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# Impact of Mitochondrial Genetic Variants in *ND1*, *ND2*, *ND5* and *ND6* Genes on Sperm Motility and Intracytoplasmic Sperm Injection (ICSI) Outcomes

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vorgelegt von: Mohammad Ahmed Alsmadi

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Dekan: Prof. Dr. Med. Michael D. Menger

Berichterstatter: Prof. Dr. Mohamed Hammadeh

Prof. Dr. Eckart Meese

## Abstract

Sperm mitochondrial dysfunction generates an insufficient amount of the energy that required for the movement of sperm flagellum. In this case, sperm cannot reach the site of fertilization, thereby reducing sperm fertilization capacity, and thus usually causing most asthenozoospermic men to need assisted reproductive techniques.

The etiology of asthenozoospermia itself remains largely unknown. The aim of this current study is therefore to investigate the effect of mitochondrial genetic variants on sperm motility and intracytoplasmic sperm injection (ICSI) outcomes. Furthermore, this study aims to investigate the role of oxidative stress in mitochondrial dysfunction by measuring the protein carbonyl levels in the sperm of asthenozoospermic men.

A total of 150 couples from the ICSI cycle were enrolled in this study. One hundred-and-five of the male partners were asthenozoospermic patients, and they were subdivided into three groups, according to their percentage of sperm motility, while forty-five of the male partners were normozoospermic. Genetic variants were screened using direct Sanger's sequencing in four mitochondrial genes Nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase 6 (*ND6*), NADH dehydrogenase 5 (*ND5*), NADH dehydrogenase 2 (*ND2*) and NADH dehydrogenase 1 (*ND1*) in the sperm of the male partners.

Protein carbonyl was used as a biomarker to detect protein oxidative damage, a phenomenon which is induced by the attacks of reactive nitrogen species (RNS) in addition to reactive oxygen species (ROS). Furthermore, protein degradation causing the formation of carbonyl groups, which is derived from the direct oxidation process of a number of amino-acids' side chains such as threonine and arginine residues. Quantitation of the protein carbonyl levels in the sperm of asthenozoospermic men was performed using the enzyme-linked immunosorbent assay (ELISA) technique.

Sperm motility was significantly positively correlated with the fertilization rate ($r=0.701$, $p<0.001$). The medians of the fertilization rate among groups (G) were: (G1 (36±1.86), G2 (40±1.63), G3 (47±13.41) and the control (67±14.69) $p< 0.001$). Sperm motility was also significantly positively correlated with the embryo cleavage score ($r=0.549$, $p<0.001$). The medians of the embryo cleavage score among the groups were: (G1 (3±0.31), G2 (3.34±0.36), G3 (3.58±0.31) and the control (3.66±0.25) $p< 0.001$). Furthermore, sperm motility was significantly positively correlated with the embryo quality score ($r=0.656$, $p<0.001$). The medians of the embryo quality score among the groups were: (G1 (1.5±0.31),

G2 (1.75±0.39), G3 (2.14±0.44) and the control (2.5±0.27) p< 0.001). On the other hand, the fertilization rate was significantly positively correlated with the embryo cleavage score (r=0.590, p<0.001) and with the embryo quality score (r=0.745, p<0.001).

The protein carbonyl levels were significantly negatively correlated with the sperm motility (r=-0.894, *P*<0.001). The medians of the protein carbonyl levels among the groups were: (G1 (2.24±0.3), G2 (1.97±0.17), G3 (1.22±0.2) and the control (0.32±0.12) p< 0.001). The protein carbonyl levels were negatively correlated with the fertilization rate (r = - 0.670, *P* <0.001), the embryo cleavage score (r = - 0.511, *P* <0.001) and the embryo quality score (r = - 0.623, *P* <0.001).

The frequency of the total variants in all genes was negatively correlated with the percentage of sperm motility, *P* <0.001.Three significant variants were identified, namely: 13708 G>A (rs28359178) in *ND5*, 4216 T>C (rs1599988) in *ND1* and a novel 12506T>A in *ND5* with *P*-values: 0.006, 0.036 and 0.013, respectively. The medians of the sperm motility, the fertilization rate, the embryo quality score and the embryo cleavage score were significantly differed between men showing 4216 T>C, 12506T>A, 13708G>A and the wild type, Mann-Whitney *P*-values for the differences in the medians were < 0.05 in all of them.

In conclusion, the sperm motility was positively correlated with the ICSI outcomes, while protein carbonyl levels were negatively correlated with sperm motility and ICSI outcomes. This demonstrates that sperm motility can predict the ICSI outcomes, and the quantification of protein carbonyl can be used as a biomarker for protein oxidative damage in sperm. The frequencies of total mitochondrial variants in *ND1*, *ND2*, *ND5* and *ND6* genes were negatively correlated with the percentages of sperm motility and the ICSI outcomes. Finally, the results from this study showed three missense variants, namely, 13708 G>A, 4216 T>C and 12506T>A, which were found to be negatively correlated with sperm motility and ICSI outcomes.

## Zusammanfassung

Die mitochondriale Dysfunktion von Spermien erzeugt eine unzureichende Menge an Energie, die für die Bewegung der Spermiengeißel erforderlich ist. In diesem Fall können die Spermien den Ort der Befruchtung nicht erreichen, wodurch die Befruchtungsfähigkeit der Spermien reduziert wird und somit die meisten asthenozoospermischen Männer in der Regel assistierte Reproduktionstechniken benötigen.

Die Ätiologie der Asthenozoospermie selbst ist noch weitgehend unbekannt. Das Ziel dieser aktuellen Studie ist es daher, den Einfluss mitochondrialer genetischer Varianten auf die Spermienmotilität und die Ergebnisse der intrazytoplasmatischen Spermieninjektion (ICSI) zu untersuchen. Darüber hinaus soll in dieser Studie die Rolle von oxidativem Stress bei der mitochondrialen Dysfunktion untersucht werden, indem der Protein-Carbonyl-Gehalt in den Spermien von asthenozoospermischen Männern gemessen wird.

Insgesamt wurden 150 Paare aus dem ICSI-Zyklus in diese Studie eingeschlossen. Einhundertfünfundfünfzig der männlichen Partner waren asthenozoospermische Patienten, und sie wurden in drei Gruppen unterteilt, je nach dem Prozentsatz ihrer Spermienmotilität, während fünfundvierzig der männlichen Partner normozoospermisch waren. Genetische Varianten wurden mittels direkter Sanger-Sequenzierung in vier mitochondrialen Genen Nicotinamid-Adenin-Dinukleotid-Hydrogenase 6 (*ND6*), NADH-Dehydrogenase 5 (*ND5*), NADH-Dehydrogenase 2 (*ND2*) und NADH-Dehydrogenase 1 (*ND1*) im Sperma der männlichen Partner untersucht.

Protein-Carbonyl wurde als Biomarker verwendet, um oxidative Schäden an Proteinen nachzuweisen, ein Phänomen, das durch den Angriff reaktiver Stickstoffspezies (RNS) zusätzlich zu den reaktiven Sauerstoffspezies (ROS) induziert wird. Darüber hinaus verursacht der Proteinabbau die Bildung von Carbonylgruppen, die aus dem direkten Oxidationsprozess einer Reihe von Seitenketten von Aminosäuren wie Threonin- und Argininresten stammen. Die Quantifizierung der Protein-Carbonyl-Gehalte in den Spermien asthenozoospermischer Männer wurde mit der Enzymimmunoassay (ELISA)-Technik durchgeführt.

Die Spermienmotilität war signifikant positiv mit der Befruchtungsrate korreliert ($r=0,701$, $p<0,001$). Die Mediane der Befruchtungsrate zwischen den Gruppen (G) waren: (G1 (36±1.86), G2 (40±1.63), G3 (47±13.41) und die Kontrolle (67±14.69) $p< 0.001$). Die Spermienmotilität war auch signifikant positiv mit dem Embryo-Cleavage-Score korreliert

(r=0,549, p<0,001). Die Mediane des Embryo-Cleavage-Scores zwischen den Gruppen waren: (G1 (3±0,31), G2 (3,34±0,36), G3 (3,58±0,31) und die Kontrolle (3,66±0,25) p<0,001). Außerdem war die Spermienmotilität signifikant positiv mit dem Embryoqualitätsscore korreliert (r=0,656, p<0,001). Die Mediane des Embryoqualitätsscores zwischen den Gruppen waren: (G1 (1,5±0,31), G2 (1,75±0,39), G3 (2,14±0,44) und die Kontrolle (2,5±0,27) p<0,001). Andererseits war die Befruchtungsrate signifikant positiv korreliert mit dem Embryo-Cleavage-Score (r=0,590, p<0,001) und mit dem Embryo-Quality-Score (r=0,745, p<0,001).

Die Protein-Carbonyl-Werte waren signifikant negativ mit der Spermienmotilität korreliert (r=-0,894, p<0,001). Die Mediane der Protein-Carbonyl-Werte zwischen den Gruppen waren: (G1 (2,24±0,3), G2 (1,97±0,17), G3 (1,22±0,2) und die Kontrolle (0,32±0,12) p<0,001). Die Protein-Carbonyl-Gehalte waren negativ korreliert mit der Befruchtungsrate (r = - 0,670, p <0,001), dem Embryo-Cleavage-Score (r = - 0,511, p <0,001) und dem Embryo-Quality-Score (r = - 0,623, p <0,001).

Die Häufigkeit der Gesamtvarianten in allen Genen war negativ mit dem Prozentsatz der Spermienmotilität korreliert, P <0,001.Drei signifikante Varianten wurden identifiziert, nämlich: 13708 G>A (rs28359178) in *ND5*, 4216 T>C (rs1599988) in *ND1* und eine neue 12506T>A in *ND5* mit P-Werten: 0,006, 0,036 und 0,013, jeweils. Die Mediane der Spermienmotilität, der Befruchtungsrate, des Embryo-Qualitäts-Scores und des Embryo-Cleavage-Scores unterschieden sich signifikant zwischen Männern mit 4216 T>C, 12506T>A, 13708G>A und dem Wildtyp, Mann-Whitney P-Werte für die Unterschiede in den Medianen waren in allen Fällen < 0,05.

Zusammenfassend lässt sich sagen, dass die Spermienmotilität negativ mit den ICSI-Ergebnissen korreliert war. Die Protein-Carbonyl-Werte waren ebenfalls negativ mit der Spermienmotilität und den ICSI-Ergebnissen korreliert. Dies zeigt, dass die Spermienmotilität die ICSI-Ergebnisse vorhersagen kann, und die Quantifizierung von Protein-Carbonyl kann als Biomarker für oxidative Proteinschäden in Spermien verwendet werden. Die Häufigkeiten der gesamten mitochondrialen Varianten in den Genen *ND1*, *ND2*, *ND5* und *ND6* waren negativ mit den Prozentsätzen der Spermienmotilität und den ICSI-Ergebnissen korreliert. Schließlich zeigten die Ergebnisse dieser Studie drei Missense-Varianten, nämlich 13708 G>A, 4216 T>C und 12506T>A, die negativ mit der Spermienmotilität und den ICSI-Ergebnissen korreliert waren.

## Dedication

To my family

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## Abbreviations

| | |
|---|---|
| Intracytoplasmic sperm injection | ICSI |
| Nicotinamide adenine dinucleotide hydrogen | NADH |
| Adenosine 5′-triphosphate | ATP |
| Adenosine 5’-diphosphate | ADP |
| NADH dehydrogenase 1 | *ND1* |
| NADH dehydrogenase 2 | *ND2* |
| NADH dehydrogenase 5 | *ND5* |
| NADH dehydrogenase 6 | *ND6* |
| Reactive oxygen species | ROS |
| Reactive nitrogen species | RNS |
| Enzyme-linked immunosorbent assay | ELISA |
| Oxidative phosphorylation | OxPhos |
| Mitochondrial DNA | mtDNA |
| Urinary tract infection | UTI |
| Follicle-stimulating hormone | FSH |
| Low-density lipoprotein | LDL |
| Polyunsaturated fatty acid | PUFA |
| Malondialdehyde | MDA |
| High-Performance Liquid Chromatography | HPLC |
| Advanced glycation end-products | AGE |
| 2,4-dinitrophenylhydrazine | DNPH |
| 2,4-dinitrophenyl | DNP |
| Two-dimensional polyacrylamide gel electrophoresis | 2DE |
| Alzheimer's disease | AD |
| Hippocampus and parahippocampus gyrus | HPG |
| Superior and middle temporal gyri | SMT |
| Coronary artery disease | CAD |
| Hepatitis C virus | HCV |
| Alanine aminotransferease | ALT |
| Relative Light Units | RLUs |

| | |
|---|---|
| Polymorphonuclear leucocytes | PMNs |
| World Health Organization | WHO |
| Phorbol myristate acetate | PMA |
| Total antioxidants | TAS |
| *In vitro* fertilization | IVF |
| Pre implantation genetic screening | PGS |
| Cystic fibrosis | CF |
| Parkinson's disease | PD |
| Machado-Joseph disease | MJD |
| Progressive motility | PR |
| Non-progressive motility | NP |
| 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid | HEPES |
| Polyvinylpyrrolidone | PVP |
| National Centre of Biotechnology Information | NCBI |
| American College of Medical Genetics and Genomics | ACMG |
| Bovine serum albumin | BSA |
| Phosphate buffer saline | PBS |
| Single nucleotide polymorphisms | SNPs |
| Intrauterine insemination | IUI |
| Assisted reproductive techniques | ART |
| Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes | MELAS |
| Schizophrenia | SCZ |
| Myoclonus epilepsy and a ragged-red fibers | MERRF |
| Leigh syndrome | LS |
| cystic fibrosis | CF |
| Autism spectrum disorder | ASD |
| juvenile neuronal ceroid-lipofuscinosis | JNCL |
| Polymerase chain reaction | PCR |
| Tris-acetate-EDTA | TAE |

# 1. Introduction

Infertility is a global dilemma threatening about 15 % of couples throughout the world (Kamel RM, 2010). A couple is diagnosed as being infertile if they have failed to achieve a normal pregnancy after one year of regular intercourse without using any contraception (Gurunath *et al*., 2011). Male infertility is in fact a main contributor to infertility cases, as about 40% of the causes of infertility are due to male factors (Maduro & Lamb, 2002).

Asthenozoospermia is the main cause of human male infertility. Furthermore sperm depends on the mitochondrial oxidative phosphorylation (OxPhos) mechanism to produce the energy required for its motility (Moscatelli *et al*., 2019); mutated mitochondrial DNA (mtDNA) thus leads to energy insufficiency, which causes a reduction in sperm motility (Spiropoulos, 2002). In comparison to the oocyte, which has 150,000 mtDNA copies, the spermatozoa have only 100 mtDNA copies (Wai *et al*., 2010).

Genetic variants in mitochondrial genes are associated with many diseases (Cohen, 2019). Most of these diseases affect organs with a high energy demand, such as the brain, the skeletal muscles, the eye and the heart (Frazier *et al*., 2019). As the mtDNA is not conserved by histones or other DNA-binding proteins, it is more prone to DNA damage caused by excess levels of ROS and free radicals present in the matrix (Piomboni *et al*., 2012). Furthermore, the mtDNA repair mechanism is less efficient compared to nuclear DNA repair (Stewart & Chinnery, 2015). Together, these factors increase the mutation rate in mtDNA, compared to nuclear DNA, by 10-100 times (O'Connell *et al*., 2002).

Sperms usually produce ROS in a natural physiological process, and ROS at low levels are essential for sperm function, as they play a significant role in sperm maturation, acrosome reaction and sperm-oocyte fusion (Robert J. Aitken & Drevet, 2020). Excess levels of ROS, however, have a damaging effect, leading to nuclear DNA strand breaks (Hammadeh *et al*., 2009). Sperms are subjected to damage caused by oxidative stress, as sperm plasma membranes are composed of polyunsaturated fatty acids, where the peroxidation of these lipids results in the production of secondary products that can oxidize sperm proteins (Dalle-Donne *et al*., 2003). Leucocytes in the semen produce ROS one thousand times more than sperms, where such a situation is common during urinary tract infections (UTI) (Ochsendorf, 1999). Mitochondrial dysfunction, caused by the generation of elevated levels of ROS from

complex I, has been shown to cause lipid peroxidative damage to the sperm midpiece and to result in a reduction in sperm motility (Koppers *et al*., 2008a).

Seminal plasma is the major source of the antioxidants that protect sperm cells from oxidative damage (Ko *et al*., 2014). Indeed, a significant decrease in the reduced to oxidized glutathione ratio and total glutathione levels in seminal plasma among groups of males with varicocele and idiopathic infertility has been reported (Micheli *et al*., 2016).

It has been thought for a long time that mtDNA was inherited solely from the mother, but a recent study has challenged this idea and provided evidence of additional paternal mtDNA transmission from fathers to offspring (Luo *et al*., 2018), where paternal mtDNA is governed by a quasi-Mendelian inheritance (Annis *et al*., 2019). Furthermore, during ICSI the whole sperm is injected into the cytoplasm of the egg and, interestingly, the mtDNA is occasionally preserved, so that the offspring may indeed share their father's mitochondrial DNA (Eker *et al*., 2019).

### 1.1. Spermatogenesis

Spermatogenesis is a highly complex process involving the production of the spermatozoa in the Seminiferous tubules in the human testes (Kretser *et al*., 1998). The duration of spermatogenesis is about 64 days: radioautographs of testicular biopsy, labeled with thymidine-H3, showed that the preleptotene spermatocytes appeared within the first hour, and then after 16 days the pachytene spermatocytes were identified, then after 32 days the immature spermatids were detected, and finally the estimated time for each cycle of seminiferous epithelium was about 16 days (Heller & Clermont, 1963). It all depends on hormonal control such as the follicle-stimulating hormone (FSH) and testosterone, the sites of which sites are located in the sertoli cells (Davidoff *et al*., 1990).

Seminiferous tubules are considered as an efficient unit of the testis, and they possess most of its volume, which is about 30 ml, and also their length, when extended, may reach 250 meters for each testis (Knobil and Neill's, 2006). However, leydig cells, which are prominent cells consisting of groups of adjacent capillaries, are found between seminiferous tubules and blood vessels and are considered to be the main source of testosterone production in men (Paniagua *et al.,* 1986). It has been reported that testosterone is an essential hormone during

the process of spermatogenesis, and that its deficiency therefore impairs sperm production and leads to male infertility; it has also been found that testosterone plays a role in the stimulation of sexual behavior (Coviello et al., 2004; Nieschlag et al., 2010). The testosterone produced by the leydig cells is kept in the intratesticular compartment;, this explains why the testosterone concentrations there are 100 times higher than the serum levels (Jarow *et al*., 2005).

Sertoli cells, which possess about 20 % of seminiferous tubules, are distinctive as they are the only somatic cells there, Sertoli cells play a significant role in spermatogenesis, where they establish a blood testes barrier, which regulates the entrance of hormones, ions and nutrients into the seminiferous tubules, in addition to their function as phagocytes where they engulf the cytoplasmic residues of the germ cells that have been subjected to apoptosis (Chui *et al*., 2011). On the other hand the hormones, which are secreted by sertoli cells, such as the mullerian inhibiting substance and inhibin B, regulate the production of sperm in the male. It has been found that the levels of these hormones in the blood are higher among boys compared to their levels in adult males, while the testosterone levels were lower in the boys, compared to adult men. This would explain why males do not produce sperm during their childhood and also why the maturation of females is relatively faster than that of males (Morgan *et al*., 2011). Sertoli cells also have the receptors of another important hormone, namely, the follicle-stimulating hormone (FSH) that induces the early phases of spermatogenesis (Sofikitis *et al*., 2008).

Spermatogenesis consists of a sequence of cell divisions; this is subdivided into the following three stages: spermatocytogenesis, at this phase spermatogonia with 46 chromosomes evolve into primary spermatocytes through mitosis, and then those spermatocytes in the sertoli cells go through meiosis to generate secondary spermatocytes with 23 chromosomes; the second stage is spermatidogenesis where the production of spermatids occurs; the final stage is spermiogenesis where the spermatids form their flagella, in the upper part of which the midpiece is contained. Germ cells are well-arranged in the seminiferous tubule from basal lamina to the lumen (Figure 1.1), where spermatogonia are located after basal lamina, then primary spermatocytes come after them, followed by secondary spermatocytes, and finally spermatids and spermatozoa move in the direction of the lumen (Sharma & Agarwal, 2011).

## 1.2. Features of human sperm

Normal human sperm includes three major parts, involving the flagellum, the midpiece and the head (Figure 1.2). The normal head has an oval shape with dimensions of width to length being about 2 x 4 µm (KATZ *et al*., 1986). What is more, the head plays a significant role during fertilization as it ensures hyaluronidase is in the acrosome region; this enzyme is essential for penetration of the zona pellucida, which is sheltering the human oocyte (Abdul-Aziz *et al*., 1995). The flagellum is divided into the following three distinguishable parts: midpiece, principal piece and endpiece. The principal piece is the longest piece and is considered as a propulsive force for sperm motility, while the midpiece is packed with mitochondria, which play a significant role in the energy production that is required for sperm movement (Selmi & Soldani, 1984).

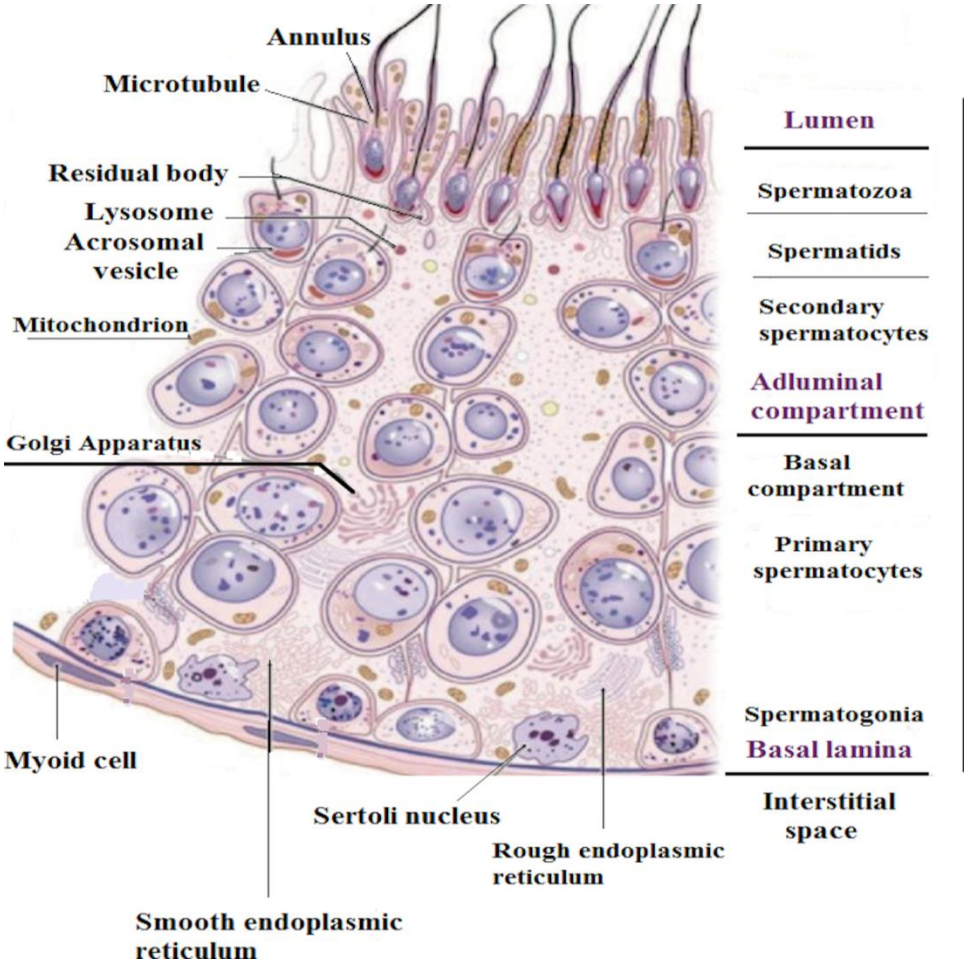


Figure 1.1: Cross-section diagram of a seminiferous tubule, where the germ cells are arranged from the basal lamina to the lumen (Adapted from Sharma & Agarwal, 2011.)

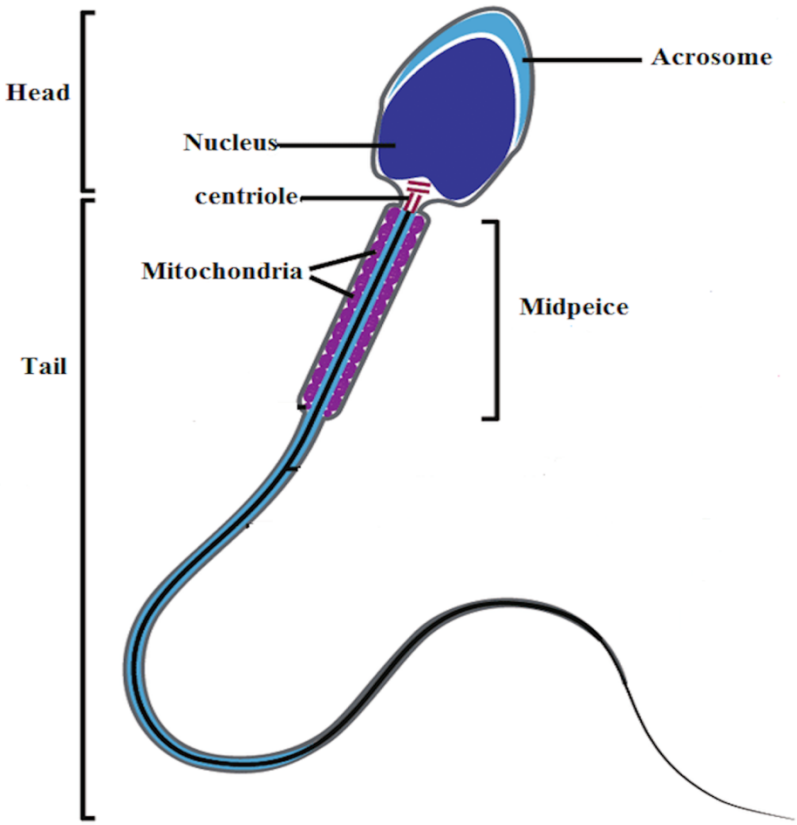


Figure 1.2: A schematic representation of a mature human sperm which contains three main sections: the tail, the midpiece and the head. Adapted from (Ding *et al*., 2015.)

### 1.3. Sperm abnormalities

There are different forms of sperm abnormalities; these include a low sperm count (oligozoospermia), weak sperm movement (asthenozoospermia) and morphological defects (teratozoospermia). These defects may appear separately in the semen, or they can appear collectively in cases such as oligo-astheno-teratozoospermia disease (Jungwirth *et al*., 2012).

According to the World Health Organization (WHO) criteria, a male is considered as oligozoospermic if he has a sperm concentration below the reference limit, which is fifteen million sperm / ml., while he is diagnosed with asthenozoospermia if he has a total sperm motility of less than 40 %, or the progressive motility of the sperm (type A and type B ) is less than 32 %. On the other hand, teratozoospermia results when abnormal forms of sperm

morphology exceed 96 %. Sperm abnormalities comprise the following three main parts: first, where the head defects involve tapered, rounded, double heads, amorphous, vacuolated and pyriform heads; second, the midpiece abnormalities involve thick, thin, cytoplasmic droplets, irregular and bent midpieces; thirdly, the tail defects involve short, coiled, broken, terminal droplets and double tails for the same sperm (WHO, 2010).

Mundy reported that asthenozoospermia may result due to deformity in the sperm tail, and it may occur because of the production of an insufficient amount of Adenosine 5′-triphosphate (ATP), which is essential for sperm movement. Also It was also found that there was shortening in the midpiece among men with reduced sperm motility on comparing them to men with normal sperm motility (Mundy *et al*., 1995).

### 1.4. Oxidative stress

Oxidative stress occurs when the generation of ROS overwhelm the production of antioxidants; this is characterized by an upset in the pro-oxidant–antioxidant equilibrium with an increment of the pro-oxidant, result in oxidative damage (Sies, 1991). The mitochondrial respiratory chain and the metabolic processes are accounted as the major sources of ROS (Brieger *et al*., 2012). Approximately 1 % of the used $O_2$ used through metabolic pathway is diverted into the oxygen radical ($O_2^-$) which functions as the precursor of most ROS. Furthermore, dismutation of $O_2^-$ by superoxide dismutases leads to the formation of $H_2O_2$ (Ott *et al*., 2007), while $OH^-$ is generated by the interaction of $H_2O_2$ and O.− 2 in a Haber-Weiss reaction, which is motivated by the decomposition of $H_2O_2$ through the Fenton reaction (Liochev & Fridovich, 2002).

The cell tries to keep a balanced state between the concentrations of ROS and antioxidants, where the antioxidants control the levels of ROS without eliminating them, because their elimination would be highly costly in terms of the required energy inside the cell, and also antioxidants cannot resist some highly reactive ROS, such as the hydroxyl radical (OH ) (Nathan, 2003). Another reason for keeping ROS in small amounts is because they are playing a significant role in the regulation of gene expression, such as nitric oxide (NO), which regulates the vascular inflammatory gene expression, involving the vascular cell adhesion gene, which is associated with the pathogenesis of atherosclerosis (Kunsch &

Medford, 1999). Moreover, NO inhibits the oxidation of low-density lipoprotein (LDL) and also inhibits the aggregation of platelets (Hogg *et al*., 1993) .

ROS with low levels are essential inside the cell because they can function as a physiological signaling messenger (Niki, 2016). It was also found that the products of lipid peroxidation – for an example Phosphatidylcholine hydroperoxide, 4-hydroxynonenal, Lysophosphatidylcholine (lysoPC) - at low levels enhance the adaptive response, and are also improving cell resistance against oxidative cell death by enhancing the expression of antioxidant (Chen *et al*., 2006). Stress can therefore be subdivided into eustress and distress, where eustress refers to a positive or good stress as it is beneficial to the cell, while distress refers to a negative or bad stress as it is harmful to the cell (Li *et al*., 2016).

Antioxidants protect cells from ROS by converting them into unreactive derivatives. They include many compounds, for an example glutathione-S-transferase, methionine sulfoxide reductase, superoxide dismutase, glutathione peroxidase, catalase, and thioredoxin reductase. They also involve several metal-binding proteins, such as ferritin and ceruloplasmin, in addition to different metabolite, cofactors and multivitamins. Moreover antioxidants include some of the metal ions, such as zinc, magnesium and manganese (Halliwell, 1996).

ROS and RNS attack the ions' channel and affect their function with several mechanisms, one of which is posttranslational changes of channel proteins involving the nitration and oxidation of the main amino-acid residues, another is by fluctuating the achievement in signaling pathways that directly affect the channel activity or channel gene expression, and the final one of which is that ROS may directly damage the $Ca^{+2}$, $K^{+1}$, $Na^{+1}$, and $Cl^{-1}$ channels (Matalon *et al*., 2003). There are several responses for the cell when it is attacked by ROS: it may proliferate or stop its division, or it may prefer to select senescence, necrosis or apoptosis (Harwell, 2007).

### 1.5. Quantification of oxidative stress

Quantifying oxidative stress needs precise quantification of unstable molecules or impaired biomolecules which involve fats, proteins and nucleic acids. There are many techniques used to measure free radicals and their chemical reaction products; one of these methods is to measure prostaglandin $F_2$-isoprostanes, which are stable, robust biomolecules measurable in

the tissues of human and bio fluids, such as blood, sweat, urine, and saliva (Morrow *et al*., 1999).

$F_2$-isoprostanes are considered the most abundant products among isoeicosanoids. Furthermore, 8-iso-Prostaglandin $F_{2\alpha}$ is a common $F_2$ -isoprostane that is secreted in human urine , and its concentration in the urine was found to be associated with cardiovascular diseases, where it was reported that patients with unstable angina had elevated levels of 8-iso-PG $F_{2\alpha}$ in their urine sample. compared to healthy individuals (Schwedhelm *et al*., 2004). The quantitation of 8-iso-PG $F_{2\alpha}$ was performed using different methods, such as the column chromatography and thin-layer chromatography techniques (Burke *et al*., 2000; Schwedhelm *et al*., 2000).

Another biomarker used to detect oxidative stress is malondialdehyde (MDA), which is produced by the peroxidation of polyunsaturated fatty acid (PUFA). MDA is a stable-reactive aldehyde and functions as a second cytotoxic messenger because it has the ability to diffuse within the cell towards far targets (Uchida, 2000). In humans MDA affects many of the physiological mechanisms for body systems, also MDA interacts with many biomolecules, like proteins and DNA, and so it is particularly beneficial as biomarker for pathogenicity (Del Rio *et al*., 2005).

Most of the assays used to detect MDA have been established upon the reaction's derivate with 2-thiobarbituric acid, where the concentration of these two molecules can be measured by a spectrophotometer (Knight *et al*., 1988). Furthermore, another method was developed to quantify MDA in breath among patients with upper respiratory tract infections, namely, the High-Performance Liquid Chromatography (HPLC) technique with fluorescence detection, where elevated levels of MDA in the breath are considered as a biomarker for inflammation (Lärstad *et al*., 2002). MDA can also be measured using a method based on HPLC with ultraviolet-visible detection (HPLC-UV/Vis). Using this technique, MDA concentrations can be assessed in the plasma; it has also been reported that MDA concentration is associated with the onset of severe renal failure (Templar *et al*., 1999).

A previous study reported that MDA levels were associated with mtDNA mutations in the sperm of asthenozoospermic patients, where they found that MDA levels were elevated among asthenozoospermic patients with 4.8, 7.2, 7.3 and 7.4- kb mtDNA deletions,

compared to normozoospermic men. In this study MDA was quantified through a thiobarbituric acid-reactive-substances assay, where the MDA levels were quantified using the molar coefficient of MDA at 534 nm. While the large scale mtDNA deletions were identified by Sanger sequencing. They explained the occurrence of mtDNA deletions among asthenozoospermic patients with high levels of MDA by the effect of ROS when attacking mtDNA, which is non-protected by histones, in addition to the presence of repeated sequences where most of the deletions take place at these sites (Abasalt *et al*., 2013).

### 1.6. Protein carbonyl as a biomarker for oxidative stress

Protein carbonyl is another biomarker used to detect protein oxidative damage, which is induced by the attacks of ROS and RNS. Furthermore protein degradation causes the generation of carbonyl groups , which are produced from the oxidation process for a number of amino-acids side chains, where the oxidation of amino acids, such as proline and lysine, generates carbonyl compounds (Stadtman & Levine, 2000).

Proteins are sensitive when they are attacked by ROS. It has also been found that there are different kinds of modifications in response to protein oxidative damage (Berlett & Stadtman, 1997). One of these modifications is glycation, which involves a reaction between free groups of amino acids (arginine and lysine) and reactive aldehyde or reducing sugar, which causes the production of advanced glycation end-products (AGE); this reaction is also known as the Maillard or browning reaction (Uribarri *et al*., 2010). AGE, which are known as glycotoxins, are considered to be a main cause of the damaging of extracellular proteins (Thornalley *et al*., 2003).

The level of change by protein oxidation depends on the type of oxidant; for example, the hydroxyl radical (OH−) is the most oxidizing ROS that attacks protein, which itself is produced by radiolysis of $H_2O$ or by Fenton reaction, the degree of modification also depends on the protein structure (Baraibar *et al*., 2013). Protein oxidative modifications affect several protein functions, such as enlarged exposure to proteolysis, and inhibit enzymatic action, where the degree of severity for enzymes' oxidative modification depends on the ratio of molecules that are altered (Oliver *et al*., 1987; Shacter, 2000).

Protein carbonyls cannot be detected directly by ultraviolet ( UV) or by spectrophotometric absorbance, and their detection requires special chemical probes to be used, such as 2,4-dinitrophenylhydrazine (DNPH), which is the most common reagent used for the quantification of protein oxidative modification (RODNEY *et al*., 1990). The chemical stability of the protein carbonyl derivatives, which include lysine, arginine, proline and threonine, support the using of protein carbonyl as a biomarker for oxidative stress. Total carbonyl groups can be detected using assays that include derivatives of the protein carbonyl group and DNPH, which in turn causes the production of a stable 2,4-dinitrophenyl (DNP). This DNP compound has the ability to absorb ultraviolet light; therefore, total protein carbonyl can be quantified using a spectrophotometer at absorption wavelength 365-375 nm. Moreover, the investigation of protein carbonyl groups is achieved with a protein immunoblot and ELISA (Fedorova *et al*., 2014).

A previous study found that protein carbonyl levels are elevated by 20 % among frozen bull sperm. compared to fresh sperm; the measurement of protein carbonyl was carried out using the Eliza technique after isolation of the total protein from the semen (Mostek *et al*., 2017). Another study reported that cryopreservation affects sperm motility after the sperm has been thawed, and enhances the production of ROS, which react with sperm DNA, lipids and proteins, leading to structural and functional damage to the sperm, the result of which being the fertilization ability of the spermatozoa will be decreased (Agarwal *et al*., 2014).

A previous study found that protein carbonyl is positively correlated with Alzheimer's disease (AD), where the levels of protein carbonyl were identified using a two-dimensional Oxyblot and Immunohistochemical analysis, followed by a applying two-dimensional polyacrylamide gel electrophoresis (2DE) with specific antibodies to determine the exact proteins that have protein carbonyl, On the other hand, the samples were collected from the tissues of the cerebellum for 12 individuals, where six individuals had been diagnosed with AD, while the rest were normal and served as a control group. According to their results, it was observed that protein carbonyl measurements were elevated among those patients diagnosed with AD compared to the normal individual patients, Moreover, they also found that protein carbonyl levels were elevated only in the hippocampus and parahippocampus gyrus (HPG) and superior and middle temporal gyri (SMT) among patients diagnosed with AD but not in the cerebellum (Aksenov *et al*., 2001).

Another study found that coronary artery disease (CAD) is associated with protein carbonyl, it was observed that the levels of protein carbonyl were elevated among patients diagnosed with CAD, compared to normal individuals, but it was also noticed that these values could not reflect the severity of artery stenosis as they did not change with an increased percentage of stenosis or with an increased number of affected arteries (Mutlu-Türkoğlu *et al*., 2005). A previous study about Eales disease, which is a rare ophthalmic disorder where the peripheral retinas of both eyes are infected and may cause vision loss, found that Eales disease was positively correlated with protein carbonyl levels and that also the content of protein carbonyl varied with the disease stages, therefore being particularly useful as a biomarker for the degree of disease severity (Rajesh *et al*., 2004). Furthermore, the hepatitis C virus (HCV) was found to be correlated with protein carbonyl, according to a previous study, the levels of protein carbonyl were elevated among patients with this disease; the study also reported an association between the alanine aminotransferease (ALT) enzyme- which is an indication of liver damage – with protein carbonyl, the protein carbonyl was positively correlated with ALT levels in the serum of HCV patients (De Maria *et al*., 1996).

### 1.7. Sperm oxidative stress

Sperms usually produce ROS in a natural physiological process, and ROS at low levels are necessary for sperm function, as they play a significant role in sperm-oocyte fusion in addition to playing a role in acrosome reaction. But excess levels of ROS have a damaging effect, leading to nuclear DNA strand breaks (Hammadeh *et al*., 2009). Homa and his colleagues determined the normal levels of ROS in the human semen to be < 24.1 Relative Light Units (RLUs) per second / $10^6$ sperm. They quantified ROS by an organic compound, known as luminol; this compound is sensitive to ROS and, when it is oxidized, leads to the formation of chemiluminescent compounds, which can emit light; then quantified these compounds with a single tube luminometer (Homa *et al*., 2015). In preparation for ICSI the semen will be treated with sperm washing media and the semen plasma will be discarded A previous study reported that ROS quantification by luminol-mediated chemiluminescence in raw semen provided a more accurate evaluation of the real level of ROS than the quantification in washed semen (Fingerova *et al*., 2009) .

ROS, present in seminal plasma, are produced from a several of exogenous and endogenous sources (Koppers *et al*., 2008a). The effect of ROS on sperm also depends on their characteristics. Some ROS, such as hydrogen peroxide, can pass through the plasma membrane of sperm, while many of them, like superoxide and hydroxyl radical, are impermeable to the plasma membrane As a result, these impermeable ROS oxidize PUFA in the plasma membrane and create a state of lipid peroxidation, which impairs both sperm motility and morphology (Henkel *et al*., 2005).

The human ejaculate is composed of different types of cells, including round cells, which originate during spermatogenesis, contaminating white blood cells and epithelial cells (Agarwal *et al*., 2012). Furthermore, leucocytes in the semen are producing ROS one thousand times more than sperms; such a situation is common during urinary tract infections (UTI) (Pasqualotto *et al*., 2000). On the other hand, polymorphonuclear leucocytes (PMNs), specifically neutrophils, are responsible for the production of 50–60% of ROS that are produced by leucocytes in the semen (König *et al*., 2006). Leukocytospermia, which is defined by the WHO as being a state in which the levels of white blood cells are more than one million leukocytes per milliliter (Gdoura *et al*., 2008). A previous study concluded that Leukocytospermia is negatively correlated with spermatozoa movement and morphology, but was positively correlated with ROS levels (Athayde *et al*., 2007).

Another study reported that ROS, generated by PMN, is inversely correlated with sperm motility and morphology; the researchers in this study they assessed the effect of ROS that are generated from PMN by incubating the sperm in three different conditions for four days: the first group of sperms was incubated alone, and this was the control group, while the second group of sperms was incubated with phorbol myristate acetate (PMA) alone and finally the third group of sperms was incubated with both PMA and PMN. According to the results from this study, the motility of the sperms treated with both PMA and PMN (group 3) was more reduced compared to that of groups two and three, What is more, the abnormalities in sperm morphology increased by 10 % in group 3 compared to groups one and two, where the most of the changes in sperm morphology were described as sperm swelling, or a defect in acrosomal integrity in addition to deficiency of the sperm- plasma membrane (Shi *et al*., 2012).

Sperms are subjected to the damage produced by the effect of oxidative stress, because the plasma membrane of the sperm is composed of polyunsaturated fats, where the oxidative degradation of lipids result in the formation of products that have the ability to oxidize the proteins in sperm (Dalle-Donne *et al*., 2003). Another study found that mitochondrial dysfunction in sperm produced an insufficient amount of energy, and was also responsible for the generation of higher levels of ROS, which negatively affected the mitochondrial function, and can even cause a breakdown for mtDNA ( St. John *et al*., 2000).

Another study reported that abnormal sperm morphology is associated with elevated levels of ROS production in the semen (Aitken *et al*., 2003). However, a previous study reported that there was no association between the measurements of total antioxidants (TAS) and ROS among the sub-fertile patients in the ICSI (group 1) and the measurements of ROS and TAS among sub-fertile patients in the *in vitro* fertilization (IVF) technique (group 2), although the semen parameters were better among group two, compared to group one. Furthermore there was no difference in the fertilization rate among the two groups. Therefore they concluded that TAS and ROS were not useful in the speculation of IVF or ICSI outcomes (Hammadeh *et al*., 2003).

A previous study concluded that life-style and several environmental conditions play a key role in the elevation of ROS production inside the cells (Wong, E. W. & Cheng, 2011). Another study also reported that ROS levels are correlated with smoking, as they measured MDA levels in the semen plasma of smoker and non-smoker infertile men by TBARS assay, by means of which they found that the MDA levels were higher among smoker-infertile patients compared to non-smoker infertile patients with a significant difference ($P$-value < 0.05) (Hammadeh *et al*., 2010).

### 1.8. The mitochondrion

Mitochondrion was first defined by Von Kölliker in 1856, during his study on muscle tissue. Mitochondria were known as “sacrosomes” in the beginning, but then they were given the name ‘mitochondria’ by Benda in 1898, who detected them in the process of spermatozoa development. The name mitochondria is of Greek origin and consists of two words, *mitos* (thread) and *chondros* (granule) (van der Giezen, 2011). Scientists believed that the origin of

mitochondria came from a symbiotic relationship between bacteria that occupied the inside of a eukaryotic cell over 1,500 million years ago (Martin, 2007).

### 1.8.1. Structure of mitochondria

Mitochondria are double membrane organelles as they have a permeable outer membrane in addition to an inner membrane, which consists of impermeable finger-like projections, called cristae (Mannella, 2006). The inner membrane is surrounding the matrix space, which contains the enzymes required for the citrate cycle (Figure 1.3); the inner membrane is comprised of the four complexes of the electron transport chain (Picard *et al*., 2011). Mitochondria have a uniform thickness from the edge to the base with a thickness ranging between 18 to 20 mμ for most of the organelles in the cell, while the ridges of mitochondria in spermatids were found to be thicker, and this thickness reaches up to 30 mμ at the beginning of the ridge and up to 75 mμ at the end (Palade, 1952).

### 1.8.2. Function of mitochondria

Mitochondria is considered as the power-house of the cell. Moreover, the production of energy by glycolysis alone provides the cell with small amounts of ATP, compared to the energy produced via oxidative phosphorylation in the mitochondria, which is considered to be the major source for ATP generating inside the cell (Saraste, 1999). The mitochondrial load in any cell depends on its required energy, which ranges widely according to the function and activity of the cell. Where they are abundant with large numbers in those cells, it requires large quantities of energy, as in the oocyte where their number may reaches 100,000 mitochondria (Scheffler, 2011).

The inner mitochondrial membrane contains DNA coding for 13 proteins. These proteins are an essential part of oxidative phosphorylation, which is considered to be the functional unit of mitochondria, where by this process a lot of energy is produced in addition to free radicals being produced. Furthermore, the electron transport chain in OxPhos involves five complexes, which they are ATP synthase, Cytochrome c oxidase, Ubiquinol-Cytochrome c oxidoreductase, Succinate dehydrogenase and NADH-Ubiquinone oxidoreductase (Schäfer *et al*., 2006). Most of the electrons transported in ETC are obtained by NADH and pass through

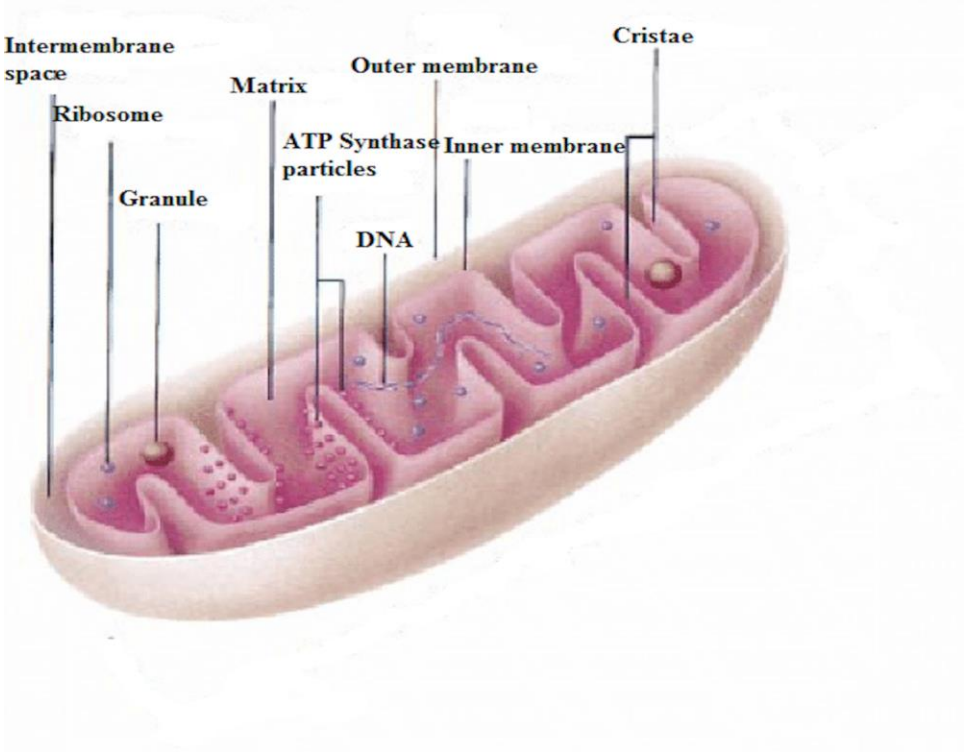


Figure 1.3: Structure of human mitochondria, the outer membrane, the inner membrane, the matrix and the cristae are indicated. Adapted from (Perkins & Frey, 2000).

the cycle by complex I. Furthermore Succinate-Q oxidoreductase transfers electrons into the ubiquinone. Then via ETS these electrons are transported to oxygen, where the pumped protons through the inner membrane of the mitochondria are responsible for generating a gradient potential. Finally, ATP synthase utilizes the stored energy, created by the proton gradient, to motivate the synthesis of ATP from adenosine 5'-diphosphate (ADP) and phosphate (Hüttemann *et al*., 2007).

In addition to OxPhos, several biochemical reactions occur in the mitochondria of aerobic eukaryotes, such as Pyruvate decarboxylation, the tricarboxylic acid cycle and ETS. Mitochondria also have an essential function in haem and iron–sulphur (FeS) biosynthesis. Furthermore, mitochondria play a significant role in the processes of calcium homeostasis and apoptosis in the sperm (Sun *et al*., 2017). Another important function for mitochondria is i in the regulation of cytosolic calcium and fatty-acids oxidation (van der Giezen & Tovar, 2005).

The mitochondria in sperm have unique characteristics that are compatible with the sperm function in terms of capacitation, hyperactivation, acrosome reaction and fertilization (Moraes & Meyers, 2018). Mitochondrial sperm also reflect their ability to perform fertilization, whereas mitochondrial dysfunction leads to the production of an insufficient amount of energy (Darr *et al*., 2016). Mitochondria may be damaged during cryopreservation, one of the common methods used to store sperm in assisted reproductive techniques (ART). The freezing-thawing processes in cryopreservation negatively affect sperm motility (Ball, 2008; González-Fernández *et al*., 2012).

The MtDNA copy number was found to affect the implantation rate during ICSI. Diez-Juan *et al*. proved that there is a strong correlation between the implantation rate and the mtDNA copy number. This study involved women being treated with the ICSI technique. Firstly, the woman followed a program of ovarian stimulation to generate more follicles, then, after follicle maturation, the oocytes were retrieved and after that, the oocytes were injected by ICSI. Furthermore, they using a hatching laser for embryos at the cleavage or blastocyst stage for genetic screening. In their research they scanned the mtDNA copy number by Pre implantation genetic screening (PGS) for euploid embryos, which they transferred at day-3 or at blastocyst stage. They also identified the mitochondrial score (Ms), which reflected the amount of mitochondrial copy number per cell. According to their results, they found that embryos with successful implantation have a lower mtDNA content; this result was valid for embryo transfer at day-3 or day-5. On the other hand, embryos with poor implantation were estimated to have a high mtDNA copy number. They explained this correlation by the requirements of the embryo for the energy in the early stage of development which they supplied through mitochondria. So they believed that the mtDNA copy number could be used for the prediction of successful implantation (Diez-Juan *et al*., 2015).

### 1.8.3. Mitochondrial genome

On 1963 Margit Nass and Sylvan Nass revealed the mtDNA in chick embryos by electron microscopy as DNA threads inside the mitochondria (NASS & NASS, 1963). Then mtDNA was extracted from mitochondria of hepatocytes and myocardiocytes in chick embryo (Rabinowitz *et al.*, 1965). Anderson *et al.* completed the sequencing of human mtDNA in 1981. Human mtDNA is composed of two closed strands with a circular shape, where the outer ring, which is forming the heavy chain, is surrounding the inner ring, which is forming

the light chain. MtDNA is approximately 16.5 kbp in length (Figure 1.4), the genes in mtDNA are encoding for 16 S and 12S, also they are encoding for twenty two transfer RNAs, in addition thirteen polypeptides (Anderson *et al*.,1981).

Exons in MtDNA are more susceptible to damage and mutations from the effect of free radicals rather than from exons of DNA in the nucleus; this refers to the absence of histones in mtDNA (Shamsi *et al*., 2008). The translation of genetic code in mtDNA differs from the universal genetic code (Anderson *et al*.,1981). Analysis of the human mitochondrial sequence of the cytochrome oxidase subunit II gene reveals that the AUA codon in human mtDNA encodes for methionine instead of isoleucine, AGG and AGA also encode for stop codons instead of arginine, and furthermore UGA in the universal genetic code encodes for stop codon while it encodes for tryptophan in the mammalian mitochondrial genetic code (Barrell *et al*., 1980).

In the mammalian mitochondrial genetic code the same amino-acids are coded by more than one codon. These codons in this case are known as synonymous codons and they are used in different frequencies (Grantham *et al*., 1981). One of the rules that govern codon usage is that abundant tRNA are usually utilized more than uncommon tRNAs (Bulmer, 1991). Moreover, codons identified by the same tRNA are predicted to form a natural Watson-Crick pair at the wobble position with the anticodon (Grosjean & Fiers, 1982). Another feature for codon usage is that strong bias is present in highly expressed genes because of the efficiency of translation or its speed selection (Gold, 1988; Tomich *et al*., 1989).

### 1.8.4. The pattern of inheritance in human mtDNA

Up until a few years ago scientists believed that human mtDNA was only maternally inherited and that paternal mtDNA disappeared after the cleave stage (Cummins *et al*., 1998). On October 2018 Luo and her colleagues provided evidence for paternal mtDNA inheritance. According to Lao *et al*., (2018), parental mtDNA can transmitted from father to offspring. Their study involved three separate families with an elevated level of mtDNA heteroplasmy and including 17 individuals (Luo *et al*., 2018).

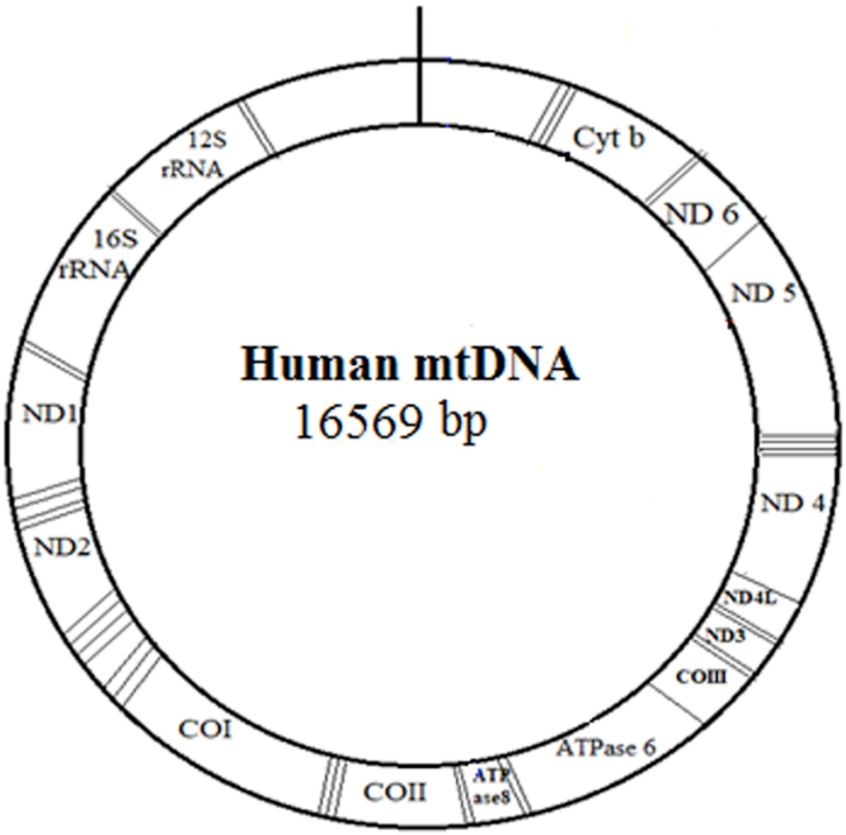


Figure 1.4: A schematic diagram for human mtDNA, with two strands, the heavy and light chains. Adapted from (L. Shen *et al*., 2010)

Among family (A) a four-year-old boy was diagnosed with hypotonia, fatigue and myalgia. The boy was assumed to have a mitochondrial illness. On the other hand, other individuals displayed varying clinical symptoms but without supposing to have mitochondrial diseases. The boy had a twin sister, who had speech difficulties although she was normal, but his grandfather was diagnosed as having had a heart attack but he was in a good physical condition as far as other situations were concerned. The older sister was not complaining about any disease. while his mother had been diagnosed with neuropathy. The mtDNA was wholly sequenced for all the family members with assumed mitochondrial diseases. The results of the sequencing indicated that the boy and his sisters had a similar pattern of

inheritance for mtDNA heteroplasmy. Maternal inheritance was responsible for 60% of the heteroplasmy, while 40% of the heteroplasmy resulted from paternal inheritance. In this study they confirmed their results by including two other separate families. The second family B involved a 35-year-–old man who had been diagnosed with developmental problems, DM and supraventricular tachycardia SVT, while the third family included a 46-year-old woman, Who was diagnosed with muscle fatigue, and recurrent fever and also having a thin skin with severe pain in her body. These findings illustrated a pattern of biparental inheritance for human mtDNA. The researchers double-checked their results by performing DNA sequencing in different laboratories (Luo *et al*., 2018).

A recent study by Annis *et al.* (2019) refuted the model of inheritance suggested by Luo *et al*. 2018 with the agreement of biparental mtDNA transmission to the offspring. They presented another model enables the alterations in the count and size of mitochondria for each cell, together with the rate of cell divisions. This model was able to give an explanation for the findings of Luo *et al.* (2018). They observed that the mtDNA, which was transmitted from the father, was considered a significant parameter in their numerical model, with variations in the number of copies for biparental mtDNA in the zygotes. Many factors are involved in this variation as if the sperm is injected wholly by ICSI, so that all the mitochondria in the midpiece enter the cytoplasm of the oocyte in this case On the other hand, during normal fertilization it is hard to conserve paternal mtDNA as the sperm flagellum with its midpiece, which contains the mitochondria, does not enter the cytoplasm of the oocyte (Annis *et al*., 2019).

Annis reported that parental mtDNA is governed by a quasi-Mendelian inheritance. This means the new offspring has pure mosaicism for embryo cell distribution. Moreover, the principle of segregation for the gatekeeper gene, as proposed by Lou, disagrees with the fundamental biology of the germline process, as the cytokinesis is still imperfect through spermatogenesis at the meiosis stages.. Furthermore, they concluded that the pattern of mtDNA inheritance is reproducible as the transmission of genes occurred among the same family individuals more than once (Annis *et al*., 2019).

## 1.9. Mitochondrial diseases

Mitochondrial defects affect specifically the tissues, the functions of which require large amounts of ATP, such as the skeletal muscle, the myocardial cells, the central nervous system and the eye, all areas where neurological diseases were found to be the most frequent among mitochondrial disorders (Scaglia, 2010).

A previous study reported that juvenile neuronal ceroid-lipofuscinosis (JNCL) disease is correlated with mitochondrial dysfunction (Boriack *et al*., 1995) . This disease is known as the most recognized neurodegenerative sickness among children (Dyken & Krawiecki, 1983). A patient who is diagnosed with this disease is suffering from vision loss, delayed motor development, and seizures and in the late stages of this disease it can lead to death . Mitochondrial impairment in this case is caused by gentamycin supplementation; it is conjugated with a great elevation in lysosomal storage material in this disease (Kohlschütter *et al*., 1993).

Other studies found that mitochondrial variants 4216 in the *ND1*gene and 13708 in the *ND5* gene are associated with Alzheimer's and Parkinson's diseases. These are neurodegenerative disorders related to a progressive loss of memory and movement problems. These studies identified two novel variants, namely, 1709 G>A and 15851 A>G in the 16s rRNA and *CytB* genes, where the frequency of these mitochondrial variants was higher among Parkinson's patients, compared to individuals unaffected with this disease. The occurrence of two other mitochondrial variants at positions 709 (rRNA 12S) and 15928 (tRNA Thr) was higher among patients diagnosed with Alzheimer's disease, compared to control (Brown *et al*., 1996; Chagnon *et al*., 1999)

Parkinson's disease (PD) was found to be correlated with a mutation in PTEN putative kinase-1 (*PINK1*) (Finsterer, 2011). This gene is broadly expressed in neurons, and it is responsible for the preservation of the mitochondrial chain; it is also considered to be an antagonist factor for mitochondrial stress (Rakovic *et al*., 2010). Moreover, PD was found to be correlated with mutation in the *PARK2* gene encodes for parkin that is enrolled from the cell cytoplasm to spoil the mitochondrion, and it then activates autophagosomes to engulf dysfunctional mitochondria (Suen *et al*., 2010). Another gene that was found to be associated with Parkinson's disease is the *DJ1* gene, which is considered as an important

factor in the existence of many types of nerve cells; it has also been found that it protects dopaminergic neurons from toxicity (Foti *et al*., 2010). A defect in the *DJ1* gene results in the accumulation of ROS and may cause the death of neurons (Irrcher *et al*., 2010). Another pathogenic mutation in the α-Synuclein gene was also associated with PD in Italy (Polymeropoulos *et al.*, 1997). The Alpha-Synuclein gene is considered to be an antiapoptotic factor as it protects the cell from death (Bayir *et al.*, 2009). Finally, a mutation in the *LRRK2* gene was correlated with PD as it affects kinase function, and the elevated activity of LRRK2 also enhances mitochondrion-dependent apoptosis, which causes death for neurons (Winklhofer & Haass, 2010). The mutations in these five genes were found to be correlated with the severity of PD (Satake *et al*., 2009; Simón-Sánchez *et al*., 2009). Another study also concluded that the frequencies of heterozygous variants among patients with PD were high in the *PARK2* and *LRRK2* genes, compared to normal individuals (Nuytemans *et al*., 2010).

Another study reported that the point mutation A3243G is correlated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) disease; the patients with this disease suffer from seizures, migraine-like headaches, lactic acidosis, episodic vomiting, hemianopia and cortical blindness (King *et al*., 1992). Furthermore, another study found an interesting correlation between the gender and stroke-like episodes in 3243A>G carriers; they found that 65% (33/51) of males with 3243A>G were diagnosed with MELAS, while only 35 % ( 18/51) of females with 3243A>G were diagnosed with this disease; also 43 % ( 32/75) of the males showing 3243A>G were not diagnosed as having MELAS (Mancuso *et al*., 2014).

A previous study also found that the A8344G mutation in in the *tRNALys* gene in the skeletal muscle is correlated with myoclonus epilepsy and a ragged-red fibers (MERRF) disease, which is described as having ataxia, seizures, myoclonus epilepsy, dementia, ragged-red fibers and hearing loss (Boulet et al., 1992; Grossman & Shoubridge, 1996). Furthermore, another study reported that the A8344G in the *tRNALys* gene is associated with MERRF, and the frequency of this variant among patients was 33.3 % (Choi *et al*., 2010), while in another study the frequency of this variant among patients diagnosed with MERRF was much higher, namely 80 % (Shoffner *et al*., 1996).

Autism spectrum disorder (ASD) was associated with the A3243G mtDNA mutation; this mutation was mentioned previously as it was correlated with MELAS (Sue *et al*., 1999). The G8363A mutation in the mtDNA *tRNALys* gene was also correlated with this disease; in this case a child with autism showed 82 % and 86 % of the mutated mitochondrial transfer RNA Lys gene in her blood and her muscles respectively (William *et al.*, 2000). Mitochondrial dysfunction leads to the accumulation of lactic acid and pyruvate, and this imbalance causes excess fatty acid accumulation. Elevated levels of lactate in the plasma are thus biomarkers for mitochondrial impairment (Haas, 2010). Furthermore, another study supports the role of mitochondrial dysfunction in autism as they found the levels of plasma lactate to be elevated in 20% of ASD (Oliveira & Clinic, 2005).

Another disease, found to be correlated with mitochondria dysfunction, is diffuse leukoencephalopathy. A case study of a male child revealed the symptoms of this disease, namely, difficulty in sucking the milk, short bouts of crying, hypotonia, and a reduction in social smiling. His head was also not correctly aligned but instead was posteriorly behind the trunk as a result of poor neck control. He also showed poor responses to visual contact and voices . He exhibited a T9176C mutation in the mitochondrial ATP synthetase 6 gene; a biopsy from his muscle also indicated mild myopathic change. This child died at the age of seven months as a result of his lungs being arrested (Hung & Wang, 2006).

Leigh syndrome (LS) is correlated with a deficiency in mitochondrial energy production. LS is a severe neurodegenerative disease with distinctive spongy degeneration and microscopic vascular proliferation being found in many cases primarily in the brain stem, the basal ganglia, the thalamus and the central nervous system (Pronicki *et al*., 2008). In a previous study it was found that mutations in the nuclear gene (*SURF1*) lead to a dysfunction in the respiratory chain complex IV, which is associated with LS (Yüksel *et al*., 2006). According to a previous study, 8593 A>G in the mtDNA *ATPase* gene is associated with LS; this was identified in patients while it was absent in the control group (Chalmers *et al*., 1997).

Another disease that is correlated with a mitochondrial defect is schizophrenia (SCZ), which is a severe mental disorder characterized by false beliefs that conflict with reality, hallucinations and disorganized thinking (Sklar, 2009).The mitochondrial variant 12027T>C in the *ND4* gene was associated with schizophrenia, where the frequency of this variant among male patients, diagnosed with schizophrenia, was 47% comparing to being only 18%

among uninfected men (Marchbanks *et al*., 2003). Another study also identified three variants, namely, 7750C>A in the *COII* gene, 8857G>A in the *ATPase6* gene and 12096T>A in the *ND4* gene, in the mtDNA of SCZ patients, while these particular variants were absent in 95 control individuals (Folch *et al*., 2006). According to another study, two mitochondrial variants, 12403 C>T and 12950 A>C in the *ND5* gene, were found to be associated with SCZ with more frequency for these variants were more often found in patients diagnosed with the disease, compared to normal individuals (Scaglia, 2010).

According to a previous study, Huntington's disease is correlated with an expanding of the CAG tandem repeat in the *IT-15* gene; this gene is encoding huntingtin protein, which plays an essential role in embryonic development (MacDonald *et al.*, 1993). In addition, there is a negative correlation between CAG tandem repeat and energy production, where CAG repeats affects negatively on the activity of complexes II/III in the mtDNA, which then leads to the production of insufficient amounts of energy (Turner *et al*., 2007).

Another case study was of a woman diagnosed with cystic fibrosis (CF); her clinical history revealed that she had been suffering from asthma throughout her childhood, and that her arms and legs twitched at 5-years-old; she also had poor hand-writing; later, at the age of 17, she was admitted to hospital due to movement disorder in her body parts, susceptibility to fatigue, swallowing difficulties and irregular emotional responses. After performing a molecular analysis for this patient, the hospital doctors concluded that CF is associated with two mutations, G15995A *in tRNApro* and A8326G in *tRNALys* with a heteroplasmic pattern. They identified these two mutations in this patient, while these same mutations were not found in 130 normal individuals (Gropman, 2002). It was also reported that mitochondrial disorder is common among patients with CF, where there is hyperactivity in the electron transport chain in cells from patients with CF; these infected cells were also found to consume more oxygen, compared to those cells from normal individuals (Shapiro, 1989).

McArdle's disease results from a deficiency in phosphorylase; this enzyme is necessary to break down glycogen, and therefore without phosphorylase glycogen cannot be utilized to generate energy during body exercise. Patients with this disease suffer from muscle fatigue, severe muscle pain and cramps (Lewis & Haller, 1986). A previous study reported that McArdle's disease is correlated with mitochondrial dysfunction; this study found that the concentration of muscle intracellular adenosine 5'-diphosphate (ADP) was higher among

patients with McArdle's compared to uninfected individuals at the beginning of exercise; furthermore they also noticed that after exercise the half-time for intracellular ADP among patients with McArdle's was slower than the half-time for intracellular ADP among the control group (Stefano *et al*., 1996).

A previous study reported another neurological disorder correlated with mitochondrial dysfunction; it is known as Machado-Joseph disease (MJD) . It is in fact one of thirty identified forms of ataxia, where the patients with this disease suffer from imbalance in their legs and upper limbs, trouble in talking and swallowing, ocular motor impairment, uncontrolled muscle contractions that lead to the twisting of body and limbs, and a short mean age of about 40.5 years. Unfortunately there is no treatment available to delay the progression of this particular disease (Lima, 1998; Y. Yu *et al*., 2009). It has also been reported that MJD/spinocerebellar ataxia type 3 (SCA3) is correlated with mitochondrial disorder, for it was found that there is a decrease in the mtDNA copy number among SCA3 patients compared to a control group The occurrence of 4977 bp deletion was also higher among individuals with this disease compared to the normal control (Padiath *et al*., 2005).

### 1.10. Sperm mtDNA mutations and infertility

There are different types of mutations, such as single nucleotide polymorphisms or large deletions, that affect sperm mtDNA. One of these deletions is the 4977-bp deletion, which is considered as the most common among mtDNA deletions (Guo *et al*., 2017). This deletion involves the removal of seven genes and five transfer RNAs in the mtDNA (Figure 1.5), which are located between 8483 bp and 13459 bp, where the site of this deletion is between two 13-bp repeated sequences (5'-ACCTCCCTCACCA-3') (Tanaka *et al*., 1989). It was reported that the 4977-bp sperm mtDNA deletion is associated with asthenozoospermia, where it was found that the percentage of this deletion in the mtDNA of sperm with normal motility was higher, compared to sperm with weak motility (Kao *et al*., 1998). A recent study in Jordan found an association between this deletion and asthenozoospermia, where the ratio of the 4977-bp deletion among patients diagnosed with asthenozoospermia was higher, compared to normozoospermic men (Al Zoubi *et al.*, 2020). However, another study did not find any significant difference between the percentage of this deletion among different groups of sperm motility (Ieremiadou & Rodakis, 2009).

Another two mtDNA deletions, namely 7599 and 7345-bp deletions ,were associated with poor semen quality. The mtDNA 7599-bp deletion is located between 8642 and 16243-bp and characterized by the presence of 7 nucleotides' direct repeats (5'-CATCAAC-3') on both

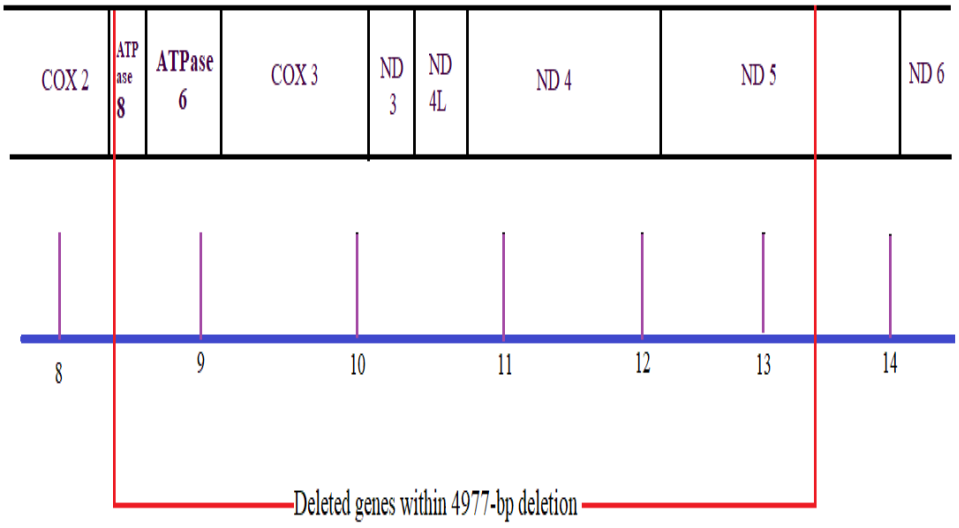


Figure 1.5: Deleted genes in 4977-bp mtDNA deletion. Adapted from (Ambulkar *et al*., 2016).

sides, whereas the 7345-bp mtDNA deletion lies between 9009 and 16354-bp (Figure 1.6). These deletions involved the removal of several mitochondrial genes; these were *ATPase8* (lost with 7599-bp only), *ATPase 6*, Cytochrome Oxidase *(COX) III*, Cytochrome b, NADH Dehydrogenase (*ND*) 3, 4, 4L, 5, and 6. These deletions also included the losing of 8 tRNA genes (See Figure 6) (Kao *et al*., 1998). The deleted genes from the mtDNA deletions perform a key role in oxidative phosphorylation inside the mitochondria; therefore removing them from the mtDNA will reduce the obtained energy, which in turn has a negatively effect on sperm-flagellum movement and leads to asthenozoospermia (Talebi *et al*., 2018a).

A previous study reported that sperm motility was negatively correlated with mtDNA deletions. According to their results, the mtDNA deletions were identified among immotile sperm, while sperm with normal motility did not possess these deletions (Carra *et al*., 2004). Another study found that the occurrence of 7436-bp sperm mtDNA deletion among asthenozoospermic men was higher compared to normozoospermic men; it was therefore

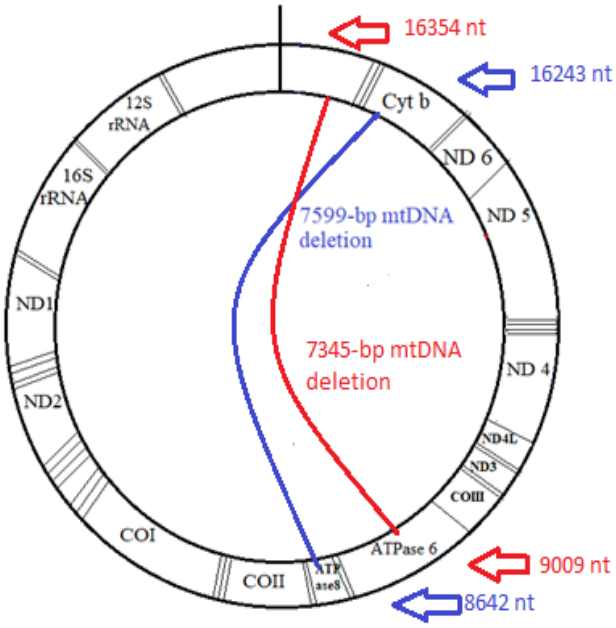


Figure 1.6: Human mtDNA with the sites of the 7345-bp and 7599-bp deletions. Red arrows indicate the site of the 7345-bp mtDNA deletion, which is located between nucleotides 9009 and 16354 bp. The blue arrows indicate the site of the 7599-bp mtDNA deletion, which is located between nucleotides 8642 and 16243 bp. Adapted from (Rajender et al., 2010).

suggested that this deletion be used as an indicator for reduced sperm motility (Ambulkar *et al*., 2016). However, in contrast, a previous study demonstrated that mtDNA deletions did not affect sperm motility, as they did not register a significant difference between the occurrence of mtDNA deletions in sperm with poor quality and in sperm with good quality (S. John *et al*., 2001). Another study also identified mtDNA deletions in the sperm of asthenozoospermic patients and normozoospermic men without any significant difference; they therefore then decided to ignore the role of these deletions in male infertility (Cummins *et al*., 1998).

A previous study reported that the existence of that large scale mtDNA deletion was associated with asthenozoospermia because it was responsible for losing some of the mitochondrial genes that are responsible for mitochondrial respiration, which provides sperm with the required energy for their motility and this consequently affects male fertility (Colagar & Karimi, 2014).

Another study in the USA of 119 infertile males involved the IVF technique and their sperms being injected by ICSI, after screening for mtDNA deletion of the semen samples by polymerase chain reactions (PCR), and assessing the embryos at day-3 and day 5 of the transfer. This reported that there was indeed a correlation between mtDNA deletions and low fertilization rate, and that the presence of mtDNA deletion also had a negative effect on the blastocyst grading at day-5. According to their research, the fertilization rate outcome was the most affected among the ICSI outcomes in terms of mtDNA deletion occurrence; they explained this by saying that fertilization very much depended on the quality of the sperm. Although their study was the first piece of research to correlate between mtDNA deletions and embryo grading, they nevertheless still found some limitations according to their results: firstly. the number of samples was from only 119 donors, and secondly, this kind of research needed to be a more international study or at least to involve a more widespread population (Wu *et al.*, 2019).

Diez-Juan proved that there is a strong correlation between the implantation rate and the mtDNA copy number in euploid embryos. This study involved women being treated with the IVF technique. Firstly, the woman followed a program of ovarian stimulation to generate more follicles, and then, after the size of the follicles was suitable, oocyte retrieval and ICSI took place, By using a hatching laser for embryos at the 8-cell or blastocyst stage for genetic screening. In their research they scanned the mtDNA copy number by PGS for euploid embryos, which they then transferred at day-3 or a the blastocyst stage. Furthermore, the mitochondrial score (Ms), which represents the ratio of mtDNA to the DNA in the nucleus, reflected the amount of mitochondrial copy numbers per cell. They found that embryos with successful implantation had a lower mtDNA content; this result was valid for the embryo transfer at either the cleavage or the blastocyst stages. On the other hand, embryos with poor implantation were estimated to have a high mtDNA copy number. They explained this correlation by the requirements of the embryo for energy in the early stage of development; this energy is supplied by mitochondria. So the researchers believed that the mtDNA copy number could be reliably used for the prediction of a successful implantation (Diez-Juan *et al.*, 2015).

According to a previous study in China involving 246 cases of fresh IVF cycle, where couples were attending ICSI because of the female factor or idiopathic infertility. Semen

samples were collected, and genomic DNA was isolated, followed by PCR and DNA sequencing. The detection of single nucleotide polymorphisms (SNPs) was identified using the Human Mitochondrial Genome Polymorphism Database (mtSNP). They found that no stop codons or frame shift mutations were in the MT-DNA gene, but they identified 32 homoplasmic variants, including 7 missense variants and 25 synonymous ones. They also found that males with haplogroup Z were more exposed to IVF failure, but their observations about fertilization failure between the fertile group and the infertile group with haplogroup D or haplogroup G did not show any significant difference. So they concluded that *MT-ND2* gene SNPS were not correlated with the total fertilization rate (J. Zhang *et al*., 2018).

A previous study in New Zealand compared the occurrence of mitochondrial variants among infertile and fertile men. They found that among a group of patients there were 11 nucleotide substitutions while among normozoospermic men there were only 7 nucleotide substitutions. Most of the identified variants were silent, while only 6 substitutions were missense. The percentage of nucleotide substitution in the control group was 2.44%, while among males with abnormal sperm criteria it was 8.43%. Furthermore They also identified two significant variants; 9055 G>A in the ATP synthase 6 gene with frequency of 10.7%, this missense variant lead to replacing alanine by threonine. They also identified another synonymous variant 11719 G>A in the *ND4* gene with a frequency of 12%. According to their results, 11 % of infertile males were found to have 9055 G>A, while the frequency of this variant among normozoospermic men was only 1.3 %. The frequency of 11719 G>A among infertile men was 12 %, while this particular variant was not identified among normozoospermic men. In their study they noticed that all infertile men who had these substitutions were diagnosed with asthenozoospermia and/or oligozoospermia, and so they concluded that these SNPs in the MT-genes can predict for sperm quality in these males (Holyoake *et al*., 2001).

Another study identified mitochondrial variants by performing next generation sequencing for the mtDNA genome in the sperm of 233 idiopathic infertile men. It reported that the mitochondrial genetic variant 11696G>A in the *ND4* gene is associated with asthenozoospermia, for it was found that the frequency of this variant among infertile patients was higher compared to normozoospermic men. This variant is missense and leads to the replacement of isoleucine by valine at amino-acid locus 313. When a software analysis for

prediction of the effect of SNP was applied, this variant was also predicted to change the secondary structure for the protein (Ji *et al*., 2017).

A previous study identified 12 mitochondrial variants in the sperm of 30 infertile men and three variants (C8927G, A9041G, C9105G) identified in the *ATPase6* gene. Six variants (15296A>C, 14969T>C, 15806G>C, 15143C>T, 15282T>G and 15804T>A) were also identified in the *Cytb* gene, and finally three variants (T4114G, G4153A and C4159) were identified in the *ND1* gene. All these variants were missense and they were predicted to affect the secondary structure of protein. According to the results in this study there was no statistical difference between the frequency of these variants among the infertile men and the control group, based on the chi-square test ($P > 0.05$), although the three variants (T4114G, G4153A and C4159) in the *ND1* gene were only identified among the infertile group and they were not found in the control group (Güney *et al*., 2012).

### 1.11. Aim of the study:

1. To investigate the correlation between sperm motility and ICSI outcomes.
2. To determine the relationship between the fertilization rate and the embryo quality.
3. To measure protein carbonyl levels in the sperm of asthenozoospermic men.
4. To find the correlation between protein carbonyl levels and reduced sperm motility.
5. To identify sperm mtDNA variants in the *ND1*, *ND2*, *ND5* and *ND6* genes among asthenozoospermic men subjected to the ICSI technique.
6. To explore the correlation between sperm mtDNA variants and sperm motility.
7. To examine the effect of sperm mtDNA variants on the fertilization rate, the embryo cleavage score and the embryo quality score.

# 2. Materials and methods

## 2.1. Materials

### 2.1.1. Subjects

From August 2018 to October 2019, samples were collected from 150 male partners, aged < 40 years, of the ICSI couples that attended the Assisted Reproductive Techniques (ART) Department at Prince Rashid Bin AL Hassan Hospital (PRBH), Irbid, Jordan.

According to the World Health Organization (WHO) laboratory manual for semen analysis, the total motility (progressive motility (PR) and non-progressive motility (NP)) for a normal male should be above 40%; otherwise, it will be considered to be asthenozoospermic. 105 of these samples were from asthenozoospermic men (PR+NP<40%) and were divided into three groups according to their percentage of sperm motility. Group one involved asthenozoospermic men with percentage of sperm motility ranged between 0% to 5%, group two included asthenozoospermic men with percentage of sperm motility ranged between 6% to 15%, and group three involved asthenozoospermic men with percentage of sperm motility ranged between 16% to 35%. Forty-five samples of normozoospermic males with an elevated levels of sperm motility, which ranged between 50% and 75% were also collected as controls. The other semen parameters were within normal ranges (Table 2.1). Patients with varicocele, and alcoholic problems, as well as cigarette smokers and patients with genetic abnormalities, such as Klinefelter's Syndrome, were excluded from this study. The study was approved by the Jordanian Royal Medical Services-Human Research Ethics Committee on 30/7/2018 with the project identification code (TF3/1/ Ethics Committee / 9126), and written consent from each couple was obtained, see supplementary (Figure 6.1) and (Figure 6.2).

Table 2.1: Semen parameters among groups

| | Semen volume (ml) Median ± SD | Sperm concentration ($10^6$ per ml) Median ± SD | Total motility (PR + NP %) Median ± SD | Morphologically normal spermatozoa (%) , Median ± SD |
|---|---|---|---|---|
| Group 1 | 2.63 ± 1.18 | 32.04 ± 15.12 | 0 ± 2.57 | 5.78 ± 3.14 |
| Group 2 | 3.1 ± 2.16 | 40.56 ± 27.88 | 9 ± 3.40 | 4.89 ± 2.45 |
| Group 3 | 2.89 ± 1.43 | 61.13 ± 39.74 | 20 ± 6.99 | 6.13 ± 5.69 |
| control | 3.2 ± 1.35 | 73.16 ± 52.21 | 58 ± 9.05 | 8.82 ± 7.03 |

### 2.1.2. Materials, chemicals and instruments used in this study

| Disposables | Manufacturer |
|---|---|
| 10 mL serological pipette | FALCON, USA |
| 5 mL disposable plastic pipette | NUNC, EU |
| 1 mL disposable plastic pipette | NUNC, EU |
| Manipulation pipette (300μm) | COOK, Ireland |
| Denuding pipette (170 μm) | COOK, Ireland |
| Denuding pipette (140 μm) | COOK, Ireland |
| Sterile single-use syringe (1 mL) | Codan, Australia |
| Handling pipette | GYNETICS, Belgium |
| Holding micropipette | ORIGIO, Costa Rica |
| ICSI micropipette | ORIGIO, Costa Rica |
| 50 mL flask | FALCON, USA |
| 15 mL polystyrene conical tube | FALCON, Mexico |
| Transfer pipette 3 mL | FALCON, Mexico |
| Slides with ground edges 90° | Porlab, Netherlands |
| Coverslips | Porlab, Netherlands |
| 60 x15 mm IVF one-well dish | FALCON, USA |
| ICSI dish | Thermo SCIENTIFIC, USA |
| 14 mL Round-bottom tubes | FALCON, USA |
| 5 mL Round-bottom tubes | FALCON, USA |
| 110 mL sterile specimen container | FALCON, USA |
| Sterile tips | Gilson, USA |
| Wallace embryo-transfer catheter | CooperSurgical Fertility Company, Denmark |
| Biosphere filter tips (0.1-20) μm | Sarstedt, Germany |
| Biosphere filter tips (200) μm | Sarstedt, Germany |
| Biosphere filter tips (1000 ) μm | Sarstedt, Germany |
| 1.5 mL sterile PCR tubes (DNA-/ DNase-/ RNase) inhibitor-free | Sarstedt, Germany |
| Tube Laboratory Rack | Sarstedt, Germany |

| | |
|---|---|
| Graduated Cylinders | VWR international, USA |
| PCR Freezer Storage Box | Sarstedt, Germany |
| Media used for ICSI | |
| QUINN'S Advantage Medium with HEPES | SAGE, USA |
| Fertilization medium | SAGE, USA |
| Cleavage medium | SAGE, USA |
| Blastocyst medium | SAGE, USA |
| Flushing medium | SAGE, USA |
| Human serum albumin | SAGE, USA |
| Polyvinylpyrrolidone (PVP) media | SAGE, USA |
| Oil for covering media during IVF | Vitrolife, Sweden |
| Sperm washing media | SAGE, USA |
| Chemicals and reagents | |
| SYBR SAFE DNA GEL STAIN | ThermoFisher SCIENTIFIC, Germany |
| Gel loading dye | New England Biolabs, Germany |
| Nuclease-free water | New England Biolabs, Germany |
| 10 Kb DNA Ladder | New England Biolabs, Germany |
| Phosphate buffer saline | ThermoFisher SCIENTIFIC, Germany |
| Absolute Ethanol | Merck, Germany |
| Agarose tablets | Bioline, United Kingdom |
| Tris-acetate-EDTA (TAE) Buffer | ThermoFisher SCIENTIFIC, Germany |
| Instruments | |
| IVF work station | IVFtech, Denmark |
| Light microscope model BX41TF | OLYMPUS, Japan |
| Galaxy CO2 Incubator model 300 | RS Biotech, United Kingdom |
| CO2 analyzer G100 | Geotech, USA |
| Makler counting chamber | Irvine Scientific, USA |
| Microscope Integra 3 micromanipulator | CooperSurgical Fertility Company, Denmark |
| Centrifuge model 5702 | Eppendorf, Germany |
| Refrigerated centrifuge model(1-14 K) | SIGMA, Germany |

| | |
|---|---|
| Nanodrop spectrophotometer ND-2000c | Thermo Scientific, USA |
| Thermocycler (C1000$^{TM}$ Thermal cycler | Bio-Rad, United States |
| Molecular Imager Gel Doc XR+ | Bio-Rad, Germany |
| Electrophoresis power supply | Sigma-Aldrich, Germany |
| Freezer – 20° c | Liebherr, Germany |
| Freezer (2-8) °c | Liebherr, Germany |
| PCR workstation | VWR international, USA |
| Vortex | Scientific industries, USA |
| Pipettes | Eppendorf, Germany |
| Multichannel pipettes | Eppendorf, Germany |
| Hotplate with magnetic stirrer | Boeco, Germany |
| Orbital Thermo-shaker | Eppendorf, Germany |
| Rotary shaker | Eppendorf, Germany |
| ELISA microplate reader | Biotek, Germany |
| Kits | |
| QIAamp DNA Mini Kit Cat. No. 51304 | Qiagen, Germany |
| REPLI-g Mitochondrial DNA Kit (151023) | Qiagen, Germany |
| PCR Master Mix (2X) | ThermoFisher SCIENTIFIC, Germany |
| Qproteome Mammalian Protein Prep Kit | Qiagen, Germany |
| Coomassie Plus(Bradford) Assay | ThermoFisher SCIENTIFIC, Germany |
| OxiSelect Protein Carbonyl ELISA | Cell Biolabs, USA |

## 2.2. Methods

### 2.2.1. Semen collection and evaluation

Semen samples were collected from all individuals by masturbation, with the period of sexual abstinence ranging between three to five days. Each male was provided with a 110 ml sterile specimen container (Falcon, USA). After ejaculation the samples were kept in the incubator at 37 °C for 30 minutes (min) to evaluate the semen liquefaction. Then the semen parameters were assessed based on WHO criteria, where the seminal fluid analysis involved the evaluation of semen volume, PH, colour, leucocytes in the semen, round

cells, aggregation, agglutination, sperm count, sperm morphology and sperm motility (Table 2.2) (WHO, 2010).

### 2.2.2. Sperm preparation for ICSI

Semen samples were transferred to a 15 ml sterile conical tube (Falcon, Mexico). Then they were treated with density gradient media (Sage, USA), which contained two different concentrations of silica particles, where the lower phase included a 90% density gradient , and the upper phase included a 45% density gradient, while the semen was added to the top of the two layers. The density gradient was achieved by centrifugation at 240 g for 22 minutes. After this, the pellet was collected and washed two times by a sperm-washing media (Sage, USA) to remove silica particles. Supernatants were discarded, and the pellet was then gently layered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (21 mM, PH=7.3) plus 0.5% human serum albumin (Sage, USA). After this, the sperm pellet was collected and placed in the $CO_2$ incubator at 37° C for 1 hour and, later on, the surface layer was aspired. This technique was necessary to discard contaminating cells in the semen, such as leucocytes. Only sperm samples with a 0% percentage of sperm motility were washed without this HEPES layering.

Table 2.2: Semen parameters with their reference values according to WHO,2010

| Semen parameter | Reference value according to WHO,2010 |
|---|---|
| Volume (mL) | $\geq 1.5$ |
| Sperm count($10^6$/mL) | $\geq 15$ |
| Total sperm count ($10^6$/mL) | $\geq 39$ |
| Total motility (%) | $\geq 40$ |
| Progressive motility | $\geq 32$ % ( type A + type B) |
| Morphology (%) | $\geq 4$ |
| Leucocyte count ($10^6$/mL) | $< 1$ |
| Round cell count ($10^6$/mL) | $< 5$ |
| PH | 7.2 - 8 |

### 2.2.3. Oocyte denudation

The oocytes were denudated after 2 hours of egg retrieval. This process involved chemical and mechanical treatments. The chemical denudation was performed using a hyaluronidase enzyme (Vitrolife, Sweden), while the mechanical denudation was done with special denuding pipettes (COOK, USA) (with a 150–300 μm inner diameter) in HEPES media covered with oil (Vitrolife, Sweden). Denudated eggs were then incubated in the fertilization media (Sage, USA) for 3 hours in the CO2 incubator at 37°C.

### 2.2.4. The Intracytoplasmic Sperm Injection (ICSI) technique

The oocytes were transferred to a special ICSI dish (Thermo SCIENTIFIC, USA) in droplets of HEPES media covered with oil (Vitrolife, Sweden). The mature oocytes at metaphase II were only selected for injection by ICSI using the microscope Integra 3 micromanipulator (CooperSurgical Fertility Company, Denmark), while immature and bad quality oocytes were not inseminated. The injecting and holding micropipettes were made from 1 mm borosilicate glass capillaries by Origio, Costa Rica. Sperms were embedded in polyvinylpyrrolidone (PVP) media (Sage, USA) to reduce their motility; this can break their tails, causing immobilization, which makes it easier to insert them inside the injection needle. After that orientation of the polar body was selected to be at the 6 o'clock or the 12 o'clock position, it was also determined that the 7 o'clock or 11 o'clock positions were preferable for oocyte holding. After breaking the sperm flagellum and aspirating it inside the injection needle, it was placed at the tip of the ICSI needle, and then compressing the zona pellucida at the 3 o'clock position, passing through the cytoplasm of the oocyte until reaching near 9 o'clock; then some of the cytoplasm outside the plasma membrane was aspirated before it was finally returned with the sperm (Griffiths *et al.*, 2000).

### 2.2.5. Fertilization assessment

After 16-18 hours post-ICSI, the zygotes were evaluated according to Scott's zygote scoring system, where the zygote was considered as grade 1 if it had the same count of nucleoli and they were aligned in their true position at the pronuclear junction. The zygotes were considered to be grade 2 if they had the same number of nucleoli with a symmetrical size for all of them, but the distribution of the nucleoli differed from one nucleus to another; ,here in

one nucleus there was alignment for nucleoli, the second nucleus might contain scattered nucleoli. In grade 3 there was no alignment at the pronuclear junction; there was only a symmetry in size and the same count for the nucleoli with a scattered pattern within the zygote. Grade 4 zygotes showed differences in size and number of nucleoli, with the difference occurring in the number only or in the size only or in both of them. Grade 5 was the worst, especially if the zygote had unequal numbers of nucleoli in both nuclei, was without symmetry in size and had a scattered distribution of nucleoli in the nucleus (Scott, 2000).

### 2.2.6. Embryo grading

Embryos were considered to be grade A if they had 6-8 symmetrical blastomeres at day 3, with a percentage of fragmentation of less than 10%. But if the percentage of fragmentation reached 20%, the embryos were evaluated as being grade B. Grade C embryos had blastomeres with less than 6-8 cells, or they had 6-8 blastomeres but of unequal size (Figure 2.1). Finally, embryos with severe fragmentation exceeding 50% of the cell size were categorized as grade D (Johansson *et al*., 2003).

### 2.2.7. Embryo cleavage score and quality score

The embryo cleavage score for each couple was determined by the total of the embryos cleavage scores / the number of all embryos, where embryos at day 3 with 8 cells were given 4 points, embryos with 6 cells were given 3 points and embryos with 4 cells were given 2. The embryo quality score for each couple was determined by the total of the embryos quality scores / the number of all embryos, where embryos with grade A were given 3 points, embryos with grade B were given 2 points, while embryos with grade C and D were given only 1 point (Nasr-Esfahani *et al*., 2007). The embryo cleavage scores and the embryo quality scores among patient group were provided in supplementary (Table 6.1). While the embryo cleavage scores and the embryo quality scores among the control group were provided in supplementary (Table 6.2).

### 2.2.8. Sperm genomic DNA extraction

Genomic DNA was extracted from the purified samples using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Germany). Sperm genomic DNA was extracted as follows:

1. Transfer 200 µl from the sample to a sterile 1.5 mL microcentrifuge tube.
2. Adding 20 µl of proteinase K to the microcentrifuge tube that containing the sample.
3. Transfer 200 µl of AL buffer to the microcentrifuge tube.
4. Mixing the mixture with vortexing for 20 seconds.
5. Incubation of the mixture at 56 ℃ for 10 minutes.
6. Centrifugation of the mixture fleetingly.
7. Adding 200 µl of absolute ethanol to the microcentrifuge tube.
8. Transfer the mixture into the QIAamp Mini spin column- which was supplied by the kit - and placing it in a 2 mL collection tube.
9. Centrifugation of the mixture with a speed of 6000 g for 60 seconds.
10. Transfer the QIAamp Mini spin column into a new sterile 2 mL microcentrifuge tube, while the old microcentrifuge tube that containing the filtrate was discarded.
11. Adding 500 200 µl of the AW1 buffer to the QIAamp Mini spin column.
12. Centrifugation of the mixture with a speed of 6000 g for 60 seconds.
13. Transfer the QIAamp Mini spin column into a new sterile 2 mL microcentrifuge tube, while the old microcentrifuge tube that containing the filtrate was discarded.
14. Adding 500 µl of the AW2 buffer to the QIAamp Mini spin column.
15. Centrifugation of the mixture with a speed of 18,000 g for 3 minutes.
16. Transfer the QIAamp Mini spin column into a new sterile 2 mL microcentrifuge tube, while the old microcentrifuge tube that containing the filtrate was discarded.
17. Adding 200 µl of the AE buffer to the QIAamp Mini spin column for DNA elution.
18. Incubation of the mixture at room temperature for 60 seconds.
19. Centrifugation of the mixture with a speed of 6000 g for 60 seconds.

Discarding the QIAamp Mini spin column, and microcentrifuge tube which contained DNA was closed and stored at -20 ℃. Only the isolated DNA with an optimal density ratio of 260/280 of 1.8 or more was chosen using the Nanodrop spectrophotometer ND-2000c (Thermo Scientific, USA).

### 2.2.9. MtDNA amplification

MtDNA was amplified using the REPLI-g Mitochondrial DNA Kit (Qiagen, Germany). MtDNA was amplified as follows:

1. Placing 10 µl of genomic DNA in a 1.5 mL microcentrifuge tube.

2. Adding 29 µl of amplification mixture to the sample and mixing by vortexing.
3. Incubation of the mixture at 75 ℃ for 5 minutes.
4. Cooling the mixture to room temperature.
5. Thawing REPLI-g Midi DNA polymerase on ice.
6. Adding 1 µl REPLI-g Midi DNA polymerase to the mixture and mixing through centrifugation fleetingly.
7. Incubation of the mixture at 33 ℃ for 8 hours.
8. Inactivation of REPLI-g Midi DNA polymerase by incubation of the mixture at 65 ℃ for 3 minutes.
9. Amplified mtDNA was stored at -20 ℃.

### 2.2.10. Polymerase chain reaction (PCR)

To amplify the *ND1, ND2, ND5 and ND6* genes, 4 sets of PCR primers (forward and reverse) were designed using the Primer 3 program flanking the region of each gene. Primers were designed using the human mitochondrial sequence, obtained from the National Centre of Biotechnology Information (NCBI). The primers were synthesized by the Microsynth Seqlab in Germany (Table 2.3). A 25 µL reaction mixture was prepared to contain 12.5 µL PCR Master Mix (2X) (ThermoFisher SCIENTIFIC, Germany), 0.8 µL of 10 mM forward primer, 0.8 µL of 10 mM reverse primer, 2 µL mtDNA (20 ng/µL) and 8.9 µL nuclease-free water. The Thermocycler (C1000$^{TM}$ Thermal cycler, Bio-Rad, United States) program was set as follows: initial denaturing at 95 ºC for 3 min., then 35 cycles of denaturation at 95 ºC for 30 sec., annealing for 40 sec. (*ND1*: 59 ºC; *ND2* and *ND6:* 61 ºC; *ND5*: 64º C), an extension of primers at 72 ºC for *ND1* and *ND2*: 1 min.; *ND5*: 2 min.; *ND6*: 45 sec.), then a final extension for 5 min. at 72º C. PCR products were stored at 4º C.

### 2.2.11. VISUALIZATION OF PCR PRODUCTS

To check the amplification, 5µL of each PCR product was run on 1% agarose gel prepared using 1X Tris-acetate-EDTA (TAE) buffer and stained with 10,000X SYBR Safe stain (ThermoFisher SCIENTIFIC, Germany) diluted 1:10,000 in TAE. Electrophoresis was carried out at 100 V for 50 min by Electrophoresis power supply (Sigma Aldrich, Germany). DNA was then visualized using Molecular Imager Gel Doc XR+ (Bio-Rad, Germany). See (Figure 2.2), (Figure 2.3), (Figure 2.4), (Figure 2.5) and (Figure 2.6).

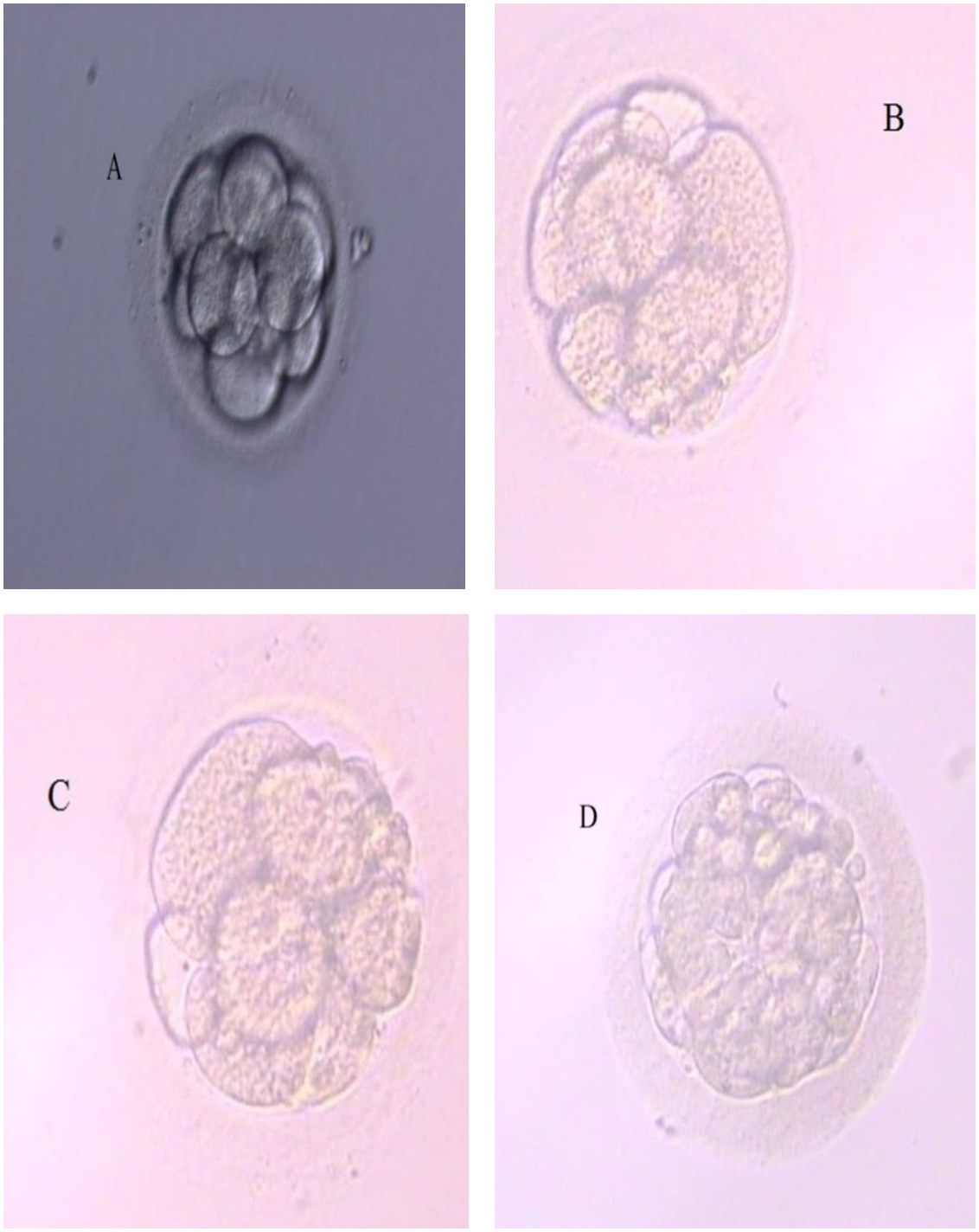


Figure 2.1: : Embryos at day 3 with different qualities (A) grade A embryo with 8 symmetrical blastomeres and without fragmentation (B) grade C embryo with 8 unequal blastomeres (C) grade C embryo with 7 unequal blastomeres and slight fragmentation (D) grade D embryo with severe fragmentation .

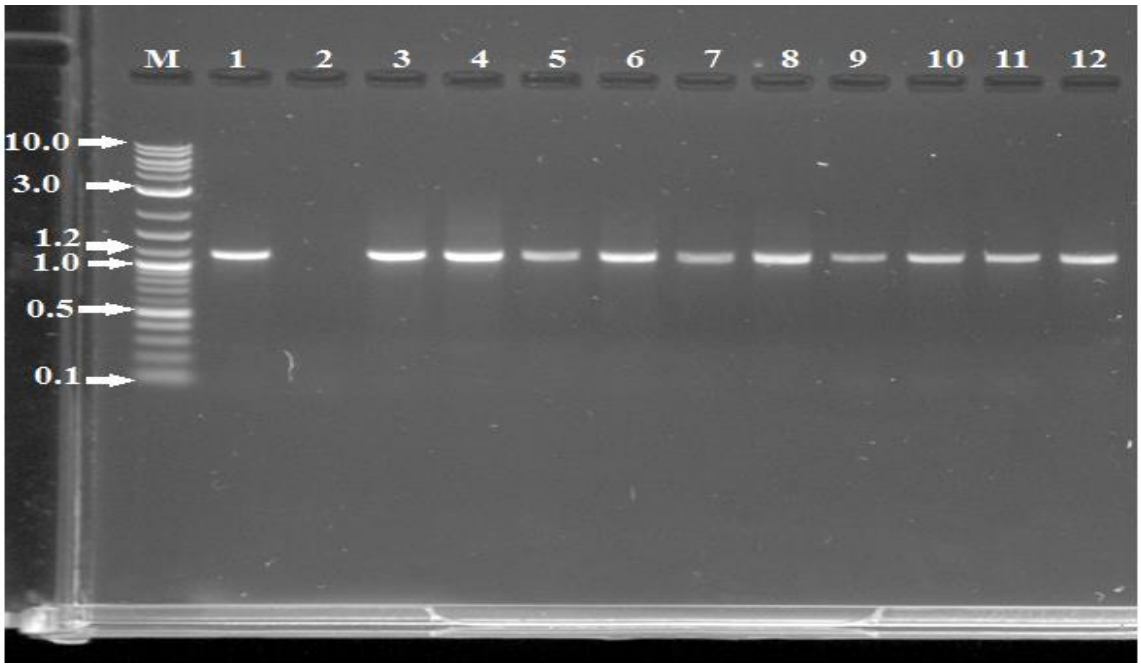


Figure 2.2: Electrophoretogram on agarose gel (1%) of PCR products for the amplification of the ND1 gene (1.155 Kb). Lane M:DNA Ladder (0.1-10.0 kb) (NE Biolabs, Germany), Lane 2 : negative control, Lane 1,3-12 : PCR sample products. The fragments of DNA were separated on an electric field of 100 V/ 50 min. (Sigma-Aldrich, Germany). The Gel Doc XR+ imaging system (BIO-RAD, USA) was used for the visualization of the gel after DNA migration.

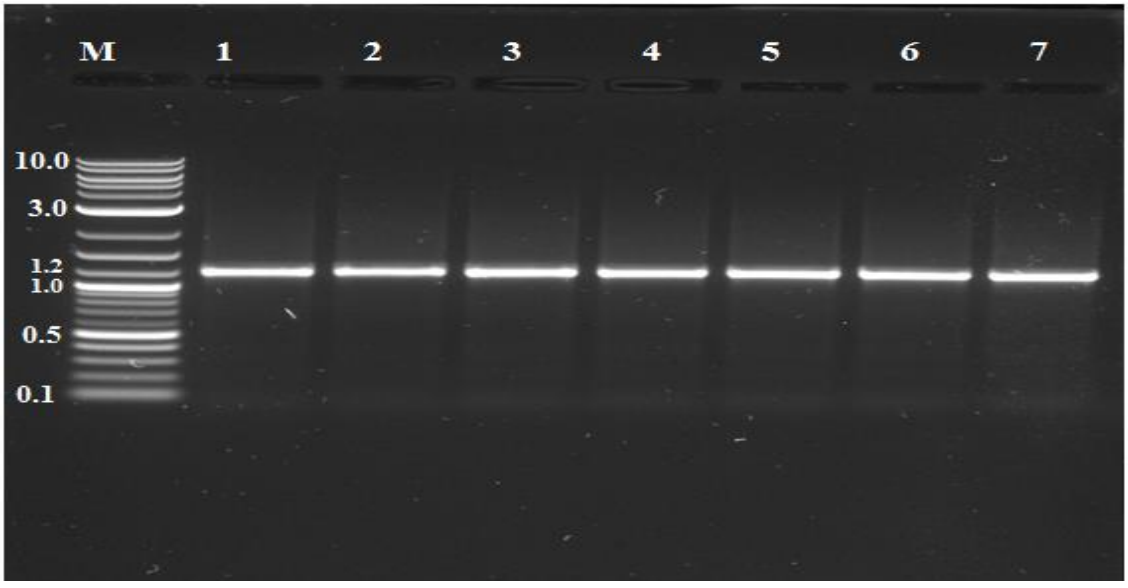


Figure 2.3: Electrophoretogram on agarose gel (1%) of PCR products for the amplification of the ND2 gene (1.2 Kb). Lane M:DNA Ladder (0.1-10.0 kb) (NE Biolabs, Germany), Lane 1-7 : PCR sample products. The fragments of DNA were separated on an electric field of 100 V/ 50 min. (Sigma-Aldrich, Germany). The Gel Doc XR+ imaging system (BIO-RAD, USA) was used for the visualization of the gel after DNA migration.

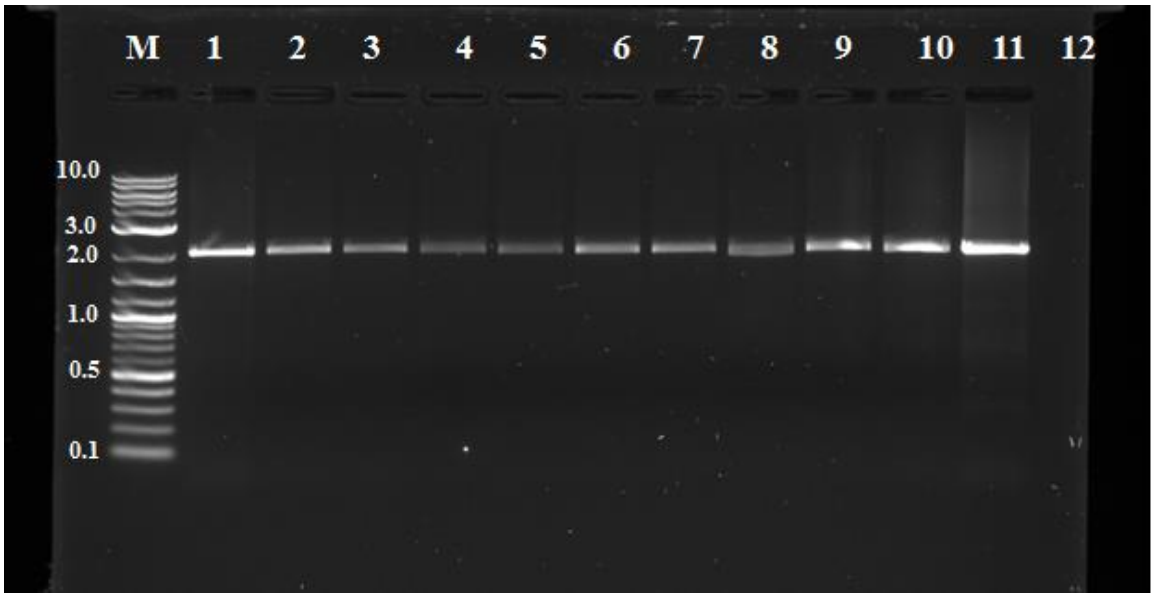


Figure 2.4: Electrophoretogram on agarose gel (1%) of PCR products for the amplification of the ND5 gene (2.043 Kb). Lane M:DNA Ladder (0.1-10.0 kb) (NE Biolabs, Germany), Lane 1-11 : PCR sample products, Lane 12: negative control. The fragments of DNA were separated on an electric field of 100 V/ 50 min. (Sigma-Aldrich, Germany). The Gel Doc XR+ imaging system (BIO-RAD, USA) was used for the visualization of the gel after DNA migration.

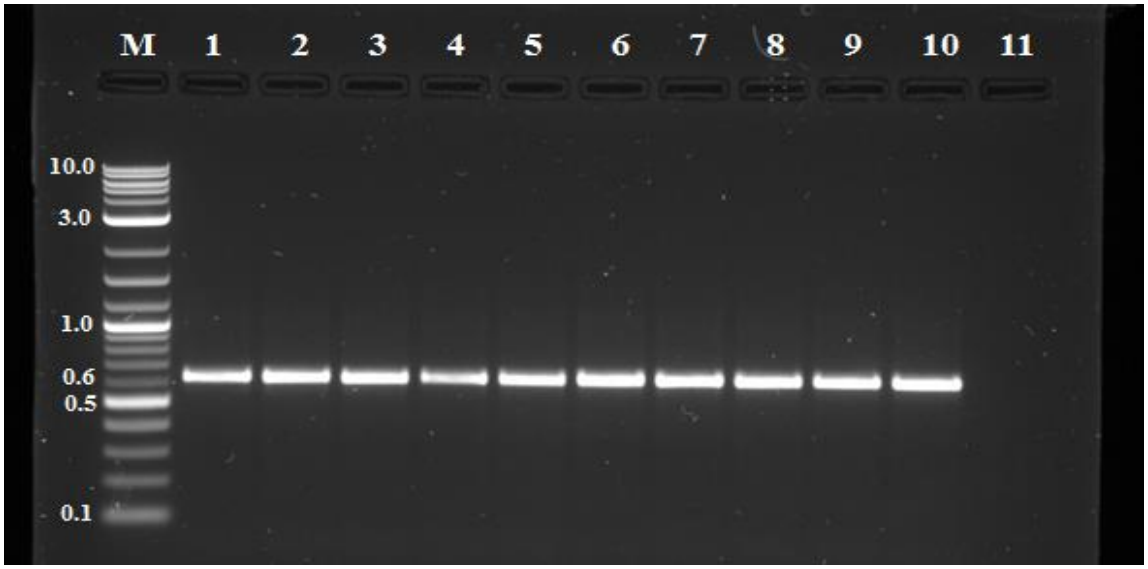


Figure 2.5: Electrophoretogram on agarose gel (1%) of PCR products for the amplification of the ND6 gene (622 bp). Lane M:DNA Ladder (0.1-10.0 kb) (NE Biolabs, Germany), Lane 1-10 : PCR samples products, Lane 11: negative control. The fragments of DNA were separated on an electric field of 100 V/ 50 min. (Sigma-Aldrich, Germany). The Gel Doc XR+ imaging system (BIO-RAD, USA) was used for the visualization of the gel after DNA migration.

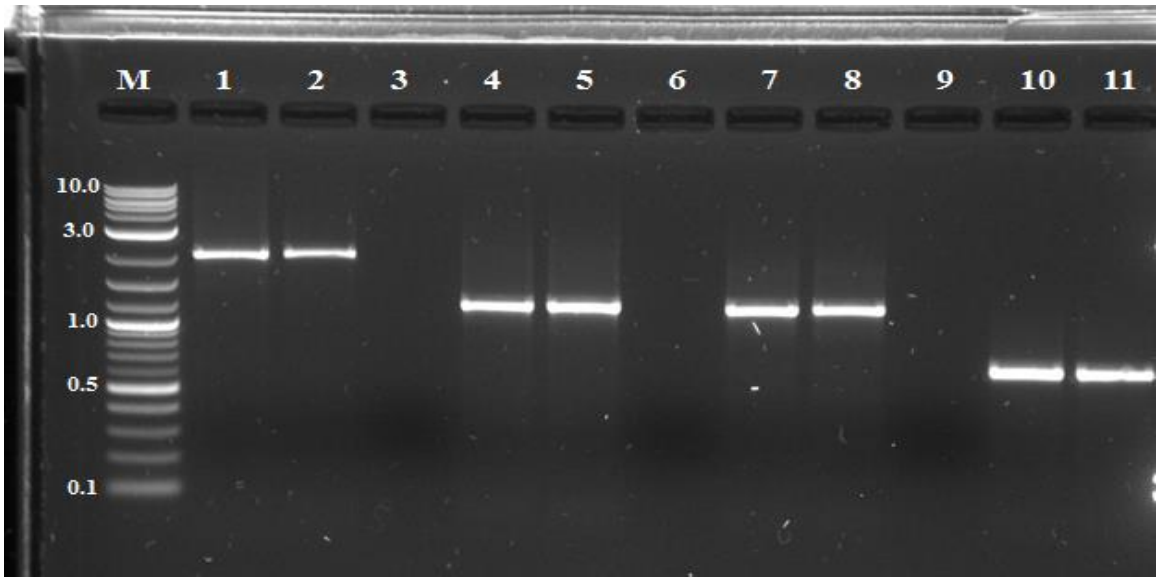


Figure 2.6: Electrophoretogram on agarose gel (1%) of PCR products for the amplification of the ND5, ND2, ND1 and ND6 genes. Lane M:DNA Ladder (0.1-10.0 kb) (NE Biolabs, Germany), , Lane 1-2 : amplification of the ND5 gene (2043 bp), Lanes (3,6 and 9) : negative control, Lane 4-5: amplification of the ND2 gene ( 1200 bp), Lane 7-8: amplification of the ND1 gene (1155 bp), Lane 10-11: amplification of the ND6gene (622 bp). The fragments of DNA were separated on an electric field of 100 V/ 50 min. (Sigma-Aldrich, Germany). The Gel Doc XR+ imaging system (BIO-RAD, USA) was used for the visualization of the gel after DNA migration.

Table 2.3: Primers list for PCR amplification and Sanger sequencing

| Primer Name | Sequence (5'->3') | Product Length |
|---|---|---|
| MT-ND1-F | CACCCACCCAAGAACAGGGT | 1155 |
| MT-ND1-R | TTCTCAGGGATGGGTTCGATTC | |
| MT-ND2-F | TCAGCTAAATAAGCTATCGGGC | 1200 |
| MT-ND2-R | GAGTGGGGTTTTGCAGTCCT | |
| MT-ND5-F | CTGCTAACTCATGCCCCCAT | 2043 |
| MT-ND5-R | GGAGGATCCTATTGGTGCGG | |
| MT-ND6-F | CCTCTCTTTCTTCTTCCCACTCA | 622 |
| MT-ND6-R | CGATGGTTTTTCATATCATTGGTCG | |
| ND5A* | CTAAACGCTAATCCAAGCC | * |
| ND5B* | CTATTACTCTCATCGCTACCTC | * |

ND5A* and ND5B* are additional internal primers were designed for Sanger sequencing only

### 2.2.12. Identification of genetic variants in the *ND1, ND2, ND5* and *ND6* genes

PCR products were purified and sequenced using the Sanger method (Microsynth Seqlab, Germany). Sequencing was carried out in both directions (forward and reverse) for each sample. For the *ND5* gene, two additional internal primers were designed, namely, ND5A and ND5B (Table 2.3).

The primary and secondary sequences for each sample were analysed using the BioEdit sequence alignment editor version 7.2.5 and aligned to the NCBI reference sequences (NC_012920.1).

### 2.2.13. Prediction of the functional effect of the mitochondrial variant

For prediction the possible effect of amino-acid substitution on the structure and function of the protein and to evaluate the possible damaging effect of genetic variants, two versions of software were used (The American College of Medical Genetics and Genomics (ACMG), **https://www.acmg.net** and Poly Phenyl-2, **http://genetics.bwh.harvard.edu/pph2**.

### 2.2.14. Protein isolation

Total protein was isolated from the semen by using the Qproteome Mammalian Protein Prep Kit (Qiagen, Germany). Before starting the isolation the sperm count was adjusted to be about 5 x $10^6$ sperm / ml, according to the kit instructions. The following steps summarize the isolation process:

1. Washing the sperm by adding 10 ml of cold phosphate buffer saline (PBS); this step involved transferring the semen to a 15 mL conical tube.
2. Centrifugation of the suspension at 450 x g for five minutes in a precooled centrifuge to 4°C.
3. Discarding the supernatants, while the pellet was kept on ice.
4. Preparation of the lysis buffer, which contained 1 µl benzonase and 10 µl of protease inhibitor in addition to 1 ml mammalian lysis buffer.
5. Adding the lysis buffer to the pellet, which formed in step 4.
6. Incubation of the suspension on a rotary shaker for 5 minutes at 4°C.
7. Transferring the suspension from the 15 mL conical tube to a new 2.0 mL tube.
8. Centrifugation of the suspension by a refrigerated microcentrifuge at 4°C, with a speed of 14,000 g for 10 minutes.

9. Finally, the supernatant was transferred into a new sterile 1.5 ml tube, which contained the total protein, and it was then stored at – 20 °C.

### 2.2.15. Total protein quantification

The protein level was measured by the Coomassie Plus (Bradford) Assay Kit from Thermo Fisher Scientific, Germany, as follows:

1. Diluted bovine serum albumin (BSA) standards were prepared, which they were set up to involve a BSA concentration ranged between 2.5 µg/mL to 25 µg/mL, in addition to the blank with 0 µg/mL.
2. Transfer 1.0 mL of each standard or unknown sample in a clean tube
3. Adding 1.0mL of the Coomassie Plus Reagent into each tube and mixing well.
4. Incubation of the samples at room temperature for 10 min.
5. Zeroing the spectrophotometer on a cuvette that contained water only, after setting the wavelength at 595 nm.
6. Preparing the standard curve through plotting the average measurement at 595nm for each BSA standard versus its concentration in µg/Ml (Table 2.4).
7. The protein concentration of each unknown sample was known by using the standard curve; also to confirm the accuracy of measurement, a triple reading for each sample was carried out.

Table 2.4: Standard curve preparation for Bradford assay with diluted BSA

| Tube Name | Diluent Volume (µL) | BSA Volume (µL) and Source | Concentration of the final BSA (µg/mL) |
|---|---|---|---|
| 1 | 3555 | 45 from the stock | 25 |
| 2 | 6435 | 65 from the stock | 20 |
| 3 | 3970 | 30 from the stock | 15 |
| 4 | 3000 | 3000 from vial 2 dilution | 10 |
| 5 | 2500 | 2500 from vial 4 dilution | 5 |
| 6 | 1700 | 1700 from vial 5 dilution | 2.5 |
| 7 | 4000 | 0 | 0 (Blank) |

### 2.2.16. Quantification of protein carbonyl

Quantification of protein carbonyl was performed using the OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs, USA), as follows:

1.The concentration of total protein for each sample was diluted to 10 µg/mL in 1X PBS before starting he assay according to the kit instructions.

2. Preparation of protein carbonyl BSA Standard Curve to have concentrations ranged between 0.375 to 7.5 (nmol/mg); this was performed by mixing the reduced BSA and the oxidized BSA in the appropriate ratios (Table 2.5).

3. Adding 100µL of 10 µg/mL protein samples in addition to the same volume from reduced/oxidized BSA standards, which were then placed in the protein-binding plate and incubated at 37ºC at 4ºC overnight.

3. Washing three times with 250 µL 1X PBS per well. After the third wash the wells were dried by tissue towel to remove the remains of the washing solution.

4. Adding 100 µL DNPH working solution to each well.

5. Incubation of the plate in a closed place far of light for 45 min. around 20ºC.

6. Washing the wells five times with 250 µL of 1X PBS/Ethanol with ratio (1:1), with incubation of the plate after each washing on an orbital shaker for 5 minutes. After the final wash the wells were dried by tissue towel to remove the remains of the washing solution.

7. Adding 200 µL of blocking solution to each well.

8. Incubation of the plate at room temperature for 1.5 hours on an orbital shaker.

9. Washing three times with 250 µL of 1X wash buffer. After the third wash the wells were dried by tissue towel to remove the remains of the washing buffer.

10. Adding 100 µL of the diluted anti-DNP antibody to each well.

11. Incubation of the plate at room temperature for 60 min. on an orbital shaker.

12. Washing the wells three times with 250 µL of 1X wash buffer.

13. Adding 100 µL of the diluted HRP conjugated secondary antibody to each well and incubating for 1 hour at room temperature on an orbital shaker.

14. Washing the wells five times with 250 µL of 1X wash buffer. After the third wash the wells were dried by tissue towel to remove the remains of the washing buffer.

15. Adding 100 µL of substrate solution to each well, which was pre-warmed to the room temperature before adding.

16. Incubation of the plate at room temperature on an orbital shaker for 20 minutes.

17. Termination of the enzyme reaction by adding 100 µL of stop solution to all wells for.

18. Measuring the absorbance of all wells using an ELISA microplate reader at 450 nm , after zeroing the microplate reader with the well filled only with reduced BSA as a blank.

Table 2.5: Construction of protein carbonyl BSA standards.

| vial | 10 µg/mL oxidized BSA (µL) | 10 µg/mL Reduced BSA (µL) | Concentration of Protein Carbonyl (nmol/mg) |
|---|---|---|---|
| A | 400 | 0 | 7.5 |
| B | 320 | 80 | 6.0 |
| C | 240 | 160 | 4.5 |
| D | 160 | 240 | 3.0 |
| E | 80 | 320 | 1.5 |
| F | 40 | 360 | 0.75 |
| G | 20 | 380 | 0.375 |
| H | 0 | 400 | 0 |

### 2.3. Statistical analysis

Statistical analysis was carried out using the OriginPro, Version 2020 (OriginLab Corporation, Northampton, MA, USA). The normality assumptions were checked for the variables in question and were found not to be fitted by a normal distribution and hence non-parametric tests were applied to our study. The Kruskal-Wallis H test alongside the Mann Whitney U test in addition to chi-square were applied to decide if the differences of variables between groups were significant. Spearman's Rho was applied to evaluate the strength of correlation between different variables. The odds ratio and their 95% confidence intervals were determined, and some descriptive statistics and graphs for the variables in question were presented. A *P*-value < 0.05 was considered to be statistically significant.

# 3. Results

## 3.1. Correlation between sperm motility and ICSI outcomes

Sperm motility and ICSI outcomes were tested for correlations in all studied samples (n=150). These outcomes included the fertilization rate, the embryo cleavage score and the embryo quality score. As illustrated in (Figure 3.1) below, the sperm motility was significantly positively correlated with the fertilization rate (r=0.701, p<0.001),the embryo cleavage score (r=0.549, p<0.001) and the embryo quality score (r=0.656, p<0.001).

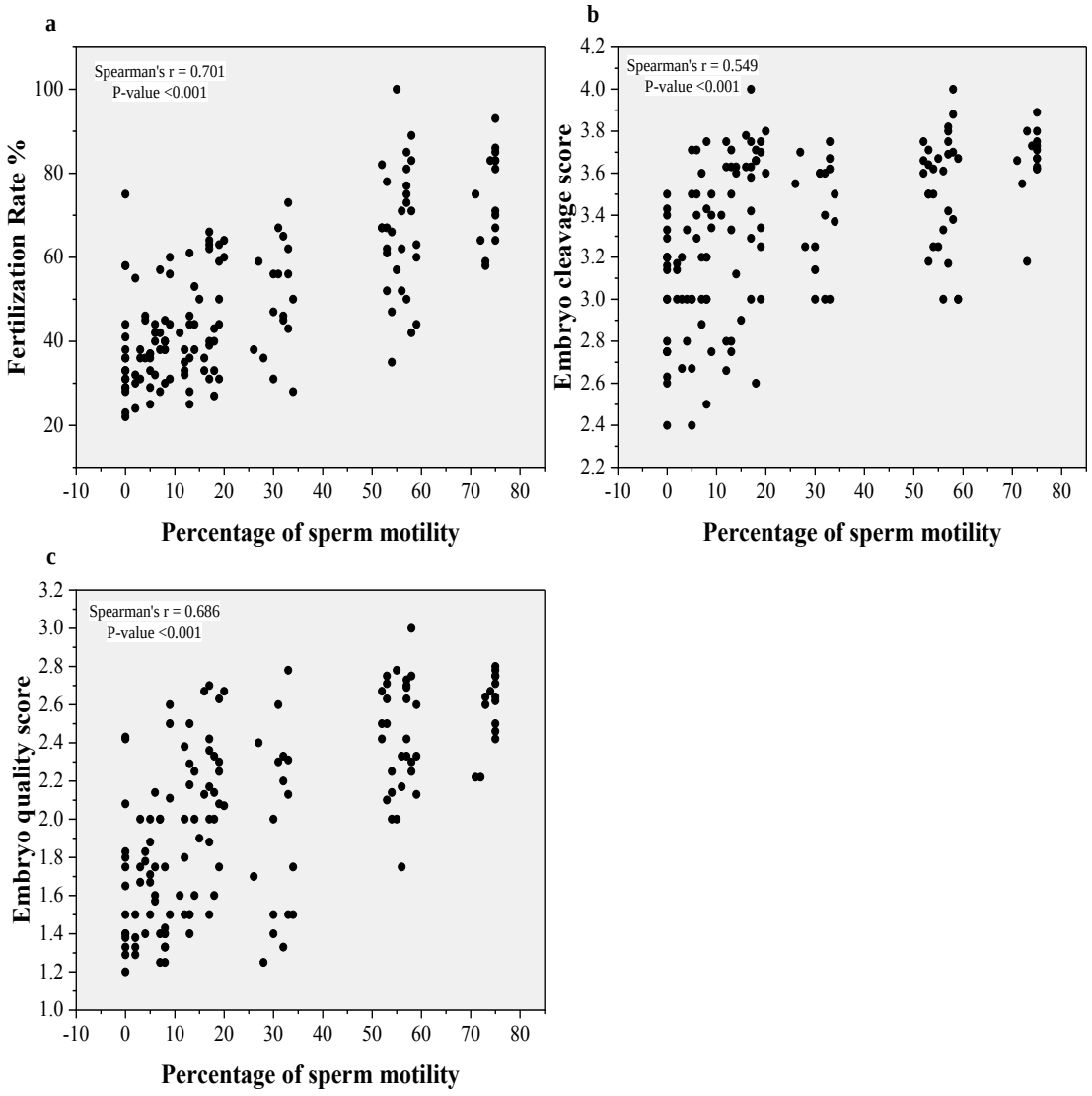


Figure 3.1: Scatterplots showing the correlations between sperm motility and ICSI outcomes: (a) fertilization rate. (b) embryo cleavage score.(c) embryo quality score. Spearman's correlation coefficients and significance levels were included in each figure.

### 3.2. Correlations between the (ICSI) outcomes

Figure 3.2 shows the correlation between all ICSI outcomes in the studied samples (n=150). These outcomes include the fertilization rate, the embryo cleavage score and the embryo quality score. The fertilization rate was significantly positively correlated with the embryo cleavage score (r=0.590, p<0.001) and the embryo quality score (r=0.745, p<0.001). The embryo quality score was significantly positively correlated with the embryo cleavage score (r=0.692, p<0.001).

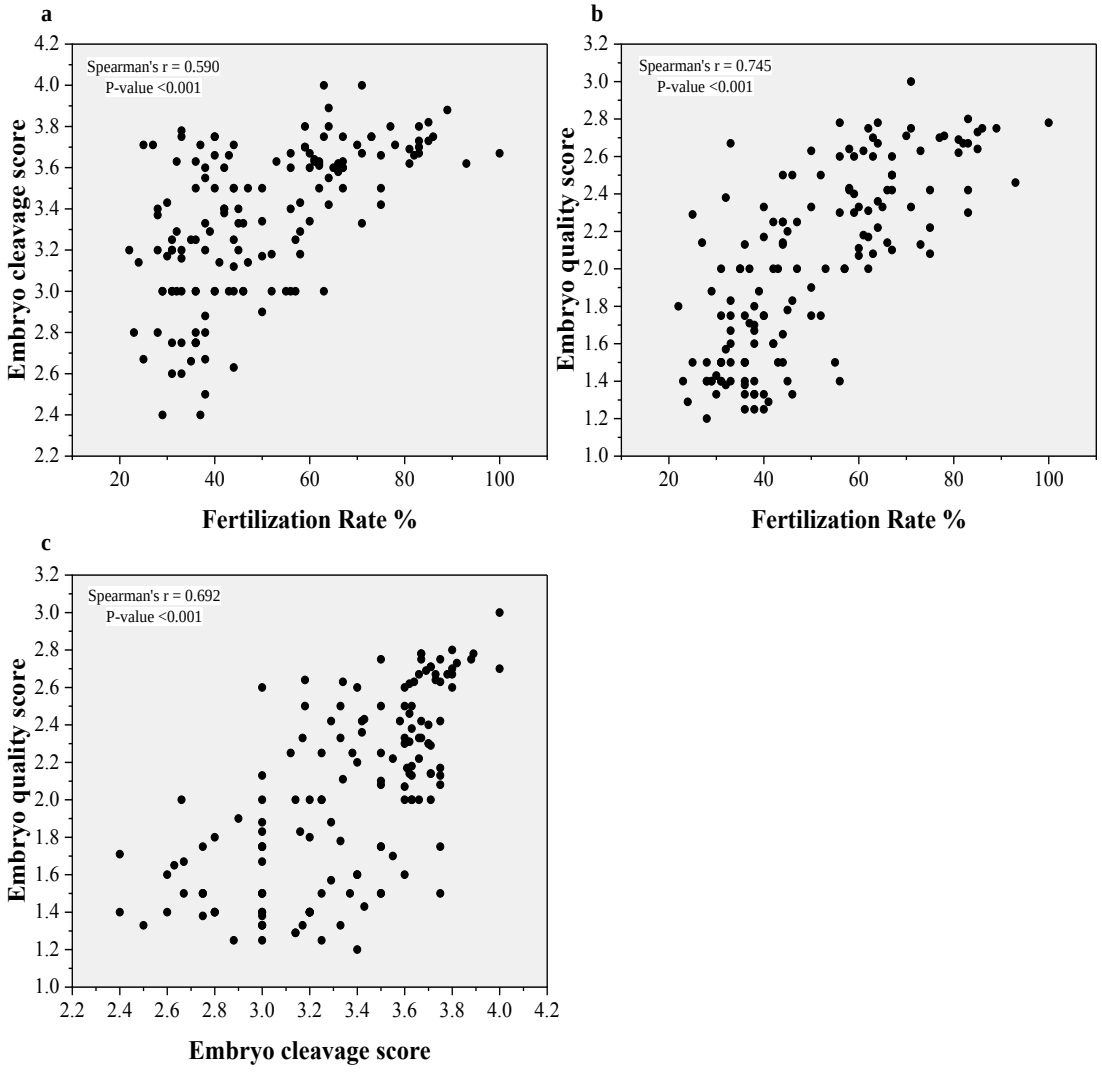


Figure 3.2: Scatterplots of the correlations between ICSI outcomes (a) scatterplots of the fertilization rate and the embryo cleavage score. (b) Scatter plots of fertilization rate and the embryo quality score. (c) scatterplots of the embryo cleavage score and the embryo quality score. Spearman's correlation coefficients and significance levels were included in each figure.

### 3.3. (ICSI) outcomes among the studied groups

#### 3.3.1. The fertilization rate among the studied groups

The median of the fertilization rate (G1 (36±1.86), G2 (40±1.63), G3 (47±13.41), and the control (67±14.69) p< 0.001), as illustrated in (Figure 3.3). See supplementary (Table 6.1).

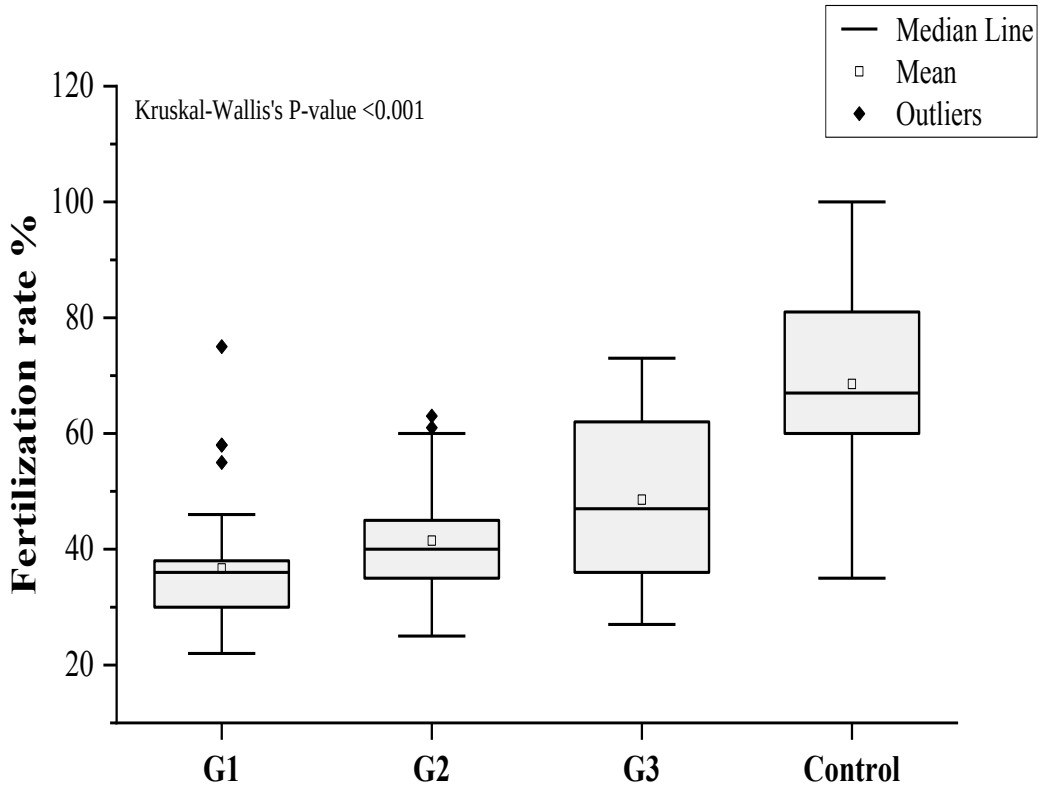


Figure 3.3 The fertilization rate among the studied groups ( 1, 2, 3 and the control), the Kruskal-Wallis's P-value, was included, the Mann-Whitney P-values for the differences in the medians between all groups <0.001

#### 3.3.2. The embryo cleavage score among the studied groups

The median of the embryo cleavage score (G1 (3±0.31), G2 (3.34±0.36), G3 (3.58±0.31), control (3.66±0.25) p< 0.001), as illustrated in (Figure 3.4). See supplementary (Table 6.2).

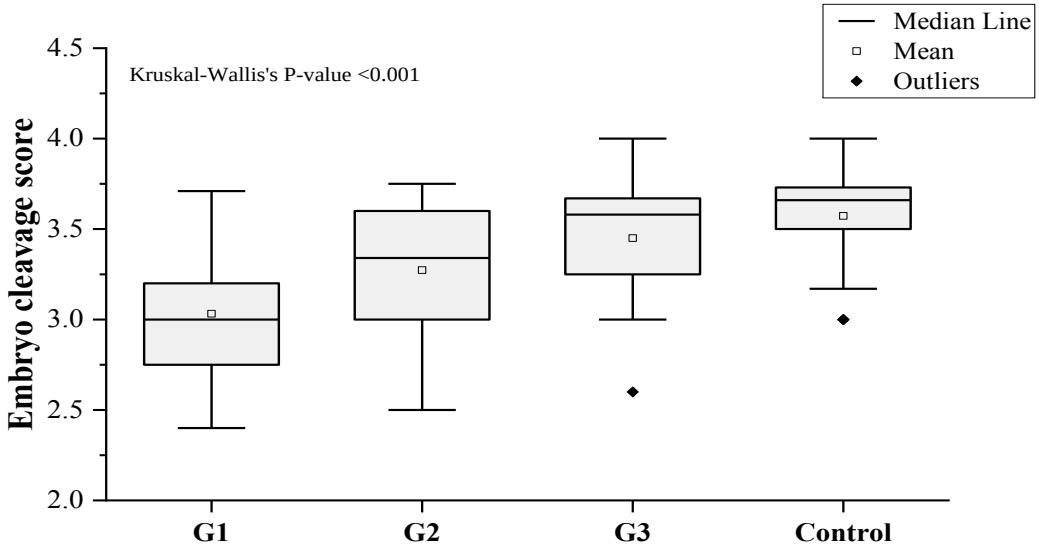


Figure 3.4: The embryo cleavage score among the studied groups ( 1, 2, 3 and the control), the Kruskal-Wallis's P- value was included, Mann-Whitney P-values for the differences in the medians between all groups <0.001

### 3.3.3. The embryo quality score among the studied groups

The median of the embryo quality score (G1 (1.5±0.31), G2 (1.75±0.39), G3 (2.14±0.44), control (2.5±0.27) p< 0.001), as illustrated in (Figure 3.5).

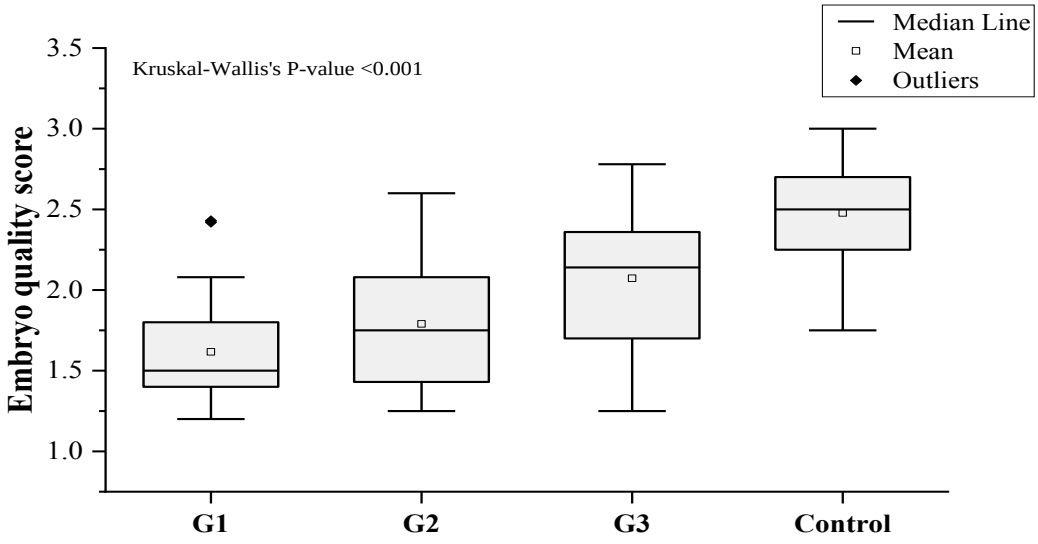


Figure 3.5: The embryo quality score among the studied groups ( 1, 2, 3 and the control), the Kruskal-Wallis's P- value was included, Mann-Whitney P-values for the differences in the medians between all groups <0.001

### 3.4. The correlation between protein carbonyl and sperm motility

The protein carbonyl levels and sperm motility were tested for correlation in all the studied samples (n=150, as illustrated in (Figure 3.6) below. The protein carbonyl levels were significantly negatively correlated with sperm motility (r=-0.894, *P*<0.001).

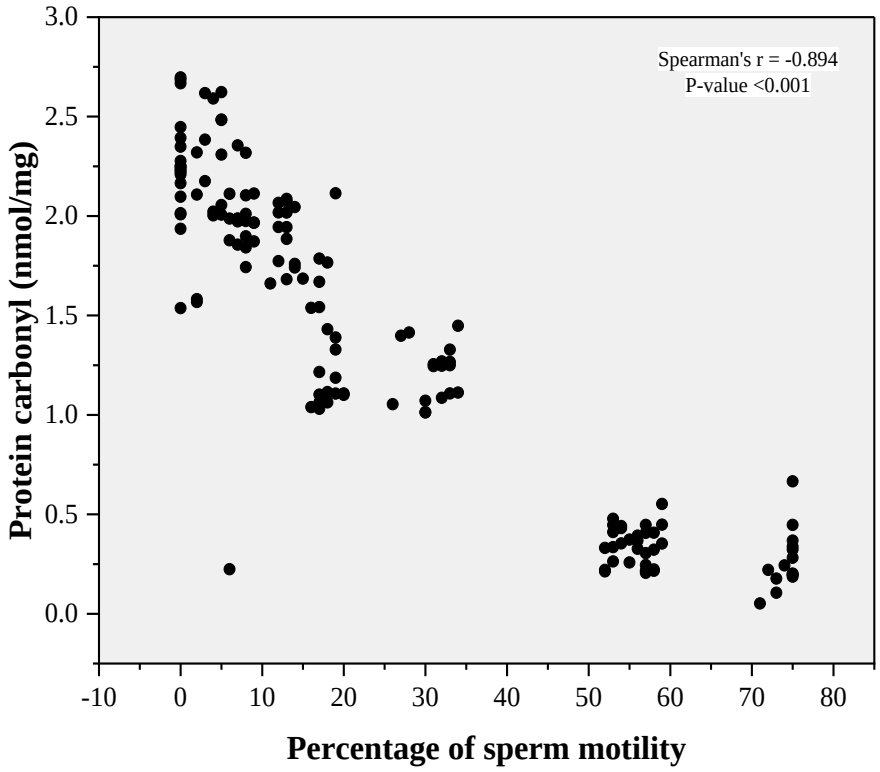


Figure 3.6: The scatterplot shows a strong negative correlation between the protein carbonyl levels and the percentage of sperm motility ( r = - 0.894, P < 0.001).

### 3.5. Protein carbonyl among the studied groups

The medians of the protein carbonyl levels among the groups were: (G1 (2.24±0.3), G2 (1.97±0.17), G3 (1.22±0.2), and the control (0.32±0.12) p< 0.001), as illustrated in (Figure 3.7) below. The highest levels of protein carbonyl (2.24± 0.30) were in group one, which included asthenozoospermic men with a percentage of motile sperm that ranged between 0 % to 5 %, while the lowest protein carbonyl levels among the patient groups were in group three, which included asthenozoospermic men with a percentage of motile sperm

that ranged between 16% to 35%. Furthermore the control group which included normozoospermic men with a percentage of motile sperm that ranged between 50% to 75% had the lowest level of protein carbonyl (0.32 ± 0.12) compared to all patient groups.

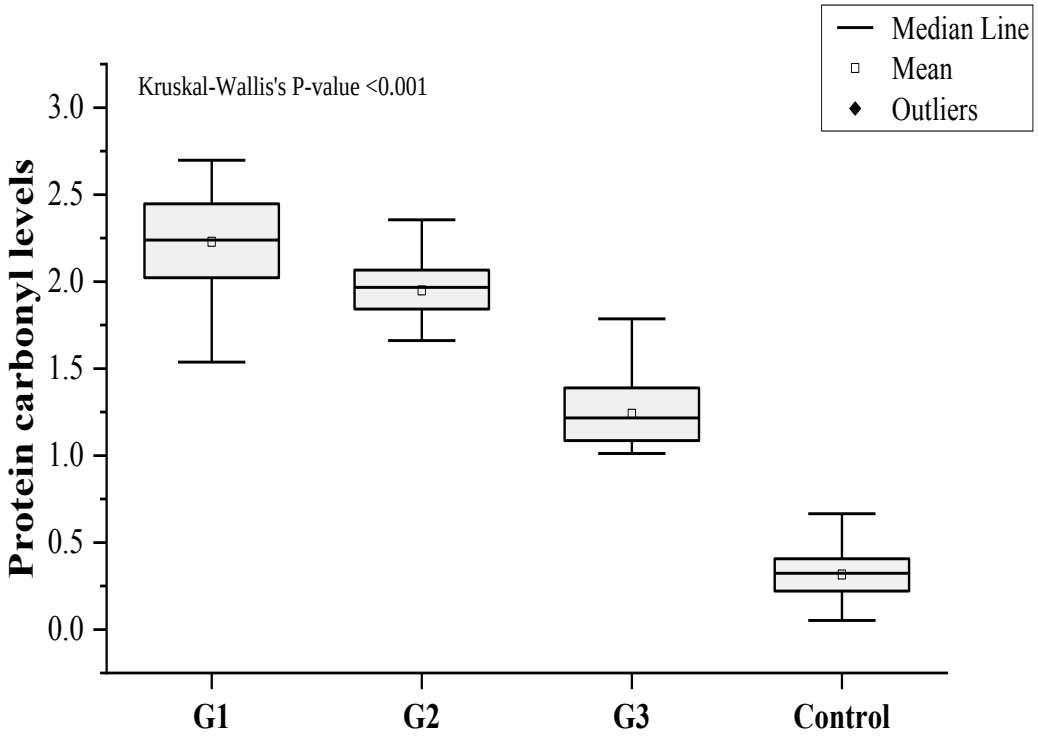


Figure 3.7: Protein carbonyl levels among groups 1, 2, 3 and the control, Kruskal-Wallis's P- value was included, the Mann-Whitney P-values for the differences in the medians between G1and G2(0.009), G1 and G3(<0.001), G1 and the control (<0.001)l, G2 and G3(30.029), G2 and the control(<0.001) , G3 and the control(<0.001).

### 3.6. Effect of protein carbonyl on ICSI outcomes

The protein carbonyl levels and the ICSI outcomes were tested for correlations in all studied samples (n=150). These outcomes included the fertilization rate, the embryo cleavage score and the embryo quality score. As illustrated in (Figure 3.8), the protein carbonyl levels were negatively correlated with the fertilization rate (r = - 0.670, *P* <0.001), the embryo cleavage score (r = - 0.511, *P* <0.001) and the embryo quality score (r = - 0.623, *P* <0.001) .

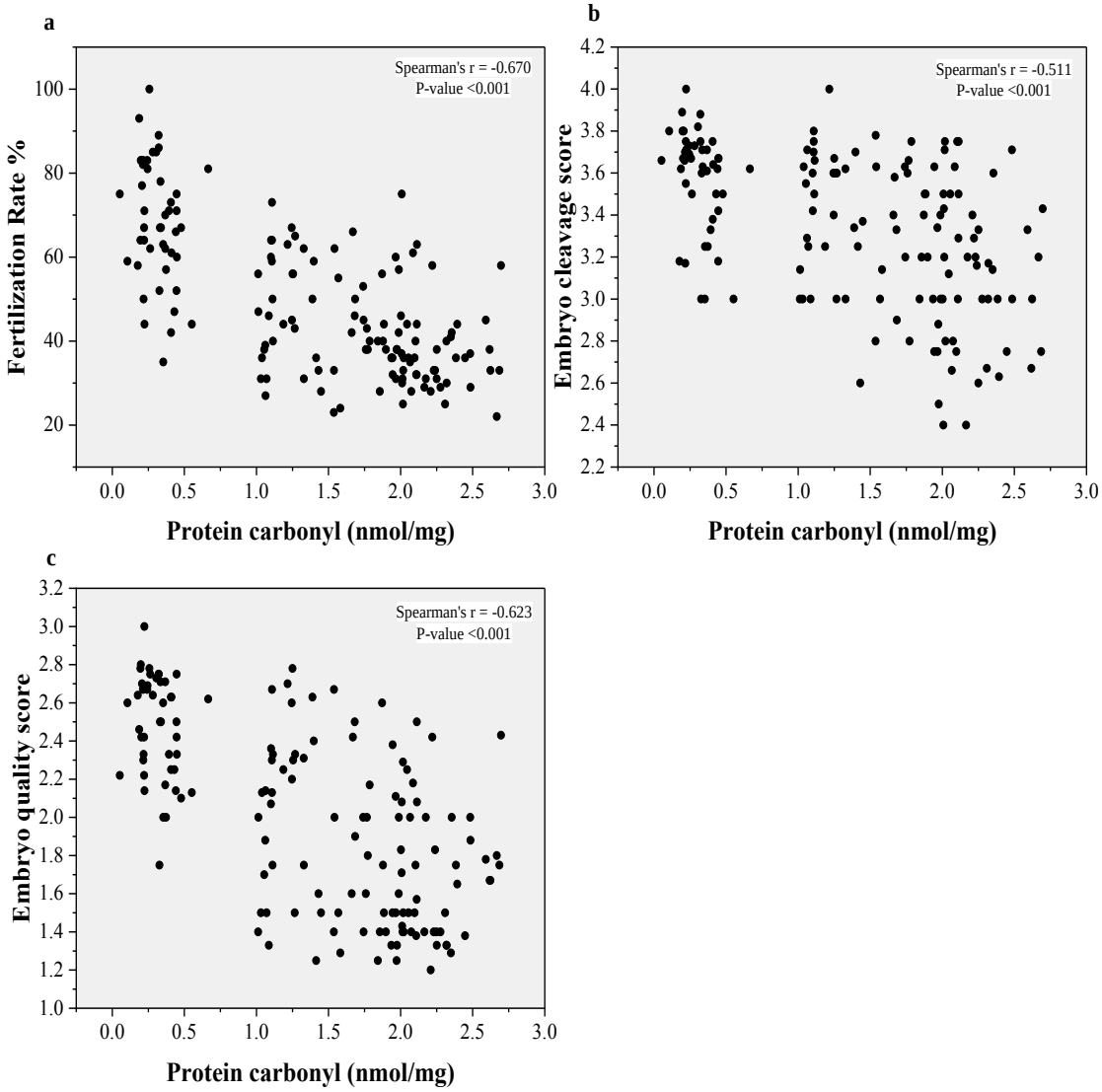


Figure 3.8: Scatter plots of protein carbonyl levels and ICSI outcomes (a) Scatter plots of the protein carbonyl levels and the fertilization rate (b) Scatter plots of the protein carbonyl levels and the embryo cleavage score (c) Scatter plots of the protein carbonyl levels and the embryo quality score. Spearman's correlation coefficients and the significance levels were included in each figure.

See supplementary tables for chapter 3.6 effect of protein carbonyl on ICSI outcomes (Table 6.3), (Table 6.4), (Table 6.5) and (Table 6.6).

## 3.7. DNA sequence analysis for the *ND1* gene

The *ND1* gene is located between 3307 and 4262 on mtDNA with a length of 956 nt and a 318 amino-acid protein length. A total of 29 variants in the *ND1* gene were identified; six of them were missense, while 23 were synonymous (Table 3.1).

Table 3.1: The mtDNA variants identified in the *ND1* gene

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in Normo-zoospermia | Frequency of variant in Astheno_ zoospermia | G Test | P Value |
|---|---|---|---|---|---|---|
| 1 | 3316 G>A | Ala>Thr | 0/45 | 5/105 | 3.64 | 0.056 |
| 2 | 3348 A>G | Leu>Leu | 2/45 | 3/105 | 0.234 | 0.629 |
| 3 | 3480 A>G | Lys>Lys | 2/45 | 6/105 | 0.104 | 0.747 |
| 4 | 3462 C>T | Ala>Ala | 0/45 | 3/105 | 2.166 | 0.141 |
| 5 | 3537 A>G | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 6 | 3594 C>T | Val>Val | 1/45 | 3/105 | 0.051 | 0.822 |
| 7 | 3720 A>G | Gln>Gln | 0/45 | 1/105 | 0.716 | 0.397 |
| 8 | 3741 C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 3826 T>C | Leu > Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 3882 G>A | Gln>Gln | 0/45 | 2/105 | 1.438 | 0.23 |
| 11 | 3921 C>T | Ser>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 12 | 4086 C>T | Val>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 13 | **4216 T>C*** | Tyr > His | 0/45 | 10/105 | 7.436 | **0.006*** |
| 14 | 4017 C>T | Leu> Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 15 | 3705 G>A | Leu>Leu | 0/45 | 3/105 | 2.166 | 0.141 |
| 16 | 3505 A>G | Thr > Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 17 | 4104 A>G | Leu>Leu | 1/45 | 4/105 | 0.266 | 0.606 |
| 18 | 3847 T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 19 | 3834 G>A | Leu>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 20 | 3843 A>G | Trp > Trp | 0/45 | 1/105 | 0.716 | 0.397 |
| 21 | 3819 C>T | His > His | 0/45 | 1/105 | 0.716 | 0.397 |
| 22 | 3335 T>C | Ile > Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 23 | 3396 T>C | Tyr > Tyr | 0/45 | 1/105 | 0.716 | 0.397 |
| 24 | 3483 G>A | Glu> Glu | 0/45 | 1/105 | 0.716 | 0.397 |
| 25 | 3666 G>A | Gly> Gly | 1/45 | 0/105 | 2.424 | 0.12 |
| 26 | 3915 G>A | Gly > Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 27 | 3992 C>T | Thr > Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 28 | 3593 T>C | Val > Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 29 | 3513 C>T | Thr > Thr | 1/45 | 1/105 | 0.354 | 0.552 |

*: statistically significant difference, *p*-value <0.05

### 3.7.1. The frequency of total variants in the *ND1* gene among the studied groups

The median of frequency of total variants in the *ND1* gene (G1 (1±0.83), G2 (0±0.74), G3 (0±0.24), and the control (0±0.34) p< 0.001), as illustrated in (Figure 3.9). The Mann-Whitney P-values for the differences in the medians between all the groups were significant except for the difference in the medians between G3 and the control ( *P*=0.267 ).

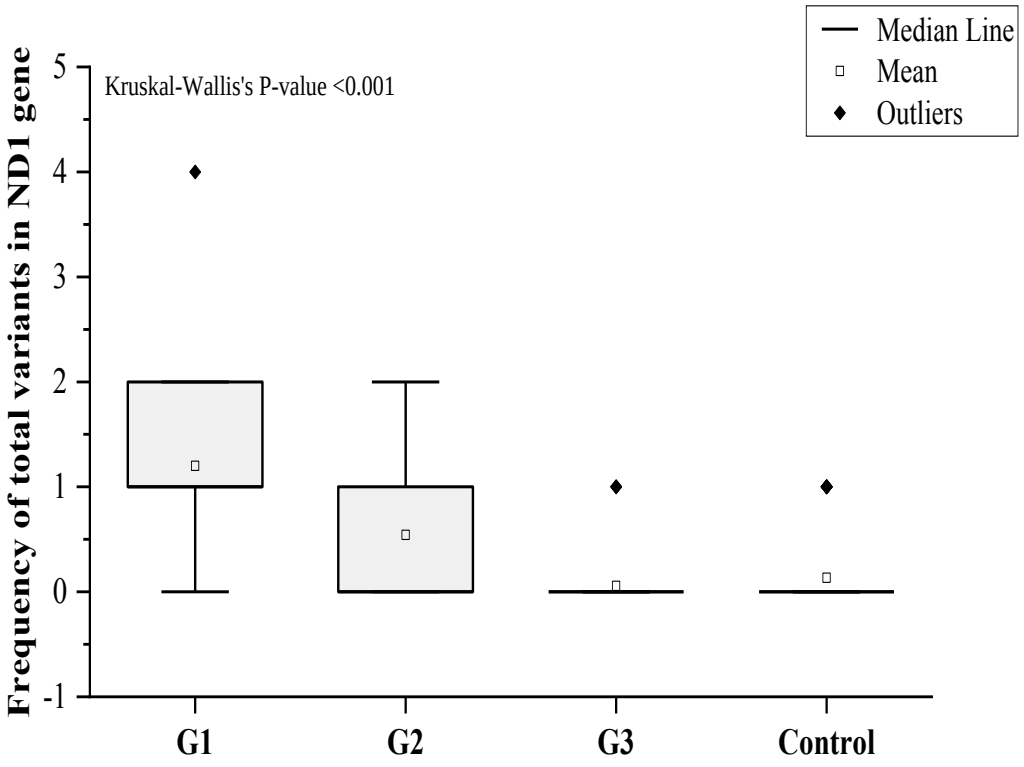


Figure 3.9: Box plots showing the frequency of total variants in the *ND1* gene among the groups. Kruskal-Wallis's P- value was included, and the Mann-Whitney P-values for the differences in the medians between G1 and G2(0.001), G1 and G3(<0.001), G1 and the control(<0.001),G2 and G3(0.001), G2 and the control(<0.001) , G3 and the control were (0.267 ).

### 3.7.2. Identification of the missense variant 4216 T>C in the *ND1* gene

Only one variant 4216 T>C (rs1599988) was significantly different between cases and controls (*P*-value = 0.006). This variant caused Tyr>His amino acid substitution (Figure 3.10). It was identified among 10 asthenozoospermic patients in a homoplasmic state (Figure 3.11).

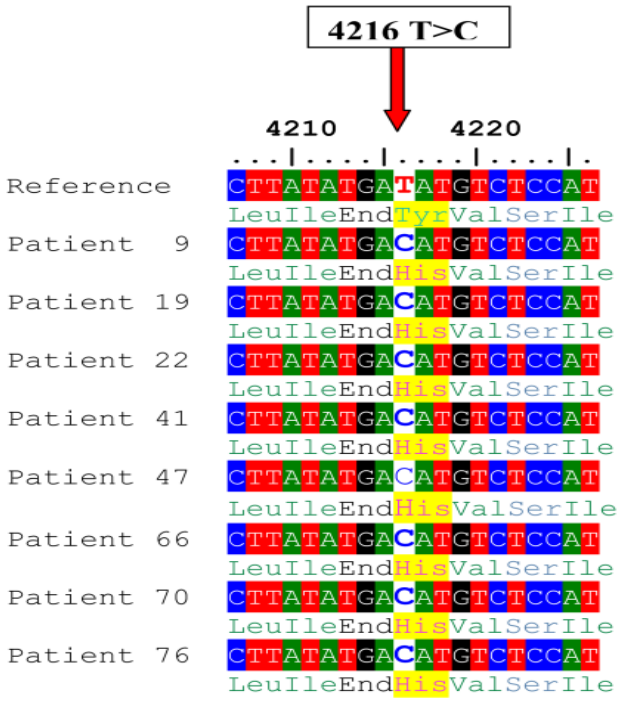


Figure 3.10: Alignment of the ND1 gene sequence for 8 patients showing a 4216 T>C nucleotide substitution. The red arrow indicates the site of the nucleotide substitution, and the highlighted yellow colour indicates the amino acid replacement (Tyr>His).

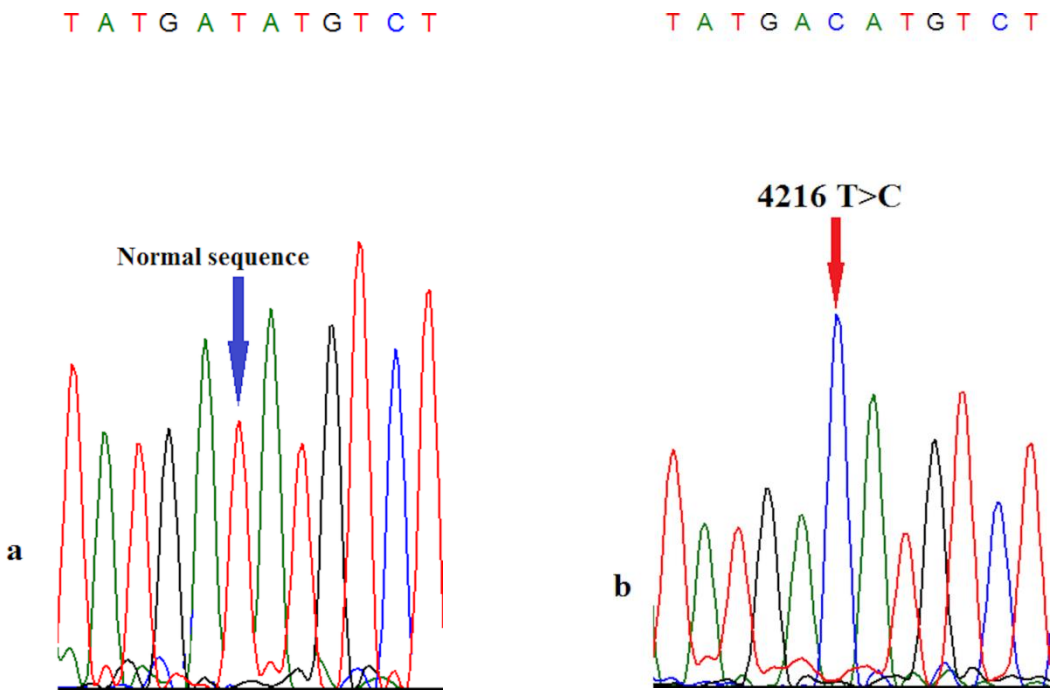


Figure 3.11: Chromatograms showing the difference between normal sequence and 4216 T>C (a) shows the chromatogram for a normozoospermic man, the blue arrow indicates for thymine as normal sequence at nt 4216, while ( b) shows the chromatogram for an asthenozoospermic patient, and the red arrow indicates the site of 4216 T>C where thymine is replaced by cytosine.

According to ACMG and Poly Phenyl-2, it is predicted that this variant is benign (score: 0.001, sensitivity: 0.99; specificity: 0.15) (Figure 3.12).

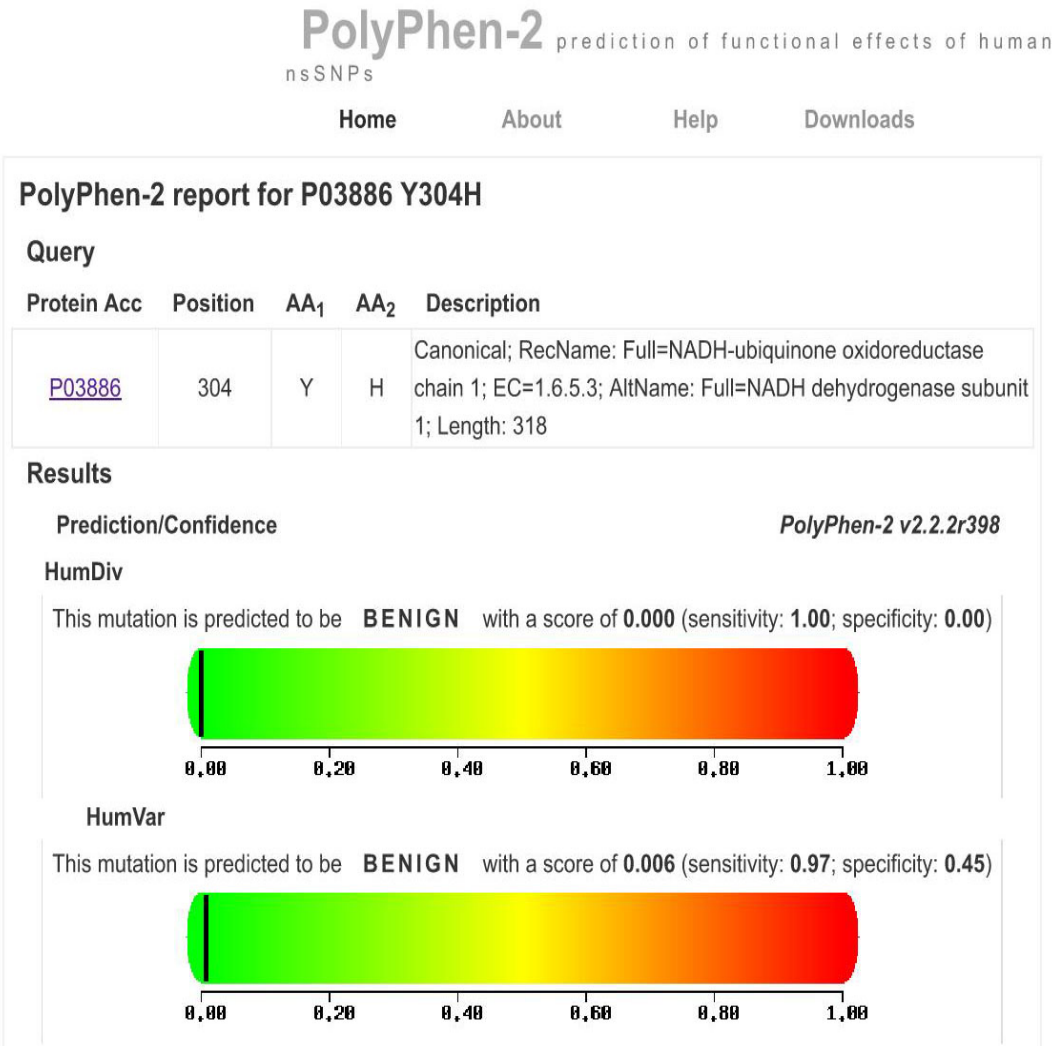


Figure 3.12: Prediction of the functional effect of 4216 T>C by Poly Phenyl-2 software. It is predicted that this variant is benign (score: 0.006, sensitivity: 0.97; specificity: 0.45).

4216 T>C was associated with a decline in sperm motility and ICSI outcomes. The medians of sperm motility (the wild type (17.5±25.21), 4216 T>C (7±6.1), $P$ = 0.012), the fertilization rate (wild type (45.5±18.08, 4216 T>C (36±7.61), $P$ = 0.013), the embryo cleavage score (wild type (3.42±0.37), 4216 T>C (3.07±0.12), $P$=0.044), and the embryo quality score (wild type (2.11±0.49), 4216 T>C (1.69±0.41), $P$ = 0.041) (Figure 3.13).

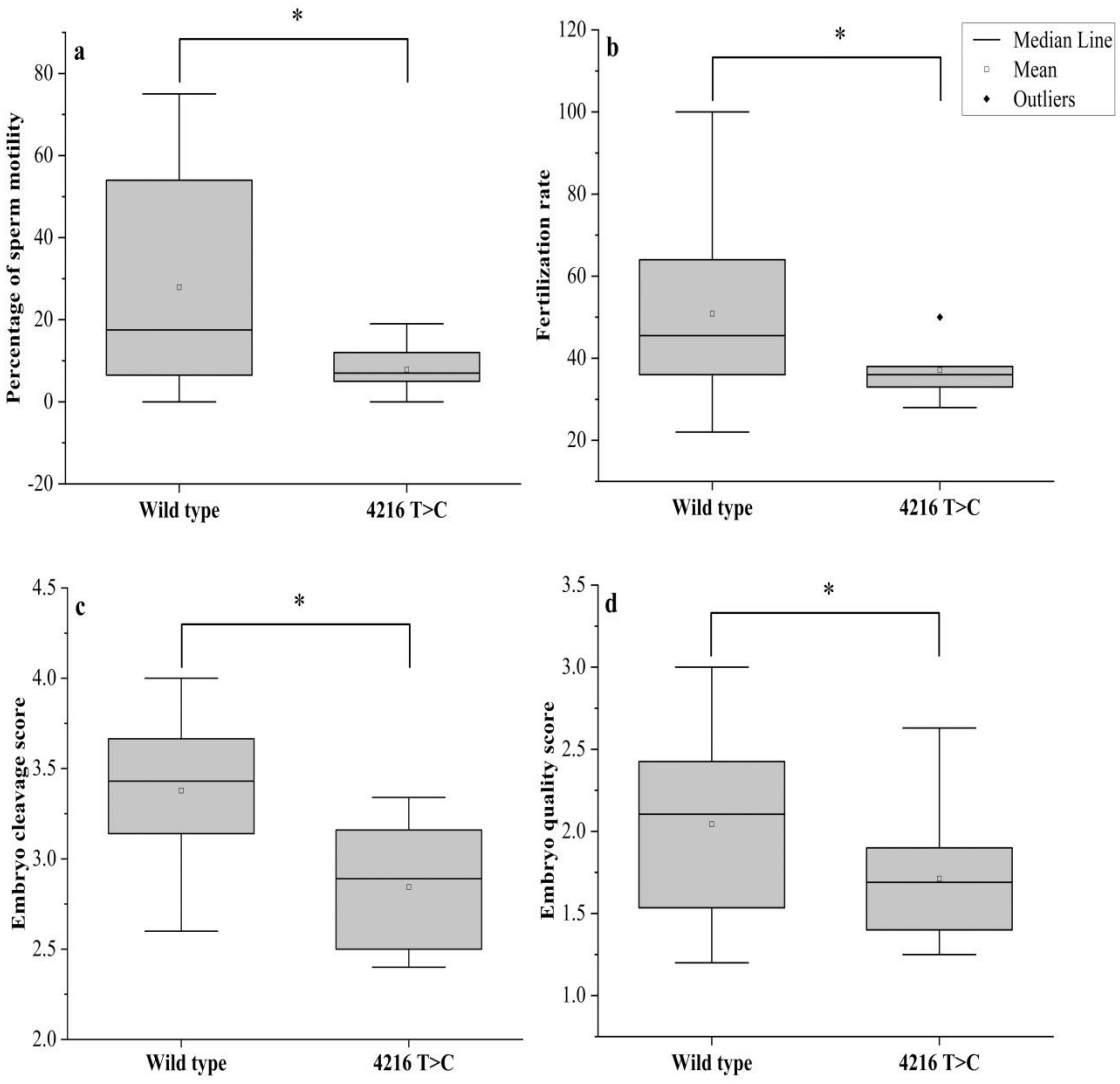


Figure 3.13: Box plots show the differences between the medians of men with or without the 4216 T>C. The Mann-Whitney P-values for the differences in the medians were (a) sperm motility (p=0.012), (b) fertilization rate(0.013), (c) embryo cleavage score(p=0.044) and (d) embryo quality score(p=0.041). *P < 0.05.

### 3.7.3. Correlation between the frequency of total variants in the *ND1* gene with sperm motility and ICSI outcomes

The frequency of total variants in the *ND1* gene was negatively correlated with the sperm motility, the fertilization rate, the embryo cleavage score and the embryo quality score (r = - 0.541, *P* <0.001), (r = - 0.466, *P* <0.001), (r = - 0.401, *P* <0.001) and (r = - 0.503, *P* <0.001) respectively (Figure 3.14).

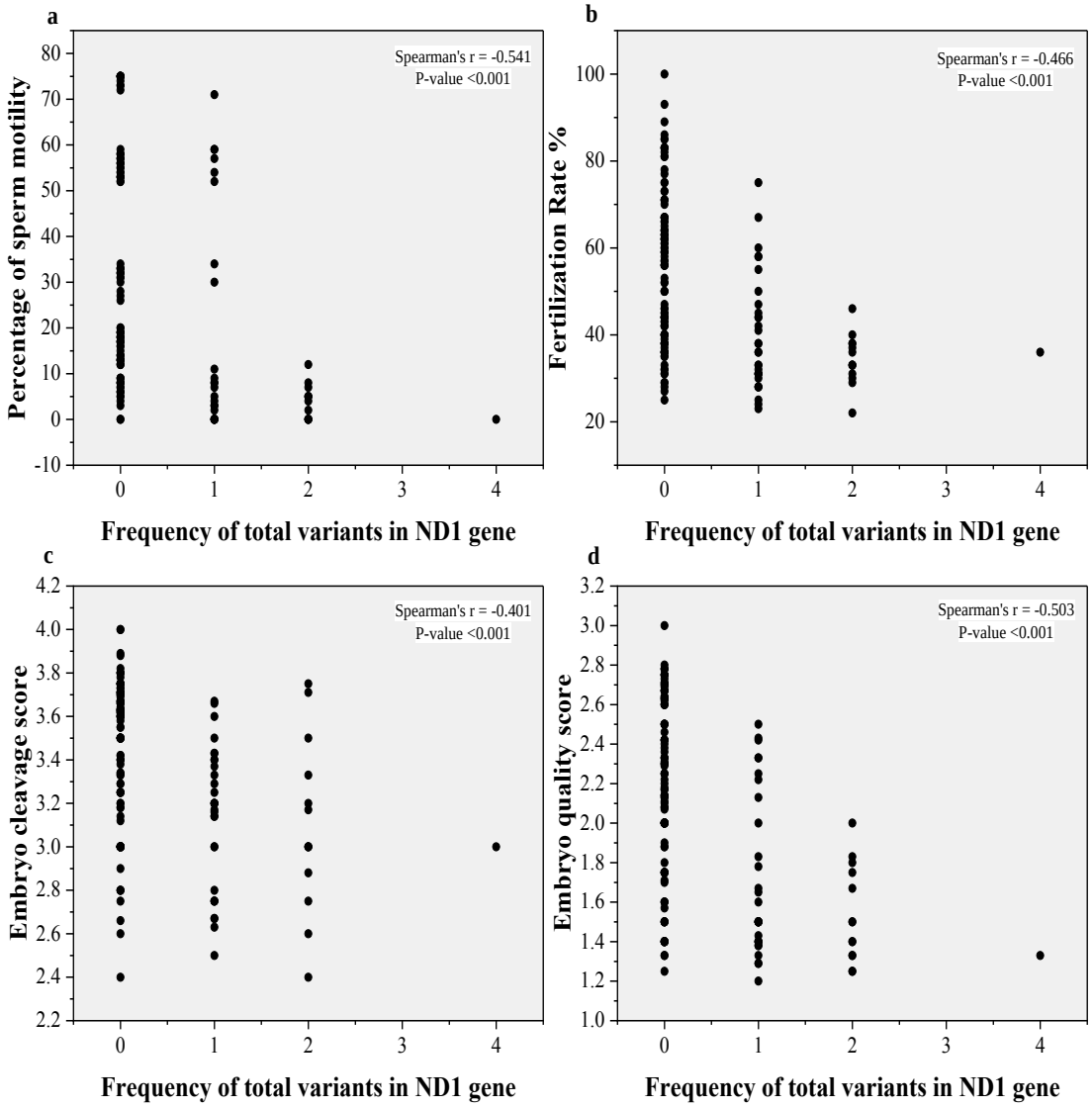


Figure 3.14: Scatter plots of the frequency of total variants in the *ND1* gene with (a) the sperm motility. (b) the fertilization rate (c) the embryo cleavage score (d) the embryo quality score. Spearman's correlation coefficients and significance levels are included in each figure.

### 3.8. DNA sequence analysis for *ND2* gene

The *ND2* gene is located between 4470 and 5511 on mtDNA with a length of 1042 nt and a 347 amino-acid protein length. A total of 21 nucleotide substitutions in the *ND2* gene were identified, six of which were missense and 15 of which were synonymous (Table 3.2). However, none of these variants were significantly different between asthenozoospermic and the control groups (P-value > 0.05).

Table 3.2: The mtDNA variants identified in the *ND2* gene

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in Normo-zoospermia | Frequency of variant in Astheno-zoospermia | G Test | P Value |
|---|---|---|---|---|---|---|
| 1 | 4769 A>G | Met > Met | 2/45 | 12/105 | 2.061 | 0.151 |
| 2 | 4917 A>G | Asn > Asp | 1/45 | 11/105 | 3.601 | 0.058 |
| 3 | 4733 T>C | Asn > Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 4 | 4967 T>C | Ser > Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 5 | 4973 T>C | Gly > Gly | 0/45 | 2/105 | 1.438 | 0.23 |
| 6 | 4991 G>A | Gln > Gln | 1/45 | 4/105 | 0.266 | 0.606 |
| 7 | 5178 C>A | Leu > Met | 0/45 | 2/105 | 1.438 | 0.23 |
| 8 | 4646 T>C | Tyr > Tyr | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 5302 T>C | Ile > Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 5417 G>A | Gln > Gln | 0/45 | 1/105 | 0.716 | 0.397 |
| 11 | 5237 G>A | Pro > Pro | 1/45 | 1/105 | 0.354 | 0.552 |
| 12 | 5331 C>A | Leu > Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 13 | 4561 T>C | Val >Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 14 | 4823 T>C | Val > Val | 1/45 | 4/105 | 0.266 | 0.606 |
| 15 | 5147 G>A | Thr > Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 16 | 4639 T>C | Ile > Thr | 0/45 | 2/105 | 1.438 | 0.23 |
| 17 | 4883 C>T | Pro > Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 18 | 4703 T>C | Asn > Asn | 1/45 | 0/105 | 2.424 | 0.12 |
| 19 | 5004 T>C | Leu > Leu | 1/45 | 0/105 | 2.424 | 0.12 |
| 20 | 4640 C>A | Ile > Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 21 | 5048 T>C | Val > Val | 1/45 | 1/105 | 0.354 | 0.552 |

### 3.8.1. The frequency of total variants in *ND2* gene among the studied groups

The frequency of the total variants in the *ND2* gene among groups one and two was higher compared to group three and the control, based on the Kruskal-Wallis test. But there was a significant difference between the sample medians among groups $P$ <0.001. Furthermore, according to Mann-Whitney, there was a significant difference between the medians of both

group one and group two, compared with the with control, while there was no significant difference between the medians of G3 with the control, with the Mann-Whitney P-values for the differences in the medians being between G1and G2, G1 and G3, G1 and the control,G2 and G3, G2 and the control, G3 and the control were 0.006, <0.001, <0.001, 0.443, 0.047 and 0.254 respectively (Figure 3.15).

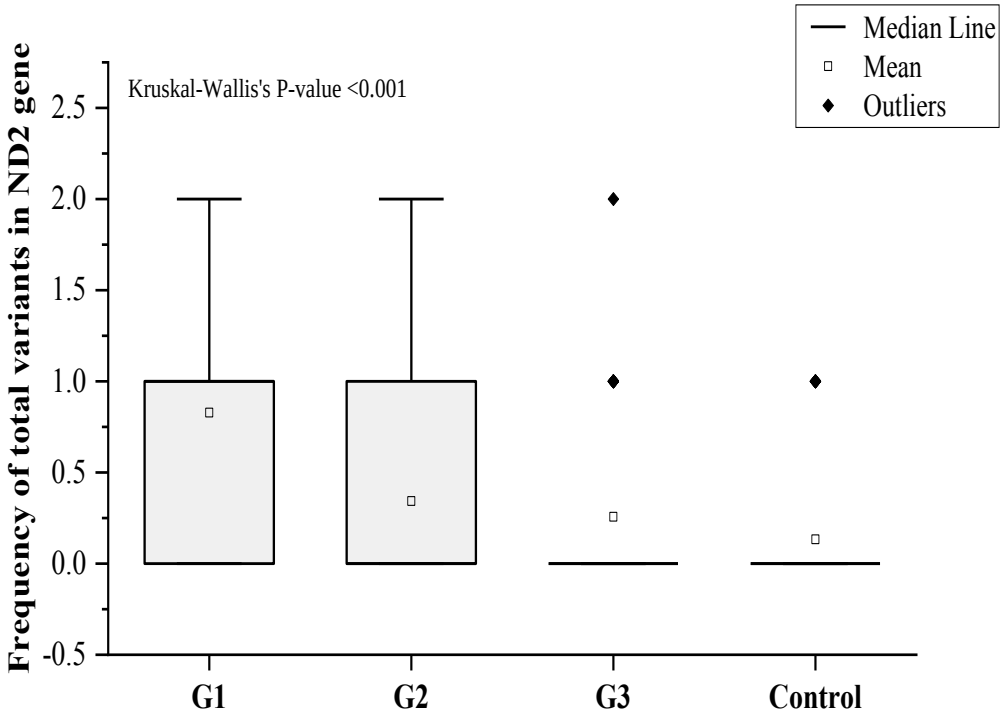


Figure 3.15: The box plots show the frequency of total variants in the *ND2* gene among the groups; Kruskal-Wallis's P- value was included, and the Mann-Whitney P-values for the differences in the medians between groups were: G1and G2 (P= 0.006), G1 and G3 (P<0.001), G1 and control (P<0.001), G2 and G3 (P= 0.443), G2 and control (0.047), G3 and the control (P= 0.254).

### 3.8.2. Correlation between frequency of total variants in *ND2* gene with sperm motility and ICSI outcomes

The frequency of the total variants in the *ND2* gene was negatively correlated with the sperm motility (r = - 0.388, *P* <0.001), the fertilization rate (r = - 0.367, *P* <0.001), the embryo cleavage score (r = - 0.349, *P* <0.001) and the embryo quality score (r = - 0.450, *P* <0.001) (Figure 3.16).

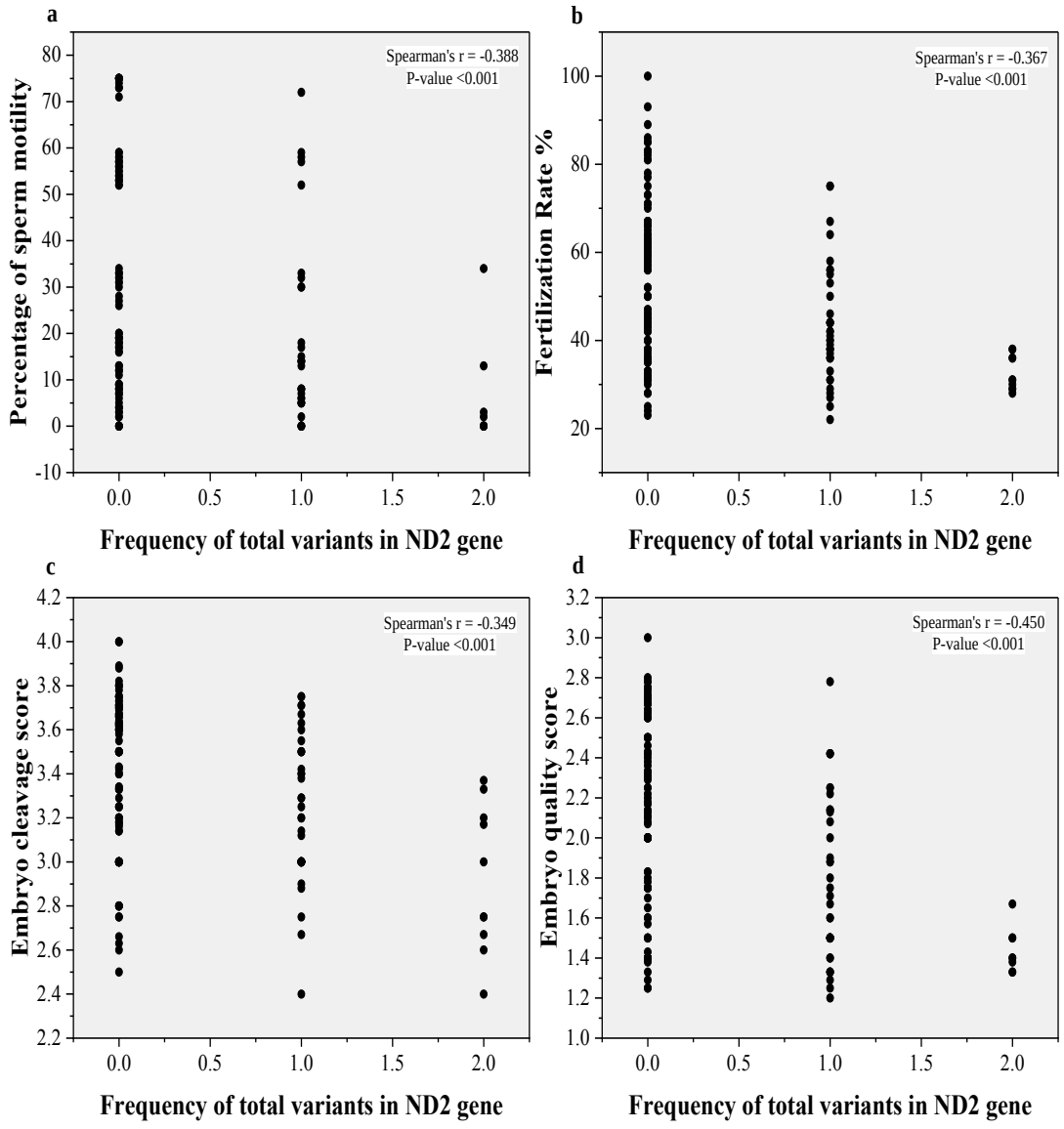


Figure 3.16: Scatter plots of the frequency of the total variants in the *ND2* gene with (a) sperm motility. (b) fertilization rate (c) embryo cleavage score (d) embryo quality score. Spearman's correlation coefficients and significance levels were included in each figure

### 3.9. DNA sequence analysis for *ND5* gene

The *ND5* gene is located between 12337 and 14148 on mtDNA with a length of 1812 nt and a 603 amino-acid protein length. A total of 19 missense variants and 37 synonymous variants were identified in the *ND5* gene (Table 3.3).

Table 3.3: The mtDNA variants identified in the *ND5* gene

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in Normozoospermia | Frequency of variant in Asthenozoospermia | G Test | P Value |
|---|---|---|---|---|---|---|
| 1 | **13708 G>A*** | Ala>Thr | 1/45 | 15/105 | 6.131 | **0.013*** |
| 2 | 13879 T>C | Ser>Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 3 | 13965 T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 4 | 13966 A>G | Thr>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 5 | 13967 C>T | Thr>Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 6 | 13928G>C | Ser>Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 7 | 13734 T>C | Phe>Phe | 0/45 | 1/105 | 0.716 | 0.397 |
| 8 | 14040 G>A | Gln>Gln | 0/45 | 4/105 | 2.9 | 0.089 |
| 9 | 14070 A>G | Ser>Ser | 1/45 | 3/105 | 0.051 | 0.822 |
| 10 | 13650 C>T | Pro>Pro | 0/45 | 3/105 | 2.166 | 0.141 |
| 11 | 13752 T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 12 | 13803 A>G | Thr>Thr | 0/45 | 2/105 | 1.438 | 0.23 |
| 13 | 14059 A>G | Ile>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 14 | 13780 A>G | Ile>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 15 | 14053 A>G | Thr>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 16 | 14110 T>C | Phe>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 17 | 13762 T>G | Ser>Ala | 0/45 | 2/105 | 1.438 | 0.23 |
| 18 | 12372G>A | Leu>Leu | 6/45 | 18/105 | 0.35 | 0.554 |
| 19 | 12705C>T | Ile>Ile | 0/45 | 14/105 | 10.593 | 0.001 |
| 20 | 12850A>G | Ile>Val | 0/45 | 3/105 | 2.166 | 0.141 |
| 21 | 12822A>G | Ala>Ala | 0/45 | 4/105 | 2.9 | 0.089 |
| 22 | 12406 G>A | Val>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 23 | 13722 A>G | Leu>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 24 | 12346C>T | His>Tyr | 0/45 | 2/105 | 1.438 | 0.23 |
| 25 | 12403C>T | Leu>Phe | 0/45 | 2/105 | 1.438 | 0.23 |
| 26 | 12414T>C | Pro>Pro | 0/45 | 2/105 | 1.438 | 0.23 |
| 27 | 12612A>G | Val>Val | 0/45 | 8/105 | 5.898 | 0.015 |

Table 3.3 ( continued)

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in Normozoospermia | Frequency of variant in Asthenozoospermia | G Test | P Value |
|---|---|---|---|---|---|---|
| 28 | 12501G>A | Met>Met | 2/45 | 3/105 | 0.234 | 0.629 |
| 29 | 12693A>G | Lys>Lys | 0/45 | 1/105 | 0.716 | 0.397 |
| 30 | 12950A>G | Asn>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 31 | 12408T>C | Val>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 32 | 13368G>A | Gly>Gly | 1/45 | 13/105 | 4.83 | 0.028 |
| 33 | 13020T>C | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 34 | 13215T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 35 | 13702C>G | Arg>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 36 | 13392T>C | Asn>Asn | 0/45 | 2/105 | 1.438 | 0.23 |
| 37 | 13104A>G | Gly>Gly | 1/45 | 5/105 | 0.589 | 0.443 |
| 38 | 13422A>G | Leu>Leu | 0/45 | 2/105 | 1.438 | 0.23 |
| 39 | 13145G>A | Ser>Asn | 1/45 | 2/105 | 0.016 | 0.9 |
| 40 | 13326T>C | Cys>Cys | 0/45 | 1/105 | 0.716 | 0.397 |
| 41 | 13188C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 42 | 13590G>A | Leu>Leu | 0/45 | 2/105 | 1.438 | 0.23 |
| 43 | 13650C>T | Pro>Pro | 0/45 | 2/105 | 1.438 | 0.23 |
| 44 | 13188C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 45 | 13780A>G | Ile>Val | 1/45 | 0/105 | 2.424 | 0.12 |
| 46 | 13981C>T | Pro>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 47 | 14025T>C | Pro>Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 48 | 14034T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 49 | 12630G>A | Trp>Trp | 0/45 | 1/105 | 0.716 | 0.397 |
| 50 | 12654A>G | Trp>Trp | 0/45 | 1/105 | 0.716 | 0.397 |
| 51 | 12681T>C | Asn>Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 52 | 13542A>G | Ser>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 53 | 13617T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 54 | 13821C>T | Phe>Phe | 0/45 | 3/105 | 2.166 | 0.141 |
| 55 | **12506T>A*** | Leu>Gln | 0/45 | 6/105 | 4.386 | **0.036*** |
| 56 | 12879T>C | Gly>Gly | 0/45 | 3/105 | 2.166 | 0.141 |

*: statistically significant difference, *p*-value <0.05

### 3.9.1. The frequency of total variants in the *ND5* gene among the studied groups

The highest frequency of total variants in the *ND5* gene was among group one while the lowest frequency was among the control, based on the Kruskal-Wallis test. There is, however, a significant difference between sample medians among groups $P$ <0.001, According to the Mann-Whitney test, there were a significant differences between the medians of all groups, The Mann-Whitney P-values for the differences in the medians between G1and G2 ($P$<0.001), G1 and G3 ($P$<0.001), G1 and the control ($P$<0.001), G2 and G3 ($P$=0.033), G2 and control($P$<0.001), G3 and control ($P$=0.017) (Figure 3.17).

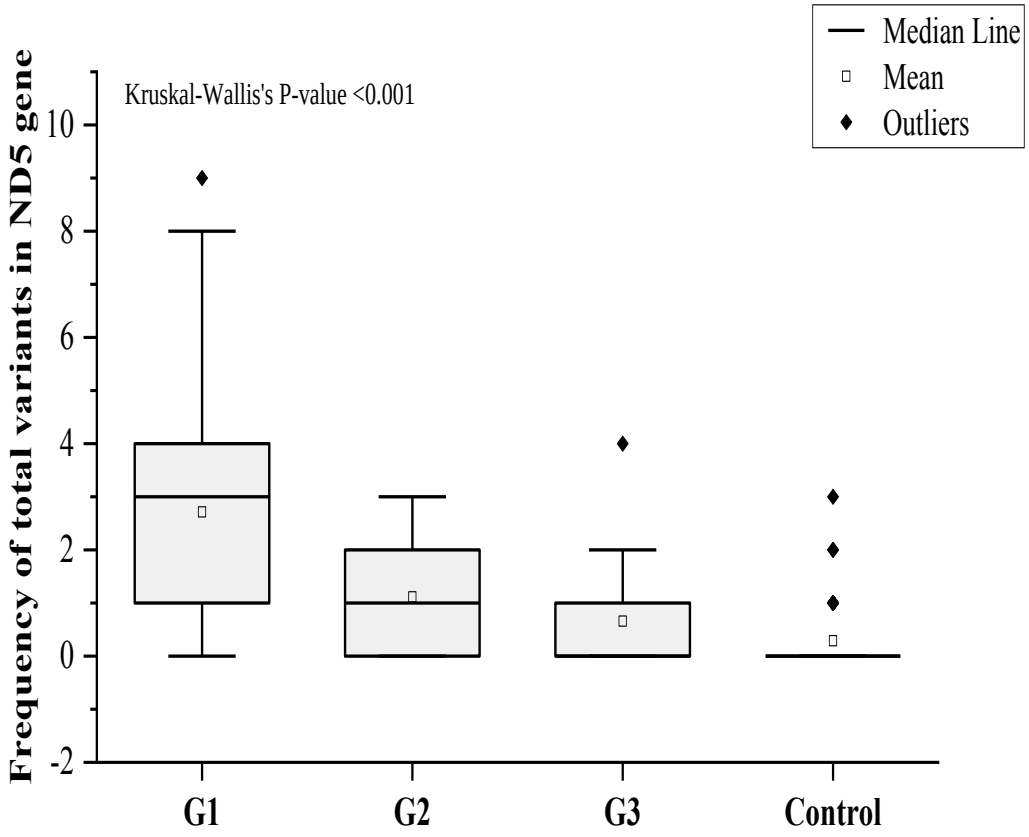


Figure 3.17: The box plots show the frequency of the total variants in the *ND5* gene among the groups; the Kruskal-Wallis's *P*-value was included.

### 3.9.2. Identification of the missense variant 12506 T>A in the *ND5* gene

All of these variants were previously reported in the NCBI https://www.ncbi.nlm.nih.gov/) and in the human mitochondrial DNA database (www.mitomap.org), except for a novel variant at the 12506 locus. The novel variant 12506T>A is a missense variant that replaced Gln by Leu (Figure 3.18). This variant was identified in 6 asthenozoospermic patients with a heteroplasmic state (Figure 3.19). This variant was significantly different between patients and the control groups ($P$ = 0.036).

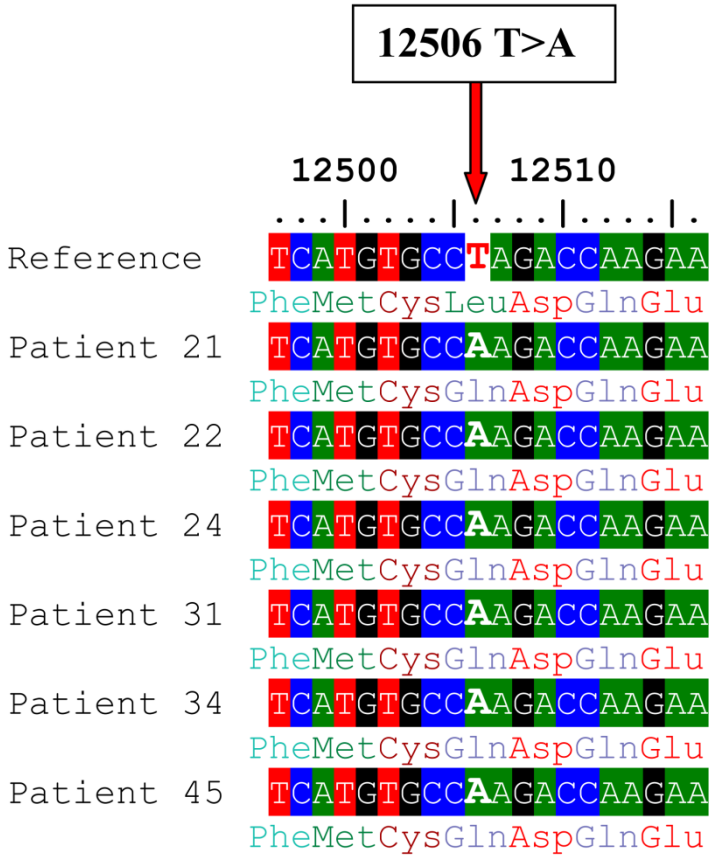


Figure 3.18: Alignment of *the ND5* gene sequence for 6 patients showing the novel 12506 T>A variant. The red arrow indicates the site of nucleotide substitution causing an amino-acid replacement (Leu>Gln).

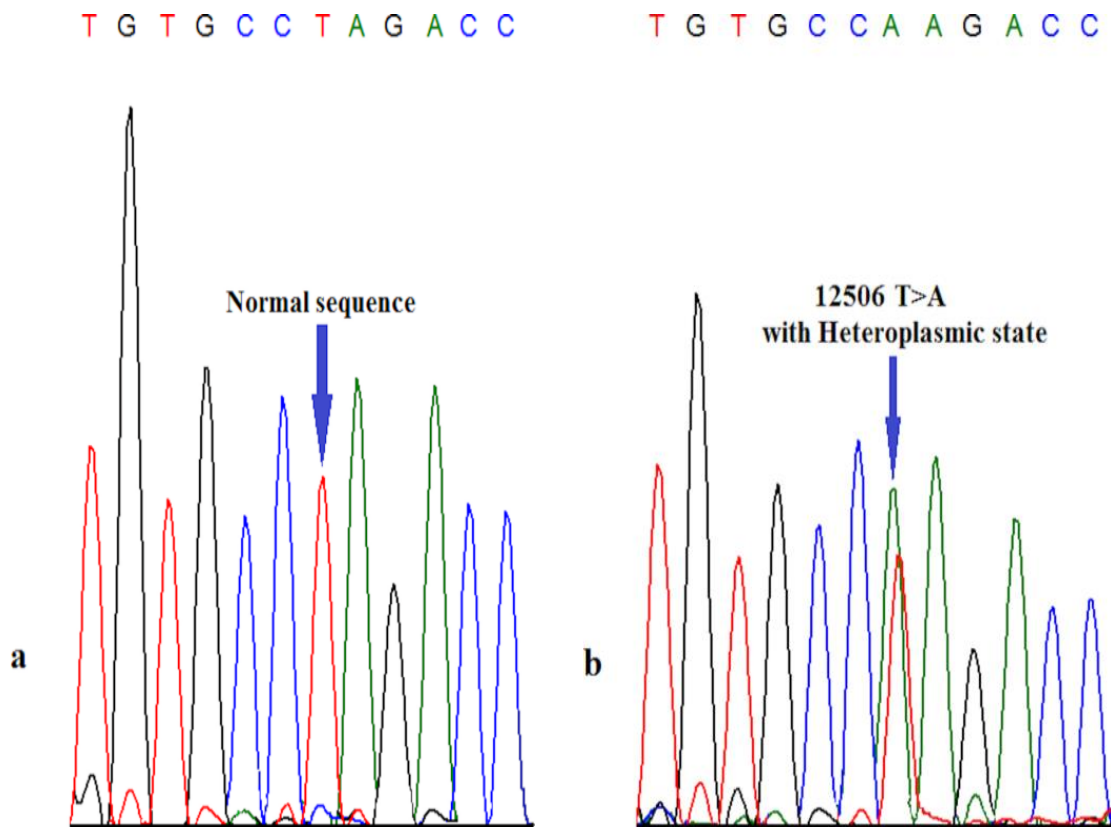


Figure 3.19: Chromatograms showing the difference between normal sequence and12506 T>A ( a) shows the chromatogram for a normozoospermic man, and the blue arrow indicates thymine as the normal sequence at nt 12506. (b) shows the chromatogram for an asthenozoospermic patient, and the blue arrow indicates the site of 12506 T>A where thymine is replaced by adenine with a heteroplasmic state.

The nucleotide sequences of 6 patients (BankIt2363991 ND5_21, BankIt2363991 ND5_22 , BankIt2363991 ND5_24, BankIt2363991 ND5_31, BankIt2363991 ND5_34 and BankIt2363991 ND5_45) were recorded by NCBI and they are given the following GenBank accession numbers respectively (MT742299, MT742300, MT742301, MT742302, MT742303 and MT742304).

12506T>A was predicted to be probably damaging according to Poly Phenyl-2 and ACMG (score: 0.663, sensitivity: 0.79; specificity: 0.84) (Figure 3.20).

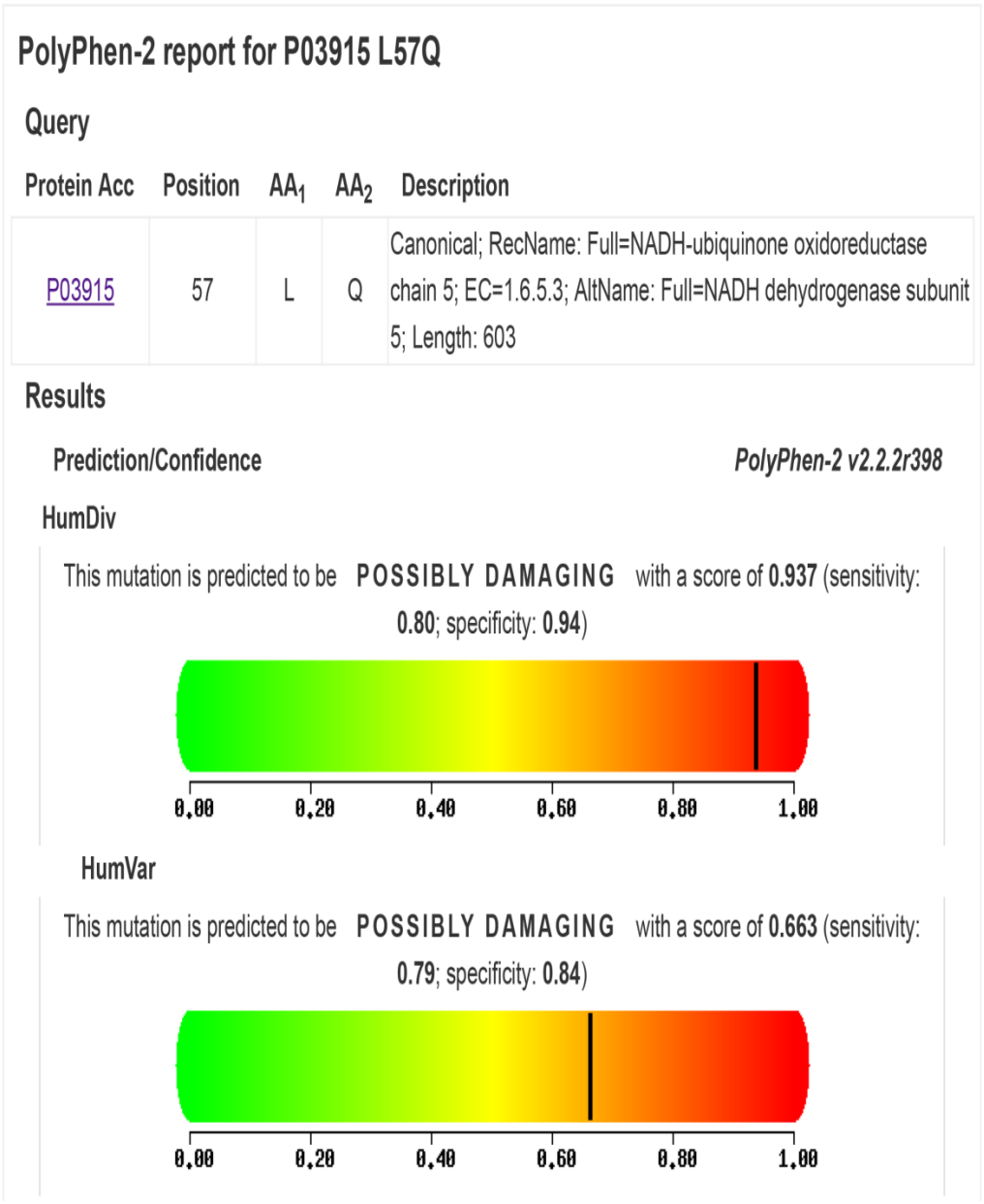


Figure 3.20: Prediction of the functional effect of the 12506 T>A substitution by Poly Phenyl-2 software

12506T>A was associated with a decline in sperm motility and ICSI outcomes. The medians of sperm motility (the wild type (17.5±24.93), 12506 T>A (4.5±24.93), $P$ = 0.009), the fertilization rate (wild type (46±17.8), 12506 T>A (32±2.13), $P$ = 0.001), the embryo cleavage score (wild type (3.42±0.37) , 12506 T>A ( 3.07±0.12), $P$ = 0.044), the embryo quality score (the wild type (2.09±0.49), 12506 T>A (1.62±0.22), $P$ = 0.028) (Figure 3.21).

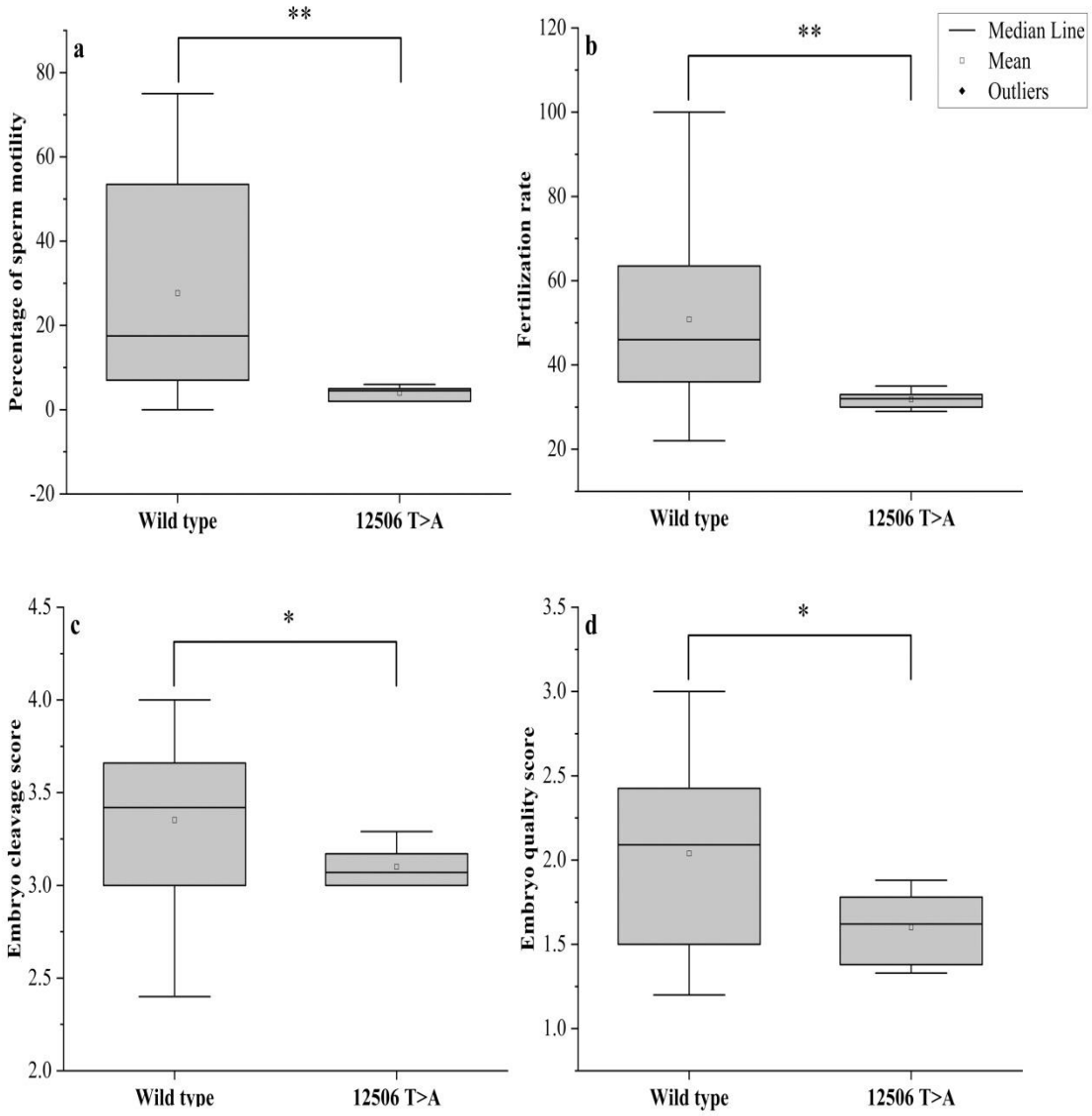


Figure 3.21: The box plots show the differences between men with or without the 12506T>A. Mann-Whitney P-values for the differences in the medians were (a) sperm motility (P= 0.009), (b) fertilization rate (P= 0.001), (c) embryo cleavage score (P= 0.044) and (d) embryo quality score (P= 0.028) .*P < 0.05, **P < 0.01

### 3.9.3. Identification of the missense variant 13708G>A in the *ND5* gene

Another variant 13708G>A (rs28359178) in the *ND5* gene was significantly different between patients and the control groups (*P* = 0.013), causing an Ala>Thr amino-acid substitution (Figure 3.22). This variant was identified among 14 asthenozoospermic men only -all were homoplasmic (Figure 3.23).

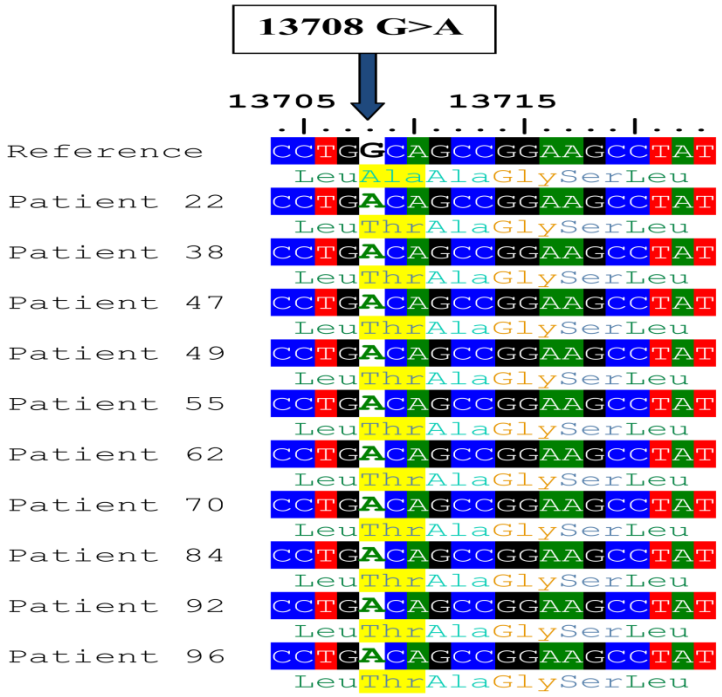


Figure 3.22 Alignment of the ND5 gene sequence for 10 patients showing a 13708 G>A variant. The blue arrow indicates the site of the nucleotide substitution, and the highlighted yellow colour indicates the amino-acid replacement (Ala>Thr)

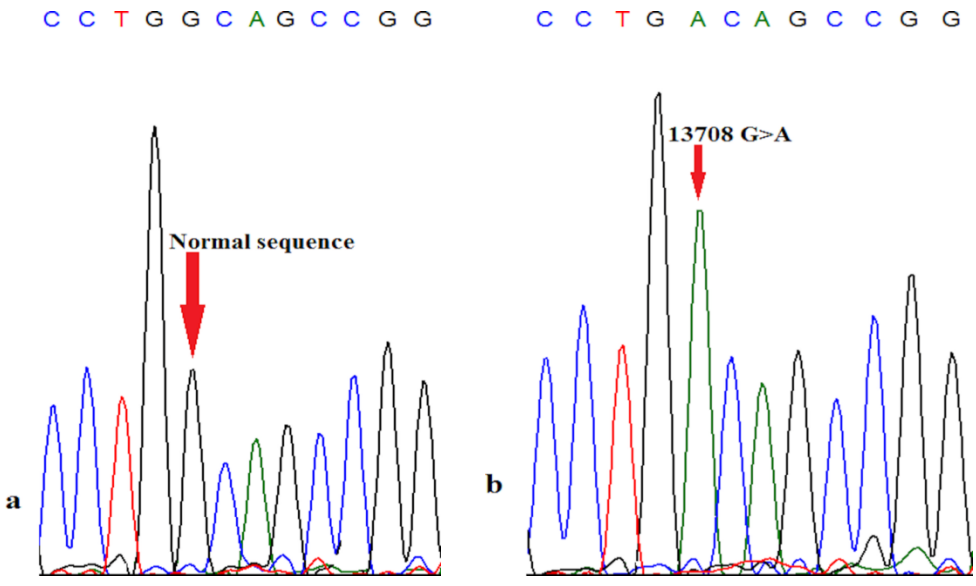


Figure 3.23: Chromatograms showing the difference between normal sequence and13708 G>A (a ) shows a chromatogram for a normozoospermic man, the red arrow indicates guanine as normal sequence at nt 13708, while ( b ) shows a chromatogram for an asthenozoospermic patient, the red arrow indicates the site of 13708 G>A where guanine is replaced by adenine in a homoplasmic state.

This variant is predicted to be benign, according to Poly Phenyl-2 and ACMG criteria (score: 0.001, sensitivity: 0.99; specificity: 0.09) (Figure 3.24).

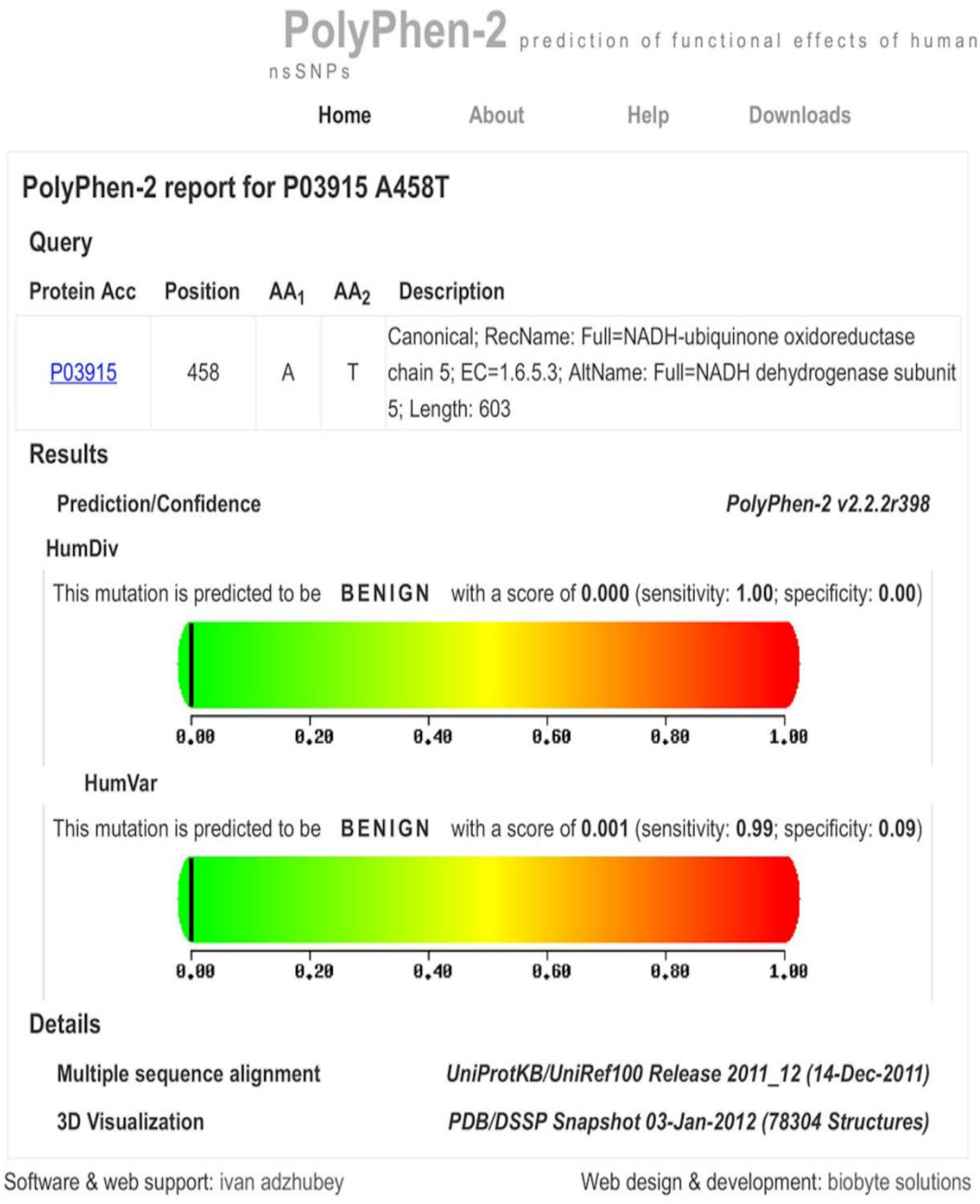


Figure 3.24: Prediction of the functional effect of the 13708 G>A substitution by Poly Phenyl-2 software

13708 G>A was associated with a decline in sperm motility and ICSI outcomes. The medians of sperm motility (the wild type (18±25.46), 13708G>A (8±7.13), *P*=0.043), the fertilization rate (wild type (47±18.09), 13708G>A (36±9.55), *P*=0.017), the embryo cleavage score (wild type (3.5±0.36), 13708G>A (3.0±0.24), *P*=0.001), the embryo quality score (wild type (2.11±0.49), 13708G>A 1.67±0.35), *P* = 0.007) (Figure 3.25).

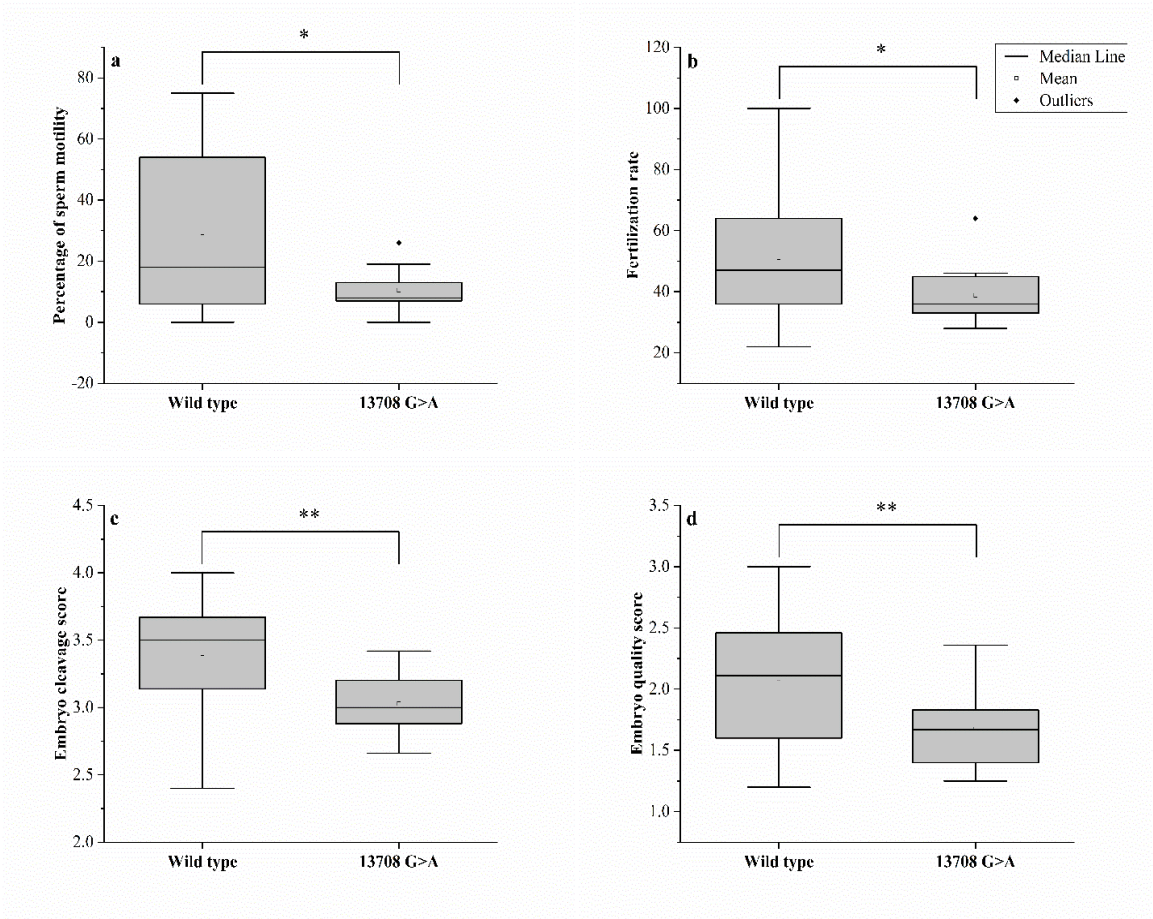


Figure 3.25: The box plots show the differences between men with or without the 13708G>A. Mann-Whitney P-values for the differences in the medians were (a) the sperm motility (P= 0.043), (b) the fertilization rate (P= 0.017), (c) the embryo cleavage score (P= 0.001) and (d) the embryo quality score (P= 0.007).*P < 0.05, **P < 0.01

### 3.9.4. Correlation between the frequency of total variants in the *ND5* gene with sperm motility and ICSI outcomes

The frequency of total variants in the *ND5* gene was negatively correlated with the sperm motility (r = - 0.563, *P* <0.001), the fertilization rate (r = - 0.646, *P* <0.001), the embryo

cleavage score (r = - 0.615, *P* <0.001) and the embryo quality score (r = - 0.679, *P* <0.001) (Figure 3.26).

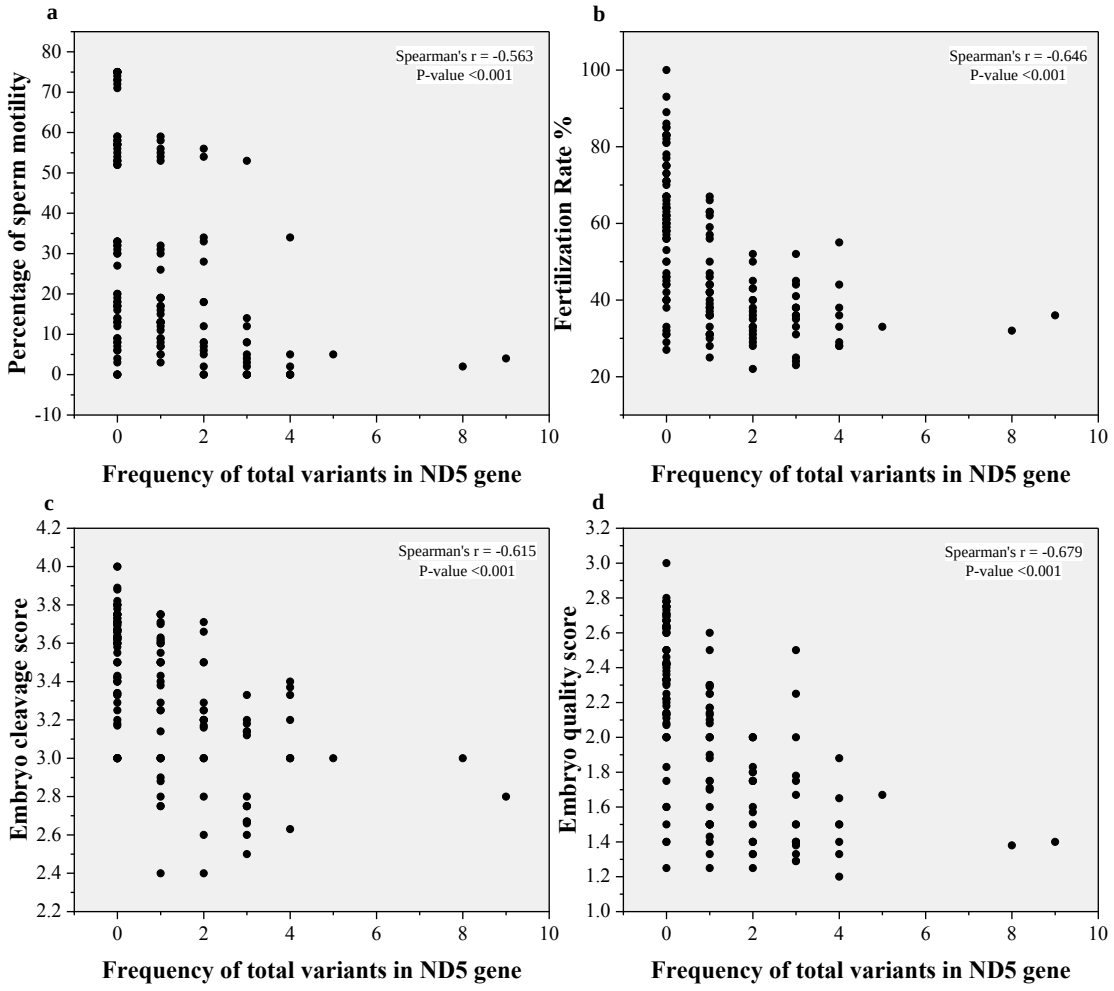


Figure 3.26: Scatter plots of the frequency of total variants in the ND5 gene with (a) the sperm motility. (b) the fertilization rate (c) the embryo cleavage score and (d) the embryo quality score, Spearman's correlation coefficients and significance levels were included in each Figure.

### 3.10. DNA sequence analysis for the *ND6* gene

The *ND6* gene is located between 14149 and 14673 on the mtDNA with a 525 nt length and a 174 amino-acid protein length. A total of 15 nucleotide substitutions were identified in the *ND6* gene; two of them were missense, while the rest were synonymous variants (Table 3.4). However, none of these variants were significantly different between the cases and the control groups.

Table 3.4: The mtDNA variants identified in the *ND6* gene

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in Normo-zoospermia | Frequency of variant in Astheno-zoospermia | G Test | P Value |
|---|---|---|---|---|---|---|
| 1 | 14167C>T | Glu>Glu | 0/45 | 4/105 | 2.9 | 0.089 |
| 2 | 14179A>G | Tyr>Tyr | 1/45 | 2/105 | 0.016 | 0.9 |
| 3 | 14182T>C | Val>Val | 0/45 | 3/105 | 2.166 | 0.141 |
| 4 | 14233A>G | Asp>Asp | 2/45 | 12/105 | 2.061 | 0.151 |
| 5 | 14323G>A | Asp>Asp | 0/45 | 3/105 | 2.166 | 0.141 |
| 6 | 14364G>A | Leu>Leu | 1/45 | 7/105 | 1.438 | 0.23 |
| 7 | 14560G>A | Val>Val | 1/45 | 1/105 | 0.354 | 0.552 |
| 8 | 14620C>T | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 14470T>C | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 14566A>G | Gly>Gly | 0/45 | 2/105 | 1.438 | 0.23 |
| 11 | 14569G>A | Ser>Ser | 0/45 | 2/105 | 1.438 | 0.23 |
| 12 | 14178T>C | Ile>Val | 0/45 | 2/105 | 1.438 | 0.23 |
| 13 | 14180T>C | Tyr>Cys | 0/45 | 2/105 | 1.438 | 0.23 |
| 14 | 14212T>C | Val>Val | 1/45 | 2/105 | 0.016 | 0.9 |
| 15 | 14305G>A | Ser>Ser | 1/45 | 1/105 | 0.354 | 0.552 |

### 3.10.1. The frequency of total variants in the *ND6* gene among the studied groups

The frequency of the total variants in the *ND6* gene among groups one and two was higher compared to groups three and the control; based on the Kruskal-Wallis test, there is a significant difference between the sample medians among groups $P$ <0.001, and according to

the Mann-Whitney test there were significant differences between the medians of both groups one and two and the control, while there was no significant difference between the medians of G3 and the control, and there was also no significant difference between G1 and G2. The Mann-Whitney P-values for the differences in the medians between the groups were: G1 and G2 ($P$=0.459), G1 and G3 ($P$<0.001), G1 and the control ($P$<0.001),G2 and G3 ($P$=0.001), G2 and the control ($P$<0.003), G3 and the control ($P$=0.388) (Figure 3.27).

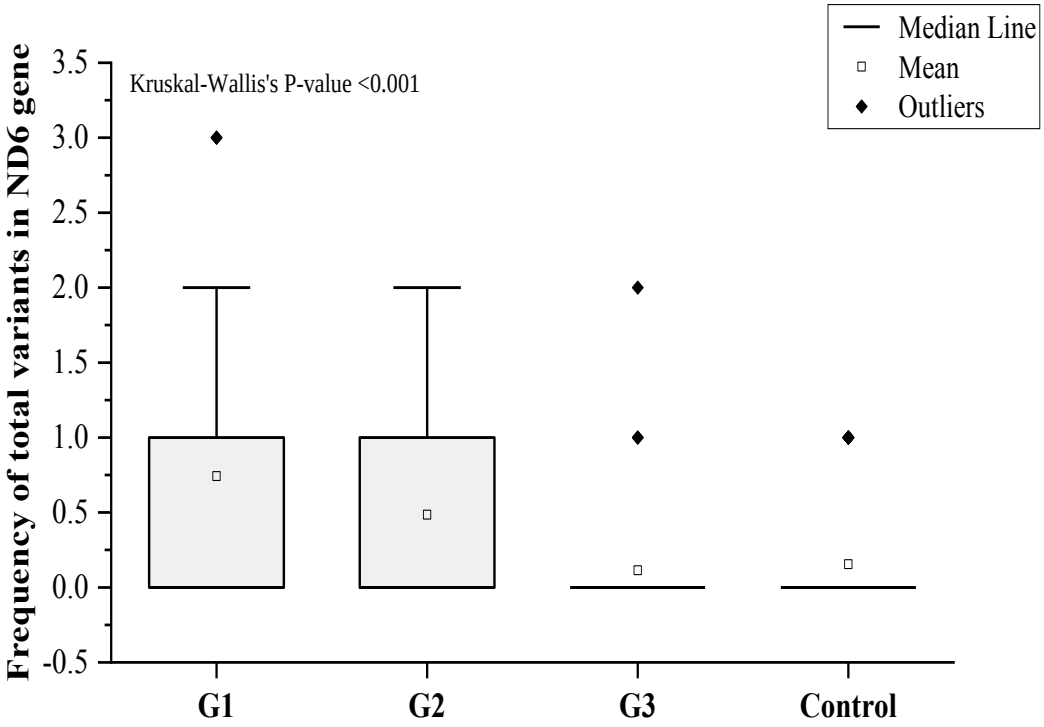


Figure 3.27: The box plots show the frequency of the total variants in the *ND6* gene among the groups; the Kruskal-Wallis's P- value was included.

### 3.10.2. The correlation between the frequency of the total variants in the *ND6* gene with sperm motility and ICSI outcomes

The frequency of the total variants in the *ND6* gene was negatively correlated with the sperm motility (r = - 0.280, $P$ <0.001), the fertilization rate (r = - 0.213, $P$ <0.001), the embryo cleavage score (r = - 0.260, $P$ <0.001) and the embryo quality score (r = - 0.291, $P$ <0.001) (Figure 3.28).

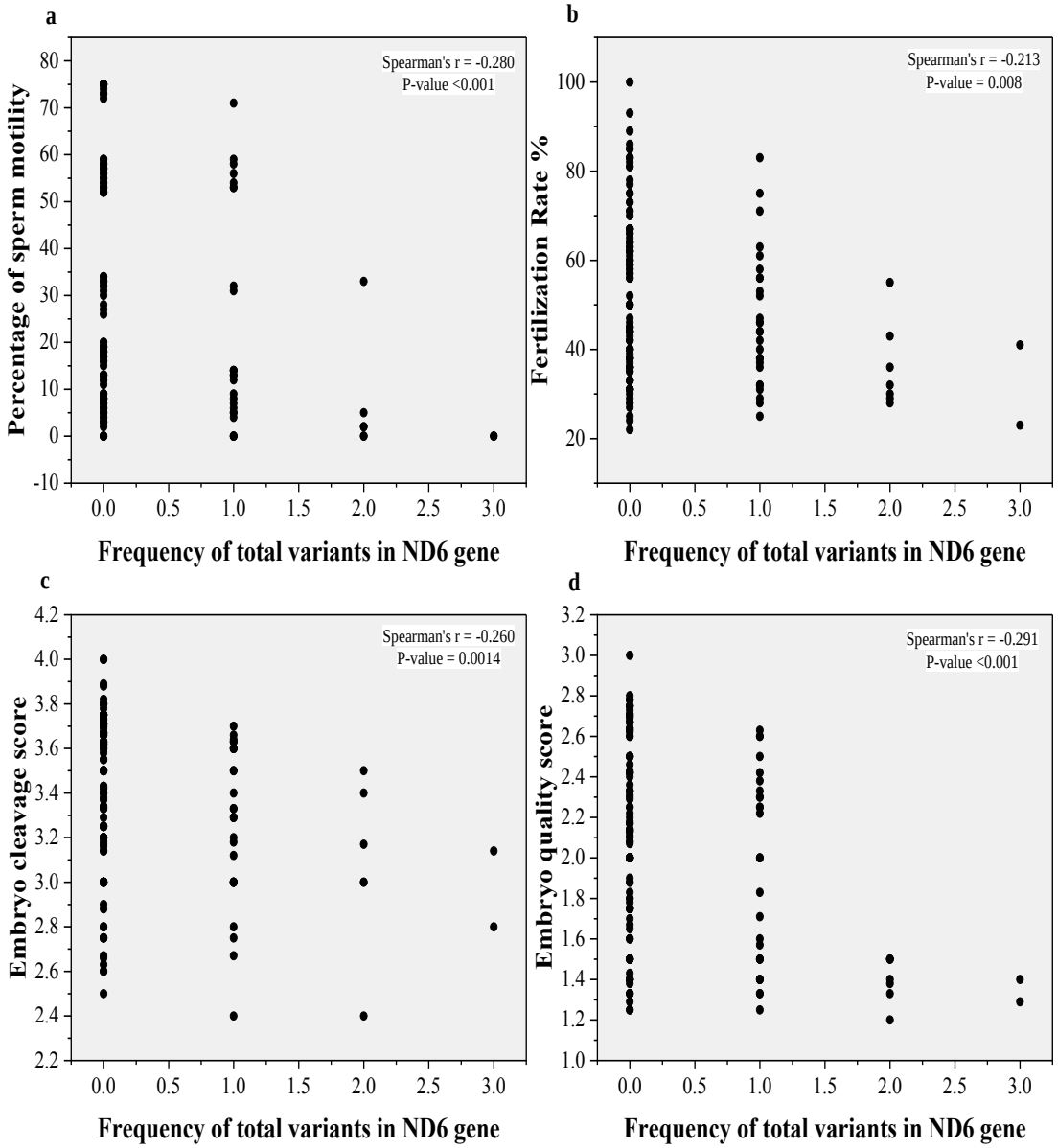


Figure 3.28: Scatter plots of the frequency of total variants in the *ND6* gene with (a) the sperm motility. (b) the fertilization rate (c) the embryo cleavage score and (d) the embryo quality score. Spearman's correlation coefficients and significance levels were included in each figure.

### 3.11. The frequency of total variants in all genes

The highest frequency of total variants in all genes was in group one while the lowest frequency was in the control; based on the Kruskal-Wallis test there is a significant difference between the sample medians among groups $P$ <0.001 Furthermore, according to Mann-Whitney, there was a significant difference between the medians of all the groups except

between G3 and the control. The Mann-Whitney P-values for the differences in the medians between the groups were: G1 and G2 ($P$<0.001), G1 and G3 ($P$<0.001), G1 and the control ($P$<0.001), G2 and G3 ($P$=0.001), G2 and the control ($P$<0.001), G3 and the control ($P$=0.304) (Figure 3.29).

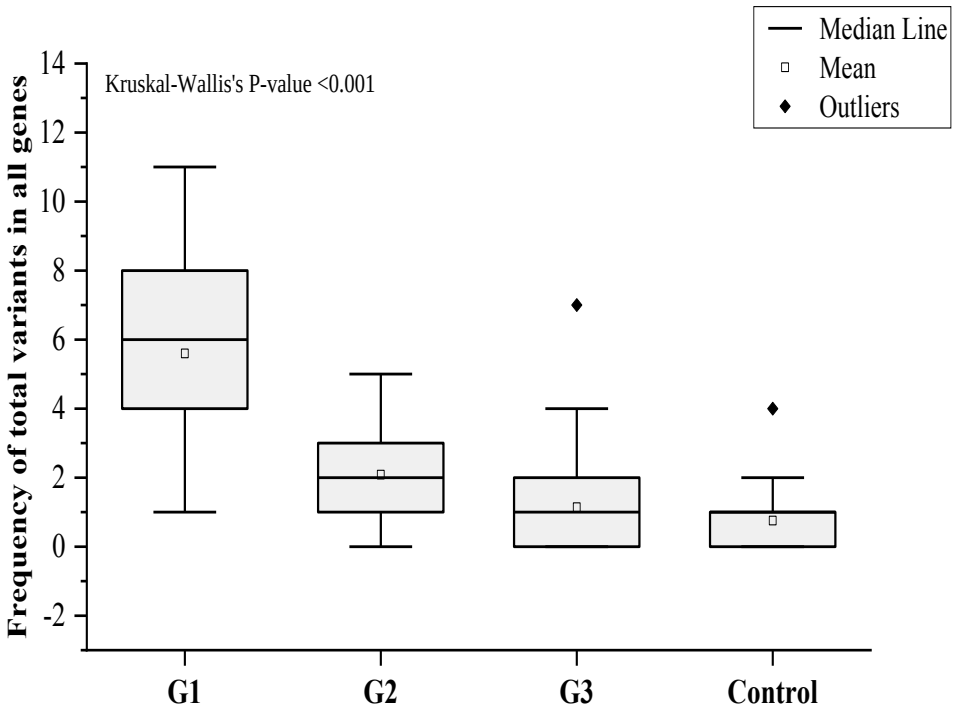


Figure 3.29: The box plots show the frequency of the total variants in all genes among the groups; the Kruskal-Wallis's P- value was included.

### 3.12. The percentages of men with total mitochondrial variants in all genes

The percentages of men with total variants in the *ND1* gene in groups 1, 2, 3 and in the control were: 80%, 25.7 %, 17.1% and 15.6 % respectively, $P < 0.001$, while the percentages of men with total variants in the *ND2* gene in groups 1, 2, 3 and the control were 60 %, 31.4 %, 22.9% and 13.3 % respectively, $P < 0.001$, and also the percentages of men with total variants in the *ND5* gene among groups 1, 2, 3 and the control were 91.4%, 77.1%, 42.9% and 17.8% respectively, $P < 0.001$, finally the percentages of men with total variants in the *ND6* gene in groups 1, 2, 3 and the control were 45.7%, 31.4, 17.1% and 13.3% respectively, $P < 0.001$ (Table 3.5).

Table 3.5: The percentages of men with total mitochondrial variants in (*ND1, ND2, ND5* and *ND6*) genes among the controls and different asthenozoospermic groups

| Gene | Group 1 (N=35) | Group 2 (N=35) | Group 3 (N=35) | Control (N=45) | $X^2$ (3, $N$ = 150) | *P*-value |
|---|---|---|---|---|---|---|
| | Number of males with total variants (Percentage) | Number of males with total variants (Percentage) | Number of males with total variants (Percentage) | Number of males with total variants (Percentage) | | |
| ND1 | 28 (80.0%) | 9 (25.7%) | 6 (17.1%) | 7 (15.6%) | 47.9 | 0.0001* |
| ND2 | 21 (60.0%) | 11 (31.4%) | 8 (22.9%) | 6 (13.3%) | 21.5 | 0.0008* |
| ND5 | 32 (91.4%) | 27 (77.1%) | 15 (42.9%) | 8 (17.8%) | 52.9 | 0.0001* |
| ND6 | 16 (45.7%) | 11 (31.4%) | 6 (17.1%) | 6 (13.3%) | 12.8 | 0.0051* |

*: statistically significant difference, *p*-value <0.05

### 3.13. The correlation between the frequency of total variants in all genes with sperm motility and ICSI outcomes

The Frequency of total variants in all genes was negatively correlated with the sperm motility (r = - 0.672, *P* <0.001), the fertilization rate (r = - 0.718, *P* <0.001), the embryo cleavage score (r = - 0.692, *P* <0.001) and the embryo quality score (r = - 0.800, *P* <0.001) (Figure 3.30).

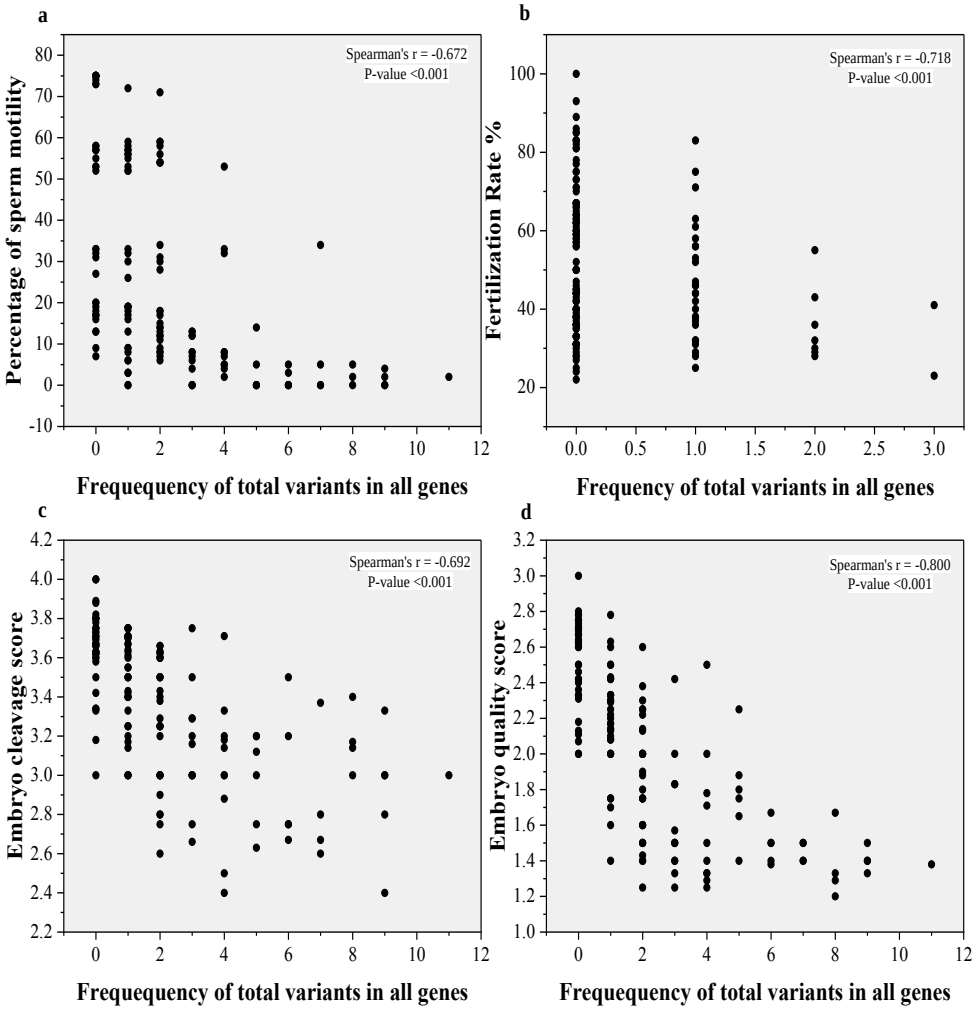


Figure 3.30: The scatter plots of the frequency of total variants in all genes with (a) the sperm motility (b) the fertilization rate (c) the embryo cleavage score and (d) the embryo quality score. Spearman's correlation coefficients and significance levels were included in each figure

### 3.14. The frequency of the three significant variants among the groups

The percentages of men with 4216 T>C among groups 1, 2 and 3 were 14.3%, 8.6% and 5.8% respectively, $P = 0.005$, while the percentages of men with 13708G>A among groups 1, 2 and 3 were 17.1 %, 14.3 % and 8.6 %, $P = 0.001$, and finally the percentages of men with 12506 T>A among groups 1, 2 and were 14.3 %, 2.9 % respectively, $P < 0.001$. These three variants were only identified among patient groups, while they were not found among the control (Table 3.6).

Table 3.6: Percentage of men with 4216, 13708 and 12506 among the groups

| | G1 | G2 | G3 | Patients (G1+G2+G3) | control | $X^2$ (3, $N$ = 150) | P-value |
|---|---|---|---|---|---|---|---|
| Percentage of men with 4216 T>C | 14.3 % | 8.6 % | 5.8 % | 9.6 % | 0 % | 12.83 | 0.005* |
| Percentage of men with 13708 G>A | 17.1 % | 14.3 % | 8.6 % | 13.3 % | 0 % | 15.95 | 0.001* |
| Percentage of men with 12506 T>A | 14 .3% | 2.9 % | 0 % | 8.6 % | 0 % | 17.38 | <0.001* |

*: statistically significant difference, $p$-value <0.05

## 4. Discussion

Asthenozoospermia is a major cause of male infertility (Heidary *et al*., 2020). Sperm motility is considered as an important factor in normal fertilization, as it enables the sperm to reach the oocyte, and then it can penetrate the zona pellucida (Chemes *et al*., 1998). Several environmental factors were associated with the decline in sperm motility; one of the most important factors that has a negative effect sperm motility is cigarette smoking (Pant *et al*., 2015; Sofikitis *et al*., 1995; Vine, 1996). Exposure to pesticides and toxic chemicals also has a negative effect on the semen quality and pregnancy rate (Cock *et al.*, 1994; Neubert, 1997; Wong, E. W., & Cheng, 2011). Age is also correlated with sperm motility, for, as the male becomes older, the sperm motility will decline; and sperm DNA will become more prone to damage (Kühnert & Nieschlag, 2004; Tissera *et al*., 2018; Vagnini *et al*., 2007).

Sperm motility is reduced by the presence of anti-sperm antibodies in the semen (Roy Choudhury & Knapp, 2001). Another factor affecting sperm motility is defective sperm flagellum, which refers to a dysfunction in the dynein arms (Sha *et al*., 2020). Several studies have reported that, as the sperm depends on generated ATP for its progressive motility, mitochondrial dysfunction is correlated with reduced sperm motility (Folgerø *et al*., 1993; Nowicka-Bauer *et al*., 2018).

Many diseases, such as varicocele, infection of the genital tract and retardation of the testicular sperm passageway, are related to male infertility (Pellati *et al*., 2008; Sheehan *et al*., 2014). Endocrine system dysfunction including an imbalance among the hormones that are necessary for spermatogenesis, such as testosterone, FSH and LH, is a contributory factor for male infertility (Ring *et al*., 2016). About 15% of male infertility cases are still without any known causes; this is known as idiopathic infertility (Ambasudhan *et al*., 2003).

One of the genetic bases for asthenozoospermia refers to sperm mtDNA deletions (Durairajanayagam *et al*., 2020). Large-scale mtDNA deletions, including (4977, 7345, 7436 and 7599) bp, are associated with asthenozoospermia (Ambulkar *et al*., 2016; Kao *et al*., 1998; Talebi *et al*., 2018b). These deletions involve a complete removal of some structural genes in the mtDNA, including *ND* genes (3-6), *ND4L*, *ATPase 6/8*, *COX 3*,*COX 2* and *CytB* (Guo *et al*., 2017; Kao *et al*., 1998). Therefore mutated mtDNA in sperm leads to

a respiratory function defect, which impairs the generated energy and leads to a reduction in motility (Nakada *et al.*, 2006).

For infertile couples attending various types of ART, intrauterine insemination (IUI) is recommended at the beginning due to its relatively low cost and to not requiring oocyte retrieval (Goverde *et al*., 2000; Van Weert *et al*., 2004). IUI is preferred when the male has progressive motility of more than one million and the wife has normal fertility conditions (Van Weert *et al*., 2004). After an IUI failure the infertile couple is recommended to have conventional IVF, where the oocyte is inseminated with 100 000 sperm/ml; conventional IVF is preferred as it enhances the natural selection among motile sperms (Dang *et al*., 2019; Zhang *et al*., 2016). On the other hand, if the male has progressive motility less than half-a-million the couple are recommended to try the ICSI technique (Tournaye, 2012).

ICSI outcomes are associated with low sperm quality, and with the fertilization rate being negatively correlated with the sperm count. Furthermore, males with severe oligozoospermia (a sperm count of less than five million /ml) and cryptozoosoermia (a sperm count of less than 100000/ml) have a lower percentage of fertilized oocytes during ICSI compared to men with a normal sperm count (Hashimoto *et al*., 2010; Strassburger *et al*., 2000). Sperm morphology is also considered to be an important factor during ICSI; an injection of the oocyte with abnormal sperm morphology has a lower fertilization rate compared to an injection with normal sperm morphology (De Vos *et al*., 2003). The present study, however, investigated the effect of sperm motility on ICSI outcomes, and so all oocytes were injected with sperms with normal morphology, and the preparation of the ICSI samples involved selecting semen with a normal sperm count.

### 4.1. The effect of sperm motility on ICSI outcomes

ICSI is currently broadly used for couples suffering from male infertility, specifically among asthenozoospermic patients, where the sperm of these infertile men usually cannot reach the fertilization site due to a deficiency in its forward progressive motility, which is essential for normal fertilization (De Mendoza *et al*., 2000).

In the present study sperm motility was positively correlated with the fertilization rate, which was lower among group one that included patients with severe asthenozoospermia (0%-5%),

compared to group three, which included asthenozoospermic men with a percentage of sperm motility that ranged between (16% to 35%). The control group, which included normozoospermic men with a percentage of sperm motility ranging between (50% to 75%) had a higher fertilization rate compared to all the other patient groups. In this study the embryo quality, including the embryo cleavage score and the embryo quality score, were positively correlated with sperm motility. The embryos in group one had the lowest quality scores, whereas the best embryo quality was in the control group.

Our results tally with those in a previous study that reported that reduced sperm motility was associated with a decline in the fertilization ability and the embryo quality in ICSI (Zheng *et al*., 2016). Another study concluded that sperm motility can predict the success of ICSI and considered this to be an important factor for the fertilization rate (S. Shen *et al*., 2003). Shoukir *et al.* demonstrated that an oocyte that had been injected by sperm with forward progressive motility had a higher chance of reaching the blastocyst stage compared to an oocyte injected with weak sperm that had poor motility. There was, however, no significant difference between blastocyst development and other semen parameters, such as the sperm morphology and the sperm count (Shoukir *et al.*, 1998). The fertilization rate was higher when ICSI was performed using motile sperms compared to the injection of the oocyte with immotile sperm in 14 IVF cycles (Nagy *et al*., 1998). Complete asthenozoospermia, also known as absolute asthenozoospermia is considered to be one of the most important causes of fertilization failure during ICSI (Ortega *et al*., 2011).

On the other hand, a previous study disagrees with our results for the researchers showed that ICSI outcomes were not affected by sperm motility, and that the injection of oocytes with immotile sperms can in fact induce a normal fertilization rate and even a high-score embryo quality (Moghadam *et al*., 2005).

The criteria used for the sample selection and the sample grouping in the current study influenced the results obtained. We excluded many environmental factors, such as smoking, varicocele, alcoholism and men more than 40 years old, that are known to affect sperm motility. These factors have already been shown to affect sperm motility, and by thus excluding them, it increases the probability of identifying the genetic aetiology (Chhabra *et al*., 2018; Tissera *et al*., 2018). In most of the previous studies, patient grouping was done by including all sperm abnormalities together as one group, while in our study

asthenozoospermic patients were divided into different categories where the reduced sperm motility varied between 0% and 35%. Samples with a sperm motility of between 36% and 40% were excluded in order to stay far from the borderline of the normal percentage (40%) of total sperm motility recommended by the WHO (WHO, 2010). We also relied on the percentage of total sperm motility rather than on the type of sperm motility because, according to WHO progressive motility, type A and type B should be greater than 32%. So, as our samples were evaluated by different embryologists, we felt it would be more reliable to depend on total sperm motility where it is easier to distinguish between motile and immotile sperm rather than determining the specific type of sperm; this also decreases the number of individual errors among technicians.

### 4.2. The effect of protein carbonyl on sperm motility and ICSI outcomes

Excess levels of ROS are associated with poor semen quality, such as reduced sperm motility and abnormal sperm shape (Iwasaki & Gagnon, 1992). ROS at high levels induces peroxidation of poly-unsaturated fatty acids in the sperm leading to a reduction in its motility. The quantification of protein carbonyl derivatives is the preferred biomarker because they are produced relatively faster than other biomarkers; the derivatives of protein carbonyl are also themselves relatively stable (Dalle-Donne *et al*., 2003).

In the present study the protein carbonyl levels were negatively correlated with the percentage of motile sperm and ICSI outcomes. The highest levels of protein carbonyl were among asthenozoospermic patients with severe reduced sperm motility, whilst the lowest levels were among the control. Moreover, the fertilization rate and the embryo quality were negatively correlated with the protein carbonyl levels. The lowest fertilization rate was among patients with severe asthenozoospermia, while the highest fertilization rate was among the control. Finally, the lowest score of embryo quality was among patients with severe asthenozoospermia, while the highest score of embryo quality was amongst the control.

Our results agree with those from a previous study that reported ROS levels in the semen to be negatively correlated with the ICSI outcomes (Hammadeh *et al*., 2006). Padron *et al*. demonstrated that ROS production is inversely correlated with sperm motility, and also that elevated levels of ROS existed among 40-80 % of infertile men (Padron *et al*., 1997).

According to another previous study, protein carbonylation was associated with sperm cryopreservation, where the levels of protein carbonyl were elevated by 20% in frozen bull sperm, compared to fresh samples (Mostek *et al*., 2017). This may refer to the fact that cryopreservation encourages the generation of ROS, which have the ability to react with proteins, sperm DNA and lipids resulting in several disorders for the cell function (Ashok Agarwal *et al.*, 2014). The freezing and thawing processes also induce changes in the positions of lipid membranes, which leads to increased fluidity and calcium ions inside the cell (Cormier & Bailey, 2003).

Another study reported that oxidative stress was negatively correlated with sperm motility; in this study 8-OHdG was a beneficial biomarker for oxidative stress, where the levels of 8-OHdG were elevated among asthenozoospermic men compared to normozoospermic men, and the lipid peroxide measurements were also higher among sperm with reduced motility, compared to normal sperm motility (Kao *et al*., 2008).

Mitochondrial dysfunction is caused by the generation of ROS at complex I in the inner membrane, thereby causing peroxidative damage to the midpiece of the sperm, leading to a reduction in its motility (Koppers *et al*., 2008b). ROS attacks different parts of the sperm, involving both mitochondrial and nuclear DNA (Ashok Agarwal *et al.*, 2008). The absence of cytoplasm in mature spermatozoa minimizes the viability of antioxidants as the cytoplasm in the cell is considered as the main source of antioxidants and leading to a deficiency in the repair mechanism (Agarwal & Allamaneni, 2004).

Hydrogen peroxide($H_2O_2$) is considered as toxic to ROS even at low concentrations; the generation of ATP in sperm treated with hydrogen peroxide (0.38 – 1.5 ) Mm for 30 minutes was reduced to ($0.10 \times 10^{-10}$) moles/$10^6$ sperm cells, compared to non-treated sperm, which had ATP levels of ($0.84 \times 10^{-10}$) moles/$10^6$ sperm cells, although the superoxide dismutase (100 U/ml) was included as an antioxidant. Furthermore, a significant reduction in the progressive motility for treated sperm was indicated, compared to the control; the effect of $H_2O_2$ on the sperm mitochondrial membrane potential(MMP) was dependent on the dose of $H_2O_2$. A low dose of $H_2O_2$ $\leq$1.5 mM had no effect on MMP while if the dose of $H_2O_2$ exceeded 5 Mm ,the MMP was affected (Armstrong *et al*., 1999).

### 4.3. The effect of mitochondrial variants on sperm motility and ICSI outcomes

MtDNA is one hundred times more susceptible to mutations than nuclear DNA is (O'Connell *et al.*, 2002). This theory refers to the lack of protection by histones or other DNA-binding proteins, thereby making the MtDNA more prone to DNA damage caused by excess levels of ROS and free radicals being present in the matrix (Piomboni *et al*., 2012). The sperm mtDNA repair mechanism is also less efficient, compared to nuclear DNA, due to the absence of cytoplasm in the sperm, which is considered to be a major source for antioxidants (Stewart & Chinnery, 2015). In addition, sperm contains relatively low copies of mtDNA about one hundred copies, compared to other cells, such as the oocyte, which contains about $10^5$ copies (Rivlin *et al*., 2004).

The studied genes are a part of Complex I, which itself plays a key role in OxPhos by receiving electrons from NADH; the captured energy from these electrons is utilized to release protons to the intermembrane space, which is later used to generate ATP (Yano *et al*., 2000). Sperm require ATP for flagellar movement, and this depends on OxPhos providing their energy requirements (Piomboni *et al*., 2012). Therefore, it is expected that pathogenic variants in the *ND* genes affect complex 1 activity, causing a deficiency in energy production, which in turn will negatively affect the sperm motility (Spiropoulos, 2002).

#### 4.3.1. The correlation between the frequency of variants in the *ND1* gene with sperm motility and ICSI outcomes

In the present study a negative correlation was found between the frequency of total variants in the *ND1* gene and the percentage of sperm motility among the patient groups. The highest frequency of mitochondrial variants in the *ND1* gene was in group one, while the lowest frequency was in group three. On the other hand, the control group showed a lower frequency, when compared with all the patient groups. In the *ND1* gene a missense variant 4216 T>C was also negatively correlated with sperm motility. This variant was identified only among the asthenozoospermic patients, while it was not identified among the control group.

Our results agree with a previous study that reported 4216 T>C is associated with reduced sperm motility among white men, while it is not correlated with oligoteratozoospermia (Ruiz-

Pesini *et al.*, 2000). While our results disagree with this previous study that concluded there was no association between mitochondrial variants in the *ND1* gene and poor semen quality, as they had identified eight variants in the *ND1* gene with no significant difference between patients and the control. However the small sample size in their study should be considered as one of the limitations of their results, as they included only 30 infertile men with 30 fertile men as the control (Güney *et al*., 2012)

There are only a few studies about the correlation between 4216 T>C and asthenozoospermia. However, 4216 T>C was already linked with other mitochondrial diseases: a previous study showed that the rate of the 4216 T>C variant among diabetic patients was higher than in controls and was statistically associated with diabetes mellitus type 2 (Crispim *et al*., 2006). Another study found an interesting male-specific association between the 4216 T>C variant and the rate of infection, leading to complicated sepsis and death (Gomez *et al*., 2009).

In the present study the ICSI outcomes were negatively correlated with the frequency of the total variants in the *ND1* gene, and the fertilization rate declined among patients with asthenozoospermia. Furthermore, the embryo quality was negatively correlated with the frequency of the total variants, where the lowest scores for both embryo quality and cleavage were among patients with severe asthenozoospermia. 4216 T>C was also negatively correlated with the ICSI outcomes. The patients with 4216 T>C showed lower rates of fertilization compared to other men without this particular variant. The embryo quality score and the embryo cleavage score among patients with 4216 T>C were lower compared to those of other men without this variant.

The effect of 4216 T>C on the ICSI outcomes had not been studied before. However, a previous study reported that another variant in the *ND1* gene, namely 3243 A>G, was positively correlated with the mtDNA copy number in the embryos during ICSI. They explained the increment in the mtDNA copy number among embryos with 3243 A>G as an adaptation for inefficient ATP production via oxidative phosphorylation due to mutated mtDNA (Monnot *et al*., 2013).

### 4.3.2. The correlation between the frequency of variants in the *ND2* gene with sperm motility and ICSI outcomes

In the present study a negative correlation was found between the frequency of the total variants in the *ND2* gene and the percentage of sperm motility. Among patient groups the highest frequency of mitochondrial variants in the *ND2* gene was in group one, while the lowest frequency was in group three. The control group showed a lower frequency compared to all the patient groups. However, all the identified variants in the *ND2* gene were non-significantly different between the control and patient groups. On the other hand, the frequency of the total variants in the *ND2* gene was negatively correlated with the ICSI outcomes.

Our results agree with a previous study that included 246 cases in an IVF cycle and found the frequencies of identified variants in *ND2* in the patient group were not significantly different from those found in the control. However, our results disagree with their conclusion that the total fertilization failure was not correlated with the frequency of total variants in the *ND2* gene (J. Zhang *et al*., 2018).

Our results disagreed with those from another study, which reported that the mtDNA mutations did not affect sperm motility. They concluded that their results were after sequencing the whole mtDNA for 20 asthenozoospermic men and 23 teratoasthenozoospermic men; they also identified 247 variants, including 4917 in the *ND2* gene. Furthermore, according to their results, the two groups were sharing the same variants As a result. asthenozoospermia was not associated with mitochondrial variants, although this study involved sequencing for the whole mtDNA but it must be noted that the low sample size (43) does not provide conclusive evidence against the role of mtDNA in sperm motility. What is more, the grouping criteria did not fit with the aim of their study, as they compared the frequency of variants among asthenozoospermia with those among asthenoteratozoospermia; it would have been more logical if they had compared the frequency of mitochondrial variants among asthenozoospermia and normozoospermia (Pereira *et al*., 2007).

### 4.3.3. The correlation between the frequency of variants in the *ND5* gene with sperm motility and ICSI outcomes

The *ND5* gene with an 1812 nt length is considered to be the tallest gene in complex I of ETC. This explains why it had the larger number of variants, compared to other genes examined in this study; 121 variants were identified in the four genes, with 56 of them located in the *ND5* gene alone.

In the present study the frequency of total variants in the *ND5* gene was negatively correlated with sperm motility. The higher frequency of the variants was in group one while the lower frequency was in the control. Furthermore, the ICSI outcomes were negatively correlated with the frequency of the total variants in *ND5*. Two variants were significantly different between patients and the control: the first variant, 13708 G>A (rs28359178), had previously been reported as being in the NCBI (https://www.ncbi.nlm.nih.gov/) and in the human mitochondrial DNA database (www.mitomap.org), while the second variant 12506 T>A was novel. Both variants were negatively correlated with the percentage of motile sperm and ICSI outcomes; men with these two variants showed a lower sperm motility, a lower fertilization rate and a lower embryo quality score, compared to other men without these variants. The novel variant 12506 T>A was predicted by poly phenyl 2 to a damaging variant and it was indeed affecting the function of protein.

Our results agree with a previous study that identified 186 variants in the *ND5* gene, seven variants of which were significantly different between the total fertilization failure group and the control (12441 T>G,12561G>C, 12735 T>G,13164 A>G, 13743 T>C,13812 T>C and 13982 G>A); as a result, they reported these seven variants as being correlated with ICSI failure. Another missense variant 13651G>A was also identified in the *ND5* gene and it was predicted by polyphenol 2 to affect the protein function (Mao *et al*., 2020).

13708 G>A has not been linked to asthenozoospermia before; however, the 13708 G>A variant has been linked to several clinical manifestations. It has been shown that the 13708 G>A variant increases the susceptibility to multiple sclerosis (X. Yu *et al*., 2008) and was also found to enhance the expression of Leber hereditary optic neuropathy (LHON) disease in another study (Brown et al., 1996). Furthermore, the 13708 G>A variant was found to increase the risk for Alzheimer's disease, specifically among male patients (Maruszak *et al*.,

2009). The frequency of 13708 G>A was higher among patients with breast cancer, compared to the control (Brandon *et al*., 2006). Another variant, 12705 T>C, is associated with the promotion of diabetes mellitus type two (Lalrohlui *et al*., 2020).

### 4.3.4. The correlation between the frequency of variants in the *ND6* gene with sperm motility and ICSI outcomes

In the present study the frequency of the total variants in the *ND6* gene was negatively correlated with sperm motility. Group one had the highest frequency of variants in the *ND6* gene, while the control group had the lowest frequency. Furthermore, the fertilization rate and embryo quality scores were negatively correlated with the frequency of the total variants in the ND6 gene. On the other hand, none of these variants were significantly different between patients and the control.

Our study disagreed with a previous study which identified 45 variants in the *ND6* gene, two variants(14172 T>C and 14368 C>T) of which showed a significant difference between the total fertilization failure group and the control (Mao *et al*., 2020), while in our study all the identified 15 variants in the *ND6* gene were non-significantly different between patients and the control. The contradiction between the results of this study and those of other studies may be attributed to population variation. In a previous study, they found that the frequency of mtDNA SNPs varies between African American, European and Asian populations for the same mitochondrial disease (Herrnstadt *et al*., 2002). Two independent studies on the association of the same SNP in the *ND4* gene, namely,11994C>T, and oligoasthenozoosperima have reached different conclusions: a strong association was found in India (Selvi Rani *et al*., 2006), while in the other study in Portugal no association was found (Pereira *et al*., 2008).

Several studies reported that *ND6* gene variants are linked to other diseases rather than to infertility. A missense variant 14439G>A in the *ND6* gene was associated with mitochondrial respiratory chain disease (Uehara *et al*., 2014). Another variant, 14459G>A, was associated with Leigh Disease (Kirby *et al*., 2000). Finally four mitochondrial variants (14459G>A, 14495A>G, 14482C>A and 14568C>T) in the *ND6* gene were correlated with LHON disease among patients from Germany, France and Italy (Achilli *et al*., 2012).

### 4.3.5. The correlation between the frequency of total mitochondrial variants in all genes with sperm motility and ICSI outcomes

In the present study the frequency of the total variants in *ND1, ND2, ND5 and ND6* genes was negatively correlated with sperm motility and ICSI outcomes. The highest frequency was in group one while the lowest frequency was in the control. Furthermore, our results demonstrated that the fertilization rate and the embryo quality score were inversely correlated with a load of mtDNA variants. The three variants (13708 G>A, 4216 T>C and 12506 T>A) were also negatively correlated with the ICSI outcomes. Our findings agree with the results of a previous study where they reported that mtDNA mutations reduce motility and negatively affect the fertilization rate (Kumar *et al*., 2009). It was reported that sperms with a high mitochondrial membrane potential have high fertilizing capacities, and this is correlated positively with sperm motility (Gallon *et al*., 2006).

## 4.4. The effect of paternal mtDNA on embryo quality

Until a few years ago, maternal-restricted mtDNA inheritance was the only accepted idea, due to the disappearance of the paternal mtDNA after the cleavage-stage of an embryo (Cummins *et al*., 1998). In 2018, Luo and co-workers presented strong evidence for a bi-parental mtDNA inheritance: following the pattern of mitochondrial disease inheritance in three separate multi-generation families, they showed evidence of parental mtDNA transmission from father to offspring (Luo *et al*., 2018). Ecker found that sons who were born by ICSI shared the same SNPs in mitochondrial genes (*COX1*, *ND1*, *ND4* and *ND5*) with their fathers (Eker *et al*., 2019). The degree of similarity reached in some cases up to 99% of the paternal mtDNA (Eker *et al*., 2019). Another study had observed that mtDNA myopathy can be transmitted from the father to the son by ICSI and found that spermatozoa mtDNA mutations were maintained in the embryo (Lestienne, 1997). This suggests that paternal mtDNA mutations can be transmitted to the offspring at least in certain situations. Keeping in mind that the severity of mitochondrial diseases depends on the level of heteroplasmy, the exhibition of a disease phenotype is also correlated with the threshold value of mutated mtDNA which is ranged between (60% to 80%) based on mutation and cell type (Stewart & Chinnery, 2015).

In the present study, the embryo quality score has a negative relationship with the frequency of the total mtDNA variants. Embryos with no or low mtDNA variants have a high probability of reaching grade A on day 3. Furthermore, we found that the embryo cleavage score was negatively correlated with the frequency of the total mtDNA variants. A recent study has reported that mtDNA variations were correlated negatively with embryo grading and that the embryo quality at the blastocyst stage was also correlated positively with sperm motility (Wu *et al.,* 2019).

A recent study presented evolutionary proof supporting the role of paternal mtDNA in fertilization, where the data collected from two families of neotropical primates (Cebidae and Atelidae) indicated that the midpiece-containing mtDNA had evolved to be larger and wider in the younger species (Atelidae family), compared to the narrower and shorter midpiece in the ancestral species (Cebidae family) (Steinberg *et al.,* 2019). Another phenomenon supporting the role of sperm mtDNA in early embryo development is the double uni-parental inheritance of mitochondria in the marine mussel, *Mytilus edulis,* as females predominately contain maternal mtDNA in their somatic cells, while males carry maternal mtDNA in their somatic cells and paternal mtDNA in their gonads (Cao *et al*., 2004). Interestingly, the behavior of sperm/paternal mtDNA in this species depends on whether the egg will develop into females or into males, where the sperm mtDNA tends to aggregate with each other within the same cell in male-producing eggs, while sperm mtDNA are scattered randomly in female-producing oocytes (Cogswell *et al*., 2006). Later on, during the early cleavage stages in these embryos, the paternal mtDNA continues to play sex-specific roles and distinctive localization supporting the role of paternal mtDNA in early development (Cogswell *et al*., 2006).

Conversely, there is potent proof against the role of mtDNA in fertilization. In one study in *Caenorhabditis elegans*, they found that paternal mtDNA was eliminated immediately after fertilization by the autophagosomes (Sato & Sato, 2011). Another study found that the whole sperm mtDNA was degraded in the embryos of pigs and monkeys post-fertilization by the ubiquitin-proteasome system, induced by a specific microtubule-associated protein, known as sequestosome 1 (SQSTM1) (Song *et al*., 2016). Furthermore, one part of the evidence against paternal inheritance refers to the dilution effect, where it is expected that the effect of paternal mtDNA variants will be diluted since the unfertilized oocyte contains around

150 000 copies of mtDNA, compared to sperm that contain around only 100 copies (Chinnery & Hudson, 2013). It remains to be determined how common paternal mtDNA inheritance is, especially in ICSI settings and the phenotypic consequences it may cause.

### 4.5. Conclusion

In conclusion, the sperm motility was positively correlated with the ICSI outcomes, while protein carbonyl levels were negatively correlated with sperm motility and ICSI outcomes. This demonstrates that sperm motility can predict the ICSI outcomes, and the quantification of protein carbonyl can be used as a biomarker for protein oxidative damage in sperm. The frequencies of the total mitochondrial variants in the *ND1*, *ND2*, *ND5* and *ND6* genes were negatively correlated with the percentage of sperm motility and ICSI outcomes. The three missense variants, namely, 13708 G>A, 4216 T>C and 12506T>A, which were found to be negatively correlated with sperm motility and ICSI outcomes. Future studies are needed to determine the functional consequence of the identified variants and to understand the mechanism of how the fertilization rate is affected by sperm mtDNA in ICSI technique, specifically in the early stages of embryo development.

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# 6. Appendices

The published article from this thesis

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REPRODUCTIVE GENETICS: ORIGINAL ARTICLE

## Impact of Mitochondrial Genetic Variants in *ND1*, *ND2*, *ND5*, and *ND6* Genes on Sperm Motility and Intracytoplasmic Sperm Injection (ICSI) Outcomes

Mohammad A. Al Smadi[1] · Mohamad Eid Hammadeh[1] · Erich Solomayer[1] · Osamah Batiha[2] · Mohammad M. Altalib[3] · Mohammad Y. Jahmani[1] · Mohammad A. Shboul[4] · Bassam Nusair[5] · Houda Amor[1]

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**Abstract**
Sperm mitochondrial dysfunction causes the generation of an insufficient amount of energy needed for sperm motility. This will affect sperm fertilization capacity, and thus, most asthenozoospermic men usually require assisted reproductive techniques. The etiology of asthenozoospermia remains largely unknown. The current study aimed to investigate the effect of mitochondrial genetic variants on sperm motility and intracytoplasmic sperm injection (ICSI) outcomes. A total of 150 couples from the ICSI cycle were enrolled in this study. One hundred five of the male partners were asthenozoospermic patients, and they were subdivided into three groups according to their percentage of sperm motility, while forty-five of the male partners were normozoospermic. Genetic variants were screened using direct Sanger's sequencing in four mitochondrial genes (nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase 1 (ND1), NADH dehydrogenase 2 (ND2), NADH dehydrogenase 5 (ND5), and NADH dehydrogenase 6 (ND6)). We identified three significant variants: 13708G>A (rs28359178) in *ND5*, 4216T>C (rs1599988) in *ND1*, and a novel 12506T>A in *ND5* with *P* values 0.006, 0.036, and 0.013, respectively. The medians of sperm motility, fertilization rate, embryo cleavage score, and embryo quality score were significantly different between men showing 4216T>C, 12506T>A, 13708G>A and wild type, Mann-Whitney *P* values for the differences in the medians were < 0.05 in all of them. The results from this study suggest that 13708G>A, 12506T>A, and 4216 T>C variants in sperm mitochondrial DNA negatively affect sperm motility and ICSI outcomes.

**Keywords** mtDNA · Asthenozoospermia · Sperm motility · Fertilization rate · Embryo cleavage score · Embryo quality score

### Introduction

Genetic variants in mitochondrial genes are associated with many diseases [1]. Most of these diseases affect organs with high energy demand, such as the brain, the skeletal muscle, the eye, and the heart [2]. Because mitochondrial DNA (mtDNA) is not protected by histones or other DNA-binding proteins, it is more prone to DNA damage caused by excess levels of reactive oxygen species (ROS) and free radicals present in the matrix [3]. Furthermore, the mtDNA repair mechanism is less efficient compared to nuclear DNA repair [4]. Together, these factors increase the mutation rate in mtDNA, compared to nuclear DNA by 10–100 times [5].

Sperm depends on the mitochondrial oxidative phosphorylation (OxPhos) mechanism to produce the required energy for its motility [6]; therefore, mutated mtDNA leads to energy insufficiency, which causes a reduction in sperm motility [7]. In comparison to the oocyte, which has around 150,000 mtDNA copies, the spermatozoa have only around 100 mtDNA copies [8].

Sperms usually produce ROS in a natural physiological process, and ROS at low levels are necessary for sperm

✉ Mohammad A. Al Smadi
m_alsmadi@yahoo.com

[1] Department of Obstetrics & Gynecology, Reproductive Medicine Unit, Saarland University, Homburg, Germany

[2] Department of Biotechnology & Genetic Engineering, Jordan University of Science and Technology, Irbid, Jordan

[3] Department of Statistics, Yarmouk University, Irbid, Jordan

[4] Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Irbid, Jordan

[5] Reproductive Endocrinology and IVF Unit, King Hussein Medical Centre, Amman, Jordan

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function, as they play a significant role in sperm maturation, acrosome reaction, and sperm-oocyte fusion [9]. Excess levels of ROS have a damaging effect, leading to nuclear DNA strand breaks [10]. Leukocytes in the semen produce ROS one thousand times more than sperms, where such a situation is common during urinary tract infection (UTI) [11]. Mitochondrial dysfunction, caused by the production of high levels of ROS from complex I, has been shown to cause lipid peroxidative damage to the sperm midpiece and to result in a reduction in the sperm motility [12].

Seminal plasma is the major source of antioxidants that protect sperm cells against oxidative damages [13]. A significant decrease in the reduced to oxidized glutathione ratio and total glutathione levels in seminal plasma among groups of males with varicocele and idiopathic infertility has been reported [14].

It has been thought for a long time that mtDNA was inherited restrictedly from the mother, but a recent study has challenged this idea and provided evidence of additional paternal mtDNA transmission from fathers to offspring [15], where paternal mtDNA is governed by a quasi-Mendelian inheritance [16]. Furthermore, during intracytoplasmic sperm injection (ICSI) the whole sperm is injected into the cytoplasm of the oocyte, interestingly, the mtDNA is occasionally preserved, so that the offspring may indeed share their father's mitochondrial DNA [17].

Sperm motility has been shown to be affected by variations in the mitochondrial genome. Two single nucleotide polymorphisms (SNPs) in *ATPase6* and *ND4* mitochondrial genes at 9055 and 11719 loci, respectively, were found to be associated with asthenozoospermia [18]. Furthermore, a recent study has found a missense variant (11696G>A) in the *MT-ND4* gene to be associated with reduced sperm motility and causing the replacement of valine residue at position 313 with isoleucine, leading to a change in the secondary structure of the protein [19].

Several studies have shown connections between mitochondrial mutations, sperm motility, fertilization rate, and pregnancy completion. A study found that the fertilization rate had a strong positive correlation with sperm motility [20]. Moreover, a point mutation in the *ND1* gene at locus 4216 has been associated with recurrent pregnancy loss [21]. On the other hand, another study found that embryo grading at day 3 can predict the pregnancy rate of the in vitro fertilization (IVF) cycle, where embryos with high-quality scores have a better chance of a successful embryo transfer compared to embryos with a low-quality score [22].

The aim of this study was to investigate the influence of genetic variations in four mitochondrial genes (*ND1*, *ND2*, *ND5*, and *ND6*) on sperm motility and ICSI outcomes. This study is part of a larger project that aims to understand the role of mitochondrial genetic variants in infertility.

## Methods

### Subjects

From August 2018 to October 2019, samples were collected from 150 male partners, aged < 40 years, of ICSI couples that attended Assisted Reproductive Techniques (ART) Department at Prince Rashid Bin AL Hassan Hospital (PRBH), Irbid, Jordan.

According to the World Health Organization (WHO) laboratory manual for semen analysis, the total motility (progressive motility (PR) and non-progressive motility (NP)) for a normal male should be above 40%; otherwise, it will be considered to be asthenozoospermic. One hundred five of these samples were from asthenozoospermic men (PR + NP < 40%) and were divided into three groups according to their percentage of sperm motility. Group 1 included patients with sperm motility from 0 to 5%, group 2 included patients with sperm motility from 6 to 15%, and group 3 included patients with sperm motility from 16 to 35%. Forty-five samples of normozoospermic men with a high percentage of sperm motility between 50% and 75% were also collected as controls. The other semen parameters were within normal ranges (Table 1), Patients with varicocele, and alcoholic problems, as well as cigarette smokers and patients with genetic abnormalities, such as Klinefelter's syndrome, were excluded from this study. The study was approved by the Jordanian Royal Medical Services-Human Research Ethics Committee on 30/7/2018 with the project identification code (TF3/1/Ethics Committee/9126), and written consent from each couple was obtained.

Semen samples were obtained from all subjects by masturbation after 3 to 5 days of sexual restraint. The samples were incubated at 37 °C for 30 min (min) to allow liquefaction. Then they were evaluated by a senior clinical embryologist according to WHO criteria (WHO, 2010).

### Semen Preparation for ICSI

Semen samples were fractionated by Percoll media (45% and 90% gradient) through centrifugation at 1000*g* for 22 min.

**Table 1** Semen parameters among groups

| | Semen volume (ml), median ± SD | Sperm concentration ($10^6$ per ml), median ± SD | Total motility (PR + NP %), median ± SD | Morphologically normal spermatozoa (%), median ± SD |
|---|---|---|---|---|
| Group 1 | 2.63 ± 1.18 | 32.04 ± 15.12 | 0 ± 2.57 | 5.78 ± 3.14 |
| Group 2 | 3.1 ± 2.16 | 40.56 ± 27.88 | 9 ± 3.40 | 4.89 ± 2.45 |
| Group 3 | 2.89 ± 1.43 | 61.13 ± 39.74 | 20 ± 6.99 | 6.13 ± 5.69 |
| Control | 3.2 ± 1.35 | 73.16 ± 52.21 | 58 ± 9.05 | 8.82 ± 7.03 |

**Table 2** Primers list for PCR amplification and Sanger sequencing

| Primer name | Sequence (5′– 3′) | Product length |
|---|---|---|
| MT-ND1-F | CACCCACCCAAGAACAGGGT | 1155 |
| MT-ND1-R | TTCTCAGGGATGGGTTCGATTC | |
| MT-ND2-F | TCAGCTAAATAAGCTATCGGGC | 1200 |
| MT-ND2-R | GAGTGGGGTTTTGCAGTCCT | |
| MT-ND5-F | CTGCTAACTCATGCCCCCAT | 2043 |
| MT-ND5-R | GGAGGATCCTATTGGTGCGG | |
| MT-ND6-F | CCTCTCTTTCTTCTTCCCACTCA | 622 |
| MT-ND6-R | CGATGGTTTTTCATATCATT GGTCG | |
| ND5A* | CTAAACGCTAATCCAAGCC | * |
| ND5B* | CTATTACTCTCATCGCTACCTC | * |

ND5A* and ND5B* are additional internal primers were designed for Sanger sequencing only

After that, the pellet was collected and washed twice with a sperm-washing medium. Supernatants were discarded, and then the pellet was gently layered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (21 mM, PH = 7.3) plus 0.5% human serum albumin (Sage, USA). After that, the sperm pellet was collected and placed in the $CO_2$ incubator at 37 °C for 1 h, and later on the surface layer was aspired. Only sperm samples with 0% percentage of sperm motility were washed without this HEPES layering.

## The ICSI Technique

The oocytes were denudated after 2 h of egg retrieval by both chemical and mechanical treatments. Chemical denudation was performed using a hyaluronidase enzyme, while the mechanical denudation was done by aspirating the oocytes through glass pipettes (with a 150–300 μm inner diameter)

**Table 3** The mtDNA variants identified in the *ND1* gene

| Serial Number | MtDNA variant | Amino Acid change | Frequency of variant in control | Frequency of variant in asthenozoospermia | G test | *P* value |
|---|---|---|---|---|---|---|
| 1 | 3316G>A | Ala>Thr | 0/45 | 5/105 | 3.64 | 0.056 |
| 2 | 3348A>G | Leu>Leu | 2/45 | 3/105 | 0.234 | 0.629 |
| 3 | 3480A>G | Lys>Lys | 2/45 | 6/105 | 0.104 | 0.747 |
| 4 | 3462C>T | Ala>Ala | 0/45 | 3/105 | 2.166 | 0.141 |
| 5 | 3537A>G | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 6 | 3594C>T | Val>Val | 1/45 | 3/105 | 0.051 | 0.822 |
| 7 | 3720A>G | Gln>Gln | 0/45 | 1/105 | 0.716 | 0.397 |
| 8 | 3741C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 3826T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 3882G>A | Gln>Gln | 0/45 | 2/105 | 1.438 | 0.23 |
| 11 | 3921C>T | Ser>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 12 | 4086C>T | Val>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 13 | 4216T>C* | Tyr>His | **0/45** | **10/105** | **7.436** | **0.006*** |
| 14 | 4017C>T | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 15 | 3705G>A | Leu>Leu | 0/45 | 3/105 | 2.166 | 0.141 |
| 16 | 3505A>G | Thr>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 17 | 4104A>G | Leu>Leu | 1/45 | 4/105 | 0.266 | 0.606 |
| 18 | 3847T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 19 | 3834G>A | Leu>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 20 | 3843A>G | Trp>Trp | 0/45 | 1/105 | 0.716 | 0.397 |
| 21 | 3819C>T | His>His | 0/45 | 1/105 | 0.716 | 0.397 |
| 22 | 3335T>C | Ile>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 23 | 3396T>C | Tyr>Tyr | 0/45 | 1/105 | 0.716 | 0.397 |
| 24 | 3483G>A | Glu>Glu | 0/45 | 1/105 | 0.716 | 0.397 |
| 25 | 3666G>A | Gly>Gly | 1/45 | 0/105 | 2.424 | 0.12 |
| 26 | 3915G>A | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 27 | 3992C>T | Thr> Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 28 | 3593T>C | Val>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 29 | 3513C>T | Thr>Thr | 1/45 | 1/105 | 0.354 | 0.552 |

* Statistically significant difference, *P* value < 0.05

in HEPES, covered with oil (Vitrolife, Sweden). After 3 h in the $CO_2$ incubator at 37 °C, only the mature metaphase II oocytes were selected for injection by ICSI, using the microscope Integra 3 micromanipulator (CooperSurgical Fertility Company, Denmark) [23].

### Fertilization and Embryo Assessment

Zygotes were evaluated and graded from 1 to 5 after 16–18 h post-ICSI, and embryos at day 3 were classified as grades A, B, C, and D according to the Scott scoring system [24]. The cleavage score of each patient was calculated as follows: the sum of cleavage scores of embryos/the total number of embryos, where embryos at day 3 with 8 cells were given 4 points, embryos with 6 cells were given 3 points, and embryos with 4 cells were given 2. The cumulative quality score of the embryos for each patient was calculated as follows: the sum of scores of embryos/the total number of embryos, where embryos with grade A were given 3 points, embryos with grade B were given 2 points, and embryos with grade C were given only 1 point [25].

### Sperm mtDNA Extraction

Genomic DNA was extracted from the purified samples using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Germany); then, mtDNA was amplified using the REPLI-g Mitochondrial DNA Kit (Qiagen, Germany). Using the Nanodrop spectrophotometer ND-2000c (Thermo Scientific, USA), only the isolated DNA with an optimal density ratio of 260/280 of 1.8 or more was chosen and stored at − 20 °C.

### PCR

To amplify the *ND1, ND2, ND5 and ND6* genes, 4 sets of polymerase chain reaction (PCR) primers (forward and reverse) were designed using the Primer 3 program, flanking the region of each gene. Primers were designed using the human mitochondrial sequence obtained from the National Centre of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The oligonucleotide primers were synthesized by Microsynth Seqlab in Germany (Table 2).

A 25 μL reaction mixture was prepared to contain 12.5 μL PCR Master Mix (2X) (Thermo Scientific), 0.8 μL of 10 mM forward primer, 0.8 μL of 10 mM reverse primer, 2 μL mtDNA (20 ng/μL) and 8.9 μL nuclease-free water. The Thermocycler (C1000™ Thermal cycler, Bio-Rad, USA) program was set as follows: initial denaturing at 95 °C for 3 min,

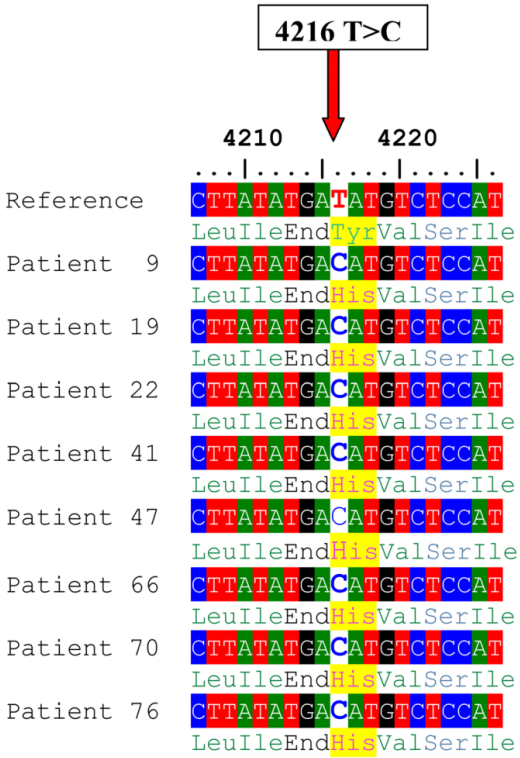


**Fig. 1** Alignment of the ND1 gene sequence for 8 patients showing 4216T>C nucleotide substitution. The red arrow indicates the site of nucleotide substitution, and the highlighted yellow colour indicates the amino-acid replacement (Tyr>His)

**Table 4** The percentages of men with total mitochondrial variants in (*ND1, ND2, ND5* and *ND6*) genes among controls and different asthenozoospermic groups

| | Group 1 (*N*=35) | Group 2 (N=35) | Group 3 (N=35) | Controls (*N*=45) | | |
|---|---|---|---|---|---|---|
| Gene | Number of males with total variants (percentage) | Number of males with total variants (percentage) | Number of males with total variants (percentage) | Number of males with total variants (percentage) | $X^2$ (3, *N*= 150) | *P* value |
| ND1 | 28 (80.0%) | 9 (25.7%) | 6 (17.1%) | 7 (15.6%) | 47.9 | 0.0001 |
| ND2 | 21 (60.0%) | 11 (31.4%) | 8 (22.9%) | 6 (13.3%) | 21.5 | 0.0008 |
| ND5 | 32 (91.4%) | 27 (77.1%) | 15 (42.9%) | 8 (17.8%) | 52.9 | 0.0001 |
| ND6 | 16 (45.7%) | 11 (31.4%) | 6 (17.1%) | 6 (13.3%) | 12.8 | 0.0051 |

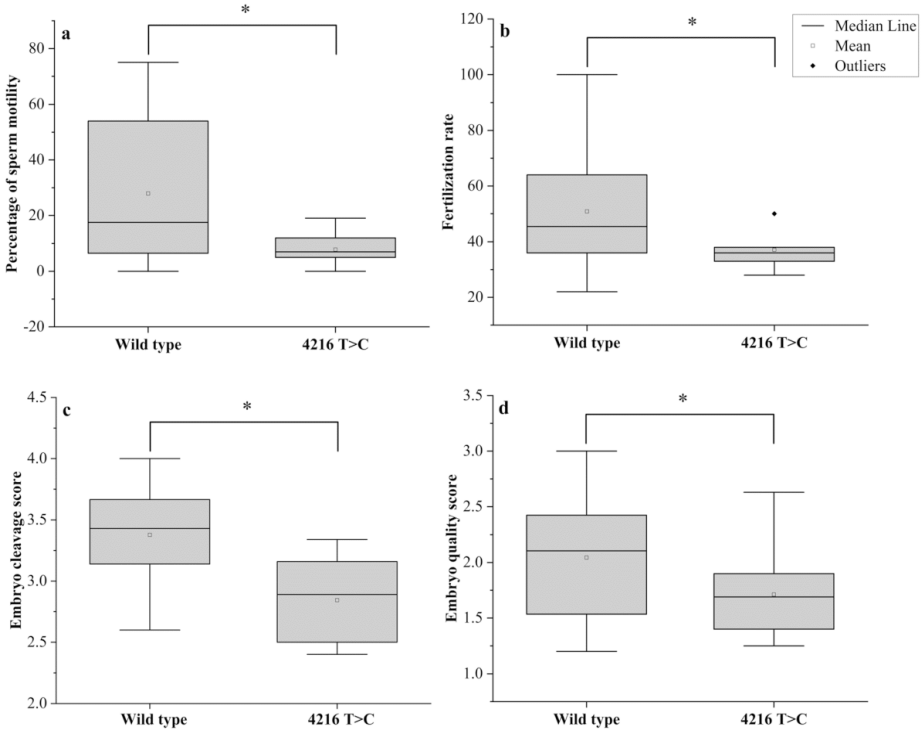


**Fig. 2** Box plots showing the differences between men with or without the 4216T>C. **a** Difference in sperm motility, **b** difference in fertilization rate, **c** difference in embryo cleavage score, and **d** difference in embryo quality score. Mann-Whitney *P* values for the differences in the medians were 0.012, 0.013, 0.044, and 0.041, respectively. *$P < 0.05$

followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 40 s (*ND1*: 59 °C; *ND2* and *ND6*: 61 °C; *ND5*: 64 °C), an extension of primers at 72 °C for *ND1* and *ND2*: 1 min; *ND5*: 2 min; *ND6*: 45 s), then a final extension for

**Table 5** The mtDNA variants identified in the *ND2* gene

| Serial number | MtDNA variant | Amino acid change | Frequency of variant in control | Frequency of variant in asthenozoospermia | G test | *P* value |
|---|---|---|---|---|---|---|
| 1 | 4769A>G | Met>Met | 2/45 | 12/105 | 2.061 | 0.151 |
| 2 | 4917A>G | Asn>Asp | 1/45 | 11/105 | 3.601 | 0.058 |
| 3 | 4733T>C | Asn>Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 4 | 4967T>C | Ser>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 5 | 4973T>C | Gly>Gly | 0/45 | 2/105 | 1.438 | 0.23 |
| 6 | 4991G>A | Gln>Gln | 1/45 | 4/105 | 0.266 | 0.606 |
| 7 | 5178C>A | Leu>Met | 0/45 | 2/105 | 1.438 | 0.23 |
| 8 | 4646T>C | Tyr>Tyr | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 5302T>C | Ile>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 5417G>A | Gln>Gln | 0/45 | 1/105 | 0.716 | 0.397 |
| 11 | 5237G>A | Pro>Pro | 1/45 | 1/105 | 0.354 | 0.552 |
| 12 | 5331C>A | Leu>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 13 | 4561T>C | Val>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 14 | 4823T>C | Val>Val | 1/45 | 4/105 | 0.266 | 0.606 |
| 15 | 5147G>A | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 16 | 4639T>C | Ile>Thr | 0/45 | 2/105 | 1.438 | 0.23 |
| 17 | 4883C>T | Pro>Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 18 | 4703T>C | Asn>Asn | 1/45 | 0/105 | 2.424 | 0.12 |
| 19 | 5004T>C | Leu>Leu | 1/45 | 0/105 | 2.424 | 0.12 |
| 20 | 4640C>A | Ile>Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 21 | 5048T>C | Val>Val | 1/45 | 1/105 | 0.354 | 0.552 |

**Table 6** The mtDNA variants identified in the *ND5* gene

| Serial number | MtDNA variant | Amino acid change | Frequency of variant in control | Frequency of variant in asthenozoospermia | G test | *P* value |
|---|---|---|---|---|---|---|
| 1 | 13708G>A* | Ala>Thr | 1/45 | 15/105 | 6.131 | 0.013* |
| 2 | 13879T>C | Ser>Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 3 | 13965T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 4 | 13966A>G | Thr>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 5 | 13967C>T | Thr>Met | 0/45 | 1/105 | 0.716 | 0.397 |
| 6 | 13928G>C | Ser>Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 7 | 13734T>C | Phe>Phe | 0/45 | 1/105 | 0.716 | 0.397 |
| 8 | 14040G>A | Gln>Gln | 0/45 | 4/105 | 2.9 | 0.089 |
| 9 | 14070A>G | Ser>Ser | 1/45 | 3/105 | 0.051 | 0.822 |
| 10 | 13650C>T | Pro>Pro | 0/45 | 3/105 | 2.166 | 0.141 |
| 11 | 13752T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 12 | 13803A>G | Thr>Thr | 0/45 | 2/105 | 1.438 | 0.23 |
| 13 | 14059A>G | Ile>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 14 | 13780A>G | Ile>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 15 | 14053A>G | Thr>Ala | 0/45 | 1/105 | 0.716 | 0.397 |
| 16 | 14110T>C | Phe>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 17 | 13762T>G | Ser>Ala | 0/45 | 2/105 | 1.438 | 0.23 |
| 18 | 12372G>A | Leu>Leu | 6/45 | 18/105 | 0.35 | 0.554 |
| 19 | 12705C>T | Ile>Ile | 0/45 | 14/105 | 10.593 | 0.001 |
| 20 | 12850A>G | Ile>Val | 0/45 | 3/105 | 2.166 | 0.141 |
| 21 | 12822A>G | Ala>Ala | 0/45 | 4/105 | 2.9 | 0.089 |
| 22 | 12406G>A | Val>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 23 | 13722A>G | Leu>Leu | 1/45 | 1/105 | 0.354 | 0.552 |
| 24 | 12346C>T | His>Tyr | 0/45 | 2/105 | 1.438 | 0.23 |
| 25 | 12403C>T | Leu>Phe | 0/45 | 2/105 | 1.438 | 0.23 |
| 26 | 12414T>C | Pro>Pro | 0/45 | 2/105 | 1.438 | 0.23 |
| 27 | 12612A>G | Val>Val | 0/45 | 8/105 | 5.898 | 0.015 |
| 28 | 12501G>A | Met>Met | 2/45 | 3/105 | 0.234 | 0.629 |
| 29 | 12693A>G | Lys>Lys | 0/45 | 1/105 | 0.716 | 0.397 |
| 30 | 12950A>G | Asn>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 31 | 12408T>C | Val>Val | 0/45 | 1/105 | 0.716 | 0.397 |
| 32 | 13368G>A | Gly>Gly | 1/45 | 13/105 | 4.83 | 0.028 |
| 33 | 13020T>C | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 34 | 13215T>C | Leu>Leu | 0/45 | 1/105 | 0.716 | 0.397 |
| 35 | 13702C>G | Arg>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 36 | 13392T>C | Asn>Asn | 0/45 | 2/105 | 1.438 | 0.23 |
| 37 | 13104A>G | Gly>Gly | 1/45 | 5/105 | 0.589 | 0.443 |
| 38 | 13422A>G | Leu>Leu | 0/45 | 2/105 | 1.438 | 0.23 |
| 39 | 13145G>A | Ser>Asn | 1/45 | 2/105 | 0.016 | 0.9 |
| 40 | 13326T>C | Cys>Cys | 0/45 | 1/105 | 0.716 | 0.397 |
| 41 | 13188C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 42 | 13590G>A | Leu>Leu | 0/45 | 2/105 | 1.438 | 0.23 |
| 43 | 13650C>T | Pro>Pro | 0/45 | 2/105 | 1.438 | 0.23 |
| 44 | 13188C>T | Thr>Thr | 0/45 | 1/105 | 0.716 | 0.397 |
| 45 | 13780A>G | Ile>Val | 1/45 | 0/105 | 2.424 | 0.12 |
| 46 | 13981C>T | Pro>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 47 | 14025T>C | Pro>Pro | 0/45 | 1/105 | 0.716 | 0.397 |
| 48 | 14034T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 49 | 12630G>A | Trp>Trp | 0/45 | 1/105 | 0.716 | 0.397 |

**Table 6** (continued)

| Serial number | MtDNA variant | Amino acid change | Frequency of variant in control | Frequency of variant in asthenozoospermia | G test | *P* value |
|---|---|---|---|---|---|---|
| 50 | 12654A>G | Trp>Trp | 0/45 | 1/105 | 0.716 | 0.397 |
| 51 | 12681T>C | Asn>Asn | 0/45 | 1/105 | 0.716 | 0.397 |
| 52 | 13542A>G | Ser>Ser | 0/45 | 1/105 | 0.716 | 0.397 |
| 53 | 13617T>C | Ile>Ile | 0/45 | 1/105 | 0.716 | 0.397 |
| 54 | 13821C>T | Phe>Phe | 0/45 | 3/105 | 2.166 | 0.141 |
| 55 | 12506T>A* | Leu>Gln | 0/45 | 6/105 | 4.386 | 0.036* |
| 56 | 12879T>C | Gly>Gly | 0/45 | 3/105 | 2.166 | 0.141 |

* Statistically significant difference, *P* value <0.05

5 min at 72 °C. To check the amplification, 5 μL of each PCR product was run on 1% agarose gel stained with SYBR Safe stain (Invitrogen) and then visualized using Molecular Imager Gel Doc XR+ (Bio-Rad).

### Identification of Genetic Variants in *ND1*, *ND2*, *ND5*, and *ND6*

PCR products were purified and sequenced using the Sanger method (Microsynth Seqlab, Germany). Sequencing was carried out in both directions (forward and reverse) for each sample. For the *ND5* gene, two additional internal primers were designed, namely, ND5A and ND5B (Table 1).

The primary and secondary sequences for each sample were analyzed using the BioEdit sequence alignment editor version 7.2.5 and aligned to the NCBI reference sequences (NC_012920.1).

To predict the possible impact of amino-acid substitution on protein structure and function, and to evaluate the possible damaging effect of genetic variants; two versions of software were used (The American College of Medical Genetics and Genomics (ACMG),https://www.acmg.net and Poly Phenyl-2, http://genetics.bwh.harvard.edu/pph2.

### Statistical Analysis

Statistical analysis was carried out using the OriginPro, Version 2020 (OriginLab Corporation, Northampton, MA, USA). The normality assumptions were checked for the variables in question and were found not to be fitted by a normal distribution, and hence, non-parametric tests were applied to our study. The Kruskal-Wallis H test alongside the Mann-Whitney *U* test in addition to chi-square were used to determine if there were statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable. Spearman's rho, a non-parametric test, was used to measure the strength of association between two variables. Odds ratio and their 95% confidence intervals were determined, and some descriptive statistics and graphs for the variables in question were presented. A *P* value < 0.05 was considered to be statistically significant.

## Results

A total of 29 nucleotide substitutions (SNPs) in the *ND1* gene were identified; six of them were missense, while 23 were synonymous (Table 3). The percentages of men with total variants in the *ND1* gene among groups 1, 2, 3, and the control were: 80%, 25.7%, 17.1%, and 15.6%, respectively, *P* = 0.0001(Table 4). Only one variant 4216T>C (rs1599988)

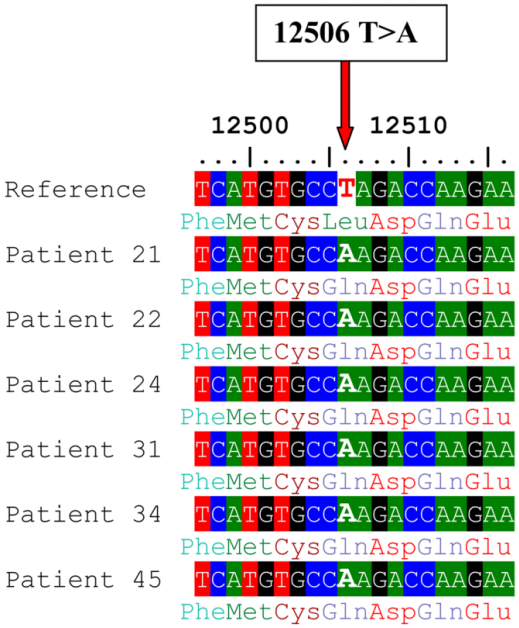


**Fig. 3** Alignment of the ND5 gene sequence for 6 patients showing the novel 12506T>A variant. The red arrow indicates the site of nucleotide substitution causing an amino acid replacement (Leu>Gln)

was significantly different between cases and controls (*P* value = 0.006). The percentages of men with 4216T>C among groups 1, 2, and 3 were 14.3%, 8.6%, and 5.8%, respectively (*P* = 0.005), while it was not found among the control. This variant caused Tyr>His amino acid substitution (Fig. 1). According to ACMG and Poly Phenyl-2, it is predicted that this variant is benign (score 0.001, sensitivity 0.99; specificity 0.15). It was identified among 10 asthenozoospermic patients in a homoplasmic state while it was not found among control group. The medians of sperm motility were (wild type (17.5 ± 25.21), 4216T>C (7 ± 6.1), *P* = 0.012), fertilization rate (wild type (45.5 ± 18.08, 4216T>C (36 ± 7.61), *P* = 0.013), embryo cleavage score (wild type (3.42 ± 0.37), 4216T>C (3.07 ± 0.12), *P* = 0.044), and embryo quality score (wild type (2.11 ± 0.49), 4216T>C (1.69 ± 0.41), *P* = 0.041) (Fig. 2).

A total of 21 nucleotide substitutions in the *ND2* gene were identified; six of them were missense, and 15 were synonymous (Table 5). The percentages of men with total variants in the *ND2* gene among groups 1, 2, 3, and in the control were 60%, 31.4%, 22.9%, and 13.3%, respectively, *P* = 0.0008 (Table 4). However, none of these variants were significantly different between the 2 groups (*P* value > 0.05).

A total of 19 missense variants and 37 synonymous variants were identified in the *ND5* gene (Table 6). The percentages of men with total variants in the *ND5* gene among groups 1, 2, 3, and the control were 91.4%, 77.1%, 42.9%, and 17.8%, respectively, *P* = 0.0001(Table 4). All of these variants had been previously reported in the NCBI (https://www.ncbi.nlm.nih.gov/) and in the human mitochondrial DNA database (www.mitomap.org), except for a novel variant at the locus 12506. This variant was identified in 6 asthenozoospermic patients (all were heteroplasmic), also the percentages of men with 12506T>A among groups 1, 2 and were 14.3%, 2.9% respectively, *P* = 0.0001 while it was not found among group 3 and the control. We reported the nucleotide sequences of 6 patients (BankIt2363991 ND5_21, BankIt2363991 ND5_22, BankIt2363991 ND5_24, BankIt2363991 ND5_31, BankIt2363991 ND5_34 and BankIt2363991 ND5_45) to the GenBank and the following accession numbers were given, respectively (MT742299,

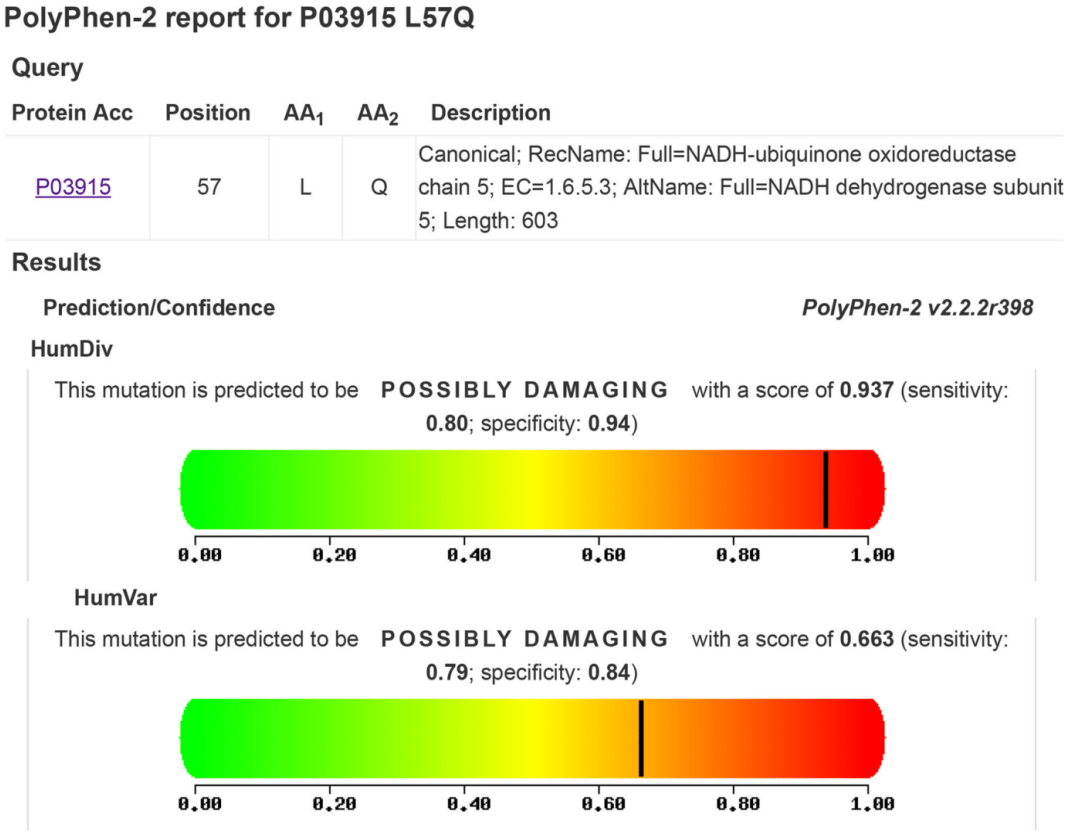


**Fig. 4** Prediction of the functional effect of 12506T>A substitution by Poly Phenyl-2 software

MT742300, MT742301, MT742302, MT742303 and MT742304). The novel SNP 12506T>A is a missense variant that replaced Gln by Leu (Fig. 3). This variant is significantly different between cases and controls ($P = 0.036$) and is predicted to be probably damaging according to the Poly Phenyl-2 and ACMG (score 0.663, sensitivity 0.79; specificity 0.84) (Fig. 4). The medians of sperm motility (wild type (17.5 ± 24.93), 12506T>A (4.5 ± 24.93), $P = 0.009$), fertilization rate (wild type (46 ± 17.8), 12506T>A (32 ± 2.13), $P = 0.001$), embryo cleavage score (wild type (3.42 ± 0.37), 12506T>A (3.07 ± 0.12), $P = 0.044$), embryo quality score (wild type (2.09 ± 0.49), 12506T>A (1.62 ± 0.22), $P = 0.028$) (Fig. 5).

Another variant 13708G>A (rs28359178) in the *ND5* gene was significantly different between cases and controls ($P = 0.013$), causing Ala>Thr amino acid substitution (Fig. 6). However, this variant is predicted to be benign according to the Poly Phenyl-2 and ACMG criteria (score 0.001, sensitivity 0.99; specificity 0.15). The 13708G>A variant was identified among 14 asthenozoospermic men only—all were homoplasmic—while it was not found among control group. The percentages of men with 13708G>A among groups 1, 2, and 3 were 17.1%, 14.3%, and 8.6%, $P = 0.0012$. The medians of sperm motility (wild type (18 ± 25.46), 13708G>A (8 ± 7.13), $P = 0.043$), fertilization rate (wild type (47 ± 18.09), 13708G>A (36 ± 9.55), $P = 0.017$), embryo cleavage score (wild type (3.5 ± 0.36), 13708G>A (3.0 ± 0.24), $P = 0.001$), embryo quality score (wild type (2.11 ± 0.49), 13708G>A 1.67 ± 0.35), $P = 0.007$) (Fig. 7).

A total of 15 nucleotide substitutions was identified in the *ND6* gene; two of them were missense, while the rest were synonymous variants (Table 7). The percentages of men with total variants in the ND6 gene among groups 1, 2, 3, and control were 45.7%, 31.4%, 17.1%, and 13.3%, respectively, $P = 0.0051$(Table 4). However, none of these variants were significantly different between the two groups.

The frequency of all missense variants had a significant inverse relationship with sperm motility, fertilization rate, embryo cleavage score, and embryo cleavage score ($r = -0.583$, $P = 0.0001$), ($r = -0.576$, $P = 0.0001$), ($r = -0.613$, $P = 0.0001$), and ($r = -0.717$, $P = 0.0001$), respectively (Fig. 8). The fertilization rate had a significant positive relationship with the embryo cleavage score and the embryo quality score ($r = 0.582$ and $P = 0.0001$) ($r = 0.739$ and $P = 0.0001$),

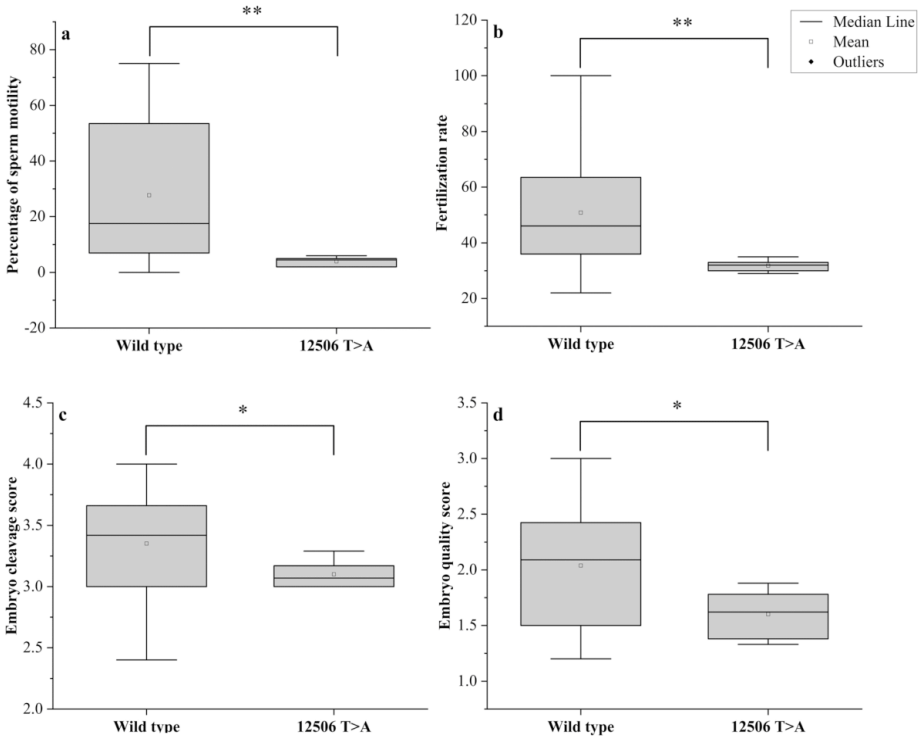


**Fig. 5** Box plots showing the differences between men with or without the 12506T>A. **a** Difference in sperm motility, **b** difference in fertilization rate, **c** difference in embryo cleavage score, and **d** difference in embryo quality score. Mann-Whitney *P* values for the differences in the medians were 0.009, 0.001, 0.044, and 0.028, respectively. $^*P < 0.05$, $^{**}P < 0.01$

respectively (Fig. 9). The median fertilization rates were as follows: (G1 (36 ± 1.86), G2 (40 ± 1.63), G3 (47 ± 13.41), control (67 ± 14.69), $P < 0.001$) as illustrated in (Fig. 10).

The average embryo quality score and cleavage score among asthenozoospermic patients were 1.83 and 3.24, respectively, compared to 2.47 and 3.58, respectively, in normozoospermic ones. A significant difference in the embryo cleavage score was detected between patients and controls ($P = 0.0001$). We also found a significant difference between the embryo quality score in the two categories ($P = 0.0001$). The median of the embryo cleavage scores were statistically different between the different groups: (G1 (3 ± 0.31), G2 (3.34 ± 0.36), G3 (3.58 ± 0.31), control (3.66 ± 0.25), $P < 0.001$), as illustrated in (Fig. 11). The median of the embryo quality scores were also statistically different: (G1 (1.5 ± 0.31), G2 (1.75 ± 0.39), G3 (2.14 ± 0.44), control (2.5 ± 0.27), $P < 0.001$) (Fig. 12).

## Discussion

In the present study, we found three missense variants correlated inversely with sperm motility and ICSI outcomes. Two variants, namely, 13708G>A (rs28359178) in the *ND5* gene and 4216T>C (rs1599988) in the *ND1*, were previously reported in the NCBI (https://www.ncbi.nlm.nih.gov/) and human mitochondrial DNA database (www.mitomap.org), while we identified 12506T>A as a novel variant in the *ND5* gene.

The variants detected in the present study have not been linked to asthenozoospermia before. However, a previous study showed that the rate of 4216T>C variant among diabetic patients was higher than in the controls and was statistically associated with diabetes mellitus type 2 [26]. Another study found an interesting male-specific association between the 4216T>C variant and the rate of infection leading to complicated sepsis and death [27].

Similarly, the 13708G>A variant has been linked to several clinical manifestations. It has been shown that the 13708G>A variant increases the susceptibility to multiple sclerosis [28] and found to enhance the expression of Leber hereditary optic neuropathy (LHON) disease in another study [29]. Furthermore, 13708G>A variant was found to increase the risk for Alzheimer's disease, specifically among male patients [30].

Our results showed a negative correlation between sperm motility and the frequency of total variants, where the highest frequency of mitochondrial variants in the four genes was among group one (asthenozoospermic patients with 0 to 5% sperm motility), while the lowest frequency was among group three (asthenozoospermic patients with 16 to 35% sperm motility). The control group with normozoospermia (sperm motility between 50 and 75%) showed a lower frequency of mitochondrial variants, compared to all patients' groups. A previous study reported that 9055G>A in the ATPase gene and 11719G>A in *ND4* were associated with poor semen quality [18]. Another variant 11994C>T in *ND4* gene was negatively correlated with oligoasthenozoosperima [31].

It is known that sperms require ATP for flagellar movement, and this depends on OxPhos to provide their energy requirements [3]. Here, we identified three missense variants with a significant association with sperm motility. These variants are located in the *ND1* and *ND5* genes, which are part of complex 1 [32]. Complex 1 plays a key role in OxPhos by receiving electrons from NADH, and the captured energy from these electrons is utilized to release protons to the intermembrane space, and these protons are used later to generate ATP [33]. Therefore, pathogenic variants in the *ND* genes are expected to affect complex 1 activity, causing a deficiency in energy production, and this will negatively affect the sperm motility [7].

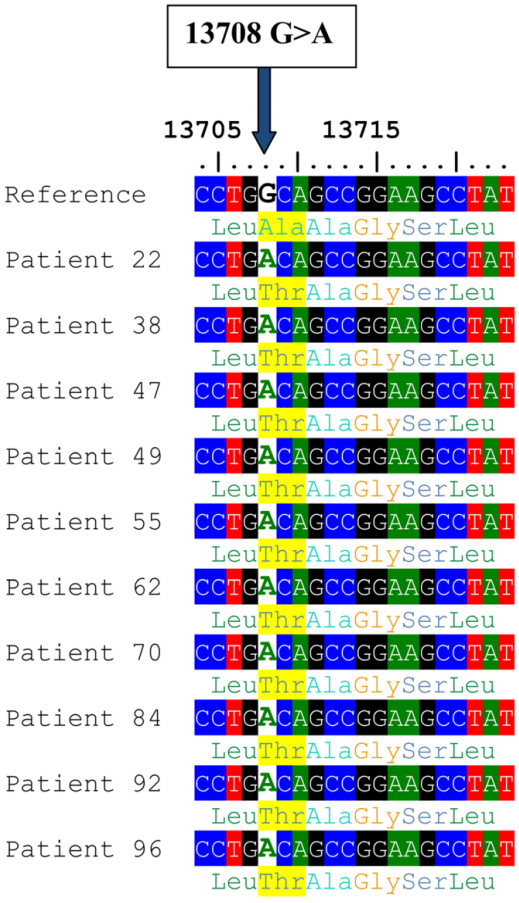


**Fig. 6** Alignment of the *ND5* gene sequence for 10 patients showing 13708G>A variant. The blue arrow indicates the site of nucleotide substitution, and the highlighted yellow colour indicates the amino acid replacement (Ala>Thr)

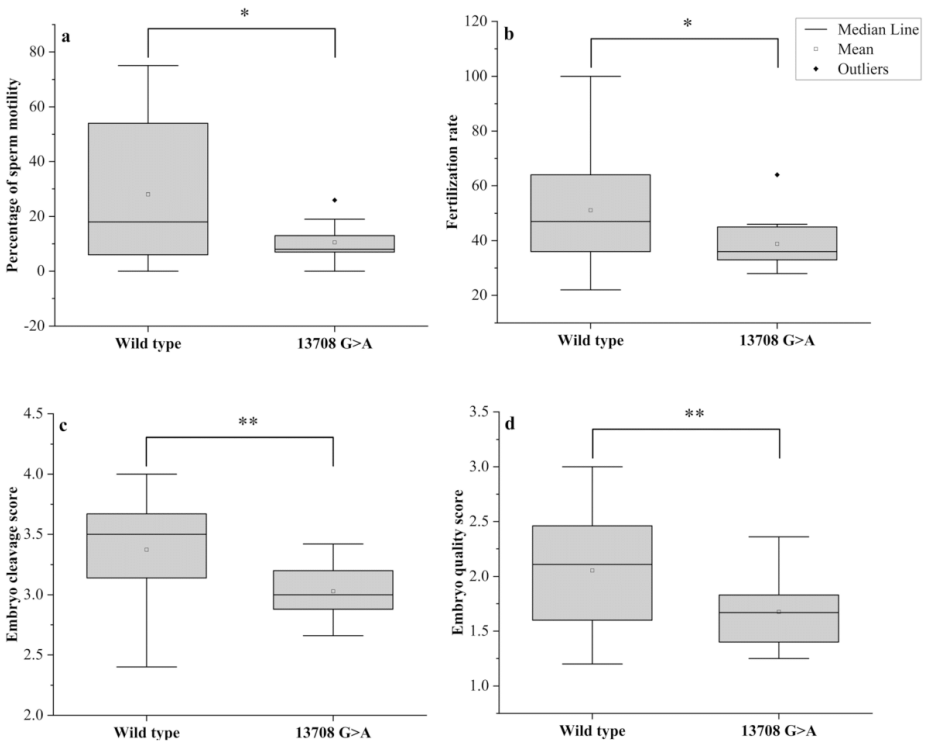


**Fig. 7** Box plots showing the differences between men with or without the 13708G>A. **a** Difference in sperm motility, **b** difference in fertilization rate, **c** difference in embryo cleavage score, and **d** difference in embryo quality score. Mann-Whitney *P* values for the differences in the medians were 0.043, 0.017, 0.001, and 0.007, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$

Our results demonstrated that the ICSI outcomes were correlated inversely with the load of mtDNA variants, while it had a strong positive correlation with the percentage of sperm motility, where the fertilization rate was lower among group one compared to group three. Embryo quality, including embryo cleavage score and embryo quality score, were positively

**Table 7** The mtDNA variants identified in the *ND6* gene

| Serial number | MtDNA variant | Amino acid change | Frequency of variant in control | Frequency of variant in asthenozoospermia | G test | *P* value |
|---|---|---|---|---|---|---|
| 1 | 14167C>T | Glu>Glu | 0/45 | 4/105 | 2.9 | 0.089 |
| 2 | 14179A>G | Tyr>Tyr | 1/45 | 2/105 | 0.016 | 0.9 |
| 3 | 14182T>C | Val>Val | 0/45 | 3/105 | 2.166 | 0.141 |
| 4 | 14233A>G | Asp>Asp | 2/45 | 12/105 | 2.061 | 0.151 |
| 5 | 14323G>A | Asp>Asp | 0/45 | 3/105 | 2.166 | 0.141 |
| 6 | 14364G>A | Leu>Leu | 1/45 | 7/105 | 1.438 | 0.23 |
| 7 | 14560G>A | Val>Val | 1/45 | 1/105 | 0.354 | 0.552 |
| 8 | 14620C>T | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 9 | 14470T>C | Gly>Gly | 0/45 | 1/105 | 0.716 | 0.397 |
| 10 | 14566A>G | Gly>Gly | 0/45 | 2/105 | 1.438 | 0.23 |
| 11 | 14569G>A | Ser>Ser | 0/45 | 2/105 | 1.438 | 0.23 |
| 12 | 14178T>C | Ile>Val | 0/45 | 2/105 | 1.438 | 0.23 |
| 13 | 14180T>C | Tyr>Cys | 0/45 | 2/105 | 1.438 | 0.23 |
| 14 | 14212T>C | Val>Val | 1/45 | 2/105 | 0.016 | 0.9 |
| 15 | 14305G>A | Ser>Ser | 1/45 | 1/105 | 0.354 | 0.552 |

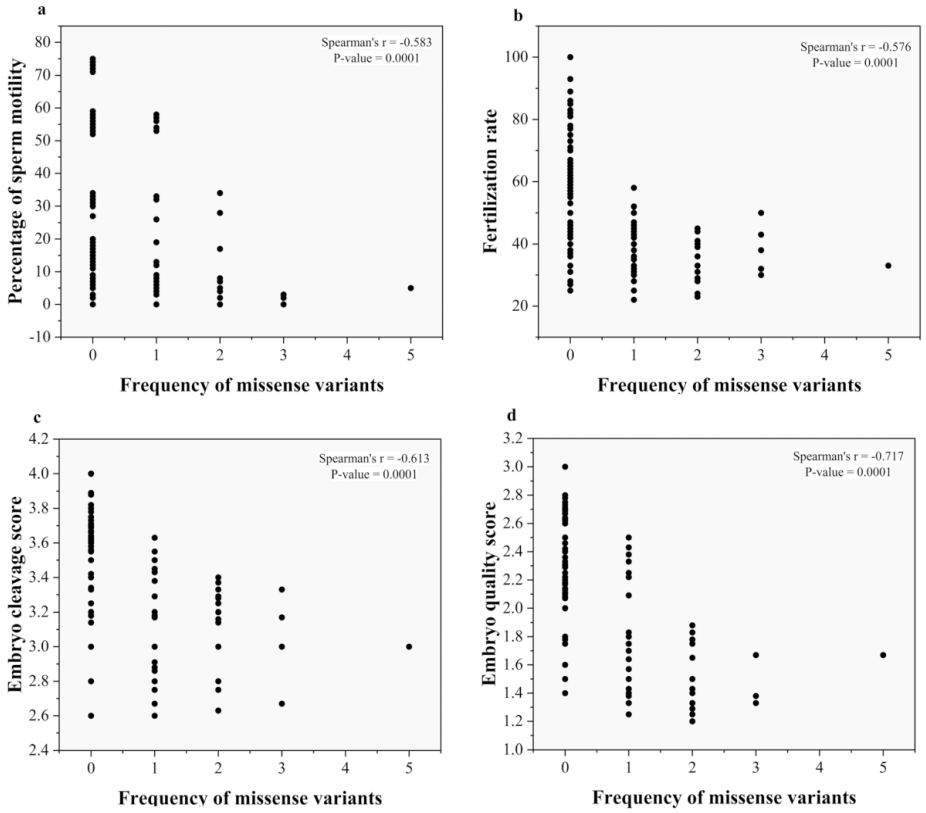


**Fig. 8** Scatter plots of frequency of missense variants with sperm motility and ICSI outcomes. **a** Frequency of missense variants with sperm motility ($r = -0.583$, $P = 0.0001$). **b** Frequency of missense variants with fertilization rate ($r = -0.576$, $P = 0.0001$). **c** Frequency of missense variants with embryo cleavage score($r = -0.613$, P = 0.0001). **d** Frequency of missense variants with embryo quality score ($r = -0.717$, $P = 0.0001$)

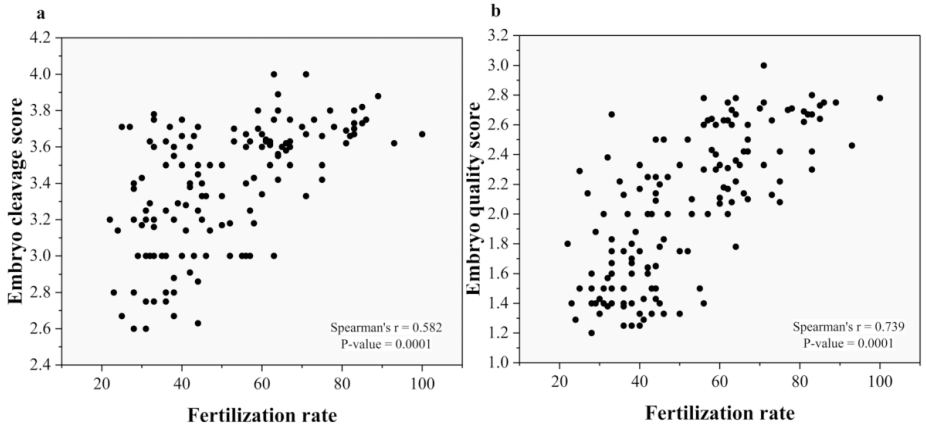


**Fig. 9** **a** Scatter plots of fertilization rate and embryo cleavage score showing a positive correlation, $r = 0.582$, $P = 0.0001$. **b** Scatter plots of fertilization rate and embryo quality score showing a positive correlation, $r = 0.739$, $P = 0.0001$

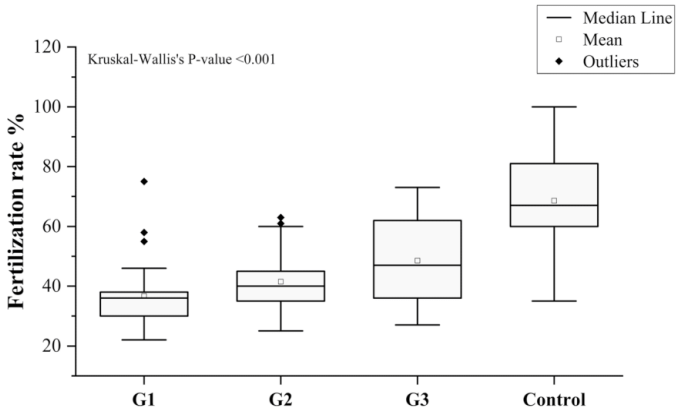


**Fig. 10** Fertilization rate among groups 1, 2, 3, and control. Kruskal-Wallis's *P* value was included. Mann-Whitney *P* values for the differences in the medians between all groups < 0.001

correlated with sperm motility. Embryos among group one had the lowest quality scores, while the best embryo quality was among controls. Furthermore, the three variants (13708G>A, 4216T>C, and 12506T>A) were negatively correlated with ICSI outcomes. Patients with those variants showed lower fertilization rate and embryo quality scores compared to other men without these variants.

Interestingly, our findings agree with the results of a previous study where they reported that mtDNA mutations reduce motility and negatively affect the fertilization rate [34]. Our results also agree with a previous study that identified statistical associations between seven variants in the *ND5* gene and fertilization failure [35]. In the present study, we determined that the embryo quality score has a negative correlation with the frequency of total mtDNA variation. Embryos with no or low mtDNA variants have a high probability of reaching grade A on day 3. A recent study has reported that mtDNA variations were correlated negatively with embryo grading, and embryo quality at the blastocyst stage was correlated positively with sperm motility [20].

However, our results disagree with previous findings where no significant difference between the frequency of variants in mtDNA among teratozoospermia and asthenozoospermia was found [36]. However, these results do not provide conclusive evidence against the role of mtDNA in sperm motility, because of the small sample (43 samples only). The contradiction between the results of this study and other studies may be attributed to population variation. In a previous study, they found that the frequency of mtDNA SNPs varies between African American, European and Asian populations for the same mitochondrial disease [37]. Two independent studies on the association of the same SNP, namely 11994C>T, and oligoasthenozoosperima have reached different conclusions, where a strong association was found in India [31], while in the other study in Portugal no association was found [38]. The severity of the mitochondrial diseases depends on the level of heteroplasmy and the threshold value mutant mtDNA should pass in order to show pathogenic effects, which ranges between 60 and 80%, based on the types of mutations and cells [4].

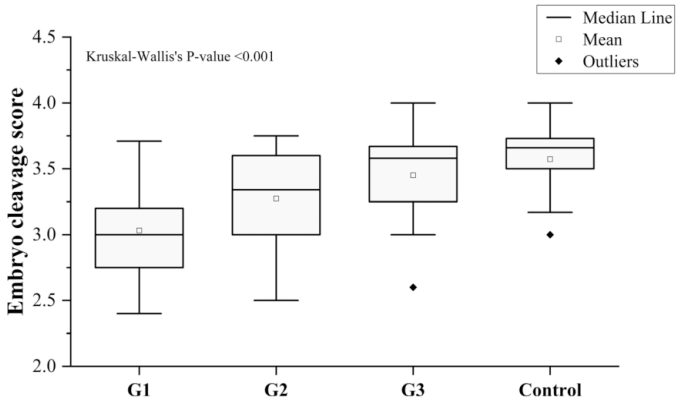


**Fig. 11** Embryo cleavage score among groups 1, 2, 3, and control. Kruskal-Wallis's *P* value was included. Mann-Whitney *P* values for the differences in the medians between all groups < 0.001

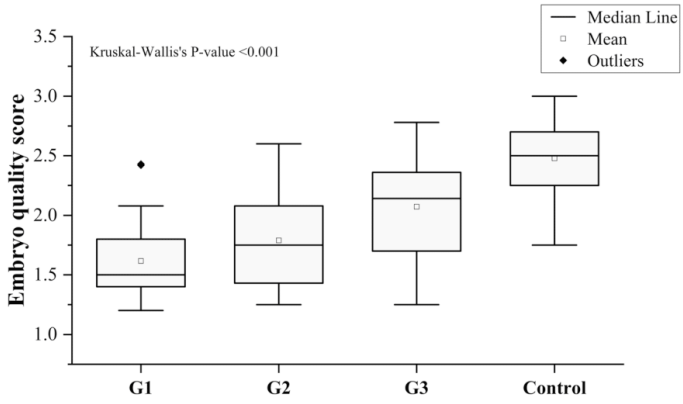


**Fig. 12** Embryo quality score among the studied groups. Kruskal-Wallis's *P* value was included, Mann-Whitney *P* values for the differences in the medians between all groups < 0.001

We think that the criteria used in the current study for sample selection and sample grouping influenced the results obtained. We excluded many environmental factors that are known to affect sperm motility, such as smoking, varicocele, alcoholism, and men older than 40 years old. These factors have already been shown to affect sperm motility, and thus excluding them increases the probability of identifying the genetic etiology [39, 40]. Most of the previous studies, the patients' grouping was done by including all sperm abnormalities together as one group, while in our study asthenozoospermic patients were divided into different categories, where the reduced sperm motility varied between 0 and 35%. Samples with sperm motility between 36 and 40% were excluded to stay far from the borderline of the normal percentage (40%) of total sperm motility recommended by the WHO [41]. We also relied on the percentage of total sperm motility rather than on the type of sperm motility, because, according to WHO, progressive motility (type A and type B) should be greater than 32%. So, as our samples were evaluated by different embryologists, it could be more reliable to depend on total sperm motility where it is easier to distinguish between motile and immotile sperm, rather than determining the specific type of sperm; this also decreases the number of individual errors among technicians.

Recent studies have provided evidence supporting the paternal transmission of mtDNA. Luo and co-workers presented a strong evidence for a bi-parental mtDNA inheritance, following the pattern of mitochondrial disease inheritance in three separate multi-generation families; they showed evidence of parental mtDNA transmission from father to offspring [15]. Ecker et al. found that sons who were born by ICSI shared the same SNPs in mitochondrial genes (*COX1*, *ND1*, *ND4*, and *ND5*) with their fathers; also, they found that the degree of similarity reached in some cases up to 99% of the paternal mtDNA [17]. Another study found that mtDNA myopathy can be transmitted from the father to the son by ICSI, and that spermatozoa mtDNA mutations were maintained in the embryo [42]. However, it is expected that the effect of paternal mtDNA variants will be diluted since the unfertilized oocyte contains around 150,000 copies of mtDNA, compared to sperm which contain around only 100 copies [43]. It remains to be determined how common paternal mtDNA inheritance is especially in ICSI settings, and the phenotypic consequences it may cause.

## Conclusions

We found that the frequencies of total mitochondrial variants in *ND1*, *ND2*, *ND5*, and *ND6* genes were negatively correlated with the percentages of sperm motility and ICSI outcomes. We also identified three variants, 13708G>A, 4216T>C, and 12506T>A, to be negatively correlated with sperm motility and ICSI outcomes. Future studies are needed to determine the functional consequences of the identified variants, and to understand the mechanism of how the fertilization rate is affected by sperm mtDNA, specifically in the early stage of embryo development. Despite the recent studies to identify the genetic basis of male infertility in Jordan [44–46], its cause remains unknown in a large number of cases. High-throughput genomic studies to identify the genetic etiology for infertility in Jordan should therefore be undertaken.

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**Authors' Contributions** MAS: conducted the experiments and generated the manuscript. MEH: designed the study and contributed to data interpretation and analysis. ES: methodology. OB: manuscript editing. MMA: statistical analysis. MYJ: methodology. MASH: bioinformatics analysis. BN: clinical supervision. HA: methodology and supervision. All authors approved the final version and the submission of this article.

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**Data Availability** Data are available upon request from the corresponding author.

## Compliance with Ethical Standards

**Competing Interests** The authors declare that they have no conflict of interest.

**Ethical Approval and Consent to Participation** The study was approved by the Jordanian Royal Medical Services- Human Research Ethics Committee on 30/7/2018 with the project identification code (TF3/1/ Ethics Committee/9126).

**Consent for Publication** Not applicable.

**Abbreviations** ICSI, Intracytoplasmic sperm injection; MtDNA, Mitochondrial DNA; ROS, Reactive oxygen species; OxPhos, Oxidative phosphorylation; SNP, Single nucleotide polymorphism; IVF, In vitro fertilization; WHO, World Health Organization; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, Polymerase chain reaction; NCBI, National Centre of Biotechnology Information; ACMG, American College of Medical Genetics and Genomics; SQSTM1, Sequestosome 1; MDPs, Mitochondrial-derived peptides; UPR, Unfolded protein response; PR, Progressive motility; NP, Non-progressive motility



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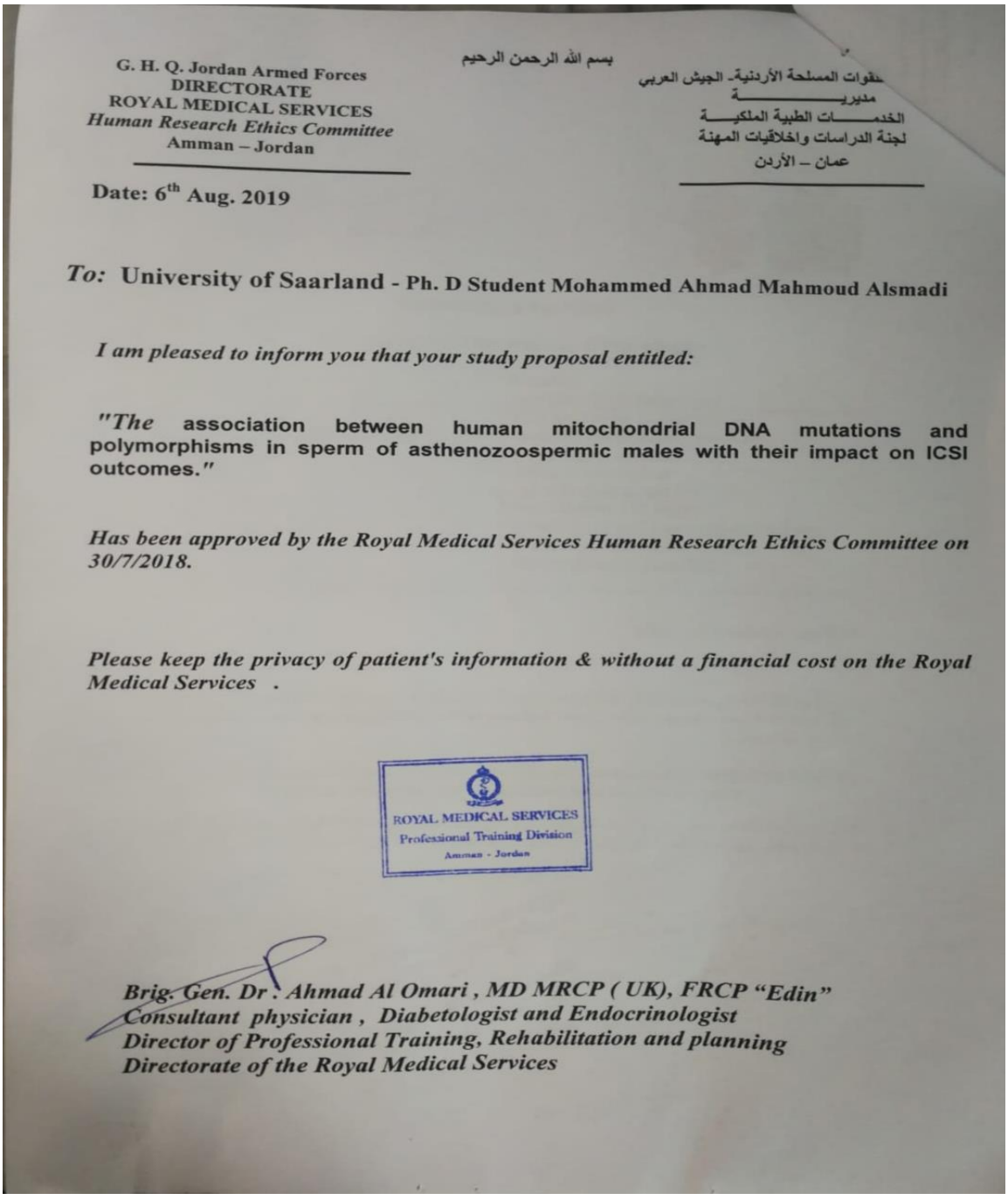
Supplementary figures for chapter 2.1.1 Subjects



بسم الله الرحمن الرحيم

G. H. Q. Jordan Armed Forces
DIRECTORATE
ROYAL MEDICAL SERVICES
*Human Research Ethics Committee*
Amman – Jordan

القوات المسلحة الأردنية- الجيش العربي
مديرية الخدمات الطبية الملكية
لجنة الدراسات واخلاقيات المهنة
عمان – الأردن

**Date: 6th Aug. 2019**

***To:*** **University of Saarland - Ph. D Student Mohammed Ahmad Mahmoud Alsmadi**

***I am pleased to inform you that your study proposal entitled:***

***"The*** **association between human mitochondrial DNA mutations and polymorphisms in sperm of asthenozoospermic males with their impact on ICSI outcomes."**

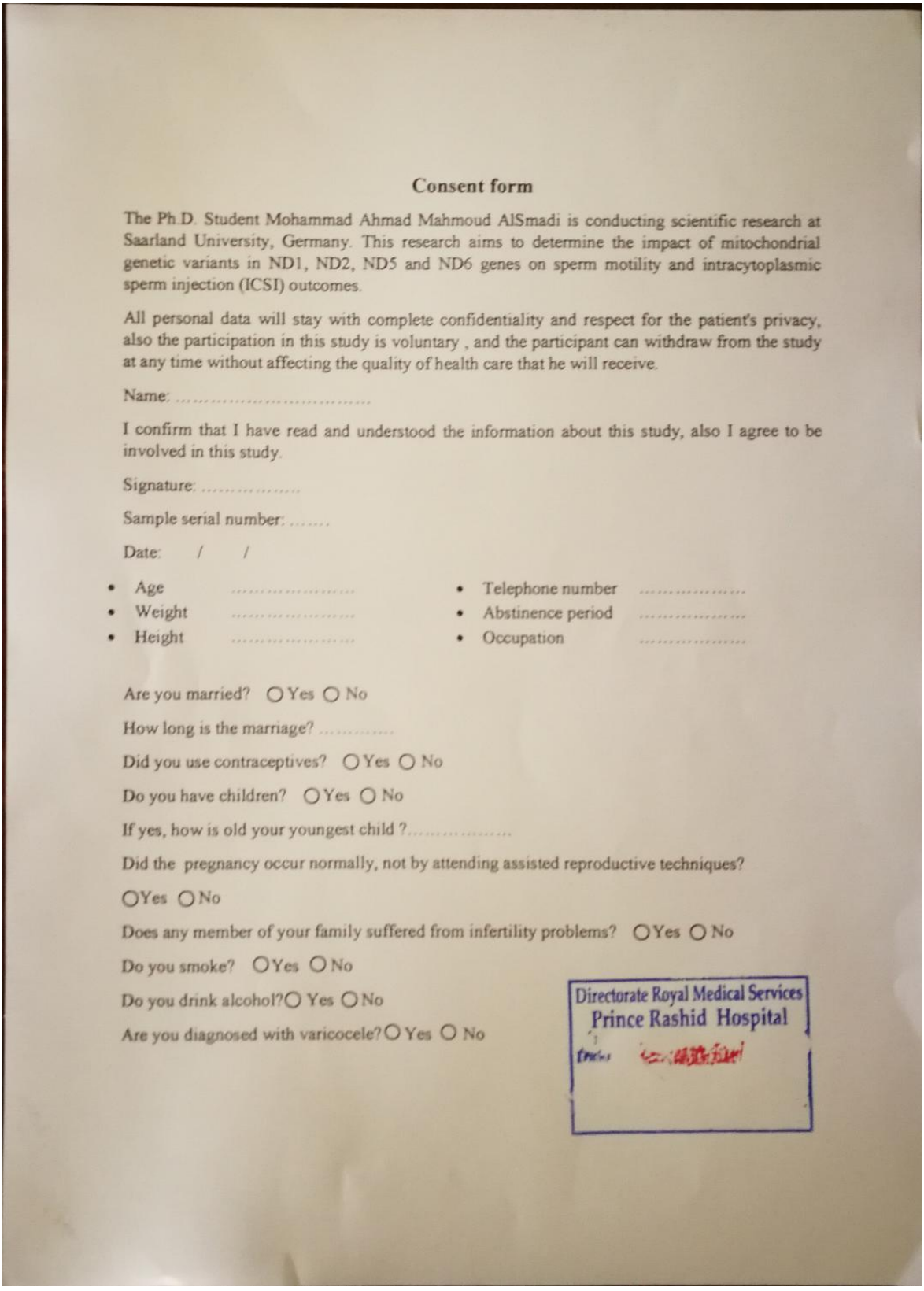
***Has been approved by the Royal Medical Services Human Research Ethics Committee on 30/7/2018.***

***Please keep the privacy of patient's information & without a financial cost on the Royal Medical Services .***

ROYAL MEDICAL SERVICES
Professional Training Division
Amman - Jordan

***Brig. Gen. Dr : Ahmad Al Omari , MD MRCP ( UK), FRCP "Edin"***
***Consultant physician , Diabetologist and Endocrinologist***
***Director of Professional Training, Rehabilitation and planning***
***Directorate of the Royal Medical Services***

Figure 6.1: The ethical approval by the Jordanian Royal Medical Services-Human Research Ethics Committee



**Consent form**

The Ph.D. Student Mohammad Ahmad Mahmoud AlSmadi is conducting scientific research at Saarland University, Germany. This research aims to determine the impact of mitochondrial genetic variants in ND1, ND2, ND5 and ND6 genes on sperm motility and intracytoplasmic sperm injection (ICSI) outcomes.

All personal data will stay with complete confidentiality and respect for the patient's privacy, also the participation in this study is voluntary , and the participant can withdraw from the study at any time without affecting the quality of health care that he will receive.

Name: .....

I confirm that I have read and understood the information about this study, also I agree to be involved in this study.

Signature: .....

Sample serial number: .....

Date: / /

- Age .....
- Weight .....
- Height .....
- Telephone number .....
- Abstinence period .....
- Occupation .....

Are you married? ○ Yes ○ No

How long is the marriage? .....

Did you use contraceptives? ○ Yes ○ No

Do you have children? ○ Yes ○ No

If yes, how is old your youngest child ?.....

Did the pregnancy occur normally, not by attending assisted reproductive techniques?

○ Yes ○ No

Does any member of your family suffered from infertility problems? ○ Yes ○ No

Do you smoke? ○ Yes ○ No

Do you drink alcohol? ○ Yes ○ No

Are you diagnosed with varicocele? ○ Yes ○ No

Directorate Royal Medical Services
Prince Rashid Hospital

Figure 6.2: Consent form that obtained from each couple in this study

Supplementary tables for chapter 3.3 (ICSI) outcomes among the studied groups

Table 6.1: ICSI outcomes among asthenozoospermic group

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 1 | 38 | 1 (8 cells(Cs)) 2 (6Cs) | 1Grade(G)B 2(GC) | 3.33 | 1.33 |
| 2 | 36 | 2 (8Cs) 2 (6Cs) 2 (4Cs) | 2 (GB) 4 (GC) | 3 | 1.33 |
| 3 | 36 | 3 (8Cs) 3 (6Cs) 2 (4Cs) | 3 (GB) 5 (GC) | 2.75 | 1.38 |
| 4 | 41 | 3 (8Cs) 2 (6Cs) 2 (4Cs) | 2 (GB) 5 (GC) | 3.14 | 1.29 |
| 5 | 28 | 2 (8Cs) 3 (6Cs) | 1 (GB) 4 (GC) | 3.4 | 1.2 |
| 6 | 36 | 1 (8Cs) 1 (6Cs) 2 (4Cs) | 2 (GB) 2 (GD) | 2.75 | 1.5 |
| 7 | 58 | 1 (8Cs) 2 (6Cs) 1 (4Cs) | 1(GA) 1(GB) 2(GD) | 3 | 1.75 |
| 8 | 31 | 2 (8Cs) 2 (6Cs) 1 (4Cs) | 2(GB) 3 (GC) | 3.2 | 1.4 |
| 9 | 29 | 3 (8Cs) 3 (6Cs) 1 (4Cs) | 3 (GB) 4 (GC) | 3.28 | 1.43 |
| 10 | 23 | 2 (8Cs) 3 (4Cs) | 2 (GB) 3 (GD) | 2.8 | 1.4 |
| 11 | 75 | 6 (8Cs) 6 (6Cs) | 3(GA) 7(GB) 2(GC) | 3.5 | 2.08 |
| 12 | 44 | 3 (8Cs) 4 (4Cs) 1 (2Cs) | 1(GA) 2(GB) 5(GC) | 2.63 | 1.65 |
| 13 | 29 | 2 (8Cs) 1(6Cs) 2 (4Cs) | 2 (GB) 3 (GC) | 3 | 1.4 |
| 14 | 31 | 1 (8Cs) 1 (6Cs) 3 4c | 1 (GA) 4 (GC) | 2.6 | 1.4 |
| 15 | 33 | 2 (8Cs) 2 (6Cs) 1 (4Cs) | 2(GB) 3(GC) | 3.2 | 1.4 |
| 16 | 58 | 3 (8Cs) 4 (6Cs) | 3 (GA) 4 (GB) | 3.43 | 2.43 |
| 17 | 22 | 3 (8Cs) 1 (6Cs) 1 (4Cs) | 1(GA) 2(GB) 2(GD) | 3.2 | 1.8 |
| 18 | 33 | 3 (8Cs) 1 (6Cs) 2 (4Cs) | 3 (GB) 1(GC) | 2.75 | 1.75 |
| 19 | 33 | 3 (8Cs) 2 (6Cs) 1 (2Cs) | 1(GA) 3(GB) 2(GD) | 3.16 | 1.83 |

Table 6.1(continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 20 | 37 | 5 (8Cs) l 2 (6Cs) | 2(GA) 3(GB) 2(GD) | 3.71 | 2 |
| 21 | 35 | 4 (8Cs) 4 (6Cs) 1 (4Cs) | 2(GA) 3(GB) 4(GC) | 3.33 | 1.78 |
| 22 | 33 | 1 (8Cs) 2 (6Cs) 1 (4Cs) | 1(GB) 3(GC) | 3 | 1.67 |
| 23 | 38 | 1 (8Cs) 2 (6Cs) 3(4Cs) | 1(GA) 2(GB) 3(GC) | 2.67 | 1.67 |
| 24 | 29 | 3 (8Cs) 2 (6Cs) 3 (4Cs) | 2(GA) 3(GB) 3(GC) | 3 | 1.88 |
| 25 | 25 | 1 (8Cs) 2 (6Cs) 3 (4Cs) | 3(GB) 3(GC) | 2.67 | 1.5 |
| 26 | 36 | 2 (8Cs) 2 (6Cs) | 2(GB) 2(GC) | 3.5 | 1.5 |
| 27 | 46 | 3 (8Cs) 3 (6Cs) | 2(GA) 1 (GB) 3(GC) | 3 | 1.83 |
| 28 | 37 | 7(8Cs) 3 (6Cs) | 4(GA) 3(GB) 3(GC) | 3.7 | 2.1 |
| 29 | 24 | 4 (8Cs) 3 (4Cs) | 2(GB) 5(GC) | 3.14 | 1.29 |
| 30 | 55 | 2 (8Cs) 2 (6Cs) 2 (4Cs) | 3(GB) 3(GC) | 3 | 1.5 |
| 31 | 32 | 3 (8Cs) 2 (6Cs) 3 (4Cs) | 3(GB) 5(GC) | 3 | 1.38 |
| 32 | 36 | 2 (8Cs) 3 (4Cs) | 2(GB) 3(GC) | 2.8 | 1.4 |
| 33 | 31 | 3(8Cs) 2 (4Cs) | 2 (GA) 1(GB) 2(GC) | 3.2 | 2 |
| 34 | 30 | 1(8Cs) 5 (6Cs) | 2(GB) 4(GC) | 3.17 | 1.33 |
| 35 | 36 | 1 (8Cs) 2 (6Cs) 1 (4Cs) | 3(GB) 1(GC) | 3 | 1.75 |
| 36 | 40 | 3 (8Cs) 1 (6Cs) | 3(GB) 1(GC) | 3.75 | 1.75 |
| 37 | 42 | 2 (8Cs) 3(6Cs) | 1(GA) 3 (GB) 1(GC) | 3.4 | 1.6 |
| 38 | 28 | 2 (8Cs) 2 (6Cs) 1(4Cs) | 2(GB) 3(GC) | 3.2 | 1.4 |

Table 6.1(continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 39 | 30 | 3 (8Cs) 4 (6Cs) | 3(GB) 4(GC) | 3.43 | 1.43 |
| 40 | 57 | 2 (8Cs) 4 (6Cs) 2 (4Cs) | 2(GA) 4(GB) 2(GC) | 3 | 2 |
| 41 | 38 | 1 (8Cs) 4 (6Cs) 2 (4Cs) | 3(GB) 4(GC) | 2.86 | 1.43 |
| 42 | 60 | 3 (8Cs) 6 (6Cs) | 3(GA) 4(GB) 2(GC) | 3.34 | 2.11 |
| 43 | 42 | 3 (8Cs) 2 (6Cs) | 1(GA) 3(GB) 1(GD) | 3.6 | 2 |
| 44 | 40 | 1 (8Cs) 4 (6Cs) 1 (4Cs) | 2(GB) 4(GC) | 3 | 1.33 |
| 45 | 32 | 3 (8Cs) 3 (6Cs) 1 (4Cs) | 1(GA) 2(GB) 4(GC) | 3.29 | 1.57 |
| 46 | 40 | 5 (8Cs) 2 (6Cs) 1 (4Cs) | 2(GA) 2(GB) 4(GC) | 3.5 | 1.75 |
| 47 | 35 | 3(8Cs) 3 (6Cs) 3 (4Cs) | 3(GA) 5(GB) 1(GC) | 3 | 2.22 |
| 48 | 44 | 2 (8Cs) 2(4Cs) 1(3c) | 3(GB) 2(GC) | 2.6 | 1.6 |
| 49 | 28 | 1 (8Cs) 2 (6Cs) 2 (4Cs) | 1(GA) 4(GC) | 2.8 | 1.4 |
| 50 | 61 | 7 (8Cs) 4 (6Cs) | 4(GA) 5(GB) 2(GC) | 3.63 | 2.18 |
| 51 | 56 | 2 (8Cs) 3 (6Cs) | 3(GA) 2(GB) | 3.4 | 2.6 |
| 52 | 63 | 7 (8Cs) 5 (6Cs) 1 (4Cs) | 4 (GA) 5 (GB) 3(GC) | 3.75 | 2.08 |
| 53 | 25 | 5 (8Cs) 2 (6Cs) | 3(GA) 3(GB) 1(GC) | 3.71 | 2.29 |
| 54 | 43 | 4 (8Cs) 2 (6Cs) | 2(GA) 2(GB) 2(GC) | 3.66 | 2 |
| 55 | 36 | 1 (8Cs) 1 (6Cs) 2 (4Cs) | 2(GB) 2(GC) | 2.75 | 1.5 |
| 56 | 38 | 2 (8Cs) 3 (4Cs) | 1(GA) 2(GB) 2(GC) | 2.8 | 1.8 |
| 57 | 33 | 3 (8Cs) 1 (6Cs) | 2(GB) 2(GC) | 3.75 | 1.5 |
| 58 | 44 | 2(8Cs) 2 (6Cs) | 2(GA) 2(GB) | 3.5 | 2.5 |

Table 6.1(continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 59 | 32 | 5 (8Cs) 3 (6Cs) | 4(GA) 3(GB) 1(GC) | 3.63 | 2.38 |
| 60 | 38 | 2 (8Cs) 2(6Cs) 1(4Cs) | 2(GB) 3(GC) | 3.2 | 1.4 |
| 61 | 44 | 2 (8Cs) 2 (6Cs) | 2(GB) 2(GC) | 3.5 | 1.5 |
| 62 | 45 | 1 (8Cs) 4 (6Cs) | 2(GB) 3(GC) | 3.2 | 1.4 |
| 63 | 38 | 3 (8Cs) 2 (6Cs) | 3(GB) 2(GC) | 3.6 | 1.6 |
| 64 | 46 | 2 (8Cs) 4 (6Cs) | 3(GA) 3(GB) | 3.33 | 2.5 |
| 65 | 53 | 5 (8Cs) 3(6Cs) | 2(GA) 4(GB) 2(GC) | 3.63 | 2 |
| 66 | 50 | 6 (8Cs) 2 (6Cs) 2(4Cs) | 2(GA) 3(GB) 4(GC) | 3.56 | 1.78 |
| 67 | 42 | 2 (8Cs) 3 (6Cs) | 1(GA) 1 (GB) 3(GC) | 3.4 | 1.6 |
| 68 | 40 | 1 (8Cs) 2 (6Cs) 1 (4Cs) | 1(GB) 3(GC) | 3 | 1.25 |
| 69 | 31 | 1 (8Cs) 1 (6Cs) 2 (4Cs) | 2GB 2(GC) | 2.75 | 1.5 |
| 70 | 38 | 2 (8Cs) 3(6Cs) 3(4Cs) | 1(GA) 2(GB) 5 (GC) | 2.88 | 1.25 |
| 71 | 40 | 9 (8Cs) 3 (6Cs) | 5(GA) 4(GB) 3(GC) | 3.75 | 2.17 |
| 72 | 66 | 11 (8Cs) 8 (6Cs) | 11(GA) 5(GB) 3(GC) | 3.58 | 2.42 |
| 73 | 62 | 5 (8Cs) 3 (6Cs) | 3(GA) 2(GB) 3(GC) | 3.63 | 2 |
| 74 | 33 | 7 (8Cs) 2(6Cs) | 6(GA) 3(GB) | 3.78 | 2.67 |
| 75 | 33 | 3 (8Cs) 2 (6Cs) | 3(GB) 2(GC) | 3.6 | 1.6 |
| 76 | 50 | 5(8Cs) 3 (6Cs) | 5(GA) 3(GB) | 3.63 | 2.63 |
| 77 | 31 | 3 (8Cs) 4 (6Cs) 4 (4Cs) | 2(GA) 3(GB) 6(GC) | 2.91 | 1.64 |
| 78 | 62 | 8 (8Cs) 5 (6Cs) | 6 (GA) 5(GB) 2(GC) | 3.62 | 2.31 |

Table 6.1(continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 79 | 28 | 3 (8Cs) 5 (6Cs) | 1(GA) 2(GB) 5 (GC) | 3.37 | 1.5 |
| 80 | 59 | 7 (8Cs) 3(6Cs) | 5(GA) 4 (GB) 1(GC) | 3.7 | 2.4 |
| 81 | 36 | 1 (8Cs) 3(6Cs) | 1(GB) 3(GC) | 3.25 | 1.25 |
| 82 | 65 | 9 (8Cs) 6 (6Cs) | 8(GA) 4(GB) 3(GC) | 3.6 | 2.33 |
| 83 | 67 | 6 (8Cs) 4 (6Cs) | 6(GA) 4(GB) | 3.6 | 2.6 |
| 84 | 45 | 2 (8Cs) 3 (6Cs) | 1(GA) 4(GB) | 3.4 | 2.2 |
| 85 | 56 | 6(8Cs) 3 (6Cs) | 5(GA) 4(GB) | 3.67 | 2.78 |
| 86 | 43 | 2(8Cs) 3 (6Cs) 1(4Cs) | 3(GB) 3(GC) | 3 | 1.5 |
| 87 | 56 | 6 (8Cs) 4 (6Cs) | 5(GA) 3(GB) 2(GC) | 3.6 | 2.3 |
| 88 | 50 | 2 (8Cs) 2 (6Cs) | 1(GA) 1(GB) 2(GC) | 3.5 | 1.75 |
| 89 | 56 | 2 (8Cs) 2 (6Cs) 1 (4Cs) | 1(GA) 1(GB) 2(GC) | 3 | 1.4 |
| 90 | 47 | 3 (8Cs) 3 (6Cs) 1 (4Cs) | 2(GA) 3(GB) 2(GC) | 3.14 | 2 |
| 91 | 31 | 1 (8Cs) 3 (6Cs) | 2(GB) 2(GC) | 3.25 | 1.5 |
| 92 | 46 | 1 (8Cs) 4 (6Cs) 1(4Cs) | 2 (GB) 4(GC) | 3 | 1.33 |
| 93 | 38 | 4 (8Cs) 3 (6Cs) 3 (4Cs) | 2(GA) 3(GB) 5(GC) | 3.55 | 1.7 |
| 94 | 64 | 6 (8Cs) 8 (6Cs) | 8(GA) 3(GB) 3(GC) | 3.42 | 2.36 |
| 95 | 64 | 10 (8Cs) 8 (6Cs) | 12 (GA) 6 (GB) | 3.8 | 2.67 |
| 96 | 59 | 7 (8Cs) 3 (6Cs) | 5(GA) 3(GB) 2(GC) | 3.7 | 2.3 |
| 97 | 63 | 7 (8Cs) 4 (6Cs) | 7 (GA) 3 (GB) | 4 | 2.7 |
| 98 | 27 | 5 (8Cs) 2 (6Cs) | 2(GA) 4(GB) 1(GC) | 3.71 | 2.14 |

Table 6.1(continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 99 | 60 | 9 (8Cs) 6 (6Cs) | 7(GA) 4(GB) 4(GC) | 3.6 | 2.07 |
| 100 | 36 | 5 (8Cs) 3 (6Cs) | 3(GA) 3(GB) 2(GC) | 3.63 | 2.13 |
| 101 | 39 | 3 (8Cs) 3(6Cs) 1 (4Cs) | 2(GA) 2(GB) 3(GC) | 3.29 | 1.88 |
| 102 | 31 | 1 (8Cs) 2 (6Cs) 1 (4Cs) | 2(GB) 2(GC) | 3 | 1.5 |
| 103 | 73 | 6 (8Cs) 2 (6Cs) | 3(GA) 3(GB) 2 (GC) | 3.75 | 2.13 |
| 104 | 40 | 4 (8Cs) 2 (6Cs) | 3(GA) 2(GB) 1(GC) | 3.66 | 2.33 |
| 105 | 44 | 1 (8Cs) 3 (6Cs) | 1 (GA) 3 (GB) | 3.25 | 2.25 |

Table 6.2: ICSI outcomes among normozoospermic group

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 1 | 81 | 8 (8Cs) 5 (6Cs) | 8 (GA) 5 (GB) | 3.62 | 2.62 |
| 2 | 70 | 5 (8Cs) 2 (6Cs) | 5 (GA) 2 (GB) | 3.71 | 2.71 |
| 3 | 86 | 9 (8Cs) 3 (6Cs) | 9 (GA) 3 (GB) | 3.75 | 2.75 |
| 4 | 85 | 8 (8Cs) 3 (6Cs) | 8(GA) 2(GB) 1(GC) | 3.73 | 2.64 |
| 5 | 75 | 6 (8Cs) 3 (6Cs) | 4(GA) 3(GB) 2(GC) | 3.66 | 2.22 |
| 6 | 59 | 8 (8Cs) 2 (6Cs) | 6(GA) 4(GB) | 3.8 | 2.6 |
| 7 | 63 | 1 (8Cs) 3 (6Cs) 1 (4Cs) | 3(GA) 2(GB) | 3 | 2.6 |
| 8 | 64 | 8 (8Cs) 1 (6Cs) | 8(GA) 1(GC) | 3.89 | 2.78 |
| 9 | 52 | 5 (8Cs) 3 (6Cs) 3 (4Cs) | 4(GA) 2(GB) 2(GC) | 3.18 | 2.5 |

Table 6.2 (continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 10 | 67 | 7 (8Cs) 5 (6Cs) 1(4Cs) | 5(GA) 7 (GB) | 3.75 | 2.42 |
| 11 | 78 | 5 (8Cs) 2 (6Cs) | 6(GA) 1(GB) | 3.71 | 2.71 |
| 12 | 71 | 8 (8Cs) 4 (6Cs) | 9(GA) 3(GB) | 3.67 | 2.75 |
| 13 | 44 | 4 (8Cs) 4 (6Cs) | 5(GA) 3(GB) | 3.5 | 2.63 |
| 14 | 67 | 5 (8Cs) 3 (6Cs) | 5(GA) 2(GB) 1(GC) | 3.63 | 2.5 |
| 15 | 47 | 4 (8Cs) 4 (6Cs) | 3(GA) 4(GB)1(GC) | 3.5 | 2.25 |
| 16 | 100 | 6 (8Cs) 3 (6Cs) | 7 (GA) 2 (GB) | 3.67 | 2.78 |
| 17 | 62 | 4 (8Cs) 4 (6Cs) | 6 (GA) 2 (GB) | 3.5 | 2.75 |
| 18 | 67 | 6 (8Cs) 4 (6Cs) | 6 (GA) 3 (GB) 1(GC) | 3.6 | 2.5 |
| 19 | 57 | 1 (8Cs) 3(6Cs) | 1(GA) 2(GB) 1(GC) | 3.25 | 2 |
| 20 | 44 | 5 (8Cs) 8c 2 (6Cs) | 3(GA) 2(GB) 2(GC) | 3.71 | 2.14 |
| 21 | 60 | 4 (8Cs) 2 (6Cs) | 4(GA) 2(GB) | 3.67 | 2.33 |
| 22 | 35 | 5 (8Cs) 6 (6Cs) | 5(GA) 2(GB) 4(GC) | 3.45 | 2.09 |
| 23 | 62 | 11 (8Cs) 7 (6Cs) | 8 (GA) 5(GB) 5(GC) | 3.61 | 2.17 |
| 24 | 66 | 13 (8Cs) 8 (6Cs) | 9(GA) 6(GB) 6(GC) | 3.62 | 2.14 |
| 25 | 67 | 5(8Cs) 5(6Cs) | 4(GA) 3(GB) 3(GC) | 3.5 | 2.1 |
| 26 | 61 | 7(8Cs) 4(6Cs) | 6(GA) 3(GB) 2(GC) | 3.64 | 2.63 |
| 27 | 42 | 3(8Cs) 5 (6Cs) | 3(GA) 2(GB) 3(GC) | 3.38 | 2.25 |
| 28 | 73 | 4 (8Cs) 4 (6Cs) 4 ( 2c) | 3(GA) 3(GB) 6(GC) | 3 | 1.75 |

Table 6.2 (continued)

| Sample number | Fertilization rate % | Number of blastomeres | Embryo grade | Embryo cleavage score | Embryo quality score |
|---|---|---|---|---|---|
| 29 | 52 | 4 (8Cs) 4 (6Cs) 4 ( 2c) | 3(GA) 3(GB) 6(GC) | 3 | 1.75 |
| 30 | 89 | 7 (8Cs) 1 (6Cs) | 6(GA) 2(GB) | 3.88 | 2.75 |
| 31 | 83 | 7 (8Cs) 3 (6Cs) | 6(GA) 1(GB) 3(GC) | 3.7 | 2.3 |
| 32 | 82 | 6 (8Cs) 3 (6Cs) | 7(GA) 1(GB) 1(GC) | 3.66 | 2.67 |
| 33 | 71 | 5 (8Cs) | 5(GA) | 4 | 3 |
| 34 | 64 | 10 (8Cs) 8 (6Cs) | 9(GA) 4(GB) 5(GC) | 3.55 | 2.22 |
| 35 | 83 | 8 (8Cs) 2(6Cs) | 8(GA) 2(GB) | 3.8 | 2.8 |
| 36 | 58 | 5 (8Cs) 3 (6Cs) 3(4Cs) | 7(GA) 4 (GB) | 3.18 | 2.64 |
| 37 | 50 | 5 (8Cs) 4 (6Cs) 3 (4Cs) | 6(GA) 4(GB) 2(GC) | 3.17 | 2.33 |
| 38 | 83 | 11 (8Cs) 4 (6Cs) | 10(GA) 5(GB) | 3.73 | 2.67 |
| 39 | 85 | 9 (8Cs) 2 (6Cs) | 8(GA) 3(GB) | 3.82 | 2.73 |
| 40 | 75 | 5 (8Cs) 7 (6Cs) | 7(GA) 3(GB) 2(GC) | 3.42 | 2.42 |
| 41 | 71 | 4 (8Cs) 8 (6Cs) | 7(GA) 2(GB) 3(GC) | 3.33 | 2.33 |
| 41 | 83 | 16 (8Cs) 8 (6Cs) | 14(GA) 6(GB) 4(GC) | 3.67 | 2.42 |
| 43 | 77 | 8 (8Cs) 2 (6Cs) | 7(GA) 3(GB) | 3.8 | 2.7 |
| 44 | 81 | 9 (8Cs) 4 (6Cs) | 9(GA) 4(GB) | 3.69 | 2.69 |
| 45 | 93 | 8 (8Cs) 5 (6Cs) | 8(GA) 3(GB) 2(GC) | 3.62 | 2.46 |

Supplementary Tables for chapter 3.6 Effect of protein carbonyl on ICSI outcomes

Table 6.3: Protein carbonyl levels and ICSI outcomes among group one

| Sample number | Protein carbonyl level (nmol/mg) | Sperm motility % | Fertilization rate % | Cleavage score for the embryo | Quality score for the embryo |
|---|---|---|---|---|---|
| 1 | 2.251 | 0 | 38 | 3.33 | 1.33 |
| 2 | 1.936 | 0 | 36 | 3 | 1.33 |
| 3 | 2.447 | 0 | 36 | 2.75 | 1.38 |
| 4 | 2.349 | 0 | 41 | 3.14 | 1.29 |
| 5 | 2.209 | 0 | 28 | 3.4 | 1.2 |
| 6 | 2.097 | 0 | 36 | 2.75 | 1.5 |
| 7 | 2.22 | 0 | 58 | 3 | 1.75 |
| 8 | 2.014 | 0 | 31 | 3.2 | 1.4 |
| 9 | 2.165 | 0 | 29 | 3.28 | 1.43 |
| 10 | 1.537 | 0 | 23 | 2.8 | 1.4 |
| 11 | 2.008 | 0 | 75 | 3.5 | 2.08 |
| 12 | 2.393 | 0 | 44 | 2.63 | 1.65 |
| 13 | 2.277 | 0 | 29 | 3 | 1.4 |
| 14 | 2.25 | 0 | 31 | 2.6 | 1.4 |
| 15 | 2.23 | 0 | 33 | 3.2 | 1.4 |
| 16 | 2.697 | 0 | 58 | 3.43 | 2.43 |
| 17 | 2.667 | 0 | 22 | 3.2 | 1.8 |
| 18 | 2.686 | 0 | 33 | 2.75 | 1.75 |
| 19 | 2.239 | 0 | 33 | 3.16 | 1.83 |
| 20 | 2.483 | 5 | 37 | 3.71 | 2 |
| 21 | 2.591 | 5 | 33 | 3.33 | 1.78 |
| 22 | 2.623 | 3 | 38 | 3 | 1.67 |
| 23 | 2.618 | 5 | 29 | 2.67 | 1.67 |
| 24 | 2.485 | 5 | 25 | 3 | 1.88 |
| 25 | 2.309 | 5 | 36 | 2.67 | 1.5 |
| 26 | 2.055 | 4 | 46 | 3.5 | 1.5 |
| 27 | 2.003 | 5 | 37 | 3 | 1.83 |
| 28 | 2.007 | 2 | 24 | 3.7 | 2.1 |
| 29 | 1.582 | 2 | 55 | 3.14 | 1.29 |
| 30 | 1.568 | 2 | 32 | 3 | 1.5 |
| 31 | 2.108 | 4 | 36 | 3 | 1.38 |
| 32 | 2.022 | 3 | 31 | 2.8 | 1.4 |
| 33 | 2.175 | 2 | 30 | 3.2 | 2 |
| 34 | 2.32 | 3 | 36 | 3.17 | 1.33 |
| 35 | 2.384 | 4 | 36 | 3 | 1.75 |

Table 6.4: Protein carbonyl levels and ICSI outcomes among group two

| Sample number | Protein carbonyl level (nmol/mg) | Sperm motility % | Fertilization rate % | Cleavage score for the embryo | Quality score for the embryo |
|---|---|---|---|---|---|
| 36 | 2.104 | 8 | 40 | 3.75 | 1.75 |
| 37 | 1.987 | 6 | 42 | 3.4 | 1.6 |
| 38 | 1.856 | 7 | 28 | 3.2 | 1.4 |
| 39 | 2.011 | 8 | 30 | 3.43 | 1.43 |
| 40 | 1.988 | 7 | 57 | 3 | 2 |
| 41 | 1.975 | 8 | 38 | 2.86 | 1.43 |
| 42 | 1.966 | 9 | 60 | 3.34 | 2.11 |
| 43 | 2.355 | 7 | 42 | 3.6 | 2 |
| 44 | 2.318 | 8 | 40 | 3 | 1.33 |
| 45 | 2.112 | 6 | 32 | 3.29 | 1.57 |
| 46 | 1.878 | 6 | 40 | 3.5 | 1.75 |
| 47 | 2.066 | 12 | 35 | 3 | 2.22 |
| 48 | 2.045 | 14 | 44 | 2.6 | 1.6 |
| 49 | 2.074 | 13 | 28 | 2.8 | 1.4 |
| 50 | 2.086 | 13 | 61 | 3.63 | 2.18 |
| 51 | 1.872 | 9 | 56 | 3.4 | 2.6 |
| 52 | 2.114 | 19 | 63 | 3.75 | 2.08 |
| 53 | 2.017 | 13 | 25 | 3.71 | 2.29 |
| 54 | 1.766 | 18 | 43 | 3.66 | 2 |
| 55 | 1.944 | 13 | 36 | 2.75 | 1.5 |
| 56 | 1.773 | 12 | 38 | 2.8 | 1.8 |
| 57 | 2.018 | 12 | 33 | 3.75 | 1.5 |
| 58 | 2.113 | 9 | 44 | 3.5 | 2.5 |
| 59 | 1.945 | 12 | 32 | 3.63 | 2.38 |
| 60 | 1.898 | 8 | 38 | 3.2 | 1.4 |
| 61 | 1.885 | 13 | 44 | 3.5 | 1.5 |
| 62 | 1.743 | 8 | 45 | 3.2 | 1.4 |
| 63 | 1.759 | 14 | 38 | 3.6 | 1.6 |
| 64 | 1.682 | 13 | 46 | 3.33 | 2.5 |
| 65 | 1.741 | 14 | 53 | 3.63 | 2 |
| 66 | 1.685 | 15 | 50 | 3.56 | 1.78 |
| 67 | 1.661 | 11 | 42 | 3.4 | 1.6 |
| 68 | 1.842 | 8 | 40 | 3 | 1.25 |
| 69 | 1.967 | 9 | 31 | 2.75 | 1.5 |
| 70 | 1.973 | 7 | 38 | 2.88 | 1.25 |

Table 6.5: Protein carbonyl levels and ICSI outcomes among group three

| Sample number | Protein carbonyl level (nmol/mg) | Sperm motility % | Fertilization rate % | Cleavage score for the embryo | Quality score for the embryo |
|---|---|---|---|---|---|
| 71 | 1.786 | 17 | 40 | 3.75 | 2.17 |
| 72 | 1.669 | 17 | 66 | 3.58 | 2.42 |
| 73 | 1.542 | 17 | 62 | 3.63 | 2 |
| 74 | 1.538 | 16 | 33 | 3.78 | 2.67 |
| 75 | 1.431 | 18 | 33 | 3.6 | 1.6 |
| 76 | 1.389 | 19 | 50 | 3.63 | 2.63 |
| 77 | 1.329 | 19 | 31 | 2.91 | 1.64 |
| 78 | 1.328 | 33 | 62 | 3.62 | 2.31 |
| 79 | 1.448 | 34 | 28 | 3.37 | 1.5 |
| 80 | 1.398 | 27 | 59 | 3.7 | 2.4 |
| 81 | 1.414 | 28 | 36 | 3.25 | 1.25 |
| 82 | 1.269 | 32 | 65 | 3.6 | 2.33 |
| 83 | 1.245 | 31 | 67 | 3.6 | 2.6 |
| 84 | 1.247 | 32 | 45 | 3.4 | 2.2 |
| 85 | 1.25 | 33 | 56 | 3.67 | 2.78 |
| 86 | 1.2663 | 33 | 43 | 3 | 1.5 |
| 87 | 1.255 | 31 | 56 | 3.6 | 2.3 |
| 88 | 1.112 | 34 | 50 | 3.5 | 1.75 |
| 89 | 1.012 | 30 | 56 | 3 | 1.4 |
| 90 | 1.014 | 30 | 47 | 3.14 | 2 |
| 91 | 1.071 | 30 | 31 | 3.25 | 1.5 |
| 92 | 1.086 | 32 | 46 | 3 | 1.33 |
| 93 | 1.054 | 26 | 38 | 3.55 | 1.7 |
| 94 | 1.102 | 17 | 64 | 3.42 | 2.36 |
| 95 | 1.108 | 20 | 64 | 3.8 | 2.67 |
| 96 | 1.107 | 19 | 59 | 3.7 | 2.3 |
| 97 | 1.216 | 17 | 63 | 4 | 2.7 |
| 98 | 1.063 | 18 | 27 | 3.71 | 2.14 |
| 99 | 1.101 | 20 | 60 | 3.6 | 2.07 |
| 100 | 1.039 | 16 | 36 | 3.63 | 2.13 |
| 101 | 1.062 | 17 | 39 | 3.29 | 1.88 |
| 102 | 1.031 | 17 | 31 | 3 | 1.5 |
| 103 | 1.108 | 33 | 73 | 3.75 | 2.13 |
| 104 | 1.115 | 18 | 40 | 3.66 | 2.33 |
| 105 | 1.187 | 19 | 44 | 3.25 | 2.25 |

Table 6.6: Protein carbonyl levels and ICSI outcomes among the control

| Sample number | Protein carbonyl level (nmol/mg) | Sperm motility % | Fertilization rate % | Cleavage score for the embryo | Quality score for the embryo |
|---|---|---|---|---|---|
| 106 | 0.666 | 75 | 81 | 3.62 | 2.62 |
| 107 | 0.368 | 75 | 70 | 3.71 | 2.71 |
| 108 | 0.323 | 75 | 86 | 3.75 | 2.75 |
| 109 | 0.282 | 75 | 85 | 3.73 | 2.64 |
| 110 | 0.052 | 71 | 75 | 3.66 | 2.22 |
| 111 | 0.106 | 73 | 59 | 3.8 | 2.6 |
| 112 | 0.353 | 59 | 63 | 3 | 2.6 |
| 113 | 0.195 | 75 | 64 | 3.89 | 2.78 |
| 114 | 0.446 | 53 | 52 | 3.18 | 2.5 |
| 115 | 0.221 | 52 | 67 | 3.75 | 2.42 |
| 116 | 0.335 | 53 | 78 | 3.71 | 2.71 |
| 117 | 0.447 | 75 | 71 | 3.67 | 2.75 |
| 118 | 0.552 | 59 | 44 | 3 | 2.13 |
| 119 | 0.338 | 75 | 67 | 3.63 | 2.5 |
| 120 | 0.431 | 54 | 47 | 3.5 | 2.25 |
| 121 | 0.258 | 55 | 100 | 3.67 | 2.78 |
| 122 | 0.263 | 53 | 62 | 3.5 | 2.75 |
| 123 | 0.331 | 52 | 67 | 3.6 | 2.5 |
| 124 | 0.373 | 55 | 57 | 3.25 | 2 |
| 125 | 0.224 | 6 | 44 | 3.71 | 2.14 |
| 126 | 0.448 | 59 | 60 | 3.67 | 2.33 |

Table 6.6 (continued)

| Sample number | Protein carbonyl level (nmol/mg) | Sperm motility % | Fertilization rate % | Cleavage score for the embryo | Quality score for the embryo |
|---|---|---|---|---|---|
| 127 | 0.354 | 54 | 35 | 3.25 | 2 |
| 128 | 0.368 | 56 | 62 | 3.61 | 2.17 |
| 129 | 0.441 | 54 | 66 | 3.62 | 2.14 |
| 130 | 0.478 | 53 | 67 | 3.5 | 2.1 |
| 131 | 0.411 | 53 | 61 | 3.64 | 2.63 |
| 132 | 0.408 | 58 | 42 | 3.38 | 2.25 |
| 133 | 0.407 | 57 | 73 | 3.75 | 2.63 |
| 134 | 0.327 | 56 | 52 | 3 | 1.75 |
| 135 | 0.322 | 58 | 89 | 3.88 | 2.75 |
| 136 | 0.216 | 58 | 83 | 3.7 | 2.3 |
| 137 | 0.213 | 52 | 82 | 3.66 | 2.67 |
| 138 | 0.223 | 58 | 71 | 4 | 3 |
| 139 | 0.221 | 72 | 64 | 3.55 | 2.22 |
| 140 | 0.198 | 75 | 83 | 3.8 | 2.8 |
| 141 | 0.177 | 73 | 58 | 3.18 | 2.64 |
| 142 | 0.217 | 57 | 50 | 3.17 | 2.33 |
| 143 | 0.243 | 74 | 83 | 3.73 | 2.67 |
| 144 | 0.305 | 57 | 85 | 3.82 | 2.73 |
| 145 | 0.447 | 57 | 75 | 3.42 | 2.42 |
| 146 | 0.393 | 56 | 71 | 3.33 | 2.33 |
| 147 | 0.202 | 75 | 83 | 3.67 | 2.42 |
| 148 | 0.206 | 57 | 77 | 3.8 | 2.7 |
| 149 | 0.245 | 57 | 81 | 3.69 | 2.69 |
| 150 | 0.187 | 75 | 93 | 3.62 | 2.46 |

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## Publications

Batiha, O., Haifawi, S., **Al-Smadi, M.**, Burghel, G. J., Naber, Z., Elbetieha, A. M., ... & Abdelnour, A. (2018). Molecular analysis of CAG repeat length of the androgen receptor gene and Y chromosome microdeletions among Jordanian azoospermic infertile males. *Andrologia*, *50*(4), e12979.

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**An article from this thesis:**

**Al Smadi**, **M. A**., Hammadeh, M. E., Solomayer, E., Batiha, O., Altalib, M. M., Jahmani, M. Y., ... & Amor, H. (2021). Impact of Mitochondrial Genetic Variants in ND1, ND2, ND5, and ND6 Genes on Sperm Motility and Intracytoplasmic Sperm Injection (ICSI) Outcomes. Reproductive Sciences, 28(5), 1540-1555.

## Curriculum vitae