PROTEOMIC APPROACH TO THE CARACTERIZATION OF UNKNOWN ORIGIN MALE INFERTILITY

RITA CAROLINA IVO PACHECO

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research (Specialization Area: Ageing and Chronic Diseases) at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

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Abstract

Infertility affects around 50 million couples worldwide, where male infertility contributes for half of all cases. When no clear causes for infertility can be found, it is designated of unknown origin (UOMI), including idiopathic (ID) and unexplained male infertility (UMI). Diagnosis of male infertility is firstly based on routine semen analysis, an evaluation that has been shown to have poor prognostic value. This is especially evident in men with UMI that, despite presenting a normal seminal analysis, are still infertile, stressing the need to deepen the analysis of sperm functionality in these patients to clarify what might be behind their infertility state.

In this project, we focused on a detailed characterization of sperm function, that goes far beyond the conventional analysis, to clarify this issue. Besides analysing functional and bioenergetic parameters such as sperm capacitation, acrosome reaction, chromatin status and mitochondrial functionality, a sperm proteomic analysis was also performed using sequential window acquisition of all theoretical mass spectra (SWATH-MS). In addition, lifestyle and medical history of patients were assessed by survey. Symptoms of anxiety and depression were also evaluated through proper surveys.

In this study, ID patients besides having significantly decreased sperm concentration, motility and morphology, also presented significantly decreased sperm viability, chromatin integrity and percentage of capacitated cells, comparing to healthy men, with the proteomic results further supporting the differences among these groups. Furthermore, ID patients had significantly increased incidence of urogenital infections and varicocele. Regarding UMI patients, we observed that their sperm functionality is very similar to that of the healthy individuals, but significantly different from ID patients, which was also mirrored at the proteomic level. UMI patients were also observed to have increased incidence of diagnosed depression, when comparing to healthy individuals and ID patients. Finally, the proteins annexin A5, α-crystallin B chain, apolipoprotein H, destrin, NADH-cytochrome b5 reductase 3, platelet-activating factor acetylhydrolase IB subunit α1 and transthyretin were found, for the first time, to significantly differentiate the 3 patient groups, hence being good candidates for further studies on UOMI.

Overall, this study entailing a unique complete and integrated analysis of 3 groups of individuals' sperm functionality and proteome, accurately categorized, provided new insights and add knowledge on these patients' infertility unknown aetiology, opening road to future studies in the field.

Keywords: Unknown origin male infertility, spermatozoa, proteomics

Resumo

A infertilidade afeta cerca de 50 milhões de casais mundialmente, sendo que a infertilidade masculina contribui para cerca de metade dos casos totais. Quando nenhuma causa clara de infertilidade consegue ser encontrada, esta é designada como sendo de origem desconhecida (UOMI), incluindo a infertilidade idiopática (ID) e inexplicada (UMI). O diagnóstico para a infertilidade masculina é baseado, inicialmente, na análise seminal básica, uma avaliação que se tem vindo a demonstrar ter fraco valor prognóstico. Isto é especialmente evidente em homens com UMI que, apesar de apresentarem uma análise seminal normal, são inférteis, realçando a necessidade de aprofundar a análise funcional espermática nestes doentes para clarificar o que pode estar por detrás da sua infertilidade.

Neste projeto, focámo-nos numa caracterização detalhada da função espermática que vai para além da análise convencional, de forma a clarificar esta questão. Assim, além de analisar parâmetros espermáticos funcionais e bioenergéticos como a capacitação, reacção acrossómica, integridade da cromatina e função mitocondrial, foi também realizada uma análise proteómica espermática utilizando "sequential window acquisition of all theoretical mass spectra" (SWATH-MS). Adicionalmente, o estilo de vida e historial médico foram analisados através de questionário. Os sintomas de ansiedade e depressão foram também avaliados através de um questionário específico para esse fim.

Neste estudo, homens com ID, além de terem diminuições significativas na concentração, mobilidade e morfologia espermática, mostraram também uma viabilidade, integridade da cromatina e percentagem de células capacitadas diminuídas, comparativamente a homens saudáveis. Estas diferenças entre grupos foram também expressas ao nível proteómico. Adicionalmente, homens com ID têm significativamente maior incidência de infeções urogenitais e varicocelo. Em relação aos homens com UMI, observamos que a sua função espermática é muito similar à de indivíduos saudáveis, mas significativamente diferente de homens com ID, o que foi também refletido na análise proteómica. Nos homens com UMI foi também observada uma maior incidência de depressão diagnosticada quando comparados com indivíduos saudáveis e com ID. Por fim, as proteínas anexina A5, α-cristalina cadeia B, apolipoproteína H, destrina, NADH-citocromo b5 redutase 3, fator ativador de plaquetas acetil-hidrolase IB subunidade α1 e transtirretina foram descritas pela primeira vez, como diferenciadoras dos 3 grupos de indivíduos, sendo assim bons candidatos para estudos futuros em UOMI.

Em suma, este estudo que consistiu numa análise completa e integrada da função espermática e proteoma de 3 grupos de indivíduos criteriosamente caracterizados, revelou dados novos, contribuindo para aumentar o conhecimento sobre estes doentes com infertilidade de etiologia desconhecida e abrindo caminho para estudos futuros na área.

Palavras-chave: Infertilidade de origem desconhecida, espermatozoide, proteómica.

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Prologue

Infertility is a disease characterized by the inability to accomplish spontaneous pregnancy after one year or more of sexual activity without contraception, as defined by the World Health Organization (WHO), and it affects 15%, representing around 50 million, couples worldwide (2). Although often disregarded, for many couples, infertility can also represent a heavy financial burden, not to mention the psychological distress withstood, which can further contribute to the couple's inability to conceive (3-5). Infertility usually manifests when both the male and the female partner have reduced fertility, since normally the healthier partner is able to compensate for the fertility problem. Nonetheless, male infertility alone can contribute up to 20% of cases (6), being that in Europe it affects 7.5% of all men (6). Despite some known causes for male infertility, nearly half of the cases remain unexplained.

1. Introduction

1.1. Spermatozoa structure and development

Spermatozoa are the mammalian male gametes, cells whose primary function is to reach the oocyte, the female gamete, and accomplish fertilization, delivering half of the parental genetic information to the new zygote and future embryo (7).

1.2. Structure

The spermatozoon has a unique cellular structure not comparable with any other human cell, with its elongated form and structured cellular compartments, allowing it to efficiently reach the oocyte through the female reproductive tract. These cells have an incomparable membrane to cellular volume ratio that contrast with most human cells, requiring different adaptations and special functionality (7). The spermatozoon can be divided in 3 main parts: the head, the midpiece and the tail [Figure 1]. The head contains the acrosome and the nucleus. The acrosome is an enzyme-filled vesicle at the tip of the spermatozoa's head, whose content is released in a process called acrosome reaction (AR). The nucleus placed at the centre of the head, contains the DNA, extremely and densely packed due to special proteins called protamines, that replace the classic histones in the latter phases of spermatogenesis, allowing for higher degree of chromatin condensation (8). Maintaining chromatin integrity is essential, since it determines the quality of the genetic and non-genetic information that will pass to the offspring (9, 10). Accordingly, sperm DNA fragmentation and other ROS-associated damages are increasing concerns that affect human fertility and could help to explain male infertility of unknown origin aetiology (11, 12). In what concerns the midpiece, this structure is composed of densely packed mitochondria that, among their numerous functions also produce energy for the spermatozoa's movement (13). Finally, the tail is mainly composed by the axoneme, a specialized motile cilium with a 9+2 microtubular structure (14). The tail is most known for performing the mechanical movement of the cell, being also a recognized site of energy production through glycolysis (15). Accordingly, genetically defective structural components of the axoneme and, more recently studied, impairments on transport and assembly of these components have been described as factors that commonly impair spermatozoa's motility (16, 17). Of the most importance are impairments on energy production, which lead to decreased motility and can ultimately hamper the spermatozoa to reach the oocyte (18).

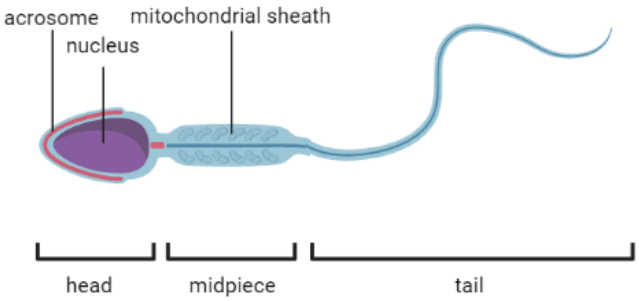

Figure 1. Spermatozoa structure. The spermatozoon is divided in 3 main parts: head, midpiece and tail. The head comprises the nucleus and the acrosome. The mitochondrial sheath is located at the midpiece.

1.3. Development

Spermatozoa are produced in the testes, through a process called spermatogenesis, whose duration varies according to species, being of approximately 74 days in the human (19). After spermatogenesis, spermatozoa can progress from the testes and are primed for ejaculation. The testes are composed by many seminiferous tubules, filled with germ cells embedded in the cytoplasm of the somatic Sertoli cells, disposed side-to-side, connected to each other by tight junctions and participating, among several other functions, in the lumen formation (20). The placement of these tight junctions divides the seminiferous epithelium in 2 regions associated to different phases of spermatogenesis: the basal compartment, where the spermatogoniogenesis occurs; and the adluminal compartment, where meiosis and subsequent spermiogenesis occur; the spermiation, the process through which the sperm cells are released, will then occur near the lumen [Figure 2] (20).

Postnatally, the prospermatogonial germ cells in the testes give rise to spermatogonial stem cells (SSCs). These cells are located on the basal compartment, and undergo mitotic proliferation, giving rise to type A spermatogonial cells, initiating the first phase of spermatogenesis. In humans there are two types of A spermatogonia: A_{dark} and A_{pale} spermatogonia, where A_{dark} constitute the regenerative reserve, proliferating only after testicular damage, whereas $A_{pale,}$ constitute the functional reserve, giving rise to B spermatogonia, that will then divide to form the primary spermatocytes (21, 22). Throughout spermatogoniogenesis (also known as spermacytogenesis), all mitotic divisions have incomplete cytokinesis, forming complexes of interconnected cells (syncytium) originated by the same A_{pale} spermatogonial cell (20) and assuring the synchrony of the seminiferous epithelium (20).

Meiosis is the second phase of spermatogenesis, also known as spermatidogenesis. Meiosis I occurs when resting primary spermatocytes duplicate its DNA content and are consequently pushed through the tight junctions (20). This is the longest phase of spermatogenesis and cells suffers several alterations essentially at the chromatin level (19). During prophase (divided in 4 stages: leptotene, zygotene,

pachytene and diplotene), the formation of synapsis starts in the zygotene stage and after that, the crossing over occurs, being the synapsis finalized during the pachytene stage (23); through this stages the chromatin will be increasingly condensed. Cells will then go through metaphase, anaphase, and telophase, being the final result the secondary spermatocytes, already haploid cells, that will then go throughout Meiosis II. After the second division of meiosis, round spermatids will be obtained and be ready to enter the next stage of spermatogenesis (24). Like in the previous phase, spermatidogenesis has incomplete cytokinesis, forming a cluster of spermatids (24).

Spermiogenesis, the third phase of spermatogenesis, is also known as cytodifferentiation phase, since the cells will go through several morphological changes to finally form the spermatozoon (20). In fact, the structure of the tail will be formed, the post-acrosomal cap region will appear on the top of the spermatozoon head, the nucleus will elongate and a residual body will be formed in result. This residual body is comprised of superfluous cytoplasm that will be separated from the cell on the spermatozoon departure from the adluminal compartment, being consequently phagocytized by the Sertoli cells (20). At this phase, spermatids become transcriptionally inactive, and chromatin is further compacted (8, 25). The first step for this DNA repackaging is to replace approximately 90% of histones by transition proteins (TPs), sperm-specific modified histones (25, 26). The second and last step occurs at the late spermatid stage, where TPs are replaced by protamines, and chromatin is densely repackaged. This histone-to-protamine process is thought to have 3 main purposes: better hydrodynamic shape, which is favoured by the higher condensation of the chromatin; higher protection from DNA damage; and epigenetic regulation (8). As the spermiogenesis ends, the spermatozoon is formed and starts rupturing the syncytium, to reach the lumen in a process called spermiation, the very last step of spermatogenesis (20).

Nonetheless, the cell now released form the seminiferous tubules, although endowed with a new and aerodynamic shape, is still not motile, neither mature nor competent to fertilize (27). Sperm cells will then enter the epididymis, a tubular organ divided in three sections: the caput, corpus and cauda (27). One of the main functions of epididymis is to further mature the spermatozoa. There, the sperm cells will acquire progressive motility and fertilization capability by altering plasma membrane composition, further increasing chromatin condensation, and acquiring surface proteins essential for oocyte-sperm interaction, such as ADAM (A disintegrin and A metalloprotease domain) family proteins (27, 28). After adequate maturation, the epididymis is also responsible for the spermatozoa transport to the vas deferens.

Finally, for spermatozoa to survive the journey to the oocyte, a liquid environment is necessary, allowing for better movement and providing nutrients to fuel their progression. The seminal plasma's function is exactly to assure those needs, adding to spermatozoa after they accomplish the epididymal journey. Seminal fluid is obtained through the combination of fluids from the seminal vesicle, prostate,

and epididymis, being the major components fructose, inositol, citric acid, glycerylphosphorylcholine, and acid phosphatase, crucial for the energy production, coagulation, osmotic pressure and pH maintenance (29). The spermatozoa together with the seminal fluid compose the semen and will then be ejaculated.

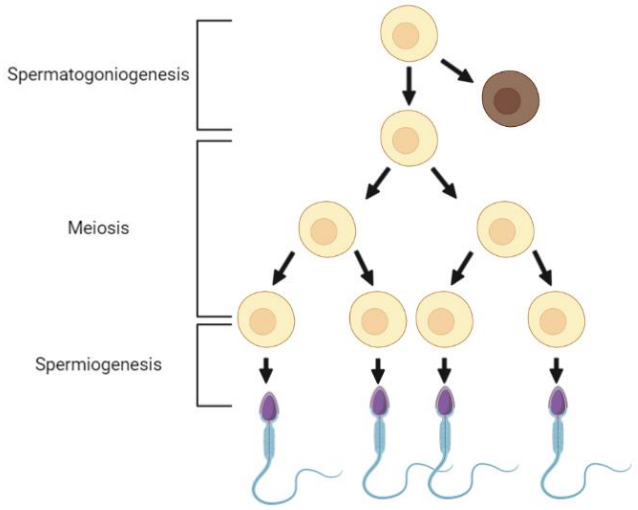

Figure 2 Schematic representation of spermatogenesis and its different stages throughout the seminiferous tubule's.

1.4. Sperm functionality

1.4.1. Sperm DNA

As previously mentioned, the quality of the DNA is determinant for the correct transmission of the paternal information to the offspring. However, DNA damage can occur due to both intrinsic and extrinsic factors and this damage can impair not only fertilization, but also the ability to carry a pregnancy to term, which even in full-term pregnancies can influence the embryo correct development (12, 30). In the nucleus, due to densely packed chromatin, the genetic content seems to have at least some level of protection against environmental factors like oxidative stress (8). Still, the fact that the spermatozoon has a low volume of cytoplasm and thus antioxidant defences, increases the vulnerability of chromatin to reactive oxygen species (ROS) attack (11). Additionally, after spermatogenesis, spermatozoa are not able to repair DNA damage, making this a permanent condition, that can only be compensated at the time of fertilization by the oocyte DNA damage repair mechanisms (31).

It is thus not surprising that the DNA fragmentation index (DFI) is now considered an important factor in determining the gamete's quality. In fact, it was already reported that men with DFI $\geq$ 26.1% have increased 2.84-fold probability to be infertile (32). In addition, sperm from non-pregnant couples who undergone *in vitro* fertilization (IVF) had significantly higher DFI, comparing to pregnant couples, and a negative correlation between high DFI and fertilization rate has also been described (33-35). Moreover, also in couples undergoing intracytoplasmatic sperm injection (ICSI), clinical pregnancy was

observed to be significantly lower, while pregnancy loss was significantly higher in individuals with high DFI sperm, comparing to that with low DFI (34). Also, infertile patients with different aetiologies have been described to have increased DNA fragmentation/damage, highlighting the relevance of the DNA integrity for fertility purposes (32, 36).

Despite the apparent importance of DNA fragmentation assessment, nowadays there is not a unique or recommended test to assess it. In fact, there are different methods to evaluate DNA and chromatin status such as sperm chromatin structure assay (SCSA), deoxynucleotidyl transferase-mediated dUTP nick end labelling assay (TUNEL), single-cell gel electrophoresis assay (Comet), and sperm chromatin dispersion test (SCDt) (37). The fact that the procedures are varied and that different thresholds have been defined for each procedure, makes the implementation of a solely universal test at andrology labs very difficult, hampering the routine evaluation of this parameter.

1.4.2. Sperm mitochondrial function

Mitochondria, with a total number varying among 22-75 in humans, are located in the sperm midpiece in a densely packed manner, being involved in diverse cellular functions such as metabolic integration, ROS synthesis and management, regulation of apoptosis, calcium balance, also supporting the energy requirements of the cell through oxidative phosphorylation (OXPHOS) (13, 38, 39). In OXPHOS, energy is produced via the electron transport chain (ETC) creating a proton gradient throughout the inner mitochondrial membrane that, together with the generated mitochondrial membrane potential (MMP), forms the electrochemical gradient which ultimately promotes ATP synthesis at the ATP synthase level (13). This organelle is also unique for containing its own DNA (mtDNA) and ribosomes allowing for nucleus-independent protein synthesis of some mitochondrial enzymes such as NADH dehydrogenase and cytochrome c reductase (39, 40).

Importantly, as the very specific location and compartmentalization of these organelles in the sperm cell might predict, the sperm mitochondrial function is intimately related to that of sperm, and over the years several were the studies reporting this association. In fact, several mitochondrial parameters have been correlated with sperm function, such as the MMP, the mitochondrial complexes activities, the mitochondrial respiration efficacy and the quality and content of mtDNA (39). In fact, the mitochondrial complexes activities, specifically succinate dehydrogenase (complex II), NADH-cytochrome c reductase (complex I and III), succinate-cytochrome c reductase (complex II and III) and cytochrome c oxidase were reported to be related to sperm concentration, progressive motility and vitality (41). The mitochondrial respiration efficacy was associated with sperm selected after swim-up comparing to before swim-up and also positively associated with sperm motility (42, 43). On the other hand, mtDNA mutations have been described to be associated with worst sperm quality and fertilization success (44-47) and the mtDNA content was also observed to be altered in low quality samples (48-50).

Regarding the MMP, this seems to be the more unanimously accepted indicator of sperm function. In fact, a study from our group on the relationship between mitochondria activity and sperm quality observed that healthy and normozoospermic men had significantly increased MMP compared with men with altered seminal parameters. Also in this study, after sperm cells sorting using proper mitochondrial fluorescent probes, it was reported that the sperm cells with more active mitochondria have better functionality, namely in terms of capacitation, acrosome intactness and chromatin integrity (51). Additionally, several other studies have shown that human sperm MMP was also positively correlated with sperm progressive motility, viability, capacitation, acrosome and chromatin integrity (51-56). Corroborating the importance of this parameter, high MMP has also been observed to be correlated with better fertilization capacity (54-56). Furthermore, a decrease in MMP is an early apoptosis signal that precedes DNA fragmentation and ROS production (57), corroborating the validity of MMP as a sensitive marker for sperm quality and fertilizing capability.

Mitochondria are also the main site of ROS production (being also one of the main targets), that in specific conditions might surpass the antioxidant defence capacity, conducing to oxidative stress (58). ROS have long been debated whether to be prejudicial or beneficial to spermatozoon's function, since low levels of ROS are known to be involved and crucial for several spermatozoon's processes such as acrosome reaction (AR), capacitation and fertilization (59, 60), while when at excess can be detrimental, having several consequences at the cellular level, impairing the general function (61). Furthermore, spermatozoa are especially vulnerable to this type of stress since they lack the necessary repair system, due the reduced cytoplasm and also due to their membrane constitution, very rich in unsaturated fatty acids, highly vulnerable to oxidation and subsequent cascades of damage (59, 61). In this context, several were the studies reporting a negative association of ROS with sperm quality and infertility. In a paper studying oxidative stress in infertile men, Dorostghoal *et al.* found that infertile men with abnormal sperm motility (asthenozoospermia) had increased oxidative stress characterized by increased seminal malondialdehyde, a known oxidative stress marker, and decreased superoxide dismutase (SOD), an important antioxidant enzyme, compared with fertile men (62). Similarly, Saleh *et al.* showed that men with abnormal sperm parameters (referred in this paper as male factor infertility) had significantly increased seminal ROS levels and decreased antioxidant capacity comparing to fertile men (35). In addition, it is also showed that ROS levels were significantly higher in seminal plasma of men from ART success couples comparing to failed ART couples (35). Oxidative stress is also one of the hallmarks of chronic diseases, which have been increasingly implicated in male infertility. For instance, type II diabetes has been suggested to decrease sperm quality by increasing oxidative stress (63, 64). The increasing relevance of sperm oxidative status for fertility is shown by a growing interest in antioxidant treatments for infertile men specially in idiopathic patients, men with abnormal sperm parameters with no clear cause and female factor ruled out. A recent systematic review of antioxidant

supplements showed vitamin E, vitamin C, carnitines and coenzyme Q10, as effective in reversing oxidative stress and improving ART success and live birth rates (65).

Furthermore, using an *in vitro* approach, Amaral *et al.* have shown that excess ROS production in sperm cells is accompanied by a decrease on MMP, lipid peroxidation and decreased sperm viability (66), compromising the general cellular function, corroborating not only the correlation between the organelle and male gamete function, but also stressing the network of interconnected reactions.

1.4.3. Capacitation

Before meeting the oocyte, spermatozoa must travel from the vagina (or cervix) to the fallopian tube. During this journey, the male gamete suffers the last maturation step called capacitation (67, 68). The ideal niche for capacitation to occur is the uterus or the oviducal isthmus, where estrogen, proteolytic enzymes, cholesterol acceptor proteins and a high ionic strength are present (24). The most documented factors able to trigger this process, either in the female tract or *in vitro*, are calcium, bicarbonate, and bovine serum albumin (BSA) (69, 70). The female tract is rich in bicarbonate, when compared with the seminal fluid, that then enters the spermatozoa's cytoplasm, increasing its pH and calcium permeability. This fast rise of calcium levels is mediated by cation channel CatSper that can also be activated by progesterone and BSA (71, 72). In the cytoplasm, bicarbonate activates soluble adenylyl cyclases, enzymes mostly present on the midpiece, that synthetize cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) [Figure 3]. The increase in cAMP levels leads the spermatozoon to a hypermotility state, where the cell acquires a wider amplitude and a stronger flagellar beat pattern (73, 74), crucial for its progression in the cervical mucus (75). cAMP also activates protein kinase A (PKA), which in turn activates a phospholipid scramblase that destabilizes the plasma membrane causing a cholesterol efflux, one of the hallmarks of capacitation (76). BSA can also play a fundamental role as cholesterol acceptor (77-79). On the other hand, PKA also activates tyrosine kinases that phosphorylate tyrosine residues in several proteins including PKA (24). At the end of capacitation active PKA activates phosphatidyl-inositol-3-kinase (PI3K), allowing it to stimulate actin polymerization in the cytosol, hence producing F-actin fibres that prevent the premature fusion of the acrosomal envelop with the plasma membrane (1, 80). Although many studies are continuously published on this topic, its regulation of is not yet fully known. Nevertheless, it seems that capacitation is affected in some types of infertility (81, 82), although when it comes to unknown origin male infertility the available information is limited, warranting further studies.

1.4.4. Acrosome reaction

After capacitation, spermatozoa are fully mature and closer to the oocyte. For the spermatozoa to fertilize the female gamete, it needs to penetrate through the cumulus cell layer and the *zona pellucida*,

which is achieved through AR, aided by the hypermotility motion acquired during capacitation (1, 83). During AR, spermatozoa will release its acrosomal content, composed mainly of proteases and other digestive enzymes maintained at an acidic pH (84). It is thought that a first wave of spermatozoa that reaches the oocyte is "sacrificed" in the sense, that its acrosomal content rich in hyaluronidase is released digesting the hyaluronan-based matrix that holds cumulus cells together, making way for the next wave of spermatozoa to contact directly with the *zona pellucida* (24).

Although, the control mechanisms behind AR is still poorly understood, humans' AR was described to be triggered by follicular fluid and secretions from both the cumulus-oocyte-complex and *zona pellucida*, the last being the most consensual trigger (85). Specifically, four sulphated glycoproteins were identified in the *zona pellucida*: ZP1, ZP2, ZP3 and ZP4 and described to be involved in this process. (85). While ZP1, ZP3 and ZP4 bind primarily to the head area of the capacitated spermatozoa, ZP2 preferentially binds to acrosome-reacted spermatozoa (86). Progesterone, highly concentrated in the cumulus mass fluids, is also a well-known trigger of the AR *in vitro*. A rise in calcium levels through voltage-dependent Ca^{2+} channels (CaV) activates phospholipase C (PLC), which catalyses phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis to inositol-1,4,5-trisphosphate (IP_3), allowing PIP_2-bound gelsolin to be activated and released to the cytosol (1) [Figure 4]. Gelsolin depolymerases F-actin fibres into G-actin monomers, allowing the outer acrosomal membrane to fuse with the plasma membrane. IP_3 also activates protein kinase C (PKC), inhibiting PKA, deactivating the cascade for actin polymerisation (80). IP_3 together with PKC activate receptors at the acrosome and the plasma membranes, respectively, to decrease calcium levels, activating the store-operated calcium channel (SOCC) leading to a sustained increase in calcium levels triggering AR (1). This process seems to be compromised in some infertile men, either by a premature AR or impaired induction of AR, hampering fertilization (87-89).

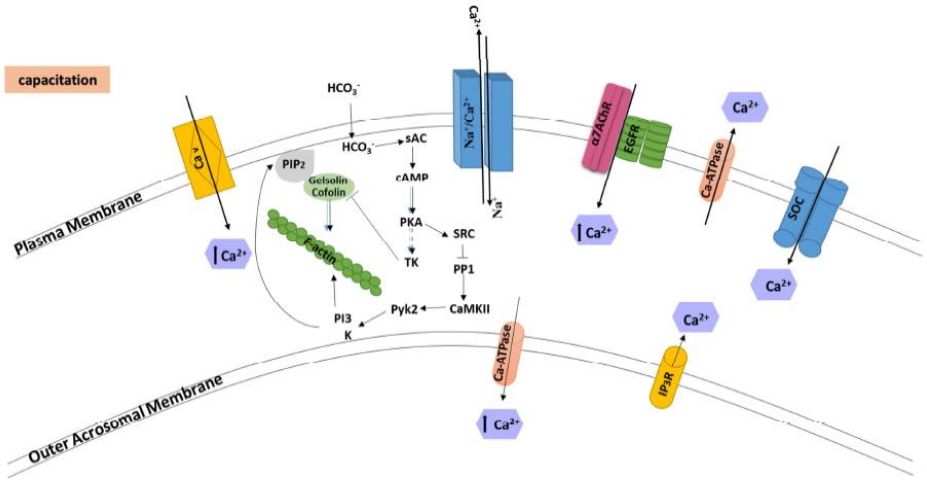

Figure 3. Schematic representation of the molecular mechanisms involved in spematozoa's capacitation. Adapted from Finkelstein et al. 2020 (1)

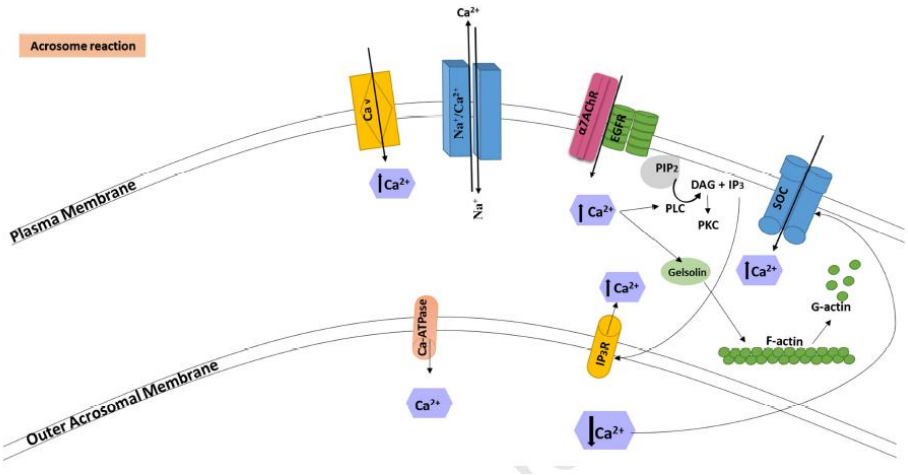

Figure 4. Schematic representation of the molecular mechanism involved in spermatozoa's acrosome reaction. Adapted from Finkelstein *et al.* 2020 (1).

1.5. Male infertility

1.5.1. The causes

There are several recognized causes for male infertility such as urogenital and genetic anomalies, genital tract infections, endocrine disorders, immunological factors, among others (90-92). Varicocele, an enlargement of the spermatic cord, is among the main causes of infertility (92, 93). Genetic anomalies are the cause for 4% of male infertility cases, being Klinefelter syndrome the most common chromosomal abnormality and is currently evaluated in the clinical setting prior to assisted reproductive techniques (ART) (94, 95). Despite this, some patients present male infertility of unknown origin. In

fact, around 37–58% of male infertility cases are of unknown origin (96, 97). This categorization can be further divided in idiopathic (ID), and unexplained infertility (UMI), that essentially differ in the seminal analysis that is abnormal in the former and normal in the later, being that in both the female factor have been ruled out (98), as we will further discuss in detail.

The pillar for male infertility diagnosis is the routine semen analysis, that being cost effective and easy to perform, constitutes the initial evaluation tool for male fertility worldwide (99, 100). This analysis includes the evaluation of several sperm parameters such as count, motility, vitality, and morphology, according to established reference values (2). However, this approach is limited when it comes to the prediction of sperm functionality and fertilization capacity (100, 101). This is especially evident in men with UMI that, despite presenting a normal seminal analysis, are still infertile. Furthermore, the opposite can also happen, and it is not rare that men with abnormal seminal analysis can father a child (102). This stresses the need to deepen the study of the male gametes, ideally focusing on more relevant functional aspects, not routinely evaluated, but that might add knowledge on the mechanisms behind male infertility, hopefully providing the scaffold to the development of new diagnostic tools and treatments options that might allow to improve fertility rates globally.

1.5.2. Lifestyle, Environmental and psychological factors effects on male fertility

Despite the well-known causes of male infertility there are also several other factors that have been described to have an impact on male reproductive health, among them age, obesity, chronic diseases, physical activity, addictive consumptions, exposure to environmental contaminants as well as psychological alterations. In this regard, several life-style changes are recommended by the European academy of Urology (EAU) to improve male infertility such as weight loss, increased physical activity, smoking cessation and alcohol consumption reduction (92)

1.5.2.1. Age and body mass index (BMI)

The general health status is known to influence semen quality in men (103) although the contrary has also been discussed with the suggestion of male (in)fertility as a window to the individuals general health status (104); yet, this seems to be a question similar to that of "who came first the chicken or the egg"? that will certainly not be answered in short time. Regardless, age is among the recognized risk factors for infertility an one of the most studied (92, 105-107), and it has been already described to decrease semen quality in volume, sperm count, motility, morphology and DNA integrity (107). Regarding the reproductive success in natural conception, this was also reported to be lower in older men (106, 108), while in the case of assisted conception, the effect of male advanced age is still not clear (109). Furthermore, age also increases the risk for several chronic diseases, such as hypertension, hypercholesterol, sleep apnea, diabetes and neurodegenerative diseases (110-113), and several of these

had also been described to have an impact on male fertility, as is the case of hypertension and diabetes (63, 64, 114-117).

Body mass index is also an important factor, since excess weight and obesity are known to impair female and male fertility (118). Couples with an obese male partner are 1.66 times more likely to be infertile than that with normal weight males (119). Overweight and obesity have been reported to affect sperm quality, particularly motility, morphology, MMP, DNA fragmentation and chromatin condensation were observed to be significantly impaired in sperm from obese men, being this associated with higher risk of infertility (119, 120). In addition, these alterations seems to be, at least partially, overcome with weight loss, after which an improvement in total sperm count, semen volume and testosterone serum level was reported (121), thus being recommended as a valid approach to overweight male patients with problems to conceive (92). Logically, healthy eating is also important for sperm quality and fertility (122). For instance, higher consumption of red meat, sweets drinks and processed meat was associated with decreased sperm quality, while higher consumption of vegetables, fruit, nuts and fish was associated with increased sperm quality (118, 123)

In line with this, recreational physical activity is also recommended for infertile men (92), as it was observed to improve sperm count, motility and morphology in infertile patients (124, 125). On the other hand, elite physical activity is significantly correlated with decreased sperm progressive motility (124). Also, total sperm motility and normal morphology showed a tendency for being decreased in men practicing elite physical activity, although not reaching statistical significance (124). Hence, only recreational physical activity is recommended to infertile men (92).

1.5.2.2. Systemic vs Reproductive diseases

Several reproduction-associated conditions can affect male fertility including urogenital infections and anomalies, testicular torsion, inguinal hernia and varicocele. As previously mentioned, urogenital anomalies and infections are among the leading causes of male infertility (90). Among the most described pathologies of the reproductive tract is varicocele, an abnormal enlargement of the veins in the spermatic cord, which can be corrected through surgery (93). Varicocele patients were observed to have decreased sperm concentration, motility and normal morphology and increased sperm ROS levels (126, 127). Despite this, not all men with varicocele are infertile (93, 128). In fact, the mechanism by which varicocele can cause infertility is still not completely understood.

Urogenital infections, among which are also included the sexually transmitted diseases (STD), are characterized by increased number of leucocytes, that being major ROS producers, expose male genital tract to oxidative stress, thus affecting fertility (129). As examples, the infection by *Chlamydia trachomatis, Eschericia coli* and C hepatitis were associated with increased sperm ROS production and DFI, as well as with a decrease in sperm parameters, mostly motility (130-134). Despite antibiotic and

anti-inflammatory treatments being available to fight these infections, these treatments are not always able to revert the consequences on sperm quality (129).

Inguinal hernia, a bulging in the lower abdominal wall, can also affect men's fertility, being one of the main hypothesis that these injuries increase inflammation, that could then affect the testes normal function, due to the proximity of the lesion and the male gonad (135). Nowadays, inguinal hernias can be easily treated through a minor surgical intervention (embolization), which was observed to significantly improve sperm parameters (135).

Another relevant condition for fertility is cryptorchidism, characterized by one or two undescended testes, being one of the most common urogenital anomalies. This condition can have negative consequences on testis formation, hormonal regulation and sperm quality, compromising fertility in adulthood (136, 137). Nonetheless, early reparative surgical procedure improves fertility outcomes in these patients (136, 138).

Several chronic diseases, such as diabetes, cardiovascular diseases and cancer that are increasing worldwide, are also thought to impair fertility. In general, these diseases are commonly associated with inflammation, metabolism deregulation and increased oxidative stress, which affect different systems in the organism, including the reproductive system, hence possibly impairing fertility (139). Hypertension, one of the most prevalent chronic diseases, was observed to be more incident in infertile patients comparing to fertile men (140). In addition, it was associated with decreased semen volume, sperm count and total motility (114). Diabetes is also known to affect men's fertilizing capability decreasing sperm concentration, progressive motility and normal morphology in humans, although the reports are not always concordant and the different types of diabetes suggested to have different impact (63, 64, 141, 142). Furthermore, in rodents, besides several histological alterations at the seminiferous tubules level and spermatogenesis itself, together with alterations in metabolism and redox balance (115, 117), it has also been observed that hyperglycaemia impairs Sertoli cells' maturation in mice in an organ culture model (108). Additionally, it was recently shown that chronic diseases including vascular and pulmonary diseases, diabetes and cancer have a cumulative and negative effect on sperm quality (139), showing the importance of analysing the global health status of infertile patients, especially when the aetiology is not known.

1.5.2.3. Environmental contaminants

Environmental factors can also have a detrimental effect in fertility. Especially harmful are endocrine-disrupting chemicals (EDC), which can be found in pesticides, paints, solvents, and metals, among others (91). The exposure to radiation or extreme temperatures have also been described to have a negative impact in terms of fertility (143, 144). In several professional activities workers are exposed to these products/situations daily, being the collection of the information regarding occupational

activities and potential exposures crucial to complete the clinical history in patients with fertility problems.

Regarding EDC, besides their effect on the endocrine pathways, these substances can also cause impairments in cell cycle, apoptosis and epigenetic regulation on male's reproductive system (145). For example, exposure to organophosphate pesticides, commonly used for insect control on agriculture and also mosquito control, was shown to decrease serum testosterone and luteinizing hormone (LH) levels in Peruvian pesticide sprayers (146). Consequently, these pesticides were also observed to have deleterious effects on both the spermatozoa and the seminal fluid, decreasing sperm count, viability and normal morphology as well as seminal fluid's volume and acidity (145-147). Additionally, p,p'-dichlorodiphenyldichloroethylene (p,p' DDE), a stable metabolite of a chlorinated hydrocarbon pesticide, was shown to impair human sperm acrosome integrity and mitochondrial function, decreasing the proportion of cells with high MMP, cellular ATP levels and lastly sperm motility (148, 149). Overall, from what has been exposed so far, it is clear the importance of avoiding the use of products containing EDC contaminants.

On the other hand, radiation constitutes another factor described to affect reproductive health. There are two types of radiation: ionizing (IR) and non-ionizing (NIR). NIR corresponds to visible light, infrared, microwaves and radiowaves radiation, while IR corresponds to higher energy radiations: α, β, γ and X (143, 150) Ultraviolet radiation presents IR and NIR properties (150). Unlike NIR, IR is able to remove electrons from atoms, which can alter molecules' function within our bodies (143). Typically, people are exposed to low levels of IR daily without drastic consequences to health. Nevertheless, IR originating from medical sources or in an occupational context, is especially dangerous, as it was observed to decrease sperm motility and normal morphology, while increasing DNA fragmentation and methylation in exposed individuals (143). In animal models, effects of IR exposure on spermatogenesis were also reported, such as decreasing testis weight and sperm count, and increased sperm DNA fragmentation (151-153). Despite its lower frequency, NIR exposure can heat tissues damaging them, hence being harmful for human health (150, 154). NIR exposure can be found in contact with WiFi, laptops and mobile phones, hence very present in our daily life (154). *In vitro* studies have shown that exposure to this type of radiation decreased sperm motility and acrosin activity while increasing DNA fragmentation (155). NIR exposure is also able to affect spermatogenesis. In studies using rodents as animal models, NIR exposure induced degeneration, necrosis, shedding of spermatogenic cells in a dose-dependent manner, decreasing the number of germ and Leydig cells and increasing DNA damage in the testis (156-158).

Finally, extreme temperatures at which men might be exposed, for instance at the workplace, being this exposure common in ceramic workers, welders and miners, can also have an impact on (in)fertility (144, 159, 160). In fact, it is well established that the spermatogenesis occurs at a specific temperature,

lower than that of the human body, being that the reason why the testes are located externally to the body (144) and several were the studies that have reported a higher scrotal temperature in infertile men, when compared to fertile ones (161, 162). It is known that high temperatures induce heat stress in the testis (162). In animal model studies, heat stress led to apoptosis, autophagy and DNA damage in germ cells (162-166). In addition, Ikeda *et al.* showed that ROS levels were also elevated after induced heat stress in isolated testicular cells from rats (167). Since ROS can induce apoptosis and DNA damage, it was hypothesised that its increased production was the trigger of heat stress response (162, 167). Not surprisingly, in humans, high temperatures were found to be correlated to a decreased seminal quality, particularly in terms of sperm count and morphology (144, 159, 168-170). Accordingly, Bonde *et al.* found that 6-week exposure to heat in a welding context significantly decreased the percentage of sperm with normal morphology, but that this alterations was reversible after a break in exposure (159). Interestingly, also idiopathic oligoasthenoteratozoospermic patients (OAT) were observed to have significantly increased scrotal temperature comparing to normozoospermic patients, a condition that was at least partially reversed by nocturnal scrotal cooling, using an air stream, that significantly increased sperm concentration and total count, being therefore suggested by the authors as a therapeutic option for these patients (171). Nevertheless, a systematic review on the potential beneficial effects of scrotal cooling to male infertility concluded that the evidences in this regard don't allow definitive conclusions (172).

On the other hand, cold temperature was also studied for its effect on the semen quality of men working in cold areas and, although no differences were observed in terms of semen routine analysis, the long term exposure to cold temperatures was observed to significantly increase sperm DNA fragmentation (173), that might further compromise the fertilization process and have deleterious consequences to the progeny (30).

Still, the presence of these expositions and their relevance for the infertility status of ID and UMI patients, is still to be determined.

1.5.2.4. Tobacco and alcohol consumption

In Portugal, 26.7% of men smoke daily, while males older than 15 years old drink on average 20.5L of pure alcohol per year (174, 175). Tobacco and alcohol consumption are long known to negatively affect health on a large spectrum of body functions, including reproduction, with alterations described at all the levels of the Hypothalamus-Hypophysis-Gonadal axis, expressed in hormonal changes, as well as alterations at the spermatogenesis levels and in the male gamete function (118, 176-178). Furthermore, several studies point to the hypothesis that both tobacco and alcohol exert their effects activating cascades of damage based on an increase of ROS and therefore an oxidative environment, promoted by the constituents of the tobacco and alcoholic beverages (178-180). Cigarette smoking, for

instance, was observed to be significantly correlated with reduced human sperm count, motility and morphology (177, 181-183). Furthermore, smoking was reported to be especially deleterious at the sperm mitochondria level, as human sperm incubation with cigarette smoke extract was observed to significantly decrease not only sperm motility and chromatin condensation, but also the number of spermatozoa with high MMP, while increasing apoptosis (184). Corroborating these observations, a study on the effects of smoking cessation showed that 3-month cessation led to an increase in sperm concentration and viability (185), stressing that the cessation might reverse, at least partially, some of the induced alterations, as described before to other compounds/expositions. Regarding alcohol consumption, this was also observed to have a negative impact at the reproductive level as a significantly decrease in semen volume, sperm concentration and increased DFI have been reported in different studies (118, 176, 178, 186). When analysing the type of consumption, in the scales of frequency (never, occasional or daily) it was reported that the detrimental effects at the sperm levels were more expressive in daily consumers, suggesting that a moderate consumption has not such adverse effects (176). Regarding the quantity itself, it was shown that the semen analysis gets worst in heavy drinkers (more than 70g of alcohol per week), when comparing to non and moderate users (178). Interestingly, a recent paper has analysed the concomitant effect of both tobacco and alcohol consumption, showing that both habits individually used lowered sperm concentration and progressive motility and their concomitant use worsen these parameters, when compared with individual habits (187). In this regard, the European academy of Urology (EAU) recommends smoking cessation and diminished alcohol consumption to improve male fertility (92). Overall, based on what has been described so far, it seems imperative to have access to smoking and drinking habits of infertile patients, especially those to whom a cause cannot be identified.

1.5.2.5. Anxiety and depression

The psychological state, though often not considered, is also an important factor in patients facing fertility problems, not only because these disorders can influence the ability to conceive a child, but also because infertility can further increase the psychological distress of a couple (5, 186, 188). In Portugal, 248 in 100000 men have depression (189).

In fact, couples that face fertility problems are under psychosocial distress (anxiety, stress, marital problems, social isolation, stigmatization), due to the social pressure to have a child and also to the frustration and depression associated with the inability to conceive, either naturally or through ART. Adding to this is also the financial component (costs from ART treatments and loss of financial income due to job quitting/renounce to better jobs and careers to focus only on having a child) that is a factor of additional distress to these couples (3, 4). Accordingly, significantly stressed men, assessed by the Hospital Anxiety and Depression Score (HADS) (190), were observed to have decreased serum levels of testosterone and increased levels of follicle-stimulating hormone (FSH) and LH, comparing to men

with normal HADS score, which was mirrored in decreased sperm count, motility and normal morphology (191). Furthermore, anxiety and depression were reported to be significantly correlated with low sperm concentration and motility in infertile men, although no significant differences were observed in terms of clinical pregnancies or live births (188, 192). On the other hand, infertility-related conditions such as varicocele, epididymal cyst and erectile dysfunction have also been described as risk factors associated with symptoms of depression and anxiety (193). However, until now, there's not enough data on the effect of psychological distress on ART outcomes to draw any coherent conclusions (188). Importantly, in the framework of unknown origin male infertility this issue seems to be essentiality unexplored, stressing the need to develop further studies in this regard. Moreover, by what has been discussed so far, this topic related to mental health it's of extreme importance and should not be disregarded, being imperative to address it in future studies, as a complementary information to assess the general health status of infertile patients and also to add knowledge on this field. Only in this way it will be possible to identify alterations in these patients and intervene in a specialized/multidisciplinary fashion, as soon as possible, working towards an improved diagnosis and the development of therapeutic strategies specifically tailored to these patients, that in the end will hopefully translate in a better reproductive performance and outcomes.

1.6. Unknown origin male infertility

This category, as mentioned above, includes ID and UMI patients. However, in the literature, it is frequent to find different terms to classify these patients, which severely compromise the interpretation of the available information, as well as the real knowledge on the details of this disease (97, 194). Also, often male patients are categorized without considering the female factor, and studies conducted without appropriate controls groups, questioning the reliability of the available information and further contributing to perpetuate the misleading classifications. It is thus essential to use congruent criteria to categorize (male) infertility and to urgently consider the evaluation of additional parameters, especially when it comes to the unknown origin category.

1.6.1. Idiopathic male infertility

By definition, ID patients, although not presenting any obvious condition affecting fertility and having normal findings on physical, endocrine, genetic and biochemical examination, with the female factor being ruled out, have abnormal sperm parameters (92, 97, 194). This type of infertility affects approximately 30 to 40% of infertile men (92). Depending on the sperm parameter that does not fit the WHO established criteria (2), these patients can be classified as having idiopathic oligozoospermia, if the total number of spermatozoa is below 15 million/mL, idiopathic asthenozoospermia, when presenting a percentage of spermatozoa with progressive motility lower than 32% and/or total motility lower than 40% or idiopathic teratozoospermia, when the percentage of morphologically normal

spermatozoa is below 4% (2). Frequently, patients can present two or more sperm parameters bellow the reference limits simultaneously, being referred as idiopathic oligoastenoteratozoospermic (OAT), when concentration, motility and morphology are affected, despite the lack of an apparent cause for theses alterations.

Idiopathic asthenozoospermia, for instance, has been associated with significantly higher number of morphologically abnormal mitochondria, in which the severity of these pathological changes increased significantly with the decrease in sperm viability (195, 196). These patients' sperm also had significantly decreased MMP and succinate dehydrogenase levels, indicating altered organelle functionality (195, 196). As previously mentioned, sperm MMP was also shown to be negatively correlated with ROS production. Accordingly, idiopathic patients were shown to have significantly increased sperm ROS levels comparing to healthy controls (197, 198) and Aydemir *et al.* found malondialdehyde, a known oxidative stress marker, increased in sperm and seminal plasma of idiopathic infertile men compared to fertile men (199). Furthermore, catalase and glutathione peroxidase, two important antioxidant enzymes, showed significantly lower activity in sperm of idiopathic patients comparing to fertile controls (197). As already discussed, oxidative stress is characterized by increased ROS levels in relation to the available antioxidants, and in sperm this could also lead to DNA fragmentation (200, 201). Furthermore, it has been suggested that genetic factors and DNA defects might be implicated in ID male infertility (202). Accordingly, in a study evaluating lipid peroxidation, antioxidant enzymes levels and activity in seminal plasma, Shamsi *et al.* observed that ID patients decreased levels in blood serum of the previously mentioned antioxidant enzymes (catalase, glutathione and superoxide dismutase) comparing to fertile controls, blood serum levels were correlated with seminal plasma levels of these enzymes (203). In addition, superoxide dismutase serum levels are correlated with sperm count, while glutathione levels are correlated with sperm progressive motility in ID patients. Malondialdehyde, a marker of lipid peroxidation was positively correlated with abnormal sperm morphology and negatively correlated with sperm motility (203). In a different study, Garcia-Segura *et al.* studied the relationship between oxidative stress and sperm DNA fragmentation (SFD), using the MiOXSYS system, designed for specifically measure the seminal oxidation-reduction potential (ORP) and have shown that although ORP was not significantly correlated with SFD in ID patients, it was negatively correlated with sperm motility and chromatin compaction (204). However, the authors did not explain in detail how patients' classification was performed neither have presented the inclusion vs exclusion criteria, which can influence the (interpretation of) obtained results. In addition, this study lacks a control group where ORP and DFI could be assessed and compared with ID patients, being in our opinion the major handicap of this work.

In line with these observations, Pourmasuni *et al.*, using two different methodological approaches, described that idiopathic patients have significantly increased DNA damage comparing to control patients, and that chromatin integrity and maturity were negatively associated with sperm count, normal morphology and progressive motility (205). Still, although these patients have sperm parameters lower

that the normozospermic group, they are still above the reference limits, what lead us to assume that actually this is a UMI group, stressing the importance of clear and strict classification of infertile patients.

Due to the sperm cell particularities already discussed, DNA damage can be further aggravated by impaired DNA damage repair system in ID patients (206, 207). DNA ligase 4 (LIG4) is an important enzyme involved in DNA repair through non-homologous end joining pathway. Ghasemi *et al.* studied the incidence of a known polymorphism in LIG4, *LIG4* ThrIle, in ID patients, observing that this polymorphism was correlated with the risk of infertility, where *LIG4* ThrIle increased infertility risk by 3.12-fold when heterozygous and 2.5-fold when homozygous (206).

Despite all the results pointing to alterations in the redox balance in ID patients that have a detrimental effect at the sperm levels, to our knowledge more functional aspects such as capacitation and acrosome integrity, have not been evaluated in these patients.

Nowadays, patients with this disease are left with scarce treatment options and can only rely on ART, in which the rate of success is disputable (92, 208), or empirical treatments that are essentially based on the assumption that this kind of infertility might be related to endocrine disruption due to environmental pollution or, alternatively due to oxidative stress, induced by a variety of agents and conditions (92, 208). Given this problematic, research has been made to find treatment options that might work for these men, although the results are not always concordant (208). One of such approaches has been giving gonadotrophins to these patients. Accordingly, highly purified FSH (hpFSH) 4-month treatment has been shown to improve sperm count in severe idiopathic oligospermia patients (209). In two other studies, 3-month hpFSH treatment was given to ID patients significantly improving not only sperm count, but also sperm motility, viability, normal morphology, and the percentage of hyaluronan-bound spermatozoa (as an indicator of sperm maturity), comparing with levels before treatment (210, 211). Hence, the authors conclude that this therapy could improve sperm maturation by enhancing spermatogenesis. Additionally, treatment also reduced sperm ROS levels and DNA damage, which, as previously discussed, are disturbed in these patients (211). Regarding conception, a study with 60 ID patients submitted to a 3-month treatment with hpFSH, showed that only 20 patients (33%) normalized their sperm parameters after treatment, and 15 patients (25%) achieved spontaneous pregnancy (212), suggesting that this treatment is not effective for the majority of ID patients. On the other hand, a recent study reported hpFSH treatment as having no significant improvements in sperm parameters after treatment, only showing a significant increase in sperm concentration when considering only the "responders", a group of patients showing a significant increase in semen fluid volume after treatment (213). Overall, the obtained results with hpFSH treatment point out to an improvement of the seminal quality in ID patients, however most of these studies do not mention ruling out female factor infertility, which could be one of the reasons why results among different studies vary, hence it would be of

importance to carefully and accurately categorize ID patients through a stricter criteria and additionally to separate the different categories of ID patients (e.g. oligozoospermia, asthenozoospermia and teratozoospermia), evaluating them in parallel to assess which patients would benefit more from hpFSH therapy. In addition, only few of these studies evaluate non routine sperm parameters, as additional functional and mitochondrial parameters such as DNA integrity, MMP, ROS production which, as previously mentioned, are deregulated in these patients and deserve further evaluations to achieve a complete characterization of the patients and the efficacy of the treatments.

Estrogen antagonists like clomiphene citrate and tamoxifen have also been given to idiopathic patients, and were observed to significantly increased sperm concentration and motility, pregnancy rates, and serum FSH and testosterone levels in these patients (214). Accordingly, a recent study, have shown that idiopathic OAT patients significantly increased testosterone, FSH and LH serum levels after 3-month tamoxifen treatment, with a 42% clinical pregnancy rate, in which only 2.6% corresponds to spontaneous conception (215). For idiopathic OA patients, the same treatment significantly increased sperm concentration, progressive motility, total motility, sperm MMP and ATP content, as well as serum and seminal total antioxidant capacity, while sperm intracellular ROS activity was significantly reduced, comparing to patients' analysis before treatment (216). Although the most recent meta-analysis reported that these treatments were not associated with increased adverse effects comparing to placebo (214), estrogen antagonist treatments were not fully deprived of some adverse effects like flush, decreased libido and headaches (215). Hence, is still not recommended by EAU guidelines to treat ID patients (92).

A different approach has been using antioxidant supplements to these patients (207), being Coenzyme Q10 (CoQ-10) and carnitine among the most used compounds to this aim. In a study were combined 6-month treatment with L-carnitine and L-acetylcarnitine was used in idiopathic oligoastenozoospermic (OA) patients, a significantly improvement in spermatozoa's progressive motility and viability together with a reduced DNA fragmentation index was observed (217). Furthermore, a recent meta-analysis involving 693 OAT idiopathic patients showed that this treatment was significantly effective on increasing motility and the number of pregnancies, although no other semen parameters were reported to be improved (218). On the other hand, CoQ-10 treatment alone, for 3-months, was able to significantly increase seminal plasma total antioxidant capacity in idiopathic OAT patients (219), indicating that it might contribute to attenuate the oxidative stress. Nevertheless, this treatment showed no significant effects on sperm parameters of the CoQ10 group, comparing to placebo group (219). Furthermore, in a different study in which the activity of several antioxidant enzymes was evaluated in idiopathic OAT patients after CoQ-10 treatment, similar results were obtained in terms of an attenuation of the oxidative environment, with the observation of significantly higher catalase and superoxide dismutase activity in the seminal plasma of the CoQ-10 treated patients, comparing to

placebo group, although in this case some improvements at the sperm level were reported (220). Accordingly, a study on 6-month treatment with CoQ-10, showed a significant increase in sperm motility in idiopathic asthenozoospermia patients (221). Finally, in the most recent study on the effectiveness of different pharmacologic approaches for idiopathic patients, performed by Shahid and colleagues in 2021 (222), it was reported that the use of supplements like carnitine and coenzyme Q10 was more effective in increasing sperm concentration and serum testosterone levels; that estrogen antagonists were more effective to increasing sperm motility and serum FSH levels and, finally, that hormonal treatments, like the FSH treatment, were more effective to increase sperm normal morphology (222).

Despite improvements on some individual sperm parameters, these are still empirical treatments that have not yet satisfactory information and results with a sufficient level of confidence (223, 224). Furthermore, most of these studies did not show these treatments as capable of increasing conception rates (208), questioning their real efficacy. All the exposed limitations have certainly contributed to the fact that, the most recent European Association of Urology (EAU) guidelines 2021 do not present clear recommendation of treatments for these patients (92).

1.6.2. Unexplained male infertility

Unexplained infertility patients, have normal physical examination, clinical history and the female factor has been ruled out (97, 99, 194). Furthermore, they present a normal seminal analysis (sperm count, motility and morphology), although being unable to accomplish spontaneous pregnancy (97, 225). Approximately 20 to 30% of infertile men have UMI, although the prevalence reported in the literature is not unanimous (92, 99, 226). Knowledge on this type of infertility is still very scarce, since this group is frequently confounded with the idiopathic infertility, as already discussed above.

As expected, most studies performed show no significant differences in conventional sperm parameters when comparing to fertile individuals (227, 228). When evaluating more functional aspects of sperm cells, these patients showed significantly increased ROS levels, such as H_2O_2, HO^-, $ONOO^-$ (165, 166) and $O2^-$ (167) (227-229) as well as increased DNA fragmentation (165,167), when comparing to fertile controls. Still, the methods to access the later parameter were not the same in the mentioned studies, as two of them used SCSA (sperm chromatin structure assay) while the other used SCD (sperm chromatin dispersion test). It was also observed that the DNA fragmentation was significantly correlated with the levels of ROS production (227, 229), suggesting that both oxidative stress and DNA fragmentation might be behind the alterations in UMI patients. Still, in one of the studies, DNA fragmentation was not significantly different between fertile men and UMI patients (228). This variability in results among different studies can be explained by the different experimental designs, including the defined groups and controls, the number of patients entailed, as well as the different

techniques and thresholds used/considered to assess these end points. Furthermore, processes such as capacitation and acrosome reaction, were not assessed in these patients, although some proteins interfering with these processes were observed to be differentially expressed in UMI patients, as we will further discuss (230).

Few studies have been performed on treatments for these patients, still some empirical treatments have been evaluated. Similarly, to what has been described for idiopathic patients, the use of antioxidant supplements to improve fertility was studied. In a study aiming to understand the effect of antioxidant supplementation (FH PRO for Men) on UMI and ID patients, sperm progressive motility, as well as sperm DNA fragmentation and seminal plasma oxidation reduction potential (ORP) were significantly improved after 3-month treatment in UMI patients, although the efficacy of the treatment was significantly higher in idiopathic patients (98).

Yet, these studies entailed a very limited number of patients properly diagnosed and categorized, the defined control groups varies and a complete characterization of UMI patients' sperm cells is missing (225, 230-232). Regarding treatment options, the only solution offered to these patients is ART, especially intrauterine insemination (233). Furthermore, despite some empirical treatments, such as expectant management (234), which consists solely in observing patients' condition, and antioxidant therapy, with some potential benefits as discussed above (98), the impact of this treatment in the patients' fertility rates has not been assessed, and to our knowledge, no other treatment options have been described in order to improve the fertility outcomes of these couples (231, 235).

Therefore, not only a diagnosis approach for these patients is missing, as is their correct categorization, as the evidence regarding potential treatments is scarse, resulting in no treatment recommendation for these patients (92).

1.7. Sperm Proteomics

Since ejaculated spermatozoa are thought to be transcriptionally (and translationally) inactive, proteins are a good bet to find relevant new information on these cells (236). In this sense, proteomic approaches constitute a valuable tool to obtain significant amount of information about the studied cells, relying on a relatively small amount of biological material and having the potential to find possible markers for different types of conditions, as is the case of groups of individuals with different fertility states/types. During the last two decades several were the studies that have used this type of analysis to better understand both spermatozoa's structure, biology and metabolism (237), as will be further discussed, aided by the fact that spermatozoa is a cell particularly easy to isolate and accessible (237-239).

Nowadays there are numerous proteomic techniques, among them, shotgun mass spectrometry (MS) techniques are the most adequate, allowing deep coverage of complex mixtures for a great number of samples (240). The most common MS techniques are matrix assisted laser desorption ionization-time of flight MS (MALDI-TOF MS) and liquid chromatography coupled to tandem MS (LC-MS/MS) (240). MALDI-TOF MS is a protein fingerprinting technique, where proteins are digested by a proteolytic enzyme and the resulting peptides are massed by the MS software. The proteins are identified by the masses of the resulting peptides in the respective databases. LC-MS/MS is a peptide sequencing technique, where proteins are also digested, with the particularity that the resulting peptides are run through a high-performance liquid chromatography (HPLC) column and the different fractions of the eluted peptides massed separately. In this method, proteins are identified by matching the acquired peptide spectrums to known peptide spectrums. Although this method is more time consuming than MALDI-TOF MS, it allows for better sensitivity and reliability, since it identifies more proteins and with higher levels of confidence (240).

On the other hand, Short GeLC-SWATH-MS is a LC-MS/MS protocol, where acquisition is performed by two distinct methods: information dependent acquisition (IDA) and sequential window acquisition of all theoretical mass spectra (SWATH) (241). This protocol is divided in three phases: sample preparation, protein identification and protein quantification. Sample preparation involves digestion of the proteins and separation in different fractions, in this case both are performed using a reduced piece of acrylamide gel that will originate three peptide fractions – "short-GeLC". Protein identification is performed using IDA and the quantification is performed using SWATH. This approach will result in deep proteome coverage and accurate quantification of various proteins (241, 242).

In the sperm cell, several approaches have been used, one of the most common being gel-based techniques including 1D-PAGE, 2D-PAGE, 3D-PAGE, 4D-PAGE and 2D-DIGE. The PAGE techniques consist in separating proteins in a SDS gel through electrophoresis by several dimensions: the first protein size, the second protein charge, the third retention time and the fourth and latest ion mobility. The DIGE technique differentiates from PAGE by using different fluorescent tags to increase sensitivity of protein spots detection (240). Still, MS remains the most sensitive proteomic approach (240).

Although the first proteomic study on human sperm dates back to the 60's (Felix, 1960 (243)) only in 1999, the first proteomic analysis on human sperm using a 2D electrophoretic analysis of a pool sample from 10 fertile man was published (244). In this paper, 7 proteins were identified: heat shock protein (HSP) 90 and 70, calreticulin, alfa-tubulin, beta-tubulin, PH-20, and gastrin-binding protein (244). Since then, advances in technology have allowed for identification of a higher number of proteins. One of the largest sperm proteomic studies, published in 2013, was able to identify 4675 different proteins, being the most represented pathways the energy metabolism, signal transduction and

cytoskeleton regulation (245). More recently, these studies in human sperm have expanded to gain novel insights on sperm development, structure, metabolism, sperm-oocyte interaction, and early embryo development (238, 239, 246).

Regarding metabolic pathways, proteomic data gave new insights supporting the metabolic versatility of the sperm cells when it comes to energy attainment, with the indication that sperm have the machinery to perform metabolic pathways as diverse as glycolysis, pentose phosphate, gluconeogenesis, Krebs cycle, OXPHOS and also nucleotide and lipids metabolism (238, 246). To the latter, important knowledge was added by Amaral and colleagues, in a proteomic study specifically focused on sperm tail, that found 3-ketoacyl-CoA thiolase (ACAA1) and peroxisomal membrane protein 11 (PEX11), two peroxisomal enzymes, to be present in this compartment, showing that spermatozoa's tail not only has peroxisomes, but also that these cells can use lipid oxidation as an additional energy source (237), an information that was a breakthrough revelation at the time, as the peroxisomes were believed to be absent in the sperm cell.

Additionally, proteomic studies have also clarified that sperm cells have the machinery for protein and RNA metabolism, as well as other processes such as cell cycle, meiosis, membrane trafficking and apoptosis (175).

Overall, the spermatozoa's unique structural features and functionality is mirrored in their proteomic composition (238, 245). Hence, proteomic profiles of spermatozoa are now being studied to a broad range of aims in the male reproductive field, including the characterization of sperm samples with high vs low quality, the profiling of sperm samples from fertile vs infertile individuals or from individuals with diseases that might affect the reproductive function (e.g diabetes), or the profiling of samples with different fertilization outcomes in ART, having the major common purpose of finding putative biomarkers for male (in)fertility, opening road to the development of tailored therapeutic interventions, or even helpful biomarkers that can further improve gamete selection for ART, contributing to higher fertilization and embryonic development rates (246, 247).

Regarding male infertility, one of the most studied phenotypes is asthenozoospermia. In this regard, although the initial studies, had identified only few differentially expressed proteins that might have a role in the regulation of sperm motility (248-250), several posterior studies added proteins to this list, such as that conducted by Paiva and collaborators in 2014, identifying 1157 proteins, with 80 differentially expressed among asthenozoospermic and normozoospermic samples (251). Further on, Saraswat and colleagues, identified 667 proteins, from which 20 proteins were down or up regulated in asthenozoospermic sperm samples. Furthermore, the sperm proteomic signature was able to separate the two classes of samples (astheno vs normozoospermic) in a PCA (Principal component analysis). In this study the authors also performed, in parallel, a proteomic analysis of the seminal fluid; yet they have

concluded that this data was not reflective of possible defects in motility and that the sperm analysis is preferable (252). Using a proteomic approach coupled to a biochemical analysis of the sperm MMP and mitochondrial ROS, Nowicka-Bauer *et al.*, found 25 differentially expressed proteins in asthenozoospermic individuals, most of them downregulated, whose samples have also shown lower sperm MMP and higher ROS production (253). In a different study using 4D-quantative proteomics, Yang *et al.* identified 4718 proteins, where 1430 were found differentially expressed between astheno and normozoospermic men, which was the higher number of proteins identified until now (254). In addition, they propose extracellular matrix protein 1 as a novel biomarker for asthenozoospermia (254). Based on the fact that protein S-sulfhydration is an important process to protect proteins from oxidative stress, Qi *et al.* focused specifically on S-sulphydrated proteins, identifying 244 of these in sperm (255). Additionally, they found two histone proteins, S-sulfhydrated H3 and S-sulfhydrated H3.3, significantly down-regulated in asthenozoospermic patients, compared with fertile men, showing that impaired S-sulfhydration could be associated with these patients' infertility (255).

Phophoproteomics, an approach that specifically focus on the phosphorylation status of sperm proteins, known to be important for several sperm functions, including motility, have also provided important information in this regard. In fact, after some initial studies, that identified some differentially expressed phosphoproteins in astenozoospermic samples (256, 257), more recently, differential phophoproteins signatures have also been associated with sperm motility, in a study analysing normozoospermic samples with low or high motility (258). In this study they found that low motility spermatozoa have phosphoproteins mainly involved in sperm metabolism (40%), while for high motility spermatozoa, they are mainly associated to spermatogenesis and sperm function- motility (48%), with only a small part associated to metabolism (8%). Furthermore, they reported that GSK3α, a kinase involved in sperm motility regulation, is one of the most abundant phosphoproteins in spermatozoa with high motility, opening road for future studies on this protein as a potential biomarker of clinical use and having concluded that protein phosphorylation is important for sperm motility regulation (258).

Overall, all the proteins identified in the mentioned studies above, are, not surprisingly, related to metabolism and energy production or belong to the cytoskeleton.

Besides astenozoospermia and motility, proteomic studies have also been performed to understand alterations related to globozospermia, oligozoospermia, DNA damage, sperm protamine content and capacitation process (175). The sperm proteomic profile of patients with diabetes versus healthy controls have also been determined and concluded to differ (259), an important information having in mind the impact of diabetes on the male reproductive function, as already mentioned (115).

Proteomic studies have also added information regarding the reproductive success after ART. In fact, recently, a study entailing 27 infertile couples (idiopathic couples, couples with known male factor

and with known female factor), reported that 18 proteins were found to be associated with embryo quality after ICSI (239). Specifically, for idiopathic infertile couples, the sperm equatorial segment protein 1 (SPESP1), a protein essential for gametes binding and fusion, was found to be significantly (and positively) correlated with good-quality ICSI-derived embryos while other 15 proteins were significantly (and negatively) correlated with poor-quality ICSI-derived embryos, those being functionally enriched in processes such as sperm binding to zona pellucida, protein folding and telomere maintenance (239). A study on idiopathic recurrent pregnancy loss (iRPL), characterized by two consecutive miscarriages before the 20th week of gestation, with no known cause associated, after identifying 1656 proteins, with 27 differentially expressed, showed that the expression of 22 proteins, including the fatty acid synthase (FASN) and mitochondrial ATP synthase subunit β, was significantly up-regulated, while 5 proteins, including lactotransferrin and superoxide dismutase, were significantly down-regulated in idiopathic RPL male's sperm (246). Furthermore, the PCA analysis based on the differentially expressed proteins among iRPL and controls sperm samples clearly separate the two groups (182). Further statistical analysis identified 7 potential biomarkers for iRPL: lactotransferrin, ATP synthase subunit beta mitochondrial, fatty acid synthase, anterior gradient protein 2 homolog, hemoglobin subunit beta, short-chain specific acyl-CoA dehydrogenase mitochondrial, cytoplasmic dynein 1 heavy chain, and 14-3-3 protein sigma. This data, together with the fact that two pathways, response to oxidative stress and response to ROS were enriched in iRPL, lead the authors to suggest that oxidative stress and a redox imbalance might also play a role in these patients' inability to carry a pregnancy to term (182).

Worth mentioning, Wang and colleagues, in a study aiming to identify the human sperm proteome of fertile men with normal seminal analysis, identified around 5000 new proteins, of which nearly 4% were testis specific, and have shown that there's little overlap among sperm proteome and transcriptome, proving the need for more of these studies in sperm cells (245). Interestingly, they also found that nearly 500 proteins were targets of known drugs and, after studying some of those drugs, the authors found an effect on sperm motility.

In sum, although proteomic studies have given great knowledge on this unique cell, still there is much more to discover, especially to define a good and functional sperm at the proteome level. The technological advances will certainly help in this pursue.

1.7.1. Proteomic studies in ID patients

Due to lack of appropriate diagnose and consequent treatment, some studies have been performed using proteomics in an attempt to better understand ID aetiology. Regarding studies performed in the seminal plasma, Wang *et al.* have identified 741 proteins, of which 45 were upregulated and 56 downregulated in the asthenozoospermic group, when comparing to controls (healthy donors), being

that the majority of these proteins have origin in the epididymis and prostate, well known to contribute with secretions to the seminal fluid (260). Importantly they found DJ-1, a protein though to be involved in the management of oxidative stress, to be downregulated in asthenozoospermic patients and that the levels of ROS were higher in this patients samples, leading the authors to suggest that the downregulation of DJ-1 might contribute to a state of oxidative stress in the semen, further affecting semen quality (260). Nonetheless, and although in most of the asthenozoospermia cases the cause it's not fully understood being thus classified as idiopathic, the authors didn't specify this detail. Furthermore, the authors based their analysis on the criteria defined in 1999 by WHO, that being stricter might include cases that are presently not considered asthenozoospermic (252). Also lipocalin-1, a scavenger protein of lipid peroxidation products, was shown to be upregulated in idiopathic OA patients' seminal plasma (231). This protein's expression is known to be induced by oxidative stress, pointing to ROS accumulation as a relevant factor in ID aetiology (261). Epididymal secretory protein E1, an important cholesterol transporter involved in cholesterol efflux during capacitation (262), was found to be downregulated in the seminal plasma from idiopathic OA patients, an alteration that can severely impact capacitation and acrosome reaction, two mechanisms dependent on cholesterol, interfering with fertilization ability (231).

Besides seminal plasma, there were also studies that focused directly on the sperm cells. In one of such studies, the spermatozoa proteomic profile of asthenozoospermic samples has identified 86 differentially expressed proteins, comparing to normozoospermic samples, with the most represented proteins being located in the mitochondria, and having a role on metabolism and energy production. Additionally, the authors have described that proteins involved in the TCA cycle, OXPHOS and fatty acid catabolism were down regulated in asthenozoospermic samples. Some examples include CPT2 (carnitine O-palmitoyltransferase 2), GOT1 (aspartate aminotransferase) and ATP5A1 (ATP synthase subunit α), all metabolic enzymes involved in fatty acids catabolism, carbon skeletons catabolism and oxidative phosphorylation, respectively (263), suggesting that this alteration in terms of bioenergetic pathways may underlie the changes in motility. Yet, this study didn't specifically clarify if the asthenozoospermic samples are from idiopathic infertile patients, as mentioned previously regarding the study of Wang et al, 2009. A different study, performing 2D-gel and tandem MS, found 16 differentially expressed proteins in idiopathic asthenozoospermia patients comparing to normozoospermic men (264). Among these proteins were β-actin and tektin 4, constituents of the cytoskeleton in sperm flagella; phosphoglycerate kinase 2, known to be associated with sperm motility; and the previously mentioned DJ-1 protein, involved in management of oxidative stress (264).

As previously mentioned, antioxidant supplementation is the main approach given to idiopathic patients, having as a major aim to improve fertilizing capacity (207). Having this in mind, Agarwal *et al.* performed a proteomic study on idiopathic patients pre and post 6-month antioxidant

supplementation (FH PRO for Men), showing 379 proteins differentially expressed before and after treatment (98, 265). Protein analysis found that these proteins were associated with activation of OXPHOS, spermatogenesis, sperm maturation and fertilization (265), functions that were previously described as being impaired in these patients.

Nevertheless, the fact that most of the studies using this promising proteomic approach for ID patients, were performed on seminal plasma, together with the dubious classification of patients in some studies, regarding the idiopathic factor, clearly rises the need to investigate more at the gamete level, aiming to find functional details that might be compromised in these patients. In this away, the combined information of the proteomic profile of the environment and the cell itself will have the potential to bring an integrated view of what might be wrong with these patients, at a molecular level, opening road for proper and tailored interventions.

1.7.2. Proteomic studies in UMI patients

There are some reports that, relying on a proteomic approach, have identified a few proteins to be differentially expressed in UMI patients' spermatozoa, when compared to fertile controls (225, 230, 232), shedding some light on the molecular mechanism that might be altered in these enigmatic patients. Frapsauce and collaborators, designed a study to understand the molecular basis of failures in IVF, comparing sperm samples from patients with a IVF failure to control individuals with successful IVF, both normozoospermic. They have identified 17 differentially expressed proteins between infertile man and controls. Laminin receptor LR67 is one of such proteins, that was found to be significantly overexpressed in UMI patients' spermatozoa. The authors hypothesised that increased levels of this protein will foment the binding of integrins to laminin, instead of binding to sperm, where it is needed for binding and fusion to the oocyte, thus affecting the gametes interaction and ultimately fertilization (225). In the same study, the expression of P34H (L-xylulose reductase), a known human sperm epididymal maturation marker important for the binding of sperm to *zona pellucida*, was found to be significantly decreased in UMI patients comparing to control men, showing that UMI patients' fertilization failure could be related to impaired sperm-oocyte binding and fusion (225). However, this study was based in the WHO 1999 guidelines, a stricter evaluation than the current one, as already discussed. Moreover, the authors have used a pool of samples and the samples used for sperm proteomic analysis were not the same that were used for IVF, an issue that might introduce some variability and influence the results. Using a different method, LC-MS/MS, Azpiazu *et al.* found 66 proteins differentially expressed in IVF failure comparing to IVF success normozosspemic patients (266). The authors found that upregulated proteins in IVF failure are associated with meiosis and chromosome maintenance. Of relevance is SRSF protein kinase 1, which is highly expressed in the testis and regulates binding of protamines to DNA. Among downregulated proteins in IVF failure are apolipoprotein E, lipase member I and lipoprotein lipase, all associated with lipoprotein metabolism. Regardless, this

study is limited by the fact that samples analysed were pooled and never analysed individually, which decreases the accuracy of data gathered.

Also focused on ART outcomes, McReynolds and coworkers. studied the contribution of sperm for blastocyst development and found 49 proteins differentially expressed between UMI patients' sperm resulting in good and bad blastocyst development, after undergoing IVF oocyte donor cycles (267). Several proteins associated with spermatogenesis were observed to be downregulated in sperm resulting in poor blastocyst development, among them were the nucleoside diphosphate kinase homolog 5 and testis-specific serine/threonine kinase 2. While the former regulates antioxidant activity of glutathione peroxidase 5, the latter regulates microtubule dynamics during spermatogenesis. In contrast, heat-shock associated proteins were upregulated in sperm resulting in poor blastocyst development, as is the case of heat shock protein β-1, involved in cytoskeletal integrity; and clusterin, a marker for seminal oxidative stress. Overall, these results points to alteration in the sperm development and the existence of an oxidative environment as probable causes to the reduced reproductive potential of these men.

In a different study, aiming to explore the proteome of sperm from normozoospermic infertile men, Xu and colleagues have identified 24 differentially expressed proteins, of which 9 were involved in cell communication, proliferation and differentiation (232). Semenogelin-1, glycodelin (also called progestogen-associated endometrial protein) and prostate-specific antigen (KLK3) were upregulated, while outer dense fiber protein 1 (ODF1) and glutathione peroxidase (GPx4) were downregulated in normozoospermic infertile men, results that were also confirmed by western blot (232). Overexpression of semenogelin-1 is associated with decreased sperm motility and capacitation, while KLK3 and ODF1 are associated with increased motility. Yet, as these parameters are not altered in these patients, these proteins' role in these men's infertility is still not clear. On the other hand, glycodelin is known to regulate fertilization through glycosylation but the effect its overexpression on fertility is not well understood. On the other hand, GPx4 downregulation, known to protect cells against oxidative stress, lipid peroxidation and ultimately apoptosis (268), could leave cells more susceptible to damage. Also peroxiredoxin 2 and 5, mitochondrial proteins known to have antioxidant activity, were reported to be increased in UMI patients comparing to normozoospermic fertile men, further corroborating the thesis that oxidative stress is a very likely player in UMI patients' sperm dysfunction (230, 232). The authors concluded that the observed differences in signalling and metabolic pathways will have implications at several levels in sperm, including on motility, capacitation, acrosomal reaction and sperm–oocyte communication, and might therefore constitute potential diagnostic markers. Yet, it is important to mention that Xu and coworkers (231) used highly selected sperm populations for analysis (after swim up), a fact that might influence the obtained results, namely decreasing the amount of information obtained. Finally, a recent study comparing the proteome of UMI patients with normozoospermic fertile men, identified 162 differentially expressed proteins between the two groups (230). Among them were

A-kinase anchoring protein 3 and 4, both upregulated in UMI, and SPA17 and SERPINA5, both under expressed in UMI, which altered expression was hypothesised by the authors to lead to impaired sperm capacitation, acrosome reaction and sperm-oocyte binding. In addition, cell death and survival and free radicals scavenging pathways were enriched in UMI patients comparing to fertile men (230).

Overall, although some proteomic analysis have been performed focusing specifically on ID and UMI patients, one might highlight that not only the proteomic approaches used were different, but also the number of patients entailed, the samples processing methods used or even the WHO reference guidelines greatly differ, warranting future studies on this topic taking advantage of the high throughput techniques that are currently available for proteomic analysis and that can improve the quantity and quality of the obtained results.

1.8. Objectives

The main goal of this study is to obtain new insights into the mechanisms beyond unknown origin male infertility (ID and UMI), performing an innovative, complete, and integrated analysis of sperm function in well characterized and defined groups of patients. Our ultimate objective is to find biomarkers specifically for these cases of infertility that can be further used to develop new tools that will improve diagnostic and tailored treatments for these patients, and/or to collect information that can be helpful in the hard task of selecting better gametes.

2. Material and methods

2.1. Chemicals

All chemicals were supplied by Sigma-Aldrich (Merck) unless stated otherwise.

2.2. Semen samples collection, preparation, and clinical diagnosis

The human sperm samples came directly from the Reproductive Medicine Unit of the University Hospitals of Coimbra, where they were obtained by masturbation after 3-5 days of abstinence according to WHO guidelines (2), from men who were undergoing routine semen analysis for fertility evaluation. A total number of 1710 samples were collected. After liquefaction, semen samples were density-gradient centrifuged (400-500*g*, 10 min) with SupraSperm (#10970060A, Medicult-Origio) using a 40% (v/v) density on top layer and 80% (v/v) density in the lower layer (2). The high-density layer recovered sperm population was consequentially resuspended in sperm preparation medium (#10690010A, Medicult-Origio). The sperm cells were then allowed to capacitate for 3h at 37ºC and 5% CO_2 (269).

All procedures were approved by the ethics committee of the University Hospitals of Coimbra (CHUC-098-18) and the participants signed informed consents. Afterwards, based on the seminal analysis and couple's clinical history, the samples were categorized in the different fertility groups under guidance of the responsible clinicians and embryologists. Therefore, all the analysis done at the scope of this thesis were completely blind.

The exclusion criteria for this study were: presence of urogenital anomalies or infections, namely untreated varicocele; leucocytospermia; azoospermia; AIDS and significant genetic anomalies (e.g. Klinefelter syndrome, Kallmann Syndrome; Y chromosome deletions). Patients under hormonal treatment, that had oncological diseases and/or underwent treatments (chemo or radiotherapy) or other major illnesses were also excluded. Individuals were further classified as control if the semen analysis presented normal concentration, viability, and morphology according to WHO reference values (2), and their female partner presented any identifiable fertility issue (e.g. ovulatory, tubar, endocrine) or conditions that could explain the couples' infertility. Then, those individuals that had failed to conceive, after one year or more of regular unprotected intercourse, were classified as ID or UMI. In the former case, individuals fall in this category if their seminal analysis presented abnormal concentration, viability, or morphology or any combination of these anomalies (e.g. AT, OA; OAT), according to WHO reference values (2), and if their female partner did not present any fertility-related anomalies or conditions. Finally, individuals were classified as UMI if having a normal seminal analysis (2), and, likewise, if their female partner presented normal findings after (in)fertility evaluation.

2.3. Sperm count, motility, and viability evaluation

Sperm count, motility and viability were evaluated by phase-contrast microscopy (DM4000B, Leica).

Sperm concentration evaluation was achieved using an improved Neubauer chamber (0,1 mm depth; Marienfeld), loaded with 10 µl of sample previously diluted and immobilized by osmotic shock, according to WHO recommendations (2) and counting the number of cells in specific squares using a 600x magnification. Concentration was then calculated based on the Neubauer chamber characteristics and sample's dilution (2). From the initial sample, a stock of 10 million/mL spermatozoa was prepared with adequate medium, the phosphate-buffered saline [PBS; Oxoid™ (BR0014G), Thermo Fisher] supplemented with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.0 mM D-glucose, 1.0 mM sodium pyruvate, 10 mM (v/v) sodium lactate, 25.0 mM $NaHCO_3$, 1% (v/v) Penicillin-Streptomycin 10,000 U/mL (#15140122, Thermo Fisher) and 3g/L BSA at 7.2-7.4 pH (269). This was our working stock for all the further evaluations of the present study.

Sperm motility was evaluated by observing a preparation with 10 µL from the previously prepared 10 million/mL stock sample, using 400x magnification. Spermatozoa's motility was classified into progressive, non-progressive (*in situ*) and immotile (2). At least 100 spermatozoa were assessed in total, in at least 5 different fields and percentages of different motility types calculated. Percentage of total motility (progressive plus non-progressive motility) was also calculated for each sample.

For the evaluation of sperm vitality, Eosin Y was used (2). Plasma membrane is impermeable to this dye, thus only dead cells with a compromised membrane could internalise the dye acquiring a pink-reddish colour in the head [Figure 5]. To assess this, 5 µL from the previously prepared 10 million/mL spermatozoa stock were mixed with 5 µL of 0.5% (w/v) Eosin Y [Sigma-Aldrich (#45380), Merck] in a glass slide and observed using 400x magnification. At least 100 cells were scored for each sample. Spermatozoa with a pink-reddish head were considered dead, while spermatozoa with white heads were considered alive (2) and results were expressed as the percentage of live spermatozoa in each sample.

2.4. Sperm's morphology and chromatin status

The Diff-Quik kit was employed to evaluate both spermatozoa's morphology and chromatin status. This kit [Millipore (#1.11674.0001), Merck] is composed by 3 solutions: methanol, a fixative reagent; eosin, an anionic dye that stains red positively charged proteins; and thiazine, a blue staining agent for DNA. For each sample a smear was performed using 5 µL of the previously mentioned sperm sample stock. After air-drying, slides were sequentially immerged in the 3 solutions for 15s each. Slides were left to airdry and then mounted with Eukitt mounting medium [Sigma-Aldrich (#03989), Merck] (270). Both spermatozoa's morphology and chromatin status were evaluated via oil-immersion bright field

microscopy (DM4000B, Leica). Spermatozoa were classified as having abnormal morphology if any significant head, midpiece or tail defects were found, according to WHO guidelines, and normal if no defects were found (2). At least 100 cells were observed per sample, in different fields, using 1000x magnification, and afterwards, the percentage of normal sperm cells was calculated.

Chromatin status was also assessed using the Diff-Quik kit, since a darker staining in the sperm nucleus was previously correlated with higher chromatin damage (270, 271). This is possible because less compacted chromatin allows for higher accumulation of thiazine, showing a darker colour on the nucleus (271, 272). For each sample at least 100 cells were observed using 1000x magnification and classified as having intact chromatin (light nucleus) or damaged chromatin (dark nucleus) [Figure 6] (270). Afterwards, the percentage of intact chromatin was calculated and results expressed as so.

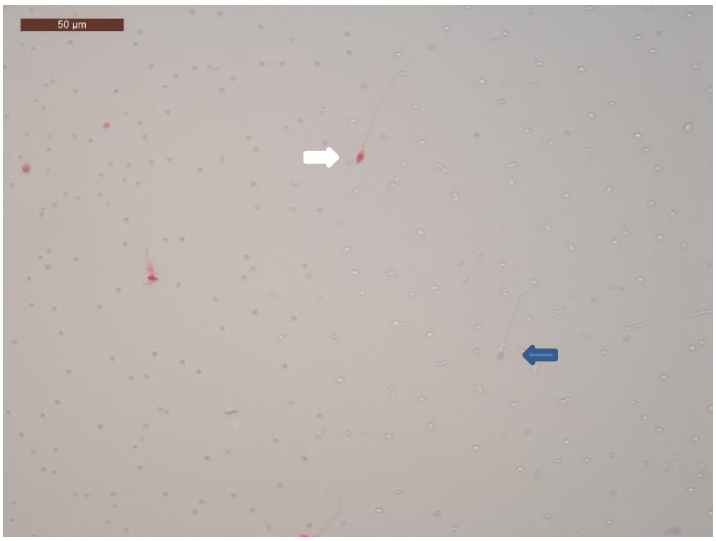

Figure 5. Sperm vitality assessed by Eosin Y, under bright field microscopy. Live sperm nucleus appear transparent (blue arrow), while dead sperm nucleus are coloured pink (white arrow). Magnification of 630x. Scale bar: 50 µm

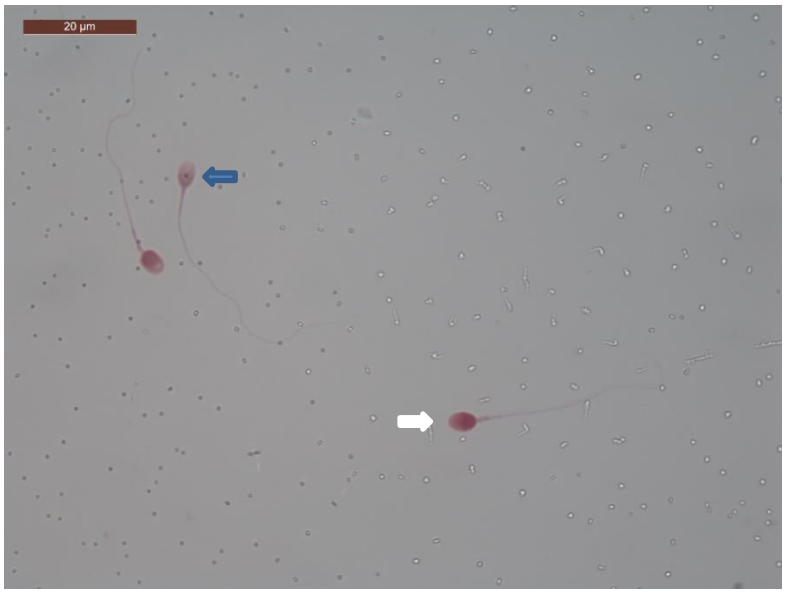

Figure 6. Sperm morphology and chromatin status detected using Diff-Quik staining kit under bright field microscopy. Cell with normal morphology and light nucleus is represented by the blue arrow, while abnormal sperm with dark nucleus is represented by the white arrow. Magnification of 1000x. Scale bar: 20 µm

2.5. Capacitation status assessment

As previously mentioned, capacitation is an essential step for spermatozoa competence. Capacitation status can be assessed by immunocytochemistry through the detection of phosphorylated tyrosines, a known hallmark of this process, using proper antibodies (269, 273). For this analysis, we have used a protocol in which approximately 200 µL of the previously prepared 10 million/mL spermatozoa stock were centrifuged (300*g*, 5 min), followed by resuspension in 300 µL of fixation solution [0.5 g/L azide in PBS (PBS-azide) with 2% (v/v) formaldehyde at 7.2-7.4 pH], and incubated at room temperature (RT) for 40 min. After this, samples were centrifuged (300*g*, 5 min) followed by resuspension of the pellet in 300 µL of permeabilization solution [1% (v/v) Triton X-100 in PBS-azide at 7.2-7.4 pH], and incubated at RT for 20 min. Then, samples were again centrifuged (300*g*, 5 min) and resuspended in 300 µL of blocking solution [1 g/L BSA and 100 mmol/L glycine in PBS-azide at 7.2-7.4 pH], and finally stored at 4ºC until further use. For phosphotyrosines detection, samples were then centrifuged (300*g*, 5 min) followed by resuspension with rabit anti-phosphotyrosine polyclonal antibody [1:10; (#61-5800), Thermo Fisher] in blocking solution and incubated overnight at 37ºC. In the next morning, after washing steps [0.1% Triton X-100 in PBS-azide at 7.2-7.4 pH] samples were incubated in 200 µL of blocking solution with goat anti-rabbit (1:200; (#T-6391), Thermo Fisher) and incubated for 1 h at 37ºC. A wet preparation was then prepared adding to the sample 5 µL of Vectashield antifade mounting media with 4',6-Diamidino-2-Phenylindole, Dihydrochloride [DAPI; (#H1200), Vector Labs] (269).

The proportion of capacitated spermatozoa in each sample was evaluated after counting at least 100 cells in different fields via oil-immersion fluorescent microscopy with a final 1000x magnification (DM4000B, Leica). DAPI was used as a counterstain, while capacitation evaluation was preformed according to the pattern of red fluorescence [Figure 7]. Spermatozoa were classified in 4 categories: homogeneously stained tail, partially stained tail, dot-stained tail, and very weak or non-existent fluorescence. Spermatozoa were considered capacitated if classified as one of the first three categories. The percentage of capacitated spermatozoa was calculated for each sample [Figure 7].

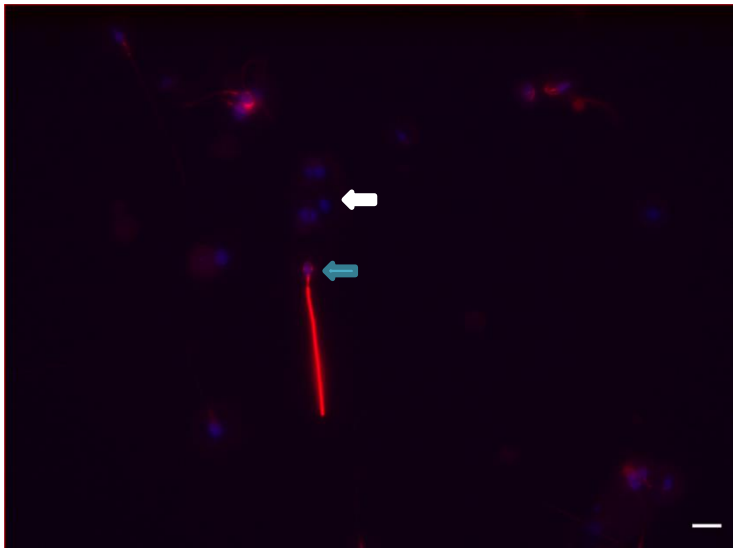

Figure 7. Capacitation status of spermatozoa. Phosporilated tyrosines were detected using rabbit anti-phosphotyrosine polyclonal primary antibody and goat anti-rabbit with red fluorophore conjugated as secondary antibody. DAPI (blue) was used as a counterstain the nucleus. Capacitated spermatozoa exhibit red bright fluorescence in the tail (blue arrow), while non-capacitated spermatozoa show no tail fluorescence (white arrow). Magnification of 1000x. Scale bar: 10 µm

2.6. Acrosomal integrity evaluation

Acrosome reaction is a crucial event that enables spermatozoa to bind and penetrate the oocyte and ultimately achieve fertilization. Nevertheless, the acrosome should be intact until in the proximity of the oocyte, assuring the fertilization process to occur in due time (274). In order to evaluate the acrosome integrity, a marker for acrosomal content, *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC) was used. For this analysis, we have used a similar protocol to that described above for capacitation status assessment, in which the same quantity of sperm cells was used, being the procedure the same until samples were stored at 4ºC, after the blocking step (269). Samples were then centrifuged (300*g*, 5 min), resuspended in blocking solution containing 5 mg/µL of PSA-FITC [Sigma-Aldrich (#L0770), Merck) and incubated 1 hour at 37ºC.

After this, samples were washed being the pellets placed in a glass slide together with 5 µL of Vectashield antifade with DAPI [DAPI (#H1200), Vector Labs] and covered with a coverslip. Spermatozoa were observed via oil-immersion fluorescent microscopy with 1000x magnification. At least 100 spermatozoa were scored from each sample, in different fields. Intact acrosomes exhibit bright green homogeneous fluorescent staining, while acrosome-reacted spermatozoa present either no signs of fluorescence or heterogeneous fluorescent spots [Figure 8]. The percentage of sperm cells with intact acrosome was calculated for each sample, expressing the obtained results (269).

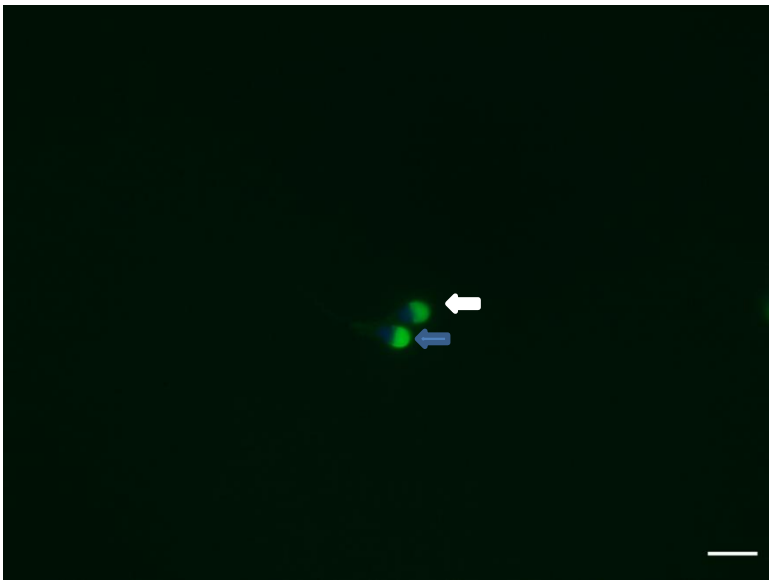

Figure 8. Acrosomal content detected using the fluorescent probe PSA-FITC (green), by fluorescent microscopy. DAPI (blue) was used to counterstain the nucleus. Intact acrosome present green homogeneous fluorescence (blue arrow), while reacted spermatozoa presented heterogeneous fluorescence due to the acrosome reaction initiation (white arrow). Magnification of 1000x. Scale bar: 10 µm.

2.7. Sperm mitochondrial status

As described hitherto, mitochondrial functionality is intimately related to that of sperm (13, 39, 59, 61, 66) and the dysfunction of this organelle has been extensively correlated to oxidative stress, a determining factor for sperm functionality and ultimately male (in)fertility (51, 61, 66, 275). Hence the importance of assessing MMP and ROS production as competent indicators of sperm mitochondrial status is huge and we did it through flow cytometry, using specific probes, as will be further detailed below.

Samples flow cytometric analysis was performed in a FACSCalibur flow cytometer (BD Biosciences), which is equipped with an argon laser with an excitation wavelength of 488 nm and three emission filters: 530/30 band pass (FL-1 green), 585/42 band pass (FL2/red) and >620 nm long pass filter (FL3/far red). Spermatozoa has specific characteristics of forward (FSC) and side scatter (SSC) and based on that, unspecific events were gated out of the analysis. A total of 15000 events were

recorded per sample. Data acquirement and consequent analysis were achieved using BD Cell Quest Pro Acquisition program.

2.7.1. Mitochondrial Membrane Potential assay

MMP was evaluated using 5,50,6,60-tetra-chloro-1,10,3,30-tetraethylbenzimidazolyl-carbocyanine iodide, JC-1® [Molecular Probes (#T3168), Thermofisher]. JC-1 accumulates in mitochondria, forming aggregates and emitting red fluorescence when MMP is high; or emitting green fluorescence, remaining in its monomeric form when the MMP is low (66, 276). JC-1 probe has dual fluorescence emission, being detected in FL-1 and FL-2 channels.

For this protocol 2.5 million/mL spermatozoa in PBS supplemented medium (see composition in the 2.3. subchapter), without BSA and $NaHCO_3$, were used. Three conditions were prepared for each sample: A) sperm sample only, as a negative control; B) sperm sample with 2 µM JC-1; and C) sperm sample with 2 µM JC-1 and 50 µM carbonyl cyanide 3 chlorophenylhydrazone, CCCP [Sigma-Aldrich (#C2759), Merck] as a positive control. Samples were incubated with JC-1 (condition B) or JC-1 and CCCP (condition C) for 15 min at 37ºC, then centrifuged (500*g*, 5 min) and resuspended in PBS supplemented medium (composition in the 2.3. subchapter) without BSA and $NaHCO_3$. In the cytometer, 15000 events were registered.

For results analysis, condition A, the negative control, was used to exclude the cells autofluorescence contibution. CCCP, is a mitochondria uncoupling factor that disrupts the MMP, which for this reason was included in condition C, the positive control. These conditions were further used to define two regions for analysis in the flow cytometry dot plot chart: R2 and R3; R3 region corresponding to the region where cells with low MMP will fit, and consequently R2 region corresponds to the region were cells with high MMP will be found [Figure 9]. Results were expressed in percentage of gated cells in the R2 region.

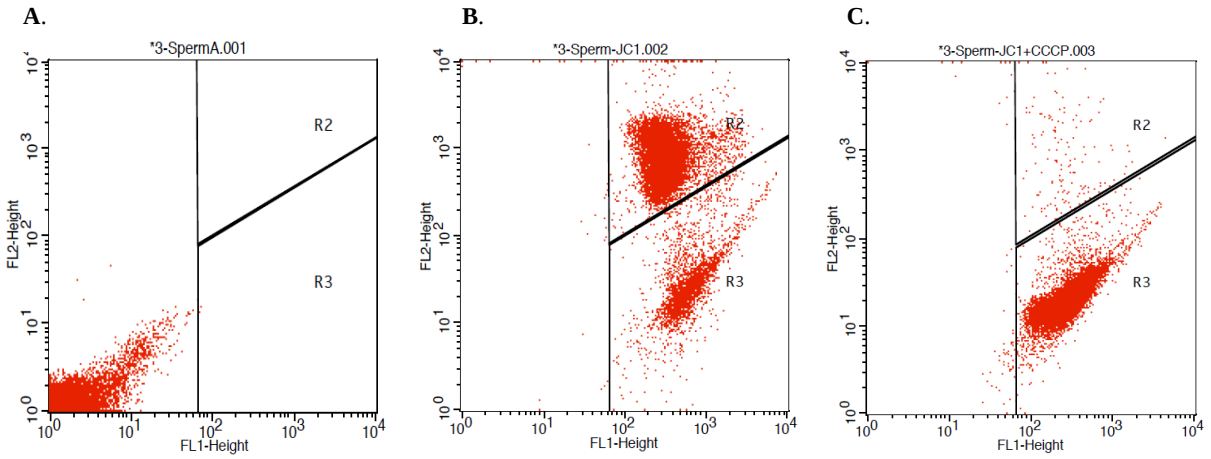

Figure 9. Flow cytometry records for JC-1 evaluation: Dot-plot chart of sperm cells only (A), incubated with JC-1 (B), and incubated with JC-1 and CCCP (C). R2 region entails the cells with high MMP and R3 region entails the cells low MMP

2.7.2. Superoxide production assay

Mitochondrial superoxide production was evaluated using MitoSOX red, a probe containing triphenylphosphonium ion (TPP^+) allowing for mitochondria specificity. Mitochondrial membrane is permeable to this probe, allowing it to be oxidize by the superoxide anion, emitting red fluorescence (66, 269, 276). High intensity of fluorescence correlates with high levels of superoxide produced in the mitochondria. MitoSOX fluorescence was detected on the FL-2 channel.

For this protocol 2.5 million/mL spermatozoa in PBS supplemented medium (composition in the 2.3. subchapter) without BSA and $NaHCO_3$ were used. Three conditions were prepared for each sample: A) sperm sample only, as negative control; B) sperm sample with 3 µM MitoSOX red® [Invitrogen (#M36008), Thermo Fisher); and C) sperm sample with 3 µM MitoSOX red and 75 µM antimycin A, positive control [Sigma-Aldrich (#A8674), Merck] (269). Samples were incubated with MitoSOX red for 15 min at 37ºC, then centrifuged (500*g*, 5 min) and resuspended in fresh media without BSA and $NaHCO_3$. In the cytometer, 15000 events were registered.

Similarly to JC-1, the controls allowed to define interest regions in the flow cytometry histogram. In fact, as Antimycin A inhibits complex III of the ETC, it will increase production of superoxide, and for this reason it was used to define the region of superoxide producing cells in the histogram (M2; (66, 276). On the other hand, the negative control condition was used to define the M1 region, corresponding to non-labelled cells [Figure 10]. Results were further expressed as the fluorescence intensity in the M2 region.

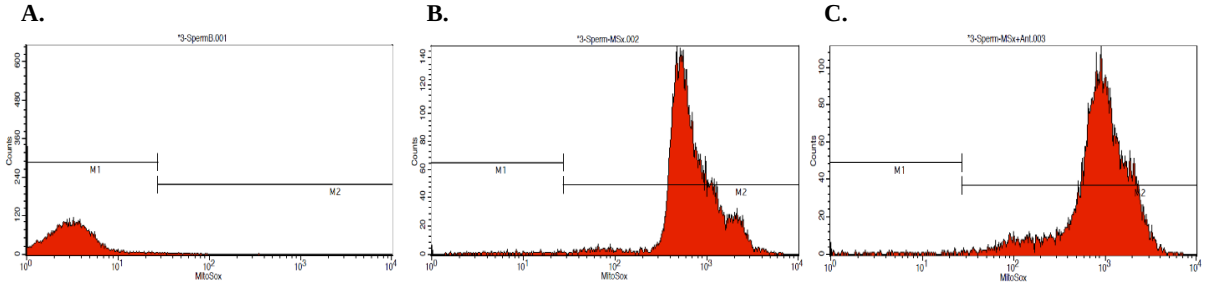

Figure 10. Flow cytometry records for MitoSox red evaluation: Histogram chart of sperm cells only (A), incubated with MitoSOX red (B), and with MitoSOX red and antimycin A (C). M1 region comprises cells without superoxide production and and M2 region comprises cells with of superoxide production.

2.8. Socio-demographic and life-style data

Complete and detailed information on the patients' habits, general health and biometric information are essential to study and better understand other possible factors that might be entailed in the infertility aetiology of ID and UMI patients, as mentioned previously. Accordingly, all patients enrolled in the study filled a survey where information like lifestyle data and health-related issues was collected.

2.9. Fertility outcomes

In this work, we accessed the fertility outcomes of 535 male patients that performed ART treatments and whose diagnose was complete. Fertility results from both IVF and ICSI were used to calculate fertility rate and embryo development rate. The first corresponds to the number of oocytes with two pronucleus per number of oocytes inseminated or injected, and the second to the number of embryos per number of oocytes with two pronucleus. These rates were expressed in percentage (272).

2.10. Psychological evaluation for depression and anxiety symptoms

Infertility can have a colossal impact on patients' mental health, especially when going through repetitive ART. Having this in mind, and intending to further explore this aspect, patients were asked to fill a self-response questionnaire "Hospital Anxiety and Depression scale"(HADS) in the Portuguese version, designed to help in the identification of emotional components of physical illness as a screening tool for anxiety and depression states (277, 278). HADS is composed of 14 questions, 7 measuring anxiety and 7 measuring depression, and to each question a score between 0 and 3 was given (277, 278). A total score of 7 or less is considered "normal", a score between 8 and 10 is considered "mild", a score between 11 and 14 is considered a "moderate", and between 15 and 21 "severe" (278).

2.11. Statistical analysis

For the statistical analysis, Statistical Package for the Social Sciences (SPSS) software version 20.0 for windows (SPSS inc, Chicago, IL, USA) was used. Normality tests, either Shapiro-Wilk (variables with $n<30$) or Kolmorogov-Smirnov test (variables with $n>30$), were performed for every variable, followed by homogeneity of variances assessment with Levene's test. For variables with normal distribution statistical relevance was assessed through one-way ANOVA and appropriated post hoc tests. For variables that did not fit a normal distribution statistical relevance was assessed through Kruskal-Wallis and Mann-Whitney tests. Spearman or Pearson correlations were performed to assess the strength of the relationship between different variables. Differences were considered statistically significant if $p \leq 0.05$. Results were expressed as mean±standard error of the mean (SEM) and the number of experiments indicated.

2.12. Sequential window acquisition of all theoretical mass spectra

Lastly, we performed a proteomic characterization of spermatozoa of the three experimental groups, aiming to find differential signatures. For this purpose, we performed Short GeLC-SWATH-MS, a protocol where protein extracts are digested in-gel, quantified, and the resulting peptides analysed by liquid chromatography mass spectra (LC-MS) using the SWATH-MS acquisition mode (241).

For this analysis, we have used a protocol in which 5 million/mL spermatozoa were centrifuged (500*g*, 5 min) followed by resuspension in 30 to 50 µL of sample buffer with recombinant green fluorescent protein fused with maltose binding protein (MBP-GFP) in accordance with the number of spermatozoa. The sample buffer is composed of 0.25 M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 1 mg bromophenol blue, 200 mM dithiothreitol (DTT) and 10 µg/mL recombinant MBP-GFP. Then, the samples were sonicated at 50% output for 1 min with 5s bursts at 3s intervals, using a Hielscher sonifier (UP100H; Hielsher ultrasonics), and boiled for 5 min at 95ºC. After, samples were centrifuged (20000*g*, 15 min) and the supernatants transferred to new tubes (241). Protein extracts were stored at -80ºC. For protein identification/library generation, 3 pooled samples were created: one from control samples, a second one from ID samples, and a third one from UMI samples. Additionally, to test the reproducibility of the method, a pool containing part of all samples studied was created to be used as technical replicate.

For in-gel digestion, samples were incubated at 95ºC for 5 min and 2 µL of acrylamide were added to each tube [Figure 11]. Samples were loaded in a SDS polyacrylamide gel (4–20% TGX Stain-Free Gel; Bio-Rad) and ran at 110 V for 20 min. The gels were immersed in fixation solution (10% ortho-phosphoric acid, 20% methanol and 13 mM $(NH_4)_2SO_4$ in double distilled water, ddwater), with Coomassie Brilliant blue (#20279, Thermofisher) and were left on a rocking platform for 1 h. To remove the staining background, the gels were placed in a new container with ddwater and were left on a rocking platform, the water was replaced with fresh ddwater a couple of times, and the gels were left in the platform overnight. The gels were cut to isolate the different samples/lanes and each lane was cut in 3 fractions of similar size, and further cut in small pieces. Gels pieces from each fraction were transferred to different wells of a 96 deep well plate with 600 µL of ddwater per well. Then, ddwater was replaced by destaining solution (50 mM ammonium bicarbonate and 30% acetonitrile) and plates were agitated (850 rpm, 15 min, RT). After this step was repeated, the destaining solution was replaced by ddwater, the plates were agitated (850 rpm, 10 min, RT). After, the water was removed, and gel pieces were dehydrated on Concentrador Plus.

Next, the same volume of TPKC-treated trypsin solution (0.01 g/L trypsin dissolved in 10 mM ammonium bicarbonate solution) was added to each well containing the dried gel pieces and plates were left at 4ºC for 15 min to dehydrate the gels. Then, we add 10 mM ammonium bicarbonate solution and plates were incubated overnight in the dark at RT. In the next morning, the excess solutions from the gel pieces were transferred to low binding microcentrifuge tubes. For the peptide extraction, 100 µL of 3 acetonitrile solutions with 1% formic acid (30%, 50% and 98% acetonitrile) were added to each well, sequentially. In between solutions, the plates were agitated in the thermomixer (1050 rpm, 15 min, RT) and the peptide solutions were recovered to the respective tubes together with the previously recovered peptide solution. At this stage, the three fraction of each individual samples were combined into a single

tube, while the three fraction of the pooled samples were maintained in separate tubes. Finally, the tubes with the extracted peptides were dried by rotary evaporation under vacuum.

Samples were analyzed on a NanoLC™ 425 System (Eksigent®, Framingham, MA, USA) couple to a TripleTOF™ 6600 System (Sciex®, Framingham, MA, USA) using information-dependent acquisition (IDA) for each fraction of the pooled samples for protein identification and SWATH-MS acquisition of each individual sample for protein quantification. Samples were loaded onto a YMC-Triart C18 Capillary Guard Column 1/32" (12 nm, S-3µm, 5 x 0.5 mm) (YMC, Kyoto, Japan) at 5 µL/min of 5% of mobile phase B during 8 min and the peptides separation was carried out by micro-flow liquid chromatography using a YMC-Triart C18 Capillary Column (12 nm, S-3µm, 150 × 0.3 mm) (YMC) at 50°C. The flow rate was set to 5 µL/min and mobile phases A and B were 5% DMSO plus 0.1% formic acid in water and 5% DMSO plus 0.1% formic acid in acetonitrile, respectively. The LC program was performed as followed: 5–30% of B (0–50 min), 30–98% of B (50–52 min), 98% of B (52-54 min), 98-95% of B (54–56 min), 95% of B (56–65 min). Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, ABSciex®, Framingham, MA, USA) with a 25 µm internal diameter hybrid PEEKsil/stainless steel emitter (ABSciex®). The ionization source was operated in the positive mode set to an ion spray voltage of 5 500 V, 25 psi for nebulizer gas 1 (GS1), 10 psi for nebulizer gas 2 (GS2), 25 psi for the curtain gas (CUR), and source temperature (TEM) at 100°C.

For IDA experiments, the mass spectrometer was set to scanning full spectra (m/z 350-2250) for 250 ms, followed by up to 100 MS/MS scans (m/z 100–1500) per cycle, in order to maintain a cycle time of 3.295s. The accumulation time of each MS/MS scan was adjusted in accordance with the precursor intensity (minimum of 30 ms for precursor above the intensity threshold of 2000). Candidate ions with a charge state between +1 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 15 seconds (mass spectrometer operated by Analyst® TF 1.8.1, Sciex®). Rolling collision was used with a collision energy spread of 5.

For SWATH-MS experiments, the mass spectrometer was operated in a looped product ion mode (279) with the same chromatographic conditions used in the IDA run described above. A set of 168 windows [Annex I] of variable width (containing a m/z of 1 for the window overlap) was constructed covering the precursor mass range of m/z 350-2250. A 50 ms survey scan (m/z 350-1250) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from the precursors ranging from m/z 350 to 1250 for m/z 100–1500 for 19 ms resulting in a cycle time of 3.291 s The collision energy (CE) applied to each m/z window was determined considering the appropriate CE for a +2 ion centred upon this window and the collision energy spread was also adapted to each m/z window.

By combining all files from the IDA experiments, a specific library containing the precursor masses and fragment ions was created and used for subsequent SWATH processing. Libraries were obtained using ProteinPilot™ software (v5.0.1, ABSciex®), in accordance with the following parameters: i) search against a database from SwissProt composed by Homo Sapiens (downloaded in August 2021) and MBP-GFP (IS) protein sequences; ii) acrylamide alkylated cysteines as fixed modification; iii) trypsin as digestion type. To assess the quality of the identifications, an independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was performed. Positive identifications were considered when identified proteins and peptides reached a 5% local FDR (280, 281).

Data processing was performed using SWATH-TM processing plug-in for PeakView™ (v2.0.01, ABSciex®) (282). After retention time adjustment using peptides from the internal standard (MBP-GFP) (283), up to 15 peptides, with up to 5 fragments each, were chosen per protein, and extracted-ion chromatograms (XIC) were attempted for all proteins in library file that were identified from ProteinPilot™ search. Only proteins with at least one confidence peptide (FDR<0.01) in no less than four replicates condition and with at least three transitions were considered. Peak areas of the target fragment ions (transitions) of the retained peptides were extracted across the experiments using an XIC window of 6 minutes with 100 ppm XIC width. The proteins' levels were estimated by summing all the transitions from all the peptides for a given protein that met the criteria described above (an adaptation of Collins *et al.* 2013 (284)) and normalized to the levels of two spermatozoid specific proteins (AKAP4+AKAP3) (283).

The statistical analysis regarding the proteomic data was done using R. A Kruskal–Wallis test was performed to identify the proteins differentially regulated between all the comparisons followed by the Dunn's test of Multiple Comparisons, with Benjamini–Hochberg p-value adjustment, to determine in which comparisons statistical differences were observed. All the analyses were performed using the normalized protein levels and a p-value of 0.05 were defined as cut-off.

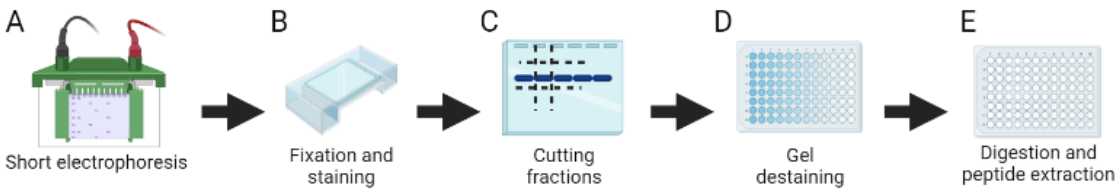

Figure 11 Simplified workflow of short-GeLC. A: Protocol starts by running the protein extracts in a short electrophoresis (20 min). B: Gel is fixated and stained with Coomassie blue. C: Gels were cut separating the different lanes and each lane in 3 fractions and placed in different well of a 96-well plate. D: Gel pieces were destained and dehydrated. E: Protein extracts were digested with trypsin and resulting peptides were collected.

2.13. Proteomic analysis

From all the altered proteins, the ones with only 1 peptide quantified and a coefficient of variation (CV) among technical replicates higher than 20% were excluded. Protein Analysis Through Evolutionary Relationships (PANTHER) was used to analyse the different biological processes present in proteins up- and down-regulated between the patient's groups (285). The Database for Annotation. Visualization and Integrated Discovery (DAVID) was used to perform cluster analysis on differentially expressed proteins between the patient's groups with a medium classification stringency (286). Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to find relevant interactions between proteins, where medium confidence (0.4) was used (287). MetaboAnalyst 5.0 was used to perform random forest to find proteins and sperm parameters capable of accurately classify samples into CTRL, ID and UMI (288).

3. Results and discussion

3.1. Sperm quality parameters

In this study several sperm parameters were evaluated to fully characterize ID and UMI patients, aiming to find parameters that could differentiate them. Below, results regarding these parameters are presented.

3.1.1. Sperm's concentration, motility and viability

Sperm concentration, viability and total motility were observed to be significantly decreased in ID patients comparing both to the CTRL ($p \leq 0.001$) and UMI patient's group ($p \leq 0.001$) [Figure 12-14]. Concentration and motility were expected to be significantly lower in ID patients, since these are typical features of ID patients, whose (idiopathic) seminal quality can vary from oligo to astheno, terato or even present the 3 abnormalities (oligoasthenoteratozoospermic), contrarily to CTRL or UMI patients, with a normal seminal analysis. Our results are in accordance with previous reports that also described that ID patients had affected sperm count and motility (197), while no alterations were reported in terms of conventional seminal analysis in UMI patients (227, 228). Interestingly, in one of the studies on UMI patients, a decrease in motility and morphology were reported in these patients, although still in the reference limits of WHO guidelines (229).

On the other hand, the sperm viability in UMI patients was comparable to CTRL, as observed in previous study (227, 228). Moreover, in all the 3 parameters, the UMI patients presented a very similar profile to that of the control group, also mirrored in statistical differences when compared to the ID group [Figure 14].

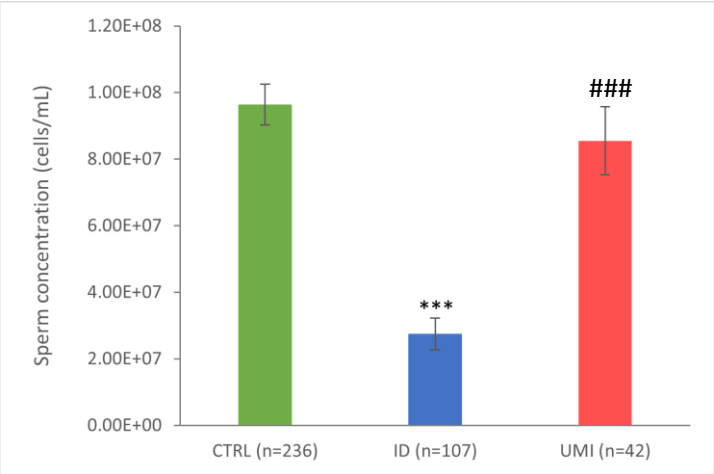

Figure 12. Sperm concentration on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. *** - $p \leq 0.001$ in comparison to CTRL; ### - $p \leq 0.001$ in comparison to ID.

In accordance with our results, in two previous studies, fertile patients (CTRL) and normozoospermic patients from the general population (GPG- with unproven infertility), show no significant differences in these sperm parameters comparing to UMI patients. However, in these study UMI patients are referred indiscriminately, as "idiopathic infertility group" and " unexplained infertility patients", being this one of the examples in which the terms used to classify these patients vary, even in the same study, making it difficult to interpret the data (228). Moreover, in our cohort, the significant difference observed in terms of viability between ID patients and the other groups, together with alterations in motility, might further contribute to explain these patients' inability to conceive. To our knowledge no other studies were found comparing viability of ID patients, classified under our precise criteria, to CTRL or UMI patients.

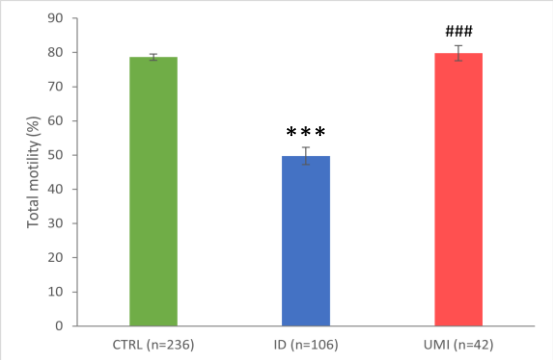

Figure 13. Percentage of motile cells on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. *** - p≤0.001 in comparison to CTRL; ### - p≤0.001 in comparison to ID.

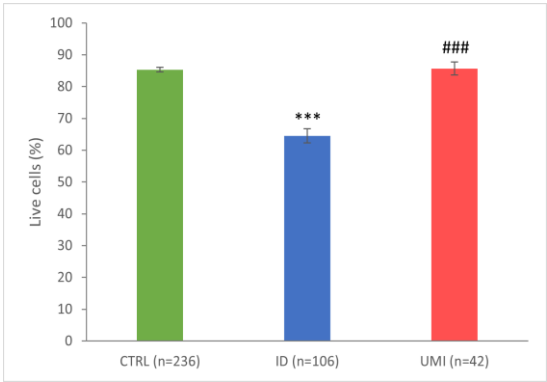

Figure 14. Percentage of live cells on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. *** - p≤0.001 in comparison to CTRL; ### - p≤0.001 in comparison to ID.

3.1.2. Sperm's morphology and chromatin status

A significantly decreased percentage of cells with normal morphology was observed in ID patients, when compared to CTRL (p≤0.001) and UMI (p≤0.001) patients [Figure 15]. This observation was not unexpected since the altered morphology is a recurrent trait in these patients' sperm.

Regarding the chromatin integrity, this was also significantly decreased in ID patients compared to CTRL (p≤0.05), although no significant differences were observed between ID and UMI or CTRL and UMI patients (p>0.05) [Figure 16]. As previously mentioned, there is great controversy on what the best method for chromatin status evaluation is, mainly because the methods used currently are various, with different results and interpretations, a fact that undeniably hampers the implementation of a universal test in all andrology labs. Host *et al.* studied infertile men with oligozoospermia and UMI, and found oligozoospermic men to have significantly increased DNA strand breaks than fertile and UMI men, which could corroborate our results (289). However, it must be taken into account that these

oligozoospermic patients were not classified as idiopathic and the methods used to access it were different from ours (an easy and quick, although indirect method to access Chromatin status). In addition, UMI patients presented increased DNA strand breaks formation compared to CTRL, contrarily to our results. Two other studies, have described differences among UMI and controls, regarding DNA fragmentation, with the former group presenting higher DNA fragmentation (227), yet again using a different methodology from ours, the SCSA and SCD, respectively (227, 229) and therefore suggesting that DNA fragmentation might have a role in these patients (in)fertility. Surprisingly, using basically the same clinical setting, one of these groups, Mayorga-Torres and collaborators, have latter described that UMI patients do not differ in terms of DNA fragmentation from the control group, now in accordance with our results (228). Yet the authors didn't present any justification for the different results among studies, although, the number of patients were lower and the female partner evaluation was not described in their initial study, facts that might interfere with the results (227). Moreover, the two studies by Mayorga-Torres and coworkers, using UMI patients, used both idiopathic and unexplained terminologies to classify them. Worth mentioning, our cohort entails more patients than any other of the mentioned studies. Although we could not find studies on DNA quality on ID patients comparing to proper defined control groups, several antioxidant treatments were described to decreased the DFI in these patients (98, 217). pointing to alterations in this parameter in ID patients.

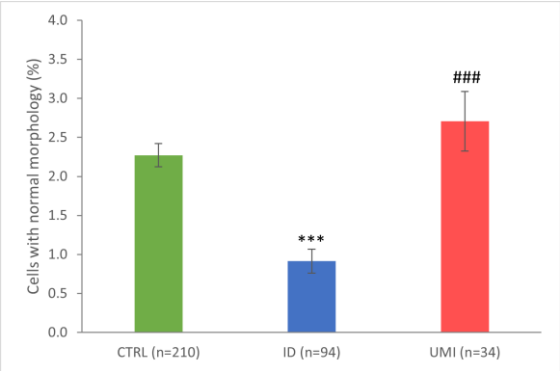

Figure 16. Percentage of normal cells on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. *** - $p \leq 0.001$ in comparison to CTRL; ### - $p \leq 0.001$ in comparison to ID

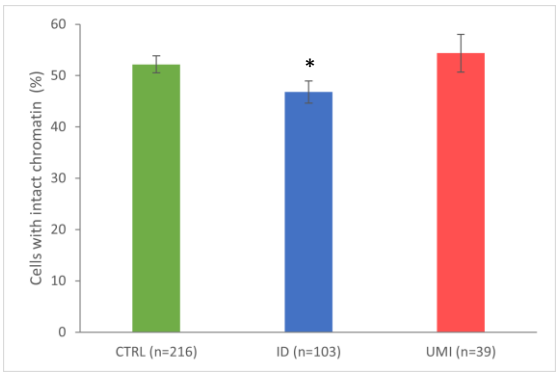

Figure 15. Percentage of cells with intact chromatin on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. * - $p \leq 0.05$ in comparison to CTRL

3.1.3. Capacitation status

The percentage of capacitated cells was significantly decreased in ID patients comparing to CTRL patients ($p \leq 0.001$), although no significant difference was observed when compared to the UMI patients [Figure 17]. Capacitation is an essential process for spermatozoa to fertilize the oocyte (67, 68) and recently, a study on the ability of capacitation status to predict pregnancy success, showed that patients with higher percentage of capacitated sperm had significantly increased IUI success comparing to patients with low percentage of capacitated sperm (290). Hence, our observations point out to a possible

aspect that might be altered and justify the infertile state of the ID patients, although the statistical significance was not reached when comparing ID and UMI patients, probably due the small size of the later. The lack of differences in this process between UMI and CTRL patients, was previous reported in a study where hyperactivation, a hall mark of the capacitation process, was evaluated. Nevertheless the authors reported that the despite the absence of differences in terms of hyperactivation rate, among the groups, 20% the men with unexplained group didn't present hyperactivation, compared to only 4% in the control group (291). Nevertheless, to our knowledge, no further studies on this topic were developed in ID or UMI patients with the exception of proteomic studies that have identified differentially expressed proteins in UMI patients whose functions might interfere with capacitation, acrosome reaction and gametes interaction (232).

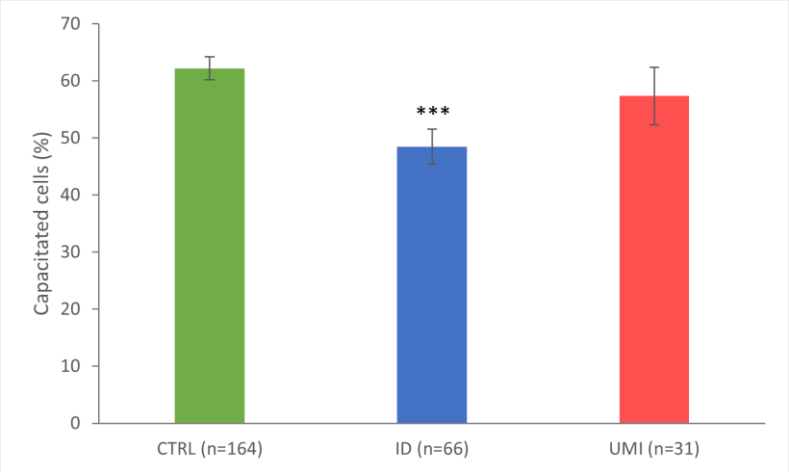

Figure 17. Percentage of capacitated cells on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. *** - $p \leq 0.001$ in comparison to CTRL

3.1.4. Acrosome integrity

The percentage of cells with intact acrosome was observed to be significantly higher in UMI patients comparing to ID patients ($p \leq 0.05$) [Figure 18]. This functional parameter, not commonly evaluated in clinical practice, provides important information on the fertilizing capacity of the sperm samples, since AR before near contact with *zona pellucida* can impair fertility.

In fact, according to our results this seems to be an aspect that differentiates ID from UMI patients. To our knowledge this constitute an important discovery since this process has not been evaluated in a similar setting to ours, involving the three patient groups. Yet, in a previous study, acrosome status was assessed in CTRL and UMI patients sperm, with no significant differences reported (291). Interestingly, a study focused on the role of X farnesoid receptor on sperm cells, a bile acid sensor that is activated upon binding of endogenous bile acids, found that this receptor is expressed in the sperm midpiece and that increasing concentration of bile acids negatively affect several sperm parameters, including capacitation and acrosome reaction, affecting sperm fertilizing capacity, leading the authors to suggest

that bile acids levels could be involved in some cases of idiopathic male infertility. Nevertheless, this study was performed in normozoospermic samples from healthy individuals (292).

More recently, interesting data was also reported by Lu and colleagues in a work aiming to explore if there is any relationship among CATSPER gene abnormalities (main calcium channel mediating Ca^{2+} efflux in sperm) and male unexplained infertility (that they called idiopathic with normal semen parameters). What they found was a novel copy number variation (CNV) in CATSPER2, that dramatically reduces the expression of CATSPER2 proteins in sperm, that despite not affecting semen parameters, had an effect in terms of capacitation-related processes and acrosome reaction, compromising fertilizing capacity, that the author claim that might explain the idiopathic infertility state (293).

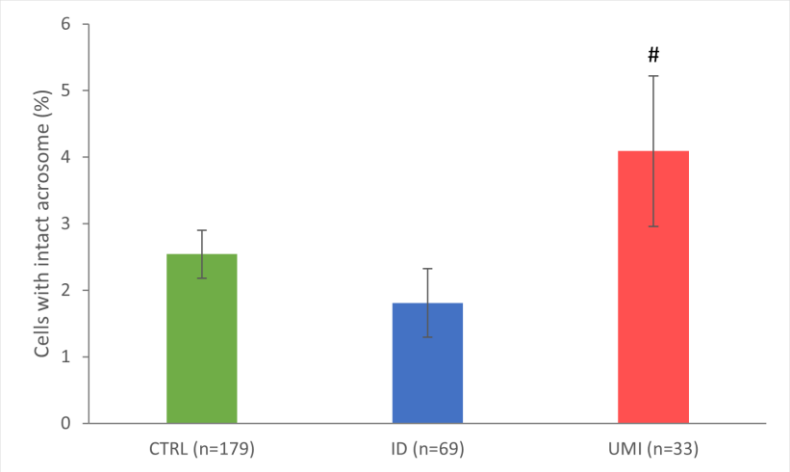

Figure 18. Percentage of cells with intact acrosome on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM. # - $p \leq 0.05$ in comparison to ID

3.1.5. Sperm mitochondrial status

Due to the strong association among sperm and mitochondrial (dys)function (66), the evaluation of mitochondrial related parameters has gained supporters in the last years as a trustworthy and easy readout of sperm quality. Yet, in this study, we have observed that the percentage of cells with high MMP and with high ROS levels were not significantly different between the experimental groups ($p>0.05$), contrarily to what we would expect. Worth mentioning, ID patients showed higher ROS levels and lower MMP [Figures 19 and 20], in agreement with our previous results. However, this observation didn't reach statistical significance, probably due to the low number of samples involved in these assays, an issue that we aim to improve in future studies. In the existing literature, these parameters were reported to be significantly altered in ID patients, especially in oligoasthenozoospermic men comparing to fertile controls (197-199). Concerning UMI patients, ROS production was unanimously seen increased in the literature, when compared to fertile controls, while MMP is frequently not significantly

different between CTRL and UMI patients (227-229), the latter in accordance with our results, although in this case our cohort was smaller. Regarding the ROS levels, our different results (with no alterations reported, only a trend), might also be due to the fact that most of the cited papers evaluated the levels of several ROS, while, in our study, we only have evaluated, mitochondrial superoxide. Nevertheless, it should not be neglected that, despite different cohort sizes, the study designs (number/type of study groups) as well as the categorization of patients and the used inclusion and exclusion criteria vary a lot among studies, a fact that might contribute to the variety of results. Still, to clarify the oxidative status of infertile patients, both parameters should be further explored with a higher number of samples.

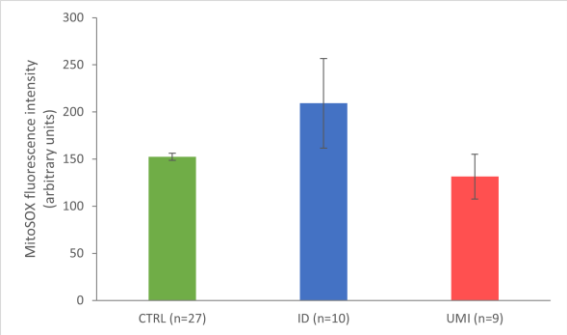

Figure 20. MitoSOX fluorescence intensity (arbitrary units) on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM

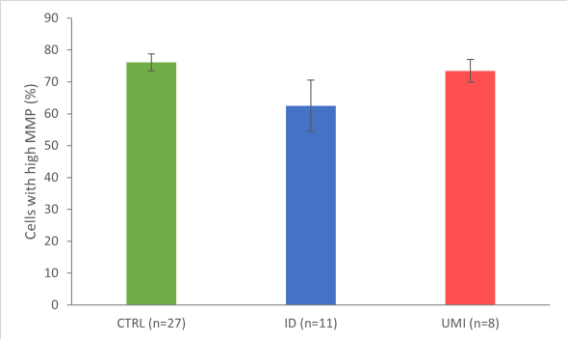

Figure 19. Percentage of cells with high MMP on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM

3.2. Fertility outcomes

Fertility outcomes constitute an important measure on the gametes quality and fertilization success being therefore expectable that the infertile groups present alterations in this regard. Nevertheless, no significant differences were detected between the experimental groups in all the calculated rates: fertilization, embryonic development, and embryonic transfer rates ($p>0.05$) [Table 1]. This can be explained by the low number of patients where this information was retrieved, especially on the infertile groups, as not all the patients that came to our clinic were submitted to ART. It is also important to highlight that these rates depend both on the male and the female partner, hence it can be more difficult to observe significant differences between the groups, that might be due only to the male side of the equation.

Table 1. Fertilization, embryonic development, and transfer rates on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM

Rates	CTRL	ID	UMI	*P* value
Fertilization rate (n=94)	0.60±0.05	0.52±0.07	0.62±0.09	0.646
Embryonic development rate (n=85)	0.76±0.05	0.92±0.03	0.83±0.06	0.210
Embryonic transfer rate (n=78)	0.23±0.04	0.23±0.06	0.31±0.11	0.606

3.3. Cohort characterization

Besides the general characterization of our experimental groups (age, BMI, diseases, lifestyle), reproductive health aspects, exposure to detrimental agents (at the scope of a professional activity) and anxiety and depression symptoms were evaluated by proper surveys, in order to have the more accurate and complete characterization possible of the experimental groups.

3.3.1. General characterization of the cohort and lifestyle habits

Age, BMI, regular physical exercise, eating habits, the existence of other diseases and allergies or chirurgical interventions, were not significantly different between the patient groups, not contributing to explain any of the differences observed in the sperm functional parameters ($p>0.05$) [Table 2]. Regarding tobacco and alcohol consumption, the fact that these factors can contribute to infertility, being negatively correlated with sperm quality, is well documented (176-178, 181, 182, 294). In our study, however, no significant differences were observed regarding these addictions' frequencies among the three individual's groups ($p>0.05$) [Table 3], leading us to conclude that these factors are not determinant for the fertility state of our patient's groups.

Table 2. Study cohort characterization; age, body mass index (BMI) and percentage of individuals performing regular physical exercise, eating healthy, presenting chronic diseases, hypercholesterolemia, COVID-19, allergies, mumps, other diseases or submitted to surgery in the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). All parameters are expressed in percentage, except age and BMI which are presented as mean±SEM. COVID 19- Corona Virus Disease; * - $p\leq0.05$ and *** - $p\leq0.001$ in comparison to CTRL; ## - $p\leq0.01$ in comparison with ID.

	CTRL	ID	UMI	*P* value
Age (n=385)	35.8±0.4	36.9±0.6	36.0±0.9	0.646
BMI (n=382)	26.4±0.2	27.1±0.4	27.4±0.7	0.166
Regular physical exercise (n=384)	56	52	60	0.658
Healthy eating (n=384)	84	83	79	0.651
Chronic diseases (n=381)	12	15	12	0.753
Hypercholesterol (n=375)	38	46	46	0.315
COVID-19 (n=128)	8	3	0	0.376
Allergies (n=385)	27	26	12*	0.109
Mumps (n=372)	23	17	27	0.371
Other diseases (n=384)	8	7	0	0.158
Surgery (n=381)	12	33***	10##	0.000

Table 3. Consumption of alcohol and tobacco among the three study groups (CTRL – healthy controls, ID - idiopathic male infertility, UMI - unexplained male infertility). Consumption of alcohol is divided in yes (daily consumption), no and occasional (oc.). Tobacco consumption is divided in yes (currently consuming), no and ex-smoker (for at least 3 months). Results are expressed in percentage of patients in each of the three groups.

Consumption	CTRL			ID			UMI			*P* value
	yes	**No**	**oc.**	**yes**	**no**	**oc.**	**yes**	**no**	**oc.**	
Alcohol (n=385)	15	18	67	15	19	66	19	12	69	0.766
	Yes	**No**	**ex-smoker**	**Yes**	**No**	**ex-smoker**	**Yes**	**No**	**ex-smoker**	
Tobacco (n=382)	29	51	20	31	16	53	29	52	19	0.874

3.3.2. Reproductive health aspects

Most of the reproductive health aspects assessed by survey (STDs, urogenital infections and pre-existing varicocele, urogenital anomalies, testicular torsion, inguinal hernia, and hormonal therapy) were not significantly different among the three groups ($p>0.05$), with the exception of urogenital infections and pre-existing varicocele which prevalence was significantly increased in ID patients' group ($p\leq0.001$), being null in the UMI patients' group [Table 4]. Although the later conditions were accessed in the same survey question, an issue that we should definitely improve in future studies, after a more detailed analysis, it has been concluded that these results represent mostly cases of pre-existing varicocele (33/106 in ID vs 6/236 in CTRL and 0/42 in UMI). Moreover, the fact that ID patients were also more frequently submitted to surgeries ($p\leq0.001$), might be related with the varicocele treatment. In addition, urogenital infections and pre-existing varicocele were significantly and negatively correlated with sperm concentration, viability, total motility and normal morphology ($p\leq0.001$) as well as chromatin damage ($p\leq0.050$), in accordance with previous studies (126, 295, 296). Furthermore, several studies reported and association of varicocele with oxidative stress, observed by increased DNA fragmentation and ROS levels (127, 296, 297), results in line with the observations made in the ID patients group in the present study (although our results didn't reach statistical significance in terms of ROS levels) (Figure 16 and 19). However, it is still to be determined for how long this oxidative environment associated with varicocele persists after the surgical treatment and if this will be relevant in the context of our work. Nevertheless, these results point out to a probable role of a pre-existing varicocele in the ID patients' fertility state. Interestingly, these patients were submitted to varicocele embolization (otherwise were excluded as explained in the exclusion criteria), that was not always 100% effective in terms of normalization of their seminal analysis, highlighting that also other factors should be involved in their infertile state.

Finally, infertile patients (ID and UMI) had more frequently undergone hormonal therapy than the individuals from the CTRL group (0/236 in CTRL vs 3/106 in ID vs 1/42 in UMI) ($p \leq 0.05$). Although, this treatment can be prescribed in an attempt to improve fertility, mainly in those cases in which an ART is being considered, this approach is not consensual, being not recommended by EAU guidelines (234).

Table 4. Percentage of patients presenting conditions known to affect fertility in the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). All parameters are expressed in percentage. STD - sexually transmitted diseases; * - $p \leq 0.05$ and *** - $p \leq 0.001$ in comparison to CTRL; ### - $p \leq 0.001$ in comparison with ID.

	CTRL	ID	UMI	*P* value
STD (n=384)	2	2	5	0.433
Urogenital infections and varicocele (n=384)	7	35***	0###	0.000
Urogenital anomalies (n=385)	1	3	0	0.248
Testicular torsion (n=381)	5	4	0	0.352
Inguinal hernia (n=382)	7	12	7	0.236
Hormonal therapy (n=384)	0	3*	2*	0.039

3.3.3. Work-related exposures to detrimental agents

Exposure, in a professional context, to different agents for more than 3 months, such as paints, solvents, pesticides, metals, high temperatures, low temperatures, radiation, and dust were not observed to be significantly different between the three studied groups ($p > 0.05$) [Table 5]. Although occupational exposures were previously associated with decreased sperm quality (143, 144, 146, 159), no studies were found entailing the three patient's groups in which the present work is focused on. Literature on the environmental exposure to detrimental agents have also reported negative effects on sperm quality (143, 145, 148). The available information, together with the obtained results in the present study, thus suggests that these exposures, although possibly impacting fertility, do not fully explain ID or UMI aetiology.

Table 5. Exposure to paints, solvents, pesticides, metals, high temperatures, low temperatures, radiation and dust among the three study groups (CTRL – healthy controls, ID - idiopathic male infertility, UMI - unexplained male infertility). Results are expressed in percentage of patients exposed in each of the three groups.

Exposures	CTRL	ID	UMI	*P* value
Paints (n=378)	29	21	15	0.089
Solvents (n=373)	24	19	13	0.187
Pesticides (n=373)	4	7	5	0.435
Metals (n=375)	33	25	33	0.270
High temperature (n=375)	25	26	15	0.337
Low temperature (n=371)	14	10	10	0.438

Radiation (n=373)	3	8	5	0.190
Dust (n=380)	40	36	34	0.595

3.3.4. Anxiety and depression

Anxiety and depression levels, evaluated by the HADS questionnaire, were not significantly different among the three study groups ($p>0.05$) although it seems that UMI patients tend to present higher anxiety and depression HADS scores, compared to the other groups [Figure 21 and 22], yet still in the normal range, according to HADS manual (277). Despite these results, diagnosed depression, when assessed by survey under the question "Do you had or have diagnosed depression?", was significantly increased in UMI patients comparing to CTRL and ID ($p\leq0.01$) [Figure 23]. However, the relevance of this observation seems to be dependent on the collection of more data and maybe a rephrasing of the question, since in the way that is presented, we might be collecting information from a long time ago, not relevant for the study purpose. Nevertheless, the existent literature points out to a detrimental effect of this psychological alteration on male fertility, although which is the cause or consequence is still to be determined (188).

Regarding anxiety, as previously mentioned, a recent meta-analysis has described some studies in which an association between decreased sperm motility, concentration and DNA fragmentation and pre-treatment ART anxiety was reported (188), although in one of the studies these relationship was also observed in the control group (298). However, these studies were not specifically focused on our patients' groups and used different tools to assess depression and anxiety. Nevertheless, worth mentioning, one of those studies was conducted in a Portuguese Reproductive Unit and included nearly 17 males with ID (among 112). However, the study didn't particularly focused on those, but instead on the significance of these psychological states before the first ART or on a repetitive one (299). Transposing what has been discussed to our results, although in our study ID patients have significantly lower motility than CTRL and UMI [Figure 13], this difference was not justified by HADS scores or frequency of diagnosed depression. It is also important to point out that high HADS scores were reported to be correlated with hormonal deregulation, namely lower testosterone levels, together with lower sperm count and motility (118), which is, by principle, not present in ID and UMI patients, by inclusion/exclusion criteria. To our knowledge, no studies were found on the incidence and/or possible role of anxiety and depression in UMI patients, warranting further works on this topic.

A topic that should also be considered when talking about anxiety and depression is the medication used to treat these conditions (often the antidepressants are also used to treat anxiety disorders) and its possible effect on seminal quality. On this regard, it has been already described that antidepressant use, mainly based on serotonin reuptake inhibitors, such as citaloram, escitalopram, fluoxetine, sertraline and

paroxetin reduced sperm quality, including at the sperm DNA level, in healthy/fertile men (300, 301), being that the described effects seems to depend on the duration of the treatment and are apparently reversible after medication cessation (300, 301). Mazzilli *et al.* studied the effect of different psychotropic medications, including the antidepressant fluoxetine, on sperm parameters in men with idiopathic and/or unexplained male infertility, showing that these treatments have a negative impact on seminal quality, being that the plasma concentration of fluoxetine was observed to be correlated with sperm number reduction (302). Still, no studies were found on antidepressant use in UMI or ID patients studied separately and/or under our classification criteria and experimental setting. In the current study, several UMI patients with self-declared diagnosed depression also reported that were taking antidepressants, which could further contribute to these patients' fertility problems. Nonetheless, it is hard to determine if this relation is due to the disease itself or to the medicines taken by this specific group of patients and we consider that our data don't allow us to take definite conclusions on this regard and further studies will be needed to be explore this data.

Overall, although data in the literature on the effect of anxiety, depression and antidepressants use on sperm functionality is still not consistent and derive from studies with different settings, varied designs (e.g, *in vitro, in vivo*) and entailing differing samples sizes, it has been suggested that serotonin reuptake inhibitors might exert the negative effects at the sperm level, through a deregulation of the tryptophan metabolism, that can impact the spermatogenesis, or alternatively by inhibiting the OXPHOS at the mitochondrial level, affecting the ATP synthesis (303). Also important will be to refer that, it is clear from the literature, and also observed in our Reproductive Unit, that while women easily admit to have these kind of psychological problems, with men, this is not always the case, and often they try to hide this kind of problems, hampering an accurate diagnosis. A fact that can also have impact when analysing these type of parameters (193, 304).

In sum, more well-controlled, complete, integrated and multidisciplinary studies are needed to clarify this issue and take more definitive conclusions.

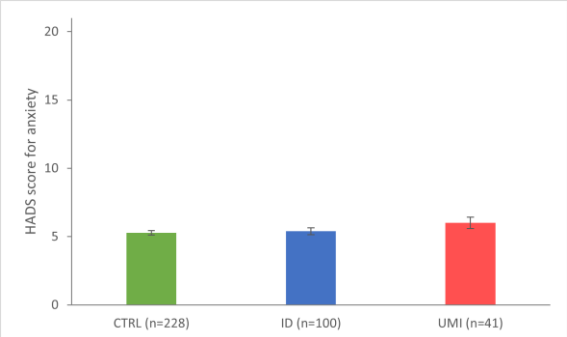

Figure 22. Hospital Anxiety and Depression Score (HADS) for anxiety on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM.

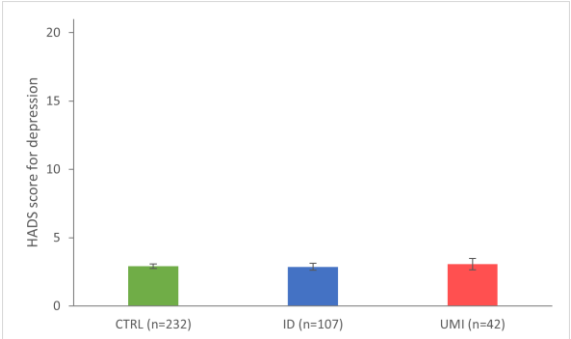

Figure 21. Hospital Anxiety and Depression Score (HADS) for depression on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM

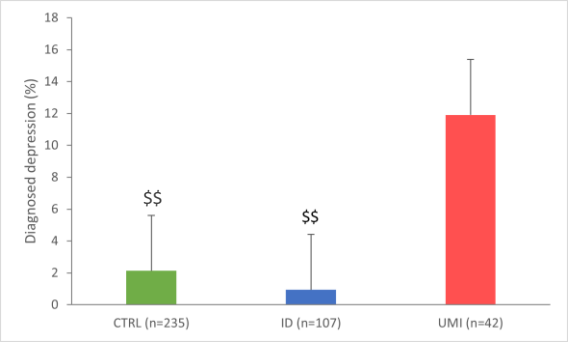

Figure 23. Percentage of individuals with diagnosed depression on the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men). Results are presented as mean±SEM.$$ - $p \leq 0.01$ in comparison with UMI

3.4. Proteomic analysis

The proteomic analysis included 79 samples: 50 CTRL, 19 ID and 10 UMI. In these samples, 295 proteins were found to be differentially expressed ($p<0.05$). Proteins where only 1 peptide was detected and the coefficient of variation on the technical replicates was higher than 20% were excluded, leaving 145 proteins for the following analysis. These proteins were then distributed in three protein sets: (1) differentially expressed proteins between CTRL and ID (CTRLvsID), containing 136 proteins; (2) differentially expressed proteins between CTRL and UMI (CTRLvsUMI), containing 14 proteins, and differentially expressed proteins between ID and UMI (UMIvsID), containing 121 proteins [Figure 24]. This shows that CTRL and UMI proteomes have a similar protein expression profile, having less proteins that differ between them than with the ID proteome, since only 14 proteins could distinguish them, while several proteins could distinguish these individuals from ID. In addition, 7 proteins were altered in all three comparisons, being, therefore, the most probable to distinguish the three study groups. These proteins are annexin 5 (ANXA5), apolipoprotein H (APOH), transthyretin (TTHY), α-crystallin B chain (CRYAB), NADH-cytochrome b5 reductase (NB5R3), destrin and platelet-activation factor acetylhydrolase IB subunit α1 (PA1B3).

3.4.1. Biological processes classification

For classification of proteins based on biological process, using PANTHER, each of the 3 previous protein sets (CTRLvsID, CTRLvsUMI and UMIvsID), were divided in two groups, depending on their type of regulation. CTRLvsID was divided in 111 proteins up-regulated in ID (CTRLvsID-UP) and 25 proteins down-regulated in ID (CTRLvsID-DW), comparing to CTRL. CTRLvsUMI was divided in 8 proteins up-regulated in UMI (CTRLvsUMI-UP) and 6 proteins down-regulated in UMI (CTRLvsUMI-DW), comparing to CTRL. UMIvsID was divided in 110 proteins up-regulated in ID (UMIvsID-UP) and 11 proteins down-regulated in ID, comparing to UMI (UMIvsID-DW).

Most of the proteins in the CTRLvsID set are involved in cellular and metabolic processes. Interestingly, while these categories represent 59% of the biological processes present in the CTRLvsID-UP, they only represent 39% of the biological processes in the CTRLvsID-DW [Figure 25A and 25B]. On the other hand, reproductive processes are 11 times more represented in CTRLvsID-DW than CTRLvsID-UP, meaning that, in general, proteins related to reproductive processes are downregulated in ID patients compared to control individuals.

The most represented biological process in CTRLvsUMI set is also cellular processes, 33% in CTRLvsUMI-DW and 40% in CTRLvsUMI-UP [Figure 25E and 25F]. The major difference is that 22.2% of the biological processes represented in the CTRLvsUMI-DW proteins are reproduction-related processes, which are not at all present on the CTRLvsUMI-UP proteins [Figure 25E and 25F].

Overall, reproduction-related proteins seem to be essentially down-regulated in the infertile patients (ID and UMI) groups, comparing to CTRL, showing that fertility-related pathways could be affected in these patients and hence contributing to explain their infertility, an aspect that deserves further investigation.

When analysing the most represented categories of biological processes in the set of UMIvsID altered proteins, we observe that metabolic processes is the second most represented category in the UMIvsID-UP proteins, and the least represented in the UMIvsID-DW [Figure 25C and 25D]. In addition, reproduction-related processes are 15 times more represented on UMIvsID-DW than on UMIvsID-UP. These results, pointing to a down-regulation of reproduction-related proteins in ID patients, is consistent with the increased dysfunction observed on the different functional parameters evaluated in this study in ID patients, when comparing to UMI.

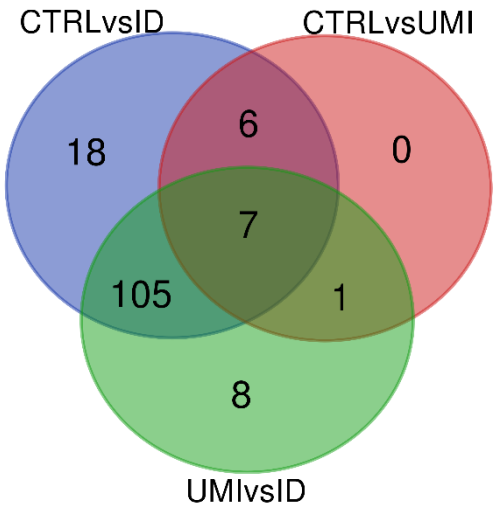

Figure 24. Venn diagram reflecting the number of differentially expressed proteins between CTRL and ID (CTRLvsID), CTRL and UMI (CTRLvsUMI) and between UMI and ID (UMIvsID) study groups. CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men.

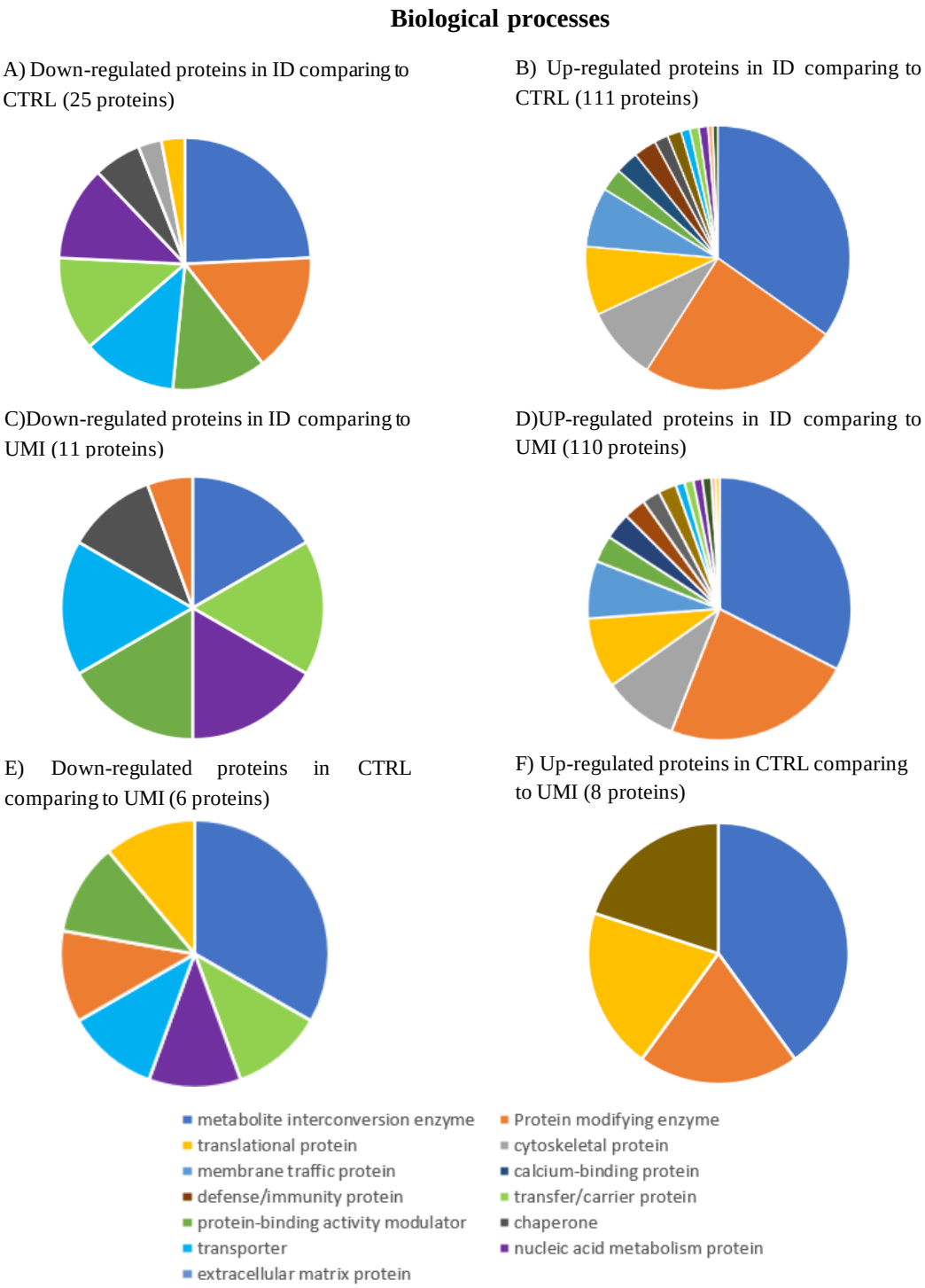

Figure 25. Biological processes expressed by up-regulated and down-regulated proteins in the three study groups (CTRL – healthy controls, ID – idiopathic infertile men, UMI – unexplained infertile men) using PANTHER. CTRLvsID-DW – down-regulated proteins in ID comparing to CTRL; CTRLvsID-UP – up-regulated proteins in ID comparing to CTRL; UMIvsID-DW – down-regulated proteins in ID comparing to UMI; UMIvsID-UP – up-regulated proteins in ID comparing to UMI; CTRLvsUMI-DW – down-regulated proteins in UMI comparing to CTRL; CTRLvsUMI-UP – up-regulated proteins in UMI comparing to CTRL.

3.4.2. Cluster analysis using DAVID bioinformatics

As previously mentioned, CTRLvsID and UMIvsID protein sets have a considerable overlap. After performing cluster analysis, we observed that several clusters were common for the two protein sets, being this the reason why they will be discussed together. In the case of the CTRLvsUMI protein set, this contained few proteins to have relevant clusters (>1 enrichment score) and were not included in this analysis [Annex II to III].

One of the clusters found in the two protein sets mentioned above was composed of glycoproteins (3.56 and 3.68 enrichment scores for CTRLvsID and UMIvsID, respectively), that being a very broad type of proteins included 60 proteins in the CTRLvsID set and 56 in the UMIvsID. Since these comprehended nearly half of the proteins in these sets, we chose to analyse the most significant gene ontology (GO) term present in the cluster named "secreted" [Annex II and III]. This GO term had a Benjamini correction (adjusted *P* value on the enrichment of sample proteins in all proteins of that GO term) of 1.9 $x10^{-5}$ containing 34 proteins in the CTRLvsID and a Benjamini correction of 3.5 $x10^{-5}$ containing 31 proteins in UMIvsID [Table 6]. Most of these secreted proteins were up-regulated in ID comparing to CRTL and UMI. However, when it comes to the physiologic/functional significance of these results, since spermatozoa are considered transcriptionally inactive cells (305), these proteins could represent reminiscences of early spermatogenesis. Nevertheless, these secreted proteins are involved in different pathways.

Progestogen associated endometrial protein (PAEP), for instance, is a glycodelin, that are secretory glycoproteins with different isoforms which can be differentiated by the glycosylation profile, being the Glycodelin-S the isoform found in the seminal plasma and one of the most abundant glycoproteins in it (306, 307). These proteins are involved in processes as diverse as proliferation, differentiation, adhesion, and motility and have been described to regulate immunosuppression, fertilization, and implantation (308). Glycodelin-S is known to bind sperm head and supress capacitation in human sperm (307) and have also been described to be upregulated in seminal plasma exosomes isolated from men with severe asthenozoospermia (309). Additionally, in a proteomic study on normozoospermic infertile men (UMI), this protein was found to be upregulated. The authors also identified the protein in the sperm head and suggested that its upregulation might be involved in the patients' infertility (232). Yet, in our study this protein expression was not significantly different between CTRL and UMI, and was significantly overexpressed in ID comparing to UMI.

Nevertheless, the existing data together with our results indicates that the upregulation of this protein in ID patients could help to explain their infertility and this might be related with the decreased percentage of capacitated spermatozoa in ID patients' sperm observed in this study [Figure 17]. However, being the capacitation a very complex process, this issue should be further analysed, focusing specifically on this protein and also taking in consideration other proteins that might be entailed in the

capacitation process. To our knowledge, there are no other studies in ID patients with identification of this protein.

Regarding semenogelin-1 and 2, these are gel-forming proteins predominant in semen after ejaculation (sperm coagulum), and highly concentrated in seminal vesicles secretions (310). They have been reported to inhibit sperm motility (311, 312), sperm capacitation (313), and to activate sperm acrosomal hyaluronidase, an enzyme essential for sperm penetration in the oocyte (314). Moreover, prostate-specific antigen (KLK3, a member of the Kallikrein family of proteins) is a serine protease known to hydrolyse semen coagulum, transforming semenogelin in less active fragments, liquefying semen and allowing for sperm motility. Still, KLK3 can be inhibited by plasma serine protease inhibitor (SERPINA5), a protein known to inactivate several serine proteases implicated in the reproductive system (315). Interestingly, these 4 proteins were observed to be upregulated in ID [Table 6], suggesting alterations in the liquefaction process that might have an impact on sperm motility. Corroborating our findings, in two previous studies, semenogelin-1was reported to be up-regulated in asthenozoospermic patients' sperm (249, 316), although one of them did not specifically classified them as idiopathic (249). In the seminal plasma, in a study aiming to compare the proteome of samples from infertile patients with different qualities, regarding concentration and morphology, this protein has been reported as upregulated in oligoteratozoospermic samples. The authors suggested that this might justify the alterations in the sperm motility that they observed in men with abnormal morphology (317). However, in sperm and seminal plasma from samples with high level of ROS, SEMGs and KLK3 were described as under-expressed. Yet in this studies samples from infertile patients and healthy donors were entailed and then divided according to the level of ROS, not considering the fertility state (317). In UMI patients, both SEMG1, SEMG2, PSA were also observed to be upregulated, when comparing to normal donors samples (232).

Additionally, SEMG1 was seen up-regulated in varicocele patients with abnormal sperm parameters compared to heathy patients and varicocele patients with normal sperm parameters. The authors suggested the overexpression of this protein was associated with oxidative stress in patients with varicocele (318). Regarding SERPINA5, although no studies were found on ID patients, in a study comparing the sperm proteome of normozospermic fertile and infertile patients (UMI), it was found that this protein was under expressed in UMI patients. A discovery that the authors defend that might contribute to the patients infertility, as they identified that SERPINA5 was interacting with FN1 (fibronectin), by Ingenuity Pathway Analysis (IPA), that is a protein known to be involved in capacitation and also under expressed in UMI patients, leading to a capacitation failure and probably compromising fertilization (230). This is in accordance with our results, that show this protein under expressed in UMI patients comparing to ID and CTRL, although the levels of SERPINA5 between CTRL and UMI was not statistically significant. However, at the light of our results, we hypothesize that the higher levels of SERPINA5 in ID patients are inhibiting hydrolysation of semenogelin, hence

decreasing sperm motility, which was also observed to be significantly decreased in these patients [Figure 13].

In addition, increased levels of annexin A2 (ANXA2), a calcium-dependent membrane binding protein, in ID patients could also be contributing to the decreased motility observed in these patients [Figure 13].

ANXA2 has been associated with several regulatory processes such as actin remodelling, signal transduction, transcription, DNA replication and repair (319, 320). The detection of this protein in several proteomic and transcriptomic studies in the testis (spermatogonia, spermatocytes, spermatids, Sertoli, Leydig and peritubular cells), on spermatozoa, seminal plasma and prostatic secretions, also suggest an important role on several reproductive processes such as spermatogenesis, sperm maturation and fertilization (320, 321). Accordingly, ANXA2 has been previously reported to have a role on sperm binding in pig and bovine oviducts (322, 323), while in rodents it was described to be involved in the blood testis-barrier integrity (321).

Munuce and coworkers, reported an inverse correlation among the mRNA levels of ANXA2 and its protein levels, with poor quality samples showing lower levels of proteins and higher levels of mRNA, an observation that the authors suggested that might be due to a regulation at the translation level in the spermatogenesis, in which the low proteins levels were compensated by more transcripts (320). Furthermore, in the same study, a correlation between ANXA2 and DNA fragmentation was also described , leading the authors to suggest that decreased protein levels of ANXA2 in samples with higher DNA fragmentation might interfere with their binding capacity (320). These results are not in agreement with what we found, as in our ID group, the ANXA2 levels were upregulated while the DNA damage was increased.

In a different study, ANXA2 transcript was described to be decreased in infertile asthenozoospermic patients, that have by definition motility below the reference values, comparing to infertile normozoospermic men (corresponding to our UMI group) and fertile donors (CTRL) (324). In the latter group of patients, ANXA2 mRNA levels were also positively correlated with progressive motility (324). However, this study didn't evaluate protein levels, but if Munuce and cowokers were right (320), this would mean higher levels of protein that will then be in accordance with our observations.

Concerning lysozyme like 4 (LYZL4) and zona pellucida binding protein (ZPBP), these are acrosomal proteins known for mediating sperm-oocyte interaction and were observed to be down-regulated in ID patient's group [Table 6]. It has been previously shown that immunoneutralization of LYZL4 decreased the number of human spermatozoa bound to zona-free hamster eggs, IVF fertilization rate and percentage of 2-cell embryos in mice (325, 326). LYZL4 was also observed to be downregulated in sperm samples (apparently from UMI patients) resulting in a poor blastocyst development after IVF (267), which was also seen in our UMI patients although it was not statistically significant. On the other hand, ZPBP is known to be an important protein for sperm normal morphology, progressive motility

and sperm-oocyte interaction (327, 328) and was observed to be downregulated in sperm from normozoospermic infertile men (UMI) who have failed a pregnancy with IVF only achieving it after a rescue ICSI (329). In this study the female factor has been ruled out, leading the author to assume that the fertilization problem has a male origin. In our study, the level of ZPBP was not significantly different between CTRL and UMI, although it was significantly increased in UMI patients comparing to ID. Having in mind the mentioned studies and our results, these proteins down-regulation in ID patients might have a negative impact on the fertilization process, which was observed as having a tendency to be decreased (fertilization rate) in ID patients [Table 1], although not reaching statistical significance.

Table 6. Proteins present in the GO term "secreted" by DAVID bioinformatics cluster analysis on the differentially expressed proteins between CTRL and ID (CTRLvsID) and UMI and ID (UMIvsID) protein sets. Benjamini correction of 1.9×10^{-5} and 3.5×10^{-5} for CTRLvsID and UMIvsID respectivelly.

Protein	Uniprot accession	Fold-change ID/CTRL	Fold-change ID/UMI
Transmembrane protease serine 2	O15393	4.93	4.82
Prostatic acid phosphatase	P15309	3.66	4.14
GNAS complex locus	Q5JWF2	3.45	2.36
Plasma serine protease inhibitor (SERPINA5)	P05154	3.10	4.11
Zymogen granule protein 16B	Q96DA0	3.00	4.52
Clusterin	P10909	2.61	3.27
Epididymal sperm-binding protein 1	Q96BH3	2.57	-
Semenogelin 2	Q02383	2.52	2.74
Prolactin induced protein	P12273	2.49	2.74
Haptoglobin	P00738	2.45	2.73
Dipiptidyl peptidase 4	P27487	2.41	2.66
Cathelicidin antimicrobial peptide	P49913	2.37	3.49
α-1-antitrypsin	P01009	2.23	2.72
Afamin	P43652	2.16	3.17
Zinc- α-2-glycoprotein	P25311	2.14	2.62
Semenogelin 1	P04279	2.12	4.46
Serine carboxypeptidase 1	Q9H3G5	2.10	3.26
Prostate-specific antigen (KLK3)	P07288	2.04	2.74
Hemopexin	P02790	2.03	2.68
Annexin A2 (ANXA2)	P07355	1.98	2.30
Gastricsin	P20142	1.93	2.59
Epididymal protein 3B	P56851	1.92	2.65
Albumin	P02768	1.89	2.45
α-1B-glycoprotein	P04217	1.74	2.48

Transthyretin	P02766	1.58	2.42
Glycodelin	P09466	1.49	1.70
Apolipoprotein H (APOH)	P02749	1.40	2.14
Zona pellucida binding protein (ZPBP)	Q9BS86	0.82	0.79
Protein MENT	Q9BUN1	0.76	-
IZUMO sperm-egg fusion protein 4	Q1ZYL8	0.74	-
Galactosidase beta 1 like	Q6UWU2	0.72	0.81
Lysozyme like 4 (LYZL4)	Q96KX0	0.64	0.77
Trypsin-1	P07477	0.59	-
Antithrombin-III	P01008	0.38	-
Laminin subunit β-2	P55268	-	2.46
Serotranferrin	P02787	-	2.10

Another interesting cluster was that related with redox activity (1.97 and 1.77 enrichment scores for CTRLvsID and UMIvsID respectfully), including 12 proteins on the CTRLvsID and 11 on the UMIvsID. All the 11 proteins present in the UMIvsID redox activity cluster were also present in the CTRLvsID equivalent cluster [Table 7]. This cluster is composed of oxidoreductase enzymes involved in lipid, amino acid and carbohydrate biosynthesis, which are mostly upregulated in ID comparing to both CTRL and UMI patients. These are crucial processes for cell metabolism, that when deregulated can lead to redox imbalance and sperm dysfunction (330).

One of them is ATP-citrate synthase, also known as ATP citrate lyase (ACLY), an enzyme that catalyses the conversion of citrate and CoA to oxaloacetate and acetyl-CoA using ATP, representing an important step connecting the carbohydrate metabolism and fatty acid biosynthesis (331) and the other, fatty acid synthase (FASN) that catalyses the de novo biosynthesis of long-chain saturated fatty acids from malonyl-CoA and a acetyl-CoA, using NAPDH (331), being also involved in pathways associated with DNA damage, exerting a protective role (331). Therefore, the upregulation of these two proteins can lead to increased synthesis of fatty acids, suggesting metabolic alterations or adaptations in the sperm from ID patients, or even compensatory mechanisms to avoid DNA damage.

Accordingly, by studying the acetylation and ubiquitination regulation of ACLY, Lin *et al.* showed that acetylation of this protein inhibited its ubiquitination, leading to its accumulation. This accumulation was suggested by the authors to increased levels of fatty acid synthesis (332).

On the other hand, FASN was observed to be upregulated in the seminal plasma from bilateral varicocele patients (332) and in seminal plasma from samples with high DNA fragmentation (333), while in sperm, intriguingly. It was reported to be exclusively expressed in samples with low DNA fragmentation (334, 335), suggesting that the mechanism of action of this enzyme might differ among the seminal plasma and the cell itself. Nevertheless, in our study, ID patients were observed to have increased expression of this protein and significantly decreased chromatin integrity, while UMI patients

have decreased expression of this protein and no significant difference in chromatin status comparing to ID or CTRL. Yet, we should keep in mind that these are two different patient groups that probably have different infertility aetiologies.

Furthermore, Peroxiredoxin-1 and 2, two other proteins present in this cluster, were also observed to be up-regulated in ID comparing to CTRL and UMI patients. Peroxiredoxins (PRDXs) are cysteine dependent peroxidases that have a crucial role on peroxides detoxification and in redox signalling (336, 337),that have already been identified in seminal plasma (338) and sperm (251). Furthermore, a role on sperm and male fertility has been reported. In fact, PRDXs inhibition was observed to impair several sperm parameters, decrease ATP levels and MMP, while increasing ROS levels and DNA fragmentation also affecting capacitation and acrosome reaction and further compromising fertilisation and early embryonic development in mice sperm (337). Furthermore, PRDXs were observed to be differentially expressed in capacitated vs non capacitated boar sperm (339), to be overexpressed mice under stress conditions, such as exposure to environmental contaminants (340), while their absence was observed to have a negative impact on mice sperm motility, capacitation and DNA integrity (341). Infertile patients were also reported to have reduced levels of PRDXs, that was associated with lower sperm quality and DNA integrity. Yet this study included both idiopathic infertile men and varicocele infertile men in the infertile group, being the alterations more evident in the varicocele group and suggesting an important role of PRDXs for sperm quality maintenance, protecting them from oxidative stress, especially increased in varcicocele (338).

PRDX1 and 2 were also reported to be upregulated in patients with varicocele, and also in normozoospermic men with increased levels of membrane lipid peroxidation (342), data that supports these proteins antioxidant role in an attempt to counteract ROS. On the other hand, Hamada et al, found that PRDX1 was overexpressed in sperm with low amount of ROS, leading the authors to suggest that together with other antioxidants detected this might be protecting cells form ROS build up (343). In a different study, Liu and co-workers reported that PRDX1 was under expressed in normozoospermc patients that failed IVF and only achieved pregnancy after a rescue ICSI (328), probably being indicative of an altered redox state in these patients that might interfere with fertilizing capacity. In our study, we observed a tendency to higher ROS levels in ID patients [Figure 19], that might explain the observed increase level of PRDX that we are reporting here.

On the other hand, lactate dehydrogenase chain C (LDHC) was observed to be downregulated in ID comparing to both CTRL and UMI. LDH is a glycolytic enzyme that catalyses the conversion of Pyruvate to lactate, being the LDHC4 isoform, specific for the testis/sperm, located in the flagellum, and representing more than 90% of the LDH activities of sperm cell (344, 345). Overall, it is now known that LDHC is necessary for sperm capacitation, motility and ultimately, its absence, as shown in KO mice, can impair fertilization (18, 346, 347), functions that could explain the low capacitation rates and motility found in ID patients in our study. Furthermore, in a study in which sperm proteome was

analysed in idiopathic patients (categorized as our ID group) after treatment with antioxidants, LDHC was observed to be overexpressed post-treatment and associated with sperm function and fertilization (265). In addition, the activity of LDHC4 per spermatozoa, in the seminal plasma, was previously described to be increased in oligozoospermic patients, when compared to CTRL and UMI (348). However, despite the fact that this was not a proteomic study, it is not clear if the entailed oligozoospermic patients have known causes of infertility, hence further investigations on patients under our strict criteria is needed.

Table 7. Proteins present in the cluster "Redox activity" by DAVID cluster analysis on the differentially expressed proteins between CTRL and ID (CTRLvsID) and UMI and ID (UMIvsID) protein sets. Enrichment score of 1.97 and 1.77 for CTRLvsID and UMIvsID respectivelly.

Protein	Uniprot accession	Fold-change ID/CTRL	Fold-change ID/UMI
Peroxiredoxin-2 (PRDX2)	P32119	2.24	3.27
Sorbitol dehydrogenase (SORD)	Q00796	2.23	3.69
Peroxiredoxin-1 (PRDX1)	Q06830	2.04	2.35
ATP-citrate synthase (ACLY)	P53396	1.97	-
D-3-phosphoglycerate dehydrogenase (PHGDH)	O43175	1.96	2.96
6-phosphogluconate dehydrogenase (PGD)	P52209	1.93	2.22
Carbonyl reductase 1 (CBR1)	P16152	1.83	1.61
Fatty acid synthase (FASN)	P49327	1.59	2.41
NADH-cytochrome b5 reductase 3 (CYB5R3)	P00387	1.43	1.70
Adenosylhomocysteinase (AHCY)	P23526	1.40	1.70
Rab GDP dissociation inhibitor beta (GDI2)	P50395	1.31	-
L-lactate dehydrogenase C chain (LDHC)	P07864	0.80	0.82

3.4.3. String analysis on CTRLvsUMI proteins

Since this protein set was too small to perform DAVID analysis, STRING software was applied to find reliable connections between the CTRLvsUMI proteins [Figure 26]. All CTRLvsUMI proteins were downregulated in UMI patients when compared to CTRL. In the first node, 3 proteins were connected: annexin A5 (ANXA5), Transthyretin (TTHY), and apolipoprotein H (APOH). As mentioned above, Annexins are calcium dependent phospholipid binding proteins that participate in several processes such as membrane fusion, exocytosis, anti-inflammatory and anti-coagulation processes and signal transduction (349, 350). ANXA5 has been detected in the human testis, epididymis, prostate and sperm in several proteomic studies (238, 351-353) and also in the seminal plasma from several species including humans (354), and its known to affect seminal quality in rabbits (355), being also described as a decapacitating factor in the seminal plasma of this specie (356). In humans, *in vitro* studies have

shown that it can improve sperm motility (357), and protect sperm form DNA damage (358). It has been reported that ANX5 was overexpressed in the seminal plasma of patients with bilateral varicocele (335), and under expressed in asthenozoospermic sperm samples when compared with donor control samples (222). Interestingly, contrarily to our results, Xu and coworkers have reported that this protein was upregulated in UMI patients, comparing to healthy donors (232), defending that it might be protecting membrane and DNA integrity. Still, as previously mentioned this study used highly selected after swim-up sperm populations, which decreases the amount of information obtained. On another note, ANX5 is also highly expressed in placenta where it exerts anti-coagulation roles (359) and decreased levels of ANXA5 were previously associated with recurrent pregnancy loss (360).

Having in mind what has been discussed so far, the decreased levels of this protein in our study might be therefore indicating a failure in counteracting a possible redox imbalance.

Regarding APOH or β2 glycoprotein I, this is a plasma protein identified in the two major lipoprotein fractions (HDL, VDL) and known to be involved in anti-coagulation and on lipoprotein metabolism, being also identified as a cofactor for anti-phospholipids antibodies formation (361-363). APOH has been previously detected in the murine testis, in the later stages of spermatogenesis, suggesting a role in apoptotic body clearance (364-366), and also in murine testicular fluid, being suggested to interact with CDC42 (cell division cycle 42) at Sertoli cell membrane, probably facilitating communication between germ and Sertoli cells. Additionally, by immunohistochemistry, APOH was observed to be present in the rat seminiferous tubules (367). More recently, this protein was also detected in human sperm (239).

APOH has also been detected in human follicular fluid, and described to have a stimulator role on sperm motility and eventually on the fertilization process (368). Interestingly, the antiphospholipid syndrome (APS), an autoimmune disorder characterized by frequent vascular thrombosis and obstetric morbidity, is associated with the presence of persistent antiphospholipid antibodies including anti-APOH (369-372) and there is significant evidence that aPL antibodies can interfere with ANXA5 binding to phospholipids and may thereby promote pregnancy losses and thrombosis in APS (360).

Based on these evidences, it seems that the downregulation of this protein in our UMI patients group, might cause problems at several levels that might compromise their fertility, nevertheless further studies will be need to confirm this.

TTHY was the other protein in this node, which is mainly synthesized by the liver, choroid plexus, and syncytiotrophoblast of the human placenta and is known to be involved in the retinol metabolism and transport of thyroid hormone in mammals (373-376). TTHY, also known as pre-albumin, has been previously identified in human sperm and its levels observed to be higher in capacitated vs ejaculated sperm (377). Furthermore, TTHY and APOH were previously reported to be up-regulated in UMI patients that achieved pregnancy undergoing ICSI, after IVF failure, comparing to UMI patients with IVF success (329). Still, in this work, the authors failed to explain how the upregulation of these proteins

might affect IVF failure. Also, a mutation in TTHY, associated with the neurodegenerative Corino de Andrade disease, is known to be associated with reproductive problems, namely sexual dysfunction (378).

Nevertheless, the role of TTHY on the thyroid hormone transport, seems to be particularly relevant for reproductive purposes. In fact, the effects of thyroid hormone on the reproductive system have been studied in humans and animal models, showing that changes in normal thyroid function resulted in decreased sexual activity and fertility (379). In the male side, thyroid hormones were described to have a role in testis, acting on different cell types, including the somatic Leydig and Sertoli cells, and germ cells (380). Alterations in thyroid hormones levels were shown to induce alterations in testis function, including semen abnormalities, being that in general, hyperthyroidism has been more frequently associated with reduced semen volume and reduced sperm density, motility, and morphology, while hypothyroidism was associated with altered sperm morphology (380). From the available literature is clear the need to include the thyroid function evaluation in the diagnostic workup of the infertile man. In females, hypothyroidism has been also associated with increased risk of miscarriage, and other obstetric complications (381, 382).

Overall, while ANXA5 and APOH have a common function, being involved in anti-coagulation (383-385) processes, and can thus be hypothesised that could have a similar role in seminal fluid (386), (although to our knowledge this has not be described so far), all the three proteins in this node, seem to be connected by several and common effects on different aspects of sperm function, and were associated with obstetric problems, including pregnancy loss, being these among the probable mechanisms by which their lower expression interfere with UMI patients fertility.

On the second node we found Citrate synthase (CS) and α-crystallin B chain (CRYAB). CS is a mitochondrial enzyme (nucleus encoded) that catalyses the opposite reaction of ACLY, using acetyl-CoA and oxaloacetate to produce citrate, without ATP consumption in first step of the Tricarboxylic acid (TCA) cycle, that will then provide reducing equivalents to the OXPHOS or precursors to other biosynthetic pathways (387, 388). Therefore, the TCA cycle in sperm may also be needed, directly or indirectly, as these cells are highly dependent on a variety of energy sources, including for motility purposes (13, 38). In this regard, though studies in rat testicular germ cells suggested that the TCA cycle may be more relevant for spermatids (389), ejaculated boar sperm seem to be capable of metabolizing exogenous citrate and lactate through the TCA cycle (390). In *Cynops pyrrhogaster* newt, injection of CS extracted from *Xenopus* and pigs was observed to induce egg activation, through a rise in intracellular Ca^{2+} (391). Moreover, CS was detected in germ cells, spermatozoa and seminal plasma (389, 392). In stallion sperm, CS expression was significantly associated with conception rate in fertile stallions (393).Accordingly, CS activity was observed to be associated with sperm concentration, vitality and motility (41) and an enrichment in CS was suggested to be a result of better spermatogenesis in humans, before the cessation of nuclear transcription (41). Yet in this study the patients' group is barely

described. On a recent study, sperm from mice lacking the *Cs-like* gene, that encodes extra-mitochondrial CS, was analysed and used for IVF procedure. 6-month-old *Cs-like* deficient mice showed significantly decreased two-cell formation comparing to wild-type mice with the same age, leading to a faster fertility decline (394). Taking all this into account, we hypothesise that CS down-regulation in UMI could decrease the flux of reducing equivalents to the electron transport chain or metabolic precursors to other pathways that might be important for the sperm, such as OXPHOS, impairing energy supply in spermatozoa, and possibly spermatogenesis.

Moreover, CS is also associated with α-crystallin B chain (CRYAB), a small heat shock protein (HSPB5) described to bound to CS during *in vitro* exposure to heat and microwaves (395). Besides spermatozoa (142), CRYAB is also expressed in the spermatids in mice (396). In Sertoli cells (TM4 mouse cell line), heat stress was observed to induce CRYAB expression, which was associated with reduced apoptosis (397). HSP are a group of structurally unrelated proteins divided into the following families: the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), and HSP27 (HSPB) families (398). HSPs are part of the cellular defence mechanism and are expressed after exposure to a variety of insults such as high temperatures, oxidative stress, heavy metals, inflammation and infection. In the male reproductive system, they are expressed from the initial stages of embryo development and throughout the spermatogenesis and they are known to influence several aspects of reproduction (399). In fact, in the mammalian testis and sperm, several HSP family members are expressed. Testis-specific HSP70 forms were first described in mice (400) and later in rat and humans (401, 402). Interestingly, when examining the HSP27 expression in human testicular biopsies with different qualities in terms of spermatogenesis (normal, maturation arrest and Sertoli cell only syndrome), a study found that HSP27 was expressed in both somatic and germ cells in normal conditions, but that this expression was altered in the pathological situations, stressing a probable role of HSP27 in those alterations (403).

Furthermore, HSPs have also been identified at the sperm surface of several mammals, including humans (404, 405). Interestingly in a study aiming to study the unfolded protein response in human sperm, a decrease in viability and motility and an increase in the levels of several HSP including HSP27, was reported after exposure to H_2O_2 (406), highlighting their activation upon oxidative stress conditions.

Additionally, seminal plasma and sperm have been reported to induce HSP gene transcription, probably to facilitate immunological responses (399). Having this in mind, there is margin to believe that some alterations in spermatogenesis might be taking place in UMI patients' contributing to their infertility aetiology. Yet, this requires further confirmation.

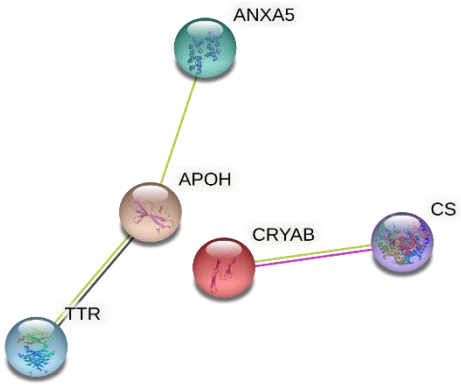

Figure 26 Representative protein-protein interaction network of CTRLvsUMI proteins using STRING. A total of 14 proteins was classified. Proteins with no interaction are not shown. ANXA5 – Annexin 5, TTHY – Transthyretin, APOH - Apolipoprotein H, CRYAB – α-crystallin B chain, CS – Citrate synthase

3.4.4. Differentially expressed proteins between all patient's groups

As previously mentioned, there are 7 differentially expressed proteins between the three experimental groups, thus being the most plausible candidates to distinguish them [Table 8]. Interestingly, all these proteins are up-regulated in ID compared to CTRL and UMI, and down-regulated in UMI compared to CTRL.

CRYAB is a small HSP, as it was previously mentioned, and this family of proteins is expressed throughout the human organism including the testis and sperm. Expression of several HSP was shown increased in stress conditions in the testis, which also decreased sperm viability and motility. Hence, it can be hypothesised that increased expression of CRYAB in ID patients could also be indicative of stress condition in the testis of these patients and justify their decreased vitality and motility. In addition, CRYAB overexpression was described to be associated to neurodegenerative diseases and diabetes, which also present increased inflammation and oxidative stress (142, 407, 408). Reaffirming that the expression of this protein could be induced by oxidative stress to restore redox homeostasis in neuroinflammation (409). CRYAB up-regulation together with elevated ROS levels [Figure 18] and decreased MMP [Figure 20] contribute to the hypothesis that ID patients have increased oxidative stress levels comparing to the other experimental groups. This situation is also commonly reported in the literature regarding ID patients (197, 204, 410, 411).

On the other hand, ANXA5 is a calcium-dependent protein that binds to negatively charged phospholipids, and which is involved in anti-coagulation and membrane repair pathways (385, 412). ANXA5 was previously observed to be down-regulated in asthenozoospermic patients including men with known and unknown cause of infertility hence not following our criteria for ID patient (249). In addition, was reported to be up-regulated in UMI patients, following the same criteria as ours, being this

reported in proteomic studies, although not confirmed by western blot (230). Contrarily, the opposite was observed in the present study, probably the different results were due to the different types of samples used (e.g swim up vs gradient treated), proteomic methodologies, classification criteria of patients and used inclusion/exclusion criteria. Also, making a parallelism with other defence systems/mechanisms, while an increase expression/activity, as seen in our ID group, can represent a bigger necessity of protection due to higher damage, the inverse, as observed in our UMI group, might indicate failure in counteracting a possible redox imbalance. Yet, it seems reasonable to suggest that both situations can result in sperm damage.

APOH is also a known anticoagulant protein present in sperm, testis and follicular fluid (368). On follicular fluid, this protein was associated with anti-phospholipids antibodies formation promoting pregnancy losses and thrombosis in APS. However.to our knowledge this has never been studied in seminal plasma, where it can have a relevance in the context of immunological infertility. This protein was also hypothesised to be involved in spermatogenesis possibly through clearance of apoptotic bodies (365), hence UMI patients down-regulation of this protein, together with that of CRYAB, discussed above, might be a sign of impaired spermatogenesis. On the other hand, in ID patients the opposite was observed. Yet, our knowledge, the consequences of APOH overexpression in sperm are not known. Nevertheless, this protein is also involved in triglyceride metabolism, increasing triglyceride hydrolysis (413), and having in mind that several lipid metabolism-related enzymes were also seen overexpressed in ID patients, it can be hypothesised that these patients' sperm cells have some metabolic alterations, more specifically on lipid metabolism, a pathway already described to be important for sperm (237).

PA1B3 is one of the two subunits of platelet-activating factor acetylhydrolase IB (PA1B), which produces a precursor of platelet-activating factor (PAF) (414). A study was performed to understand the effect of disrupting the two PA1B subunits (PA1B3 and PA1B subunit α2) on spermatogenesis using mice. Mice with the double knockout of these proteins were infertile and showed significantly decreased testis weight and number of germ cell in the seminiferous tubules comparing to wild-type mice, still the single knockout of PA1B3 did not show these effects possibly due to a compensation mechanism through α2 subunit expression (414). Therefore, PA1B3 seems to have a role in spermatogenesis, and the downregulation of this protein observed in UMI patients could contribute to explain their infertility in accordance with the previous mentioned alterations on the expression of APOH and CRYAB, proteins. Furthermore, PAF is an important lipid messenger, that at the human sperm level is known to increase motility and to induce AR (415-417). Unexpectedly, the ID patients, although presenting decreased motility, comparing to the other groups, had increased levels of PA1B3. One explanation for these results might be that the PA1B3 over-expression in ID represents a compensatory mechanism to further increase PAF levels, yet it might not be enough to increase PAF production or being effective, the PAF increased production might not be sufficient to counteract the sperm motility impairments of these patients.

Destrin, is also included in these 7 proteins, being an actin depolymerizing protein (ADF/cofilin family) with a testis specific isoform, identified at the anterior head of mature bovine spermatozoa and known to suffer rearrangements during epididymal maturation and acrosome reaction, that are associated with concomitant changing levels of actin polymerization, suggesting a role in sperm maturation and acrosome reaction in bovine spermatozoa (418). In humans, this protein has also been detected in sperm (237, 245), although its role in this context is not known. Importantly, the ADF/cofilin family is known to regulate F-fibres depolymerization facilitating the acrosome reaction in human sperm (419). Based on what has been exposed, the downregulation of this protein in UMI patients, together with that of APOH, CRYAB and PA1B3, strengthens the hypothesis that spermatogenesis might be impaired in these patients, although this needs further confirmation with new studies. In addition, actin polymerization at the end of capacitation is essential to prevent premature AR (80), hence decreased level of destrin in UMI patients can be contributing for the maintenance of acrosomal integrity, that is higher than in the other groups (Figure 17). In the case of ID patients, the opposite can be happening, and increased levels of destrin could be inducing the depolymerization of F-actin fibres inducing premature AR, that will further compromise the fertilization process.

NADH-cytochrome b5 reductase 3 (NB5R3), in its turn, is an oxidoreductase involved in cholesterol biosynthesis and fatty acids elongation (420, 421). This protein has been previously identified in human sperm (237, 245, 422-425). However, the relevance of this protein for fertility is unknown. A study on a patient with congenital methemoglobinemia, a disease characterised by a deficiency in NB5R3, showed that lack of this protein is associated with decreased levels of cholesterol on brain cells comparing to a healthy control, demonstrating the importance of this protein on cholesterol biosynthesis (420). Cholesterol is known to maintain fluidity in the plasma membrane (426), a feature especially important for sperm function, as processes such as capacitation begins with plasma membrane remodelling, leading to the efflux of cholesterol (76-78, 426). So, it can be hypothesised that the downregulation of this protein in UMI patients could lead to cholesterol depletion in spermatozoa, impairing capacitation, a necessary step for sperm to be able to accomplish fertilization. On the other hand, the opposite, as observed in ID patients, might lead to a situation where the excess of fluidity might be counterproductive. Yet in our work, these alterations were not mirrored in terms of capacitation (Fig. capacitation). Moreover, NB5R3 also contributes to the formation of nitric oxide, that could lead to increasing formation of NOS and ROS which further result in oxidative stress (427). It can be thus hypothesised that over-expression of this protein in ID patients might be behind the observed increase in ROS levels that might compromise mitochondrial function, in accordance with the decreased MMP observed in this group, altogether contributing for the sperm's dysfunction, including at the mitochondrial level, observed in these patients.

Finally, TTHY (transthyretin) is a transporter of thyroxine, a thyroid hormone, on bloodstream, which is essential for the normal functioning of the testis, sexual activity and fertility. Hence,

downregulation of TTHY in UMI patients could impair thyroxin's transport and consequently decrease fertility in these patients.

TTHY is also known for promoting amyloid fibres formation in several human tissues including the epididymis and, interestingly, also in semen (428, 429). Although, in the only three patients with epididymal amyloid fibres formation observed until now, it caused no pathology, in other tissues like peripheral nerves and cardiac muscle was shown to cause disease (430, 431). In addition, hyperthyroidism was shown to decreased sperm quality, as we observed ID patients. Hence, the TTHY expression pattern in ID patients' sperm could lead to protein aggregation and further cellular dysfunction leading to decreased sperm parameters and fertility, contributing for reproductive system dysfunction.

3.4.5. Predictive patient classification value of selected proteins

The random forest algorithm was applied to find the effect of different factors on patients' classification, that is their categorization in the different groups. For that aim, the weight of sperm functional parameters and the expression of the 7 differentially expressed proteins between CTRL, ID and UMI were considered [Figure 26]. Taken together, these parameters had an out-of-bag error of 0.253 with 4%, 42% and 100% error on CTRL, ID and UMI classification, respectively, meaning these parameters taken together had a 25% chance of failing to predict the patient's group. This is mainly due to the low number of samples in the infertile groups, as the lower the number of samples, the higher will be the error.

Among all the factors considered, the PA1B3 expression was the one with more weight on the patients' classification [Figure 27], meaning that this aspect is more capable of distinguishing the three experimental groups. Considering the function of this protein described above, although these results seem to be reliable, more studies need to be made to accurately define the specific role of this protein in these patients' (in)fertility. Concentration was the sperm functional parameter with more capability of differentiating the three patient groups, being higher in the CTRL and UMI group and lower in the ID group, as expected having in mind the previous results on this parameter and also the spermiogram characteristics typical of each experimental group [Figure 12]. Acrosome status, a parameter seen significantly increased in the UMI patients compared to ID patients [Figure 18], was the third best parameter in differentiating the 3 patient groups, corroborating that this functional parameter can distinguish ID from UMI patients. Still, due to the proteomic analysis having smaller cohort of patients than the cohort used for seminal analysis, the percentage of intact acrosome in the proteomic cohort was lower in CTRL than ID patients, although we cannot find an explanation for these findings. Vitality had a similar capability to differentiate the three experimental groups as concentration and acrosome status, being higher in CTRL and UMI patients, and lower in the ID patients, as previously observed [Figure

14]. Overall, sperm parameters seem to have a bigger weight on the patient's classification than the altered proteins, reinforcing the idea that non routine parameters need to be evaluated to accurately diagnose these patients.

Table 8 Differentially expressed proteins between all 3 patient groups CTRL, ID and UMI.

Protein	Uniprot accession	Fold-change ID/CTRL	Fold-change ID/UMI	Fold-change UMI/CTRL
α-crystallin B chain (CRYAB)	P02511	1.44	2.86	0.50
Annexin A5 (ANXA5)	P08758	1.56	2.74	0.57
Apolipoprotein H (APOH)	P02749	1.40	2.14	0.65
Destrin (DEST)	P60981	1.50	2.06	0.73
NADH-cytochrome b5 reductase 3 (NB5R3)	P00387	1.43	1.70	0.84
Platelet-activating factor acetylhydrolase IB subunit α1 (PA1B3)	Q15102	1.52	2.22	0.68
Transthyretin (TTHY)	P02766	1.58	2.42	0.65

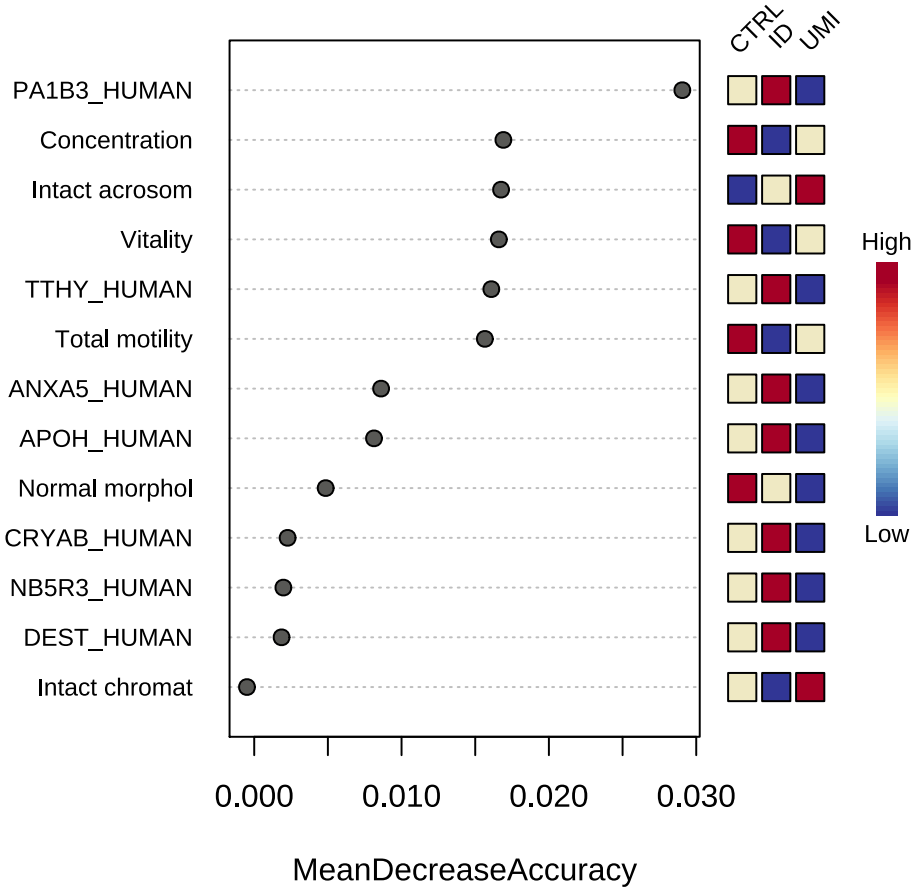

Figure 27. Mean decreased accuracy for sperm functionality parameters (concentration, vitality, total motility, normal morphology, chromatin status and acrosome status) and expression of the 7 proteins differentially expressed among the 3 groups [α-crystallin B chain (CRYAB), Annexin 5 (ANXA5), Apolipoprotein H (APOH), Destrin (DEST) NADH-cytochrome b5 reductase 3 (NB5R3), Platelet-activating factor acetylhydrolase IB subunit α1 (PA1B3), Transthyretin (TTHY)].

4. Conclusion

In this integrated study, that was unique in the sense of all the performed analysis and by the thorough categorization of the experimental groups, we were able to find some aspects that might differentiate ID from UMI patients, not only by the different sperm functionality parameters, but also by their proteome. ID patients besides having significantly decreased sperm concentration, motility and morphology, also presented significantly decreased viability, chromatin integrity and percentage of capacitated cells. Furthermore, through the surveys, we concluded that ID patients had significantly increased incidence of urogenital infections and varicocele, which can of course contribute to the decreased sperm quality, as already reported (126, 295, 296). Focusing on the proteomic analysis, the observed up-regulation of SERPINA5, semenogelin-1, semenogelin-2 and ANXA2 seems to justify, at least partially, the decreased motility observed in these patients. Also glycodelin, having in mind that it was described to inhibit capacitation, seems a good candidate to explain the decreased capacitation observed in ID patients. In addition, ID patients had altered levels of several oxidoreductase enzymes, such as PRDX1, PRDX2, ACLY and FASN, that might be upregulated as a response to oxidative stress and that can possibly explain the decreased chromatin integrity observed in these patients and the observed trend to altered mitochondrial function, including higher ROS levels. Yet this requires further confirmation in a bigger cohort of patients. Additionally, the proteomic results also point out to several metabolic alterations in the spermatozoa of these patients, that might as well explain the general dysfunctionality of their gametes. Examples of this include the ATP citrate synthase, FASN, PA1B3, APOH, NB5R3, all related to lipid and fatty acids metabolism, or TTHY involved in retinol metabolism and CS in the TCA cycle, all over expressed in ID and under expressed in UMI; with the exception of LDHC that was under expressed in ID. All these proteins are involved in metabolic pathways, that seem to be prone to alterations in our study groups. Finally, the proteomic data also provide evidence to believe that sperm structure and processes related to the gamete competence, interaction and binding, might be altered in our patient's groups, as is the case of the altered expression of the proteins ANX2, SERPINA5, ZBPZ, LYZ4, Gycodelin, CRYAB and Destrin, with opposing levels of expression in ID and UMI patients. Therefore, it seems crucial to continue this study, with experiments specifically designed for this aim, being the final objective to clarify and prove the value of our discoveries. Regarding the UMI patients, our results indicate that their sperm are functionality very similar to that of the CTRL individuals, but significantly different from ID patients. The proteomic results also support the functional observations, as only 14 proteins that can potentially differentiate UMI from CTRL patients were identified, while 121 proteins differentiated UMI from ID. Nevertheless, several spermatogenesis-related proteins were observed to be down-regulated in UMI patients, showing that despite presenting normal sperm parameters, their proteome can differentiate them from heathy men (CTRL), hence possibly explaining their, so far unexplained, infertility. In addition, the surveys analysis indicate that these patients had increased incidence of diagnosed depression, when comparing to CTRL

and ID patients. These aspects need to be further investigated, in the sense of clarifying the riddle depression-infertility and which is actually the cause and the consequence.

Remarkably, in this study, the proteins CRYAB, ANXA5, APOH, destrin, NB5R3, PA1B3 and TTHY were found, for the first time, to significantly differentiate the three patient groups, hence being good candidates for further studies on male infertility of unknown origin. In the future, it would be of particular interest to assess the cellular localization of these proteins by immunocytochemistry assays as well as their levels by Western blot. In sum, this study presented a unique complete and integrated analysis of these patients' sperm functionality and proteome, providing new insights and adding knowledge on these patients' unknown aetiology, opening road to future studies in the field.

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5. References

1. Finkelstein M, Etkovitz N, Breitbart H. Ca2+ signaling in mammalian spermatozoa. Molecular and Cellular Endocrinology. 2020:110953.
2. Organization WH. WHO laboratory manual for the examination and processing of human semen. 2010.
3. Dyer SJ, Patel M. The economic impact of infertility on women in developing countries - a systematic review. Facts Views Vis Obgyn. 2012;4(2):102-9.
4. Kawwass JF, Penzias AS, Adashi EY. Fertility-a human right worthy of mandated insurance coverage: the evolution, limitations, and future of access to care. Fertil Steril. 2021;115(1):29-42.
5. Dooley M, Dineen T, Sarma K, Nolan A. The psychological impact of infertility and fertility treatment on the male partner. Hum Fertil (Camb). 2014;17(3):203-9.
6. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reproductive biology and endocrinology. 2015;13(1):1-9.
7. De Jonge CJ, Barratt C. The sperm cell: production, maturation, fertilization, regeneration: Cambridge University Press; 2006.
8. Rathke C, Baarends WM, Awe S, Renkawitz-Pohl R. Chromatin dynamics during spermiogenesis. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 2014;1839(3):155-68.
9. Ioannou D, Miller D, Griffin DK, Tempest HG. Impact of sperm DNA chromatin in the clinic. Journal of assisted reproduction and genetics. 2016;33(2):157-66.
10. Immler S. The sperm factor: paternal impact beyond genes. Heredity. 2018;121(3):239-47.
11. Simon L, Emery B, Carrell DT. Sperm DNA fragmentation: consequences for reproduction. Genetic Damage in Human Spermatozoa: Springer; 2019. p. 87-105.
12. Haddock L, Gordon S, Lewis SE, Larsen P, Shehata A, Shehata H. Sperm DNA fragmentation is a novel biomarker for early pregnancy loss. Reproductive BioMedicine Online. 2021;42(1):175-84.
13. Ramalho-Santos J, Amaral S. Mitochondria and mammalian reproduction. Molecular and cellular endocrinology. 2013;379(1-2):74-84.
14. Freitas MJ, Vijayaraghavan S, Fardilha M. Signaling mechanisms in mammalian sperm motility. Biology of Reproduction. 2017;96(1):2-12.
15. Lehti MS, Sironen A. Formation and function of sperm tail structures in association with sperm motility defects. Biol Reprod. 2017;97(4):522-36.
16. Liu H, Li W, Zhang Y, Zhang Z, Shang X, Zhang L, et al. IFT25, an intraflagellar transporter protein dispensable for ciliogenesis in somatic cells, is essential for sperm flagella formation. Biology of reproduction. 2017;96(5):993-1006.
17. Liu C, Miyata H, Gao Y, Sha Y, Tang S, Xu Z, et al. Bi-allelic DNAH8 variants lead to multiple morphological abnormalities of the sperm flagella and primary male infertility. The American Journal of Human Genetics. 2020;107(2):330-41.
18. Odet F, Duan C, Willis WD, Goulding EH, Kung A, Eddy EM, et al. Expression of the gene for mouse lactate dehydrogenase C (Ldhc) is required for male fertility. Biology of reproduction. 2008;79(1):26-34.
19. Amann RP. The cycle of the seminiferous epithelium in humans: a need to revisit? Journal of andrology. 2008;29(5):469-87.
20. Holstein A-F, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. Reproductive Biology and Endocrinology. 2003;1(1):1-16.
21. Ehmcke J, Wistuba J, Schlatt S. Spermatogonial stem cells: questions, models and perspectives. Human reproduction update. 2006;12(3):275-82.
22. Jahnukainen K, Stukenborg J-B. Present and future prospects of male fertility preservation for children and adolescents. The Journal of Clinical Endocrinology & Metabolism. 2012;97(12):4341-51.
23. Cobb J, Handel MA, editors. Dynamics of meiotic prophase I during spermatogenesis: from pairing to division. Seminars in cell & developmental biology; 1998: Elsevier.
24. Johnson MH. Essential reproduction: John Wiley & Sons; 2018.
25. Wang T, Gao H, Li W, Liu C. Essential role of histone replacement and modifications in male fertility. Frontiers in genetics. 2019;10:962.

26. Bao J, Bedford MT. Epigenetic regulation of the histone-to-protamine transition during spermiogenesis. Reproduction (Cambridge, England). 2016;151(5):R55.
27. James ER, Carrell DT, Aston KI, Jenkins TG, Yeste M, Salas-Huetos A. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. International Journal of Molecular Sciences. 2020;21(15):5377.
28. Wong GE, Zhu X, Prater CE, Oh E, Evans JP. Analysis of fertilin α (ADAM1)-mediated sperm-egg cell adhesion during fertilization and identification of an adhesion-mediating sequence in the disintegrin-like domain. Journal of Biological Chemistry. 2001;276(27):24937-45.
29. Toragall MM, Satapathy SK, Kadadevaru GG, Hiremath MB. Evaluation of seminal fructose and citric acid levels in men with fertility problem. Journal of human reproductive sciences. 2019;12(3):199.
30. Simon L, Murphy K, Shamsi M, Liu L, Emery B, Aston K, et al. Paternal influence of sperm DNA integrity on early embryonic development. Human Reproduction. 2014;29(11):2402-12.
31. González-Marín C, Gosálvez J, Roy R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. International journal of molecular sciences. 2012;13(11):14026-52.
32. Zeqiraj A, Beadini S, Beadini N, Aliu H, Gashi Z, Elezaj S, et al. Male infertility and sperm DNA fragmentation. Open access Macedonian journal of medical sciences. 2018;6(8):1342.
33. Lewis SE, Simon L. Clinical implications of sperm DNA damage. Human Fertility. 2010;13(4):201-7.
34. Borini A, Tarozzi N, Bizzaro D, Bonu M, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Human reproduction. 2006;21(11):2876-81.
35. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril. 2003;79 Suppl 3:1597-605.
36. Wang YJ, Zhang RQ, Lin YJ, Zhang RG, Zhang WL. Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: a meta-analysis. Reprod Biomed Online. 2012;25(3):307-14.
37. Komiya A, Kato T, Kawauchi Y, Watanabe A, Fuse H. Clinical factors associated with sperm DNA fragmentation in male patients with infertility. The scientific world journal. 2014;2014.
38. Boguenet M, Bouet P-E, Spiers A, Reynier P, May-Panloup P. Mitochondria: Their role in spermatozoa and in male infertility. Human Reproduction Update. 2021.
39. Amaral A, Lourenço B, Marques M, Ramalho-Santos J. Mitochondria functionality and sperm quality. Reproduction. 2013;146(5):R163-74.
40. St John JC, Facucho-Oliveira J, Jiang Y, Kelly R, Salah R. Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. Hum Reprod Update. 2010;16(5):488-509.
41. Ruiz-Pesini E, Lapeña AC, Díez C, Álvarez E, Enríquez JA, López-Pérez MJ. Seminal quality correlates with mitochondrial functionality. Clinica Chimica Acta. 2000;300(1-2):97-105.
42. Stendardi A, Focarelli R, Piomboni P, Palumberi D, Serafini F, Ferramosca A, et al. Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa. Int J Androl. 2011;34(3):247-55.
43. Ferramosca A, Provenzano SP, Coppola L, Zara V. Mitochondrial respiratory efficiency is positively correlated with human sperm motility. Urology. 2012;79(4):809-14.
44. Holyoake AJ, McHugh P, Wu M, O'Carroll S, Benny P, Sin IL, et al. High incidence of single nucleotide substitutions in the mitochondrial genome is associated with poor semen parameters in men. Int J Androl. 2001;24(3):175-82.
45. Wu H, Whitcomb BW, Huffman A, Brandon N, Labrie S, Tougias E, et al. Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting. Hum Reprod. 2019;34(1):163-70.
46. Mughal IA, Irfan A, Jahan S, Hameed A. Male infertility is significantly associated with multiple deletions in an 8.7-kb segment of sperm mtDNA in Pakistan. Turkish journal of medical sciences. 2017;47(3):928-33.

47. Bahrehmand Namaghi I, Vaziri H. Sperm mitochondrial DNA deletion in Iranian infertiles with asthenozoospermia. Andrologia. 2017;49(3).
48. May-Panloup P, Chrétien MF, Savagner F, Vasseur C, Jean M, Malthièry Y, et al. Increased sperm mitochondrial DNA content in male infertility. Hum Reprod. 2003;18(3):550-6.
49. Tian M, Bao H, Martin FL, Zhang J, Liu L, Huang Q, et al. Association of DNA methylation and mitochondrial DNA copy number with human semen quality. Biol Reprod. 2014;91(4):101.
50. Zhang G, Wang Z, Ling X, Zou P, Yang H, Chen Q, et al. Mitochondrial Biomarkers Reflect Semen Quality: Results from the MARCHS Study in Chongqing, China. PLoS One. 2016;11(12):e0168823.
51. Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, et al. Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. PLoS One. 2011;6(3):e18112.
52. Alamo A, De Luca C, Mongioì LM, Barbagallo F, Cannarella R, La Vignera S, et al. Mitochondrial Membrane Potential Predicts 4-Hour Sperm Motility. Biomedicines. 2020;8(7).
53. Zhang G, Yang W, Zou P, Jiang F, Zeng Y, Chen Q, et al. Mitochondrial functionality modifies human sperm acrosin activity, acrosome reaction capability and chromatin integrity. Hum Reprod. 2019;34(1):3-11.
54. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod. 2002;17(5):1257-65.
55. Gallon F, Marchetti C, Jouy N, Marchetti P. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil Steril. 2006;86(5):1526-30.
56. Marchetti P, Ballot C, Jouy N, Thomas P, Marchetti C. Influence of mitochondrial membrane potential of spermatozoa on in vitro fertilisation outcome. Andrologia. 2012;44(2):136-41.
57. Kroemer G. Mitochondrial control of apoptosis: an introduction. Biochem Biophys Res Commun. 2003;304(3):433-5.
58. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol. 2007;47:143-83.
59. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health. 2014;32(1):1-17.
60. Mannucci A, Argento FR, Fini E, Coccia ME, Taddei N, Becatti M, et al. The Impact of Oxidative Stress in Male Infertility. Front Mol Biosci. 2021;8:799294.
61. Park Y-J, Pang M-G. Mitochondrial functionality in male fertility: From spermatogenesis to fertilization. Antioxidants. 2021;10(1):98.
62. Dorostghoal M, Kazeminejad SR, Shahbazian N, Pourmehdi M, Jabbari A. Oxidative stress status and sperm DNA fragmentation in fertile and infertile men. Andrologia. 2017;49(10).
63. Condorelli RA, La Vignera S, Mongioì LM, Alamo A, Calogero AE. Diabetes Mellitus and Infertility: Different Pathophysiological Effects in Type 1 and Type 2 on Sperm Function. Front Endocrinol (Lausanne). 2018;9:268.
64. Tavares RS, Escada-Rebelo S, Silva AF, Sousa MI, Ramalho-Santos J, Amaral S. Antidiabetic therapies and male reproductive function: where do we stand? Reproduction. 2018;155(1):R13-R37.
65. Majzoub A, Agarwal A. Systematic review of antioxidant types and doses in male infertility: Benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth rate. Arab J Urol. 2018;16(1):113-24.
66. Amaral S, Redmann K, Sanchez V, Mallidis C, Ramalho-Santos J, Schlatt S. UVB irradiation as a tool to assess ROS-induced damage in human spermatozoa. Andrology. 2013;1(5):707-14.
67. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature. 1951;168(4277):697-8.
68. Austin CR. The 'capacitation' of the mammalian sperm. Nature. 1952;170(4321):326-.
69. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development. 1995;121(4):1129-37.
70. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote. 1994;2(4):371-2.

71. Luque GM, Dalotto-Moreno T, Martín-Hidalgo D, Ritagliati C, Puga Molina LC, Romarowski A, et al. Only a subpopulation of mouse sperm displays a rapid increase in intracellular calcium during capacitation. Journal of cellular physiology. 2018;233(12):9685-700.
72. Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, et al. The CatSper channel mediates progesterone-induced Ca 2+ influx in human sperm. Nature. 2011;471(7338):382-6.
73. White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete research. 1989;22(2):163-77.
74. Katz DF, Vanagimachi R. Movement characteristics of hamster spermatozoa within the oviduct. Biol Reprod. 1980;22(4):759-64.
75. Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod Update. 2006;12(1):23-37.
76. Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J, Kopf GS. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. Molecular human reproduction. 1999;5(11):1017-26.
77. Visconti PE, Ning X, Fornés MW, Alvarez JG, Stein P, Connors SA, et al. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Developmental biology. 1999;214(2):429-43.
78. Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, et al. Cholesterol efflux-mediated signal transduction in mammalian sperm: β-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. Journal of Biological Chemistry. 1999;274(5):3235-42.
79. Fraser LR. The "switching on" of mammalian spermatozoa: molecular events involved in promotion and regulation of capacitation. Molecular Reproduction and Development: Incorporating Gamete Research. 2010;77(3):197-208.
80. Ickowicz D, Finkelstein M, Breitbart H. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. Asian J Androl. 2012;14(6):816-21.
81. Sharara F, Seaman E, Morris R, Schinfeld J, Nichols J, Sobel M, et al. Multicentric, prospective observational data show sperm capacitation predicts male fertility, and cohort comparison reveals a high prevalence of impaired capacitation in men questioning their fertility. Reprod Biomed Online. 2020;41(1):69-79.
82. Buffone MG, Calamera JC, Verstraeten SV, Doncel GF. Capacitation-associated protein tyrosine phosphorylation and membrane fluidity changes are impaired in the spermatozoa of asthenozoospermic patients. Reproduction. 2005;129(6):697-705.
83. Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, Garbers DL. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. Proc Natl Acad Sci U S A. 2003;100(25):14869-74.
84. Ito C, Toshimori K. Acrosome markers of human sperm. Anat Sci Int. 2016;91(2):128-42.
85. Hirohashi N, Yanagimachi R. Sperm acrosome reaction: its site and role in fertilization. Biol Reprod. 2018;99(1):127-33.
86. Gupta SK. The human egg's zona pellucida. Current topics in developmental biology. 2018;130:379-411.
87. Takahashi K, Wetzels AM, Goverde HJ, Bastaans BA, Janssen HJ, Rolland R. The kinetics of the acrosome reaction of human spermatozoa and its correlation with in vitro fertilization. Fertil Steril. 1992;57(4):889-94.
88. Oehninger S, Blackmore P, Morshedi M, Sueldo C, Acosta AA, Alexander NJ. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. Fertil Steril. 1994;61(2):349-54.
89. Egeberg Palme DL, Rehfeld A, Bang AK, Nikolova KA, Kjærulff S, Petersen MR, et al. Viable acrosome-intact human spermatozoa in the ejaculate as a marker of semen quality and fertility status. Hum Reprod. 2018;33(3):361-71.

90. Rusz A, Pilatz A, Wagenlehner F, Linn T, Diemer T, Schuppe H, et al. Influence of urogenital infections and inflammation on semen quality and male fertility. World journal of urology. 2012;30(1):23-30.
91. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocrine reviews. 2009;30(4):293-342.
92. Minhas S, Bettocchi C, Boeri L, Capogrosso P, Carvalho J, Cilesiz NC, et al. European Association of Urology Guidelines on Male Sexual and Reproductive Health: 2021 Update on Male Infertility. European Urology. 2021.
93. Jensen CFS, Østergren P, Dupree JM, Ohl DA, Sønksen J, Fode M. Varicocele and male infertility. Nat Rev Urol. 2017;14(9):523-33.
94. Houston BJ, Riera-Escamilla A, Wyrwoll MJ, Salas-Huetos A, Xavier MJ, Nagirnaja L, et al. A systematic review of the validated monogenic causes of human male infertility: 2020 update and a discussion of emerging gene–disease relationships. Human reproduction update. 2022;28(1):15-29.
95. Hawksworth DJ, Szafran AA, Jordan PW, Dobs AS, Herati AS. Infertility in Patients With Klinefelter Syndrome: Optimal Timing for Sperm and Testicular Tissue Cryopreservation. Rev Urol. 2018;20(2):56-62.
96. Pierik FH, Van Ginneken AM, Dohle GR, Vreeburg JT, Weber RF. The advantages of standardized evaluation of male infertility. International journal of andrology. 2000;23(6):340-6.
97. Hamada A, Esteves SC, Agarwal A. Unexplained male infertility: potential causes and management. Human Andrology. 2011;1(1):2-16.
98. Arafa M, Agarwal A, Majzoub A, Panner Selvam MK, Baskaran S, Henkel R, et al. Efficacy of antioxidant supplementation on conventional and advanced sperm function tests in patients with idiopathic male infertility. Antioxidants. 2020;9(3):219.
99. Hamada A, Esteves SC, Nizza M, Agarwal A. Unexplained male infertility: diagnosis and management. International braz j urol. 2012;38(5):576-94.
100. Wang C, Swerdloff RS. Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests. Fertility and sterility. 2014;102(6):1502-7.
101. Esteves SC. Clinical relevance of routine semen analysis and controversies surrounding the 2010 World Health Organization criteria for semen examination. Int Braz J Urol. 2014;40(4):443-53.
102. Izhar R, Masood Z, Husain S, Tahir S. The effect of suboptimal semen parameters on male partner's ability to conceive. Is he really subfertile because the test says so? Journal of human reproductive sciences. 2019;12(4):294.
103. Capogrosso P, Ventimiglia E, Boeri L, Cazzaniga W, Chierigo F, Montorsi F, et al. Male infertility as a proxy of the overall male health status. Minerva Urol Nefrol. 2018;70(3):286-99.
104. Choy JT, Eisenberg ML. Male infertility as a window to health. Fertil Steril. 2018;110(5):810-4.
105. Amaral S, Amaral A, Ramalho-Santos J. Aging and male reproductive function: a mitochondrial perspective. Front Biosci (Schol Ed). 2013;5:181-97.
106. Ford W, North K, Taylor H, Farrow A, Hull M, Golding J. Increasing paternal age is associated with delayed conception in a large population of fertile couples: evidence for declining fecundity in older men. Human Reproduction. 2000;15(8):1703-8.
107. Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S. Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. Ageing research reviews. 2015;19:22-33.
108. Hassan MA, Killick SR. Effect of male age on fertility: evidence for the decline in male fertility with increasing age. Fertil Steril. 2003;79 Suppl 3:1520-7.
109. Mazur DJ, Lipshultz LI. Infertility in the Aging Male. Curr Urol Rep. 2018;19(7):54.
110. Sierra F, Kohanski R. Advances in geroscience: Springer; 2016.
111. Virani SS, Alonso A, Aparicio HJ, Benjamin EJ, Bittencourt MS, Callaway CW, et al. Heart Disease and Stroke Statistics-2021 Update: A Report From the American Heart Association. Circulation. 2021;143(8):e254-e743.
112. Hou Y, Dan X, Babbar M, Wei Y, Hasselbalch SG, Croteau DL, et al. Ageing as a risk factor for neurodegenerative disease. Nat Rev Neurol. 2019;15(10):565-81.
113. Shochat T, Pillar G. Sleep apnoea in the older adult. Drugs & aging. 2003;20(8):551-60.

114. Guo D, Li S, Behr B, Eisenberg ML. Hypertension and Male Fertility. World J Mens Health. 2017;35(2):59-64.
115. Amaral S, Oliveira PJ, Ramalho-Santos J. Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. Curr Diabetes Rev. 2008;4(1):46-54.
116. La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE. Diabetes mellitus and sperm parameters. J Androl. 2012;33(2):145-53.
117. Amaral S, Moreno AJ, Santos MS, Seiça R, Ramalho-Santos J. Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology. 2006;66(9):2056-67.
118. Durairajanayagam D. Lifestyle causes of male infertility. Arab J Urol. 2018;16(1):10-20.
119. Campbell JM, Lane M, Owens JA, Bakos HW. Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. Reprod Biomed Online. 2015;31(5):593-604.
120. La Vignera S, Condorelli RA, Vicari E, Calogero AE. Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters. J Androl. 2012;33(1):53-8.
121. Håkonsen LB, Thulstrup AM, Aggerholm AS, Olsen J, Bonde JP, Andersen CY, et al. Does weight loss improve semen quality and reproductive hormones? Results from a cohort of severely obese men. Reprod Health. 2011;8:24.
122. Skoracka K, Eder P, Łykowska-Szuber L, Dobrowolska A, Krela-Kaźmierczak I. Diet and nutritional factors in male (in) fertility—underestimated factors. Journal of clinical medicine. 2020;9(5):1400.
123. Nassan FL, Chavarro JE, Tanrikut C. Diet and men's fertility: does diet affect sperm quality? Fertil Steril. 2018;110(4):570-7.
124. Ibañez-Perez J, Santos-Zorrozua B, Lopez-Lopez E, Matorras R, Garcia-Orad A. An update on the implication of physical activity on semen quality: a systematic review and meta-analysis. Archives of gynecology and obstetrics. 2019;299(4):901-21.
125. Hajizadeh Maleki B, Tartibian B. Moderate aerobic exercise training for improving reproductive function in infertile patients: A randomized controlled trial. Cytokine. 2017;92:55-67.
126. Damsgaard J, Joensen UN, Carlsen E, Erenpreiss J, Jensen MB, Matulevicius V, et al. Varicocele is associated with impaired semen quality and reproductive hormone levels: a study of 7035 healthy young men from six European countries. European urology. 2016;70(6):1019-29.
127. Agarwal A, Prabakaran S, Allamaneni SS. Relationship between oxidative stress, varicocele and infertility: a meta-analysis. Reprod Biomed Online. 2006;12(5):630-3.
128. de Castro MP, Mastrorocco DA. Reproductive history and semen analysis in prevasectomy fertile men with and without varicocele. J Androl. 1984;5(1):17-20.
129. Agarwal A, Rana M, Qiu E, AlBunni H, Bui AD, Henkel R. Role of oxidative stress, infection and inflammation in male infertility. Andrologia. 2018;50(11):e13126.
130. Gallegos G, Ramos B, Santiso R, Goyanes V, Gosálvez J, Fernández JL. Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. Fertil Steril. 2008;90(2):328-34.
131. Boguen R, Treulen F, Uribe P, Villegas JV. Ability of Escherichia coli to produce hemolysis leads to a greater pathogenic effect on human sperm. Fertil Steril. 2015;103(5):1155-61.
132. La Vignera S, Condorelli RA, Vicari E, D'Agata R, Calogero AE. Sperm DNA damage in patients with chronic viral C hepatitis. Eur J Intern Med. 2012;23(1):e19-24.
133. Domes T, Lo KC, Grober ED, Mullen JB, Mazzulli T, Jarvi K. The incidence and effect of bacteriospermia and elevated seminal leukocytes on semen parameters. Fertil Steril. 2012;97(5):1050-5.
134. Moretti E, Capitani S, Figura N, Pammolli A, Federico MG, Giannerini V, et al. The presence of bacteria species in semen and sperm quality. J Assist Reprod Genet. 2009;26(1):47-56.
135. Gupta S, Krishna A, Jain M, Goyal A, Kumar A, Chaturvedi P, et al. A three-arm randomized study to compare sexual functions and fertility indices following open mesh hernioplasty (OMH),

laparoscopic totally extra peritoneal (TEP) and transabdominal preperitoneal (TAPP) repair of groin hernia. Surg Endosc. 2021;35(6):3077-84.
136. Thorup J, Cortes D. Surgical Management of Undescended Testis - Timetable and Outcome: A Debate. Sex Dev. 2019;13(1):11-9.
137. Kojima Y, Hayashi Y, Mizuno K, Kurokawa S, Nakane A, Maruyama T, et al. Future treatment strategies for cryptorchidism to improve spermatogenesis. Hinyokika kiyo Acta urologica Japonica. 2007;53(7):517-22.
138. Rossi V, Sartori A, Bordin G, Parolini F, Morandi A, Arnoldi R, et al. [Cryptorchidism: medium- and long-term follow-up]. Minerva Pediatr. 2013;65(3):261-9.
139. Shiraishi K, Matsuyama H. Effects of medical comorbidity on male infertility and comorbidity treatment on spermatogenesis. Fertil Steril. 2018;110(6):1006-11.e2.
140. Eisenberg ML, Li S, Behr B, Pera RR, Cullen MR. Relationship between semen production and medical comorbidity. Fertil Steril. 2015;103(1):66-71.
141. Tavares RS, Portela JMD, Sousa MI, Mota PC, Ramalho-Santos J, Amaral S. High glucose levels affect spermatogenesis: an in vitro approach. Reprod Fertil Dev. 2017;29(7):1369-78.
142. An T, Wang YF, Liu JX, Pan YY, Liu YF, He ZC, et al. Comparative analysis of proteomes between diabetic and normal human sperm: Insights into the effects of diabetes on male reproduction based on the regulation of mitochondria-related proteins. Mol Reprod Dev. 2018;85(1):7-16.
143. Wdowiak A, Skrzypek M, Stec M, Panasiuk L. Effect of ionizing radiation on the male reproductive system. Annals of Agricultural and Environmental Medicine. 2019;26(2).
144. Ashiru OA, Odusanya OO. Fertility and occupational hazards: review of the literature. African journal of reproductive health. 2009;13(1).
145. Sifakis S, Androutsopoulos VP, Tsatsakis AM, Spandidos DA. Human exposure to endocrine disrupting chemicals: effects on the male and female reproductive systems. Environmental Toxicology and Pharmacology. 2017;51:56-70.
146. Yucra S, Rubio J, Gasco M, Gonzales C, Steenland K, Gonzales GF. Semen quality and reproductive sex hormone levels in Peruvian pesticide sprayers. International journal of occupational and environmental health. 2006;12(4):355-61.
147. Tavares RS, Escada-Rebelo S, Correia M, Mota PC, Ramalho-Santos J. The non-genomic effects of endocrine-disrupting chemicals on mammalian sperm. Reproduction. 2016;151(1):R1-R13.
148. Tavares RS, Mansell S, Barratt CL, Wilson SM, Publicover SJ, Ramalho-Santos J. p, p′-DDE activates CatSper and compromises human sperm function at environmentally relevant concentrations. Human Reproduction. 2013;28(12):3167-77.
149. Tavares RS, Amaral S, Paiva C, Baptista M, Ramalho-Santos J. In vitro exposure to the organochlorine p, p′-DDE affects functional human sperm parameters. Chemosphere. 2015;120:443-6.
150. Omer H. Radiobiological effects and medical applications of non-ionizing radiation. Saudi J Biol Sci. 2021;28(10):5585-92.
151. Haines GA, Hendry JH, Daniel CP, Morris ID. Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia. Mutat Res. 2001;495(1-2):21-32.
152. Cordelli E, Eleuteri P, Grollino MG, Benassi B, Blandino G, Bartoleschi C, et al. Direct and delayed X-ray-induced DNA damage in male mouse germ cells. Environ Mol Mutagen. 2012;53(6):429-39.
153. Haines GA, Hendry JH, Daniel CP, Morris ID. Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoaa after testicular X-irradiation. Biol Reprod. 2002;67(3):854-61.
154. Gautam R, Priyadarshini E, Nirala J, Rajamani P. Impact of nonionizing electromagnetic radiation on male infertility: an assessment of the mechanism and consequences. Int J Radiat Biol. 2021:1-22.
155. Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y, Mostafa T. In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. Int J Fertil Steril. 2015;9(1):129-36.

156. Wang SM, Peng RY, Gao YB, Ma JJ, Chen HY, Zhou HM, et al. [Pathological study of testicular injury induced by high power microwave radiation in rats]. Zhonghua Nan Ke Xue. 2006;12(6):486-9, 95.
157. Oh JJ, Byun SS, Lee SE, Choe G, Hong SK. Effect of Electromagnetic Waves from Mobile Phones on Spermatogenesis in the Era of 4G-LTE. Biomed Res Int. 2018;2018:1801798.
158. Atasoy HI, Gunal MY, Atasoy P, Elgun S, Bugdayci G. Immunohistopathologic demonstration of deleterious effects on growing rat testes of radiofrequency waves emitted from conventional Wi-Fi devices. J Pediatr Urol. 2013;9(2):223-9.
159. Bonde JP. Semen quality in welders exposed to radiant heat. Occupational and Environmental Medicine. 1992;49(1):5-10.
160. Figà-Talamanca I, Dell'Orco V, Pupi A, Dondero F, Gandini L, Lenzi A, et al. Fertility and semen quality of workers exposed to high temperatures in the ceramics industry. Reprod Toxicol. 1992;6(6):517-23.
161. Durairajanayagam D, Sharma RK, Plessis SSd, Agarwal A. Testicular heat stress and sperm quality. Male infertility: Springer; 2014. p. 105-25.
162. Durairajanayagam D, Agarwal A, Ong C. Causes, effects and molecular mechanisms of testicular heat stress. Reprod Biomed Online. 2015;30(1):14-27.
163. Zhang M, Jiang M, Bi Y, Zhu H, Zhou Z, Sha J. Autophagy and apoptosis act as partners to induce germ cell death after heat stress in mice. PLoS One. 2012;7(7):e41412.
164. Lue YH, Hikim AP, Swerdloff RS, Im P, Taing KS, Bui T, et al. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. Endocrinology. 1999;140(4):1709-17.
165. Lue YH, Lasley BL, Laughlin LS, Swerdloff RS, Hikim AP, Leung A, et al. Mild testicular hyperthermia induces profound transitional spermatogenic suppression through increased germ cell apoptosis in adult cynomolgus monkeys (Macaca fascicularis). J Androl. 2002;23(6):799-805.
166. Yin Y, Hawkins KL, DeWolf WC, Morgentaler A. Heat stress causes testicular germ cell apoptosis in adult mice. J Androl. 1997;18(2):159-65.
167. Ikeda M, Kodama H, Fukuda J, Shimizu Y, Murata M, Kumagai J, et al. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heat stress. Biol Reprod. 1999;61(2):393-9.
168. Wang C, McDonald V, Leung A, Superlano L, Berman N, Hull L, et al. Effect of increased scrotal temperature on sperm production in normal men. Fertil Steril. 1997;68(2):334-9.
169. Hjollund NH, Bonde JP, Jensen TK, Olsen J. Diurnal scrotal skin temperature and semen quality. The Danish First Pregnancy Planner Study Team. Int J Androl. 2000;23(5):309-18.
170. Hjollund NH, Storgaard L, Ernst E, Bonde JP, Olsen J. Impact of diurnal scrotal temperature on semen quality. Reprod Toxicol. 2002;16(3):215-21.
171. Jung A, Eberl M, Schill W. Improvement of semen quality by nocturnal scrotal cooling and moderate behavioural change to reduce genital heat stress in men with oligoasthenoteratozoospermia. Reproduction (Cambridge, England). 2001;121(4):595-603.
172. Nikolopoulos I, Osman W, Haoula Z, Jayaprakasan K, Atiomo W. Scrotal cooling and its benefits to male fertility: a systematic review. J Obstet Gynaecol. 2013;33(4):338-42.
173. Ding X, Yan S, Zhang N, Dong W, Lu H, Tang J, et al. Study on sperm quality of males living in cold area. Zhonghua liu xing bing xue za zhi= Zhonghua liuxingbingxue zazhi. 2003;24(3):206-9; discussion 9.
174. Leite A, Machado A, Pinto S, Nunes B, Matias Dias C. Daily tobacco consumption and associated socioeconomic factors in the Portuguese population: National Health Interview Survey data 1987-2014. Rev Port Cardiol (Engl Ed). 2019;38(8):583-93.
175. Organization WH. Global status report on alcohol and health 2018: World Health Organization; 2019.
176. Ricci E, Al Beitawi S, Cipriani S, Candiani M, Chiaffarino F, Viganò P, et al. Semen quality and alcohol intake: a systematic review and meta-analysis. Reproductive biomedicine online. 2017;34(1):38-47.
177. Sharma R, Harlev A, Agarwal A, Esteves SC. Cigarette smoking and semen quality: a new meta-analysis examining the effect of the 2010 World Health Organization laboratory methods for the examination of human semen. European urology. 2016;70(4):635-45.

178. Anifandis G, Bounartzi T, Messini C, Dafopoulos K, Sotiriou S, Messinis I. The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm®. Archives of gynecology and obstetrics. 2014;290(4):777-82.
179. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril. 2002;78(3):491-9.
180. Aboulmaouahib S, Madkour A, Kaarouch I, Sefrioui O, Saadani B, Copin H, et al. Impact of alcohol and cigarette smoking consumption in male fertility potential: Looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage. Andrologia. 2018;50(3).
181. Colagar AH. Cigarette Smoking and the Risk of Male Infertility* A. Hosseinzadeh Colagar,* GA Jorsaraee and" E. Tahmasbpour Marzony" Department of Biology, Faculty of Basic Science, Mazandaran University, Babolsar, Iran "Department of Fertility and Infertility, Fateme Zahra Hospital. Pakistan journal of biological sciences. 2007;10(21):3870-4.
182. Liu R, Gao J, Zhang H, Wang R, Zhang Z, Liu X. Seminal plasma zinc level may be associated with the effect of cigarette smoking on sperm parameters. Journal of International Medical Research. 2010;38(3):923-8.
183. Rehman R, Zahid N, Amjad S, Baig M, Gazzaz ZJ. Relationship Between Smoking Habit and Sperm Parameters Among Patients Attending an Infertility Clinic. Front Physiol. 2019;10:1356.
184. Calogero A, Polosa R, Perdichizzi A, Guarino F, La Vignera S, Scarfia A, et al. Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis. Reproductive biomedicine online. 2009;19(4):564-71.
185. Prentki Santos E, López-Costa S, Chenlo P, Pugliese M, Curi S, Ariagno J, et al. Impact of spontaneous smoking cessation on sperm quality: case report. Andrologia. 2011;43(6):431-5.
186. Li Y, Lin H, Cao J. Association between socio-psycho-behavioral factors and male semen quality: systematic review and meta-analyses. Fertil Steril. 2011;95(1):116-23.
187. Boeri L, Capogrosso P, Ventimiglia E, Pederzoli F, Cazzaniga W, Chierigo F, et al. Heavy cigarette smoking and alcohol consumption are associated with impaired sperm parameters in primary infertile men. Asian journal of andrology. 2019;21(5):478.
188. Peaston G, Subramanian V, Brunckhorst O, Sarris I, Ahmed K. The impact of emotional health on assisted reproductive technology outcomes: a systematic review and meta-analysis. Hum Fertil (Camb). 2020:1-12.
189. Coelho IL, Sousa-Uva M, Pina N, Marques S, Matias-Dias C, Rodrigues AP. [Economic Crisis in Portugal: Trajectory of the Incidence of Depression and Correlation With Unemployment]. Acta Med Port. 2020.
190. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand. 1983;67(6):361-70.
191. Bhongade MB, Prasad S, Jiloha RC, Ray PC, Mohapatra S, Koner BC. Effect of psychological stress on fertility hormones and seminal quality in male partners of infertile couples. Andrologia. 2015;47(3):336-42.
192. Merari D, Chetrit A, Modan B. Emotional reactions and attitudes prior to in vitro fertilization: an inter-spouse study. Psychology and Health. 2002;17(5):629-40.
193. Yang B, Zhang J, Qi Y, Wang P, Jiang R, Li H. Assessment on Occurrences of Depression and Anxiety and Associated Risk Factors in the Infertile Chinese Men. Am J Mens Health. 2017;11(3):767-74.
194. Hamada A, C Esteves S, Agarwal A. The role of contemporary andrology in unraveling the mystery of unexplained male infertility. The Open Reproductive Science Journal. 2011;3(1).
195. Sun ZM, Ding CF, Yan ZZ, Bao YZ. [Ultrastructure and function of mitochondria in idiopathic asthenospermia: study of 151 cases]. Zhonghua Yi Xue Za Zhi. 2007;87(18):1263-5.
196. Pelliccione F, Micillo A, Cordeschi G, D'Angeli A, Necozione S, Gandini L, et al. Altered ultrastructure of mitochondrial membranes is strongly associated with unexplained asthenozoospermia. Fertil Steril. 2011;95(2):641-6.
197. Kumar R, Venkatesh S, Kumar M, Tanwar M, Shasmsi M, Gupta N, et al. Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. 2009.
198. Pasqualotto FF, Sharma RK, Pasqualotto EB, Agarwal A. Poor semen quality and ROS-TAC scores in patients with idiopathic infertility. Urol Int. 2008;81(3):263-70.

199. Aydemir B, Onaran I, Kiziler AR, Alici B, Akyolcu MC. Increased oxidative damage of sperm and seminal plasma in men with idiopathic infertility is higher in patients with glutathione S-transferase Mu-1 null genotype. Asian J Androl. 2007;9(1):108-15.
200. Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reprod Biomed Online. 2014;29(1):32-58.
201. Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod. 1998;13(4):896-900.
202. Ferlin A, Foresta C. New genetic markers for male infertility. Curr Opin Obstet Gynecol. 2014;26(3):193-8.
203. Shamsi MB, Venkatesh S, Kumar R, Gupta NP, Malhotra N, Singh N, et al. Antioxidant levels in blood and seminal plasma and their impact on sperm parameters in infertile men. Indian J Biochem Biophys. 2010;47(1):38-43.
204. Garcia-Segura S, Ribas-Maynou J, Lara-Cerrillo S, Garcia-Peiró A, Castel AB, Benet J, et al. Relationship of Seminal Oxidation-Reduction Potential with Sperm DNA Integrity and pH in Idiopathic Infertile Patients. Biology (Basel). 2020;9(9).
205. Pourmasumi S, Khoradmehr A, Rahiminia T, Sabeti P, Talebi AR, Ghasemzadeh J. Evaluation of Sperm Chromatin Integrity Using Aniline Blue and Toluidine Blue Staining in Infertile and Normozoospermic Men. J Reprod Infertil. 2019;20(2):95-101.
206. Ghasemi H, Khodadadi I, Fattahi A, Moghimbeigi A, Tavilani H. Polymorphisms of DNA repair genes XRCC1 and LIG4 and idiopathic male infertility. Syst Biol Reprod Med. 2017;63(6):382-90.
207. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod. 2011;26(7):1628-40.
208. Agarwal A, Baskaran S, Parekh N, Cho C-L, Henkel R, Vij S, et al. Male infertility. The Lancet. 2020.
209. Aljuhayman A, Almardawi A, Fallatah M, Alhathal N. Efficacy of follicle-stimulating hormone as a treatment of severe idiopathic oligospermia: A retrospective study. Urology annals. 2020;12(1):69.
210. Casamonti E, Vinci S, Serra E, Fino MG, Brilli S, Lotti F, et al. Short-term FSH treatment and sperm maturation: a prospective study in idiopathic infertile men. Andrology. 2017;5(3):414-22.
211. Palomba S, Falbo A, Espinola S, Rocca M, Capasso S, Cappiello F, et al. Effects of highly purified follicle-stimulating hormone on sperm DNA damage in men with male idiopathic subfertility: a pilot study. J Endocrinol Invest. 2011;34(10):747-52.
212. La Vignera S, Condorelli RA, Duca Y, Mongioi LM, Cannarella R, Giacone F, et al. FSH therapy for idiopathic male infertility: four schemes are better than one. Aging Male. 2020;23(5):750-5.
213. Mongioì LM, Condorelli RA, Alamo A, Cannarella R, Musso N, La Vignera S, et al. Follicle-Stimulating Hormone Treatment and Male Idiopathic Infertility: Effects on Sperm Parameters and Oxidative Stress Indices according to FSHR c. 2039 A/G and c.-29 G/A Genotypes. Journal of Clinical Medicine. 2020;9(6):1690.
214. Chua M, Escusa K, Luna S, Tapia L, Dofitas B, Morales M. Revisiting oestrogen antagonists (clomiphene or tamoxifen) as medical empiric therapy for idiopathic male infertility: a meta-analysis. Andrology. 2013;1(5):749-57.
215. Paffenholz P, Votteler S, Nazari S, Nestler T, Salem J, Grabbert M, et al. Efficacy of the Oestrogen Antagonist Tamoxifen on Sperm Parameters in Patients with Idiopathic Oligoathenoteratozoospermia. Urol Int. 2019;103(1):108-15.
216. Guo L, Jing J, Feng YM, Yao B. Tamoxifen is a potent antioxidant modulator for sperm quality in patients with idiopathic oligoasthenospermia. Int Urol Nephrol. 2015;47(9):1463-9.
217. Micic S, Lalic N, Djordjevic D, Bojanic N, Bogavac-Stanojevic N, Busetto GM, et al. Double-blind, randomised, placebo-controlled trial on the effect of L-carnitine and L-acetylcarnitine on sperm parameters in men with idiopathic oligoasthenozoospermia. Andrologia. 2019;51(6):e13267.
218. Zhang X, Cui Y, Dong L, Sun M, Zhang Y. The efficacy of combined l-carnitine and l-acetyl carnitine in men with idiopathic oligoasthenoteratozoospermia: A systematic review and meta-analysis. Andrologia. 2020;52(2):e13470.

219. Nadjarzadeh A, Sadeghi M, Amirjannati N, Vafa M, Motevalian S, Gohari M, et al. Coenzyme Q 10 improves seminal oxidative defense but does not affect on semen parameters in idiopathic oligoasthenoteratozoospermia: A randomized double-blind, placebo controlled trial. Journal of endocrinological investigation. 2011;34(8):e224-e8.
220. Nadjarzadeh A, Shidfar F, Amirjannati N, Vafa MR, Motevalian SA, Gohari MR, et al. Effect of Coenzyme Q10 supplementation on antioxidant enzymes activity and oxidative stress of seminal plasma: a double-blind randomised clinical trial. Andrologia. 2014;46(2):177-83.
221. Balercia G, Buldreghini E, Vignini A, Tiano L, Paggi F, Amoroso S, et al. Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double-blind randomized trial. Fertil Steril. 2009;91(5):1785-92.
222. Shahid MN, Khan TM, Neoh CF, Lean QY, Bukhsh A, Karuppannan M. Effectiveness of Pharmacological Intervention Among Men with Infertility: A Systematic Review and Network Meta-Analysis. Front Pharmacol. 2021;12:638628.
223. Omar MI, Pal RP, Kelly BD, Bruins HM, Yuan Y, Diemer T, et al. Benefits of empiric nutritional and medical therapy for semen parameters and pregnancy and live birth rates in couples with idiopathic infertility: a systematic review and meta-analysis. European urology. 2019;75(4):615-25.
224. Smits RM, Mackenzie-Proctor R, Yazdani A, Stankiewicz MT, Jordan V, Showell MG. Antioxidants for male subfertility. Cochrane Database Syst Rev. 2019;3:CD007411.
225. Frapsauce C, Pionneau C, Bouley J, Delarouziere V, Berthaut I, Ravel C, et al. Proteomic identification of target proteins in normal but nonfertilizing sperm. Fertility and sterility. 2014;102(2):372-80.
226. Collins JA, Crosignani PG. Unexplained infertility: a review of diagnosis, prognosis, treatment efficacy and management. Int J Gynaecol Obstet. 1992;39(4):267-75.
227. Mayorga-Torres BJ, Cardona-Maya W, Cadavid Á, Camargo M. [Evaluation of sperm functional parameters in normozoospermic infertile individuals]. Actas Urol Esp. 2013;37(4):221-7.
228. Mayorga-Torres BJM, Camargo M, Cadavid Á, du Plessis SS, Cardona Maya WD. Are oxidative stress markers associated with unexplained male infertility? Andrologia. 2017;49(5).
229. Zandieh Z, Vatannejad A, Doosti M, Zabihzadeh S, Haddadi M, Bajelan L, et al. Comparing reactive oxygen species and DNA fragmentation in semen samples of unexplained infertile and healthy fertile men. Ir J Med Sci. 2018;187(3):657-62.
230. Panner Selvam MK, Agarwal A, Pushparaj PN, Baskaran S, Bendou H. Sperm proteome analysis and identification of fertility-associated biomarkers in unexplained male infertility. Genes. 2019;10(7):522.
231. Giacomini E, Ura B, Giolo E, Luppi S, Martinelli M, Garcia RC, et al. Comparative analysis of the seminal plasma proteomes of oligoasthenozoospermic and normozoospermic men. Reproductive biomedicine online. 2015;30(5):522-31.
232. Xu W, Hu H, Wang Z, Chen X, Yang F, Zhu Z, et al. Proteomic characteristics of spermatozoa in normozoospermic patients with infertility. Journal of proteomics. 2012;75(17):5426-36.
233. Tournaye H. Male factor infertility and ART. Asian J Androl. 2012;14(1):103-8.
234. Ljungberg B, Albiges L, Bensalah K, Bex A, Giles R, Hora M. EAU guidelines. Edn. presented at the EAU annual congress Barcelona 2019. EAU Guidelines Office, Arnhem.
235. Buckett W, Sierra S. The management of unexplained infertility: an evidence-based guideline from the Canadian Fertility and Andrology Society. Reprod Biomed Online. 2019;39(4):633-40.
236. Kierszenbaum A, Tres LL. Structural and transcriptional features of the mouse spermatid genome. The Journal of cell biology. 1975;65(2):258-70.
237. Amaral A, Castillo J, Estanyol JM, Ballescà JL, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. Molecular & Cellular Proteomics. 2013;12(2):330-42.
238. Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. Hum Reprod Update. 2014;20(1):40-62.
239. Jodar M, Attardo-Parrinello C, Soler-Ventura A, Barrachina F, Delgado-Dueñas D, Cívico S, et al. Sperm proteomic changes associated with early embryo quality after ICSI. Reprod Biomed Online. 2020;40(5):700-10.

240. Aslam B, Basit M, Nisar MA, Khurshid M, Rasool MH. Proteomics: technologies and their applications. Journal of chromatographic science. 2017;55(2):182-96.
241. Anjo SI, Santa C, Manadas B. Short GeLC-SWATH: A fast and reliable quantitative approach for proteomic screenings. Proteomics. 2015;15(4):757-62.
242. Ludwig C, Gillet L, Rosenberger G, Amon S, Collins BC, Aebersold R. Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. Molecular systems biology. 2018;14(8):e8126.
243. FELIX K. Protamines. Adv Protein Chem. 1960;15:1-56.
244. Shetty J, Naaby-Hansen S, Shibahara H, Bronson R, Flickinger CJ, Herr JC. Human sperm proteome: immunodominant sperm surface antigens identified with sera from infertile men and women. Biology of reproduction. 1999;61(1):61-9.
245. Wang G, Guo Y, Zhou T, Shi X, Yu J, Yang Y, et al. In-depth proteomic analysis of the human sperm reveals complex protein compositions. J Proteomics. 2013;79:114-22.
246. Naglot S, Tomar AK, Singh N, Yadav S. Label-free proteomics of spermatozoa identifies candidate protein markers of idiopathic recurrent pregnancy loss. Reprod Biol. 2021;21(3):100539.
247. Légaré C, Droit A, Fournier F, Bourassa S, Force A, Cloutier F, et al. Investigation of male infertility using quantitative comparative proteomics. J Proteome Res. 2014;13(12):5403-14.
248. Zhao C, Huo R, Wang FQ, Lin M, Zhou ZM, Sha JH. Identification of several proteins involved in regulation of sperm motility by proteomic analysis. Fertil Steril. 2007;87(2):436-8.
249. Martínez-Heredia J, de Mateo S, Vidal-Taboada JM, Ballesca JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. Human reproduction. 2008;23(4):783-91.
250. Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, et al. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. Molecular human reproduction. 2010;16(7):452-62.
251. Amaral A, Paiva C, Attardo Parrinello C, Estanyol JM, Ballescà JL, Ramalho-Santos J, et al. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. J Proteome Res. 2014;13(12):5670-84.
252. Saraswat M, Joenväärä S, Jain T, Tomar AK, Sinha A, Singh S, et al. Human Spermatozoa Quantitative Proteomic Signature Classifies Normo- and Asthenozoospermia. Mol Cell Proteomics. 2017;16(1):57-72.
253. Nowicka-Bauer K, Lepczynski A, Ozgo M, Kamieniczna M, Fraczek M, Stanski L, et al. Sperm mitochondrial dysfunction and oxidative stress as possible reasons for isolated asthenozoospermia. J Physiol Pharmacol. 2018;69(3).
254. Yang J, Liu Q, Yu B, Han B, Yang B. 4D-quantitative proteomics signature of asthenozoospermia and identification of extracellular matrix protein 1 as a novel biomarker for sperm motility. Mol Omics. 2022;18(1):83-91.
255. Qi Q, Pan H, Jiang N, Zhang M, Sun S, Wan X, et al. A novel posttranslational modification of histone, H3 S-sulfhydration, is down-regulated in asthenozoospermic sperm. J Assist Reprod Genet. 2021;38(12):3175-93.
256. Parte PP, Rao P, Redij S, Lobo V, D'Souza SJ, Gajbhiye R, et al. Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC–MSE) reveals altered proteomic signatures in asthenozoospermia. Journal of proteomics. 2012;75(18):5861-71.
257. Chan CC, Shui HA, Wu CH, Wang CY, Sun GH, Chen HM, et al. Motility and protein phosphorylation in healthy and asthenozoospermic sperm. J Proteome Res. 2009;8(11):5382-6.
258. Martin-Hidalgo D, Serrano R, Zaragoza C, Garcia-Marin LJ, Bragado MJ. Human sperm phosphoproteome reveals differential phosphoprotein signatures that regulate human sperm motility. J Proteomics. 2020;215:103654.
259. Paasch U, Heidenreich F, Pursche T, Kuhlisch E, Kettner K, Grunewald S, et al. Identification of increased amounts of eppin protein complex components in sperm cells of diabetic and obese individuals by difference gel electrophoresis. Mol Cell Proteomics. 2011;10(8):M110.007187.
260. Wang J, Wang J, Zhang H-R, Shi H-J, Ma D, Zhao H-X, et al. Proteomic analysis of seminal plasma from asthenozoospermia patients reveals proteins that affect oxidative stress responses and semen quality. Asian journal of andrology. 2009;11(4):484.

261. LECHNER M, WOJNAR P, REDL B. Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system. Biochemical Journal. 2001;356(1):129-35.
262. Sullivan R, Legare C, Thabet M, Thimon V. Gene expression in the epididymis of normal and vasectomized men: what can we learn about human sperm maturation? J Androl. 2011;32(6):686-97.
263. Moscatelli N, Lunetti P, Braccia C, Armirotti A, Pisanello F, De Vittorio M, et al. Comparative proteomic analysis of proteins involved in bioenergetics pathways associated with human sperm motility. International journal of molecular sciences. 2019;20(12):3000.
264. Shen S, Wang J, Liang J, He D. Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia. World J Urol. 2013;31(6):1395-401.
265. Agarwal A, Panner Selvam MK, Samanta L, Vij SC, Parekh N, Sabanegh E, et al. Effect of Antioxidant Supplementation on the Sperm Proteome of Idiopathic Infertile Men. Antioxidants (Basel). 2019;8(10).
266. Azpiazu R, Amaral A, Castillo J, Estanyol JM, Guimerà M, Ballescà JL, et al. High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction. Hum Reprod. 2014;29(6):1225-37.
267. McReynolds S, Dzieciatkowska M, Stevens J, Hansen KC, Schoolcraft WB, Katz-Jaffe MG. Toward the identification of a subset of unexplained infertility: a sperm proteomic approach. Fertil Steril. 2014;102(3):692-9.
268. Seiler A, Schneider M, Förster H, Roth S, Wirth EK, Culmsee C, et al. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. Cell Metab. 2008;8(3):237-48.
269. Portela J, Tavares R, Mota P, Ramalho-Santos J, Amaral S. High glucose concentrations per se do not adversely affect human sperm function in vitro. Reproduction. 2015;150(1):77-84.
270. Sousa APM, Tavares RS, Velez de la Calle JF, Figueiredo H, Almeida V, Almeida-Santos T, et al. Dual use of Diff-Quik-like stains for the simultaneous evaluation of human sperm morphology and chromatin status. Human Reproduction. 2009;24(1):28-36.
271. Mota PC, Ramalho-Santos J. Comparison between different markers for sperm quality in the cat: Diff-Quik as a simple optical technique to assess changes in the DNA of feline epididymal sperm. Theriogenology. 2006;65(7):1360-75.
272. Tavares R, Silva A, Lourenço B, Almeida-Santos T, Sousa A, Ramalho-Santos J. Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization. Andrology. 2013;1(6):830-7.
273. Ramalho-Santos J, Amaral A, Sousa AP, Rodrigues AS, Martins L, Baptista M, et al. Probing the structure and function of mammalian sperm using optical and fluorescence microscopy. Modern research and educational topics in microscopy. 2007;1:394-402.
274. Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. Gamete Research. 1986;15(3):213-26.
275. Marques M, Sousa AP, Paiva A, Almeida-Santos T, Ramalho-Santos J. Low amounts of mitochondrial reactive oxygen species define human sperm quality. Reproduction. 2014;147(6):817-24.
276. Sousa MI, Amaral S, Tavares RS, Paiva C, Ramalho-Santos J. Concentration-dependent Sildenafil citrate (Viagra) effects on ROS production, energy status, and human sperm function. Syst Biol Reprod Med. 2014;60(2):72-9.
277. Snaith RP, Zigmond AS. The hospital anxiey and depression scale with the irritability-depression-anxiety scale and the leeds situational anxiety scale: manual: Nfer-Nelson; 1994.
278. Pais-Ribeiro J, Silva I, Ferreira T, Martins A, Meneses R, Baltar M. Validation study of a Portuguese version of the Hospital Anxiety and Depression Scale. Psychol Health Med. 2007;12(2):225-35; quiz 35-7.
279. Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol Cell Proteomics. 2012;11(6):O111 016717.
280. Tang WH, Shilov IV, Seymour SL. Nonlinear fitting method for determining local false discovery rates from decoy database searches. Journal of proteome research. 2008;7(9):3661-7.

281. Sennels L, Bukowski-Wills JC, Rappsilber J. Improved results in proteomics by use of local and peptide-class specific false discovery rates. BMC bioinformatics. 2009;10:179.
282. Lambert J-P, Ivosev G, Couzens AL, Larsen B, Taipale M, Lin Z-Y, et al. Mapping differential interactomes by affinity purification coupled with data-independent mass spectrometry acquisition. Nature methods. 2013.
283. Anjo SI, Simões I, Castanheira P, Grãos M, Manadas B. Use of recombinant proteins as a simple and robust normalization method for untargeted proteomics screening: exhaustive performance assessment. Talanta. 2019;205:120163.
284. Collins BC, Gillet LC, Rosenberger G, Röst HL, Vichalkovski A, Gstaiger M, et al. Quantifying protein interaction dynamics by SWATH mass spectrometry: application to the 14-3-3 system. Nature methods. 2013;10(12):1246-53.
285. Mi H, Muruganujan A, Huang X, Ebert D, Mills C, Guo X, et al. Protocol Update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). Nat Protoc. 2019;14(3):703-21.
286. Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
287. Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res. 2021;49(D1):D605-D12.
288. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. Curr Protoc Bioinformatics. 2019;68(1):e86.
289. Høst E, Lindenberg S, Ernst E, Christensen F. DNA strand breaks in human spermatozoa: a possible factor, to be considered in couples suffering from unexplained infertility. Acta Obstet Gynecol Scand. 1999;78(7):622-5.
290. Schinfeld J, Sharara F, Morris R, Palermo GD, Rosenwaks Z, Seaman E, et al. Cap-Score™ prospectively predicts probability of pregnancy. Mol Reprod Dev. 2018;85(8-9):654-64.
291. Peedicayil J, Deendayal M, Sadasivan G, Shivaji S. Assessment of hyperactivation, acrosome reaction and motility characteristics of spermatozoa from semen of men of proven fertility and unexplained infertility. Andrologia. 1997;29(4):209-18.
292. Malivindi R, Santoro M, De Rose D, Panza S, Gervasi S, Rago V, et al. Activated-farnesoid X receptor (FXR) expressed in human sperm alters its fertilising ability. Reproduction. 2018;156(3):249-59.
293. Luo T, Chen HY, Zou QX, Wang T, Cheng YM, Wang HF, et al. A novel copy number variation in CATSPER2 causes idiopathic male infertility with normal semen parameters. Hum Reprod. 2019;34(3):414-23.
294. Künzle R, Mueller MD, Hänggi W, Birkhäuser MH, Drescher H, Bersinger NA. Semen quality of male smokers and nonsmokers in infertile couples. Fertility and sterility. 2003;79(2):287-91.
295. Santana VP, James ER, Miranda-Furtado CL, de Souza MF, Pompeu CP, Esteves SC, et al. Differential DNA methylation pattern and sperm quality in men with varicocele. Fertility and Sterility. 2020;114(4):770-8.
296. Tahamtan S, Tavalaee M, Izadi T, Barikrow N, Zakeri Z, Lockshin RA, et al. Reduced sperm telomere length in individuals with varicocele is associated with reduced genomic integrity. Scientific reports. 2019;9(1):1-7.
297. Ammar O, Tekeya O, Hannachi I, Sallem A, Haouas Z, Mehdi M. Increased sperm DNA fragmentation in infertile men with varicocele: relationship with apoptosis, seminal oxidative stress, and spermatic parameters. Reproductive Sciences. 2021;28(3):909-19.
298. Vellani E, Colasante A, Mamazza L, Minasi MG, Greco E, Bevilacqua A. Association of state and trait anxiety to semen quality of in vitro fertilization patients: a controlled study. Fertil Steril. 2013;99(6):1565-72.
299. Bártolo A, Reis S, Monteiro S, Leite R, Montenegro N. Psychological Adjustment of Infertile Men Undergoing Fertility Treatments: An Association With Sperm Parameters. Arch Psychiatr Nurs. 2016;30(5):521-6.

300. Safarinejad MR. Sperm DNA damage and semen quality impairment after treatment with selective serotonin reuptake inhibitors detected using semen analysis and sperm chromatin structure assay. J Urol. 2008;180(5):2124-8.
301. Tanrikut C, Feldman AS, Altemus M, Paduch DA, Schlegel PN. Adverse effect of paroxetine on sperm. Fertil Steril. 2010;94(3):1021-6.
302. Mazzilli R, Curto M, De Bernardini D, Olana S, Capi M, Salerno G, et al. Psychotropic Drugs Levels in Seminal Fluid: A New Therapeutic Drug Monitoring Analysis? Front Endocrinol (Lausanne). 2021;12:620936.
303. Sylvester C, Menke M, Gopalan P. Selective Serotonin Reuptake Inhibitors and Fertility: Considerations for Couples Trying to Conceive. Harv Rev Psychiatry. 2019;27(2):108-18.
304. Peterson BD, Newton CR, Feingold T. Anxiety and sexual stress in men and women undergoing infertility treatment. Fertil Steril. 2007;88(4):911-4.
305. Steger K, Balhorn R. Sperm nuclear protamines: A checkpoint to control sperm chromatin quality. Anat Histol Embryol. 2018;47(4):273-9.
306. Seppälä M, Koistinen H, Koistinen R, Mandelin E, Oehninger S, Clark GF, et al. Glycodelins: role in regulation of reproduction, potential for contraceptive development and diagnosis of male infertility. Hum Reprod. 1998;13 Suppl 3:262-9l; discussion 70.
307. Chiu PC, Chung M-K, Tsang H-Y, Koistinen R, Koistinen H, Seppala M, et al. Glycodelin-S in Human Seminal Plasma Reduces Cholesterol Efflux andInhibits Capacitation ofSpermatozoa. Journal of Biological Chemistry. 2005;280(27):25580-9.
308. Uchida H, Maruyama T, Nishikawa-Uchida S, Miyazaki K, Masuda H, Yoshimura Y. Glycodelin in reproduction. Reprod Med Biol. 2013;12(3):79-84.
309. Murdica V, Cermisoni GC, Zarovni N, Salonia A, Viganò P, Vago R. Proteomic analysis reveals the negative modulator of sperm function glycodelin as over-represented in semen exosomes isolated from asthenozoospermic patients. Hum Reprod. 2019;34(8):1416-27.
310. Lilja H, Abrahamsson PA, Lundwall A. Semenogelin, the predominant protein in human semen. Primary structure and identification of closely related proteins in the male accessory sex glands and on the spermatozoa. J Biol Chem. 1989;264(3):1894-900.
311. Mitra A, Richardson RT, O'Rand MG. Analysis of recombinant human semenogelin as an inhibitor of human sperm motility. Biology of reproduction. 2010;82(3):489-96.
312. Yoshida K, Yamasaki T, Yoshiike M, Takano S, Sato I, Iwamoto T. Quantification of seminal plasma motility inhibitor/semenogelin in human seminal plasma. J Androl. 2003;24(6):878-84.
313. de Lamirande E, Yoshida K, Yoshiike TM, Iwamoto T, Gagnon C. Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. J Androl. 2001;22(4):672-9.
314. Mandal A, Bhattacharyya AK. Andrology: Sperm hyaluronidase activation by purified predominant and major basic human seminal coagulum proteins. Human Reproduction. 1995;10(7):1745-50.
315. Sun W, Grassi P, Engström A, Sooriyaarachchi S, Ubhayasekera W, Hreinsson J, et al. N-glycans of human protein C inhibitor: tissue-specific expression and function. PLoS One. 2011;6(12):e29011.
316. Heidary Z, Zaki-Dizaji M, Saliminejad K, Khorramkhorshid HR. Expression Analysis of the CRISP2, CATSPER1, PATE1 and SEMG1 in the Sperm of Men with Idiopathic Asthenozoospermia. J Reprod Infertil. 2019;20(2):70-5.
317. Sharma R, Agarwal A, Mohanty G, Jesudasan R, Gopalan B, Willard B, et al. Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. Reproductive Biology and Endocrinology. 2013;11(1):1-20.
318. Zylbersztejn DS, Andreoni C, Del Giudice PT, Spaine DM, Borsari L, Souza GHMF, et al. Proteomic analysis of seminal plasma in adolescents with and without varicocele. Fertil Steril. 2013;99(1):92-8.
319. Ayala-Sanmartin J, Zibouche M, Illien F, Vincent M, Gallay J. Insight into the location and dynamics of the annexin A2 N-terminal domain during Ca(2+)-induced membrane bridging. Biochim Biophys Acta. 2008;1778(2):472-82.

320. Munuce MJ, Marini PE, Teijeiro JM. Expression profile and distribution of Annexin A1, A2 and A5 in human semen. Andrologia. 2019;51(2):e13224.
321. Chojnacka K, Bilinska B, Mruk DD. Annexin A2 is critical for blood-testis barrier integrity and spermatid disengagement in the mammalian testis. Biochim Biophys Acta Mol Cell Res. 2017;1864(3):527-45.
322. Teijeiro JM, Ignotz GG, Marini PE. Annexin A2 is involved in pig (Sus scrofa)sperm-oviduct interaction. Mol Reprod Dev. 2009;76(4):334-41.
323. Ignotz GG, Cho MY, Suarez SS. Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. Biol Reprod. 2007;77(6):906-13.
324. Jodar M, Kalko S, Castillo J, Ballescà JL, Oliva R. Differential RNAs in the sperm cells of asthenozoospermic patients. Human reproduction. 2012;27(5):1431-8.
325. Sun R, Shen R, Li J, Xu G, Chi J, Li L, et al. Lyzl4, a novel mouse sperm-related protein, is involved in fertilization. Acta Biochim Biophys Sin. 2011;43(5):346-53.
326. Huang P, Qian N, DU WC, Shi WJ, Sun QW, Zhang N. [The role of human lysozyme-like protein 4 in fertilization and its enzymatic properties]. Zhonghua Nan Ke Xue. 2018;24(2):109-15.
327. Lin Y-N, Roy A, Yan W, Burns KH, Matzuk MM. Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Molecular and cellular biology. 2007;27(19):6794-805.
328. Yatsenko AN, O'Neil DS, Roy A, Arias-Mendoza PA, Chen R, Murthy LJ, et al. Association of mutations in the zona pellucida binding protein 1 (ZPBP1) gene with abnormal sperm head morphology in infertile men. Molecular human reproduction. 2012;18(1):14-21.
329. Liu X, Liu G, Liu J, Zhu P, Wang J, Wang Y, et al. iTRAQ-based analysis of sperm proteome from normozoospermic men achieving the rescue-ICSI pregnancy after the IVF failure. Clinical proteomics. 2018;15(1):1-15.
330. Peña FJ, Ortiz-Rodríguez JM, Gaitskell-Phillips GL, Gil MC, Ortega-Ferrusola C, Martín-Cano FE. An integrated overview on the regulation of sperm metabolism (glycolysis-Krebs cycle-oxidative phosphorylation). Animal Reproduction Science. 2021:106805.
331. Wu X, Dong Z, Wang CJ, Barlow LJ, Fako V, Serrano MA, et al. FASN regulates cellular response to genotoxic treatments by increasing PARP-1 expression and DNA repair activity via NF-κB and SP1. Proc Natl Acad Sci U S A. 2016;113(45):E6965-E73.
332. Lin R, Tao R, Gao X, Li T, Zhou X, Guan KL, et al. Acetylation stabilizes ATP-citrate lyase to promote lipid biosynthesis and tumor growth. Mol Cell. 2013;51(4):506-18.
333. Intasqui P, Camargo M, Antoniassi MP, Cedenho AP, Carvalho VM, Cardozo KHM, et al. Association between the seminal plasma proteome and sperm functional traits. Fertil Steril. 2016;105(3):617-28.
334. Intasqui P, Camargo M, Del Giudice PT, Spaine DM, Carvalho VM, Cardozo KH, et al. Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation. J Assist Reprod Genet. 2013;30(9):1187-202.
335. Panner Selvam MK, Agarwal A, Baskaran S. Proteomic analysis of seminal plasma from bilateral varicocele patients indicates an oxidative state and increased inflammatory response. Asian J Androl. 2019;21(6):544-50.
336. Quijano C, Trujillo M, Castro L, Trostchansky A. Interplay between oxidant species and energy metabolism. Redox Biol. 2016;8:28-42.
337. Ryu DY, Kim KU, Kwon WS, Rahman MS, Khatun A, Pang MG. Peroxiredoxin activity is a major landmark of male fertility. Sci Rep. 2017;7(1):17174.
338. Gong S, San Gabriel MC, Zini A, Chan P, O'Flaherty C. Low amounts and high thiol oxidation of peroxiredoxins in spermatozoa from infertile men. J Androl. 2012;33(6):1342-51.
339. Kwon WS, Rahman MS, Lee JS, Kim J, Yoon SJ, Park YJ, et al. A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics. 2014;15:897.
340. Rahman MS, Kwon WS, Lee JS, Yoon SJ, Ryu BY, Pang MG. Bisphenol-A affects male fertility via fertility-related proteins in spermatozoa. Sci Rep. 2015;5:9169.

341. Ozkosem B, Feinstein SI, Fisher AB, O'Flaherty C. Absence of Peroxiredoxin 6 Amplifies the Effect of Oxidant Stress on Mobility and SCSA/CMA3 Defined Chromatin Quality and Impairs Fertilizing Ability of Mouse Spermatozoa. Biol Reprod. 2016;94(3):68.
342. Cannarella R, Barbagallo F, Crafa A, La Vignera S, Condorelli RA, Calogero AE. Seminal Plasma Transcriptome and Proteome: Towards a Molecular Approach in the Diagnosis of Idiopathic Male Infertility. Int J Mol Sci. 2020;21(19).
343. Hamada A, Sharma R, du Plessis SS, Willard B, Yadav SP, Sabanegh E, et al. Two-dimensional differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress. Fertil Steril. 2013;99(5):1216-26.e2.
344. ZINKHAM WH, BLANCO A, CLOWRY LJ. AN UNUSUAL ISOZYME OF LACTATE DEHYDROGENASE IN MATURE TESTES: LOCALIZATION, ONTOGENY, AND KINETIC PROPERTIES. Ann N Y Acad Sci. 1964;121:571-88.
345. Goldberg E. The sperm-specific form of lactate dehydrogenase is required for fertility and is an attractive target for male contraception (a review). Biol Reprod. 2021;104(3):521-6.
346. Liu X, Li Q, Wang W, Liu F. Aberrant expression of sperm-specific glycolytic enzymes are associated with poor sperm quality. Mol Med Rep. 2019;19(4):2471-8.
347. Odet F, Gabel S, London RE, Goldberg E, Eddy EM. Glycolysis and mitochondrial respiration in mouse LDHC-null sperm. Biol Reprod. 2013;88(4):95.
348. Velasco JAN, Zapata IT, Hernandez PM, Albacete MP, Oltra JT, Paricio JJP. Lactic dehydrogenase-C4 activity in seminal plasma and male infertility. Fertility and sterility. 1993;60(2):331-5.
349. Römisch J, Pâques EP. Annexins: calcium-binding proteins of multi-functional importance? Med Microbiol Immunol. 1991;180(3):109-26.
350. Gerke V, Moss SE. Annexins: from structure to function. Physiol Rev. 2002;82(2):331-71.
351. Li J, Liu F, Liu X, Liu J, Zhu P, Wan F, et al. Mapping of the human testicular proteome and its relationship with that of the epididymis and spermatozoa. Mol Cell Proteomics. 2011;10(3):M110.004630.
352. Guyonnet B, Dacheux F, Dacheux JL, Gatti JL. The epididymal transcriptome and proteome provide some insights into new epididymal regulations. J Androl. 2011;32(6):651-64.
353. Haigler HT, Christmas P. Annexin 1 is secreted by the human prostate. Biochem Soc Trans. 1990;18(6):1104-6.
354. Casares-Crespo L, Talaván AM, Viudes-de-Castro MP. Can the Genetic Origin Affect Rabbit Seminal Plasma Protein Profile along the Year? Reprod Domest Anim. 2016;51(2):294-300.
355. Arruda-Alencar J, Viana-Neto A, Souza C, Martins J, Moreno F, Moreira A, et al., editors. Major proteins of the seminal plasma of New Zealand white rabbits and association with semen criteria. 10th World Rabbit Congress World Rabbit Science Association, Sharm El-Sheikh, Egypt; 2012.
356. Okabe M, Kishi Y, Ying X, Kohama Y, Mimura T, Li SS. Characterization of capacitation inhibitory protein from rabbit seminal plasma: homology with human annexins. Biol Pharm Bull. 1993;16(5):453-6.
357. Tao XQ, Liu HY, Shi SS, Han XF, Lin CY, Yao B. [Expression of rat annexin 5 and its effect on human sperm motility in vitro]. Zhonghua Nan Ke Xue. 2010;16(5):400-4.
358. Lu R, Guo C, Tao XQ, Liu HY, Shi SS, Lin CY, et al. [Protective effect of Annexin 5 on human sperm membrane and DNA integrity]. Zhonghua Nan Ke Xue. 2011;17(1):17-20.
359. Lan Y, Wang J, Zhang Q, Yang X, Li L, Yin J, et al. Genetic variations and haplotypes in the annexin A5 gene are associated with the risk of recurrent pregnancy loss. J Cell Physiol. 2019;234(10):18308-16.
360. Rand JH, Wu XX, Quinn AS, Taatjes DJ. The annexin A5-mediated pathogenic mechanism in the antiphospholipid syndrome: role in pregnancy losses and thrombosis. Lupus. 2010;19(4):460-9.
361. Schousboe I. beta 2-Glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. 1985.
362. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proceedings of the National Academy of Sciences. 1990;87(11):4120-4.

363. Polz E, Kostner GM. The binding of beta 2-glycoprotein-I to human serum lipoproteins: distribution among density fractions. FEBS Lett. 1979;102(1):183-6.
364. Jégou B, Pineau C, Dupaix A. Paracrine control of testis function. Male reproductive function: Springer; 1999. p. 41-64.
365. Giuffrida V, Pezzino FM, Romano F, Litrico L, Garofalo MR, Nicotra G, et al. Gene expression in mouse spermatogenesis during ontogenesis. International journal of molecular medicine. 2006;17(3):523-8.
366. Shima JE, McLean DJ, McCarrey JR, Griswold MD. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod. 2004;71(1):319-30.
367. Chalmel F, Com E, Lavigne R, Hernio N, Teixeira-Gomes AP, Dacheux JL, et al. An integrative omics strategy to assess the germ cell secretome and to decipher sertoli-germ cell crosstalk in the Mammalian testis. PLoS One. 2014;9(8):e104418.
368. Aleporou-Marinou V, Pappa H, Yalouris P, Patargias T. Purification of apolipoprotein H (beta2-glycoprotein I)-like protein from human follicular fluid. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2001;128(3):537-42.
369. Pignatelli P, Ettorre E, Menichelli D, Pani A, Violi F, Pastori D. Seronegative antiphospholipid syndrome: refining the value of "non-criteria" antibodies for diagnosis and clinical management. Haematologica. 2020;105(3):562.
370. Lopes MRU, Danowski A, Funke A, Rêgo J, Levy R, Andrade DCO. Update on antiphospholipid antibody syndrome. Rev Assoc Med Bras (1992). 2017;63(11):994-9.
371. Khefacha L, Rassas R, Bergaoui H, Mustapha H, Faleh R, Sakly N, et al. Antiphospholipid antibodies in the obstetric antiphospholipid syndrome: Detection and antibodies profile at the Maternity and Neonatal Medicine Center of Monastir, Tunisia. J Obstet Gynaecol Res. 2022.
372. Pleguezuelo DE, Cabrera-Marante O, Abad M, Rodriguez-Frias EA, Naranjo L, Vazquez A, et al. Anti-Phosphatidylserine/Prothrombin Antibodies in Healthy Women with Unexplained Recurrent Pregnancy Loss. J Clin Med. 2021;10(10).
373. Ingenbleek Y, Young V. Transthyretin (prealbumin) in health and disease: nutritional implications. Annu Rev Nutr. 1994;14:495-533.
374. Vranckx R, Savu L, Maya M, Nunez EA. Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. Biochem J. 1990;271(2):373-9.
375. Wolf G. Multiple functions of vitamin A. Physiol Rev. 1984;64(3):873-937.
376. Goodman DS. Overview of current knowledge of metabolism of vitamin A and carotenoids. J Natl Cancer Inst. 1984;73(6):1375-9.
377. Secciani F, Bianchi L, Ermini L, Cianti R, Armini A, La Sala GB, et al. Protein profile of capacitated versus ejaculated human sperm. J Proteome Res. 2009;8(7):3377-89.
378. Lopes RA, Coelho T, Barros A, Sousa M. Corino de Andrade disease: mechanisms and impact on reproduction. JBRA Assist Reprod. 2017;21(2):105-14.
379. Krajewska-Kulak E, Sengupta P. Thyroid function in male infertility. Front Endocrinol (Lausanne). 2013;4:174.
380. La Vignera S, Vita R. Thyroid dysfunction and semen quality. Int J Immunopathol Pharmacol. 2018;32:2058738418775241.
381. Leung AM. Thyroid function in pregnancy. J Trace Elem Med Biol. 2012;26(2-3):137-40.
382. Roncaglia N, Avagliano L, Crippa I, Cameroni I, Malberti S, Sala F, et al. Preeclampsia is strongly associated with maternal hypothyroidism. American Journal of Obstetrics & Gynecology. 2006;195(6):S109.
383. Schousboe I. Binding of beta 2-glycoprotein I to platelets: effect of adenylate cyclase activity. Thromb Res. 1980;19(1-2):225-37.
384. Nimpf J, Wurm H, Kostner GM. Beta 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. Atherosclerosis. 1987;63(2-3):109-14.
385. van Genderen HO, Kenis H, Hofstra L, Narula J, Reutelingsperger CP. Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. Biochim Biophys Acta. 2008;1783(6):953-63.

386. Lwaleed BA, Greenfield R, Stewart A, Birch B, Cooper AJ. Seminal clotting and fibrinolytic balance: a possible physiological role in the male reproductive system. Thromb Haemost. 2004;92(4):752-66.
387. Beeckmans S. Some structural and regulatory aspects of citrate synthase. Int J Biochem. 1984;16(4):341-51.
388. Verschueren KHG, Blanchet C, Felix J, Dansercoer A, De Vos D, Bloch Y, et al. Structure of ATP citrate lyase and the origin of citrate synthase in the Krebs cycle. Nature. 2019;568(7753):571-5.
389. Bajpai M, Gupta G, Setty BS. Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol. 1998;138(3):322-7.
390. Medrano A, Fernández-Novell JM, Ramió L, Alvarez J, Goldberg E, Montserrat Rivera M, et al. Utilization of citrate and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa. Mol Reprod Dev. 2006;73(3):369-78.
391. Harada Y, Matsumoto T, Hirahara S, Nakashima A, Ueno S, Oda S, et al. Characterization of a sperm factor for egg activation at fertilization of the newt Cynops pyrrhogaster. Dev Biol. 2007;306(2):797-808.
392. Chen L, Wen CW, Deng MJ, Ping-Li, Zhang ZD, Zhou ZH, et al. Metabolic and transcriptional changes in seminal plasma of asthenozoospermia patients. Biomed Chromatogr. 2020;34(3):e4769.
393. Novak S, Smith TA, Paradis F, Burwash L, Dyck MK, Foxcroft GR, et al. Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. Theriogenology. 2010;74(6):956-67.
394. Kang W, Harada Y, Yamatoya K, Kawano N, Kanai S, Miyamoto Y, et al. Extra-mitochondrial citrate synthase initiates calcium oscillation and suppresses age-dependent sperm dysfunction. Lab Invest. 2020;100(4):583-95.
395. George DF, Bilek MM, McKenzie DR. Non-Thermal effects in the microwave induced unfolding of proteins observed by chaperone binding. Bioelectromagnetics: Journal of the Bioelectromagnetics Society, The Society for Physical Regulation in Biology and Medicine, The European Bioelectromagnetics Association. 2008;29(4):324-30.
396. Kato K, Shinohara H, Kurobe N, Inaguma Y, Shimizu K, Ohshima K. Tissue distribution and developmental profiles of immunoreactive alpha B crystallin in the rat determined with a sensitive immunoassay system. Biochim Biophys Acta. 1991;1074(1):201-8.
397. Sun J, Yin B, Tang S, Zhang X, Xu J, Bao E. Vitamin C mitigates heat damage by reducing oxidative stress, inducing HSP expression in TM4 Sertoli cells. Mol Reprod Dev. 2019;86(6):673-85.
398. Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. J Reprod Immunol. 2010;84(1):32-40.
399. Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS. The role of heat shock proteins in reproduction. Hum Reprod Update. 2000;6(2):149-59.
400. Allen RL, O'Brien DA, Jones CC, Rockett DL, Eddy EM. Expression of heat shock proteins by isolated mouse spermatogenic cells. Mol Cell Biol. 1988;8(8):3260-6.
401. Eddy EM. Role of heat shock protein HSP70-2 in spermatogenesis. Rev Reprod. 1999;4(1):23-30.
402. Son WY, Hwang SH, Han CT, Lee JH, Kim S, Kim YC. Specific expression of heat shock protein HspA2 in human male germ cells. Mol Hum Reprod. 1999;5(12):1122-6.
403. Adly MA, Assaf HA, Hussein MR. Heat shock protein 27 expression in the human testis showing normal and abnormal spermatogenesis. Cell Biol Int. 2008;32(10):1247-55.
404. Boulanger J, Faulds D, Eddy EM, Lingwood CA. Members of the 70 kDa heat shock protein family specifically recognize sulfoglycolipids: role in gamete recognition and mycoplasma-related infertility. J Cell Physiol. 1995;165(1):7-17.
405. Miller D, Brough S, al-Harbi O. Characterization and cellular distribution of human spermatozoal heat shock proteins. Hum Reprod. 1992;7(5):637-45.
406. Santiago J, Silva JV, Fardilha M. First Insights on the Presence of the Unfolded Protein Response in Human Spermatozoa. Int J Mol Sci. 2019;20(21).
407. Renkawek K, Voorter C, Bosman G, Van Workum F, De Jong W. Expression of αB-crystallin in Alzheimer's disease. Acta neuropathologica. 1994;87(2):155-60.
408. Iwaki T, Kume-Iwaki A, Liem RK, Goldman JE. αB-crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. Cell. 1989;57(1):71-8.

409. Zhang J, Liu J, Wu J, Li W, Chen Z, Yang L. Progression of the role of CRYAB in signaling pathways and cancers. OncoTargets and therapy. 2019;12:4129.
410. Shamsi MB, Venkatesh S, Tanwar M, Talwar P, Sharma RK, Dhawan A, et al. DNA integrity and semen quality in men with low seminal antioxidant levels. Mutat Res. 2009;665(1-2):29-36.
411. Wang X, Sharma RK, Gupta A, George V, Thomas AJ, Falcone T, et al. Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. Fertil Steril. 2003;80 Suppl 2:844-50.
412. Bouter A, Carmeille R, Gounou C, Bouvet F, Degrelle SA, Evain-Brion D, et al. Review: Annexin-A5 and cell membrane repair. Placenta. 2015;36 Suppl 1:S43-9.
413. Nakaya Y, Schaefer EJ, Brewer Jr HB. Activation of human post heparin lipoprotein lipase by apolipoprotein H (β2-glycoprotein I). Biochemical and biophysical research communications. 1980;95(3):1168-72.
414. Yan W, Assadi AH, Wynshaw-Boris A, Eichele G, Matzuk MM, Clark GD. Previously uncharacterized roles of platelet-activating factor acetylhydrolase 1b complex in mouse spermatogenesis. Proc Natl Acad Sci U S A. 2003;100(12):7189-94.
415. Sengoku K, Tamate K, Takaoka Y, Ishikawa M. Effects of platelet activating factor on human sperm function in vitro. Hum Reprod. 1993;8(9):1443-7.
416. Jarvi K, Roberts KD, Langlais J, Gagnon C. Effect of platelet-activating factor, lyso-platelet-activating factor, and lysophosphatidylcholine on sperm motion: importance of albumin for motility stimulation. Fertil Steril. 1993;59(6):1266-75.
417. Krausz C, Gervasi G, Forti G, Baldi E. Effect of platelet-activating factor on motility and acrosome reaction of human spermatozoa. Hum Reprod. 1994;9(3):471-6.
418. Howes E, Hurst S, Jones R. Actin and actin-binding proteins in bovine spermatozoa: potential role in membrane remodeling and intracellular signaling during epididymal maturation and the acrosome reaction. Journal of andrology. 2001;22(1):62-72.
419. Megnagi B, Finkelstein M, Shabtay O, Breitbart H. The role and importance of cofilin in human sperm capacitation and the acrosome reaction. Cell Tissue Res. 2015;362(3):665-75.
420. Hirono H. Lipids of myelin, white matter and gray matter in a case of generalized deficiency of cytochrome b5 reductase in congenital methemoglobinemia with mental retardation. Lipids. 1980;15(4):272-5.
421. Martin-Montalvo A, Sun Y, Diaz-Ruiz A, Ali A, Gutierrez V, Palacios HH, et al. Cytochrome b 5 reductase and the control of lipid metabolism and healthspan. npj Aging and Mechanisms of Disease. 2016;2(1):1-12.
422. Martínez-Heredia J, Estanyol JM, Ballescà JL, Oliva R. Proteomic identification of human sperm proteins. Proteomics. 2006;6(15):4356-69.
423. Baker MA, Reeves G, Hetherington L, Müller J, Baur I, Aitken RJ. Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. Proteomics Clin Appl. 2007;1(5):524-32.
424. Baker MA, Naumovski N, Hetherington L, Weinberg A, Velkov T, Aitken RJ. Head and flagella subcompartmental proteomic analysis of human spermatozoa. Proteomics. 2013;13(1):61-74.
425. de Mateo S, Castillo J, Estanyol JM, Ballescà JL, Oliva R. Proteomic characterization of the human sperm nucleus. Proteomics. 2011;11(13):2714-26.
426. Leahy T, Gadella BM. New insights into the regulation of cholesterol efflux from the sperm membrane. Asian J Androl. 2015;17(4):561-7.
427. Sparacino-Watkins CE, Tejero J, Sun B, Gauthier MC, Thomas J, Ragireddy V, et al. Nitrite reductase and nitric-oxide synthase activity of the mitochondrial molybdopterin enzymes mARC1 and mARC2. J Biol Chem. 2014;289(15):10345-58.
428. Roan NR, Sandi-Monroy N, Kohgadai N, Usmani SM, Hamil KG, Neidleman J, et al. Semen amyloids participate in spermatozoa selection and clearance. Elife. 2017;6.
429. Díaz-Flores L, Gutiérrez R, García MDP, Gayoso MJ, Carrasco JL, Álvarez-Argüelles H. Localized amyloidosis of the epididymis: a previously unreported phenomenon. Diagn Pathol. 2017;12(1):58.

430. Adams D, Ando Y, Beirão JM, Coelho T, Gertz MA, Gillmore JD, et al. Expert consensus recommendations to improve diagnosis of ATTR amyloidosis with polyneuropathy. J Neurol. 2021;268(6):2109-22.
431. Conceição I, González-Duarte A, Obici L, Schmidt HH, Simoneau D, Ong ML, et al. "Red-flag" symptom clusters in transthyretin familial amyloid polyneuropathy. J Peripher Nerv Syst. 2016;21(1):5-9.

6. Annex

Annex I – Sequential window acquisition of all theoretical mass spectra method

	m/z range	Width (Da)	CES
Window 1	349.5-394.1	44.6	5
Window 2	393.1-415.3	22.2	5
Window 3	414.3-427	12.7	5
Window 4	426-431.9	5.9	5
Window 5	430.9-436	5.1	5
Window 6	435-439.6	4.6	5
Window 7	438.6-443.2	4.6	5
Window 8	442.2-446.3	4.1	5
Window 9	445.3-449.9	4.6	5
Window 10	448.9-453.1	4.2	5
Window 11	452.1-456.2	4.1	5
Window 12	455.2-459.4	4.2	5
Window 13	458.4-462.4	4	5
Window 14	461.1-465.2	4.1	5
Window 15	464.2-468.4	4.2	5
Window 16	467.4-471.4	4	5
Window 17	470.1-474.2	4.1	5
Window 18	473.2-477.2	4	5
Window 19	475.9-480.1	4.2	5
Window 20	479.1-483.1	4	5
Window 21	481.8-485.8	4	5
Window 22	484.5-488.6	4.1	5
Window 23	487.6-491.6	4	5
Window 24	490.3-494.9	4.6	5
Window 25	493.9-499	5.1	5
Window 26	498-503.5	5.5	5
Window 27	502.5-507.5	5	5

Window 28	506.5-512	5.5	5
Window 29	511-516.1	5.1	5
Window 30	515.1-520.1	5	5
Window 31	519.1-523.7	4.6	5
Window 32	522.7-527.8	5.1	5
Window 33	526.8-530.9	4.1	5
Window 34	529.9-534.1	4.2	5
Window 35	533.1-537.1	4	5
Window 36	535.8-539.8	4	5
Window 37	538.5-542.5	4	5
Window 38	540.7-544.7	4	5
Window 39	543.7-547.7	4	5
Window 40	546.7-550.7	4	5
Window 41	549.7-553.7	4	5
Window 42	552.7-556.7	4	5
Window 43	555.7-559.7	4	5
Window 44	558.7-562.7	4	5
Window 45	561.7-565.7	4	5
Window 46	564.7-568.7	4	5
Window 47	567.7-571.7	4	5
Window 48	570.7-574.7	4	5
Window 49	573.7-577.7	4	5
Window 50	576.7-580.7	4	5
Window 51	579.7-583.7	4	5
Window 52	582.7-586.7	4	5
Window 53	585.7-589.7	4	5
Window 54	588.7-592.7	4	5
Window 55	591.7-595.7	4	5
Window 56	594.7-598.7	4	5
Window 57	597.7-601.7	4	5

Window 58	600.7-604.7	4	5
Window 59	603.7-607.7	4	5
Window 60	606.7-610.7	4	5
Window 61	609.7-613.7	4	5
Window 62	612.7-616.7	4	5
Window 63	615.7-619.7	4	5
Window 64	618.7-622.7	4	5
Window 65	620.9-624.9	4	5
Window 66	623.1-627.1	4	5
Window 67	625.8-629.8	4	5
Window 68	628.1-632.1	4	5
Window 69	630.8-634.8	4	5
Window 70	633-637	4	5
Window 71	635.7-639.7	4	5
Window 72	638.4-642.4	4	5
Window 73	641.1-645.1	4	5
Window 74	643.8-648	4.2	5
Window 75	647-651	4	5
Window 76	649.7-653.7	4	5
Window 77	652.4-656.5	4.1	5
Window 78	655.5-659.7	4.2	5
Window 79	658.7-663.3	4.6	5
Window 80	662.3-666.9	4.6	5
Window 81	665.9-670.5	4.6	5
Window 82	669.5-674.1	4.6	5
Window 83	673.1-677.7	4.6	5
Window 84	676.7-681.3	4.6	5
Window 85	680.3-684.9	4.6	5
Window 86	683.9-688.5	4.6	5
Window 87	687.5-692.1	4.6	5

Window 88	691.1-696.1	5	5
Window 89	695.1-700.6	5.5	5
Window 90	699.6-704.7	5.1	5
Window 91	703.7-708.7	5	5
Window 92	707.7-712.3	4.6	5
Window 93	711.3-715.5	4.2	5
Window 94	714.5-719.1	4.6	5
Window 95	718.1-722.7	4.6	5
Window 96	721.7-725.8	4.1	5
Window 97	724.8-729.4	4.6	5
Window 98	728.4-733	4.6	5
Window 99	732-736.2	4.2	5
Window 100	735.2-739.2	4	5
Window 101	737.9-742	4.1	5
Window 102	741-745	4	5
Window 103	743.7-747.9	4.2	5
Window 104	746.9-751	4.1	5
Window 105	750-754	4	5
Window 106	752.7-756.9	4.2	5
Window 107	755.9-760	4.1	5
Window 108	759-763.2	4.2	5
Window 109	762.2-766.2	4	5
Window 110	764.9-769	4.1	5
Window 111	768-772.6	4.6	5
Window 112	771.6-775.8	4.2	5
Window 113	774.8-779.4	4.6	5
Window 114	778.4-783	4.6	5
Window 115	782-786.1	4.1	5
Window 116	785.1-789.3	4.2	5
Window 117	788.3-792.4	4.1	5

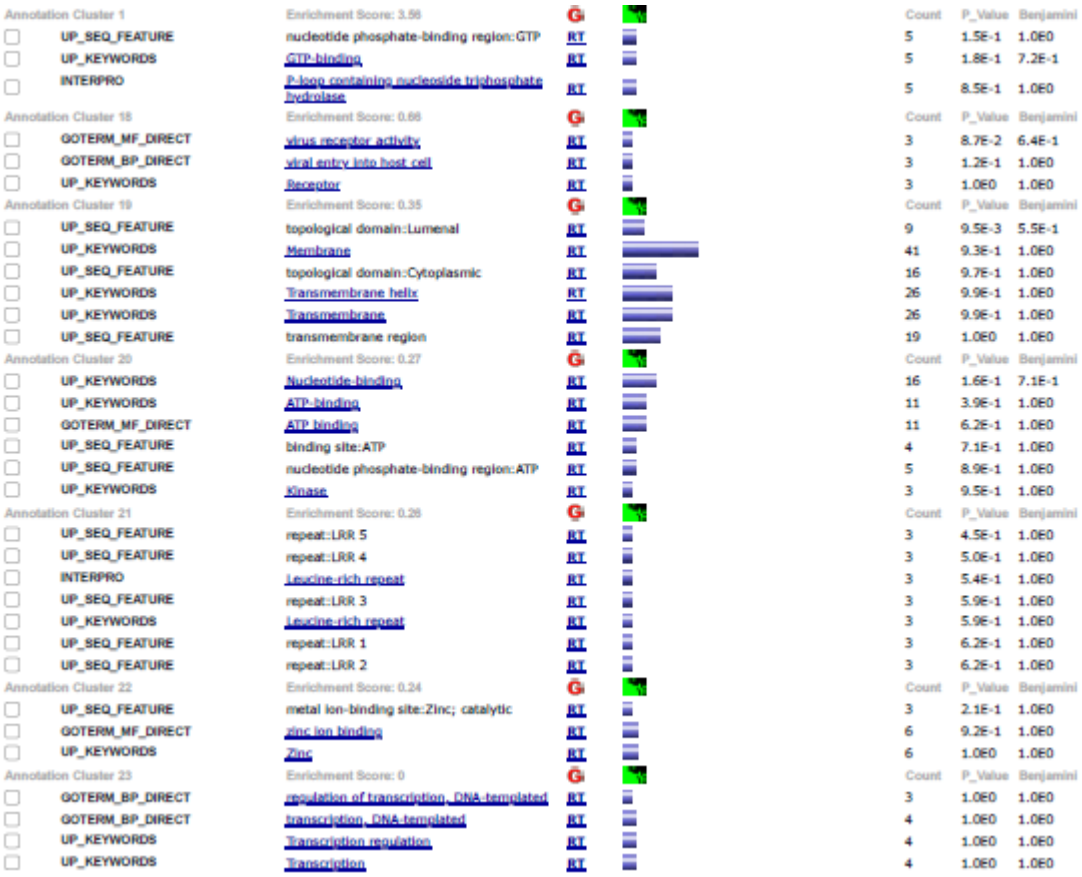
Window 118	791.4-795.6	4.2	5
Window 119	794.6-799.2	4.6	5
Window 120	798.2-802.8	4.6	8
Window 121	801.8-807.3	5.5	8
Window 122	806.3-811.3	5	8
Window 123	810.3-815.8	5.5	8
Window 124	814.8-820.3	5.5	8
Window 125	819.3-824.8	5.5	8
Window 126	823.8-829.3	5.5	8
Window 127	828.3-833.8	5.5	8
Window 128	832.8-838.3	5.5	8
Window 129	837.3-843.3	6	8
Window 130	842.3-848.2	5.9	8
Window 131	847.2-853.2	6	8
Window 132	852.2-857.7	5.5	8
Window 133	856.7-861.7	5	8
Window 134	860.7-866.2	5.5	8
Window 135	865.2-870.7	5.5	8
Window 136	869.7-875.2	5.5	8
Window 137	874.2-880.2	6	8
Window 138	879.2-884.7	5.5	8
Window 139	883.7-889.2	5.5	8
Window 140	888.2-894.1	5.9	8
Window 141	893.1-898.6	5.5	8
Window 142	897.6-903.1	5.5	8
Window 143	902.1-908.1	6	8
Window 144	907.1-913	5.9	8
Window 145	912-919.3	7.3	8
Window 146	918.3-927.9	9.6	8
Window 147	926.9-936.4	9.5	8

Window 148	935.4-945.4	10	8
Window 149	944.4-955.3	10.9	8
Window 150	954.3-965.2	10.9	8
Window 151	964.2-975.6	11.4	8
Window 152	974.6-986.8	12.2	8
Window 153	985.8-999.4	13.6	8
Window 154	998.4-1011.6	13.2	10
Window 155	1010.6-1023.3	12.7	10
Window 156	1022.3-1036.8	14.5	10
Window 157	1035.8-1051.6	15.8	10
Window 158	1050.6-1067.4	16.8	10
Window 159	1066.4-1084.5	18.1	10
Window 160	1083.5-1103.4	19.9	10
Window 161	1102.4-1121.4	19	10
Window 162	1120.4-1139.8	19.4	10
Window 163	1138.8-1159.6	20.8	10
Window 164	1158.6-1181.7	23.1	10
Window 165	1180.7-1205.1	24.4	10
Window 166	1204.1-1228	23.9	10
Window 167	1227-1249.6	22.6	10
Window 168	1248.6-1252.6	4	10

Annex II – Results of functional annotation clustering by DAVID on CTRLvsID protein set

Annotation Cluster 1	Enrichment Score: 3.56			Count	P_Value	Benjamini
UP_KEYWORDS	Secreted	RT		34	1.5E-7	1.9E-5
UP_SEQ_FEATURE	signal peptide	RT		44	4.4E-6	2.0E-3
UP_KEYWORDS	Signal	RT		49	9.4E-6	6.1E-4
UP_KEYWORDS	Glycoprotein	RT		44	3.0E-3	5.3E-2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		40	1.2E-2	5.7E-1
UP_SEQ_FEATURE	disulfide bond	RT		29	2.2E-2	7.5E-1
UP_KEYWORDS	Disulfide bond	RT		32	2.5E-2	2.9E-1
Annotation Cluster 2	Enrichment Score: 3.01			Count	P_Value	Benjamini
SMART	ANX	RT		4	8.1E-5	5.1E-3
UP_KEYWORDS	Annexin	RT		4	8.7E-5	3.6E-3
INTERPRO	Annexin repeat, conserved site	RT		4	1.1E-4	1.3E-2
INTERPRO	Annexin repeat	RT		4	1.1E-4	1.3E-2
INTERPRO	Annexin	RT		4	1.1E-4	1.3E-2
UP_KEYWORDS	Calcium/phospholipid-binding	RT		4	1.1E-4	3.8E-3
UP_SEQ_FEATURE	repeat:Annexin 1	RT		4	1.2E-4	9.1E-3
UP_SEQ_FEATURE	repeat:Annexin 2	RT		4	1.2E-4	9.1E-3
UP_SEQ_FEATURE	repeat:Annexin 3	RT		4	1.2E-4	9.1E-3
UP_SEQ_FEATURE	repeat:Annexin 4	RT		4	1.2E-4	9.1E-3
GOTERM_MF_DIRECT	phospholipase inhibitor activity	RT		3	2.6E-3	6.9E-2
GOTERM_MF_DIRECT	calcium-dependent phospholipid binding	RT		4	8.0E-3	1.4E-1
GOTERM_BP_DIRECT	negative regulation of catalytic activity	RT		4	1.8E-2	5.5E-1
GOTERM_MF_DIRECT	calcium-dependent protein binding	RT		3	6.3E-2	5.3E-1
UP_KEYWORDS	Calcium	RT		8	3.3E-1	9.8E-1
GOTERM_MF_DIRECT	calcium ion binding	RT		6	5.7E-1	1.0E0
Annotation Cluster 3	Enrichment Score: 2.78			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	translational elongation	RT		4	2.9E-4	5.4E-2
UP_KEYWORDS	Elongation factor	RT		4	9.0E-4	2.0E-2
GOTERM_MF_DIRECT	translation elongation factor activity	RT		4	1.7E-3	6.2E-2
UP_KEYWORDS	Protein biosynthesis	RT		5	1.6E-2	2.2E-1
Annotation Cluster 4	Enrichment Score: 1.97			Count	P_Value	Benjamini
INTERPRO	NAD(P)-binding domain	RT		8	2.2E-4	2.1E-2
UP_KEYWORDS	NAD	RT		6	5.3E-3	8.1E-2
UP_KEYWORDS	Oxidoreductase	RT		9	3.3E-2	3.4E-1
GOTERM_MF_DIRECT	oxidoreductase activity	RT		5	5.3E-2	4.9E-1
GOTERM_BP_DIRECT	oxidation-reduction process	RT		9	6.9E-2	1.0E0
Annotation Cluster 5	Enrichment Score: 1.87			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	proteolysis	RT		11	3.7E-3	2.8E-1
UP_SEQ_FEATURE	propeptide:Activation peptide	RT		4	1.4E-2	5.7E-1
UP_KEYWORDS	Zymogen	RT		5	4.8E-2	3.8E-1
Annotation Cluster 6	Enrichment Score: 1.84			Count	P_Value	Benjamini
INTERPRO	Thioredoxin-like fold	RT		6	1.9E-3	1.4E-1
GOTERM_BP_DIRECT	cell redox homeostasis	RT		4	1.9E-2	5.7E-1
INTERPRO	Thioredoxin domain	RT		3	3.2E-2	4.9E-1
UP_KEYWORDS	Redox-active center	RT		3	3.9E-2	3.5E-1
Annotation Cluster 7	Enrichment Score: 1.75			Count	P_Value	Benjamini
KEGG_PATHWAY	Protein processing in endoplasmic reticulum	RT		9	2.6E-4	3.6E-2
UP_KEYWORDS	Chaperone	RT		7	1.8E-3	3.8E-2
GOTERM_BP_DIRECT	regulation of mRNA stability	RT		5	6.9E-3	3.7E-1
GOTERM_MF_DIRECT	unfolded protein binding	RT		5	7.6E-3	1.4E-1
UP_KEYWORDS	Stress response	RT		4	2.6E-2	2.9E-1
GOTERM_BP_DIRECT	protein folding	RT		5	4.3E-2	8.5E-1
KEGG_PATHWAY	Estrogen signaling pathway	RT		4	7.7E-2	1.0E0
GOTERM_BP_DIRECT	regulation of cellular response to heat	RT		3	1.0E-1	1.0E0
KEGG_PATHWAY	MAPK signaling pathway	RT		3	7.3E-1	1.0E0
Annotation Cluster 8	Enrichment Score: 1.75			Count	P_Value	Benjamini
INTERPRO	Thioredoxin-like fold	RT		6	1.9E-3	1.4E-1
INTERPRO	Glutathione S-transferase, N-terminal	RT		3	1.3E-2	4.9E-1
UP_SEQ_FEATURE	domain:GST C-terminal	RT		3	2.4E-2	7.5E-1
GOTERM_MF_DIRECT	glutathione transferase activity	RT		3	2.5E-2	3.3E-1
INTERPRO	Glutathione S-transferase, C-terminal-like	RT		3	3.2E-2	4.9E-1
GOTERM_BP_DIRECT	glutathione metabolic process	RT		3	6.3E-2	9.5E-1
Annotation Cluster 9	Enrichment Score: 1.41			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	serine-type peptidase activity	RT		5	1.0E-3	4.6E-2

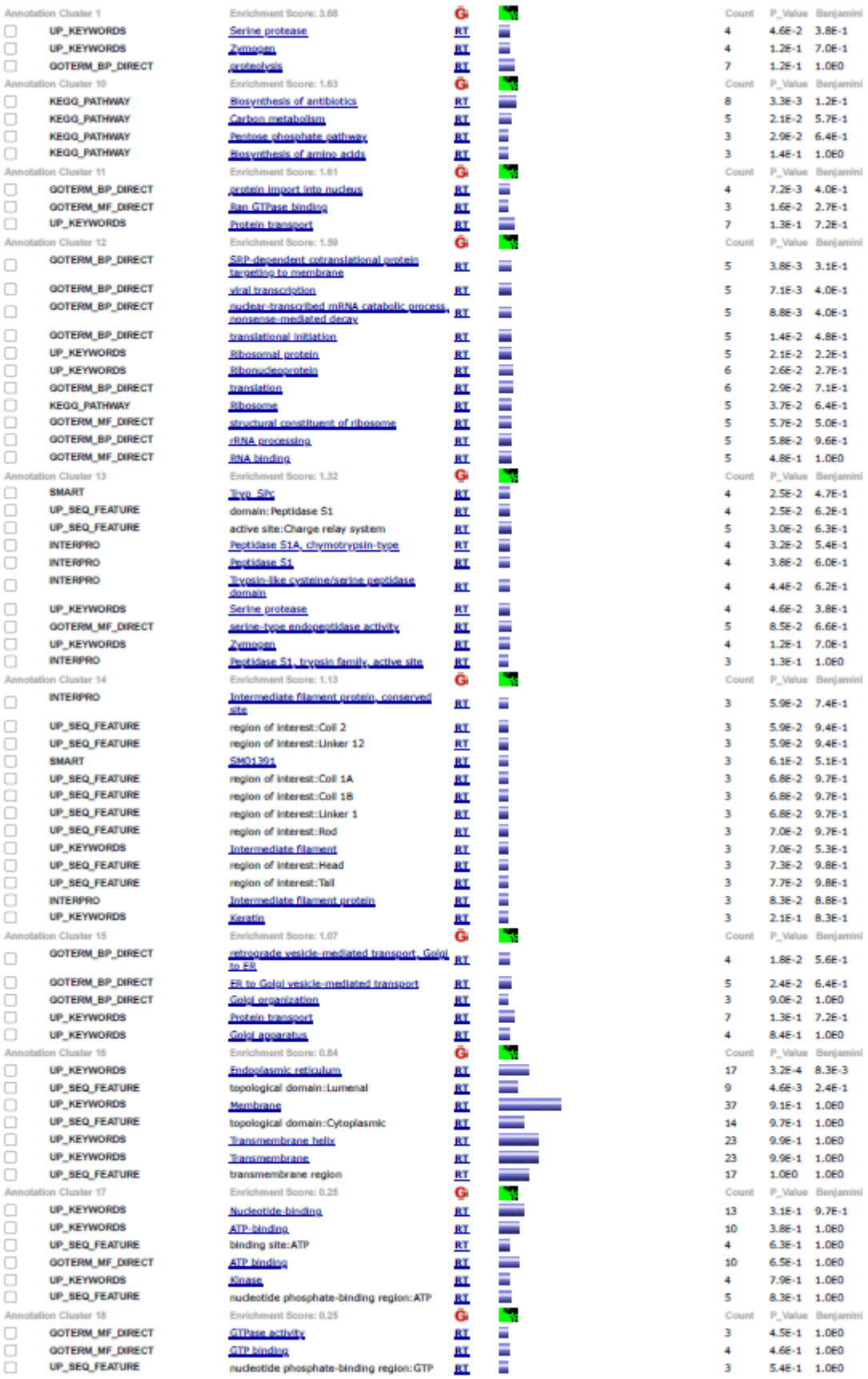
Annotation Cluster 1	Enrichment Score: 3.56			Count	P_Value	Benjamini
UP_SEQ_FEATURE	domain:Peptidase S1	RT		4	3.5E-2	8.6E-1
SMART	Tryp_SPc	RT		4	3.8E-2	5.9E-1
INTERPRO	Peptidase S1A, chymotrypsin-type	RT		4	4.2E-2	5.7E-1
UP_SEQ_FEATURE	active site:Charge relay system	RT		5	4.4E-2	9.1E-1
UP_KEYWORDS	Zymogen	RT		5	4.8E-2	3.8E-1
INTERPRO	Peptidase S1	RT		4	4.8E-2	6.2E-1
INTERPRO	Trypsin-like cysteine/serine peptidase domain	RT		4	5.7E-2	6.5E-1
UP_KEYWORDS	Serine protease	RT		4	6.1E-2	4.6E-1
GOTERM_MF_DIRECT	serine-type endopeptidase activity	RT		5	1.1E-1	7.2E-1
INTERPRO	Peptidase S1, trypsin family, active site	RT		3	1.5E-1	1.0E0
Annotation Cluster 10	Enrichment Score: 1.4			Count	P_Value	Benjamini
UP_KEYWORDS	Hemostasis	RT		3	3.7E-2	3.5E-1
UP_KEYWORDS	Blood coagulation	RT		3	3.7E-2	3.5E-1
GOTERM_BP_DIRECT	blood coagulation	RT		5	4.6E-2	8.5E-1
Annotation Cluster 11	Enrichment Score: 1.37			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	protease binding	RT		6	7.4E-4	4.2E-2
INTERPRO	Protease inhibitor I4, serpin, conserved site	RT		3	2.1E-2	4.9E-1
SMART	SERPIN	RT		3	2.2E-2	5.2E-1
INTERPRO	Serpin domain	RT		3	2.7E-2	4.9E-1
INTERPRO	Serpin family	RT		3	2.7E-2	4.9E-1
UP_SEQ_FEATURE	site:Reactive bond	RT		3	3.5E-2	8.6E-1
GOTERM_BP_DIRECT	blood coagulation	RT		5	4.6E-2	8.5E-1
UP_KEYWORDS	Serine protease inhibitor	RT		3	1.0E-1	6.1E-1
GOTERM_MF_DIRECT	serine-type endopeptidase inhibitor activity	RT		3	1.5E-1	8.9E-1
KEGG_PATHWAY	Complement and coagulation cascades	RT		3	1.5E-1	1.0E0
UP_KEYWORDS	Protease inhibitor	RT		3	1.9E-1	7.2E-1
GOTERM_BP_DIRECT	negative regulation of endopeptidase activity	RT		3	2.2E-1	1.0E0
Annotation Cluster 12	Enrichment Score: 1.36			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	hydrolase activity, hydrolyzing O-glycosyl compounds	RT		3	1.9E-2	2.6E-1
UP_SEQ_FEATURE	active site:Nucleophile	RT		5	4.0E-2	8.8E-1
INTERPRO	Glycoside hydrolase, superfamily	RT		3	5.2E-2	6.3E-1
UP_KEYWORDS	Glycosidase	RT		3	9.6E-2	6.1E-1
Annotation Cluster 13	Enrichment Score: 1.15			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	ER to Golgi vesicle-mediated transport	RT		6	6.5E-3	3.7E-1
GOTERM_BP_DIRECT	Golgi organization	RT		4	1.7E-2	5.5E-1
GOTERM_BP_DIRECT	retrograde vesicle-mediated transport, Golgi to ER	RT		4	2.2E-2	6.2E-1
UP_KEYWORDS	Protein transport	RT		7	1.9E-1	7.2E-1
GOTERM_BP_DIRECT	protein transport	RT		5	3.3E-1	1.0E0
UP_KEYWORDS	Golgi apparatus	RT		5	7.6E-1	1.0E0
Annotation Cluster 14	Enrichment Score: 1.04			Count	P_Value	Benjamini
INTERPRO	Intermediate filament protein, conserved site	RT		3	7.0E-2	7.2E-1
UP_SEQ_FEATURE	region of interest:Coil 2	RT		3	7.3E-2	1.0E0
UP_SEQ_FEATURE	region of interest:Linker 12	RT		3	7.3E-2	1.0E0
SMART	SM01391	RT		3	8.0E-2	6.0E-1
UP_SEQ_FEATURE	region of interest:Linker 1	RT		3	8.4E-2	1.0E0
UP_SEQ_FEATURE	region of interest:Coil 1A	RT		3	8.4E-2	1.0E0
UP_SEQ_FEATURE	region of interest:Coil 1B	RT		3	8.4E-2	1.0E0
UP_SEQ_FEATURE	region of interest:Rod	RT		3	8.6E-2	1.0E0
UP_KEYWORDS	Intermediate filament	RT		3	8.6E-2	6.1E-1
UP_SEQ_FEATURE	region of interest:Head	RT		3	9.0E-2	1.0E0
UP_SEQ_FEATURE	region of interest:Tail	RT		3	9.4E-2	1.0E0
INTERPRO	Intermediate filament protein	RT		3	9.8E-2	8.1E-1
UP_KEYWORDS	Keratin	RT		3	2.5E-1	8.6E-1
Annotation Cluster 15	Enrichment Score: 1.01			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	SRP-dependent cotranslational protein targeting to membrane	RT		4	3.2E-2	8.2E-1
GOTERM_BP_DIRECT	viral transcription	RT		4	4.9E-2	8.5E-1
GOTERM_BP_DIRECT	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	RT		4	5.7E-2	8.9E-1
GOTERM_BP_DIRECT	translational initiation	RT		4	8.0E-2	1.0E0
UP_KEYWORDS	Ribosomal protein	RT		4	1.1E-1	6.1E-1
GOTERM_BP_DIRECT	translation	RT		5	1.2E-1	1.0E0
UP_KEYWORDS	Ribonucleoprotein	RT		5	1.2E-1	6.1E-1
KEGG_PATHWAY	Ribosome	RT		4	1.6E-1	1.0E0
GOTERM_MF_DIRECT	structural constituent of ribosome	RT		4	2.1E-1	1.0E0
GOTERM_BP_DIRECT	rRNA processing	RT		4	2.1E-1	1.0E0
Annotation Cluster 16	Enrichment Score: 0.99			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	peptide catabolic process	RT		3	1.1E-2	4.9E-1
COG_ONTOLOGY	Amino acid transport and metabolism	RT		3	3.9E-2	1.8E-1
GOTERM_MF_DIRECT	metalloendopeptidase activity	RT		3	1.1E-1	7.4E-1
UP_KEYWORDS	Metalloprotease	RT		3	2.5E-1	8.6E-1
GOTERM_MF_DIRECT	zinc ion binding	RT		6	9.2E-1	1.0E0
Annotation Cluster 17	Enrichment Score: 0.72			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	GTPase activity	RT		5	8.4E-2	6.4E-1
GOTERM_MF_DIRECT	GTP binding	RT		6	1.4E-1	8.7E-1



				Count	P_Value	Benjamini
Annotation Cluster 1	Enrichment Score: 3.56					
UP_SEQ_FEATURE	nucleotide phosphate-binding region:GTP	RT		5	1.5E-1	1.0E0
UP_KEYWORDS	GTP-binding	RT		5	1.8E-1	7.2E-1
INTERPRO	P-loop containing nucleoside triphosphate hydrolase	RT		5	8.5E-1	1.0E0
Annotation Cluster 18	Enrichment Score: 0.66			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	virus receptor activity	RT		3	8.7E-2	6.4E-1
GOTERM_BP_DIRECT	viral entry into host cell	RT		3	1.2E-1	1.0E0
UP_KEYWORDS	Receptor	RT		3	1.0E0	1.0E0
Annotation Cluster 19	Enrichment Score: 0.35			Count	P_Value	Benjamini
UP_SEQ_FEATURE	topological domain:Lumenal	RT		9	9.5E-3	5.5E-1
UP_KEYWORDS	Membrane	RT		41	9.3E-1	1.0E0
UP_SEQ_FEATURE	topological domain:Cytoplasmic	RT		16	9.7E-1	1.0E0
UP_KEYWORDS	Transmembrane helix	RT		26	9.9E-1	1.0E0
UP_KEYWORDS	Transmembrane	RT		26	9.9E-1	1.0E0
UP_SEQ_FEATURE	transmembrane region	RT		19	1.0E0	1.0E0
Annotation Cluster 20	Enrichment Score: 0.27			Count	P_Value	Benjamini
UP_KEYWORDS	Nucleotide-binding	RT		16	1.6E-1	7.1E-1
UP_KEYWORDS	ATP-binding	RT		11	3.9E-1	1.0E0
GOTERM_MF_DIRECT	ATP binding	RT		11	6.2E-1	1.0E0
UP_SEQ_FEATURE	binding site:ATP	RT		4	7.1E-1	1.0E0
UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	RT		5	8.9E-1	1.0E0
UP_KEYWORDS	Kinase	RT		3	9.5E-1	1.0E0
Annotation Cluster 21	Enrichment Score: 0.26			Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:LRR 5	RT		3	4.5E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 4	RT		3	5.0E-1	1.0E0
INTERPRO	Leucine-rich repeat	RT		3	5.4E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 3	RT		3	5.9E-1	1.0E0
UP_KEYWORDS	Leucine-rich repeat	RT		3	5.9E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 1	RT		3	6.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 2	RT		3	6.2E-1	1.0E0
Annotation Cluster 22	Enrichment Score: 0.24			Count	P_Value	Benjamini
UP_SEQ_FEATURE	metal ion-binding site:Zinc; catalytic	RT		3	2.1E-1	1.0E0
GOTERM_MF_DIRECT	zinc ion binding	RT		6	9.2E-1	1.0E0
UP_KEYWORDS	Zinc	RT		6	1.0E0	1.0E0
Annotation Cluster 23	Enrichment Score: 0			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	regulation of transcription, DNA-templated	RT		3	1.0E0	1.0E0
GOTERM_BP_DIRECT	transcription, DNA-templated	RT		4	1.0E0	1.0E0
UP_KEYWORDS	Transcription regulation	RT		4	1.0E0	1.0E0
UP_KEYWORDS	Transcription	RT		4	1.0E0	1.0E0

Annex III – Results of functional annotation clustering by DAVID on UMIvsID protein set

Annotation Cluster 1	Enrichment Score: 3.68			Count	P_Value	Benjamini
UP_KEYWORDS	Secreted	RT		31	3.0E-7	3.5E-5
UP_SEQ_FEATURE	signal peptide	RT		39	1.6E-5	6.7E-3
UP_KEYWORDS	Signal	RT		44	1.9E-5	1.1E-3
UP_KEYWORDS	Glycoprotein	RT		42	7.6E-4	1.6E-2
UP_SEQ_FEATURE	disulfide bond	RT		29	4.1E-3	2.4E-1
UP_KEYWORDS	Disulfide bond	RT		32	4.2E-3	6.1E-2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		36	1.4E-2	4.7E-1
Annotation Cluster 2	Enrichment Score: 3.12			Count	P_Value	Benjamini
SMART	ANX	RT		4	5.1E-5	2.9E-3
UP_KEYWORDS	Annexin	RT		4	6.1E-5	2.4E-3
UP_KEYWORDS	Calcium/phospholipid-binding	RT		4	7.6E-5	2.5E-3
INTERPRO	Annexin	RT		4	7.8E-5	9.1E-3
INTERPRO	Annexin repeat, conserved site	RT		4	7.8E-5	9.1E-3
INTERPRO	Annexin repeat	RT		4	7.8E-5	9.1E-3
UP_SEQ_FEATURE	repeat:Annexin 1	RT		4	8.1E-5	6.7E-3
UP_SEQ_FEATURE	repeat:Annexin 2	RT		4	8.1E-5	6.7E-3
UP_SEQ_FEATURE	repeat:Annexin 3	RT		4	8.1E-5	6.7E-3
UP_SEQ_FEATURE	repeat:Annexin 4	RT		4	8.1E-5	6.7E-3
GOTERM_MF_DIRECT	phospholipase inhibitor activity	RT		3	2.2E-3	5.7E-2
GOTERM_MF_DIRECT	calcium-dependent phospholipid binding	RT		4	6.4E-3	1.5E-1
GOTERM_BP_DIRECT	negative regulation of catalytic activity	RT		4	1.4E-2	4.8E-1
GOTERM_MF_DIRECT	calcium-dependent protein binding	RT		3	5.5E-2	5.0E-1
UP_KEYWORDS	Calcium	RT		7	3.7E-1	1.0E0
GOTERM_MF_DIRECT	calcium ion binding	RT		6	5.0E-1	1.0E0
Annotation Cluster 3	Enrichment Score: 2.91			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	translational elongation	RT		4	2.3E-4	4.2E-2
UP_KEYWORDS	Elongation factor	RT		4	6.3E-4	1.5E-2
GOTERM_MF_DIRECT	translation elongation factor activity	RT		4	1.4E-3	4.9E-2
UP_KEYWORDS	Protein biosynthesis	RT		5	1.1E-2	1.4E-1
Annotation Cluster 4	Enrichment Score: 2.05			Count	P_Value	Benjamini
KEGG_PATHWAY	Protein processing in endoplasmic reticulum	RT		8	9.1E-4	4.6E-2
UP_KEYWORDS	Chaperone	RT		6	5.7E-3	7.8E-2
GOTERM_BP_DIRECT	protein folding	RT		5	3.4E-2	7.6E-1
GOTERM_MF_DIRECT	unfolded protein binding	RT		4	3.5E-2	3.9E-1
Annotation Cluster 5	Enrichment Score: 1.95			Count	P_Value	Benjamini
INTERPRO	Thioredoxin-like fold	RT		6	1.2E-3	8.7E-2
GOTERM_BP_DIRECT	cell redox homeostasis	RT		4	1.5E-2	4.9E-1
INTERPRO	Thioredoxin domain	RT		3	2.6E-2	5.3E-1
UP_KEYWORDS	Redox-active center	RT		3	3.1E-2	3.0E-1
Annotation Cluster 6	Enrichment Score: 1.92			Count	P_Value	Benjamini
UP_SEQ_FEATURE	active site:Proton donor	RT		8	1.6E-4	1.1E-2
GOTERM_MF_DIRECT	hydrolase activity, hydrolyzing O-glycosyl compounds	RT		3	1.6E-2	2.7E-1
UP_SEQ_FEATURE	active site:Nucleophile	RT		5	2.7E-2	6.2E-1
INTERPRO	Glycoside hydrolase, superfamily	RT		3	4.4E-2	6.2E-1
UP_KEYWORDS	Glycosidase	RT		3	7.8E-2	5.7E-1
Annotation Cluster 7	Enrichment Score: 1.85			Count	P_Value	Benjamini
INTERPRO	Thioredoxin-like fold	RT		6	1.2E-3	8.7E-2
INTERPRO	Glutathione S-transferase, N-terminal	RT		3	1.1E-2	5.3E-1
UP_SEQ_FEATURE	domain:GST C-terminal	RT		3	1.9E-2	5.9E-1
GOTERM_MF_DIRECT	glutathione transferase activity	RT		3	2.2E-2	3.4E-1
INTERPRO	Glutathione S-transferase, C-terminal-like	RT		3	2.6E-2	5.3E-1
GOTERM_BP_DIRECT	glutathione metabolic process	RT		3	5.5E-2	9.4E-1
Annotation Cluster 8	Enrichment Score: 1.77			Count	P_Value	Benjamini
INTERPRO	NAD(P)-binding domain	RT		7	8.3E-4	7.3E-2
GOTERM_MF_DIRECT	NAD binding	RT		4	1.9E-3	5.4E-2
UP_KEYWORDS	NAD	RT		6	3.1E-3	4.9E-2
UP_KEYWORDS	Oxidoreductase	RT		9	1.7E-2	1.9E-1
GOTERM_BP_DIRECT	oxidation-reduction process	RT		8	1.1E-1	1.0E0
GOTERM_MF_DIRECT	oxidoreductase activity	RT		4	1.4E-1	9.7E-1
UP_KEYWORDS	NADP	RT		3	2.8E-1	9.4E-1
Annotation Cluster 9	Enrichment Score: 1.65			Count	P_Value	Benjamini
UP_KEYWORDS	Protease	RT		11	1.0E-3	2.0E-2
GOTERM_MF_DIRECT	serine-type peptidase activity	RT		4	8.1E-3	1.7E-1



Annotation Cluster 1	Enrichment Score: 3.68			Count	P_Value	Benjamini
UP_KEYWORDS	Serine protease	RT		4	4.6E-2	3.8E-1
UP_KEYWORDS	Zymogen	RT		4	1.2E-1	7.0E-1
GOTERM_BP_DIRECT	proteolysis	RT		7	1.2E-1	1.0E0
Annotation Cluster 10	Enrichment Score: 1.83			Count	P_Value	Benjamini
KEGG_PATHWAY	Biosynthesis of antibiotics	RT		8	3.3E-3	1.2E-1
KEGG_PATHWAY	Carbon metabolism	RT		5	2.1E-2	5.7E-1
KEGG_PATHWAY	Pentose phosphate pathway	RT		3	2.9E-2	6.4E-1
KEGG_PATHWAY	Biosynthesis of amino acids	RT		3	1.4E-1	1.0E0
Annotation Cluster 11	Enrichment Score: 1.61			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	protein import into nucleus	RT		4	7.2E-3	4.0E-1
GOTERM_MF_DIRECT	Ran GTPase binding	RT		3	1.6E-2	2.7E-1
UP_KEYWORDS	Protein transport	RT		7	1.3E-1	7.2E-1
Annotation Cluster 12	Enrichment Score: 1.59			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	SRP-dependent cotranslational protein targeting to membrane	RT		5	3.8E-3	3.1E-1
GOTERM_BP_DIRECT	viral transcription	RT		5	7.1E-3	4.0E-1
GOTERM_BP_DIRECT	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	RT		5	8.8E-3	4.0E-1
GOTERM_BP_DIRECT	translational initiation	RT		5	1.4E-2	4.8E-1
UP_KEYWORDS	Ribosomal protein	RT		5	2.1E-2	2.2E-1
UP_KEYWORDS	Ribonucleoprotein	RT		6	2.6E-2	2.7E-1
GOTERM_BP_DIRECT	translation	RT		6	2.9E-2	7.1E-1
KEGG_PATHWAY	Ribosome	RT		5	3.7E-2	6.4E-1
GOTERM_MF_DIRECT	structural constituent of ribosome	RT		5	5.7E-2	5.0E-1
GOTERM_BP_DIRECT	rRNA processing	RT		5	5.8E-2	9.6E-1
GOTERM_MF_DIRECT	RNA binding	RT		5	4.8E-1	1.0E0
Annotation Cluster 13	Enrichment Score: 1.32			Count	P_Value	Benjamini
SMART	Tryp_SPc	RT		4	2.5E-2	4.7E-1
UP_SEQ_FEATURE	domain:Peptidase S1	RT		4	2.5E-2	6.2E-1
UP_SEQ_FEATURE	active site:Charge relay system	RT		5	3.0E-2	6.3E-1
INTERPRO	Peptidase S1A, chymotrypsin-type	RT		4	3.2E-2	5.4E-1
INTERPRO	Peptidase S1	RT		4	3.8E-2	6.0E-1
INTERPRO	Trypsin-like cysteine/serine peptidase domain	RT		4	4.4E-2	6.2E-1
UP_KEYWORDS	Serine protease	RT		4	4.6E-2	3.8E-1
GOTERM_MF_DIRECT	serine-type endopeptidase activity	RT		5	8.5E-2	6.6E-1
UP_KEYWORDS	Zymogen	RT		4	1.2E-1	7.0E-1
INTERPRO	Peptidase S1, trypsin family, active site	RT		3	1.3E-1	1.0E0
Annotation Cluster 14	Enrichment Score: 1.13			Count	P_Value	Benjamini
INTERPRO	Intermediate filament protein, conserved site	RT		3	5.9E-2	7.4E-1
UP_SEQ_FEATURE	region of interest:Coil 2	RT		3	5.9E-2	9.4E-1
UP_SEQ_FEATURE	region of interest:Linker 12	RT		3	5.9E-2	9.4E-1
SMART	SM01391	RT		3	6.1E-2	5.1E-1
UP_SEQ_FEATURE	region of interest:Coil 1A	RT		3	6.8E-2	9.7E-1
UP_SEQ_FEATURE	region of interest:Coil 1B	RT		3	6.8E-2	9.7E-1
UP_SEQ_FEATURE	region of interest:Linker 1	RT		3	6.8E-2	9.7E-1
UP_SEQ_FEATURE	region of interest:Rod	RT		3	7.0E-2	9.7E-1
UP_KEYWORDS	Intermediate filament	RT		3	7.0E-2	5.3E-1
UP_SEQ_FEATURE	region of interest:Head	RT		3	7.3E-2	9.8E-1
UP_SEQ_FEATURE	region of interest:Tail	RT		3	7.7E-2	9.8E-1
INTERPRO	Intermediate filament protein	RT		3	8.3E-2	8.8E-1
UP_KEYWORDS	Keratin	RT		3	2.1E-1	8.3E-1
Annotation Cluster 15	Enrichment Score: 1.07			Count	P_Value	Benjamini
GOTERM_BP_DIRECT	retrograde vesicle-mediated transport, Golgi to ER	RT		4	1.8E-2	5.6E-1
GOTERM_BP_DIRECT	ER to Golgi vesicle-mediated transport	RT		5	2.4E-2	6.4E-1
GOTERM_BP_DIRECT	Golgi organization	RT		3	9.0E-2	1.0E0
UP_KEYWORDS	Protein transport	RT		7	1.3E-1	7.2E-1
UP_KEYWORDS	Golgi apparatus	RT		4	8.4E-1	1.0E0
Annotation Cluster 16	Enrichment Score: 0.84			Count	P_Value	Benjamini
UP_KEYWORDS	Endoplasmic reticulum	RT		17	3.2E-4	8.3E-3
UP_SEQ_FEATURE	topological domain:Lumenal	RT		9	4.6E-3	2.4E-1
UP_KEYWORDS	Membrane	RT		37	9.1E-1	1.0E0
UP_SEQ_FEATURE	topological domain:Cytoplasmic	RT		14	9.7E-1	1.0E0
UP_KEYWORDS	Transmembrane helix	RT		23	9.9E-1	1.0E0
UP_KEYWORDS	Transmembrane	RT		23	9.9E-1	1.0E0
UP_SEQ_FEATURE	transmembrane region	RT		17	1.0E0	1.0E0
Annotation Cluster 17	Enrichment Score: 0.25			Count	P_Value	Benjamini
UP_KEYWORDS	Nucleotide-binding	RT		13	3.1E-1	9.7E-1
UP_KEYWORDS	ATP-binding	RT		10	3.8E-1	1.0E0
UP_SEQ_FEATURE	binding site:ATP	RT		4	6.3E-1	1.0E0
GOTERM_MF_DIRECT	ATP binding	RT		10	6.5E-1	1.0E0
UP_KEYWORDS	Kinase	RT		4	7.9E-1	1.0E0
UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	RT		5	8.3E-1	1.0E0
Annotation Cluster 18	Enrichment Score: 0.25			Count	P_Value	Benjamini
GOTERM_MF_DIRECT	GTPase activity	RT		3	4.5E-1	1.0E0
GOTERM_MF_DIRECT	GTP binding	RT		4	4.6E-1	1.0E0
UP_SEQ_FEATURE	nucleotide phosphate-binding region:GTP	RT		3	5.4E-1	1.0E0

Annotation Cluster 1	Enrichment Score: 3.68			Count	P_Value	Benjamini
UP_KEYWORDS	GTP-binding	RT		3	5.8E-1	1.0E0
INTERPRO	P-loop containing nucleoside triphosphate hydrolase	RT		4	9.1E-1	1.0E0
Annotation Cluster 19	**Enrichment Score: 0**			**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Transcription regulation	RT		3	1.0E0	1.0E0
GOTERM_BP_DIRECT	transcription, DNA-templated	RT		3	1.0E0	1.0E0
UP_KEYWORDS	Transcription	RT		3	1.0E0	1.0E0