Aus dem Institut für Humangenetik
Theoretische Medizin und Biowissenschaften
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Veränderungen von Expressionsmustern auf Transkriptomebene, auf Ebene der post-transkriptionellen Regulation durch miRNAs und auf Proteinebene in Spermien von Männern mit Infertilitäts-assoziierten Anomalien

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INHALTSVERZEICHNIS

I. PUBLIKATIONSLISTE

Diese kumulative Promotion wurde am Institut für Humangenetik der Medizinischen Fakultät der Universität des Saarlandes unter der Leitung von Herrn Prof. Dr. Eckart Meese durchgeführt und basiert auf den folgenden drei Publikationen. Die Auflistung erfolgt chronologisch. Erstautoren, die gleichermaßen zur Publikation beigetragen haben, wurden mit einem (*) - Symbol markiert. Weitere Publikationen, die im Rahmen meiner Promotion entstanden sind, sind unter 7. Erweiterte Publikationsliste aufgeführt.

Becker LS*, Abu-Halima M*, Ayesh BM, Meese E. MicroRNA-targeting in male infertility: Sperm microRNA-19a/b-3p and its spermatogenesis related transcripts content in men with oligoasthenozoospermia. Front Cell Dev Biol. (2022); 10:973849. doi: 10.3389/fcell.2022.973849.

Becker LS, Al Smadi MA, Raeschle M, Rishik S, Abdul-Khaliq H, Meese E, Abu-Halima M. Proteomic Landscape of Human Sperm in Patients with Different Spermatogenic Impairments. Cells. (2023); 12(7):1017. doi: 10.3390/cells12071017.

Becker LS, Al Smadi MA, Koch H, Abdul-Khaliq H, Meese E, Abu-Halima M. Towards a More Comprehensive Picture of the MicroRNA-23a/b-3p Impact on Impaired Male Fertility. Biology. (2023); 12(6):800. doi: 10.3390/biology12060800.

II. ABBILDUNGSVERZEICHNIS

III. TABELLENVERZEICHNIS

IV. ABKÜRZUNGSVERZEICHNIS

3'UTR	3' untranslatierten Region (engl. 3' untranslated region)
A	Asthenozoospermie (engl. Asthenozoospermia)
AGO	Argonaut – Protein
AN	Abnormal
ART	Assistierte Reproduktionstechnik (engl. Assisted Reproductive Technology)
ASRM	engl. American Society for Reproductive Medicine
AUC	engl. Area Under the Curve
AZF	Azoospermiefaktor
BCA	engl. Bicinchoninic Acid
C	Celsius
cDNA	engl. Complementary DNA
CEP41	engl. Centrosomal Protein 41
CFTR	engl. Cystic Fibrosis Transmembrane Conductance Regulator
CI	Konfidenzintervall (engl. Confidence Interval)
Ct	engl. Cycle Threshold
CV	engl. Cross-Validation
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonukleinsäure (engl. Deoxyribonucleic Acid)
DTT	engl. Dithiothreitol
EAU	engl. European Association of Urology
ECL	engl. Enhanced Chemiluminescence
FASP	engl. Filter-Aided Sample Preparation-Method
FC	engl. Fold Change
FDR	engl. False Discovery Rate
FSH	Follikel-stimulierendes Hormon (engl. Follicle Stimulating Hormone)
G2E3	engl. G2/M-Phase Specific E3 Ubiquitin Protein Ligase
GO	engl. Gene Ontology
GOLGA6B	engl. Golgin A6 Family Member B
GOLGA6C	engl. Golgin A6 Family Member C
HEK-293T	menschliche embryonale Nierenzellen (engl. Human Embryonic Kidney Cells)
HRP	engl. Horseradish Peroxidase
ICSI	Intrazytoplasmatischen Spermieninjektion (engl. Intracytoplasmic Sperm Injection)
IVF	In-Vitro Fertilisation (engl. In-Vitro Fertilization)
LC-MS/MS	Flüssigchromatographie mit Massenspektrometrie-Kopplung (engl. Liquid Chromatography with Tandem Mass Spectrometry)
LFQ	engl. Label-Free Quantified
LMLN	engl. Leishmanolysin Like Peptidase
lncRNA	lange nicht-kodierende RNA (engl. long non-coding RNA)
MCS	engl. Multiple Cloning Site
miRNA	mikroRNA (engl. microRNA)
miRTC	engl. miRNA Reverse Transcription Control
ml	Milliliter
mRNA	Boten-RNAs (engl. messenger RNA)
MTIs	miRNA-Zielgen-Interaktion (engl. MiRNA-Target Interaction)

mut	Mutante (engl. mutant)
ncRNA	Nicht-kodierenden RNAs (engl. non-coding RNA)
NOA	Nicht-obstruktive Azoospermie (engl. Non-Obstructive Azoospermia)
NOL4	engl. Nucleolar Protein 4
NTC	engl. No Template Control
OA	Oligoasthenozoospermie (engl. Oligoasthenozoospermia)
OAT	Oligoasthenoteratozoospermie
PAPOLB	engl. Poly(A) Polymerase Beta
PBS	engl. Phosphate-Buffered Saline
PCDHA9	engl. Protocadherin Alpha 9
piRNA	piwi-interacting RNA
pre-miRNA	Vorläufer-miRNA (engl. precursor microRNA)
pri-miRNA	primäre miRNA (engl. primary microRNA)
PVDF	engl. Polyvinylidene Difluoride
RAN-GTP	Ras-related Nuclear Protein - Guanosintriphosphat
rasiRNA	repeat-associated small interfering RNA
RGPD1	engl. RANBP2 Like and GRIP Domain Containing 1
RISC	engl. RNA-Induced Silencing Complex
RNA	Ribonukleinsäure (engl. Ribonucleic Acid)
RNAi	engl. RNA Interference
ROC	engl. Receiver Operating Characteristic
RT	Reverse Transkription (engl. Reverse Transcription)
RT-qPCR	Echtzeit quantitative – Polymerase - Kettenreaktion (engl. real-time quantitative polymerase chain reaction)
SD	engl. Standard Deviation
SDF	DNA-Fragmentierungsrate des Spermas (engl. Sperm DNA Fragmentation)
SDS-PAGE	engl. Sodium Dodecyl Sulfate – PolyAcrylamid Gel Electrophoresis
SEM	engl. Standard Error of Mean
sncRNA	kurze nicht-kodierende RNA (engl. small non-coding RNA)
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SOX6	engl. SRY-Box Transcription Factor 6
SRY	engl. Sex-determining Region Y
SSC	Spermatogonien Stammzellen (engl. Spermatogonial Stem Cell)
TBS-T	engl. Tris Buffered Saline with Tween 20
WHO	Weltgesundheitsorganisation (engl. World Health Organization)
WT	Wildtyp (engl. Wild Type)
ZNF492	engl. Zinc Finger Protein 492
ZNF695	engl. Zinc Finger Protein 695
Δ	Delta

1. ZUSAMMENFASSUNG IN DEUTSCHER UND ENGLISCHER SPRACHE

1.1. Zusammenfassung

Infertilität ist weit verbreitetet und betrifft weltweit jedes sechste Paar. Diesen Paaren ist es nicht möglich trotz ungeschützten Geschlechtsverkehrs innerhalb eines Jahres Nachwuchs zu empfangen. Die aktuelle Forschung zeigt, dass die männliche Infertilität komplex und multifaktoriell ist und in 30 bis 40 % der Fälle idiopathisch bleibt. Auch wenn die assistierte Reproduktionstechnik trotz diagnostizierter Infertilität eines Paares eine Schwangerschaft ermöglicht, bleibt die Frage nach den Ursachen für männliche Infertilität erhalten. Zur Verbesserung unseres Verständnisses der genetischen Faktoren, die zur Entstehung männlicher Infertilität beitragen, wurde nach Veränderungen auf Proteom- und Transkriptomebene gesucht. Darüber hinaus ist die Rolle der mikroRNAs (miRNAs) als post-transkriptionelle Regulatoren von besonderem Interesse für die Aufklärung der mit Infertilität verbundenen Mechanismen. MiRNAs sind kurze, nicht-kodierende RNA-Moleküle, die eine entscheidende Rolle bei der Genregulation spielen und an der Entstehung der männlichen Infertilität beteiligt sind. In dieser Arbeit habe ich mich speziell auf miRNAs und ihre Interaktionen mit Zielgenen fokussiert, um ihre Rolle bei der männlichen Infertilität zu untersuchen. Hierbei sollten molekulare Signalwege aufgedeckt werden, die bei männlicher Infertilität gestört sind und die Grundlage neuer diagnostischer Marker und maßgeschneiderter Therapien bilden könnten. Darüber hinaus habe ich eine umfassende Analyse der Veränderungen im Proteom von Spermien durchgeführt, die von Männern mit Infertilitäts-assoziierten Anomalien stammen. Hierbei sollten spezifische Proteinveränderungen identifiziert werden, die helfen zugrundeliegende Mechanismen der männlichen Infertilität zu entschlüsseln und dabei neue potentielle Biomarker zu identifizieren. Die Untersuchungen der miRNAs, ihrer Zielgene und des Proteoms sollte einen Beitrag zum besseren Verständnis der Dysregulation bei männlicher Infertilität und der männlichen Reproduktion im Allgemeinen leisten.

Im Speziellen habe ich die miR-23a-3p und miR-23b-3p (miR-23a/b-3p) im Kontext der Oligoasthenozoospermie untersucht, einer Infertilitäts-assoziierten Anomalie, die durch die Reduktion der Spermienanzahl und –motilität charakterisiert ist. Ich untersuchte 16 Testis-exprimierte Zielgene, deren Interaktion mit der miR-23a/b-3p *in-silico* vorhergesagt wurden. Zur Identifikation der miRNA-Zielgen-Interaktionen der durch miR-23a-3p und miR-23b-3p regulierten Zielgene, habe ich einen automatisierten Luciferase-Assay durchgeführt und acht Zielgene der miR-23a-3p (*NOL4, SOX6, GOLGA6C, PCDHA9, G2E3, ZNF695, CEP41* und *RGPD1*) und drei Zielgene der miR-23b-3p (*SOX6, GOLGA6C* und *ZNF695*) identifiziert. Zur Bestätigung der direkten Interaktion zwischen den miRNAs und ihren Zielgenen, habe ich die

miRNA-Bindestelle der Zielgene gezielt mutiert und somit fünf direkte Zielgene der miR-23a-3p (*NOL4, SOX6, GOLGA6C, PCDHA9* und *CEP41*) und drei direkte Zielgene der miR-23b-3p (*NOL4, SOX6* und *PCDHA9*) bestätigt. Zur Feststellung der Expressionslevel der Zielgene der miR-23a/b-3p habe ich Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit normalen Samenparametern (Normozoospermie) mittels Echtzeit - Polymerase - Kettenreaktion untersucht. Die Ergebnisse zeigten eine Reduktion der Expressionslevel in zehn Zielgenen (*NOL4, SOX6, GOLGA6C, PCDHA9, G2E3, ZNF695, CEP41, RGPD1, GOLGA6B* und *LMLN*) bei Männern mit Oligoasthenozoospermie. Diese veränderten Expressionslevel zeigten eine positive Korrelation mit den Samenparametern, der Spermienanzahl, -motilität und –morphologie. Dies lässt vermuten, dass die Dysregulation dieser Gene mit den veränderten Samenparametern bei Oligoasthenozoospermie assoziiert ist.

Darüber hinaus habe ich den Zusammenhang zwischen der miR-19a-3p und miR-19b-3p (miR-19a/b-3p) und ihre *in-silico* vorhergesagten, Testis-exprimierten Zielgene im Kontext der Oligoasthenozoospermie untersucht. Zur Analyse der Expressionslevel der miR-19a/b-3p und ihrer 82 Zielgene wurden Spermien von Männern mit Oligoasthenozoospermie mit Spermien von Männern mit Normozoospermie mittels quantitativer Echtzeit – Polymerase - Kettenreaktion verglichen. Die Ergebnisse zeigten die erhöhte Expression der miR-19a/b-3p und die gleichzeitige reduzierte Expression von 51 Zielgenen. Zwei Zielgene zeigten eine erhöhte Expression. Zur Untersuchung der Zusammenhänge zwischen der Expressionslevel der miRNAs und der Zielgene mit den Samenparametern habe ich eine Korrelationsanalyse durchgeführt. Die Ergebnisse zeigten positive Korrelationen der 51 vermindert exprimierten Zielgene mit den Samenparametern, Spermienanzahl, -motilität, und -morphologie, sowie negative Korrelationen der miR-19a-3p und miR-19b-3p mit den Samenparametern, Spermienanzahl, -motilität, -vitalität und -morphologie. Außerdem wurde eine negative Korrelation zwischen der veränderten Expression der miR-19a/b-3p und den 51 Zielgenen festgestellt. Zur Validierung dieser Beobachtungen auf Proteinebene, habe ich Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit Normozoospermie mittels Western Blot untersucht. Es zeigte sich eine Reduktion der Proteinlevel zweier Gene (STK33 und DNAI1). Die Ergebnisse dieser Studie deuten auf eine mögliche Beteiligung von miR-19a/b-3p und ihren Zielgenen am Erhalt der normalen Spermienfunktion hin und legen eine regulatorische Rolle der miR-19a/b-3p in der Kontrolle der Expressionslevel ihrer Zielgene im Kontext der Oligoasthenozoospermie nahe.

Des Weiteren habe ich das Spermien Proteom von Männern mit Normozoospermie, Oligoasthenozoospermie und Asthenozoospermie, die durch eine Verminderung der Spermienmotilität charakterisiert ist, analysiert. Mittels Flüssigchromatographie mit Tandem

Massenspektrometrie Kopplung wurden insgesamt 4412 Proteine identifiziert, von denen 1336 Proteine in mindestens 70 % der Spermienproben gefunden wurden. Zur Analyse der differentiellen Expression der Spermienproteine, habe ich das Proteom von Spermien subfertiler Männer, d.h. Männer mit Oligoasthenozoospermie und Asthenozoospermie, mit Spermien von Männern mit Normozoospermie verglichen. Die Ergebnisse zeigten in Spermien von subfertilen Männern gegenüber Männern mit Normozoospermie eine reduzierte Expression von 32 Proteinen und eine erhöhte Expression von 34 Proteinen. Bei Männern mit Asthenozoospermie verglichen mit Normozoospermie wiesen 95 Proteine eine geringere Expression auf, während 86 Proteine eine höhere Expression zeigten. Hingegen zeigten bei Männern mit Oligoasthenozoospermie verglichen mit Normozoospermie acht Proteine eine geringere Expression und ein Protein eine höhere Expression. Zur Untersuchung der Zusammenhänge zwischen den Proteinlevel mit der Spermienmotilität sowie der Spermienanzahl habe ich eine Korrelationsanalyse durchgeführt. Die Ergebnisse zeigten, dass 13 Proteine negativ und 37 Proteine positiv mit der Spermienanzahl und 35 Proteine negativ und 20 Proteine positiv mit der Spermienmotilität korrelierten. Darüber hinaus habe ich Kombinationen aus Proteinen identifiziert, die potentiell als Biomarker für die Vorhersage männlicher Subfertilität oder die spezifische Diagnose von Asthenozoospermie und Oligoasthenozoospermie dienen könnten. Außerdem könnten identifizierte Proteine potentiell zwischen Asthenozoospermie und Oligoasthenozoospermie differenzieren und so weitere Einblicke in die unterschiedlichen molekularen Profile geben, die mit diesen Erkrankungen verbunden sind.

Zusammenfassend zeigten die Ergebnisse meiner Forschung den regulatorischen Zusammenhang zwischen miR-23a-3p, miR-23b-3p, miR-19a-3p und miR-19b-3p und ihren jeweiligen Zielgenen bei Oligoasthenozoospermie. Darüber hinaus wurden veränderte Proteinexpressionsmuster identifiziert, die mit Subfertilität und spezifischen Infertilitäts-assoziierten Anomalien in Zusammenhang stehen. Die Korrelationsanalyse zeigte den Einfluss der entsprechenden miRNAs, der Zielgene und spezifischer Proteine auf die Spermienparameter, insbesondere auf die Spermienzahl, die Spermienmotilität und die Spermienmorphologie. Die Ergebnisse tragen zu einem besseren Verständnis der molekularen Mechanismen bei, die bei der männlichen Fortpflanzung eine Rolle spielen und der männlichen Infertilität zugrunde liegen, und können bei der Entwicklung von neuer Diagnose- und individueller Therapiestrategien helfen.

1.2. Summary

Alterations in expression patterns at the transcriptome level, at the level of post-transcriptional regulation by miRNAs, and at the protein level in sperm from men with infertility-associated impairments

Infertility is widespread and affects one in six couples worldwide. These couples are unable to achieve conception within a year of unprotected sexual intercourse. Current research shows that male infertility is complex and multifactorial, and remains idiopathic in 30–40% of cases. Although assisted reproductive technology enables pregnancy even if a couple has been diagnosed with infertility, the causes of male infertility remain to be elucidated. To gain a deeper comprehension of the genetic factors contributing to male infertility, investigations have delved into changes occurring at both the proteome and transcriptome levels. Furthermore, the role of microRNAs (miRNAs) as post-transcriptional regulators has been of particular interest in elucidating the mechanisms associated with infertility. MiRNAs are small non-coding RNA molecules that play a crucial role in gene regulation, and they have been found to have potential implications in the development of male infertility. In this thesis, I have specifically focused on miRNAs and their interactions with target genes, exploring their involvement in infertility-associated impairments. The aim was to uncover molecular signaling pathways that may be disrupted in male infertility and to lay the ground for identifying new diagnostic markers and customized therapies. Additionally, I have conducted an in-depth analysis of changes occurring in the proteome of sperm samples obtained from men with infertility-associated impairments. The aim was to determine specific protein alterations, to unravel the underlying mechanisms of male infertility and to help identify new potential biomarkers. By combining investigations of miRNAs and their target genes with proteomic analyses, I aimed to deepen our understanding of the genetic intricacies involved in male infertility.

Specifically, I focused on exploring the involvement of miR-23a-3p and miR-23b-3p (miR-23a/b-3p) in the context of oligoasthenozoospermia, a condition characterized by reduced sperm count and motility. I investigated 16 testis-expressed target genes that were predicted to interact with miR-23a/b-3p. To identify the miRNA-targeting by miR-23a/b-3p, I employed an automated luciferase assay and identified eight target genes for miR-23a-3p, including *NOL4, SOX6, GOLGA6C, PCDHA9, G2E3, ZNF695, CEP41,* and *RGPD1*. Additionally, three target genes, namely *SOX6, GOLGA6C*, and *ZNF695*, were identified as targets of miR-23b-3p. To confirm the direct interaction between miRNAs and their target genes, I performed mutagenesis experiments targeting the binding sites of these genes. I validated five genes (*NOL4, SOX6, GOLGA6C, PCDHA9*, and *CEP41*) that were directly regulated by miR-23a-3p. Three genes (*NOL4, SOX6*, and *PCDHA9*) were directly regulated by miR-23b-3p. To assess the expression levels of the target genes, I conducted real-time quantitative polymerase chain reaction (RT-qPCR) analysis on sperm samples obtained from

men with oligoasthenozoospermia and compared them to samples from men with normal sperm parameters (normozoospermia). The results revealed a significant reduction in the expression levels of ten target genes, including *NOL4, SOX6, GOLGA6C, PCDHA9, G2E3, ZNF695, CEP41, RGPD1, GOLGA6B,* and *LMLN* in men with oligoasthenozoospermia. The observed differential expression levels showed a positive correlation with semen parameters, such as sperm count, motility, and morphology. This suggests that the dysregulation of these genes may play a role in the altered sperm characteristics associated with oligoasthenozoospermia.

In addition, I investigated the relationship between miR-19a-3p and miR-19b-3p (miR-19a/b-3p) and their *in-silico* predicted testis-expressed target genes, which are associated with oligoasthenozoospermia. To analyze the expression levels of miR-19a/b-3p and its 82 predicted target genes, I performed RT-qPCR on sperm samples obtained from men with oligoasthenozoospermia in comparison to sperm from men with normozoospermia. The comparison revealed higher expression of miR-19a/b-3p and lower expression of 51 target genes. Two target genes displayed higher expression levels. To explore the relationship between the expression levels of these genes and semen parameters, I performed a correlation analysis. The findings demonstrated a positive association between the 51 target genes and semen parameters, such as sperm count, motility, and morphology. A negative correlation was observed between miR-19a/b-3p expression and sperm count, motility, vitality, and morphology. A negative correlation was also observed between miR-19a/b-3p and its 51 target genes. To validate these observations at the protein level, Western blot analysis was performed on sperm samples from men with oligoasthenozoospermia and normozoospermia. I identified reduced protein levels of two genes (STK33 and DNAI1). These findings indicate the potential involvement of miR-19a/b-3p and their target genes in maintaining normal sperm function and suggest a regulatory role of these miRNAs in controlling the expression levels of their target genes in the context of oligoasthenozoospermia.

I analyzed the sperm proteome of men with normozoospermia, oligoasthenozoospermia, and asthenozoospermia, which is characterized by reduced sperm count. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS), a total of 4412 proteins were identified. Among these, 1336 proteins were found to be present in at least 70% of the analyzed sperm samples. To analyze the differential expression of sperm proteins, I compared the proteome of sperm from subfertile men, i.e., men with oligoasthenozoospermia and asthenozoospermia, with sperm from men with normozoospermia. The results exhibited reduced expression of 32 proteins and increased expression of 34 proteins in sperm from subfertile men compared to men with normozoospermia. In the case of asthenozoospermia, 95 proteins demonstrated lower expression, whereas 86 proteins displayed higher expression. Sperm samples from men with oligoasthenozoospermia showed lower expression in eight

proteins and higher expression in one protein. To investigate the relationship between protein levels and sperm motility and count, I performed a correlation analysis. The results showed that 13 proteins exhibited a negative correlation, while 37 proteins displayed a positive correlation with sperm count. Similarly, 35 proteins showed a negative correlation, and 20 proteins displayed a positive correlation with sperm motility. Moreover, I identified combinations of proteins that could potentially serve as biomarkers for generally predicting male subfertility or specifically diagnosing asthenozoospermia and oligoasthenozoospermia. Additionally, I identified proteins that potentially differentiate between astheno- and oligoasthenozoospermia, providing further insights into the distinct molecular profiles associated with these conditions.

In conclusion, my research shows the association between miR-23a-3p, miR-23b-3p, miR-19a-3p, and miR-19b-3p and their respective target genes in oligoasthenozoospermia. Additionally, a proteomic analysis identified altered protein expression patterns associated with subfertility and specific subtypes of male infertility. The correlation analysis demonstrated the impact of these miRNAs, target genes, and specific proteins on semen parameters, particularly sperm count, motility, and morphology. The findings contribute to a better understanding of the molecular mechanisms underlying male infertility and may help in the development of diagnostic and therapeutic strategies.

2. EINLEITUNG

2.1. Infertilität – Definition und Epidemiologie

Infertilität ist laut Weltgesundheitsorganisation (engl. World Health Organization, WHO) durch die Unfähigkeit definiert, innerhalb eines Jahres, trotz regelmäßigem Geschlechtsverkehrs und ohne die Anwendung von Empfängnisverhütungsmitteln, Nachwuchs zu empfangen (WORLD HEALTH ORGANIZATION, 2022, 2023). Bei aktivem Kinderwunsch erreichen ungefähr 75 bis 80 % der Paare im reproduktiven Alter in weniger als sechs Monaten eine Empfängnis (GNOTH et al., 2003; SCHLEGEL et al., 2020). Insgesamt 85 % der Paare empfangen innerhalb eines Jahres und 90 % der Paare nach Ablauf von mindestens zwei Jahren Nachwuchs (SCHLEGEL et al., 2020). Demnach ist weltweit jedes sechste Paar im reproduktiven Alter von einem unerfüllten Kinderwunsch betroffen (WORLD HEALTH ORGANIZATION, 2023). Ursachen für die ungewollte Kinderlosigkeit können auf Seiten des Mannes, der Frau, oder beider Partner begründet liegen (AGARWAL et al., 2015; SHARLIP et al., 2002). In circa 50 % aller Fälle tragen Faktoren auf Seiten des Mannes zur Infertilität des Paares bei, wovon in 20 bis 30 % der Fälle allein der männliche Faktor der Infertilität zugrunde liegt (AGARWAL et al., 2015).

2.2. Männliche Infertilität

2.2.1. Definition, Ätiologie und Diagnostik

Männliche Infertilität geht in den meisten Fällen mit Problemen der Ejakulation, der Abwesenheit oder Einschränkung funktioneller Eigenschaften der Spermien, und/oder morphologischen Veränderungen der Spermien einher (WORLD HEALTH ORGANIZATION, 2022, 2023). Die Ätiologie der männlichen Infertilität ist vielfältig. Die Ursachen für männliche Infertilität sind angeboren oder können erworben werden. Urologische und genetische Anomalien, Störungen der Hormonsynthese, aber auch Folgeerscheinungen von Erkrankungen und Therapien, einschließlich chirurgischer Eingriffe, sowie immunologische Veränderungen können zur männlichen Infertilität führen (ROWE, WORLD HEALTH ORGANIZATION, 2000; SALONIA et al., 2023). Aufgrund des breiten Spektrums der Ursachen, muss die Beurteilung der männlichen Fertilität auf unterschiedlichen Ebenen erfolgen. Die Routinediagnostik bei männlicher Infertilität wird u.a. in den Leitlinien der European Association of Urology (EAU) und der American Society for Reproductive Medicine (ASRM) zusammengefasst (PRACTICE COMMITTEE OF THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023). Neben der Feststellung der individuellen Reproduktions- und Krankheitsgeschichte, sowie einer anatomischen Untersuchung, wird eine Analyse des Ejakulates durchgeführt (PRACTICE COMMITTEE OF

THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023). Eine endokrinologische Untersuchung des Blutes und eine genetische Analyse kann bei entsprechender Indikation zusätzlich vorgenommen werden (POZZI et al., 2023; PRACTICE COMMITTEE OF THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023; WORLD HEALTH ORGANIZATION, 2021).

2.2.2. Analyse des Ejakulates und Spermiogramm

Parameter des Ejakulates und der enthaltenen Spermien werden idealerweise nach zwei bis sieben Tagen sexueller Abstinenz analysiert, quantifiziert und im Spermiogramm zusammengefasst (WORLD HEALTH ORGANIZATION, 2021). Zu den Basisparametern des Ejakulates zählen Ejakulatvolumen, pH-Wert, die Spermienkonzentration, die sich daraus ergebende Gesamtzahl der Spermien, sowie die prozentuale Angabe zur Motilität, Vitalität und veränderten Morphologie der Spermien (WORLD HEALTH ORGANIZATION, 2021). Bei der Motilität wird zwischen der Gesamtmotilität und der progressiven Motilität unterschieden. Ein Spermium mit linearer Vorwärtsbewegung gilt als progressiv motil. Nicht-progressiv motile Spermien zeigen eine aktive Bewegung des Spermienschwanzes, bewegen sich jedoch nicht linear oder mit geringer Geschwindigkeit. Die Gesamtmotilität ergibt sich aus progressiver und nicht-progressiver Motilität der Spermien (WORLD HEALTH ORGANIZATION, 2021). Die Quantifizierung von morphologisch „normal“-aussehenden Spermien wird mittels Lichtmikroskopie erreicht (WORLD HEALTH ORGANIZATION, 2021).

Die WHO liefert Leitlinien zur Untersuchung und Prozessierung von humanem Sperma, die regelmäßig reevaluiert und fortlaufend aktualisiert werden. Aktuell gelten die unteren Referenzwerte zur Beurteilung der Samenparameter der WHO aus dem Jahr 2021 (CAMPBELL et al., 2021; WORLD HEALTH ORGANIZATION, 2021). Die unteren Referenzwerte, die in den Publikationen meiner Arbeit berücksichtigt wurden, wurden den vorangegangenen Leitlinien der WHO aus dem Jahr 2010 entnommen (COOPER et al., 2010; WORLD HEALTH ORGANIZATION, 2010). Die unteren Referenzwerte zur Beurteilung der Samenparameter der Jahre 2010 und 2021 sind in **Tabelle 1** aufgelistet.

Tabelle 1: Untere Referenzwerte zur Beurteilung der Samenparameter im Spermiogramm festgelegt durch das Laborhandbuch der WHO zur Untersuchung und Prozessierung von humanem Sperma aus dem Jahr 2010 und 2021.

Samenparameter	Untere Referenzwerte 2010 (95 % Konfidenzintervall)	Unterer Referenzwerte 2021 (95 % Konfidenzintervall)
Ejakulatvolumen	1,5 (1,4 – 1,7) ml	1,4 (1,3 – 1,5) ml
pH-Wert	> 7,2	> 7,2
Gesamtzahl der Spermien	39 (33 – 46) × 10^6	39 (35 – 40) × 10^6
Spermienkonzentration	15 (12 – 16) × 10^6/ml	16 (15 – 18) × 10^6/ml
Gesamtmotilität	40 (38 – 42) % motil	42 (40 – 43) % motil
Progressive Motilität	32 (31 – 34) % progressiv motil	30 (29 – 31) % progressiv motil
Vitalität	58 (55 – 63) % vital	54 (50 – 56) % vital
Morphologie	4 (3.0 – 4.0) % morphologisch normal	4 (3,9 – 4,0) % morphologisch normal

Festgelegt anhand des unteren fünften Perzentils und dessen 95 % Konfidenzintervall nach (CAMPBELL et al., 2021; COOPER et al., 2010; WORLD HEALTH ORGANIZATION, 2010, 2021).

2.2.2.1. Infertilitäts-assoziierte Anomalien auf Basis des Spermiogramms

Liegen die Samenparameter des Spermiogramms oberhalb der Referenzwerte, spricht man von Normozoospermie. Auf Grundlage des Spermiogramms unter Berücksichtigung der unteren Referenzwerte zur Beurteilung der Samenparameter aus dem Jahr 2010 können folgende grundlegende Anomalien unterschieden werden: Oligozoospermie, Asthenozoospermie und Teratozoospermie. Oligozoospermie ist definiert durch eine Spermienkonzentration von weniger als 15 Millionen Spermien pro Milliliter Ejakulat. Hierbei kann zusätzlich zwischen Azoospermie, dem vollständigen Fehlen von Spermien im Ejakulat, und Kryptozoospermie, einer sehr geringen Spermienkonzentration, die erst nach Konzentration des Ejakulates nachweisbar ist, unterschieden werden. Asthenozoospermie zeichnet sich durch eine reduzierte Spermienmotilität von weniger als 32 % progressiv motiler Spermien im Ejakulat aus. Teratozoospermie liegt vor, wenn weniger als 4 % morphologisch „normal“-aussehende Spermien im Ejakulat vorhanden sind (COOPER et al., 2010; WORLD HEALTH ORGANIZATION, 2010). Mischformen dieser Anomalien, wie Oligoasthenozoospermie (OA) und Oligoasthenoteratozoospermie (OAT), sind weit verbreitet (WORLD HEALTH ORGANIZATION, 2010). Die Feststellung dieser Anomalien ist jedoch kein eindeutiger Indikator für die männliche Infertilität, sondern deutet auf eine mögliche

Einschränkung der Fertilität des Mannes hin (SALONIA et al., 2023; WORLD HEALTH ORGANIZATION, 2010). Diese wird auch als Subfertilität bezeichnet.

2.2.3. Idiopathische männliche Infertilität und ungeklärte Infertilität

Bei 30 bis 40 % der Männer mit Samenparameter unterhalb der Referenzwerte lässt sich keine Ursache für die männliche Infertilität bestimmen (OLESEN et al., 2017; SALONIA et al., 2023). Dieses Phänomen wird idiopathische männliche Infertilität genannt. Ursachen für schwere Beeinträchtigungen der Spermienbildung, wie Aspermie, das vollständige Fehlen des Ejakulates, Azoospermie, und Kryptozoospermie sind besser verstanden als Ursachen für Oligozoospermie, die in ungefähr 75 % der Fälle idiopathisch bleibt (PUNAB et al., 2017). Die ungeklärte Infertilität ist von der idiopathischen männlichen Infertilität abzugrenzen und liegt vor, wenn weder auf Seiten des Mannes noch der Frau eine Ursache für die diagnostizierte Infertilität festgestellt werden kann und demnach auch die Samenparameter des Mannes oberhalb der Referenzwerte liegen. Ungeklärte Infertilität betrifft insgesamt 20 bis 30 % der Paare mit diagnostizierter Infertilität (SALONIA et al., 2023).

2.2.4. Schwächen der bisherigen Diagnostik und Notwendigkeit der Ursachenforschung bei männlicher Infertilität

Der hohe Anteil der ungeklärten Infertilität von Paaren und der idiopathischen männlichen Infertilität deutet darauf hin, dass die bisherige Routinediagnostik unzureichend zur adäquaten Erfassung der Ursachen von männlicher Infertilität ist (CALOGERO et al., 2023; PANDRUVADA et al., 2021; TUTTELMANN et al., 2018). Vor allem im genetischen Bereich sind die diagnostischen Tests begrenzt. Patienten werden routinemäßig bei entsprechender Indikation auf chromosomale Anomalien, wie beispielsweise spezifische Mikrodeletionen in der Azoospermiefaktor (AZF) – Region des Y-Chromosoms und auf wenige Infertilitäts-assoziierte Genmutationen, wie beispielsweise im Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-Gen, getestet (PRACTICE COMMITTEE OF THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023). Die Routinediagnostik bei männlicher Infertilität, hat sich über 20 Jahre hinweg nur marginal verändert (TUTTELMANN et al., 2018). Währenddessen hat sich das Methodenspektrum der genetischen Forschung im Allgemeinen und in der männlichen Reproduktionsmedizin deutlich erweitert. Die aktuelle Forschung fokussiert sich zunehmend auf spezifische Gene, die mit Infertilität assoziiert sind. So beschäftigen sich im Jahr 2020 circa 50 % aller genetischen Studien im Bereich der männlichen Infertilität mit spezifischen Genen. Somit steigt auch die Anzahl der jährlich „neu“-identifizierten Infertilitäts-assoziierten Gene stetig an (HOUSTON et al., 2021). Spezifische Gene könnten sich als Kandidaten zur Diagnose männlicher Infertilität

eignen und werden bereits in der Diagnostik vereinzelt angewendet (BIENIEK et al., 2016; TUTTELMANN et al., 2018). Die Leitlinien der WHO zur Untersuchung und Prozessierung von humanem Sperma wurden zwar im Jahre 2021 im Bereich der genetischen Diagnostik durch die DNA-Fragmentierungsrate der Spermien ergänzt, jedoch fanden bisher keine weiteren Tests spezifischer infertilitäts-assoziierter Gene weder Eingang in die Leitlinien der WHO, der EAU oder der ASRM (CALOGERO et al., 2023; PRACTICE COMMITTEE OF THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023; WORLD HEALTH ORGANIZATION, 2021).

Ursachen für die marginalen Fortschritte der Routinediagnostik und der Ursachenforschung bei männlicher Infertilität in den letzten Jahrzehnten könnten mit der Entwicklung der assistierten Reproduktionstechnik (ART), vor allem die der intrazytoplasmatischen Spermieninjektion (engl. Intracytoplasmic sperm injection, ICSI) in den 90er Jahren, zusammenhängen (AITKEN, 2018). Die ART umfasst Verfahren in denen Spermien und Eizellen bzw. Embryos genutzt werden, um die diagnostizierte Infertilität eines Paares zu überwinden und eine Schwangerschaft zu erzielen (ZEGERS-HOCHSCHILD et al., 2009). Im Rahmen der Behandlung der Infertilität von Paaren werden Methoden der ART weltweit und alltäglich angewendet (CHAMBERS et al., 2021). Im Jahr 2021 wurden 41% aller ICSI- und In-Vitro Fertilisation (IVF) - Behandlungen in Deutschland aufgrund einer Indikation allein auf Seiten des Mannes durchgeführt (DEUTSCHES IVF REGISTER, 2022). Der männliche Fertilitätsstatus bleibt bei der Anwendung der ART jedoch unverändert. Zugleich beschränken sich die aktuellen Therapiemöglichkeiten bei männlicher Infertilität hauptsächlich auf die Umstellung des Lebensstils, Hormontherapien und operative Eingriffe zur Behebung obstruktiver Anomalien (SALONIA et al., 2023).

Trotz der Erfolge der ART bringt sie, neben der physischen und psychischen Belastung des betreffenden Paares, auch Risiken für den Nachwuchs mit sich, da funktionelle und morphologische Defekte der Spermien, die eine natürliche Empfängnis verhindern oder erschweren, umgangen werden (CAIRO CONSENSUS WORKSHOP GROUP, 2020; CALOGERO et al., 2023; TURNER et al., 2020). Angeborenen Anomalien, wie beispielsweise angeborene Herzfehler und urogenitale Anomalien der Nachkommen, die mit Hilfe von ART empfangen wurden, könnten mit der zugrundeliegenden männlichen Infertilität zusammenhängen (JWA et al., 2019; MASSARO et al., 2015; TARARBIT et al., 2013).

Die Verbesserung der Diagnose, der Aufklärung der Ursachen und der Entwicklung neuer Therapien männlicher Infertilität würde zum einen den gezielteren Einsatz der ART ermöglichen und zum anderen die Anwendung der ART obsolet machen.

Aufgrund der aufgeführten Problematiken wird seit Jahren in unzähligen Studien die Ausweitung der Routinediagnostik und die Notwendigkeit der Ursachenforschung männlicher Infertilität thematisiert und diskutiert (BOITRELLE et al., 2021; CAIRO CONSENSUS

WORKSHOP GROUP, 2020; CALOGERO et al., 2023; PANDRUVADA et al., 2021; TURNER et al., 2020; TUTTELMANN et al., 2018).

2.3. Die Rolle der Genetik in der männlichen Reproduktion

Die männliche Reproduktion ist komplex. So werden beispielsweise 77 % aller Protein-kodierenden Gene im Testis exprimiert und mindestens 2300 Gene sind an der Spermatogenese beteiligt (HOTALING, CARRELL, 2014; UHLEN et al., 2015).

Neue „Omics"-Technologien können unser Verständnis für die Physiologie und Pathologie der männlichen Reproduktion drastisch verbessern, indem sie die Betrachtung der Gesamtheit des Genoms, Transkriptoms, Proteoms, sowie des Epigenoms und des Metaboloms zulassen. In meiner Arbeit betrachte ich hauptsächlich Veränderungen auf Transkriptom- und Proteomebene in Spermien. Diesbezüglich werden grundlegende Zusammenhänge und bisherige Erkenntnisse in den folgenden Abschnitten zusammengefasst.

Ich möchte an dieser Stelle darauf hinweisen, dass mit den Begriffen miRNA-Expression, mRNA-Expression und Protein-Expression im Rahmen meiner Arbeit keine Transkriptions- bzw. Translationsprozesse impliziert werden. Vielmehr beziehen sich die Begriffe miRNA-Expression, mRNA-Expression und Protein-Expression auf die zu einem Zeitpunkt gemessene Menge an miRNA, mRNA bzw. Protein. Hierbei handelt es sich nicht um Angaben zu absoluten Mengen, sondern um Angaben zu relativen Mengen.

2.3.1. Humanes Spermien Transkriptom

2.3.1.1. Transkriptionelle Aktivität in Spermien

Die Transkription in Spermien ist nahezu inaktiviert (GRUNEWALD et al., 2005; KIERSZENBAUM, TRES, 1975; REN et al., 2017). Deren Stilllegung erfolgt während der Spermatogenese, dem Prozess in dem die Spermien in den Samenkanälchen des Hodens gebildet werden (HECHT, 1998). Die Spermatogenese unterteilt sich in drei Phasen. Die erste Phase ist die Proliferation bei der mittels mitotischer Zellteilung aus Keimzellstammzellen, den Spermatogonien, diploide primäre Spermatozyten entstehen. In der zweiten Phase werden durch meiotische Zellteilung erst haploide, sekundäre Spermatozyten und im Anschluss runde Spermatiden gebildet. In der dritten Phase, der post-meiotischen Spermiogenese, differenzieren sich die Spermatiden zu Spermatozoen aus, die aus Kopf, Mittelstück und Spermienschwanz aufgebaut sind (DE KRETSER et al., 1998). Im Verlauf dieser dritten Phase wird das Zytoplasma der Zellen reduziert und das Spermien Genom kondensiert, sodass die Transkription schrittweise heruntergefahren wird (DE KRETSER et al., 1998; REN et al.,

2017). Während in Spermatozyten und Spermatiden noch eine RNA-Synthese stattfindet, konnte in reifen ejakulierten Spermien keine Transkription nachgewiesen werden (DADOUNE et al., 2004; GEREMIA et al., 1978; GRUNEWALD et al., 2005; HAMATANI, 2012; MONESI, 1965). Trotz der weitgehend inaktiven Transkription enthält jedes einzelne Spermium zwischen 10 – 100 Femtogramm RNA (KRAWETZ, 2005; PESSOT et al., 1989). Spermien enthalten eine komplexe Zusammensetzung aus kodierenden Boten-RNAs (engl. messenger RNA, mRNA), und nicht-kodierenden RNAs (engl. non-coding RNA, ncRNA) (HAMATANI, 2012; KRAWETZ et al., 2011; MILLER et al., 1999; OSTERMEIER et al., 2002).

2.3.1.2. Kodierende Messenger RNAs in Spermien

Viele der mRNAs in ejakulierten Spermien sind testikulären Ursprungs und verbleiben untranslatiert im Spermium (HAMATANI, 2012). Jedoch geschieht diese Retention nicht zufällig, stattdessen werden mRNAs selektiv im sich entwickelnden Spermium zurückgehalten (HAMATANI, 2012; MILLER et al., 1999; MILLER, OSTERMEIER, 2006). Nach abgeschlossener Spermatogenese sind Spermien funktionell inaktiv und reifen erst durch die Anwesenheit von extrazellulären Vesikeln, RNAs, Proteinen, Ionen und Nährstoffe während des Transits durch den Epididymis weiter heran, wodurch sie motil werden und die Fähigkeit zur Fertilisation erlangen (SANTIAGO et al., 2021; SULLIVAN, 2016). Einige mRNAs scheinen dabei durch extrazelluläre Vesikel in das ejakulierte Spermium zu gelangen (JODAR et al., 2016; SANTIAGO et al., 2021).

In Spermien enthaltene mRNAs verschiedenen Ursprungs können an spezifischen Prozessen der Reproduktion beteiligt sein. Dazu zählen die post-testikuläre Spermienreifung im Epididymis, die Motilität, der Reifeprozess der Spermien im weiblichen Genitaltrakt, genannt Kapazitation, die Fertilisation und die frühe Entwicklung des Embryos (HERNANDEZ-SILVA et al., 2022; KRAWETZ, 2005; LAMBARD et al., 2004; OSTERMEIER et al., 2004; SILVA et al., 2022; VALLET-BUISAN et al., 2023). Die differentielle Expression einzelner oder mehrerer mRNAs in Spermien im Zusammenhang mit männlicher Infertilität und Subfertilität wurde in den letzten Jahrzehnten vielfach untersucht (SANTIAGO et al., 2021). Zudem wurde das Spermien Transkriptom in infertilen und fertilen Männern untersucht und insgesamt wurden mehr als 60.000 Transkripte identifiziert. Mehr als 11.000 Transkripte waren dabei in Spermien von infertilen Männern im Vergleich zu fertilen Männern differentiell exprimiert (JOSHI et al., 2022a). Einige dieser in Spermien identifizierten mRNAs wurden als potentielle Biomarker identifiziert und könnten sich einzeln oder in Kombination als potentielle Kandidaten zur Diagnose von männlicher Infertilität, besonders der idiopathischen Infertilität, eignen (HERNANDEZ-SILVA et al., 2022).

2.3.1.3. Nicht-kodierende RNAs in Spermien

Neben den kodierenden RNAs enthalten Spermien auch lange, nicht-kodierende RNAs (engl. long non-coding RNAs, lncRNAs) und kurze, nicht-kodierende RNAs (engl. small non-coding RNAs, sncRNAs) (HAMATANI, 2012; SANTIAGO et al., 2021). Zu den in Spermien identifizierten sncRNAs zählen mikroRNAs (miRNAs), piwi-interacting RNAs (piRNAs), repeat-associated small interfering RNAs (rasiRNAs), small nucleolar RNA (snoRNAs), small nuclear RNAs (snRNAs) und kurze RNAs in der Transkriptionsstart/Promotor Region. MiRNAs sind dabei mit 6,9 %, piRNAs mit 16,9 %, rasiRNAs mit 65 %, snoRNAs mit 0,3 %, snRNAs mit 0,1 % und kurze RNAs in der Transkriptionsstart/Promotor Region mit 10,8 % in Spermien vertreten (KRAWETZ et al., 2011). Die am besten charakterisierte Klasse der sncRNAs sind miRNAs. Diese sind an der männlichen Reproduktion und der Entstehung männlicher Infertilität beteiligt (DANESHMANDPOUR et al., 2020; KHAWAR et al., 2019; PAGES et al., 2018; SANTIAGO et al., 2021).

2.3.1.4. MikroRNA Biogenese und post-transkriptionelle Regulation

Die Biogenese kanonischer miRNAs beginnt mit der Transkription im Zellkern durch die RNA-Polymerase II von miRNA-kodierenden Genen, die entweder intergenisch, d.h. zwischen einzelnen Genen, oder intragenisch, d.h. innerhalb von Genen, lokalisiert sind (LEE et al., 2002; LEE et al., 2004; WANG, 2010). Es entsteht eine primäre miRNA (engl. primary-microRNA, pri-miRNA), die aufgrund komplementär angeordneter Sequenzmotive auf demselben Strang eine Haarnadel-Struktur ausbildet (CAI et al., 2004; LEE et al., 2002). Eine Endonuklease mit zwei RNAse III Domänen, Drosha genannt, sowie zwei Moleküle eines Partnerproteins bilden den Mikroprozessor, der anschließend das offene Ende der Haarnadel-Struktur mit einem 2-Nukleotid großen Überhang einkürzt (LEE et al., 2003; NGUYEN et al., 2015). Die entstandene Vorläufer-miRNA (engl. precursor microRNA, pre-miRNA) im Zellkern ist circa 70 Nukleotide lang und wird von Exportin-5 und Ras-related nuclear protein - Guanosintriphosphat (RAN-GTP) ins Zytoplasma transportiert (LEE et al., 2003; LUND et al., 2004; YI et al., 2003). Dort trennt eine Endonuklease mit RNAse III Domäne, genannt Dicer, die Schleife der Haarnadel mit einem zwei Nukleotid großen Überhang ab (BERNSTEIN et al., 2001; ZHANG et al., 2004). Die entstandene doppelsträngige RNA, der miRNA-Duplex, besteht aus der circa 22 Nukleotid langen reifen miRNA und einen passenger-Strang, der degradiert wird (BARTEL, 2018; LEE et al., 2003; MATRANGA et al., 2005; ZHANG et al., 2004). Die reife miRNA wird von Argonauten (AGO) – Proteinen, meist AGO2, rekrutiert und in den RNA-induced silencing complex (RISC) eingelagert, mit dessen Hilfe die miRNA ihr Zieltranskript findet (LEUSCHNER et al., 2006; MATRANGA et al., 2005). Zielgene werden in der Regel durch komplementäre Basenpaarung der Seed-Region der miRNA, die sich an der

Nukleotidposition zwei bis sieben am 5‘-Ende der miRNA befindet, an der basenkomplementären Bindestelle im Bereich der 3’ untranslatierten Region (3’UTR) des Zieltranskriptes gebunden (BARTEL, 2009, 2018). Die Bindung der miRNA am Zieltranskript verhindert die Proteinexpression durch die Degradation der mRNA selbst oder durch die Inhibierung der Translationsmaschinerie (BARTEL, 2009). Die meisten Protein-kodierenden Gene werden durch miRNAs reguliert und damit besitzen miRNAs ein enormes regulatorisches Potential (BARTEL, 2018; FRIEDMAN et al., 2009). MiRNAs werden, ähnlich zu mRNAs, gewebespezifisch exprimiert und variieren in Entwicklungsprozessen (IVEY, SRIVASTAVA, 2015; LUDWIG et al., 2016).

2.3.1.5. MikroRNA Zielgenidentifizierung und -validierung

Ein besseres Verständnis der regulatorischen Netzwerke der miRNAs und ihrer Zielgene ist essentiell und wird bedingt durch die Aufklärung der miRNA-Zielgen-Interaktionen. Bioinformatische Algorithmen sind in der Lage miRNA-Zielgen-Interaktionen vorherzusagen und basieren vor allem auf Charakteristika der miRNA Seed-Sequenz, Charakteristika der Bindestelle innerhalb der mRNA des Zielgens und bekannten miRNA-Zielgen-Interaktionen (RIOLO et al., 2020). Circa 100 bioinformatische Online-Anwendungen sind zur Vorhersage von miRNA-Zielgen-Interaktionen verfügbar (KERN et al., 2020). Durch die Unterschiede in den zugrundeliegenden bioinformatischen Algorithmen variieren die vorhergesagten Zielgene je nach Anwendung zum Teil stark (RIFFO-CAMPOS et al., 2016). Potentielle Zielgene sollten daher mittels direkter und/oder indirekter Methoden experimentell validiert werden (HUANG et al., 2022; RIFFO-CAMPOS et al., 2016). Direkte Methoden, wie Reportergen-Assays, betrachten die direkte Basenpaarbindung der miRNA-Seed-Region zur Bindestelle in der 3’UTR des Zieltranskripts durch den Effekt auf den Reporter. Indirekte Methoden stellen inverse Korrelationen von miRNA, deren Zieltranskripte bzw. deren Zielproteine her. Dazu zählen beispielsweise Echtzeit quantitative – Polymerase - Kettenreaktion (engl. real-time quantitative polymerase chain reaction, RT-qPCR) und Western Blot (HUANG et al., 2022).

Die Entwicklung von Hochdurchsatzverfahren, wie beispielsweise Next-Generation-Sequencing und Massenspektrometrie, hat die Effizienz der Validierung von miRNA-Zielgen-Interaktionen verbessert. Jedoch ersetzen sie nicht gänzlich die klassischen Methoden der experimentellen Validierung und dienen eher als Bindeglied zwischen bioinformatischer Vorhersage und klassischer Validierungsmethoden, wie Reportergen-Assay, RT-qPCR und Western Blot (KUHN et al., 2008; RIOLO et al., 2020). Technische Entwicklungen haben jedoch auch den Durchsatz dieser klassischen Methoden deutlich erhöht. Hochdurchsatz RT-qPCR mittels Mikrofluidik Technologie ermöglicht beispielsweise die parallele Analyse einer Vielzahl von Zielgenen und Proben mit sehr geringen Volumina (ABU-HALIMA et al.,

2022). Auch Reportergen-Assays können automatisiert werden und standardisiert mehrere direkte miRNA-Zielgen-Interaktionen parallel validieren (KERN et al., 2021).

Bis heute wurden ungefähr 2300 humane reife miRNAs identifiziert (ALLES et al., 2019), die mehr als 60 % aller Protein-kodierender Gene regulieren können (FRIEDMAN et al., 2009). Die Datenbank für experimentell validierte miRNAs, miRTarBase, listet für den Menschen mehr als 17.000 Zielgene und mehr als vier Millionen miRNA-Zielgen-Interaktionen, von denen bisher nur ein geringer Anteil mittels klassischer Methoden experimentell validiert wurde (HUANG et al., 2022).

2.3.1.6. Die Rolle der MikroRNAs in der männlichen Reproduktion

MiRNAs sind an der männlichen Reproduktion beteiligt und wurden in ejakulierten Spermien, in Seminalplasma, in testikulären Gewebe, sowohl in somatischen Zellen als auch in sich differenzierenden Keimzellen, sowie im Epididymis identifiziert (KOTAJA, 2014; PRATT, CALCATERA, 2016; SALAS-HUETOS et al., 2020; VASHISHT, GAHLAY, 2020). Akteure der miRNA-Biogenese in Keim- und somatischen Zellen des Testis sind essentiell für die Spermatogenese (KOTAJA, 2014). Ein Fehlen von Drosha oder Dicer in diesen Zellen führt zur Beeinträchtigung der Spermatogenese und meistens zur Infertilität (HAYASHI et al., 2008; KIM et al., 2010; KORHONEN et al., 2011; MAATOUK et al., 2008; PAPAIOANNOU et al., 2011; PAPAIOANNOU et al., 2009; ROMERO et al., 2011; WU et al., 2012a; ZIMMERMANN et al., 2014). In der Spermatogenese werden miRNAs, ähnlich wie kodierende RNAs, phasen-spezifisch exprimiert und sind essentielle Regulatoren bei der Spermatogonien Selbsterneuerung und deren Differenzierung zu reifen Spermien (KOTAJA, 2014; WALKER, 2022). Die meisten miRNAs werden in späten meiotischen Keimzellen und haploiden Keimzellen, d.h. Spermatozyten und Spermatiden, produziert (KOTAJA, 2014; RO et al., 2007). Einige miRNAs und deren Zielgene sind funktional mit der Spermatogenese assoziiert (WALKER, 2022).

Beim Transit der Spermien durch das männliche Genitalsystem kommen die Spermien mit extrazellulären Vesikeln, wie Epididymosomen, Prostasomen und Liposomen, in Kontakt (SULLIVAN, SAEZ, 2013). Epididymosomen, beispielsweise, enthalten miRNAs, die sich je nach Region des Epididymis in ihrem miRNA-Expressionsprofil unterscheiden (ALVES et al., 2020; BELLEANNEE et al., 2013; REILLY et al., 2016; SHARMA et al., 2018). Die Epididymosomen verschmelzen mit der Membran der Spermien und verändern das miRNA-Expressionsprofil der Spermien entlang des Transits durch den Epididymis (ALVES et al., 2020; ZHOU et al., 2019). Ein Knock-out der Akteure der miRNA-Biogenese in spezifischen Zellen des Epididymis resultiert in einer Deregulation des Differenzierungsprozesses der epithelialen Zellen, wodurch die Spermienreifung und folglich die Fertilität beeinträchtigt wird (ALVES et al., 2020; BJORKGREN et al., 2012).

Aus Spermien-stammende miRNAs sind an der Fertilisation der Eizelle und an der embryonalen Entwicklung beteiligt (YUAN et al., 2016). MiRNA-defiziente Spermien von Mäusen weisen nach ICSI ein verringertes Potential zur embryonalen Differenzierung auf, welches durch die Gabe von wildtypischen Spermien-RNA wieder ausgeglichen werden konnte (YUAN et al., 2016). Jedoch ist die Beteiligung einzelner spezifischer miRNAs an der Fertilisation und der embryonalen Entwicklung wenig erforscht und wird zum Teil kontrovers diskutiert (LIU et al., 2012; YUAN et al., 2015).

Neben ihrer physiologischen Rolle in den oben genannten Prozessen ist die Deregulation von einzelnen miRNAs, miRNA-Familien und miRNA-Clustern mit männlicher Infertilität assoziiert (ABU-HALIMA et al., 2021; KHAWAR et al., 2019). Die differentielle Expression von miRNAs in Spermien von Männern mit verschiedenen Infertilitäts-assoziierten Anomalien und beeinträchtigter Spermatogenese wurde vielfach untersucht (ABU-HALIMA et al., 2021; ABU-HALIMA et al., 2013; BARBU et al., 2021; DANESHMANDPOUR et al., 2020; VASHISHT, GAHLAY, 2020). Die Expressionslevel einiger miRNAs sind mit Samenparametern, wie Spermienanzahl, -motilität und -morphologie korreliert (ABU-HALIMA et al., 2021; DANESHMANDPOUR et al., 2020). Einzelne miRNAs und Kombinationen aus verschiedenen miRNAs wurden als potentielle Biomarker zur Diagnose von männlicher Infertilität bzw. von Infertilitäts-assoziierten Anomalien identifiziert und könnten sich zur Prognose einer erfolgreichen Empfängnis mittels ART eignen (ALVES et al., 2020; KHAWAR et al., 2019; VASHISHT, GAHLAY, 2020). Darüber hinaus könnten miRNAs zukünftig in der Therapie männlicher Infertilität angewendet werden (VASHISHT, GAHLAY, 2020). Konkrete Studien zum Einsatz von miRNAs als Therapeutikum bei männlicher Infertilität gibt es bisher nicht.

2.3.1.6.1. MiR-23a/b-3p und miR-19a/b-3p als potentielle Regulatoren in der männlichen Infertilität

Die miR-23 und miR-19 Familie, die zentraler Bestandteil meiner Arbeit sind, wurden bereits mit männlicher Infertilität in Zusammenhang gebracht. Die miR-23 Familie wurde mittels Mikroarray in Spermien, testikulärem Gewebe und Exosomen, die dem Seminalplasma entstammen, untersucht. Verglichen wurde hierbei die Expression der Mitglieder der miR-23 Familie bei Männern mit verschiedenen Infertilitäts-assoziierten Anomalien im Vergleich zu Männern mit Normozoospermie (ABU-HALIMA et al., 2014; ABU-HALIMA et al., 2013; ABU-HALIMA et al., 2016). Die Expression einzelner Mitglieder miR-23 Familie in Spermien von Männern mit Oligoasthenozoospermie und Asthenozoospermie ist erhöht (ABU-HALIMA et al., 2013). Die erhöhten Expressionslevel der miR-23a-3p und miR-23b-3p (miR-23a/b-3p) in Spermien von Männern mit Oligoasthenozoospermie wurden kürzlich mittels RT-qPCR validiert (ABU-HALIMA et al., 2019; ABU-HALIMA et al., 2021). Zudem wurden erste

Spermatogenese-assoziierte Zielgene der miR-23a/b-3p und deren miRNA-Zielgen Bindung untersucht. Dabei wurden *PFKFB4*, *HMMR*, *SPATA6*, *TEX15, ODF2 und UBQLN3* als direkte Zielgene der miR-23a/b-3p identifiziert und validiert (ABU-HALIMA et al., 2019; ABU-HALIMA et al., 2021).

Auch Mitglieder der miR-19 Familie sind in Spermien, testikulärem Gewebe und Seminalplasma von Männern mit Infertilitäts-assoziierten Anomalien verglichen mit Männern mit Normozoospermie differentiell exprimiert (ABU-HALIMA et al., 2014; ABU-HALIMA et al., 2013; ABU-HALIMA et al., 2020; WU et al., 2012b). Sie sind im miRNA-Cluster 17-92 im Genom lokalisiert, das als essentieller Regulator an der Spermatogenese beteiligt ist (TONG et al., 2012; XIE et al., 2016). Außerdem ist die miR-19b-3p in Kulturmedium, das nach Embryonen Transfer im Rahmen einer IVF oder ICSI zurückbleibt, erhöht exprimiert und könnte sich als prognostischer Biomarker für den Erfolg der Einnistung des Embryos nach IVF bzw. ICSI eignen (ABU-HALIMA et al., 2020; CIMADOMO et al., 2019; MCCALLIE et al., 2010; ZHOU, DIMITRIADIS, 2020).

2.3.2. Humanes Spermien Proteom

Ähnlich wie die Transkription ist auch die Translation in Spermien nahezu inaktiv. Erst während der Kapazitation wird die Translation weniger mRNAs wiederaufgenommen (GUR, BREITBART, 2006). Die meisten Spermien Proteine werden während der Spermatogenese synthetisiert (CASTILLO et al., 2018). Zusätzlich gelangen andere Proteine aus dem Seminalplasma in die Spermien, wie beispielsweise während des Transits durch den Epididymis mittels Epididymosomen (BAKER et al., 2012; CASTILLO et al., 2018; MARTIN-DELEON, 2015; SAEZ et al., 2003). Funktionell wirken Spermien Proteine an der vorangegangenen Spermatogenese und der Erhaltung der Spermien Funktionen, wie beispielsweise der Motilität, mit (AMARAL et al., 2014; CASTILLO et al., 2018). Andere Spermien Proteine sind an der Kapazitation beteiligt, spielen bei der Fertilisation der Eizelle eine Rolle oder werden zum Embryo transportiert und sind dort an der embryonalen Entwicklung beteiligt (CASTILLO et al., 2019; CASTILLO et al., 2018; SAUNDERS et al., 2002).

Bisher wurden insgesamt mehr als 6000 Proteine in Spermien in verschiedenen Studien identifiziert (CASTILLO et al., 2018). Nur wenige Studien hingegen betrachteten bisher jedoch das gesamte Spermien Proteom fertiler Männer mittels Hochdurchsatzverfahren, wie beispielsweise Massenspektrometrie (BAKER et al., 2007; JOHNSTON et al., 2005; LI et al., 2007; MARTINEZ-HEREDIA et al., 2006; WANG et al., 2013).

Männliche Infertilität bzw. Subfertilität und Veränderungen in den Samenparametern von Männern sind mit der aberranten Expression einzelner Proteine assoziiert (AGARWAL et al., 2020). Auch im Kontext Infertilitäts-assoziierter Anomalien wurden wenige Studien bisher

publiziert, deren Fokus auf das gesamte Spermien Proteom gerichtet ist (AGARWAL et al., 2016; LIANG et al., 2021; MARTINEZ-HEREDIA et al., 2008; SARASWAT et al., 2017; YANG et al., 2022; ZHAO et al., 2007). Noch weniger Studien beschreiben die differentielle Expression des Spermien Proteoms von Männern mit Oligoasthenozoospermie im Speziellen (AGARWAL et al., 2020; GREITHER et al., 2023).

Erkenntnisse dieser Studien können genutzt werden, um bisher unbekannte Ursachen der männlichen Infertilität bzw. Subfertilität aufzudecken und neue Biomarker zur Diagnose von männlicher Infertilität oder zur Vorhersage einer erfolgreichen Empfängnis mittels ART zu identifizieren. In der Vergangenheit wurden hierzu bereits in vielen Studien potentielle Biomarker-Kandidaten identifiziert (BUCKMAN et al., 2013; INTASQUI et al., 2018; ROSA et al., 2023; SCHIZA et al., 2014).

2.4. Zielsetzung

Die grundlegenden zellbiologischen Mechanismen der männlichen Fertilität sind bis heute wenig verstanden. In der vorliegenden Arbeit werden entsprechende Veränderungen auf der Transkriptomebene, auf der Ebene der post-transkriptionellen Regulation durch miRNAs und auf der Proteinebene bei männlicher Infertilität untersucht. Mit diesen Arbeiten soll neben einem verbesserten Grundlagenverständnis der zellulären Dysregulation in Spermien bei männlicher Infertilität auch ein Beitrag zur Entwicklung einer verbesserten Diagnostik der männlichen Infertilität geleistet werden.

1. Das erste Ziel meiner Arbeit war die Identifizierung von 16 *in-silico* vorhergesagten, Testis-exprimierten Zielgenen der Infertilitäts-assoziierten miR-23a-3p und miR-23b-3p, die Validierung der direkten miRNA-Zielgen-Interaktionen, sowie die Bestimmung der Expressionslevel von Zielgenen in Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit Normozoospermie.
2. Das zweite Ziel meiner Arbeit war die Bestimmung der Expressionslevel der miR-19a-3p und miR-19b-3p und die Identifizierung und Validierung von 82 *in-silico* vorhergesagten, Testis-exprimierten Zielgenen dieser miRNAs in Spermien von Männern mit Oligoasthenozoospermie, im Vergleich zu Männern mit Normozoospermie.
3. Das dritte Ziel meiner Arbeit war die Identifizierung des Proteoms in Spermien von Männern mit Subfertilität, d.h. spezifisch von Männern mit Oligoasthenozoospermie und Asthenozoospermie, sowie von Männern mit Normozoospermie. Aufbauend auf diesen Proteomanalysen sollten potentielle Biomarker für eine verbesserte Diagnose der Subfertilität und hierbei spezifisch der Oligoasthenozoospermie und der Asthenozoospermie bestimmt werden.

3. ERGEBNISSE

Diese kumulative Promotion basiert auf den folgenden drei Publikationen (ABU-HALIMA et al., 2022; BECKER et al., 2023a; BECKER et al., 2023b). Die in dieser Dissertation gebundenen Publikationen sind vollständig abgebildet und identisch zu den veröffentlichten Versionen durch die entsprechenden Verlage.

3.1. Towards a More Comprehensive Picture of the MicroRNA-23a/b-3p Impact on Impaired Male Fertility

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Towards a More Comprehensive Picture of the MicroRNA-23a/b-3p Impact on Impaired Male Fertility

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Simple Summary: This study focused on the relationship between microRNA-23a/b-3p and genes involved in human spermatogenesis, with the aim of understanding how this microRNA targets these genes and how it affects male fertility. Through computational prediction and experimental assays, we identified specific genes that are targeted by microRNA-23a/b-3p. By intentionally modifying the binding sites of microRNA-23a/b-3p in these genes, we confirmed the direct targeting. To validate their findings, we analyzed sperm samples from men with low sperm count and motility compared to a control group. The results revealed that the men with fertility issues had lower expression levels of the target genes. Furthermore, we found a positive correlation between basic semen parameters and the reduced expression levels of the target genes. This indicates that microRNA-23a/b-3p plays a significant role in spermatogenesis by regulating the expression of target genes associated with male fertility problems, and it also has an impact on basic semen parameters. Overall, this study provides important insights into the involvement of microRNA-23a/b-3p in spermatogenesis and its potential influence on male fertility.

Abstract: The expression levels of various genes involved in human spermatogenesis are influenced by microRNAs (miRNAs), specifically microRNA-23a/b-3p. While certain genes are essential for spermatogenesis and male germ cell function, the regulation of their expression remains unclear. This study aimed to investigate whether microRNA-23a/b-3p targets genes involved in spermatogenesis and the impact of this targeting on the expression levels of these genes in males with impaired fertility. In-silico prediction and dual-luciferase assays were used to determine the potential connections between microRNA-23a/b-3p overexpression and reduced expression levels of 16 target genes. Reverse transcription-quantitative PCR (RT-qPCR) was conducted on 41 oligoasthenozoospermic men receiving infertility treatment and 41 age-matched normozoospermic individuals to verify the lower expression level of target genes. By employing dual-luciferase assays, microRNA-23a-3p was found to directly target eight genes, namely *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, *G2E3*, *ZNF695*, *CEP41*, and *RGPD1*, while microRNA-23b-3p directly targeted three genes, namely SOX6, *GOLGA6C*, and *ZNF695*. The intentional alteration of the microRNA-23a/b binding site within the 3′ untranslated regions (3′UTRs) of the eight genes resulted in the loss of responsiveness to microRNA-23a/b-3p. This confirmed that *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41* are direct targets for microRNA-23a-3p, while *NOL4*, *SOX6*, and *PCDHA9* are direct targets for microRNA-23b-3p. The sperm samples of oligoasthenozoospermic men had lower expression levels of target genes than age-matched normozoospermic men. Correlation analysis indicated a positive correlation between basic semen parameters and lower expression levels of target genes. The study suggests that microRNA-23a/b-3p plays a significant role in spermatogenesis by controlling the expression of target genes linked to males with impaired fertility and has an impact on basic semen parameters.

Keywords: male subfertility; oligoasthenozoospermia; miRNA-target-interaction; expression level; microRNA-23a/b-3p

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1. Introduction

MicroRNAs (miRNAs) have become a prominent field of genetic research since their discovery, owing to their significant role in regulating gene expression post-transcriptionally. These small, non-coding RNAs, which are approximately 22 nucleotides in length, primarily function through complementarity with the 3′ untranslated region (3′UTR) of the target messenger RNA (mRNA) [1]. MiRNAs play a vital role in numerous cellular and molecular processes, including development, cell differentiation, and physiological homeostasis [2]. It is, therefore, not unexpected that the aberrant expression of certain miRNAs can disrupt critical processes and lead to various pathologies [3]. Moreover, miRNAs' disease-specific expression patterns make them suitable prognostic biomarkers [4]. As of today, 2300 mature human miRNAs have been validated [5], and more than 60% of protein-coding genes are predicted to contain target sites for miRNA binding [6]. In the case of humans, miRTarBase, a database for experimentally validated microRNA–target interactions, has documented over 17,000 target genes and more than 4 million miRNA–target interactions (MTIs) [7]. However, the majority of predicted MTIs have yet to be studied experimentally, despite the high number of confirmed target genes. MiRNA has been identified as a crucial regulator during different stages of spermatogenesis and early embryonic development in male human reproduction, as reviewed by Salas-Huetos et al. [8]. Additionally, the involvement of miRNAs in male infertility development is widely accepted, with observed alterations in miRNA expression levels in human sperm, seminal plasma, and testicular tissue of men with various patterns of non-obstructive azoospermia [8]. In recent studies, a correlation has been observed between the differential expression of miRNAs and/or their target mRNAs levels, and basic semen parameters, notably sperm motility [9,10]. This correlation suggests that miRNAs and/or their target mRNAs can potentially serve as diagnostic, prognostic, and even therapeutic biomarkers for male infertility. Specifically, higher expression levels of microRNA-23a/b-3p have been linked to lower expression of a group of testis-specific target genes in male patients with subfertility [11,12], which helps in comprehending the complexity of miRNA regulation during spermatogenesis and/or sperm function. However, although prediction tools for MTIs are continually improving, they still lack the ability to confidently validate miRNA target genes. Therefore, the development and improvement of algorithms rely on experimental validation and expanding the verified target genes is of utmost importance. This study aims to experimentally validate a possible connection between the level of microRNA-23a/b-3p and another set of 16 testis-specific targets using dual-luciferase assays and RT-qPCR validation in human spermatozoa from healthy and oligoasthenozoospermic individuals.

2. Materials and Methods

2.1. Ethics Approval

The study adhered to the principles of the Declaration of Helsinki and received approval from the Saarland Medical Association Ethical Board (Ha 195/11/updated June 2021). All participants, including both patients and healthy donors, provided written and informed consent before participating in the study.

2.2. In-Silico Prediction of MicroRNA-23a/b-3p Target Genes

The miRWalk algorithm 2.0 was utilized for an in silico analysis to identify potential target genes of microRNA-23a/b-3p [13]. To narrow down the selection, only those target genes predicted in at least 4 out of 12 prediction algorithms incorporated into the miRWalk algorithm were considered, which yielded around 13,000 potential target genes. Further refinement was done by cross-matching these predicted target genes with genes specifically expressed in the testes, based on the Human Protein Atlas (proteinatlas.org, 2237 genes), resulting in 1097 potential target genes [14]. These target genes, which were identified based on the miRWalk algorithm and the Human Protein Atlas, respectively, were found to bear a microRNA-23a/b-3p and play a role in spermatogenesis. Among the 1097 potential target genes, 16 target genes namely *NOL4*, *SOX6*, *GOLGA6C*, *PDCHA9*, *G2E3*, *ZNF695*,

CEP41, *RGPD1*, *GOLGA6B*, *LMLN*, *ZNF492*, *GNAT1*, *REEP1*, *CSNK1G1*, *FAM169A*, and *TMEM215* were selected for further investigation.

2.3. Cloning of MiRNA Expression and 3'UTR Reporter Vector Constructs

In order to overexpress microRNA-23a/b-3p, expression vectors pSG5-miR-23a-3p and pSG5-miR-23b-3p were employed. The overexpression of microRNA-23a/b-3p using the pSG5 expression vector was confirmed by Northern blotting [11]. To generate pMIR-RNL-TK reporter vectors containing the 3′UTR of the chosen 16 target genes, a cloning procedure was performed. This involved amplifying and inserting 3′UTR fragments from the genomic DNA template into the multiple cloning site (MCS) downstream of the luciferase gene. This was achieved using sequence-specific forward and reverse primers, which had an additional 5′ SpeI (5′-ACTAGT-3′) and SacI (5′-GAGCTC-3′) restriction enzyme cleavage site, as outlined in Table S1. Mutagenesis was carried out to modify the binding sites within the 3′UTR of vector constructs by using overlap extension PCR and specific primers listed in Table S1. The vector constructs containing the native and mutated binding site were designated as wild-type (WT) and mutant (mut), respectively. Supplementary Table S2 provides information regarding the reference nucleotide positions within the 3′UTR, the restriction enzymes used, the position of the binding site, and the size of the reporter constructs.

2.4. Cell Line and Cell Culture

For our experiments, we employed the human embryonic kidney cell line HEK-293T, which was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Biochrom, Cambridge, UK), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma, Burlington, MA, USA). The HEK-293T cells were maintained at 37 °C and 5% CO_2 and subcultured after trypsinization with 1x Solution of 0.05% trypsin-EDTA (Thermo Fisher Scientific).

2.5. Automated Dual-Luciferase Reporter Assay

To enhance the reproducibility and replicability of results, we utilized the automatic liquid handling system epMotion 5075 (Eppendorf, Hamburg, Germany) for the Dual-Luciferase Reporter Assay in this study. Specifically, 3.6×10^4 HEK-293T cells were seeded per well of the 96-well culture plates (Eppendorf) and were transfected 24 h later with 1 µL/well PolyFect transfection reagent (Qiagen, Hilden, Germany) containing 50 ng/well of each reporter vector and 200 ng/well of each expression vector. For each run, empty vector constructs (pMIR-empty and pSG5-empty) were included as a control, and all plasmid combinations were transfected in technical duplicates. After 48 h, cells were lysed and measured using the Dual-Luciferase Reporter Assay System manual with the GlowMax navigator microplate luminometer (Promega, Madison, WS, USA). We performed Luciferase assays of the WT 3′UTRs in four independent experiments and compared WT to mutated binding sites within the 3′UTR in three independent experiments. As a positive control, we re-evaluated the downregulation of four target genes (*PFKFB4*, *HMMR*, *UBQLN3*, and *ODF2*) previously studied by overexpressing microRNA-23a/b-3p using this automated technique, as shown in Figure S1.

2.6. RNAs Collection and Prepaartion

In this study, purified RNAs were obtained from a cohort of 82 men who were aged between 18 and 35 years old (with a mean age ± SD of 25.66 ± 4.21 years) [15]. This cohort included 41 subfertile men with oligoasthenozoospermia who sought infertility treatment at the IVF lab, as well as 41 age-matched normozoospermic control men with confirmed fertility. All participants underwent analysis of primary semen parameters according to the World Health Organization's 2010 guidelines. It is worth noting that none of the individuals had abnormal cell morphology (<4%) or any known medical conditions that could cause infertility, such as Y chromosome microdeletions or chromosomal abnormalities. The detailed clinical characteristics of individuals are listed in Abu-Halima et al. [15].

2.7. cDNA Conversion, and qPCR

Reverse transcription (RT) was conducted using 75 ng of total RNA in a 5 µL reaction volume, employing the Reverse Transcription Master Mix from Fluidigm Corporation, following the manufacturer's instructions. Subsequently, 1.5 µL of cDNA was mixed with 1 µL of PreAmp Master Mix (Fluidigm Corporation, San Francisco, CA, USA) and 0.5 µL of pooled DELTAgene Assay Mix (500 nM each primer, Fluidigm Corporation) in a 5 µL reaction volume for pre-amplification. Exonuclease I treatment, as recommended by Fluidigm, was performed using a mixture of Exonuclease I (New England Biolabs, Ipswitch, MA, USA) and Exonuclease I Reaction Buffer added to the preamplification reaction. The preamplification process was then inactivated by incubation at 80 °C for 15 min. The samples were diluted with DNA suspension buffer and stored at −20 °C until RT-qPCR, following automation using the QIAcube™ Robotic Workstation (Qiagen).

For RT-qPCR of multiplex mRNA, the Biomark™ HD system from Fluidigm Corporation was employed. After preamplification, RT-qPCR was carried out using a 96.96 Dynamic Array™ IFC for Gene Expression (Fluidigm Corporation). The pre-amplified samples were mixed with SsoFast EvaGreen Supermix with low ROX (Bio-Rad Laboratories, Hercule, CA, USA) and DNA Binding Dye (Fluidigm), and the assay mix contained forward and reverse primers. The chip with the sample and assay mix was loaded into the Biomark™ HD system, and the specific thermal cycling protocol for mRNA analysis was performed. No-template controls (NTC) were included, and *GAPDH* was used for normalization. The data were analyzed using the Real-Time PCR Analysis Software (Fluidigm Corporation).

2.8. Statistical Analysis

GraphPad Prism 9 software from GraphPad Software in La Jolla, USA, and R Software from R Core Team in Vienna, Austria, was utilized for statistical analysis and visualization of the luciferase assay and RT-qPCR data. The ΔCt values and fold change (FC) of each target gene were determined relative to *GAPDH*, which served as the endogenous control for RT-qPCR. The differences in ΔCt values between oligoasthenozoospermic men and normozoospermic controls were assessed using Student's unpaired two-tailed *t*-test. Spearman's correlation was applied to evaluate the relationship between basic semen parameters and the expression levels of the target genes. The luciferase assay data were normalized to the empty expression vector (pSG5) and presented as mean ± standard error of the mean (SEM). *p*-values were calculated using Student's unpaired two-tailed *t*-test, and for the wild-type experiments, Welch correction was applied. The levels of statistical significance were denoted as follows: * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; *** = $p \leq 0.001$.

3. Results

3.1. Target Gene Validation by Dual-Luciferase Reporter Assay

The dual-luciferase reporter assay confirmed the binding of microRNA-23a/b-3p within the 3′UTR of the 16 potential target genes. MicroRNA-23a-3p overexpression significantly decreased the luciferase activity of ten target genes (*NOL4*, $p < 0.0001$; *SOX6*, $p = 0.0002$; *GOLGA6C*, $p = 0.0007$; *PCDHA9*, $p = 0.0007$; *G2E3*, $p = 0.0009$; *ZNF695*, $p = 0.0013$; *CEP41*, $p = 0.0014$; *RGPD1*, $p = 0.0023$; *GOLGA6B*, $p = 0.0031$; *LMLN*, $p = 0.0322$), as depicted in Figure 1. Conversely, six target genes (*ZNF492, GNAT1, REEP1, CSNK1G1, FAM169A,* and, *TMEM215*, Figure S2) did not show a significant reduction in luciferase activity. Of the ten target genes with reduced luciferase activity, eight showed a high-confidence decrease of more than 20% (*NOL4*, 39.91%; *SOX6*, 30.11%; *GOLGA6C*, 36.19%; *PCDHA9*, 27.54%; *G2E3*, 24.60%; *ZNF695*, 29.83%; *CEP41*, 22.21%; *RGPD1*, 24.24%, Figure 1).

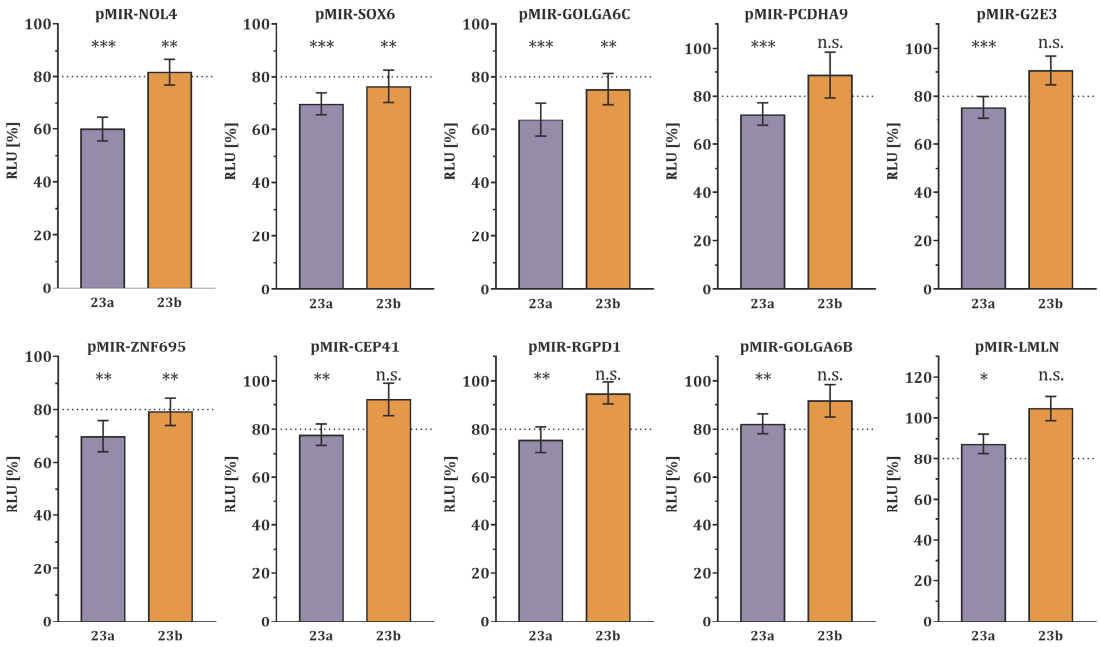

Figure 1. Dual-luciferase reporter gene assays were performed on the 3′ untranslated regions (UTRs) of ten genes, namely *NOL4, SOX6, GOLGA6C, PCDHA9, G2E3, ZNF695, CEP41, RGPD1, GOLGA6B,* and *LMLN*. HEK 293T cells were transfected with the reporter gene constructs (pMIR) and miRNA-expression plasmids (23a, pSG5-miR-23a-3p/ 23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of four independent experiments carried out in duplicates. The data are presented as mean ± SEM, and *p*-value was calculated using Welch *t*-test. A *p*-value of less than 0.05 was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, n.s. non-significant). Target genes with luciferase activity of less than 80% are considered high confidence target genes as indicated by the dotted line.

The overexpression of microRNA-23b-3p significantly decreased the luciferase activity of four target genes (*NOL4*, $p = 0.0084$; *SOX6*, $p = 0.0062$; *GOLGA6C*, $p = 0.0041$; *ZNF695*, $p = 0.0056$), as shown in Figure 1. Conversely, twelve target genes (*PCDHA9, G2E3, CEP41, RGPD1, GOLGA6B, LMLN, ZNF492, GNAT1, REEP1, CSNK1G1, FAM169A, TMEM215*, Figures 1 and S1) did not show a significant reduction in luciferase activity. Three of the four target genes that had a decrease in luciferase activity by more than 20% were considered high-confidence target genes (*SOX6*, 23.54%; *GOLGA6C*, 24.61%; *ZNF695*, 20.65%).

3.2. Expression Level of MiR-23a/23b Target Genes in Spermatozoa by RT-qPCR

To validate the effect of microRNA-23a-3p and microRNA-23b-3p on target genes, RT-qPCR analysis was performed on ten genes that showed a significant reduction in the luciferase activity. Results indicated that the expression level of all the ten target genes was significantly lower in oligoasthenozoospermic men compared to normozoospermic controls (Figure 2). Among these, four genes showed high statistical significance (*G2E3*, $p = 0.0031$, FC = 1.9; *ZNF695*, $p = 0.0096$, FC = 2.0; *CEP41*, $p = 0.0023$, FC = 2.2; *RGPD1*, $p = 0.0031$, FC = 2.9), whereas the remaining six genes were highly significant (*NOL4*, $p = 0.0003$, FC = 3.1; *SOX6*, $p = 0.0003$, FC = 3.2; *GOLGA6C*, $p = 0.0002$, FC = 5.9; *PCDHA9*, $p = 0.0001$, FC = 11; *GOLGA6B*, $p = 0.0009$, FC = 4.1; *LMLN*, $p < 0.0001$, FC = 3.4).

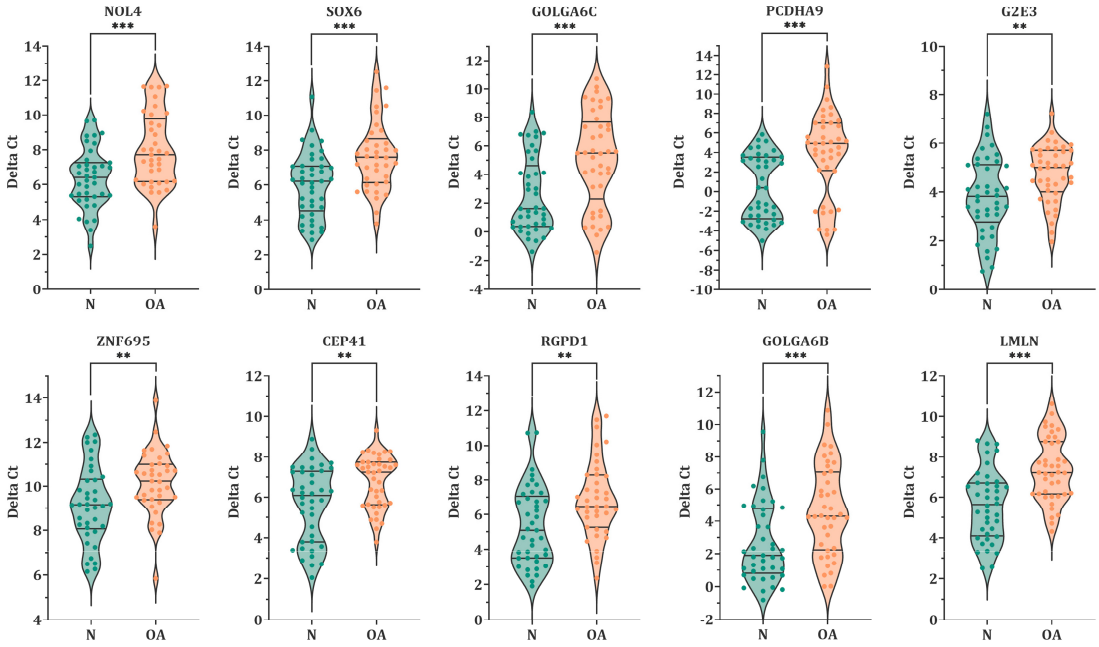

Figure 2. Violin plots display the expression levels of 10 chosen target genes in sperm samples from oligoasthenozoospermic men (n = 41) compared to normozoospermic controls (n = 41), as assessed by RT-qPCR. The data points are presented as ΔCt, and the plot lines depict the median and quartiles of ΔCt. The *p*-value was calculated using an unpaired two-tailed Student's *t*-test, with a significance level set at $p < 0.05$ (*** $p < 0.001$; ** $p < 0.01$). N: normozoospermic controls, OA: oligoasthenozoospermic men.

Figure 3 summarizes the correlation analysis between lower expression levels of the target genes and basic semen parameters. All ten target genes showed a weak to moderate positive correlation with sperm count and motility (*p*-value < 0.05), and eight genes (*NOL4*, *SOX6*, *GOLGA6C*, *PCDIIA9*, *ZNF695*, *RGPD1*, *GOLGA6B*, and *LMLN*) showed a weak positive correlation with normal morphology of sperm.

3.3. Validation of Binding-Site Specificity by Mutagenesis Dual-Luciferase Reporter Assay

The mutagenesis dual-luciferase assay was used to determine the binding site specificity. The 3'UTR of eight target genes, which were high-confidence targets for at least one of the miRNAs (microRNA-23a-3p and microRNA-23b-3p) and contained binding sites complementary to the seed region of the miRNAs, were mutated. The wild-type and mutant constructs were then subjected to overexpression of microRNA-23a-3p or microRNA-23b-3p, and the relative luciferase activity was compared. For microRNA-23a-3p, the luciferase activity of the wild-type construct was significantly reduced compared to the mutant in five target genes (*NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41*). Four of these target genes showed a reduction of more than 20% (*NOL4*, *SOX6*, *PCDHA9*, and *CEP41*), while GOLGA6C was borderline with an 18.95% reduction. The remaining three target genes (*G2E3*, *ZNF695*, and *RGPD1*) did not exhibit significant reductions in luciferase activity. Similarly, for microRNA-23b-3p, the luciferase activity of the wild-type construct was significantly reduced by more than 20% compared to the mutant in three target genes (*NOL4*, *SOX6*, and *PCDHA9*), and the wild-type construct of *PCDHA9* showed significant reduction only in this experiment. The remaining five target genes (*GOLGA6C*, *CEP41*, *G2E3*, *ZNF695*, and *RGPD1*) did not exhibit significant reductions in luciferase activity. Additionally, some of the target genes that were not significantly reduced in luciferase activity had mutants

that showed reduced activity, indicating an off-target effect where the miRNA bound to a different binding site within the cloned fragment of the 3′UTR. Specifically, *G2E3* and *ZNF695* for microRNA-23a-3p, and *GOLGA6C*, *CEP41*, *G2E3*, *ZNF695*, and *RGPD1* for microRNA-23b-3p had reduced luciferase activity in their mutants. This suggests that the miRNAs may have additional targets beyond the identified ones. All these findings are summarized in Figures 4 and S3.

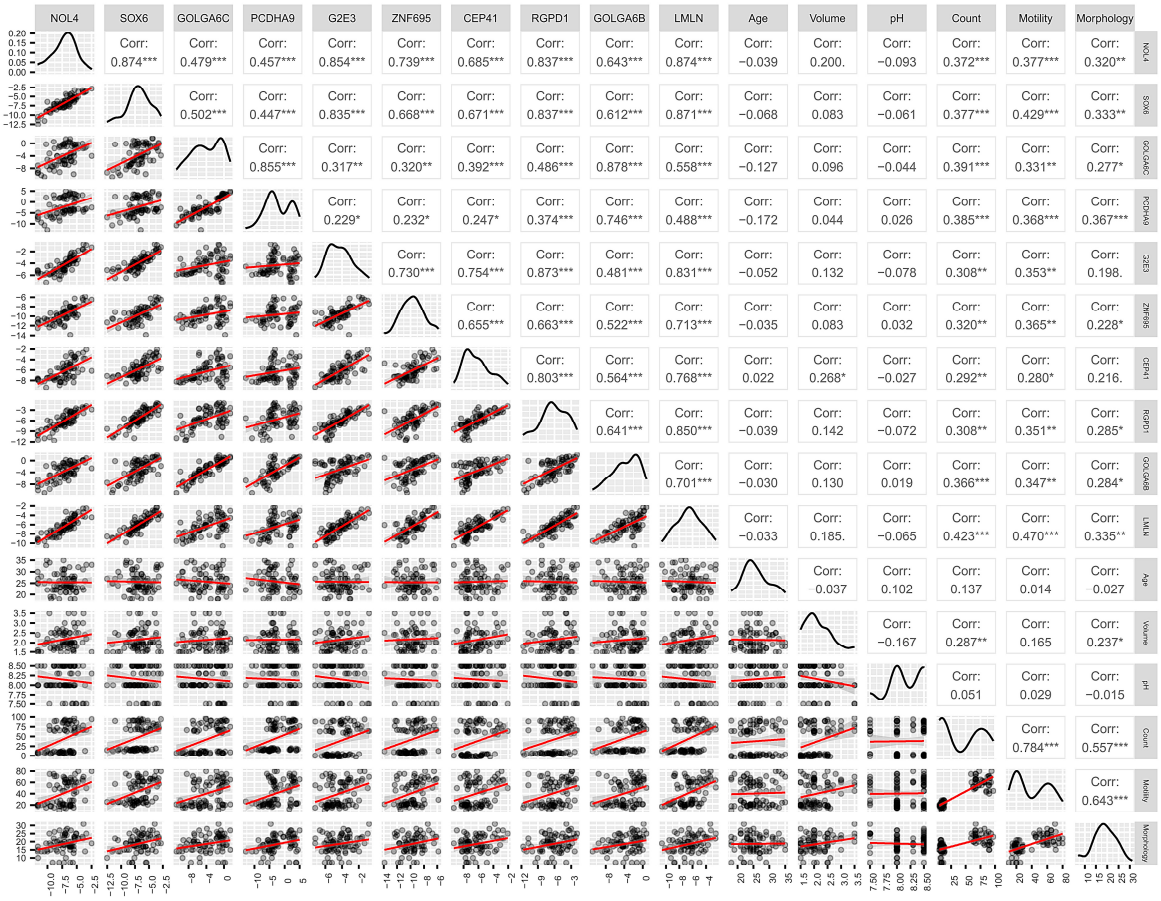

Figure 3. Correlation analysis of target gene abundance levels as determined by RT-qPCR (ΔCt) with age and basic semen parameters volume, pH, count, motility, and morphology. The lower and upper triangle show the scatter plot of the variables, the correlation coefficient and the significance. The diagonal shows the distribution of samples in density plots. Spearman correlation analysis and unpaired two-tailed *t*-test was performed. *p*-value < 0.05 was considered as statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

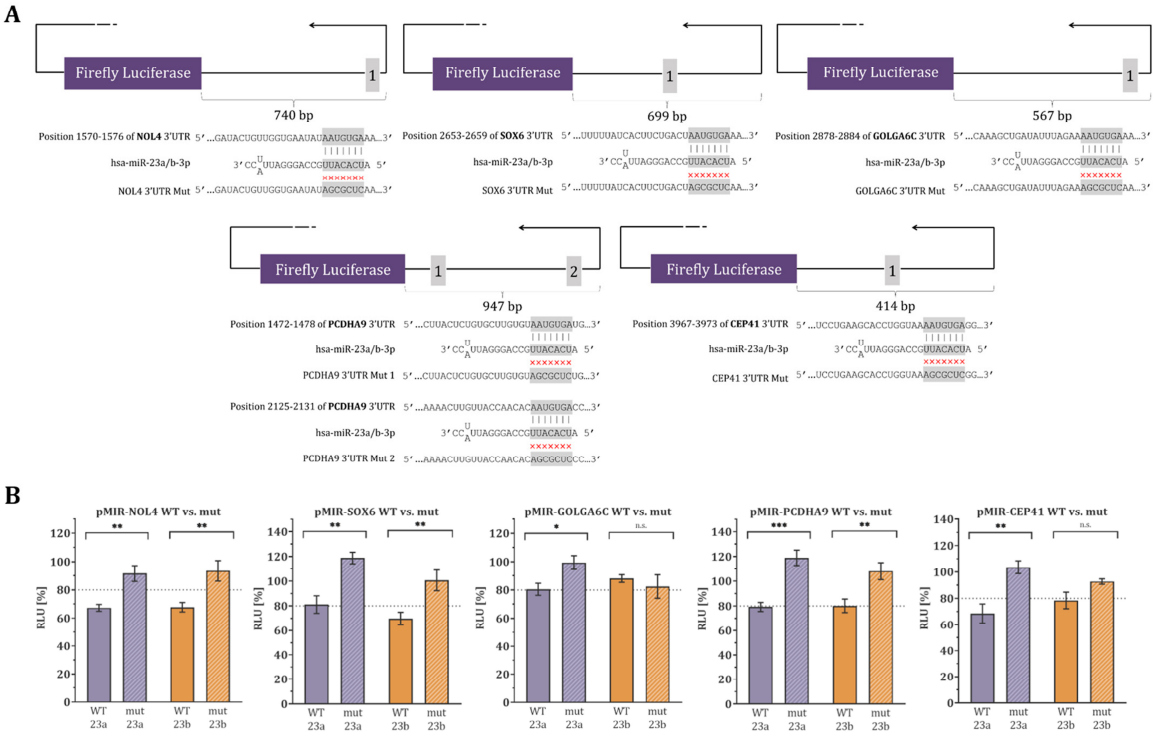

Figure 4. (**A**) Schematic diagram of the reporter vector constructs including microRNA-23a/b-3p binding sites. The localization of the predicted binding sites in the 3′UTRs of *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41*, the sequences of the microRNA-23a/b-3p binding sites, and the mutated binding sites (Mut) are shown [16]. (**B**) Mutagenesis dual-luciferase reporter gene assays of the 3′UTRs of *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41*. HEK 293T cells were transfected with the wild-type (WT) and mutated (mut) reporter gene constructs (pMIR) and the miRNA-expression plasmids (23a, pSG5-miR-23a-3p/23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of three independent experiments carried out in duplicates. Data are represented as mean ± SEM, and the *p*-value was calculated using an unpaired two-tailed Student's *t*-test, and $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant).

4. Discussion

In this study, miRNA target genes were predicted using in silico analysis to identify 16 testis-specific potential target genes of microRNA-23a/b-3p, which we previously found to be more abundant in sperm from oligoasthenozoospermic men than normozoospermic controls [11,12]. These genes were cloned, and luciferase assays revealed that ten and four of them were target genes of microRNA-23a-3p and microRNA-23b-3p, respectively, with significantly reduced luciferase activity (Figure 1). To confirm the lower expression levels of these high-confidence target genes, RT-qPCR was conducted in sperm of oligoasthenozoospermic men compared to normozoospermic controls, and the results were positively correlated with the basic semen parameters (Figures 2 and 3). To verify the binding-site specificity, we mutated the binding sites within the 3′UTR of these target genes and compared them to the wild type. Specifically, overexpression of microRNA-23a-3p and microRNA-23b-3p had an adverse effect on *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41*, as well as *NOL4*, *SOX6*, and *PCDHA9* in the mutant compared to the wild type (Figure 4). Using the single-cell type transcriptomic map from the Human Protein Atlas (proteinat-

las.org), we found that *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41* were expressed in various stages of spermatogenesis, including spermatogonia, spermatocytes, early and/or late spermatids [17]. These genes are known to have testis-specific expression [17,18], and some of them have been implicated in physiological processes related to male reproduction. For example, SRY-Box Transcription Factor 6 (*SOX6*), which is a cancer-testis antigen with a high mobility group box, is directly associated with the sex-determining region Y (*SRY*) and is involved in various physiological and developmental processes [19–22]. *SOX6* has been identified as a co-factor of the testis-expressed centromere-associated protein (called Solt), which is essential for cell cycle progression and inactivation of cell division in mouse spermatogenesis [23–25]. Moreover, SOX6 is thought to play a role in sperm maturation and cycles during spermatogenesis in rodents [23,26]. However, the precise function of *SOX6* in human spermatogenesis remains unclear [23]. Golgin Subfamily A Member 6C (*GOLGA6C*) is predicted by the Gene Ontology consortium and the Human Protein Atlas to be involved in spermatogenesis, particularly in flagellum and Golgi organization, and was computationally identified as an interactor of *GSK3A*, which affects human sperm motility [17,27,28]. The Centrosomal Protein 41 kDa (CEP41) is localized on the primary cilia and plays a functional role in post-translational modifications, regulating the glutamylation of tubulins at the cilium [29]. Even if the relationship between *CEP41* ciliary function and sperm has not yet been explored, the Centrosomal Protein 192 (*CEP192*) is a well-known essential protein required for spermatogenesis. Recent evidence is emerging about "moonlighting" functions of cell division, i.e., meiosis players actively involving in spermatogenesis, which seems to be evolutionary conserved from *drosophila melanogaster* to human [30,31].

Regarding the mutant *G2E3*, *ZNF695*, and *RGPD1* 3′UTRs that demonstrated lower luciferase activities compared to the wild type (Figure S3), it is suggested that the miRNA may still activate the RNA interference (RNAi) mechanism. Previously, it was thought that only seed binding was essential, but now it is known that compensatory binding 3′ to the miRNA seed sequence also plays a role in miRNA binding [32,33]. Despite the mutated binding site being designed to prevent the binding of microRNA-23a/b-3p to the site, it is speculated that miRNA binding may have inadvertently shifted, causing single base pairing outside the seed and binding site. Other techniques including miR-CLIP can also be used to detect miRNA target interactions and these off-target effects [34].

It is noteworthy that even though microRNA-23a-3p and microRNA-23b-3p have identical seed sequences, there are differences in post-translational regulation strength, resulting in varying definitions of high-confidence target genes. MiRNAs with identical seed sequences typically belong to miRNA families, such as the miR-23 family [35,36]. While differences in target spectra and miRNA regulation strength of miRNA-family members using in vivo techniques are widely acknowledged, they are not well understood [37].

Our previous work indicated that there were differences in the targeting characteristics of microRNA-23a-3p and microRNA-23b-3p in sperm samples, as determined by RT-qPCR and luciferase assays. These findings are consistent with the results of the current study [11,12]. Previous research on other miRNA families has suggested a link between miRNA expression levels, MTI construction, and miRNA regulation strength and specificity [32,37]. It has been reported that supplementary base pairing outside the seed can influence the effectiveness of miRNA activity and the compensatory potential of imperfect base pairing within the seed [32,37]. We would like to emphasize that our study was not designed to identify differences in the functions of microRNA-23a-3p and microRNA-23b-3p. Further functional targeting experiments conducted in vivo will be necessary to fully comprehend the mechanisms of miRNA targeting.

We would like to acknowledge certain limitations of our study. An important aspect is the absence of fresh testicular biopsy samples and specific testicular cell lines for further analysis. Nonetheless, our findings revealed the impact of the selected microRNA-23a/b-3p on spermatogenesis and the testicular microenvironment. It is quite uncommon to obtain fresh testicular biopsies, particularly from fertile males. To some extent, we overcame this

limitation by examining sperm samples, which represent the final product of spermatogenesis. Another limitation is that we did not validate the target gene protein levels in sperm samples obtained from subfertile men and healthy controls. We recently conducted a study of the proteomic landscape of human sperm obtained from oligoasthenozoospermic men, comparing these samples with samples from normozoospermic men using LC-MS/MS. Despite the detection of 4412 proteins and the analysis of 53 samples, we did not detect any of the miRNA-23a/b targets [38]. In the present study, we performed Western blot that also failed to detect the proteins that are encoded by the miRNA-23a/b target genes (Anti-NOL4, PA5-103986, Invitrogen and Anti-SOX6, ab64946, Abcam). The absence of miRNA-23a/b target proteins in our Western blot and mass spectrometry data might be attributed to the unique biology of sperm with its nearly silent transcription and translation processes [39]. It is widely recognized that certain mRNAs are remnants from earlier spermatogenesis events and that such miRNA can remain untranslated in sperm [39–41]. Other proteins that are transcribed during spermatogenesis can be removed as part of the removal of most cytoplasm during sperm development [42]. Additionally, proteins can be transported by extracellular vesicles that are frequently found in the seminal fluid [42]. These factors combined complicate the comparison of mRNAs and proteins in sperm.

5. Conclusions

This study has affirmed that men with oligoasthenozoospermia exhibit reduced expression levels of specific genes such as *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, *G2E3*, *ZNF695*, *CEP41*, *RGPD1*, *GOLGA6B*, and *LMLN* when compared to men with normal sperm. Additionally, the study has identified particular interactions between microRNA-23a-3p and *NOL4*, *SOX6*, *GOLGA6C*, *PCDHA9*, and *CEP41*, as well as between microRNA-23b-3p and *NOL4*, *SOX6*, and *PCDHA9*. These findings suggest that the increased levels of microRNA-23a/b-3p and subsequent decrease in target gene expression are related to male subfertility, potentially by impacting key semen parameters. The study lays a foundation for future research focused on developing therapies for male infertility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology12060800/s1, Figure S1: Dual-luciferase reporter gene assays of previously tested genes *PFKFB4*, *HMMR*, *UBQLN3*, and *ODF2*. HEK 293T cells were transfected with the reporter gene constructs (pMIR), and the miRNA-expression plasmids (23a, pSG5-miR-23a-3p/23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of four independent experiments carried out in duplicates. Data are represented as mean ± SEM, and *p*-values were calculated using Welch *t*-test, and $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant). Figure S2: Dual-luciferase reporter gene assays of the 3′UTRs of *ZNF492*, *GNAT1*, *REEP1*, *CSNK1G1*, *FAM169A*, and *TMEM215* that were not significantly reduced in luciferase activity. HEK 293T cells were transfected with the reporter gene constructs (pMIR), and the miRNA-expression plasmids (23a, pSG5-miR-23a-3p/ 23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of four independent experiments carried out in duplicates. Data are represented as mean ± SEM, and the *p*-value was calculated using Welch *t*-test. $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant). Figure S3: Mutagenesis dual-luciferase reporter gene assays of the 3′UTRs of *G2E3*, *ZNF695*, and *RGPD1* that were not significantly reduced in luciferase activity comparing wild-type (WT) and mutated (mut) 3′UTR. HEK 293T cells were transfected with the WT and mut reporter gene constructs (pMIR), and the miRNA-expression plasmids (23a, pSG5-miR-23a-3p/ 23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of three independent experiments carried out in duplicates. Data are represented as mean ± SEM, and the *p*-value was calculated using unpaired two-tailed Student's *t*-test. $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant). Table S1: Sequences of cloning and mutagenesis primers. Table S2:

Constructs with NCBI Reference Sequence, restriction enzymes for cloning, nucleotide position in 3′UTR, binding site position, and size of the amplified fragment. Table S3: Sequences of RT-qPCR primers and their respective design reference sequence.

Author Contributions: Conceptualization, M.A.-H.; methodology, M.A.-H., L.S.B. and H.K.; validation, M.A.-H. and L.S.B.; formal analysis, M.A.-H. and L.S.B.; investigation, M.A.-H. and L.S.B.; resources, M.A.-H., M.A.A.S., H.A.-K. and E.M.; data curation, M.A.-H. and L.S.B.; writing—original draft preparation, M.A.-H. and L.S.B.; writing—review and editing, M.A.-H., H.A.-K. and E.M.; visualization, M.A.-H. and L.S.B.; supervision, M.A.-H., H.A.-K., E.M.; project administration, M.A.-H. and E.M.; funding acquisition, M.A.-H. and L.S.B. All authors have read and agreed to the published version of the manuscript.

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3.2. MicroRNA-targeting in male infertility: Sperm microRNA-19a/b-3p and its spermatogenesis related transcripts content in men with oligoasthenozoospermia

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MicroRNA-targeting in male infertility: Sperm microRNA-19a/b-3p and its spermatogenesis related transcripts content in men with oligoasthenozoospermia

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Objective: To elucidate and validate the potential regulatory function of miR-19a/b-3p and its spermatogenesis-related transcripts content in sperm samples collected from men with oligoasthenozoospermia.

Methods: Men presenting at an infertility clinic were enrolled. MicroRNA (miRNA) and target genes evaluation were carried out using *in silico* prediction analysis, Reverse transcription-quantitative PCR (RT-qPCR) validation, and Western blot confirmation.

Results: The expression levels of miRNA-19a/b-3p were significantly up-regulated and 51 target genes were significantly down-regulated in oligoasthenozoospermic men compared with age-matched normozoospermic men as determined by RT-qPCR. Correlation analysis highlighted that sperm count, motility, and morphology were negatively correlated with miRNA-19a/b-3p and positively correlated with the lower expression level of 51 significantly identified target genes. Furthermore, an inverse correlation between higher expression levels of miRNA-19a/b-3p and lower expression levels of 51 target genes was observed. Consistent with the results of the RT-qPCR, reduced expression levels of STK33 and DNAI1 protein levels were identified in an independent cohort of sperm samples collected from men with oligoasthenozoospermia.

Conclusion: Findings suggest that the higher expression of miRNA-19a/b-3p or the lower expression of target genes are associated with oligoasthenozoospermia and male infertility, probably through influencing basic semen parameters. This study lay the groundwork for future studies focused on investigating therapies for male infertility.

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KEYWORDS

male subfertility, oligoasthenozoospermia, expression analysis, miR-19a-3p, miR-19b-3p

Introduction

About 15% of couples seeking pregnancy are infertile (Sharlip et al., 2002). The defect in about half of them (7%) is associated with the inability of the male partner to produce functional sperms that are capable of fertilization (Krausz and Riera-Escamilla, 2018). The aetiology of male infertility could be attributed to a plethora of congenital and acquired factors (Jungwirth et al., 2018). About 20% of azoospermia and 25% of oligozoospermia cases result from known genetic causes, while the cause is unknown in about a third (known as idiopathic male infertility) (Krausz and Riera-Escamilla, 2018). Alterations in the sequence or expression of single or multiple genes were held responsible; however, their role and precise molecular pathology remain obscure (Zorrilla and Yatsenko, 2013). An intensive effort has been invested to elucidate how those genes are involved in regulating spermatogenesis and sperm function. Recent transcriptome analyses showed that hundreds of genes are predominantly or exclusively expressed in the male germ cells (Djureinovic et al., 2014; Uhlen et al., 2015). These germ cell-specific genes play a crucial role during spermatogenesis and/or sperm function, and their aberrant expression could contribute to male infertility. During spermatogenesis, the gene expression is highly dynamic and strictly regulated via several regulators including microRNAs (miRNAs) (Salas-Huetos et al., 2020). MiRNAs are small non-coding RNAs (~22 nucleotides in length). These small RNAs are transcribed by RNA Polymerase II from miRNA genes located within protein-coding genes to form a characteristic hairpin structure called primary miRNA. Primary miRNA is processed by Drosha, Dicer, and DGCR8 to form the mature miRNA, which is incorporated into the argonaute protein (AGO) and forms the RNA-induced silencing complex (RISC) (Bartel, 2018; O'Brien et al., 2018). Mature miRNAs regulate gene expression post-transcriptionally through base-pairing with the 3′untranslated region (3′UTR) of messenger RNAs (mRNA) (Bartel, 2018; O'Brien et al., 2018). This interaction results in gene silencing either by destabilization of the mRNA or inhibition of the translation (Bartel, 2018; O'Brien et al., 2018). To date, 2,300 miRNAs have been reported as'' true and/or real'' human mature miRNAs (Alles et al., 2019). These miRNAs are involved in many, if not all, cellular and biological processes, including germ cell development (Bhin et al., 2015), spermatogenesis (Abu-Halima et al., 2013; Abu-Halima et al., 2014a; Abu-Halima et al., 2014b; Abu-Halima et al., 2016; Abu-Halima et al., 2019; Abu-Halima et al., 2020a; Abu-Halima et al., 2021a), early stages of embryonic development (Abu-Halima et al., 2017; Abu-Halima et al., 2020b), women undergoing infertility treatment (Salas-Huetos et al., 2019; Abu-Halima et al., 2021b), and germ cell apoptosis (Reza et al., 2019). Loss of the miRNA processing machinery and/or lack of miRNA biogenesis impair the reproduction process and that loss in function is highly dependent on the stage in which the Dicer and/or Drosha was deleted (Abu-Halima et al., 2021b). Studies reported that the testicular-expressed miRNA changes depending on the stage of spermatogenesis (Hayashi et al., 2008; Maatouk et al., 2008) and that the late meiotic stage and haploid germ cells are the main sources of miRNA production during spermatogenesis (Bouhallier et al., 2010). The haploid mature sperm are stored in the epididymis, whose miRNA expression profile differs from that of the other spermatogenesis stages (Nixon et al., 2015a; Reilly et al., 2016; Reza et al., 2019). Thus, epididymal miRNA might contribute to the maturation and motility of spermatozoa (Reza et al., 2019). Of these miRNAs, hsa-miR-19a-3p and/or hsa-miR-19b-3p (miR-19a/b-3p) were differentially abundant in the sperm, seminal plasma, and testicular tissue of men with different types of unexplained infertility (Wu et al., 2012; Abu-Halima et al., 2013; Abu-Halima et al., 2014a; Abu-Halima et al., 2020b). We have previously suggested that miR-19b-3p might be a potential biomarker for predicting the pregnancy outcome of artificially fertilized embryos (Abu-Halima et al., 2020b). Therefore, the identification of the potential targets of miR-19a/b-3p and the nature of their molecular action will provide insight into the complex downstream effects of miR-19a/b-3p in male fertility/subfertility. It will also allow for identifying potential diagnostic biomarkers and therapeutic targets for male infertility/subfertility. Therefore, in this study, we investigated the differential expression of miR-19a/b-3p and their predicted target genes in men with oligoasthenozoospermia and normozoospermic controls.

Materials and methods

Collection and preparation of human sperm samples

A total of 82 men were recruited for the study (mean age ± SD, 25.66 ± 4.21 years; range, 18—35), including 41 oligoasthenozoospermic (subfertile) who attended the IVF lab for infertility treatment and 41 age-matched proven fertile normozoospermic control men. The study complies with the declaration of Helsinki and was approved by the Institutional Review Board (Ha 195/11/updated June 2021) of the Saarland

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Medical Association. Ethical guidelines were also followed in the conduction of the research. All participants gave their written informed consent before enrolment. Semen samples were obtained from participants by masturbation after 3 days of sexual abstinence, liquefied at 37°C for 30 min, and then processed immediately. All samples were analyzed according to the World Health Organization (WHO) 2010 guidelines for primary semen parameters (liquefaction time, volume, pH, viscosity, agglutination, sperm motility, sperm viability, sperm density, and sperm morphology). These parameters, when taken together, have determined our tested subgroups, i.e., normozoospermia and oligoasthenozoospermia, and none of the included subjects exhibited cells with abnormal morphology (<4%). We excluded samples with a known medical reason for their infertility including Y chromosome microdeletions and other chromosomal abnormalities.

Purification of sperm samples was performed by discontinuous PureSperm® density gradient (Nidacon). Briefly, liquefied semen samples were layered upon a 45:90% density gradient and centrifuged at 500 × *g* for 20 min at room temperature. The recovered pellet was washed twice with Ham–F10 medium supplemented with 5 mg/mL human serum albumin and 0.1 mg/mL penicillin G/streptomycin sulphate (PAN Biotech) and carefully overlaid with 0.75 ml of the same medium. The sample was then kept at 37 °C for 45 min in 5% CO2. One ml of supernatant containing actively motile sperm was then removed and placed in a different tube. After purification of semen samples with density gradient and swim-up, the concentration and progressive motility were evaluated once more. To eliminate possible somatic cells in the ejaculate, semen samples are processed using the somatic cell lysis (SCL) method. This involves incubating the cells in SCL buffer (0.1% sodium dodecyl sulfate and 0.5% Triton X–100 in MilliQ water) on ice for 30 min. A microscopic examination was performed to confirm the absence of residual somatic contaminants with the use of a Makler counting chamber (Irvine Scientific).

RNA isolation, reverse transcription (RT), and miRNA RT-qPCR

The miRNeasy Mini Kit (Qiagen) was used with a minor modification to extract total RNA including miRNA from sperm. Briefly, 200 µL of the purified sperm samples were homogenized in 700 µL QIAzol® Lysis Reagent (Qiagen) mixed with Dithiothreitol (DTT) (80 mmol/L, Sigma) for 15 min. Subsequently, the procedure was completed according to the manufacturer's instructions for Qiagen miRNeasy Mini Kit using QIAcube™ Robotic Workstation (Qiagen). DNase I treatment (Qiagen) was performed during the isolation to eliminate any contaminating genomic DNA. Total RNA concentration and purity were determined using Nanodrop-2000 (Thermo Fisher Scientific). Complementary DNA (cDNA) was generated in 20 µL reactions from 75 ng total RNA including miRNA using the *mi*Script Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The 5x *mi*Script HiSpec Buffer was used to generate cDNA from total RNA including miRNA by reverse transcriptase using oligo-dT primers. After RT, the cDNA was diluted with *mi*Script SYBR Green PCR Master Mix (Qiagen) and amplified to detect hsa-miR-19a-3p and hsa-miR-19a-3p. All RT-qPCR experiments were performed using the Liquid Handling Robot QIAgility™ (Qiagen) before performing RT-qPCR using the StepOnePlus™ Real-Time PCR system (Applied Biosystems). Moreover, RT negative controls and no template controls (NTC) were included.

Prediction of miR-19a/b-3p target genes and network construction

An *in-silico* prediction was carried out to predict the potential target genes of miR-19a-3p (MIMAT0000073) and miR-19b-3p (MIMAT0000074) abbreviated (miR-19a/b-3p), using miRWalk 2.0, which combines 12 target gene prediction algorithms (Dweep and Gretz, 2015). The number of potential target genes of miR-19a/b-3p was shortlisted by including only the target genes predicted by at least 5 different algorithms of the miRWalk. These predicted targets were then checked for the inverse expression levels with miRNA (i.e., miR-19a/b-3p) and mRNA (i.e., inversely correlated expressions of miRNA and mRNA in the RT-qPCR data). As a result, 3,066 potential target genes were predicted. Testis-specific target genes were identified by cross-matching the predicted targets to the Human Protein Atlas (2,237 genes) (www.proteinatlas.org/humanproteome/tissue/testis) and the ToppGene Suite (Chen et al., 2009) algorithms. From this analysis, 130 potential target genes were identified. Out of these 130 potential targets, 82 were selected for RT-qPCR validation based on their functional role in spermatogenesis and/or sperm function, context score of miRNA-mRNA interactions, seed sequence (7-mer sequence or more), and miRNA binding site position within the 3′UTR (left, middle, and right). Using Cytoscape version 3.8.2, the regulatory network was constructed for target genes that have been validated by qPCR and the target genes predicted by at least 5 different algorithms of the miRWalk 2.0.

Reverse-transcription and pre-Amplification of target genes in the sperm samples

The expression level of multiplex mRNA was quantified by RT-qPCR using the Biomark HD System (Fluidigm Corporation). The Fluidigm® DELTAgene™ Assays (Fluidigm Corporation) was used for the detection of selected target genes

according to the manufacturer's recommendations. Briefly, cDNA was generated in 5 µL reactions from 75 ng total RNA using the Reverse Transcription Master Mix (Fluidigm Corporation) containing both oligo-dTs and random primers, according to the manufacturer's instructions. Following reverse transcription, 1.5 µL of the generated cDNA was pre-amplified by mixing 1.0 µL of PreAmp Master Mix (Fluidigm Corporation) and 0.5 µL of the Pooled DELTAgene™ Assays mix (500 nM, Fluidigm Corporation) in 5 µL reaction volume. A clean-up step with Exonuclease I (New England Biolabs) was carried out as indicated in Fluidigm's protocol PN 100–5875 C1. A mixture of 2 µL Exo I containing 0.4 µL Exonuclease I (20 U/µL), 0.2 µL Exonuclease I Reaction Buffer, and 1.4 DNase-free water was added to each 5-µL preamplification reaction using QIAcube™ Robotic Workstation (Qiagen). The preamplification reaction was allowed to digest at 37°C for 30 min before inactivating at 80°C for 15 min using TProfessional Thermocycler (Biometra). Lastly, each sample was diluted with 30.5 µL DNA suspension buffer using the QIAcube™ Robotic Workstation (Qiagen) and was then stored at −20°C for the Biomark™ HD High-throughput RT-qPCR (Fluidigm).

High-throughput RT-qPCR of target genes in the sperm samples

Following the pre-amplification, RT-qPCR was carried out with 96.96 Dynamic Array™ IFC for Gene Expression (Fluidigm Corporation) as recommended in Fluidigm's protocol for mRNA (PN 100–9792B1). For each sample, a 6 µL sample mix was prepared, containing 3 µL of 2X SsoFast EvaGreen Supermix with low ROX (Bio-Rad Laboratories), 0.3 µL of 20X DNA Binding Dye (Fluidigm), and 2.7 µL of the pre-amplified sample. A primer stock (100 µM combined forward and reverse primers) was prepared for each assay, and 0.3 µL of the stocks was mixed with 3 µL of 2X Assay Loading Reagent (Fluidigm) and 2.7 µL 1X DNA Suspension Buffer to make assay mixes. Finally, 5 µL of each Assay and Sample Mix were transferred into the appropriate inlets according to the Fluidigm's recommendation. After loading, the array was placed in the Biomark HD instrument for quantification and detection using a specific thermal cycling protocol for mRNA analysis. GAPDH was used as a reference endogenous control for normalization. The data were then analyzed with Real-Time PCR Analysis Software, Version 4.7.1 (Fluidigm Corporation) as recommended by the provider. A No-Template Control (NTC) and RT-negative control were included in each run.

Western blot analysis

Western blot analysis was carried out as a small "Proof-of-Concept" to further validate the RT-qPCR using a different set of samples from normozoospermic controls (n = 4) men and oligoasthenozoospermic men (n = 4). Semen samples were thawed on ice and were washed three times with Phosphate-Buffered Saline (PBS). The samples were then centrifuged at 14,000 × *g* at 4°C for 15 min. The pellet was suspended in RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitor (Sigma–Aldrich). Then, the samples were sonicated at 20 J for 2 s × 10 at intervals of 10 s and incubated in ice overnight inside the fridge. All samples were then gently mixed for one minute and centrifuged at 14,000 x g at 4°C for 10 min, after which the supernatant was transferred to a new microcentrifuge for Western blot analysis. The concentration of solubilized protein was determined using the bicinchoninic acid assay (BCA assay) (Thermo Fisher Scientific). Twenty-five micrograms of total protein were denatured with Laemmli buffer mixed with β-mercaptoethanol (1:4) (Bio-Rad Laboratories), and the denatured proteins were separated by gel electrophoresis (SDS-PAGE) in a Mini-Protean® TGX Precast Gel (Bio-Rad Laboratories) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Whatman). After that, membranes were blocked for 1 h at RT with TBS Blotto A (Santa Cruz Biotechnology). Membranes were exposed to two primary antibodies namely anti-STK33 antibody [EPR15343] and anti-Dynein intermediate chain 1/DNAI1 antibody [EPR11244-61] diluted in TBS Blotto A overnight at 4°C with agitation. After three washes, each for 15 min, with 1X-Tris buffered Saline with Tween 20 (TBS-T) (Santa Cruz Biotechnology), membranes were exposed to the horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000, A0545, Sigma-Aldrich) for 1 h. The membranes were cut based on size or stripped and then probed again with GAPDH (14C10) rabbit mAb antibody (1:1,000, 2118S, Cell Signalling Technology), followed by incubation in anti-rabbit secondary antibody. Membranes were then washed three times in 1X-TBS, each for 15 min, developed with enhanced chemiluminescence (ECL) reagent (Cell Signalling Technology), and were exposed to ChemiDoc™ MP Imaging System to detect the chemiluminescence signals (Bio-Rad Laboratories).

Statistical analysis

GraphPad Prism Software version 9.3.1 (GraphPad Software) was used for statistical analysis. Semen characteristics and expression levels of miRNA and mRNA data were presented as mean ± SD and SE, respectively. To determine the sample size for each group, *a priori* power analysis with α and β error *p* probability of 0.05 was performed, showing that > 30 samples per group are

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TABLE 1 Semen characteristics of the enrolled men.

Parameters	Normozoospermic controls	Oligoasthenozoospermic men	*p*-value
Age (year)	25.7 ± 4.35	25.6 ± 4.16	0.9871
Volume (mL)	2.32 ± 0.58	1.96 ± 0.35	0.0047
pH	8.15 ± 0.37	8.16 ± 0.31	0.8367
Count (10^6/mL)	77.3 ± 11.3	9.39 ± 2.41	0.0001
Motility (% motile)	60.5 ± 10.2	20.4 ± 4.3	0.0001
Sperm vitality (eosin) (%)	65.44 ± 5.13	59.90 ± 7.22	0.0002
Morphology (%)	7.29 ± 2.09	5.71 ± 2.21	0.0012

Normozoospermic controls (n − 41) and oligoasthenozoospermic men (n − 41).
An unpaired two-tailed t-test was used to calculate the p-values.
Data were presented as mean ± standard deviation.
$p < 0.05$ was considered statistically significant.

needed for a reliable statistical analysis. An unpaired two-tailed t-test test was used to evaluate the differences in miRNA and mRNA expression levels between subfertile and fertile controls. The relative quantitative method was used to measure the dynamic change of miRNA and mRNA expression levels (Livak and Schmittgen, 2001). RNU6B small nuclear RNA (snRNA) as an endogenous reference miRNA as previously validated for this type of sample (Abu-Halima et al., 2013; Abu-Halima et al., 2014b; Salas-Huetos et al., 2015; Salas-Huetos et al., 2016; Abu-Halima et al., 2019; Abu-Halima et al., 2020a; Abu-Halima et al., 2020b; Abu-Halima et al., 2021a), and only miRNAs with a cycle threshold value (Ct) of < 35 were included in the analysis. Additionally, Spearman correlation analysis was performed to find the association between basic semen parameters and the expression levels of miR-19a/b-3p and significant target genes that showed a lower expression level. Adjustment for multiple testing was performed by controlling the false discovery rate (FDR) according to the approach of Benjamini and Hochberg and a p-value of less than 0.05 was considered statistically significant. Finally, the Cytoscape Software (v3.9.1) was used to study the functional enrichment of the DEGs from the dataset.

Enrichment and network analysis of target genes

The target genes for the miR19a/b-3p were predicted using multiple bioinformatics databases which were comparatively analyzed using the miRWalk algorithm online software (available at: http://mirwalk.uni-hd.de/). Then, we combined the outcomes from the top predicted target genes as predicted by miRWalk and 53 genes which were validated by RT-qPCR to Cytoscape software to find out the interactions among the predicted and validated target genes.

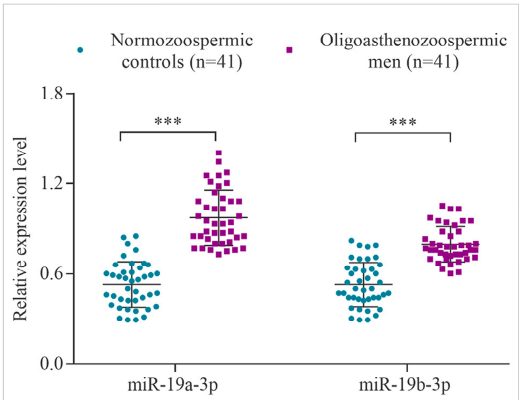

FIGURE 1
Expression levels of miR-19a/b-3p in the sperm samples collected from men with oligoasthenozoospermia (n = 41) and normozoospermia (n − 41) as determined by RT-qPCR. Data were presented as the relative expression level $2^{-\Delta\Delta Ct}$ of oligoasthenozoospermic and normozoospermic men. The p-value was calculated using an unpaired two-tailed Student's t-test and $p < 0.05$ was considered statistically significant (***$p < 0.001$).

Results

Characteristics of the study population

A total of 82 participants (including 41 subfertile men with oligoasthenozoospermia and 41 normozoospermic controls) were recruited for this study. The detailed clinical characteristics of individuals are listed in Table 1. Compared to fertile controls, the subfertile men with oligoasthenozoospermia were significantly different in terms of semen volume (p = 0.0047), sperm count ($p < 0.0001$), % motility ($p < 0.0001$), and % sperms exhibiting normal morphology (p = 0.0012). Other parameters, such as age and pH were not significantly different.

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TABLE 2 Significantly abundant levels of mRNAs in the sperm samples collected from men with oligoasthenozoospermia compared to men with normozoospermia as determined by RT-qPCR.

Human symbol and name	Fold change	log2	*p*-value	Adjusted *p*-value	Regulation
2A) target genes with lower expression level					
DPYSL5 (dihydropyrimidinase like 5)	0.21	−2.24	1.99×10^{-6}	1.47×10^{-4}	Lower
BOD1L2 (biorientation of chromosomes in cell division 1 like 2)	0.20	−2.33	8.07×10^{-6}	2.03×10^{-4}	Lower
MED26 (mediator complex subunit 26)	0.31	−1.68	1.36×10^{-5}	2.03×10^{-4}	Lower
UBQLNL (ubiquilin-like)	0.15	−2.77	1.37×10^{-5}	2.03×10^{-4}	Lower
ATF7IP2 (activating transcription factor 7 interacting protein 2)	0.30	−1.72	2.20×10^{-5}	2.72×10^{-4}	Lower
GOLGA6D (golgin A6 family member D)	0.09	−3.43	3.73×10^{-5}	3.94×10^{-4}	Lower
SENP8 (SUMO peptidase family member, NEDD8 specific)	0.14	−2.80	5.47×10^{-5}	4.31×10^{-4}	Lower
COX8C (cytochrome c oxidase subunit 8C)	0.12	−3.02	5.63×10^{-5}	4.31×10^{-4}	Lower
CCDC87 (coiled-coil domain containing 87)	0.12	−3.10	6.22×10^{-5}	4.31×10^{-4}	Lower
CSMD1 (CUB and Sushi multiple domains 1)	0.24	−2.06	6.70×10^{-5}	4.31×10^{-4}	Lower
RFX4 (regulatory factor X4)	0.28	−1.86	7.31×10^{-5}	4.31×10^{-4}	Lower
DCAF12L1 (DDB1 and CUL4 associated factor 12 like 1)	0.11	−3.14	7.58×10^{-5}	4.31×10^{-4}	Lower
CCER1 (coiled-coil glutamate-rich protein 1)	0.11	−3.16	1.06×10^{-4}	5.15×10^{-4}	Lower
SAMD4A (sterile alpha motif domain containing 4A)	0.27	−1.88	1.17×10^{-4}	5.15×10^{-4}	Lower
FAM104A (family with sequence similarity 104 member A)	0.21	−2.27	1.18×10^{-4}	5.15×10^{-4}	Lower
TKTL2 (transketolase like 2)	0.12	−3.03	1.31×10^{-4}	5.15×10^{-4}	Lower
TMEM215 (transmembrane protein 215)	0.26	−1.93	1.32×10^{-4}	5.15×10^{-4}	Lower
HSPA2 (heat shock protein family A (Hsp70) member 2)	0.13	−2.92	1.43×10^{-4}	5.30×10^{-4}	Lower
PCDHA7 (protocadherin alpha 7)	0.11	−3.15	2.63×10^{-4}	8.29×10^{-4}	Lower
REEP1 (receptor accessory protein 1)	0.11	−3.16	2.69×10^{-4}	8.29×10^{-4}	Lower
CSNK1G1 (casein kinase 1 gamma 1)	0.40	−1.33	2.75×10^{-4}	8.29×10^{-4}	Lower
POC1A (POC1 centriolar protein A)	0.44	−1.18	2.75×10^{-4}	8.29×10^{-4}	Lower
SPATA12 (spermatogenesis associated 12)	0.25	−1.98	2.80×10^{-4}	8.29×10^{-4}	Lower
ZNF280B (zinc finger protein 280B)	0.35	−1.52	3.01×10^{-4}	8.56×10^{-4}	Lower
AQP5 (aquaporin 5)	0.30	−1.75	4.43×10^{-4}	1.21×10^{-3}	Lower
TTLL2 (tubulin tyrosine ligase like 2)	0.30	−1.74	5.06×10^{-4}	1.30×10^{-3}	Lower
MYBL1 (MYB proto-oncogene like 1)	0.33	−1.60	5.09×10^{-4}	1.30×10^{-3}	Lower
TDRD10 (tudor domain containing 10)	0.35	−1.53	5.96×10^{-4}	1.39×10^{-3}	Lower
ELAVL2 (ELAV like RNA binding protein 2)	0.34	−1.56	6.04×10^{-4}	1.39×10^{-3}	Lower
ASAP2 (ArfGAP with SH3 domain, ankyrin repeat and PH domain 2)	0.40	−1.33	6.20×10^{-4}	1.39×10^{-3}	Lower
PRSS54 (serine protease 54)	0.30	−1.74	6.26×10^{-4}	1.39×10^{-3}	Lower
CPEB1 (cytoplasmic polyadenylation element binding protein 1)	0.30	−1.75	6.40×10^{-4}	1.39×10^{-3}	Lower
SPIRE1 (spire type actin nucleation factor 1)	0.44	−1.19	1.05×10^{-3}	2.15×10^{-3}	Lower
HSF5 (heat shock transcription factor 5)	0.36	−1.47	1.36×10^{-3}	2.72×10^{-3}	Lower
USP6 (ubiquitin specific peptidase 6)	0.33	−1.61	2.24×10^{-3}	4.36×10^{-3}	Lower
GOLGA6A (golgin A6 family member A)	0.31	−1.69	2.41×10^{-3}	4.54×10^{-3}	Lower
GNAT1 (G protein subunit alpha transducin 1)	0.43	−1.23	2.45×10^{-3}	4.54×10^{-3}	Lower
UBN2 (ubinuclein 2)	0.53	−0.92	2.93×10^{-3}	5.17×10^{-3}	Lower
C2orf42 (chromosome 2 open reading frame 42)	0.50	−1.01	3.22×10^{-3}	5.54×10^{-3}	Lower
C22orf31 (chromosome 22 open reading frame 31)	0.34	−1.56	5.00×10^{-3}	8.41×10^{-3}	Lower
FHL5 (four and a half LIM domains 5)	0.44	−1.19	5.21×10^{-3}	8.57×10^{-3}	Lower
DNAI1 (dynein axonemal intermediate chain 1)	0.41	−1.27	5.50×10^{-3}	8.85×10^{-3}	Lower
MCHR2 (melanin-concentrating hormone receptor 2)	0.42	−1.26	1.02×10^{-2}	1.61×10^{-2}	Lower
SCML2 (Scm polycomb group protein-like 2)	0.53	−0.90	1.11×10^{-2}	1.71×10^{-2}	Lower
DEPDC1 (DEP domain containing 1)	0.58	−0.78	1.16×10^{-2}	1.75×10^{-2}	Lower

(Continued on following page)

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TABLE 2 (*Continued*) Significantly abundant levels of mRNAs in the sperm samples collected from men with oligoasthenozoospermia compared to men with normozoospermia as determined by RT-qPCR.

Human symbol and name	Fold change	log2	*p*-value	Adjusted *p*-value	Regulation
STK33 (serine/threonine kinase 33)	0.40	−1.31	1.56×10^{-2}	2.31×10^{-2}	Lower
DDHD1 (DDHD domain containing 1)	0.54	−0.89	2.74×10^{-2}	3.97×10^{-2}	Lower
FAM169A (family with sequence similarity 169 member A)	0.67	−0.58	2.99×10^{-2}	4.25×10^{-2}	Lower
FSHR (follicle stimulating hormone receptor)	0.50	-0.99	3.09×10^{-2}	4.31×10^{-2}	Lower
ODF4 (outer dense fiber of sperm tails 4)	0.45	−1.16	3.27×10^{-2}	4.48×10^{-2}	Lower
BRCA2 (BRCA2 DNA repair associated)	0.55	−0.86	4.32×10^{-2}	*5.82 × 10^{-2}*	Lower
2B) target genes with higher expression level					
WNK3 (WNK lysine deficient protein kinase 3)	1.89	0.92	1.26×10^{-4}	5.15×10^{-4}	Higher
MBNL3 (muscleblind like splicing regulator 3)	2.21	1.14	8.96×10^{-4}	1.89×10^{-3}	Higher

Normozoospermic controls ($n = 41$) and oligoasthenozoospermic men ($n = 41$).
An unpaired two-tailed t-test was used to calculate the *p*-values.
False Discovery Rate (FDR) correction was used to adjust the *p*-values.
Significant changes in abundance levels are shown with an adjusted *p*-value < 0.05 or a border-line *p*-value (*italic font*).

The expression level of miR-19a/b-3p in the sperm samples of subfertile and fertile men

The expression levels of the hsa-miR-19a-3p and hsa-miR-19b-3p were determined using RT-qPCR in a cohort of 41 subfertile men with oligoasthenozoospermia and 41 normozoospermic controls. The levels of miR-19a-3p and miR-19b-3p were significantly increased in oligoasthenozoospermic men compared to the normozoospermic controls ($p = 1.21 \times 10^{-9}$ and 2.49×10^{-6}, respectively) (Figure 1). The mean expression level was 3.11-fold higher for miR-19a-3p and 2.78-fold higher for miR-19b-3p in subfertile oligoasthenozoospermic men compared to the normozoospermic controls.

The expression level of target genes in the sperm samples of subfertile and fertile men

RT-qPCR was conducted to confirm the differential expression level of the 82 targets in the same cohort of 41 oligoasthenozoospermic men and 41 normozoospermic controls used for the validation of the higher expression of miR-19a/b-3p. As shown in Supplementary Table S1, out of 82 tested target genes, 53 target genes showed a significant expression level, 19 target genes showed a non-significant expression level, and 10 target genes were omitted from further analysis due to either showing a Ct value of >35 and/or Ct value was not determined. Considering the direction of regulation along with the adjusted significant *p*-value, the differences were significant for 53 target genes (adjusted $p < 0.05$) including 51 targets with lower expression levels and 2 target genes (namely *WNK3* and *MBNL3*, $p = 5.15 \times 10^{-4}$ and 1.89×10^{-3}, respectively) with higher expression levels (Table 2). Moreover, a borderline significant expression level was observed for only one target gene, namely *BRCA2* ($p = 4.32 \times 10^{-2}$ and adjusted *p*-value $= 5.82 \times 10^{-2}$). Using hierarchical clustering with the Euclidean distance measure, we analyzed how the subfertile men and matched fertile controls were related to each other. For this task, we included the significant differential expression target genes (Supplementary Figure S1). We observed two not highly distinct clusters between the subfertile oligoasthenozoospermic men and fertile normozoospermic controls. The first cluster contains lower expression levels detected mostly in fertile controls, and the expression levels ranged from lower to higher in the subfertile men. A more detailed distinction between the tested groups based on the clustering dendrogram was, however, not conclusive.

Western blot analysis

To further confirm the results of RT-qPCR, a new set of semen samples collected from oligoasthenozoospermic men (n = 4) and four normozoospermic controls (n = 4) was analyzed by Western blot. Western blot results showed that the expression levels of anti-STK33 and anti-DNAI1 were consistent with the results of the RT-qPCR analysis. Specifically, a reduction in STK33 and DNAI1 expression levels was observed, as shown in Figure 2 and Supplementary Figure S2. The average quantified protein level of STK33 was 2.15 ± 0.82 in normozoospermic men versus 0.59 ± 0.10 in oligoasthenozoospermic men ($p = 0.017$), and for DNAI1 it was 0.9 ± 0.22 in normozoospermic men versus 0.31 ± 0.13 in oligoasthenozoospermic men ($p = 0.007$) Figure 2.

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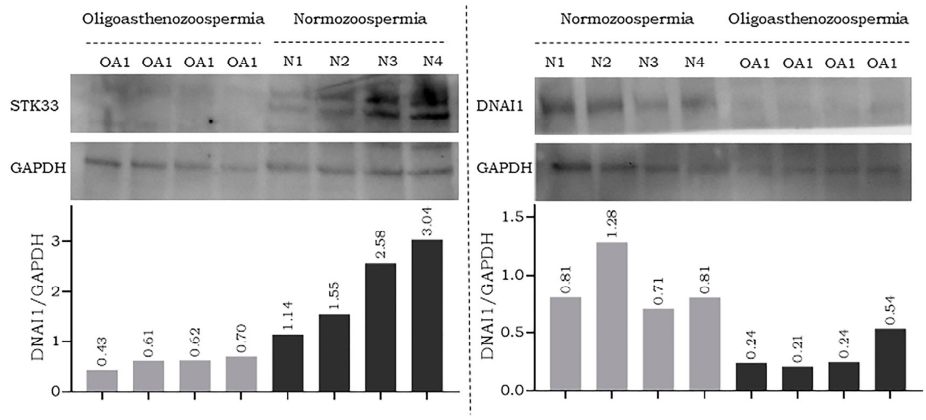

FIGURE 2
Western blot analysis and quantification of flagellar and cilia assembly proteins STK33 (approximately 58 kDa), DNAI1 (approximately 79 kDa), and the endogenous control GAPDH (approximately 37 kDa) in semen samples collected from men with oligoasthenozoospermia (OA) and normozoospermia (N).

TABLE 3 Correlation analysis between the expression level of miRNAs and basic semen parameters.

Spearman's correlation		Count (10^6/mL)	Motility (% motile)	Sperm vitality (eosin) (%)	Morphology (%)
miR-19a-3p	Correlation coefficient	−0.76	−0.73	−0.45	−0.29
	p value	0.0001	0.0001	0.0001	0.0076
miR-19b-3p	Correlation Coefficient	−0.64	−0.66	−0.46	−0.25
	p value	0.0001	0.0001	0.0001	0.0249

Spearman correlation analysis.
An Unpaired two-tailed t-test was used to calculate the *p*-value.
A significant change in abundance level was considered with a *p*-value < 0.05.

Correlation of expression levels (ΔCt) of miRNAs and target genes with basic semen parameters

We next analyze correlations between the expression level of significant genes and the basic semen parameters i.e., sperm count, motility, and morphology. Spearman correlation analysis showed a strong correlation between the miR-19a/b-3p and the sperm count and motility, and a weak correlation with the number of cells exhibiting normal morphology and sperm vitality (eosin, %) (Table 3). Furthermore, a weak to moderate correlation between the significant target genes associated with sperm motility and basic semen parameters. Specifically, correlation analysis showed that the lower the expression level of the 51 genes, the lower the sperm count, motility, and the number of cells exhibiting normal morphology ($p < 0.0001$) (Supplementary Table S2). By contrast, the higher the expression level of the 2 genes namely (*WNK3* and *MBNL3*), the lower the sperm count, motility, and the number of cells exhibiting normal morphology ($p < 0.0001$) (Supplementary Table S2). Similarly, a weak to moderate correlation was observed between the expression level of miR-19a/b-3p and the significant target genes associated with sperm motility (Supplementary Table S3).

Regulatory network construction

Using Cytoscape software, a regulatory network was constructed between the potential target genes which were predicted by at least 5 different algorithms of the miRWalk, and the 53 genes which were validated by RT-qPCR. A clear distinction between the predicted potential target genes and the validated target by RT-qPCR based on the regulatory networking

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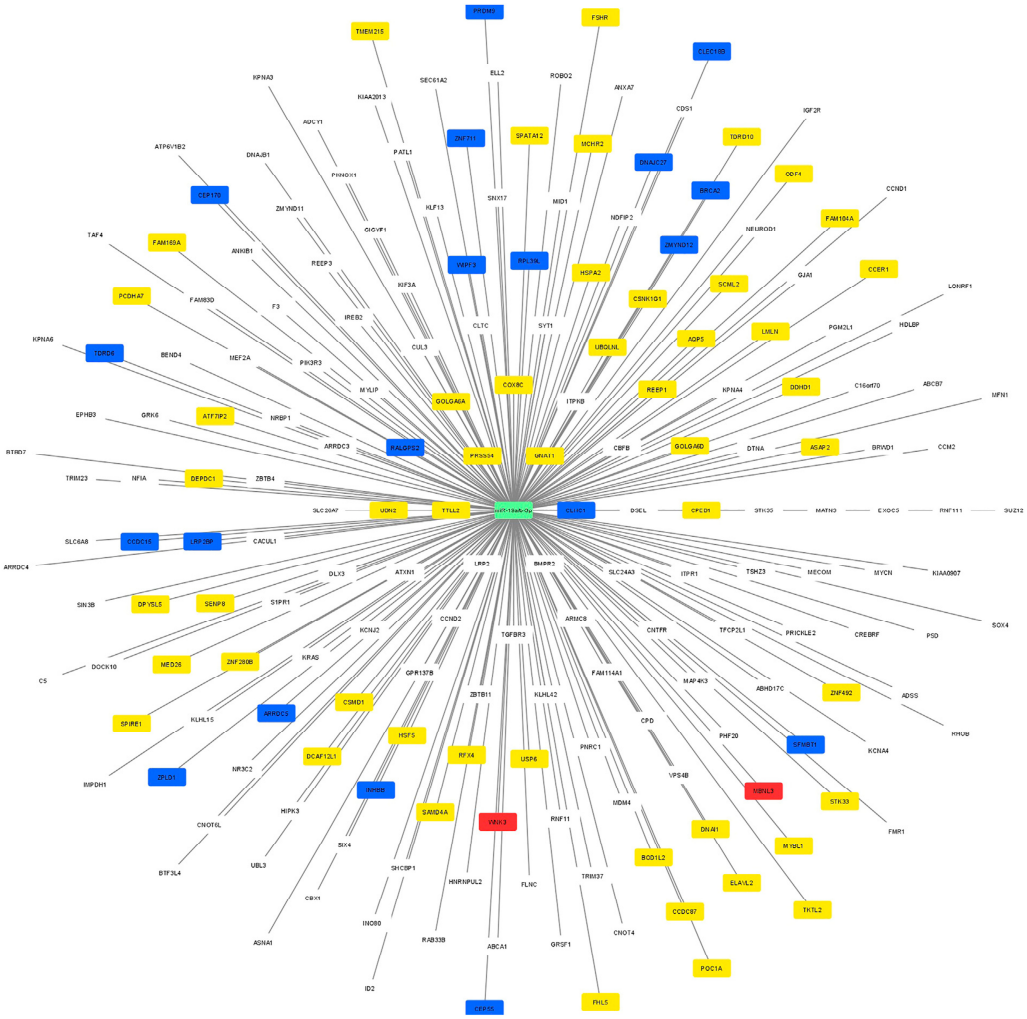

FIGURE 3
A regulatory network between the potential target predicted genes and the 74 genes which were included in the RT-qPCR. The yellow nodes showed the significantly lower expressed genes, the red nodes showed the significantly higher expressed genes, and the blue nodes represent the non-significant genes, as resulted from RT-qPCR.

was, however, not possible. Therefore, the regulatory network was constructed based on the highest differential 250 target genes and 2 miRNAs (miR-19a/b-3p). The significant differential expression value (log2 fold change and adjusted *p*-value) for each validated gene is shown in Figure 3. The yellow nodes showed the significantly lower expressed genes, the red nodes showed the significantly higher expressed genes, and the blue nodes represent the non-significant genes, as resulted from RT-qPCR.

Discussion

Since their discovery, miRNAs have been characterized in several cell types and tissues, and their biomolecular functions are being defined in an expanding number of biological processes (Bartel, 2018). Despite their differential expression in hundreds of species and their role as a crucial regulator during the developmental processes and diseases, the comprehension of their functions in detail needs to be gathered. Update to date,

many hundred genes are involved in the regulation of the spermatogenesis process, some of which are not exclusively expressed in the testis and control multiple physiological processes, and many other genes are exclusively expressed (Djureinovic et al., 2014; Uhlen et al., 2015). Aberration of gene expression at any stage of this complex and highly regulated process can lead to male subfertility. Additionally, it is not surprising that miRNAs also play a crucial role in regulating the process of sperm production at different levels, and their aberrant expression can lead to male subfertility. Thus, it is legitimate to hypothesize that differential miRNA patterns can be used to a large extent as promising tools for the treatment and diagnosis of male infertility (Salas-Huetos et al., 2020). Recently, hsa-miR-19a and hsa-miR-19b were highly dysregulated in purified sperm (Abu-Halima et al., 2013), seminal plasma (Wu et al., 2012), and testicular tissue (Abu-Halima et al., 2014a) of men with different types of unexplained infertility in comparison to control fertile men. The miR-19 family members, miR-19a and miR-19b-1 are located within the miR-17–92 gene cluster, which regulates spermatogenesis (Novotny et al., 2007; Tong et al., 2012; Liu et al., 2015; Xie et al., 2016). Specifically, miR-19a-3p was reported as an important regulator of the immune system within the testis (Parker and Palladino, 2017). In agreement with other studies (Cimadomo et al., 2019; Zhou and Dimitriadis, 2020), miR-19b-3p was differentially expressed in the spent culture media after embryo transfer and showed an AUC value for predicting positive pregnancy outcomes, both, from spent culture media and sperm collected from infertile couples attending infertility treatment (Abu-Halima et al., 2020b). These findings lead us to hypothesize that the miR-19 family could play an essential role in reproduction, especially in human spermatogenesis and embryonic development, and could influence male fertility when aberrantly expressed.

In our study, the higher expression level of miR-19a/b-3p was observed in sperm samples collected from oligoasthenozoospermic men compared to normozoospermic men using RT-qPCR validation analysis. To appraise the downstream effects of miR-19a/b-3p, we selected 82 potential target genes for further analysis using high throughput RT-qPCR. These target genes were selected by cross-match between the potential target genes predicted by miRWalk (3,066 potential target genes), testis-specific genes annotated in Human Protein Atlas (2,237 protein-coding genes), and the testis-specific genes that are related to spermatogenesis and male infertility as identified by the ToppGene Suite algorithm (Chen et al., 2009). The overlap of genes (130 target genes identified) was further filtered according to their functional role in spermatogenesis and sperm function, context score of mRNA-miRNA interaction, their binding site within the 3′UTR of the transcript, and seed sequence (7mer, 8mer). A total of 53 genes were significantly confirmed as downstream targets, including 51 target genes with lower expression and two genes with higher expression in sperm samples collected from oligoasthenozoospermic men compared to normozoospermic men. Taking into consideration the simultaneous higher expression of miR-19a/b-3p and lower expression of 51 target genes, we can assume a direct interaction between miRNA at the binding site in the 3′UTR of the target genes. Identification of spermatogenesis dysfunctions is usually done through the evaluation of the end product of the process; thus, sperm count, motility and morphology are typically the means of determining whether the spermatogenesis process is being completed as it should (excluding men with obstructive azoospermia). Even a small interruption of the spermatogenesis process can create major difficulties for couples attempting to conceive. Therefore, without proper spermatogenesis occurring, a male human will be subfertile or infertile. In our study, the identified higher miRNAs and lower mRNAs expression levels may lead to male subfertility and/or infertility. However, it is remarkable, that our studied miRNAs and their experimentally identified mRNA targets have previously been reported to play an essential role in the early stages of testicular differentiation and development, including a functional role in sperm motility and morphology.

A close look into the dysregulated target genes, some genes play a wide variety of functions within the testes, some other genes have an unknown or poorly characterized function and some other target genes are involved in the basal cellular processes without any clear function in spermatogenesis or infertility. The aberrant expression of some, if not most of these gene within the testis may disrupt some important cellular processes and ultimately lead to male subfertility or in worse cases lead to infertility and/or sterility. Furthermore, some other genes have functions that have been clarified only in model organisms, especially in mice. We argue if these genes could potentially show similar effects in humans. Based on previous studies, many genes were found to be associated with infertility or are dysregulated in various phenotypes, but it is still not clear their exact function in the male infertility. For example, *FAM104A* was down-regulated in men with non-obstructive azoospermia (NOA) (Hu et al., 2021), and *TMEM215* was up-regulated in spermatozoa of infertile men (Cheung et al., 2019) as determined by microarray and sequencing analyses, respectively. *MCHR2* has been recognized as one of 371 clinically relevant genes for infertility as determined by high-resolution chromosome ideograms analysis (Butler et al., 2016).

In our study, we reported several lower expressed genes that are associated with the early stages of sperm production during the spermatogenesis process. Of these target genes, *SPATA12* is one of the most well-known genes involved in the regulation of spermatogenesis and is mainly expressed in spermatocytes, spermatids, and spermatozoa (Dan et al., 2007). The amount of these precursor and mature cells were directly correlated with the expression level of *SPATA12* (Dan et al., 2007). The *UBQLNL* gene encodes Ubiquilin and is particularly active in the post-meiotic stages of spermatogenesis (Marin, 2014). Nevertheless, little is known about the exact function of *UBQLNL* in male fertility (Marin, 2014). Another spermatogenesis-associated gene

is *ELAVL2* which regulates the proliferation of spermatogonia and apoptosis (Yang et al., 2021). *ELAVL2* is conserved in spermatogonia stem cells (SSCs) and was down-regulated in NOA (Yang et al., 2021). SPIRE1 acts as a regulator of ectoplasmic specialization to modulate spermatogenesis by supporting germ cell development (Wen et al., 2018). Moreover, *SPIRE1* supports epithelial and endothelial cell function by the initiation of actin nucleation for building actin microfilaments efficiently (Wen et al., 2018). Of the target genes that were differentially down-regulated in sperm of subfertile as compared to fertile controls and play a role in the meiosis stage of spermatogenesis is *SCML2*. It is expressed in both gonads, and functions as a regulator of heterochromatin after the meiosis stage during spermatogenesis (Maezawa et al., 2018). *SCML2* has globally suppressed the somatic gene expression in late spermatogenesis, during meiosis, and into post-meiotic stages, while a distinct class of late spermatogenesis genes, is being activated (Hasegawa et al., 2015; Sin et al., 2015). *HSPA2* plays a key regulator role in the sperm maturation within the testis and capacitation in the reproductive tract of females. Additionally, *HSPA2* is a member of the 70 kDa heat shock protein family, that is, associated with the IVF outcome (Nixon et al., 2015b). Its main function is to support the folding, transport, and formation of protein complexes (Nixon et al., 2015b). A reduction of its expression leads to complications with sperm-egg recognition and fertilization, as reviewed in Nixon *et al.* (Nixon et al., 2015b). The reduction of HSPA2 protein expression level was observed in seminal plasma samples of men with oligozoospermia as compared to the controls (Nowicka-Bauer et al., 2022), suggesting the possibility of using *HSPA2* genes as a biomarker for spermatogenesis status, especially in cryptozoospermic males, and as a predictive biomarker for the success of assisted reproductive techniques (ART) (Nowicka-Bauer et al., 2022). Moreover, the expression of the *HSPA2* gene was down-regulated in human testes with abnormal spermatogenesis (Son et al., 2000). These findings indicate that these genes are potentially required for the completion of normal spermatogenesis and their loss of function in the case of aberrant expression could lead to a reduction of fecundity.

A portion of our identified lower expressed genes are not yet associated with the maturation of sperm but have structural functions and their absence may reduce sperm motility. *ODF4* gene, for example, encodes the outer dense fibers of the sperm tail, that surround the axoneme and protect the elastic rigidity of the sperm flagellum (Cao et al., 2006). The down-regulation of *ODF4* might result in abnormal sperm structure and motility. Another identified gene, DNAI1 originates from a dynein family, which is responsible for the cilia movement. Mutation of this gene leads to ciliary dyskinesia and male infertility (Knowles et al., 2012; Pereira et al., 2015; Precone et al., 2020). A reduction in the *DNAI1* gene might lead to ciliary loss of function, resulting in immotile sperm and ultimately infertility. Like *ODF4* and *DNAI1*, the absence of *STK33* leads to reduced motility and morphological changes in the flagellum. The encoded serine/threonine kinase seems to play a crucial role in the organization of flagellar ultrastructure (Ma et al., 2021). *AQP5* is one of the aquaporin family and is required for cell volume, by regulating the permeability of the cell membrane (Liu et al., 2006). In sperm, aquaporins protect cells from harmful swelling to maintain normal sperm function and survival (Chen and Duan, 2011). Downregulation of *AQP5*, as shown in our results, might decrease sperm function and could lead to apoptosis. In addition to spermatogenesis and structural properties, hormonal regulation also plays a major role in the development of infertility. Our results indicated that *FSHR* is down-regulated in patients with oligoasthenozoospermia. *FSHR* functions as a receptor of follicle-stimulating hormone (FSH), which stimulates several pathways to control the trophic and mitotic effects of Sertoli cells (Casarini et al., 2020). Previous studies indicated that a loss of FSHR reduced the FSH-dependent intracellular signaling (Casarini et al., 2014) and the volume of testes (Grigorova et al., 2013). Therefore, we assume that a reduction in FSHR expression might reduce the fertility of men.

In summary, we would like to point out that this study has also a number of limitations. The major limitation is the lack of miRNA-target interaction validation experiments to elucidate the complex downstream effects of miR-19a/b-3p and to lay the ground for an experimentally validated network in sperm. For the validation procedure, the Luciferase-Assay was globally used to confirm the miRNA binding site within the 3′UTR regions of the target gene. Experimentally, transfection of the human embryonic kidney cell line (HEK-293T) with both, the expression vector (pSG5), which includes the precursor miRNA of interest, and the reporter plasmid (pMiR-RNL-TK), which encodes the binding site of the miRNA within the 3′UTR of the target gene. The direct interactions between the over-expressed miRNA (miR-19a/b-3p) and 3′UTR of mRNAs (significant 51 target genes with lower expression levels), down-regulate the firefly luciferase protein resulting in the reduction of luminescence that will be relatively quantified. In our study, we were unable to produce the expression plasmid, that has the ability to over-expresses the miR-19a-3p and/or miR-19b-3p. We designed a variety of plasmid inserts, which encodes the precursor miRNAs with 50 bp up and downstream, the sequence which forms the hairpin structure, only the sequence of precursor, the full length 17–92 cluster, and the miR-19b precursor sequence with the surrounding miRNA of the cluster (data not shown). Furthermore, the precise miRNA-target interaction validation experiments should be evaluated in human germ cell-specific cell lines, not HEK-293T, However, human germ cell line has not yet been available on the market. Nevertheless, consistent with the idea of post-transcriptional down-regulation of mRNA while miRNA is over-expressed at the same time, our results give us, however, an indication of the complex downstream effects of miR-19a/b-3p and their target genes. The outcome of our study provides further insights into

the miRNA-target networks and might contribute to a deeper understanding of idiopathic infertility and the future use of miRNA as a biomarker for infertility.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Saarland Medical Association (Ha 195/11/updated June 2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design: MA-H, Sample collection and clinical data: MA-H, Experimental work, and data analysis: MA-H and LB, Study supervision: MA-H and EM, Writing of the original version: MA-H and LB (discussion section), Revision of the version: MA-H, LSB, BA, and EM, Approval of the article: all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.973849/full#supplementary-material

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3.3. Proteomic landscape of human sperm in patients with different spermatogenic impairments

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Proteomic Landscape of Human Sperm in Patients with Different Spermatogenic Impairments

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Abstract: Although the proteome of sperm has been characterized, there is still a lack of high-throughput studies on dysregulated proteins in sperm from subfertile men, with only a few studies on the sperm proteome in asthenozoospermic and oligoasthenozoospermic men. Using liquid chromatography–mass spectrometry (LC-MS/MS) along with bioinformatics analyses, we investigated the proteomic landscape of sperm collected from subfertile men (n = 22), i.e., asthenozoospermic men (n = 13), oligoasthenozoospermic men (n = 9) and normozoospermic controls (n = 31). We identified 4412 proteins in human sperm. Out of these, 1336 differentially abundant proteins were identified in 70% of the samples. In subfertile men, 32 proteins showed a lower abundance level and 34 showed a higher abundance level when compared with normozoospermic men. Compared to normozoospermic controls, 95 and 8 proteins showed a lower abundance level, and 86 and 1 proteins showed a higher abundance level in asthenozoospermic and oligoasthenozoospermic men, respectively. Sperm motility and count were negatively correlated with 13 and 35 and positively correlated with 37 and 20 differentially abundant proteins in asthenozoospermic and oligoasthenozoospermic men, respectively. The combination of the proteins APCS, APOE, and FLOT1 discriminates subfertile males from normozoospermic controls with an AUC value of 0.95. Combined APOE and FN1 proteins discriminate asthenozoospermic men form controls with an AUC of 1, and combined RUVBL1 and TFKC oligoasthenozoospermic men with an AUC of 0.93. Using a proteomic approach, we revealed the proteomic landscape of sperm collected from asthenozoospermic or oligoasthenozoospermic men. Identified abundance changes of several specific proteins are likely to impact sperm function leading to subfertility. The data also provide evidence for the usefulness of specific proteins or protein combinations to support future diagnosis of male subfertility.

Keywords: sperm; male subfertility; oligoasthenozoospermia; asthenozoospermia; proteome

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1. Introduction

Infertility defined by the inability to achieve pregnancy within one year of unprotected intercourse is a widespread problem, affecting about 15% of couples worldwide [1]. Infertility can be caused by either male or female reproductive issues. Various medical conditions including malignancies, infections, urogenital conditions, or genetic causes can contribute to male infertility. According to the guidelines of the European Association of Urology (EAU), 30–40% of men in their reproductive age are affected by idiopathic infertility [1]. The routine semen parameters evaluated according to the World Health Organization such as sperm count, motility, and the number of sperm with normal morphology and vitality guidelines are insufficient to fully understand the mechanisms of male subfertility. Towards a better functional understanding, RNAs have been correlated

to sperm motility, count, and to a lesser extent to sperm morphology [2–5]. These studies showed a direct link between the messenger RNAs (mRNAs) and different classes of non-coding RNAs such as microRNAs (miRNAs) in men undergoing infertility treatment. As for the more holistic approaches, a total of 60,505 transcripts have been identified for the sperm transcriptome including 11,688 differentially expressed transcripts in infertile and fertile men (2022) [6]. A total of 6871 proteins have been identified for the sperm proteome, as reviewed by [7]. There is, however, a lack of high-throughput studies on sperm proteome and specifically on dysregulated proteins in sperm of subfertile men. Only few studies focus on the sperm proteome in asthenozoospermic men and no proteomic studies on oligoasthenozoospermic men [8–16]. The understanding of male subfertility will largely benefit from a better knowledge of the sperm proteome in general and of the proteins that show differential abundance in specific types of subfertility. Likewise, the function of most sperm proteins awaits further elucidation although first studies on functional clustering did already associated proteins with sperm motility, the capacity of fertilization, structural sperm composition, and sperm energy metabolism [15,17,18]. Here, we employed liquid chromatography mass-spectrometry (LC-MS/MS)-based proteomics to investigate the proteomic landscape and to identified differential abundant proteins of human sperm collected from asthenozoospermic and oligoasthenozoospermic men as compared to normozoospermic controls. Our findings lay the basis to further the discovery of new diagnostic biomarkers for specific forms of male infertility. In the long run, our proteomic data will also help to find therapeutic targets for the treatment of male infertility.

2. Materials and Methods

2.1. Study Population and Sample Collection

Proteomics analysis was performed using sperm samples collected from a total of 53 men attending infertility treatment. Semen samples were classified for primary semen parameters based on WHO 2010 guidelines. These parameters, when taken together, have specified our groups. These groups were specifically classified based on the sperm count ($\geq 15 \times 10^6$/mL) and progressive motility ($\geq$32%, motile), resulting in a total of 31 normal men (normozoospermic men, N) and a total of 22 men with at least one abnormal semen parameter (subfertile men, AN). Subfertile men were additionally subdivided into oligoasthenozoospermia (OA, $n = 9$) and asthenozoospermia (A, $n = 13$) as summarized in Figure 1A. More detailed information on the sperm motility of the participants is given in Table S5. All the included men exhibited sperm morphology with an average percentage of $\geq$4. Semen samples were collected after at least three days of sexual abstinence and immediately liquefied for 30 min at 37 °C. Sperm were purified using discontinuous PureSperm® density gradient (Nidacon international AB, Mölndal, Sweden) to eliminate somatic cells, round cells, and leukocytes as previously described [19,20]. The study complies with the declaration of Helsinki and was approved by the Institutional Review Board (Ha 195/11/updated June 2021) of the Saarland Medical Association. Ethical guidelines were also followed in the conduction of the research, with patients' and controls' written informed consent obtained before experiments.

2.2. Protein Lysis

Sperm samples were thawed on ice and washed three times with 1× Phosphate-Buffered Saline (PBS) to eliminate contaminants and discontinuous density gradient residues. Samples were mixed with 100 µL of Lysis Buffer [4% SDS, 100 mM Tris/HCl pH 7.6, 0.1 M DTT] and incubated for 5 min at 95 °C. Afterward, each sample was sonicated on ice 10 times each 10 s at 20 joules for 2 s. After 30 min of incubation on ice, samples were centrifuged at 14,000× *g* for 10 min and the supernatant was collected in a separate collection tube and stored at −80 °C.

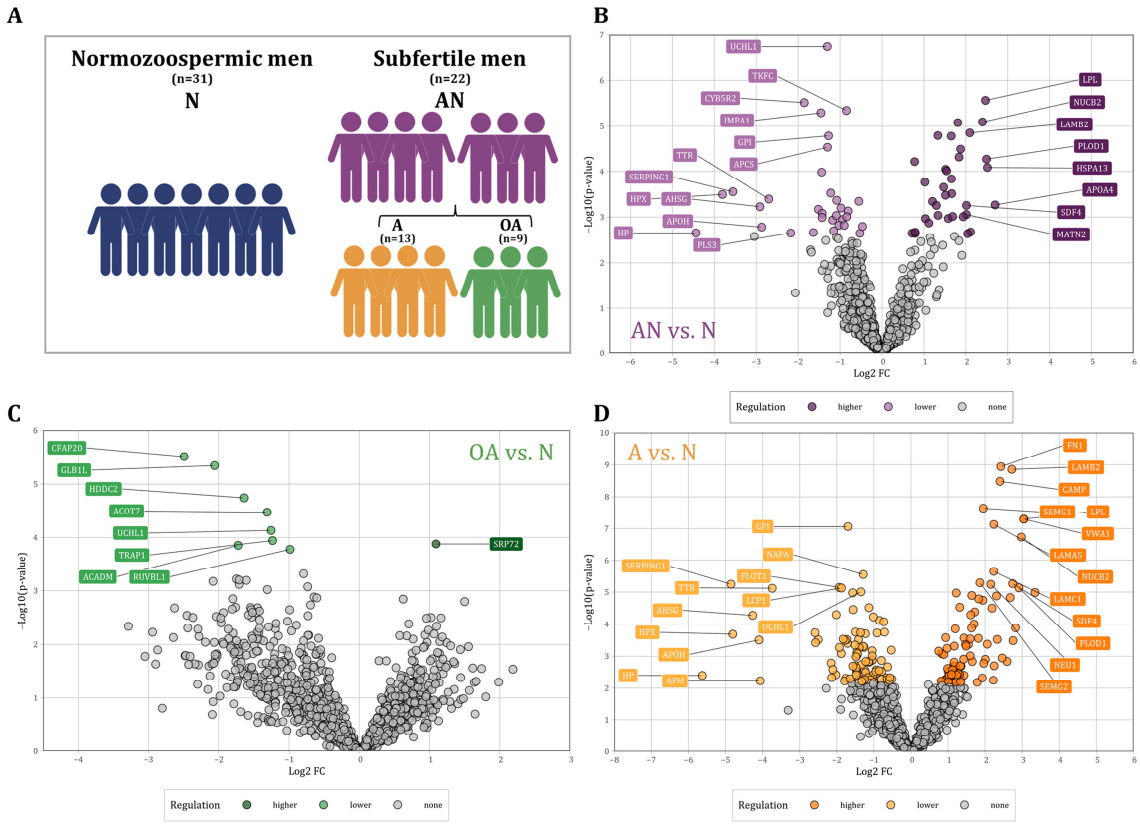

Figure 1. (**A**) Schematic of the study population and their classification into normozoospermic men (N), subfertile men (abnormal, AN) and their specific phenotypes, asthenozoospermic men (A), and oligoasthenozoospermic men (OA). (**B**–**D**): Volcano plot showing the differential abundance levels, i.e., the log2 Fold Change (FC) plotted against the −log10 *p*-value of proteins in sperm collected from (**B**) the subfertile men (n = 22) compared to the normozoospermic controls (n = 31, AN vs. N), (**C**) the oligoasthenozoospermic men (n = 9) compared to the normozoospermic controls (n = 31, OA vs. N), and (**D**) the asthenozoospermic men (n = 13) compared to the normozoospermic controls (n = 31, A vs. N). Significantly abundant proteins were highlighted by color (adjusted p-value < 0.05).

2.3. Peptide Preparation and LC-MS/MS Analysis

To remove SDS from protein lysates, a filter-aided sample preparation (FASP)-method was carried out as described by Wisniewski et al. [21]. Ninety microliters of protein lysate were mixed with 600 µL freshly prepared UA solution [8 M Urea in 0.1 M Tris/HCl pH 8.5], transferred to the Microcon-centrifugal filter unit (MRCF0R030, Merck-Millipore, Darmstadt, Germany) and centrifuged at 14,000× *g* for 15 min at room temperature (RT). Then, the filter unit was washed twice with 200 µL of UA solution at 14,000× *g* for 15 min to remove the remaining SDS. The carbamidomethylation of thiols was achieved with 100 µL of IAA solution (0.05 M iodoacetamide in UA) in a 30-min incubation step in the dark. Residual IAA was eliminated by centrifugation at 14,000× *g* for 15 min at RT and three following washing steps with 100 µL UA solution. After washing the filter units three times with 100 µL of ABC buffer (0.05 M NH4HCO3 in H_2O), each at 14,000× *g* for 15 min at RT, proteins were digested with 40 µL ABC buffer containing trypsin (trypsin to protein ratio 1:100) overnight in a wet chamber at 37 °C for approximately 18 h. After removing the ABC-trypsin mixture by centrifugation at 14,000× *g* for 15 min, 50 µL of 0.5 M NaCl was added to elute the peptides from the centrifugal filter unit by centrifuging at 14,000× *g*

for 15 min at RT. The obtained peptides were acidified with CF3COOH (Trifluoroacetic acid, TFA; Final Conc. 1%) and tested for their acidity with pH paper (pH < 2). Before applying the peptides to the MS-System, digested peptides were cleaned, desalted, and concentrated using the "Stage Tips technique" with self-made C18 filter tips, as described by Rappsilber et al. [22]. After Elution, the peptides were vacuum dried, resuspended in 20 µL of 0.1% Formic Acid (Buffer A) and the concentration of peptides was determined using the Pierce™ Quantitative Colorimetric Peptide Assay kit (Thermo Fisher Scientific Inc., Waltham, Mass., USA). For MS analysis, desalted peptides were resuspended in buffer A (0.1% (*v*/*v*) formic acid) and separated on a 50 cm reverse phase column with an inner diameter of 75 mm (New Objective from Woburn, MA, USA) packed in-house with 1.8 mm ReproSil-Pur 120 C18-AQ particles (Dr. Maisch GmbH, Ammerbruch-Entrigen, Germany) using a 180 min non-linear gradient of 2–95% buffer B (0.1% (*v*/*v*) formic acid, 80% (*v*/*v*) acetonitrile) at a flow rate of 250 nL/min. All MS data were recorded with a data-dependent acquisition strategy. Survey scans were acquired with a resolution of 60,000 at m/z = 200. The top 15 most abundant precursors with a charge >2 were selected for fragmentation. MS/MS scans were acquired with a resolution of 15,000 at m/z = 200. All other parameters can be obtained from raw files available at the ProteomExchange repository.

2.4. Data Processing and Statistical Analysis

The processing of LC-MS/MS data was performed with MaxQuant software (v1.6.3.3, Max Planck Institute of Biochemistry, Martinsried, Germany) [23]. All data were matched with the human reference proteome database (UniProt: UP000005640) with protein and peptide FDR < 1%. The mass spectrometry proteomics data (raw data, MaxQuant Output parameters, and tables) have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD039703. Label-free quantified (LFQ) intensities of all protein groups along the 53 samples were preprocessed using R software (v4.2.1, R Core Team, Vienna, Austria). R Script available upon request. Downstream analysis and visualization of results was performed using the R packages operators (v0.1–8), tidyverse (v1.3.2), readxl (v1.4.0), ggplot2 (v3.3.6), VennDiagram (v1.7.3), UpSetR (v1.4.0), pheatmap (v1.0.12), plotly (v4.10.0), gplots (v3.1.3), gridExtra (v2.3), grid (v4.2.1), lattice (v0.20-45), ggrepel (v0.9.1), extrafont (v0.18), showtext (v0.9-5), Rttf2pt1 (v1.3.10), systemfonts (v1.0.4), RColorBrewer (v1.1-3) and/or viridis (v0.6.2). LFQ intensities were log2 transformed and protein groups were filtered to eliminate contaminants, reverse hits, and proteins identified by site only. Protein groups that were identified in at least 70% of all samples were kept for further processing. An unequal variance *t*-test was used to call proteins with significant enrichment in the subfertile men (AN) and their respective subgroups (OA and A) compared to the normozoospermic men (N) and *p*-values were adjusted for multiple testing with the Benjamini–Hochberg procedure [25] and fold changes of the mean LFQ intensities of the respective comparisons of samples were determined. Spearman's correlation between the LFQ intensities and selected semen parameters (sperm count and progressive sperm motility) was generated using the R package Hmisc (v4.7-2). The correlation coefficients of all proteins are provided in Table S3. To perform ROC and logistic regression analysis, missing values were replaced with values drawn from a normal distribution centered around the detection limit of the MS instrument with a width of 0.3 and a downshift of 1.8 with respect to the standard deviation and mean of all protein intensities of each sample [26]. ROC and logistic regression analysis were performed using the R package pROC (v1.16.0), glmnet (v2.0_18), caret (v6.0_86), broom (v0.5.6), and Matrix (v1.2_18). To increase the predictive potential of a single protein as a biomarker, we build statistical models combining 2 or 3 proteins. Feature selection for a binary logistic regression classification model with L1 regularization was used to reduce the number of proteins tested and obtain the best biomarker combination (LASSO Method). L1 regularization reduces the coefficients of the model features such that some of the features have coefficients of 0, resulting in feature selection. The data were split into a training set (80%) and a test set (20%) for each of the compared classes. The model was fit to the training

set and the performance of the model was evaluated against the test set. After selecting features for each of the comparison groups, combinations of size 2 and 3 were selected from the protein subsets, and a logistic regression model was fitted to each of these combinations. Three-fold cross-validation was used to calculate the AUC of the ROC for each of these models to identify the protein combinations that yielded the highest values.

3. Results

3.1. Basic Semen Characteristics of Patients with Subfertility Compared to Normozoospermic Men

We analyzed sperm samples from normozoospermic (n = 31) and subfertile men (n = 22) including 9 oligoasthenozoospermic and 13 asthenozoospermic men (Table 1). Normozoospermic men were significantly different in sperm count and progressive motility compared to subfertile men (i.e., oligoasthenozoospermia and asthenozoospermia). Oligoasthenozoospermic and asthenozoospermic men were significantly different in combined sperm count and motility and in combined sperm count, motility, and morphology compared to normozoospermic men (Table 1). Other parameters including age and volume, were not significantly different in these comparisons.

Table 1. Basic semen characteristics of the comparisons subfertile vs. normozoospermic controls (AN vs. N), oligoasthenozoospermic men vs. normozoospermic controls (OA vs. N) and asthenozoospermic men vs. normozoospermic controls (A vs. N).

Parameters	N (n = 31)	AN (n = 22)	OA (n = 9)	A (n = 13)	N vs. AN	N vs. OA	N vs. A
Age	35.8 ± 7.3	33.5 ± 8.3	33.2 ± 7.2	33.7 ± 9.3	n.s.	n.s.	n.s.
Volume (mL)	3.3 ± 1.8	4.1 ± 2.8	4.5 ± 3.4	3.8 ± 2.4	n.s.	n.s.	n.s.
Count (10^6/mL)	86.7 ± 39.6	26.9 ± 19.6	9.1 ± 3.4	39.2 ± 16.3	***	***	***
Motility (% motile)	52.0 ± 10.6	12.3 ± 9.0	14.9 ± 11.3	10.5 ± 7.0	***	***	***
Morphology (%)	7.0 ± 8.2	9.6 ± 5.7	6.2 ± 3.8	11.9 ± 5.8	n.s.	n.s.	*

Data were presented as mean ± standard deviation. Unpaired two-tailed t-test was performed. p-value < 0.05 was considered as statistically significant. *** $p < 0.001$; * $p < 0.05$; n.s. = not significant.

3.2. Differentially Abundant Proteins in Sperm as Determined by LC-MS/MS

Proteomic analysis of sperm samples collected from subfertile men (n = 22) and normozoospermic men (n = 31) was performed using LC-MS/MS analysis. We found a total of 4412 distinct proteins that were present in at least one of the samples. In the following, we restrict our analysis to 1336 proteins that were present in at least 70% of the samples. A total of 204 proteins were differentially abundant in subfertile men as compared to normozoospermic men with a p-value < 0.05. (Figure 1). Out of these proteins, 75 proteins showed a higher and 129 a lower abundance level in sperm of subfertile men as compared to normozoospermic men. After adjustment for multiple testing [25], we identified 66 proteins including 34 proteins with significantly higher and 32 proteins with significantly lower abundance levels (Figure 1B). The comparison between oligoasthenozoospermic men (n = 9) and normozoospermic men (OA vs. N) revealed 271 differentially abundant proteins with a p-value < 0.05 including 203 proteins with lower and 68 proteins with higher abundance levels in sperm of oligoasthenozoospermic men. After adjusting for multiple testing, eight proteins had significantly lower, and one protein had a significantly higher abundance level in oligoasthenozoospermic men (Figure 1C). The comparison between asthenozoospermic men (n = 13) and normozoospermic men (A vs. N) identified a total of 417 differentially abundant proteins including 245 proteins with lower and 172 proteins with higher abundance levels in asthenozoospermic men. After adjusting for multiple testing, we identified 181 proteins including 95 proteins with lower and 86 proteins with higher abundance levels in asthenozoospermic men (Figure 1D). These results are summarized in Table S1.

3.3. Dysregulated Proteins between Subfertile and Normozoospermic Men

We next differentiated between proteins that were dysregulated only in a single, in several, or in all of the above-mentioned comparisons. We restricted these analyses to proteins that were differentially abundant after adjustment for multiple testing (adjusted p-value < 0.05). As illustrated in Figure 2, UCHL1 protein was the only protein, which showed a lower abundant level in all comparisons. The HDDC2 protein showed a lower abundant level in both, the specific comparison of OA vs. N and the general comparison AN vs. N. Far more proteins, i.e., 55 proteins were differentially abundant in both, the specific comparison A vs. N and the general comparison AN vs. N, including 22 lower and 33 higher abundant proteins. Likewise, a larger number of proteins (n = 125), including 72 lower and 53 higher abundant proteins, were found only in the comparison A vs. N. Few proteins were exclusively found in the comparison OA vs. N (n = 7), including six lower abundant proteins (CFAP20, GLB1L, ACADM, ACOT7, TRAP1, RUVBL1) and one (SRP72) higher abundant protein. A total of eight proteins including STOM, PPP3CC, FHL1, TEX101, PRKACG, TSPAN16, RPS27A, and CCT7 were exclusively found with a lower abundant level in the general comparison AN vs. N. CANX was the only protein found in a higher abundant level in the comparison AN vs. N (Figure 2A,B). A detailed list on the proteins is given in Table S2.

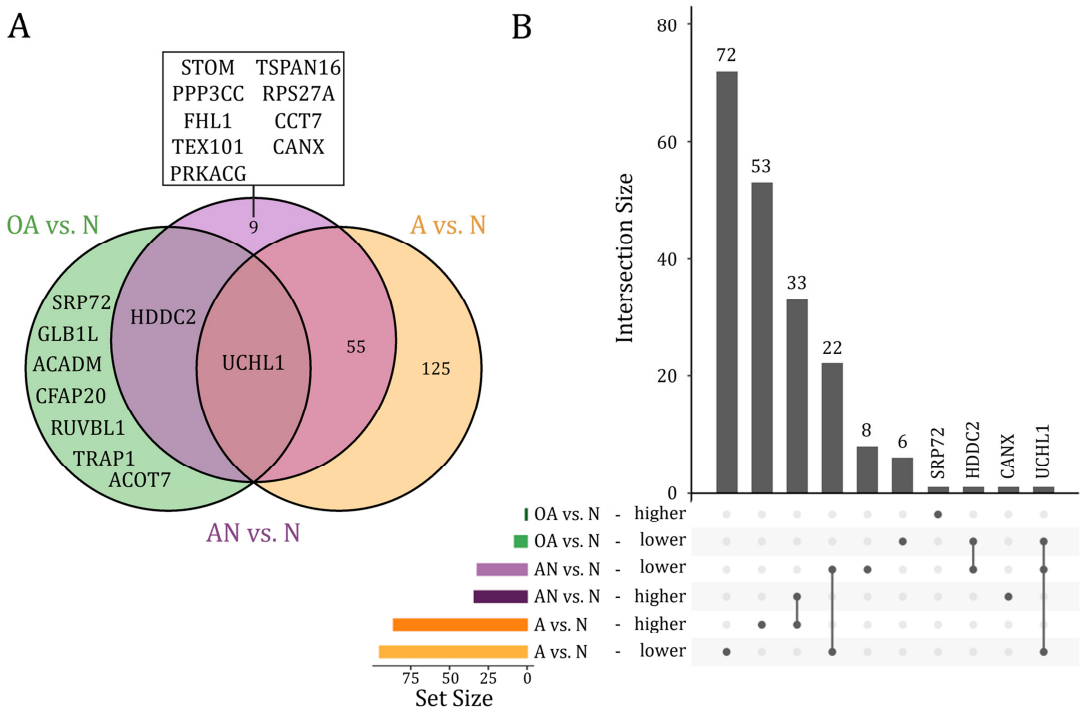

Figure 2. (**A**) Venn diagram and (**B**) Upset plot of proteins that showed significantly different abundance levels (adjusted p-value < 0.05) in the comparison of subfertile men compared to the normozoospermic controls (AN vs. N), the oligoasthenozoospermic men compared to the normozoospermic controls (OA vs. N), and the asthenozoospermic men compared to the normozoospermic controls (A vs. N).

3.4. Correlation of Proteins with Progressive Sperm Motility and Sperm Count

We next performed correlation analysis to examine the relationship between protein abundance levels and sperm motility and count. Considering only proteins with a correlation coefficient of $r \leq -0.5$ and $r \geq 0.5$, we observed 35 proteins that were negatively and 20 proteins that were positively correlated with sperm motility (Figure 3A and Table 2, $p < 0.05$). Figure 3B shows heatmaps for both the positively and negatively correlated proteins with the proteins vertically clustered by their z-score intensity and horizontally sorted by the sperm motility. As expected, the samples of the normozoospermic men showed higher sperm counts and were clearly separated from the samples of the subfertile men. However, the oligoasthenozoospermic and the asthenozoospermic samples were not separated into two different clusters. Using the same correlation coefficients as above, we observed 13 proteins that were negatively and 37 proteins that were positively correlated with sperm count (Figure 3C and Table 2, $p < 0.05$). Figure 3D shows heatmaps for the positively and negatively correlated proteins vertically clustered by their z-score intensity and horizontally sorted by the sperm motility. In contrast to sperm count, the samples of normozoospermic and subfertile men were not clearly separated by sperm motility with normozoospermic samples interspersed within the cluster of the samples from subfertile men and vice versa subfertile samples interspersed within the cluster of normozoospermic samples. The results of the correlation analyses are summarized in Table S3.

3.5. Directions of Deregulation and Evidence for Diagnostic Accuracy

Considering the significant dysregulated proteins listed in Table S1 ($p < 0.05$), we further analyzed the identified proteins based on the direction of regulation of the general (i.e., AN vs. N) and the specific comparisons (OA vs. N, and A vs. N). As shown in Figure 4A, most proteins that were upregulated in AN vs. N were also upregulated in OA vs. N. Likewise, most proteins downregulated in AN vs. N were also regulated in the same direction in OA vs. N. Several proteins showed the same fold change of deregulation in both comparisons. Similarly in Figure 4B, most proteins that were upregulated in AN vs. N were also upregulated in A vs. N. Likewise, most proteins downregulated in AN vs. N were also regulated in the same direction in A vs. N. Several proteins showed the same fold change of deregulation in both comparisons. The correlation between the two comparisons A vs. N and AN vs. N was even more pronounced. Here, we found an extended number of proteins that were deregulated with the same fold change in both comparisons. Several commonly deregulated proteins were strongly downregulated with a fold change below −3. The comparison of proteins, which are significantly deregulated in both or one of the specific comparisons A vs. N and/or OA vs. N, reveals contradictory findings (Figure 4C). Many proteins are inversely regulated depending on the comparison. A total of 14 proteins (FAM209A, COX7C, MLF1, COX5B, PDHB, MDH2, H1FNT, SUN5, HADHA, SPAM1, ATP5B, ETFA, PTPMT1, TUBB4B), which are significant in both comparisons are lower in their abundance level in the OA vs. N comparison and higher in their abundance level in the A vs. N comparison (Figure 4C). A total of eight proteins (ABHD14B, PSME1, RAB3D, RAB10, RAB27A, CYB561, MLPH, DOPEY2) showed a higher abundance level in OA vs. N comparison and a lower abundance level in A vs. N comparison (Figure 4C).

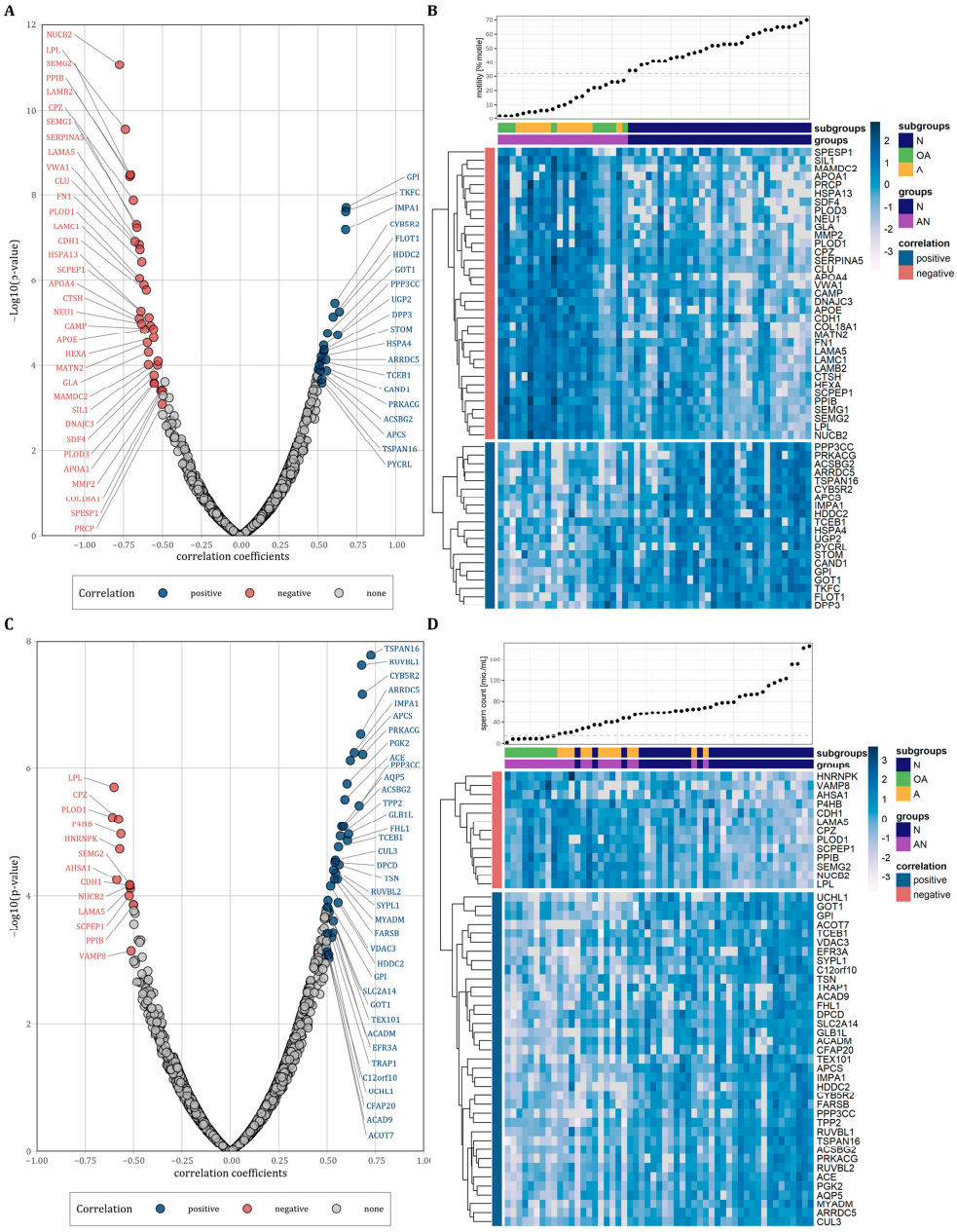

Figure 3. (**A**,**C**): Volcano plots of correlations between protein abundance levels of all identified proteins and sperm motility or sperm count plotted against the −log10 *p*-value, respectively. Correlated proteins were highlighted by color (r > 0.5 and r < −0.5). (**B**,**D**): Heatmaps representing vertically hierarchical clustering of the abundance levels of proteins that were correlated with sperm motility or sperm count, respectively (r > 0.5 and r < −0.5). Horizontally, the proteins were sorted by increasing sperm motility or sperm count, respectively. The colour of rectangles represents *z*-scored protein abundance levels (white, lower abundant proteins; dark blue, higher abundant proteins; light blue, no change).

Table 2. Correlation of protein abundance level as determined by LC-MS/MS with the basic semen parameters sperm motility and sperm count.

A: Negatively Correlated Proteins							
Experimental ID	**Gene Symbol**	**Motility (% motile)**			**Count (10^6/mL)**		
		r	***p*-Value**	**Correlation**	**r**	***p*-Value**	**Correlation**
3707	NUCB2	−0.78	8.65×10^{-12}	negative	−0.52	6.90×10^{-5}	negative
2530	LPL	−0.74	2.86×10^{-10}	negative	−0.6	1.97×10^{-6}	negative
3510	LAMB2	−0.71	3.75×10^{-9}	negative	−0.5	1.70×10^{-4}	-
2938	PPIB	−0.71	3.53×10^{-9}	negative	−0.5	1.37×10^{-4}	negative
3774	SEMG2	−0.71	3.38×10^{-9}	negative	−0.52	6.75×10^{-5}	negative
4530	CPZ	−0.69	1.33×10^{-8}	negative	−0.58	6.18×10^{-6}	negative
4609	VWA1	−0.68	1.24×10^{-7}	negative	−0.42	3.04×10^{-3}	-
2475	SEMG1	−0.67	4.83×10^{-8}	negative	−0.49	1.84×10^{-4}	-
2497	SERPINA5	−0.66	5.81×10^{-8}	negative	−0.5	1.38×10^{-4}	-
2521	APOA4	−0.65	7.96×10^{-6}	negative	−0.39	1.50×10^{-2}	-
1961	LAMA5	−0.65	1.45×10^{-7}	negative	−0.52	7.64×10^{-5}	negative
3782	PLOD1	−0.65	8.84×10^{-7}	negative	−0.61	5.77×10^{-6}	negative
2673	CLU	−0.64	1.89×10^{-7}	negative	−0.4	3.29×10^{-3}	-
3308	HSPA13	−0.64	5.39×10^{-6}	negative	−0.49	1.00×10^{-3}	-
5763	NEU1	−0.64	1.07×10^{-5}	negative	−0.5	1.04×10^{-3}	-
2431	FN1	−0.63	3.64×10^{-7}	negative	−0.46	4.95×10^{-4}	-
2420	APOE	−0.62	1.47×10^{-5}	negative	−0.33	3.44×10^{-2}	-
2675	LAMC1	−0.62	1.30×10^{-6}	negative	−0.42	1.99×10^{-3}	-
2722	CDH1	−0.6	1.71×10^{-6}	negative	−0.52	6.87×10^{-5}	negative
1832	MATN2	−0.6	2.98×10^{-5}	negative	−0.33	3.12×10^{-2}	-
2516	GLA	−0.59	4.95×10^{-5}	negative	−0.37	1.60×10^{-2}	-
4767	MAMDC2	−0.59	9.70×10^{-5}	negative	−0.45	4.17×10^{-3}	-
6191	SCPEP1	−0.59	7.62×10^{-6}	negative	−0.52	9.98×10^{-5}	negative
131	CTSH	−0.58	1.19×10^{-5}	negative	−0.47	4.93×10^{-4}	-
1642	CAMP	−0.56	1.42×10^{-5}	negative	−0.28	4.04×10^{-2}	-
2531	HEXA	−0.56	2.26×10^{-5}	negative	−0.47	5.30×10^{-4}	-
2419	APOA1	−0.55	2.68×10^{-4}	negative	−0.32	4.91×10^{-2}	-
2096	PLOD3	−0.55	2.57×10^{-4}	negative	−0.42	7.00×10^{-3}	-
5836	SDF4	−0.55	1.70×10^{-4}	negative	−0.26	1.06×10^{-1}	-
3918	DNAJC3	−0.53	9.86×10^{-5}	negative	−0.41	3.37×10^{-3}	-
6045	SIL1	−0.53	8.01×10^{-5}	negative	−0.46	8.97×10^{-4}	-
3180	COL18A1	−0.51	4.09×10^{-4}	negative	−0.32	3.42×10^{-2}	-
2572	MMP2	−0.51	3.76×10^{-4}	negative	−0.26	8.85×10^{-2}	-
3228	PRCP	−0.5	8.23×10^{-4}	negative	−0.43	5.04×10^{-3}	-
4643	SPESP1	−0.5	4.02×10^{-4}	negative	−0.11	4.58×10^{-1}	-
2536	P4HB	−0.48	2.41×10^{-4}	-	−0.56	1.05×10^{-5}	negative
5898	VAMP8	−0.4	1.04×10^{-2}	-	−0.51	7.20×10^{-4}	negative
3601	HNRNPK	−0.38	7.34×10^{-3}	-	−0.57	1.81×10^{-5}	negative
2304	AHSA1	−0.35	2.47×10^{-2}	-	−0.59	5.60×10^{-5}	negative

Table 2. *Cont.*

B: positively correlated proteins							
Experimental ID	**Gene symbol**	**Progressive sperm motility**			**Sperm count**		
		r	***p*-value**	**Correlation**	**r**	***p*-value**	**Correlation**
4245	TKFC	0.68	2.41×10^{-8}	positive	0.49	1.83×10^{-4}	-
2526	GPI	0.68	1.97×10^{-8}	positive	0.5	1.18×10^{-4}	positive
3026	IMPA1	0.68	6.53×10^{-8}	positive	0.64	5.63×10^{-7}	positive
4778	HDDC2	0.64	5.55×10^{-6}	positive	0.56	1.27×10^{-4}	positive
3299	PPP3CC	0.63	1.96×10^{-5}	positive	0.67	3.80×10^{-6}	positive
4544	CYB5R2	0.61	3.49×10^{-6}	positive	0.68	6.85×10^{-8}	positive
2227	FLOT1	0.6	7.35×10^{-6}	positive	0.3	3.84×10^{-2}	-
2823	GOT1	0.56	1.80×10^{-5}	positive	0.51	1.55×10^{-4}	positive
2923	PRKACG	0.56	1.34×10^{-4}	positive	0.68	5.98×10^{-7}	positive
632	ARRDC5	0.55	7.40×10^{-5}	positive	0.67	2.93×10^{-7}	positive
6391	DPP3	0.54	4.40×10^{-5}	positive	0.28	5.02×10^{-2}	-
2997	STOM	0.54	4.85×10^{-5}	positive	0.46	7.69×10^{-4}	-
4198	UGP2	0.54	3.44×10^{-5}	positive	0.37	5.79×10^{-3}	-
6619	TSPAN16	0.53	2.09×10^{-4}	positive	0.73	1.64×10^{-8}	positive
3116	HSPA4	0.52	6.24×10^{-5}	positive	0.4	2.70×10^{-3}	-
4301	PYCRL	0.52	2.58×10^{-4}	positive	0.29	5.51×10^{-2}	-
1079	TCEB1	0.52	7.86×10^{-5}	positive	0.56	1.68×10^{-5}	positive
4332	ACSBG2	0.51	1.31×10^{-4}	positive	0.59	8.01×10^{-6}	positive
4838	CAND1	0.51	9.96×10^{-5}	positive	0.4	3.24×10^{-3}	-
2426	APCS	0.5	1.27×10^{-4}	positive	0.62	7.41×10^{-7}	positive
3976	FHL1	0.49	7.70×10^{-4}	-	0.61	1.34×10^{-5}	positive
5742	MYADM	0.49	4.10×10^{-4}	-	0.55	5.50×10^{-5}	positive
3025	TPP2	0.48	3.03×10^{-4}	-	0.57	1.13×10^{-5}	positive
3972	CUL3	0.47	3.59×10^{-4}	-	0.54	2.72×10^{-5}	positive
4179	SYPL1	0.47	5.68×10^{-4}	-	0.54	4.37×10^{-5}	positive
4655	GLB1L	0.46	1.67×10^{-3}	-	0.61	1.06×10^{-5}	positive
6705	RUVBL1	0.45	8.14×10^{-4}	-	0.68	2.41×10^{-8}	positive
5910	DPCD	0.43	1.28×10^{-3}	-	0.54	2.94×10^{-5}	positive
6313	FARSB	0.43	1.94×10^{-3}	-	0.54	5.55×10^{-5}	positive
2616	UCHL1	0.43	5.71×10^{-3}	-	0.51	8.25×10^{-4}	positive
1810	ACOT7	0.41	9.95×10^{-3}	-	0.51	9.30×10^{-4}	positive
6860	CFAP20	0.4	1.00×10^{-2}	-	0.51	8.26×10^{-4}	positive
5950	TEX101	0.4	7.97×10^{-3}	-	0.53	2.43×10^{-4}	positive
1189	TSN	0.39	6.80×10^{-3}	-	0.56	3.21×10^{-5}	positive
6190	C12orf10	0.37	1.04×10^{-2}	-	0.5	3.79×10^{-4}	positive
2535	PGK2	0.37	5.69×10^{-3}	-	0.6	1.75×10^{-6}	positive
6698	RUVBL2	0.36	7.23×10^{-3}	-	0.53	3.91×10^{-5}	positive
2721	ACE	0.34	1.32×10^{-2}	-	0.59	3.06×10^{-6}	positive
436	EFR3A	0.34	2.81×10^{-2}	-	0.53	3.71×10^{-4}	positive
6708	VDAC3	0.34	1.20×10^{-2}	-	0.52	7.06×10^{-5}	positive
5290	SLC2A14	0.33	1.81×10^{-2}	-	0.5	1.49×10^{-4}	positive
2690	ACADM	0.31	2.80×10^{-2}	-	0.51	1.79×10^{-4}	positive
3883	TRAP1	0.29	6.17×10^{-2}	-	0.52	4.47×10^{-4}	positive
3502	AQP5	0.24	9.10×10^{-2}	-	0.58	8.00×10^{-6}	positive
6150	ACAD9	0.23	1.49×10^{-1}	-	0.5	7.45×10^{-4}	positive

Spearman correlation analysis was performed. $r \leq -0.5$ was considered as negatively correlated. $r \geq 0.5$ was considered as positively correlated. Unpaired two-tailed *t*-test was performed. *p*-value < 0.05 was considered as statistically significant.

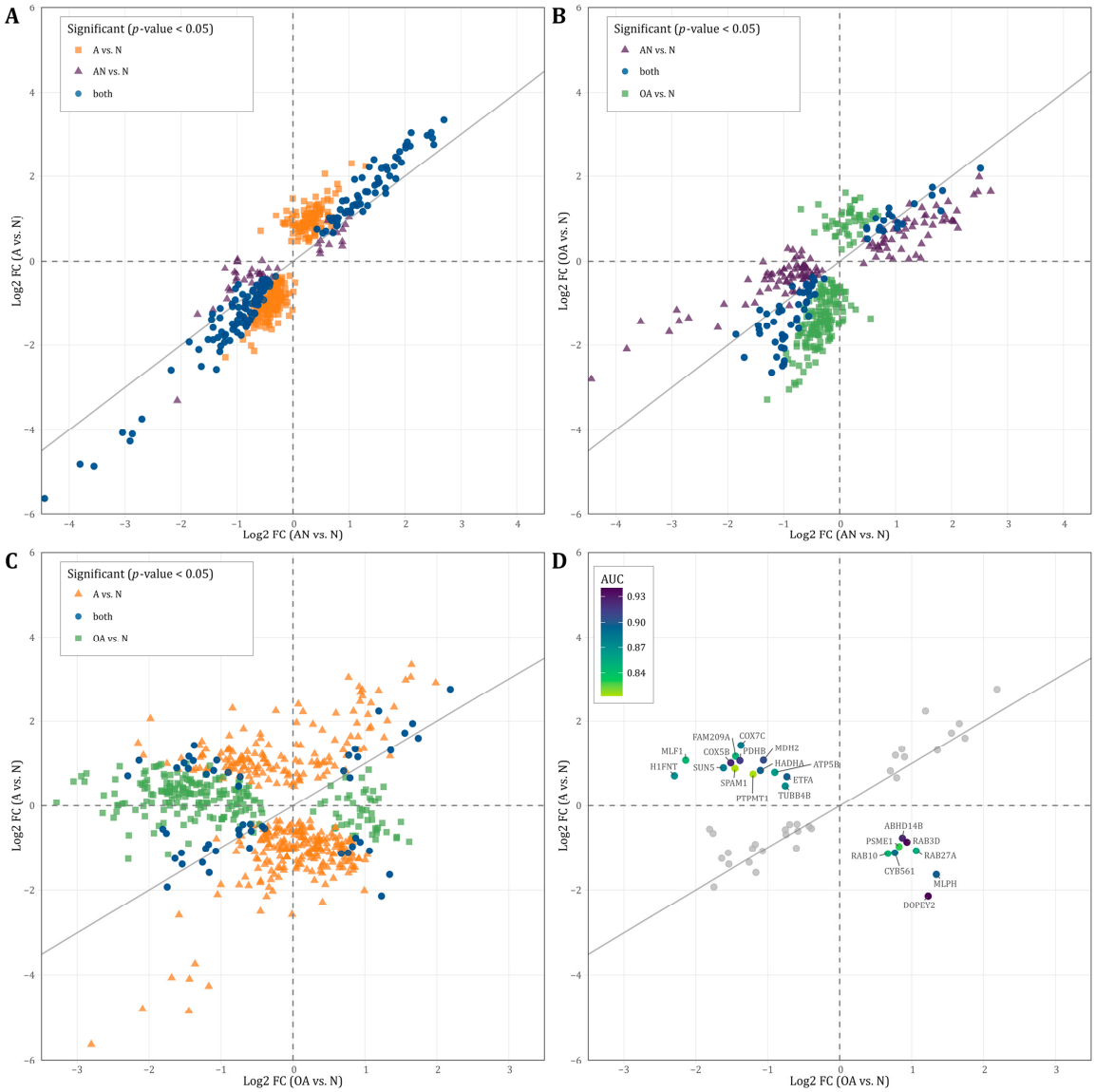

Figure 4. (**A**–**C**): Scatterplot displaying the direction of regulation (log2 fold change) of proteins that showed significantly different abundance levels (p-value < 0.05) in the (**A**): AN vs. N and/or OA vs. N, (**B**): AN vs. N and/or A vs. N, and (**C**): A vs. N and/or OA vs. N. (**D**): Scatterplot displaying the direction of regulation (log2 fold change) of proteins that showed significantly different abundance levels (p-value < 0.05) in both A vs. N and OA vs. N. The color represents the area under the curve (AUC) value of proteins that were oppositely regulated.

To explore the diagnostic value of the identified proteins, which are shown in Figure 4C, we tested these proteins for their predictive value to discriminate between the different subgroups. As shown in Figure 4D, the AUC values of these proteins ranged between 0.81–0.94 indicating that these proteins can be used to distinguish men with oligoasthenozoospermia and asthenozoospermia (Figure 4D).

3.6. Prediction of Subfertility and Their Specific Phenotypes Oligoasthenozoospermia and Asthenozoospermia

Proteins with adjusted significant *p*-values in at least one comparison were cross-matched with the proteins that were correlated with either sperm count and/or motility (i.e., $r \leq -0.5$ or $r \geq 0.5$, *p*-value < 0.05). The crossmatch yielded 62 proteins. To determine the predictive potential value of these proteins, we employed feature selection along with 3-fold cross-validation (CV). Table S4 lists the AUC values of single proteins and combinations of 2 or 3 proteins, the mean CV AUC, the confidence interval, and the *p*-values for each comparison, i.e., AN vs. N, A vs. N, and OA vs. N. The combination of the three proteins APCS, APOE, and FLOT1 showed the best AUC value to discriminate subfertile from normozoospermic controls with a mean AUC of 0.95 (Figure 5A). In addition, 5 combinations of two or three proteins showed a perfect AUC value to predict men with asthenozoospermia in the A vs. N comparison with a mean AUC of 1 including the combination of the two proteins APOE and FN1 with a mean AUC of 1 (Figure 5B). As for the discrimination between men with oligoasthenozoospermia and normozoospermic men, the combination of RUVBL1 and TKFC proteins showed the best AUC value with a mean AUC of 0.93 (Figure 5C).

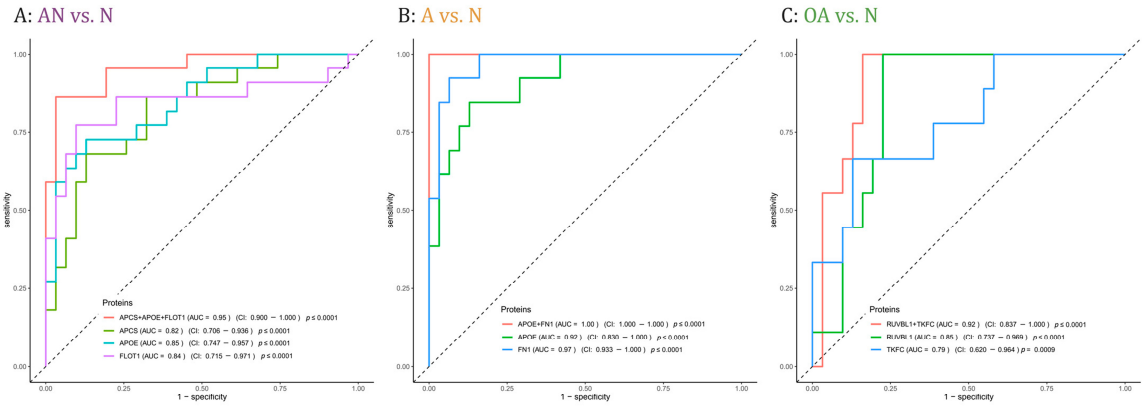

Figure 5. Receiver operating characteristic (ROC) curves comparing sensitivity and specificity of single proteins and protein combinations in predicting (**A**): subfertility (AN), (**B**): asthenozoospermia (**A**,**C**): oligoasthenozoospermia (OA) as compared to normozoospermic controls (N). Area under the curve (AUC) values, the confidence interval (CI), and the *p*-values of single proteins and protein combinations were indicated.

4. Discussion

Large-scale MS-based proteomics was used for a comprehensive view on the proteomic landscape of human sperm collected from asthenozoospermic and oligoasthenozoospermic men. In total, we identified 1336 proteins that were present in at least 70% of all sperm samples. The comparison between subfertile men and controls (AN vs. N) showed 66 dysregulated proteins, the comparison between asthenozoospermic with the normozoospermic men (A vs. N) showed 181 proteins, and the comparison between oligoasthenozoospermic and normozoospermic men (OA vs. N) revealed nine proteins.

Notably, the sum of the results obtained from asthenozoospermic and oligoasthenozoospermic men compared to normozoospermic controls (A vs. N and OA vs. N) is not equivalent to the results obtained from subfertile men and controls (AN vs. N). In detail, nine proteins were exclusively identified by comparing abnormal and normal men (Figure 2A). Most of these proteins, namely STOM, PPP3CC, FHL1, TEX101, PRKACG, and TSPAN16, are correlated with sperm motility and/or count (Figure 3/Table 2). Some of the above-mentioned proteins have previously been found to be associated with infertility. Testis-expressed protein 101 (TEX101) is well-studied glycoprotein and highly

related to male fertility (as reviewed in [27]). Knockout models of TEX101 and its counterpart protein contributes to the maintenance of spermatogenesis and production of fertile sperm [28]. Similarly, Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform (PPP3CC) is a catalytic subunit of the sperm-specific isoform of calcineurin and is highly related to male fertility [29]. Specifically, the knockout of PPP3CC in mice reduced the sperm motility and leads to male infertility [29]. Protein Kinase cAMP-Activated Catalytic Subunit Gamma (PRKACG) is a catalytic subunit of protein kinase A (PKA), which is involved in energy metabolism, hyperactivation, and capacitation of sperm [30,31].

The proteins found in our study are largely consistent with the proteins identified in sperm of unaffected men (as reviewed by [7]). We, however, identified 70 additional proteins including nine proteins, namely ESPN (B1AK53), MATN2 (O00339), SERPING1 (P05155), NEU1 (Q99519), GLIPR2 (Q9H4G4), ANKFY1(Q9P2R3), GPR64 (Q8IZP9-9), MYO1C (O00159-2), CABYR (iso3) (O75952-3, O75952-5, O75952-4, G9BQT7) that were dysregulated in at least one of our comparisons. According to the Human Protein Atlas (proteinatlas.org) [32], these proteins are also expressed in the developing germ cells and/or in the testicular somatic cells, i.e., Sertoli cells. We would like to point out that our stringent inclusion criteria for the identified proteins and the relation to testicular cells and/or tissues provided by the Human Protein Atlas strongly suggest that these proteins essentially contribute to the sperm proteome. Additionally, the crossmatch between the proteins identified in this study and the proteins, which were identified by others in asthenozoospermic men [8,9,14,16] yielded at least 10 matched proteins. Of these identified proteins, four proteins, namely ELSPB1, ECH1, GK2, and HSPA9, showed contradictory directions of regulation and six proteins, namely SEMG1, IMPA1, PARK7, LTF, SDHA, and CLU showed the same direction of regulation for asthenozoospermic men. These findings underline the complexity of proteomic regulations in sperm: the small overlap indicates that further studies are likely to identify additional deregulated proteins and the differences in the regulation direction suggest that the direction of regulation depends on further variables such as the biological context.

Sperm do not only deliver paternal DNA but also RNA and proteins to the maternal oocyte [7,33,34]. Our analysis of the sperm proteome contributes to deepen our understanding of the functions and the networks of sperm proteins. Some of the identified proteins are known to play a role specifically in the maintenance of physiological sperm functions, such as energy metabolism and in sperm tail structural composition and mechanics. While most of the proteins were dysregulated in the same direction in all comparisons, we also found proteins that were inversely regulated in specific comparisons such as in asthenozoospermic vs. oligoasthenozoospermic samples (Figure 4D). The proteins PDHB, ATP5B, COX7C, COX5B, MDH2, ETFA, and HADHA all of which showed a lower abundance level in oligoasthenozoospermic men and a higher abundance level in asthenozoospermic men, were involved in metabolism (HSA-1430728) [35] and partly in the generation of precursor metabolites and energy (GO:0006091) [36,37]. The proteins RAB10, RAB3D, RAB27A, and MLPH that showed a lower abundance level in asthenozoospermia and a higher abundance level in oligoasthenozoospermia were either Ras-related or were associated with sperm motility and capacitation [38,39].

Out of the 1336 proteins 55 proteins were correlated with sperm motility including 35 negatively and 20 positively correlated proteins. Likewise, 50 proteins were correlated with sperm count including 13 negatively and 37 positively correlated proteins (Figure 3). The hierarchical clustering of the proteins negatively correlated with motility indicates a cluster within the subfertile men (Figure 3B). This subcluster is composed of samples with a motility below 32%. Notably, the decline in male fertility potential is globally discussed and the semen parameters contributing to male fertility have been declined in recent decades [40]. The sperm count decreased by 50–60% between 1973 and 2011 [41] and sperm motility diminished by 10% between 2002 and 2017 [42]. In the future such proteomic analysis may help to define a new lower limit for sperm motility and may improve the subgrouping of subfertile men.

Current diagnostic methods including physical examination, endocrine, genetic and biochemical testing and semen analysis fail in 30–40% of infertility cases [43]. Protein-based biomarkers may help to characterize and to better understand the biological causes of these cases of idiopathic male infertility. We identified three sets of proteins that may serve as potential biomarkers to predict subfertility and specifically asthenozoospermia and oligoasthenozoospermia (Figure 5). In detail, the combination of the three proteins APCS, APOE, and FLOT1 predicts male subfertility in general, the two combined proteins APOE and FN1 predicts asthenozoospermia, and the two combined proteins RUVBL1 and TFKC oligoasthenozoospermia. The power and robustness of these prediction need of course to be validated by an independent and extended set of samples. As well as their potential as biomarkers, APCS, APOE, FLOT1, FN1, and RUVBL1 proteins have been recognized to play a crucial function in the development of male subfertility. By contrast, the TFKC protein has not yet been related to defined biological processes in sperm production or sperm function. Our findings show a lower abundance level of serum amyloid P-component (APCS) in sperm of asthenozoospermic men, and a positive correlation between APCS and sperm motility and sperm count. These findings are in agreement with previous studies reporting a correlation between APCS and sperm motility and count in seminal plasma. Previous studies also suggested that APCS may be used as a biomarker for low sperm concentrations [44]. APCS is localized on the surface of mature sperm specifically in the tail of spermatozoa and plays a physiological role in reproduction, more precisely in tail-associated functions such as sperm motility [45]. APCS protein was identified in sperm, seminal plasma, and testicular tissues collected from the male reproductive tract [45]. With no APCS-deficiency reported for humans, the role of this protein in human reproduction awaits further evaluation. Likewise, we found a lower abundance level of flotillin-1(FLOT1) in asthenozoospermic men and a positive correlation of FLOT1 with sperm motility. FLOT1 is a scaffolding protein of the tyrosine kinase family, which is involved in the capacitation and acrosome reaction of sperm [46]. As shown for the porcine and mouse sperm acrosome, FLOT1 that is organized in lipid rafts changes its dispersed pattern to an accumulation at the apical ridge of the acrosome during capacitation [46–48]. However, the exact function of FLOT1 in sperm and their potential involvement in sperm motility is still not well understood and needs further elucidation. The apolipoprotein E (APOE) was also found in higher abundance levels in asthenozoospermic men and was negatively correlated with sperm motility. The impact of APOE on male infertility was extensively studied on different genotypes and its potential impact on steroidogenesis has been investigated [49,50]. Specifically, the lower fertility potential of specific genotypes accompanied by the decrease in APOE concentration suggests an association with male infertility [49,50]. Thus, it has been suggested that the APOE is involved in sperm maturation during epididymal transit [51]. Very recently, Liu et al. observed a markedly lower total and progressive motility sperm in ApoE-knockout mice compared to wild type control mice and concluded that ApoE knockout effect male reproductive capacity [52]. In addition to the protein abundance levels, post-translational modifications such as APOE glycosylation likely influence the sperm function thereby complicating the search of suitable biomarkers of male fertility [49,53,54]. Additionally, the importance of APOE Receptor 2 (apoER2) for sperm development was previously demonstrated [55]. The apoER2 protein was expressed in the epididymis and its knockout leads to male infertility in mice [55]. Our results showed that sperm-derived Fibronectin 1 (FN1) was negatively correlated with sperm motility and showed a higher abundance level in men with asthenozoospermia. In agreement with our findings, Wennemuth et al. (1997) observed a negative correlation between sperm motility and seminal fibronectin concentration in men with oligoasthenoteratozoospermia [56]. The glycoprotein FN that is expressed on the sperm surface plays a role in sperm-oocyte interaction and fertilization, and was used as a biomarker for selection in assisted reproductive medicine [57]. Notably, FN binds to sperm-specific integrins that can trigger intracellular signaling pathways. The increase in intracellular Ca^{2+} in turn triggers the cyclic adenosine monophosphate/protein kinase A pathway (cAMP/PKA)

and enables the sperm capacitation and ultimately the fertilization [58,59]. Hyperactivation of sperm during sperm capacitation alters the movement pattern and bending of the sperm flagellum. Since transcription is silenced in sperm, hyperactivated sperm motility is achieved through post-translational modification, primarily phosphorylation. PKA can phosphorylate AMP-activated protein kinase (AMPK), which regulates sperm motility in a Ca^{2+}-dependent manner [31]. Calle-Guisado et al. (2017) have observed that activation of AMPK beyond physiological levels results in a reduction in sperm motility [60]. We suggest that the higher abundance level of FN1 in asthenozoospermic men could lead to an overactivation of the cAMP/PKA pathway and ultimately to a reduction in sperm motility.

Our data also show that the RuvB Like AAA ATPase 1 (RUVBL1) protein has a lower abundance level in oligoasthenozoospermic men and is only positively correlated with sperm count. RUVBL1 functions as a DNA-dependent ATPase with ATP-dependent DNA helicase activity and is involved in several transcription complexes and histone modifications [61–63]. RUVBL1 interacts with the testis/sperm-specific Small Kinetochore-Associated Protein (SKAP1) and is located in the sperm flagellum [64]. In addition, RUVBL1 is a part of the R2TP complex, which acts as a co-chaperon together with the heat shock protein 90 (HSP90) [65]. HSP90 is also located in the sperm flagellum and is crucial to maintain male fertility. It was hypothesized that a co-chaperon complex is formed in sperm thereby contributing to the maturation of sperm [64,65]. The RUVBL1 protein was positively correlated with sperm count. The sperm count reduction can be caused by environmental factors, testis-related diseases, infections, and obstructions of the male reproductive system [66–69]. However, cellular mechanisms that reduced the number of sperm in the ejaculate are not well understood. We speculate that defects, lower levels, or even the absence of structural proteins that contribute to the ciliary axoneme or flagellum might stimulate the apoptotic machinery or disrupt spermatogenesis, ultimately leading to a decrease in sperm count in men.

As well as their potential as diagnostic biomarkers, several identified proteins might be used as new therapeutic targets. In agreement with others, our study suggests that the lower or higher abundance of specific proteins impact male fertility. Some of the above-mentioned proteins (APOE, TEX101, PPP3CC) were previously functionally validated in knockout-studies of mice. The absence of these proteins leads to impaired spermatogenesis and/or reduced motility and ultimately to infertility. Current studies are investigating whether such proteins are suitable for the treatment of infertility. For example, Kim et al. developed a nanoparticle complex with PIN1 proteins inside to treat infertile mice that were lacking the PIN1 protein. These experiments successfully restore the fertility of the mice [70]. It has recently been suggested that proteins offer advantages over other small molecules regarding their use as therapeutic agents. They are highly specific and bear less risks of adverse effects as compared to gene therapies [70,71].

5. Conclusions

In conclusion, we report the proteomic landscape of human sperm collected from asthenozoospermic and oligoasthenozoospermic men as compared to normozoospermic controls. We identified numerous proteins with differential abundance levels between subfertile and normozoospermic men, between asthenozoospermic and normozoospermic men, and between oligoasthenozoospermic and normozoospermic men. Furthermore, we found numerous proteins correlated with sperm motility and sperm count. Several of these proteins were involved in sperm lipid composition and remodelling, the extracellular matrix, the glycocalyx, the ciliary movement, and/or energy metabolism. There are also several proteins with known involvement in sperm-specialized functions, such as sperm capacitation and fertilization. We identified protein combinations of potential diagnostic value for subfertility in general and asthenozoospermia and oligoasthenozoospermia, specifically. As well as their diagnostic potential, these proteins may offer themselves as possible new therapeutic targets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12071017/s1, Table S1: Detailed *t*-test results of proteins comparing subfertile men vs. normozoospermic controls (AN vs. N), asthenozoospermic men vs. normozoospermic controls (A vs. N), and oligoasthenozoospermic men vs. normozoospermic controls (OA vs. N) as determined by LC-MS/MS.; Table S2: Detailed listing of upset plot results in Figure 2B.; Table S3: Detailed results of correlation analysis of all included proteins as determined by LC-MS/MS with the basic semen parameters motility and count.; Table S4: Detailed results of ROC curve analysis of all determined potential biomarker combinations for subfertility in general (AN vs. N), asthenozoospermia (A vs. N), and oligoasthenozoospermia (OA vs. N). Data were presented as the mean of the three-fold cross-validation area under the curve value (Mean CV AUC), the area under the curve (AUC) value of the full data set, the lower and upper confidence interval (CI) with 95% confidence level, and the *p*-value, respectively.; Table S5: More detailed information of participants sperm motility.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Saarland Medical Association (Ha 195/11/updated June 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available via ProteomeXchange with the identifier PXD039703.

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4. DISKUSSION

In meiner Thesis habe ich mich auf Infertilitäts-assoziierte miRNAs, deren Interaktionen mit Zielgenen und deren Zusammenhang mit Infertilitäts-assoziierter Anomalien fokussiert. Darüber hinaus habe ich Veränderungen im Spermien Proteom von Männern mit Infertilitäts-assoziierten Anomalien analysiert.

Im Speziellen untersuchte ich die Infertilitäts-assoziierten miRNAs miR-23a-3p und miR-23b-3p sowie miR-19a-3p und miR-19b-3p und insgesamt 98 ihrer *in-silico* vorhergesagten, Testis-exprimierten Zielgene. Die miR-19a-3p und die miR-19b-3p waren in Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit Normozoospermie höher exprimiert. Die erhöhte Expression der miR-23a-3p und miR-23b-3p in Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit Normozoospermie wurde bereits im Vorfeld validiert. Insgesamt zehn Zielgene der miR-23a/b-3p und 51 Zielgene der miR-19a/b-3p zeigten eine reduzierte Expression in Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Männern mit Normozoospermie. Zusätzlich wurden negative Korrelationen der miRNAs und positive Korrelationen der Zielgene mit Spermienanzahl, –motilität und/oder –morphologie nachgewiesen. Die Zielgene der miR-23a/b-3p wurden darüber hinaus hinsichtlich ihrer miRNA-mRNA-Interaktion untersucht. Es konnten acht direkte Zielgene der miR-23a-3p und drei direkte Zielgene der miR-23b-3p nachgewiesen werden. Nach gezielter Mutagenese der miRNA-Bindestellen wurden fünf Zielgene der miR-23a-3p und drei Zielgene der miR-23b-3p bestätigt. Für zwei Zielgene der miR-19a/b-3p habe ich zusätzlich eine verminderte Proteinexpression in Spermien von Männern mit Oligoasthenozoospermie im Vergleich zu Normozoospermie nachgewiesen.

Des Weiteren wurde das Spermien Proteom im Kontext Infertilitäts-assoziierter Anomalien untersucht. Insgesamt wurden 4412 Proteine in Spermienproben von Männern mit Normozoospermie, Oligoasthenozoospermie und Asthenozoospermie identifiziert, wovon 1336 Proteine in mindestens 70 % der Spermienproben nachgewiesen wurden. Von diesen Proteinen waren in Spermien von subfertilen Männern, d.h. Männern mit Oligoasthenozoospermie und Asthenozoospermie, 32 Proteine geringer exprimiert und 34 Proteine höher exprimiert als in Spermien von Männern mit Normozoospermie. In Spermien von Männern mit Asthenozoospermie verglichen mit Spermien von Männern mit Normozoospermie wiesen jeweils 95 Proteine eine reduzierte und 86 Proteine eine erhöhte Expression auf, während in Spermien von Männern mit Oligoasthenozoospermie verglichen mit Spermien von Männern mit Normozoospermie acht Proteine eine reduzierte Expression aufwiesen und ein Protein eine erhöhte Expression aufwies. Außerdem korrelierten 20 Proteine positiv und 35 Proteine negativ mit der Spermienmotilität, während 37 Proteine

positiv und 13 Proteine negativ mit der Spermienanzahl korrelierten. Zusätzlich habe ich drei Kombinationen aus Proteinen identifiziert, die sich als potentielle diagnostische Biomarker zur Unterscheidung zwischen subfertilen Männern, bzw. Männern mit Asthenozoospermie oder Oligoasthenozoospermie, gegenüber Normozoospermie eigenen könnten.

Spezifische Gesichtspunkte meiner Arbeiten werden in meinen unter Punkt drei aufgeführten Publikationen diskutiert. In diesen Publikationen werden Interaktionen zwischen einzelnen miRNAs und ihren Zielgenen bzw. einzelnen identifizierten Proteinen im Zusammenhang mit physiologischen Mechanismen der männlichen Reproduktion und im Zusammenhang mit Infertilitäts-assoziierter Anomalien diskutiert. Weiterhin wird in meinen Publikationen das Potential spezifischer Proteine, miRNAs und Transkripte als Biomarker zur Diagnose der männlichen Infertilität diskutiert. Weitere Aspekte und Details sind in den entsprechenden Publikationen zu finden. Die nachfolgende Diskussion bezieht sich deshalb nicht auf die genannten Punkte, sondern versucht vor allem übergeordnete Aspekte meiner gesamten Ergebnisse zu thematisieren.

4.1. Vergleichbarkeit von Veränderungen im Transkriptom und Proteom in Spermien

Anhand meiner Ergebnisse werden Aspekte erläutert, die beim direkten Vergleich von Transkriptom und Proteom generell und speziell in Spermien zu berücksichtigen sind.

Meine Ergebnisse zeigten beim Vergleich der insgesamt 98 vorhergesagten Zielgene der miRNA-Studien (ABU-HALIMA et al., 2022; BECKER et al., 2023a) mit den 4412 identifizierten Proteinen der Spermien Proteom-Studie (BECKER et al., 2023b) eine Überschneidung von zehn Transkripten bzw. Proteinen (AQP5, ARRDC5, BOD1L2, CSNK1G1, DNAI1, HSPA2, RALGPS2, STK33, TKTL2 und ZMYND12). Mit Ausnahme von ARRDC5, ZMYND12 und RALGPS2, sind die Transkripte in Spermien von Männern mit Oligoasthenozoospermie verglichen mit Normozoospermie niedriger exprimiert. Für die Untersuchung der differentiellen Expression der Spermien-Proteine wurden lediglich Proteine analysiert, deren Expression in mindestens 70 % aller Spermienproben identifiziert wurden. Somit konnte für fünf der zehn überlappenden Transkripte bzw. Proteine kein Vergleich zwischen der Expression von Protein und Transkript angestellt werden (BOD1L2, CSNK1G1, RALGPS2, STK33 und ZMYND12). Die verbleibenden fünf Proteine (AQP5, ARRDC5, DNAI1, HSPA2, TKTL2) waren weder in Spermien von Männern mit Subfertilität im Allgemeinen noch in spezifischen Formen der Subfertilität differentiell exprimiert. Jedoch waren AQP5 und

ARRDC5 Proteine positiv mit der Spermienanzahl, bzw. mit Spermienanzahl und Spermienmotilität korreliert.

Den geringen Überschneidungen, sowie den Unterschieden in der Expression, können verschiedene Ursachen zugrunde liegen (JODAR et al., 2016). Zum einen können technisch methodische Umstände sich auf die Sensitivität, Spezifität und letztendlich auf die Vergleichbarkeit der Ergebnisse auswirken. LC-MS/MS bietet gegenüber anderen Techniken vor allem den Vorteil des erhöhten Durchsatzes. Jedoch ist auch diese Technik in ihrer Sensitivität limitiert (LEUNG, FONG, 2014; SEGER, 2012; VOGESER, SEGER, 2010). Dies könnte erklären, dass Proteine mittels Western Blot identifiziert und validiert wurden, jedoch nicht mittels LC-MS/MS erfasst werden konnten. Zusätzlich können die unterschiedlichen Halbwertszeiten von mRNAs und Proteinen die Vergleichbarkeit von Proteom und Transkriptom erschweren. Während mRNAs generell eine Halbwertszeit von 10 bis 20 Stunden aufweisen, bleiben Proteine mit einer Halbwertszeit von 48 bis 72 Stunden länger stabil (HARGROVE, SCHMIDT, 1989).

Auch die Regulierung durch miRNAs könnte bei der Vergleichbarkeit von Transkriptom und Proteom eine Rolle spielen, so können Transkript-basierte Techniken, wie RT-qPCR, miRNA-induzierte Expressionsveränderungen erkennen, die aus einer Degradation der Transkripte resultieren, jedoch keine Expressionsveränderungen durch Inhibition der Translationsmaschinerie. Protein-basierte Techniken würden bei beiden miRNA-Regulationsmechanismen eine Reduzierung der Protein-Expression feststellen (KERN et al., 2020).

Differenzen im Proteom und Transkriptom können auch in der Natur der Spermien und der Spermatogenese begründet liegen. Anders als somatische Zellen, sind reife ejakulierte Spermien transkriptionell und translationell größtenteils inaktiv (GRUNEWALD et al., 2005; GUR, BREITBART, 2006; KIERSZENBAUM, TRES, 1975; REN et al., 2017). Einige Transkripte werden während der Spermatogenese oder in späten Phasen der Spermiogenese gebildet, jedoch nicht translatiert (HAMATANI, 2012; MILLER et al., 1999; MILLER, OSTERMEIER, 2006). Außerdem können Transkripte, sowie Proteine, durch extrazelluläre Vesikel aus dem Seminalplasma aufgenommen oder ins Seminalplasma ausgeschleust werden und verändern somit die Komposition der verbleibenden Moleküle in den Spermien (JODAR et al., 2016; MARTIN-DELEON, 2015; SAEZ et al., 2003; SANTIAGO et al., 2021).

Letztendlich ist der direkte Vergleich des Transkriptomebene und Proteomebene aufgrund technischer und methodischer Gegebenheiten generell nur bedingt möglich. Vor allem aber in Spermien sollte gegenüber anderen Zelltypen in Frage gestellt werden, ob sich beobachtete Veränderungen auf Transkriptomebene zwingend auch auf Proteinebene widerspiegeln, da zellbiologische Gegebenheiten, die in der Physiologie der Spermien und der Spermatogenese begründet liegen, diesen Vergleich zusätzlich erschweren.

4.2. Einsatz von miRNAs, mRNAs und Proteinen als Biomarker für männliche Infertilität

Der hohe Anteil der Paare mit ungeklärter Infertilität und idiopathischer männlicher Infertilität verdeutlicht den Bedarf neuer diagnostischer Ansätze in der Reproduktionsmedizin, die ergänzend zur bisherigen Routinediagnostik bei männlicher Infertilität eingesetzt werden können. In meiner Arbeit wurden miRNAs, mRNAs und Proteine in Spermien untersucht, die zukünftig als Biomarker zur Diagnose männlicher Infertilität genutzt werden könnten. Die jüngsten Entwicklungen molekulargenetischer Methoden, vor allem der Hochdurchsatzverfahren in der Transkriptomik und Proteomik, begünstigen die Identifizierung von potentiellen Biomarker-Kandidaten für männliche Infertilität (CALOGERO et al., 2023). In einer Vielzahl an Publikationen wurden bereits verschiedene Proteine, mRNAs sowie miRNAs in Spermien oder Seminalplasma als Biomarker für männliche Infertilität vorgeschlagen (BARBU et al., 2021; BEERAM et al., 2019; LALANCETTE et al., 2009; VASHISHT, GAHLAY, 2020).

Viele der Studien zur Identifizierung von Biomarkern weisen Limitierungen auf, die der Qualifizierung der Biomarker-Kandidaten für eine Anwendung in der klinischen Diagnostik im Wege stehen. Die mangelnde Standardisierung bei der Probengewinnung, Lagerung, Prozessierung und Validierung, vor allem im Bereich der miRNAs, behindert oftmals die Weiterverfolgung potentiell vielversprechender Biomarker-Kandidaten (BARBU et al., 2021; ETHERIDGE et al., 2011; VASHISHT, GAHLAY, 2020).

Trotz dieser Problematik ist mittlerweile die Entwicklung einzelner Biomarker-Kandidaten zur praktischen Anwendung der Biomarker in der Diagnostik bei männliche Infertilität weit fortgeschritten (BIENIEK et al., 2016). Beispielsweise wurden spezifische miRNAs und miRNA-Paare mittels Metaanalysen als Biomarker-Kandidaten identifiziert und ihre differentielle Expression in verschiedenen Infertilitäts-assoziierter Anomalien reevaluiert und validiert (CORRAL-VAZQUEZ et al., 2019; JOSHI et al., 2022b). Allerdings werden diese bisher nicht in der Diagnostik männlicher Infertilität eingesetzt. Die Protein-Biomarker TEX101, ECM1, ACRV1, PTGDS und LGALS3BP hingegen werden bereits vor allem zur Beurteilung nicht-obstruktiver und/oder obstruktiver Azoospermie verwendet (BIENIEK et al., 2016). Auch spezifische Genmutationen in *NR5A1*, *DMRT1* und *TEX11* eignen sich zur Diagnose von Azoospermie und werden bereits in der Diagnostik vereinzelt eingesetzt (TUTTELMANN et al., 2018). Trotz der vereinzelten Anwendung spezifischer Biomarker in der Diagnostik, finden sich diese bisher weder in den aktuellen Leitlinien der WHO, der ASRM, noch in den Leitlinien der EAU wieder (PRACTICE COMMITTEE OF THE AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, 2015; SALONIA et al., 2023; WORLD HEALTH ORGANIZATION, 2021).

Generell ist, trotz der Limitierungen, in näherer Zukunft eine deutliche Verbesserung der Diagnostik bei männlicher Infertilität, vor allem im Bereich der Genetik, zu erwarten, nicht zuletzt aufgrund der technisch methodischen Entwicklungen der letzten Jahrzehnte im Bereich der genetischen Forschung, sondern auch aufgrund des enormen Potentials der Biomarker im Kontext der männlichen Infertilität.

4.3. Schlussfolgerung

Die vorliegende Arbeit beschreibt die Expressionsmuster von miRNAs, Transkripten und Proteinen in Spermien im Kontext Infertilitäts-assoziierter Anomalien. Zudem wurden Zielgene der Infertilitäts-assoziierten miR-23a-3p, miR-23b-3p, miR-19a-3p und miR-19b-3p identifiziert und direkte miRNA-Zielgen-Interaktionen der miR-23a-3p und miR-23b-3p mit ihren Zielgenen validiert. Darüber hinaus wurde ein Zusammenhang zwischen miRNAs, Zielgenen und Proteinen mit verschiedenen Samenparametern hergestellt. Außerdem wurden Proteine identifiziert, die sich als potentielle Biomarker in der Diagnostik männlicher Infertilität eigenen könnten.

Die Ergebnisse meiner Arbeit leisten einen Betrag zum besseren Verständnis der regulatorischen Prozesse in der männlichen Reproduktion, vor allem bei der Entstehung der männlichen Infertilität. Sie tragen zur Verbesserung der Diagnostik bei männlicher Infertilität und zur Entwicklung maßgeschneiderter Therapien in der Zukunft bei.

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6. ANHANG

Nachfolgend werden die ergänzenden Tabellen und Abbildungen der entsprechenden Publikationen aufgelistet. Die Tabellen und Abbildungen wurden im Original in englischer Sprache belassen, entsprechend der Publikation gekennzeichnet und lediglich dem Layout angepasst.

6.1. Towards a More Comprehensive Picture of the MicroRNA-23a/b-3p Impact on Impaired Male Fertility

Supplementary Table 1 (BECKER et al., 2023a): Sequences of cloning and mutagenesis primers.

Target Gene	Sequence (restriction sites SpeI and SacI are in underline bold and mutated sites are shown in bold and italic)
CEP41_for	GG**ACTAGT**GGGTAGTAATACTTTCCTAG
CEP41_rev	C**GAGCTC**GGTGTGTGACCTTTGCGTGC
CEP41_for_mut	CCTGAAGCACCTGGTAA***AGCGCTC***GGCAAAGAGAGGCC
CEP41_rev_mut	GGCCTCTCTTTGCC***GAGCGCT***TTACCAGGTGCTTCAGG
GOLGA6C_for	GG**ACTAGT**GGAATGATCTTCAGCTAGCC
GOLGA6C_rev	C**GAGCTC**CTATATTATGCATAGGGCTG
GOLGA6C_rev_mut	C**GAGCTC**CTATATTATGCATAGGGCTGATTTATATTT***GAGCGCT***TTCTAAATATCAGC
GOLGA6B_for	GG**ACTAGT**CGGACAATAATGTGTTCATC
GOLGA6B_rev	C**GAGCTC**GCAGTTACACTTATGAATAG
GOLGA6B_for_mut	CCAAAGCTGATATTTAGAA***AGCGCTC***AAATGTAAATCAGCCC
GOLGA6B_rev_mut	GGGCTGATTTACATTT***GAGCGCT***TTCTAAATATCAGCTTTGG
G2E3_for	GG**ACTAGT**GATATTTGAATGATCTCTGTGG
G2E3_rev	C**GAGCTC**GGTATGTTAGTGTACAGATTG
G2E3_for_mut	CAAAGTGCTAAC***AGCGCTC***AAATATATAGTCCC
G2E3_rev_mut	GGGACTATATATTT***GAGCGCT***GTTAGCACTTTG
NOL4_for	GG**ACTAGT**GCACTCTTGTGTGTAG
NOL4_rev	C**GAGCTC**CAGAGAAAATTATACCAGCCC
NOL4_for_mut	GTGATACTGTTGGTGAATAT***AGCGCTC***AAAATCTCATTGAAATATGAG
NOL4_rev_mut	CTCATATTTCAATGAGATTTT***GAGCGCT***ATATTCACCAACAGTATCAC
PAPOLB_for	GG**ACTAGT**GCTATCAAAGCTCCATATTC

PAPOLB_rev	C**GAGCTC**GCAAGGCTAATTGGAAAGCC
PAPOLB_rev_mut	C**GAGCTC**GCAAGGCTAATTGGAAAGCCTGTTAAGTTTGCTGTCTAGATTAAGAAAGACTCAACTTTCATTCATTCACATCTGTTGC CCC***GAGCGCT***TCC
PCDHA9_for	GG**ACTAGT**GAGCAGATTTTTCAATCTAC
PCDHA9_rev	C**GAGCTC**GATTCTCCTAGTTCAAAAGGC
PCDHA9_for_mut	CTCTGTGCTTGTGT***AGCGCTC***TGTTAATGCAACTATTACC
PCDHA9_rev_mut_1	GGTAATAGTTGCATTAACA***GAGCGCT***ACACAAGCACAGAG
PCDHA9_rev_mut_2	C**GAGCTC**GATTCTCCTAGTTCAAAAGGCACGTAAGGTCAGATACATGGGCTTCAGTGGGGCAAAATGATCACAACCTACAATGTA GCAATGG***GAGCGCT***GTG
RGPD1_for	GG**ACTAGT**GAGATCACTTGATGATAC
RGPD1_rev	C**GAGCTC**CCAAACAAAAAAGAGCTTTG
RGPD1_rev_mut	C**GAGCTC**CCAAACAAAAAAGAGCTTTGTTTCTTT***GAGCGCT***CATTTCTC
SOX6_for	GG**ACTAGT**CCTCTCCTCCTCAGTTCACTC
SOX6_rev	C**GAGCTC**GGATATTTAGAGCCAAACTTGGC
SOX6_for_mut	GGTATTTTTTATCACTTCTGACT***AGCGCTC***AACTGTTGTACG
SOX6_rev_mut	CGTACAACAGTT***GAGCGCT***AGTCAGAAGTGATAAAAAATACC
ZNF695_for	GG**ACTAGT**CCTATTCACAACTTCACAGC
ZNF695_rev	C**GAGCTC**AGAGATGAGGTTTTGCCATG
ZNF695_for_mut	CTGTTTTACAGATGCAGTA***AGCGCTC***AAAAATGTCTAATC
ZNF695_rev_mut	GATTAGACATTTTT***GAGCGCT***TACTGCATCTGTAAAACAG
CSNK1G1_for	GG**ACTAGT**CCTCCAGGAGAGATATTTGTGC
CSNK1G1_rev	C**GAGCTC**GAAGTGACTCACAGGAGAAG
FAM169A_for	GG**ACTAGT**GACCATTGGGGGCTAACAG
FAM169A_rev	C**GAGCTC**GACTAGATGTTTTCCTTACC

GNAT1_for **GNAT1_rev**	GG**ACTAGT**GGCACACTCACCTTGGGTGC C**GAGCTC**GAGATTTGTACACCCATGTTC
LMLN_for **LMLN_rev**	C**GAGCTC**CAAGAAAGTCTCACTCTGTCA G**GACTAG**TGCTCAACCTGCGTAATGGTC
REEP1_for **REEP1_rev**	GG**ACTAGT**GCTTACACAGCTTCTTAGC C**GAGCTC**GCCTGTGTCTGTAAGTCAGC
TMEM215_for **TMEM215_rev**	GG**ACTAGT**CTTCTTACACCTGAGTTTCC C**GAGCTC**GAGACAGAATGATATAAAGG
ZNF492_for **ZNF492_rev**	GG**ACTAGT**GGTGAGAAATAATAGAAATATG C**GAGCTC**CATTTACAGGGTTTTTCTTCG

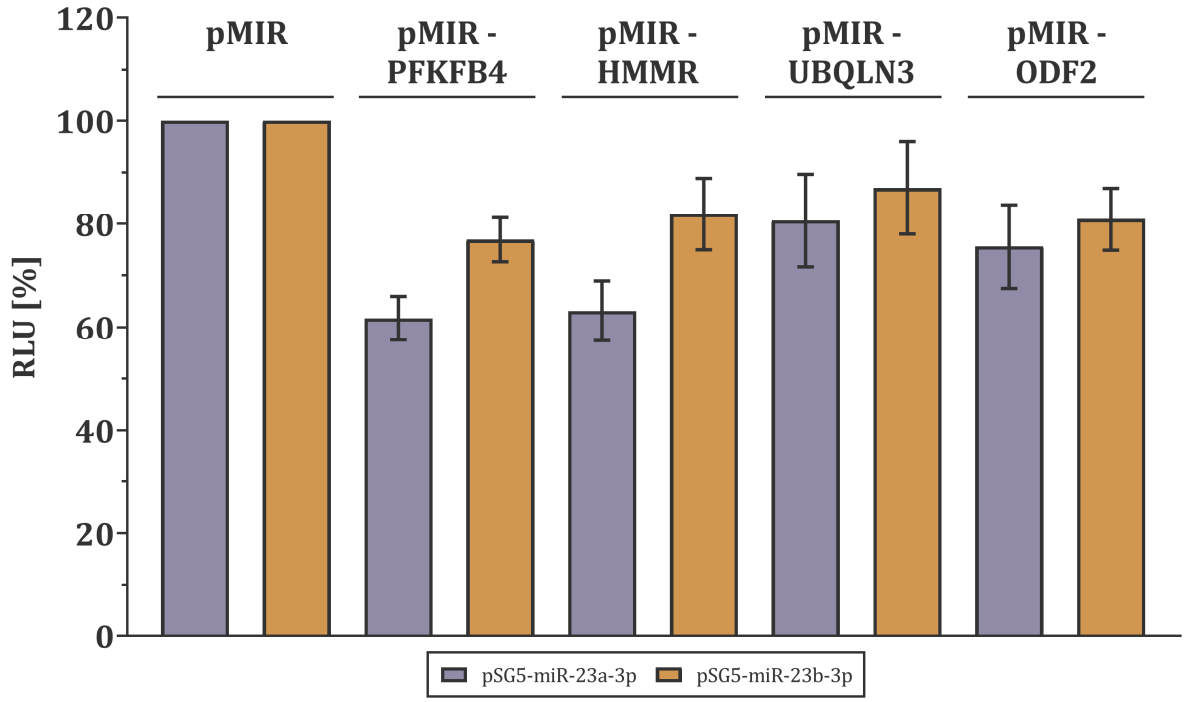
Supplementary Table 2 (BECKER et al., 2023a): Constructs with NCBI Reference Sequence, restriction enzymes for cloning, nucleotide position in 3'UTR, binding site position, and size of amplified fragment

Construct	NCBI Reference Sequence	Restriction enzymes for cloning	Nucleotide position in 3'UTR	Binding site position in 3'UTR	Size of amplified fragment
pMIR-CEP41	NM_001257158.2	SpeI - SacI	3688 - 4401	3967 - 3973	714 bp
pMIR-GOLGA6C	NM_001164404.2	SpeI - SacI	2348 - 2914	2878 - 2884	567 bp
pMIR-GOLGA6B	NM_018652.5	SpeI - SacI	2391 - 2984	2883 - 2889	593 bp
pMIR-G2E3	NM_001308097.2	SpeI - SacI	934 - 1637	1262 - 1268	704 bp
pMIR-NOL4	NM_001198546.1	SpeI - SacI	963 - 1702	1570 - 1576	750 bp
pMIR-PAPOLB	NM_020144.5	SpeI - SacI	937 - 1604	1516 - 1522	668 bp
pMIR-PCDHA9	NM_031857.2	SpeI - SacI	1270 - 2216	1472 - 1478, 2125 - 2131	947 bp
pMIR-RGPD1	NM_001024457.4	SpeI - SacI	920 - 1398	1365 - 1371	497 bp

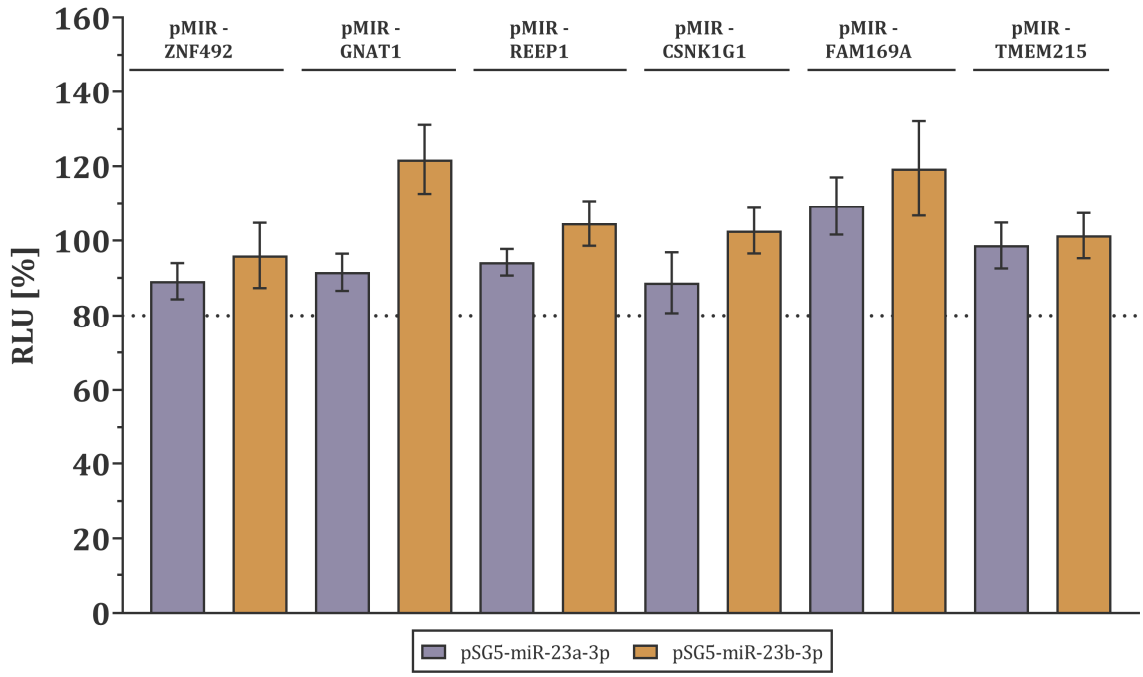
pMIR-SOX6	NM_001145811.2	SpeI - SacI	2293 - 2991	2653 - 2659	699 bp
pMIR-ZNF695	NM_020394.5	SpeI - SacI	44 - 716	400 - 406	673 bp
pMIR-CSNK1G1	NM_001329605.2	SpeI - SacI	8 - 418	145 - 151	418 bp
pMIR-FAM169A	NM_015566.3	SpeI - SacI	2262 - 2965	2593 - 2599	704 bp
pMIR-GNAT1	NM_144499.3	SpeI - SacI	1715 - 2428	2167 - 2173	714 bp
pMIR-LMLN	NM_001136049.3	SpeI - SacI	2935 - 3720	3379 - 3385	786 bp
pMIR-REEP1	NM_001164730.2	SpeI - SacI	2077 - 2576	2482 - 2488	500 bp
pMIR-TMEM215	NM_212558.3	SpeI - SacI	1616 - 2323	1975 - 1981	708 bp
pMIR-ZNF492	NM_020855.3	SpeI - SacI	-12 - 468	11 - 17	480 bp

Supplementary Table 3 (BECKER et al., 2023a): Sequences of RT-qPCR primers and their respective Design Reference Sequence.

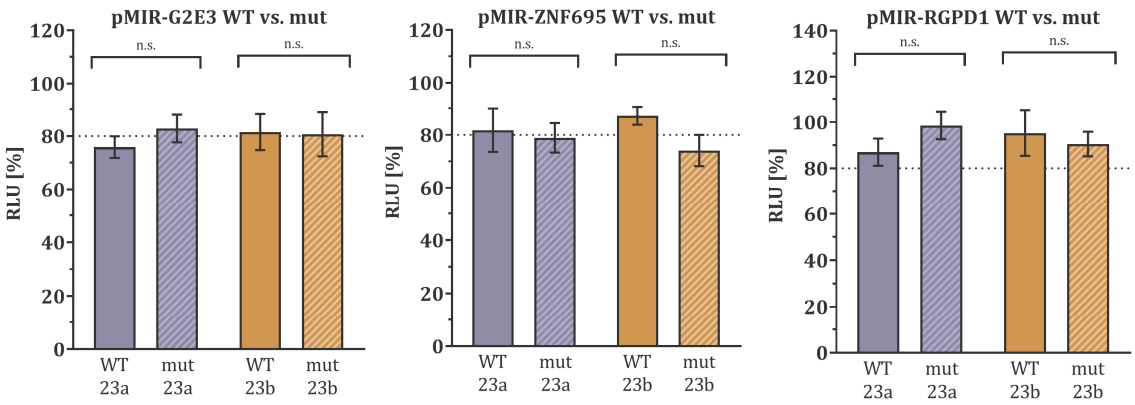
Target gene	**Forward Primer**	**Reverse Primer**	**Design RefSeq**
CEP41	AACCCGTGAGGCTAGAACC	TGGGTTCTGTGGTATCCTTTTCA	NM_018718.N
G2E3	TTGCAAAGAAGGGCGAGACTA	TGCCACTGGAACCACAACA	NM_017769.N
GOLGA6B	CTCCACCGGCGGTTACA	TCGCTCTAGCTGGACTTGTTTTA	NM_018652.N
GOLGA6C	GCTGAACGCACACGTGAC	TTTAGCATATTCGTCCCGCTCTA	NM_001164404.N
LMLN	AGCTCTGTCCTCCAGAAACA	GCTCGAGGAACAGGAACAAA	NM_001136049.N
NOL4	GGAACGAGAGGCGAGAGAA	TCCTCAGCTCCTCGGTCTA	NM_001353233.N
PAPOLB	AGCCTGCCATTTCTCCATCA	AGGTCTGGATGGCTTATGAGAC	NM_020144.N
PCDHA9	ACGCTCTCGGTTCCTGTAAA	TCTGCGTCTAGGTCGATCAC	NM_014005.N
RGPD1	AGACCGCTTAGCATGGACTA	TTCTTTAGCTTCATAATACAGCTTTGC	NM_001024457.N
SOX6	CAGCAGCAACTTCTGCAACA	TGAGCGGAGGCATGTGAC	NM_001367873.N
ZNF492	ACCTGTCTGGAGCAAGGAAA	GGTCTCGGGCAAAATAAGAACA	NM_020855.N
ZNF695	CCCTTGGTGAGGATAGCTTCAA	TCTTTCCTTGCCTCCAGACA	NM_020394.N
ACTB	CCAACCGCGAGAAGATGAC	TAGCACAGCCTGGATAGCAA	NM_001101.N
GAPDH	GAGAACGGGAAGCTTGTCATCA	TGGACTCCACGACGTACTCA	NM_002046.



Supplementary Figure 1 (BECKER et al., 2023a): Dual-luciferase reporter gene assays of previously tested genes PFKFB4, HMMR, UBQLN3, and ODF2. HEK 293T cells were transfected with the reporter gene constructs (pMIR), and the miRNA-expression plasmids (23a, pSG5-miR23a-3p/23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of four independent experiments carried out in duplicates. Data are represented as mean ± SEM, and p-values were calculated using Welch t-test, and $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant).



Supplementary Figure 2 (BECKER et al., 2023a): Dual-luciferase reporter gene assays of the 3'UTRs of ZNF492, GNAT1, REEP1, CSNK1G1, FAM169A, and TMEM215 that were not significantly reduced in luciferase activity. HEK 293T cells were transfected with the reporter gene constructs (pMIR), and the miRNA-expression plasmids (23a, pSG5-miR-23a3p/ 23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of four independent experiments carried out in duplicates. Data are represented as mean ± SEM, and the p-value was calculated using Welch t-test. $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant).



Supplementary Figure 3 (BECKER et al., 2023a): Mutagenesis dual-luciferase reporter gene assays of the 3'UTRs of G2E3, ZNF695, and RGPD1 that were not significantly reduced in luciferase activity comparing wild-type (WT) and mutated (mut) 3'UTR. HEK 293T cells were transfected with the WT and mut reporter gene constructs (pMIR), and the miRNAexpression plasmids (23a, pSG5-miR-23a-3p/ 23b, pSG5-miR-23b-3p) in the indicated combinations. The relative luciferase activity (RLU) was normalized to the empty control vectors (pMIR-empty and pSG5-empty). The results represent the mean of three independent experiments carried out in duplicates. Data are represented as mean ± SEM, and the p-value was calculated using unpaired two-tailed Student's t-test. $p < 0.05$ was considered statistically significant (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non-significant).

6.2. MicroRNA-targeting in male infertility: Sperm microRNA-19a/b-3p and its spermatogenesis related transcripts content in men with oligoasthenozoospermia

Supplementary Table 1 (ABU-HALIMA et al., 2022): The primers and design RefSeq of the genes included in the study.

Target	Forward Primer	Reverse Primer	Design RefSeq	Results
AQP5	ACTTCACTGGCTGCTCCA	GATGGGCCCTACCCAGAAA	NM_001651.N	Significant
ASAP2	CTGACCAACAAAGGCCAACC	ACCATAGCATTGGACAGAGGAC	NM_003887.N	Significant
ATF7IP2	TGGCAGTCATCACTTGACAC	GCTCTGAAGTTTTCATGCGTTTAA	NM_001352120.N	Significant
BOD1L2	TCCAGCAAACGACAACCAAC	TGTCCACTCCAGCTTCTGAC	NM_001257964.N	Significant
BRCA2	AGAGCCGATTACCTGTGTACC	TCTTGACCAGGTGCGGTAAA	NM_000059.N	Significant
C22ORF31	TCCCCTTACTAAGCTAGCGAAA	CTGGGGTCTCGTCTCACA	NM_015370.N	Significant
C2ORF42	CTGTGGTTGCTTCCTCGTTAAA	CCAGCCAGTCTTGGAAGGATA	NM_001348758.N	Significant
CCDC87	ACTGAATCAGCCACCCTCAC	ACGGTAGACGTTGGGACTAGTA	NM_018219.N	Significant
CCER1	AACCACCCGTGTGCTCTAA	GCAGGGGAATTTCCAGAATCC	NM_152638.N	Significant
COX8C	AGCACTGGAGCTTGCGTTA	ATCGCGTCAGGTGAGACAAA	NM_182971.N	Significant
CPEB1	CCCAAAGACCCCTTCAGCATA	AGGTACAGGTGGCTTCATTCA	NM_001365240.N	Significant
CSMD1	AGCAAAACCAGGCCACTCTA	CTCTGACCTCCAAGGTGTCATAA	NM_033225.N	Significant
CSNK1G1	TTGACCTCTGTGACCGAACA	GTGCACGTATTCCATTCGAGAA	NM_022048.N	Significant
DCAF12L1	AACAAGGTGTTCGCGTCAC	CCACCACGAAAAGCGTGTTA	NM_178470.N	Significant
DDHD1	CCGCCGACACTATGGAGAA	TCCAAGTCCCTTTCCAATGCTA	NM_001160148.N	Significant
DEPDC1	TATCAAAGGGAGGTGGGGATCA	TAAGTGGCGAAGTTGCAGGAA	NM_001114120.N	Significant
DNAI1	GGGAAGATGGCCATGAGGAA	TGATCTTAGCAGCTTGGGACAA	NM_001281428.N	Significant
DPYSL5	GAGGGACCTTCACGAATCCA	GCTGAAGCTCGCTTTGGAA	NM_020134.N	Significant
ELAVL2	AGAGAAAGCTATCAACACCCTGAA	AACTTGGGCGAGCATAGGAA	NM_001351455.N	Significant
FAM104A	CCCACTTCCACAGCCTACA	TCCCTTCACAGAAGGCAAGAA	NM_001098832.N	Significant

FAM169A	AGGAGAGGCCATTGGGTTTTA	CTGGCAATTGGTAACTTTGGGTA	NM_015566.N	Significant
FHL5	CCACTGCAAGAGGACCATCA	CAGGTTTCATGCCAGTAGTTTCC	NM_020482.N	Significant
FSHR	GATGTGTTCTCCAACCTTCCC	AAGGCCTCAGGGTTGATGTA	NM_000145.N	Significant
GNAT1	ACCACTGGCATCATCGAGAC	CCCGCCCACATCGAACA	NM_144499.N	Significant
GOLGA6A	GCAGTTGGAGCAGCAAGTAAA	GGTGGCTGGCAGCTTCTA	NM_001038640.N	Significant
GOLGA6D	ATGAGCGCTGCACTATGGTA	GCTGCAGCTCATGATGCAATA	NM_001145224.N	Significant
HSF5	TACACACCCTCAGCACAGTA	TCCATGTGGGTAGGAGAACA	NM_001080439.N	Significant
HSPA2	GTGTCATGACCCCACTCATCA	GTTGTCCGAGTAGGTGGTGAA	NM_021979.N	Significant
MCHR2	AATTTGCCTGTAGTGCCATCA	CTCCAACGTGTCAGTCGAAA	NM_001040179.N	Significant
MED26	CCAGCCTGGAGAAATACCCTA	ACGTCGTTGATGAGCTTCC	NM_004831.N	Significant
MYBL1	CCTTGTGCAGCTATGGATCA	CAGGTGACACATACTGATACCC	NM_001080416.N	Significant
ODF4	GTTGGCTGGTGCTTATCCTA	GTGGCTCAGAATCAGACTCC	NM_153007.N	Significant
PCDHA7	TATTCCAGAGGACGCCCAAC	ACCTGTCCGTTGACTCCAAA	NM_018910.N	Significant
POC1A	CGGGAATGTGTCCACTCGTA	GTGGAAGTCCACATAGGTGACA	NM_001161581.N	Significant
PRSS54	ACACCACCAGTCTTGCAGAA	TCTGGAGTTTGTATAGGGGACAC	NM_001080492.N	Significant
REEP1	GTCAAATGGATGATGTACTGGATTA	CAACAAAGGAAGATGTCTGTGAA	NM_001371279.N	Significant
RFX4	TGGCTGGATACCATGGTTGAC	TGCTGGGCCACTTTCTTCAA	NM_213594.N	Significant
SAMD4A	CTTTCCCTCGGAAAACCCTTCTA	AGGGAGTTGGATTGCCCAAA	NM_015589.N	Significant
SCML2	TCGCTTCTGGGCCATGTAA	CAGATGAGGGCCAAAGTTTCC	NM_006089.N	Significant
SENP8	GAGCAACTCAGTTCACGCAAA	CACAAAGGCCAGTTTGTCTCC	NM_001172111.N	Significant
SPATA12	GCCTGCAGTATCAGACCTCAA	TGTCCACTTTCACAGCTTCCC	NM_181727.N	Significant
SPIRE1	GAGCGATGAATCTAGCACAGAC	CCTCATCACCTGTACCCAGAA	NM_001128626.N	Significant
STK33	ACTATAGCCAGCAGTGTGAC	AGGGTGGTTCTCCACGTA	NM_001352398.N	Significant
TDRD10	GGTGTATGTTGGCAATCTTCC	ACATCAAGAGGGTTGAAGTCC	NM_001098475.N	Significant
TKTL2	AGTGCCTCAACGTGGGAAAA	CGGCTGCTATAATGTGTCTGGTA	NM_032136.N	Significant

TMEM215	TGGAGAGGGCAGGAAACC	CGCATGTTTCACTCCATCCA	NM_212558.N	Significant
TTLL2	ACCTGTGTTCCTCCACACAA	CCTCGGATGGAATGTTAAGGGTA	NM_031949.N	Significant
UBN2	TAAGGAAAGCCGGAGTGTTCA	TGGGTTTAGGTGCAGGAATCA	NM_173569.N	Significant
UBQLNL	TGGCAGGACAAGTGCTAGAA	GTGAGCTGGTCTTGTTGTTCC	NM_145053.N	Significant
USP6	GCCACTACCAATGGACAGTTAC	TAGTCCATACCGTACAGGGGTA	NM_001304284.N	Significant
ZNF280B	CGGCAGCTCGCGAACA	CTCAGGTTTATTACAACGCAACTTTTAA	NM_080764.N	Significant
WNK3	TTTCACAACCCCAGGTTTCC	AGCTCCAACTGGTTGGGATA	NM_020922.N	Significant
MBNL3	GCCCAAAACTGATGCGTTCA	CAATCATTCTCCCCACGGGTA	NM_001170704.N	Significant
RALGPS2	AGATGACACCTTGCATTCCCTA	TGCTGCCAGTTGATGGGTA	NM_001286247.N	Borderline significant
ZNF711	CCCGAAGGTATGAAGATTGTCA	AAGGTACTGTGCTGCTGTAC	NM_001330574.N	Borderline significant
ZPLD1	TGCTCAGTTCAACGGCTACA	TAGCCTGCACTCCACAATAGAC	NM_001329788.N	Borderline significant
SFMBT1	GGGTGGTGTGAGCAGAATAA	GCAGAAACTCATCCCAGTCA	NM_016329.N	Borderline significant
ARRDC5	GGAACTTCCACCTTCCACAA	GGCAGCAGTTGTAGGAGAC	NM_001367189.N	Non-Significant
C5ORF49	GACCCCAAAGAGCACAGCTA	TGATCATAATCTAGGCGCCTCTTA	NM_001089584.N	Non-Significant
CCDC15	TCCTGATACCTGTGCCAACAA	TGAATGAATGTAGTGCCCGAGTA	NM_025004.N	Non-Significant
CEP170	ACAGGCTGCCACGAGAAA	GCGTGTTGCTTATCCACACTAC	NM_014812.N	Non-Significant
CEP55	GGAGCTCCGAAAAGCAAGAA	TGGCTCTGTGATGGCAAAC	NM_018131.N	Non-Significant
CLEC18B	ACCTGGGCAGAGGTGTG	CAAGAAACTCTCCTTCCTGTTCAG	NM_001011880.N	Non-Significant
CLHC1	TGCAGCAAACAGTCCTAGAA	AATGGAAGAGGCTTTCCTCTAA	NM_001353781.N	Non-Significant
DNAJC27	AGCTTGGACCTCATGGAAAC	ACACAGCGATGTTTGGTACA	NM_016544.N	Non-Significant
INHBB	GAGCGCGTTTCCGAAATCA	TGCCTTCGTTGGAGATGAAGAA	NM_002193.N	Non-Significant
LRP2BP	GGGCATCACCAGGGATGAAA	ATCTGCCAATGCGGGATTCA	NM_018409.N	Non-Significant
PRDM9	GCAGCCAACAATGGATACTCC	ATCCAGTTGGCCCAGGATTTA	NM_020227.N	Non-Significant
RPL39L	AGCATCTTTGCCTCTTTGGAGTA	ACATGGCGAGAAACAGAGTCA	NM_052969.N	Non-Significant
TDRD6	GCAGTGATGGAAGCAAGCA	GACCACACAACACATTTAGATCCA	NM_001010870.N	Non-Significant

WIPF3	GAGGTCAACTGCGAAATGGAA	CTTCCACAGAATGGAACGTGAA	NM_001080529.N	Non-Significant
ZMYND12	ACTATGAAGAGGCCCGTTATCA	GGTGGAAGTAGCCTCCTGAA	NM_001146192.N	Non-Significant
SH2D6	AGGAAGGAAATCGTCTCTTCCC	CTGGGGCGCACGGTATA	NM_201594.N	Omitted
TCL1B	TGGTCGTGCGGTTCAATCC	AGGAGAGTAGCTCCCGGGTA	NM_004918.N	Omitted
CALCR	CCATCTACTGCTTCTGCAACA	CAACGCTGGTTCCACTGAA	NM_001164737.N	Omitted
EPHA10	GGACTGCACTGCCAAGTAA	GCACATTGCACACTTGGTAC	NM_001099439.N	Omitted
FAM9A	GCCGCTACTTGAGCAATTAC	CTTCACTGGAAGAAATGATGCA	NM_001171186.N	Omitted
GABRR1	GCCTGATGGGAAAGTGCTCTA	TCCAAGGGAAATCGGCTGAA	NM_001256704.N	Omitted
MAGEA10	CAGGCTTGAGATCGGCTGAA	CGACCCACTGTGTCTGTAGAA	NM_001011543.N	Omitted
NYAP1	AAGGTCCTCTATGGAGGGAGAA	TCGGCACTGCCATTCCA	NM_173564.N	Omitted
POU4F1	TCACTTTGCCATGCATCCC	GGCGAAGAGGTTGCTCTG	NM_006237.N	Omitted
SCN9A	GGTGTTGGCTGGATTTCCTA	ATGGGGCCAAGATCTGAGTA	NM_002977.N	Omitted
ACTB	CCAACCGCGAGAAGATGAC	TAGCACAGCCTGGATAGCAA	NM_001101.N	Endogenous Control
GAPDH	GAGAACGGGAAGCTTGTCATCA	TGGACTCCACGACGTACTCA	NM_002046.	Endogenous Control

Supplementary Table 2 (ABU-HALIMA et al., 2022): Correlation analysis between the expression level of genes and basic semen parameters.

Gene	Count (106/mL)			Motility (% motile)			Morphology (%)		
	r	95 % confidence interval	P value	r	95 % confidence interval	P value	r	95 % confidence interval	P value
AQP5	0.34	0.527 to 0.125	0.0019	0.36	0.543 to 0.147	0.0010	0.29	0.488 to 0.073	0.0081
ASAP2	0.34	0.52 to 0.122	0.0020	0.39	0.561 to 0.179	0.0003	0.28	0.471 to 0.0571	0.0118
ATF7IP2	0.48	0.634 to 0.286	<0.0001	0.43	0.598 to 0.231	<0.0001	0.32	0.508 to 0.106	0.0032
BOD1L2	0.45	0.611 to 0.25	<0.0001	0.47	0.628 to 0.276	<0.0001	0.32	0.508 to 0.105	0.0033
BRCA2	0.21	0.44 to 0.0537	0.1082	0.25	0.472 to 0.013	0.0553	0.27	0.494 to 0.0162	0.0325
C22ORF31	0.34	0.527 to 0.128	0.0017	0.31	0.499 to 0.0914	0.0049	0.25	0.453 to 0.031	0.0222
C2ORF42	0.32	0.511 to 0.109	0.0029	0.36	0.539 to 0.147	0.0009	0.26	0.453 to 0.0345	0.0204
CCDC87	0.42	0.584 to 0.211	0.0001	0.39	0.563 to 0.181	0.0003	0.34	0.524 to 0.128	0.0017
CCER1	0.42	0.587 to 0.216	<0.0001	0.38	0.552 to 0.165	0.0005	0.33	0.517 to 0.118	0.0023

COX8C	0.43	0.594 to 0.226	<0.0001	0.39	0.563 to 0.182	0.0003	0.32	0.51 to 0.109	0.0030
CPEB1	0.32	0.507 to 0.096	0.0045	0.38	0.557 to 0.164	0.0006	0.28	0.473 to 0.0511	0.0141
CSMD1	0.37	0.546 to 0.154	0.0008	0.44	0.602 to 0.234	<0.0001	0.36	0.541 to 0.148	0.0010
CSNK1G1	0.37	0.548 to 0.16	0.0006	0.43	0.596 to 0.228	<0.0001	0.30	0.49 to 0.0818	0.0063
DCAF12L1	0.42	0.59 to 0.219	<0.0001	0.37	0.551 to 0.165	0.0005	0.32	0.507 to 0.105	0.0034
DDHD1	0.27	0.467 to 0.0516	0.0135	0.32	0.509 to 0.106	0.0032	0.22	0.423 to 0.0031	0.0468
DEPDC1	0.27	0.468 to 0.0539	0.0128	0.27	0.465 to 0.0497	0.0142	0.21	0.41 to 0.0181	0.0635
DNAI1	0.35	0.532 to 0.129	0.0018	0.35	0.536 to 0.135	0.0015	0.25	0.448 to 0.0188	0.0295
DPYSL5	0.45	0.608 to 0.244	<0.0001	0.52	0.668 to 0.336	<0.0001	0.42	0.589 to 0.216	<0.0001
ELAVL2	0.36	0.543 to 0.15	0.0009	0.38	0.557 to 0.17	0.0005	0.27	0.464 to 0.0458	0.0157
FAM104A	0.42	0.592 to 0.222	<0.0001	0.40	0.568 to 0.188	0.0002	0.32	0.508 to 0.106	0.0032
FAM169A	0.25	0.447 to 0.0263	0.0247	0.30	0.49 to 0.0818	0.0063	0.22	0.421 to 0.00518	0.0488
FHL5	0.28	0.474 to 0.0581	0.0117	0.28	0.478 to 0.0636	0.0101	0.19	0.393 to 0.041	0.0978
FSHR	0.37	0.603 to 0.0682	0.0146	0.43	0.651 to 0.145	0.0035	0.25	0.513 to 0.0641	0.1077
GNAT1	0.27	0.487 to 0.0313	0.0235	0.42	0.604 to 0.196	0.0004	0.36	0.553 to 0.121	0.0029
GOLGA6A	0.29	0.481 to 0.0675	0.0092	0.34	0.522 to 0.122	0.0021	0.26	0.457 to 0.0369	0.0194
GOLGA6D	0.44	0.604 to 0.241	<0.0001	0.42	0.588 to 0.218	<0.0001	0.36	0.541 to 0.15	0.0009
HSF5	0.35	0.536 to 0.14	0.0012	0.36	0.543 to 0.15	0.0009	0.25	0.449 to 0.0262	0.0248
HSPA2	0.43	0.595 to 0.228	<0.0001	0.38	0.56 to 0.176	0.0004	0.32	0.504 to 0.0996	0.0038
MCHR2	0.47	0.682 to 0.177	0.0021	0.48	0.692 to 0.194	0.0015	0.19	0.476 to 0.136	0.2398
MED26	0.41	0.578 to 0.202	0.0001	0.48	0.631 to 0.281	<0.0001	0.37	0.544 to 0.154	0.0008
MYBL1	0.35	0.532 to 0.132	0.0016	0.39	0.565 to 0.178	0.0004	0.28	0.478 to 0.0607	0.0110
ODF4	0.27	0.469 to 0.0401	0.0184	0.18	0.398 to 0.0483	0.1089	0.17	0.386 to 0.0616	0.1366
PCDHA7	0.38	0.553 to 0.167	0.0005	0.35	0.531 to 0.136	0.0013	0.32	0.507 to 0.104	0.0034
POC1A	0.32	0.509 to 0.0975	0.0043	0.33	0.52 to 0.112	0.0028	0.36	0.539 to 0.139	0.0013
PRSS54	0.34	0.528 to 0.121	0.0023	0.38	0.562 to 0.168	0.0006	0.30	0.497 to 0.0788	0.0072
REEP1	0.38	0.558 to 0.174	0.0004	0.36	0.542 to 0.152	0.0008	0.31	0.497 to 0.0909	0.0049
RFX4	0.38	0.556 to 0.171	0.0004	0.45	0.609 to 0.248	<0.0001	0.34	0.526 to 0.129	0.0016
SAMD4A	0.39	0.562 to 0.179	0.0003	0.44	0.605 to 0.242	<0.0001	0.30	0.491 to 0.0831	0.0060
SCML2	0.34	0.562 to 0.0816	0.0094	0.28	0.513 to 0.0118	0.0358	0.28	0.515 to 0.0146	0.0340
SENP8	0.45	0.608 to 0.247	<0.0001	0.44	0.604 to 0.241	<0.0001	0.37	0.548 to 0.159	0.0006

SPATA12	0.37	0.548 to 0.16	0.0006	0.40	0.574 to 0.197	0.0002	0.25	0.451 to 0.0319	0.0217
SPIRE1	0.34	0.521 to 0.123	0.0020	0.38	0.559 to 0.175	0.0004	0.26	0.454 to 0.0354	0.0200
STK33	0.28	0.477 to 0.0557	0.0126	0.27	0.465 to 0.041	0.0178	0.15	0.365 to 0.0801	0.1869
TDRD10	0.36	0.537 to 0.142	0.0011	0.39	0.564 to 0.18	0.0003	0.30	0.489 to 0.0775	0.0071
TKTL2	0.42	0.587 to 0.215	<0.0001	0.39	0.562 to 0.18	0.0003	0.34	0.527 to 0.131	0.0016
TMEM215	0.39	0.562 to 0.176	0.0004	0.42	0.592 to 0.22	<0.0001	0.29	0.481 to 0.0678	0.0091
TTLL2	0.37	0.552 to 0.16	0.0007	0.39	0.563 to 0.176	0.0004	0.31	0.498 to 0.0865	0.0057
UBN2	0.29	0.478 to 0.0663	0.0094	0.37	0.545 to 0.156	0.0007	0.25	0.447 to 0.0266	0.0245
UBQLNL	0.44	0.606 to 0.244	<0.0001	0.44	0.602 to 0.238	<0.0001	0.35	0.532 to 0.138	0.0012
USP6	0.31	0.504 to 0.094	0.0046	0.33	0.514 to 0.108	0.0032	0.25	0.447 to 0.0207	0.0281
ZNF280B	0.35	0.534 to 0.137	0.0013	0.40	0.576 to 0.197	0.0002	0.29	0.479 to 0.0651	0.0098
WNK3	-0.32	-0.0845 to -0.521	0.0070	-0.44	-0.224 to -0.618	0.0001	-0.40	-0.18 to -0.588	0.0005
MBNL3	-0.33	-0.0985 to -0.523	0.0047	-0.34	-0.113 to -0.534	0.0032	-0.27	-0.0385 to -0.478	0.0196

Spearman correlation analysis. An Unpaired two-tailed t-test was used to calculate the p-value. Significant change in abundance level was considered with a p-value < 0.05

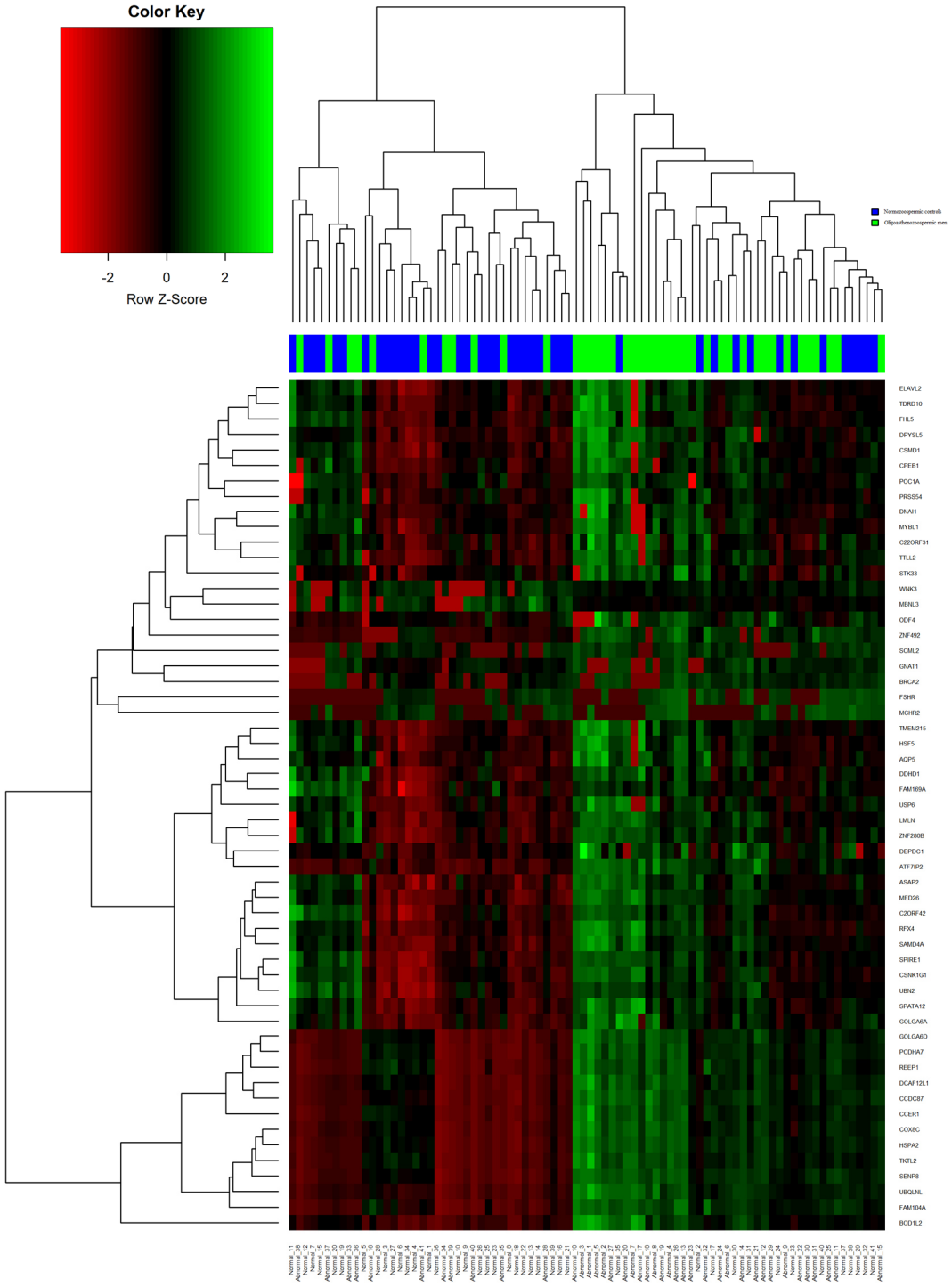
Supplementary Table 3 (ABU-HALIMA et al., 2022): Correlation analysis between the expression level of miRNAs and target genes.

Gene	miR-19a-3p			miR-19-b-3p		
	r	95 % confidence interval	P value	r	95 % confidence interval	P value
AQP5	-0.41	-0.579 to -0.199	0.0002	-0.29	-0.48 to -0.0636	0.0103
ASAP2	-0.47	-0.627 to -0.275	<0.0001	-0.40	-0.571 to -0.192	0.0002
ATF7IP2	-0.49	-0.645 to -0.303	<0.0001	-0.34	-0.521 to -0.122	0.002
BOD1L2	-0.53	-0.674 to -0.349	<0.0001	-0.39	-0.565 to -0.185	0.0003
BRCA2	-0.33	-0.539 to -0.077	0.0094	-0.14	-0.383 to 0.121	0.2778
C22ORF31	-0.47	-0.629 to -0.276	<0.0001	-0.37	-0.55 to -0.16	0.0006
C2ORF42	-0.45	-0.612 to -0.253	<0.0001	-0.35	-0.53 to -0.135	0.0014
CCDC87	-0.49	-0.64 to -0.295	<0.0001	-0.37	-0.549 to -0.162	0.0006
CCER1	-0.46	-0.616 to -0.259	<0.0001	-0.35	-0.531 to -0.136	0.0013
COX8C	-0.47	-0.623 to -0.269	<0.0001	-0.37	-0.548 to -0.16	0.0006

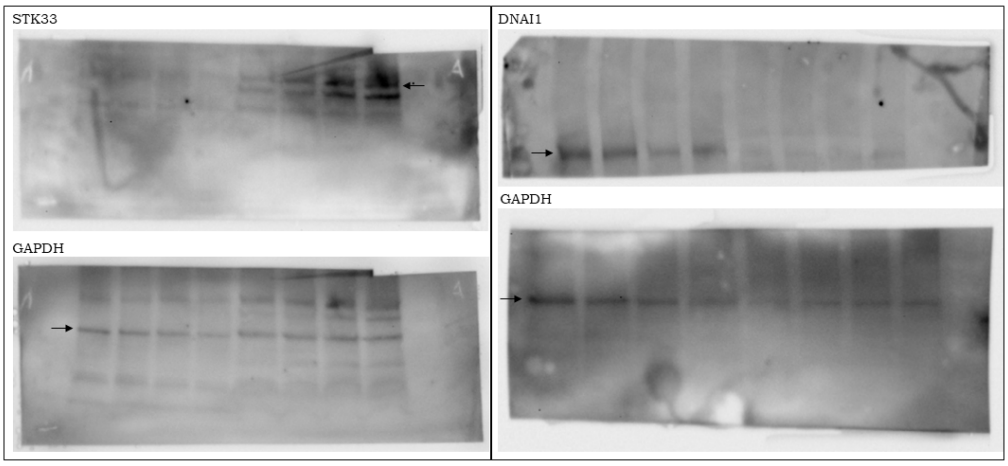
CPEB1	-0.44	-0.61 to -0.241	<0.0001	-0.35	-0.532 to -0.13	0.0017
CSMD1	-0.51	-0.656 to -0.318	<0.0001	-0.40	-0.577 to -0.198	0.0002
CSNK1G1	-0.50	-0.651 to -0.312	<0.0001	-0.42	-0.584 to -0.211	0.0001
DCAF12L1	-0.46	-0.619 to -0.262	<0.0001	-0.36	-0.54 to -0.15	0.0009
DDHD1	-0.43	-0.597 to -0.23	<0.0001	-0.35	-0.527 to -0.132	0.0015
DEPDC1	-0.47	-0.63 to -0.279	<0.0001	-0.42	-0.588 to -0.218	<0.0001
DNAI1	-0.42	-0.589 to -0.21	0.0001	-0.34	-0.522 to -0.116	0.0026
DPYSL5	-0.55	-0.692 to -0.375	<0.0001	-0.41	-0.584 to -0.209	0.0001
ELAVL2	-0.48	-0.634 to -0.283	<0.0001	-0.36	-0.537 to -0.142	0.0012
FAM104A	-0.44	-0.603 to -0.239	<0.0001	-0.31	-0.501 to -0.0961	0.0042
FAM169A	-0.39	-0.562 to -0.18	0.0003	-0.31	-0.497 to -0.0912	0.0049
FHL5	-0.40	-0.576 to -0.197	0.0002	-0.28	-0.473 to -0.0566	0.0121
FSHR	-0.43	-0.647 to -0.14	0.0039	-0.25	-0.515 to 0.0604	0.1026
GNAT1	-0.44	-0.615 to -0.212	0.0002	-0.24	-0.46 to 0.00319	0.0466
GOLGA6A	-0.50	-0.653 to -0.313	<0.0001	-0.43	-0.597 to -0.227	<0.0001
GOLGA6D	-0.47	-0.627 to -0.275	<0.0001	-0.37	-0.545 to -0.156	0.0007
HSF5	-0.45	-0.616 to -0.255	<0.0001	-0.34	-0.521 to -0.12	0.0022
HSPA2	-0.47	-0.626 to -0.273	<0.0001	-0.37	-0.551 to -0.164	0.0005
MCHR2	-0.43	-0.654 to -0.128	0.0054	-0.33	-0.586 to -0.0166	0.0345
MED26	-0.53	-0.669 to -0.341	<0.0001	-0.41	-0.584 to -0.211	0.0001
MYBL1	-0.46	-0.623 to -0.264	<0.0001	-0.32	-0.51 to -0.103	0.0037
ODF4	-0.24	-0.443 to -0.00723	0.0378	-0.12	-0.337 to 0.118	0.3168
PCDHA7	-0.41	-0.579 to -0.204	0.0001	-0.32	-0.504 to -0.1	0.0038
POC1A	-0.47	-0.63 to -0.272	<0.0001	-0.35	-0.531 to -0.127	0.0019
PRSS54	-0.35	-0.536 to -0.132	0.0017	-0.26	-0.461 to -0.032	0.0221
REEP1	-0.38	-0.555 to -0.17	0.0005	-0.30	-0.493 to -0.086	0.0056
RFX4	-0.50	-0.651 to -0.313	<0.0001	-0.39	-0.561 to -0.178	0.0003
SAMD4A	-0.50	-0.649 to -0.309	<0.0001	-0.39	-0.561 to -0.178	0.0003
SCML2	-0.34	-0.555 to -0.0718	0.0115	-0.21	-0.452 to 0.0672	0.1261

SENP8	-0.48	-0.637 to -0.29	<0.0001	-0.38	-0.554 to -0.168	0.0005
SPATA12	-0.52	-0.665 to -0.334	<0.0001	-0.43	-0.594 to -0.226	<0.0001
SPIRE1	-0.50	-0.654 to -0.316	<0.0001	-0.39	-0.566 to -0.185	0.0003
STK33	-0.29	-0.482 to -0.0632	0.0105	-0.20	-0.409 to 0.0283	0.0766
TDRD10	-0.47	-0.631 to -0.279	<0.0001	-0.34	-0.526 to -0.127	0.0018
TKTL2	-0.44	-0.605 to -0.243	<0.0001	-0.36	-0.538 to -0.147	0.001
TMEM215	-0.52	-0.663 to -0.329	<0.0001	-0.40	-0.574 to -0.195	0.0002
TTLL2	-0.53	-0.679 to -0.351	<0.0001	-0.47	-0.63 to -0.274	<0.0001
UBN2	-0.44	-0.602 to -0.237	<0.0001	-0.38	-0.555 to -0.17	0.0005
UBQLNL	-0.51	-0.654 to -0.317	<0.0001	-0.38	-0.554 to -0.168	0.0005
USP6	-0.50	-0.653 to -0.311	<0.0001	-0.39	-0.564 to -0.177	0.0004
ZNF280B	-0.51	-0.661 to -0.325	<0.0001	-0.43	-0.595 to -0.224	<0.0001
MBNL3	0.45	0.239 to 0.62	<0.0001	0.37	0.143 to 0.555	0.0014
WNK3	0.41	0.185 to 0.592	0.0005	0.27	0.027 to 0.478	0.0255

Spearman correlation analysis. An Unpaired two-tailed t-test was used to calculate the p-value. Significant change in abundance level was considered with a p-value < 0.05



Supplementary Figure 1 (ABU-HALIMA et al., 2022).



Supplementary Figure 2 (ABU-HALIMA et al., 2022).

6.3. Proteomic Landscape of Human Sperm in Patients with Different Spermatogenic Impairments

Die ergänzenden Tabellen 1, 3, und 4 können aufgrund ihres Ausmaßes nicht in dieser Arbeit abgebildet werden. Sie stehen jedoch online freizugänglich zur Verfügung (https://www.mdpi.com/article/10.3390/cells12071017/s1; abgerufen am 18.07.2023). Die ergänzenden Tabellen 2 und 5 sind im Folgenden dargestellt.

Supplementary Table 2 (BECKER et al., 2023b): Detailed listing of upset plot results in Figure 2B.

Intersection	Size	Proteins
Only in A vs. N lower	72	AFM, DBI, MYO1C, DOPEY2, GPR64, MYO1D, SELENBP1, ESPN, SERPINA3, ATP8A1, MGAM, SYTL1, GALE, BASP1, DPP3, SLC44A4, GDI2, FBP1, CD9, GDI1, AZGP1, ANKFY1, GNB1, GLIPR2, SRC, ATP9A, GSS, GNAI2, MON2, IDH1, VAT1, DNAJC5, TSPAN6, CAMK1, MPI, CLIC4, CSTB, NAPA, EZR, ATP6V0A1, CNP, ATP6V1B2, LTA4H, CAP1, QDPR, RAP1A, RAB10, GSR, RAB27A, SYT7, EFHD2, TXNDC17, WDR1, PGD, SERPINB6, ACTN4, GNAS, RAC1, RAB7A, AHCY, MDH1, PARK7, RAB8A, SCFD1, ATP6V1H, RAB5C, RAB18, APEH, TMEM30A, PSMD13, PYCRL, EIF4A1
Only in A vs. N higher	53	SAR1A, SEP7, HMOX2, GAPDHS, GAA, TMED4, ENKUR, HSPA9, SCCPDH, PLA1A, HSPD1, LTF, ECH1, MLF1, ATP5O, NFS1, DLD, HADHB, AK8, ACAT1, RPL22, SDHA, FAM209A, CYC1, CABYR, CABYR (iso3), CTSF, CLU, ACO2, SSBP1, OGDH, GK2, ELSPBP1, CAPZA3, COX7C, CPD, NUP210L, CHID1, APOE, H2AFX, HSPE1, SLPI, LRRC37B, CSNK2A1;CSNK2A3, GPC4, PLOD3, DNAH2, IZUMO4, HIST1H1T, SPESP1, CAMP, MMP2, APOA1
AN vs. N higher & A vs. N higher	33	P4HB, GANAB, CALR, CDH1, TUBA1A, CPQ, CTSH, HEXA, PPIB, DNAJC3, SIL1, SCPEP1, SEMG1, SEMG2, COL18A1, CPZ, SERPINA5, LAMC1, NEU1, LAMA5, MAMDC2, FN1, GLA, MATN2, SDF4, MXRA5, LAMB2, VWA1, NUCB2, LPL, PLOD1, HSPA13, APOA4
AN vs. N lower & A vs. N lower	22	HP, HPX, SERPING1, AHSG, APOH, TTR, PLS3, CYB5R2, ALDH1A1, LCP1, IMPA1, GOT1, APCS, GPI, FLOT1, SYPL1, PGAM2, MYADM, TKFC, ACTG1, HSPA4, CAND1
Only in AN vs. N lower	8	STOM, PPP3CC, FHL1, TEX101, PRKACG, TSPAN16, RPS27A, CCT7
Only in OA vs. N lower	6	CFAP20, GLB1L, ACADM, ACOT7, TRAP1, RUVBL1
Only in OA vs. N higher	1	SRP72
AN vs. N lower & OA vs. N lower	1	HDDC2
Only in AN vs. N higher	1	CANX
AN vs. N lower & A vs. N lower & OA vs. N lower	1	UCHL1

Supplementary Table 5 (BECKER et al., 2023b): More detailed information of participants' sperm motility.

Parameters	N (n=31)	AN (n=22)	OA (n=9)	A (n=13)	N vs. AN	N vs. OA	N vs. A
Total motility (PR + NP, %)	60.2 ± 9.8	50.6 ± 16.0	41.9 ± 12.3	56.7 ± 15.8	*	n.s.	**
Progressive motile (PR, %)	52.0 ± 10.6	12.3 ± 9.0	14.9 ± 11.3	10.5 ± 7.0	***	***	***
Non-progressive motile (NP, %)	7.9 ± 7.0	38.3 ± 20.7	27.0 ± 22.1	46.2 ± 16.2	***	*	***
Immotile (IM, %)	39.8 ± 9.8	49.1 ± 16.2	58.1 ± 12.3	42.8 ± 15.9	*	**	n.s.

PR = progressive, NP = non-progressive, IM = immotile. Data were presented as mean ± standard deviation. Unpaired two-tailed t-test was performed. p-value < 0.05 was considered as statistically significant. *** $p<0.001$; ** $p<0.01$; * $p<0.05$; n.s. = not significant.

7. ERWEITERTE PUBLIKATIONSLISTE

Neben den Publikationen, die Bestandteil meiner kumulativen Dissertation sind, wurden weitere sechs Publikationen im Rahmen meiner Promotion am Institut für Humangenetik der Medizinischen Fakultät der Universität des Saarlandes veröffentlicht. Diese werden nachfolgend chronologisch aufgelistet. Erstautoren, die gleichermaßen zur Publikation beigetragen haben, wurden mit einem (*) - Symbol markiert.

Abu-Halima M*, Belkacemi A*, Ayesh BM, **Becker LS**, Sindiani AM, Fischer U, Hammadeh M, Keller A, Meese E. MicroRNA-targeting in spermatogenesis: Over-expressions of microRNA-23a/b-3p and its affected targeting of the genes ODF2 and UBQLN3 in spermatozoa of patients with oligoasthenozoospermia. Andrology. (2021); 9(4):1137-1144. doi: 10.1111/andr.13004.

Abu-Halima M, Wagner V, **Becker LS**, Ayesh BM, Abd El-Rahman M, Fischer U, Meese E, Abdul-Khaliq H. Integrated microRNA and mRNA Expression Profiling Identifies Novel Targets and Networks Associated with Ebstein's Anomaly. Cells. (2021); 10(5):1066. doi: 10.3390/cells10051066.

Abu-Halima M, **Becker LS**, Ayesh BM, Baus SL, Hamza A, Fischer U, Hammadeh M, Keller A, Meese E. Characterization of micro-RNA in women with different ovarian reserve. Sci Rep. (2021); 11(1):13351. doi: 10.1038/s41598-021-92901-w.

Abu-Halima M*, Keller A*, **Becker LS**, Fischer U, Engel A, Ludwig N, Kern F, Rounge TB, Langseth H, Meese E, Keller V. Dynamic and static circulating cancer microRNA biomarkers - a validation study. RNA Biol. (2023); 20(1):1-9. doi: 10.1080/15476286.2022.2154470.

Becker LS*, Abu-Halima M*, Al Smadi MA, Kunz LS, Gröger L, Meese E. Expression of SPAG7 and its regulatory microRNAs in seminal plasma and seminal plasma-derived extracellular vesicles of patients with subfertility. Sci Rep. (2023); 13(1):3645. doi: 10.1038/s41598-023-30744-3.

Abu-Halima M, **Becker LS**, Al Smadi MA, Abdul-Khaliq H, Raeschle M, Meese E. Sperm Motility Annotated Genes: Are They Associated with Impaired Fecundity? Cells. (2023), 12(9): 1239. doi: 10.3390/cells12091239.

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9. LEBENSLAUF

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.