Universidade de
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Bruno Miguel Neiva Queirós

# IMPACTO DA TRADUÇÃO ESPERMÁTICA DE PROTEÍNAS NA MOTILIDADE

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Carlos Pedro Fontes Oliveira, Professor Auxiliar do Departamento de Química, Universidade de Aveiro e da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas, Universidade de Aveiro

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**palavras-chave** Espermatozoide, tradução, inibição da tradução, motilidade

**resumo**

Os espermatozoides são células haploides que sofrem transformações, culminando, teoricamente, no silenciamento da expressão génica. No entanto, estudos mais recentes, mostraram tradução residual em espermatozoides capacitados, particularmente nos mitoribossomas. No entanto, foi também demonstrado que esta atividade é fortemente influenciada por inibidores da tradução citoplasmática e que a inibição da síntese proteica afeta negativamente a motilidade.

O objetivo desta dissertação centra-se em caracterizar e avaliar a atividade de tradução nos espermatozoides e o seu impacto na motilidade, através de uma análise experimental e bioinformática.

Neste sentido, avaliou-se a motilidade dos espermatozoides quando tratados com inibidores da tradução citoplasmática e mitocondrial. No geral, não foram encontrados resultados significativos, mas observou-se um padrão de diminuição de espermatozoides com motilidade progressiva rápida e um aumento de espermatozoides com motilidade progressiva média, não progressiva e imóveis, sugerindo que a atividade de tradução pode desempenhar um papel na manutenção da motilidade dos espermatozoides durante a capacitação. Por outro lado, não foi possível localizar a síntese proteica no interior da célula através da incorporação de puromicina e da técnica de imunocitoquímica.

Na abordagem bioinformática, foram recolhidas 13216 proteínas de espermatozoide, das quais 654 estavam relacionadas com a tradução e 197 estavam diferencialmente expressas em espermatozoides astenozoospérmicos e de baixa motilidade. Em seguida, para encontrar proteínas altamente relacionadas e exclusivas do processo de tradução, realizaram-se análises de redes de interação proteína-proteína e de enriquecimento funcional, resultando em 71 proteínas. Dessas 71 proteínas, 12 foram encontradas associadas à infertilidade e a fenótipos e doenças relacionados com a infertilidade.

No seu conjunto, estes resultados sugerem um papel da atividade de tradução na motilidade dos espermatozoides e na fertilidade masculina. No entanto, mais estudos são necessários para compreender com maior detalhe os mecanismos associados à tradução e ao seu impacto na função dos espermatozoides, em particular a motilidade.

**keywords**

Spermatozoa, translation, inhibition of translation, motility

**abstract**

Spermatozoa are haploid cells that undergo important transformations, culminating, theoretically, in gene expression silencing. However, recent studies report residual translational activity in capacitated spermatozoa, particularly in the mitoribosomes. Nevertheless, it has been demonstrated that this activity is heavily influenced by cytoplasmic translation inhibitors and that protein synthesis inhibition negatively affects motility patterns.

The aim of this dissertation is to characterize and evaluate the translational activity in spermatozoa and its impact on sperm motility, through an experimental and bioinformatic analysis.

Firstly, sperm motility when treated with cytoplasmic and mitochondrial translation inhibitors was assessed. Overall, no statistically significant results were found, but it was possible to observe a pattern of decreased fast-progressive spermatozoa and increased medium-progressive, non-progressive, and immotile spermatozoa, suggesting that translation activity may play a role in maintaining sperm motility during capacitation. On the other hand, it was not possible to locate protein synthesis within the cell by puromycin incorporation and immunocytochemistry detection.

Concerning the bioinformatic approach, 13216 sperm proteins were collected, from which 654 were translation-related and 197 differentially expressed in asthenozoospermic and low motility spermatozoa. Next, to find highly-related proteins exclusively associated with translation, a protein-protein interaction and functional enrichment analysis were performed, resulting in 71 proteins. From those 71 proteins, 12 were associated with infertility and infertility-related phenotypes and diseases.

Together, these findings suggest a role of translational activity in sperm motility and male fertility, but further studies are needed to fully understand the mechanisms underlying translation's contribution to sperm function, particularly motility.

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## List of abbreviations:

**5'UTR:** 5′ untranslated region

**A site:** aminoacyl site

**AAA:** mitochondrial stop codon

**AAG:** mitochondrial stop codon

**Ad:** A dark spermatogonia

**AID:** artificial insemination by donor

**Ap:** A pale spermatogonia

**ATP:** adenosine triphosphate

**AUA:** mitochondrial initiation codon

**AUG:** initiation codon (cytosolic and mitochondrial)

**AUU:** mitochondrial initiation codon

**AZS:** asthenozoospermia

**B:** B spermatogonia

**cAMP:** cyclic adenosine monophosphate

**CASA:** computer-assisted sperm analysis

**$CC_i$:** clustering coefficient

**CHX:** cycloheximide

**D-CP:** chloramphenicol

**DEP:** differentially expressed proteins

**DM:** Diabetes Mellitus

**E site:** exit site

**Em:** emetine

**FR:** fertility rate

**FS:** fibrous sheath

**FUNDC2**: FUN14 Domain Containing 2

**GO:** gene ontology

**GSK3B:** glycogen synthase kinase 3 beta

**HPA:** Human Protein Atlas

**iBiMED:** Institute for biomedicine

**LM:** low motility

**MGI:** Mouse Genome Informatics

**mRNA:** messenger RNA

**MS:** mitochondrial sheath

**NZS:** normozoospermia

**OAT:** oligoasthenozoospermia

**ODF:** outer dense fiber

**ORF:** open reading frame

**OZS:** oligozoospermia

**P site:** peptidyl site

**PAGE:** polyacrylamide gel electrophoresis

**PBS:** phosphate Buffered Saline

**PFA:** paraformaldehyde

**PKA:** protein kinase A

**PKC:** protein kinase C

**Post-TC:** post-termination ribosomal complexes

**PPI:** protein-protein interactions

**QRICH2:** Glutamine Rich 2

**RM:** reduced fertility

**RP:** ribosomal proteins

**RT:** room temperature

**SD:** standard deviation

**SDS:** sodium dodecyl sulfate

**SG:** spermatogonia

**SPC I:** spermatocyte I

**SPC II:** spermatocyte II

**SPM:** sperm preparation medium

**STs:** seminiferous tubules

**STD:** spermatid

**SUnSET:** Surface Sensing of Translation

**TBS-T:** Tris-buffered saline with tween 20

**TC:** tetracycline

**tRNA:** transfer RNA

**UAA:** eukaryotic stop codon

**UAG:** eukaryotic stop codon

**UGA:** eukaryotic stop codon

**ZP:** zona pellucida

# I.

# INTRODUCTION

# 1. Spermatozoa – a rather queer cell

Spermatozoa were first discovered in 1677 by Van Leeuwenhoek [1]. Soon after, scientists started to study this cell type in mammals, learning that it varies in shape and size, but has a relatively well-conserved main structure: head, neck, and tail (**Figure 1A**).

## 1.1. Sperm head – nucleus, acrosome, and neck

The sperm head comprehends the nucleus and the acrosome enclosed in the cytoplasmatic membrane. The nucleus consists of condensed chromatin and, in species such as *Homo sapiens*, irregular, and abnormal nuclear vacuoles [1,2]. The acrosome covers up to two-thirds of the sperm head by forming a cap-like structure around the nucleus, as seen in **Figure 1A**. It consists of an inner and outer membrane that surrounds hydrolytic enzymes required for oocyte penetration and fertilization [1,3].

The sperm neck divides authors as to whether it should be included in the head or the tail, as it is a connecting piece between both parts. This connecting piece is composed by a ‘‘capitulum’’, a structure made of segmented columns and dense fibrous (as in sperm tail) and an almost insignificant number of mitochondria-derived from the mitochondrial sheath of the sperm midpiece [1,4].

## 1.2. Tail - midpiece, principal piece, and end piece

The head and neck are rather small structures standing to the sperm cell tail, as this is the longest sperm structure. The sperm tail can be divided into the midpiece, principal piece, and end piece [1]. Together, they are responsible for the sperm motility.

The tail axoneme is the main structure in sperm motility, being composed by tubulin microtubules: two central microtubules and nine peripherical microtubule pairs. The microtubule pairs include a circular microtubule subunit A and a C-shaped subunit B that attaches to the end of subunit A. Subunit A helps maintain the stability of the complex through nexin links with adjacent microtubule pairs, connections with central microtubules via radial spokes, and formation of dynein arms (**Figure 1B**) [4]. The sperm cell tail also contains outer dense fibers (ODF) arranged in a 9+2+2 pattern [1], which originate from major and minor columns of dense fiber and attach longitudinally to the corresponding microtubule pair [4,5].

In the midpiece, the mitochondria form a tight helix around the layer of ODF – mitochondrial sheath (MS) (**Figure 1B1**). Those mitochondria are responsible to provide

energy to providing energy and during spermatozoa's movement [1,4].It is important to note that midpiece mitochondria are more regular, numerous, and organized than the ones seen in the neck [2]. At the distal extremity of the MS, the annulus divides the midpiece from the principal piece and it is necessary for the normal movement of the tail, avoiding mitochondria displacement [1,4,6].

The principal piece is the longest part of the tail and is characterized by a fibrous sheath (FS) that begins right above the annulus (**Figure 1 B2**) [1,4]. The FS consists of two longitudinal columns, ventral and dorsal, connected by ribs that go around the tail. This structure follows the tail length becoming smaller and leaner until ending just before the end piece [1].

The end piece is very similar to a normal flagellum, whereas it is just the axoneme surrounded by the plasma membrane (**Figure 1 B3**) [2]. However, as the tail gets thinner, some elements of the main structure get lost, such as dynein, and consequently, the nine pairs of microtubules divide into eighteen single microtubules, giving up the pattern 9+2+2 seen in the other tail pieces [7].

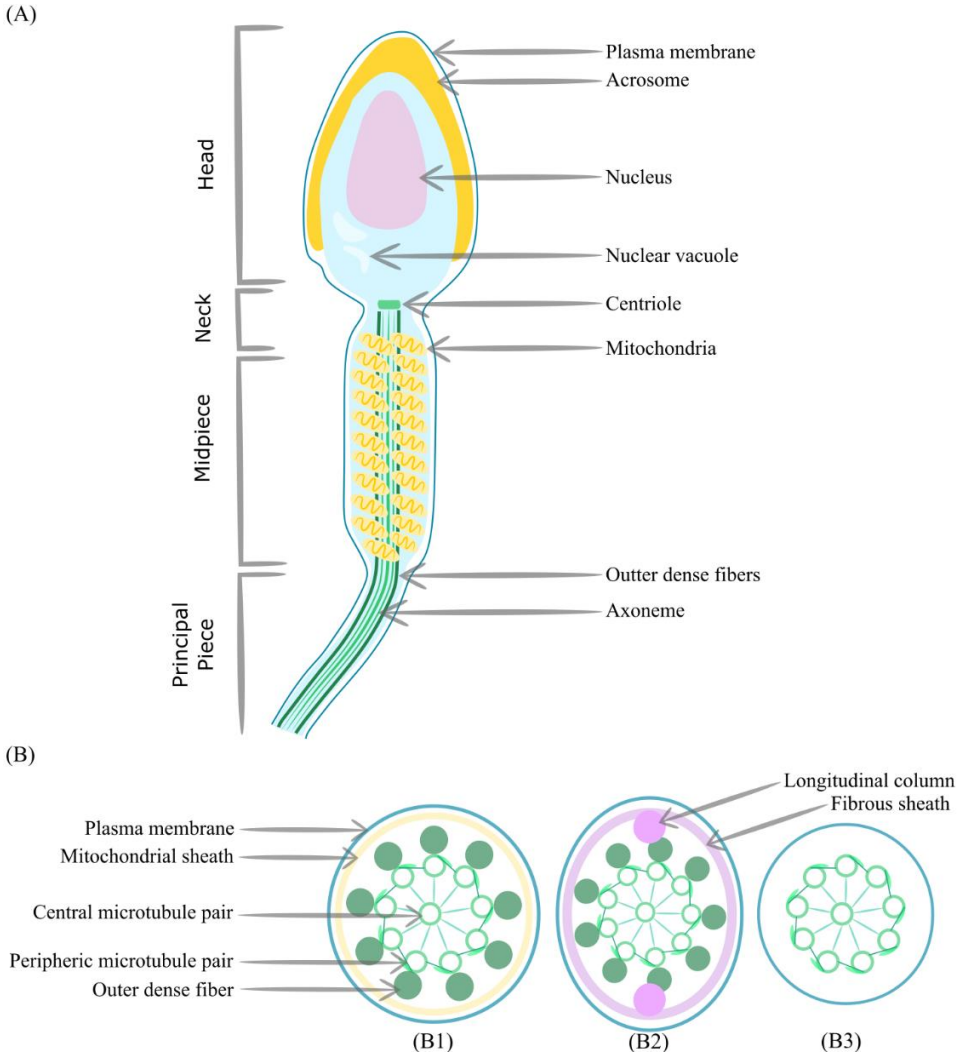

**Figure 1 - Representation of the spermatozoa's structure. (A)** The spermatozoa are morphologically divided into the head, which comprises the nucleus and the acrosome, and the tail, which is divided into midpiece, principal piece, and end piece. **(B)** The sperm tail's main structure is the axoneme enclosed by outer dense fibers (ODF). **(B1)** Transversal cross-section of the midpiece presents the axoneme and the ODF, surrounded by a mitochondrial sheath responsible for providing energy to this cell motility. **(B2)** Similarly, the cross-section of the principal piece maintains the main structure of the tail, but instead of a mitochondrial sheath, it has a fibrous sheath. **(B3)** The end piece only presents the axoneme.

## 2. Spermatogenesis

Spermatozoa are formed in a process named spermatogenesis, a complex process that includes mitosis, meiosis, and spermiogenesis [8,9]. Spermatogenesis is a highly regulated process that occurs in seminiferous tubules (STs) within testis, and in humans takes approximately 64 days leading to the formation of up to 200 million spermatozoa each day [8–10]. This process is summarized in **Figure 2**.

### 2.1 Proliferation, differentiation and meiosis

The primordial germ cells migrate from extra-embryonic tissues to the gonadal ridge where they differentiate into G0 gonocytes [10]. These cells are enclosed by immature Sertoli cells and are mitotically inactive [9,10]. When puberty starts, spermatogonia undergo 3 main events: proliferation, apoptosis and differentiation into A pale, A dark and B spermatogonia, according to their heterochromatin content [8,9,11,12]. Spermatogonia are diploid cells that have distinct roles based on Sertoli cell-derived factors: B spermatogonia proceed to meiosis, A pale spermatogonia can multiply or differentiate into B spermatogonia, and A dark spermatogonia are quiescent cells that act as a backup cell population [10,13–16] . In order for meiosis to start, the B spermatogonia undergo one more mitotic division and then enters meiosis [14,15].

Diploid B spermatogonia undergo meiosis to generate haploid round spermatids. After proliferative steps, the diploid B spermatogonia (2N) is converted to a primary spermatocyte, which enters meiosis [9]. Prophase I is initiated, where chromosome condensation, homologous chromosome pairing, and crossing-over events occur [8,17]. In metaphase I, the homologous chromosomes align on the metaphase plate and migrate to opposite sides of the cell, followed by telophase I and cytokinesis, resulting in two secondary spermatocytes [8,17–19]. Meiosis II then breaks down each chromosome into

single chromatids, resulting in four haploid round spermatids [8,17–19]. The process of meiosis is complex and variable in duration, with meiosis I being the slowest part and meiosis II taking about 6 hours to convert a diploid spermatocyte into a haploid round spermatid [9]. Nevertheless, the process of meiosis is important for genetic variability and human evolution [10,20].

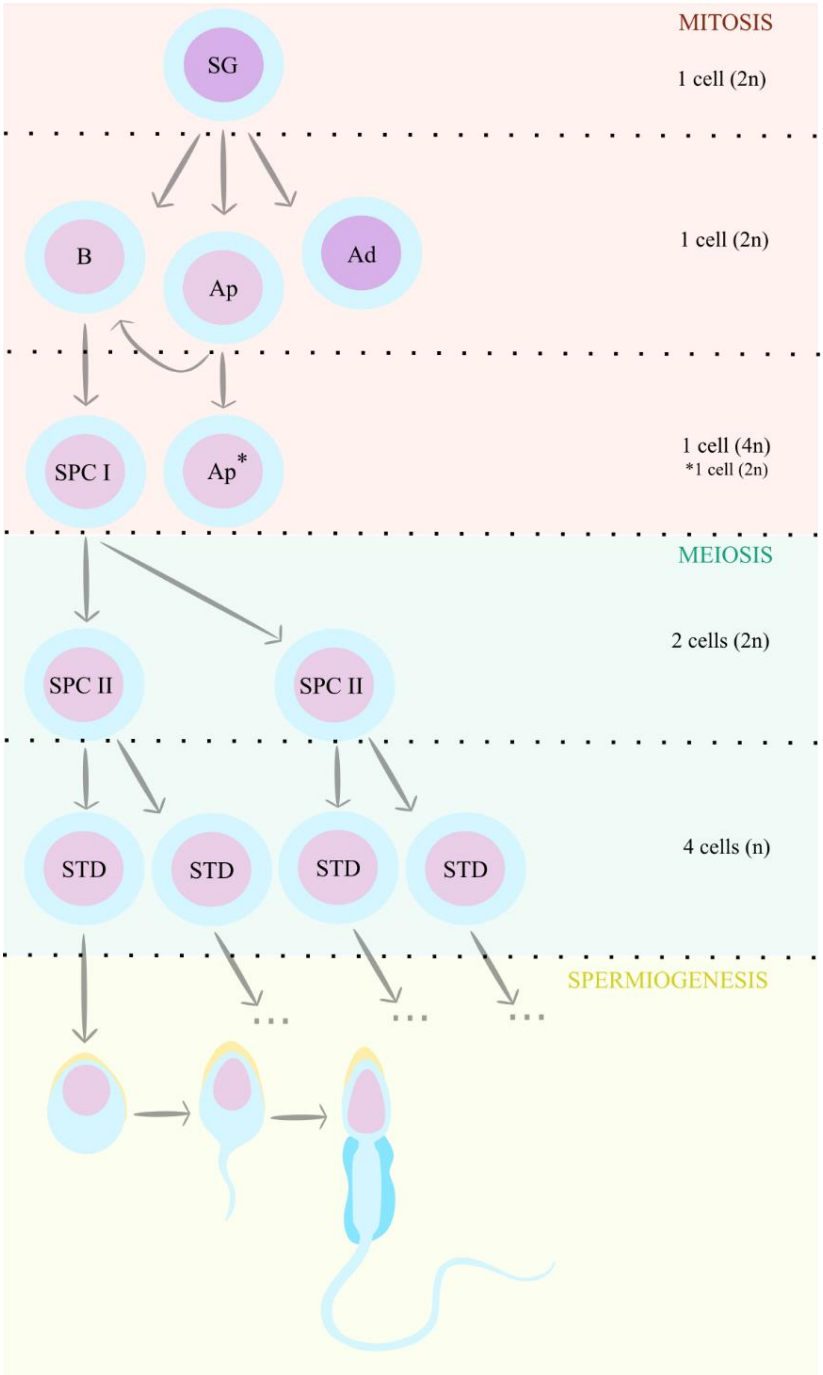


**Figure 2 - Schematic representation of the spermatogenesis events.** Spermatogonia (SG) are responsible to self-renew and differentiate into spermatogonia B (B), A pale (Ap) and A dark (Ad). Spermatogonia B then differentiates into spermatocyte I (SPC I), which enters meiosis and differentiated into spermatocyte II (SPC II) and, ultimately, becomes a spermatid (STD). Finally,

the STD undergoes nuclear rearrangement, acrosome and tail formation, and cytoplasm/organelles remotion, becoming a spermatozoon.

### 2.2 Spermiogenesis

When the spermatid is completely formed the cell undergoes a set of transformations to become a spermatozoon – spermiogenesis [9]. These complex modifications consist of nucleus rearrangement, tail formation, organelle alterations, and cytoplasm remotion [8].

The chromatin in the nucleus undergoes significant rearrangement, with histones being replaced by protamines, resulting in highly condensed chromatin [8,21–23]. This substitution supports the theory that sperm cells are transcriptionally silenced. However, some recent studies showed that, during spermatogenesis, mRNA molecules are present [24,25] and transcription occurs until the very last step of spermatozoa maturation [26,27]. In addition to proving transcription and mRNA presence, authors defend that RNA molecules can be translated long after they have been transcribed, which opened the door for new studies and theories that will be discussed in the translation section below [24,28,29].

Additionally, beginning in prophase I and continuing through spermiogenesis, Golgi vesicles fuse together, forming the acrosome near the nuclear envelope with assistance from the perinuclear theca [8,30,31]. The acrosome increases in size and becomes more flattened as additional vesicles are assembled, while the Golgi apparatus adopts a spheric shape and moves to the caudal regions, leaving a cytoplasmic droplet [8,17]. The nucleus migrates to the periphery of the cell and becomes adjacent to the acrosome, which is then fully formed [8].

The round spermatid has two centrioles, a proximal and a distal one [8]. From the distal centriole, the axoneme starts building up with the assembly of the microtubules and other structural elements and, as this happens, the round spermatid is now an elongated spermatid [8,10,32]. Additionally, the ODF, the fibrous sheath, and the mitochondria sheath start to form along the axoneme, giving the sperm tail the characteristic structure discussed in the section 1.2 [10,32] .

As the tail is formed, the spermatid gains a shape that facilitates its movement, however, the cell motility is highly increased with the remotion of the cytoplasm and other organelles, forming the residual body that is later phagocytized by Sertoli cells [10,32]. Part of the cytoplasm can be preserved as a factor for sperm maturation, being named the cytoplasmatic droplet. The cytoplasm droplet is located between the sperm head base (as seen in humans) and the annulus, according to the maturation stage [8].

Lastly, when the spermatid has completed all these transformations to become a spermatozoon, spermiation occurs in which the newly formed spermatozoa are separated from the Sertoli cells, leaving the seminiferous epithelium, and moving to the seminiferous tubule (ST) lumen [10,33].

## 3. Sperm cell maturation and capacitation

After spermatogenesis and spermiogenesis, spermatozoa are functional but still lack motility and fertilization capability. The spermatozoa leave the ST and enter the epididymis, where they maturate. Spermatozoa reach their full fertilizing potential in the female reproductive system, where they undergo capacitation [34].

### 3.1 Sperm maturation in epididymis

The epididymis comprehends the caput, corpus, and the cauda, which is connected to the vas deferens. Each epididymis segment contribute differentially to sperm maturation since different genes, proteins and ion concentrations are found [34].

Caput sperm cells have in vitro inducible motility, but different flagellar bending. This suggests that spermatozoa arriving in epididymis have functional flagellar machinery, but they undergo changes along the epididymis, which allow them to naturally move once they enter the vas deferens [35,36].

The cytoplasmatic droplet has water and ion channels that allow it to regulate the cytoplasmatic volume along the epididymal journey [34]. Additionally, it possesses a set of enzymes responsible for energy metabolism. Morphologically, the droplet moves from the sperm neck to the annulus [34]. It is important to note that not all mammalian species retain the cytoplasmatic droplet during cell development, and its function is not fully understood. However, it has been reported that sperm cells without this structure are less motile, which supports the hypothesis that cytoplasmic droplet has a role in spermatozoa maturation [37].

Along with these prominent changes, major remodeling events occur at which alter the content of proteins, lipids, and sugars. Assuming spermatozoa are translationally silenced this content alteration must occur through epididymal proteins incorporation into the sperm cell [34]. The most accepted mechanism for this transference is the epididymosomes, extracellular vesicles secreted from the epididymal epithelium and contain proteins, RNAs, and lipids [38,39]. Other hypothesized mechanisms claim that this

protein transference occurs directly by absorption of soluble epididymal proteins or by interactions between the sperm cell and the apical epididymal epithelial membrane [34,40]. Either way, the acquired proteins have major roles in sperm motility,[41,42] capacitation,[43] acrosome reaction [44] and fertilization,[45,46] but also in defective sperm targeting [47,48]. Along with the protein acquisition, proteins in the spermatozoa also undergo post-translational modifications, mainly phosphorylation and thiol groups oxidation [34].

Regarding lipid and sugar remodeling, it occurs on the sperm surface [34]. Sugar remodeling adds negatively charged components [49–51], while lipid remodeling reduces cholesterol and increases fluidity [52,53]. Both the charge change in the plasma membrane and the fluidity increase may be essential to oocyte recognition and fertilization.

### 3.2 Capacitation

Capacitation is a process that begins after ejaculation when spermatozoa encounter the female reproductive tract. There, sperm capacitation is induced by the female tract environment, particularly by the $HCO_3^-$ and $Ca^{2+}$ concentrations and pH changes [54,55].

One of the capacitation's first steps is the removal of seminal plasma along with the surface-adhered decapacitation factors [8]. Then, the elevated concentration of $HCO_3^-$ activates the soluble adenyl cyclase, increasing the amount of cyclic adenosine monophosphate (cAMP), and, consequently, protein kinase A activity (PKA) [56,57]. The PKA signaling pathway has a main role in the changes undergoing these events. For instance, activation of PKA triggers a phospholipid translocase, which removes cholesterol molecules and rearranges lipids from the sperm's plasma membrane [55,58]. These changes significantly increase membrane fluidity and permeability, allowing the intake of $Ca^{2+}$, the increase of intracellular pH, and the further activation of intracellular signaling pathways [8,57,59].

A hallmark of capacitation is the intense protein phosphorylation, which is carried out by PKA, but also protein kinase C (PKC) and glycogen synthase kinase-3 beta (GSK3B) [8]. Briefly, these proteins phosphorylate tyrosine and serine/threonine residues on ionic channels, structural proteins, enzymes, and members of other signaling pathways [60]. These events seem to have a major impact on the tail's principal piece, which is associated with hyperactivated motility [61].

Even before capacitation, the asynchronous phosphorylation and dephosphorylation of dynein molecules cause the axoneme pairs of microtubules to slide along each other, bending the sperm's tail and allowing it to move [62,63]. However, with the changes mentioned here, spermatozoa develop hyperactivated motility, which is essential to

leave the oviduct's epithelium and fuse with the oocyte [64–66]. This type of movement consists of an asymmetrical whip-like motion of the sperm tail by a high-amplitude beating pattern that is activated upon $Ca^{2+}$ entry in the cell [50,67,68]. This enhanced motility is due to $Ca^{2+}$, $HCO_3^-$ and cAMP,[69,70] but also requires the stimulation of effector molecules, such as progesterone,[55] and an optimal cytoplasmatic pH, such as 7.8 in human case [71].

Interestingly, the phosphorylation patterns shift from the sperm tail to the acrosomal region along capacitation steps [61]. This indicates that phosphorylation of the tail is important for initial capacitation steps, such as hyperactivated motility, whereas as the sperm approaches the oocyte, phosphorylation plays a critical role in zona pellucida binding and the acrosome reaction [61].

In short, by encountering the female reproductive tract, spermatozoa's plasma membrane undergoes remodeling, and several intracellular signaling pathways are activated. This leads to extensive protein phosphorylation, which is crucial for spermatozoa's environment adaptation, as well as its ability to penetrate and reach the oocyte.

# 4. Translation

### 4.1 Cytosolic Translation - from AUG to UAA (or UAG or UGA)

Translation occurs after transcription as part of gene expression in cells. It starts with the recruitment and attachment of translational machinery to a mature mRNA (initiation), leading to a newly formed protein (elongation and termination).

The ribosome is a ribonucleoprotein complex responsible for mRNA translation into protein [72]. This supramolecular complex is well-conserved among all living beings but is rather larger and more intricate in eukaryotic species [73]. Eukaryotic ribosomes include 2 subunits, a small subunit (40S) and a large subunit (60S), 4 different rRNA molecules, and 79 core ribosomal proteins (RP) [74,75]. Internally, the ribosome has 3 main translation-related sites, each with its own role in protein translation: the aminoacyl-transfer RNA (tRNA) (A site), peptidyl-tRNA (P site), and deacylated tRNA (exit or E site) [76,77].

The ribosome is the main effector in the protein-making process, but several translation factors are needed, acting directly along translational steps, or maintaining the stability and fidelity of these events.

### 4.1.1 Translation mechanism

The process of mRNA translation involves the attachment of the small ribosome subunit to mRNA, which may require the unwinding of the 5'UTR region [78]. Several factors collaborate to recruit the 40S subunit, which then proceeds to scan the mRNA for the start codon (**Figure 3A**) [79,80]. This step is particularly highly regulated to ensure the fidelity of initiation, avoiding base-pairing in the 5'UTR region and promoting the recognition of the exact start codon by discriminating between non-AUG triplets and AUG triplets [80–83]. Once the start codon has been identified, the translation complex adopts a "closed" conformation onto the mRNA that is being translated [84–86]. Finally, the 60S subunit assembles with the 40S submit the form the 80S machinery, and the elongation steps begin **(Figure 3A)** [78,84].

In addition to being the critical step in mRNA translation, initiation is highly regulated, either through mechanisms that impact translation machinery or that impact mRNA molecule itself [87]. For one thing, the availability of translation factors can be controlled through reversible phosphorylation or protein binding, inhibiting their participation, and reducing the translation rate [78,88]. On the other hand, through the action of RNA-binding proteins (RBPs), which bind to specific RNA motifs, the attachment of the 40S subunit is prevented, thus inhibiting translation [87].

At the beginning of the elongation phase, the ribosome has encountered the start codon AUG and recruits a Met-tRNA$_i^{Met}$ – the tRNA (transfer RNA) transporting methionine – to the A site (**Figure 3B**) [89]. The ribosome then recruits an aminoacyl-tRNA complementary to the second codon of the open reading frame (ORF), which is accommodated in the A site. The ribosome rotates, allowing the formation of a peptide bond between the first and second amino acids, and moves the recent codon-anticodon bond to the P site (**Figure 3C**) [90–94]. This process is repeated as the ribosome moves along the mRNA, adding new amino acids to the growing peptide chain [90–94.]. The ribosome changes between open/closed conformations and new aminoacyl-tRNA is added to the A site while deacylated tRNA is moved to the E site, later being released from the translation machinery (**Figure 3C**) [95]. The elongation process continues until the entire protein sequence has been synthesized.

The process of translational elongation continues until a stop codon is encountered, signalling the beginning of the termination steps (**Figure 3D**) [96]. When the ribosome reaches the end of the ORF and encounters a stop codon, eRF1, a protein shaped like a tRNA, is recruited to the translation complex and terminates the protein synthesis by releasing the nascent peptide [97–99].

To start the synthesis of a new protein, the ribosomal complexes from the previous cycle need to be recycled meaning that all ligands must be released, and the subunits dissociated (**Figure 3E**) [100]. Therefore, mRNA and tRNA molecules are released from the post-termination ribosomal complexes (post-TCs) along with unnecessary translation factors. Eventually, however, some factors remain bound to the 40S subunit to prevent its reassociation with the 60S subunit, but the ribosome's subunits are, at this point, ready for a new translation cycle [84,100,101].

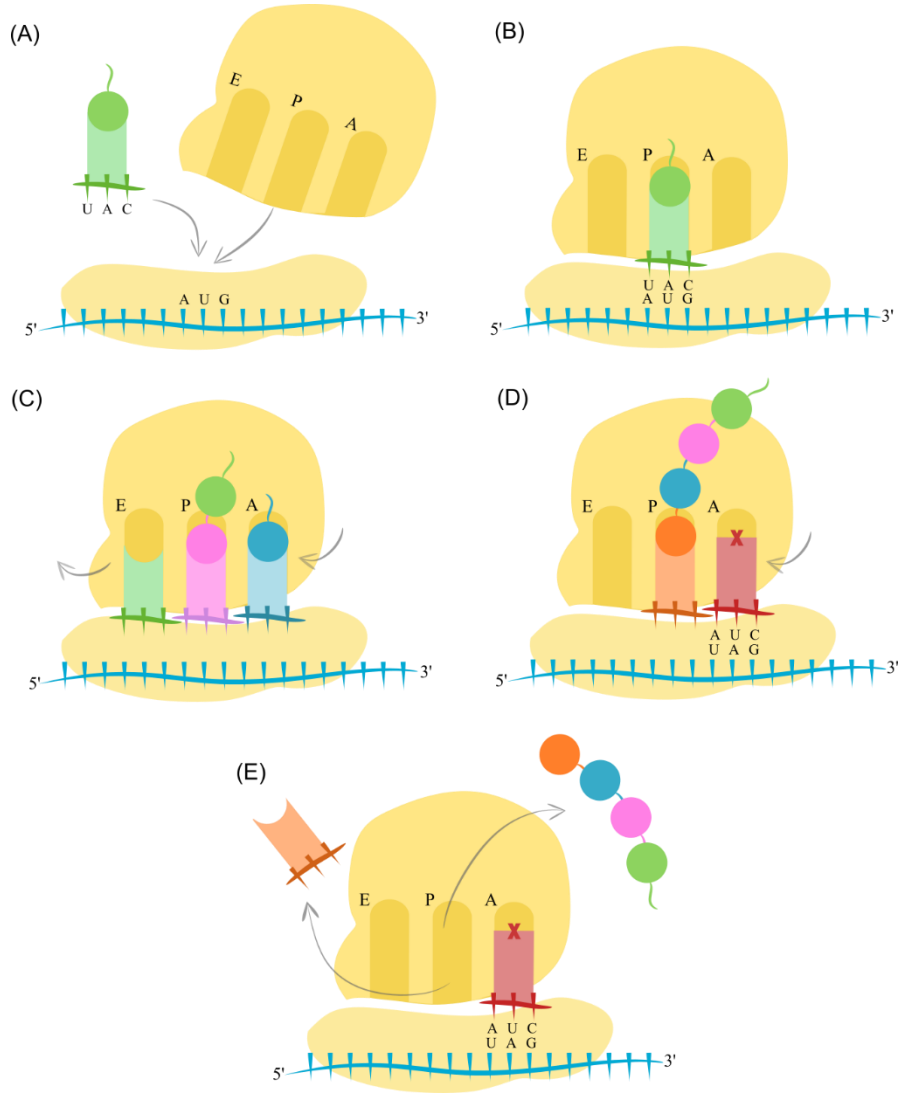


**Figure 3 - Schematic representation of the protein synthesis. (A)** The ribosome 40S subunit attaches itself to mRNA and scans the molecule for the start codon, AUG, which **(B)** recruits methionine to the ribosomal P site. **(C)** The ribosome proceeds to read the mRNA molecule and recruits the aminoacyl-tRNA that corresponds to the second codon into the A site. Methionine and the newly added amino acid form a peptide bond, which is moved to the P site while the deacylated Met-tRNA to the E site. **(D)** The ribosome moves along the mRNA molecule reading each codon and adding new amino acids to the peptide until a stop codon is found. **(E)** When a

stop codon is found eRF1 terminates the protein synthesis and liberates the newly synthesized peptide.

### 4.2 Mitochondrial translation vs. cytosolic translation

Eukaryotic cells perform translation in both the cytoplasm and mitochondria. While mitochondrial translation shares similarities with cytoplasmic translation, it retains characteristics of bacterial translation due to its bacterial origin [102].

Mitoribsomes have lower sedimentation coefficient (55S), being composed by a 39S large subunit and a 28S small subunit [103,104]. During translation initiation the mRNA binds to the small ribosome subunit [104] and is scanned for an initiation codon, as occurs in cytosolic translation [105]. Curiously, mitochondrial mRNA doesn't have 5'UTR, contrarily to what occurs in cytoplasmic mRNAs. Still on the initiation steps, unlike cytoplasmic translation, the initiation amino acid in mitochondrial translation is formylated methionine, rather than methionine itself [106].

During mitochondrial translation elongation, the ribosome moves and rotates, incorporating new amino acids and moving along the mRNA molecule [107,108]. The 39S subunit of the mitoribosome has two exit sites from which the peptide can emerge, either the conventional E site (as in cytoplasmic translation) or a site before it that prematurely exposes the peptide to the mitochondrial matrix [109]. The process continues until a stop codon is reached, followed by recycling. In mitochondria UAA and UAG codons still codify for translation termination, but the mitochondrial genetic code has some specificities where UGA encodes tryptophan and AAA and AAG are stop codons as well [110,111].

It is worth noting that about 98% of mitochondrial proteins are encoded by genes from the cell's nucleus as the mitochondria itself lost or transferred its genetic information during evolution and the origin of the eukaryotic cell [112–115]. This exemplifies the need for import mechanisms and active exchange between cytoplasm and mitochondria. Structural studies showed the presence of protein translocation channels along the mitochondrial membranes [116–118], explaining how nuclear-encoded proteins can move from the cytosol to mitochondria.

## 5. Protein translation in sperm cell: myth of fact?

For several years the scientific community believed that transcription and translation were silenced in germ cells as these processes were shut down during meiosis and spermiogenesis to allow the nucleus and chromatin rearrangement [119]. This is considered a biology dogma. As new techniques emerge and get optimized, scientists began to note that spermatozoa carry specific populations of RNAs (long-term RNA synthesized by spermatozoa or incorporated epididymosome's RNA ), hypothesizing that are either left behind during cell development or stay with a purpose in oocyte fertilization and embryonic development [120–123]. However, it has been shown that transcription activity and RNA content varies with sperm motility, cryopreservation, and even the carried sex chromosome, meaning sperm RNA may have more roles than first hypothesized [121].

The first observation of protein synthesis in spermatozoa occurred when protein localization changes were seen due to total protein content augmentation and not intracellular movements, as expected [104]. Gur and Breitbart [104,124] utilized labeled amino acids to examine the effect of chloramphenicol, an inhibitor of protein chain elongation, in nuclear-encoded sperm-specific proteins and showed that translation started not long after capacitation initiation in mitochondrial ribosomes. Mitochondria are able to synthesize mitochondrial transmembrane proteins, but to function correctly they import several nuclear-encoded proteins through contact sites between the mitochondrial membranes [104,125]. The fact that mitochondria and the cytosol establish exchanges, with the results discussed before, suggests that this organelle is likely responsible for co-translation and translocation of nuclear-encoded proteins [104,126,127].

Capallo-Obermann (2011)[119] studied highly purified spermatozoa RNA and showed that, contradictorily to past results, human spermatozoa had rRNA. In the results, she presents evidence that both 18S and 28S rRNA from cytosolic ribosomes, and 16S and 12S rRNA from mitochondrial ribosomes, are present in ejaculated spermatozoa. Additionally, with transmission electron microscopy and immunohistochemistry techniques, the existence of cytoplasmic ribosomes was proved in ejaculated spermatozoa, but these were not functionally assembled, unlike mitochondrial ribosomes.

Moved by this information, Nunes J (2018)[128] and Corda P (2019)[129] carried out their experiments using the Surface Sensing of Translation (SUnSET) method. They experimented with puromycin, a tRNA analog, and protein synthesis inhibitors, D-chloramphenicol (D-CP) and cycloheximide (CHX), showing that translation levels are lower than translation seen in somatic cells, but they are still positive corroborating Gur

and Breitbart's studies. However, with the inhibitors chosen they were able to assess whether translation occurs in cytosolic or mitochondrial ribosomes, and even though both seem to be active, the cytosolic translation machinery was the most affected by the inhibitor's action, contradicting Gur and Breitbart and corroborating Capallo-Obermann's work.

Asthenozoospermia is an infertility condition characterized by decreased progressive motility in spermatozoa, whose underlying mechanisms are still unclear [130]. However, evidence suggests that altered RNA content and phosphoprotein profile may contribute to impaired motility, which is not surprising considering that RNA content has already been associated with sperm motility, as mentioned before [121,130]. Besides, other studies report impaired motility when protein synthesis is inhibited. The inhibition of cytoplasmatic protein synthesis had a spermicidal effect, with a strong impact on flagellar growth suppression [131,132]. Similarly, the mitochondrial translation inhibitors led to a decrease in sperm motility without affecting ATP production, thus suggesting a necessity for the synthesis of motility-related proteins within the mitochondria [133,134].

Interestingly, bacteria with mitochondrial translation inhibitors showed downregulation of motility-related genes and flagellar assembly, leading to flagella abnormalities, low motility, and cell agglutination [135,136]. Considering the prokaryotic origin of mitochondria, this evidence further supports and helps understand the impact of mitochondrial translation on sperm metabolism, particularly motility.

Altogether, this information suggests that spermatozoa may synthesize motility-related proteins, such as flagellar proteins, to replace degraded proteins during sperm capacitation, as proposed by Gur et Breitbart [124]. This proposition also finds support in a proteomic analysis of spermatozoa treated with chloramphenicol (D-CP), which revealed a differential expression of FUN14 Domain Containing 2 (FUNDC2) protein, a component of the outer mitochondrial membrane, and Glutamine Rich 2 (QRICH2) protein, a testis-specific protein involved in sperm tail development [133]. Curiously, this observation also provides further evidence for the hypothesis on mitochondrial importation and co-translation of nuclear-encoded proteins [124].

## 6. Goals

Spermatozoa undergo an extensive set of changes and specializations to become structurally and functionally able to fertilize the egg but become theoretically unable to transcribe DNA and translate mRNA. However, recent studies show that translation occurs as a residual process, and it may impact sperm's motility. Together, these studies give rise to several questions and hypothesis: even though translation in spermatozoa is residual, does it occur by chance, or does it have a role to play? What impact does translation has in sperm cell's function, particularly in motility? Does translation occur in cytoplasm or mitochondria? Which proteins are being translated and what role do they play? New studies are needed not only to corroborate the previous findings but also to answer these questions and all raised hypotheses.

Therefore, in the present study we aimed to evaluate spermatozoa translation activity with three main goals:

i. To assess the protein synthesis inhibition's impact on sperm motility.
ii. To locate puromycin incorporation in spermatozoa.
iii. To identify, *in silico,* translation-related differentially expressed proteins in asthenozoospermic and low-motility spermatozoa.

# II.

# LOCATING TRANSLATION IN SPERM AND ASSESSING ITS IMPACT ON MOTILITY – AN EXPERIMENTAL APPROACH

# 1. Material and Methods

The experimental procedures described below were performed in the Signal Transduction Laboratory, at the Institute for Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal).

## 1.1 Motility Assay

### 1.1.1 Bovine sperm preparation

Fresh bovine semen samples (n=3) were kindly given by LusoGenes, Lda (Aveiro, Portugal). We firstly isolated spermatozoa from the seminal plasma by centrifugation (500g, 10 min, room temperature (RT)) and washing with 1x Phosphate Buffered Saline (PBS). The spermatozoa concentration was assessed using the Sperm Class Analyzer (CASA) system (Microptic S L, Barcelona, Spain) with SCA® software (v6.2.0.16).

### 1.1.2 Impact of translation inhibition in sperm motility

To understand the impact of sperm translation inhibition on sperm motility, experiments using cytoplasmic – cycloheximide (CHX) and emetine (Em) – and mitochondrial – chloramphenicol (D-CP) and tetracycline (TC) – translation inhibitors were conducted. The main goal was to determine whether protein synthesis is important for maintaining sperm motility and whether there are differences between mitochondria and cytoplasm contributions. The followed protocol is briefly represented in **Figure 4**.

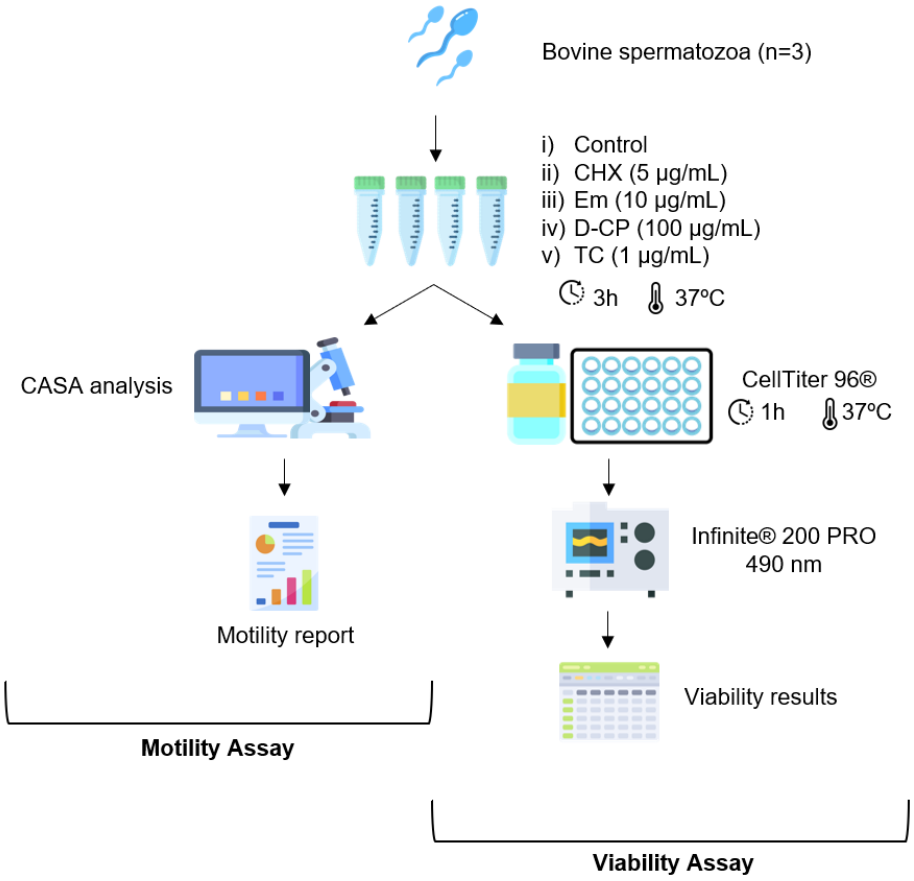


**Figure 4 - Schematic representation of the motility and viability protocols.** Bovine semen samples were isolated and analyzed using the Sperm Class Analyzer CASA System to assess concentration. Spermatozoa concentration was adjusted and incubated with various translation inhibitors and motility parameters were evaluated using the CASA system. Viability was assessed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation method.

### 1.1.3 Spermatozoa treatment with translation inhibitors

To evaluate the impact of sperm translation inhibition in sperm motility, the bovine sperm ($25x10^6$ sperm/mL) was incubated in Sperm Preparation Medium (SPM; Origio, Denmark). The SPM was used to induce *in vitro* capacitation of mammalian spermatozoa. We used the following translation inhibitors: i) CHX (5 µg/mL), ii) Em (10 µg/mL), iii) D-CP (100 µg/mL) and iv) TC (1 µg/mL). A control condition was performed in abscess of translation inhibitors. Samples were incubated at 37ºC for 3 hours. After that, spermatozoa from each condition were used for motility and viability assays and to western blot analysis.

### 1.1.4 Sperm motility assay

After capacitation incubation, sperm motility parameters were evaluated through the CASA system. Samples (2 µL) were loaded onto preheated (37°C) chambers of Leja Standard Count 8 chamber slide with 20 µm depth (Leja Products B. V., The Netherlands) and at least 500 cells per condition were evaluated, assessing number of the fast-progressive, medium progressive, non-progressive and immotile spermatozoa.

### 1.1.5 Viability assay

To evaluate if translation inhibitors had an impact in bovine spermatozoa viability, the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation (Promega, Madison, USA) kit was used according to manufacturer guidelines. Briefly, we added 20 µL of Cell Titer 96 to 100 µL of suspended spermatozoa for 1h at 37°C. After, the absorbance of each sample was assessed at 490 nm in the Infinite® 200 PRO spectrophotometer (Tecan, Switzerland).

### 1.1.6 Capacitation-induction evaluation

To confirm that sperm capacitation was induced, the analysis of the phosphotyrosine levels of ejaculated *versus* capacitated bovine sperm was performed. After washing with 1xPBS and centrifugate three times to discard seminal plasma and other components, the sperm pellet was lysed in 1% Sodium Dodecyl Sulfate (SDS) for 30 minutes on ice. Then, the sperm lysates were centrifuged at 4ºC (16000g for 10 min), collecting and storing the supernatant at -20ºC. The proteins were separated in a 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 120 V, and were transferred onto a nitrocellulose membrane at 200 mA for 2 hours. To verify the successful protein transfer, the membrane was stained with Ponceau S solution (Sigma Aldrich, 0.1% [w/v] in 5% acetic acid) with agitation for 15 minutes. This also allowed to normalize the quantification for obtaining more accurate results. Then the membrane was washed three times with 1x Tris-buffered saline containing 0.1% Tween 20 (TBS-T).

Next, the membrane was blocked with 5% Bovine Serum Albumin/TBS-T for 1 hour to prevent non-specific binding of the antibodies. Next, the membrane was incubated with anti-phosphotyrosine antibody (clone 4G10, Merck) (1:1000 in blocking solution) for 1 hour, followed by three washes with 1x TBST and incubation with the RDye anti-mouse antibody (LI-COR Biosciences – GmbH; 1:5000 in blocking solution) for 1 hour. Then, the membrane was washed two more times with TBS-T for 10 minutes each, and a final

wash with 1x TBS. The fluorescence was detected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

Finally, pixel intensity quantification was performed using Image Studio Lite software (version 5.2.5). To obtain accurate measurements, all lanes were normalized to the Ponceau control, ensuring consistent comparisons.

### 1.2 Immunofluorescence

Puromycin, a structural analogue of aminoacyl-tRNAs, can be incorporated into nascent peptides during elongation steps [137]. This property makes puromycin a partial inhibitor of translation that in small quantities allows to track translation rate, while also enabling the assessment and localization of protein synthesis. In this study, taking advantage of puromycin's capabilities, here, the main goal was to determine the subcellular location of newly synthesised peptides. The main steps of this protocol are summarized in **Figure 5**.

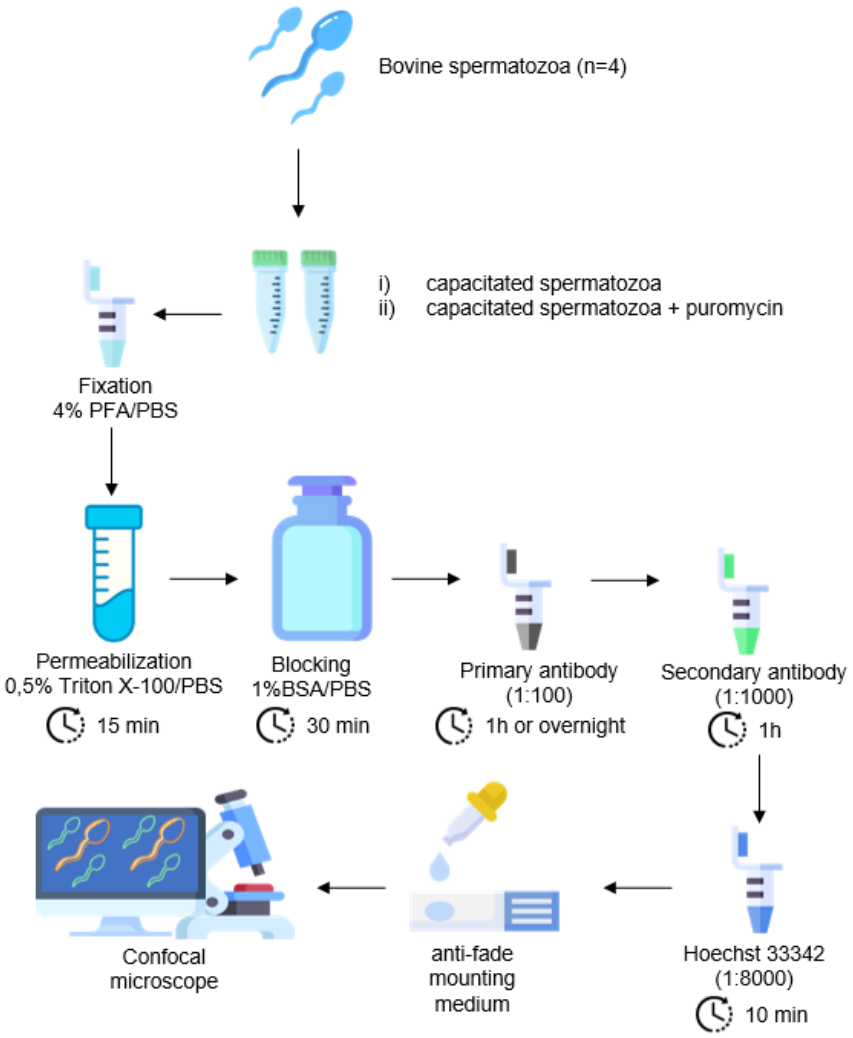

**Figure 5 - Schematic representation of the immunocytochemistry protocol.** Spermatozoa samples were obtained through centrifugation and washes with PBS. They were then capacitated with SPM and treated with puromycin to create two study conditions. After treatment, the samples were washed and prepared for immunofluorescence analysis. The spermatozoa were fixed, permeabilized, and blocked before being incubated with mouse anti-puromycin Alexa clone 12D10. Two approaches were taken with the addition of goat anti-mouse Alexa Fluor Plus 488, and experimentation with anti-mouse Alexa Fluor 561 was also performed. The cells were then incubated with Hoechst 33342 and mounted with an anti-fade mounting medium. The slides were stored in the dark until image capture in the confocal microscope.

### 1.2.1 Spermatozoa preparation and treatment with puromycin

Ejaculated semen samples (n=3) were obtained by masturbation into a sterile container. Samples were centrifuged (500g for 10 minutes at RT) and washed with 1x PBS, discarding the seminal plasma. Next, human spermatozoa (1,5 x $10^5$ cells/mL) were incubated in SPM for 3 hours, 37ºC. After that, puromycin (Sigma-Aldrich) was added (7,5 µg/mL). A control condition was performed without the incubation of puromycin. After treatment, the samples were centrifuged (500g for 10 minutes at RT) and washed twice with 1x PBS.

### 1.2.2 Immunofluorescence labeling protocol for detecting puromycin incorporation in spermatozoa

To prepare the samples for immunofluorescence analysis, spermatozoa were fixed with 4% Paraformaldehyde (PFA)/PBS and 1.5 x $10^5$ cells were smeared onto coverslip at RT until dry. After each step, the coverslips were washed three times with ice-cold 1x PBS for 5 minutes. The cells were permeabilized with 0.5% Triton X-100/PBS for 15 minutes at room temperature, followed by blocking with 1% BSA/PBS for 30 minutes at room temperature.

Then, the coverslips were incubated with mouse anti-puromycin (Alexa clone 12D10, Merck) (1:100 in blocking solution) for 1 hour at RT, or overnight at 4ºC. On a first approach, after the primary antibody incubation, goat anti-mouse IgG Alexa Fluor Plus 488 (1:1000 in blocking solution) was added to each sample for 1 hour at RT. Due to spermatozoa's autofluorescence the protocol was optimized; thus, on a second approach, goat anti-mouse Alexa Fluor Plus 488 (Invitrogen; 1:1000 in blocking solution) was still used, but experiments with tetramethylrhodamine (TRITC, ThermoFisher Scientific) (1:1000 in blocking solution) were also performed. The cells were then

incubated with Hoechst 33342 (1:8000) for 10 minutes and mounted with a drop of anti-fade mounting medium (Enzo life Sciences). The slides were stored in the dark at 4°C until image capture in the Zeiss LSM 880 with Airyscan confocal microscope.

### 1.2.3 Image capture and processing

Images were captured in the Zeiss LSM 880 with Airyscan confocal microscope (Zen Black (version 2.3) software) with a Plan-Apochromat 63x objective, with a total ampliation of 630x. For the detection of Hoechst 33342 we used the Diode 405 (405 nm) laser; to detect the goat anti-mouse Alexa Fluor Plus 488 antibody we used the Argon (488 nm) laser; lastly to detect the TRITC antibody we used the DPSS 561-10 (561 nm) laser. The images were then processed in ImageJ (version 1.54D).

### 1.2.4 Anti-puromycin incorporation confirmation

To confirm the proper incorporation of puromycin in spermatozoa, the reminiscent treated cells were lysed. A western blot protocol was performed as described in section 1.5. The membrane was blocked with 5% non-fat dry milk solution in 1x TBS-T and washed three times with TBS-T (5min, RT). Next the membrane was incubated with primary antibody mouse anti-puromycin (1:10000) and goat anti-mouse Alexa Fluor Plus 488 (Invitrogen; 1:10000). The fluorescence was once again detected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

Finally, pixel intensity quantification was performed using Image Studio Lite software (version 5.2.5). To obtain accurate measurements, we normalized every lane to the Ponceau control, ensuring consistent comparisons.

### 1.1.1 Statistical analysis

The statistical analysis was performed using GraphPad Prism (version 8.0.1, GraphPad Software, San Diego). To assess normality a Shapiro-Wilk test was performed. As all the requirements were not met, a nonparametric method using Mann-Whitney U Test to compare two independent conditions at a time was carried out. The significance level was set at 0.05.

# 1. Results

## 1.1 Motility assay

We used the two cytoplasmic - CHX and Em - and two mitochondrial - D-CP and TET - translation inhibitors to assess the impact on sperm motility. Incubation with CHX, EM, and D-CP led to a decreased percentage of spermatozoa with progressive motility compared to the capacitated control (**Figure 6A**). Additionally, sperm cells incubated with CHX, EM and D-CP presented an increment in medium progressive and non-progressive motility **(Figure 6B** and **6C)**. Also, in CHX and D-CP treated conditions there was an increment in the percentage of immotile spermatozoa (**Figure 6D**). Nevertheless, the TET incubation had a low impact on sperm motility parameters (**Figure 6A-D**). Additionally, we also evaluated if sperm viability was impacted by the inhibitor's treatment. As observed in **Figure 6E**, the inhibitors treatment had no impact in sperm viability.

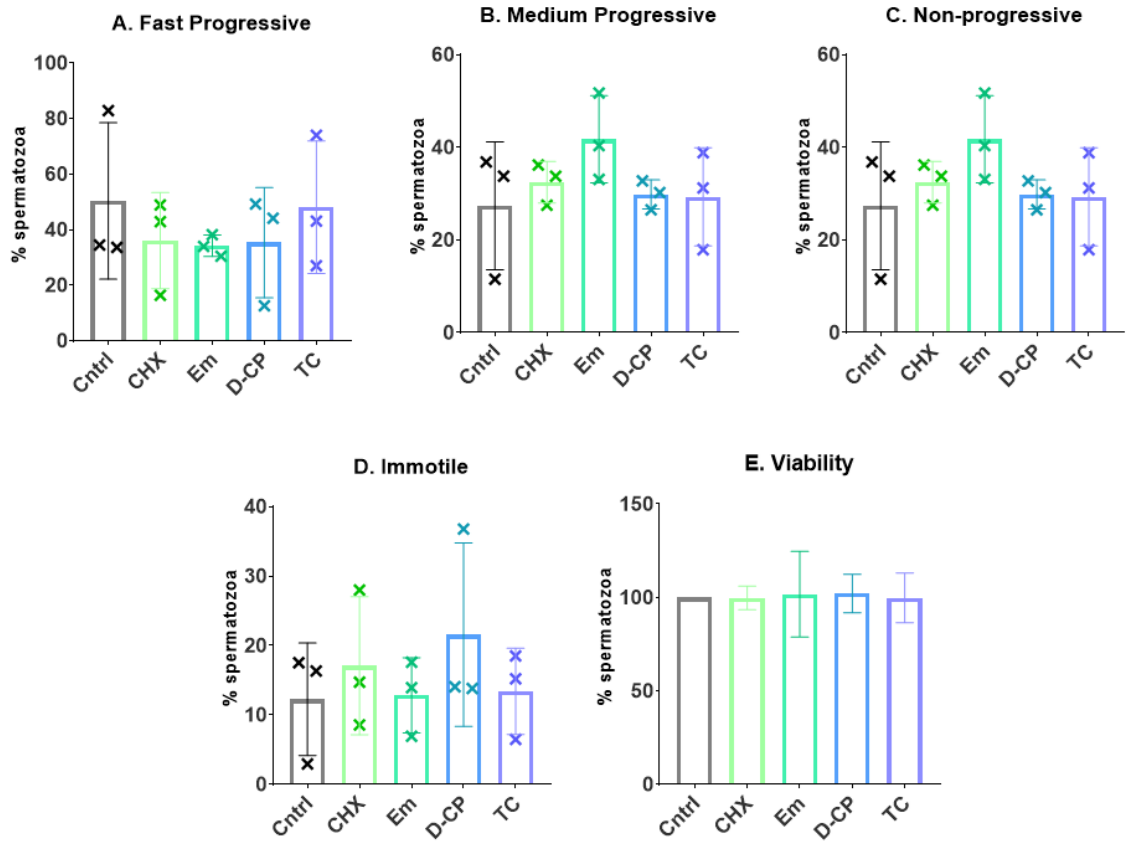


**Figure 6 - Impact of translation inhibitors on sperm motility and viability. (A)** fast progressive sperm, **(B)** medium progressive sperm, **(C)** non-progressive sperm, and **(D)** immotile sperm parameters. **(E)** Sperm viability. The graph bars depict the average percentage of

spermatozoa with error bars representing the standard deviation (SD) from 3 individual samples. Cntrl: Control; CHX: cycloheximide; Em: emetine; D-CP: chloramphenicol; TC: tetracycline.

We also assessed the effectiveness of capacitation induction. The results in **Figure 7**, show that peptides derived from capacitated samples had a higher degree of tyrosine phosphorylation compared to fresh samples peptides, suggesting a successful induction of capacitation.

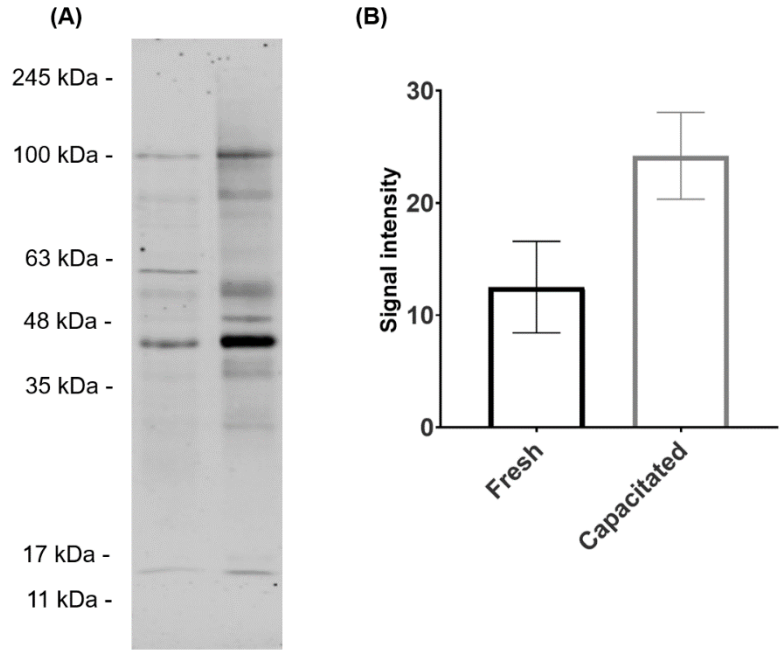


**Figure 7 - Western-blot results of phosphorylated peptides using anti-phosphotyrosine antibody. (A)** Bovine spermatozoa lysates were separated by electrophoresis, followed by a western blot with anti-phosphotyrosine antibody. **(B)** Pixel intensity quantification with Image Studio Lite software, using Ponceau S staining as a loading control. Graph bars represent the signal intensity with error bars representing the SD.

### 1.2 Immunocytochemistry

In this study, one of the objectives was to determine the subcellular location of protein synthesis in human spermatozoa. Initially, control and puromycin-treated spermatozoa were fixated, permeabilized, blocked, and immunostained with Alexa clone 12D10 primary antibody and Alexa Fluor Plus 488 secondary antibody, which peak of emission is 518 nm (green).

Either at 1h or overnight incubations, no specific signal was detected (**Figure 8**). To avoid nonspecific signals from spermatozoa's autofluorescence, we also attempted incubations with TRITC, which peak of emission is 576 nm (orange), but no significant signal was observed either (**Figures 9 and 10**).

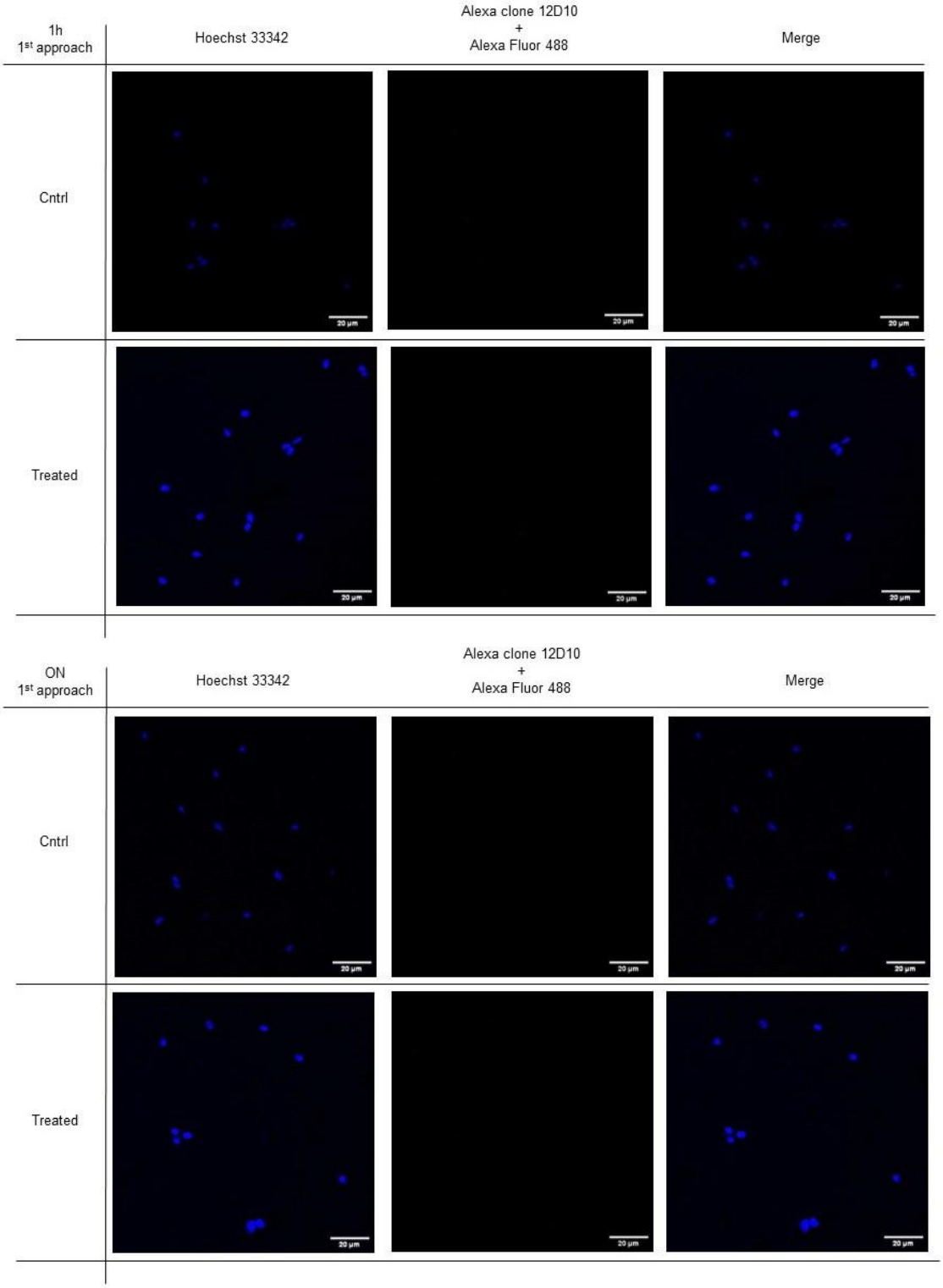


**Figure 8 - Subcellular location of puromycin incorporation in human spermatozoa (1st approach).** Control and puromycin-treated human spermatozoa were immunostained with mouse

anti-puromycin (1:100) and Alexa Fluor Plus 488 secondary antibody (1:1000). Sperm nuclei were stained with Hoescht 33342 staining. Ampliation: 63x. Scale bars correspond to 20 µm.

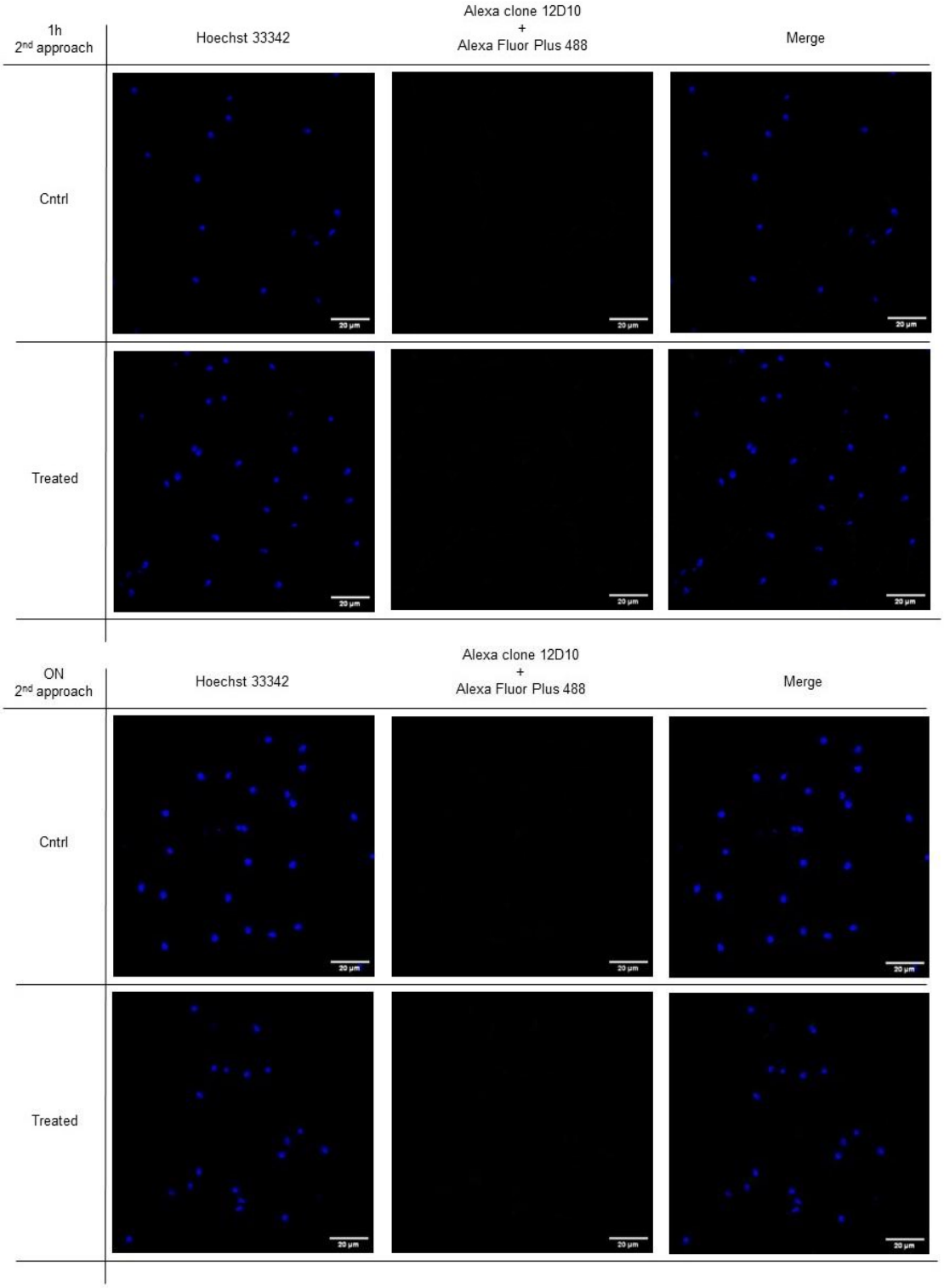


**Figure 9 - Immunocytochemistry detection of puromycin in human spermatozoa with Alexa Fluor Plus 488 secondary antibody (2nd approach).** Ampliation: 63x. Bars correspond to 20 µm.

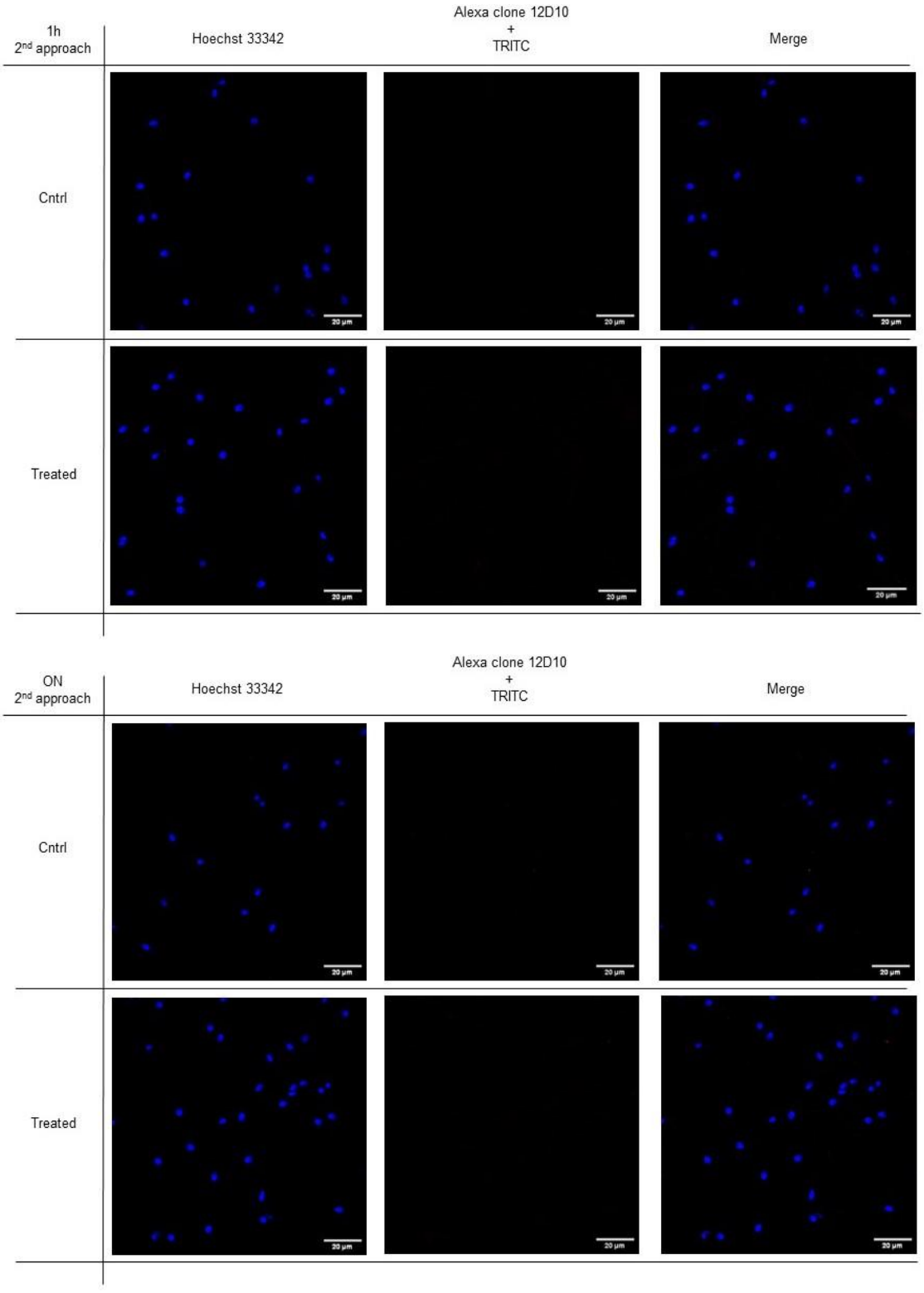


**Figure 10 - Immunocytochemistry detection of puromycin in human spermatozoa with TRITC secondary antibody (2nd approach).** Capacitated-only and capacitated spermatozoa with puromycin were stained with Alexa clone 12D10 primary antibody and Alexa Fluor Plus 561 secondary antibody, which emits light in the red wavelength. Ampliation: 63x. Bars correspond to 20 µm.

To confirm the proper incorporation of puromycin into spermatozoa, we extracted proteins from the same sperm samples. The results showed the presence of puromycylated peptides among the sperm proteins (data not shown), ruling out the possibility of non-incorporation of puromycin as the reason for the lack of fluorescent signal (**Figure 16**).

# III.

# IDENTIFICATION OF TRANSLATION-RELATED DEPS IN ASTHENOZOOSPERMIC AND LOW-MOTILITY SPERMATOZOA – A BIOINFORMATIC ANALYSIS

# 1. Material and Methods

The protein content of mammalian spermatozoa appears to be more closely linked to their motility than previously believed and our results suggest that inhibition of protein synthesis may impact sperm motility [130]. To deepen our knowledge and understand which translation-related proteins are present in human spermatozoa, and how those proteins are differentially expressed in asthenozoospermic sperm samples, a bioinformatic workflow was performed (**Figure 11**).

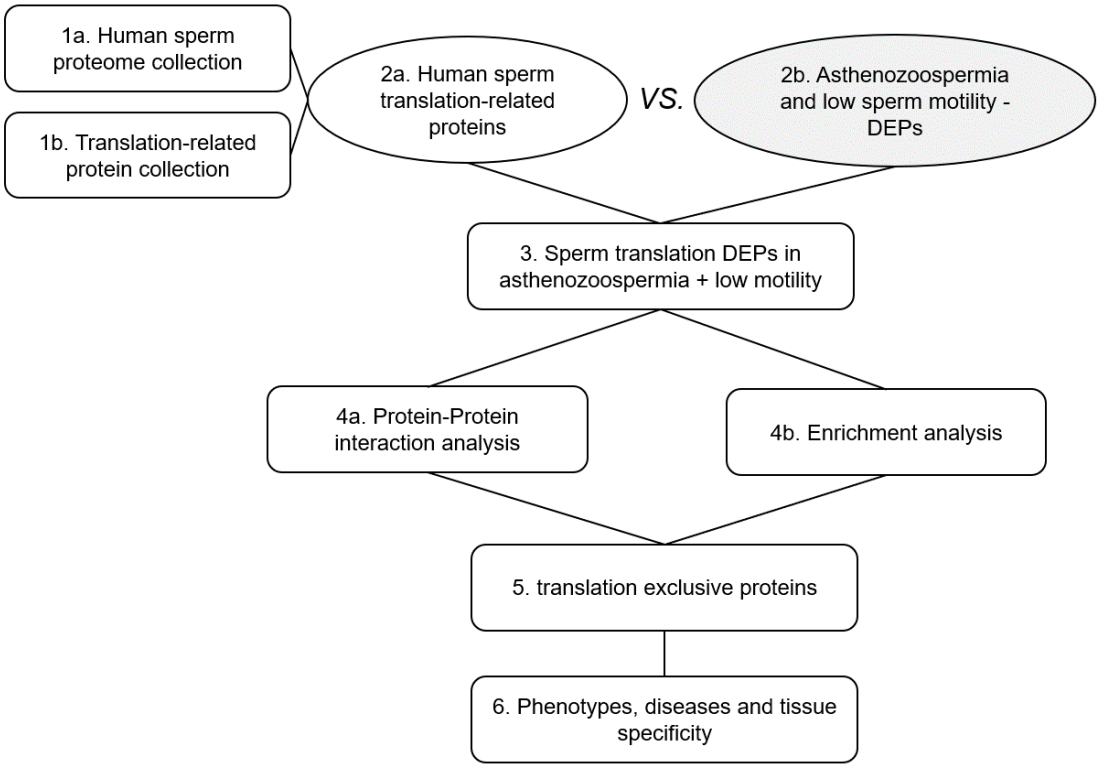


**Figure 11 - Bioinformatic workflow.** From 3 databases (Pubmed, Scopus, and Web of Science), proteomic studies were collected to compile the human sperm proteome (1a). Alongside this step, a search for translation-related proteins was also retrieved (1b). Through cross-comparison, the human sperm translation-related proteins were listed (2a). The resulting protein' list was compared with the differentially expressed proteins (2b) form asthenozoospermic and low motility sperm samples. Thus, dysregulated sperm-translation proteins in asthenozoospermia and low sperm motility were identified (3). The interaction among those proteins were retrieved (4a) and an enrichment analysis was carried out (4b) to identify translation exclusive proteins (5). Finally, fertility-related phenotypes and diseases were searched on MGI and DisGeNET platforms, and tissue expression specificity was assessed in the HPA site (6).

### 1.1 Human spermatozoa proteome collection

An exhaustive literature search was performed on PubMed, Scopus, and Web of Science databases until January 23, 2023. The articles were found by searching the three

databases for combinations of the terms “sperm” or “spermatozoa” or “spermatozoon”, and “proteomics” or “proteome” or “protein profile” or “proteomic analysis”, and “human” or “*homo sapiens*”. Only English and Portuguese studies performed in human ejaculated spermatozoa were used. Disclosure of UniProtKB/Swiss-Prot ID or gene name was also required. After retrieving the articles, duplicate records were eliminated using Excel. Studies in other languages, performed with epididymal sperm or with other animal models were not included.

When analyzing the retrieved articles, a list of included and excluded articles was created. In the eligible articles table, it was annotated the respective title, authors, PMID/DOI, and year of publication along with the study condition, number of controls and cases, sample preparation protocol, proteomic technique, protein identification parameters, and number of reported, collected and mapped proteins; In the excluded articles table, additionally to the reference information, each study was categorized under the respective exclusion criteria.

The protein data from the selected articles was compiled, and, to avoid redundancy, all sperm proteins were mapped in UniProt [138] database using the Retrieve/ID mapping tool and annotated using the UniProtKB AC/ID (downloaded on 14th February 2023).

### 1.2 Translation-related proteins collection

To identify translation-related proteins, a search in the Gene Ontology (GO) database [139] was conducted on February 13, 2023, using the expression "protein translation". Based on this search, the gene ontology terms *translation* (GO:0006412), *cytoplasmic translation* (GO:0002181), and *mitochondrial translation* (GO:0032543) were selected. The output lists were filtered to *Homo sapiens* (organism) and *protein* (type) to retrieve only human translation-related proteins. To avoid redundancy, translation-related proteins were also mapped in UniProt database (17th February 2023).

### 1.3 Sperm-translation proteins

To identify sperm translation proteins in human sperm, the sperm proteome and translation-related proteins were cross compared using the JVenn tool [140]. Then, the sperm translation-related proteins were cross-compared with cytoplasmic- and mitochondrial-related proteins’ lists (GO: GO:0002181 and GO:0032543, respectively). Thus, it was possible to distinguish between cytoplasmatic translation proteins and mitochondrial translation proteins.

### 1.4 Sperm translation-related proteins with differential expression in asthenozoospermic and low-motility spermatozoa

Recently, Corda *et al.* reviewed the sperm proteomic studies to identify differently expressed proteins (DEPs) in different male infertility conditions or poor-sperm quality, including in asthenozoospermia and low sperm motility [141]. Sperm translation proteins were cross compared with the asthenozoospermia and low motility DEPs through the JVenn tool.

### 1.5 Identification differentially expressed specific translation proteins in asthenozoospermic and low-motility spermatozoa

Next the objective was to understand which of the translation related DEPs were more intimately related or exclusively involved in protein translation. To identify the specific translation proteins in sperm, a network analysis using Cytoscape (version 3.9.1)[142] and Cytoscape's app STRING [143] was conducted.

#### 1.5.1 Identification of proteins highly related

The translation-related DEPs obtained in step 1.4 (step 3 in **Figure 11**), were imported to collect protein-protein interactions (PPIs) and create a PPI network (18th April 2023). The results were filtered by selecting edges with an interaction score from "experiments" equal to or greater than 0.75. From the resulting network, a subnetwork was created. To cluster the proteins that were closely related or acted as functional modules, ClusterViz (version 1.0.3) [144] was used. Only clusters associated with translation initiation, elongation, and termination steps were considered; proteins associated with other events that contribute to protein synthesis but are not directly involved were not included. The resulting subset of proteins was listed with the corresponding UniProtKB/Swiss-Prot accession numbers in an Excel sheet for further analysis.

#### 1.5.2 Enrichment analysis

Concurrently, the translation-related DEPs from step 1.4 were also analyzed on the Cytoscape application ClueGo (version 2.5.9) [145], allowing the option GO term perfusion and setting the showing pathways to $p < 0.05$, GO tree interval between 8 and 15, and

perfuse force directed layout. Only proteins that exclusively participated in translation processes were maintained in the functional enrichment network.

### 1.6 Translation-related protein's role in infertility-related phenotypes and diseases

To investigate whether the identified proteins play a role in human infertility or disease, an extensive search was carried on the Mouse Genome Informatics (MGI)[146] and DisGeNET[147] databases. Each protein of interest was manually queried against these databases using the UniProt/SwissProt accession number. Furthermore, a search in the Human Protein Atlas (HPA)[148] was carried out to determine the tissue-specific expression of these proteins. All data were carefully compiled and organized in an Excel spreadsheet for further analysis.

# 2. Results

## 2.1 Translation-related proteins in human spermatozoa

An exhaustive literature search was conducted to collect the human sperm proteome from PubMed, Scopus, and Web of Science databases. The search initially yielded 1687 articles, of which 632 were duplicates (**Figure 12**). From those, 128 articles were excluded and only 927 articles were full text evaluated. From those, only 80 articles were included to retrieve sperm proteins (**table 1**). The excluded articles were recorded along wise with exclusion motif in Table S1. From the included articles, 78101 proteins were retrieved corresponding to 13216 unique proteins (**Table S2** and **S3**).

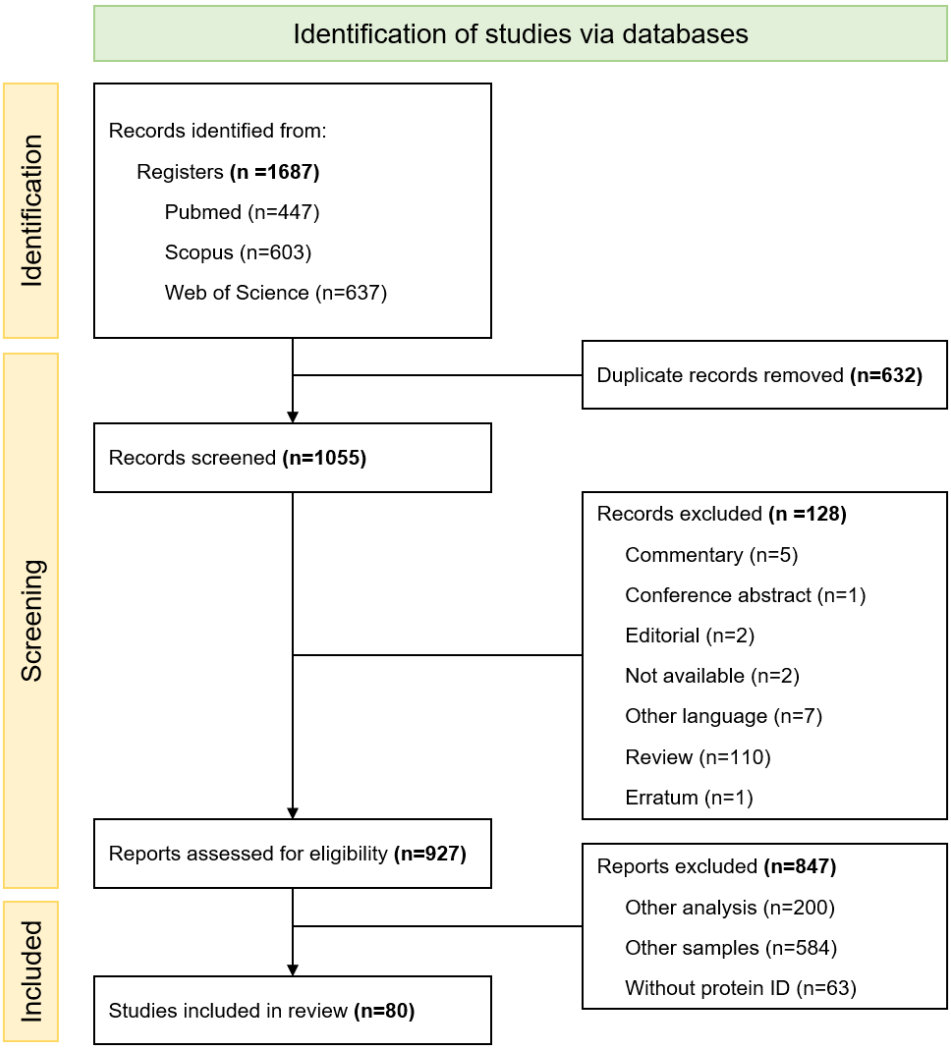


**Figure 12 - Flowchart of literature analysis.** A search on the PubMed, Scopus, and Web of Science databases was conducted, which initially yielded 1687 articles. From the selected articles, only 80 articles met all of our eligibility criteria for inclusion in our study.

**Table 1 - Summary of the included proteomic studies**

| | | | Participants (#) | | | Proteins (#) | |
|---|---|---|---|---|---|---|---|
| No | Reference | Condition | Controls | Cases | Proteomic method | Reported | Mapped |
| 1 | Yang *et al.* (2022) [149] | AZS | NZS men (n=7) | Asthenozoospermic men (n=11) | UHPLC | 3753 | 1430 |
| 2 | Chhikara *et al.* (2022) [150] | AZS | NZS men (n=3) | Asthenozoospermic men (n=12) | label-free quantitative + LC-MS/MS | 1088 | 1087 |
| 3 | Li *et al.* (2022) [151] | Defective sperm tail | NZS men (n=3) | Men with defective sperm tail (n=3) | TMT + HPLC | 1262 | 1262 |
| 4 | Fernandez-Encinas *et al.* (2022) [152] | Recurrent miscarriage | Fertile Men (n=5) | Recurrent miscarriage men (n=5) before and after treatment | TMT + LC−MS/MS | 607 | 33 |
| 5 | Hezavehei *et al.* (2022) [153] | Cryopreservation | NZS men (n=10) | Same men (n=10) | TMT + LC-MS/MS | 2912 | 2896 |
| 6 | Xin *et al.* (2022) [154] | Glycosylation | NZS men (n=10) | N/A | LC-MS/MS | 968 | 968 |
| 7 | Bisconti *et al.* (2022) [155] | Translation inhibition | NZS men | N/A | UHPLC-HRMS/MS + MRM | 1371 | 692 |
| 8 | Grande *et al.* (2022) [156] | Secondary Hypogonadism | Normogonadic NZS fertile men (n=5) | Men w/secondary hypogonadism (n=6) | TMT + LC−MS/MS | 986 | 43 |
| 9 | Liu *et al.* (2022) [157] | LM | Hight motility spermatozoa from fertile men (n=20) | LM spermatozoa from same men (n=20) | 2DE + MS | 21 | 21 |
| 10 | Luo *et al.* (2022) [158] | Protein extraction method | NZS men (n=10) | Same men (n=10) | SDS-PAGE + LC-MS/MS | 4470 | 4470 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 11 | Grassi *et al.* (2022) [159] | Capacitation and HSP70 isoforms | AT, NZS, and TZS men (n=20) | Same men (n=20) | MRM | 10 | 10 |
| 12 | Staicu *et al.* (2021) [160] | Capacitation and NO impact | Capacitated Sperm w/out NO | Capacitated Sperm w/ NO donors | HPLC-ESI-Q-TOF–MS/MS | 29 | 29 |
| 13 | Teke *et al.* (2021) [161] | Varicocele (grade II-III) | NZS fertile men (n=14) | Men w/ varicocele grade II–III (n=20) before and after varicocelectomy | 2DE + SDS-PAGE + MALDI-TOF-MS | 11 | 11 |
| 14 | Pini *et al.* (2021) [162] | DNA fragmentation | Low sperm DNA fragmentation (n=7) | High sperm DNA fragmentation (n=6) | LC-MS/MS + SDS-PAGE | 78 | 78 |
| 15 | Liang *et al*. (2021) [163] | OAT | NZS men (n=2) | OAT men (n=2) | iTRAQ + 2D LC–MS/MS | 3444 | 938 |
| 16 | Naglot *et al.* (2021) [164] | RM | NZS fertile men (n=3) | NZS men w/ idiopathic RPL (n=3) | LC–MS/MS | 2165 | 2165 |
| 17 | Qi *et al*. (2021) [165] | S-sulfhydration in AZS | NZS men (n=26) | AZS men (n=24) | IP + SDS-PAGE + LC-MS/MS | 244 | 244 |
| 18 | Wang *et al.* (2021) [166] | Cryopreservation | NZS men (n=30) | Same men (n=30) | TMT + LC-MS/MS | 1108 | 1104 |
| 19 | Torra-Massana *et al.* (2021) [167] | FR | Men w/high FR [FR>75%] (n=8) | Men with low FR [FR<20%] (n=4) | TMT + 2D-LC-MS/MS + SDS-PAGE | 231 | 231 |
| 20 | Rivera-Egea *et al.* (2021) [168] | ZP binding failure | Successful pregnancy men (n=4); Library: fertile men (n=2) + infertile men (n=2) | Unsuccessful pregnancy men (n=4) | SDS-PAGE + SWATH LC-MS/MS; Library: 1D SDS-PAGE + LC-MS/MS | 5174 | 5294 |
| 21 | Hernandez-Silva *et al.* (2020) [169] | Capacitation | Capacitated Sperm (n=8) | Same men (n=8) | 2DE + LC-MS/MS | 29 | 24 |
| 22 | Wu *et al.* (2020) [170] | Hyperthermia | NZS men (n=10) | Same men (n=10) exposed to heat treatment | iTRAQ + 2D-LC–MS/MS | 3446 | 3446 |
| 23 | Jodar *et al.* (2020) [171] | Embryo development | N/A | idiopathic infertility (n=16) + male factor infertility (n=4) + female factor infertility (n=7) | TMT + LC−MS/MS | 1409 | 1409 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 24 | Pini *et al.* (2020) [172] | Obesity | NZS men (n=5) | NZS obese men (n=5) | Label-free quantification + LC-MS/MS | 2034 | 1972 |
| 25 | Mohanty *et al.* (2019) [173] | RM | NZS fertile men (n=5) | NZS men w/miscarriage history (n=5) | SDS-PAGE + LC-MS/MS | 340 | 435 |
| 26 | Netherton *et al.* (2020) [174] | Defective sperm head | Normal men (n=8) | Same men (n=8) | Nuclei Precipitation + SWATH-MS | N/A | 1575 |
| 27 | Mohanty *et al.* (2020) [175] | RM | Fertile men (n=20) | Men w/ RM (n=16) | Cy dye labelling + 2D SDS-PAGE + MALDI-TOF/MS | 7 | 7 |
| 28 | Moscatelli *et al.* (2019) [176] | AZS and severe AZS | NZS men (n=3) | AZS men (n=3) and severe AZS men (n=3) | label-free + LC−MS/MS | 1962 | 146 |
| 29 | Guo and Yu *et al.* (2019) [177] | AZS | NZS men (n=5) | AZS men (n=5) | TMT + LC−MS/MS | 2413 | 2388 |
| 30 | Sinha *et al.* (2019) [178] | AZS | NZS men (n=5) | AZS men (n=5) | 2DE + MALDI-TOF MS | 7 | 7 |
| 31 | Castillo *et al.* (2019) [179] | Capacitation and acrosome reaction | NZS fertile men (n=3) | Same men (n=3) | TMT + LC−MS/MS | 484 | 484 |
| 32 | Fu *et al.* (2019) [180] | Cryopreservation | Healthy men (n=9) | Same men (n=9) | LC-MS/MS | 174 | 174 |
| 33 | Li *et. al* (2019) [181] | Cryopreservation | Healthy men (n=11) | Same men (n=11): cryostraw group (CS) and cryovial group (CV) | iTRAQ + LC–MS/MS | 3294 | 3199 |
| 34 | Guo and Zhang *et al.* (2019) [182] | Globozoospermia | Normozoospermia (n=3) | Globozoospermic men (n=3) | TMT + LC-MS/MS | 2510 | 2415 |
| 35 | Xue *et al.* (2019) [183] | RM | NZS fertile men (n=7) | NZS men w/miscarriage history (n=10) | iTRAQ + LC–MS/MS | 2350 | 2318 |
| 36 | Urizar-Arenaza *et al.* (2019) [184] | Phosphorylation | NZS men (n=3) | Same men (n=3) | TMT + LC−MS/MS | 5070 | 5063 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 37 | Samanta *et al.* (2019) [185] | Sperm maturation in infertility | Mature sperm from infertile men (n=11) | Immature sperm from same men (n=11) | 1DE + LTQ-Orbitrap Elite hybrid MS | 1585 | 350 |
| 38 | Shen *et al.* (2019) [186] | Ejaculatory abstinence period | NZS fertile men (n=5) | Same men (n=5) | LC-MS/MS | 4959 | 4957 |
| 39 | Schiza *et al.* (2019) [187] | Idiopathic infertility | Fertile men (n=4) | Subfertile men (n=4) (idiopathic infertility or OZS) | LC-MS/MS + SDS-PAGE | 8046 | 8026 |
| 40 | Nowicka-Bauer and Lepczynski *et al.* (2018) [188] | AZS | NZS men (n=10) | AZS men (n=4) | 2DE + MALDI-TOF MS | 25 | 23 |
| 41 | An *et al.* (2018) [189] | DM I | Healthy men (n=6) | Diabetic men (n=6) | iTRAQ + HPLC-MS/MS | 1114 | 1088 |
| 42 | Schiza *et al.* (2018) [190] | Sperm physiology | Healthy fertile men | N/A | CO-IP + LC-MS/MS | 74 | 181 |
| 43 | Nowicka-Bauer and Ozgo *et al.* (2018) [191] | Sperm physiology | NZS men (n=10) | N/A | 2 DE + MALDI-TOF MS | 73 | 73 |
| 44 | Wang *et al.* (2018) [192] | AID treatment | High FR sperm (n=20) | Low FR sperm (n=20) (n=20) | 2DE + MALDI-TOF MS | 26 | 26 |
| 45 | Gholami *et al.* (2018) [193] | Cryopreservation | Healthy men (n=210) | Same men (n=210) | 1D + LC−MS/MS | N/A | 14 |
| 46 | Liu *et al*. (2018) [194] | ZP binding failure | NZS men with IVF pregnancy (n=20) | NZS men with R-ICSI pregnancy (n=20) | iTRAQ + RPLC separation + MALDI-TOF/TOF | 450 | 450 |
| 47 | Saraswat *et al.* (2017) [195] | AZS | NZS men (n=5) | AZS men (n=8) | Label-Free + UPLC-MS | 667 | 659 |
| 48 | Bogle *et al.* (2017) [196] | Cryopreservation | NZS men (n=3) | Same men (n=3): (i)protein-free cryoprotectant (ii) semen temperatures after thawing maintained at 0°C vs. 23°C | TMT + LC−MS/MS | 769 | 229 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 49 | Cui *et al.* (2016) [197] | Immature Spermatozoa | Fertile men (n=12) | Same men (n=12) | SDS-PAGE + LC-MS | 1469 | 80 |
| 50 | Torabi *et al.* (2017) [198] | ZP binding proteins | N/A | NZS men (n=4) | Affinity purification + SDS-PAGE + LC-MS/MS | 785 | 583 |
| 51 | Hetherington *et al.* (2016) [199] | Idiopathic infertility | Fertile men (n=4) | Infertile men (n=2) | LC-MS/MS | 80 | 80 |
| 52 | Agarwal *et al.* (2016) [200] | Unilateral and bilateral varicocele | Fertile healthy men (n=5) | Men w/ unilateral varicocele (n=5) and bilateral varicocele (n=3) | 1D SDS-PAGE + HPLC-MS/MS | 1250 | 99 |
| 53 | Liu *et al.* (2015) [201] | Ageing | Young men who fathered (n=60) | Old men who fathered (n=60) | 2DE + MALDI-TOF MS | 22 | 22 |
| 54 | Liu *et al.* (2014) [202] | AZS and obesity | NZS men (n=3) | Asthenozoospermic obese men (n=3) | label-free quantitative + LC-MS/MS | 1975 | 1905 |
| 55 | Wang *et al.* (2015) [203] | Capacitation | NZS men (n=32) | Same men (n=32) | IMAC + LC–MS/MS | 986 | 993 |
| 56 | Amaral *et al.* (2014) [204] | a) Asthenozoospermia and b) low motility | a) NZS men (n=5) and b) higher motile sperm (n=5) | a) AZS men (n=5) and b) LM sperm (n=5) | TMT + SDS-PAGE + LC−MS/MS | 2044 | 1263 |
| 57 | Azpiazu *et al.* (2014) [205] | IVF failure | NZS men w/ successful IVF (n=10) | NZS men w/out successful IVF (n=10) | TMT + 1D-LC MS/MS | 1717 | 65 |
| 58 | Frapsauce *et al.* (2014) [206] | IVF failure | NZS men w/ successful IVF (n=3) | NZS men w/out successful IVF (n=3) | IEF + 2D-DIGE + MS/MS | 17 | 12 |
| 59 | Castillo *et al.* (2014) [207] | Sperm chromatin | N/A | NZS men (n=9) | 1D-SDS-PAGE or 1D-acid-urea-PAGE + LC-MS/MS | 495 | 494 |
| 60 | Légaré *et al.* (2014) [208] | i) Idiopathic infertility, ii) IVF failure | NZS fertile men (n=6) | NZS infertile men (n=6) and IVF failure (n=4) | iTRAQ + LC MS-MS + LC−MRM/MS | 348 | 45 |
| 61 | McReynolds *et al.* (2014) [209] | Unexplained infertility | Infertile men w/ blastocyst development (n=6) | Infertile men w/ poor blastocyst development (n=6) | SDS-PAGE + 1D + LC-MS/MS | 529 | 49 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 62 | Intasqui *et al.* (2013) [210] | DNA fragmentation | Low sperm DNA fragmentation (n=11) | High sperm DNA fragmentation (n=6) | 2DE + nano UPLC-ESI-MS | 257 | 220 |
| 63 | Shen *et al.* (2013) [211] | Asthenozoospermia | NZS men (n=30) | AZS men (n=30) | 2DE + MS-MS | 16 | 12 |
| 64 | Wang *et al.* (2013) [212] | Sperm physiology | NZS men (n=32) | N/A | LC-MS/MS | 4675 | 4470 |
| 65 | Kichine *et al.* (2013) [213] | Sperm physiology (head) | Fertile men (n=3) | Same men (n=3) | 1D SDS-PAGE + LC-MS/MS | 686 | 684 |
| 66 | Baker *et al.* (2013) [214] | Sperm physiology | NZS men | N/A | SDS page + UPLC-MS/MS | 1605 | 1401 |
| 67 | Amaral *et al.* (2013) [215] | Sperm physiology (tail) | NZS men (n=41) | N/A | SDS page + LC-MS/MS | 1049 | 1047 |
| 68 | Hosseinifar *et al.* (2013) [216] | Varicocele (grade III) and OZS | NZS men (n=20) | OZS men w/ grade 3 varicocele | 2DE + MALDI-TOF/TOF-MS | 10 | 10 |
| 69 | Parte *et al.* (2012) [217] | Severe AZS | NZS men (n=4) | AZS men (n=4) | IMAC + Nano UPLC–MS | 666 | 81 |
| 70 | de Mateo *et al.* (2011) [218] | Sperm physiology (nucleus) | NZS men (n=4) | N/A | 1DE or 2DE + MALDI-TOF MS | 404 | 404 |
| 71 | Paasch *et al*. (2011) [219] | Obesity, type I diabetes and type II diabetes | NZS men (n=21) | i) Men w/DM-I (n=8); ii) DM-II (n=7); iii) obesity (n=13) | DIGE + MALDI-TOF/TOF MS-MS | 96 | 43 |
| 72 | Redgrove *et al.* (2011) [220] | Sperm-ZP interaction | N/A | Capacitated NZS spermatozoa | 1D or 2D BN-PAGE + 1D nano LC-ESI MS/MS | N/A | 22 |
| 73 | Siva *et al.* (2010) [221] | AZS | NZS men (n=20) | AZS men (n=17) | 2DE + MALDI-TOF MS | 75 | 63 |
| 74 | Secciani *et al.* (2009) [222] | Capacitation | NZS men (n=120) | Same men (n=120) | 2DE + MALDI-TOF MS | N/A | 58 |

**Table 1 – continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 75 | Kriegel *et al.* (2009) [223] | Obesity and DM-I | NZS men (n=5) | i) Men w/DM-I (n=2); ii) obesity (n=2) | DIGE + MALDI-TOF/TOF MS-MS | ≤ 2700 | 26 |
| 76 | Chan *et al.* (2009) [224] | AZS | NZS men (n=20) | AZS men (n=20) | 2DE + MALDI-TOF/TOF-MS | 12 | 12 |
| 77 | Martınez-Heredia *et al.* (2008) [225] | AZS | NZS men (n=20) | AZS men (n=10) | 2DE | 17 | 15 |
| 78 | de Mateo *et al.* (2007) [226] | DNA fragmentation | Donors (n=10) | Patients (n=47) | 2DE + MALDI-TOF MS | 98 | 38 |
| 79 | Lefièvre *et al.* (2007) [227] | S-nitrosylation | NZS men | N/A | 1DE + ESI MS - MS | 240 | 233 |
| 80 | Martínez-Heredia *et al.* (2006) [228] | Sperm physiology | NZS men (n=11) | N/A | 2DE + MALDI-TOF MS | 98 | 89 |

Abbreviations: 1DE: One-dimensional electrophoresis; 2DE: Two-dimensional gel electrophoresis; AID: Artificial insemination by donor; AT: Asthenoteratozoospermia; AZS: Asthenozoospermia; ESI: Electrospray ionization; FR: Fertility rate; HPLC: High-Performance Liquid Chromatography; HRMS: High-resolution mass spectrometry; ICSI: Intracytoplasmic sperm injection; IEF: Isoelectric focusing; IMAC: Immobilized metal affinity chromatography; IMAC: Immobilized metal affinity chromatography; IP: Immunoprecipitation; iTRAQ: Isobaric tags for relative and absolute quantitation; IVF: in vitro fertilization; LC: Liquid-chromatography; LM: Low motility; MALDI: Matrix-assisted laser desorption/ionization; MRM: Multiple-reaction monitoring; MS: Mass spectrometry; MS/MS: tandem mass spectrometry; NZS: Normozoospermia; OAT: Oligoasthenozoospermia; OZS: Oligozoospermia; PAGE: Polyacrylamide gel electrophoresis; RM: recurrent miscarriage; RPL: Recurrent pregnancy lost; TMT: Tandem mass tag labeling; TOF: Time-of-flight; TZS: Teratozoospermia; UHPLC: Ultra-High-Performance Liquid Chromatography; ZP: Zona pellucida.

Alongside with the human sperm proteome collection, the translation-related were collect from the Gene Ontology database. The GO terms “translation” (GO:0006412), “cytoplasmic translation” (GO:0002181), “mitochondrial translation” (GO:0032543) resulted in 860, 123, and 111 proteins, respectively (**Table S4**). This corresponds to 860 unique proteins.

The cross-comparison between sperm proteome and translation-related proteins leaded to the identification of 654 common proteins (**Figure 13A**) (**Table S5**). Among sperm translation proteins, 118 are associated with cytoplasmatic translation and 70 are associated with mitochondrial translation (**Figure 13B**)(**Table S5**). Thus, ~5% of the proteins in human sperm appears to be associated with protein translation.

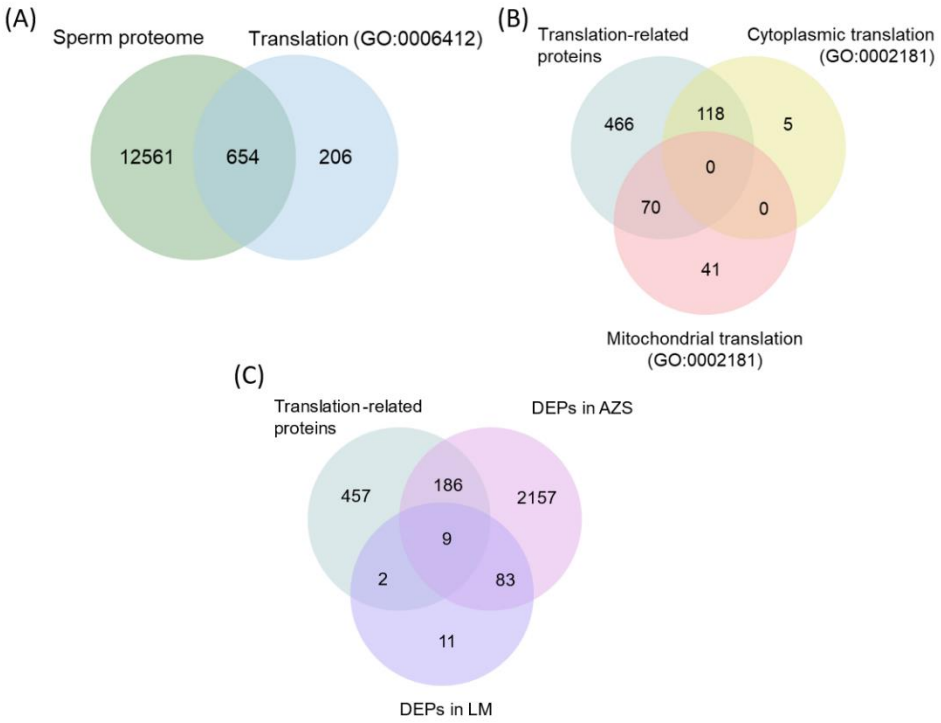


**Figure 13 - Venn diagrams illustrating common proteins among different analyses. (A)** highlights translation-related proteins in sperm; **(B)** further differentiates the translation-related proteins between cytoplasmic and mitochondrial translation; **(C)** focuses on the differentially expressed translation-related proteins in asthenozoospermic and low motility sperm.

### 2.2 Translation-related DEPs

From the work of Corda *et al.*[141] we retrieved 2435 proteins differentially expressed in asthenozoospermia and 105 proteins in sperm with low motility (**Table S6**). The cross-comparison with the sperm translation proteins led to the identification of 195 sperm translation DEPs in spermatozoa from asthenozoospermic men and 11 sperm translation

DEPs in sperm with low motility (**Figure 13C**) (**Table S6**). These two conditions shared only 9 DEPs. Among the 197 DEPs, 129 were increased, 48 were decreased, and 20 were both decreased/increased in asthenozoospermia and/or low motility.

Of the 118 cytoplasmatic translation-related proteins, 39.0% had increased expression and 5.9% had decreased expression, while of the 70 mitochondrial translation-related proteins, 1.4% had increased expression and 10% had decreased expression. These results suggest that cytoplasmic translation is more sensitive to sperm motility, culminating in increased expression of several proteins.

### 2.3 Translation specific proteins

Since the same protein has different functions, we analyzed which of the sperm translation DEPs would be exclusively associated to the translation process. Thus, for the 197 DEPs a PPI network was created. After filtering the interactions collected, a PPI network of 131 nodes and 1442 edges was generated. The resulting network was rather complex with several interactions thus, using ClusterViz, a search for protein clusters was carried out. This step allowed us to assess which of the proteins were more closely related and led to the generation of 4 protein clusters with 47, 8, 7, and 3 proteins, respectively. The cluster containing 7 proteins corresponded to proteins that do not act directly in the steps of protein translation and was not included. Nonetheless, the clustering coefficient ($CC_i$) and the network density, both measures of possible clustering and relationships between nodes, increased relative to the initial network (**Table 2**). The retrieved clusters were merged into a single network of 58 nodes and 1095 edges.

On the other hand, from the 197 imported proteins, the ClueGo approach resulted in a functional enrichment network of 182 proteins associated with 74 GO terms. However, we only wanted to look into events that are directly involved in initiation, elongation, and termination steps. For that, we manually removed GO terms of minor translation events and their respective associated proteins. Additionally, some proteins were related to more than one process, but we only maintained proteins exclusively related to the main steps of protein synthesis. Ultimately, this analysis resulted in 35 proteins associated with 8 steps of protein synthesis (**Figure 14**). In total, the two analysis resulted in 71 unique proteins, from which 20 proteins were found in both networks.

**Table 2 - Summary of the ClusterViz PPI network analysis, detailing the number of proteins and interactions, the clustering coefficient (CCi) and the network density of each cluster/network.**

| | Proteins | Interactions | $CC_i$ | Network density |
|---|---|---|---|---|
| **Initial Network** | 131 | 1442 | 0,63 | 0,23 |
| **Cluster 1** | 47 | 1064 | 0,99 | 0,99 |
| **Cluster 2** | 8 | 28 | 1,0 | 1 |
| **Cluster 3** | 3 | 3 | N/A | N/A |
| **Merged Network** | 58 | 1095 | 0,99 | 0,99 |

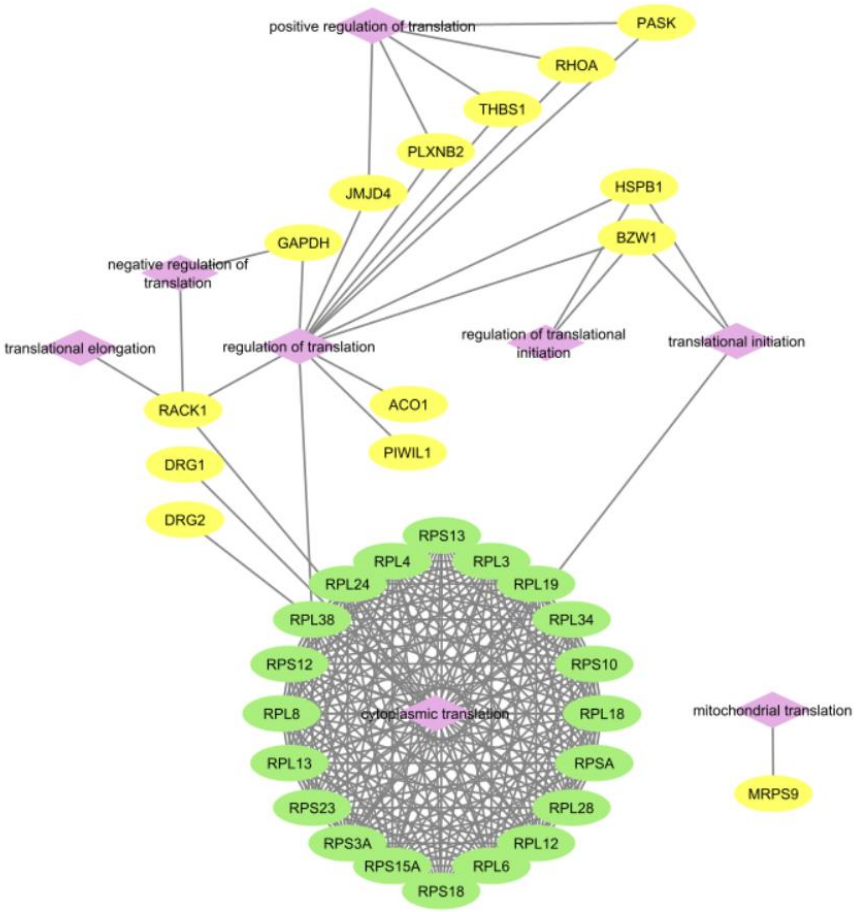


**Figure 14 - Network resulting from merging the PPI network analysis and the functional enrichment analysis.** The violet diamond-shaped nodes correspond to GO terms and are

associated with their respective proteins. The common proteins between the two analysis are represented in green and the functional enrichment analysis exclusive proteins are represented in yellow.

### 2.4 Infertility-related phenotypes, diseases and tissue expression

Next, we wonder if the specific translation proteins altered in AZS and Low motility were previously associated with male infertility phenotypes. We searched the 71 specific proteins in the MGI and DisGeNET databases (**Table S7 and S8**). Our analysis yielded 12 proteins of interest: RPS3, RPS23, RPL38, RPS21, HBS1L, EIF2S2, and EIF4G1, BZW1, GAPDH, HSPB1, PIWIL1 and THBS1 (**table 3**). RPS3 and RPS23 were associated with teratozoospermia; RPL38, RPS21, HBS1L, BZW1, PIWIL1, and THBS1 were associated with reduced fertility or infertility; EIF4G1, GAPDH, and PIWIL1 were associated with asthenozoospermia; and HBS1L was associated with oligozoospermia. We also examined tissue expression patterns for 58 translation specific proteins. HBS1L is enriched in spermatocytes and spermatogonia, EIF2S2 and BZW1 are enriched in spermatids, and PIWIL1 is enriched in spermatids, spermatocytes, and also in testis tissue.

**Table 3 - List of sperm's exclusive translation-related proteins that had been associated with infertility-related phenotypes and diseases and/or are enriched in the male reproductive tract.**

| | **Phenotypes (MGI)** | **Diseases (DisGeNET)** | **Expression (AZS and LM)** | **Tissue/ Cell Enrichment (HPA)** |
|---|---|---|---|---|
| RPS3 | - | TZS | ↓ | - |
| RPS23 | - | TZS | ↑ | - |
| RPL38 | RF | - | ↑ | - |
| RPS21 | RMF | - | ↑ | - |
| HBS1L | OZS and RMF | - | ↑ | SPC and SG |
| EIF2S2 | DTW and DPG | - | ↑ | STD |
| EIF4G1 | - | AZS | ↑ | - |
| BZW1 | MI | - | ↑ | STD |
| GAPDH | - | AZS | ↓ | - |

| | | | | |
|---|---|---|---|---|
| HSPB1 | - | MI | ↑ | - |
| PIWIL1 | - | AZS and MI | ↑ | Testis, STD and SPC |
| THBS1 | RF | - | ↓ | - |

Abbreviations: AZS: asthenozoospermia; DPG: decreased proliferation of germinative cells; DTW: decreased testis weight; LM: low-motility; MI: male infertility; OZS: oligozoospermia; RF: reduced fertility; RMF: reduced male fertility; SG: spermatogonia; SPC: spermatocyte; STD: spermatid.

# IV.

# DISCUSSION

## 1. The Effects of Translation Inhibitors on Sperm Motility

Gur and Breitbart suggested that protein translation is needed to replace degraded proteins during the intense phosphorylation events of capacitation [124]. On the other hand, studies suggest that both RNA content and protein synthesis are related to sperm motility [121,131–134]. In order to gain a deeper understanding of the role of sperm translation in motility, we performed experiments utilizing inhibitors of cytoplasmic and mitochondrial translation. The objective was to investigate the impact of those inhibitors on sperm motility.

CHX and emetine, the two cytoplasmatic translation inhibitors, had a very similar yet different impact on spermatozoa's motility. After incubating the cells with CHX, the percentage of fast progressive spermatozoa decreased, and the percentage of medium-progressive, non-progressive and immotile sperm increased. CHX has the capability of suppressing flagellar growth, as reported by previous studies [131]. In fact, CHX inhibits protein translation by interfering with aminoacyl-tRNA or by preventing ribosome recycling and reassembly which can lead to the depletion of the flagellar protein pools and to impaired tail function [131,229]. Having in mind Gur and Breitbart's hypothesis, CHX may disrupt the normal function of the sperm's tail, by preventing the reposition of degraded proteins, thus compromising the cell's motility [124]. Em decreased the percentage of fast-progressive spermatozoa and increased the percentage of non-progressive sperm. However, unlike any other used inhibitor, it had no effect on the percentage of immotile sperm and had particular higher increased on medium-progressive spermatozoa. Alkaloids such as Em have an initial metabolism stimulation followed by inhibition, as described in other studies [132]. However, this is rather odd considering that our samples were incubated with Em for a relatively long period. There is not much information about Em as a translation inhibitor and sperm motility, but the literature suggests that it has spermicidal potential by affecting sperm kinematics [132].

On the other hand, D-CP and tetracycline, the two mitochondrial translation inhibitors, had very different impacts on sperm motility. Contrarily to D-CP, tetracycline showed little to no effect in sperm motility. As seen for CHX, incubation with D-CP lead to a decreased percentage of fast and medium-progressive spermatozoa, an increased percentage of non-progressive sperm, and a greater increase of immotile sperm cells. This decreased sperm motility is consistent with the literature, and some studies also showed that translation inhibition did not affect mitochondrial respiratory chain complex and ATP production [133,134]. This suggests that D-CP inhibits the production of motility-related proteins within the mitochondria.

Tetracycline did not show any impact on sperm motility, which can be due to a set of factors. Some studies report that a tetracycline concentration of 2.5 µg/mL is enough to interfere with sperm viability by chelating $Ca^{2+}$ and inducing oxidative stress, which can prevent the cell's motility [230,231]. In our approach we used tetracycline in a concentration of 1 µg/mL, which did not impact either motility or viability. This calls for the need to study the dose-response relationship and establish an optimal concentration that doesn't affect sperm viability but still allows to assess its impact on motility. Nonetheless, this mitochondrial inhibitor of translation was also reported to bind to eukaryotic ribosomes but at different binding sites [232]. This could mean that, in spermatozoa, tetracycline could bind to cytoplasmatic ribosomes before getting to mitochondrial ribosomes, thus being unable to inhibit protein translation.

## 2. Locating protein translation in sperm: limitations of puromycin incorporation and confocal microscopy

SUnSET is a method that allows monitoring the incorporation of puromycin in neosynthesized proteins, thus reflecting the rate of mRNA translation[137]. Our group has previously optimized this technique so it could be applied to spermatozoa [128]. Through this analysis, Nunes J (2018)[128] and Corda P (2019)[129] showed that translation occurs in both bovine and human spermatozoa after capacitation. However, the location of puromycin's incorporation remained unknown. Therefore, we attempted to observe the subcellular location where puromycin incorporation occurs in spermatozoa.

In puromycin-treated spermatozoa, no specific anti-puromycin signal was detected. In fact, one limitation observed was the high levels of spermatozoa autofluorescence which coincided with the emission spectrum of the secondary antibody used. To avoid this redundancy in the detection of signals, we incubated spermatozoa with a secondary antibody whose emission spectrum did not coincide with spermatozoa autofluorescence, but this did not result in a significant signal. However, protein extraction from the same sperm samples confirmed the presence of puromycylated peptides, ruling out the possibility of non-incorporation of puromycin as the reason for the lack of fluorescent signal.

The results show that the implemented immunofluorescence protocol did not work even though we could assess puromycin incorporated through western blot (data not shown). This suggests that the chosen technique might be limited to this end. In fact, even though confocal microscopy has high sensitivity, the absolute fluorescence sensitivity is rather low

as the out-of-focus signals are not considered [233]. Furthermore, by focusing on one plane only the rest of the sample can become photobleached interfering in the detection of signals from other planes of focus [233]. On the other hand, puromycin has its own limitations and although it can faithfully represent the rate of translation, it might not be the right molecule to locate protein synthesis. Firstly, the puromycylated peptides dissociate from the ribosome and diffuse very rapidly across the cell, which prevents the pinpointing of the exact site of translation [234]. Additionally, the composition of the cell membranes can negatively influence the detection of puromycylated peptides, which, if the translation was occuring in mitochondria, it could possibly be undetected [235].

This way, flow cytometry may be a better alternative to efficiently measure translation rate by puromycin incorporation, as it is a more sensitive intensity-based technique [234]; as for the location of protein synthesis, the detection method needs optimization, either by selecting another technique or another target molecule.

## 3. Identification of Translation-Related Proteins and DEPs in the Human Sperm Proteome and Their Association with Infertility-Related Phenotypes and Diseases

Although we were unable to locate protein synthesis within the spermatozoa, we showed a pattern of decreased motility when this process is inhibited, as described in the literature. This piqued our curiosity to identify translation-related proteins that were differentially expressed in asthenozoospermic and low-motility spermatozoa, through an *in silico* analysis.

Firstly, the literature search performed allowed the inclusion of 80 proteomic studies to compile the human sperm proteome. By merging the proteins identified in those studies, a list of 13,216 unique proteins was obtained. Notably, this shows the significant advancement in sperm proteomics, surpassing a previous study from our group, which identified 7,131 proteins in human sperm [129]. Also, 860 translation-related proteins were collected, and the cross-comparison with human sperm proteome identified 654 common proteins. Among those, 118 proteins associated with cytoplasmic translation and 70 proteins associated with mitochondrial translation. This study collected new sperm proteins and new translation-related sperm proteins comparing to our group's previous work [129], which is due to the advancement and expansion of proteomics, particularly proteomics of male (in)fertility.

From the work of Corda *et al.*[141] we retrieved proteins present in asthenozoospermic and low-motility sperm. A cross-comparison with sperm translation proteins identified 197 sperm translation DEPs which were mainly related to increased expression and cytoplasmic translation.

To identify sperm translation DEPs that were intimately related or specifically linked to translation, we created a PPI network using the Cytoscape app. Using ClusterViz we generated four protein clusters that were merged into a single network of 58 proteins. Meanwhile, the ClueGo approach resulted in a functional enrichment network of 35 proteins associated with 8 steps of protein synthesis. Interestingly, 20 proteins were found in both networks. Further analysis showed that only one out of the 71 unique proteins was involved in mitochondrial translation, supporting the hypothesis that protein synthesis, specifically cytoplasmic translation, plays a crucial role in sperm motility.

The identified proteins were searched against the MGI and DisGeNET databases to investigate their role in human infertility or disease. The HPA database was also used to determine their tissue-specific expression. This analysis identified 12 proteins associated with infertility and infertility-related phenotypes and diseases, such as teratozoospermia, asthenozoospermia, and oligozoospermia. Additionally, the tissue expression patterns of some of these proteins suggest that they play a crucial role in the reproductive system.

Overall, this analysis provides new insights into the human sperm proteome and identifies translation-related proteins closely related to sperm motility. As some of these proteins had increased expression under poor motility phenotypes or were even associated with infertility-related phenotypes, we believe that protein synthesis has an important role in spermatozoa and its dysregulation bears consequences for the cell's normal physiology. Further studies are needed but these findings have the potential for a better understanding of male (in)fertility.

# V.

# CONCLUSION AND FUTURE PERSPECTIVES

# 1. Conclusion

The main goals of this study were to locate the subcellularly protein synthesis events within the spermatozoa and assess the relationship between translation and motility through bioinformatic and experimental approaches.

To understand where, in sperm, translation occurs, we implemented an immunocytochemistry protocol with the use of puromycin. Our results didn't allow us to draw any conclusions, and, as puromycin incorporation successfully occurred, we suggest that the detection method needs optimization, either by selecting another technique or another target molecule.

We carried out a motility assay to assess the impact of translation inhibition on sperm motility, treating spermatozoa with cytoplasmic translation inhibitors (CHX and Em) and mitochondrial translation inhibitors (D-CP and TC). Overall, sperm motility decreased under the effect of the two types of inhibitors. In the light of literature, our results suggest that flagellar assembly and other motility-related proteins are synthesized in the cell's cytoplasm. Nonetheless, the inhibition of both these processes leads to impaired motility.

On the other hand, through a bioinformatic approach, we searched for differentially expressed translation-related proteins in AZS and LM. We collected 13,216 sperm proteins and 860 translation-related proteins, which corresponded to 654 translation-related proteins in sperm. Moreover, of the 654 translation-related proteins in sperm, 197 were differentially expressed in AZS and LM, being mainly associated with increased expression and cytoplasmatic translation. Next, we carried out a PPI network analysis and a functional enrichment analysis, to identify highly related proteins and proteins that exclusively participate in protein synthesis. From the analysis, we retrieved 71 proteins. Finally, by searching these 71 proteins against MGI, DisGeNET, and HPA databases, we found that 12 proteins were associated with infertility and infertility-related phenotypes and diseases. Overall, this approach showed an advancement and expansion of proteomics in male (in)fertility and that protein synthesis in spermatozoa may be more relevant than previously hypothesized.

Altogether, with these results we propose that protein synthesis occurs by translation of long-term mRNAs in both cytoplasmic and mitochondrial ribosomes. Both types of translation may be needed for proper spermatozoa physiology, either through cooperation or side events that serve the same purpose.

## 2. Future Perspectives

In a future approach, the results from the motility assay would benefit from more replicas, particularly with human spermatozoa to assess if the same results occur from the effect of translation inhibitors. Using other translation inhibitors is relevant as some of these substances impact more than the protein synthesis pathways, but also dose-response relationships should be studied to apply optimal concentrations of inhibitors without affecting the cells viability.

In addition, some studies claim the presence of disassembled translation machinery in the cytoplasm, but little is known about mitochondrial translation; characterization of the translation-related proteome of mitochondria would give valuable information about the translation machinery status of this organelle.

Finally, we report 12 translation-related proteins seemingly having an impact on motility when dysregulated; new studies are needed to validate these results, but also to understand the role of these proteins concerning sperm translation and motility.

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## Supplementary tables

**Supplementary table S1***

Table S1 - Excluded articles from literature search

**Supplementary table S2***

Table S2 – Total sperm proteins collected

**Supplementary table S3***

Table S3 – Total sperm proteins collected w/out duplicates

**Supplementary table S4***

Table S4 – Proteins collect from AmiGO search

**Supplementary table S5***

Table S5 – Translation-related proteins in spermatozoa

**Supplementary table S6***

Table S6 – Translation-related DEPs in asthenozoospermic and low motility spermatozoa

**Supplementary table S7***

Table S7 – Proteins resulting from the PPI-network analysis and respectively associated phenotypes, diseases, expression, and tissue specificity

**Supplementary table S8***

Table S8 – Proteins resulting from the functional enrichment analysis and respectively associated phenotypes, diseases, expression, and tissue specificity

*Available at: https://tinyurl.com/20230606

# Supplementary figures

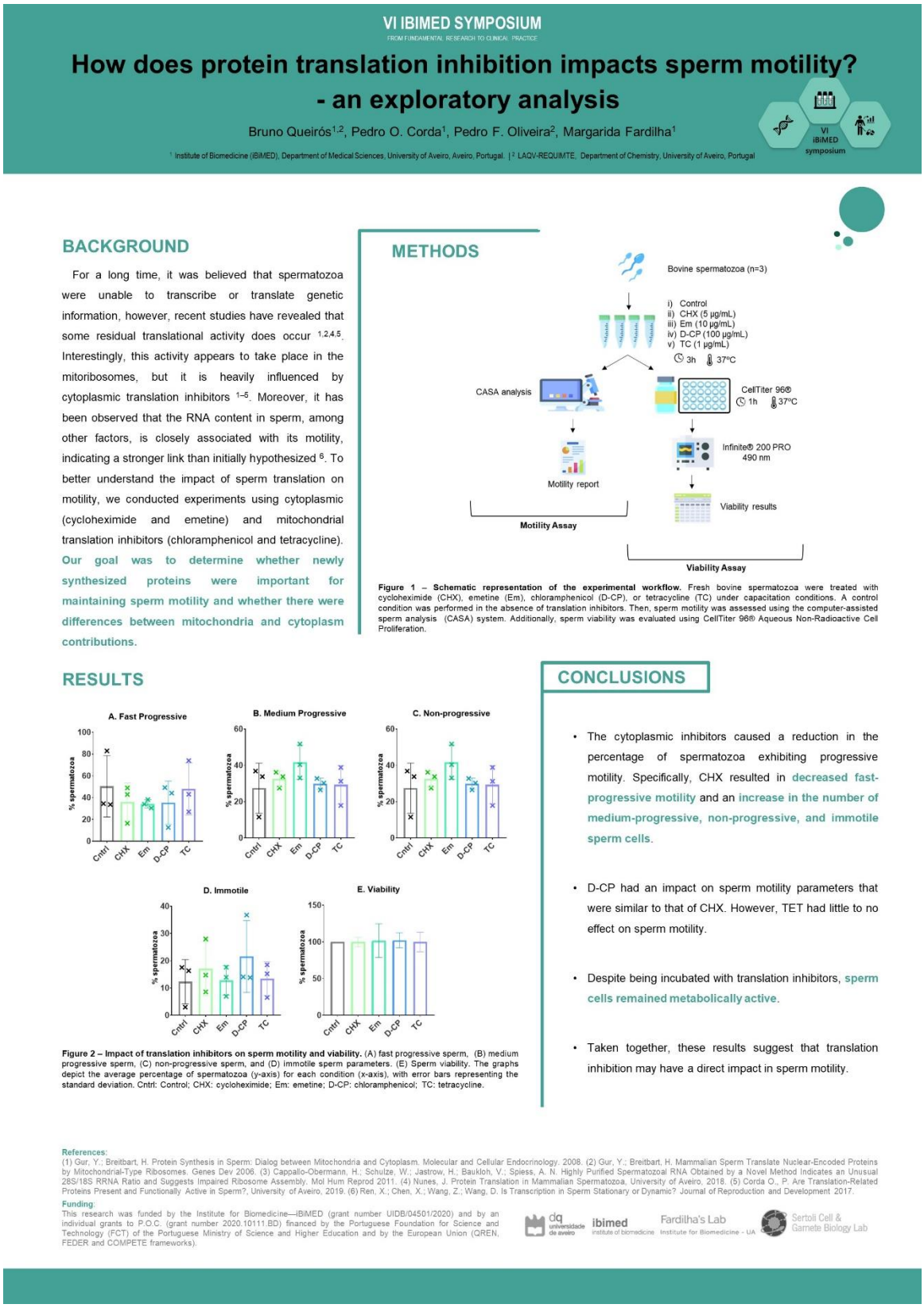


**Figure S1 –** How does protein translation inhibition impacts sperm motility? – an exploratory analysis, in VI iBiMED Symposium, 25-26th May 2023, Aveiro, Portugal.