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ORIGINAL RESEARCH

Mitochondrially encoded NADH dehydrogenase subunit 3 (*MT-ND3*) gene variants in asthenozoospermia-associated male infertility

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Abstract

Background: Male infertility is a complex, multifactorial condition contributing to approximately 50% of infertility cases. Among these, asthenozoospermia (reduced sperm motility) accounts for around 13% of male infertility cases. This study aims to investigate the *mitochondrial ND3* gene variants in asthenozoospermic infertile men in Jordan. **Methods**: 188 semen samples (117 asthenozoospermic and 71 normozoospermic) were collected from the *in-vitro* fertilization (IVF) unit at the Jordanian Royal Medical Services. Mitochondrial DNA (mtDNA) was extracted and amplified, and the *Mitochondrially encoded NADH dehydrogenase subunit 3* (*MT-ND3*) gene was sequenced, followed by the identification of genetic variants. **Results**: Seventeen single-nucleotide polymorphisms (SNPs) were detected in the *MT-ND3* gene in the examined samples. One novel synonymous variant (m.10313 A>T) was identified exclusively in the normozoospermic group, while the remaining variants were previously reported in the National Center for Biotechnology Information (NCBI) databases. Six missense variants were found: two in the asthenozoospermic group (rs41487950 T>C (Ile9Thr) and rs1603222800 G>A (Ala103Thr)), three in the normozoospermic group (rs202131419 G>A (Gly29Ser), rs193302928 T>C (Val88Ala), and rs1603222776 T>C (Met89Thr)), and one common variant (rs2853826 A>G (Thr114Ser)) in both groups. Additionally, eleven synonymous variants were identified in the study population. **Conclusions**: Although no statistically significant associations were found between the identified *MT-ND3* gene SNPs and asthenozoospermia, several detected variants in asthenozoospermic samples are predicted to alter the protein's structure. Sample size is a limitation of the current study; therefore, further investigations are required to assess the potential impact of these variants on male infertility across diverse populations.

Keywords

mtDNA; Asthenozoospermia; Sperm motility; *MT-ND3* gene

Variantes del gen de *la subunidad 3 de la NADH deshidrogenasa* (*ND3*) codificado mitocondrialmente en la infertilidad masculina asociada a astenozoospermia

Resumen

Antecedentes: La infertilidad masculina es una afección compleja y multifactorial que contribuye a aproximadamente el 50% de los casos de infertilidad. Entre estos, la astenozoospermia (reducción de la movilidad espermática) representa alrededor del 13% de los casos de infertilidad masculina. Este estudio tiene como objetivo investigar las variantes *mitocondriales del gen ND3* en hombres infértiles astenozoospérmicos en Jordania. **Métodos**: Se obtuvieron 188 muestras de semen (117 astenozoospérmicos y 71 normozoospérmicos) de unidades de (la Unidad de Fertilización *In Vitro*) FIV de los Servicios Médicos Reales de Jordania. Se extrajo y amplificó el ADN mitocondrial (ADNmt), se secuenció el gen (Gen de *la subunidad 3 de la NADH deshidrogenasa codificado mitocondrialmente*) *MT-ND3* y se identificaron las variantes genéticas. **Resultados**: Se detectaron diecisiete polimorfismos de un solo nucleótido (SNP) en el gen MT-ND3 en las muestras examinadas. Se identificó una nueva variante sinónima (m.10313 A>T) exclusivamente en el grupo normozoospérmico, mientras que las variantes restantes se habían reportado previamente en las bases de datos del Centro Nacional para la Información Biotecnológica (NCBI). Se encontraron seis variantes sin sentido: dos en el grupo astenozoospérmico (rs41487950 T>C (Ile9Thr) y rs1603222800 G>A (Ala103Thr)), tres en el grupo normozoospérmico (rs202131419 G>A (Gly29Ser), rs193302928 T>C (Val88Ala) y rs1603222776 T>C (Met89Thr)), y una variante común (rs2853826 A>G (Thr114Ser)) en ambos grupos. Además, se identificaron once variantes sinónimas en la población del estudio. **Conclusiones**: Aunque no se encontraron asociaciones estadísticamente significativas entre los SNP identificados del gen ND3 y la astenozoospermia, se predice que varias variantes detectadas en muestras astenozoospérmicas alteran la estructura de la proteína. El tamaño de la muestra es una limitación del presente estudio; por lo tanto, se requieren más investigaciones para evaluar el posible impacto de estas variantes en la infertilidad masculina en diversas poblaciones.

Palabras Clave

ADNmt; Astenozoospermia; Motilidad espermática; Gen *MT-ND3*

1. Introduction

The inability of couples to conceive after one year of regular, unprotected sexual intercourse is medically known as infertility [1]. According to the World Health Organization (WHO), infertility affects approximately 8–12% of couples worldwide, with male-related factors contributing to nearly 50% of cases [2]. Male infertility often arises from a variety of causes, including congenital defects, anatomical abnormalities, immune responses, environmental exposures, and genetic reasons [3]. However, certain factors remain undetectable despite extensive scientific efforts [4]. Among the known causes, genetic alterations such as chromosomal abnormalities, deletions in the Y chromosome, and mutations in mitochondrial DNA have been recognized as contributing to male infertility [5].

Notably, mitochondrial dysfunction has attracted significant attention due to its critical role in energy metabolism, particularly in sperm motility [6]. Mitochondria are indispensable for sperm functionality, with approximately 70–80 mitochondria located in the sperm's midpiece, supplying the energy required for motility and successful fertilization [7]. Mutations in mitochondrial genes such as *Mitochondrial ATP Synthase 6* (*MT-ATP6*), *Mitochondrial ATP Synthase 8* (*MT-ATP8*), *Mitochondrial NADH Dehydrogenase 1* (*MT-ND1*), *Mitochondrial NADH Dehydrogenase 4* (*MT-ND4*), and *Mitochondrial NADH Dehydrogenase* (*MT-ND5*), particularly during developmental phases, have been linked to asthenozoospermia, a condition characterized by reduced sperm motility, defined as total motility below 40% or progressive motility less than 32%, which severely impacts male fertility [8]. The total mitochondrial variant frequencies in the *MT-ND1*, *MT-ND2*, *MT-ND5*, and *MT-ND6* genes have shown a negative correlation with sperm motility, and several studies have reported genetic alterations in the mitochondrial genome related to asthenozoospermia [9–14].

The *MT-ND3* gene encodes a key subunit of Complex I in the mitochondrial respiratory chain, playing an essential role in ATP synthesis and electron transport [15]. Variations in *MT-ND3* have been associated with several disorders, including male infertility, neurodegenerative diseases, and diabetes [11, 16, 17]. For instance, a study involving a Chinese population identified a homozygous mutation at position 10397 of the *MT-ND3* gene, suggesting a potential impact on *in vitro* fertilization outcomes [18].

Based on the hypothesis that genetic alterations in mtDNA may contribute to deficient energy production and consequently reduced sperm motility, we aimed to investigate the genetic variants of the *MT-ND3* gene and their possible association with asthenozoospermia in the Jordanian population. This will enhance the understanding of the genetic factors underlying male infertility, which could support the development of improved diagnostic tools and effective therapeutic approaches.

2. Materials and methods

2.1 Sample collection

Before starting this study, we obtained ethical approval from the Institutional Review Board at Yarmouk University under IRB number (IRB/2022/28) (Issued in 19 May 2022). A total

of 188 semen samples, comprising 117 samples from individuals diagnosed with asthenozoospermia (sperm motility <40%) and 71 from normozoospermic individuals, were collected from the *in-vitro* fertilization unit at Prince Rashid Military Hospital from 2022 to 2024 [9]. The inclusion criteria for the study population included men aged <40 years, who were non-smokers, did not consume alcohol, and were free from varicocele [19]. Meanwhile, individuals with chronic diseases, cancer, alcoholism, varicocele, smokers, and under treatment were excluded from the study.

Semen specimens were collected as described before [20]. Briefly, the participants provided semen samples by masturbation, considering the international standards of semen collection, such as sexual abstinence for 3 to 5 days before sample collection. A 100 mL sterile plastic container was given to each participant for semen sample collection [20]. The collected semen samples were incubated at 37 °C for 30 minutes for the semen liquefaction assay, followed by analysis of semen parameters based on WHO criteria. Generally, sperm motility, morphology, concentration, and other semen parameters were measured following the guidelines set by the WHO (2021) [21].

2.2 mtDNA extraction and purification

Genomic DNA extraction was carried out using a commercially available kit (QIAamp DNA Mini Kit Cat. No. 51304, Qiagen, Hilden, NRW, Germany). Enrichment of the extracted DNA was performed using the REPLI-g Mitochondrial DNA Kit (Cat. No. 151023, Qiagen, Hilden, NRW, Germany) according to the manufacturer's instructions. The purified mtDNA concentration and purity were measured using a Nanodrop and stored at −20 °C until subsequent analyses were conducted.

2.3 Primer's design and amplification

Primers targeting the *MT-ND3* gene were designed using PRIME 3 software (Prime 3 Software Inc., Norfolk, VA, USA) (https://primer3.ut.ee/), utilizing the human mitochondrial DNA reference sequence (NC_012920) obtained from the NCBI. The primers were synthesized by Integrated DNA Technologies (IDT), USA. Table 1 shows the primer designed for the *MT-ND3* gene. The *MT-ND3* gene was amplified using the MiniAmp™ Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Singapore). Polymerase Chain Reaction (PCR) was carried out in 30 μL, comprising 15 μL of 2X myPOLS master mix (Lot 020320KLA, myPOLS Biotec, Konstanz, BW, Germany), 0.7 μL of each forward and reverse primer, 3 μL of diluted mtDNA, and 10.6 μL of nuclease-free water. The quality of DNA was assessed on 2% gel electrophoresis to verify the specific amplification with a 100-bp DNA ladder, and visualized using a UV gel documentation system to confirm the presence of the specific bands. Table 1 shows the PCR conditions for amplification of the *MT-ND3* gene.

An *in-silico* specificity check of the primers was performed using the BLASTN tool against the human reference genome (GRCh38.p14), which revealed homology to nuclear mitochondrial DNA segments (Numts). Although the mtDNA enrichment step significantly reduces nuclear DNA, the potential for co-amplification of homologous nuclear mitochondrial DNA sequences cannot be entirely ruled out. To ensure reaction fidelity, a negative control (nuclease-free water) was included in every PCR run to monitor for contamination. The specificity of the amplification was initially confirmed by visualizing a single band of the expected size (420 bp) on a 2% agarose gel.

2.4 Sequencing and data analysis

To detect potential variations in the *MT-ND3* gene, the PCR products and Sanger sequencing were provided by an external service laboratory (Biotrust Laboratory, Jordan). Sequencing was carried out in both forward and reverse directions to ensure the reliability of the data. The resulting sequences were analyzed using BioEdit software (version 7.2.5, Tom Hall, Raleigh, NC, USA) (https://bioedit.software.informer.com/7.2/), where they were aligned against the reference mitochondrial DNA sequence (NC_012920) to identify mutations or polymorphisms. Variants detected in the *MT-ND3* gene were annotated and assessed for pathogenic potential using established databases, such as MITOMAP and dbSNP.

2.5 *In silico* pathogenicity prediction

The potential functional impact of the identified missense variants was assessed using a computational prediction tool: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). Variants can be classified as benign, probably damaging, or deleterious based on the respective tools' default score thresholds.

2.6 Statistical analysis

GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA) was used to calculate all statistical values, based on the chi-square test and Fisher's exact test to compare genotype frequencies and allele frequencies between asthenozoospermic and normozoospermic groups. Associations were

TABLE 1. Primer sequences, amplicon size, and thermal cycling conditions for amplification of the *MT-ND3* gene.

Primer	Sequences (5′ → 3′)	Product size (bp)	Thermal cycler conditions
Forward	CCAATTAACTAGTTTTG	420	Initial denaturation 95 °C 3min Denaturation 95 °C 30 s Annealing 50 °C 30 s Extension 72 °C 1 min Final extension 72 °C 5 min
Reverse	GAGTCGAAATCATTCGT		

considered statistically significant at p-value ≤ 0.05, and all computations and visualizations were carried out in GraphPad Prism 6.

3. Results

The subjects were categorized into two groups based on sperm mobility. Normozoospermic samples (n = 71), serving as the control group, had a total sperm motility percentage above 40% and/or progressive motility of more than 32%, while asthenozoospermic samples (n = 117), constituting the case group, had a total sperm motility percentage below 40%. A comparative analysis of semen parameters, including age and motility, between the two groups is illustrated in Fig. 1. The mean sperm motility was significantly lower in the asthenozoospermic group (12.29 ± 10.71) compared with the normozoospermic group (60.82 ± 9.31), with a highly significant p-value of 0.001. Conversely, there was no statistically significant difference in the mean age between the two groups ($p > 0.05$).

The sequencing analysis of the *MT-ND3* gene identified a total of 17 genetic variants, including one novel variant detected in a normozoospermic sample at position m.10313 A>T (Fig. 2). This novel variant is synonymous, meaning it does not alter the amino acid sequence (Fig. 3). Additionally, the DNA sequence analysis revealed six missense variants, which are as follows: (rs41487950 T>C (Ile9Thr), rs202131419 G>A (Gly29Ser), rs193302928 T>C (Val88Ala), rs1603222776 T>C (Met89Thr), rs1603222800 G>A (Ala103Thr), and rs2853826 A>G (Thr114Ser)). Additionally, 11 synonymous variants were identified, namely: (rs3899188 T>C (Ile19), rs1603222690 A>G (Leu24), rs878969753 C>T (Asn28), rs2068720641 C>A (Val49), rs1556423786 A>G (Met53), rs193302927 T>C (Ile60), rs1603222757 C>T (Leu75), rs1556423796 A>G (Trp77), rs163222794 A>G (Leu98), rs1603222805 A>G (Trp106), and rs28358278 C>T (Thr114)). Table 2 shows the genotype distributions for variants. Interestingly, rs2853826 showed a tendency of significant difference between the asthenozoospermic and normozoospermic groups, where the p-value = 0.08. The Minor Allele Frequency (MAF) (G allele) ≈ was 12.0% and ≈ 10.6% in the asthenozoospermic group and normozoospermic group, respectively.

We compared genotypes and allele frequencies of MT-ND3 between asthenozoospermic and normozoospermic individuals to investigate its potential role in male infertility, but no significant association was observed. Table 2 summarizes the genotype frequencies of the *MT-ND3* gene in the asthenozoospermic group and normozoospermic group.

The potential pathogenicity of the six identified missense variants was evaluated using PolyPhen-2 (Polymorphism Phenotyping v2). All missense variants were found to be benign, as shown in Fig. 4, which requires experimental functional studies to reveal the exact role of these variants on the function of the MT-ND3 protein.

For a better understanding of the potential role of the detected alterations in the *MT-ND3* gene, we generated a 3D illustration using (https://www.ncbi.nlm.nih.gov/Structure/icn3d/) that displays both missense and synonymous variants. Fig. 5 illustrates the location of the ND3 subunit in complex I, demonstrating different locations of the detected genetic alterations, proposing their possible role in the protein structure alteration since they are located in the alpha-helices of the ND3 protein.

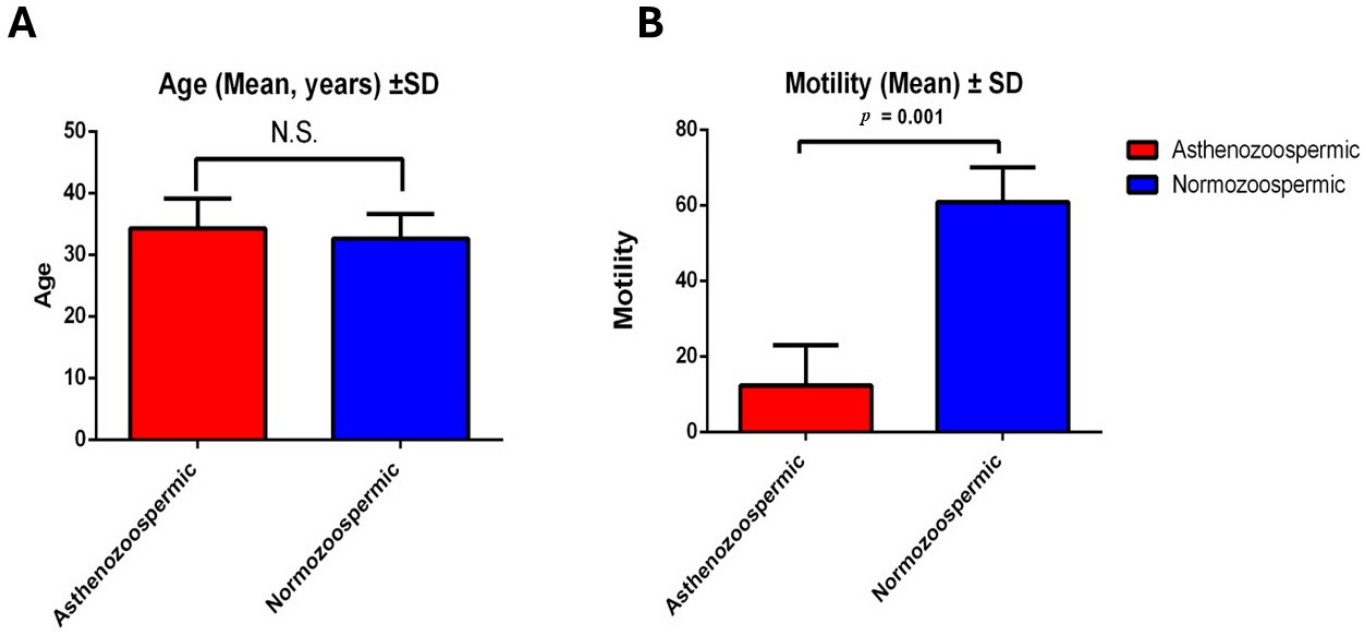

FIGURE 1. Age and motility differences between Asthenozoospermic and Normozoospermic groups. (A) Age groups did not show any significant difference. (B) Significant difference in motility is shown between asthenozoospermic and normozoospermic groups. SD: Standard Deviation; N.S.: Not Significant.

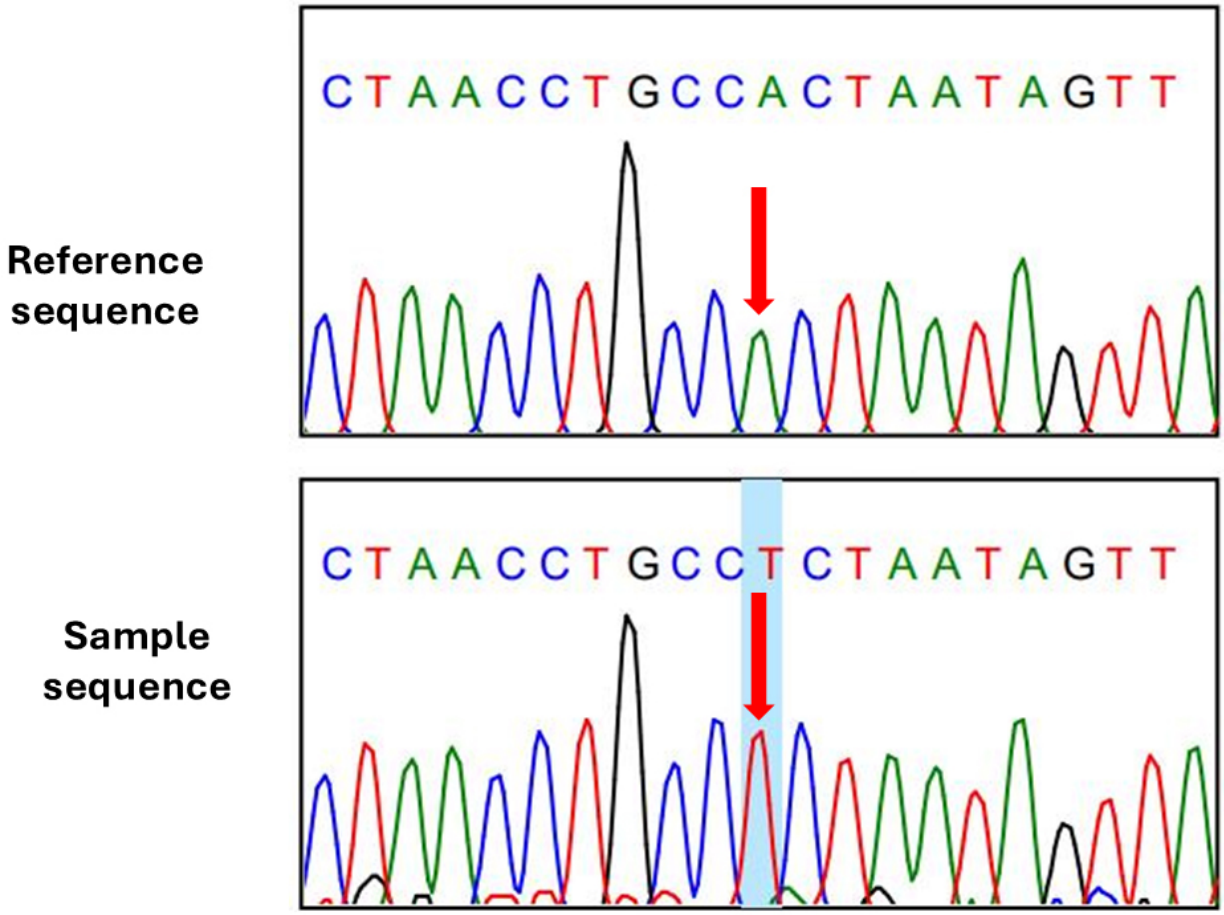

FIGURE 2. Sequence chromatogram of a novel variant at position M.10313 A>T, the wild type in the upper panel and the novel variant in the lower panel.

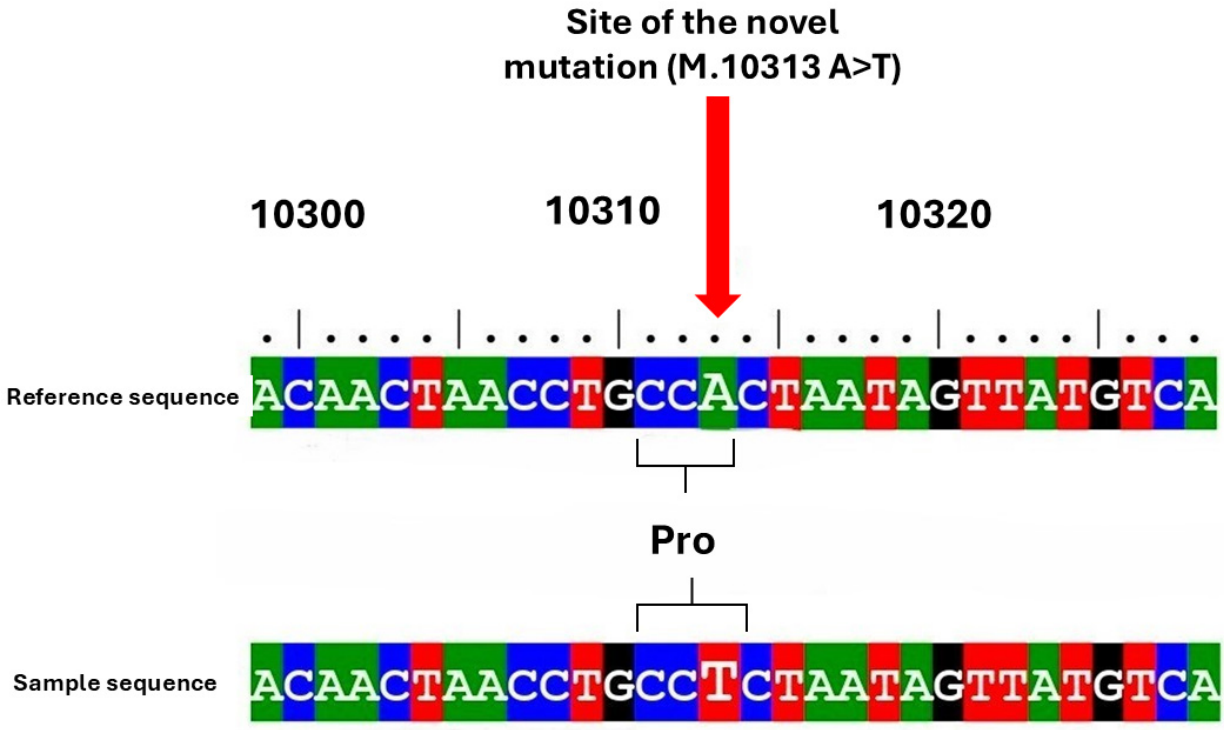

FIGURE 3. Alignment of the *MT-ND3* reference gene sequence (NC_012920.1) with the sample sequence showing the synonymous mutation 10313 A>T. The red arrow indicates the site of the nucleotide substitution.

TABLE 2. Distribution of genotype frequency of the *MT-ND3* gene polymorphisms in the study population.

SNPs Rs#	Position	Codon Change	A.A change	Mutation type	Genotype	Asthenozoospermia (117)	Normozoospermia (71)	*p* value By (χ^2-test)
					T	116	71	
rs41487950	10084 T>C	ATC>ACC	Ile9Thr	Missense	T, C	0	0	0.4348
					C	1	0	
					T	113	70	
rs3899188	10115 T>C	ATT>ATC	Ile19	Synonymous	T, C	0	0	0.4062
					C	4	1	
					A	116	71	
rs1603222690	10130 A>G	CTA>CTG	Leu24	Synonymous	A, G	0	0	0.4348
					G	1	0	
					C	116	70	
rs878969753	10142 C>T	AAC>AAT	Asn28	Synonymous	C, T	0	0	0.7200
					T	1	1	
					G	117	70	
rs202131419	10143 G>A	GGC>AGC	Gly29Ser	Missense	G, A	0	0	0.1980
					A	0	1	
					C	117	70	
rs2068720641	10205 C>A	GTC>GTA	Val49	Synonymous	C, A	0	0	0.1980
					A	0	1	
					A	113	69	
rs1556423786	10217 A>G	ATA>ATG	Met53	Synonymous	A, G	0	0	0.8199
					G	4	2	
					T	113	69	
rs193302927	10238 T>C	ATT>ATC	Ile60	Synonymous	T, C	0	0	0.8199
					C	4	2	
					C	117	70	
rs1603222757	10281 C>T	CTA>TTA	Leu75	Synonymous	C, T	0	1	0.1980
					T	0	0	
					A	115	70	
rs1556423796	10289 A>G	TGA>TGG	Trp77	Synonymous	A, G	0	0	0.8732
					G	2	1	
					T	117	70	
rs193302928	10321 T>C	GTT>GCT	Val88Ala	Missense	T, C	0	0	0.1980
					C	0	1	

TABLE 2. Continued.

SNPs Rs#	Position	Codon Change	A.A change	Mutation type	Genotype	Asthenozoospermia (117)	Normozoospermia (71)	*p* value By (χ^2-test)
					T	117	70	
rs1603222776	10324 T>C	ATG>ACG	Met89Thr	Missense	T, C	0	0	0.1980
					C	0	1	
					A	116	71	
rs163222794	10352 A>G	CTA>CTG	Leu98	Synonymous	A, G	0	0	0.4348
					G	1	0	
					G	116	71	
rs1603222800	10365 G>A	GCC>ACC	Ala103Thr	Missense	G, A	0	0	0.4348
					A	1	0	
					A	117	70	
rs1603222805	10376 A>G	TGA>TGG	Trp106	Synonymous	A, G	0	0	0.1980
					G	0	1	
					A	89	58	
rs2853826	10398 A>G	ACC>GCC	Thr114Ser	Missense	A, G	0	2	0.0824
					G	28	11	
					C	115	70	
rs28358278	10400 C>T	ACC>ACT	Thr114	Synonymous	C, T	0	0	0.8732
					T	2	1	

SNPs: Single Nucleotide Polymorphisms; A.A: Amino Acid.

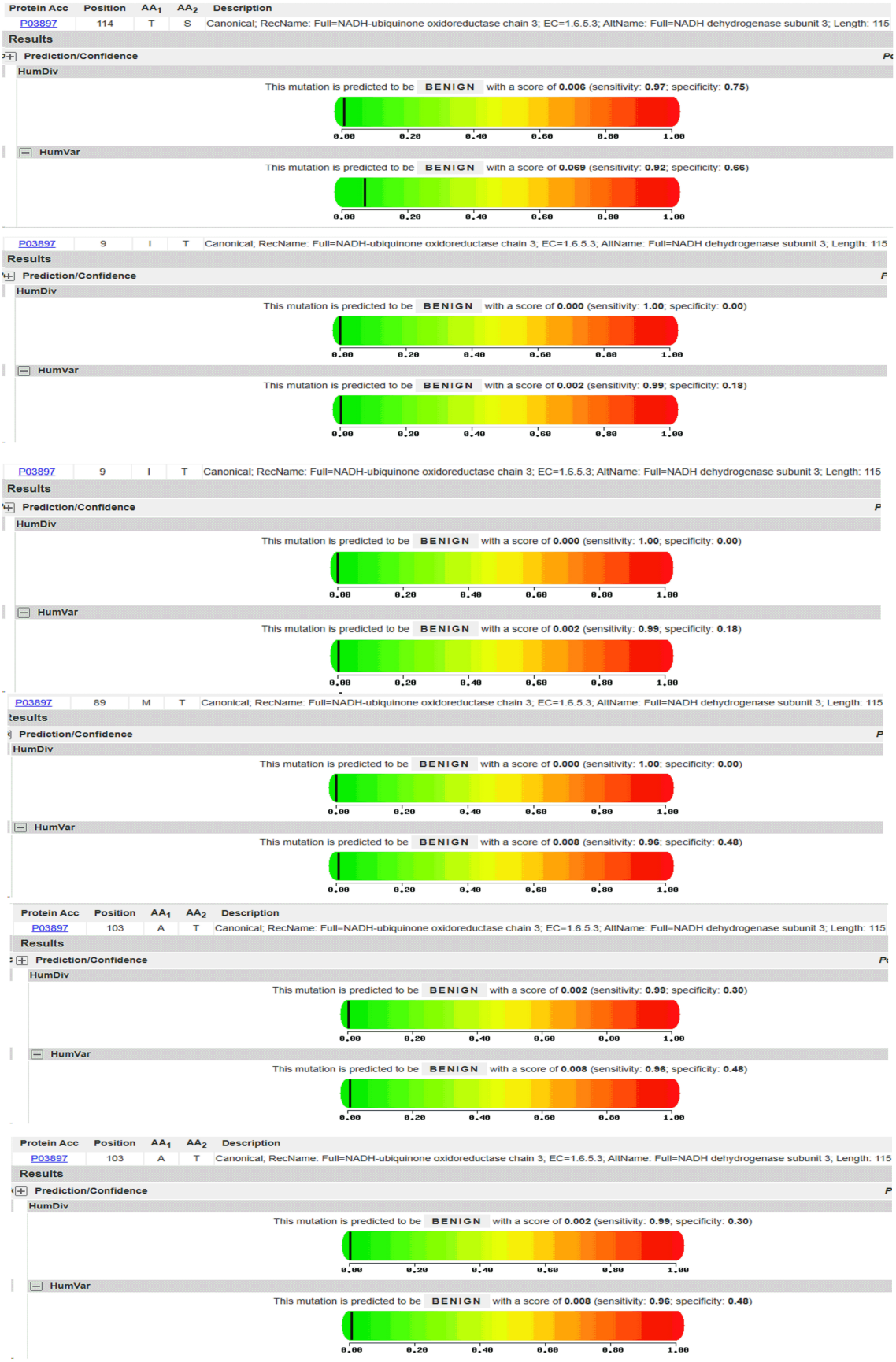

FIGURE 4. PolyPhen prediction of the detected missense variants in the *MT-ND3* gene shows the benign effect of these variants. AA: Amino Acids; NADH: Nicotine Amide Dinucleotide Hydride; EC: Enzyme Commission.

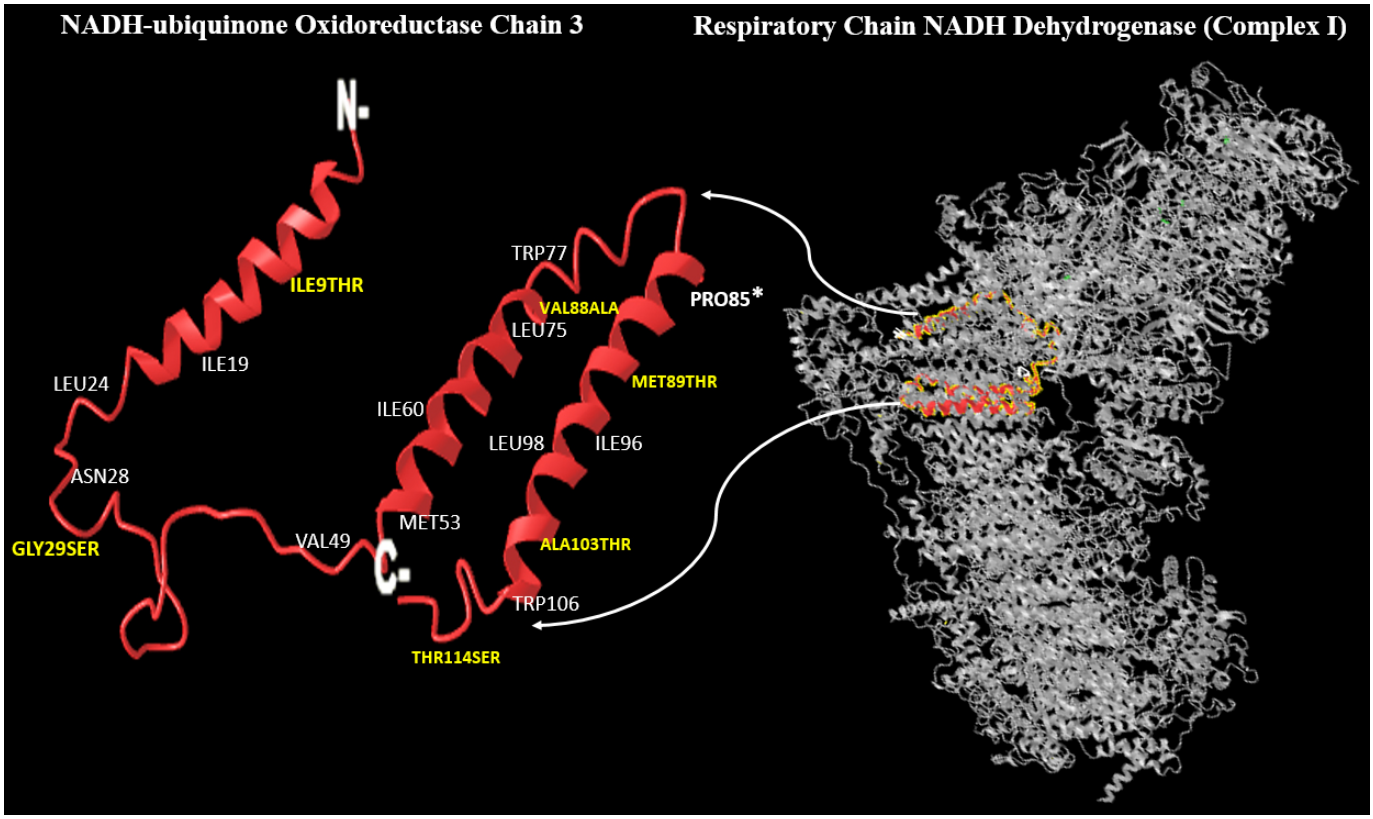

FIGURE 5. 3D structure of mitochondrial ND3 protein showing its location in complex I (right) and the places of the detected variants (Left). Yellow: missense mutations, White: synonymous alterations. Generated using (https://www.ncbi.nlm.nih.gov/Structure/icn3d/). NADH: Nicotine Amide Dinucleotide Hydride.

4. Discussion

Sperm motility is critically dependent on mitochondrial function, which provides ATP, the primary energy source necessary for motility. Impaired mitochondrial function or flagellar abnormalities can, therefore, contribute to infertility [22]. Compared to nuclear DNA, mtDNA lacks the repair systems that sustain DNA stability and integrity. Normally, Oxidative Phosphorylation (OXPHOS) reactions in mitochondria generate reactive oxygen species (ROS) and free radicals, which can lead to mtDNA genetic alterations and DNA damage [23, 24]. In addition, mtDNA lacks nucleosome structures and an efficient repairing system, reducing its capacity for DNA protection [25]. Collectively, genetic alterations in the mtDNA may result in deficient proteins of the electron transport system and impaired ATP production, suggesting a role for these genetic alterations in male asthenozoospermic infertility [26].

mtDNA variations, particularly in the *MT-ND3* gene, are suggested to play a pivotal role in energy production through the electron transport chain and may impact male infertility [27]. In this study, we identified several mtDNA variants, some of which demonstrated potential links to sperm motility. A novel synonymous variant, M.10313 A>T, was identified exclusively in normozoospermic samples. While synonymous mutations generally do not alter the amino acid sequence, they can impact the efficiency of translation or the stability of mRNA, potentially affecting ATP production [28]. The presence of this variant solely in normal samples suggests that its effect on infertility is minimal [29].

Missense variants were more informative in distinguishing between normozoospermic and asthenozoospermic samples. Variants rs41487950 T>C (Ile9Thr) and rs1603222800 G>A (Ala103Thr), found exclusively in asthenozoospermic samples, result in changes from nonpolar to polar amino acids. This likely disrupts the protein's three-dimensional structure, impairing its function and potentially reducing ATP production, which is critical for sperm motility. In contrast, rs202131419 G>A (Gly29Ser), rs193302928 T>C (Val88Ala), and rs1603222776 T>C (Met89Thr) were detected only in normozoospermic samples. The occurrence of MT-ND3 variants among normozoospermia could be attributed to the Heteroplasmy state, defined as not all sperm mtDNA copies are affected. The occurrence of potentially damaging MT-ND3 variants in some normozoospermic individuals could be explained by several factors. While heteroplasmy is a common mechanism for modulating the expression of a mitochondrial mutation, our Sanger sequencing methodology lacks the sensitivity to detect low-level heteroplasmy. Alternative explanations include the presence of compensatory nuclear genetic factors, epigenetic modifications, or environmental influences that mitigate the functional impact of these variants in fertile individuals.

In this study, the presence of missense variants identified in the MT-ND3 gene in healthy samples suggests that they alter the polypeptide chain and do not negatively impact mitochondrial function or energy production. The variant rs2853826 A>G (Thr114Ser) was detected in both groups and exhibited a high prevalence in the population (≈22%). In particular, the

MAF (G allele) ≈ was 12.0% and ≈ 10.6% in the asthenozoospermic group and normozoospermic group, respectively. These findings showed a higher MAF for the G allele of rs2853826 in the asthenozoospermic group compared with the normozoospermic group; however, this difference did not reach a statistically significant level, suggesting a potential that the G allele might be associated with the development of asthenozoospermia. Further studies in a larger population are needed to confirm this borderline correlation. Despite modifying the polypeptide chain, the retention of hydrophobic properties likely preserves the protein's structural integrity and function. Its widespread occurrence further indicates a limited role in male infertility. Several of these variants have been associated with other health conditions. For instance, rs41487950 T>C has been linked to aging through the production of reactive oxygen species [30], while rs2853826 A>G has been associated with type 2 diabetes, gastric cancer, and breast cancer [31].

Our results agree with previous studies that reported a lack of significant associations between mtDNA variants and sperm motility [32], highlighting the complex and context-dependent nature of mtDNA variants. The synonymous variants also present a nuanced picture. Variants rs1603222690 A>G and rs163222794 A>G were identified only in asthenozoospermic samples; conversely, rs2068720641 C>A, rs1603222757 C>T, and rs1603222805 A>G were found exclusively in normozoospermic samples, while synonymous variants such as rs3899188 T>C and rs878969753 C>T were shared between the both groups. The presence of synonymous variants in different distributions and frequencies in asthenozoospermic and normozoospermic individuals can be attributed to polymorphic variations between individuals; however, these single-nucleotide polymorphisms may be associated with specific genetic functions, such as gene regulation. Although the synonymous variants identified do not alter the amino acid sequence, they should not be dismissed as functionally inert. A change in nucleotide sequence can influence mRNA stability, secondary structure, and translation kinetics by creating or abolishing codon preferences [33]. While the functional impact of mitochondrial tRNA codon usage is less well-characterized in the context of sperm function, this represents a potential mechanism by which synonymous variants could subtly affect OXPHOS efficiency and warrants further investigation. Therefore, further studies are necessary to elucidate the precise role of these variations on cellular function through functional investigations. In addition, a larger sample size will be very helpful to explore the presence of similar SNPs in other populations. The current study cannot confirm the association of these SNPs with the pathogenesis of asthenozoospermia; nevertheless, it highlights the presence of these SNPs in the study population.

Although allele frequency analysis did not reveal significant associations between mitochondrial genetic variants in the MT-ND3 gene and sperm motility, trends suggest that an increased prevalence of wild-type alleles or reduced mutant alleles may preserve male fertility. Conversely, higher mutant allele frequencies could contribute to infertility. These findings underscore the importance of further investigations to elucidate the structural and functional impacts of MT-ND3 protein variants on mitochondrial function and male fertility. Expanding the focus to include other mitochondrial genes is also essential, as mitochondrial dysfunction may result from multiple genetic factors, not solely from the MT-ND3 gene.

The current study provides further information about the genetic alteration in the mtDNA in the development of asthenozoospermia, which is consistent with several previous works. For instance, the mtDNA 7345bp, 7599bp, and 4977 macro-deletions showed a significant association with asthenozoospermia. In another study, single-nucleotide variants of the *mitochondrial cytochrome B* gene (MT-CYB) were associated with male infertility. Moreover, on one side, a study showed a correlation between the *MT-ND4* gene polymorphisms and male infertility [9, 10, 12, 13, 34]. On the other hand, several studies have reported an association between single polymorphic variants in certain mtDNA genes, such as *mitochondrial nicotinamide adenine dinucleotide dehydrogenase 3* (*MT-ND3*), *4L* (*MT-ND4L*), and *mitochondrial cytochrome C oxidase* genes, and male infertility. Collectively, pieces of evidence support the hypothesis of the genetic bases behind the development of certain types of male infertility, such as asthenozoospermia [11, 14]. A limitation of this study is the sample size, which may lack the statistical power to detect significant associations for low-frequency variants.

Our *in-silico* analysis provided a more nuanced view of the missense variants. However, the PolyPhen prediction tool did not find any probability of a damaging effect of the detected missense variants, which can be attributed to the tool's settings, which require the conduct of functional studies on these variants.

Another limitation of this study is the potential for co-amplification of nuclear mitochondrial DNA segments, despite the mtDNA enrichment step. The primers used show *in silico* homology to a nuclear mitochondrial DNA segment on chromosome 1. Therefore, we cannot definitively rule out that a minority of the sequenced products originated from the nucleus, which could theoretically influence variant calling. Future studies would benefit from long-range PCR or RNA sequencing strategies that more effectively avoid nuclear mitochondrial DNA segments contamination.

5. Conclusions

The current study identified various variants in the *MT-ND3* gene in asthenozoospermic and normozoospermic individuals. Certain variants in asthenozoospermic samples are predicted to alter the protein's structure, suggesting a possible role of these variants in male infertility. While a novel synonymous variant and other variants were detected in the normozoospermic individuals, which can be attributed to the Heteroplasmy of the mitochondrial genome. Therefore, further genetic studies are required to understand the role of these variants in male infertility in different populations with a larger sample size. While our case-control analysis did not find a statistically significant association between specific *MT-ND3* SNPs and asthenozoospermia in our Jordanian cohort, *in silico* predictions suggest that two variants found exclusively in asthenozoospermic men may be functionally deleterious. Important limitations of this study include the potential for nuclear mi-

tochondrial DNA segments co-amplification and the inability to detect low-level heteroplasmy. Therefore, the role of MT-ND3 requires further elucidation through larger, multi-ethnic studies employing more sensitive sequencing technologies and functional assays to validate the computational predictions presented here.

AVAILABILITY OF DATA AND MATERIALS

The corresponding author will provide the required data upon reasonable request.

AUTHOR CONTRIBUTIONS

MYJ and MSAZ—Conceptualization, experimental design, supervision, and manuscript drafting and approval. MAA, AA and AAS—Supervision and experimental data collection and analysis. SFM—Laboratory experiments, data collection, data analysis, and manuscript drafting. MSAZ, MYJ, MAS, KMAB and RMAZ—Manuscript drafting, editing, and final version approval. The final version of the manuscript is approved by all contributing authors.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Institutional Review Board approval, at number (IRB/2022/28), was provided by the Deanship of Scientific Research and Graduate Studies at Yarmouk University. All participants signed consent forms after explaining the aim of the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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