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UNIVERSIDADE D
COIMBRA

Carolina Morais da Silva

RNA AND SPERM MOTILITY: WHAT IS THE RELATION?

VOLUME 1

Dissertação no âmbito do mestrado em Investigação Biomédica, área de infeção e imunidade, orientada pela Professora Doutora Rute Ribeiro Pereira e pelo Professor Doutor Mário Manuel da Silva Leite de Sousa e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Agosto de 2022

Agradecimentos

À minha orientadora, Professora Doutora Rute Ribeiro Pereira, por todos os ensinamentos e pela elevada exigência, que me fizeram aprender e crescer não só como futuro profissional de investigação, mas também como pessoa.

Ao Professor Doutor Mário Manuel da Silva Leite de Sousa, co-orientador, por toda a disponibilidade ao longo desta minha etapa, e por toda a partilha de conhecimentos, que me permitiu desenvolver diversas competências na área, completando bastante a minha formação académica.

Aos meus pais e irmão, que são um suporte imprescindível na minha vida, que me apoiam incondicionalmente e, sem os quais, nada do que tenho conquistado seria possível.

Ao Nuno por todo o amor, amizade, cumplicidade, paciência, compreensão, apoio e segurança, em todos os momentos.

A todos os meus amigos(as), com especial referência à Mariana, Raquel, Beatriz, Joana, Ana Sofia, Gabriela, João Luís, Miguel, Carlos, Zé Pedro, Mariana Calado, Salomé, Inês e Matilde, por toda a amizade, companheirismo e fidelidade que sempre tiveram para comigo ao longos destes largos anos e, que são parte importante na minha vida e no meu equilíbrio.

Ao Instituto de Ciências Biomédicas Abel Salazar, pela oportunidade de desenvolvimento da minha dissertação numa área que me desperta bastante interesse, sendo um objetivo no futuro profissional.

À Universidade de Coimbra e a todos os professores que nela exercem funções, por estes dois anos de Mestrado, que termino agora com muito orgulho e com sentimento de dever cumprido.

Muito obrigado a todos!

Resumo

A astenozoospermia é uma das principais causas de infertilidade masculina e é caracterizada por uma diminuição da motilidade dos espermatozoides (motilidade progressiva <30%). Sabe-se que fatores genéticos são uma causa significativa de astenozoospermia, sendo que várias mutações em genes que codificam constituintes estruturais ou funcionais dos espermatozoides foram já identificadas como causas conhecidas de astenozoospermia. No entanto, o papel do RNA na motilidade dos espermatozoides não é tão claro. Consequentemente, com o presente trabalho pretendemos contribuir para aumentar o conhecimento sobre a expressão do RNA nos espermatozoides e a sua relação com a motilidade.

Foram investigados, por PCR em tempo real, a expressão de um grupo de mRNAs (*CATSPER3, CFAP44, CRHR1, HIP1, IQCG KRT34, LRRC6, QRICH2, RSPH6A, SPATA33* e *TEKT2*) e seus microRNAs alvo correspondentes, com maior score, nos espermatozoides de indivíduos com astenozoospermia e normozoospermia. Observámos que todos os mRNAs e microRNAs dos espermatozoides estavam regulados negativamente nos indivíduos com astenozoospermia.

O presente estudo fornece informação adicional sobre a função do RNA nos espermatozoides, comprovando a sua importância na motilidade, uma vez que nos foi possível observar uma diminuição da expressão em todos os pacientes com reduzida motilidade dos espermatozoides, sendo esta mais acentuada nos pacientes com motilidade progressiva inferior a 15%.

Futuros trabalhos serão necessários para validar os presentes resultados, nomeadamente pelo uso de um maior número de pacientes. A utilização de modelos animais poderá permitir, adicionalmente, alcançar uma melhor compreensão da influência, tanto dos mRNAs como dos microRNAs correspondentes, na motilidade dos espermatozoides, principalmente em relação aos genes *KTR34* e *CRHR1*, cuja informação relativa aos espermatozoides é ainda escassa.

Palavras-Chave: Expressão genética; infertilidade masculina; motilidade dos espermatozoides; mRNA; microRNA; PCR quantitativo.

Abstract

Asthenozoospermia is one of the main causes of male infertility and is characterized by reduced sperm forward motility (progressive motility <30%). Several gene pathologic variants (mutations) that code for structural or functional constituents of the sperm having already been identified as a cause asthenozoospermia. In contrast, the role of sperm RNA in the regulation of sperm motility is still not fully understood. To help fill this gap in knowledge, we here evaluated the expression of sperm RNA and its relationship with sperm motility.

We investigated by real-time PCR the expression of a group of mRNAs (*CATSPER3, CFAP44, CRHR1, HIP1, IQCG KRT34, LRRC6, QRICH2, RSPH6A, SPATA33* and *TEKT2*) and its highest score corresponding target microRNA, in sperm from subjects presenting asthenozoospermia and normozoospermia. We observed that all sperm mRNA and microRNAs were downregulated in patients with asthenozoospermia.

Our work provided further insights regarding the role of RNA in sperm function, proving its importance to sperm motility as it was observed a reduced expression of all in patients with reduced sperm motility, with a more accentuated reduction in patients with rapid progressive motility lower than 15%.

Studies with a higher number of patients can, in future work, validate this data. Additionally, studies using animal models might further help to understand how these genes and their corresponding microRNA act in sperm motility, particularly regarding genes *KTR34* and *CRHR1* whose information on spermatozoa is still scarce.

Keywords: Gene expression; male infertility; mRNA; microRNA; quantitative PCR; RNA expression; sperm motility.

Articles Published Under the Scope of this Master Thesis:

Silva C, Viana P, Barros A, Sá R, Sousa M, Pereira R. Further Insights on RNA Expression and Sperm Motility. Genes (Basel). 2022 Jul 21;13(7):1291. doi: 10.3390/genes13071291. PMID: 35886074; PMCID: PMC9319021.

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Abbreviations and/or acronyms

AKAP - Protein kinase anchoring proteins

ATP - Adenosine triphosphate (ATP)

ATZ – Asthenozoospermia

BRD2 - Bromodomain 2 protein

Ca^{2+} - Calcium

CFAPs - Cilia and flagella associated proteins

CGR - Centre of Reproductive Genetics Prof. Alberto Barros

Cp - Central pair

CPC - Central pair complex

CP - Connecting piece

CRISP2 - Cysteine-rich secretory protein 2

DA - Dynein arms

DMT - Doublet microtubules

DRC - Dynein regulatory complex

dsRNAs - Double-stranded RNAs

EP - Endpiece

FS - Fibrous sheath

FSH - Follicle- stimulating hormone

GnRH - Hypothalamic gonadotropin releasing hormone

IDAs - Inner Dynein Arms

IFs - intermediate filaments

LH - Luteinizing hormone

lncRNAs - Long non-coding RNAs

MCR - Mean-Centering Restricted

miRNAs – MicroRNAs

miRNP - miRNA-containing ribonucleoprotein

MMAF - Multiple morphological abnormalities of the sperm flagella

MP - Midpiece

mRNAs - Messenger RNAs

MS - Mitochondrial sheath

NCBI - National Center for Biotechnology Information

ncRNAs - Non-coding RNAs

NDRC - Nexin-dynein regulatory complex

OAT - Oligoasthenoteratozoospermia

ODAs - Outer Dynein Arms

ODFs - Outer dense fibers

OXPHOS - Oxidative phosphorylation

PAS - Postacrosomal sheath

PBS - Phosphate-buffered saline

PCD - Primary ciliary dyskinesia

PCR - Polymerase chain reaction

piRNA - PIWI-interacting RNA

PKA - cAMP-dependent protein kinase

PP - Principal piece

PRM1 - Protamine 1

PRM2 - Protamine 2

RISC - RNA-induced silencing complex

RPM – Rapid Progressive Motility

rNRAs - Ribosomal RNAs

ROS - Reactive oxygen species

RP - Ribosomal protein

RS - Radial spokes

RT-PCR - Real-time PCR

SCLB - Somatic cell lysis buffer

SPM- Slow Progressive Motility

siRNAs - Small interfering RNA

snRNAs - Small nuclear RNAs

SP - Seminal plasma

TFAM - Mitochondrial transcription factor A

tRNAs - Transfer RNAs

3'-UTR - 3'-untranslated region

5'-UTR - 5'-untranslated region

WHO - World Health Organization

Introduction

1. Spermatogenesis

Human spermatogenesis [1–3] is a biological process involving molecular and cellular events that leads, from spermatogonia stem cells to the production of spermatozoa. This process takes place within the seminiferous tubules of the testis and includes three major steps: 1) spermatogonia proliferation by mitosis; 2) meiosis, through a first meiotic division of primary spermatocytes that originates secondary spermatocytes and a second meiotic division of secondary spermatocytes that originates round spermatids; 3) differentiation of round spermatids into spermatozoa. The total spermatogenic cycle takes about 76 days [4]. In the seminiferous tubules reside, besides germ cells, the somatic Sertoli cells, which play and essential role in spermatogenesis [5]. The seminiferous tubules are supported by a loose connective tissue filled with blood and lymphatic vessels, nerves, immunologic cells and Leydig cells. Seminiferous tubules are separated from the connective tissue by a basal lamina encircled with peritubular myoid cells [1–3].

Germ cells are spatial and structurally divided into two compartments, basal and adluminal, by the blood-testis barrier (BTB), which provides a privileged microenvironment for the completion of spermatogenesis [6]. The BTB is formed by intercellular junctions between Sertoli cells. The function of the BTB is to separate spermatogonia and preleptotene spermatocytes, which reside in the basal compartment, from primary and secondary spermatocytes, spermatids and spermatozoa, which reside in the adluminal compartment.

Spermatogenesis initiates during the fetal life, with differentiation of primordial germ cells into gonocytes (non-migrating germ cells). After birth and up to 6 months of life, gonocytes differentiate into spermatogonia, which stay quiescent until the age of 5-7 years. Spermatogonia can be subdivided morphologically into three subtypes based on their heterochromatin content: A-dark, A-pale and B-spermatogonia [7]. In puberty, in parallel with the proliferation process that takes place in the nutrient rich basal compartment, spermatogonia begin a differentiation process. First, A-dark spermatogonia divide mitotically to originate one A-dark spermatogonia (stem cell) and one A-pale spermatogonia. A-pale spermatogonia then originate B-spermatogonia

and these enter a multiple mitotic process. B-spermatogonia then differentiate into pre-leptotene primary spermatocytes and meiosis begins. Meiosis attains sequential primary spermatocyte steps, namely, leptotene, zygotene and pachitene. After crossing-over in pachitene spermatocytes (4n), the cell enters the first meiotic division (separation of homologue chromosomes), giving origin to secondary spermatocytes (2n), which rapidly finish the second meiotic division (separation of homologue chromatids), originating haploid (1n) round spermatids [1–3].

Haploid round spermatids then undergo a complex series of differentiation events (spermiogenesis) to originate testicular spermatozoa that will be released from seminiferous tubules through a spermiation process (release from Sertoli cell pouches). With the aid of the seminiferous fluid flow and contractions of the mioid cells, testicular spermatozoa are continuously conveyed to the rete testis, then to the efferent ducts and finally to the epididymis, where they will reside and mature until ejaculation [1–3,8]. During spermiogenesis, extensive changes occur, particularly in the spermatid nucleus and cytoplasm. These include chromatin compaction through histone replacement by transient proteins and then protamines [1–3,8]. The round spermatid nucleus, assisted by the manchette, a microtubule-based bundle [9], becomes progressively elongated. Concomitantly with nucleus elongation, the acrosome vesicle, originated from the fusion of Golgi vesicles, attaches to the upper pole of the nucleus, and suffers a similar elongation process, covering the upper 2/3 of the nucleus [1–3,10]. One of the centrioles attaches to basal nuclear envelope region and becomes the proximal centriole. The other centriole (distal centriole) establishes the distal pole of the cell and originates the axoneme of the flagellum [11]. During this process of flagellum elongation, mitochondria migrate to the anterior flagellum region (midpiece), and the axoneme becomes surrounded by accessory fibers [12].

The maintenance of spermatogenesis is provided by luteinizing hormone (LH) and follicle- stimulating hormone (FSH), whose secretion from the anterior hypophysis is stimulated by the hypothalamic gonadotropin-releasing hormone (GnRH). FSH binds to Sertoli cell receptors. LH binds to Leydig cell receptors, promoting testosterone secretion. Testosterone enters the blood circulation, being responsible for the development of secondary male characteristics. Testosterone also binds to Sertoli cell receptors. Sertoli cell stimulation by FSH and testosterone activate several metabolic

pathways that end in the release of nutrients and paracrine factors that stimulate and regulate spermatogenesis [1–3,13,14].

The estimated daily sperm production per man ranges from 150-275 million spermatozoa, and several testicular structures and cells play important roles during spermatogenesis, while an ample array of factors can influence their quality and quantity. Spermatogenesis is summarized in figure 1.

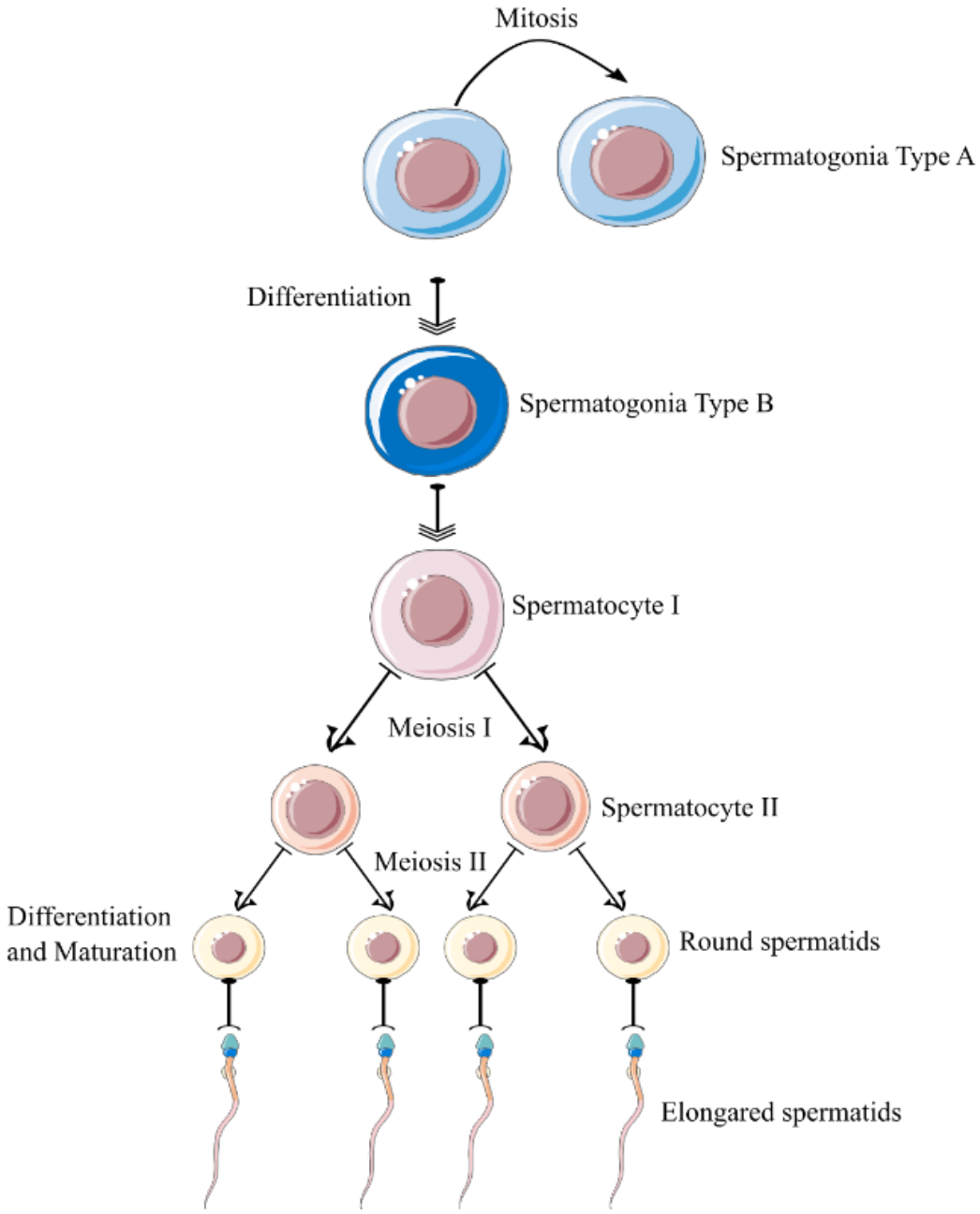

Figure 1. Representation of the main steps occurring in male germ cells during spermatogenesis. Spermatogenesis occurs in the seminiferous tubules in the testis. The process begins with the formation of spermatogonia (Type A and B spermatogonia) through germ cells. Then, they differentiate into primary spermatocytes (Spermatocyte I), which, through the process of meiosis, differentiate into secondary spermatocytes (Spermatocyte II). Through the second meiosis, the secondary spermatocytes differentiate into round spermatids (Immature spermatid), which through a maturation and differentiation process originate elongated spermatids (Differentiating spermatid). Once they are out the seminiferous tubules and along the epididymis, elongated spermatids form mature sperm (Spermatozoa). Image provided by Rute Pereira

2. Sperm Structure

The spermatozoon is a highly specialized cell with a unique structure, crucial for the different stages of fertilization. It can be divided in two principal parts, the head, and the flagellum (or tail) (Fig. 2 and 3) [15].

The sperm head contains the genetic information (nucleus) and an acrosome containing enzymes responsible for gamete interaction. The head is further divided in two regions, acrosome region and post-acrosome region.

The sperm flagellum is responsible for motility. It is subdivided in four principal parts. The neck, or connecting piece (CP), connects the sperm nucleus to the flagellum, being constituted by a basal plate (fibers that attach the proximal centriole to the basal nuclear envelope), the striated columns (structural fibers that surround the proximal centriole) and the modified distal centriole. The midpiece (MP) is formed by the axoneme, surrounded by nine structural outer dense fibers (ODF), being both encapsulated by a mitochondrial helix of about 70 mitochondria. The ATP generated by mitochondria is mainly used for the molecular changes occurring in the sperm membrane during maturation in the epididymis and during capacitation in the uterine cavity. At the end of the midpiece, the flagellum is encircled by a ring (annulus) of fibers that prevent the descent of mitochondria, marking the beginning of the principal-piece (PP). The PP is subdivided into proximal PP (PPP) and distal PP (DPP). The proximal PP contains the axoneme and the ODF, encircled by ribbons of fibers of the fibrous sheath (FS). Besides being a structural component of the flagellum, the FS contains the proteins responsible for glycolysis, believed to be the main source of ATP for sperm motility during the transit through the female genital tract [15]. The distal PP contains de axoneme surrounded only by the FS. From the end of the distal PP, the axoneme loses the FS and the axoneme disintegrates into isolated microtubules that progressively decrease in number, constituting the flagellum end-piece (EP) [15].

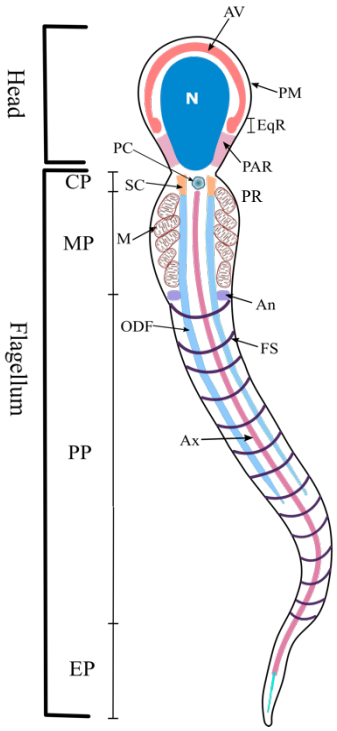

Figure 2. Schematic drawing of a human spermatozoon. As any other cell, the spermatozoon is delimited by a plasma membrane (PM). The spermatozoon can be morphologically divided into two principal parts: head and flagellum. In the head, the acrosome (AV) surrounds the nucleus (N); the region bellow the AV is the postacrosomal region (PAR); the head is physically separated from the flagellum by a posterior ring (PR). The sperm flagellum is divided in four principal parts, the connecting or neck piece (CP), the midpiece (MP), the principal piece (PP) and the end piece (EP). The MP includes the proximal centriole (PC), segmented columns (SC), mitochondria (M) and the annulus (An). The PP can be subdivided into proximal PP and distal PP. The outer dense fibers (ODF) and the axoneme (Ax) are both present in the MP and in the proximal PP. The proximal PP also includes the rings of the fibrous sheath (FS), while the distal PP only contains the Ax and FS. Finally, the EP only contains the Ax. Image provided by Rute Pereira.

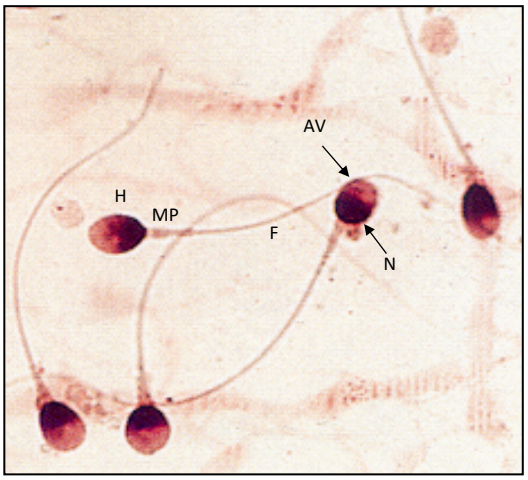

Figure 3. Spermatozoa observed after conventional staining under a light microscope. Head (H), with the acrosome (AV) and nucleus (N). Flagellum (F), with midpiece (MP).
Image provided by Mário Sousa

2.1. Sperm Head Structure

The sperm head (Fig. 4) has about 2.5-3.5 µm in diameter, 4.0-5.5 µm length and a smooth oval shape [16]. It contains two main structures, the nucleus, and the acrosome (acrosomal vesicle). The plasma membrane surrounding the head is designed for several adhesion and fusion events ultimately leading to fertilization. The sperm head plasma membrane is domain-separated from the MP plasma membrane by the posterior ring, and the MP plasma membrane is domain-separated from the PP plasma membrane by the annulus ring. The MP and PP plasma membranes have specific domains involved in sperm osmotic and motility regulation [17].

The sperm head can be divided into an apical acrosome region, an equatorial acrosome region and a post-acrosome region. The acrosome covers the anterior 2/3 of the nucleus, being slightly indented and enlarged towards the nuclear equatorial region. Below the acrosome, there is a short cytoplasmic area, the post-acrosome region (PAR). A thin fibrillar matrix surrounds the nuclear envelope, constituting the perinuclear matrix (theca) [15,18].

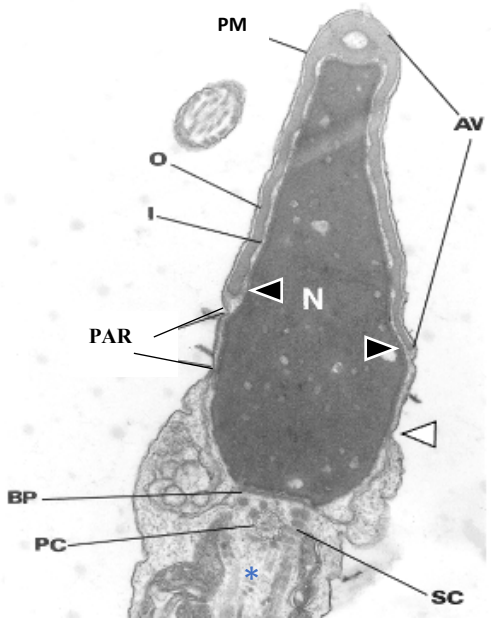

Figure 4. Ultrastructure of the sperm head. The sperm head is surrounded by the plasma membrane (PM) and contains the acrosome (AV), with outer (O) and inner (I) membrane domains, and the nucleus (N). The acrosome ends at the equatorial nuclear region (black arrows). The cytoplasmic region bellows the acrosome is the postacrosomal region (PAR). The membrane of the head is physical separated from the flagellum membrane by a nuclear ring (white arrowhead). The neck region contains the basal plate (BP), the segmented columns (SC) and the proximal (PC) and distal (*) centrioles.
Image provided by Mário Sousa

During spermatogenesis, histones are progressively replaced by transition proteins and then by protamines, which promotes a toroid organization, enabling a high-condensed chromatin. In epididymis, bisulfite bridges are established, giving a final highly condensed chromatin structure [15,19]. This is supposed to prevent against DNA insults that could occur during sperm migration along male and female tracts [20]. Sperm condensed chromosomes are at least six times more condensed than mitotic somatic chromosomes [21]. During this process, the nuclear matrix undergoes remodeling. The nuclear matrix consists of a network of fibers distributed throughout the nuclear interior and a peripheral nuclear lamina, which is composed by intermediate filament proteins, lamins A, B and C [22]. Chromosomes display different territories in the nucleus, which are supposed to protect gene-rich areas from external insults but also facilitate transcription after fertilization [23–25].

After sperm-oocyte fusion, and once in the ooplasm, protamines are replaced by female histones, enabling chromatin decondensation, followed by transcription and development of the male pronucleus [26].

2.2. Sperm Flagellum Structure

The sperm flagellum is responsible for motility and, therefore, indispensable for migration of spermatozoa in the female reproductive tract and passage through the zona pellucida, the extracellular matrix that surrounds the oocyte [27–29]. Formation of the sperm flagellum is a complex process that includes the preassembly and transport of sperm tail components, the structural assembly of the axoneme and the structural assembly of accessory fibers. The flagellum arises during early spermiogenesis, extending as the nucleus and acrosome become elongated [30].

2.2.1. Axoneme

During spermatogenesis, a microtubule-based bundle, the manchette, surrounds the nucleus, promoting nuclear and acrosome elongation [29]. The manchette also captures other molecules that are delivered to assist tail development. The axoneme and related structures start to polymerize in the cytoplasm near the nuclear base, followed by migration of the distal centriole to the periphery to enable flagellum extrusion [31]. The manchette is connected to the nucleoskeleton and is involved in nucleocytoplasmic transport. Regarding the flagellum, some proteins required at the base (centriole) of the flagellum are transported by a process named intra-manchette transport (IMT), using the motor proteins kinesins and dynein along manchette microtubules, whereas proteins needed in flagellum extension are transported through a process named intra-flagellar transport (IFT), using the motor protein myosin along manchette actin tracks [29].

The axoneme is a highly conserved microtubule-based structure that extends throughout the entire flagellum. The axoneme extends from the distal centriole (DC) and is the central structure of the sperm flagellum. It consists of microtubules and hundreds of associated proteins, which contribute a scaffolding structure and motile apparatus. The axoneme is surrounded by accessory structures, the mitochondrial sheath (MS), the ODF and the FS. The axoneme is a continuity of the centriole structure, being a cylinder, whose wall is composed by nine microtubule doublets. Peripheral doublets are connected by radial spokes (RS) to two central single microtubules. Central microtubules, named C1 and C2, are interlinked by a series of

regularly spaced linkages (central bridge), and surrounded by a fibrillar sheath (central sheath), forming the central pair complex (CPC), previously known as the central apparatus. The structure of the axoneme thus present a 9d+2s pattern (Fig. 5) [32].

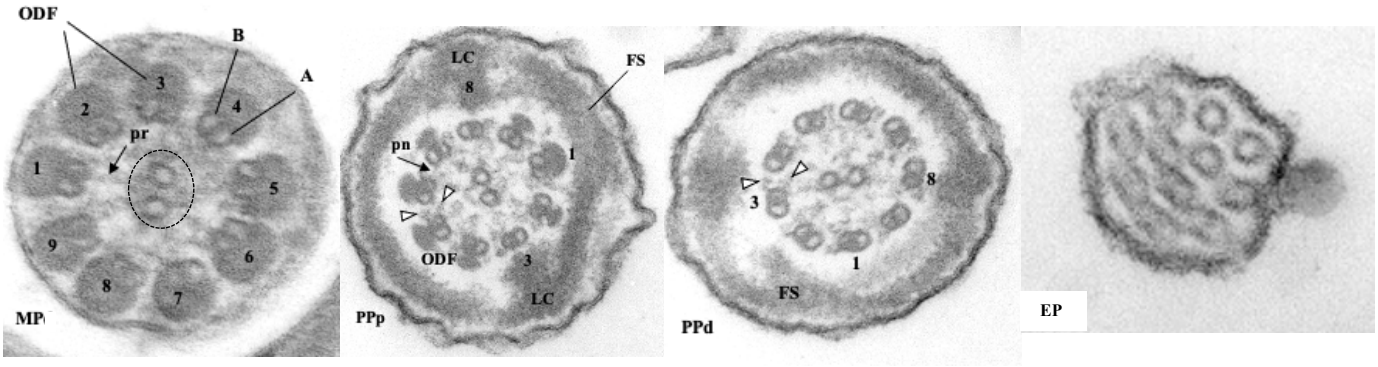

Figure 5. Ultrastructure of the axoneme**.** The axoneme is formed by nine microtubule doublets (1-9) and two central microtubules (dotted circle). Each doublet has an inner (A) and outer (B) microtubule, are linked by nexin bridges (pn) and connected to the central microtubules by radial spokes (pr). The doublet whose radial projection is perpendicular to the central microtubules is named 1, with numbering up to 9 following the clockwise direction. From the A microtubule two dynein arms arise, outer and inner (white arrow-heads). Transverse sections through the midpiece (MP), proximal principal-piece (PPP), distal principal-piece (DPP) and end-piece (EP). ODF: outer dense fibers; FS: fibrous sheath. The ribbons of the FS at positions 3 and 8 form two longitudinal columns.
Image provided by Mário Sousa.

Peripheral doublets are made of an inner complete A-microtubule (13-protofilaments) and an outer incomplete B-microtubule (10-protofilaments). The numbering of these protofilaments is formally specified due to their unique positions for the attachment of different motor and regulatory proteins [33]. The A-Microtubule contains a pair of projections named dynein arms (DA). Dyneins are motor proteins that convert the chemical energy released from adenosine triphosphate (ATP) hydrolysis into mechanical work allowing the sliding of doublets, which is converted into flagellar beating [34]. The two DA, the Inner Dynein Arm (IDA) and the Outer Dynein Arm (ODA), are attached to the A-microtubule of each doublet and directed to the B-microtubule of the next doublet in a clockwise direction [35].

Peripheral doublets are connected to each other by the nexin-dynein regulatory complex (N-DRC) and to the CPC by RS. Radial spokes are a multiprotein structure, with a T-shaped globular structure attached to the A-microtubule. The interaction between RS and CPC plays an important role on dynein motor proteins activity and, consequently, on the bending motion of the flagellum, with motor triggering signals starting in the CPC being directed to the DRC through the RS [36].

Genetic variants (mutations) in ODA genes, such as *DNAH5* and *DNAI1*, were demonstrated to cause reduced sperm motility [37,38], whereas mutations in IDA/CPC genes, such as *CCDC39* and *CCDC40*, cause reduced sperm motility due to the absence of IDA and displacement or abnormal CPC [27–29,39].

2.2.2. Connecting piece

The connecting piece (Fig. 6), also known as neckpiece, is located between the sperm head and the beginning of the MP. It is composed by rigid proteins in the form of a capitellum structure that gives shape to the SC [15]. The SC are nine cross-striated longitudinal columns held together by their confluence with each other, being attached to the BP [40]. Below the SC, nine ODF columns arise. The ODF extend though the entire flagellum up to the DPP, each being connected to one of the peripheral doublets [41,42].

During spermiogenesis, the PC (perpendicular to the sperm long axis) links to the nucleus BP, while the DC gives rise to the axoneme. The C-microtubule of the DC is generally shorter (in other cases its polymerizing pole is blocked by capping proteins). In consequence, during the process of axoneme extension, only microtubules A and B expand, originating the 9-microtubule doublets of the axoneme [43]. In the mature sperm, the triplet microtubules of the DC disaggregate and form a vault with the cone apex facing the PC [11]. After sperm-oocyte fusion, both sperm centrioles originate the zygote asters [44] [45].

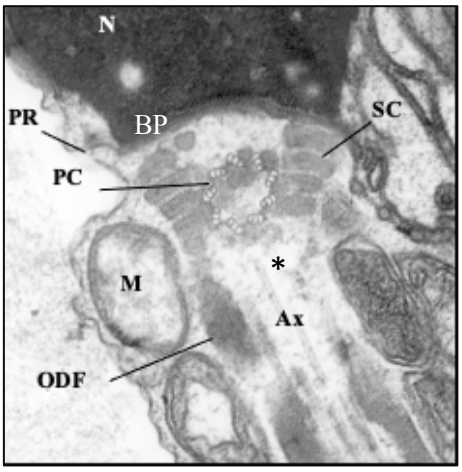

Figure 6. Ultrastructure of the sperm neckpiece (NP). The membrane of the neckpiece is physically separated from the head membrane by the posterior ring (PR). The NP contains the basal plate (BP), the segmented columns (SC), the proximal centriole (PC), the Distal centriole (*) and the axoneme (Ax). Image provided by Mário Sousa.

The CP not only confers mechanically support to the sperm head but also mediate coordination of sperm movement. When doublets slide or experience any tension, they are stabilized by their connection with ODF, with forces being transmitted to the neckpiece via ODF [46].

2.2.3. Midpiece

The MP region (Fig. 7) of the spermatozoon is 5–7 μm long, being located between the CP and the beginning of the PP. the MP is distinguishable due to the presence of the MS, which surrounds the axoneme. The MS is composed by an organized helical spiral of mitochondria strongly linked to each other [47]. During spermiogenesis, a large part of the cell cytoplasm is phagocyted by Sertoli cells, and only 50-70 mitochondria remain in spermatozoa. The MS produces the ATP necessary for the major membrane changes occurring during maturation (in the epididymis) [48], capacitation (in the uterine cavity) [49] and fertilization. It also confers mechanical resistance [50,51]. Mitochondria ATP production is not sufficient to diffuse through the entire flagellum in order to provide the energy needed for motility [52,53]. Whereas the ATP produced by mitochondria in the MP is through the oxidative phosphorylation (OXPHOS) process, the main ATP used in the PP is provided by anaerobic glycolysis performed through the activity of glycolytic enzymes [54]. Mitochondria are responsible for producing reactive oxygen species (ROS) which, at physiological concentrations, are important triggers for several mechanisms in the sperm, such as maturation, capacitation, acrosome reaction and oocyte fusion [55].

The distal limit of the MP is established by the presence of the annulus, a structure composed of several septins, which are cytoskeletal molecules with conserved GTPase activity [15,56]. The annulus is an important and key structure acting as a morphological organizer guiding the growth of the flagellum and the alignment of the mitochondria along the axoneme [57]. It also has a direct role in restricting the diffusion of membrane proteins, thus acting as a sperm diffusion barrier [58]. Pathologic variants in septin genes or other genes associated to annulus formation are known to cause a disorganized or absent annulus, with formation of abnormal sperm tails, such as bent tails, midpiece-principal piece disjunction, abnormal mitochondrial organization, FS dysplasia and reduced motility [59,60].

2.2.4. Principal Piece

The PP of the sperm flagellum (Fig. 7) makes up about three-fourths of the length of the entire flagellum (45–50 μm long), being defined by the presence of a FS [15,61], which underlines the plasma membrane and surrounds the ODF. The FS consists of two longitudinal columns, connected by a series of semi-circumferential ribs, which form a ring around the axoneme [62]. The FS is attached to ODF 3 and 8 in the PPP. At the DPP, the FS becomes associated with doublets 3 and 8. The assembly of the FS progresses from distal to a proximal direction along the axoneme throughout spermiogenesis. The FS contains several structural proteins, such as AKAP4 and AKAP3. These proteins are cAMP-dependent protein kinase (PKA) anchoring proteins (AKAP), which function as scaffolds for signaling pathway components [63,64]. The FS also plays an important role in providing energy for sperm flagellum motility [53], serving as a scaffold for glycolytic enzymes. By trapping several constituents of signaling cascades, it also plays a role in sperm motility regulation. The attachment of the longitudinal columns of the FS to doublets 3 and 8 restricts the doublet sliding movement, which is responsible for the flagellar bending movement [64].

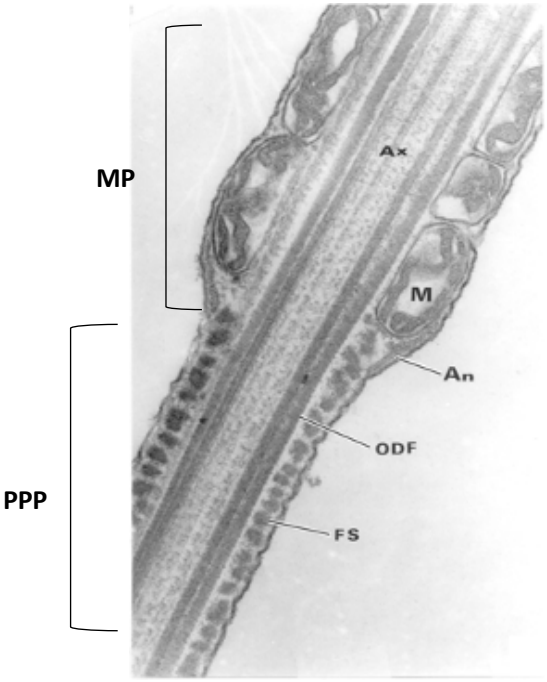

Figure 7. Ultrastructure of the sperm midpiece (MP) and of the proximal principal piece (PPP). In the MP, the axoneme (Ax) is surrounded by outer dense fibers (ODF) and mitochondria (M). The MP is separated from the PPP by the annulus (An). In the PPP, the Ax is surrounded by ODF and the rings of the fibrous sheath (FS). Image provided by Mário Sousa.

2.2.5. End Piece

The EP of the sperm flagellum has about 6 μm length and contains only the axoneme. This last part of the sperm flagellum begins when the FS structure ends. The FS becomes progressively thinner in the distal direction and does not extend to the last microns of the sperm flagellum [65,66].

3. Sperm Pathologies

Infertility is a disease of the male and female reproductive system that is defined by the inability to achieve pregnancy after 12 months of unprotected sexual intercourses [16]. It is estimated that about 48 million couples and 186 million individuals globally are infertile, with a global prevalence of 10-15% in the Caucasian population [16]. Male infertility accounts for about half of the infertility cases [67]. One main cause of male infertility is due to abnormal semen parameters, especially those related to concentration, motility and morphology [68].

In a normal ejaculate, the normal volume is ≥1.4 ml, the total sperm count is ≥39 million, the sperm concentration is ≥16 million per mL, the total motility is ≥30%, the total progressive motility is ≥30%, the normal morphology is ≥4%, and the Vitality is ≥54% [16].

Men with normal values are considered normozoospermic. Any deviation to this pattern is considered pathological and is assumed to cause infertility or subfertility. Abnormal semen parameters are categorized as follows: Aspermia; Hypospermia; Azoospermia; Oligozoospermia; Asthenozoospermia; Teratozoospermia; Necrozoospermia and Leukocytospermia.

Aspermia is characterized by the absence of semen during ejaculation. This can occur in men with retrograde ejaculation, where due to incomplete urethral sphincter closure, semen is introduced into the bladder. Other main cause is the obstruction of the ejaculatory duct, which blocks semen to reach the urethra.

Hypospermia is characterized by an ejaculate volume under 1.4 mL. Azoospermia is characterized by absence of sperm in the ejaculate. This can be caused by genital duct obstruction (rete testis, efferent ducts, epididymis) or absence of the vas deferens (Obstructive azoospermia), or absence/reduction of testicular sperm production (Secretory azoospermia/non-Obstructive azoospermia).

Oligozoospermia (OZ) is characterized by a lower sperm concentration (less than 16 million sperm/mL). Asthenozoospermia (AZ) is characterized by the presence of sperm progressive motility lower than 30%. AZ can also be defined by a rapid progressive motility (RPM) lower than 25% (< 25% RPM). Total PM includes slow progressive motility (SPM) and RPM. It is believed that only spermatozoa with RPM have the capacity to move along the female genital tract (vaginal channel, cervix

ostium, uterine cavity, tube cavity) and penetrate oocyte vestments (cumulus follicular cells, zona pellucida), thus enabling full fertilization. Teratozoospermia (TZ) is observed when less than 4% of morphologically normal spermatozoa are observed in the semen sample. Teratozoospermia can be subdivided into two categories, polymorphic teratozoospermia (more than one type of abnormality) or monomorphic teratozoospermia (unique abnormality) [69–71]. Nevertheless, most often, a combination of sperm anomalies is observed (OA, OT, AT, OAT). When patients show abnormalities in the three major parameters, sperm concentration, motility and morphology, the situation is named as oligoasthenoteratozoospermia (OAT). Necrozoospermia is characterized by the presence of dead sperm. Leukocytospermia is characterized by the presence of $>10^6$ leukocytes in the ejaculate.

The identification of the exact reason of a given sperm abnormality is challenging due to the heterogeneity and complexity of the factors that underlie sperm biogenesis, development and function. In recent years, research has focused on the possible factors leading to male infertility and revealed the existence of many cellular and molecular defects during sperm production and maturation. More than 4000 genes are thought to be involved in sperm production and as regulators of different aspects of sperm development, maturation and function [72–76].

3.1. Asthenozoospermia

Asthenozoospermia is characterized by the presence of abnormal sperm motility, being that motility is a sperm function of highest relevance for reproduction. Reduction in sperm motility has been profoundly studied, mainly focusing on the determination of the possible causes leading to infertility. Many important cellular and molecular factors involved in the onset of asthenozoospermia have been reported, such as the calcium pathway, cAMP-dependent protein kinase pathway and radical oxygen species (ROS) [77].

The normal spermatozoon displays two types of physiological motility: activated motility, as observed in sperm recovered from the ejaculate, and hyperactivated motility, as observed in sperm retrieved from the uterine cavity or tubal cavity. An effective motility is necessary for spermatozoa to transit through the female reproductive tract, and hyperactivated motility is required to capacitation and the acrosome reaction, the preliminary stage for fertilization. A decrease of both forms of sperm motility considerably reduces the ability of spermatozoa to reach the site of fertilization and to penetrate the oocyte, and consequently influence male fertility [78].

The current WHO guidelines, categorize sperm motility into four grades (a-d) when analyzed by computerized measures of the sperm velocity. Grade a, represents rapid progressive motility (>25 μm/s), with spermatozoa moving actively forward, either linearly or in a large circle. Grade b, represents slow (5-25 μm/s), with spermatozoa still moving actively, either linearly or in a large circle. Grade c, corresponds to non-progressive motility (<5 μm/s), in which sperm presents tail movements with absence of progression. Grade d, acts for total sperm immotility (no active tail movements).

According to WHO criteria, asthenozoospermia is defined as sperm total motility (<42%), corresponding to grades a+b+c of sperm motility, or sperm progressive motility (<30%), corresponding to grades a+b of sperm motility [16].

To move properly, the spermatozoon needs to have a proper flagellum structure, with sperm motility being directly affected by flagellum defects [79].

Multiple morphological abnormalities of the sperm flagellum (MMAF), abnormalities of the head–neck attachment, dysplasia of the FS, missing or poorly developed ODF and mitochondrial defects, have been reported as causes of sperm motility disorders.

These morphological anomalies have a genetic or an acquired origin, the latter being associated with varicocele, infections, lifestyle styles and environmental factors [80].

Regarding the genetic causes of asthenozoospermia, a ciliopathy named primary ciliary dyskinesia (PCD) raises as an important cause. PCD is an autosomal recessive disease caused by dysfunction of motile cilia. Motile cilia are highly conserved structures, which share the same central core structure, the axoneme. Motile cilia are specifically located in epithelial cells of the Fallopian tubes, rete testis, efferent ducts, brain ependymal cells, respiratory airways and embryonic node. As the sperm flagellum has the same axoneme structure, sperm is included in the motile cilia group. Consequently, any structural or functional anomaly in axonemes can lead to a PCD phenotype that culminates in respiratory, reproductive and/or laterality issues. In rare cases, PCD patients can also present hydrocephalus [81,82]. The hallmark clinical features of PCD are upper and lower respiratory tract symptoms from an early age, with development of chronic rhinitis, sinusitis and otitis media, bronchitis, bronchiectasis and pneumonia.

Males with PCD are infertile or sub-fertile due to total/partial sperm immotility, which is explained by structural deficiencies at the different components of the axoneme [83]. Some patients may also develop obstructive azoospermia, due to anomalies in motile cilia present in the rete testis and efferent ducts, which are responsible by fluid flow and sperm transport from the seminiferous tubules to the epididymis [84].

Currently, about 45 genes were already associated with the PCD phenotype, which include proteins either involved in cilia/flagellum cytoplasmic assembly as well as in the axoneme structure and associated fibers [85]. Due to high degree of conservation of the axoneme and its associated mechanisms, genes associated to PCD are also associated with asthenozoospermia.

Besides axonemal anomalies associated to PCD, defects in sperm mitochondrial ultrastructure are also associated with a decrease in sperm motility in humans [51]. In fact, comparative proteomic outcomes suggested that the expression of several sperm mitochondrial proteins may be altered in ATZ patients. A normal mitochondrial function is an imperative for sperm membrane events, regarding maturation, capacitation, chemotaxis, sperm penetration through cumulus cells [86].

Another structure of the sperm flagellum that is correlated with energy production is the FS. The FS contains several glycolytic enzymes and signaling molecules that are essential for sperm motility from ejaculation to fertilization, since the necessary energy is produced using glycolysis. Thus, a functional FS structure plays an important role in sperm motility, and so defects in genes that are associated with this structure may affect energy production and consequently sperm motility [87].

The effort to identify and understand the effects of genomic mutations and mechanisms contributing to sperm motility and ATZ remains urgent. Recent advancements in sequencing technologies have greatly accelerated the identification of the genes that underlie ATZ, which has improved our understanding of the physiopathology of ATZ and have provided candidates for biomarkers for the treatment of patients with ATZ. In addition, an early predictive diagnosis by genetic testing may also be helpful for treatment. However, considering the range of phenotypic and genetic heterogeneity of ATZ, only a limited number of mutations have been identified, indicating that additional studies are still needed.

Considering the complexity of the spermatozoon, the knowledge of ATZ causes is still very limited, the same applying to the mechanisms that regulate sperm motility. Thus, the identification and study of different RNA expression markers, presents an extremely actual importance for the understanding, discovery of new biomarkers and development of treatment strategies in ATZ.

4. Role of RNA in Sperm Motility

During spermatogenesis, which is a highly regulated transcriptional process, a high level of DNA packing within the sperm head occurs. Also, throughout the transformation of spermatids in spermatozoa most of the cytoplasm is phagocytosed by Sertoli cells [88]. Consequently, most of the transcripts that are present in round spermatids, which are produced during spermatogenesis and stored in the spermatid cytoplasm, are removed when cytoplasm is phagocytosed. Nevertheless, it was already described that RNAs are present in the mature spermatozoa, but at an extremely low level, being estimated that a single human spermatozoon contains about 10 to 20 fg of RNA [89,90].

Initially RNA was thought to be localized in sperm head region, more specifically at the periphery of the nucleus, close to the nucleus envelope and constitutes a structural part of the nuclear matrix [91] However, subsequent works described that RNA is also deposited in the sperm MP, within the mitochondria and in PP [92]. Later investigations using RT-PCR technology for sets of RNAs [93], as well as microarrays [94] and single cell analysis [95], have allowed the discovery of several transcripts present in human mature sperm. Microarrays and the first general RNA profiles suggested that human spermatozoa contain, approximately, 3000 to 7000 different coding transcripts [96]. Besides, the mammalian sperm cell also contains a large proportion of non-coding RNAs [97,98].

c-MYC mRNA, basic helix-loop-helix transcription factor, was the first specific mRNA identified in mature human spermatozoa, by RT-PCR analysis and *in situ* hybridization in the post-acrosomal MP and tail [99]. At begin, sperm RNA was thought to be merely remaining form the spermatogenesis process, and throughout the years this have been topic of scientific debate. Currently, some functions were already proposed for the sperm RNA. The exact role of sperm RNA is still largely unknown, but it was shown in mouse that sperm can transmit some of these RNAs during oocyte fertilization and suggested a role of paternal RNAs in early embryogenesis [100,101].

According to Zhao *and co-works,* which performed a serial analysis of gene expression in human ejaculated sperm, the *GA17 (*a dendritic cell protein), *SPATA7 (Spermatogenesis-related protein 7), COX5B (*Cytochrome c oxidase subunit Vb),

Cysteine-rich secretory protein 2 (*CRISP2*), mitochondrial transcription factor A (*TFAM*), Protamine 2 (*PRM2*) are among 10 most abundant unique transcripts in human ejaculated spermatozoa [102].

Among the coding RNA present in human sperm, some were already shown to be differentially expressed in low motile sperm samples. For instance, lower levels of Annexin A2 mRNA, which codes for a calcium-dependent phospholipid-binding protein, are associated with low sperm motility [103–105] and bromodomain 2 protein (*BRD2*) mRNA, which encodes a transcriptional regulator, showed higher levels of mRNA expression in fertile patients when compared to infertile ones [106]. *BRD2* is believed to be involved in chromatin remodeling during spermatogenesis - modifications on chromatin structures are known to have impact in spermatogenesis, and potentially lead to infertility [107]. Another set of transcripts that are widely study in male infertility is protamine 1 (*PRM1*) and *PRM2*, which have shown a higher expression in fertile controls when compared with infertile patients [108], an abnormal abundance of protamine mRNAs is related to an abnormal chromatin condensation and increased DNA strand breaks [109].

With the advance of novel sequence technologies, many transcripts have been identified and more may be identified in near future. For instance, a study from Bansal and coworkers found that *RPL24, HNRNPM, RPL4, PRPF8, HTN3, RPL11, RPL28, RPS16, SLC25A3, C2orf24, RHOA, GDI2, NONO, PARK7* were specifically up-regulated and the genes *HNRNPC, SMARCAD1, RPS24, RPS24, RPS27A, KIFAP3* were specifically down-regulated in asthenospermic patients [110].

4.1. Non-Coding RNA and Sperm Motility

Non-coding RNAs (ncRNA) are all functional RNA molecules that are not translated into proteins. ncRNAs include small nuclear RNAs (snRNAs) that are mainly involved in splicing events of messenger RNAs (mRNAs), transfer RNAs (tRNAs) that decode the mRNA sequence into peptide or protein, and ribosomal RNAs (rRNAs) that are the primary component of ribosomes and thought to represent the most abundant RNA molecules in the sperm cell [111]. Double-stranded RNAs (dsRNAs) have shown to mediate post-translational gene silencing of complementary mRNAs by a process called RNA interference. Another class of regulatory ncRNAs are the long non-coding RNAs (lncRNAs), which are characterized by at least 200 nucleotides, they perform multiple functions in mammals and regulate gene expression at several levels, more than 10 000 lncRNA transcripts have been reported in humans [112,113]. Genome imprinting, cell differentiation, apoptosis and nuclear transport are included in the variety of biological processes that lncRNAs are strongly involved [114–116]. In what concerns sperm cells, lncRNAs are related to the development of the testes, differentiation of spermatogonial stem cells and regulation of spermatocyte meiosis [117,118].

Small non-coding RNAs have emerged as key regulators of gene expression in many different cellular pathways, such as contributing to gene regulation, chromatin structure and inhibit transposition. Two of the most studied classes are the small interfering RNA (siRNAs) and the microRNA (miRNA) which are endogenous dsRNAs. The presence of these small RNAs have already been demonstrated in spermatogenic cells and revealed a crucial role in spermatogenesis [119].

PIWI-interacting RNAs (piRNA) have also demonstrated their importance. Initially, it was discovered that piRNAs are related to transposon silencing and gene integrity maintained in germline cells [120]. Nowadays, it is known that they also play a crucial role in regulating gene expression [121–123] as same as their role in sperm development. A recent study has described how piRNAs act as regulators of acrosome biogenesis during spermiogenesis [124].

Among all ncRNAs, the most well characterized non-coding sperm RNAs are miRNAs. MiRNAs are short (18–23 nucleotides in length), noncoding, endogenous, single-stranded RNA molecules involved in posttranscriptional regulation of gene expression and have been demonstrated to modulate several stages of spermatogenesis [125],

being their main biological function to block the translation of the mRNA. Most miRNAs base-pair with sequences in the 3'- untranslated region (3'-UTR), this partial complementary often facilitates translational repression and/or target the mRNA for degradation [126]. MiRNA guide the RNA-induced silencing complex (RISC) to specifically recognize the target mRNAs containing perfect or almost perfect complementary sites. When translational repression occurs, it implicates that the target mRNA is not cleaved, this mechanism involves multiple binding sites for the same or different miRNAs in the mRNA 3'UTR and cooperation among different miRNA-containing ribonucleoprotein (miRNP) attached to these binding sites on the target messages. These miRNPs may prevent initiation of translation or block active translation [127]. Alternatively, the complex miRNA-RISC can direct the cleavage of mRNA by the activation of different mechanisms, such as deadenylation, decapping, and exonucleolytic digestion of mRNA [128,129]. After cleavage of the mRNA, the miRNA remains intact and can guide the recognition and destruction of additional mRNAs. Besides the 3' UTR, the miRNA has also been detected to bind to other mRNA regions including the 5'- untranslated region (5'-UTR) and coding sequence, as well as within promoter regions [130]. It is believed that when the binding of miRNAs occurs in 3'UTR, 5' UTR and coding regions, it has leads to a block in mRNA expression (i.e has silencing effects) [131,132], but when miRNA interaction occurs within the promoter region it has been reported to have the opposite effect, to induce transcription [133].

Some miRNAs have already been identified and studied as sperm miRNAs in humans, such as *miR-34c-5p, miR-888-3p* and *miR-27a/b*. *Mir-34c-5p* is highly conserved among different species and is associated with cell proliferation, apoptosis and invasiveness of malignant tumors [134,135]. The role of *miR-34c* in later steps of spermatogenesis is indispensable, as its main function is to suppress cell proliferative ability through down-regulation of multiple targets [136,137]. *MiR-888-3p* was validated by RT-PCR and significantly overexpressed in male patients with ATZ, involved in the tumorigenic process of several cancers, defined as a cancer testis antigen and a possible inducer of an aggressive endometrial tumor phenotype [138]. *MiR-27a* and -*27b* negatively regulate expression of CRISP2 and downregulation of CRISP2 protein was significantly associated with low sperm abnormal morphology, progressive motility, and infertility in ATZ patients [139].

4.2. RNAs in Human Seminal Plasma

Semen, also known as seminal liquid, is a fluid from the male reproductive tract and is composed by sperm cells, which are capable of fertilizing the egg of the female and by seminal plasma, which is the major component and helps to maintain the viability of sperm cells. Seminal plasma is a mixture of several incompletely mixed components, including secretions from testes, epididymis and other accessory sex glands, and contains several proteins which contribute to sperm maturation, metabolism, motility, modification of sperm membranes and capacitation [140]. Recent studies showed the presence of DNA, long single stranded RNA and small RNAs (miRNAs and piRNAs) [141,142]. Even though biological role of RNA in seminal plasma remains unclear, it is believed that RNA may provide a regulation role of gene expression in the testis and epididymis, which is crucial for a normal spermatogenesis and sperm maturation process [143].

Exosomes, detected in different body fluids, have demonstrated to participate in intercellular communication through the selective incorporation of their cargo (energy substrates, ions, proteins, RNAs, lipids, among others) into the target cell. Thus, exosomes released by epididymis (epididymosomes) - which are capable of modifying the lipid composition of the sperm membrane and, consequently, contribute to the acquisition of sperm motility and the ability to penetrate the zona pellucida [144] and accessory glands could play a role in the communication between seminal plasma and sperm. The acquisition of new RNAs by the spermatozoon during testicular maturation comes from the active communication between sperm and epididymal fluid, most likely through the incorporation of RNAs within epididymosomes [145]. Since seminal plasma also interacts with the female reproductive tract, additional functions for seminal plasma RNAs have been proposed. Seminal plasma can modulate ovulation in mammals including rabbits, cats and camels [146], some seminal proteins can increase ovulation rate through neuromodulatory machinery induction in the female or promoting the storage of sperm by the induction of uterine contractions, as well as the female immune response to the developing embryo [147], RNAs from seminal exosomes could regulate gene expression in cells within female genital tract, probably by modulating critical events.

4.3. Sperm RNAs as Biomarkers

The study of sperm RNA profiles in both humans and domestic animals is currently providing the framework for the discovery of novel clinical biomarkers and potential diagnostic tools for fertility evaluation. Comparative analysis on human sperm RNA profiles has helped to delineate possible aberrant mechanisms underlying male infertility, revealing sperm molecular disturbances occurring during either spermatogenesis or sperm maturation [148].

In 1994, evaluating protamine transcript expression in the human ejaculate was used as a marker for spermatozoa in post-vasectomized individuals. Sperm mRNAs coding for protamines may be useful biomarkers for predicting male infertility as neither the nature of chromatin organization nor the extent of chromatin compaction differs dramatically in human sperm with head-shape abnormalities. Later was demonstrated that RNA profiles obtained from mature ejaculated spermatozoa reflect spermatogenic gene expression [149].

The application of microarray technology to spermatozoal RNA has provided a unique opportunity to assess alterations in male fertility. Large-scale microarray analysis in sperm from both fertile and infertile men patients with normal semen parameters has revealed a significant difference in spermatozoa transcripts, demonstrating a diagnostic and therapeutic potential [150,151].

Thus, human sperm RNAs might predict the success of specific assisted reproductive technologies, this relies on the capacity of a set of sperm RNA elements to discern idiopathic infertile patient groups according to the likelihood of achieving pregnancy using less invasive assisted reproductive technologies [152]. Besides, sperm RNA can be suitable molecular markers for the diagnosis of cell lines in spermatogenesis, in future eventually they can provide a generalized picture of spermatogenesis in the infertile testis instead of invasive testicular biopsy and, more importantly, could be used to develop effective therapies for ATZ.

Objectives

The current knowledge about the sperm transcriptome in men and animals have allowed the identification of common differentially expressed genes, which might serve as molecular diagnostic platform to assist in screening for male infertility causes

Considering the urge of a better understanding of ATZ mechanisms, to develop effective therapies and treatments for ATZ and other pathologies associated with male infertility and the possible role of RNAs as biomarkers.

With this work, we aim to contribute to increase knowledge regarding the relationship between the dysregulation of sperm RNA and reduced sperm immotility, by evaluate the expression profiles of a set of RNA (including mRNA and their high score correspondent miRNAs) in human sperm from patients with ATZ.

Materials and Methods

Literature review and database search

To select the genes to be included in the study a literature review was performed in PubMed, using key words: asthenozoospermia or sperm immotility, flagellar/ciliary anomalies, genetics of male infertility, sperm RNA and male infertility. Firstly, a list of seventy genes was defined and after, through inclusion and exclusion parameters (exclusion criteria: existence of multiple studies reporting gene expression analysis in human spermatozoa and high expression in blood cells according to the Human Protein Atlas Database (https://www.proteinatlas.org/); inclusion criteria: no reported gene expression analysis in human spermatozoa, high expression in germ cells and ciliated cells) the list was reduced to twenty genes (*BSCL2, CATSPER2, CATSPER3, CCDC40, CFAP43, CFAP44, CRHR1, DRC1, HIP1, IQCG, KRT34, LRRC6, PLAG1, QRICH2, RSPH6A, SPATA33, TEKT2, TTC21A, USP11, ZMYND10*).

Biological sample collection

According to the National Law on Medically Assisted Procreation (Law 32/2006) and the National Council for Medically Assisted Procreation guidelines (2018), surplus gametes for research were used under strict individual anonymity and after patient written informed consent. The study was approved by the University Hospital Ethics Committee, with authorization number Project: 2019/CE/P017 (266/CETI/ICBAS). Ejaculate samples from 75 patients with reduced rapid RPM (cases) and with normal semen parameters (controls) were obtained at the Centre of Reproductive Genetics Prof. Alberto Barros. In all cases, only surplus ejaculates from men undergoing routine spermiogram evaluation were used.

Control nasal cells and peripheral blood were obtained from healthy university volunteers. These cells derived from own research RNA bank. Nasal cells were collected by nasal brushing at the hospital under specialized medical personnel. White blood cells were collected from peripheral blood at the hospital under specialized nurse per-sonnel using EDTA containing tubes (VACUETTE, Porto, Portugal).

Control testicular tissue derived from own research RNA bank. This testicular tissue was obtained from excedentary testicular tissue of men with obstructive azoospermia

under infertility treatments. Cases with obstructive azoospermia can be used as controls as they present conserved spermatogenesis. Obstructive azoospermic men had normal karyotypes and absence of Y microdeletions and CFTR mutations

Spermatozoa isolation

To obtain a population of spermatozoa from the surplus ejaculates, we first separate the seminal plasma from spermatozoa. For that, ejaculate samples were washed with 1 ml of HEPES buffer (Fisher Bio Reagents, Maharashtra, India) and centrifuged at 600 × g for 10 min at 24 C°, with this step being repeated three times. Supernatants, containing seminal plasma, were stored at -80°C until use. To eliminate somatic cells from the pellets, these were suspended in somatic cell lysis buffer (SCLB) and incubated on ice for 30 min. SCLB contains 0.1% SDS (TCI Chemicals, California, USA), 0.5% Triton X (Sigma-Aldrich, Missouri, USA) and H20-DEPC (Sigma-Aldrich). Obtainment of a purified spermatozoa pellet was checked by light microscopy to verify elimination of somatic cells. In cases of somatic cell persistence, samples were retreated with SCLB. The pellet was then resuspended in HEPES and centrifuged at 600 × g for 10 min at 24 C°. The purified spermatozoa were stored at -80°C until use.

Genes and primer design

The reference sequences for the selected genes were retrieved using the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/) or Ensembl (https://www.ensembl.org/). Primer design was performed with Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Parameters used for primer design were as follow: primer length: 18-25 bp; GC content: 40-60%; amplification length: 80-200 bp; and melting temperature: 58-62 C°. Each designed primer pair was tested for the presence of dimer formation using the FastPCR software (version 3.7.7; Institute of Bi-otechnology, University of Helsinki, Finland) and for their specificity towards the re-gions of interest using the PrimerBlast tool (NCBI, Bethesda, USA). The primers used in this study are list in supplemental table A1.

RNA extraction and cDNA conversion

The extraction of total RNA from purified spermatozoa was performed using the Single Cell RNA Purification Kit (Norgen, Thorold, Canada). RNA was quantified using the NanoDrop ND-1000 Spectrophotometer. The sperm RNA includes both mRNA and miRNA, as the extraction kit that was used is designed to extract the total RNA from the cell. Part of the extracted RNA was used to cDNA conversion using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA), according to the manufacturer instructions. Other part of sperm RNA was used to cDNA conversion using the kit NZY First-Strand cDNA Synthesis (NZYThech, Lisbon, Portugal) specifically to study microRNA, because it includes a Oligo dT primer, used for the production of cDNA from RNA containing poly(A) tail. The total RNA from nasal, white blood cells and testis to use as positive controls were extracted with the NZY Total RNA Isolation Kit (MB13402, NZYTech, Lisbon, Portugal), according to manufacturer instructions, and quantified using a NanoDrop spectrophotometer ND-1000. The synthesis of cDNA, from extracted RNA, was per-formed with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems), according to the manufacturer instructions.

Polymerase chain reaction (PCR)

The PCR technique was used to confirm the expression of the previously selected genes in spermatozoa RNA. As a positive control, a sample in which the presence of each gene was previously confirmed (human testis, human nasal ciliated cells (obtained by nasal brushing) or White Blood cells (obtained from peripheral blood)) was included. The reaction mixture used (20 µl) contained: 4 µl of PCR Master Mix (5xFIREPol, Tartu, Estonia), 13 µl of DEPC treated water; 1 µl of each primer (Eurofins Genomics, Ebersberg, Germany) at 10 pmol/µl each and 1 µl of cDNA at 40 Ngl/µl. PCR conditions were optimized for each primer pair, and it was performed in a PCR T100™ thermal cycler (BioRad, California, USA). PCR products were analyzed by 1,5% agarose gel electrophoresis: mix of TAE 1x SeaKem LE Agarose (Lonza, Rockland, USA), and 5 µl/100 ml of GreenSafe (NZYTech).

Definition of a list of miRNAs

After gene expression in spermatozoa had been confirmed, a list of microRNAs was generated based on their predicted interaction with the genes previously selected according to the mirDIP database (https://ophid.utoronto.ca/mirDIP/) and CSmiRTar database – Condition-Specific miRNA Targets database - (http://cosbi4.ee.ncku.edu.tw/CSmiRTar/). The selection was made using the highest average normalized score, with microRNA with average normalized score higher than 0,9 and whenever possible supported by more than one database defined (supplemental table A2). For genes CRHR1, KRT34 and QRICH2, at the time of the study, no microRNA with defined average normalized score was found in these databases, and thus microRNAs from these genes were not included. The miRNA sequences were retrieved using the miRbase (https://www.mirbase.org) and primers were designed using the Mirprimer software [153]. The primers used in this study are list in supplemental table A3.

Real-time PCR (RT-PCR)

The mRNA of the selected genes and miRNA was analyzed by quantitative real-time PCR, with SYBR green, using the previously designed primers. qPCR was performed in a Bio-Rad CFX96 (Bio-Rad, Hercules, USA) and amplifications were prepared with the NZY qPCR Green (NZYTech), according to the manufacturer instructions. The cDNA concentration was optimized prior to initiate the experiences, by making an amplification efficiency determination via standard curve with a r^2 closer to 1. A concentration of 40 ngl/µl was chosen. Three technical replicates for each individual were performed in each PCR assay and a calibration sample was always included in each 96-well plate set-up. For mRNA analysis the *B2M* and *GAPDH* genes were used as housekeeping genes to normalize gene expression levels. For miRNA analysis *miR-30a-5p* and *miR-100-5p* were used as normalizers. Fold variation of gene expression levels was calculated following a mathematical model using the formula $2^{-\Delta\Delta Ct}$ [154]. The statistical significance was determined using the non-parametric statistical test Krustall-Wallis test, with alpha <0.05. Tests were performed in the GraphPad Prism (version 6.01, GraphPad Software, California, USA).

Immunofluorescence microscopy

Immunostaining was used to determine the presence of KRT34 and CRHR1 in the sperm samples. Sperm cell suspensions were spread onto glass slides (STARFROST, Knittel-Glass, Germany), air dried and stored at −80 °C until use. Then, glass slides containing the spread cells were washed in phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (Merck) at room temperature for 30 min. Cells were washed twice in PBS, followed by a permeabilization treatment with 0,2% Triton X-100 (SigmaAldrich) in PBS at room temperature for 10 min. After another wash in PBS, cells were incubated with 5% non-fat milk in PBS at room temperature for 60 min, to inhibit non-specific binding. After a final PBS wash, cells were incubated with primary antibodies rabbit anti-KRT34 or CRHR1 from Biorbyt (Cambridge, United Kingdom) at a 1:70 dilution and mouse antiacetylated α-tubulin (Santa Cruz Biotechnology) at a 1:150 dilution overnight at 4C°. For each experiment, a negative control, through the omission of the primary antibody, was included. Then, after PBS wash, cells were incubated with 1:200 DyLight-488 anti-rabbit (Biolegend, California, USA) and Texas Red anti-mouse (Santa Cruz Biotechnology) secondary antibodies at room temperature for 90 min. After another PBS wash, cells were counterstained with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI: Vector Laboratories, California, USA) at room temperature for 20 min. Slides were examined under an epifluorescence microscope (Eclipse E400; Nikon, Tokyo, Japan).

Results

SCLB efficiently removes somatic cells

In the seminiferous tubules, the spermatogenic process is supported by somatic Sertoli cells [155]. As sperm RNA concentrations are very reduced, somatic cell RNA contamination could interfere with the gene expression data. Therefore, is important to remove somatic cells prior initiated the gene expression studies. Firstly, using ejaculated samples from normospermic individuals, we have processed the semen samples following the density gradient centrifugation, according to Bansal and co-workers [156]. However, the gradient method is based on the selective isolation of the high-motility fraction or the morphologically normal sperm cells. Consequently, we observed a significant loss of sperm cells during the process, as a wide fraction of spermatozoa with low motility and/or abnormal morphology was lost during the processing. When aiming to obtain the best sperm sample for assisted reproductive techniques density gradient centrifugation is indeed crucial. However, our objective is to characterize the sperm expression profile of a set of RNA in patients suffering from low sperm motility, thus the RNA of the sperm that were discarded by the selective method would be highly informative. Besides, as sperm RNA is very low, we needed to have as many sperm cells as possible.

In that sense SCLB treatment was selected to apply in all samples that were used for gene expression analysis. SCLB has the advantage that the obtained cell fraction maintains the original proportion of sperm cells (no sperm selection is performed). Besides it is ease of use and lack specialized equipment or costly reagents, therefore it has been applied with satisfactory results in several studies [157–159].

Human ejaculate, purified spermatozoa and seminal plasma were examined by light microscopy (Fig. 8). The first examination by light microscopy was done at an initial stage, with no treatment done to the ejaculate sample (named T0). Then, after the centrifugation step seminal plasma (SP) was separated from sperm cells. The sperm cell pellet was wash with HEPES, and then sperm cells were treated with SCLB, a minimum of three times until eliminate the contamination with somatic cells. With this approach, we observed a minimal loss of sperm cells and an effective removal of the present somatic cells, which allowed to obtain purified spermatozoa.

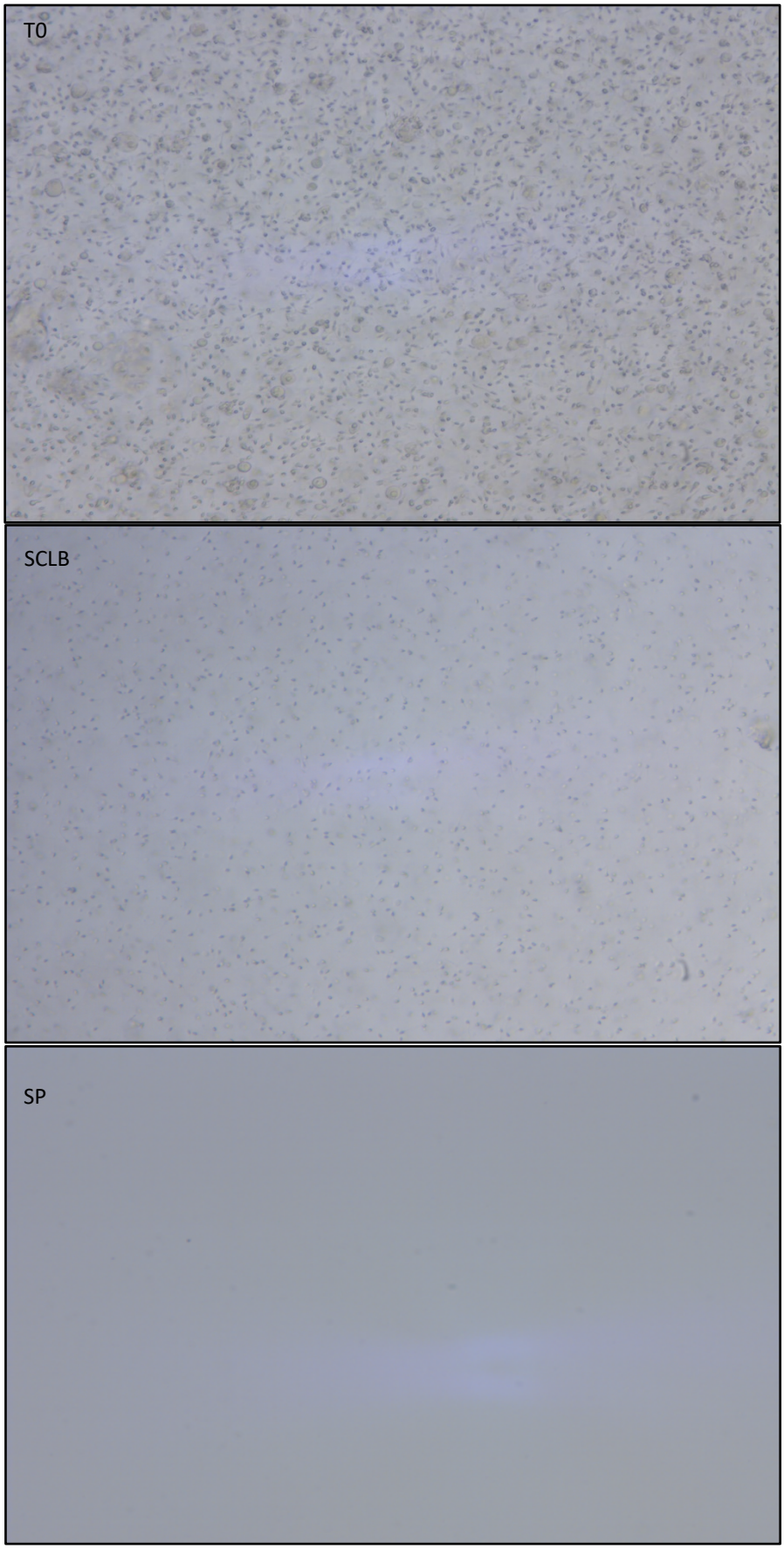

Figure 8. Human ejaculate sample from a normozoospermic patient by light microscopy (T0), sperm cells treated with SCLB (SCLB) and seminal plasma separated from sperm cells (SP).

The presence of mRNA in the purified spermatozoa

After literature search, 20 genes were selected for PCR analyses to confirm their expression in spermatozoa RNA. So, we designed specific primers for each of them and optimization was achieved by performing several PCR reactions under different conditions, using a cDNA sample from a control tissue (blood, testis, or ciliated cells), depending on the gene. Once PCR conditions were optimized, we tested the presence of the 20 initial transcripts in purified spermatozoa from a normozoospermic patient, using the same cDNA sample for all.

The transcripts of *CATSPER3, CFAP44, CRHR1, HIP1, IQCG, KRT34, LRRC6, QRICH2, RSPH6A, SPATA33* and *TEKT2* were found in purified spermatozoa from a human sperm sample by PCR (Fig. 9), whereas there was no expression of *BSCL2, CATSPER2, CCDC40, CFAP43, DRC1, PLAG1, TTC21A, USP11* and *ZMYND10* mRNAs in the same purified spermatozoa, even after changing the PCR cycle number and/or temperature (Fig. 10). These results could be mean either that the selected transcripts (see supplemental table 1) were not present in sperm or that they are present at reduced levels and thus were not detected.

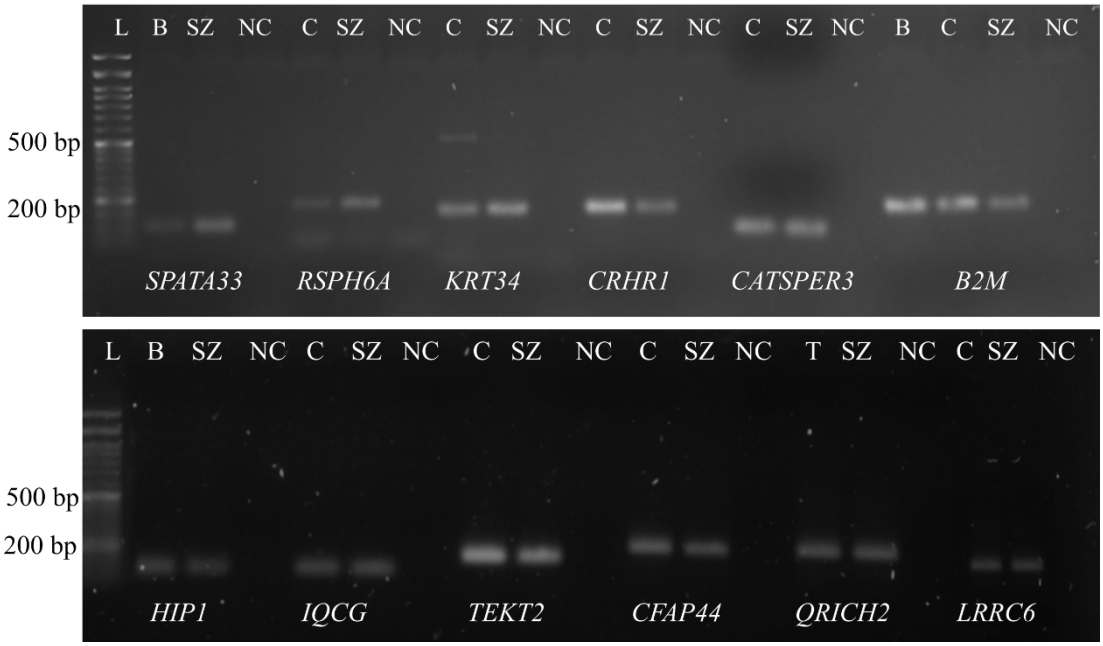

Figure 9. The presence of *SPATA33, RSPH6A, KRT34, CRHR1, CATSPER3* (A), *HIP1, IQCG, LRCC6, QRICH2, TEKT2* and *CFAP44* (B) transcripts in human ejaculated spermatozoa identified by PCR and running on a 1.5% agarose gel. *B2M* was used as control for the different tissues (A). L, DNA Ladder; B, blood; C, ciliated cells; T, testis; SZ, purified spermatozoa; NC, negative control.

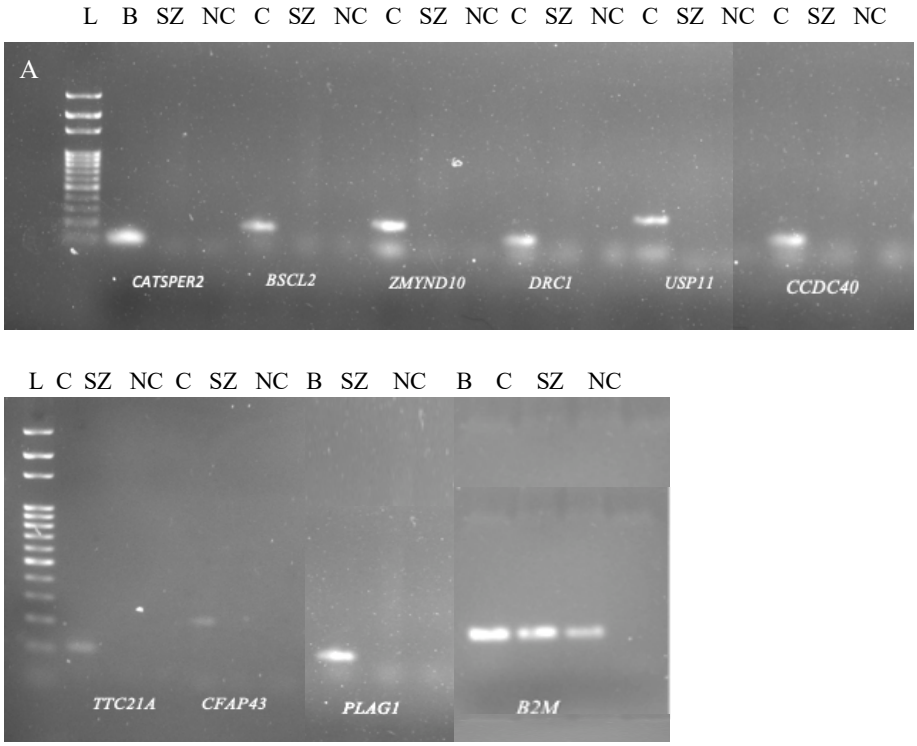

Figure 10. No expression of *CATSPER2, BSCL2, ZMYND10, DRC1, USP11, CCDC40* (A), *TTC21A, CFAP43* and *PLAG1* (B) transcripts in human ejaculated spermatozoa identified by PCR and running on a 1.5% agarose gel. *B2M* was used as control for the different tissues (A). L, DNA Ladder; B, blood; C, ciliated cells; T, testis; SZ, purified spermatozoa; NC, negative control.

Analysis of mRNA and microRNA expression profiles by quantitative RT-PCR

After validating the expression of the eleven transcripts in purified spermatozoa through PCR, quantitative RT-PCR was performed. Each transcript was tested in all 33 samples, which were divided into three groups according to the % of RPM: 4 samples of purified spermatozoa with RPM <15%, 10 samples of purified spermatozoa with RPM between 15-25%, and 19 samples of purified spermatozoa with RPM >25%. Samples from the RPM <15% and RPM 15-25% groups were considered our target samples (AZ group) and samples from the RPM >25% group were considered our control samples. GAPDH and B2M were used as reference genes. However, as GAPDH showed high variability only B2M results was used as a reference (Fig. 11).

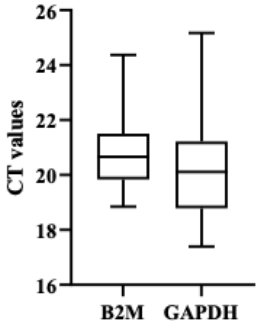

Figure 11. Box Plot of Ct values of *B2M* and *GAPDH* genes.

After RT-PCR, quantification of the expression profiles was performed using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was determined using the Krustall-Wallis test, with alpha <0.05.

Using *B2M* as a reference gene, we observed that all mRNA were downregulated in patients with reduced RPM, <15% and 15-25% groups (Fig. 12 and 13). In the case of *QRICH2, TEKT2, KRT34, CRHR1, HIP1* and I*QCG* was observed that higher RPM rate means a higher expression of the transcript. *QRICH2, KTR34, TEKT2* and *HIP1*, were the genes that show a high fold-decrease, particularly in the <15% group. In the case of *RSPH6A, CATSPER3, CFAP44* and *SPATA33* the expression of the transcripts is lower in patients with low sperm motility, when compared to the normozoospermic patients, however there is not direct relation between the RPM rate and the expression level of

the target transcript. For the *LRRC6* transcript, a reduced expression was observed in the ATZ group, however this reduction was not statistically significant.

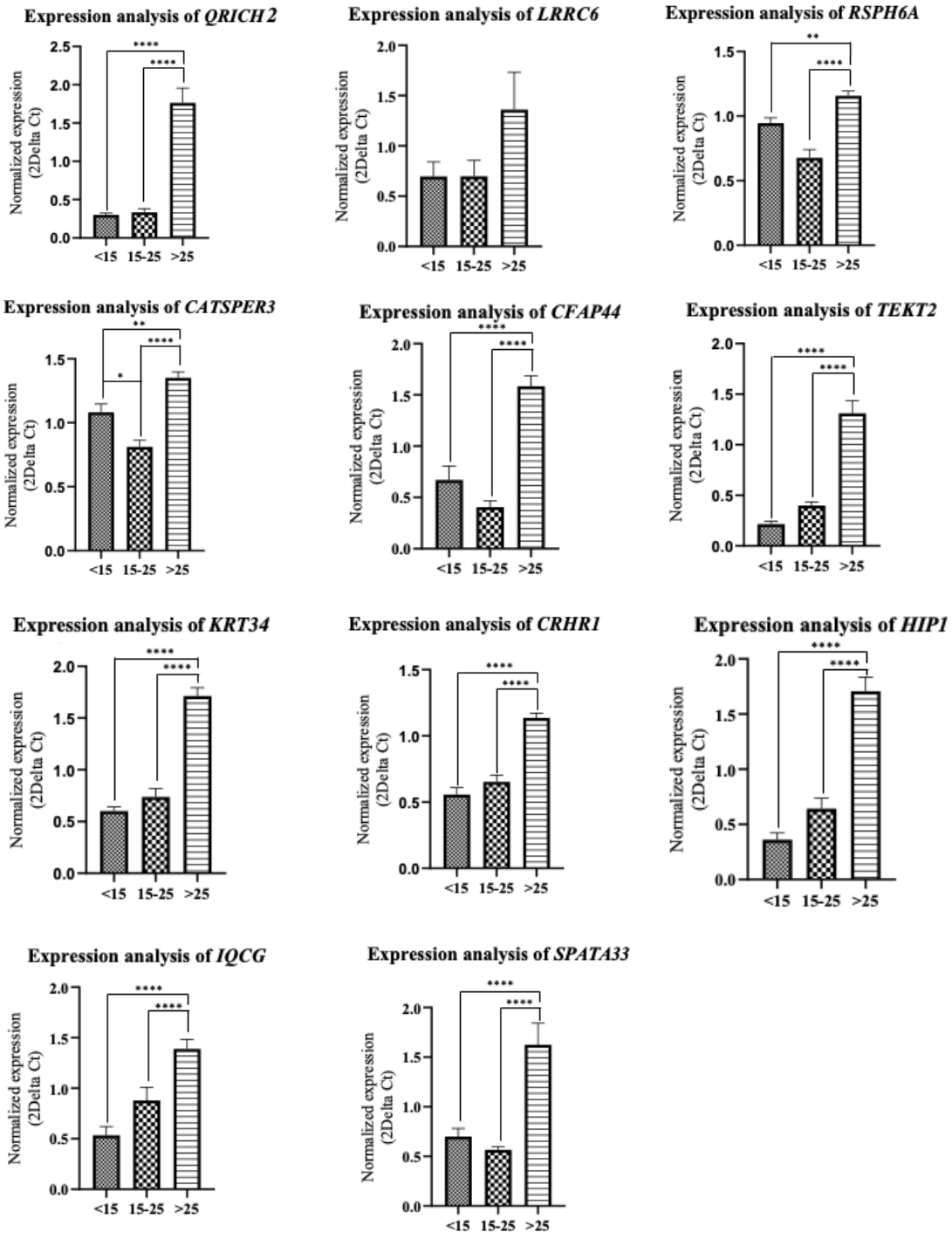

Figure 12. RT-PCR analysis of the mRNA expression levels of each transcript in sperm cells from patients with RPM lower than 15% and between 15 and 25%. Statistical significance was determined using the Krustall-Wallis test, with alpha <0.05.**p<0.01 and ***p<0.0001. *B2M* was used as reference gene.

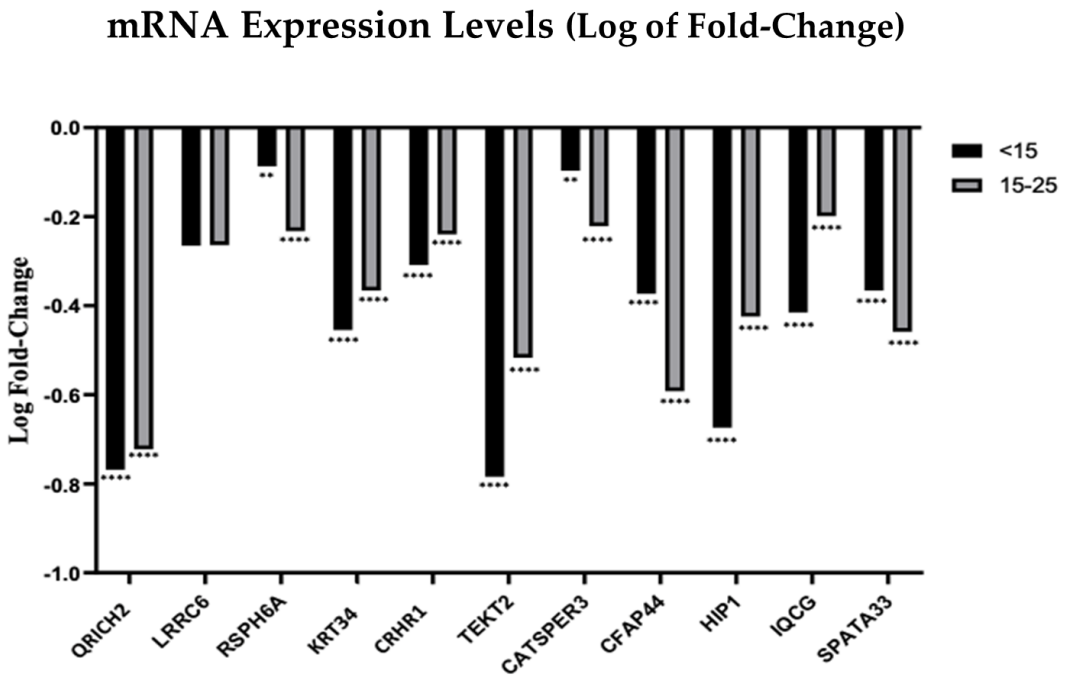

Figure 13. Log of fold change of the mRNA expression levels in sperm cells from patients with RPM lower than 15% and between 15 and 25%. Statistical significance was determined using the Krustall-Wallis test, with alpha <0.05.**p<0.01 and ****p<0.0001. *B2M* was used as reference gene.

Relatively to the microRNAs, two normalizers, miR-30a-5p and miR-100-5p, were used to assess gene expression. We studied the expression profiles of miR-3664-5p (*LRRC6*), miR-2110 (*RSPH6A*), miR-4660 (*TEKT2*), miR-492 (*CATSPER3*), miR-4425 (*CFAP44*), miR-4731 (*HIP1*), miR-4514 (*IQCG*) and miR-518c-5p (*SPATA33*). For genes *CRHR1*, *KRT34* and *QRICH2*, at the time of the study, no microRNA with defined average normalized score (>0.9) was found in databases, and thus microRNAs from these genes were not included. Using both, all microRNAs were also downregulated in patients with low sperm motility, when compared to the normozoospermic patients. Except for miR-3664-5p, all microRNAs demonstrated a more accentuated down regulation in patients with the lower RPM rate (<15%) when compared to 15-25% group or with >25% group. The mir-4660 and mir-2110 were the ones with a high fold-decrease, in both normalizers used, in the group of <15% (Fig. 14 and 15).

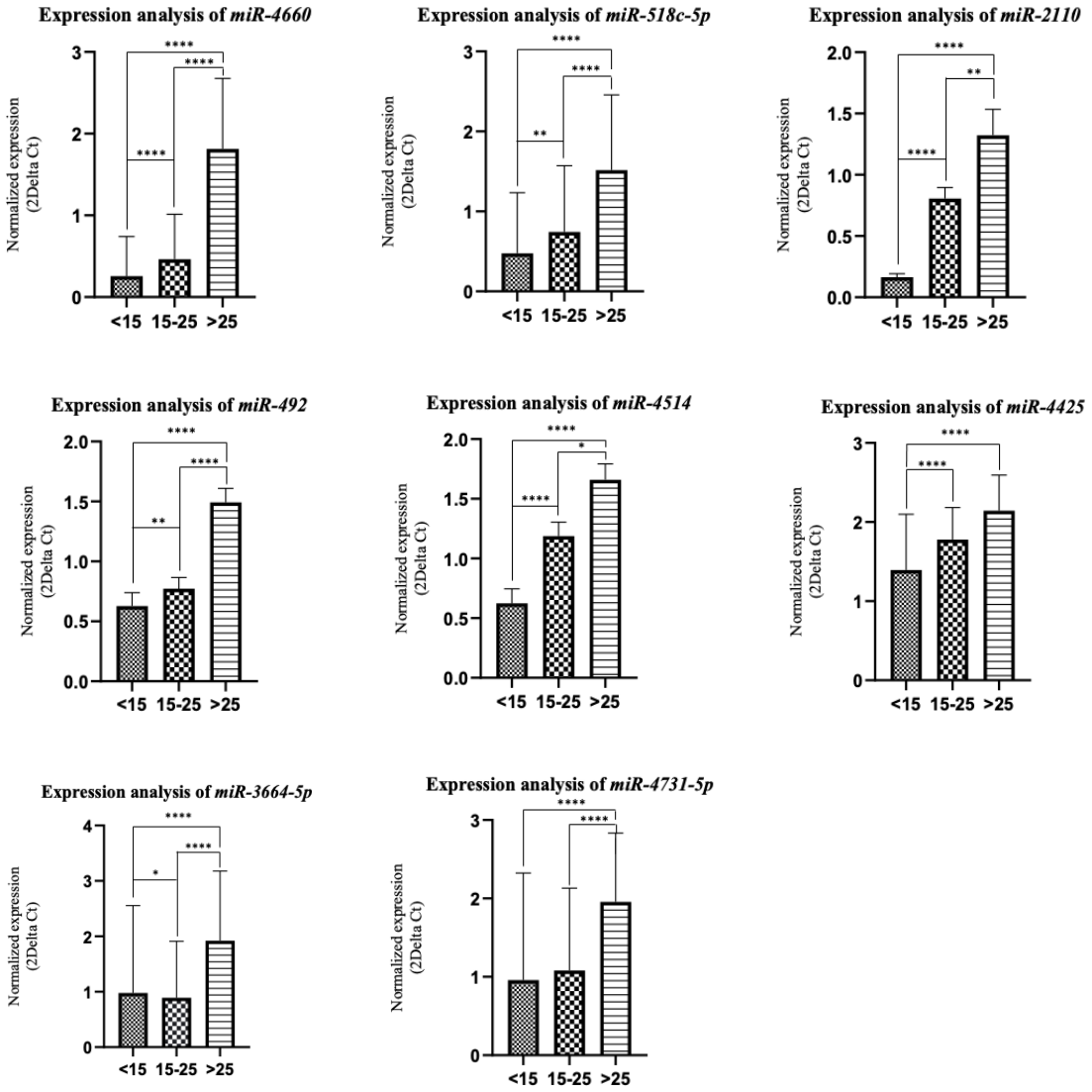

Figure 14. RT-PCR analysis of the microRNA expression levels of each microRNA in sperm cells from patients with progressive motility lower than 15% and between 15 and 25%. Statistical significance was determined using the Krustall-Wallis test, with alpha <0.05.**p<0.01 and ***p<0.0001. miR-30a-5p and miR-100-5p were used as reference genes.

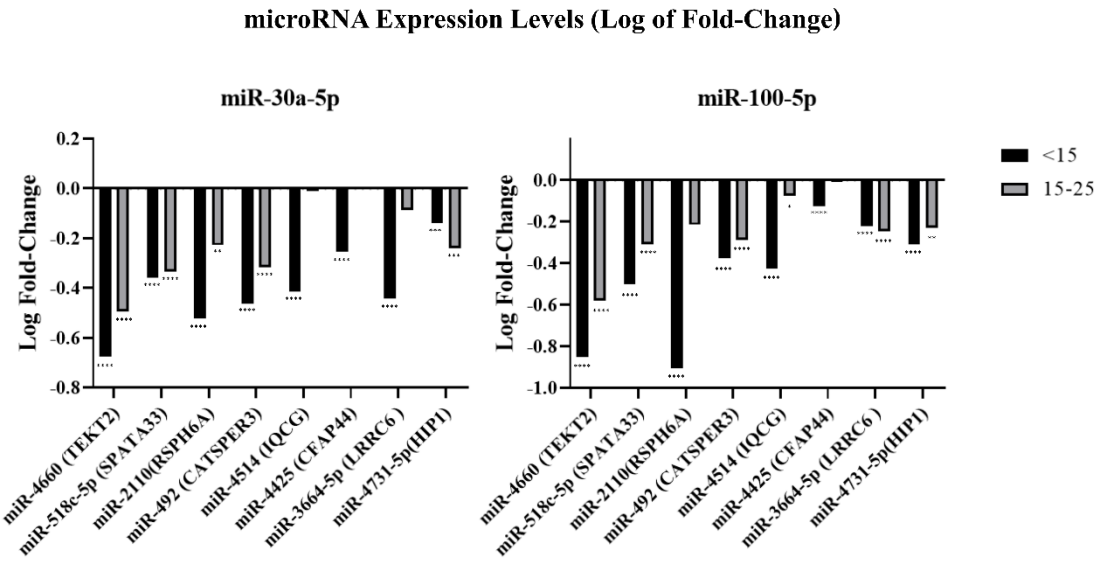

Figure 15. Log of fold change of the microRNA expression levels in sperm cells from patients with progressive motility lower than 15% and between 15 and 25%. Statistical significance was determined using the Krustall-Wallis test, with alpha <0.05. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. miR-30a-5p (left) and miR-100-5p (right) were used as reference genes.

To get further knowledge regarding CRHR1 and KRT34, we decide to preform immunofluorescence analysis to understand where those proteins could be in sperm cells from normospermic individuals. By immunodetection we observed expression of the proteins in the head region, presumably in the perinuclear matrix (techa) and midpiece for CRHR1 and in the techa and slightly over the midpiece for KRT34 (Fig. 16).

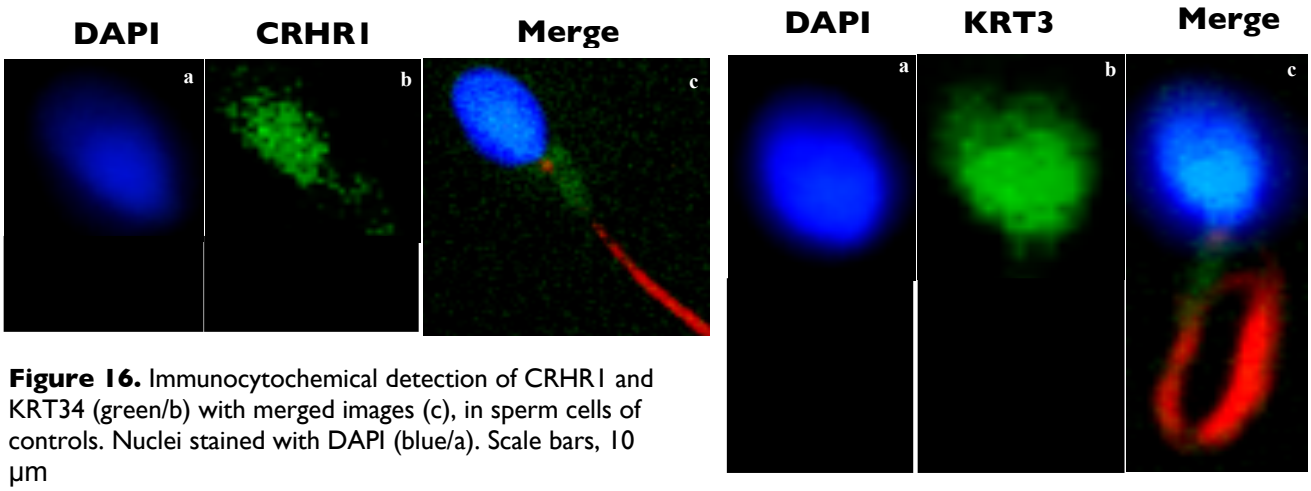

Figure 16. Immunocytochemical detection of CRHR1 and KRT34 (green/b) with merged images (c), in sperm cells of controls. Nuclei stained with DAPI (blue/a). Scale bars, 10 µm

Discussion

The goal of this work was to investigate the expression profiles of a set of sperm RNA, including mRNA and ncRNAs (miRNAs), in human sperm from patients with ATZ. After an extensive literature review and expression analyses through PCR, eleven genes associated with sperm immotility were selected, namely *CATSPER3, CFAP44, CRHR1, HIP1, IQCG, KRT34, LRRC6, QRICH2, RSPH6A, SPATA33* and *TEKT2*. Then, the expression profiles of the mentioned mRNAs were analyzed. Using *B2M* as a reference gene, we observed that all mRNA were downregulated in patients with reduced sperm motility, <15% and 15-25% groups (Fig. 13).

Our results showed that the expression of the mRNAs *CATSPER3, CFAP44, RSPH6A* and *SPATA33* was reduced in low motility samples, however the lowest expression was in 15-25% group. *CATSPER3* is a member of a sperm cation channel-like protein family, named Cation channel of Sperm (CatSper). *CATSPER3* was proposed to have a role in acrosome reaction, which is a calcium (Ca^{2+})-dependent secretory event, an essential to early steps of the fertilization process, being essential for the regulation of the positive loaded calcium ions that go into the sperm cell and for sperm hyperactivation [160]. A high expression levels of *CATSPER3* mRNA have been observed in high-motile human spermatozoa, when compared to low-motile spermatozoa [161]. Further, *CATSPER3* gene expression was decreased in spermatozoa from patients with ATZ and OAT, a condition that includes oligozoospermia (low number of sperm), ATZ, and teratozoospermia (abnormal sperm shape) [162]. Cilia and flagella associated proteins (CFAPs) play a vital role in the biogenesis of axoneme and are responsible for multiple morphological abnormalities of the sperm flagella (MMAF). Patients with mutations in *CFAP44* (also known as *WDR52*) gene have shown a higher rate of spermatozoa with short and absent flagella and a lower motility rate, furthermore, *Cfap44*$^{-/-}$ male mice have also showed flagellar immotility, leading to infertility [163]. Our results also show a reduced expression in low motility samples, specifically, we observed a lower expression in the 15-25% group. However, these studies considered low motile when motility is lower than 30%, thus we could not exactly know if these studies also note some difference regarding the motility rate.

RSPH6A (Radial spoke head 6 homolog A) is a conserved protein present in eukaryotic cells implicated in ciliary and flagellar motility. It has been described that *RSPH6A* is

testis-specific in humans and localized throughout the entire flagellum [164]. In mouse, in the absence of *RSPH6A* sperm flagellar elongation may become unstable [164]. RSPH6A protein, together with other RS proteins, play a role in the connection of the Cp of microtubules with the outer ones and is essential for the characteristic bending pattern of any given axoneme [165]. The RS is important for axoneme stabilization, thus damage in RS complex makes the axoneme unstable, potentially leading to a stop in elongation process before a proper mitochondrial and FS formation [166]. Studies in mice have shown that *RSPH6A* interacts with *RSPH1, RSPH4A, RSPH9* and *RSPH10B*, being essential for male fertility due to its role in sperm flagellum formation [164]. *SPATA33* (Spermatogenesis associated 33) gene encodes a Ser-rich protein with many potential phosphorylation sites, suggesting its role through kinase signaling during spermatogenesis [167]. *SPATA33* interacts with sperm calcineurin, which is a calcium-dependent phosphatase composed by two subunits, a catalytic (PPP3CCC) and a regulatory (PPP3R2) and localized in both MP and PP in mice [168–170]. Its regulatory subunit is crucial for regulating sperm protein phosphorylation status, an important mechanism linked with sperm maturation and capacitation. On the other hand, in the absence of the regulatory subunit sperm flagella appears to be bent or even broken during capacitation [169,171]. A recent study in mice, revealed that *SPATA33* is important in localizing sperm calcineurin to the mitochondria and regulating sperm motility, being that *SPATA33* mutant spermatozoa presented inflexible MPs. [172]. This reveals that the lack of calcineurin-*SPATA33* interaction is an important cause of sperm immotility. We could not find studies evaluating the gene expression of *SPATA33* and *RSPH6A* in human sperm, thus the present work is the first to shown SPATA33 and RSPH6A gene expression in human sperm, and a decreased expression in ATZ patients. Our results support the established role of these genes in sperm motility by showing a reduction in gene expression in ATZ patients. The observation that the lowest expression was on 15-25% patients' group in mRNAs *CATSPER3, CFAP44, RSPH6A* and *SPATA33,* may suggest that these genes may belong to a cascade of genetic events related with sperm motility, but are not the master players in the process and a better knowledge of the interactions between these genes and sperm motility is needed to fully understand these results.

LRRC6 (leucine-rich repeat (LRR)-containing 6) is mainly expressed in testis and respiratory epithelial cells and is also described as a PCD-causing gene. A nonsense

mutation that caused the absence of *LRRC6* protein was reported to resulted in a defective flagellar ultrastructure, specifically in a defective axonemal dynein arm, leading to sperm immotility phenotype [173]. The present results for the *LRRC6* transcript showed a reduced expression in the ATZ groups, however this reduction was not statistically significant, suggesting that *LRRC6* is potentially specific of a PCD associated infertility and should not be used as general biomarker of ATZ.

The remain transcripts, *CRHR1, HIP1, IQCG, KRT34, QRICH2* and *TEKT2* have showed a reduced expression in patients with low sperm motility, with a more accentuated reduction in patients with RPM lower than 15% (<15%), which demonstrates a direct correlation between their expression and RPM rate in human spermatozoa.

Regarding the *CRHR1* (corticotropin releasing hormone receptor 1) and *KRT34* (keratin 34) gene, there is not any direct correlation between these genes and spermatozoa. However, those genes were observed to be differentially expressed in a PCD patient [174]. Therefore, we decide to explore the gene expression in ATZ patients and to analyze by immunofluorescence the location of those proteins in sperm cells from normospermic individuals.

CRHR1 encodes for a G-protein coupled receptor which binds to neuropeptides of the corticotropin-releasing hormone (CRH) family, leading to *CRHR1* activation [175]. *CRHR1* has been observed to be down regulated in ciliary cells of a PCD patient, suggesting that *CRHR1* could be involved in the ciliary beating [174]. Further, *CRHR1* was also shown have a role in female fertility. CRH increases the apoptosis of activated T lymphocytes, participating in the processes of both implantation – contributes to the implantation of the fertilized egg by inhibiting local maternal immune response [176,177] - and early pregnancy tolerance, being this specifically mediated through CRHR1 [178]. *KRT34* gene is a type I cuticular Ha4 keratin, representing a subtype of intermediate filaments (IFs), whose associated proteins are expressed primarily in epithelial cells. It is believed that keratins have other functions than structural support, however *KRT34* specific function remains unclear [179]. It has already been described the expression of *KRT34* ciliated cells from a PCD patient and suggested that an upregulation of *KRT34* gene and a reduction of its protein expression in ciliated cells may be associated with PCD [174]. Here, we report for the first time the association of *CRHR1* and *KRT34* with sperm immotility,

specifically by showing a reduced expression in patients with ATZ, corroborating the hypothesis that *CRHR1* and *KRT34* could be involved in ciliary/flagellar beating. To get further knowledge regarding these genes, we perform immunolocalization study by immunofluorescence. We observed expression of the proteins in the head region and sperm flagellum midpiece. Based on the specific localization in normal nasal cilia of both proteins, previously observed [174] and, being the KRT34 an IF subtype, we assume that the observed fluorescence in the head region mean that the proteins are localized in the perinuclear matrix and midpiece for CRHR1 and in the techa and slightly over the midpiece for KRT34. Further studies are needed to understand the exact role of these genes in motility process.

HIP1 (Huntingtin interacting protein) is known to be an endocytic adaptor protein and a component of clathrin-coated vesicles at the plasma membrane that binds to cytoplasmatic proteins, such as F-actin, tubulin, and huntingtin [180,181]. Since it binds to both actin and microtubules, *HIP1* might also play a role in cytoskeletal-based vesicular trafficking and/or act as a linking protein between actin and microtubule networks. A study in mice revealed that in the absence of *HIP1*, deformations on the heads, flagella and acrosomes are observed in post-meiotic spermatids, resulting in alterations in sperm motility, count, and fertility [182].

IQCG (Human IQ motif containing G) is a regulator of Ca^{2+} signaling which binds to a ubiquitous calcium-binding protein, CaM [183]. Studies in mice have shown that *IQCG* is essential for sperm flagellum formation, being proposed to be associated with manchette[1] in the developing spermatids, which is important for the correct formation of sperm heads, through calcium signaling. However, the molecular mechanisms underlying the sperm flagellum formation remain unclear [184,185]. Nevertheless, a malformed manchette can lead to abnormal spermatozoon head morphology, tail abnormalities and decapitation [186].

QRICH2 (Glutamine rich 2) exact function have not been characterized so far, however it is known that is localized in the sperm flagellum. A recent study proved that *QRICH2* is a functional molecule essential for sperm flagellar development by regulating the genes associated with the accessory structure of sperm flagella. Shen and co-workers showed that mutations of this gene result in MMAF and consequently

[1] Manchette is a perinuclear mantle of microtubules emanating from the perinuclear ring and linked to the nuclear membrane

cause male infertility [187]. Another study revealed other homozygous mutations in *QRICH2* in MMAF individuals, confirming that these mutations although are rare, seem to be recurrent in MMAF phenotype [188]. Furthermore, *QRICH2* mutations were also associated to low sperm concentration and immotile sperm [189]. So, however its exact role is not fully understood, it was already proved its correlation with MMAF and its importance for the correct formation of the accessory structures of the sperm flagellum.

Human *TETK2*, also known as Tektin-t, is a membrane protein localized in the PP of spermatozoa and play a crucial role in the formation and development of the cilia and flagella of spermatozoa [190]. Studies have already demonstrated that *TEKT2* deficient mice show defects in mobility and structural disruption of the dynein inner arms, once the absence of *TEKT2* in tektin profilaments may distort the A tubule structure of the microtubule, which can affect the construction of the IDAs. This also suggest that *TEKT2* might play a role in the assembly of inner arms to the microtubules. On the other hand, absence of *TEKT2* also affected the flagellar formation leading to an ineffective sperm movement and consequently to immotile sperm [191,192].

From the best of our knowledge, we firstly report a reduced gene expression of *HIP1, IQCG, QRICH2* and *TEKT2* in patients with low sperm motility. Our results further corroborate the previous hypothesis relating these genes with sperm motility, suggesting that they could be used as potential biomarkers of ATZ.

Relatively to the housekeeping genes, studies that have compared the expression of the most widely used housekeeping genes showed that individually, the expression levels of these genes differ dramatically between tissues. For that reason, the use of more than one control gene for each experiment has been suggested [193,194]. In this study, we have used *GAPDH* and *B2M* as reference genes. *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) is one of the most common housekeeping genes and its expression is related with the regulation of proliferation, activation status and differentiation. Some studies have revealed that this gene is localized in the cytoplasm, vesicles, mitochondria, and the nucleus [195,196] and it has already been described as one of the most stable reference gene in boar spermatozoa samples [197]. However, our results, demonstrated that *B2M*, is more stable to be used as reference gene in human spermatozoa samples than *GAPDH*, which showed a

high variability comparing with *B2M*. This might suggest that *GAPDH* is not an appropriated reference gene for human spermatozoa gene expression studies.

In what concerns microRNAs, their expression profile is considered as a key in gene expression regulation processes, being recognized as vital regulatory factors. As said before, this regulation results in the alteration of their target mRNAs translation, being that microRNAs are involved in many biological functions namely cellular proliferation, differentiation, and apoptosis. A single miRNA can have hundreds of target mRNAs and mRNA target can bind to one or different miRNAs. This way, miRNAs constitute a powerful regulatory network that controls several targets and physiological processes and diseases [198]. Thus, miRNAs can have specific expression patterns depending on its location. The observation that some miRNAs have alterations in their expression profiles in mature spermatozoa and SP in patients with spermatogenic problems highlights their potential as biomarkers for diagnosis and classification of male fertility [199]. These molecules represent a tiny function within the total RNA from samples, which can vary significantly across samples, so its normalization in RT-PCR analyses represents a massive challenge. Among the developed strategies to measure the expression levels of miRNAs across samples and identify a normalizer with a uniform and ubiquitous expression, the Mean-Centering Restricted (MCR) strategy stands out [200]. The *miR-100-5p* and *miR-30a-5p* have demonstrated positive results for uniformity and ubiquitous patterns across samples and display a high proximity rate to MCR [201], therefore were used as normalizers in the present work. We observed reduced variability among these normalizers, thus corroborating its value in miRNA expression studies of human sperm samples.

It was expected that a downregulation of the mRNAs meant an upregulation of the correspondent miRNAs. So, if all the studied mRNAs are downregulated, we were expecting to observe an upregulation of all miRNAs, meaning that the mRNAs were repressed by the expression of miRNAs. However, the present results show that all miRNAs are also downregulated in patients with ATZ, with a more accentuated reduction in patients from the <15% group, with the exception of *miR-3664-5p*. *MiR-3664-5p* corresponds to the *LRRC6* transcript, which results were not statistically significant.

Despite the stablished role of miRNA in translation repression, studies have emerged reporting that miRNAs can interact with the promoter regions and activate

gene expression, a mechanism known as RNA activation [202]. It was reported that *miR-10a* binds in the 5'-UTR and facilitates translational enhancement rather than repression, with the overexpression of *miR-10a* enhancing ribosomal protein (RP), specifically the RP Rps16, Rps6, and Rpl9, synthesis and ribosome biogenesis and the blockage of *miR-10a* results in reduced production of these RP proteins [203]. In addition, *miR-24-1* was found to be an unconventional mediator for transcriptional gene activation through chromatin remodeling at enhancer regions [204].

So, to justify the absence of an inverted correlation regarding the expression of the mRNA and the correspondent miRNA, two hypotheses could be proposed. First, it is possible that the miRNAs here studied could in sperm cells act under the mechanism of RNA activation and thus instead of repressing RNA translation, they may stimulate the RNA translation of the present mRNAs. Therefore, a lower expression of those miRNAs would mean negative feedback in the RNA translation and consequently a lower expression of theirs correspondent mRNAs, as were observed. The other hypothesis is related with the selection process of the miRNA. The studied miRNAs selection was made through database search based on highest score of the target miRNA for each mRNA, thus it is possible that the chosen miRNA might not be the specific target for the sperm cells mRNAs. Due to alternative splicing different mRNAs could be found in distinct tissues, thus it is possible that the same could occur with miRNAs, meaning that although this miRNA could be indeed regulators of the mRNA, but in other tissues. Besides, it is also possible that the selected mRNA had multiple target miRNA to regulate their expression, thus the effects of this upregulation were neutralized by the action of the other target miRNA. To validate this hypothesis, further studies should be conducted to analyze other miRNAs that are listed in the databases and also to evaluate this relation mRNA-miRNA in other tissues.

Conclusion

To conclude, our work has provided further insights regarding the role of RNA in sperm function, proving for the first-time evidence that associate the genes *CRHR1* and *KRT34* to sperm motility and report, also for the first time, a reduced expression of *HIP1, IQCG, QRICH2* and *TEKT2* in patients with low sperm motility, with a more accentuated reduction in patients with RPM lower than 15% (<15%). These results demonstrate a direct correlation between the expression of these genes and progressive motility rate in human spermatozoa.

We also firstly report that the miRNAs *miR-3664-5p (LRRC6), miR-2110 (RSPH6A), miR-4660 (TEKT2), miR-492 (CATSPER3), miR-4425 (CFAP44), miR-4731 (HIP1), miR-4514 (IQCG)* and *miR-518c-5p (SPATA33)* were downregulated in ATZ patients and suggesting that this miRNA could act under the mechanism of RNA activation.

Certainly, these data need to be further investigated. Further studies with a higher number of patients can further validate this data and studies using animal models are important for future research on the functions of these genes and how these genes and its corresponding miRNA act in sperm motility, particularly, regarding the genes *CRHR1* and *KRT34* of which information in sperm cells is scare.

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Attachments

Attachment 1

This attachment contains the primers and the PCR conditions that were used to sequence the following genes: *BSCL2, CATSPER2, CATSPER3, CCDC40, CFAP43, CFAP44, CRHR1, DRC1, HIP1, IQCG, KRT34, LRRC6, PLAG1, QRICH2, RSPH6A, SPATA33, TEKT2, TTC21A, USP11*and *ZMYND10*.

The standard primer design parameters were: primer length 18-25 bp; percentage of GC 40%-60%; amplification length 80-200 bp; Tm 58-62 C°. However, in some regions, some punctual modifications were made.

The PCR conditions applied were an initial denaturation at 95C° for 3 minutes, followed by thirty-eight cycles of 95C° for 45 seconds, specific Ta for each primer (tables below) for 30 seconds and 72C° for 1 minute with a final extension at 72C° for 10 minutes.
The standard PCR reaction mixture (20 µl) contained: 4 µl of PCR Master Mix (5xFIREPol, Tartu, Estonia), 13 µl of DEPC treated water; 1 µl of each primer (Eurofins Genomics, Ebersberg, Germany) at 10 pmol/µl each and 1 µl of cDNA at 40 pmol/µl.

Table A1. List of the primers used to amplify the exonic regions of each gene, including the PCR conditions used.

Primer	Transcript	Sequence	Lengh (BP)	Tm (ºC)	%GC	Fragment size	Expression	PCR condition
ZMYND10-F	NM_015896.4	TGGAGCGAGAAAACAGAGGC	20	60.32	55.00	209	Ciliated cells	60ºC
ZMYND10-R		ATTCTGGCATCGTGAGCAGC	20	61.09	55.00			
TEKT2-F	NM_014466.3	AACCATCGCTGCCCTGAAG	19	60.38	57.89	112	Ciliated cells	58ºC
TEKT2-R		CATGGAGTTGGCCTTGCAG	19	59.11	57.89			
SPATA33-F	NM_001271907.2	CTGATGTAAAGCAAAAGTCCAGC	23	58.52	43.48	95	PBMC	60ºC
SPATA33-R		GGAACTGCAACTGACTAGCG	20	58.93	55.00			
RSPH6A-F	NM_030785.4	CGTGAACGCCCGAAAGATC	19	59.29	57.89	176	Testis	60ºC
RSPH6A-R		CGCCCTCCTCCTCACTAAAC	20	59.82	60.00			
CATSPER3-F	NM_178019.3	TCTCAACACCGTCACCAGC	19	59.93	57.89	188	Testis	60ºC
CATSPER3-R		GCATTCGATGTGACAGTGC	19	57.41	52.63			
CATSPER2-F	NM_172095.4	CCCTCAAGAGCATGACCTTCC	21	60.41	57.14	87	PBMC	58/60ºC
CATSPER2-R		ACTCTGAGAAGACGTAGACACC	22	58.92	50.00			
BSCL2-F	NM_001122955.4	TCCCTGTTGCCAATGTCTCG	20	60.32	55.00	179	Ciliated cells	60ºC
BSCL2-R		AGAAGTGGAGATGATTCGGCC	21	59.86	52.38			
IQCG-F	NM_032263.5	ATCCCGGAAACTCTAGAGCC	22	60.29	50.00	100	Ciliated cells	59ºC
IQCG-R		TGATTGTTGAGGCCATTGGAAG	21	59.59	52.38			
CFAP43-F	NM_025145.7	AAACGCCGACCAAGGATTTC	20	59.12	50.00	81	Ciliated cells	60ºC
CFAP43-R		AGATCCTGGTAGTTCTCCGAAAG	23	59.30	47.83			
PLAG1-F	NM_002655.3	CAACAAGACTGCACCAAGGC	20	59.97	55.00	193	PBMC	60ºC
PLAG1-R		AGTTCTTGCCACATTCTTCGC	21	59.46	47.62			
CFAP44-F	NM_001164496.2	GCCTTGGACTGCTCTGTTCG	20	61.29	60.00	122	Ciliated cells	58ºC
CFAP44-R		GCTCCAGTGAAGTTTACCATTCG	23	59.87	47.83			

Table A1 (continuation). List of the primers used to amplify the exonic regions of each gene, including the PCR conditions used.

Primer	Transcript	Sequence	Lengh (BP)	Tm (ºC)	%GC	Fragment size	Expression	PCR condition
QRICH2-F	NM_001388453.1	CCATCAGGTCAGCACGCT	18	59.73	61.11	195	Testis	60ºC
QRICH2-R		TGCTGGTGGTGATGTTGAGC	20	60.89	55.00			
TTC21A-F	NM_001366900.1	CATGAAACTGGACAAGGATGGC	22	59.83	50.00	246	Ciliated cells	60ºC
TTC21A-R		CCTTGCATGCTGGAGAAGTGAAG	23	61.97	52.17			
KRT34-F	NM_021013	GCTGACGGAGAGCGAGGCCCAC	22	65	72	148	Ciliated cells	62ºC
KRT34-R		CCGGGCACGCACGTCCAGCA	20	65	75			
CRHR1-F	NM_001145146	AGCACGCATGTCCCTCCAAGGCTGT	25	65	60	129	Ciliated cells	62ºC
CRHR1-R		TCACGAGTTGCCCATGATGCCCCA	24	65	58			
LRRC6-F	NM_012472.6	GCCATGGGCTGGATCAC	17	57.68	64	95	Ciliated cells	60ºC
LRRC6-R		TGCTGATGCAACGAGAGTTC	20	58.57	50			
HIP1-F	NM_005338.7	CATGAGAAAGGGGCACAGAC	21	58.35	47.62	94	PBMC	59ºC
HIP1-R		TTCAGGTAGATGCTGCACAG	19	58.43	57.89			
CCDC40-F	NM_017950.4	TTAGGCCCGTCGGAGCAAATGG	22	59.22	59	182	Ciliated cells	60ºC
CCDC40-R		ACTGGCTCCTGCGAGACGAACT	22	59.92	59			
DRC1-F	NM_145038.5	GAGCCTTTGATGTGGACAGG	20	58.54	55	126	Ciliated cells	60ºC
DRC1-R		TCTGTGTGGCGGACTTCTG	19	59.63	57			
USP11-F	NM_004651.3	CCGCAAGCCAGAGCAGCACC	20	66	70	275	Ciliated cells	60ºC
USP11-R		TGCGCTCCCGCAGGTAGACA	20	66	65			

Attachment 2

This attachment contains the list of microRNAs that are predicted to interact with the studied genes (table A2), the primers and the RT-PCR conditions that were used.

The RT-PCR was performed with SYBR green, with the enzyme NZY qPCR Green (NZYZTech), using the respective primers and PCR conditions (table A3) and was performed in a Bio-Rad CFX96 (Bio-Rad, Hercules, USA) and amplifications were prepared according to the manufacturer instructions.

Table A2. List of the mRNA highest score corresponding target microRNA

mRNA	Highest score corresponding target microRNA
QRICH2	-
LRRC6	*miR-3664-5p*
RSPH6A	*miR-2110*
KRT34	-
CRHR1	-
TEKT2	*miR-4660*
CATSPER3	*miR-492*
CFAP44	*miR-4425*
HIP1	*miR-4731*
IQCG	*miR-4514*
SPATA33	*miR-518c-5p*

Table A3. List of the primers used for the selected microRNAs

Primer	Sequence	Lengh (BP)
miR-4660-F	GCAGCTCTGGTGGAAAATG	19
miR-4660-R	GGTCCAGTTTTTTTTTTTTTTTCTC	25
miR-518c-5p-F	TCTCTGGAGGGAAGCAC	17
miR-518c-5p-R	GTCCAGTTTTTTTTTTTTTTTCAGAAAG	28
miR-2110-F	GGAAACGGCCGCTGA	15
miR-2110-R	GGTCCAGTTTTTTTTTTTTTTTCACT	26
miR-492-F	GACCTGCGGGACAAG	15
miR-492-R	GGTCCAGTTTTTTTTTTTTTTTAAGAATC	29
miR-4514-F	GCAGACAGGCAGGATTG	17
miR-4514-R	GGTCCAGTTTTTTTTTTTTTTTCCC	25
miR-4425-F	CAGTGTTGGGATTCAGCAG	19
miR-4425-R	GGTCCAGTTTTTTTTTTTTTTTATGGT	27
miR-572-F	CCGCTCGGCGGT	12
miR-572-R	AGTTTTTTTTTTTTTTTGGGCCA	23
miR-3664-5p-F	GCAGAACTCTGTCTTCACTCA	21
miR-3664-5p-R	CAGGTCCAGTTTTTTTTTTTTTTTACT	27
miR-4731-5p-F	TGGGGGCCACATGAG	15
miR-4731-5p-R	GTCCAGTTTTTTTTTTTTTTTCACAC	26