general circulation. GnRH modulates its own production via an intra-hypothalamic feedback loop, as GnRH receptors are expressed in the hypothalamus (Cheng and Leung, 2005).

a. Endocrine regulation:

FSH and LH act directly on the testis via specific receptors (Ramaswamy and Weinbauer, 2015). Sertoli cells express FSH receptors, while interstitial Leydig cells express LH receptors (Neto et al. 2016). FSH is important for the maturation of Sertoli cells, optimal spermatogonial proliferation and survival and spermatocyte development (Weinbauer et al. 2010). Spermatogenesis can occur in the absence of FSH; however, testis size is reduced due to decreased Sertoli cell proliferation during puberty, leading to a significant reduction in sperm production in adulthood, reviewed in (O'Donnell et al. 2017). LH stimulates production of the androgen testosterone in Leydig cells, which is essential for gametogenesis (Ramaswamy and Weinbauer, 2015). Testosterone acts on androgen receptors within the Sertoli cells, and to a lesser extent the peritubular myoid cells, to support spermatogenesis (reviewed in Smith and Walker, 2014). In the absence of Sertoli cell androgen receptor, spermatogenesis is arrested at early meiosis, indicating that androgen action on Sertoli cells is required for spermatogenesis (De Gendt, 2014). Testosterone in turn feeds back to suppress the secretion of gonadotropins and GnRH by the hypothalamic-pituitary secretory unit. Elevated levels of Testosterone reduce LH production, and high levels of systemic estrogen suppresses gonadotropin secretion by the pituitary gland (Corradi et al. 2016).

b. Autocrine and paracrine regulation:

In addition to endocrine control, locally produced steroids, proteins and peptides are also important for sperm production (Weinbauer et al. 2010). As well as testosterone produced by the Leydig cells as described above, other regulators include estrogen, growth factors, IGF-1, TGF- α and β), activin, inhibin B and cytokines (Boepple et al. 2008, Roser, 2008). Testosterone acting via the androgen receptor (AR) is involved in a wide range of functions, including maintenance of blood–testis barrier formation and function, inducing meiosis and post-meiotic development of germ cells (Larose et al. 2020), inhibiting germ cell apoptosis, as well as modulating the gene expression and regulation of steroidogenic enzymes in Sertoli and Leydig cells (Meng et al. 2005, De Gendt, 2014, O'Hara and Smith, 2015). Estrogen is produced by the enzymatic conversion of androgens to estrogens via the aromatase enzyme (Robertson et al. 1999). The secretion of this hormone is influenced by FSH in the Sertoli cells and by LH in Leydig cells (Weinbauer and Wessels, 1999). Estrogen is important because indirectly it facilitates normal germ cell development and Sertoli cell function by increasing the diameter of seminiferous tubules and affecting fluid resorption in the efferent ducts (Hess et al. 1997, Fisher et al. 1998, O'Donnell et al. 2001). Various growth factors are expressed in Sertoli, Leydig and germ cells. They bind to the surface receptors of target cells to activate cell division and cell-specific differentiation (Niederberger et al. 1993). Inhibin B and activin, which are members of the superfamily TGF- β, affect testosterone production, spermatogonia number and Sertoli cell numbers (Weinbauer and Wessels, 1999). Insulin-like growth factor I (IGF-1) stimulates spermatogonia DNA synthesis and has a maintaining effect on premeiotic DNA synthesis, besides being involved in spermatogonia differentiation and having anti-apoptotic effects in Leydig and germ cells (Colón et al. 2006), in addition to its role in the paracrine regulation of GnRH secretion (Cheng and Leung, 2005).Various cytokines (e.g., interleukins, interferons, tumour necrosis factors) attach to the cell surface receptors and induce cell proliferation and differentiation by interacting with Sertoli cells, and modulating DNA synthesis and differentiation in spermatogonia (Weinbauer and Wessels, 1999).

II. Male infertility and azoospermia:

The World Health Organization defines infertility as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al. 2009). In recent decades, total fertility rates of populations from industrialized countries have been decreasing (Nargund,

2009). For instance, in the USA, the average number of live births per woman was around 3.7 in 1960, while in 2018 that value was below 1.8 (DataBank, 2020). A great portion of this decline is related to economic and social factors combined with access to contraceptive methods and legal abortions. However, infertility still represents a major health problem worldwide. It has been estimated that there are approximately 49 million infertile couples worldwide, representing 15% of the total couples globally (Mascarenhas et al. 2012). Male factor infertility affects 20-30% of couples and contributes to about 50% of the infertility cases overall (Agarwal et al. 2016).

Multiple studies have pointed to an increased prevalence of male reproductive disorders in the last few decades, including testicular germ cell cancer, hypospadias, and infertility caused by abnormal sperm parameters (oligozoospermia, asthenozoospermia, teratozoospermia, azoospermia, etc) (Skakkebaek et al. 2015, Öztekin et al. 2019). Levine et al. (2017) performed an extensive meta-regression analysis of sperm concentration data from Western populations spanning the previous 40 years and reported a decrease of nearly 60% in sperm counts. However, these findings were recently challenged by Boulicault et al. (2021), who cited the limitations of the study (missing information such as the age of subjects/ dates of sample collection/ developments in semen analysis protocols and quality control schemes that were unaccounted for) and postulated that the reported change in sperm counts is not necessarily indicative of decreased fertility in the "Western" male population, and could be explained by the evolution of measurement methods and high inter-individual variability.

II.1. Azoospermia:

Azoospermia is defined as the complete absence of spermatozoa in the ejaculated semen (Luigi et al. 2012). Azoospermia is present in approximately 1% of all men, and around 15% of infertile men (Jarvi et al. 2010). Initially, the diagnosis for azoospermia is made when no spermatozoa can be detected on high-powered microscopic observation of centrifuged seminal fluid on at least two occasions (Luigi et al., 2012). Furthermore, this diagnosis can be further classified as obstructive azoospermia (OA) or non-obstructive azoospermia (NOA), each having distinct aetiologies and treatments, although in clinical practice both types can be present in a single patient (Wosnitzer et al. 2014).

II.2.1. Obstructive azoospermia (OA):

OA represents approximately 40% of azoospermia cases and occurs when spermatozoa produced by the testes are unable to reach the semen (Luigi et al. 2012, Practice Committee of American Society for Reproductive Medicine, 2008). This is a consequence of the physical

blockage to the male excurrent ductal system and may occur in any region between the rete testis and the ejaculatory ducts (Jow et al. 1993, Lin et al. 2020). Moreover, the obstruction of OA can be attributed to a wide range of factors, which include genetic abnormalities, infections, iatrogenic injuries, and congenital factors, such as the bilateral absence of the vas deferens (CBAVD) or congenital unilateral absence of the vas deferens (CUAVD), often related with mutations of the cystic fibrosis transmembrane regulator (CFTR) protein gene, known as Wolffian duct abnormalities (Lenk and Oesterwitz, 1994). OA is diagnosed using a combination of history/physical examination, laboratory testing, genetics, and imaging studies (Baker and Sabanegh, 2013). Although OA is detrimental for sperm count, endocrine, and exocrine functions remain unchanged and normal (Luigi et al. 2012).

a. CBAVD:

Congenital bilateral absence of the vas deferens (CBAVD) is a rare obstructive anomaly that contributes to male infertility and causes OA. CBAVD prevalence represents roughly 1–2% of all infertile males (Lin and Huang, 2020). This genetic anomaly is related to malformations in the epididymis and seminal vesicles, where transrectal ultrasonography and scrotal ultrasonography are some of the techniques used for diagnosis (Lin and Huang, 2020). CBAVD is not amenable to surgical intervention, therefore microsurgical epididymal sperm aspiration combined with *in vitro* fertilization/intracytoplasmic sperm injection is often the treatment used (Anger et al. 2004).

b. Secondary obstruction:

When any obstruction to the vas deferens or efferent ducts is caused by non-natural factors, such as iatrogenic injuries and infection, it is considered a secondary obstruction (Junge et al. 2008). For instance, tissue injuries resulting from procedures like paediatric hernia repair, renal transplant, and appendectomy were cited as the main causes of azoospermia in 8-19% of patients (Berardinucci et al. 1998, Schiff et al. 2005, Junge et al. 2008). Acute epididymitis, brought on by bacterial, viral or fungal infections, can also induce an obstruction of the epididymis, vas deferens and ejaculatory ducts in about 10% of cases (Berardinucci et al. 1998, Nicholson et al. 2010, Schuppe et al. 2017). Chronic urethritis stemming from sexually transmitted infections can also lead to obstruction and a reduced ejaculate volume (Rusz et al. 2012). When possible, microsurgical vasoepididymostomy and vasovasostomy are considered the most efficient treatments for tissue restoration in these types of obstructions (Song et al. 2019, Wosnitzer et al. 2014).

II.2.2. Non-obstructive azoospermia (NOA):

NOA affects around 60% of azoospermic males and is the result of a lack of spermatozoa production due to primary, secondary, or incomplete/ambiguous testicular failure (Jarow et al., 1989). Primary testicular failure, which manifests as decreased testosterone production, elevated FSH and LH serum levels, and impaired spermatogenesis is the most common cause of NOA (Barak et al. 2016). Several testicular histopathologic phenotypes are associated with primary spermatogenic failure, including hypospermatogenesis, mixed atrophy, spermatogenic arrest, and Sertoli cell only syndrome (Behre et al. 2015). Secondary hypogonadism stems from a dysfunctional hypothalamic-pituitary axis, resulting in low serum testosterone levels, low to normal levels of LH and FSH, and decreased spermatogenesis (Richard-Eaglin, 2018). NOA diagnosis includes laboratory testing, genetic testing (e.g., karyotype, Y chromosome microdeletion), analysing the testis consistency/volume, and performing biopsies (Wosnitzer et al. 2014). Genetic conditions such as Kallman syndrome (short stature, polydactyly, anosmia) and Prader-Willi syndrome (extreme obesity) have to be investigated in cases of secondary hypogonadism, whereas Klinefelter syndrome (47, XXY) is usually investigated in cases of primary hypogonadism (Bhasin et al. 2010, Richard-Eaglin, 2018). In a subset of patients suffering from hypogonadism, Testosterone therapy can be recommended to restore fertility by increasing T serum levels (Bhasin et al. 2010, Silveira and Latronico, 2013). Compared to men with obstructive azoospermia, men with non-obstructive azoospermia generally have lower rates of successful sperm retrieval (Corona et al. 2019).

a. Hypospermatogenesis and mixed atrophy:

Hypospermatogenesis is a phenotype of reduced spermatogenesis when all types of germ cells are present in the testis and are visible in most seminiferous tubules, but germ cell numbers are greatly reduced, leading to reduced sperm counts or azoospermia (Behre et al. 2015). This disorder is often related to hormonal dysregulation, congenital germ cell deficiency, androgen insensitivity, chemical exposure, and exposure to heat and radiation (Nistal and Paniagua, 1999). Hypospermatogenesis represents around 29% and 25% of the total histopathological patterns found in testicular biopsies according to two different studies (Abdullah and Bondagji, 2011, Jamal and Mansoor, 2001). Hypospermatogenesis has been linked to the presence of multinucleated spermatocytes or “megalospermatocytes”, which are indicative of missing synaptonemal complexes during meiosis (Johanisson et al. 2003). In addition to reduced mitosis and impaired spermiogenesis (Steger et al. 2001), hypospermatogenesis is also associated with increased germ cell apoptosis (Steger et al. 1998, Kandirali et al. 2009). Mixed atrophy (MA) of the seminiferous tubules is another testicular

disorder that is observed in around 25% of testicular biopsies performed in infertile males (Nistal et al. 2007). Mixed atrophy is associated with a range of seminiferous tubule phenotypes within the same testis biopsy, ranging from tubules containing only Sertoli cells, those showing no cells and a high level of hyalinization, to tubules with some or all germ cells present (Schulze et al. 1999, Bergmann and Kliesch, 2010, Behre et al. 2015). Although the mechanisms that cause this disorder are relatively unknown, Behr et al. (2007) noted that it can be caused by mutations in the FOXA3 locus, which is a transcription factor important for organ development. In incomplete germ cell aplasia, FSH therapy has been suggested as a remedy for residual sperm production. There is some evidence of an increase in sperm concentration in the ejaculate and in pregnancy rates based on clinical studies (Santi et al. 2015).

b. Sertoli cell only syndrome:

Sertoli cell-only syndrome (SCOS), also known as germ cell aplasia, was first described by Del Castillo et al. in 1947. Histologically, SCOS manifests as a complete lack of germ cells, with only Sertoli cells lining the seminiferous tubules, reduced tube diameter, and thicker tubular walls (Luigi et al., 2012). A patient with SCOS will present with azoospermia, normal virilization, and atrophic to normal testes. Although radiation, chemotherapy, and genetic anomalies can all contribute to the SCOS phenotype, the underlying molecular causes are still unclear (Ghanami-Ghashti et al. 2021). This syndrome is irreversible and can be a consequence of many factors including steroids therapy, chemotherapy or radiation therapy, orchitis (testis infection), or genetic factors (Carrara et al. 2004, Krausz and Riera- Escamilla, 2018, Pilatz et al. 2019). The most prevalent anomaly detected in Caucasian azoospermic males with Sertoli cell-only syndrome is karyotype abnormalities (24 %), particularly Klinefelter syndrome and AZF (Stouffs et al. 2016). Infertile men with nonobstructive azoospermia or severe oligozoospermia are frequently tested for AZF deletions, karyotype analysis, and Yq microdeletions. On average, Sertoli cell only syndrome can represent 15% of the total abnormalities from testicular biopsies (Ghanami-Ghashti et al. 2021). Vloeberghs et al. (2015) looked at the success rate of TESE ICSI cycles in patients with NOA. A total of 464 of the 714 participants were diagnosed with “SCOS.” Mature sperm cells were found in 38.4 percent of these patients. This meant that for the vast majority of SCO patients, there were no spermatozoa to be recovered. Although men with smaller testes and higher FSH have worse testicular function overall, neither testis volume nor FSH can be used to accurately predict the reproductive potential in NOA subjects (Jarvis et al.2010). Berookhim et al. (2014) found no correlation between testis volume and sperm retrieval rates (SRRs) in SCO men, though earlier research has shown a lower testis volume to be associated with poor reproductive outcomes

(Bromage et al. 2007). In fact, a subgroup of men with normal testis volume (>= 15 mL) and intermediate FSH levels (10–15 mU/mL) had the lowest SRR (Berookhim et al. 2014). According to the same study, SCO men with high serum FSH and small-volume testes are more likely to have heterogenous testicular tissue, and therefore some sperm production sites. On the other hand, normal testis volume in SCO is indicative of a homogenous cellular pattern throughout, leading to a reduced probability of locating spermatogenesis sites and lower SRRS.

c. Spermatogenic arrest:

Spermatogenic arrest occurs when the germ cell maturation is halted at a specific cell stage, including at the level of spermatogonia, spermatocytes, or spermatids. Some cases are also characterized by the presence of seminiferous cords with immature Sertoli cells in some cases (McLachlan et al. 2007, Behre et al. 2015). This phenotype has multiple causes, including genetic defects such as trisomy and Y chromosome microdeletions (Yao et al. 2001, Hung et al. 2007), copy number variations of meiotic cell-specific genes (Eggers et al. 2015) as well as exposure to genotoxic substances, radiotherapy, or heat (Martin-du Pan and Campana, 1993). Clinical parameters such as FSH and inhibin B levels and testicular volume are usually within normal range, therefore testicular biopsy is needed for differential diagnosis (Behre et al. 2015). Uniform spermatogenic arrest is widely considered to be irreversible as there are no proven therapies. FSH treatment was deemed ineffective in increasing sperm counts in SA patients (Foresta et al. 2000), although a more recent case study reported the success of a 9-month recombinant FSH treatment in a 36-years old man diagnosed with maturation arrest at spermatocyte/spermatid stages (Barbotin et al. 2017).

III. Diagnosis and management of male infertility:

III.1. Clinical evaluation of the infertile man:

An extensive clinical examination encompassing medical history and physical examination is essential for the diagnosis of male infertility. The gathered historical data includes present and past illnesses or injuries affecting the testes, pubertal development, sexual performance, occupational and lifestyle hazards, and habits like smoking. The physical examination includes assessment of virilization, body proportions and BMI, scrotal examination, testes and epididymides size and condition, and presence of varicocele (Jarvi et al. 2010). Several other methods are routinely used for the differential diagnosis of male infertility, the main ones being semen analysis, hormonal investigation, genetic testing, and testicular biopsy. Whether all or just some of these methods are called for is determined by the clinician on a case-to-case basis.

III.1.1. Semen analysis:

Semen analysis is the primary test for the determination of male reproductive potential, and the basis for subsequent diagnosis and treatment strategies. During ejaculation, epididymal spermatozoa are mixed with accessory gland secretions, from the seminal vesicles, prostate, and bulbourethral glands. Therefore, the two main attributes of the ejaculate are the total volume and sperm concentration, usually expressed as 10^6 cells/ ml (WHO, 5th edition, 2010). Other important parameters are sperm motility, morphology, and vitality. The sample is typically collected via masturbation after a period of abstinence of 2-5 days. The patient is provided with a sterile non-toxic collection jar and proper guidelines on sample collection and delivery. The sample should not be exposed to extreme temperatures and delivered to the laboratory within an hour of collection. Given the high variability observed for human samples collected on different days, a repeat test should be done fourteen days later when the results of the first test show abnormal semen parameters (Barak and Baker, 2016). The aspects examined during semen analysis are determined by the World Health Organization for Semen analysis (WHO, 2021) and are summarized in Table 1. Reduced semen volume can be suggestive of incomplete collection or abstinence from ejaculation for a period shorter than recommended, but more often it is a sign of the absence or impairment of the seminal vesicles/ vas deferens, retrograde ejaculation (usually confirmed by testing post-ejaculatory urine), or emission failure (Jarvi et al. 2010). Hemospermia or the presence of discoloration in the semen are indicative of infections of the urethra or accessory glands (Barak and Baker, 2016). Sperm counts below the reference value are categorized as “oligozoospermic” (WHO, 2010). A sperm concentration ($<2 \times 10^6$/mL) is classified as “severe oligozoospermia” (Colbi et al. 2018). According to WHO nomenclature, asthenozoospermia is described as a lower-than-normal percentage of progressively motile spermatozoa, teratozoospermia as a percentage of morphologically normal spermatozoa lower than the reference limit. A combination of two or more of these sperm conditions are often observed in subfertile and infertile men, most commonly referred to as oligoasthenozoospermia, oligoteratozoospermia or oligoasthenoteratozoospermia (OAT) (WHO, 2010, Colbi et al. 2018). When the analysis shows a total absence of sperm from the semen, known as azoospermia, repeated testing with vigorous centrifugation and careful examination of the pellet is necessary in order to confirm this observation (Blickenstorfer et al. 2019, Cooper et al. 2006).

Table 1: Lower reference limits (5th centiles and their 95% confidence intervals) for semen characteristics (World Health Organization, 2021)

Parameter	**Lower reference limit (CI)**
Semen volume (mL)	1.4 (1.3-1.5)
Total sperm number (10^6 per ejaculate)	39 (35-40)
Sperm concentration (10^6 per mL)	16 (15-18)
Total motility (Progressive and Non-progressive, %)	42 (40-43)
Progressive motility (%)	30 (29-31)
Non-progressive motility (%)	1 (1-1)
Vitality (live spermatozoa, %)	54 (50-56)
Immotile spermatozoa (%)	20 (19-20)
Sperm morphology (normal forms, %)	4 (3.9-4.0)
pH	> 7.2
MAR test (motile spermatozoa with bound particles, %)	< 50
Immunobead test (motile spermatozoa with bound beads, %)	< 50
Seminal zinc (µmol/ejaculate)	> 2.4
Seminal fructose (µmol/ejaculate)	> 13
Seminal neutral glucosidase (mU/ejaculate)	> 20

III.1.2. Hormonal investigation:

Hormonal screening for FSH, LH, and testosterone levels is required in cases of abnormal semen parameters. The normal adult male range is 2 – 12 mU/mL for serum FSH and 2 – 9 mU/mL for LH. Measurements of serum FSH levels are usually indicative of the state of the seminiferous epithelium. Elevated FSH concentrations correlate with cellular damage in the seminiferous tubules (Schoor et al. 2002). Although less predictive than FSH, elevated LH and low testosterone (normal testosterone range: 280- 1.100 ng/dL) indicate some degree of testicular failure (Nieschlag, 2001). Elevated serum LH coupled with low-to-normal testosterone levels can be a sign of compensated Leydig cell insufficiency, whereby Leydig cells are less effective at responding to LH and/or at producing androgens (Jørgensen et al. 2016). Prolactin, a pituitary hormone that modulates testicular activity through specific receptors that are present on Sertoli and Leydig cells, has a normal range of 2 to 18 ng/mL in the adult male. Prolactin is routinely measured as part of hormonal profiling of subfertile and infertile men. Hyperprolactinemia is responsible for infertility in 11% of men diagnosed with oligospermia, as it inhibits the pulsatile secretion of the GnRH and subsequently the release of FSH and LH, resulting in spermatogenic arrest and impaired sperm morphology and motility (Masud et al. 2007).

In cases of azoospermia, low LH and FSH levels coupled with low to normal serum testosterone point to a hypothalamic or pituitary aetiology. However, normal FSH, LH or testosterone concentrations can be observed both in men with obstructive azoospermia and non-obstructive azoospermia (Jarvi et al. 2010). In such instances, hormonal investigation is not conducive to differential diagnosis. On the other hand, Inhibin B serum levels are generally lower in men with severe testicular dysfunction and are undetectable in men showing Sertoli cell only syndrome (Hu et al. 2003, Kumanov et al. 2006). However, inhibin B levels in men with maturation arrest and hypospermatogenesis may be identical to those found in men with full spermatogenesis (von Eckardstein et al. 1999). Inhibin B levels are a good indicator of spermatogenic impairment but are not sufficient to predict the outcome of sperm extraction (Ruiz Plazas et al. 2010).

III.1.3. Genetic testing:

Genetic abnormalities have the potential to cause infertility in men by impairing spermatogenesis, affecting sperm quality, or preventing or the transit of sperm from the testes to the ejaculated semen. Three types of genetic tests are commonly undertaken:

- Screening for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene mutations is recommended for men with suspected congenital obstruction, normal testicular volume and serum FSH. CFTR mutations can be the underlying cause of congenital bilateral absence of the vas deferens (CBAVD) (Lewis-Jones et al. 2000).
- Karyotype analysis of peripheral blood cells is recommended during the diagnosis for men who are azoospermic or have severe oligozoospermia. For these individuals, the cytogenetic screening is mandatory before resorting to assisted reproductive technologies (ARTs) procedures. Numeric (chromosome gain/loss) and structural abnormalities (reciprocal translocations, inversions) are associated with approximately 6% of all male infertility cases (reference). Particularly, the loss or the addition of sex chromosomes such as Klinefelter and XXYY syndromes are associated with reduced fertility and sterility (Nieschlag et al. 2010).
- Screening for Y-Chromosome Microdeletions (YCMDs) is recommended in cases of primary testicular failure (low testicular volume, high FSH, and low testosterone (Simoni et al. 2008, Wosnitzer, 2014). YCMDs occur as a result of deletions on the long arm of the Y chromosome (Yq), affecting three azoospermia factor (AZF) regions which code several genes essential for spermatogenesis: AZFa (USP9Y and DDX3Y), AZFb (EIF1AY, HSFY, SMCY, RPS4Y2, PRY), and AZFc (BPY2, CDY, DAZ). The most common microdeletion of the Y chromosome among infertile men is AZFc which is found in 1:4,000 men, with

two out of three cases presenting with severe oligospermia whereas the rest are azoospermic (Reijo et al. 1995). This test has a prognostic value in guiding the choice of therapeutic options (surgical sperm retrieval vs. use of donor sperm).

III.1.4. Testicular Biopsy:

Testicular biopsy has long been regarded as the cornerstone of the diagnosis of idiopathic male infertility. Diagnostic testicular biopsy is performed under local or general anesthesia. A small incision of approximately 5 mm is made into the tunica albuginea and a biopsy of 3x3x3 mm of testicular tissue is excised (Jungwirth et al. 2012). During the procedure, special care is taken to avoid compromising the structural integrity of the seminiferous tubules to allow for the accurate evaluation of spermatogenesis in the collected biopsies (Schlegel and Li, 1998). Testicular histology can reveal the presence of spermatozoa even in subjects with signs of severe testicular failure (high serum FSH, azoospermia) (Jungwirth et al. 2018). Testicular biopsy is also routinely used for sperm retrieval both in obstructive and nonobstructive azoospermia cases to varying success rates (50-60 % in NOA, 100% in OA) (Jungwirth et al. 2012). Conventional testicular sperm extraction causes transient pockets of acute inflammation or hematoma in a majority of patients (Schlegel et al. 1997) resulting in tissue scarification and calcification. In rare cases, more permanent effects such as impaired blood flow and complete devascularization of the testis can result from the ligation or division of subtunical arteries (Schlegel and Su, 1997, Schlegel and Li, 1998).

- ***Microsurgical TESE:***

Microsurgical testicular sperm extraction (micro-TESE) is a surgical technique first described by Schlegel and Li in 1998 as a means for sperm retrieval in non-obstructive azoospermia. The procedure requires the use of an operating microscope to identify and avoid subtunical blood vessels when making an incision to the surface of the testis, and to spot dilated seminiferous tubules that are more likely to contain viable spermatozoa (Schlegel and Li, 1998). Micro-TESE was initially touted for having a higher success rate compared to random biopsy, limiting the amount of excised testicular parenchyma, and reducing the loss of testicular volume (Schlegel and Li, 1998). However, a more recent systematic review reported similar rates of clinical complication between micro and conventional TESE (c-TESE), with a non-significant tendency for higher sperm retrieval rates in micro-TESE (Saccà et al. 2015). In cases of NOA, the European Urology Association (EAU) guidelines recommend performing microdissection TESE and testicular biopsy simultaneously for a better chance of accurate diagnosis and sperm retrieval for use in ICSI (Jungwirth et al. 2018).

IV. Seminal plasma: overview

Seminal plasma (SP) is the acellular fraction of semen and accounts for 95-98% of the seminal fluid (Vitku et al. 2017). SP is an alkaline biological fluid that is primarily formed by accessory gland secretions (Agrawal et al. 2016). In addition to providing nutrition, protecting sperm from infection, seminal plasma proteins allow sperm cells to resist the biochemical environment of the female reproductive tract (Rodriguez-Martinez et al. 2011). Quantitatively, SP is principally composed of a few high-abundance seminal vesicle proteins (semenogelin, fibronectin), with the ten most abundant proteins representing more than 80% of the total protein content (Drabovich et al. 2014). Nonetheless, seminal plasma contains thousands of proteins originating from the prostate, testis, and epididymis at significant concentrations, making it exceptionally reflective of the entirety of the male reproductive tract and a promising source of markers of male reproductive disorders (Agarwal et al. 2016, Bieniek et al. 2016). In the past few decades, an increasing number of studies have attempted to find biomarkers in seminal plasma that could accurately predict the reproductive potential in men.

IV.1. Gonadotropins and sex hormones:

IV.1.1. Gonadotropins:

Seminal FSH levels are lower than serum FSH and do not correlate with sperm count and fertility status (Fossati et al. 1979, Milbradt et al. 2009). Conversely, LH concentration is reported to be within a similar range or slightly higher in seminal plasma than in blood (Milbradt et al. 2009, Vitku et al. 2017). Seminal LH concentrations could be positively correlated with sperm count and motility (Fossati et al. 1979, Sharma et al. 1992). Human chorionic gonadotropin (hCG) levels are significantly higher in seminal fluid than in serum (Berger et al. 2007). Lower levels of hCG in seminal plasma correlate with decreased sperm concentration and quality (Caroppo et al. 2003). A similar reduction of seminal hCG concentrations in non-obstructive azoospermia and post-vasectomy men can be explained by the fact that hCG is secreted both by the testis and the prostate (Saito et al. 1988). FSH and LH amounts in seminal plasma are not correlated to serum levels and are of limited added value in the diagnosis of male infertility (Vitku et al. 2017).

IV.1.2. Steroids:

The first studies of hormones in semen date back to the late 1970s. However, steroid hormones in seminal plasma have not been investigated as extensively as in blood serum (Vitku et al. 2017, Zuffrey et al. 2018). Typically, sex steroids including testosterone, progesterone, prolactin, and oestradiol are lower in the seminal plasma of men with differing levels of

testicular failure, oligo- and asthenozoospermia, compared to those presenting with normal spermatogenesis (Zhang and Jin, 2010, Vitku et al. 2017). Dihydrotestosterone was proposed as a potential seminal plasma marker to differentiate between OA and NOA (García Díez et al. 1983). Most studies of sex steroids in seminal plasma report an association between qualitatively and quantitatively altered spermatogenesis and higher concentrations of Dehydroepiandrosterone (DHEA), estrogen, estradiol (Vitku et al. 2015), as well as 5α-androstane-3α17β-diol, and progesterone (Vitku et al. 2017). Conversely, impaired spermatogenesis is linked to diminished levels of dihydrotestosterone and androstenedione (Vitku et al. 2015). Overall, no significant differences are seen in seminal testosterone levels between fertile men and those with abnormal semen parameters (Zhang et al. 2010, Zuffrey et al. 2018).

IV.1.3. Anti-Mullerian hormone:

Anti-Mullerian hormone (AMH) is a dimeric glycoprotein secreted in the adult male by Sertoli cells into the seminiferous lumen, which explains its higher concentration in the seminal fluid compared to blood levels (Fujisawa et al. 2002). Given its testis-specific expression, AMH is usually undetectable in OA seminal plasma (Duvilla et al. 2008). Seminal AMH was reported as being positively correlated with sperm count and motility in non-azoospermic fertile and subfertile male subjects when adjusted for other clinical and experimental parameters such as age, BMI, abstinence period, and site of sample collection (Andersen et al. 2016). AMH in semen also correlates positively to the number of spermatozoa in men with sperm counts lower than 15.10-6 /mL (Kucera et al. 2016). However, seminal AMH is a poor predictor of sperm retrieval in NOA cases (Mostafa et al. 2006, Duvilla et al. 2008).

IV.1.4. Inhibin B:

Inhibin is a Sertoli-cell produced dimeric glycoprotein hormone, composed of an 18kDa α-subunit and a 14 kDa βB-subunit (Kumanov et al. 2006, Wang et al. 2017). Inhibin B is a blood biomarker with a considerable predictive value for the differential diagnosis of male infertility, especially when coupled with serum FSH measurements (Kumanov et al. 2006), although wide overlaps with fertile subjects can often be seen (Tsametis et al. 2011). Inhibin B is more abundant in seminal plasma than in serum, and it is measured at higher concentrations in the seminal plasma of fertile men compared to azoospermic men (Duvilla et al. 2008). Seminal Inhibin B levels cannot differentiate between OA and NOA but might be useful in predicting TESE outcome in NOA patients (Nagata et al. 2005, Duvilla et al. 2008).

IV.2. Micro-RNA:

MicroRNAs (miRNAs) are small non-coding single-stranded RNA particles involved in the post-transcriptional regulation of gene expression (Ha and Kim, 2014). MiRNAs can silence specific transcripts by base-binding to complementary sequences of mRNA in the cytoplasm (Barceló et al., 2018). MiRNAs act mainly as intracellular modulators of gene expression but also released in exosomes into extracellular biological fluids, including semen (Hu et al., 2014, Abu Halima et al., 2014). The potential of miRNAs as biomarkers of disease, in particular gynocologic and prostate cancer, has been reported in numerous studies over the last two decades (reviewed in Galvão-Lima et al. 2021). Compared to proteomic biomarkers, mi-RNAs present a number of advantages as diagnostic tools including better accessibility in liquid biopsies and a lower cost of extraction and detection methods (Condrat et al. 2020). The mi-RNA content of seminal exosomes arising from testicular and epididymal cells present in seminal plasma can shed light on the pathophysiological processes taking place in the originating organs (Vojtech et al., 2014, Barceló et al., 2018). Several miRNAs have been proposed as markers for the non-invasive diagnosis of male reproductive disorders. MiR-7-1-3p, miR-141, miR-200a, and miR-429's expression levels in seminal plasma were found to be positively correlated with sperm concentration in normozoospermic, asthenozoospermic, and oligozoospermic men (Mokánszki et al., 2020). MiR-31-5p's expression in seminal plasma can potentially differentiate between OA and NOA, whereas the combined expression values of miR-539-5p and miR-941 can help predict the presence of residual spermatogenesis pockets in cases of severe testicular failure (Barceló et al., 2018). On the other hand, seminal miR-192a was proposed as a marker for the improvement of testicular function in NOA patients after varicocele repair (Zhi et al., 2018). Despite the large number of promising studies, very few mi-RNA biomarkers have reached the clinical trial phase as validation of the initial results is often unsuccessful (Saliminejad et al. 2019). Low reproducibility and high inter-lab/ inter-cohort variability have been attributed to methodological discrepancies (sample processing and storage, data normalization) and the high susceptibility of mi-RNAs to confounding factors such as age, ethnicity, and substance use (Saliminejad et al. 2019, Condrat et al. 2020).

IV.3. Proteomic biomarkers of fertility:

Proteins are the main actors of cellular function. Thus, abnormal protein expression can be an excellent indicator of pathophysiological states (Hedge et al. 2003). Due to the post-transcriptional control of gene expression, mRNA levels do not always correlate to protein abundance. Therefore, proteomics allows for a more accurate evaluation of cell function than

other omics technologies (Twyman, 2005). Proteomic investigation of human tissues and biological fluids produced a wealth of new information and contributed to our understanding of human physiology and pathology (Graves and Haystead, 2002, Horgan and Kenny, 2011). In the last two decades, there has been a remarkable growth in the number of studies centered around the proteomic analysis of seminal plasma, accompanied by an emergence of potential male infertility and prostate cancer biomarkers. The high dynamic range of protein expression in seminal plasma can impede the detection of lower abundance proteins by mass spectrometry, but protein pre-fractionation and enrichment can improve the detection limit substantially (Crutchfield et al. 2016). Proteomic methods, such as two- dimensional separation by gel electrophoresis and identification by both matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) permit the identification of thousands of protein and peptide components in human seminal plasma (Agarwal et al. 2016). In an early study, Milardi and team attempted the characterization of the human seminal plasma proteome in healthy, fertile men and identified 83 proteins using high-resolution MS, a few of which were known to be involved in fertility and reproduction (Milardi et al. 2013). Several studies focused on the seminal plasma proteomics of azoospermia with the goal of discovering markers of differential diagnosis (summarized in Table 2). Some of these proteins could in theory be useful in clinical settings following validation (Milardi et al., 2013, Batruch et al., 2012).

V. Proteomics of testicular Interstitial fluid (TIF):

V.1. Role of TIF in spermatogenesis:

Testicular interstitial fluid is essential for the optimal proliferation and differentiation of spermatogonial cells in mice. Growth factors secreted by interstitial cell types as well as Sertoli cells play important roles in spermatogenesis: Insulin-like growth factors (IGFs) and seminiferous growth factor (SCF) are essential for the proliferation and survival of germ cells in general and SSCs in particular (Wang et al; 2014). Supplementation of mouse SSC culture media with TIF results in an increased proliferation rate, suggesting that TIF contains concentrations of hormones and growth factors that are ideal for the support of the spermatogenic process (Wang et al., 2014). In addition to Leydig cell-secreted testosterone, TIF contains interleukin-alpha (IL-α), transforming growth factors 1 and 2, and SCF/ c-KIT ligand, all of which have established roles in promoting cell proliferation and survival in rodents (Wang et al., 2014, Yoshinaga et al., 1991). In an in vitro study, testicular peritubular myoid cells (PTMCs) were demonstrated to be essential in maintaining the SSCs niche in the human testis

by releasing growth factors that play a role in the vascularisation of the spermatogonial niche. Connective tissue growth factor (CTGF) and vascular endothelial growth factor C (VEGFC) were particularly important. PTMCs also secrete proteins that promote cell adhesion and stem cell migration and renewal, namely C–C motif chemokine 2 (CCL2), stromal cell-derived factor 1 (CXCL12), and laminin A1 (LAMA1) (Flenkenthaler et al., 2014).

V.2. The TIF proteome:

The testicular interactome network encompasses complex interactions between various genes and gene products across different cell types (Sertoli cells, peritubular cells, Germ cells, Leydig cells). These interactions are essential for spermatogenesis but many of the involved molecular pathways remain undiscovered (Rolland et al. 2013, Chalmel et al. 2014, Stanton, 2016). Analysis of the rat and ram seminiferous tubule fluid proteome and transcriptome with the goal of characterizing Sertoli-germ cell interactions in mammalian spermatogenesis identified 177 proteins preferentially expressed in adluminal germ cell and 150 that were preferentially expressed in Sertoli cells (Chalmel et al., 2014). A significant percentage of these proteins were secreted factors (37% of germ cell transcripts vs 40% of Sertoli cell transcripts), confirming the significant extent of cellular crosstalk between the two cell types. Two relevant cell-cell interactions were highlighted and validated by the authors. The first is between germ-cell secreted Apolipoprotein H (APOH) and Sertoli-cell membrane-localized protein CDC42, potentially involved in the modulation of residual body phagocytosis by Sertoli cells during spermiogenesis. The second interaction, which likewise plays a role in the late stages of germ cell development, is between post-meiotic germ cells Nerve Growth Factor Receptor (NGFR) and Amyloid-beta precursor protein (APP), which is expressed only in SCs in the human testis. (Chalmel et al., 2014).

Analysis of the TIF proteome in Sprague-Dawley rats resulted in the identification of 276 proteins of varying abundance, originating from various testicular cell types. Coagulation and acute inflammatory response were the most enriched biological processes within this protein list. Rat TIF contained only 36% of the proteins identified in rat seminiferous tubule fluid (STF), enriched in luminal post-meiotic cell proteins and a higher percentage of proteins typically secreted by basal compartment cells (Spermatogonia, SCs). The comparison between TIF and STF proteomic contents confirmed the directional secretion of testicular cells. Nevertheless, TIF contains several proteins that localize specifically to adluminal germ cells in the rat testis, including Inter-alpha-trypsin inhibitor heavy chain H3 (Itih3), Histidine-rich glycoprotein (Hrg), and Microtubule-associated protein tau (Mapt) (Stanton, 2016). In a

subsequent study, 1728 proteins were identified in rat TIF. Spermatogenic disruption by heat treatment is reflected in the TIF proteome by the downregulation of spermatogenesis-implicated proteins (HSPA2, SPATA20, BAG6) and those needed for the function of the seminiferous epithelium (EB1, TUBA3A), demonstrating that the characterization of TIF proteome can be useful in gauging testicular function and potentially detecting spermatogenic impairment (Aldahan et al., 2019).

Recently a study in mice compared the TIF proteome in normal mice vs those in which Sertoli cells had been acutely ablated (for one week) from the seminiferous epithelium. A total of 3551 proteins were quantified in mouse TIF, and Sertoli cell ablation caused a significant reduction in 41% of the proteins identified in TIF, indicating the considerable contribution of Sertoli cells to TIF proteome. Also, 141 of the proteins showing major decreases after Sertoli cell ablation were determined to be likely of post-meiotic germ cell origin. The analysis of human TIF from 3 fertile men by high-resolution mass spectrometry identified 4720 proteins. Using transcriptomic and proteomic databases, 33 proteins were determined to be enriched or specifically expressed in advanced germ cells (pachytene spermatocytes, round and elongating spermatids). These include proteins with well-investigated roles in sperm motility and fertilization and putative markers of spermatogenesis (AKAP4, GAPDHS, ACR, CLGN) (O'Donnell et al., 2021a). Thus, human TIF contains proteins indicative of testicular somatic cell (including Leydig and Sertoli cell) function as well as proteins that likely arise from germ cells within the seminiferous tubules.

Table 2: Comparative proteomic analyses of seminal plasma and identification of potential biomarkers of Azoospermia

Study reference	Diagnosis	Potential Biomarkers identified	Proteomics method
Yamakawa et al. (2007)	Azoospermia	STAB2, CP135, GNRP, PIP	Nanoscale capillary LC-MS/MS
Heshmat et al. (2008)	Azoospermia	L-PGDS	ELIZA
Drabovich et al. (2011)	Azoospermia	LDHC, MUC15, SPAG11B, TEX101	MALDI-TOF MS/MS
Batruch et al. (2012)	Azoospermia	ELSPBP1; COL6A2; LDHC; PGK2	Strong cation exchange chromatography-LTQ-Orbitrap MS/MS
Drabovich et al. (2013)	Azoospermia (Obstructive vs non-obstructive)	TEX101, ECM1	Selected Reaction Monitoring MS/MS
Freour et al. (2013)	Azoospermia	LGALS3BP	1D-NanoLC-MS/MS
Korbakis et al. (2015)	Azoospermia	TEX101	Immunocapture-SRM
Cui et al. (2018)	Non-obstructive Azoospermia	HSPA4L and HSPA4 HSP90AB1, HSPA1B, HSP90AA1	LC- MS

Aims:

Despite significant advances in our understanding of the aetiology of male infertility, there are still significant gaps in our knowledge of the underlying causes. The clinical management of azoospermia usually involves invasive procedures (testicular biopsy, testicular sperm extraction) with uncertain outcomes. The constant progress of high-throughput omics technologies allows for deeper, more exhaustive characterization of biological samples. Proteomic analysis of seminal plasma and testicular interstitial fluid from men diagnosed with obstructive azoospermia and with different types of testicular failure would not only offer new insights into the molecular mechanisms involved in these forms of infertility but could also lead to the discovery of biomarkers to assist with the differential diagnosis of azoospermia and better inform clinical decisions. The aims of this study were:

- The discovery of proteomic biomarkers for the differential diagnosis of azoospermia in seminal plasma
- The characterization of the proteome of normal human testicular interstitial fluid
- Uncovering markers of testicular function in testicular interstitial fluid
- The identification of promising seminal plasma and TIF biomarkers of successful sperm retrieval in non-obstructive azoospermia

Ethics statement:

This study was approved by the appropriate ethics committee (Ethik-Kommission am FB 11 “Humanmedizin”, Justus-Liebig-Universität Giessen; Ref. No. 26/11). All human procedures performed were in accordance with the principles of Good Clinical Practice. All patients provided written consent for testicular surgery and for the collection and use of samples for research purposes.

Patient selection criteria:

Patients with azoospermia were subjected to an exhaustive clinical examination, including hormonal profiling and medical history. Histological scoring of testicular tissues obtained during microsurgical testicular sperm extraction (m-TESE) was used to determine the aetiology of azoospermia. Collected testicular tissue specimens were immediately fixed in Bouin’s solution pending histological evaluation. Histological scoring was performed according to Bergmann and Kliesch (2010): the number of tubules containing elongated spermatids is divided by the total number of tubules examined x 10. For the OA/ normal spermatogenesis group, patients with a score range between 8 and 10. Patients diagnosed with Congenital bilateral absence of the vas deferens (CBAVD) were excluded. For the SCO and MA groups, cases with karyotypic anomalies such as Kleinefelter syndrome, history of testicular cancer or varicocele were disregarded. Also excluded were subjects suffering from comorbidities such as diabetes mellitus and cancer. Clinical data, including hormonal profiles, are contained in supplementary table 1. Histological scores corresponding to the samples used in the various proteomic analyses are listed in supplementary table 2.

Chapter 1:

Study of the seminal plasma proteome: discovery of azoospermia biomarkers

I. Experimental design:

In this study, we sought to identify seminal plasma biomarkers for use in the non-invasive differential diagnosis of azoospermia. Using label-free LC-MS/MS, we compared proteomic profiles of seminal plasma from fertile men (healthy controls, HC) and men diagnosed with three different forms of azoospermia: obstructive (OA), mixed atrophy (MA), and Sertoli cell-only syndrome (SCO) (all groups n=8) (Fig. 1). Antibody-based assays (Western Blot, immunohistochemistry) were used to validate the mass spectrometry results for a select number of proteomic markers.

II- Materials and Methods:

II.1. Mass spectrometry analysis of seminal plasma:

II.1.1. Sample preparation:

Semen samples were collected by the study participants after an abstinence period of 48 to 72h and left to liquefy at RT for 1-2h. After liquefaction, semen samples were centrifuged for 10 min at 13000 g. The supernatants were subsequently aliquoted in 2 mL polypropylene vials and kept at -80°C pending analysis. Total protein content was estimated using the Pierce BCA Protein Assay (Cat. # PI23227). Seminal plasma aliquots were thawed on ice and diluted in MilliQ water. From each sample, a volume equivalent to 30 µg of total protein content was pipetted into a numbered tube. 12 µl of SDS lysis buffer (5% SDS/50 mM Tris-HCl, pH 7.5/50 mM Tris(2-carboxyethyl) phosphine-hydrochloride (pH 7), /200 mM 2-chloroacetamide) was added to each diluted sample. Samples were then boiled for 3 min at 95°C and left to cool at RT for 5 min. Carboxy agarose beads were pre-washed twice in 500 µl of MilliQ H2O and then reconstituted in an equal volume of water. 20 µl of the Carboxy agarose beads solution was pipetted into each tube, and neat acetonitrile added resulting in a final 70% v/v, then incubated at RT in a ThermoMixer C (Eppendorf) at 400 rpm for 20 min. This step allows the proteins

and peptides in the sample to bind to the surface of the beads. Next, the samples were placed on a magnetic rack for 2 min causing the protein-covered beads to adhere to the bottom of the wells/tubes. The beads were washed twice with 200 µl of 70% Ethanol and once with 200 µl of neat acetonitrile.

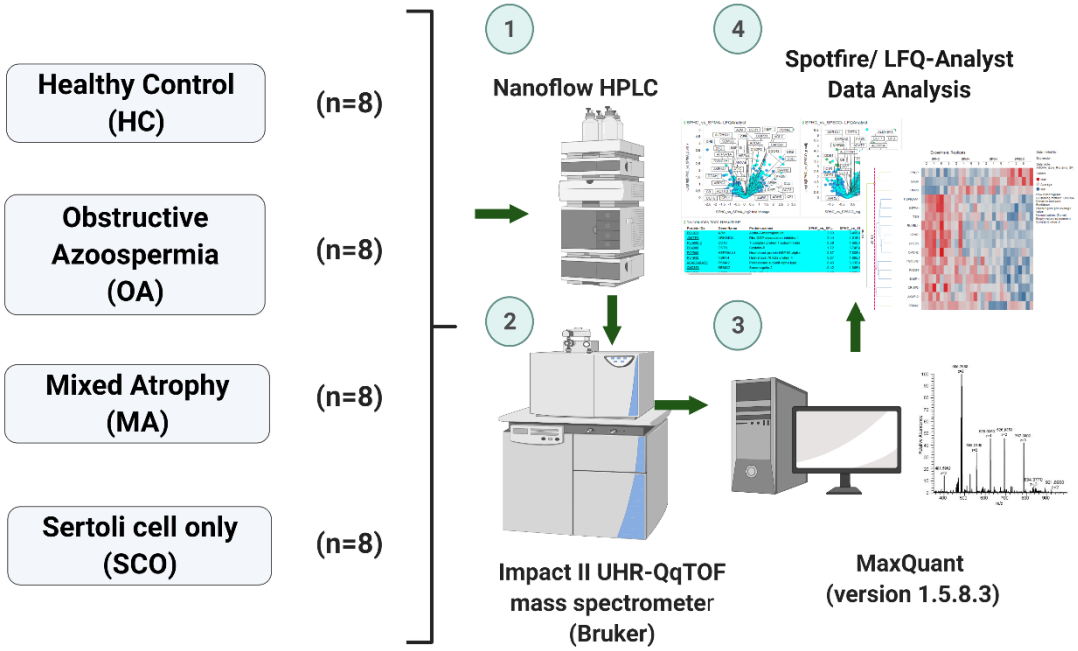


Figure 1: LC-MS/MS analysis of seminal plasma; Samples were separated by reverse phase high performance liquid chromatography (HPLC), paired with an Impact II ultra-high resolution Qq Time Of Flight Mass Spectrometer (Bruker). Peak scoring and protein identification were performed using the MaxQuant suite (version 1.5.8.3). Proteomic data was analysed using the LFQ-Analyst platform (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/).

After lyophilization to ensure the complete removal of all traces of acetonitrile, 50 µl of a digestion buffer containing Trypsin and Lysine C was added to each sample and the mixture was sonicated in an Ultrasonic bath for 2 min before incubation in ThermoMixer C for 1 hour at 37°C, shaking at 400 rpm. After the end of the digestion step, the samples were again placed on the magnetic rack and 50 µl of MilliQ water was added to each well. The samples were sonicated for 1 min to detach the peptides from the beads, and the supernatants were collected. 5µl of each supernatant was transferred into a LoBind Eppendorf tube to form four pools for the different groups (OA, MA, SCO, SA). C18 stage tips were prepared by adding two plugs of 3M Empore resin (#2215) to 200 µl plastic pipette tips. To pre-equilibrate, 20 µl of Isopropanol was added and the tips were centrifuged for 2 min at 1000 x g. Next, 50 µl of the following buffers (80% Acetonitrile/5% formic acid, 50% Acetonitrile/5% formic acid, 5% formic acid

in MQ water) were added in order to the tips before spinning at 1000 x g for 2-3 min each time, ensuring that a small volume remains on top of the resin and discarding the flowthrough. The individual peptide samples were loaded into the pre-equilibrated tips and centrifuged at 800 x g for 2 min until only a small volume remained on top of the resin plug. After two washes with 5% formic acid, the tips were placed into Lobind peptide tubes, and the peptides were eluted with 50 µl of 50% Acetonitrile/5% formic acid followed by 50 µl of 80% Acetonitrile/5% formic acid for 1-2 min at 800 x g. The eluate was transferred into mass spectrometry vials, frozen at 80°C then lyophilized down to dryness in preparation for the analysis.

II.1.2. Mass spectrometry analysis:

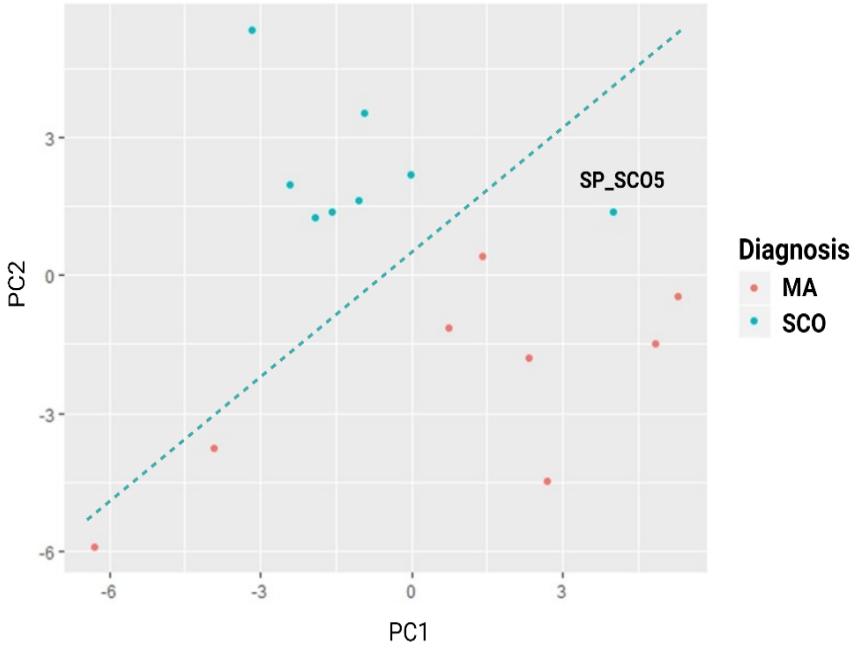
The resulting peptides were separated through a C18-fused silica column (O.D. 75µm) packed into an emitter tip (IonOpticks, Middle Camberwell, VIC, Australia) by reverse-phase chromatography using a nano-flow HPLC (M-class; Waters, Wilmslow, UK). The peptide mixture was loaded directly onto the column at a constant flow rate of 400 nl/min with 0.1% formic acid in Milli-Q water and eluted with a 99.9%acetonitrile and 0.1% formic acid linear-gradient (from 2 to 34%, 90 min). The chromatography setup was connected to an Impact II UHR-QqTOF mass spectrometer (Bruker, Billerica, MA, USA) via a CaptiveSpray source and nanoBooster at 0.20 Bar using acetonitrile. Data-dependent acquisition of mass spectra occurred by switching between MS and MS/MS scans automatically using a 4 Hz rate for MS1 spectra scans and 8–20 Hz for MS/MS scans according to precursor intensity. The set mass range for spectra acquisition was 200–2000m/z. Peptide fragmentation was achieved by collision-induced dissociation. Protein identification was accomplished by analysing the raw files of MS/MS spectra in MaxQuant (version 1.5.8.3) using the Andromeda search engine. The derived peaks were matched with the reviewed homo sapiens database (UniProt, July 2019). To determine the false discovery rate (FDR), the peak lists were compared to a reverse decoy database, using strict trypsin specificity and allowing up to two missed cleavages.

II.2. Data analysis and Data base research:

Proteomic data was analysed using the LFQ-Analyst platform (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/). To decrease the false discovery rate in group comparisons, p-values were corrected using the Benjamini-Hochberg procedure. p-values< 0.05 were considered significantly differentially expressed. Gene names of the identified proteins were matched to a list of 2237 testis-enriched proteins and 94 epididymis-enriched proteins downloaded from the Human Protein Atlas database.

II.3. Principal components analysis:

New developments in statistical methods for mass spectrometry data analysis were introduced by our colleagues in the mass spectrometry laboratory at the WEHI in March 2019 during the course of the current study. These developments were retrospectively applied to the raw data for more precise relative quantification of proteins and more accurate protein ratios and has resulted in a reduction in the level of significance observed for most group comparisons. To address potential reasons for the loss of significant proteins, a principal components analysis of the mass spectrometry data was conducted and revealed two main patient-based outliers. In the first comparison: SCO vs MA (n = 8 patients/group), SCO patient sample #5 clustered with the MA group (Fig. 2). It is not uncommon for patients with severe hypospermatogenesis to be diagnosed with SCO given the heterogeneity of the testicular phenotype. In the second comparison: SCO vs OA sample SP_OA4 showed a proteomic profile distinct to the rest of the samples within the group (Fig. 3). A subsequent examination of the mass spectrometry-abundance (iBAQ) values of individual samples revealed higher levels of epididymis-enriched proteins in OA4 compared to the other samples in the group (Fig. 4). This result indicates that the obstruction site for this patient in particular might be at the level of the rete testis, contrary to the rest of the OA men, for which the drop in the relative abundance of epididymal markers suggests a vas deferens obstruction. In summary, use of an unbiased PCA approach based on molecular data now shows that the existing clinical samples are likely heterogeneous, and provide a rationale for re-analysis of the patient grouping at the mass spec level. Re-analysis of the data at the mass spectrometry level, disregarding SP_OA4 and SP_SCO5 was subsequently performed, and the results were used for biomarker selection.



Sample ID	PC1	PC2
SP_SCO1	-1.04932421	1.614331
SP_SCO2	-3.16638328	5.33644186
SP_SCO3	-1.90373968	1.24334698
SP_SCO4	-2.42493478	1.97033376
SP_SCO5	**3.99422698**	**1.37364163**
SP_SCO6	-0.93422644	3.52536359
SP_SCO7	-0.01261709	2.19168358
SP_SCO8	-1.58273969	1.38091139
SP_MA1	5.29020086	-0.45287024
SP_MA2	0.7364847	-1.14671673
SP_MA3	2.7024031	-4.47480959
SP_MA4	-6.30497649	-5.90579601
SP_MA5	4.82991498	-1.49106376
SP_MA6	1.40199876	0.40883032
SP_MA7	-3.91416826	-3.76075117
SP_MA8	2.33788056	-1.81287663

Figure 2: Principal components analysis of iBAQ data from the SCO (n=8) and MA (n=8) groups. Principal components were extracted using the 'prcomp' function in RStudio (version 1.2.1335-1), PC1 and PC2 were represented using the ggplot2 package.

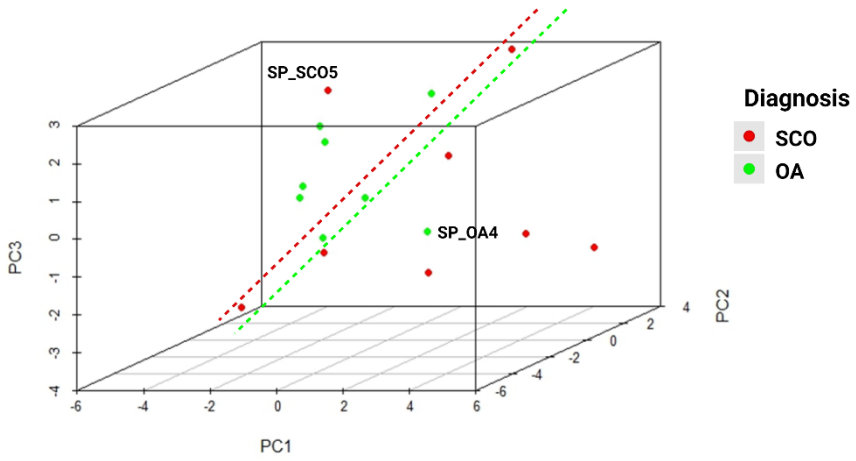


Figure 3: Principal components analysis of iBAQ data from the SCO (n=8) and OA (n=8) groups. Principal components were extracted using the 'prcomp' function in RStudio (version 1.2.1335-1), and the 3D scatterplot was rendered using the 'rgl' package.

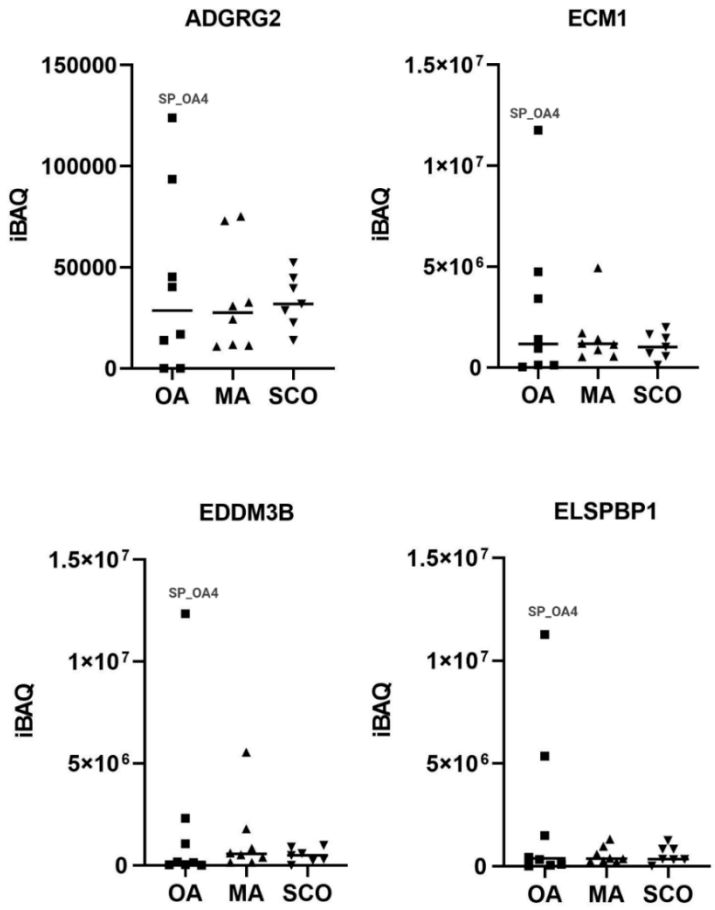


Figure 4: iBAQ (intensity based absolute quantification values) of four epididymis enriched proteins: ADGRG2, ECM1, EDDM3B and ELSPBP1 in the seminal plasma of OA, MA and SCO men, showing SP_OA4 as a clear outlier. Bar represents the median

II.4. Biomarker selection:

Mass spectrometry data were analysed to identify differences in proteomic profiles between groups and identify candidate markers for the differential diagnosis of azoospermia. Two sets of potential markers were selected: the first, designated as "markers of obstruction", was composed of epididymal and testicular proteins significantly less abundant in the seminal plasma of men diagnosed with obstructive azoospermia (OA) compared to the two non-obstructive azoospermia (NOA) groups (mixed atrophy (MA) and Sertoli cell-only (SCO) as well as the control group; the second included proteins that were, according to mass spectrometry data, significantly higher in seminal plasma from fertile men and the mixed atrophy group compared to the pure SCO group. Some of these proteins are ubiquitously expressed but were selected as candidate biomarkers of successful sperm retrieval based on i)

mass spectrometry data, ii) higher abundance in seminiferous tubules compared to the rest of male tissues, and iii) links to spermatogenic signalling pathways.

II.5. Q-PCR:

Testis tissue samples were collected during TESE procedures on azoospermic patients (as described in "Patient selection criteria", p. 33). Epididymis tissue was obtained from orchiectomies performed on testicular cancer patients. Histological sections were microscopically examined to verify the phenotype of testicular tissue samples and the absence of abnormal/ cancerous cells in epididymal samples.

II. 5.1. RNA extraction and treatment:

- ***RNA extraction using the PEQGOLD Total RNA isolation protocol***

Tissue samples were taken from liquid nitrogen, cut with a blade on a sterile Petri dish, and homogenized mechanically in 500 µl of RNA lysis buffer. The lysate was then transferred into a DNA removing column placed in a 2.0 mL column and centrifuged at 12.000 x g for 1 minute at room temperature. The flow-through was transferred into a 1.5 ml tube, 600 µl of 70% Ethanol (in sterile RNAse free water) was added. The solution was vortexed vigorously. A PerfectBind RNA column was placed in a new 2.0 ml collection tube, and the lysate was added directly onto the membrane. The column-collection tube was centrifuged at 10.000 x g for 1 minute. The collection tube and flow-through were subsequently discarded. For the first wash, the PerfectBind RNA column was placed in a clean collection tube. 500 µl of RNA wash buffer was added to the column, followed by a 15s centrifugation at 10.000 x g. The flow-through was discarded but the collection tube was re-used for the following step. DNase digestion was performed by pipetting the DNase mix (73.5 µl of DNase digestion buffer + 1.5 µl RNase-free DNase I) onto the column surface and incubating the tubes at RT for 15 minutes. The PerfectBind column was then placed into a fresh collection tube and 400 µl of RNA wash buffer I. was added; the columns were incubated on the benchtop for 5 min and then centrifuged at 10.000 x g for 15 s. The flow-through was discarded and the collection tube was re-used for the next step. Second wash: 600 µl of RNA wash buffer II was added to the column and centrifuged at 10.000 x g for 15s. The flow-through was discarded and the wash step was repeated. Drying the column (important step): the columns were centrifuged uncovered in empty collection tubes at 10.000 x g for 2 min prior to RNA elution: The PerfectBind RNA columns were put into fresh 1.5 mL microcentrifuge tubes and 30 µl of RNase-free dH2O was added directly to the matrix and the tubes were centrifuged at 5000 x g for 1 min. The step was repeated for a total volume of 60 µl of eluted RNA. The RNA samples were stored at -80°C.

- ***DNase treatment:***

To eliminate remaining traces of DNA, 3.25 µl of DNase mix (2 µl of DNase buffer, 1 µl of DNase I, RNA free, 0.25 µl of RNase inhibitor) were added to 6.65 µl of RNA for a total volume of 9.9 µl/ vial. The tubes were briefly centrifuged to mix then put into the thermocycler:

Program: DNase Roche	
37°C; 10 min;	DNase I activity
75 °C; 5 min;	Arrest of enzymatic digestion
4°C, forever	Cooling down

II. 5.2. Q-RT-PCR:

- ***Reverse transcription:***

To each 9 µl of DNase treated RNA solution were added 12 µl of MgCl2 (catalyzer), 6 µl of PCR buffer, 6 µl of dNTP mix, 3 µl of random hexamer solution, 3 µl of RNase inhibitor, 18 µl of RNase free water, and 3µl of MultiSkribe RT (retro-transcriptase) (Invitrogen, Cat. No: 4311235). The tubes were capped tightly and placed in the thermocycler.

PCR program: RT-gold	
10 min; 21°C	Annealing of random hexamer primers
15 min; 42°C	Reverse transcription
5 min; 99°C	Reaction stop
5 min; 5°C	Cooling down
Forever; 4°C	Storage

- ***Checking DNA quality:***

To 1µl of cDNA were added 11.5 µl of primer mix, containing 0.5 µl of MgCl2 (catalyzer), 1 µl of PCR buffer, 0.25 µl of dNTP mix, 0.25 µl of forward-primer, 0.25 µl of reverse-primer (Housekeeping gene RPL19), 9.175 µl of RNase free water, and 0.075 µl of Gold Amplitaq (DNA polymerase). The tubes were incubated in the thermocycler for 36 PCR cycles (9 min at 95°C, 45s at 94°C, 45s at 60°C, 45s at 72°C, 7 min at 72°C), followed by cooling at 4°C.

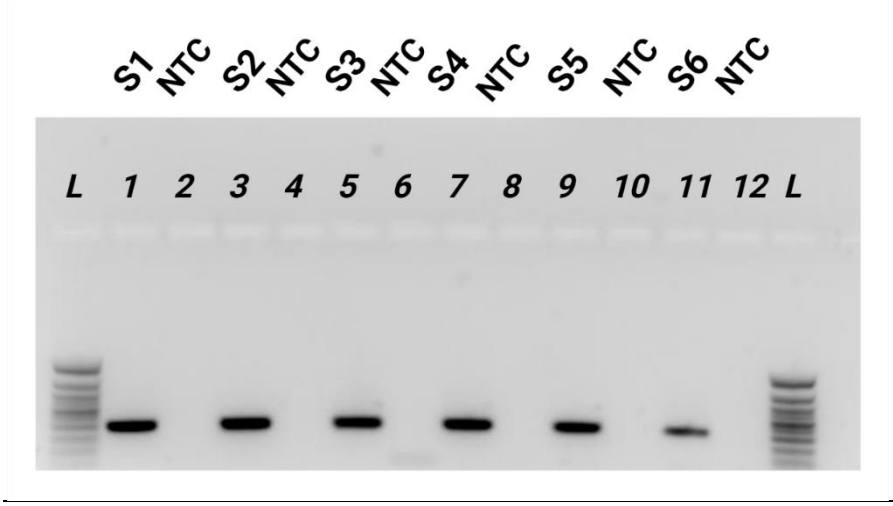


Figure 5: Checking cDNA quality: PCR products for cDNA samples (S1-S6 using the GeneAmp Gold RNA PCR Core Kit 4308207, PE Biosystems and RPL19 primer mix. 2.5 µl of loading dye were added to each sample before loading onto a 1.5% agarose gel and resolving at 110V for 70 min. Into the 'L' lane, 5µl of GeneRuler 50 bp DNA Ladder (Thermofisher) were loaded), NTC: No Template Control.

- ***Primer testing:***

The same primer mix used for controlling DNA quality was used for running a 36 cycle PCR, with the addition of no-template controls (NTCs).

Validated primers for epididymal genes:	
Primer	**Sequence**
hu_DEFB129_139bp_for	CAAGAAATGCTAAAACCTGCCA
hu_DEFB129_139bp_rev	TCATAGGGGTGGCATTTGGAA
hu_EL149_200bp_for	GGGCCAAAGCCATTCAACCA
hu_EL149_200bp_rev	TGCATCCCTCCACTTGACTC
hu_ADGRG2_113bp_for	GGAGATGTGTGCCTTCACGA
hu_ADGRG2_113bp_rev	CTTCCCCGCTTTGAAGTCCT

Validated primers for testis-enhanced genes	
Primer	**Sequence**
hu_DNAH2_229bp_for	AAAGTACGAGGTGCCAGTCG
hu_DNAH2_229bp_rev	GTATCCCACGTTGCTGGTGA
hu_SLC52A3_106bp_for	CATATACCACTGCCCCGCTG
hu_SLC52A3_106bp_rev	CCATTCCGAAGACGCAGACC
hu_CSE1L_167bp_for	AGCGATACCTGCCTCGTTTT
hu_CSE1L_167bp_rev	ACTTGTCAGCGTGTTCTGGT
hu_PFKP_125bp_for	ACCAACCTGTGTGTGATCGG
hu_PFKP_125bp_rev	TTCTGCACGGCCTCCTTATC

- ***Real-time PCR:***

Onto a clear Multiplate™ Low-Profile Unskirted 96-Well PCR Plate (Biorad), 1 µl of cDNA was pipetted in each well and 19 µl of the primer mix (containing 13.8 µl of RNase free water, 4 µl of SsoFast EvaGreen Supermix (Biorad; Cat. N: 1725200), 0.6 µl of the forward primer and 0.6 µl of the reverse primer of the selected gene. The plate was subsequently placed into a CFX Connect Real-Time PCR System (Biorad) and the Evagreen protocol (40 cycles: 95°C for 15 s, 60°C for 30 s; 72°C for 20s) was selected.

II.6. Western Blot:

The relative abundance of three proteins (LDHC, HSPA2, and ELSPBP1) selected from the mass spectrometry data was investigated by immunoblotting. Seminal plasma samples were thawed on ice and vortexed lightly before dilution using Bolt LDS Sample Buffer (1X) (Life Technologies, B0007) to the desired concentration with a final volume of 12µl/tube. 14 µl of the Sample buffer master mix (1:2.5 v/v Bolt Reducing Agent (10X) ((Life Technologies, B0009), Bolt LDS Sample Buffer (4X)) was then added. The samples were then heated at 70°C for 10 min and loaded onto a 15-well Bolt 4-12% Bis-Tris Plus Gel (Invitrogen/Thermo Fisher Scientific, NW04125BOX). In wells 1 and 15, 8µl of the SeeBlue Plus2 Pre-Stained Protein Standard (Life Technologies, #LC5925) were pipetted. The electrophoresis chamber was filled with 1X Bolt MES SDS Running Buffer (Life Technologies, B0002) and samples were separated at 100V for 60 min at room temperature. After the end of the run, the gel cassette was opened, and the wells were trimmed. The gel was washed twice for 5 min in Transfer Buffer (0.45% Tris base, 2.2% Glycine, 20% Methanol) and a PVDF membrane (Immobilon-P 0.45 µm, Merck, IPVH00010) was soaked in Methanol for 15 min prior to assembling the gel holder cassette. A Mini Trans-Blot® Cell (BIORAD, 1703930) was filled with the Transfer Buffer and the transfer apparatus was inserted into the electrode assembly module. The proteins were transferred from the gel onto the membrane overnight (16-17h) at 30V at 4°C. The membrane was then gently removed and incubated for 1 hour at room temperature in Blocking Buffer (1%bSA/TBS,0.05% Tween), after which it was probed with anti-LDHC, anti-HSPA2 and anti-ELSPBP1 polyclonal antibodies produced in Rabbit (Invitrogen, # PA5-30079 and # PA5-29083 and Sigma-Aldrich #HPA044256, diluted 1:5000, 1:5000 and 1:2500, respectively). After 3 washes of 10 min each with Washing Buffer (TBS/0.05% Tween), the membrane was incubated in 1:10,000 Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody-HRP conjugate (Invitrogen, Cat. # G-21234) in 5% skim milk powder, PBS 0.1% Tween for 1 hour

at RT. The membrane was incubated on a shaker 4 x 10 min in Washing Buffer. Bands were developed by incubating the membrane for 45s with 2 mL Lumi-LightPLUS Western Blotting Substrate (Merck,12015196001) and visualized in a ChemiDoc XRS+ imaging system. Image Lab 6.0 (BIO-RAD) was used to acquire blot images and quantify protein bands. Statistical significance was determined using the Mann-Whitney test (GraphPad Prism 8).

II. 7. Immunohistochemistry:

Histological sections were prepared from formalin-fixed, paraffin-embedded human testicular tissue obtained after micro-TESE procedures. Tissue sections were deparaffinized by two consecutive 10-minute incubations in xylene. They were then hydrated in a series of ethanol baths (100%, 95%, 80%, 70% EtOH) for 5 min each, followed by a 5-min incubation in distilled water. For antigen retrieval, the slides were placed in sodium citrate buffer (10 mM $Na_3C_6HO_7$, 0.05% Tween 20, pH 6.0) and brought to boiling temperature in a microwave for 20 min. 3% BSA (Thermo Scientific, Cat# 134731000) in PBST (0.05% Tween 20) was used for blocking (1h, RT). Antibodies directed against LDHC (Invitrogen # PA5-30079), HSPA2 (Invitrogen, # PA5-29083), and HSPA4L (Atlas Antibodies, #HPA039149) were diluted 1:300, 1:500 and 1:400, respectively. Probed sections were incubated with a biotinylated Goat anti-rabbit IgG (Vector Laboratories, #BA-1000), diluted 1:200 in 10% Goat serum in PBS. For detection, the VECTASTAIN® Elite ABC-HRP Kit, Peroxidase (Vector Laboratories, PK-6100) was used. DAB chromogen (DAKO, K3468) was added as an HRP substrate and colour development was monitored under a light microscope. Slides were imaged on an Olympus model BX41 fluorescence microscope. Photos were captured with the coupled Olympus DP-12 microscope camera and processed using the CellSens imaging software (Olympus Life Sciences).

III. Results:

- ***Protein identification by mass spectrometry*:**

Seminal plasma samples from fertile men (HC, control) and men diagnosed with OA, MA and SCO (n=8/ group) were analysed by label-free LC-MS/MS. One sample from the OA group (SP_OA4) and one sample from the SCO group (SP_SCO5) were excluded from the final data analysis for ambiguous diagnosis (see II.3. Principal components analysis). In total, 917 proteins were identified across the remaining samples. Given the stringent statistical analysis used to estimate protein ratios, proteins with adjusted p-values < 0.05 and fold changes > 1.5 were considered significant.

The control/SCO comparison showed a significant change in the abundance of a total of 42 proteins. Fold changes and p-values corresponding to these 42 proteins are contained in table 1, and their relative abundances (iBAQ values) in all samples are presented in Fig1. A. Of these, 24 proteins were upregulated more than 2-fold and 8 proteins (ANGPT1, TIMP3, ATPGAP1, CNTN3, SPR, SEMG2, HS6ST1, and SERPINA5) were down-regulated more than 2-fold in the control compared to the SCO group. Twelve proteins were found to be significantly altered in the OA group relative to control (listed in Table 2), and the majority (11/12) were more than 2-fold higher in the control compared to OA. In the HC/MA comparison, LDHC and TIMP3 were the only proteins to exhibit a significant difference in abundance (FC HC/MA= 10.63 and 0.12, respectively). No statistically significant protein ratios were uncovered in the comparison between the three azoospermia groups, althIhr jetziger Vermieter:ough several proteins appear to be similarly decreased in SCO and OA compared to the control, namely LDHC, A2M, DPEP3, HSPA2, HSPA4 and HSP90AA1. These changes in seminal protein abundance could be interpreted as a direct result of altered gene expression in SCO but not in OA, given the self-evident impact of vas deferens obstruction on the composition of the seminal fluid.

Table 1: Proteins with significantly different abundances in Sertoli cell only (SCO, n=7) seminal plasma relative to fertile controls (HC, n=8), arranged by decreasing order of fold change (HC/SCO). Fold changes are estimated based on spectral counts from mass spectrometry; p-values were corrected using the Benjamin-Hochberg procedure.

Protein IDs	Gene Name	Protein Names	Fold change HC/ SCO	Adjusted p-value HC vs SCO
P07864	LDHC	L-lactate dehydrogenase C chain	25.11	9.89E-05
Q9H4B8	DPEP3	Dipeptidase 3	11.00	0.0135
P01023	A2M	Alpha-2-macroglobulin	9.92	0.0217
E9PGT1	TSN	Translin	7.41	0.00144
P54652	HSPA2	Heat shock-related 70 kDa protein 2	7.31	0.0217
P07205	PGK2	Phosphoglycerate kinase 2	5.78	0.0143
P04080	CSTB	Cystatin-B	4.96	0.0322
P16562	CRISP2	Cysteine-rich secretory protein 2	4.08	0.0042
Q9Y266	NUDC	Nuclear migration protein nudC	4.08	0.0138
P34932	HSPA4	Heat shock 70 kDa protein 4	3.89	9.89E-05
P15374	UCHL3	Ubiquitin carboxyl-terminal hydrolase	3.78	0.0233
A0A087X1V8	OVCH2	Ovochymase-2	3.71	0.00731
P50990-2	CCT8	T-complex protein 1 subunit theta	3.63	0.0135

Q9NZJ9	NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 4	3.63	0.0217
O95757	HSPA4L	Heat shock 70 kDa protein 4L	3.25	0.0217
B3GLJ2	PATE3	Prostate and testis expressed protein 3	3.18	0.011
Q99832	CCT7	T-complex protein 1 subunit eta	3.05	0.0418
Q9Y265	RUVBL1	RuvB-like 1	3.05	0.0119
Q7L266	ASRGL1	Isoaspartyl peptidase/L-asparaginase	2.95	0.0425
Q02952-3	AKAP12	A-kinase anchor protein 12	2.55	0.00731
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	2.50	0.0119
F8VY35	NAP1L1	Nucleosome assembly protein 1-like 1	2.45	0.0229
P37802	TAGLN2	Transgelin-2	2.35	0.0299
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	2.31	0.00731
J3KQ45	TGOLN2	Trans-Golgi network integral membrane protein 2	2.19	0.0138
P80723	BASP1	Brain acid soluble protein 1	2.04	0.0217
P08238	HSP90AB1	Heat shock protein HSP 90-beta	1.81	0.0217
H0Y7A7	CALM2	Calmodulin	1.58	0.0425
P42785	PRCP	Lysosomal Pro-X carboxypeptidase	0.65	0.0217
G5E9W0	PLA1A	Phospholipase A1 member A	0.55	0.0372
Q9GZN4	PRSS22	Brain-specific serine protease 4	0.54	0.0322
A0A0G2JNJ8	MUC6	Mucin-6	0.54	0.0285
Q14393-2	GAS6	Growth arrest-specific protein 6	0.51	0.00731
P02766	TTR	Transthyretin	0.51	0.0425
P05154	SERPINA5	Plasma serine protease inhibitor	0.49	0.0264
O60243	HS6ST1	Heparan sulfate 6-O-sulfotransferase 1	0.40	0.0299
Q02383	SEMG2	Semenogelin-2	0.39	0.0217
P35270	SPR	Sepiapterin reductase	0.39	0.0326
Q9P232	CNTN3	Contactin-3	0.28	0.0217
Q15904	ATP6AP1	V-type proton ATPase subunit S1	0.23	0.03
P35625	TIMP3	Metalloproteinase inhibitor 3	0.23	0.0403
Q15389-2	ANGPT1	Angiopoietin-1	0.19	0.0299

Table 2: Proteins with significantly different abundances in obstructive azoospermia (OA) relative to the control (HC) seminal plasma. Fold changes are estimated based on spectral counts; p-values were corrected using the Benjamin-Hochberg procedure.

Protein IDs	Gene Name	Protein Names	Fold change HC/OA	Adjusted p-value HC vs OA
P07864	LDHC	L-lactate dehydrogenase C chain	21.11	0.000294
P01023	A2M	Alpha-2-macroglobulin	12.21	0.0256
P41222	PTGDS	Prostaglandin-H2 D-isomerase	11.71	0.0412
J3KTF8	ARHGDIA	Rho GDP-dissociation inhibitor 1	10.34	0.0256
Q9H4B8	DPEP3	Dipeptidase 3	9.51	0.0361
P54652	HSPA2	Heat shock-related 70 kDa protein 2	8.94	0.0256
P25787	PSMA2	Proteasome subunit alpha type-2	6.73	0.000606
E7ER45	MGAM	Maltase-glucoamylase	6.32	0.0256
P78371	CCT2	T-complex protein 1 subunit beta	2.73	0.0256
P34932	HSPA4	Heat shock 70 kDa protein 4	2.48	0.0256
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	2.04	0.0362
P35625	TIMP3	Metalloproteinase inhibitor 3	0.19	0.0362

- ***Tissue specificity of differentially expressed proteins:***

The Human Protein Atlas (HPA) tissue database (proteinatlas.org) was queried to determine the most likely cellular origin of significant seminal plasma proteins, with this comparison based on mRNA expression patterns. From a total of 26 proteins of significantly higher abundance in the control compared to SCO seminal plasma (FC(HC/SCO)>= 1.5), Table 1), 5 were enriched in the testis (LDHC, DPEP3, PGK2, DPEP3 and HSPA4L). A further two proteins, HSPA2 and AKAP12 are classified as spermatocyte-enhanced in the HPA database. RUVBL1, HSPA4, and TSN are deemed to be proteins of low tissue-specificity, but their highest levels of expression (TPM, Transcript Per Million) appear to be in the seminiferous tubules (HPA Tissue Atlas). CCT2 and A2M are expressed throughout most human tissues but are cell-type enhanced in early spermatids and spermatocytes, respectively. Tissue and cell expression patterns for 32 proteins significantly elevated levels in HC compared to SCO, OA, and or MA are summarized in Table 3. A considerable number of these proteins are reported to be mainly or exclusively expressed in male reproductive tissues. Several proteins, including ASRGL1, CRISP2, HSPA4L, LDHC, and HSPA2 exhibit enhanced levels of expression in post-meiotic germ cells. Notably, proteins of testicular origin (LDHC, DPEP3, HSPA2) are undetected in both OA and SCO, whereas epididymis-enriched OVCH2, and epididymis-enhanced BASP1 are down-regulated in SCO and undetected in OA.

- ***Distribution of iBAQ values: intra-group variability in MA***

Variability of relative protein abundances in the MA group is noticeably higher than in OA and SCO (Fig.1. A). For testis-enriched LDHC, DPEP3, HSPA4L and PGK2, single outlier values appear to be the only difference between MA and the other two azoospermia groups (SCO, OA) (Fig.1. B). Despite showing relatively higher abundances of a number of proteins compared to the rest of the samples in the group (Fig. 1. A), SP_MA8 was not removed from the analysis. Apart from CRISP2, the proteins present at higher concentrations in SP_MA8 are either of low tissue specificity (CALM2, GAS6, HSPA90AB), are enhanced/ enriched in the epididymis or accessory glands (BASP1, SEMG2), or are highly expressed by somatic testicular cells (SERPINA5, TIMP3). For most germ-cell specific, spermatogenesis-essential gene products, abundance values in SP_MA8 are comparable to those observed in other mixed atrophy samples. Contrarily, SP_MA3 shows slightly higher abundances of post-meiotic germ cell-enriched proteins (HSPA4L, DPEP3, PGK2), pointing to the possible presence of more pockets of preserved spermatogenesis in the testis. The heterogeneity of the MA group can be explained by varying degrees of residual testicular function resulting in similar diagnoses. However, there is no apparent correlation between histological scores (Supp. Table 2) and the abundance of germ cell enriched proteins in seminal plasma. By comparison, the SCO and OA groups are more homogenous in regard to the measured levels of epididymal and testicular proteins (Fig. 1. A).

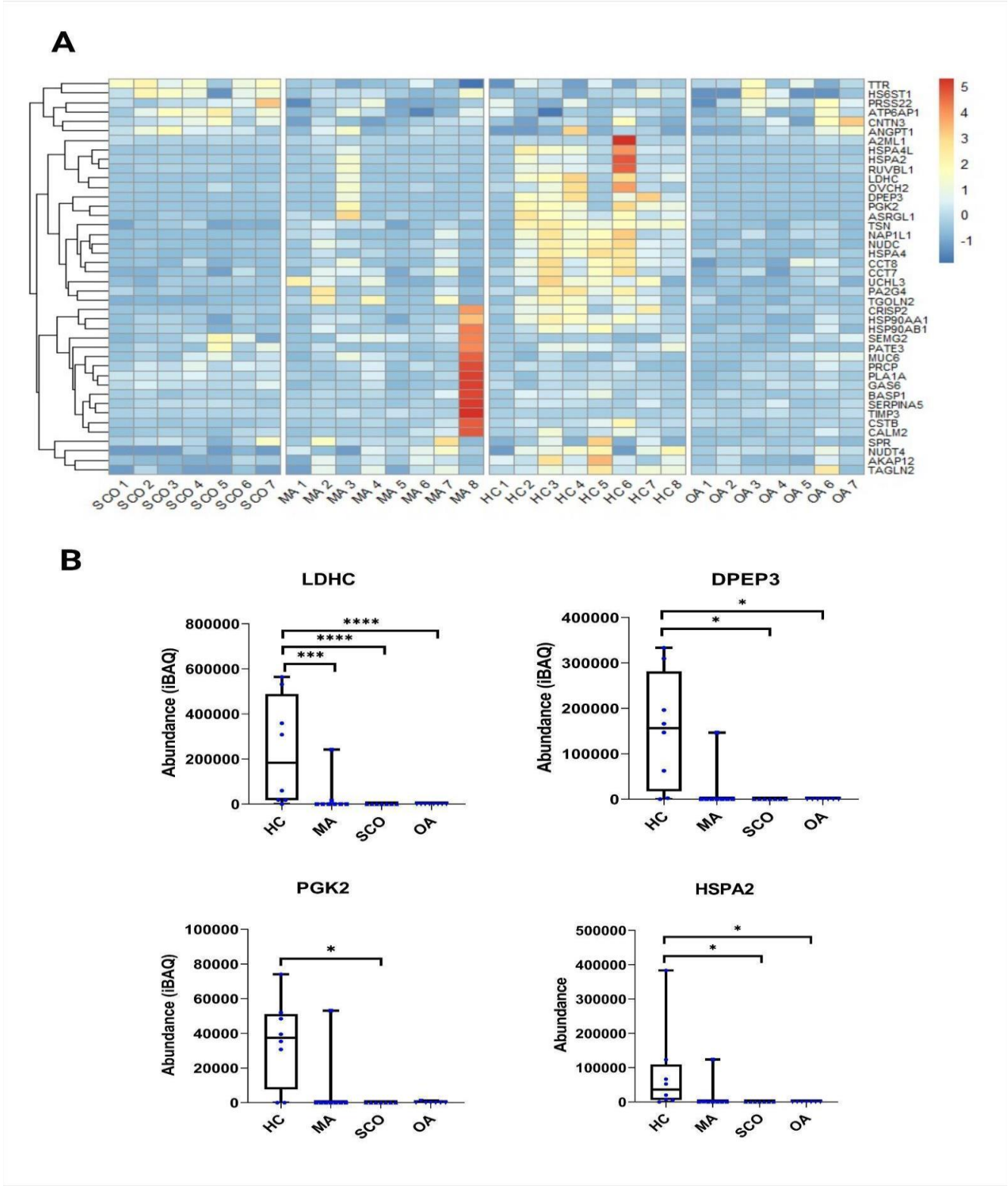


Figure 1: ***A:*** Scaled abundance values of 42 differentially expressed proteins in seminal plasma from 7 obstructive azoospermia men (OA 1- OA 7), 8 fertile men (HC 1- HC 8), 8 men diagnosed with mixed atrophy (positive micro-TESE outcome) (MA 1- MA 8) and 7 men diagnosed with Sertoli cell only syndrome (SCO). Clustering method= UPGMA, rows clustering distance measure = Euclidian. ***B:*** relative abundances of testis-enriched LDHC, DPEP3, HSPA4L, and PGK2, boxes represent

median, 1st and 3rd quartiles, whiskers represent maximum and minimum, statistical significance was determined by the Mann-Whitney test. *: p-value >0.01, ***: p-value <0.001, ****: $p<10^{-4}$

Table 3: Expression in male reproductive tissues of 32 proteins significantly downregulated in one or more of the azoospermia groups (SCO, OA, MA) relative to the control. Protein distribution and localization were extracted from the Tissue Atlas database (www.proteinatlas.org), which integrates omics data from antibody-based imaging (immunohistochemistry), mass spectrometry, and RNA seq data to map protein distribution across the various cell types, tissues, and organs of the human body. Specificity categories: tissue/ cell type enriched: expression level is at least four-times any other tissue/ cell type; tissue/ cell type enhanced: expression levels of a group of 1-5 tissues or 1-10 cell types at least 4-times the mean of any other tissue/ region/ cell type.

Gene name	Detected in SCO	Detected in OA	Detected in MA	Expression in male tissues
A2M	yes	yes	yes	Cell type enhanced: Spermatocytes
AKAP12	yes	yes	yes	Cell type enhanced: Sertoli cell
ARHGDIA	yes	yes	yes	Highest in Leydig cells
ASRGL1	yes	yes	yes	Testis enhanced, Cell type enhanced: Late Spermatids, Spermatocytes, Early Spermatids
BASP1	yes	yes	yes	Epididymis enhanced
CCT2	yes	yes	yes	Cell type enhanced: early spermatids
CCT7	yes	yes	yes	Low tissue specificity
CCT8	yes	yes	yes	Low tissue specificity, highest in spermatogonia
CRISP2	yes	yes	yes	Testis enhanced, Cell type enhanced: Late Spermatids, Early Spermatids
CSTB	yes	yes	yes	Low levels of expression in all male tissues
DPEP3	no	no	yes	Testis enriched, Cell type enriched: Spermatogonia, Spermatocytes, Early Spermatids
HSP90AA1	yes	yes	yes	Highest in Testis, epididymis; enhanced in female tissues
HSP90AB1	yes	yes	yes	Low tissue specificity, highest in glandular cells of epididymis and prostate, spermatogonia
HSPA2	no	no	yes	Cell type enriched: Spermatocytes
HSPA4	yes	yes	yes	Low tissue specificity, highest in seminiferous epithelium
HSPA4L	yes	no	yes	Testis enhanced, Cell type enriched: Late Spermatids, Spermatocytes, Early Spermatids
LDHC	no	no	yes	Testis enhanced, Cell type enriched: Spermatocytes, Early Spermatids
MGAM	yes	yes	yes	Cell type enhanced: Late Spermatids, Early spermatids
NAP1L1	no	yes	yes	Low tissue specificity

NUDC	yes	yes	yes	Low tissue specificity; highest in germ cells
NUDT4	yes	yes	yes	Expressed in all male tissues, highest in the epididymis
OVCH2	yes	no	yes	Epididymis enriched
PA2G4	yes	yes	yes	Expressed in all male tissues, highest in prostate glandular cells
PATE3	yes	no	yes	Epididymis enriched
PGK2	no	yes	yes	Testis enriched, Cell type enriched: Late spermatids, Early Spermatids
PSMA2	yes	yes	yes	Low tissue specificity
PTGDS	yes	yes	yes	Cell type enhanced: Leydig cells
RUVBL1	yes	yes	yes	Expressed in all male tissues, highest in spermatocytes
TAGLN2	yes	yes	yes	Low tissue specificity
TGOLN2	yes	yes	yes	Low tissue specificity
TSN	yes	yes	yes	Low tissue specificity, highest in the seminiferous epithelium
UCHL3	yes	yes	yes	Expressed in all male tissues, highest in testis, epididymis

- ***Seminal plasma testis-enriched proteins: Confirmation of mass spectrometry results by antibody-based assays:***

LDHC and HSPA2 were selected for confirmation of mass spectrometry results using Western Blot for relative quantification in seminal plasma samples. Densitometric analyses revealed a clear reduction in the abundance of both proteins in the two NOA groups (SCO and MA) compared to the control group (Fig.3, Fig. 4). Similar to mass spectrometry data, LDHC and HSPA2 were significantly downregulated in SCO ($p=0.0025$ and 0.0003, respectively) and MA ($p= 0019$ and $p= 0.0006$, respectively) compared to the normozoospermia control samples (Table 1). Interestingly, LDHC levels were undetectable in SCO patients by mass spectrometry (Fig. 1, B), yet according to Western blot, LDHC was detectable in all SCO samples, albeit at a relatively low level (Fig. 3. A). Additionally, the expression pattern of HSPA2, HSPA4L, and LDHC in testicular tissue was investigated by comparing histological sections from 'normal spermatogenesis' cases compared to sections from men with severely impaired spermatogenesis (SCO). In normal testicular tissue, the staining was strongest towards the lumen of the tubules as HSPA2 appears to be highly expressed in pachytene spermatocytes, round and elongating spermatids, but undetected in testicular somatic cells (peritubular, Sertoli, Leydig) (Fig. 4. A). Conversely, in SCO, the tubules are clear, but the peritubular cells are stained, indicating a significant level of expression of

HSPA2 (Fig. 4. B). Similarly, the expression of LDHC was highest in spermatids in normal human testis (Fig. 3. B). However, low to moderate staining of Sertoli cells was seen in both normal spermatogenesis and SCO (Fig. 3. C), which might explain the presence of LDHC, albeit at low levels, in the seminal plasma of SCO men. The immunolocalization of HSPA4L resulted in strong staining of post-meiotic cells (round and elongating spermatids) and low signal in spermatocytes and spermatogonia (Fig. 5. A). In normal spermatogenesis, HSPA4L appears to be undetectable in Sertoli and Leydig cells. By contrast, in SCO and MA sections, Sertoli cells are moderately stained (Fig. 5).

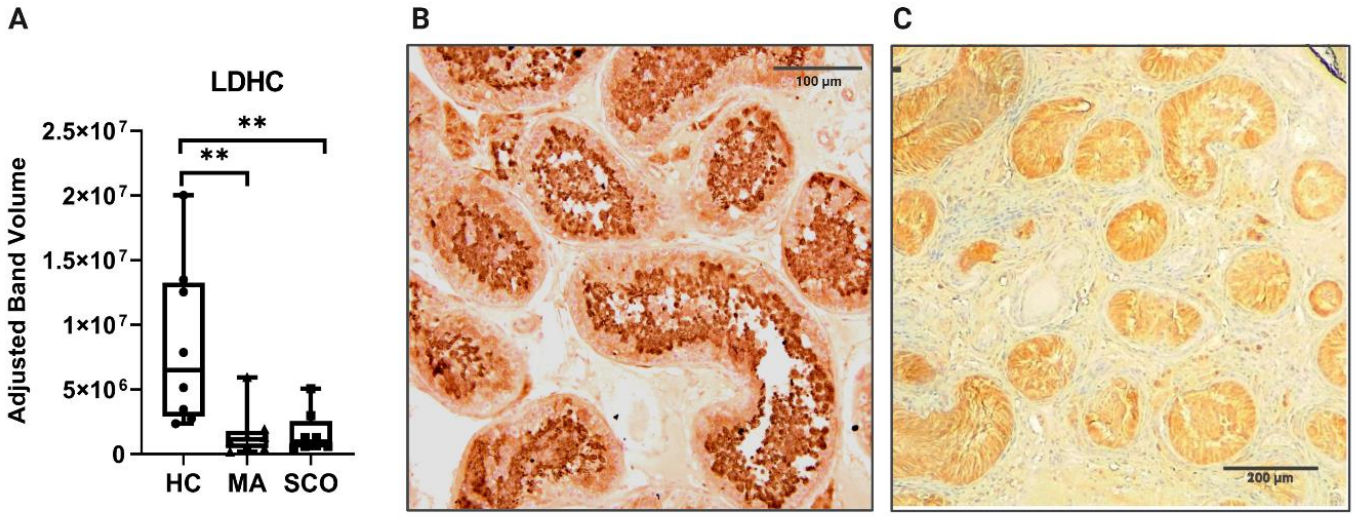


Figure 3: Differential expression of LDHC in non-obstructive azoospermia (A) Relative quantitation of LDHC in seminal plasma by Western Blot. Seminal plasma samples were resolved on a 4-12% Tris-Glycine gradient gel. Band intensities were measured using Image Lab 6.0 (Bio-Rad) and corrected for total protein content. Boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum. Statistical significance was determined by the Wilcoxon Rank Sum Test. (B) Expression of LDHC in normal testicular tissues, showing high intensity staining of germ cells (C) Expression of LDHC in testicular tissue exhibiting germ cell aplasia and seminiferous tubule atrophy (SCO): medium staining of Sertoli cells

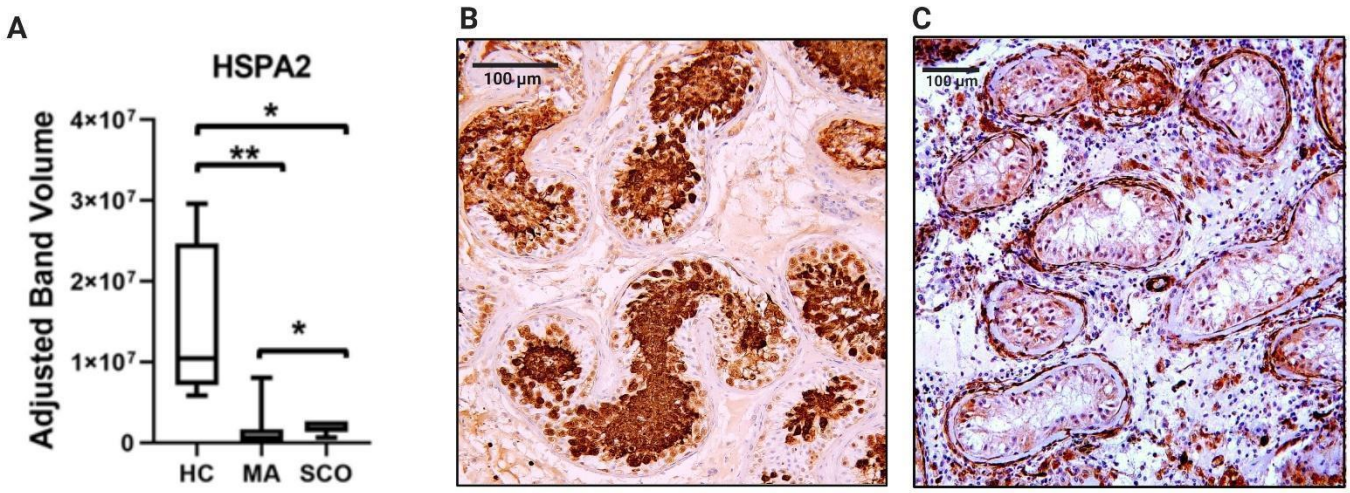


Figure 4: Differential expression of HSPA2 in testicular failure (A) Relative quantitation of HSPA2 in seminal plasma by Western Blot. Seminal plasma samples were resolved on 4-12% Tris-Glycine gradient gels. Band intensities were measured using Image Lab 6.0 (Bio-Rad) and corrected for total protein content. Boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum. Statistical significance was determined by the Wilcoxon Rank Sum Test. (B) Expression of HSPA2 in testicular tissue showing normal spermatogenesis, (C) Expression of HSPA2 in the testicular epithelium from an azoospermic patient showing SCO

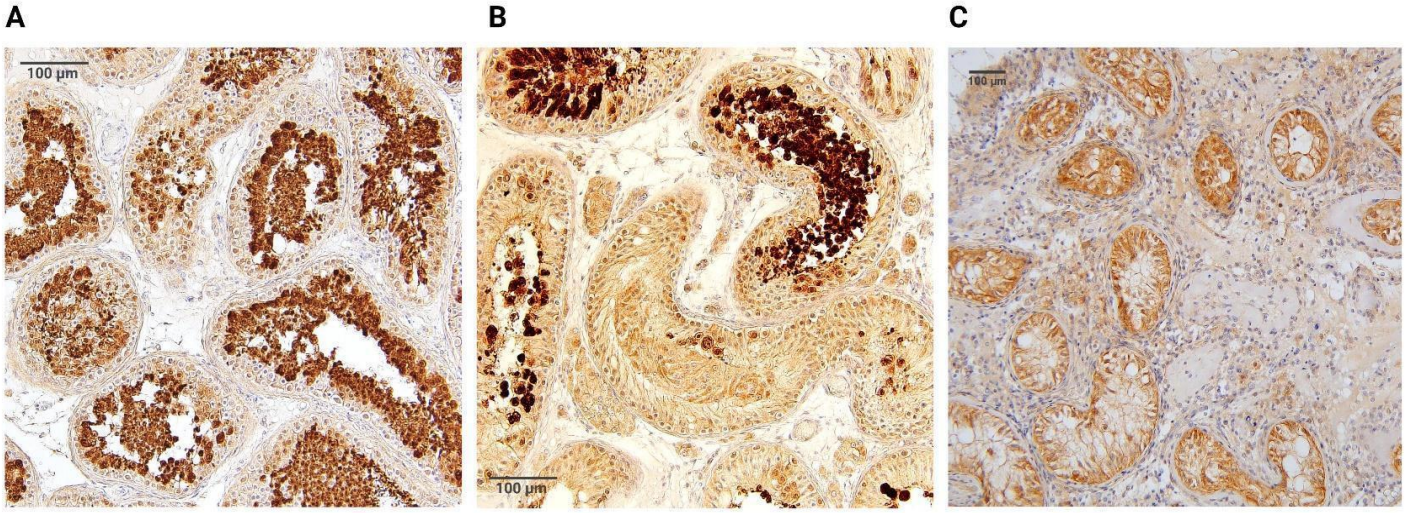


Figure 5: Immunolocalization of HSPA4L in testicular tissue showing (A) normal spermatogenesis, (B) mixed atrophy, (C) Sertoli cell only. Paraffin-embedded histological sections were probed with 1:250 anti-HSPA4L antibody produced in rabbit (HPA039149, Sigma Aldrich). In normal seminiferous epithelium, HSPA4L is highly produced by round and elongated spermatids and is present to a lesser extent in pachytene spermatocytes and spermatogonia. In mixed atrophy, the same intense signal from post-meiotic cells is seen but Sertoli cells show medium staining. The SCO section is also characterized by medium staining of Sertoli cells.

- ***Differentiating MA and SCO at the transcriptional level: q-RT-PCR of 4 testis-enhanced genes***

A number of relevant biomarkers were selected based on preliminary results of MS analysis of SP (unpublished data), previously published SP datasets (Batruch et al. 2011, Batruch et al. 2012), and gene expression in male reproductive tissues (Human Protein Atlas). The selection criteria were downregulation in NOA or OA and high relative abundance in seminal plasma. Gene expression levels for testis-enhanced DNAH2, SLC52A3, spermatid-enhanced PFKP, and spermatocyte-enhanced CSE1L, were evaluated in SCO and MA testicular tissue to investigate the possibility of differentiating the two aetiologies at the transcriptomic level and confirm that the variance in abundance in seminal plasma correlates to differential expression in the testis rather than in other parts of the male reproductive tract. To investigate differences at the transcriptional level between MA and SCO, two forms of NOA characterized by different degrees of testicular failure, the expression of four testis-enhanced genes was evaluated by RT-Q-PCR. Transcription levels of PFKP and DNAH2 were significantly higher in MA compared to SCO. CSE1L showed a tendency to be more elevated in MA without reaching the significance threshold. Meanwhile, SLC52A3 appeared to be expressed at similar levels in the two groups. These results indicate a possible correlation between the expression of PFKP and DNAH2 in the testis and the extent of spermatogenic activity. The presence of considerable differences between MA and SCO at the transcriptional level indicates they could be distinguishable at the protein level as well with the use of a more targeted approach.

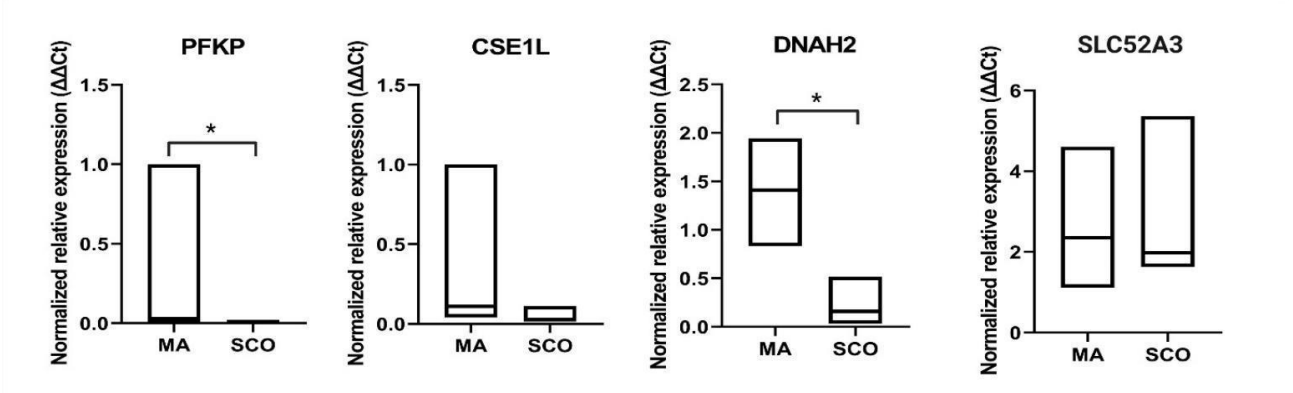


Figure 6: Real-time reverse transcription polymerase chain reaction: DNase treated RNA samples (PFKP & CSE1L: MA (n=4), SCO (n=4); DNAH2: MA(n=5), SCO(n=3); SLC52A3: MA(n=5), SCO(n=4)) were reverse transcribed using a recombinant Moloney murine leukemia virus reverse transcriptase (MultiSkribe, Invitrogen). The resulting cDNA was added to the tested primers and a q-

PCR supermix containing the Evagreen dye (equivalent to SYBR green) and a *Taq hotstart* DNA polymerase) and amplified for 40 cycles. Experimental setup and data analysis, including determination of Ct values and expression levels, were processed by the CFX manager software (Bio-Rad). RPL19 Ribosomal protein L19 was used as a housekeeping gene, statistical significance was calculated using the Mann-Whitney test (p-values = 0.028, 0.11, 0.035, and 0.9 for the PFKP, CSE1L, DNAH2, and SLC52A3 comparisons, respectively. Boxes represent the median, maximum, and minimum.

- ***Epididymal proteins* significantly decreased in OA seminal plasma:**

PTGDS (Prostaglandin D2 synthase) was significantly more abundant in normal seminal plasma compared to OA (FC= 11.71, p=0.041). According to the FANTOM5 dataset resulting from Cap Analysis of Gene Expression (CAGE) of healthy tissues (Takashi et al., 2012), PTGDS is more present in the testis at the transcriptional level (12683.2 TPM compared to 3626.7 in the epididymis). However, on the protein level (Tissue Atlas, https://www.proteinatlas.org/humanproteome/tissue), PTGDS is expressed higher in the epididymis, with "medium" levels in the testis (Leydig cells, peritubular cells enriched) and seminal vesicles and low expression in the prostate. The semi-ubiquitous expression of PTGDS in testicular cells could explain the tendency towards lower abundances in SCO and MA relative to the control (Fig. 7).

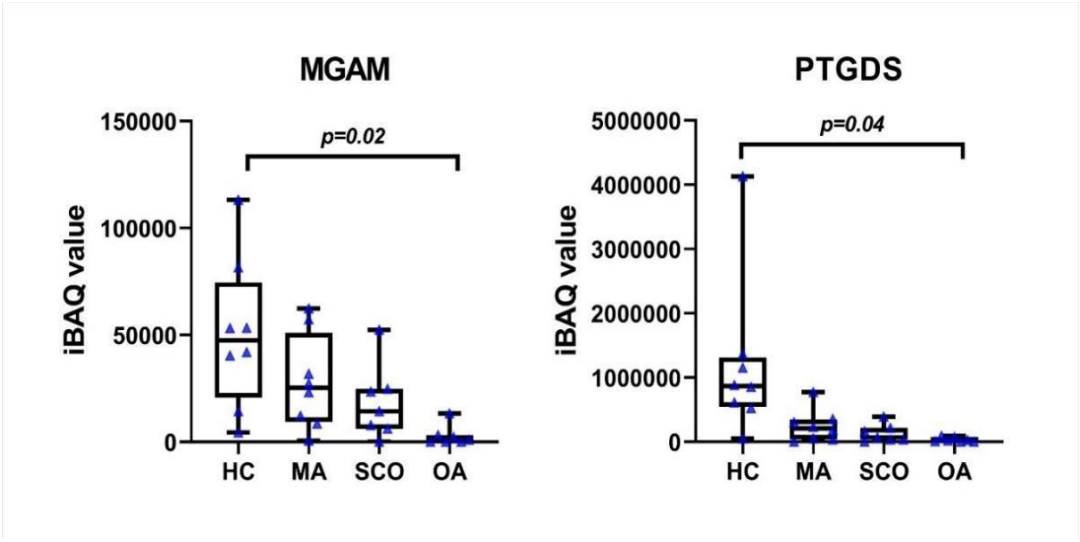


Figure 7: iBAQ (intensity-based absolute quantification values) of MGAM and PTGDS in the seminal plasma of OA, MA, SCO, and fertile (HC) men measured by LC-MS/MS; boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum.

The abundance of MGAM (Maltase-glucoamylase) by mass spectrometry was also significantly higher in HC vs OA (FC= 6.32, p=0.025). MGAM is detected in the testis at low levels and only in spermatids but is expressed at an increased level in the epididymis (201.9 TPM vs 30.9 TPM in the testis) (Human Protein Atlas). Based on its expression pattern in male tissues, the majority of MGAM present in seminal plasma likely comes from epididymal glandular cells, with only a small fraction originating from spermatids. This is consistent with the markedly lower concentration of MGAM in OA by mass spectrometry and the non-significant decrease in MA and SCO compared to the control (Fig.7). Undetectable levels of both MGAM and PTGDS could indicate an obstruction of the vas deferens.

- ***Epididymis-enriched proteins significantly downregulated in SCO:***

Ovochymase-2 (OVCH2) and Prostate and testis expressed protein 3 (PATE3) are two epididymis-enriched proteins that are mostly absent from other tissues of the male reproductive tract (Tissue Atlas, https://www.proteinatlas.org/humanproteome/tissue). Mass spectrometry results show OVCH2 and PATE3 are undetectable in OA seminal plasma and are decreased more than 3-fold in the SCO group compared to the control (Fig. 8). However, iBAQ value distribution of PATE3 in SCO, MA and HC does not appear to support a clear association between testicular failure and the downregulation of PATE3. On the other hand, OVCH2's expression seems to be altered in SCO and MA, but further experiments are needed to establish a link between NOA and diminished levels of OVCH2.

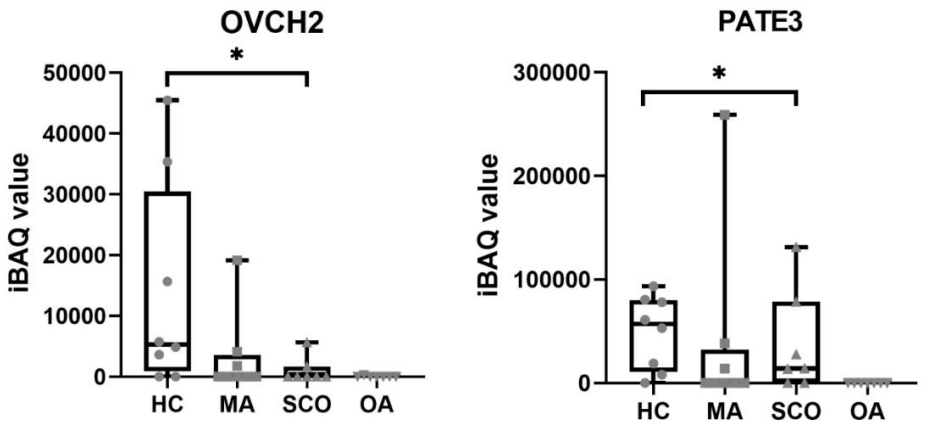


Figure 8: Relative abundance of OVCH2 and PATE3 in the seminal plasma of OA, MA, SCO and fertile (HC) men; OVCH2 and PATE3 are significantly down-regulated in SCO relative to the control ($FC_{HC/SCO}$ = 3.71, p= 0.007 and $FC_{HC/SCO}$= 3.18, p= 0.011 respectively); Boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum

- ***Markers of epididymal function: abundance in seminal plasma vs gene expression in the testis and epididymis:***

With the initial assumption that testicular failure would not significantly affect epididymal function and secretions, the expression of epididymis enriched proteins in the testis was compared to that in the epididymis to confirm the potential of these markers in differentiating OA and NOA. Beta-Defensin 129 (DEFB129), Adhesion G protein-coupled receptor G2 (ADGRG2) and Epididymal sperm binding protein 1 (ELSPBP1) were selected as epididymal markers since they appear to be expressed only in the epididymis according to transcriptomic and proteomic databases. Q-RT-PCR was used to confirm tissue-specificity of these three gene products. ADGRG2, DEFB129, and ELSPBP1 were found to be highly expressed in the epididymis and except for ADGRG2, almost undetectable in the testis (Fig. 9. A) These gene expression results align with the immunostaining profiles of the corresponding proteins from the Human Protein Atlas. Measurements of the relative abundance of ADGRG2, DEFB129, and ELSPBP1 in seminal plasma by mass spectrometry show a clear decrease in OA, but no statistically significant differences were seen for this dataset (Fig. 9. B). The abundances of all three proteins appear to be highly variable within the NOA (SCO, MA) and control (HC) groups. Although the levels of these proteins in MA and SCO show a tendency to be lower compared to the control, these results cannot be interpreted as NOA impacting epididymal function.

On the other hand, antibody-based relative quantification of ELSBP1 in seminal plasma using Western Blot resulted in a significant difference between OA and NOA ($p=0.01$) (Fig.10. A). Similar to the MS results, the range of abundance values for ELSPBP1 in NOA seminal plasma samples is wider than in OA. These findings suggest that using antibody-based assays to measure epididymis specific proteins in seminal plasma can be a simple and cost-effective way to distinguish OA and NOA cases.

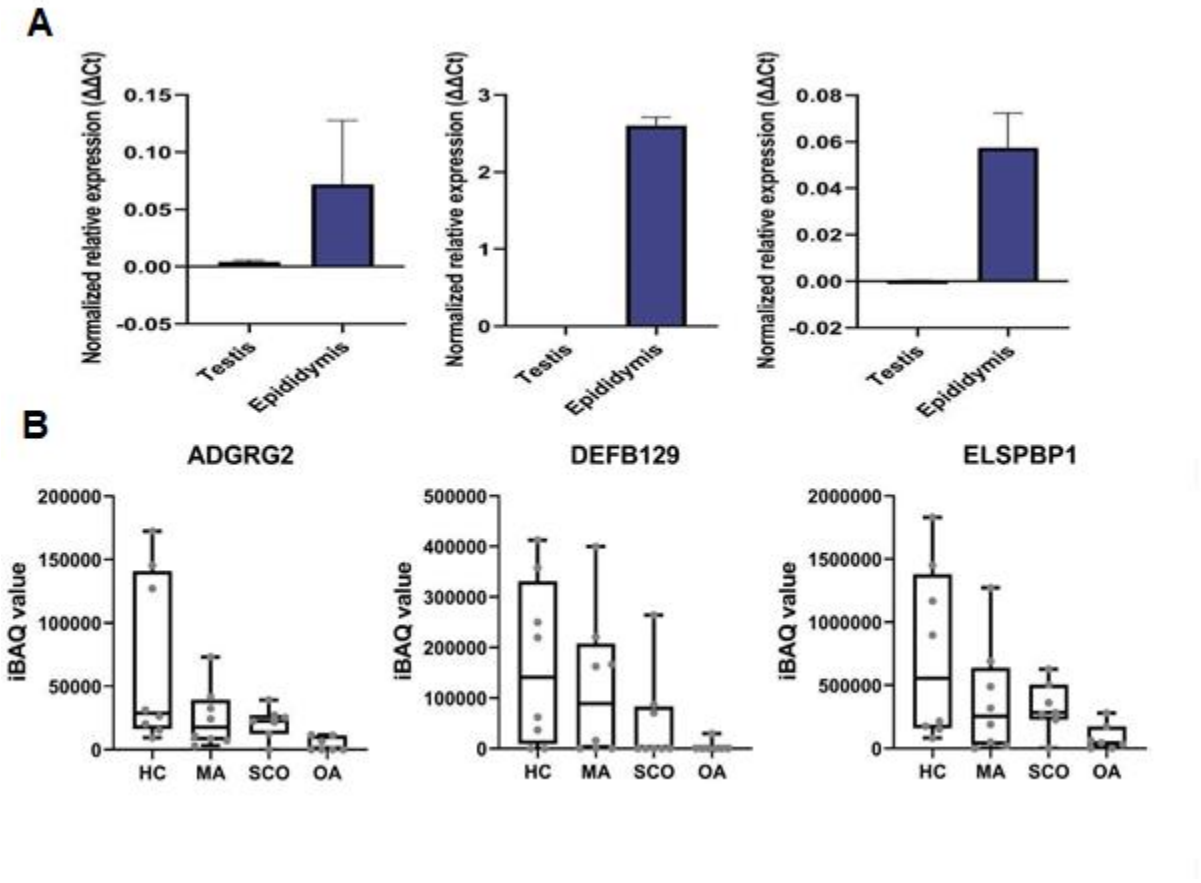


Figure 9: Expression of epididymis specific genes in the testis and abundance in seminal plasma **A**: Normalized relative expression of ADGRG2, DEFB129, and ELSPBP1 in normal testicular and epididymal tissue. mRNA was extracted from healthy epididymal (n=3) and testicular (n=3) tissue samples. RPL19 was used as a housekeeping gene, bars represent mean and standard deviation.**B**: iBAQ (intensity-based absolute quantification values) of ADGRG2, DEFB129, and ELSPBP1 in the seminal plasma of OA, MA, SCO, and fertile (HC) men measured by LC-MS/MS; boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum,

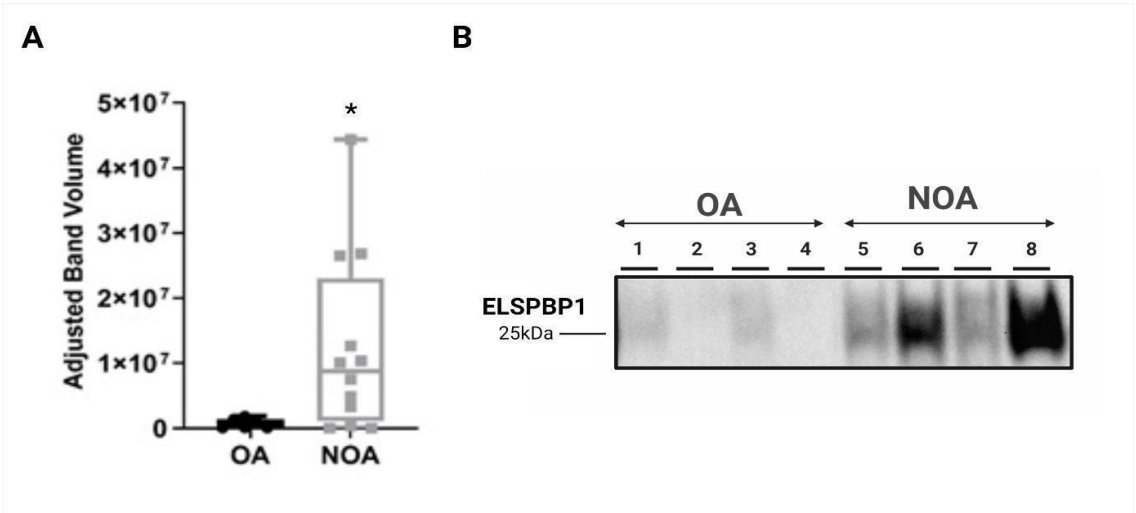


Figure 10: Relative abundance of ELSPBP1 in seminal plasma. **A:** Relative abundance of epididymis-specific ELSPBP1 measured by immunoblotting in seminal plasma from obstructive azoospermia (OA, n=8) and non-obstructive azoospermia (NOA, n=12) men, NOA samples include hypospermatogenesis, MA and SCO cases diagnosed by histological scoring, boxes represent the median, 1st and 3rd quartiles,

whiskers represent maximum and minimum; *: p= 0.01; statistical significance was determined by the Wilcoxon Rank Sum Test. **B**: Western Blot showing the abundance of ELSPBP1 in seminal plasma from OA and NOA men. Samples were resolved on a 4-12% gradient gel, blots were incubated with 1:250 anti-ELSPBP1 antibody produced in rabbit (Sigma-Aldrich, HPA044256) and imaged after signal development in a ChemiDoc XRS+ imaging system and bands were detected using the 'High Resolution" function in Image Lab 6.0 (Bio-Rad). Band intensities were corrected for the total protein content of individual samples.

IV. Discussion:

In order to tailor the treatment to the patient's needs, an accurate diagnosis of azoospermia is of paramount importance. Cases of obstructive azoospermia could benefit from reconstructive surgery to facilitate natural conception, whereas men with hypospermatogenesis/mixed atrophy are likely to have sperm present in their testes that could be surgically retrieved (Vloeberghs et al.,2015). On the other hand, patients with an SCO diagnosis are much less likely to have sperm present for retrieval (Ramasamy and Schlegel, 2007). Accurate prediction of the presence of sperm in the testes would allow infertile couples to avoid ineffective and costly therapies and surgeries (Sharlip et al., 2002). Azoospermia poses a unique clinical challenge: the absence of sperm limits the use of a number of measurable parameters, including sperm count, morphology and motility, and function tests, as only the acellular fraction of the ejaculate is available for analysis. Gonadotropin and testosterone serum levels have a considerable predictive value but are insufficient for a conclusive diagnosis in 60% of azoospermia cases (Jarvi et al., 2010). In the past two decades, many proteomic studies have aimed to correlate the proteomic profile of seminal plasma with male reproductive potential, using techniques ranging from SDS-PAGE and 2-D electrophoresis to high-resolution mass spectrometry (Agarwal et al., 2016). Bai et al. identified 28 proteins with differential expression in the seminal plasma of fertile men in comparison to that of 6 NOA volunteers, 24 of which were downregulated in azoospermia (Bai et al., 2007). In a subsequent study, the same group reported larger differences in SP proteomes between severe oligozoospermia and NOA, than between oligozoospermia and normal spermatogenesis, theorizing that the molecular mechanisms affected in NOA differ significantly from those implicated in oligozoospermia (Bai et al., 2008). Other proteins like ubiquitously expressed laminin and pituitary-gland-enriched prolactin were investigated in seminal plasma as potential markers of infertility, with negative or inconclusive results in regard to the correlation of their abundance and different male reproductive disorders (El-Dakhly et al., 2005, Arowojulu et al. 2007). In other instances,

the proteins being investigated were reported to be correlated to sperm quality or general reproductive potential. Down-regulation of prolactin inducible protein (PAP) in the seminal plasma of azoospermic men compared to oligozoospermia and normozoospermia has been previously described (Tomar et al., 2012). Roshdy and colleagues estimated the concentration of apoptosis inhibitor survivin (BIRC5) in human seminal plasma, and found it to be decreased in azoospermia, showing a lower abundance in cases with a negative TESE outcome compared to those where sperm retrieval was a success (Roshdy et al.,2009). In an earlier study of seminal plasma proteome using mass spectrometry, Batruch et al. identified 34 proteins of significantly higher abundance in fertile men and 16 increased in post-vasectomy men compared to NOA. Several of these proteins were of testicular or epididymal origin, with potential or proven involvement in spermatogenesis (Batruch et al. 2012). Epididymal marker ECM1 and testis-enriched TEX101 were subsequently investigated by the same team as potential markers for the differential diagnosis of azoospermia (Drabovich et al. 2013). Despite being primarily composed of a few highly abundant accessory gland proteins (semenogelin, fibronectin), with the ten most abundant proteins representing more than 80% of the total protein content (Drabovich et al. 2014), seminal plasma contains thousands of tissue-specific proteins at relatively high concentrations, making it exceptionally reflective of the entirety of the male reproductive tract (Batruch et al. 2011) and a promising source of biomarkers of male fertility (Bieniek et al., 2016). In this study, we attempted to sidestep some of the limitations of past studies (Batruch et al. 2011, Batruch et al. 2012) such as a smaller sample size (n=5/ group), using samples from post-vasectomy men instead of idiopathic OA patients, and NOA samples with unspecified testicular phenotype. We used label-free mass spectrometry analysis coupled with a stringent statistics pipeline to compare the proteomic content of seminal plasma from patients diagnosed with three types of azoospermia, obstructive (OA), mixed atrophy (MA), and Sertoli cell-only (SCO) to that of fertile men (HC) to ascertain the differences in protein expression between the four groups. Furthermore, we performed a principal components analysis to disregard samples with an uncertain diagnosis, and confirmed MS findings for selected biomarkers using antibody-based assays.

- ***Up-regulation of accessory gland proteins in SCO***

Proteins significantly up-regulated more than 2-fold in SCO relative to the control include smooth muscle cell-enriched Angiopoietin-1 (ANGPT1), which is expressed in the epididymis and accessory glands but not-detected in the testis on the protein level (Tissue Atlas, https://www.proteinatlas.org/), and seminal vesical enriched semenogelin II (SEMG2).

Semenogelins I and II (SEMG1, SEMG2), involved in semen coagulation and liquefaction are the predominant proteins in seminal plasma; they are secreted principally by the seminal vesicles, with low expression in the rest of male tissues (Lundwall et al. 2002). Increased seminal levels of SEMG1 and SEMG2 have been reported in relation to male infertility (Milardi et al, 2013). SERPINA5, also up-regulated 2-fold in the SCO group, has a regulatory role in the degradation of SEMG1 and SEMG2 during the transfer of spermatozoa to the female reproductive tract, and its inactivation in seminal plasma could be linked to infertility (Kise et al., 1996, He et al., 1999). Sertoli cells of SCO men also exhibit patterns of protein expression distinct from normal spermatogenesis (Ma et al., 2013). The SCO seminal plasma displays some distinct characteristics, which have been described as a deviation from the "normal seminal plasma homeostatic state" (Antoniassi et al., 2016). Milardi et al. noted a loss of enzymatic regulators and catalytic activity, as well as sperm binding capacity in SCO seminal plasma (Milardi et al., 2013). Further research is needed to establish a link between Sertoli cell only syndrome and the increased expression of SEMG2 and SERPINA5 and to investigate their potential as SCO biomarkers.

- ***Down-regulation of germ-cell enriched proteins in SCO***

Several of the proteins of lower abundance in SCO (Table 1) have confirmed or potential roles in spermatogenesis and male fertility in general. LDHC is a testis-specific isoenzyme from the lactate dehydrogenase family, enriched in pachytene spermatocytes, round spermatids, and the central piece in spermatozoa (Goldberg et al., 2010). The role of LDHC in lactate metabolism and sperm motility has been established in mice (Tang et al., 2013). LDHC has been reported previously as undetectable or highly decreased in the seminal plasma of post-vasectomy and NOA men (Batruch et al., 2011; Batruch et al., 2012), and proposed as a marker of male fertility based on an aggregation of proteomic, transcriptomic, and genomic data (Rolland et al., 2013). DPEP3 and PGK2 were also among the proteins that were described by Batruch et al. as unique to the seminal plasma of fertile men (Batruch et al., 2011). The role of DPEP3 in spermatogenesis has been linked to its metalloproteinase action on the ADAM protein family and its activity in the human testis is modulated by a previously validated marker of male fertility, TEX101 (Schiza et al., 2018, Korbakis et al., 2017). The expression of PGK2 is specific to the spermatogenic glycolytic pathway and is required for sperm motility (Danshina et al., 2010, Yoshioka et al., 2007). CRISP2 is also involved in sperm motility, in addition to facilitating the adhesion between Sertoli cells and germ cells and cytoplasm removal during spermiogenesis (Jamsai et al., 2007, McReynolds et al., 2014) and is significantly decreased in

the seminal plasma of men with asthenozoospermia, teratozoospermia, and teratoasthenozoospermia (Gholami et al., 2020).

- ***Proteins of the HSP70 kDa family as potential markers of successful sperm retrieval***

Heat shock proteins (HSPs) act as molecular chaperones, and via downstream signalling, assisting the assembly and folding of newly formed peptides and protecting the structural integrity of proteins by decreasing stress-induced denaturation, or promoting renaturation and restoration of their correct tertiary structure. Proteomic data from our mass spectrometry analysis shows Heat shock 70 kDa proteins 2, 4, and 4L (gene names: HSPA2, HSPA4, and HSPA4L) to be significantly depleted in SCO. These results were confirmed for HSPA2 by Western Blot (Fig. 4. A), and for HSPA4L by immunostaining (Fig. 5) indicating the potential of these proteins as biomarkers of severe testicular failure. Proteins of the HSP 70 kDa protein family play an essential role in male fertility (Meinhardt et al. 1999, Neuer et al. 2000). HSPA2 is involved in the post-testicular remodelling of the sperm head, necessary for oocyte recognition and binding, and essential for the progression of spermatogenesis, both in meiosis and germ cell differentiation (Huszar et al., 2000; Huszar et al., 2006). HSPA4 acts as a co-factor of other 70 kDa HSPs as part of the cytosolic protein folding mechanism (Dragovic et al. 2006). HSPA4 deficient mice suffer from reduced fertility, manifested by a majority of pachytene spermatocytes failing to advance beyond the first meiotic prophase, indicating a crucial role in spermatogenesis (Held et al, 2011). Similarly, HSPA4L -/- mice show a clear decrease in sperm quantity, with fewer active spermatogenic sites and increased infertility (Held et al. 2006). HSPA4L mRNA suppressing miR-429 is up-regulated in spermatozoa from asthenozoospermia men, suggesting a role of HSPA4L in sperm viability and motility (Chen et al., 2016). In a previous study of seminal plasma proteome by mass spectrometry, HSPA4L was undetected in NOA, but quantifiable in the seminal plasma of fertile men (Cui et al., 2018).

- ***Proteins of lower abundance in OA as potential markers of obstruction***

The depletion of proteins of testicular origin (LDHC, DPEP3, HSPA2, HSPA4) in the seminal plasma of OA men can be attributed to the physical obstruction of the ductus deferens preventing spermatozoa and testicular secretions from passing into the seminal fluid. Prostaglandin-H2 D-isomerase (PTGDS) is expressed in all male tissues, yet significantly decreased only in OA seminal plasma. PTGDS has been previously suggested as a marker of obstructive azoospermia (Chen et al., 2004, Heshmat et al., 2008). In like manner, Maltase-glucoamylase (MGAM) is reduced in OA but not in NOA seminal plasma and was reported by

Batruch et al. to be of significantly lower abundance in the seminal plasma of post-vasectomy men (Batruch et al., 2011). The abundance of PTGDS and MGAM in the seminal plasma of NOA men (MA, SCO) shows high variability but is overall closer to the control group levels (Fig. 7). Our results also showed epididymis enriched OVCH2 as significantly downregulated in SCO, and completely undetected in OA. (Fig. 8). Epididymis-specific ADGRG2, DEFB129, and ELSPBP1, despite being present at very low to undetectable quantities in OA samples, are present at varying abundances in NOA seminal plasma (Fig. 9). Taken together, these results indicate that the secretion of some epididymis-enriched proteins could be affected in cases of testicular failure. Therefore, some of these tissue-specific markers are potentially more useful than others in differentiating between OA and NOA. Previous studies have reported a decrease in epididymis-specific proteins in the seminal plasma of post-vasectomy men (Batruch et al. 2011). Epididymis-enhanced Extracellular matrix protein 1 (ECM1) was validated as a marker of obstruction (Drabovitch et al. 2013). However, our data shows that ECM1 is not significantly decreased in OA compared to SCO or MA (supplementary data table I). More downstream studies are needed to determine which of these candidate biomarkers are the most suitable for differentiating OA and NOA in a clinical setting. In addition to the possibility of surgical correction of the site of obstruction (Du et al., 2010), the TESE/TESA success rate for OA is close to 100% (Tournaye et al. 1997). The probability of sperm retrieval in NOA is substantially lower (20 to 45%) and up to 60% using micro-TESE (Bernie and Ramasamy, 2013).

- ***Challenges in the discovery and validation of biomarkers of male infertility in seminal plasma***

High variability in protein expression in seminal plasma compared to other tissues is one of the main hurdles in linking certain male reproductive disorders/ testicular phenotypes to seminal plasma biomarkers. Yamakawa et al. 2007 pointed out the high interindividual variability in protein expression in SP from fertile men. For 63 proteins detected in all 10 samples, the median CV value for abundance was 63.1%. We observed similar intra-group variability when performing a principal components analysis of our proteomic data (Methods, Fig. 2 and 3). Relative abundances of the majority of the 900 detected proteins do not correlate with the patient's histological scores and can vary largely within each group. Davalliera et al. (2012) addressed the relatively low overlap in data between independent studies of seminal plasma proteomics, which can be explained by the high variability between individuals and cohorts, in addition to the differences in sample handling and analytical methods. Gilany and

colleagues compiled a list of 2168 non-redundant proteins detected in seminal plasma by aggregating proteomic data from several studies encompassing different proteomics methods (2-DE, SDS-PAGE, LC-MS/MS) and described a set of proteins identified in multiple studies as a basis for the human seminal plasma proteome. However, the significant proteins identified as candidate biomarkers of male infertility in seminal plasma differ vastly from one study to another. This could be attributed to the variation in analytical methods, inter-cohort variability, and the lack of downstream confirmation studies which could determine which biomarkers are more promising for validation (Camargo et al., 2018).

Conclusion

The quantification of a selection of biomarkers of epididymal and testicular function in seminal plasma, coupled with routine hormonal investigation, could help direct the differential diagnosis of azoospermia without having to resort to testicular biopsy. Normal FSH and Testosterone levels, together with complete depletion of testis-specific and epididymis-specific proteins in SP are signs of vas deferens obstruction. Above a certain threshold, the presence of post-meiotic germ cell enriched proteins such as LDHC and HSPA2 in the SP of azoospermic men can potentially rule out both OA and SCO. Higher concentrations of epididymis-specific and germ cell-specific proteins, coupled with normal FSH levels strongly indicates a case of MA, where a sperm retrieval procedure is a viable option. Conversely, low or undetectable concentrations of spermatogenic markers, coupled with normal abundance of epididymal proteins and high FSH levels could be indicative of a case of severe hypospermatogenesis or SCO, where sperm extraction is unlikely to have a positive outcome. Our study of the seminal plasma proteome uncovered several candidate biomarkers of sperm retrieval in azoospermia. After clearing the validation phase, these biomarkers could be of great value in the clinical setting. The recent emergence of better multiplex protein-based assays (Luminex platform) opens new possibilities for the development of a test that can simultaneously measure several markers of spermatogenic function (LDHC, PGK2, HSPA2, HSPA4L) and markers of vas deferens obstruction (ELSPBP1, DEFB129, PTGDS). Such an assay could be an excellent tool for the differential diagnosis of azoospermia in combination with routine clinical tests.

Chapter 2:

Proteomic characterization of normal human testicular interstitial fluid

I. Overview of experimental design:

Deep profiling of normal human TIF using mass spectrometry-based proteomics was conducted to identify proteins involved in testicular function. Post-meiotic germ cell-specific proteins, which could serve as biomarkers of spermatogenesis, were of particular interest. Two separate mass spectrometry analyses were performed of TIF samples collected from obstructive azoospermia patients with quantitatively and qualitatively normal spermatogenesis (NS). First, 3 NS TIF samples were analysed by High Performance Liquid Chromatography (HPLC-MS/MS) to explore the proteomic composition of human TIF. For the second analysis, Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS/MS), the number of samples and resolution were increased to maximize proteome coverage. The identified proteins were matched against transcriptomic and proteomic databases to determine the best candidate biomarkers of male reproductive potential.

II. Materials and methods:

II.1. Characterization of the human TIF proteome by HPLC-MS/MS:

II.1.1. Sample preparation:

TIF samples were collected during micro-TESE procedures by experienced surgeons. Before dissecting the tubules, TIF was recovered adjacent to the tubules by applying gentle pressure on the tissue, collected using a micro-syringe and snap frozen over dry ice (1ml) before storing at -80° pending analysis. The USP3 protocol (Hughes et al. 2014) was used to prepare 200 µg of protein from three individual TIF samples collected from patients diagnosed with obstructive azoospermia/ normal spermatogenesis. For treatment, samples were transferred to a 2 ml LoBind deep well plate (Eppendorf, Hamburg, Germany). Samples were reduced with 2 M dithiothreitol (DTT, 50 mM final concentration) for 1 h at 37 °C, followed by alkylation with 1M iodoacetamide (100 mM final concentration) for 30 minutes at RT. The samples were quenched with 2M DTT (250 mM final concentration). A fresh stock solution of Sera-Mag carboxylate beads (Thermo Fisher Scientific) was prepared beforehand at a concentration of 20

g / L and stored at 4 °C. To each sample, 4 µl of the bead solution was added, followed by acetonitrile (ACN) to a final concentration of 70% (v/v). Sample-bead mixtures were incubated for 20 minutes at RT to allow the proteins to adsorb onto the beads. The plate was then placed on a magnetic rack (Ambion, Thermo Fisher Scientific) to pellet the beads in the bottom of the wells. The beads were washed twice with 500 µl of 70% ethanol and once with 500 µl of ACN.

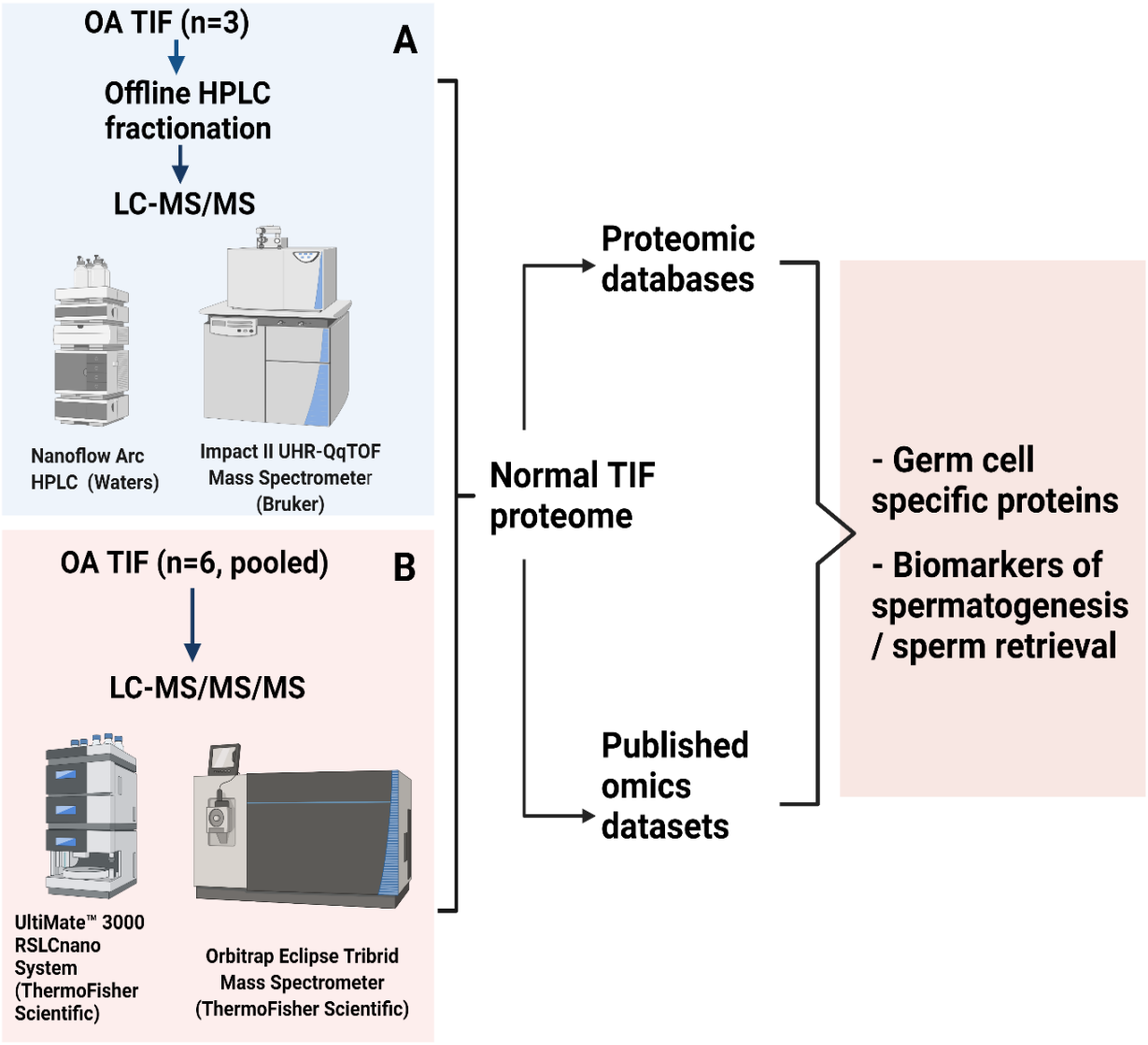


Figure 1: Mass spectrometry-based characterization of normal TIF proteome and in-silico discovery of candidate biomarkers of testicular activity. ***A:*** offline reversed phase high performance liquid chromatography (HPLC) fractionation is used to increase proteome coverage/ number of proteins identified by tandem mass spectrometry in 3 'normal' TIF samples. ***B:*** A high-throughput LC-MS/MS/MS workflow is used to analyse a pool of 6 TIF samples. An advanced feature, "Real-Time Search", interrogates every MS2 spectrum against a database of choice, in parallel with the acquisition of the next MS2 scan, and directs MS3 scans.

A CentriVap (Labconco, Kansas City, MO, USA) was then used to completely evaporate the ACN from the wells. For enzymatic digestion, 40 µl of the digestion buffer (10% 2-2-2-trifluoroethanol /100 mM NH_4HCO_3) containing 4 µg Trypsin-gold (Promega, V5280) and 4 µg Lys-C (Wako) were added to each sample. The plate was then incubated on a shaking dry block (ThermoMixer C, Eppendorf) at 37 °C for 1 h (1200 rpm). The plate was placed on the magnetic rack and the resulting peptide mixture contained in the supernatant was collected. 50 µl of 2% dimethyl sulfoxide (Sigma) was added to the beads to elute any remaining peptides. 200 µl unfiltered pipette tips were prepared with six plugs of C18 resin (3M Empore, 66883-U) and pre-equilibrated by sequential washes with 100 µl 80% ACN/5% formic acid (FA), 50% ACN/5% FA, and 5% FA. The peptide mixtures were then added to the prepared tips, placed into fresh Eppendorf tubes. Peptides bound to the resin matrix were washed twice with 5% FA. Sequential elutions with 50 µl of 50% ACN/5% FA, then 50 µl of 80% ACN/5% FA were performed on a benchtop centrifuge at 500× g. The eluates were centrifuged to dryness in Centrivap MS vials prior to offline HPLC fractionation.

II.1.2. High-pH HPLC fractionation:

The peptide samples were reconstituted in 40 µl 5 mM ammonium formate buffer, pH 10 before undergoing high pH reversed-phase analysis on an Agilent 1100 Series HPLC system equipped with a variable wavelength detector (280 nm). An XBridgeTM Shield C18 column (10 x 100 mm, 3.5 µm bead size, Waters) was used for the fractionation. Peptides were separated on a gradient of mobile phase A (5 mM ammonium formate, pH 10) and a mobile phase B (100% ACN), from 3% to 35% over 60 mins at a flow rate of 0.1 ml/min. Fractions were collected every minute across the gradient length and concatenated into 24 fractions, then lyophilized in a SpeedVac concentrator.

II.1.3. Mass spectrometry and data analysis:

Peptides were reconstituted in MS loading buffer (2% ACN/0.1% FA) and separated using a nano-flow HPLC (M-class, Waters) coupled to an Impact II UHR-QqTOF mass spectrometer (Bruker, Bremen, Germany) (Figure 1A) and data processing and analysis were conducted as described in Chapter I, Methods, p.31).

II.2. Investigation of the expression of AKAP4 and AKAP3 by RT-PCR:

We used RT-PCR to evaluate the expression of two testis-enriched A-kinase anchoring proteins (spermatid-specific AKAP3 and sperm flagellum-localized AKAP4) in testicular tissue collected during microsurgical TESE procedures from azoospermic men diagnosed with OA (normal spermatogenesis), MA and SCO. mRNA was extracted, treated, and reverse transcribed

as described in Chapter 1 (Methods, p.12). Primer mix was prepared by adding together 8 µl of PCR buffer, 4 µl of $MgCl_2$, 2 µl of dNTP mix, 0.6 µl of the Gold Amplitaq DNA polymerase (GeneAmp Gold RNA PCR Core Kit 4308207, PE Biosystems), 73.4 µl of RNase free water, 2 µl of the forward-primer and 2 µl of the reverse primer. Primer sequences were as follows: AKAP3 forward primer: 5'- TGGCCCGCAAAGAGATCAAT- 3', AKAP3 reverse primer: 5'- CTGGAACATGCAGAGACGGT- 3', AKAP4 forward primer: 5'- TGGCGTACTCTGATACTACAATG- 3', AKAP4 reverse primer: 3'- AGCTGGAACTAGCAGCATCC-3' 0.2 mL Eppendorf PCR tubes containing 1µl of cDNA and 11.5 µl of primer mix each were incubated in the thermocycler for 36 PCR cycles (9 min at 95°C, 45s at 94°C, 45s at 60°C, 45s at 72°C, 7 min at 72°C), followed by cooling at 4°C for 1hr. PCR products were onto a 1.5% agarose gel and resolved at 110V for 70 min. Gels were imaged using a Uvidoc D55 Gel Documentation System (UVITEC).

II.3. Label-free high-resolution analysis of "normal" TIF:

II.3.1. Sample extraction and digestion:

The protein content of the samples was determined by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). A pool of 6 OA TIF samples was used to prepare 10 µg of proteins, which were subsequently dissolved in 25 µl of ammonium bicarbonate (Fluka) containing 0.1 % ProteasMax TM (Promega). Cysteines were reduced with 5 mM DTT (30 min at 50°C) and modified with 10 mM iodoacetamide (30 min at 24°C). The reaction was quenched with an excess of cysteine and trypsin was added to a final concentration of 25 ng/µl in a total volume of 100 µl. After an incubation of 16 hours at 37°C, the digestion was stopped by adding TFA to a final concentration of 1%. The sample was purified using a C18-ZipTip (Millipore), then vacuum dried and finally dissolved in 10 µl of 0.1% TFA prior to MS analysis.

II.3.2. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS/MS) and data analysis:

The peptide mixture was loaded onto a 50 cm µPAC^{TM} C18 column (Pharma Fluidics, Gent, Belgium) in 0.1% formic acid (Fluka) at 35°C. Peptides were eluted with a linear gradient of ACN from 3% to 44% over 240 min followed by a wash with 72% acetonitrile at a constant flow rate of 300 nl/min (ThermoScientific™UltiMate™3000RSLCnano) and infused via an Advion TriVersa NanoMate ionising source (Advion BioSciences, Inc. New York, USA) into an Orbitrap Eclipse Tribrid mass spectrometer (ThermoScientific). The mass spectrometer was operating in positive-ionization mode with a spray voltage of the NanoMate system set to 1.6 kV and source temperature at 300°C. Using the data-dependent acquisition mode, the

instrument performed full MS scans every 2.5 seconds over a mass range of m/z 400–1600, with the resolution of the Orbitrap set to 120000. The RF lens was set to 30%, auto gain control (AGC) was set to standard with a maximum injection time of 50 ms. In each cycle, the most intense ions (charge state 2-6) above a threshold ion count of 5.000 were selected with an isolation window of 0.7 m/z for CID-fragmentation at a normalised collision energy of 35% and an activation time of 10 ms. Fragment ion spectra were acquired in the linear IT with a scan rate set to "rapid" and mass range to normal and a maximum injection time of 100 ms. After fragmentation, the selected precursor ions were excluded for 20s for further fragmentation. From each MS/MS cycle, up to ten fragment ions were selected with an isolation window of 3 m/z for HCD-fragmentation at normalized collision energy of 55%. MS3-fragment ion spectra were acquired in the Orbitrap with a resolution of 50.000. The mass range was set to 100 – 500 m/z, maximum injection time to 100 ms, and AGC to 300. Data was acquired with Xcalibur 4.3.73.11. (Thermo Fisher Scientific) and analyzed with Proteome Discoverer 2.4.0.305 (Thermo Fisher Scientific). Sequest HT (Proteome Discoverer version 2.5.0.400; ThermoFisher) was used to search against the Uniprot_human database (*20201007, 70502241 residues, 214889 sequences*). Q-values (minimum false discovery rate) were deduced from peptide spectra matches (PSMs). Protein identification confidence ranges were defined as follows (High: confidence better than 99% (q-value <= 0.01), Medium: confidence better than 95% (q-value <=0.05, Low: confidence below 95% (q-value >0.05). The resulting dataset was exported from Xcalibur and processed with RStudio (v.1.2.5042-1). Gene names were extracted from the 'description' column using the 'grepl' function and non-annotated proteins were filtered out. Rows with different accession numbers and redundant gene names and gene descriptions were excluded using the anti_join function from the R package dplyr.

III. Results:

- ***Characterization of normal human TIF proteome:***

To characterize the human TIF proteome, samples from 3 men with normal testicular function were analysed. From this analysis, a total of 4720 proteins were identified. The complete dataset has been published by O'Donnell et al. 2021 (SI Dataset 5). By comparison with the lists of tissue and cell-type enriched proteins from the Human Protein Atlas, 84 testis-enriched proteins were identified, 30 of which are enriched in post-meiotic germ cells (Fig. 2 A). The Plasma Proteome Database (Muthusamy et al. 2005, Nanjappa et al. 2014) was queried to determine whether these adluminal germ cell markers are detectable in blood by mass

spectrometry. A total of 22 proteins, including ACTL7B, AKAP3, AKAP4, HSPA4L, and LDHC were found to be present in human blood plasma (Fig. 2 A).

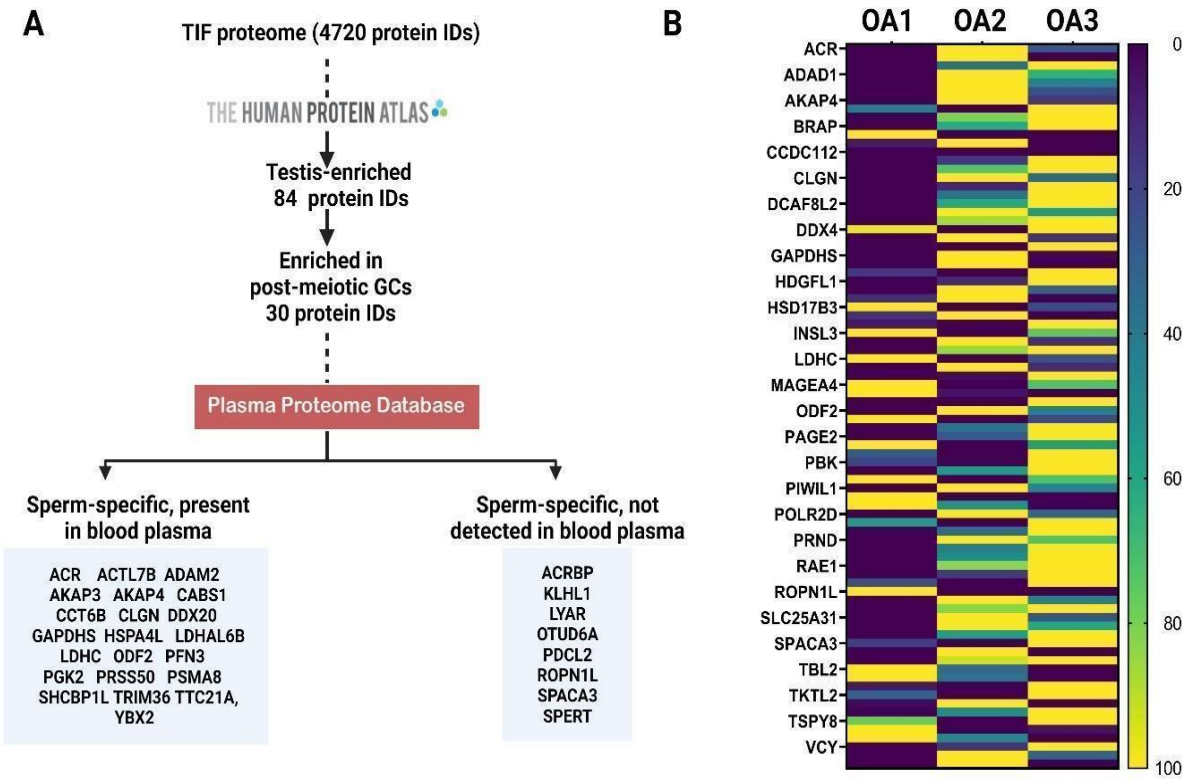


Figure 2: Identification of biomarkers of post-meiotic germ cells in TIF. A: Testis and germ cell-enriched proteins were determined by matching identified gene names to downloadable datasets from https://www.proteinatlas.org/. Presence in blood plasma was verified by querying the Plasma Proteome database http://www.plasmaproteomedatabase.org/ B: Normalized relative abundance of the 84 testis-enriched proteins (labels shown for 28 proteins) highlighting inter-sample variability

AKAP3 and AKAP4 were among TIF proteins present in blood plasma (Fig. 2. A) and highly specific to the spermatozoa in the testis (Human Protein Atlas), making them good candidate markers of sperm retrieval. The expression of AKAP3 and AKAP4 was evaluated in testicular tissue by RT-PCR (Fig.3). At the transcriptional level, AKAP4 appears to be decreased compared to MA and normal spermatogenesis. For AKAP3, the difference between the three groups is more noticeable as stronger bands are seen for NS samples compared to MA and SCO. There is also a noticeable difference in band signal between the MA and SCO samples. These results indicate that AKAP3 and AKAP4 are present at the mRNA level in SCO, albeit at a low level.

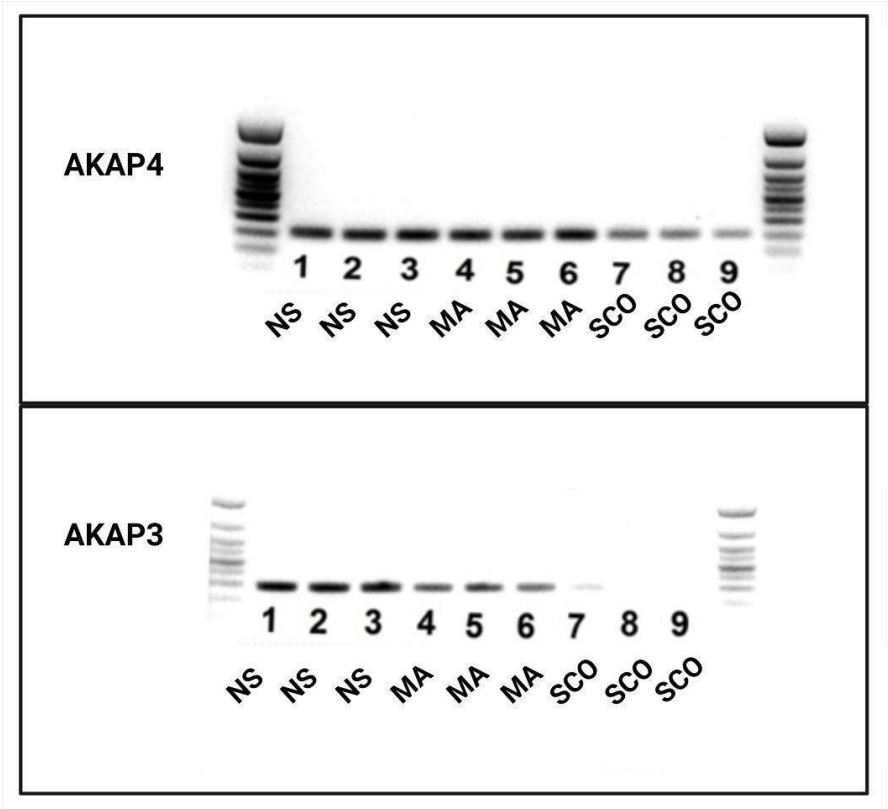


Figure 3: Expression of the AKAP4 and AKAP3 genes in testicular tissue from men diagnosed with mixed atrophy (MA), Sertoli cell only (SCO), and normal spermatogenesis (NS). RT-PCR products were resolved on a 1.5% agarose gel at 110V for 70 min.

- ***Deep proteomic profiling of normal human TIF by LC-MS/MS/MS***

A novel mass spectrometry workflow was used for global mapping of the TIF proteome (n=6, pooled). Over 25,000 proteins were identified, including 16,390 annotated proteins with a wide range of peptide spectra matches (PSMs) (Fig. 4 A). Among these, 1,578 proteins were found to be testis-enriched (Fig. 5, Fig. 4 B), amounting to 70% of all proteins listed as testis-enriched by the Human Protein Atlas. Proteins were then further categorized according to their expression in different germ cell types. Genes with mRNA levels at least four-fold higher in a particular cell type compared to the average level in all other cells and tissues were considered 'elevated'. In total, 213 proteins were found to be elevated in spermatogonia, 482 in spermatocytes, 927 in early spermatids and 727 in late spermatids (Fig. 5) with significant overlap in enriched proteins between post-meiotic germ cells. Additionally, 27 proteins were categorized as Sertoli cell enhanced (Table 1) and 11 were Leydig cell enhanced (Table 2).

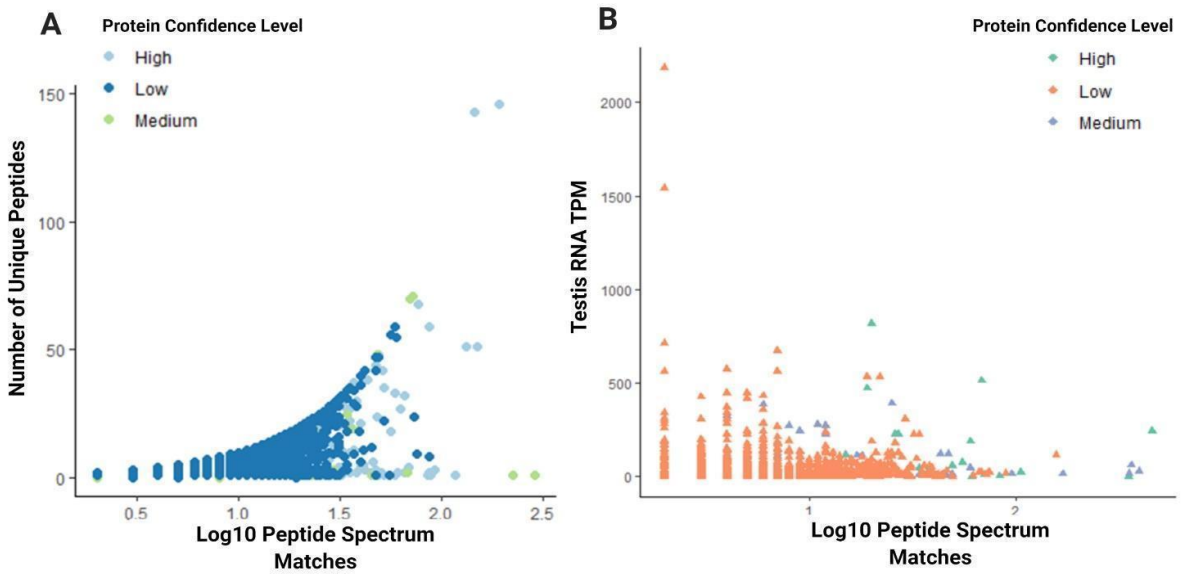


Figure 4: Deep analysis of normal TIF proteome (n=6, pooled) A: Log 10 of peptide spectral matches (PSM) of 16930 identified proteins plotted against the number of unique peptides. B: Distribution of testis mRNA levels (Human Protein Atlas, Tissue Atlas) and protein false discovery rate confidence level of 1578 testis-enriched genes.

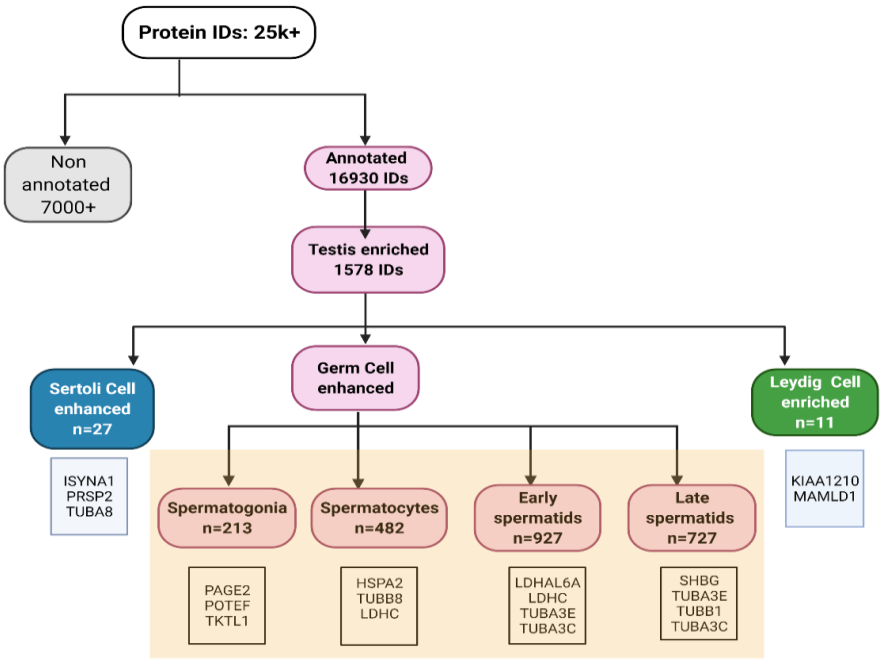


Figure 5: Testis enhanced proteins in TIF. Normal TIF proteome was matched with downloadable cell-type elevated protein lists from https://www.proteinatlas.org/, accession numbers and gene names corresponding to proteins enhanced in early spermatids (n=927) and late spermatids (n=727) are listed in supplementary table 3.

Table 1: Sertoli cell enhanced proteins detected in normal human TIF (Single cell type Atlas, https://www.proteinatlas.org/)

Gene	Gene description	Expressed in Germ cells	Expressed in Leydig cells
ACE2	Angiotensin I converting enzyme 2	No	Yes
BEND2	BEN domain containing 2	Yes, spermatocyte / spermatogonia enhanced	Yes, cell type enhanced
C16orf95	Chromosome 16 open reading frame 95	Enriched in round spermatids	Yes
C5orf34	Chromosome 5 open reading frame 34	Yes	Yes
CCDC81	Coiled-coil domain containing 81	Yes	No
CD300LG	CD300 molecule like family member g	Yes	Yes, cell type enriched
CDCA2	Cell division cycle associated 2	Yes, germ cell enriched	No
CILP2	Cartilage intermediate layer protein 2	Yes	Yes
DMC1	DNA meiotic recombinase 1	Yes	Yes
DNMT3B	DNA methyltransferase 3 beta	Yes	Yes
FAM153 B	Family with sequence similarity 153 member B	Yes	Yes
FAM170 A	Family with sequence similarity 170 member A	Yes	No
FATE1	Fetal and adult testis expressed 1	Yes, spermatocyte / spermatid enhanced	No
FBXO47	F-box protein 47	Yes, spermatocyte/ spermatogonia enhanced	No
HUS1B	HUS1 checkpoint clamp component B	Yes, spermatocyte/ spermatogonia enhanced	Yes
ISYNA1	Inositol-3-phosphate synthase 1	Yes, spermatocyte/ spermatogonia enriched	Yes
MAGEC1	MAGE family member C1	Yes, spermatocyte/ spermatogonia enhanced	No
NPTX2	Neuronal pentraxin 2	Yes	Yes
NUP210L	Nucleoporin 210 like	Yes, germ cell enriched	Yes
PCDHA1	Protocadherin alpha 1	No	No
PRPS2	Phosphoribosyl pyrophosphate synthetase 2	No	Yes, cell type enriched
RIMS4	Regulating synaptic membrane exocytosis 4	Yes	Yes
SSX1	SSX family member 1	Yes, spermatocyte /spermatogonia enriched	No
SYCE2	Synaptonemal complex central element protein 2	Yes, germ cell enriched	No
TCF15	Transcription factor 15	Yes	No
TDRD15	Tudor domain containing 15	Yes, germ cell enhanced	No
TOPAZ1	Testis and ovary specific PAZ domain containing 1	Yes, spermatocyte/ spermatogonia enriched	No
TUBA8	Tubulin alpha 8	Yes, round / elongated spermatid enriched	No

ZNF99	Zinc finger protein 99	Yes, spermatocyte enriched	No

Table 2: Leydig cell enhanced proteins detected in normal human TIF (Single cell type Atlas, https://www.proteinatlas.org/)

Gene	Gene description	Expressed in germ cells	Expressed in Sertoli cells
MAMLD1	Mastermind like domain containing 1	Yes	Yes
CD300LG	CD300 molecule like family member G	Yes	Yes, cell type enhanced
KCNG3	Potassium voltage-gated channel modifier subfamily G member 3	Yes	No
CST9L	Cystatin 9 like	Yes, germ cell enhanced	No
KIAA1210	KIAA1210	Yes, germ cell enhanced	No
GREB1L	Growth regulation by estrogen in breast cancer 1 like	Yes	Yes
CHODL	Chondrolectin	Yes	No
BEND2	BEN domain containing 2	Yes, spermatocyte / spermatogonia enhanced	Yes, cell type enhanced
LRRN3	Leucine rich repeat neuronal 3	Yes	No
LHCGR	Luteinizing hormone/choriogonadotropin receptor	No	No
OBP2B	Odorant binding protein 2B	Yes, spermatid enhanced	No

- ***Selection of biomarkers of spermatogenesis:***

From the intersection of the two mass spectrometry datasets, 20 candidate biomarkers of testicular function in TIF were selected based on a number of criteria, including relative abundance, expression in germ cells at the mRNA and protein level (Human Protein Atlas), presence in seminal plasma of fertile men (mass spectrometry data, Chapter 1), and presence in blood plasma (Plasma Proteome Database, Muthusamy et al. 2005, Nanjappa et al. 2014). Proteins that are highly expressed in adluminal germ cells, are very highly enriched, and have been detected in human blood plasma were picked as candidate markers for male fertility/ sperm retrieval (Table 3). Testis-enriched proteins that are not testis-specific, are expressed in all testicular cell types, are expressed in all germ cells, or are unlikely to be present in blood plasma, were disregarded.

Table 3: Candidate biomarkers of testicular function in normal human TIF. *Relative abundance was estimated based on the average iBAQ value (LC-MS/MS analysis of OA, n=3), ** False discovery rate confidence was determined by q-values of peptide spectral matched (LC-MS/MS analysis of OA, n=6, pooled), ***: proteins expressed in round and elongated spermatids but mostly undetected in spermatogonia, spermatocytes and somatic cells

Gene name	Gene description	Mw (kDa)	# Peptides	# Unique Peptides	Relative abundance 20 fold ranges*	Protein FDR Confidence**	Testis specific	Spermatid specific***
ACR	Acrosin	45.84	2	2	++	Low	Yes	Yes
ACRBP	Acrosin binding protein	57.57	5	5	++	Low	Yes	Yes
ACTL7B	Actin like 7B	45.23	6	6	++	Low	Yes	Yes
ADAM2	ADAM metallopeptidase domain 2	75.58	5	5	++	Low	Yes	Yes
AKAP3	A-kinase anchoring protein 3	94.75	12	12	++	Low	Yes	Yes
AKAP4	A-kinase anchoring protein 4	93.44	11	11	++	Medium	Yes	Yes
CABS1	Calcium binding protein, spermatid associated 1	43.00	3	3	++	Low	Yes	Yes
CETN1	Centrin 1	19.57	5	5	++	Medium	Yes	Yes
GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	44.50	5	4	++	Medium	Yes	Yes
KLHL10	Kelch like family member 10	68.94	11	11	++	Low	Yes	Yes
LDHAL6B	Lactate dehydrogenase A like 6B	41.94	7	6	++	Medium	Yes	Yes
LDHC	Lactate dehydrogenase C	36.31	9	5	+++	High	Yes	Yes
ROPN1B	Ropporin-1B (Fragment) OS=Homo sapiens OX=9606	14.8	4	4	++	Low	Yes	Yes
SHCBP1L	SHC binding and spindle associated 1 like	72.63	5	1	+++	Low	Yes	Yes
YBX2	Y-box binding protein 2	38.51	6	2	+++	High	Yes	Yes
DDX4	DEAD-box helicase 4	75.82	7	4	+++	Medium	Yes	No
HIST1H1T	Histone cluster 1 H1 family member t	22.019	5	5	+++	Medium	Yes	No
MAGEA4	MAGE family member A4	34.89	3	3	+++	Low	Yes	No
SMC1B	Structural maintenance of chromosomes 1B	135.84	8	1	++	Low	Yes	No
STK31	Serine/threonine kinase 31	113.01	11	1	++	Medium	Yes	No

IV. Discussion:

Using high-resolution mass spectrometry, we identified thousands of proteins in 'normal' human testicular interstitial fluid. The first analysis by HPLC-MS/MS of 3 individual NS TIF samples resulted in the identification of 4720 proteins. Despite evident inter-sample variability (Fig. 2. B), many adluminal germ cell proteins were detected at relatively high levels (see Table 3). The second analysis of a pool of 6 NS TIF samples by LC-MS/MS/MS led to the identification of over 16000 annotated proteins. Although sample pooling can have considerable drawbacks in quantitative/ comparative proteomics by affecting the ratios of certain analytes (Molinari et al. 2018), it is less disadvantageous when the only objective of the MS run is protein identification. Given the high resolution of the used MS technology, we were able to detect a large number of proteins despite the complexity of the pooled sample. By comparing the two resulting datasets to cell-type enriched gene lists from the Human Protein Atlas (https://www.proteinatlas.org/), we were able to determine germ-cell enriched gene products present in TIF. During micro-TESE, very special care was taken to aspire TIF without damaging the adjacent seminiefeorus tubules. Contamination of TIF samples with luminal fluid during collection is therefore unlikely. Rodent studies which recovered TIF by percolation through a small incision in the tunica albuginea, leaving the seminiferous tubules completely untouched, also reported the presence of proteins specific to adluminal germ cells (Stanton et al. 2016, Aldahhan et al. 2019). Post-meiotic germ cell proteins found in TIF would in theory have access to the general circulation, and therefore could be blood markers of male infertility (O'Donnell et al. 2021a). Many of these are Cancer / Testis antigens (CTAs); these are products of genes that are exclusively expressed in male germ cells in healthy individuals but are aberrantly expressed in various cancers (Simpson et al. 2005). The abnormal expression of these proteins in certain tumours could be linked to their role in meiosis and chromosome segregation, making them potential therapeutic targets for cancer treatment (Whitehurst et al. 2014). Spermatid-enhanced proteins present in TIF (Supp. Table 3) include several over-represented protein families with reported roles in spermatogenesis and male fertility:

- ***Tubulins:***

Early and late spermatid-enriched tubulins TUBA3C, TUBA3E, TUBA8, TUBB1, and TUBB8 were identified in normal TIF (Supp. Table 3, Fig. 5). Tubulins are the main components of microtubules. Axonemes of spermatozoa and manchettes of spermatids contain microtubules, composed of diverse post-translationally modified tubulins (Kierszenbaum et al. 2002). Furthermore, alpha and beta tubulins are involved in spindle formation during cell

division (Mochida et al., 1998). There is mounting evidence that the structure and function of microtubules are affected by multiple αβ-tubulin isotypes (Chawan et al., 2020). Bhagwat et al. reported that the sperm of asthenozoospermic individuals contain less acetyl tubulin than normal sperm (Bhagwat et al. 2014). TUBB8 (Tubulin beta 8 class VIII) is cell-type enhanced in spermatogonia, spermatocytes, and round spermatids, and has now been found in TIF. Moreover, TUBB8 is essential for oogenesis, fertilization, and female fertility, as mutation of the Tubb8 gene results in meiotic arrest in human oocytes (Feng et al., 2016).

●***Solute carrier protein family:***

A total of 13 solute carrier proteins are present in TIF (SLC1A6, SLC22A14, SLC22A16, SLC25A52, SLC26A8, SLC2A5, SLC30A3, SLC35E4, SLC35E4, SLC36A3, SLC45A2, SLC6A16, SLC9B1) (Supp. Table 3). A number of solute carrier proteins are highly expressed in germ cells and have been investigated for possible roles in spermatogenesis. HCO3-membrane transporters from solute carrier 4-SLC4 and solute carrier 26-SLC26 protein family are abundantly present in the male reproductive tract, where they contribute significantly to the ionic equilibrium of tubular fluids (Barnardino et al., 2019). Maintaining an ideal ionic balance is crucial for sperm production. HCO3- and H+ play an important role in pH homeostasis in male tissues (Pastor-Soler et al. 2005). SLC32 and SLC36 protein families are involved in the transport of neutral amino acids and were reported to be highly expressed in the testis in mouse models (Sundberg et al. 2008). SLC22A14 has a crucial role in spermatogenesis and in male fertility and is essential for flagellar formation and sperm motility in mice (Maruyama et al. 2016). The expression of Solute carrier family 9, subfamily A, member 3, regulator 1 (SLC9A3R1), involved in sperm ion regulation, in ejaculated spermatozoa correlates negatively to litter size in boars (Kim et al. 2019).

●***Spermatogenesis-associated protein family:***

Spermatogenesis-associated proteins are encoded by a number of genes with developmental expression in the testis, often regulated by FSH (Onisto et al. 2001). Our analysis showed TIF contains numerous late spermatid-enriched spermatogenesis-associated proteins, including SPATA3, SPATA4, SPATA6, SPATA9, SPATA12, SPATA16, SPATA19, and SPATA24 (Supp. Table 5). SPATA4, also known as TSARG2, is a seminiferous tubule-specific protein expressed in spermatogonia and spermatocytes (Jiang et al., 2015). SPATA4 has an apoptosis suppressing activity and could be a factor in testicular oncogenesis and cryptorchidism (Liu et al. 2004). SPATA9 is a post-meiotic cell-enriched membrane protein. In humans, SPATA9 is absent from the testis of SCO men, and the mouse homologous gene, NYD-SP16 is developmentally regulated in the testis, hinting at an important role in male

fertility (Cheng et al., 2003). SPATA19 was also reported to be “over-expressed” in mouse post-meiotic cells (Nourashrafeddin et al. 2014).

●***Sperm-associated antigens:***

Sperm-associated antigens (SPAGs) are a grouping of testis-enriched proteins, involved in the formation and maintenance of the mitotic spindle and axonemal ciliary dynein arms in the flagellum (Gruber et al., 2002, Shao et al. 2001). SPAG 1, enriched in spermatocytes, regulates the GTP and PKC-dependent ERK1/2 signal transduction pathways in spermatogenesis and fertilization (Lin et al., 2001) in addition to a role in the orchestration of mitotic cytoskeletal architecture in mouse oocytes (Huang et al. 2016). An increased expression of SPAG1 is associated with an unfavourable prognosis in prostate cancer and might play a role in tumour expansion to pelvic lymph nodes (Shamsara and Shamsara, 2020). SPAG1 was also reported to promote the motility of cancer cells in pancreatic ductal adenocarcinoma (Neesse et al. 2006). SPAG5, also referred to as astrin, is enriched in early and late spermatids. SPAG5 plays a role in chromatid segregation and binds to ODF1 in the outer dense fibres of the sperm tail (Shao et al. 2001, Fitzgerald et al., 2006). Similar to SPAG1, SPAG5 has oncogenic effects in several human cancers (liver, renal, pancreatic) (He et al. 2020). It was found to exert a tumour-promoting activity in triple-negative breast cancer, with its upregulation being linked to lymph-node metastasis and poor prognosis (Li et al. 2019). Spermatid-enriched SPAG6 is another component of the sperm tail axoneme, essential for sperm motility (Sapiro et al. 2002, Ye et al., 2019). SPAG6 induces tumour proliferation in certain cancers (Wang et al. 2008, Altenberger et al. 2017). Increased SPAG6 expression is associated with poor prognosis in Burkitt Lymphoma (aggressive B-cell tumor) (Zhang et al., 2020). SPAG17 is also enriched in elongating spermatids (Supp. Table 5). Like SPAG5 and SPAG6, SPAG17 is crucial for sperm tail motility. Spag17 knockout mice are infertile because of spermatogenic arrest at the spermatid stage and head and tail deformities of the produced spermatozoa (Kazarian et al. 2018). The expression of SPAG17 was also reported to be essential for male fertility and associated with litter size in goats (Zhang et al., 2020).

●***Testis expressed proteins (TEX):***

Testis expressed proteins are products of germ-cell specific genes with established roles in meiosis (Boroujeni et al. 2015). Testis expressed 101 (TEX101) is a testis-enriched glycoprotein highly expressed in germ cells (Yoshitake and Araki, 2020). TEX101 is essential for spermatogenesis, the structure of sperm plasma membrane, and fertilization (Endo et al., 2016) and was validated as a biomarker of testicular function/ diagnosis of azoospermia (Drabovich et al. 2014, Korabakis et al. 2015). TEX11 is highly expressed in the testis and is

involved in the maintenance of chromosome synapsis. In men, mutations of the TEX11 gene are linked to non-obstructive azoospermia and the lack of TEX11 expression causes meiotic arrest (Yatsenko et al. 2015, Yang et al. 2015, Sha et al. 2018). TEX15 (Testis expressed 15, meiosis and synapsis associated) mutations were also found in a case study of two infertile brothers affected by idiopathic NOA (Colombo et al. 2017). Moreover, TEX11, TEX12, TEX14, and TEX15 were reported to be down-regulated in SCOS testicular tissue (Boroujeni et al. 2018). TEX13 is germ cell-specific and plays a potential role in gene regulation during spermatogenesis (Kwon et al. 2016, Kim et al. 2021).

- ***Tektins:***

Tekins 1, 3 and 4 are spermatid enriched filamentous proteins of the sperm flagellum (Larsson et al. 2000, Angshumoy et al. 2008, Roy et al. 2009). In mice post-meiotic germ cells, the cellular localization of TEKT1 shifts from the centrosome in round spermatids to the caudal end in elongating spermatids, hinting at a role in the formation of the flagellar axoneme (Larsson et al. 200). Given their established functions in supporting sperm progressive motility and coordinated beating of the flagellum in rodent models, TEKT3 and TEKT4 have been suggested as potential markers for asthenozoospermia (Roy et al. 2007, Angshumoy et al. 2008, Takiguchi et al. 2011, Gao et al. 2017).

- ***Transketolase like 1 and 2:***

TKTL1 is a spermatogonia enriched enzyme implicated in glucose metabolism, which has been identified as a spermatogenesis biomarker in seminal plasma (Rolland et al. 2013). There has been an association between TKTL1 and carcinogenesis, as the protein was found to promote human cancer cell proliferation by activating the pentose phosphate pathway (Xu et al. 2009). High expression of TKTL1 is associated with lower disease-free survival rates in urothelial cancer (Langbein et al. 2006), non-small cell lung cancer (Kayser et al. 2011), and colorectal cancer patients (Peltonen et al. 2020). TKTL2, enriched in spermatocytes and spermatids, has similar enzymatic activity to TKTL1 and an equal potential as a cancer therapeutic target (Zhao and Zhong, 2009, Deshpande et al. 2019). TKTL2 is associated with a poor prognosis for overall survival in ovarian cancer (Zhao et al. 2019).

Conclusion:

Spermatogenesis involves the epigenetic, transcriptomic, and post-transcriptomic regulation of over 2300 genes (Carrell et al. 2016; Agarwal et al.; 2016). The temporally regulated expression of these genes in spermatogonial stem cells; spermatozoa and testicular somatic cells is essential for the spermatogenic process (Oatley and Brinster, 2008, Carrell et al. 2016). The past two decades have seen many omics-driven advances in our understanding of the molecular mechanisms of mammalian spermatogenesis. This study adds to the existing knowledge by deep profiling of the human TIF proteome using two mass spectrometry workflows to maximize the sensitivity and reliability of protein identification. Hundreds of spermatogenic proteins are present in TIF, which suggests that it can be reflective of spermatogenic function and serve as a source of biomarkers of male reproductive potential. Many of these proteins were reported in blood plasma (http://www.plasmaproteomedatabase.org/), and could potentially be investigated as blood markers of male fertility. Furthermore, the characterization of the TIF proteome could offer new insights into Sertoli cell-germ cell interactions and the underlying molecular mechanisms of testicular failure.

Chapter 3:

Discovery of biomarkers of spermatogenesis and male fertility in TIF

I. Overview of the experimental design:

Using comparative proteomics, we compared the proteomic profile of TIF collected from men with non-obstructive azoospermia (MA, SCO) and normal TIF (NS). Sample selection criteria and clinical parameters are summarized in supplementary tables 1 and 2. In the first analysis, LC-MS/MS was performed on 8 individual samples per group, to determine the relative abundance of detectable proteins in NS, MA, and SCO. For better proteome coverage and statistical significance, we performed a second analysis with a sample size of 12 per group and added an offline HPLC fractionation step of pooled samples from each group (Figure 1). A third analysis was carried out using Tandem Mass Tags (TMT) isobaric labelling LC-MS/MS/MS, which allows for multiplexing and offers more accurate relative quantification (Figure 2). Tissue/ cell specificity of significantly differentially expressed proteins was determined using proteomic databases in order to identify biomarkers of testicular failure and sperm retrieval.

II. Materials and methods:

II.1. LC-MS/MS analysis:

Sample preparation and mass spectrometry analysis of TIF samples from patients diagnosed with OA/ normal spermatogenesis (NS), MA, and SCO (n=8/ group) were performed as described in Chapter 1, methods section. Proteomic data was analysed using the LFQ-Analyst platform (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/). To decrease the false discovery rate in group comparisons, p-values were corrected using the Benjamini-Hochberg procedure. p-values< 0.05 were considered significantly differentially expressed. Gene names of the identified proteins were matched to lists of testis and testicular cell type enriched proteins downloadable from the Human Protein Atlas database (https://www.proteinatlas.org/).

II.2. Label-free high-resolution LC-MS/MS:

- ***Sample preparation and protein digestion:***

Total protein concentrations of all TIF samples were determined using the BCA (Pierce) protein assay kit, with BSA as standard. The samples were thawed on ice and diluted in MilliQ water. A volume equivalent to 30 g of total protein content was pipetted from each sample into a numbered tube. 12 ul of SDS lysis buffer (5% SDS/50 mM Tris-HCl, pH 7.5/50 mM Tris(2-carboxyethyl) phosphine -hydrochloride (pH 7), /200 mM 2-chloroacetamide) were added to each diluted sample. After boiling for 3 minutes at 95°C, the samples were left to cool at room temperature for 5 minutes. Carboxy agarose beads were pre-washed twice in 500 µl of MilliQ H2O and then reconstituted in an equal volume of water. 20 ul of the Carboxy agarose beads solution were pipetted into each tube, and neat acetonitrile was added resulting in a final 70% v/v, then incubated at RT in a ThermoMixer C (Eppendorf) at 400 rpm for 20 min. During this stage, the peptides in the sample bind to the surface of the beads. For the next 2 minutes, the samples were placed on a magnetic rack in order to cause the protein-covered beads to adhere to the bottom of the tubes. The beads were washed twice with 200 µl of 70% ethanol, then once with 200 µl of neat acetonitrile. To ensure the complete removal of all acetonitrile, samples were lyophilized. Each sample was then treated with 50 µl of digestion buffer containing Trypsin and Lysine C, then sonicated in an ultrasonic bath for 2 minutes prior to incubation in ThermoMixer C for 1 hour at 37°C, shaking at 400 rpm. Following digestion, the sample rack was repositioned on the magnetic rack and 50 µl of MilliQ water is added to each tube. To remove the peptides from the beads, the samples were sonicated for 1 minute, and the supernatants were collected. 5µl of each supernatant was transferred into a LoBind Eppendorf tube to form four pools for the different groups (OA, MA, SCO, SA) (Figure 1). C18 stage tips were prepared by adding two plugs of 3M Empore resin (#2215). 20 µl of Isopropanol was added and the tips were centrifuged for 2 min at 1000 x g to pre-equilibrate. Next, 50 µl of the following buffers (80% acetonitrile/5% formic acid, 50% acetonitrile/5% formic acid, 5% formic acid in MQ water) were added in order to the tips before spinning at 1000 x g for 2-3 min each time, ensuring that a small volume remains on top of the resin and discarding the flowthrough. The individual peptide samples were loaded into the pre-equilibrated tips and centrifuged at 800 x g for 2 min until only a small volume remained on top of the resin plug. After two washes with 5% formic acid, the tips are placed into Lobind peptide tubes and the peptides were eluted with 50 µl of 50% acetonitrile/5% formic acid then with 50 µl of 80% Acetonitrile/5% formic acid for 1-2 min at 800 x g. The eluate was transferred into mass spectrometry vials and frozen at 80°C before lyophilizing down to dryness before analysis.

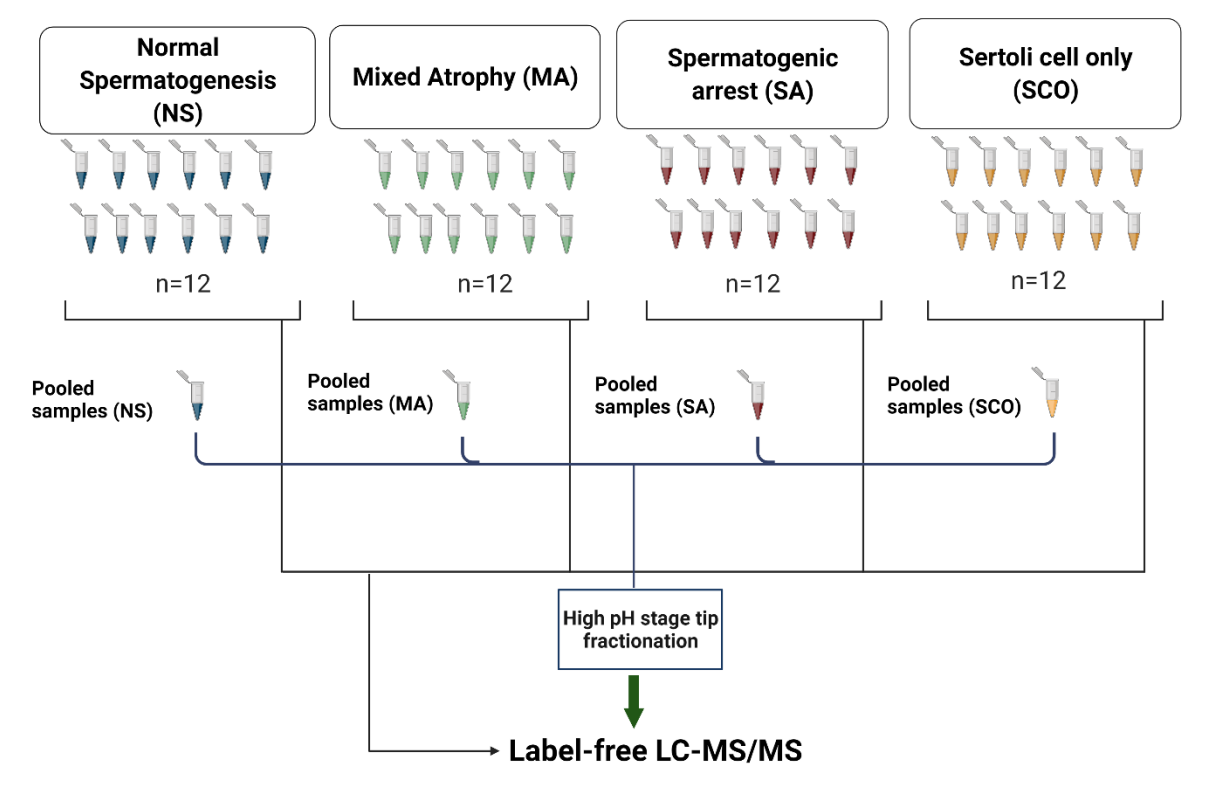


Figure 1: Label-free high-resolution LC-MS/MS analysis of testicular interstitial fluid from men diagnosed with obstructive azoospermia/ normal spermatogenesis (NS), mixed atrophy (MA), spermatogenic arrest (SA), and Sertoli cell only (SCO). Pooled samples from each group were subjected to an additional fractionation step prior to mass spectrometry analysis to produce a library of peptides to enhance protein identification in individual sample runs.

- ***Offline high pH Stage tip fractionation***

After the digestion step, 5 µl of supernatant was transferred from each sample to prepare a pool for each of the four groups (OA, MA, SA, SCO). Each of the pooled samples was subjected to high pH fractionation before MS analysis and used to prepare a library of peptides to which the MS spectra resulting from individual samples would be matched. High pH fractionation allows for deeper proteome analysis and increases the number of identified peptides (Hughes et al. 2014). To start, 200 µl tips were packed with four C18 plugs and equilibrated by spinning at 1000 x g with 20 µl of Isopropanol until only a small volume of liquid remains on top of the resin. The centrifugation step was repeated with the addition of 50 µl of 60% ACN, 10 mM ammonium formate, pH 10, then 50 µl of 10 mM ammonium formate, pH 10. In preparation for fractionation, 50 µg of lyophilized peptides were reconstituted in 50 µl of 10 mM ammonium formate, pH 10, and added to the pre-equilibrated tip. The tip was then washed twice with 50 µl of 10 mM ammonium formate, pH 10 before transferring into a new collection tube. The peptides were then eluted sequentially using buffers 1-12 (see Table 1). The collection tube was changed every time to isolate the different fractions.

Table 1: Acetonitrile gradient buffers for high-pH fractionation of peptide mixtures

Fraction #	% ACN	ACN volume (ul) for 1 ml	Volume of MQ water (ul)	Volume of 50 mM Amm. Formate, pH 10 (10 mM final conc.) (ul)
1	2.75	27.5	772.5	200
2	3.75	37.5	762.5	200
3	5	50	750	200
4	6	60	740	200
5	7	70	730	200
6	8	80	720	200
7	9	90	710	200
8	10	100	700	200
9	13	130	670	200
10	17.5	175	625	200
11	21	210	590	200
12	60	600	200	200

- ***Mass spectrometry and data analysis:***

Reverse-phase chromatography was used to separate peptides on a 1,6 cm C18 fused silica column (I.D. 75 µm, O.D. 360 µm x 25 cm length) packed into an emitter tip (IonOpticks, Australia), using a nano-flow HPLC (M-class, Waters) coupled to an Impact II UHR-QqTOF mass spectrometer (Bruker, Bremen, Germany). Data acquisition and analysis was performed as described in Chapter 1, methods section.

II.3. Tandem Mass Tag (TMT) mass spectrometry analysis:

A total of 36 TIF samples (12 samples/ group) were subject to relative protein quantification using Tandem Mass Tag labelling MS/MS/MS. Isobaric labeling of peptide mixtures allows for multiplexing (10 samples/ run). A pooled sample was used as a standard to normalize measures between runs (Figure 2).

- ***Sample extraction and digestion***

Samples were prepared according to the SPEED protocol with modifications (Doellinger et al. 2020). Briefly, 0.5-1.0 µl of human testis serum were dissolved in 10 µl of trifluoroacetic acid (TFA, Applied Biosystems™, Warrington, UK). After incubation for 10 mins at room temperature, the samples were neutralized with a nine-fold volume of 2 M Tris-base and centrifuged at 13000 x g for 10 min. The cysteines of 25 µg protein sample were reduced by the addition of dithiothreitol (DTT; Fluka, Seelze, Germany) to a final concentration of 5 mM and incubation at 50 °C for 30 min followed by carbamidomethylation for 30 min at room temperature using iodoacetamide (IAA; Sigma, Taufkirchen, Germany) at a final concentration of 5 mM. The reaction was quenched by the addition of cysteine (7.5 mM final concentration) and proteolytic digestion was carried out for 20 h at 37 °C using trypsin (sequencing grade,

Promega, Mannheim, Germany) in 25 mM ammonium bicarbonate (Fluka) at a protein/enzyme ratio of 50:1.

- ***Peptide desalting and purification***

The tryptic peptides were desalted using Chromabond C18ec cartridges (Macharey-Nagel, Germany; 100 mg adsorbent weight, 1 ml bed volume). After acidification by addition of an equal volume of 1% (v/v) TFA the samples were applied to the activated cartridges, the retained peptides were washed with 0.1% TFA and eluted with 80% acetonitrile (ACN) in 0.1% TFA. The eluate was lyophilized overnight, redissolved in 50 µL of a 50 mM HEPES buffer (pH 8.5; Sigma) and amounts were quantified using a NanoDrop 2000c (Thermo Scientific, MA, USA).

- ***TMT labelling of peptides***

Equal amounts of peptides from each sample were labeled using the TMT10plexIsobaric Label Reagent Set (Thermo Scientific, MA, USA) to quantify simultaneously up to 10 samples in the same LC-ESI-MS analysis. The 36 individual samples were split into four sets, so each set comprised nine samples plus one average across all 36 samples. For isobaric labelling, 100 µg from each label (dissolved in 20 µL of ACN) were added to 10 µg of the tryptic peptides (dissolved in 25 µl of 50 mM HEPES, pH 8.0). After 60 mins of incubation at room temperature, unused labelling reagents were quenched by the addition of 4.2 µL of 5% L-cysteine (Sigma) prepared in HEPES buffer. Finally, every ten samples of the four different sets were mixed together to yield four master samples and dried under vacuum.

- ***High pH reversed-phase peptide fractionation***

To increase the number of peptide identifications in the LC-ESI-MS analysis, the four labelled master samples were fractionated using the High pH Reversed-Phase Peptide Fractionation Kit (Pierce™, Thermo Scientific, MA, USA), according to the manufacturer's protocol. Briefly, the dried labelled peptides were resuspended in 300 µL of a 0.1% TFA and loaded to the pre-conditioned C18 spin column. After washing once with water and once with 5% ACN in 0.1% trimethylamine (TEA), labelled peptides were eluted by eight steps of increasing concentration of ACN in 0.1% TEA (10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25% and 50%). Each fraction was then lyophilized overnight and reconstituted with 15µL of 0.1% TFA and analysed by LC-MS.

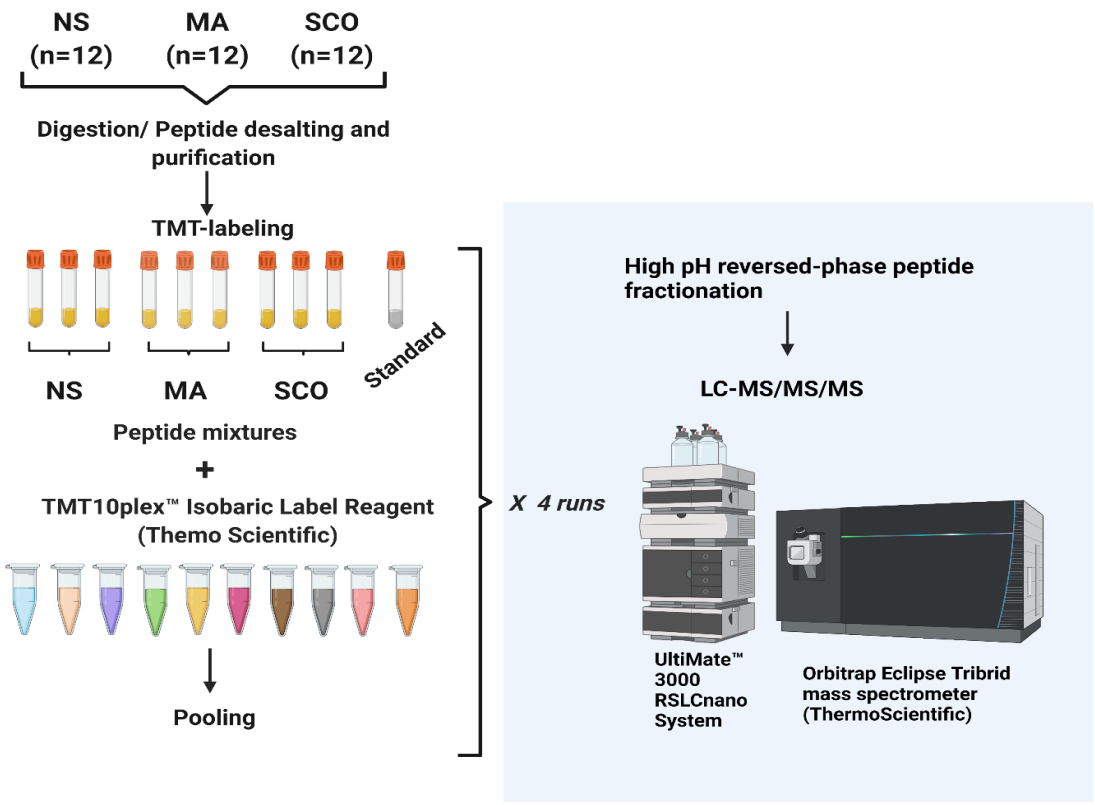


Figure 2: Tandem Mass Tag (TMT) mass spectrometry analysis of TIF samples from men with normal spermatogenesis (NS, OA), mixed atrophy (MA), and Sertoli cell only (SCO) (n=12/ group). Every individual run included 3 samples from each group, plus a mix of all the samples used as an internal standard for the normalization of abundance values between runs.

- ***Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS/MS)***

For analysis, 1 µg of each of the 32 fractions was loaded onto a 50 cm µPAC™ C18 column (Pharma Fluidics, Gent, Belgium) in 0.1% formic acid (Fluka) at 35°C. Peptides were eluted with a linear gradient of ACN from 3% to 44% over 240 min followed by a wash with 72% acetonitrile at a constant flow rate of 300 nl/min (ThermoScientific™UltiMate™3000RSLCnano) and infused via an Advion TriVersa NanoMate (Advion BioSciences, Inc. New York, USA) into an Orbitrap Eclipse Tribrid mass spectrometer (ThermoScientific). The mass spectrometer was operating in positive-ionization mode with a spray voltage of the NanoMate system set to 1.6 kV and source temperature at 300°C. Using the data-dependent acquisition mode, the instrument performed full MS scans every 2.5 seconds over a mass range of m/z 400–1600, with the resolution of the Orbitrap set to 120000. The RF lens was set to 30%, auto gain control (AGC) was set to standard with a maximum injection time of 50 ms. In each cycle, the most intense ions (charge state 2-6) above

a threshold ion count of 5.000 were selected with an isolation window of 0.7 m/z for CID-fragmentation at normalized collision energy of 35% and an activation time of 10 ms. Fragment ion spectra were acquired in the linear IT with a scan rate set to rapid and mass range to normal and a maximum injection time of 100 ms. After fragmentation, the selected precursor ions were excluded for 20s for further fragmentation. From each MS/MS cycle, up to ten fragment ions were selected with an isolation window of 3 m/z for HCD-fragmentation at a normalized collision energy of 55%. MS3-fragment ion spectra were acquired in the Orbitrap with a resolution of 50.000. The mass range was set to 100 – 500 m/z, maximum injection time to 100 ms and AGC to 300.

- ***Protein Identification and Quantitation***

Data was acquired with Xcalibur 4.3.73.11. (Thermo Fisher Scientific) and analyzed with Proteome Discoverer 2.4.0.305 (Thermo Fisher Scientific). Sequest HT (Proteome Discoverer version 2.5.0.400; ThermoFisher) was used to search against Uniprot_human database (20201007, 70502241 residues, 214889 sequences) which also extracted the quantitation data from the 10 TMT tags and performed the label-based quantitative analysis. A precursor ion mass tolerance of 10 ppm was used, and one missed cleavage was allowed. Carbamidomethylation of cysteines and labelling with TMT10plex at peptide N-termini and lysine side chains were defined as static modifications with optional oxidation of methionine. The fragment ion mass tolerance was set to 0.6 Da for the linear IT MS2 detection. The FDR for peptide identification was limited to 0.01 by using a decoy database. Protein identifications were accepted with a decoy false discovery rate (FDR) cut-off of less than 1%. Protein identifications were accepted if they could be established with at least 1 unique identified peptide of a length between 6 and 144 amino acids. TMT reporter ion values were quantified from MS3 scans with an integration tolerance of 20 ppm. The reporter ion values were normalized to the TMT 131 channel. Proteins were defined as differentially expressed when the fold-change (FC) between different groups was > 1.5 or < -1.5 and the p-value of the Mann Whitney U test was < 0.05.

II.4. Western Blot:

Western Blotting was performed for the quantification of LDHC in human testicular interstitial fluid. Samples containing 50 µg of protein were mixed with 6:5 (v/v) NuPAGE™ LDS Sample Buffer (4X) and 3:1 (v/v) of NuPAGE™ Sample Reducing Agent (10X). Proteins were resolved on a 4-12% Tris-Glycine gel, at 110V for 90 min, using the Invitrogen min-gel

system. After transfer onto an Immobilon-P PVDF membrane (Merck KGaA, Germany), proteins were fixed by drying at 37°C for 20 min. LDHC was detected using a rabbit polyclonal antibody (Invitrogen, Cat Nr. PA5-30079) and the IRDye® 800CW Goat anti-Rabbit IgG secondary antibody (Cat Nr. 326-32211; LI-COR, NE, USA) diluted 1:5,000 and 1:10,000 respectively in the Odyssey Blocking Buffer (PBS) (LI-COR). The blot was imaged in an Odyssey® Fc Imaging System and the intensity of the bands was measured after a 2-min exposure time by Image Studio Quantification Software (LI-COR) and expressed in arbitrary units. For HSPA2 detection, the membrane was first blocked with 5% skim milk powder in PBS 0.01% Tween 20, for 1h at RT then probed with 1:600 anti-HSPA2 rabbit polyclonal antibody (Invitrogen, # PA5-29083) in blocking buffer for 1h at. After 3 washes of 10 min each with Washing Buffer (TBS/0.05% Tween), the membrane was incubated in 1:10.000 goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (Invitrogen, Cat. # G-21234) in 1:10,000 goat anti-rabbit IgG in 3% normal goat serum for 1h at RT. The membrane was incubated on a shaker 4 x 10 min in PBS 0.01% Tween 20. Bands were developed by incubating the membrane for 45s with 2 mL Lumi-LightPLUS Western Blotting Substrate (Merck,12015196001) and visualized in a ChemiDoc XRS+ imaging system. Image Lab 6.0 (BIO-RAD) was used to acquire blot images and quantify protein bands. Statistical significance was determined using the Mann-Whitney test (GraphPad Prism 8).

III. Results:

- ***Results of LC-MS/MS analysis of TIF:***

To study the expression of TIF proteins in NS, MA, and SCO, we analysed 24 TIF samples distributed equally between three groups by LC-MS/MS. Data analysis revealed a total of 709 proteins IDs and a large number of differentially expressed proteins: 58 in the MA-NS comparison, and 5 in the MA-SCO comparison, and 81 in the SCO-NS comparison (Table 2). Only 4 proteins were significantly increased in the normal spermatogenesis group compared to SCO: Actin, alpha cardiac muscle 1 (ACTC1), Transketolase-like protein 1 (TKTL1), Immunoglobulin heavy variable 3-72 (IGHV3-72), and Hydroxymethylglutaryl-CoA synthase, cytoplasmic (HMGCS1). Out of these, only TKTL1 is a testis-enriched germ cell marker (see Chapter 2 discussion). The remaining 77 significant proteins were up-regulated more than 1.5-fold in SCO (Table 2). A majority of these have low cell-type specificity in the testis, apart from testis-enhanced Pyridoxal kinase (PDXK) which is only expressed in Sertoli and Leydig cells at the protein level, and Leydig cell-enhanced Heat shock protein family B (small) member 6 (HSPB6). The expression of several other SCO-increased proteins seems restricted to somatic

cells in the testis, despite not being testis-specific. These include Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta (YHWAB), Lactate dehydrogenase B chain (LDHB), Macrophage migration inhibitory factor (MIF), Annexins 2 and 5 (ANXA2, ANXA5), which are found in Sertoli cells and Leydig cells, 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2) and Apolipoprotein M (APOM) present only in Leydig cells, Myosin light chain 6 (MYL6) and Spectrin beta chain, non-erythrocytic 1 (SPTBN1) which are enhanced in peritubular cells.

In the MA comparison with NS/OA samples, only TKTL1 and ACTC1 were present at a significantly higher abundance in normal TIF compared to MA samples, whereas the remaining 56 significant proteins were increased in MA. 41 of the proteins that were significantly down-regulated in SCO compared to the control (above) were also down-regulated in MA compared to NS. Adenine phosphoribosyltransferase (APRT), annexin 2 (ANXA2), and cytochrome B5 reductase 3 (CYB5R3) were significantly upregulated in SCO compared to MA. These proteins are ubiquitously expressed, but in the testis, they are more concentrated in somatic cells: APRT is most abundant in Leydig cells, ANXA2 can be detected on the protein level only in Sertoli cells and peritubular myoid cells, while CYB5R3 is predominant in Leydig cells and is involved in cholesterol/ steroid metabolism. Few germ-cell enriched proteins were quantified, most likely because their abundances were below the detection limit of the mass spectrometry analysis method used. However, several Sertoli cell and Leydig cell proteins appear to be upregulated by testicular failure, especially in the SCO group. With the exception of TKTL1, no germ cell specific proteins were identified as differentially expressed in this TIF analysis. This can be explained by the low abundance of these markers compared to more ubiquitously expressed proteins, the high dynamic range of TIF and the LOD of this particular MS method.

- ***Results of label-free high-resolution LC-MS/MS:***

With the objective of investigating the TIF proteome in a larger sample size and a more sensitive quantification method, high-resolution LC-MS/MS was used to analyse 48 TIF samples divided equally between 4 groups (NS, SA, MA, SCO). 2083 proteins were identified in total, although the number of identifications per individual samples ranged between 600 and 1200 (data not shown). Figure 3 presents the average relative abundances of the 30 proteins that are most abundant in TIF from OA/NS patients and that were undetected in all SCO samples. No statistically significant differences were observed between the groups, however, several germ-cell enriched proteins were quantified. Testis enriched PAGE family member 2 (PAGE2) expressed mainly in spermatogonia was detected in all groups except

Table 2: Differentially expressed proteins in SCO (n=8) compared to NS (n=8) testicular interstitial fluid, data from LC-MS/MS analysis of TIF. FC= Fold Change, p-values were corrected using the Benjamini-Hochberg procedure. Tissue/ cell type specificity was determined based on the Human Protein Atlas available IHC data (https://www.proteinatlas.org/humanproteome/tissue)

Gene Name	Protein Names	FC NS/SCO	p-value NS vs SCO	FC NS/ MA	p-value NS vs MA	FC MA/SCO	p-value MA vs SCO	Testis specific/ enriched
ACTC1	Actin, alpha cardiac muscle 1	2,93	1,07E-02	2,77	2,46E-02	0,16	9,89E-01	No
TKTL1	Transketolase-like protein 1	2,58	2,74E-04	1,84	1,60E-02	0,74	7,27E-01	Yes, Germ cell enriched
IGHV3-72		1,73	1,07E-02	0,42	7,87E-01	1,31	2,39E-01	No
HMGCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	1,60	1,86E-02	0,75	4,79E-01	0,85	5,42E-01	No
ADD1	Alpha-adducin	0,64	1,26E-02	0,08	9,72E-01	0,61	7,35E-02	No
GALM	Aldose 1-epimerase	0,64	6,43E-03	0,71	2,14E-02	6,71	9,89E-01	No
PPP2R4	Serine/threonine-protein phosphatase 2A activator	0,64	2,42E-02	0,56	1,60E-02	0,20	9,89E-01	No
PGLS	6-phosphogluconolactonase	0,63	2,45E-02	1,04	3,06E-01	1,60	8,46E-01	No
SERPINA10	Protein Z-dependent protease inhibitor	0,62	7,60E-04	0,76	1,21E-02	3,45	9,75E-01	No
C1QA	Complement C1q subcomponent subunit A	0,62	2,40E-02	1,40	5,31E-01	1,11	5,42E-01	No
ESD	S-formylglutathione hydrolase	0,62	2,81E-02	0,83	1,79E-01	2,39	9,89E-01	No
MYH9	Myosin-9	0,60	7,78E-03	1,11	2,67E-01	1,32	5,67E-01	No
TXNRD1	Thioredoxin reductase 1, cytoplasmic	0,60	1,62E-02	0,40	8,74E-04	0,80	6,24E-01	No
SPTBN1	Spectrin beta chain, non-erythrocytic 1	0,59	2,42E-02	1,37	5,42E-01	1,05	5,42E-01	No, enhanced in peritubular cells
LDHB	L-lactate dehydrogenase B chain;L-lactate dehydrogenase	0,59	3,70E-03	0,88	1,02E-01	1,75	7,97E-01	Highest TPM in Leydig/ Sertoli cells
EEF1B2	Elongation factor 1-beta	0,58	2,44E-02	0,86	2,23E-01	1,75	9,06E-01	No
RPLP0	60S acidic ribosomal protein P0;60S acidic ribosomal protein P0-like	0,56	1,26E-03	0,95	1,08E-01	1,39	5,42E-01	No
IPO5	Importin-5;Ran-binding protein 6	0,56	9,81E-03	0,72	7,96E-02	2,42	9,75E-01	No
FBP1	Fructose-1,6-bisphosphatase 1	0,56	9,67E-03	0,50	5,81E-03	0,22	9,89E-01	No

PROC	Vitamin K-dependent protein C;	0,55	1,24E-03	0,83	5,96E-02	1,63	6,93E-01	No
CYB5R3	NADH-cytochrome b5 reductase 3	0,54	2,65E-02	0,53	7,69E-01	0,42	3,52E-02	Highest TPM in Leydig/ Sertoli cells
SNCA	Alpha-synuclein;Beta-synuclein	0,54	1,04E-02	0,23	8,97E-01	0,48	3,52E-02	No
NPM1	Nucleophosmin	0,54	7,78E-03	0,66	5,33E-02	2,83	9,89E-01	No
FLNB	Filamin-B;Filamin-C	0,53	3,42E-02	0,77	2,47E-01	1,69	9,34E-01	No
G6PD	Glucose-6-phosphate 1-dehydrogenase	0,52	1,28E-02	0,78	1,70E-01	1,59	8,89E-01	No
RPLP2	60S acidic ribosomal protein P2	0,51	1,04E-02	0,81	1,83E-01	1,40	8,09E-01	No
ACO1	Cytoplasmic aconitate hydratase	0,51	1,76E-02	0,47	1,54E-02	0,18	9,89E-01	No
ANXA2	Annexin A2;Annexin;Putative annexin A2-like protein	0,50	4,77E-04	0,38	7,63E-01	0,42	5,21E-04	No
PLS3	Plastin-3;Plastin-1	0,50	1,24E-03	1,58	5,40E-01	0,72	1,83E-01	No
KPNB1	Importin subunit beta-1	0,50	5,64E-04	1,48	4,62E-01	0,75	1,66E-01	No
AOX1	Aldehyde oxidase	0,50	5,33E-04	0,65	1,57E-02	2,13	9,01E-01	No
SMS	Spermine synthase	0,50	2,62E-03	0,70	5,95E-02	1,71	8,58E-01	No
VAT1	Synaptic vesicle membrane protein VAT-1 homolog	0,50	9,81E-03	0,50	1,60E-02	50,51	9,98E-01	No
CAPN2	Calpain-2 catalytic subunit	0,49	2,74E-04	0,78	3,37E-02	1,36	5,38E-01	No
APEH	Acylamino-acid-releasing enzyme	0,49	7,78E-03	5,13	9,31E-01	0,54	1,37E-01	No
C2	Complement C2;Complement C2b fragment;Complement C2a fragment	0,48	4,77E-04	0,41	1,79E-04	0,35	9,82E-01	No
USP5	Ubiquitin carboxyl-terminal hydrolase 5	0,47	5,02E-03	0,81	1,79E-01	1,15	6,19E-01	No
NACA	Nascent polypeptide-associated complex subunit alpha	0,47	7,58E-04	1,04	2,33E-01	0,87	2,93E-01	No
C1QB	Complement C1q subcomponent subunit B	0,47	3,60E-03	0,56	2,68E-02	2,68	9,89E-01	No
BLVRB	Flavin reductase (NADPH)	0,46	2,37E-02	0,76	2,99E-01	1,17	8,39E-01	No
TPT1	Translationally-controlled tumor protein	0,45	4,77E-04	0,63	1,95E-02	1,68	8,19E-01	No
ACAT2	Acetyl-CoA acetyltransferase, cytosolic	0,44	2,34E-02	0,52	8,54E-02	3,09	9,89E-01	No
CUTA	Protein CutA	0,44	2,74E-02	0,68	2,72E-01	1,25	9,06E-01	No

MYL6	Myosin light polypeptide 6;Myosin light chain 6B	0,44	2,29E-02	0,64	1,95E-01	1,40	9,06E-01	No
ALDH1L1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	0,44	3,35E-04	0,57	1,18E-02	1,90	8,89E-01	No
C1QC	Complement C1q subcomponent subunit C	0,43	6,68E-03	0,47	2,07E-02	5,10	9,89E-01	No
AARS	Alanine--tRNA ligase, cytoplasmic	0,43	1,35E-04	0,62	1,54E-02	1,38	6,01E-01	No
IGLL5	Immunoglobulin lambda-like polypeptide 5;	0,42	3,16E-02	0,36	1,63E-02	0,42	9,89E-01	No
TMSB4X	Thymosin beta-4	0,41	7,31E-03	0,54	6,63E-02	1,77	9,57E-01	No
CMBL	Carboxymethylenebutenolidase homolog	0,41	2,80E-04	0,60	2,14E-02	1,30	6,25E-01	No
ANK1	Ankyrin-1	0,40	1,89E-02	1,32	7,26E-01	0,58	3,45E-01	No
CNDP2	Cytosolic non-specific dipeptidase	0,40	5,29E-05	0,45	5,45E-04	4,15	9,89E-01	No
TXNL1	Thioredoxin-like protein 1	0,40	9,18E-08	0,45	1,18E-06	4,10	9,89E-01	No
PAPSS2	Bifunctional 3-phosphoadenosine 5-phosphosulfate synthase 2;Sulfate adenylyltransferase;Adenylyl-sulfate kinase	0,40	8,05E-04	0,43	3,83E-03	6,25	9,89E-01	No
AKR1B1	Aldose reductase;Aldo-keto reductase family 1 member B15	0,40	2,57E-02	1,57	8,06E-01	0,53	3,38E-01	No
GLUL	Glutamine synthetase	0,40	3,35E-04	0,51	1,02E-02	1,82	9,06E-01	No
IGKV1-12		0,39	4,89E-04	0,53	1,76E-02	1,52	8,46E-01	No
PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	0,39	1,33E-04	0,51	5,70E-03	1,71	8,46E-01	No
AKR1C1	Aldo-keto reductase family 1 member C1	0,39	4,89E-04	0,51	1,54E-02	1,66	9,00E-01	No
YWHAB	14-3-3 protein beta/alpha	0,39	5,23E-03	0,85	3,67E-01	0,71	4,20E-01	No
ARHGDIA	Rho GDP-dissociation inhibitor 1	0,38	2,89E-03	0,69	1,84E-01	0,85	5,38E-01	No
ARHGAP1	Rho GTPase-activating protein 1	0,38	5,05E-05	0,87	1,16E-01	0,68	1,37E-01	No
RBP4	Retinol-binding protein 4	0,38	4,77E-04	0,41	3,01E-03	5,46	9,89E-01	No
TLN1	Talin-1	0,37	3,91E-05	0,57	1,21E-02	1,02	4,20E-01	No
IGJ	Immunoglobulin J chain	0,36	2,62E-03	0,34	3,01E-03	0,20	9,89E-01	No
LBP	Lipopolysaccharide-binding protein	0,36	5,29E-05	0,38	2,28E-04	8,00	9,89E-01	No
AKR1C3	Aldo-keto reductase family 1 member C3	0,35	9,06E-05	0,46	3,83E-03	1,55	8,39E-01	No

HSPB6	Heat shock protein beta-6	0,34	2,57E-05	0,49	3,83E-03	1,18	5,42E-01	No
IGKV2D-28	Ig kappa chain V-II region TEW	0,34	9,82E-05	0,34	2,28E-04	98,04	9,98E-01	No
HUWE1	E3 ubiquitin-protein ligase HUWE1	0,34	1,34E-04	0,45	5,96E-03	1,44	8,41E-01	No
PCBP1	Poly(rC)-binding protein 1	0,32	1,53E-05	0,56	1,50E-02	0,78	2,55E-01	No
APRT	Adenine phosphoribosyltransferase	0,32	1,74E-05	1,22	4,43E-01	0,43	8,91E-03	No
LRG1	Leucine-rich alpha-2-glycoprotein	0,32	1,32E-03	0,31	3,01E-03	0,05	9,98E-01	No
IGLV3-21		0,32	8,21E-04	0,80	3,55E-01	0,52	2,27E-01	No
CNDP1	Beta-Ala-His dipeptidase	0,31	9,18E-08	0,32	3,06E-07	11,24	9,89E-01	No
C4A	Complement C4-A	0,29	1,24E-03	0,54	1,46E-01	0,65	4,71E-01	No
APOM	Apolipoprotein M	0,29	1,03E-04	0,37	3,59E-03	1,49	9,06E-01	No
ANXA5	Annexin A5;Annexin	0,29	3,03E-04	0,36	5,70E-03	1,63	9,50E-01	No
PDXK	Pyridoxal kinase	0,28	2,67E-13	0,30	2,66E-12	4,88	9,89E-01	Yes, Sertoli/ Leydig cells enhanced
APOC2	Apolipoprotein C-II;Proapolipoprotein C-II	0,26	7,36E-05	0,38	1,21E-02	0,81	5,42E-01	No
MIF	Macrophage migration inhibitory factor	0,25	1,11E-04	0,31	3,83E-03	1,18	8,96E-01	No

SCO. PAGE2B and PAGE5, enriched in spermatogonia and spermatocytes, were present only in the NS and SA groups. PAGE2, PAGE2B, and PAGE5 are cancer-testis antigens with anti-apoptotic activity: PAGE2 and PAGE2B were proposed as potential therapeutic targets in colorectal cancer (Yilmaz-Ozcan et al. 2014), while PAGE5 was associated with melanoma progression (Nylund et al., 2012). Germ cell-enriched Tubulin alpha 3e (TUBA3E) and spermatocytes/ spermatid enhanced Asparaginase like 1 (ASRGL1) were also absent in SCO TIF and quantifiable in the 3 remaining groups, whereas LDHC was only measured in NS and MA. ASRGL1 has not been classified as a testis cancer antigen but is a prognosis biomarker in endometrial carcinoma (Edqvist et al., 2015). Overall, this analysis uncovered more testis-specific proteins than the previous one (see paragraph III.1 above), but due to limited resolution and considerable intra-group variability, no statistically significant proteins were identified. Low and highly variable numbers of identified proteins in each sample are likely caused by inefficient peptide elution. Manually packed tips were used for the elution of individual samples and for the high-pH fractionation of pooled samples. Differences in C18 resin plug size and position in the tip can affect the efficiency and time of elution and lead to considerable sample loss.

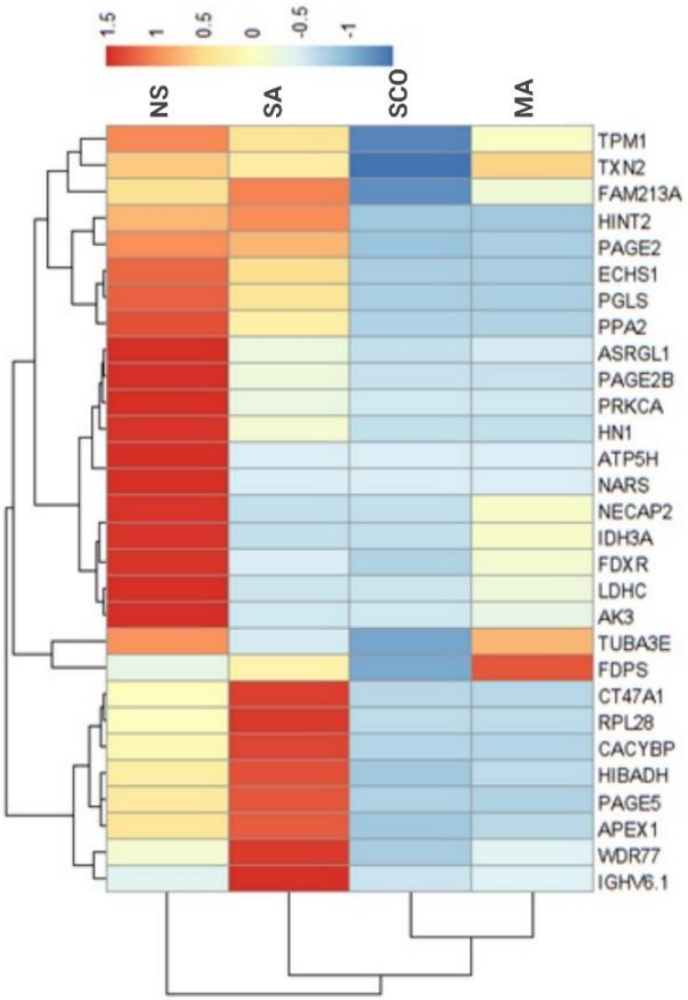


Figure 3: Normalized average relative abundance in TIF of 30 proteins undetectable in the SCO group. iBAQ values were obtained by LC-MS/MS analysis of TIF samples from men diagnosed based on histological scoring and m-TESE outcome with obstructive azoospermia/ normal spermatogenesis (NS), spermatogenic arrest (SA), mixed atrophy (MA), and Sertoli cell only (SCO), n=12/ group. Dendrogram: Rows and Columns; Normalization method: log transformation; Clustering method= UPGMA, clustering distance measure = Euclidean.

- ***Relative quantification of TIF proteins by isobaric labelling mass spectrometry:***

For a more robust comparison of TIF proteomic profiles, we analysed a total of 36 samples: 12 from men with normal spermatogenesis (NS), 12 from SCO patients, and 12 from MA patients using LC-MS/MS/MS with TMT labelling. Across all samples, 1418 proteins were quantified as a result of the analysis. LDHC and HSPA2 were significantly downregulated in both non-obstructive azoospermia groups (MA and SCO) compared to normal spermatogenesis, but not between MA and SCO. The results are consistent with seminal plasma mass spectrometry and immunoblotting data (see Chapter I, Results).

The abundance of germ cell-enriched Tubulin alpha-3D (TUBA3D) appears to correlate better to the spermatogenic state as it was significantly decreased in SCO compared to NS, MA relative to NS, and SCO relative to MA (p= 0.0257,0.046, and 0.0139 respectively). The most significant differences between groups, and the greatest fold-changes within testis-enriched proteins, are seen for spermatogonia-enriched Transketolase-like 1 (TKTL1). A clearer view of iBAQ value distribution for LDHC, HSPA2, TKTL1, and TUBA3D is presented in Figure 5. Leucine zipper transcription factor-like protein 1 (LZTFL1) is specifically enhanced in spermatocytes and significantly less abundant in SCO and MA compared to NS. Tropomyosin 2 (TPM2), also downregulated in SCO and MA, is expressed in several types of testicular cells at the protein level, including Sertoli cells, pachytene spermatocytes, and round spermatids. Using the Human Protein Atlas' downloadable datasets, the complete list of identified proteins was matched to cell type elevated proteins. 86 proteins were found to be enhanced in elongated spermatids, 81 in round spermatids, 21 in spermatocytes, and 11 in spermatogonia. Proteins present at higher levels in late spermatids include sperm tail A-kinase anchor protein 4 (AKAP4), cAMP-regulated phosphoprotein 19 (ARPP19), Cytosolic acyl coenzyme A thioester hydrolase (ACOT7), Acyl-protein thioesterase 1 (LYPLA1) (Figure 4). Among the proteins elevated in Sertoli cells and Leydig cells compared to other cell types, some tend to be more abundant in SCO TIF: Sertoli cell-enriched Phosphoribosyl pyrophosphate synthetase 2 (PRPS2) and Leydig cell enhanced Acrosomal protein KIAA1210 are both down-regulated 2.5-fold in NS compared to SCO. Alpha-1,4 glucan phosphorylase (PYGB) and Angiotensin I converting enzyme 2 (ACE2), which are expressed exclusively in Leydig and Sertoli cells in the testis, are up-regulated by a factor of ten and two in SCO compared to NS. Overall, this study has identified a considerable number of testis-specific proteins, including germ cell-enriched proteins, and confirmed the differential expression of several potential markers of sperm production (LDHC, HSPA2, TKTL1, TUBA3D) in testicular failure (Figure 4).

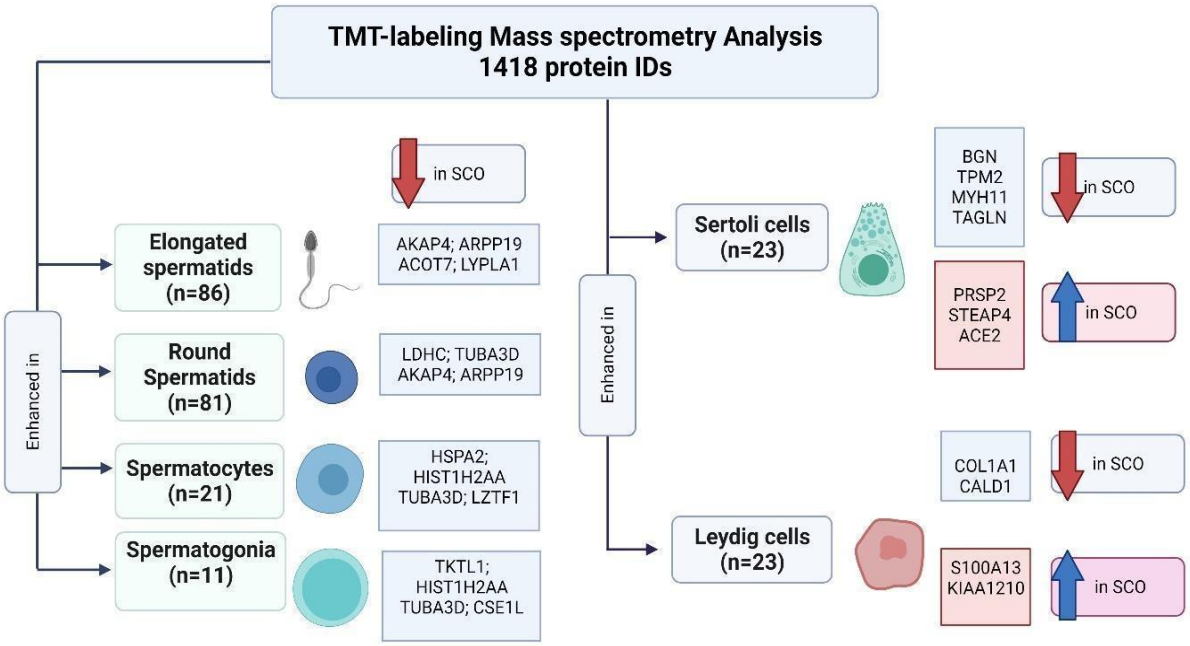


Figure 4: Distribution of TIF proteins identified by Tandem Mass Tag mass spectrometry in testicular cell types. Gene names were matched to lists of germ cell, Sertoli cell, and Leydig cell elevated proteins downloaded from the Single Cell Type Atlas (https://www.proteinatlas.org/). Specificity and distribution classification of cell type enhanced genes is based on single-cell RNA sequencing (scRNAseq) and immunohistochemistry data from normal human tissues. Red/ blue arrows indicate a down/ up-regulation of the listed proteins at least 1.5-fold in SCO relative to NS.

- ***Estimation of the relative abundances of LDHC and HSPA2 in TIF by immunoblotting:***

Western Blot was used to measure LDHC and HSPA2 in NS, MA, and SCO TIF samples. The comparison of protein-specific adjusted band volumes showed a significant difference between NS and SCO (p= 0.012 and 0.0006, respectively) and a non-significant tendency towards lower abundance in MA (Figure 6). However, a clear difference in value distribution was observed between the two proteins: LDHC was detected in all samples, albeit at different levels, while HSPA2 was undetectable in most MA and SCO samples. These findings are consistent with the results of relative quantification of LDHC and HSPA2 by Western Blot in seminal plasma (Chapter 1): LDHC is less abundant but detectable in a majority of SCO samples, which could be explained by residual expression in Sertoli cells (Chapter 1, Results, Figure 3). There is a greater difference in HSPA2 abundance between normal spermatogenesis and non-obstructive azoospermia in both TIF and seminal plasma, likely due to its germ-cell specific expression in the seminiferous epithelium (Chapter 1, Results, Figure 4, Figure 6).

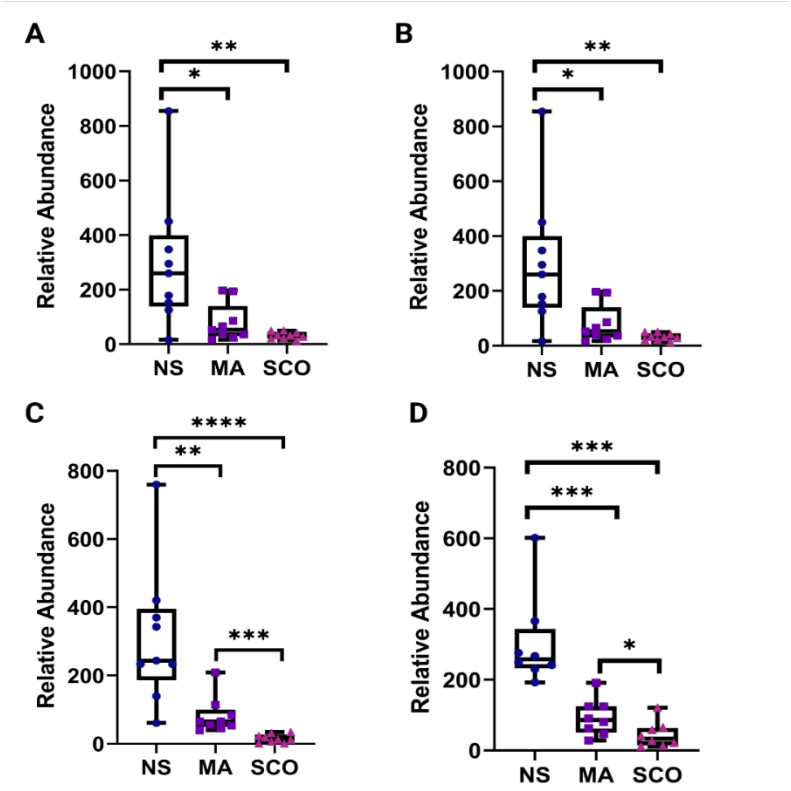


Figure 5: Relative abundance of LDHC (A) and HSPA2 (B), TKTL1 (C) and TUBA3E (D) in the testicular interstitial fluid of mixed atrophy (MA, n=12), Sertoli cell only (SCO, n=12), and fertile men (NS, n=12); *: p >0.01: **: p<=0.01; ***: p<= 0.001. Boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum.

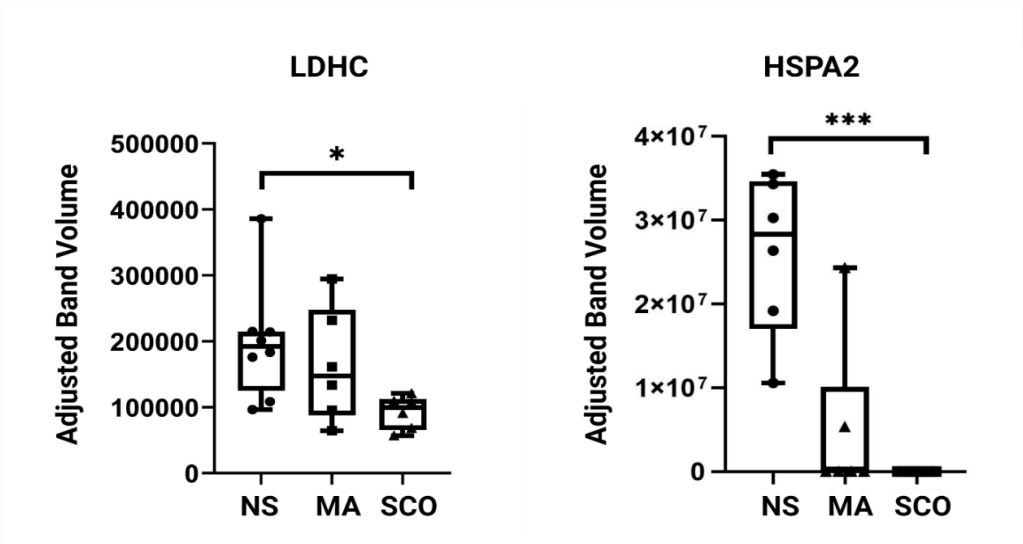


Figure 6: Relative abundance of LDHC and HSPA2 measured by immunoblotting in testicular interstitial fluid. Western blotting for LDHC was performed on 8 TIF samples from normal spermatogenesis (NS), 6 mixed atrophy (MA), and 6 Sertoli cell only (SCO) samples. Western blotting for HSPA2 was performed on 6 NS TIF samples, 6 mixed atrophy (MA), and 7 Sertoli cell only (SCO) samples. Boxes represent median, 1st and 3rd quartiles, whiskers represent maximum and minimum; *: p= 0.0127, ***: p=0.0006; statistical significance was determined by the Mann-Whitney test.

V. Discussion:

Spermatogenesis is an intricate process involving thousands of genes, of which some have a ubiquitous expression pattern while others are specific to reproductive tissues. In this study, we focus on two distinct testicular phenotypes which result in nonobstructive azoospermia (NOA): Sertoli cell-only syndrome and mixed atrophy. The comprehensive characterization of the TIF proteome led to the identification of numerous proteins involved in spermatogenesis and testicular function in general. By comparing the relative abundance of these proteins between normal spermatogenesis and NOA, we can gain new insights into the causes of severe testicular failure. Study participants included azoospermic patients with well-documented clinical, hormonal, and genetic evaluations, as well as TESE results with histological confirmation of the presence or absence of preserved pockets of spermatogenesis. By conducting three separate mass spectrometry experiments with varying protocols and resolutions, we were able to identify distinct sets of differentially expressed proteins in the compared diagnoses.

- ***Label-free vs isobaric labelling mass spectrometry for biomarker discovery in TIF:***

Mass spectrometry (MS) is an invaluable tool for the analysis of complex protein mixtures. Paired with chromatographic separation, MS allows for high resolution, sensitive protein identification. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a widely used technology that provides the possibility of optimizing multiple facets of the analytical process: sample preparation, fractionation, chromatographic parameters, ionization conditions, and m/z transitions (Crutchfiled et al. 2016). Relative or absolute protein quantification by MS is done using one of two methods: label-free quantification or stable isotope labelling. Several comparative studies point to the fact that the label-free method leads to the identification of a higher number of proteins, while isotopic labelling produces more accurate and reproducible results (Bachor et al. 2019). Isobaric labelling proteomics offer other advantages, including multiplexing, reducing inter-run variability and a shorter analysis time (Frost et al. 2018), but remain too cost-prohibitive for use in the clinic (Bachor et al. 2019, Zecha et al. 2019). In this study of TIF proteome in NS, MA and SCO, we used both methods: the relatively less expensive label-free tandem MS to obtain higher coverage and process a larger sample size, and the more specific and reproducible TMT-labelling MS to identify robust biomarkers using lower protein amounts

of carefully selected TIF samples. The first label-free mass spectrometry analysis (n=8/group) produced the least protein identifications (709), but identified a considerable number of differentially expressed proteins, especially those up-regulated in SCO. The second label-free second MS analysis preceded by high-pH peptide fractionation resulted in a larger number of protein IDs (2083) but due to high inter-sample variability and loss of protein content during pre-analytical sample handling, no significant differences in protein abundance were observed. This analysis did however detect more germ-cell specific proteins that are more suitable potential markers of sperm retrieval. The third experiment, TMT-labelling tandem MS (n=12/ group) culminated in the identification of over 1400 proteins. Despite the low number of differentially expressed proteins, this analysis uncovered promising biomarkers of testicular function that could potentially distinguish between MA and SCO cases. Although there is limited overlap between the three datasets when it comes to proteins of statistically different abundances in group comparisons, we were able to discern certain patterns of protein expression in azoospermia-inducing spermatogenic impairment (discussed below).

- ***Up-regulation of somatic cell proteins in SCO TIF:***

The expression of several SCO-increased proteins appears to be enhanced in, or limited to, somatic cells in the testis. The first label-free mass spectrometry analysis uncovered a significant number of significantly increased proteins in SCO (n=77), hinting to big changes in gene regulation. The germ cell depletion-induced up-regulation of Sertoli cell and Leydig cell transcripts has been reported in animal and human studies. Soffientini et al. used a Cre-inducible diphtheria toxin receptor (iDTR) transgenic mouse model and busulfan injections for Sertoli and germ cell ablation. The Resulting RNAseq data showed up-regulation of Sertoli cell transcripts and to a lesser extent Leydig cell transcripts following germ cell ablation (Soffientini et al. 2017). Pyridoxal kinase (PDXK), only detected in Leydig cells and Sertoli cells at the protein level in the human testis (immunohistochemistry data, Human Protein Atlas), is 3.5-fold more abundant in SCO TIF compared to normal spermatogenesis (Table 4). In mouse testis, Pdxk is up-regulated 4-fold after germ cell depletion. Phosphoribosyl pyrophosphate synthetase 2 (PRPS2) and Lactate dehydrogenase B (LDHB) have a similar expression pattern: up-regulated in the germ-cell ablated mouse testis (5 and 6-fold respectively), and present at higher abundances in SCO TIF compared to the control (2-fold and 3-fold increase, respectively). The overexpression of PRPS2 has been previously observed in SCO men compared to normal spermatogenesis and was linked to apoptosis inhibition via the p53/Bcl-2/caspase-9/caspase-3/caspase-6/caspase-7 signalling pathway (Lei et al. 2015). Noveski et al. reported 52

differentially expressed microRNAs in Sertoli cell only syndrome patients, including mi-R517a which regulates cell proliferation, migration and survival, and an overrepresentation of genes involved in the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) signalling pathway, which regulates apoptosis in the normal testis (Noveski et al. 2016) and tumour suppression in other tissues (Thorburn et al. 2007). Other apoptosis-modulating SCO-increased genes include galectins 1 and 3 (LGALS1, LGALS3) (Wollina et al. 1999); Fas ligand (FASLG), and caspase 3 (Kim et al., 2007). Several of the proteins that show increased expression in SCO TIF (Table 4), (i.e. in the SCO testis), are linked to inflammation or cancer progression. Complement 2 (C2) and complement subcomponent subunits A, B, and C (C1QA, C1QB, C1QC) are involved in the regulation of the complement cascade (Kishore et al, 2020), and might play a role in tumorigenesis (Bulla et al. 2016) and tumour microenvironment remodelling (Chen et al. 2021). Annexin 2 (ANXA2), up-regulated 2-fold in SCO TIF, is a phospholipid-binding protein with widespread expression (Gerke et al. 2005). ANXA2 plays a role in membrane domain organization and angiogenesis, but in disturbed homeostasis, it can contribute to chronic inflammation (Dallacasagrande et al. 2020). ANXA2 is overexpressed in several types of cancer including non-small cell lung cancer, breast cancer, glioblastoma and plays a role in tumour progression (Liu et al. 2015, Christensen et al. 2017). Elevated activity of Acylamino Acid Releasing Enzyme (APEH), also increased 2-fold in SCO relative to normal spermatogenesis (Table 2), is a marker of inflammation in the lung (Komatsu et al. 2016). Vesicle amino transport protein 1 (VAT1) is classified as an oncogene in multiple types of tumours, including glioblastoma and gastric cancer (Shan et al. 2019, Yang et al. 2020), and has been reported as a pathogenic factor in benign prostatic hyperplasia (Mori et al. 2011). These substantial changes in the expression of Sertoli cell enriched transcripts may underlie reported changes in the ultrastructure and Raman spectrum of SCs in testicular failure and the dysregulation of their spermatogenesis-supporting activities (Ma et al. 2013). Our comparative analysis of SCO, MA, and normal spermatogenesis testicular interstitial fluid sheds light on a number of genes whose expression might be affected by the depletion of spermatogenic cells from the seminiferous tubules and serve as markers of severe testicular failure.

- ***Potential biomarkers of sperm retrieval in TIF:***

The presence of a small number of active spermatogenesis sites in MA and the near absence of germ cells in SCO accounts for the difference in protein expression between the two phenotypes. The limited number of differentially expressed proteins both in the label-free and isobaric labelling MS analysis shows that the distinction between MA and SCO is often not

discernible at the molecular level. Transketolase like I (TKTL1) could be a candidate for differentiating mixed atrophy and SCO as it is expressed at a significantly higher level in MA according to TMT-labelling MS ($FC_{MA/SCO}$= 5.46, p= 0.0027). Germ-cell enriched Tubulin alpha-3D (TUBA3D) also shows a significantly lower abundance in MA relative to SCO. TKTL1 was previously identified as a germ cell marker, specifically spermatocytes and spermatids by transcriptional analysis of male reproductive tissues (Reynolds et al. 2013). Other germ cell enriched proteins are undetectable in all SCO samples, yet they are present in some or all MA and NS samples (LDHC, HSPA2, ASRGL1). This could lead to the determination of a concentration threshold at which SCO can be ruled out and a positive TESE result is more likely. TEX101 has been proposed in a similar way as an indicator of the presence of sperm in the testis when quantified in seminal plasma (Drabovich et al. 2014, Korabakis et al. 2015). Other adluminal germ cell proteins could be useful indicators of the presence of sperm in the testis. Our isobaric labelling mass spectrometry analysis also identified over 80 elongated-spermatid enriched proteins in TIF, such as A-kinase anchoring protein 4 (AKAP4), Lysophospholipase I (LYPLA1), and RuvB like AAA ATPase 2 (RUVBL2) (Figure 4), many of which were only detectable in a few samples. Although the abundance of these sperm-specific proteins is relatively low, they could potentially be measured in TIF using targeted proteomics (Gillette et al. 2013) and serve as markers of normal spermatogenesis. Data from functional studies and comparative transcriptomics could be used to select gene products essential for germ cell development and more likely to be only present in late spermatids. Sheng Cheng et al. reported that testis miRNAs have distinct expression patterns in SCO compared to normal spermatogenesis, categorizing miRNAs whose expression is upregulated in SCO as "sterility miRNAs" which cause spermatogenic failure by suppressing genes involved in germ cell growth and differentiation. Of these, miR-630 was chosen randomly for validation by determining the number of transcripts of its target genes: spermatocyte-enhanced SPAG1 and early spermatid enriched SOX30 were significantly reduced in SCO, while HMGCR, SPAM1, CRISP3, and GNAS gene expression was similar between normal and SCOS testes (Sheng Cheng et al. 2015). Round spermatid enhanced Septin12 (SEPTIN12) is another potential biomarker for ruling out SCO, as single nucleotide polymorphisms in the gene are associated with the syndrome (Miyakawa et al. 2012).

The results of our TMT-labelling mass spectrometry analysis of TIF highlight the high inter-sample variability and the presence of markers of normal testicular function Despite similar proteomic profiles, there remains a possibility to find proteomic biomarkers that are differentially expressed between MA and SCO when using the right analytic methods. The

Blood-Testis-Barrier (BTB), composed of cellular tight-junctions formed by somatic Sertoli cells, creates an immune-privileged luminal compartment where advanced germ cells gradually evolve into highly specialized sperm, isolated from the interstitial vasculature (Meinhardt et al. 2011). Recent experimental findings have challenged the dogma of sperm-proteins being completely sequestered on the adluminal side of the BTB (O'Donnell et al. 2021). The deposition of proteins specific to post-meiotic germ cells into the interstitium and subsequent access to the bloodstream offers new possibilities for non-invasive clinical diagnostics to monitor testicular function in the bloodstream.

Conclusion:

In-depth characterization of the TIF proteome allowed for the identification of proteins involved in spermatogenesis and testicular function in general. These included proteins specific to adluminal germ cells. We used a comparative proteomic approach to find molecular indicators of testicular function in patients with normal spermatogenesis and NOA. In this study, we determined several candidate biomarkers of non-obstructive azoospermia in TIF and identified proteins whose expression levels in the testicular interstitial fluid of MA and SCO patients are distinct and that could reflect sperm production in the testes. A non-invasive test that indicates with certainty whether spermatozoa are present prior to TESE can improve NOA patient counselling and improve sperm retrieval rates by referring only patients with residual spermatogenesis (Batruch et al. 2011, Freour et al. 2013). Further research is needed to validate the differential expression of these proteomic biomarkers of spermatogenesis in different forms of testicular failure and to confirm their detectability in blood plasma.

General discussion:

In the last few years, there has been considerable progress in the understanding and treatment of male infertility. Men have been seeking treatment for infertility at an increasing rate in recent years (Levine et al. 2017). Even men with little viable sperm might be able to conceive thanks to advances in sperm retrieval methods as well as IVF and ICSI (Corona et al. 2019). The invasiveness of assisted reproduction procedures as well as the uncertainty of the outcomes can pose a significant mental burden on infertile couples. Couples undergoing fertility treatment should be offered counselling to reduce psychological stress, according to NICE (2013). Research is currently focused on improving sperm retrieval rates, by advancing micro-surgical techniques and diagnosis tools (for review, see Leaver, 2016).

Despite major breakthroughs in clinical biochemistry and medical imaging techniques, testicular biopsy results are still indispensable for the accurate diagnosis of severe forms of male infertility such as azoospermia (Schlegel, 2004). It's not uncommon for patients with varying degrees of spermatogenic failure to have comparable physical and hormonal profiles (Jarvi et al. 2010, Zufferey et al. 2018). There is an unmet clinical need for molecular biomarkers that can predict sperm retrieval outcomes in idiopathic non-obstructive azoospermia (NOA) (Agarwal et al. 2020). Sertoli cell-only syndrome (SCO), also known as germ cell aplasia, manifests as a complete lack of germ cells, with only Sertoli cells lining the seminiferous tubules, reduced tube diameter, and thicker tubular walls. A patient with SCOS will present with azoospermia, normal virilization, and atrophic to normal testes (Del Castillo et al. 1947). Mixed atrophy, also referred to as focal SCOS, is characterized by the presence of residual pockets of normal spermatogenesis (Ghanami Ghashti et al. 2020). Mixed atrophy patients can benefit from sperm extraction and ICSI for conception, but sperm retrieval is usually unsuccessful in the majority of Sertoli cell only syndrome patients as no mature sperm cells can be found (Vloeberghs et al. 2015). The most prevalent genetic anomaly detected in Caucasian azoospermic males with Sertoli cell-only syndrome is karyotype abnormalities (24 %), particularly Klinefelter syndrome and AZF deletions, which are routinely investigated in men with non-obstructive azoospermia (Stouffs et al. 2016). However, no genetic factors are found in approximately 40% of cases of testicular failure. The underlying molecular mechanisms of idiopathic spermatogenic impairment in SCOS are poorly understood but microRNA deregulation (Noveski et al. 2016) and X-chromosome copy number variations

(Stouffs et al. 2016), and chronic testicular parenchymal hypoxia (Gat et al. 2010) have been investigated as possible contributing factors.

In the last three decades, numerous studies have focused on the discovery of biomarkers for the differential diagnosis of azoospermia in seminal plasma as a means to predict the success rate of microsurgical TESE (Milardi et al. 2013, Agarwal et al. 2016). Testis-specific proteins drastically decreased in NOA have been proposed as potential biomarkers for sperm retrieval (Drabovich et al. 2011, Batruch et al. 2012, Rolland et al. 2013, Freour et al. 2013, Drabovich et al. 2014, Korbakis et al. 2015). The translation of these research findings into clinical diagnostics has been slow. However, the accumulation of discovery proteomics data resulted in the emergence of a number of robust candidate biomarkers of sperm retrieval, as certain proteins were reported as differentially regulated in seminal plasma from NOA men across multiple studies originating from different labs. Some of these include LDHC, HIST1H2BA, HSPA2, and PGK2, which are downregulated in NOA, and semenogelins 1 and 2 (SEMG1, SEMG2), which are overexpressed in testicular failure (Agarwal et al. 2016).

Our comparative analysis of seminal plasma proteome from fertile and azoospermic men showed a significantly lower abundance of some epididymis-enriched proteins (PTGDS, ELSPBP1) in obstructive azoospermia, and of certain germ cell-specific proteins in Sertoli cell only (LDHC, HSPA2, DPEP3, PGK2, HSPA4L). These findings are consistent with previously published mass spectrometry data (Drabovich et al. 2011, Batruch et al. 2012). By analysing testicular interstitial fluid (TIF), a less accessible biological sample that is more reflective of testicular function, we aimed to gain a better understanding of the molecular mechanisms involved in severe spermatogenic failure and determine markers for differentiating between MA and SCO cases. The presence of adluminal germ cell-specific proteins in human TIF, along with similar findings from animal studies, might indicate they have been able to enter the general circulation (O'Donnell et al. 2021a). Thus, new tests could potentially be developed for monitoring testicular function in blood plasma. The results of high-resolution proteomic analysis of TIF reflected substantial changes in gene expression in SCO that are not limited to the depletion of spermatid-enriched proteins (LDHC, TKTL1, HSPA2, TUBA3D), but also include the significant up-regulation of proteins exclusively expressed in somatic cells.

Overall, differentiating mixed atrophy and SCO at the molecular level can be challenging. The two conditions are characterized by very low to undetectable levels of germ cell specific proteins in seminal plasma and TIF. Nevertheless, the prediction of the presence of sperm in

the testis could be possible by the targeted quantification of a select number of post-meiotic germ cell proteins using immunoassays. By combining mass spectrometry data, results of antibody-based assays (immunoblotting, immunohistochemistry), omics databases and recent literature, we were able to select a few proteins (LDHC, HSPA2, TKTL1, TUBA3D) whose expression patterns, cell specificity and abundance make them ideal biomarkers for the presence of viable sperm in the testes of azoospermic men. The validation of these candidate biomarkers must pass through the development of suitable immunoassays and the quantification of the target proteins in an adequate number of clinical samples (Looney et al. 2015). Further research is needed to determine the best methods for the detection and quantitation of these low abundance proteins in seminal and blood plasma, including sample pre-treatment and protein enrichment strategies. Mass spectrometry-based target proteomics can be useful for confirming the presence of germ cell markers in the blood of healthy men and the subsequent development of diagnostic tests. Constant progress in omics technologies has improved our understanding of the aetiology of male infertility by enabling high-throughput analysis of complex biological samples and the discovery of biomarkers of physiological processes and disorders of the male reproductive tract. Translating these research findings into clinical applications is essential for the improvement of the diagnosis and treatment of male infertility.

Appendix

Supplementary table 1:
Clinical data on Sertoli cell only, mixed atrophy, spermatogenic arrest, and obstructive azoospermia/ normal spermatogenesis patients from whom TIF and seminal plasma samples were used for proteomic analysis

	Non-obstructive azoospermia			Obstructive azoospermia (OA)
	SCO	Mixed atrophy (MA)	Spermatogenic arrest (SA)	Normal spermatogenesis
	n=18	n=17	n=12	n=18
Demographics				
Patient's age (years)	34 (27- 45)	36 (30- 50)	34 (25- 41)	33 (27- 98)
Body size (cm)	180 (165- 194)	180 (169- 195)	183 (150- 190)	175 (170- 186)
Body weight (kg)	84 (64- 124)	81 (62- 100)	88 (67- 128)	80 (68- 127)
BMI (kg/m^2)	25.2 (20.2- 38.3)	24.5 (21.3- 31.6)	27.8 (23.7- 37.8)	26.1 (22.2- 37.4)
Semen parameters				
Volume (ml)	3.6 (2.0- 5.9)	2.3 (1.6- 4.8)	1.2 (0.5- 4.4)	2.7 (1.5- 4.0)
pH value	7.6 (7.4 - 7.9)	7.6 (7.4 - 7.8)	7.9 (7-9.3)	7.7 (7.4 - 7.8)
Sperm concentration (mio/ml)	0 (0 - 0.0)	0 (0 - 0.0)	0 (0-0)	0 (0 - 0.0)
Peroxidase- positive Leukocytes (mio/ml)	0.1 (0 - 0.2)	0.2 (0 - 0.3)	0.1 (0-0.8)	0.0 (0 - 0.0)
Fructose (µmol/ejaculate)	57.3 (35.5 - 118.6)	35.0 (16.8 - 48.6)	18.78 (3-86.4)	43.2 (14.6 - 76.5)
Glucosidase (mU/ejaculate)	37.8 (27.8 - 59.4)	26.5 (13.7 - 46.5)	17.59 (8.15 -98)	19.2 (11.4 - 29.8)
Elastase (ng/ml)[a]	27 (15 - 130)	87 (21 - 226)	19 (10-245)	68 (12 - 152)
Zinc (µmol/ejaculate)[b]	14.7 (4.7 - 24.1)	11.4 (6.3 - 21.8)	6.08 (0.264-36.4)	8.6 (4.6 - 13.4)
Hormones				
FSH (mU/ml)	22.5 (16.2-29.7)	27.3 (15.5 - 41.8)	8.55 (5.6-53.8)	4.5 (2.6 - 8.8)
LH (mU/ml)	7.9 (4.3 - 10.3)	9.4 (5.0 - 13.3)	6.15 (1.6-19.8)	3.6 (2.2 - 6.1)
Testosterone (nmol/l)	12.0 (9.9 - 17.1)	14.0 (12.5 - 18.1)	253.5(131-551)	14.3 (10.2 - 17.5)
Free testosterone (pmol/l)	229.7 (178.8 - 276.6)	257.8 (168.6 - 335.4)	5.195 (2.66-10.99)	269.3 (204.7 - 320.4)

SHBG (nmol/l)	37.5 (24.6 - 53.0)	43.6 (30.4 - 62.7)	28.65 (6.7-78.1)	31.2 (21.3 - 46.4)
Albumin (g/l)	45.6 (43.7 - 47.7)	47.1 (44.0 - 49.0)	47.3(41.8-50.4)	45.7 (44.2 - 48.1)
Estradiol (pmol/l)	30 (23 - 36)	34 (27 - 40)	32 (19-38)	29 (22 - 39)
Prolactin (uIU/ml)	228 (186 - 262)	171 (121 - 339)	141.5 (109-485)	167 (118 - 189)
Ultrasound				
Total testicular volume (ml)	14.2 (10.6 - 19.7)	13.2 (10.5 - 15.7)	17.45 (4.35-31.78)	22.9 (19.4 - 31.9)
Median testicular volume (ml)	7.1 (5.3 - 9.8)	6.6 (5.3 - 7.9)	8.85 (1.88-17.68)	12.1 (10.1 - 17.3)
Epididymal head thickness (mm)[b]	6.8 (5.9 - 7.8)	8.0 (7.2 - 9.7)	7.35(4.65-11.5)	8.3 (7.0 - 9.5)
data presented as median, (min- max)				

Supplementary table 2:

Histological scores on testis biopsies from patients diagnoses with Sertoli cell only, mixed atrophy, spermatogenic arrest, and obstructive azoospermia/ normal spermatogenesis whom TIF and seminal plasma samples were used for proteomic analyses

Sample ID	Score TESE top right	Score TESE middle right	Score TESE bottom right	Score Micro-TESE right	Score TESE top left	Score TESE middle left	Score TESE bottom left	Score Micro-TESE left	Seminal plasma Label-free LC MS/MS	TIF Label-free LC MS/MS	TIF High resolution LC MS/MS	TIF TMT labelling LC MS/MS/MS
MA1	1,00	0.9	2,00	0,00	0,00	0,00	0,00	0,00	X	X	X	
MA2	0,00	1,00	5,00	0.6	0,00	0.1	5,00	2,00	X	X	X	
MA3	0,00	0.1	2,00	1,00	4,00	2,00	0,00	0.6	X	X		
MA4	0,00	0,00	0,00	3,00	0,00	0,00	0,00	0.2	X	X		
MA5	2,00	0.2	0.7	0.6	0.9	0.09	0.5	0.5	X	X	X	
MA6	4,00	0.4	0.4	0.7	0,00	0,00	0,00	0,00	X	X		
MA7	6,00	2,00	0.7	0.7	4,00	0.8	0.4	2,00	X	X	X	
MA8	2,00	0.3	0,00	0,00	0,00	0,00	0,00	0,00	X	X	X	
MA9	0,00	0,70	2,00	7,00	0,50	0,00	0,00	0,60			X	
MA10	3,00	0,50	0,00	0,30	1,20	0,00	0,00	0,00			X	
MA11	6,60	0,00	0,00	3,70	1,60	0,00	9,00	1,50			X	
MA13	0,00	0,30	0,80	0,30	1,30	1,00	0,30	0,10			X	X
MA14	3,00	4,00	2,00	7,70	0,80	4,00	0,00	6,60			X	X
MA15	0,00	0,00	0,50	0,00	0,00	0,60	3,60	2,60			X	X
MA16	2,00	7,00	3,00	0,20	3,00	2,00	1,00	1,00			X	X
MA17	0,00	0,00	0,00	2,00	0,00	0,50	0,00	*NA*				X
MA18	2,00	2,00	0,00	3,00	0,10	0,00	0,00	0,00				X
OA1	10,00	10,00	7,00	10,00	9,00	9,00	10,00	10,00	X	X	X	

OA2	10,00	10,00	10,00	10,00	10,00	10,00	10,00	10,00	X	X	X	X
OA3	10,00	10,00	10,00	10,00	10,00	10,00	10,00	10,00	X	X	X	
OA4	10,00	10,00	10,00	10,00	10,00	10,00	10,00	10,00	X	X	X	
OA5	10,00	10,00	10,00	10,00	10,00	10,00	10,00	10,00	X	X	X	X
OA6	10,00	10,00	10,00	10,00	Single testis	Single testis	Signle testis	Single testis	X	X	X	X
OA7	10,00	7,00	10,00	10,00	10,00	10,00	10,00	10,00	X	X	X	X
OA8	9,00	9,00	9,00	10,00	7,00	9,00	8,00	8,00			X	
OA9	9,00	9,50	9,20	*NA*	9,20	10,00	10,00	*NA*			X	X
OA10	9,00	9,60	9,40	10,00	9,00	9,30	10,00	10,00			X	X
OA11	10,00	9,80	10,00	9,30	10,00	10,00	10,00	10,00			X	X
OA12	10,00	10,00	9,50	0,50	10,00	10,00	9,00	*NA*			X	X
OA13	8,00	8,00	9,00	9,00	*NA*	*NA*	*NA*	*NA*				X
OA14	10,00	10,00	9,00	9,00	8,00	9,00	8,00	10,00				X
OA15	9,00	10,00	9,00	10,00	9,00	10,00	9,00	10,00				X
OA16	10,00	10,00	10,00	NA	10,00	10,00	5,00	NA				X
OA17	3,00	4,00	9,00	9,00	*NA*	*NA*	*NA*	*NA*				
OA18	7,00	9,00	10,00	NA	8,00	9,00	10,00					X
SCO1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X		X
SCO2	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X	X	X
SCO3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X		
SCO4	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X		
SCO5	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X	X	X
SCO6	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X		

SCO7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	X	X	X	X
SCO8	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO9	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO12	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO13	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	X
SCO14	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SCO15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SCO16	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00				X
SCO17	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00				X
SCO18	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00				X
SA1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA2	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA4	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA5	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA6	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA8	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA9	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			X	
SA12	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00			X	

Supplementary table 3: TIF proteins (listed by gene names, Uniprot accession numbers) that are enhanced/ enriched in early and late spermatids. Proteins were identified in a pool of 6 OA/ normal spermatogenesis TIF samples by LC-MS/MS/MS. Cell type specificity data was downloaded from https://www.proteinatlas.org/ , August 2021

Enhanced in late spermatids (n=727)									
Accession N	**Gene**	**Accession N**	**Gene**	**Accession N**	**Gene**	**Accession N**	**Gene**	**Gene**	**Accession N**
Q96J65	ABCC12	I3L073	CDRT1	Q4G1C9	GLIPR1L2	H0YFW3	PACRG	H0YGV1	TIAM2
P10323	ACR	Q96T59	CDRT15	Q96MS3	GLT1D1	Q6QHF9	PAOX	Q8IY51	TIGD4
E7EP66	ACRBP	H0Y5L8	CENPJ	Q7Z4J2	GLT6D1	A0A1L4AAP0	PAX3	Q96H15	TIMD4
Q9Y615	ACTL7A	Q71F23	CENPU	A6NDN3	GOLGA6B	E5RFX4	PBK	Q9H0I9	TKTL2
Q9Y614	ACTL7B	J3QL98	CEP112	P0CG33	GOLGA6D	Q9Y5H5	PCDHA9	H0YMD4	TMCO5A
Q8TC94	ACTL9	O94986	CEP152	A0A087X1J3	GOLGA6L10	K7EJB6	PCSK4	D6R911	TMEM108
Q8TDG2	ACTRT1	H0YB92	CEP170	Q8N9W4	GOLGA6L2	F2Z2B1	PCYT1B	A6NGA9	TMEM202
Q96M93	ADAD1	Q96MC4	CEP295NL	A0A0G2JRP8	GOLGA6L22	Q8N4E4	PDCL2	Q6GV28	TMEM225
Q8NCV1-2	ADAD2	Q9BYV8	CEP41	A8MZA4	GOLGA6L6	P29803	PDHA2	A6NEH6	TMEM247
Q9Y3Q7-2	ADAM18	H0Y432	CEP55	P0CJ92	GOLGA8H	Q8N807	PDILT	Q9P1W3	TMEM63C
O43506	ADAM20	Q5SZL2	CEP85L	H3BSY2-2	GOLGA8M	A0A087X0X8	PHF7	A2RUT3	TMEM89
Q9UKJ8	ADAM21	Q12798	CETN1	A6NCC3	GOLGA8O	Q5TCQ3	PHTF1	P0DMS9	TMIGD3
Q9UKF5	ADAM29	Q494V2	CFAP100	Q8NHS2	GOT1L1	A0A087WUY7	PIAS2	F8WBX2	TMPRSS12
Q8TBZ7	ADAM30	Q6P656	CFAP161	Q9HCN6-3	GP6	Q8WWB5	PIH1D2	Q8IU80	TMPRSS6
A0A0J9YWN9	ADAM32	Q8IYR0	CFAP206	Q8N158	GPC2	Q8TDX9	PKD1L1	L8E7F2	TMPRSS7
Q96PN6	ADCY10	Q4G0U5-3	CFAP221	Q8IZ08	GPR135	Q9NZM6-2	PKD2L2	A0A3B3IU58	TMPRSS9
Q8N7X0	ADGB	Q96M91	CFAP53	Q8NFN8	GPR156	Q9NTG1	PKDREJ	P23510	TNFSF4
A0A3B0IT49	ADIG	Q9H4Y4	CFAP58	A0A087X043	GRID2	Q4KWH8-2	PLCH1	Q05952	TNP2
Q8NDL9	AGBL5	Q8NHU2	CFAP61	P32298	GRK4	H0YGH7	PLCZ1	Q6XPS3-4	TPTE2
D6RF73	AHRR	Q6ZU64	CFAP65	A6NFK2	GRXCR2	O00444	PLK4	Q9Y577	TRIM17
Q96MA6	AK8	Q6ZQR2	CFAP77	Q9UNN4	GTF2A1L	H0YFP6	PMFBP1	Q9NQ86-3	TRIM36
Q86UN6	AKAP14	A0A087WWY8	CFAP99	P52657	GTF2A2	Q8NBT0-2	POC1A	Q8IWZ5	TRIM42

O75969	AKAP3	A0A0C4DFS2	CHODL	Q8IZA3	H1FOO	Q9HBV1	POPDC3	Q86XT4	TRIM50
Q5JQC9	AKAP4	Q8IVW0	CHRM5	Q96MB7	HARBI1	A8MPX8	PP2D1	A0A0G2JPI4	TRIM69
Q5T1N1-3	AKNAD1	P30926	CHRNB4	Q7Z4H7	HAUS6	Q8WY54	PPM1E	Q8N9V2	TRIML1
Q96JD6	AKR1E2	A0A087WU70	CHST13	Q9Y3Q4	HCN4	S4R3I3	PPM1J	J3KNI5	TRIML2
B6D4Y8	ALK	K7EPJ4	CILP2	Q86WZ0	HEATR4	Q7Z5V6	PPP1R32	Q15645	TRIP13
Q8N6M5	ALLC	M0R1J6	CLDND2	A2RTY3	HEATR9	Q96LQ0	PPP1R36	F8W1E3	TROAP
Q8TCU4	ALMS1	O14967-2	CLGN	Q9BXL5	HEMGN	Q96LZ3	PPP3R2	F8WA32	TSGA10
Q7Z5J8	ANKAR	Q8NHS4	CLHC1	H0Y3X7	HFM1	D2K7F0	PRDM9	Q3SY00	TSGA10IP
J3KQ21	ANKMY1	Q6NUQ2	CLMN	Q6UWX4	HHIPL2	J3QQU2	PROCA1	Q96PP4	TSGA13
A0A0F6MY87	ANKRD18B	P53675	CLTCL1	Q8NE63	HIPK4	P21108	PRPS1L1	Q9UJT2	TSKS
A0A590UK20	ANKRD30B	Q8IZ96-5	CMTM1	Q16695	HIST3H3	A6NJB7	PRR19	E7ENJ7	TSNAXIP1
Q8N9B4	ANKRD42	Q8TAZ6	CMTM2	Q8NCD3	HJURP	Q8IZ63	PRR22	Q86VY4	TSPYL5
Q5TZF3	ANKRD45	Q8NA66	CNBD1	O75330	HMMR	C9JVA3	PRR30	Q96PF2	TSSK2
Q8N9V6	ANKRD53	Q96M20	CNBD2	C9JSF2	HSF2BP	Q5T870	PRR9	Q96PN8	TSSK3
D6RBD3	ANKRD55	B4DYQ8	CNGA4	Q9BQS6	HSPB9	A4D1T9	PRSS37	Q6SA08	TSSK4
Q9BZ19	ANKRD60	Q9NRU3	CNNM1	P34969-2	HTR7	A0A3B3ISV0	PRSS51	Q8IWY7	TTBK2
A6NGH8	ANKRD61	K7ERX8	CNTD1	Q96M11	HYLS1	Q6PEW0	PRSS54	Q8NEE8	TTC16
Q92527	ANKRD7	P38432	COIL	P15814	IGLL1	A0A0C4DGJ2	PSORS1C1	Q8NDW8	TTC21A
F1T0L7	ANO2	A0A140TA54	COL11A2	D0EFR8	IL13RA2	Q4JDL3-12	PTPN20	A0A3B3IS63	TTC23L
A6NF34	ANTXRL	Q9BXS0	COL25A1	P01583	IL1A	F8W122	PTPRQ	Q96NG3	TTC25
P55064	AQP5	Q14050	COL9A3	Q5VWK5	IL23R	Q2VPE7	PTTG1	D6R945	TTC29
Q9P2N2-2	ARHGAP28	Q6YFQ2	COX6B2	A0A494C190	IL31RA	Q9UJV8	PURG	A0A1C7CYW7	TTC34
A8MVX0	ARHGEF33	Q8N123	CPXCR1	Q0VD86-2	INCA1	A1KZ92	PXDNL	A0A0A0MSW9	TTC39A
Q5H913	ARL13A	H7BXU2	CSMD1	P14616	INSRR	H0YDB9	QRICH2	G3V3A5	TTC6
Q6T311	ARL9	Q7Z407	CSMD3	A0A087WXK3	INTS7	A0A1B0GTL5	RAB11FIP5	H7C055	TTC7A
Q5T9G4	ARMC12	Q8N752	CSNK1A1L	H0YN07	IPO4	H0YHP4	RAB3IP	P33981	TTK
H0Y4P5	ARMC2	Q8IXA3	CSNK1G1	E0ZS59	IQCA1L	Q9H4I0	RAD21L1	Q6ZVT0	TTLL10

B4DXS3	ARMC3	Q9H4G1	CST9L	F8VZV9	IQCD	I6L9E5	RASGEF1C	Q9BWV7	TTLL2
A0A5F9ZI13	ARMC4	Q8NEG8	CTAGE1	A8MTL0	IQCF5	Q8NHQ8	RASSF8	G3V2J9	TTLL5
A6NEK1	ARRDC5	A4FU28	CTAGE9	A0A590UJ54	IQCF6	O75526	RBMXL2	F8WDP8	TTLL6
H7C1W0	ART5	C9IZ88	CTNNA2	B9EG57	IQCG	K7ELF7	RDH8	A0A1C7CYW9	TTLL8
K7ELW9	ASF1B	A0A3B3ISQ8	CXorf58	Q86VS3	IQCH	O75679-2	RFPL3	A0A087WYL2	TTLL9
Q9UHC3-2	ASIC3	Q5JRM2	CXorf66	Q8NA54	IQUB	P48378	RFX2	P0DPH7	***TUBA3C***
Q13733	ATP1A4	E9PRM4	CYB5R2	Q6NXR0	IRGC	P48380-2	RFX3	Q6PEY2	***TUBA3E***
Q96A05	ATP6V1E2	Q3ZCV3	CYLC1	Q24JQ5	ISLR2	Q33E94	RFX4	C9J2C0	***TUBA8***
Q9NTI2	ATP8A2	Q14093	CYLC2	M0QXA9	IZUMO2	A0A286YEQ5	RGPD1	Q9H4B7	***TUBB1***
Q5Y191	AURKC	Q9UBU7	DBF4	S4R3E6	IZUMO3	A0A1W2PNU4	RGPD2	Q3ZCM7	***TUBB8***
Q96A70	AZIN2	Q5VU92	DCAF12L1	Q1ZYL8-3	IZUMO4	J3KNE0	RGPD3	O00295	TULP2
Q9UL15	BAG5	J3QKL2	DCC	B2RDY3	KATNAL1	Q7Z3J3	RGPD4	Q86VQ3	TXNDC2
A0A0B4J2D7	BBOF1	M0R2J8	DCDC1	K7EIJ8	KATNAL2	O14715	RGPD8	V9GYA6	UBE2U
K7EMX4	BCL2L14	A8MYV0	DCDC2C	E5RHP1	KCNU1	A0A087WV61	RGS22	Q6ZU65	UBN2
H3BLT4	BIRC5	A0A1B0GTZ4	DCLK3	Q68DU8	KCTD16	B7Z7N5	RGS6	Q9H347	UBQLN3
Q96P09	BIRC8	Q5T197	DCST1	Q6B0I6	KDM4D	A5PLK6	RGSL1	Q8IYU4	UBQLNL
F5GY04	BNC1	Q5T1A1	DCST2	L8EC47	KHDRBS3	Q9H4K1	RIBC2	Q3SY77	UGT3A2
Q8IYS8	BOD1L2	Q8NEL9-4	DDHD1	Q8NCT3	KIAA0895	Q8IZP6	RNF113B	Q96C45	ULK4
Q8N9W6-3	BOLL	Q8WTU0	DDI1	Q9ULL0	KIAA1210	Q8N7C7	RNF148	Q5DID0-2	UMODL1
Q7Z569-2	BRAP	Q8TDR3	DDX20	B7ZVZ4	KIAA1211	A0A0C4DFQ4	RNF151	Q8NB66	UNC13C
Q58F21-5	BRDT	E9PR46	DDX25	A0A2R8YFM9	KIAA1257	A8MTL3	RNF212B	Q9P2D8	UNC79
J3QQP5	BRIP1	O15523	DDX3Y	Q9NS87-2	KIF15	Q9H0A6	RNF32	O75604	USP2
Q32M84	BTBD16	Q9NQI0	DDX4	Q8NI77	KIF18A	E9PI44	RNFT1	A1L447	USP29
Q5VZT2	C10orf113	Q86TM3	DDX53	Q5T7B8	KIF24	D6RCR2	ROPN1B	A4D2N7	USP42
Q5SQS8	C10orf120	H0YES2	DEPDC1	Q86VH2	KIF27	G3V3F7	RPGRIP1	Q9H0E7	USP44
B7Z368	C10orf142	Q96QD5	DEPDC7	Q8N4N8	KIF2B	Q96L21	RPL10L	H0YNB9	USP50
Q8WW14	C10orf82	Q9NSV4-6	DIAPH3	Q5JT82	KLF17	B2RC85	RSPH10B2	P35125	USP6
H7C3F8	C10orf90	M0R1E5	DKKL1	Q8NEP7-2	KLHDC9	H7C3W6	RSPH14	F8WBX4	VWA3B
Q8NCR3	C11orf65	H0YBY6	DLGAP2	Q6JEL2	KLHL10	M0R103	RSPH6A	E9PNX8	VWA5B1

C9JXX5	C11orf94	O95886	DLGAP3	Q9NVR0	KLHL11	F8WC89	SAC3D1	L8E9M8	WBP2NL
A0A1B0GVM6	C11orf97	H3BN61	DMRT1	Q6PF15	KLHL35	Q9P1V8	SAMD15	B1ANS9	WDR64
Q86WS4	C12orf40	A0A0S2Z5I3	DMRTB1	H0YM11	KNSTRN	Q9UPU9	SAMD4A	H0YEH4	WDR78
F8VV63	C12orf42	Q8N9W5	DNAAF3	P50748	KNTC1	F6S232	SAXO1	Q6ZMY6	WDR88
Q8NA57	C12orf50	A0A087WV07	DNAH10	Q7Z3Z0	KRT25	F8VWH5	SCNN1D	Q6P2C0	WDR93
Q8IXR9	C12orf56	Q0VDD8	DNAH14	O76009	KRT33A	Q5TEA6	SEL1L2	B7ZMN2	XKR3
Q5U649	C12orf60	K7ELN3	DNAH17	Q14CN4-2	KRT72	H7C188	SERINC4	I3L2D6	YBX2
Q6P387	C16orf46	Q8TD57	DNAH3	O95447	LCA5L	Q8N6R1	SERP2	A0A087WUA6	YJEFN3
Q8WTQ4	C16orf78	Q9C0G6	DNAH6	Q6ZMR3	LDHAL6A	P58005	SESN3	B9EG67	ZAR1
H3BSP6	C16orf92	Q8WXX0	DNAH7	P07864	LDHC	A6NKC9	SH2D7	B7ZMD2	ZBBX
Q9H693	C16orf95	A0A075B6F3	DNAH8	J3KP02	LEKR1	Q99963	SH3GL3	A0A2R8Y5N3	ZBED9
A6NNT2	C16orf96	J3QRG2	DNAI2	E9PMA4	LETM2	B4DYU0	SHBG	Q9Y2Y4	ZBTB32
A0A075B7C2	C17orf50	H0YFX2	DNAJB13	Q3ZCV2-2	LEXM	Q9BZQ2	SHCBP1L	J3KMY6	ZC2HC1C
Q86WR6	C17orf64	Q9H819	DNAJC18	Q9H9Z2	LIN28A	A0A087WVB7	SHOX2	Q504Y3	ZCWPW2
C9J6K1	C19orf81	Q8N7S2-2	DNAJC5G	Q8TD35	LKAAEAR1	***P48664***	***SLC1A6***	Q8WVZ1	ZDHHC19
Q5JVX7	C1orf141	Q8IYX4	DND1	Q6UX01	LMBR1L	***Q9Y267***	***SLC22A14***	Q9C0A1	ZFHX2
Q5SNV9	C1orf167	A0A024R627	DOC2A	Q96KR4	LMLN	***Q86VW1***	***SLC22A16***	L8E970	ZFHX4
Q5VU69	C1orf189	A0A2X0SFH6	DOCK3	Q8N9Z9	LMNTD1	***Q8N5S1***	***SLC25A41***	Q96MM3	ZFP42
B1ALJ5	C1orf194	Q9NYP3-2	DONSON	A1A4G5	LNP1	***I3L0B8***	***SLC25A52***	Q9UPR6	ZFR2
Q6P1W5	C1orf94	H0YF77	DPY19L2	A0A2R8Y7K4	LOXHD1	***Q96RN1***	***SLC26A8***	Q9NZV7	ZIM2
Q86Z23	C1QL4	Q9BPU6	DPYSL5	A0A2R8YEJ5	LRGUK	***P22732-2***	***SLC2A5***	O75800-2	ZMYND10
Q96LM9	C20orf173	Q96MC2	DRC1	Q9P2V4	LRIT1	***C9JV68***	***SLC30A3***	Q9H091	ZMYND15
Q9BZE7	C22orf23	H3BRN2	DRC7	Q9P2M1	LRP2BP	***Q6ICL7***	***SLC35E4***	V9H066	ZNF177
O95567	C22orf31	Q13167	DRD3	Q8N456	LRRC18	***Q495N2***	***SLC36A3***	P98182	ZNF200
Q6IC83	C22orf42	Q9NZJ0	DTL	Q8IZ02	LRRC34	***A0A0G2JQN1***	***SLC45A2***	A6NK53	ZNF233
C9JG08	C2orf16	Q5QP64	DUSP15	J3QSG3	LRRC36	***Q9GZN6-2***	***SLC6A16***	A0A3B3ITE2	ZNF385C
Q9NWW7	C2orf42	Q9H596	DUSP21	A6NM11	LRRC37A2	***Q4ZJI4***	***SLC9B1***	Q6S9Z5	ZNF474
B7ZM12	C2orf73	E0Z3F1	DUX4	A0JLV1	LRRC37A3	***Q86UG4***	***SLCO6A1***	A0A087WTN2	ZNF479

A6NCI8	C2orf78	Q8WWB3	DYDC1	Q8N309-3	LRRC43	Q499Z3-2	SLFNL1	B1APH4	ZNF487
L0R591	C3orf20	Q8TF09	DYNLRB2	Q96FV0	LRRC46	A6NFE2	SMCO2	Q86UE3	ZNF546
A0A5F9ZI70	C3orf49	A0A0A0MTH5	DYRK4	Q8N7C0	LRRC52	M0R0U0	SMG9	Q96MR9	ZNF560
C9J3M8	C3orf67	Q86YF9	DZIP1	Q8IYG6	LRRC56	J9JIF2	SMIM21	Q8TA94	ZNF563
H3BNL1	C3orf84	F8VSE7	E2F7	J3KQU2	LRRC63	H3BMG3	SMKR1	Q68EA5	ZNF57
Q53FE4	C4orf17	P0CF75	EBLN1	Q6ZNQ3	LRRC69	Q9NY99-2	SNTG2	Q3MI94	ZNF578
Q96KX1	C4orf36	Q9HAE3	EFCAB1	Q8N4P6	LRRC71	A0A087X1Q5	SOCS7	Q8TC21	ZNF596
D6RCA9	C4orf47	A0A2R8YDC4	EFCAB10	A6NJI9	LRRC72	Q9NX45-3	SOHLH2	Q8N9Z0-2	ZNF610
D6RHK3	C5orf49	Q6NXP0	EFCAB12	Q5JTD7	LRRC73	O94993-2	SOX30	Q5EBL2	ZNF628
Q5TEZ5	C6orf163	Q8IY85	EFCAB13	Q0VAA2	LRRC74A	P35711	SOX5	O15015-1	ZNF646
Q7Z4U5	C6orf201	Q8N7B9-2	EFCAB3	A0A2R8Y4H4	LRRC9	P35712-2	SOX6	A0A3B3ITU2	ZNF676
A0A024RA13	C7orf31	A4FU69	EFCAB5	Q9C099	LRRCC1	Q6Q759	***SPAG17***	Q8IYX0	ZNF679
H7C3K1	C7orf61	Q5THR3-5	EFCAB6	A4D1F6	LRRD1	K7ELG0	***SPAG5***	Q5T142	ZNF683
Q96LL4	C8orf48	A0A096LNH2	EFCAB8	H0YCJ9	LRRIQ1	O75602-3	***SPAG6***	Q8N972	ZNF709
Q6P047	C8orf74	Q8N7U6	EFHB	H0Y5F9	LRRIQ3	H7C4J6	***SPAG8***	Q6ZMV8	ZNF730
A0A087WUN2	C8orf89	C9J7B5	EIF5A2	A6NIV6	LRRIQ4	P38567	SPAM1	A0A0C4DH12	ZNF821
Q5VYM1	C9orf131	P26378	ELAVL4	Q6UWN0	LYPD4	A0FGV4	SPANXC	Q3KNS6-3	ZNF829
H0YD79	C9orf135	A0A5H1ZRS3	ENO4	H7C5C8	MAATS1	Q4KRI2	SPANXD	M0QXL2	ZNF85
A0A3B3ISE1	C9orf153	Q8TC92	ENOX1	Q96JY0	MAEL	Q5VSR9	SPANXN1	M0QZH9	ZNF98
H0Y454	C9orf24	J3KQG3	EPHA10	P20794-3	MAK	Q5MJ09	SPANXN3	Q8WWF5	ZNRF4
Q5SZB4	C9orf50	Q9UF33-3	EPHA6	Q56UN5	MAP3K19	Q5MJ07	SPANXN5	Q8NEG5	ZSWIM2
Q96KC9	CABS1	P29322	EPHA8	Q8WWY6	MBD3L1	Q7Z6I5	***SPATA12***	Q9H900	ZWILCH
J3KTE8	CABYR	P54762-5	EPHB1	Q86YR7	MCF2L2	A0A494BZR9	***SPATA16***	Q12834	CDC20
D6RCC6	CAGE1	Q9NQ60	EQTN	Q4G0Z9	MCMDC2	Q96L03	***SPATA17***	P30307-3	CDC25C
Q96L12	CALR3	Q5RHP9	ERICH3	Q7Z553-3	MDGA2	Q8TC71	***SPATA18***	H7BZ91	CDC45
F8WBB8	CAND2	Q7L0X2	ERICH6	Q5I0G3	MDH1B	Q7Z5L4	***SPATA19***	Q69YH5	CDCA2
Q9UMQ6	CAPN11	Q5W0A0	ERICH6B	O95402	MED26	Q7Z572	***SPATA21***	Q8IVW4	CDKL3
Q96KX2	CAPZA3	A0A348FV91	ESR2	Q4G0I1	MEI1	H0YAD3	***SPATA24***	Q5MAI5	CDKL4

F8W8F9	CASC1	E7EWD9	ESRRB	A0A1P8YWJ3	MEIG1	Q6ZQQ2	***SPATA31D1***	A0A087WW25	CDKN3
P23435	CBLN1	Q5SXX8	ETNK2	A2RUB1	MEIOC	P0C874	***SPATA31D3***	Q49AH0	CDNF
Q14781	CBX2	O00321-2	ETV2	Q6ZN04	MEX3B	Q6ZUB1	***SPATA31E1***	Q9NY28	GALNT8
Q6DHV5-2	CC2D2B	Q8NHP7-3	EXD1	D6RDM6	MFAP3L	Q96LK8	***SPATA32***	Q7Z4T8	GALNTL5
Q8IYK2	CCDC105	H0Y3Q4	EYS	Q8IWD5	MFSD6L	Q8NEY3	***SPATA4***	Q49A17	GALNTL6
A0A096LNP5	CCDC110	Q15910-2	EZH2	Q9UBM8	MGAT4C	Q9NWH7	***SPATA6***	Q6W3E5	GDPD4
Q9H0I3	CCDC113	A6NFH5	FABP12	H0Y908	MGAT4D	Q9BWV2	***SPATA9***	Q86UU5	GGN
C9JW89	CCDC116	J3KSI9	FAM104A	A0A494C175	MLF1	Q76KD6	SPATC1	A0A494C112	GGTLC2
Q6ZUS5-2	CCDC121	Q49AJ0	FAM135B	O60882	MMP20	Q496A3	SPATS1	A0A097IW80	GJA8
H7C4R2	CCDC136	H0YMR7	FAM153B	A0A2Z2E9D2	MMS22L	Q5MJ68	SPDYC	Q14410	GK2
Q96M89	CCDC138	Q3B820	FAM161A	Q8NEH6	MNS1	A0A494C1S0	SPDYE1	Q6UWM5-2	GLIPR1L1
A2RUR9	CCDC144A	Q96GL9	FAM163A	E5RI85	MOK	J3KT05	SPDYE4	A0A126GWL0	OR4N2
Q8IYE0	CCDC146	A1A519	FAM170A	Q86VD1	MORC1	A6NIY4	SPDYE5	Q96RD0	OR8B2
Q8NFR7	CCDC148	A6NMN3	FAM170B	H7C1D5	MOV10L1	Q08AE8	SPIRE1	H7C428	OSBP2
Q0P6D6	CCDC15	A6NE01	FAM186A	Q7Z745-2	MROH2B	A0A0G2JNG8	SPPL2C	Q6ZRI0	OTOG
Q8NCX0	CCDC150	H0YIB0	FAM186B	F8WFA2	MROH7	H0Y8T2	SPSB4	Q96DC9	OTUB2
A0A590PWR5	CCDC154	Q17R55	FAM187B	H0Y3S5	MROH8	Q9BXG8	SPZ1	Q7RTY7	OVCH1
M0QZW6	CCDC155	Q6ZU69	FAM205A	H0Y687	MROH9	Q8WWF3	SSMEM1	A0A087X210	P2RX3
Q569K6	CCDC157	A6NFA0	FAM205C	H0YD53	MS4A14	Q9Y2M2	SSUH2	Q5JQF8	PABPC1L2A
Q5M9N0	CCDC158	Q5JX69	FAM209B	H0YD29	MS4A5	Q9Y2D8	SSX2IP	Q9P2T0	THEG
Q8NDH2	CCDC168	A0A1W2PRP4	FAM217A	Q6ZTZ1	MSANTD1	H0Y7M0	ST7L	Q8IWB6	***TEX14***
H0Y701	CCDC171	F8W8N9	FAM221B	O43347	MSI1	P59095	STARD6	A0A1W2PS94	***TEX15***
Q0VFZ6	CCDC173	Q5U5X8	FAM222A	A0A140VK87	MYCBPAP	A0A2R8Y693	STAT4	Q8N6G2	***TEX26***
P0C221	CCDC175	H7C3M9	FAM228A	A7E2Y1	MYH7B	Q8NE28	STKLD1	Q8N6K0	***TEX29***
F8W7A7	CCDC178	Q5JRC9	FAM47A	A0A087WYA1	MYO15A	H0Y4J3	STPG1	O43247	***TEX33***
Q5TID7	CCDC181	Q8NA70	FAM47B	A0A140TA25	MYO1H	A0A590UJF1	STRA8	Q5T0J5	***TEX35***
Q5T5S1	CCDC183	Q5HY64	FAM47C	Q8NEV4	MYO3A	H7BZA7	SUN3	Q6PEX7	***TEX38***

Q8N715	CCDC185	H0Y9F1	FAM53A	E9PKX5	NAALAD2	A9Z1W8	SUN5	Q8IUE0	TGIF2LY
A0A1W2PQX5	CCDC187	Q8IYT1	FAM71A	Q9H094	NBPF3	Q9H5I1	SUV39H2	Q96PF1	TGM7
H7C350	CCDC188	Q8TC56	FAM71B	H7C000	NEK10	Q5T5J6	SWT1	E7EUW0	CCSER1
J3QKX2	CCDC27	Q8NEG0	FAM71C	F6U4U2	NEK2	Q6PIF2	SYCE2	Q92526-2	CCT6B
A0A590UK19	CCDC30	G3V4K8	FAM71D	G5E9C2	NFKBIB	A1L190	SYCE3	A0A087WT28	CD200R1L
Q8N5R6-2	CCDC33	Q6IPT2	FAM71E1	Q5M9Q1	NKAPL	O14994	SYN3	C9JSP6	CDC14A
Q96HJ3	CCDC34	Q8N5Q1	FAM71E2	Q9H2Z4	NKX2-4	Q17RD7	SYT16	Q8NA03	FSIP1
Q8IYA8-2	CCDC36	Q96KD3	FAM71F1	P59045	NLRP11	A0A494C117	TACC3	Q5CZC0	FSIP2
F8VTU7	CCDC38	Q6NXP2	FAM71F2	Q86W24	NLRP14	Q8IZX4	TAF1L	F8VY49	G2E3
Q96M95	CCDC42	H0Y979	FAM81B	A0A0G2JNS7	NLRP7	Q5H9L4-2	TAF7L	Q99928	GABRG3
Q8NEL0	CCDC54	Q8IYD8	FANCM	P28336	NMBR	Q7RTX1-4	TAS1R1	Q8IXM7	ODF3L1
Q8IWA6	CCDC60	Q8TC84-2	FANK1	P56597	NME5	Q9NYW7	TAS2R1	O14841	OPLAH
Q6P9F0	CCDC62	Q969F0	FATE1	H7C0E4	NODAL	A0A0A0MSR7	TBATA	Q6U736	OPN5
Q8NA47	CCDC63	C9JI88	FBXL13	O94818-2	NOL4	H3BTA9	TBC1D21	P35372-10	OPRM1
Q8IXS2	CCDC65	J3KRT3	FBXO15	Q5JYC0	NOL4L	B9A6M8	TBC1D26	Q9BXF9	***TEKT3***
Q96M83	CCDC7	O75426	FBXO24	C9JNM7	NPHP1	A0A0B4J2F4	TBC1D3I	Q8WW24	***TEKT4***
F8WEI8	CCDC74A	Q8N4B4	FBXO39	A6NHN6	NPIPB15	F8WEC3	TBL2	Q96M29	***TEKT5***
H7C1C8	CCDC74B	Q5XX13-2	FBXW10	A0A494C1T1	NPIPB7	J3KTL9	TCP10L	Q8IYF3	***TEX11***
Q6ZN84	CCDC81	Q6X9E4	FBXW12	Q9NQ35	NRIP3	D6RGY5	TCP11	Q8TC90	CCER1
Q8IWF9	CCDC83	B1AJZ9	FHAD1	A0A087WT36	NSUN4	Q8IZS6	TCTE3	M1TIV4	CCHCR1
Q9NVE4	CCDC87	O75344-3	FKBP6	Q86YG4	NT5DC4	Q9BXT4	TDRD1	Q13939	CCIN
Q8N998	CCDC89	Q5VTL7	FNDC7	O75694	NUP155	Q5VZ19	TDRD10	H0YGS4	FOXM1
Q2M329	CCDC96	Q8TC99	FNDC8	Q5VU65	NUP210L	Q587J7	TDRD12	C9JUX3	FRG2C
Q86Y26-3	NUTM1	Q8NAT2	TDRD5	Q5BJF6	ODF2	O60522	TDRD6	P23945	FSHR
F8W6Z3	***ODF3***	***I3L122***	***TEKT1***						
Enhanced in early spermatids (n=927)									
Accession N	Gene	Accession N	Gene	Accession N	Gene	Accession N	Gene	Accession N	Gene
F5GYI2	ABCB9	Q14093	DCC	F5GYI3	LRRCC1	A0A3B3ISE2	PRR9	Q8NEL9-5	SSX2IP

Q96J65	ABCC12	Q5VU92	DCST1	Q96J66	LRRD1	H0Y455	PRSS21	Q8WTU1	ST7L
E7EP66	ACRBP	J3QKL2	DCST2	E7EP67	LRRIQ1	Q5SZB5	PRSS37	Q8TDR4	STARD6
Q9Y615	ACTL7A	Q5T197	DCUN1D1	Q9Y616	LRRIQ3	Q96KC10	PRSS51	O15524	STAT4
Q8TC94	ACTL9	Q5T1A1	DDHD1	Q8TC95	LRRIQ4	J3KTE9	PRSS54	H0YES3	STK33
Q8TDG2	ACTRT1	Q0VD86-3	DDI1	Q8TDG3	LYPD4	H0Y7I9	PSORS1C1	M0R1E6	STKLD1
Q96M93	ADAD1	Q8NEL9-4	DDX20	Q96M94	MAATS1	D6RCC7	PTPN20	O95887	STPG1
Q9Y3Q7-2	ADAM18	Q8WTU0	DDX3Y	Q9Y3Q7-3	MAEL	Q9UMQ7	PTPRQ	A0A0S2Z5I4	SUV39H2
Q9UKJ8	ADAM21	Q8TDR3	DDX4	Q9UKJ9	MAGEB3	Q96KX3	PURG	Q8N9W6	SWT1
Q9UKF5	ADAM29	O15523	DEPDC1	Q9UKF6	MAP3K19	F8W8F10	QRICH2	A0A087WV08	SYCE2
A0A0J9YWN9	ADAM32	Q9NVH2-3	DKKL1	A0A0J9YWN10	MBD3L1	P23436	RAB11FIP5	K7ELN4	SYN3
Q96PN6	ADCY10	H0YES2	DLGAP2	Q96PN7	MED26	Q14781-3	RAB3IP	A0A075B6F4	SYT16
Q8N7X0	ADGB	M0R1E5	DLGAP3	Q8N7X1	MEI1	A0A096LNP6	RAD21L1	Q9UI47	TACC3
A0A3B0IT49	ADIG	H0YN08	DMRTB1	A0A3B0IT50	MEIOC	Q9H0I4	RASGEF1C	J3QRG3	TAF7L
Q8NDL9	AGBL5	O95886	DNAAF3	Q8NDL10	MEX3B	Q8NA55	RASSF8	H0YFX3	TAS1R1
D6RF73	AHRR	A0A0S2Z5I3	DNAH10	D6RF74	MFAP3L	Q8IYX3-4	RBMXL2	Q9H820	TAS2R1
Q96MA6	AK8	Q8N9W5	DNAH17	Q96MA7	MFSD6L	Q6ZUS5-3	REEP6	Q8N7S2-3	TBATA
Q86UN6	AKAP14	A0A087WV07	DNAH8	Q86UN7	MGAT4C	H7C4R3	RFX2	A0A024R628	TBC1D21
O75969	AKAP3	K7ELN3	DNAI1	O75970	MGAT4D	Q8NCX1	RFX3	H0YF78	TBC1D26
Q9BQS7	AKAP4	A0A075B6F3	DNAI2	Q96JD7	MLF1	Q569K7	RFX4	Q9BPU7	TBL2
Q96JD6	AKR1E2	Q9UI46	DNAJB13	B6D4Y9	MORC1	Q6TFL3-5	RGPD2	H3BRN3	TBX22
B6D4Y8	ALK	J3QRG2	DNAJC18	Q8TCU5	MOV10L1	F8W7A8	RGPD4	Q13168	TCP10L
Q8TCU4	ALMS1	H0YFX2	DNAJC5G	P03972	MROH2B	Q5T5S2	RGS22	Q9H1R2-4	TCP11
P03971	AMH	Q9H819	DOC2A	Q7Z5J9	MROH7	A0A1W2PQX6	RGS6	Q9H597	TDRD10
Q7Z5J8	ANKAR	Q8N7S2-2	DPY19L2	J3KQ22	MROH8	H7C351	RGSL1	E0Z3F2	TDRD15
J3KQ21	ANKMY1	A0A024R627	DPYSL5	A0A590UK21	MS4A14	J3QKX3	RNF113B	Q8WWB4	TDRD5
A0A590UK20	ANKRD30B	H0YF77	DRC1	Q5TZF4	MS4A5	Q8IYA8-3	RNF151	A0A0A0MTH6	TDRKH
Q5TZF3	ANKRD45	Q9BPU6	DRC7	Q8N9V7	MSANTD1	F8VTU8	RNF212B	Q86YF9-4	***TEKT1***
Q8N9V6	ANKRD53	E0ZS60	DRD3	Q9BZ20	MSI1	Q96M96	RNF32	F8VSE8	***TEKT3***

Q9BZ19	ANKRD60	H3BRN2	DUSP15	A6NGH9	MYCBPAP	Q8NEL1	ROPN1B	P0CF76	***TEKT4***
A6NGH8	ANKRD61	Q13167	DUSP21	F1T0L8	MYH7B	Q8IWA7	RPGRIP1	Q9HAE4	***TEX11***
F1T0L7	ANO2	Q9H1R2-3	DUX4	A6NF35	MYO15A	Q6P9F1	RPL10L	A0A2R8YDC5	***TEX15***
A6NF34	ANTXRL	Q9H596	DYDC1	P55065	MYO1H	Q8NA48	RSPH10B2	Q6NXP1	***TEX26***
P55064	AQP5	E0Z3F1	DYNLRB2	Q9P2N2-3	MYO3A	Q96M84	RSPH14	Q8IY86	***TEX35***
Q9P2N2-2	ARHGAP28	Q8WWB3	DYRK4	A8MVX1	NEK10	F8WEI9	SAC3D1	A4FU69-6	TEX38
A8MVX0	ARHGEF33	F8VZV10	DZIP1	Q8IVW6-5	NFKBIB	H7C1C9	SAMD15	Q5THR3-6	TGIF2LY
Q8IVW6-4	ARID3B	A0A0A0MTH5	E2F7	Q5H914	NKX2-4	Q8IWF10	SAMD4A	P26379	THEG
Q5H913	ARL13A	Q86YF9-3	EBLN1	Q6T312	NLRP11	Q2M330	SATL1	Q8TC93	TIAM2
Q6T311	ARL9	F8VSE7	EFCAB1	Q5T9G5	NLRP7	M1TIV5	SDK2	J3KQG4	TIMD4
Q5T9G4	ARMC12	P0CF75	EFCAB10	Q8NEN0-3	NMBR	E7EUW1	SEL1L2	Q6NXR1	***TKTL2***
Q8NEN0-2	ARMC2	Q9HAE3	EFCAB12	Q5W041-5	NME5	P05154	SERINC4	A0A0B4J1T9	TMCO5A
Q5W041-4	ARMC3	A0A2R8YDC4	EFCAB13	A0A5F9ZI14	NODAL	A0A087WT29	SERP2	P29323	TMEM108
A0A5F9ZI13	ARMC4	Q6NXP0	EFCAB5	H7C1W1	NOL4	C9JSP7	SERPINA5	P54762-6	TMEM202
H7C1W0	ART5	Q8IY85	EFCAB6	Q9UHC3-3	NOL4L	P30307-4	SESN3	Q5RHP10	TMEM247
Q9UHC3-2	ASIC3	A4FU69-5	EIF5A2	H0YFS2	NPIPB15	B4DYU0	SH2D7	Q7L0X3	TMEM63C
P34970	ATP6V1E2	Q5THR3-5	ELAVL4	Q96A71	NRIP3	Q69YH6	SH3GL3	Q5W0A1	TMEM89
H0YFS1	ATP8B3	A8MTL1	ENOX1	Q9UL16	NSUN4	Q8IVW5	SHBG	A0A0A0MSQ5	TMIGD3
Q96A70	AZIN2	P26378	EPHA10	A0A0B4J2D8	NUP155	Q5MAI6	***SLC1A6***	A0A348FV92	TMPRSS6
Q9UL15	BAG5	Q8TC92	EPHA6	Q9BZR8-4	NUP210L	Q49AH1	***SLC22A14***	E7EWD10	TMPRSS9
A0A0B4J2D7	BBOF1	J3KQG3	EPHA8	Q96P10	NXF3	I3L074	***SLC22A16***	Q5SXX9	TNP2
Q9BZR8-3	BCL2L14	A0A0B4J1T8	EPHB1	F5GY05	OAZ3	Q96T60	***SLC25A52***	O00321-3	TPPP2
Q96P09	BIRC8	P29322	ERICH3	Q8IYS9	OBP2B	Q71F24	***SLC26A8***	Q8NHP7-4	TRIM17
F5GY04	BNC1	P54762-5	ERICH6	Q8N9W6-4	ODF2	J3QL99	***SLC2A5***	A6NFH6	TRIM36
Q8IYS8	BOD1L2	Q5RHP9	ERICH6B	Q7Z569-3	ODF3L2	H0YB93	***SLC30A3***	J3KSI10	TRIM42
Q8N9W6-3	BOLL	Q7L0X2	ESPN	J3QQP6	OPLAH	Q96MC5	***SLC35E4***	Q49AJ1	TRIP13
Q7Z569-2	BRAP	Q5W0A0	ESR2	Q32M85	OPN5	Q9BYV9	***SLC36A3***	Q3B821	TROAP
J3QQP5	BRIP1	A0A0A0MSQ4	ESRRB	Q5VZT3	OPRM1	Q5SZL3	***SLC45A2***	Q96GL10	TSGA10
Q32M84	BTBD16	A0A348FV91	ETNK2	B7Z369	OR4N2	P27545	***SLC6A16***	A1A520	TSGA10IP

Q5VZT2	C10orf113	E7EWD9	ETV2	A0A3B3IRX5	OR8B2	Q494V3	***SLC9B1***	Q8IXR6	TSGA13
B7Z368	C10orf142	Q5SXX8	EXD1	Q8WW15	OSBP2	Q4G0U5-4	SLFNL1	H0YIB1	TSKS
A0A3B3IRX4	C10orf67	O00321-2	FABP12	H7C3F9	OTOG	Q96M92	SMCO2	Q17R56	TSNAXIP1
Q8WW14	C10orf82	Q8NHP7-3	FAM104A	C9JXX6	OTUB2	Q9H4Y5	SMG9	Q24JQ6	TSPYL5
H7C3F8	C10orf90	A6NFH5	FAM135B	Q86WS5	OVCH1	Q8NHU3	SMIM21	A6NFA1	TSSK2
C9JXX5	C11orf94	J3KSI9	FAM161A	F8VV64	P2RX3	Q6ZU65	SMKR1	A0A1W2PRP5	TSSK3
Q86WS4	C12orf40	Q49AJ0	FAM163A	Q8NA58	PABPC1L2A	Q9C0B3	SNTG2	F8W8N10	TSSK6
F8VV63	C12orf42	Q3B820	FAM170A	Q6X4T1	PACRG	Q6ZQR3	SOCS7	Q8NA71	TTBK2
Q8NA57	C12orf50	Q96GL9	FAM178B	Q8IXR10	PAOX	A0A087WWY9	SOHLH2	Q8IYT2	TTC16
Q6X4T0	C12orf54	A1A519	FAM186B	E9PFP8	PARD6A	A0A0C4DFS3	SOX5	Q8TC57	TTC21A
Q8IXR9	C12orf56	Q8IXR5	FAM187B	Q6P388	PAX3	Q8IVW1	***SPAG17***	Q8NEG1	TTC23L
Q96M12	C15orf39	H0YIB0	FAM205C	Q8WTQ5	PCBP3	P30927	***SPAG8***	G3V4K9	TTC29
Q6P387	C16orf46	Q17R55	FAM217A	H3BSP7	PCP2	K7EPJ5	SPAM1	Q6IPT3	TTC34
Q8WTQ4	C16orf78	A6NFA0	FAM221B	Q9H694	PCSK4	Q8NHS5	SPANXC	Q8N5Q2	TTC39A
H3BSP6	C16orf92	A0A1W2PRP4	FAM47B	A6NNT3	PCYT1B	Q6NUQ3	SPANXD	Q96KD4	TTC6
Q9H693	C16orf95	F8W8N9	FAM71A	A0A075B7C3	PDCL2	Q9H079	SPANXN1	Q6NXP3	TTC7A
A6NNT2	C16orf96	Q8NA70	FAM71B	Q8N3J4	PDHA2	Q8IZ96-6	SPANXN3	H0Y980	TTLL10
A0A075B7C2	C17orf50	Q8IYT1	FAM71C	Q86WR7	PHF24	Q8TAZ7	SPANXN5	Q8NEG5	TTLL2
Q8N3J3	C17orf53	Q8TC56	FAM71D	Q5JVX8	PHF7	Q8NA67	***SPATA12***	Q8IYD9	TTLL5
Q86WR6	C17orf64	Q8NEG0	FAM71E1	Q5SNV10	PHOSPHO1	Q96M21	***SPATA16***	Q8TC84-3	TTLL6
Q5JVX7	C1orf141	G3V4K8	FAM71E2	Q5VU70	PHTF1	B4DYQ9	***SPATA17***	Q969F1	TTLL8
Q5SNV9	C1orf167	Q6IPT2	FAM71F1	B1ALJ6	PIAS2	Q9NRU4	***SPATA18***	A0A0R4J2E5	TTLL9
Q5VU69	C1orf189	Q8N5Q1	FAM71F2	Q6P1W6	PIH1D2	K7ERX9	***SPATA19***	P0DPH7	***TUBA3C***
B1ALJ5	C1orf194	Q96KD3	FAM81B	Q86Z24	PKD1L1	P38433	***SPATA21***	Q6PEY2	***TUBA3E***
Q6P1W5	C1orf94	Q6NXP2	FAM83F	Q96LM10	PKDREJ	Q9BXS1	***SPATA24***	C9J2C0	***TUBA8***
Q86Z23	C1QL4	H0Y979	FANCM	Q9BZE8	PLCZ1	Q14051	***SPATA3***	Q9H4B7	***TUBB1***
Q96LM9	C20orf173	Q8NEG4	FANK1	O95568	PLK4	Q6YFQ3	***SPATA31E1***	C9JI89	TULP2
Q9BZE7	C22orf23	Q8IYD8	FATE1	Q6IC84	PMFBP1	Q5T046	***SPATA32***	O75427	TXNDC2

O95567	C22orf31	Q8TC84-2	FBF1	Q9NWW8	POC1A	Q8N753	***SPATA4***	Q8N4B5	UBE2U
Q6IC83	C22orf42	Q969F0	FBXL13	B7ZM13	POLN	Q8IXA4	***SPATA6***	Q5XX13-3	UBN2
Q9NWW7	C2orf42	A0A0R4J2E4	FBXO24	L0R592	POLR2D	Q9H4G2	***SPATA9***	Q6X9E5	UBQLN3
B7ZM12	C2orf73	C9JI88	FBXO39	A0A5F9ZI71	POPDC3	C9IZ89	SPATC1	B1AJZ10	UBQLNL
L0R591	C3orf20	O75426	FBXW10	C9J3M9	PP2D1	E9PRM5	SPATS1	Q6QHK5	UGT3A2
A0A5F9ZI70	C3orf49	Q8N4B4	FBXW12	H3BNL2	PPM1J	Q3ZCV4	SPDYC	O75345	ULK4
C9J3M8	C3orf67	Q5XX13-2	FHAD1	Q96KX2	PPP1R32	Q14094	SPDYE1	Q8TC100	UMODL1
H3BNL1	C3orf84	Q6X9E4	FIGLA	D6RHK4	PPP1R36	Q5VU93	SPDYE5	H0YGS5	UNC13C
Q96KX1	C4orf36	B1AJZ9	FKBP6	Q7Z4U6	PPP3R2	J3QKL3	SPIRE1	C9JUX4	UNC79
D6RHK3	C5orf49	Q6QHK4	FNDC8	A0A024RA14	PRDM9	O15020	SPPL2C	Q9NQT6-3	USP2
Q8IVU2	C6orf163	O75344	FOXM1	H7C3K2	PROCA1	Q5T198	SPSB4	P23946	USP50
Q7Z4U5	C6orf201	Q8TC99	FRG2C	Q96LL5	PRR19	Q5T1A2	SPTBN2	M0QXA10	VWA3B
A0A024RA13	C7orf31	H0YGS4	FSCN3	Q6P048	PRR22		SPZ1	Q8NA04	VWA5B1
H7C3K1	C7orf61	C9JUX3	FSHR	Q5VYM2	PRR30		SSUH2	F8VY50	WBP2NL
Q96LL4	C8orf48	Q9NQT6-2	FSIP1	Q8N9Z9	LRIT1	A0A097IW80	GLT1D1	Q99929	WDR62
Q6P047	C8orf74	P23945	G2E3	Accession	LRP2BP	Q14410	GLT6D1	Q9NY29	WDR64
Q5VYM1	C9orf131	Q8NA03	GABRG3	P49659	LRRC36	Q96MS3	GP6	Q7Z4T9	WDR88
A0A3B3ISE1	C9orf153	F8VY49	GALNT8	P101326	LRRC43	Q7Z4J2	GPR135	Q49A18	WDR93
H0Y454	C9orf24	Q99928	GALNTL5	Q7Z3Z1	LRRC46	Q9HCN6-3	GPR156	S4R3E7	YBX2
Q5SZB4	C9orf50	Q9NY28	GALNTL6	Q6ZMR4	LRRC56	Q8IZ08	GRID2	Q9BWX6	YJEFN3
Q96KC9	CABS1	Q7Z4T8	GAPDHS	E9PFP9	LRRC63	Q8NFN8	GRK4	Q6W3E6	ZAR1
J3KTE8	CABYR	Q49A17	GATA5	P05155	LRRC69	A0A087X043	GRXCR2	Q86UU6	ZBBX
H0Y7I8	CACNA1B	A0A590UJ55	GDPD4	B4DYU1	LRRC71	P32298	GSG1	A0A494C113	ZBTB32
D6RCC6	CAGE1	Q9BWX5	GGN	O15021	LRRC74A	A6NFK2	GTF2A1L	A0A097IW81	ZBTB37
Q9UMQ6	CAPN11	Q6W3E5	GGTLC2	P0DPH8	LRRC9	Q2KHT4-2	GTF2A2	Q14411	ZC2HC1C
Q96KX2	CAPZA3	Q86UU5	GJA8	Q9UGL1-2	KIAA0895	Q9UNN4	HARBI1	Q96MS4	ZDHHC19
F8W8F9	CASC1	A0A494C112	GK2	L8EC47	KIAA1210	P52657	HBZ	Q7Z4J3	ZFHX2
P23435	CBLN1	Q71F23	CENPU	Q8NCT3	KIAA1211	Q96MB7	HCN4	Q9HCN6-4	ZIM2
Q14781-2	CBX2	J3QL98	CEP112	Q9ULL0	KIAA1257	P02008	HEATR9	Q8IZ09	ZMYND10

A0A096LNP5	CCDC110	H0YB92	CEP170	B7ZVZ4	KIF15	Q9Y3Q4	HEMGN	Q8NFN9	ZNF177
Q9H0I3	CCDC113	Q96MC4	CEP295NL	A0A2R8YFM9	KIF24	A2RTY3	HHIPL2	A0A087X044	ZNF474
Q8IYX3-3	CCDC116	Q9BYV8	CEP41	Q9NS87-2	KIF27	Q9BXL5	HIPK4	P32299	ZNF479
Q6ZUS5-2	CCDC121	Q5SZL2	CEP85L	Q5T7B8	KIF2B	Q6UWX4	HIST3H3	A6NFK3	ZNF487
H7C4R2	CCDC136	P27544	CERS1	Q86VH2	KLF17	Q8NE63	HSF2BP	Q2KHT4-3	ZNF57
Q8NCX0	CCDC150	Q5VWK6	CETN1	Q8N4N8	KLHDC9	B9EG58	HSPB9	Q9UNN5	ZNF628
Q569K6	CCDC157	Q494V2	CFAP100	Q5JT82	KLHL10	C9JSF2	HTR7	P52658	ZNF679
P15815	CCDC158	Q4G0U5-3	CFAP221	Q8NEP7-2	KLHL11	Q9BQS6	HYLS1	Q96MB8	ZNF683
Q6TFL3-4	CCDC171	Q96M91	CFAP53	Q6JEL2	KLHL35	P34969	IGDCC3	Q9Y3Q5	ZNF709
F8W7A7	CCDC178	Q9H4Y4	CFAP58	Q9NVR0	KNSTRN	Q96M11	IGLL1	A2RTY4	ZNF730
Q5T5S1	CCDC183	Q8NHU2	CFAP61	Q6PF15	KRT25	Q8IVU1	IL13RA2	Q9BXL6	ZNRF4
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H7C350	CCDC188	Q9C0B2	CFAP74	Q7Z3Z0	KRT72	D0EFR8	IL23R	Q8NE64	ZSWIM2
J3QKX2	CCDC27	Q6ZQR2	CFAP77	O76009	LCA5L	P01583	INCA1	C9JSF3	ZWILCH
D0EFR9	CCDC33	A0A087WWY8	CFAP99	Q14CN4-2	LDHAL6A	Q5VWK5	INTS7	Q86VS3	IRGC
Q8IYA8-2	CCDC36	A0A0C4DFS2	CHODL	O95447	LEKR1	Q0VD86-2	IPO4	Q8NA54	ISLR2
F8VTU7	CCDC38	Q8IVW0	CHRM5	Q6ZMR3	LETM2	Q9NVH2-2	IQCA1L	Q6NXR0	IZUMO2
Q96M95	CCDC42	P30926	CHRNB4	J3KP02	LEXM	H0YN07	IQCD	Q24JQ5	IZUMO3
Q8NEL0	CCDC54	K7EPJ4	CILP2	E9PMA4	LIN28A	E0ZS59	IQCF5	M0QXA9	IZUMO4
Q8IWA6	CCDC60	Q8NHS4	CLHC1	Q3ZCV2-2	LKAAEAR1	F8VZV9	IQCF6	S4R3E6	KATNAL2
Q6P9F0	CCDC62	Q6NUQ2	CLMN	Q9H9Z2	LMBR1L	A8MTL0	IQCG	Q1ZYL8-3	KCNU1
Q8NA47	CCDC63	Q9H078	CLPB	Q8TD35	LMNTD1	A0A590UJ54	IQCH	K7EIJ8	KDM4D
Q96M83	CCDC7	P53675	CLTCL1	Q6UX01	LOXHD1	B9EG57	IQUB	E5RHP1	KDM5B
F8WEI8	CCDC74A	Q8IZ96-5	CMTM1	Q9BXS0	COL25A1	C9JSP6	CDC14A	Q6B0I6	KHDRBS3
H7C1C8	CCDC74B	Q8TAZ6	CMTM2	Q14050	COL9A3	P30307-3	CDC25C	E9PRM4	CYB5R2
Q8IWF9	CCDC83	Q8NA66	CNBD1	Q6YFQ2	COX6B2	Q69YH5	CDCA2	Q3ZCV3	CYLC1
Q2M329	CCDC96	Q96M20	CNBD2	Q5T045	CRIP3	Q8IVW4	CDKL3	CYLC2	Q9H4B8
M1TIV4	CCHCR1	B4DYQ8	CNGA4	Q8N752	CSNK1A1L	Q5MAI5	CDKL4	DCAF12L1	Q86VS4

E7EUW0	CCSER1	Q9NRU3	CNNM1	Q8IXA3	CSNK1G1	Q49AH0	CDNF		
P01584	CCT6B	K7ERX8	CNTD1	Q9H4G1	CST9L	I3L073	CDRT1		
A0A087WT28	CD200R1L	P38432	COIL	C9IZ88	CTNNA2	Q96T59	CDRT15		
F8VTU7	CCDC38	Q8IVW0	CHRM5	Q6ZMR3	LETM2	Q9NVH2-2	IQCA1L	Q6NXR0	IZUMO2
Q96M95	CCDC42	P30926	CHRNB4	J3KP02	LEXM	H0YN07	IQCD	Q24JQ5	IZUMO3
Q8NEL0	CCDC54	K7EPJ4	CILP2	E9PMA4	LIN28A	E0ZS59	IQCF5	M0QXA9	IZUMO4
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Q6P9F0	CCDC62	Q6NUQ2	CLMN	Q9H9Z2	LMBR1L	A8MTL0	IQCG	Q1ZYL8-3	KCNU1
Q8NA47	CCDC63	Q9H078	CLPB	Q8TD35	LMNTD1	A0A590UJ54	IQCH	K7EIJ8	KDM4D
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F8WEI8	CCDC74A	Q8IZ96-5	CMTM1	Q9BXS0	COL25A1	C9JSP6	CDC14A	Q6B0I6	KHDRBS3
H7C1C8	CCDC74B	Q8TAZ6	CMTM2	Q14050	COL9A3	P30307-3	CDC25C	E9PRM4	CYB5R2
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Q2M329	CCDC96	Q96M20	CNBD2	Q5T045	CRIP3	Q8IVW4	CDKL3	CYLC2	Q9H4B8
M1TIV4	CCHCR1	B4DYQ8	CNGA4	Q8N752	CSNK1A1L	Q5MAI5	CDKL4	DCAF12L1	Q86VS4
E7EUW0	CCSER1	Q9NRU3	CNNM1	Q8IXA3	CSNK1G1	Q49AH0	CDNF		
P01584	CCT6B	K7ERX8	CNTD1	Q9H4G1	CST9L	I3L073	CDRT1		
A0A087WT28	CD200R1L	P38432	COIL	C9IZ88	CTNNA2	Q96T59	CDRT15		

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