**DEPARTMENT OF OBSTETRICS, GYNECOLOGY & REPRODUCTIVE MEDICINE**

**FACULTY OF MEDICINE OF SAARLAND UNIVERSITY HOMBURG/ SAAR, GERMANY**

# The association between Mitochondrial NADH Dehydrogenase (*MTND3*, *MTND4L*, *MTND4*) polymorphisms and male infertility

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# List of Contents

## ABSTRACT

Male infertility has been related to many factors and about 15 - 30% of the cases are related to genetic predisposition. The purpose of the present study was to determine the relationship between infertility and the polymorphism of mitochondrial NADH dehydrogenase subunit 3, 4L, and 4 (*MT-ND3*, *MT-ND4L*, and *MT-ND4*) genes. Direct sequencing of the target genes in the mitochondrial DNA was carried out on semen samples of 68 subfertile and 44 fertile men. Forty single nucleotide polymorphisms in the *MT-ND3*, *MT-ND4L*, and *MT-ND4* genes were identified and genotyped as follows: eight SNPs in *MTND3* rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954, seven SNPs in *MTND4L* rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881, and twenty five SNPs in *MTND4* in the cases and controls: rs2853495, rs2857284, rs2853496, rs2853497, rs3087901, rs2853493, rs2853490, rs3088053, rs2853491, rs2857285, rs28358282, rs28594904, rs28669780, rs28415973, rs28471078, rs55714831, rs28358283, rs75214962, rs28529320, rs2853494, rs28609979, rs28358286, rs28359168, rs28384199, and rs869096886. The genotypes frequencies of the study population showed that rs2853495 G>A (Gly320Gly) in the *MT-ND4* gene was statistically associated with male infertility *(P* = 0.0351). In the allele frequency test, the results showed that rs2853495 G>A (Gly320Gly) and rs869096886 A>G (Leu164Leu) in *MT-ND4* were significantly associated with male infertility (adjusted OR = 2.616, 95% CI = 1.374 - 4.983, *P* = 0.0028; adjusted OR = 2.237, 95% CI = 1.245 - 4.017, *P* = 0.0073, respectively). On the other hand, no statistically significant association difference was reported between the asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia, oligoteratozoospermia subgroups of subfertile males and the fertile ones. In conclusion, our findings suggested that male infertility was correlated to rs2853495 and rs869096886 SNPs in the *MTND4* gene. More studies on the subfertile males in different populations are required to develop a clear understanding of the role of these SNPs in male infertility. In addition, functional studies will be very helpful to elucidate the molecular role of these SNPs in the function of these genes.

## ZUSAMMENFASSUNG

Männliche Unfruchtbarkeit wurde mit vielen Faktoren in Verbindung gebracht und ungefähr 15 - 30% der männlichen Unfruchtbarkeit ist mit genetischer Prädisposition verbunden. Der Zweck dieser Studie war es zu bestimmen, die Beziehung zwischen der Unfruchtbarkeit und dem Polymorphismus der mitochondrialen NADH-Dehydrogenase-Untereinheit 3, 4L, und 4 (*MTND3*, *MT-ND4L*, und *MT-ND4*) durch Analyse von Spermium bei fruchtbaren und unfruchtbaren Männern. Sanger-Sequenzierung der Ziel Gene in der mitochondrialen DNA wurde an Spermaproben von 68 unfruchtbare und 44 fruchtbare Männer. Vierzig einzelne Nukleotid Polymorphismen (SNPs) in den Genen *MT-ND*3, *MT-ND4L* und *MT-ND4* wurden identifiziert und genotypisiert wie folgt: acht SNPs in MTND3 rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 und rs28673954, sieben SNPs in *MTND4L* rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 und rs28532881, und fünfundzwanzig SNPs in MTND4 in den Fällen und Kontrollgruppen: rs2853495, rs2857284, rs2853496, rs2853497, rs3087901, rs2853493, rs2853490, rs3088053, rs2853491, rs2857285, rs28358282, rs28594904, rs28669780, rs28415973, rs28471078, rs55714831, rs28358283, rs75214962, rs28529320, rs2853494, rs28609979, rs28358286, rs28359168, rs28384199, und rs869096886. Die Genotyp Frequenzen der Studienpopulation hat gewiesen, dass rs2853495 G>A (Gly320Gly) in *MT-ND4* wurde statistisch mit männlicher Unfruchtbarkeit korreliert (P = 0.0351). Die Ergebnisse im Allel Frequenz Test haben gewiesen, dass rs2853495 G>A (Gly320Gly) und rs869096886 A>G (Leu164Leu) in *MT-ND4* wurden signifikant mit männlicher Unfruchtbarkeit korreliert (adjustiert OR = 2.616, 95% CI = 1.374 - 4.983, P = 0.0028; adjustiert OR = 2.237, 95% CI = 1.245 - 4.017, P = 0.0073, Beziehungsweise). Andererseits wurde kein statistisch signifikanter Zusammenhang zwischen der Asthenozoospermie, Oligozoospermie, Teratozoospermie, Asthenoteratozoospermie, Oligoasthenoteratozoospermie, Oligoteratozoospermie und den Untergruppen von unfruchtbaren und fruchtbaren Männern. Zusammenfassend legen unsere Ergebnisse nahe, dass männliche Unfruchtbarkeit mit rs2853495 and rs869096886 SNPs im Gen korreliert wurde. Weitere Studien an unfruchtbaren Männern in verschiedenen Populationen sind erforderlich, um ein klares Verständnis für die Rolle dieser SNPs bei der männlichen Unfruchtbarkeit zu entwickeln. Darüber hinaus werden funktionelle Studien sehr hilfreich sein, um die molekulare Rolle dieser SNPs für die Funktion dieser Gene aufzuklären.

## LIST OF ABBREVIATIONS

ADP: Adenosine Diphosphate

Ala: Alanine

AMD: Age-Related Macular Degeneration

ART: Assisted Reproductive Technology

ART: Antiretroviral Therapy Initiation

Arg: Arginine

Asn: Asparagine

ATP: Adenosine Triphosphate

AZF: Azoospermia Factor Region

Bp: Base Pair

CBVAD: Congenital bilateral absence of vas defense

CFTR: Conducted Regulator Gene

CI: Confidence Interval

Cys: Cysteine

DAZ: Deleted in Azoospermia

DFFRY: Drosophila Fat Facet Related Y

DNA: Deoxyribonucleic Acid

ETC: Electron Transfer Chain

F: Forward Primer

GC: Gastric Cancer

Gln: Glutamine

Glu: Glutamic acid

Gly: Glycine

H1: heavy strand 1

H2: heavy strand 2

HIV: Human Immunodeficiency Virus

HWE: Hardy-Weinberg Equilibrium

I: Internal Primer

ICSI: Intracytoplasmic Sperm Injection

Ile: Isoleucine

IUI: Intrauterine Insemination

IVF: In Vitro Fertilization

Kb: Kilobase

kDa: Kilodalton

L: light strand

Leu: Leucine

LHON: Leber's Hereditary Optic Neuropathy

Lys: Lysine

Met: Methionine

Min: Minute

Ml: Millilitre

mRNA: Messenger Ribonucleic Acid

mtDNA: Mitochondrial Deoxyribonucleic Acid

MTLE: Mesial Temporal Lobe Epilepsy

Mt-ND3: Mitochondrial NADH Dehydrogenase Subunit 3

Mt-ND4: Mitochondrial NADH Dehydrogenase Subunit 4

Mt-ND4L: Mitochondrial NADH Dehydrogenase Subunit 4L

MtRNAP: DNA-dependent RNA polymerase

NADH: Nicotinamide Adenine Dinucleotide Hydride

NP: Non-progressive Motility

OR: Odds Ratio

OXPHOS: Oxidative Phosphorylation

PCR: Polymerase Chain Reaction

PPS: Phosphate Buffer Saline

PR: Progressive Motility

PR + NP: Total Motility

Pro: Proline

R: Reverse Primer

RBM: RNA Binding Motif

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: Ribosomal Ribonucleic Acid

Sec: Second

SCLB: Somatic Cell Lysis Buffer

SD: Standard Deviation

Ser: Serine

SNP: Single Nucleotide Polymorphism

T2DM: Type 2 Diabetes Mellitus

TBE: Tris-borate-EDTA

Thr: Threonine

tRNA: Transfer Ribonucleic Acid

Trp: Tryptophan

Tyr: Tyrosine

UV: Ultraviolet

V: Volt

Val: Valine

WHO: World Health Organization

ZP: Zona Pellucida

## LIST OF TABLES

## LIST OF FIGURES

# 1. INTRODUCTION

## 1.1. The Mitochondria

Mitochondria, the powerhouse of the cell, are semiautonomous double membrane-bound cell organelles and are located in the cytoplasm of eukaryotic organisms, therefore, they are segregated from the nucleus and nuclear DNA. Eukaryotic cells have many mitochondria and differ highly in size and structure (Darley-Usmar *et al*., 1994).

### 1.1.1. Mitochondrial structure

The mitochondria are quite preserved in various organisms. There are two membranes on each mitochondrion; the internal membrane and the outer membrane **(Figure 1)**. The internal membrane is folded to form cristae that seem to be finger-like projections. Cristae are impenetrable and have essential enzymes that are important for metabolic functions like Adenosine triphosphate (ATP) production and cellular respiration. The mechanism responsible for ATP generation is oxidative phosphorylation (OXPHOS) (Cummins *et al*., 1998). The original host membrane is included in the outer membrane, which acts as a cation-penetration barrier (Cummins *et al*., 1998).

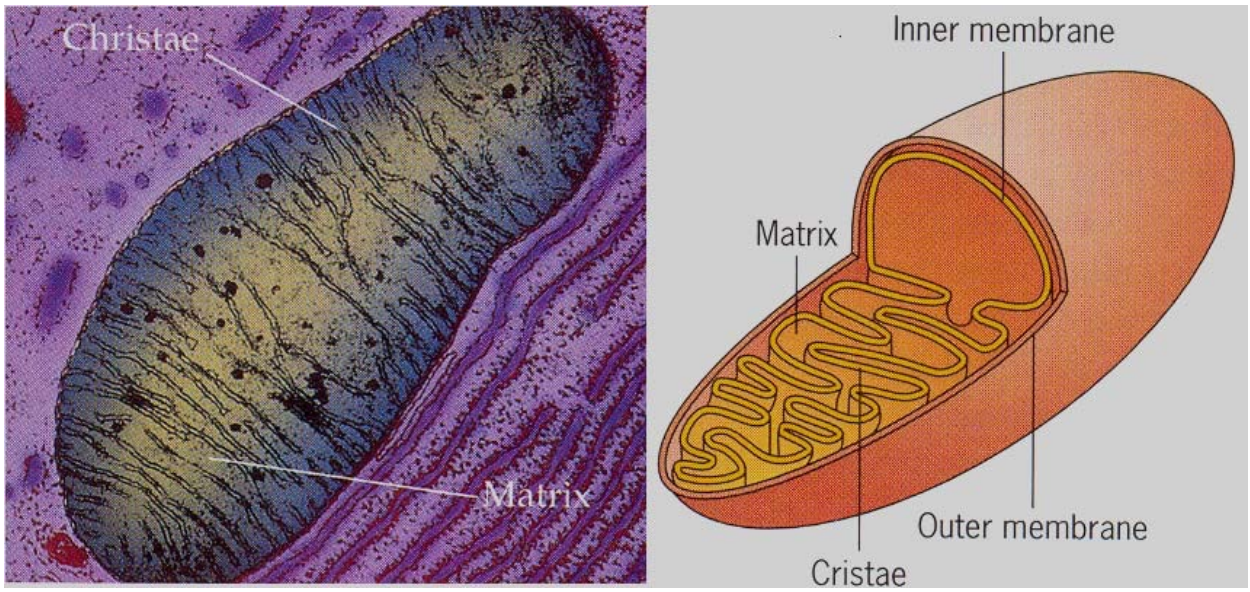



**Figure 1:** The human mitochondrion.
An electron microscope image of a mitochondrion (left) and a graphical model of a mitochondrion's inner form (right) [Adapted from Snustad and Simmons, 2000].

### 1.1.2. Transcription and translation of the human mitochondrial genes

The oxidative phosphorylation necessary for ATP generation involves five complexes with several subunits located in the organelle's internal membrane. The mitochondrial genome, known as mtDNA, encodes thirteen complexes' subunits. For this reason, for the oxidative phosphorylation complexes gathering and functionality, mitochondrial DNA (mtDNA) expression is essential. Furthermore, Imbalances in the assembly of these complexes are related to defects in the pathways monitoring mtDNA gene expression, resulting in mitochondrial disorders. Several factors implicated in these mechanisms have recently been recognized and characterized, resulting in a better explanation of the pathways underlying mitochondrial diseases (Garone *et al*., 2018).

#### 1.1.2.1. Mitochondrial transcription

The transcription of mitochondria involves three stages: initiation, elongation, and termination. Transcription of mitochondria in humans is initiated from the non-coding region, heavy strand 1 and 2 (H1 and H2), and light strand (L) promoters. The H2 strand promoter generates a transcript that extended the whole genome. Moreover, the L strand promoter transcribes eight tRNAs and *MT-ND6* gene. In addition, the H1 strand promoter triggers the 12S and 16S mitochondrial rRNA transcription (Shokolenko and Alexeyev, 2017; Garone *et al*., 2018).

Human mitochondria include lots of copies of a small double-stranded DNA that encode 13 of the RNA components and electron-transport chain complexes which are necessary for the mitochondrial translation. For the transcription of the mitochondrial genome, a specialized transcription machinery is involved. This machinery includes a monomeric DNA-dependent RNA polymerase (mtRNAP) and its Ancillary factors: the mitochondrial transcription factor A (TFAM), the mitochondrial transcription factor B1 (TFB1M), and the mitochondrial transcription factor B2 (TFB2M). Structural studies have elaborated the cooperation between mtRNAP and its associated transcription factors in RNA synthesis: the mitochondrial transcription initiation includes the association of the mtRNAP, TFAM, TFB1M, and TFB2M whereas the elongation factor TEFM enhances the processivity of RNA polymerase to the rates needed for long polycistronic mtRNA transcripts to be synthesized (**Figure 2)** (Hillen *et al*., 2018; Garone *et al*., 2018).

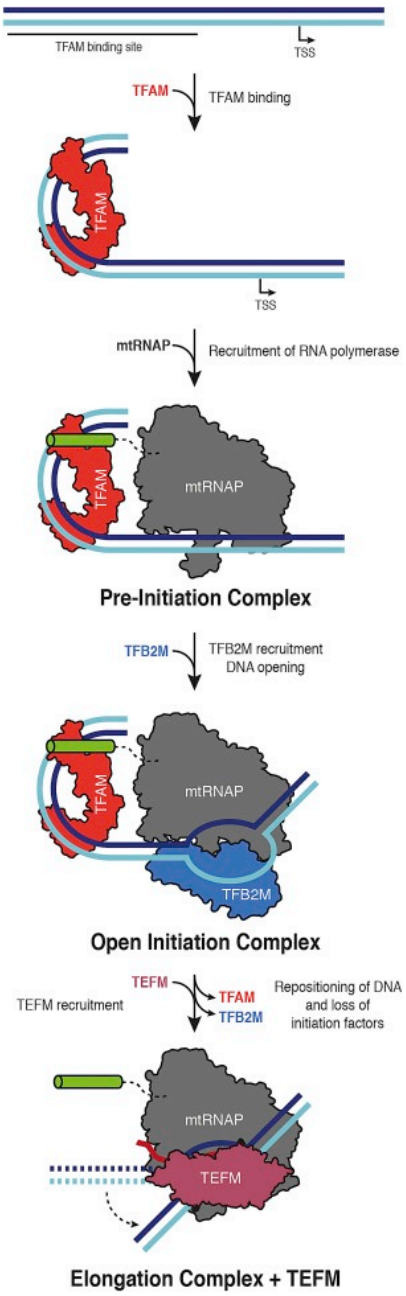



**Figure 2:** Human mitochondrial transcription initiation model. [Adapted from Hillen *et al*., 2017].

### 1.1.2.2. Mitochondrial translation

The mitochondrial translation is crucial to the cell energy balance maintenance during protein synthesis in oxidative phosphorylation. This is needed for properly adenosine triphosphate (ATP) production and cristae folding. Therefore, dysfunctional mitochondrial translation results in a greater combined respiratory chain impairment leading to lower ATP generation and an eventual cellular energy depletion (Aibara *et al*., 2020).

The basic mechanism of mitochondrial translation includes mtDNA-encoded rRNAs and tRNAs and several nuclear genome-encoded proteins: (1) translation factors of initiation, elongation, and termination; (2) ribosomal proteins of mitochondria (MRPs); (3) methionyl-tRNA transformylase and mitochondrial aminoacyl-tRNA synthetases (Smits *et al*., 2010).

As in most other protein synthesis processes, mitochondrial translation is initiated by a methionine residue. Though the difference that mitochondria uses, for both initiation and elongation, only a single $tRNA^{Met}$. The elongation is regulated by a variety of elongation factors in the mitochondria. Moreover, the translation termination of mitochondria is eventually initiated by the existence of a stop codon. Ultimately, after the polypeptide releasement, mitochondrial ribosomal recycling factors induce the releasing of the mRNAs, ribosomal subunits, and the deacetylated tRNAs Garone *et al*., 2018).

### 1.1.3. Mitochondrial functions

The major prominent role of mitochondria is to produce (ATP) by oxidative phosphorylation (OXPHOS). The OXPHOS is carried-out through the respiratory chain. The respiratory chain of mitochondria involves five protein complexes located in the internal membrane of mitochondria "nicotinamide adenine dinucleotide coenzyme Q reductase (NADH-CoQ reductase) (complex I), succinate CoQ reductase (complex II), ubiquinol cytochrome b reductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V)" (**Figure 3**). Complex I as well as complex II receive electrons resulted from "NADH and FADH2" reduction. These electrons flow down an electrochemical gradient, delivered to complex III and IV. The released energy is utilized by complexes I, III, and IV to pump protons (H+) from the matrix into the inner membrane space of the mitochondria. Thereafter, complex V uses this proton gradient to

generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP). ATP is the high-energy source that must be produced from the mitochondrion and used for all cellular metabolic processes (Chinnery and Schon, 2003).

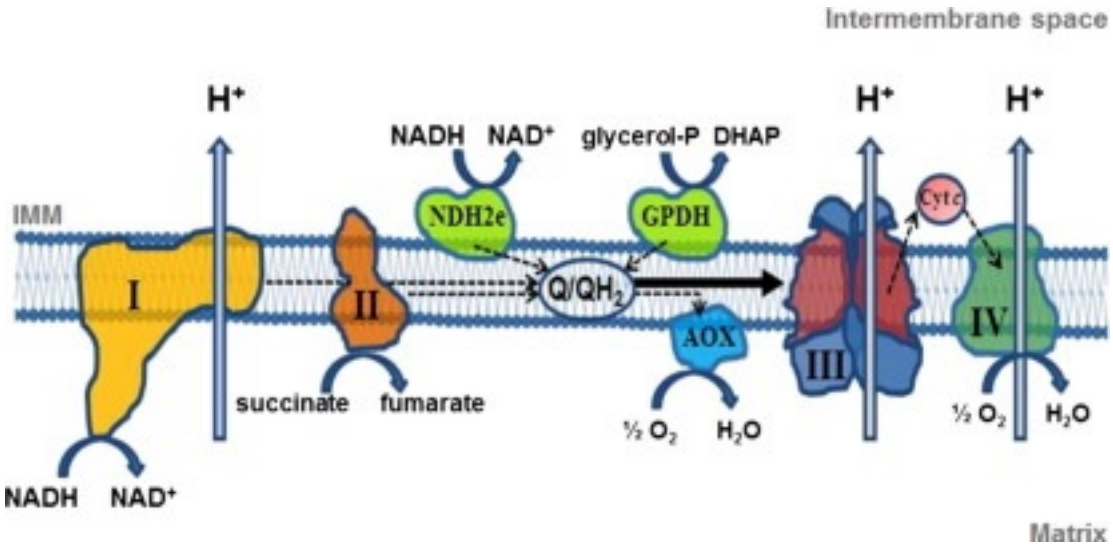



**Figure 3:** The mitochondrial respiratory chain.
[Adapted from Cabrera-Orefice *et al*., 2014].

### 1.1.4. Mitochondrial dysfunction

OXPHOS is an effective way to release energy; nevertheless, it generates reactive oxygen species (ROS) during mitochondrial activity. Large amounts of ROS are detrimental to cells (Khan *et al*., 2016). The generation of reactive oxygen species occurs as a natural physiological process in sperm. However, only a small quantity of ROS is needed for normal sperm functioning. ROS including nitric oxide (NO) and superoxide anion can affect the capacitation and the acrosome reaction. Moreover, ROS are involved in sperm-oocyte communication, but the disproportionate levels in ROS production can negatively affect the sperm quality and impair the capacity of their fertilization (Garrido *et al*., 2004).

### 1.1.5. Human mitochondrial genome and mitochondrial genetics

Mitochondria have their genome, which is separated from the nuclear DNA. The mitochondrial DNA genome in humans is considerably small and circular in structure, and double-stranded

DNA, a light strand “cytosine rich” and a heavy strand “guanine rich”. Anderson *et al* (1981) had previously identified the mitochondrial genome's entire sequence of 16,569 base pairs (bp). MtDNA encodes two rRNAs (16S and 12S) which are important for the expression of the mRNA, 22 tRNAs, and 13 polypeptides of the mitochondrial ATP-synthesis pathway (Shoffner and Wallace, 1994).

The mitochondrial genome has maternal inheritance as a distinguishing feature. Around 100,000 mtDNA are found in the mammalian egg, while 100-150 mtDNA are found in sperm cells. (Chen *et al*., 1995). MtDNA contains no introns and is unprotected by histones or DNA binding proteins, therefore, it replicates rapidly without DNA repair mechanisms (Shamsi *et al.*, 2008). The lack of histone proteins and DNA repair mechanisms in mitochondria increases the mitochondrial mutation rates about 10-100 times higher than nuclear DNA. Mutations, that occur in the mitochondrial genome, are involved in a variety of human genetic disorders (Baklouti-Gargouri *et al*., 2013).

Several studies showed a strong correlation between the impaired mtDNA and the occurrence of male infertility conditions such as asthenozoospermia, oligozoospermia and teratozoospermia. For instance, large-scale deletions in the mtDNA have been identified in asthenozoospermia in various populations (Bahrehmand Namaghi *et al*., 2017; Kao *et al.*, 1998; Al Zoubi *et al*., 2020). Other studies established an association between the CAG repeats and the development of oligozoospermia and teratozoospermia in infertile males (Al Zoubi *et al*., 2020).

Moreover, the removal of certain structural genes and tRNA genes may result in a large number of mtDNA deletions. Sperm containing defective mitochondria cannot effectively produce ATP and more likely to produce free radical/reactive oxygen (ROS), thereby causing a defect in mtDNA, make trouble energy and defect the motility and fertility (JOHN *et al*., 2000; Spiropoulos *et al*., 2002). Mitochondrial genes play an important role in the mature sperm construction and flagella movement after ejaculation (Nakada *et al.,* 2006).

### 1.1.6. The mitochondrial DNA inheritance

Each spermatozoa and oocyte has its own mitochondria. After fertilization, the zygote gets both paternal and maternal mtDNA. However, in most mammals, the mtDNA is inherited exclusively from the mitochondria of the oocytes. The maternal inheritance of mitochondrial DNA is

commonly recognized in several eukaryotes. Sperm-derived paternal mitochondria and their mtDNA reach the cytoplasm of oocyte upon fertilization and usually disappear through early embryogenesis and never transferred to the offspring. Though, the molecular mechanism involving this paternal mitochondria clearance has remained relatively unclear (Sato, M and Sato, K, 2012; Sato, M and Sato, K, 2013).

Mitochondria are important intra-cellular organs that are the main energy source in the form of ATP. Mitochondrial DNA can lead to a wide variety of human pathologies. For several decades, it has been widely agreed that mtDNA is inherited solely from the maternal line in human beings. The occurrence of both mutant and wild-type variant alleles in the same individual (heteroplasmy) and rapid shifts that occurring in allele frequency will lead to offspring with varying disease severity (Wei and Chinnery, 2020).

The human mitochondrial genome mutations are proven to cause several diseases, mostly are maternally inherited, and all of which are related to deficiencies in the metabolism of oxidative energy. It is now evident that somatic mutations in mitochondrial DNA are also associated with other diverse traits, involving neurodegenerative diseases, aging, and cancer (Schon *et al*., 2012).

## 1.2. Human infertility

Infertility is known as the inability to achieve pregnancy after 12 months of unprotected sexual intercourse. The global prevalence of infertility varies between 2.5%–15% and is associated with at least 30 million infertile males worldwide (Agarwal *et al*., 2015). Previous studies reveal that men account for 50% of infertility cases (Talebi *et al*., 2018). In Males, many reasons affect the sperm and play a role in preventing conception including hormone abnormalities, disorders, reproductive anatomy trauma and obstruction and sexual dysfunction. These disorders will be difficult to treat if they are left untreated for a long time (Kumar and Sangeetha, 2009).

According to the guidelines of the World Health Organization, male infertility can be diagnosed by the traditional semen analysis which can assess semen volume and liquefaction, antisperm antibodies, sperm count, morphology and motility (Agarwal *et al*., 2008).

Sperm motility is essential for normal fertilization (Rajender *et al*., 2010). Approximately, 75% of infertile men have oligozoospermia, a reduced sperm number or asthenozoospermia, in which most of the sperm are immotile (Kumar and Sangeetha, 2009; Talebi *et al*., 2018). The movement of sperm to the fertilization site requires energy provided by sperm mitochondria. Therefore, genetic alterations in the mitochondrial DNA are expected to be associated with asthenozoospermia and eventually the infertility phenotype (Rajender *et al*., 2010).

## 1.3. Spermatogenesis

The production of large numbers of mature spermatozoa is produced daily in the male testis through a complex process called spermatogenesis. Spermatogenesis undergoes three stages: several divisions on diploid cells (2n) take place through the first stage to produce haploid immature cells called spermatids, during the second stage, many processes of maturation and differentiation of spermatids occurs. This stage carries out three phases: the Golgi bodies formation phase, Acrosomes condensing phase, and centrioles formation and tail elongation phase, where the spermatids lose the excess of cytoplasm and form an immotile mature sperm. The third stage is called spermiation and occurs in the lumen of the seminiferous tubule. Sperm then spend a few days in the epididymis layer to gain motility. Therefore, motile sperm can undergo the fertilization process (**Figure 4**) (Kretser *et al*., 1998; Amann and Howards, 1980).

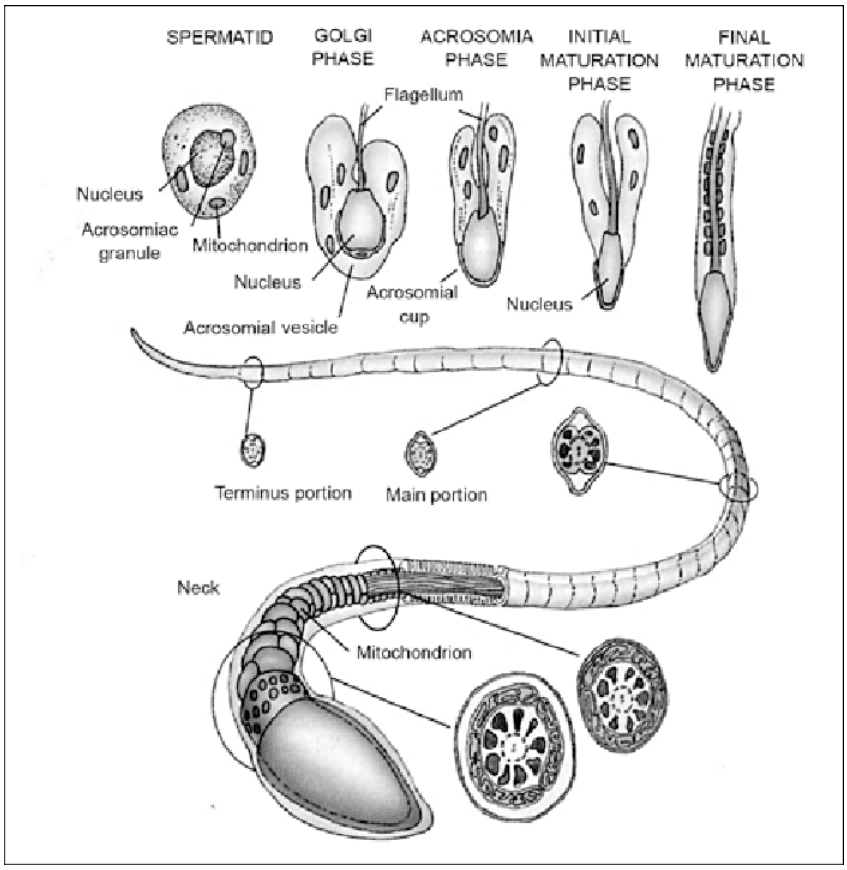



**Figure 4:** Spermatogenesis in human.
[Taken from "Histology", Gartner].

Spermatozoa divided into two main parts that play a role in the fertilization process: head and tail **(Figure 5)**. The head consists of a nucleus containing the genomic DNA and an acrosome that play an important role in the binding to the zona pellucida (ZP) and exocytosis. The tail, called also flagellum, is subdivided into three pieces: mitochondrial midpiece, the principal piece, and the end piece. This part is important for the energy production which is necessary to guide the sperm through the female reproductive tract. (Esposito *et al*., 2004).

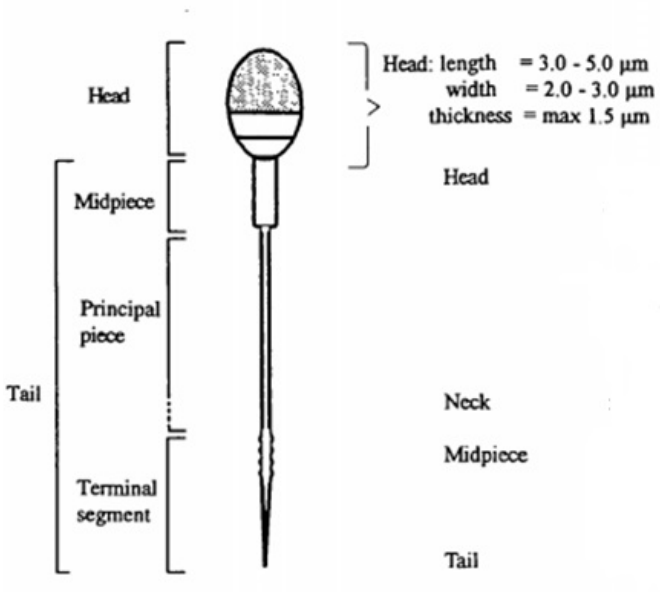



**Figure 5:** Illustration of the mature sperm cell of human.

Showing its several portions: head, neck, middle piece and tail [Adapted from Kruger *et al*., 1993].

## 1.4. Normal semen parameters

According to WHO 2010, the normal semen parameters include complete liquefaction in 60 minutes, 1.5 mL volume, grey-white color, PH> 7.1, concentration more than 15 million sperm/ mL, motility > 32%, and morphology > 4%. Infertile males usually have lower sperm parameters (WHO, 2010).

## 1.5. Sperm abnormalities

Infertility cases are diagnosed according to three categories: count, morphology and motility.

### 1.5.1. Oligozoospermia

Sperm concentration less than 15 million sperm/ml is subdivided into three types of sperm abnormalities including oligozoospermia in which the number of sperm is between 100 000 to 15 million, severe oligozoospermia where the number of sperm is less than 100 000, and azoospermia when there is no sperm in 1 ml of seminal fluid (WHO, 2010; Dajanı, 2016).

### 1.5.2. Teratozoospermia

Sperm morphology refers to the shape and structure of spermatozoon, any defect in sperm morphology can contribute to male infertility (WHO, 2010). Different forms of morphological abnormalities are shown in **(Figure 6)**.

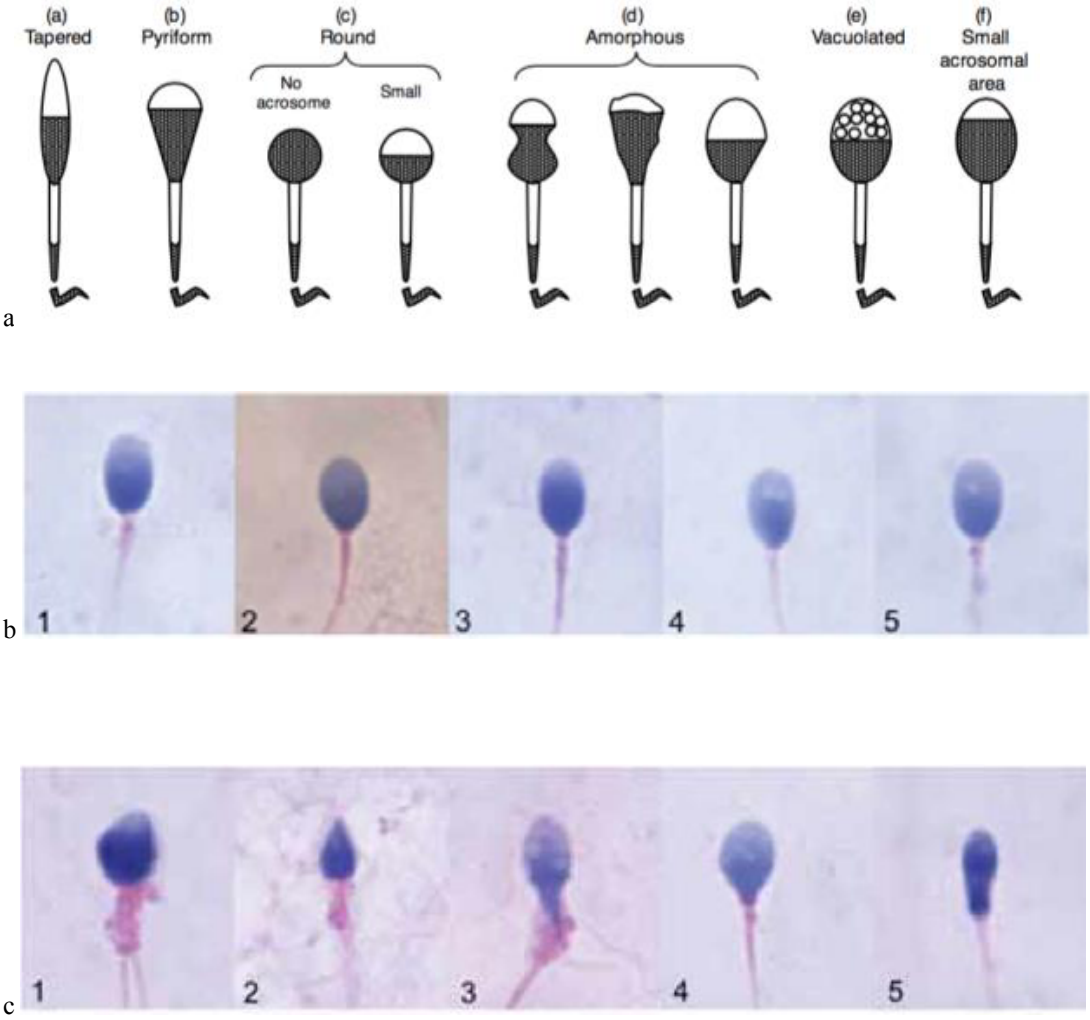



**Figure 6:** (a) Abnormal sperm-head morphology, (b) Normal-shaped sperm, (c) Abnormal-shaped sperm. (Standard WHO, 2010).

### 1.5.3. Asthenozoospermia

The motility of spermatozoa is defined as the ability of sperm to move. Four types of motility are taken into consideration during diagnosis:
Type 1 is known as progressive motility in which the sperm move in a fast and straightforward movement.
Type 2 is known as non-progressive motility in which the movement form is fast and sluggish.
Type 3 is known as local motility in which the sperm is shaking and vibrates in the same place.
Type 4 is known as immotile sperm.

When the sum of progressive and non-progressive motility is less than 32%, the case is considered as Asthenzoospermia (WHO, 2010).

## 1.6. Assisted reproductive technologies

Assisted reproductive technology (ART) developed the treatment of infertility worldwide. ARTs, the hope technologies, are also being used by individuals who are not technically infertile but need to conceive a child (Franklin, 1997).

### 1.6.1. In Vitro Fertilization (IVF)

In vitro fertilization (IVF) was the first developed ART. IVF includes hormonal stimulation of the ovaries to produce a number of eggs, which are then retrieved and placed in a petri dish contains the male's sperm, where the fertilization takes place. The fertilized embryos are then transferred to the female's uterus. Moreover, IVF is currently used to treat idiopathic infertility problems.

### 1.6.2. Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic Sperm Injection, the novel technology, allows embryologists to inject a single spermatozoon into an egg by using a high-powered microscope, increasing the fertilization effectiveness of eggs by even weak sperm. ICSI has developed the treatment of male infertility and has enabled sterile males to transmit their genetic makeup.

IVF and ICSI are both used with the freezing, storage, and posterior thawing of sperm, eggs, and embryos to allow long-term fertility conservation. Freezing has opened up many novel reproductive options (Franklin, 2006).

### 1.6.3. Intrauterine Insemination (IUI)

Intrauterine insemination (IUI) is an inexpensive option of assisted reproductive technologies, non-invasive, and efficient first-line therapy for infertility treatment of patients with cervical factor, non-severe male factor, idiopathic infertility, immune infertility, infertility caused by ejaculatory disorders, and ovulatory disorders. To obtain a higher chance of pregnancy, IUI is usually coincided with ovulation, either in a natural cycle or stimulation cycle. It also includes fractionating or washing motile sperm, before injecting it into the uterine cavity (Duran *et al*., 2002; Allahbadia, 2017).

## 1.7. Factors associated with male infertility

### 1.7.1. Age

A study reported that infertile males above 40 years old have a higher percentage of sperm DNA fragmentation which may raise the sperm DNA damage risk (Alshahrani *et al*., 2014). Age also affects hormone production, sperm production, and sexual functions in males (Belloc *et al*., 2014).

### 1.7.2. Stress

An increase in the time of pregnancy has been reported in a nurse population, who are working for long hours (more than 40 hours per week), which indicates a relationship between the long hours of work or stress and the decreased fertility. Moreover, the quality of semen is affected by mental stress in men. In fact, a severe depression seems to have a relation with lower testosterone levels, which affects the spermatogenesis and testicular paracrine interactions (Gaskins *et al*., 2015; Jozkow and Medras, 2012).

### 1.7.3. Cigarette Smoking

In the European Union, the prevalence of smoking among female populations of reproductive age has elevated over the recent decades to 33% in 2006 (Huisman *et al*., 2005). In the United

States, at the same time, the smoking prevalence rate reached 28% (CDC, 2008). According to the WHO, about one-third of the world's population, aged 15 years and older, smokes tobacco. According to reports, smoking is a common health hazard. Evidence shows that both men and women have a greater impact on reproductive health than caffeine or alcohol intake (Curtis *et al*., 1997).

The health risks of cigarette smoking have a known impact on fertility in both male and female partners. A cigarette smoking female has an impairment in each stage of reproductive function, folliculogenesis, steroidogenesis, embryo transport, endometrial receptivity, endometrial angiogenesis, uterine blood flow and uterine myometrium, as the smoke contains several cytotoxic substances such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), nitrosamines, and aromatic amines (Dechanet *et al*., 2011). In men, it has been reported that cigarette smoking can negatively impact sperm production, motility and morphology and has been linked to increased sperm DNA damage (Kunzle *et al*., 2003).

## 1.8. Primary and secondary infertility

According to World Health Organization (WHO), primary infertility describes women who have never been pregnant, while secondary infertility describes the failure to conceive in couples who have successfully conceived at least once in the past (Tabong and Adongo, 2013). Infertility can be attributed to abnormalities related to the male or female reproductive system or two partners. Several factors can interfere with the reproductive process at any step. For example, female infertility may be related to one more causes like polycystic ovary syndrome (Lash *et al*., 2008), hormonal disorders (Kazerooni and Dehghan, 2003), premature ovarian failure (Shen *et al*., 2014), genital infections (Goundry *et al*., 2013), endometriosis (Wang *et al*., 2009), fallopian tube obstruction (Wallach *et al*., 1987), congenital uterine anomalies (Celik *et al*., 2012), uterine synechiae (Umdagas *et al*., 2006), or other medical complications (diabetes and thyroid disorders) (Codner *et al*., 2012, Menif *et al*., 2008).

## 1.9. Genetic basis of male infertility

Genetic disorders are responsible for about 15%–30% of male factor infertility. The proper understanding of the genetic basis of reproductive problems is substantial to manage an infertile couple (Kretser, 1997; Ferlin *et al*., 2007). Genetic disorders involving male infertility may be caused by "chromosomal abnormalities, autosomal gene defects, or Y chromosome mutation".

### 1.9.1. Chromosomal abnormalities and male infertility

The karyotype variant of Klinefelter's syndrome, 47, XXY, is the most common numerical chromosomal abnormality associated with male infertility and occurs in 1 in 500 newborn males (Singh et al., 2006; Seli and Sakkas, 2005). 11% of azoospermic and 0.7% of oligozoospermic males are probable to show this abnormality (Seli and Sakkas, 2005). Autosomal anomalies in men may lead to a congenital bilateral absence of vas defense (CBVAD) that is a form of obstructive azoospermia resulted from the disconnection between the epididymis and the ejaculatory duct. CBVAD exhibits a positive correlation with cystic fibrosis caused by transmembrane conducted regulator gene (CFTR) (O'Brien et al., 2010, Tahmasbpour et al., 2014). This is found to be responsible for about 1-2% of infertility in males (Jequier et al. 1985; Weiske et al. 2000). About four percent of infertile men had sex-chromosomal disorders, whereas approximately 1% had autosomal anomalies (Singh *et al*., 2006).

### 1.9.2. Y-Chromosome variations and males deficiency

The human Y-chromosome is found to be associated with male infertility. The greatest impact on spermatogenesis is caused by Y-linked mutations (Hargreave *et al*., 2000; Singh *et al*., 2006). Microdeletions of Y-chromosome have been reported among patients with poor sperm quality (7.4%) (Erasmuson *et al*., 2003). Microdeletions in the azoospermia factor region (AZF) on the long arm of the Y-chromosome are recognized as the most frequent abnormalities related to male infertility (Vogt *et al*., 1996). The AZF locus is subdivided into four loci subregions "AZFa, AZFb, AZFc and AZFd" (Brinton-Jones *et al*., 2000). These regions are also involved 3 genes: Deleted in azoospermia (DAZ) gene, RNA binding motif (RBM) gene, and Drosophila fat facet related Y (DFFRY) gene.

The human DAZ gene is found in the AZFc area on the Y chromosome and is identified in approximately 10-15% of oligozoospermic and azoospermic males. The RBM gene is located in the AZFb area (Moore *et al*., 2000). The azoospermic and oligozoospermic males have deletions of this region. Likewise, the DFFRY gene in the AZFa area is also related to male infertility. The other azoospermic factor, AZFd, has also been recently described to be related to moderate oligozoospermia or normal count of spermatozoa with abnormalities in sperm shape (Brinton-Jones *et al*., 2000).

## 1.10. The role for mtDNA and sperm survival

Mitochondria are found in both animal and plant cells' cytoplasm. Most of the chemical reactions that produce adenosine triphosphate (ATP) occurs within Mitochondria. The double membranes of mitochondria modulate the molecules' transportation. the outer membrane allows the transmission of large molecules via nonspecific porin channels; the inner membrane is invaginated and forms cristae. Within cristae, the enzymes of the electron transfer chain (ETC) are located (JOHN *et al*., 2000).

The mitochondrial ATP Generation is critical for most cells viability and contributes to cell homeostasis (Moyes *et al.*, 1998). The main contribution to ATP in the cell comes from oxidative phosphorylation reactions, in which electrons are released from hydrogen and transferred from one electron-acceptor molecule to the other, thereby oxidizing cytochromes, resulting in the loss of a lot of energy. This energy is used to pump protons into the surrounding medium to establish an electrochemical gradient, thereby providing energy for ATP synthesis. Electrons passing down the ETC combine with protons and react with molecular oxygen to generate H2O. This process realizes the conversion of redox energy into a proton gradient and then works as a driving force for ATP synthesis. Indeed, conventional experiments using mitochondrial replacement inhibitors have demonstrated the importance of oxidative phosphorylation for this process (Slater, 1979).

The cell's demand for oxygen reflects its metabolic activity and mitochondrial products are usually indicative of the ATP level of cell function (Moyes *et al*., 1998). Cells with a huge oxidative capability, such as those in the liver, muscle, heart, and neural tissue, have thousands of mitochondria for each cell (Brown and Wallace, 1994). The lungs have a high demand for

oxygen absorption, which is reflected in the oxygen tension of 160 mm Hg, while sperm are produced in the testis even under almost anoxic conditions with a partial pressure of 4 to 16 mm Hg (Setchell, 1978). The sperm has 22 to 28 mitochondria per cell, and their number is relatively low considering their motility requirements. In addition, sperm mitochondria tend to respond to the glucose levels of the female reproductive tract (Storey, 1980).

The mitochondrial respiratory chain consists of 13 proteins encoded by mtDNA. Complex I includes seven Nicotinamide Adenine Dinucleotide Hydride (NADH) Dehydrogenase subunits (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*), complex III contains cytochrome B, complex IV contains three subunits: cytochrome oxidase subunit I (*COX I*), cytochrome oxidase subunit II (*COX II*), and cytochrome oxidase subunit III (COX III), and complex V contains *ATPase 6* and *ATPase 8* **(Figure 7)** (Smeitink *et al*., 2001).

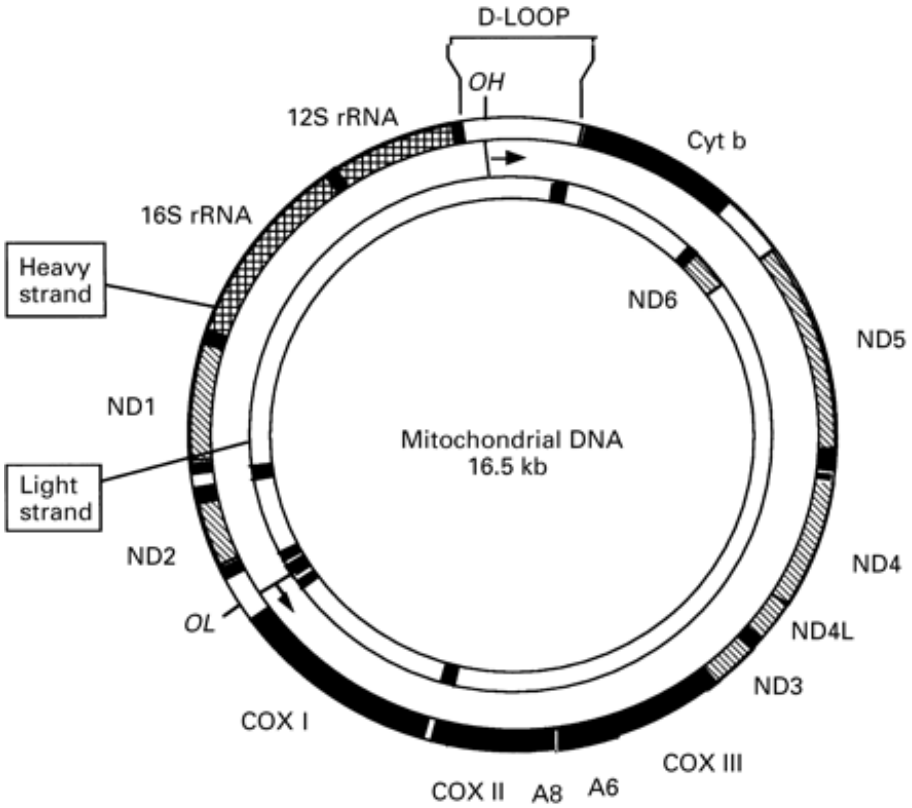



**Figure 7:** The mitochondrial respiratory chain.
[Adapted from Niaudet, 1998].

Each of the complexes of the Electron transport chain (ETC), except complex II, has genes encoded by the mitochondrial genome, while the remainder of the subunits of the ETC is encoded by the nucleus. Unlike nuclear DNA, there are no or only a few non-coding sequences between mitochondrial genes, therefore, lack introns. Nevertheless, there is one noncoding sequence, the displacement loop (D- loop), which is essential for mtDNA replication.

The mitochondrial genes *ATPase 6*, *ATPase 8*, *COX 3*, *COX 2*, *CytB*, *ND3*, *ND4*, *ND5*, and *ND6* play a substantial role in the mature sperm construction and progressive flagellar movement after ejaculation (Nakada *et al.,* 2006). The sperm with defective mitochondria produce less amount of ATP and more reactive oxygen species (ROS) and free radicals. Therefore, further damage to mitochondria and mtDNA occurs, subsequently; result in energy trouble and lowering of sperm motility (John *et al*., 2000).

There are three distinct types of mtDNA mutations that have already been related to various disorders. They are single nucleotide deletions in the genes of tRNA and rRNA as well as in protein-coding genes (Shanske *et al*., 2001; DiMauro *et al*., 2001; 2003). These mtDNA mutations have an effect on mitochondrial protein synthesis. However, mitochondrial single nucleotide deletions are sporadic in the majority of cases (DiMauro *et al*., 2001; Shanske *et al*., 2001).

Recently, mitochondrial DNA mutations and men's infertility, especially Asthenospermia, have aroused widespread concern. There is increasing evidence indicates the importance of mtDNA mutations with low sperm motility and male infertility. Mitochondrial DNA mutations are associated with Asthenozoospermia or Oligoasthenozoospermia (Folgero *et al*., 1993; Lestienne *et al*., 1997). It has been reported that mutations in the mitochondrial DNA polymerase (POLG) locus are associated with male infertility (Rovio *et al*., 2001). The high incidence of single nucleotide substitutions (SNPs) in mtDNA is observed in semen samples of poor sperm quality (Holyoake *et al*., 2001). Several studies have shown that sperm mtDNA mutations lead to reduced sperm motility which is unrelated to infertility. There was an association between the presence of the mutations and low motile sperm, however, was fertile (Thangaraj *et al*., 2003).
Mitochondrial oxidative phosphorylation (OXPHOS) is the main metabolic pathway and is essential for sperm to do its function properly by generating ATP. Any changes may disturb its normal activity (Ferramosca *et al*., 2008; Ferramosca and Zara, 2014).

## 1.11. Human Complex I

Complex I, the first enzyme of the chain, oxidizes NADH which is generated through the Krebs cycle in the matrix of mitochondria. Complex I is the main entry point for electrons to the respiratory chain and is suggested as the rate-limiting step in overall respiration. Therefore, it plays a critical role in energy metabolism (Sharma *et al*., 2009).

### 1.11.1. Mt-ND3

Mitochondrial NADH dehydrogenase subunit 3 (*Mt-ND3*) is a complex I gene encoded by mtDNA. (Chomyn *et al*., 1986). Several mutations in mtDNA genes have been identified, one of these mutations is the single nucleotide polymorphism at locus rs2853826 in *MTND3* that raises

ROS production in type 2 diabetes mellitus (T2DM) (van der Walt *et al*., 2003). To date, last studies have reported a significant association between *MTND3* polymorphisms and the risk of Parkinson's disease, T2DM, and breast and esophageal cancers, but not with Gastric Cancer (Chomyn *et al*., 2010; van der Walt *et al*., 2003; Pezzotti *et al*., 2009). *MT-ND3* is located in human mitochondrial DNA from base pair 10,059 to 10,404 (Nguyen *et al*., 2020). The *MT-ND3* gene produces a 13 kDa protein consisting of 115 amino acids (Zong *et al*., 2013).

### 1.11.2. MT-ND4L

The *MT-ND4L* gene is found in human mtDNA between base pairs 10,469 and 10,765 (Nguyen *et al*., 2020). The *MT-ND4L* gene codes an 11 kDa protein that included 98 amino acids (Zong *et al*., 2013). Several mutations in the *MT-ND4L* gene were found to be associated with clinical disorders like Leber's Hereditary Optic Neuropathy (LHON) (Achilli *et al*., 2012).

The structure of *MT-ND4*, *MT-ND3*, and *MT-ND4L* are L-shaped that contains two domains: a hydrophobic transmembrane domain and a hydrophilic peripheral arm domain which includes all redox centers of the complex and the NADH binding site. *MT-ND3*, *MT-ND4L*, and *MT-ND4* as well as the majority of the encoded-subunits in mitochondria are the most hydrophobic subunits of Complex I and shape the core of the transmembrane-domain (Voet *et al*., 2016).

### 1.11.3. MT-ND4

*MT-ND4* is one of the core mitochondrial-encoded subunits of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) (Yagi and Matsuno, 2003; Hirst *et al*., 2003). MT-ND4 gene codes for the NADH-ubiquinone oxidoreductase chain 4 (*ND4*) protein (Valdivieso *et al*., 2007). The *ND4* protein is located in the mitochondrial inner membrane and is the largest of the five complexes of the electron transport chain (Voet *et al*., 2016).

MT-ND4 plays an important role in the oxidative phosphorylation process and it has been reported that is associated with sperm motility (Holyoake *et al*., 2001; Rani *et al*., 2006; Spiropoulos *et al*., 2002). Variations in the *MT-ND4* gene are associated with age-related macular degeneration (AMD), Leber's hereditary optic neuropathy (LHON), mesial temporal lobe epilepsy (MTLE) and cystic fibrosis (Valdivieso *et al*., 2007; Gurses *et al*., 2014; Wallace *et al*., 1988; Restrepo *et al*., 2014). The *MT-ND4* gene is located in human mtDNA between base

pairs 10,760 and 12,137 (Nguyen *et al*., 2020). The *MT-ND4* gene codes a 52 kDa protein consisted of 459 amino acids (Zong *et al*., 2013; Cwerman-Thibault *et al*., 2015).

## 1.12. The purpose of the study

The purposes of the current study are:

1- To examine whether *MT-ND3*, *MT-ND4L*, and *MT-ND4* polymorphisms contribute to male infertility.

2- To study the distribution of genotype frequency and allele frequency between subfertile and fertile males.

3- To identify the SNPs in *MT-ND3*, *MT-ND4L*, and *MTND4* by Sanger sequencing and determined the association between mitochondrial gene polymorphisms and male infertility.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Study population

One hundred and twelve semen samples were collected from subfertile and fertile males attending the in-vitro fertilization clinic. The informed consent of all individuals was obtained before collecting samples. The study population aged between 26- 48 years had been divided into two groups according to semen analysis (WHO, 2010) to 68 subfertile and 44 fertile males. The individuals with age over 55 years old, men exposed to chemotherapy or radiotherapy, varicocele or any surgical procedure in the reproductive tract, diabetes, blood pressure and any chronic disease, hormonal imbalance, Y chromosome microdeletion were excluded from this study.

### 2.1.2. Reagents, chemicals, kits and equipment in the experimental part

| **Reagent or chemical** | **Company** |
|---|---|
| Absolute Ethanol | Merck, Germany |
| phosphate buffer saline | Sigma-Aldrich, Germany |
| Agarose tablets (DNase/RNase free) | Biocat, Germany |
| DNA Ladder (1kb) | New England BioLabs, USA |
| Nuclease-free water | New England BioLabs, USA |
| Safe- Red | Biocat, Germany |
| Tris-Borate-EDTA buffer (TBE) | Thermo Fisher Scientific, Germany |
| **Kits** | |
| QIAamp DNA Mini Kit | QIAGEN, Germany |
| REPLI-g Mitochondrial DNA Kit | QIAGEN, Germany |
| PCR primers (*MTND3*, *MTND4L*, and *MTND4*) | MicrosynthSeqLab, Germany |
| ThermoScientific Dream Taq Green PCR master mix (2x) | Thermo Fisher Scientific, Germany |

| **Instruments** | |
|---|---|
| Centrifuge CM-6MT | ELMI, Latvia |
| Consort EV 243 Electrophoresis power supply | Sigma-Aldrich, Germany |
| EasyCast B2 Mini Gel Electrophoresis System | Thermo Scientific, USA |
| Eppendorf Bench-top centrifuge | Eppendorf, Germany |
| Freezer, -20°C | Liebherr, Germany |
| Freezer, -80°C | Thermo Scientific, USA |
| Freezer, 8°C | Liebherr, Germany |
| Laboratory timer | Qiagen, Germany |
| Light Microscope | Carl Zeiss Microscopy, Germany |
| Manual counter | Karl Hecht "Assistent", Germany |
| Microcentrifuge | VWR International, USA |
| MolecularImager Gel Doc XR & System with Image Lab Software | Bio-Rad, Germany |
| Nanodrop spectrophotometer ND-2000c | Thermo Scientific, USA |
| PCR workstation pro (peqlab) | VWR international, USA |
| Thermal Cycler C100 | Bio-Rad, Germany |
| Thermomixer comfort | Eppendorf, Germany |
| Vortex-Genie 2 | Scientific industries, USA |
| **Disposables** | |
| 96-well PCR Plate 0.2 mL, non-skirted | Nippon Genetics Europe, Germany |
| Biosphere Filter tips (10-100-1000 microliter) | Sarstedt, Germany |
| Biosphere plus SafeSeal Micro Tubes (1.5 mL/ 2 mL) | Sarstedt, Germany |
| Eppendorf Conical Tubes, 15 mL | Eppendorf, Germany |
| MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml) | MicrosynthSeqLab, Germany |

| | |
|---|---|
| PCR Soft tubes, 0.2 ml (DNA, DNase, RNase free) | Sarstedt, Germany |
| Parafilm | American National Can, USA |
| Pipettes | Eppendorf, Germany |
| Racks | Sarstedt, Germany |
| Single Scale Graduated Cylinders | VWR International, USA |
| Storage boxes | Sarstedt, Germany |

## 2.2. Methods

### 2.2.1. Sperm sample collection and preparation

Semen samples were obtained by masturbation after 3 days of abstinence; the semen had been collected in a sterile, wide-mouthed, non-toxic, and special container, and then had been allowed to liquefy at 37°C for 30 minutes before assessment. Clinical history was collected including age and Sperm Parameters (ejaculate volume, viscosity, count, percentage of motility, and percentage of morphology) by using Makler counting chamber according to World Health Organization (WHO., 2010) guidelines (Table 1).

**Table 1: Semen characteristics according to the WHO (2010)**

| Parameters (Unit) | Reference value |
|---|---|
| Semen volume (ml) | 1.5 |
| Total sperm number ($10^6$ per ejaculate) | 39 |
| Sperm concentration ($10^6$ per ml) | 15 |
| Total motility (PR + NP, %) | 40 |
| Sperm morphology (normal forms, %) | 4 |

The semen samples were processed by discontinuous pure sperm gradient (45% and 90%) technique before DNA extraction. Briefly, semen samples were loaded at the upper level of the gradient and centrifuged at 250 g for 20 minutes. Thereafter, the pellet was collected and washed twice with a sperm washing medium. The absence of all other cells was confirmed by microscopic examination. Finally, the sperm pellet was kept at -20°C for DNA extraction.

### 2.2.2. Mitochondrial DNA extraction

Sperm cells were incubated with somatic cell lysis buffer (SCLB) for 30 min on ice, and then sperm had been washed twice with phosphate buffer saline (PPS) for 10 min (Ieremiadou and Rodakis, 2009; Jenkins *et al*., 2016). The QIAamp DNA Mini Kit was used to extract the whole genome from the sperma, then the REPLI-g Mitochondrial DNA Kit was used for the mitochondrial DNA amplification, as recommended by the kit instruction manual (QIAGEN, Hilden, Germany). A 260/280 ratio with 1.8 density or more was used for the quality selection of the extracted DNA. Then DNA was kept at -80°C for further analysis later.

## 2.3 PCR assay of mitochondrial genes

The following mitochondrial DNA genes that encode for NADH dehydrogenase - CoQ reductase (*ND3*, *ND4L*, *ND4*) - complex I, were chosen for SNPs genotyping in this study.

The polymerase chain reaction was applied to determine the gene variant by using self-designed pairs of Primers using PRIME 3 software for the target genes (*MTND3*, *MTND4L*, and *MTND4*) as shown in **Table (2)**. An additional internal primer, *Nd4*.I, was designed for sequencing. The primers were designed using the human mitochondrial sequence; accession number NC_012920, which was established by the National Center of Biotechnology Information (NCBI) and ordered from Microsynth seq lab- Germany.

**Table 2: Oligonucleotides primers of *Nd3*, *Nd4L*, *Nd4* mtDNA genes used for PCR amplification**

| Primers | Sequences (5′ → 3′) | Cycling conditions | The length of amplified product (bp) |
|---|---|---|---|
| **Nd3.F** | CCAATTAACTAGTTTTG | 95 °C 3 Min<br>95 °C 30 Sec<br>48.8 °C 30 Sec (30x cycles)<br>72 °C 1 Min<br>72 °C 5 Min | 420 bp |
| **Nd3.R** | GAGTCGAAATCATTCGT | | |
| **Nd4L.F** | GATTTCGACTCATTAAATT | 95 °C 3 Min<br>95 °C 30 Sec<br>45.9 °C 30 Sec (30x cycles)<br>72 °C 1 Min<br>72 °C 5 Min | 376 bp |
| **Nd4L.R** | CATGTCAGTGGTAGTAATAT | | |
| **Nd4.F** | CTACGTACATAACCTAAACC | 95 °C 3 Min<br>95 °C 30 Sec<br>49 °C 40 Sec (35x cycles)<br>72 °C 1 Min<br>72 °C 5 Min | 1432 bp |
| **Nd4.I** | CTTAAAACTAGGCGGCTATGG | | |
| **Nd4.R** | CTGATGTTTTGGTTAAAC | | |

**F**: Forward primer, **R**: Reverse primer, **I**: Internal primer, **bp**: base pair position related to the reference human mitochondrial sequence (accession number NC_012920).

The amplification reaction was carried out in a 30 µL mixture using ThermoScientific Dream Taq Green PCR master mix (2x), according to manufacturer instructions. The amplification conditions for the genes of this study were optimized to obtain good results. To confirm the presence of an amplified PCR product, 5 µL of each PCR sample was investigated by 1% agarose gel electrophoresis using 1 × TBE buffer and a DNA ladder (1 kb) (NE Biolabs, USA) as a reference. Electrophoresis was carried out at 100 V for 45 min. Gels were stained with red-safe stain and then DNA was visualized by ultra-violet (UV) transilluminator with Image LabTM Software (BIO-RAD, USA) **(Figures 8, 9, and 10).**

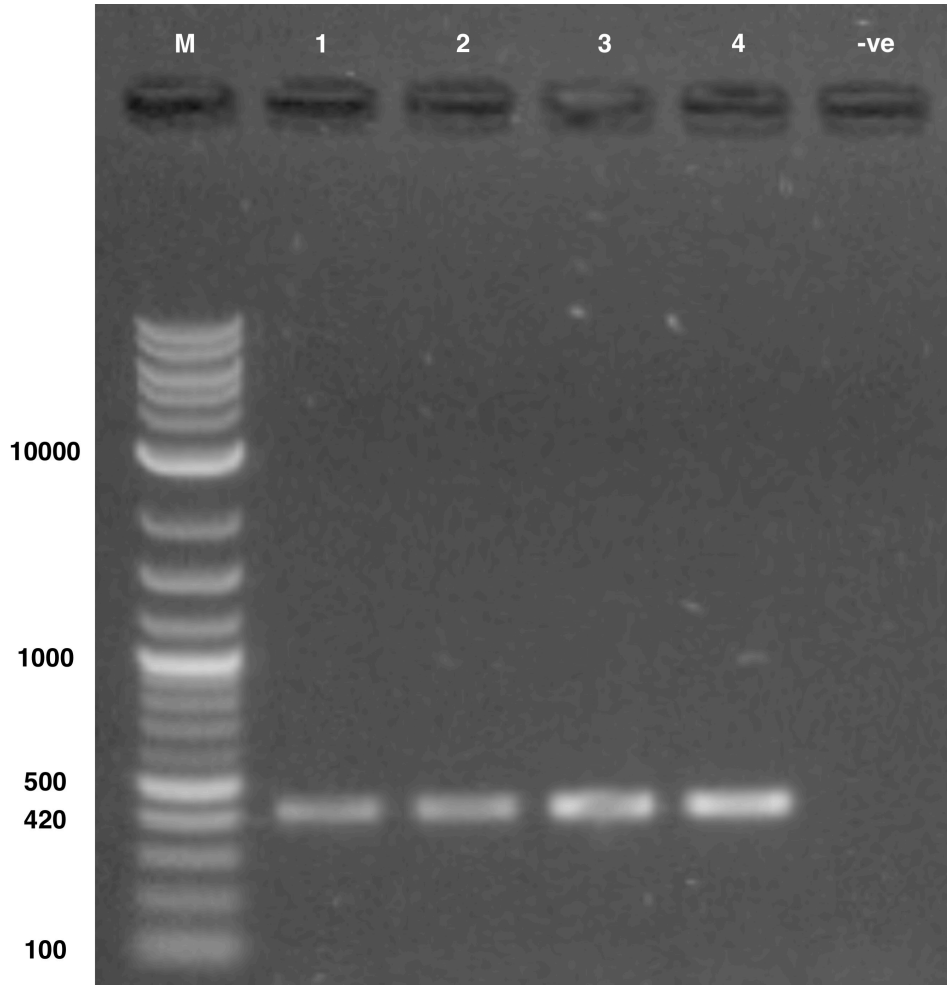



**Figure 8:** PCR products of the *MT-ND3* gene (420 bp) on 1% agarose gel electrophoresis. Lane M: DNA Ladder (100-10000 bp) (NE Biolabs, USA), Lane 1-4: PCR samples products, lane -ve: negative control. Electrophoresis was carried out at 100 V for 45 min. Gels were stained with red-safe stain and then DNA was visualized by ultra-violet (UV) transilluminator using Image LabTM Software (BIO-RAD, USA).

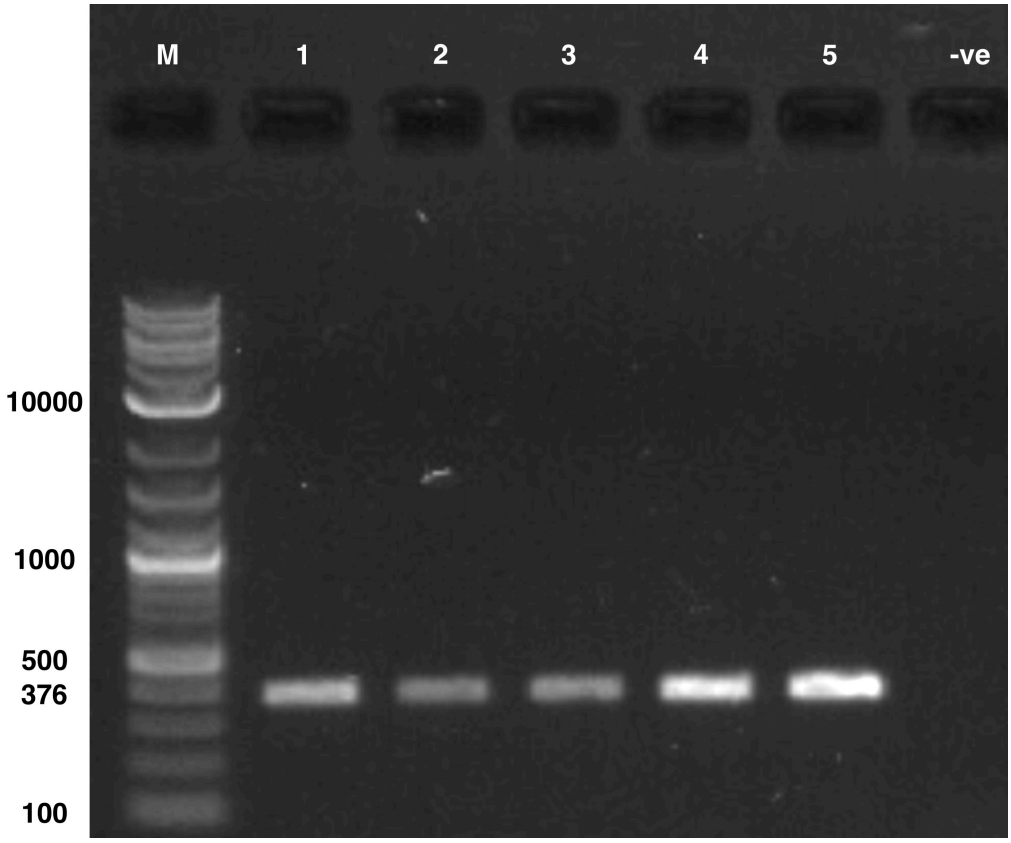



**Figure 9:** PCR products of the *MT-ND4L* gene (376 bp) on 1% agarose gel electrophoresis. Lane M: DNA Ladder (100-10000 bp) (NE Biolabs, USA), Lane 1-4: PCR samples products, lane -ve: negative control. Electrophoresis was carried out at 100 V for 45 min. Gels were stained with red-safe stain and then DNA was visualized by ultra-violet (UV) transilluminator using Image LabTM Software (BIO-RAD, USA).

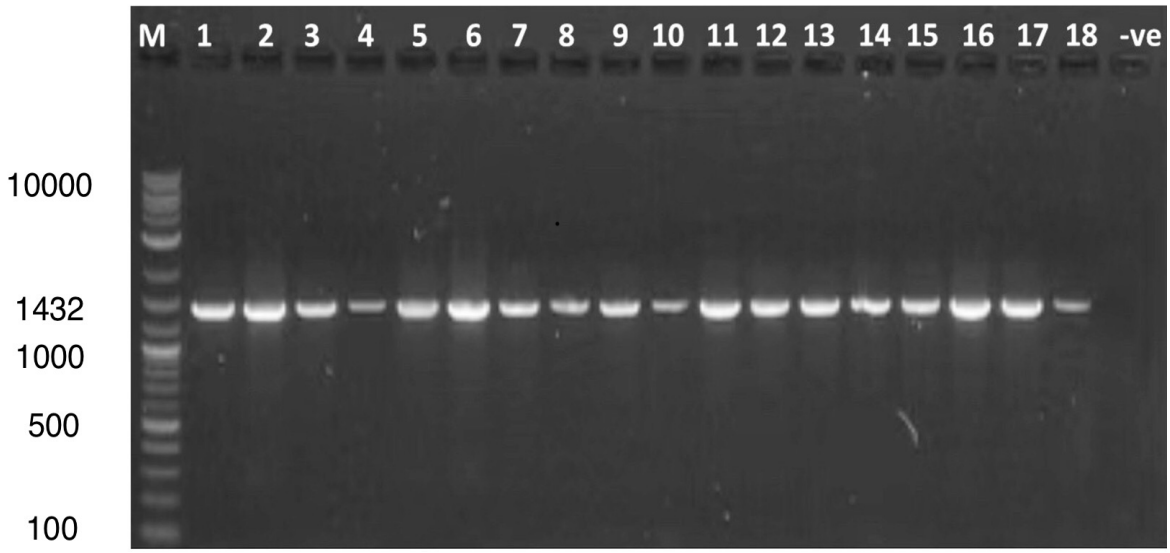



**Figure 10:** PCR products of the *MT-ND4* gene (1432 Bp) on 1% agarose gel electrophoresis. Lane M: DNA Ladder (100-10000 bp) (NE Biolabs, USA), Lane 1-18: PCR samples products, lane -ve: negative control. Electrophoresis was carried out at 100 V for 45 min. Gels were stained with red-safe stain and then DNA was visualized by ultra-violet (UV) transilluminator using Image LabTM Software (BIO-RAD, USA).

## 2.4 DNA Sequencing

Samples were purified and sequenced by the Sanger method at Microsynth Seq lab in Germany. PCR products were sequenced using the same PCR primers. The SNPs of *MTND3*, *MTND4L*, *MTND4* were detected by sequence analysis based on the reference sequence of human MT (GenBank accession number: NC_012920). The sequenced DNA samples were analyzed using FinchTV software after determined Mt-DNA variants by Mutation Surveyor software.

## 2.5 Statistical Analysis

Genotypes and allele frequencies between the subfertile (case) and fertile (control) groups were performed using the Chi-square test and Fischer's exact test respectively. The identified SNPs were also tested for the Hardy Weinberg equilibrium test to determine the genotype frequencies and to describe statistically significant deviations from the Equilibrium. The allele frequencies between the subfertile (case) and fertile (control) groups were measured according to odds ratios (ORs) and 95% confidence intervals (CIs). *P*-value was considered statistically significant if $\leq$ 0.05. Statistical analyses were performed using the SPSS Version 22 for Mac.

# 3. RESULTS

## 3.1 Investigated parameters for all studied males

The study subjects were divided into two groups: a control group (fertile, n=44) and a case group (subfertile, n= 68). The subfertile group was classified into 6 subgroups (asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia, oligoteratozoospermia). The study population did not show any significant difference between the ages of the subfertile and fertile group ($P$= 0.247). On the other hand, the semen analysis showed significant differences in the mean percentage of sperm concentration, total motility, and morphologically normal spermatozoa between the fertile and subfertile individuals ($P < 0.0001$) **(Table 4)**.

**Table 3: Descriptive statistic of studied parameters for all males (N=112)**

| Parameter | M ± SD | Median |
|---|---|---|
| **Age** | 35 ±5.51 | 34 |
| **Sperm concentration ($10^6$ x1ml)** | 54.86 ± 42.11 | 45 |
| **Total Motility (PR+NP%)** | 54.68 ± 21.62 | 57 |
| **Morphologically normal spermatozoa (%)** | 18.55 ± 7.42 | 18.5 |

**M**: mean; **SD**: standard deviation.

**Table 3** illustrates the descriptive statistics: mean ± standard deviation, and median of the different studied parameters. The mean of age and the sperm parameters for all population: age, sperm concentration ($10^6$ per ml), total motility (PR + NP. %), and morphologically normal spermatozoa were (35 ±5.51; 54.86 ± 42.11; 54.68 ± 21.62; 8.99 ± 8.77; 18.55 ± 7.42 respectively).

**Table 4: Comparison of the parameters of the sperm analysis between the fertile and subfertile groups**

| Parameter | Fertile (n=44) Medien (range) | Subfertile (n=68) Medien (range) | *P*-VALUE |
|---|---|---|---|
| **Age** | 34 (26-48) | 34 (26-48) | 0.247 |
| **Sperm concentration ($10^6$x1ml)** | 78.5 (17-185) | 28 (0.6-135) | **< 0.0001** |
| **Total Motility (PR+NP %)** | 67.5 (44-90) | 48.5 (2-88) | **< 0.0001** |
| **Morphologically normal spermatozoa (%)** | 24.5 (20-30) | 15 (0-28) | **< 0.0001** |

**N:** Number

### 3.2 Genotypes and allelic frequencies

We identified eight SNPs in *MTND3* rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954, seven SNPs in *MTND4L* rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881, and twenty five SNPs in *MTND4* in the cases and controls: rs2853495, rs2857284, rs2853496, rs2853497, rs3087901, rs2853493, rs2853490, rs3088053, rs2853491, rs2857285, rs28358282, rs28594904, rs28669780, rs28415973, rs28471078, rs55714831, rs28358283, rs75214962, rs28529320, rs2853494, rs28609979, rs28358286, rs28359168, rs28384199, and rs869096886.

To determine whether the variations of *MTND3*, *MTND4L*, and *MTND4* were related to infertility, we compared each of the genotypes and allele frequencies between the case and control groups. The genotype distributions for the SNPs in *MTND3*, *MTND4L*, and *MTND4* are shown in **Tables (5-10)**. For *MTND4*, the allele frequency, the rs2853495 and the rs869096886 were significantly different between subfertile and fertile males ($P$ = 0.0028, $P$ = 0.0073 respectively) **(Figure 11 (a), 12)**. Whereas the remaining SNPs showed no significant association. For genotype frequency, only the rs2853495 showed a significant association ($P$ = 0.0351) (**Figure 11 (b)**). All SNPs were tested for the Hardy-Weinberg genotype frequency test. All of these SNPs showed a significant deviation from HWE ($P < 0.0001$).

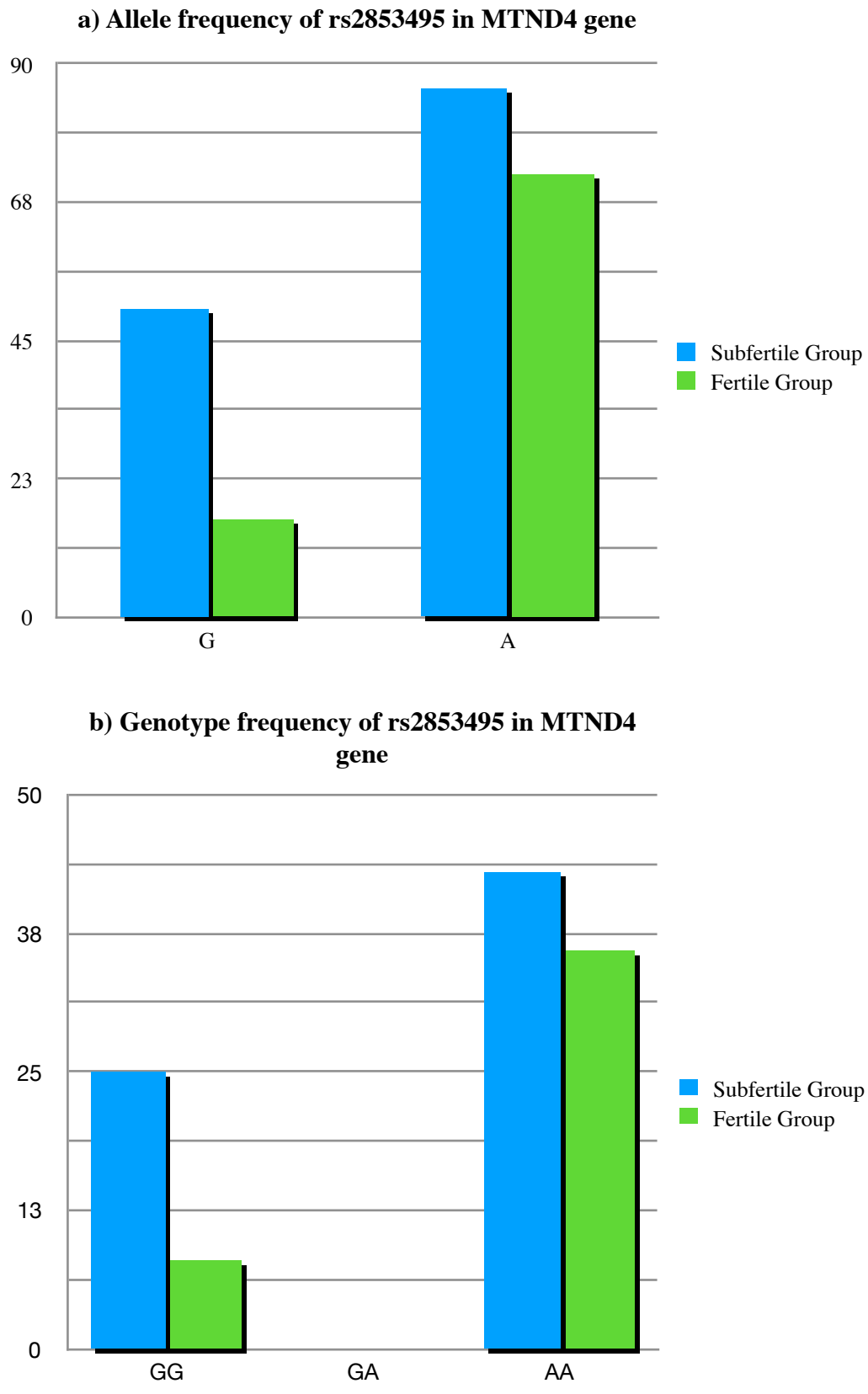



**Figure 11: a)** allele frequency of rs2853495 in *MTND4* gene (*P*= 0.002), **b)** genotype frequency of rs2853495 in *MTND4* gene (*P*= 0.0351).

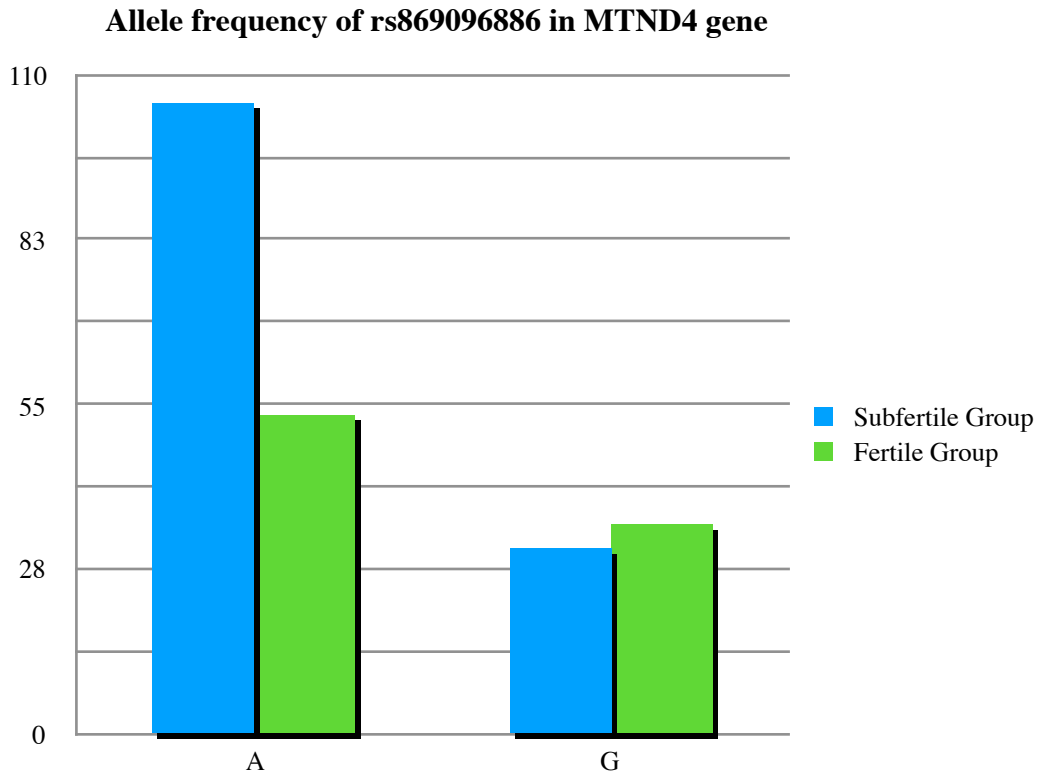



**Figure 12:** allele frequency of rs869096886 in *MTND4* gene (*P*= 0.0073).

**Table 5: Genotypes frequency of *MTND3* polymorphisms between subfertile males and control (fertile)**

| SNP | Contig position | Codon change | Amino acid change | Type of mutation | Genotype | Subfertile (N) | Fertile (N) | *P*-value |
|---|---|---|---|---|---|---|---|---|
| rs2853826 (A>G,T) | 10398 | [ACC]>[GCC] | Thr114 Ala | Missense variant | AA | 37 | 21 | 0.768 |
| | | | | | AG | 1 | 1 | |
| | | | | | GG | 30 | 22 | |
| rs28435660 (G>A) | 10353 | [GCC]>[ACC] | Ala99Thr | Missense variant | GG | 61 | 40 | 0.825 |
| | | | | | GA | 4 | 3 | |
| | | | | | AA | 3 | 1 | |
| rs193302927 (T>C) | 10238 | [ATT]>[ATC] | Ile60Ile | Synonymous variant | TT | 62 | 40 | 0.959 |
| | | | | | TC | 2 | 1 | |
| | | | | | CC | 4 | 3 | |
| rs28358278 (C>T) | 10400 | [ACC]>[ACT] | Thr114Thr | Synonymous variant | CC | 65 | 44 | 0.158 |
| | | | | | CT | 0 | 0 | |
| | | | | | TT | 3 | 0 | |
| rs41467651 (G>A) | 10310 | [CTG]>[CTA] | Leu84Leu | Synonymous variant | GG | 65 | 42 | 0.9320 |
| | | | | | GA | 1 | 1 | |
| | | | | | AA | 2 | 1 | |
| rs3899188 (T>C) | 10115 | [ATT]>[ATC] | Ile19Ile | Synonymous variant | TT | 67 | 43 | 0.754 |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 1 | 1 | |
| rs28358277 (G>A) | 10373 | [GAG]>[GAA] | Glu105Glu | Synonymous variant | GG | 66 | 44 | 0.517 |
| | | | | | GA | 1 | 0 | |
| | | | | | AA | 1 | 0 | |
| rs28673954 (T>C) | 10370 | [TAT]>[TAC] | Tyr104Tyr | Synonymous variant | TT | 67 | 44 | 0.419 |
| | | | | | TC | 1 | 0 | |
| | | | | | CC | 0 | 0 | |

**Table 6: Allele frequency of *MTND3* polymorphisms between subfertile males and fertile groups**

| SNP | Contig position | Allele | Subfertile (N,%) | Fertile (N,%) | OR (95% CI)* | *P*-value |
|---|---|---|---|---|---|---|
| rs2853826 (A>G,T) | 10398 | A | 75 (34%) | 43 (19%) | 1.287 ( 0.751 - 2.203) | 0.411 |
| | | G | 61 (27%) | 45 (20%) | | |
| rs28435660 (G>A) | 10353 | G | 126 (56%) | 83 (37%) | 0.759 (0.25 - 2.3) | 0.7865 |
| | | A | 10 (5%) | 5 (2%) | | |
| rs193302927 (T>C) | 10238 | T | 126 (56%) | 81 (36%) | 1.089 (0.398 - 2.977) | 1.000 |
| | | C | 10 (5%) | 7 (3%) | | |
| rs28358278 (C>T) | 10400 | C | 130 (58%) | 88 (39%) | 0.1134 ( 0.006-2.041) | 0.0837 |
| | | T | 6 (3%) | 0 (0%) | | |
| rs41467651 (G>A) | 10310 | G | 131 (59%) | 85 (38%) | 0.924 (0.215- 3.972) | 1.000 |
| | | A | 5 (2%) | 3 (1%) | | |
| rs3899188 (T>C) | 10115 | T | 134 (60%) | 86 (38%) | 1.558 (0.2153 - 11.275) | 0.6466 |
| | | C | 2 (1%) | 2 (1%) | | |
| rs28358277 (G>A) | 10373 | G | 133 (60%) | 88 (39%) | 0.2155 (0.0109 - 4.226) | 0.2812 |
| | | A | 3 (1%) | 0 (0%) | | |
| rs28673954 (T>C) | 10370 | T | 135 (61%) | 88 (39%) | 0.5104 ( 0.0205 - 12.679) | 1.000 |
| | | C | 1 (0%) | 0 (0%) | | |

**Table 7: Genotypes frequency of *MTND4L* polymorphisms between subfertile males and control (fertile)**

| SNP | Contig position | Codon change | Amino acid change | Type of mutation | Genotype | Subfertile (N) | Fertile (N) | *P*-value |
|---|---|---|---|---|---|---|---|---|
| rs28358280 (A>G) | 10550 | [ATA]>[ATG] | Met27 Met | Synonymous variant | AA | 67 | 42 | 0.325 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 1 | 2 | |
| rs28358281 (G>A,C) | 10586 | [TCG]>[TCA] | Ser39Ser | Synonymous variant | GG | 62 | 43 | 0.3335 |
| | | | | | GA | 2 | 0 | |
| | | | | | AA | 4 | 1 | |
| rs28358279 (T>A,C) | 10463 | N/A | N/A | Synonymous variant | TT | 64 | 42 | 0.759 |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 4 | 2 | |
| rs2853487 (G>A) | 10589 | [CTG]>[CTA] | Leu40Leu | Synonymous variant | GG | 66 | 43 | 0.8306 |
| | | | | | GA | 0 | 0 | |
| | | | | | AA | 2 | 1 | |
| rs2853488 (G>A) | 10688 | [GTG]>[GTA] | Val73Val | Synonymous variant | GG | 66 | 43 | 0.2416 |
| | | | | | GA | 2 | 0 | |
| | | | | | AA | 0 | 1 | |
| rs193302933 (C>T) | 10664 | [GTC]>[GTT] | Val65Val | Synonymous variant | CC | 68 | 43 | 0.2118 |
| | | | | | CT | 0 | 0 | |
| | | | | | TT | 0 | 1 | |
| rs28532881 (C>A) | 10763 | [TGC]>[TGA] | Cys98Trp | Missense variant | CC | 68 | 44 | N/A |
| | | | | | CA | 0 | 0 | |
| | | | | | AA | 0 | 0 | |

**N/A**: Not applicable.

**Table 8: Allele frequency of *MTND4L* polymorphisms between subfertile males and fertile groups**

| SNP | Contig position | Allele | Subfertile (N,%) | Fertile (N, %) | OR (95% CI)* | *P*-value |
|---|---|---|---|---|---|---|
| rs28358280 (A>G) | 10550 | A | 134 (60%) | 84 (37%) | 3.190 ( 0.571 - 17.810) | 0.214 |
| | | G | 2 (1%) | 4 (2%) | | |
| rs28358281 (G>A,C) | 10586 | G | 126 (56%) | 86 (38%) | 0.2883 ( 0.0616 - 1.350) | 0.131 |
| | | A | 10 (5%) | 2 (1%) | | |
| rs28358279 (T>A,C) | 10463 | T | 128 (57%) | 84 (37%) | 0.7619 ( 0.2223 - 2.611) | 0.768 |
| | | C | 8 (4%) | 4 (2%) | | |
| rs2853487 (G>A) | 10589 | G | 132 (59%) | 86 (38%) | 0.7674 (0.1375 - 4.283) | 1,000 |
| | | A | 4 (2%) | 2 (1%) | | |
| rs2853488 (G>A) | 10688 | G | 134 (60%) | 86 (38%) | 1.558 ( 0.215 - 11.275) | 0.6466 |
| | | A | 2 (1%) | 2 (1%) | | |
| rs193302933 (C>T) | 10664 | C | 136 (61%) | 86 (38%) | 7.890 (0.374 - 166.44) | 0.1533 |
| | | T | 0 | 2 (1%) | | |
| rs28532881 (C>A) | 10763 | C | 136 (61%) | 88 (39%) | N/A | N/A |
| | | A | 0 (0%) | 0 (0%) | | |

**N/A**: Not applicable.

**Table 9: Genotypes frequency of *MTND4* polymorphisms between subfertile males and control (fertile)**

| SNP | Contig position | Codon change | Amino acid change | Type of mutation | Genotype | Subfertile (N) | Fertile (N) | *P*-value |
|---|---|---|---|---|---|---|---|---|
| rs2853495 G>A | 11719 | [GGG]>[GGA] | Gly320Gly | Synonymous variant | GG | 25 | 8 | **0.0351** |
| | | | | | GA | 0 | 0 | |
| | | | | | AA | 43 | 36 | |
| rs2857284 T>C | 10873 | [CCT]>[CCC] | Pro38Pro | Synonymous variant | TT | 49 | 35 | 0.0995 |
| | | | | | TC | 2 | 4 | |
| | | | | | CC | 17 | 5 | |
| rs2853496 G>A,C | 11914 | [ACG]>[ACA] | Thr385Thr | Synonymous variant | GG | 56 | 40 | 0.597 |
| | | | | | GA | 3 | 1 | |
| | | | | | AC | 1 | 0 | |
| | | | | | AA | 8 | 3 | |
| rs2853497 G>A | 12007 | [TGG]>[TGA] | Trp416Trp | Synonymous variant | GG | 63 | 39 | 0.598 |
| | | | | | GA | 3 | 4 | |
| | | | | | AA | 2 | 1 | |
| rs3087901 T>A,C,G | 11944 | [CTT]>[CTC] | Leu395Leu | Synonymous variant | TT | 63 | 42 | 0.548 |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 5 | 2 | |
| rs2853493 A>G | 11467 | [TTA] >[TTG] | Leu236Leu | Synonymous variant | AA | 66 | 40 | 0.158 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 2 | 4 | |
| rs2853490 G>A | 11176 | [CAG]>[CAA] | Gln139Gln | Synonymous variant | GG | 66 | 40 | 0.183 |
| | | | | | GA | 0 | 2 | |
| | | | | | AA | 2 | 2 | |
| rs3088053 A>C,G | 11812 | [CTA] >[CTG] | Leu351Leu | Synonymous variant | AA | 64 | 42 | 0.758 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 4 | 2 | |
| rs2853491 C>T | 11335 | [AAC]>[AAT] | Asn192Asn | Synonymous variant | CC | 66 | 42 | 0.655 |
| | | | | | CT | 0 | 0 | |
| | | | | | TT | 2 | 2 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs2857285 T>C,G | 10915 | [TGT] >[TGC] | Cys52Cys | Synonymous variant | TT | 66 | 43 | 0.241 |
| | | | | | TC | 0 | 1 | |
| | | | | | CC | 2 | 0 | |
| rs28358282 T>C | 10810 | [CTT] >[CTC] | Leu17Leu | Synonymous variant | TT | 67 | 42 | 0.434 |
| | | | | | TC | 1 | 1 | |
| | | | | | CC | 0 | 1 | |
| rs28594904 G>A,C | 11016 | [AGT] >[AAT] | Ser86Asn | Missense variant | GG | 67 | 42 | 0.434 |
| | | | | | GA | 0 | 1 | |
| | | | | | AA | 1 | 1 | |
| rs28669780 C>A | 11603 | [CTA] >[ATA] | Leu282Met | Missense variant | CC | 67 | 42 | 0.434 |
| | | | | | CA | 0 | 1 | |
| | | | | | AA | 1 | 1 | |
| rs28415973 T>C | 12091 | [ATT] >[ATC] | Ile444Ile | Synonymous variant | TT | 67 | 42 | 0.434 |
| | | | | | TC | 0 | 1 | |
| | | | | | CC | 1 | 1 | |
| rs28471078 T>C | 11722 | [CTT] >[CTC] | Leu321Leu | Synonymous variant | TT | 67 | 43 | 0.754 |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 1 | 1 | |
| rs55714831 C>T | 11332 | [GCC]>[GCT] | Ala191Ala | Synonymous variant | CC | 67 | 43 | 0.754 |
| | | | | | CT | 1 | 1 | |
| | | | | | TT | 0 | 0 | |
| rs28358283 A>G | 10819 | [AAA]>[AAG] | Lys20Lys | Synonymous variant | AA | 67 | 44 | 0.419 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 1 | 0 | |
| rs75214962 C>T | 11197 | [GGC]>[GGT] | Gly146Gly | Synonymous variant | CC | 67 | 44 | 0.419 |
| | | | | | CT | 0 | 0 | |
| | | | | | TT | 1 | 0 | |
| rs28529320 T>C | 11485 | [GGT]>[GGC] | Gly242Gly | Synonymous variant | TT | 68 | 43 | 0.211 |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 0 | 1 | |
| Rs2853494 A>G | 11641 | [ATA] >[ATG] | Met294Met | Synonymous variant | AA | 68 | 43 | 0.211 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 0 | 1 | |
| rs28609979 T>C | 11365 | [GCT]>[GCC] | Ala202Ala | Synonymous variant | TT | 68 | 44 | N/A |
| | | | | | TC | 0 | 0 | |
| | | | | | CC | 0 | 0 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs28358286 C>T | 11674 | [ACC]>[ACT] | Thr305Thr | Synonymous variant | CC | 68 | 43 | 0.211 |
| | | | | | CT | 0 | 0 | |
| | | | | | CC | 0 | 1 | |
| rs28359168 A>G | 11947 | [ACA]>[ACG] | Thr396Thr | Synonymous variant | AA | 68 | 43 | 0.211 |
| | | | | | AG | 0 | 0 | |
| | | | | | GG | 0 | 1 | |
| rs28384199 C>A,G | 11777 | [CGC]>[GGC] | Arg340Gly | Missense variant | CC | 67 | 44 | 0.419 |
| | | | | | CG | 0 | 0 | |
| | | | | | GG | 1 | 0 | |
| rs869096886 A>G | 11251 | [CTA] >[CTG] | Leu164Leu | Synonymous variant | AA | 52 | 26 | 0.147 |
| | | | | | AG | 1 | 1 | |
| | | | | | GG | 15 | 17 | |

**Table 10: Allele frequency of *MTND4* polymorphisms between subfertile males and fertile groups**

| SNP | Contig position | Allele | Subfertile (N, %) | Fertile (N, %) | OR (95% CI)* | *P*-value |
|---|---|---|---|---|---|---|
| rs2853495 G>A | 11719 | G | 50 (23%) | 16 (7%) | 2.616 (1.374-4.983) | **0.002** |
| | | A | 86 (38%) | 72 (32%) | | |
| rs2857284 T>C | 10873 | T | 100 (45%) | 74 (33%) | 0.5255 (0.264-1.044) | 0.071 |
| | | C | 36 (16%) | 14 (6%) | | |
| rs2853496 G>A,C | 11914 | G | 115 (52%) | 81 (36%) | 0.496 (0.200-1.230) | 0.145 |
| | | A | 20 (9%) | 7 (3%) | | |
| rs2853497 G>A | 12007 | G | 129 (58%) | 82 (36%) | 1.348 (0.437-4.155) | 0.771 |
| | | A | 7 (3%) | 6 (3%) | | |
| rs3087901 T>A,C,G | 11944 | T | 126 (56%) | 84 (37%) | 0.6000 (0.18-1.97) | 0.573 |
| | | C | 10 (5%) | 4 (2%) | | |
| rs2853493 A>G | 11467 | A | 132 (59%) | 80 (35%) | 3.300 (0.962-11.31) | 0.066 |
| | | G | 4 (2%) | 8 (4%) | | |
| rs2853490 G>A | 11176 | G | 132 (59%) | 82 (36%) | 2.415 (0.661-8.817) | 0.196 |
| | | A | 4 (2%) | 6 (3%) | | |
| rs3088053 A>C,G | 11812 | A | 128 (57%) | 84 (37%) | 0.761 (0.222-2.611) | 0.768 |
| | | G | 8 (4%) | 4 (2%) | | |
| rs2853491 C>T | 11335 | C | 132 (59%) | 84 (37%) | 1.571 (0.382-6.456) | 0.714 |
| | | T | 4 (2%) | 4 (2%) | | |
| rs2857285 T>C,G | 10915 | T | 132 (59%) | 87 (39%) | 0.379 | 0.650 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | C | 4 (2%) | 1 (0%) | (0.041-3.453) | |
| rs28358282 T>C | 10810 | T | 135 (61%) | 85 (38%) | 4.765 | 0.302 |
| | | C | 1 (0%) | 3 (1%) | (0.487-46.58) | |
| r s 2 8 5 9 4 9 0 4 G>A,C | 11016 | G | 134 (60%) | 85 (38% | 2.365 | 0.383 |
| | | A | 2 (1%) | 3 (1%) | (0.386-14.45) | |
| rs28669780 C>A | 11603 | C | 134 (60%) | 85 (38%) | 2.365 | 0.383 |
| | | A | 2 (1%) | 3 (1%) | (0.386-14.45) | |
| rs28415973 T>C | 12091 | T | 134 (60%) | 85 (38%) | 2.365 | 0.383 |
| | | C | 2 (1%) | 3 (1%) | (0.386-14.45) | |
| rs28471078 T>C | 11722 | T | 134 (60%) | 86 (38%) | 1.558 | 0.646 |
| | | C | 2 (1%) | 2 (1) | (0.215-11.27) | |
| rs55714831 C>T | 11332 | C | 135 (61%) | 87 (39%) | 1.552 | 1.000 |
| | | T | 1 (0%) | 1 (0%) | (0.095-25.15) | |
| rs28358283 A>G | 10819 | A | 134 (60%) | 88 (39%) | 0.304 | 0.520 |
| | | G | 2 (1%) | 0 (0%) | (0.014-6.411) | |
| rs75214962 C>T | 11197 | C | 134 (60%) | 88 (39%) | 0.304 | 0.520 |
| | | T | 2 (1%) | 0 (0%) | (0.014-6.411) | |
| rs28529320 T>C | 11485 | T | 136 (61%) | 86 (38%) | 7.890 | 0.153 |
| | | C | 0 | 2 (1%) | (0.37-166.44) | |
| rs2853494 A>G | 11641 | A | 136 (61%) | 86 (38%) | 7.890 | 0.153 |
| | | G | 0 | 2 (1%) | (0.37-166.44) | |
| rs28609979 T>C | 11365 | T | 136 (61%) | 88 (39%) | - | N/A |
| | | C | 0 (0%) | 0 (0%) | | |
| rs28358286 C>T | 11674 | C | 136 (61%) | 86 (38%) | 7.890 | 0.153 |
| | | T | 0 (0%) | 2 (1%) | (0.37-166.44) | |
| rs28359168 A>G | 11947 | A | 136 (61%) | 86 (38%) | 7.890 | 0.153 |
| | | G | 0 (0%) | 2 (1%) | (0.37-166.44) | |
| rs28384199 C>A,G | 11777 | C | 134 (60%) | 88 (39%) | 0.304 | 0.520 |
| | | G | 2 (1%) | 0 (0%) | (0.01-6.41) | |
| r s 8 6 9 0 9 6 8 8 6 | | A | 105 (47%) | 53 (24%) | 2.237 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| A>G | 11251 | G | 31 (14%) | 35 (15%) | (1.24-4.01) | **0.007** |

**SNP**: single nucleotide polymorphism; **OR**: odds ratio; **CI**: confidence interval; **N/A**: Not applicable. The significant results are in bold.

## 4. DISCUSSION

Complex I encodes seven Nicotinamide Adenine Dinucleotide Hydride (NADH) dehydrogenase subunits. The mitochondrial genes *MTND3*, *MTND4L*, and *MTND4* encode the subunits (3, 4L, and 4 respectively) of Nicotinamide Adenine Dinucleotide Hydride (NADH) dehydrogenase and are considered part of the mitochondrial oxidative phosphorylation system (OXPHOS) complex I (Smeitink *et al*., 2001; Koopman *et al*., 2010). OXPHOS is the main metabolic pathway and is essential for spermatozoa to properly perform their function by producing ATP (Ferramosca *et al*., 2008; Ferramosca and Zara, 2014).

The role of complex I as the first step of the electron transport chain is to remove electrons from Nicotinamide Adenine Dinucleotide Hydride (NADH) dehydrogenase and add them to ubiquinone. This reaction releases energy that is used to transport protons through the inner membrane of the mitochondria. In this way, complex I help in maintaining the proton gradient that drives mitochondrial ATP production and many other mitochondrial functions (Koopman *et al*., 2010).

The mitochondrial DNA of sperm does not contain introns and lacks histones protection or DNA-binding proteins. Therefore, it multiplies rapidly without DNA repair mechanisms (Shamsi *et al*., 2008). The mutation rates of mitochondria are about 10-100 times higher than those of nuclear DNA. Mutations that occur in the mitochondrial genome play an important role in some human genetic disorders (Baklouti-Gargouri *et al*., 2013).

The sperm with defective mitochondria produce less ATP and more reactive oxygen species (ROS) and free radicals. This leads to further damage to the mitochondria and the mtDNA and consequently to energy troubles and reduced sperm motility (John *et al*., 2000).

The purpose of the current study was to investigate whether the mitochondrial genes *MTND3*, *MTND4L*, and *MTND4* polymorphisms are correlated with male infertility. It has previously been reported that the polymorphisms of these genes are associated with many diseases. Among the identified *MTND3* SNPs, rs2853826 (A10398G) (*MT-ND3*) was reported to be related to increased mitochondrial reactive oxygen species production and leading to oxidative stress and mitochondrial DNA damage (Pezzotti *et al*., 2009).

Moreover, the rs2853826 was found to be associated with an Earlier Age at Onset in Male Machado-Joseph disease patients, breast cancer, type 2 diabetes (T2D), GC development, esophageal cancer, Parkinson's disease, and metabolic/cardiovascular complications in HIV-

infected, ART-treated individuals (Chen *et al*., 2016; Pezzotti *et al*., 2009; Rai *et al*., 2012; Bhat *et al*., 2007; Jin *et al*., 2018; Darvishi *et al*., 2007; Van Der Walt *et al*., 2003; Hulgan *et al*., 2013). Furthermore, rs28358278 and rs41467651 (*MT-ND3*) were associated with gastric cancer (Jin *et al*., 2018).

For the first time, a significant association between rs28358280 (A10550G) (*MT-ND4L*) and Body Mass Index (BMI) has been identified, where the increase in G alleles leads to a higher BMI than if only A alleles were present (Flaquer *et al*., 2014).

Several studies have reported that rs2853495 is related to ulcerative colitis and pancreatic cancer among the established *MTND4* SNPs (Dankowski *et al*., 2016; Wang *et al*., 2007), and rs869096886 is associated with schizophrenia (Gonçalves *et al*., 2018), whereas rs2857285 is associated with a more invasive form of ovarian cancer (Earp *et al*., 2013). Furthermore, rs28384199 is related to late-onset encephalopathy and is considered a highly pathogenic mutation (Deschauer *et al*., 2003).

Numerous studies have shown that many males with ulcerative colitis, a principal form of Inflammatory bowel disease, are unable to control their patterns of smoking, drinking, and eating, which can contribute to sexual dysfunction and infertility (Park and Kim, 2020). Moreover, it has been repeatedly reported that the fertility of schizophrenia patients is lower than that of people with other psychiatric illnesses and the general population (Srinivasan and Padmavati, 1997).

In the current study, we scanned the polymorphisms of subfertile and fertile males by direct sequencing of the *MTND3*, *MTND4L*, and *MTND4* genes. Therefore, eight MTND3 SNPs have been identified: *MTND3*: rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277, and rs28673954 as well as seven SNPs in *MTND4L*: rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933, and rs28532881. Missense variants include rs2853826, rs28435660 (*MTND3*), and rs28532881 *(MTND4L)*. The remaining SNPs in both *MTND3* and *MTND4L* are synonymous variants.

In addition, twenty-five SNPs were identified in *MTND4* as follow: rs2853495, rs2857284, rs2853496, rs2853497, rs3087901, rs2853493, rs2853490, rs3088053, rs2853491,rs2857285, rs28358282, rs28594904, rs28669780, rs28415973, rs28471078, rs55714831, rs28358283, rs75214962, rs28529320, rs2853494, rs28609979, rs28358286, rs28359168, rs28384199, and rs869096886. The rs28594904 (Ser86Asn), rs28669780 (Leu282Met) and rs28384199

(Arg340Ser or Arg340Gly) SNPs are missense variants, whereas the rest of SNPs are synonymous coding variants.

Overall, we observed a significant association between the *MTND4* SNP rs2853495 and male infertility in the genotype frequency test. In the allele frequency test, the rs2853495 (G11719A) and rs869096886 (A11251G) in *MTND4* were also associated with male infertility, indicating that the presence of the allele itself could be associated with male infertility regardless of its genotype (**Figure 13,14**).

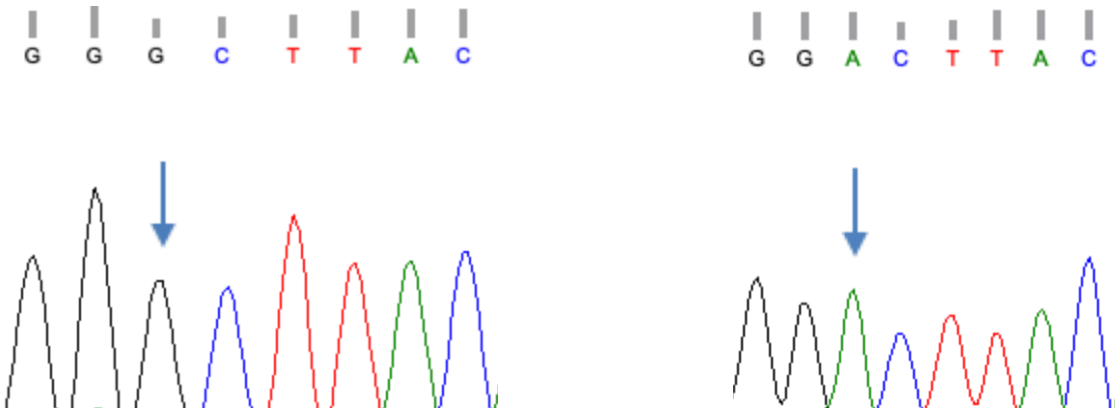



**Figure 13:** Sequencing electropherogram results (GG, AA) of the rs2853495 of *MT-ND4*.

The nucleotide transition at the position 11719 (G>A) resulted in a synonymous variant (Gly>Gly) at codon 320.

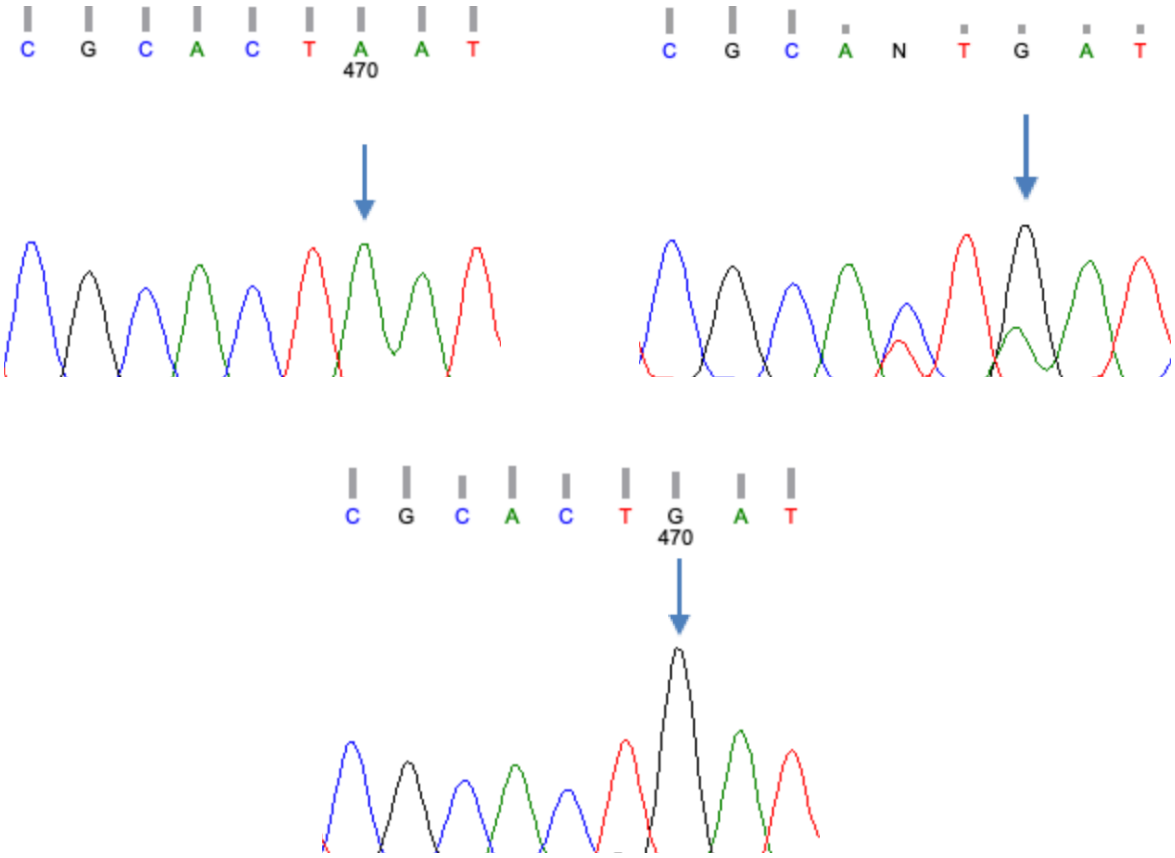



**Figure 14:** Sequencing electropherogram results (AA, AG, GG) of the rs869096886 of *MT-ND4*. The nucleotide transition at the position 11251 (A>G) resulted in a synonymous variant (Leu> Leu) at codon 164.

No significant findings have been reported so far for the *MTND3*, *MTND4L* gene polymorphisms with male infertility. Moreover, All SNPs were tested for the Hardy-Weinberg genotype frequency test. All SNPs showed a significant deviation from HWE ($P < 0.0001$), which means that the genotype distribution was not following Hardy-Weinberg and biased to one group.

Moreover, the OR of rs2853495 SNP was associated with a 2.61-times increased risk of subfertile males than fertile ones. Furthermore, the OR of rs869096886 (2.237) was also higher

for subfertile males than for control. These results demonstrated that although the rs2853495 and the rs869096886 SNPs are synonymous variants and do not cause an amino acid change, they can be related to male infertility. The rs2853495 SNP is a synonymous variant, as the codon substitution from [GG**G**] to [GG**A**] at position 11719 does not change the encoded amino acid (Glycine). Additionally, the rs869096886 is also a synonymous variant that changes from [CT**A**] to [CT**G**] at position 11251. Therefore, the amino acid remains leucine, which means that this change does not affect the resulting product protein sequence (NCBI).

In other words, increasing the number of wild-type alleles (G) (or decreasing mutant alleles (A)) at G11719A in males can help to preserve male fertility while increasing the number of G alleles (or decreasing A alleles) at A11251G can play a role in causing male infertility.

In particular, non-synonymous variations can affect the respiratory chain and the pathways of oxidative phosphorylation (OXPHOS), resulting in low ATP production and increased levels of reactive oxygen species (ROS) (Kumar *et al*., 2012).

On the other hand, there was no statistically significant association between asthenozoospermia, oligozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenoteratozoospermia, oligoteratozoospermia subgroups of subfertile males and the fertile ones.

Less is known about mitochondrial gene polymorphisms in male infertility. Therefore, we identified the genetic associations between mitochondrial polymorphisms and male infertility. Our study is the first to explore the association between *MTND3*, *MTND4L*, and *MTND4* SNPs and male infertility. Although the sample size was too small, our findings suggest that the rs2853495 and rs869096886 SNPs in *MTND4 might* be associated with male infertility. However, analysis of a larger sample is needed and will be allowed a better understanding and clarifying of the role of *MTND3*, *MTND4L*, and *MTND4* SNPs in male infertility.

## 5. CONCLUSION

In conclusion, we identified a yet unknown association between mitochondrial gene polymorphisms in *MTND3*, *MTND4L*, and *MT-ND4* and male's infertility as follows: eight SNPs in *MTND3* rs2853826, rs28435660, rs193302927, rs28358278, rs41467651, rs3899188, rs28358277 and rs28673954, seven SNPs in *MTND4L* rs28358280, rs28358281, rs28358279, rs2853487, rs2853488, rs193302933 and rs28532881, and twenty five SNPs in *MTND4* in the cases and controls: rs2853495, rs2857284, rs2853496, rs2853497, rs3087901, rs2853493, rs2853490, rs3088053, rs2853491, rs2857285, rs28358282, rs28594904, rs28669780, rs28415973, rs28471078, rs55714831, rs28358283, rs75214962, rs28529320, rs2853494, rs28609979, rs28358286, rs28359168, rs28384199, and rs869096886. In the genotype frequency test, we found a significant association between the *MT-ND4* SNP rs2853495 and male infertility. In the allele frequency test, rs2853495 and rs869096886 in the *MT-ND4* were also associated with male infertility. This indicates that mitochondrial genetics might help to give a better understanding of the correlation between the presence of these SNPs and the male's infertility. Moreover, larger prospective studies are required to confirm these associations of mitochondrial gene polymorphisms and male infertility and to clarify the definite effect of the mitochondrial genetic variations in male infertility.

## 6. REFERENCES


- Talebi, E., Karimian, M., & Nikzad, H. (2018). Association of sperm mitochondrial DNA deletions with male infertility in an Iranian population. *Mitochondrial DNA Part A*, *29*(4), 615-623.
- Kumar, D. P., & Sangeetha, N. (2009). Mitochondrial DNA mutations and male infertility. *Indian journal of human genetics*, *15*(3), 93.
- Agarwal, A., Bragais, F. M., & Sabanegh, E. (2008). Assessing sperm function. *Urologic Clinics of North America*, *35*(2), 157-171.
- Gavella, M., & Lipovac, V. (1992). Pentoxifylline‐mediated reduction of superoxide anion production by human spermatozoa. *Andrologia*, *24*(1), 37-39.
- Rajender, S., Rahul, P., & Mahdi, A. A. (2010). Mitochondria, spermatogenesis and male infertility. *Mitochondrion*, *10*(5), 419-428.
- Al Zoubi, M. S., Al‐Batayneh, K., Alsmadi, M., Rashed, M., Al‐Trad, B., Al Khateeb, W., ... & Batiha, O. (2020). 4,977‐bp human mitochondrial DNA deletion is associated with asthenozoospermic infertility in Jordan. *Andrologia*, *52*(1), e13379.
- Al Zoubi, M.S., et al., *CAG Repeats in the androgen receptor gene is associated with oligozoospermia and teratozoospermia in infertile men in Jordan.* Andrologia, 2020. **52**(9): p. e13728.
- Bahrehmand Namaghi, I. and H. Vaziri, *Sperm mitochondrial DNA deletion in Iranian infertiles with asthenozoospermia.* Andrologia, 2017. **49**(3): p. e12627.
- Kao, S.-H., H.-T. Chao, and Y.-H. Wei, *Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa.* Molecular Human Reproduction, 1998. **4**(7): p. 657-666.
- Curi, S. M., Ariagno, J. I., Chenlo, P. H., Mendeluk, G. R., Pugliese, M. N., Sardi Segovia, L. M., ... & Blanco, A. M. (2003). Asthenozoospermia: analysis of a large population. *Archives of andrology*, *49*(5), 343-349.
- Shoffner, J. M., & Wallace, D. C. (1994). Oxidative phosphorylation diseases and mitochondrial DNA mutations: diagnosis and treatment. *Annual review of nutrition*, *14*(1), 535-568.

- Shamsi, M. B., Kumar, R., Bhatt, A., Bamezai, R. N. K., Kumar, R., Gupta, N. P., ... & Dada, R. (2008). Mitochondrial DNA mutations in etiopathogenesis of male infertility. *Indian journal of urology: IJU: journal of the Urological Society of India*, *24*(2), 150.
- Baklouti-Gargouri, S., Ghorbel, M., Mahmoud, A. B., Mkaouar-Rebai, E., Cherif, M., Chakroun, N., ... & Ammar-Keskes, L. (2013). Mitochondrial DNA mutations and polymorphisms in asthenospermic infertile men. *Molecular biology reports*, *40*(8), 4705-4712.
- Spiropoulos, J., Turnbull, D. M., & Chinnery, P. F. (2002). Can mitochondrial DNA mutations cause sperm dysfunction?. *MHR: Basic science of reproductive medicine*, *8*(8), 719-721.
- Nakada, K., Sato, A., Yoshida, K., Morita, T., Tanaka, H., Inoue, S. I., ... & Hayashi, J. I. (2006). Mitochondria-related male infertility. *Proceedings of the National Academy of Sciences*, *103*(41), 15148-15153.
- Guo, Z. S., Jin, C. L., Yao, Z. J., Wang, Y. M., & Xu, B. T. (2017). Analysis of the mitochondrial 4977 Bp deletion in patients with hepatocellular carcinoma. *Balkan Journal of Medical Genetics*, *20*(1), 81-86.
- Shoffner, J. M., Lott, M. T., Voljavec, A. S., Soueidan, S. A., Costigan, D. A., & Wallace, D. C. (1989). Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. *Proceedings of the National Academy of Sciences*, *86*(20), 7952-7956.
- St John, J. C., Jokhi, R. P., & Barratt, C. L. (2001). Men with oligoasthenoteratozoospermia harbour higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology. *Molecular human reproduction*, *7*(1), 103-111.
- Kao, S. H., Chao, H. T., & Wei, Y. H. (1995). Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biology of reproduction*, *52*(4), 729-736.
- Jenkins, T. G., Aston, K. I., Hotaling, J. M., Shamsi, M. B., Simon, L., & Carrell, D. T. (2016). Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. *Andrology*, *4*(5), 843-849.
- Wang, J., & Lü, Y. Y. (2009). Mitochondrial DNA 4977-bp deletion correlated with reactive oxygen species production and manganese superoxidedismutase expression in gastric tumor cells. *Chinese medical journal*, *122*(4), 431-436.

- Smeitink, J., van den Heuvel, L., & DiMauro, S. (2001). The genetics and pathology of oxidative phosphorylation. *Nature Reviews Genetics*, *2*(5), 342.
- Kao, S. H., Chao, H. T., & Wei, Y. H. (1998). Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Molecular Human Reproduction*, *4*(7), 657-666.
- Garrido, N., Meseguer, M., Simon, C., Pellicer, A., & Remohi, J. (2004). Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian journal of andrology*, *6*(1), 59-66.
- Amann, R. P., & Howards, S. S. (1980). Daily spermatozoal production and epididymal spermatozoal reserves of the human male. *The Journal of urology*, *124*(2), 211-215.
- 23. de Kretser, D. M., Loveland, K. L., Meinhardt, A., Simorangkir, D., & Wreford, N. (1998). Spermatogenesis. *Human reproduction*, *13*(suppl_1), 1-8.
- Esposito, G., Jaiswal, B. S., Xie, F., Krajnc-Franken, M. A., Robben, T. J., Strik, A. M., ... & Gossen, J. A. (2004). Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proceedings of the National Academy of Sciences*, *101*(9), 2993-2998.
- World Health Organization (WHO) (2010). *Laboratory manual for the examination and processing of human semen*, 5th ed. Geneva, Switzerland: WHO Press.
- Franklin, Sarah. 1997. Embodied Progress: A Cultural Account of Assisted Conception. New York: Routledge.
- Franklin, Sarah, and Celia Roberts. 2006. Born and Made: An Ethnography of Preimplantation Genetic Diagnosis. Princeton: Princeton University Press.
- Duran, E. H., Morshedi, M., Taylor, S., & Oehninger, S. (2002). Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Human Reproduction, 17(12), 3122-3128.
- Allahbadia, G. N. (2017). Intrauterine insemination: Fundamentals revisited. *The Journal of Obstetrics and Gynecology of India*, *67*(6), 385-392.
- de Kretser DM. Male infertility. Lancet 1997;349: 787–90.
- Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G, Foresta C. Male infertility: role of genetic background. Reprod Biomed Online 2007;14: 734–45.

- Alshahrani, S., Agarwal, A., Assidi, M., Abuzenadah, A. M., Durairajanayagam, D., Ayaz, A., ... & Sabanegh, E. (2014). Infertile men older than 40 years are at higher risk of sperm DNA damage. *Reproductive Biology and Endocrinology*, *12*(1), 103.
- Belloc, S., Hazout, A., Zini, A., Merviel, P., Cabry, R., Chahine, H., ... & Benkhalifa, M. (2014). How to overcome male infertility after 40: Influence of paternal age on fertility. *Maturitas*, *78*(1), 22-29.
- Moyes CD, Battersby BJ, Leary SC. Regulation of muscle mitochondrial design. J Exp Biol. 1998;201:299–307.
- St John, J. C., Sakkas, D., & Barratt, C. L. (2000). A role for mitochondrial DNA and sperm survival. *Journal of andrology*, *21*(2), 189-99.
- Slater EC. Measurement and importance of phosphorylation potentials: calculation of free energy of hydrolysis in cells. Methods Enzymol. 1979;55:235–245.
- Brown, M. D., & Wallace, D. C. (1994). Molecular basis of mitochondrial DNA disease. *Journal of bioenergetics and biomembranes*, *26*(3), 273-289.
- Storey, B. T. (1980). Strategy of oxidative metabolism in bull spermatozoa. *Journal of Experimental Zoology*, *212*(1), 61-67.
- Anderson S, Bankier AT, Barrell BG, de Brujin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. Nature. 1981;290:457–465.
- Fryer A, Appleton R, Sweeney MG, Rosenbloom L, Harding AE. Mi- tochondrial DNA 8993 (NARP) mutation presenting with a hetero- geneous phenotype including cerebral palsy. Arch Dis Child. 1994; 71:419 – 422.
- Shih K-D, Yen TC, Pang C-Y, Wei Y-H. Mitochondrial DNA mutation in a Chinese family with myoclonic epilepsy and ragged-red fibre disease. Biochem Biophys Res Comm. 1991;174:1109–1116.
- Brown MD, Voljavec AS, Lott MT, Torroni A, Yang CC, Wallace DC. Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. Genetics. 1992;130:163–173.
- Schon EA, Rizzuto R, Moraes CT, Nakase H, Zeviani M, DiMauro S. A direct repeat is a hotspot for large-scale deletion of human mitochon- drial DNA. Science. 1989;244:346–349.

- Zeviani M, Servidei S, Gellera C, Bertini E, DiMauro S, DiDonato S. An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. Nature. 1989;339:309–311.
- Zhang C, Baumer A, Maxwell RJ, Linnane AW, Nagley P. Multiple mi- tochondrial DNA deletions in an elderly human individual. FEBS Lett. 1992;297:34 –38.
- Folgero, T., Bertheussen, K., Lindal, S., Torbergsen, T., Oian, P. (1993) Mitochondrial disease and reduced sperm moti- lity. Hum Reprod 8(11): 1863–1868.
- Lestienne, P., Reynier, P., Chretien, M.F., Penisson-Besnier, I., Malthiery, Y., Rohmer, V. (1997) Oligoasthenospermia associated with multiple mitochondrial DNA rearrange- ments. Mol Hum Reprod 3(9): 811–814.
- Ferramosca, A., Focarelli, R., Piomboni, P., Coppola, L., Zara, V. (2008) Oxygen uptake by mitochondria in demembra- nated human spermatozoa: a reliable tool for the evalua- tion of sperm respiratory efficiency. Int J Androl 31(3): 337–345.
- Ferramosca, A., Zara, V. (2014) Bioenergetics of Mammalian Sperm Capacitation. Biomed Res Int 2014: 902953.
- Holyoake, A.J., McHugh, P., Wu, M., O'Carroll, S., Benny, P., Sin, I.L., et al. (2001) High incidence of single nucleotide substitutions in the mitochondrial genome is associated with poor semen parameters in men. Int J Androl 24(3): 175–182.
- Rani, D.S., Vanniarajan, A., Gupta, N.J., Chakravarty, B., Singh, L., Thangaraj, K. (2006) A novel missense mutation C11994T in the mitochondrial ND4 gene as a cause of low sperm motility in the Indian subcontinent. Fertil Steril 86 (6): 1783–1785.
- Yagi, T., Matsuno-Yagi, A. (2003) The proton-translocating NADH- quinoneoxidoreductase in the respiratory chain: the secret unlocked. Biochemistry 42(8): 2266–2274.
- Hirst, J., Carroll, J., Fearnley, I.M., Shannon, R.J., Walker, J.E. (2003) The nuclearencoded subunits of complex I from bovine heart mitochondria. Biochim Biophys Acta 1604 (3): 135–150.
- Sharma, L. K., Lu, J., & Bai, Y. (2009). Mitochondrial respiratory complex I: structure, function and implication in human diseases. *Current medicinal chemistry*, *16*(10), 1266-1277.
- Thangaraj K., Joshi M. B., Reddy A. A., and Singh L. (2003). Sperm mitochondrial mutations as a cause of low sperm motility. Journal of Andrology. 24. 388-392.
- Darley-Usmar, V. M. (1994). The proteins of the mitochondrial inner membrane and their role in oxidative phosphorylation. *Mitochondria: DNA, proteins and disease*, 1-27.

- Cummins J. M., Jaquier A. M., Martin R., Mehmet D., Goldblatt J. (1998). Semen levels of mitochondrial DNA deletions in men attending an infertility clinic do not correlate with phenotype. International Journal of Andrology. 21(1): 47-52.
- Shanske A. L, Shanske S. and DiMauro S. (2001). The other human genome. Archives of Pediatrics and Adolescent Medicine. 155: 1210- 1217.
- DiMauro S and Schon E. A. (2001). Mitochondrial DNA mutations in Human disease. American Journal of Medical Genetics (Semin. Med. Genet.) 106: 18-26.
- DiMauro S and Schon, E. A. (2003). Mitochondrial respiratory chain disease. The New England Journal of Medicine. 348: 2656-2668.
- Chomyn A, Cleeter MW, Ragan CI, Riley M, Doolittle RF, Attardi G. URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. Science. 1986; 234: 614-8.
- Chomyn, A., Cleeter, W. J., Ragan, C. I., Riley, M., Doolittle, R. F., Attardi, G. URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. Science 234: 614-618, 1986.
- Hung WY, Wu CW, Yin PH, et al. Somatic mutations in mitochondrial genome and their potential roles in the progression of human gastric cancer. Biochim Biophys Acta. 2010; 1800: 264-70.
- van der Walt JM, Nicodemus KK, Martin ER, et al. Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease. Am J Hum Genet. 2003; 72: 804-811.
- Pezzotti A, Kraft P, Hankinson SE, Hunter DJ, Buring J, Cox DG. The mitochondrial A10398G polymorphism, interaction with alcohol consumption, and breast cancer risk. PLoS One. 2009; 4: e5356.
- Valdivieso, Á. G., Marcucci, F., Taminelli, G., Guerrico, A. G., Álvarez, S., Teiber, M. L., ... & Santa-Coloma, T. A. (2007). The expression of the mitochondrial gene MT-ND4 is downregulated in cystic fibrosis. *Biochemical and biophysical research communications*, *356*(3), 805-809.
- Voet, D., Voet, J. G., & Pratt, C. W. (2016). *Fundamentals of biochemistry: life at the molecular level*. John Wiley & Sons.
- Gurses, C., Azakli, H., Alptekin, A., Cakiris, A., Abaci, N., Arikan, M., ... & Ustek, D. (2014). Mitochondrial DNA profiling via genomic analysis in mesial temporal lobe epilepsy patients with hippocampal sclerosis. *Gene*, *538*(2), 323-327.

- Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M., ... & Nikoskelainen, E. K. (1988). Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*, *242*(4884), 1427-1430.
- Restrepo, N. A., Mitchell, S. L., Goodloe, R. J., Murdock, D. G., HAINES, J. L., & Crawford, D. C. (2014). Mitochondrial variation and the risk of age-related macular degeneration across diverse populations. In *Pacific Symposium on Biocomputing Co-Chairs* (pp. 243-254).
- Rovio, A. T., Marchington, D. R., Donat, S., Schuppe, H. C., Abel, J., Fritsche, E., ... & Harrison, R. F. (2001). Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility. *Nature Genetics*, *29*(3), 261-262.
- Zong, N. C., Li, H., Li, H., Lam, M. P., Jimenez, R. C., Kim, C. S., ... & Liem, D. (2013). Integration of cardiac proteome biology and medicine by a specialized knowledgebase. *Circulation research*, *113*(9), 1043-1053.
- Cwerman-Thibault, H., Augustin, S., Lechauve, C., Ayache, J., Ellouze, S., Sahel, J. A., & Corral-Debrinski, M. (2015). Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss. *Molecular Therapy-Methods & Clinical Development*, *2*, 15003.
- Achilli, A., Iommarini, L., Olivieri, A., Pala, M., Kashani, B. H., Reynier, P., ... & Barboni, P. (2012). Rare primary mitochondrial DNA mutations and probable synergistic variants in Leber's hereditary optic neuropathy. *PLoS One*, *7*(8), e42242.
- Singh A. R., Vrtel R., Vodicka. R., Dhaifalah I., Konovalinka D. and Santavy J. (2006). Genetic factors in male infertility and their implications. International journal of human genetics. 6(2): 163-169.
- Seli, E., & Sakkas, D. (2005). Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Human reproduction update*, *11*(4), 337-349.
- Tahmasbpour, E., Balasubramanian, D., & Agarwal, A. (2014). A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART). *Journal of assisted reproduction and genetics*, *31*(9), 1115-1137.
- O'Brien, K. L. F., Varghese, A. C., & Agarwal, A. (2010). The genetic causes of male factor infertility: a review. *Fertility and sterility*, *93*(1), 1-12.
- Jequier, A. M., Ansell, I. D., & Bullimore, N. J. (1985). Congenital absence of the vasa deferentia presenting with infertility. *Journal of Andrology*, *6*(1), 15-19.

- Weiske, W. H., Sälzler, N., Schroeder−Printzen, I., & Weidner, W. (2000). Clinical findings in congenital absence of the vasa deferentia. *Andrologia*, *32*(1), 13-18.
- Brinton-Jones C, Haines C.J. (2000). Microdeletions on the long arm of the Y chromosome and their association with male-factor infertility. Hong Kong Medical Journal. 6:184-9.
- Erasmuson T., Sin, I. L. and Sin F. Y. T. (2003) Absence of association of androgen receptor trinucleotide expansion and poor semen quality. International Journal of Andrology. 26, 46-51.
- Vogt P. H. (1996). Molecular basis of male (in)fertility. Advances in Andrology. ESHRE Campus Course, March, 1996, Freiburgi. Britain. International Journal Of Andrology., 20(3): 2-10.
- Hargreave T. B. (2000). Genetic basis of male infertility. British Medical Bulletin. 56. 650-671.
- Moore F. L. and Reijo-Pera R. A. (2000). Male sperm motility dictated by mother's mtDNA. American Journal of Human Genetics. 67: 543- 548.
- Tabong, P. T. N., & Adongo, P. B. (2013). Infertility and childlessness: a qualitative study of the experiences of infertile couples in Northern Ghana. BMC pregnancy and childbirth, 13(1), 72.
- Lash, M. M., Yaghamee, A., Strohsnitter, W., & Lalwani, S. (2008). Association between secondary infertility and fallopian tube obstruction on hysterosalpingography. The Journal of reproductive medicine, 53(9), 677.
- Kazerooni, T., & Dehghan-Kooshkghazi, M. (2003). Effects of metformin therapy on hyperandrogenism in women with polycystic ovarian syndrome. Gynecological endocrinology, 17(1), 51-56.
- Shen, S. Y., Huang, S. Y., Hsieh, C. H., Hsu, M. I., Cheng, C. Y., & Hsu, C. S. (2014). Clinical and biochemical characteristics of women with menstrual disturbance. Taiwanese Journal of Obstetrics and Gynecology, 53(2), 178-182.
- Goundry, A. L. R., Finlay, E. R., & Llewellyn, C. D. (2013). Talking about links between sexually transmitted infections and infertility with college and university students from SE England, UK: a qualitative study. Reproductive Health, 10(1), 47.
- Wang, P. H., Su, W. H., Sheu, B. C., & Liu, W. M. (2009). Adenomyosis and its variance: adenomyoma and female fertility. Taiwanese Journal of Obstetrics and Gynecology, 48(3), 232-238.

- Wallach, E. E., Lavy, G., Diamond, M. P., & DeCherney, A. H. (1987). Ectopic pregnancy: its relationship to tubal reconstructive surgery. Fertility and sterility, 47(4), 543-556.
- Celik, N. Y., & Mulayim, B. (2012). A mullerian anomaly ‘‘without classification’’: Septate uterus with double cervix and longitudinal vaginal septum. Taiwanese Journal of Obstetrics and Gynecology, 51(4), 649-650.
- Umdagas, H. A., Kawuwa, B. M., Hajara, U. S., & Mohammed, S. (2006). Prevalence of uterine synechiae among infertile females in a Nigerian teaching hospital. Journal of obstetrics and gynaecology, 26(4), 351-352.
- Codner, E., Merino, P. M., & Tena-Sempere, M. (2012). Female reproduction and type 1 diabetes: from mechanisms to clinical findings. *Human Reproduction Update*, *18*(5), 568-585.
- Menif, O., Omar, S., Feki, M., & Kaabachi, N. (2008, January). Hypothyroidism and pregnancy: impact on mother and child health. In *Annales de Biologie Clinique* (Vol. 66, No. 1, pp. 43-51).
- Gaskins, A. J., Rich-Edwards, J. W., Lawson, C. C., Schernhammer, E. S., Missmer, S. A., & Chavarro, J. E. (2015). Work schedule and physical factors in relation to fecundity in nurses. *Occupational and environmental medicine*, *72*(11), 777-783.
- Jóźków, P., & Mędraś, M. (2012). Psychological stress and the function of male gonads. *Endokrynologia Polska*, *63*(1), 44-49.
- Dechanet, C., Anahory, T., Mathieu Daude, J. C., Quantin, X., Reyftmann, L., Hamamah, S., ... & Déchaud, H. (2011). Effects of cigarette smoking on reproduction. *Human reproduction update*, *17*(1), 76-95.
- Künzle, R., Mueller, M. D., Hänggi, W., Birkhäuser, M. H., Drescher, H., & Bersinger, N. A. (2003). Semen quality of male smokers and nonsmokers in infertile couples. *Fertility and sterility*, *79*(2), 287-291.
- Huisman, M., Kunst, A. E., & Mackenbach, J. P. (2005). Educational inequalities in smoking among men and women aged 16 years and older in 11 European countries. *Tobacco control*, *14*(2), 106-113.
- Centers for Disease Control and Prevention (CDC. (2008). Smoking prevalence among women of reproductive age--United States, 2006. *MMWR. Morbidity and mortality weekly report*, *57*(31), 849.

- Curtis, K. M., Savitz, D. A., & Arbuckle, T. E. (1997). Effects of cigarette smoking, caffeine consumption, and alcohol intake on fecundability. *American journal of epidemiology*, *146*(1), 32-41.
- Agarwal, A., Ahmad, G., & Sharma, R. (2015). Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. *Journal of assisted reproduction and genetics*, *32*(12), 1721-1729.
- Snustad D. P. and Simmons M. J. (2000). Principles of genetics. Second edition. *John Wiley and sons, Inc. NY.*
- Kruger, T. F., DuToit, T. C., Franken, D. R., Acosta, A. A., Oehninger, S. C., Menkveld, R., & Lombard, C. J. (1993). A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. *Fertility and sterility*, *59*(1), 202-209.
- Dajanı, M. N. (2016). Do Infections Cause Oligospermia and Could Empiric Antibiotics Antiprotozoal and Antifungal Treat Oligospermia?. *Gynecology Obstetrics & Reproductive Medicine*, *20*(1), 34-37.
- *Homo sapiens* mitochondrion, complete genome. "Revised Cambridge Reference Sequence (rCRS): accession NC_012920", *National Center for Biotechnology Information*. Retrieved on 30 January 2016.
- Ieremiadou, F., & Rodakis, G. C. (2009). Correlation of the 4977 bp mitochondrial DNA deletion with human sperm dysfunction. *BMC research notes*, *2*(1), 18.
- Niaudet, P. (1998). Mitochondrial disorders and the kidney. *Archives of disease in childhood*, *78*(4), 387-390.
- Chen, S., Gan, S. R., Cai, P. P., Ni, W., Zhou, Q., Dong, Y., ... & Wu, Z. Y. (2016). Mitochondrial NADH dehydrogenase subunit 3 polymorphism associated with an earlier age at onset in male Machado–Joseph disease patients. *CNS neuroscience & therapeutics*, *22*(1), 38-42.
- Mohideen, A. M. S. H., Dicks, E., Parfrey, P., Green, R., & Savas, S. (2015). Mitochondrial DNA polymorphisms, its copy number change and outcome in colorectal cancer. *BMC research notes*, *8*(1), 272.
- Blein, S., Berndt, S., Joshi, A. D., Campa, D., Ziegler, R. G., Riboli, E., ... & Diver, W. R. (2014). Factors associated with oxidative stress and cancer risk in the Breast and Prostate Cancer Cohort Consortium. *Free radical research*, *48*(3), 380-386.

- Rai, E., Sharma, S., Kaul, S., Jain, K., Matharoo, K., Bhanwer, A. S., & Bamezai, R. N. (2012). The interactive effect of SIRT1 promoter region polymorphism on type 2 diabetes susceptibility in the North Indian population. *PLoS One*, *7*(11), e48621.
- Bhat, A., Koul, A., Sharma, S., Rai, E., Bukhari, S. I. A., Dhar, M. K., & Bamezai, R. N. K. (2007). The possible role of 10398A and 16189C mtDNA variants in providing susceptibility toT2DM in two North Indian populations: a replicative study. *Human genetics*, *120*(6), 821-826.
- Jin, E. H., Sung, J. K., Lee, S. I., & Hong, J. H. (2018). Mitochondrial NADH Dehydrogenase Subunit 3 (MTND3) Polymorphisms are Associated with Gastric Cancer Susceptibility. *International Journal of Medical Sciences*, *15*(12), 1329.
- Hulgan, T., Stein, J. H., Cotter, B. R., Murdock, D. G., Ritchie, M. D., Dube, M. P., ... & Torriani, for the AIDS Clinical Trials Group A5152s and DACS 252 Study Teams, F. J. (2013). Mitochondrial DNA variation and changes in adiponectin and endothelial function in HIV-infected adults after antiretroviral therapy initiation. *AIDS research and human retroviruses*, *29*(10), 1293-1299.
- Kumar, M., Kaur, P., Kumar, M., Saxena, R., Sharma, P., & Dada, R. (2012). Clinical characterization and mitochondrial DNA sequence variations in Leber hereditary optic neuropathy. *Molecular vision*, *18*, 2687.
- Flaquer, A., Baumbach, C., Kriebel, J., Meitinger, T., Peters, A., Waldenberger, M., ... & Strauch, K. (2014). Mitochondrial genetic variants identified to be associated with BMI in adults. *PloS one*, *9*(8), e105116.
- Pezzotti, A., Kraft, P., Hankinson, S. E., Hunter, D. J., Buring, J., & Cox, D. G. (2009). The mitochondrial A10398G polymorphism, interaction with alcohol consumption, and breast cancer risk. *PloS one*, *4*(4), e5356.
- Darvishi, K., Sharma, S., Bhat, A. K., Rai, E., & Bamezai, R. N. K. (2007). Mitochondrial DNA G10398A polymorphism imparts maternal Haplogroup N a risk for breast and esophageal cancer. *Cancer letters*, *249*(2), 249-255.
- Koopman, W. J., Nijtmans, L. G., Dieteren, C. E., Roestenberg, P., Valsecchi, F., Smeitink, J. A., & Willems, P. H. (2010). Mammalian mitochondrial complex I: biogenesis, regulation, and reactive oxygen species generation. *Antioxidants & redox signaling*, *12*(12), 1431-1470.
- Park YE, Kim TO. Sexual dysfunction and fertility problems in men with inflammatory bowel disease. World J Mens Health. 2020;38(3), 285.

- Srinivasan TN, Padmavati R. Fertility and schizophrenia: Evidence for increased fertility in the relatives of schizophrenic patients. Acta Psychiatr Scand. 1997;96(4), 260-264.
- Dankowski T, Schröder T, Möller S, Yu X, Ellinghaus D, Bär F, Fellermann K, Lehnert H, Schreiber S, Franke A SC. Male-specific association between MT-ND4 11719 A/G polymorphism and ulcerative colitis: a mitochondria-wide genetic association study. BMC Gastroenterol. 2016;16(1), 118.
- Wang L, Bamlet WR, De Andrade M, Boardman LA, Cunningham JM, Thibodeau SN, et al. Mitochondrial genetic polymorphisms and pancreatic cancer risk. Cancer Epidemiol Biomarkers Prev. 2007;16(7), 1455-1459.
- Gonçalves VF, Giamberardino SN, Crowley JJ, Vawter MP, Saxena R, Bulik CM, et al. Examining the role of common and rare mitochondrial variants in schizophrenia. PLoS One. 2018;13(1), e0191153.
- Earp MA, Brooks-Wilson A, Cook L, Le N. Inherited common variants in mitochondrial DNA and invasive serous epithelial ovarian cancer risk. BMC Res Notes. 2013;6(1), 425.
- Deschauer M, Bamberg C, Claus D, Zierz S, Turnbull DM, Taylor RW. Late-onset encephalopathy associated with a C11777A mutation of mitochondrial DNA. Neurology. 2003;60(8), 1357-1359.
- Cabrera-Orefice, A., Chiquete-Félix, N., Espinasa-Jaramillo, J., Rosas-Lemus, M., Guerrero-Castillo, S., Peña, A., & Uribe-Carvajal, S. (2014). The branched mitochondrial respiratory chain from Debaryomyces hansenii: components and supramolecular organization. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1837*(1), 73-84.
- Chinnery, P. F., & Schon, E. A. (2003). Mitochondria. *Journal of Neurology, Neurosurgery & Psychiatry*, *74*(9), 1188-1199.
- Khan, A. U. H., Rathore, M. G., Allende-Vega, N., Vo, D. N., Belkhala, S., Orecchioni, S., ... & Villalba, M. (2016). Human leukemic cells performing oxidative phosphorylation (OXPHOS) generate an antioxidant response independently of reactive oxygen species (ROS) production. *EBioMedicine*, *3*, 43-53.
- Garone, C., Minczuk, M., & D'Souza, A. R. (2018). Mitochondrial transcription and translation: overview. *Essays in biochemistry*, *62*(3), 309-320.
- Shokolenko, I. N., & Alexeyev, M. F. (2017). Mitochondrial transcription in mammalian cells. *Frontiers in bioscience (Landmark edition)*, *22*, 835.

- Hillen, H. S., Temiakov, D., & Cramer, P. (2018). Structural basis of mitochondrial transcription. *Nature structural & molecular biology*, *25*(9), 754-765.
- Hillen, H. S., Morozov, Y. I., Sarfallah, A., Temiakov, D., & Cramer, P. (2017). Structural basis of mitochondrial transcription initiation. *Cell*, *171*(5), 1072-1081.
- Aibara, S., Singh, V., Modelska, A., & Amunts, A. (2020). Structural basis of mitochondrial translation. *Elife*, *9*, e58362.
- Smits, P., Smeitink, J., & van den Heuvel, L. (2010). Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. *Journal of Biomedicine and Biotechnology*, *2010*.
- Sato, M., & Sato, K. (2012). Maternal inheritance of mitochondrial DNA: degradation of paternal mitochondria by allogeneic organelle autophagy, allophagy. *Autophagy*, *8*(3), 424-425.
- Sato, M., & Sato, K. (2013). Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1833*(8), 1979-1984.
- Wei, W., & Chinnery, P. F. (2020). Inheritance of mitochondrial DNA in humans: implications for rare and common diseases. *Journal of Internal Medicine*, *287*(6), 634-644.
- Schon, E. A., DiMauro, S., & Hirano, M. (2012). Human mitochondrial DNA: roles of inherited and somatic mutations. *Nature Reviews Genetics*, *13*(12), 878-890.

# 7. APPENDICES

**APPENDIX 1:** Supplementary figures for Chapter **2.4**. Sequencing results of all detected SNPs.

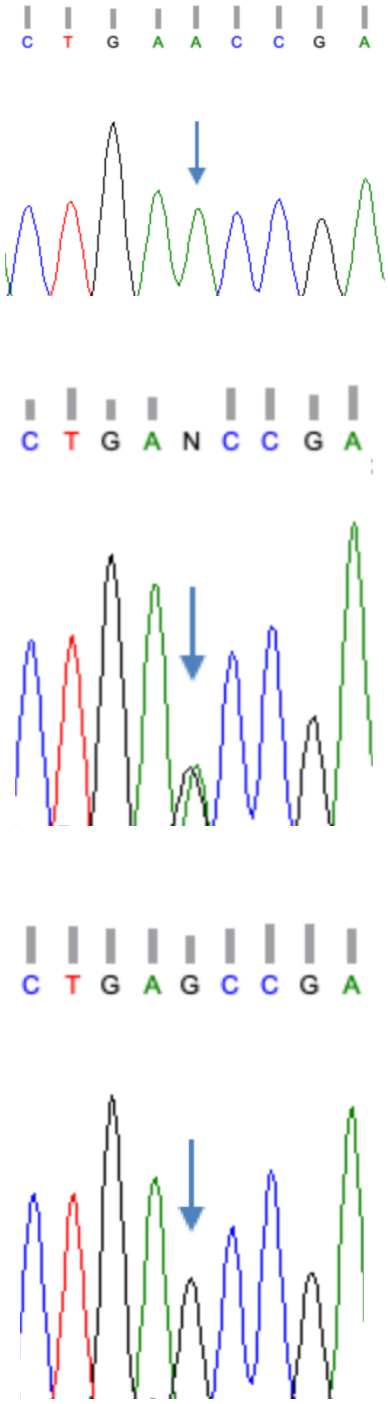



**Figure 15:** Sequencing electropherogram results (AA, AG, GG) of the rs2853826 of *MT-ND3*. The nucleotide transition at the position 10398 (A>G) resulted in a missense variant (Thr>Ala) at codon 114.

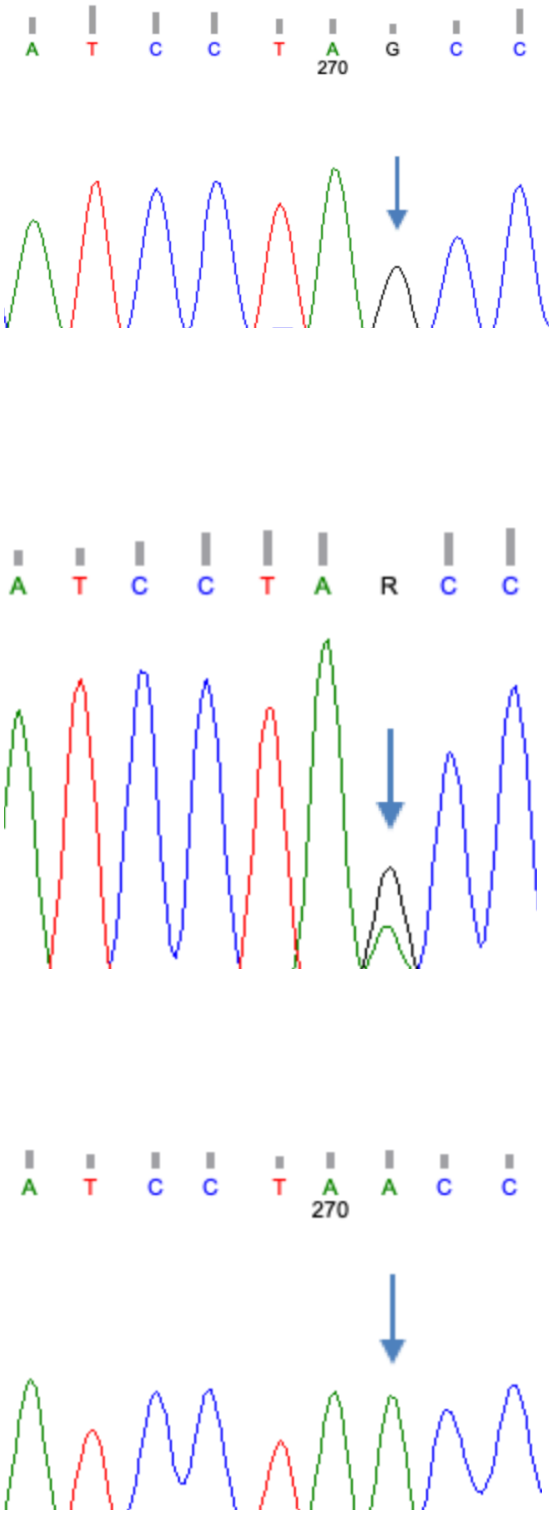



**Figure 16:** Sequencing electropherogram results (GG, GA, AA) of the rs28435660 of *MT-ND3*. The nucleotide transition at the position 10353 (G>A) resulted in a missense variant (Ala>Thr) at codon 99.

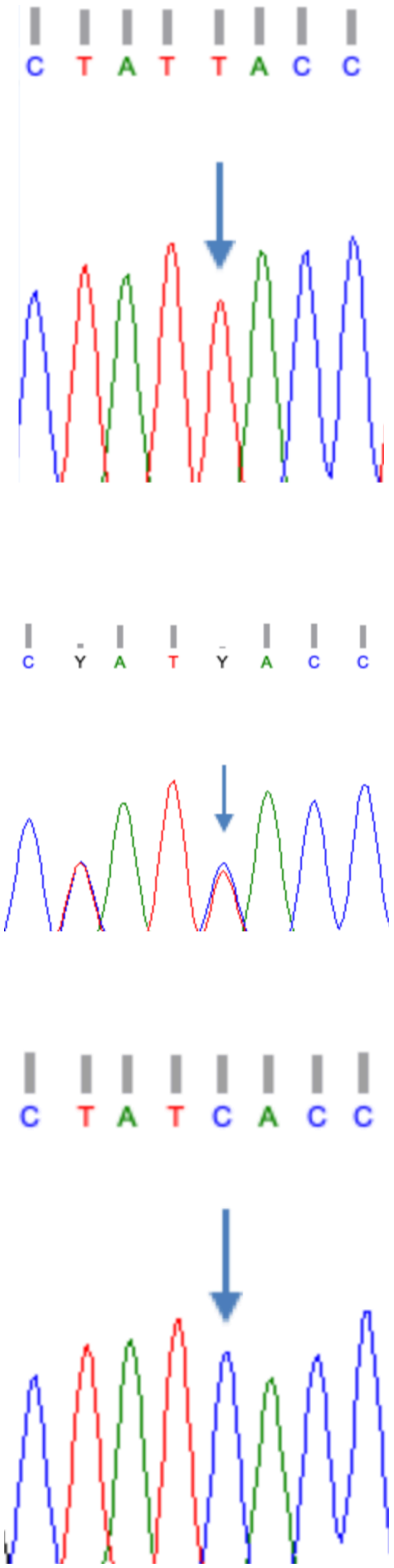



**Figure 17:** Sequencing electropherogram results (TT, TC, CC) of the rs193302927 of *MT-ND3*. The nucleotide transition at the position 10238 (T>C) resulted in a synonymous variant (Ile>Ile) at codon 60.

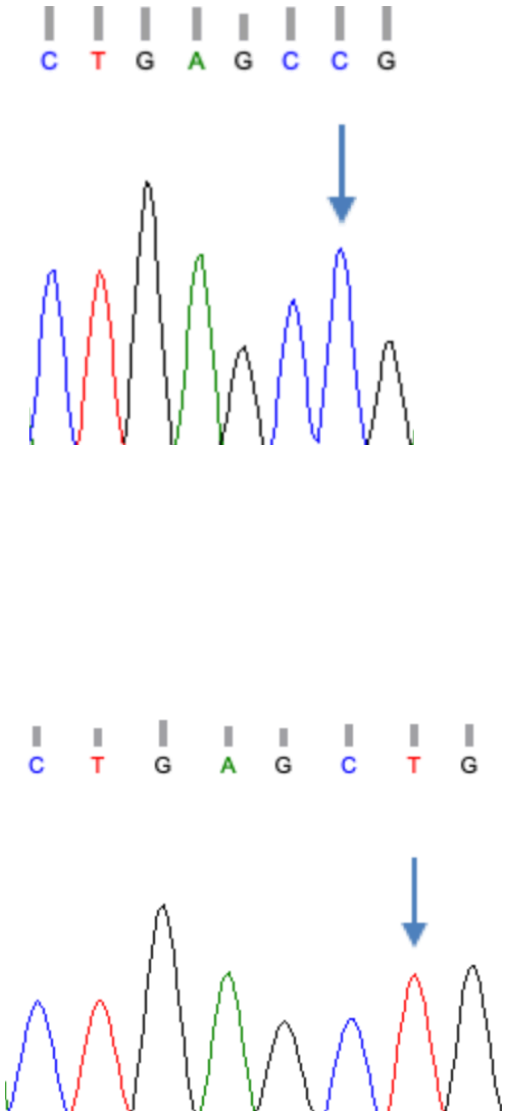



**Figure 18:** Sequencing electropherogram results (CC, TT) of the rs28358278 of *MT-ND3*.
The nucleotide transition at the position 10400 (C>T) resulted in a synonymous variant (Thr>Thr) at codon 114.

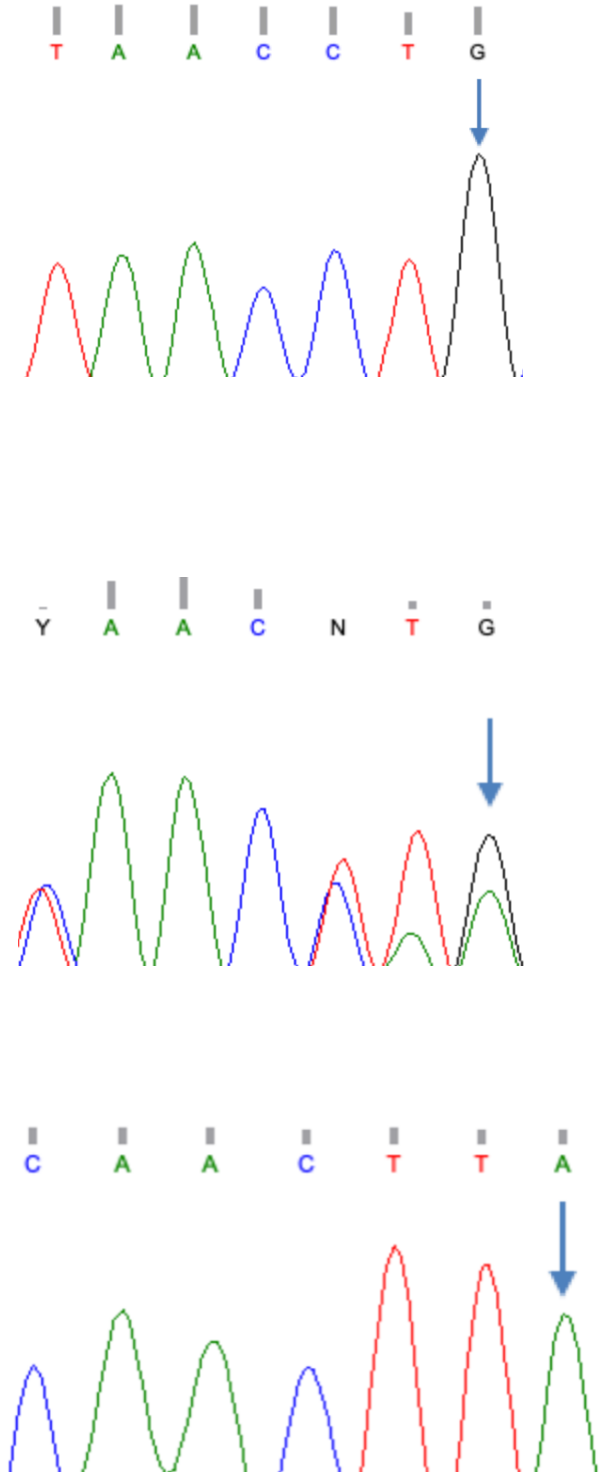



**Figure 19:** Sequencing electropherogram results (GG, GA, AA) of the rs41467651 of *MT-ND3*. The nucleotide transition at the position 10310 (G>A) resulted in a synonymous variant (Leu>Leu) at codon 84.

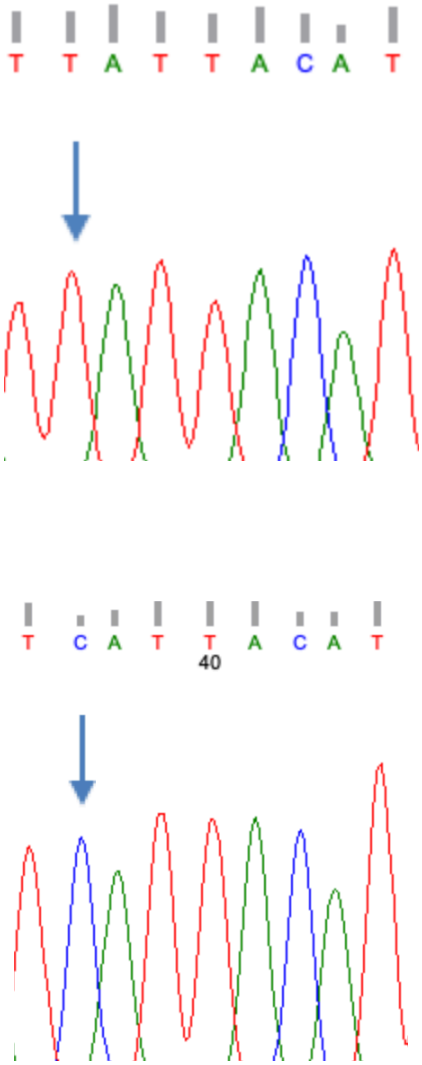



**Figure 20:** Sequencing electropherogram results (TT, CC) of the rs3899188 of *MT-ND3*.
The nucleotide transition at the position 10115 (T>C) resulted in a synonymous variant (Ile>Ile) at codon 19.

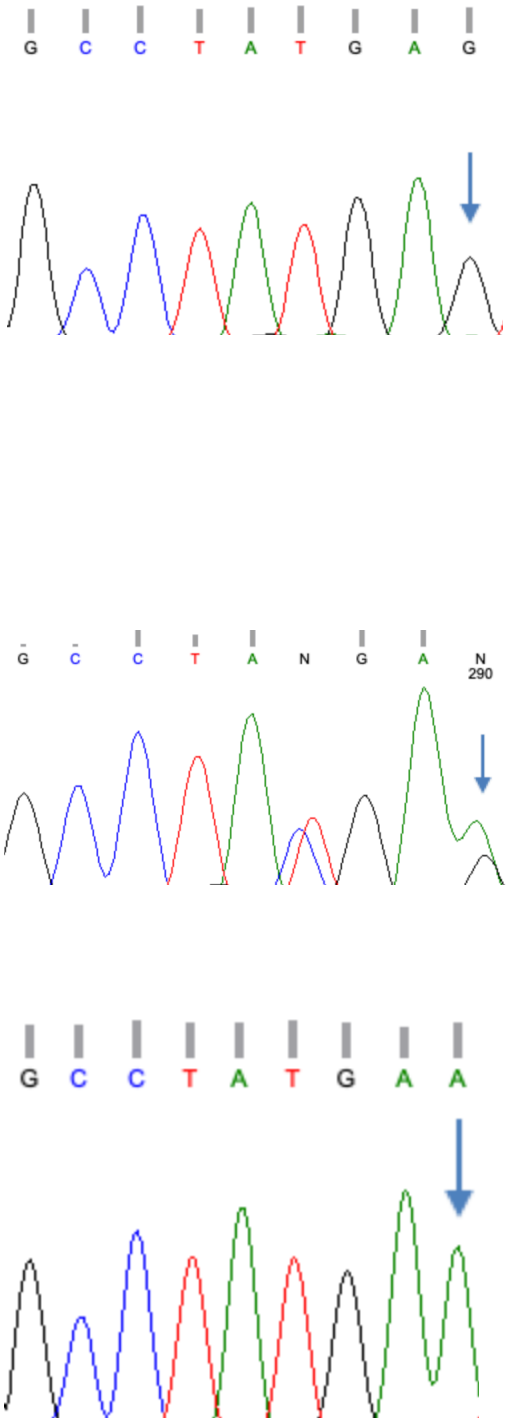



**Figure 21:** Sequencing electropherogram results (GG, GA, AA) of the rs28358277 of *MT-ND3*. The nucleotide transition at the position 10373 (G>A) resulted in a synonymous variant (Glu>Glu) at codon 105.

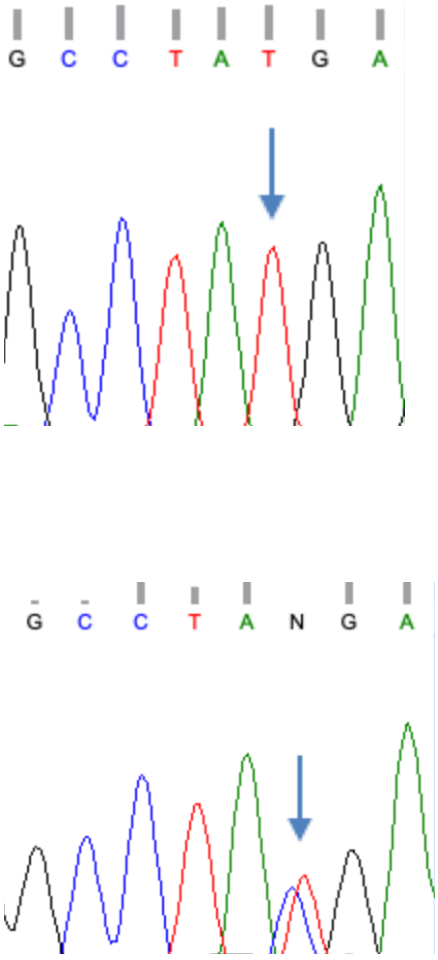



**Figure 22:** Sequencing electropherogram results (TT, TC) of the rs28673954 of *MT-ND3*.

The nucleotide transition at the position 10370 (T>C) resulted in a synonymous variant (Tyr>Tyr) at codon 104.

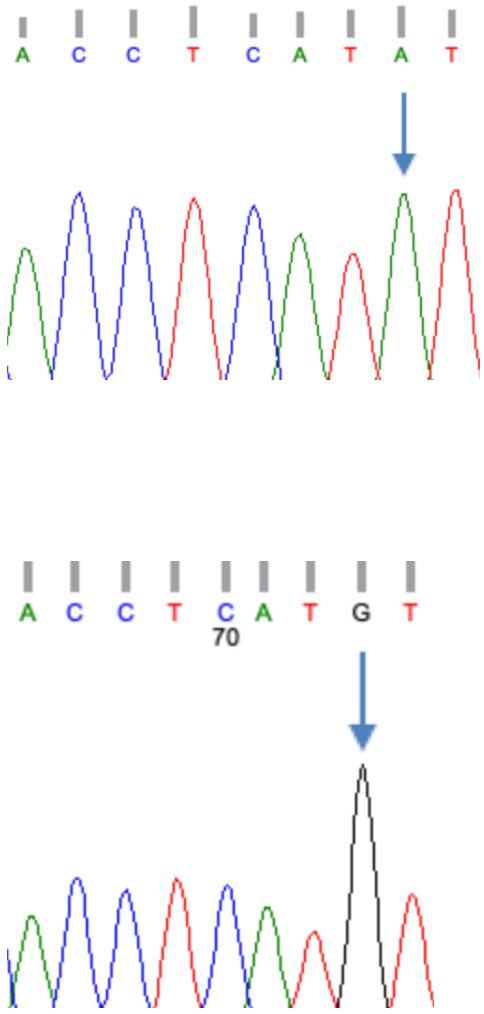



**Figure 23:** Sequencing electropherogram results (AA, GG) of the rs28358280 of *MT-ND4L*.

The nucleotide transition at the position 10550 (A>G) resulted in a synonymous variant (Met>Met) at codon 27.

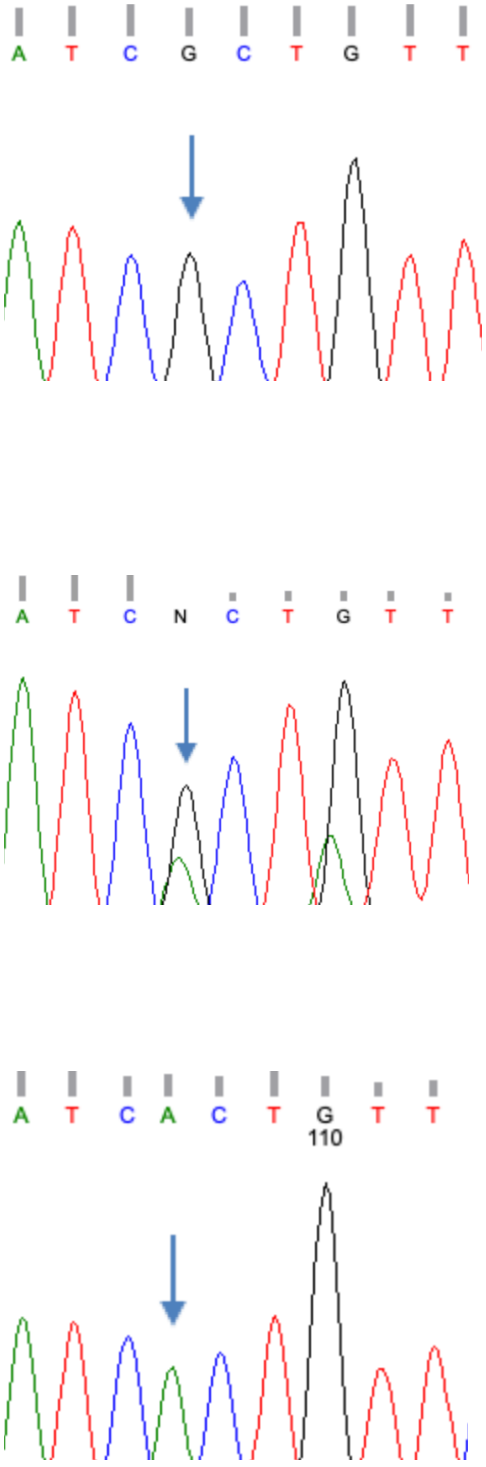



**Figure 24:**Sequencing electropherogram results (GG, GA, AA) of the rs28358281 of *MT-ND4L*. The nucleotide transition at the position 10586 (G>A) resulted in a synonymous variant (Ser>Ser) at codon 39.

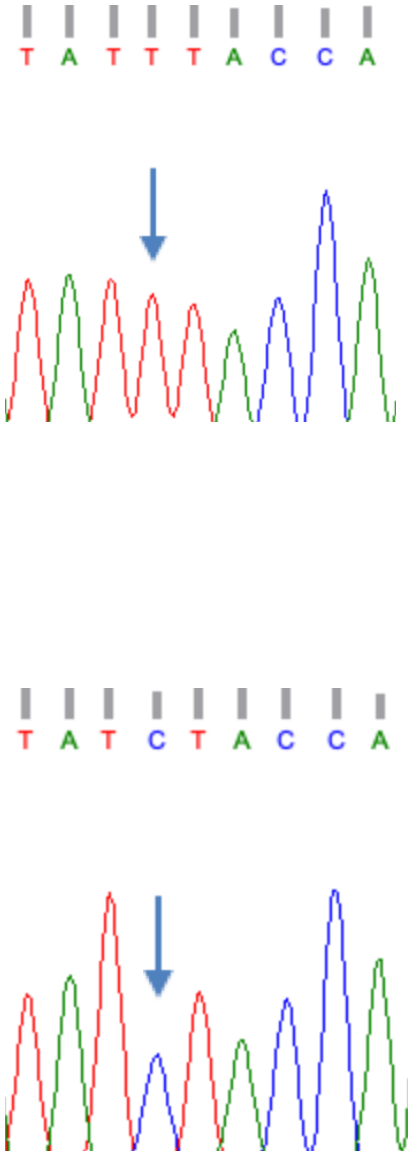



**Figure 25:** Sequencing electropherogram results (TT, CC) of the rs28358279 of *MT-ND4L*. The nucleotide transition at the position 10463 (T>C).

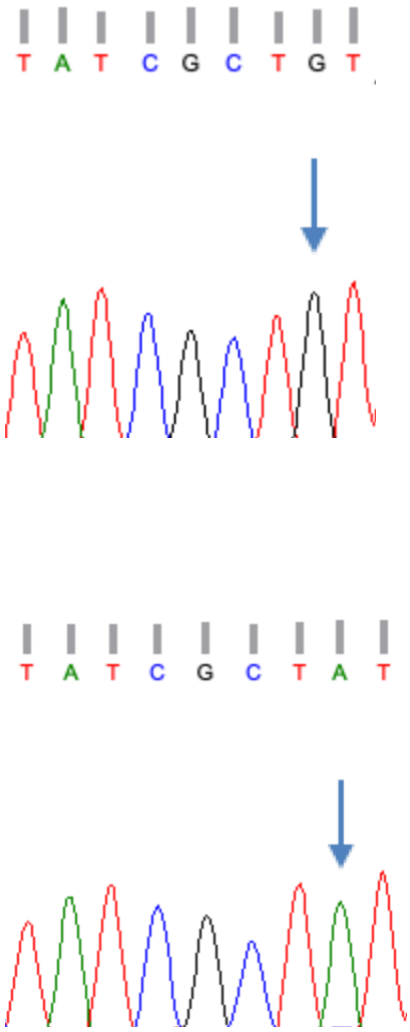



**Figure 26:** Sequencing electropherogram results (GG, AA) of the rs2853487 of *MT-ND4L*.

The nucleotide transition at the position 10589 (G>A) resulted in a synonymous variant (Leu>Leu) at codon 40.

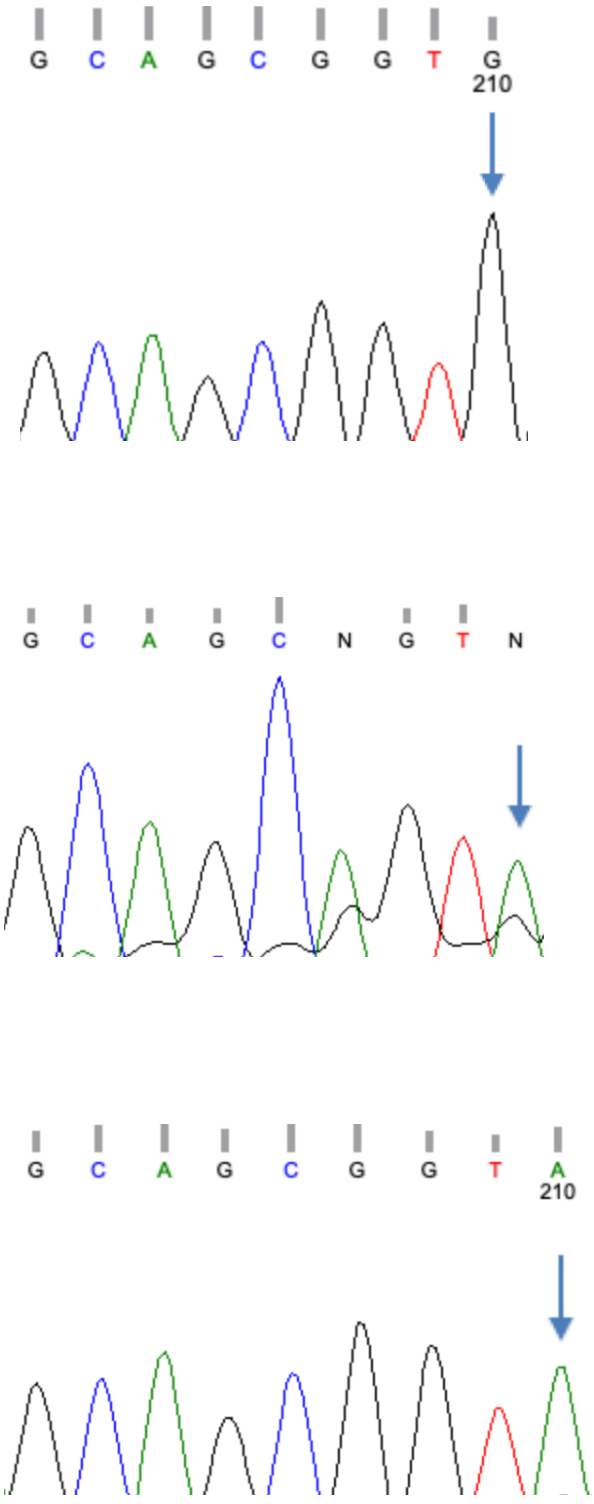



**Figure 27:** Sequencing electropherogram results (GG, GA, AA) of the rs2853488 of *MT-ND4L*. The nucleotide transition at the position 10688 (G>A) resulted in a synonymous variant (Val>Val) at codon 73.

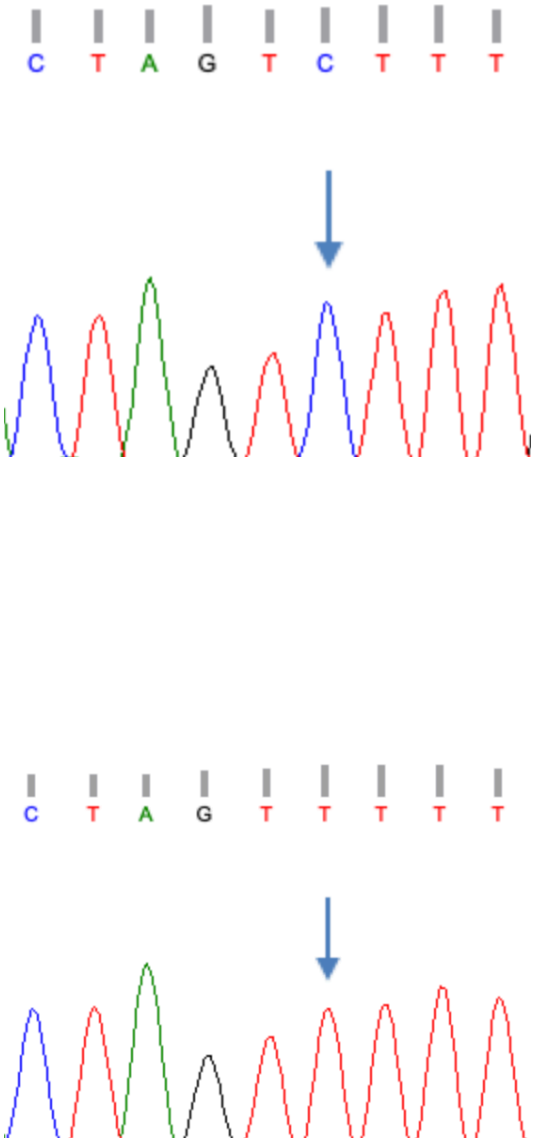



**Figure 28:** Sequencing electropherogram results (CC, TT) of the rs193302933 of *MT-ND4L*. The nucleotide transition at the position 10664 (C>T) resulted in a synonymous variant (Val>Val) at codon 65.

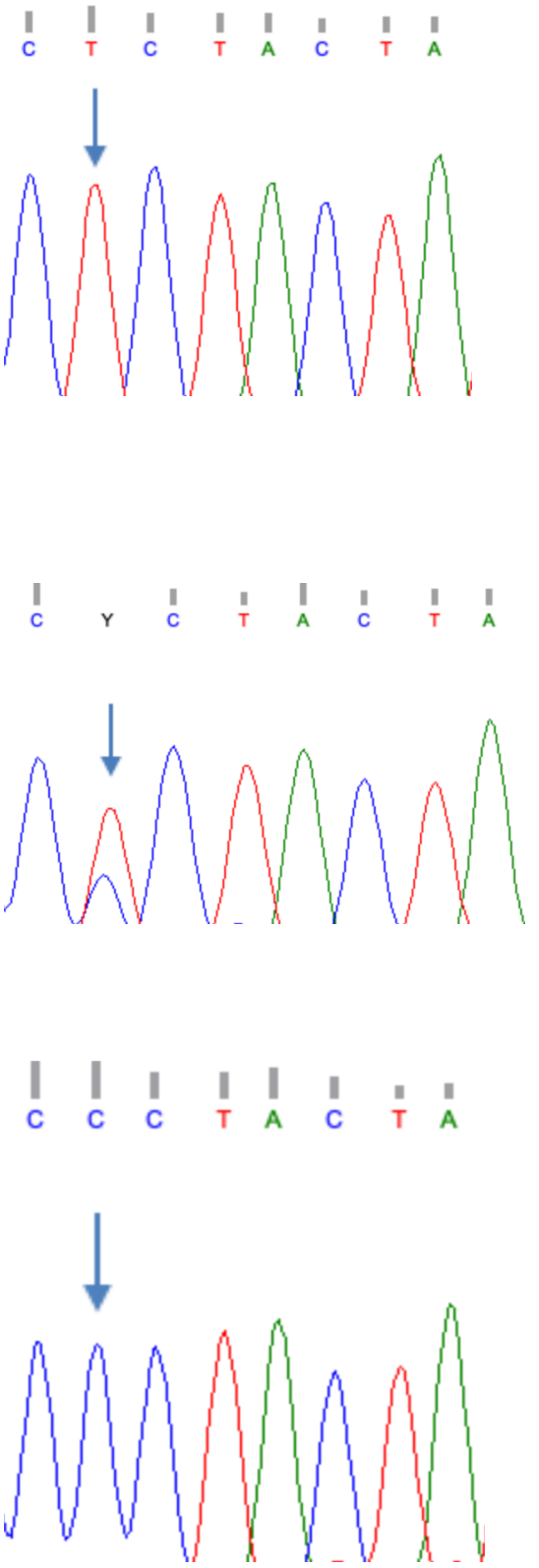



**Figure 29:** Sequencing electropherogram results (TT, TC, CC) of the rs2857284 of *MT-ND4*. The nucleotide transition at the position 10873 (T>C) resulted in a synonymous variant (Pro>Pro) at codon 38.

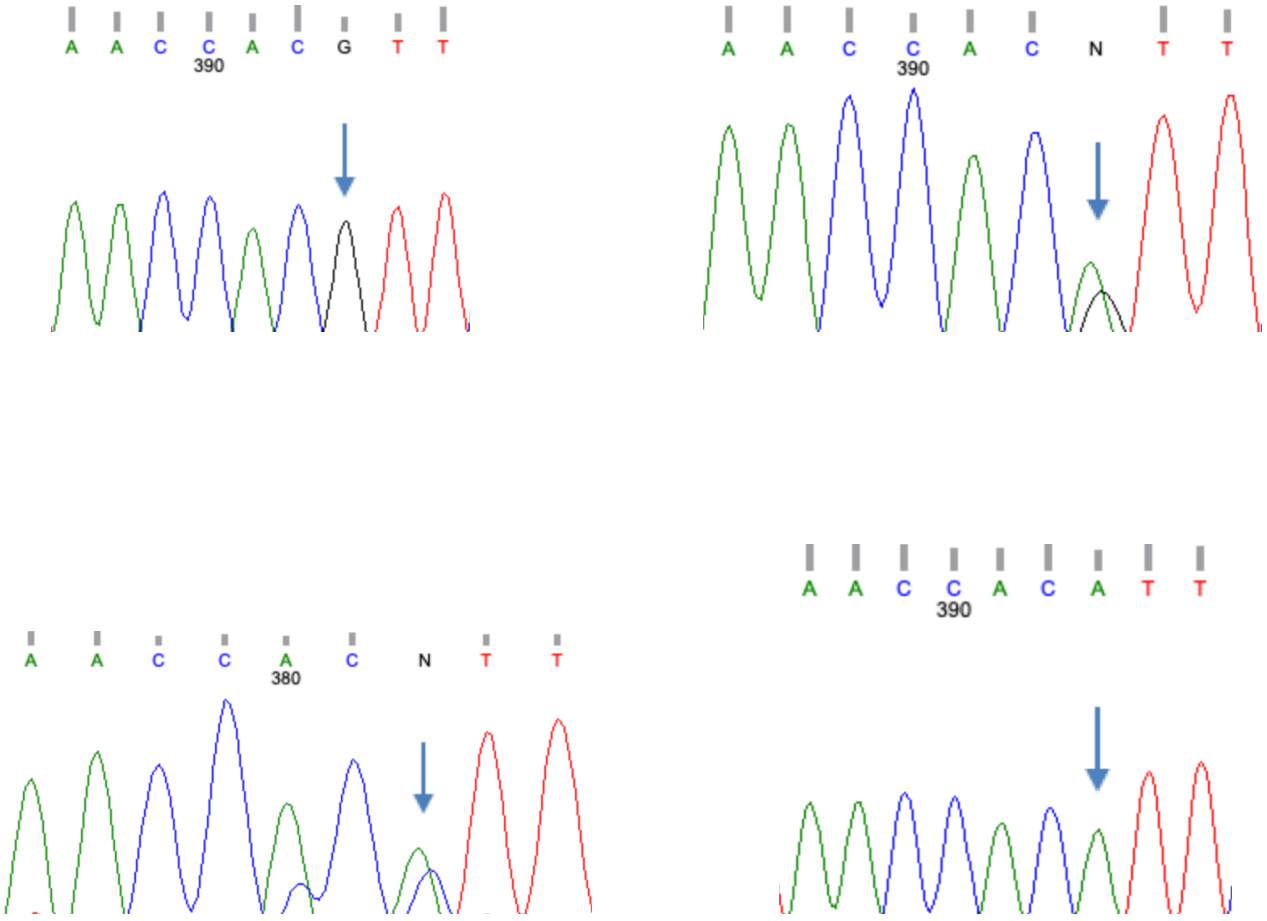



**Figure 30:** Sequencing electropherogram results (GG, GA, AC, AA) of the rs2853496 of *MT-ND4*.

The nucleotide transition at the position 11914 (G>A,C) resulted in a synonymous variant (Thr>Thr) at codon 385.

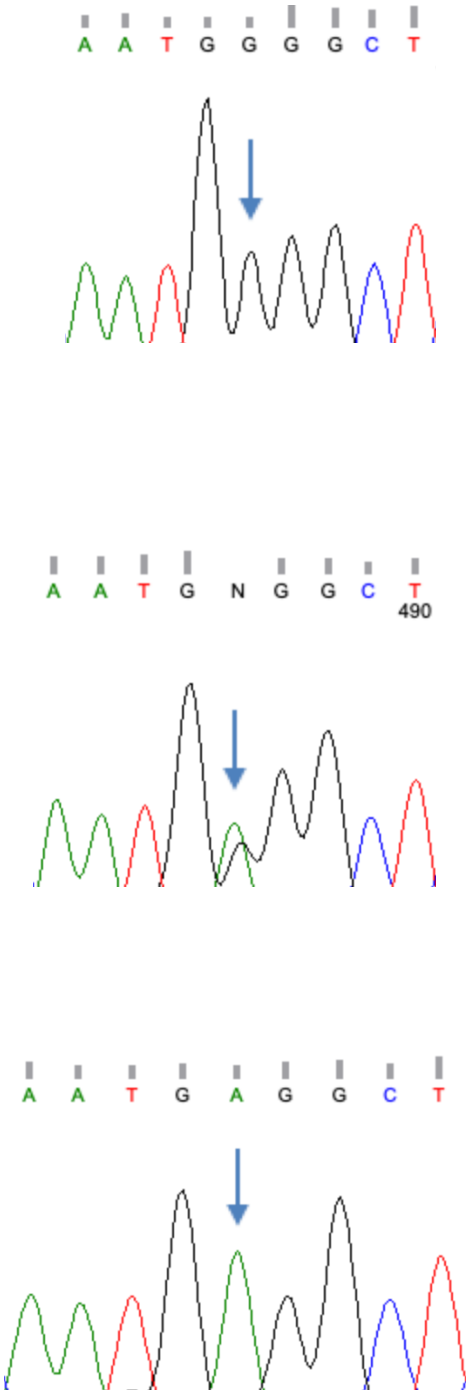



**Figure 31:** Sequencing electropherogram results (GG, GA, AA) of the rs2853497 of *MT-ND4*. The nucleotide transition at the position 12007 (G>A) resulted in a synonymous variant (Trp>Trp) at codon 416.

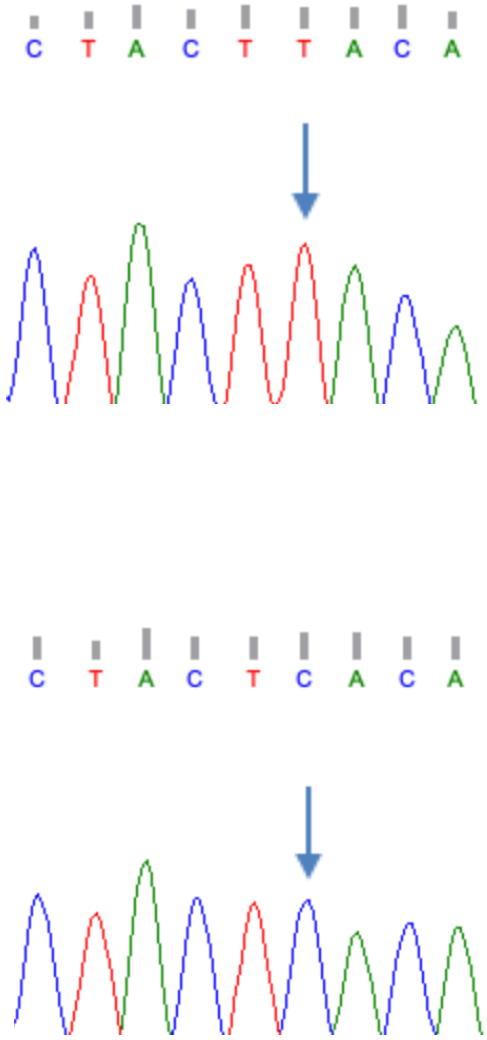



**Figure 32:** Sequencing electropherogram results (TT, CC) of the rs3087901 of *MT-ND4*.
The nucleotide transition at the position 11944 (T>C) resulted in a synonymous variant (Leu>Leu) at codon 395.

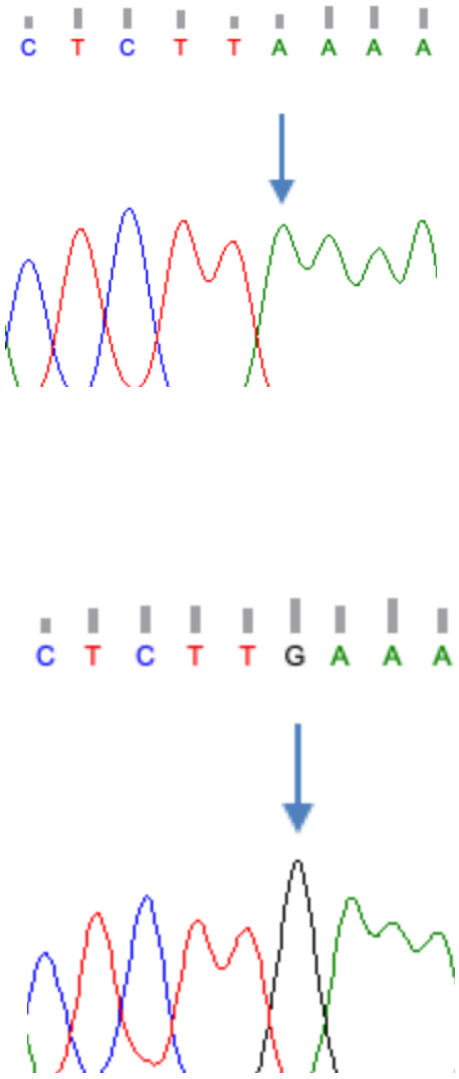



**Figure 33:** Sequencing electropherogram results (AA, GG) of the rs2853493 of *MT-ND4*.

The nucleotide transition at the position 11467 (A>G) resulted in a synonymous variant (Leu>Leu) at codon 236.

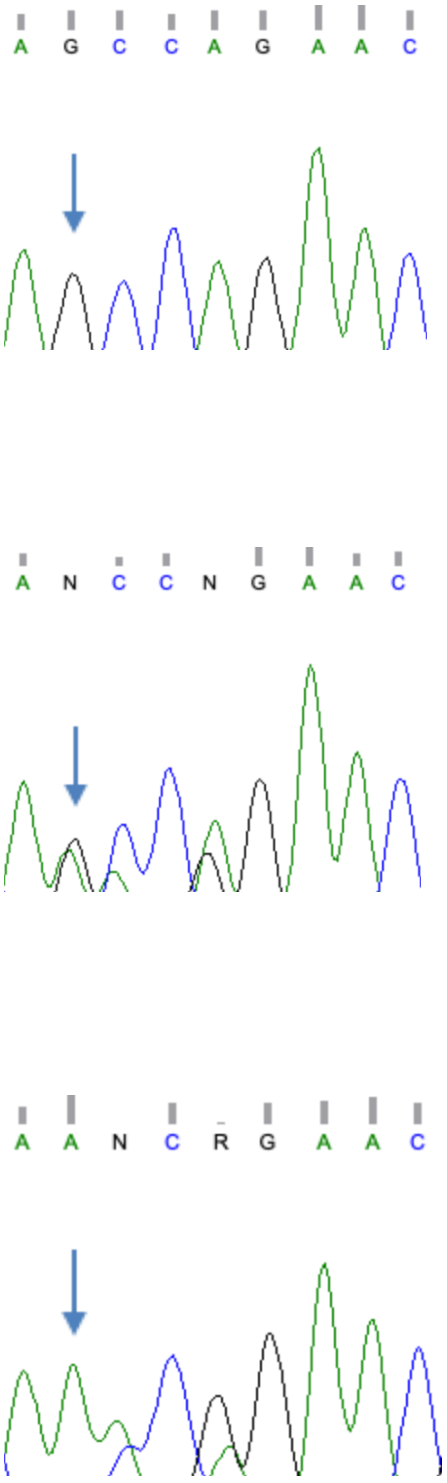



**Figure 34:** Sequencing electropherogram results (GG, GA, AA) of the rs2853490 of *MT-ND4*. The nucleotide transition at the position 11176 (G>A) resulted in a synonymous variant (Gln>Gln) at codon 139.

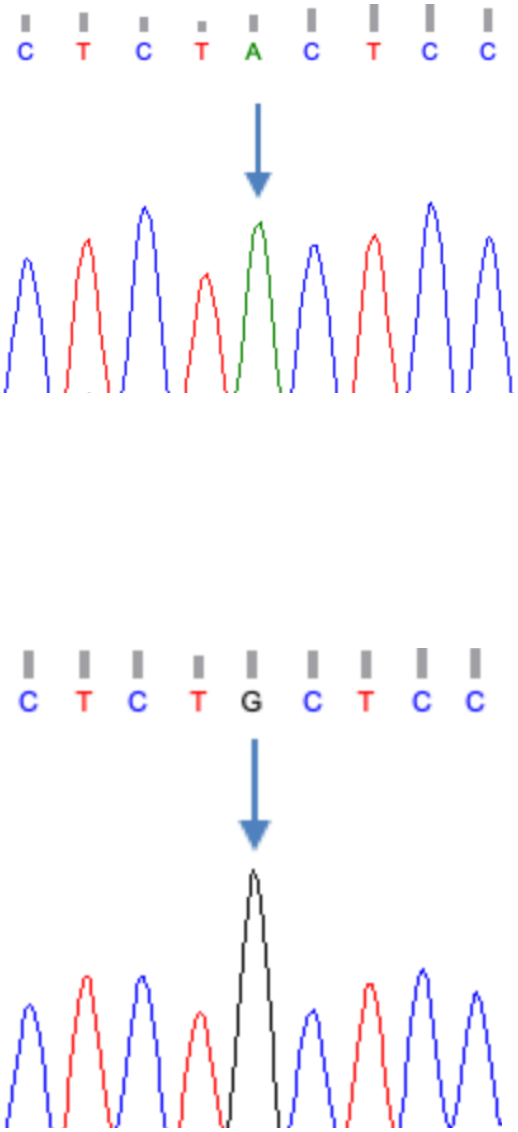



**Figure 35:** Sequencing electropherogram results (AA, GG) of the rs3088053 of *MT-ND4*.

The nucleotide transition at the position 11812 (A>G) resulted in a synonymous variant (Leu>Leu) at codon 351.

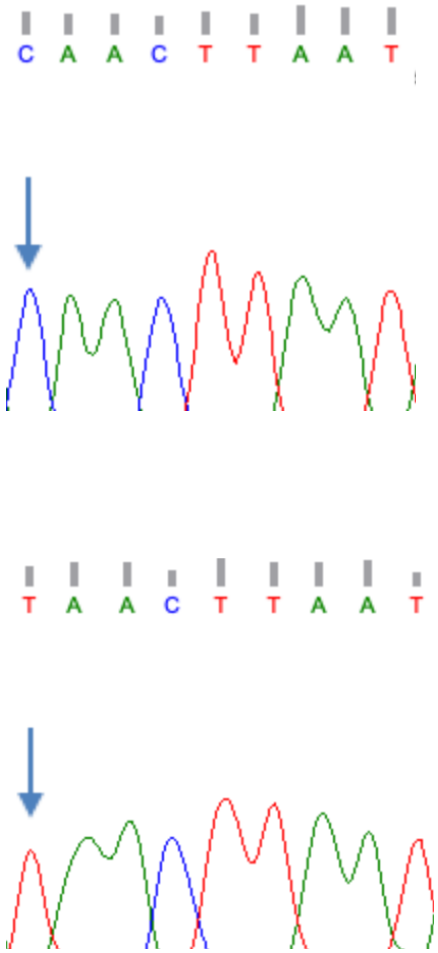



**Figure 36:** Sequencing electropherogram results (CC, TT) of the rs2853491 of *MT-ND4*.
The nucleotide transition at the position 11335 (C>T) resulted in a synonymous variant (Asn>Asn) at codon 192.

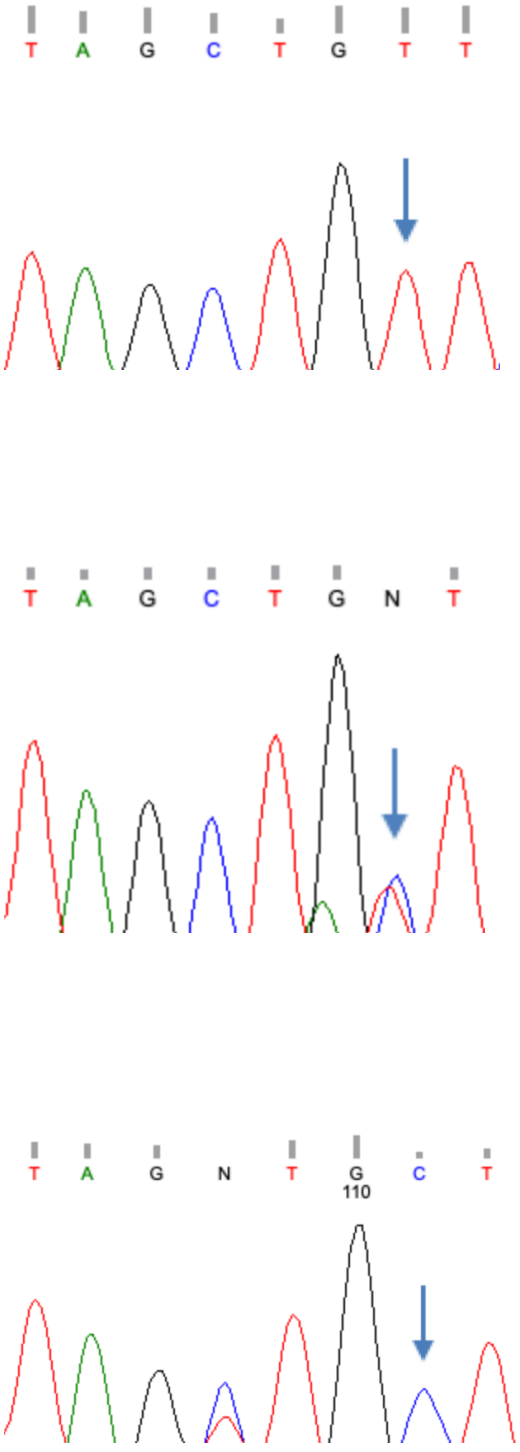



**Figure 37:** Sequencing electropherogram results (TT, TC, CC) of the rs2857285 of *MT-ND4*. The nucleotide transition at the position 10915 (T>C) resulted in a synonymous variant (Cys>Cys) at codon 52.

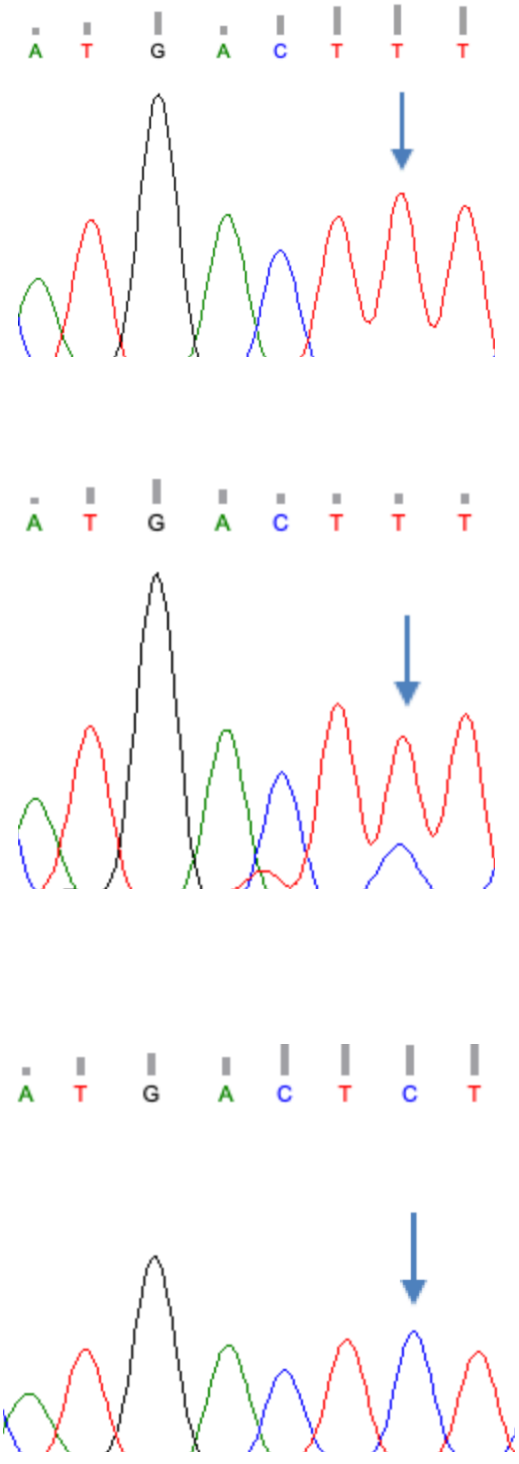



**Figure 38:** Sequencing electropherogram results (TT, TC, CC) of the rs28358282 of *MT-ND4*. The nucleotide transition at the position 10810 (T>C) resulted in a synonymous variant (Leu>Leu) at codon 17.

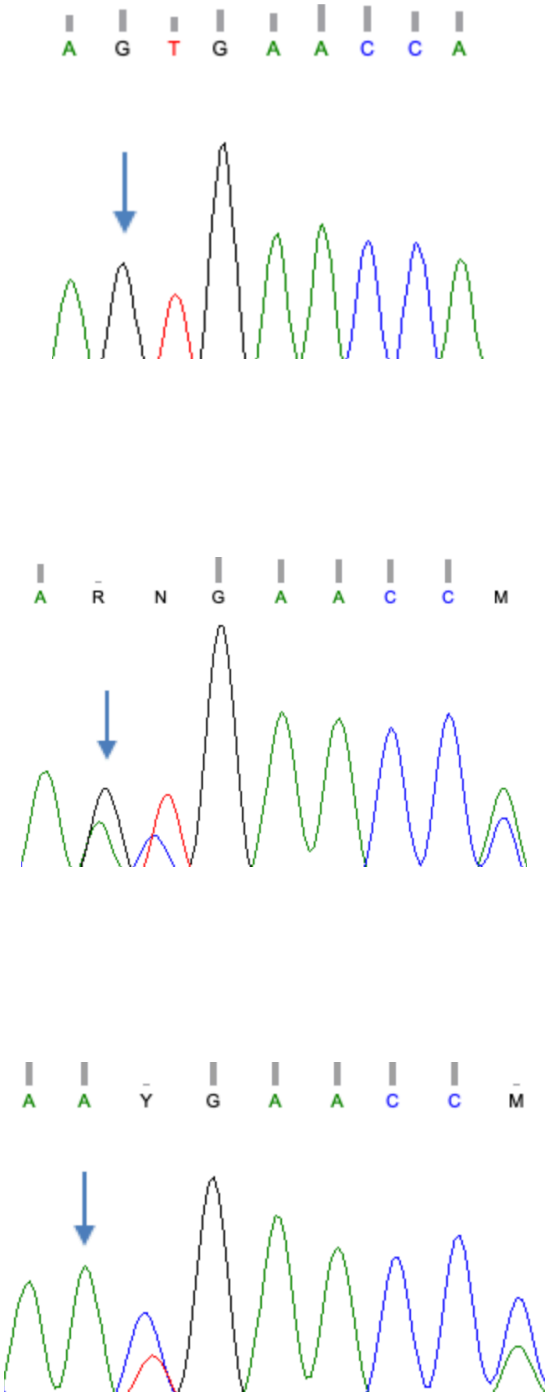



**Figure 39:** Sequencing electropherogram results (GG, GA, AA) of the rs28594904 of *MT-ND4*. The nucleotide transition at the position 11016 (G>A) resulted in a missense variant (Ser>Asn) at codon 86.

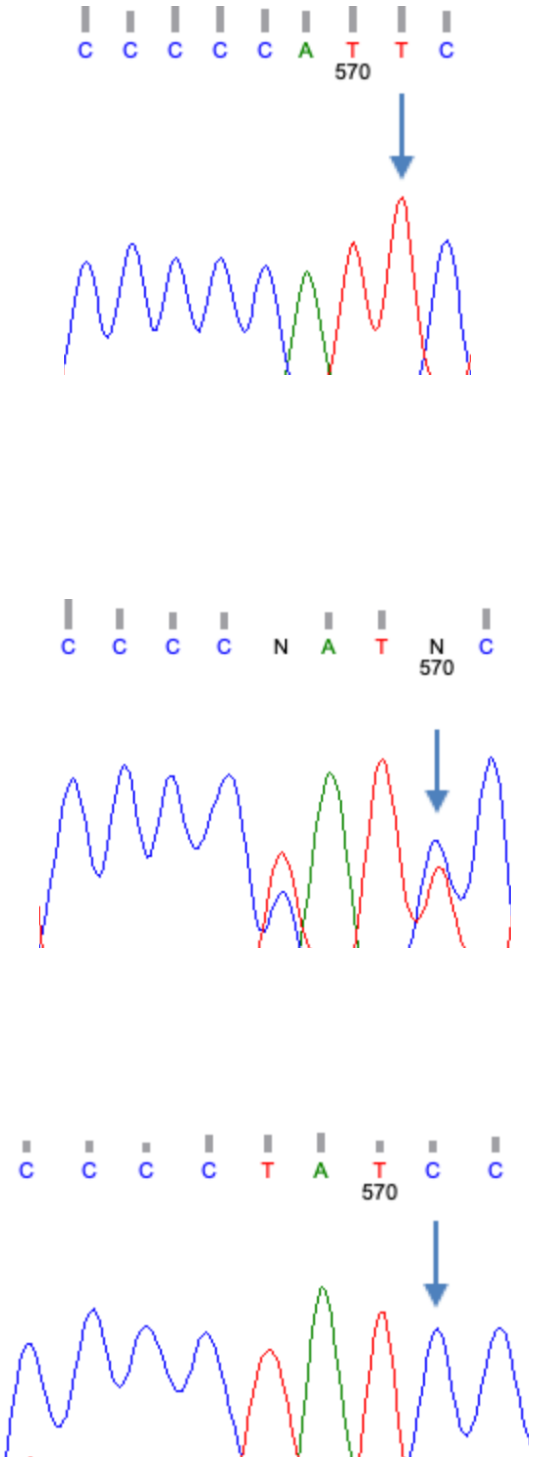



**Figure 40:** Sequencing electropherogram results (TT, TC, CC) of the rs28415973 of *MT-ND4*. The nucleotide transition at the position 12091 (T>C) resulted in a synonymous variant (Ile>Ile) at codon 444.

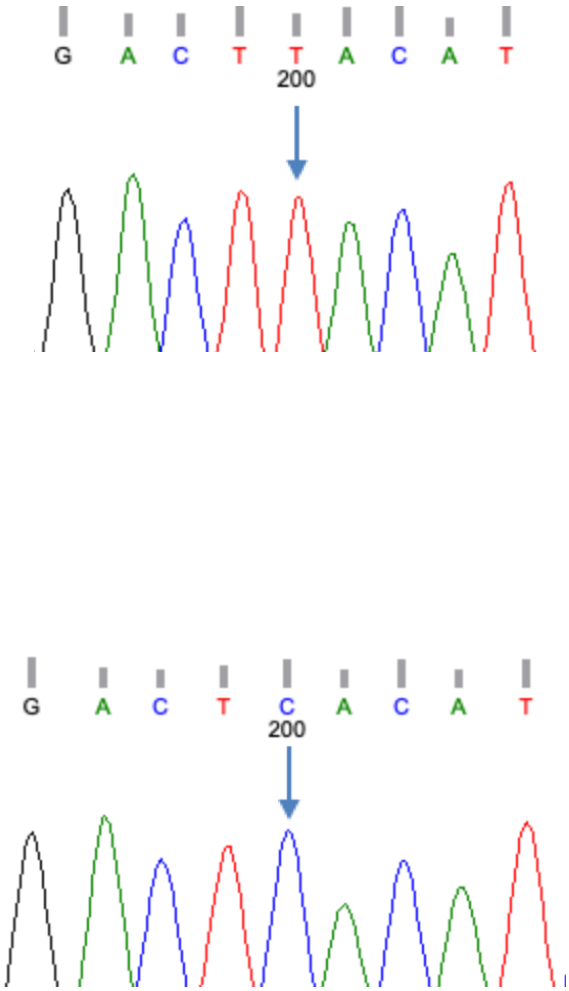
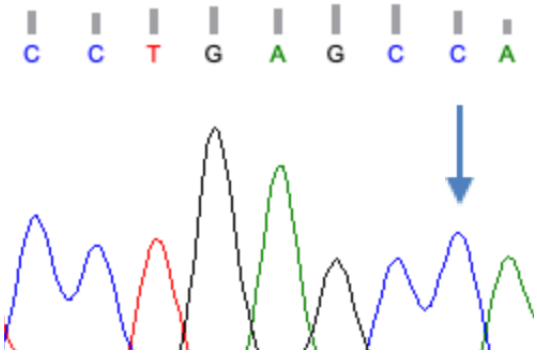



**Figure 41:** Sequencing electropherogram results (TT, CC) of the rs28471078 of *MT-ND4*.

The nucleotide transition at the position 11722 (T>C) resulted in a synonymous variant (Leu>Leu) at codon 321.



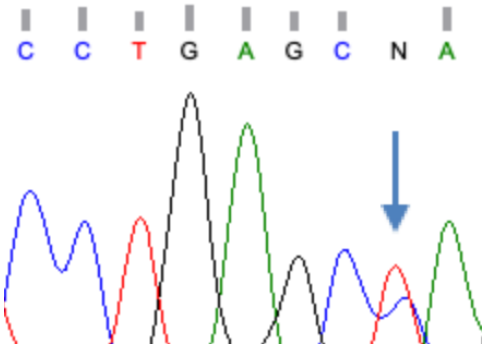




**Figure 42:** Sequencing electropherogram results (CC, CT) of the rs55714831 of *MT-ND4*.

The nucleotide transition at the position 11332 (C>T) resulted in a synonymous variant (Ala>Ala) at codon 191.

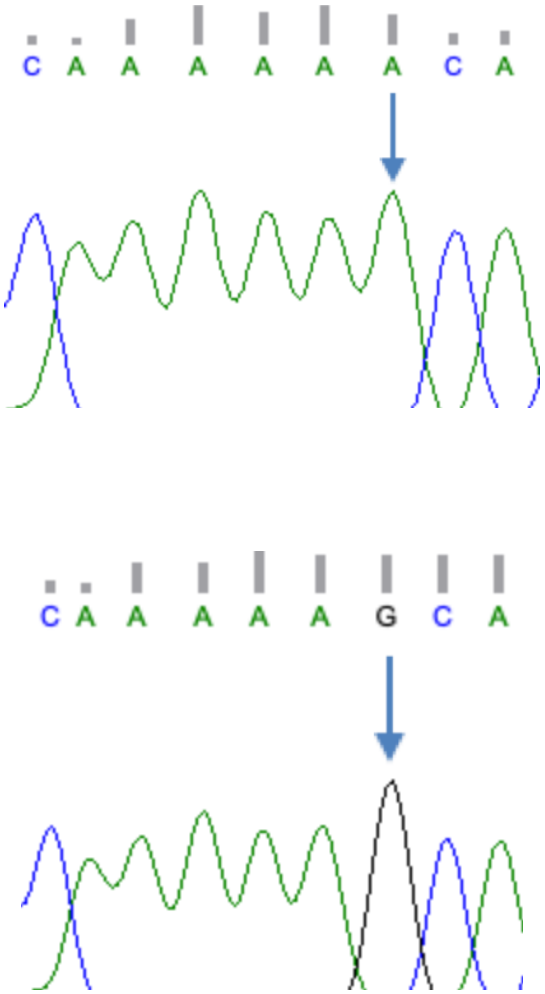



**Figure 43:** Sequencing electropherogram results (AA, GG) of the rs28358283 of *MT-ND4*.
The nucleotide transition at the position 10819 (A>G) resulted in a synonymous variant (Lys>Lys) at codon 20.

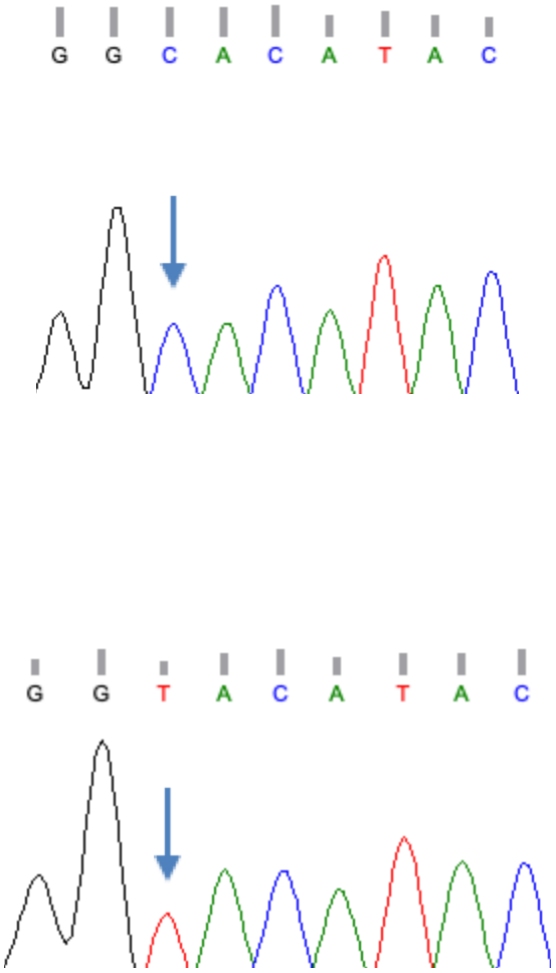



**Figure 44:** Sequencing electropherogram results (CC, TT) of the rs75214962 of *MT-ND4*.

The nucleotide transition at the position 11197 (C>T) resulted in a synonymous variant (Gly>Gly) at codon 146.

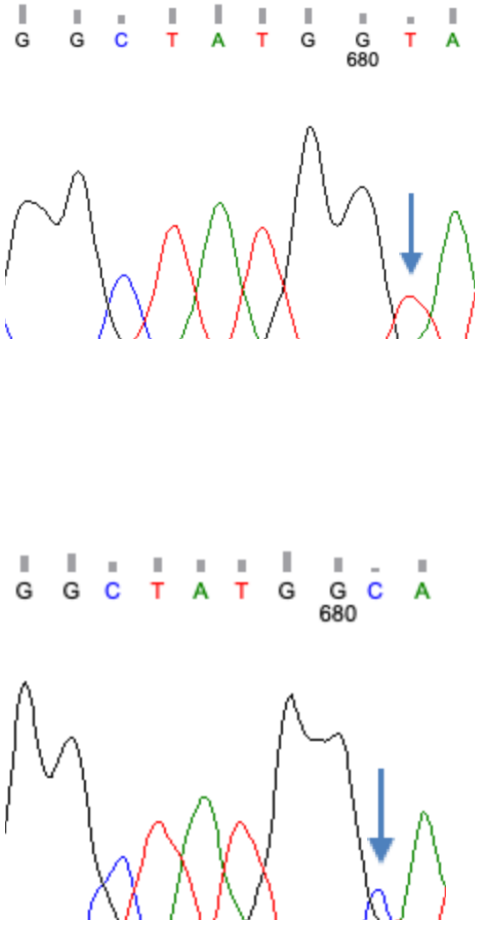



**Figure 45:** Sequencing electropherogram results (TT, CC) of the rs28529320 of *MT-ND4*.

The nucleotide transition at the position 11485 (T>C) resulted in a synonymous variant (Gly>Gly) at codon 242.

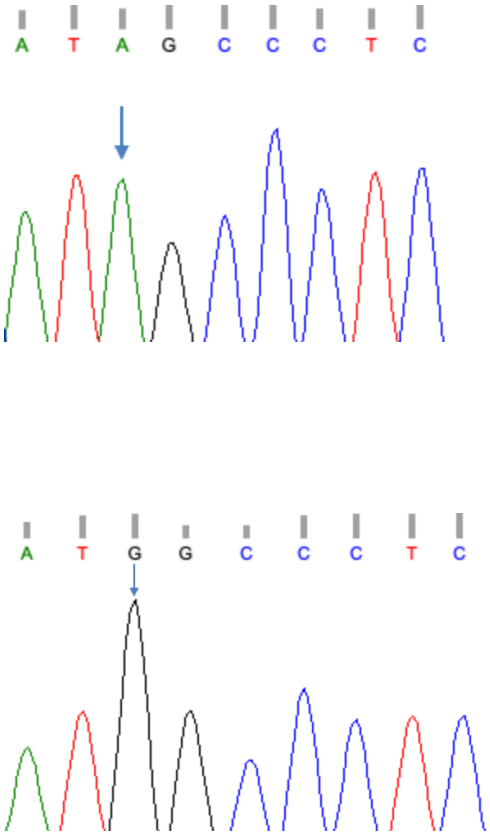



**Figure 46:** Sequencing electropherogram results (AA, GG) of the rs2853494 of *MT-ND4*.

The nucleotide transition at the position 11641 (A>G) resulted in a synonymous variant (Met>Met) at codon 294.

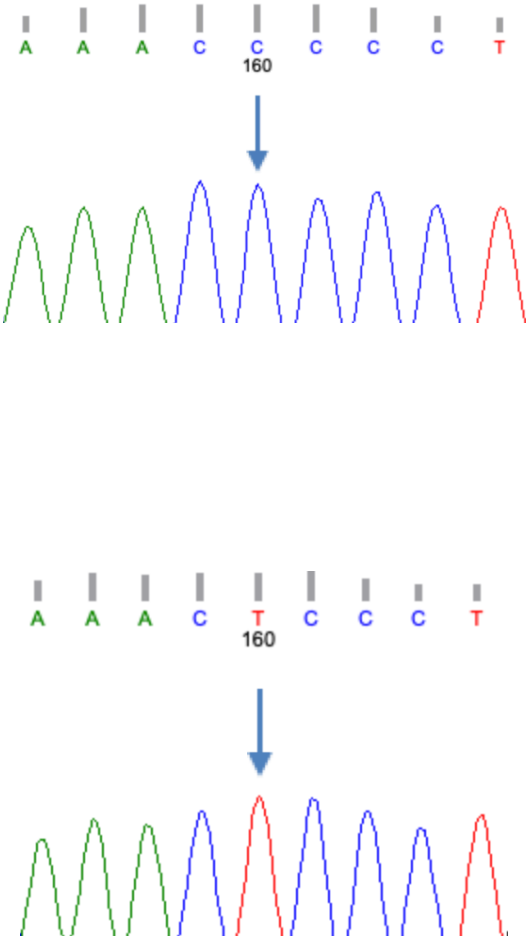



**Figure 47:** Sequencing electropherogram results (CC, TT) of the rs28358286 of *MT-ND4*.

The nucleotide transition at the position 11674 (C>T) resulted in a synonymous variant (Thr>Thr) at codon 305.

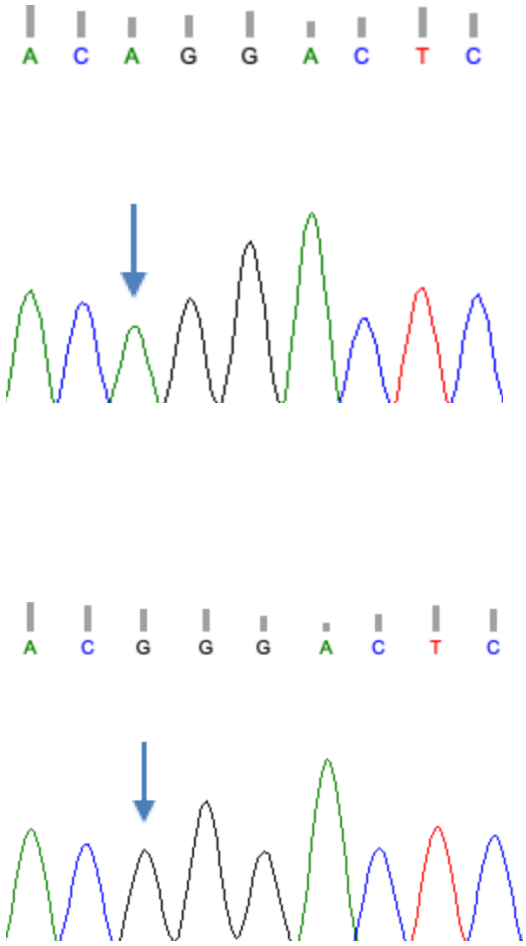



**Figure 48:** Sequencing electropherogram results (AA, GG) of the rs28359168 of *MT-ND4*.
The nucleotide transition at the position 11947 (A>G) resulted in a synonymous variant (Thr>Thr) at codon 396.

**APPENDIX 2:** Supplementary figures for Chapter 3.2 Genotypes and allelic frequencies

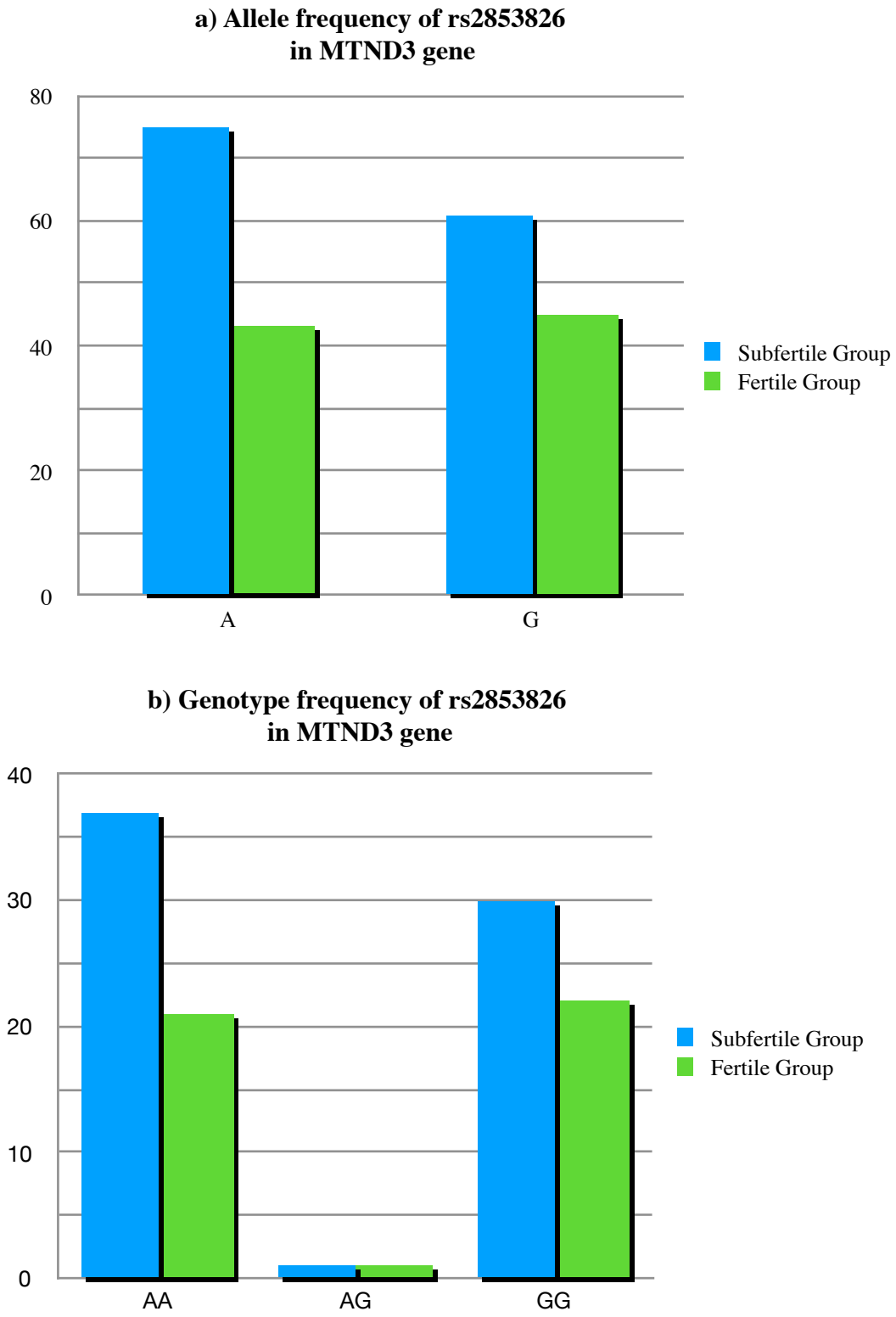



**Figure 49: a)** allele frequency of rs2853826 in *MTND3* gene (*P*= 0.411), **b)** genotype frequency of rs2853826 in *MTND3* gene (*P*= 0.768).

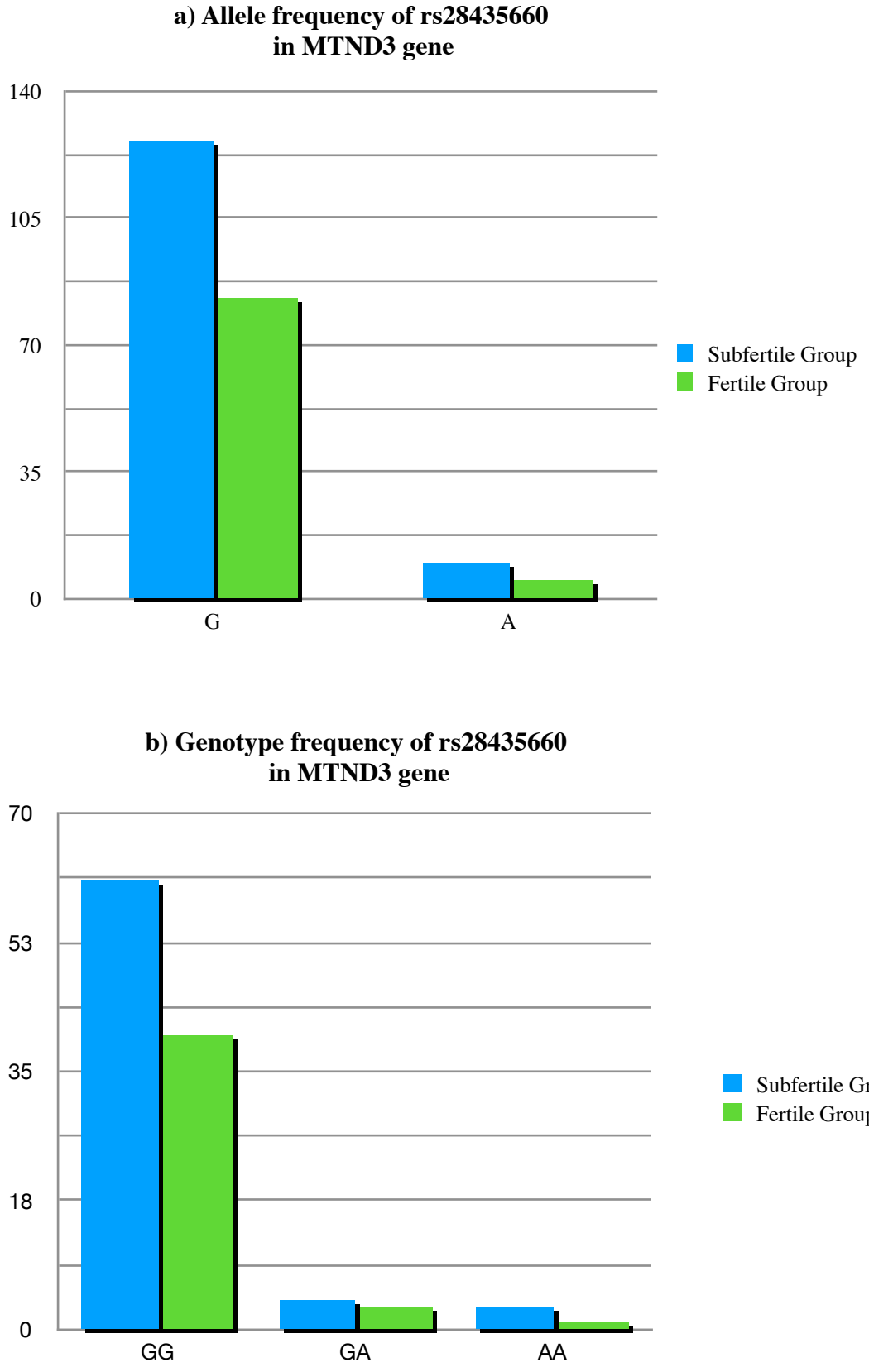



**Figure 50: a)** allele frequency of rs28435660 in *MTND3* gene (*P*= 0.7865), **b)** genotype frequency of rs28435660 in *MTND3* gene (*P*= 0.825).

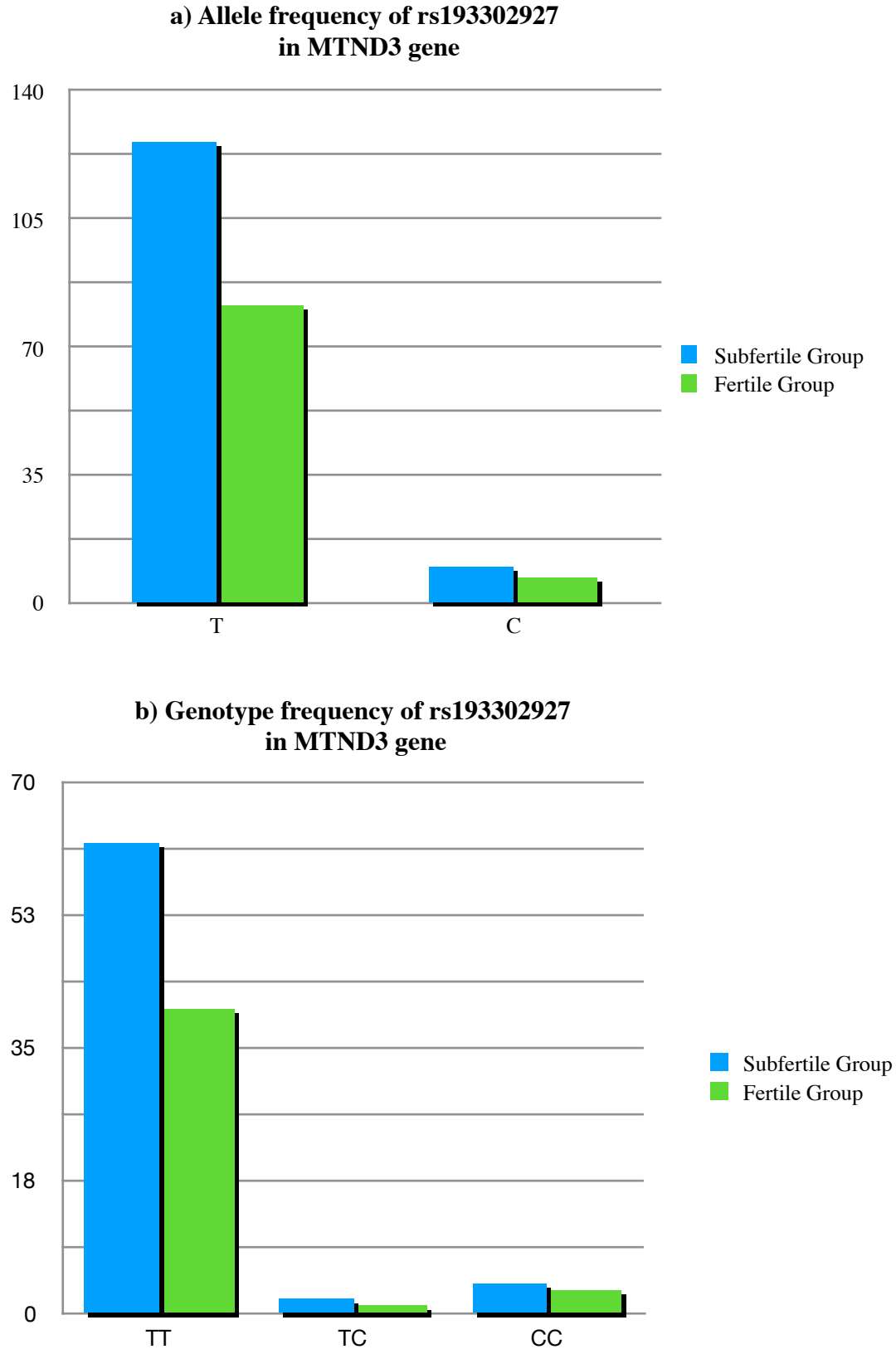



**Figure 51: a)** allele frequency of rs193302927 in *MTND3* gene (*P*= 1.000), **b)** genotype frequency of rs193302927 in *MTND3* gene (*P*= 0.959).

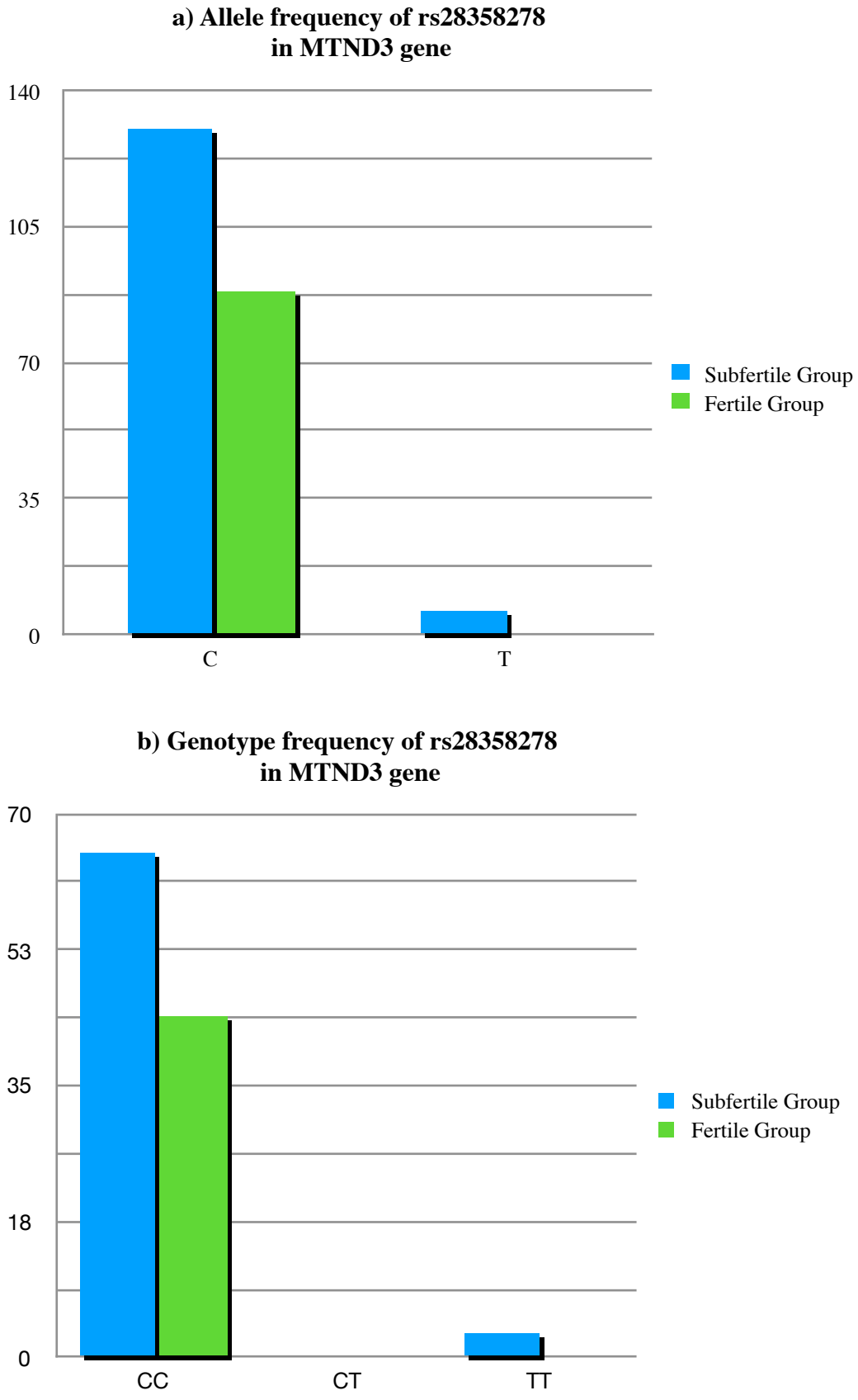



**Figure 52: a)** allele frequency of rs28358278 in *MTND3* gene (*P*= 0.0837), **b)** genotype frequency of rs28358278 in *MTND3* gene (*P*= 0.158).

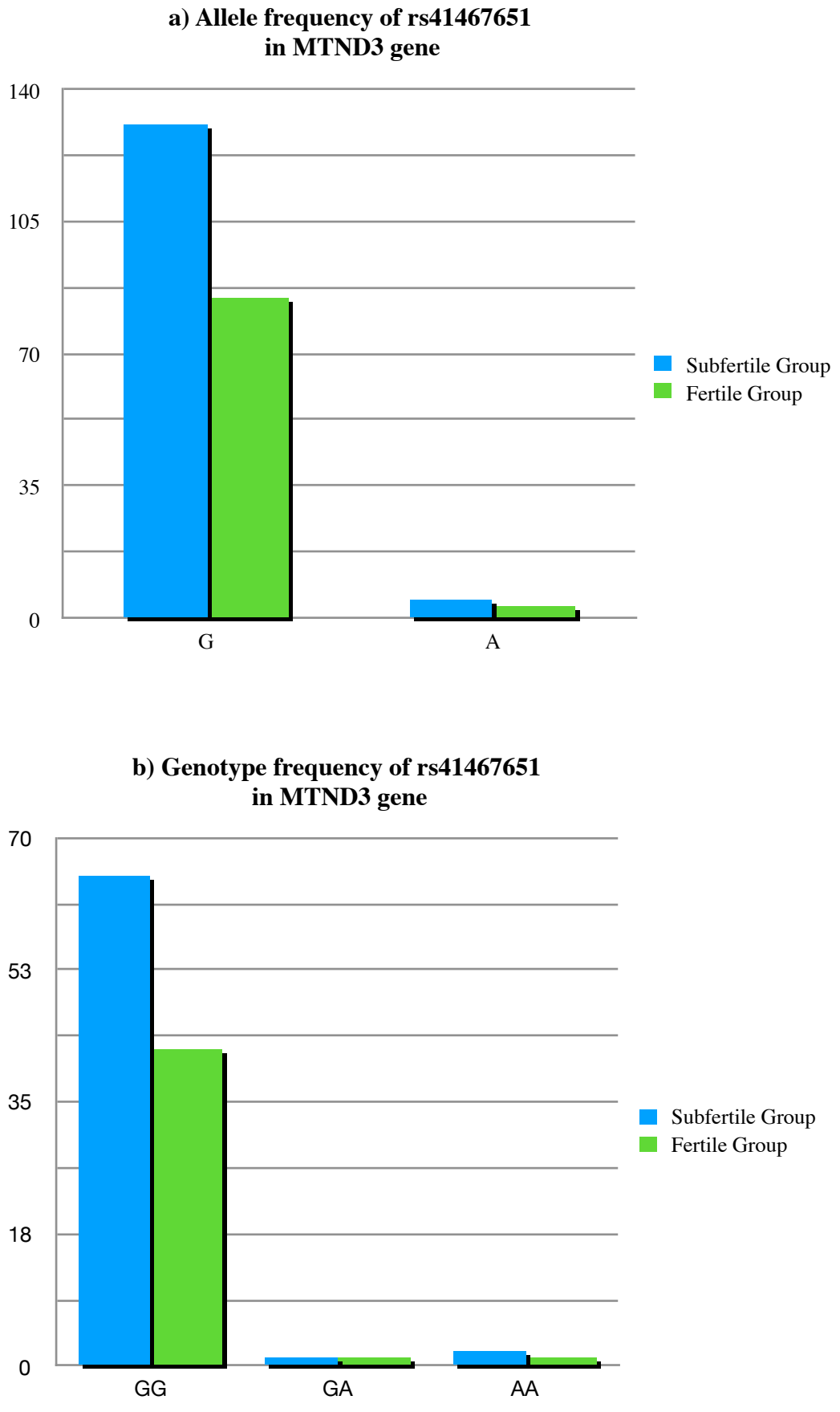



**Figure 53: a)** allele frequency of rs41467651 in *MTND3* gene (*P*= 1.000), **b)** genotype frequency of rs41467651 in *MTND3* gene (*P*= 0.9320).

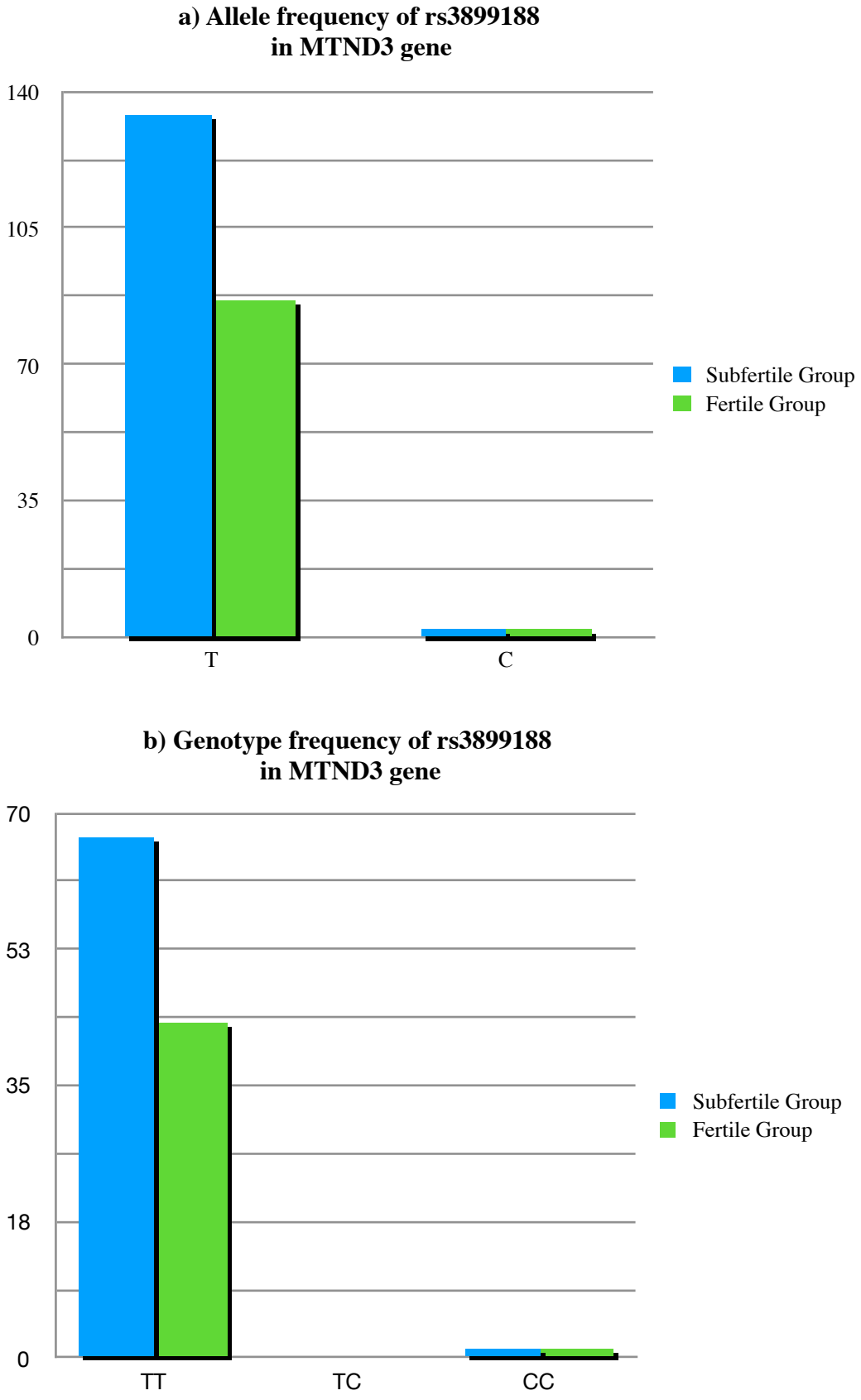



**Figure 54: a)** allele frequency of rs3899188 in *MTND3* gene (*P*= 0.6466), **b)** genotype frequency of rs3899188 in *MTND3* gene (*P*= 0.754).

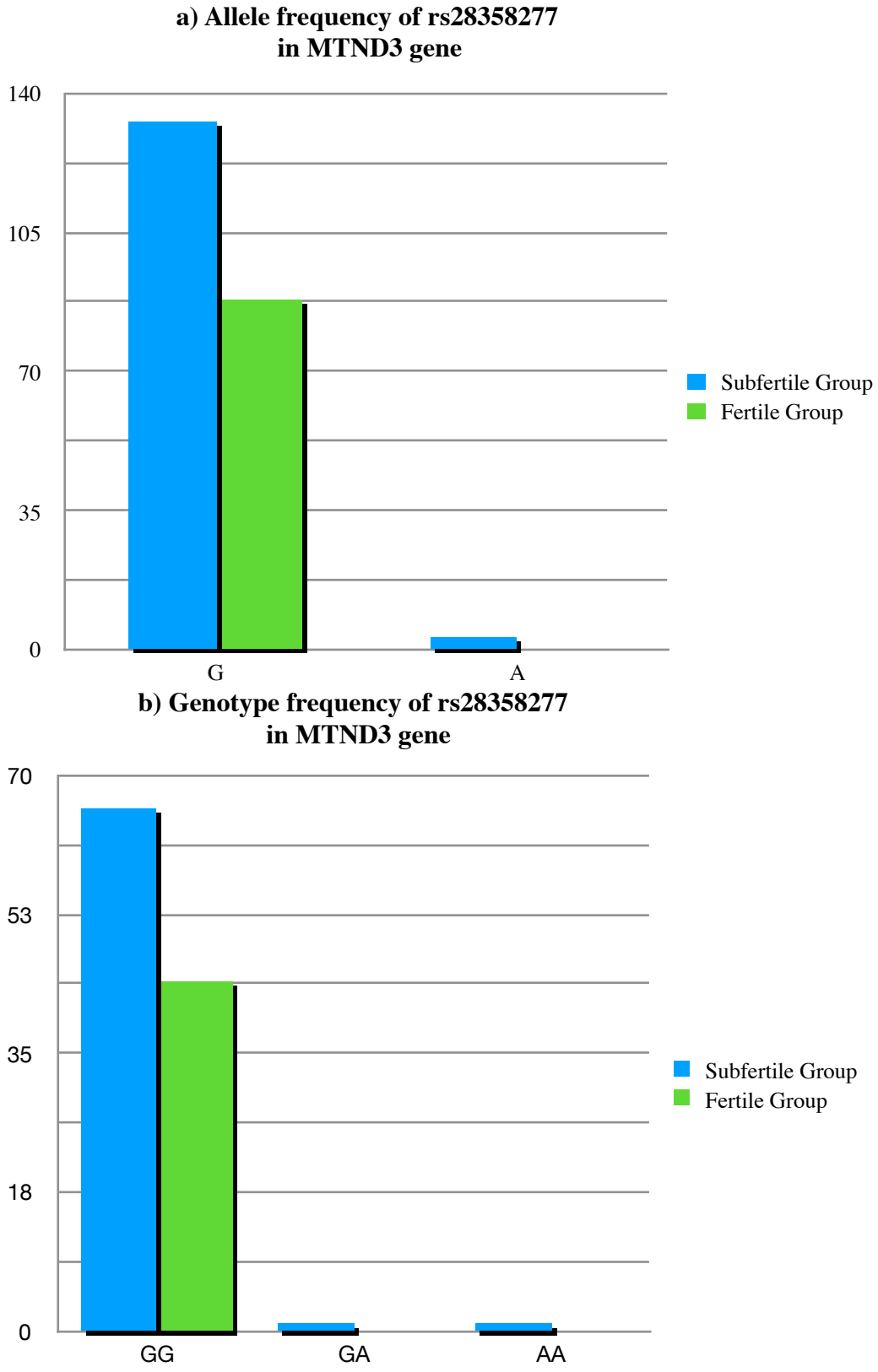



**Figure 55: a)** allele frequency of rs28358277 in *MTND3* gene (*P*= 0.2812), **b)** genotype frequency of rs28358277 in *MTND3* gene (*P*= 0.517).

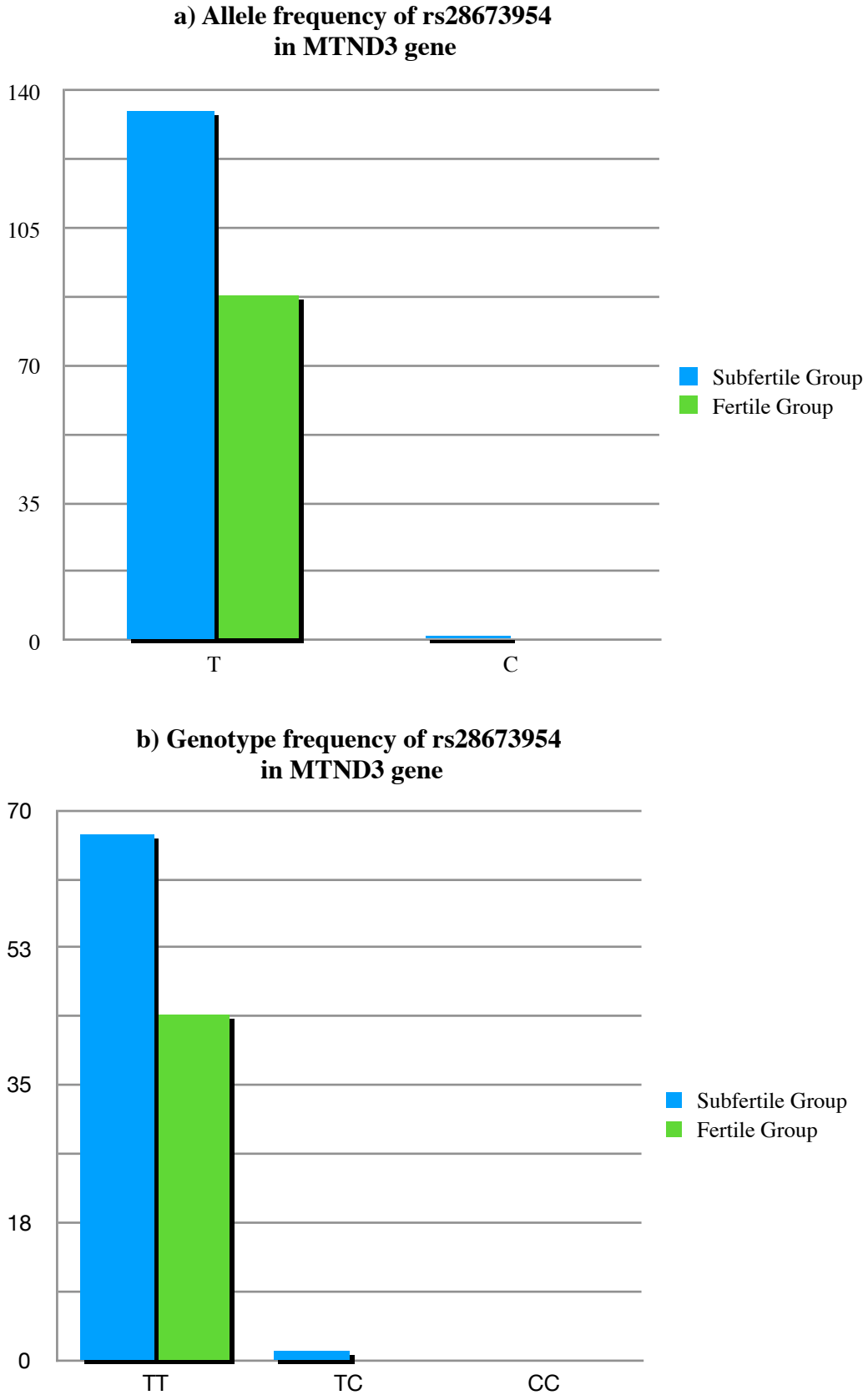
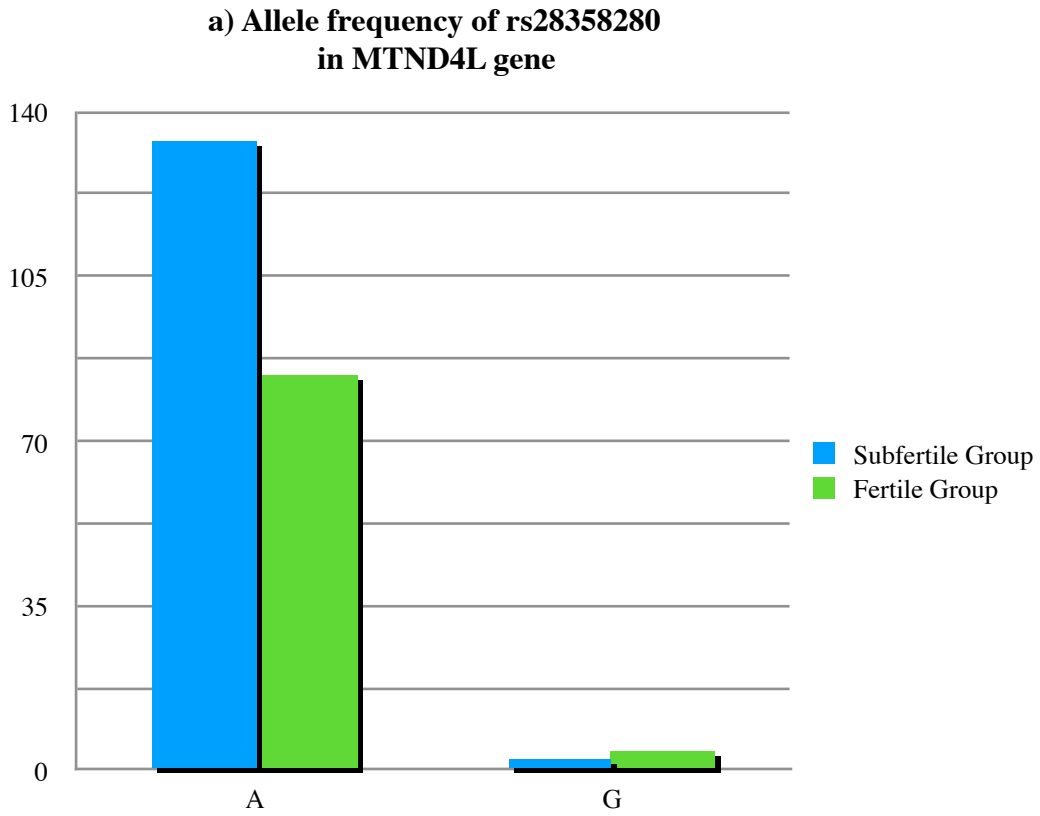



**Figure 56: a)** allele frequency of rs28673954 in *MTND3* gene (*P*= 1.000), **b)** genotype frequency of rs28673954 in *MTND3* gene (*P*= 0.4191).



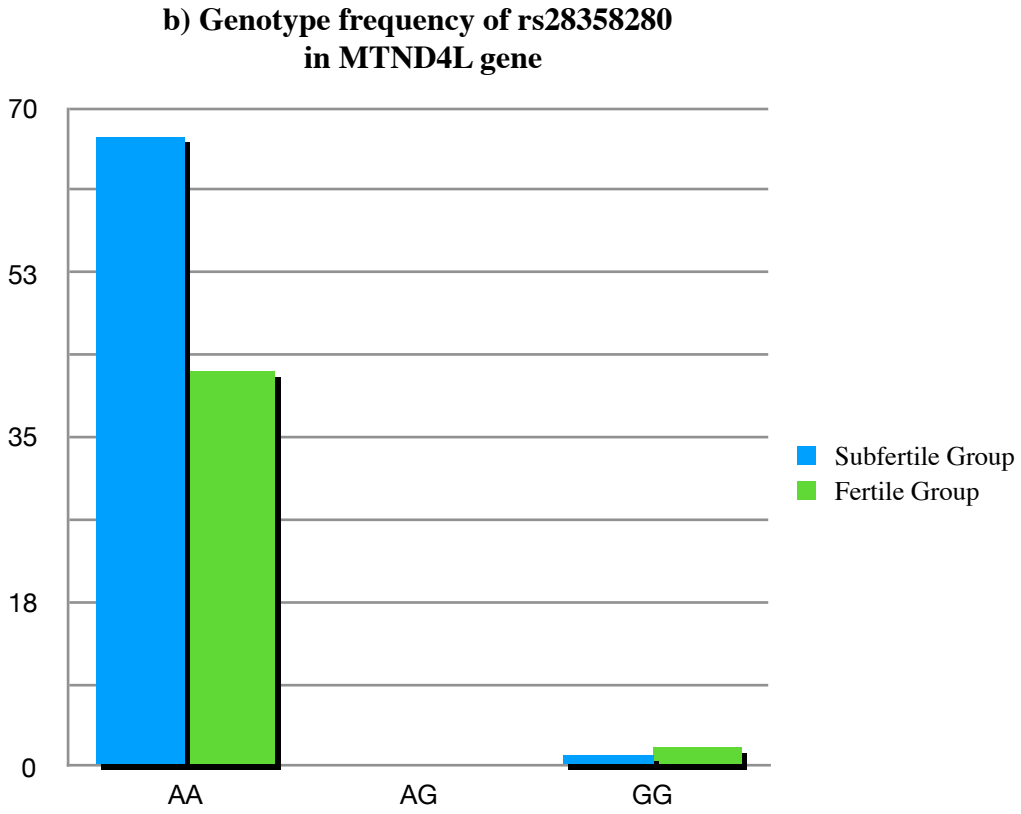




**Figure 57: a)** allele frequency of rs28358280 in *MTND4L* gene (*P*= 0.214), **b)** genotype frequency of rs28358280 in *MTND4L* gene (*P*= 0.325).

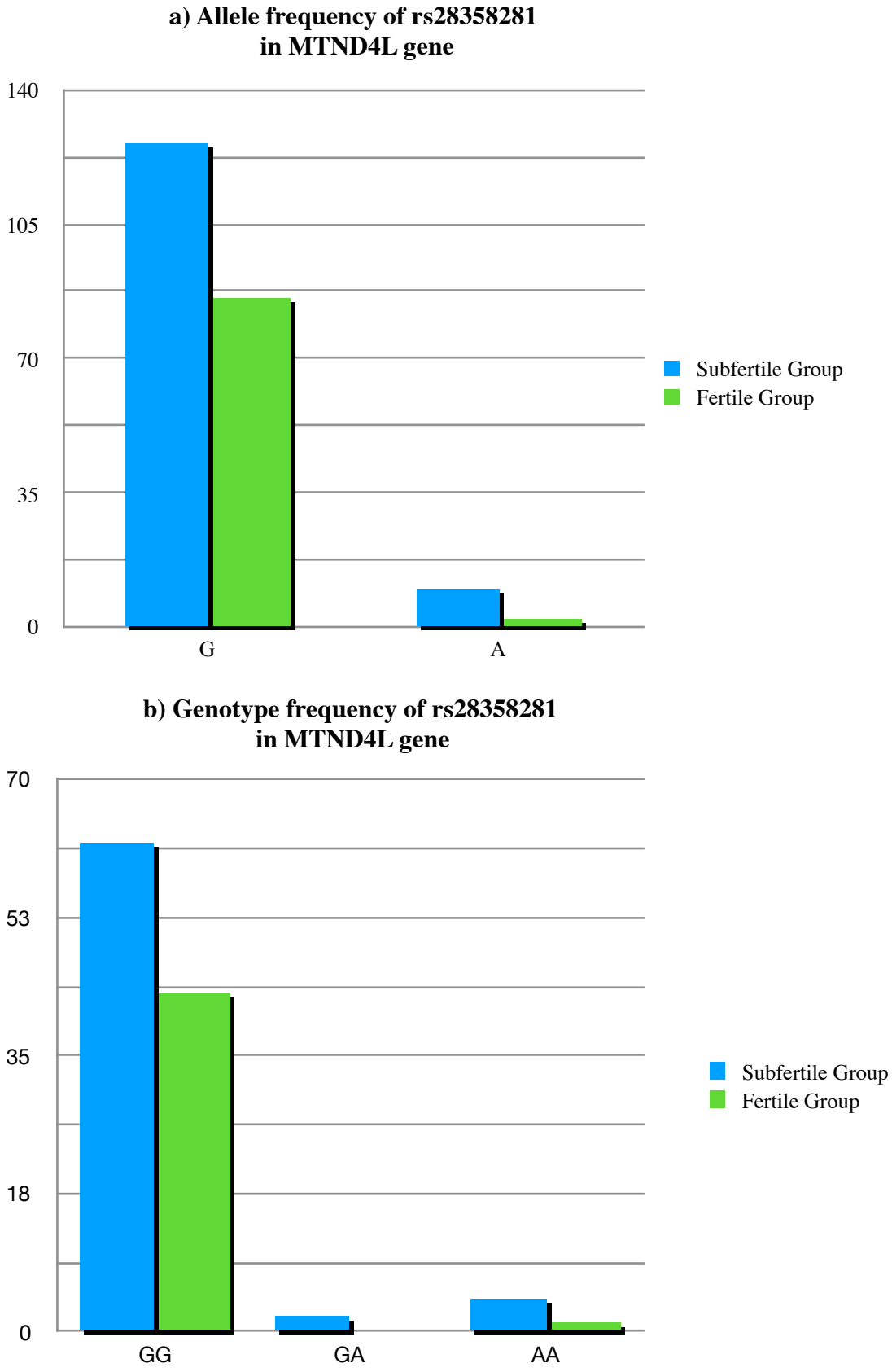
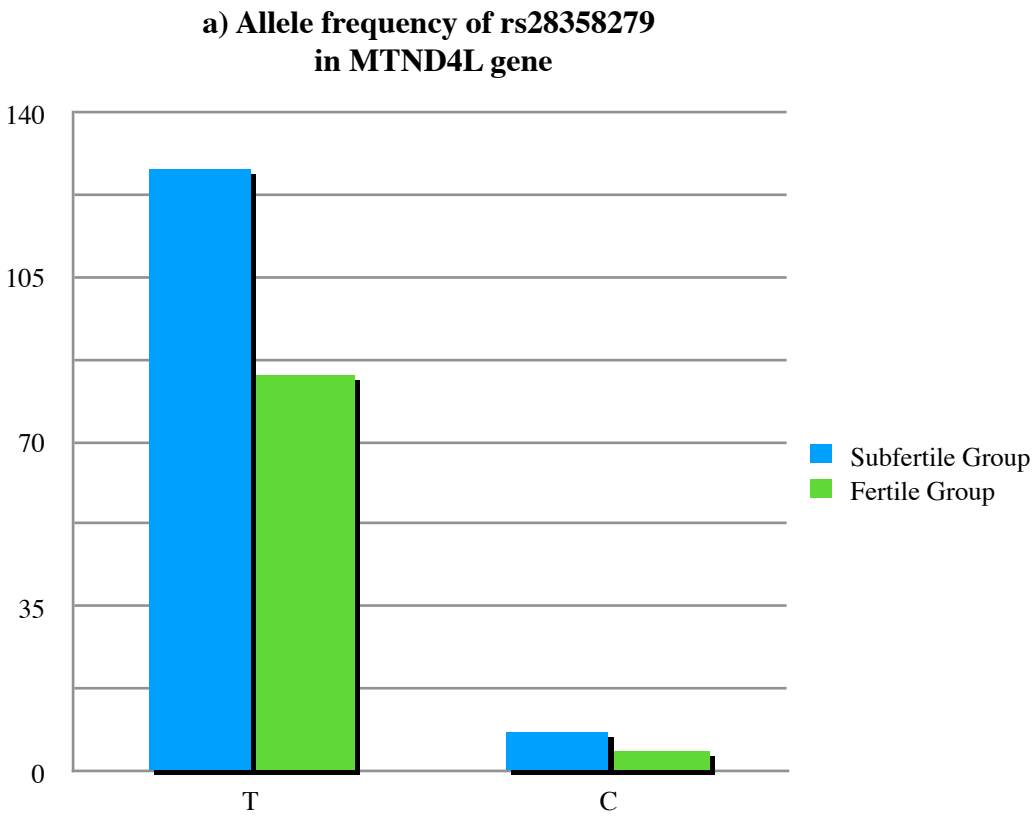



**Figure 58: a)** allele frequency of rs28358281 in *MTND4L* gene (*P*= 0.131), **b)** genotype frequency of rs28358281 in *MTND4L* gene (*P*= 0.3335).



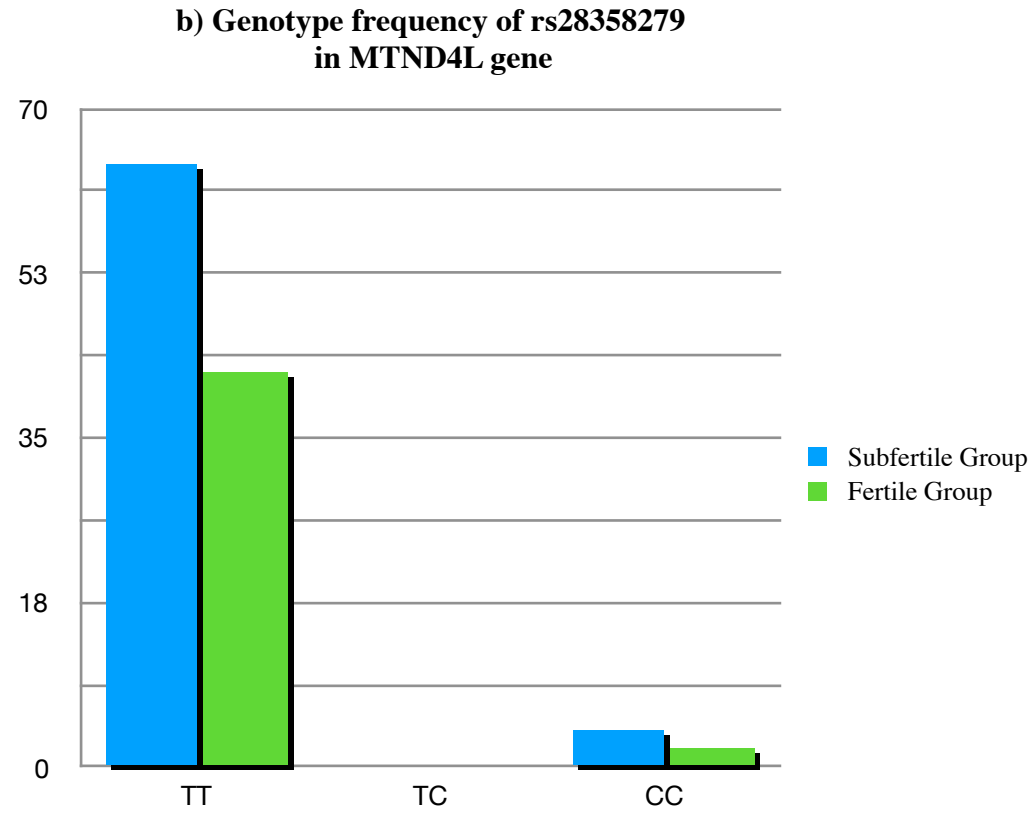




**Figure 59: a)** allele frequency of rs28358279 in *MTND4L* gene (*P*= 0.131), **b)** genotype frequency of rs28358279 in *MTND4L* gene (*P*= 0.768).

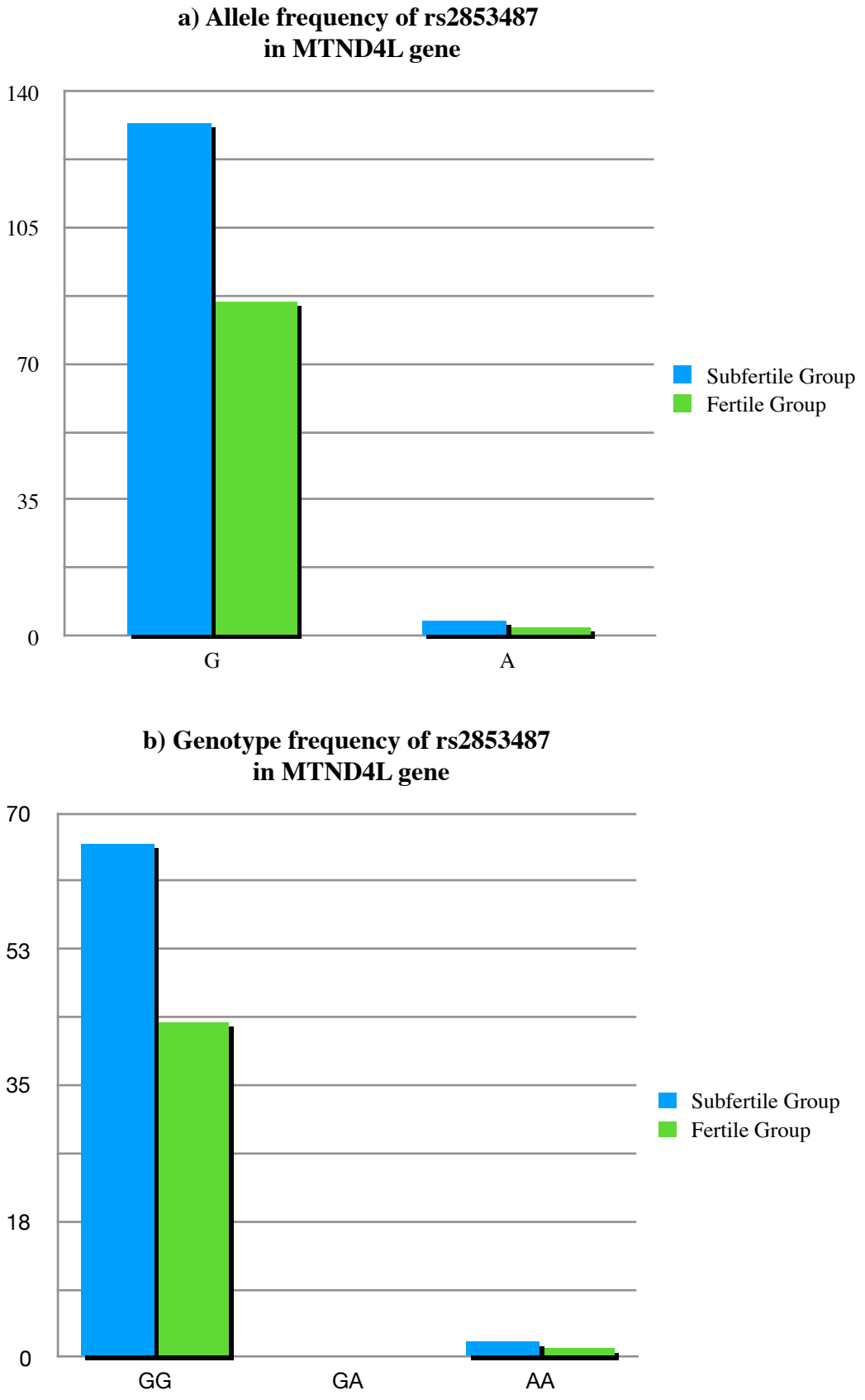



**Figure 60: a)** allele frequency of rs2853487 in *MTND4L* gene (*P*= 1.000), **b)** genotype frequency of rs2853487 in *MTND4L* gene (*P*= 0.8306).

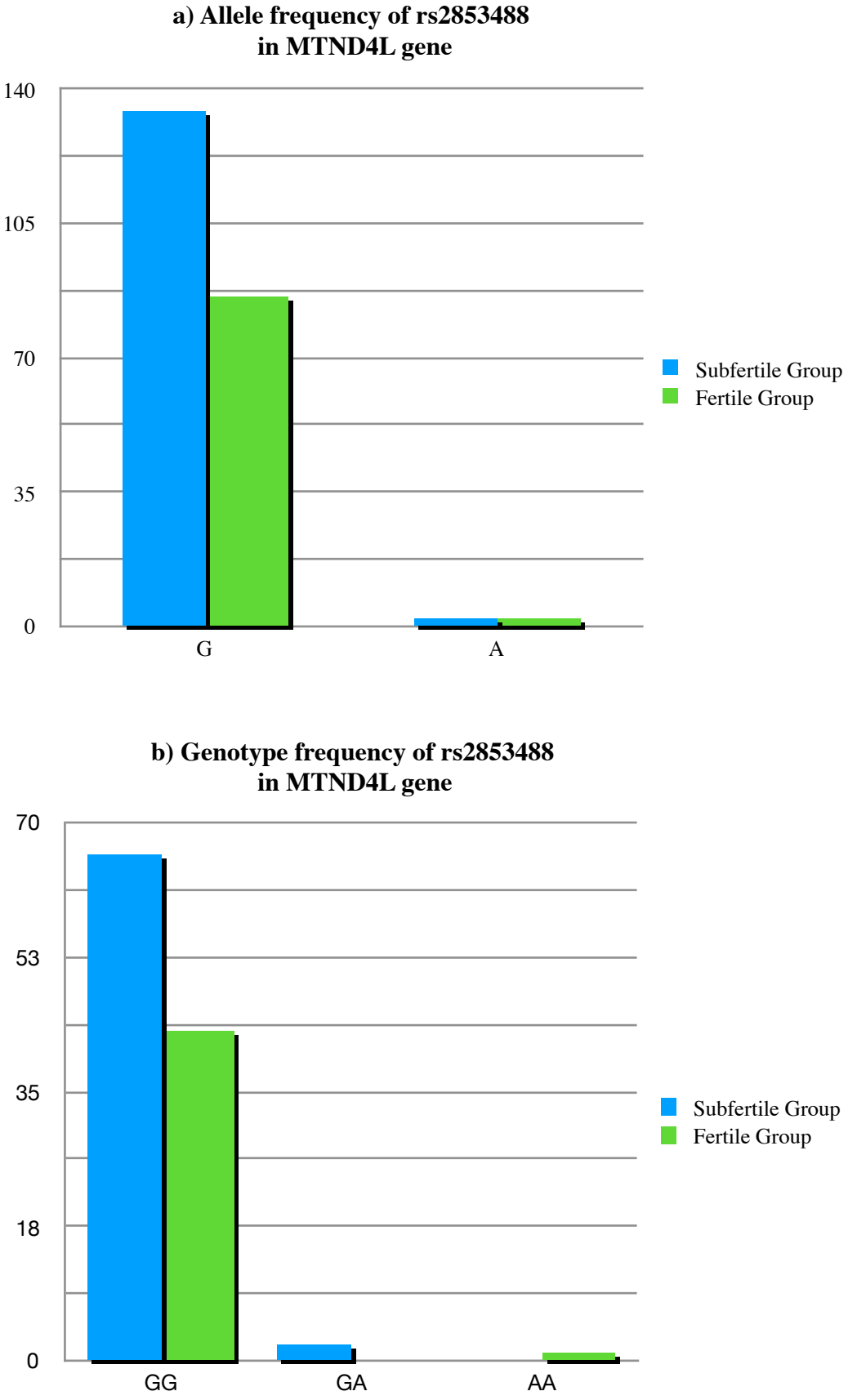



**Figure 61: a)** allele frequency of rs2853488 in *MTND4L* gene (*P*= 0.6466), **b)** genotype frequency of rs2853488 in *MTND4L* gene (*P*= 0.2416).

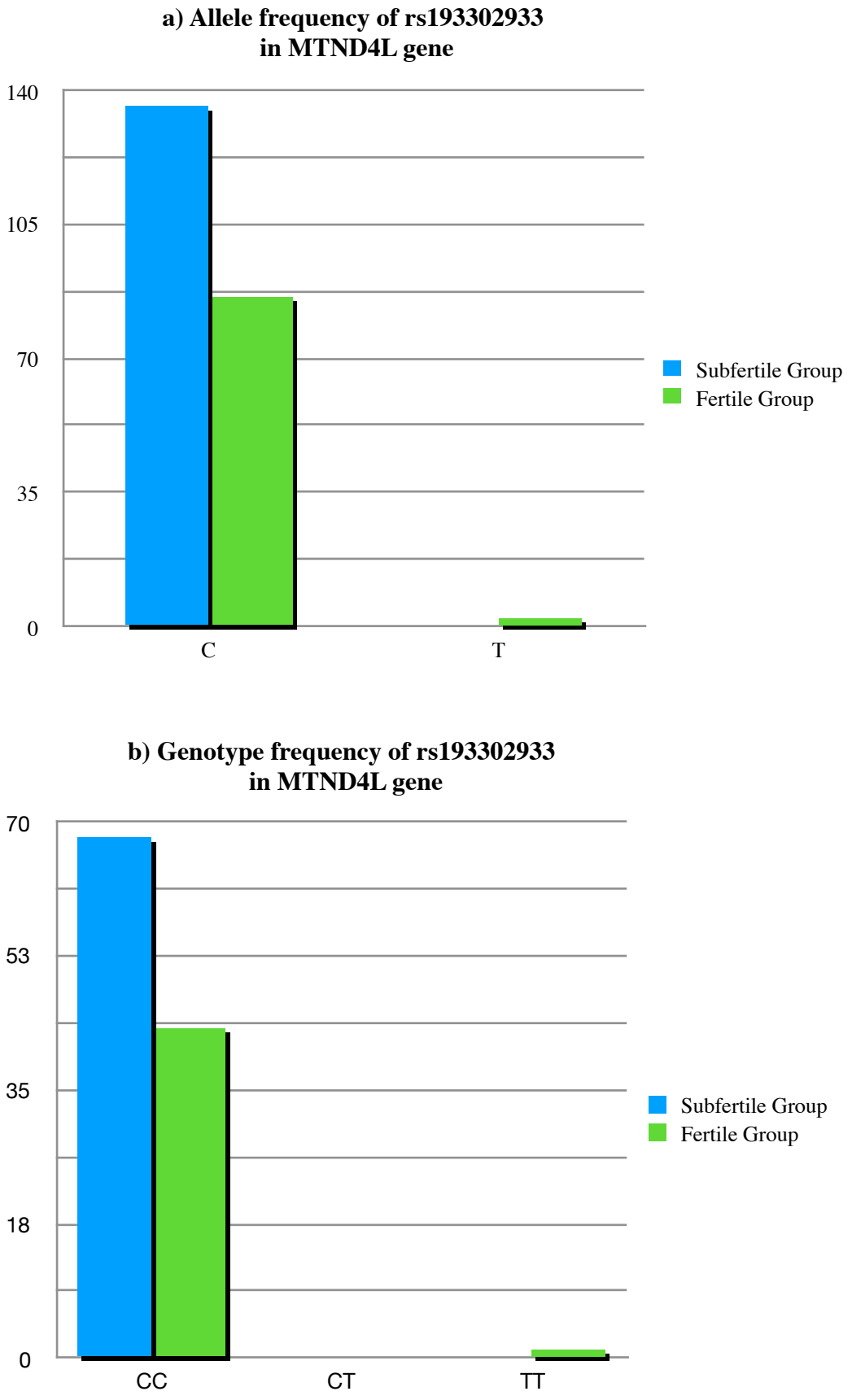



**Figure 62: a)** allele frequency of rs193302933 in *MTND4L* gene (*P*= 0.1533), **b)** genotype frequency of rs193302933 in *MTND4L* gene (*P*= 0.2118).

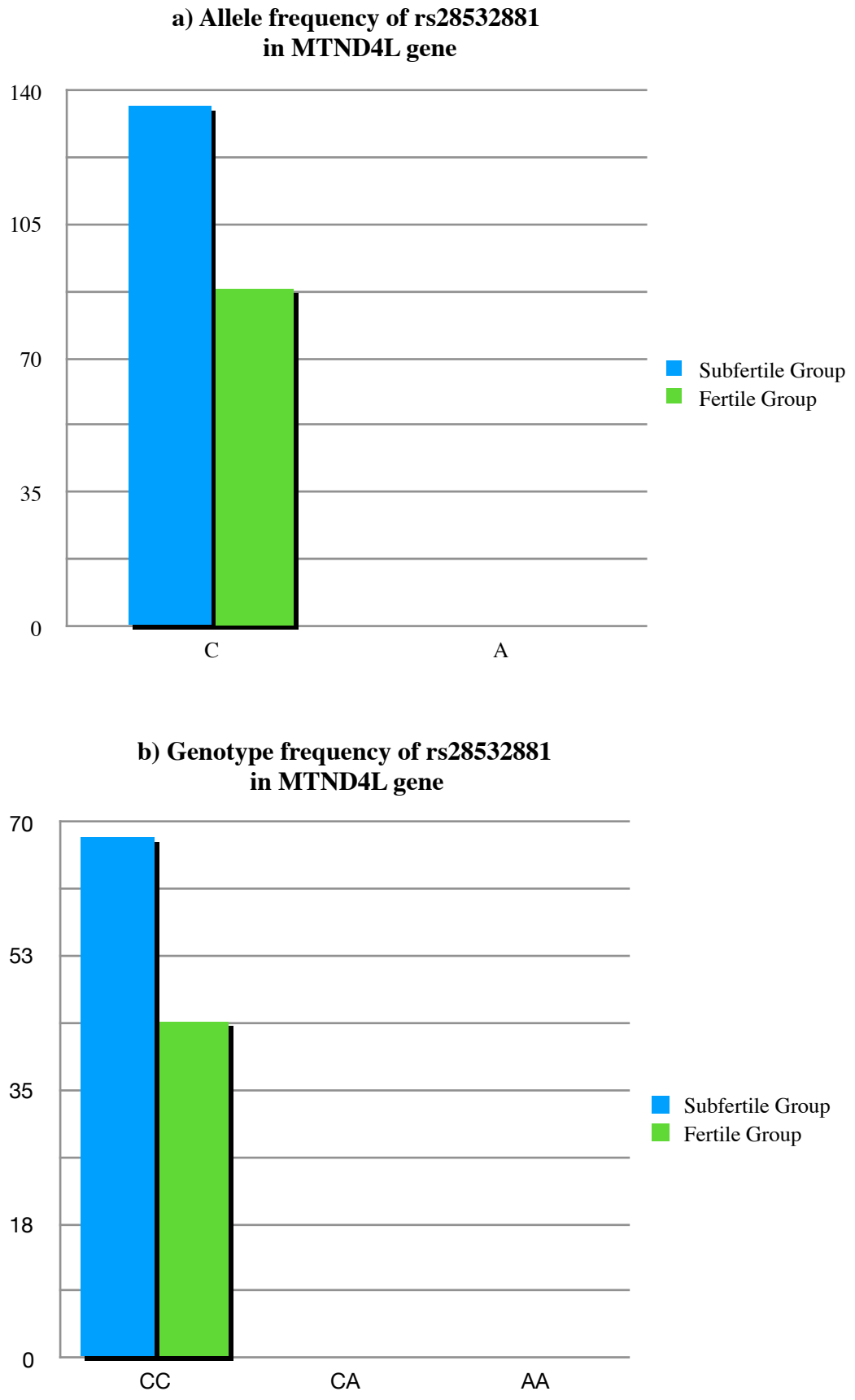



**Figure 63: a)** allele frequency of rs28532881 in *MTND4L* gene, **b)** genotype frequency of rs28532881 in *MTND4L* gene.

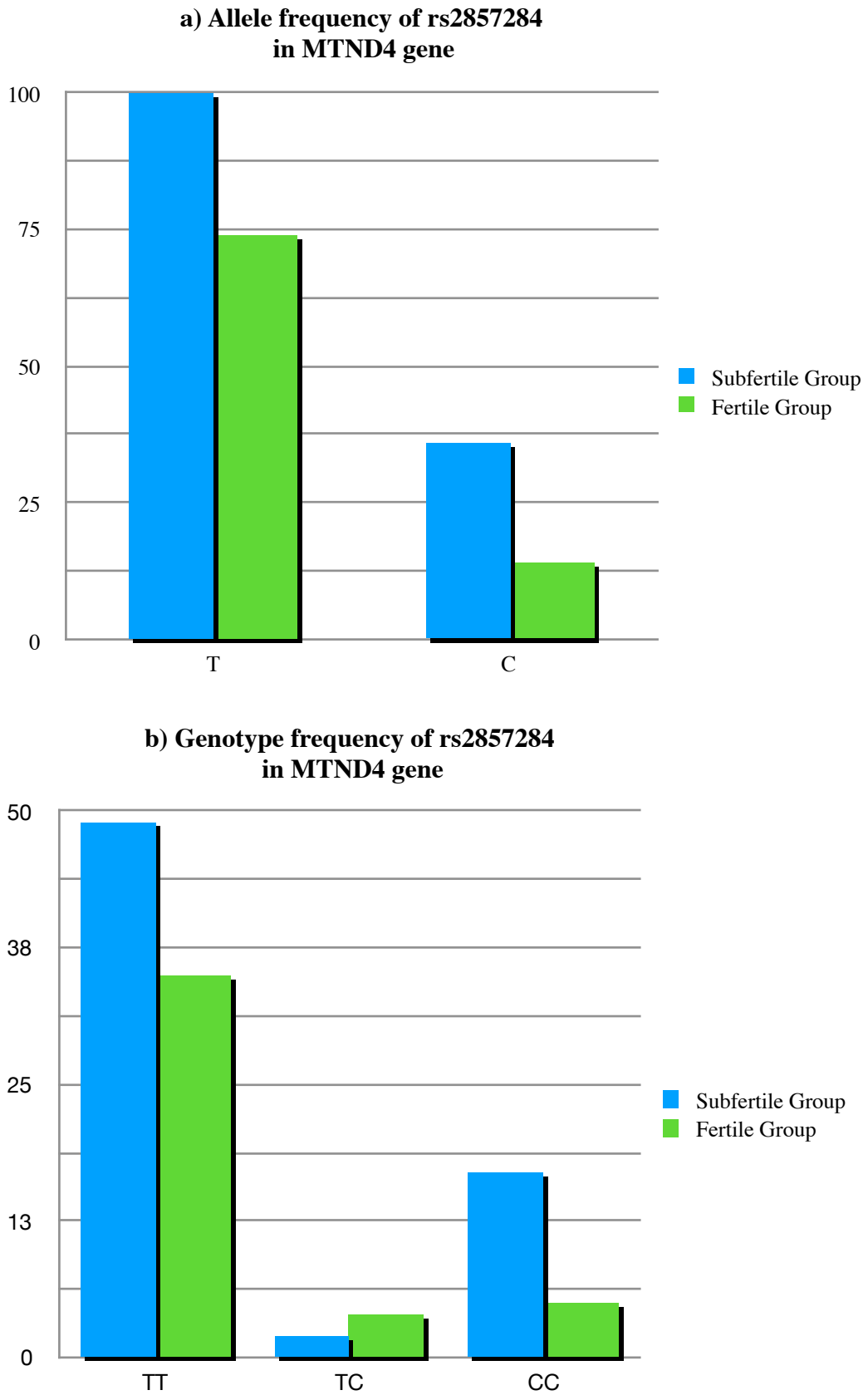



**Figure 64: a)** allele frequency of rs2857284 in *MTND4* gene (*P*= 0.071), **b)** genotype frequency of rs2857284 in *MTND4* gene (*P*= 0.0995).

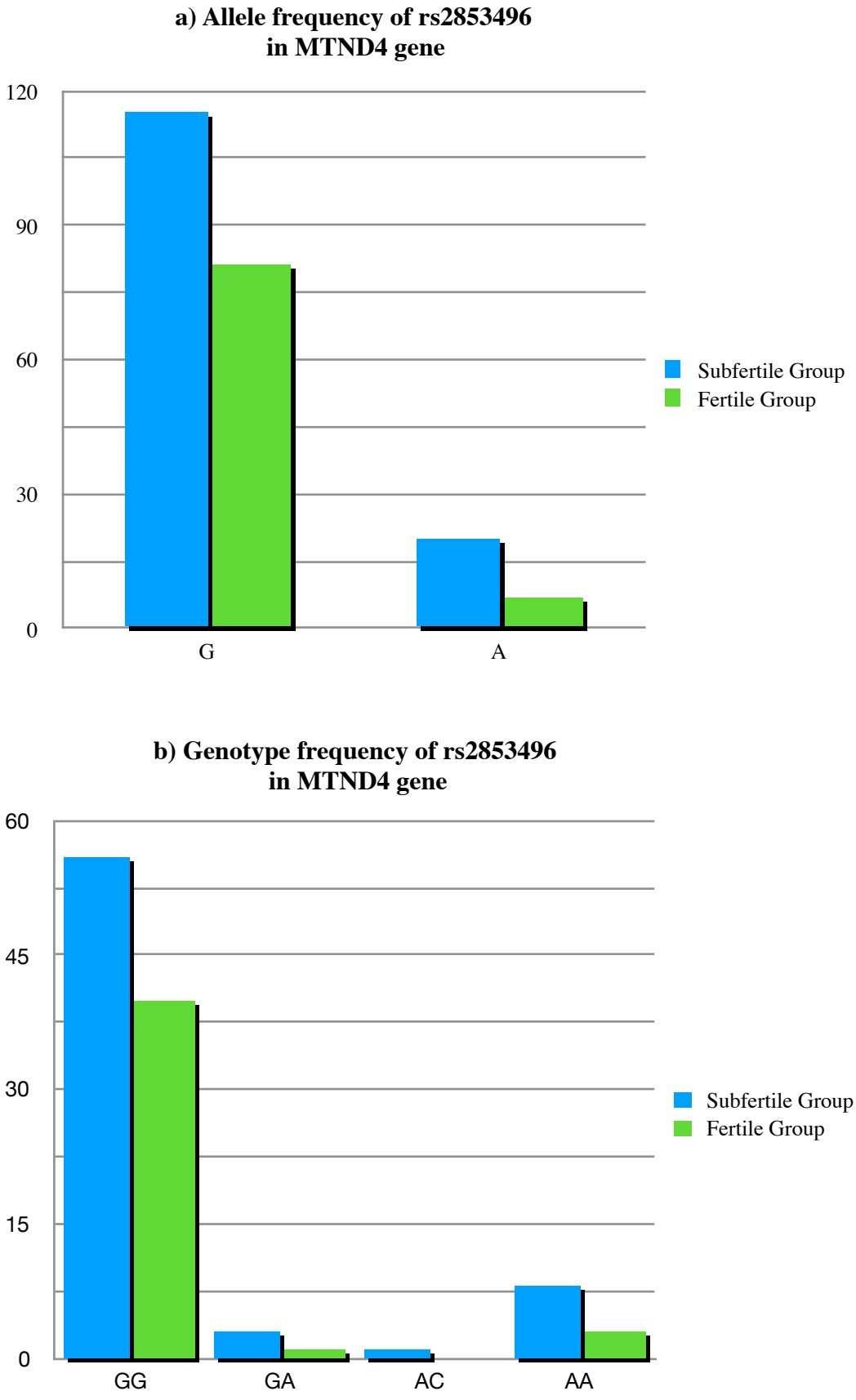



**Figure 65: a)** allele frequency of rs2853496 in *MTND4* gene (*P*= 0.145), **b)** genotype frequency of rs2853496 in *MTND4* gene (*P*= 0.597).

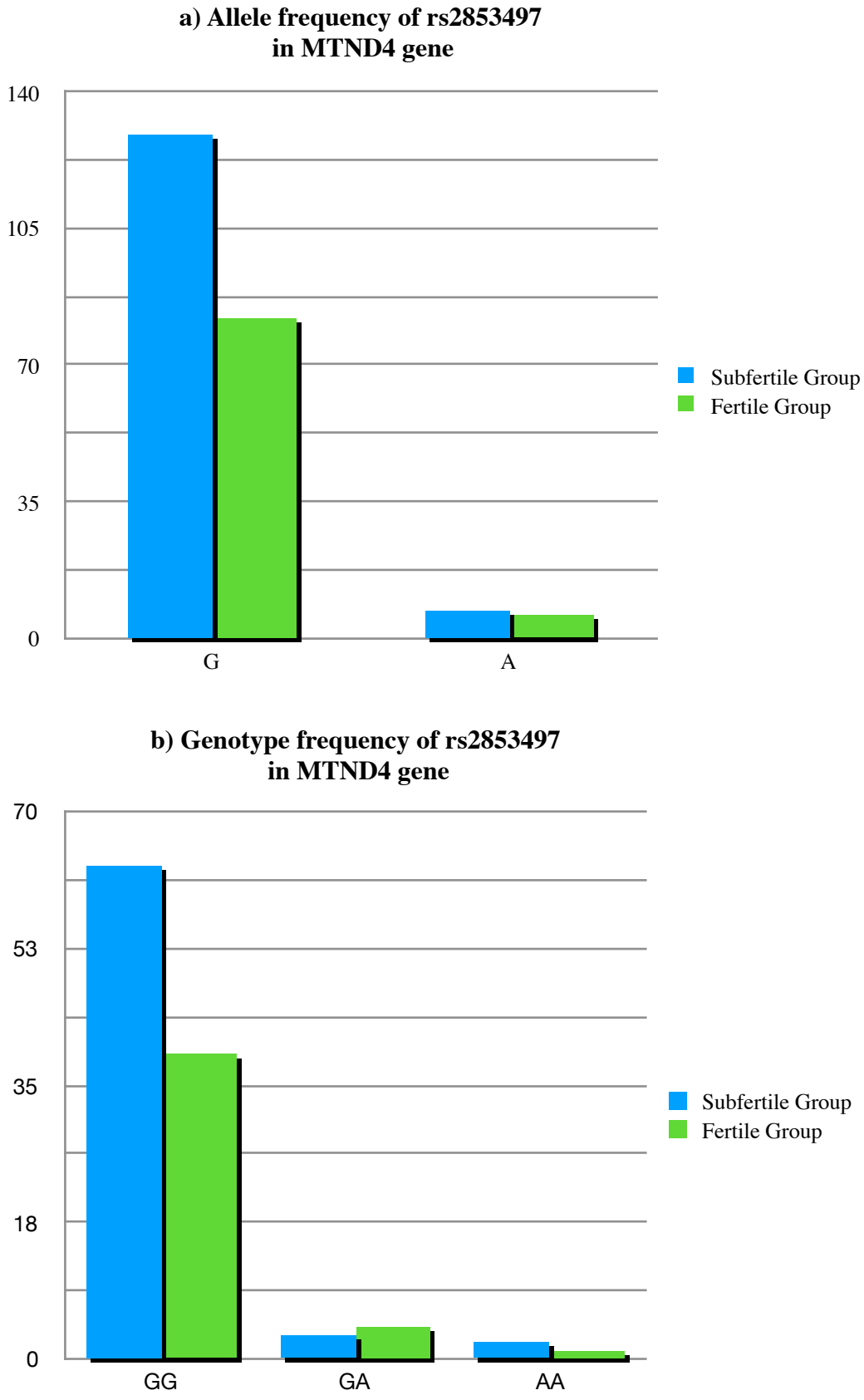



**Figure 66: a)** allele frequency of rs2853497 in *MTND4* gene (*P*= 0.771), **b)** genotype frequency of rs2853497 in *MTND4* gene (*P*= 0.598).

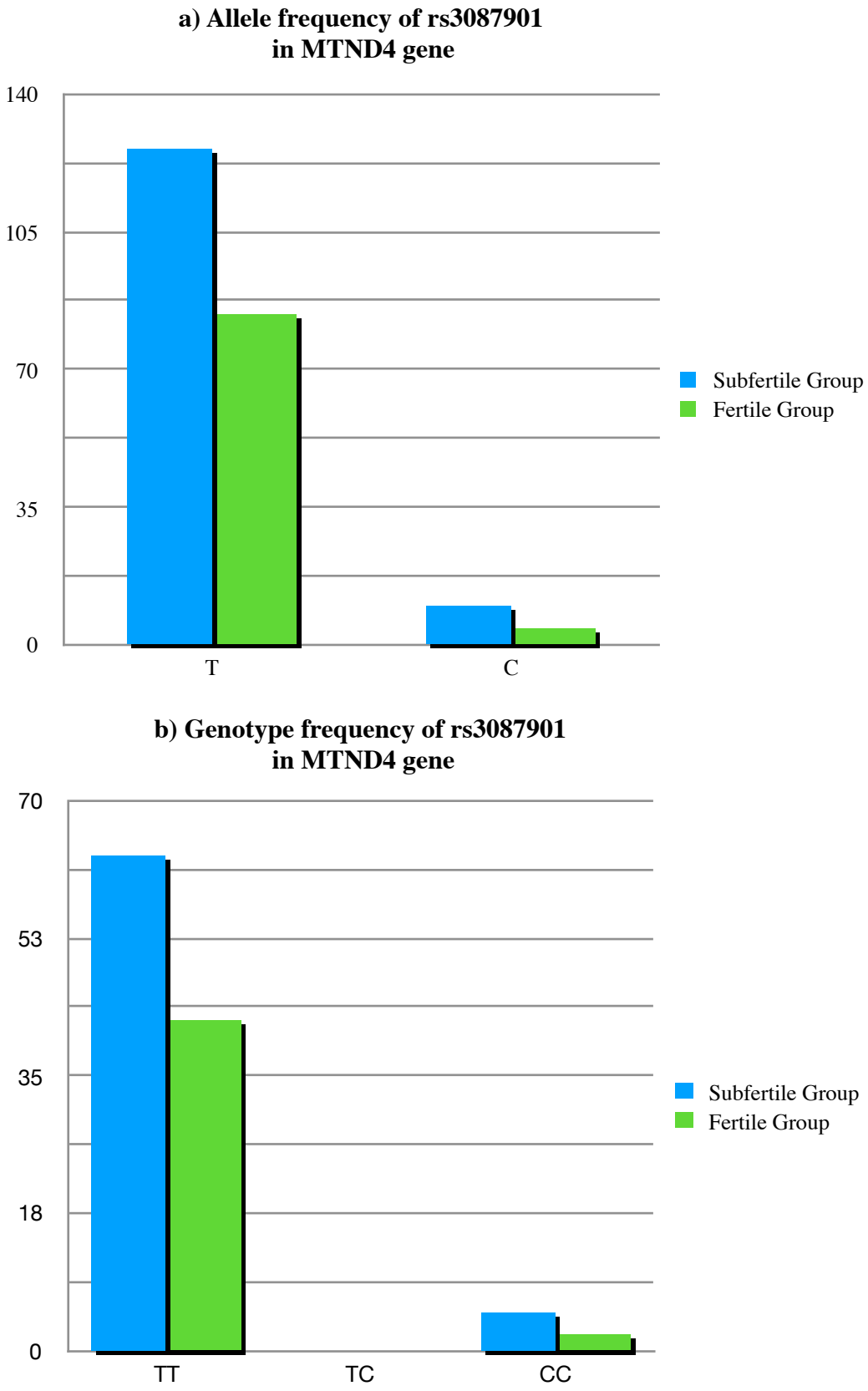



**Figure 67: a)** allele frequency of rs3087901 in *MTND4* gene (*P*= 0.573), **b)** genotype frequency of rs3087901 in *MTND4* gene (*P*= 0.548).

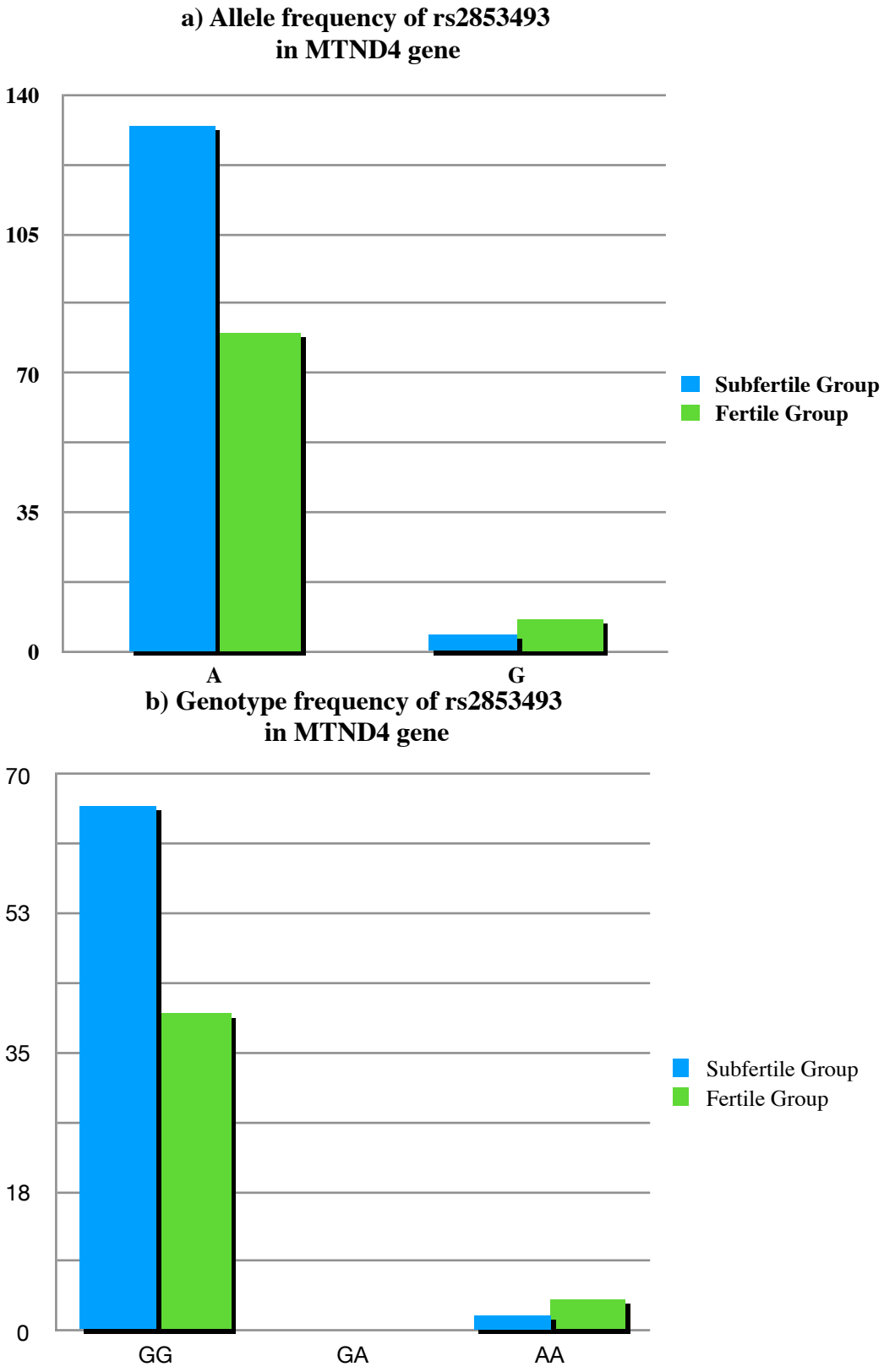



**Figure 68: a)** allele frequency of rs2853493 in *MTND4* gene (*P*= 0.066), **b)** genotype frequency of rs2853493 in *MTND4* gene (*P*= 0.158).

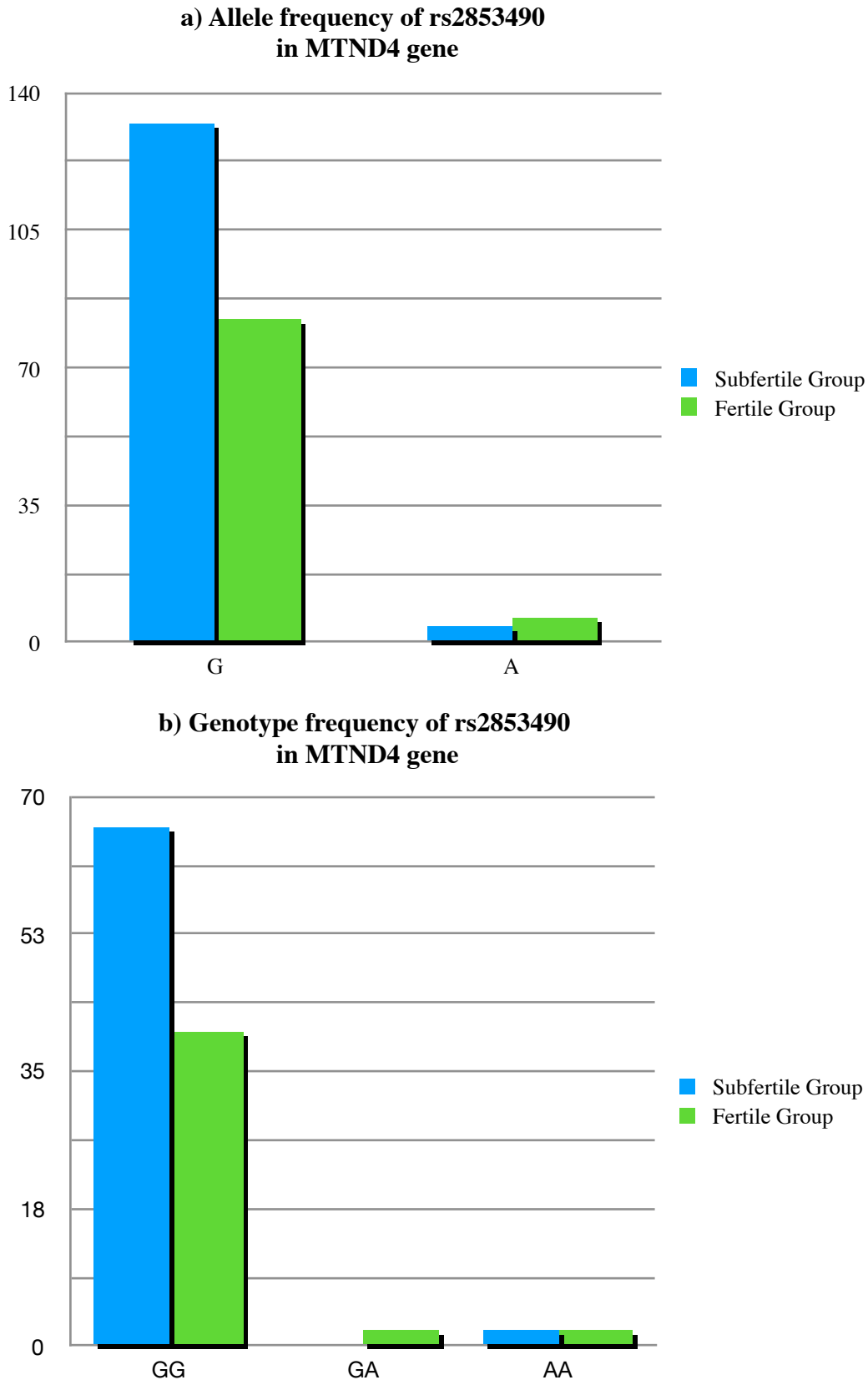



**Figure 69: a)** allele frequency of rs2853490 in *MTND4* gene (*P*= 0.196), **b)** genotype frequency of rs2853490 in *MTND4* gene (*P*= 0.183).

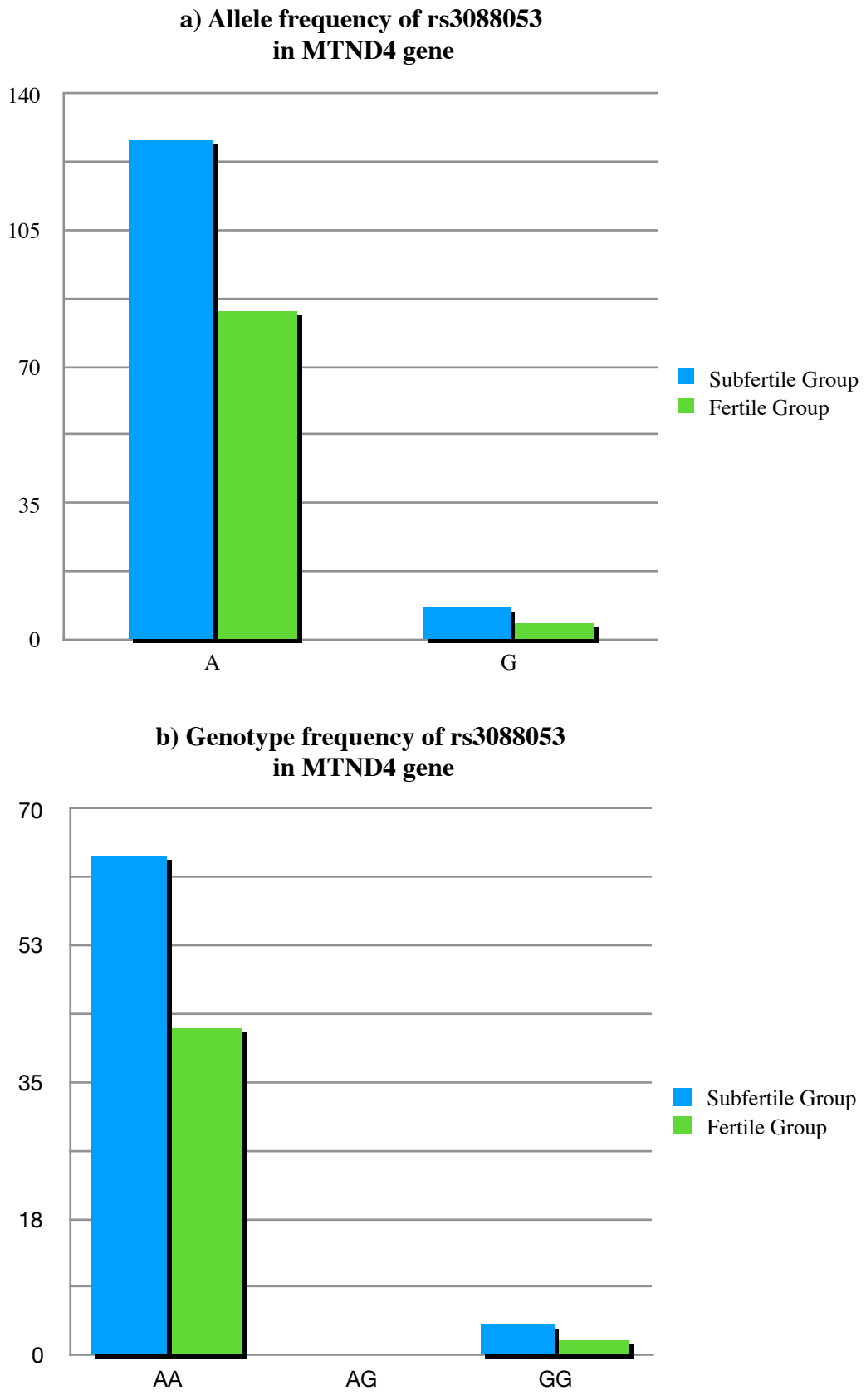



**Figure 70: a)** allele frequency of rs3088053 in *MTND4* gene (*P*= 0.758), **b)** genotype frequency of rs3088053 in *MTND4* gene (*P*= 0.183).

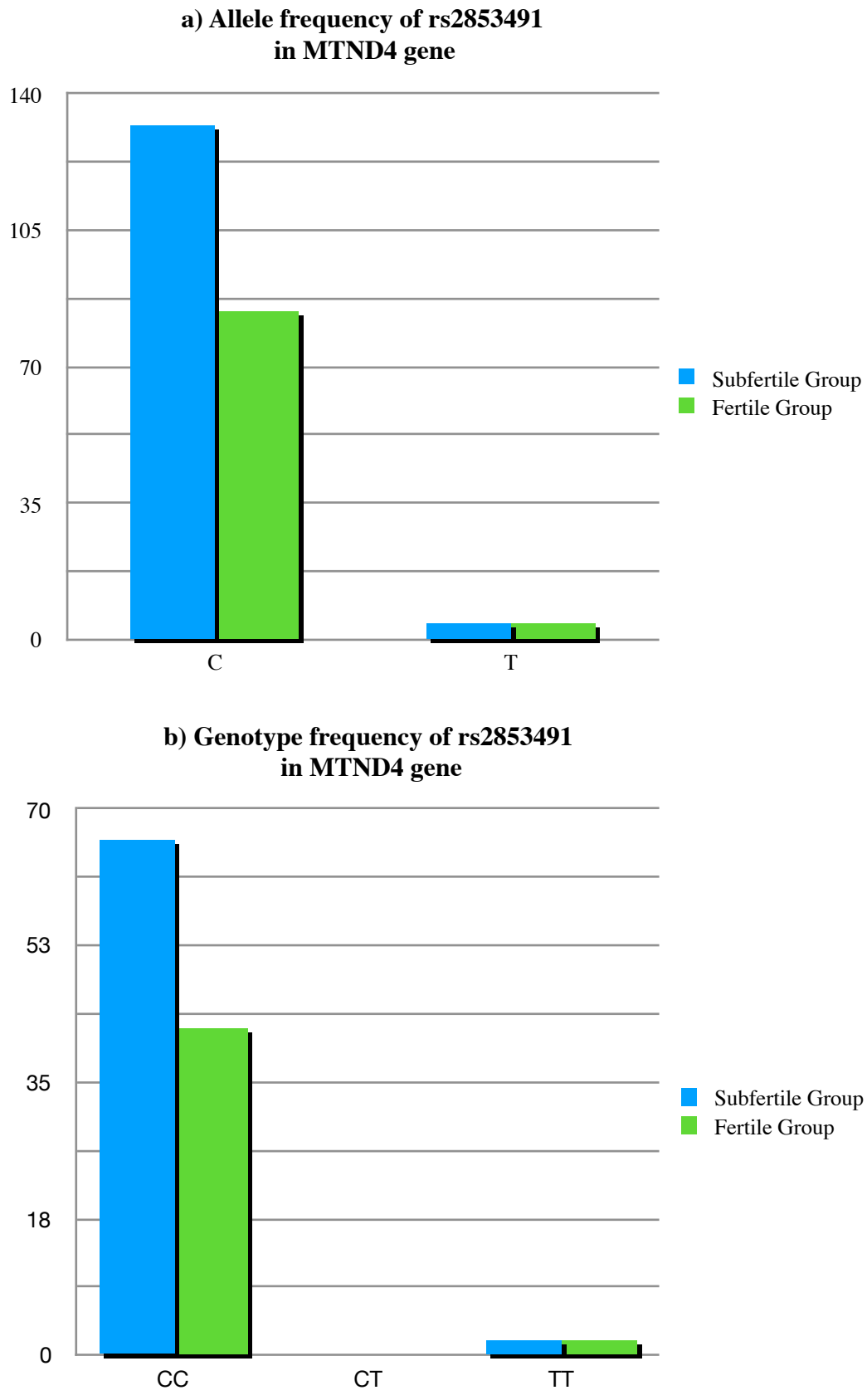



**Figure 71: a)** allele frequency of rs2853491 in *MTND4* gene (*P*= 0.714), **b)** genotype frequency of rs2853491 in *MTND4* gene (*P*= 0.655).

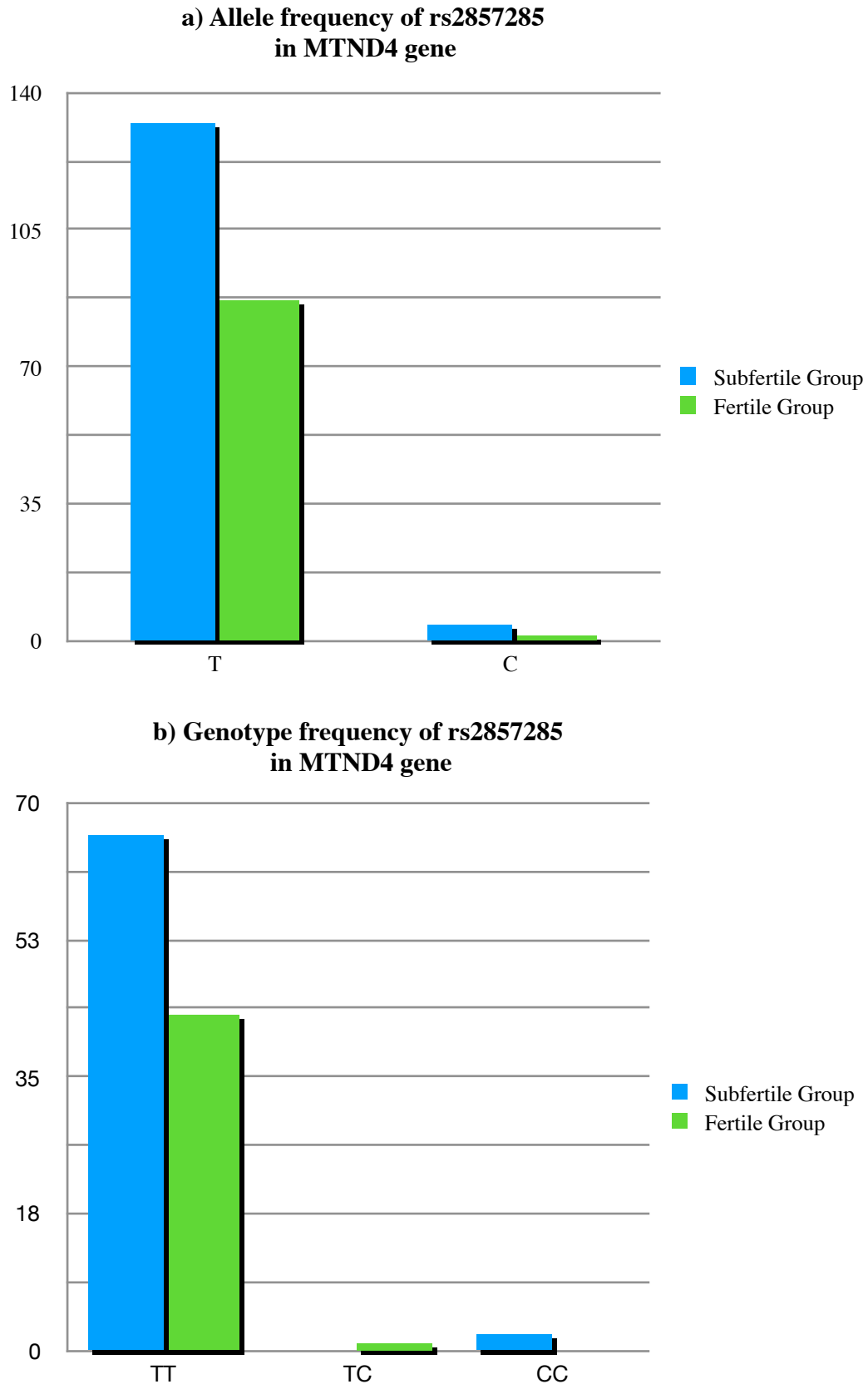



**Figure 72: a)** allele frequency of rs2857285 in *MTND4* gene (*P*= 0.650), **b)** genotype frequency of rs2857285 in *MTND4* gene (*P*= 0.241).

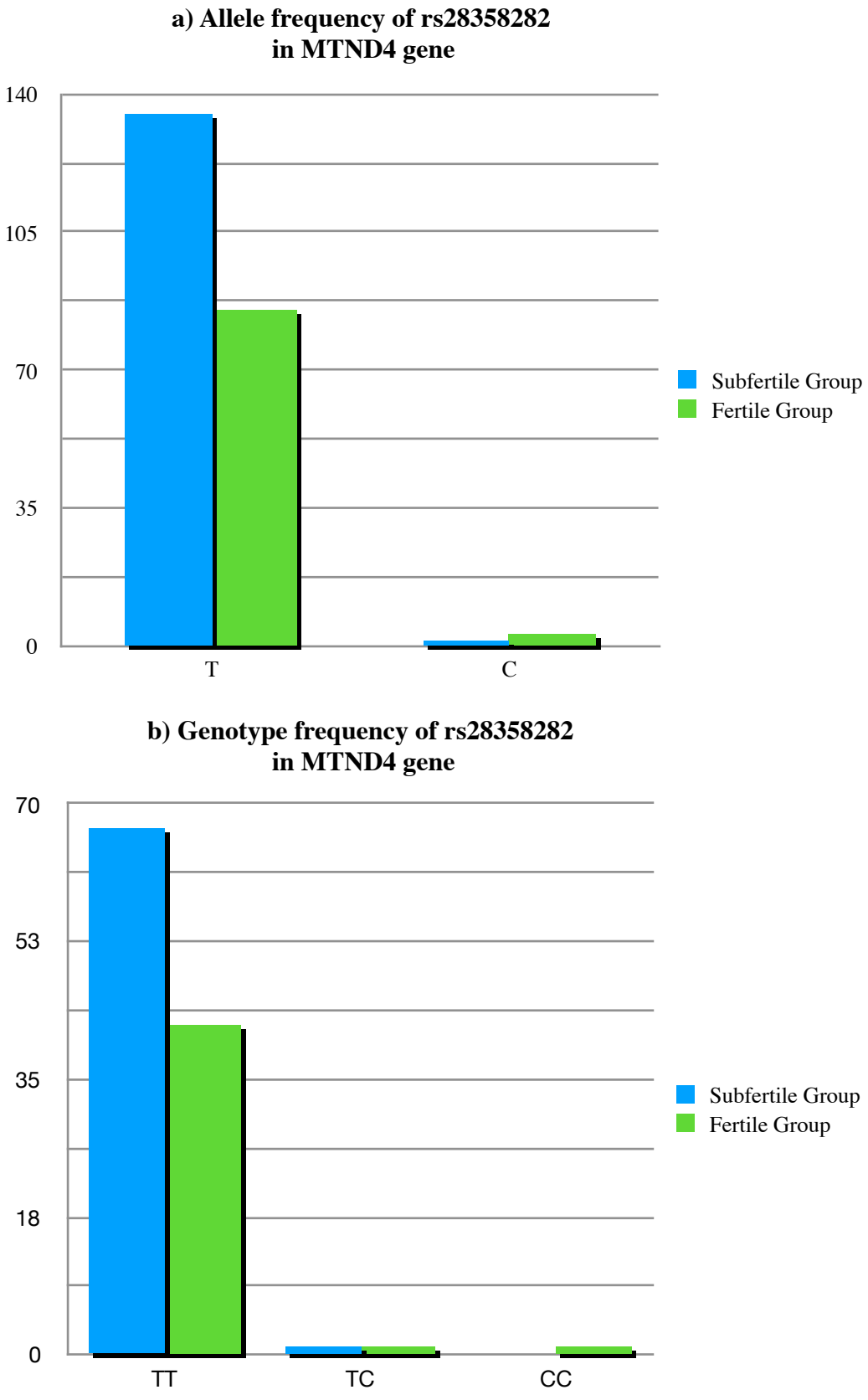



**Figure 73: a)** allele frequency of rs28358282 in *MTND4* gene (*P*= 0.302), **b)** genotype frequency of rs28358282 in *MTND4* gene (*P*= 0.434).

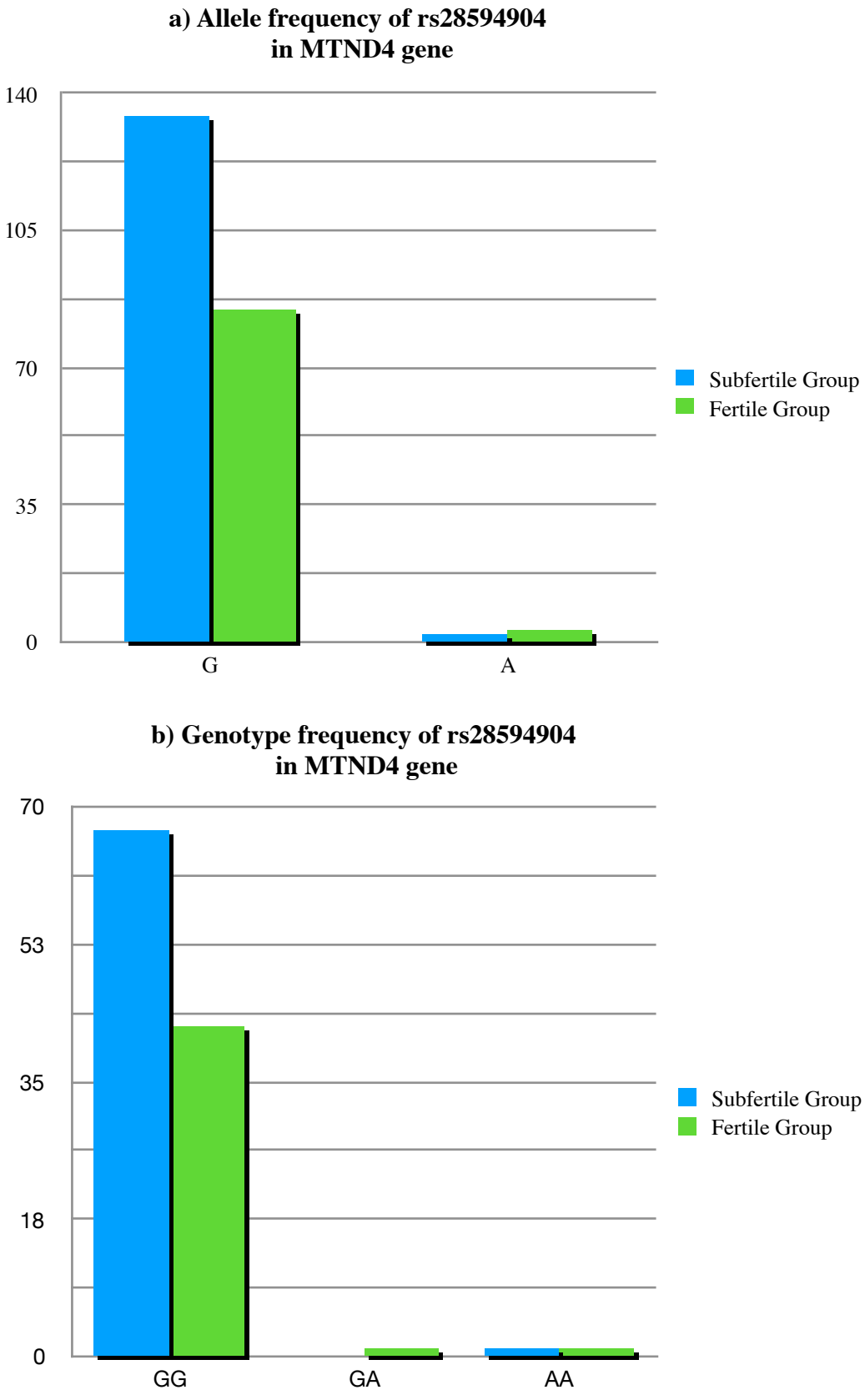



**Figure 74: a)** allele frequency of rs28594904 in *MTND4* gene (*P*= 0.383), **b)** genotype frequency of rs28594904 in *MTND4* gene (*P*= 0.434).

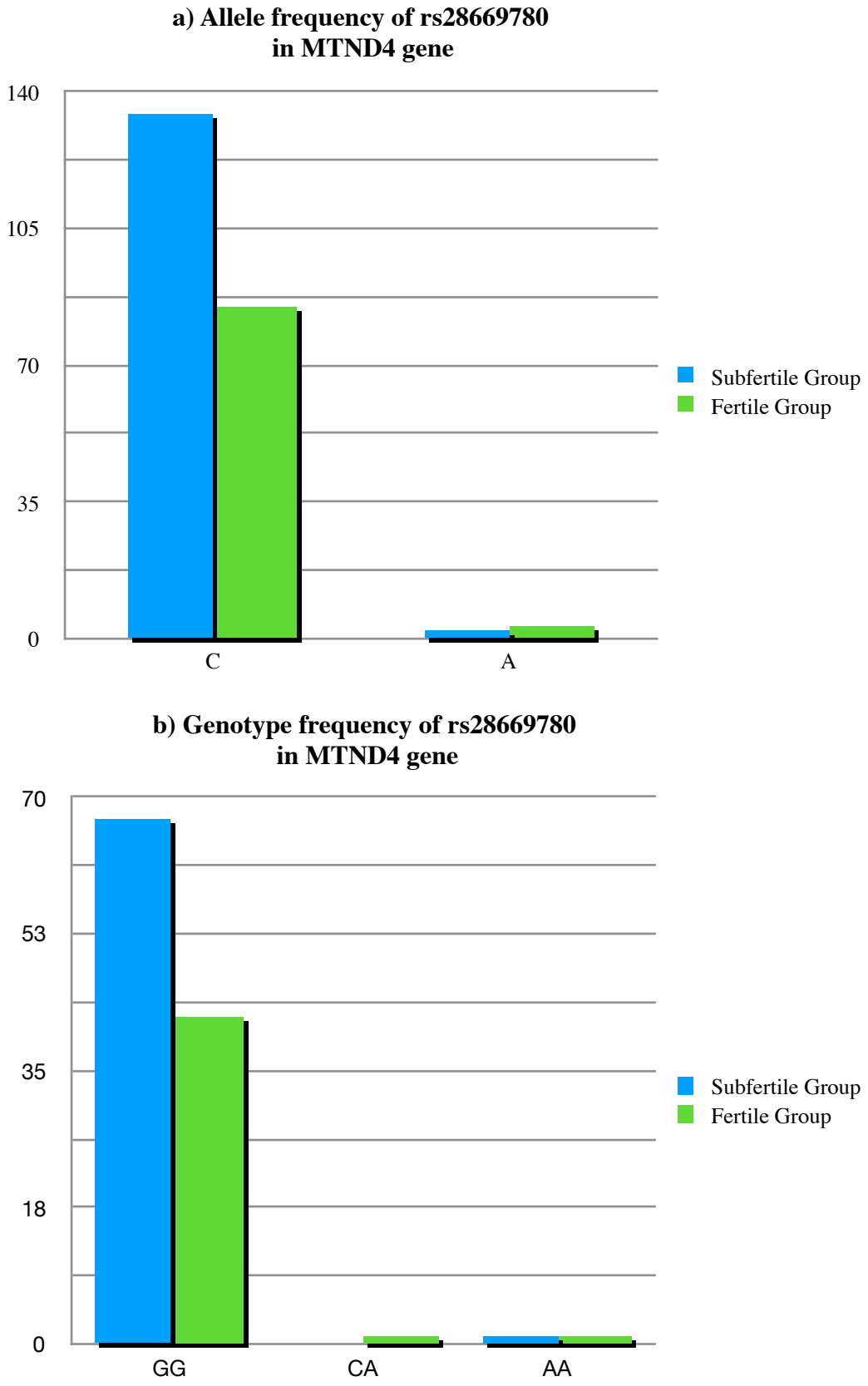



**Figure 75: a)** allele frequency of rs28669780 in *MTND4* gene (*P*= 0.383), **b)** genotype frequency of rs28669780 in *MTND4* gene (*P*= 0.434).

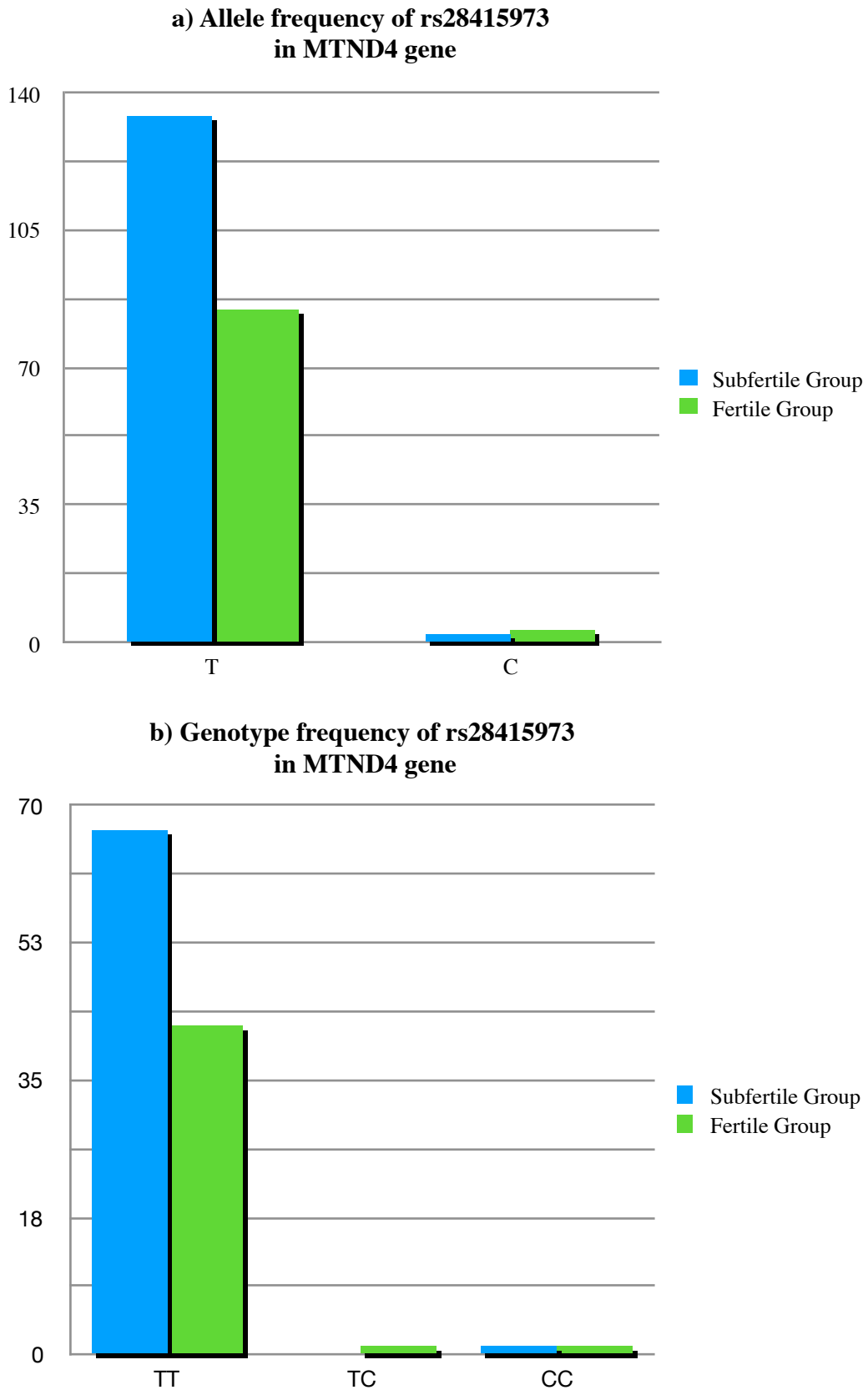



**Figure 76: a)** allele frequency of rs28415973 in *MTND4* gene (*P*= 0.383), **b)** genotype frequency of rs28415973 in *MTND4* gene (*P*= 0.434).

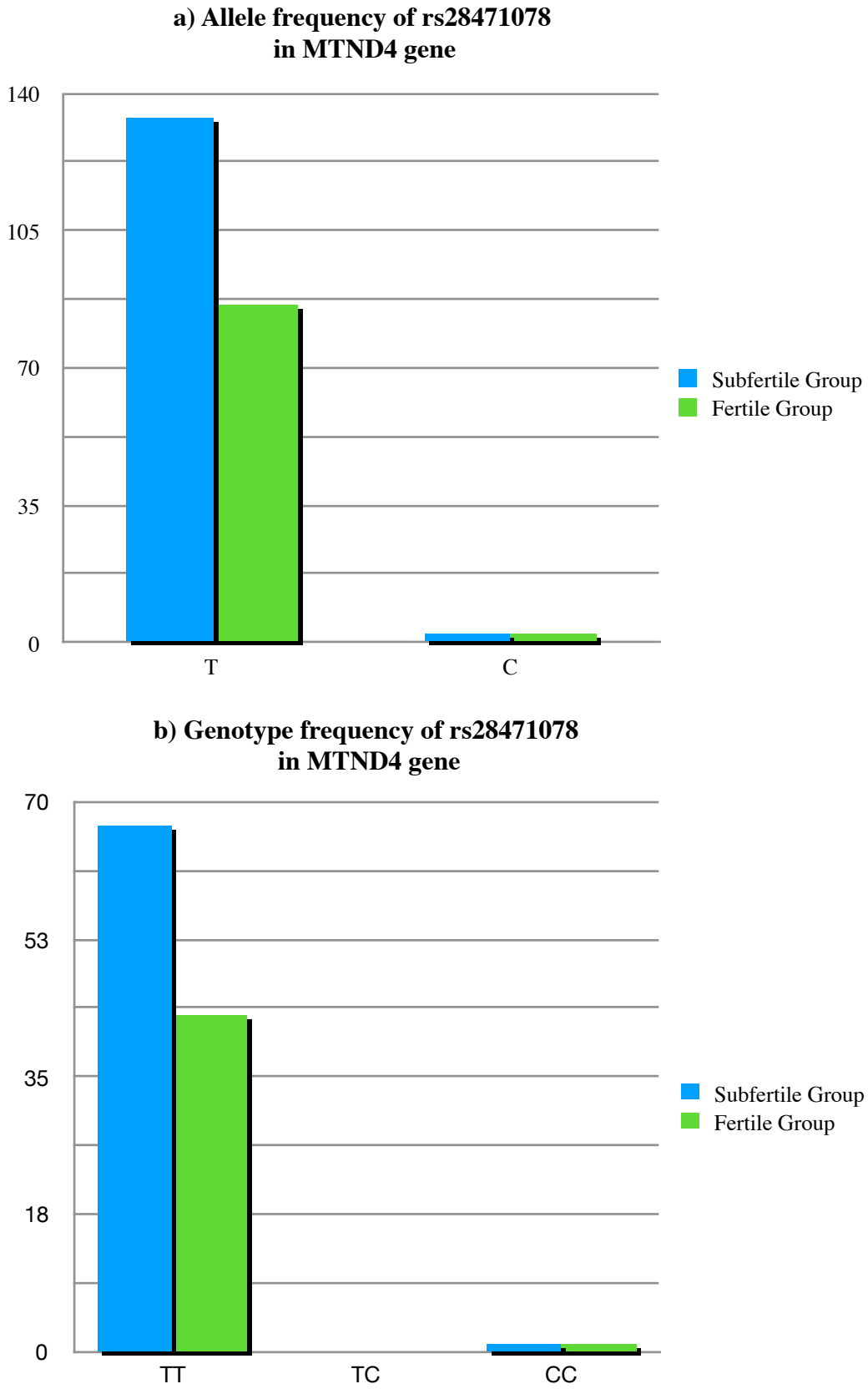



**Figure 77: a)** allele frequency of rs28471078 in *MTND4* gene (*P*= 0.646), **b)** genotype frequency of rs28471078 in *MTND4* gene (*P*= 0.754).

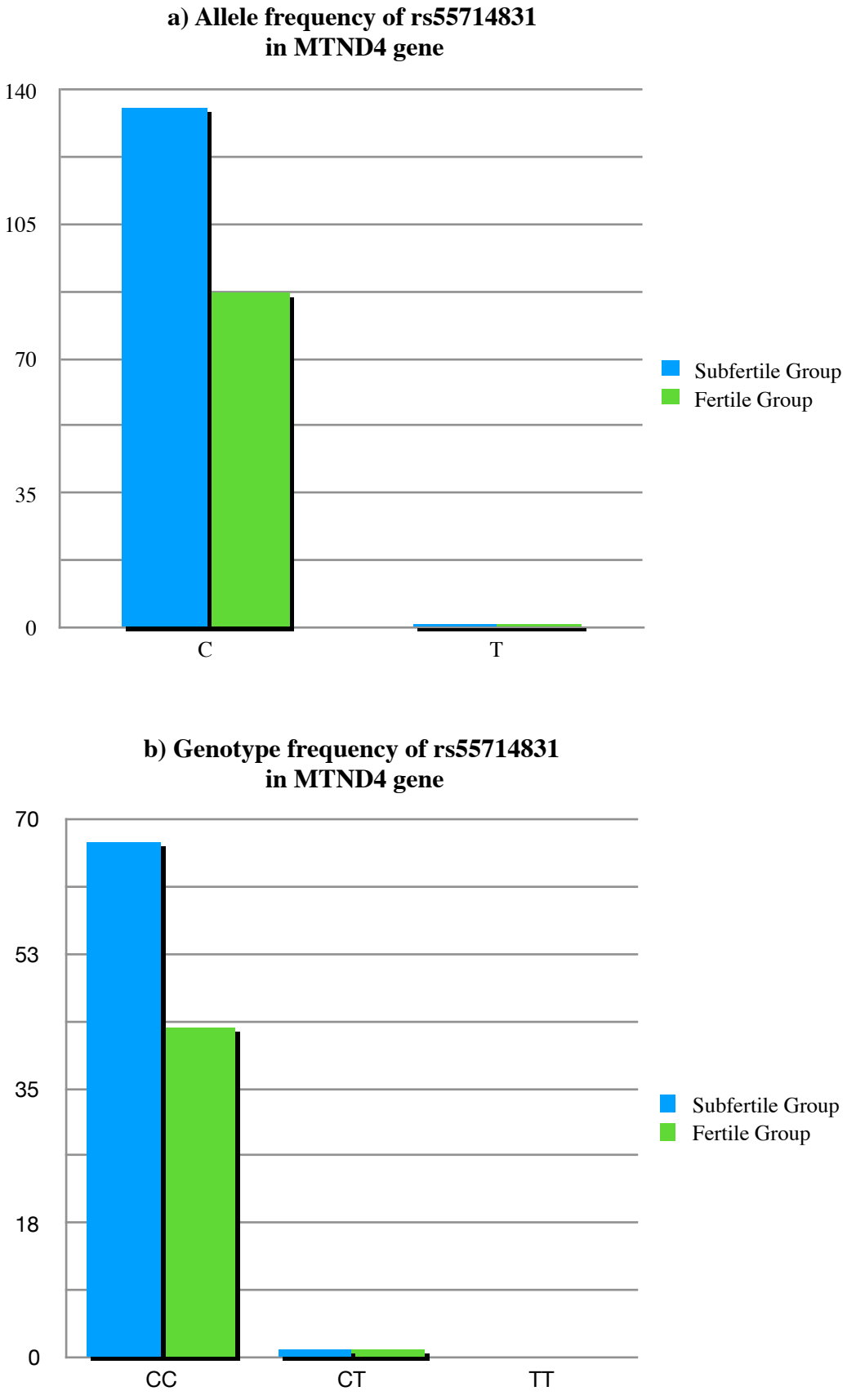



**Figure 78: a)** allele frequency of rs55714831 in *MTND4* gene (*P*= 1.000), **b)** genotype frequency of rs55714831 in *MTND4* gene (*P*= 0.754).

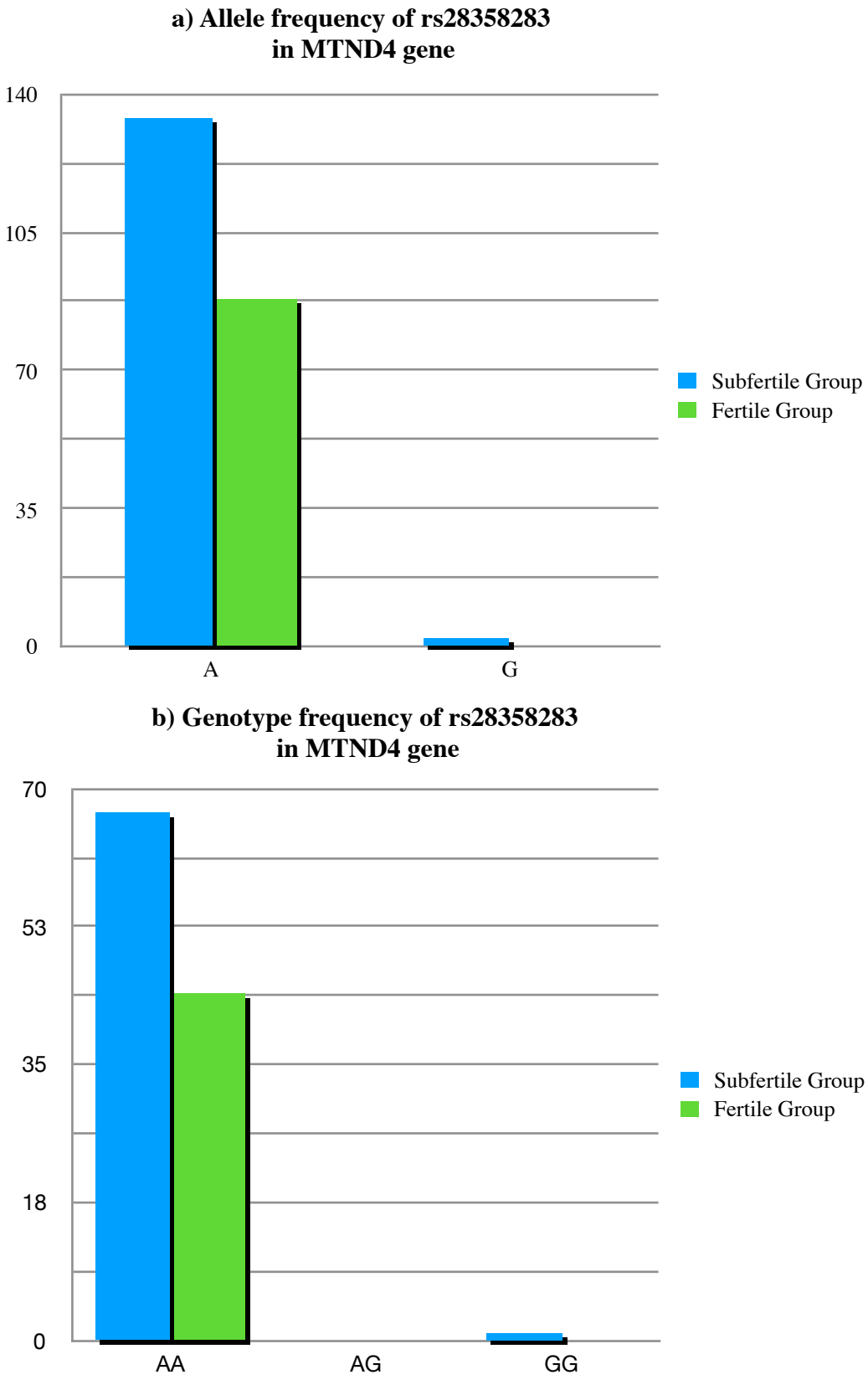



**Figure 79: a)** allele frequency of rs28358283 in *MTND4* gene (*P*= 0.520), **b)** genotype frequency of rs28358283 in *MTND4* gene (*P*= 0.419).

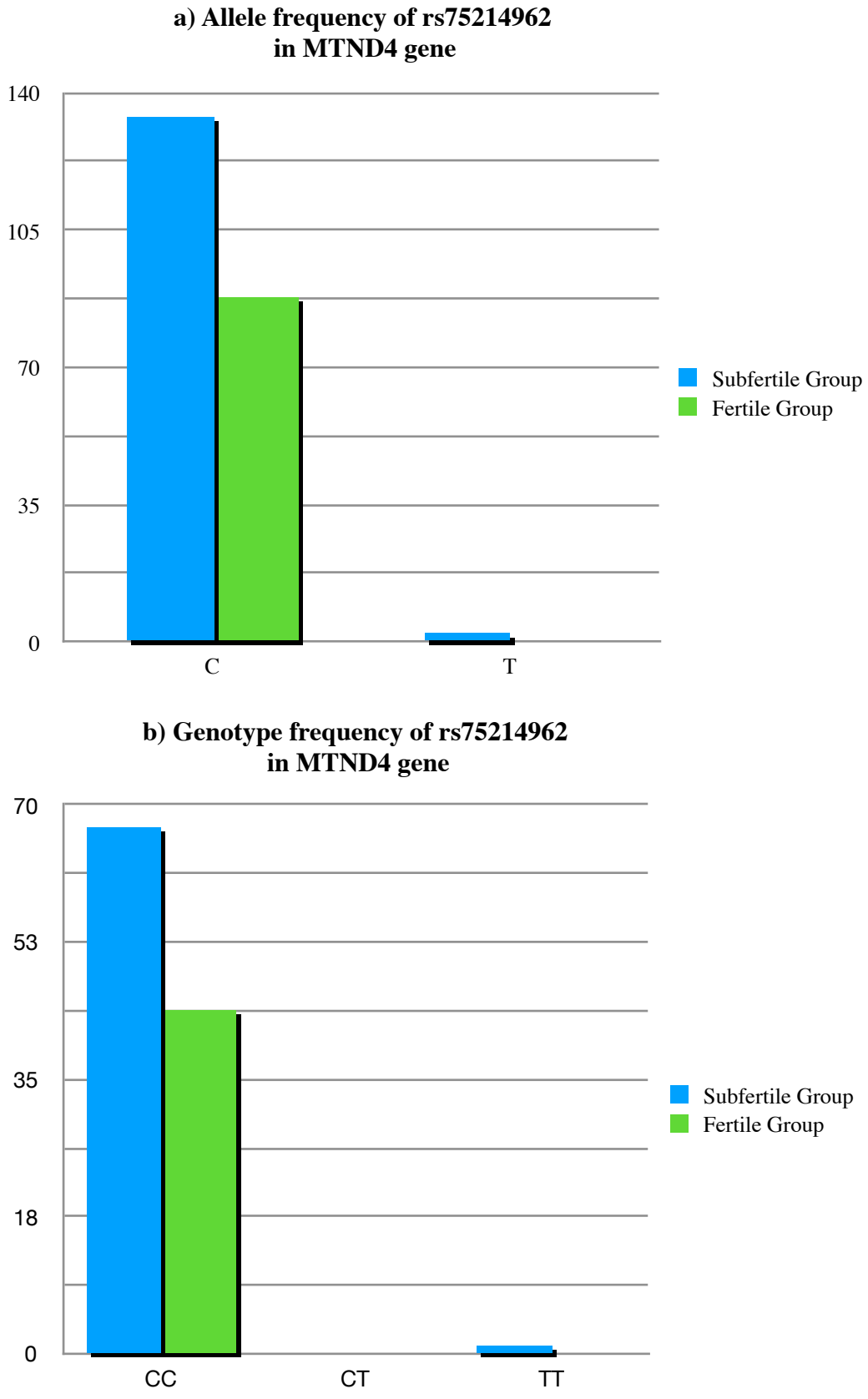



**Figure 80: a)** allele frequency of rs75214962 in *MTND4* gene (*P*= 0.520), **b)** genotype frequency of rs75214962 in *MTND4* gene (*P*= 0.419).

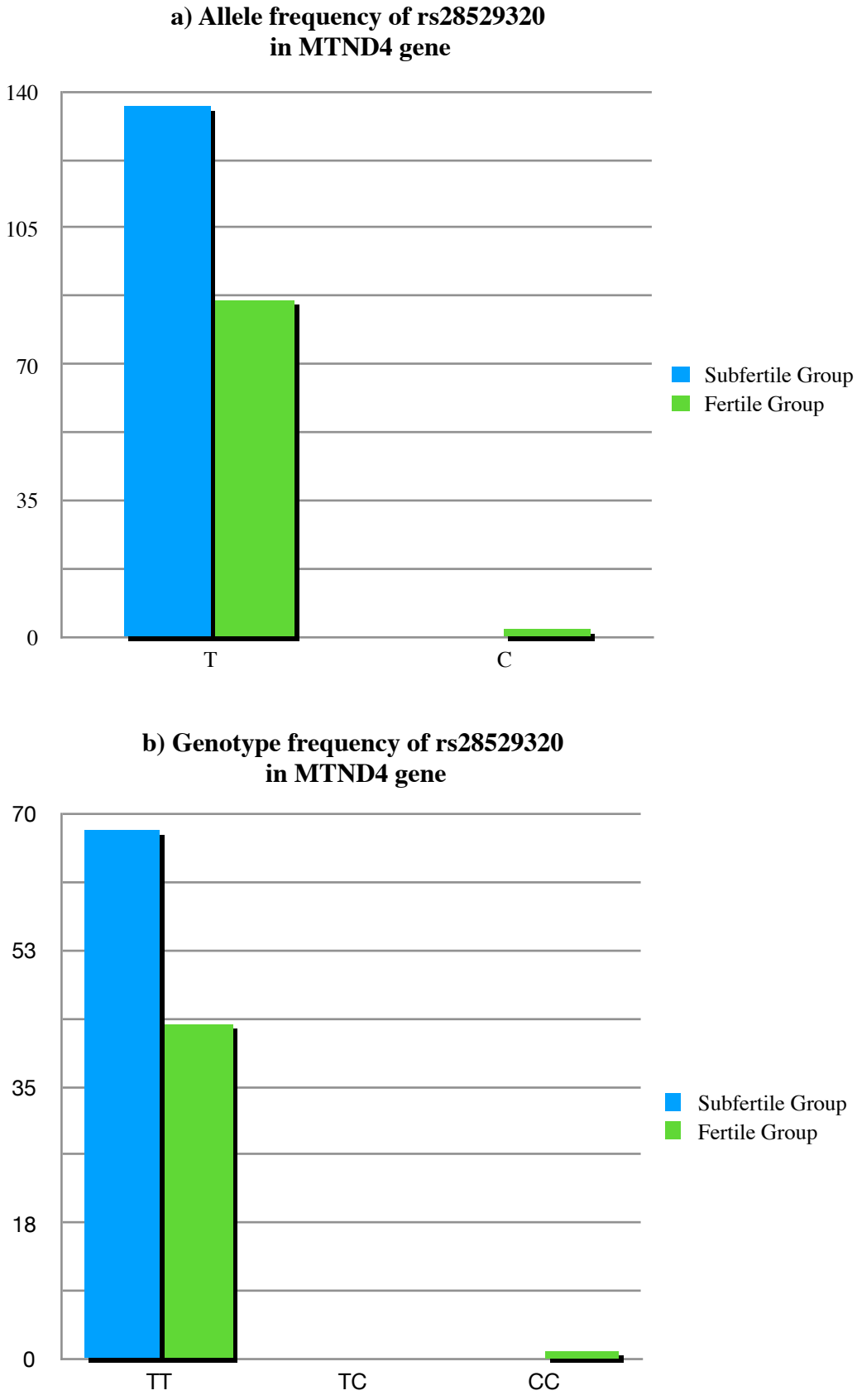



**Figure 81: a)** allele frequency of rs28529320 in *MTND4* gene (*P*= 0.153), **b)** genotype frequency of rs28529320 in *MTND4* gene (*P*= 0.211).

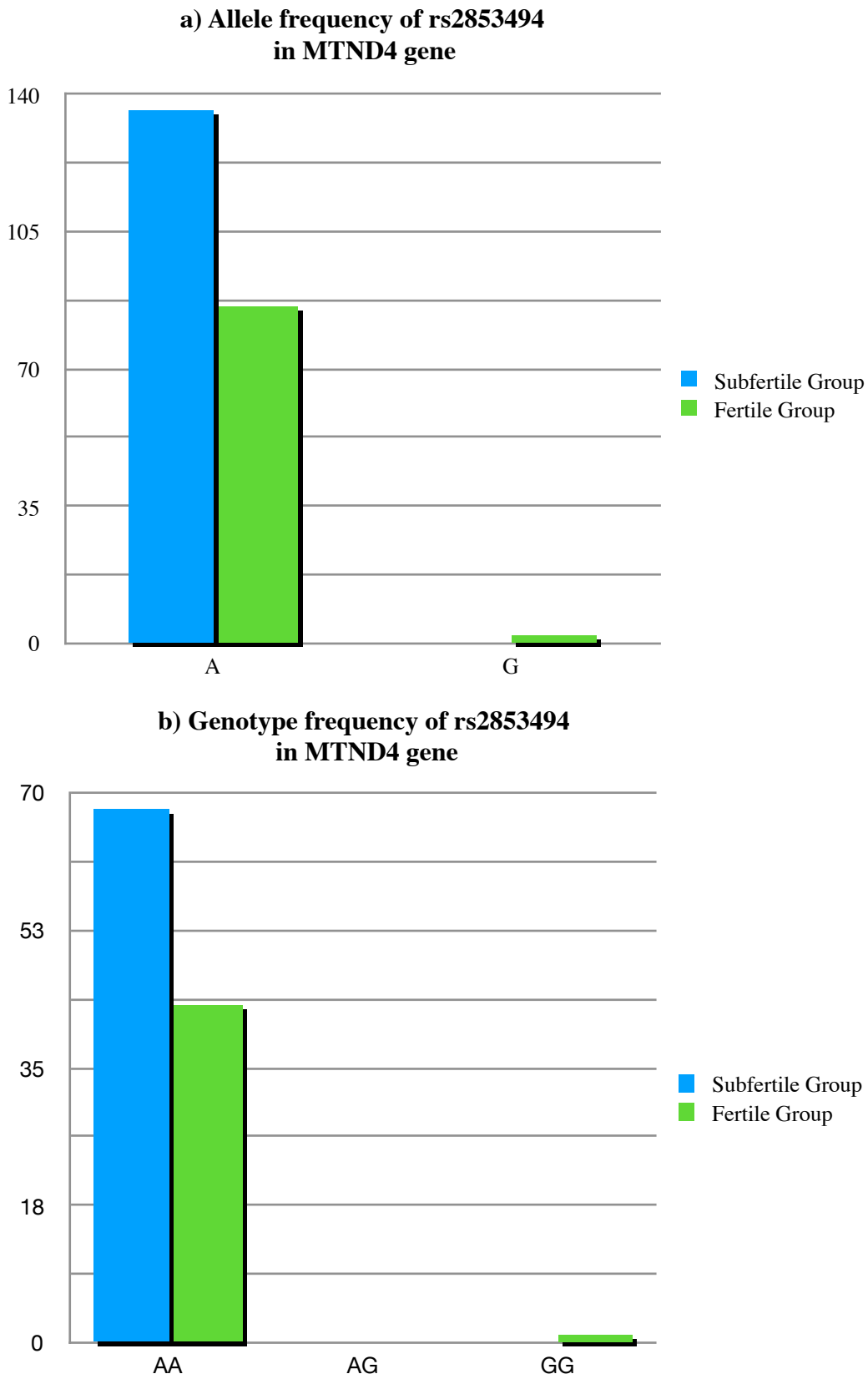



**Figure 82: a)** allele frequency of rs2853494 in *MTND4* gene (*P*= 0.153), **b)** genotype frequency of rs2853494 in *MTND4* gene (*P*= 0.211).

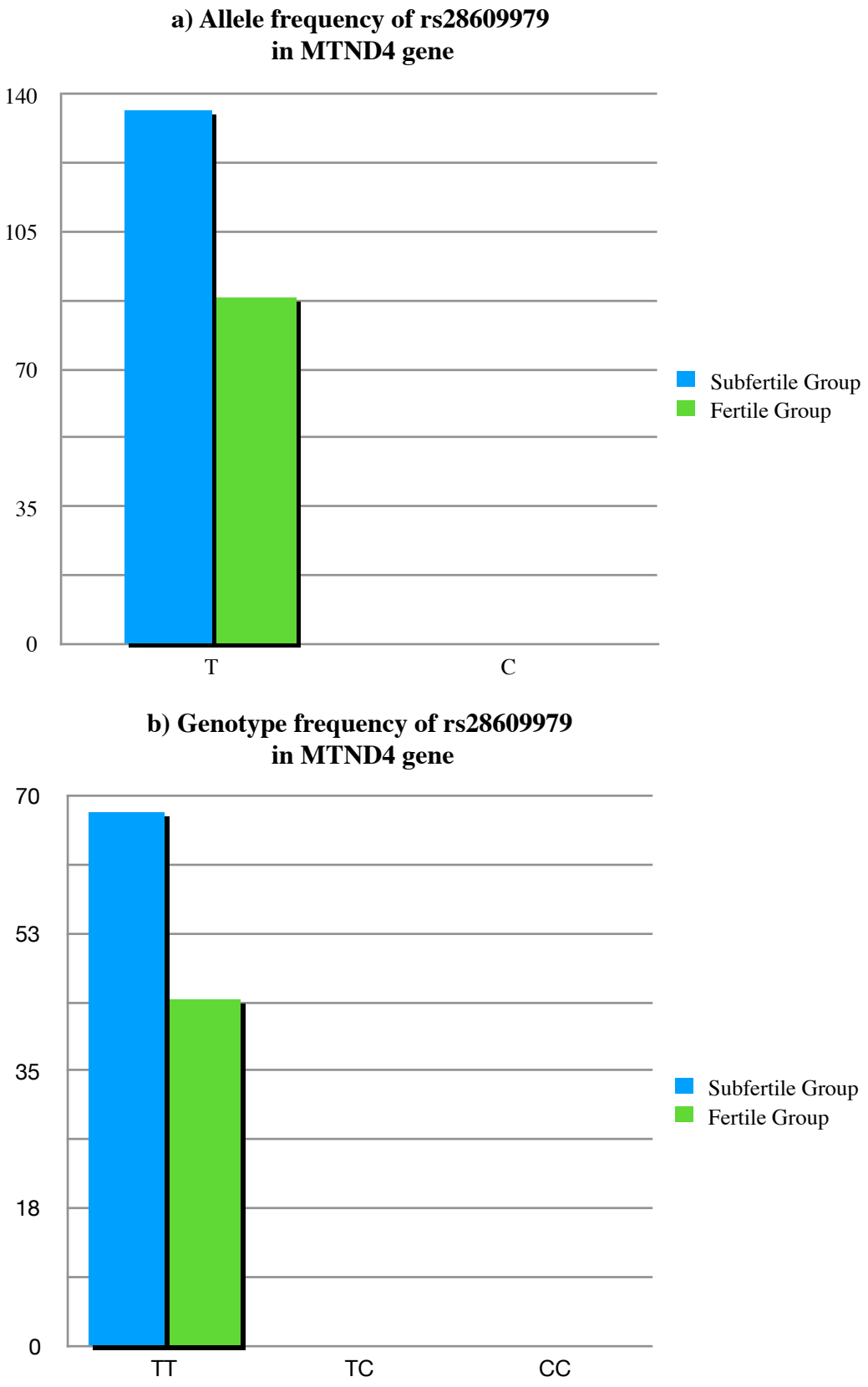



**Figure 83: a)** allele frequency of rs28609979 in *MTND4* gene, **b)** genotype frequency of rs28609979 in *MTND4* gene.

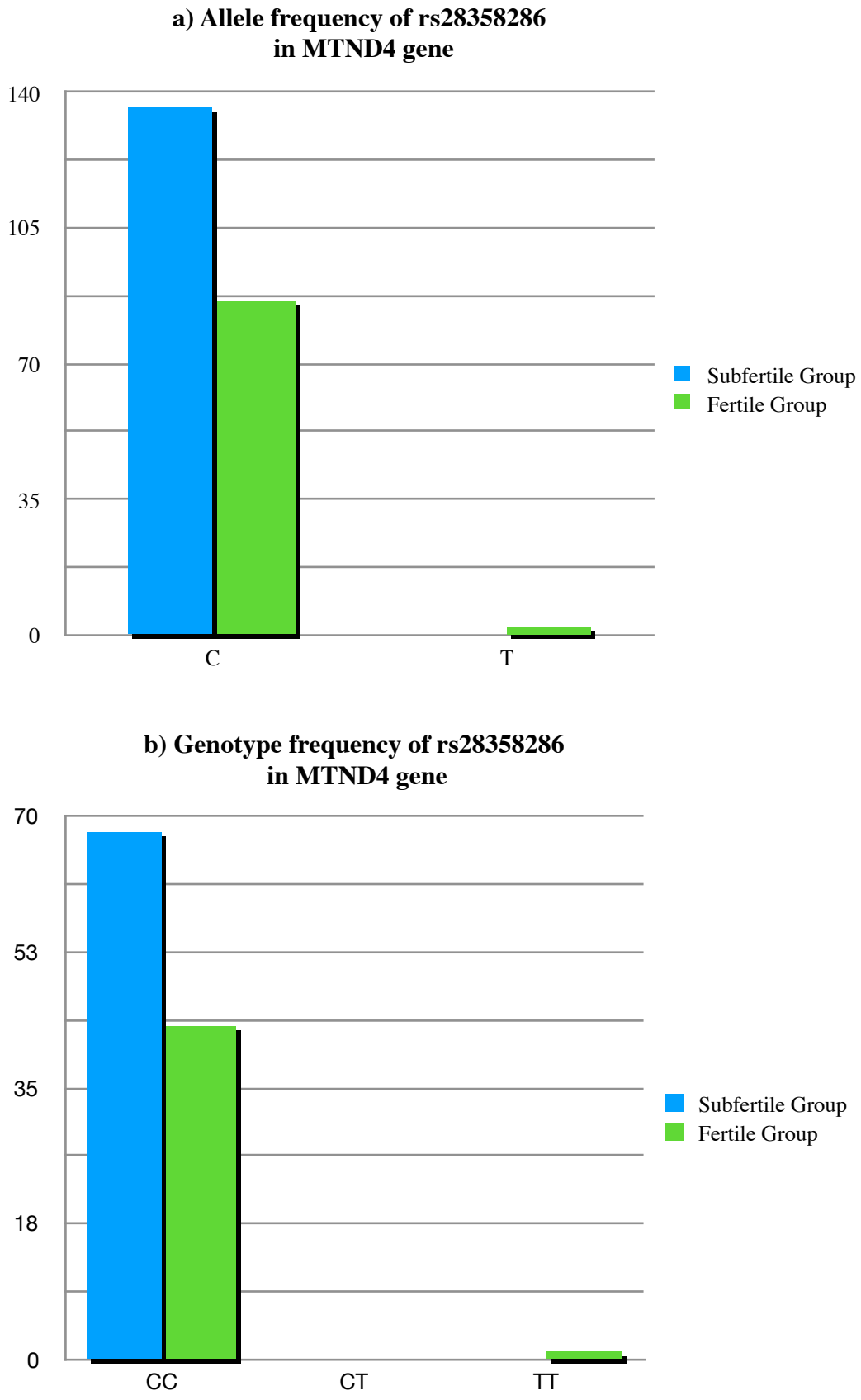



**Figure 84: a)** allele frequency of rs28358286 in *MTND4* gene (*P*= 0.153), **b)** genotype frequency of rs28358286 in *MTND4* gene (*P*= 0.211).

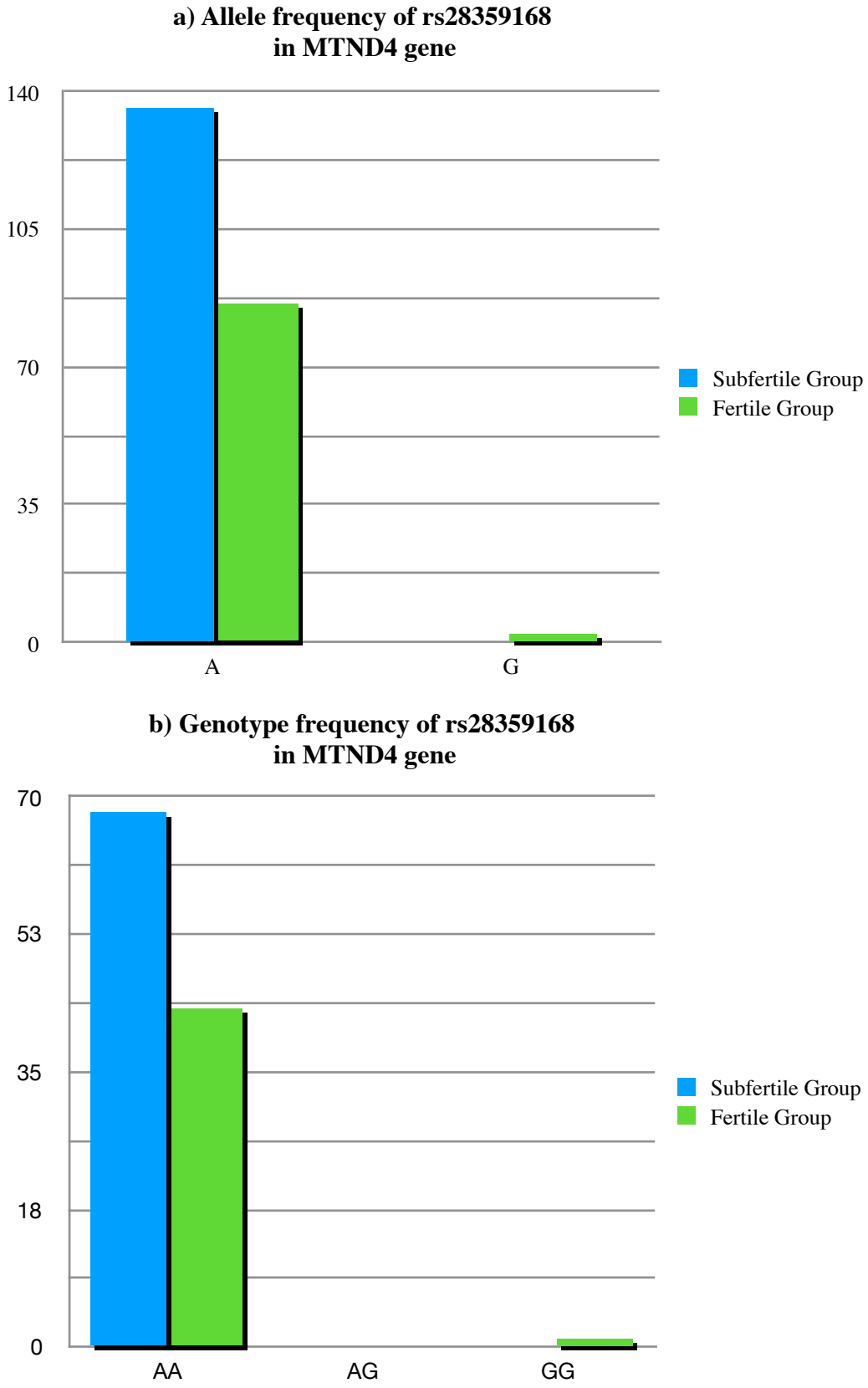



**Figure 85: a)** allele frequency of rs28359168 in *MTND4* gene (*P*= 0.153), **b)** genotype frequency of rs28359168 in *MTND4* gene (*P*= 0.211).

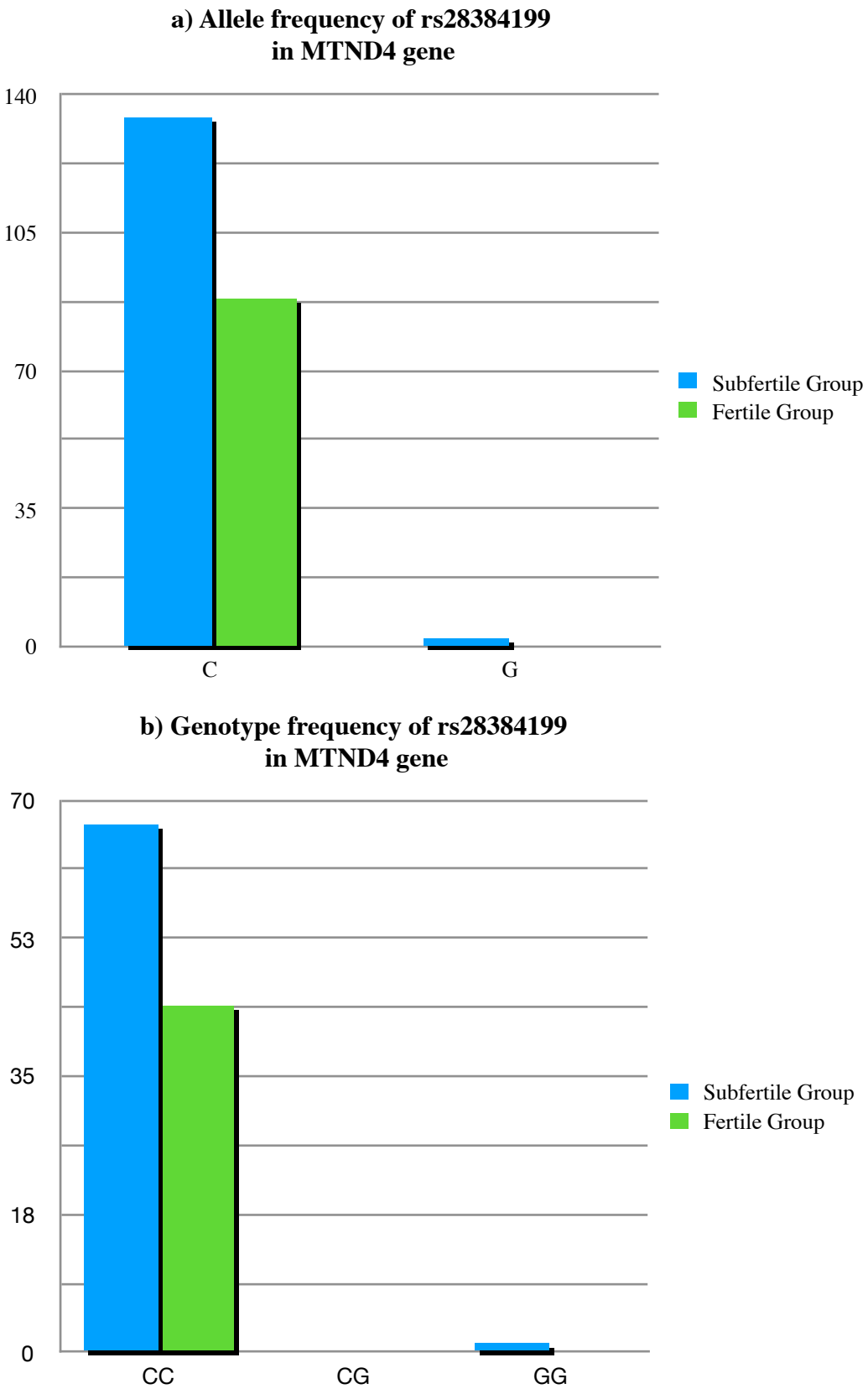



**Figure 86: a)** allele frequency of rs28384199 in *MTND4* gene (*P*= 0.520), **b)** genotype frequency of rs28384199 in *MTND4* gene (*P*= 0.419).

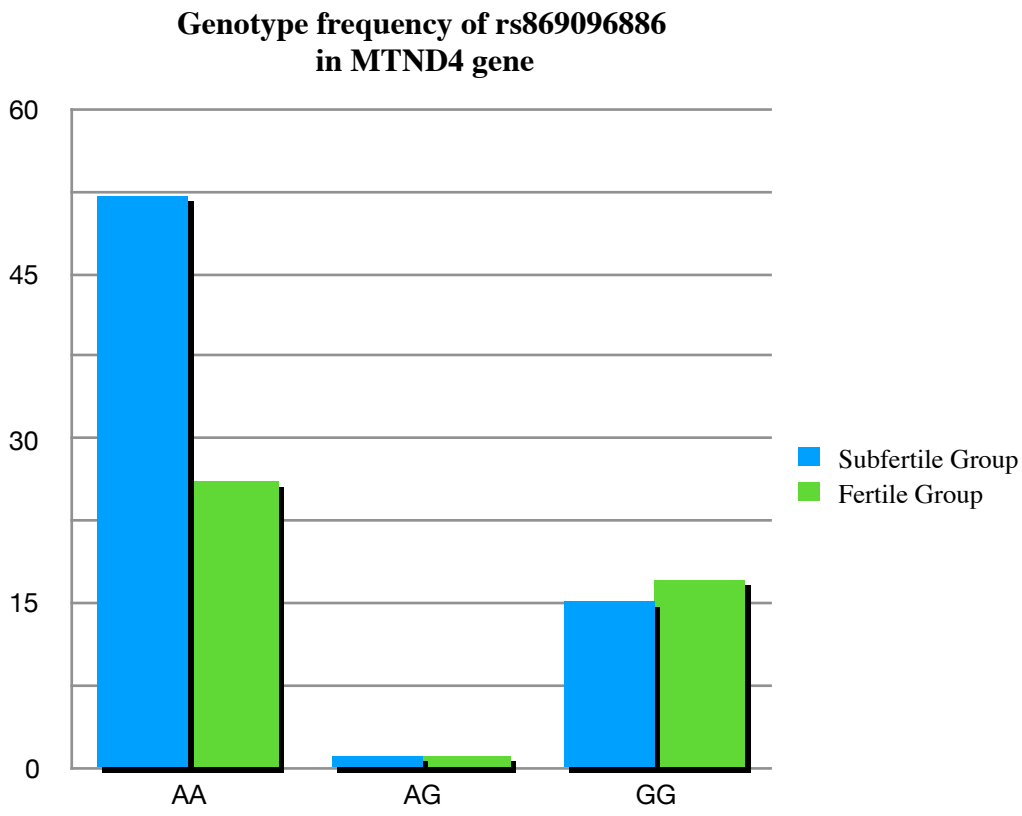



**Figure 87:** genotype frequency of rs869096886 in *MTND4* gene (*P*= 0.147).

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# PUBLICATIONS

**Dahadhah, F. W.**, Jaweesh, M. S., Al Zoubi, M. S., Abu Alarjah, M. I., Hammadeh, M.E., Amor, H. (2021). Mitochondrial Nicotinamide Adenine Dinucleotide Hydride dehydrogenase (NADH) Subunit 4 (*MTND4*) polymorphisms and their association with male infertility. Journal of Assisted Reproduction and Genetics. **(Accepted)**

**Dahadhah, F. W.**, Jaweesh, M. S., Al Zoubi, M. S., Abu Alarjah, M. I., Hammadeh, M.E., Amor, H. (2021). Association between polymorphic variants of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase 3 and 4L *(MT-ND3* and *MT-ND4L*) and male infertility. Andrologia. **(Submitted, under review)**

Hussein, E. I., Al-Batayneh, K., Masadeh, M. M., **Dahadhah, F. W.**, Al Zoubi, M. S., Aljabali, A. A., & Alzoubi, K. H. (2020). Assessment of Pathogenic Potential, Virulent Genes Profile, and Antibiotic Susceptibility of Proteus mirabilis from Urinary Tract Infection. *International journal of microbiology*, *2020*.

# CURRICULUM VITAE