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Mapping UPR elements in male reproductive system: A bioinformatics approach

Proteínas *UPR* no sistema reprodutor masculino: Uma abordagem bioinformática

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e co-orientação do Doutor Rui Miguel Pinheiro Vitorino, Investigador Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

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palavras-chave

UPR, fertilidade masculina, espermatozoides, testículo, plasma seminal, proteómica, proteoma, bioinformática

resumo

A *Unfolded Protein Response* (UPR) é um mecanismo de defesa crucial que protege as células contra o enrolamento incorreto de proteínas, através da ativação de três sensores principais: ATF6, PERK e IRE1. Cada sensor guia a célula em diferentes mecanismos de transdução de sinal culminando na produção de fatores de transcrição que, por sua vez, regulam genes que aumentam a capacidade da célula corrigir a conformação de proteínas mal enoveladas, impedindo, em último caso, a sua agregação. Nos últimos anos a UPR tem sido associada a várias patologias. Na infertilidade masculina, poucos estudos se têm focado na influência dos componentes da UPR, sendo importante numa primeira abordagem, a identificação destes componentes no sistema reprodutor masculino. Através de pesquisa de bases de dados e com abordagens bioinformáticas, com o objetivo de identificar potenciais candidatos associados a fenótipos de infertilidade, foi realizada uma recolha de proteínas UPR no testículo, espermatozoide e plasma seminal. De forma a determinar possíveis alvos envolvidos na infertilidade masculina, as interações proteína-proteína foram analisadas, destacando-se 6 proteínas com elevado grau de interação: HSP90AA1, HSPA5, SEC61A1, VCP, PERK e ATF4. Considerando ainda a sua importância funcional, as proteínas efetoras da via PERK, a GADD34 e a eIF2 foram destacadas para estudos de deteção experimentais. Neste sentido, foi confirmada pela primeira vez a presença das proteínas PERK e GADD34 em espermatozoides humanos. Estes resultados constituem o primeiro passo fundamental para avançar para estudos mais aprofundados relativamente à expressão e níveis de atividade destes candidatos, procurando perceber a contribuição dos mesmos na via de sinalização UPR e a sua eventual desregulação na infertilidade masculina.

keywords

Unfolded protein response, male fertility, spermatozoa, testis, seminal plasma, proteomics, proteome, bioinformatics.

abstract

The unfolded protein response (UPR) is an essential cell defense response against defects in protein folding and it is mainly triggered by the activation of ATF6, PERK and IRE1. Each sensor leads to different signal transduction mechanisms through the production of transcription factors that, in turn, regulate genes that increase the cell's ability to correct conformation of poorly folded proteins, ultimately hindering their aggregation. The past years shed light on the role of the UPR in several diseases. Regarding male infertility, few studies have focused on the implications of UPR components, hence the need to a prior approach concerning the presence of these components on the male reproductive system. Through a database search and using bioinformatics approaches, with the aim of identifying potential candidates associated with infertility phenotypes, a collection of UPR proteins in the testis, spermatozoa and seminal plasma was performed. To determine potential targets to scrutinize possible involvement in male infertility, a protein-protein interaction network analysis was performed, depicting 6 key proteins highly interconnected: HSP90AA1, HSPA5, SEC61A1, VCP, PERK and ATF4. Considering their functional value, the effector proteins of the PERK pathway, GADD34 and eIF2 were highlighted for experimental studies. Thus, the presence of the PERK and GADD34 were confirmed for the first time in human spermatozoa. These results constitute the first fundamental step towards further studies on the expression and activity levels of these candidates and understand their contribution to the UPR signaling pathway and their possible deregulation in male infertility.

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LIST OF ABBREVIATIONS

1D-LC | 1-dimensional liquid chromatography

1D-PAGE | 1-dimensional polyacrylamide gel electrophoresis

2D-LC | 2-dimesional liquid chromatography

2D-PAGE | 2-dimensional polyacrylamide gel electrophoresis

ADP | Adenosine diphosphate

ASK1 | Apoptosis signal-regulating kinase 1

ATF4 | Cyclic AMP-dependent transcription factor ATF-4

ATF6 | Cyclic AMP-dependent transcription factor ATF-6

ATP | Adenosine triphosphate

BAK | Apoptosis regulator BAK

BAP | BiP associated protein

BAX | Apoptosis regulator BAX

BCA | Bicinchoninic acid

BCL2 | Apoptosis regulator Bcl-2

BSA | Bovine serum albumin

BiP | Binding immunoglobulin protein

CHOP | C/EBP-homologous protein

COPII | Coat protein complex II

DMSO | Dimethyl sulfoxide

DNAJA1 | DnaJ homolog subfamily A member 1

EDEM | ER degradation-enhancing α-mannosidase-like protein

EDTA | Ethylenediamine-tetra acetic acid

EIF2AK3 | Eukaryotic translation initiation factor 2-alpha kinase 3

ER | Endoplasmic reticulum

ERAD | Endoplasmic reticulum associated degradation pathway

FSH | Follicle-stimulating hormone

GADD34 | Growth arrest and DNA damage-inducible protein GADD34

GDP | Guanosine diphosphate

GO | Gene ontology

GRP94 | 94 kDa glucose-regulated protein

GTP | Guanosine triphosphate

HPA | Human protein atlas

HCL | Hydrogen chloride

HSF1 | Heat shock factor 1

HSF2 | Heat shock factor 2

HSP40 | Heat shock 40 kDa

HSP60 | Heat shock 60 kDa

HSP70 | Heat shock 70 kDa

HSP90AA1 | Heat shock protein HSP 90-alpha

HSPA1L | Heat shock 70 kDa protein 1-like

HSPA2 | Heat shock-related 70 kDa protein 2

HSPA4 | Heat shock-related protein 4

HSPA4L | Heat shock 70 kDa protein 4L

HSPA5 | Heat shock 70 kDa protein 5

IRE1 | Serine/threonine-protein kinase/endoribonuclease IRE1

IRES | Internal ribosome entry site

JNK | JUN N-terminal kinase

LC-MS/MS | Liquid chromatography tandem-mass spectrometry

LMNA | Lamin A/C

MGI | Mouse genomics informatics

MS | Mass spectrometry

NaCl | Sodium Chloride

NF-KB | Factor nuclear kappa B

NP-40 | Nonyl Phenoxypolyethoxylethanol

NPM | Non-progressive motility

ODF | Outer dense fibers

OMIM | Online mendelian inheritance in man

OVOL1 | Putative transcription factor Ovo-like 1

P-eIF2alpha | Eukaryotic translation initiation factor 2-alpha kinase 2 phosphorylated

PAGE | Polyacrylamide gel electrophoresis

PBS | Phosphate-buffered saline

PDI | Protein disulfide isomerase

PERK | PRKR-like endoplasmic reticulum kinase

PM | Progressive motility

PMSF | Phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride

PP1C | Protein phosphatase 1C

PPI | Protein-protein interaction

RIDD | IRE1-dependent degradation

ROS | Reactive oxygen species

S1P | Sphingosine-1-phosphate

S2P | Sphingosine-2-phosphate

SDS | Sodium dodecyl sulfate

SEC61A1 | Protein transport protein Sec61 subunit alpha isoform 1

TBS | Tris-buffered saline

TBST | Tris-buffered saline containing 0,1% Tween 20

TNF | Tumor necrosis factor

TRAF2 | Receptor-associated factor 2

TRIS | Tris (hydroxymethyl) aminomethane

UPR | Unfolded protein response

UPS | Ubiquitin-proteasome system

VCP | Transitional endoplasmic reticulum ATPase

WHO | World health organization

XBP1 | X-box-binding protein 1

1. Introduction

One of the most important aspects in evolution is the ability of cells to detect, respond and adapt to numerous intracellular and/or extracellular stress signals, through defensive mechanisms taking place in the different cellular organelles. These stress responses help the entire organism maintaining its capacity to evolve and reproduce (1). The protein quality control system is one of the cornerstones of the cell stress responses, because proteins are key effectors and regulators of cell activity. Thus, understanding the systems that contribute to cellular protein quality control is crucial to unveil the ways that the cell respond to several stresses. One of these systems is the unfolded protein response (UPR), which plays a fundamental role in the maintenance of cellular homeostasis and thus is essential to keep normal cell activity. Several perturbations that disrupt endoplasmic reticulum (ER) homeostasis may lead to protein misfolding and ER stress, activating the UPR pathways (2). In most diseases affecting these mechanisms, proteins are transformed from their native soluble forms into insoluble fibrils or aggregated plaques that accumulate in a variety of organs (3). Although UPR effectors are remarkably capable of avoiding protein aggregation, if stressors remain chronic, cells begin to fail maintaining proteostasis. Moreover, proteostasis disruption and subsequent protein aggregation may be caused or aggravated by translational errors, presence of polymorphisms and oxidative stress, among others. These aggregates tend to accumulate in elderly organisms and contribute to loss-of-function and loss of viability both at cell and tissue levels (4–6).

It is well settled that the age of paternity is rising over the years. Therefore the age hypothesis has been gaining relevance, adding to the many causes or potential causes that lead to male infertility (7). Given the association between aging and proteostasis decline, it is imperative to investigate the presence and understand the role of UPR elements in spermatozoa, before attempting to study any causal relationship between UPR decline with age and male infertility. Thus, this study primary goal was to evaluate the presence of UPR components in human sperm and to look over already described associations to male infertility phenotypes.

1.1(IN)FERTILITY

Infertility is recognized as a global public health issue by the World Health Organization (WHO) and is defined as the "failure to achieve a pregnancy after twelve months or more of regular unprotected sexual intercourse" (8). A recent review of Virtanen *et al.* (9) points to at least thirty million men worldwide being infertile, affecting fifteen percent of couples globally that have unprotected intercourse, with the highest rates found in Africa and Eastern Europe (9).

Mammalian fertility depends on an orchestrated complex set of reactions that begin with spermatogenesis in the testis, and ends with sperm-oolemma penetration, leading to sub-fertility or infertility if any defect within these reactions occurs (10). There are several analyses that are widely used to evaluate semen quality in laboratories and hospitals. Various features such as total and progressive motility, morphology and DNA quality can be studied for that purpose. However, these analyses cannot reliably diagnose infertility or even reveal upstream causes of abnormal sperm function or molecular defects in spermatozoa. Hence the great need for more research related to sperm function and male fertility in order to accomplish more accurate diagnostic and prognostic methods for the management of the daunting rates of male infertility (11–13).

As fifty percent of infertility is associated with the male partner, molecular scrutiny of sperm and seminal plasma can lead to new insights on male infertility (14). Although many questions regarding the etiology of male infertility remain unanswered with idiopathic infertility as the most common type of male infertility, many efforts have been made to reverse this issue, for instance, by comparing abnormal sperm (e.g. asthenozoospermic, globozoospermic or oligoasthenoteratozoospermic sperm) with normozoospermic sperm by proteomic approaches (11). For instance, Siva *et al.* (15) discovered eight deregulated proteins in asthenozoospermia, compared to normozoospermia samples, and Liao *et al* (16) a total of nine upregulated and twenty-six downregulated proteins in round-head spermatozoa, when compared with normal spermatozoa. Continuing the quest for new targets and the investigation of their effect in the male reproductive components can reveal new insights on the causes that lead to male infertility.

1.2 Male reproductive system

The causes for male infertility can be found in the intra-testicular and/or in the extra-testicular environments. Therefore, it is important to review the main components and functions of the male reproductive system. Its main function is to provide means for the production and maturation of gametes, as well as to transport them to the female reproductive system, being also responsible for the production of sexual hormones. The male reproductive system is composed by testes, several channels, including epididymis, vas deferens, ejaculatory ducts, and urethra, accessory glands and support structures, which are the penis and the scrotum. The accessory glands are a set of seminal vesicles, prostate and bulbourethral gland, and collectively are responsible for the production of secretions, that together with spermatozoa results in the semen that is projected from the urethra (figure 1) (17).

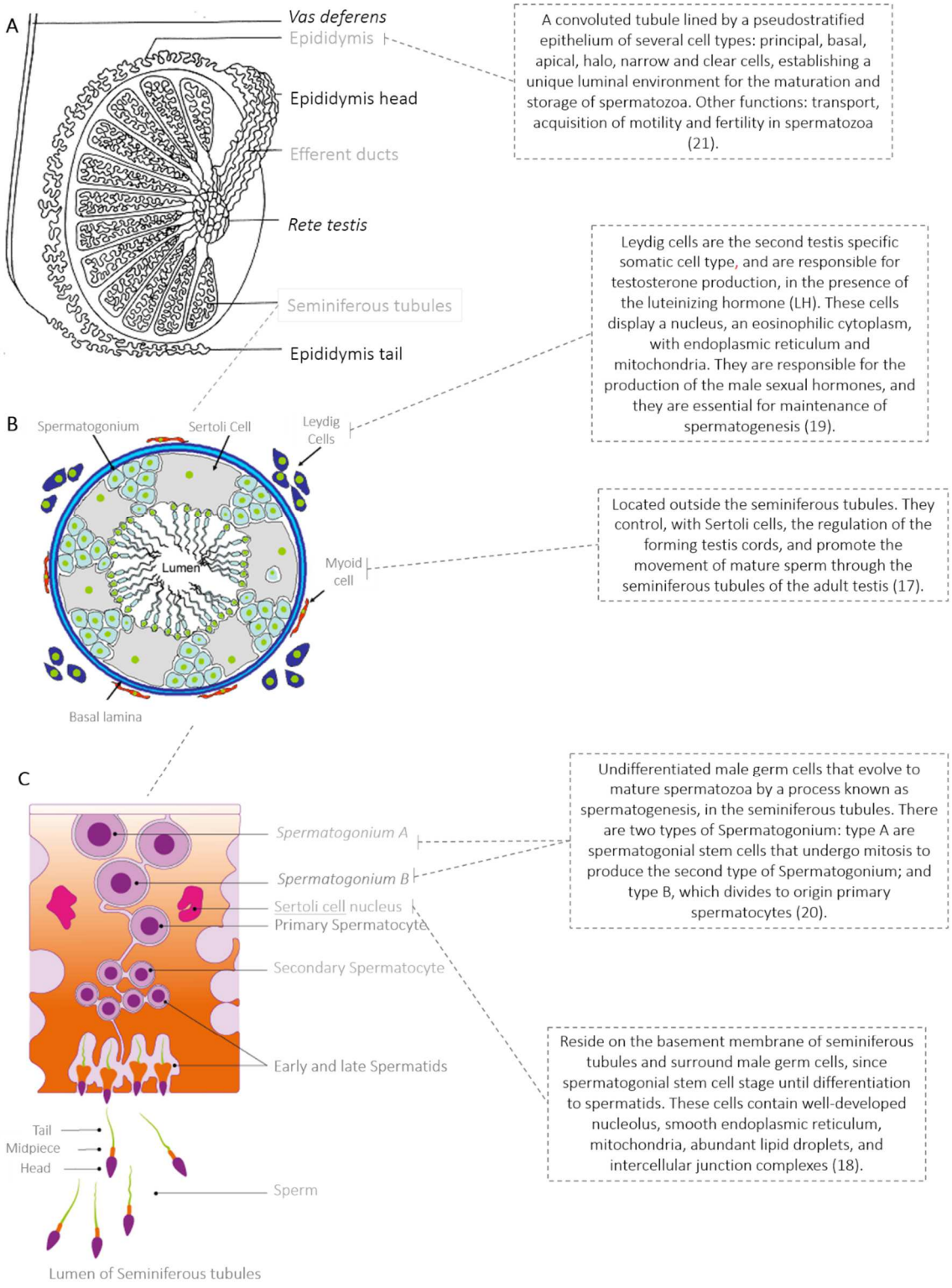


Figure 1 - Testis (A) Cross-section showing the location of seminiferous tubules, vas deferens and epididymis. (B) Cross-section of a seminiferous tubule. (C) Cross-section of a seminiferous tubule illustrating the process of spermatogenesis from spermatogonia until spermatozoa adapted from (17–21).

1.2.1 Spermatogenesis

In males, spermatogenesis - formation of the male gametes - do not begin until puberty. After that, this process go on continuously in the seminiferous tubules where immature germ cells proliferate by mitosis. The precursors of all germ cells are called spermatogonia and perform three key functions: proliferation, differentiation and apoptosis. Several daughter cells stop proliferating and differentiate to diploid primary spermatocytes, which in turn enter the first meiotic division, leading to haploid secondary spermatocytes. These spermatocytes undergo a second meiotic division creating four rounded spermatids that, in turn, undergo morphological differentiation resulting in mature spermatozoa. This differentiation is regulated primarily by follicle-stimulating hormone (FSH) from the anterior pituitary and by androgens from the testis (17,22).

The process by which spermatids evolve to mature spermatozoa is known as spermiogenesis, where the rounded spermatids become elongated. Subsequently, maturation is achieved by chromatin condensation, with the replacement of histones by protamines in DNA supercoiling, and the development of the acrosome, flagellum and other sperm organelles. Spermiogenesis, regulated by testosterone, has four phases: the Golgi phase, the cap/acrosome phase, the cauda formation and the maturation phase. The Golgi phase is where spermatids acquire polarity and the head is formed, consisting of the acrosome and the haploid nucleus surrounded by the cell membrane. The Golgi creates enzymes that will become the acrosome. The midpiece, that connects the head and tail, bears mitochondria for energy production, around the axoneme. The cap/acrosome phase is played by the Golgi apparatus that surrounds the nucleus to form the acrosome. The acrosome is the membrane-bound vesicle at the tip of the head of the spermatozoa and contain several enzymes, such as proteases, that have the capacity to digest the outer coat of the egg cell, allowing the sperm to inject its haploid sperm nucleus at fertilization. The cauda formation phase has the help of one of the centrioles, and in this phase spermatozoa begin to reorient to the lumen, away from the epithelium (22). The maturation phase requires Sertoli cells that phagocyte residual bodies. These cells release mature spermatozoa to the seminiferous tubules lumen giving rise to the spermiation phase in which the organelles and unwanted cytoplasm are discharged (17). Sertoli cells create a blood-testis barrier, sustain spermatozoa and do not replicate after puberty. The blood-testis barrier splits the germinal epithelium into a basal and adluminal

compartment, where different phases of germ cell development take place. While the mitotic phases occur in the basal compartment, in the adluminal compartment, the meiotic and postmeiotic germ cell differentiation stages are observed. Sertoli cells produce specific products that are necessary for germ cell survival, such as glycoproteins, growth and paracrine factors, and those combined generate an exclusive and essential environment in the adluminal compartment, thus regulating testicular size, germ cell numbers and spermatozoa output (23).

This process demands extensive protein synthesis during mitosis and meiosis to differentiate spermatogonia into spermatozoa. Additionally, after ejaculation, spermatozoa undergo intracellular, membranous and biochemical changes during capacitation to achieve fertilization in the female tract. These changes have been linked with hyper-activated motility, zona pellucida binding and acrosome reaction, and include increases in tyrosine phosphorylation and intracellular Ca^{2+} concentration (24–26). Several studies identified the role of ER stress in spermatogenesis, where an increase of protein synthesis and/or degradation was revealed to compensate these changes during spermatogenesis (27–30). Figure 2 illustrates these studies, by exposing testis to toxic environments there is an induction of ER-to-nucleus signaling 1 (IRE1) phosphorylation and DNA damage-inducible transcript 3 protein (CHOP) expression in rat spermatozoa (31). Another study by Kim *et al.* (27) observed the activation of UPR by increases of eukaryotic translation initiation factor 2 subunit 1 phosphorylated (p-eIF2α), activating transcription factor 4 (ATF4), Growth arrest and DNA damage-inducible protein GADD34 (GADD34) and phosphorylated IRE1 and presence of ER stress-mediated apoptosis of spermatocytes, in response to testicular hyperthermia. These studies suggest that ER-stress induces UPR signaling mechanisms that contribute to keep the normal function of reproductive tissues.

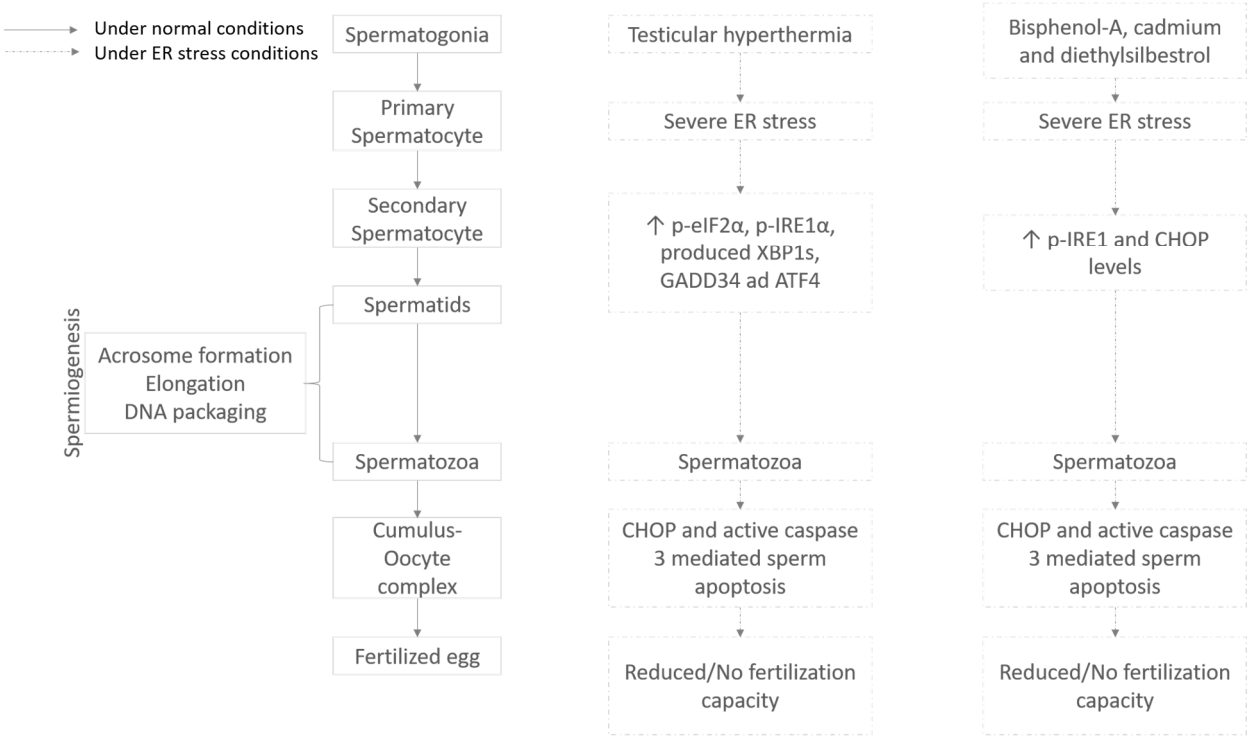


Figure 2 - ER stress and relation to infertility. On the left, the normal spermatogenesis, spermiogenesis and fertilization processes are depicted. On the right, spermatogenesis, spermiogenesis and fertilization processes under ER stress conditions. These changes activate UPR signaling that may impair spermatogenesis. Two studies show that inducing testicular hyperthermia and treating cells with chemicals that leave the cells in a toxic environment lead to severe ER stress increasing IRE1 phosphorylation and CHOP expression in spermatozoa. Note that the increase of CHOP expression triggers apoptosis in sperm, reducing or eliminating fertilization capacity. Adapted from (31).

1.2.2 The sperm cell

The spermatozoon is divided in head and tail (figure 3). The head comprises the acrosome and a condensed haploid nucleus, whereas the tail includes the midpiece, principal piece and the end piece, being both surrounded by the plasma membrane (32). The acrosome contains an inner and outer membrane consisting of hydrolytic enzymes essential for lysis of the zona pellucida and consequently, the penetration into the oocyte. The acrosome covers the first two thirds of the sperm head in a cap-like structure. The nucleus contains condensed chromatin with the genetic information and is tightly packed with highly positively charged protamines, instead of histones (33). The perinuclear theca works as a protective matrix of the nucleus composed of disulfide bond-stabilized structural proteins along with other types of proteins (34,35). The neck represents a short link between the flagellum and the sperm head, and serves as connecting, articular piece (32). The tail is the longest component of the

sperm and is also known as flagellum. The midpiece consists of a mitochondrial sheath surrounding the axonemal complex and nine outer dense fibers (ODF) (34). The axonemal complex is a central bundle of microtubules, nine microtubules supporting the axoneme from within, surrounding two single microtubules. The nine ODFs of the midpiece surround the nine microtubules doublets, giving support during flagellar movement, and the mitochondrial sheath surround the ODFs and the axoneme, generating energy for sperm flagellar motility (36). The Jensen's ring separates the principal and midpiece, and it stabilizes the mitochondrial sheath during the tail's movement. The principal piece is covered by fibrous sheaths and the end piece contains axonemal doublets, while the fibrous sheaths and ODFs occasionally are absent or containing only the ends (34,36).

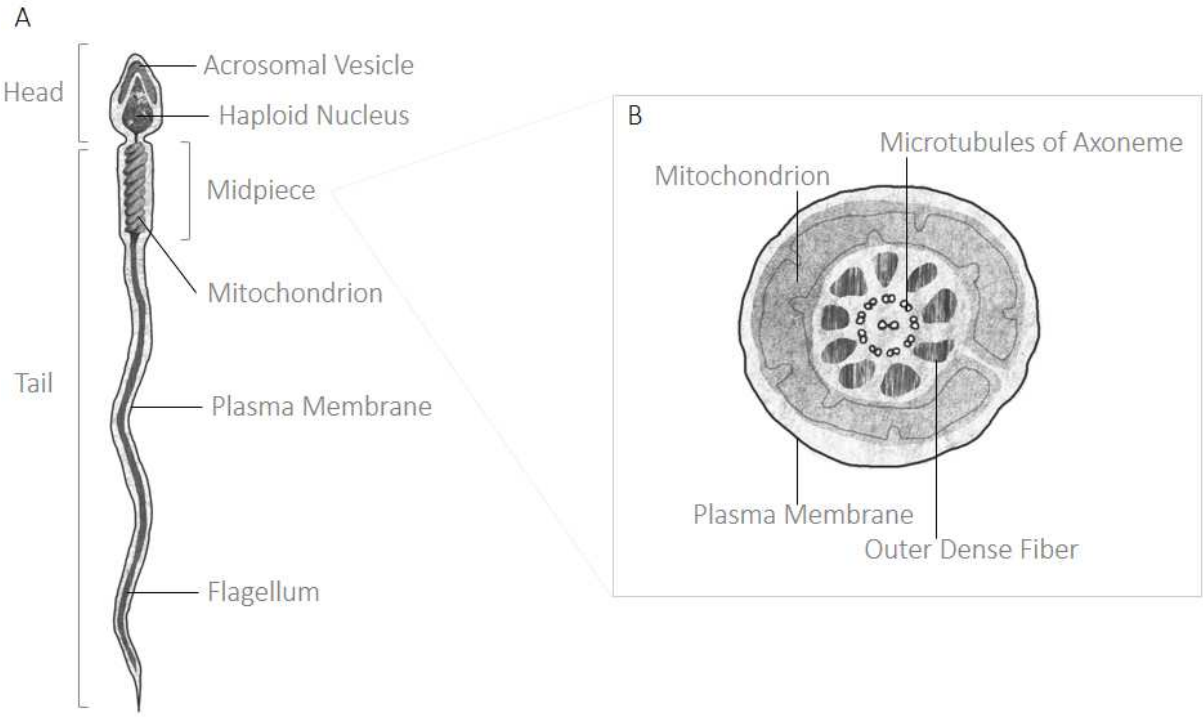


Figure 3 - The sperm cell in longitudinal section: two distinct regions enclosed by a plasma membrane. Tail propels the sperm to the egg and the head contains a condensed haploid nucleus. (A). Drawing of the midpiece of a mammalian sperm in cross section. Flagellum is composed of an axoneme surrounded by nine dense fibers. The axoneme is composed of two singlet microtubules surrounded by nine microtubule doublets. Mitochondrion provided ATP for flagellar movement. (B). Adapted from: (37)

1.3 UPR: an essential pathway to keep proteostasis

To preserve the biogenesis, folding, trafficking and degradation of proteins present within and outside the cell, it is crucial that protein homeostasis, also known as proteostasis, is sustained. Upon stress, cells trigger a series of complementary adaptive mechanisms to handle protein-folding variations and taken together are defined as UPR. For instance, testicular hyperthermia may activate UPR pathways promoting survival and adaptation of germ cells (27). UPR takes place in the cytosol (cytosolic heat shock response), in the endoplasmic reticulum as well as inside mitochondria (UPRmit) and requires communication with the nucleus. Any disturbance in proteostasis may result in protein aggregation and more serious age-related conditions, such as neurodegenerative, inflammatory and metabolic diseases (38–40).

1.3.1 Protein synthesis, folding and degradation

Most cellular proteins undergo folding after translation in ribosomes with the assistance of molecular chaperones, whereas secreted and transmembrane proteins fold and mature in the lumen of the ER. The ER is the first compartment of the protein secretory pathway and is involved in the biosynthesis and processing of about one third of the genome products, including secreted proteins, plasma membrane proteins, and membrane or soluble proteins of the different organelles of the secretory pathway itself (encompassing ER, Golgi apparatus, lysosomes, endosomes and secretory vesicles). As soon as they are synthesized, folded and assembled in the ER, proteins are translocated to the Golgi apparatus, from which they are addressed to their final compartment (1,41).

Protein folding in the ER is facilitated by the oxidizing environment, promoting the formation of disulfide bonds, by specific enzymes (protein disulfide isomerases and peptidyl prolyl cis-trans isomerases, for example) and by molecular chaperones. Chaperones help un- or misfolded proteins to achieve their native state, but do not belong to the final folded protein structure. There are several categories of ER molecular chaperones: the heat shock family (binding immunoglobulin protein (BiP), 94 kDa glucose-regulated protein (GRP94), and co-chaperones), lectins (calreticulin, calnexin, and ER degradation-enhancing α-mannosidase-like protein (EDEM)), the protein disulfide isomerase (PDI) and peptidyl-prolyl *cis/trans* isomerases. These ER chaperones, along with folding enzymes and

proteases, prevent inappropriate molecular interactions and recognize misfolded proteins, thus targeting them to the ER associated degradation (ERAD) pathway. This regulation is key for ER quality control and it comprises two mainly mechanisms of action (42,43). The first requires two lectin chaperones, calnexin and calreticulin, and depends on the presence of monoglucosylated *N*-linked glycans and unfolded regions on nascent glycoproteins. When entering the ER, proteins acquire multiple *N*-glycans groups, changing not only the properties of the protein, but their transport, interaction with other proteins and degradation. The second ER chaperone system only requires the presence of unfolded regions on proteins with hydrophobic residues. These residues (tryptophan, phenylalanine, or leucine) are normally in the core of properly folded proteins but become exposed in misfolded proteins. BiP binds to the exposed hydrophobic regions of unfolded proteins in its adenosine triphosphate (ATP)-bound conformation. Then, heat shock protein 40 kDa (DnaJ/HSP40) binds to BiP, and sometimes to the BiP associated unfolded protein, leading to the rapid hydrolysis of ATP to APD, leaving BiP to be locked onto the unfolded substrate. Adenosine triphosphate (ADP) release from BiP, and consequent unlocking, is stimulated by an ER nucleotide exchange factor, known as BiP associated protein (BAP) promoting substrate (protein) folding and release. These mechanisms are imperative to prevent dysfunctional or nonfunctional proteins transportation to cytosol or to any other cell compartment, which could result in a deleterious phenotype or in cell's lack of function (44–47).

When proteins recognized as terminally misfolded are retained in the calnexin-calreticulin cycle longer, they are trimmed by ER mannosidase I, allowing their recognition by EDEM which acts as a degradation machinery that is responsible for delivering calnexin substrates to the ERAD pathway. EDEM accepts misfolded proteins by interacting with calnexin, and continues the trimming of mannose group on the surface of those proteins allowing the retro translocation out of the ER and subsequent degradation of misfolded proteins in the cytosol by the ubiquitin-proteasome system (UPS) (46).

As mentioned previously, UPR can be activated in several compartments. Cytosolic heat shock response governs denatured proteins in the cytosol, and the crucial element in this mechanism is the transcription factor heat shock factor 1 (HSF1). HSF1 is activated during cellular stress and induces transcription of chaperones and other protective genes. These genes and chaperones will dissociate from the HSF1 when the load of unfolded proteins increases, to promote their refolding. Upon dissociation, HSF1 trimerizes and

translocates to the nucleus, where it is post-translationally modified, activating the transcription of more heat shock proteins (1,48,49).

In addition to ER, mitochondrion performs numerous functions in the cell, including energy harvesting, programmed cell death, regulation of Ca^{2+} levels, and biosynthesis or metabolism of lipids, amino acids and iron sulfur (50). The mitochondrial proteome is composed of approximately 1500 proteins, encoded by both mitochondrial and nuclear genomes, but most of them are encoded in the latter and must be imported from the cytoplasm (51). Since mitochondrion has separate matrix and intermembrane space compartments it must have special machineries to deliver the proteins to their correct mitochondrial destinations. The two major mitochondrial chaperones are the mitochondrial heat shock 70 kDa protein (mtHSP70), which directly folds incoming proteins, and multimeric heat shock 60 kDa (HSP60)-HSP10 machinery in the matrix. Additionally, there are proteases specific to each mitochondrial compartment assuring protein quality control (52,53). Hence, mitochondrial UPR can sense perturbations that would, eventually, result in the accumulation of unfolded proteins, through the transcription activation of nuclear-encoded protective genes, re-establishing mitochondrial homeostasis (1,48,49).

In the testis, there is a high rate of protein synthesis and the UPR is responsible for detecting misfolding proteins since they are translocated to the ER. In the case of spermatozoa, since they are deprived of ER, the UPR will probably be more prominent in the cytosol and mitochondria. Thus, mitochondria seems to be an essential organelle in spermatozoa, restricted to the midpiece to provide energy needed for motility (54). As figure 1 depicts, the mature spermatozoa undergo several modifications during spermatogenesis ending with components such as flagellum containing mitochondria. Elements, e.g. ER, are removed during spermiogenesis thus leaving the sperm cell uncappable of doing protein synthesis as somatic cells do. However, a recent study collected all evidences that have been addressing the possibility of sperm mitochondria bearing an alternative protein synthesis mechanism, along with mitochondria chaperones (55).

1.3.2 The UPR Signaling Pathways

The adaptive response to ER stress, known as UPR, can transduce information about protein folding status in the ER to the nucleus and cytosol to buffer fluctuations in unfolded protein load. Only when ER stress is not alleviated nor is homeostasis restored, the UPR triggers apoptosis. In addition, this mechanism can control other pathways such as lipid and cholesterol metabolism, energy homeostasis, inflammation and cell differentiation (41,56).

Prolonged interaction of a folding protein with the chaperone machinery leads to the activation of three stress sensors present in the ER (figure 4): activating transcription factor 6 (ATF6, both α and β isoforms), the inositol requiring kinase 1 (IRE1, both α and β isoforms), and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), also known as eukaryotic initiation factor 2α (eIF2α) kinase (PEK) that transduce an unfolded protein signal across ER membrane. In the absence of stress, the ER chaperone BiP binds to luminal domains of these regulators keeping them inactive. When ER stress becomes permanent, there is release of Ca^{2+} from the ER, activating apoptotic signaling pathways (41,57,58). The role of these proteostasis-sensing molecules and their interplay in cellular signaling pathways is detailed in the following sub-sections.

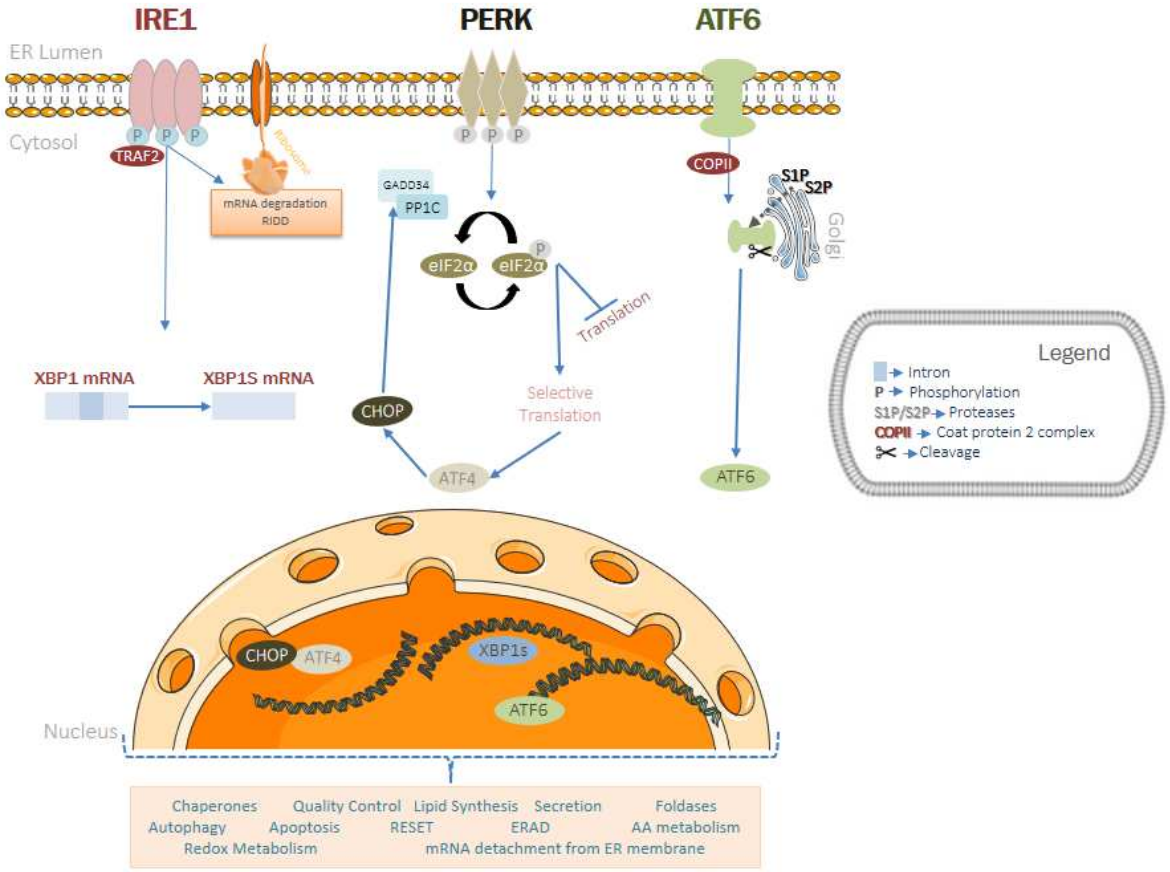


Figure 4 - The Unfolded Protein Response (UPR). Accumulation of abnormally folded proteins in the ER leads to a stress response known as UPR, where three major stress sensors can be activated: IRE1α, PERK and ATF6. These sensors induce a signaling cascade that results in the production of transcription regulators and these initiate the transcription of UPR target genes that promote ER homeostasis and cell survival. Each sensor follow a different signal transduction: IRE1 reduces folding load by degrading mRNAs and thus arresting protein synthesis; PERK by attenuating translation by phosphorylation-induced-eIF2α inactivation and ATF6 by regulated proteolysis. Additionally, IRE1α processes an mRNA that encodes XBP1, a transcription factor that upregulates UPR essential genes involved in folding, ERAD, organelle biogenesis, autophagy and protein quality control. eIF2α phosphorylation by PERK increases the translation ATF4 mRNA encoding transcription factors that induces the expression of genes involved in autophagy, antioxidant responses, apoptosis and amino acid metabolism. After being processed in the Golgi apparatus, ATF6 increases the expression of ER chaperones, ERAD-related genes and proteins involved in ER expansion. Adapted from (59)

1.3.2.1 PERK

Upon overt ER stress, a rapid and transient reduction of protein synthesis is ensued with the help of PERK. PERK has a luminal sense domain that controls the balance between unfolded proteins and chaperones, and can also go through oligomerization in response to ER stress. PERK has also a cytosolic effector domain, a kinase, which undergoes trans-autophosphorylation activation. When PERK is activated, it recruits and phosphorylates eIF2 at S51 of its alpha subunit. In basal conditions, the Guanosine-5'-triphosphate (GTP)-bound dephosphorylated eIF2 isoform plays a role in translation initiation. Whenever its

alpha subunit is phosphorylated, the Guanosine diphosphate (GDP)-GTP switch is inhibited and, thus, the protein synthesis. Translation attenuation is followed by an increase in the clearance of accumulated proteins by ERAD pathway and expression of pro-survival genes (coding for cellular inhibitors of apoptosis, for example (60)). However, decreased protein translation is not unbiased: genes with internal ribosome entry site (IRES) sequences avoid eIF2 translational block. One example is the translation of the ATF4 mRNA that encodes a transcription factor, which controls the levels of pro-survival genes related to redox balance, amino acid metabolism, protein folding, autophagy and apoptosis (61). ATF4 controls the expression of pro-apoptotic components such as C/EBP-homologous protein (CHOP), which downregulates the anti-apoptotic protein BCL-2 and induces the expression of BCL-2 homology 3 (BH3)-only proteins that are essential initiators of apoptosis. Hence, both extrinsic and intrinsic cell death signals are propagated and growth is arrested (62). CHOP also upregulates DNA damage-inducible 34 (GADD34), which can induce eIF2 dephosphorylation (GADD34 binds to protein phosphatase 1C (PP1C) yielding a phosphatase complex) that, in turn, fails to prevent protein synthesis, thus overloading cells with unfolded proteins (41,63). The absence of PERK leads to the exposure of cells to the risk of protein misfolding in the ER and to higher levels of other UPR stress sensors (64).

1.3.2.2 IRE1

The oldest and most conserved arm of the UPR is mediated by IRE1. IRE1 is a transmembrane protein and, similarly to PERK, contains a luminal domain that senses stress through an interaction with BiP and a cytosolic effector containing a protein kinase domain. Under ER stress, BiP dissociates allowing IRE1 activation by homo-oligomerization followed by autophosphorylation of the C-terminal kinase domain at S724. IRE1 activation unmasks a second catalytic function, the cleavage of pre-existing X-box-binding protein 1 (XBP1) mRNAs. The spliced XBP1 mRNA encodes a transcription factor that activates UPR target genes, products of which operate in ERAD via and allows the entry of protein in the ER and subsequent folding. XBP1s can also modulate phospholipid synthesis which is required for ER membrane expansion under ER stress (65). IRE1 has also RNase activity for a specific subset of mRNAs, in a process known as regulated IRE1-dependent

degradation (RIDD) and acts in parallel with PERK decreasing the load of unfolded proteins that will enter the ER by degrading their encoding mRNAs (66).

Additionally, phosphorylated IRE1 can recruit the adaptor protein tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) activating downstream apoptosis signal-regulating kinase 1 (ASK1) and JUN N-terminal kinase (JNK) or caspases, linking ER stress to cell death and dysfunction (41,63).

1.3.2.3 ATF6

Other stress sensor of the UPR is ATF6, which is a latent transcription factor and is tethered to the ER membrane. Under ER stress, ATF6 is released by a regulated intra-membrane proteolysis orchestrated by sphingosine 1-phosphate (S1P) and sphingosine 2-phosphate (S2P), and migrates to the nucleus to join ATF4 and other mediators such as XBP1 and CHOP, activating UPR target genes. Before ATF6 cleavage and activation, ATF6 migrates from the ER to the Golgi where it is a target of proteases, releasing its cytosolic domain, as mentioned before. ATF6 is co-activated with the other two arms of UPR, and BiP binding retain ATF6, thus playing a role in repressing its trafficking to the Golgi in unstressed cells (1,41).

1.4 Consequences of persistent ER stress: UPS, ER-stress Induced Autophagy and Apoptosis

Overall, UPR has three goals: adaptation, alarm and apoptosis. During adaptation, UPR tries to reestablish folding homeostasis by inducing the expression of chaperones, and, simultaneously, by attenuating global translation to reduce ER folding load while degradation rate of unfolded proteins is increased. Ultimately, if adaptation fails, UPR induces a cellular alarm and mitochondrial-mediated apoptosis program. The two major pathways responsible for the degradation of most long-lived or aggregated proteins is the UPS and autophagy. These two degradative systems act either during acute starvation for the UPS, or in chronic starvation for autophagy. Consequently, autophagy can protect cells from toxicity of proteasome inhibitors (67,68). Autophagy can be triggered in response to starvation but also in response to the accumulation of prolonged and misfolded proteins, which can be potentially detrimental cellular substances. This mechanism acts as a source

of energy for the biosynthesis of macromolecules by recycling metabolites produced by intralysosomal proteolysis. In addition, autophagy can regulate the energy balance of entire organisms through the improvement of metabolic activity (63,69). However, if the stress is too severe or persists, decompensation of ER function can induce cell death.

The UPS is responsible for the degradation of 80-90% of proteins, including regulated, short-lived, denaturated, abnormal or in general, damaged proteins. This catabolic pathway belongs to the cellular control of protein quality, where it senses misfolded or damaged proteins, tags them, and finally, degrades them. UPS is involved in the regulation of transcription, cell cycle progression, DNA repair, receptor-mediated endocytosis, cell stress response and apoptosis, and represent the predominant method of targeting proteins to degradation (70).

Autophagy contain numerous proteins involved in it regulation which are activated during starvation conditions and environmental stresses (71,72). However, this mechanism is also involved in several pathophysiological processes such as myopathies, neurodegenerative disorders and cancer (73). Autophagy is initiated by the formation of an autophagosome which undergoes cargo selection and packaging, expansion of the phagophore membrane and closure. Then this organelle fuses with endolysosomal vesicles creating an autolysosome. The autolysosome content is then degraded by lysosomal hydrolases, followed by the efflux of the breakdown products (63).

The three established branches of the UPR regulate autophagy in different ways during ER stress. Other than its involvement in ER stress response, the UPR enables cells to cope with a high demand of protein load by remodeling and expanding the ER membranes. In the last years, studies have shown a correlation between ER expansion caused by ER stress and autophagosome formation, by revealing that ER volume increases 5-fold under ER stress, and cells undergoing such stress display an increase in autophagosome abundance (74).

In the male reproductive system, autophagy remains unclear. A study by Zhang *et al.* detected autophagy markers, LC3-II and autophagosomes, in mice germ cells, after testes heat treatment (75). Another study by Wang *et al.* (76) and Shang *et al.* (77) reported a critical function for Atg7 in processes such acrosome formation in mice, spermatozoa flagellum biogenesis and cytoplasm removal during spermiogenesis. More recently, a study by Aparicio *et al.* (78) concluded that autophagy activation may have a positive role in

human spermatozoa physiology, assuring viability and mobility since it has the machinery required for such phenomenon.

Cell death under ER stress relies on the core mitochondrial apoptosis pathway, which is regulated by the proteins described above (79). Activation of both PERK and IRE1 pathways lead to apoptosis through regulation of the Apoptosis regulator Bcl-2 (Bcl2) expression and activity. Bcl2 is localized in mitochondrion's outer membrane promoting cell survival and inhibiting the activity of pro-apoptotic proteins. Activation of the apoptosis regulators Bax and Bak (Bax and Bak), proteins of the Bcl2 family, leads to the permeabilization of mitochondrial membrane and consequent release of cytochrome C and reactive oxygen species (ROS), essential elements in the apoptosis cascade.

One of the biomarkers of apoptosis is the activation of aspartate-specific proteases, known as caspases. Such enzymes dismantle cell cycle, cytoskeletal and organelle proteins by proteolytic cleavage. There are two main ways that lead to caspase activation, in response to apoptotic signals, namely ligand activation of death-receptors on the plasma membrane and stress-mediated pathways, e.g. ER stress (80).

1.5 UPR and Infertility phenotypes

The UPR is already implicated in many neurodegenerative diseases (81) as well as several cancers (82) and a host of inflammatory diseases (38). This way, it can be hypothesized that there is some link between these defense mechanisms and infertility. In the last years, some proteins have been linked with infertility phenotypes. Heat shock-related 70 kDa protein 2 (HSPA2) has been reported in several studies where its absence resulted in failed meiosis, pachytene spermatocytes undergoing massive apoptosis and in male infertility (83). Heat shock-related protein 4 (HSPA4) is increased in the sperm of male groups with varicocele and oligozoospermia (84). Moreover, a study by Shen *et al.* (85) showed that the knockdown of lamin A/C (LMNA) proteins leads to the deformation and fragmentation of the spermatozoa's acrosome (85). Thus, HSPA2, HSPA4 and LMNA take all part in UPR signaling pathway, and are related to infertility phenotypes such as male infertility, more specifically varicocele, abnormal morphology and oligozoospermia. These results show how important it is to scrutinize the presence, expression and role of all UPR

proteins in the male reproductive system as well as to investigate if their activation is somehow related to infertility.

1.6 Aims

The aim of this study was to map UPR signaling components in three components of the male reproductive system (testis, spermatozoa and seminal plasm), to explore their association to male infertility phenotypes and to explore the presence of (yet) obscure UPR elements in spermatozoa and their potential activation upon external insults (hyperthermia). To that end proposed to:

- Retrieve and map UPR elements to different components of the male reproductive system (sperm, testis and seminal plasma) in order to detect potential proteins whose expression, localization and role remains to be investigate in these biological specimens.
- Explore the implication of UPR elements in male infertility phenotypes.
- Investigate the presence of UPR elements in spermatozoa, following the findings of bioinformatics analysis.
- Evaluate the degree of UPR activation in spermatozoa subjected to heat stress (hyperthermia, 42ºC) by assessing total eIF2-α and phospho-eIF2-α activation.

2. Material and methods

Experimental procedures were performed in Signal Transduction Laboratory, Institute for Research in Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal). The details of the solutions used in this work are stated in the appendix. This section is divided in two phases: bioinformatics and experimental procedures. In the former, the aim was to map possible key players in UPR signaling and spermatozoa, testis and seminal plasma by bioinformatic tools to translate to the second phase. The second phase aimed to test the presence of UPR elements derived from human spermatozoa samples and to test their activation upon exposure to hyperthermia.

2.1 Collection of proteomes

To fulfill the first goal of this work, which was to map UPR elements in different components of the male reproductive system (testis, sperm and seminal plasma), a bioinformatics approach was followed using publicly available tools, as summarized in Figure 5.

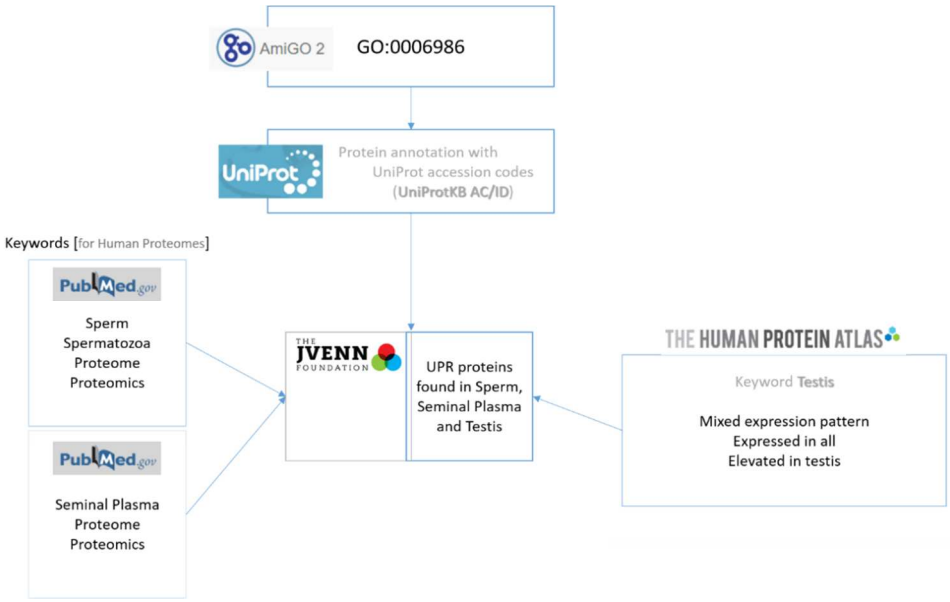


Figure 5 - Schematic representation of the bioinformatics study. First, AmiGO2 was used to retrieve all proteins already annotated to unfolded protein response (GO: 0006986). Then, proteins expressed in the three components of the male reproductive system at scope (testis, sperm and seminal plasma) were obtained either by Human Protein Atlas database search (testis) or by manually collecting all proteomes of spermatozoa and seminal plasma, in humans, reported in PubMed. Finally, all proteomes were crossed with the list of UPR to find common proteins, using JVENN tool.

2.1.1 Collection of UPR proteins

In order to retrieve human proteins already associated to UPR, a search was carried out on a Gene Ontology database, AmiGO2, (amigo.geneontology.org/amigo – version 2.4.24 – amigo2b). This database gathers a controlled vocabulary of terms covering biological concepts and a great number of genes or gene products whose attributes have been annotated as gene ontology (GO) terms. The term "unfolded protein response" was searched in AmiGO2 query resulting in several annotations, such as mitochondrial unfolded protein response (GO:0034514), IRE1-mediated unfolded protein response (GO:0036498) and PERK-mediated unfolded protein response (GO:0036499). The GO:0006986 was selected because it was the most general biological process term related to unfolded protein response. Next, the protein list was restricted to *Homo sapiens* (downloaded at Dec 20th, 2016) and the output was revised to exclude duplicates, using Excel for that end.

2.1.2 Collection of Sperm and Seminal Plasma proteins

An exhaustive literature search was conducted using the PubMed database to retrieve human sperm proteomic studies. The search was conducted up to Dec 20th, 2016. A combination of the keywords "proteomics", "proteome", "sperm" and "spermatozoa" was used. A list of all the sperm proteins identified was extracted from each paper and compiled in Excel spreadsheets. As exclusion criteria, all studies published in languages other than English and/or addressing the characterization of human epididymal sperm proteome or animal models were not included. Also, only proteomics studies in which a false discovery rate < 5% of protein identification was set were considered. Finally, only proteins identified with at least two peptides were considered.

The same method was used for collection of Seminal Plasma proteins, including all proteomic studies in which a false discovery rate < 5% was set and with only proteins identified with at least two peptides. In this case, a combination of the keywords "seminal plasma", "proteomics" and "proteome" was used. The search was also conducted up to Dec 20th, 2016. The main inclusion criterion was studies in which collection of seminal plasma was followed by centrifugation and separation of the supernatant seminal plasma. As exclusion criteria, all studies that did not follow the centrifugation and supernatant separation protocol were not included.

To bypass redundancy, all proteins were annotated using the UniProtKB/Swiss-Prot accession number by using the Retrieve/ID mapping tool of the UniProt knowledgebase. This tool only requires the list of identifiers, the selection of the original and desired identifiers and the selection of the organism "Homo Sapiens [9606]". Common original identifiers included gene symbols, protein names and Ensembl, and the final identifier was defined as UniProtKB AC/ID.

2.1.3 Collection of Testis proteins

The Human Protein Atlas (HPA) is a research platform with the purpose of exploring the expression and localization of the whole human proteome using an antibody-based approach (86). HPA gathers information for the large majority of human protein-coding genes based on both RNA and protein data obtained at the tissue level. For that reason, the list of proteins expressed in testis was obtained by searching on HPA using "testis" as the keyword. Data included proteins categorized as "elevated in testis", containing only proteins with elevated expression in the testis when compared with other tissues, "mixed expression pattern", being expressed in several but not in all tissue types and proteins "expressed in all" which refers to proteins expressed in all tissues including testis, to cover all proteins whose expression in testis was confirmed. Data was downloaded at Jan 18th, 2017 in the format of gene names.

2.2 Compilation of the UPR proteins with expression in testis and sperm and present in seminal plasma

To solve redundancy, all proteins were annotated using the UniProtKB/Swiss-Prot accession number by using the Retrieve/ID mapping tool available on Uniprot database. To identify common proteins between studies, the proteins were identified by their accession numbers, since as gene names they have several synonyms. However, several studies presented proteins in the format of gene names, whilst others as protein names. For those presented as protein names, a conversion for accession numbers was made. Next, all proteomes from sperm, seminal plasma and testis were merged individually. Data collected in HPA was added to the latter. Duplicates were then removed in Excel. Finally, to obtain the lists of proteins involved in UPR in the three elements of the male reproductive systems, Venn diagram analysis was performed, using the Jvenn tool (www.jvenn.toulouse.inra.fr).

2.3 UPR gene annotation and inspection of the involvement in male infertility-related phenotypes

To identify possible modulable UPR targets in male infertility, the UniProt database was used to gather information regarding biological process, cellular component and molecular function (data was downloaded at Dec 21st, 2016). This way it was possible to describe each protein in terms of their associated biological processes, cellular components and molecular functions. Additionally, it was also annotated the subcellular location of each protein using the GO vocabulary stored in UniProt database.

Then, to collect the current knowledge on the involvement of UPR elements in disease, particularly regarding male infertility, three databases, namely Online Mendelian Inheritance in Man (OMIM), Mouse Genome Informatics (MGI) and DisGeNET were explored. Each UPR protein compiled in section 2.2 was searched against OMIM and DisGeNET, using the respective UniProt code and the associated phenotypes/diseases were obtained. To acquire mammalian phenotypes in the MGI database, it was required the use of the BioMart search engine incorporated in Ensembl, to predict human orthologues for ENSEMBL human gene identifiers. After the acquisition of the identifiers, the search for mammalian phenotypes in the MGI database was made. The identifiers, in the form of UniProt/SwissProt accession numbers, were uploaded in the MGI and the output data selected was mammalian phenotype and human disease.

2.4 Network analysis

Aiming to take the global scenario of the UPR knowledge in the male reproductive system and its relation with male infertility, several network analyses were performed with Cytoscape (version 3.4.0.) application. To that end, the associations of UPR elements to the three biological components at scope (testis, sperm and seminal plasma), as well as the associations between UPR and the infertility sub-phenotypes were compiled in Excel files. In spite of the previous mapping to UniProt/SwissProt accession numbers, for Cytoscape analysis, UPR proteins from all components (testis, spermatozoa and seminal plasma) were mapped to the respective gene names in order to ease their recognition in the networks. After importing the table, the target and source nodes were identified, and the network main statistics were obtained through the “Network Analyzer” application, treating the network

as undirected. This approach allowed the visualization of the molecular interactions according to the degree of node association. Visual parameters were defined as follows: node size and color were mapped to "Betweeness centrality"; node size was set as low values to small sizes; edge size and color were mapped to "Edge betweeness"; edge thickness was set as low values to small sizes and node and edge colors were defined by attributing low values to dark colors. This way it was possible to visually identify more interconnected nodes by darker color and bigger size. Thus, UPR proteins whose expression is found across the three components and already associated with infertility will present bigger nodes and vice-versa.

Aiming to look over potential hub proteins, two extra networks were made with the use of a Cytoscape app, the stringApp (version 10.5). In order to build the protein-protein interaction (PPI) network, StringApp was used setting the species as *Homo Sapiens*. In the first network the source of proteins (sperm, testis and seminal plasma) was made evident, while in the second the infertility phenotypes were highlighted. In both networks the same type of network analysis was performed as described before, to elicit nodes with higher degree of association. To highlight protein source or the associated phenotype, pie charts were added to the nodes by using the "Image/chart" style option. The distribution of the slices reflected the presence of attributes that were added to the initial table: sample (sperm, testis and seminal plasma) and infertility phenotypes (for e.g. oligozoospermia, varicocele and azoospermia).

Afterwards, the biological background of the network was interpreted based on the reports found in the literature and some proteins, whose expression in spermatozoa and role in UPR and whose involvement in male infertility remains to confirm and investigate, were selected for further experimental characterization.

2.5 Sperm sample processing

The sections described above are referred to the bioinformatics study. The elements defined as potential after bioinformatic analysis were used in experiments with biological samples, as described below.

Five ejaculated human semen samples from volunteer donors were collected by masturbation into a sterile container. All donor signed an informed consent allowing the use of the samples for scientific proposes. Basic semen analysis was conducted in accordance

with World Health Organization (WHO) guidelines. Briefly, after complete liquefaction of the semen samples at 37 ˚C, during approximately 30 minutes, a microscopic examination was performed. The microscopic examination included the analysis of spermatozoa motility, concentration and morphology. All microscopic analyses were performed using a Zeiss Primo Star microscope (Carl Zeiss AG, Germany). Human spermatozoa were isolated and washed three times from seminal plasma by centrifugation (500g for 10 minutes at room temperature) using ALLGrad Wash medium (LifeGlobal, Brussels, Belgium).

Sperm cells were lysed in 1x RIPA buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) (Millipore Iberica S.A.U., Madrid, Spain) supplemented with protease inhibitor (1 mM PMSF) for 30 minutes on ice and centrifuged at 16000 × *g* for 15 minutes at 4 ºC. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, USA) following the manufacturer instructions and the final absorbance was measured at 562 nm in a microplate reader (TECAN, Genius, Männedorf, Switzerland).

2.6 Evaluation of UPR elements in human spermatozoa (by Western blotting)

Sperm extracts (see section 2.5), corresponding to 30 µg of protein, were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were electrotransferred onto nitrocellulose membranes (Amersham, 0.45 µm) for 2 hours at 200 mA. The system was cooled with ice to avoid overheating. Non-specific protein-binding sites on the membrane were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The blots were then washed with TBST and incubated with primary antibodies (see Table 1) overnight at 4 ºC. After the incubation, the blots were washed three times for 10 minutes each with TBST and then incubated with the appropriate secondary antibody for 1 hour at room temperature. Then, blots were washed three times for 10 minutes with TBST and once with Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl) and immunodetected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US). The results were analyzed in the Odyssey software (LI-COR, Biosciences, US). This system is equipped with two infrared channels for direct fluorescence detection on membranes (700 and 800 nm, anti-rabbit and anti-mouse respectively).

Table 1 - Description of the primary antibodies used in this study.

Antibody	Isotype	Predicted MW	Company
Phospho-PERK (Thr980) (16F8)	Rabbit	170 kDa	Cell Signaling
PERK (C33E10)	Rabbit	140 kDa	Cell Signaling
Phopho-eIF2a (Ser51) (119A11)	Rabbit	38 kDa	Cell Signaling
eIF2a	Rabbit	38 kDa	Cell Signaling
GADD34 (10449-1-AP)	Rabbit	100 kDa	Proteintech

2.7 Assess the impact of hyperthermia in human spermatozoa

To determine the effects of hyperthermia (42 ºC) on human spermatozoa, an assay with different time points and temperatures was conducted. After the processing step, described in section 2.5, approximately 12 million of cells per condition were used. As a control, several conditions (figure 6 - conditions C, E, G and I) were pre-treated with a specific inhibitor, salubrinal, a drug used to study stress responses linked with eIF2α. This drug inhibits complexes that de-phosphorylate eIF2α. Salubrinal (SML0951, obtained from Sigma-Aldrich) was reconstituted with dimethyl sulfoxide (DMSO). According to literature, the optimal concentration of salubrinal to inhibit dephosphorylation of eIF2α, is 75 µM (58,87). Except for negative control, all the remaining conditions with no salubrinal, were treated with the same volume of DMSO.

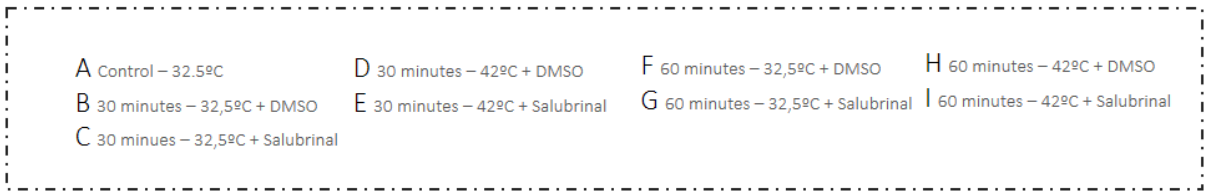


Figure 6 - Conditions of the hyperthermia assay: Conditions B and C were incubated for 30 minutes at 32,5 ºC; conditions D and E were incubated for 30 minutes at 42ºC; conditions F and G were incubated for 60 minutes at 32,5 ºC; conditions H and I were incubated for 60 minutes at 42 ºC. Conditions C, E, G and I are the controls containing the inhibitor salubrinal. Conditions B, D, F and H were diluted in DMSO since salubrinal was reconstituted in DMSO.

Before exposing the cells to hyperthermia, cells containing salubrinal were incubated for a period of 30 minutes at 32.5 ºC. Next, in conditions A, B, C, F and G, sperm cells were kept at 32.5 ºC for 30 minutes, while conditions H and I were kept at a 42 ºC for 60 minutes. Conditions D and E were exposed to hyperthermia for 30 minutes. Finally, the medium was removed by centrifugation (500 g, 5 minutes) and cells were washed with PBS. Before loading the samples in the gel, the sample was treated with DNase (NZY DNase I, MB19901, NZYTECH). First, a 10x buffer solution was prepared with 100 mM Tris-HCl (pH 7.6), 25 mM MgCl2 and 5 mM CaCl2. Next, 10 µl of the 10x buffer solution was added to 1 µl of diluted enzyme. After adding 1/10 volume of the buffer reaction mix to each condition, cells were incubated for 10 minutes at 37 ºC with agitation. All cells were re-suspended in 30 µl of SDS 1%. Next, all lysate protein fractions were resolved in an SDS-PAGE assay as described in section 2.6. After the proteins were electrotransferred onto nitrocellulose membrane (Amersham, 0.45 µm) for 2 hours at 200 mA, the membrane was blocked with 5% BSA in TBST for 1 hour at room temperature. The blot was then washed with TBST and incubated with primary antibodies (see Table 1, eIF2α and phospho-eIF2α) overnight at 4 ºC. After the incubation, the blot was washed three times for 10 minutes each with TBST and then incubated with the appropriate secondary antibody for 1 hour at room temperature. Next, the blot was washed three times for 10 minutes with TBST and once with TBS. The immunodetection was made with the Odyssey Infrared Imaging System (LI-COR® Biosciences, US). The results were analyzed in the Odyssey software (LI-COR®, Biosciences, US). This system is equipped with two infrared channels for direct fluorescence detection on membranes (700 and 800 nm, anti-rabbit and anti-mouse respectively).

3. Results and Discussion

All tables containing the proteomes can be found in the appendix.

UPR activation has been linked to cell survival, or cell death if the stress is chronic or severe. To unveil the possible presence of the main components of UPR in the male reproductive system, a bioinformatics methodology was followed and the potential proteins were then investigated in human samples. Mapping proteins associated with UPR and detecting them in human spermatozoa is an important step in uncovering possible links to male infertility.

3.1 Retrieving UPR proteins

Aiming to collect all proteins known to date to be involved in UPR, a search was conducted in AmiGO2, using the accession GO:0006986 "response to unfolded protein", because it entails any process that result in a change in the state or activity of a cell or an organism, in terms of movement, secretion, gene expression, enzyme production, as a result of an unfolded protein stimulus, as defined in QuickGO (www.ebi.ac.uk/QuickGO/) and not only a specific type of UPR (figure 7). Also, the GO terms above the GO:0006986 were not chosen since they comprise other non-directly associated biological processes. The entry GO:0006986 retrieved initially 237 annotations; restricted to *Homo sapiens*, however, after excluding all

duplicates turned to be only 167 different proteins. Therefore, this collection was defined as the human UPR elements henceforth.

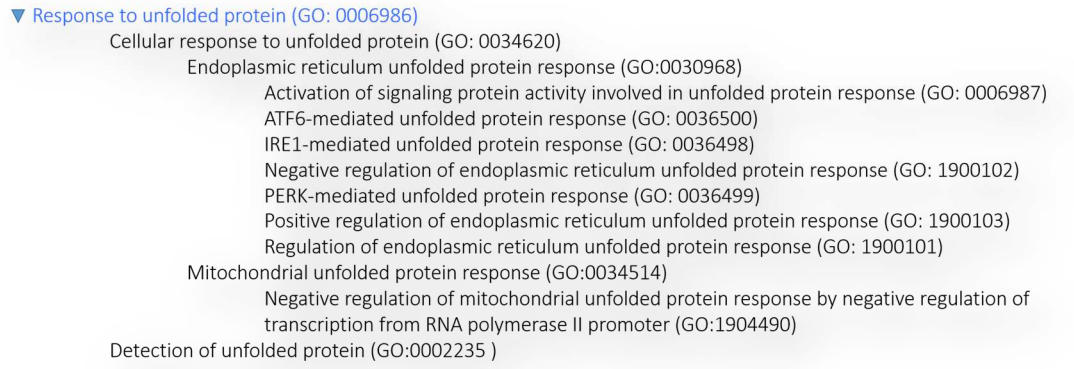


Figure 7 - Tree view of all gene ontology (GO) terms returned when searching with the keywords unfolded protein response. The GO term selected for this study was GO: 0006986, corresponding to response to unfolded protein, which contain both endoplasmic reticulum and mitochondrial unfolded protein response. Adapted from AMIGO2.

Table 2 - Summary of all studies used for the collection of testis, sperm and seminal plasma's proteomes. Also detailed is the number of proteins collected for each sample and for "UPR" term as found in AmiGO2. The number of UPR elements in each component of the male reproductive system, after duplication and Venn analysis, is also indicated in the last column.

Reviews composed of several studies of spermatozoa proteomics.

Sample	Reference	Total Proteins In each Study	Total Proteins w/out Duplicates	Sample VS UPR	Sample VS UPR (w/out duplicates)
Sperm	(88)*	6198	6198	88	**89**
	(89)	74	72	6	
	(90)	94	94	0	
	(91)*	2132	2132	39	
	(92)	264	227	8	
	(93)	235	235	0	
	(94)	111	67	0	
Sum of studies	7	9108	9025	141	
Protein sum w/out duplicates			6792		
Seminal Plasma	(95)	1463	571	15	**44**
	(96)	535	535	14	
	(97)	2545	2545	42	
	(98)	83	83	1	
	(99)	372	372	5	
Sum of studies	5	4998	4106	77	
Proteins sum w/out duplicates			2750		
Testis	Human Protein Atlas	Elevated in testis: 2200	2196	159	**159**
		Expressed in all: 7367	7364		
		Mixed Expression pattern: 6499	6498		
Sum of studies		16066	16058		
Without duplicates			16034		
UPR	AmiGO2	167		159	**159**

3.2 Collection of Sperm proteins

As illustrated in Table 2, five independent studies and two reviews with several studies of proteomics were selected to generate the collection of sperm proteins. All studies used ejaculated sperm and followed common steps for protein extraction (figure 8) by targeting the whole cell, except several studies inserted in the review by Amaral *et al.* (88,90). The study by Amaral *et al.* (88) used several procedures of subcellular fractionation, namely, the Percoll fractionation, *PureSperm* fractionation, Isolate fractionation and other mechanical alternatives to isolate compartments of the sperm cell (e.g. sperm nuclei, fibrous sheath, and head and tails (100–102)). Moreover, different strategies were followed to characterize sperm proteome. For instance, Bogle *et al.* (92) and Sharma *et al.* (89) have digested the proteins without previous separation (shotgun proteomics), right after the initial sperm cell isolation steps. Instead, Vanderbrouck *et al.* (93) and Garin-Muga *et al.* (94) have separated the proteins by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) before protein digestion and subsequent MS analysis (bottom up proteomics). A similar bottom-up approach was followed by Jumeau *et al.* (90). However, these authors separated the proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) prior to liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis. Exceptionally, in the studies by Naaby-Hansen *et al.* (103) and Kim *et al.* (100), collected in the review by Amaral *et al.* (88), other proteomics' approaches were performed, namely LC-MS followed by Edman degradation, where the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds. In the study by Parte *et al.* (104), also collected from the review of Amaral *et al.* (88), after protein separation performed nano ultra-high-performance liquid chromatography mass spectrometry (HPLC-MS), contrary to all remaining studies whose proteomic approach was either LC-MS/MS and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) or just LC-MS/MS. Figure 5 summarized the main approaches used to characterize sperm proteome. To improve coverage and enhance the range and sensitivity in the proteins identified, before analysis peptides can be separated by 1 or 2-dimensional liquid chromatography (1D-LC; 2D-LC), however, only a minority of the studies analyzed followed this strategy (104–106). Together, all proteomes combined, and after removal of duplicates, a list of 6850 different proteins was achieved, which were defined as the human sperm proteome.

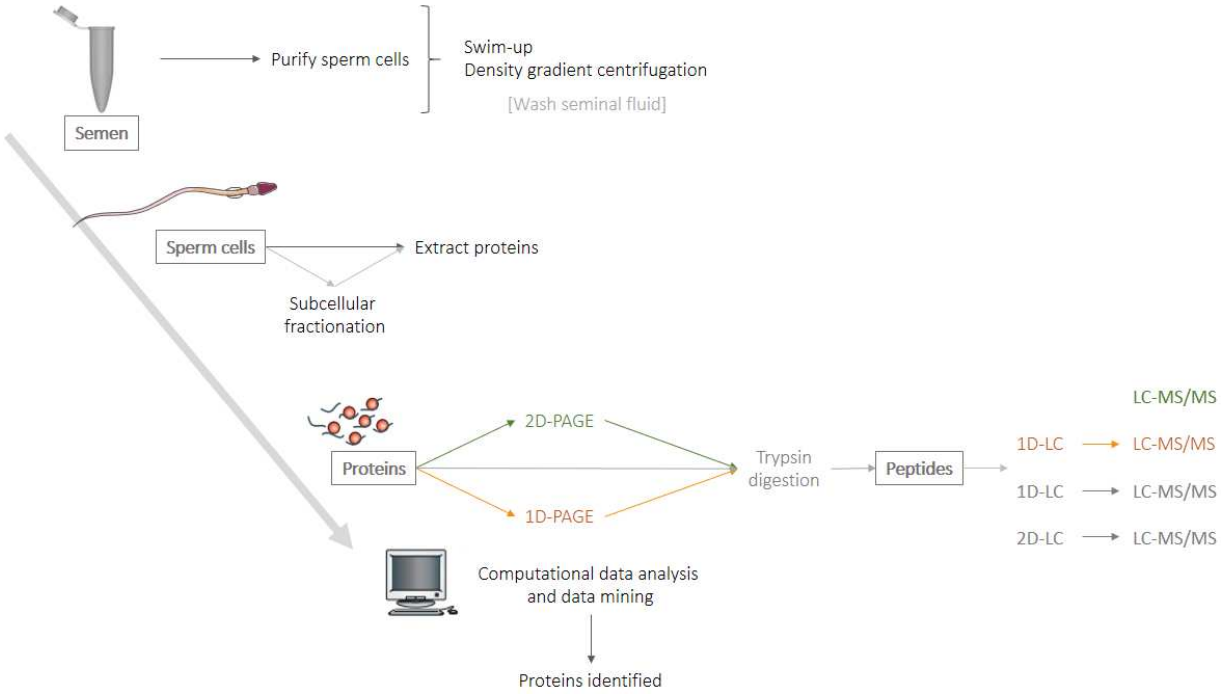


Figure 8 - Human sperm proteomics strategies used to identify proteins detected in the human sperm proteome. The first step is the isolation of sperm cells from the seminal fluid and from the remaining cells present in the semen. Next, the proteins are extracted from the whole cell or from specific subcellular sections. Following extraction, two possible approaches are generally followed: separation (1D-PAGE or 2D-PAGE) and digestion, or digestion without previous separation. Also, peptides can be first separated by 1D-LC or 2D-LC before fragmentation and analysis by MS/MS.

3.3 Seminal plasma proteins

Similar to the strategy followed for sperm proteome collection, five studies were selected to create the seminal plasma reference proteome. The studies by Intasqui *et al.* (95,96), Yang *et al.* (99), Roland *et al.* (97) and Milardi *et al.* (98) all employed common processing steps before characterization of the proteomes. First, semen samples were liquefied and centrifuged to eliminate cells and any solid materials. Seminal plasma was then extracted and the proteins digested samples with trypsin. Resulting peptides were then subjected to LC-MS/MS analysis. Combining all proteomes obtained from each study, a list of 2750 non-redundant proteins was obtained and was used as the reference seminal plasma proteome from hereinafter.

3.4 Testis proteins

Unlike the sperm and the seminal plasma whose proteomes had to be collected by literature search and data-mining, the testis proteome could be directly collected from HPA. This is a database that explores the whole human proteome by an antibody-based approach, mapping the proteins in all major organs and tissues in the human body (86). The different protein sets from HPA are illustrated in the figure 9, in which the proteins are distributed in testis and in other tissues as well. Although 82% of all human proteins are expressed in the testis, only 11% of those are elevated in testis compared to other tissue types. Unlike the other human tissues, the testis has far more highly expressed genes with more than 1000 genes showing at least 5-fold higher expression. A study by Djureinovic *et al.* (107) shows that 77% of all protein-coding genes are expressed in testis, more than any of the remaining 26 tissues analyzed. These results confirm the value of such a large abundance of enriched genes in the testes, so they can be able to fulfill their main function in terms of meiosis, to generate haploid cells.

Since all data from this project has been converted to the format UniProtKB/Swiss-Prot accession number, the data downloaded from HPA was also converted from Gene Name to UniProtKB/Swiss-Prot accession number, in order to keep data consensus. Out of the initial 32.116 genes, a total of 16.034 testis proteins were retrieved, creating the reference human testis proteome.

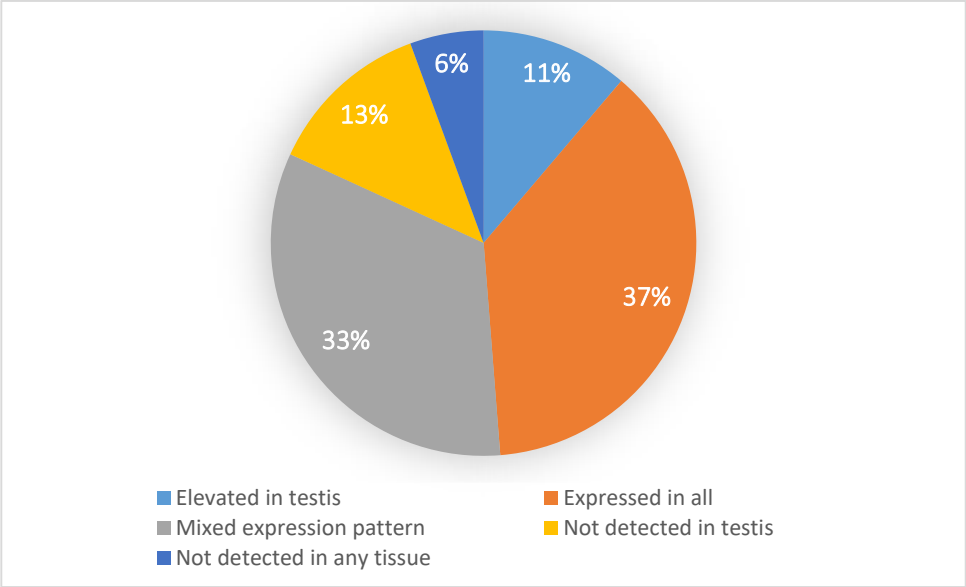


Figure 9 - Distribution of all genes across the five categories of transcript's abundance/detection: only 11% of all proteins in testis are specifically elevated in testis; 33% represent proteins in both testis and in other several tissues; 37% of all testis' proteins are also expressed in all the remaining tissues; 13% were not detected in testis and 6% in any tissue at all. Source: Human Protein Atlas.

3.5 Distribution of UPR elements across testis, spermatozoa and seminal plasma, and their characterization with respect to protein class and subcellular localization

The collection and mapping of the UPR key elements in a first approach is important to establish whether they are already described in the male reproductive system or not. Gathering all proteomes obtained in the previous sections for sperm, testis and seminal plasma, and crossing them with the UPR proteins' list created in section 2.1, resulted in a list of 159 different proteins (see figure 10). Interestingly, as it is illustrated in figure 10, none of the UPR proteins could be found exclusively in either sperm and/or seminal plasma. To date, however, 67 UPR elements were found to be exclusively expressed in the testis. This may indict little knowledge about UPR in sperm cells and the involvement of its elements in the spermatozoa function, but it also may reflect the demand of UPR proteins for protein synthesis in testis, unlike the spermatozoa and seminal plasma where this is virtually absent. In the last years, the research of UPR in the male reproductive system has been mainly focused in the role of UPR signaling in testis (27,108,109). One of those studies showed that UPR signaling is induced by testicular hyperthermia, as mouse testes exposed to several cycles of hyperthermia exhibit apoptotic spermatocytes, induced by ER stress

(27). Figure 10 also makes clear that none of the UPR proteins identified in sperm were also in the seminal plasma and that only three of them were both in seminal plasma and in the testis, namely, HSPA2, Heat shock 70 kDa protein 4L (HSPA4L) and Heat shock 70 kDa protein 1-like (HSPA1L)). These three proteins are highly expressed in testis, and since all of them are heat shock proteins, they are often present in stress conditions, where their upregulation is often described as part of the stress response (110,111). These proteins are also important mediators of protein folding and in the prevention of protein aggregation, by transporting proteins across the membranes into the endoplasmic reticulum or mitochondria (112). The observation that spermatozoa lack the expression of specific UPR proteins, points to the need for an in-depth study of UPR proteins present in spermatozoa, and more important, the need to clarify if their activity in spermatozoa is indeed related to common UPR triggers.

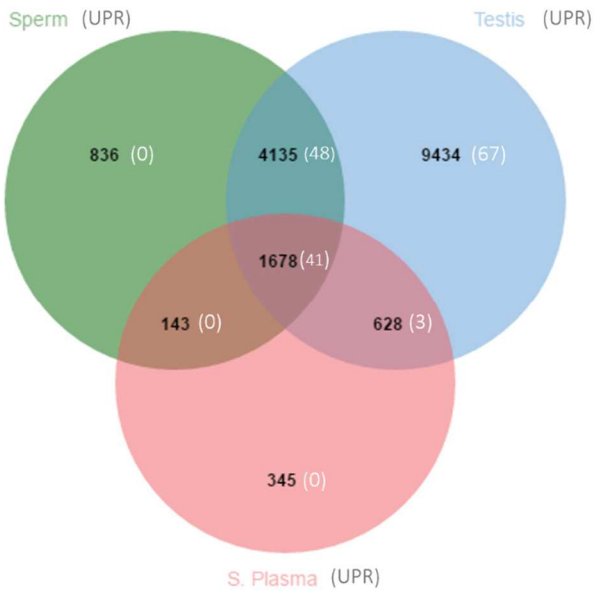


Figure 10 - Venn's diagram illustrating common proteins between sperm, testis and seminal plasma and all UPR proteins found in each tissue (number in brackets). S. Plasma: Seminal Plasma.

Aiming to shed light onto the protein classes and cellular localization of the UPR proteins, a gene ontology analysis was performed with the UniProt knowledgebase. As expected (figure 11), the majority of the 159 UPR proteins present in the male reproductive system are chaperones, which makes sense since one of the first responses when a cell is under stress is the release and activation of chaperones to manage the load of mis- or

unfolded proteins (112). Also, 13% of these chaperones are present in testis and seminal plasma, whereas in spermatozoa only 9%, possibly since translation is absent in these cells. Hydrolases and transferases are also very prominent in testis (6% and 8%, respectively) when compared to spermatozoa, due to the higher rates of metabolism in testis. However, as enzymes, they play an important role in sperm cells during fertilization (17).

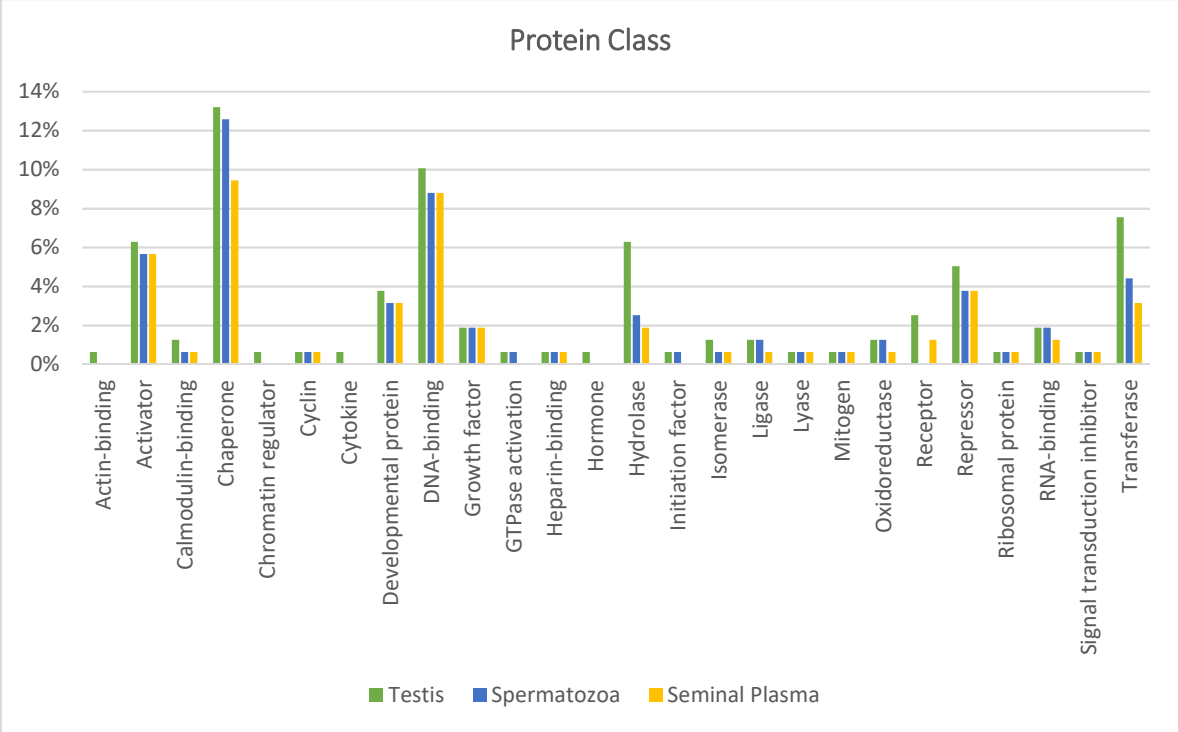


Figure 11 - Distribution of the 159 UPR proteins according to the functional class, for each component of the male reproductive system: seminal plasma (yellow), spermatozoa (blue) and testis (green). All proteins are identified in testis. Several proteins are not present in spermatozoa and/or seminal plasma. Source: Uniprot database.

Regarding the other two main classes among spermatozoa, testis and seminal plasma, DNA-binding and activator proteins, such as the transcription factors heat shock factor 2 (HSF2) and putative transcription factor Ovo-like 1 (OVOL1), they are involved in gene expression regulation and have been linked with spermatogenesis regulation (113). In general, transcription factors are essential to maintain the proteostasis and remain in a repressed or inactive state that is distinct for each stress response pathway (UPRmit, UPR or UPRcyt) (114).

Another important aspect of these proteins is their subcellular location (figure 12) to understand where these proteins may perform their activity.

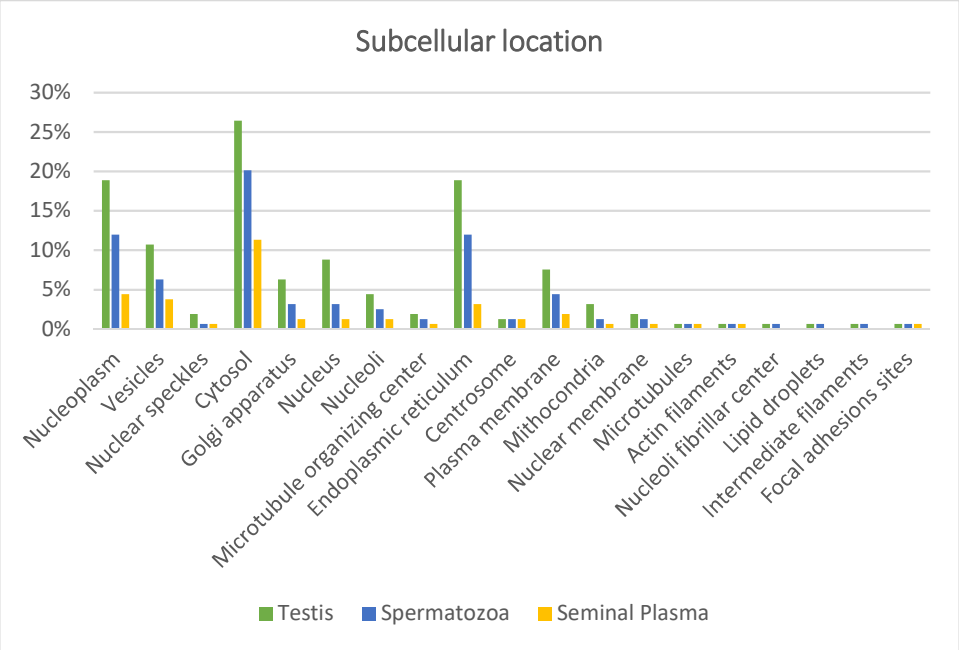


Figure 12 - Subcellular location of the 159 UPR proteins distributed in the three components spermatozoa, seminal plasma and testis. Source: Human Protein Atlas.

According to the HPA and UniProt databases, in figure 12, the common locations of UPR proteins are the nucleoplasm, cytosol and endoplasmic reticulum. Due to the absence of ER in spermatozoa, it is plausible to hypothesize that these proteins occupy a different subcellular location in these cells and possibly playing different roles (115). 12% of the proteins identified in spermatozoa are present in nucleoplasm, however, this organelle is also absent in the sperm nucleus. Thus, due to the absence of UPR key organelles, this pathway may act differently in sperm cells. Vesicles also occupy a prominent place in the top of the locations list. This is maybe because they are involved in metabolism, transport, storage of enzymes and have key roles in gamete maturation and fertilization (116).

The proteostasis network comprises the translational machinery, molecular chaperones and co-chaperones, the UPS and the autophagy machinery and spans all subcellular compartments to maintain cell viability, including both membrane trafficking organelles as well as energy-producing organelle mitochondria (117). The percentage of proteins in mitochondria (1%) was expected to be higher in spermatozoa since it is one of the few organelles present in these cells. Mitochondria is also believed to play a central role in the signal transmissions to the nucleus, under stress conditions (118). As some of the locations

are not present in sperm cells, it is vital to study all UPR proteins in this component to understand their main functions and locations.

3.6 Association of UPR elements to male infertility phenotypes

Aiming to retrieve the current knowledge on the association of UPR players to male infertility phenotypes, three databases were explored, namely OMIM, MGI and DisGeNET. OMIM is a continuously updated collection of human genes and genetic disorders and traits, with aim on gene-phenotype relationship (119). MGI is an international database resource for the laboratory mouse, providing integrated genetic, genomic and biological data to facilitate the study of human health and disease. It also provides the search for phenotype and disease associations by mouse genes (120). DisGeNET, in turn, stores and sorts protein-disease or gene-disease associations depending on the degree and the number of evidences (121). The desired data was searched in all three databases since these provide supplementary data.

In OMIM, all proteins were individually searched and 12 out of 159 proteins were found associated to diseases, such as Parkinson disease. Nevertheless, none was related to fertility phenotypes.

As MGI is a database devoted to mice models, a previous mapping of human proteins (159) to their corresponding mouse orthologue was ensued resulting in 158 mouse proteins. Overall, 20 knock-out male infertility phenotypes could be associated to 100 proteins out of the 158 inputted proteins. These included infertility; reduced male fertility; arrest of spermatogenesis; abnormal spermatogenesis; male meiosis; asthenozoospermia; azoospermia; oligozoospermia; abnormal spermiation; small seminiferous tubules; seminiferous tubules degeneration; abnormal reproductive system; abnormal male meiosis; abnormal male germ cells apoptosis; decreased male germ cell number; abnormal sertoli cell morphology; abnormal spermatocyte morphology; abnormal spermatid morphology; abnormal seminal vesicle morphology and/or abnormal vas deferens morphology.

Finally, 11 infertility-related conditions could be associated through DisGeNET database search to 68 out of the 159 inputted proteins. These included the more general male infertility term, but also more particular conditions such as varicocele, azoospermia, reduced male fertility and infertility.

Next, three distinct analyzes were carried out to: illustrate the UPR proteins distributed by testis, spermatozoa, and seminal plasma; distribute all UPR proteins according to their protein-protein interactions, also illustrating to either what component each protein is associated to or to what infertility phenotypes are associated. The correspondent tables were imported to Cytoscape application, resulting in three different networks (figures 13, 14 and 15). Figure 13 shows all 159 proteins and their association to sperm, testis and seminal plasma, reflecting whose proteins have already been detected in each of the samples at scope. As expected, all 159 different proteins are associated with testis. To find potential hubs that may have important roles among all 159 UPR proteins, an additional tool was used in Cytoscape, the STRING tool. After a PPI analysis, it was obvious that it would be enriching to add protein data to the network, that is, to add to what elements of the male reproductive system and infertility phenotypes these proteins were associated. The resulted networks (Figures 14 and 15) illustrate information on UPR proteins in testis, spermatozoa and/or seminal plasma, as well as which of these interacting proteins already have associated infertility phenotypes. The STRING database provide protein-protein interactions, including direct (physical) and indirect (functional) associations (122). Figure 14 represents a network with all 159 proteins and each node contains different colors for each sample (blue for sperm, pink for testis and yellow for seminal plasma) identified in the proteomes obtained in the sections 2.3, 2.4 and 2.5. According to the network analyzer and STRING tools, the most relevant proteins in this network, as represented by the larger nodes higher interconnectivity are heat shock protein HSP 90-alpha (HSP90AA1), heat shock 70 kDa protein 5 (HSPA5), VCP, SEC61A1, ATF4 and eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3). The latter, EIF2AK3 (synonym: PERK) is very important in UPR signaling, as it is one of the key sensors activated in ER when cells are under stress. After identifying possible key players in the network, it is important to link this information with male infertility phenotypes. Thus, the next step was to explore all 159 different proteins in databases that retrieve to which phenotype each protein introduced is or not related, resulting in the network illustrated in figure 15, where all 159 UPR proteins contain different inner colors that represent infertility phenotypes already found associated, according to MGI and DisGeNET databases. Indeed, only 19 out of the 159 proteins displayed such association, however 9 of them are linked with general phenotypes such as reduced fertility and infertility. These results show that only 12% of UPR proteins were found to be associated

with infertility phenotypes, meaning, on one hand, that most UPR proteins are not involved in infertility or, on the other hand, may reflect lack of knowledge of their possible connection to infertility, requiring more research in the area.

Some of the proteins represented with bigger nodes and, thus, highly interconnected, were also associated with more infertility phenotypes (Figures 14 and 15). See, for instance, the proteins HSP90AA1, DnaJ homolog subfamily A member 1 (DNAJA1), ATF4 and HSPA4. Accordingly, it is expected that other UPR proteins displayed with bigger nodes may play a role in at least some infertility conditions, due to their central interplay. For instance, the protein coded by the EIF2AK3 gene (PERK), whose presence in sperm cells remain to address (Figure 14), would be an interesting target to investigate in an experimental basis. Additionally, downstream effectors of this kinase (e.g. eIF2 and GADD34) deserve special attention because they are also associated to another key protein found in network analysis, the ATF4, as previously mentioned. In fact, ATF4 is important in the PERK pathway, as it controls the expression of pro-apoptotic components, such as CHOP, that in turn upregulates GADD34, inducing eIF2 dephosphorylation (123).

For all these reasons, the PERK pathway was chosen to be studied experimentally in human spermatozoa. This kind of basic science studies is important to pave the way to studies addressing the association between these players and infertility phenotypes.

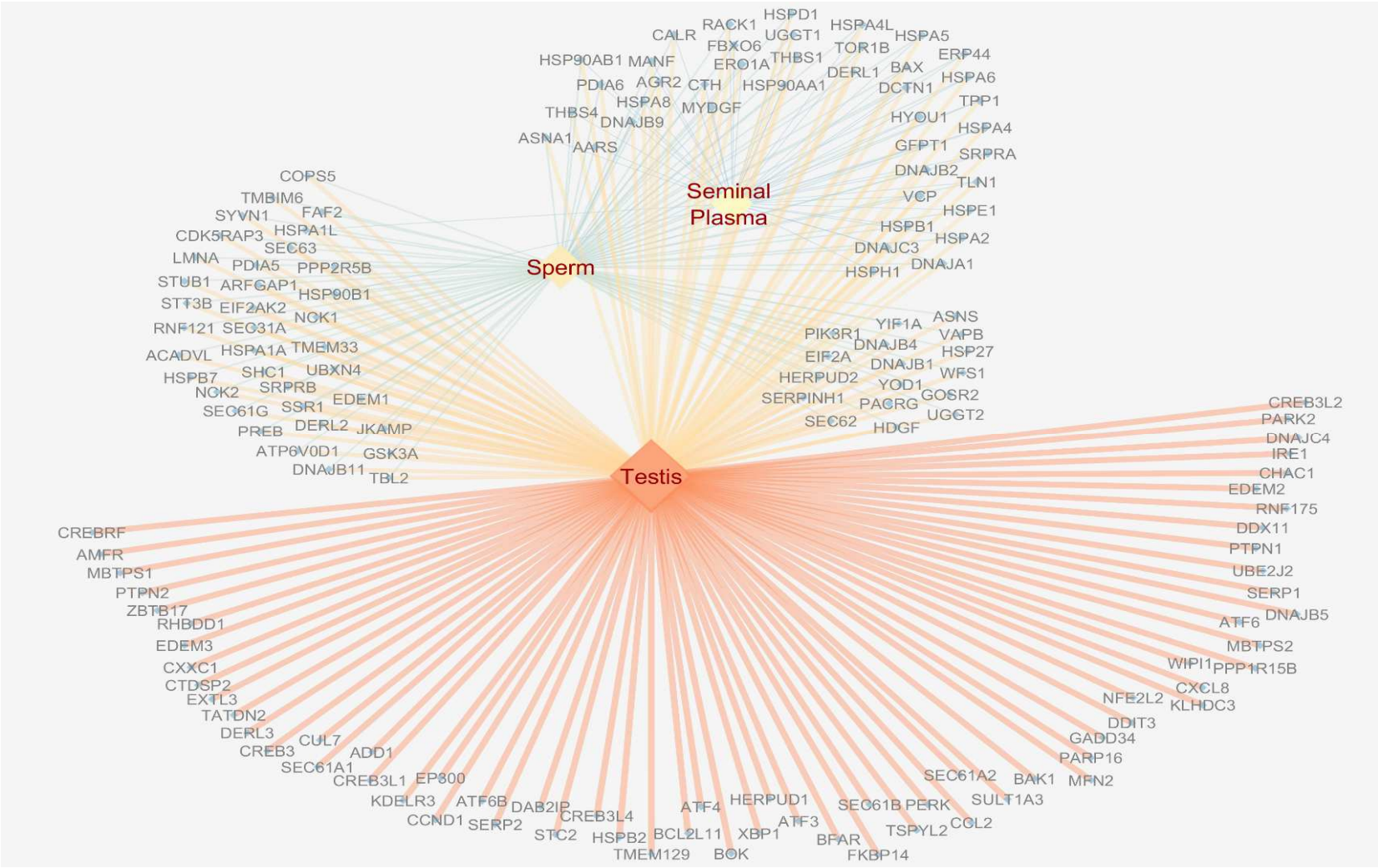


Figure 13 - Protein-sample association network obtained through Cytoscape analysis and Network Analyzer. All 159 UPR proteins are presented, and dispersed according to their presence in spermatozoa, testis and/or seminal plasma. All UPR proteins are mapped in testis, contrary to seminal plasma and spermatozoa.

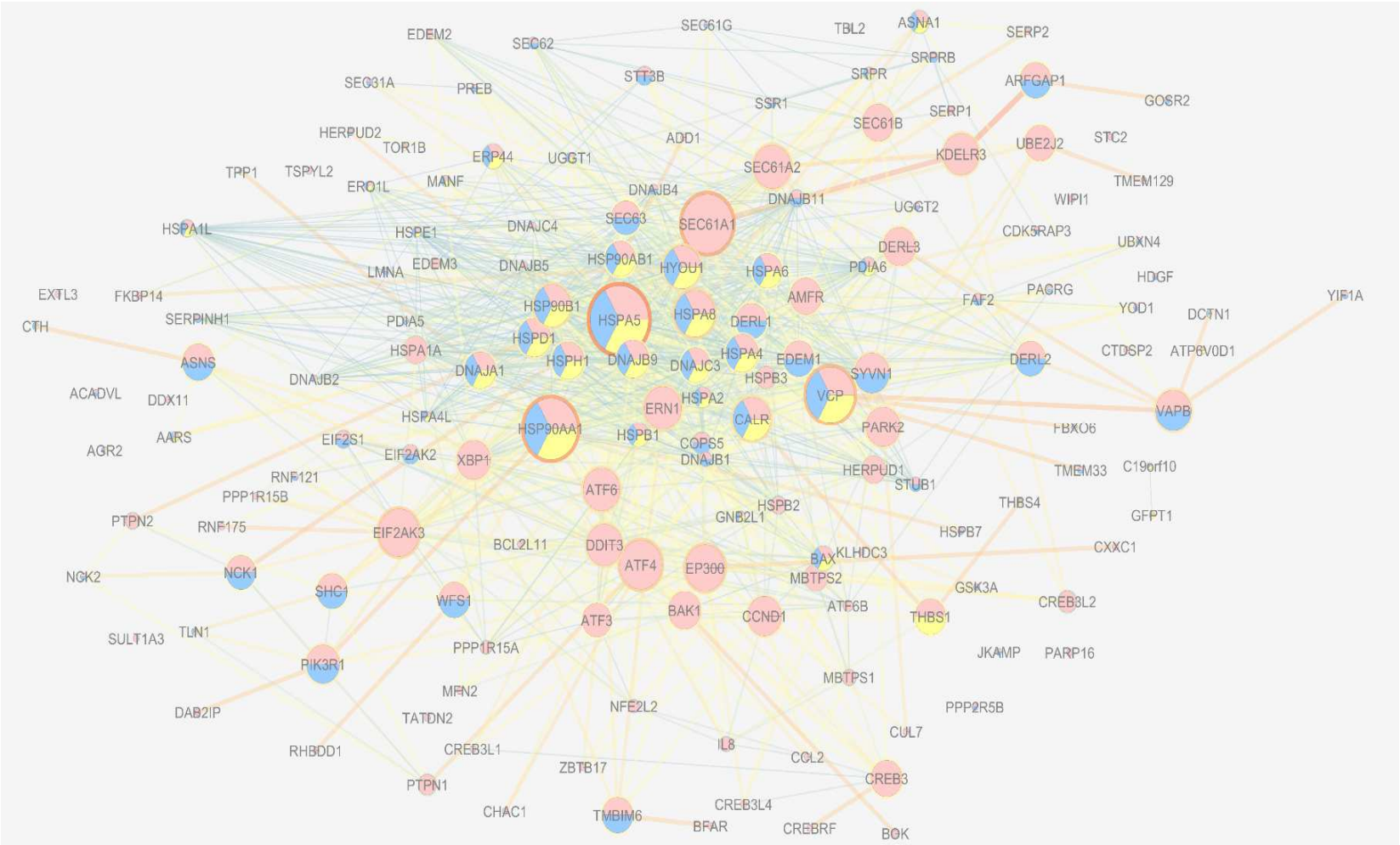


Figure 14 - Protein-protein interaction network relative to the 159 UPR proteins mapped to spermatozoa (blue), seminal plasma (yellow) and testis (rose). Interactions were retrieved from STRING database, using the Cytoscape application, StringApp. The proteins HSP90AA1, HSPA5, SEC61A1, VCP, EIF2AK3 and ATF4 have the highest interactivity in the network.

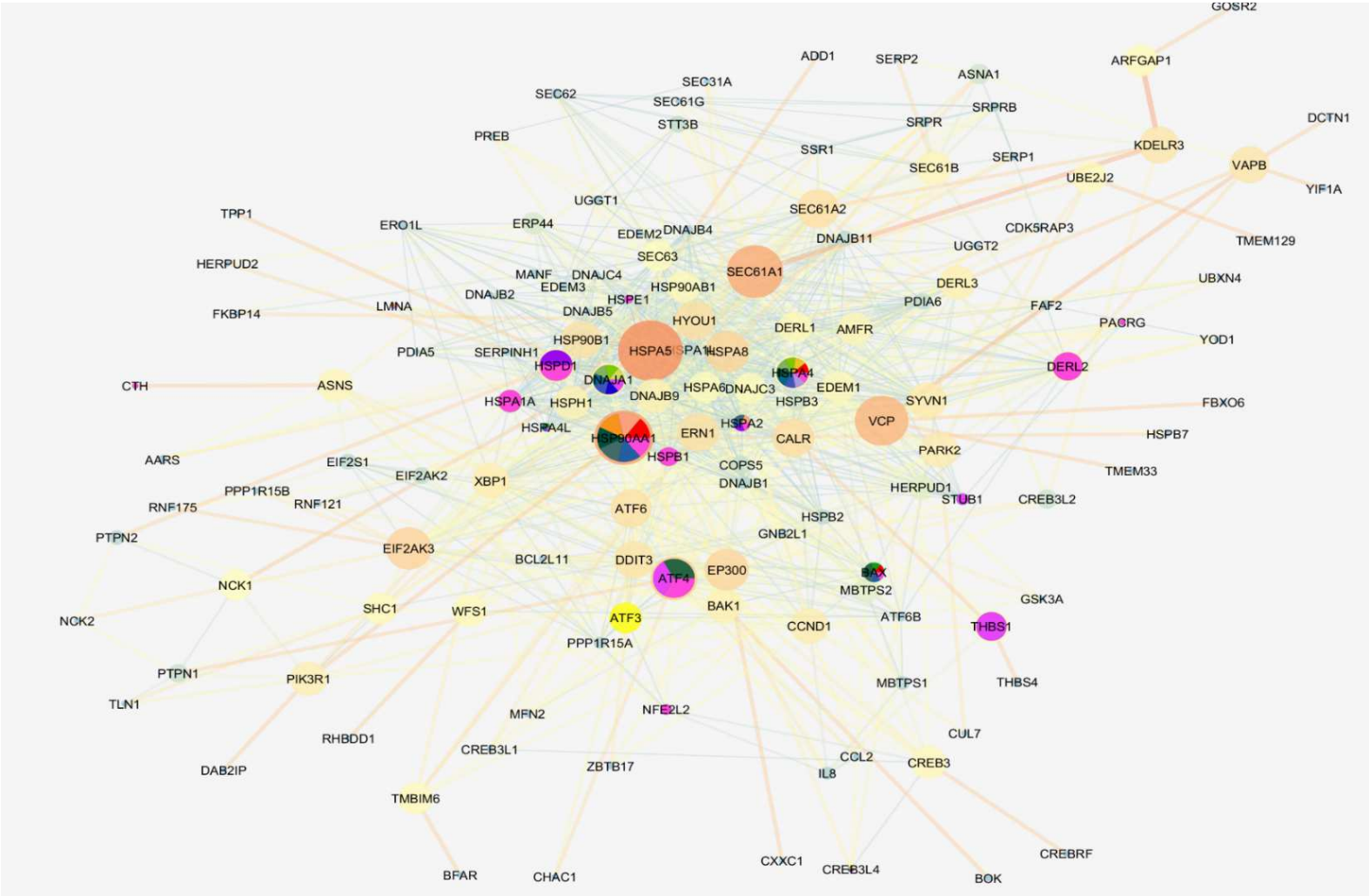


Figure 15 - Protein-protein interaction network relative to the 159 UPR proteins. Each node contains a pie-chart relative to all infertility phenotypes already associated to each protein. Interactions were retrieved from STRING database, using the Cytoscape application, StringApp.

The network analysis revealed potential proteins to study with more detail, namely the PERK, a key sensor in UPR signaling. As figure 16 illustrates, there is a lack of information regarding UPR components presence in spermatozoa (color: green) and their roles in the male reproductive system, contrary to testis (color: blue) in which all proteins were already identified (see also figure 10 and 13). A study by Amaral *et al.* (88) demonstrate that eIF2α was already present in the spermatozoa proteome, however, there is still no information regarding its activation or function in these cells. Although eIF2α in the network (figure 14) does not represent a potential hub, it is important in stress conditions and has a role in the regulation of translation, a process (virtually) absent in spermatozoa, but highly evident in testis.

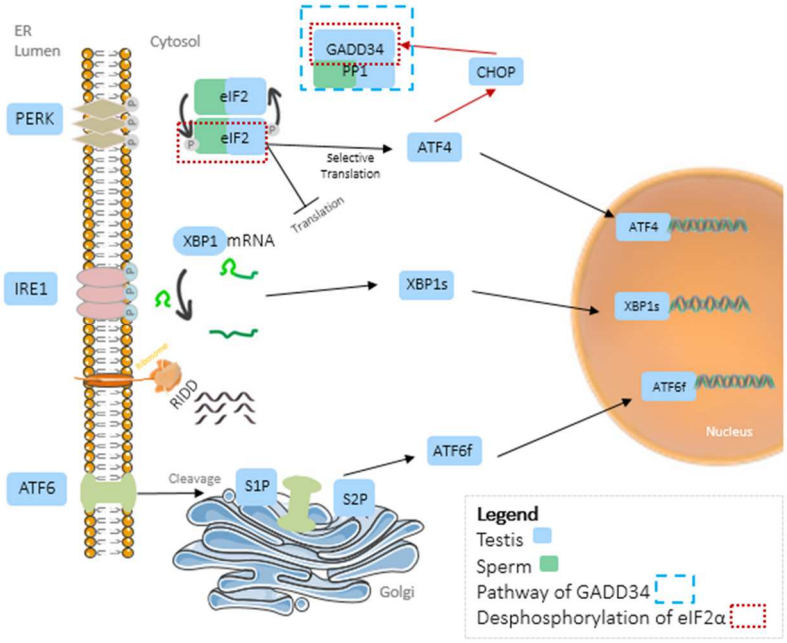


Figure 16 - Upon ER stress, unfolded and misfolded proteins bind and sequester immunoglobulin heavy-chain binding protein (BiP), thereby activating the UPR. The UPR comprises three parallel signaling branches: PERK-eukaryotic translation initiation factor 2 alpha (eIF2α), IRE1a-XBP1 and ATF6. PERK activation leads to attenuation of protein synthesis by phosphorylating eIF2α. The latter, however, allows the translation of ATF4 mRNA that in turn controls the expression of the pro-apoptotic components growth arrest and DNA damage-inducible 34 (GADD34) and C/EBP-homologous protein (CHOP). ATF6 translocates to the Golgi apparatus where it is processed by a site 1 protease (S1P) and site 2 protease (S2P) releasing its cytosolic domain (ATF6f). Inositol-requiring enzyme 1 (IRE1) produces either adaptive or death signals by splicing of XBP1 mRNA and regulated IRE1-dependent decay of mRNA (RIDD), respectively. The potential outcome of UPR activation is an increase in protein folding and transport or alternatively, the activation of ER-associated degradation (ERAD) pathway and an attenuation of protein synthesis. This image illustrates which proteins from UPR branches are present in sperm and testis proteomes resulted from this study. Adapted from (59).

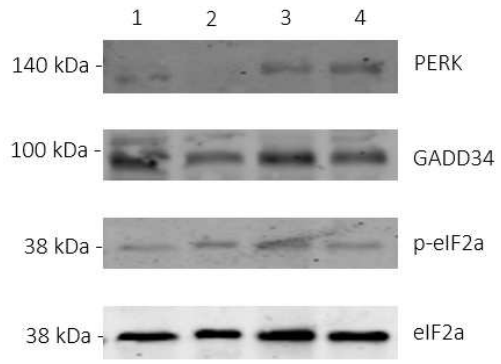
3.7 Detection of PERK, GADD34, phospho-eIF2α and eIF2α in human spermatozoa

PERK, GADD34, phospho-eIF2α and eIF2α are proteins that play important roles when somatic cells are under stress, in a process known as UPR. PERK is known to be located in the endoplasmic reticulum in most tissues, where it is activated by ER stress due to misfolded proteins (124). GADD34's location is either in the ER membrane and in mitochondrion outer membrane and recruits the serine/threonine-protein phosphatase PP1 to dephosphorylate eIF2α (125). According to HPA and UniProt immunocytochemistry studies, eIF2α is known to be located at cytosol and predicted at cytoplasmic granules, which are stress granules that appear when the cell is under stress. Regarding eIF2α, only a study by Amaral *et al.* (88) identified, in a proteomic study, this protein in human spermatozoa.

PERK and GADD34 were here identified for the first time in human spermatozoa (Figure 17-A). GADD34 expression revealed variations in the samples 1 and 3, when compared to 2 and 4, maybe due to the lower concentration levels in these last samples (12 and 24 x10^6/mL). The expression of both eIF2α and p-eIF2α appear to be consistent in all four samples.

As mentioned before, when PERK is activated, due to the accumulation of un- or misfolded proteins in the lumen of ER under stress conditions, eIF2α is phosphorylated attenuating protein synthesis (126). However, these proteins may play other functions, hence the need for further investigation to study their activation and function in human spermatozoa.

A – Protein expression in human spermatozoa



B – Basic seminal parameters of the human samples

	Age	Volume (mL)	Concentration (x10^6/mL)	PM (%)	NPM (%)	I (%)	Normal morphology (%)
1	32	3,5	229	30	14	56	12
2	33	23,7	12	25	24	51	5
3	35	2,1	60	56	15	29	18
4	36	4,3	24	23	18	59	8

Figure 17 - Western blot analysis of PERK, eIF2α and GADD34 expression in soluble fractions of ejaculated human spermatozoa; Sperm preparations corresponding to 30 µg of protein were loaded in the lanes. PM progressive motility; NPM: Non-progressive motility.

3.8 Effect of hyperthermia on human sperm cells

A study by Kim et al. (27) showed that testicular hyperthermia induces the activation of UPR signaling via phospho-eIF2α, ATF4, GADD34 and IRE1α-XBP1 and that repetitive cycles of this stress lead to apoptosis of spermatocytes in mouse testis. In this study, to understand if the components of the PERK pathway of UPR were present in human spermatozoa under stress conditions, the human spermatozoa were exposed to hyperthermia.

To mimic stress conditions and according to the study by Sabés-Alsina *et al.* (127), the optimal temperature to achieve hyperthermia conditions in sperm cells is 42º C. The remaining cells were incubated at 32,5 ºC since it is the temperature at which the sperm cells are when leaving the testis. The sperm cells were exposed for different period of times, 30 and 60 minutes, and the expression of p-eIF2α and eIF2α was evaluated (figure 19).

Previous studies indicated that salubrinal inhibits the protein complex that dephosphorylates eIF2α, maintaining eIF2α phosphorylated (figure 18) (128). In this study,

the sperm cells were treated with salubrinal 75µm, as a control with protective effects under stress.

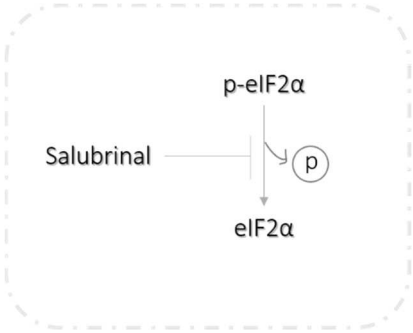
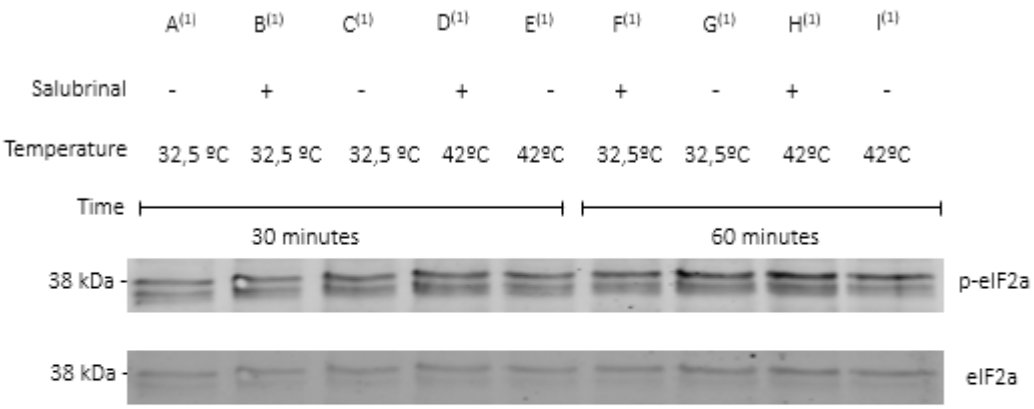


Figure 18 - Salubrinal, a selective inhibitor of cellular complexes that dephosphorylate eukaryotic translation initiation factor 2 subunit alpha (eIF2a). Under ER stress, eIF2α is dephosphorylated by a complex containing the serine/threonine phosphatase PP1 and its non-enzymatic cofactor GADD34. GADD34 recruits PP1 to dephosphorylate eIF2α, thus, reactivating protein synthesis. In this scenario, salubrinal inhibits GADD34:PP1 complex, rendering protein synthesis inactive. Adapted from (128).

A – Protein expression in human spermatozoa



B – Basic seminal parameters of the human samples

Sample	Age	Volume (mL)	Concentration (x10^6/mL)	PM (%)	NPM (%)	I (%)	Normal morphology (%)
1	35	4,4	40	56	13	31	10

Figure 19 - a) Western blot analysis of eIF2α and p-eIF2α in soluble fractions of ejaculated human spermatozoa in several conditions. Sperm preparations corresponding to 30 µg of protein were loaded in the lanes. 19-b) shows the basic seminal parameters of the human samples from the sample 1. PM progressive motility; NPM: Non-progressive motility.

The results of Western blotting revealed that salubrinal slightly induces the expression of eIF2α phosphorylated, in the presence of hyperthermia (figure 19-A). To

quantify these p-eIF2α alterations, the optical densities of the proteins signals were normalized to the optical density of the total Ponceau S staining. After that, the ratio of p-eIF2α and eIF2α was calculated and compared between control and experimental samples (figure 20).

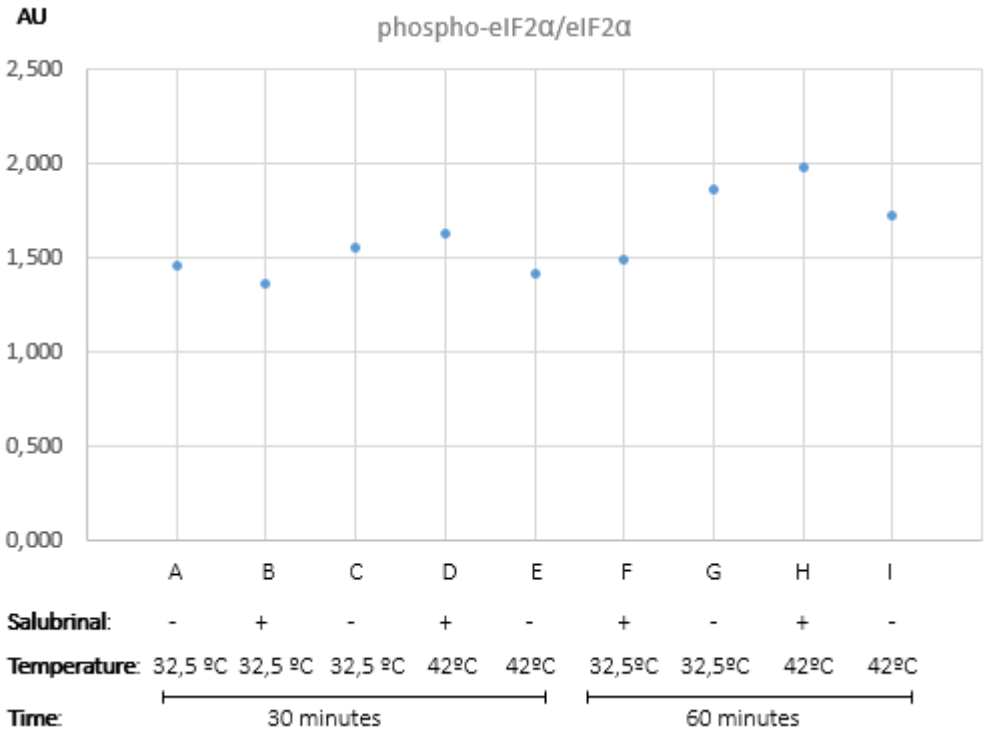


Figure 20 - Sperm cells were exposed to hyperthermia conditions (42ºC) at different time points (30 and 60 minutes). The graph illustrates the ratio phosphorylated eIF2α/ eIF2α.

In this study, temperature and different time points were considered. Here we show, for the first time, changes in the ratio of p-eIF2α and total eIF2α in spermatozoa under stress conditions (hyperthermia).

As expected, the expression of p-eIF2α was increased in the conditions where salubrinal and hyperthermia were present. Increasing the incubation period always increased the proportion of phosphorylated eIF2α (B and F, C and G, D and H, E and I). Also, the increase in the temperature resulted in a decrease of the ratio of phosphorylated eIF2α/ total eIF2α (conditions C and E, G and I), which may indicate the restore of protein synthesis in order to increase the presence of heat shock proteins to cope with the stress in these cells (hyperthermia). In the conditions where salubrinal was present, there are indeed alterations

of p-eIF2α levels when temperature is increased (B and D, F and H). Interestingly, when conditions A to B, C to B and G to F are compared, there was, in fact a decrease of p-eIF2α in the presence of salubrinal. These results show that the inhibitory effect of salubrinal was only effective in conditions where temperature reached 42 ºC, and not at 32,5 ºC.

The described results, however, are not sufficient to reliably conclude the described changes. Figure 20 illustrates a hyperthermia assay of a single individual (n = 1), hence the need for a future work with more samples to conclude with precision what happens in the sperm cells when under stress conditions. Here, it is speculated that sperm cells under stress respond by the activation of PERK through phosphorylation of eIF2α. However, a lot of issues remain to be resolved. It is poorly understood if the increased levels are related to hyperthermia conditions and for how long this phosphorylation is sustained and if it is beneficial for the sperm cells. For a better understanding of how these components are activated and the long-term effects, this work and other assays with different temperatures (50 ºC and 60 ºC, for e.g.) and different time points (several days), in all main sensors of the UPR signaling (PERK, IRE1 and ATF6) should be performed.

4. Final Conclusions

From the moment that the sperm cell is produced until fecundation, protein synthesis and the resulted proteins, some of which are secreted, play fundamental roles in guaranteeing fertilization. For this process to be completed it requires the synthesis of proteins from pre-existing mRNAs. Most proteins that a cell secretes need to be properly assembled to advance from the ER to the cell surface, otherwise, they tend to accumulate in the cytosol. If the capacity of protein-refolding is exceeded, a series of signal transduction pathways is activated, and taken together are known as UPR. Along the male reproductive system, UPR has been described for some stages of the production of sperm cells as an important ruler in proteostasis and in guaranteeing that the needed proteins for this system are presented in a proper conformation. However, the prolonged activation of UPR means that the change that activated the UPR cannot be reduced and homeostasis cannot be reestablished, ultimately leading to cell death. UPR has three principal players: ATF6, IRE1alpha and PERK, and all operate in parallel. These key sensors and their downstream effectors work towards proteostasis, and any disruption of their activity/expression can harm the cell, thus being implicated in a variety of diseases (129). The importance of compiling, mapping and analyzing these known players in testis, spermatozoa and seminal plasma is crucial for unveiling their specific mechanisms of action in the male reproductive system components. Studying these key proteins may also reveal potential biomarkers or therapeutic targets for male infertility.

The results from the bioinformatics study revealed a group of UPR proteins present in testis, spermatozoa and/or seminal plasma proteomes and established that all UPR proteins are present in testis, contrary to spermatozoa and seminal plasma. Moreover, the main proteins identified in the spermatozoa, testis and seminal plasma are chaperones, proteins that help stabilizing maturing polypeptides reducing the probability of incorrect folding and stress in the cell. The compilation of the infertility phenotypes already described for the group of 159 UPR proteins may reflect the lack of knowledge regarding these proteins in male infertility, or ultimately the absence of these proteins in this system. Still, these results gave more insights in which key proteins this work should focus on the next steps. The network analysis (with Cytoscape) and the protein-protein interaction analysis (with its app, StringApp) contributed with visual mappings across the obtained data and helped to find potential relevant proteins in the networks. One of the UPR key sensors, the EIF2AK3 (synonym: PERK), for instance, showed itself with high interactivity in the network

obtained, thus it was studied in detail by experimental approaches. The detection of PERK, eIF2α, phospho-eIF2α and GADD34 in human spermatozoa confirms that one of the branches of UPR could be activated in these cells, maybe in an alternative mechanism and conditions, given the lack of several important components for protein synthesis in the mature spermatozoa. Nevertheless, the detection of these proteins should be repeated.

Regardless, the other UPR components must be identified in both human spermatozoa and seminal plasma by Western blot, and all proteins identified should be analyzed by Immunocytochemistry to determine subcellular locations, in a future work. For assessing UPR proteins activation, the assay of spermatozoa hyperthermia demonstrated in this work should be repeated, and other kind of stressors should be tested in human spermatozoa, as well as other temperatures and cycles of hyperthermia.

Another possible approach would be to compare between normozoospermic samples and samples with atypical sperm cells, such as oligozoospermia for e.g., and identify UPR proteins and assess their expression to understand if they are somehow related to male infertility.

In conclusion, the results obtained from several bioinformatic approaches allowed a first step in the connection between key proteins of UPR pathway and male infertility phenotypes. These results illustrate through protein-protein interactions, potential targets absent in human spermatozoa and seminal plasma. Studying with more emphasis these players could give answers regarding the presence and activation of the UPR in the male reproductive system and associate them with infertility phenotypes.

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Appendix

SOLUTIONS FOR WESTERN BLOT

10% APS (ammonium persulfate)	**In 10 ml of deionized H_2O dissolve 1 g of APS**	
10% SDS (sodium dodecilsulfate)	In 10 ml of deionized H_2O dissolve 1 g of SDS	
4x Loading gel buffer	1M Tris solution (pH 6.8)	2.5 ml (250 mM)
	SDS	0.8 g (0.8%)
	Glycerol	4 ml (40%)
	B-Mercaptoethanol	2 ml (2%)
	Bromophenol blue	1 mg (0.01%)
	Adjust the volume to 10 ml with deionized H_2O. Store in darkness at RT.	
1 M Tris (pH 6.8) solution	To 150 ml of deionized H_2O add:	
	Tris base 30.3 g	
	Adjust the pH to 6.8 and adjust the final volume to 250 ml	
10x Running buffer	Tris	30.3 g (250 mM)
	Glycine	144.2 g (2.5 M)
	SDS	10 g (1%)
	Dissolve in deionized H_2O, adjust the pH to 8.3 and adjust the volume to 1L	
10x Transfer buffer	Tris	3.03 g (250 mM)
	Glycine	14.41 g (192 mM)
	Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionized H_2O. Just prior to use add 200 ml of methanol (20%)	
10x TBS (Tris buffered saline)	Tris	12.11 g (10 mM)
	NaCl	87.66 g (150 mM)

	Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O	
10x TBST (TBS + Tween)	Tris	12.11 g (10 mM)
	NaCl	87.66 g (150 mM)
	Tween 20	5 ml (0.05%)
	Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O	
Resolving gel	Deionized H_2O	
	Tris 1,5 M pH 8.8	
	Acrylamide 40%	
	Bisacrylamide 2%	
	SDS 10%	
	APS 10%	
	TEMED	
Stacking gel	Deionized H_2O	
	Tris 0.5 M pH (6.8)	
	Acrylamide 40%	
	Bisacrylamide 2%	
	SDS 10%	
	APS 10%	
	TEMED	

TABLES: COLLECTED PROTEOMES

UPR PROTEOME

Available at: https://drive.google.com/open?id=0B4fboJDy_PQDclFFeGZGdjRwUms

Two columns for Accession numbers and Gene names of every UPR protein identified.

TESTIS PROTEOME

Available at: https://drive.google.com/open?id=0B4fboJDy_PQDd2lqSzVlX0xnaUU

Five excel sheets: proteins elevated in testis (columns for gene names and gene description); proteins expressed in all (columns for gene names and gene description); proteins with mixed expression pattern (column for gene names); all proteins converted to accession numbers; list with UPR proteome (columns for accession numbers and gene names of UPR proteins and the resulted proteins' accession numbers in common with Testis proteome); all proteins in common between UPR proteome and Testis proteome (columns for accession numbers; gene names; protein names; data from OMIM, DisGeNET and MGI - IDs and terms from each database; gene ontology from UniProt and subcellular location).

SPERMATOZOA PROTEOME

Available at: https://drive.google.com/open?id=0B4fboJDy_PQDUjFWZzRoVjFneE0

Eight excel sheets: the first seven sheets contain all proteomes selected for the bioinformatic study (accession numbers, gene names and resulted proteins in common between each proteome and UPR proteome). The eighth sheet contain all data acquired for each UPR protein identified in spermatozoa: columns for accession numbers; gene names; protein names; data from OMIM, DisGeNET and MGI - IDs and terms from each database; gene ontology from UniProt and subcellular location.

SEMINAL PLASMA PROTEOME

Available at: https://drive.google.com/open?id=0B4fboJDy_PQDR29YMWlRZW1ZMlU

Six excel sheets: the first four sheets contain all proteomes extracted from each selected study for the construction of the seminal plasma proteome (note that the first sheet is a compilation of several studies - includes a column with all references for the studies collected in that review). Again, the last sheet contains all information acquired in the

bioinformatic study of UPR proteins included in seminal plasma - columns for accession numbers; gene names; protein names; data from OMIM, DisGeNET and MGI - IDs and terms from each database; gene ontology from UniProt and subcellular location.

FINAL LIST OF 159 DIFFERENT PROTEINS

Available at: https://drive.google.com/open?id=0B4fboJDy_PQDWEo5d3pOQmxaQTA

All 159 UPR proteins: columns for accession numbers; gene names; protein names; data from OMIM, DisGeNET and MGI - IDs and terms from each database; gene ontology from UniProt and subcellular location.

DATA USED FOR NETWORK (FIGURE 13)

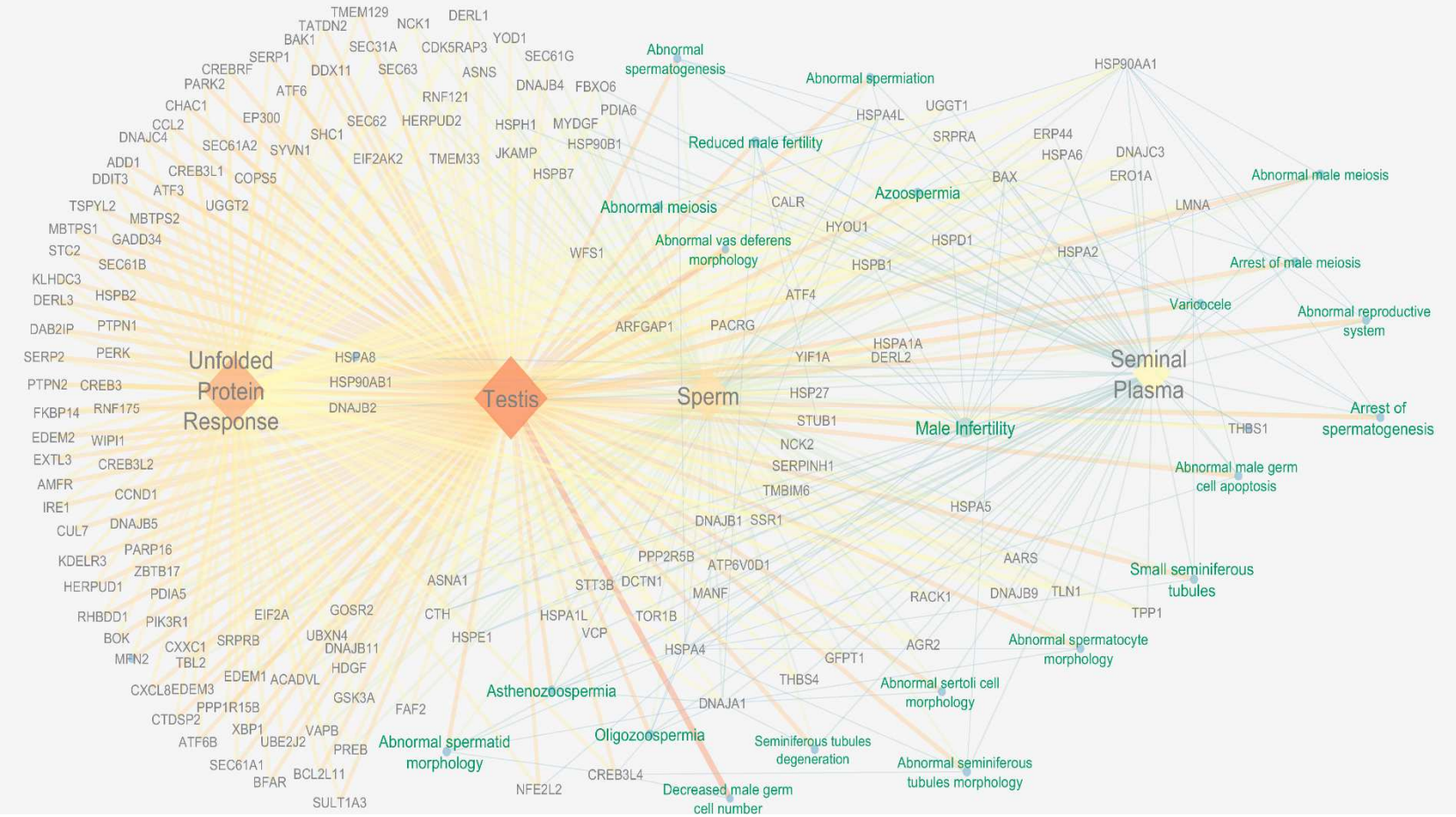
Available at: https://drive.google.com/open?id=0B4fboJDy_PQDX2YxR1FFWk1wSkU

Columns for gene names and presence in sperm, testis or spermatozoa.

FIRST NETWORK WITH ALL DATA OBTAINED FROM CHAPTER 2

DATA AVAILABLE AT: https://drive.google.com/open?id=0B4fboJDy_PQDVFROaG1iUEhPUjQ

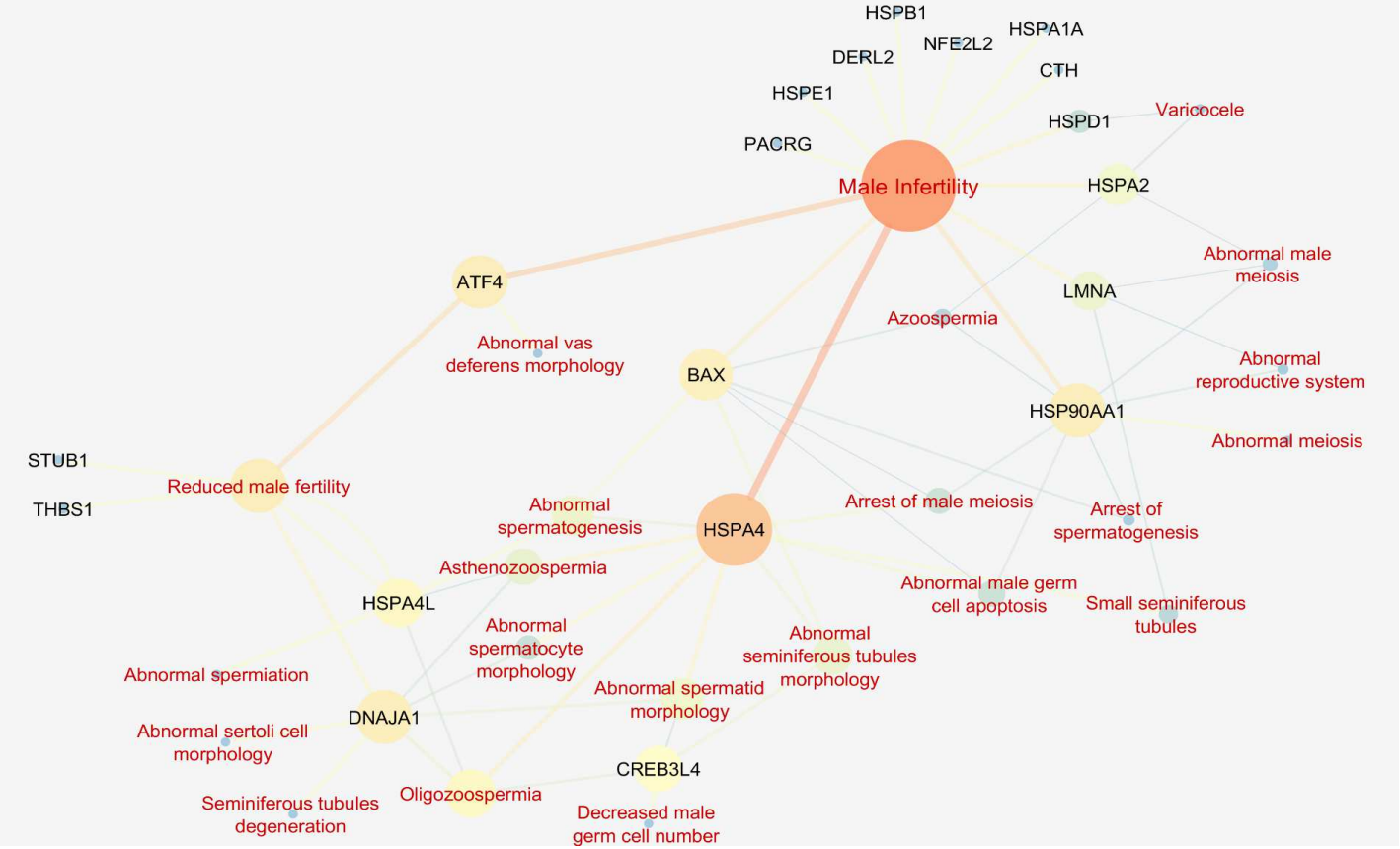
Columns for gene names, unfolded protein response and infertility phenotype.



SUB-NETWORK OBTAINED FROM NETWORK IN FIGURE 15

DATA AVAILABLE AT: https://drive.google.com/open?id=0B4fboJDy_PQDTkJIekVpX2NJenM

Columns for gene names and the infertility phenotypes associated.



Protein-phenotype association network obtained through Cytoscape analysis and Network Analyzer. Only UPR proteins already found associated to male infertility phenotypes are depicted. Only 19 out of 159 proteins have infertility phenotypes associated – 22 different phenotypes identified.

PONCEAU USED FOR NORMALIZATIONS OF EIF2A AND P-EIF2A