Received: 18 March 2021 | Revised: 3 May 2021 | Accepted: 17 May 2021

DOI: 10.1111/and.14139

ORIGINAL ARTICLE

First International Journal of Andrology
aNDROLOGIa
WILEY

Lack of association between single polymorphic variants of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase 3, and 4L (*MT-ND3* and *MT-ND4L*) and male infertility

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Funding information
This work is funded by the Department of Obstetrics and Gynaecology, Saarland University, Germany.

Abstract

Male infertility is a multifactorial condition associated with different genetic abnormalities in at least 15%–30% of cases. The purpose of this study was to identify suspected correlations between infertility and polymorphisms in mitochondrial NADH dehydrogenase subunits 3 and 4L (*MT-ND3* and *MT-ND4L*) in subfertile male spermatozoa. Sanger sequencing of the mitochondrial DNA target genes was performed on 68 subfertile and 44 fertile males. Eight single nucleotide polymorphisms (SNPs) in *MT-ND3* (rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954) and seven SNPs in *MT-ND4L* (rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881) were detected and genotyped. The genotypes and allele frequencies of the study population have shown a lack of statistically significant association between *MT-ND3* and *MT-ND4L* SNPs and male infertility. However, no statistically significant association was found between the asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia and oligoteratozoospermia subgroups of subfertile males. However, rs28358278 genotype of the *MT-ND3* gene was reported in the subfertile group but not in the fertile group, which implies a possible role of this SNP in male infertility. In conclusion, the investigated polymorphic variants in the *MT-ND3* and *MT-ND4L* genes did not show any significant association with the occurrence of male infertility. Further studies are required to evaluate these findings. Moreover, the subfertile individuals who exhibit a polymorphism at rs28358278 require further monitoring and evaluation.

KEYWORDS

male infertility, mtDNA, *MT-ND3*, *MT-ND4L*, SNP

1 | INTRODUCTION

Malefactors represent up to 50% of couples' infertility worldwide (De Kretser & Baker, 1999; Navarro-Costa et al., 2010). Different factors have been related to male infertility such as abnormal spermatogenesis leading to abnormal and low-quality spermatozoa including azoospermia, asthenozoospermia, oligozoospermia and teratozoospermia (Grimes & Lopez, 2007). However, the abnormalities that were mentioned to diagnose by conventional semen analysis can also be caused by genetic predisposition. Semen analysis could only reveal the abnormalities but it could not explain the reason; therefore, molecular approaches have been applied to reveal the aetiology in such cases also (Jenkins et al., 2016; Mobasseri et al., 2018; Poongothai et al., 2009). The sperm efficiency to fertilise the ovum is related to the energy level which is provided by the mitochondria. Therefore, mitochondrial DNA (mtDNA) damage will affect mitochondrial energy production and leads to low quality of spermatozoa (Okutman et al., 2018). Normally, mtDNA is more vulnerable compared to nuclear DNA due to the lack of an efficient repairing system and high exposure to oxidative species produced by the mitochondria. Eventually, this leads to abnormal sperm function, structure and even infertility (Hsia et al., 2003; Nakane et al., 2008). Many studies reported different molecular causes of male infertility by studying the mtDNA and genomic DNA integrity (Gunes et al., 2016; Jungwirth et al., 2015; Poplinski et al., 2010); however, the aetiology of idiopathic male infertility (IMI) is not completely elucidated.

MtDNA sequencing in infertile males has shown the importance of genetic predisposition in the development of idiopathic male infertility in different populations (Carrell et al., 2006). MtDNA is coded for thirteen proteins that are part of the mitochondrial respiratory chain. These proteins are localised in major complexes as the following: Complex I contains seven subunits of Nicotinamide Adenine Dinucleotide Hydride (NADH) dehydrogenase (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6*), complex III includes cytochrome B, complex IV contains three subunits: subunit I of cytochrome oxidase (*COX I*), subunit II of cytochrome oxidase (*COX II*) and subunit III of cytochrome oxidase (*COX III*), and Complex V contains *ATPase 6* and *ATPase 8* (Smeitink et al., 2001). Therefore, abnormalities in any of these proteins are expected to affect the quality of mitochondrial function which could affect sperm function and fertility in males. Consequently, screening of mtDNA for genetic variations has been suggested to elucidate the molecular impact on male infertility.

Previous studies have demonstrated a strong association between impaired mtDNA and the development of male infertility conditions such as asthenozoospermia, oligozoospermia and teratozoospermia (Dahadhah et al., 2021). For instance, large-scale deletions in the mtDNA were reported in asthenozoospermia in different populations (Al Zoubi et al., 2020; Bahrehmand Namaghi & Vaziri, 2017; Kao et al., 1998). These reported mutations revealed a loss of vital genes in the mtDNA such as *ATPases 6* and *8* and *ND3* and *ND4L* genes (Ambulkar et al., 2016; Karimian & Babaei, 2020). However, the molecular bases of male infertility are still not completely understood. Therefore, other genetic alterations have been suggested to be related to idiopathic male infertility including metabolic and structural enzymes such as methylenetetrahydrofolate reductase (MTHFR) and cystic fibrosis transmembrane conductance regulator (CFTR) (Cuppens & Cassiman, 2004; Wei et al., 2012).

MTHFR is a key enzyme that plays an essential role in spermatogenesis (Cuppens & Cassiman, 2004). MTHFR enzyme converts 5,10 Methyltetrahydrofolate (5,10 MTHF) into 5 Methyltetrahydrofolate (5 MTHF) (Zhang et al., 2012). Then, the 5 MTHF acts as a methyl donor for the methionine synthase enzyme which converts homocysteine into methionine (Leonhartsberger et al., 2005). Defects in the *MTHFR* gene increase homocysteine levels in blood plasma resulting in hyperhomocysteinemia (Altmäe et al., 2010). Elevated levels of homocysteine as well polymorphisms in the *MTHFR* gene were reported to have an association with male infertility in several populations (Dhillon et al., 2007; Lee et al., 2006; Mfady et al., 2014; Tetik et al., 2008). CFTR is vital for sperm fertilising capacity and is associated with sperm quality in humans. Around 97% of cystic fibrosis males are infertile due to congenital bilateral absence of the vas deferens (CBAVD) with resultant obstructive azoospermia. Other causes of azoospermia involve abnormalities of the seminal vesicles and congenital unilateral absence of the vas deferens (Li et al., 2014).

Nevertheless, polymorphic variation in the mtDNA genes has not been well studied in subfertile males. Therefore, we aimed to elucidate the possible association between the *MT-ND3* and *MT-ND4L* genes' polymorphisms and the development of male infertility.

2 | MATERIALS AND METHODS

2.1 | *Sperm sample collection*

One hundred and twelve semen samples were collected from males attending the in-vitro fertilisation clinic (IVF). Informed consent was obtained from all males before sample collection. The study population, aged between 26 and 48 years, was divided into two groups: 68 subfertile and 44 fertile men. Males who had one child or more, and had normal semen parameters: volume: 1.5 ml, sperm count: 15 million spermatozoa/ml; normal forms: 4%; vitality: 58% live; progressive motility: 32%; total (progressive +non-progressive) motility: 40%, according to WHO guideline 2010, were considered as the fertile group and those who failed to have children after 12 months or more of regular unprotected sexual intercourse and had at least one sperm parameter under WHO (2010) criteria were considered as the subfertile group.

Individuals over 50 years of age, males exposed to chemo- or radiotherapy, varicocele or any surgical intervention in the reproductive tract, diabetes, blood pressure and all chronic disease, hormonal imbalance and Y chromosome microdeletion were excluded from the study.

TABLE 1 Oligonucleotides primers used for PCR amplification of *Nd3* and *Nd4L* mtDNA genes

Primers	Sequences (5′ → 3′)	Cycling conditions	The length of the amplified product (bp)
MT-Nd3.F MT-Nd3.R	CCAATTAACTAGTTTTG GAGTCGAAATCATTCGT	95°C 3 min 95°C 30 s 48.8°C 30 s (30x cycles) 72°C 1 min 72°C 5 min	420 bp
MT-Nd4L.F MT-Nd4L.R	GATTTCGACTCATTAAATT CATGTCAGTGGTAGTAATAT	95°C 3 min 95°C 30 s 45.9°C 30 s (30x cycles) 72°C 1 min 72°C 5 min	376 bp

Abbreviations: bp, base pair; F, forward primer; R, reverse primer.

Semen samples were obtained by masturbation after 3 days of abstinence; collected in a sterile, wide-mouthed, non-toxic and special container; and then was allowed to liquefy at 37°C for 30 min before assessment. Before DNA extraction, the semen samples were processed by the discontinuous pure sperm gradient (45% and 90%) technique (Nidacon International, Sweden). Briefly, semen samples were loaded at the upper level of the gradient and centrifuged at 250 g for 20 min. Subsequently, the pellet was collected and washed twice with a sperm washing medium. The absence of all other cells was confirmed by microscopic examination. Finally, the sperm pellet was stored at −20°C for DNA extraction. The discontinuous pure sperm gradient technique is used to exclude any somatic source of the extracted DNA. The technique is based on the purification of the spermatozoa which provides a more representable specimen for the affected spermatozoa.

2.2 | *Mitochondrial DNA extraction*

Genomic DNA was extracted from the spermatozoa using a QIAamp DNA Mini Kit; then, the mitochondrial DNA was amplified by using the REPLI-g Mitochondrial DNA Kit (QIAGEN, Hilden, Germany), as recommended by the kit instruction manual. Isolated DNA with an optimal density ratio of 260/280 of 1.8 or more was selected for subsequent assays and preserved at −80°C.

2.3 | *Polymerase chain reaction*

The polymerase chain reaction was performed to identify the gene variant using self-designed pairs of unique primers employing the PRIME 3 software for the target genes (*MT-ND3* and *MT-ND4L*) as illustrated in Table 1. The primers were based on the human mitochondrial sequence; accession number NC_012920 provided by the National Centre of Biotechnology Information (NCBI) and ordered from Microsynth seq laboratory, Germany. The amplification reaction was carried out in a 30 μl mixture using Thermo Scientific Dream Taq Green PCR master mix (2x), according to manufacturer instructions. To confirm the presence of an amplified PCR product, 5 μl of each PCR sample was investigated by 1% agarose gel electrophoresis using 1× TBE buffer and a DNA ladder (1kb) (NE Biolabs, USA) as a reference. Electrophoresis was performed at 100V for 45 min. Gels were stained with red-safe stain, and thereafter, DNA was visualised by ultra-violet (UV) transilluminator using Image Lab TM Software (BIO-RAD, USA) (Figures 1 and 2).

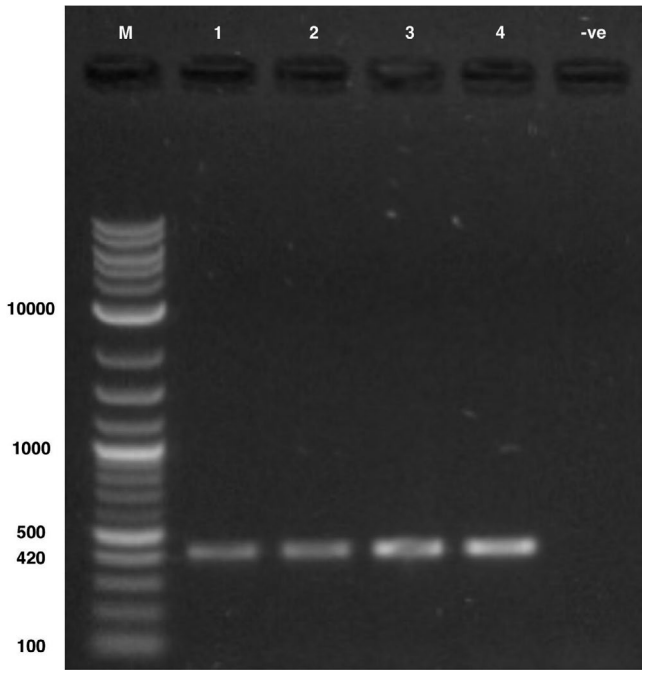

FIGURE 1 Representative gel electrophoresis on 1% agarose gel of PCR products for the amplification of the *MT-ND3* gene (420 bp). Lane M: DNA Ladder (100–10,000 bp) (NE Biolabs, USA), Lane 1–4: PCR samples products, lane -ve: negative control

2.4 | *DNA sequencing*

Samples were purified and sequenced using the Sanger method in the laboratory Microsynth Seq in Germany. The SNPs of *MT-ND3*

and *MT-ND4L* were identified by sequence analysis based on the reference sequence of human mitochondria (GenBank accession number: NC_012920). The sequenced DNA samples were analysed with Mutation Surveyor software to determine the mitochondrial DNA variants.

2.5 | *Statistical analysis*

Genotypes and allele frequencies between the subfertile (case) and fertile (control) groups were identified using the chi-square test and Fisher's exact test respectively. The defined SNPs were also tested for the Hardy–Weinberg equilibrium test to determine genotype frequencies and to describe statistically significant deviations from the equilibrium. Allele frequencies between the subfertile (case) and fertile (control) groups were measured according to odds ratios (ORs) and 95% confidence intervals (CIs). The *P*-value was regarded as statistically significant if ≤0.05. Statistical analyses were carried out using the SPSS Version 22 for Mac.

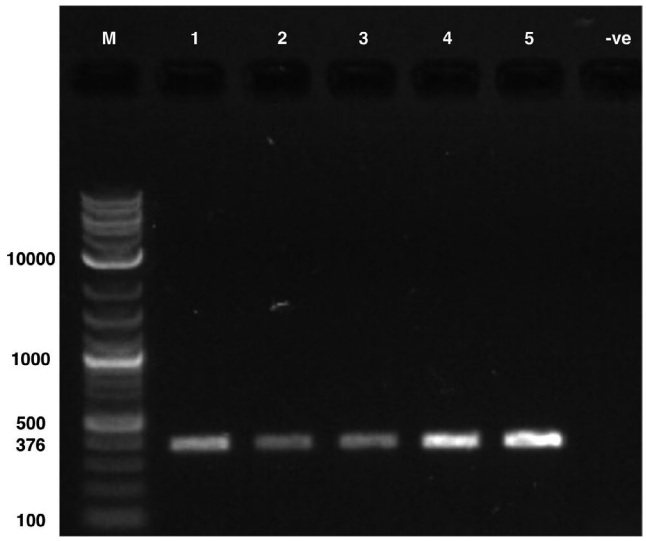

FIGURE 2 Representative gel electrophoresis on 1% agarose gel of PCR products for the amplification of the *MT-ND4L* gene (376 bp). Lane M: DNA Ladder (100–10000 bp) (NE Biolabs, USA), Lane 1–4: PCR samples products, lane −ve: negative control

3 | RESULTS

The participants in this study were divided into two groups: a control group (fertile, $n = 44$) and a case group (subfertile, $n = 68$). The subfertile group was divided into the following 6 subgroups (asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia and oligoteratozoospermia). The study population showed no significant difference between the age of the subfertile and the fertile group ($p = .247$). Furthermore, the semen analysis did show significant differences in the mean percentage of sperm concentration, total motility and morphologically normal spermatozoa between the fertile and subfertile males ($p < .0001$) (Table 2).

3.1 | *Genotypes and allelic frequencies*

We identified eight SNPs in *MT-ND3* (rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954) and seven SNPs in *MT-ND4L* (rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881). To determine whether the variations of *MT-ND3* and *MT-ND4L* were related to infertility, we compared each of the genotypes and allele frequencies between the case and control groups. The genotype distributions for the SNPs in *MT-ND3* and *MT-ND4L* are shown in Tables 3–6. There was no statistically significant association found in frequencies of genotypes and alleles between the present MT-SNPs and male infertility. Moreover, all SNPs were tested for the Hardy–Weinberg genotype frequency test. Each of these SNPs showed a significant deviation from HWE ($p < .0001$). On the other hand, there was no statistically significant association between asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia and oligoteratozoospermia subgroups of subfertile males and the fertile ones ($p > .05$).

4 | DISCUSSION

The sperm**atozoa** with impaired mitochondria are expected to face insufficient ATP production and more reactive oxygen species (ROS) or free radicals. Production of ROS and free radicals in an unbalanced

Parameter	Fertile ($n = 44$) Median (range)	Subfertile ($n = 68$) Median (range)	(*t*-test) *P*-value
Age	34 (26–48)	34 (26–48)	.247
Sperm concentration (106 x 1 ml)	78.5 (17–185)	28 (0.6–135)	<.0001
Total motility (PR + NP %)	67.5 (44–90)	48.5 (2–88)	<.0001
Morphologically normal Spermatozoa (%)	24.5 (20–30)	15 (0–28)	<.0001

Abbreviation: *n*, number.

TABLE 2 Comparison of the semen analysis parameters between the fertile and subfertile groups

TABLE 3 Genotype frequency of *MTND3* polymorphisms between subfertile and fertile males

SNP	Contig position	Codon change	Amino acid change	Type of mutation	Genotype	Subfertile (*N*)	Fertile (*N*)	(Chi-square test) *P*-value
rs2853826 (A > G,T)	10,398	[ACC]>[GCC]	Thr114 Ala	Missense variant	AA	37	21	.768
					AG	1	1	
					GG	30	22	
rs28435660 (G>A)	10,353	[GCC]>[ACC]	Ala99Thr	Missense variant	GG	61	40	.825
					GA	4	3	
					AA	3	1	
rs193302927 (T>C)	10,238	[ATT]> [ATC]	Ile60Ile	Synonymous variant	TT	62	40	.959
					TC	2	1	
					CC	4	3	
rs28358278 (C>T)	10,400	[ACC]>[ACT]	Thr114Thr	Synonymous variant	CC	65	44	.158
					CT	0	0	
					TT	3	0	
rs41467651 (G>A)	10,310	[CTG]>[CTA]	Leu84Leu	Synonymous variant	GG	65	42	.9320
					GA	1	1	
					AA	2	1	
rs3899188 (T>C)	10,115	[ATT]> [ATC]	Ile19Ile	Synonymous variant	TT	67	43	.754
					TC	0	0	
					CC	1	1	
rs28358277 (G>A)	10,373	[GAG]>[GAA]	Glu105Glu	Synonymous variant	GG	66	44	.517
					GA	1	0	
					AA	1	0	
rs28673954 (T>C)	10,370	[TAT]> [TAC]	Tyr104Tyr	Synonymous variant	TT	67	44	.4191
					TC	1	0	
					CC	0	0	

Abbreviations: MV, missense variant; *N*, number; SNP, single nucleotide polymorphism; SV, synonymous variant.

TABLE 4 Allele frequency of *MTND3* polymorphisms between subfertile and fertile males

SNP	Contig position	Allele	Subfertile (*N*, %)	Fertile (*N*, %)	OR (95% CI)*	(Fisher's exact test) *P*-value
rs2853826 (A>G,T)	10,398	A	75 (34%)	43 (19%)	1.287 (0.751 – 2.203)	0.411
		G	61 (27%)	45 (20%)		
rs28435660 (G>A)	10,353	G	126 (56%)	83 (37%)	0.759 (0.25 – 2.3)	0.7865
		A	10 (5%)	5 (2%)		
rs193302927 (T>C)	10,238	T	126 (56%)	81 (36%)	1.089 (0.398 – 2.977)	1.000
		C	10 (5%)	7 (3%)		
rs28358278 (C>T)	10,400	C	130 (58%)	88 (39%)	0.1134 (0.006 – 2.041)	0.0837
		T	6 (3%)	0 (0%)		
rs41467651 (G>A)	10,310	G	131 (59%)	85 (38%)	0.924 (0.215 – 3.972)	1.000
		A	5 (2%)	3 (1%)		
rs3899188 (T>C)	10,115	T	134 (60%)	86 (38%)	1.558 (0.2153 – 11.275)	0.6466
		C	2 (1%)	2 (1%)		
rs28358277 (G>A)	10,373	G	133 (60%)	88 (39%)	0.2155 (0.0109 – 4.226)	0.2812
		A	3 (1%)	0 (0%)		
rs28673954 (T>C)	10,370	T	135 (61%)	88 (39%)	0.5104 (0.0205 – 12.679)	1.000
		C	1 (0%)	0 (0%)		

Abbreviations: CI, confidence interval; *N*, number; OR, odds ratio; SNP, single nucleotide polymorphism.

TABLE 5 Genotype frequency of *MTND4L* polymorphisms between subfertile and fertile males

SNP	Contig position	Codon change	Amino acid change	Type of mutation	Genotype	Subfertile (*N*)	Fertile (*N*)	(Chi-square test) *P*-value
rs28358280 (A>G)	10,550	[ATA]>[ATG]	Met27Met	Synonymous variant	AA	67	42	.325
					AG	0	0	
					GG	1	2	
rs28358281 (G>A,C)	10,586	[TCG]>[TCA]	Ser39Ser	Synonymous variant	GG	62	43	.3335
					GA	2	0	
					AA	4	1	
rs28358279 (T>A,C)	10,463	*N*/A	*N*/A	Synonymous variant	TT	64	42	.759
					TC	0	0	
					CC	4	2	
rs2853487 (G>A)	10,589	[CTG]>[CTA]	Leu40Leu	Synonymous variant	GG	66	43	.8306
					GA	0	0	
					AA	2	1	
rs2853488 (G>A)	10,688	[GTG]>[GTA]	Val73Val	Synonymous variant	GG	66	43	.2416
					GA	2	0	
					AA	0	1	
rs193302933 (C>T)	10,664	[GTC]>[GTT]	Val65Val	Synonymous variant	CC	68	43	.2118
					CT	0	0	
					TT	0	1	
rs28532881 (C>A)	10,763	[TGC]>[TGA]	Cys98Trp	Missense variant	CC	68	44	*N*/A
					CA	0	0	
					AA	0	0	

Abbreviations: MV, missense variant; *N*, number; *N*/A, not applicable; SNP, single nucleotide polymorphism; SV, Synonymous variant.

TABLE 6 Allele frequency of *MTND4L* polymorphisms between subfertile and fertile males

SNP	Contig position	Allele	Subfertile (*N*, %)	Fertile (*N*, %)	OR (95% CI)*	(Fisher's exact test) *P*-value
rs28358280 (A>G)	10,550	A	134 (60%)	84 (37%)	3.190 (0.571 – 17.810)	0.214
		G	2 (1%)	4 (2%)		
rs28358281 G>A,C	10,586	G	126 (56%)	86 (38%)	0.2883 (0.0616 – 1.350)	0.132
		A	10 (5%)	2 (1%)		
rs28358279 (T>A,C)	10,463	T	128 (57%)	84 (37%)	0.7619 (0.2223 – 2.611)	0.768
		C	8 (4%)	4 (2%)		
rs2853487 (G>A)	10,589	G	132 (59%)	86 (38%)	0.7674 (0.1375 – 4.283)	1.000
		A	4 (2%)	2 (1%)		
rs2853488 (G>A)	10,688	G	134 (60%)	86 (38%)	1.558 (0.215 – 11.275)	0.6466
		A	2 (1%)	2 (1%)		
rs193302933 (C>T)	10,664	C	136 (61%)	86 (38%)	7.890 (0.374 – 166.44)	0.1533
		T	0	2 (1%)		
rs28532881 (C>A)	10,763	C	136 (61%)	88 (39%)	*N*/A	*N*/A
		A	0 (0%)	0 (0%)		

Abbreviations: CI, confidence interval; *N*, number; *N*/A, not applicable; OR, odds ratio; SNP, single nucleotide polymorphism.

mechanism will lead to severe damage to the mitochondria and mtDNA which will affect sperm motility and eventually lead to the development of infertility in males (St John et al., 2000). Many studies have been conducted to reveal the molecular causes of idiopathic male infertility by sequencing the mtDNA and genomic DNA. Some of these studies reported a significant association between large-scale mtDNA deletion and the occurrence of male infertility in different populations (Al Zoubi et al., 2020; Karimian & Babaei, 2020).

However, there is still an incomplete molecular portrait to describe all types of male infertility.

The purpose of the current study was to investigate a possible correlation between polymorphisms in the mitochondrial genes *MT-ND3* and *MT-ND4L* and the development of male infertility. Among the identified *MT-ND3* SNPs, rs2853826 (A10398G) (*MT-ND3*) has been reported to be related to increased mitochondrial reactive oxygen species (ROS) production and leads to oxidative stress and mitochondrial DNA damage (Pezzotti et al., 2009). It has previously been reported that polymorphisms in these genes are associated with many other diseases. For instance, the rs2853826 was found to be associated with an earlier age onset of male Machado-Joseph disease, breast cancer, type 2 diabetes, gastric cancer development, oesophageal cancer, Parkinson's disease, metabolic/cardiovascular complications in HIV-infected and ART-treated individuals (Bhat et al., 2007; Chen et al., 2016; Darvishi et al., 2007; Jin et al., 2018; Pezzotti et al., 2009; Rai et al., 2012). Furthermore, rs28358278 and rs41467651 (*MT-ND3*) were associated with gastric cancer (Jin et al., 2018). In addition, a significant association between rs28358280 (A10550G) (*MT-ND4L*) and body mass index (BMI) has been identified, where the increase in G alleles is correlated with a higher BMI than if only A alleles were present (Flaquer et al., 2014).

In the current study, we scanned the polymorphisms in the *MT-ND3* and *MT-ND4L* genes of subfertile and fertile males by direct sequencing. Eight *MT-ND3* SNPs have been identified (rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954) and seven SNPs in the *MT-ND4L* gene (rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881). Missense variants include rs2853826, rs28435660 (*MT-ND3*) and rs28532881 (*MT-ND4L*). The remaining SNPs in both *MT-ND3* and *MT-ND4L* genes are synonymous variants.

The results of the current study reported a lack of significant association between the SNPs in the *MT-ND3* and *MT-ND4L* genes and male infertility. Moreover, all SNPs were tested for the Hardy–Weinberg genotype frequency test. All SNPs showed a significant deviation from HWE ($p < .0001$), indicating that the genotype distribution was not following Hardy–Weinberg and biased to one group.

The allele frequency of rs28358278 SNP (C10400T) in *MT-ND3* showed a non-significant association with male infertility ($p = .08$). This might be indicating that an increase in the number of wild-type alleles (C) or the decrease of mutant alleles (T) at C10400T in males can help to preserve male fertility while increasing the number of T alleles (or decrease C alleles) can cause male infertility. Despite the lack of significant association between the rs28358278 of the *MT-ND3* gene and the occurrence of infertility, the polymorphism was reported in the subfertile group solely. Therefore, the subfertile individuals with rs28358278 SNP need further monitoring and future studies to evaluate the possible role of this SNP in the development of male infertility or maybe other disorders. Interestingly, in a previous study, the rs28358278 polymorphism showed an association with the occurrence of gastric cancer (Jin et al., 2018). On the other hand, there was no statistically significant association between the reported SNPs and asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia and oligoteratozoospermia subgroups of subfertile and fertile males.

Further analysis in a larger and broader population is necessary and might shed more light on and elucidate the effect of *MT-ND3* and *MT-ND4L* genes SNPs in male infertility. The current findings indicated that there is no statistically significant association between *MT-ND3* and *MT-ND4L* genes SNPs and male infertility. Nevertheless, previous reports supported the role of large-scale deletions in the mtDNA to be involved in the development of male infertility. For instance, 4,977 and 7,599 bp deletions of mtDNA have been related to male infertility in different populations (Kumar & Sangeetha, 2009; Talebi et al., 2018). Other large-scale mutations such as 7436-bp and 4866-bp deletion have been described to be related to the possible association with male infertility (Chari et al., 2015; Gholinezhad et al., 2019; Karimian & Babaei, 2020). Therefore, the complete portrait of the molecular markers related to male infertility needs to be developed by screening the high-risk variants in different populations to elucidate the most common genetic mutations and variations that are related to the development of male infertility. Other genes should also be investigated for possible roles in male infertility.

In summary, we investigated a possible association between mitochondrial gene polymorphisms in *MT-ND3* and *MT-ND4L* and male infertility. However, no significant association between the *MT-ND3* and *MT-ND4L* SNPs and male infertility was found. This indicates that larger prospective studies would be helpful to probe the associations of mitochondrial gene polymorphisms and male infertility and to clarify the effect of the mitochondrial genetic variations on male infertility. In addition, the subfertile individuals who exhibited a polymorphism at rs28358278 require further monitoring and evaluation.

ACKNOWLEDGEMENTS

We would like to thank the Department of Obstetrics, Gynaecology and Reproductive Medicine, University Hospital of Saarland, for their financial support. We thank Dr. Greg Eaton for his help in the proofreading of the manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

All authors declare there is no conflict of interest in this work.

ETHICAL APPROVAL

Ethical approval for this study was obtained from the Institutional Ethics Committee of Saarland University. All the subjects provided written informed consent before participation in this research.

DATA AVAILABILITY STATEMENT

Data are available on request due to privacy/ethical restrictions.

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How to cite this article: Dahadhah FW, Saleh Jaweesh M, Al Zoubi MS, Issam Abu Alarjah M, Hammadeh ME, Amor H. Lack of association between single polymorphic variants of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase 3, and 4L (*MT-ND3* and *MT-ND4L*) and male infertility. *Andrologia*. 2021;53:e14139. https://doi.org/10.1111/and.14139