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

Fatina W. Dahadhah1 | Mayyas Saleh Jaweesh1 | Mazhar Salim Al Zoubi2 | Manal Issam Abu Alarjah2 | Mohamad Eid Hammadeh1 | Houda Amor1

1 Department of Obstetrics & Gynaecology, Saarland University, Homburg/Saar, Germany
2 Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan

Correspondence
Fatina W. Dahadhah, Department of Obstetrics & Gynaecology, Saarland University, 66421 Homburg/Saar, Germany. Email: fwdhadha11@outlook.com

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
Male factors 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 (Ambulka 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 & Cassim, 2004; Wei et al., 2012).

MTHFR is a key enzyme that plays an essential role in spermatogenesis (Cuppens & Cassim, 2004). MTHFR enzyme converts 5,10 Methylene-tetrahydrofolate (5,10 MTHF) into 5 Methyl-tetrahydrofolate (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-ND1 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.
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 1x 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).

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](image)

| 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.

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 spermatozoa 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
## 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 | 10,398          | [ACC]>[GCC]  | Thr114           | Missense variant | AA       | 37             | 21           |                   | .768    |
|           |                 |              | Al  a           |                  | AG       | 1              | 1            |                   |         |
|           |                 |              |                 |                  | GG       | 30             | 22           |                   |         |
| rs28435660| 10,353          | [GCC]>[ACC]  | Ala99Thr         | Missense variant | GG       | 61             | 40           |                   | .825    |
|           |                 |              |                 |                  | GA       | 4              | 3            |                   |         |
|           |                 |              |                 |                  | AA       | 3              | 1            |                   |         |
| rs193302927| 10,238         | [ATT]>[ATC]  | Ile60Ile         | Synonymous variant| TT       | 62             | 40           |                   | .959    |
|           |                 |              |                 |                  | TC       | 2              | 1            |                   |         |
|           |                 |              |                 |                  | CC       | 4              | 3            |                   |         |
| rs28358278| 10,400          | [ACC]>[ACT]  | Thr114Thr        | Synonymous variant| CC       | 65             | 44           |                   | .158    |
|           |                 |              |                 |                  | CT       | 0              | 0            |                   |         |
|           |                 |              |                 |                  | TT       | 3              | 0            |                   |         |
| rs41467651| 10,310          | [CTG]>[CTA]  | Leu84Leu         | Synonymous variant| TT       | 67             | 43           |                   | .754    |
|           |                 |              |                 |                  | TC       | 0              | 0            |                   |         |
|           |                 |              |                 |                  | CC       | 1              | 1            |                   |         |
| rs28358277| 10,373          | [GAG]>[GAA]  | Glu105Glu        | Synonymous variant| TT       | 66             | 44           |                   | .517    |
|           |                 |              |                 |                  | 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) |
|-----------|-----------------|--------|-------------------|----------------|--------------|------------------------|
| rs2853826 | 10,398          | A      | 75 (34%)          | 43 (19%)       | 1.287       | (0.751 – 2.203)        |
|           |                 | G      | 61 (27%)          | 45 (20%)       |             |                        |
| rs28435660| 10,353          | G      | 126 (56%)         | 83 (37%)       | 0.759       | (0.25 – 2.3)           |
|           |                 | A      | 10 (5%)           | 5 (2%)         |             |                        |
| rs193302927| 10,238         | T      | 126 (56%)         | 81 (36%)       | 1.089       | (0.398 – 2.977)        |
|           |                 | C      | 10 (5%)           | 7 (3%)         |             |                        |
| rs28358278| 10,400          | C      | 130 (58%)         | 88 (39%)       | 0.1134      | (0.006 – 2.041)        |
|           |                 | T      | 6 (3%)            | 0 (0%)         |             |                        |
| rs41467651| 10,310          | G      | 131 (59%)         | 85 (38%)       | 0.924       | (0.215 – 3.972)        |
|           |                 | A      | 5 (2%)            | 3 (1%)         |             |                        |
| rs3899188 | 10,115          | T      | 134 (60%)         | 86 (38%)       | 1.558       | (0.2153 – 11.275)      |
|           |                 | C      | 2 (1%)            | 2 (1%)         |             |                        |
| rs28358277| 10,373          | G      | 133 (60%)         | 88 (39%)       | 0.2155      | (0.0109 – 4.226)       |
|           |                 | A      | 3 (1%)            | 0 (0%)         |             |                        |
| rs28673954| 10,370          | T      | 135 (61%)         | 88 (39%)       | 0.5104      | (0.0205 – 12.679)      |
|           |                 | C      | 1 (0%)            | 0 (0%)         |             |                        |

Abbreviations: CI, confidence interval; N, number; 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).

### 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        | 0.214                       |
|              |                 | G      | 2 (1%)            | 4 (2%)         |              |                             |
| rs28358281 (G>A,C) | 10,586          | G      | 126 (56%)         | 86 (38%)       | 0.2883       | 0.132                       |
|              |                 | A      | 10 (5%)           | 2 (1%)         |              |                             |
| rs28358279 (T>A,C) | 10,463          | T      | 128 (57%)         | 84 (37%)       | 0.7619       | 0.768                       |
|              |                 | C      | 8 (4%)            | 4 (2%)         |              |                             |
| rs2853487 (G>A) | 10,589          | G      | 132 (59%)         | 86 (38%)       | 0.7674       | 1.000                       |
|              |                 | A      | 4 (2%)            | 2 (1%)         |              |                             |
| rs2853488 (G>A) | 10,688          | G      | 134 (60%)         | 86 (38%)       | 1.558        | 0.6466                      |
|              |                 | A      | 2 (1%)            | 2 (1%)         |              |                             |
| rs193302933 (C>T) | 10,664          | C      | 136 (61%)         | 88 (39%)       | 7.890        | 0.1533                      |
|              |                 | T      | 0                 | 0 (0%)         |              |                             |
| 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.
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.

ORCID
Fatina W. Dahadhah https://orcid.org/0000-0003-2673-2691
Mayyas Saleh Jaweesh https://orcid.org/0000-0002-5847-0465
REFERENCES

Al Zoubi, M. S., Al-Batayneh, K., Alsmadi, M., Rashed, M., Al-Trad, B., Al Kateeb, W., Aljabali, A., Otoum, O., Al-Talib, M., & Batıha, O. (2020). 4977-bp human mitochondrial DNA deletion is associated with asthenozoospermic infertility in Jordan. Andrologia, 52(1), e13379. https://doi.org/10.1111/and.13379

Altmäe, S., Stavreus-Evers, A., Ruiz, J. R., Laanpere, M., Syvänen, T., Yngue, A., Salumets, A., & Nilsson, T. K. (2010). Variations in folate pathway genes are associated with unexplained female infertility. Fertility and Sterility, 94(1), 130–137. https://doi.org/10.1016/j.fertnstert.2009.02.025

Ambulkar, P. S., Chuadhari, A. R., & Pal, A. K. (2016). Association of large scale 4977-bp "common" deletions in sperm mitochondrial DNA with asthenozoospermia and oligoasthenoteratozoospermia. Journal of Human Reproductive Sciences, 9(1), 35. https://doi.org/10.4103/0974-4208.176835

Bahremaneh Namagli, I., & Vaziri, H. (2017). Sperm mitochondrial DNA deletion in Iranian infertile women with asthenozoospermia. Andrologia, 49(3), e12627. https://doi.org/10.1111/and.12627

Bhat, A., Koul, A., Sharma, S., Rai, E., Bukhari, S., Dhar, M., & Bamezai, Y. (2007). The possible role of 10398A and 16189C mtDNA variants in providing susceptibility toT2DM in two North Indian populations: A replicative study. Human Genetics, 120(6), 821–826. https://doi.org/10.1007/s00439-006-0272-4

Carrell, D., De Jonge, C., & Lamb, D. (2006). The genetics of male infertility: A field of study whose time is now. Archives of Andrology, 52(4), 269–274. https://doi.org/10.1080/0148550050053603

Chari, M. G., Colagro, A. H., & Bîdîmîshkipoaur, A. (2015). A novel large-scale deletion of the mitochondrial DNA of spermatozoa of men in north Iran. International Journal of Fertility & Sterility, 8(4), 453.

Chen, S., Gan, S. R., Cai, P. P., Ni, W., Zhou, Q., Dong, Y., Wang, N., & Wu, Z.-Y. (2016). Mitochondrial NADH dehydrogenase subunit 3 polymorphism associated with an earlier age at onset in male MachadoJoseph disease patients. CNS Neuroscience & Therapeutics, 22(1), 38–42. https://doi.org/10.1111/cns.12443

Cuppens, H., & Cassiman, J. J. (2004). CFTR mutations and polymorphisms in male infertility. International Journal of Andrology, 27(5), 251–256. https://doi.org/10.1111/j.1365-2605.2004.00485.x

Dahdahah, F. W., Jawees, M. S., Al Zoubi, M. S., Aljarah, M. I. A., Hammadah, M. E., & Amor, H. (2021). Mitochondrial nicotinamide adenine dinucleotide hydride dehydrogenase (NADH) subunit 4 (MTND4) polymorphisms and their association with male infertility. Journal of Assisted Reproduction and Genetics, 1–9.

Darvishi, K., Sharma, S., Bhat, A. K., Rai, E., & Bamezai, R. (2007). Mitochondrial DNA G10398A polymorphism imparts maternal haplogroup N a risk for breast and esophageal cancer. Cancer Letters, 249(2), 249–255. https://doi.org/10.1016/j.canlet.2006.09.005

De Kretser, D., & Baker, H. (1999). Infertility in men: Recent advances and continuing controversies. The Journal of Clinical Endocrinology & Metabolism, 84(10), 3443–3450.

Dhillon, V. S., Shahid, M., & Husain, S. A. (2007). Associations of MTHFR DNM3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. MHR: Basic Science of Reproductive Medicine, 13(4), 213–222. https://doi.org/10.1093/molehr/gal118

Flaquer, A., Baumbach, C., Kriebel, J., Meitinger, T., Peters, A., Waldenberger, M., Grallert, H., & Strauch, K. (2014). Mitochondrial genetic variants identified to be associated with BMI in adults. PLoS One, 9(8), e105116. https://doi.org/10.1371/journal.pone.0105116

Gholinezhad, M., Yousefnia-pasha, Y., Hosseinzhadeh Colagar, A., Mohammad-Khorasani, M., & Bidmeshkipour, A. (2019). Comparison of large-scale deletions of the sperm mitochondrial DNA in normozoospermic and asthenoteratozoospermic men. Journal of Cellular Biochemistry, 120(2), 1958–1968. https://doi.org/10.1002/jcb.27492

Grimes, D. A., & Lopez, L. M. (2007). "Oligozoospermia","azoospermia", and other semen-analysis terminology: The need for better science. Fertility and Sterility, 88(6), 1491–1494.

Gunes, S., Arslan, M. A., Hekim, G. N. T., & Asci, R. (2016). The role of epigenetics in idiopathic male infertility. Journal of Assisted Reproduction and Genetics, 33(5), 553–569. https://doi.org/10.1007/s10816-016-0682-8

Hsia, K.-T., Millar, M. R., King, S., Selfridge, J., Redhead, N. J., Melton, D. W., & Saunders, P. T. (2003). DNA repair gene Ercc1 is essential for normal spermatogenesis and oogenesis and for functional integrity of germ cell DNA in the mouse. Development, 130(2), 369–378.

Jenkins, T., Aston, K., Hotaling, J., Shamsi, M., Simon, L., & Carrell, D. (2016). Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. Andrology, 4(5), 843–849. https://doi.org/10.1111/and.12231

Jin, E.-H., Sung, J. K., Lee, S.-I., & Hong, J. H. (2018). Mitochondrial NADH dehydrogenase subunit 3 (MTND3) polymorphisms are associated with gastric cancer susceptibility. International Journal of Medical Sciences, 15(12), 1329.

Jungwirth, A., Diemer, T., Dohle, G., Giwercman, A., Kopa, Z., Krausz, C., & Tournaye, H. (2015). Guidelines on male infertility. European Urology, 62, 324–332.

Kao, S.-H., Chao, H.-T., & Wei, Y.-H. (1998). Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. Molecular Human Reproduction, 4(7), 657–666. https://doi.org/10.1093/molehr/4.7.657

Karimian, M., & Babaei, F. (2020). Large-scale mtDNA deletions as genetic biomarkers for susceptibility to male infertility: A systematic review and meta-analysis. International Journal of Biological Macromolecules, 158, 85–93. https://doi.org/10.1016/j.ijbiomac.2020.04.216

Kumar, D. P., & Sangeetha, N. (2009). Mitochondrial DNA mutations and male infertility. Indian Journal of Human Genetics, 15(3), 93. https://doi.org/10.4103/0971-6866.60183

Lee, H.-C., Jeong, Y.-M., Lee, S. H., Cha, K. Y., Song, S.-H., Kim, N. K., & Lee, S. (2006). Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. Human Reproduction, 21(12), 3162–3170. https://doi.org/10.1093/humrep/dei280

Leonhartsberger, N., Tosun, K., Pinggara, G.-M., Mitterberger, M., Rehder, P., Gozzi, C., ... Herwig, R. (2005). 1367: Plasma homocysteine as a possible marker for male infertility: Does nutrition influence sperm quality? The Journal of Urology, 173(4S), 371.

Li, S., Li, J., Xiao, Z., Ren, A., & Jin, L. (2014). Prospective study of MTHFR genetic polymorphisms as a possible etiology of male infertility. Genetics and Molecular Research, 13(3), 6367–6374. https://doi.org/10.4238/2014.March.24.26

Mfady, D. S., Sadiq, M. F., Khabour, O. F., Fararjeh, A. S., Abu-Awad, A., & Khader, Y. (2014). Associations of variants in MTHFR and MTRR genes with male infertility in the Jordanian population. Gene, 536(1), 40–44. https://doi.org/10.1016/j.gene.2013.12.001

Mobasseri, N., Babaei, F., Karimian, M., & Nikkaz, H. (2018). Androgen receptor (AR)-CAG trinucleotide repeat length and idiopathic male infertility: A case-control trial and a meta-analysis. EXCLI Journal, 17, 1167.

Nakane, H., Hirota, S., Brooks, P. J., Nakabeppu, Y., Nakatsu, Y., Nishimune, Y., lino, A., & Tanaka, K. (2008). Impaired spermatogenesis
and elevated spontaneous tumorigenesis in xeroderma pigmentosum group A gene (Xpa)-deficient mice. *DNA Repair*, 7(12), 1938–1950. https://doi.org/10.1016/j.dnarep.2008.08.003

Navarro-Costa, P., Plancha, C. E., & Gonçalves, J. (2010). Genetic Dissection of the AZF Regions of the Human Y Chromosome: Thriller or Filler for Male (Inf)ertility? *Journal of Biomedicine and Biotechnology*, 2010, 1-18. https://doi.org/10.1155/2010/936569

Okutman, O., Rouma, M. B., Benkhalifa, M., Muller, J., & Viville, S. (2018). Genetic evaluation of patients with non-syndromic male infertility. *Journal of Assisted Reproduction and Genetics*, 35(11), 1939–1951. https://doi.org/10.1007/s10815-018-1301-7

Pezzotti, A., Kraft, P., Hankinson, S. E., Hunter, D. J., Buring, J., & Cox, D. G. (2009). The mitochondrial A10398G polymorphism, interaction with alcohol consumption, and breast cancer risk. *PLoS One*, 4(4), e5356. https://doi.org/10.1371/journal.pone.0005356

Poplinski, A., Tüttelmann, F., Kanber, D., Horsthemke, B., & Gromoll, J. (2010). Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *International Journal of Andrology*, 33(4), 642–649.

Rai, E., Sharma, S., Kaul, S., Jain, K., Matharoo, K., Bhanwer, A. S., & Bamezai, R. N. (2012). The interactive effect of SIRT1 promoter region polymorphism on type 2 diabetes susceptibility in the North Indian population. *PLoS One*, 7(11), e48621. https://doi.org/10.1371/journal.pone.0048621

St John, J. C., Sakkas, D., & Barratt, C. L. (2000). A role for mitochondrial DNA and sperm survival. *Journal of Andrology*, 21(2), 189–199.

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;00:e14139. https://doi.org/10.1111/and.14139