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ORIGINAL ARTICLE

Genetic variants in *QRICH2* gene among Jordanians with sperm motility disorders

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ABSTRACT

Sperm motility, a key determinant of male fertility, is often impaired by genetic variations affecting flagellar formation. The *glutamine-rich protein 2* (*QRICH2*) gene encodes a protein essential for sperm flagella biogenesis and structural integrity. This study investigates genetic variations within exon 3 of the *QRICH2* gene, identifying novel heterozygous variants associated with sperm tail-specific abnormalities and motility impairments. Among 34 individuals diagnosed with asthenozoospermia (ASZ) and 26 individuals with normal sperm parameters (NZ) from Jordan, eight unique heterozygous variants (c.123 G>T, c.133 G>C, c.138A>G, c.170A>C, c.189C>G, c.190T>C, c.195A>T, and c.204A>T) were exclusive to the ASZ group, while four variants (c.136 G>A, c.145A>C, c.179T>G, and c.180T>G) were found only in NZ. These variants were absent from major genetic databases, suggesting their potential novelty, while two variants (c.206C>T and c.189C>T) were linked to known SNP cluster IDs rs73996306 and rs1567790525, respectively. Four non-synonymous SNPs (c.136 G>A, c.145A>C, c.170A>C, and c.204A>T) were predicted to be functionally and structurally damaging, underscoring their significance. Additionally, five variants overlapped with previously reported mutation sites, indicating potential mutation hotspots. Statistical analysis revealed a significant association between *QRICH2* mutations and tail defects ($p < 0.021$). These findings highlight the critical role of heterozygous *QRICH2* mutations in mild-to-moderate ASZ, even in NZ individuals. Despite some carriers meeting WHO criteria for NZ, notable morphological abnormalities suggest the need for refined diagnostic benchmarks. Screening for *QRICH2* mutations is essential for accurate molecular diagnosis and should be integrated into genetic counseling, particularly in regions like Jordan. Further research into the cumulative effects of heterozygous mutations and their environmental interactions is needed to expand our understanding of idiopathic male infertility and to enhance diagnostic and therapeutic strategies for male infertility.

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1. Introduction

Infertility is characterized by a couple's inability to conceive after 12 months or more of regular attempts to start a family [1]. Globally, 16.5–17.8% of the population grapple with infertility [2] and male factors contribute to half of these cases [3,4].

Male infertility constitutes a multifactorial syndrome involving diverse disorders, which may be either congenital or acquired [5]. The ability of men to achieve fertilization is markedly affected by factors such as sperm concentration, motility, morphology, and DNA integrity [6]. Semen analysis continues to stand out as the paramount indicator of overall male fertility status; it remains the cornerstone of the evaluation of male and couple fertility [7]. Various impact factors, including climate, pollution, diet, lifestyle behaviors, chronic diseases, genetics, and even some folk herbal medicines, have been previously demonstrated their potential negative effects on the male reproductive system [8–11].

ASZ is a condition characterized by the presence of less than 32% progressive motile sperm in the ejaculate, based on the normal values established by the WHO [12]. It is associated with various structural abnormalities in the sperm tail, including abnormal mitochondrial sheath, head–tail or midpiece–principal piece junction, bending or coiling of the tail, and irregular tail caliber or aberrant residual cytoplasm [13].

Research by Curi et al. (2003) revealed that 82% of individuals diagnosed with ASZ concurrently exhibited abnormalities such as teratozoospermia and oligozoospermia [14]. According to the criteria

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established by the WHO in 2010, a patient is diagnosed with teratozoospermia when more than 96% of the sperm they produce exhibit abnormal shapes [12]. Teratozoospermia encompasses many kinds of sperm deformities that arise during spermiogenesis, the ultimate phase of spermatogenesis. These conditions are globozoospermia (which is characterized by sperm with round heads), multiple morphological abnormalities of the sperm flagella (MMAF, which involves aberrant flagellar phenotypes), and acephalic spermatozoa syndrome (ASS, which is defined by a disruption in the head-tail junction of sperm) [15].

Numerous reports suggest an association between low sperm motility and genetic defects [16], highlighting the role of both nuclear and mitochondrial genetic variations in male infertility. Defects in genes involved in spermatogenesis, flagellar structure, and energy metabolism have been linked to ASZ [16]. Mutations in dynein arm-related genes such as DNAH1, DNAH3, DNAH12, and DNALI1 have been implicated in primary ciliary dyskinesia (PCD), which affects sperm tail movement and results in ASZ [17–21]. Similarly, mutations in QRICH2, a gene crucial for sperm flagellar assembly, have been associated with severe sperm motility disorders [22]. In addition, a study by Al Zoubi et al. (2019) highlights a significant association between mitochondrial DNA (mtDNA) deletions and sperm motility impairments, particularly in asthenozoospermic men in the Jordanian population. Their findings emphasize that a 4,977-bp mtDNA deletion leads to disruptions in oxidative phosphorylation (OXPHOS), impairing ATP production— a critical energy source for sperm motility. The study detected this deletion in 79.2% of ASZ individuals, compared to only 10% in normozoospermic controls, underscoring its potential role as a molecular biomarker for male infertility [23].

The Glutamine-rich protein 2, encoded by the *Glutamine-rich protein 2 (QRICH2)* gene (Gene ID: 84074), is a human protein that originally comprised 1663 amino acids (NCBI Reference Sequence: NP_115510.1) with three conserved domains: Glutenin_hmw, SMC_N super family, and DUF4795 [18]. Thereafter, this sequence has been updated to 1829 amino acids (NCBI Reference Sequence: NP_115510.2). The QRICH2 gene is located on human chromosome 17 (NC_000017.11) and consists of 19 exons. The QRICH2 protein has been found to co-localize with α-tubulin, a microtubule protein within the axoneme of the sperm flagellum. This co-localization indicates that QRICH2 is essential for sperm flagellum development and function [24].

A recent study identified QRICH2 as a glutamine sensor involved in regulating glutamine and glutamate metabolism, playing a significant role in maintaining appropriate α-tubulin glutamylation levels required for microtubule stability and function in sperm flagella. Flow cytometry analysis displayed reduced levels of glutamylation in the sperm from ASZ patients, which could be partially restored *in vitro* by adding purified N-terminal QRICH2 protein along with 2 mm glutamine [25]. An additional related study on *Qrich2* knockout (KO) mice displayed impaired mitochondrial protein localization and function, leading to diminished mitochondrial activity and energy production, which ultimately led to reduced sperm motility, increased reactive oxygen species (ROS), DNA damage in spermatids, increased autophagy, apoptosis, and significantly lower sperm counts. The antioxidant properties of the QRICH2 protein, particularly the N-terminal glutenin-rich domain, were shown to counteract ROS-induced damage, improving sperm survival and motility [26].

These findings emphasize the importance of the conserved N-terminal domain of QRICH2 in sperm motility and highlight the potential for developing therapeutic strategies targeting QRICH2 mutations for addressing male infertility. Since there are no studies that investigate the correlation between the *QRICH2* gene and sperm motility disorders in the Jordanian population, the main aim of this study is to identify the genetic variations in exon 3 of the *QRICH2* gene among Jordanians diagnosed with sperm motility disorder (asthenozoospermia, ASZ) and to investigate the relationship between these genetic variations and ASZ in Jordanian men.

2. Methods

2.1. Study participants

A total of 60 Jordanian individuals underwent standard seminal fluid analysis (SFA) at Al-Arabi Medical Laboratories, an accredited facility for SFA, according to the guidelines of the WHO 5th edition reference values [12]. These participants were included in the study depending on how well their SFA findings matched the study's inclusion criteria.

The study employed specific inclusion and exclusion criteria; ASZ inclusion criteria include abnormal sperm motility (<32% of sperm with progressive motility), normal sperm concentration (≥15 million/ml), and normal sperm morphology (≥4% of sperm with normal morphology). Individuals with normal sperm motility (≥32% of sperm with progressive motility), normal sperm concentration, and normal sperm morphology are classified as the normozoospermia group (NZ) and served as the control group for the cohort study. Patients with varicocele, primary ciliary dyskinesia (PCD) syndrome, and individuals undergoing chemotherapy or with a history of chemotherapy were excluded from the study.

Informed consent was obtained from all participants in accordance with the local guideline and the

ethical principles outlined in the Declaration of Helsinki [26]. In addition, participants completed a questionnaire survey providing details about their general information, including age, medical history, presence of first-degree relatives with fertility issues, consumption of drugs and vitamins, smoking and alcohol habits, and dietary and physical activities. The present study was approved by local ethics committees, the Institutional Review Board (IRB) of the Al-Ahliyya Amman University, Faculty of Allied Medical Sciences (IRB: AAU/20/11/2022–2023).

2.2. DNA extraction and Sanger sequencing

Genomic DNA was isolated from peripheral-blood samples of the subjects using a whole blood DNA purification kit (Quick-DNA™ Miniprep kit, USA), according to the manufacturer's protocol. DNA quantification was conducted with the Nabi® UV/Vis NANO spectrophotometer. The 260/280 ratio of the DNA samples was 1.8 ± 0.1. Exon number 3 and flanking intronic regions of *QRICH2* gene were targeted by polymerase chain reaction (PCR) using specific primers described previously [18]. PCR Reaction is composed of 20 µl total volume PCR reaction containing 10 ng genomic DNA, 10 µl of 2× PCR Master mix Solution (DreamTaq DNA polymerase) (Thermo Scientific™, USA), 1 µM forward and reverse primers. PCR amplification was performed using a thermal cycler (Applied Biosystems®, USA). The amplification program started by initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 10 min. The anticipated size of 533 bp PCR products was confirmed by gel electrophoresis on 1.5% agarose. The PCR products were purified and subsequently subjected to Sanger sequencing using the BigDye terminator v3.1 cycle sequencing kit and the ABI PRISM 3730XL automated sequencing analyzer from Applied Biosystem, Foster City, CA.

2.3. Mutations identification

At first, Sanger sequencing reads were tested to be matched with reference sequences for the gene Homo sapiens *QRICH2* (NCBI Reference sequence gene on chromosome 17; NC_000017.11:76304168–76304671) by using Finch TV (version 1.4.0), which was developed by Geospiza, Inc. (https://digitalworldbiology.com/FinchTV). Initially, *the Nagahama* server [27] was used to align Sanger sequencing reads to the Homo sapiens *QRICH2* gene, and the resulting mutations were compared to the NM_032134.2 and NP_115510.1 Refseq. The UniPro *UGENE* program [28] (version 48.1) was then used to verify and to identify genetic variants.

2.4. In silico prediction of missense variant affects

To determine the functional consequences of missense variants, two bioinformatics-based web tools were used, which are:

Sorting Intolerant from Tolerant (SIFT): is a computational sequence homology-based tool that predicts the effect of amino acid changes on the protein function [29]. For this study, the native QRICH2 protein sequence (Accession: NP_115510.2) was used. Missense variants were categorized as tolerant (score is >0.05) or deleterious (score <0.05) based on their prediction score. The SIFT tool is an accessible online tool at http://sift-dna.org.

Polymorphism Phenotyping v2 (PolyPhen-2) version 2.0.17 is another computational tool that predicts the possible impact of amino acid substitutions on protein structure based on physical and structural properties [30]. Sequences were submitted in FASTA format, specifying the positions of the native and mutant amino acids. The PolyPhen calculated the position-specific independent count (PSIC) score for each variant and categorized the predicted effects into three groups: benign, possibly damaging, and probably damaging. Benign results indicate that the variant is not likely to affect protein function, while the possibly damaging indicates that the variant may affect protein function. The result of probably damaging suggests a high likelihood of functional disruption. The tool is available online at. (http://genetics.bwh.harvard.edu/pph2/)

In our study, we used both PolyPhen-2 with the HumDiv classifier and PolyPhen-2 with the HumVar classifier. HumDiv targets rare alleles in complex disease phenotypes and natural selection, while HumVar focuses on Mendelian disorders, distinguishing disease-causing mutations from benign polymorphisms more stringently [31].

The structural impact of missense variants on the QRICH2 protein was analyzed using two web servers, including HOPE and MudPred2, which relied on the prediction of phenotypic effects

MutPred2 is a standalone web application designed to classify amino acid substitutions in humans as pathogenic or benign [32]. In addition, it predicts the impact of these substitutions on over 50 different protein properties, thus offering insights into the molecular mechanisms underlying pathogenicity. The overall score is obtained from the neural network ensemble and represents the likelihood of a variant being pathogenic. A score cutoff of 0.50 is used to determine pathogenicity, with scores ranging from 0 to 1; higher values indicate a greater probability of the variant being pathogenic. MutPred2 is accessible online at https://mutpred.mutdb.org.

Have (y) Our Protein Explained (HOPE) is a next-generation web-based tool for automated mutation analysis [33]. HOPE integrates information from data from the protein's 3D structure and the UniProt database, storing it in a PostgreSQL-based information system. A decision scheme is used to process these data and to predict the impact of the mutations on protein structure and function.

A report includes details on the mutated residue contacts, structural domains, modifications targeting this residue, and known variants associated with the mutated residue. The HOPE Web server is freely accessible at http://www.cmbi.ru.nl/hope/.

2.5. Protein secondary structure and three-dimensional protein structure prediction

Protein secondary structure was predicted using PSIPRED [34]. It is a popular and cutting-edge protein secondary structure method. We used the web server PSIPRED version 4, which is available via the following URL: http://bioinf.cs.ucl.ac.uk/psipred.

To evaluate the impact of these mutations, we used the SWISS-MODEL (https://swissmodel.expasy.org/) software, a fully automated server for protein structure homology modeling [35].

2.6. Statistical analysis

The data were analyzed using IBM SPSS Statistics version 26 (IBM Corp., Chicago, IL, USA). Descriptive statistics, including mean and standard deviation (SD) for continuous variables and frequency and percentages for categorical variables, were used to summarize semen parameters and mutation characterization among the study participants. Differences between groups for continuous variables were assessed using the independent sample t-test and non-parametric independent samples test, while the chi-square test was employed for categorical variables. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of the sample

The semen parameters of 60 study participants, comprising 35 individuals with ASZ and 25 with NZ, were analyzed. There were no statistically significant differences in demographic data between the two groups regarding age, smoking habits, family history of late conception and infertility, consanguinity of parents, or the presence of chronic diseases such as high blood pressure, cardiovascular diseases, eye disorders, prostate inflammation, H. pylori/IBS, nervous system disorders, and diabetes, as shown in Table 1.

General semen parameters such as ejaculate volume, liquefaction time, viscosity, aggregation, and pH did not show significant ($p > 0.05$) differences between the two groups. However, significant variations were observed in parameters related to sperm motility and morphology.

The ASZ group demonstrated a significantly lower sperm concentration (55.91 ± 39.03 vs. 87.13 ± 59.48 M/ml, $p = 0.027$) and sperm count per sample (150.66 ± 127.08 vs. 235.53 ± 174.03 M, $p = 0.045$) compared to the NZ group. Total motility ($37.94 \pm 11.20\%$ vs. $64.05 \pm 14.04\%$, $p < 0.001$) and progressive motility ($18.20 \pm 7.34\%$ vs. $42.62 \pm 10.66\%$, $p < 0.001$) were significantly reduced in the ASZ group, while immotile sperm was significantly higher ($62.06 \pm 11.20\%$ vs. $35.95 \pm 14.04\%$, $p < 0.001$). Morphological analysis revealed a lower percentage of normal morphology in the ASZ group ($14.87 \pm 5.76\%$ vs. $20.84 \pm 6.40\%$, $p < 0.001$) and a corresponding increase in abnormal morphology.

Based on the questioner's inquiry, none of the participants displayed respiratory health issues or symptoms associated with primary ciliary dyskinesia (PCD), such as sinusitis, pneumonia, or bronchitis. Therefore, it can be concluded that these individuals exhibited isolated ASZ.

Tail-specific abnormalities were more prevalent in the ASZ group, with a higher percentage of sperm without tails compared to the NZ group ($p = 0.036$). Despite meeting WHO criteria for normozoospermia, individuals in the NZ group showed varying degrees of abnormal morphology and reduced motility. These results highlight distinct impairments in motility and morphology among ASZ individuals compared to those in the NZ group.

3.2. Identification of the genetic variants detected in the QRICH2 gene

The sequencing was conducted on a subset of 56 individuals from the entire cohort. Out of the 35 cases of AZN and 25 cases of NZ, four samples had insufficient DNA volume, resulting in their inability to be sequenced due to their short length.

Our investigation identified a total of 14 heterozygous missense and synonymous mutations in both ASZ and NZ. Additionally, a singular frameshift mutation was detected in NZ; however, its impact on the encoded protein remains unidentified. Table 2 provides a comprehensive inventory of all identified genetic variants, including specific information regarding their respective chromosome locations.

Our investigation identified two known variants, specifically the missense variant c.206C>T and the synonymous variant c.189C>T, which are documented as known variants with reference SNP cluster IDs rs73996306 and rs1567790525, respectively. Both

Table 1. Semen parameters of study groups.

Parameters	Sample (N = 60) Asthenozoospermia ASZ (n = 35)	Normozoospermia NZ (n = 25)	p-value
Volume of ejaculate (ml)	2.87 ± 1.52	3.11 ± 1.61	0.562
Appearance (normal color)	35 (100)	25 (100)	1.000
Liquefaction time	15 (42.9)	13 (52.0)	0.485
Complete	15 (42.9)	11 (44.0)	
Incomplete	5 (14.3)	1 (4.0)	
Less than 60 min			
Viscosity	19 (54.3)	13 (52.0)	1.000
Normal	9 (25.7)	7 (28.0)	
Abnormal	7 (20.0)	5 (20.0)	
Slight			
Aggregation	35 (100)	25 (100)	1.000
Agglutination	35 (100)	25 (100)	1.000
Cellular elements	35 (100)	25 (100)	1.000
pH	8.81 ± 0.39	8.86 ± 0.37	0.646
Sperm Concentration (M/ml)	55.91 ± 39.03	87.13 ± 59.48	**0.027***
Sperm Number (M/sample)	150.66 ±127.08	235.53 ± 174.03	**0.045***
Total Motility (%)	37.94 ± 11.20	64.05 ± 14.04	**< .001***
Immotile sperms (%)	62.06 ±11.20	35.95 ± 14.04	**< .001***
Progressive motility (%)	18.20 ± 7.34	42.62 ± 10.66	**< .001***
Rapid progressive sperms (%)	14.03 ±6.30	37.52 ± 12.59	**< .001***
Medium progressive sperms (%)	16.05 ± 6.53	19.16 ± 7.17	0.085
Slow sperms (%)	7.86 ± 3.41	7.36 ± 2.77	0.547
Rapid progressive type A (%)	4.79 ± 3.59	12.98 ± 9.38	**< .001***
Medium progressive type B (%)	13.41 ± 4.71	29.64 ± 11.35	**< .001***
Non-progressive type C (%)	19.74 ± 7.11	21.42 ± 7.45	0.378
Immotile (%) type D	62.06 ±11.20	35.95 ± 14.04	**< .001***
Normal morphology (%)	14.87 ± 5.76	20.84 ± 6.40	**< .001***
Abnormal morphology (%)	85.13 ± 5.76	79.16 ± 6.40	**< .001***
Head defects (%)	27.67 ± 30.64	28.39 ± 26.09	0.925
Midpiece defects (%)	11.96 ± 14.29	14.60 ± 13.57	0.479
Tail defects (%)	77.01 ± 16.89	75.55 ± 10.11	0.684
Head + Midpiece + Tail	9.31 ± 12.17	13.87 ± 12.65	0.194
Head + Midpiece	2.18 ± 5.32	0.57 ± 2.67	0.160
Head + Tail	9.91 ± 13.17	10.55 ± 11.08	0.855
Midpiece + Tail	0.0 ± 0.0	0.0 ± 0.0	-
Cytoplasmic defects (%)	4.99 ± 11.66	3.01 ± 10.45	0.505
Teratozoospermia index (%)	1.42 ± 0.44	1.53 ± 0.45	0.345
Deformity index (%)	1.57 ± 0.76	1.51 ± 0.62	0.782
Multiple anomalities index (%)	1.82 ± 0.88	1.95 ± 0.74	0.556
Normal tail	21.24 ± 14.74	24.44 ± 10.65	0.391
Abnormal tail	73.24 ± 25.86	73.50 ± 17.39	0.968
Short tail	0.75 ± 2.97	0.35 ± 1.64	0.567
Without tail	2.85 ± 7.09	0.0 ± 0.00	0.036*
Rolled tail	1.91 ± 6.23	1.70 ± 8.00	0.916
Regular, Multiple, Angulation	0.0 ± 0.0	0.0 ± 0.0	-

Sperm characteristics according to the WHO 5th edition manual. *indicates statistical significance with p-value < 0.05, t-test.

variants are classified as single nucleotide variations (SNVs), and neither has any known clinical significance in ClinVar, according to the NCBI dbSNP database of short genetic variations (https://www.ncbi.nlm.nih.gov/clinvar/). The c.206C>T missense mutation was the most frequent mutation and was identified in both ASZ and NZ cases, with a frequency of 8.93% across all research groups.

The chromatograms of each detected variant, as revealed by the UGENE server, are displayed in supplementary Figure S1. The precise position of each missense variant in exon 3, using the Ensembl genome browser, is illustrated in Figure 1.

Additional variants have been identified in patients with ASZ include synonymous mutations (c.189C>G, c.138A>G) and five missense mutations (c.133 G>C, c.170A>C, c.190T>C, c.195A>T, and c.204A>T).

The NZ group showed a synonymous mutation at position c.189C>T, along with five unique missense variants at positions c.123 G>T, c.136 G>A, c.145A>C, c.179T>G, and c.180T>G. Three of them were detected in one subject, which are c.145A>C and c.180T>G, in addition to the c.189C>T mutation.

3.3. Determination of functional consequences of missense variants

The predicted consequences of all detected variants are summarized in Table 3. SIFT and PolyPhen2 prediction tools anticipated 6 (54.5%) out of 11 missense variants as potentially deleterious and probably damaging ones. These variants are c.136 G>A, c.145A>C, c.170A>C, c.179T>G, c.180T>G, and c.204 A>T.

Table 2. Genetic variants detected in exon 3 of the *QRICH2* gene.

Variant type	Coding sequence Variation*	Amino acid variation NP_115510.1 ENST00000262765.10	Amino acid variation NP_115510.2 ENST00000636395.1	Genomic location	Number (Frequency %) ASZ (n = 33)	Number (Frequency %) NZ (n = 23)	Allelic status	Known Variant dbSNP ID
Missense	c.123G>T	p.Leu41Phe	p.Leu207Phe	Chr17:76304499	1 (3.0)	0 (0.0)	Hetero	none
Frameshift	c.128_129insC	NA	NA	Chr17:76304493	0 (0.0)	1 (4.3)	Homo	none
Missense	c.133 G>C	p.Ala45Pro	p.Ala211Pro	Chr17:76304489	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.136 G>A	p.Glu46Lys	p.Glu212Lys	Chr17:76304486	0 (0.0)	1 (4.3)	Hetero	none
Synonymous	c.138A>G	p.Glu46=	p.Glu212=	Chr17:76304484	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.145A>C	p.Thr49Pro	p.Thr215Pro	Chr17:76304477	0 (0.0)	1 (4.3)	Hetero	none
Missense	c.170A>C	p.Glu57Ala	p.Glu223Ala	Chr17:76304452	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.179T>G	p.Ile60Ser	p.Ile226Ser	Chr17:76304443	0 (0.0)	1 (4.3)	Hetero	none
Missense	c.180T>G	p.Ile60Met	p.Ile226Met	Chr17:76304442	0 (0.0)	1 (3.85)	Hetero	none
Synonymous	c.189C>G	p.Gly 63=	p.Gly229=	Chr17:76304433	1 (3.0)	0 (0.0)	Hetero	none
Synonymous	c.189C>T	p.Gly 63=	p.Gly229=	Chr17:76304433	0 (0.0)	1 (4.3)	Hetero	rs1567790525
Missense	c.190T>C	p.Trp64Arg	p.Trp230Arg	Chr17:76304432	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.195A>T	p.Arg65Ser	p.Arg231Ser	Chr17:76304427	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.204A>T	p.Gln68His	p.Gln234His	Chr17:76304418	1 (3.0)	0 (0.0)	Hetero	none
Missense	c.206C>T	p.Ala69Val	p.Ala235Val	Chr17:76304416	4(12.1)	1 (4.3)	Hetero	rs73996306

*The accession number for *QRICH2* gene is GenBank transcript ID: NM_032134.2; ASZ: asthenozoospermia; NZ: normozoospermia; None: Variant was not found in ExAC, 1000Genome, or gnomAD. The number of the nucleotide is from the first nucleotide in exon 3.

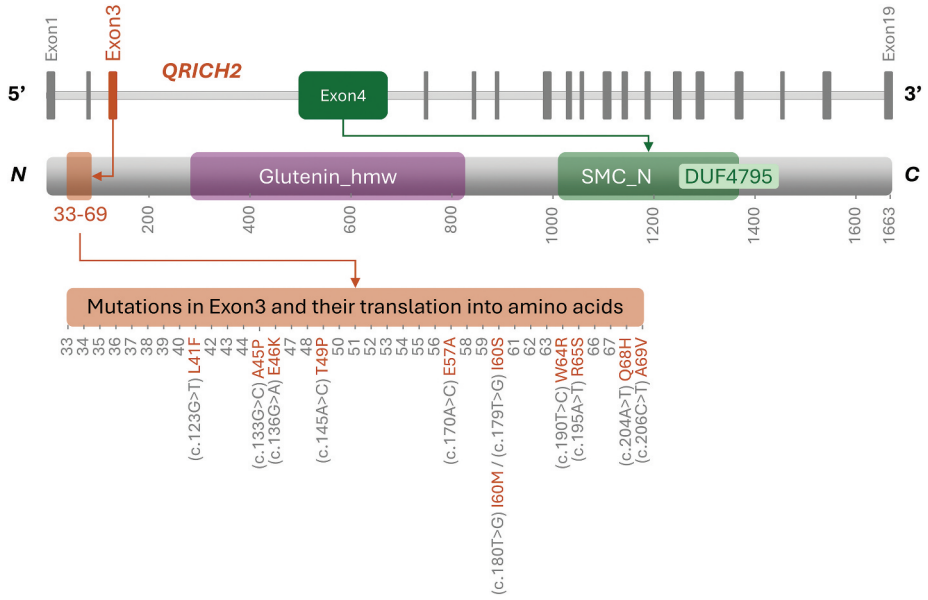

Figure 1. Genomic and structural location of detected missense variants. Locations of the *QRICH2* mutation sites and the location of the affected amino acids. transcript: ENST00000262765.10 (QRICH2–201) - protein summary - Homo_sapiens - ensembl genome browser 110.

3.4. Determination of structural impact of missense variants

The impact of point mutations on the structural stability of the QRICH2 protein was analyzed using bioinformatics tools. The results indicated structural changes such as loss of loop (c.136 G>A), altered coiled coil (c.170A>C, c.179T>G, c.190T>C), gain of intrinsic disorders (c.179T>G, c.190T>C), loss of helix (c.179T>G), altered stability (c.145A>C, c.179T>G), changes in transmembrane protein structure, and gain of a phosphorylation site (c.190T>C) (Table 4).

In addition, according to MutPred2, five missense mutations may alter protein confirmation and be pathogenic, which are c.170A>C, and c.190T>C detected in participants with ASZ, and c.136 G>A, c.145A>C, and c.179T>G detected in participants with normal sperm motility.

The mutations were analyzed using HOPE to evaluate their potential structural and functional effects on the QRICH2 protein. However, due to the lack of an experimentally resolved 3D structure or a suitable homology modeling template, HOPE relied on sequence-based annotations from the UniProt database to assess mutation impact. While this provides valuable insights into possible functional consequences, further studies utilizing predictive structural tools, such as AlphaFold or homology modeling approaches, may be needed to better understand the molecular effects of these mutations.

Table 3. The predicted consequences of all detected missense variants.

CDS	Protein change NP_115510.1	SIFT	PolyPhen-2_{Div}	PolyPhen-2_{var}
c.123G>T	p. Leu41Phe	Tolerated 0.11	Possibly damaging 0.737	Benign 0.364
c.133 G>C	p. Ala45Pro	Tolerated 0.06	Benign 0.002	Benign 0.003
c.136 G>A	p. Glu46Lys	Affect protein function <0.001	Probably damaging 0.992	Possibly damaging 0.876
c.145 A>C	p. Thr49Pro	Affect protein function <0.001	Probably damaging 0.999	Probably damaging 0.984
c.170 A>C	p. Glu57Ala	Affect protein function <0.001	Probably damaging 0.974	Probably damaging 0.758
c.179 T>G	p. Ile60Ser	Affect protein function 0.01	Probably damaging 0.996	Probably damaging 0.936
c.180 T>G	p. Ile60Met	Affect protein function <0.001	Probably damaging 0.996	Probably damaging 0.952
c.190 T>C	p. Trp64Arg	Affect protein function 0.05	Benign 0.09	Benign 0.112
c.195 A>T	p. Arg65Ser	Affect protein function <0.001	Benign 0.123	Benign 0.023
c.204 A>T	p. Gln68His	Affect protein function 0.01	Probably damaging 1.000	Probably damaging 0.982
c.206 C>T	p. Ala69Val	Tolerated 0.33	Benign 0.216	Benign 0.039

SIFT: sorting intolerant from tolerant predicts substitutions with scores less than 0.05 as deleterious, MutPred2 score>0.5 suggests pathogenicity; polyPhem: Polymorphism Phenotyping. Benign: No protein features affected. The PolyPhen-2 Div model is the preferred choice for analyzing rare alleles, conducting dense mapping of regions identified through genome-wide association studies, and studying natural selection. In contrast, the PolyPhen-2 Var model is better suited for diagnosing Mendelian diseases, as it focuses on distinguishing mutations with severe effects from other human variations, including common mildly deleterious alleles.

HOPE analysis (Table 4) suggests that four mutations (p.Glu46Lys, p.Glu57Ala, p.Trp64Arg, p.Arg65Ser) alter the charge of the amino acid residues, which could impact electrostatic interactions within the protein. Charge modifications may lead to disruptions in molecular binding or create repulsive forces that destabilize specific regions. Additionally, four mutations (p.Thr49Pro, p.Glu57Ala, p.Trp64Arg, p. Arg65Ser) were predicted to increase or decrease hydrophobicity, which plays a crucial role in protein folding and stability. Changes in hydrophobic properties may interfere with hydrophobic core interactions, potentially leading to misfolding or reduced structural integrity. Moreover, 10 mutations showed differences in residue size, a factor that could cause steric clashes or alter the local conformational structure. Mutations such as p.Leu41Phe and p.Ala45Pro introduced larger side chains, which may create spatial hindrance, affecting neighboring residues and secondary structural elements. Conversely, mutations that replaced larger residues with smaller ones (e.g., p.Ile60Ser, p. Gln68His) might lead to loss of stabilizing interactions, making the protein more flexible or unstable.

While these findings suggest that these mutations could influence protein function, it is important to note that HOPE analysis is based on sequence-derived predictions and does not generate actual 3D structural models. The absence of a resolved QRICH2 protein structure limits the ability to definitively confirm these structural disruptions. To strengthen these predictions, future studies could apply molecular dynamics simulations, homology modeling, or experimental protein stability assays to assess how these mutations impact protein conformation, interactions, and functional outcomes.

Additionally, the significance of these mutations should be considered in a broader biological context, particularly in relation to QRICH2's role in spermatogenesis. Given that QRICH2 is essential for sperm tail development and motility, even subtle structural alterations may compromise its function, potentially contributing to asthenozoospermia. Further research integrating functional assays, evolutionary conservation studies, and clinical validation is needed to determine how these mutations correlate with sperm motility defects and overall male fertility.

HOPE also assessed the pathogenicity of these mutations based on conservation, revealing that the wild-type residues Glu46, Glu57, Ile60, Trp64, and Gln68 are either fully conserved or highly conserved at their respective positions. Consequently, their mutated forms (p.Glu46Lys, p.Glu57Ala, p.Ile60Ser, p. Ile60Met, p.Trp64Arg, and p.Gln68His) are predicted to be likely damaging to protein function.

Furthermore, specific amino acid substitutions, such as those involving proline (p.Thr49Pro and p. Ala45Pro), associated with c.145A>C and c.133G>C variants, may destabilize the local protein conformation. The unique rigidity of proline can disrupt proper protein folding by breaking secondary structures, highlighting its significant structural impact [36].

3.5. Modelling the secondary structure of QRICH2 protein

The secondary structure of the QRICH2 protein (1–140 residues) was predicted by PSIPRED 4.0 server. The prediction results indicated that this region comprised α-helices, β-strand, and coils. As shown in Table 5, we found that the c.145A>C (p. Thr49Pro) occurred in the predicted β-strand which could make the strand shorter or reorganized, while c.170A>C (p. Glu57Ala), c.179T>G (p. Ile60Ser), and c.180T>G (p. Ile60Met) occurred in helix region. The remaining mutations occurred in the coil regions. Of them c.133 G>C(p. Ala45Pro) and c.136 G>A (p. Glu46Lys) mutations which affect the adjacent β-strand and α-helix regions and make these regions shorter or reorganized.

Table 4. An analysis of the phenotypic effect of missense variants predicted by MutPred2 and project HOPE.

CDS	Amino acid variation	HOPE	MutPred2
c.123G>T	p. Leu41Phe	• Based on conservation, wild-type residue is frequently observed at this position in the sequence. The mutant residue has not been observed in homologous sequences. Despite this, residues with similar properties have been detected, suggesting that the mutant residue may still be tolerated at this position. • Based on amino acid properties, the mutant residue is bigger in size than the wild-type residue, potentially causing steric clashes or structural disruptions due to spatial constraints.	0.287
c.133 G>C	p. Ala45Pro	• Based on conservation analysis, the wild-type residue at this position is not conserved, although the mutant residue is situated near a highly conserved region. • In homologous sequences, another different residue is more commonly observed at this position. This suggests that the mutation is possibly damaging. • The mutant residue is bigger than the wild-type residue, which could lead to bumps.	0.285
c.136 G>A	p. Glu46Lys	• This position is occupied only by the wild-type residue, which is 100% conserved. Therefore, Mutation is usually damaging to the protein. • This mutation might occur in rare cases, but it is more likely to have a damaging effect on the protein's structure or function. • The mutant residue is bigger than the wild-type residue, potentially causing steric clashes or structural disturbances due to its size. • The wild-type residue carries a negative charge, whereas the mutant residue charge is positive. This may lead to electrostatic repulsion with other residues or ligands, disrupting the protein's interactions.	0.708 Loss of Loop Altered Coiled coil
c.145 A>C	p. Thr49Pro	• Wild-type residue often occurred at this position in the sequence. • Neither the mutant residue nor any residue with similar properties has been observed at this position in homologous sequences, suggesting that the mutation might be damaging to the protein. • The wild-type residue is predicted to be located within its preferred secondary structure, a β-strand, while the mutant residue prefers a different secondary structure. Therefore, the local protein conformation will be slightly destabilized. • The mutant residue is more hydrophobic than the wild-type residue, which may disrupt correct folding and result in the loss of hydrogen bonds.	0.556 Altered Coiled coil & Altered Stability
c.170 A>C	p. Glu57Ala	• This position is entirely occupied by the wild-type residue, which is 100% conserved. Thus, mutation is probably damaging to the protein. • The mutant residue is located near a region that is highly conserved. • The mutant residue is smaller than the wild-type residue, potentially causing loss of critical interactions with other residues. • The wild-type residue has a negative charge, while the mutant residue is neutral, this may disrupt interactions with other molecules or residues. • The mutant residue exhibits greater hydrophobicity than the wild-type residue, which may result in loss of hydrogen bonds and disrupt the correct folding of the protein.	0.555 Altered Coiled coil
c.179 T>G	p. Ile60Ser	• This location is solely occupied by the wild-type residue, which is 100% conserved. Therefore, mutation is probably damaging to the protein. • The mutant residue is located close to a highly conserved region. • The mutant residue is smaller than the wild-type residue, this might lead to loss of interactions. • The wild-type residue demonstrates increased hydrophobicity relative to the mutant residue, which may interfere with hydrophobic interactions within the protein core or on its surface.	0.535 Gain of Intrinsic disorder, Altered Coiled coil, Altered Stability, Loss of Helix
c.180 T>G	p. Ile60Met	• The wild-type residue, which is 100% conserved, occupies this position exclusively. Therefore, mutation is usually damaging for protein. Nonetheless, the mutant residue has some properties in common with the wild-type residue. Although this mutation might rarely happen, it is more likely to be damaging on the protein. • The mutant residue is located close to a highly conserved region. • The mutant residue is bigger than the wild-type residue, which could result in steric clashes or structural disruptions within the protein	0.185
c.190 T>C	p. Trp64Arg	• Only this residue type was found at this position, which is 100% conserved. Mutating a conserved residue is probably damaging to the protein. • The mutant residue is near a highly conserved region. • The mutant residue is smaller than the wild-type residue, potentially leading to the loss of crucial interactions. • The wild-type residue is neutral, whereas the mutant residue carries a positive charge, which may lead to repulsion with similarly charged ligands or nearby residues. • The wild-type residue demonstrates increased hydrophobicity relative to the mutant residue, resulting in diminished hydrophobic interactions, whether in the protein core or on its surface.	0.507 Altered Coiled coil, Gain of Intrinsic disorder, Gain of Phosphorylation at S67, Altered Transmembrane protein

(*Continued*)

Table 4. (Continued).

CDS	Amino acid variation	HOPE	MutPred2
c.195 A>T	p. Arg65Ser	• The wild-type residue is highly conserved and exhibits properties similar to those of our mutant residue, which has also been observed in homologous proteins at this position. This indicates that, in rare instances, mutation may occur without detrimental effects to the protein. • The mutant residue is located near a highly conserved region. • The mutant residue is smaller than the wild-type residue, which may result in a loss of essential interactions. • The wild-type residue possesses a positive charge, whereas the mutant residue is neutral, potentially disrupting interactions with other molecules or residues. • The mutant residue exhibits greater hydrophobicity compared to the wild-type residue, which may lead to loss of hydrogen bonds and/or interfering with proper protein folding.	0.290
c.204 A>T	p. Gln68His	• The wild-type residue is highly conserved, and neither our mutant residue nor any other residue with similar properties has been observed at this position in homologous sequences. Conservation scores indicate that this mutation is probably damaging. • The mutant residue is positioned close to a highly conserved region. • The mutant residue is larger than the wild-type residue, which could cause steric clashes or structural disruptions within the protein.	0.426
c.206 C>T	p. Ala69Val	• The wild-type residue at this position is not conserved. • The mutant residue has not been observed at this position in homologous sequences, which could suggest that the mutation is possibly damaging. • The mutant residue is larger than the wild-type residue, potentially causing steric clashes or structural disruption	0.088

Table 5. The location of wild type and mutant type residues in the predicted secondary structures.

CDS	Amino acid variation	Location of the Wild-type residue	Location of the Mutant residue
c.123G>T	p. Leu41Phe	Coil region	Coil region
c.133 G>C	p. Ala45Pro	Coil region	Coil region
c.136 G>A	p. Glu46Lys	Coil region	Coil region
c.145 A>C	p. Thr49Pro	Strand	Coil region
c.170 A>C	p. Glu57Ala	Helix region	Helix region
c.179 T>G	p. Ile60Ser	Helix region	Helix region
c.180 T>G	p. Ile60Met	Helix region	Helix region
c.190 T>C	p. Trp64Arg	Coil region	Coil region
c.195 A>T	p. Arg65Ser	Coil region	Coil region
c.204 A>T	p. Gln68His	Coil region	Coil region
c.206 C>T	p. Ala69Val	Coil region	Coil region

Table 6. Clinical semen manifestations in individuals harboring *QRICH2* mutations.

	Results (N = 60) Median (1st −3rd quartile)		
Parameters	Mutation carriers (n = 14)	Mutation non-carriers (n = 46)	p-value*
QRICH 2 (n = 14)			
Tail defects	83.33 (77.57–88.13)	78.89 (70.36–83.33)	**0.032**
Abnormal tail	83.33 (77.57–88.13)	78.89 (71.07–82.60)	**0.016**

*p-value < 0.05 Mann−–Whitney U test (non-parametric).

The WT and the mutated protein secondary structure prediction results are illustrated in supplementary Figure S2.

To gain deeper insight into the impact of these mutations, we used SWISS-MODEL to investigate the conformational changes in the QRICH2 protein. However, no suitable homology templates were available for the region spanning residues 1–70. As a result, the effect of these mutations on the 3D structure could not be determined.

Upon examination of the clinical semen characteristics in individuals with *QRICH2* mutations, it was found that those with such mutations were statistically more prone to tail defects in comparison to individuals without mutations in the *QRICH2* gene, as shown in Table 6.

4. Discussion

More than 4,000 genes are thought to play a vital role in sperm production, with defects in any of these genes potentially disrupting spermatogenesis and resulting in a range of sperm abnormalities [37]. Impaired sperm motility, primarily due to abnormalities in the sperm flagellum, is a major cause of male infertility. Genetic variations affecting flagellum formation can result in reduced sperm motility, leading to ASZ [22]. According to clinical classification, pathogenic variants in at least 22 known genes are responsible for up to 50% of severe sperm motility disorder cases [38]. Furthermore, genetic research has indicated that abnormalities in several genes, specifically those related to energy metabolism, energy generation, and ion channels, are significant contributors to the decline in sperm motility, leading to asthenozoospermia [39].

The *QRICH2* gene in humans produces a QRICH2 protein that is evenly distributed along the entire length of the sperm flagella. The QRICH2 protein is deemed essential for the stabilization of proteins crucial to the biogenesis of sperm flagella. In doing so, it helps create and maintain the structural integrity of sperm flagella [40]. Reduced levels of QRICH2 protein result in dysplasia of the fibrous sheath, which impairs sperm motility and ultimately results in male infertility [22].

A study conducted on the Jordanian population identified several key genes and proteins that play an essential role in the development of sperm flagella at both molecular and biological levels. Among these, QRICH2 demonstrated altered expression levels in individuals with oligoasthenozoospermia compared to those with normozoospermia [39]. The findings indicate that changing the expression levels of specific genes and proteins, including QRICH2 May 2001, leads to spermatogenic dysfunction, decreased sperm motility, and complications associated with capacitation and fertilization [39]. In particular, the N-terminal glutenin-rich domain of the QRICH2 protein exhibits significant antioxidant activity *in vitro* and *in vivo*, enhancing the viability and motility of spermatozoa [25]. Moreover, treatment of human and mouse sperm with the purified N-terminal glutenin-rich domain showed a significant improvement in both viability and motility [25]. Additional research from the same group highlights the critical role of QRICH2 in regulating glutamine/glutamate metabolism, stabilizing α-tubulin in sperm flagella through tubulin glutamylation, and maintaining mitochondrial function to support sperm motility in both mice and humans [24].

Additionally, patients with asthenozoospermia exhibited reduced levels of tubulin glutamylation in sperm, along with increased reactive oxygen species (ROS) and oxidative damage to sperm DNA [25].

In our study, we have sequenced exon 3 of the QRICH2 gene in ASZ patients because it likely encodes a crucial part of the N-terminal domain, which plays a significant role in regulating sperm motility and mitochondrial function. Additionally, this region may be a mutation hotspot; prior research has identified a critical nonsense mutation in this exon linked to male infertility and indicated that this exon is particularly relevant in male infertility [22], making it a prime target for identifying variants linked to reduced sperm motility. Given that exon 3 encodes part of the functionally critical N-terminal domain, which plays a role in sperm motility regulation and mitochondrial function, and that previous studies have identified a nonsense mutation in this region linked to male infertility, this exon represents a highly relevant target for identifying variants associated with reduced sperm motility [22,24,25]. However, further functional studies are needed to fully elucidate its role in male infertility.

ASZ is categorized as mild or severe according to sperm motility and the percentage of immotile sperm. Mild grade ASZ has 60–75% immotile or poorly motile sperm, while severe cases exceed 75–80% of immotile spermatozoa [41]. In our study, only 7 participants (20%) exhibited severe ASZ, with over 75% immotile spermatozoa, while the remaining participants displayed mild asthenozoospermia. Curi et al. (2003) found that 82% of ASZ cases also had teratozoospermia or oligozoospermia [14]. Teratozoospermia, characterized by over 85% abnormal sperm morphology according to Kruger's criteria [42], was identified in 21 participants in this study 17 from the ASZ group and 4 from the NZ group. Furthermore, out of the subjects in our investigation, only nine exhibited anomalies in the flagella morphology, with seven from ASZ and two from NZ. All of them showed head deformities except for one from the NZ group, which had no head defects. These findings align with previous observations [43]. Additionally, individuals in our study with genetic variants in their *QRICH2* gene do not exhibit MMAF syndrome in their sperm flagella. Our DNA sequencing analysis revealed no homozygous mutations in either the ASZ or NZ patient groups. This finding aligns with the established autosomal recessive inheritance pattern of MMAF, as reported by Shen et al. (2019) [22].

Our sequencing analysis identified seven unique heterozygous missense and synonymous genetic variants (c.133 G>C, c.138A>G, c.170A>C, c.189C>G, c.190T>C, c.195A>T, c.204A>T), which were exclusively detected within the ASZ category. On the other hand, six heterozygous missense and synonymous genetic variants (c.189C>T, c.123 G>T, c.136 G>A, c.145A>C, c.179T>G, and c.180T>G) were identified solely in participants with normal sperm motility. Notably, three variants (c.145A>C, c.180T>G, and c.189C>T) were observed in a single individual. None of these genetic variants correspond to any previously reported SNVs in the NCBI dbSNP database, underscoring their potential novelty and relevance in sperm motility phenotypes.

Our study identified the c.206C>T (rs73996306) missense variant in exon 3 of the *QRICH2* gene, resulting in the p.Ala69Val substitution, with an overall frequency of 8.93% across participants. This variant was more common in ASZ cases (12.1%) than in NZ cases (4.3%) and was associated with significantly higher rates of aberrant sperm tails ($p = 0.041$), emphasizing its potential role in sperm morphology abnormalities. Despite this association, its clinical significance is unreported in ClinVar, and computational tools (SIFT and PolyPhen-2) classify it as tolerated and benign, suggesting limited functional impact. Noteworthy, the literature reports another missense variant, c.205 G>A (rs1481373615), at the same amino acid residue (Ala69) in the QRICH2 protein, also classified as tolerated and benign. Additionally, the synonymous or splice region variant c.207 G>A (rs1478053584) has been documented, with no predicted functional or clinical consequences. The identification of multiple variants at the Ala69 residue indicates that this location may be a genetic hotspot. Further investigation is needed to understand the biological significance of these variants and their contribution to sperm morphology abnormalities.

Our study identified five genetic variants, namely the missense variants c.133 G>C, c.136 G>A, c.145A>C, c.170 A>C, c.204A>T, and the synonymous variant c.138A>G, which are located at the same position as previously reported variants with assigned RS numbers and different mutation types, suggesting these loci may serve as mutational hotspots.

The c.133 G>A corresponds to the position of the known variant c.133 G>C (rs1470635848), which has a tolerated effect according to SIFT predictions. Similarly, our detected missense variant c.136 G>A aligns with the location of the c.140_142del (rs763544502; p.Glu47del) in-frame deletion mutation. The c.145A>C variant identified in our study is located at the same position as the pathogenic frameshift variant c.145dup (rs760133954; p.Thr49Asnfs*31) previously reported in an infertile man with severe sperm motility disorders (motility 2%) [38], suggesting its potential impact on QRICH2 function and sperm motility Our analysis predicts this mutation to be damaging to the QRICH2 protein, as indicated by PolyPhen and MutPred2 tools. The individual carrying this mutation was classified as NZ but exhibited borderline sperm parameters, including low sperm concentration

(17.45 M/mL), reduced progressive motility (33.79%), and a high deformities index (TZI = 2.0). Morphological abnormalities were predominantly observed in the sperm tail, accounting for 83.33% of the defects.

Moreover, this individual harbored four genetic variants in *the QRICH2* gene, which are c.189C>T (p.Gly63=), c.145A>C (p.Thr49Pro), c.180T>G (p.Ile60Met), and frameshift c.128_129insC. These findings suggest the c.145A>C mutation, along with other variants in the *QRICH2* gene, may contribute to subtle sperm dysfunction, even in NZ classifications, warranting further investigation.

Additionally, our c.170A>C (Glu57Lys) variant aligns with the c.169 G>A (p.Glu57Lys) homozygous missense mutation reported by Oud et al. (2021) in a patient with severe infertility, characterized by very low sperm concentrations, poor motility, and 0% normal sperm morphology [38], indicating its possible role in sperm dysfunction. In contrast, the individual carrying our detected variant exhibited higher sperm concentrations (93 M/mL), moderate motility (42%), and 12.5% normal morphology, suggesting a potentially milder phenotypic effect of the heterozygous mutation compared to its homozygous counterpart. This distinction highlights the possible impact of zygosity on the severity of sperm abnormalities associated with this variant.

Lastly, the synonymous variant c.204A>G (rs1269588860) overlaps with our detected missense variants c.204A>T (p.Gln68His), further emphasizing the significance of this region as a potential mutation hotspot.

The alignment of our detected variants with previously reported mutations at the same or nearby positions underscores the potential significance of these genomic regions in sperm motility and morphological abnormalities. The recurrence of different mutation types at the same loci indicates that these positions might be particularly prone to genetic alterations, potentially impacting the function of the QRICH2 protein. This reinforces the importance of these loci and highlights the need for further functional studies to elucidate their biological roles in asthenozoospermia and related sperm dysfunctions. Several studies have utilized in silico structural modeling, *in vitro* functional assays, animal models, and patient-derived samples to investigate the role of QRICH2 in sperm function and male infertility. While specific studies detailing in silico structural modeling of QRICH2 are limited, computational modeling and molecular docking techniques have been widely employed to predict the structural and functional impact of genetic mutations, aiding in understanding their biological effects [44]. These approaches are particularly useful for assessing the potential pathogenicity of novel QRICH2 variants. Shen et al. (2019) conducted *in vitro* experiments, demonstrating that QRICH2 stabilizes and enhances the expression of proteins involved in sperm flagellar development [22]. To further validate the role of QRICH2, Zhang et al. (2024) generated QRICH2 knockout (KO) male mice using CRISPR-Cas9 technology, which resulted in multiple morphological abnormalities of the sperm flagella (MMAF) and infertility, confirming QRICH2's essential function in sperm flagellar formation [24]. Similarly, Raza et al. (2024) identified a novel homozygous missense variant (c.4618C>T) in QRICH2 in infertile patients with MMAF, where sperm analysis revealed severe morphological abnormalities and reduced expression of QRICH2 in patient-derived sperm samples, further supporting its role in male infertility [45].

These findings emphasize the importance of QRICH2 mutations in sperm motility and structural integrity, highlighting the need for computational modeling, in vitro functional validation, and clinical genetic analysis to clarify its role in spermatogenesis and male infertility, while future advancements in genetic diagnostics, including CRISPR-based screening, could enhance the precise identification of pathogenic QRICH2 variants, improving early detection and potential therapeutic interventions [46].

Noteworthy, these five genetic variants identified in our study (c.133 G>C, c.136 G>A, c.145A>C, c.170A>C, and c.204A>T) demonstrate potential novelty, as they are not documented in prominent genetic databases such as the NCBI SNP database, ClinVar-NCBI, the Genome Aggregation Database, or SNPedia. This suggests these variants may represent previously unidentified genetic alterations. Among them, four non-synonymous SNPs (c.136 G>A, c.145A>C, c.170A>C, and c.204A>T) were predicted to be functionally and structurally damaging to the QRICH2 protein by all bioinformatics tools used, emphasizing their potential importance. These findings further highlight the need for additional investigation to elucidate the biological and clinical implications of these novel variants.

Noteworthy, all participants with c.136 G>A, c.170 A>C, and c.204A>T mutations display morphological abnormalities exclusively in the sperm tail, with no defects observed in the head or midpiece. Interestingly, while the non-synonymous SNPs c.136 G>A and c.145A>C were identified in a normospermia participant, seminal analysis revealed that their progressive motility percentages (34.9% and 33.8%, respectively) were comparable to those observed in ASZ patients, suggesting a potential link between these mutations and impaired sperm function despite a normospermia classification, warranting further investigation.

Our analysis identified two genetic variants, c.190T>C (p.Trp64Arg) and c.195A>T (p.Arg65Ser), in

the ASZ group. These variants are located near the previously reported pathogenic stop-gain mutation c.192 G>A (p.Trp64Ter) in the QRICH2 gene, which has been associated with spermatogenic failure [40]. The c.192 G>A mutation results in a truncated QRICH2 protein lacking three domains, leading to abnormal sperm flagella development [22]. This suggests that this particular region is likely to be highly susceptible to mutations in this gene. Furthermore, the conservation of the p. Trp64 residue, as indicated by HOPE analysis, supports the prediction that the p.Trp64Arg mutation is probably damaging to the protein. Notably, an ASZ patient with c.190T>C (p. Trp64Arg) mutation in *QRICH2* gene also harbors an additional missense variant c.133 G>C (p.Ala45Pro). Semen analysis revealed 22.57% progressive motility, with 8.0% demonstrating rapid progressive (type A) motility, and a high prevalence of morphological abnormalities predominantly affecting the sperm tail (81.82% abnormal morphology; 72.7% tail defects), followed by head defects (27.2%), and midpiece deformities (9.09%). Despite this, the WHO deems it to be within the parameters of morphological normalcy. These findings underscore the potential contribution of *QRICH2* mutations on sperm structure and function.

Our study demonstrates a clear association between *QRICH2* mutations and sperm tail-specific morphological abnormalities in both NZ and ASZ groups. All individuals in the NZ group carrying *QRICH2* mutations exhibited multiple abnormalities, predominantly characterized by tail defects. Similarly, in the ASZ group, participants with heterozygous mutations in exon 3 of the *QRICH2* gene displayed mild asthenozoospermia, with 63.3% showing a teratozoospermy index of 1, indicating that all morphological defects were confined to the sperm tail. These findings highlight the essential function of QRICH2 in preserving sperm tail integrity and function.

Importantly, this study underscores the significance of identifying heterozygous mutations in the *QRICH2* gene, as evidenced by our findings and supported by prior research. Shen et al. (2019) demonstrated that heterozygous *QRICH2* mutations impair sperm motility in both mice and humans, resulting in pure ASZ. This finding highlights the gene's crucial role in sperm function and its contribution in the development of ASZ. Similarly, Kherraf et al. (2019) reported that heterozygous *QRICH2* mutations may exert a mild impact on spermatogenesis adequate for normal reproduction, yet they may also contribute to moderate forms of ASZ [40].

The implications of heterozygous mutations are not limited to the *QRICH* gene. Recent findings by Martinez et al. (2022) revealed that multiple heterozygous mutations in functionally related genes associated with flagellar defects result in progressively deteriorating sperm morphology and motility. This supports the concept of oligogenic inheritance, where the cumulative effect of heterozygous mutations, rather than specific combinations, drives changes in sperm parameters [43]. Their findings suggest that idiopathic male infertility and various cases of asthenoteratozoospermia may be linked to heterozygous deleterious variants impacting multiple proteins within the same intracellular pathways, resulting in progressive dysfunction that manifests once a critical threshold is attained [43]. Additionally, Coutton et al. (2018) demonstrated that KO heterozygous mouse model exhibited a slight decline in sperm motility and morphology. This suggests that heterozygous mutations in key spermatogenesis genes may contribute to milder but more common phenotypes such as mild to intermediate oligoasthenozoospermia, particularly when combined with other genetic or environmental factors [47].

In summary, these studies, along with our findings presented here, demonstrate how important it is to identify heterozygous mutations in the *QRICH2* gene and associated genes, because they may play a crucial role in both common and mild cases of asthenozoospermia as well as rare severe sperm abnormalities. This emphasizes the necessity of more research on the cumulative effects of heterozygous mutations and how they affect male fertility.

Ultimately, it is important to highlight that the two study groups, ASZ and NZ, exhibited similarity in all sperm parameters, except for the progressive motility. Both groups complied with WHO 5th edition criteria for normal morphology, which defines normal sperm as having ≥4% with a normal structure, despite the presence of morphological abnormalities in up to 95% of the sperm. Consequently, the identification of QRICH2 mutations in normozoospermic individuals with semen parameters within WHO reference limits is therefore not surprising. The clinical relevance of these evidence-based limits is debated, particularly in cases where genetic mutations are accompanied by significant abnormalities in sperm morphology and motility. These findings suggest potential limitations in current diagnostic criteria that surpass conventional reference limits, highlighting the necessity for additional studies to enhance male fertility assessment protocols and explore the broader clinical implications of such genetic variations [48].

Finally, our study focused on identifying heterozygous QRICH2 variants and their potential association with sperm motility disorders; however, segregation analysis within affected families was not conducted. This represents a limitation, as familial analysis could provide further insights into the inheritance pattern and clinical significance of these variants, particularly in distinguishing heterozygous and homozygous effects. Future studies incorporating segregation

analysis in larger family cohorts will be essential to validate the genetic contribution of QRICH2 mutations to male infertility and to explore possible oligogenic interactions with other fertility-related genes.

5. Conclusions

This study identifies novel heterozygous QRICH2 variants associated with sperm tail abnormalities and motility impairments. These findings suggest that even in individuals with normal semen parameters, such mutations may contribute to mild-to-moderate asthenozoospermia, highlighting the importance of genetic screening in male infertility diagnosis, particularly in populations like Jordan, where these variants may have clinical significance.

Further research is needed to identify mutation hotspots and novel variants within QRICH2 and to explore the combined effects of genetic and environmental factors on sperm dysfunction. Functional and bioinformatics studies are essential to assess the protein-level impact of these variants, while larger clinical investigations could validate their role in infertility. Additionally, segregation analysis and parental DNA assessments may provide deeper insights into inheritance patterns and the broader genetic landscape of QRICH2-related male infertility.

This study underscores the importance of genetic screening in reproductive health and highlights the need for comprehensive clinical and molecular research to improve diagnostic and therapeutic strategies. Future investigations incorporating segregation analysis in larger family cohorts will be essential to confirm the genetic contribution of QRICH2 mutations and explore potential oligogenic interactions with other fertility-related genes.

Disclosure statement

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