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SpermChannels: CFTR and Aquaglyceroporins as key modulators of sperm function

João Pedro Cardoso Ribeiro

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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SpermChannels: CFTR and Aquaglyceroporins as key modulators of sperm function

Tese de candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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“Well, how shall I start speaking to them? What word should I begin with, so that they understand at least something?' I was so afraid, but I was more afraid for you, terribly, terribly afraid! And yet how could I be afraid, wasn't it shameful to be afraid? What of it, if for one advanced person there are such myriads of backward and unkind ones? This is precisely my joy, that I'm now convinced that it's not so at all, and that there is living material! Nor is there any embarrassment in the fact that we're ridiculous, isn't that true? For it's actually so, we are ridiculous, light-minded, with bad habits, we're bored, we don't know how to look, how to understand, we're all like that, all, you, and I, and they! Now, you're not offended when I tell you to your face that you're ridiculous? And if so, aren't you material? You know, in my opinion it's sometimes even good to be ridiculous, if not better: we can the sooner forgive each other, the sooner humble ourselves; we can't understand everything at once, we can't start right out with perfection! To achieve perfection, one must first begin by not understanding many things! And if we understand too quickly, we may not understand well. This I tell you, you, who have already been able to understand...and not understand ...so much." – **Prince Myshkin**

In “*The Idiot”*
by Fyodor Dostoyevsky

Declaração de Honra

Declaro que a presente tese é de minha autoria com apenas edição da minha equipa de orientação e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras de atribuição e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio constitui um ilícito académico.

(João Pedro Cardoso Ribeiro)

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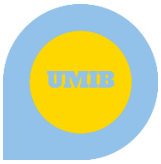

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List of Publications

Publications included in this thesis:

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Martins, A. D*., **J. C. Ribeiro***, R. Ferreira, M. G. Alves and P. F. Oliveira (2023). "Understanding the age-related alterations in the testis-specific proteome." Expert Review of Proteomics 20(12): 331-343. https://doi.org/10.1080/14789450.2023.2274857.
*Both authors contributed equally.

Ribeiro, J. C., R. L. Bernardino, A. Gonçalves, A. Barros, G. Calamita, M. G. Alves and P. F. Oliveira (2023). Aquaporin-7-Mediated Glycerol Permeability Is Linked to Human Sperm Motility in Asthenozoospermia and during Sperm Capacitation. Cells.; 12(15):2003. https://doi.org/10.3390/cells12152003

Ribeiro, J. C., R. L. Bernardino, D. F. Carrageta, G. Soveral, G. Calamita, M. G. Alves and P. F. Oliveira (2022). "CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells." Cellular and Molecular Life Sciences 79(12): 592. https://doi.org/10.1007/s00018-022-04619-1

Ribeiro, J. C., R. Nogueira-Ferreira, F. Amado, M. G. Alves, R. Ferreira and P. F. Oliveira (2022). "Exploring the Role of Oxidative Stress in Sperm Motility: A Proteomic Network Approach." Antioxidants & Redox Signaling 37(7-9): 501-520. https://doi.org/10.1089/ars.2021.0241

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Abstract

The decline in fertility potential, particularly in the male reproductive outcomes, has been a worrying trend in developed countries over the past decades. Idiopathic male infertility is the most prevalent type of infertility and is characterized by the lack of a clear cause that justifies it. It is believed that most cases of idiopathic male infertility are due to genetic mutations. The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, when mutated, is the root cause of cystic fibrosis, the most prevalent genetic disorder among Europeans. *CFTR* mutations impair the function of epithelial tissue in various organs, especially in the male reproductive tract. Reduced CFTR availability can lead to infertility or subfertility in multiple ways, demonstrating its essential role in spermatogenesis, sperm maturation, and capacitation. CFTR functions as a chloride (Cl^-) and bicarbonate (HCO_3^-) ion channel thus, regulating the power of hydrogen (pH) in intracellular and extracellular environments and indirectly modulating water and solute diffusion through cellular membranes. To assist in these processes, cells express aquaporins (AQPs). AQPs play an important role in the fluid composition of the male reproductive tract and sperm osmoadaptation. Aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) are a class of AQPs that are permeable to small solutes such as glycerol. CFTR and aquaglyceroporins have been found to form protein complexes in epithelial cells of the epididymis and other tissues. However, the existence of such complexes in epithelial cells of the seminiferous tubules and sperm cells remains unknown, as does their role in sperm function. The main goal of this project was to elucidate the role of the CFTR-aquaglyceroporin interaction in regulating water and solute exchange between intracellular and extracellular environments, a process essential for male fertility, with a particular focus on Sertoli and sperm cells. To do so, we divided the main goal into three different objectives. The first was to assess the ability of CFTR to modulate aquaglyceroporin permeability in Sertoli cells and to explore the possibility of a protein complex forming at the cell membrane. This mechanism could be crucial in regulating the composition of the seminiferous tubule lumen during spermatogenesis, potentially revealing a novel mechanism of dysregulation in individuals with CFTR mutations. The second objective was to study the importance of aquaglyceroporin permeability in sperm function, namely, in sperm motility. This objective

aimed to clarify the importance of aquaglyceroporins in sperm maturation and motility development. The third objective was to study the capability of CFTR to modulate aquaglyceroporin-mediated glycerol permeability in sperm cells, the presence of the complex CFTR/aquaglyceroporin, and its impact on sperm motility. This objective aimed to isolate the potential dysregulation of aquaglyceroporin permeability modulation by CFTR, independent of the influence of common *CFTR* gene variants within the sample.

To answer the first objective, we used mouse Sertoli cell line (TM4) as a model and assessed the expression of CFTR, AQP3, AQP7, and AQP9 using quantitative polymerase chain reaction (qPCR), western blot, and immunofluorescence techniques. To investigate the functional role of CFTR, we incubated the cells with a specific CFTR inhibitor (CFTRinh-172) and measured the glycerol permeability using stopped-flow light scattering. The data showed that CFTR inhibition decreased cellular glycerol permeability by 31% in TM4 Sertoli cells. Additionally, we used a proximity ligation assay (PLA) to assess endogenous protein-protein interactions between CFTR and the identified aquaglyceroporins. The results showed that CFTR is in close proximity to AQP3, AQP7, and AQP9 in these cells. This suggests the formation of a protein complex that includes two or more of these proteins, allowing the CFTR to influence AQP-mediated glycerol permeability in Sertoli cells through potential physical interactions. The dysregulation of this mechanism could impair the homeodynamics of water and solute between Sertoli cells, seminiferous tubules lumen environment, and germ cells, being a potential link between male infertility and CFTR dysfunction.

Following spermatogenesis, sperm cells are transported into the epididymis, where they gain motility and where glycerol may serve as a crucial energy substrate. Shifting our attention to sperm cells and the second objective, we investigated the importance of AQP-mediated glycerol permeability in sperm motility. The characteristic morphology of sperm cells highlights the importance of understanding the expression patterns of the different aquaglyceroporins. Thus, we used immunofluorescence techniques to evaluate the presence and location of AQP3 and AQP7 in human sperm. Glycerol permeability in spermatozoa from normozoospermic individuals was estimated by stopped-flow light scattering after incubation with specific inhibitors targeting AQP3 (DFP00173) and AQP7 (Z433927330). Sperm from asthenozoospermic men were also evaluated to compare AQP7-mediated glycerol permeability with that of normozoospermic men. In order to further understand the connection between sperm glycerol permeability and motility, sperm cells from normozoospermic samples were incubated in a capacitating medium until hypermotility was reached. We then assessed glycerol permeability and AQP7 expression levels in these sperm. Our results indicated that AQP7 plays a key role in glycerol permeability since its inhibition resulted in a 55% decrease in glycerol diffusion across the sperm membrane. The

impairment of this mechanism was evident in spermatozoa from asthenozoospermic individuals, suggesting dysregulation of AQP7-mediated glycerol permeability despite showing similar AQP7 protein levels. Conversely, AQP7 expression increased in capacitated sperm compared to non-capacitated sperm. Hence, AQP7-mediated permeability may serve as a valuable indicator of sperm motility and be crucial in sperm function.

The results obtained drove us to investigate the nature of CFTR's modulation of AQP7-mediated glycerol permeability in sperm and whether this could be affected in asthenozoospermic men, even without major *CFTR* gene mutations. This led us to our third objective. We first evaluated the effect of CFTR inhibition on sperm motility in normozoospermic men. Then, using stopped-flow light scattering, we assessed aquaglyceroporin-mediated glycerol permeability in sperm cells from both normozoospermic and asthenozoospermic men, with and without CFTR inhibition. To study potential physical modulation, the co-localization of CFTR and AQP7 was studied by immunofluorescence, and the possible formation of a protein complex was confirmed by PLA. The results show that CFTR inhibition decreased aquaglyceroporin-mediated glycerol permeability in sperm to 48%. Complying with these results, CFTR co-localizes with AQP7 in the midpiece and in the equatorial segment of the sperm's head, where they were also found to be in close proximity allowing the formation of a protein complex. Despite not reaching statistical significance, CFTR inhibition in sperm from asthenozoospermic men showed a diminishing trend on aquaglyceroporin-dependent glycerol diffusion, suggesting a possible dysregulation in this mechanism in men with low sperm motility. Thus, CFTR can modulate aquaglyceroporin-mediated glycerol permeability in human sperm and this mechanism can be dysregulated in sperm from asthenozoospermic men without major *CFTR* mutations. The pathway of CFTR activation might be of particular interest to understanding the mystery of isolated asthenozoospermia.

Our results demonstrate a novel mechanism through which CFTR can influence aquaglyceroporin-mediated glycerol permeability in both Sertoli cells and spermatozoa. This mechanism appears to be compromised in sperm cells from men with asthenozoospermia. Further research is needed to validate these findings. However, they open the door for additional investigation regarding the role of CFTR modulation in aquaglyceroporin permeability and its impact on spermatogenesis and sperm function.

Resumo

O declínio do potencial de fertilidade, particularmente nos resultados reprodutivos masculinos, tem sido uma tendência preocupante em países desenvolvidos nas últimas décadas. A infertilidade masculina idiopática é o tipo de infertilidade mais prevalente e caracteriza-se pela ausência de uma causa clara que a justifique. Acredita-se que a maioria dos casos de infertilidade masculina idiopática seja devido a mutações genéticas. O gene regulador de condutância transmembranar da fibrose quística (*CFTR*), quando mutado, é a causa da fibrose quística, a doença genética mais prevalente entre os europeus. As mutações no *CFTR* prejudicam o funcionamento do tecido epitelial em vários órgãos, especialmente no trato reprodutivo masculino. A disponibilidade reduzida da CFTR pode levar à infertilidade ou subfertilidade por vários caminhos, demonstrando o seu papel essencial na espermatogénese, maturação e capacitação dos espermatozoides. A CFTR atua como um canal de iões de cloreto (Cl^-) e bicarbonato (HCO_3^-), regulando assim o potencial de hidrogénio (pH) nos ambientes intra e extracelulares e modulando indiretamente a difusão de água e solutos através das membranas celulares. Para auxiliar nestes processos, as células expressam aquaporinas (AQPs). As AQPs desempenham um papel importante na composição dos fluidos no trato reprodutivo masculino e na osmo-adaptação dos espermatozoides. As aquagliceroporinas (AQP3, AQP7, AQP9 e AQP10) são uma classe de AQPs permeáveis a pequenos solutos, como o glicerol. Verificou-se que a CFTR e as aquagliceroporinas formam complexos proteicos em células epiteliais do epidídimo e de outros tecidos. No entanto, a existência de tais complexos em células epiteliais dos túbulos seminíferos e nos espermatozoides permanece desconhecida, tal como o seu papel na função espermática.

O objetivo principal deste projeto foi elucidar o papel da interação CFTR-aquagliceroporina na regulação da troca de água e solutos entre os ambientes intra e extracelulares, um processo essencial para a fertilidade masculina, com particular foco nas células de Sertoli e nos espermatozoides. Para tal, dividimos o objetivo principal em três objetivos distintos. O primeiro foi avaliar a capacidade da CFTR em modular a permeabilidade das aquagliceroporinas nas células de Sertoli e explorar a possibilidade da formação de um complexo proteico na membrana celular. Este mecanismo poderá ser crucial na regulação

da composição do lúmen dos túbulos seminíferos durante a espermatogénese, potencialmente revelando um novo mecanismo de desregulação em indivíduos com mutações no *CFTR*. O segundo objetivo foi estudar a importância da permeabilidade das aquagliceroporinas na função dos espermatozoides, nomeadamente na motilidade espermática. Este objetivo visa clarificar a importância das aquagliceroporinas na maturação dos espermatozoides e no desenvolvimento da motilidade. O terceiro objetivo foi estudar a capacidade da CFTR de modular a permeabilidade ao glicerol mediada pelas aquagliceroporinas nos espermatozoides, a presença do complexo CFTR/aquagliceroporina e o seu impacto na motilidade espermática. Este objetivo pretende isolar a potencial desregulação da modulação da permeabilidade das aquagliceroporinas pela CFTR, independentemente da influência de variantes comuns do gene *CFTR* na amostra.

Para responder ao primeiro objetivo, utilizámos uma linha celular de Sertoli de rato (TM4) como modelo e avaliámos a expressão do CFTR, AQP3, AQP7 e AQP9 utilizando técnicas de reação em cadeia da polimerase quantitativa (qPCR), western blot e imunofluorescência. Para investigar o papel funcional do CFTR, incubámos as células com um inibidor específico da CFTR (CFTRinh-172) e medimos a permeabilidade ao glicerol utilizando dispersão de luz de fluxo interrompido. Os dados mostraram que a inibição de CFTR diminuiu a permeabilidade celular ao glicerol em 31% nas células de Sertoli TM4. Adicionalmente, utilizámos um ensaio de ligação de proximidade (PLA) para avaliar interações endógenas entre a CFTR e as aquagliceroporinas identificadas. Os resultados mostraram que a CFTR está em proximidade com a AQP3, AQP7 e AQP9 nestas células. Isto sugere a formação de um complexo proteico que inclui duas ou mais destas proteínas, permitindo à CFTR influenciar a permeabilidade ao glicerol mediada por AQP nas células de Sertoli através de potenciais interações físicas. A desregulação deste mecanismo poderá prejudicar a homeodinâmica da água e dos solutos entre as células de Sertoli, o ambiente do lúmen dos túbulos seminíferos e as células germinativas, sendo um possível elo entre a infertilidade masculina e a disfunção da CFTR.

Após a espermatogénese, os espermatozoides são transportados para o epidídimo, onde adquirem motilidade e onde o glicerol pode servir como um substrato energético crucial. Mudando o nosso foco para os espermatozoides e o segundo objetivo, investigámos a importância da permeabilidade ao glicerol mediada por AQP na motilidade dos espermatozoides. A morfologia característica dos espermatozoides acentua a importância de compreender os padrões de expressão das diferentes aquagliceroporinas. Assim, utilizámos técnicas de imunofluorescência para avaliar a presença e localização da AQP3 e AQP7 em espermatozoides humanos. A permeabilidade ao glicerol nos espermatozoides de indivíduos normozoospérmicos foi estimada por dispersão de luz de fluxo interrompido

após incubação com inibidores específicos direcionados a AQP3 (DFP00173) e AQP7 (Z433927330). Também foram avaliados espermatozoides de homens astenozoospérmicos para comparar a permeabilidade ao glicerol mediada por AQP7 com a dos homens normozoospérmicos. Para entender melhor a conexão entre a permeabilidade ao glicerol e a motilidade, espermatozoides de amostras normozoospérmicas foram incubados num meio capacitante até atingirem hipermotilidade. Avaliámos então a permeabilidade ao glicerol e os níveis de expressão de AQP7 nestes espermatozoides. Os nossos resultados indicaram que a AQP7 desempenha um papel crucial na permeabilidade ao glicerol, uma vez que a sua inibição resultou numa diminuição de 55% na difusão do glicerol através da membrana dos espermatozoides. A desregulação deste mecanismo foi evidente nos espermatozoides de indivíduos astenozoospérmicos, sugerindo uma desregulação da permeabilidade ao glicerol mediada pela AQP7, apesar de apresentarem níveis de AQP7 semelhantes. Por outro lado, a expressão da AQP7 aumentou nos espermatozoides capacitados em comparação com os espermatozoides não capacitados. Assim, a permeabilidade mediada pela AQP7 pode servir como um indicador valioso da motilidade dos espermatozoides e ser crucial na sua função.

Os resultados obtidos levaram-nos a investigar a natureza da modulação de CFTR na permeabilidade ao glicerol mediada pela AQP7 nos espermatozoides e se isso poderia ser afetado em homens astenozoospérmicos, mesmo sem mutações significativas no gene *CFTR*. Isso conduziu-nos ao nosso terceiro objetivo. Avaliámos primeiro o efeito da inibição da CFTR na motilidade dos espermatozoides em homens normozoospérmicos. Depois, utilizando dispersão de luz de fluxo interrompido, avaliámos a permeabilidade ao glicerol mediada por aquagliceroporinas em espermatozoides de homens normozoospérmicos e astenozoospérmicos, com e sem inibição da CFTR. Para estudar uma potencial modulação física, a co-localização de CFTR e AQP7 foi estudada por imunofluorescência, e a possível formação de um complexo proteico foi confirmada por PLA. Os resultados mostram que a inibição da CFTR diminuiu a permeabilidade ao glicerol mediada por aquagliceroporinas nos espermatozoides em 48%. De acordo com estes resultados, a CFTR co-localiza com AQP7 na peça intermédia e no segmento equatorial da cabeça do espermatozoide, onde também foram encontrados em proximidade, permitindo a formação de um complexo proteico. Apesar de não ter atingido significância estatística, a inibição da CFTR nos espermatozoides de homens astenozoospérmicos mostrou uma tendência decrescente na difusão do glicerol dependente das aquagliceroporinas, sugerindo uma possível desregulação deste mecanismo em homens com baixa motilidade espermática. Assim, o CFTR pode modular a permeabilidade ao glicerol mediada pelas aquagliceroporinas nos espermatozoides humanos e este mecanismo pode estar desregulado nos espermatozoides de homens astenozoospérmicos sem grandes mutações no *CFTR*. A via

de ativação da CFTR pode ser de particular interesse para entender o mistério da astenozoospermia isolada.

Os nossos resultados demonstram um novo mecanismo através do qual o CFTR pode influenciar a permeabilidade ao glicerol mediada por aquagliceroporinas tanto nas células de Sertoli como nos espermatozoides. Este mecanismo parece estar comprometido nos espermatozoides de homens com astenozoospermia. Mais investigação é necessária para validar estes achados. No entanto, abrem-se portas para investigações adicionais sobre o papel da modulação da CFTR na permeabilidade das aquagliceroporinas e o seu impacto na espermatogénese e função espermática.

.

Table of Contents

List of Tables

Chapter 1: Introduction

Chapter 3: CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells

Chapter 4: Aquaporin 7-Mediated Glycerol Permeability Is Linked to Human Sperm Motility in Asthenozoospermia and during Sperm Capacitation

Chapter 5: CFTR-AQP7 protein complex modulates glycerol transport in human spermatozoa

Annexes A: Supplemental Data

List of Figures

Chapter 1: Introduction

Chapter 3: CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells

List of Abbreviations

A

ABC	Adenosine triphosphate binding cassette
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AQP	Aquaporin
ART	Assisted reproductive technology
ATP	Adenosine triphosphate

B

BSA	Bovine serum albumin
BTB	Blood-testis barrier

C

cAMP	Cyclic adenosine 3’,5’-monophosphate
CBAVD	Congenital bilateral absence of the *vas deferens*
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CTR	Control vehicle

D

DAPI	Diamidino-2-phenylindole
DHA	Dihydroxyacetone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNAI2	Dynein axonemal intermediate chain 2

E

ENaC	Epithelial sodium channel
ERM	Ezrin, radixin, and moesin binding motif

F

FBS	Fetal bovine serum
G	
GAPDS	Glyceraldehyde 3-phosphate dehydrogenase, spermatogenic
GK2	Glycerol kinase 2
GPD	Glycerol 3-phosphate dehydrogenase
H	
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human papillomavirus
I	
iAQP	Aquaporin inhibitor
IBMX	3-Isobutyl-1-methylxanthine
K	
KCNMA1	Calcium-activated potassium channel subunit alpha-1
L	
LDH	Lactate dehydrogenase
M	
mRNA	Messenger ribonucleic acid
N	
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBD	Nucleotide binding domain
NHERF	Sodium and hydrogen exchanger regulatory factor
P	
PBS	Phosphate-buffered saline
PDZ	Postsynaptic density 95; Discs large; Zonula occludens
pH	Power of hydrogen
PK	Protein kinase
PLA	Proximity ligation assay
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
Q	
qPCR	Quantitative polymerase chain reaction

R

R	Regulatory domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species

S

sAC	Soluble adenylyl cyclase
SEM	Standard error of the mean
SLC	Solute carrier

T

TAT1	Testis anion transporter 1
TMD	Transmembrane Domain
TPI1	Triosephosphate isomerase
tRNA	Transfer ribonucleic acid

W

WHO	World Health Organization

Chapter 1

Introduction

This chapter was adapted from sections of the published works:

Ribeiro, J. C., B. C. Rodrigues, R. L. Bernardino, M. G. Alves and P. F. Oliveira (2024). “The interactome of cystic fibrosis transmembrane conductance regulator and its role in male fertility: a critical review.” Journal of Cell Physiology e31422. https://doi.org/10.1002/jcp.31422

Martins, A. D*., **J. C. Ribeiro***, R. Ferreira, M. G. Alves and P. F. Oliveira (2023). "Understanding the age-related alterations in the testis-specific proteome." Expert Review of Proteomics 20(12): 331-343. https://doi.org/10.1080/14789450.2023.2274857.
*Both authors contributed equally.

Ribeiro, J. C., N.-F. R., F. Amado, M. G. Alves, R. Ferreira and P. F. Oliveira (2022b). "Exploring the Role of Oxidative Stress in Sperm Motility: A Proteomic Network Approach." Antioxidants & Redox Signaling 37(7-9): 501-520. https://doi.org/10.1089/ars.2021.0241

Ribeiro, J. C., M. G. Alves, M. Yeste, Y. S. Cho, G. Calamita and P. F. Oliveira (2021). "Aquaporins and (in)fertility: More than just water transport." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1867(3): 166039. https://doi.org/10.1016/j.bbadis.2020.166039

1.1. Human (In)Fertility

The ability to pass one's genetic material to the next generation is the primary objective of all species. One example of that is the case of *Attacus atlas*, also known as the atlas moth. Despite being considered one of the bigger moth species, this moth is not able to feed itself during its reproductive stage due to incomplete mouth development (Majumder, 2011), serving as a description for the prioritization of evolution towards certain species. Fortunately, humans took a different evolutionary path which led to different sexual outcomes and principles. In recent decades, these outcomes have changed due to changes in life choices, particularly in developed nations. A career-oriented lifestyle, an increase in life expectancy, and the postponement of family planning are some of the examples that contribute to the rise seen in the average paternal age (Lian et al., 1986, Cedars, 2015). Advanced paternal age correlates with an enhanced risk of fertility complications (Rowe, 2006, Khandwala et al., 2017, Martins et al., 2023), and is contributing to the decline in fertility rates. The number of births per woman has been decreasing in developed countries and has been a matter of concern since 1978 (Westoff, 1978). According to the World Health Organization (WHO), infertility is defined as the failure to achieve pregnancy after a year of regular and unprotected sex (WHO, 2023). WHO also reports that lifetime infertility prevalence in human females is 17.5% (based on 37 studies) and in males is 12.4% (based on 12 different studies) (WHO, 2023). Given the number of studies that sustain the WHO findings, it is fair to assume that the scientific community has a higher focus on women's fertility. Approximately 50% of infertility cases are attributed to women, with the remaining 50% attributed to men or a combination of both partners (Agarwal et al., 2021). Thus, both sexes have different contributions to the infertility problem, which can explain the different attention. However, it is within the male sex that the decline in fertility outcomes is more apparent. Between 1973 and 2018, there was a decrease of 51.6% in sperm concentration. The result of this analysis also shows that the declining rate of sperm concentration doubled from the year of 1973 to 2000 (a decrease of 1.16% per year) compared to the period between 2000 and 2018 (a decrease of 2.64% per year) (Levine et al., 2023), making it even more worrisome. On the other hand, men experience a decline in the percentage of motile sperm during their aging, which is a matter of concern taking into consideration the increase in paternal age already mentioned (Auger et al., 1995, Khandwala et al., 2017). Sperm motility is essential for natural fertilization and its decrease can be an evident cause for infertility. Asthenozoospermia is characterized by the presence of a subnormal percentage of motile sperm (below 42% of motile sperm) in the ejaculate (WHO, 2021). Impaired sperm motility is present in approximately 82% of infertility cases (Curi et al., 2003). This number includes cases of isolated asthenozoospermia but also cases associated with oligozoospermia (subnormal sperm concentration) and/or

teratozoospermia (subnormal sperm morphology) (Curi et al., 2003). These conditions are mostly caused by a dysregulation in the process of spermatogenesis and spermiogenesis but also a deficiency in sperm maturation.

1.1.1. Spermatogenesis and Sertoli cells

Males experience substantial changes during puberty. One notorious example is the emergence of reproductive capabilities. It is within this developmental phase that the male testis starts the continuous process of gametes production, known as spermatogenesis. Spermatogenesis involves a series of complex events, starting with spermatogonium suffering a series of differentiation events that result in a spermatogonium type B. Then, spermatogonium type B differentiates into a pachytene spermatocyte. A strict balance between self-renewal and differentiation of spermatogonial stem cells is necessary to ensure the production of mature spermatozoa (Oliveira et al., 2015a). After this, the meiotic phase takes place (Bellve et al., 1977, Hess et al., 2008). Pachytene spermatocyte goes through two meiotic processes giving origin to four round spermatids (Luk et al., 2014). These developing germ cells then suffer a series of morphologic differentiation stages during spermiogenesis, and finally detach from the Sertoli cells as immature spermatozoa (Figure 1.1.) in a process called spermiation (Bellve et al., 1977, Sprando et al., 1988, O'Donnell, 2014). Throughout these phases, different cellular processes take place within the developing germ cells but also in the adjacent Sertoli cells. Sertoli cells are somatic cells of the seminiferous tubules that are known as ‘nurse’ cells since they surveil every step of spermatogenesis. Their functions include the sustenance (structural and nutritional) of developing germ cells, the formation of the blood-testis barrier (BTB), secretion of hormones and signaling factors, response to hormonal signals, the control of the seminiferous tubule fluid composition, modulation of germ cells apoptosis and phagocytosis of apoptotic germ cells (Griswold, 1998, O’Donnell et al., 2022). Sertoli cells are large cells with an extensive cytoplasm and a flattened surface communicating with the basement membrane of the seminiferous tubule (Russell et al., 1990, O’Donnell et al., 2022). The large Sertoli cell cytoplasmatic membrane is dynamic and can adjust to support several germ cells in different stages of development, from spermatogonia to spermatozoa, that are released into the seminiferous tubule lumen (O’Donnell et al., 2022). Sertoli cell structural support of germ cells is due to extensions of extracellular matrix components, with this cellular cooperation presenting well-organized cytoskeleton interconnections (Russell et al., 1985, Mruk et al., 2004). Germ cell nutritional support is provided by Sertoli cells through the production and release of nutrients and regulatory factors essential to spermatogenesis (Ribeiro et al., 2022b). Glucose is the preferred substrate metabolized by Sertoli cells. They present a highly glycolytic metabolic profile resembling a Warburg-like effect metabolism (Oliveira et

al., 2015b) to ensure the production and secretion of lactate for the developing germ cells, which is their favorite energy substrate (Meneses et al., 2016). Sertoli cells not only secret energy substrates into the seminiferous tubules' lumen but also other metabolites necessary for every step of the developing germ cells like acetate and glycerol, needed for lipid synthesis (Martins et al., 2015, Ribeiro et al., 2022a). In fact, Sertoli cells are known for their metabolic plasticity by being able to metabolize multiple substrates to ensure nutritional support for spermatogenesis (Martins et al., 2019, Ribeiro et al., 2022b). In this way, proper assembly and metabolic functioning of Sertoli cells is vital for normal progress of the spermatogenic process and male fertility. On the other hand, Sertoli cells present a well conserved metabolism and overall function between most mammal species studied (Oliveira et al., 2015b).

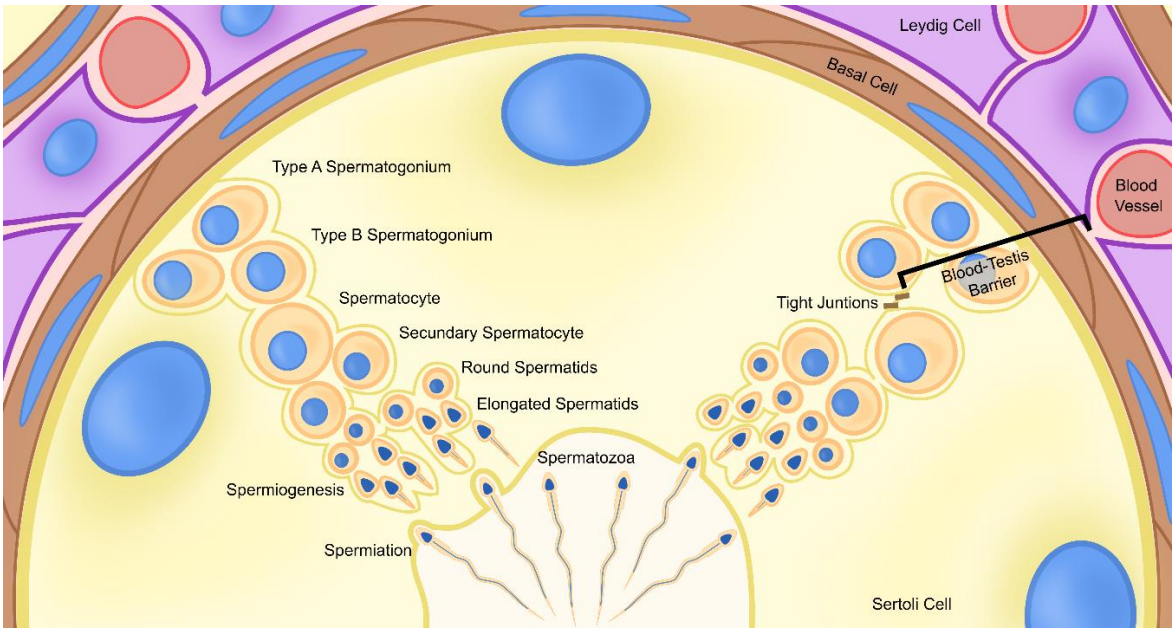

Figure 1.1.: Schematic representation of a seminiferous tubule structure and stages of the developing germ cells during spermatogenesis.

Spermiogenesis occurs during the last stages of spermatogenesis and is characterized by drastic morphological changes needed for the specialization of the sperm cells to achieve the ability to swim (Tanaka et al., 2003). During this phase, takes place the most recognizable characteristics of the sperm cells, like the further chromatin condensation, loss of cytoplasmatic volume, and, of course, the development of the acrosome and flagellum (Calamita et al., 2001a, Huang et al., 2020). It is during this phase that many of the morphological defects that impair sperm motility occur. One of the most worrisome defects is the ones that cause flagellum abnormalities, which is the part responsible for the mechanical thrusting of the sperm but also contains many of the glycolytic enzymes responsible for adenosine triphosphate (ATP) production and sperm motility (Li et al., 2010, Touré et al., 2021). On the other hand, mid-piece malformations and mitochondria

impairment also highly impact sperm motility (Coutton et al., 2018, Díaz-Miranda et al., 2020). Even if everything goes as planned during spermatogenesis, sperm cells still lack the ability to be motile. That will be developed downstream, in the epididymis.

1.1.2. Epididymis and sperm maturation

After the separation of the immature sperm cells from the Sertoli cells, the former will be released into the seminiferous tubule lumen and guided into the efferent ducts. The epididymis is their next stop and here is where the sperm cells attain their motility (Haidl et al., 1994). The epididymis can be divided into three different sections: the caput (closer to the efferent ducts), the corpus, and the cauda (closer to the *vas deferens*). All these sections have different properties and cell composition ratios. The epididymis is thicker in the caput section and is gradually decreasing in thickness reaching its lowest in the cauda. However, the opposite happens with its lumen, reaching its bigger diameter in the final section where sperm cells are stored (Sullivan et al., 2019). It can be found three different cell types in the epididymis epithelia: principal cells, clear cells, and basal cells and their distribution changes throughout the tubule. Basal cells are the base of the epithelium and form a clear circle around the other cell types and the lumen of the tubule in transversal cuts (Sullivan et al., 2019). Principal and clear cells are the ones in contact with the lumen environment and the main secretor of its constituents (Sullivan et al., 2019). The main functions of the epididymis are to initiate sperm maturation, sperm concentration (mostly in the caput), and sperm storage (in the cauda section) (Marchiani et al., 2017). The bypassing of the epididymis results in immature, immotile sperm unable to fertilize (Bedford, 1967). Taking into consideration that sperm cells reach the epididymis immotile and are quiescent in the cauda section (Bedford, 1967, Devi et al., 2021), it is fair to assume that the gaining of motility capabilities takes place between the caput and the corpus. The processes that take place within the epididymis are still one of the biggest mysteries regarding male fertility. Thus, epididymis and sperm maturation malfunction are an easy scapegoat for asthenozoospermia, especially when the morphology of the sperm samples is within normal parameters. What we do know is the morphologic changes that happen to the sperm cells during the epididymis transit. Those changes comprise: lipid remodeling of the sperm's membrane (increasing its fluidity) (Rejraji et al., 2006), as well as modifications of surface glycoproteins and antigens (Belmonte et al., 2000, Tulsiani, 2006); increase in the micro ribonucleic acid (microRNA) and transfer ribonucleic acid (tRNA) library, further condensation of the sperm cells chromatin (Haidl et al., 1994, Belleannée, 2015, Sharma et al., 2016); proteome changes (Skerget et al., 2015), and a metabolic reprograming and activation/inactivation of multiple pathways that ultimately result on sperm motility (Tash, 1989, Cooper et al., 2003, Somanath et al., 2004, Baker et al., 2012, Vadnais et al., 2013).

All these modifications result from the crosstalk between the epididymal epithelia and the sperm cells through the epididymal lumen (De Jonge et al., 2017). Thus, any dysregulation on these mechanisms can result in the impairment of sperm motility even after an incident-free spermatogenic process.

As highlighted above, seminiferous tubules and epididymis epithelia (Sertoli cells, and principal and clear cells, respectively) are vital for the proper development and maturation of the sperm cells due to their control of the extracellular medium. These environments can manipulate sperm cells' metabolism and signaling pathways and transform them into one of the most specialized cells in the human body.

1.1.3. Influence of extracellular and intracellular environment on sperm physiology

The medium within the testicular and epididymal lumen facilitates the linkage between the epithelial cells and the sperm cells. Sertoli cells and epididymal epithelial cells secrete metabolites, ions, proteins, or nucleic acids to the immature sperm cells which fuel the changes in these cells. During spermatogenesis, metabolite secretion by Sertoli cells allows the energy production necessary for the proper development of the germ cells (Rato et al., 2014, Martins et al., 2015, Crisóstomo et al., 2017). Downstream, in the epididymis, the power of hydrogen (pH) regulation through ion homeodynamics is fundamental for the regulation of sperm cell motility during maturation (Yeung et al., 2004, Joseph et al., 2010, Weissgerber et al., 2012, Zhou et al., 2018). That is not only the case in sperm maturation and quiescence in the male reproductive tract but also during sperm capacitation in the female reproductive tract. Sperm cells possess an amplitude of channels and pores within their cell membrane that allow the influence of the extracellular medium within the sperm cell’s cytoplasm. In the next topic, it will be discussed the metabolites and ions within the microenvironment during the processes of spermatogenesis, sperm maturation, and sperm capacitation and its influence in the intracellular medium of sperm/germ cells.

1.1.3.1. Microenvironment of spermatogenesis

Sertoli cells are responsible for the regulation of the germ cells’ development microenvironment by secreting proteins, metabolites, and ions (Levine et al., 1971, Itoh et al., 1994, Ribeiro et al., 2022b). Even though spermatogonia are in the basal section of the seminiferous tubules and closer to the circulatory system, Sertoli cells still have a major role in providing support and secreting exosomes for spermatogonia development (Salek et al., 2021). This role further increases downstream where the isolation of developing stem cells is increased due to the BTB. With the advancement of the spermatogenic stages, the metabolic requirement of the developing germ cells also changes (Rato et al., 2012). While spermatogonia obtains most of its energy through glycolysis, spermatocytes and

spermatids obtain most of their substrates for energy production from lactate and pyruvate (Bajpai et al., 1998). In fact, lactate is essential for the spermatogenic development and expression of key spermiogenic proteins (Nakamura et al., 1981). Lactate also has anti-apoptotic properties for spermatocytes and spermatids (Rato et al., 2014). Acetate is also secreted by Sertoli cells and is thought to be essential for the lipid reorganization of the developing germ cells (Martins et al., 2015). Glycerol, another constituent of phospholipids, is also able to be secreted by Sertoli cells into the lumen's microenvironment. This substrate and its direct metabolites are thought to have an important role in spermatogenesis and Sertoli cell metabolism as a metabolic connecter between glycolysis, lipid metabolism, and oxidative phosphorylation (Crisóstomo et al., 2017), which is especially important for cells with metabolic plasticity like Sertoli cells. However, glycerol is only useful in tightly regulated concentrations and can provoke spermatogenic arrest at high concentrations (Wiebe et al., 1989).

The fluid from the seminiferous tubule's lumen is formed by Sertoli and germ cell (to a lesser extent) secretions and has an acidic pH (7.3) when compared to the plasma in circulation (Levine et al., 1971, Sprando et al., 1987). It is hypertonic in relation to the plasma despite having a lower concentration of sodium, compensating it with a higher concentration of potassium ion (K^+) and chloride ion (Cl^-) (Levine et al., 1971). The presence of such gradients may be necessary for the development of spermatogenesis, namely, during the loss of cytoplasmatic volume that happens during spermiogenesis (Calamita et al., 2001b). A study noted that around 50% of the cytoplasmatic volume from early elongated spermatids is lost in the transition to late elongated spermatids (Sprando et al., 1987). To assist in this mechanism, Sertoli and germ cells present the expression of multiple ion and substrate channels that may allow the creation of such specialized gradients (Bernardino et al., 2019).

1.1.3.2. Microenvironment role in sperm motility

Sperm cells acquire motility in the epididymis as was already mentioned. However, in the female reproductive tract, sperm cells can enter a new motility state known as hypermotility, which occurs during the capacitation process (Boerke et al., 2013). This upshift in sperm motility is needed for the final stages of the sperm journey and is essential for unassisted fertilization of the oocyte. For either the acquirement of motility in the epididymis or its upgrade in the female reproductive tract, the medium that contains the sperm cells modulates its development.

Each epididymis section has its own tightly controlled lumen microenvironment noted by the change in osmolarity and composition (Levine et al., 1971). Fluid reabsorption between the seminiferous tubules and the caput section of the epididymis could explain this difference but, the continuous anion and cation changes between the different sections show that the

epididymal epithelium could be specialized to generate and maintain specific ion gradients across the apical membrane (Levine et al., 1971). In fact, changes in osmolarity, ion composition, and pH of the epididymal microenvironment can lead to infertility (Yeung et al., 2004, Joseph et al., 2010, Weissgerber et al., 2012, Zhou et al., 2018). The decreased concentration of solutes in water and the ability of sperm cells to respond to that osmotic stress is already used to access sperm viability (Hecht et al., 2022). Sperm cells must overcome the osmotic stress created by the passage from the male reproductive tract fluid (340 mmol/kg) to the female reproductive tract fluid (280 mmol/kg) (Yeung et al., 2006). Osmotic shocks are regarded as a trigger for the activation of different pathways that change cell functioning (Ortiz et al., 2022), and in sperm cells, it is believed that can be one of the triggers for motility development (Chen et al., 2011). To resist such shocks, sperm cells express proteins capable of diffusing water and other solutes, but it is also believed that sperm cells can acquire osmolytes during the high osmolarity of the epididymal fluid useful to resist the hypotonicity of the female reproductive tract fluid (Cooper et al., 2003).

Microenvironment pH is particularly important for sperm motility. Bicarbonate ion (HCO_3^-) and pH reach their lowest levels in the caput of the epididymis (Levine et al., 1971). This is also the section where it is believed that the sperm cells acquire their ability to swim. It is possible that these specific conditions of the extracellular medium can have a role in the metabolic reprogramming that allows sperm flagellar movement, but the mechanism is yet to be studied. On the other hand, the acidification of the cauda section is resultant of lactate accumulation causing sperm quiescence and motility arrest during epididymal storage (Carr et al., 1985, Matsuzaki et al., 2015, Battistone et al., 2019).

The alkalinization of the sperm cytoplasm, mediated by multiple membrane channels, is the trigger for the capacitation process and the increase in sperm motility. This mechanism is already well studied and involves a change in protein phosphorylation profile after the activation of the soluble adenylyl cyclase (sAC) and an increase in cytoplasmatic cyclic adenosine 3',5'-monophosphate (cAMP) (Carr et al., 1989). Sperm capacitation is a complex process that is believed to be initiated by an increase in HCO_3^- cytoplasmatic levels, followed by proton extrusion *via* the Hv1 proton channel, and calcium (Ca^{2+}) entry *via* cation channel sperm-associated protein (Navarrete et al., 2015, Jin et al., 2017). This is rapidly followed by an increase in cAMP production by sAC, whose activity is also related to the redox balance of the sperm cell and, therefore, reactive oxygen species (ROS) production (Parrish et al., 1994, Aitken, 2017, Chang et al., 2021). Then, activation of protein kinase (PK)-A, PKC, and mitogen-activated PK develops an activation of tyrosine kinases that greatly increase the levels of tyrosine phosphorylation and other motifs such as threonine/ glutamine/tyrosine and arginine-X-X-(serine/threonine), setting in motion the process to fully capacitate the sperm cell (Rivlin et al., 2004). Also, ROS are involved in

cholesterol oxidation and removal of cholesterol from the sperm plasma membrane, enhancing the fluidity of the plasmatic membrane that promotes the development of the hyperactivation state (Boerke et al., 2013). Sperm hyperactivation is characterized by a high amplitude and torque force flagellar movement. This process is crucial for the final stage of the sperm journey when extra thrust force is necessary to propel the sperm cell through the zona pellucida (Aitken et al., 2013, Ritagliati et al., 2018). Likewise, acrosome reaction also benefits from controlled ROS levels (Ichikawa et al., 1999) responsible for the increase in membrane fluidity by cholesterol efflux and Ca^{2+} intake (Lucchesi et al., 2016, Rahban et al., 2020), however, its mechanism is not yet fully understood (Figure 1.2.). Another process that highlights the importance of the intra and extracellular microenvironments on sperm motility is the development of sperm plasmatic membrane hyperpolarization (López-González et al., 2014). The process is resultant of a combination of the inhibition of sodium channels (epithelial sodium channels ENaC) and the activation of K^+ channels (Ca^{2+}-activated K^+ channel subunit alpha-1 KCNMA1), having a preponderant role in protein phosphorylation, maintenance of alkaline cytosolic pH, and acrosome reaction (Espinosa et al., 1995, Hernández-González et al., 2006, Ritagliati et al., 2018).

1.1.4. Idiopathic Male Infertility

Male infertility can be due to pre-testicular, testicular, or pos-testicular causes (Dimitriadis et al., 2017). Pre-testicular causes mainly englobe hypogonadotropic hypogonadism cases due to comorbidities such as diabetes or obesity, or due to drug prescription such as testosterone supplementation (Kolettis et al., 2015, Braga et al., 2020). Testicular infertility comprises cancers and inflammation in the testis and epididymis, varicocele, and cryptorchidism (Alahmar, 2019). Pos-testicular infertility can be caused by reproductive tract malformations or inflammation causing obstructive azoospermia (Dimitriadis et al., 2017). All these sources of male infertility can be due to genetic factors that affect the hypothalamus-pituitary-gonads axis, spermatogenesis/sperm maturation, or incomplete formation of the male reproductive tract (Krausz et al., 2018). Most of these genetic causes are diagnosed as idiopathic male infertility. Idiopathic male infertility is the most prevalent category of male infertility. A study showed that its incidence makes up for 30-40% of infertility cases (Vander Borght et al., 2018) and affects 10-15% of men of reproductive age (Kothandaraman et al., 2016). Standardized semen analysis protocols are not sufficient to diagnose the cause of idiopathic male infertility, being genetic screening necessary to understand the origin of the dysregulation (Krausz et al., 2015). As highlighted throughout this chapter, sperm cell formation and maturation are dependent on multiple intricate processes. The presence of mutations in genes encoding proteins involved in sperm function and spermatogenesis is involved in pre-testicular, testicular, and post-testicular

forms of infertility (Krausz et al., 2015). One example of a gene in which mutations are responsible for such consequences is the cystic fibrosis transmembrane conductance regulator (*CFTR*) (Vander Borght et al., 2018).

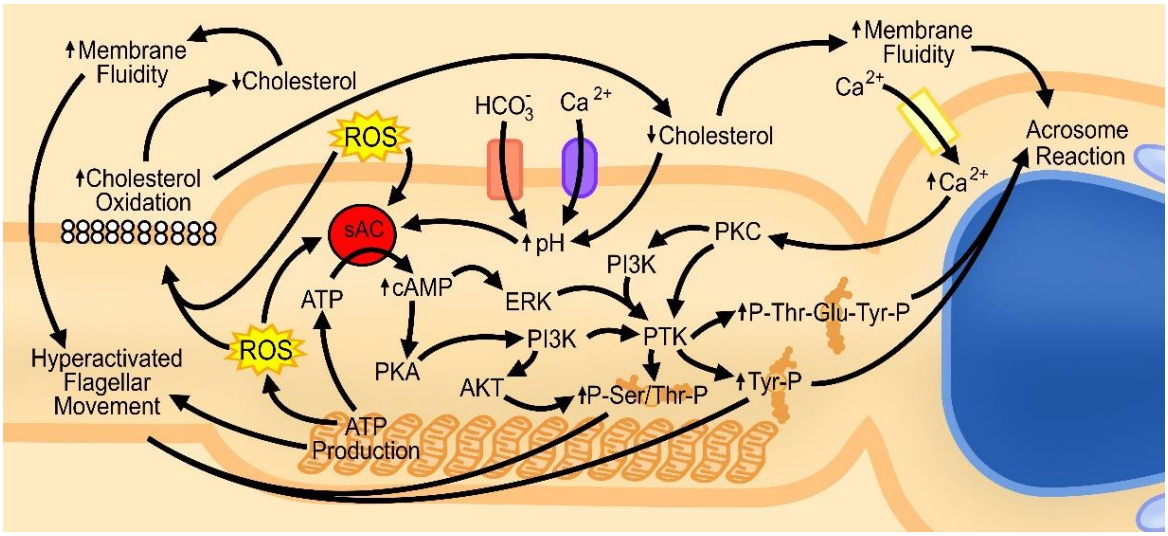

Figure 1.2.: Schematic representation of the mechanism of sperm capacitation. Under normal physiological ROS conditions and after an increase of sperm cytoplasmatic pH by influx of bicarbonate ion (HCO_3^-), activating the soluble adenylyl cyclase (sAC). This enzyme converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) that, in turn, activates the protein kinase A (PKA) and mitogen-activated protein kinase (ERK) pathway. On the other hand, the protein kinase C (PKC) pathway is activated by the increase in calcium intake (Ca_2^+). PKA, PKC, and ERK lead to the activation of other protein kinases such as PI3K, RAC-alpha serine/threonine-protein kinase (AKT), and protein tyrosine kinases (PTK). These proteins are responsible for the phosphorylation of tyrosine (Tyr-P), threonine-glutamine-tyrosine (P-Thr-Glu-Tyr-P), and serine/threonine (P-Ser/Thr-P) motifs which are responsible for the development of the acrosome reaction and the hyperactive state. Moreover, physiological ROS enhances sperm membrane cholesterol oxidation and consequent cholesterol efflux, which results in increased membrane fluidity that facilitates sperm motility.

1.2. Cystic fibrosis transmembrane conductance regulator

CFTR is a transmembrane protein that acts as a Cl^- and HCO_3^- ion channel. It is directly and indirectly responsible for water and ion diffusion between the cytoplasm and the extracellular space, having a particularly important role in secretion formation, such as mucus (Hasegawa et al., 1992, McCarty, 2000). Maintaining and regulating a dynamic balance between CFTR activation and inactivation is essential to ensure body homeostasis. Once this balance is disrupted, one of two major outcomes may occur: hypofunction of CFTR, mainly due to genetic defects, which may lead to cystic fibrosis; or hyperfunction of CFTR, caused by bacterial infections, which may lead to secretory diarrhea (Chao et al., 1994, Choi et al., 2001).

Since its discovery, in 1989, the *CFTR* gene and its respective protein have been the target of extensive research (Rommens et al., 1989, Cai et al., 2019, Wang et al., 2024), particularly because mutations in the *CFTR* gene are responsible for the most common genetic disease in the European Caucasian population: cystic fibrosis (Corvol et al., 2016). Cystic fibrosis manifests in approximately 1 in 3000 births of northern European descendants and it affects primarily tissues in the lungs and pancreas (Quinton, 1990, Rowe et al., 2005). Interestingly, cystic fibrosis also affects the male reproductive tract (Kaplan et al., 1968), with 97% of male patients with cystic fibrosis being subfertile or infertile (Kaplan et al., 1968, Chen et al., 2012a).

Despite being the target of many studies (Cai et al., 2019), the exact role of CFTR in cellular function remains to be fully disclosed, due to its involvement in a complex network of interactions. Thus, it is pivotal to understand CFTR interactome and how it may influence the physiology of tissues and organs responsible for the creation of the male reproductive potential.

1.2.1. CFTR and its network

CFTR belongs to the ATP-binding cassette (ABC) transporters superfamily, despite being the only one in the family with ion channel capabilities (Gadsby et al., 2006). As an ABC transporter, CFTR has a cytosolic hydrophilic region that binds to ATP, the nucleotide-binding domain (NBD), connected to two repeated motifs, each one containing a hydrophobic transmembrane domain (TMD) with six α-helices (Riordan et al., 1989). TMD allows the pore formation for ion diffusion, while the NBD allows the opening and closing of the pore through ATP binding (Levring et al., 2023).

Another particularity of CFTR is the presence of the regulatory (R) domain (Figure 1.3.). The R domain can be classified as an intrinsically disordered protein, due to its lack of stable secondary or tertiary structure, while still being biologically active (Hwang et al., 2018). This makes it difficult to understand the phosphorylation pattern of its multiple phosphorylation

sites. On the other hand, this loose configuration allows sections of the R domain to take part in protein-protein interactions, and interactions with multiple small and big molecules, thus having the ability to take part in the signaling and regulation of multiple pathways (Hwang et al., 2018). Notwithstanding, R-domain phosphorylation is fundamental for CFTR opening, without which, CFTR is in an auto-inhibitory stage (Levring et al., 2023). R domains, like many intrinsically disordered proteins, are a target for posttranscriptional modifications. However, the phosphorylation sites (serines) are highly conserved (Ostedgaard et al., 2001). This suggests that the spacing between phosphorylation sites is the main factor in CFTR regulation, not the R domain sequence itself. Still, plenty remains to be elucidated about the R domain of CFTR (Ostedgaard et al., 2001).

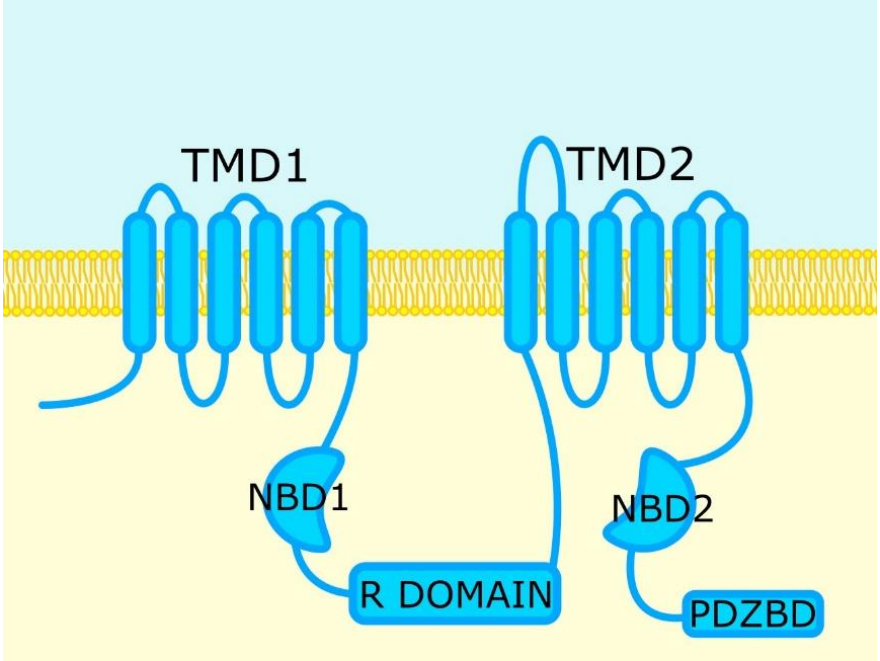

Figure 1.3.: Schematic representation of the domains of cystic fibrosis transmembrane conductance regulator (CFTR) structure.TMD: Transmembrane domain. NBD: Nucleotide binding domain. R: regulatory. PDZBD: Postsynaptic density 95; Discs large; Zonula occludens-binding domain.

After phosphorylation of the R domain by PKA, both NBD domains (NBD1 and NBD2) can change conformation and start CFTR gating (Cheng et al., 1991). For that, ATP must be bonded to the Walker region in both NBD1 and NBD2 domains, causing their dimerization (Carson et al., 1995). This transforms the CFTR conformation into a state that allows ion diffusion. For channel closure, ATP is rapidly hydrolyzed in the NBD2 site, causing the inorganic phosphate to detach and the pore to close. After that, adenosine diphosphate (ADP) unbinds from NBD2, but the dimerization of the NBD domains may continue (Aleksandrov et al., 2002, Aleksandrov et al., 2007, Levring et al., 2023). A recent article by Levring *et al.* demonstrated that ATP rebinding to NBD2, and subsequent CFTR reopening, leads to two possible conformations: one in which the NBD domains separate, and another

in which the NBD dimerization continues (Levring et al., 2023). The ATP binding site in the NBD1 domain is more stable and maintains the ATP interaction for a longer period (Aleksandrov et al., 2002). Adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylates the R domain in a different pattern to PKA (Kongsuphol et al., 2009), transitioning CFTR to an auto-inhibitory state through the R domain (Figure 1.4.). AMPK is activated in response to metabolic stress caused by a low ATP/AMP ratio (Carling et al., 2011), thus, phosphorylating numerous proteins and activating catabolic pathways that preserve ATP (Hardie et al., 2006, Motoshima et al., 2006).

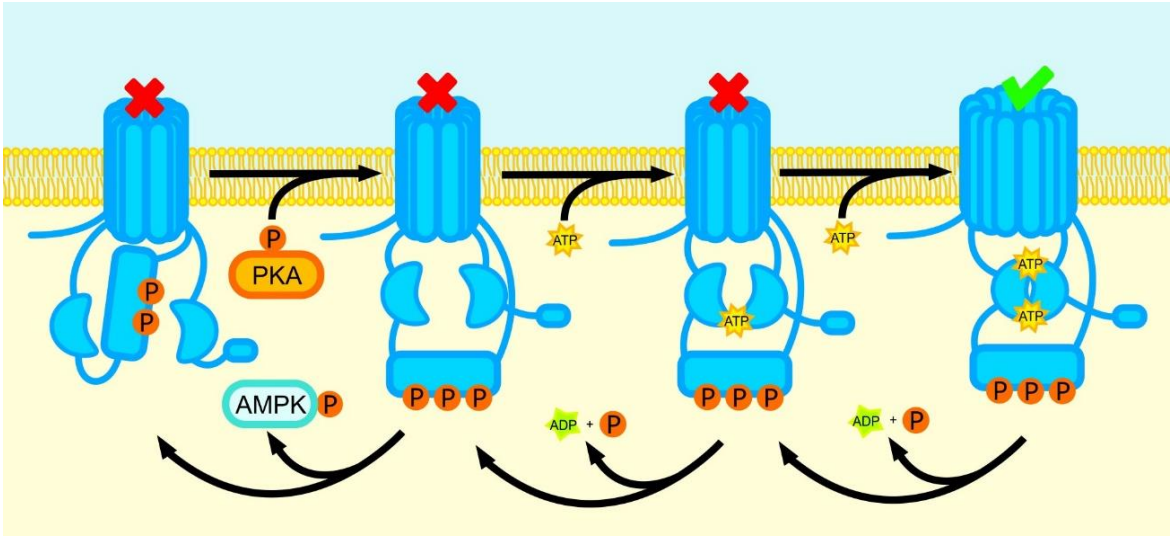

Figure 1.4. : Schematic representation of cystic fibrosis transmembrane conductance regulator (CFTR) gating.Native CFTR is phosphorylated (P) by Protein Kinase A (PKA), enabling nucleotide binding to the Nucleotide binding domain (NBD)-1. Adenosine triphosphate (ATP) then promotes dimerization, but CFTR continues closed. Once another ATP molecule binds to NBD2, full dimerization of both NBD occurs and, consequently, the CFTR pore opens. This second interaction (ATP-NBD2) is less stable than the first, and so ATP hydrolysis happens resulting in adenosine diphosphate (ADP). From there, CFTR can either bind ATP again to reopen the pore; or hydrolyze the remaining ATP molecule (ATP-NBD1). CFTR can be further inhibited by a different phosphorylation pattern via adenosine monophosphate (AMP)-activated protein kinase (AMPK).

Both the amino (N) and carboxyl (C) terminal tails of CFTR are oriented to the cytoplasm and mediate its interaction with a variety of other proteins (Li et al., 2005, Guggino et al., 2006). The last 4 amino acid residues of the CFTR C-terminus sequence are regarded as a Postsynaptic density 95; Discs large; Zonula occludens (PDZ)-binding motif, constituted by aspartate-threonine-arginine-leucine (Guggino, 2004). This sequence allows the interaction of CFTR with proteins containing PDZ domains, revealing a broader role of CFTR that goes beyond Cl^- and HCO_3^- diffusion. The discovery of the first interaction of CFTR with a PDZ domain protein involved sodium (Na^+) and hydrogen (H^+) exchanger regulatory factor (NHERF)1 (Hall et al., 1998, Short et al., 1998, Wang et al., 1998). Soon after, NHERF2, NHERF3, and NHERF4 were also shown to interact with CFTR (Guggino et al., 2006). These proteins have two or more PDZ domains that allow for a connection between CFTR and its network of interactors (Amacher et al., 2014). One example of an interaction that allows the anchoring of the network and CFTR to the cell's cytoplasm is the

CFTR-NHERF1-ezrin interaction. NHERF1 interacts with CFTR through the PDZ domain and with ezrin through the ezrin, radixin, and moesin (ERM) binding motif. Ezrin, in turn, interacts with actin and cell cytoskeleton (Short et al., 1998) (Figure 1.5.). In fact, mutations in the CFTR PDZ-binding C-terminus motif prevent the creation of this scaffolding and CFTR membrane integration, leading to CFTR degradation and cystic fibrosis (Moyer et al., 2000). Thus, these interactions grant CFTR proper function and allow it to indirectly interact, and directly modulate, other transmembrane channels, signaling-producing proteins, and the cytoskeleton (Short et al., 1998). The CFTR network of interactions is involved in a great number of processes and its dysregulation can impact different systems, such as the respiratory, digestive, and reproductive. In the next topic, we will explore the established role of CFTR in male fertility and the consequences of its dysfunction.

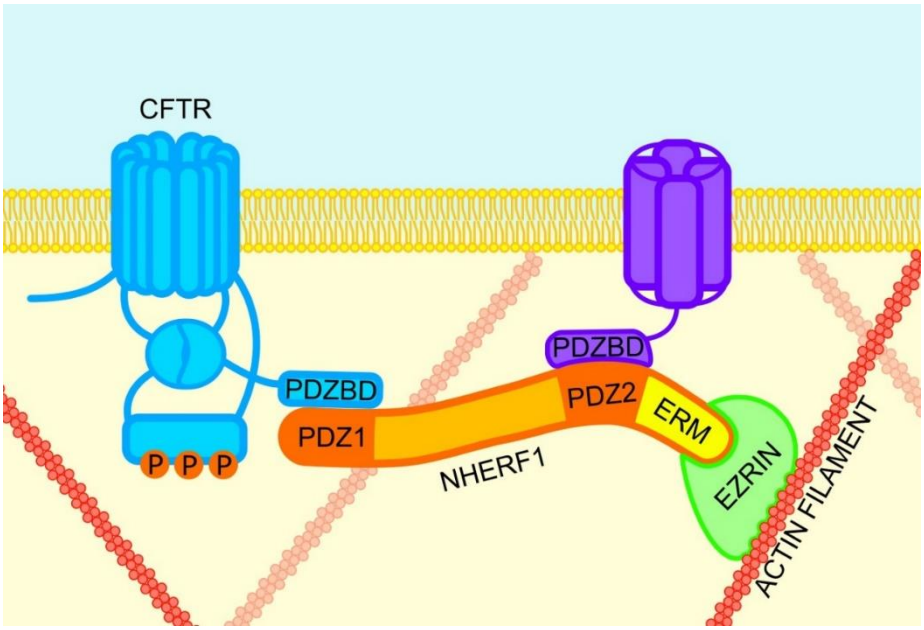

Figure 1.5: Schematic representation of a cystic fibrosis transmembrane conductance regulator (CFTR) scaffolding.CFTR binds to sodium (Na^+) and hydrogen (H^+) exchanger regulatory factor 1 (NHERF1) through the interaction of Postsynaptic density 95; Discs large; Zonula occludens-binding domain (PDZBD) with PDZ1. PDZ2 of NHERF1 can bind to a series of other proteins with their own PDZBD. NHERF1 also has an ezrin, radixin, and moesin (ERM) binding domain that binds to ezrin, anchoring this complex to the cytoskeleton. P: phosphorylation.

1.2.2. CFTR and male fertility

The male reproductive tract is composed of highly heterogeneous tissues that establish a proper environment to produce spermatozoa and provide a vehicle for their development, transit, and maturation. Throughout this process, Sertoli cells rely on the flux of water and electrolytes through several membrane transporters to ensure a highly regulated environment (Rato et al., 2010), among which CFTR is known to be essential (Boockfor et al., 1998).

The importance of CFTR is evidenced in its wide distribution throughout the male reproductive tract (Tizzano et al., 1994, Xu et al., 2011, Teixeira et al., 2013, Ribeiro et al.,

2022a). In the seminiferous tubules, CFTR was shown to have a wide expression among Sertoli cells and all germ cell types, particularly in primary spermatocytes and elongating spermatids (Boockfor et al., 1998, Xu et al., 2011, Teixeira et al., 2013, Ribeiro et al., 2022a). Sertoli cells are dependent on proper CFTR function to maintain a specific fluid and solute balance within the seminiferous lumen (Boockfor et al., 1998, Ribeiro et al., 2022a). CFTR is thought to be involved in: the activation of the pathways that result in CREB phosphorylation and consequent transcription of key spermatogenic genes (Xu et al., 2011); HCO_3^- entry into Sertoli cells and secretion/absorption into/from the seminal lumen for pH regulation; formation of the tight junctions between adjacent Sertoli cells (Chen et al., 2012a, Chen et al., 2012b). These tight junctions form the BTB and are essential for the physical support and proper development of the maturing germ cells (Nicander, 1967). As we will explore in the next paragraph, obstructive azoospermia is commonly observed in patients with cystic fibrosis. However, certain CFTR mutations (IVS8−T5 and TG12−T5−V470) have been identified as being positively associated with non-obstructive azoospermia and spermatogenic failure (Sharma et al., 2014, Jiang et al., 2017). According to a meta-analysis by Levkova *et al.*, the IVS8−5T variant is associated with spermatogenic dysfunction that may be due to an impairment in Sertoli cell function (Xu et al., 2011, Chen et al., 2019, Levkova et al., 2022).

In line with what is observed in the seminiferous tubules, the epithelial lining of the epididymis and *vas deferens* also exhibits abundant CFTR expression (Harris et al., 1989, Harris et al., 1991, Leir et al., 2020). In these structures, CFTR is thought to be responsible for directly and indirectly controlling water diffusion and luminal solute concentration (Hasegawa et al., 1992). It has a particularly important role in sperm maturation in the epididymis (Leir et al., 2020), where a tight control of the pH (especially in the cauda of the epididymis) is crucial for sperm storage (Acott et al., 1984, Carr et al., 1985). Additionally, it is impossible to overlook the role of CFTR in the development of the male reproductive tract tubules. In fact, patients with cystic fibrosis, caused by CFTR dysfunction, commonly present abnormal epididymis and *vas deferens* tubules (Yu et al., 2012). CFTR is described to be essential for the morphogenesis and differentiation of the epididymis epithelia (Breton et al., 2016). Despite that, *CFTR* gene and protein expression in the cells from the epididymis epithelium shows some contradictory results, possibly indicating a dynamic CFTR expression in epidydimal clear cells in relation to the others (Breton et al., 2016, Leir et al., 2020). Still, CFTR undoubtedly plays a crucial role in regulating luminal fluid composition, working alongside other transmembrane transporters (Pietrement et al., 2008, Ruan et al., 2012, Leir et al., 2020). pH acidification of the epididymis lumen, due to decreased HCO_3^- secretion, is crucial for arresting sperm motility during maturation and preventing the sperm from initiating the capacitation process prematurely.

CFTR also has a role in the formation of the *vas deferens*. Most cystic fibrosis patients show congenital bilateral absence of *vas deferens* (CBAVD) (Yu et al., 2012), which results in obstructive azoospermia. Men diagnosed with CBAVD often exhibit a high frequency of CFTR mutations, even in the absence of typical cystic fibrosis symptoms. (Tamburino et al., 2008, Tomaiuolo et al., 2011). Apart from this anatomical alteration, CFTR mutations are also found in men with non-obstructive azoospermia, oligozoospermia, teratozoospermia, and asthenozoospermia (Stuppia et al., 2005, Schulz et al., 2006, Sharma et al., 2014, Jiang et al., 2017), evidencing that CFTR dysfunction impacts spermatogenesis, spermiogenesis, and sperm maturation.

Although the significance of CFTR in Sertoli cells for spermatogenesis has been well-documented, its role in developing germ cells remains unexplored. Nevertheless, it is known that *CFTR* has a stage-specific expression pattern and can be found in human pachytene, spermatocytes, and elongated spermatids (Hihnala et al., 2006). The *CFTR* expression pattern during these specific stages could highlight an involvement of CFTR in spermiogenesis, during the decrease in cytoplasmatic volume (Gong et al., 2001). Moreover, HCO_3^- transport by CFTR increases cAMP levels in germ cells, which leads to the activation of the cAMP-CREM pathway via sAC activation (De Cesare et al., 2000, Jaiswal et al., 2003). This pathway is responsible for the expression of multiple spermatogenic genes in germ cells that allow them to complete their differentiation and specialization. After that, CFTR continues to be present in matured human sperm. It is expressed in a very specific location, known as the equatorial section of the sperm's head (Xu et al., 2007, Li et al., 2009, Diao et al., 2013, Sun et al., 2018, De Geyter et al., 2022), but its expression in the midpiece has also been noted (Diao et al., 2013).

CFTR expression levels in human sperm have been related to their motility. Asthenozoospermic men were shown to have decreased CFTR expression when compared to healthy fertile donors (Li et al., 2009). CFTR expression was also shown to be down-regulated in sperm from aged males, following the tendency of decreased sperm motility noted in this group. In this study, CFTR expression was positively correlated with forward motility (Diao et al., 2013). Furthermore, the presence of polymorphisms in the *CFTR* gene is also seen as a predictor of low progressive motility in idiopathic infertile men (Ventimiglia et al., 2016). From another perspective, CFTR inhibition is able to prevent sperm hyperactivation and acrosome reaction, possibly by inhibiting the increase in HCO_3^- levels, decreasing cytoplasmatic pH and consequently, decreasing cAMP production and PKA pathway activation (Xu et al., 2007, Li et al., 2009, Puga Molina et al., 2017).

1.2.3. Aquaporins interaction with CFTR

The water movement in the male reproductive tract is a vital process that not only helps the correct transport of spermatozoa through the rete testis and the epididymal ducts, but also defines the composition of the luminal fluids that fill the ducts in which germ cells develop, maturate and are stored (Voglmayr et al., 1970, Rato et al., 2010). For a long time, it was assumed that the water transport through the lipid bilayer was only due to simple diffusion. Further investigation showed that this theory was incomplete, bringing to light the pivotal role of a specific class of water channels: aquaporins (AQPs) (Verkman et al., 2000, Agre et al., 2002). AQPs are a family of small, hydrophobic, integral membrane channel proteins that are essential for the regulation of water homeostasis and for providing a continuous and rapid movement of water across tight junction epithelia (Brown et al., 1993, Verkman et al., 2000, Agre et al., 2002). In these types of epithelia, CFTR helps the creation of an ionic gradient that promotes water diffusion to the extracellular medium (Saint-Criq et al., 2017). Taking into consideration the roles of CFTR in male fertility, its role on water and solute diffusions presents a big role on the formation of proper fertilizing capable sperm cells. There is some evidence that shows that AQPs assist CFTR in those functions.

Several studies have highlighted the interaction between CFTR and certain AQPs (Schreiber et al., 1997, Schreiber et al., 2000, Pietrement et al., 2008, Ribeiro et al., 2022a), particularly in the male reproductive system (Pietrement et al., 2008, Jesus et al., 2014, Ribeiro et al., 2022a). In fact, CFTR has been shown to interact with AQP3, AQP7, and AQP9 in rodent Sertoli cells, suggesting that CFTR might serve as a regulator of water homeodynamics in these cells and in the lumen of the seminiferous tubules. Additionally, it was shown that during mammalian spermatogenesis, the reduction of volume of differentiating germ cells is remarkable, mainly due to the osmotically driven fluid efflux (Huang et al., 2006), being expected that AQPs and CFTR are implicated in this process. Also, since AQP7 and AQP8 are expressed in rat testis with a remarkably similar distribution to CFTR, an involvement of CFTR in the volume reduction observed in germ cells has been hypothesized (Gong et al., 2001). Moreover, the co-expression of AQP9 and CFTR in the luminal membrane of the epididymis, and their interaction through NHERF1 seems to be pivotal for the establishment of the epidydimal luminal fluid for sperm maturation and storage (Cheung et al., 2003, Pietrement et al., 2008). CFTR could also be involved in the regulation of AQP9-mediated secretion of glycerol from the epididymal epithelial cells into the maturing sperm cells, a substrate that is thought to have a role in this process (Pietrement et al., 2008). All these findings support the role of CFTR in regulating AQPs' water and solute transport in the reproductive system and corroborate the impact of such interactions in cystic fibrosis-related infertility. However, it is clear that many is yet to be brought into light about the modulation and interaction of CFTR and AQPs.

1.3. Role of aquaporins in male fertility

Cellular membranes are permeable to water, small nutrients, and toxins. However, the exchange through the membrane bilayer is rather slow due to its lipidic composition. To maintain cellular health and proper functioning, cells have to transport several substances (e.g. nutrients and water) faster than through simple diffusion (Yeste et al., 2017). In 1992, the group of Peter Agre identified a transmembrane channel protein responsible for facilitating water diffusion, later called AQP (Preston et al., 1992).

In mammalian cells, 13 different AQPs have already been described (AQP0-12), which were organized into groups (Figure 1.6.). The first group englobes the so-called orthodox AQPs represented by seven different homologs (AQP0-2, AQP4-6, and AQP8). These AQPs are described to be most selective for water, due to their low permeability to other small molecules. The second group includes AQP3, AQP7, AQP9, and AQP10, which are called aquaglyceroporins. They are characterized by a larger pore size (Sales et al., 2013), enabling them to be permeable to glycerol, urea, and other small neutral solutes, in addition to water (Laforenza et al., 2016a). The third group of AQPs is composed of AQP11 and AQP12 and is called superaquaporins. This group of AQPs is not yet fully understood, but research suggests that these AQPs are responsible for the movement of water and neutral solutes in intracellular compartments (Ishibashi et al., 2014). Still, these classifications are somewhat outdated, as new nomenclatures are being proposed for some AQPs. For example, AQP3, AQP4, AQP6, AQP7, AQP8, and AQP9 are also known as ammoniaporins for allowing the transportation of ammonia (Tesse et al., 2018). Additionally, AQPs capable of transporting hydrogen peroxide (H_2O_2; AQP3, AQP5, AQP8, AQP9, and AQP11) are known as peroxiporins (Tesse et al., 2018, Bestetti et al., 2020).

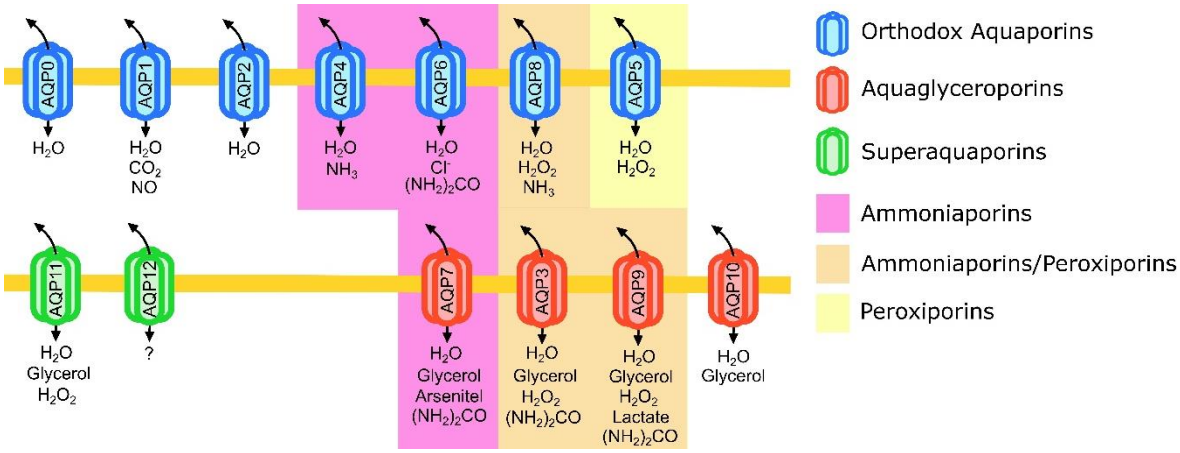

Figure 1.6.: Aquaporins (AQPs) classification based on their homology and biophysical properties. Up to date, thirteen distinct aquaporins have been identified in mammals (AQP0-12) and they have been classified into three main groups: orthodox aquaporins, aquaglyceroporins and superaquaporins. Although high homology exists among homologues of the same group, each may exhibit a different specificity in its permeability to solutes. For that reason, some AQPs have also been classified according to their permeability to particular solutes (ammoniaporins and peroxiporins).

Hence, AQPs are central in the regulation of cellular volume and in the movement of nutrients and/or elimination of metabolic residues (Yeste et al., 2017). They are involved in proper organ/tissue function, with male and female reproductive systems not being an exception (Laforenza et al., 2016a). The development of male and female gametes is a sum of multiple complex processes in which water regulation is essential (Zhang et al., 2012, Carrageta et al., 2020). Water homeostasis is pivotal for spermatogenesis (Yang et al., 2005) and sperm motility (Saito et al., 2004, Chen et al., 2011). Herein, we present the available research concerning the expression and function of the different AQPs on the male reproductive tract, as well as their role in gametes development until fertilization.

1.3.1. Aquaporins in the male reproductive system

AQPs are present in all human tissues, with some specificity regarding their distribution. In this section, we present the data available concerning the expression pattern of AQPs in each cell type/tissue (Table 1.1. and Figure 1.7.) and their role (if known) throughout the male reproductive tract.

1.3.1.1. Aquaporins in the testis

The testes participate in the creation of the microenvironment that allows the occurrence of spermatogenesis, with the consequential development of germ cells (Eto, 2015). The testicular environment can be divided into two main areas: the interstitial tissue and the seminiferous tubules (Setchell, 1986).

1.3.1.1.1. Aquaporins in Interstitial tissue

Leydig cells are the predominant cells (Wing et al., 1982) in the testicular interstitial tissue and produce and secrete testosterone (Su et al., 2011). Rat Leydig cells express AQP0 (Hermo et al., 2004) which seems to be involved in the water equilibrium between the extracellular and the intracellular medium (Hermo et al., 2004). AQP1 expression has also been confirmed in Leydig cells of human adolescents with varicocele, but that expression was not observed in healthy subjects (Nicotina et al., 2005). AQP1 expression exclusivity in pathological conditions highlights the extra need for fluid reabsorption/excretion characteristic of the varicocele disease (Nicotina et al., 2005). For AQP5, although mRNA microarrays predict its presence in human Leydig cells, protein expression is yet to be confirmed (Lizio et al., 2015). Another AQP described in mammal Leydig cells is AQP9. In rats, AQP9 has been suggested to mediate intensive transport of water (Tsukaguchi et al., 1998, Nihei et al., 2001) and non-charged solutes (Hermo et al., 2004), between cells and blood vessels and/or interstitial space (Nihei et al., 2001), assisting in the maintenance of cell homeodynamics.

Moreover, AQP1 expression was reported in endothelial cells of the microvessels net throughout the testis. It is believed that this AQP is directly involved in the water diffusion of

endothelial cell membranes between interstitial tissue and the circulatory system (Nicotina et al., 2004).

1.3.1.1.2. Aquaporins in the cells of the seminiferous tubules

The seminiferous epithelium is mainly composed of Sertoli cells and germ cells. AQPs present in Sertoli cells are suggested to contribute to the creation of a specific environment that allows spermatogenesis and serves as a vehicle for immature sperm motion through the tubules (Setchell et al., 1969). Similarly to what is described for Leydig cells, the orthodox AQP0 is also present in rodent Sertoli cells. However, its expression pattern is rather complex and not yet fully understood (Hermo et al., 2004).

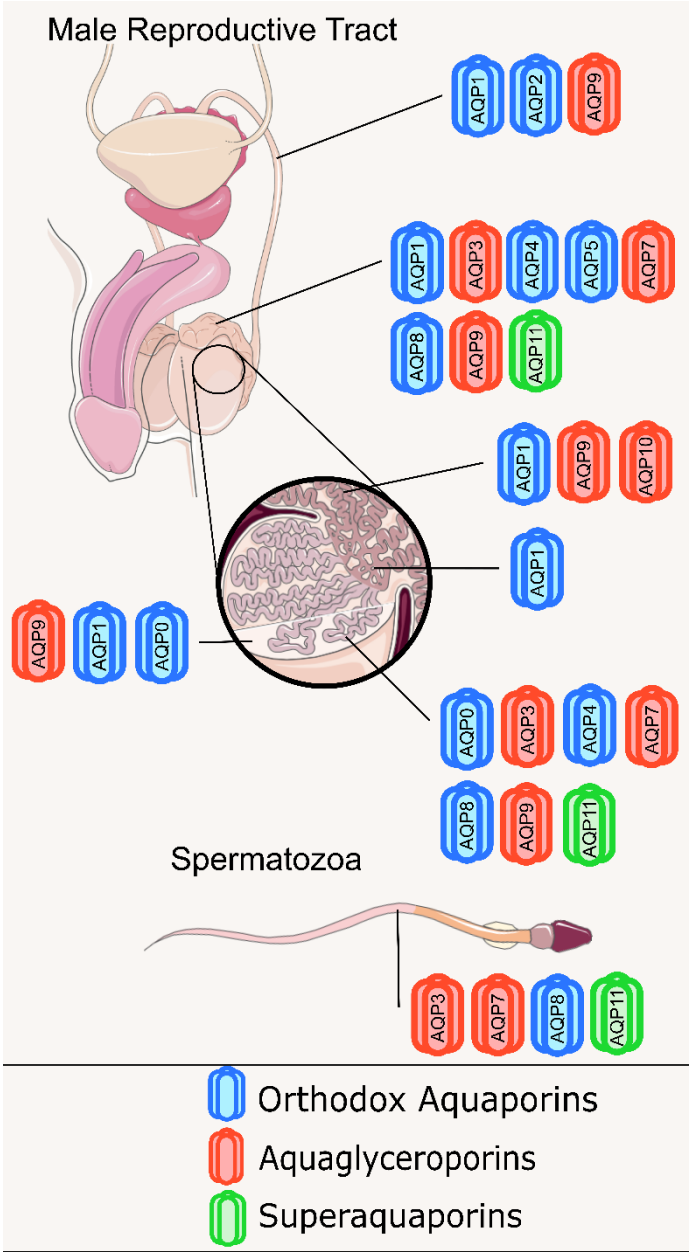

Figure 1.7.: Aquaporins (AQPs) distribution throughout the male reproductive tract.Ten AQPs have been identified in the male reproductive tract and spermatozoa (AQP0 to AQP5 and AQP7 to AQP11), among which orthodox aquaporins (in blue), aquaglyceroporins (in red) and superaquaporins (in green).

Table 1.1.: Aquaporin expression in the male reproductive tract and gametes of mammals.

Tissue		AQPs	Species	References
Interstitial tissue	Leydig cells	AQP0	R	(Hermo et al., 2004)
		AQP1	H*	(Nicotina et al., 2005)
		AQP9	R	(Tsukaguchi et al., 1998, Nihei et al., 2001)
Seminiferous tubules	Sertoli Cells	AQP0	R	(Hermo et al., 2004)
		AQP3	M, R	(Bernardino et al., 2018), (Hermo et al., 2004)
		AQP4	H, R	(Mobasheri et al., 2007), (Jesus et al., 2014)
		AQP8	R, M	(Elkjaer et al., 2001, Badran et al., 2002), (Yang et al., 2005)
		AQP9	M, H	(Bernardino et al., 2018), (Yeung et al., 2010a)
		AQP11	R	(Yeung et al., 2010b)
	Germ cells	AQP7	R, M, H	(Calamita et al., 2001a),(Sohara et al., 2007), (Kageyama et al., 2001)
		AQP8	R, M, H	(Calamita et al., 2001a), (Yang et al., 2005)
		AQP9	H	(Arena et al., 2011)
		AQP11	R	(Yeung et al., 2010b)
Excurrent ducts	*Rete testis*	AQP1	M, R	(Zhou et al., 2001), (Brown et al., 1993)
	Efferent ducts	AQP1	M, R	(Zhou et al., 2001), (Badran et al., 2002)
		AQP9	R, H	(Badran et al., 2002), (Pastor-Soler et al., 2001)
		AQP10	R	(Hermo et al., 2004)
	Epididymis	AQP1	R	(Brown et al., 1993)
		AQP3	R	(Hermo et al., 2004)
		AQP4	H	(Mobasheri et al., 2007)
		AQP5	R	(da Silva et al., 2006b)
		AQP7	R	(Hermo et al., 2011)
		AQP8	R	(Elkjaer et al., 2001)
		AQP9	R, H	(Pastor-Soler et al., 2001), (da Silva et al., 2006a)
		AQP11	R	(Hermo et al., 2008)
	Vas deferens	AQP1	R	(Brown et al., 1993)
		AQP2	R	(Stevens et al., 2000)
		AQP9	R, M	(da Silva et al., 2006a), (Hashem, 2010)
Spermatozoa		AQP3	H,P,Ho	(Chen et al., 2011), (Prieto-Martinez et al., 2017), (Bonilla-Correal et al., 2017)
		AQP7	H, M, P	(Ishibashi et al., 1997, Saito et al., 2004, Moretti et al., 2012), (Sohara et al., 2007), (Prieto-Martinez et al., 2017)
		AQP8	H, M	(Yeung et al., 2010a, Laforenza et al., 2016b), (Yang et al., 2005)
		AQP11	H, M, P, Ho	(Laforenza et al., 2016b), (Yeung et al., 2009) ,(Prieto-Martinez et al., 2016) , (Bonilla-Correal et al., 2017)

Legend: H-Human, M-Mice, R-Rat, P-Pig, Ho-Horse; * Expression reported in non-healthy individuals

Histological cuts of rat seminiferous tubules showed that AQP0 expression levels in Sertoli cells changed in different stages of the seminiferous epithelium cycle. Higher expression is seen in the intermediate stages (Hermo et al., 2004), explaining their detection only in half

the circle of the histological cuts. This pattern can be due to the spermatogenic wave and its involvement in specific spermatogenic phases. In fact, AQP0 expression in Sertoli cells was associated with the detachment process of elongated spermatids from these somatic cells into the lumen of the seminiferous tubules (Hermo et al., 2004). As far as AQP3 is concerned, its expression was confirmed in mice (Bernardino et al., 2018) and rat Sertoli cells (Hermo et al., 2004). This aquaglyceroporin is suggested to mediate the transport of glycerol (Bernardino et al., 2018), which is thought to be an important substrate for germ cell development and thus, spermatogenesis (Crisostomo et al., 2017). AQP4 is also present in human seminiferous tubules (Mobasheri et al., 2007) and rat Sertoli cell membranes (Jesus et al., 2014). In rodent Sertoli cells, AQP4 is situated in direct contact with CFTR (Jesus et al., 2014). This interaction indicates a possible role of AQP4 and CFTR in regulating water and ion homeostasis in the seminiferous tubules.

Regarding AQP7, its expression was confirmed in germ cells, particularly Sertoli cells and germ cells in later stages of development (secondary spermatocytes and elongated spermatids) (Kageyama et al., 2001). A concurrent study described AQP7 expression also on spermatocytes of rodents (Calamita et al., 2001a). Authors hypothesized that AQP7 may have a role in the drastic decrease in cellular volume in spermatids during spermiogenesis, by modulating water efflux. Interestingly, studies in AQP7 knockout mice showed no alteration in male fertility, particularly in daily sperm production, motility, or even in the number of offspring when compared to wild-type animals (Sohara et al., 2007). These results suggest that other AQPs may compensate for the absence of AQP7 expression. For example, AQP8 expression was also confirmed in rat elongated spermatids. This highlights the involvement of both AQPs in spermatids cytoplasmic condensation (Calamita et al., 2001a). Moreover, AQP8 expression was also noted in the cytoplasm of primary spermatocytes. AQP8 was also confirmed in rat Sertoli cells. Studies showed that AQP8 immunolocalization delineates the adluminal section of Sertoli cells plasmatic membrane (Elkjaer et al., 2001, Badran et al., 2002) and is also present in human Sertoli cells (Yeung et al., 2010a). Interestingly, AQP8-null mice (like AQP7 knockout mice) are fertile. Once more, it suggests versatility regarding AQPs function and their ability to compensate other AQPs function. However, it was noticed that AQP8-null mice had greater sized testes when compared to wild type (Yang et al., 2005).

AQP9 expression was detected in human Sertoli cells (Yeung et al., 2010a), while in germ cells, AQP9 protein expression is present in human primary spermatocytes and adluminal haploid germ cells (Arena et al., 2011). Functional studies performed in mice Sertoli cells showed that estrogen treatment caused *Aqp*9 gene down-regulation accompanied by a decrease in glycerol permeability (Bernardino et al., 2018), evidencing that aquaglyceroporin expression is essential for Sertoli cells and germ cell physiology and thus,

male fertility (Bernardino et al., 2018). A study performed in the testis of adolescents suffering from varicocele showed that Sertoli cells had decreased AQP9 expression (Arena et al., 2011). These authors suggested that AQP9 might have a role in lactate transport from Sertoli cells to the developing germ cells, causing lactate deprivation in developing germ cells of varicocele patients (Arena et al., 2011). Lactate is known to be the preferable substrate for developing germ cells thus, lactate deficiency due to AQP9 down-regulation may explain the slow spermatogenic rate reported in varicocele patients (Arena et al., 2011). Evaluation of AQP11 expression by immunohistochemistry on rat testis showed a signal in germ cells, exclusively in the distal tail section of elongated spermatids and in residual bodies inside Sertoli cells, probably originated by unviable elongated spermatids phagocytosis and intracellular organelles (Yeung et al., 2010b). The particular location in the tail distal section of elongated spermatids, where the cytoplasm is scarce, and no organelle is known to be located makes it difficult to assign a function to this AQP. However, the authors hypothesized that AQP11 could be located in the sperm membrane to help eliminate the excess water for the completion of flagellum formation (Yeung et al., 2010b).

1.3.2. Aquaporins in the male excurrent ducts

After spermatozoa are released into the lumen of the seminiferous tubules, the fluid created by Sertoli cells assists in the transportation of the immature spermatozoa to the *rete testis* (Holstein et al., 2003). In this portion of the male reproductive tract, AQP1 expression was confirmed in both mice and rats (Brown et al., 1993, Zhou et al., 2001). In a study performed with *Aqp1*-knockout mice, it was reported that these animals presented a dilated *rete testis* when compared to wild-type animals. This suggests that AQP1 is crucial for fluid reabsorption in this section (Zhou et al., 2001), possibly due to its expression in the vascular endothelium that could be involved in transcellular water transportation. This transcellular transportation is necessary since 90% of fluid reabsorption in the testis is done by the epithelial cells in the efferent ducts (Zhang et al., 2012). Here, is where the concentration of sperm cells is mostly achieved and, for that, water channels are mostly needed. Like in *rete testis*, rat efferent ducts also express AQP1 (Badran et al., 2002). Ciliated cells expressed AQP1 on the cilia, while the non-ciliated cells expressed this AQP in the microvilli, apical endosomes, and basolateral cellular membrane (Badran et al., 2002). However, unlike *rete testis*, efferent ducts of *Aqp*1-knockout mice do not show any histological alteration, when compared to the efferent ducts of wild-type animals (Zhou et al., 2001). This is suggested to be due to the expression of multiple AQPs in the efferent ducts. In fact, AQP9 expression was reported in the microvilli of the non-ciliated cells of rat efferent ducts (Badran et al., 2002). In humans, AQP9 expression was also reported in the apical membrane of non-ciliated cells of the efferent ducts (Pastor-Soler et al., 2001). The

importance of different AQPs expression in the same tissue (especially from different groups such as AQP1 and AQP9) can be noted in tissues like the efferent ducts. While the orthodox AQP1 seems to be specialized in transporting water, the expression of the aquaglyceroporin AQP9 may assist in the movement of glycerol and other neutral solutes into the lumen of the tubule (Cooper et al., 1981). To assist in these events, AQP10 is also present in ciliated and non-ciliated cells of rodent efferent ducts (Hermo et al., 2004).

Epididymis is the subsequent duct located downstream to the efferent ducts. The literature points to specific AQPs expression patterns in the different sections of the epididymis. Moreover, many AQPs were confirmed to be expressed in the epididymis of animal models, but few were studied in human male tissue. Since epididymis supports, concentrates, and stores the maturing sperm cells, one would assume that this tissue expresses multiple AQPs. In fact, it was suggested that the multiple cells that constitute the epididymis have cooperative reabsorption of water and solutes between the more luminal cell types (principal and clear cells) and the basal cells increasing the flux of water through the entire width of the epididymis epithelium (Hermo et al., 2004), a process enhanced by the expression of multiple AQPs.

As reported in the testis, AQP1 expression was described in the vascular endothelium adjacent to the epididymis epithelium in rats (Brown et al., 1993). There, AQP1 can assist in the final reabsorption of water into the circulation. This process, with the contribution of other AQPs, allows the exchange of water from the lumen of the tubule and the circulatory system and the creation of the microenvironments that permit the maturation, concentration, and storage of sperm cells. However, specific mechanisms are yet to be revealed.

AQP3 expression was confirmed in basal cells across all sections of rodent epididymis (Hermo et al., 2004) whereas AQP4 expression was only confirmed in human epididymis (Mobasheri et al., 2007), with no further information available on the cell-specific expression of this AQP on the epididymis. Contrastingly, AQP5 expression has not yet been described in human tissue but is present in principal cells in the corpus and cauda sections of rat epididymis (da Silva et al., 2006a). AQP7 expression is also present in rat epididymis (Hermo et al., 2011). The presence of this protein was confirmed in the basolateral membrane of adjacent principal cells in the caput section of the epididymis. In the corpus and cauda epididymis section, AQP7 expression was found in the plasma membrane of principal cells and delineating some basal cells (Hermo et al., 2011). Moreover, a study performed in rat models confirmed the expression of AQP8 in basal cells of the epididymis (Elkjaer et al., 2001).

AQP9 seems to be one of the most abundant AQPs in the epididymis, and its expression was already described in humans with similar expression patterns to what is reported in rodents (Pastor-Soler et al., 2001). AQP9 is expressed through all sections of epididymal

epithelium, especially in the apical microvilli of principal cells (da Silva et al., 2006a), likely to maintain metabolic substrate supply for sperm cells (Cooper et al., 1981). Similarly, AQP11 expression was also confirmed in the microvilli of principal cells of rat epididymis, although in the more distal region of the caudal section (Hermo et al., 2008).

After their maturation in the epididymis, spermatozoa are then guided to the *vas deferens*. This section of the male reproductive tract can be divided into three different sections (proximal, middle, distal), and like the epididymis, separate regions have different AQP expression patterns. AQP1, for example, is only expressed in the luminal and basolateral cell membrane of principal cells of rat *vas deferens* distal section (Brown et al., 1993). Similarly to what was described for the efferent ducts, this AQP is responsible for the movement of large amounts of water possibly for the purpose of concentrating sperm cells in the *vas deferens* (da Silva et al., 2006a). In adult rats, AQP2 is also differentially expressed throughout the sections of the *vas deferens*. It has a more intense expression in the principal cells of the middle and distal sections, when compared to that of the principal cells of the proximal section, where no expression is apparent (Stevens et al., 2000). Interestingly, AQP2 expression levels change through postnatal development in rats, that at four weeks of age exhibit the pattern of expression detected in adult animals. This finding suggests that AQP2 expression is coordinated via translational or post-transcriptional mechanisms (da Silva et al., 2006a). Rodent principal cells also exhibit AQP9 expression (da Silva et al., 2006a), probably to ensure fast uptake of glycerol by sperm cells (Cooper et al., 1981) as AQP9 does in the liver (Calamita et al., 2012). A study conducted using an *Aqp9*-knockout mouse model showed an increased concentration of glycerol in serum although these animals were fertile, highlighting the ability of AQP9 to transport this substrate (Hashem, 2010). The role of AQP9 in the excurrent ducts related to its peroxiporin activity is not ruled out.

1.3.3. Aquaporins in spermatozoa

Throughout their journey from the seminiferous tubules until reaching the oocyte, spermatozoa encounter multiple microenvironments created by the epithelial cells of both male and female reproductive tracts, in order to ensure maximum fertilization capacity. However, all the effort of the epithelial cells would be in vain if the sperm cells were not able to withstand the changes in the composition of the various milieu and accommodate the passage of substances from the extracellular to the intracellular medium (and vice-versa).

To assist in those events, multiple AQPs have been described in the membranes of mammalian spermatozoa. In humans, AQP3 expression was described on the tail of sperm cells (Chen et al., 2011), whereas AQP7, AQP8, and AQP11 expression, already described in developing germ cells (Calamita et al., 2001a, Kageyama et al., 2001, Yeung et al.,

2010b), have been non-consensually noted in mature spermatozoa. While AQP7 expression is most commonly situated in the middle piece, some authors also reported less intense expression through all sections of the spermatozoa, suggesting different pattern expressions in different individuals (Ishibashi et al., 1997, Saito et al., 2004, Moretti et al., 2012). In the case of AQP8, its expression is mostly described in the middle piece of human spermatozoa (Yeung et al., 2010a, Laforenza et al., 2016b). Finally, AQP11 seems to be expressed in the intracellular organelles of the head and middle piece, although its expression has also been referred to throughout the membrane of the tail (Laforenza et al., 2016b).

The functional role of AQPs in spermatozoa has been related to: changes in cell volume occurring during spermiation; and changes in the osmolality of the surrounding environment that spermatozoa experience during their journey throughout the male and female reproductive tracts. Throughout their passage, sperm are exposed to the mild hypertonic environment of the cauda epididymis (~415 mOsm/Kg in mice) and to the isotonic one in the uterine body (~310 mOsm/Kg in mice), which is crucial for motility activation. In the latter case, this osmoadaptation/osmoregulation is related to a proper osmotic balance allowing sperm to have adequate shape and function. Not only does this adaptation rely on ion channels but also water channels (Yeung et al., 2010a). Thus, various AQPs have different roles and functions in the processes that spermatozoa undergo during their journey to fertilization, which ultimately allows fertilization.

As aforementioned, osmoadaptation is crucial during the transition of sperm from the male to female reproductive tracts, due to the hypertonic environment of the epididymal cauda and the isotonic environment of the uterine body that activates sperm motility (Chen et al., 2011). This was described for mice, which are known to ejaculate into the uterus and not into the vagina like humans. In such an osmoadaptation, there is an increase in cell volume, so AQPs are needed to protect spermatozoa from detrimental swelling. In this osmoadaptation, AQP3 would play a pivotal role (Chen et al., 2011). This was studied in much detail using knockout mice. Mouse sperm with deficient AQP3 were seen to activate their motility adequately when exposed to a hypotonic environment, but they showed an impaired ability to regulate cell volume upon entering the uterus and the oviduct, which resulted in an increased tail bending (Chen et al., 2011). Specifically, the absence of AQP3 was found to lead the tail to deform, forming a hairpin-like structure due to mechanical membrane stretch. Therefore, AQP3 was suggested to serve as an osmosensor specialized in the efflux of water that is involved in the osmoadaptation of sperm cells during their transit throughout male and female reproductive tracts, and a deficiency in this protein results in reduced fertilizing ability due to impaired motility in the oviduct (Chen et al., 2011).

Most of the functional studies about AQPs in mammalian sperm have been conducted with AQP7. This includes knockout studies in mice, and the relationship of AQP7 with sperm quality and fertilizing ability in humans. With regard to knockout studies, Sohara and colleagues surprisingly reported no differences between the *Aqp7* knockout model and the wild-type control specimen regarding testis and epididymis morphology, sperm quality (daily sperm production and motility) and fertilizing ability (*in vitro*) and offspring (Sohara et al., 2007). This suggests that AQP7 knockout mice were neither sterile nor had abnormal sperm function and morphology. On the other hand, blocking K^+ channels with quinine results in sperm volume increase, which is prevented when AQPs are inhibited with mercuric chloride ($HgCl_2$) (Sohara et al., 2007). With regard to the relationship between the relative content of AQP7 and fertilizing ability in humans, Saito and colleagues compared fertile controls and infertile patients and associated the absence of AQP7 in ejaculated spermatozoa with impaired motility and infertility (Saito et al., 2004). In another trial, Yeung and colleagues compared fertile controls and infertile patients and also found that the relative content of AQP7 in sperm evaluated with flow cytometry was correlated with sperm motility and was higher in fertile donors than in infertile patients (Yeung et al., 2010a). As far as the relationship between AQP7 and sperm quality is concerned, Moretti and colleagues found that human sperm selected through swim up and separated into motile and immotile fractions have different AQP7-localization. Indeed, a higher percentage of sperm cells from the motile population showed staining of AQP7 labeled in the pericentriolar area, midpiece, equatorial segment and in the tail while the abnormal and immotile fraction had AQP7 labeling in the cytoplasmatic residues, coiled tails, and entire head and acrosome. In addition, spermatozoa with normal morphology and progressive motility showed clear dotted AQP7 staining, whereas weak, diffuse AQP7 immunoreactivity was observed in sperm cells with cytoplasmic residues (translucent vacuoles and swollen and badly assembled mitochondria), cytoplasmic droplets (immature) and coiled tails (Moretti et al., 2012). Therefore, this link with sperm morphology indicates that defective spermatogenesis and epididymal maturation led to an abnormal localization and expression of AQP7. In light of all the aforementioned, current evidence supports the idea that AQP7 is crucial during spermatogenesis, differentiation into spermatozoa (spermiation) and epididymal maturation, glycerol metabolism in sperm and volume reduction of spermatids (Suzuki-Toyota et al., 1999, Yeung et al., 2010a).

Regarding AQP8, earlier studies suggested that since phloretin, an inhibitor of GLUT transporters, affected the permeability of human and sheep sperm to water, these transporters could be involved in water transport across the sperm plasmalemma (Curry et al., 1995). This hypothesis was later dismissed by Callies and colleagues (2008), who did not find evidence that sodium-dependent solute and glucose transporters were involved in

the transport of water in mouse spermatozoa (Yeung et al., 2009). In fact, phloretin does not inhibit orthodox AQPs but rather aquaglyceroporins (Delgado-Bermudez et al., 2019). Since AQP8 is an orthodox AQP, it was reasonable that when Yeung and colleagues used two inhibitors ($HgCl_2$ and phloretin) to determine the role of AQPs in the regulation of sperm volume, they observed that $HgCl_2$ but not phloretin was effective in blocking quinine-induced swelling (Yeung et al., 2010a). This suggested that AQP8 rather than aquaglyceroporins (targeted by phloretin) is involved in the regulation of sperm volume. These results were also in agreement with Callies and colleagues who found that mercury ion (Hg^{2+}) and silver ion (Ag^{+})-sensitive channels are involved in the transport of water, thus supporting a role for AQP8 (Yeung et al., 2009). Taking this evidence into consideration, it is conceivable to assume that AQP8 is the major permeation route of water in the middle piece of spermatozoa (Yeung et al., 2009). It is also possible that this peroxiporin is responsible for the elimination of ROS (especially H_2O_2) from sperm mitochondria, as demonstrated in a study made in seabreams (Chauvigne et al., 2015). In fact, the inactivation of an ortholog of AQP8 (AQP8b) and consequent ROS accumulation in sperm mitochondria, resulted in mitochondrial membrane depolarization, which reduced the production of ATP and diminished progressive sperm motility (Chauvigne et al., 2015). Moreover, decreased ATP production suggested the inability of sperm to go through capacitation (high ATP levels are a marker for sperm hyperactivation), which is a crucial process for sperm to achieve fertilization. Still, it is worth noticing that there is not a total homology between the AQP8b and mammalian AQP8 (Chauvigne et al., 2015) and its expression pattern. For instance, in rats, while found at the inner mitochondrial membrane of hepatocytes and Sertoli cells, AQP8 is absent in the mitochondria of spermatozoa (Calamita et al., 2005). However, in a recent study, its expression was reported in the mitochondrial membrane of human spermatozoa (Laforenza et al., 2016b), highlighting AQP8 involvement in ROS management in spermatozoa, as was reported in other cell types (Medrano-Fernandez et al., 2016). In fact, low H_2O_2 concentration is important for sperm capacitation in order to initiate the cascade of events that allow oocyte fertilization. However, if too low, H_2O_2 inhibits this process (Rivlin et al., 2004). This opens up the possibility of peroxiporins, like AQP8, to regulate the concentrations of H_2O_2 to initiate the process of sperm capacitation. Interestingly *Aqp8*-knockout mice are fertile, with no differences being found in sperm concentration and morphology between AQP8 null and wild-type mice (Yang et al., 2005), although sperm motility and ability to fertilize were not evaluated. Because of the crucial role of AQPs on sperm function, it has also been investigated whether infection with human Papillomavirus (HPV) could affect the expression and function of AQPs in human spermatozoa. Interestingly, while neither AQP3 nor AQP7 were found to interact with HPV, AQP8 was found to co-localize (laser scanning confocal microscopy) and co-

immunoprecipitate with HPV-L1 protein, which could explain why HPV reduces the sperm permeability to water and increases their sensitivity to oxidative stress via interaction/inhibition of AQP8 (Pellavio et al., 2020). The same study also described that AQP8, as well as AQP3 and AQP7, are found expressed in the head of human spermatozoa (Pellavio et al., 2020) around the acrosome which could highlight a possible role of these AQPs in acrosomal reaction. Despite not being yet fully understood, sperm capacitation need tightly regulated H_2O_2 concentrations to allow hypermotility and acrosome reaction, being both these processes critical for natural fertilization (Rivlin et al., 2004). In fact, acrosome swelling is required for the proper completion of acrosomal reaction (Zanetti et al., 2009). Taking into consideration that 2 of the 3 AQPs found in human spermatozoa head are capable of monitoring H_2O_2 concentrations and acrosomal swelling (AQP3 and AQP8) one could assume that these water pores are involved in acrosome reaction. However, we could not find any studies that were able to confirm this possibility.

In spite of all the aforementioned, Yeung and colleagues compared fertile controls and infertile patients and found that while sperm cells with higher levels of AQP8 showed less sperm tail coiling in response to the induction of swelling, there were no significant differences regarding the relative content of this protein. Therefore, AQP8 is now suggested to play a vital role in water transport (Yeung et al., 2010a), but more research is needed to address its relationship with sperm fertilizing ability in humans and animal models.

The last AQP encountered in the mammalian spermatozoa is AQP11. Unfortunately, knockout animal studies of this particular AQP are, to this day, impossible to perform due to kidney failure and consequent premature death (Matsuzaki et al., 2017). Nevertheless, its expression pattern indicates some important functions. AQP11 presence on the membrane of intracellular organelles (Laforenza et al., 2016b) could be important for the maintenance of microenvironments essential for sperm function. As an example, AQP11 expression in the endoplasmic reticulum is essential for H_2O_2 transportation and thus, in the assistance of redox homeostasis and pathways signaling in kidney cells (Bestetti et al., 2020). A possible role of AQP11 in the elimination of H_2O_2 and other metabolic waste from mitochondria and other organelles, similar to what was discussed for AQP8, would be interesting to explore. For now, a study performed with pig sperm cells, that have a similar expression pattern to the one seen in human sperm, noted that AQP11 expression was correlated with higher sperm quality (Laforenza et al., 2016b). The spermatozoa with higher AQP11 expression also had higher membrane integrity and motility, further evidencing the relevance of AQP11 on sperm function. However, more studies are needed to assess the function of AQP11 and other AQP homologs in sperm physiology. This information is of extreme relevance and valuable as it could help in the development of technologies capable of enhancing assisted reproductive technologies (ARTs).

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Chapter 2

Aims

The trend of delayed parenthood is associated with the rise of male subfertility and infertility. A significant number of these cases are diagnosed as idiopathic, reflecting the difficulty in locating a specific cause through standard fertility diagnosis. Genomic screening is often used to obtain additional information for these infertility cases, however, it is normally limited to the identification of known pathogenic mutations. Individuals with cystic fibrosis, the most common genetic disorder among Europeans, are characterized by having mutations in the *CFTR* gene and frequently exhibit fertility impairments, highlighting the crucial role of CFTR in male reproductive function. While CFTR mutations are known to reduce CFTR protein expression and function at the cell membrane, the precise mechanisms underlying male infertility in this condition remain to be fully elucidated.

The generation of healthy spermatozoa capable of fertilization depends on the proper development and coordination of a series of intricate processes within the male reproductive system. Spermatozoa formation during spermatogenesis in the testis, maturation in the epididymis, and capacitation in the female reproductive tract are the major processes that must be completed to allow natural fertilization. For context, most ejaculated spermatozoa are not fit for fertilization even in healthy fertile individuals. All three processes are crucial for the development of one of the more evident characteristics of the spermatozoa: their ability to swim. However, four in five infertile men have an impairment in sperm motility. The increase in cytoplasmatic pH is one of the triggers that start the pathway for sperm motility, which CFTR is an integral part of. On the other hand, CFTR is also involved in multiple other mechanisms due to its vast interactome. One family of proteins found in the CFTR scaffolding complex is the aquaglyceroporins. Aquaglyceroporins are the primary channel for water and small solutes between the cytoplasmatic and extracellular environments. Recognizing the critical role of the balance between intracellular and extracellular

environments in male fertility, we focused our research on exploring the potential existence and importance of the CFTR/aquaglyceroporin complex. To do so, we have formulated 3 different objectives:

- **Study of the impact of CFTR function on aquaglyceroporin-mediated permeability in Sertoli cells.** The impact of CFTR dysfunction on male fertility is multifaceted. Severe cases can lead to obstructive azoospermia, whereas milder forms are associated with non-obstructive azoospermia and compromised spermatogenesis. Recent investigation revealed that the negative effects on male fertility in men with *CFTR* gene mutations are not solely due to decreased CFTR conductance but also involve the impairment of its interactors. CFTR and aquaglyceroporins are crucial in many processes essential for male fertility, including spermatogenesis. Given CFTR's ability to modulate the permeability of other membrane channels, we will study the effect of CFTR inhibition on aquaglyceroporin permeability. The structure of CFTR allows it to interact with other transmembrane channels and exert its modulation by protein-protein interaction. Thus, it is relevant to understand if CFTR can modulate aquaglyceroporin permeability in Sertoli cells since these are responsible for spermatogenesis. and a possible factor for spermatogenic impairment. This could highlight a novel mechanism that contributes for spermatogenic impairment
- **Investigate the implications of aquaglyceroporin-mediated membrane permeability in the motility of human sperm.** Increasing our focus on sperm cells, the specific roles and significance of the various aquaglyceroporins remain poorly understood. Current literature suggests a correlation between the expression of certain AQPs and sperm parameters, highlighting their role in sperm osmoadaptation. While compensatory behaviors of different aquaglyceroporins in somatic cells of knock-out models are well-documented, the same is not true regarding sperm cells. Aquaglyceroporins have a strong influence on sperm osmoadaptation. Thus, it is plausible that these pores may also be useful for solute diffusion during sperm maturation and motility development. Herein, we aimed to study a potential link between sperm aquaglyceroporin-mediated glycerol permeability and sperm motility.
- **Evaluate the impact of CFTR function on aquaglyceroporin-mediated permeability in human sperm and its relationship with sperm motility.** The final objective was to evaluate the impact of CFTR function on aquaglyceroporin-mediated permeability in human sperm and its relationship with sperm motility. Literature indicates that CFTR dysfunction and down-expression are related with decreased sperm motility. With this objective, we aimed to investigate the potential modulation

of aquaglyceroporin-mediated glycerol permeability by CFTR dysfunction in sperm from normozoospermic men. This research may reveal a new mechanism of CFTR in sperm function and emphasize the importance of CFTR and aquaglyceroporin interaction in sperm motility, highlighting its function, or lack thereof, in sperm from asthenozoospermic men.

Chapter 3

CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells

This chapter was adapted from the published work:

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel that is crucial for fluid homeodynamics throughout the male reproductive tract. Previous evidence shed light on a potential molecular partnership between this channel and aquaporins (AQPs). Herein, we explore the role of CFTR on AQPs-mediated glycerol permeability in mouse Sertoli cells. We were able to identify the expression of CFTR, AQP3, AQP7, and AQP9 in mouse Sertoli cells by qPCR, western blot, and immunofluorescence techniques. Cells were then treated with CFTRinh-172, a specific CFTR inhibitor, and its glycerol permeability was evaluated by stopped-flow light scattering. We observed that CFTR inhibition decreased glycerol permeability in mouse Sertoli cells by 30.6% when compared to the control group. A DUOLINK proximity ligation assay was used to evaluate the endogenous protein-protein interactions between CFTR and the various aquaglyceroporins we identified. We positively detected that CFTR is in close proximity with AQP3, AQP7, and AQP9 and that, through a possible physical interaction, CFTR can modulate AQP-mediated glycerol permeability in mouse Sertoli cells. As glycerol is essential for the control of the blood-testis barrier (BTB) and elevated concentration in testis results in the disruption of spermatogenesis, we suggest that the malfunction of CFTR and the consequent alteration in glycerol permeability is a potential link between male infertility and cystic fibrosis.

3.1. Introduction

CFTR is a transmembrane channel that is responsible for the transport of HCO_3^- and Cl^-. CFTR absence or malfunction is the main cause of cystic fibrosis, one of the most prevalent and lethal monogenic disorders among Caucasians (Csanady et al., 2019). The absence or malfunction of CFTR leads to impaired transport of Cl^- and HCO_3^- and, consequently, impaired fluid and ionic regulation in the affected tissues. Of particular importance, cystic fibrosis greatly affects the male reproductive tract. For instance, CFTR mutations are the main cause of CBAVD (de Souza et al., 2018). In addition and due to the wide expression of CFTR in the male reproductive tract, cystic fibrosis results in a debilitated pH regulation and fluid homeodynamics, which culminates in male subfertility or even infertility (Alves et al., 2015, Bernardino et al., 2019).

CFTR has been shown to directly impact water movement between the intra and extracellular space, which is especially evident in epithelial cells, but CFTR itself is not the primal route for water across cell membranes (Hasegawa et al., 1992, Morrison et al., 2022). Water diffusion is driven by the establishment of solute gradients that are transported by CFTR, being mostly facilitated by another family of transmembrane proteins, AQPs. Like CFTR, AQPs are also widely expressed in the male reproductive tract, where they assist in the fluid homeostasis essential for spermatogenesis (Yeste et al., 2017). Alterations in the expression or activity of AQPs are linked with male subfertility or infertility scenarios. Among the AQPs family, aquaglyceroporins are one of the three subgroups essential for the testis function due to their capacity to mediate the movement of glycerol across membranes (Crisóstomo et al., 2017). Glycerol is reported as necessary for spermatogenesis, although

only at specific concentrations (Crisóstomo et al., 2017). Acute exposition to high concentrations of glycerol in the testis can lead to a temporary arrest of spermatogenesis, while chronic exposure to high concentrations of glycerol may lead to definitive damage in the BTB, death of germ cells, and, consequently, permanent oligospermia or even azoospermia (Wiebe et al., 1989).

Compelling evidence suggests that CFTR acts as a molecular partner of AQPs, regulating their activity (Alves et al., 2015). For instance, our group described a molecular interaction between CFTR and both AQP4 and AQP9 in primary cultured rat Sertoli cells (Jesus et al., 2014b, Jesus et al., 2014a). Sertoli cells are responsible for the subsistence and regulation of the BTB, conferring crucial mechanical and nutritional support to germ cells (Crisóstomo et al., 2018). Since AQP9 is reported as crucial for water and solute homeostasis in several tissues, it is also expected that this AQP regulates water homeostasis in Sertoli cells and consequently inside seminiferous tubules (Pastor-Soler et al., 2010, Yeung et al., 2010, da Silva et al., 2022). Recently, our group identified for the first time the expression of AQP3 and AQP9 in mouse Sertoli cells (Bernardino et al., 2018). In that study, we showed that both these aquaglyceroporins are responsible for the glycerol entry into Sertoli cells, whereas AQP9 was observed as the main glycerol transmembrane channel. Since CFTR modulates AQP-mediated water transport (Cheung et al., 2003), we hypothesize that CFTR would also modulate the transport of other AQP-mediated solutes. Following these results, we hypothesized that CFTR physically interacts with aquaglyceroporins in mouse testis, specifically in mouse Sertoli cells, where it may directly modulate aquaglyceroporins-mediated glycerol transport. Thus, this study aimed to evaluate the effect of CFTR on the membrane glycerol permeability of mouse Sertoli cells. We also aimed to determine a possible physical interaction of CFTR with mice aquaglyceroporins AQP3, AQP7, or AQP9 (*Aqp10* gene is a pseudogene in mice) (Morinaga et al., 2002).

3.2. Materials and methods

3.2.1. Chemicals

NZY Total RNA Isolation kit, NZY M-MuLV Reverse Transcriptase, and NZYSpeedy qPCR Green Master Mix were purchased from NZYtech (Lisboa, Portugal), fetal bovine serum (FBS) from Biochrom AG (Berlin, German), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States of America) unless stated otherwise.

3.2.2. Cell culture and experimental groups

Mouse Sertoli cell line TM4 was purchased from ATCC (Manassas, VA, United States of America) and cultured as previously described (Bernardino et al., 2018). Briefly, cells were seeded in 75 cm^2 flasks (SPL7005, SPL Life Sciences, Gyeonggi, South Korea) in Dulbecco's modified Eagle medium and Ham's nutrient mixture F12 (DMEM:F12) (1:1)

supplemented with 10% FBS, 1.2 g/L sodium bicarbonate ($NaHCO_3$), 15 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, and 50 ug/mL gentamicin. Cells were handled in a laminar flow chamber and maintained in an atmosphere of 5% carbon dioxide (CO_2) at 37 ºC. Experiments were carried out with n=6.

3.2.3. Reverse Transcriptase Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

The extraction of total RNA from mouse Sertoli cells and mouse testis was conducted using the NZY Total RNA Isolation Kit according to the manufacturer's instructions. The extracted RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, United States of America). Then, RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase. The resultant complementary deoxyribonucleic acid (cDNA) was used with primer sets designed to amplify cDNA fragments of interest. Conventional RT-PCR was executed to identify *Aqp3*, *Aqp7*, and *Aqp9* mRNA in mouse Sertoli cells and mouse testis. Quantitative Real-Time PCR (qPCR) was performed to evaluate the *Aqp3, Aqp7, and Aqp9* mRNA abundance in mouse Sertoli cells, as previously described (Bernardino et al., 2016a). Specific primers were designed for the amplification of the *Aqp3*, *Aqp7*, *Aqp9*, and β-*2-microglobulin* transcripts (Table 3.1.). qPCR conditions were previously optimized and the specificity of the amplicons was determined by melting curves. Primers' efficiency was set to 90-110%. Amplification conditions: 5 min at 95 °C, followed by 30 or 40 runs of a 3-step cycle: 10 s at 95 °C; 30 s with a specific temperature for each set of primers, and 10 s at 72 °C. β-2-microglobulin transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels was calculated following the model proposed by Pfaffl (Pfaffl, 2001), using the formula $2^{-\Delta\Delta Ct}$.

3.2.4. Western Blotting

Proteins from mouse Sertoli cells and mouse testis were extracted using radioimmunoprecipitation assay (RIPA) buffer and then quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, United States of America). Western blotting was performed using standard methods (Bernardino et al., 2016b). Protein samples (20 µg) were denatured for 10 min at 55 ºC. Then, protein samples were fractionated on a 10% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, United Kingdom) and transferred to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hemel Hempstead, United Kingdom) using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, United Kingdom). Membranes were blocked with a solution of 5% bovine serum albumin (BSA) solution in Tris-buffered saline solution. Then, membranes were incubated overnight at 4 ºC separately with primary antibodies rabbit anti-AQP3 (1:1000, ab125219, Abcam, Cambridge, United

Kingdom), rabbit anti-AQP7 (1:1000, NBP1-30862, Novus Biologicals, CO, United States of America), mouse anti-AQP9 (1:500, sc-74409, Santa Cruz Biotechnology, Heidelberg, Germany), or mouse anti-CFTR (1:500, sc-376683, Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were separately detected upon incubation with goat anti-mouse (1:5000, AP308P, Merck Millipore, Burlington, MA, EUA) or goat anti-rabbit (1:5000, AP307P, Merck Millipore, Burlington, MA, United States of America) and reaction with Clarity™ Western ECL Substrate (Bio-Rad, Hemel Hempstead, United Kingdom) following manufacturer's instructions. Membranes were read using a Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, United Kingdom).

Table 3.1.: Oligonucleotides and cycling conditions for PCR amplification of Aquaporin-3 (AQP3), Aquaporin-7 (AQP7), Aquaporin-9 (AQP9), and β-2-microglobulin.

Gene	Sequence 5′-3′	Annealing T°	C
Aqp3 **(NM_016689.2)**	FWD: GGACCCTCATCCTTGTGATGTT RVS: TCGTAGTACAGCCCAAAAACAA	63 °C	40
Aqp7 **(NM_001378639.1)**	FWD: CCTTGTTACCGTCCTTGGGG RVS: ATGAAAGTGAACAACCGGGGA	65 °C	37
Aqp9 **(NM_022026.3)**	FWD: CCTTGGATGTGGCTCTATTGC RVS: GGCCATGAGTCCGTCATAGTAAA	60 °C	40
β-2-microglobulin **(NM_009735.3)**	FWD: GCTTCAGTCGTCAGCATGGC RVS: GGATTTCAATGTGAGGCGGGT	58 °C	30

Legend: FWD: Forward; RVS: Reverse; Tº: temperature; C: Number of cycles.

3.2.5. Immunofluorescence

Immunofluorescence was performed to further observe the cellular localization of CFTR, AQP3, AQP7, and AQP9. Cells were cultured on glass coverslips inside 12-well plates until reaching 70% confluence. Then, mouse Sertoli cells were fixed with 4% paraformaldehyde for 60 min and permeabilized by incubation with a solution of 0.1% Triton X-100 in phosphate-buffered saline (PBS), both at room temperature. Cells were blocked in 0.1% gelatine in PBS for 60 min at 37 ºC and incubated with primary antibodies rabbit anti-AQP3 (1:50), rabbit anti-AQP7 (1:50), mouse anti-AQP9 (1:50), or rabbit anti-CFTR (1:50, ACL-006, Alomone, Jerusalem, Israel), overnight at 4 ºC. Cells were incubated without primary antibodies as the negative control. Afterward, cells were washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1500, A-11001, Thermo Fischer Scientific, Waltham, MA, United States of America) or Alexa Fluor 488 goat anti-rabbit IgG (1:1500, A-11008, Thermo Fischer Scientific, Waltham, MA, United States of America) for 1 h at room temperature. Coverslips were mounted and nuclei were stained with VECTASHIELD® Antifade Mounting Medium with diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, United States of America). For observation by confocal microscopy, all preparations were

also co-incubated with anti- Na^+/ K^+ ATPase antibody (1:50, [EP1845Y] - Plasma Membrane Marker - Alexa Fluor 647, ab198367, Abcam, Cambridge, United Kingdom), overnight at 4 ºC. Fluorescence was observed in a Zeiss LSM 510 META confocal microscope. Images were obtained with Zen Black Software (Carl Zeiss, Jena, Germany). For visualization of the proximity ligation assay slides, fluorescence was observed in a Nikon Eclipse E400 microscope equipped with a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). Images were obtained with NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

3.2.6. Stopped-Flow Light Scattering

Stopped-flow light scattering was performed to measure the membrane permeability of mouse Sertoli cells to glycerol, following a previously described method (Bernardino et al., 2018). Cultured mouse Sertoli cells were detached with trypsin and centrifuged at 300x g to obtain a pellet. Then, cells were resuspended in an isotonic medium (300 mOsm, pH 7.4, constituted by 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 10 mM disodium phosphate (Na_2HPO_4), and 1.8 mM monopotassium phosphate (KH_2PO_4), and 1 mM phenylmethylsulphonyl fluoride (PMSF) and left for 10 min to reach the equilibrium. Cells were checked under light microscopy for homogeneity and spherical shape in suspension. The diameter of cells was measured with pictures obtained by light microscopy using the ImageJ software.

Glycerol permeability was measured in mouse Sertoli cells after 15 min incubation with a specific CFTR inhibitor (1 μM CFTRinh-172), a general inhibitor of mediated transport of glycerol (0.7 mM phloretin) or the control vehicle (CTR) with dimethyl sulfoxide (DMSO) at 1% (Calamita et al., 2012, Rodríguez et al., 2014). Stopped-flow light scattering experiments were performed on a Stopped-Flow SX20 apparatus (Applied Photophysics, Leatherhead, Surrey, United Kingdom), which has a 1 ms dead time and temperature controlled at 25 °C. The osmotic shock was performed with a hyperosmotic glycerol solution (isotonic medium supplemented with 500 mM glycerol). A minimum of seven runs were analyzed in each experimental condition. In each run, 100 μL of cellular suspension was mixed with an equal amount of hyperosmotic glycerol solution to reach inwardly directed gradients of solute. In the first instance, the hyperosmotic solution leads to fast cell shrinkage due to water outflow. Then, a glycerol influx occurs in response to its chemical gradient, which is followed by water influx and subsequent cell re-swelling. The tracing of the curves obtained agrees with the specifications of the equipment used (SX20). The kinetics of cell re-swelling were measured from the time course of 90° scattered light intensity at 450 nm until a stable light scatter signal was attained. Glycerol permeability (P_{gly}) was calculated as:

$$P_{gly} = \frac{1}{\left(\frac{A_0}{V_0} * \tau\right)}$$

where τ is equal to 1/k which is the exponential rate coefficient (s^{-1}) obtained by fitting the light scattering signal of glycerol influx; A_0/V_0 is the initial cell area to volume ratio. Isotonic solution osmolarity was determined from freezing point depression on an Osmometer Basic (Löser, Berlin, Germany) using standards of 300 and 900 mOsm.

3.2.7. Proximity ligation assay

Proximity ligation assay (PLA) was carried out to study a potential physical interaction of CFTR with AQP3, AQP7, and AQP9. To do so, it was used a Duolink® In Situ Red Starter Kit Mouse/Rabbit (DUO92101) and the technique was performed using the manufacturer's instructions. Briefly, cells were cultured on glass coverslips inside 12-well plates until reaching 70% confluence. Then, mouse Sertoli cells were fixed with 4% paraformaldehyde for 60 min and permeabilized by incubation with a solution of 0.1% Triton X-100 in PBS, both at room temperature. Cells were incubated with the kit's Blocking Solution for 60 min at 37 ºC and then with antibodies from two different species (mouse and rabbit) overnight at 4 ºC. For CFTR and AQP3 interaction, the antibodies used were rabbit anti-AQP3 (1:50) and mouse anti-CFTR (1:50). CFTR and AQP7 interaction was studied with mouse anti-CFTR (1:50) and rabbit anti-AQP7 (1:50) whereas CFTR and AQP9 interaction was studied with mouse anti-AQP9 (1:50) and rabbit anti-CFTR (1:50). Then, cells were incubated with the Duolink PLUS and MINUS probes for 1 h at 37 ºC. Ligation of the probes was conducted by incubating the cells with a ligase for 30 min and followed by a Polymerase for 100 min, both at 37ºC. Finally, cells were covered with Duolink® In Situ Mounting Media with DAPI and analyzed in a Nikon Eclipse E400 microscope equipped with a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). Images were obtained with NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

3.2.8. Statistical Analysis

Experimental results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, United States of America). The statistical significance of the mRNA abundance was assessed with the Kruskal-Wallis test followed by Dunn's multiple comparison test since the data do not follow a Gaussian distribution according to the Shapiro-Wilk normality test. The statistical significance of the glycerol permeability groups was assessed by one-way ANOVA, followed by Tukey's multiple comparisons test, with single pooled variance. $p < 0.05$ was considered significantly different.

3.3. Results

3.3.1. *Aqp3, Aqp7,* and *Aqp9* mRNA and proteins are expressed in mouse testis and mouse Sertoli cells

The presence of aquaglyceroporins in the male reproductive tract and their role in male fertility remains to be fully elucidated. In our previous study (Bernardino et al., 2018), we were able to identify, for the first time, the expression of *Aqp3* and *Aqp9* in mouse Sertoli cells. Herein, we were able to identify the expression of *Aqp3*, *Aqp9,* and *Aqp7* in mouse testis (as a positive control) and in mouse Sertoli cells by RT-PCR (Figure 3.1.A). Then, the relative abundance of mRNA expression of the different AQPs was accessed by qPCR. In mouse Sertoli cells, the relative abundance of *Aqp3* mRNA (0.0024 ± 0.0014 arbitrary units) was similar to the one of *Aqp7* mRNA (0.0039 ± 0.0013 arbitrary units). However, *Aqp9* mRNA relative abundance (0.018 ± 0.0018 arbitrary units) was approximately 6-fold greater than both the other AQPs (Figure 3.1.B). Protein expression of AQP3, AQP7, AQP9, and CFTR was also confirmed in mouse Sertoli cells and mouse testis through Western blot (Figure 3.1.C). The blot band representative of AQP9 expression had an expected molecular weight of ~33 kDa. Our results show a band at a higher molecular weight (~70 kDa) that is due to AQP9 dimerization, a process that is in common with AQP9 (Badaut et al., 2001).

3.3.2. CFTR, AQP3, AQP7, and AQP9 are expressed in the plasma membrane of mouse Sertoli cells

The localization of the different AQPs and CFTR in mouse Sertoli cells was assessed by immunofluorescence through confocal microscopy. CFTR (Figure 3.2.A), AQP7 (Figure 3.2.C), and AQP9 (Figure 3.2.D) presented a wide expression in all the cell's body. AQP3 also presented diffuse expression throughout the cell body, however, together with CFTR, also presented expression in the cytoplasmatic perinuclear region; yet this information does not allow their specific localization (Figure 3.2.A and 3.2.B).

Images resulting from the z-stacks from confocal microscopy further support the presence of CFTR (Figure 3A), AQP3 (Figure 3B), AQP7 (Figure 3.3.C), and AQP9 (Figure 3.3.D) in the plasma membrane by co-localizing with specific plasma membrane protein Na^+/ K^+ ATPase (Figure 3.3.), although at some extension some expression was observed in the cells cytoplasm, particularly as observed for CFTR and AQP3, which show a signal in the perinuclear region of mouse Sertoli cells. No labeling was observed in negative control preparations where the primary antibodies were omitted.

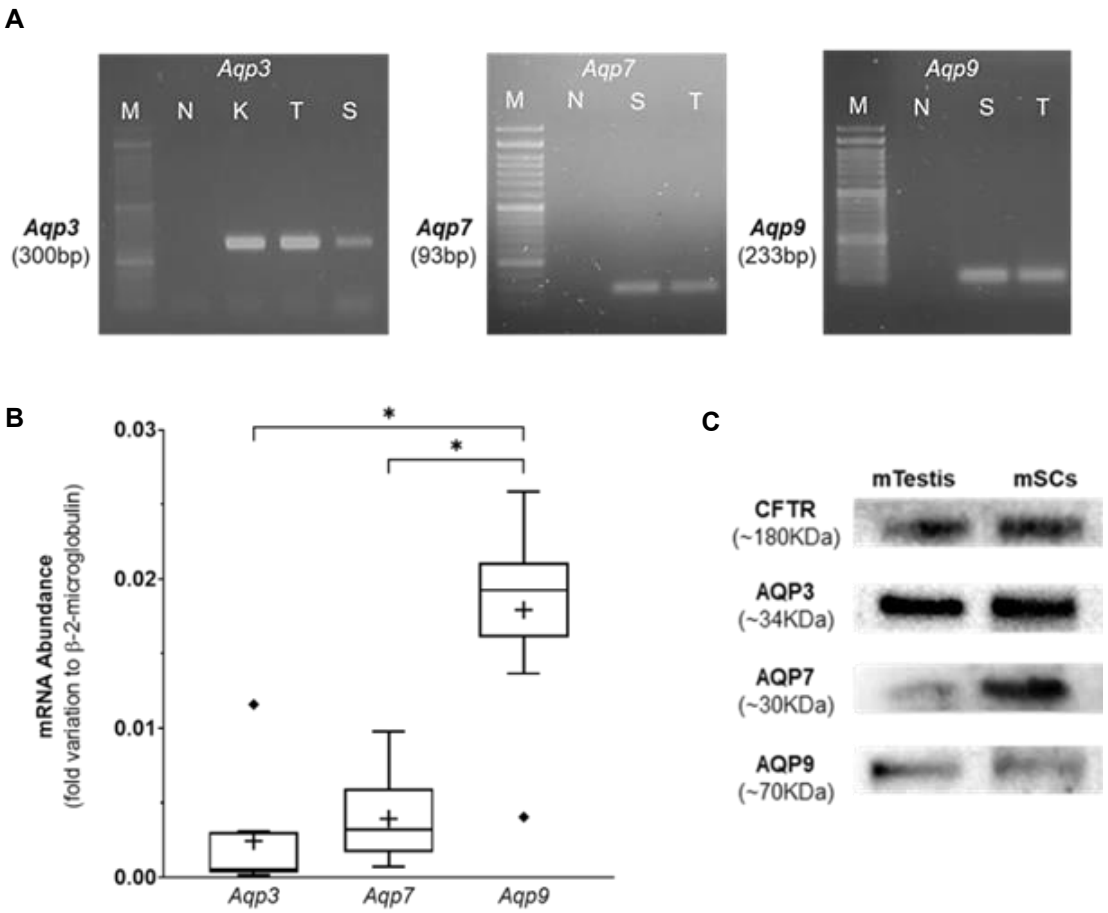

Figure 3. 1.: Identification and quantification of mRNA abundance of Aqp3, Aqp7, and Aqp9 and assessment of AQP3, AQP7, and AQP9 protein expression in mouse Sertoli cells.(A) Representative RT-PCR experiment for identification of Aqp3, Aqp7, and Aqp9 mRNA on mouse testis and mouse Sertoli cells. Mouse testis and kidney cDNA were used as the positive control. (B) Relative abundance of Aqp3, Aqp7, and Aqp9 mRNA in mouse Sertoli cells. Significantly different results ($p < 0.05$) are indicated as: *. (C) Representative blots of AQP3, AQP7, and AQP9 protein expression in mouse Sertoli cells from Western blot technique. Results from mRNA abundance experiment are expressed as Tukey boxplot (n = 6) with 3 repetitions for each gene studied. N – negative control; K – kidney; T – testis; S – Sertoli cells.

3.3.3. CFTR inhibition decreases glycerol permeability in mouse Sertoli cells

It is known that CFTR can modulate water movement across the plasma membrane by creating a gradient through Cl^- and HCO_3^- transport (Deignan et al., 2020). To study if CFTR malfunction can affect AQPs glycerol permeability, mouse Sertoli cells were treated with a specific CFTR inhibitor (CFTRinh-172) and then exposed to an osmotic stress created by a hyperosmotic solution of glycerol as described in Materials and Methods. Cells were also incubated with phloretin, a non-specific inhibitor of glycerol-facilitated transport across membranes known to decrease cellular glycerol permeability (de Oliveira, 2016). Glycerol permeability was obtained by analyzing the decrease in light scattering intensity associated with the osmotic influx of water following the entry of glycerol into cells that moved based on its chemical gradient. Before the permeability experiment, cells were suspended in osmotic equilibrium in a 300 mOsm solution and treated with CFTRinh-172, phloretin, or respective vehicles. Then, the diameter of cells was measured by optical microscopy (no

difference was found between the treatments, data not shown). The mean diameter of mouse Sertoli cells was 21.50 ± 0.32 µm. Scattered light-intensity representative curves of mouse Sertoli cells from the control, CFTRinh-172-treated, and phloretin-treated groups are illustrated in Figure 3.4. (A, B, and C, respectively). We further observed that the inhibition of CFTR by CFTRinh-172 led to a 30.6% reduction in glycerol permeability when compared to the control group ($5.04 \times 10^{-6} \pm 4.9 \times 10^{-7}$ cm/s and $7.26 \times 10^{-6} \pm 5.9 \times 10^{-7}$ cm/s, respectively, $p < 0.05$) (Figure 3.4.d). In addition, phloretin reduced glycerol permeability in mouse Sertoli cells ($3.96 \times 10^{-6} \pm 4.8 \times 10^{-7}$ cm/s, $p < 0.01$) by 54.5%, when compared with the cells of the control group.

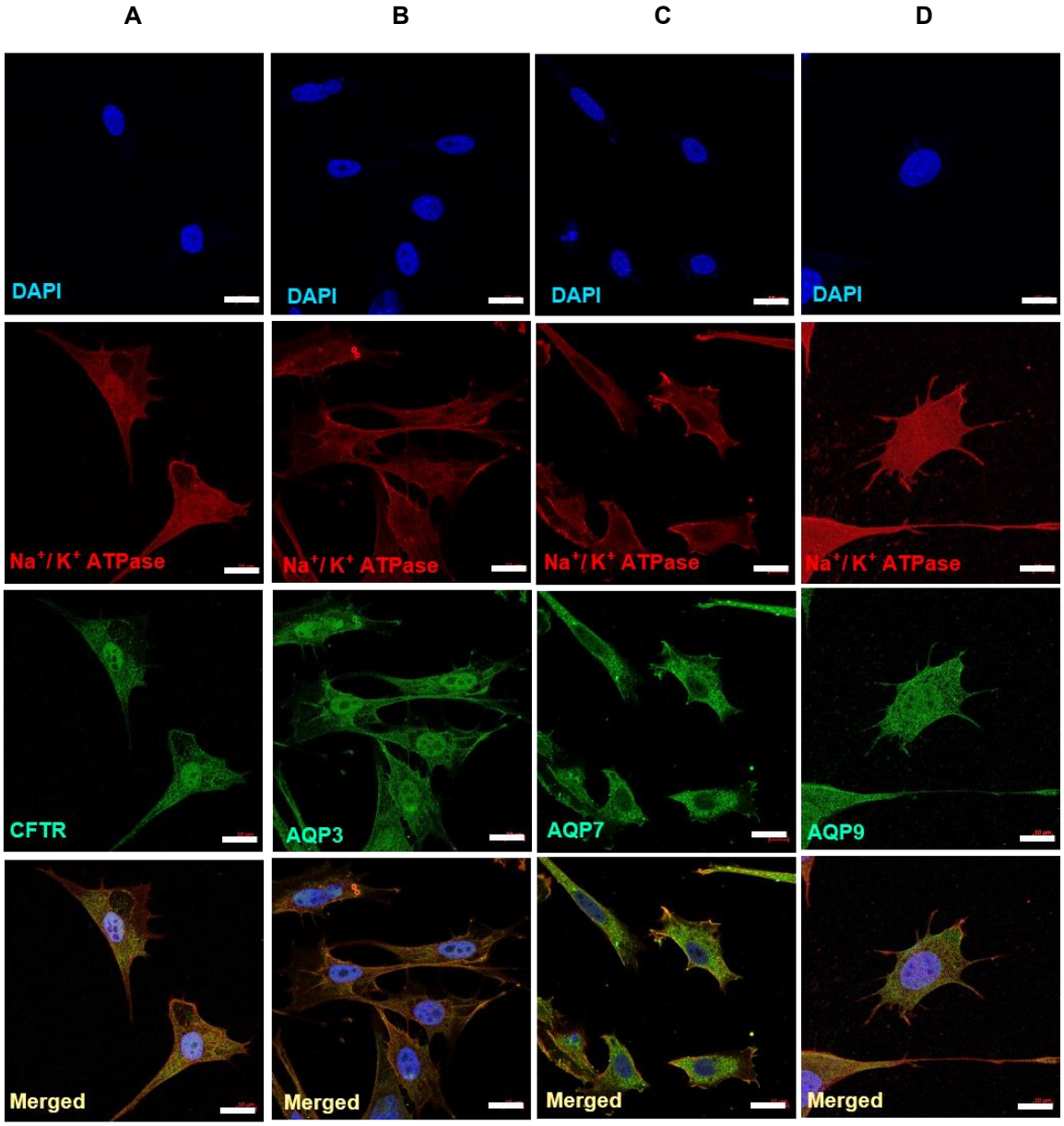

Figure 3. 2.: Immunofluorescence analysis of CFTR, AQP3, AQP7, AQP9, and membrane marker Na^+/ K^+ ATPase, in mouse Sertoli cells by confocal microscopy.Immunolabeling of Na^+/ K^+ ATPase (red) CFTR (A), AQP3 (B), AQP7 (C), and AQP9 (D) (green) is seen in mouse Sertoli cells. Negative controls were carried out without primary antibody for each protein. The nuclei of cells were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 25 µm.

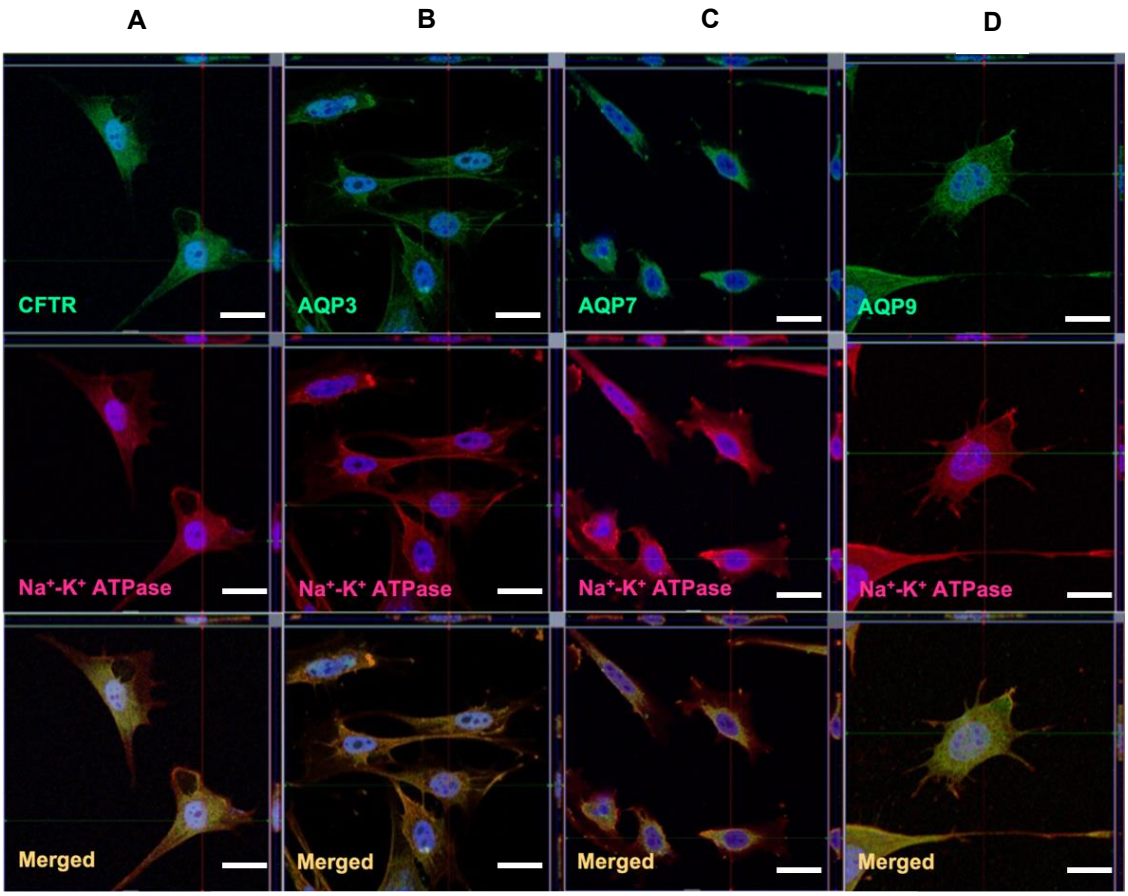

Figure 3. 3.: Immunofluorescence analysis of co-localization of CFTR, AQP3, AQP7, and AQP9 with membrane marker Na^+/ K^+ ATPase in mouse Sertoli cells plasma membrane by confocal microscopy Z-stacks.Immunolabeling of Na^+/ K^+ ATPase (red) and CFTR (A), AQP3 (B), AQP7 (C), and AQP9 (D, green) is seen in mouse Sertoli cells as well as, its co-localization (yellow) is also represented in z-stacks. The nuclei of cells were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 25 µm.

3.3.4. AQP3, AQP7, and AQP9 are in close proximity with CFTR in mouse Sertoli cells

CFTR can modulate the function of various proteins (Lim et al., 2018) and even physically interact with and control the function of other membrane transporters (Gentzsch et al., 2010). To shed light on the mechanism by which the noted decrease in glycerol permeability after CFTR inhibition was caused by physical modulation, we used a protein ligation assay (Duolink Proximity Ligation Assay). The results obtained show that CFTR is adjacent to AQP3, AQP7, and AQP9 in mouse Sertoli cells (Figure 3.5.). The vast majority of CFTR and AQP3 interactions were localized above the DAPI-marked nucleus but few were also present in other zones of the cell's membrane (Figure 3.5.A). CFTR and AQP7 or CFTR and AQP9 interactions are evident throughout the mouse Sertoli cell membrane (Figure 3.5.B and 3.5.C, respectively). These results are in concordance with the ones obtained from the immunofluorescence preparations that indicated an expression of CFTR, AQP7, and AQP9 in the plasmatic membrane of mouse Sertoli cells. On the other hand, CFTR interaction with AQP3 in the perinuclear region also indicates the presence of CFTR in

intracellular membranes. Not only do the results herein obtained from confocal microscopy point to that, but it also has already been described in previous works (Dalemans et al., 1992, Lukasiak et al., 2021). No labeling was observed in negative control preparations where the primary antibodies were omitted (Figure 3.5.D).

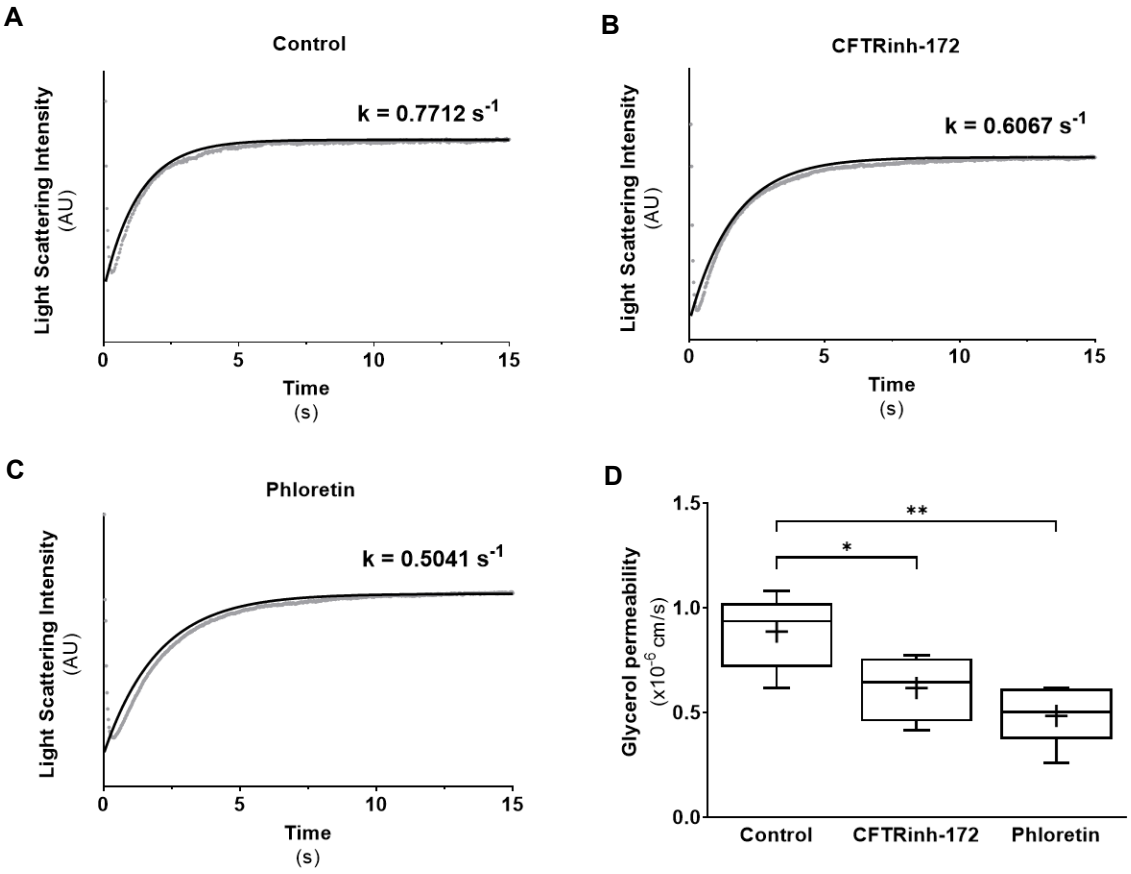

Figure 3.4.: Effect of 1 μM CFTRinh-172 or 0.7 mM phloretin on mouse Sertoli cells glycerol permeability.Representative light scattering intensity curves of control group, k = 0.7712 s−1 (A); group treated with CFTRinh-172, ki = 0.6067 s−1 (B); and group treated with phloretin, ki = 0.5041 s−1 (C) are represented. Data resulting from the calculation of mouse Sertoli cells glycerol permeability (d) are expressed as Tukey boxplot. The experiment was carried out with a n=6 with a minimum of 7 repeated curves for each condition and n, presented curves are the average of the repetitions of the first n. Significantly different results are indicated as (*) when $p < 0.05$ and (**) when $p < 0.01$.

3.4. Discussion

Solute and fluid regulation in the male reproductive tract is essential for the production, movement, and maturation of competent spermatozoa (Bernardino et al., 2019). CFTR is regarded as one of the major regulators of fluid dynamics and ion homeostasis in the male reproductive tract due to its direct and indirect activity on the modulation of the function of multiple proteins (Lim et al., 2018), including other membrane transporters (Gentzsch et al., 2010). Aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) are a subgroup among the AQPs family with the specific biophysical property of allowing facilitated transport of glycerol in addition to other solutes and water. Glycerol is usually described as a non-toxic molecule, which is important for the structure of several lipids and, at adequate levels, for healthy

spermatogenesis (Crisóstomo et al., 2017). Although glycerol may be used as a metabolic substrate, high concentrations and its overaccumulation in the testis have adverse effects which could lead to definitive oligozoospermia and even azoospermia (Wiebe et al., 1989). Taking this into consideration, this work intended to unravel if CFTR can modulate aquaglyceroporin glycerol permeability and if a physical interaction between CFTR and aquaglyceroporins occurs in mouse Sertoli cells. This would shed light on the direct involvement of CFTR in aquaglyceroporin-mediated solute permeability and on the potential importance of this interaction in male fertility.

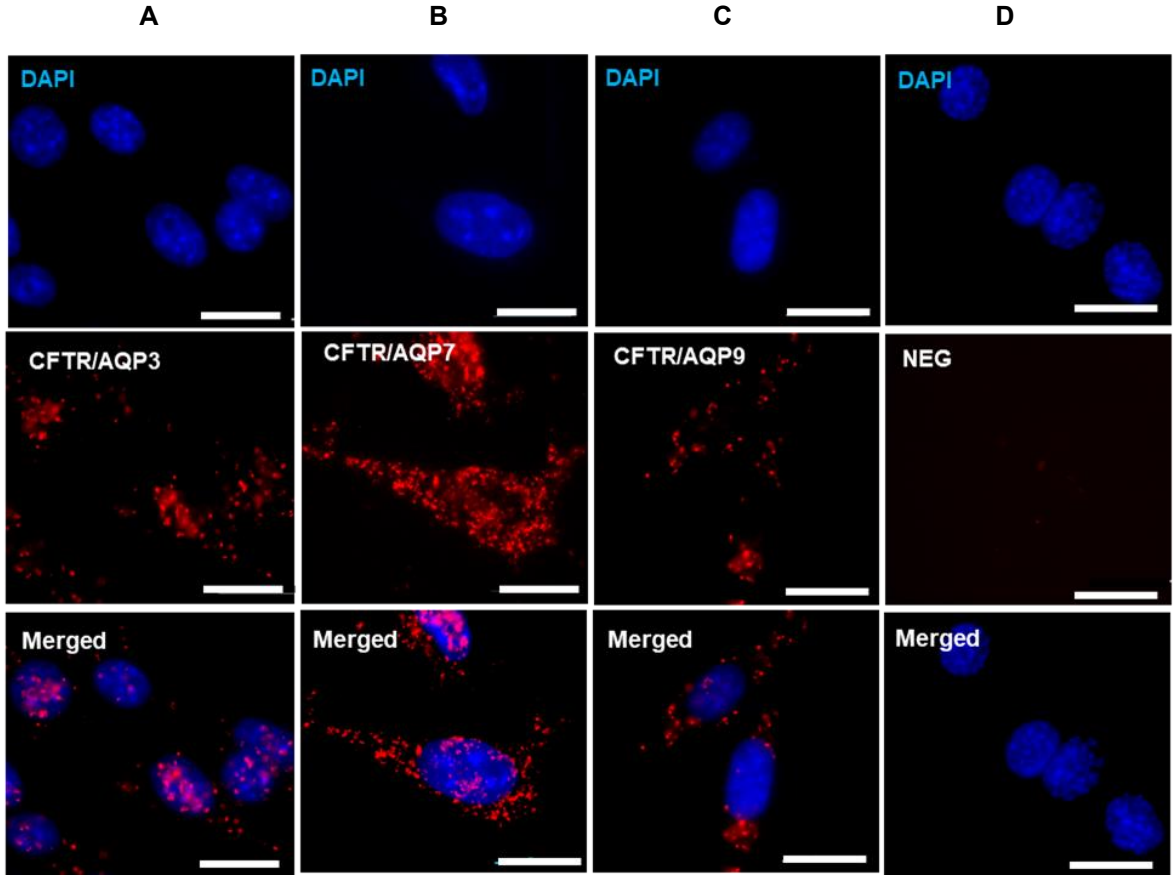

Figure 3.5.: Molecular interaction between AQP3, AQP7, AQP9, and CFTR as determined by proximity ligation assay.Red signal is representative of each interaction between CFTR and AQP3 (A), CFTR and AQP7 (B), and CFTR and AQP9 (C). Negative control (d) was carried out by omitting primary antibodies. The cell nuclei was stained with Duolink® In Situ Mounting Media with DAPI. White bar, 10 µm.

To accomplish the objective of this work, CFTR, AQP3, AQP7, and AQP9 gene and protein expressions were evaluated in mouse Sertoli cells. To the best of our knowledge, the expression of AQP7 in mouse Sertoli cells is herein described for the first time. A previous report had already described the presence of AQP7 in rat's seminiferous tubules (Calamita et al., 2001) but no information was available on mouse Sertoli cells. Moreover, qPCR results indicate that *Aqp9* seems to be the most predominantly expressed gene of all aquaglyceroporins found in mouse Sertoli cells. That is in concordance with a previous work from our group that suggested that AQP9 is the most important aquaglyceroporin in mouse

Sertoli cells (Bernardino et al., 2018). CFTR was also previously reported to be expressed in testis, where it was mainly identified in Sertoli cells (Boockfor et al., 1998). In humans with Sertoli cell-only syndrome, CFTR is present throughout the Sertoli cells's plasma membrane, with more expression found in the basal region (Teixeira et al., 2013). The expression pattern in healthy individuals, however, is yet to be clarified. Our results suggest that CFTR is expressed throughout the plasma membrane of mouse Sertoli cells but also in the membrane of cytoplasmatic organelles. Likewise, we show that AQP3, AQP7, and AQP9 expression patterns are also throughout the plasma membrane and in the cytoplasmatic region. CFTR is known to be found in multiple subcellular localizations, namely in the Golgi apparatus, phagosomes, lysosomes, and endoplasmic reticulum (for review see: (Lukasiak et al., 2021)). In the case of AQPs, their subcellular localization has been a topic of great interest in the past decades, with some showing the ability to move from the cytoplasm to the plasma membrane and vice-versa in response to external stimuli (Kamsteeg et al., 2000, Kitchen et al., 2020). However, this was only explored in orthodox AQPs (AQP2 and AQP4) and not in aquaglyceroporins. Our results also show the presence of CFTR and AQP3 in the cytoplasmatic perinuclear region. Human CFTR expression was previously described in perinuclear regions (Dalemans et al., 1992). In the case of AQP3, this interesting expression pattern was already described by Mirabella *et al.* in dog testis (Mirabella et al., 2021). In gonadotrophs of the tree frog, an AQP3 homolog (AQP-h3BL) was also found in the nuclear membrane and cytoplasm. The authors of this study hypothesized that these subcellular localizations were a "storage area" of the AQP3 homolog, which can be translocated as a response to volume regulation and osmotic adaptation of secretory granules (Sato et al., 2011). Taken together, we hypothesize that AQP3 could be involved in the secretion of vesicles since Sertoli cells are known for secreting many substrates and exosomes in the seminiferous tubule's lumen (Li et al., 2021, Salek et al., 2021). Despite being an interesting proposition, more studies are needed to fully unravel the role of AQP3 in mouse Sertoli cells. However, this indicates that different aquaglyceroporins are specialized in different processes of water and solutes diffusion. It was described that AQP7 are specialized in the outward diffusion of glycerol in mice adipocytes (Hara-Chikuma et al., 2005), whereas AQP9 were found to have a greater role in the inward glycerol diffusion in mice hepatocytes (Calamita et al., 2012). The presence of the two AQPs in mouse Sertoli cells could indicate the importance of glycerol homeodynamics in these cells. On the other hand, it is also known that AQP7- and AQP9-*knockout* mice are fertile, demonstrating the ability of other AQPs to compensate for the absence of one single aquaglyceroporin (Sohara et al., 2007, Hashem, 2010).

In Sertoli cells, CFTR is mainly described as an HCO_3^- transporter, which highlights its function in pH regulation (Xu et al., 2011). CFTR, however, may act as an HCO_3^-/Cl^-

exchanger, which generates an osmotic driving force for water movement that occurs either paracellularly or through AQPs (Saint-Criq et al., 2017). One of the earliest pieces of evidence concerning the molecular partnership between CFTR and AQPs was observed in the airway epithelial cells of human lungs. In this study, Schreiber *et al.* (Schreiber et al., 1999) described that CFTR promotes AQP3-mediated water transport. Herein, our results show that CFTR modulates aquaglyceroporin permeability to glycerol. CFTR inhibition by its specific inhibitor CFTRinh-172 caused a reduction of 30.6% in membrane glycerol permeability in mouse Sertoli cells. On the other hand, inhibition conferred by phloretin, a general aquaglyceroporins inhibitor, presented an even higher effect (-54.5%) on glycerol permeability, suggesting that CFTR is only partially able to modulate aquaglyceroporins activity. Compelling evidence suggests that the functional modulation conferred by CFTR on AQPs could be due to the occurrence of physical interaction in different tissues of the male reproductive tract, although the mechanisms are still unclear. For instance, both CFTR and AQP9 are co-localized in the luminal membrane of the principal cells in the epididymis of both rats and humans (Cheung et al., 2003, Ruz et al., 2004). In addition, our group previously observed that CFTR co-immunoprecipitated with AQP4 and AQP9 in cultured rat primary Sertoli cells (Jesus et al., 2014b, Jesus et al., 2014a). In agreement with our previous results, Pietrement *et al.* observed that CFTR co-immunoprecipitates with AQP9 in the principal cells of the epididymis and *vas deferens* of rats (Pietrement et al., 2008). This study also demonstrated that the inhibition of CFTR decreased glycerol permeability, concluding that CFTR modulates AQP9 activity on the epididymis and *vas deferens*. AQP9 is the major aquaglyceroporin isoform expressed in mouse Sertoli cells. Herein, we demonstrate through the proximity ligation assay that, under native cellular conditions, CFTR is not only adjacent to AQP9 in mouse Sertoli cells, but also to AQP3 and AQP7. Taken together, these results further support the hypothesis that CFTR is a direct regulator of AQP-mediated glycerol permeability. In addition, and equally important, it highlights that CFTR can modulate the transport of other uncharged molecules that are transported by AQPs, opening the possibility for new research. One example is the already described ability of AQP9 to transport lactate. Decreased AQP9 permeability could be a factor in lactate secretion for germ cells. This hypothesis has already been raised by Arena *et al.* (Arena et al., 2011) in a study performed on Sertoli cells of varicocele patients. Lactate is the preferred substrate for germ cell energy production (Rato et al., 2012). Thus, CFTR-mediated AQP9 permeability impairment could also disrupt lactate supply for germ cell development.

In conclusion, although these proteins were present in other cellular locations (apart from their presence in the plasma membrane), when we focused on the functional aspect of aquaglyceroporins (glycerol permeability through the plasma membrane) we observed that

CFTR appears to be a direct functional modulator of AQP3, AQP7, and AQP9 in, being able to modulate glycerol permeability through these channels. While further studies will be needed to enlighten the relevance of the intracellular presence of these aquaglyceroporins, it is known that glycerol is essential for the control of the BTB, and its accumulation results in the disruption of spermatogenesis. Thus, our results suggest that the malfunction of CFTR and the consequent decrease in glycerol permeability is a potential link between male infertility and cystic fibrosis.

3.5. Authors contributions

Pedro F. Oliveira, Marco G. Alves, Graça Soveral, and Giuseppe Calamita contributed to the study conception and design. Material preparation, data collection, and analysis were performed by João C. Ribeiro, Raquel L. Bernardino, and David F. Carrageta. The first draft of the manuscript was written by João C. Ribeiro, Raquel L. Bernardino, and David F. Carrageta. João C. Ribeiro, Raquel L. Bernardino, David F. Carrageta, Pedro F. Oliveira, Marco G. Alves, Graça Soveral, and Giuseppe Calamita contributed to the writing-review and editing of the manuscript. The funding was obtained by Marco G. Alves, Pedro F. Oliveira, and Raquel L. Bernardino. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Chapter 4

Aquaporin 7-mediated glycerol permeability is linked to human sperm motility in asthenozoospermia and during sperm capacitation

This chapter was adapted from the published work:

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Abstract

Osmoregulation plays a vital role in sperm function, encompassing spermatogenesis, maturation, and fertilization. Aquaglyceroporins, a subclass of aquaporins (AQPs), facilitate the transport of water and glycerol across the sperm membrane, with glycerol serving as an important substrate for sperm bioenergetics. This study aimed to elucidate the significance of AQP-mediated glycerol permeability in sperm motility. The presence and localization of AQP3 and AQP7 in human sperm were assessed using immunofluorescence. Subsequently, the glycerol permeability of spermatozoa obtained from normozoospermic individuals (n = 30) was measured, using stopped-flow light scattering, after incubation with specific aquaporin inhibitors targeting AQP3 (DFP00173), AQP7 (Z433927330), or general aquaglyceroporin (phloretin). Sperm from asthenozoospermic men (n = 30) were utilized to evaluate the AQP7-mediated glycerol permeability, and to compare it with that of normozoospermic men. Furthermore, hypermotile capacitated sperm cells were examined, to determine the AQP7 expression and membrane glycerol permeability. AQP3 was predominantly observed in the tail region, while AQP7 was present in the head, midpiece, and tail of human sperm. Our findings indicate that AQP7 plays a key role in glycerol permeability, as the inhibition of AQP7 resulted in a 55% decrease in glycerol diffusion across the sperm membrane. Importantly, this glycerol permeability impairment was evident in spermatozoa from asthenozoospermic individuals, suggesting the dysregulation of AQP7-mediated glycerol transport, despite similar AQP7 levels. Conversely, the AQP7 expression increased in capacitated sperm, compared to non-capacitated sperm. Hence, AQP7-mediated permeability may serve as a valuable indicator of sperm motility, and be crucial in sperm function.

4.1. Introduction

Fertility rates have been decreasing in the last decades in developed countries (Skakkebaek et al., 2016). According to the WHO guidelines, one of the parameters used to assess male fertility is the motility percentage of the ejaculated sperm (WHO, 2021). Sperm cells acquire their motility during the maturation process in the epididymis that allows the sperm cells to move through the female reproductive tract, and to develop the state of hypermotility in the final stages of their journey, an activation called capacitation. During the journey of the sperm cells through the male and female reproductive tract, medium osmolarity is in constant change, thus osmoregulation is important for proper sperm maturation and fertilization (Calamita et al., 2001, Yeung et al., 2006, Yeung et al., 2010, Cerdà et al., 2017).

AQPs are transmembrane proteins that assist in the diffusion of water and small neutral solutes through the plasma membrane. Humans possess 13 different AQPs, of which 4 (AQP3, AQP7, AQP8, AQP11) are present in human sperm cells (Saito et al., 2004, Chen et al., 2011, Moretti et al., 2012, Laforenza et al., 2017, Pellavio et al., 2020). It is worth mentioning that two of the four different AQPs expressed are aquaglyceroporins (AQP3 and AQP7), which are also capable of allowing the diffusion of small solutes, such as glycerol (Ribeiro et al., 2021). The role of glycerol in male fertility is still a mystery. However, it has been hypothesized that this polyol could have an important role in epididymal sperm

maturation, and be utilized by sperm cells as an energy source (Mann et al., 1956, Cooper et al., 1981, Jones et al., 1996, Jones et al., 1997). In fact, aquaglyceroporin expression in the epididymal epithelial cells has been suggested as a marker of male fertility (Ribeiro et al., 2021). If this is the case, then the significance of glycerol and its permeability in sperm function and, consequently, male fertility, demands awareness and care, considering that sperm motility is acquired during the epididymal transit. However, the understanding of this relationship remains incomplete and requires further investigation. Thus, this work aims to unravel the relationship between AQP-mediated glycerol permeability and sperm motility.

4.2. Materials and methods

4.2.1. Chemicals

Specific inhibitors for AQP3 and AQP7 (DFP00173 and Z433927330, respectively) were purchased through MedChemExpress (Monmouth Junction, NJ, United States of America). All other chemicals were purchased from Merck KGaA (Darmstadt, Germany), unless stated otherwise.

4.2.2. Patient characterization and study design

Human sperm samples were collected from Centro de Genética da Reprodução Professor Alberto Barros, located in Porto, Portugal, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]), between September 2020 and July 2022. All patients included in this study signed informed written consent forms. Semen samples were obtained from male patients through masturbation after 2–4 days of abstinence and were placed in sterile tubes. Additionally, patients were asked to provide information on their consumption of tobacco, alcohol, and other substances that could interfere with the experiment. The fresh semen samples were left to liquefy at 37 °C for at least 30 min and were then used for sperm parameter measurements. Afterward, sperm cells were centrifuged at 500x *g* for 5 min at room temperature. The seminal fluid was discarded, and the pellet was washed with PBS solution, with the osmolarity corrected to 300 mOsm. After two series of washing cycles performed at 500x *g* for 5 min, the supernatant was discarded, and the pellet was finally resuspended in PBS. The sperm samples were characterized according to the WHO guidelines for the laboratory examination and processing of human sperm (WHO, 2021). Samples with the following semen parameters, according to the WHO criteria, were utilized in this study: sperm concentration > 15 million/mL, and sperm viability > 56%. After this selection, samples were further classified as normozoospermic when the total motility was ≥ 42%. These samples were used to study the role of each aquaglyceroporin on sperm glycerol permeability. To do so, the sperm cells were resuspended in an isotonic medium, corrected to 300 mOsm, comprising 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, and 1 mM

PMSF at a concentration of 20–30 million sperm cells per mL, and left for 10 min for stabilization. Then, sperm cells from 30 normozoospermic men were incubated for 10 min, with a specific AQP3 inhibitor (iAQP3, 200 nM of DFP00173), a specific AQP7 inhibitor (iAQP7, 200 nM of Z433927330), a general inhibitor of glycerol diffusion phloretin (0.35 mM), or the CTR (DMSO at 1%) (de Oliveira, 2016, Sonntag et al., 2019). Sperm cells from 30 asthenozoospermic men (when the total motility was <40%) were also collected (Table 4.1.), and incubated in the presence and absence of iAQP7, for comparison with the samples from normozoospermic men. Then, the sperm membrane glycerol permeability was measured using a stopped-flow light scattering apparatus, as described below, and the AQP7 protein content was determined using Western blotting, as described below.

Table 4.1.: Characterization of samples from normozoospermic and asthenozoospermic men.

	Concentration (**10^6 Cells/mL**)	**Viability** (%)	**Motility** (%)
Normozoospermia	117.8 ± 15.94	70.20 ± 1.54	49.89 ± 1.63 *
Asthenozoospermia	112.8 ± 17.60	66.40 ± 1.21	19.93 ± 1.34 *

* $p < 0.05$.

4.2.3. Sperm motility and viability measurement

Human sperm motility was measured with the assistance of a Makler counting chamber, pre-heated at 37 °C. An aliquot of 10 µL of sperm suspension was removed from the semen, to assess the motility percentage, and proceed with the categorization of normo- or asthenozoospermic samples (Table 4.1.). Likewise, 10 µL of sperm cell solution was used to measure the motility percentage after treatment with the different inhibitors. A total of 100 sperm cells were counted per sample/condition, and the number of sperm with tail movement was regarded as motile sperm, thus including progressive and non-progressive motility. The sperm cells were filmed using a Nikon Eclipse C*i* microscope (Nikon, Shinagawa, Tokyo, Japan). Video was obtained using NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

The percentage of viable and nonviable sperm cells was determined according to an established protocol (Rato et al., 2013). Briefly, 10 µL of semen was mixed with an equal volume of eosin–nigrosin solution, and then was used to conduct smears. A total of 100 sperm cells were counted per slide, in continuous and random fields, under a bright-field optical microscope. Pink-stained sperm cells were considered nonviable, whereas white sperm cells were considered viable.

4.2.4. Spermatozoa gradient separation

To unravel the possible role of glycerol permeability in sperm motility, we separated the highly motile sperm from the sperm that were immature or showed low motility. A total of 12 semen samples from normozoospermic men were used for separation with the GemsR

Sperm Wash Gradient Set (45% and 90%) (SWG-01, Genea, Sydney, Australia), used according to the manufacturer's instructions. In brief, 1.5 mL of 90% solution was dropped into a 15 mL tube, and flowed with an equal volume of 45% solution and semen. Afterward, sperm cells were separated via centrifugation of 300x *g* for 20 min. High-motile sperm were extracted from the 95% fraction, and low-motile sperm were extracted from the 45% fraction. Each fraction was washed in PBS two times, for further usage in the glycerol permeability measurement through stopped-flow light scattering.

4.2.5. Spermatozoa capacitation

To capacitate the sperm cells, spermatozoa from normozoospermic samples (N = 12) were washed and then incubated in the presence or absence of a capacitation medium. The capacitation medium comprised NaCl 94.5 mM, KCl 4.8 mM, calcium chloride ($CaCl_2$) 1.7 mM, KH_2PO_4 1.17 mM, $MgSO_4$ 1.22 mM, HEPES 20 mM, glucose 11 mM, $NaHCO_3$ 25 mM, and BSA 0.4 mM. Non-capacitation spermatozoa were obtained by placing cells in a similar medium, without $NaHCO_3$ and BSA. The spermatozoa were incubated for 3 h at 37 °C, and the capacitation was confirmed by evaluating the total tyrosine phosphorylation levels through Western blot analysis. Capacitated and non-capacitated sperm were used in assessing the AQP7 quantification via Western blotting, and the glycerol permeability via stopped-flow light scattering.

4.2.6. Immunofluorescence

Immunofluorescence was performed to further observe the cellular localization of AQP3 and AQP7 in human sperm. Sperm cells from a normozoospermic healthy donor were washed and smeared on a slide and left to dry. Then, the sperm cells were fixed with 10% paraformaldehyde for 30 min, and permeabilized via incubation with a solution of 0.1% Triton X-100 in PBS, both at room temperature. The sperm cells were blocked in 0.1% gelatine in PBS for 60 min at 37 °C, and incubated with the primary antibodies anti-AQP3 (1:50, ab125219, Abcam, Cambridge, United Kingdom), and mouse anti-AQP7 (1:50, sc-376407, Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4 °C. Cells were incubated without primary antibodies as the negative control. Afterward, the cells were washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1500, A-11001, Thermo Fischer Scientific, Waltham, MA, United States of America), or Alexa Fluor 488 goat anti-rabbit IgG (1:1500, A-11008, Thermo Fischer Scientific, Waltham, MA, United States of America) for 1 h at room temperature. Coverslips were mounted, and the nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, United States of America). Images were obtained using Zen Black Software (Carl Zeiss, Jena, Germany). For the visualization of the slides, the fluorescence was observed using a Nikon Eclipse C*i* microscope (Nikon, Shinagawa, Tokyo, Japan)

equipped with a CoolLed pE-300 lite (CoolLed, Andover, England). Images were obtained using NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan). The specificity of the antibodies used was tested via Western blot (Supplementary Data Figure A.1.).

4.2.7. Western Blotting

Sperm samples from 20 normozoospermic and 20 asthenozoospermic men, as well as 12 samples of non-capacitated and capacitated sperm from normozoospermic men, were precipitated via centrifugation at 5000x *g* for 5 min. The sperm pellet was resuspended, and its protein was extracted via incubation with a solution of sodium dodecylsulfate at 1% for 30 min, at room temperature, and under constant agitation. After that, the remaining cellular residues were centrifugated via centrifugation at 15000x *g* for 20 min. The supernatant was collected, and its protein content was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, United States of America). Western blotting was performed using standard methods (Rato et al., 2013). The protein samples (10 µg) plus Laemmli buffer were incubated for 10 min at 37 °C. Then, the protein samples were fractionated on a 10% polyacrylamide gel from the TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, United Kingdom), and transferred to a low-fluorescence PVDF membrane (Bio-Rad, Hemel Hempstead, United Kingdom), using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, United Kingdom). The membranes were blocked with a solution of 5% BSA in Tris-buffered saline for 90 min. Then, the membranes were incubated overnight at 4 °C separately, with the primary antibodies rabbit anti-AQP3 (1:1000, ab125219, Abcam, Cambridge, United Kingdom), mouse anti-AQP7 (1:500, sc-376407, Santa Cruz Biotechnology, Heidelberg, Germany), and mouse anti-phosphotyrosine (1:1000, 05–32, Merck Millipore, Burlington, MA, EUA). Proteins were separately detected upon incubation using goat anti-mouse (1:5000, AP308P, Merck Millipore, Burlington, MA, EUA) or goat anti-rabbit (1:5000, AP307P, Merck Millipore, Burlington, MA, United States of America), and via reaction with Clarity™ Western ECL Substrate (Bio-Rad, Hemel Hempstead, United Kingdom), following the manufacturer's instructions. Membranes were read using a Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, United Kingdom).

4.2.8. Stopped-Flow Light Scattering

Stopped-flow light scattering was performed to measure the membrane permeability of human sperm cells to glycerol, following a method previously described, with slight optimizations for sperm cells (Ribeiro et al., 2022a). The stopped-flow light scattering experiments were performed using a Stopped-Flow SX20 apparatus (Applied Photophysics, Leatherhead, Surrey, United Kingdom), which has a 1 ms dead time, and the temperature controlled at 25 °C. The osmotic shock was performed with a hyperosmotic

glycerol solution (isotonic medium supplemented with 250 mM glycerol). A minimum of seven runs were stored and analyzed in each experimental condition. In each run, 100 µL of sperm suspension was mixed with an equal amount of hyperosmotic glycerol solution. The kinetics of the adaptation of sperm volume were measured from the time course of 90° scattered light intensity at 450 nm, until a stable light scatter signal was attained. The isotonic solution osmolarity was determined from freezing point depression on an Osmometer Basic (Löser, Berlin, Germany), using standards of 300 and 900 mOsm. Treatment with AQP inhibitors did not change the spermatozoa volume or viability without an osmotic shock. Since sperm cells are not spherical, the results are presented as the exponential rate coefficient of the volume change, and that can be assumed as proportional to the sperm glycerol permeability.

4.2.9. Statistical analysis

Experimental results are presented as mean ± SEM if the data followed a Gaussian distribution, or as median [interquartile range] otherwise. Statistical analysis was performed using GraphPad Prism 8 (v8.0.1.244, GraphPad Software, United States of America). All data sets were screened for outliers using the ROUT method with Q = 1%. The statistical significance of the permeability assay was assessed with the Kruskal–Wallis test, followed by Dunn's multiple comparison test, as the data do not follow a Gaussian distribution according to the Shapiro–Wilk normality test. If the data passed the normality test, a mixed-effects analysis was performed, with the Geisser–Greenhouse correction, followed by Dunnett's multiple comparison tests, with individual variances computed for each comparison. Comparisons between normozoospermic and asthenozoospermic were performed using an unpaired *t*-test. $p < 0.05$ was considered significantly different.

4.3. Results

4.3.1. AQP3 and AQP7 are localized in complementary locations in human sperm

The literature available on the localization of the different aquaglyceroporins is still a controversial topic, especially that of AQP7, particularly in sperm cells. To clarify this information, our first step was to determine the localization of both aquaglyceroporins in human sperm. AQP3 was found in the tail and the base of the head and was noted to show a distinct lack of signal in the midpiece of the sperm cells (Figure 4.1.A). AQP7 was found to be present in the head and the midpiece of human sperm, with a weak signal in the tail of some spermatozoa (Figure 4.1.B). Thus, both aquaglyceroporins are present throughout the sperm membrane in a complementary fashion. It is worth mentioning that while the AQP3 protein pattern was consistent among all sperm cells analyzed (Figure 1C), the AQP7

protein pattern seemed inconsistent between spermatozoa within a sample from the same individual (Figure 4.1.B, 4.1.D).

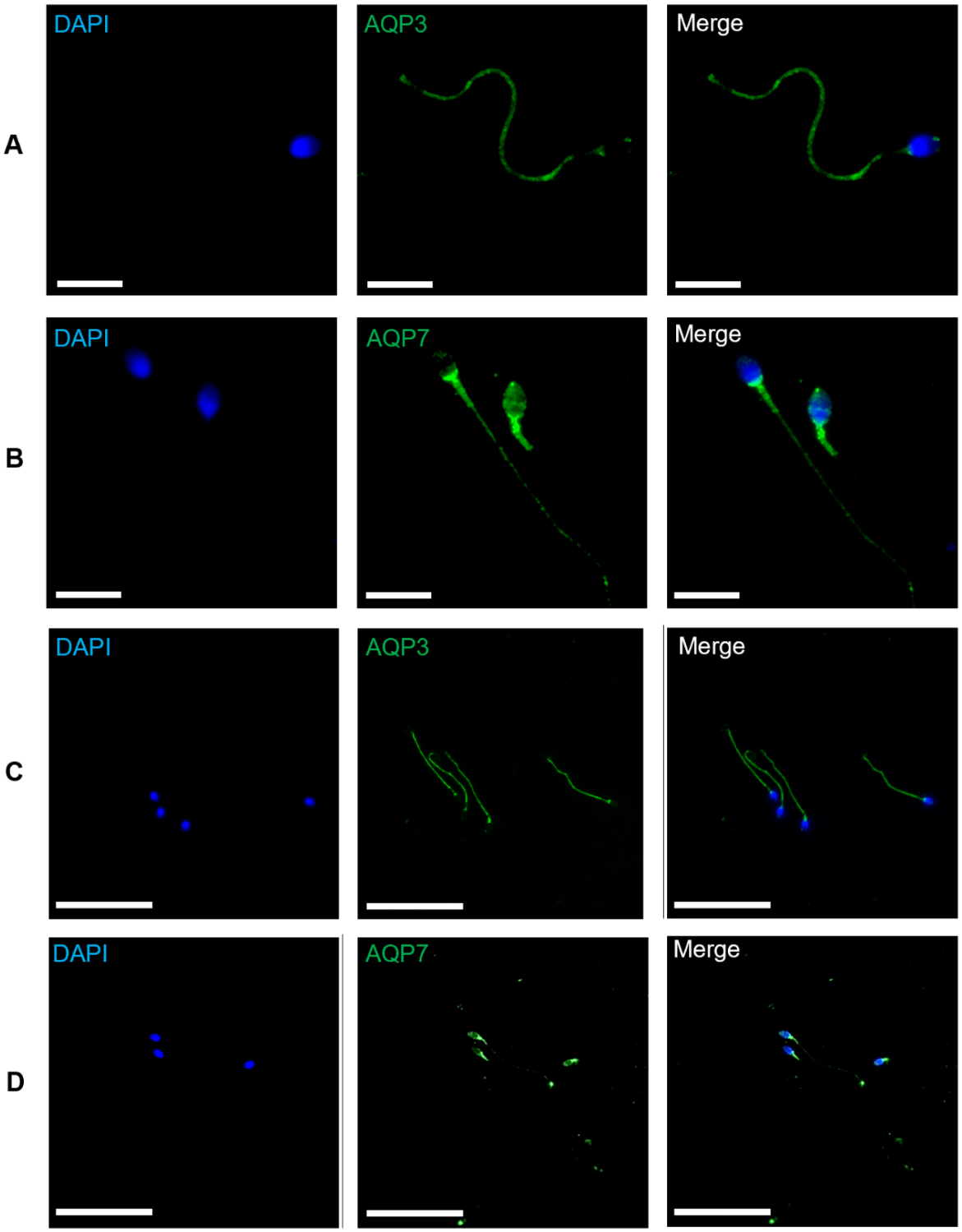

Figure 4.1.: Immunofluorescence staining of AQP3 and AQP7 in human sperm.Green immunolabeling of AQP3 (A, C) and AQP7 (B, D) at two different amplifications. Negative controls were carried out, without a primary antibody, for each protein. Cell nuclei were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. (A, B) White bar, 10 µm; (C, D) white bar, 50 µm.

4.3.2. Highly motile sperm cells have higher glycerol permeability than sperm with low motility

Gradient separation allowed the separation of the most homogeneous and motile sperm (90% phase) from the immature and lower-motility sperm cells (45% phase) in the normozoospermic samples, and the measurement of their glycerol permeability. Low-motility sperm were less permeable to glycerol (0.84 ± 0.05—fold variation to 90% phase) when compared to high-motility sperm (1.00 ± 0.06—fold variation to 90% phase) (Figure 4.2.A). Despite our results showing a correlation between motility and glycerol permeability in sperm from the same individual, when we opened the research to sperm from asthenozoospermic men versus normozoospermic men, our results did not show such a correlation. The glycerol permeability of the total sperm from the asthenozoospermic (0.94 ± 0.09—fold variation to the normozoospermic group) and normozoospermic samples (1.00 ± 0.09—fold variation to the normozoospermic group) was statistically similar (Figure 4.2.B). Thus, we branched out and studied the role of each aquaglyceroporin in sperm motility.

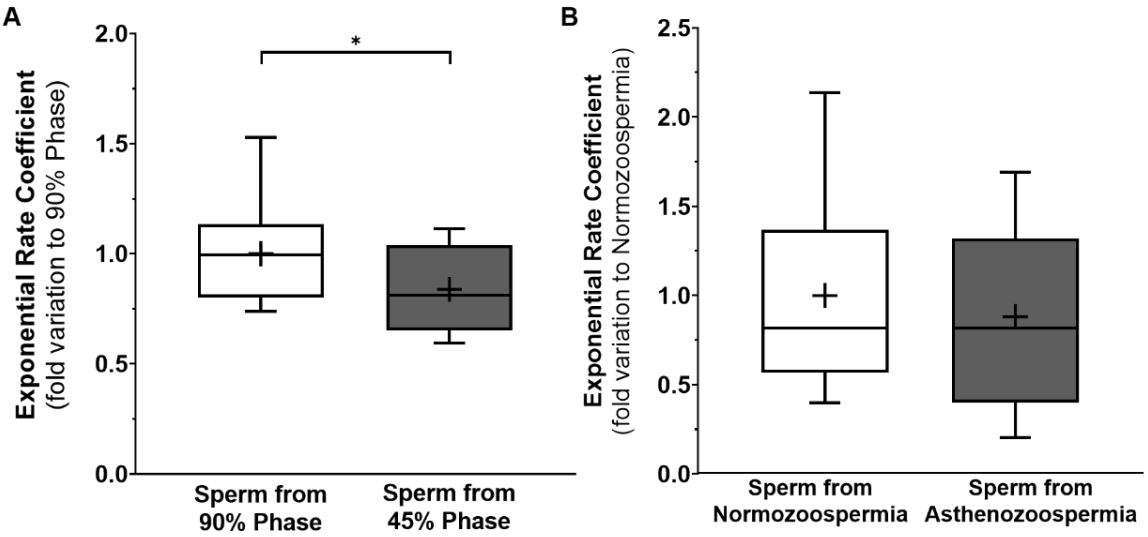

Figure 4.2.: Membrane glycerol permeability (exponential rate coefficient) of human sperm from 90% phase versus 45% phase, after gradient sperm separation (A); and sperm from men with normo-zoospermia versus sperm from men with asthenozoospermia (B).Results presented in min. to max. graphs, with a cross representing the mean of the sample size. Significantly different results ($p < 0.05$) are indicated as: *. A, n = 12; B, n = 30.

4.3.3. AQP7 is the primary aquaporin for glycerol diffusion in human sperm

Our results show no alterations in sperm motility when exposing sperm cells to the already-mentioned concentrations of specific aquaglyceroporin inhibitors for 10 min in the absence of osmotic stress. Concerning the cells of the control group (1.00 ± 0.21—fold variation to the control group), neither AQP3 inhibition (0.73 ± 0.11—fold variation to the control group) nor AQP7 inhibition (0.89 ± 0.19—fold variation to the control group) had any effect on sperm motility. However, incubation with the general aquaglyceroporin inhibitor phloretin caused a decrease in sperm motility (0.73 ± 0.11—fold variation to the control group), in

comparison to that of cells from the control group (Figure 4.3.A), without compromising sperm viability (Supplementary Data Figure A.2.).

Different aquaglyceroporins have different affinities to glycerol, changing their overall permeability to this solute (Sonntag et al., 2019). Thus, we studied the role of each aquaglyceroporin in sperm glycerol permeability after a hyperosmotic shock created by a glycerol solution, with the help of specific AQP3 and AQP7 inhibitors. In sperm from normozoospermic samples, the glycerol permeability was significantly reduced in sperm with inhibited AQP7 (0.39 [0.25;0.59]—fold variation to the control group), in relation to that seen with the AQP3 permeability inhibited (0.64 [0.41;1.13]—fold variation to control group), and the control group (0.82 [0.57;1.37]—fold variation to control group). The incubation of the sperm with the non-selective inhibitor of aquaglyceroporins (phloretin) also reduced the glycerol permeability of the sperm's membrane (0.42 [0.22;0.59]—fold variation to control group) in relation to the AQP3 inhibition group and the control group, but not to the group where AQP7 was inhibited (Figure 4.3.B). That indicates that AQP7 is the AQP most responsible for glycerol import in sperm from normozoospermic men.

4.3.4. AQP7-mediated glycerol permeability is impaired in sperm from asthenozoospermic men

To further explore the link between sperm motility and glycerol permeability, we studied the effect of AQP7 inhibition in sperm from normozoospermic and asthenozoospermic samples. Our results showed that AQP7 inhibition in sperm from asthenozoospermic samples is less effective in decreasing sperm glycerol permeability (1.29 [0.92;2.17]—fold variation to control group) than in sperm from normozoospermic samples (0.87 [0.55;1.31]—fold variation to control group) (Figure 4.3.C). Despite this, our results did not show any difference in the protein expression levels of AQP7 between sperm from normozoospermic samples (1.00 ± 0.19—fold variation to the normozoospermic group) and asthenozoospermic samples (0.87 ± 0.16—fold variation to the normozoospermic group) (Figure 4.3.D). On the other hand, a negative correlation was evident between the effect of AQP7 inhibition on sperm glycerol permeability and sperm motility ($r = -0.3599$) (Figure 4.3.E). That indicates that the spermatozoa with a lower AQP7 permeability (or that have a higher glycerol permeability after AQP7 inhibition, in our results) have lower sperm motility; therefore, AQP7 could be a key factor in sperm motility.

4.3.5. Sperm capacitation is accompanied by an increase in AQP7 levels in human spermatozoa

To further study the relation between AQP7 and sperm motility, we studied the levels of this membrane pore during the capacitation process of sperm cells, and the development of hypermotility. The occurrence of the capacitation process was confirmed using tyrosine phosphorylation quantification. The non-capacitated sperm tyrosine phosphorylation (1.00

± 0.27—fold variation to the non-capacitated group) was decreased, in relation to the one seen in the group of capacitated sperm (2.60 ± 0.32—fold variation to the non-capacitated group) (Figure 4.4.C). A representative blot is demonstrated in Figure 4.4.D.

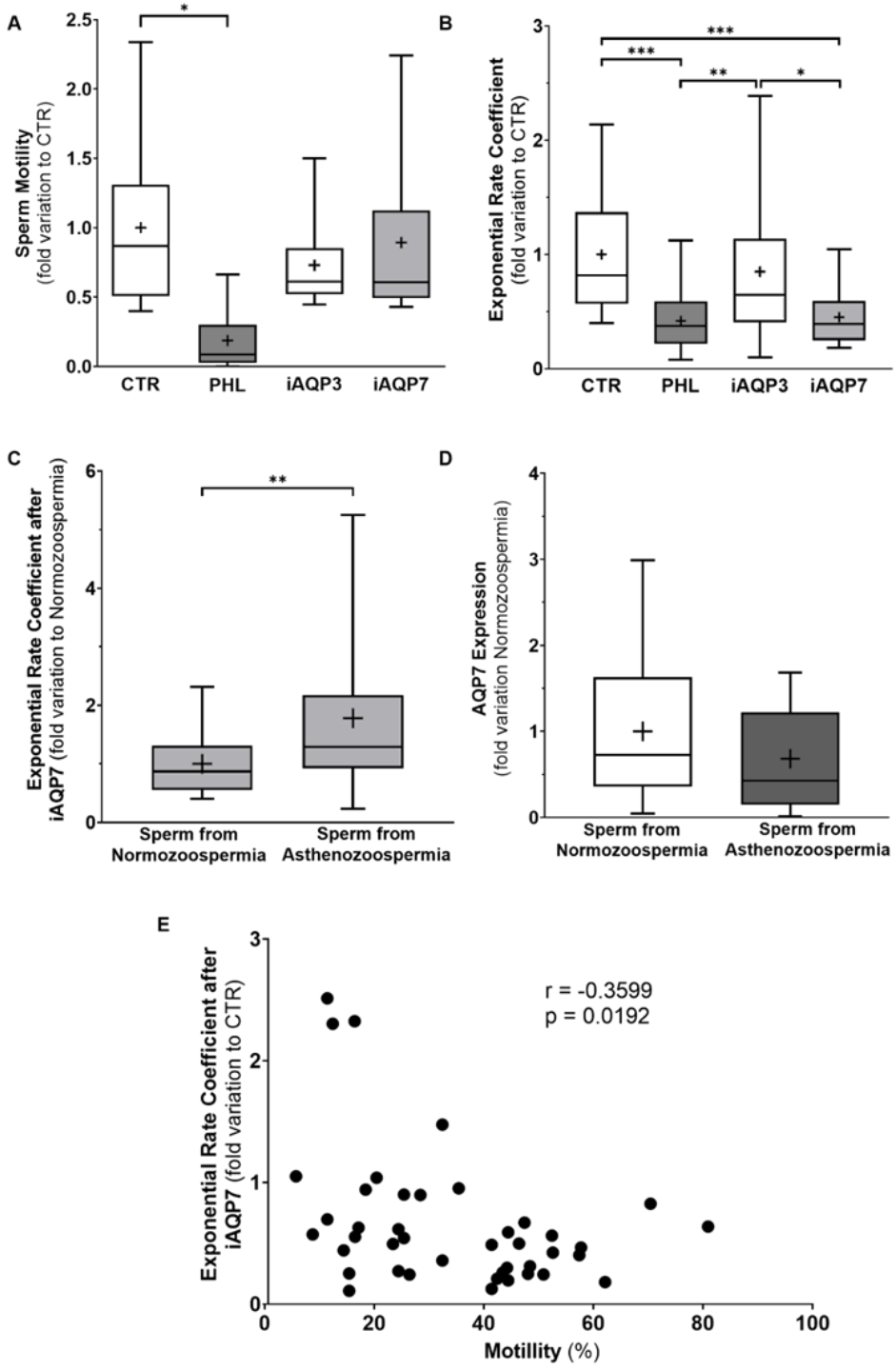

Figure 4.3.: Representation of the effect of AQP3 and AQP7 inhibition with 200 nM of Z433927330 (iAQP3) and DFP00173 (iAQP7), respectively, and both AQGPs with phloretin (PHL) in sperm motility (A) and membrane glycerol permeability (exponential rate coefficient) (B) of spermatozoa from normozoospermic men. Effect of AQP7 inhibition in membrane glycerol permeability (C) and AQP7 expression levels (D) of sperm from normozoospermic versus asthenozoospermic men.Results presented in min. to max. graphs, with a cross representing the mean of the sample size. Significantly different results with $p < 0.05$ are indicated as: *; $p < 0.01$ are indicated as: **; and $p < 0.001$ are indicated as: ***. Graphical representation of the negative correlation between the effect of AQP7 inhibition on sperm glycerol permeability and the percentage of sperm motility (E). A, n = 12; B, n = 30; C, n = 30; D, n = 20; E, n= 42.

The AQP7 expression in capacitated sperm (1.53 ± 0.16—fold variation to the non-capacitated group) was higher than in the non-capacitated sperm (1.00 ± 0.08—fold variation to the non-capacitated group) (Figure 4.4.A). Additionally, the increase in AQP7 protein expression resulted in an increase in the total glycerol permeability in capacitated sperm cells (Figure 4.4.B). Non-capacitated sperm (1.00 ± 0.19—fold variation to the non-capacitated group) had a decreased membrane glycerol permeability, compared to capacitated sperm (2.72 ± 0.73—fold variation to the non-capacitated group).

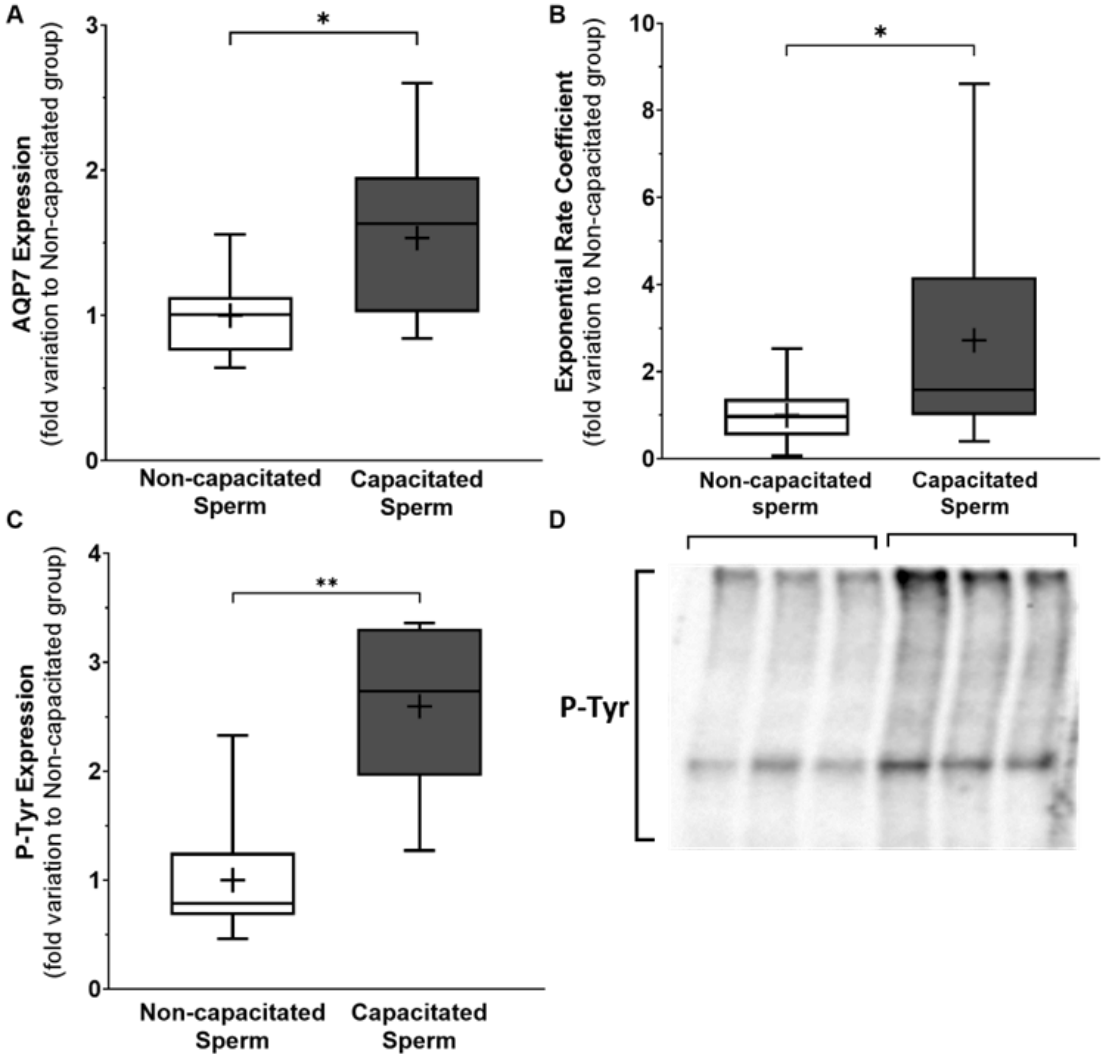

Figure 4.4.: Protein expression levels of AQP7 (A), membrane glycerol permeability (exponential rate coefficient) (B), level of tyrosine phosphorylation (p-Tyr) (C), and representative blot (D) of non-capacitated sperm versus capacitated sperm.Results presented in min. to max. graphs, with a cross representing the mean of the sample size. Significantly different results with $p < 0.05$ are indicated as: *; $p < 0.01$ are indicated as: **. n = 12.

4.4. Discussion

Sperm membrane permeability and, more specifically, glycerol permeability, have been explored in multiple studies. However, most of those studies' concern was its impact on animal sperm cryopreservation (Mazur et al., 1976, Gao et al., 1992). Thus, the role of sperm glycerol permeability in sperm function has been on the back burner. It is already

known that AQP9 is crucial for the diffusion of glycerol in the epididymal epithelia (Pietrement et al., 2008). Interestingly, the lumen glycerol concentration is greater (1.15 mM) than the plasma glycerol concentration (0.35 mM) in rats, suggesting that this glycerol arises from epithelial glycerol-based lipid degradation (Cooper et al., 1981). It was even found that glycerol-based lipid metabolism disruption in epididymal epithelial cells is linked to male infertility in mice (Liu et al., 2022). Taking that into consideration, as well as the fact that multiple studies suggest that sperm cells can metabolize glycerol (Mann et al., 1956, Jones et al., 1996, Jones et al., 1997), we challenged ourselves to enlighten the role of glycerol permeability in human sperm motility.

The first step was to determine the localization of both AQP3 and AQP7 in human spermatozoa. Our results showed that AQP3 is present in the tail and the base of the head of human sperm. AQP3 has already been described in the tail and head of human sperm (Chen et al., 2011, Pellavio et al., 2020). On the other hand, our results showed a strong signal of AQP7 in the head and midpiece, and a weaker signal throughout the tail of human spermatozoa. Previous studies have already described the localization of AQP7 in human spermatozoa. However, the results were somewhat different between studies. One report showed AQP7 presence in the midpiece and anterior portion of the tail (Saito et al., 2004), whereas another study demonstrated AQP7 in the equatorial segment of the head, pericentriolar area and midpiece, with weak labeling in the tail (Moretti et al., 2012). Yet another showed AQP7 localization only in the head of human sperm (Pellavio et al., 2020). As referred to previously, while AQP3 expression was consistent within all the sperm cells analyzed, the AQP7 expression pattern seemed inconsistent between spermatozoa within a sample from the same individual, which might explain the conflicting results described in previously published studies. This heterogeneity can arise due to differences in the developmental or maturation process of sperm cells during their transit through the epididymis, where they acquire motility and functional changes. This maturation process can involve modifications in the protein amount, and post-translational modifications. The timing and extent of these changes may vary among individual sperm cells, leading to inconsistent protein expression patterns (Saito et al., 2004).

The compartmentalized nature of aquaglyceroporin expression in human sperm is an interesting concept and worth exploring. It opens up the possibility that different aquaglyceroporins might have different roles in glycerol permeability. To assess that, we used specific AQP3 and AQP7 inhibitors to unravel the relevance of each aquaglyceroporin in the glycerol permeability of sperm from normozoospermic men (Sonntag et al., 2019). Our results showed that AQP3 inhibition did not have any effect on sperm glycerol permeability. However, AQP7 inhibition decreased the sperm glycerol permeability by 55%; a similar magnitude is seen in the group treated with phloretin, the general aquaglyceroporin

inhibitor. Thus, AQP7 appears to be the main aquaglyceroporin responsible for the facilitated diffusion of glycerol into sperm cells from normozoospermic men. This tendency was also noted in different cell types, in which AQP7-expressing cells were shown to have a considerably higher glycerol permeability than AQP3-expressing cells (Sonntag et al., 2019). On the other hand, AQP3 has been suggested to be an important osmosensor, specialized in the efflux of water out of the cell after a hypotonic osmotic shock, in the tail of mice sperm (Chen et al., 2011). These results could indicate that while AQP7 is more devoted to mediating the import of glycerol, AQP3 might be more permeable to water, meaning that both aquaglyceroporins demonstrate a complementary function and localization in human sperm. It should also be considered that AQP3 is also a peroxiporin, unlike AQP7. Peroxiporins can play a role in H_2O_2 diffusion, which triggers various signaling pathways in sperm capacitation (Chauvigné et al., 2015, Laforenza et al., 2017, Pellavio et al., 2021). Nevertheless, it is worth mentioning that AQP7 has a strong presence in the head and midpiece of human sperm, and that the change in sperm volume noted by the stopped-flow light scattering apparatus could be higher in magnitude than that seen in the tail of human sperm, where AQP3 is mainly expressed.

Taking into consideration the results described, an interesting comparison can be made. AQP9 is the main aquaglyceroporin in the liver, as it is in the male reproductive tract epithelia. However, AQP9 is known to be mainly responsible for the import of glycerol into the liver, while being the main actor in cellular glycerol export in the male reproductive tract epithelia into the lumen (Rodríguez et al., 2014, Ribeiro et al., 2021, da Silva et al., 2022). On the other hand, AQP7 is thought to be the main actor in glycerol efflux in adipose tissue (Hibuse et al., 2005, MacDougald et al., 2005, Rodríguez et al., 2011) whereas, according to our results, it is the main actor in glycerol influx in human sperm. This is one more indication that aquaglyceroporins are versatile in their role. It is also fair to assume that, as with aquaglyceroporins in the adipose tissue and liver, sperm aquaglyceroporins could also have a role in lipid/fatty acid metabolism. The literature on this subject is still scarce, but glycerol has been found to be used in *de novo* phospholipid synthesis in bull sperm (Vasquez et al., 1997). The role of lipids and glycerol on sperm bioenergetics is still a topic of discussion. Sperm cells have been found to express the machinery necessary for the fatty acid β-oxidation (Amaral et al., 2013). Further studies also point to a significant role of the fatty acid β-oxidation on ATP production and sperm motility (Zhu et al., 2020, Islam et al., 2021, Li et al., 2023). However, the linkage between sperm aquaglyceroporin expression and lipid or fatty acid metabolism still eludes the scientific community, but is certainly something worth exploring.

Many have agreed that AQP7 expression is a good indicator of sperm quality (Saito et al., 2004, Moretti et al., 2012). Herein, we tried to study the relationship between sperm motility

and glycerol permeability within the same individual, by separating high-motility sperm, and low-motility and immature sperm, through gradient centrifugation. Our results showed that the high-motile phase spermatozoa presented a higher glycerol permeability than the low-motile spermatozoa phase. Previous reports showed that AQP7 expression is also down-regulated in sperm from the low-motile phase after a similar protocol of sperm separation (Moretti et al., 2012). One must bear in mind that the low-motility phase also has a higher level of immature spermatozoa, compared to the high-motility sperm phase, which could influence (to some extent) the results obtained in the stopped-flow light scattering device. Contrastingly, with these results having been obtained from the same individual, we were able to remove all lifestyle and comorbidity variants that are impossible to escape when comparing different individuals. Regardless of this, we demonstrated that AQP7-mediated glycerol permeability is decreased in sperm from asthenozoospermic men, compared to sperm from normozoospermic men, despite the same levels of AQP7 being presented between cells from both groups. The different results seen between function and expression are interesting and should be further explored in future works. One possible reason for this mystery may lie in the aquaglyceroporin interactome (Törnroth-Horsefield et al., 2022). It is interesting to note that the total sperm membrane glycerol permeability is equal between the sperm from normozoospermic and asthenozoospermic men, but this is not the case for the AQP7-mediated glycerol permeability, which is reduced in asthenozoospermic men. In fact, our results indirectly indicate that AQP7 glycerol permeability is positively correlated to sperm motility.

As stated above, AQP7 expression in human sperm is a target of some scientific discussion, as the literature shows that its pattern can change among and within individuals. As the expression pattern of AQP7 changes during spermatogenesis, more specifically during spermiogenesis (Saito et al., 2004), it could be fair to assume that some dysregulation in this process can account for these changes. On the other hand, the AQP7 pattern changes from testicular to ejaculated sperm (Saito et al., 2004), possibly during sperm maturation, as referred to. Fish homolog AQPs have also been shown to be able to be translocated from the sperm membrane of seabream to new membrane sections after capacitation (Chauvigné et al., 2013), emphasizing the dynamic nature of AQP expression. Sperm protein synthesis is another topic that has been discussed for decades, but each year it becomes harder not to acknowledge it. Multiple studies have indicated that sperm can perform *de novo* protein synthesis, especially during capacitation (Naz, 1998, Gur et al., 2006, Rajamanickam et al., 2017, Hou et al., 2019). Our results showed that AQP7 might be one of the proteins that are upregulated during that process. This increase in AQP7 expression is accompanied by the development of sperm hypermotility, comprising one more link in the relationship between AQP7 and sperm motility. The increase in AQP7

expression did translate into an increased sperm membrane glycerol permeability, in our results. The sperm plasma membrane undergoes structural changes during capacitation that theoretically render it less stable and more fluid (Bernecic et al., 2019). Evidence suggests that this process aids the development of hypermotility, and facilitates the frantic tail movement that is characteristic of this capacitation phase (Boerke et al., 2013, Ribeiro et al., 2022b). Perhaps glycerol could have an important role in the final stages of the sperm's journey. The removal of human sperm from a glycerol-containing medium, after cryopreservation, to a glycerol-free medium was described to decrease the motility and capacitation rate (Jeyendran et al., 1985). On the other hand, female arousal is accompanied by an increased vaginal glycerol secretion (Preti et al., 1979), which is thought to be responsible for increasing lubrification. As multiple aquaglyceroporins have already been confirmed throughout the female reproductive tract (Ribeiro et al., 2021), it would be pertinent to study whether the secretion of glycerol also increases upstream of the vaginal canal, where lubrification is less important, which could indicate another symbiotic process between the female reproductive tract and the sperm cells, to potentiate fertilization. However, this is just a theory, and clear studies are necessary to fuel this hypothesis. It is also worth noting that this study was performed with samples that were all obtained from a fertility clinic, and that our sample does not represent the global male population, due to the criteria for sperm sample selection that we applied. Moreover, the measurement of glycerol permeability is only possible assuming that the entry of water and glycerol in the sperm cell is proportional to the change in cell volume, and that the latter is proportional to the intensity of the light scattering. The sperm cells are highly specialized, and thus do not have the best shape for this type of measurement, not allowing the calculation of glycerol permeability *per se*. Herein, we compared the exponential rate coefficient between the different groups, assuming that the viability of the sperm is similar between samples, as all of them show a normal and similar sperm viability. One limitation of this method is that we did not measure or classified the morphology of the sperm cells within our samples. It has already been reported that sperm from asthenozoospermic samples have a higher probability of having tail defects than those from normozoospermic samples (Lehti et al., 2017, Touré et al., 2021). It is possible that such morphological changes can influence the results of the glycerol permeability measurement.

In conclusion, our results indicate that AQP7 is the aquaporin responsible for glycerol permeability in sperm from normozoospermic men, and that this mechanism is impaired in sperm from asthenozoospermic men. However, that could lead us down two different routes: glycerol diffusion is important for sperm's bioenergetic and thus, sperm motility; or AQP7 is the key modulator of unknown pathways or mechanisms. With each passing year, there is more information regarding AQP7 and aquaglyceroporins, not only as pores for

small solutes but as real contributors to signaling pathways. That could be the reason for the results herein obtained; however, more work is needed to study such events.

4.5. Authors contributions

João C. Ribeiro, Raquel L. Bernardino, Pedro F. Oliveira, and Marco G. Alves contributed to the study conception and design. Material preparation, data collection, and analysis were performed by João C. Ribeiro. The first draft of the manuscript was written by João C. Ribeiro. João C. Ribeiro, Raquel L. Bernardino, Pedro F. Oliveira, Marco G. Alves, and Giuseppe Calamita contributed to the writing-review and editing of the manuscript. Ana Gonçalves and Alberto Barros contributed with resources. The funding was obtained by Marco G. Alves, Pedro F. Oliveira, and Raquel L. Bernardino. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Chapter 5

CFTR-AQP7 protein complex modulates glycerol transport in human spermatozoa

The data from this chapter is unpublished.

Abstract

Idiopathic male infertility remains a clinical challenge since it affects a substantial proportion of infertile men and still lacks a definitive etiology. The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene is known for its involvement in cystic fibrosis and also plays a crucial role in male fertility by modulating aquaporins (AQPs), particularly aquaglyceroporins. These channels are crucial for water and glycerol diffusion in the male reproductive tract and for sperm motility. Thus, this study investigates the functional and physical interaction between CFTR and AQP7 in human sperm. Our results revealed a direct link between CFTR inhibition and reduced sperm motility in sperm samples from normozoospermic men (n=10). These findings highlight the impact of the dysregulation of CFTR activity on sperm motility. This effect on sperm motility might be mediated through the disruption of glycerol diffusion since CFTR inhibition decreased sperms' aquaglyceroporin-mediated glycerol permeability by 52% in sperm from normozoospermic men (n=13). Since AQP7 is the major glycerol diffuser in human sperm, we searched for and identified a CFTR-AQP7 protein complex localized in the sperm head, using a proximity ligation assay (PLA). This suggests a novel mechanism capable of regulating sperm function. Our data challenges the conventional image of CFTR as just a chloride (Cl^-) and bicarbonate (HCO_3^-) channel and demonstrates that CFTR can modulate water and glycerol transport through AQP7, being this mechanism crucial for sperm motility. This study increases our understanding of a new molecular mechanism involved in sperm motility, providing insights into isolated asthenozoospermia even in the absence of common *CFTR* variants.

5.1. Introduction

In roughly 30-40% of infertile men, the cause of infertility is unknown. This is termed idiopathic male infertility. In many of those cases, standardized semen analysis alone is insufficient for diagnosis, and thus, genome sequencing may be required for a more comprehensive evaluation (Kothandaraman et al., 2016). Genome sequencing has revealed the genetic basis of many diseases, providing information about their mechanisms. One such disease is cystic fibrosis which is caused by mutations in the *CFTR* gene and leads to a dysregulation of multiple tissues, including those responsible for male fertility (Tarín et al., 2015). Indeed, 97% of cystic fibrosis patients present subfertility or infertility (Kaplan et al., 1968, Tomaiuolo et al., 2011, Chen et al., 2012), with CFTR being a key player in many pathways and mechanisms linked to male fertility (Cai et al., 2019). In fact, CFTR can directly modulate the function of many other proteins responsible for critical steps in male fertility (Pietrement et al., 2008, Dirami et al., 2013). One example of such proteins are aquaglyceroporins, a family of AQPs responsible for the diffusion of water and glycerol, whose permeability is regulated by CFTR in epithelial cells of the male reproductive tract (Pietrement et al., 2008, Ribeiro et al., 2022). However, the interaction between CFTR and aquaglyceroporins in human sperm remains a mystery. CFTR is known to play a role in sperm capacitation and acrosome reaction (Li et al., 2009), but its role in sperm motility development is yet to be fully elucidated. On the other hand, AQP7 is the main diffuser of glycerol in human sperm and its permeability has been linked to sperm motility (Ribeiro et

al., 2023). While the role of glycerol in sperm function needs further investigation, there is research suggesting it may contribute to sperm acquiring and maintaining its motility (Mann et al., 1956, Cooper et al., 1981, Jones et al., 1996, Jones et al., 1997, Liu et al., 2022). Thus, this study focused on the study of the functional and physical interaction between CFTR and AQP7 in human sperm, aiming to discover a novel mechanism responsible for sperm motility and a potential contribution to explain idiopathic male infertility.

5.2. Materials and Methods

5.2.1. Chemicals

All chemicals were purchased from Merck KGaA (Darmstadt, Germany) unless stated otherwise.

5.2.2. Patient characterization and study design

Human sperm samples were collected from the Centro de Genética da Reprodução Professor Alberto Barros, Porto, Portugal, from September 2020 and July 2022, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]). Semen samples were collected from male patients through masturbation and were placed in sterile tubes after 2-4 days of abstinence. Additionally, patients were required to provide information regarding their use of tobacco, alcohol, and other substances that could have an impact on results interpretation. All participants in this study signed an informed written consent. The freshly obtained semen samples were left to liquefy at 37ºC, and aliquots were removed for sperm parameters measurement before being centrifuged at 500x *g* for 5 min at room temperature. The seminal fluid was discarded, and the pelleted sperm cells were washed with PBS solution at 300 mOsm. Subsequently, two more series of washing cycles were performed at 500x *g* for 5 min, the supernatant was discarded, and the pellet was resuspended in an isotonic medium corrected to 300 mOsm constituted by 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at a concentration of 20-30 million sperm cells per mL and left 10 min for stabilization. The sperm parameters of the samples (Table 5.1.) were assessed following the WHO guidelines for laboratory examination and processing of human sperm (WHO, 2021). Only samples meeting the following semen parameters were used in this project: sperm concentration ≥ 15 million/mL, sperm viability ≥ 56%, and absence of the most common cystic fibrosis-inducing *CFTR* variants in genomic DNA (Supplementary Data Table A.1.). After characterization, samples were divided into two different groups: 13 normozoospermic (when total motility was ≥ 42%) and 14 asthenozoospermic (when total motility was < 42%). The first group was used to study the role of CFTR inhibition on sperm motility and glycerol permeability mediated by AQPs in healthy sperm. To do so, characterized normozospermic sperm cells were incubated for 10 min with a specific CFTR inhibitor (1 μM of CFTRinh-172), a general inhibitor of

aquaglyceroporin-mediated glycerol diffusion phloretin (PHL, 350 μM), and unspecific CFTR activators (forskolin 5 μM plus IBMX 100 μM) or the vehicle (DMSO at 1%). Sperm cells from men with asthenozoospermia were also collected and incubated in the presence and absence of CFTRinh-172 for comparison with normozoospermic samples. Ten samples from the normozoospermic group were used to study the effect of CFTR inhibition and activation on sperm motility after 10 min of incubation. Then, sperm membrane glycerol permeability was measured with a stopped-flow light scattering apparatus as described below.

Table 5.1.: Characterization of samples from normozoospermic and asthenozoospermic men.

	Concentration (**10^6 Cells/mL**)	**Viability** **(%)**	**Motility** **(%)**
Normozoospermia	122.5 ± 17.3	69.0 ± 1.6	49.9 ± 1.6 *
Asthenozoospermia	109.4 ± 19.0	65.3 ± 1.1	21.9 ± 1.3 *

* $p < 0.05$.

5.2.3. Sperm viability and motility measurement

The viability of sperm cells within semen samples was assessed according to an established protocol (Rato et al., 2013). Briefly, 10 μL of semen was mixed with an equal volume of eosin-nigrosin solution and used to prepare sperm smears. At least 100 sperm cells were counted per slide in continuous and random fields under a bright field in a Nikon Eclipse C*i* microscope (Nikon, Shinagawa, Tokyo, Japan). Pink-stained sperm cells were considered nonviable whereas white sperm cells were considered viable.

Sperm motility was assessed using a pre-heated Makler counting chamber set to 37 ºC. An aliquot of 10 μL of semen suspension was extracted to determine the human sperm motility percentage and proceed with its categorization of samples from normozoospermia or asthenozoospermia. A minimum of 100 sperm cells were counted per sample/condition and the number of sperm with tail movement was regarded as motile sperm. Sperm cells were filmed using a Nikon Eclipse C*i* microscope. Video was obtained with NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

5.2.4. DNA extraction from sperm cells

Sperm cells from each semen sample were washed and approximately 20 million were taken for DNA extraction. Genomic DNA extraction was performed using a commercial kit NZY Tissue gDNA Isolation kit (NZYtech, Lisbon, Portugal) following the manufacturer's instructions. Briefly, sperm cells were resuspended and lysed with a detergent-based buffer complemented with proteinase K and incubated at 56º C for 1 hour. After, 100% ethanol was added followed by vigorously mixing. DNA was isolated by using the kit's silica-based columns after centrifugation and was eluted in water after a series of washing steps. DNA

quantification was performed using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, NC, United States of America).

5.2.5. *CFTR* gene screening

Screening genomic DNA of the sperm samples for the presence of 50 of the most common *CFTR* variants that cause cystic fibrosis in Europe (Supplementary Data Table A.1.) was performed with the kit Yourgene® Cystic Fibrosis Base (Yourgene Health, Manchester, United Kingdom). Briefly, 15 ng of sperm DNA was added to a mix containing primers for all variants, Taq DNA polymerase, and nucleotides. The mix was then put through an enzymatic activation step for 20 min at 94º C in a 9800 Fast Thermal Cycler (Applied Biosystems, Massachusetts, United States of America), followed by a PCR following the kit instructions. Capillary electrophoreses were performed using a 3500 DX Genetic Analyzer (Applied Biosystems, Massachusetts, United States of America) after a denaturation step at 94º C for 3 min followed by a 30 sec incubation at 4º C. The results were analyzed using GeneMapper™ Software 6 (Applied Biosystems, Massachusetts, United States of America).

5.2.6. Immunofluorescence

Immunofluorescence was performed to further observe the cellular localization of CFTR and AQP7. Whole semen from a healthy donor was smeared on a slide and let to dry. Then, sperm cells were fixed with 4% paraformaldehyde for 10 min. After, permeabilization of sperm cells was performed by incubation with a solution of 0.1% Triton X-100 in PBS, at room temperature. Sperm cells were blocked in 5% BSA in PBS supplemented with 0.01% Triton X-100 for 60 min at room temperature and incubated with primary antibodies mouse anti-CFTR (1:50, MAB25031, R&D systems, Minneapolis, MN, United States of America) or rabbit anti-AQP7 (1:50, E-AB-17680, Elabscience, Houston, TX, United States of America), overnight at 4 ºC. Sperm cells were incubated without primary antibodies as the negative control. Afterward, cells were washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500, A-11001, Thermo Fischer Scientific, Waltham, MA, United States of America) or Alexa Fluor 568 goat anti-rabbit IgG (1:500, A-11008, Thermo Fischer Scientific, Waltham, MA, United States of America) for 1 h at room temperature. Nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, United States of America). For visualization of the slides, fluorescence was observed in a ZEISS Axio Observer equipped with a ZEISS colibri 7 Multicolor LED light Source (ZEISS, Oberkochen, Germany). Images were obtained with ZEISS ZEN blue and edited in ImageJ (Fiji 1.54f).

5.2.7. Proximity ligation assay

PLA was carried out to study the potential physical interaction of CFTR with AQP7. To do so, it was used a NaveniFlex TM Cell MR Red (Navinci, Uppsala, Sweden) and the

technique was performed using the manufacturer's instructions. Briefly, human sperm cells were fixed with 4% paraformaldehyde for 10 min and permeabilized by incubation with a solution of 0.1% Triton X-100 in PBS, both at room temperature. Cells were incubated with a blocking solution of BSA at 5% and Triton X-100 at 0.01% in PBS, for 60 min at room temperature. CFTR and AQP7 interaction was studied with mouse anti-CFTR (1:50) and rabbit anti-AQP7 (1:50) after overnight incubation at 4 ºC. Positive control was performed by incubating sperm cells with mouse anti-CFTR (1:50) and rabbit anti-testis anion transporter 1 (TAT1, L2CL4, 1:200) antibodies used previously (Touré et al., 2001, Touré et al., 2007, Rode et al., 2011). As a negative control, sperm cells were incubated with rabbit anti-AQP7 (1:50) and with mouse anti-dynein intermediate chain 2 (DNAI2, 1:200, H00064446-M01, Novus Biologicals, Centennial, CO, United States of America). Then, cells were incubated with the Navenibody M1 and R2 (both at 1:40) antibodies for 1 h at 37 ºC. Ligation of the probes was conducted by incubating the cells with a ligase for 30 min, followed by a polymerase incubation for 90 min, both at 37ºC. Finally, cells were covered with a VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, United States of America) and analyzed in a ZEISS Axio Observer equipped with a ZEISS colibri 7 Multicolour LED light Source (ZEISS, Oberkochen, Germany). Images were obtained with ZEISS ZEN blue and edited in ImageJ (Fiji 1.54f). The intensity and contrast of the signal from all images were normalized to the one of the images from the positive control. Then, the percentage of spermatozoa was measured. Only sperm cells with more than one dot indicative of an interaction were counted as positives.

5.2.8. Stopped-Flow Light Scattering

Stopped-flow light scattering was performed to measure the membrane permeability of human sperm to glycerol, following a previously described method (Bernardino et al., 2018). Sperm cells were washed and resuspended in an isotonic medium and left for 10 min to reach equilibrium. Sperm cells' glycerol permeability was measured after 10 min incubation with a specific CFTR inhibitor (1 µM CFTRinh-172), and phloretin, a general inhibitor of glycerol diffusion (0.7 mM phloretin; PHL) or the vehicle (DMSO at 1 %, CTR) (Calamita et al., 2012, Rodríguez et al., 2014). Stopped-flow light scattering experiments were performed on a Stopped-Flow SX20 apparatus (Applied Photophysics, Leatherhead, Surrey, United Kingdom), which has a 1 millisecond dead time and temperature controlled at 25 °C. The osmotic shock was performed with a hyperosmotic glycerol solution (isotonic medium supplemented with 500 mM glycerol). A minimum of seven runs were stored for analysis in each experimental condition. In each run, 100 µL of sperm suspension was mixed with an equal volume of hyperosmotic glycerol solution. The kinetics of sperm re-swelling were measured from the time course of 90° scattered light intensity at 450 nm until a stable light scatter signal was attained. Isotonic solution osmolarity was determined from freezing point

depression on an Osmometer Basic (Löser, Berlin, Germany) using standards of 300 and 900 mOsm. Since sperm cells are not spherical the results are presented as the exponential rate co-efficient of the volume change and that can be assumed as proportional to the sperm glycerol permeability (Holde et al., 1998, Maggio et al., 2018, Ribeiro et al., 2023).

5.2.8. Statistical Analysis

Experimental results are presented as mean ± SEM for data following a Gaussian distribution or as median [interquartile range] for non-Gaussian data. Statistical analysis was conducted using GraphPad Prism 8 (v8.0.1.244, GraphPad Software, United States of America). Outliers were identified using the ROUT method with Q = 1%. The statistical significance was assessed by the Kruskal–Wallis test, followed by Dunn's multiple comparison test, as the data do not follow a Gaussian distribution according to the Shapiro–Wilk normality test. If the data passed the normality test, a repeated measures one-way ANOVA analysis was performed, with the Geisser–Greenhouse correction, followed by Tukey's multiple comparison tests, with individual variances computed for each comparison. Comparisons between normozoospermic and asthenozoospermic groups were performed using an unpaired two-tailed Mann-Whitney test. $p < 0.05$ was considered significantly different.

5.3. Results

5.3.1. CFTR inhibition decreases human sperm motility in normozoospermic men

Firstly, we evaluated the effect of pharmacological CFTR inhibition and activation on sperm motility of samples from normozoospermic men. Sperm cells incubated with CFTRinh-172 showed decreased motility (0.53 ± 0.11–fold variation to control) in relation to the one measured in the sperm cells of the control group. However, CFTR activation did not have any effect on sperm motility when compared to the control group. Incubation of sperm with the forskolin and IBMX (5 μM and 100 μM, respectively) had a sperm motility of 1.09 ± 0.11–fold variation to control, which was significantly higher than the one measured for the group with the CFTR inhibited (Figure 5.1.A). Sperm cells incubated with phloretin were used as a positive control for the inhibition of total membrane glycerol diffusion. This group also displayed diminished sperm motility (0.15 ± 0.05–fold variation to control) in relation to the control group and to the group with activated CFTR.

5.3.2. CFTR inhibition impairs AQP-mediated glycerol permeability in human sperm.

Of the 30 semen samples screened (15 normozoospermic, 15 asthenozoospermic) three were excluded due to the presence of heterozygous cystic fibrosis-causing *CFTR* gene variants: one with c.1521_1523del and two with c.1210-12T[5]. Four samples carried the

benign intronic c.1210-12T[9] variant in heterozygosity. Given the consensus that this variant does not alter CFTR structure or activity (Ding et al., 2024), these samples were retained for further analysis.

Glycerol permeability of spermatozoa from men with normozoospermia (0.728 [0.526;1.33]—fold variation to the normozoospermic group) and asthenozoospermia (1.07 [0.423;1.54]—fold variation to the normozoospermic group) did not present any statistical difference (p = 0.744, Figure 5.1.B).

A previous work from our group showed that AQP-mediated glycerol permeability of human sperm is mostly provided by AQP7 (Ribeiro et al., 2023). Herein, our results show that CFTR inhibition can modulate the AQP-mediated glycerol permeability. Glycerol permeability of human sperm incubated with CFTRinh-172 (0.459 [0.314;0.537]—fold variation to control) was decreased in relation to the one of the control group (0.728 [0.526;1.33]—fold variation to control), to similar levels measured in the group incubated with phloretin (0.388 [0.204;0.618]—fold variation to control). Sperm incubation with CFTR activators did not have any effect on glycerol permeability (0.693 [0.454;1.33]—fold variation to control) in comparison to the control group (Figure 5.1.C).

Then, the samples were used for the comparison between the effect of CFTR inhibition on sperm glycerol permeability of normozoospermic (0.448 [0.316;0.523]—fold variation to control of normozoospermic group) and asthenozoospermic (0.621 [0.375;0.884]—fold variation to control of asthenozoospermic group) men. The analysis did not show any statistical significance (p = 0.068), but a tendency is apparent (Figure 5.1.D).

5.3.3. CFTR inhibition impairs AQP-mediated glycerol permeability in human sperm.

Since CFTR inhibition can modulate sperm glycerol permeability, it is valid to study the relationship between the location of CFTR with AQP7 to validate a possible interaction. Our results showed a strong AQP7 signal in the head and midpiece of human sperm cells, with some presenting diffuse signal in the tail. CFTR signal was found in the midpiece of all sperm cells and the equatorial segment of the head. However, the latter was only found in some sperm cells highlighted by the white arrows in Figure 5.2.A. The merge of both signals showed co-localization in the equatorial segment in the head of some sperm (yellow arrows in Figure 5.2.A) and midpiece of all mature sperm cells.

PLA revealed close proximity between CFTR and AQP7 in the equatorial segment of the human sperm head, a region crucial for fertilization, suggesting the formation of a protein complex. Additional interactions were also observed in the anterior part of the midpiece (Figure 5.3.A, Supplementary Data Figure A.3.). These findings suggest a potential functional interaction between these proteins, with implications for sperm motility and fertility In the analyzed sample, 77% of sperm cells presented more than one dot indicative of an

interaction of AQP7 and CFTR, from those 46% presented more than 3 interaction dots. Interaction between CFTR and TAT1 (Supplementary Data Figure A.4.) was found in 55% of sperm cells with at least one dot and in 21% of sperm cells with more than 3 dots. Biological (interaction between AQP7 and DNEI2) and technical negative (only CFTR) controls had 0% and 12% of sperm cells with more than one dot and 0% and 8% with more than 3 dots, respectively (Figure 5.3.E).

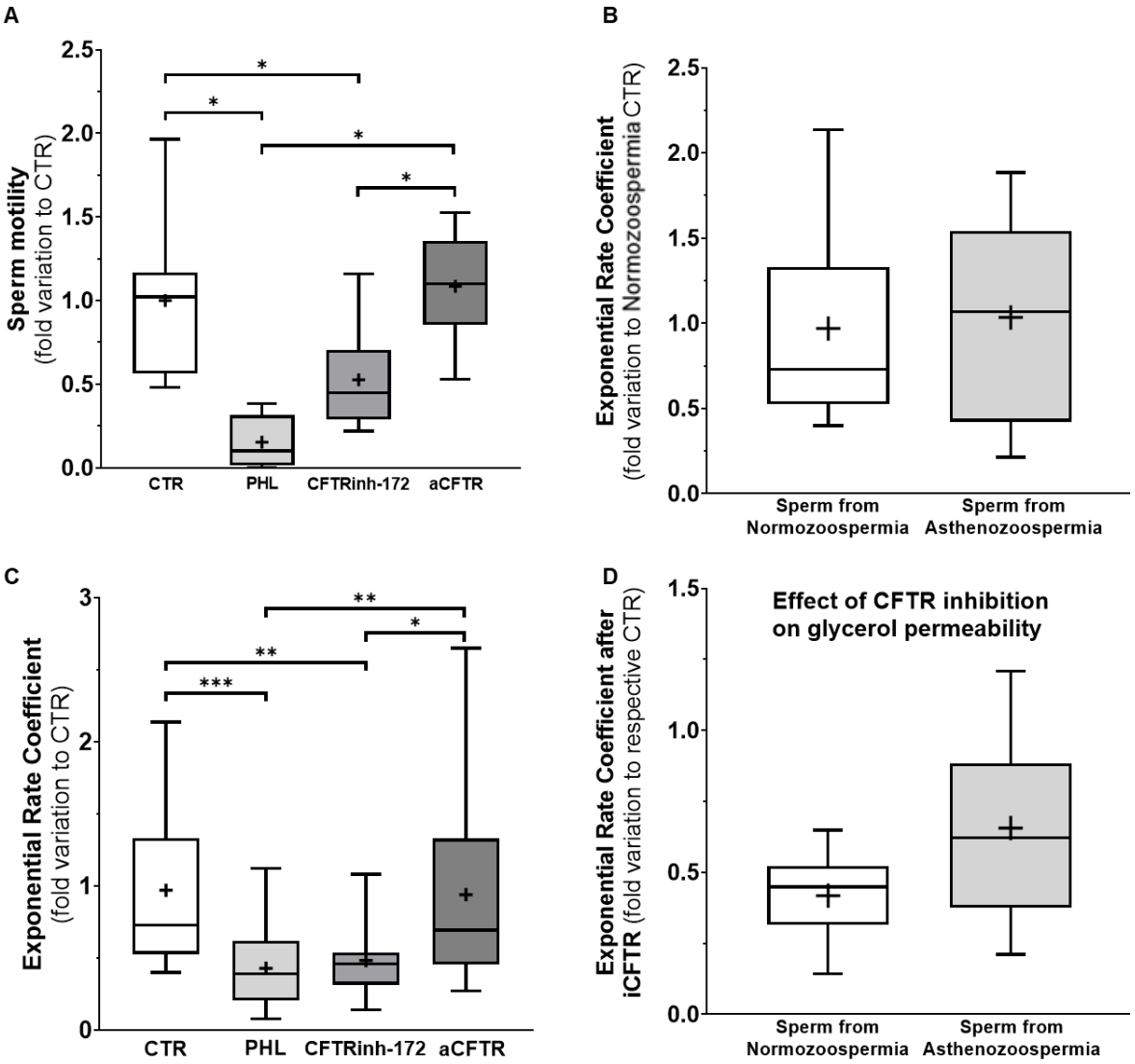

Figure 5.1.: Effect of human sperm incubation with a CFTR inhibitor (CFTRinh-172), aquaglyceroporin inhibitor phloretin (PHL), and CFTR activators forskolin and IBMX (aCFTR) on sperm motility (A) and membrane glycerol permeability (exponential rate coefficient) (C) of spermatozoa from normozoospermic men. Representation of the data of membrane glycerol permeability (exponential rate coefficient) between sperm from samples from normozoospermic men and asthenozoospermic (B) and the effect of CFTR inhibition in the glycerol permeability of sperm from normozoospermic men and asthenozoospermic men (D).Results presented in min. to max. graphs, with a cross representing the mean of the sample size. Significantly different results with $p < 0.05$ are indicated as: *; $p < 0.01$ are indicated as: **; and $p < 0.001$ are indicated as: ***. A, n = 10; B, n = 13; C, n = 23; D, n = 13

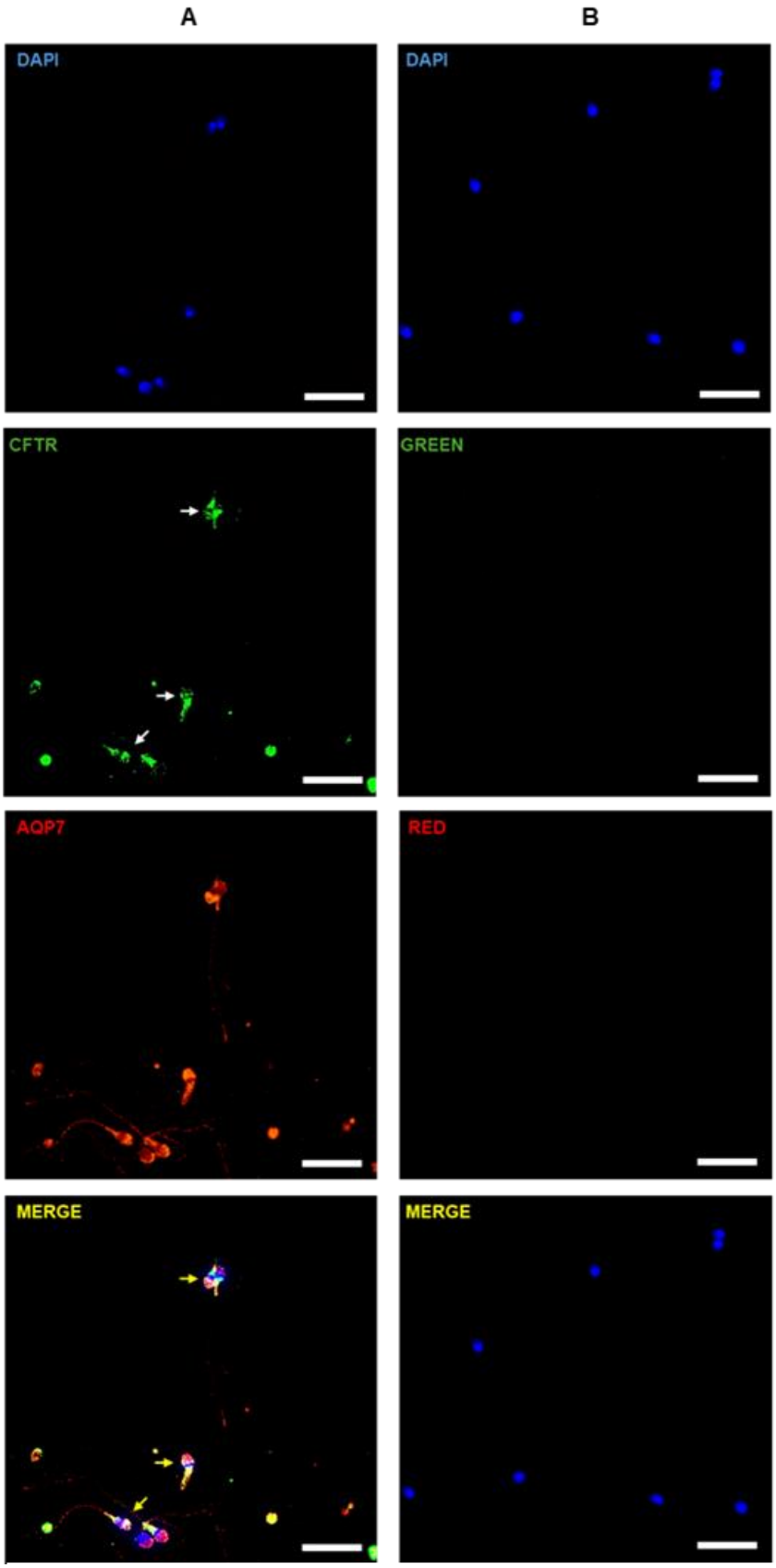

Figure 5.2: Co-immunofluorescence staining of CFTR and AQP7 in human sperm (A) and respective negative controls (B).White and yellow arrows show CFTR staining and co-localization of CFTR and AQP7, respectively, in the equatorial segment of the sperm head. Negative controls were carried out, without a primary antibody, for each protein. Cell nuclei were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 20 µm.

5.4. Discussion

A considerable proportion (30-40%) of infertile men suffer from idiopathic male infertility, a condition that lacks a definitive cause to justify it. The *CFTR* gene is associated with cystic fibrosis and is emerging as a crucial factor in male fertility beyond this disease. CFTR is involved in multiple pathways essential for male fertility and is also able to modulate many key proteins. AQPs, particularly aquaglyceroporins, are one example of such proteins that can be modulated by CFTR within the male reproductive tract. These proteins regulate water and glycerol diffusion and are vital for sperm function. While the role of CFTR in sperm capacitation and the acrosomal reaction is recognized, the information available regarding its impact on sperm motility is still to be elucidated. AQP7 is another membrane protein whose permeability is linked to sperm motility (Ribeiro et al., 2023). CFTR and aquaglyceroporins (like AQP7) work synergistically for water and solute diffusion (Ribeiro et al., 2022). CFTR is able to create an ion gradient that allows AQPs to facilitate water diffusion, responding to the gradient creation. Hence, this study focused on the potential functional and physical interaction between CFTR and AQP7 in human sperm, aiming to describe a possible novel mechanism that contributes to idiopathic male infertility. Our study presents new evidence on other mechanisms that may lead to male infertility by revealing a connection between the CFTR protein and human sperm motility. These results may help understanding some causes of idiopathic male infertility by exploring the role of CFTR beyond its well-established function in cystic fibrosis.

An important result of our study was the relation between CFTR inhibition and the decrease in sperm motility noted in samples from men with normozoospermia. Our experimental design was designed to capture the immediate impact of CFTR on sperm function. Thus, we used a 10-minute incubation with CFTRinh-172 at 1 μM, taking into consideration the time for complete CFTR inhibition and the normalization time for the stopped-flow technique (Ma et al., 2002). This finding contrasts with the ones of previous studies that used longer incubation times and did not detect significant changes in sperm motility after CFTR inhibition (Li et al., 2009, Puga Molina et al., 2017). These studies revealed that CFTR inhibition did not change human sperm motility when incubated with the same inhibitor at a concentration range of 0.1 μM to 5 μM for 5 hours (Li et al., 2009, Puga Molina et al., 2017). However, these studies focused on the impact of CFTR inhibition on sperm capacitation, which explains the longer incubation time. With our approach, we were able to identify an acute time point where CFTR activity significantly influences sperm motility. This decrease in motility may be due to the sudden decrease in cytoplasmatic pH after CFTR inhibition and decreased transport of HCO_3^-. This is described to impair cAMP production by sAC (Puga Molina et al., 2017) and, as a consequence, leads to impaired PKA activation and

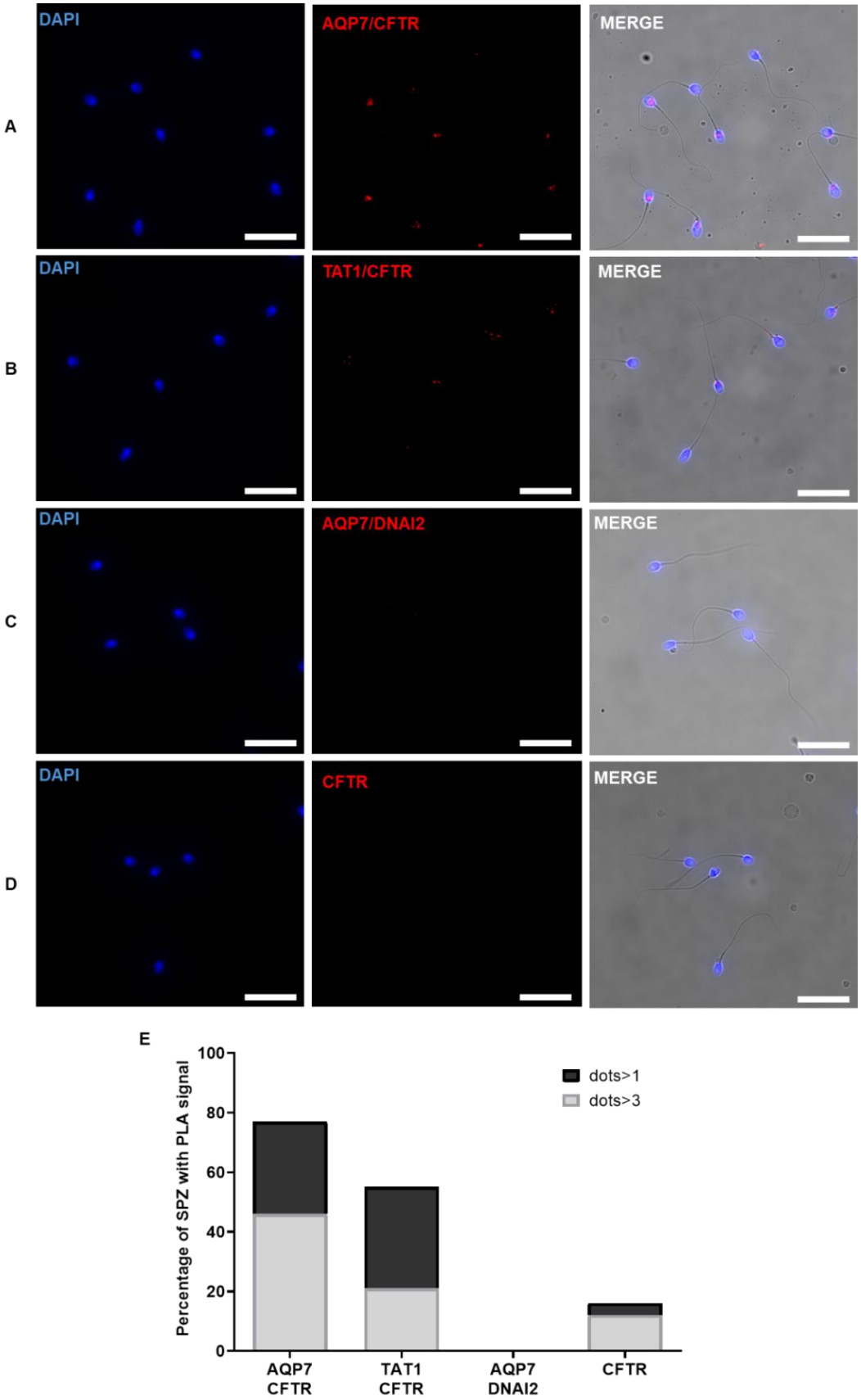

Figure 5.3.: Molecular interaction between CFTR and AQP7 (A), CFTR and TAT1 (B) as a positive control, and AQP7 and DNAI2 (C) as a negative control. Quantification of the red dots representative of each interaction between the proteins (E).Negative control (D) was carried out by only CFTR antibody incubation. The cells' nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 20 µm.

flagellar movement (Esposito et al., 2004). Interestingly, our study also revealed the potent effect of phloretin, a known inhibitor of glycerol diffusion, on sperm motility. Phloretin is known as an inhibitor of glycerol diffusion but is unspecific (de Oliveira, 2016), being also capable of inhibiting cAMP-activated Cl^- current, probably due to inhibitory CFTR properties (Fan et al., 2001). Phloretin also dysregulates calcium homeostasis and glucose transport which are crucial for sperm motility (de Oliveira, 2016, Pereira et al., 2017, Hytti et al., 2023). In our study, it is not clear if the effect of phloretin is directly due to the disruption of glycerol permeability or by a pathway that involves the inhibition of CFTR and other transporters.

CFTR demonstrates a broader influence beyond Cl^- transport by indirectly modulating water and solute diffusion through the membranes (Ribeiro et al., 2022). This mechanism affects both paracellular and transcellular water movement, notably through AQPs. Our findings extend this concept by demonstrating that CFTR inhibition disrupts the glycerol permeability of aquaglyceroporins in human sperm. This observation is supported by previous research that shows similar CFTR-dependent modulation of aquaglyceroporin-mediated glycerol permeability in human airway epithelial cells (Schreiber et al., 1999) and male reproductive tract epithelia (Pietrement et al., 2008, Ribeiro et al., 2022). However, our results did not show any difference in membrane glycerol permeability in sperm incubated with the CFTR activators. Forskolin and IBMX work by increasing cytoplasmatic cAMP levels (Thompson et al., 2019), which, in turn, increases CFTR activation through PKA (Nguyen et al., 2021). Previous studies showed that these activators can increase CFTR's Cl^- conductivity while increasing AQP3 and AQP9 water permeability (Cheung et al., 2003, Jourdain et al., 2014), but our results show that it does not seem to have an impact on AQP7-mediated glycerol permeability. In fact, CFTR was found to modulate AQP3 (in Chinese hamster ovary cells) and AQP9 (in intact rat epididymis) water permeability in a cAMP-dependent manner (Cheung et al., 2003, Jourdain et al., 2014). Interestingly, further evidence showed that this modulation seems to be independent of CFTR's Cl^- channel function (Llinares et al., 2020). This indicates that the mechanism for the modulation goes beyond ion gradient creation and consequent water/solute movement, probably involving a physical modulation. In fact, there is already data regarding the formation of CFTR/aquaglyceroporin protein complexes in male reproductive tract epithelia (Pietrement et al., 2008, Ribeiro et al., 2022). Building upon this, we investigated the potential formation of these complexes in human sperm by focusing on the localization of CFTR and AQP7, since the latter is the one responsible for glycerol permeability in sperm (Ribeiro et al., 2023).

CFTR expression in human sperm cells has already been noted in the equatorial segment of the head and midpiece (Xu et al., 2007, Diao et al., 2013), being in concordance with our results. Both studies have also shown that the pattern is not common to all sperm cells. The study by Xu and collaborators showed a high incidence of signal in the vast majority of

sperm cells however, the cells utilized were subjected to a gradient centrifugation which resulted in the enrichment of highly motile sperm (Xu et al., 2007). This hypothesis is supported by the correlation between CFTR down-expression and abnormal motility in aged men (Diao et al., 2013). The pattern of CFTR expression in the equatorial segment of the sperm head seemed to be the one that is dysregulated with aging and related to decreased sperm motility (Diao et al., 2013). AQP7 expression also seems to be found in the equatorial segment of the sperm's head (Moretti et al., 2012, Pellavio et al., 2020), although our results showed its presence throughout the head. The results regarding the interaction involving CFTR and AQP7 showed the most signal in the equatorial segment of the sperm head. However, co-immunofluorescence results indicated a co-localization in the midpiece as well as in the equatorial segment of the head. This could point to a favored protein complex formation involving CFTR and AQP7 in this location known to be involved in many processes of sperm functioning. It is known that it is in the equatorial zone of the sperm head that many proteins responsible for oocyte interaction and fertilization are situated (Yelumalai et al., 2015, Vondrakova et al., 2022). Channels like mitochondrial uncoupling protein 1 and TAT1 are also present in this specific location. The dysregulation of these proteins is known to impact sperm motility (Touré et al., 2007, Carrageta et al., 2023). Thus, this location in the sperm's head might be essential for motility by being the location of a major protein complex responsible for the synergistic control of cytosolic pH and ion/water homeodynamics. NHERF1 was found to mediate the interaction between CFTR and TAT1 (also known as SLC26A8) and two other members of the SLC26 family (SLC26A3 and SLC26A6) in mouse sperm (Chávez et al., 2012, El Khouri et al., 2014). Given the evidence presented, it is reasonable to hypothesize that NHERF1 could also serve as a scaffolding protein linking CFTR and AQP7, potentially playing a pivotal role in regulating sperm function and contributing to male fertility. The C-terminal sequence of each AQP7 monomer can be qualified as a class III PDZ-binding domain, allowing the interaction with the PDZ domain of NHERF1 (Lee et al., 2010). Concurrent results have already been demonstrated with the C-terminal of AQP9 in epithelial cells of epididymis (Pietrement et al., 2008). Moreover, when integrated within the plasma membrane, AQP7 forms a tetramer potentially facilitating multiple interactions with other PDZ-binding proteins through NHERFs. This could enhance their stability in the sperm plasma membrane, possibly even during the fluidity changes of the membrane associated with capacitation. Thus, our findings reveal the co-localization of CFTR and AQP7 in the equatorial segment of the sperm head, a region crucial for sperm-egg fusion during fertilization. This specific localization suggests that the CFTR-AQP7 interaction may play a pivotal role not only in sperm motility but also in the complex process of fertilization. Moreover, the presence of a CFTR-AQP7 protein complex

in the sperm head opens new possibilities for molecular mechanisms important for sperm function.

It is important to note that the measurement of glycerol permeability assumes that the entry of water and glycerol into sperm cells is proportional to the change in cell volume, which is in turn proportional to the intensity of light scattering (Maggio et al., 2018). However, the calculation of the glycerol permeability is easier with round cells (Ribeiro et al., 2022). Due to the specialized shape of sperm cells being segmented and with a flagellum, direct calculation of glycerol permeability is not practical. In this study, we compared the exponential rate coefficient between different groups, assuming similar sperm morphology and viability across samples. Notwithstanding, our results indicate that CFTR inhibition disrupts aquaglyceroporin-mediated glycerol permeability in sperm, revealing a novel mechanism of CFTR interaction with AQP7 in the head of human sperm. Additionally, our data shows that CFTR inhibition decreases sperm motility. The reduced effect of CFTR-induced modulation on AQP7 permeability observed in sperm from asthenozoospermic men suggests that the functional interaction between CFTR and AQP7 might be compromised in these individuals, even without the presence of common *CFTR* variants detected by a standardized cystic fibrosis screening kit. Despite not reaching statistical significance, the tendency is clear. The reason for this possible modulation can have multiple origins that allow us to hypothesize. One can be attributed to impaired glycerol permeability, a metabolite believed to have a role in sperm energy metabolism and maturation process (Mann et al., 1956, Cooper et al., 1981, Jones et al., 1996, Jones et al., 1997, Liu et al., 2022). Another could be an impairment on the pathway of CFTR activation, weakening this interaction by changing the conformation of CFTR. Further investigation into this physical interaction could lead to the development of novel therapeutic targets for idiopathic male infertility. Our results are not limited to sperm cells. They suggest, together with the available literature, a conserved mechanism of CFTR-dependent regulation of aquaglyceroporins across various tissues. Moreover, our results further challenge the view of CFTR simply as a channel. The recent literature describes the CFTR as a key player and modulator of many pathways and our data strengthens its ability to modulate water and glycerol transport through aquaglyceroporins. This understanding of CFTR's functions has implications for both basic science and clinical applications. Finally, the identification of the CFTR-AQP7 interaction and its localization in the sperm head allows speculation of a new molecular mechanisms that may cause some cases of idiopathic male infertility. While the exact cause of this condition remains elusive, our findings suggest that subtle differences in CFTR function potentially caused by *CFTR* gene variations that are not detected by standard diagnostic tests could contribute to impaired sperm motility. One must keep in mind that this study only screened for the most common variants that induce cystic fibrosis. Many

assumed benign variants are not screened in this kit as well as in clinics that use this approach. Non-synonymous but assumed benign variants could impair CFTR's protein-protein interactions and impair its role in highly complex processes like the ones responsible for developing spermatogenesis, sperm maturation, and capacitation. This highlights the value of further research for better understand the impact of these *CFTR* variants on male reproductive health.

In conclusion, our study provides evidence for a novel CFTR-AQP7 interaction in human sperm that has implications for basic science and clinical applications. These results not only increase our comprehension of sperm function but also bring new challenges worth exploring for a better understanding of idiopathic male infertility. The continuation of the study about the interaction between CFTR and aquaglyceroporins can be a promising theme to improve the probability of many infertile couples conceiving.

4.5. Authors contributions

João C. Ribeiro, Raquel L. Bernardino, Paula Jorge, and Pedro F. Oliveira contributed to the study conception and design. Material preparation, data collection, and analysis were performed by João C. Ribeiro, Paula Jorge, and Emília Vieira. The first draft of the manuscript was written by João C. Ribeiro. João C. Ribeiro, Raquel L. Bernardino, Pedro F. Oliveira, Marco G. Alves, and Paula V. Jorge contributed to the writing-review and editing of the manuscript. Alberto Barros, Rosário Santos, and Aminata Touré contributed with resources. The funding was obtained by Marco G. Alves, Pedro F. Oliveira, Aminata Touré, and Raquel L. Bernardino. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Chapter 6

Conclusion

Dysregulation of CFTR function significantly impairs male fertility by disrupting processes such as spermatogenesis, sperm maturation, and capacitation. The proper development of these processes is the basis for sperm motility and for their ability to fertilize. Frequently, infertile men exhibit compromised sperm motility. CFTR plays a key role in regulating the cytoplasmic pH necessary for the initiation and maintenance of sperm motility. Additionally, CFTR is involved in many other processes due to its wide range of interactors. This enables CFTR to both influence the activity and permeability of other transmembrane proteins and, in turn, be regulated by those same proteins. Aquaglyceroporins, one of the known CFTR interactors, are essential for water and solute balance, and thus, important for male fertility development. Taking this into consideration, the main goal of this thesis was to study the interaction between CFTR and aquaglyceroporins, and its relevance to the modulation of water and glycerol permeability on the cells that most directly contribute to the male reproductive potential: spermatozoa and Sertoli cells.

Our findings demonstrated that CFTR dysfunction impairs glycerol and water transport mediated by aquaglyceroporins in both Sertoli and sperm cells. In Sertoli cells, this disruption can compromise spermatogenesis and, consequently, male reproductive potential. Sertoli cell function and permeability are crucial for maintaining a healthy reproductive environment since they are the primary barrier between circulating plasma and developing germ cells (Russell et al., 1985, Mruk et al., 2004). It is known that glycerol concentration needs to be tightly regulated and maintained in a tight range of concentrations for the proper development of spermatogenesis (Wiebe et al., 1989). The source of these abnormal glycerol levels can be increased circulating glycerol due to metabolic diseases or lipid metabolism in Sertoli cells due to germ cell phagocytosis. On the other hand, glycerol can be used as an energy source by the ejaculated sperm (Mann et al., 1956, Jones et al., 1996, Jones et al., 1997). However, it is still to be elucidated if immature sperm cells can

metabolize glycerol and use it to maintain viability until reaching epididymis. This would be of great relevancy taking into consideration that Sertoli cells are highly glycolytic, being unlikely to share glucose into the lumen of the seminiferous tubules since the vast majority of it is used for lactate production for germ cell development (Oliveira et al., 2015). In fact, dysregulation of AQP9-mediated lactate permeability has been linked to impaired spermatogenesis (Arena et al., 2011), highlighting the importance of the permeability of these pores to male fertility. Thus, our results indicate that these hypothetical processes can be in jeopardy in men with abnormal CFTR function due to dysregulated CFTR/aquaglyceroporin interaction.

Concerning sperm cells, other processes that can be suboptimal in these men are sperm maturation, before ejaculation, and osmoadaptation to the feminine tract fluid, after ejaculation. Focusing on the processes before ejaculation, sperm maturation is characterized by the development of sperm progressive motility. As previously noted, lipid metabolism and glycerol secretion into the epididymal lumen appear to play a crucial role in sperm maturation and the development of motility (Cooper et al., 1981, Liu et al., 2022). Consequently, sperm glycerol permeability is vital for allowing glycerol diffusion between the intra and the extracellular environment of the sperm cells. Our findings revealed that sperm from asthenozoospermic men exhibit reduced glycerol permeability, primarily due to the diminished permeability of AQP7. This observation highlights the importance of glycerol permeability for normal sperm motility, suggesting a potential role in the mechanism for the initiation of sperm flagellar movement. Furthermore, our results demonstrate that CFTR inhibition can decrease AQP7-mediated glycerol permeability in human sperm. Given the prevalence of CFTR dysfunction in the European population (Chen et al., 2012), it could be a contributing factor to the impaired AQP7 permeability observed in sperm from asthenozoospermic men. Although our results did not reach statistical significance, they indicate a tendency towards reduced AQP7-mediated glycerol permeability caused by CFTR dysfunction in these individuals. This suggests that the control of AQP7 function by CFTR may be suboptimal in men with low sperm motility, potentially due to impaired physical interaction between the two proteins. This theory gains relevancy when joined with the fact that AQP7 protein amounts were similar between samples from fertile and subfertile men. Contrastingly, our findings suggested that the expression of AQP7 in sperm is dynamic and subject to regulation. The observed changes in AQP7 amount during capacitation, coupled with the increased glycerol permeability in hypermotile sperm, suggest that aquaglyceroporins may play a role in preparing sperm for their final push toward the oocyte. Thus, the functional relationship between CFTR and aquaglyceroporins can be a key player in maintaining key physiological processes related to male fertility. CFTR is able to create ion gradients and modulate intracellular pH, and by influencing the activity of

aquaglyceroporins it can regulate the flow of water and glycerol across cell membranes. This synergistic partnership is evident in both Sertoli cells and sperm cells, where CFTR's modulation of aquaglyceroporin-mediated glycerol permeability can have serious implications for sperm development and maturation. However, our results suggest that CFTR modulation towards aquaglyceroporin's permeability goes beyond gradient creation, and it is made through physical interaction and the creation of a protein complex. The discovery of a physical interaction between CFTR and AQP7 in human sperm further strengthens the notion of a close functional relationship between these proteins. This interaction localized on the sperm head, suggests a direct mechanism by which CFTR can influence AQP7 permeability and, consequently, sperm glycerol diffusion. The precise nature of this interaction and the signaling pathways involved remain to be fully elucidated, but our findings push for future investigation and allows speculation. CFTR activity is also modulated by other transporters, showing an interdependency of functionality in its interactions (Wang et al., 2017, Puga Molina et al., 2018). It is possible for the CFTR to not be the original culprit for the dysregulation of the CFTR-AQP7 interaction. Rather, its activity can be modulated by other transporters which may lead to a dysfunction in other interactors like aquaglyceroporins. Thus, the ability of CFTR to control and modulate the activity of other proteins can be a double-edged sword as a central player for health but also for disease.

Our findings show that AQP7-mediated glycerol permeability is impaired in sperm from men with asthenozoospermia, though the exact mechanism remains unclear. Our follow-up study suggested that the interaction between AQP7 and CFTR may cause this impairment. CFTR may modulate aquaglyceroporin activity in Sertoli and sperm cells through physical interactions, as our results indicate the assembly of a protein complex formed by CFTR and aquaglyceroporins. This research points to a new molecular mechanisms that may cause male idiopathic infertility, highlighting the roles of CFTR and aquaglyceroporins in sperm function. The observed decrease in AQP7-mediated glycerol permeability in sperm from asthenozoospermic men, along with evidence of a functional interaction between CFTR and AQP7, suggests that abnormal activity or expression of these proteins may contribute to impaired sperm motility and fertility. This hypothesis is strengthened by our confirmation of the relationship between aquaglyceroporin permeability with sperm motility and its dysregulation in asthenozoospermia, together with the fact of the established CFTR modulation of aquaglyceroporin permeability.

Thus, our results challenge the traditional view of CFTR as only a Cl^- channel, showing its role in regulating water and solute transport through aquaglyceroporins. This understanding has important implications for both science and clinical practice. By studying the interactions between CFTR, aquaglyceroporins, and other factors in sperm physiology, we are closer to

understanding male fertility and developing new strategies for treating idiopathic male infertility. The potential link between variations in CFTR function and idiopathic male infertility highlights the need for more studies to identify *CFTR* variants that affect sperm function. This knowledge could lead to new diagnostic tools and treatments for isolated asthenozoospermia. By further exploring the CFTR/aquaglyceroporin interaction, we might have identified a new target to improve treatments and diagnostics for male infertility, offering hope to those struggling to conceive.

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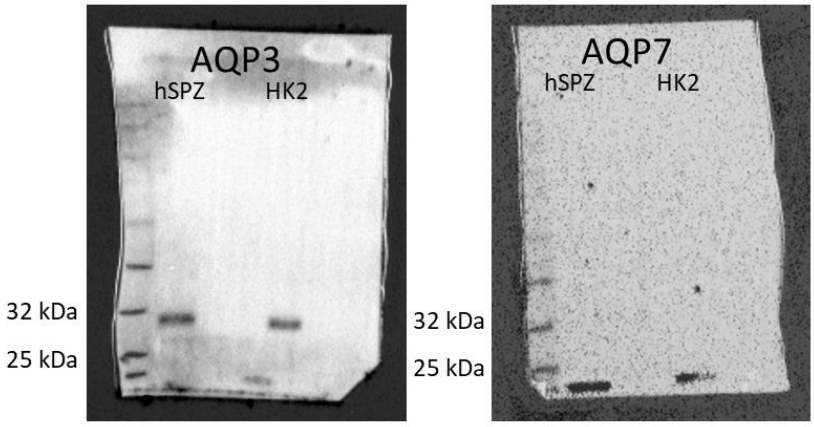
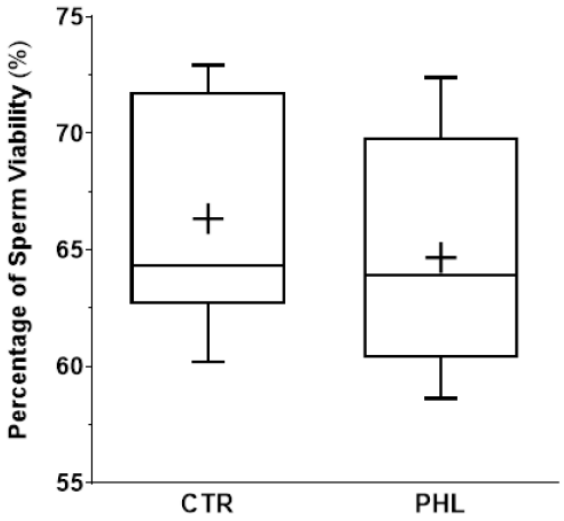
Annexes A: Supplemental Data

A.1. Chapter 4 Supplementary Data



Supplementary Data Figure A.1.: Representative blots of immunodetection of AQP3 and AQP7 in human spermatozoa (hSPZ) and human kidney cell line (HK2) for antibody specificity validation.

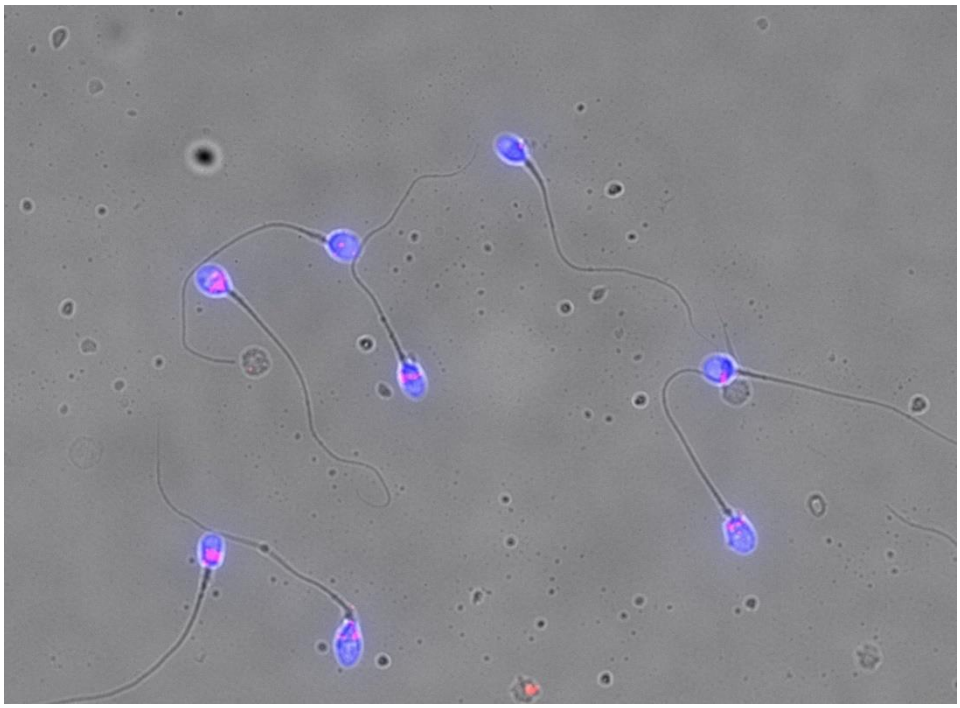


Supplementary Data Figure A.2.: Percentage of sperm viability after incubation of isosmotic medium with (CTR) and without phloretin (PHL) for 10 min. N=12

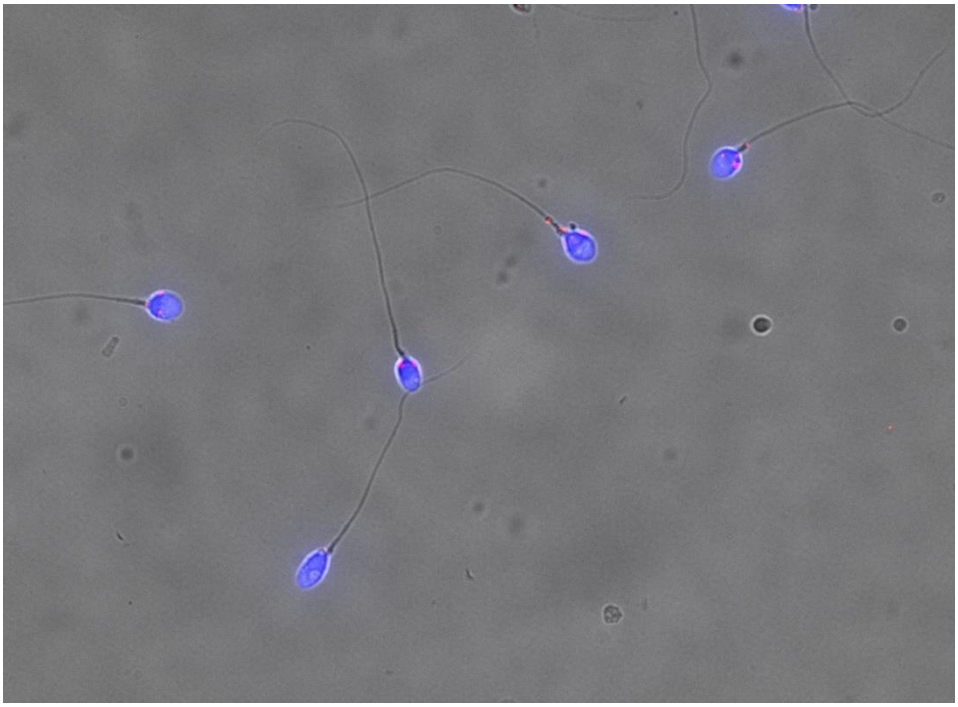
A.2. Chapter 5 Supplementary Data

Supplementary Data Table A1: CFTR variants screened in the genomic sperm DNA of the study participants.

Nomenclature	cDNA name	Protein name
CFTRdele2,3	c.54-5940_273+10250del (c.54-5940_273+10250del21080)	
E60X	c.178G>T	p.Glu60X
P67L	c.200C>T	p.Pro67Leu
G85E	c.254G>A	p.Gly85Glu
394delTT	c.262_263del (c.262_263delTT)	p.Leu88IlefsX22
444delA	c.313del (c.313delA)	p.Ile105SerfsX2
R117C	c.349C>T	p.Arg117Cys
R117H	c.350G>A	p.Arg117His
Y122X	c.366T>A	p.Tyr122X
621+1G>T	c.489+1G>T	
711+1G>T	c.579+1G>T	
L206W	c.617T>G	p.Leu206Trp
1078delT	c.948del(c.948delT)	p.Phe316LeufsX12
R334W	c.1000C>T	p.Arg334Trp
R347P	c.1040G>C	p.Arg347Pro
R347H	c.1040G>A	p.Arg347His
A455E	c.1364C>A	p.Ala455Glu
I507del	c.1519_1521del (c.1519_1521delATC)	p.Ile507del
F508del	c.1521_1523del (c.1521_1523delCTT)	p.Phe508del
1677delTA	c.1545_1546del (c.1545_1546delTA)	p.Tyr515X
V520F	c.1558G>T	p.Val 520Phe
1717-1G>A	c.1585-1G>A	
G542X	c.1624G>T	p.Gly542X
S549R(T>G)	c.1647T>G	p.Ser549Arg
S549N	c.1646G>A	p.Ser549Asn
G551D	c.1652G>A	p.Gly551Asp
R553X	c.1657C>T	p.Arg553X
R560T	c.1679G>C	p.Arg560Thr
1811+1.6kbA>G	c.1680-886A>G	
1898+1G>A	c.1766+1G>A	
2143delT	c.2012del (c.2012delT)	p.Leu671X
2184delA	c.2052del (c.2052delA)	p.Lys684AsnfsX38
2347delG	c.2215del (c.2215delG)	p.Val739TyrfsX16
W846X	c.2538G>A	p.Trp846X
Q890X	c.2668C>T	p.Gln890X
R1066C	c.3196C>T	p.Arg1066Cys
Y1092X(C>A)	c.3276C>A	p.Tyr1092X
M1101K	c.3302T>A	p.Met1101Lys
D1152H	c.3454G>C	p.Asp1152His
R1158X	c.3472C>T	p.Arg1158X
R1162X	c.3484C>T	p.Arg1162X
3659delC	c.3528del (c.3528delC)	p.Lys1177SerfsX15
S1251N	c.3752G>A	p.Ser1251Asn
3905insT	c.3773dup (c.3773dupT)	p.Leu1258PhefsX7
W1282X	c.3846G>A	p.Trp1282X
N1303K	c.3909C>G	p.Asn1303Lys
2789+5G>A	c.2657+5G>A	
3120+1G>A	c.2988+1G>A	
3272-26A>G	c. 3140-26A>G	
3849+10kbC>T	c.3718-2477C>T	



Supplementary Data Figure A.3.: Full size image of the interactions between CFTR and AQP7 in human sperm.



Supplementary Data Figure A.4.: Full size image of the interactions between CFTR and TAT1 in human sperm.

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B.4. CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells

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