**Deanship of Graduate Studies**
**Al-Quds University**

# Screening of mutations in Polynucleotide Kinase 3' - Phosphatase gene causing Microcephaly, Seizures, and Developmental Delay in Palestine

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**M.Sc. Thesis**

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## Thesis Approval

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**Jerusalem – Palestine**

**1445 – 2024**

## Dedication

This work is dedicated to my father and mother, who have been my inspiration and source of support during the process of this work, to my sisters and brothers, to my friends and knowledge seekers.
I dedicate my thesis to the experiences I never expected and the paths that were redirected.

Maysa "Mohammad Amer" Kamal Natsheh

## Declaration

I certify that this thesis submitted for the degree of master's is the result of our research; the content of the thesis is the result of work that has been carried out since the date of approval of the research program. All ethics procedures and guidelines have been appropriately followed while preparing the thesis.

**Signature:**

**Maysa "Mohammad Amer" Kamal Natsheh**

Date: 18- Jan-2025

## Acknowledgments

At the beginning, I would like to thank God for granting me the determination and strength to complete this humble work.

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All the patients who have given their time and effort to enable me to examine them, and consented to their approval to be part of this study.

And to all my professors and teachers throughout my studies, Thank You!

## Abstract

**Background:** Microcephaly, Seizures, and Developmental Delay (MCSZ) is an uncommon genetic disorder with an undetermined prevalence. It is inherited in an autosomal recessive pattern associated with either a homozygous or compound heterozygous mutation in the PNKP gene. This gene plays a crucial role in various DNA repair mechanisms, and its mutation leads to the continuous activation of the DNA damage response. This persistent activation is a consequence of the accumulation of double-strand breaks within the affected cells, which may result in the death of sensitive neuronal cells, potentially contributing to the pathogenesis of MCSZ. The disorder is primarily characterized by microcephaly, or an unusually small head size, along with a range of neurological impairments.

**Purpose:** This research aims to screen PNKP mutations in several Microcephaly, Seizure and Developmental Delay (MCSZ) disorder patients from Palestine.

**Methods:** Three patients from different families were studied, who fulfilled our inclusion criteria: (1) Patients (males and females of different ages) having the general characteristics of MCSZ. (2) Their parents, especially if they are consanguineous in marriage determine if they are carriers for PNKP mutation, and/or family members. Genomic DNA was extracted after obtaining a blood samples which drawn for PNKP gene detection using PCR and Sanger sequencing. PNKP mutations were screened and targeted the most common published exons (Exon 11, 14 and 15).

**Results:** In this research, we identified a female patient exhibiting microcephaly, significant developmental delays, and early-onset refractory seizures, attributed to a homozygous mutation in the PNKP gene. This mutation, classified as likely pathogenic, is a missense variant denoted as NM_007254.4: c.968 C > T: p. (Thr323Met),located in exon 11 of the PNKP genes. The screening of first-degree relatives revealed that this genetic variant was inherited from both the father and mother who were identified as heterozygous for the MCSZ variant (GA). A pedigree was constructed following the screening of the affected individuals' first-degree relatives. In contrast, samples from the other two patients did not exhibit any mutations in the PNKP gene. These samples were subsequently subjected to Whole Exome Sequencing, which confirmed the absence of mutations in the PNKP gene for both patients.

**Conclusion:** Microcephaly, Seizures, and Developmental Delay (MCSZ) is underdiagnosed and undertreated in our population. Even simple knowledge among families toward consanguineous marriages, genetic counseling and cascade screening are essentialfor the diagnosis and prevention of MCSZ early in life. The results in this research will be considered as preliminary results on that disease among our population and more cohorts studiesand advanced genetic analysis are still needed.



**Keywords:** Microcephaly, Seizures, and Developmental Delay, PNKP, genetic counseling.

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## Abbreviation

Abbreviation Term

MCSZ: Microcephaly, Seizures, and Developmental Delay

PNKP: Polynucleotide Kinase 3' – Phosphatase

p. (Thr323Met): Threonine 323 Methionine

G: Guanine

A: Adenine

C: Cytosine

T: Thymine

DNA: Deoxyribonucleic acid

SSBs: Single-Strand DNA Breaks

DSBs: Double-Strand DNA Breaks

DPC: DNA-Protein Crosslink

KD: Kilo Dalton

AOA4: Ataxia with oculomotor apraxia 4

PNK: Polynucleotide Kinase

FHA: Fork Head-Associated

OH: Hydroxide

BER: Base excision repair

Glu326Lys: Glutamic acid 326 Lysine

Thr424Gly: Threonine 424 Glycine

Arg 462Pro: Arginine 462 Prolin

Dup: Duplication

Del: Deletion

DDR: DNA damage response

APE1: Apurinic/apyrimidinic endonuclease 1

PARP1: poly(ADP-ribose) polymerase 1

NAD+: Oxidised form of Nicotinamide adenine dinucleotide

ADP: Adenosine diphosphate

DSBR: Double-strand break repair

HR: Homologous Recombination

NHEJ: Nonhomologous end-joining

NER: Nucleotide excision repair

MMR: Mismatch repair enzymes

ROS: Reactive oxygen species

mtDNA: Mitochondrial DNA

Kbp: Kilo base pair

tRNA: Transfer ribonucleic acid

rRNA: Ribosomal ribonucleic acid

TDP1: Tyrosyl-DNA phosphodiesterase 1

CK2: Casein kinase 2

XRCC1: X-ray repair cross-complementing 1

XRCC4: X-ray repair cross-complementing 4

p. G375W: P. Glycine 375 Tryptophan

p. T408del: P.Threonine 408 deletion

p. R439fs: p.Arginine 439 frameshift

p. T442fs: P.Threonine 442 frameshift

p. Thr424Glyfs49: p.Threonine 424Glycine frameshift 49

p. Tyr515: p.Tyrosine 515

p. L176F: p.Leucine176 Phenylalanine

p. E326K: p.Glutamic acid 326 Lysine

p. G292R/p. A55S: p.Glycine 292 Arginine/ p. Alanine 55 Serine

p. T424GfsX48: p.Threonine 424 Glycine frameshift X48

p. P20S, p. A55S: p.Proline 20Serine, p.Alanine 55 Serine

OMIM#: Online Mendelian Inheritance in Man number

DEE: Developmental and epileptic encephalopathy

COA: Coenzyme A

EDTA: Ethylenediamine tetra acetic acid

USA: The United States of America

NCBI: National Center for Biotechnology Information

SNPs: Single nucleotide polymorphisms

PCR: Polymerase Chain Reaction

TAE: Tris-Acetate-EDTA

ABI: Applied Biosystems

BLAST: Basic Local Alignment Search

LBW: Low Birth Weight at trem

IUGR: Intrauterine Growth Restriction

HC: Head Circumference

EEG: Electroencephalogram

IVH: Intraventricular hemorrhage

GSSW: Generalized slow spike-wave

BMI: Body Mass Index

MV: Mechanical Ventilation

Chr19: Chromosome 19

SIFT: Scale invariant feature transform

PROVEAN: Protein Variation Effect Analyzer

PolyPhen-2: Polymorphism Phenotyping v2

LRT: Likelihood ratio test

CONDEL: CONsensusDELeteriousness score

MetaSVM: Meta-analytic support vector

qPCR: quantitative polymerase chain reaction

RNAi: RNA interference

cDNA: Complementary DNA

# Chapter One

# Introduction

## 1.1 Background

DNA genetic information plays a crucial role in every living cell, and its integrity and stability are crucial for sustaining life. Nevertheless, as a chemical structure, it is vulnerable to environmental assaults. If any damage occurs and remains undiagnosed and addressed it can result in mutations that may contribute to the development of diseases (Clancy et al.,2008).

The maintenance of DNA integrity is a crucial process for all cell types, especially for neurons, which are particularly vulnerable to mutations that can alter DNA repair mechanisms (Hayman et al ., 2021). DNA damage process is a continual risk to cells, arising either spontaneously during DNA replication which is highly accurate due to proofreading and repair mechanisms, it is not 100% safe because of enzyme limitations, environmental damage, and the need to balance speed with accuracy. This small error rate is an inevitable trade-off in biology systems (TM, B et al ., 2019) .Defects in transcription can lead to DNA damage through the formation of R-loops, transcription-replication conflicts, and impaired DNA damage response (DDR)(Gaillard H et al ., 2016), or internally through ROS generated during oxidative metabolism (Poetsch et al ., 2020 & Timmins et al ., 2023). Cells rely on an integrated system of antioxidants, DNA repair mechanisms, and adaptive stress responses to minimize and repair DNA damage caused by ROS, a ensuring genomic stability and cellular health (Evans MD et al ., 2004).Several forms of DNA

damage exist, such as single-strand DNA breaks (SSBs). Double-strand DNA breaks (DSBs), DNA-protein crosslinks (DPCs), bulky adducts, and base mismatches (mutation) (Moon et al ., 2023).

The Polynucleotide kinase3'-phosphatase protein (PNKP) positioned on chromosome 19q13.3-q13.4 as shown in (Fig. 1.1) has a 57.1-kD molecular mass, contains 17 exons and provides instructions for creating the polynucleotide kinase-phosphatase (PNKP) enzyme (Jilani et al ., 1999; Poulton et al ., 2013 & Islam et al ., 2024).

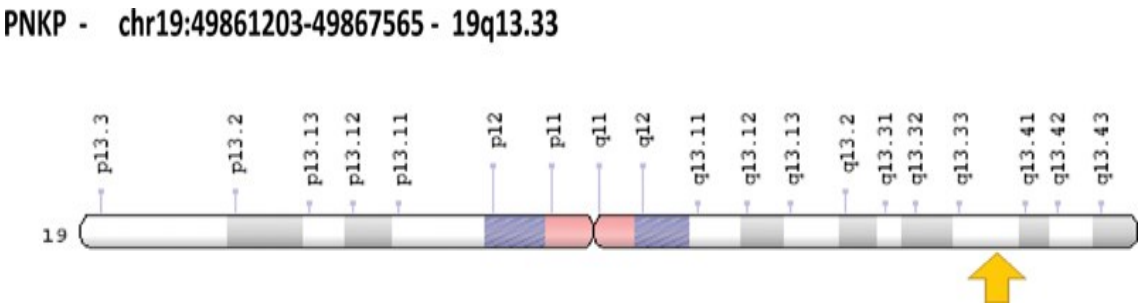


**Figure 1.1** An illustration of chromosome 19 highlighting the position of the PNKP gene, as adapted from (Marcilla Vázquez et al ., 2021).

The yellow arrow denotes the specific location of the PNKP gene on chromosome 19, situated between q13.33 and q13.41.

PNKP gene has other designations: AOA4, bi-functional polynucleotide phosphatase/kinase, Homo sapiens polynucleotide kinase 3'-phosphatase (PNKP), DNA 5'-kinase/3'-phosphatase, MCSZ, EIEE10 and PNK as available in a database reference (medchemexpress, n.d)

Polynucleotides kinase 3' phosphatase is an enzyme characterized by its bifunctional role in DNA end processing. It features a C-terminal catalytic domain that houses a fused bimodal phosphatase and kinase domain, while its N-terminus is equipped with a Fork Head -Associated (FHA) domain (Dumitrache et al ., 2017 & Islam et al .,2024). The enzyme's 3' phosphatase and 5' kinase activities facilitate the generation of the essential 3' hydroxyl and 5' phosphate ends, which are crucial for the gap filling and ligation processes necessary for the repair of single-strand breaks (SSB) in DNA (Chatterjee et al ., 2015).

The PNKP gene plays a crucial role in various DNA repair mechanisms, including base excision repair (BER) for single-strand breaks and double-strand break repair through the nonhomologous end-joining pathway (Shimada et al ., 2015). Consequently, the lack of this gene within the nucleus results in the continuous activation of the DNA damage response, stemming from the ongoing accumulation of double-strand breaks in mutant cells. This phenomenon factor in neurodegeneration (Islam et al ., 2023).

Microcephaly, seizures, and developmental delay (MCSZ) is a medical condition characterized by an unusually small head size (microcephaly) and neurological issues associated with impaired brain development occurring prior to birth (Reynolds et al ., 2012 ). Individuals with MCZS are distinguished by the occurrence of recurrent seizures (epilepsy) that typically commence in infancy, along with a delay in the development of motor skills, including sitting and walking. Additionally, speech development is often delayed and some individuals affected by this condition may never acquire the ability to speak. Intellectual disabilities and behavioral issues, particularly hyperactivity, are prevalent characteristics associated with MCSZ. In uncommon instances, patients with MCSZ may exhibit difficulties with balance and coordination, known as ataxia (Dumitrache & McKinnon, 2017; J. Shen et al., 2010).

This condition is classified as a rare disorder, with its prevalence remaining undetermined. It is inherited in an autosomal recessive pattern, associated with either a homozygous or compound heterozygous mutation affecting three domains of the PNKP gene (Bitarafan et al ., 2021 & Kalasova et al ., 2019). Diagnosed by symptom appearances, laboratory abnormalities in genetics testing display that patient cells exhibit impaired DNA repair mechanisms when subjected to irradiation and damage caused by free radicals and neuroimaging abnormalities in children appear as microcephaly (Aggarwal et al ., 2013). MCSZ patients are managed by physical Therapy which is often helpful for children with delays in gross motor skills, occupational Therapy can address fine motor skills, sensory processing and self-help issues, speech and language therapy is typically used to address problems in the areas of understanding and producing language and speech sounds, early childhood special education provides stimulation for early developmental skills, including play skills and behavioral therapy needed in some children for behavioral difficulties that affect socially appropriate behaviors (Liverpool John Moor University, ND).

By searching the PubMed utilizing the term "PNKP" revealed 38 unique PNKP variants across 21 publications. The pathogenic impact was predominantly associated with mutations occurring in exons 11 to 15, particularly exon 14 (Shen et al ., 2010; Poultan et al ., 2013 & Nakashima et al ., 2014). Table(1.1) provides a concise overview of the cases identified in the literature pertaining to the Arab world, which involve mutations in the PNKP gene, alongside a phenotype characterized by microcephaly, seizures, and developmental delay(Jiang et al., 2022; Nair et al., 2016; J. Shen et al., 2010).

**Table1.1 :**Overview of the cases identified in the bibliographic research within the Arab world, featuring mutations in the PNKP gene associated with a phenotype characterized by microcephaly, seizures, and developmental delay.

| **Article Ref.** | **Patient** | **Consanguinity** | **Mutation** | **Exon** | ***PNKP* Mutation** | | |
|---|---|---|---|---|---|---|---|
| | | | | | **DNA change** | **Amino acid change** | **Domains** |
| (Shen et al ., 2010) | X3 Palestinian (homozygous) | Yes | Substitution | 11 | c.975 G > A | Glu326Lys | Phosphatase |
| (Shen et al ., 2010) | X1Saudi Arabia (homozygous) | No | Duplication | 14 | c.1250_1266dup | Thr424Gly | Kinase |
| (Bitarafan et al ., 2021) | X1Iranian Child (Two heterozygous) | Not available | Frameshift | 14 | c.1298 + 33_129924del<br>c.1253_1269dup | Thr424Gly | Kinase |
| ( Nair et al ., 2016) | X1 Emirati (homozygous) | Yes | Missense | 15 | c.1385G > C | Arg462Pro | Kinase |

## 1.2 Problem Statement

In Palestine, MCSZ is an obscure and insufficiently acknowledged condition, marked by its rare and uncertain prevalence, various pathogenic variants of the PNKP gene, absence of comprehensive screening, restricted access to specialized medical care, and a general lack of awareness. These factors collectively lead to underdiagnosis and inadequate management of this genetic disorder. MCSZ is characterized by microcephaly, or an unusually small head size, along with neurological issues stemming from disrupted brain development during gestation. Consequently, early diagnosis and intervention may significantly improve the prognosis for individuals affected by MCSZ.

This problem statement emphasizes the necessity for better screening and diagnostic methods, increased access to suitable medical care, and heightened awareness among both the public and healthcare providers. These measures are essential to effectively manage this genetic condition and mitigate its related neurological risks within the Palestinian population.

Few studies have been carried out on MCSZ and PNKP mutation in Palestine. Studying the candidate gene (PNKP) that might be responsible for MCSZ within the Palestinian population, will allow understanding of the cause of that disease and would be considered as counseling in the Palestinian community due to the prevalence of consanguineous marriageswhich is set at a rate of ~ 40% according to the central bureau of Statistics in Palestine (PCBS, 2023), (Zawahrah et al., 2019).

## 1.3 Study Hypothesis

The study hypothesizes that the screening of  mutations which resulting in the absence of PNKP within the nucleus lead to the persistent activation of the DNA damage response, which may cause the death of susceptible brain cells, potentially contributing to the development of MCSZ.

## 1.4 Study Aims

The overall aim of this research is to screen PNKP gene mutation associated with Microcephaly, Seizure and Developmental Delay (MCSZ) disorder patients finding in Palestine.

## 1.5 Study Objectives

- Spot the light on the genetic causes of Microcephaly, Seizure and Developmental Delay disorder.
- Identification of mutations responsible for MCSZ in proband.
- To study the genotype-phenotype correlation between the affected patients.
- Recommend genetic counseling for families having affected patients.

# Chapter Two

# Literature Review

## 2.1 DNA damage and repair Mechanism

DNA structural damage significantly impairs its essential functions, such as replication and transcription, which are critical in the context of age-related diseases and cancer(Srinivas et al ., 2019 ). A sophisticated and intricate network of DDR mechanisms, encompassing various DNA repair pathways, damage tolerance strategies, and cell-cycle checkpoints, is essential for preserving genomic integrity (Biology, 1943) . Both exogenous and endogenous mechanisms serve as modification processes for the genetic material across all organisms. These mechanisms are associated with a heightened risk of single-strand breaks, which may occur directly or arise as intermediates during DNA repair(X. Shen & Gates, 2019). Consequently, the maintenance of genome integrity is a dynamic process that involves numerous distinct cellular pathways, including methylation maintenance, homologous recombination, DNA replication, DNA repair, and cell cycle regulation. A malfunction in one or more of these pathways can jeopardize genomic integrity(Rastogi et al ., 2014 & cshperspectives ., 2024). Various forms of DNA damage and corresponding repair mechanisms are illustrated in (Fig. 2.1). Among these, SSBs are prevalent, with cells possessing efficient systems for the swift identification and repair of it. These breaks represent the most frequent type of lesions encountered within cells, occurring

approximately once every one to two seconds in each cell (Caldecott et al ., 2024). The occurrence of SSBs is often a consequence of oxidized deoxyribose, which leads to the removal of the associated nucleobase and subsequent cleavage of the remaining oxidized basic site by AP endonuclease (APE1) (Xu et al ., 1998). Single-strand breaks (SSBs) can also be induced indirectly through the BER mechanism, which addresses small 'non-distorting' base lesions. This process may involve the activity of bi-functional DNA glycosylases or the action of APE1 subsequent to base excision or loss (Caldecott et al ., 2020).The persistence of unrepaired SSBs can lead to hyper activation of the SSB sensor PARP1, which can lead to toxic reduction of $NAD^+$ and/or toxic accumulation of poly (ADP-ribose) (Caldecott et al ., 2024).DSBs arise from exposure to external factors such as radiation and specific chemicals, as well as from internal mechanisms, including DNA replication and repair processes. Furthermore, meiosis I involves the intentional creation of DSBs, which triggers homologous recombination, thereby ensuring proper chromosome segregation (Cannan et al ., 2016).The cell subsequently undergoes a sequence of repair mechanisms, including DSBR, which can occur through either HR or NHEJ(Stinson et al ., 2021). DSBs pose significant challenges as they may result in cell death if left unrepaired. Furthermore, improper repair of DSBs can lead to various genetic alterations, including deletions, translocations, and fusions within the DNA (Negritto et al ., 2010).DPCs consist of DNA, proteins, and the bonds formed between them. These components can be addressed by various repair mechanisms. Numerous studies have confirmed that NER and HR are capable of interacting with DNA molecules to facilitate nuclease-dependent repair of DPCs(Zhang et al ., 2020). When a protein becomes covalently and irreversibly attached to DNA, it can obstruct various DNA-related processes, including transcription and replication. The formation of DPCs is prevalent in cells, as it can result from internal factors, such as aldehydes generated during cellular metabolism, or external factors like ionizing radiation, ultraviolet light, and chemotherapeutic substances (Stingele et al ., 2017). A mutation is a broad term that refers to any permanent alteration in the DNA sequence of an organisms genome. These changes can range from a single nucleotide variation to large- scale alterations in the structure or number of chromosomes. It can be classified into several types, including: SNPs a most common type of genetic variation, involving a change in a single nucleotide and variants which refers to any change in the DNA sequence, encompassing benign, pathogenic, or uncertain significance. The predominant form of mutation is the point mutation, which replaces one nucleotide with another.

Other types of mutations may include the insertion or deletion of one or several nucleotides. These mutations can arise from errors during DNA replication or from the harmful effects of mutagens, including chemicals and radiation, which interact with DNA and alter the structures of individual nucleotides (Ray et al ., 2022). Mismatch repair enzymes (MMR) serve as a core of DNA-repair mechanism present in all cells, aimed at reducing the frequency of mutations. These enzymes operate through two distinct processes. Certain enzymes function in a pre-replicative capacity, scrutinizing the DNA for nucleotides exhibiting atypical structures, which are corrected before to the replication process. Conversely, other enzymes act in a post-replicative manner, reviewing the newly synthesized DNA for inaccuracies and rectifying any errors identified. (Brown et al ., 2002 & Clancy et al ., 2008).

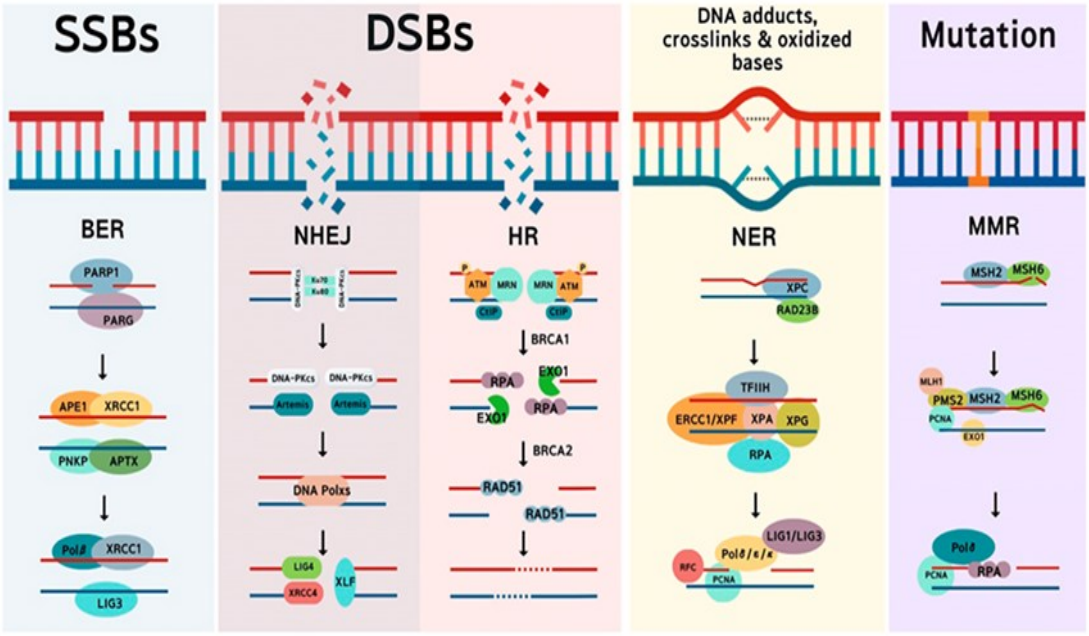


**Figure 2.1** Categories of DNA damage and their corresponding repair mechanisms, adapted from(Moon et al ., 2023)

There are four primary types of DNA damage: SSBs which are repaired via the (BER) pathway involving APE1, XRCC1, and various other enzymes; DSBs which are addressed by (NHEJ) and (HR), each utilizing specific enzymes; DNA adducts, crosslinks, and oxidized bases

(including DNA-Protein Crosslinks (DPC)), which are repaired through (NER) involving multiple enzymes; and mutations, which are corrected by (MMR), a general DNA repair enzyme.

Abbreviations: (SSBs): Single strand breaks; (DSBs): Double strand breaks; (BER): Base excision repair; (NHEJ): Non-homologous end joining; (HR): Homologous recombination; (NER): Nucleotide excision repair; (MMR): Mismatch repair enzymes are all critical components in the DNA repair process.

Congenital defects in single-strand break repair mechanisms exclusively impact the nervous system, resulting in neurodegeneration (McKinnon et al ., 2017) Neural cells exhibit a significant level of oxygen consumption, which correlates with their considerable energy demands and metabolic processes. Neurons are characterized by elevated transcription rates and the production of reactive oxygen species (ROS). The accumulation of DNA damage can lead to the formation of R-loops, which obstruct transcription and contribute to genomic instability. This instability may directly impair neuronal function. Given the longevity of neural cells and their heightened transcriptional activity, it is crucial to ensure that their genomes remain devoid of harmful DNA lesions throughout their lifespan (Caldecott et al ., 2008).

The nervous system is also influenced by unrepaired DNA double-strand breaks (Weinfeld et al ., 2011). These breaks can be addressed through two primary repair mechanisms: Homologous Recombination, which requires a sister chromatid as a template and is predominantly active during the S/G2 phases in replicating cells, and Non-Homologous End Joining, which plays a crucial role in repairing double-strand breaks in mature (non-cycling) neurons. The latter mechanism facilitates direct ligation of DNA ends and is operational throughout the entire cell cycle (Madabhushi et al ., 2014 & Zhou et al ., 2015).

Mitochondrial DNA (mtDNA) is also vulnerable to DNA damage, alongside nuclear DNA. The mtDNA is a circular molecule measuring 16.5 kbp, which encodes a total of 37 genes, comprising 13 proteins, 22 tRNAs, and 2 rRNAs. Eukaryotic cells can possess over 100 mitochondria, with each mitochondrion potentially containing up to 10 mtDNA molecules (Weinfeld et al ., 2011 ; Tahbaz et al ., 2012 & Saito et al ., 2019).

Mitochondrial DNA (mtDNA) damage appears during the process of respiration due to the generation of reactive oxygen species (ROS) in significant amounts within the mitochondria, which serve as the primary source of mtDNA lesions. If this damage remains undiagnosed, it may lead to mutations that are linked to various diseases, including diabetes, cancer, neurodegenerative disorders, and the aging process (Wallace et al ., 2005 & Brandon et al ., 2006). The base excision repair (BER) pathway serves as the primary mechanism for repairing DNA damage in mitochondria caused by reactive oxygen species. Furthermore, topoisomerase 1 and TDP1 are exists and operational within the mitochondrial environment (Hegde et al ., 2008 & Aryaman et al ., 2019).

Unaddressed DNA damage resulting from various mutations which can be resulted in a range of consequences, such as developmental abnormalities, degenerative diseases, aging, or cancer (Ribezzo et al ., 2016). In particular, failures in DNA repair mechanisms can hasten the process of cell death, leading to insufficient brain development and/or neurodegenerative conditions (Poulton et al ., 2013).

**2.2 Polynucleotide kinase3' - Phosphatase (PNKP) gene**

Neuronal cells exhibit a heightened sensitivity to DNA damage, primarily because of their extended lifespan, especially concerning endogenous DNA damage. Numerous studies have highlighted the crucial role of enzymes involved in the processing of DNA strand breaks in preserving the genetic stability of neuronal cells (Jiang et al ., 2017; Dumitrache et al ., 2017 & Islam et al ., 2024). One enzyme that is essential for the processing of DNA strand breaks is polynucleotide kinase/phosphatase (PNKP). The human variant of PNKP is a bifunctional enzyme with a molecular weight of 57.1 kD, exhibiting both DNA 3′-phosphatase and DNA 5′-kinase activities. These functions are crucial for the repair of strand break termini resulting from reactive oxygen species, ionizing radiation, and the action of topoisomerase I inhibitors (Karimi-Busheri et al ., 1999 & Weinfeld et al ., 2011). The PNKP protein in mammals comprises an N-terminal Fork Head-Associated (FHA) domain and a catalytic subunit, which are connected by a flexible linker (Bernstein et al ., 2005). The FHA domain, as illustrated in (Fig. 2.2), is connected to the catalytic domain via a flexible polypeptide segment. This domain selectively binds to phosphorylated regions of acidic casein kinase 2 (CK2), a serine/threonine-selective protein

kinase involved in various cellular processes, including cell cycle regulation, DNA repair, and circadian rhythm control. The phosphorylated regions are found in XRCC1 and XRCC4, which serve as the primary scaffolding proteins in the repair mechanisms for DNA single-strand breaks (SSBs) and double-strand breaks (DSBs), respectively, along with their independent DNA 3′-phosphatase and 5′-kinase domains (Weinfeld et al ., 2011 & Tahbaz et al ., 2012 ). The function of PNKP is regulated through its interaction with DNA repair scaffold proteins (Breslin et al ., 2017).

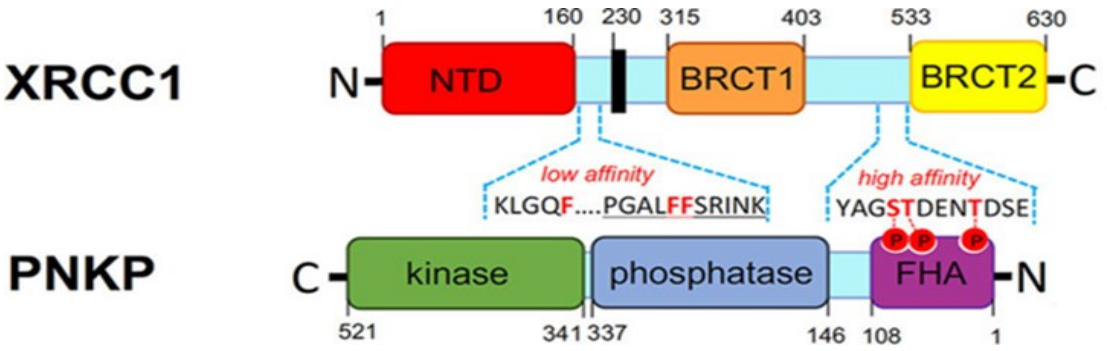


**Figure 2.2** The protein domains in XRCC1 and PNKP, adapted from (Islam et al .2024 ).

The linkage between the FHA- PNKP domain and XRCC1 in high affinity by serine(S)/threonine(T)-selective protein kinases phosphorylation during repair mechanism. The phosphatase domain of PNKP protein has a low-affinity linkage with XRCC1 due to the abundance of phenylalanine (F) amino acid which does not undergo phosphorylation.

Abbreviations: XRCC1: X-ray repair cross-complementing 1; PNKP: Polynucleotide kinase/phosphatase; FHA: Forkhead associated.

Fig. (2.3) illustrates the regulation of PNKP protein levels by the Cul4A–DDB1–STRAP complex in the context of oxidative stress. The synthesis of PNKP protein occurs through the processes of mRNA transcription and translation. In instances where PNKP is not needed for DNA repair, it undergoes polyubiquitylation mediated by the E3 ubiquitin ligase complex Cul4A–DDB1–STRAP, leading to its recognition and degradation by the proteasome. Conversely, in response to oxidative stress, which induces DNA strand breaks and activates ATM, PNKP becomes phosphorylated. This phosphorylation event inhibits its ubiquitylation and subsequent degradation by the proteasome, resulting in an increase in PNKP protein levels that are essential for effective DNA repair.

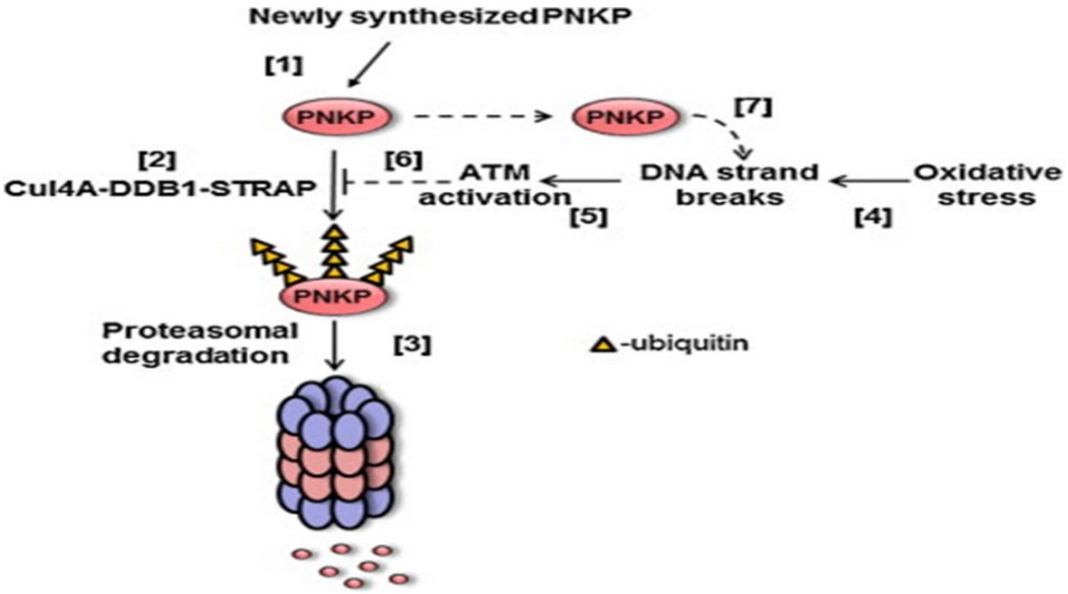


**Figure 2.3** The modulation of PNKP protein levels by the Cul4A–DDB1–STRAP complex and its response to oxidative stress, as adapted from (Parsons et al ., 2013)

The downregulation of PNKP ends to a reduction in both mitochondrial and nuclear PNKP levels, resulting in the accumulation of DNA damage in mitochondrial DNA (mtDNA) as well as an increase in damage to nuclear DNA (Das et al ., 2010 & Tahbaz et al ., 2012).

Mutations in the PNKP gene, which can be as loss-of-function, substitutions, frame-shifts, and missense mutations, are associated with a variety of neurological disorders characterized by differing degrees of epilepsy, microcephaly, psychomotor retardation, cerebellar atrophy, and peripheral neuropathy(Kalasova et al., 2019 & Furones García et al., 2021). These include microcephaly accompanied by seizures and developmental delays (MCSZ) (Shen et al ., 2010), Progressive cerebellar atrophy and polyneuropathy (Poulton et al ., 2013). Ataxia with Oculomotor Apraxia type 4 (AOA4) is characterized by the progressive degeneration of the cerebellum(Bras et al ., 2015) although Some individuals may exhibit both microcephaly and progressive cerebellar atrophy(Rudenskaya et al ., 2019 & Furones García et al ., 2021). Charcot-Marie-Tooth disease 2B2 is associated with mild axonal peripheral polyneuropathy and a relatively late onset of cerebellar ataxia(Pedroso et al ., 2015 & Previtali et al ., 2019). Mutation of PNKP also causes a flaw in cortical development (Shimada et al ., 2015 & Lu et al., 2019).

Microcephaly and neurodegenerative diseases occur as a result of specific inherited mutations in the PNKP gene. Microcephaly is characterized by a head circumference that is less than three standard deviations (SD) below the mean, or a Z score of less than -3 (Aggarwal et al ., 2013). Microcephaly with Seizures and developmental delay (MCSZ) is an autosomal recessive neurodevelopmental disorder that manifests in infancy and exhibits a spectrum of phenotypic severity. This condition arises from various hypomorphic mutations in the PNKP gene(Bernstein et al ., 2005 & Shen J et al ., 2010). Ataxia with oculomotor apraxia 4 (AOA4) is a progressive and intricate movement disorder characterized by hyperkinetic symptoms, abnormalities in eye movements, polyneuropathy, varying levels of cognitive dysfunction, and obesity (frontiersin ., n.d & Paucar et al ., 2016). Consequently, additional causative factors affect the neural phenotype in the absence of PNKP (Bras et al ., 2015). Epilepsy encompasses a range of serious neurological disorders that have diverse causes and consequences, which can be identified by the type of seizures, the age at which they begin, and associated comorbid conditions.(Stafstrom et al ., 2015). Epilepsy is predominantly observed in individuals with intellectual disability (ID), with a prevalence rate of around 22.2%. The probability of developing epilepsy escalates in correlation with the severity of the ID (Yang et al., 2021). It is estimated that 70–80% of epilepsy cases can be attributed to one or more genetic factors, taking into account the assumed genetic and phenotypic variabilities (Thakran et al ., 2020).

Various regions of the PNKP protein are linked to distinct diseases. The kinase domain of PNKP is connected to neurodegeneration related to AOA4, as evidenced by the variants p. G375W, p. T408del, p. R439fs, p. T442fs, p. Thr424Glyfs49, and p. Tyr515 (Bras et al ., 2015). Mutations associated with MCSZ are predominantly located within the phosphatase domain (p. L176F and p. E326K), although they are distributed throughout the entire gene. Specifically, one MCSZ patient exhibits a compound heterozygous genotype of p. G292R/p. A55S, which corresponds to the phosphatase and FHA domains, respectively. In contrast, another MCSZ patient is homozygous for the mutation p. T424GfsX48, situated in the kinase domain (Campopiano et al ., 2020). Variants in the FHA domain have, to date, not been linked to AOA4, neurodegeneration, or microcephaly. Instead, they have only been documented in individuals who exhibit seizures, specifically the p. P20S and p. A55S variants (Campopiano et al ., 2020).

Four mutations in PNKP have been identified in MCSZ (Shen et al ., 2010). L176F and E326K represent point mutations occurring at highly conserved residues within the DNA phosphatase domain, while T424Gfs48X and exon15Δfs4X are frame-shift mutations located in the DNA kinase domain. These mutations lead to the formation of premature stop codons and result in truncated PNKP polypeptides, as illustrated in (Fig. 2.4)

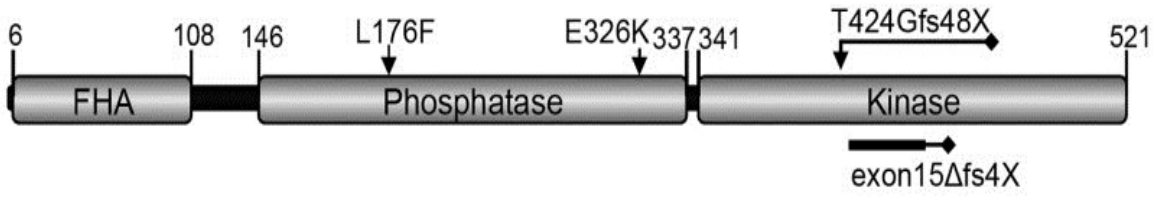


**Figure 2.4** Illustrates a schematic representation of the four PNKP mutations discovered in MCSZ, as adapted from (Reynolds et al ., 2012 ).

The mutations T424Gfs48X, 15Δfs4X, L176F, and E326K are marked with a black arrow.

T424Gfs48X arises from a 17-base pair duplication located within exon 14 of the kinase domain, producing a polypeptide consisting of 471 amino acids, with the final 48 residues being out-of-frame. Exon15Δfs4X is characterized by a 17-base pair deletion in intron 15, which results in an mRNA transcript that omits exon 15, yielding a polypeptide of 436 amino acids, where the last four residues are also out-of-frame. The mutations E326K and T424Gfs48X are the most frequently observed in MCSZ (Bernstein et al ., 2005 ; Shen J et al ., 2010 & NCBI ., 2025).

Three Palestinian families exhibited a homozygous base pair substitution in exon 11 (326G>A), leading to a non-conservative amino acid alteration from E326 to K. Families from Saudi Arabia and Turkey shared a homozygous 17 bp duplication (17 bp dup) in exon 14 (1250_1266dup), which caused a frameshift mutation resulting in T424GfsX48. The European family presented two heterozygous mutations: the same 17 bp duplication in exon 14 as observed in the Saudi and Turkish families, along with a point mutation in exon 5 (526C>T) that resulted in an L176F change. Moderately affected members of the European family demonstrated compound heterozygosity, possessing both the 17 bp duplication in exon 14 and a 17 bp deletion in intron 15, which interferes with proper mRNA splicing (Shen et al ., 2010).

Other research has indicated that three out of the four mutations associated with MCSZ significantly diminish or completely eliminate the DNA kinase activity of recombinant PNKP at 30°C (specifically L176F, T424Gfs48X, and exon15Δfs4X). However, only one of these mutations, L176F, also leads to a reduction in DNA phosphatase activity under the same temperature conditions. The fourth mutation, E326K, appears to have minimal effect on both DNA kinase and DNA phosphatase activities at 30°C, yet it exhibits lower stability compared to the wild-type enzyme at physiological temperatures. Consistent with these findings, the three MCSZ cell extracts demonstrated markedly decreased 3′-phosphatase activity on both single-stranded and duplex DNA substrates at 30°C, as evidenced by the presence of the dephosphorylated (17-OH) product, in contrast to extracts derived from wild-type or heterozygous individuals (Reynolds et al ., 2012).

In the United Arab Emirates, Whole Exome Sequencing was employed to detect homozygosity for a missense mutation c.1385G > C (p. Arg462Pro) located in exon 15 of the PNKP gene in the patient, while her consanguineous parents exhibited heterozygosity for the same mutation. The Arg 462 residue is integral to the lid subdomain helix of the P-loop Kinase domain.( Nair et al ., 2016).

Gatti and his research team (Gatti et al ., 2019) reported that over 40 patients have been identified with PNKP mutations affecting both the phosphatase and kinase domains. The clinical manifestations of these mutations reveal a diverse phenotypic spectrum, ranging from congenital microcephaly and early-onset intractable seizures to adult-onset, slowly progressive sensory-motor neuropathy and cerebellar ataxia. They identified two heterozygous mutations in the PKNP gene: a novel intronic frameshift variant, c.1298 + 33_1299-24del, and a previously

documented duplication, c.1253_1269dup; p. Thr424Glyfs49, located in exon 14. Both mutations impact the DNA kinase domain of PKNP, while L176F, G292R, and Glu326Lys mutations influence the phosphatase domain (Bermúdez-Guzmán L et al ., 2020 & Bitarafan et al ., 2021).

A case-control study revealed that Exome Sequencing conducted on the affected patient identified a causative variant in the PNKP gene, which was the sole variant detected by the annotation and prioritization pipeline. This variant was a homozygous c.968C > T mutation located in exon 11, predicted to lead to a p. Thr323Met amino acid alteration in the PNKP protein (Marcilla Vázquez et al ., 2021). Importantly, all MCSZ mutations identified thus far have been associated with approximately a tenfold decrease in cellular levels of PNKP protein, as well as diminished rates of chromosomal DNA strand break repair (Bernstein et al ., 2005 ; Shen J et al ., 2010 & NCBI ., 2025).

### 2.3 Microcephaly, Seizures, and Developmental Delay (MCSZ)

Microcephaly, Seizures, and Developmental Delay (MCSZ) (OMIM # 613402) is a rare condition with an undetermined prevalence. It is classified as an autosomal recessive neurodevelopmental disorder, resulting from homozygous or compound heterozygous mutations in three domains of the PNKP gene located on chromosome 19 (Kalasova et al ., 2019 & Bitarafan et al ., 2021). This disorder is characterized by an unusually small head size (microcephaly) and neurological issues stemming from impaired brain development during the prenatal period. The onset of symptoms may occur either prenatally or at the time of birth. In some cases, patients may experience a more prolonged course of the disorder, which can include neurodegeneration (Reynolds et al ., 2012 ).

Individuals affected by MCSZ generally exhibit recurrent seizures, known as epilepsy, which typically commence in infancy. They also experience delays in the development of motor skills, such as sitting and walking, primarily due to muscular atrophy. Additionally, speech development is often delayed, with some individuals potentially never acquiring the ability to speak. Common characteristics of MCSZ include intellectual disability and behavioral issues, particularly hyperactivity and a loss of the ability to walk independently. In rare instances, individuals may also show signs of poor balance and coordination, known as ataxia, as well as

severe cognitive impairment. Other associated symptoms involve the central nervous system, which may present with a simplified gyral pattern, a thin corpus callosum, and enlarged ventricles. The peripheral nervous system may exhibit sensorimotor polyneuropathy and hyporeflexia, which is observed in some patients (Bernstein et al ., 2005 & Shen J et al ., 2010).

A spectrum of phenotypic severity exists among patients: certain individuals experience refractory seizures during infancy, indicative of a developmental and epileptic encephalopathy (DEE), whereas others exhibit better-controlled seizures and a more prolonged clinical course linked to cerebellar atrophy and peripheral neuropathy (Bernstein et al ., 2005 & Shen J et al ., 2010).

Neurometabolic disorders are associated with MCSZ, a notable category of diseases predominantly affecting neonates and infants. These disorders primarily arise from the absence or malfunction of an enzyme or its cofactors that are crucial for specific biochemical reactions, resulting in a deficiency of vital metabolites in the brain. Consequently, this deficiency can give rise to various neurometabolic diseases. The disruption of metabolic pathways, along with inhibition at initial stages, may result in the accumulation of reaction intermediates, which are frequently toxic to the developing brain (Karimzadeh et al ., 2015).

A patient diagnosed with MCSZ typically presents with a range of inherited metabolic disorders, including Tyrosinemia type-2, Menkes disease, mitochondrial diseases, hyperphenylalaninemia, 3-methylcrotonyl-CoA carboxylase deficiency, phenylketonuria, biotinidase deficiency, glutaricaciduriatype 1, and arginase deficiency (Aggarwal et al ., 2013 & Kempińska et al ., 2022).

# Chapter Three

# Materials and Methods

## 3.1 Subjects

This research was granted approval by the ethics committee at Al-Quds University. Hebron Charitable Rehabilitation Society, along with other private clinics in West Bank permitted us to visit their clinics in March/2024. The Patients that were invited to participate in our study were based on clinical symptoms that appeared on them. The inclusion criteria: (1) Patients: males and females of different ages; having the general characteristics of MCSZ. (2) Their parents, especially if they are consanguineous in marriage to determine if they are carriers for PNKP mutation, and/or family members. Patients with other features and disorders were reported as exclusion criteria.

Three female participated patients met our inclusion criteria and consented to participate in our study. The patients, along with ten family members (including parents and siblings), were categorized as follows: eight participants belonged to the same family (Family no. I), two participants were from a different family (Family no. II), and three participants were associated with another family (Family no. III). Patients were coded from P1 to P14. Additionally, a healthy

individual without any mutations in the PNKP gene was included as a non-carrier, designated with the code P9.

Patient 7 (PNKP-7) from family I is a 4-year-old female with consanguineous parents. Patient 10 (PNKP-10) from family II is a 14-year-old female with non-consanguineous parents, while Patient 12 (PNKP-12) from family III is a 25-year-old female, also with non-consanguineous parents. Clinical presentations of these patients revealed dysmorphic craniofacial characteristics, including severe microcephaly, a depressed nasal bridge, a sloping forehead, and a short neck. Additionally, they exhibited recurrent seizures (epilepsy), delays in motor skill development such as sitting and walking, an inability to speak, loss of independent ambulation, and poor balance and coordination (ataxia).

Participated patients had signed a consent form to elucidate the study procedures and obtain their agreement for the collection of blood samples as well as for the publication of the findings (refer to appendix). Additionally, each participant filled out a questionnaire designed to gather information regarding their family and medical history (refer to appendix).

### 3.2 Blood samples

A volume of 5 milliliters of whole blood was collected in a tube containing EDTA as an anticoagulant for the purpose of DNA extraction, then stored at a temperature of -20°C until the extraction process.

### 3.3 DNA extraction

Genomic DNA was isolated from EDTA whole blood utilizing the Epicenter Master Pure TM DNA Purification Kit for Blood Version II (USA), following the guidelines provided by the manufacturer. The purity and concentration of the DNA from all participants were assessed using a NANODROP spectrophotometer (Nanodrop 1000 spectrophotometer; Thermo Scientific, USA), with DNA purity exceeding 1.7. The extracted DNA was subsequently preserved and stored at -20 °C (NCBI ., 2025)

### 3.4 Primers used in this study

We screened the PNKP gene mutations encompassing the more frequently related to the mutation's world widely on Exons 11, 14 and 15. These primers were published in (Neuser et al ., 2022). Table (3.1) shows the primer sequences.

All primers were examined using Primer 3 software from Homo sapiens polynucleotide kinase 3'-phosphatase (PNKP), mRNA NCBI Reference Sequence: NM_007254.4from Gene Bank for these exons (NCBI ., 2025).

**Table 3.1:** Primer sequences used for the PNKP mutation typing in exon (11, 14 and 15).

| | **Primer name**<br>**F:forward, R: reverse** | **Primer sequence (5'-3')** | **Length/bp** |
|---|---|---|---|
| **1** | PNKP-11F | CTCACCGGATCAAAGGCTG | **130** |
| | PNKP-11R | GCCTGTGTCTGATGTTCGTC | |
| **2** | PNKP -14F | GCCGATCTGTTTGTGACCTC | **209** |
| | PNKP-14R | AAGGAGCTGGATGTGCAGG | |
| **3** | PNKP-15F | CCTGCACATCAGCTCCTCT | **241** |
| | PNKP-15 R | GGAGTCCGTCATCTCTCGAA | |

### 3.5 Polymerase Chain Reaction Amplification Conditions

Polymerase chain reaction (PCR) was conducted in a 25 µl total reaction volume using the PCR-Biosystems Ready mix (2X PCRBIO HS Taq Mix Red, Jerusalem, Israel) using a BIO-RAD PCR-system T100 thermal cycler. For the PCR, two microliters of eluted DNA (20 ng) from the blood sample were used. Primers listed above were used to amplify each exon in a single PCR. The reaction conditions were controlled with a preliminary pre-denaturation phase at 95°C for a duration of 5 minutes. This was succeeded by a denaturation step at 95°C lasting 30 seconds, an

annealing phase at 58°C for 30 seconds, and an extension period at 72°C for 60 seconds. These steps were repeated for a total of 35 amplification cycles. Also, a reaction buffer devoid of human DNA was incorporated as a negative control for the PCR process.

### 3.6 Agarose gel electrophoresis

The amplicons for the exons 11,14, and 15 PNKP gene were subjected to electrophoresed on 2% agarose gels (SeaKem® LE agarose gel) at 100 V in 1x Tris-Acetate-EDTA buffer

(TAE) supplemented with 8-microliter of ethidium bromide and visualized under UV light using a gel documentation system (The Bio-Imaging Systems MiniLumitransilluminator). To ascertain the molecular weight of the amplicon, a 100 bp molecular weight standard ladder (ThermoScientificGeneRuler) was uploaded on the gel as a reference for each run.

### 3.7 Sanger Sequencing

All PCR products that were visible through gel electrophoresis were dispatched for Sanger Sequencing at Hylabs in Jerusalem, Israel. The sequencing was performed in both directions utilizing forward and reverse primers on an ABI 3730xl DNA Analyzer (Hylabs, Rehovot), which had previously been used for DNA amplification. The obtained sequences were aligned with reference sequences available in the Gene Bank database using the BLAST tool. The chromatograms were verified, and the sequences were assembled using BioEdit sequence alignment editor version 6.0.7. Additionally, the sequences were aligned using the Multalin Multiple sequence alignment tool available at (Multalin, 1988).

# Chapter Four

# Results and Discussion

## 4.1 Results

### 4.1.1Samples and study population

Study populations were collected based on the clinical manifestations, which included a small anterior fontanel and head circumference, intractable epilepsy and growth retardation. Three female patients shared the same symptom based on this, they were primarily diagnosed with the MCSZ and then the samples from their family members were collected.

Three female participated patients met our inclusion criteria and consented to participate in our study. The patients, along with ten family members (including parents and siblings), were categorized as follows: eight participants belonged to the same family (Family no. I), two participants were from a different family (Family no. II), and three participants were associated with another family (Family no. III). (P9) person used as a control (healthy individual without any mutations in the PNKP gene was included as a non-carrier).

### 4.1.2 Screening of PNKP gene mutations

We screened PNKP mutation in all three families on the most common publication exons (Exon 11, 14 and 15). Members of the family I were positive for the mutation on exon 11 but not on exon 14 and 15 variants. On the other hand, the two patients from families II and III were negative for any mutation on exons 11, 14 and 15.

### 4.1.3 Sanger Sequencing and Analysis

The PNKP sequences for exons 11, 14 and 15 of that gene were uploaded from the Gene Bank were downloaded from the National Center for Biotechnology Information (NCBI) nucleotide database and ensemble (Ensembl, 2024) to compare the results of PCR sequencing on the that three exons. The following sequences were used as references for this NM_007254.4.Variants were standardized to the reference transcript NM_007254.4 (GRCh38/hg19) using Mutalyzer 2.0.32 (Nature, 2006).

The amplicons derived from the tested exons of the three patients were aligned with the sequence NM_007254.4 through Multiple Sequence Alignment, and the results were visualized and analyzed utilizing the CLUSTAL program (Multalin, 1988).

The proband from family I has a missense mutation homozygous **c.968 C > T**. This mutation was examined on ensemble (Ensembl, 2024) and showed that it is likely pathogenic and this resulted in the substitution of amino acid **p. (Thr323Met), rs372148913** variant in exon 11 of the PNKP gene as shown in (Fig. 4.1) This variant was described previously and was observed to be associated with microcephaly, seizure and developmental disorder. No variants were seen in exon 14 or exon 15.


**Figure 4.1** Position of rs372148913 SNP in exon 11 of the PNKP gene. rs372148913 affect C>T nucleotide result substitution mutation using (Ensembl, 2024)

The other two patients from families II and III were negative for the c.968 C > T (Thr323Met) variant and no variants were seen in exon 14 or 15. Their blood were sent for Whole Exome Sequencing showed no mutation in the PNKP gene.

Patient PNKP7 is female a product of 38+3wk pregnancy, LBWT 1525g, has lissencephaly, IUGR, severe microcephaly (HC at birth, 24.5 cm <-3SD) on GUSS & DUSS. EEG was abnormal, slow at the delta range, and had high amplitude posterior spike and sharp waves with GSSW. Brain CT report showed multiple artifacts, no IVH. At age 2 yrs.' old she has a height of 72cm, weight of 7.5kg, BMI of 14.5 and a head of 29. At age 3 yrs.' old with a height of 74.5cm, weight of 8.3kg, BMI of 15 and head of 29.8 cm.

She has uncontrolled epilepsy, she developed many times status epileptics that were initially aborted by phenytoin loading, phenobarbital loading, Keppra loading and assival stats but due to recurrent epileptic status within the same day. She was incubated in a hospital connected to MV low moderate setup and started on dormicon drip initially was 3mic/kg/min, and morphine at 10mic/kg/hr but after two days she developed status epilepticusassival twice, aborted, then occurred again, midazolam dose raised to 6. Now she is on antiepileptic medications: Keppra, Tegretol and phenobarbital. She has abnormal limb movements with searching eyes, many episodes daily, each episode for less than 2-3 minutes.

As for the other two patients, little information was obtained about their medical history due to the loss of their medical files in Hebron Charitable Rehabilitation Society. Patient PNKP10 is 14 yrs.' old with height of 110, weight 23, BMI of 19 and head of 46 cm. She has uncontrolled epilepsy for intermittent and long periods. Now she is on risperidal 2 mg antiepileptic medications. The patient exhibit abnormal limb movements with searching eyes, significant delays in the development of motor skills, such as sitting and walking, delayed speaking, severe loss of independent ambulation and poor balance and coordination (ataxia). She also suffers from intestinal laziness, malabsorption, and chronic constipation.

Patient 12 at age 25 yrs.' old with height of 140, weight of 36, BMI of 18.37 and head of 57 cm. She has slightly controlled recurrent epilepsy for intermittent and short periods and has not taken anyantiepileptic medications. She has abnormal limb movements with searching eyes several times, delayed development of motor skills, such as sitting and walking, as well as delayed

speech. Additionally, she has experienced a severe decline in her ability to walk independently, along with poor balance and coordination (ataxia). Furthermore, she suffers from mental disability and behavioral problems, most notably hyperactivity, weakness, malabsorption, and loss of appetite.

Conversely, members of the family I was positive for the variant which directed us towards continuing cascade screening for this family in the future. Table (4.1) shows the sequence results for three families. Fig.( 4.2) represents multiple sequence alignment for the family I members and (P9) a control sample (normal person having no mutation on the PNKP gene). Fig. (4.3) represents chromatograms for 3 selected participants, respectively.

**Table 4.1:** Sanger sequence results of exon 11 of the PNKP gene.

| PCR code | Sequence result |
|---|---|
| **P1** | G/A** |
| **P2** | G/A |
| **P3** | G/A |
| **P4** | G/A |
| **P5** | G/A |
| **P6** | G/A |
| **P7** | A/A*** |
| **P8** | G/A |
| **P9** | G/G* |
| **P10** | G/G |
| **P11** | G/G |
| **P12** | G/G |
| **P13** | G/G |
| **P14** | G/G |

G/G*: Wildtype, G/A**: Heterozygous, A/A***: Homozygous c.968 C > T (G >A) substitution.

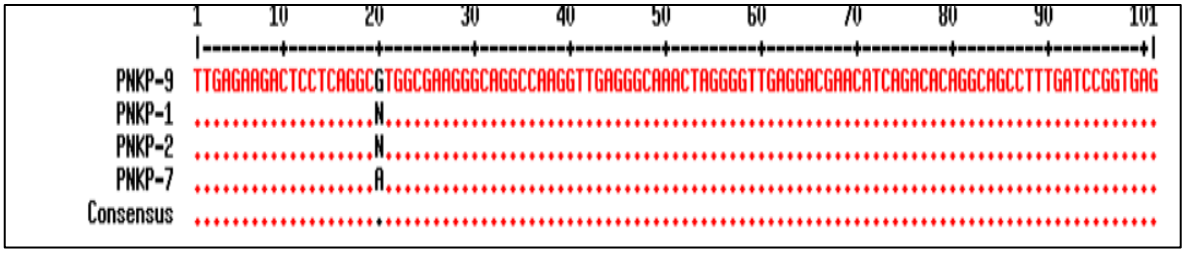


**Figure 4.2** Alignment of Multiple sequence for family I members and control sample.

c.968C > T (P. Thr323Met), rs372148913 homozygous mutation position (19:50365689 Polynucleotide Kinase 3′ Phosphatase (PNKP) gene) related to the syndrome of microcephaly, seizure and developmental delay in three families (I, II, III) members. (PNKP 9): Normal person without mutation in PNKP. PNKP 1, 2 relative members of patient PNKP 7.

**G**: Wild type, **N**: Heterozygous, **A**: Homozygous.

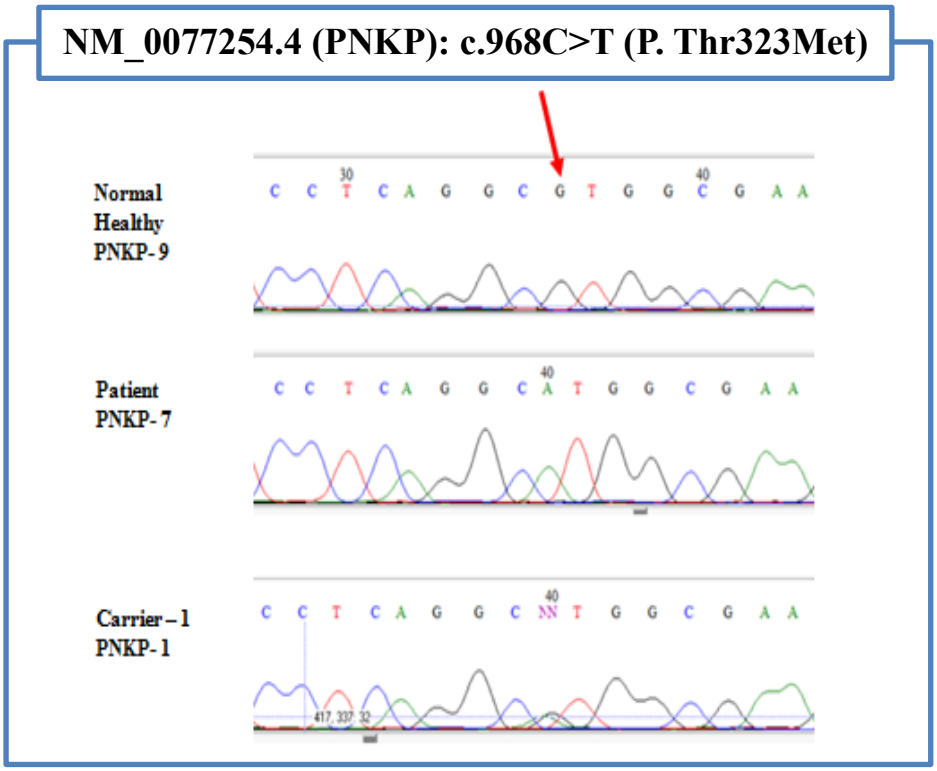


**Figure 4.3** Representative chromatograms for 3 selected participants.

c.968C>T (P. Thr323Met) missenses substitution leads to G to A transition (similar to C to T transition) as shown by the red arrow.

**G**: Wild type, **N**: Heterozygous, **A**: Homozygous.

### 4.1.4 Segregation Analysis

After the mutation was known the family members of the proband (PNKP 7) both parents and relatives were checked by PCR and sequencing for exon 11 to determine heterozygous or normal status regarding mutation. Segregation was confirmed through Sanger sequencing in all (Nature, 2006). Results showed that the proband's both parents, Grandfather, Grandmother, Aunt, and two brothers have the variant in heterozygous genotype (G/A).

### 4.1.5 c.968 C > T: p. (Thr323Met) variant interpretation

The variant found at genomic position chr19:50,365,689 was identified as a homozygous c.968C > T mutation within exon 11 which is anticipated to lead to a p.Thr323Met amino acid alteration in the PNK protein (NM_007254.4), which generates a missense mutation in the phosphatase domain of the protein. This mutation was inherited from both parents, carriers of a heterozygous mutation. The pathogenicity of this variant using the Franklin website (Franklin, 2023) and ClinVar were confirmed. This variant causes a single base pair substitution that alters the genetic code and produces a different amino acid in the sequence. This variant substitutes Cytosine (Guanine in a complementary sequence) for Thymine (Adenine in a complementary sequence), substituting the amino acid threonine for methionine at position 323 in the PNKP phosphatase domain (C>T is similar to G>A substitution).

The resulted variant has been documented in the Exome Aggregation Consortium database, exhibiting an allele frequency of less than 0.02%. Various tools, including SIFT, PROVEAN, PolyPhen-2, Mutation Taster, Mutation Assessor, LRT, CONDEL, and MetaSVM, have classified the mutation as "probably damaging." It is anticipated that this mutation will lead to a reduction in the stability of the PNKP protein.

### 4.1.6 Family pedigree

Finally, after obtaining the sequence results for our sample, we were able to construct the family pedigree for MCSZ among the members of Family I who took part in our study. The pedigree is illustrated in (Fig. 4.4).

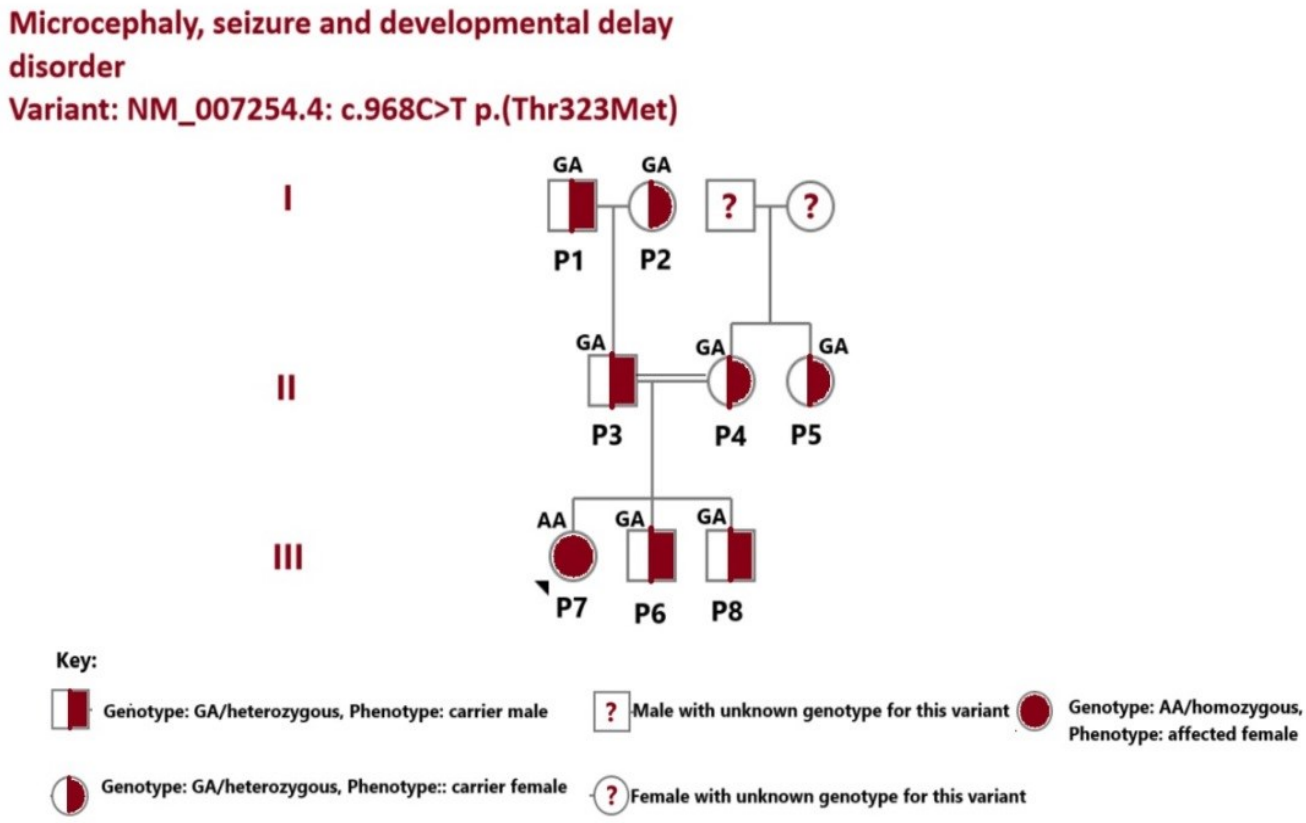


**Figure 4.4** Family I pedigree for MCSZ according to cascade screening.

## 4.2 Discussion

Genome stability is vital for the proper development and function of an organism. In humans, two key systems are fundamental to maintaining genome integrity: DNA repair mechanism and apoptosis(Bernstein et al ., 2002). This suitable procedure is concluded to prevent the formation and retention of damaged cells that could ultimately harm the organism (Ovejero S et al ., 2020). Cells that are deficient in DNA repair mechanisms tend to accumulate excess DNA damage. Despite their advanced capabilities, which include mechanisms involving DNA surveillance and repair proteins, these cells struggle to preserve the integrity of the inherited nucleotide sequence of genomic DNA over time (Yao Y et al ., 2014). The DNA surveillance and repair mechanism is activated following DNA damage, which may occur during replication or as a result of genotoxic stress. In response, cells initiate intracellular pathways that can detect the damage, halt the cell cycle, recruit DNA repair proteins, rectify the damage, or initiate apoptosis (Ray et al .,

2022). Cells that are impaired in the process of apoptosis often survive with an accumulation of DNA damage, allowing DNA replication to proceed despite the presence of this damage. This can result in mutations that contribute to the development of cancer (Jacobs WB et al ., 2004 & Springer., 2023).

There are five primary DNA repair mechanisms HR, NHEJ, NER, BER, and MMR. Each of these pathways features essential proteins that play dual roles in both the detection/repair of DNA damage, as well as in the process of apoptosis. Among these proteins is PNKP, which participates in various DNA repair processes, including BERfor single-strand breaks and double-strand break repair through the NHEJ. The lack of this element in the nucleus results in the continuous activation of the DNA damage response, as there is a persistent accumulation of double-strand breaks in the mutant cells (Nature, 2006).This phenomenon likely contributes to the death of susceptible brain cells, which may be a significant factor in neurodegeneration (Islam et al ., 2023).

Polynucleotide kinase 3′ phosphatase serves as a crucial dual-function enzyme within the diverse mechanisms of DNA damage repair. It features a C-terminal catalytic domain that houses a fused bimodal phosphatase and kinase domain, complemented by a Fork Head-Associated domain located at its N-terminus (Dumitrache et al ., 2017 ). This protein possesses both a polynucleotide 3′-phosphatase domain and a polynucleotide 5′-hydroxyl kinase domain, playing a crucial role in the repair of single-strand and double-strand breaks. The phosphatase domain is responsible for the removal of 3′ phosphates, while the kinase domain facilitates the phosphorylation of 5′-hydroxyl groups, a process that is vital for DNA ligation (Bernstein et al ., 2005 & Shen J et al ., 2010). On a genomic scale, PNKP is notable due to its unique structure, characterized by a higher number of small introns located at the 3′ end, in contrast to the typical size of human introns (Abebrese EL et al ., 2017). Smaller introns are linked to an increased probability of intron retention (Monteuuis G et al ., 2019).

PNKP has been linked to a broad phenotypic spectrum, which may be attributed to its multi-domain structure. The FHA domain facilitates the return of PNKP to sites of DNA damage, where it participates in the repair of both single- and double-strand breaks through its active kinase and phosphatase domains (Neuser S et al ., 2022). The FHA domain interacts with either XRCC1 or XRCC4 scaffold proteins, which are also crucial for the repair processes of single-

and double-strand breaks, respectively (Tsukada K et al ., 2020). Among the various types of endogenous DNA damage, single-strand breaks are the most prevalent, and PNKP is instrumental in the majority of the repair steps for these breaks, primarily due to the high occurrence of 3′-P termini ( Weinfeld et al ., 2011 & Gokben S et al ., 2017).

Mutations in PNKP can lead to a variety of distinct disease manifestations (Aggarwal et al ., 2013). To date, at least 34 variants have been documented in humans, including missense, in-frame indel, nonsense, frameshift, large deletion, and complex rearrangement types. The relationship between this gene and disease is substantiated by case-level data, segregation studies, and experimental findings, with additional support from in vitro functional assays (Reynolds et al ., 2012).

A specific number of genetic syndromes have been linked with mutations in the PNKP gene. Among these, Microcephaly with seizures and developmental delay (MCSZ) was initially documented in 2010 (Shen et al ., 2010). Furthermore, Progressive cerebellar atrophy and polyneuropathy were identified in 2013 (Poulton et al ., 2013). Ataxia with Oculomotor Apraxia type 4 (AOA4) is characterized by progressive cerebellar atrophy (Bras et al ., 2015), and it is noteworthy that some individuals may exhibit both microcephaly and progressive cerebellar atrophy (Rudenskaya et al ., 2019 & Furones García et al ., 2021). Furthermore, Charcot-Marie-Tooth disease type 2B2 is associated with mild axonal peripheral polyneuropathy and a relatively late onset of cerebellar ataxia (Pedroso et al ., 2015 & Previtali et al ., 2019). Mutations in PNKP also lead to abnormalities in cortical development (Shimada et al ., 2015).

Both MCSZ and AOA4 are classified as disorders that specifically impact the nervous system without involving other organs or systems (Bras et al ., 2015). These two conditions can be readily differentiated; the phenotype of MCSZ resembles that observed in other disorders characterized by altered mechanisms of double-strand break repair, while AOA4 is associated with deficiencies in the repair of single-strand breaks (Dumitrache et al ., 2017 ).

MCSZ (OMIM # 613402), also known as early infantile epileptic encephalopathy, is an autosomal recessive disorder associated with either a homozygous or compound heterozygous mutation in the PNKP gene located at 19q13. This genetic condition arises when a child inherits one mutated gene from each parent. Typically, the parents of a child with an autosomal recessive

disorder do not exhibit the condition themselves. These unaffected parents are referred to as carriers, as they each possess one copy of the mutated gene, which they can transmit to their offspring. MCSZ is characterized by microcephaly, intractable seizures, developmental delays, and behavioral issues, particularly hyperactivity (Reynolds et al ., 2012).The clinical manifestations of MCSZ vary widely in severity; the classical presentation involves a severe early-onset form of infantile epileptic encephalopathy without signs of brain atrophy. Conversely, other clinical variants may present with manageable seizures and a prolonged course, accompanied by cerebellar atrophy and peripheral neuropathy (Nakashima, M et al ., 2014). Clinical observations have not indicated an increased incidence of infections among MCSZ patients, suggesting that this condition is not associated with immunodeficiencies (Shen et al ., 2010). Given that microcephaly is related to defects in the repair of double-strand breaks, such defects are likely implicated in MCSZ. However, the absence of neurodegenerative changes indicates that issues with the repair of single-strand breaks are not relevant to this disorder, despite PNKP's involvement in both DNA repair mechanisms (Dumitrache et al ., 2017 ).

AOA4 (OMIM # 616267) had been classified as an autosomal recessive neurological disorder. A hallmark of this condition is the emergence of dystonia and ataxia during the first ten years of life. Other symptoms may include oculomotor apraxia characterized by abnormal saccadic eye movements, cerebellar degeneration, sensorimotor axonal neuropathy, and extrapyramidal symptoms. Additionally, cognitive deficits may be observed in certain individuals. The progression of this disorder is gradual, and by the second or third decade of life, the majority of patients may become dependent on a wheelchair for mobility(Marcilla Vázquez et al ., 2021).

We previously mentioned that PNKP is a modular protein characterized by three distinct domains: an amino-terminal FHA domain, a DNA phosphatase domain, and a DNA kinase domain (Bitarafan et al ., 2021). Various mutations impacting these domains have been implicated in MCSZ disorder. Notably, the pathogenic effects are predominantly associated with mutations occurring in exons 11 to 15, particularly exon 14 for MCSZ and exon 12 for AOA4 (Marcilla Vázquez et al ., 2021). Among the most frequently observed mutations in MCSZ is T424Gfs48X, which arises from a 17-bp duplication within exon 14 of the kinase domain, leading to a polypeptide of 471 amino acids, with the final 48 residues being out-of-frame. Additionally, Exon15Δfs4X results from a 17-bp deletion in intron 15, causing the mRNA

transcript to lack exon 15, which produces a polypeptide of 436 amino acids, with the last four residues also being out-of-frame. Furthermore, the L176F mutation, a point mutation in exon 5 (526C>T) within the DNA phosphatase domain, and the E326K base pair substitution in exon 11 (326G>A) lead to a non-conservative amino acid change within the DNA phosphatase domain (Bernstein et al ., 2005 & Shen J et al ., 2010).

The severity of the phenotype is likely linked with the specific types of mutations; however, the various phenotypes linked to mutations in PNKP do not seem to correlate with the mutation's location (Bras et al ., 2015). The homozygous variant, p. Thr424Glyfs*49 (c.1253_1269dup), found within the kinase domain of the protein, has been identified in individuals with MCSZ (Shen et al ., 2010)as well as in those with the neurodegenerative disorder AOA4 (Poulton et al., 2013). In the case of AOA4, (Bras et al ., 2015)reported the same PNKP mutation, c.1123G > T (p. Gly375Trp), in three families studied, and this mutation was subsequently noted in another patient (Rudenskaya et al ., 2019). All variants associated with AOA4 were located in or near the kinase domain of the protein, while mutations in the phosphatase or FHA domain have not been observed in AOA4 patients (Bras et al ., 2015). (Shen et al ., 2010) indicated that a splicing mutation correlates with more moderate symptoms of MCSZ and proposed a common homozygous region on chromosome 19q13 in exon 14 (1250_1266dup c.526 C > T) for four out of five families, which also recurred in the family described by (Poulton et al ., 2013). Other research identified additional regions affecting exons 11 and 15 primarily for MCSZ, noting that alterations in the mechanisms responsible for repairing double-strand breaks lead to microcephaly, whereas mutations that affect the integrity of single-strand break repair processes are associated with neurodegeneration ( Nair et al ., 2016).

The screening of PNKP mutations in our study involving various patients with Microcephaly, Seizures, and developmental disorders from the West Bank highlights the significance of this condition. The majority of samples were gathered based on clinical presentations observed in private clinics and the Hebron Charitable Rehabilitation Society in Hebron. This organization offers medical services to individuals with special needs and serves as a referral center, accommodating patients from across the West Bank. Our cohort exhibited dysmorphic craniofacial characteristics, including pronounced microcephaly, a depressed nasal bridge, a sloping forehead, and a short neck. Additionally, they experienced recurrent seizures (epilepsy),

delays in motor skill development such as sitting and walking, an inability to speak, loss of independent ambulation, and poor balance and coordination (ataxia). Furthermore, patient P10 presented with intestinal laziness, malabsorption, and chronic constipation, while patient P12 exhibited weakness, malabsorption, and a diminished appetite; these symptoms are predominantly associated with neurometabolic disorders. Nonetheless, additional metabolic assessments are necessary to establish a definitive diagnosis.

Diagnosing and managing Microcephaly with Seizures (MCSZ) in Palestine presents significant challenges due to a combination of genetic, healthcare infrastructure that faces several systemic challenges that impede the effective diagnosis and management of complex genetic disorders like MCSZ such as limited access to advanced genetic testing,comprehensive genetic services, including Whole Exome sequencing, are not widely available, making accurate diagnosis difficult, and resource constraints in which there is a shortage of specialized medical equipment and trained healthcare professionals, which hampers the provision of specialized care required for managing MCSZ. Socio-political factors such as movement restrictions and supply chain disruptions. Addressing these challenges requires a multifaceted approach such as capacity building by make an investing in healthcare infrastructure and training to enhance genetic diagnostic capabilities, international collaboration which partnering with global health organizations to facilitate access to advanced diagnostic tools and treatments, and finally policy advocacy working towards easing movement and import restrictions to improve healthcare access and resource availability.A comprehensive epidemiological data specific to Palestine is limited and detailed statistics on the number of affected individuals are not available.

The c.968C>T (P. Thr323Met) mutation, identified as rs372148913, occurs at location 19:50365689 within the PNKP gene. This missense mutation, found in our patient (P7) on exon 11, leads to a p. Thr323Met alteration in the PNKP protein. The affected residue is highly conserved and situated within the phosphatase domain. Notably, this mutation was present in a heterozygous state in both unaffected parents and other family members involved in the study. Previous reports have documented mutations in this domain in three patients, all exhibiting the MCSZ phenotype (Gatti et al ., 2019), while another patient with the same phenotype was noted in a separate study (Marcilla Vázquez et al ., 2021).

The variant c.968C>T p. (Thr323Met) has been found and validated in our proband. This alteration results in the replacement of the amino acid threonine with methionine at position 323 within the PNKP protein. Notably, this variant has neither been reported nor submitted to the ClinVar database (Marcilla Vázquez et al ., 2021). Consequently, the clinical implications and functional characterization of this variant have not been previously documented.

In last October 2024, the variant NM_007254.4: c.968 C > T: p. (Thr323Met), rs372148913 was identified as Likely pathogenic in the ClinVar database (Clinvar, 2024). While in our study, the substitution of threonine with methionine may impact the structure and functionality of the PNKP protein. Threonine, characterized by its hydroxyl functional group, is classified as uncharged amino acid and is subject to phosphorylation by threonine kinase. In contrast, methionine is a nonpolar uncharged amino acid that does not participate in phosphorylation. The distinct chemical properties of these two amino acids can influence the overall structure and function of the protein.

Other two female affected patients had approximately the same symptoms of P7, when attempting to screen c.968C>T p. (Thr323Met) on exon 11 on both them and their family members no results were found also no mutation results on exon 14 and 15, then efforts were directed to screen another mutation on other exons. Whole Exome Sequencing where these two patients showed no mutation in the PNKP gene. The similarity of the MCSZ phenotype to that observed in other disorders characterized by altered mechanisms of double-strand break repair can be identified as a contributing factor.

This finding substantiated the study's hypothesis, which posits that mutations resulting in the absence of PNKP within the nucleus lead to the persistent activation of the DNA damage response, ultimately causing the demise of susceptible brain cells, a potential contributor to MCSZ.

The study highlights the phenotypic and genotypic variability observed in patients with Microcephaly, Seizure, and developmental Delay (MCSZ), who possess various mutations in the PNKP gene. As previously noted, MCSZ is an autosomal recessive condition primarily transmitted through consanguineous unions. Such marriages increase the likelihood that both

carrier parents may pass on a homozygous mutation to their offspring, potentially resulting in a severely affected MCSZ phenotype. Consequently, there is an urgent necessity to enhance the methodologies employed by clinical genetic laboratories for the accurate detection and reporting of MCSZ cases, as well as to foster greater awareness among families regarding the implications of consanguineous marriages.

The implementation of measures to control and limit the emergence and transmission of new affected cases is crucial. This underscores the significance of genetic counseling in mitigation the transmission of hereditary diseases across generations. Achieving this objective necessitates a comprehensive awareness campaign regarding MCSZ, aimed at all segments of society, including healthcare professionals, educators, students, and families. It is imperative for physicians to remain vigilant in referring any patient exhibiting clinical signs of MCSZ for genetic counseling just as they would for other genetic disease . Additionally, the development of informational brochures detailing the disease, its modes of transmission, symptoms, and diagnostic procedures is advisable. Findings should also emphasize the importance of avoiding consanguineous marriages and encourage individuals to report cases to the appropriate health authorities rather than concealing them. Such transparency will enhance the accuracy of diagnoses and facilitate the documentation of cases, thereby allowing for a better assessment of the disease's prevalence and spread in Palestine. These straightforward initiatives could significantly alleviate the disease burden within our population.

# Chapter five

# Conclusion, Limitations, Recommendation and Future Plan

## 5.1 Conclusion

In this investigation study, we discovered a homozygous missense mutation in the PNKP gene, specifically NM_007254.4: c.968 C > T: p.(Thr323Met), situated in exon 11. This variant, which has been documented in previous literature, is linked to the microcephaly, seizures, and developmental delay (MCSZ) disorder. Findings provide initial data regarding the occurrence of this mutation within our population, highlighting the necessity for further deep research to enhance our understanding of the disease.

Our findings, in conjunction with earlier studies, underscore the clinical variability and epidemiological aspects of MCSZ disorder in Palestine, where there is a notably high carrier frequency of the PNKP mutation. This variability poses challenges for clinical diagnosis, thereby emphasizing the essential role of genetic testing in uncovering the root causes of this disorder. The established connection between PNKP mutations and autosomal recessive MCSZ has been

consistently validated in both research and clinical settings, further emphasizing its significance in the comprehension and management of this condition.

## 5.2 Limitations

This study encountered various limitations, mainly as arising from the infrequency of the disease and difficulties in securing collaboration from private clinics and the community for sample acquisition. The majority of samples were obtained from private clinics located in the West Bank, while certain demographic information was either not available or difficult to access. The most critical limitation was the small sample size, which was linked to the limited number of diagnosed cases, likely due to misdiagnosis or challenges in identifying and reaching affected individuals.

## 5.3 Recommendations

The infrequency and rarity of the disease, coupled with the existence of various mutations across different exons of the PNKP gene responsible for the condition, necessitates further research to yield significant findings. Accurate identification of PNKP mutations is strongly advised for the diagnosis of MCSZ disorder, as it serves as the most dependable indicator. This can be accomplished through sophisticated methodologies, including:

1. Mitochondrial DNA (mtDNA) testing: Implementing cell culture and transfection techniques.

2. mtDNA repair assay: Employing extra-large quantitative PCR (q-PCR).

3. Immunoprecipitation and Western blotting techniques.

4. Development of constructs and site-directed mutagenesis: Utilizing RNAi-resistant PNKP (cDNA).

These approaches highlight the importance of deepest future surveillance studies to reduce the likelihood of undiagnosed, raise awareness regarding the dangers of consanguineous marriages, and address the considerable implications for family and community health. Such initiatives are particularly vital, given that this disorder predominantly impacts infants, thereby emphasizing the critical need for timely intervention and educational programs to be conducted.

## 5.4 Future Plan

We will continue our efforts to cascade screening for Family I to identify a maximum number of MCSZ cases. In addition, we intend to explore other families in various regions of Palestine for this variant and, where possible, for other globally acknowledged variants. Moreover, we plan to organize community-based lectures to highlight the significance of preventing consanguineous marriages as a strategy to mitigate this disorder. These sessions will also aim to enhance awareness regarding the screening and treatment of genetic diseases.

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# Appendices

**Appendix 1:** Ethical Approval

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

**Research Ethics subcommittee of Faculty of medicine**

**Letter of Ethical approval**

Date:23/3/2024

Ref#: Re1-23-24

Dear Applicants: Dr. Kifaya Suleiman and Miss. Maysa Natsheh

Biochemistry and Molecular Biology master program

The Research Ethics subcommittee of faculty of medicine has recently reviewed your proposal entitled "Screening of mutation in Polynucleotide Kinase 3 Phosphatase gene causing Microcephaly, Seizures, and Developmental Delay in Palestine"
Your proposal is deemed to meet the requirements of research ethics subcommittee at Al-Quds University.

Note: This letter can be used to apply for the central Al-Quds University research ethics committee if needed

**Best of luck,**

**Dr. Suheir Ereqat**
**Head of research ethics subcommittee**
**Biochemistry and Molecular Biology master program**
**Faculty of Medicine-Al-Quds University**

P.O Box 20002

20002 ص.ب

**Appendix 2:** Consent form

## الموافقة على البحث

### عنوان الدراسة

فحص الطفرات في الجين المسؤول عن Polynucleotide Kinase 3' phosphatase- المسبب لصغر الرأس والنوبات وتأخر النمو في الاسرة الفلسطينية.

### الباحث الأولي

**الاسم**: ميساء "محمد عامر" نتشه

طالبة ماجستير في جامعة القدس- ابو ديس

**العنوان**: نمرة – الخليل – فلسطين

**الهاتف**: ٠٥٩٨٦٢٢٨٠٤

**عنوان البريد الإلكتروني**: maysaa98amer@gmail.Com

### الغرض من الدراسة

ندعوك للمشاركة معنا في دراسة بحثية تقودها الباحث/ة في (كلية الطب/ جامعة القدس- ابو ديس). نرجو منك قراءة هذه الوثيقة قبل أن تقرر المشاركة و سؤال الباحث/ة إذا كان هناك أي شيء غير واضح أو إذا كنت بحاجة إلى مزيد من المعلومات.

تهدف هذه الدراسة إلى فحص وجود طفرات في جين Polynucleotide Kinase 3' phosphatase (PNKP) في العديد من مرضى صغر الرأس والنوبات واضطرابات النمو العصبي (MCSZ) من فلسطين و هو مرض وراثي.

وتشمل الأهداف الرئيسية ما يلي:

١. تسليط الضوء على أسباب صغر الرأس والنوبات واضطرابات النمو العصبي.
٢. تحديد الطفرات المسؤولة عنMCSZ.
٣. دراسة الارتباط بين النمط الجيني والنمط الظاهري بين المرضى المصابين.
٤. التوصية بالاستشارات الوراثية للعائلات التي لديها افراد مصابون.

1

**الاجراءات**

سيتم اختيار المشاركين في هذه الدراسة بناء على معايير الشمول والاستبعاد: الافراد المشمولون: مرضى ذكور وإناث من مختلف الأعمار الذين لديهم الخصائص العامة ل MCSZ , والديهم و افراد العائلة لتحديد ما إذا كانوا حاملين لطفرة PNKP . تم استبعاد المرضى الذين يعانون من ميزات واضطرابات أخرى.

تتمحور الدراسة على استقبال المشاركين و عيناتهم بشكل رئيسي من جمعية التأهيل الخيرية في الخليل، إلى جانب حالات أخرى من العيادات الخاصة. كما سيتم جمع عينات من مناطق مختلفة في الضفة الغربية بالتعاون مع وزارة الصحة من خلال أنظمة المقابلات بمساعدة الكمبيوتر. نود منك أن تساهم في التبرع بكمية قليلة من الدم سواء كنت مريض في المراكز المذكورة او احد افراد عائلة المريض ( احد الوالدين و/ أو احد افراد العائلة) ثم الاجابة عن الاسئلة المرفقة في الاستبانة الورقية. سيتم عمل الإجراءات الجينية على كل عينة دم بما في ذلك استخراج الحمض النووي وتضخيم تفاعل البولیمیراز المتسلسل والتسلسل الجيني. سيتم ترميز جميع البيانات ثم إدخالها وتحليلها باستخدام الأداة الإحصائية (SPSS) و تحليلها باختبارات دقيقة لتقييم الاختلافات المحتملة بين المشاركين في الدراسة.

ستتطلب هذه الدراسة عشرون دقيقة فقط من كل مشارك ، وسيستغرق انجازها عشرة أشهر.

**المخاطر**

سيتم تنفيذ جميع بروتوكولات السلامة والبروتوكولات الفنية ، لذلك لا توجد مخاطر على صحة المريض أو والديه أو احد افراد العائلة.

**الفوائد**

قد تتحقق لك الفوائد التالية حيث توفر هذه الدراسة التشخيص المبكر لمرضى MCSZ من خلال دراسة جين PNKPالذي قد يكون مسؤولاً عن المرض بين السكان الفلسطينيين مما يسمح بفهم سبب ذلك المرض و بالتالي التقليل من أي مضاعفات تهدد الحياة. بالإضافة إلى ذلك ، تنصح هذه الدراسة بإجراء الاستشارة الوراثية وهو أمر ضروري لأي عائلة لديها فرد (أفراد) مصاب ب MCSZ, على وجه التحديد في العائلات الفلسطينية المنتشر فيها زواج الأقارب الذي تم تحديده تقريبا بمعدل ٤٠٪ بحسب الجهاز المركزي للإحصاء في فلسطين, وهذا بدوره يقلل من ظهور المرض بين أطفالهم.

**السرية**

يرجى عدم كتابة أي معلومات تعريفية.

سيبذل الباحث قصارى جهده للحفاظ على سريتك بما في ذلك ما يلي:

- استخدام الترميز للمشاركين وعيناتهم و سيتم استخدامه في جميع الملاحظات والوثائق البحثية فقط.

•حفظ الملاحظات ونسخ المقابلات وأي معلومات أخرى تحدد هوية المشارك في خزانة ملفات مقفلة في حوزة الباحث/ة الشخصية و لن تستخدم هذه البيانات في المنشورات او المقالات العلمية.

سيتم الحفاظ على سرية بيانات المشاركين إلا في الحالات التي يكون فيها الباحث ملزم قانونا بالإبلاغ عن حوادث محددة. تشمل هذه الحوادث ، على سبيل المثال لا الحصر ، حوادث سوء المعاملة وخطر الانتحار.

## معلومات الاتصال

إذا كانت لديك أسئلة أو استفسارات في أي وقت حول هذه الدراسة أو واجهت آثارا ضارة نتيجة للمشاركة في هذه الدراسة بالإمكان التواصل مع أي من التالية أسماؤهم :

اسم الباحث/ة أو الطالب/ة : ميساء" محمد عامر" نتشه على البريد الالكتروني: maysaa98amer@gmail.Com و/أو على الهاتف: ٠٥٩٨٦٢٢٨٠٤.

ويمكنكم التواصل مع مشرف الدراسة الدكتور/ة: كفاية عزمي سليمان الدكتور/ة في كلية الطب/جامعة القدس- ابو ديس، على الهاتف٠٥٢٢٩٧٥٣٥٩ أو البريد الالكتروني: ksuleiman@staff.alqusa.edu.

## المشاركة الطوعية

مشاركتك في هذه الدراسة طوعية. الأمر متروك لك لتقرر ما إذا كنت ستشارك في هذه الدراسة أم لا. إذا قررت المشاركة ، فسيطلب منك التوقيع على نموذج الموافقة. بعد التوقيع على نموذج الموافقة, لا يزال بإمكانك الانسحاب في أي وقت ودون إبداء السبب. فلو رغبت في الانسحاب من الدراسة بعد ذلك بإمكانك إعلامنا بذلك عن طريق الهاتف أو البريد الإلكتروني المذكورين اعلاه ولن يلحق بك أي ضرر مادي أو معنوي. الانسحاب من هذه الدراسة لن يؤثر على العلاقة التي تربطك بالباحث إن وجدت و إذا انسحبت من الدراسة قبل اكمال جمع البيانات , سيتم إرجاع بياناتك إليك أو إتلافها.

## موافقه

| رمز المشارك/العينة..... |
|---|

أقر أنا الموقع أدناه بأنّي قد وافقت على مشاركتي في دراسة فحص الطفرات في الجين المسؤول عن Polynucleotide Kinase 3' phosphatase- المسبب لصغر الرأس والنوبات وتأخر النمو في الاسرة الفلسطينية, وأنني على معرفة بالإجراءات المتبعة, وأني على علم بالفوائد والأخطار المترتبة على المشاركة, وأني كذلك على علم بالأشخاص الذين يمكن الاتصال بهم في حالة وجود أي أسئلة أو استفسارات أخرى. كما أنني حصلت على نسخة موقعة من هذا النموذج.

3

| | | |
|---|---|---|
| اسم المشارك/ة ..... | التوقيع ..... | التاريخ ..... |
| رقم الهاتف ..... | | رقم الهاتف البديل ..... |
| اسم شخص مقرب لديك لغرض التواصل معك في حال عدم التمكن من التواصل معك ..... | | رقم الهاتف ..... |

| | | |
|---|---|---|
| اسم ولي/ة امر المريض/ة ..... | التوقيع ..... | التاريخ ..... |
| رقم الهاتف ..... | | رقم الهاتف البديل ..... |
| اسم شخص مقرب لديك لغرض التواصل معك في حال عدم التمكن من التواصل معك ..... | | رقم الهاتف ..... |

| | | |
|---|---|---|
| اسم الباحث/ة ..... | التوقيع ..... | التاريخ ..... |

4

**Appendix 3:** Questioner

جامعة القدس

الدراسات العليا

كلية الطب

برنامج الماجستير في الكيمياء الحيوية والبيولوجيا الجزيئية

## استبيان عن صغر الرأس والنوبات وتأخر النمو

## (MCSZ)

إعداد: ميساء "محمد عامر " نتشه

(٢٠٢٤)

أنت مدعو للمشاركة في دراسة بحثية بعنوان **فحص الطفرات في الجين المسؤول عن Polynucleotide Kinase 3' phosphatase-المسبب لصغر الرأس والنوبات وتأخر النمو في الاسرة الفلسطينية**. تجري هذه الدراسة من قبل الباحثة ميساء "محمد عامر" ننتشه، استكمالا لمتطلبات الحصول على درجة الماجستير في كلية الطب في الكيمياء الحيوية والبيولوجيا الجزيئية من جامعة القدس- ابو ديس.

قبل أن تقرر المشاركة في هذه الدراسة، من المهم أن تفهم سبب إجراء البحث وما الذي سيتضمنه. يرجى قراءة المعلومات التالية بعناية. يرجى سؤال الباحث إذا كان هناك أي شيء غير واضح أو إذا كنت بحاجة إلى مزيد من المعلومات على هاتف رقم ٠٥٩٨٦٢٢٨٠٤ او على البريد الإلكتروني(maysaa98amer@gmail.com) .

ستستغرق الإجابة على كل سؤال أقل من دقيقتين تقريبًا، وقد تحتاج إلى ٢٠ دقيقة للإجابة على جميع الأسئلة.

**أهداف الدراسة**

الهدف الرئيسي من هذه الدراسة هو فحص طفرة PNKP في العديد من مرضى صغر الرأس والنوبات واضطرابات النمو (MCSZ) في فلسطين.

وتشمل الأهداف الرئيسية ما يلي:

١ .تسليط الضوء على أسباب صغر الرأس والنوبات واضطرابات النمو.

٢ .تحديد الطفرات المسؤولة عنMCSZ .

3 .دراسة الارتباط بين النمط الجيني والنمط الظاهري بين المرضى المصابين.

4.التوصية بالاستشارات الوراثية للعائلات التي لديها افراد مصابون.

عند مشاركتك في هذه الدراسة، نأمل أن توفر المعلومات التي تم الحصول عليها فائدة كبيرة لك أو للآخرين أو للمجتمع.

**موافقة**

لقد قرأت وفهمت المعلومات المقدمة و أتيحت لي الفرصة لطرح الأسئلة. أدرك أن مشاركتي طوعية وأنني حر في الانسحاب في أي وقت، دون إبداء سبب ودون تكلفة. أدرك أنه سيتم إعطائي نسخة من نموذج الموافقة هذا. أوافق طوعًا على المشاركة في هذه الدراسة.

توقيع المشارك/ة _____ التاريخ _____

توقيع ولي/ة الامر _____ التاريخ _____

**القسم (١): المعلومات الشخصية للمرضى**

يرجى قراءة كل سؤال بعناية ووضع علامة ( X) في المربع □ بجانب إجابتك:

س١- ما الجنس؟

□ ذكر
□ أنثى

س٢- ما هو العمر ؟

---

س٣- ما هو الوزن والطول؟

---

**القسم (٢): صغر الرأس والنوبات وتأخر النمو(MCSZ)**

هذه الحالة هي اضطراب نادر، وانتشارها غير معروف و هو مرض وراثي سببه وجود طفرة في جين PNKP.

س٤- هل الوالدان متزوجان زواج أقارب؟

□ نعم
□ لا

س٥- هل يوجد أفراد آخرين من الأسرة مصابون، كم عددهم وما علاقتهم بالمريض؟

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س٦- MCSZهي حالة تتميز بصغر حجم الرأس بشكل غير طبيعي ومشاكل عصبية تتعلق بضعف نمو الدماغ قبل الولادة.

وفيما يلي مجموعة من الأسئلة التي تشرح السمات و الاعراض العامة للمرض. يشترط قراءة كل سؤال بعناية ووضع علامة (X) مكان الإجابة التي تناسب اختيارك حسب الاعراض الظاهرة على المريض و خطورتها:

| المتغير | الرقم | الاسئلة | وجود الاعراض و خطورتها | | | |
|---|---|---|---|---|---|---|
| | | | نعم | | | لا |
| | | | طبيعي | خطر | شديد الخطورة | |
| الاعراض العامة للمرض | ١ | هل يعاني المريض من صغر حجم الرأس بشكل غير طبيعي ؟ | | | | |
| | ٢ | هل يعاني المريض من نوبات متكررة من الصرع؟ | | | | |
| | ٣ | هل يعاني المريض من تأخر في تطور المهارات الحركية مثل الجلوس والمشي؟ | | | | |
| | ٤ | هل تأخر كلام المريض؟ | | | | |
| | ٥ | هل يعاني المريض من إعاقة ذهنية ومشاكل سلوكية وعلى رأسها فرط النشاط؟ | | | | |
| | ٦ | هل يعاني المريض من ضعف التوازن والتنسيق (الترنح)؟ | | | | |

س٧- هل هناك اعراض أخرى تظهر على المريض ؟

☐ نعم
☐ لا

إذا كانت الإجابة بنعم يرجى توضيح الأعراض ----------
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س٨- بشكل عام، يُظهر المريض المصاب بـ MCSZ اضطرابات أيضية موروثة هي حالات طبية ناجمة عن تغيرات في جينات معينة تؤثر على عملية التمثيل الغذائي مما يؤثر سلبًا على توزيع المغذيات الكبيرة مثل البروتينات والدهون والكربوهيدرات في الجسم , هل يوجد لديك / او لدى المريض أي اضطراب ؟

☐ نعم
☐ لا

إذا كانت الإجابة بنعم يرجى ذكر الاضطراب ----------
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س٩- كيف تم تشخيص المريض؟

☐ بناء على الخصائص الخارجية و السمات العامة للمرض
☐ بالاعتماد على الاختبارات الجينية
☐ كلاهما

**فحص الطفرات في الجين المسؤول عن Polynucleotide Kinase 3' phosphatase– المسبب لصغر الرأس والنوبات وتأخر النمو في فلسطين**

**إعداد:** ميساء "محمد عامر" كمال نتشة

**المشرفة:** د. كفاية عزمي

**الملخص**

**المقدمة:** يعد صغر الرأس والنوبات وتأخر النمو (MCSZ) اضطرابًا نادرًا، وانتشاره غير معروف ويتم توريثه بطريقة جسمية متنحية لنقل الأمراض الجينية، وهو ناتج عن طفرات في جين PNKP المشارك في مسارات إصلاح الحمض النووي المتعددة التي تؤدي إلى تنشيط الاستجابة المستمرة لتلف الحمض النووي، الذي يؤدي إلى موت خلايا الدماغ الضعيفة وهو سبب محتمل لاضطرابMCSZ.يتميز MCSZ بصغر حجم الراس بشكل غير طبيعي (صغر الرأس) ومشاكل عصبية.

**هدف الدراسة:** فحص الطفرات في جين PNKP في العديد من مرضى صغر الرأس والنوبات وتأخر النمو (MCSZ)في فلسطين.

**طرق البحث:** تمت دراسة ثلاثة مرضى من عائلات مختلفة، الذين استوفوا معايير الاشتمال الخاصة بالدراسة و هي: (1) مرضى (كلا الجنسين من مختلف الأعمار) لديهم الخصائص العامة لـ (2)MCSZ . آباؤهم، خاصة إذا كانوا متزوجين زواج اقارب لتحديد ما إذا كانوا حاملين لطفرة في جينPNKP ،و/ أو افراد الاسرة. تم سحب عينات الدم للكشف عن وجود طفرات في جين PNKP باستخدام PCR و Sanger

Sequencingحيث تم فحص طفرات جين PNKP في الإكسونات (11 و14 و15)الأكثر انتشارا و شيوعًا.

**النتائج:** في هذه الدراسة تمكنا من ايجاد طفرة عند أنثى مصابة بصغر الرأس، وتأخر شديد في النمو، ونوبات صرع مبكرة، ناجمة عن طفرة متماثلة الزيجوت فيجين PNKPمن المحتمل أنها مسببة للمرض (NM_007254.4: c.968 C > T: p.(Thr323Met,وهو متغير في إكسون 11 في جين PNKP.وأظهرت نتائج فحص أقاربها من الدرجة الأولى أن هذا المتغير انتقل من والدها وأمها بمعنى انهم حاملين الطفرة من غير ان يظهر عليهم الاعراض. تم فحص أقاربها من الدرجة الأولى وتم رسم النسب. لم تظهر عينتا المريضتين الآخرتين أي طفرات في جين PNKP وتم إرسال عيناتهم إلى فحص Whole Exome Sequencing و لم ينتج لديهم أي طفرة في جينPNKP.

**الملخص:** نستنتج أن صغر الرأس والنوبات وتأخر النمو (MCSZ) لا يتم تشخيصه وعلاجه بشكل كافٍ بين سكاننا. لذلك تعد المعرفة العميقة بين العائلات تجاه زواج الأقارب والاستشارة الوراثية والفحص المتتالي عمليات مناسبة لتشخيصه والوقاية منه في وقت مبكر من الحياة. سيتم اعتبار نتائج هذه الدراسة كنتيجة أولية لهذا المرض بين سكاننا وما زالت هناك حاجة لمزيد من الدراسات.

**الكلمات المفتاحية:** مرض صغر الرأس والنوبات وتأخر النمو،PNKP،الاستشارة الوراثية.