DEPARTMENT OF OBSTETRICS, GYNAECOLOGY & REPRODUCTIVE MEDICINE

FACULTY OF MEDICINE OF SAARLAND UNIVERSITY HOMBURG/ SAAR, GERMANY

The association between Mitochondrial *CYB*, *CO3*, *ATP6* and *ATP8* polymorphisms and male infertility

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List of Contents

ABSTRACT ..... **I**

ZUSAMMENFASSUNG ..... **II**

LIST OF ABBREVIATIONS ..... **III**

LIST OF FIGURES ..... **VI**

LIST OF TABLES ..... **X**

1. INTRODUCTION **1**

1.1 Sperm structure 1

1.2 Spermatogenesis 2

1.3 The human mitochondria 4

1.3.1 The structure of mitochondria 4

1.3.2 The function of mitochondria 6

1.3.2.1 Cellular respiration 6

1.3.2.2 Mitochondrial oxidative phosphorylation 6

1.3.3 Mitochondrial membrane potential (ΔΨm) 8

1.3.4 Mitochondria and oxidative stress 9

1.3.5 Mitochondrial DNA (mtDNA) 10

1.3.5.1 Mitochondrial DNA inheritance 11

1.3.5.2 Mitochondrial genome transcription and translation 11

1.3.5.3 Mitochondrial DNA mutation and male infertility 13

1.4 Male Infertility 13

1.5 Sperm abnormalities 15

1.6 Factors related to male fertility 16

1.6.1 Environmental and lifestyle factors 16

1.6.2 Hormonal disorders 17

1.6.3 Sexual dysfunction 17

1.6.4 Genetic factors 17

1.6.4.1 Chromosomal abnormalities 17

1.6.4.2. Autosomal gene mutations 18

1.6.4.3. X-Linked genes....18
1.7 Human Mitochondrial Genes....18
1.7.1 MT-CYB....18
1.7.2 MT-CO3....19
1.7.3 MT-ATP6....19
1.7.4 MT-ATP8....19
1.8 The purpose of the study....21
2. MATERIALS AND METHODS....22
3. RESULTS....33
4. DISCUSSION....52
5. CONCLUSION....62
6. REFERENCES....63
7. APPENDICES....77
SUPPLEMENTARY FIGURES OF GENOTYPES AND ALLELE FREQUENCIES FOR CHAPTER 3.2....77
ACKNOWLEDGMENT....126
PUBLICATIONS....127

ABSTRACT

A genetic predisposition that affects sperm function and performance may account for idiopathic male infertility. Specific conditions of male infertility and abnormal sperm function are linked to genetic changes in the mitochondrial DNA (mtDNA). Certain deficiencies in mitochondrial function may be caused by mutations of the mitochondrial genes *MT-CYB, MT-CO3, MT-ATP6* and *MT-ATP8*. Therefore, the current study objective was to examine how mutations in the *MT-CYB, MT-CO3, MT-ATP6* and *MT-ATP8* genes affected sperm motility and male infertility.

A total of 111 men had their sperm samples collected, of which 67 were subfertile and 44 were fertile. The mitochondrial DNA was isolated and amplified using QIAGEN's QIAamp DNA Mini Kit and REPLI-g Mitochondrial DNA Kit. Following that, PCR and Sanger sequencing were used to find the target sequence in the *MT-CYB*, *MT-CO3*, *MT-ATP6*, and *MT-ATP8* genes. In the present research, a total of 49 single nucleotide polymorphisms (SNPs) were identified and genotyped: thirteen SNPs in *MT-CYB* rs28357685, rs2853508, rs41518645, rs35070048, rs2853507, rs28357376, rs2853506, rs28660155, rs527236194, rs28357373, rs28357369, rs41504845, and rs2854124, twelve SNPs in *MT-CO3* gene rs2248727, rs7520428, rs3134801, rs9743, rs28358272, rs2853824, rs2856985, rs2854139, rs41347846, rs28380140, rs3902407, and 28411821, fourteen SNPs in *MT-ATP6* rs2001031, rs2000975, rs2298011, rs7520428, rs9645429, rs112660509, rs6650105, rs6594033, rs6594034, rs6594035, rs3020563, rs28358887, rs2096044, and rs9283154, and ten SNPs in *MT-ATP8*: rs9285835, rs9285836, rs9283154, rs8179289, rs121434446, rs1116906, rs2153588, rs1116905, rs1116907, and rs3020563. The genotypes frequency between fertile and subfertile groups was significantly different for three variants in *MT-CYB*: rs28357373 (T15629C) (P = 0.0439), rs41504845 (C15833T) (P = 0.0038), and rs527236194 (T15784C) (P = 0.0005). Additionally, two SNPs demonstrated an association of significance between infertility in men and allele frequency: rs41504845 (C15833T) (P = 0.0147) and rs527236194 (T15784C) (P = 0.0014). Moreover, in *MT-CO3* and *MT-ATP6*, Only the rs7520428 demonstrated a significant difference statistically between subfertile and fertile groups in the genotype and allele frequency test ($P < 0.0001$ for both). In conclusion, the current study showed that SNPs rs527236194, rs28357373 and rs41504845 in *MT-CYB*, and rs7520428 in the *MT-CO3* and *MT-ATP6* were correlated with male infertility. To determine the correct activity of these genes in male infertility, further studies on larger populations are needed.

ZUSAMMENFASSUNG

Die idiopathische männliche Unfruchtbarkeit kann auf genetische Veranlagungen zurückgeführt werden, die die Leistung und Funktion der Spermien beeinträchtigen. Genetische Veränderungen in der mitochondrialen DNA (mtDNA) wurden mit bestimmten Arten männlicher Unfruchtbarkeit und abnormaler Spermienfunktion in Verbindung gebracht. Mutationen in den Genen *MT-CYB*, *MT-CO3*, *MT-ATP6* und *MT-ATP8* können zu einigen Mängeln der mitochondrialen Funktion führen. Daher wollten wir in der aktuellen Studie die Wirkung von Mutationen in den *MT-CYB*, *MT-CO3*, *MT-ATP6* und *MT-ATP8* Genen auf die Spermienmotilität und männliche Unfruchtbarkeit untersuchen. Es wurden Samenproben von 111 Männern entnommen, wobei 67 Männer subfertil und 44 fruchtbar waren. QIAamp DNA-Mini Kit und REPLI-g Mitochondrial DNA Kit von QIAGEN wurden verwendet, um die mitochondriale DNA zu isolieren und zu amplifizieren. Gefolgt von PCR und Sanger-Sequenzierung für die Zielsequenz in den *MT-CYB*, *MT-CO3*, *MT-ATP6*, und *MT-ATP8* Genen. Insgesamt wurden neunundvierzig Einzelnukleotidpolymorphismen (SNPs) identifiziert und wie folgt genotypisiert: dreizehn SNPs in *MT-CYB rs2853508, rs28357685, rs41518645, rs2853507, rs28357376, rs35070048, rs2853506, rs28660155, rs527236194, rs28357373, rs28357369, rs41504845,* und *rs2854124*, zwölf SNPs im *MT-CO3* Gen rs2248727, rs7520428, rs3134801, rs9743, rs28358272, rs2853824, rs2856985, rs2854139, rs41347846, rs28380140, rs3902407 und 28411821, vierzehn SNPs in *MT-ATP6* rs2001031, rs2000975, rs2298011, rs7520428, rs9645429, rs112660509, rs6650105, rs6594033, rs6594034, rs6594035, rs3020563, rs28358887, rs2096044, und rs9283154, und zehn SNPs in *MT-ATP8*: rs9285835, rs9285836, rs9283154, rs8179289, rs121434446, rs1116906, rs2153588, rs1116905, rs1116907, und rs3020563. Bei *MT-CYB* zeigten drei Varianten einen signifikanten Unterschied in der Häufigkeit der Genotypen zwischen subfertilen und fruchtbaren Gruppen: rs527236194 (T15784C) (P = 0,0005), rs28357373 (T15629C) (P = 0,0439) und rs41504845 (C15833T) (P = 0,0038). Darüber hinaus zeigten zwei SNPs einen signifikanten Zusammenhang zwischen den Allelfrequenzen von rs527236194 (T15784C) (P = 0,0014) und rs41504845 (C15833T) (P = 0,0147) und männlicher Unfruchtbarkeit. Darüber hinaus zeigte nur der rs7520428 bei *MT-CO3* und *MT-ATP6* einen statistisch signifikanten Unterschied zwischen subfertilen und fruchtbaren Gruppen im Genotyp- und Allel-Frequenztest (P < 0,0001 für beide). Zusammenfassend zeigte die aktuelle Studie, dass die SNPs rs527236194, rs28357373 und rs41504845 bei *MT-CYB* und rs7520428 bei *MT-CO3* und *MT-ATP6* mit männlicher Unfruchtbarkeit korreliert waren. Weitere Studien an größeren Populationen sind erforderlich, um die genaue Rolle dieser Gene bei der Entwicklung der männlichen Unfruchtbarkeit aufzudecken.

LIST OF ABBREVIATIONS

ADP: Adenosine Diphosphate

Ala: Alanine

AR: Androgen Receptor

Asn: Asparagine

ATP: Adenosine Triphosphate

Bp: Base Pair

CBVAD: Congenital bilateral absence of vas-deference

CFTR: Conducted Regulator Gene

CI: Confidence Interval

DNA: Deoxyribonucleic Acid

ETC: Electron Transfer Chain

F: Forward Primer

FSH: Follicular stimulating hormone

Gln: Glutamine

GTP: Guanosine Triphosphate

H: heavy strand

ICSI: Intracytoplasmic Sperm Injection

Ile: Isoleucine

IUI: Intrauterine Insemination

IVF: In Vitro Fertilization

Kb: Kilobase

kDa: Kilodalton

L: light strand

LH: Luteinizing hormone

Leu: Leucine

LHON: Leber's Hereditary Optic Neuropathy

MELAS: Mitochondrial Encephalomyopathy with Stroke-Like Episodes

Min: Minute

Ml: Millilitre

µl: Microliter

mRNA: Messenger Ribonucleic Acid

mtDNA: Mitochondrial Deoxyribonucleic Acid

MT-ATP6: Mitochondrial ATP synthase membrane subunit 6

MT-ATP8: Mitochondrial ATP synthase membrane subunit 8

MT-CYB: Mitochondrial Cytochrome B

MT-CO3: Mitochondrial Cytochrome C Oxidase III

NADH: Nicotinamide adenine dinucleotide

NADH: Nicotinamide Adenine Dinucleotide Hydride

NARP: Neuropathie, Ataxie, Retinitis Pigmentosa Syndrome

NP: Non-progressive Motility

OR: Odds Ratio

OS: Oxidative stress

OXPHOS: Oxidative Phosphorylation

PCR: Polymerase Chain Reaction

Phe: Phenylalanine

PR: Progressive Motility

PR + NP: Total Motility

Pro: Proline

R: Reverse Primer

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: Ribosomal Ribonucleic Acid

Sec: Second

SD: Standard Deviation

Ser: Serine

SNP: Single Nucleotide Polymorphism

TAE: Tris-Acetate-EDTA

Thr: Threonine

tRNA: Transfer Ribonucleic Acid

Trp: Tryptophan

UQCR: Ubiquinol Cytochrome c Reductase

V: Volt

Val: Valine

WHO: World Health Organization

ZP: Zona Pellucida

LIST OF FIGURES

Figure 1: A mature human sperm cell. 2

Figure 2: Overview of spermatogenesis. 3

Figure 3: Diagrammatic structural features of a mitochondrion. 5

Figure 4: Oxidative phosphorylation system. 7

Figure 5: The involvement of mitochondria in fundamental cellular pathways and processes. 8

Figure 7: Overview of gene expression in human mitochondria. 12

Figure 8: Standardized WHO values as a reference for normal semen parameters. 14

Figure 9: Abnormal parameters of human spermatozoa. 16

Figure 10: Steps of QIAamp® DNA Mini for MT-DNA amplification. 27

Figure 11: Illustrative gel electrophoresis on 1.5% agarose gel of PCR products for the amplification of the MT-CYB gene in 8 samples (1-8) along with DNA Ladder (M) (1246 bp). 30

Figure 12: Illustrative gel electrophoresis on 1.5% agarose gel of PCR products for the amplification of the MT-CO3 gene in 8 samples (1-8) along with DNA Ladder (M) (974 bp). 30

Figure 13: Illustrative gel electrophoresis on 2% agarose gel of PCR products for the amplification of the MT-ATP6 gene in 8 samples (1-8) along with DNA Ladder (M) (786 bp). 31

Figure 14: Illustrative gel electrophoresis on 2% agarose gel of PCR products for the amplification of the MT-ATP8 gene in 8 samples (1-8) along with DNA Ladder (M) (343 bp). 31

Figure 15: Sequencing electropherogram results (TT, CC) of the rs527236194 of MT-CYB.……. 55

Figure 16: Sequencing electropherogram results (CC, CT, TT) of the rs41504845 of MT-CYB.…The nucleotide transition at position 15,833 (C>T) resulted in a synonymous variant (Leu>Leu) at codon 363. 55

Figure 17: Sequencing electropherogram results (CC, CT, TT) of the rs28357373 of MT-CYB. 56

Figure 18: Sequencing electropherogram results (AA, GG) of the rs7520428 of MT-ATP6.……. 57

Figure 19: Sequencing electropherogram results (AA, AG, GG) of the rs7520428 of MT-CO3. 58

Figure 20: a) MT-CYB gene allele frequency for rs2853508, b) MT-CYB gene genotype frequency for rs2853508.....77

Figure 21: a) MT-CYB gene allele frequency for rs28357685, b) MT-CYB gene genotype frequency for rs28357685.....78

Figure 22: a) MT-CYB gene allele frequency for rs527236194, b) MT-CYB gene genotype frequency for rs527236194 in MT-CYB gene. 79

Figure 23: a) MT-CYB gene allele frequency for rs41518645, b) MT-CYB gene genotype frequency for rs41518645.....80

Figure 24: a) MT-CYB gene allele frequency for rs2853507 , b) MT-CYB gene genotype frequency for rs2853507.....81

Figure 25: a) MT-CYB gene allele frequency for rs28357373, b) MT-CYB gene genotype frequency for rs28357373.....82

Figure 26: a) MT-CYB gene allele frequency for rs28357376, b) MT-CYB gene genotype frequency for rs28357376.....83

Figure 27: a) MT-CYB gene allele frequency for rs35070048, b) MT-CYB gene genotype frequency for rs35070048.....84

Figure 28: a) MT-CYB gene allele frequency for rs28357369, b) MT-CYB gene genotype frequency for rs28357369 in.....85

Figure 29: a) MT-CYB gene allele frequency for rs41504845, b) MT-CYB gene genotype frequency for rs41504845.....86

Figure 30: a) MT-CYB gene allele frequency for rs2853506, b) MT-CYB gene genotype frequency for rs2853506.....87

Figure 31: a) MT-CYB gene allele frequency for rs2854124, b) MT-CYB gene genotype frequency for rs2854124.....88

Figure32: a) MT-CYB gene allele frequency for rs28660155, b) MT-CYB gene genotype frequency for rs28660155.....89

Figure 33: a) MT-CO3 gene allele frequency for rs2248727, b) MT-CO3 gene genotype frequency for rs2248727.....90

Figure 34: a) MT-CO3 gene allele frequency for rs7520428, b) MT-CO3 gene genotype frequency for rs7520428.....91

Figure 35: a) MT-CO3 gene allele frequency for rs3134801, b) MT-CO3 gene genotype frequency for rs3134801.....92

Figure 36: a) MT-CO3 gene allele frequency for rs9743, b) MT-CO3 gene genotype frequency for rs9743.....93

Figure 37: a) MT-CO3 gene allele frequency for rs28358272, b) MT-CO3 gene genotype frequency for rs28358272.....94

Figure 38: a) MT-CO3 gene allele frequency for rs2853824, b) MT-CO3 gene genotype frequency for rs2853824.....95

Figure 39: a) MT-CO3 gene allele frequency for rs2856985, b) MT-CO3 gene genotype frequency for rs2856985.....96

Figure 40: a) MT-CO3 gene allele frequency for rs2854139, b) MT-CO3 gene genotype frequency for rs2854139.....97

Figure 41: a) MT-CO3 gene allele frequency for rs28380140, b) MT-CO3 gene genotype frequency for rs28380140.....98

Figure 42: a) MT-CO3 gene allele frequency for rs3902407, b) MT-CO3 gene genotype frequency for rs3902407.....99

Figure 43: a) MT-CO3 gene allele frequency for rs28411821, b) MT-CO3 gene genotype frequency for rs28411821.....100

Figure 44: a) MT-CO3 gene allele frequency for rs41347846, b) MT-CO3 gene genotype frequency for rs41347846.....101

Figure 45: a) MT-ATP6 gene allele frequency for rs2000975, b) MT-ATP6 gene genotype frequency for rs2000975.....102

Figure 46: a) MT-ATP6 gene allele frequency for rs2001031, b) MT-ATP6 gene genotype frequency for rs2001031.....103

Figure 47: a) MT-ATP6 gene allele frequency for rs9645429, b) MT-ATP6 gene genotype frequency for rs9645429.....104

Figure 48: a) MT-ATP6 gene allele frequency for rs2298011, b) MT-ATP6 gene genotype frequency for rs2298011.....105

Figure 49: a) MT-ATP6 gene allele frequency for rs7520428, b) MT-ATP6 gene genotype frequency for rs7520428.....106

Figure 50: a) MT-ATP6 gene allele frequency for rs112660509, b) MT-ATP6 gene genotype frequency for rs112660509.....107

Figure 51: a) MT-ATP6 gene allele frequency for rs6650105, b) MT-ATP6 gene genotype frequency for rs6650105.....108

Figure 52: a) MT-ATP6 gene allele frequency for rs6594033, b) MT-ATP6 gene genotype frequency for rs6594033.....109

Figure 53: a) MT-ATP6 gene allele frequency for rs6594034, b) MT-ATP6 gene genotype frequency for rs6594034.....110

Figure 54: a) MT-ATP6 gene allele frequency for rs6594035, b) MT-ATP6 gene genotype frequency for rs6594035.....111

Figure 55: a) MT-ATP6 gene allele frequency for rs28358887, b) MT-ATP6 gene genotype frequency for rs28358887. 112

Figure 56: a) MT-ATP6 gene allele frequency for rs2096044, b) MT-ATP6 gene genotype frequency for rs2096044. 113

Figure 57: a) MT-ATP6 gene allele frequency for rs9283154, b) MT-ATP6 gene genotype frequency for rs9283154. 114

Figure 58: a) MT-ATP6 gene allele frequency for rs3020563, b) MT-ATP6 gene genotype frequency for rs3020563. 115

Figure 59: a) MT-ATP8 gene allele frequency for rs9285835, b) MT-ATP8 gene genotype frequency for rs9285835. 116

Figure 60: a) MT-ATP8 gene allele frequency for rs9285836, b) MT-ATP8 gene genotype frequency for rs9285836. 117

Figure 61: a) MT-ATP8 gene allele frequency for rs9283154, b) MT-ATP8 gene genotype frequency for rs9283154. 118

Figure 62: a) MT-ATP8 gene allele frequency for rs8179289, b) MT-ATP8 gene genotype frequency for rs8179289. 119

Figure 64: a) MT-ATP8 gene allele frequency for rs1116906, b) MT-ATP8 gene genotype frequency for rs1116906. 121

Figure 65: a) MT-ATP8 gene allele frequency for rs2153588, b) MT-ATP8 gene genotype frequency for rs2153588. 122

Figure 66: a) MT-ATP8 gene allele frequency for rs1116905, b) MT-ATP8 gene genotype frequency for rs1116905. 123

Figure 67: a) MT-ATP8 gene allele frequency for rs1116907, b) MT-ATP8 gene genotype frequency for rs1116907. 124

Figure 68: a) MT-ATP8 gene allele frequency for rs1116907, b) MT-ATP8 gene genotype frequency for rs1116907. 125

LIST OF TABLES

Table 1: Semen parameters reference ranges 25

Table 2: Preparation of amplification mixture 28

Table 3: Oligonucleotide primers for MT-CYB, MT-CO3, MT-ATP6, and MT-ATP8 genes.....32

Table 4: Descriptive statistical analysis of studied parameters for all the population (N=111) 33

Table 5: Comparing parameters of the semen analysis between Fertile (control group) and Sub-fertile (study group).....34

Table 6: The MT-CYB polymorphism genotypes between subfertile patients and controls 36

Table 7: The MT-CYB polymorphism alleles between subfertile patients and controls. 38

Table 8: The MT-CO3 polymorphism genotypes between subfertile patients and controls. 40

Table 9: The MT-CO3 polymorphism alleles between subfertile patients and controls. 42

Table 10: The MT-ATP6 polymorphism genotypes between subfertile patients and controls. 44

Table 11: The MT-ATP6 polymorphism alleles between subfertile patients and controls. 46

Table 12: The MT-ATP8 polymorphism genotypes between subfertile patients and controls. 48

Table 13: The MT-ATP8 polymorphism alleles between subfertile patients and controls. 50

1. INTRODUCTION

1.1 Sperm structure

Male sperm has a well-defined structure, consisting of a head, a mid-piece, and a tail **(Figure 1)**. A morphologically normal spermatozoon is defined as follows: The head includes the nucleus, which has dense chromatin fibers that covers most of the space of the sperm head. The acrosome, which is located in the anterior part of the sperm head, occupies around 40–70% of its area. It contains the digestive enzymes needed in fertilization process. These enzymes are needed to breakdown the zona pellucida (ZP) layer that surrounds the oocyte (Sunanda *et al.,* 2018).

The middle piece consists of the mitochondria. They form a spiral sheet that surrounds the axonemal fibers of the midpiece (Malhotra, 2020). Each mature sperm contains around 50 to 75 mitochondria in its midpiece, along with one copy of mitochondrial deoxyribonucleic acid (mtDNA) (Kao *et al.,* 2004). The mitochondria are considered as the energy source for sperm. Adenosine triphosphate (ATP) is utilized particularly by spermatozoa to sustain their intracellular environment and for cellular functions such motility, capacitation, and the acrosome reaction, all of which are necessary for successful fertilization (du Plessis *et al.,* 2015). The last part is the tail, which usually is the longest part of human sperm. It is about 55 μm long and it is divided into two parts: the principal part, which is made up of axial filaments and an end part which is a small tapering portion of the tail (Malhotra, 2020).

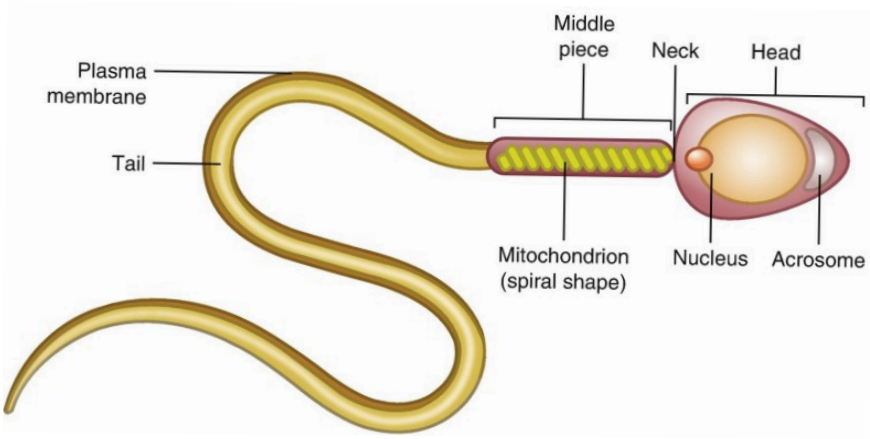

Figure 1: A mature human sperm cell.
[Adapted from (Malhotra, 2020)]

1.2 Spermatogenesis

Spermatogenesis is the production of large numbers of normal sperm by several complex processes, and is a critical requirement for male fertility. Spermatogenesis is subdivided into three steps: the multiplication of spermatogonia through the mitosis process; the reduction of diploid chromosomes to haploids through meiosis divisions; and the spermatogenesis phase, in which the round spermatid converts to a complex structure of the spermatozoon. Each of these steps plays a key role in the spermatogenic process; therefore, any defect that occurs in any of them can result in an unsuccessful process and lead to the production of defective forms of spermatozoa and reduction or loss of sperm production. Regulation of spermatogenesis occurs at two major levels: hormonal and endocrine, and paracrine/autocrine (Allais-Bonne & Pailhoux, 2014) **(Figure 2)**.

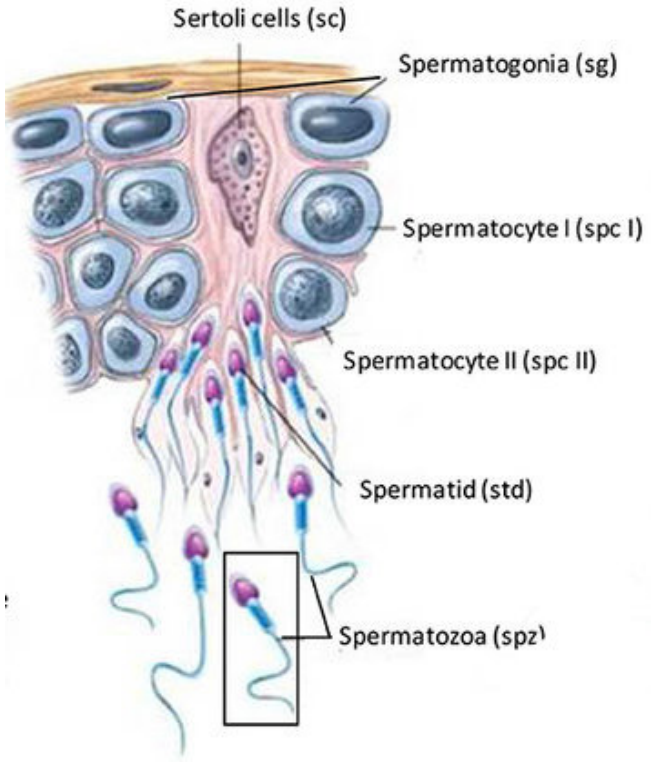

Figure 2: Overview of spermatogenesis.
[Adapted from (Allais-Bonnet & Pailhoux, (2014)].

Type A spermatogonia are the spermatogonial stem cell reserves. They only undergo mitosis under certain conditions. Type A spermatogonia have self-renewing abilities but also differentiate to committed spermatogonia once every 16 days. Successive mitotic divisions and differentiation give rise to type B spermatogonia and preleptotene primary spermatocytes. Diploid primary spermatocytes cross the blood-testis barrier before undergoing meiosis I to produce haploid secondary spermatocytes. Secondary spermatocytes undergo meiosis II to produce round spermatids. During spermiogenesis, round spermatids form tails and acrosome. Finally, the spermatozoa are released from the Sertoli cells into the lumen of the seminiferous tubule. The process is only shown for one member of a pair of committed type Ap spermatogonia (Greenbaum *et al.,* 2011).

1.3 The human mitochondria

Mitochondria originated about two billion years ago, when a precursor of the modern eukaryotic cell engulfed an α-proteobacterium. Since then, mitochondria have maintained the double membrane structure of their ancestors, but their overall form and composition have changed radically. Moreover, they have acquired many additional functions within the cell. For instance, most of the genomic material of the α-proteobacterium has been lost or transferred to the nuclear genome (Friedman & Nunnari, 2014).

Mitochondria of spermatozoa differ significantly in structure and function from the corresponding organelles of somatic cells (Ferramosca & Zara, 2017).

1.3.1 The structure of mitochondria

Mitochondria are the powerhouses of the cell. They are the major source of adenosine triphosphate (ATP), the energy-rich molecule that drives essential cell activities, in all eukaryotes that do not rely on photosynthesis (Rizzuto *et al.,* 2012). The structure of mitochondria includes a double membrane that forms four distinct compartments within the organelle: the outer membrane, the inter-membrane space, the inner membrane, and the matrix (McCarron *et al.,* 2013). Each compartment serves different functions. The outer membrane detaches the mitochondrion from the cytosol, and an inner membrane involves several invaginations that form the cristae which surround the matrix region (Mannella, 2008) **(Figure 3)**. As semi-autonomous cellular organelles, the outer mitochondrial membrane separate the mitochondria from the cytoplasm. The outer membrane is porous. Ions and small uncharged molecules are freely transported through the membrane through membrane proteins that form pores. Because of its porosity, there is no membrane potential across the outer membrane (Kühlbrandt, 2015). The inner membrane, on the other hand, acts as a tight diffusion barrier for all ions and molecules. Only specialized membranes, each of which is selective for a specific ion or molecule, transport proteins. An electrochemical membrane potential of around 180 mV builds up across the inner mitochondrial membrane because of its ion selectivity. In a suite of membrane protein complexes that produce the electrochemical gradient across the inner membrane or use it for ATP production, oxidative phosphorylation takes place in the inner membrane (Kühlbrandt, 2015).

The matrix is the site of mitochondrial DNA replication, transcription, protein biosynthesis, and numerous enzymatic reactions (Kukat *et al.,* 2011). The most specialized sub-compartments are the inner membrane and the matrix where many enzymes, generally organized as multi-subunit complexes, can be found. It has been reported that mitochondria contain about a thousand distinct proteins involved in various metabolic pathways (Sickmann *et al.,* 2003; Pagliarini *et al.,* 2008).

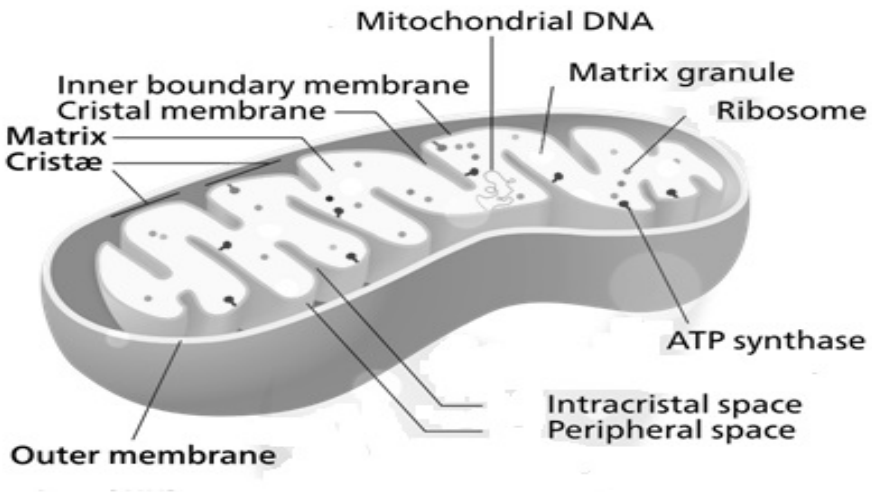

Figure 3: Diagrammatic structural features of a mitochondrion.

[Adapted from (Scheffler, 2011)]

The outer membrane separates the cytoplasm from the mitochondria. It surrounds the inner membrane, which divides the protein-dense core matrix from the inter-membrane space. The inner boundary membrane and the cristae are two types of inner membrane. At the crista junctions, the two parts merges into one. The cristae are the major sites of mitochondrial energy conversion, extending far into the matrix. The ATP synthase in the cristae membranes is driven by a low proton gradient between the intermembrane space (pH 7.2–7.4) and the matrix (pH 7.9–8) (Scheffler, 2011).

1.3.2 The function of mitochondria

The key enzyme systems required to complete the oxidation of carbohydrates, lipids, and proteins to create useable energy in the form of ATP are located in the mitochondria (Ryan & Hoogenraad, 2007).

1.3.2.1 Cellular respiration

In glycolysis, which takes place in the cytosol, glucose undergoes a variety of chemical reactions before being transformed into two pyruvate molecules. During this process, nicotinamide adenine dinucleotide (NAD+) is converted to nicotinamide adenine dinucleotide hydrate (NADH), yielding one molecule of ATP. Pyruvate then enters the matrix of the mitochondria and the enzyme Pyruvate dehydrogenase aids in the conversion of pyruvate to acetyl-CoA (Maechler, 2006).

In the citric acid cycle (Krebs cycle), acetyl-CoA is transferred to citrate. In a series of several enzymatic stages, citrate is oxidized and the electrons removed during this reaction are passed to the NADH and flavin adenine dinucleotide (FADH2). The free energy generate is then transferred by NADH and FADH2 to the electron transport chain (Alabduladhem & Bordoni, 2021). The last step in the cellular respiration process is the oxidative phosphorylation cycle.

1.3.2.2 Mitochondrial oxidative phosphorylation

Oxidative phosphorylation is the main system for mitochondria to produce ATP, which consists of the ATP synthase complex (complex V) and four oxidoreductase complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), and the cytochrome c oxidase (complex IV). All these complexes are located within the inner mitochondrial membrane. Complexes I and II transfer electrons from NADH or FADH2 onto ubiquinone while complex III transfers electrons from ubiquinol to cytochrome c, which is located in the space between the outer and inner mitochondrial membrane. Finally, complex IV transfers electrons from cytochrome c onto molecular oxygen (Chaban *et al.,* 2014) **(Figure 4)**.

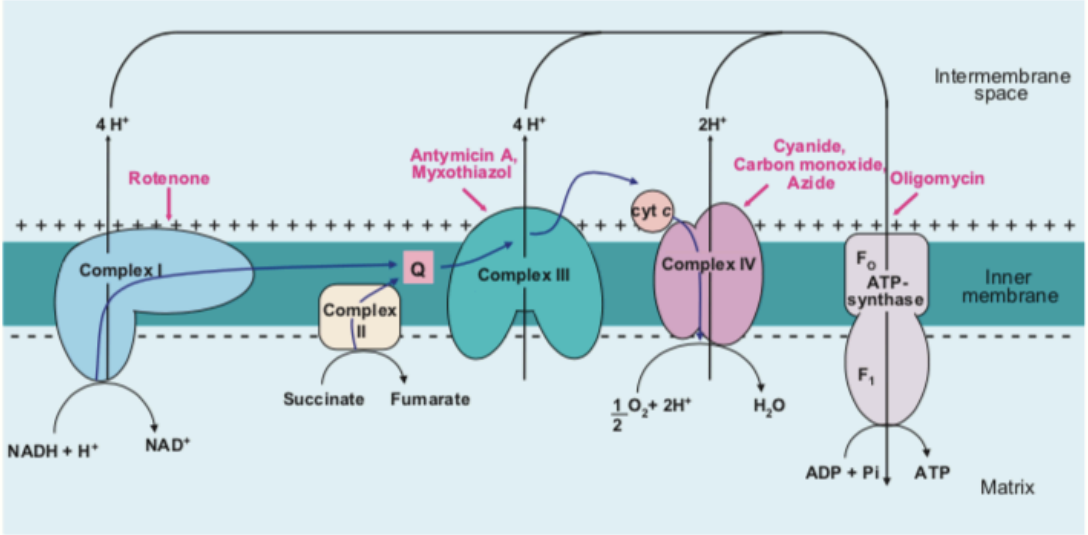

Figure 4: Oxidative phosphorylation system.
[Adapted from (Piomboni *et al.,* 2012)]

Adenosine triphosphate (ATP) is an energy-rich molecule that fuels basic cell processes. These include force generation (for example, in muscle contraction and cell division); protein biosynthesis, folding, and degradation; and the formation and maintenance of membrane potentials are all examples of these tasks. The human body produces a massive amount of ATP around 50 kilogram each day in a healthy adult. The mitochondrial ATP synthase converts ADP (adenosine diphosphate) and phosphate ions into ATP (Osellame *et al.,* 2012). At the places in the cell where energy is required, ADP and Pi are the results of ATP hydrolysis. Apart from cellular respiration and ATP synthesis, mitochondria also perform a variety of other important tasks, such as formation of NADH and guanosine triphosphate (GTP) through the citric acid cycle; the biosynthesis of amino acids, heme groups, and iron-sulfur clusters, and the synthesis of phospholipids for membrane biogenesis. They also act in calcium signaling and oxidative stress responses, and in general as cellular signaling hubs (Friedman & Nunnari 2014) **(Figure 5).**

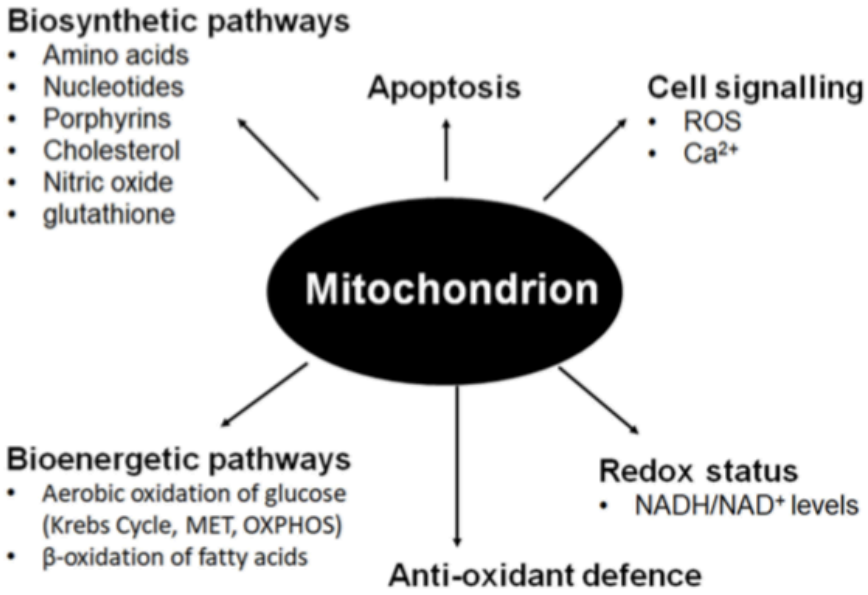

Figure 5: The involvement of mitochondria in fundamental cellular pathways and processes. [Adapted from (Herst *et al.,* 2017)].

1.3.3 Mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential (ΔΨm) is formed by redox reactions linked with Krebs cycle activity. It acts as an intermediate type of energy storage for ATP synthase enzyme to produce ATP molecules. Proton pumps (Complexes I, III, and IV of the mitochondria respiratory complexes) create the mitochondrial membrane potential (ΔΨm), which is critical for energy storage during the oxidative phosphorylation cycle (Zorova *et al.,* 2018)

These redox reactions produce both an electrical potential (due to charge separation) and a proton gradient, which combine to create the hydrogen ion transmembrane potential. According to available evidence, the signaling processes triggered by ATP and ΔΨm are distinct. Cells normally maintain steady intracellular ATP and ΔΨm levels. The maintenance of these levels is regarded as a requirement for optimal cell activity (Zorov *et al.,* 2014).

It is crucial to keep ATP and ΔΨm levels in the cell within a stable range. ΔΨm plays an important role in mitochondrial homeostasis through selective elimination of dysfunctional mitochondria. ΔΨm (rather than H+) is also a driving force in the transport of ions and proteins that are essential for healthy mitochondrial functioning (Zamzami *et al.,* 1995).

1.3.4 Mitochondria and oxidative stress

Oxidative stress (OS) is a condition that occurs due to an imbalance between reactive oxygen species (ROS) and antioxidant levels. This condition can cause mutations in the DNA of the mitochondria. It may also disrupt the respiratory chain and can affect the permeability of the mitochondrial membrane. Moreover, ROS molecules disrupt Ca2+ homeostasis, cause protein and lipid breakdown and harm the defense mechanisms of the mitochondria (Guo *et al.,* 2013).

During mitochondrial respiration, complexes I and III are thought to be the major sources of superoxide and other reactive oxygen species (Hollensworth et al., 2000). In mitochondria, hydroxyl radicals can damage macromolecules including proteins, lipids, and DNA. Therefore, the presence of damaged mitochondrial DNA leads to defective complex I and/or III function. Superoxide is formed from increased electron reduction of O2 (Van Houten et al., 2006). Mitochondrial DNA is particularly a vulnerable target for oxidative damage. Superoxides generated by mitochondrial DNA lesions may be responsible for genomic instability, metabolic oxidative stress, as well as cellular injury (Guo et al., 2013). Oxidative stress can be increased by decreased expression of essential electron transport chain proteins as a result of mitochondrial DNA damage. This leads to a destructive cycle of ROS and organelle failure that eventually induces apoptosis (Van Houten et al., 2006). It should be noted that the electron transport chain of the mitochondria is particularly vulnerable to Nitroxyl (NO^-) and peroxynitrite ($ONOO^-$) mediated damage (Sas et al., 2007). Nitration can change the structure of mitochondrial proteins. Many metabolic enzymes in the mitochondrial electron-transport chain, such as cytochrome c oxidase, NADH dehydrogenase, and ATP synthase, are affected by protein oxidation and nitration (Andreazza et al., 2010). The iron-sulfur (Fe-S) centers of complexes I, II, and III of the electron-transport chain can be inactivated by chronic exposure to ROS, resulting in the shut-down of mitochondrial energy production (Ghezzi & Zeviani, 2012).

ROS can damage several parts of spermatozoa including nuclear and mitochondrial DNA. Therefore; elevated levels of ROS can disrupt sperm physiological function. They can also impair sperm motility and subsequently the fertilization process (Kumar *et al.,* 2009).

1.3.5 Mitochondrial DNA (mtDNA)

Mitochondrial DNA (mtDNA) is located in the mitochondrial matrix, and only transmitted through the female germ line (Chiaratti *et al.,* 2020). The structure and gene arrangement of mtDNA is highly conserved through mammals (Farge *et al.,* 2019). It includes around 16.6 kilobase (kb) of close circular double-stranded DNA (heavy and light) strands. The heavy strand (H) codes for two ribosomal ribonucleic acid (rRNAs), 14 transfer ribonucleic acid (tRNAs), and 12 polypeptides, while the light strand (L) codes for 8 tRNAs and a single polypeptide. All 13 encoded proteins are subunits of the enzyme complexes of the oxidative phosphorylation system (Schon *et al.,* 2012). A map of human mtDNA is given in **Figure 6**.

Mitochondrial DNA (mtDNA) replicates rapidly, without an efficient repair mechanism. Therefore, the rate of mutations is 10-100 times more than that of nuclear DNA (Bahrehmand Namaghi & Vaziri, 2017). Moreover, sperm cells lack endogenous antioxidant activity, which increases susceptibility to damage from oxidants (Ashok, Gurpriya, Chloe, & Stefan, 2014).

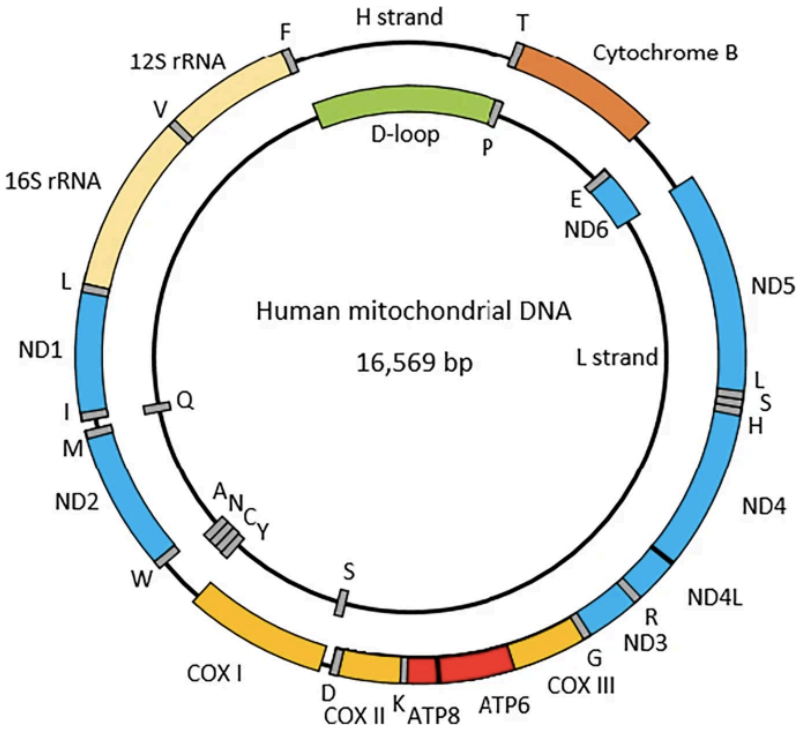

Figure 6: Map of the human mitochondrial DNA genome (16569 bp).

[Adapted from (Govers *et al*., 2021)]

1.3.5.1 Mitochondrial DNA inheritance

Human mtDNA is maternally inherited (Wei & Chinnery 2020). Although the sperm-derived paternal mitochondria enter the oocyte cytoplasm during fertilization process in humans, their mtDNA is never passed on to the offspring. The mechanism by which paternal mitochondria and mtDNA are eliminated from the cytoplasm of gametes or zygotes remains a mystery (Sato & Sato 2013).

1.3.5.2 Mitochondrial genome transcription and translation

Gene expression in mitochondria is uniquely controlled by two sets of RNAs and protein factors originating in both the mitochondria and the nucleus. There are two non-coding regions in the mitochondrial genome in which transcription originates: the L-strand (LSP) and the H-strand (H1 and H2) (D'Souza & Minczuk, 2018). The H2 strand's promoter creates a transcript that covers the whole genome. Eight tRNAs, as well as the MT-ND6 gene, are transcribed via the L strand promoter. In addition, the H1 strand promoter activates the transcription of 12S and 16S mitochondrial rRNA (Shokolenko & Alexeyev, 2017).

A. Transcription

The transcription process is done in three stages: initiation, elongation, and termination (Ringel *et al.,* 2011). POLRMT is a DNA-dependent RNA polymerase that drives transcription in human mitochondria. In order for transcription to initiate, POLRMT needs to be associated with mitochondrial transcription factors A (TFAM) and B2 (TFB2M) (Kanki *et al.,* 2004, Falkenberg *et al.,* 2002). TFAM is a DNA-binding protein that does more than only activate transcription — it also compresses DNA in the nucleoid. In vitro, recombinant TEFM enhances POLRMT processivity by stimulating the production of longer transcripts (Posse *et al.,* 2015). MTERF1 at the 3′-end of the mt-rRNA coding region has recently been found to prematurely terminate transcription from the LSP. MTERF1 binding to this location inhibits the replication fork from proceeding into the mt-rRNA genes while they are being transcribed, as well as the antisense sequence of the rRNA being transcribed (D'Souza & Minczuk, 2018) **(Figure 7)**.

B. Translation

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

This process is crucial for maintaining cell energy balance during protein synthesis in oxidative phosphorylation. It is essential for adenosine triphosphate (ATP) generation and cristae folding. In the case of mitochondrial dysfunction, the combined respiratory chain is impaired, resulting in a lower generation of ATP and eventual energy depletion within the cell (Aibara *et al.,* 2020).

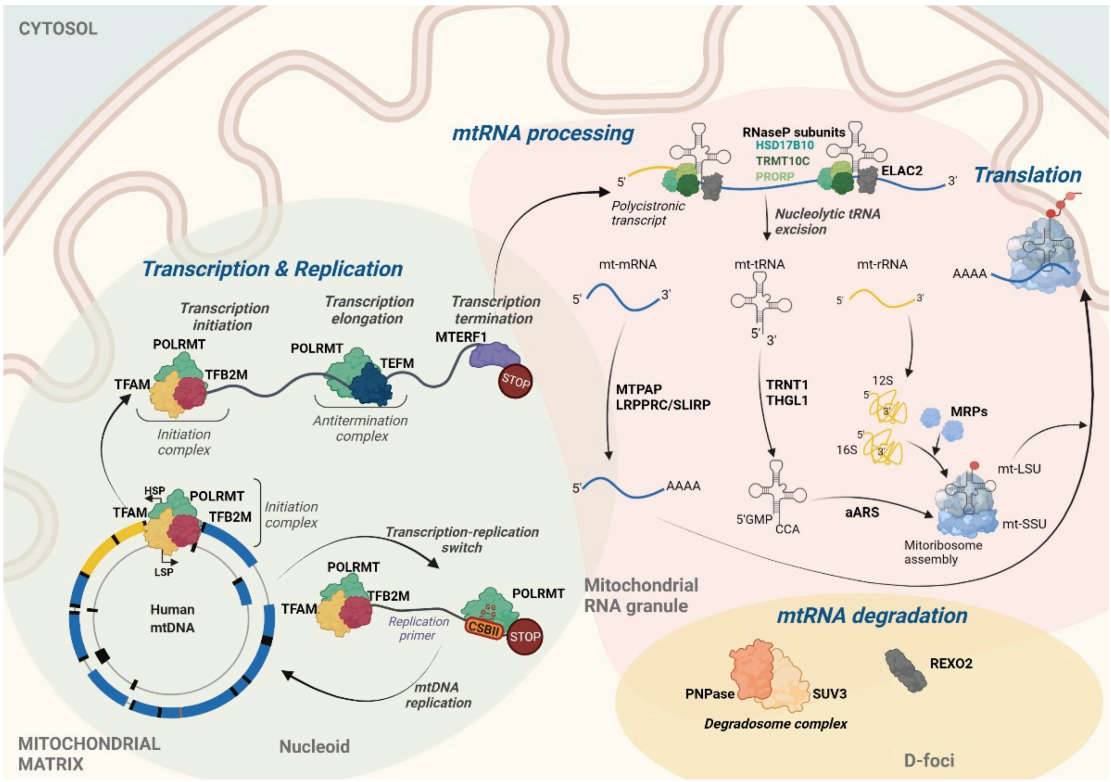

Figure 7: Overview of gene expression in human mitochondria.
[Adapted from (Jedynak-Slyvka *et al.,* 2021)]

1.3.5.3 Mitochondrial DNA mutation and male infertility

Mutations in the nuclear or mitochondrial DNA encode mitochondrial proteins, leading to mitochondrial cytopathies. Different mitochondrial cytopathies result in different types of disorders caused by mitochondrial dysfunction. Mitochondrial dysfunction causes physical, mental, and developmental disabilities, as well as a variety of organ pathologies, primarily those affecting the nervous and muscular systems. Numerous mtDNA mutations linked to the onset and progression of human illnesses have been discovered recently (Ryzhkova *et al.,* 2018).

Any aberration in mitochondrial respiratory activity has an impact on the spermatogenesis process, especially the component of pachytene phase progression during meiosis and sperm generation. Spermatogenic cells will undergo mitochondrial respiratory failure if large amounts of pathogenic mutant mtDNA accumulate in the testicular tissue. Any reduction in the mitochondrion's ability to generate energy during spermatogenesis causes meiotic arrest. Spermatocytes with a lack of oxygen will most likely not complete meiosis and will be killed by apoptosis (Shamsi *et al.,* 2008).

1.4 Male Infertility

According to existing data given by the World Health Organization (WHO), infertility affects roughly 48 million couples and 186 million people globally (Agarwal *et al.,* 2015). Briefly, infertility is a malady of reproductive systems of either the male or female, or both. It is characterized by not being successful in achieving pregnancy after exactly or more than 1 year of frequent, unprotected sexual intercourse (WHO website. 2021). Infertility has now become one of the most common and widespread problems. Although this issue has existed for a long time, several factors, including the development of science and the increased need for treatment, have led to more focus and attention being devoted to it now.

Since the birth of the first in vitro fertilization (IVF) baby in July 1978, the number of studies on the diagnosis and treatment of infertility has expanded considerably, with most of them focusing on enhancing the efficiency of infertility diagnosis and increasing the success rate of treatment (Eskew & Jungheim, 2017).

Infertility affects around 15% of couples globally (De Berardis *et al.,* 2014). Semen quality parameters established by World Health Organization (WHO) are used to diagnose male infertility **(Figure 8)**. Male factor infertility is considered to be caused by genetic defects in about 15%–30% of cases (Krausz *et al.,* 2015).

In order to manage infertility, researchers must understand and clarify the genetic basis of reproductive failure including several physiological processes like spermatogenesis, hormonal homeostasis, and sperm quality (Krausz & Giachini, 2007).

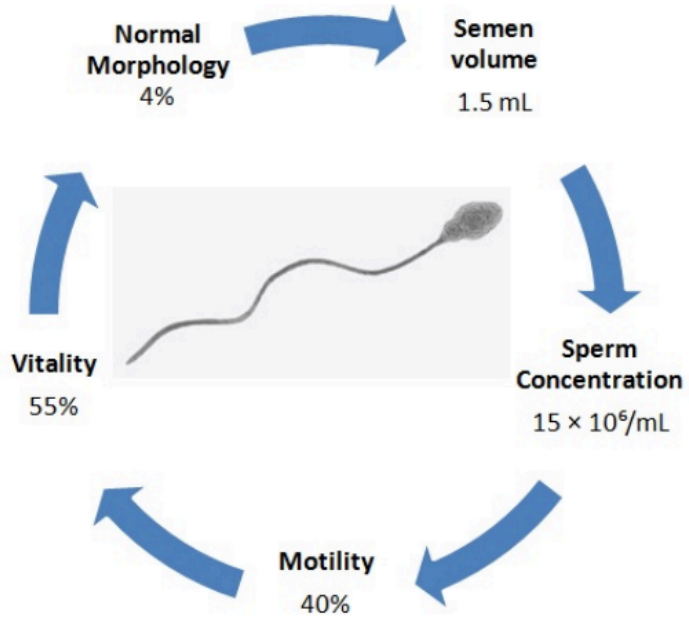

Figure 8: Standardized WHO values as a reference for normal semen parameters.

[Adapted from (Silva, 2018)]

1.5 Sperm abnormalities

Sperm quantity and quality are the major parameters in measuring the ability of sperm to achieve fertilization, and therefore in measuring sperm fertility (Rurangwa *et al.,* 2004). The evaluation of these parameters is achieved through a semen fluid analysis, which is also called a seminogram (Lozano *et al.,* 2009). Semen volume and color and sperm count, motility, and morphological forms are the major characteristics to evaluate. The normal ranges of these parameters have been determined by the World Health Organization (WHO) as more than 15 million sperm per 1 millilitre (ml), 40% sperm motility, and 4% normal morphology, respectively (WHO, 2010).

Male infertility results in decreased sperm parameters, and therefore, results in several types of defects, namely oligozoospermia, asthenozoospermia, and teratozoospermia. The term oligozoospermia refers to a low sperm count in one ml of seminal fluid. Oligozoospermia is usually classified into three classes: mild, with concentrations from 10 to 15 million sperm/ml; moderate, with concentrations from 5 to 10 million sperm/ml; and severe oligozoospermia, with concentrations of less than 5 million sperm/ml (Dajanı, 2016). Men with no sperm in one ml of seminal fluid are diagnosed as azoospermia patients (Berookhim & Schlegel, 2014) **(Figure 9a)**.

Asthenozoospermia is a medical term for men with reduced sperm motility (Vaiarelli *et al.,* 2019). Healthy sperm move in forward progression motion. There are different types of sperm motility includes: progressive motility (PR), local motility, and no motile sperms (Rurangwa *et al.,* 2004) **(Figure 9b).**

Teratozoospermia is a condition in which sperm have abnormal morphology. Sperm with defective shapes can affect male fertility by preventing the transport in the cervix or preventing the sperm from penetrating the ovum layers; therefore, successful fertilization and pregnancy may be difficult (Jungwirth *et al.,* 2012) **(Figure 9c).**

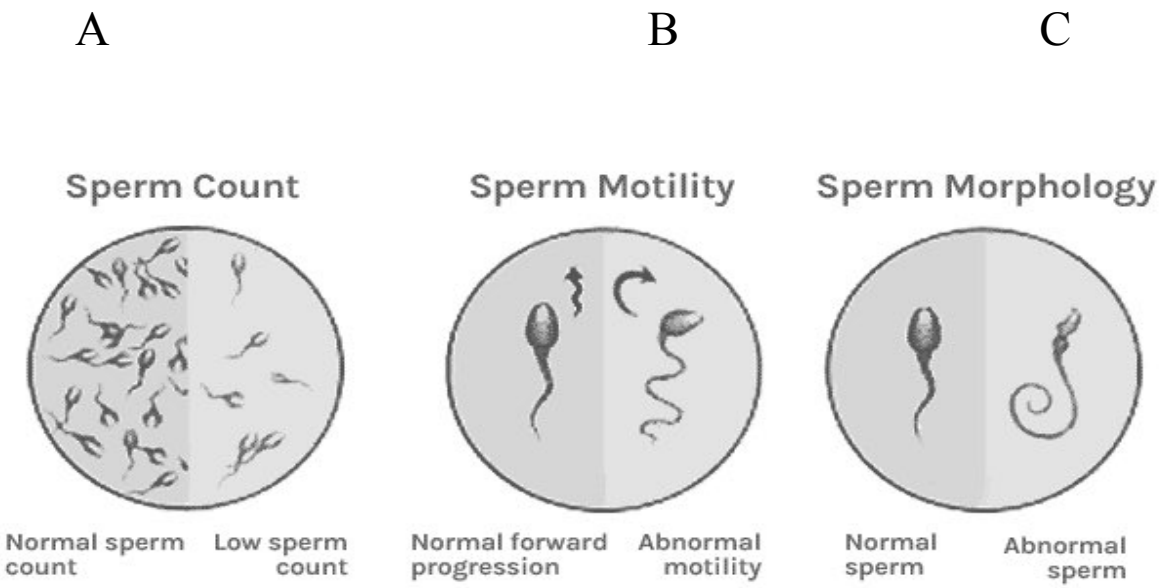

Figure 9: Abnormal parameters of human spermatozoa.

1.6 Factors related to male fertility

Sperm analysis is the most common way of diagnosing infertility in men. Semen is characterized by its concentration and appearance, as well as the motility of sperm. A number of factors contribute to infertility in men, including environmental and lifestyle problems, hormonal disorders, physical reasons, sexual dysfunction, and genetic factors (chromosomal abnormalities and single-gene disorders). Researchers have made numerous attempts to identify the causes of male infertility, but about 70% of these remain unknown. Male infertility is a complex issue that is not well understood (Babakhanzadeh *et al.,* 2020).

1.6.1 Environmental and lifestyle factors

Exposure to hazardous substances and radiation can lead to fertility problems. Those substances may include silicones, insecticides, adhesives, and metals (Rim 2017). Moreover, a study has reported that humans exposed to high levels of lead had a non-significantly longer time-to-pregnancy interval (Apostoli, Bellini, Porru, & Bisanti, 2000).

Radioactivity can cause sperm production to decrease, and extreme radiation can cause infertility (Liu *et al.,* 2017).

There are no definite conclusions as to the effects of alcohol and smoking on sperm parameters and fertility (Gaur *et al.,* 2010). A poor diet can also be a factor in male infertility, as an increase in saturated fat consumption has been reported to decrease sperm concentration in men (Tsai *et al.,* 2013).

1.6.2 Hormonal disorders

A dysfunctional hormonal system can lead to infertility. A lack of gonadotropic releasing hormone (GnRH) results in insufficient testosterone and stopped sperm production (Monaco *et al.,* 2015). Additionally, the failure of the pituitary gland to produce sufficient amount of both luteinizing hormone (LH) and follicular stimulating hormone (FSH) results in a failure to stimulate the testes to produce testosterone and subsequently initiate spermatogenesis (Wdowiak *et al.,* 2014) .

1.6.3 Sexual dysfunction

Varicocele, or enlarged sperm vessels, is one of the most common male infertility issues affecting approximately 40% of men (Sengupta *et al.,* 2018). Male infertility can also be caused by chronic or acute genital tract infections (Fallahi *et al.,* 2018).

1.6.4 Genetic factors

Genetics related to fertility impact a number of processes including hormonal homeostasis, spermatogenesis, and sperm quality.

1.6.4.1 Chromosomal abnormalities

Approximately, 5% of male infertility cases are due to chromosomal abnormalities, while that percentage increases up to 15% in azoospermia cases (Ferlin *et al.,* 2007). Chromosomal abnormalities include Y chromosome microdeletions which are considered as the major cause of azoospermia and severe oligozoospermia cases (Carrell, De Jonge, & Lamb, 2006).

Y chromosome consists of genes that are critical for spermatogenesis process and the development of male gonads. The exact cause of several infertile phenotypes is difficult to identify due to the variation on the Y chromosome and the occurrence of deletions of multiple genes (Reynolds & Cooke, 2005). Therefore, several different deletions or mutations may produce the same phenotype. Studies have reported that microdeletions are more common in men with azoospermia and severely oligozoospermia disorders (Katagiri *et al.,* 2004).

1.6.4.2. Autosomal gene mutations

Autosomal gene mutations had been investigated in several studies due to its roles in male fertility. The Conducted Regulator Gene (CFTR) is the most common autosomal gene that has been found to be mutated. Mutations have occurred in approximately 60%—90% patients with congenital bilateral absence of the Vas deferens (CBAVD) (Georgiou *et al.,* 2006), a form of obstructive azoospermia.

1.6.4.3. X-Linked genes

Large numbers of X-linked genes have a critical roles in gametogenesis (Nuti & Krausz, 2008). The most common X-linked gene that related to male infertility is the androgen receptor (AR) gene, which is located in the long arm of the X chromosome (Nuti & Krausz, 2008). The androgen receptor gene is involved in meiosis and plays a role in the conversion of spermatocytes to round spermatids during spermatogenesis (De Gendt *et al.,* 2004). A case-control study reported that 2% of infertile men had mutations in their AR gene, while none were shown in control group (Ferlin *et al.,* 2006).

1.7 Human Mitochondrial Genes

1.7.1 *MT-CYB*

Cytochrome B is a MT-DNA gene that codes cytochrome B protein. As a respiratory chain protein, the cytochrome B protein belongs to the Ubiquinol Cytochrome C Reductase(complex III, cytochrome bc1 complex, or UQCR) complex. Position 12 of the mitochondrial genome is the location of the *MT-CYB* gene. It has 1,140 base pairs that code for a 42.7 kDa protein with 380 amino acids (Zong *et al.,* 2013).

MT-CYB is necessary for the proper operation of complex III of the mitochondrial respiratory chain (Olpin *et al.,* 2015).

Mutations in *MT-CYB* can cause a range of mitochondrial deficiencies, which result in a variety of illnesses. Complex III deficiency, which is a defect in the mechanism of electron transport catalyzation via the respiratory chain of the mitochondria, is the most prevalent form. Mitochondrial encephalomyopathy, Leber hereditary optic neuropathy, mitochondrial myopathy,

muscle weakness, myoglobinuria, blood acidosis, renal tubulopathy, and others conditions can also occur as phenotypic disorders (Melchionda *et al.,* 2014).

1.7.2 *MT-CO3*

Mitochondrial cytochrome C oxidase III gene codes cytochrome c oxidase subunit III (*COX3*) enzyme, which is one of the major transmembrane subunits of cytochrome c oxidase (Strogolova *et al.,* 2012). Its 261 amino acids compose a 30 kDa protein that is located on the inner mitochondrial membrane(Consortium, 2018).

Mutations in *COX3* gene have been found to be associated with recurrent myoglobinuria, Leber hereditary optic neuropathy, severe encephalomyopathy, isolated myopathy, and mitochondrial complex IV deficiency (Horvath *et al.,* 2005).

1.7.3 *MT-ATP6*

MT-ATP6 gene codes the ATP synthase membrane subunit 6 enzyme. This enzyme contributes to the last step in the electron transport chain (ETC), oxidative phosphorylation (Anderson *et al.,* 1981). The *MT-ATP6* gene produces a protein with 24.8 kDa and is composed of 226 amino acids (Zong *et al.,* 2013).

MT-ATP6 subunit plays a role in the proton channel. It is considered as a key component in the translocation of proton across the membrane (Carbajo *et al.,* 2005).

MT-ATP6 gene mutations have been identified in about 10 to 20% of patients with Leigh syndrome. Furthermore, an association was reported between these mutations and a range of cardiovascular and neurodegenerative disorders, including Leber's hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy with stroke-like episodes (MELAS), retinitis pigmentosa syndrome (NARP), mitochondrial complex V deficiency, neuropathy, and ataxia (Blanco−Grau *et al.,* 2013).

1.7.4 *MT-ATP8*

MT-ATP8 is a mitochondrial gene that codes for the synthesis of ATP synthase membrane subunit 8 (Anderson *et al.,* 1981). This protein contains 68 amino acids that form a 8 kDa protein (Zong *et al.,* 2013), and is located within the thylakoid membrane and the inner mitochondrial membrane. It catalyzes ATP synthesis through utilizing an electrochemical gradient of protons across the inner membrane during the oxidative phosphorylation process(Urbani *et al.,* 2013).

Mutations in the *MT-ATP8* are correlated with several disorders such as Leber's hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy with stroke-like episodes (MELAS), Leigh syndrome, and NARP syndrome (Blanco−Grau *et al.*, 2013).

1.8 The purpose of the study

The objectives of the present study are as follows:

1-To identify the role of the variations in the mitochondrial genes *MT-CYB*, *MT-CO3*, *MT-ATP6*, and *MT-ATP8* in male infertility.

2-To determine the genotype and allele frequency distributions in subfertile and fertile men.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. study population

For the purpose of our research, a total of 111 samples of semen were collected from fertile and subfertile men. All participants submitted complete informed consent before any samples were collected. After 3-5 days of abstinence, participants used sterile containers to produce semen by masturbation. Upon a physical examination of the candidates, the following data was also recorded: (Age, duration of infertility, health status, existence of chronic diseases, and history of varicoceles). All participants were healthy males in reproductive age (25–55) who had no history of varicoceles and were free of any chronic conditions (diabetes, high blood pressure, etc.). In this study, men without a history of hormonal imbalance (hypogonadotropic hypogonadism), exposure to chemotherapy or radiation, surgery on their reproductive organs, or genetic disorders (such as Klinefelter's syndrome or Y-chromosome microdeletion) were involved.

2.1.2. Chemicals, Reagents, equipment and kits

Disposables	Manufacturing Company
1 ml insulin Syringe sterile U-40	BD Medical, USA
Biosphere Filter tips (10-20-200-100-1000 ml)	Sarstedt, Germany
Conical centrifuge tube,15 ml	Vitrolife, Sweden
Pipettes	Eppendorf, Germany
3 ml disposable sterile transfer pipette	Vitrolife, sweden
Cover slips	R. Langenbrinck, Germany

Glass slides	R. Langenbrinck, Germany
96-well PCR Plate 0.2 mL, non-skirted	Nippon Genetics Europe, 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
Reagents	
Sperm wash (Global total w/ Hepes w/ HSA)	Cooper surgical, Denmark
Immersion oil	Merck, Germany
Sperm stain Kit	Ferti Pro, Belgium
Sperm freezing solution	Cooper surgical, Denmark
Absolute Ethanol	Merck, Germany
Agarose tablets (DNase/RNase free)	Biocat, Germany
DNA Ladder (1kb)	New England BioLabs, USA
Instruments	
Bench-top centrifuge	Sigma-Aldrich, Germany
Binocular light microscope	Olympus, Japan
Blockthermostat	Labotect, Germany
Heraeus horizontal laminar flow cabinet	Heraeus, Germany
Makler Counting Chamber	Origio, Germany

Racks	Sarstedt, Germany
MolecularImager Gel Doc XR & System with Image Lab Software	Bio-Rad, Germany
Microcentrifuge	Eppendorf, Germany
Thermal Cycler C100	Bio-Rad, Germany
Vortex-Genie 2	Scientific industries, USA
Kits	
QIAamp DNA- Mini Kit	QIAGEN, Germany
REPLI-g mitochondrial DNA Kit	QIAGEN, Germany
PCR primers (*MT-CYB*, *MT-CO3*, *MT-ATP6* and *MT-ATP8*)	MicrosynthSeqLab, Germany
PCR master mix (2x)	Thermo Fisher Scientific, Germany

2.2. Methods

2.2.1 Sample preparation

Semen samples were evaluated and tested in the IVF laboratory of the University of Saarland at the department of Obstetrics and Gynecology. After semen collection, all samples were allowed to liquify in room temperature for 30 minutes (min) then semen analysis was carried out according to WHO laboratory manual for the examination and processing of human semen, 5th edition (2010); macroscopic (semen appearance, viscosity, pH, and volume) and microscopic (sperm concentration, motility, and morphology) evaluations were done **(Table 1)**.

After that, sperm wash media (Global Total HEPES with HSA) used to prepare semen samples, which were following centrifuged at 1200 rpm x 10 minutes. The resulting pellets were resuspended in 0.5 ml of the washing buffer.

Finally, samples kept in - 20 ºC freezer to be used in the following step for extracting the DNA.

Table 1: Semen parameters reference ranges

Semen parameters	Reference range
Volume	1.5 ml
Sperm concentration	15 × 10^6 spermatozoa per ml
Sperm motility	40%
Progression	32%
Morphology	4%

2.2.2. Extraction of the Mitochondrial DNA

For the preparation of the total DNA and MT-DNA isolation, frozen semen samples were thawed and prepared using the 40%–80% Puresperm discontinuous gradients (Nidacon International, Sweden) as a purification step to remove cells other than spermatozoa. This step was carried by layering each sample on the top of the previously prepared density media tubes, then centrifuged at (500 g x20 min) at room temperature.

Two steps were performed for isolating the mitochondrial DNA from sperm. Initially, QIAGEN's QIAamp DNA Mini Kit was used, this kit is frequently used for genomic and mitochondrial DNA isolation. The extraction was done by following the recommended guidelines provided with the kit **(**QIAamp spin procedure) **(Figure 10)**. First, 20 Microliter (µl) Protease K enzyme pipetted into the bottom of microcentrifuge tube (a 1.5 ml). Then, 200 µl of the washed semen sample added on the same tube and mixed thoroughly, the mixture incubated at 56°C overnight

to yield the maximum of the MT-DNA. Next, 200 µl of Buffer AL added to the reaction mixture and vortex for 15 sec till it yielded a homogenous solution.

next, 200 µl of the absolute ethanol (96–100%) added to the mixture, and vortex again for 15 s. After that, for the removal of drops from the inside of the lid, microcentrifuge tubes were briefly centrifuged. The mixture from previous step applied to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 min.

Following, the QIAamp Mini spin column placed in a new 2 ml collection tube (provided by the kit), and the tube containing the filtrate discarded. Make sure to close all spin columns during centrifugation to avoid aerosol formation. The column Opened and 500 µl Buffer AW1 added and centrifuged at 6000 x g (8000 rpm) for 1 min. Again, the QIAamp Mini spin column placed in a clean 2 ml collection tube provided in the kit, then the Mini spin columns opened and 500 µl Buffer AW2 added and centrifuged at full speed (20,000 g; 14,000 rpm) for 3 min.

Finally, the Mini spin columns transferred to clean 1.5 ml microcentrifuge tubes (not provided in the kit), and the tubes containing the filtrate discarded, then 200 µl Buffer AE or distilled water added and all incubated at room temperature (15–25°C) for 1 min to increase MT-DNA yield, and centrifuged at 6000 x g (8000 rpm) for 1 min.

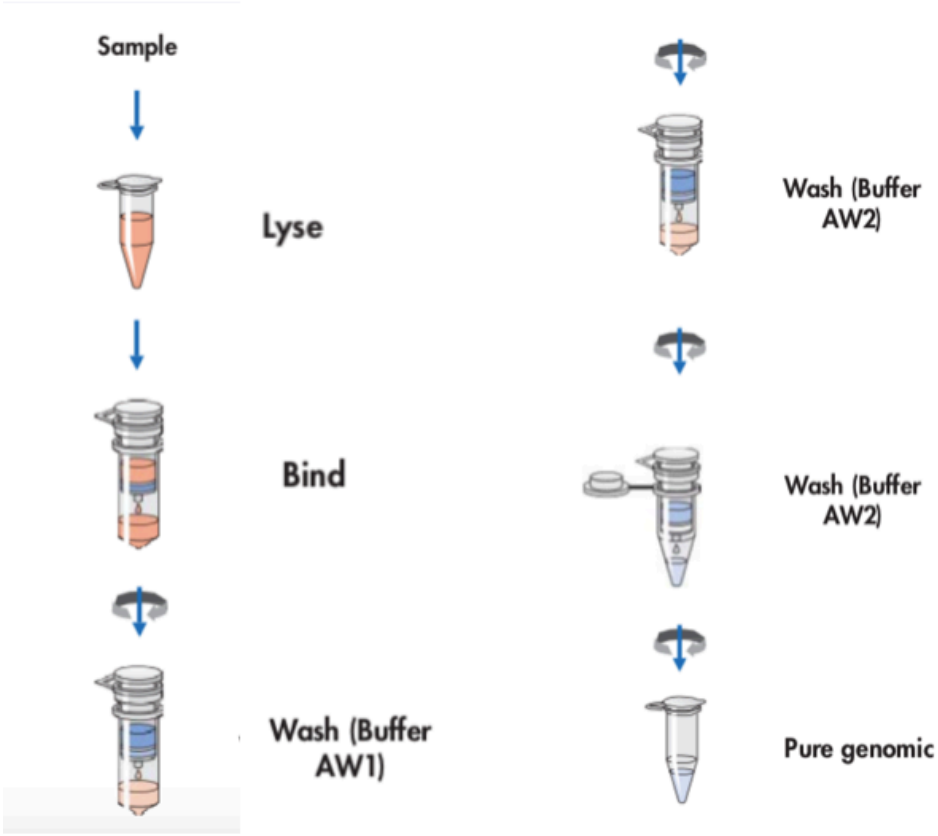

Figure 10: Steps of QIAamp® DNA Mini for MT-DNA amplification.
(From QIAamp® DNA Mini Kit). (Handbook, B. D. P. (2005). Qiagen. Gmbh, Germany)

2.2.3. MT-DNA amplification

The second step we used QIAGEN's REPLI-g Mitochondrial DNA Kit to extract and amplify only mitochondrial DNA from the samples, the amplification was done in accordance with the guidelines and recommendations provided with the kit **(Table 2).**

Table 2: Preparation of amplification mixture

Reagent	Volume
REPLI-g mt Reaction Buffer	27 µl
REPLI-g Human mt Primer Mix	2 µl
Total volume	29 µl

First, a microcentrifuge tube was filled with 10 µl of the extracted DNA sample. RNase-Free water then added to adjust the sample volume to 20 µl. A fresh amplification mixture prepared (see Table) and the mixture centrifuged briefly. Next step, 29 µl from the mixture of amplification pipetted to the DNA samples tubes, again vortex and centrifuged. Using the thermocycler, the reaction mixture incubated at 75°C X 5 min, then they left to cool till the reach room temperature. One µl of the REPLI-g Midi Polymerase pipetted into the mixture of the DNA that yielded from the last step, then incubated for 8 h at 33°C.

Following, inactivation of the enzyme was done by heating the sample at 65°C for 3 min. After that, DNA concentration in the isolated samples were calculated using the Nano-drop machine and an optimal density ratio 260/280 of 1.8 or more were only chosen. Finally, the amplified MT- DNA solutions were kept in - 80 ºC freezer to be used later for the PCR process.

2.3 Polymerase chain reaction (PCR)

For this study 4 genes of the MT DNA - encodes for parts of the proteins included in the respiratory chain of the mitochondria — were chosen to be examined for any mutation in their sequence. These genes are *CYB*, *CO3*, *ATP6* and *ATP8*.

Primers for the forward and reverse reaction were ordered from the Microsynthlab company according to the sequences designed using the PRIME 3 software and the UCSC website **(Table 3)**.

Using a thermocycler, the following protocol was used for the PCR procedure: The initial denaturation step was done at 95 ºC for 3 minutes, followed by 35 cycles of Denaturation step (at 95 ºC X 40 sec), the Annealing step (at 57 ºC X 30 sec) , and next the extension (at 72 ºC X 1 min) was done. The last extension step was done at 72 ºC for 5 min **(Table 3).**

The resulting mixture containing 30 µL of the PCR products were run on gel electrophoresis stained with ethadium bromide. For each sample, 5 µL of the PCR product were run using a DNA Ladder (0.1-10.0 kb) (NE Biolabs, USA) and 2 different concentrations of gel. Both were run at 75-volt (V) for 1 hour in 1× Tris-Acetate-EDTA (TAE) buffer. Samples visualizing done using the Molecular Imager® Gel Doc™ XR (BIO-RAD, USA).

Finally, Sanger Sequencing was done for the samples to investigate the presence of any mutation in the DNA sequence **(Figures 11, 12, 13, 14)**.

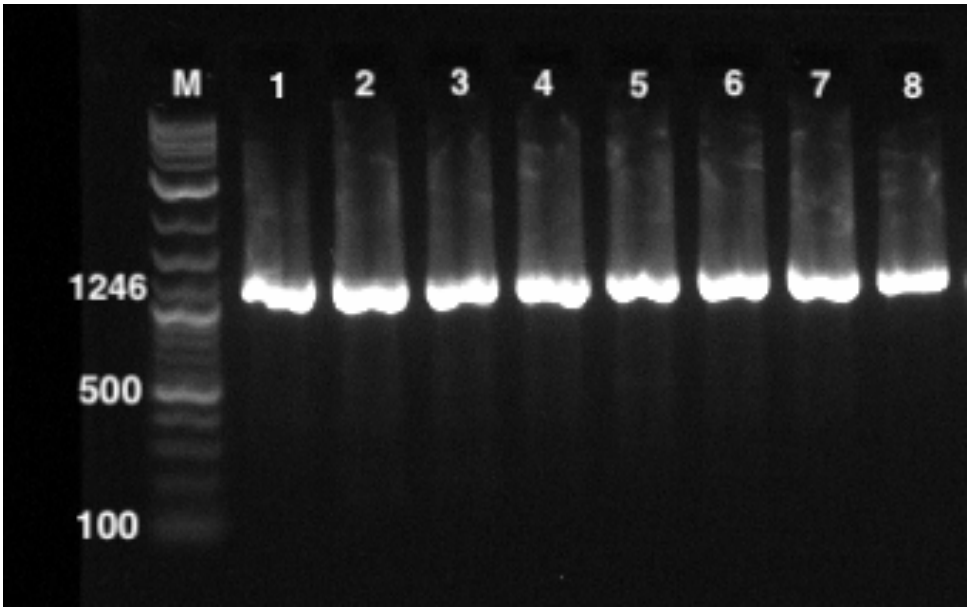

Figure 11: Illustrative gel electrophoresis on 1.5% agarose gel of PCR products for the amplification of the *MT-CYB* gene in 8 samples (1-8) along with DNA Ladder (M) (1246 bp).

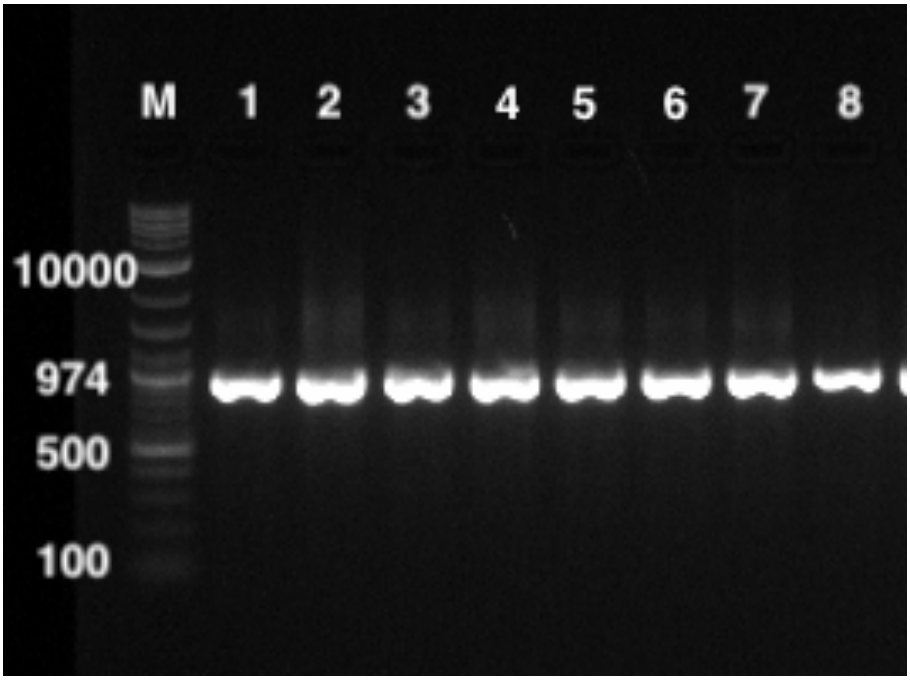

Figure 12: Illustrative gel electrophoresis on 1.5% agarose gel of PCR products for the amplification of the MT-CO3 gene in 8 samples (1-8) along with DNA Ladder (M) (974 bp).

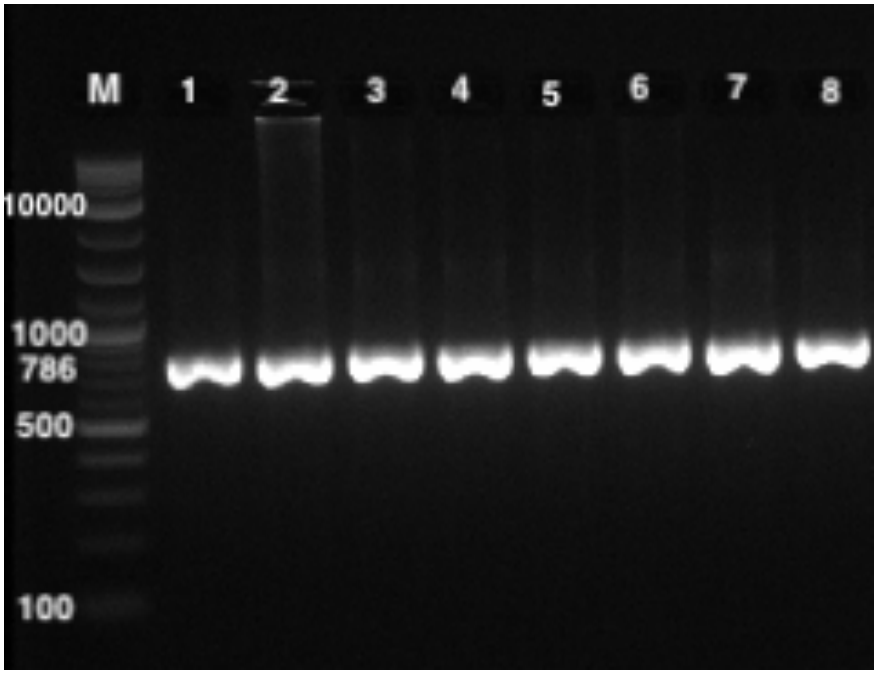

Figure 13: Illustrative gel electrophoresis on 2% agarose gel of PCR products for the amplification of the MT-ATP6 gene in 8 samples (1-8) along with DNA Ladder (M) (786 bp).

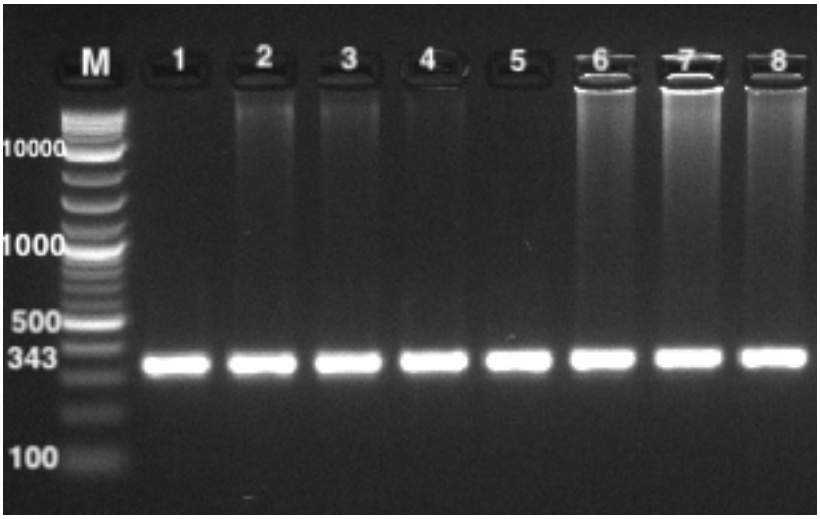

Figure 14: Illustrative gel electrophoresis on 2% agarose gel of PCR products for the amplification of the MT-ATP8 gene in 8 samples (1-8) along with DNA Ladder (M) (343 bp).

Table 3: Oligonucleotide primers for *MT-CYB*, *MT-CO3*, *MT-ATP6*, and *MT-ATP8* genes.

Gene	Primer direction	Sequence (5'->3')	Product length	PCR program
MT-CYB	MT-CYB forward -F	CGGACTACAACCACGACCAA	1246 bp	95 ºC x 3 min 95 ºC X 40 sec 57 ºC X 30 sec (35 cycles) 72 ºC X 1 min 72 ºC for 5 min.
	MT-CYB reverse -R	TCCGGTTTACAAGACTGGTGT		
MT-CO3	MT-CO3 forward -F	CCTAGAAATCGCTGTCGCCT	974 bp	
	MT-CO3 reverse -R	AAGGCTAGGAGGGTGTTGAT		
MT-ATP6	MT-ATP6 forward -F	CCTCCCTCACCAAAGCCCAT	786 bp	
	MT-ATP6 reverse -R	GGTCATGGGCTGGGTTTTACTA		
MT-ATP8	MT-ATP8 forward -F	CCCCTCTAGAGCCCACTGTAA	343 bp	
	MT-ATP8 reverse -R	GTGGGGATCAATAGAGGGGGA		

2.4 Sanger Sequencing

In Seqlab, Göttingen, GmbH, samples were purified and sequenced using the Sanger Cycle Sequencing technique. All the SNPs detected in the *MT-CYB, MT-CO3 ,MT-ATP6,* and *MT-ATP8* genes were discovered using Mutation Surveyor software. Following that, finch TV program was used to genotype all SNPs that were discovered.

2.5 Statistical analysis

Statistical data analysis was carried out by SPSS program version 23, using the chi-square and fischer's exact test. All odds ratios were estimated together with their 95% confidence intervals (CI). Statistics were considered significant if the p-value was < .05. The Hardy Weinberg equilibrium test was performed on the identified SNPs to demonstrate genotype frequencies and statistically significant deviations from equilibrium with the SNPs identified.

3. RESULTS

3.1 Parameters examined for the study population

According to WHO guidelines from 2010, the control group consisted of 44 men with normal semen analysis (Concentration: 15 × 10^6 spermatozoa/ml, total motility: ≥40%, progressive motility: ≥32%, and normal morphology: ≥4%), whereas the subfertile group consisted of 67 men with abnormal semen analysis (Concentration < 15 × 10^6 spermatozoa/ml, motility< 40%, progressive motility < 32%), [Oligozoospermia, n=10, Asthenozoospermia, n= 23, Teratozoospermia, n=19, OAT, n= 15].

Table 4: Descriptive statistical analysis of studied parameters for all the population (N=111)

Parameter	M ± SD	Median
Age (years)	35.04 ±5.51	34
Sperm concentration (10^6 per ml)	54.9 ± 42.29	44
Total Motility (PR + NP. %)	54.84 ± 21.64	57
normal morphology of spermatozoa (%)	18.59 ± 7.43	19

M: Mean; **SD**: Standard deviation; **PR**: progressive motility; **NP**: non progressive motility.

Table 4 Demonstrates the descriptive statistics: mean ± standard deviation, and median of the different studied parameters. The mean of the sperm parameters: age, sperm concentration, total motility (PR + NP. %), and morphology of normal spermatozoa (%) were (35.04 ±5.51 years ; 54.9 ± 42.29 (10^6 per ml); 54.84 ± 21.64 %; 18.89 ± 7.43 % % respectively).

Table 5: Comparing parameters of the semen analysis between Fertile (control group) and Sub-fertile (study group).

Parameter	Fertile (n=44) Median	Sub-fertile (n=67) Median	*P*-value
Age (yrs)	34 (26–48)	34 (26–48)	0.225
Sperm concentration (million/ml)	78.5 (17–185)	28 (0.6–135)	**< 0.0001**
Total Motility (%)	67.5 (44–90)	50 (2–88)	**< 0.0001**
normal morphology of spermatozoa (%)	24.5(20–30)	15 (0–28)	**< 0.0001**

We compared the semen parameters between fertile and subfertile groups, there was no significant difference in age between the two groups ($P = 0.225$). However, the mean percentage of sperm concentration, motility, and morphology of normal spermatozoa were significantly higher in the group of fertile patients in comparison to subfertile patients group ($P < 0.0001$) **(Table 5)**.

3.2 Genotypes and Allele frequency

We defined thirteen SNPs in the *MT-CYB* gene: rs2853508, rs28357685, rs41518645, rs2853507, rs28357376, rs35070048, rs2853506, rs28660155, rs527236194, rs28357373, rs28357369, rs41504845, and rs2854124, twelve SNPs in the *MT-CO3* gene: rs2248727, rs7520428, rs3134801, rs9743, rs28358272, rs2853824, rs2856985, rs2854139, rs41347846, rs28380140, rs3902407, and 28411821, fourteen SNPs in the *MT-ATP6*: rs2001031, rs2000975, rs2298011, rs7520428, rs9645429, rs112660509, rs6650105, rs6594033, rs6594034, rs6594035, rs3020563, rs28358887, rs2096044, and rs9283154, and ten SNPs in the *MT-ATP8*: rs9285835, rs9285836, rs9283154, rs8179289, rs121434446, rs1116906, rs2153588, rs1116905, rs1116907, and rs3020563.

We compared the genotype and allele frequencies between subfertile and fertile groups for *MT-CYB, MT-CO3, MT-ATP6*, and *MT-ATP8* in order to determine their association with male infertility **(Tables 6 -13)**. Among the 13 *MT-CYB* identified SNPs, three of them displayed a difference of significance in the genotype's frequency test when comparing fertile and subfertile groups: rs28357373 (P=0.0439), rs527236194 (P=0.0005), and rs41504845 (P=0.0038). Two SNPs showed significant in the allele's frequency test: rs41504845 (P=0.0147) and rs527236194 (P=0.0014). Moreover, the rs7520428 in the *MT-CO3* and *MT-ATP6* showed statistical significant difference between subfertile and fertile groups in the genotypes and alleles frequency test (P <0.0001 for both).

Five of the SNPs were detected in the *MT-CYB*, nine in the *MT-CO3* gene, four in *MT-ATP6* gene, and two in *MT-ATP8* gene were Synonymous variants. Whereas the following SNPs of the *MT-CYB* (rs2853508, rs28357685, rs41518645, rs2853507, rs28357376, rs35070048, rs2853506, rs28660155) *MT-ATP6* (rs2001031, rs2000975) and *MT-ATP8* (rs1116906, rs1116905) were located to cause a missense variation in the coding protein.

Table 6: The *MT-CYB* polymorphism genotypes between subfertile patients and controls

SNP	Contig position	Protein position	Amino Acid Change	Genotype	Subfertile N	Control N	*P*- Value
rs2853508 A>G	15326	Thr194Ala	Missense variant	AA	0	0	N/A
				AG	0	0	
				GG	67	44	
rs28357685 G>A	15110	Ala122Thr	Missense variant	GG	0	0	1.0000
				GA	2	1	
				AA	65	43	
rs527236194 T>C	15784	Pro346	Synonymous variant	TT	67	40	**0.0005**
				TC	0	0	
				CC	0	4	
rs41518645 G>A	15257	Asp171Asn	Missense variant	GG	62	41	1.0000
				GA	0	0	
				AA	5	3	
rs2853507 G>A	15317	Ala191Thr	Missense variant	GG	67	43	0.1560
				GA	0	0	
				AA	0	1	
rs28357373 T>C	15629	Leu295	Synonymous variant	TT	63	44	**0.0439**
				TC	1	0	
				CC	3	0	
rs28357376 A>G	15824	Thr360Ala	Missense variant	AA	66	44	0.5193
				AG	0	0	
				GG	1	0	
rs35070048				AA	66	44	

rs35070048 A>G	15311	Ile189Val	Missense variant	AG	0	0	0.5193
				GG	1	0	
rs28357369 A>G	15244	Gly166	Synonymous variant	AA	64	43	0.7060
				AG	1	0	
				GG	2	1	
rs41504845 C>T	15833	Leu363	Synonymous variant	CC	61	44	**0.0038**
				CT	1	0	
				TT	5	0	
rs2853506 A>G	15218	Thr158Ala	Missense variant	AA	67	43	0.1604
				AG	0	0	
				GG	0	1	
rs2854124 C>T	15136	Gly130	Synonymous variant	CC	67	43	0.3964
				CT	0	1	
				TT	0	0	
rs28660155 T>C	14871	Ile42Thr	Missense variant	TT	63	43	0.0909
				TC	0	1	
				CC	4	0	

Table 7: The *MT-CYB* polymorphism alleles between subfertile patients and controls.

SNP	Contig position	Protein position	Alleles	subfertile (N, %)	Fertile (N,%)	OR (95% CI) *	*P*- Value
rs2853508 A>G	15326	Thr194Ala	A	0 (0%)	0 (0%)	N/A	N/A
			G	134 (60%)	88 (40%)		
rs28357685 G>A	15110	Ala122Thr	G	2 (1%)	1 (0%)	1.318 (0.1177 to 14.769)	0.8221
			A	132 (59%)	87 (39%)		
rs527236194 T>C	15784	Pro346	T	134 (60%)	80 (36%)	28.404 (1.617 to 499.07)	**0.0014**
			C	0 (0%)	8 (4%)		
rs41518645 G>A	15257	Asp171Asn	G	124 (56%)	82 (37%)	0.9073 (0.3175 to 2.593)	0.8559
			A	10(4%)	6 (3%)		
rs2853507 G>A	15317	Ala191Thr	G	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			A	0 (0%)	2 (1%)		
rs28357373 T>C	15629	Leu295	T	127 (57%)	88 (40%)	0.09605 (0.005412 to 1.705)	0.0741
			C	7 (3%)	0 (0%)		
rs28357376 A>G	15824	Thr360Ala	A	132 (59%)	88 (40%)	0.2994 (0.01419 to 6.316)	0.6707
			G	2 (1%)	0 (0%)		
rs35070048 A>G	15311	Ile189Val	A	132 (59%)	88 (40%)	0.2994 (0.01419 to 6.316)	0.6707
			G	2 (1%)	0 (0%)		
rs28357369 A>G	15244	Gly166	A	129 (58%)	86 (39%)	0.6000 (0.1138 to 3.164)	0.8292
			G	5 (2%)	2 (1%)		

rs41504845 C>T	15833	Leu363	C	123 (55%)	88 (40%)	0.06067 (0.003526 to 1.044)	**0.0147**
			T	11 (5%)	0 (0%)		
rs2853506 A>G	15218	Thr158Ala	A	134 (60%)	86 (39%)	7.599 (0.3603 to 160.28)	0.3130
			G	0 (0%)	2 (1%)		
rs2854124 C>T	15136	Gly130	C	134 (60%)	87 (39%)	4.611 (0.1856 to 114.58)	0.8319
			T	0 (0%)	1 (0%)		
rs28660155 T>C	14871	Ile42Thr	T	126 (57%)	87 (39%)	0.1810 (0.02223 to 1.474)	0.1503
			C	8 (4%)	1 (0%)		

Table 8: The *MT-CO3* polymorphism genotypes between subfertile patients and controls.

SNP	Contig position	Protein position	Amino acid change	Genotype	infertile, N	CONT, N	*P*- Value
rs2248727 T>C	9540	Leu112	Synonymous variant	TT	59	34	0.1315
				TC	0	0	
				CC	8	10	
rs7520428 A>G	634390	N/A	N/A	AA	67	38	**< 0.0001**
				AG	0	0	
				GG	0	6	
rs3134801 T>C	9950	Val248	Synonymous variant	TT	67	43	0.1560
				TC	0	0	
				CC	0	1	
rs9743 T>C	9698	Leu164	Synonymous variant	TT	63	43	0.3221
				TC	0	0	
				CC	4	1	
rs28358272 C>T	9449	Tyr81	Synonymous variant	CC	65	44	0.1540
				CT	0	0	
				TT	2	0	
rs2853824 A>G	9347	Leu47	Synonymous variant	AA	67	43	0.1560
				AG	0	0	
				GG	0	1	
rs2856985 G>A	9755	Glu183	Synonymous variant	GG	67	43	0.1560
				GA	0	0	
				AA	0	1	
rs2854139 C>T	9818	His204	Synonymous variant	CC	67	44	0.1560
				CT	0	0	
				TT	0	0	
rs41347846 T>C	10034	N/A	N/A	TT	67	44	0.1560
				TC	0	0	
				CC	0	0	
				AA	67	43	

rs28380140 A>G	9377	Trp57	Synonymous variant	AG	0	0	0.1560
				GG	0	1	
rs3902407 T>C	N/A	N/A	N/A	TT	67	44	0.1560
				TC	0	0	
				CC	0	0	
rs28411821 T>A,C	9824	Leu206	Synonymous variant	TT	67	43	0.1560
				TC	0	0	
				CC	0	1	

Table 9: The *MT-CO3* polymorphism alleles between subfertile patients and controls.

SNP	Contig position	Protein position	Alleles	infertile, %	CONT, %	OR (95% CI)*	*P*- Value
rs2248727 T>C	9540	Leu112	T	118 (53%)	68 (31%)	2.169 (1.054 to 4.466)	0.0516
			C	16 (7%)	20 (9%)		
rs7520428 A>G	634390 pesodugene	N/A	A	134 (60%)	76 (34%)	43.954 (2.565 to 753.29)	**< 0.0001**
			G	0 (0%)	12 (6%)		
rs3134801 T>C	9950	Val248	T	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			C	0 (0%)	2 (1%)		
rs9743 T>C	9698	Leu164	T	126 (57%)	86 (39%)	0.3663 (0.07590 to 1.768)	0.3328
			C	8 (3%)	2 (1%)		
rs28358272 C>T	9449	Tyr81	C	130 (58%)	88 (40%)	0.1638 (0.008706 to 3.083)	0.2628
			T	4 (2%)	0 (0%)		
rs2853824 A>G	9347	Leu47	A	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			G	0 (0%)	2 (1%)		
rs2856985 G>A	9755	Glu183	G	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			A	0 (0%)	2 (1%)		
rs2854139 C>T	9818	His204	C	134 (60%)	88 (40%)	N/A	N/A
			T	0 (0%)	0 (0%)		
rs41347846 T>C	N/A	N/A	T	134 (60%)	88 (40%)	N/A	N/A
			C	0 (0%)	0 (0%)		
			A	134 (60%)	86 (39%)	7.775	

rs28380140 A>G	9377	Trp57	G	0 (0%)	2 (1%)	(0.3685 to 164.02)	0.3044
rs3902407 T>C	N/A	N/A	T	134 (60%)	88 (40%)	N/A	N/A
			C	0 (0%)	0 (0%)		
rs28411821 T>A,C	9824	Leu206	T	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			C	0 (0%)	2 (1%)		

Table 10: The *MT-ATP6* polymorphism genotypes between subfertile patients and controls.

SNP	Contig position	Protein position	Amino acid change	Genotype	infertile, N (%)	CONT, N (%)	*P*- Value
rs2001031 A>G	8860	Thr112Ala	Missense variant	AA	0	0	N/A
				AG	0	0	
				GG	67	44	
rs2000975 A>G	8701	Thr59Ala	Missense variant	AA	50	33	1.0000
				AG	3	1	
				GG	14	10	
rs2298011 A>G	9180	Val218	Synonymous variant	AA	67	43	0.3964
				AG	0	1	
				GG	0	0	
rs7520428 A>G	634390	N/A	N/A	AA	67	38	**< 0.0001**
				AG	0	1	
				GG	0	5	
rs9645429 G>A,C	634224	N/A	N/A	GG	63	43	0.3221
				GA	0	0	
				AA	4	1	
rs112660509 T>A,C	633824	N/A	N/A	TT	64	42	0.7155
				TC	2	0	
				CC	1	2	
rs6650105 G>A,T	633887	N/A	N/A	GG	64	43	1.0000
				GA	2	0	
				AA	1	1	
rs6594033 T>A,C	634112	N/A	N/A	TT	66	43	0.5647
				TC	1	0	
				CC	0	1	

rs6594034 A>C,T	634229	N/A	N/A	AA	65	43	0.6497
				AC	2	0	
				CC	0	1	
rs6594035 T>A,C	634244	N/A	N/A	TT	65	42	0.6497
				TC	2	2	
				CC	0	0	
rs3020563 A>G	8566	Gln67	Synonymous variant	AA	67	43	0.1560
				AG	0	0	
				GG	0	1	
rs28358887 G>A,T	8994	Leu156	Synonymous variant	GG	66	43	1.0000
				GA	0	1	
				AA	1	0	
rs2096044 T>A,C,G	634337	N/A	N/A	TT	66	44	1.0000
				TC	1	0	
				CC	0	0	
rs9283154 A>C,G,T	633714	N/A	N/A	AA	66	44	1.0000
				AG	1	0	
				GG	0	0	

Table 11: The *MT-ATP6* polymorphism alleles between subfertile patients and controls.

SNP	Contig position	Protein position	Alleles	infertile, %	CONT, %	OR (95% CI)*	*P*- Value
rs2001031 A>G	8860	Thr112Pro	A	0 (0%)	0 (0%)	N/A	N/A
			G	134 (60%)	88 (40%)		
rs2000975 A>G	8701	Thr59Pro	A	103 (46%)	67 (30%)	1.041 (0.5526 to 1.963)	0.9001
			G	31 (14%)	21 (10%)		
rs2298011 A>G	9180	Val218	A	134 (60%)	87 (39%)	4.611 (0.1856 to 114.58)	0.8319
			G	0 (0%)	1 (1%)		
rs7520428 A>G	634390	N/A	A	134 (60%)	77 (35%)	39.916 (2.318 to 687.23)	**0.0001**
			G	0 (0%)	11(5%)		
rs9645429 G>A,C	634224	N/A	G	126 (57%)	86 (38%)	0.3663 (0.07590 to 1.768)	0.3328
			A	8 (4%)	2 (1%)		
rs112660509 T>A,C	633824	N/A	T	130 (59%)	84 (37%)	1.548 (0.3767 to 6.359)	0.8087
			C	4 (2%)	4 (2%)		
rs6650105 G>A,T	633887	N/A	G	130 (59%)	86 (38%)	0.7558 (0.1354 to 4.219)	0.7489
			A	4 (2%)	2 (1%)		
rs6594033 T>A,C	634112	N/A	T	132 (59%)	86 (39%)	3.070 (0.2740 to 34.397)	0.7168
			C	2 (1%)	2 (1%)		
rs6594034 A>C,T	634229	N/A	A	132 (59%)	86 (39%)	1.535 (0.2121 to 11.108)	0.6690
			C	2 (1%)	2 (1%)		
rs6594035	634244	N/A	T	132 (59%)	86 (39%)	1.535	0.6690

T>A,C	634244	N/A	C	2 (1%)	2 (1%)	(0.2121 to 11.108)	0.0690
rs3020563 A>G	8566	Gln67	A	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			G	0 (0%)	2 (1%)		
rs28358887 G>A,T	8994	Leu156	G	132 (59%)	87 (39%)	0.7586 (0.06771 to 8.500)	0.8221
			A	2 (1%)	1 (1%)		
rs2096044 T>A,C,G	634337	N/A	T	133 (59%)	88 (40%)	0.5028 (0.02024 to 12.493)	0.4167
			C	1 (1%)	0 (0%)		
rs9283154 A>C,G,T	633714	N/A	A	133 (59%)	88 (40%)	0.5028 (0.02024 to 12.493)	0.4167
			G	1 (1%)	0 (0%)		

Table 12: The *MT-ATP8* polymorphism genotypes between subfertile patients and controls.

SNP	Contig position	Protein position	Amino acid change	Genotype	infertile, N (%)	CONT, N (%)	*P*- Value
rs9285835 T>A,C	633624	N/A	N/A	TT	62	41	1.0000
				TC	4	3	
				CC	1	0	
rs9285836 T>C	633630	N/A	N/A	TT	62	40	0.7565
				TC	4	3	
				CC	1	1	
rs9283154 A>C,G,T	633714	N/A	N/A	AA	62	41	1.0000
				AG	5	3	
				GG	0	0	
rs8179289 A>C,G,T	633561	N/A	N/A	AA	60	40	1.0000
				AG	5	2	
				GG	2	2	
rs121434446 G>A	8392	Trp9	Synonymous variant	GG	67	44	
				GA	0	0	
				AA	0	0	
rs1116906 A>G	8460	Asn32Ser	Missense variant	AA	67	43	0.1560
				AG	0	0	
				GG	0	1	
rs2153588 C>T	633672	N/A	N/A	CC	63	43	1.0000
				CT	4	0	
				TT	0	1	
rs1116905 C>A,T	8428	Phe21Leu	Missense varian	CC	67	43	0.1560
				CT	0	0	
				TT	0	1	
rs1116907 C>T	8468	Leu35	Synonymous variant	CC	67	43	0.1560
				CT	0	0	

				TT	0	1	
rs3020563 A>G	8566	Ile14Val	Synonymous variant	AA	67	43	0.1560
				AG	0	0	
				GG	0	1	

Table 13: The *MT-ATP8* polymorphism alleles between subfertile patients and controls.

SNP	Contig position	Protein position	Alleles	infertile, %	CONT, %	OR (95% CI)*	*P*- Value
rs9285835 T>A,C	633624	N/A	T	128 (58%)	85 (38%)	0.7529 (0.1833 to 3.094)	0.9625
			C	6 (3%)	3 (1%)		
rs9285836 T>C	633630	N/A	T	128 (58%)	83 (37%)	1.285 (0.3799 to 4.348)	0.9296
			C	6 (3%)	5 (2%)		
rs9283154 A>C,G,T	633714	N/A	A	129 (58%)	85 (38%)	0.9106 (0.2120 to 3.912)	0.8997
			G	5 (3%)	3 (1%)		
rs8179289 A>C,G,T	633561	N/A-	A	125 (56%)	82 (37%)	1.016 (0.3485 to 2.963)	0.9764
			G	9 (4%)	6 (3%)		
rs121434446 G>A	8392	Trp9	G	134 (60%)	88 (40%)	N/A	N/A
			A	0 (0%)	0 (0%)		
rs1116906 A>G	8460	Asn32Ser	A	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			G	0 (0%)	2 (1%)		
rs2153588 C>T	633672	N/A	C	130 (58%)	86 (39%)	0.7558 (0.1354 to 4.219)	0.7489
			T	4 (2%)	2 (1%)		
rs1116905 C>A,T	8428	Phe21Leu	C	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			T	0 (0%)	2 (1%)		
rs1116907 C>T	8468	Leu35	C	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			T	0 (0%)	2 (1%)		

rs3020563 A>G	8566	Ile14Val	A	134 (60%)	86 (39%)	7.775 (0.3685 to 164.02)	0.3044
			G	0 (0%)	2 (1%)		

4. DISCUSSION

Male infertility is a worldwide medical condition that accounts for around 40% of total infertility in couples seeking medical assistance to conceive. Infertility affects around one out of every four couples trying to conceive (Lotti & Maggi 2015).

It is thought that 30–40% of men in their reproductive years have abnormalities in sperm production, either qualitatively or quantitatively, or both. In approximately half of cases, abnormal sperm motility (asthenozoospermia) or abnormal sperm count (oligozoospermia) is the cause of infertility (Nakada *et al*., 2006). Infertility is a complicated condition with a wide variety of phenotypic features. Up to 15% of cases of infertility in men can be attributed to genetic factors (Agarwal *et al*., 2015).

Recent sperm physiology research has raised concerns about mitochondria as a sperm health and fertility indicator (Barbhuiya *et al*., 2016, Pereira *et al.,* 2008). Normal sperm function is an important prerequisite for successful male fertility. Several studies have focused on sperm mitochondria, showing that they have a vital role when considering sperm motility and the fertilization ability of the sperm, since they are responsible for energy generation (Aitken *et al*., 2010). Some experiments have shown a link between sperm motility and respiratory chain enzymatic activity, indicating that asthenozoospermia is caused by alterations in the mitochondrial function (Paoli *et al*., 2011).

Mitochondria is the powerhouse of eukaryotic cells because of its capacity to create ATP through oxidative phosphorylation (Hill *et al*., 2012). Any imbalance in mitochondrial respiratory activity will have a negative impact on spermatogenesis, especially pachytene phase progression during meiosis and sperm generation. If substantial levels of pathogenic mutant mtDNA accumulate in the testicular tissue, spermatogenic cells will experience mitochondrial respiratory failure. Meiotic arrest occurs when the mitochondrion's ability to produce energy is reduced during spermatogenesis. In the absence of oxygen, spermatocytes will most likely not complete meiosis and will be destroyed by apoptosis (Shamsi *et al*., 2008).

The mitochondria possess a semi-autonomous DNA that does not recombine. It goes through a fast replication cycle with no effective proofreading or DNA repair capabilities. Mitochondrial DNA mutates at a rate 10–20 times faster than nuclear DNA due to its unique replication process and placement in a highly oxidative environment (Kumar & Sangeetha, 2009). Alterations in

mitochondrial DNA are expected to induce disorders through a variety of mechanisms, the most prominent of which is interference with the oxidative phosphorylation function (Tuppen *et al*., 2010). Such mutations can result in a variety of severe diseases, ranging from modest clinical symptoms to life-threatening mitochondrial pathophysiology problems (Giacchetti *et al*., 2004).

Because of the proximity of the mtDNA to the inner mitochondrial membrane, in human sperm it is prone to oxidative damage and mutations, which have been reported to have a significant role in male infertility (Andrews *et al*., 1999). It is widely recognized that mtDNA is a major generator of ATP in sperm via the OXPHOS process through encoding for protein components in the respiratory chain. Furthermore, during the OXPHOS process, reactive oxygen species are produced, and DNA ROS-induced damage could occur (Kumar *et al.,* 2009). A deficiency in mitochondrial DNA during spermatogenesis might increase the risk of free radical production and creating an oxidative stress environment that could disrupt sperm development and maturation (Venkatesh *et al*., 2009).

Additionally, since sperm motility is largely dependent on the mitochondrial OXPHOS pathway for ATP synthesis, sperm mtDNA polymorphisms lead to the production of functionally useless proteins (Xavier *et al.*, 2019).

According to research, spermatozoa with mitochondrial impairments have inadequate ATP synthesis and higher amounts of reactive oxygen species (ROS) or free radicals. The mitochondria and mtDNA will be damaged in a malfunctioning system, reducing sperm motility and eventually leading to male infertility (Dahadhah *et al*., 2021).

In addition to mitochondrial dysfunction, seminal oxidative stress can also be responsible for the pathogenesis of male infertility (Moraes & Meyers, 2018).

It remains difficult to develop a complete molecular profile specific to all types of male infertility. Because the bioenergetic activity of mitochondria is critical for sperm motility, any quantitative or qualitative variations in mtDNA have an impact on the spermatozoa's cellular functioning. Sperm with specific mtDNA deletions are associated with deficiencies in sperm motility. Multiple 7345 and 7599 bp mutations in sperm are associated with poor sperm motility (Talebi *et al*., 2018).

The aim of this work was to investigate whether there is an association between polymorphisms of the sperm mitochondrial genes *MT-CYB*, *MT-CO3*, *MT-ATP6*, and *MT-ATP8* and the occurrence of male infertility.

In the present research, 13 SNPs in the *MT-CYB* gene were detected in the studied group. There were five synonymous SNPs: rs527236194, rs28357373, rs28357369, rs41504845, and rs2854124. Eight non-synonymous SNPs were detected (missense variants) were detected: rs2853508 (Thr194Ala), rs28357685 (Ala122Thr), rs41518645 (Asp171Asn), rs2853507 (Ala191Thr), rs28357376 (Thr360Ala), rs35070048 (Ile189Val), rs2853506 (Thr158Ala), and rs28660155 (Ile42Thr).

Moreover, the polymorphisms of the *MT-CO3*, *MT-ATP6* and *MT-ATP8* genes of subfertile and fertile men were also scanned by direct sequencing. Twelve SNPs have been identified in the *MT-CO3* gene (rs2248727, rs7520428, rs3134801, rs9743, rs28358272, rs2853824, rs2856985, rs2854139, rs41347846, rs28380140, rs3902407, and 28411821), 14 SNPs in the *MT-ATP6* rs2001031, rs2000975, rs2298011, rs7520428, rs9645429, rs112660509, rs6650105, rs6594033, rs6594034, rs6594035, rs3020563, rs28358887, rs2096044, and rs9283154, and 10 SNPs in the *MT-ATP8* rs9285835, rs9285836, rs9283154, rs8179289, rs121434446, rs1116906, rs2153588, rs1116905, rs1116907, and rs3020563.

The study findings suggested a link between the incidence of mutations in the previously listed mitochondrial genes and men's infertility. Among the identified SNPs of *MT-CYB* the following SNPs showed a statistically significant link to male infertility: rs527236194 (P=0.0005), rs28357373 (P=0.0439), and rs41504845 (P=0.0038). Both the *MT-CO3* and *MT-ATP6* genes had rs7520428, which revealed a significant difference in the genotype's and allele frequency tests between subfertile and fertile groups in both cases (P<0.0001) (**Figures 15, 16, 17**).

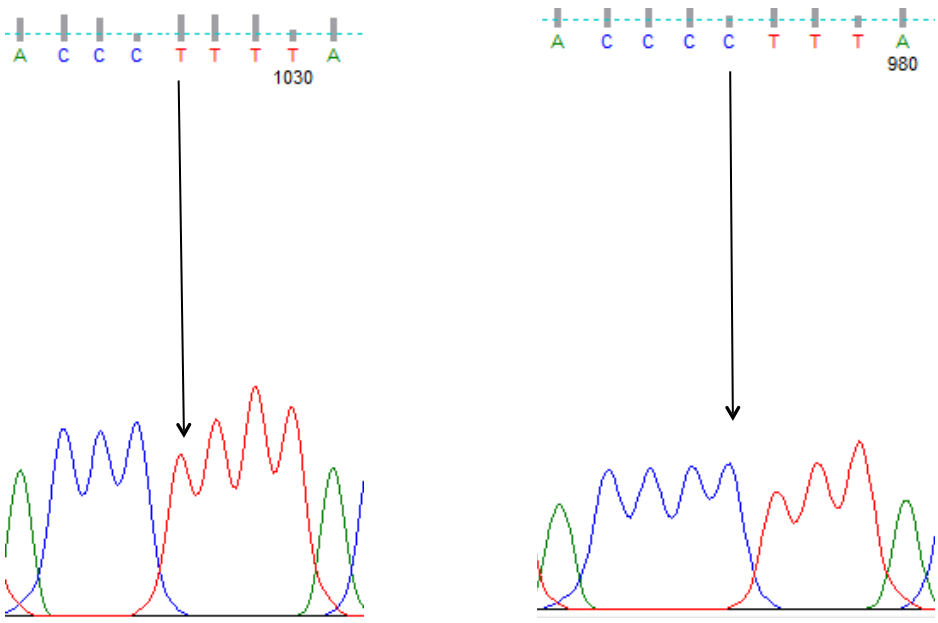

Figure 15: Sequencing electropherogram results (TT, CC) of the rs527236194 of *MT-CYB*. The nucleotide transition at position 15,784 (T>C) resulted in a synonymous variant (Pro>Pro) at codon 346.

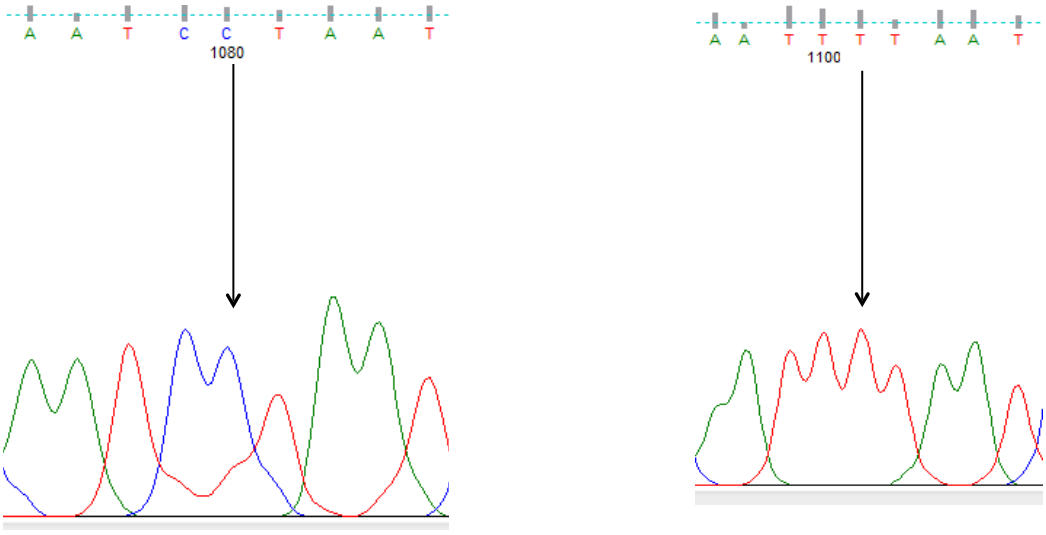

Figure 16: Sequencing electropherogram results (CC, CT, TT) of the rs41504845 of *MT-CYB*. The nucleotide transition at position 15,833 (C>T) resulted in a synonymous variant (Leu>Leu) at codon 363.

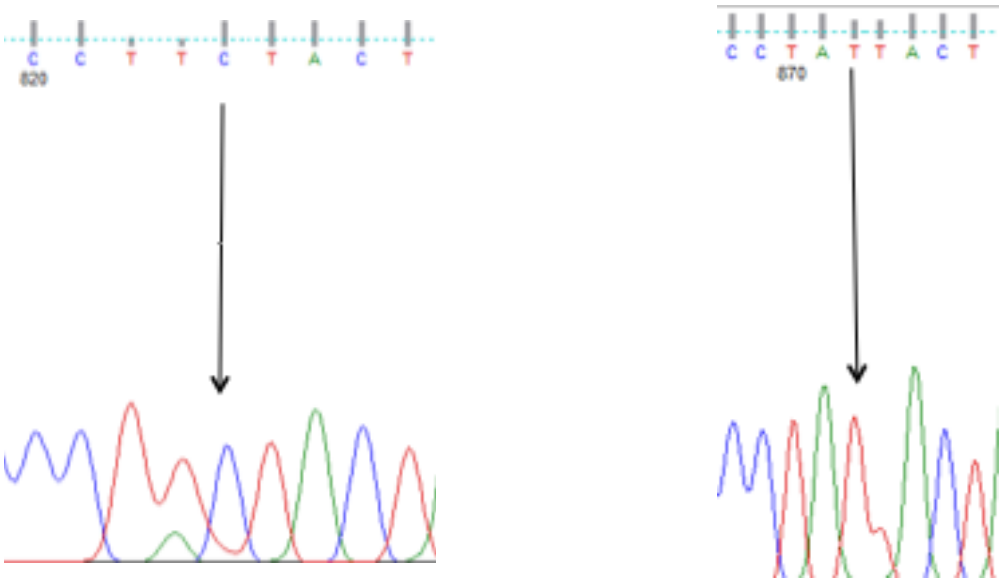

Figure 17: Sequencing electropherogram results (CC, CT, TT) of the rs28357373 of *MT-CYB*. The nucleotide transition at position 15,629 (C>T) resulted in a synonymous variant (Leu>Leu) at codon 295.

In the allelic frequency test two synonymous SNPs the *MT-CYB* gene demonstrated a significant relationship with infertility in men: rs527236194 (*P*=0.0014) and rs41504845 (*P*=0.0147). The detected substitution mutations in the above-mentioned codons were synonymous. In particular, the rs527236194 variant altered [CCT] to [CCC] at position 15,784 (Proline), while the rs41504845 variant changed [CTA] to [TTA] at 15,833 (Leucine) (NCBI) (Saleh Jaweesh *et al.,* 2022).

Synonymous mutations have been hypothesized to have an impact in gene control and the establishment of disorders (Sauna & Kimchi-Sarfaty, 2011). Nine of the SNPs were detected in the *MT-CO3* gene, four of the SNPs in *MT-ATP6* gene and two of the SNPs in *MT-ATP8* gene were synonyms variants. It has been found that synonymous variants possibly affect messenger ribonucleic acid (mRNA) stability (Duan *et al*., 2003). As a result, functional investigations on

these synonymous variants in mtDNA are needed to uncover their potential involvement in sperm function and male infertility.

The allele frequency of rs7520428 SNP (A634390G) in *MT-CO3* and *MT-ATP6* showed a significant association with male infertility (P<0.0001). Moreover, the OR of rs7520428 SNP was associated with about 40 times increased risk in subfertile males group than fertile men. This might show that an increase in the number of wild type alleles (A) or the decrease of mutant type alleles (G) at A634390G could be beneficial in preserving male fertility while increasing the number of G alleles (or decreasing A alleles) could cause male infertility (**Figures 18,19**) .

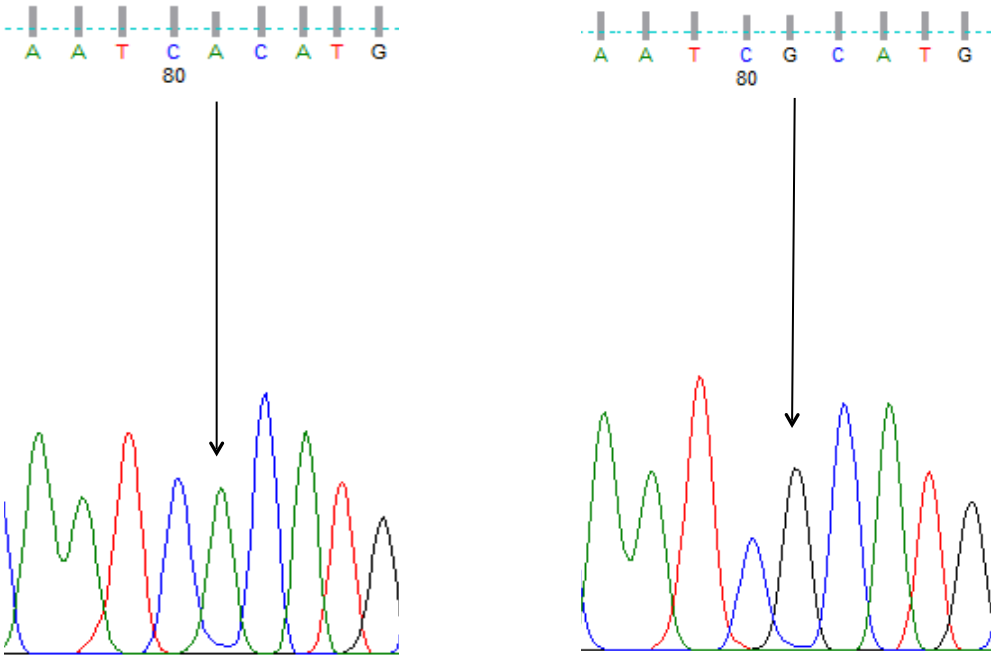

Figure 18: Sequencing electropherogram results (AA, GG) of the rs7520428 of *MT-ATP6*. The nucleotide transition at the position 634390 (A>G).

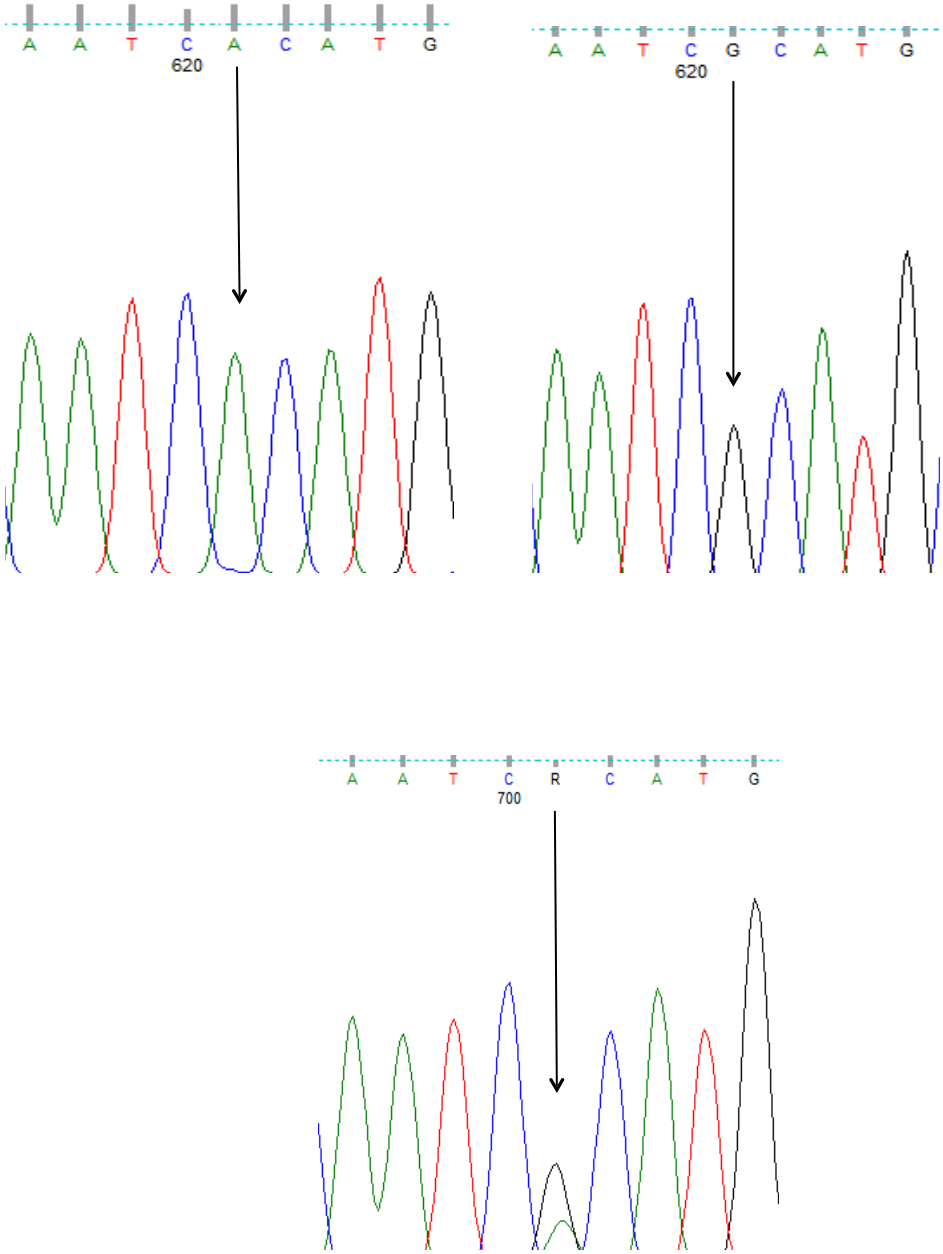

Figure 19: Sequencing electropherogram results (AA, AG, GG) of the rs7520428 of *MT-CO3*. The nucleotide transition at the position 634390 (A>G).

It has been established that mtDNA base alterations have a significant influence on the quality and motility of sperm (La Vignera *et al.,* 2019). The mtDNA rearrangement has been stated in asthenozoospermic patients (Thangaraj *et al*., 2003). A novel 4866-bp deletion was discovered in the spermatozoa mtDNA. The frequency of this mtDNA deletion was considerably greater in the abnormal motility group than in the normal motility group (Chari *et al*., 2015).

While mitochondrial mutations and their correlation to male infertility have been broadly investigated, there are still contradictions in data. Several studies have informed the association between male infertility and mtDNA mutations in certain genes. Barbhuiya *et al*., (2016) discovered that the highest number of point mutations resulting in base substitution — that is, single-nucleotide polymorphisms (SNPs) — were detected in the *ATP6* gene (21 SNPs), followed by the *ND2* gene (12 SNPs) and *ATP8* gene (9 SNPs). *ND4* gene showed the least number of point mutations (1 SNP).

Until 2005, no convincing evidence was presented for the effect of mtDNA substitutions, either directly on the sperm (as other mutations than deletions) or indirectly through haplogroup background predisposing to reduced motility (Pereira *et al*., 2008). A remarkable finding was published by Selvi Rani *et al*. (2006). They found that all 34 individuals with oligoasthenozoospermia (OA) have a homoplasmic mutation of C11994T.

Despite the presence of synonymous coding SNPs, understanding their role in gene regulation may enable researchers to relate these genetic alterations to male infertility (Cariati *et al.,* 2019). The structure or function of proteins is not affected by synonymous changes. Moreover, in earlier research, codon bias was postulated as a method for regulating gene expression (Quax *et al.,* 2015). The high number of synonymous mutations can reduce the total translation rate since the choice of codon or which codon is selected depends on which codon is translated more quickly, promptly, and smoothly. Many synonymous mutations may not shift amino acids as a result of code degeneracy. However, they may influence the efficiency of the translation machinery and thus may reduce the rate of production of ATP (Kumar *et al.,* 2012).

Consequently, functional investigations are needed to determine the precise significance of synonymous changes in gene function and regulation.

The complex III subunit of the respiratory chain is encoded by the *MT-CYB* gene only (Mancuso *et al.,* 2014). Earlier research has found a link between MT-CYB gene variations and a variety of

illnesses. For example, the rs2853508 mutation has been linked to breast cancer (Fasterius *et al.,* 2019). There is also evidence that rs41518645 SNP contributes to Leber hereditary optic neuroretinopathy (LHON) (Huoponen *et al.,* 1993). Similarly, epileptogenesis has been linked to the rs2853506 polymorphism (A15218G) (Blein *et al.,* 2015).

The current findings are consistent with and support prior findings on the involvement of *MT-CYB* gene mutations in male infertility. Studies have shown that deletions and mutations in the *MT-CYB* and *MT-ATP6* genes contribute to sperm motility (Feng *et al.,* 2008). In previous research, genetic changes in the *MT-CYB* gene have been involved in the establishment of male infertility. Three polymorphism locations in the *MT-CYB* gene, G15301A, A15326G, and A15487T, are considered to be linked to men's fertility. In addition, researchers reported a novel synonymous substitution mutation (A to G) in the *MT-CYB* gene at position 15,472 in an infertile patient (Mao *et al.,* 2015).

Deletions, point mutations, and the presence of a certain mtDNA haplogroup have all been linked to low sperm quality. In different studies, deletions of the mtDNA have been linked with asthenozoospermia and male infertility (Kumar *et al.,* 2009). Many more studies have been performed in diverse groups to thoroughly understand the molecular background and genetic variables that might be associated to male infertility and sperm quality (Rezgoune *et al.,* 2021). The cause of idiopathic male infertility, on the other hand, remains unknown. As a result, mtDNA genetic analysis can be a useful method for determining the function of mtDNA variation in a cohort of infertile males (Saleh Jaweesh *et al.,* 2022).

Despite the fact that mitochondrial DNA abnormalities and their link to male infertility have been thoroughly researched, there are still differences in the findings. Some research has discovered a link between infertility in men and mtDNA abnormalities in particular genes (Álvarez-Iglesias *et al.,* 2011, Barbhuiya *et al.,* 2016), while other research has not (Pereira *et al.,* 2008).

Five non-synonymous SNPs in the nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase 1 gene (ND1), located at nucleotides T3398C, T3821C, G4048A, T4169TT, and T4216C, were discovered in a recent research. A secondary structure prediction of T3398C and T3821C proteins indicated a negative change in protein function, suggesting that these changes might lead to sperm motility reduction and male infertility (Abd Elrahman *et al.,* 2021).

Additionally, our laboratory conducted two studies, the first one reported negative correlation between sperm motility value and Intracytoplasmic sperm injection (ICSI) outcomes and the frequencies of mutations in four mitochondrial genes (nicotinamide adenine dinucleotide hydrogen [NADH] dehydrogenase 1 [ND1], NADH dehydrogenase 2 [ND2], NADH dehydrogenase 5 [ND5], and NADH dehydrogenase 6 [ND6]), namely 13708 G>A, 4216 T>C and 12506 T>A (Al Smadi *et al.,* 2021, Abd Elrahman *et al.,* 2021).

The rs2853495 (G 11719A) and rs869096886 (A11251G) in nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase 4 (MTND4) were linked to male infertility in the second research (Dahadhah *et al*., 2021).

Furthermore, previous research has linked asthenozoospermia to patients with the A3243G mtDNA mutation and large-scale mtDNA deletions (Fadic *et al.,* 1997; Lestienne *et al.,* 1997). Subfertile men may also have a number of mtDNA mutations acquired throughout their lifetimes (Kao *et al.,* 1998). These mutations can accumulate to high levels in individual sperm progenitor cells, resulting in sperm motility problems and infertility. (Kumar *et al.,* 2009).

Other research found a genetic mutation caused by a change in the SNP T4216C in both fertile and infertile males (Khan *et al.,* 2016; Stenson *et al.,* 2017). SNP C3398T, on the other hand, has a decreased risk of asthenozoospermia, due to its low frequency and small sample size (Zhang *et al.,* 2017). Mughal *et al.* (2017, 2016) found a strong link between the 15bp deletion of cytochrome c oxidase III (at location 9390 to 9413) and human male infertility (P=0.033).

In our study, RS2000975 (A8701G) showed no significant association with male infertility in the genotype frequency (P = 1.0000), while a previous study has demonstrated a significant association between A8701G variants and increased risk of fertilization failure (Mao *et al.,* 2015).

To emphasize on the effect of the mentioned SNPs and explain their significance in male infertility, more research in a bigger and more diverse population is required.

5. Conclusion

In conclusion, we identified the association between mitochondrial gene polymorphisms in *MT-CYB*, *MT-CO3*, *MT-ATP6* and *MT-ATP8* genes and male infertility as follows: 13 SNPs in *MT-CYB* (rs2853508, rs28357685, rs41518645, rs2853507, rs28357376, rs35070048, rs2853506, rs28660155, rs527236194, rs28357373, rs28357369, rs41504845, and rs2854124); 12 SNPs in *MT-CO3* gene (rs2248727, rs7520428, rs3134801, rs9743, rs28358272, rs2853824, rs2856985, rs2854139, rs41347846, rs28380140, rs3902407, and 28411821); 14 SNPs in *MT-ATP6* (rs2001031, rs2000975, rs2298011, rs7520428, rs9645429, rs112660509, rs6650105, rs6594033, rs6594034, rs6594035, rs3020563, rs28358887, rs2096044, and rs9283154); and 10 SNPs in *MT-ATP8* (rs9285835, rs9285836, rs9283154, rs8179289, rs121434446, rs1116906, rs2153588, rs1116905, rs1116907, and rs3020563). In *MT-CYB*, three variants showed a significant difference in the frequency of the genotypes between subfertile and fertile groups: rs527236194 (T15784C) ($P = 0.0005$), rs28357373 (T15629C) ($P = 0.0439$), and rs41504845 (C15833T) ($P = 0.0038$). Moreover, two SNPs showed a significant association between allelic frequencies of rs527236194 (T15784C) ($P = 0.0014$) and rs41504845 (C15833T) ($P = 0.0147$) and male infertility. Moreover, in *MT-CO3* and *MT-ATP6*, only rs7520428 showed a statistically significant difference between subfertile and fertile groups in the genotype's and allele's frequency test ($P < 0.0001$ for both). This indicates that mitochondrial genetics might help to give a better understanding of the correlation between the presence of these SNPs and male infertility. Moreover, further studies on larger populations are required to reveal the exact role of these genes in the development of male infertility.

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7. APPENDICES

Supplementary Figures of Genotypes and Allele Frequencies for Chapter 3.2

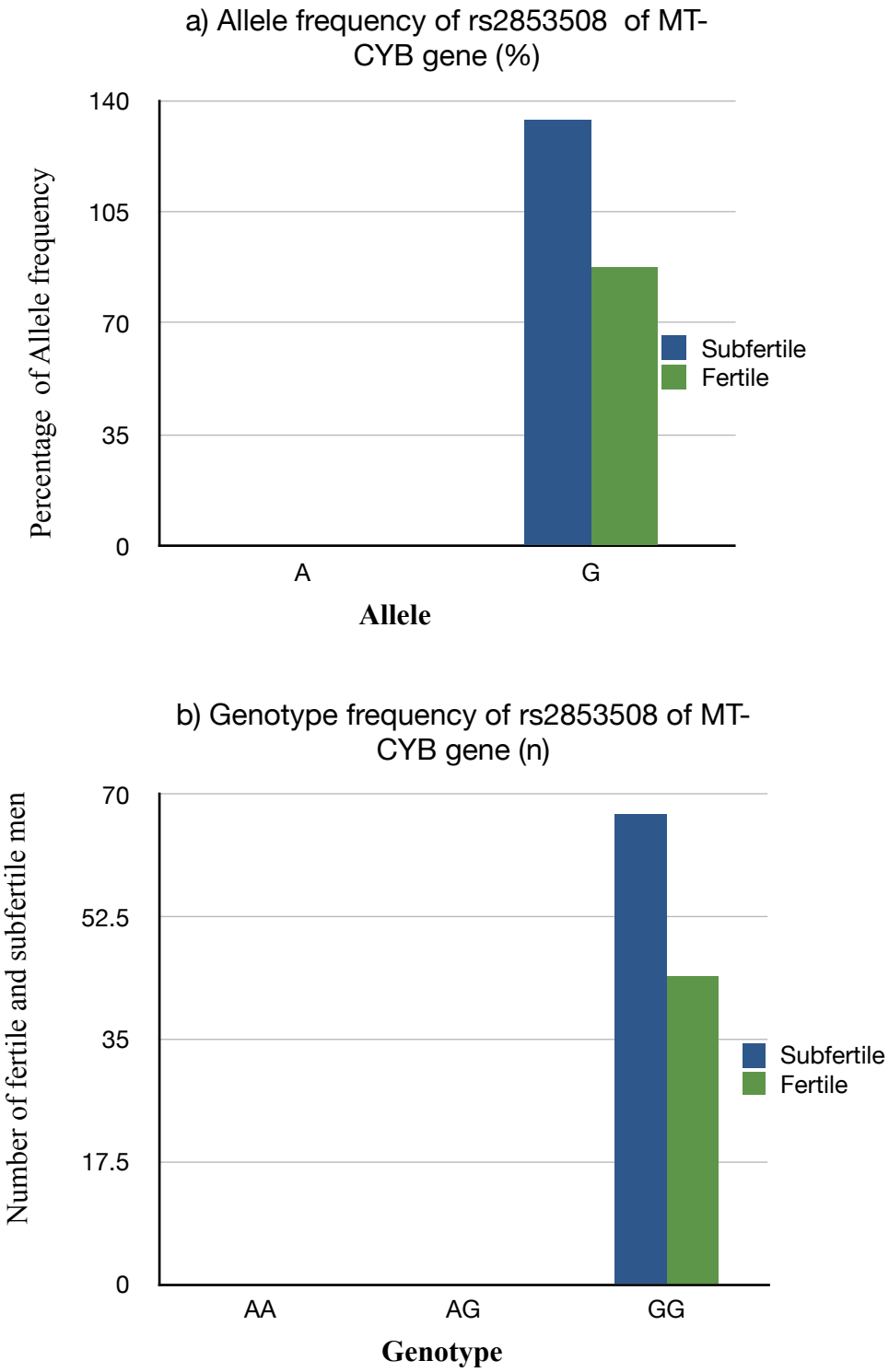


Figure 20: a) *MT-CYB* gene allele frequency for rs2853508, **b)** *MT-CYB* gene genotype frequency for rs2853508.

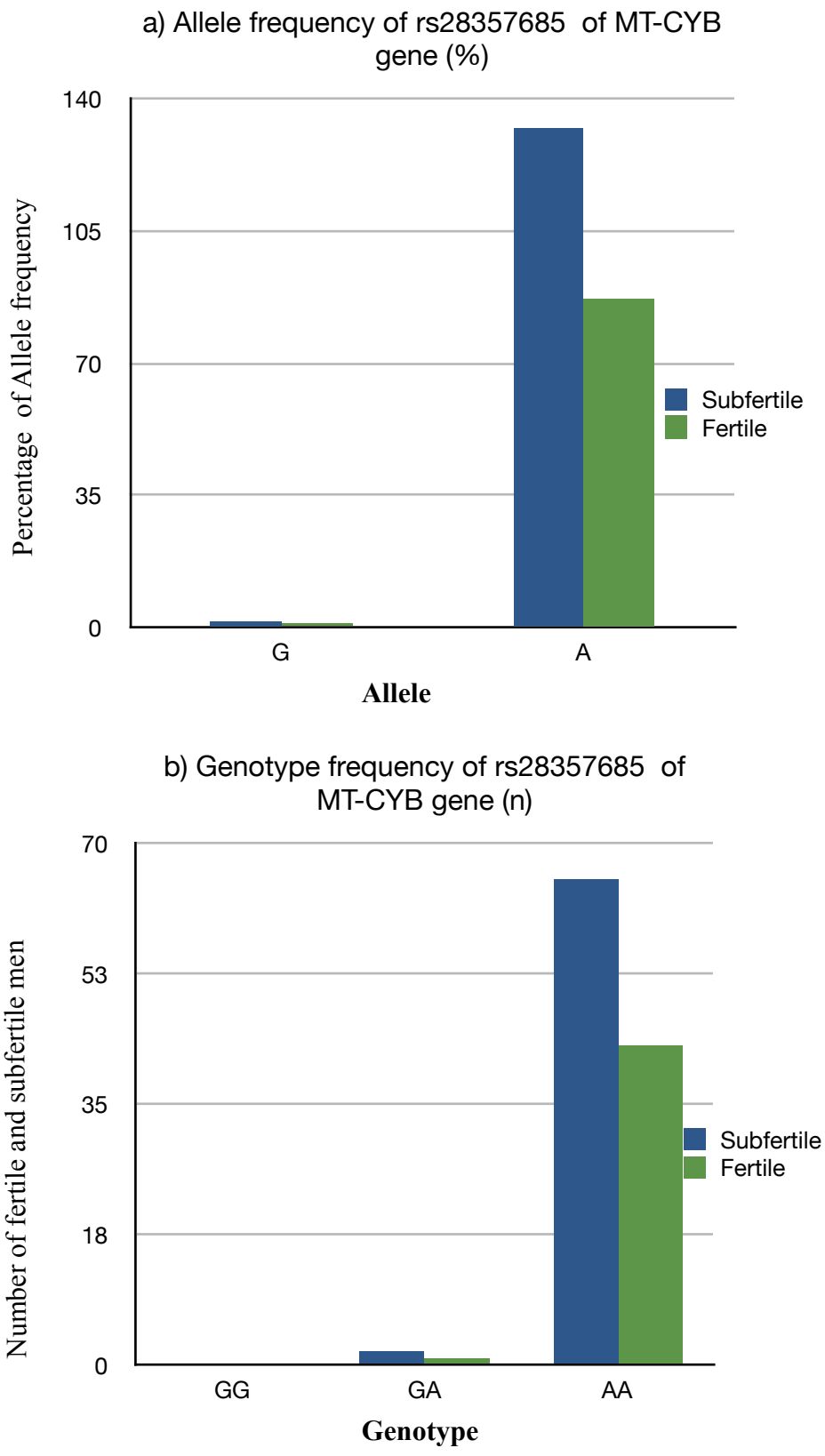
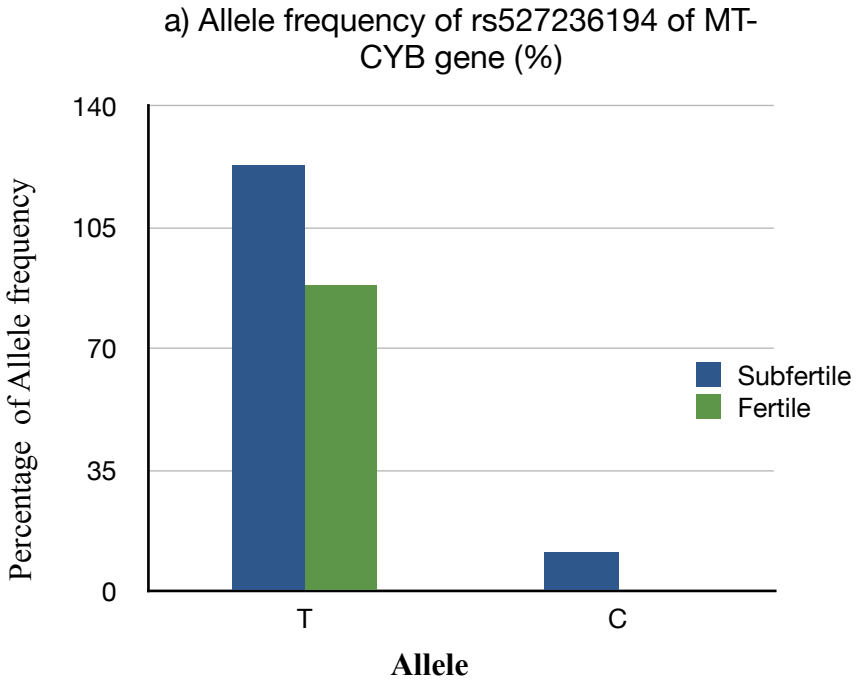


Figure 21: a) *MT-CYB* gene allele frequency for rs28357685, **b)** *MT-CYB* gene genotype frequency for rs28357685.



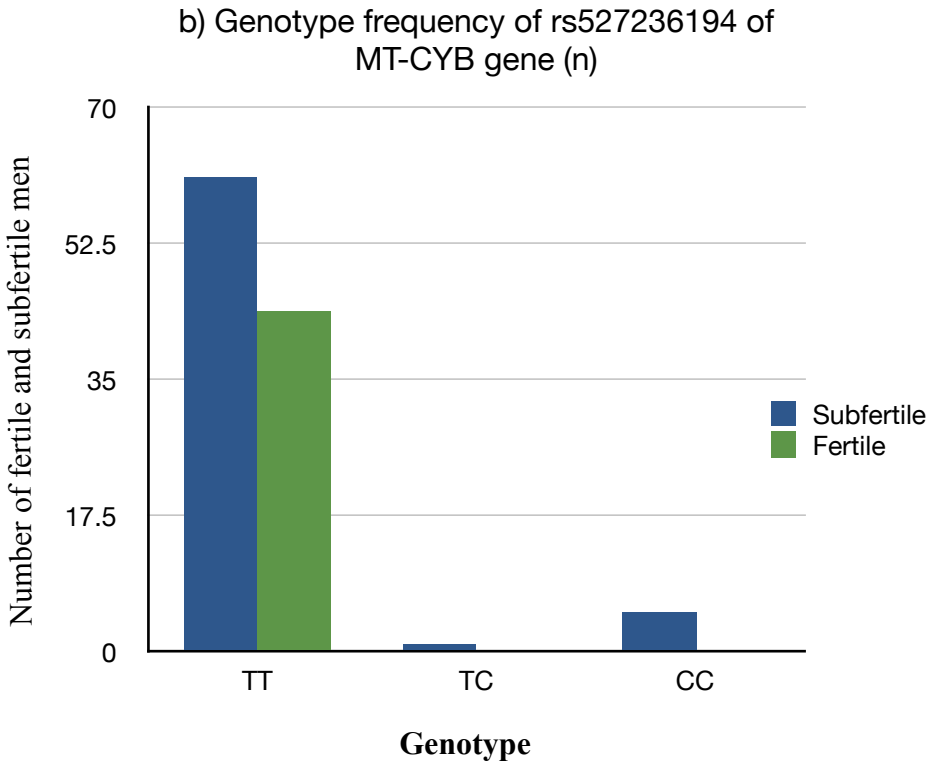


Figure 22: a) *MT-CYB* gene allele frequency for rs527236194, **b)** *MT-CYB* gene genotype frequency for rs527236194 in *MT-CYB* gene.

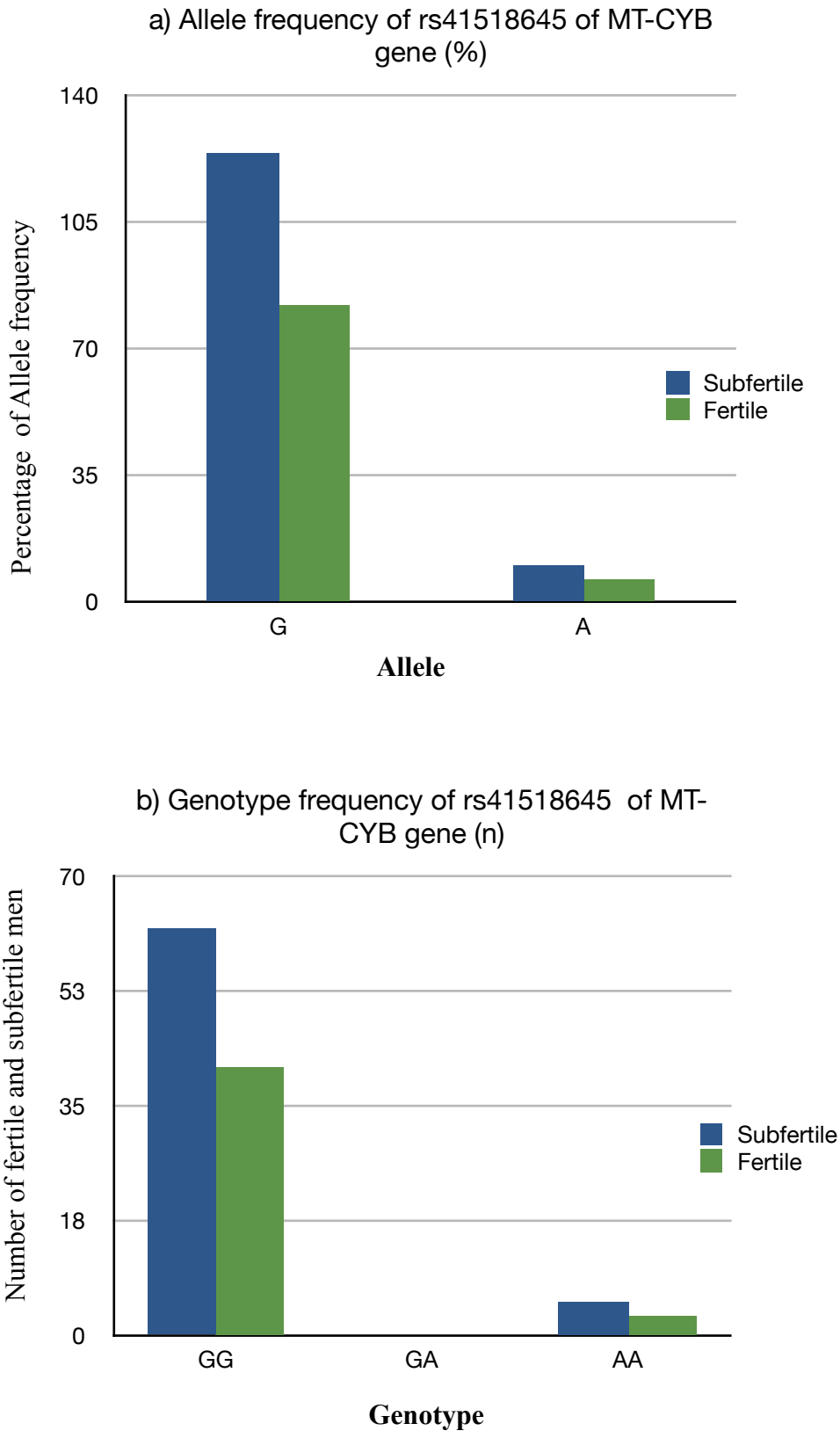


Figure 23: a) MT-CYB gene allele frequency for rs41518645, **b)** *MT-CYB* gene genotype frequency for rs41518645.

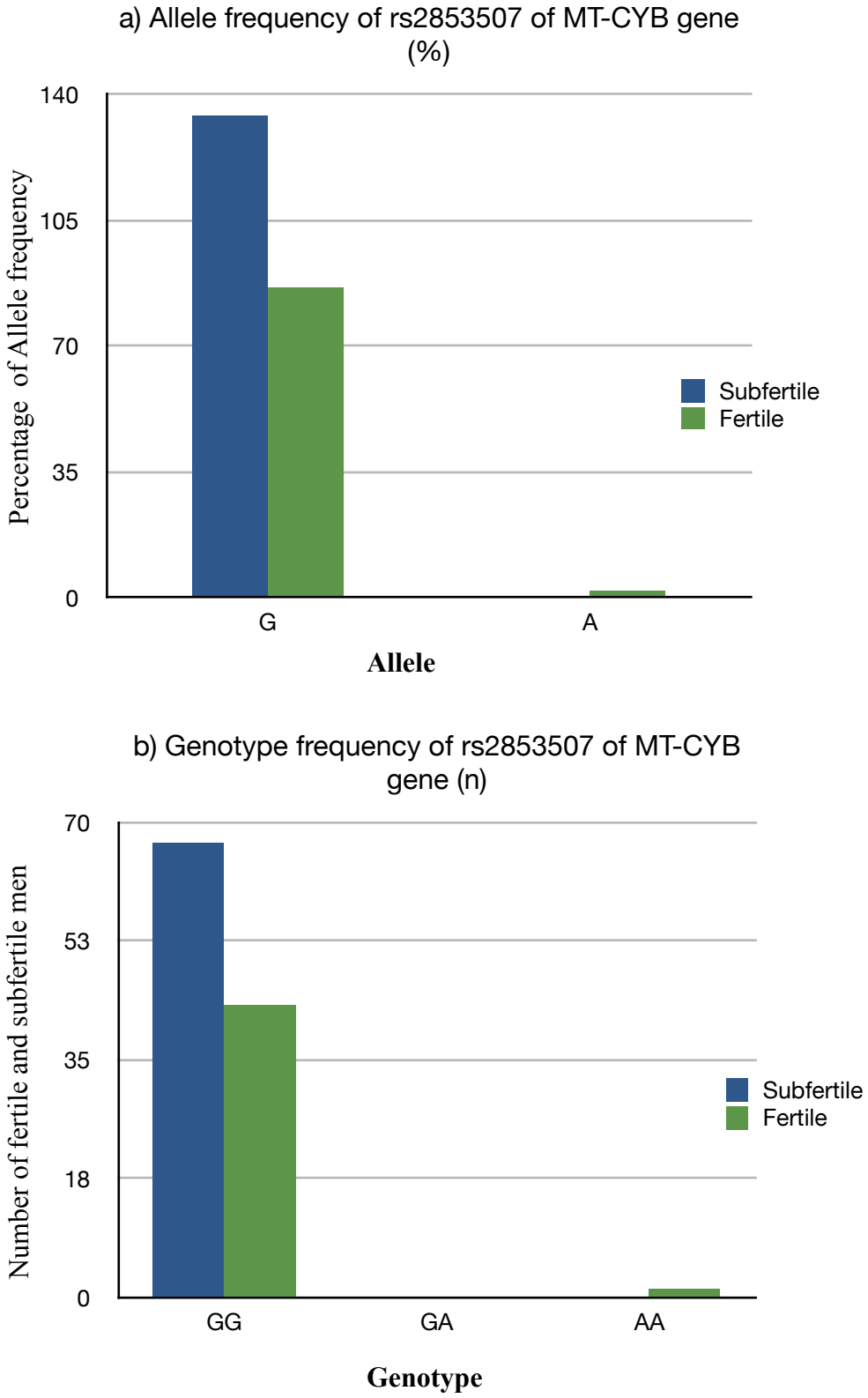
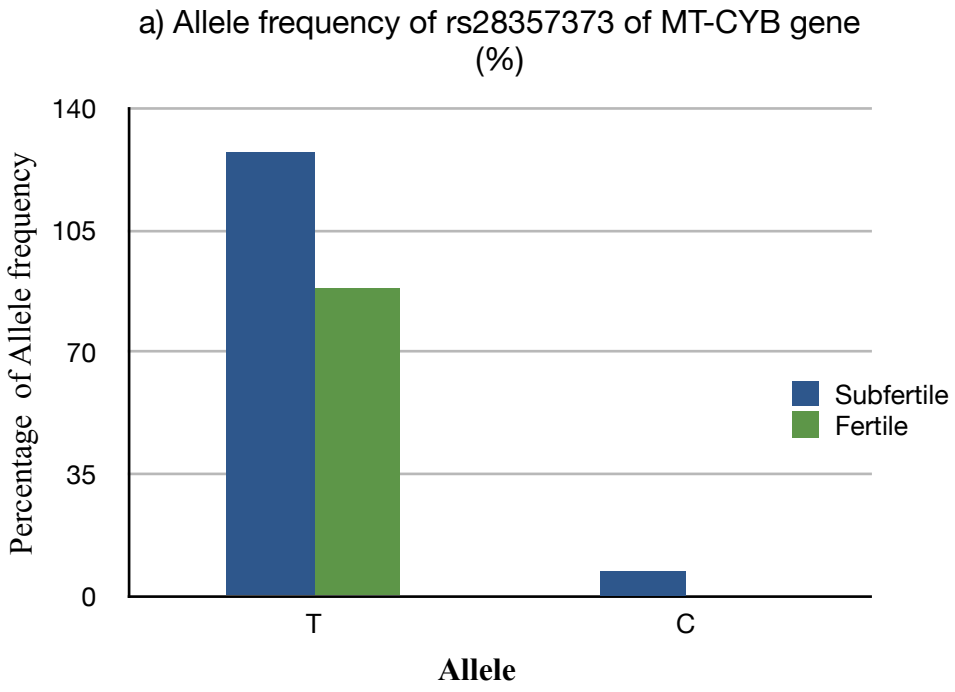


Figure 24: a) MT-CYB gene allele frequency for rs2853507 , **b)** *MT-CYB* gene genotype frequency for rs2853507.



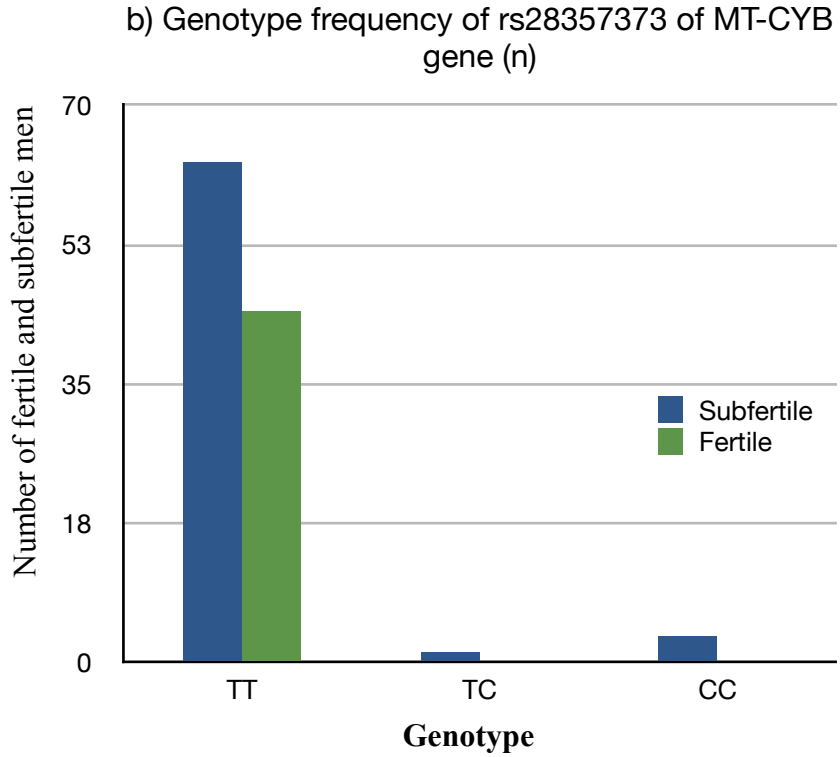


Figure 25: a) MT-CYB gene allele frequency for rs28357373, **b)** *MT-CYB* gene genotype frequency for rs28357373.

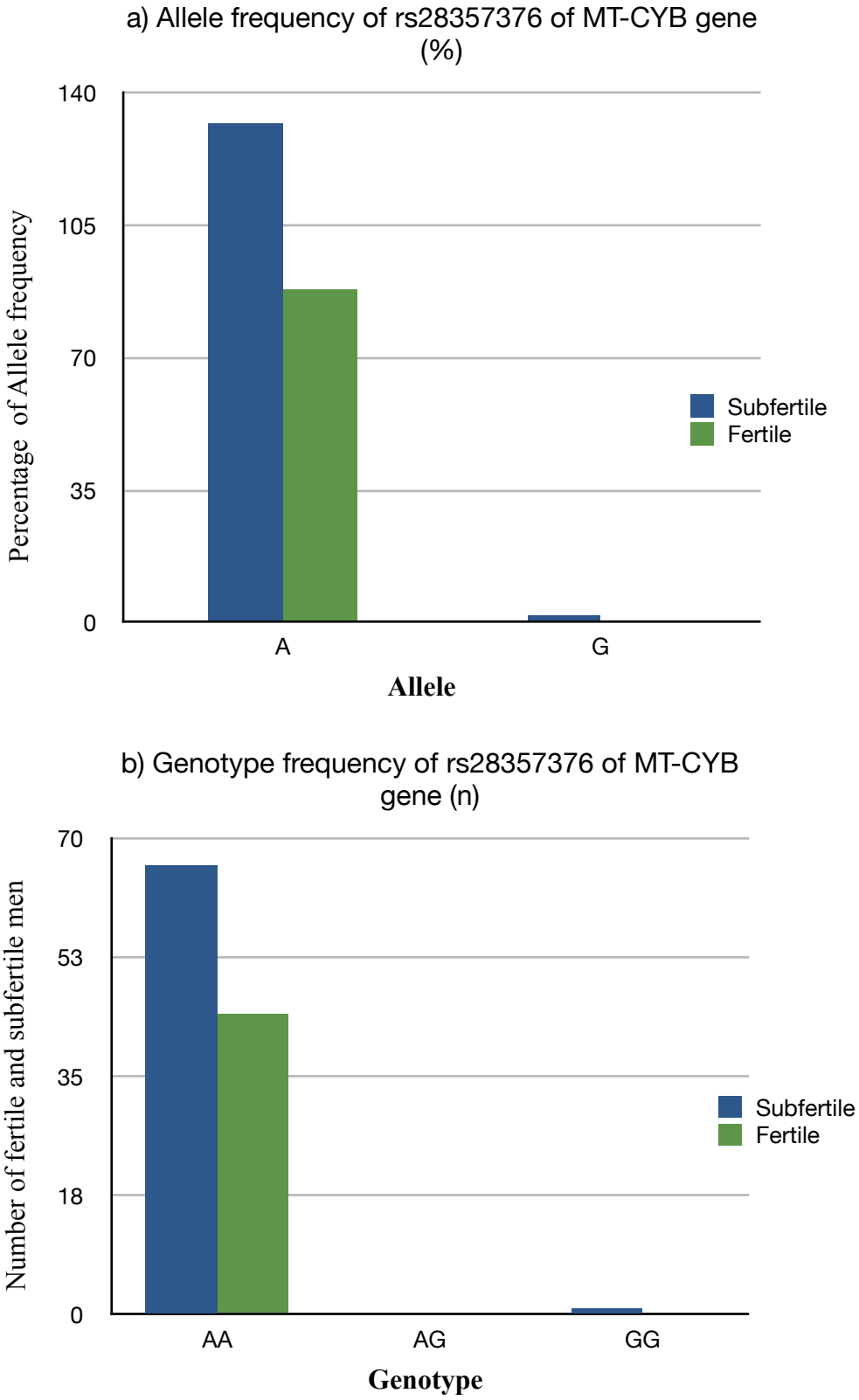


Figure 26: a) MT-CYB gene allele frequency for rs28357376, **b)** *MT-CYB* gene genotype frequency for rs28357376.

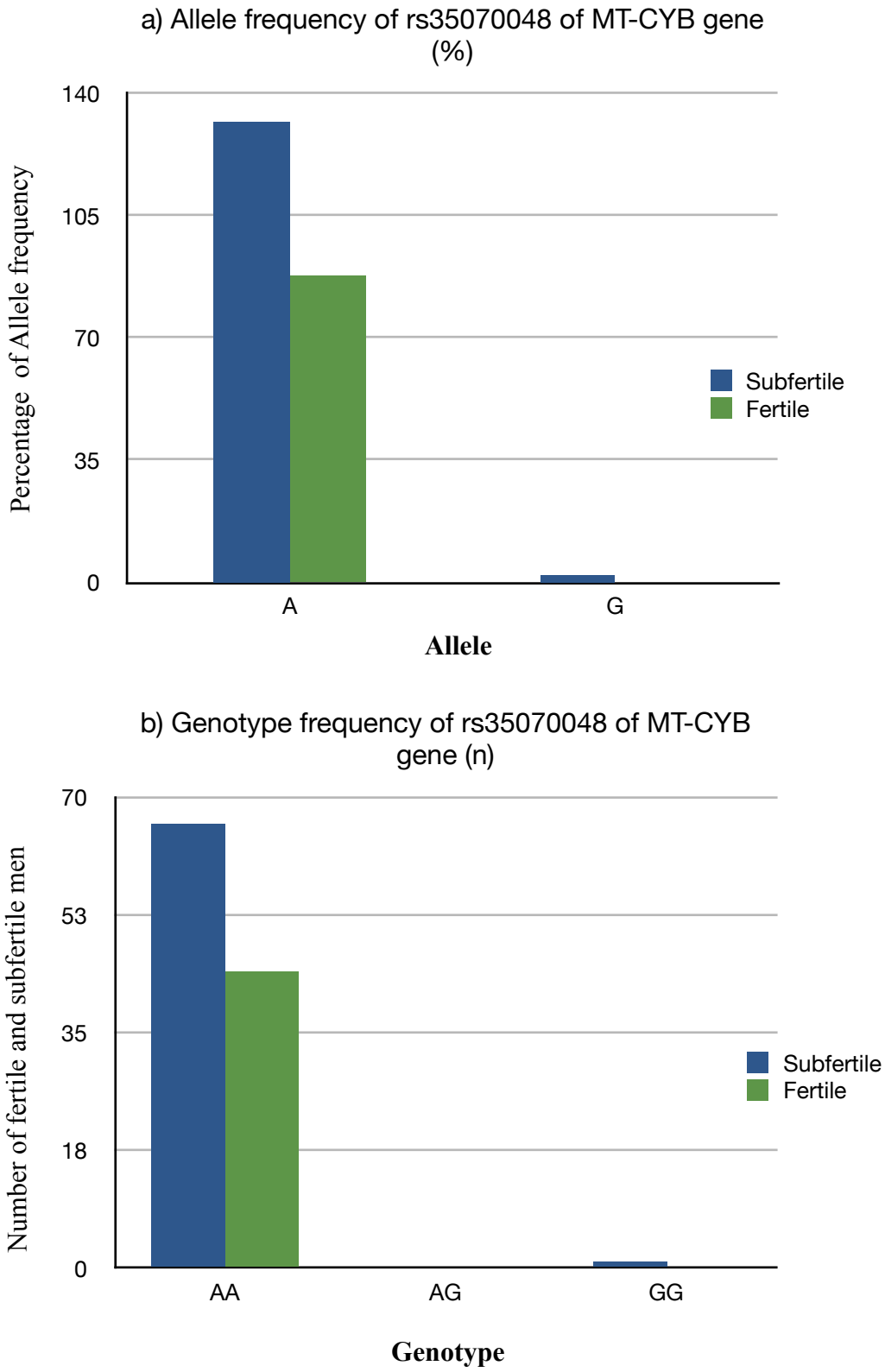


Figure 27: a) MT-CYB gene allele frequency for rs35070048, **b)** *MT-CYB* gene genotype frequency for rs35070048.

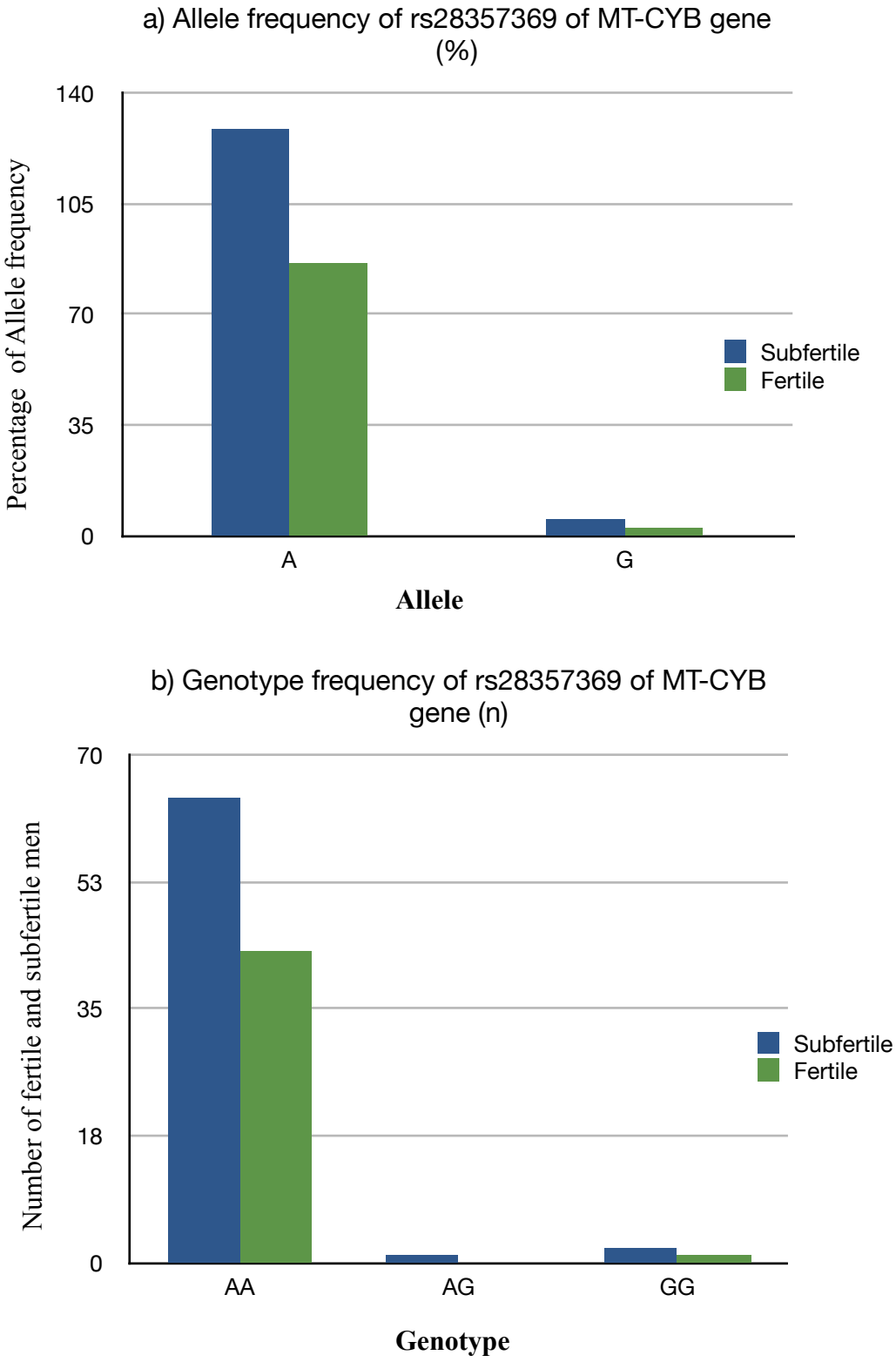
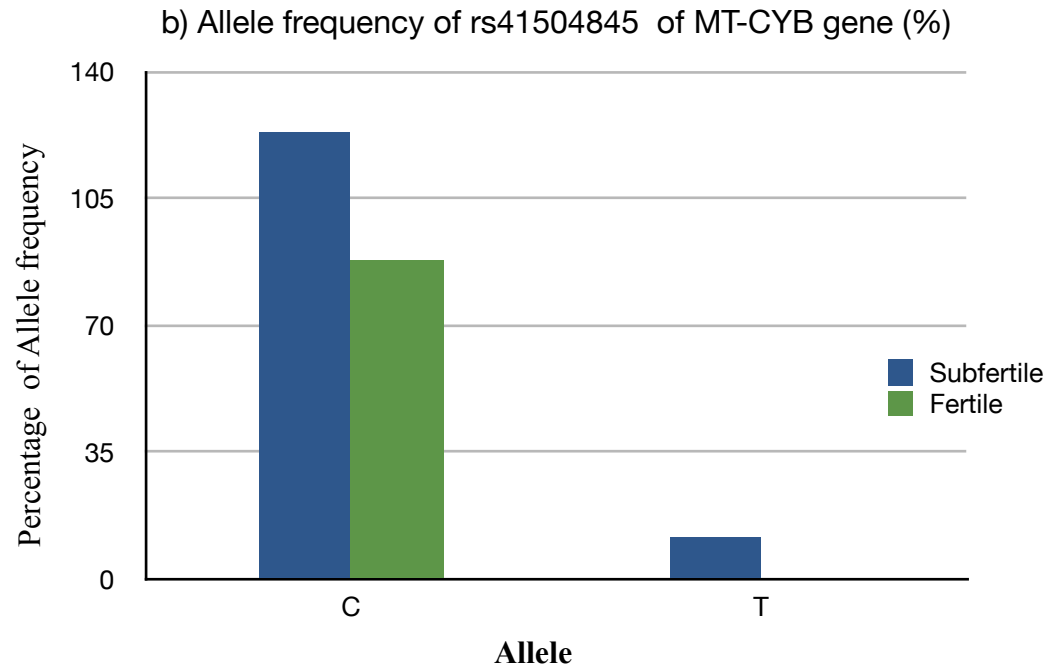


Figure 28: a) *MT-CYB* gene allele frequency for rs28357369, **b)** *MT-CYB* gene genotype frequency for rs28357369 in.



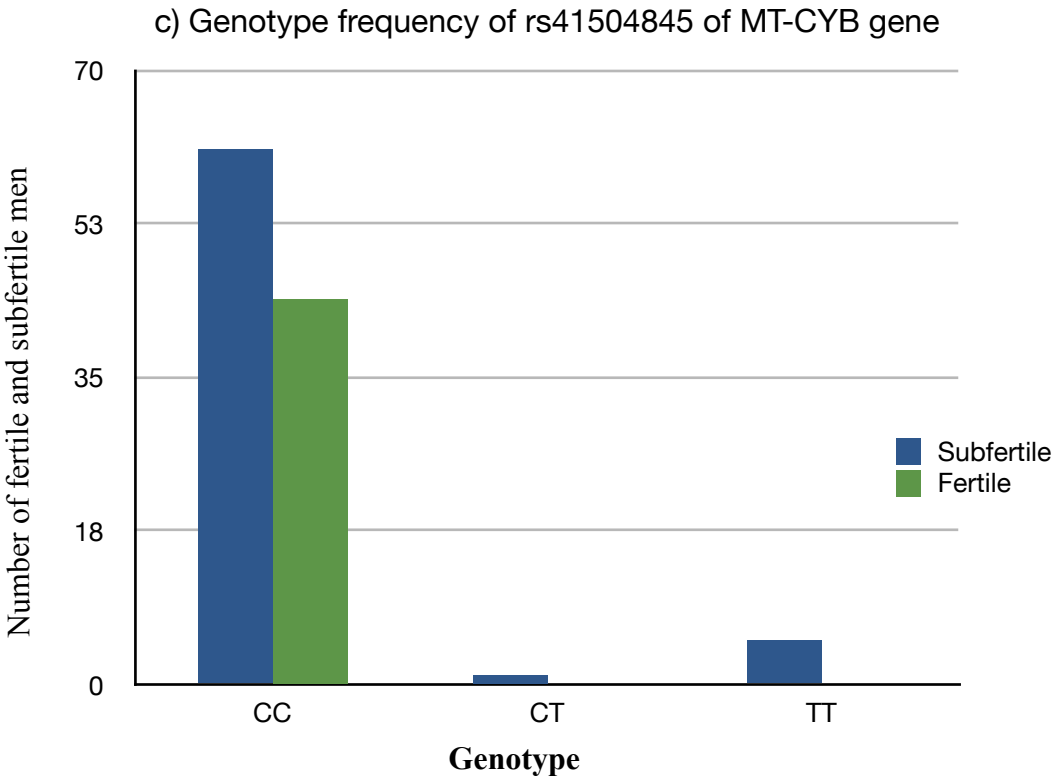


Figure 29: a) *MT-CYB* gene allele frequency for rs41504845, **b)** *MT-CYB* gene genotype frequency for rs41504845.

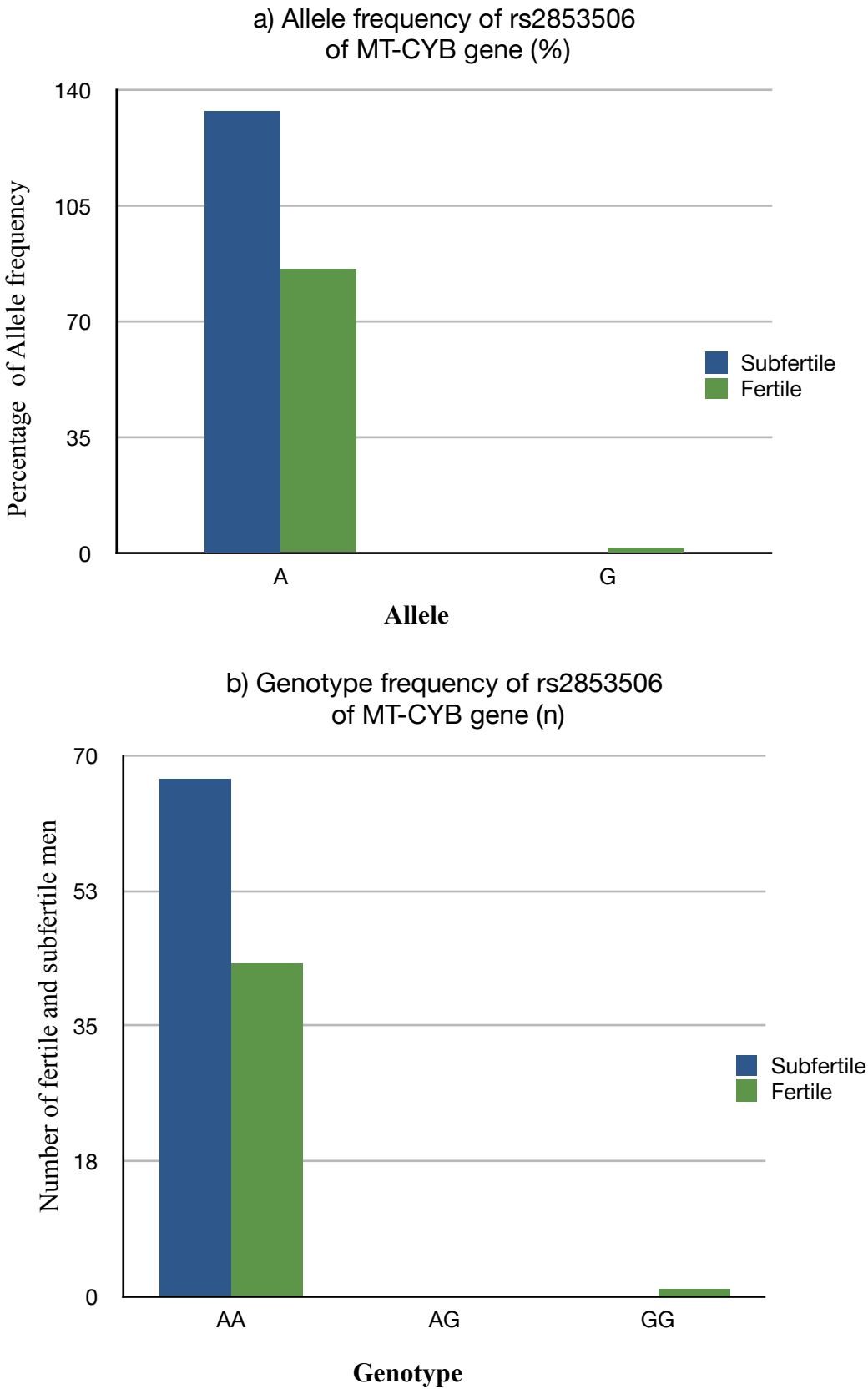


Figure 30: a) *MT-CYB* gene allele frequency for rs2853506, **b)** *MT-CYB* gene genotype frequency for rs2853506.

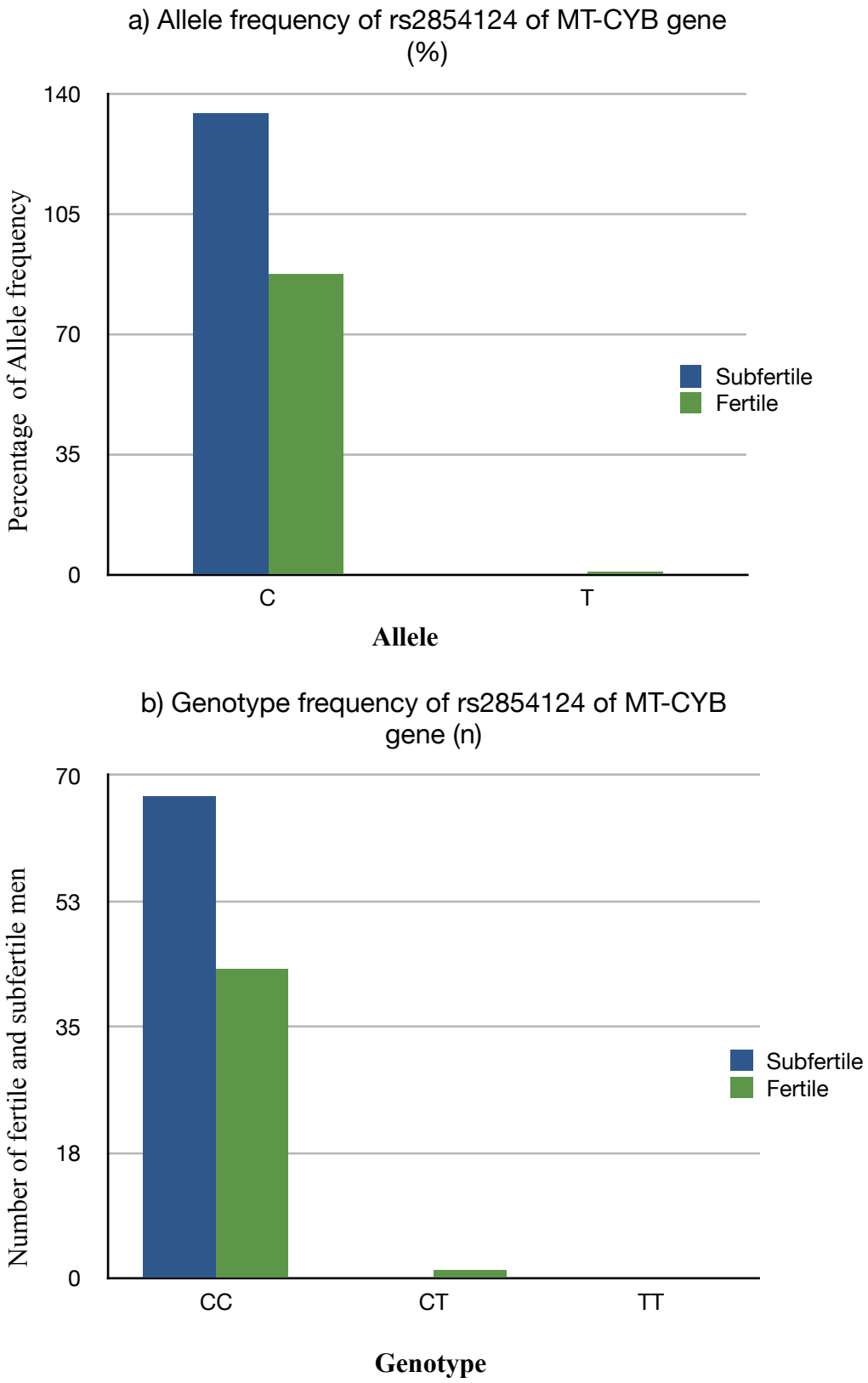


Figure 31: a) *MT-CYB* gene allele frequency for rs2854124, **b)** *MT-CYB* gene genotype frequency for rs2854124.

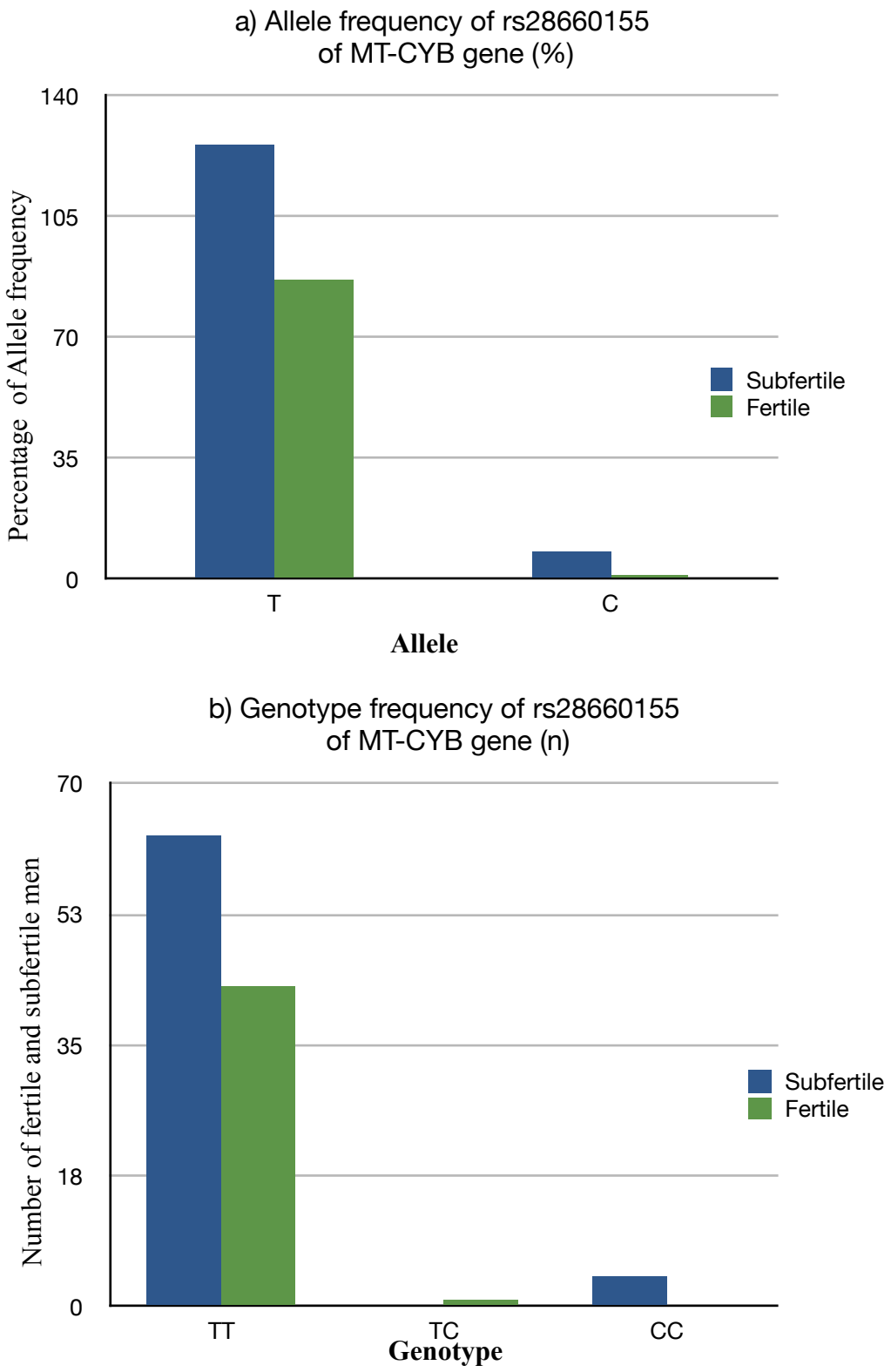
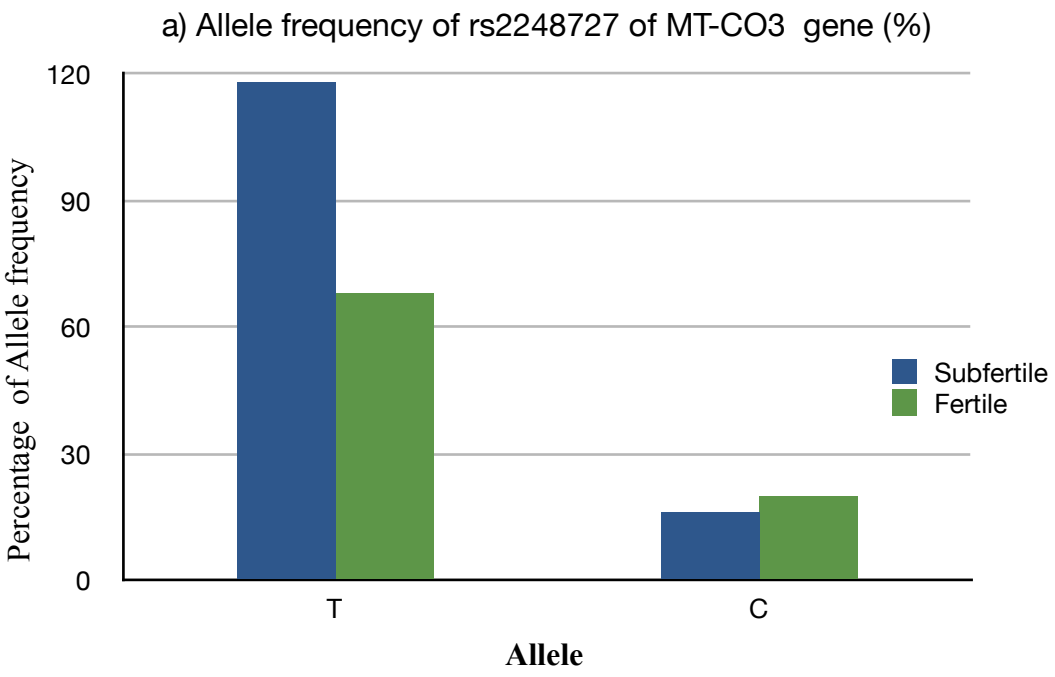


Figure32: a) *MT-CYB* gene allele frequency for rs28660155, **b)** *MT-CYB* gene genotype frequency for rs28660155.



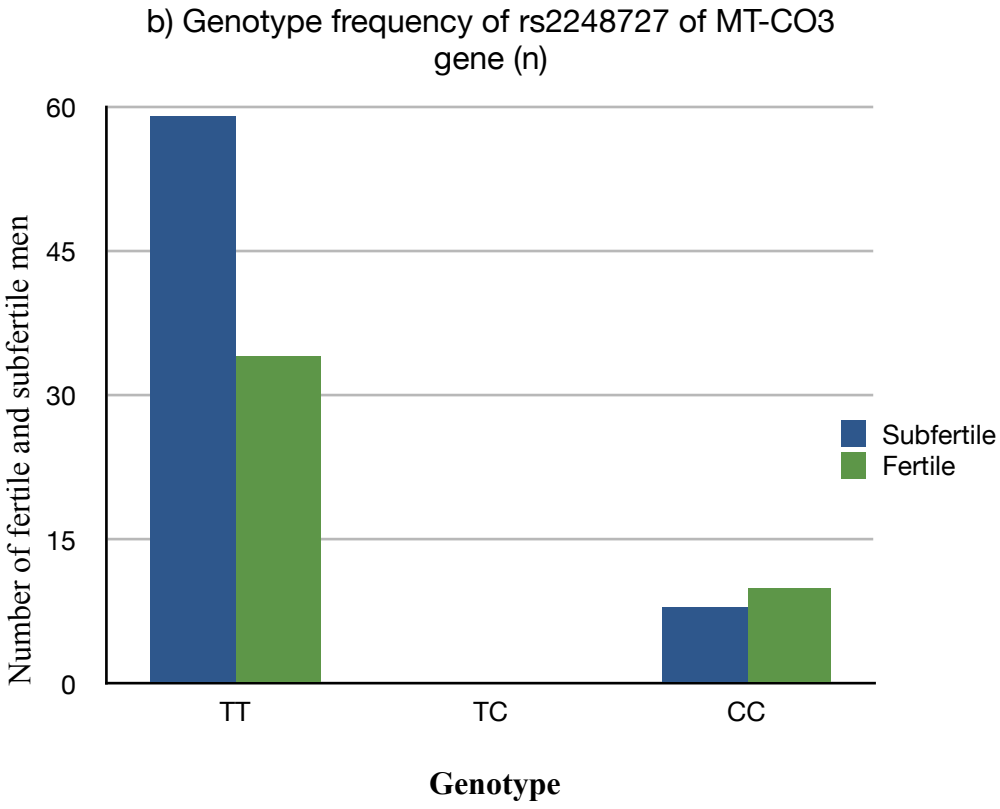


Figure 33: a) *MT-CO3* gene allele frequency for rs2248727, **b)** *MT-CO3* gene genotype frequency for rs2248727.

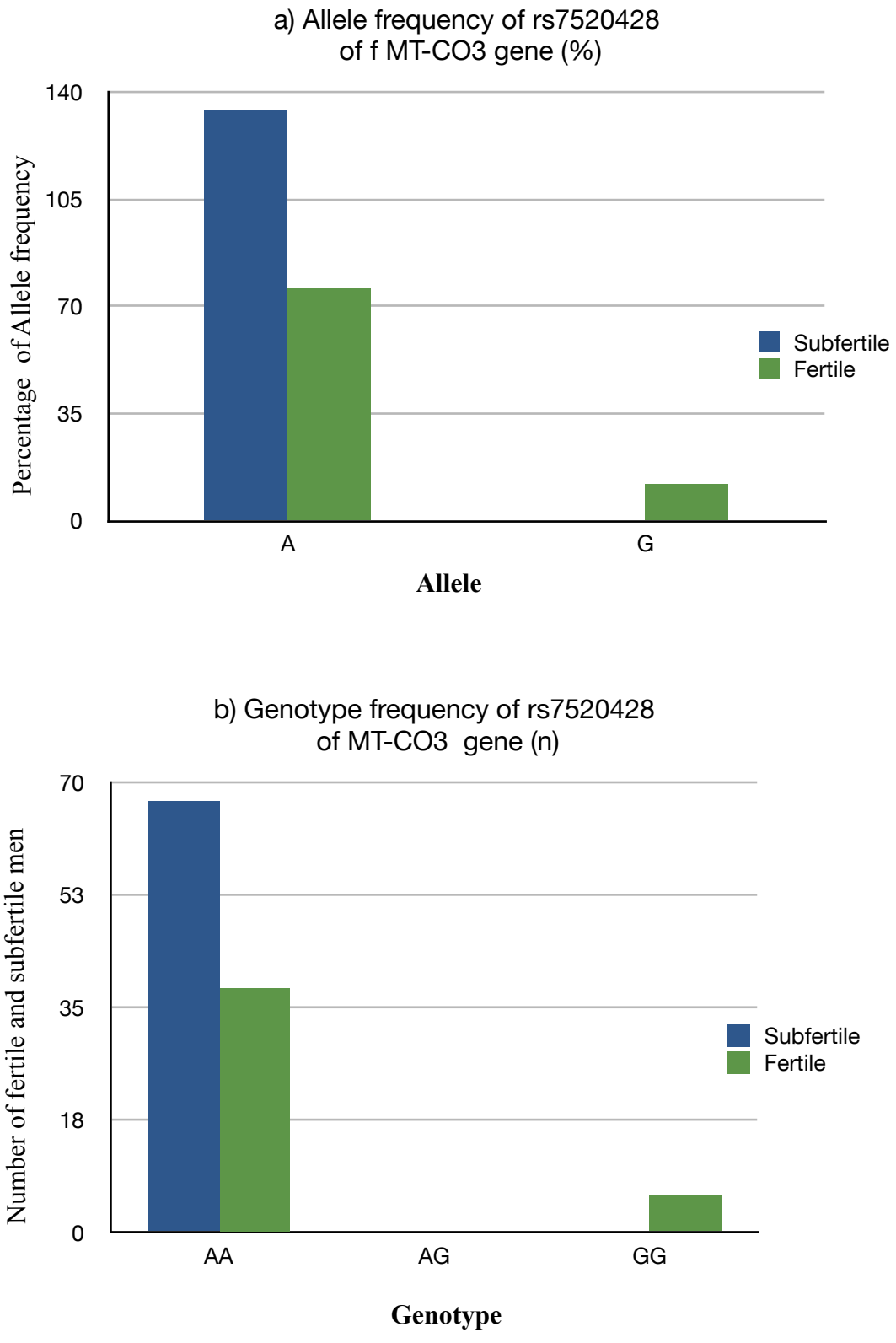
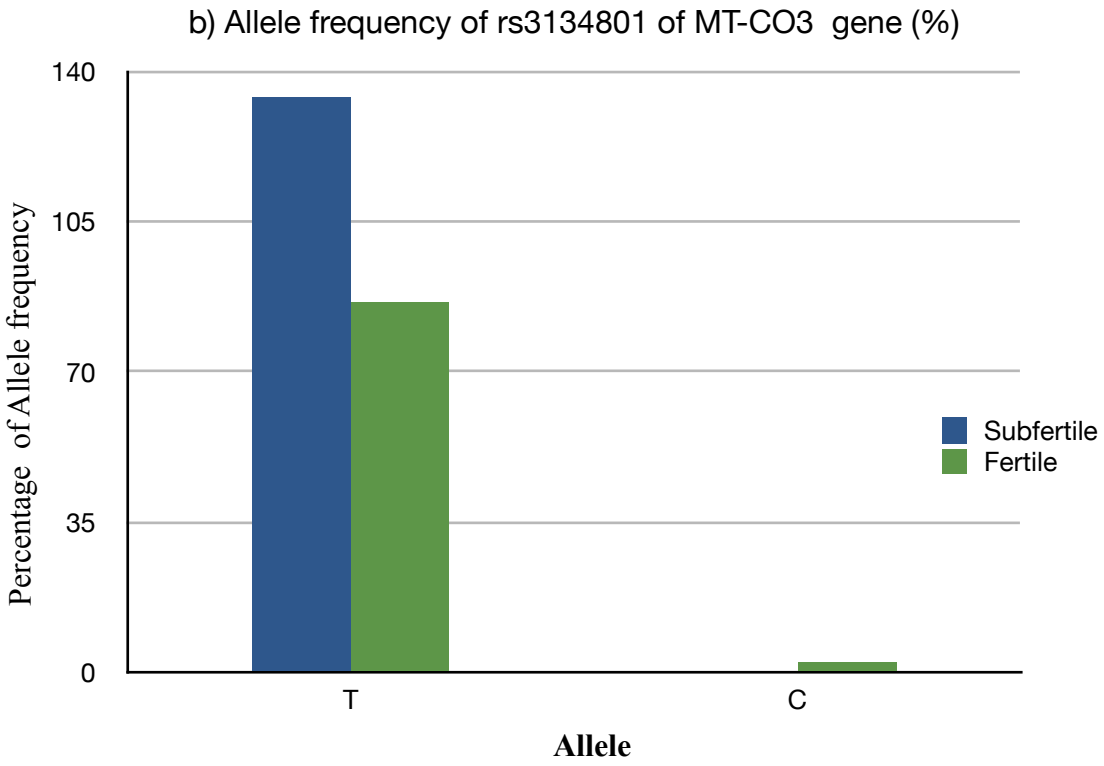


Figure 34: a) *MT-CO3* gene allele frequency for rs7520428, **b)** *MT-CO3* gene genotype frequency for rs7520428.



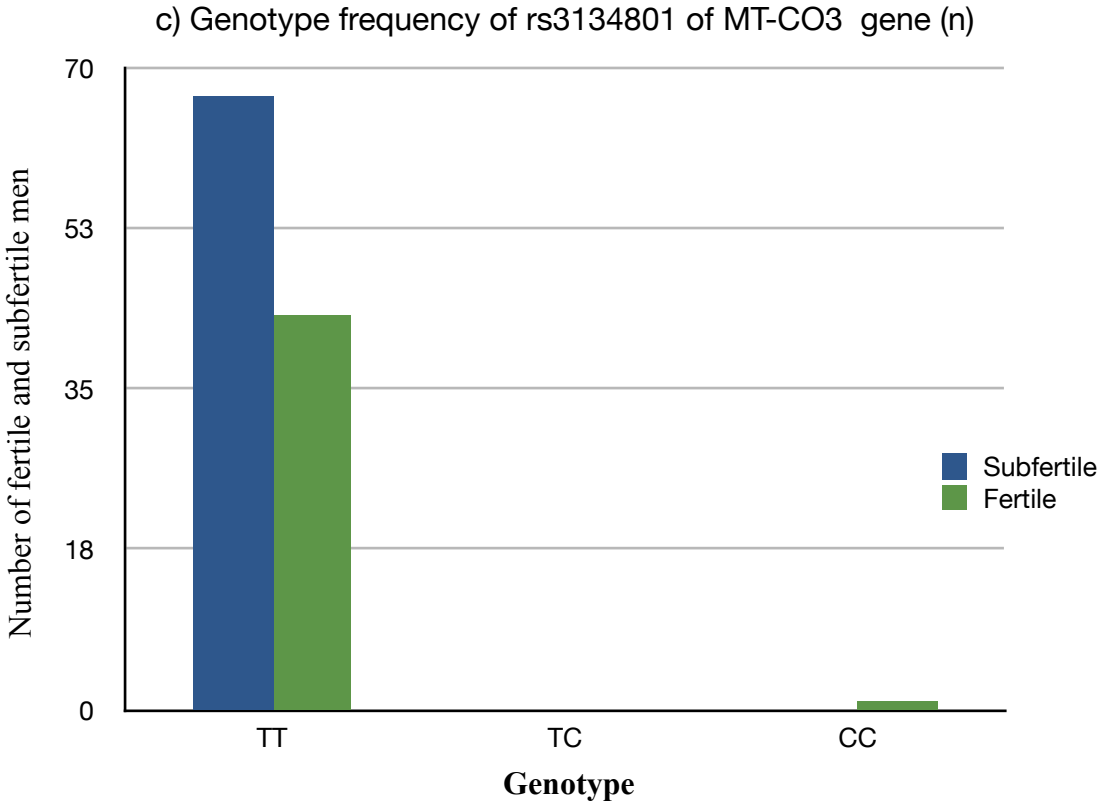


Figure 35: a) *MT-CO3* gene allele frequency for rs3134801, **b)** *MT-CO3* gene genotype frequency for rs3134801.

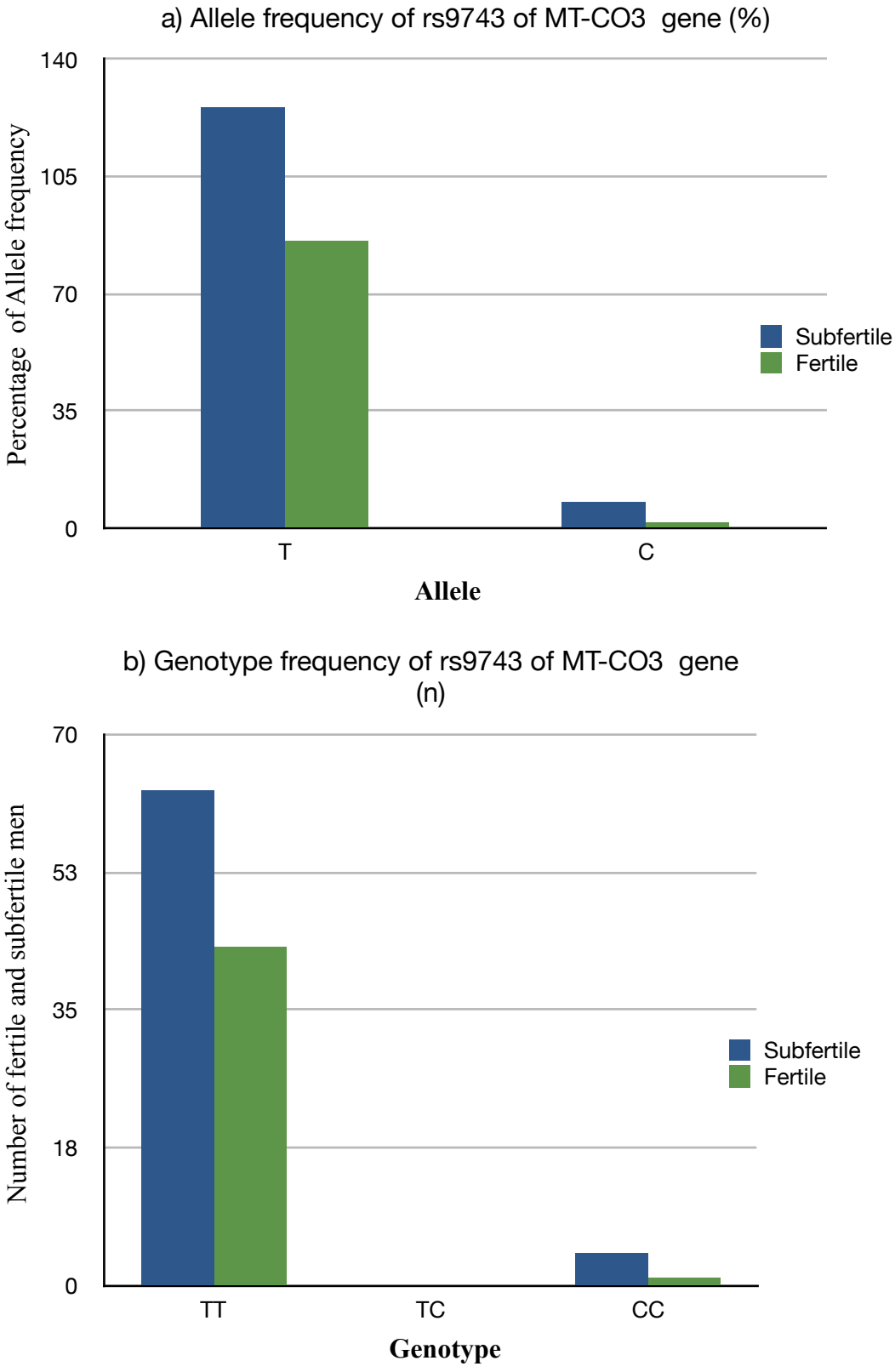


Figure 36: a) *MT-CO3* gene allele frequency for rs9743, **b)** *MT-CO3* gene genotype frequency for rs9743.

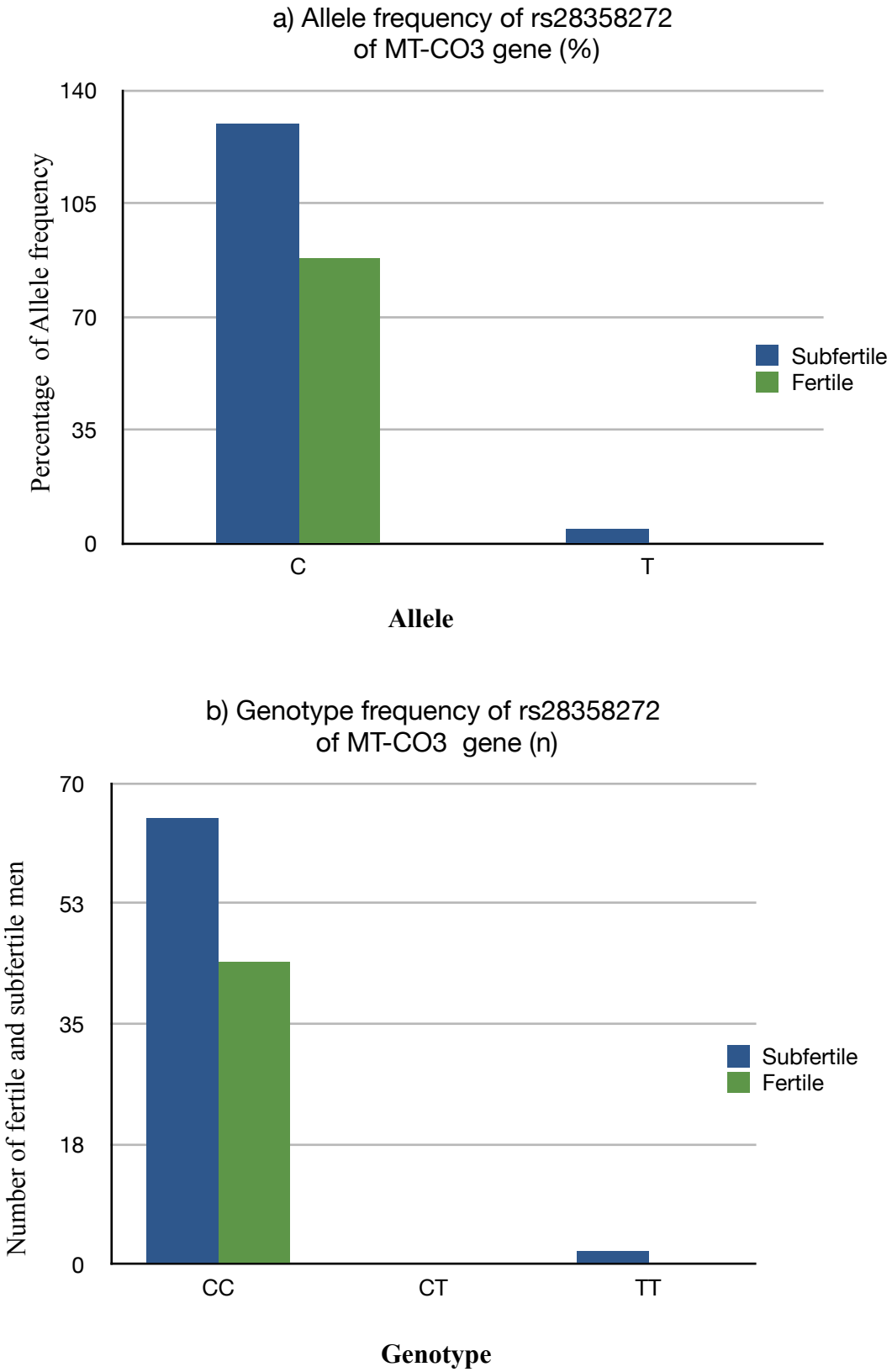


Figure 37: a) *MT-CO3* gene allele frequency for rs28358272, **b)** *MT-CO3* gene genotype frequency for rs28358272.

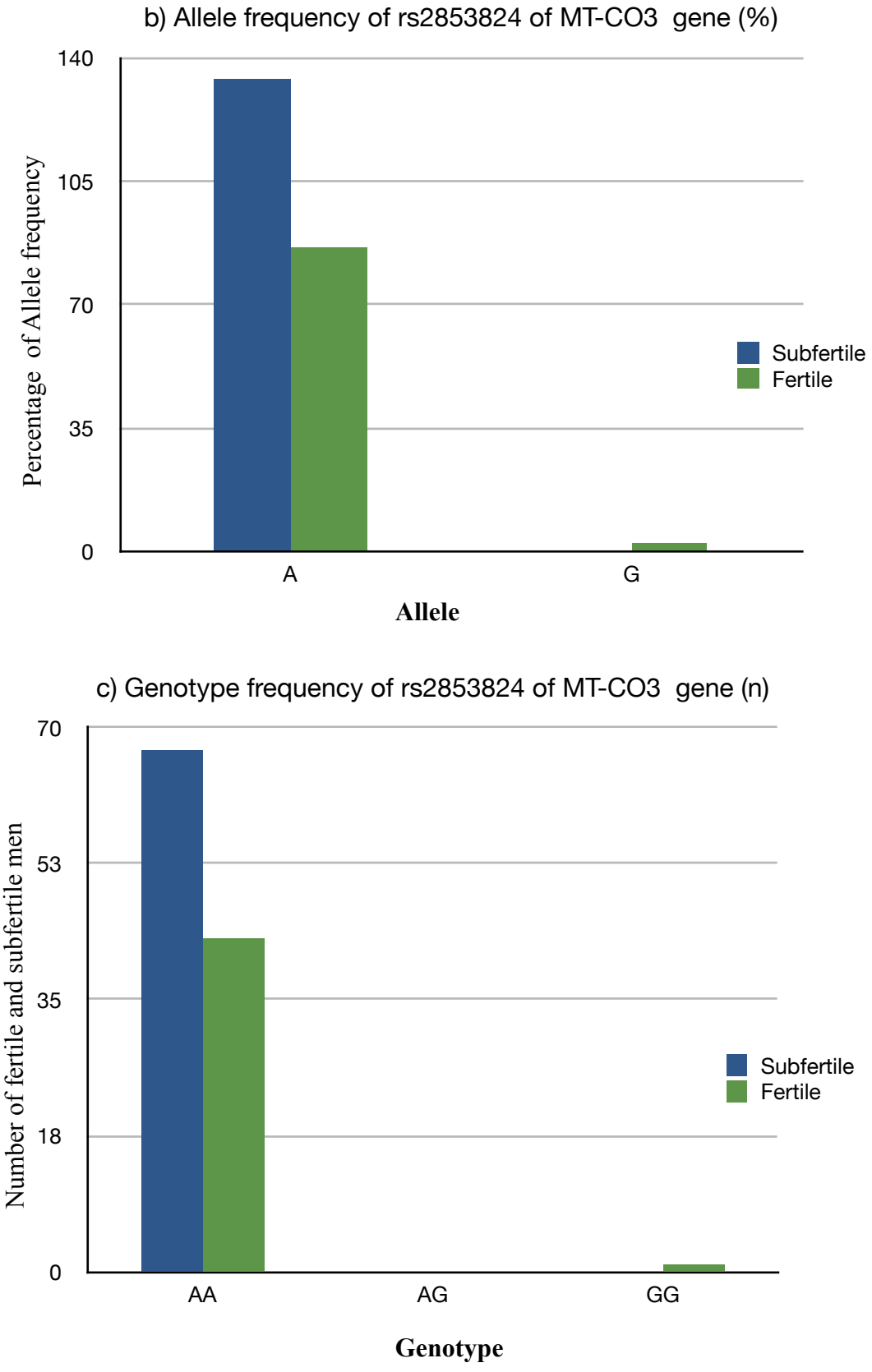


Figure 38: a) *MT-CO3* gene allele frequency for rs2853824, **b)** *MT-CO3* gene genotype frequency for rs2853824.

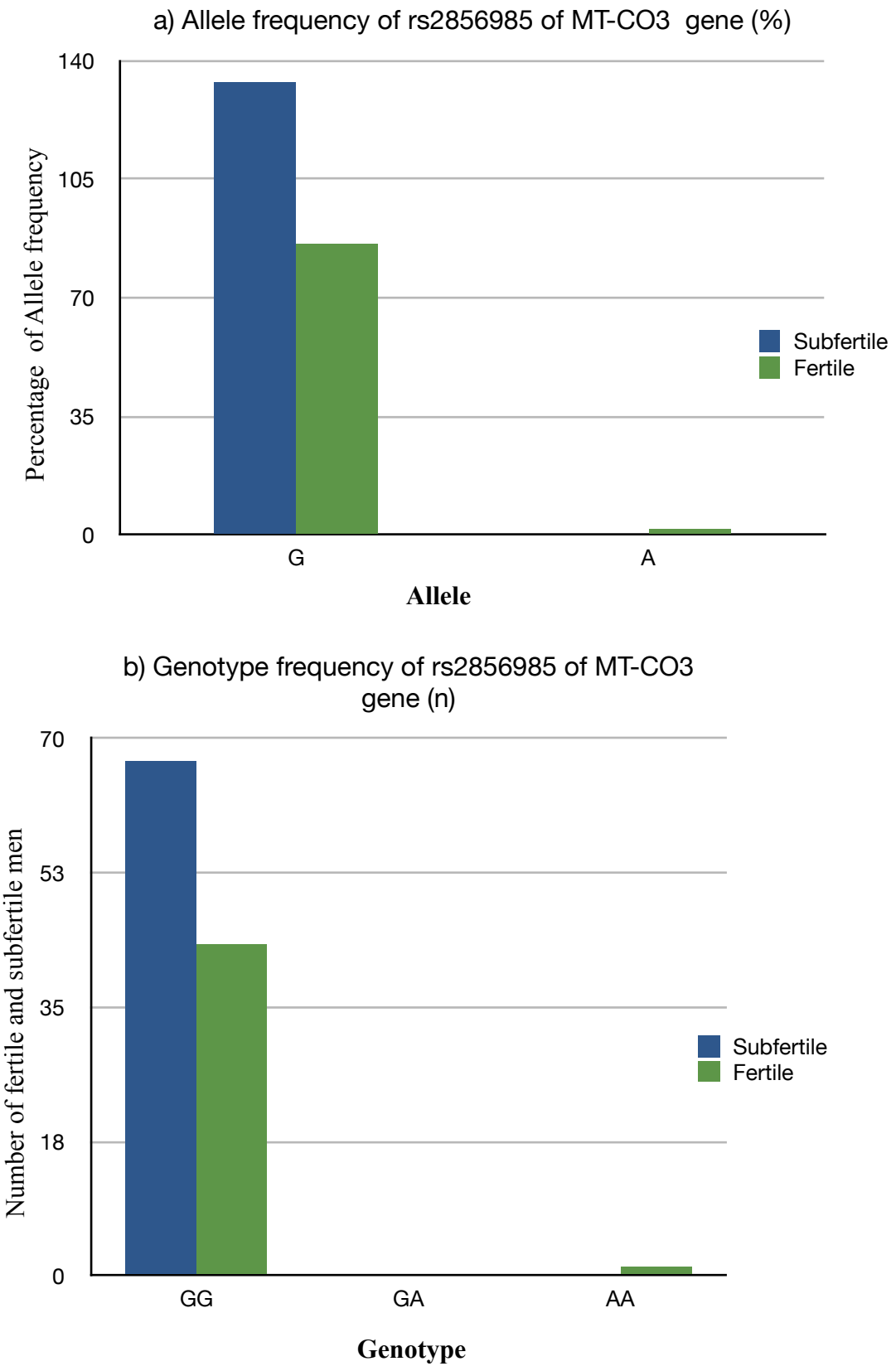
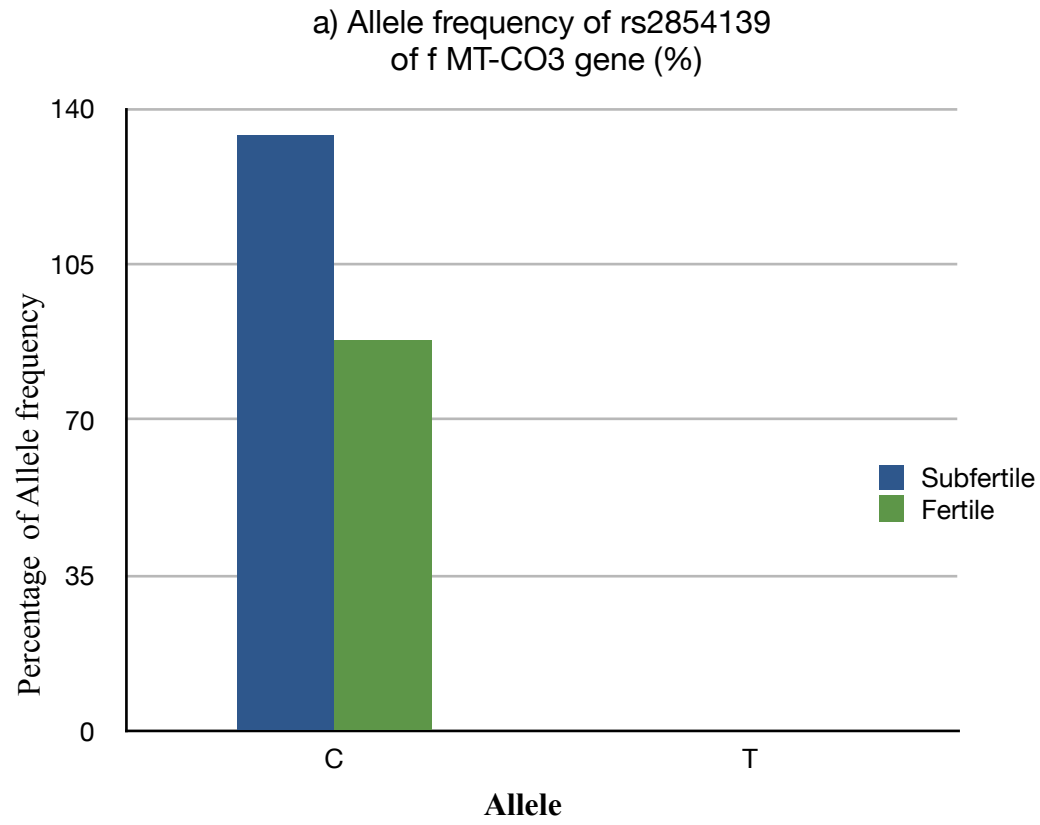


Figure 39: a) *MT-CO3* gene allele frequency for rs2856985, **b)** *MT-CO3* gene genotype frequency for rs2856985.



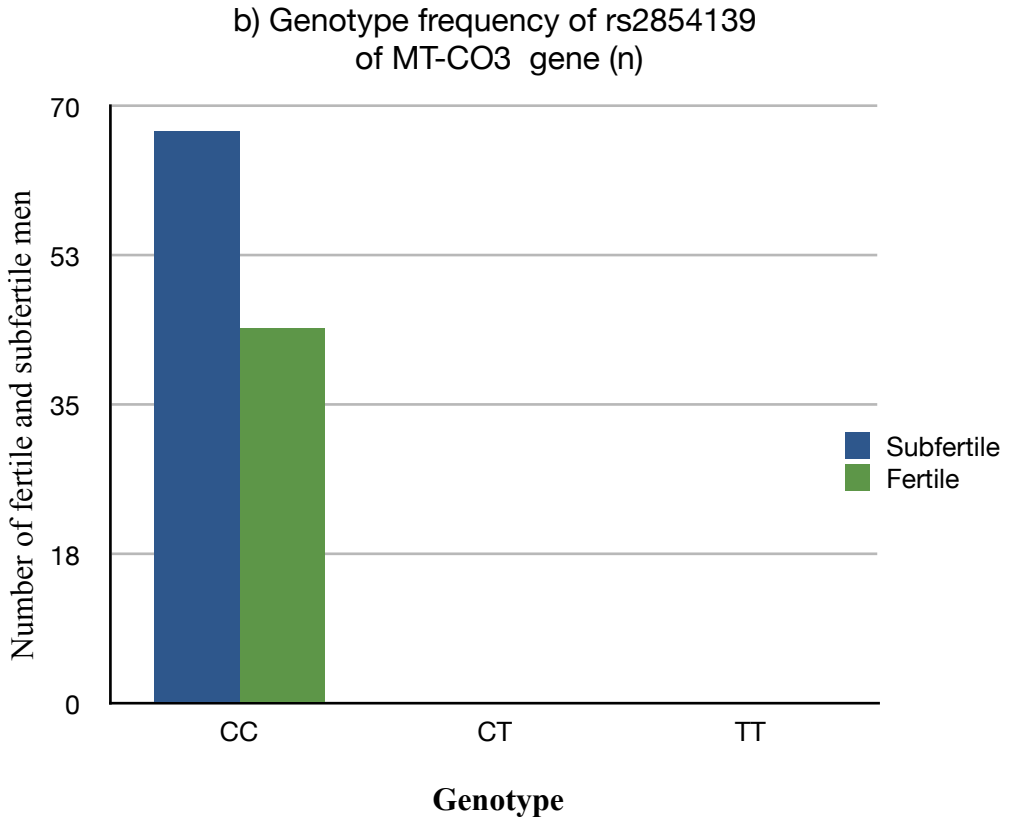
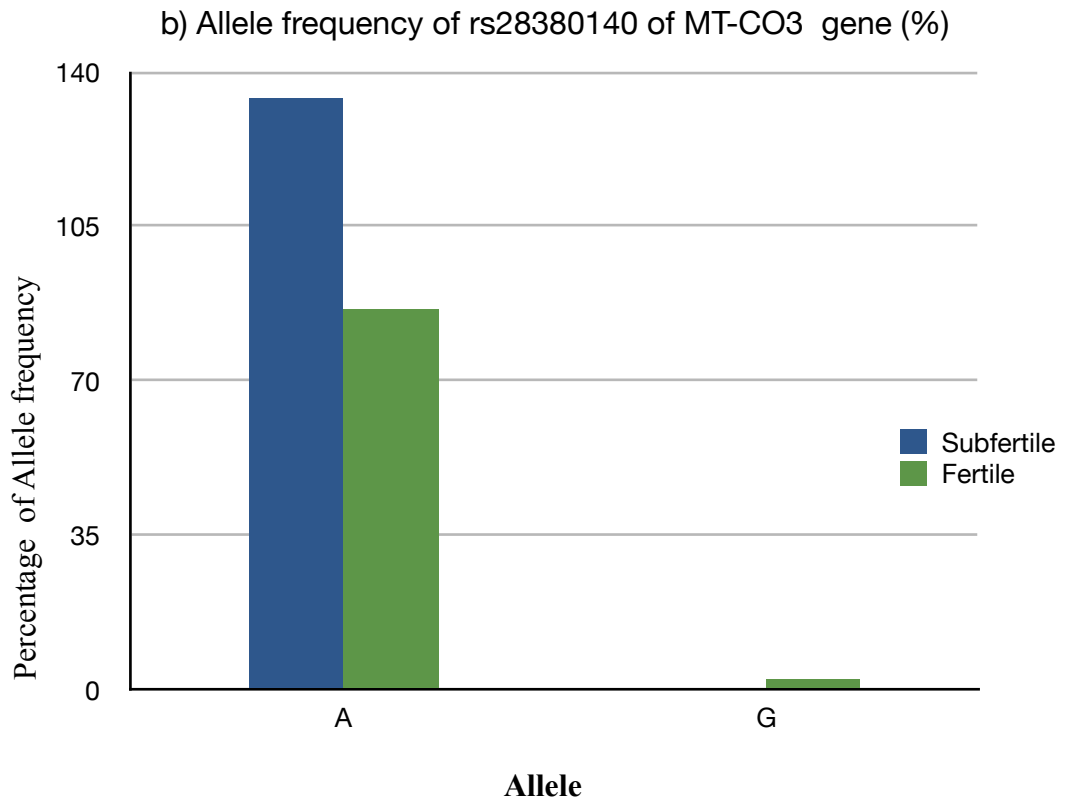


Figure 40: a) *MT-CO3* gene allele frequency for rs2854139, **b)** *MT-CO3* gene genotype frequency for rs2854139.



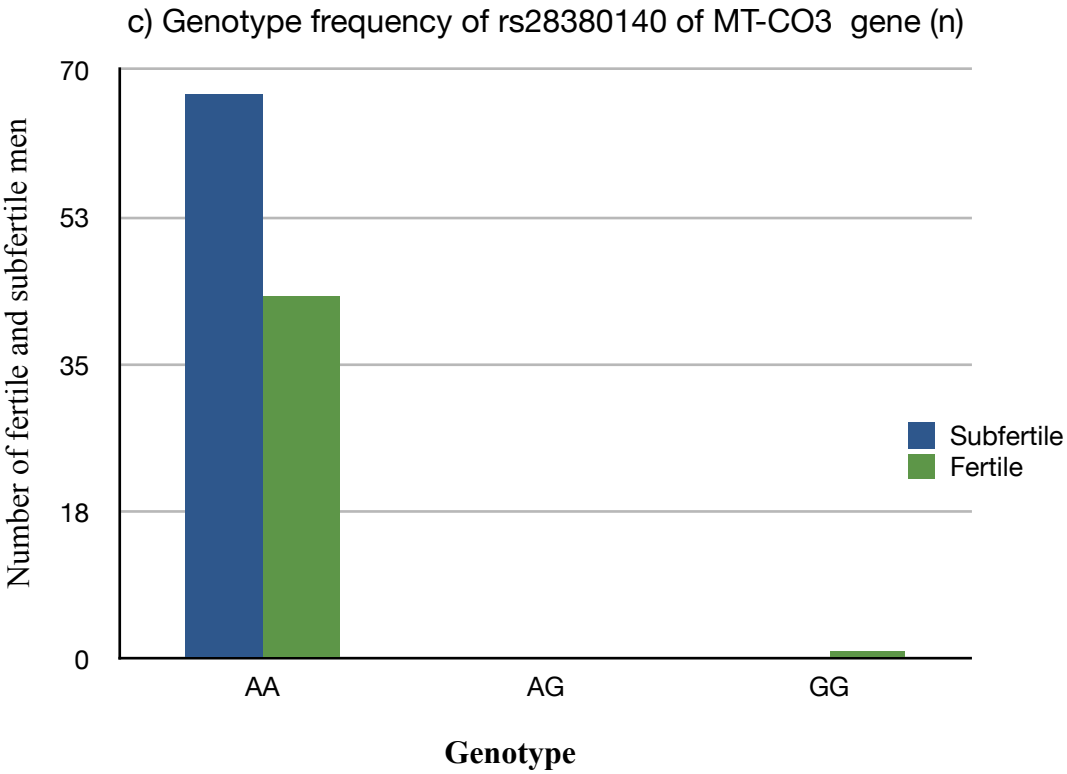


Figure 41: a) *MT-CO3* gene allele frequency for rs28380140, **b)** *MT-CO3* gene genotype frequency for rs28380140.

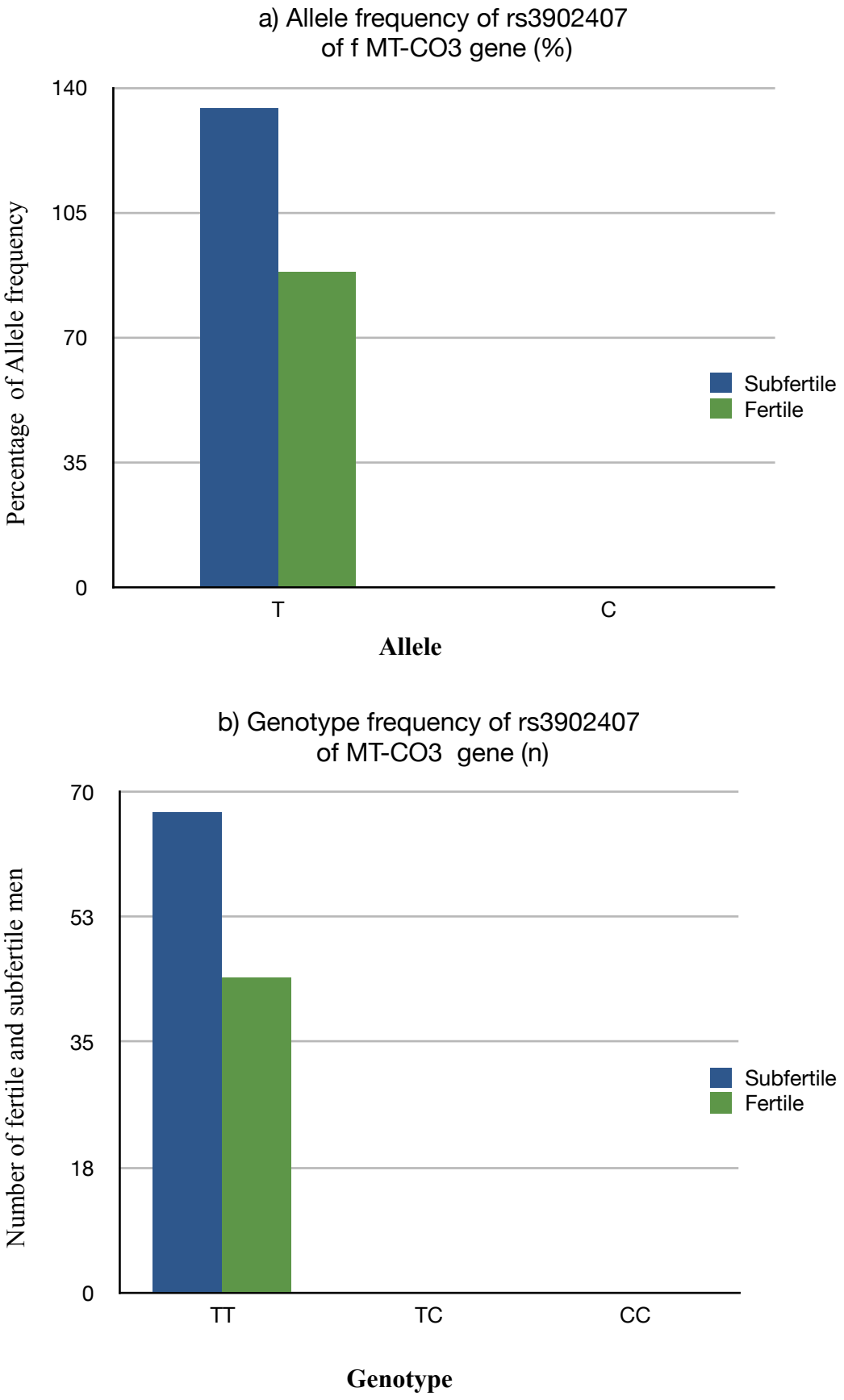
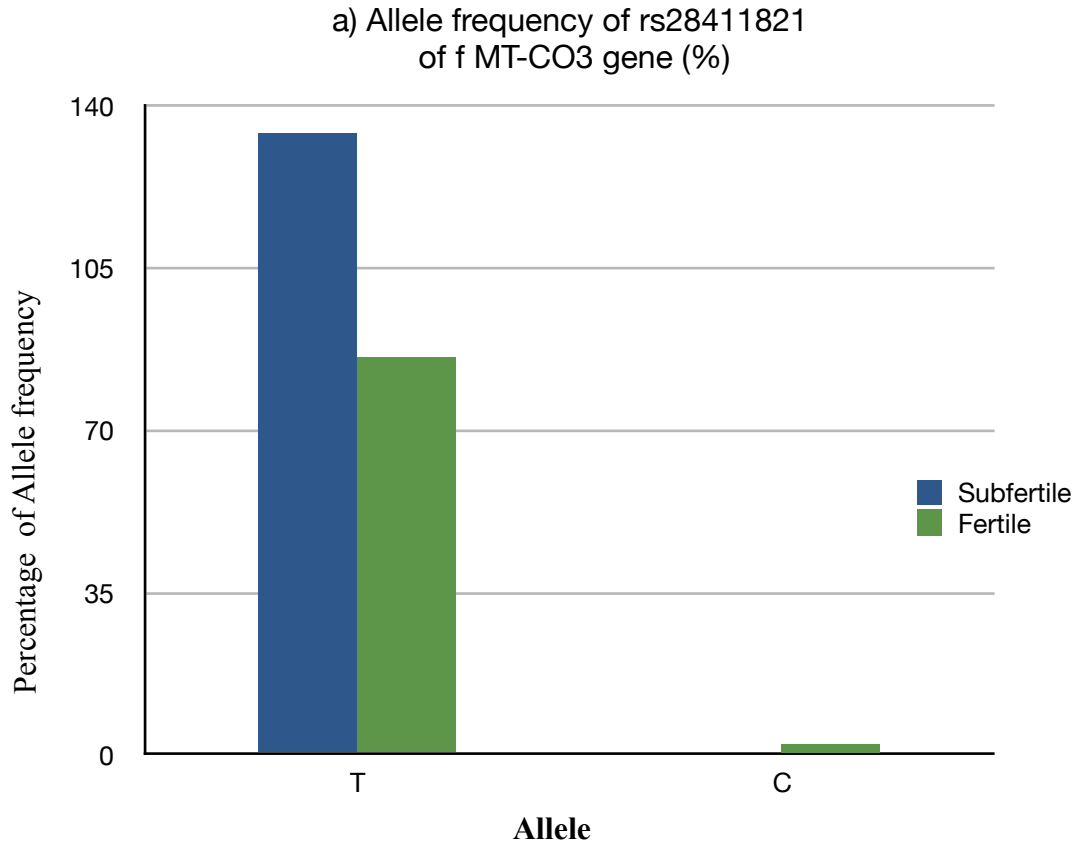


Figure 42: a) *MT-CO3* gene allele frequency for rs3902407, **b)** *MT-CO3* gene genotype frequency for rs3902407.



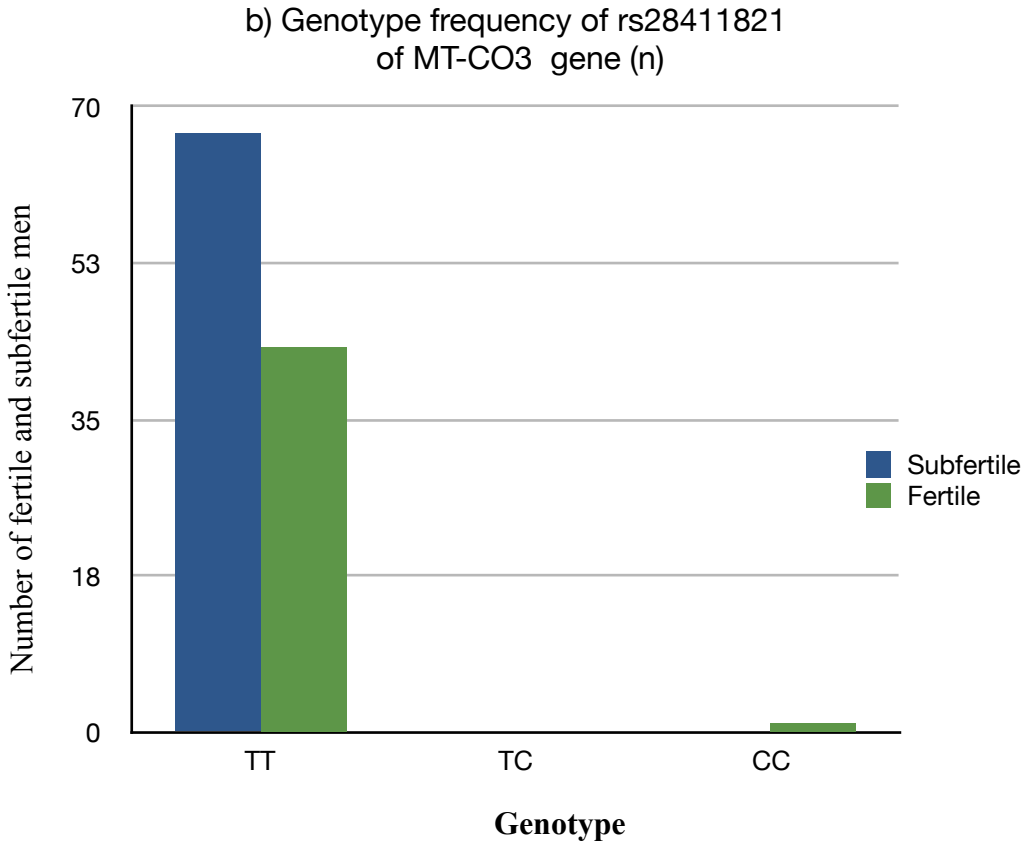
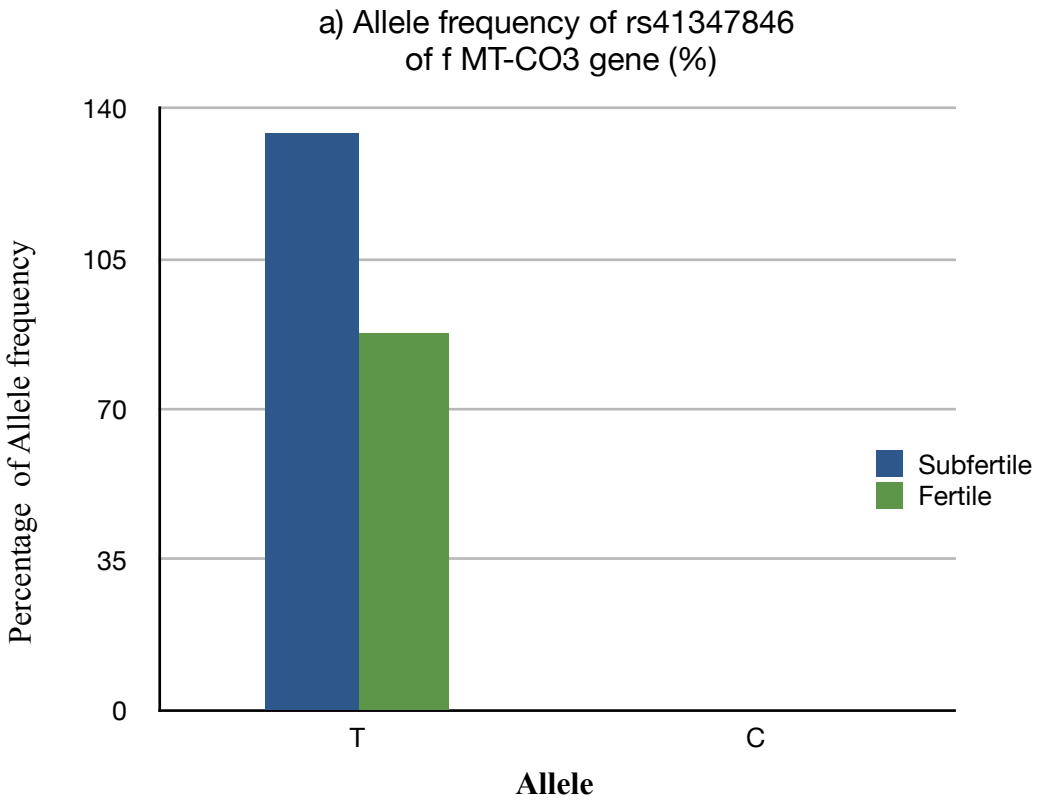


Figure 43: a) *MT-CO3* gene allele frequency for rs28411821, **b)** *MT-CO3* gene genotype frequency for rs28411821.



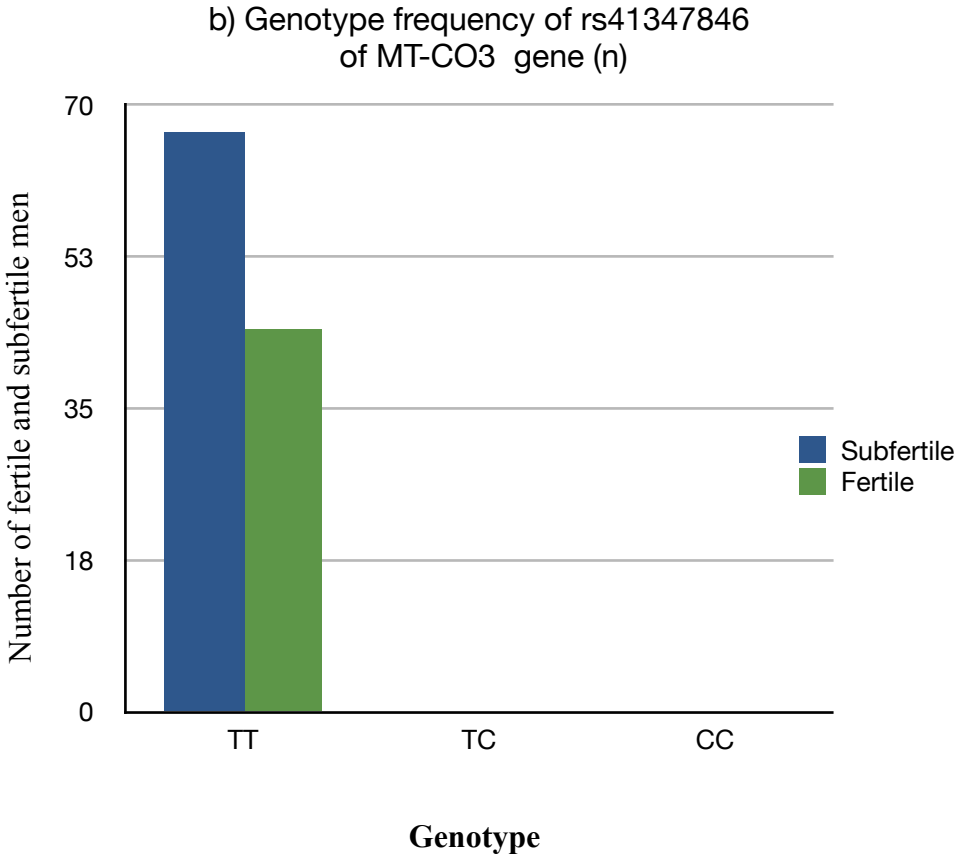
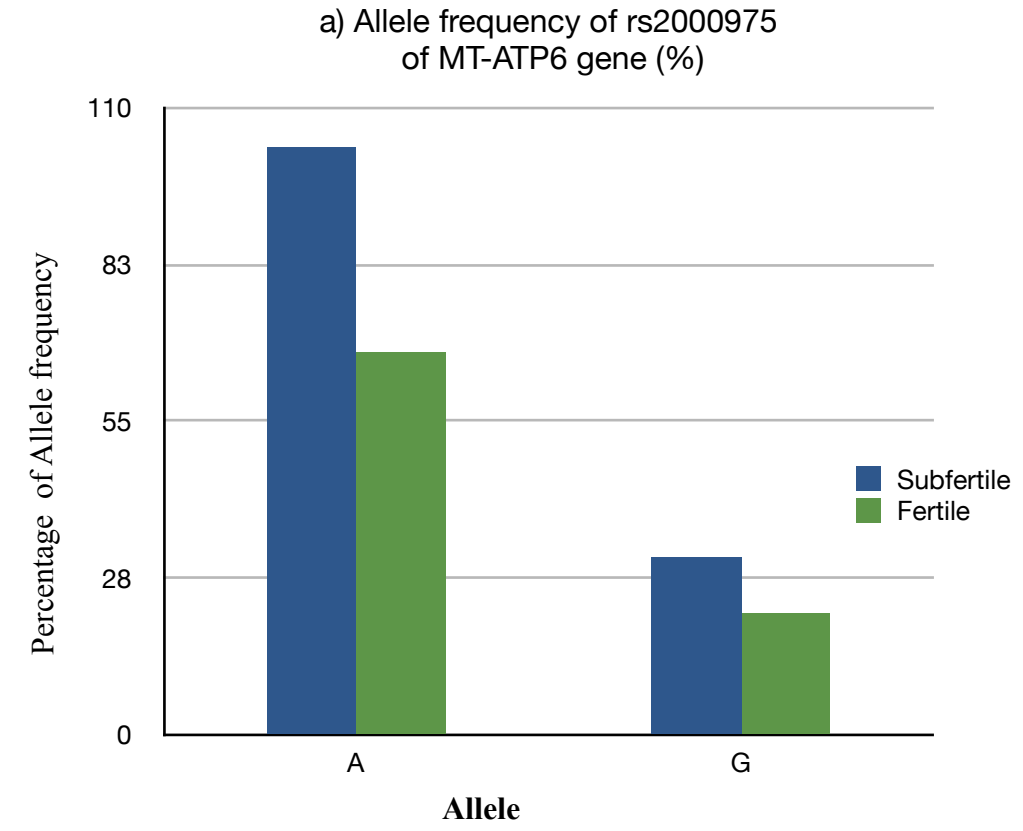


Figure 44: a) *MT-CO3* gene allele frequency for rs41347846, **b)** *MT-CO3* gene genotype frequency for rs41347846.



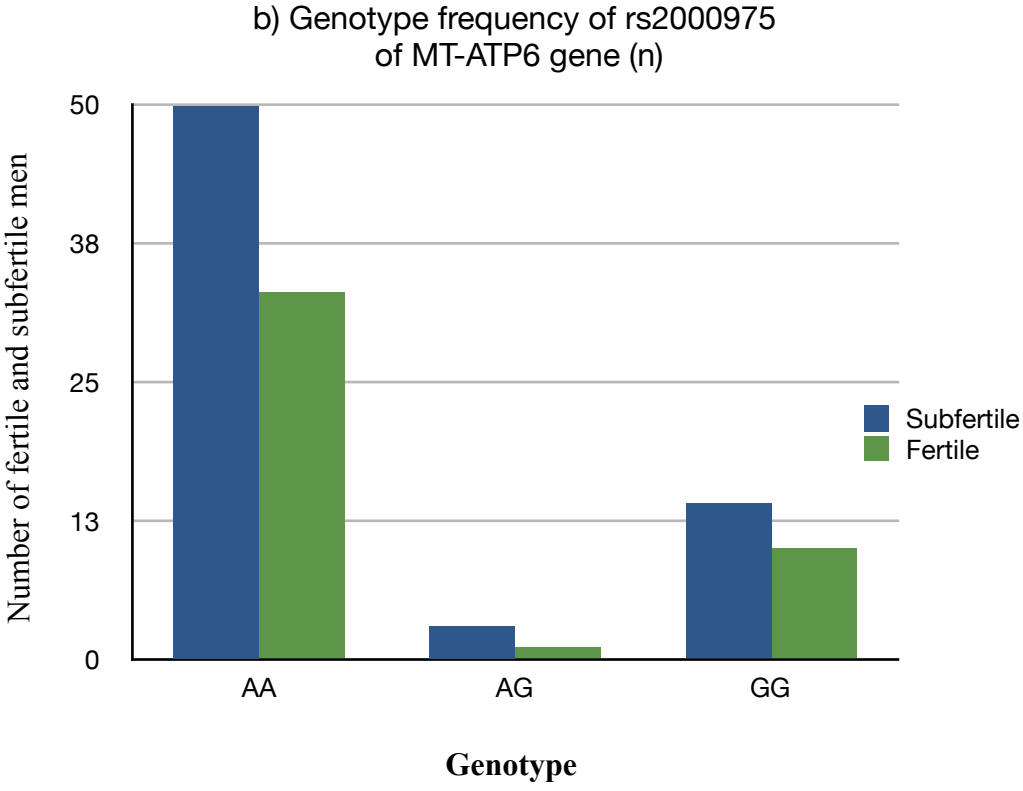


Figure 45: a) *MT-ATP6* gene allele frequency for rs2000975, **b)** *MT-ATP6* gene genotype frequency for rs2000975.

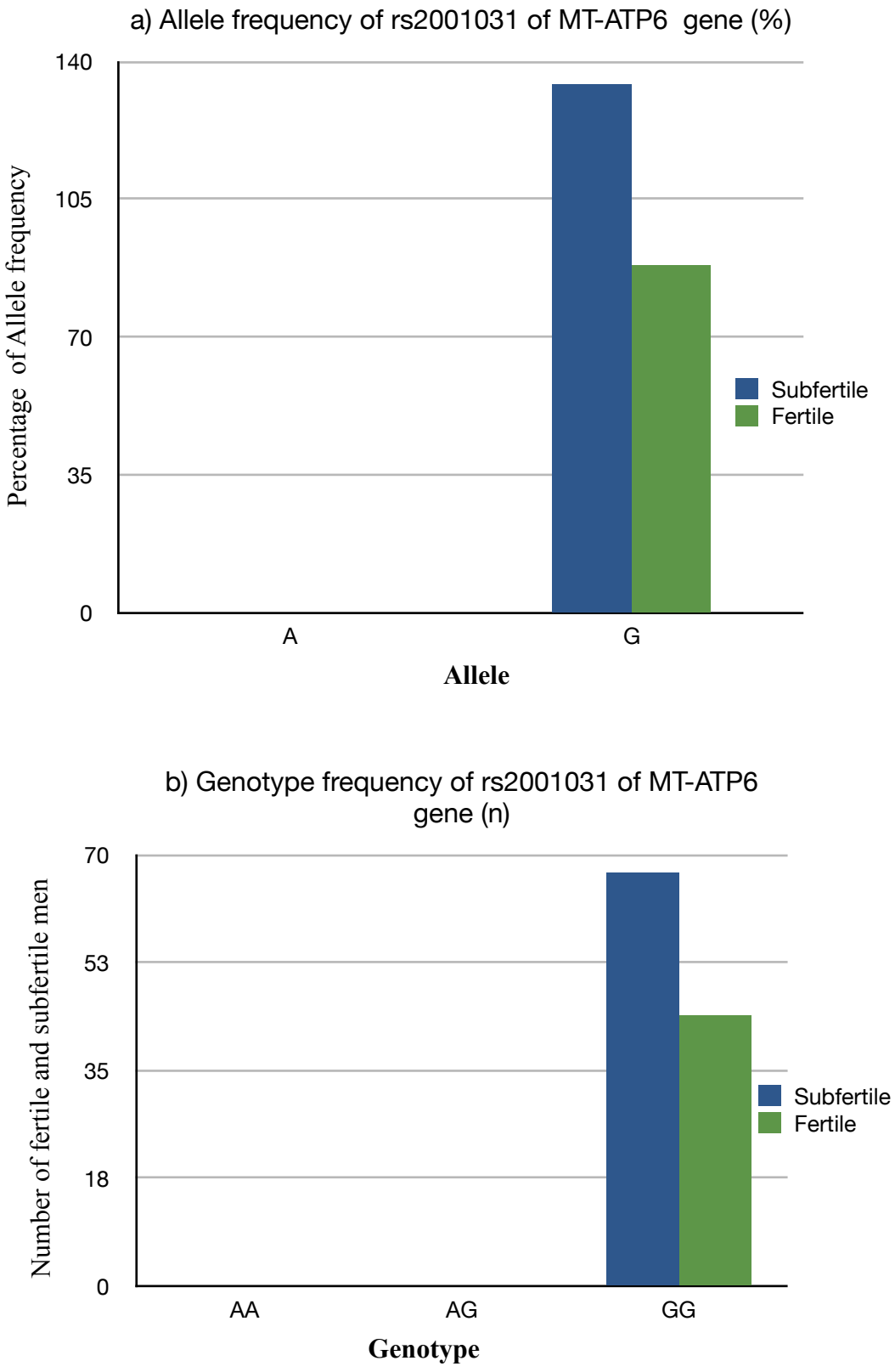


Figure 46: a) *MT-ATP6* gene allele frequency for rs2001031, **b)** *MT-ATP6* gene genotype frequency for rs2001031.

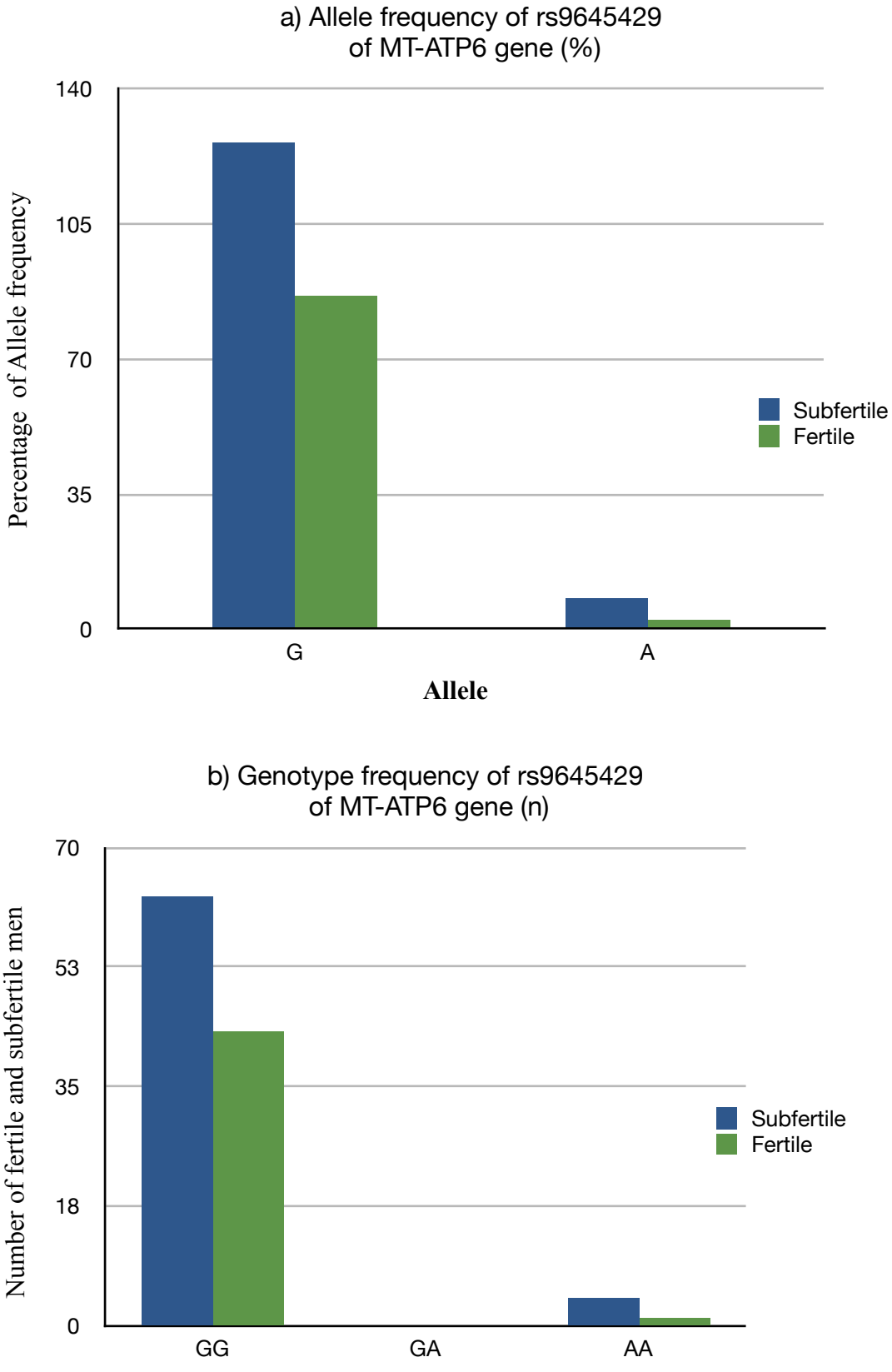
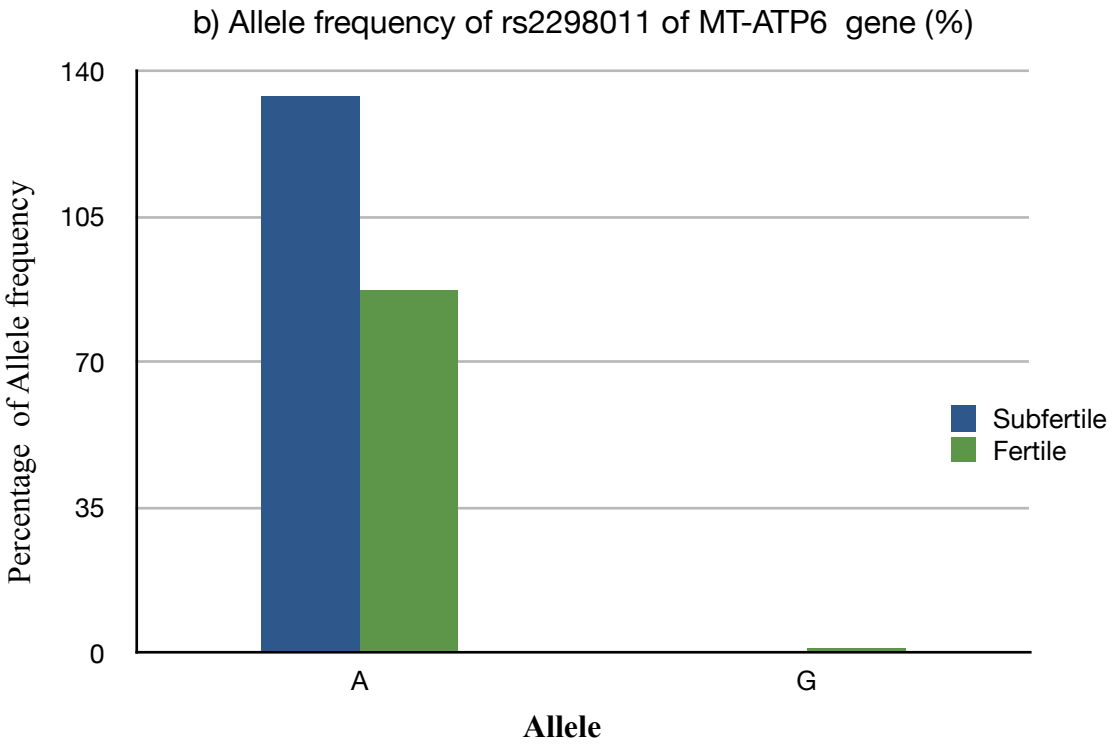


Figure 47: a) *MT-ATP6* gene allele frequency for rs9645429, **b)** *MT-ATP6* gene genotype frequency for rs9645429.



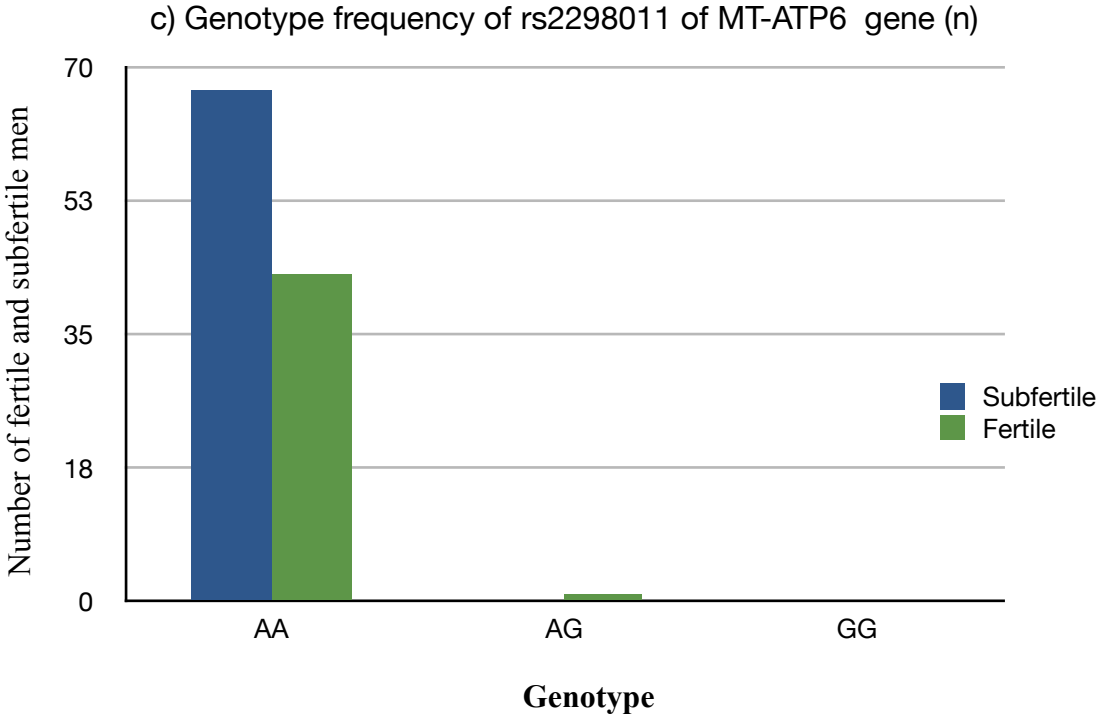
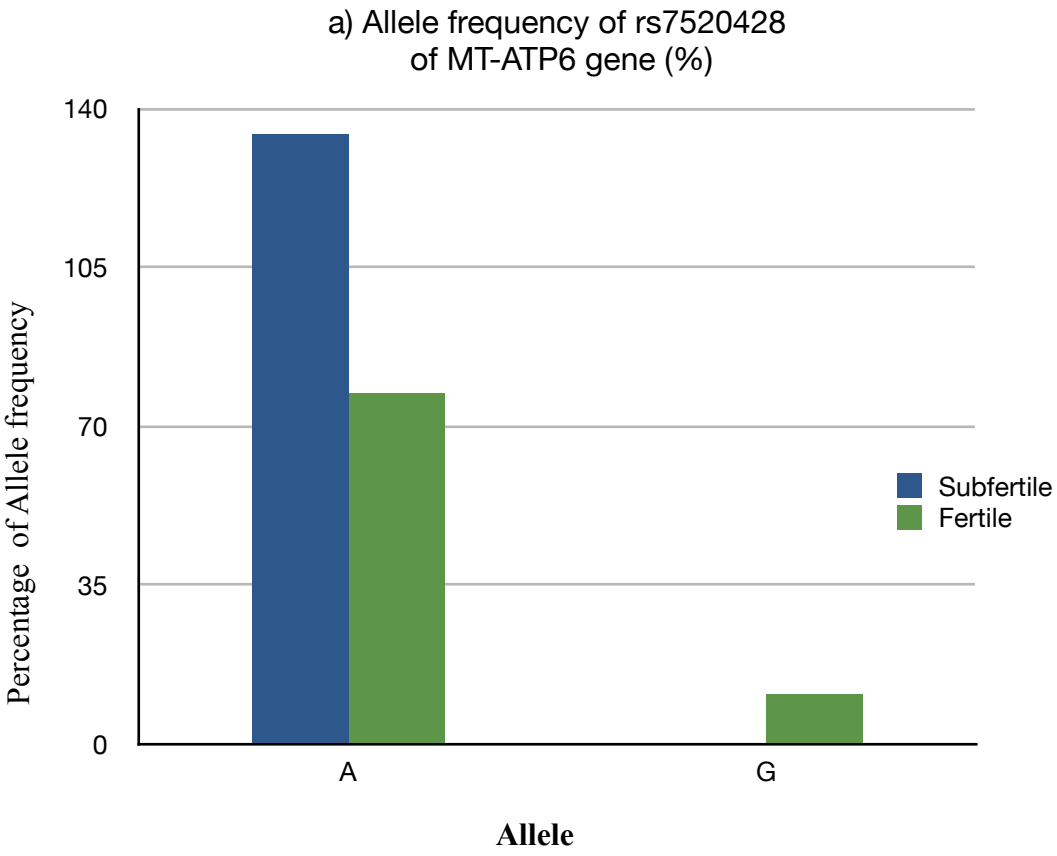


Figure 48: a) *MT-ATP6* gene allele frequency for rs2298011, **b)** *MT-ATP6* gene genotype frequency for rs2298011.



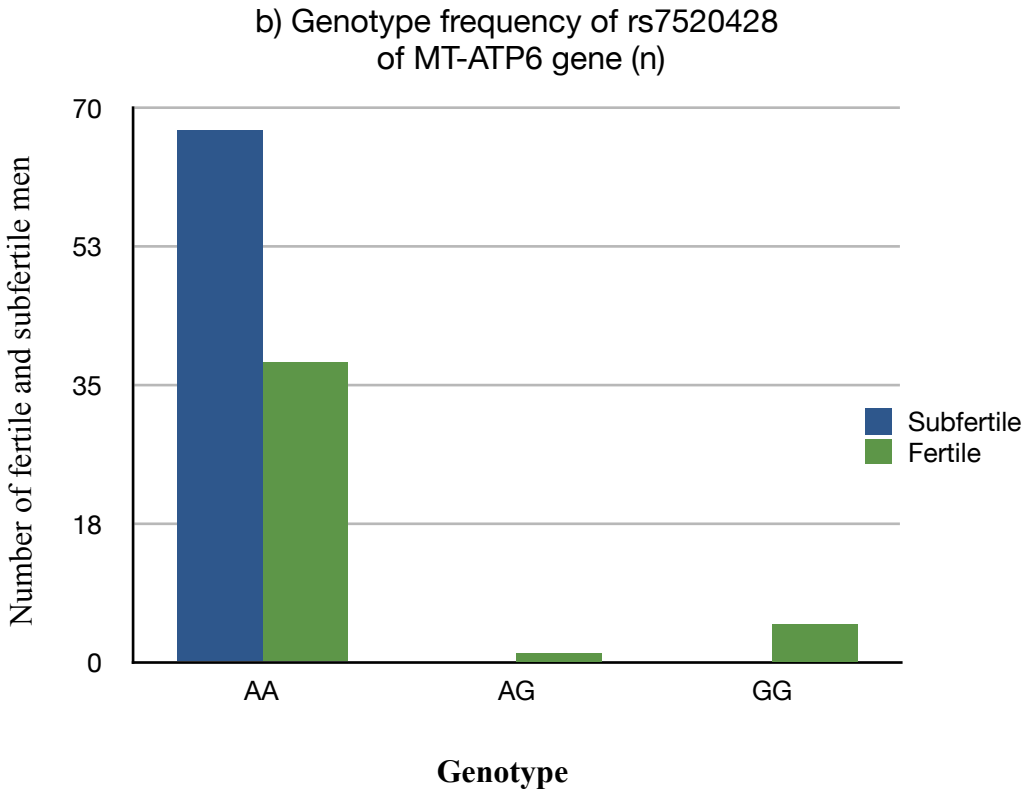


Figure 49: a) *MT-ATP6* gene allele frequency for rs7520428, **b)** *MT-ATP6* gene genotype frequency for rs7520428.

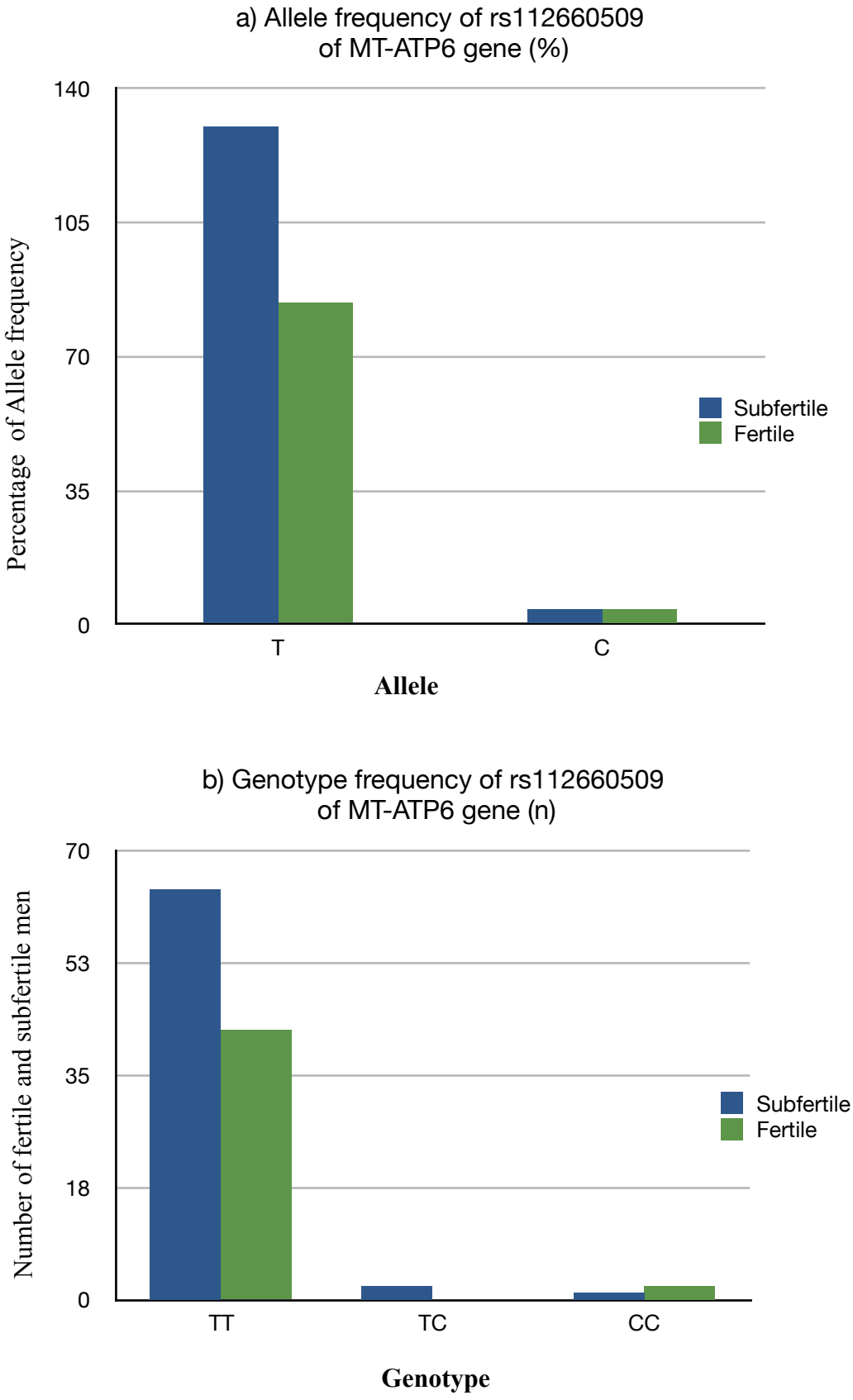


Figure 50: a) *MT-ATP6* gene allele frequency for rs112660509, **b)** *MT-ATP6* gene genotype frequency for rs112660509.

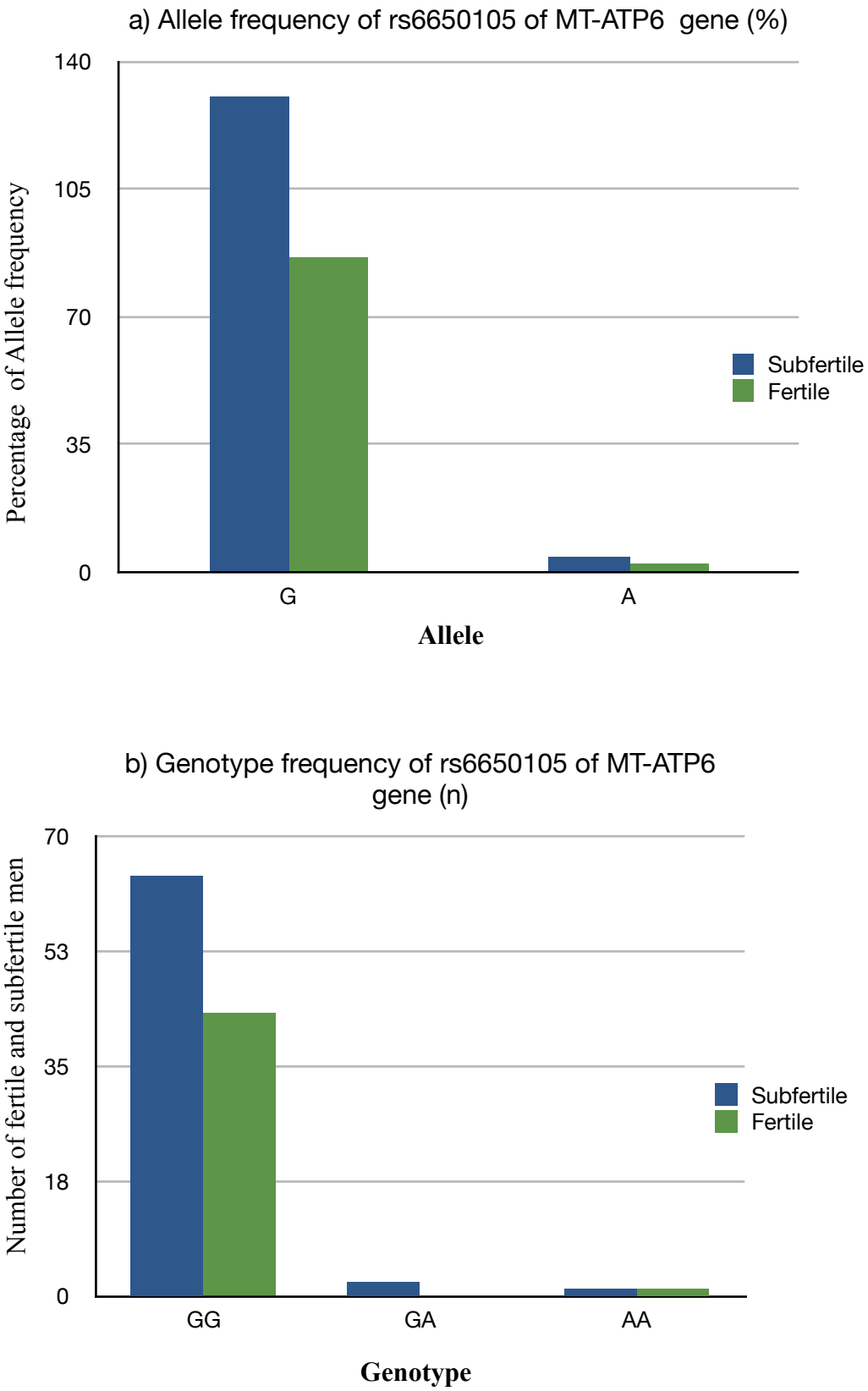


Figure 51: a) *MT-ATP6* gene allele frequency for rs6650105, **b)** *MT-ATP6* gene genotype frequency for rs6650105.

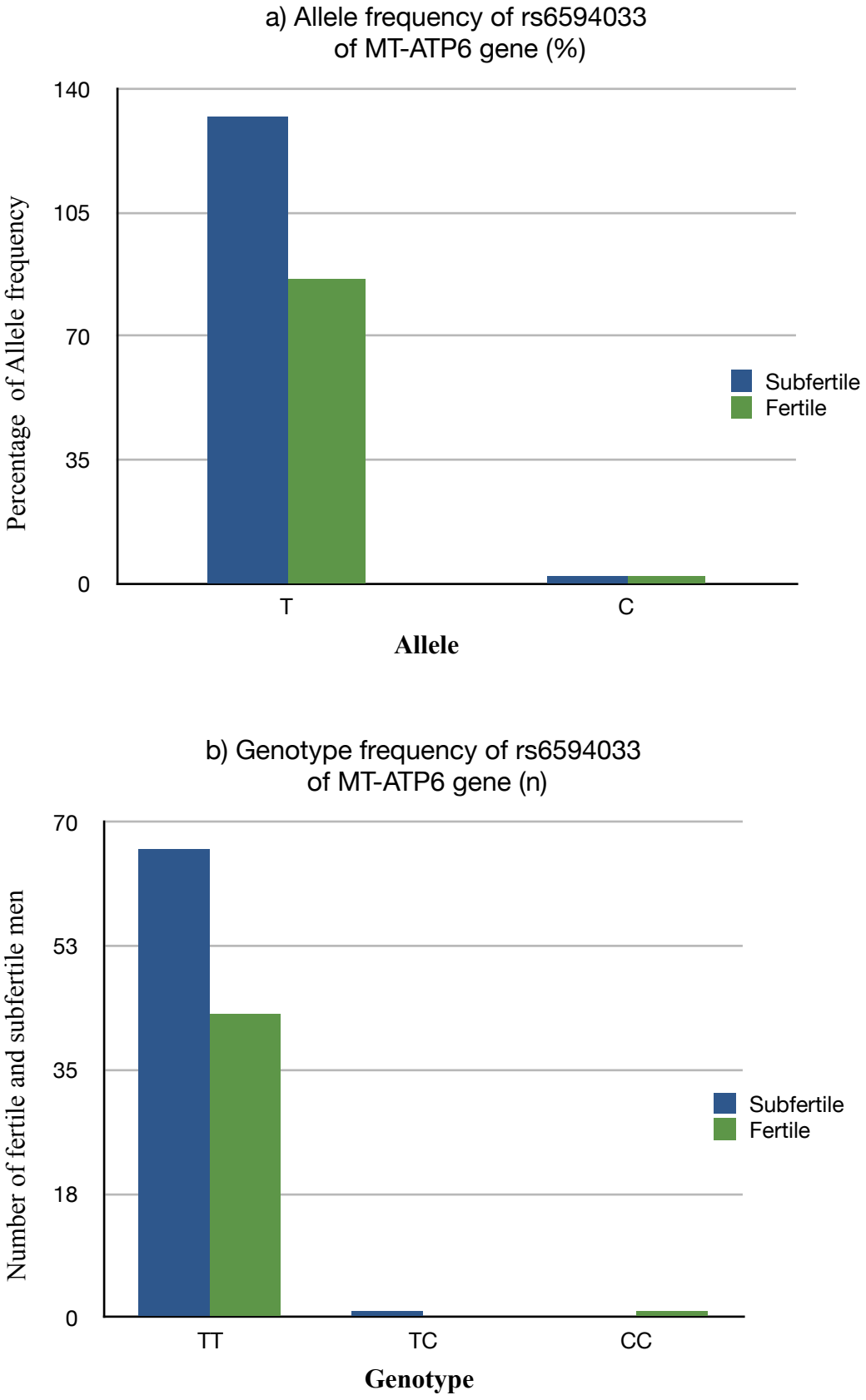
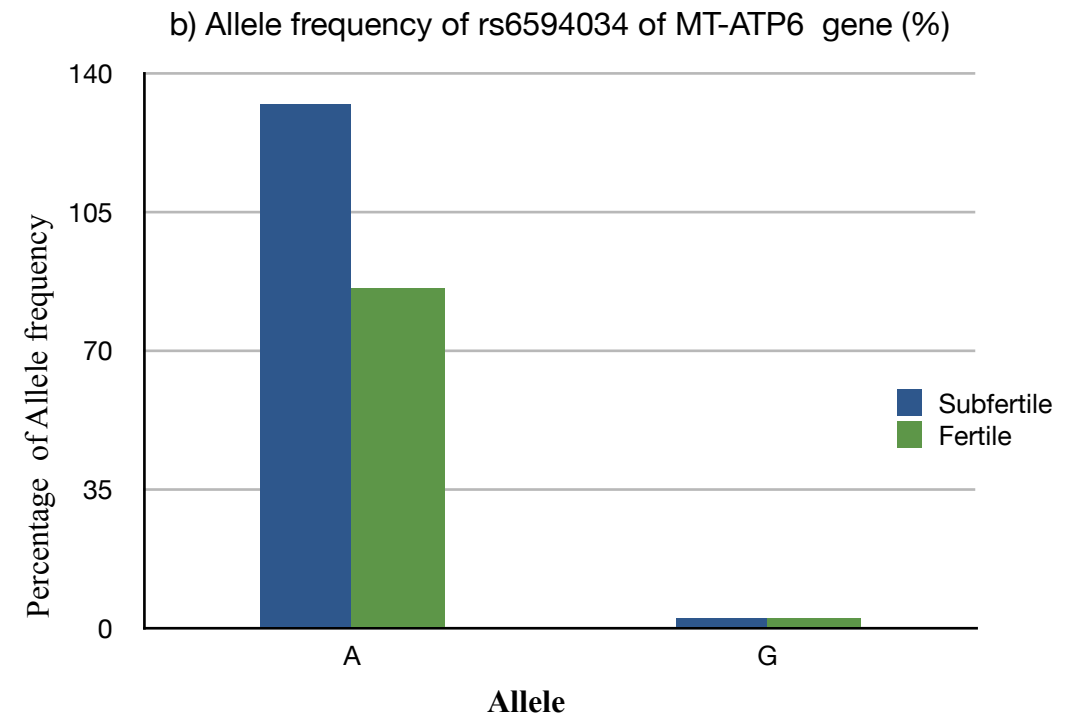


Figure 52: a) *MT-ATP6* gene allele frequency for rs6594033, **b)** *MT-ATP6* gene genotype frequency for rs6594033.



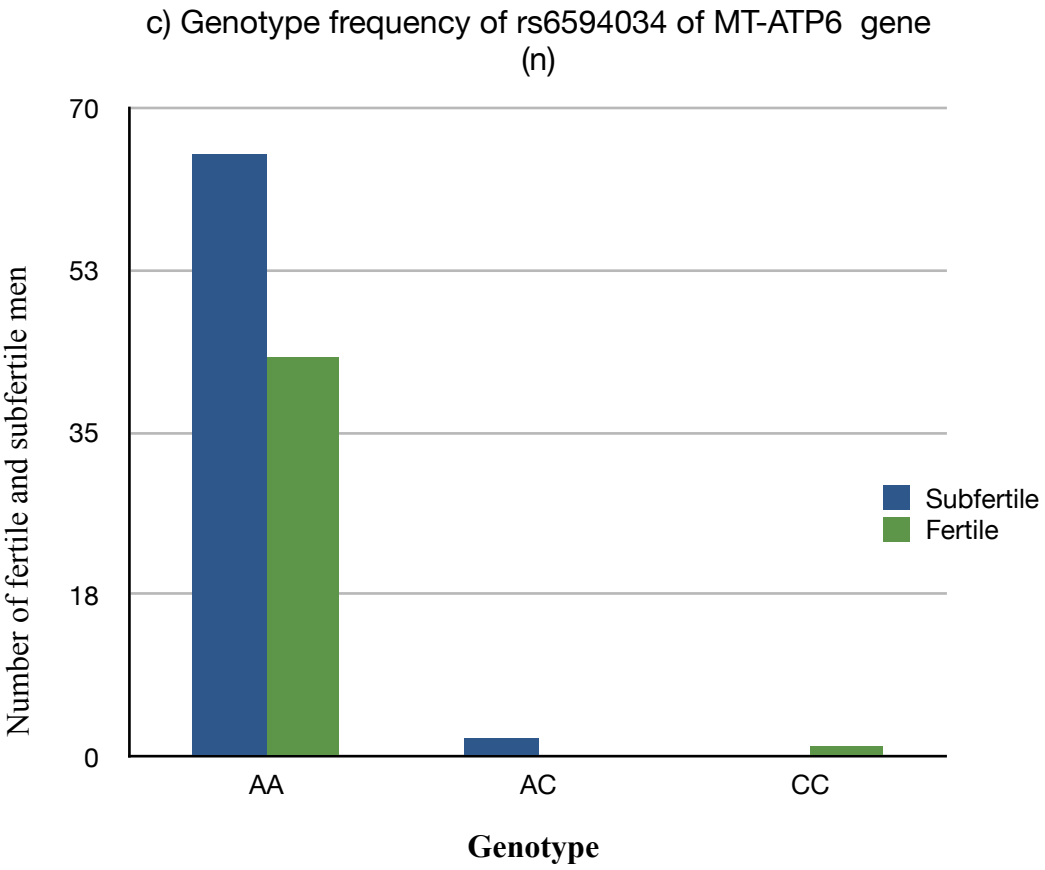


Figure 53: a) *MT-ATP6* gene allele frequency for rs6594034, **b)** *MT-ATP6* gene genotype frequency for rs6594034.

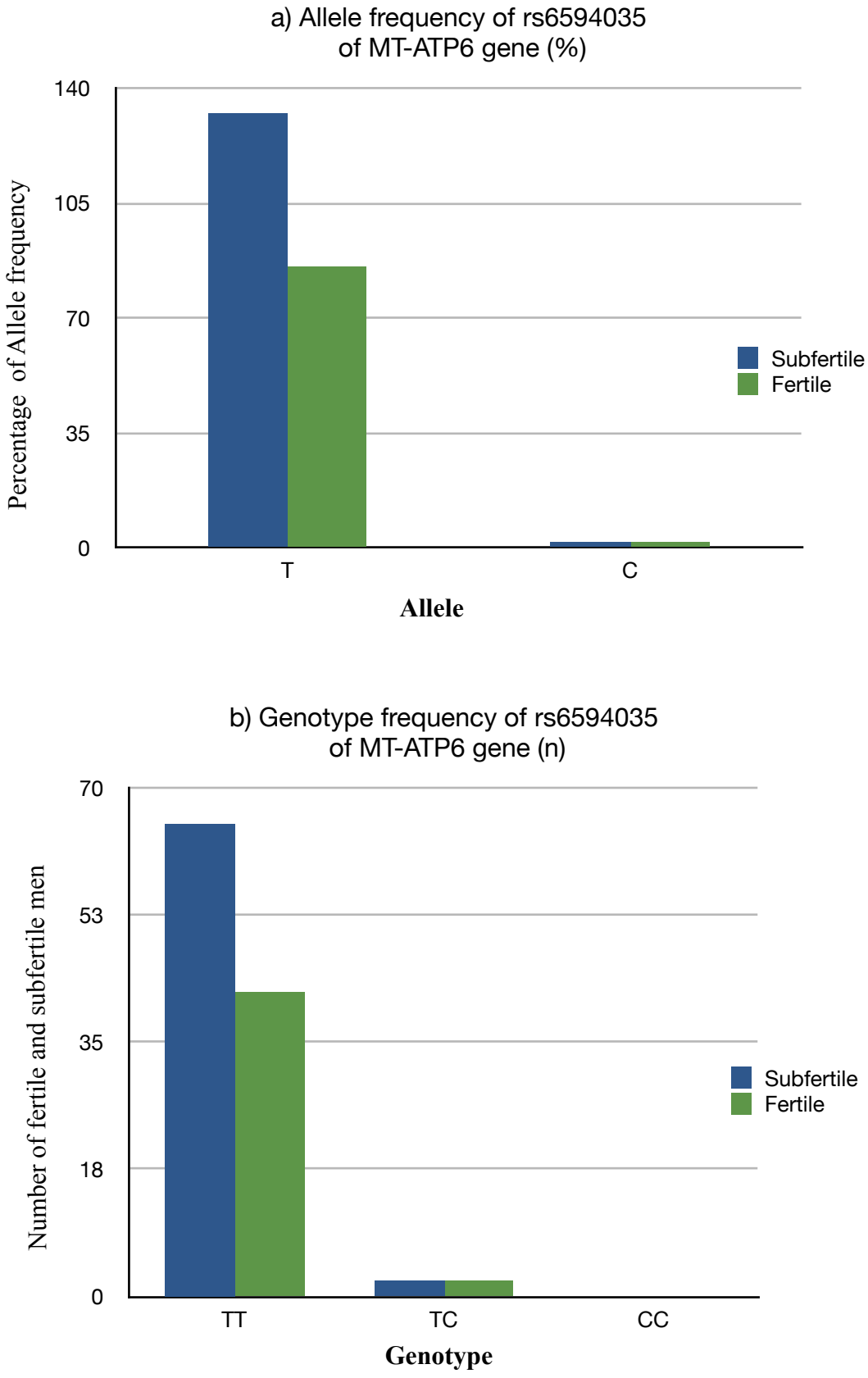


Figure 54: a) *MT-ATP6* gene allele frequency for rs6594035, **b)** *MT-ATP6* gene genotype frequency for rs6594035.

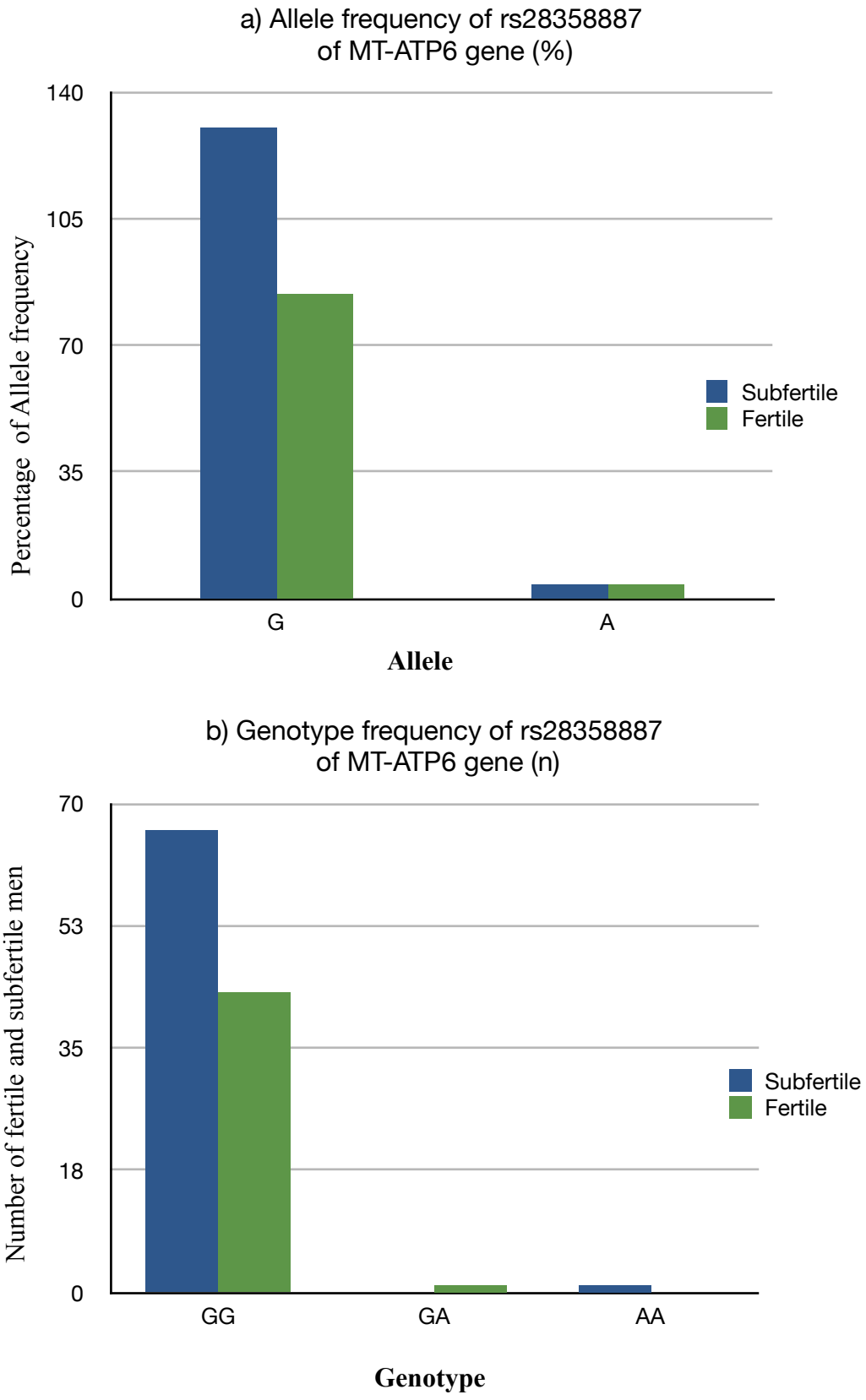


Figure 55: a) *MT-ATP6* gene allele frequency for rs28358887, **b)** *MT-ATP6* gene genotype frequency for rs28358887.

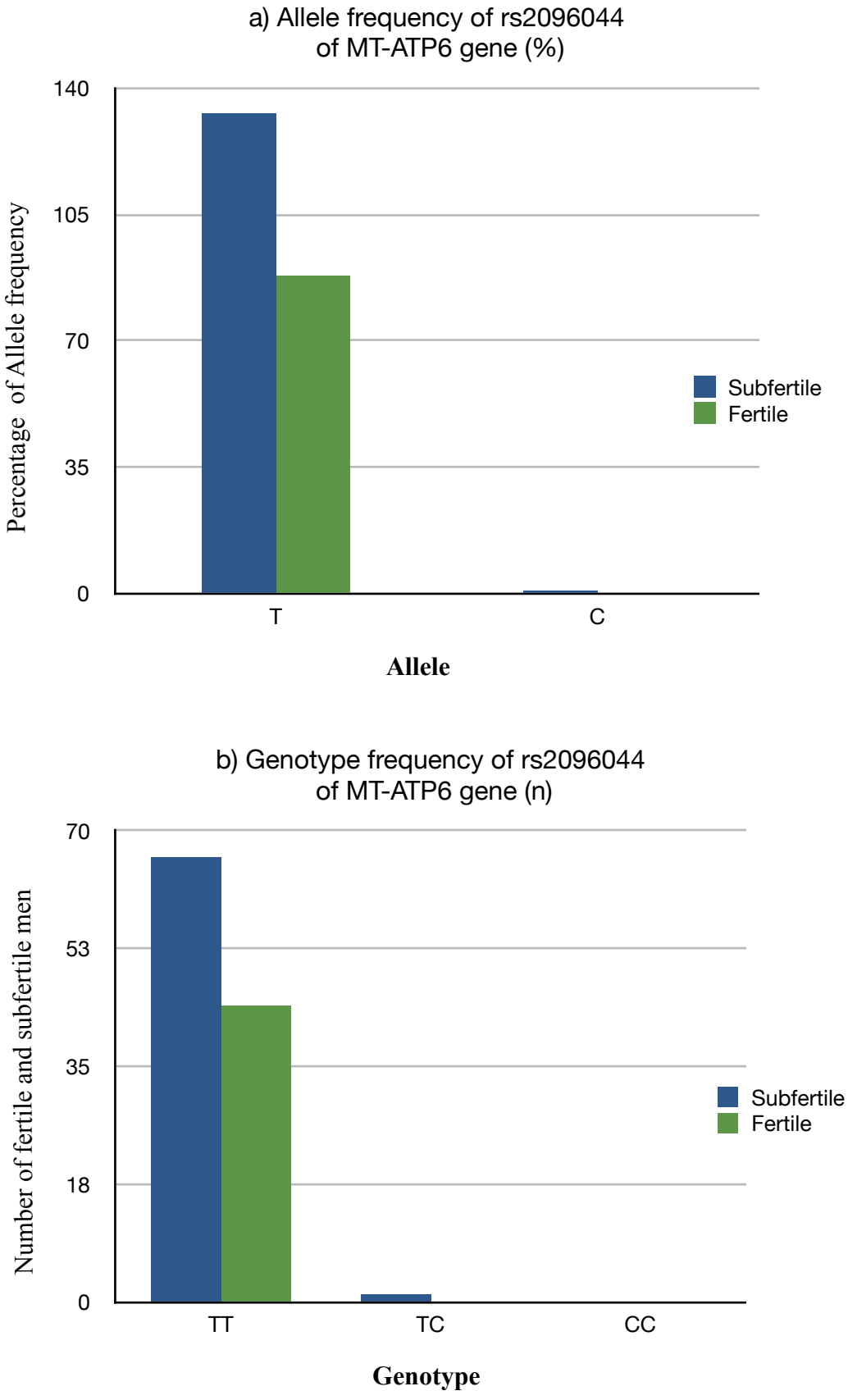
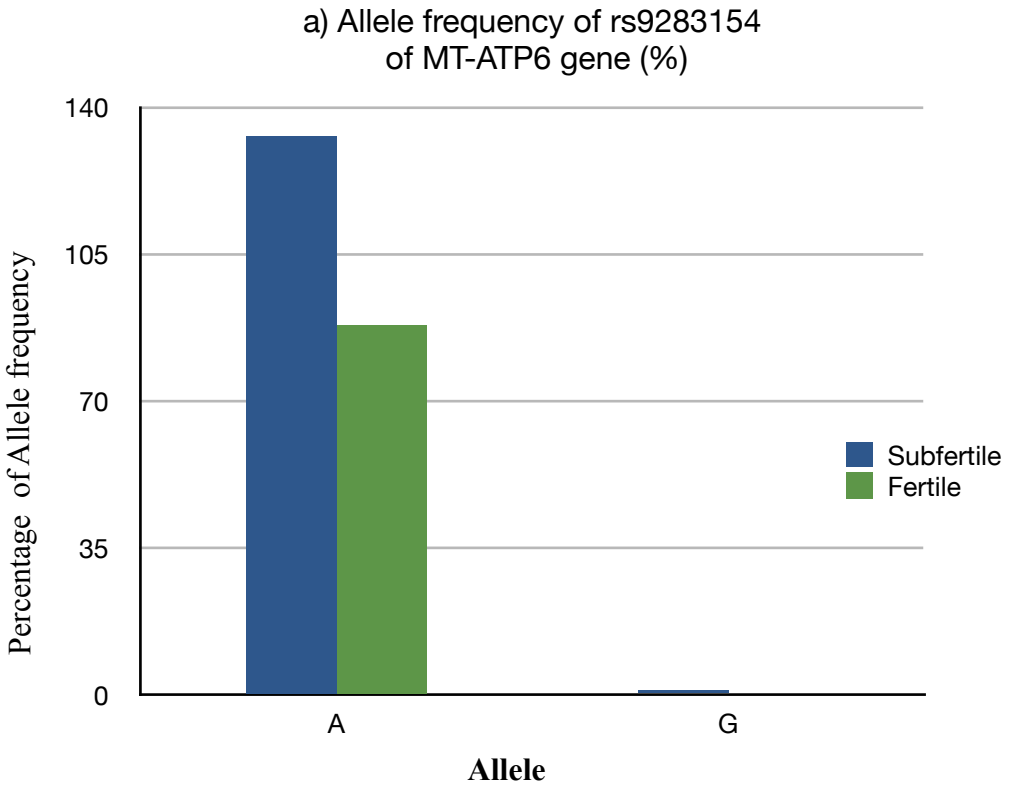


Figure 56: a) *MT-ATP6* gene allele frequency for rs2096044, **b)** *MT-ATP6* gene genotype frequency for rs2096044.



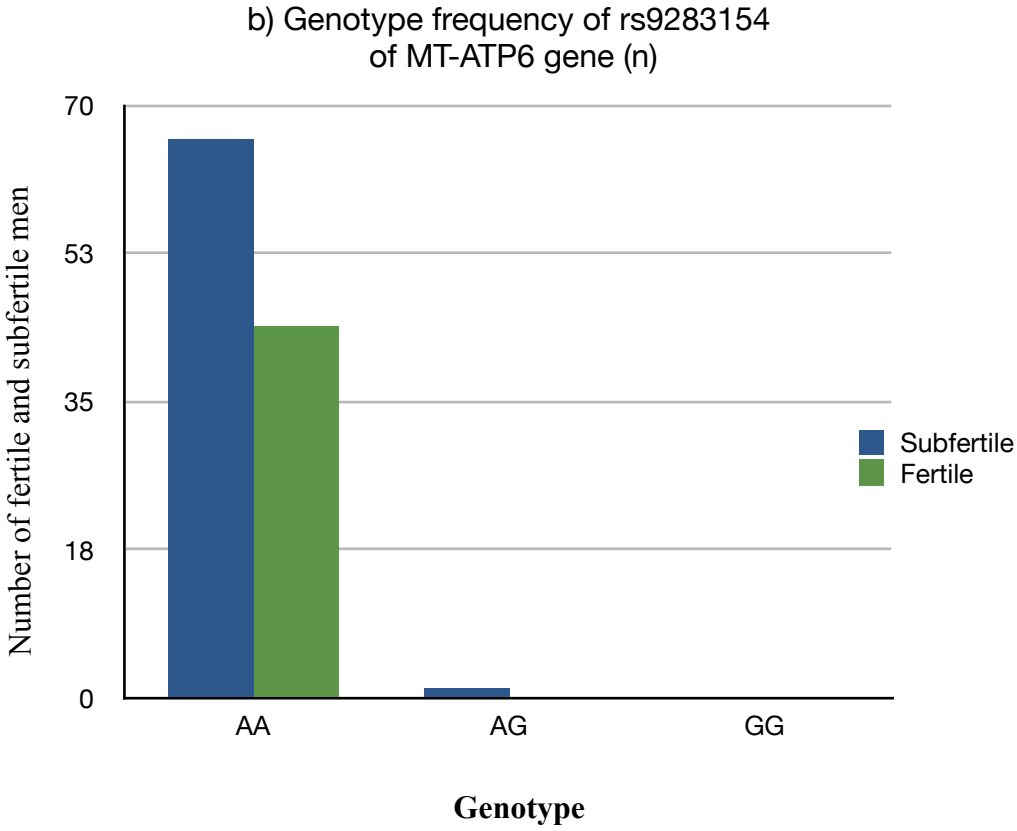


Figure 57: a) *MT-ATP6* gene allele frequency for rs9283154, **b)** *MT-ATP6* gene genotype frequency for rs9283154.

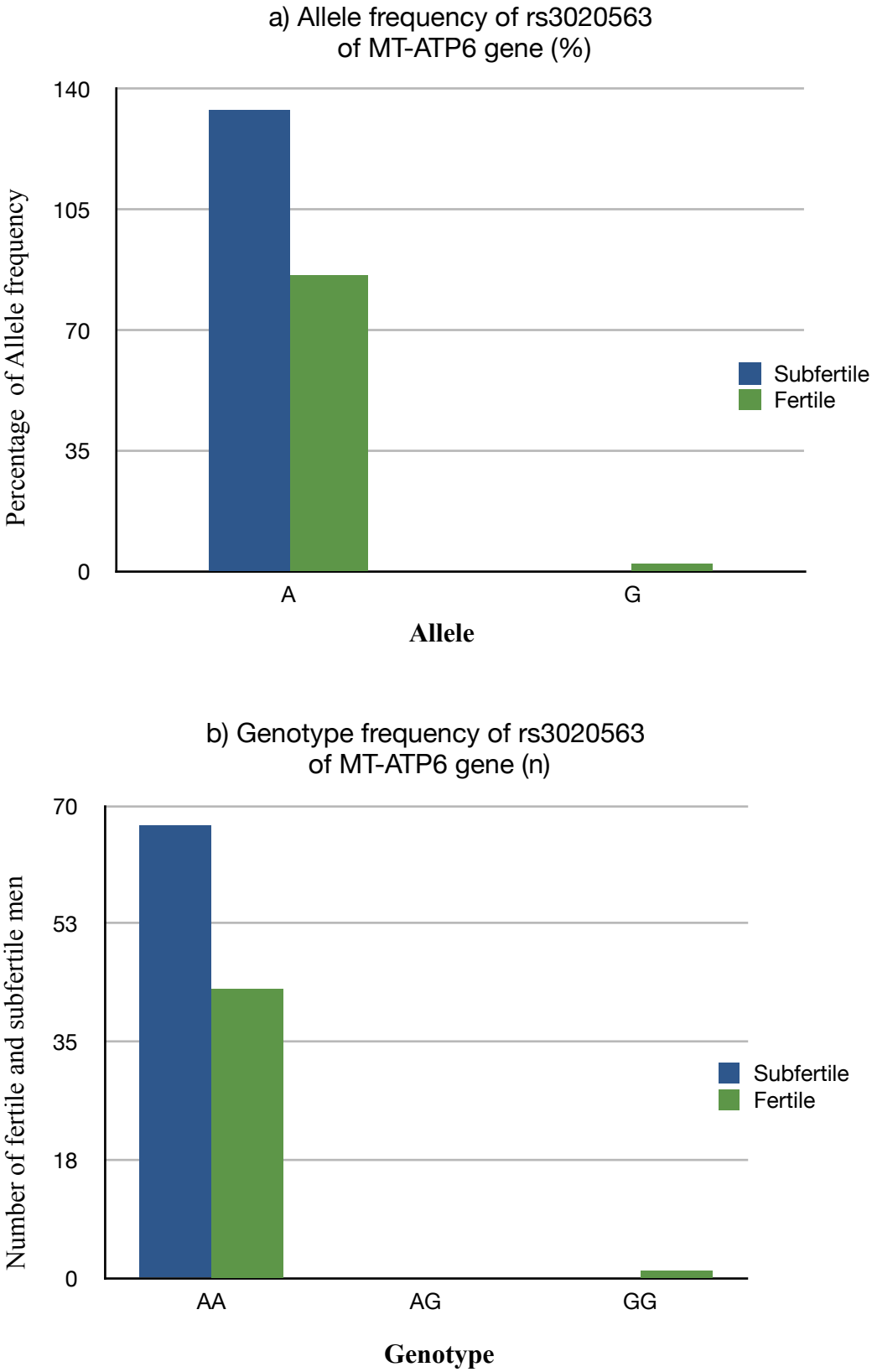


Figure 58: a) *MT-ATP6* gene allele frequency for rs3020563, **b)** *MT-ATP6* gene genotype frequency for rs3020563.

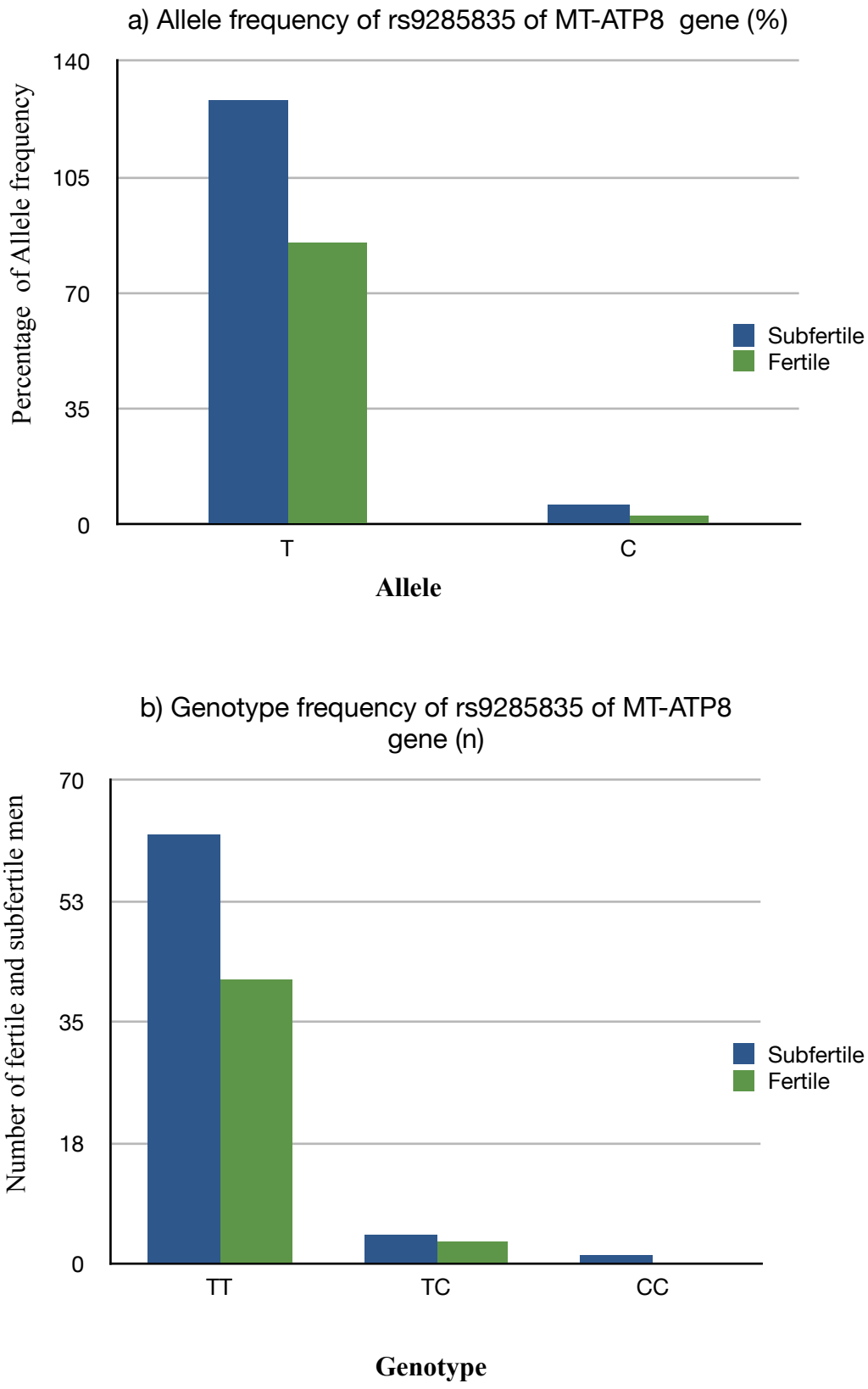
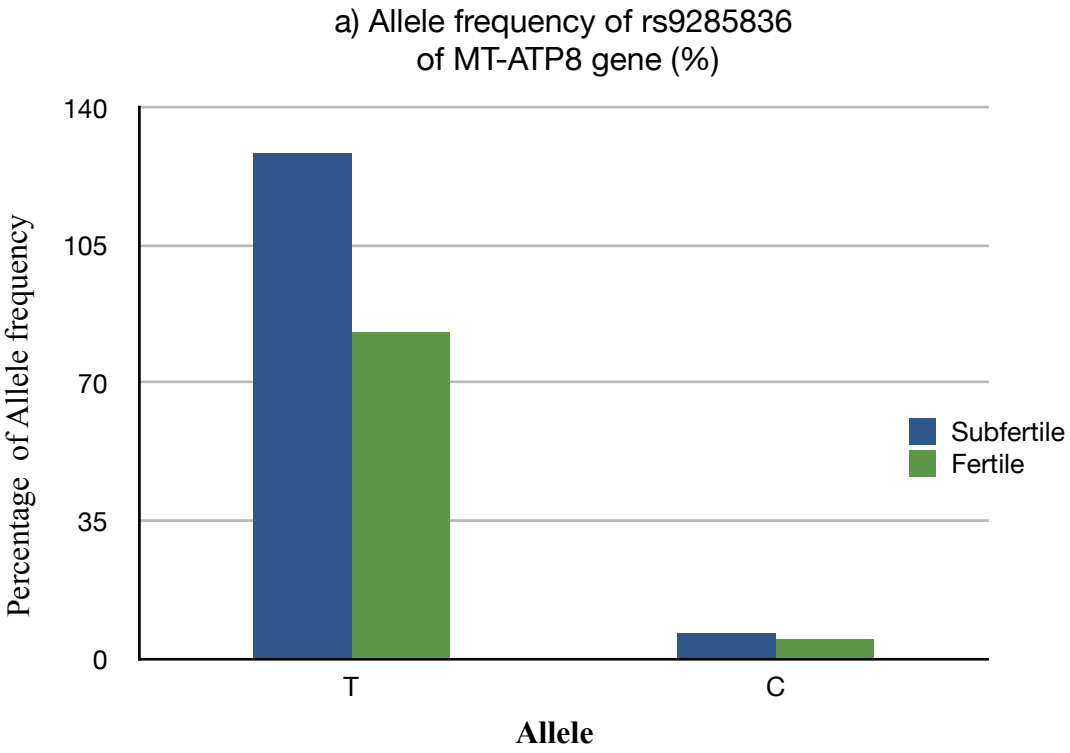


Figure 59: a) *MT-ATP8* gene allele frequency for rs9285835, **b)** *MT-ATP8* gene genotype frequency for rs9285835.



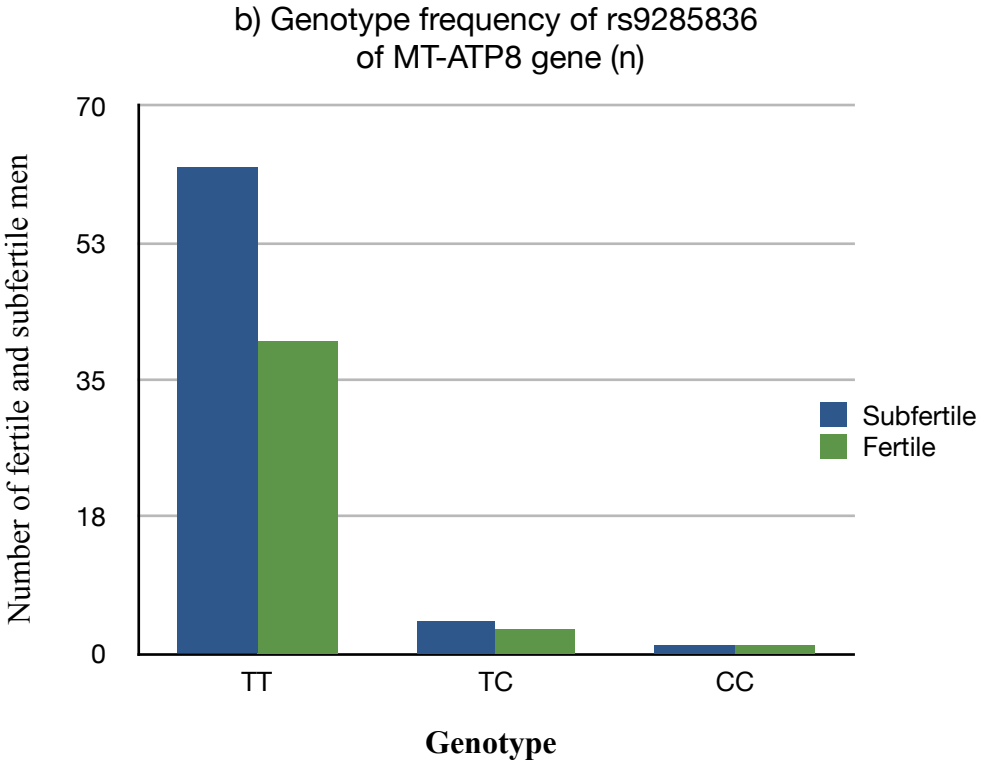
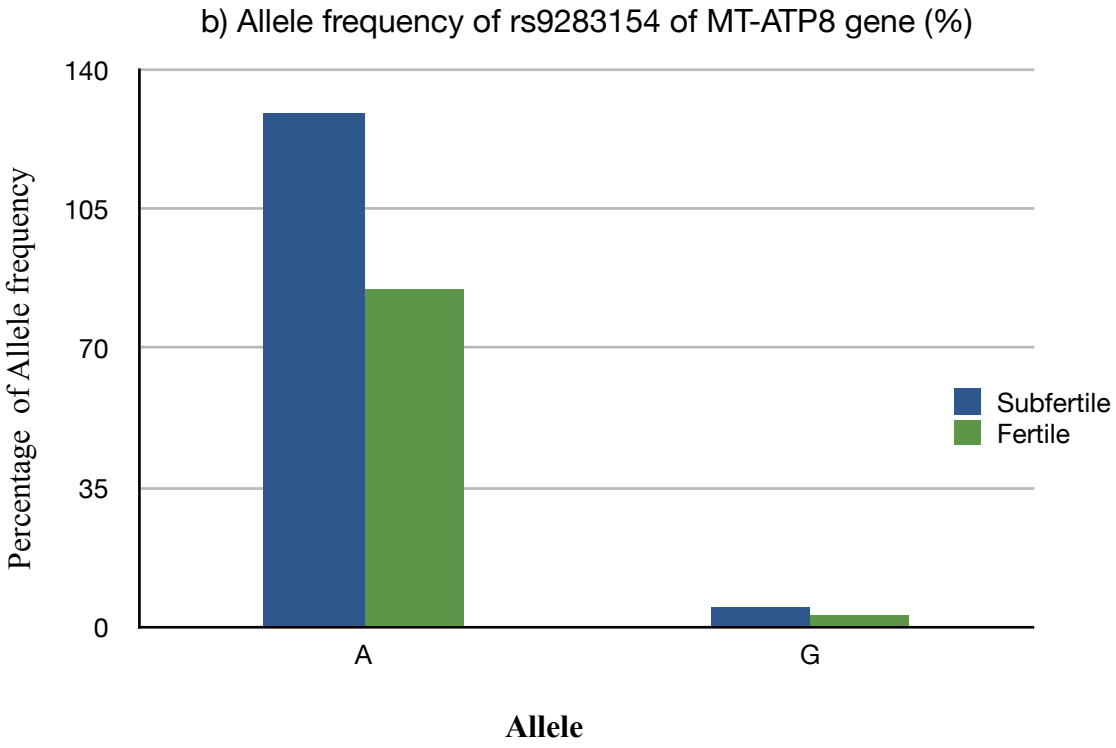


Figure 60: a) *MT-ATP8* gene allele frequency for rs9285836, **b)** *MT-ATP8* gene genotype frequency for rs9285836.



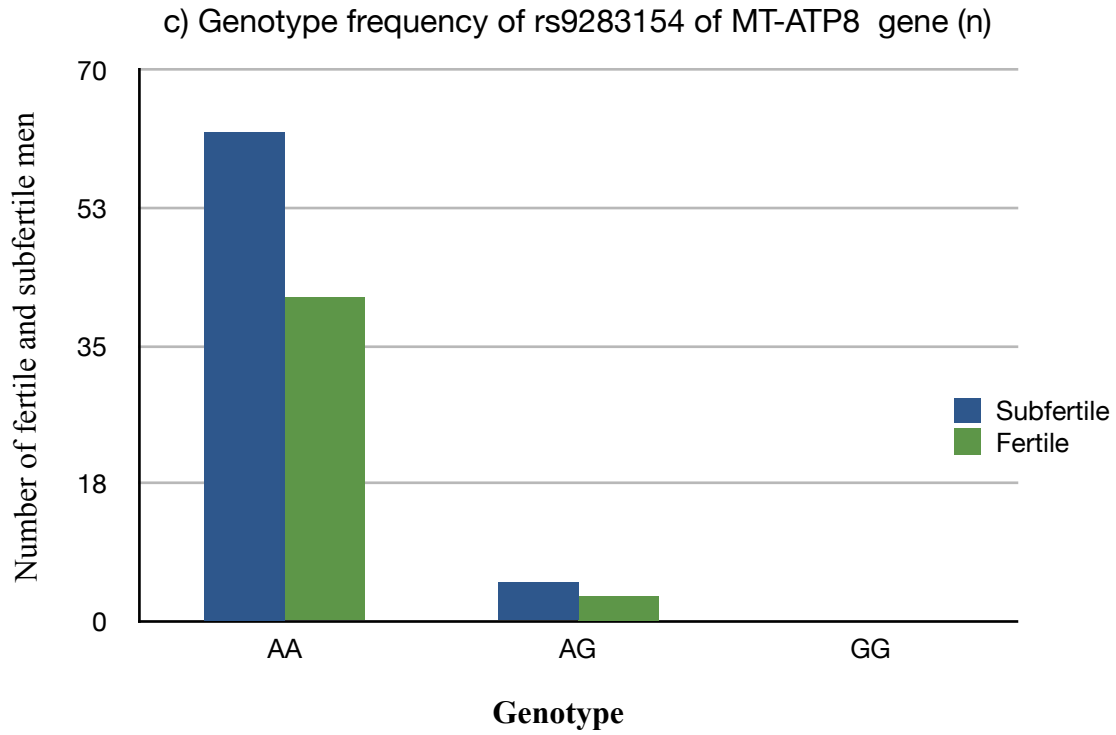


Figure 61: a) *MT-ATP8* gene allele frequency for rs9283154, **b)** *MT-ATP8* gene genotype frequency for rs9283154.

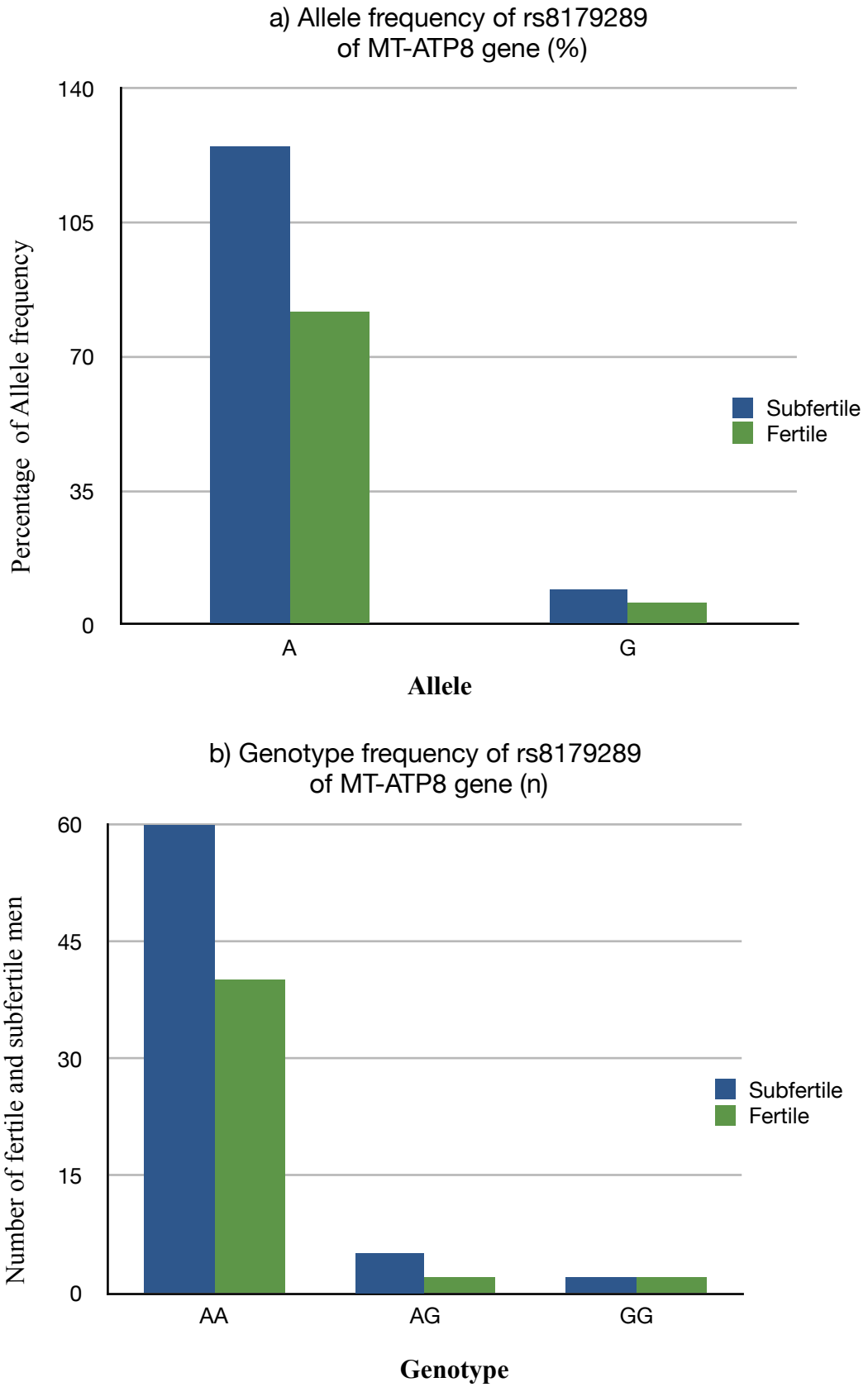
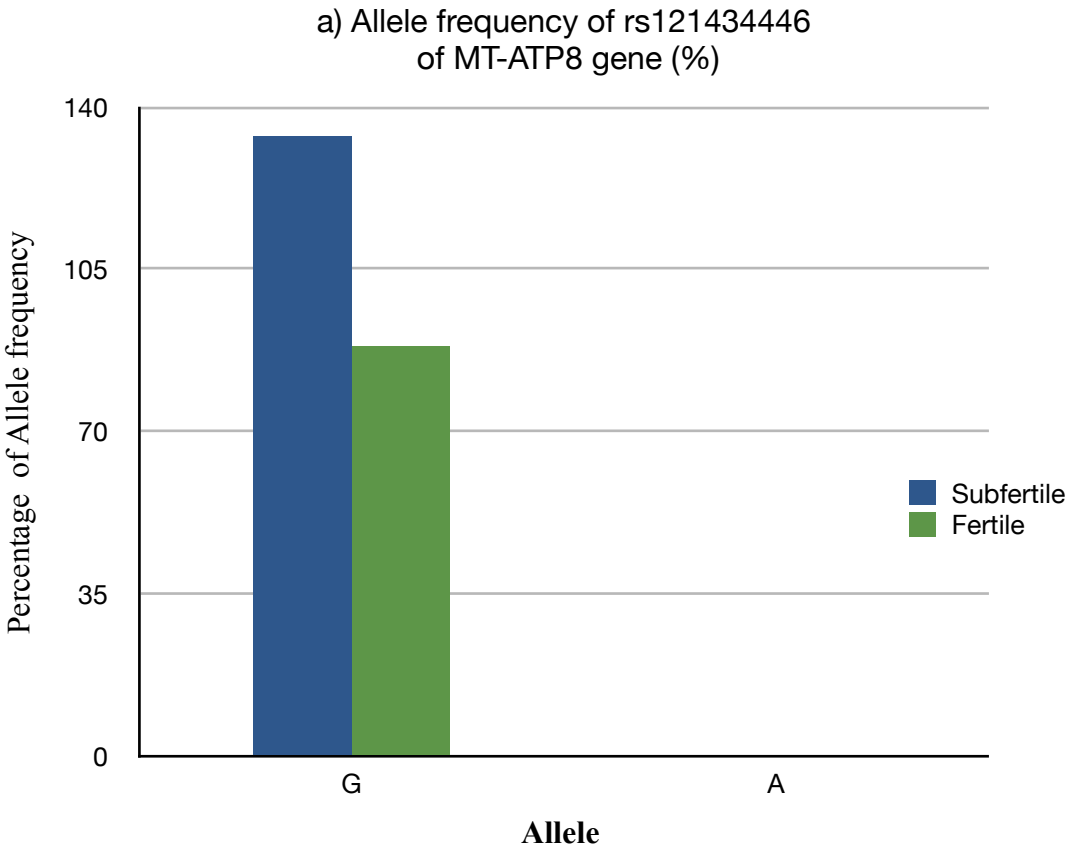


Figure 62: a) *MT-ATP8* gene allele frequency for rs8179289, **b)** *MT-ATP8* gene genotype frequency for rs8179289.



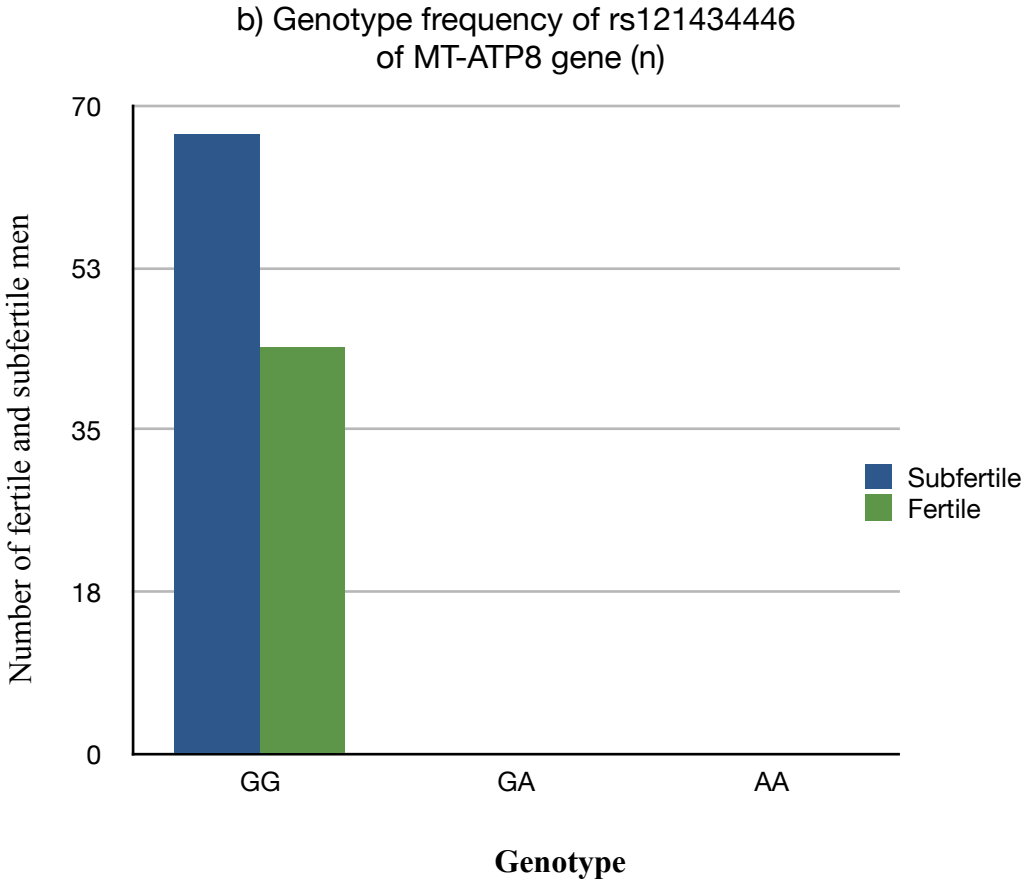


Figure 63: a) *MT-ATP8* gene allele frequency for rs121434446, **b)** *MT-ATP8* gene genotype frequency for rs121434446.

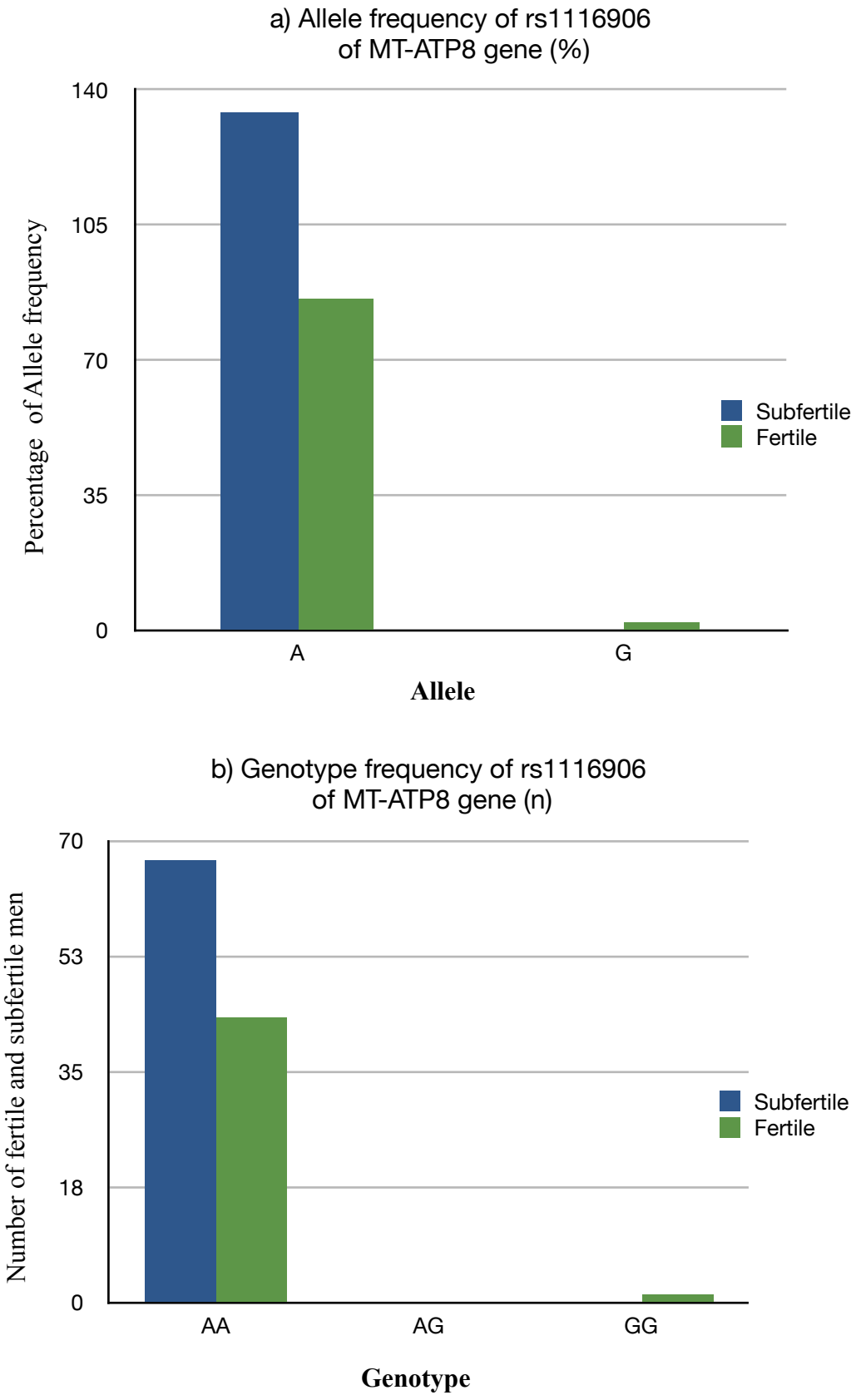


Figure 64: a) *MT-ATP8* gene allele frequency for rs1116906, **b)** *MT-ATP8* gene genotype frequency for rs1116906.

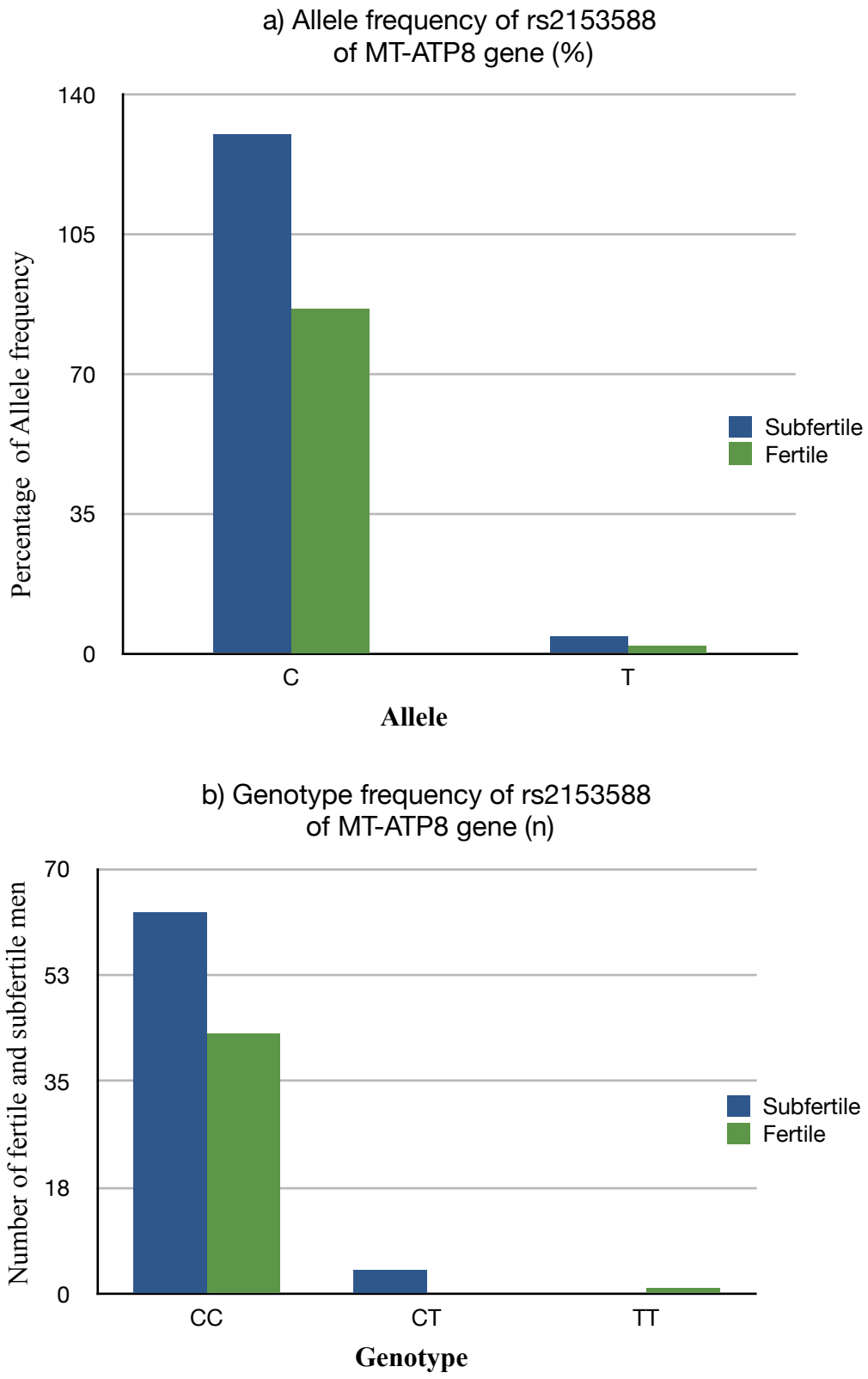


Figure 65: a) *MT-ATP8* gene allele frequency for rs2153588, **b)** *MT-ATP8* gene genotype frequency for rs2153588.

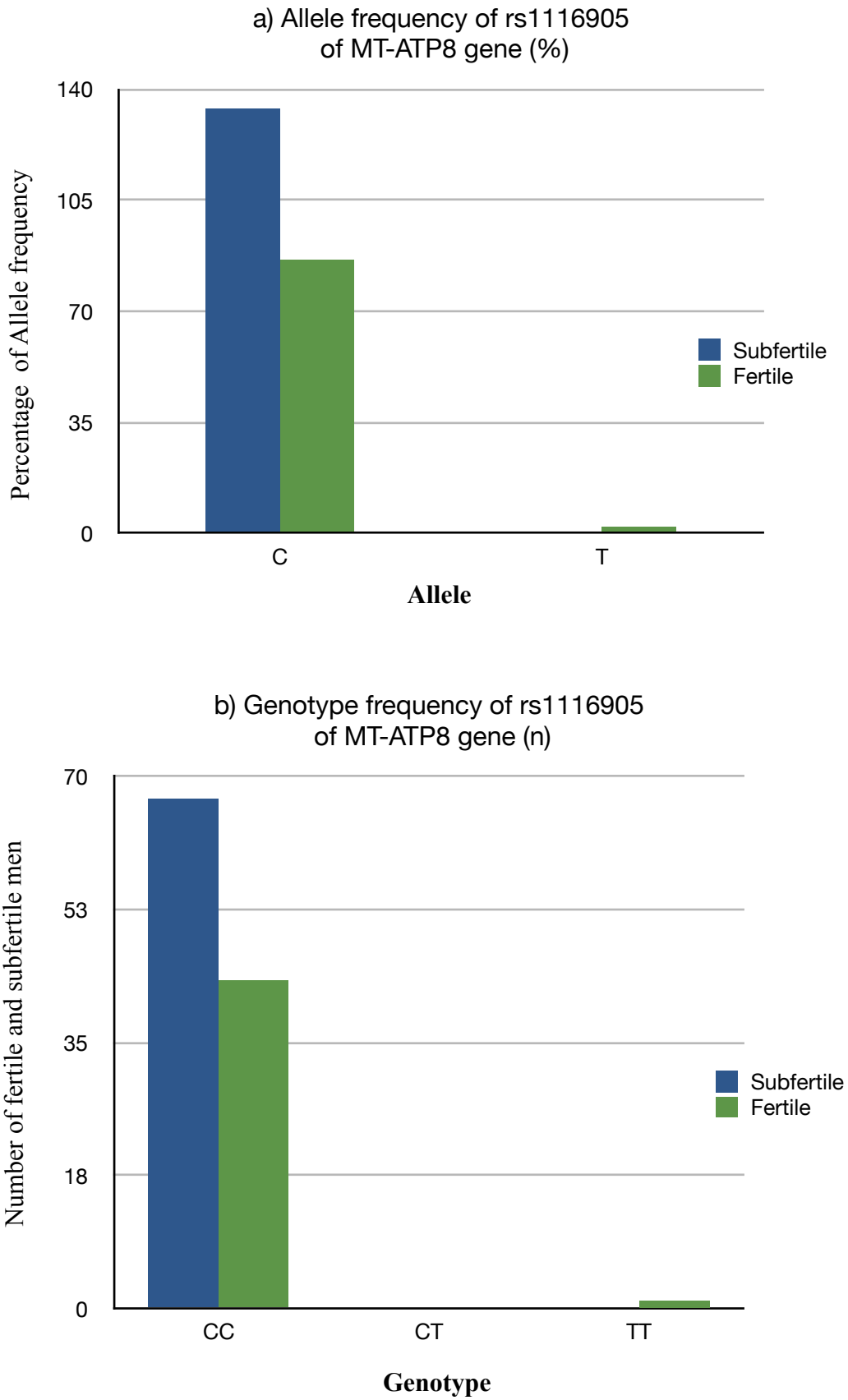


Figure 66: a) *MT-ATP8* gene allele frequency for rs1116905, **b)** *MT-ATP8* gene genotype frequency for rs1116905.

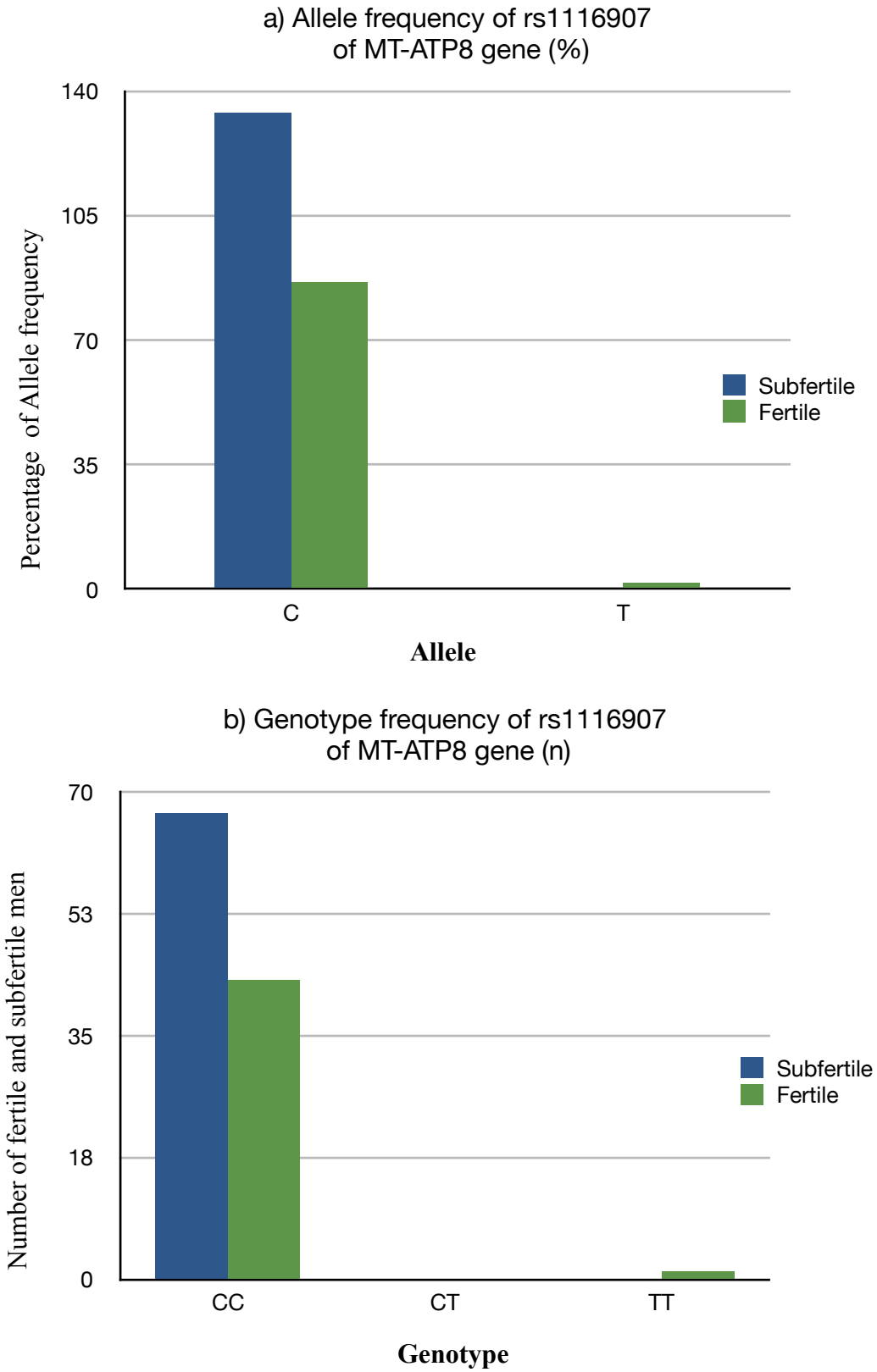


Figure 67: a) *MT-ATP8* gene allele frequency for rs1116907, **b)** *MT-ATP8* gene genotype frequency for rs1116907.

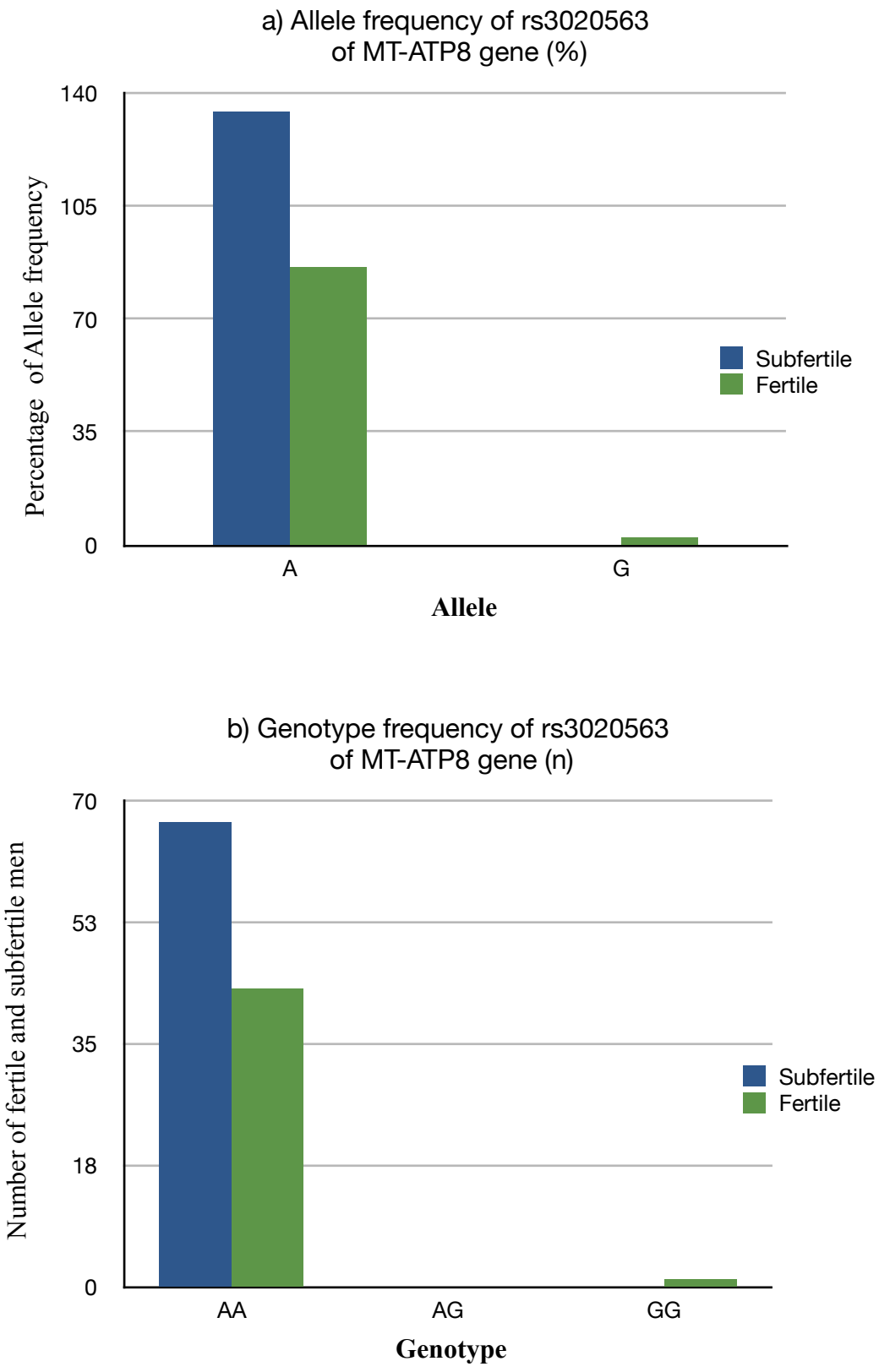


Figure 68: a) *MT-ATP8* gene allele frequency for rs1116907, **b)** *MT-ATP8* gene genotype frequency for rs1116907.

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