UNIVERSIDADE D COIMBRA

Artur Manuel Freitas Martins

IS THE PRESENCE OF DEPRESSIVE AND ANXIETY SYMPTOMS ASSOCIATED WITH SPERM QUALITY?

Dissertation submitted in fulfilment of the requirements for the Master's Degree in Biochemistry, supervised by Doctor Sandra Catarina Gomes Amaral and Professor João Ramalho-Santos, presented at Department of Life Sciences from the Faculty of Science and Technology of the University of Coimbra.

February 2023

Department of Life Sciences in University of Coimbra

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Abstract

The concerning trend on male infertility global prevalence, together with the unexplainable causes in half of those cases highlights that there are still aspects of this disease to be understood. To address this issue, one should not only be aware of the limitations of the implemented diagnostic tools, but also understand the sperm cell in depth, structurally, biochemically, and molecularly in order to develop reliable and ready-to-be new/improved diagnostic tools, as well as taking into account external and internal factors that might exert direct and indirect effects upon the male reproductive system.

In this sense, evaluating patients' psychological status undergoing fertility treatment seems to be an important aspect to consider. In fact, even though, evidence clearly implies a tight connection and crosstalk between neuroendocrine signalling and the male reproductive system in response to psychological stress, the clinical relevance, the extent and severity of the effects that this might exert over sperm quality and fertility have yet to be elucidated. Secondly, the sperm cells metabolism, highly related to its functionality, is also an aspect that deserves further attention. Though there is much information on the human sperm metabolism, there is still a lack of a quick integrated and comprehensive analysis that may be introduced with the potential to reveal innovative clinically relevant information.

In this work, it was therefore our objective to explore and study the relationship that might exist between psychological well-being and fertility-state as well as study deeper the sperm metabolism, aiming to find metabolic details that might be used to improve male infertility diagnosis and treatments. To achieve our aims, we have first performed a retrospective analysis of a previously established data basis with 3 well-defined groups of subjects (Control, ID, and UMI), in which information regarding anxiety and depression symptoms, obtained by the "Hospital Anxiety and Depression Scale" (HADS) questionnaire was available. Secondly, we have optimized the Seahorse Metabolic Flux Analyzer (SFA) methodology to human sperm cells.

Although we have found no evident relationship among psychological distress and men's fertility state, we have found that in patients presenting psychologic stress Motility *in Situ* and Acrosome integrity seems to be affected. Additionally, we have obtained standard records on human sperm cells' OCR and ECAR, that together with the

metabolic metrics, provided information on sperm cells' oxidative and glycolytic metabolism.

Overall, our achievements reinforce the need to improve the male infertility diagnostic assessment, identifying new aspects to be considered.

Keywords: Idiopathic male infertility, unexplained male infertility, psychologic stress, depression, anxiety, sperm metabolism, Seahorse Flux Analyzer, OCR, ECAR

Resumo

A preocupante tendência crescente da prevalência global da infertilidade masculina, em conjunto com as causas inexplicadas em metade desses casos, evidencia que há ainda aspetos desta doença por compreender. Para resolver este problema, há que ter em conta não só as limitações das ferramentas de diagnóstico já implementadas, mas também entender melhor e mais detalhadamente o gâmeta masculino estrutural, bioquímica e molecularmente, de forma a desenvolver ferramentas de diagnóstico mais fidedignas. Por fim, devem também ser tidos em consideração outros factores externos e internos que poderão ter efeitos diretos e indiretos sobre o aparelho reprodutor masculino.

Neste sentido, avaliar o estado psicológico dos pacientes que estão a fazer tratamentos de fertilidade parece ser um aspeto importante a considerar. De facto, apesar de haver evidências da relação estreita e interligação entre as vias de sinalização neuroendócrinas e o sistema reprodutor masculino em resposta ao stress psicológico, a relevância clínica, extensão e severidade destes efeitos sobre a qualidade espermática e fertilidade mantêm-se por elucidar. Em segundo lugar, o metabolismo dos espermatozóides, intimamente relacionado ao funcionamento destas células, é também um aspeto que merece mais atenção. De facto, apesar de existir muita informação sobre o metabolismo de espermatozóides humanos, existe ainda a falta de uma análise rápida, integrada e completa que possa ser introduzida com o potencial de revelar aspetos clinicamente relevantes e inovadores.

Neste trabalho, o nosso objetivo foi assim explorar a relação entre bem-estar psicológico e estado de fertilidade, bem como aprofundar o estudo do metabolismo espermático no sentido de encontrar detalhes metabólicos que possam ser usados para melhoria do diagnóstico e tratamento da infertilidade masculina. Para alcançar os nossos objetivos, primeiramente realizámos uma análise retrospectiva numa base de dados anteriormente estabelecida com 3 grupos de participantes bem definidos (Control, ID e UMI), na qual estava contida informação relativa a sintomatologia de ansiedade e depressão, obtida pelo questionário "Escala Hospitalar de Ansiedade e Depressão" (HADS). Por fim, otimizámos a metodologia do Seahorse Flux Analyzer (SFA) para espermatozóides humanos.

Apesar de não termos encontrado uma relação evidente entre o stress psicológico e o estado de fertilidade masculina, detetámos que em participantes que apresentavam stress psicológico, a Mobilidade *in Situ* e a integridade do Acrossoma parecem estar afetadas. Adicionalmente, obtivemos registos standard de OCR e ECAR para espermatozóides humanos, que juntamente com as métricas metabólicas, forneceram informação relativa aos perfis metabólicos glicolítico e oxidativo dos espermatozóides.

No geral, os nossos resultados reforçam a necessidade de melhorar a avaliação e diagnóstico da infertilidade masculina, identificando novos aspetos a serem considerados.

Palavras-chave: Infertilidade masculina idiopática, infertilidade masculina inexplicada, stress psicológico, depressão, ansiedade, metabolismo espermático, Seahorse Flux Analyzer, OCR, ECAR

Abbreviations

2DG	2-Deoxyglucose
A	Asthenozoospermia
Acrosome (NI)	Non-intact acrosome
ANOVA	Analysis of Variance
AR	Acrosome Reaction
ATP	Adenosine Triphosphate
ATPT	ATP Turnover
BO	Basal OCR
BSA	Bovine Serum Albumins
BTB	Blood-Testicular Barrier
C	Concentration
cAMP	cyclic Adenosine Monophosphate
Capacitation (NM)	Non-marked cells
CCM	XFe24 Cell Culture Microplate
CE	Coupling Efficiency
Chromatin (Dark)	Dark stained cells
CHUC	Centro Hospitalar Universitário de Coimbra
ConA	Concanavalin A
DGS	Direcção Geral de Saúde
DNA	Desoxyribonucleic Acid
ECAR	Extracellular Acidification Rate

FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FSH	Follicular Stimulating Hormone
G	Glycolysis
GnRH	Gonadotrophin Releasing Hormone
GC	Glycolysis Capacity
GR	Glycolysis Reserve
GRC	Glycolysis Reserve Capacity
HADS	Hospital Anxiety and Depression Scale
HIV	Human Immunodeficiency Viruses
HPA	Hypothalamic-Pituitary-Adrenal axis
HPG	Hypothalamic-Pituitary-Gonadal axis
ID	Idiopathic male infertility
IRB	Internal Review Board
L	Live cells
LH	Luteinizing Hormone
MANOVA	Multivariate analysis of variance
MiS	Motility *in Situ*
MR	Maximal Respiration
NGA	Non-Glycolytic Acidification
NM	Normal Morphology
NMO	Non-mitochondrial respiration
O	Oligozoospermia
OAT	Oligoasthenoteratozoospermia
OCR	Oxygen Consumption Rate

OS	Oxidative Stress
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate-Buffered Saline solution
PKA	Protein Kinase A
PL	Proton Leak
PM	Progressive Motility
PTK	Protein Tyrosine Kinase
PTM	Post-Translational Modifications
PTP	Phosphotyrosine Phosphatase
PVN	Paraventricular Nucleus
ROS	Reactive Oxygen Species
SC	Spare Capacity
SCFC	Spare capacity
SD	Standard Deviation
SFA	Seahorse Flux Analyzer
SPM	Sperm Preparation Medium
T	Teratozoospermia
TM	Total Motility
TPs	Transition Proteins
UMI	Unexplained Male Infertility
WHO	World Health Organization

Introduction

1. Preface

According to the World Health Organization (WHO), infertility is a disease defined as the inability to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual activity. The most recent estimates point to a global incidence of 8% - 12% among couples in reproductive age, (Ashok Agarwal et al., 2021; Inhorn & Patrizio, 2015; Starc, 2019; Vander Borght & Wyns, 2018), numbers that in Portugal reach 15-20%, according to the Portuguese Fertility Association.

Dramatically, in the last century, a growing body of evidence supports a tendency of increasing rates of infertility throughout the globe (Ashok Agarwal et al., 2015; Carlsen et al., 1992; Inhorn & Patrizio, 2015; Mehra et al., 2018; Mishra et al., 2018; Swan et al., 2000), making of infertility an issue of global and social proportions. Additionally, although not yet well-established, psychological impact in the (in)fertility status is also predicted to be significant (Nargund, 2015; Sánchez González et al., 2023; Zorn et al., 2008).

Despite being a couples' problem, it is acknowledged that the male factor alone can contribute up to 20% of cases and although several causes for male infertility have already been identified, in nearly half of the cases the identification of a cause is not possible. This highlights the need to develop novel tools to evaluate the sperm function beyond the routine analysis and to further develop new treatment strategies for infertile patients.

2. Introduction

2.1. Sperm cell: the central player

Sperm cells are the male gametes, whose primary function is to reach and fertilize the oocyte, the female gamete. Sperm cells are haploid, carrying half of the parental genetic information to the new zygote and future offspring (Niederberger, 2017).

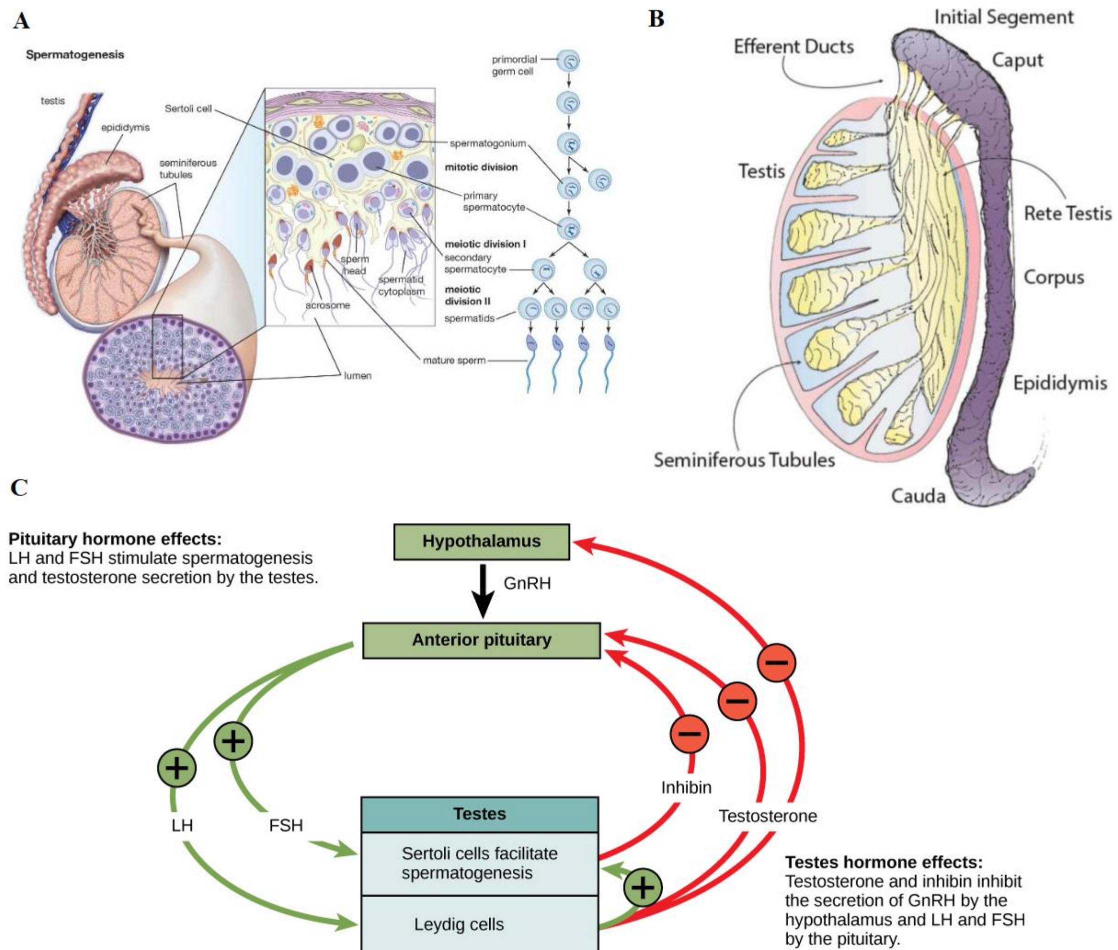

Figure 1 – A: Schematic representation of the testis, its cellular components and the spermatogenesis process (From: https://www.britannica.com/science/spermatogenesis/images-videos#/media/1/559418/143155). **B:** Schematic representation of testis and epididymis morphology (From: James et al., 2020). **C:** Schematic representation of spermatogenesis hormonal regulation (From: https://opentextbc.ca/biology/wp-content/uploads/sites/96/2015/03/Figure_43_04_01.jpg).

2.1.1. Development and maturation

Sperm cells are produced in the testes by a process known as spermatogenesis, which in male humans takes approximately 74 days (Amann, 2008). This process begins and takes place in the seminiferous tubules, the functional unit of the testes, whose epithelium is composed by germ and Sertoli cells (Holstein et al., 2003; **Figure 1A**). Ultimately, germ cells will evolve into sperm cells, by direct mediation of Sertoli cells activity and other important cells located outside the tubules, as is the case of the Leydig cells, in a highly hormonally-regulated process involving hormones such as

gonadotrophin releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinising hormone (LH), testosterone and inhibin (**Figure 1C**), altogether forming the Hypothalamus-Pituitary-Gonadal (HPG) axis. After suffering a deep morphologic change in the late stage of spermatogenesis, immature sperm cells phenotypically elongated and almost devoid of cytoplasm (Neto et al., 2021) will be released into the lumen of seminiferous tubules and further conducted to the epididymis (Simoni & Huhtaniemi, 2017; **Figure 1A and Figure 1B**).

The epididymis is a tubular organ that connects the testis to the *vas deferens* composed of three segments: the caput, corpus, and cauda (James et al., 2020; **Figure 1B**). In this organ, the sperm will suffer a maturational process through which the cells will acquire progressive motility and fertilization capability, as a result of several structural, biochemical and molecular alterations (Bonomi et al., 2017; James et al., 2020).

In fact, sperm maturation occurs in a stage-like process by the interaction between sperm cells and the unique characteristics of epididymis' luminal environment in its different segments (Bonomi et al., 2017; James et al., 2020).

Epididymal epithelium is covered with cells that have high endocytic, secretory, and metabolic activity, which are mostly regulated by the presence of androgens, like testosterone (Cornwall, 2008). In turn, androgens mediate most of the protein synthesis that occurs within the epididymis (Brooks, 1983). Cargo- and protein-coated vesicles, segment-specific, are secreted and released into the lumen exposing sperm cells to different environments (Sullivan & Saez, 2013). As sperm cells transit through each section of the epididymis, they will undergo several steps of further nuclear compaction, alterations in plasma membrane composition, cytoskeleton structural changes, and protein and RNA payloads. As a result, by the time sperm cells reach the cauda region, most cells have already acquired progressive motility (Bonomi et al., 2017; Cornwall, 2008; Gervasi & Visconti, 2017; Sullivan & Mieusset, 2016).

Finally, for survival and motility purposes a liquid environment is needed for sperm cells to reach the oocyte. The seminal plasma completely meet that end, as a combination of secretions from the prostate, epididymis, and seminal vesicle, that provide nutrients and facilitate their mechanical movement (Druart & de Graaf, 2018; James et al., 2020). The sperm cells along with the seminal fluid compose the semen, which will then be ejaculated.

In the female reproductive tract, sperm cells will only become competent after undergoing capacitation, a determinant process that will further enable fertilization (Hirohashi & Yanagimachi, 2018).

2.1.2. Sperm structure

Spermatozoa presents a very distinctive structure when compared to any other human cells (Neto et al., 2021). It is an elongated cell comprising three main parts: the head, the midpiece, and the tail (Neto et al., 2021; **Figure 2**). Altogether, these components will enable spermatozoa to be motile and reach the oocyte, completing its primary function of fertilization (Neto et al., 2021; Touré et al., 2021).

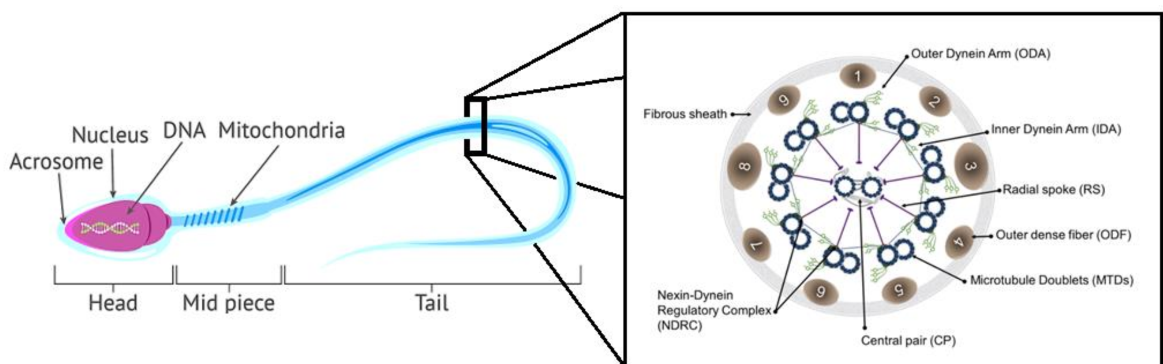

Figure 2 - Schematic representation of human sperm cell structure (left) and of mammalian sperm axoneme structure (right) (Adapted from: https://www.invitra.com/en/wp-content/uploads/2015/03/Structure-of-a-mature-human-sperm-cell.png and Touré et al., 2021, respectively).

The head contains the acrosome and the nucleus. The latter, placed at the centre of the head, is where the deeply packed genetic information (DNA) is, enabled by the presence of special proteins called protamines (Steger & Balhorn, 2018). On the other hand, the acrosome, at the very tip of the head, is a vesicle filled with enzymes, whose content is released in a process called acrosome reaction, without which the fertilization will not occur (Neto et al., 2021).

The midpiece is the structure immediately posterior to the head, mostly characterized by densely packed mitochondria that vary in number according to species

(Ramalho-Santos et al., 2009). Mitochondria, among their many functions, have a major role as producers of energy for sperm cells (Ramalho-Santos et al., 2009).

At last, the tail is the structure responsible for sperm cells' mechanical movement and is mainly composed by the axoneme. The axoneme is a specialized circular structure enwrapped by a fibrous sheath arranged in a 9+2 microtubular conformation of tubulin and dynein arms (Lehti & Sironen, 2017; Touré et al., 2021; **Figure 2**).

2.1.3. Sperm functionality

Many features have been described as crucial for sperm function and quality, such as DNA integrity status, membrane status, mitochondrial function (Ashok Agarwal et al., 2015; A. Amaral et al., 2013; Esteves et al., 2011), redox state (Baker & Aitken, 2004), capacitation potential, acrosome integrity or the ability to interact with female counterpart (Ashok Agarwal et al., 2015; A. Amaral et al., 2013; Esteves et al., 2011).

2.1.3.1. Sperm DNA

During spermatogenesis, chromatin undergoes a complex remodelling process of packing and rearrangement, in order to obtain a rigid and densely compacted sperm nucleus (Hao et al., 2019; Steger & Balhorn, 2018; Wang et al., 2019; **Figure 3**), a process that begins in the testis and is finished at the cauda of the epididymis (Simoni & Huhtaniemi, 2017). This process enables the sperm cells to become more aerodynamic, as well as to protect the DNA integrity (Steger & Balhorn, 2018), being the latter especially important to assure that half of the information to be transmitted to the next generation will not be compromised nor have detrimental consequences to the offspring (Aktan et al., 2013; Gong et al., 2012; Simoni & Huhtaniemi, 2017).

This rearrangement is only possible due to the replacement of histone proteins by protamines, but also the number of disulfide bridges that are formed between DNA and proteins (Hao et al., 2019; Steger & Balhorn, 2018; T. Wang et al., 2019) . This is a multi-step process, occurring mostly during spermiogenesis (late phase of

spermatogenesis) and dependent on the environment that the sperm cells are subjected to (Hao et al., 2019; Steger & Balhorn, 2018; T. Wang et al., 2019).

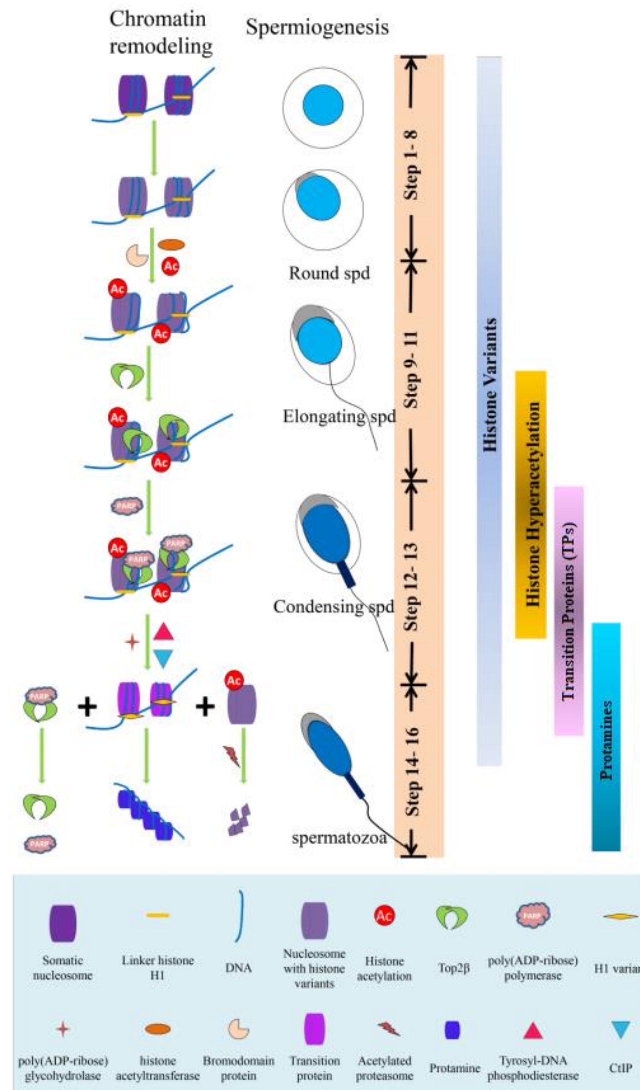

Figure 3 - Schematic representation of Histone-TP-Protamine replacement process in human sperm (From Hao et al., 2019)

Thus, the highest the efficiency on each step, the highest is the likelihood of protecting the chromatin integrity, and also the highest will be the compaction of the nucleus (Hao et al., 2019; Simoni & Huhtaniemi, 2017; Steger & Balhorn, 2018).

2.1.3.2. Membrane integrity

As mentioned, sperm cells are very different cells when compared to somatic cells, and their membranes are not an exception. Once sperm cells leave the testes, proteins and lipids, whose synthesis has ceased, are organized into a lateral polarized distribution in the sperm head surface (Flesch & Gadella, 2000; B T Storey, 1997). This lateral polarized distribution is of special importance for female-male gametic interaction (Flesch & Gadella, 2000; B T Storey, 1997).

Biochemically, the sperm membrane is characterized for presenting an unusually high content of polyunsaturated fatty acids, conferring it more fluidity but also high susceptibility to lipid peroxidation (Ashok Agarwal et al., 2018; S. Amaral et al., 2013; Barati et al., 2020; Bisht et al., 2017; Cocuzza et al., 2007; Flesch & Gadella, 2000; B T Storey, 1997).

Lipid peroxidation occurs mainly due to oxidative stress (OS), eventually leading to loss of sperm membrane integrity and quality (Ashok Agarwal et al., 2018; Barati et al., 2020; Bisht et al., 2017; Cocuzza et al., 2007; Flesch & Gadella, 2000; Hamada et al., 2011; B T Storey, 1997). Membrane damage will inevitably lead to reduced motility and fertilization rates (Hamada et al., 2011), stressing the paramount importance of membrane integrity maintenance for sperm cells viability and function (Flesch & Gadella, 2000; Hamada et al., 2011; B T Storey, 1997).

2.1.3.3. Capacitation and acrosome reaction

Capacitation is an essential process by which sperm cells become competent for fertilization and only occurs once the sperm cells are in the female reproductive tract (Puga Molina et al., 2018). In this particular environment, proteins like albumin change the sperm cells' membrane cholesterol content, altering its fluidity (Bailey, 2010; Puga Molina et al., 2018; Redgrove et al., 2012). Potassium (K^+) channels, sensitive to membrane fluidity changes, open extruding K^+ and leading to a hyperpolarization of the sperm's membrane (Puga Molina et al., 2018; Redgrove et al., 2012). Following these events, bicarbonate (HCO_3^-) and Calcium (Ca^{2+}) channels will open. After entrance, these

ions will both function as cofactors to activate adenyl cyclase enzyme to produce cyclic Adenosine Mono Phosphate (cAMP). Growing concentration of cAMP will then activate Protein Kinase A (PKA), which in turn will have two functions: activate Protein Tyrosine Kinase (PTK) and inhibit Phosphotyrosine Phosphatase (PTP), resulting in the phosphorylation of tyrosine residues throughout the cell, inducing capacitation (Bailey, 2010; Puga Molina et al., 2018; Redgrove et al., 2012; **Figure 4**).

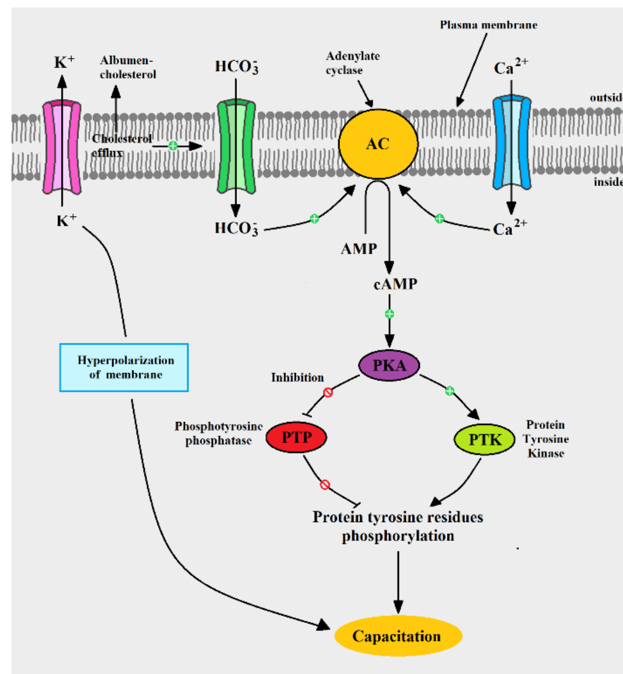

Figure 4 - Schematic representation of the main events and cellular pathways occurring during sperm capacitation (Adapted from: Visconti y Kopf, 1998).

As a result of this process, the sperm cells will suffer a shift in the motility pattern, passing from simple progressive movement to hyperactivation, and the attainment of competence (Hidalgo et al., 2020; Puga Molina et al., 2018). Once competent, sperm cells are enabled to accomplish their secretory role known as acrosome reaction (AR; Bailey, 2010; Hidalgo et al., 2020; Hirohashi & Yanagimachi, 2018; Puga Molina et al., 2018).

As mentioned above, the acrosome is a vesicle located at the tip of the sperms' head, which contains several hydrolytic enzymes essential for fertilization to occur (Mack et al., 1983; Moreno et al., 2000). Amongst this cocktail of enzymes is hyaluronidase, responsible for the decomposition of the *corona radiata* around the oocyte, or acrosin,

responsible for the dissolution of the *zona pellucida* also around the oocyte, among many others (Mack et al., 1983; **Figure 5**). The acrosome will undergo exocytosis once the sperm cell reaches a specific layer of the oocyte's *zona pellucida*, taking place the AR (Hirohashi & Yanagimachi, 2018; Mack et al., 1983; Puga Molina et al., 2018).

This exocytosis of several digestive enzymes will further ease the sperm cell to pierce through the outer layers that cover the oocyte in order to reach its membrane and fertilize the oocyte (Hirohashi & Yanagimachi, 2018; **Figure 5**).

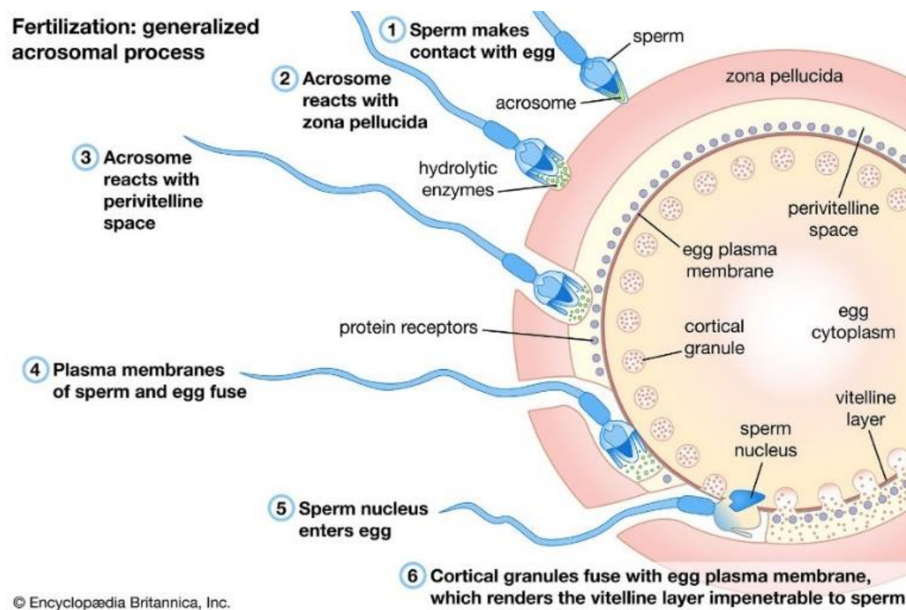

Figure 5 - Schematic representation of the acrosome reaction and fertilization process (From: https://www.britannica.com/science/fertilization-reproduction/Events-of-fertilization#/media/1/205305/229000).

2.1.3.4. Sperm mitochondria: the good and the bad

Mitochondria have a very precise localization on the sperm cells, occupying the middle region (**Figure 6A** and **Figure 6B**), and play a key role in several processes, such as metabolic and cellular homeostasis, apoptosis, Ca^{2+} homeostasis and redox state, just to mention a few. Yet, their metabolic role is the most known, as these organelles are the major producers of Adenosine Triphosphate (ATP; S. Amaral et al., 2013; Ramalho-

Santos & Amaral, 2013; Nussdorfer et al., 2018), through the oxidative phosphorylation (OXPHOS; A. Amaral, Lourenço, et al., 2013).

The latter is a process of Krebs cycle-derived molecules' breakdown that involves electron transport and transference, mediated by a series of carriers and protein complexes, until a final acceptor, the molecular oxygen (Gahan, 2005; Martínez-Reyes & Chandel, 2020; Nolfi-Donegan et al., 2020; Zhao et al., 2019; **Figure 6C**).

All these transferences are associated with the pumping of protons from the mitochondria's matrix into the intermembrane space (Nolfi-Donegan et al., 2020; Zhao et al., 2019) , having as a final result the creation of a proton and electric gradients across the inner membrane, known as electrochemical gradient. This gradient will then be used to drive ATP synthetase (or complex V) to produce ATP molecules, as protons are pumped back into the mitochondria's matrix (Gahan, 2005; Nolfi-Donegan et al., 2020; Zhao et al., 2019).

Regardless of the metabolic role of the cells' powerhouse, mitochondria seem to play a major role in sperm function overall (A. Amaral, Lourenço, et al., 2013; Ramalho-Santos et al., 2009). Mitochondria structural and functional integrity, as well as abundance in the midpiece, have been strongly associated with sperm quality and functionality (Durairajanayagam et al., 2021; Mundy et al., 1995; Pelliccione et al., 2011; Ruiz-Pesini et al., 2000). In fact, several were the studies that have methodologically correlated compromised mitochondrial ultrastructure and/or function with altered sperm motility, viability, capacitation, and fertilization ability (Aitken et al., 2012; A. Amaral, Lourenço, et al., 2013; Gallon et al., 2006; Marchetti et al., 2002; Nakada et al., 2006; Ramalho-Santos et al., 2009; Ramalho-Santos & Amaral, 2013; Ruiz-Pesini et al., 1998, 2000; Shivaji et al., 2009; A. P. Sousa et al., 2011; St John et al., 2005; Stendardi et al., 2011), ultimately leading to male infertility (Durairajanayagam et al., 2021; St John et al., 2005).

On the other hand, mitochondria are both the major producers (and targets) of ROS, that despite being important for several physiological processes, such as capacitation, acrosome reaction and fertilization (Ashok Agarwal et al., 2018; Baker & Aitken, 2004), will also have detrimental effects when in excess (Aktan et al., 2013; Aydemir et al., 2007; Barati et al., 2020; Cocuzza et al., 2007; Escada-Rebelo et al., 2020; Kowalczyk, 2022).

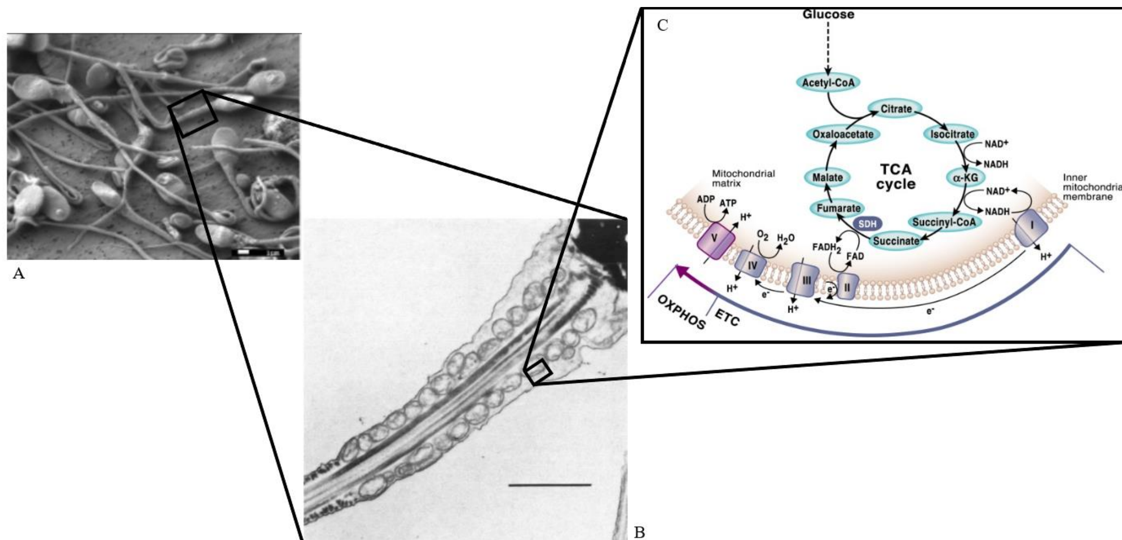

Figure 6 - Sperm mitochondria. **A:** Scanning electron micrograph of human spermatozoa using a Field Emission Scanning Electron Microscope 7500F (Bar = 1µm; From: Nussdorfer et al., 2018). **B:** Transmission electron micrograph of a longitudinal section of a human sperm cell midpiece with closely packed mitochondria, 18-21x10^3 magnification (Bar = 1µm; From: Mundy et al., 1995). **C:** Schematic representation of the TCA (Tricarboxilic acid or Krebs) cycle and OXPHOS at the mitochondria (From: Martínez-Reyes & Chandel, 2020)

2.1.3.5. Sperm metabolism

During their lifespan, sperm cells are not subjected to a homogenous environment and constant supply of the same substrates, experiencing major changes in oxygen availability and exogenous substrates availability and abundance, from the onset of spermatogenesis until fertilization (A. Amaral, 2022; Foutouhi & Meyers, 2022; Ruiz-Pesini et al., 2007; Bayard T. Storey, 2008; Tourmente et al., 2015), a situation that will inevitably be mirrored in metabolic terms. As a result, when ejaculated, sperm cells must be equipped with the necessary machinery to survive on its own and maximize the likelihood of reaching and successfully fertilize the oocyte (A. Amaral, 2022; Foutouhi & Meyers, 2022; Ruiz-Pesini et al., 2007; Bayard T. Storey, 2008; Tourmente et al., 2015).

Is not therefore surprising, that the most recent evidence in this regard support the existence of a metabolic versatility in the sperm cell. In fact, and despite the debate on which is the preferred pathway to energy attainment, namely OXPHOS and glycolysis, greatly fomented by the compartmentalization of these processes to specific regions of

the sperm cell, the midpiece and the tail, respectively (A. Amaral et al., 2014; Sandra Amaral et al., 2013; Dias et al., 2014a; Ramalho-Santos & Amaral, 2013; Ruiz-Pesini et al., 2007; Tourmente et al., 2015), is now recognized that the sperm cells can obtain energy from a variety of pathways, depending mainly on the available substrates, determined by the circumstances and surrounding environment, namely the one in the female reproductive tract (A. Amaral et al., 2013, 2014; Ramalho-Santos et al., 2009). For instance, it has been shown that sperm cells have all the machinery needed to perform fatty acids β-oxidation, as well as ketone bodies and glycerol catabolism (Moreno et al., 2000; Ramalho-Santos et al., 2009; A. Amaral, 2022).

Adding to this complex issue is the fact that the preferred metabolic pathway has been described as species specific, being set *in vivo* by the natural conditions and substrate availability of the correspondent female oviduct (A. Amaral, 2022; Bajpai et al., 1998; Foutouhi & Meyers, 2022; Peña et al., 2022; Ramalho-Santos et al., 2009; Bayard T. Storey, 2008; Tourmente et al., 2015).

Nonetheless, it seems consistent that alterations in the sperm metabolism will certainly have important consequences for the sperm cell, namely in terms of motility and functionality, further mirrored in the ability to fertilize the oocyte (A. Amaral, 2022; Dias et al., 2014a; Ruiz-Pesini et al., 2007; Bayard T. Storey, 2008; Tourmente et al., 2015; Wada & Nakatsuka, 2016). Given all the complexity, debate, and importance, it seems essential to gain a deeper understanding of sperm cells bioenergetics.

2.2. Male infertility: prevalence and aetiology

As mentioned above, reproductive health has been reported to be declining around the globe (Ashok Agarwal et al., 2015; Carlsen et al., 1992; Inhorn & Patrizio, 2015; Mehra et al., 2018; Mishra et al., 2018; Swan et al., 2000).

Although alone male infertility accounts for 20% to 30% of all cases of infertility, when associated with the female factor it reaches an impact on 50% of the cases (Ashok Agarwal et al., 2015; Imamovic Kumalic & Pinter, 2014; Vander Borght & Wyns, 2018).

Concerning solely the male factor, several studies have corroborated the thesis of a global declining trend of sperm count (Carlsen et al., 1992; Inhorn & Patrizio, 2019; Levine et al., 2017; Mehra et al., 2018; Mishra et al., 2018; Swan et al., 2000). The latest estimate, points to a reduction of approximately 52.4% in sperm count and 59.3% in total sperm count for the period between 1973 and 2011 (Levine et al., 2017).

Furthermore, male infertility has been suggested to be a window for a man's health, being the implications of this scenario even worse at a global scale (Choy & Eisenberg, 2018; Eisenberg et al., 2014, 2016; Jensen et al., 2009; Skakkebaek et al., 2016).

The causes behind male infertility have been extensively studied and are currently categorized as congenital or acquired urogenital anomalies, varicocele, genetic abnormalities, cancer, infections in the genital tract, endocrine disorders, amongst others (Barratt et al., 2017; Hamada et al., 2011). Yet, in nearly 50% of the cases no apparent cause for the infertility state is acknowledged. In these cases, the disease is categorized as male infertility of unknown origin (Ashok Agarwal et al., 2015, 2021; Barratt et al., 2017; Hamada et al., 2011) that can be further divided in unexplained male infertility (UMI), with an incidence of 6% to 37%, and idiopathic male infertility (ID), with an incidence of 30% to 40%, that mainly differ in the seminal analysis that is normal in the former, contrarily to the latter (Ashok Agarwal et al., 2015; Hamada et al., 2011).

Worth mentioning, in the literature these two terms are often confounded and used indiscriminately, making it difficult to characterize and distinguish between these groups, highlighting the need to further study these cases in more detail (Corsini et al., 2022).

2.2.1. Other hypothetical causes for male infertility

Besides the well-established causes, other hypothetical conditions have been advanced as likely players in male fertility, such as age, obesity, chronic diseases (such as diabetes), addictive consumptions (smoking, drugs, alcohol, etc), environmental exposures, oxidative stress, psychological stress, among others (Ashok Agarwal et al., 2018; Sandra Amaral, Oliveira, et al., 2008; Sandra Amaral et al., 2013; Dias et al., 2014a; Xinyi Gu et al., 2021; Komiya et al., 2014; Starc, 2019; Wada & Nakatsuka, 2016).

While for some of these, a relation has already been established, for others this is more of an assumption, as the existent studies are not congruent enough to draw definitive conclusions. Still, we cannot exclude that they might be involved in the aetiology of the male infertility of unknown origin (Ashok Agarwal et al., 2018; Aktan et al., 2013; Sandra Amaral, Oliveira, et al., 2008; Dias et al., 2014a; Nargund, 2015). At the scope of this thesis we will give a special focus to the psychological state.

2.2.1.1. Lifestyle factors

The general health status is acknowledged to play a relevant role in semen quality (Capogrosso et al., 2018), as well as the male fertility status being suggested as a likely indicator of a person's health condition (Choy & Eisenberg, 2018; Skakkebaek et al., 2016). Thus it seems crucial that lifestyle factors, that directly influence general health status, ought to be assessed when evaluating patients' clinical history (Rakesh Sharma et al., 2013; Skakkebaek et al., 2016) in a clinical setting of fertility evaluation. Below we focus on some of these factors, based on the reported detrimental effects on male fertility and also on the relevance to the present thesis.

Age and Obesity:

Age(ing) is known to be a risk factor in fertility status throughout one's life (S Amaral, Amaral, et al., 2013; S Amaral, Mota, et al., 2008; Ford et al., 2000). On the male side, aging men tend to present decreased seminal parameters, such as volume, concentration, motility, morphology, among others (S. L. Johnson et al., 2015). Additionally, age is also associated with the development of other diseases, such as diabetes, neurodegenerative diseases, among others (S Amaral, Oliveira, et al., 2008; Benjamins et al., 2017; Hou et al., 2019; Sesti et al., 2018), that together will reduce the likelihood of reproductive success in natural conception (Sandra Amaral et al., 2006; Sandra Amaral, Oliveira, et al., 2008; Ford et al., 2000; Hassan & Killick, 2003; La Vignera et al., 2012).

Obesity is also an important factor to consider, given that excess weight is known to decrease male fertility (Campbell et al., 2015; Durairajanayagam, 2018; Nätt et al., 2019; Rakesh Sharma et al., 2013) with several reports on the altered sperm quality, motility, morphology, chromatin condensation and DNA fragmentation (Campbell et al., 2015; Leisegang et al., 2021).

Environmental factors and additions:

Environmental factors are also seen as harmful players upon fertility, especially endocrine-disrupting chemicals, radiation, and extreme temperatures (Diamanti-Kandarakis et al., 2009; Wdowiak et al., 2019; Zhou et al., 2020).

Endocrine-disrupting chemicals can be found in substances such as pesticides, metals, paints, food containers, etc (Diamanti-Kandarakis et al., 2009; Frederiksen et al., 2007). Exposure to these chemicals impairs normal cellular functions and endocrine pathways, with special relevance in reproductive health concerning testosterone levels, sperm count, viability, morphology, ATP levels, and sperm motility (Sifakis et al., 2017; R. S. Tavares et al., 2016; R. S. Tavares et al., 2013, 2015).

On the other hand, some types of high energy radiation can alter molecules and their functions at all levels in our bodies, not being the male reproductive system an exception existing several reports on their detrimental effects at this level (Kesari et al., 2018; Truong et al., 2018; Wdowiak et al., 2019). Thus, radiation exposure on a regular basis constitutes another relevant factor when assessing one's reproductive health (Kandeel & Swerdloff, 1988; Kesari et al., 2018; Oh et al., 2018; Truong et al., 2018; Zalata et al., 2015).

On the subject of temperatures, it is known that spermatogenesis is highly sensitive to temperature, occurring optimally at nearly 35ºC (Kandeel & Swerdloff, 1988; Zhou et al., 2020). In fact, in human males the testes are located outside of the abdominal cavity, in the scrotum, to ensure a lower temperature than that of the human body (Kandeel & Swerdloff, 1988; Zhou et al., 2020). When thermoregulation is impaired, heat stress will occur and defects in spermatogenesis are expected, further reflected in seminal quality and fertility. In fact, altered concentration, motility, increase in morphological anomalies and DNA damage have been described in this context (Durairajanayagam et

al., 2014, 2015; Kandeel & Swerdloff, 1988; C. Wang et al., 1997; Zhou et al., 2020). The heat stress can be due to internal and external factors. In the latter category lifestyle and behavioural as well as occupational and environmental factors are included (Durairajanayagam et al., 2015), with several reports on the detrimental reproductive effects of heating in professional activities such as long course drivers, or those demanding prolonged sitting, as well as due to unproper clothing that impede air flow, or even due to lifestyle choices such as the use of saunas and heat baths or also exercises as cycling and even obesity (Durairajanayagam et al., 2015; Hoang-Thi et al., 2022). Regarding the internal factors, diseases such as cryptorchidism, characterized by undescendant testis, and varicocele, characterized by abnormal dilatation of the pampiniform plex veins in the spermatic cord, are the best studied in this context, being both associated with negative effects on sperm quality, function and fertilizing capacity (Durairajanayagam et al., 2015).

On the other hand, smoking and alcohol consumption are among the most common addictions (Addiction Center, 2014) and, have been extensively described by their negative roles upon one's general health status, including at the reproductive level (Amor et al., 2022; Asare-Anane et al., 2016; Joo et al., 2012; Ricci et al., 2017; Reecha Sharma et al., 2016) where these habits have been described to affect the normal functioning of the Hypothalamus-Hypophysis-Gonadal (HPG) axis causing hormonal alterations, as well as in spermatogenesis and gametic function (Amor et al., 2022; Asare-Anane et al., 2016; Joo et al., 2012; Ricci et al., 2017; Reecha Sharma et al., 2016).

Oxidative stress: the cause, the consequence or simply a mediator?

Oxidative stress can be defined as an imbalance between the amounts of antioxidants and oxidative species, in which an excessive accumulation of Reactive Oxygen Species (ROS) or a shortage of antioxidants will happen.

In semen, the main sources of ROS are the leukocytes and sperm (Tremellen, 2008). Within sperm cells, mitochondria and oxidase-based systems account for the major sources of oxidative species (Nolfi-Donegan et al., 2020; Tremellen, 2008). As mentioned, when in normal amounts ROS are fundamental for sperm functioning, participating in signalling cascades, and several processes such as capacitation, acrosome

reaction and fertilization (Barati et al., 2020; Cocuzza et al., 2007). Yet, when imbalanced, ROS can be highly detrimental to sperm function and integrity (Ashok Agarwal et al., 2018). In fact, sperm is very sensitive to oxidative stress, that will affect their membranes and DNA's integrity, compromising paternal genome transmission (Ashok Agarwal et al., 2018).

Damage to the sperm DNA has been significantly studied and a growing body of evidence found a strong relation to fertility problems, reduction of fertilization rates and spontaneous abortions (Ashok Agarwal et al., 2019; Esquerré-Lamare et al., 2018; Nolfi-Donegan et al., 2020).

Worth mentioning, oxidative stress has been found elevated or associated with many of the aforementioned lifestyle risk factors (Ashok Agarwal et al., 2018; Birch-Machin et al., 2013; Finelli et al., 2021; Fraczek et al., 2022; García-Díaz et al., 2015; Hauck et al., 2019; Kumar et al., 2015; Virant-Klun et al., 2022). It seems therefore likely that oxidative stress is not so much as an isolated risk factor, but more as a mediator effector common to several conditions.

2.2.1.2. Psychological stress

According to Nargund (2015), psychological stress is any uncomfortable emotional experience associated with foreseeable biochemical, physiological, and behavioural shifts or responses. Thus, psychological stress initiates a chain of events that leads to a disturbance in general homeostasis directly caused by the effects of the stress on the minds.

Untreated and/or enduring psychological stress may lead to a pathological stress response causing shifts and new adjustments in body homeostasis (Giblin et al., 1988; Nargund, 2015). These new adjustments include alterations in metabolism, vascular function, immune function, depression and anxiety disorders, among other conditions (Xinyi Gu et al., 2021; Nargund, 2015; Sánchez González et al., 2023; Starc, 2019). Thus, psychological stress is deeply intertwined with the body's capability to remain healthy and effects at the reproductive level are not unexpected (Al-Khatib et al., 2022;

Allen et al., 2018; Giblin et al., 1988; Gollenberg et al., 2010; Lutter & Elmquist, 2009; Moradi et al., 2021; Nargund, 2015; Sánchez González et al., 2023)

In Portugal the latest estimations, according to DGS (Direcção Geral de Saúde), point to 7.9% and 16.5% of the population having felt depression or anxiety symptoms, respectively (DGS, 2013). Globally, according to WHO, there is 4.4% of incidence of depression and 3.4% of anxiety (WHO, 2017).

In an infertility context, even though not often considered, during the last years more attention has been given to the impact that psychological stress might have at this level and several have been the studies that aimed to clarify the link between psychological stress and semen quality. Accordingly, some correlations with seminal or physiologic parameters, such as sperm concentration, serum total testosterone, altered levels of Follicular Stimulating Hormone (FSH) and Luteinizing Hormone (LH), among others have been described (Giblin et al., 1988; Gollenberg et al., 2010; Sánchez González et al., 2023; Zorn et al., 2008). Nonetheless it is still hard to determine what comes first and what causes what. In fact, not only psychological disorders can influence the ability to conceive, but also infertility itself can further increase the psychological distress of a couple (Evans-Hoeker et al., 2018; Fernandes et al., 2021; Nargund, 2015; Sánchez González et al., 2023).

Psychological stress plays a role upon many aspects of the male reproductive system, for instance on sexual function. It is well-established that psychological stress may lead to erectile dysfunction (Makhlouf et al., 2007; Nargund, 2015; S. N. Seidman, 2002). This is especially relevant in the context of a couple facing fertility difficulties, which maybe undergoing fertility treatment or engaging in intercourse in a strictly scheduled manner, adding up more pressure (Byun et al., 2013; Fernandes et al., 2021; Gdańska et al., 2017; Nargund, 2015). This pressure to conceive and achieve a clinical pregnancy might prove stressful, triggering anxiety and frustration symptoms, potentially leading to erectile and ejaculatory dysfunctions in men (Byun et al., 2013; Makhlouf et al., 2007; S. Seidman, 2006; S. N. Seidman, 2002), further compromising the whole process outcome. Furthermore, according to Bhongade (2015), male partners of infertile couples presenting signs of depression and/or anxiety presented lower serum testosterone levels, sperm count, motility and normal morphology, and higher serum FSH and LH, when compared to those who showed no signs for the disorders.

On the other hand, aged-reproductive couples faced with an infertility diagnosis are also more likely to develop psychological stress conditions, such as depression and anxiety disorders, due to the struggle or inability to conceive (Fallahzadeh et al., 2019; Fernandes et al., 2021; Gdańska et al., 2017).

2.2.1.2.1. Theoretical underlying mechanisms for hypothetical impact of Depression and Anxiety in the male reproductive system

A general consensus has established that the most significant centre responsible for regulating stress-related responses is the paraventricular nucleus (PVN) of the hypothalamus (Wills & Havard, 1983). The PVN is known to have broad connections with regions that control metabolism, growth, reproduction, and other autonomic functions (Nargund, 2015). The peripheral stress response mechanisms are connected to three main pathways: the sympathetic-adrenal system pathway, the hypothalamic-pituitary-adrenal (HPA) axis, and the hypothalamic-pituitary-gonadal (HPG) axis (Ferguson et al., 2008; Nargund, 2015; Sánchez González et al., 2023).

Having in mind this information, it is not difficult to predict the possible effects at the reproductive level. In fact, the chronic psychological stress, in which the stressor is present for an extended period of time, eventually leads to a reorganization of the HPA axis (Nargund, 2015). The constant activation of the HPA axis by the stressors ultimately leads to irregular glucocorticoid activity, affecting activity and causing apoptosis in Leydig cells (Nargund, 2015). In turn, serum testosterone levels become decreased leading to Sertoli cells and Blood-Testicular Barrier (BTB) impairment and spermatogenesis arrest (Nargund, 2015; Sánchez González et al., 2023; Smith & Walker, 2014).

The HPG axis is responsible for the regulation of reproductive function in both sexes (Asimakopoulos, 2012; Nargund, 2015). The hypothalamus produces GnRH, that will stimulate the anterior pituitary gland to secrete key hormones such as FSH and LH that regulate, through a feedback loop system, the male spermatogenesis and testicular function (Nargund, 2015). FSH acts upon Sertoli cells stimulating inhibin secretion and supporting spermatogenesis, while LH stimulates Leydig cells to produce testosterone (Kirby et al., 2009; Nargund, 2015).

The increased activity of HPA axis, and therefore also glucocorticoids levels, have a direct inhibitory effect on the activity of HPG and on FSH and LH secretion (Ferguson et al., 2008; Kirby et al., 2009; Nargund, 2015), being this one of the proposed mechanisms by which the three diseases might be related.

Considering the aforementioned mechanisms, serum testosterone levels fluctuations seem to play direct and indirect effects over spermatogenesis and/or tissues that are also key players in the orchestra of mature and functional sperm cell production. This is particularly evident in patients diagnosed with Cushing's syndrome, where excessive levels of cortisol (stressors) and glucocorticoids present in the bloodstream of men have been linked with testosterone production suppression (Nargund, 2015; Vierhapper et al., 2000).

Evidence clearly implies a tight connection and crosstalk between neuroendocrine signalling and the male reproductive system in response to stressors (psychological stress). However, the clinical relevance, the extent and severity of the effects that might exert over sperm production have yet to be elucidated.

2.3. Diagnosis

2.3.1. Diagnostic work up

When a couple seeks medical help in order to achieve a clinical pregnancy, several exams are performed in both to assess if there are any problems (Carson & Kallen, 2021; Evans-Hoeker et al., 2018; Leaver, 2016; Szamatowicz, 2016).

Concerning male infertility, the diagnosis usually involves general physical examination, hormonal analysis, and medical history that are followed by a seminal analysis to assess sperm quality (Elshal et al., 2009; Heidary et al., 2019; Kanannejad & Gharesi-Fard, 2019; Marzano et al., 2020), performed according WHO guidelines for human semen examination. According to this, a sample will only be considered normal (normozoospermic) if the following reference limits are met: a concentration equal or above to 15 million sperm cells per millilitre, viability equal or above to 58%, total motility equal or above to 40%, and sperm cells with normal morphology equal or above

to 4%. Hence, a sample may be categorized as oligo (O), astheno (A), teratozoospermic (T), or OAT when concentration, motility, and morphology, or all, are below the reference limits, respectively (Heidary et al., 2019; Marzano et al., 2020; WHO, 2010).

Unexplained male infertility (UMI) cases occur when men are normozoospermic (Kanannejad & Gharesi-Fard, 2019; WHO, 2010) while idiopathic male infertility (ID) occurs when seminal quality is decreased and no explanation can be found for that, translating in abnormal seminal parameters, such as concentration, motility, and/or morphology (Arcaniolo et al., 2014).

Even though seminal analysis is globally established as an important pillar for assessing male fertility status, being easy and cost effective, there are still concerns regarding its value on the prediction of the exact cause of infertility and pregnancy likelihood (Hamada et al., 2011; Heidary et al., 2019; Jafarzadeh et al., 2015). In fact, sperm cells' function and fertilization potential depend on many other features that are frequently not assessed. A proving point of these limitations resides in the fact that UMI males are normozoospermic and yet unable to father biologic offspring (Hamada et al., 2011; Heidary et al., 2019; Jafarzadeh et al., 2015; Kanannejad & Gharesi-Fard, 2019; Thonneau et al., 1991). In addition, there are men who can father a child despite being not normozoospermic (Esteves et al., 2011; Hamada et al., 2011).

The limitations of seminal analysis emphasize the need to undertake a broader and more integrated analysis that combines the evaluation of new sperm parameters, based on more reliable functional aspects, such as metabolism, chromatin integrity, transmembrane channels' function, important molecules for gamete interaction, as well as on new/improved methodologies that enable the precise assessment of these aspects. This approach may elucidate and shed light on still unknown molecular mechanisms that might help to explain unknown origin male infertility, also having the potential to develop new diagnosis tools as well as treatment options, ultimately contributing to a global improvement in reproductive health and fertility rates.

2.3.2. New approaches to be considered

One important aspect of sperm function that could be more factored in terms of possible analysis/diagnostics is metabolism. Metabolically speaking, as mentioned above, the sperm is a very versatile cell, and despite the debate on which is the preferred pathway to energy attainment (glycolysis versus OXPHOS ; A. Amaral et al., 2014; S. Amaral et al., 2013; Dias et al., 2014), greatly fomented by the compartmentalization of the two main ATP producing pathways in the mid and principal piece of the sperm cells (A. Amaral, 2022; Ruiz-Pesini et al., 2007; Bayard T. Storey, 2008), the most recent studies support an wide versatility of these cells at a metabolic level.

In fact, alternative and unexpected metabolic pathways were recently described in human sperm, as is the case of fatty acids oxidation, glycerol and ketone bodies catabolism (A. Amaral, 2022). Making a (very) long story short, the sperm cell is able to use the most convenient fuels and metabolic pathways depending on the available substrates determined by the circumstances and surrounding environment, namely the one in the female reproductive tract (A. Amaral et al., 2014; A. Amaral & Ramalho-Santos, 2013; Carrageta et al., 2022; Ramalho-Santos et al., 2009).

There are several methods to infer on the metabolic state of a cell, ranging from the evaluation of the activity of specific metabolic enzymes, or the quantification of medium and intracellular metabolites, to the use of fluorescent probes directed to a specific mitochondrial or metabolic trait. Regarding metabolic enzymes, usually the approaches include the evaluation of their activity or levels, by spectrophotometry (Gomez et al., 1998), immunocytochemistry (X. Liu et al., 2019), Western blot or proteomic approaches (A. Amaral et al., 2013; A. Amaral, Paiva, et al., 2014b), while the metabolites levels are frequently assessed by NMR (Carrageta et al., 2022; Paiva et al., 2015) or by spectrophotometry, as is the case for reactive oxygen metabolite quantification in serum (Ferramosca et al., 2013).

Among the fluorescent probes, *Mito Tracker green* (MTG; Cottet-Rousselle et al., 2011) and *JC-1* (Cottet-Rousselle et al., 2011), that provide information on mitochondrial content and membrane potential (MMP; Connolly et al., 2018), respectively, or DCFDA and *Mitosox Red*, that measure ROS production, intimately related to oxidative stress and indirectly to infertility (Escada-Rebelo et al., 2022), are commonly used.

Even though these methods are very useful, they fall short on providing a more holistic view on cellular metabolism. More recently, some promising technologies have been developed with the specific aim of evaluating different metabolic aspects, based on dynamic and real-time analysis and using less samples' quantity as is the cases of Oroboros, which permits real-time measurement of mitochondrial respiratory kinetics [an improved version of the former Clark electrodes (Garrett et al., 2008)], yet providing no relevant information regarding other metabolic processes (Inhorn & Patrizio, 2019); On the other hand, Myoxsys, specifically designed for male infertility assessment in a clinical setting is an apparently robust tool, that assessing the balance between oxidants and antioxidants provides a measure of oxidative stress that might be related to the men's infertility state. Though, besides the fact of being performed on seminal fluid (and not on sperm cells), provides no relevant metabolic information (Kavoussi et al., 2022); Finally, the recent *Seahorse Flux Analyser* (SFA) is a high throughput technology with the potential to obtain considerable information, using few cells. The SFA was designed to study cell metabolism, by measuring the immediate surrounding environment of the sampled cells in real-time. SFA specifically measures the oxygen consumption rate (OCR) and/or extracellular acidification rate (ECAR), indirect readouts of the two main metabolic pathways for energy achievement (mitochondrial respiration and glycolysis, respectively), essentially relying on drug injection strategies at specific stages, allowing to assess how the cells' metabolism responds. Additionally, the measurement of OCR provides information on important metabolic metrics such as basal respiration, ATP production, proton leak, maximal respiration, and non-mitochondrial respiration of cells (**Figure 8A**; Agilent Tecnologies, 2017). On the other hand, ECAR provides indirect information on glycolysis, due to the acidic nature of the glycolytic resulting products (pyruvate or lactate), also allowing to collect information on the glycolytic reserve, capacity and non-glycolytic acidification (**Figure 8B**; Agilent Tecnologies, 2017). Due to its versatility, SFA seems to be the ideal candidate for human sperm cells metabolic analysis. Yet, although this technology has been described in mouse sperm (Balbach, Gervasi, et al., 2020), anticipating its power to provide relevant information on sperm function, in humans, there were no studies until march 2022, when a publication came out, but oddly only focusing on the OCR and using a reduced number of samples (Taniguchi et al., 2022), warranting further studies.

3. Objectives

The current study is composed by two main goals:

- Understand, throughout a retrospective analysis of an extensive data base, if the infertility diagnosis in a well-characterized and defined groups of patients (namely idiopathic, unidentified male infertility, and control groups) is correlated with the Hospital Anxiety and Depression Scale (HADS) scores on depression and anxiety symptoms
- Optimize the Seahorse Flux Analyzer technology to human sperm cells, aiming to characterize human sperm metabolism and evaluate the potential of this approach to further improve diagnostic and treatment for infertile patients.

Material and methods

1. Chemicals

Unless stated otherwise, all chemicals and reagents used were from Sigma-Aldrich.

Part 1. Retrospective study

In this part of the study, we have used a previously established data basis in which several information was collected regarding 3 groups of individuals, the control group (CTRL), the Idiopathic infertile group (ID) and the unexplained infertile group (UMI). For the group's categorization, several aspects were taken into account namely the couple's clinical history, results from physical examination and the hormonal and seminal analysis, done together with the clinicians and embryologist of the Reproductive Medicine Unit.

Furthermore, exclusion criteria were strictly applied, namely the presence of urogenital anomalies or infections, existence of sexual transmitted diseases, varicocele, leucocytospermia, genetic abnormalities, azoospermia, past or present oncologic diseases (and/or chemo or radiotherapy treatment), among other major diseases.

Individuals were classified as CTRL if normozoospermic, according to WHO reference values, and when there is a clear and identifiable presence of a female factor.

Individuals who failed to conceive after 12 months or more of regular unprotected sexual intercourse and in which a potential female factor has been ruled out, were grouped either as ID or UMI. In ID group, individuals presented at least one or a combination of abnormal seminal parameters (concentration, viability, motility, and morphology), according to WHO reference values while in UMI cases, individuals were normozoospermic.

A. Data basis available information

The data basis information corresponds to data gathered on human semen samples collected at the Reproductive Medicine Unit of the University Hospitals of Coimbra (CHUC; Coimbra, Portugal), between January 2018 and July 2022, from men that were undertaking routine semen analysis for fertility assessment. All the participants signed informed consents and the biological material was used in accordance with the proper ethical and Internal Review Board (IRB) guidelines provided by CHUC.

Semen samples were obtained and processed according to WHO guidelines (WHO, 2010) and after that several functional parameters were assessed in the collected sperm samples namely concentration, motility, morphology and viability, as previously described (Portela et al., 2015). Additionally this data basis also comprises data on the chromatin status, evaluated by a colorimetric approach as previously described by our group (Mota & Ramalho-Santos, 2006; R. S. Tavares et al., 2013), as well as on the capacitation status and acrosome integrity. The capacitation status was evaluated by immunocytochemistry while the acrosome integrity was evaluated by fluorescence, as we have previously reported (Portela et al., 2015).

Furthermore, the data basis also includes information collected by survey on different lifestyle aspects as well as on the psychological state. At the scope of the present thesis, we will focus on the latter, specifically on the Psychological assessment for depression and anxiety symptomatology.

As previously mentioned, fertility status can play a leading role on patients' mental health, especially when undergoing fertility treatment. In order to explore and study the relationship that might exist between psychological well-being and fertility-related biologic features, patients were asked to fill in a self-response questionnaire, the "Hospital Anxiety and Depression Scale" (HADS). The HADS questionnaire presented to patients, in the European Portuguese version, is one of the most robust and used scales for evaluating anxiety and depression in hospital context amongst patients undergoing illnesses and/or treatments (J. Pais-Ribeiro et al., 2007; J. L. Pais-Ribeiro et al., 2018). This questionnaire is comprised of 14 items distributed in two subscales being 7 items designed to assess anxiety and 7 items to assess depression, with 4 possible answers each. To each of the 4 answers a score is ascribed, which can range from 0 to 3. Summing the

scores for each factor will result in a total score, one for anxiety and another for depression, each can range from 0 to 21. In the Portuguese version, patients who score from 0 to 7 are considered “Normal”, from 8 to 11 considered “Borderline abnormal”, and from 12 to 21 considered “Abnormal” (Almeida et al., 2015; Hinz & Brähler, 2011; J. Pais-Ribeiro et al., 2007; J. L. Pais-Ribeiro et al., 2018; Sánchez González et al., 2023; Zigmond & Snaith, 1983).

Due to the different number of participants across groups (a mirror of the prevalence of the infertility type), participants were grouped in two groups. One group called ‘No’ with participants scoring 7 or less (Normal) in both subscales and the other called ‘Yes’ scoring 8 or more (Borderline abnormal and Abnormal) in at least one of the subscales.

B. Statistical analysis

For the statistical analysis, RStudio software version 2022.07.2+576 "Spotted Wakerobin" for windows was used. Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene tests, respectively. All variables violated normality assumption (**Supplementary table 1**). As for homogeneity of variance, Concentration, Viability, Motility in Situ, Imotile, and Abnormal morphology variables violated homogeneity of variances assumption for fertility status, as well as for fertility status in interaction with diagnosis (**Supplementary table 1**). For diagnosis, only Motility in Situ violated homogeneity of variances assumption (**Supplementary table 1**). The number of participants varied across parameters and, thus, MANOVA with all parameters was not allowed (**Supplementary table 2**). Thus, mean differences were assessed through factorial ANOVA with each parameter separately considering two factors: fertility status (i.e., ID, UMI, or Control) and diagnosis status (Yes or No). Bootstrapping (1000 resampling; 95% confidence interval BCa) was used to correct violations of statistical assumptions and provide more reliable confidence intervals. Hochberg and Games-Howell tests were implemented for post-hoc comparisons in fertility status only considering variables with statistical significance in ANOVA. Whenever homogeneity of variances was violated, Games-Howell test was employed, otherwise Hochberg test was employed. The magnitude of differences was assessed by

partial eta square for fertility status (0.01≤partial ηp2<0.06 small; 0.06≤partial ηp2<0.14 medium; and partial ηp2>0.14 large) and *d* of Cohen for diagnosis status (*d*<0.2 negligible; 0.2≤*d*<0.5 small; 0.5≤*d*<0.8 moderate; and *d*>0.8 large). Differences were considered statistically significant if p≤0.05.

Part 2. Seahorse flux analyser optimization on human sperm cells - Metabolic assessment

Given the metabolic plasticity of the sperm cells, and the importance of the connection between metabolism and function, we have used a new approach, that to our knowledge is the only one able to measure, simultaneously, two main metabolic pathways, the glycolysis and OXPHOS, both crucial for the sperm cell, as previously described (A. Amaral et al., 2013; Sandra Amaral et al., 2013; Dias et al., 2014a; du Plessis et al., 2015; Ramalho-Santos et al., 2009; Ruiz-Pesini et al., 2000; Ruiz-Pesini et al., 2007), the Seahorse Flux Analyzer (SFA).

A. Seahorse Flux Analyzer assay

As previously mentioned, SFA specifically measures the oxygen consumption rate (OCR) and/or extracellular acidification rate (ECAR), in an experimental setting ruled by drug injection strategies (**Figure 7**; Agilent Tecnologies, 2017) whose response will give information on the two main metabolic pathways of ATP production (OXPHOS and glycolysis, respectively), (**Figure 8A** e **Figure 8B**, respectively).

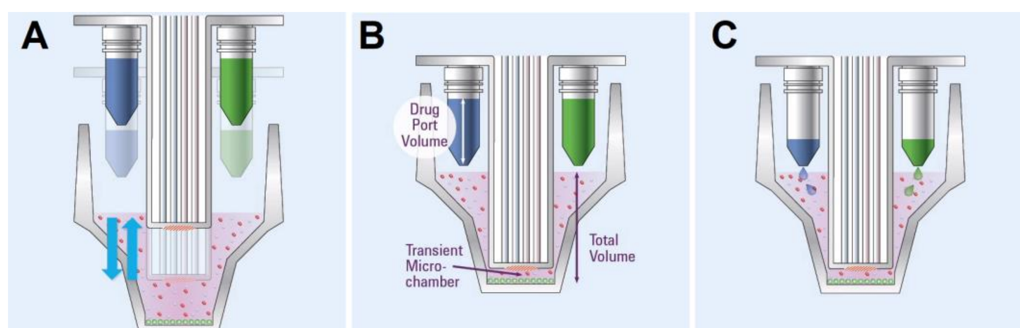

Figure 7 - Seahorse Flux Analyzer sensors stages during assay. **A:** Mix stage: in which the well content is homogeneized. **B:** Measure stage: measurent of OCR and ECAR. **C:** Injection stage: in which a specific drug is injected into the well. (From: https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-sensor-cartridges-cell-culture-microplates/seahorse-fluxpaks-740883)

The drugs injected during OCR assay were oligomycin, FCCP, Antimycin A and Rotenone, by that order. Oligomycin inhibits ATP synthase, reducing oxygen consumption by halting oxidative phosphorylation. FCCP is an uncoupler of oxidative phosphorylation that by transporting protons across the inner mitochondria's membrane disrupts the proton gradient, increasing OCR to a maximal value. Finally, Antimycin A inhibits ETC complex III and Rotenone inhibits Complex I, stopping ETC workflow, and therefore compromising OCR.

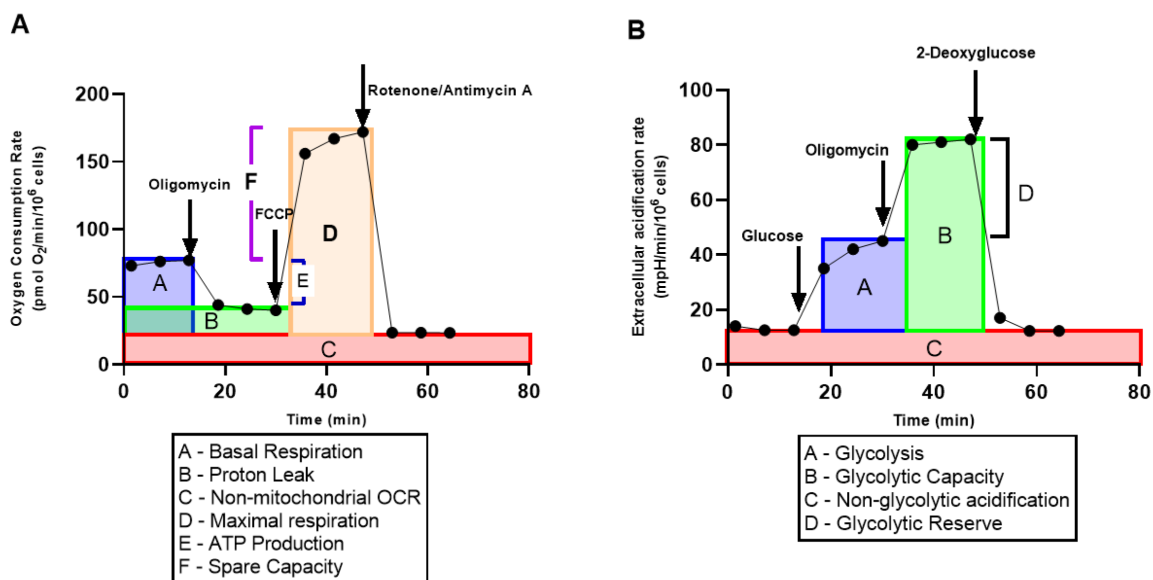

Figure 8 - Standard metabolic profile for a Seahorse Flux Analyzer assay and relevant metabolic metrics regarding OCR (**A**) and ECAR (**B**) measurements (designed using GraphPad Prism 9.0.0.)

The measurement of OCR allows to further calculate several useful metrics such as the basal respiration, ATP production, proton leak, maximal respiration, non-mitochondrial respiration of cells, and spare capacity (**Figure 8A**;**Table 1**).

Table 1 – OCR-derived metrics

OCR metrics	Bioenergetic meaning
Basal respiration	Minimal rate of metabolism needed to support basic cellular functions.
ATP production	Provides an indication/prevision on the respiration that was being used to drive ATP production under basal conditions.
Proton leak	Mirrors the natural leakage of protons across the mitochondrial inner membrane, in favour of gradient, independently of complex V activity.
Maximal respiration	Obtained following the injection of FCCP, this metric corresponds to the highest rate at which the mitochondria can work.
Non-mitochondrial respiration	Refers to the oxygen consumption that is not dependant of the mitochondria, but instead of other cytoplasmatic enzymes that also consume oxygen, (as the membrane NAPDH oxidase).
Spare capacity and Spare Capacity as fold-increase	Important indicator on the reserve capacity of the organelle to deal with a scenario of energetic crises that, as a fold increase is an expression of how close to its maximal capacity is the electron transport chain working during basal respiration.

On the other hand, during ECAR assays, the injected drugs were glucose, oligomycin and 2-Deoxyglucose (2DG). Thus, after the injection of glucose, ECAR increases due to the production of pyruvate/lactate. Following the injection of oligomycin a metabolic pressure will be put upon glycolysis to compensate for the lack of mitochondria's ATP production, further increasing ECAR. Finally, injecting 2DG, a structural analogue of glucose that blocks hexokinase enzyme (first enzyme of glycolysis pathway), will halt glycolysis pathway, resulting in substantial decrease of ECAR (Agilent Tecnologies, 2017).

The measurement of ECAR provides indirect information on glycolysis, due to the acidic nature of the glycolytic resulting products (pyruvate or lactate), also allowing to calculate several functionally-relevant metrics, such as the glycolytic reserve (and fold increase), capacity and non-glycolytic acidification (**Figure 8B**; **Table 2**; Agilent Tecnologies, 2017).

Table 2 – ECAR-derived metrics

ECAR metrics	Bioenergetic meaning
Glycolysis	Indicates the contribution of glucose breakdown to pyruvate or lactate, both acidic by-products of glycolysis, by the ECAR increase.
Glycolytic capacity	Measure on how cells meet the metabolic demands after inhibition of mitochondrial ATP synthesis. Glycolysis will increase to meet the energetic demands of sperm cells, and so does ECAR, revealing the maximal glycolytic capacity.
Glycolytic reserve and Glycolytic reserve as fold increase	These metrics allow to infer how close is basal glycolysis to the maximum glycolytic capacity of the cell, mirroring the needed increase in glycolysis to meet cellular energetic requirements without mitochondrial ATP production and being therefore indicative of the reserve capacity.
Non-glycolytic acidification	Indicative of the ECAR measurements not attributable to glucose metabolism.

A. Assay's samples preparation

a. Collection and processing of human seminal samples

Semen samples were obtained from men that were undertaking routine semen analysis for fertility assessment at the Reproductive Medicine Unit of the University Hospitals of Coimbra (CHUC; Coimbra, Portugal) and processed according to WHO guidelines (WHO, 2010). All the participants signed informed consents and the biological material was used in accordance with the proper ethical and Internal Review Board (IRB) guidelines provided by CHUC.

After liquefaction, sperm cells were isolated by density-gradient centrifugation at 400-500g for 10 mins (SupraSperm; Medicult-Origio) and allowed to capacitate in sperm preparation medium (SPM; Medicult-Origio) for at least 3 h at 5% CO_2 and 37 °C before initial experiments were performed. Only normozoospermic samples for concentration, motility, and viability were used, with no visible leukocytes or other round cells.

b. Concentration, motility and morphology evaluation

These parameters were assessed according to WHO recommendations (2010) and examined by bright field optical microscopy for concentration and morphology and phase-contrast optical microscopy for motility and viability (Leica DB400B; Leica microsystems).

Sperm concentration assessment was performed using a Neubauer chamber, loaded with 10 µL of sample formerly diluted and immobilized in double distilled water due to osmotic shock, according to WHO recommendations. The counting of the cells was performed by looking at specific regions (squares) of the Neubauer chamber at 500x magnification. Concentration was then calculated according to the number of cells counted and the sample's dilution factor.

Sperm cells motility was assessed by the observation of a preparation containing 10 µL of homogenized sample, using 400x magnification. Three categories for motility were considered for the cells' counting process, progressive, *in situ*, and immotile.

Sperm cells viability evaluation was performed using Eosin Y (WHO, 2010). Cells' plasma membranes are impermeable to Eosin Y, hence only dead cells with compromised membrane integrity will be stained in the head. To assess viability, 5 µL of homogenized sample were mixed with 5 µL of 0.5% (w/v) Eosin Y and further assessed using 400x magnification. Sperm cells stained with red/reddish/pink head were deemed dead, whilst non-stained cells were considered alive.

For both motility and viability at least 100 spermatozoa were evaluated in different fields, and results were expressed as percentage.

For morphology analysis, the Diff-Quik coloration kit was employed. This method entails 3 solutions: methanol, a fixative reagent; eosin, an anionic dye, which stains positively-charged proteins red; and thiazine, a blue staining agent, which stains the negatively-charged DNA. Smears for each sample were prepared using 10 µL of the samples, and left to air-dry. Subsequentially, slides were immerged in each of the 3 solutions for 15 seconds, methanol, eosin, and thiazine, respectively. Slides were left to air-dry and then mounted with Eukitt mounting medium. Morphology was assessed by oil-immersion bright field microscopy at 1000x magnification.

Morphologically, sperm cells were categorized, according to WHO guidelines, as normal if no defects were observed and abnormal if any major defects on the head, midpiece or tail were detected (WHO, 2010). Results were expressed as a percentage of morphologic normal/abnormal sperm cells.

c. Sperm samples preparation for Seahorse assays

Once samples' concentration was determined, the volume of needed sperm cells was calculated having in consideration that for optimal response in XFe24, $4x10^6$ cells/well are needed.

Sperm samples were divided in two aliquots and centrifuged at 300g for 5 mins. The resulting pellets were then resuspended in the two different assay media: (1) OXPHOS+BSA (OBm) medium (PBS, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.0 mM D-glucose, 1.0 mM sodium pyruvate, 10 mM sodium lactate, 3 g/dm^3 BSA, and 2 mM L-glutamine, pH 7.2) for OCR assay, and (2) ECAR (ECm) medium (PBS, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 2 mM L-glutamine, pH 7.2) for ECAR assay. Samples were then rinsed twice (300g, 5 mins) using the respective media.

B. Seahorse Flux Analyzer set up

a. On the day prior to the assay:

The sensor cartridge was carefully removed from the Extracellular Flux assay kit and each well of the utility plate was filled with 1 mL of Seahorse XF Calibrant Solution (**Figure 9**). The sensor cartridge was put back into the utility plate, submerging the sensors onto the XF Calibrant, making sure that no air bubbles were covering the sensors. Then, the sensor cartridge must be incubated overnight, in a non-CO_2 incubator at 37º C.

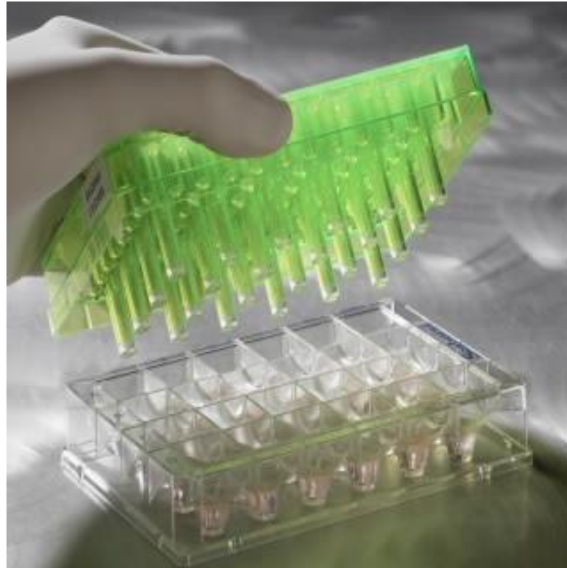

Figure 9 - Seahorse extracellular flux Assay Kit (XFe24): the sensor cartridge (green) and the utility plate (transparent) - (From: https://www.agilent.com/cs/publishingimages/XF24-cartridge-plate%20crop.jpg)

b. On the day of the assay:

i. XFe24 Cell Culture Microplate (CCM): Coating and loading

1h prior to cell seeding, the CCM wells were coated with 20 µL of Concanavalin A solution (ConA; 0.5 mg/mL), ensuring that the entire surface of the well was evenly and thinly covered by the solution. After this, the CCM was incubated at 37ºC in a non-CO_2 incubator for 30 mins. After incubation, media and sperm samples were loaded in the CCM wells obtaining a final volume of 450 µL per well. The loaded CCM was then centrifuged at 200g for 1 mins, rotated 180º and centrifuged again. Cells in the CCM were then incubated for 1h at 37ºC in a non-CO_2 incubator.

The working stocks of the drugs were prepared, according to the concentrations for which we have obtained an optimal response (**Table 3**), loaded into the respective injection ports from the sensor cartridge (**Figure 10**) and left for at least 20 mins at 37ºC in a non-CO_2 incubator. After this, the CCM and sensor cartridge were ready for analysis.

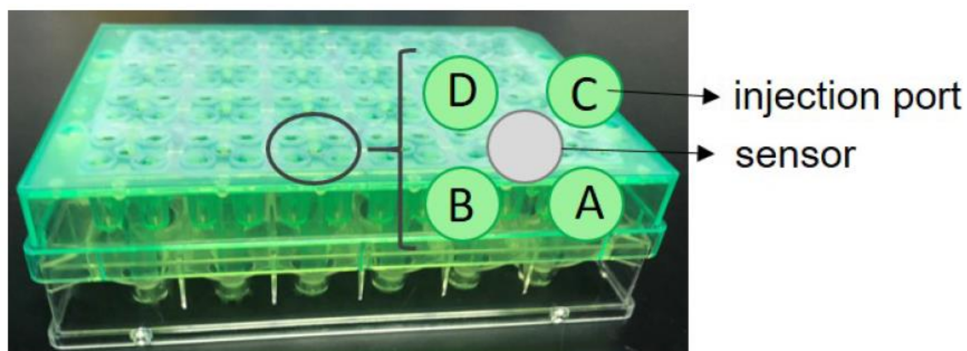

Figure 10 - Sensor cartridge**'s** Injection ports distribution (From: Plitzko & Loesgen, 2018)

Final results were obtained using Wave – software Seahorse version 2.6.3.5 Agilent Technologies using a Seahorse Flux Analyzer XFe24, and graphs by Graph Prism 9.0.0.

Table 3 - Drug concentrations with optimal responses for ECAR and OCR assays

	ECAR			OCR	
Drug	Concentration	Injection Port	Drug	Concentration	Injection Port
Glucose	5 µM	A	Oligomycin	1 µM	A
Oligomycin	1 µM	B	FCCP	0.5 µM	B
2DG	100 mM	C	Antimycin	1 µM	C
			Rotenone	1 µM	C

ii. Wave Software settings

Seahorse Flux Analyzer assay was performed using 'WAVE' software with slight modifications from the standard protocol (**Table 4**). The following adjustments were done to achieve an optimal response:

Table 4 – Modifications on the standard protocol from 'WAVE' software, concerning 'Mix', 'Wait', and 'Measure' timings for readout cycles.

Stage	Standard (mins)	Adapted (mins)
Mix	3	1.5
Wait	2	0.5
Measure	3	3

Furthermore, the option 'Equilibrate' at 'Initialization' was deselected and 'Measure after injection' option was selected for all injection stages.

C. Statistical Analysis

For the statistical analysis, RStudio software version 2022.07.2+576 "Spotted Wakerobin" for windows was used. Due to the reduced number of samples, Spearman correlations were implemented to assess possible associations between variables.

Results

Chapter 1 – Is the presence of depressive and anxiety symptoms associated with sperm quality?

1. Seminal quality in the 3 study groups:

Concerning the effect of fertility status, significant statistical differences were found for Concentration (p<0.001), Viability (p<0.001), Total Motility (p<0.001), Motility in Situ (p<0.001), Morphology (p<0.001), and Capacitation (p<0.01) variables, reported in **Figure 11** (**Supplementary table 3**). Considering partial eta square values, the magnitude of differences was small for Viability, Motility in Situ, and Capacitation; medium for Concentration and Morphology; and large for Total Motility (**Supplementary table 3**).

In comparison to Control and UMI groups, ID group presented lower Concentration, Viability, and Motility, while the percentage of morphologically abnormal cells was higher (**Supplementary table 4**). Considering d of Cohen, the magnitude of differences was large for all comparisons except for the difference between Control and ID groups in Abnormal Morphology, which met moderate magnitude. Lastly, concerning Capacitation, differences were only found between Control and ID groups, with a moderate magnitude (**Supplementary table 4**).

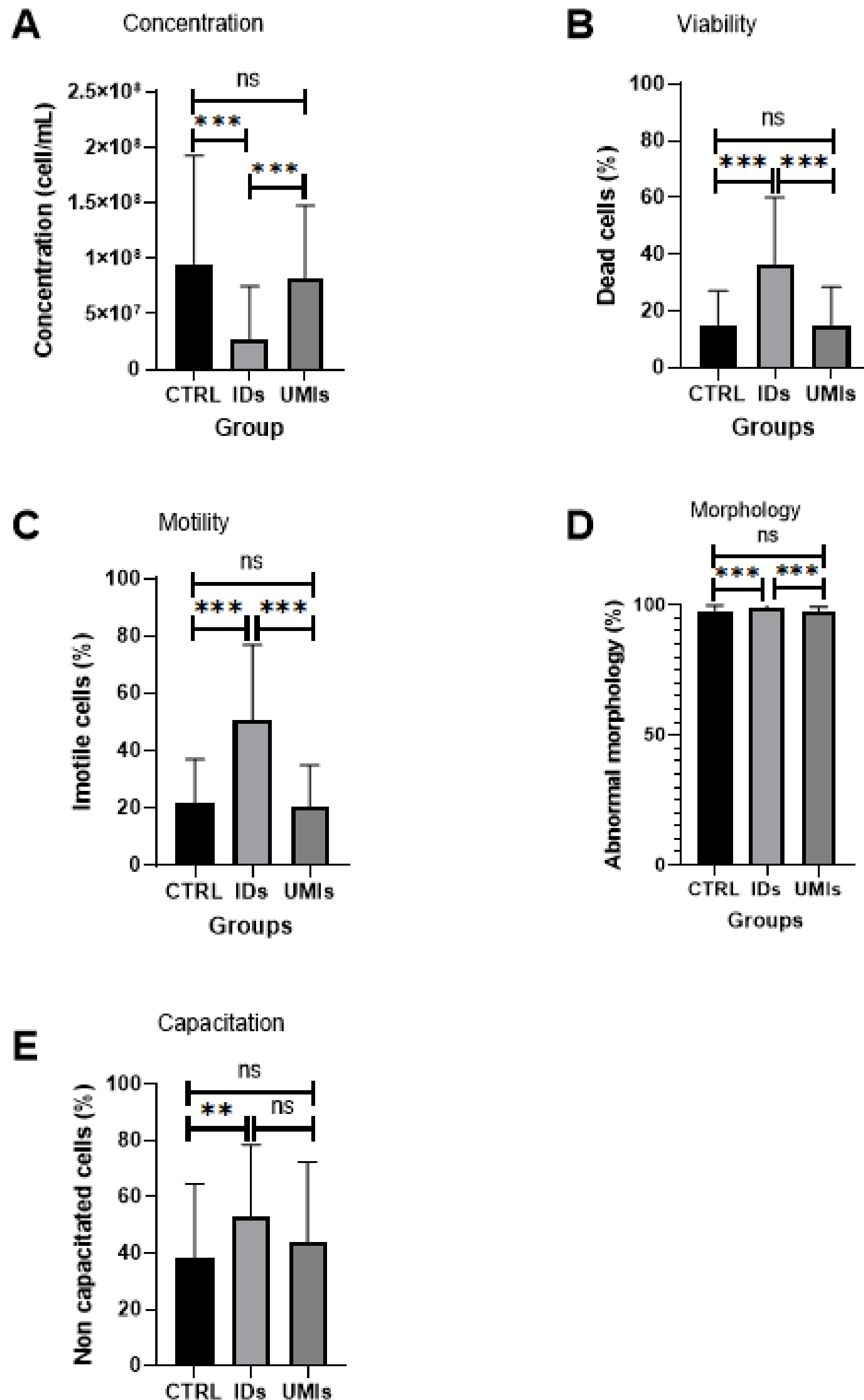

Figure 11 – Seminal quality in the three study groups (CTRL, IDs, and UMIs). **A:** Concentration (n=396; CTRL group=246; ID group=107; UMI group=43); **B:** Viability (n=395; CTRL group=246; ID group=106; UMI group=43); **C:** Motility (n=395; CTRL group=246; ID group=106; UMI group=43); **D:** Morphology (n=377; CTRL group=233; ID group=104; UMI group=40); and **E:** capacitation status (n=272; CTRL group=175; ID group=66; UMI group=31). The results are expressed as mean+SD for each of the assessed variables in which statistical differences were fond regarding the fertility status. Graphs were obtained by Graph Prism 9.0.0.; ns $p>0.05$, *$p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$. **CTRL:** Control group; **ID:** Idiopathic male infertility group; **UMI:** unexplained male infertility group.

2. Fertility status vs Psychologic state:

As for the effect of the psychological diagnosis status, significant statistical differences were found for Motility in Situ ($p \leq 0.05$) and Acrosome ($p \leq 0.05$), reported in **Figure 12** and **Supplementary table 3**. For both variables, the magnitude of differences was low. No statistical differences were found regarding the interactions between factors (**Supplementary table 3**).

The effect size was small for both Motility in Situ and Acrosome, with symptomatic participants (Yes) showing higher Motility in Situ and lower for Acrosome, reported in **Figure 12** (**Supplementary table 5**).

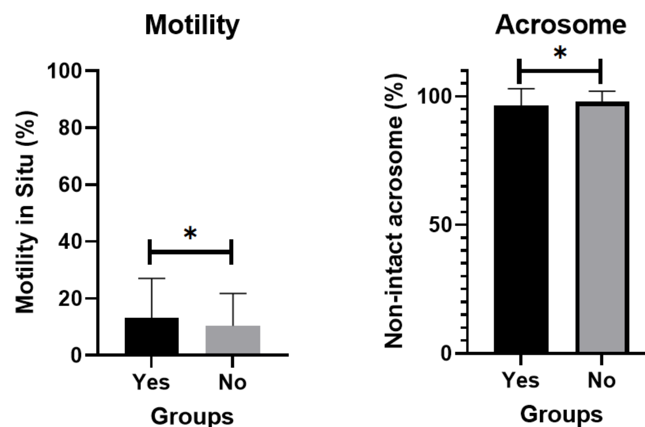

Figure 12 – Sperm parameters affected in men presenting anxiety and/or depression symptoms. Results are presented by mean+SD and between the two study groups (Yes and No) for each of the assessed variables in which statistical differences were found for diagnosis status, namely Motility (n=395; Yes group=104; No group=291) and Acrosome (n=291; Yes group=76; No group=215). Graphs were obtained by Graph Prism 9.0.0.; ns $p>0.05$, *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. **Yes:** symptomatic group; **No:** non-symptomatic group.

Chapter 2 – Seahorse Flux Analyzer optimization for sperm cells

1. Human sperm samples characterization

As previously mentioned, to ensure consistency in terms of standardizing the assay, only normozoospermic samples were used in this study, with all the parameters measured above the reference values established by WHO, except for morphology, where samples N3, N5, and N6 did not meet the normality (**Table 5**; WHO, 2010).

Table 5 - Human sperm samples characterization (concentration, motility, viability and morphology)

Sample	Concentration (cells/mL)	Motility (%)			Viability (%)		Morphology (%)	
		Progressive	In Situ	Imotile	Live	Dead	Normal	Abnormal
N1	$80x10^6$	62	19	19	84	16	5	95
N2	$59x10^6$	66	11	23	83	17	4	96
N3	$255x10^6$	75	14	11	86	14	1	99
N4	$102.5x10^6$	67	18	15	92	8	7	93
N5	$122.5x10^6$	34	36	30	77	23	3	97
N6	$137.5x10^6$	73	8	19	81	19	0	100
N7	$49x10^6$	68	4	28	82	18	4	96

2. Sperm metabolic profile

From the 7 samples used for OCR assay, only 3 were used in ECAR due to slight final optimizations, namely samples N3, N4, and N5. After optimization, concerning drugs concentration, amount of ConA solution per well, cell number, and Wave software modifications, it was concluded that the final established conditions (**Table 3** and **Table 4**) have allowed to obtain standard records for OCR and ECAR in human sperm cells. Although it is possible to observe an interindividual variation, typical of sperm samples

(**Figure 13** and **Figure 14**; Auger et al., 2000; A. P. Sousa et al., 2011), the records were still homogeneous.

3. OCR Assay

As expected, sperm cells responded by the decrease in OCR following oligomycin, antimycin A, and rotenone injections while an increase in OCR was observed following FCCP injection (**Figure 13A and C**).

Taking a closer look at the group, it is possible to observe that the aspect in which the response of the cells varies the most was after the FCCP injection stage (**Figure 13C**), with samples 1, 5 and 7 showing the highest responses.

Regarding the OCR metrics (**Table 1**), they indicate that sperm cells are bioenergetically healthy and efficient as the maximal respiration indicates that the cells can respond well to energetic challenges, the proton leak is low not affecting ATP production, and the coupling efficiency, indicative of the overall organelle functionality, is high (**Figure 13B** and **Figure 13D-K**).

Nevertheless, when looking at individual records, it was observed that samples were more homogeneous concerning basal OCR and maximal respiration, with the exception of sample N1, while the remaining metrics were more variable. Furthermore, sperm spare capacity was 2.35 to 4 times higher than basal respiration. Worth noting, once again, for samples 1, 5, and 7 higher values were recorded on this parameter.

Finally, in **Figure 13L**, it is possible to observe the ECAR measurements during the OCR assay. Although at first glance this seems to be out of importance, there still is relevant information that can be taken from this data. In fact, when analysing the stage following oligomycin injection, OCR decreases and ECAR increases.

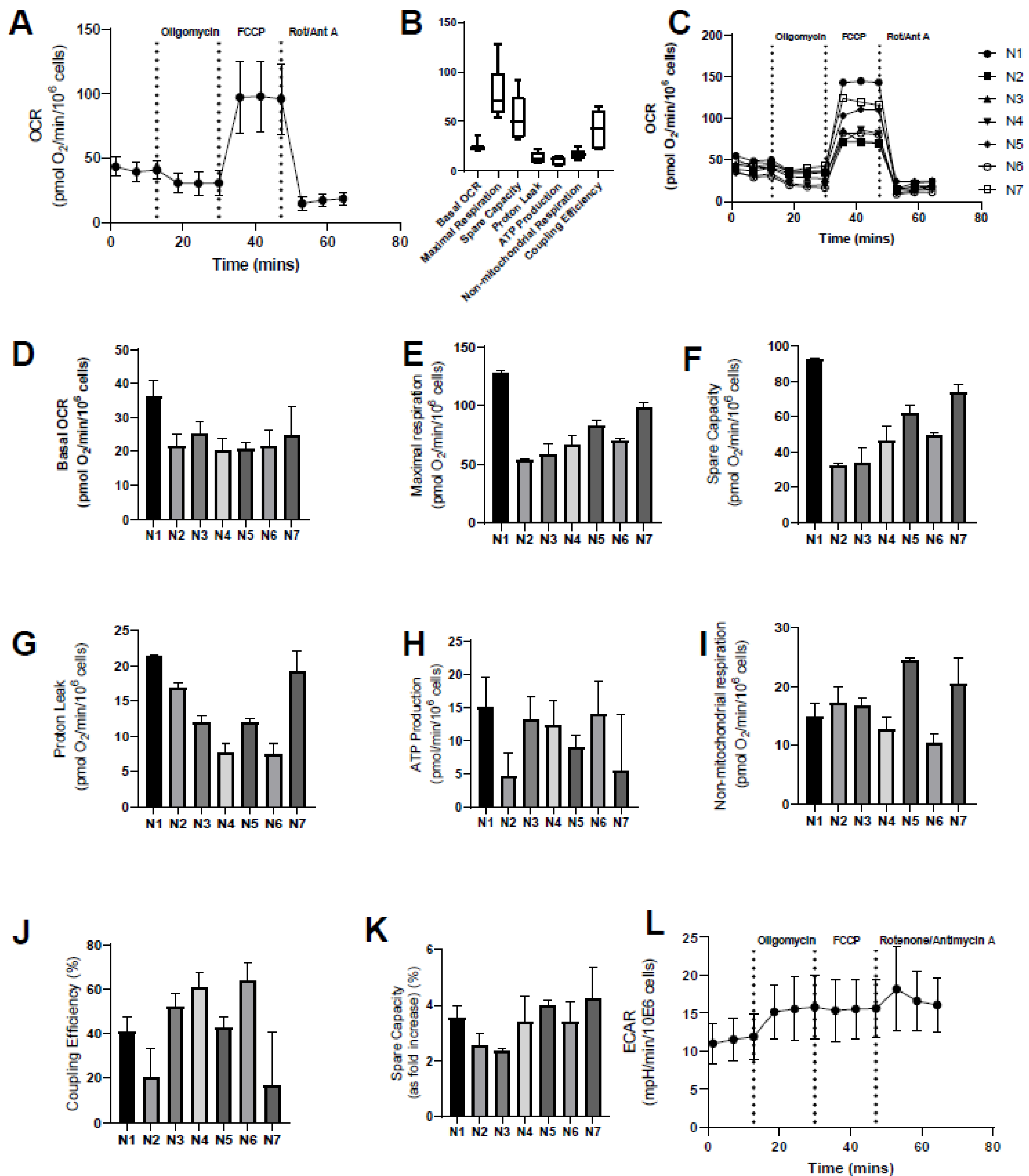

Figure 13 – Sperm oxidative metabolism was assessed through the OCR monitoring using the Seahorse Flux Analyzer. **A:** OCR profile of the 7 human sperm samples as a group (mean+SD); **B:** OCR-derived metrics of the 7 analysed samples as a group (mean+SD), namely Basal OCR, Maximal Respiration, Spare Capacity, Proton Leak, ATP Production, Non-mitochondrial Respiration, and Coupling Efficiency; **C:** individual OCR profile of the 7 analysed samples; **D-K:** individual OCR-derived metrics for the 7 analysed samples (mean+SD), namely Basal OCR, Maximal Respiration, Spare Capacity, Proton Leak, ATP Production, Non-mitochondrial Respiration, Coupling Efficiency, and Spare Capacity (as fold increase), respectively; **L:** ECAR profile correspondent to the OCR assay of the 7 analysed samples as a group (mean+SD).

4. ECAR Assay

In this assay, sperm cells have responded with an increase in ECAR following both glucose and oligomycin injections and a decrease after 2DG injection (**Figure 14A** and **Figure 14C**).

Regarding the ECAR metrics (**Table 2**), overall, they indicate that our cells are glycolytically active with a good glycolytic capacity and reserve (**Figure 14B-F**).

Looking at the group it is possible to observe that despite the typical interindividual variances, the metabolic parameters such as glycolysis and glycolysis capacity seem to be homogeneous. Regarding glycolytic reserve, the variations among samples seem to be higher (**Figure 14F**) with samples N3 and N4 showing the highest and the smallest values, respectively.

As for glycolytic reserve, records show that samples range from 1.25 to 1.56 times higher than that their respective measurement for basal ECAR. Worth noting, sample 2 presented the highest record on this parameter.

In **Figure 14I**, similarly to what we have shown above, it is possible to observe the OCR measurements for the same wells. Analysing the stage following glucose injection, it is possible to observe that while ECAR increases the OCR slightly decreases. Following the oligomycin injection, ECAR is further increased and the respective OCR decreases pronouncedly.

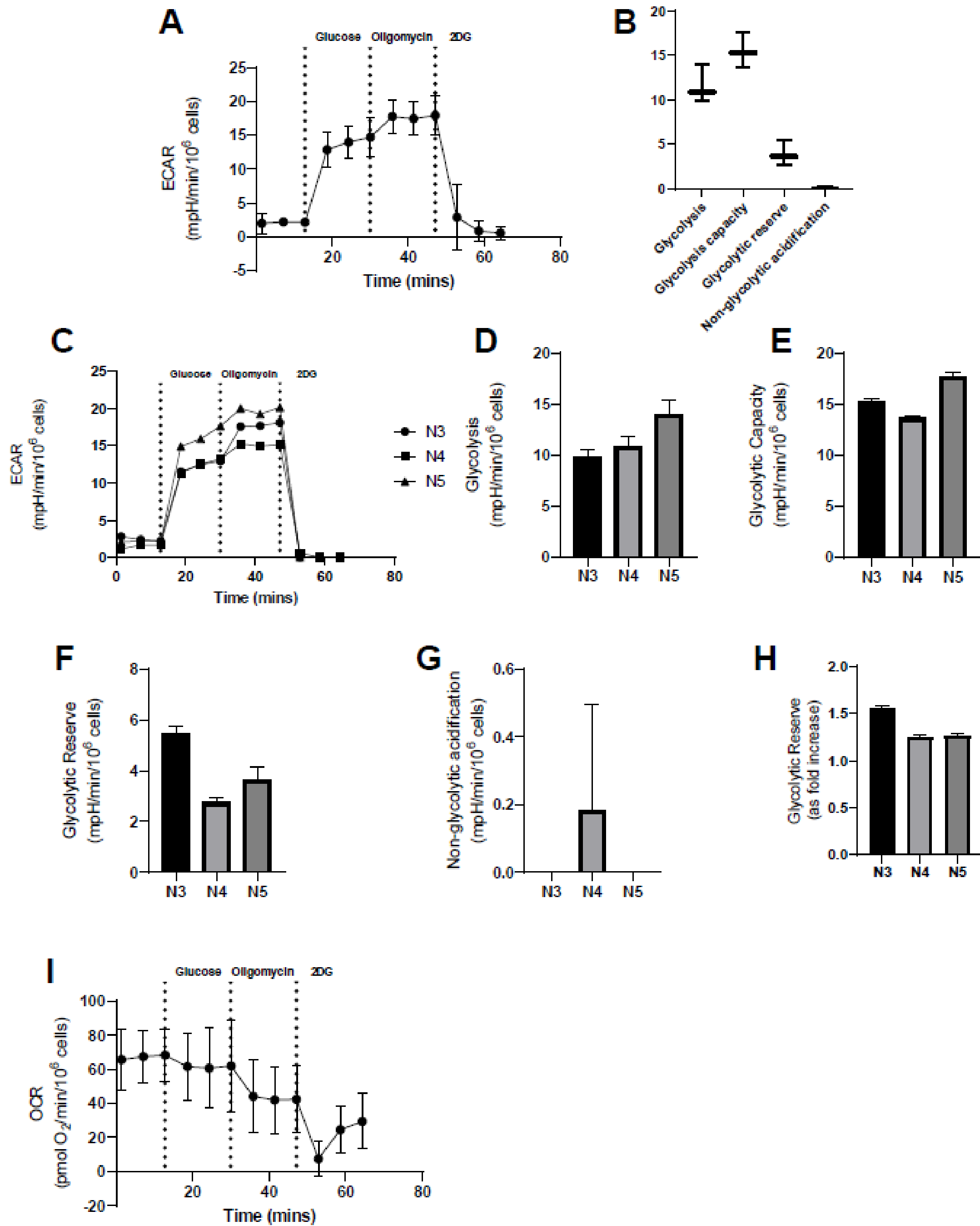

Figure 14 - Sperm glycolytic metabolism was assessed through the ECAR monitoring using the Seahorse Flux Analyzer. **A:** ECAR profile of the 3 analysed samples as a group; **B:** ECAR-derived metrics of the 3 analysed samples as a group, namely Glycolysis, Glycolytic capacity, Glycolytic reserve, and Non-glycolytic acidification; **C:** individual ECAR profile of the 3 analysed samples; **D-H:** individual ECAR-derived metrics of the 3 analysed samples, namely Glycolysis, Glycolytic capacity, Glycolytic reserve, Non-glycolytic acidification, and Glycolytic reserve (as fold increase), respectively; **I:** OCR profile correspondent to the ECAR assay of the 3 analysed samples as a group.

5. Correlations

After the analysis from the obtained records for OCR and ECAR, we looked for any existing underlying correlations between seminal parameters and OCR- and ECAR-derived metrics (**Supplementary table 6** and **Supplementary table 7**).

Correlations with statistical significance concerning OCR were identified between: Concentration and Coupling Efficiency ($p \leq 0.05$); Basal OCR and Proton Leak ($p \leq 0.05$); Spare capacity with both Maximal Respiration ($p \leq 0.05$) and Spare Capacity ($p \leq 0.05$); and Proton Leak with Coupling Efficiency ($p \leq 0.05$).

Regarding ECAR metrics, we found statistically significant correlations between: Concentration with both Glycolytic Reserve ($p \leq 0.001$) and Glycolytic Reserve (as fold increase) ($p \leq 0.001$); Glycolysis with Progressive Motility ($p \leq 0.001$), Motility *in Situ* ($p \leq 0.001$), and Total Motility ($p \leq 0.001$); Glycolytic Capacity with Live cells ($p \leq 0.001$); and Normal Morphology with both Glycolytic Reserve ($p \leq 0.001$) and Glycolytic Reserve (as fold increase) ($p \leq 0.001$).

Discussion

As stated before, male infertility can be seen in two dimensions, one related to the cases to which a cause has been acknowledged, and another to the cases of unknown origin infertility (Ashok Agarwal et al., 2021; Hamada et al., 2011; Marzano et al., 2020; Ramalho-Santos et al., 2009; Thonneau et al., 1991). The current available diagnosis tools are not able to provide enough answers that allow to find the infertility cause of the individuals falling into the latter dimension (Barratt et al., 2017; Pandruvada et al., 2021). It seems therefore clear that a more comprehensive assessment of the sperm function is in need for diagnosis purposes (Barratt et al., 2017; Pandruvada et al., 2021).

In this study, we report the use of a recent methodology on human sperm that provides, in real time, direct insights into sperm cells metabolism, a crucial aspect for sperm functionality, as several studies have already demonstrated (Sandra Amaral et al., 2013; du Plessis et al., 2015; Ramalho-Santos et al., 2009; Ramalho-Santos & Amaral, 2013; Ruiz-Pesini et al., 2000; A. P. Sousa et al., 2011; Taniguchi et al., 2022). Furthermore, concerning the psychological side of the equation, the existing studies have reported contradictory findings (Fernandes et al., 2021; Sánchez González et al., 2023; Schneid-Kofman & Sheiner, 2005; Zorn et al., 2008), and even though an overlap of regulatory pathways and regulatory centres exists (Nargund, 2015; Sánchez González et al., 2023), the connection between psychological stress and sperm features remains to be clarified (Nargund, 2015; Schneid-Kofman & Sheiner, 2005). Hence the need to systematically assess patients' psychological status to further clarify this connection and eventually apply this knowledge to better diagnose patients in an infertility setting. This will certainly allow to have a broader picture of the inner workings of the male reproductive system and fertility status.

Chapter 1 – Is the presence of depressive and anxiety symptoms associated with sperm quality?

In this part of the study, we have relied on a retrospective analysis in order to understand how the various sperm parameters assessed in three groups of individuals

with different fertility states (Control, ID, and UMI) correlate with the presence of psychological symptoms of anxiety and depression, assessed by the HADS survey answered by all the entailed individuals.

Regarding the sperm functionality, we took advantage of this data basis, that is unique in the sense that compares well-characterized groups of individuals, including controls and takes in consideration the female factor, to study deeper aspects that might be altered in unknown origin male infertility. In fact, the existent literature on this type of infertility is not very clear as the control groups are not always well defined, or when they are they are only compared with one of the types of Unknown origin male infertility (either ID or UMI), and also because the terms ID and UMI are often used indiscriminately, not always meaning the same. Finally the fact that the female factor is sometimes disregarded also contributes to the gaps and riddles in the literature on this topic, compromising the real knowledge on both types of infertility (Corsini et al., 2022).

Yet, although the data set used in here results from the analysis of several parameters that are not routinely evaluated, none of them have provided strong evidence as a differentiating factor among CTRL and UMI Group, leaving margin for further studies focused on more molecular aspects, such as proteomics. Nevertheless, and according to the literature, the ID group presented the worst scenario in terms of seminal analysis, when compared to Control and UMI groups. This was expected, given that ID group, as mentioned before, is a group typically characterized by an abnormal seminal analysis under the reference values (WHO, 2010) and these results were further corroborated by the results obtained in the new parameters evaluated, such as for the capacitation status, also affected in ID patients.

Additionally, although no clear differences were identified when comparing the CTRL and the UMI groups, it seems to exist an indication that the capacitation might be an aspect in which they differ, although this needs further clarification in a bigger cohort.

In the literature, although some alterations have been identified in ID and UMI patients, to our knowledge only one study that came out last year have studied these three groups simultaneously, in a case control retrospective analysis similar to the one presented here at the scope of this thesis (Corsini et al., 2022). In that study, and supporting our aim and results, Corsini (2022) and colleagues, have stressed the need to further distinguish between male infertility groups (ID and UMI), to better address each one clinically, and also that hidden female factors are frequently disregarded. Concerning

ID cases, several studies have reported impaired seminal parameters (Heidary et al., 2019; Jayaraman et al., 2014; Khadem et al., 2014) in accordance to our results. Furthermore, alteration on DNA integrity (A. Agarwal, 2003; Elshal et al., 2009), as well as increased oxidative stress and reduced antioxidant machinery (Sandra Amaral et al., 2006; Baker & Aitken, 2004; Fraczek et al., 2022), altered endocrine profiles, namely on what concerns testosterone levels (Bobjer et al., 2016; Skakkebaek et al., 2016; Yucra et al., 2006) have also been reported. Additionally, proteomic studies have identified some differentially expressed proteins, mainly related to flagellum structural aspects, metabolic and oxidative stress-related events. However, the results were obtained in an experimental setting only comparing to control individuals (Fu et al., 2019; Gholami et al., 2018; Hernández-Silva et al., 2020). Regarding UMI cases, the existing literature is shorter and often contradictory, but it is believed that an increase in ROS levels and DNA damage in these patients might be behind their infertility state, aspects not evaluated in the present work (Mayorga-Torres et al., 2013; Zandieh et al., 2018). Nevertheless, the most well-designed studies were based on proteomic analysis (A. Amaral et al., 2013; A. Amaral, Paiva, et al., 2014b; Fu et al., 2019; Gholami et al., 2018; Hernández-Silva et al., 2020) with the identification of several differently expressed proteins, comparing to control groups, essentially related to sperm functional aspects (e.g. capacitation), fertilization ability and also to oxidative stress related pathways. Yet, once again not in a 3 front comparison also including the ID group.

Worth mentioning, our laboratory is now conducting a thorough proteomic analysis that has so far identified nearly 145 differentially expressed proteins (data not shown) among the 3 study groups, having the potential to add information on these topics. Also, future studies, gathering more UMI samples might further help to clarify what distinguish those two groups.

Herein, our central question however, was to clarify if the fertility status might somehow be related to the psychological one, namely in terms of the presence of anxiety and depression symptoms.

Much has been the debate as to whether depression and anxiety play a role in male fertility, especially considering the overlap of signalling and processing centres in specific brain regions. Even though, in this study it was not possible to study depression and anxiety separately due to a very reduced number of participants in each of the subscales and statistical differences were only found for those who are 'Borderline

Abnormal' or 'Abnormal' to suffer from at least one of the psychological distress conditions. It would be expected to see clear differences between individuals who can produce offspring and those who cannot, yet that was not the case, warranting further studies in larger cohorts and considering more molecular targets and approaches.

Nevertheless, the conducted studies so far have described decreased serum testosterone levels and sperm concentration (Delhez et al., 2003; Zorn et al., 2008) for those suffering from depression, whereas for anxiety increased FSH and LH levels (Bak et al., 2012) and AR inhibition were reported (Sánchez González et al., 2023). Even though, associations have been identified, to our knowledge no study have yet pinpointed a clear connection between infertility diagnosis and psychological stress. Also, the cohorts on the existing studies are usually reduced and/or biased due to self-reporting.

Herein, after performing a detailed statistical analysis we have concluded that patients from 'Yes' group (symptomatic) showed higher proportion of Motility *in Situ* and intact acrosomes, compared to those pertaining to the 'No' group (non-symptomatic). Concerning Motility *in Situ* results, it was expected to be higher in the context of psychological stress, for example, Bhongade (2015) have observed overall reduced motility in patients scoring 8 or higher in HADS. Even though Bhongade (2015) have only reported for reduced progressive motility, meaning that non-progressive motility (*in Situ*) and immotile sperm cells are favoured in psychological stress context.

Regarding the acrosome integrity, Sánchez González (2023) and collaborators have very recently demonstrated that the presence of circulating cortisol molecules in patients with anxiety has an inhibitory effect upon AR and might explain the compromised AR in patients facing psychological stress, in line with our current results (Sánchez González et al., 2023).

Although this study presents promising results and conclusions, certain limitations are present. First, the different number of participants across groups (reaching a minimum in the UMI group) prevented a more thorough and robust analysis. Second, it was not possible to assess the effects that depression and anxiety separately exert over the reported biologic parameters. Third, in the current study it was not possible to further clarify the aspects in which the Control and UMI groups differ, justifying further studies.

Chapter 2 – Seahorse Flux Analyzer optimization for sperm cells

Regarding the second part of this work, that has as a connection point to the first part in the clear need to improve the evaluation and diagnosis of infertile men, especially for those in which a cause to their infertility was not found, our results were very satisfactory.

Sperm cells are one of the few cell types submitted to environmental dramatic changes throughout their life span, in addition of being motile and presenting species-specific particularities. These idiosyncrasies present additional difficulties/challenges in thoroughly studying sperm metabolism changes in each phase. Sperm cells are initially stored in an inactive state in epididymis, metabolically aided in this environment by several cargo-delivery molecules (Brooks, 1983). Once ejaculation occurs, sperm cells must instantly begin producing their own energy to sustain motility and basic functions. Therefore, it is of paramount importance to assess sperm metabolism as this seems to be a likely factor determining sperm functionality, viability and survival, and consequently fertilization capacity, which might help to uncover underlying molecular mechanisms of infertility.

In this sense, the SFA offering the possibility to monitor and study in detail the two main metabolic pathways for energy attainment, in real-time, constitutes an unvaluable tool. Furthermore, the optimization of this technique can be seen as a powerful tool to aid not only in the distinction of fertility statuses or to provide information on metabolic changes associated with other diseases or environmental factors impacting fertility, but also in the future development of new and more targeted therapies to different types of male infertility.

Although previous studies have used this technique to characterize or assess bioenergetic profiles of other cell types (Abe et al., 2010; Xia Gu et al., 2021; Jin et al., 2019; Mookerjee et al., 2016; Plitzko & Loesgen, 2018; M. I. Sousa et al., 2020; van der Windt et al., 2016; H. Zhang et al., 2021; J. Zhang & Zhang, 2019), a comparison to our study on sperm cells is not linear, as besides the fact that other cell types are different in terms of ploidy and motility, the presented results are not 100% comparable mainly due to the performed normalizations that are highly variable. Moreover, although in general the obtained records are similar (Abe et al., 2010; Jin et al., 2019; Mookerjee et al., 2016;

Plitzko & Loesgen, 2018; Taniguchi et al., 2022; van der Windt et al., 2016; H. Zhang et al., 2021), it seems that sperm cells have overall lower metabolic rates than most cellular types. Indeed, sperm cells appear to have lower metabolic rates than not only undifferentiated and highly proliferative cells, such as stem cells or cancer cells (Choi et al., 2020; Ju et al., 2020; Y. Liu et al., 2022; Mitov et al., 2017; Varum et al., 2011), but also differentiated somatic cells, such as neuronal or cardiac cells (Kokkinaki et al., 2019; Meyer et al., 2020; Sun et al., 2017; Xu et al., 2014). Interestingly, their metabolic metrics seem more similar to the ones from quiescent cells (O'Brien et al., 2021; Son et al., 2017). Further work should be developed to draw more definitive conclusions, but it is still important to stress the need of normalizing the presentation of these results.

Regarding the existing studies using this approach on sperm cells they are very limited, with some publications on murine sperm to study metabolic alterations during important processes such as capacitation (Balbach, Buck, et al., 2020; Balbach, Gervasi, et al., 2020; Hidalgo et al., 2020; Tourmente et al., 2022), or to study metabolic details such as the importance of certain metabolites to infertility, as betaine (A. R. Johnson et al., 2012). On bovine sperm (Magdanz et al., 2019; Santos et al., 2022) the SFA has confirmed the oxidative nature of sperm metabolism in this species (Garrett et al., 2008; Madeja et al., 2021; Moraes et al., 2021). In humans, this scenario is even worst, and, to our knowledge, this methodology has only been described in one study (Taniguchi et al., 2022).

Herein, we believe that we have optimized and importantly take benefit, for the first time, from all the potential of the seahorse metabolic flux analyser for the human sperm cells analysis, characterizing both their oxidative and glycolytic profile.

Our results were comparable to those obtained by Taniguchi (2022) and colleagues, in which the impact of different storage temperatures on sperm metabolism was studied. However, besides the different experimental setting, the authors have only used 3 samples and uniquely assessed OCR.

Importantly, we have clearly identified some particularities of human sperm, that completely fit what has been described so far on their metabolism. First, the previously reported interindividual variation in terms of samples (Auger et al., 2000; A. P. Sousa et al., 2011) was clearly mirrored in metabolic terms. Secondly, we have obtained indications that corroborate the active use of both pathways as well as some metabolic particularities on the sperm cell. In fact, following oligomycin injection we have

observed that a decrease in OCR was accompanied by an increase in ECAR, suggesting that the blocking on OXPHOS, will be compensated by a higher glycolytic activity. Furthermore, our ECAR records have highlighted a detail not evident in other reports. In fact, although the sperm cells have responded to oligomycin in the OCR assays, that did not happen always/or so expressively in the ECAR assays. We believe that this is a singular metabolic trait of the human sperm cells, that are probably running glycolysis already at a higher rate, warranting further studies in the topic to completely confirm this indication.

Furthermore, in general we have concluded that the metabolic profile of our cells was not much different from that of other species such as the mouse or the bull, with typical responses after the several drugs injections (Balbach, Gervasi, et al., 2020; Madeja et al., 2021; Moraes et al., 2021; Tourmente et al., 2015). This is not unexpected as, similarly to the human sperm cells, and despite controversies on the preferred/main pathway for energy attainment (Balbach, Gervasi, et al., 2020; Madeja et al., 2021; Moraes et al., 2021; Tourmente et al., 2015), mouse and bull's sperm cells, are capable of using both OXPHOS and glycolysis (Balbach, Gervasi, et al., 2020; Madeja et al., 2021; Moraes et al., 2021; Tourmente et al., 2015).

Nevertheless, this kind of analysis will always provide important information. As an example of its potential, it has been show, by SFA, that in the rodent genus Mus, the relative relevance of the OXPHOS and glycolysis is species-specific and that the oxidative pathway was favoured in species with higher sperm competition with the suggestion that, in this competitive scenario, adaptations in sperm metabolism will be promoted favouring the use of OXPHOS in relation to glycolysis, as this pathway will assure the energetic fulfilments associated with the need to move more and faster (Tourmente et al., 2015). Nevertheless, in other species, such as the bull, the OXPHOS seems to be the main energy source for sperm in better quality samples (Garrett et al., 2008; Madeja et al., 2021; Moraes et al., 2021). In either case, one should not forget that the oviduct environment has a crucial role in the final choice of the pathway for energy generation and that the quantity and how quickly the ATP is needed might also have a role on the preferred metabolic pathway choice (A. Amaral, 2022). This data definitely opens the road for several future studies on humans.

Altogether, the results suggest that both pathways are properly functioning in the human sperm cells, becoming noticeably clear after oligomycin injection in both assays.

But, even though more studies are needed to clarify this riddle (Ramalho-Santos et al., 2009), based on the present results, and accordingly to the most recent literature, it might be suggested that sperm cells benefit from a balance between both pathways, which seems the most plausible hypothesis given their natural metabolic versatility.

Importantly, the results obtained in this study might have several applications/implications in the future, contributing for the implementation of a more systematic assessment of depression and anxiety on patients undergoing fertility treatments, as well as to apply seahorse methodology to human sperm cells in various contexts, including in patients undergoing psychologic stress.

Notably, the application of the seahorse methodology to human sperm cells may become a highly valuable tool to further study metabolic shifts and/or alterations in different contexts such as specific types of infertility, after environmental contaminants exposure, after or during urogenital infections, under the effect of different medications and therapies, among others.

Finally, our global results will certainly help to further characterize and understand the molecular mechanisms of infertility of unknown origin working on the way to develop new therapies, further promoting reproductive health and better fertilization rates worldwide.

- **Limitations:**

Even in the presence of promising results and conclusions, this study has certain limitations. First, sperm cells are highly heterogeneous cells, given their sensitive development and nature. Second, the number of samples are very reduced, not representative of a population, especially in ECAR assay. Third, the current experimental design does not mimic effects *in vivo* or on large timescales.

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Supplementary table 1 - Normality and homogeneity of variances across the assessed seminal parameters

	S-W test		Levene (fertility)				Levene (diagnosis)				Levene (fertility status*diagnosis)			
	W	p	Z	df1	df2	p	Z	df1	df2	p	Z	df1	df2	p
Concentration	0.76	.000***	15.80	2	393	.000***	1.02	1	394	.313	6.69	5	390	.000***
Viability (Dead)	0.84	.000***	36.00	2	392	.000***	0.41	1	393	.524	14.10	5	389	.000***
Motility in Situ	0.80	.000***	8.69	2	392	.000***	4.17	1	393	.042*	5.36	5	389	.000***
Imotile	0.88	.000***	35.90	2	392	.000***	0.40	1	393	.529	14.60	5	389	.000***
Abnormal morphology	0.85	.000***	13.00	2	374	.000***	0.31	1	375	.577	4.35	5	371	.000***
Chromatin (Dark)	0.97	.000***	1.01	2	375	.364	0.16	1	376	.693	0.54	5	372	.745
Acrosome (NI)	0.57	.000***	2.19	2	288	.114	4.49	1	289	.035	1.84	5	285	.105
Capacitation (NM)	0.95	.000***	0.17	2	269	.845	0.22	1	270	.637	0.69	5	266	.635

Supplementary table 2 - Descriptive statistics for fertility status groups and diagnosis groups across the assessed seminal parameters

		Fertility Status (Group*diagnosis)											
		Control			ID			UMI			Total		
		n	M	SD	n	M	SD	n	M	SD	n	M	SD
Concentration													
Diagnosis	Yes	64	102226563.0	104484674.00	26	44001923.0	79089655.00	14	99321429.0	87863226.00	104	87379327	98957717
	No	182	95985440.0	96893122.00	81	22163580.0	32925868.00	29	77337931.0	53217689.00	292	73655479	86378347
	Total	246	97609146.0	98742115.00	107	27567290.0	48839561.00	43	84495349.0	66228633.00			
Viability: Dead													
Diagnosis	Yes	64	15.9	14.29	26	37.3	21.86	14	14.0	14.76	104	21.0	18.93
	No	182	14.8	11.29	80	35.0	23.31	29	14.1	12.61	291	20.3	18.05
	Total	246	15.1	12.12	106	35.6	22.88	43	14.1	13.17			
Mobility in Situ													
Diagnosis	Yes	64	16.4	15.71	26	6.8	7.54	14	10.4	8.32	104	13.2	13.83
	No	182	12.3	11.40	80	7.4	11.74	29	6.7	7.11	291	10.4	11.39
	Total	246	13.4	12.75	106	7.2	10.83	43	7.9	7.63			
Immotile													
Diagnosis	Yes	64	21.5	13.74	26	51.5	24.01	14	19.9	17.07	104	28.8	21.57
	No	182	21.8	14.76	80	50.0	26.91	29	20.0	13.50	291	29.4	22.65
	Total	246	21.7	14.48	106	50.4	26.13	43	20.0	14.55			
Abnormal morphology													
Diagnosis	Yes	59	98.3	2.36	26	99.0	1.39	13	97.1	2.10	98	98.3	2.17
	No	174	97.7	1.98	78	99.2	1.45	27	97.2	2.20	279	98.1	2.00
	Total	233	97.8	2.09	104	99.1	1.43	40	97.2	2.14			
Chromatin (Dark)													
Diagnosis	Yes	59	53.8	23.08	26	51.6	21.95	13	45.3	19.64	98	52.1	22.32
	No	175	46.1	23.73	78	53.6	21.68	27	46.3	24.61	280	48.2	23.43
	Total	234	48.0	23.76	104	53.1	21.66	40	46.0	22.86			
Acrosome (NI)													
Diagnosis	Yes	44	96.9	5.99	19	96.9	6.94	13	94.2	7.85	76	96.5	6.57
	No	145	97.7	4.32	50	98.7	2.62	20	97.1	5.36	215	97.9	4.12
	Total	189	97.5	4.76	69	98.2	4.28	33	95.9	6.50			
Capacitation (NM)													
Diagnosis	Yes	41	38.5	30.22	18	50.7	19.88	12	39.3	30.43	71	41.8	28.12
	No	134	37.9	25.40	48	51.7	26.74	19	44.7	27.27	201	41.9	26.43
	Total	175	38.1	26.52	66	51.4	24.91	31	42.7	28.15			

Supplementary table 3 - Analysis of variance to assess differences between fertility status groups, differences between diagnosis groups, and interaction between these two factors for each of the assessed seminal parameters

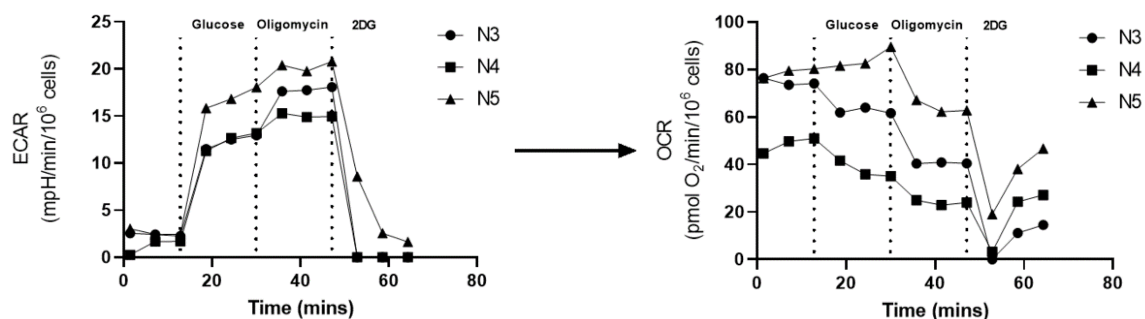
	F	df1	df2	p	Bootstrap Adj.p	Part. ηp2
Concentration						
Intercept	232.74	1	390	.000		
Fertility status	21.23	2	390	.000	.000***	.116
Diagnosis	0.26	1	390	.613	.207	.004
Interaction	0.32	2	390	.728	.717	.002
Viability (Dead)						
Intercept	157.13	1	389	.000		
Fertility status	47.55	2	389	.000	.000***	.000
Diagnosis	0.24	1	389	.628	.512	.512
Interaction	0.08	2	389	.925	.932	.932
Imotile						
Intercept	255.35	1	389	.000		
Fertility status	69.27	2	389	.000	.000***	.329
Diagnosis	0.01	1	389	.913	.950	.000
Interaction	0.06	2	389	.937	.932	.000
Motility in Situ						
Intercept	199.18	1	389	.000		
Fertility status	6.47	2	389	.002	.000***	.058
Diagnosis	5.86	1	389	.016	.037*	.012
Interaction	1.13	2	389	.324	.322	.006
Abnormal Morphology						
Intercept	442900.00	1	371	.000		
Fertility status	19.28	2	371	.000	.000***	.106
Diagnosis	4.21	1	371	.041	.208	.005
Interaction	1.32	2	371	.269	.284	.007
Chromatin (Dark)						
Intercept	699.51	1	372	.000		
Fertility status	2.97	2	372	.052	.107	.012
Diagnosis	4.90	1	372	.027	.147	.006
Interaction	1.43	2	372	.240	.246	.008
Acrosome (NI)						
Intercept	58641.97	1	285	.000		
Fertility status	1.08	2	285	.341	.085	.017
Diagnosis	0.82	1	285	.366	.038*	.014
Interaction	0.70	2	285	.496	.495	.005
Capacitation (NM)						
Intercept	275.33	1	266	.000		
Fertility status	4.93	2	266	.008	.001**	.044
Diagnosis	0.02	1	266	.898	.847	.000
Interaction	0.16	2	266	.856	.851	.001

Supplementary table 4 - Post-Hoc tests to assess differences amongst fertility status groups for all seminal variables

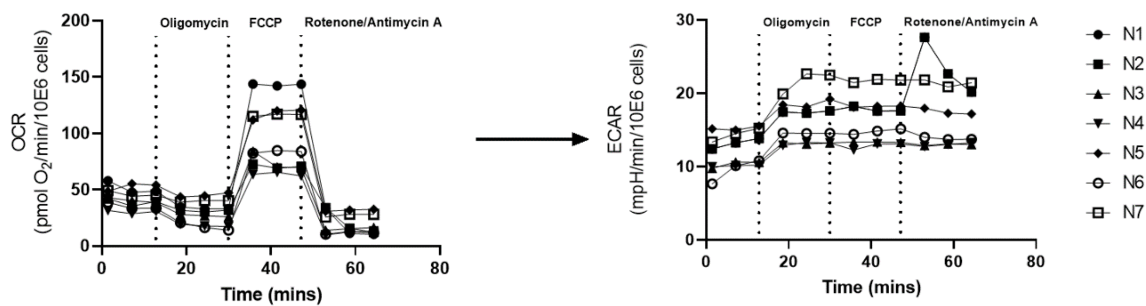
	Post-Hoc Test			Bootstrap 95% CI	
	Mean differences	p	d	Lower	Upper
Concentration					
Control*ID	-70041857.00	.000***	.899	0.62	1.08
Control*UMI	-13113798.00	.516	.156	-0.19	0.41
ID*UMI	56928059.00	.000***	-.978	-1.30	-0.50
Viability (Dead)					
Control*ID	20.50	.000***	-1.12	-1.37	-0.89
Control*UMI	-0.95	.898	0.08	-0.29	0.41
ID*UMI	-21.50	.000***	1.15	0.79	1.50
Imotile					
Control*ID	28.60	.000***	-1.35	-1.61	-1.07
Control*UMI	-1.75	.747	0.12	-0.23	0.43
ID*UMI	-30.40	.000***	1.44	1.05	1.80
Motility in Situ					
Control*ID	-6.14	.000***	0.52	0.15	0.76
Control*UMI	-5.48	.001**	0.52	0.23	0.79
ID*UMI	0.66	.908	-0.07	-0.47	0.25
Abnormal morphology					
Control*ID	1.31	.000***	-0.73	-0.96	-0.49
Control*UMI	-0.67	.165	0.32	-0.02	0.69
ID*UMI	-1.98	.000***	1.09	0.66	1.55
Capacitation (NM)					
Control*ID	13.40	.002**	-0.52	-0.82	-0.22
Control*UMI	4.57	.373	-0.17	-0.56	0.28
ID*UMI	-8.79	.252	0.33	-0.17	0.80

Supplementary table 5 - d of Cohen for diagnosis

	d	Bootstrap 95% CI Lower	Bootstrap 95% CI Upper
Motility in Situ	-.22	-0.43	-0.01
Acrosome (NI)	.26	0.02	-0.50



Supplementary figure 1 - Sperm glycolytic metabolism was assessed through the ECAR monitoring using the Seahorse Flux Analyzer. Glycolysis assay on the left (mean+SD) and corresponding OCR measurements on the right (mean+SD) both by samples.



Supplementary figure 2 - Sperm oxidative metabolism was assessed through the OCR monitoring using the Seahorse Flux Analyzer. OXPHOS assay on the left (mean+SD) and corresponding ECAR measurements on the right (mean+SD) both by samples.

Supplementary table 6 - Correlations between important seminal parameters and OCR metrics

	C	PM	MiS	TM	L	NM	BO	MR	SC	PL	ATPT	NMO	SCFC	CE
C		.39	.29	.52	.04	-.63	-.14	-.29	-.29	-.64	.54	-.36	-.46	.82*
PM			-.68	.61	.29	-.47	.18	-.36	-.36	-.29	.18	-.43	-.43	.36
MiS				.00	.11	.29	-.14	.18	.18	.00	.29	.14	.00	.18
TM					.81*	.04	.18	-.38	-.38	-.22	.56	-.72	-.74	.58
L						.56	.18	-.32	-.32	.14	.21	-.43	-.61	.14
NM							-.05	.20	.20	.41	-.13	-.02	.13	-.34
BO								.29	.29	.79*	.32	.07	-.04	-.46
MR									1.00	.39	.39	.11	.86*	-.25
SC										.39	.39	.11	.86*	-.25
PL											-.11	.43	.25	-.86*
ATPT												-.68	-.07	.57
NMO													.39	-.71
SCFC														-3.9
CE														

Notes: n=7. The seminal parameters are Concentration (C), Progressive Motility (PM), Motility in Situ (MiS), Total Motility (TM), Live cells (L), and Normal Morphology (NM). The OCR metrics are Basal OCR (BO), Maximal Respiration (MR), Spare Capacity (SC), Proton Leak (PL), ATP Turnover (ATPT), Non-mitochondrial respiration (NMO), Spare capacity (SCFC), and Coupling Efficiency (CE). (*p≤0.05, ** p≤0.01, *** p≤0.001).

Supplementary table 7 - Correlations between important seminal parameters and ECAR metrics

	C	PM	MiS	TM	L	NM	G	GC	GR	GRC	NGA
C		.39	.29	.52	.04	-.63	-.50	.50	1.00***	1.00***	-.87
PM			-.68	.61	.29	-.47	-1.00***	-.50	.50	.50	.00
MiS				.00	.11	.29	1.00***	.50	-.50	-.50	.00
TM					.81*	.04	-1.00***	-.50	.50	.50	.00
L						.56	-.50	-1.00***	-.50	-.50	.87
NM							.50	-.50	-1.00***	-1.00***	.87
G								.50	-.50	-.50	.00
GC									.50	.50	-.87
GR										1.00***	-.87
GRC											-.87
NGA											

Notes: n=3. The seminal parameters are Concentration (C), Progressive Motility (PM), Motility in Situ (MiS), Total Motility (TM), Live cells (L), and Normal Morphology (NM). The ECAR metrics are Glycolysis (G), Glycolysis Capacity (GC), Glycolytic Reserve (GR), Glycolytic reserve (GRC), and Non-Glycolytic Acidification (NGA). (*p≤0.05, ** p≤0.01, *** p≤0.001).