Genetic Screening of Families with Neurological and Metabolic Disorders Using Next-Generation Sequencing

PHD THESIS

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DEDICATION

To my unwavering pillars of support, without whom this journey would have been a solitary climb, I dedicate this thesis.

To my family, for the boundless love, encouragement, and sacrifices that fueled my pursuit of knowledge. Your unwavering belief in my abilities sustained me through the challenges, and this achievement is as much yours as it is mine.

To my friends, whose camaraderie brought balance to the intense academic endeavors. Your laughter, shared struggles, and encouraging sessions turned what could have been a daunting path into a memorable journey.

To my mentors and advisors, thank you for your guidance, patience, and wisdom. Your expertise and encouragement molded my academic perspective, and I am grateful for the lessons learned under your mentorship.

This work stands as a testament to the collective strength of these connections, each thread contributing to the intricate tapestry of my academic achievement. Your roles in this endeavor do not go unnoticed, and I am profoundly grateful for the impact you've had on this academic chapter of my life.

With heartfelt gratitude,

Zantasha Khalid

DECLARATION

I certify that the work done on this Ph.D. Biotechnology research thesis titled "***Genetic Screening of Families with Neurological and Metabolic Disorders Using Next-Generation Sequencing***" has been solely conducted by me. All the material mentioned in the thesis is my original work and has not been plagiarized from any source. However, some tests and figures have been included in the thesis, which are appropriately referenced.

Zantasha Khalid

Date: 10.07.2025

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ABSTRACT

In the last three decades, inherited disorders characterized by autosomal recessive inheritance, particularly neurological and metabolic conditions, have been granted significant attention to investigating consanguineous Pakistani families in uncovering the genetic keystones of inherited disorders.

Neurological abnormalities can result in congenital or acquired disorders marked by cognitive impairment, seizures, paralysis, muscular and coordination abnormalities, and unstable disposition, encompassing conditions like autism spectrum disorders, intellectual disability, and microcephaly. Conversely, the human metabolic system involves the biochemical processes essential for growth, reproduction, DNA repair, and environmental interrelationship. Metabolism plays a crucial role in catabolism and anabolism. Dysregulation of metabolic pathways leads to enzyme deficiencies upsetting the chemical reactions within the body, collectively designated as metabolic disorders.

For this research, we enlisted eight multiplex and consanguineous families and conducted exome sequencing to pinpoint pathogenic variants accountable for their conditions. In ***Family A*** we identified a previously reported mutation in ***RNASEH2C*** (c.205C>T), in ***Family B*** we identified a single homozygous nonsense variant in ***ASPM*** gene (c.3978G>A), in ***Family C*** we identified a homozygous missense mutation in the ***COPBI*** (c.2693G>T) gene, no candidates were identified in ***Family D***, in ***Family E,*** missense variants in ***GPSM1*** (c.620C>T) and ***IARS*** gene (c.337A>T) were identified however they failed to cosegregate in the family. Within ***Family F***, potentially pathogenic variant was ***ZSWIM5*** (c.2509T>C) gene. Similarly, no candidates were identified in ***Family G*** and ***H.***

This study is the first to report the involvement of the ***COPBI*** (c.2693G>T) variant in the Pakistani population and the first to associate ***ZSWIM5*** with a neurological disorder. These findings highlight the critical importance of investigating diverse populations to uncover the genetic underpinnings of neurodevelopmental disorders. The identification of novel variants not only sheds light on rare genetic contributors to neurological conditions but also underscores the intricate genetic architecture of syndromic intellectual disabilities (ID). This work opens avenues for further research into the genetic diversity underlying neurodevelopmental disorders, ultimately advancing diagnosis and potential therapeutic strategies.

Key words: Consanguineous families, Exome sequencing, Neurodevelopmental disorders, Intellectual disability, Novel gene variants

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION & LITERATURE REVIEW

1.0 Introduction

1.1 Nervous System

The human nervous system is arguably the most intricate biological structure, facilitating communication between the brain and the body through signal transmission (Miller, 2009). To better understand its complexity, it is categorized into two primary components: The Central Nervous System (CNS) and the Peripheral Nervous System (PNS) (Tata et al., 2015).

1.2 Central Nervous System

The Central Nervous System (CNS) acts as the central hub for relaying and interpreting signals within the body. It serves as the origin of excitatory signals, which are then transmitted to the Peripheral Nervous System (PNS). The CNS is a highly sophisticated structure that includes the brain, spinal cord, and the optic, olfactory, and auditory systems (Mai & Paxinos, 2011). To communicate effectively with peripheral systems, the CNS works closely with the PNS to ensure coordinated bodily functions.

1.3 Peripheral Nervous System

The Peripheral Nervous System (PNS) consists of a complex network of nerves, receptors, and ganglia that actively transmit sensory and excitatory signals between the Central Nervous System (CNS) and peripheral structures. Moreover, the PNS is distributed throughout muscle tissues, making it one of the most extensive and multifaceted systems in the human body (Mai & Paxinos, 2011).

1.4 Structure of the PNS and CNS

1.4.1 Peripheral Nerve

Body tissues with an ample blood supply provide the optimal environment for the motor and sensory structures to form and sustain the peripheral nerve (Mai &. Paxinos, 2011).

1.4.2 Spinal Cord

Similarly, the spinal cord, another crucial part of the nervous system, is organized into two distinct regions. The central gray matter is composed of neuronal cell bodies, dendrites, axons, glial cells,

and capillaries. Surrounding and protecting this central area is the white matter, which primarily consists of glial cells and myelinated axons (Eccles, 1973).

1.4.3 Brain

The human brain can be seen as having three distinct parts: The forebrain, which further consists of the cerebrum, thalamus, hypothalamus, and pineal gland. Then we have the midbrain which has a brainstem further extending into the third region of the brain: the hindbrain which in addition to the brainstem also consists of the pons and cerebellum. The brainstem has the responsibility to connect the brain and spinal cord (Blakemore & Frith, 2005) (Figure 1.1 and 1.2).

Neurological Disorders

1.5 Neurological Disorders

The nervous system is involved in governing all functions of our body whether they are physiological functions or psychological aspects of our lives therefore, any defects in any component of the nervous system can contribute to disruption in how the body performs its functions ultimately evolving into wide-ranging neurological disorders. While some neurological disorders are subtle, others can render a human dependent on others for their day-to-day bearing (Chandra et al., 2006).

1.5.1 Causes of Neurological Disorders

Neurological disorders can manifest as either congenital or acquired disorders. Congenital neurological disorders are present at birth and may result from genetic abnormalities, prenatal injuries, or infections. Acquired neurological disorders, on the other hand, develop later in life due to factors such as trauma, infections, degenerative diseases, or environmental influences (Chaudhari & Ho, 2022).

1.5.1.1 Congenital Defects

1.5.1.1.1 Gene and Chromosomal Abnormalities

These are essentially inherited from parents as gene defects such as mutations leading to conditions like Cystic Fibrosis, Sickle-cell Anemia, and Phenylketonuria or chromosomal abnormalities which can either be structural defects or abnormal chromosomal numbers (less or more) leading to conditions such as Down Syndrome, and Turner Syndrome (Kaplanis et al., 2020; Berglund et al., 2020; Silberberg & Katabira, 2011).

1.5.1.1.2 Metabolic Disorders

Defects in any component of the metabolic system can have a devastating impact on the well-being of an individual. Imbalance in the chemical processes of the body can directly impact the brain and in many cases cause irreversible damage such as in Wilson disease and Gaucher disease (Silberberg & Katabira, 2011; Mulligan & Bronstein, 2020; Castillon et al, 2022).

1.5.1.1.3 Congenital Malformation

Congenital malformation results when genetic, environmental, and behavioral aspects interact and interrupt the normal functioning of the body systems (Silberberg & Katabira, 2011).

1.5.1.2 Pre/Perinatal Defects

1.5.1.2.1 Toxins and Environmental Factors

Fetal development is a very sensitive process and in the event of placental breach by neurotoxins such as alcohol, tobacco, or even some food additives, it can lead to development and intellectual defects that can compromise a child's chance at a healthy life (Chandra et al., 2006).

1.5.1.2.2 Nutritional Factors

Nutrients are crucial for a child's healthy development and growth. Adequate intake of nutrients, like folic acid, is vital because a deficiency can result in a reduced number of brain cells, leading to abnormal brain function (Chandra et al., 2006).

1.5.1.2.3 Infections

While the placenta offers protection to the developing fetus, some infections, including sexually transmitted infections, can infect and therefore interrupt the normal development of the fetus. This often results in a child being born with birth defects (Chandra et al., 2006).

1.5.1.2.4 Hypoxia

Oxygen through sufficient blood supply is an essential part of fetal development. In the event of a lack of sufficient blood flow, it can deprive the developing fetus of ample oxygen then it can result in neural damage (Chandra et al., 2006).

1.5.1.2.5 Complications during birth

In the event of a birth where the skull is underdeveloped, the risk of acquiring a physical injury during the process of birth is highly likely. There is also the likelihood of disrupted blood supply hence oxygen supply due to damage to the umbilical cord which can cause neurological defects (Kvalvik et al., 2020).

1.5.1.2.6 Premature birth

Premature births are indicative of fetal growth problems and increase the probability of impaired functions, especially those involving social skills, learning capacity, and communication skills (Chandra et al., 2006).

1.5.1.3 Acquired Defects

Most neurological disorders develop because of congenital errors however exploring the acquired causes after normal development as a contributor is no less important (Watkins et al., 2002).

1.5.1.3.1 Immune Disorders

Abnormalities in the immune system can result in autoimmune complications due to infections which can compromise the body and its functions and present challenges in leading a normal life. Such individuals are often observed to have conditions like seizures, and emotional and psychological abnormalities (Haider & von Oertzen, 2013; Lee & Choi et al., 2020).

1.5.1.3.2 Postnatal Infections

These infections often involve viral infections which essentially result in the inflammation of the brain. Depending on the severity of the infection, it can leave permanent defects in the individual (Haider& von Oertzen, 2013; Weimer et al., 2020).

1.5.1.3.3 Brain Injury

Severe trauma to the head leading to brain damage constitutes traumatic brain injury (TBI) which has three categories: (i) Closed head injuries where the damage is internal and not perceptible to the naked eye. (ii) Open wounds which involve exposure of the brain to a damaging object and (iii) Crushing injuries of the head resulting in brain damage (Haider& von Oertzen, 2013).

1.5.1.3.4 Spinal cord Injuries

Like the brain, injuries to the spinal cord can lead to neurological disorders, depending on the location of the injury and the bodily functions it regulates. Such injuries are often caused by car accidents or sports-related incidents (Haider & von Oertzen, 2013; Kirshblum et al., 2020).

1.5.1.3.5 Neoplasm

Like the rest of the body, tumors of a malignant or benign nature can originate in the brain or spinal cord. While it is a given that malignant tumors are the most dangerous; benign tumors are no less important when it comes to neurological consequences (Haider& von Oertzen, 2013). Benign tumors can strongly impact the pressure in the brain therefore damaging the organ and leaving long-term damages such as learning impairments, vision or hearing-related defects and even impairing the regular body movements among other defects (Boutry et al., 2020).

1.5.1.3.6 Toxins

Chemicals or toxins in the environment especially during childhood can also impair the growing individual neurologically. These appear as closed-head injuries (Silberberg & Katabira, 2011).

1.5.2 Prevalence of Neurological Disorders

Neurological disorders are widely known to impact brain-related, behavioral, and cognitive abilities of an individual which hamper functions like walking, speaking, learning, and moving rendering an individual dependent on someone else for their day-to-day activities and even, in many cases, basic survival (Feigin et al., 2021).

Neurological disorders are the most prevalent compared to other life-threatening disorders with approximately six hundred reported neurological disorders (Dumurgier & Tzourio, 2020). Globally, the prevalence rate has been reported to be 10.2%, in comparison, the casualty rate caused by these ailments is around 16.8% (Gautam & Sharma, 2020) which is why the World Health Organization considers neurological disorders a major health threat (Pan et al., 2021).

1.5.3 Types of Neurological Disorders

Defects in the neurological configuration of the nervous system can cause several wide-ranging congenital or acquired conditions. Essentially it manifests with characteristics such as impaired cognition, seizures, paralysis, muscle defects, coordination irregularities, and unstable disposition (Khan et al., 2019). As opposed to 1.4 in 1000 for autosomal dominant disorders, genetic disorders resulting from autosomal recessive inheritance patterns are known to affect approximately 1.7-5 in 1,000 neonates (Xiao & Lauschke, 2021).

1.5.3.1 Intellectual Disability

Intellectual disability (ID), also referred to as intellectual developmental disorder (IDD), involves impairments in intellectual and adaptive functioning resulting from developmental defects that occur early in life (Patel & Brown, 2017). ID can be categorized into syndromic and non-syndromic types and is a key characteristic of approximately 1,700 disorders associated with intellectual disability (Schalock, 2010; Maia et al., 2021). The severity of ID ranges from mild to severe and profoundly impaired cases, with a global prevalence of 1 to 3%. Severe and profound cases, which account for about 0.3 to 0.5% of all cases, are particularly significant for understanding the underlying causes of ID (Patel et al., 2020). Given its many associations, intellectual disability is

best understood through its medical, social, and functional dimensions, rather than through a rigid classification.

1.5.3.1.1 Medical Classification:

From a medical viewpoint, neurological disorders are defined as the debilitation in human performance on an individual or social level due to an underlying disease or health condition. This debilitation can affect various aspects of daily living, including motor skills, cognitive functions, emotional regulation, and social interactions. Effective management and rehabilitation strategies are crucial to help individuals maintain independence and achieve the best possible quality of life (Smart & Smart, 2006; Harris, 2006).

1.5.3.1.2 Social Classification:

On the contrary, social implications include the environmental and societal contributions toward disability (Smart & Smart, 2006; Harris, 2006).

1.5.3.1.3 Functional Classification:

The functional model entails standard sociocultural and environmental expectations about an individual's compulsions towards them (Smart & Smart, 2006; Harris, 2006).

Additionally, ID is also classified based on its severity:

1.5.3.1.4 Mild to Moderate Intellectual Disability:

Most individuals with intellectual disability have a mild form, where it is less likely to identify an underlying biological cause (Patel et al., 2020; Chiurazzi & Pirozzi, 2016).

1.5.3.1.5 Severe to Profound Intellectual Disability:

Individuals with severe to profound intellectual disability typically have an identifiable underlying biological cause, which is often detected after the onset of intellectual and adaptive deficits, usually occurring between infancy and adolescence (Patel et al., 2020; Chiurazzi & Pirozzi, 2016). Severe intellectual disability has been linked to a range of conditions including chromosomal and genetic abnormalities, congenital brain malformations, neurodegenerative diseases, inherited metabolic disorders, congenital CNS infections, maternal illnesses during pregnancy, in utero exposure to toxins, and birth injuries (Mitra, 2006; Maia et al., 2021).

In recent years, advances in exome sequencing have enabled researchers to identify several genes associated with intellectual disability. This technology has led to the discovery of numerous ID-related genes, including DDHD domain-containing 2 (*DDHD2*) (Schuurs-Hoeijmakers et al., 2012), methyltransferase-like 23 (*METTL23*) (Bernkopf et al., 2014), calpain 10 (*CAPN10*), serine/threonine/tyrosine interacting-like 1 (*STYXL1*), and solute carrier family 6, member 17 (*SLC6A17*) (Iqbal et al., 2015). Additionally, genes such as AT-rich interactive domain 1B (*SWI1-like*) (*ARID1B*) and DEAD box helicase 3, X-linked (*DDX3X*) may each account for more than 1% of ID cases (Patel et al., 2020). Despite these advances, the clinical and molecular diversity of intellectual disability means that only a partial genetic understanding of the disorder has been achieved (Moudi et al., 2022; Vissers et al., 2016).

1.5.3.2 Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a clinical term used to describe individuals who exhibit a range of phenotypic and genetic anomalies leading to impairments in social interaction, cognitive processes, behavioral patterns, and sensory activities (Lord et al., 2020). ASD is a heterogeneous neurodevelopmental disorder that can be diagnosed as early as 18 months of age and affects approximately 1 in 59 children worldwide (Theoharides et al., 2019; Matson & Kozlowski, 2011). Prominent features of ASD include compromised fine motor skills, metabolic defects involving amino acids or fatty acids, intellectual disability, and epilepsy, which can result in individuals relying on others for basic daily activities (Chen et al., 2022; Lauritsen, 2013).

The genetic basis of autism is complex, with a significant hereditary component (Havdahl et al., 2021). Monogenic disorders and chromosomal abnormalities are identified in at least 10% of ASD patients (Lin et al., 2020), but the underlying causes remain unknown for the majority, suggesting that the etiological diversity of autism may be impeding progress in identifying susceptibility variants in idiopathic cases. Exome sequencing studies have revealed an increased prevalence of copy number variations (CNVs) ranging from tens of thousands to several million nucleotides, confirming six notable risk loci: 1q21.1, 3q29, 7q11.23, 16p11.2-13, and 22q11.2 (Manoli & State, 2021). Similarly, these studies have identified a higher rate of potentially damaging de novo single nucleotide variations (SNVs), with several significant ASD-associated genes overlapping with these CNVs (Sanders et al., 2015).

Key genes frequently associated with autism include the oxytocin receptor (*OXTR*), N-methyl-D-aspartate receptor (*NMDA*; *GRIN2B*), gamma-aminobutyric acid (*GABA*) A receptor, beta 3 (*GABRB3*), engrailed homeobox 2 (*EN2*), serotonin transporter (*SLC6A4*), arginine vasopressin receptor 1A (*AVPR1A*), reelin (*RELN*), met proto-oncogene (hepatocyte growth factor receptor; *MET*), contactin-associated protein-like 2 (*CNTNAP2*), integrin beta 3 (*ITGB3*), *SCN2A*, *CHD8*, and *GRIN2B* (Satterstrom et al., 2020).

1.5.3.3 Microcephaly

Microcephaly is a neurological condition characterized by an abnormal head circumference that fails to reach a normal size. This can occur either congenitally, where a child is born with a smaller head, or postnatally, where the head fails to grow normally during development (Ribeiro et al., 2023; Asif et al., 2023). Medically, an individual is classified as microcephalic if their head circumference is >2 standard deviations (SD) below the mean for their age and gender, which affects approximately 2% of the global population (O'Connor, 2021). Severe microcephaly is defined as a head circumference >3 SD below the mean for age and gender, and it is present in about 0.1% of the global population (Jayaraman et al., 2018).

Microcephaly ranges from mild to severe and is often associated with mild to moderate developmental delays (Kempińska et al., 2022). The condition is characterized by a distinct facial phenotype, including upslanting palpebral fissures, a broad nose with a rounded tip, a long philtrum with a thin upper lip, a prominent chin, and prominent ears (Ostergaard et al., 2012).

Microcephaly is a complex condition that may have three possible origins: a) genetic, b) associated with syndromes - more than 800 syndromes and over 900 OMIM conditions have been linked to microcephaly; and c) induced by secondary factors that cause disruptions in neuronal development, such as toxins, metabolites or infections, leading to proportional microcephaly (Hanzlik & Gigante, 2017).

Studies conducted on microcephaly discovered that anomalous neuronal development and relocation involving genes might be important in disease pathology. Consequently, research on autosomal recessive primary microcephaly (*MCPH*) has revealed variations in distinct genes which are sufficient to cause microcephaly, often with the absence of any other phenotypes and a normal magnetic resonance imaging (MRI) (Hanzlik & Gigante, 2017). Currently 17 genes (*MCPH-17*) have been associated with Microcephaly essentially forming premature stop codons impairing functions and structures like: cell cycle progression or centromere abnormalities leading to

apoptosis. This information however, continues to grow with the advent of exome sequencing (Sokol & Lahiri, 2023).

Metabolic Disorders

1.6 Metabolic Disorders

The human metabolic system encompasses all the biochemical processes that are involved in growth, reproduction, DNA repair, and the body's ability to interact with the environment (Dai et al., 2020). Metabolism plays a crucial role in maintaining the body's ability to break down larger molecules into smaller ones through catabolism to produce energy or utilize energy to produce larger essential molecules in the body through anabolism. Any defect in the metabolic system can cause dysregulation in its chemical reactions, essentially leading to enzyme deficiency, which is collectively known as a metabolic disorder (Kivimaki et al., 2023; Xiong et al., 2023). This can further lead to secondary conditions associated with tissue defects in skeletal muscle, liver, adipose tissues, pancreatic islets, hypothalamus, vascular system defects, and defects in the nervous system (Kitamura, 2023).

1.6.1 Causes of Metabolic Disorders

Metabolic disorders can manifest due to genetic defects, organ dysfunction or even mitochondrial defects.

1.6.1.1 Genetic Defects

Metabolic disorders arise from genetic mutations affecting the genes involved in the biochemical processes of metabolism (Čolak & Pap, 2021). Most disorders related to intermediary metabolism are inherited in an autosomal recessive pattern, though there are also cases of X-linked recessive, autosomal dominant, or X-linked dominant inheritance. Defects in energy production through oxidative phosphorylation or the electron transport chain, or impaired glucose transport, can lead to brain damage or dysfunction. Alterations in gene expression may result in neurological conditions, such as Rett syndrome (Petriti et al., 2023). Additionally, abnormal neurotransmitter levels, as seen in serine disorders, or disturbances in substrate availability, such as those found in Phenylketonuria, can disrupt nervous system function (Kahler & Fahey, 2003).

1.6.1.2 Organ Dysfunction

Metabolic dysfunction can happen when an organ fails to perform its natural function, which disrupts the biochemical balance of the body. An example of this would be when the pancreas is unable to produce enough insulin to meet the body's demand, resulting in abnormal glucose levels (Macvanin et al., 2023).

1.6.1.3 Mitochondrial Defects

Mitochondria are responsible for providing energy to the cells of the body and are commonly referred to as the powerhouse of the cells. Mutations in the mitochondria or cell DNA, as well as environmental factors, can impact their integrity, rendering them incapable of functioning properly. This can cause defects such as myocardial damage (Lemos et al., 2023; Li et al., 2023).

1.6.2 Prevalence of Inherited Metabolic Disorders

Inherited metabolic disorders (IMDs) are a group of genetic diseases caused by impairments in specific biochemical pathways critical to the disease's pathophysiology (Vara & Rahman, 2022). Although each IMD is individually rare, their combined prevalence is estimated at 50.9 per 100,000 live births, with over 1,450 known disorders of this type (Tiivoja et al., 2022).

IMDs were first identified by Sir Archibald Garrod in the early 20th century through his research on alkaptonuria (OMIM: #203500). Newborn screening (NBS) began with phenylketonuria (PKU) (OMIM: #261600) in the 1960s and was significantly advanced with the introduction of tandem mass spectrometry in the 1990s (Ramoser et al., 2022). Tandem mass spectrometry has enabled the screening of a broad range of IMDs, facilitating earlier diagnosis and treatment (Sklyarov, 2022). The 21st century has seen further advancements with the development of various “-omics” technologies, such as exome sequencing (ES) and genome sequencing, which have improved the detection and diagnosis of new IMDs (Ramoser et al., 2022).

1.6.3 Types of Metabolic Disorders

1.6.3.1 Wilson Disease

Wilson's disease (WD) is an autosomal recessive disorder that disrupts copper metabolism in the body, leading to varying degrees of hepatic damage and neurological disturbances. It has an estimated global prevalence of 1 in 30,000, with higher incidence in consanguineous populations (Poujois et al., 2019).

Wilson's disease primarily manifests through copper accumulation in the liver and brain. Hepatic symptoms can vary from mild hepatitis to severe liver failure or cirrhosis, while neurological symptoms may include dystonia, tremors, dysarthria, and psychiatric disturbances (Fernando et al., 2020; Masełbas et al., 2019).

Copper is an essential trace element involved in several critical physiological processes. It interacts with various amino acids and proteins in the blood to maintain cellular copper balance and is

transported to the liver, a key organ for copper uptake, distribution, and excretion (Schroeder et al., 2021). Copper's redox potential allows it to act as a cofactor for numerous metabolic enzymes, including cytochrome c oxidase (essential for mitochondrial respiration), copper-zinc superoxide dismutase (important for antioxidant defense), lysyl oxidase (involved in collagen cross-linking), dopamine beta-hydroxylase (necessary for catecholamine production), and ceruloplasmin and hephaestin (crucial for iron metabolism) (Członkowska et al., 2018; Huster, 2010).

The *ATP7B* gene encodes a copper-transporting P-type ATPase that regulates copper metabolism in the liver. Mutations in the *ATP7B* gene, located on the long arm of chromosome 13 (13q14–q21), cause Wilson's disease by leading to copper-induced hepatotoxicity and neurological degeneration. The *ATP7B* gene was identified and cloned in 1993 (Palumbo & Schilsky, 2019), and over 300 mutations have since been identified (Sandahl et al., 2020).

1.6.3.2 Gaucher Disease

Gaucher disease (GD) is a genetic disorder that affects the lysosomes, leading to the buildup of lipids in the body. It is the most common type of lysosomal storage disorder worldwide, and consanguineous families are more susceptible to this condition (Fan et al., 2023). According to research, lysosomal disorders affect approximately one in every 5000 live-born infants (Nagral, 2014; Riboldi & Fonzo, 2019).

The lysosome is a cell organelle responsible for digesting and recycling large organic molecules. It performs this function through autophagy, where it processes materials generated within the cell, and phagocytosis, which involves breaking down substances acquired from the external environment (Settembre & Perera, 2023). Gaucher disease (GD) arises from a congenital deficit of the acid hydrolase glucocerebrosidase. This deficiency leads to the accumulation of glucosylceramide in macrophages, resulting in the formation of Gaucher cells (Hughes et al., 2019).

Gaucher disease is a condition that can present itself in different types.

1.6.3.2.1 Type 1 Gaucher Disease (GD1)

Type 1 Gaucher disease is a frequently occuring form and is characterized by painless enlargement of the liver and spleen, which can lead to significant abdominal distension. Additional symptoms include anemia, thrombocytopenia, fatigue, nosebleeds, and easy bruising, all resulting from a low blood cell count. This low count is due to hypersplenism, bone marrow infiltration by Gaucher cells, and an inherent hematopoietic defect. Symptoms can manifest from infancy through late adulthood

and are particularly prevalent among the Ashkenazi Jewish population (Elstein et al., 2022; Arturo-Terranova et al., 2025).

1.6.3.2.2 Type 2 Gaucher Disease (GD2)

Type 2 Gaucher disease (GD2), referred to as acute neuronopathic Gaucher disease or infantile cerebral Gaucher disease, is a rare and severe variant that primarily affects the nervous system (Weinreb et al., 2022). Representing about 1% of all Gaucher disease cases, GD2 is characterized by the rapid onset of severe neurological symptoms. The disease is typically fatal by the age of 2 years (Schiffmann et al., 2022).

1.6.3.2.3 Type 3 Gaucher Disease (GD3)

Type 3 Gaucher disease (GD3), also known as chronic neuronopathic Gaucher disease, is observed in approximately 5% of all Gaucher disease cases (Grabowski et al., 2021). This form is predominantly found in Northern Europe, Egypt, and East Asia, with a notably higher prevalence in the Swedish province of Norrbotten. GD3 typically has a later onset compared to GD2, though it can appear before the age of two and progresses slowly. While GD3 presents with milder neurological symptoms compared to GD2, it still affects the visceral organs and bone marrow, similar to Type 1 Gaucher disease (Daykin & Ryan, 2021).

Gaucher disease results from a deficiency in the lysosomal enzyme glucocerebrosidase, also known as acid beta-glucosidase (GBA) (Dardis et al., 2022). This enzyme is crucial for breaking down glucosylceramide, a key component of cell membranes. Normally, saposin C presents glucosylceramide to GBA, activating the enzyme. Proper breakdown of glucosylceramide is essential for maintaining cellular health (Arévalo et al., 2022). However, a deficiency in GBA leads to the accumulation of glucosylceramide and other glycolipids in lysosomes, particularly within macrophages. This buildup mainly occurs in the spleen, liver, bone marrow, brain, and osteoclasts, and to a lesser extent in the lungs, skin, kidneys, conjunctivae, and heart (Vieira & Schapira, 2021). In neuronopathic Gaucher disease, levels of glucosylsphingosine, a deacylated form of glucosylceramide, are elevated and correlate more strongly with disease severity than the accumulation of glucosylceramide itself (Horowitz et al., 2022).

Gaucher disease (GD) is an autosomal recessive disorder caused by mutations in the GBA gene, located on chromosome 1q21 and consisting of 11 exons (Malekkou et al., 2020). Over 200 mutations in the GBA gene are associated with GD (Mozafari et al., 2021). The mainstream mutations are single nucleotide substitutions, with the N370S missense substitution being the most

common. This mutation results in partial enzyme activity. The second and third most frequent mutations are the L444P substitution and the 84 GG substitution, respectively (Dimitriou et al., 2020). Individuals who are compound heterozygotes for these mutations are at increased risk for developing GD. Approximately 70% of all GD cases are attributable to the N370S and L444P mutations (Dardis et al., 2022).

1.7 Interconnection between Neurological and Metabolic Disorders

Neurological and metabolic disorders are often interconnected, with metabolic disturbances potentially leading to neurological symptoms and conditions, and vice versa. Here are some ways in which these two types of disorders are related:

1.7.1 Metabolic Disorders Leading to Neurological Symptoms

1.7.1.1 Inborn Errors of Metabolism:

Genetic disorders like phenylketonuria (PKU) or maple syrup urine disease result in the accumulation of toxic substances that can affect brain function, leading to cognitive impairment, seizures, and other neurological issues (Warmerdam et al., 2020).

1.7.1.2 Mitochondrial Disorders:

These disorders affect the mitochondria, the energy powerhouses of cells, leading to insufficient energy supply, which is particularly detrimental to the energy-demanding brain. Symptoms can include muscle weakness, developmental delays, and neurological decline (Schiller et al., 2020).

1.7.1.3 Hypoglycemia and Hyperglycemia:

Severe fluctuations in blood sugar levels can cause neurological symptoms (McCrimmon, R. J. 2021). Hypoglycemia (low blood sugar) can lead to confusion, seizures, and loss of consciousness, while chronic hyperglycemia (high blood sugar) in diabetes can lead to diabetic neuropathy and cognitive decline (Martínez-Piña et al., 2022).

1.7.1.4 Lipid Storage Diseases:

Disorders such as Tay-Sachs and Gaucher disease involve the accumulation of harmful substances in the brain and other tissues, leading to severe neurological symptoms like seizures, muscle rigidity, and developmental regression (Pará et al. 2020).

1.7.2 Neurological Disorders Influencing Metabolism

1.7.2.1 Hypothalamic Dysfunction:

The hypothalamus regulates many metabolic processes, including hunger, thirst, and body temperature (López et al., 2010). Damage or disorders affecting the hypothalamus, such as tumors or trauma, can lead to significant metabolic disturbances like obesity, diabetes insipidus, or thermoregulation issues (López. M, 2018).

1.7.2.2 Neurodegenerative Diseases:

Conditions such as Alzheimer's disease and Parkinson's disease can interfere with normal metabolic processes. For example, Alzheimer's disease has been associated with metabolic syndrome and insulin resistance, often termed type 3 diabetes (Lynn et al., 2022; Santiago & Potashkin, 2013).

1.7.2.3 Neuropathies:

Peripheral neuropathies, often resulting from metabolic disorders like diabetes, can lead to impaired sensory and motor function. This further complicates the metabolic condition by reducing mobility and activity levels, contributing to weight gain and worsening metabolic control (Jennekens, 1987).

1.7.3 Shared Pathophysiological Mechanisms

1.7.3.1 Oxidative Stress:

Both neurological and metabolic disorders can be linked to increased oxidative stress, which damages cells and tissues. For example, oxidative stress is a common feature in both diabetes and neurodegenerative diseases (Faria & Persaud, 2017).

1.7.3.2 Inflammation:

Chronic inflammation is another common pathway. Inflammatory processes are involved in the development of metabolic syndrome and also contribute to neurodegenerative processes (Xu et al., 2013).

1.7.3.3 Insulin Resistance:

Insulin resistance not only affects glucose metabolism but is also implicated in cognitive decline and neurodegenerative diseases (Grillo et al., 2019). The brain requires a steady supply of glucose, and impaired insulin signaling can adversely affect brain function (Arnold at al., 2018).

Understanding these relationships is crucial for diagnosing and managing conditions that span both metabolic and neurological domains. Effective treatment often requires a comprehensive approach addressing both metabolic and neurological aspects of a patient's health.

1.8 Disease Gene Identification Techniques

Identifying genetic causes of hereditary diseases can be done using various technological approaches. One such approach is linkage analysis, also known as homozygosity mapping (Pavan et al., 2012). This technique involves mapping the candidate region as closely as possible in larger families having many affected individuals. However, this method has some limitations, such as the existence of several genes in the defined region and genetic heterogeneity. To overcome these limitations, next-generation sequencing (NGS) has been recently developed. NGS is a more advanced technique that allows for faster and more accurate analysis of genetic data. With NGS, the entire genome can be sequenced in a short period, enabling the identification of the genetic cause of a disease more efficiently (Muzzey et al., 2015; Collins, 1995).

Linkage analysis is employed to map recessive traits in large consanguineous families. This technique utilizes naturally occurring variations, such as microsatellites or single nucleotide polymorphisms (SNPs), as DNA markers. These markers, which involve variations in the number of repeat base pairs or single base pair changes, are not located within functional genes but are found at specific, known locations within the genome. Prior to the availability of the human reference genome, targeted gene sequencing involved cloning the region of interest or the linked region, followed by sequencing of these clones. This approach, known as positional cloning, relies heavily on homozygosity mapping (Ali, 2017; Martin et al., 2014; Sukumaran et al., 2017).

Sanger sequencing, the traditional DNA sequencing method developed by Sanger and his colleagues in 1977, remains the gold standard for variant analysis. This technique allowed for the precise determination of nucleotide sequences in DNA molecules. With subsequent technological advancements, next-generation sequencing has emerged, enabling faster and more cost-effective re-sequencing of the human genome with greater accuracy. It is important to note that only 1% of the human genome encodes proteins, yet 85% of genetic mutations occur within these protein-coding regions. Despite this, around 3,000 Mendelian disorders still lack a specific genetic cause. Consequently, whole-exome sequencing or panel sequencing provides a valuable alternative for identifying genetic diseases beyond what can be discovered through homozygosity mapping (Sanger et al., 1977).

1.8.1 Next-Generation Sequencing

Next-generation sequencing (NGS), also known as high-throughput sequencing, involves the simultaneous sequencing of a large number of DNA molecules, a key feature shared by all NGS platforms. The process begins with the amplification of DNA, followed by sequencing on a flow cell using either repetitive polymerase chain reaction cycles or successive oligonucleotide ligation (Voelkerding et al., 2009; Behjati & Tarpey, 2013). Each NGS technique employs distinct protocols and chemistries. NGS has a wide range of applications, including whole exome sequencing (WES) and whole genome sequencing (WGS).

1.8.2 Whole Exome Sequencing

Advancements in whole exome sequencing (WES) and analytical tools have significantly expanded researchers' ability to identify causative variants for complex genetic disorders, including those beyond the more commonly known conditions (Zech & Winkelmann, 2024). Since most disease-causing variants are located in the exonic regions of the genome, WES is extensively utilized to screen for rare sequence variants within these protein-coding areas. This progress has enabled the discovery of novel candidate variants and genes associated with inherited disorders. Researchers have found WES to be exceptionally effective in pinpointing causative variants for complex genetic conditions that were previously challenging to diagnose (Seaby & Ennis, 2020).

1.8.3 Whole Genome Sequencing

Whole genome sequencing (WGS) is a cutting-edge application of Next-Generation Sequencing (NGS) that involves sequencing both coding and non-coding regions to cover the entire human genome. WGS is increasingly favored for its ability to generate vast amounts of data at relatively low sample processing costs. Research has indicated that non-coding variants and introns can influence copy number variations, which may contribute to various neurodegenerative diseases. WGS offers comprehensive and unbiased data coverage, making it a superior option to Whole Exome Sequencing (WES) for resolving complex patient data. As sequencing costs continue to decrease, WGS is expected to find even broader applications in the future (MacArthur et al., 2014).

Given the complexity of neurological and metabolic disorders, Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) techniques are essential in advancing our knowledge for managing these disorders (Souche et al., 2022; Chung et al., 2020). Analyzing the entire genome or exome provides comprehensive data for identifying essential genetic variants and detecting genetic risk factors at an early stage. These tools are crucial in developing an accurate diagnosis, treatment

plans, and genetic counseling for patients and their families (Chung et al., 2023). Additionally, these tools assist in identifying novel genetic variations that offer valuable insights into the disease pathophysiology and novel therapeutic targets (Woerner et al., 2021). In conclusion, by identifying the complexities of these disorders at the molecular level, these tools open up vast possibilities for curbing the impact of these life-degrading disorders (Stranneheim et al., 2021).

It is estimated that genetic factors contribute to around 30-40% of the disease burden, with about 62% of cases having an autosomal recessive pattern (Jamra, 2018). The development of Next Generation Sequencing (O'Roak et al., 2011) has been incredibly useful in understanding the diverse genetic influences that can lead to neurological complications.

To gain deeper insights into the genetic defects linked to various neurological disorders, Whole Exome Sequencing was utilized, followed by conventional Sanger sequencing to examine candidate genes associated with the disease phenotype. The study involved eight families, with the following conditions represented: two families with microcephaly, three with intellectual disability, one with autism spectrum disorder, one with Wilson's disease, and one with Gaucher disease.

1.9 Aims and Objectives

1. To enlist families coping with Neurological and Metabolic Disorders.
2. To establish a liaison between the clinical manifestations and the inheritance patterns.
3. To identify the disease-causing genes involved in these disorders to reveal their genetic origins.
4. To assess the relationship between specific mutations in candidate genes and their corresponding phenotypes using Next Generation Sequencing (NGS).

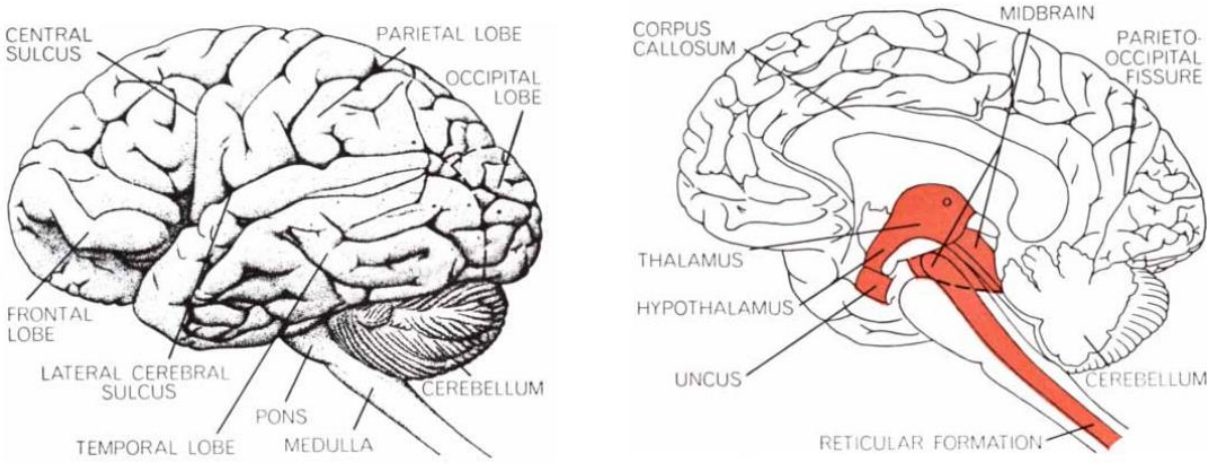

Figure 1.1 Diagrammatic representation of the anatomy of the brain (Luria, A. R., 1997).

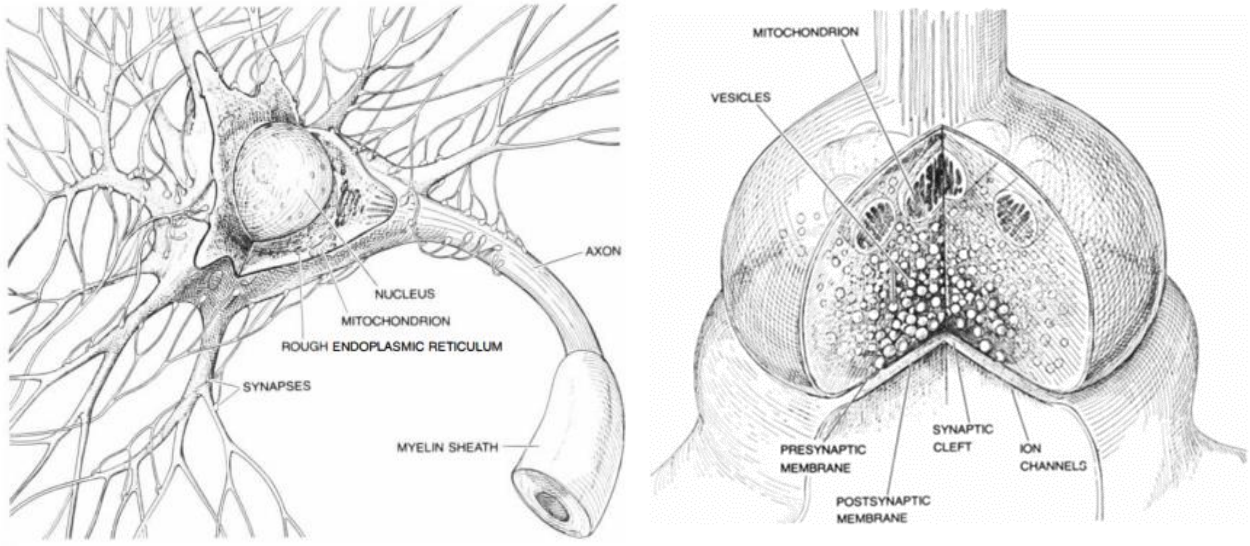

Figure 1.2 Diagrammatic representation of cell body of a neuron (Left) and synapse (Right) (Steven, C. F., 1979).

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Ethics Statement

This study was initiated following authorization from the Institutional Review Board of International Islamic University, Islamabad, Pakistan. Prior to the commencement of laboratory work, the necessary COSHH (Control of Substances Hazardous to Health) risk assessment forms were completed and signed at the Research, Innovation, Learning, and Development (RILD) Wellcome Wolfson Centre, Royal Devon & Exeter NHS Foundation Trust, and the Medical University of Exeter in the United Kingdom. Families participating in the study were provided with information in their local languages, and written approval was acquired from parents or guardians.

2.2 Recruitment of Subjects and Overview of Sequencing Approach

In this study, eight consanguineous Pakistani families affected by neurological and metabolic disorders were recruited. These families were from remote regions of Pakistan. Detailed interviews were conducted with the parents or guardians of the patients to collect comprehensive medical histories. Based on this information, pedigrees were constructed according to the methods described in Bennett et al. (1995). Various genetic studies were then performed, including Sanger sequencing, whole exome sequencing, bioinformatics analyses, cosegregation analysis, and control analyses, in collaboration with partners at the University of Exeter Medical School.

2.3 Clinical Evaluation

The pre-clinical evaluation was conducted using a standard proforma, which was included in the appendix. The medical history of each proband was obtained from their parents or guardians before and after birth to rule out any responsible environmental factors. Digital pictures of all patients were taken to analyze the presence of dysmorphic features. Additionally, the age, weight, height, and head circumference of all available members of each family were recorded for analysis. Neurological symptoms and brain dysmorphia were evaluated using MRI and CT scans in the proband of each family.

2.4 Collection of Blood Samples

Samples, including blood and buccal swabs, were collected from affected individuals, siblings, and other family members with informed consent. Adults had their blood drawn in EDTA tubes using

5mL syringes, while children under the age of 2 had their blood collected using butterflies and standard potassium EDTA vacutainer tubes. Written consent was obtained from each healthy participant, as well as from the parent or guardian of each patient with an intellectual disability (ID).

2.5 Genomic DNA Extraction

2.5.1 Human Genomic DNA Extraction from Whole Blood

Peripheral blood samples were collected from family members. DNA was then extracted using the phenol-chloroform method (Sambrook et al., 1989) and quantified following established procedures.

2.5.1.1 Phenol-Chloroform Method

DNA was extracted using the standard phenol-chloroform method for DNA extraction (Wagner & Goldstrohm, 2019) and was quantified using the Qubit® 2.0 Fluorometer (Invitrogen Inc., CA, USA).

2.5.1.2 Genomic DNA Extraction by Kit Method

Blood samples from affected families were stored at -20°C, thawed, and mixed before DNA extraction using the ReliaPrep™ Blood gDNA Miniprep System. The process involved adding 20 µL of proteinase K and 200 µL of whole blood to a microcentrifuge tube, followed by vortexing and incubation at 56°C for 10 minutes. After adding 200 µL of Cell Lysis Buffer and vortexing, the lysate was briefly centrifuged.

Then, 250 µL of Binding Buffer A was added, vortexed, and the mixture was transferred to a Reliaprep binding column for centrifugation at 13,000 rpm for 1 minute. The column was washed three times with 500 µL of Column Wash Solution, discarding the flow-through each time.

Finally, DNA was eluted with 200 µL of nuclease-free water and collected by centrifugation. The DNA concentration and purity were measured using a NanoDrop spectrophotometer, and the DNA was diluted to a concentration of 10-30 ng/µL.

2.6 Spectrophotometry

Spectrophotometry was used to evaluate the integrity of the isolated DNA. The wavelength was set to 280nm, 230nm, 260nm, and 320nm to determine the absorbance ratios, A260/A280 and A260/A230. The purpose was to identify any impurities in the DNA that may have resulted from the extraction process.

2.7 Exome Sequencing

Whole Exome Sequencing (WES) was conducted on high-quality genomic DNA extracted from one affected individual per family. DNA libraries were prepared using the Agilent SureSelect Human All Exon V6 kit, following the manufacturer's protocol. Sequencing was performed on the Illumina HiSeq 2500 platform using 150 bp paired-end reads. The raw sequence data were aligned to the GRCh37/hg19 human reference genome using BWA-MEM v0.7.17 (http://bio-bwa.sourceforge.net/). PCR duplicates were marked and removed using Picard tools v2.15 (https://broadinstitute.github.io/picard/). Downstream processing, including base quality score recalibration and indel realignment, was performed using GATK v3.7.0. Variant calling was conducted with GATK HaplotypeCaller (https://gatk.broadinstitute.org/), and only variants passing the standard hard filters set by GATK were retained for further analysis.

Variant annotation was carried out using Alamut batch v1.10 and SnpEff (http://snpeff.sourceforge.net/SnpEff_manual.html), and candidate variants were filtered based on their functional consequence (e.g., nonsynonymous, splice site, frameshift, stop-gain/loss), read depth of at least 10×, Phred quality score ≥30, and mapping quality ≥40. Variants with a minor allele frequency (MAF) of less than 0.01 in major population databases, including gnomAD, ExAC, and the 1000 Genomes Project, were prioritized. The average sequencing depth was approximately 100×, with more than 95% of targeted exonic regions covered at 20× or greater. All raw and processed sequencing data were stored on a secure institutional server, and data files are available upon reasonable request to ensure transparency and reproducibility.

2.8 Designing Primers

Gene sequences were sourced from the Ensembl Genome Browser (GRCh37 assembly, Feb 2009) (http://www.ensembl.org/Homo_sapiens/Info/Index?db=core). PCR primers were designed using Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/), with the following design criteria:

1. Primer Length and Specificity: Primers were extended to 20-24 bases, with annealing temperatures adjusted to ensure specificity.

2. Melting Temperature: Primers were chosen to have a melting temperature of 59-62°C to enhance PCR efficiency and productivity.

3. GC Content: The GC base count was maintained between 40-60% when possible.

4. Secondary Structures: Primers with more than 3 bases of inter/intra-primer binding potential were avoided to prevent secondary structure formation and primer dimers.

5. Sequence Matching: Primers were designed to have a 100% match to the target sequence to ensure accurate amplification.

6. Bioinformatics Analysis: UCSC Genome Browser was used for BLAST searches and in silico PCR analysis (http://genome.ucsc.edu/).

2.9 Sanger Sequencing

To validate the identified candidate variants and assess their segregation within each family, Sanger sequencing was performed. Polymerase chain reaction (PCR) amplifications were carried out in 25 µL reactions containing 50 ng of genomic DNA, 10× FastStart PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, and 1 U of FastStart Taq DNA Polymerase (Roche). Thermal cycling was conducted using an ABI Veriti Thermal Cycler under standard cycling conditions. The amplified PCR products were then purified using ExoSAP-IT (Thermo Fisher Scientific), with enzymatic digestion at 37°C for 15 minutes followed by inactivation at 80°C for another 15 minutes.

Bidirectional sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit, and the resulting products were analyzed on an ABI 3730xl DNA Analyzer. The electropherograms were processed and visualized using Sequencer v5.4.6 software. Variants were confirmed by comparing the sequences of the affected individuals to those of their unaffected family members, ensuring precise segregation patterns consistent with the inheritance models observed in each pedigree.

Table 2.1 Reaction mixture for one tube.

No.	Reagents	Amount µL
1.	Forward Primer	0.4
2.	Reverse Primer	0.4
3.	dNTP (5mM)	0.4
4.	Dream Taq Green Buffer	1.0
5.	Dream Taq polymerase	0.1
6.	Double distilled water (ddH_2O)	6.9
7.	DNA (control & sample)	0.8
	Total	**10**

Table 2.2 PCR Cycle Conditions.

Steps	Segment	Temperature (°C)	No. of Cycles	Time (Minutes)
1.	Denaturation Annealing Extension	95 TD +4 72	2	30
2.	Denaturation Annealing Extension	95 TD +2 72	2	30
3.	Denaturation Annealing Extension	95 TD +2 72	45	30

Table 2.3 Composition of EXOSAP solution.

No.	Reagents	Volume (µL)
1.	Exonuclease-I	2.5
2.	Shrimp Alkaline Phosphatase	25
3.	dH_2O	972.5

Table 2.4 EXOSAP Programme Conditions.

EXOSAP Programme	Temperature (°C)	Time (Minutes)
	37	30

Table 2.5 Components of Sequencing Amplification.

No.	Reagents	Volume (µL)
1.	Forward/Reverse primer	1.0
2.	Purified PCR product	2.0
3.	Big dye	1.0
4.	Big dye buffer	2.0
5.	ddH_2O	4.0
6.	Total	10

Table 2.6 Conditions for Sequencing Reaction.

No.	Segment	Temperature (°C)	Time	Cycles
1.	Denaturation	95	30 secs	25
2.	Annealing	50	15 secs	
3.	Extension	60	4 mins	

Table 2.7 Components of a 50µL PCR reaction.

No.	Component	Volume (µL)
1.	Primer mix 45mM	1.0
2.	50xdNTP mix	1.0
3.	Recombinant RNase inhibitor (400/µL)	0.5
4.	Thermostabilizing reagent	25.0
5.	GC melt	10.0
6.	Oligo (dT) primer	1.0
7.	50x Titanium Taq RT enzyme mix	1.0

Chapter 3

Results

Families with Neurological Disorder

(Family A-F)

3.0 Results

3.1 Family A

A large, multi-generational, consanguineous family (Family A) from a remote region of Khyber Pakhtun Khuwa (KPK) province, Pakistan, was recruited for the study. The family was visited at their residence, and the pedigree analysis revealed an autosomal recessive pattern of inheritance for microcephaly. Blood samples were collected for genetic analysis from two affected individuals (V-3 and V-4) as well as from three healthy individuals (IV-8, IV-9, and V-5) (Figure 3.1).

3.1.1 Clinical Findings

AGS family has two affected females (V-3 and V-4) and a normal male sibling (V-5) born to a normal consanguineous Pakistani couple (IV-8 and IV-9) (Figure 3.1 & 3.2). The oldest sibling (V-3) was a normal birth after 36 weeks of conception and displayed signs of microcephaly since birth. The second born (V-4) was a normal birth and displayed no signs of neurological inadequacy until she was two and a half months old. The head circumference at the time of sampling was 41cm and 42cm; and age was 7 years and 2 years, respectively. The clinical features of both affected individuals include microcephaly, dysmorphic features, seizures, no motility, poor vision, spasticity, and profoundly compromised visual, hearing, and motor skills functions which have since advanced progressively since birth. Based on observation over detailed interviews, it was established that both individuals suffer from severe intellectual disability (Table 3.1).

3.1.2 Whole Exome Sequencing

Whole exome sequencing (WES) was performed on DNA from one affected individual (V-4) using the Agilent 2100 Bioanalyzer and Illumina HiSeq2000. Exome enrichment was done with SureSelect Human All Exon V4, targeting a 30X mean read depth. Data were aligned with BWA-MEM, and further processed with Picard and GATK for variant calling and quality recalibration. SNVs and indels were identified with GATK HaplotypeCaller, and annotations were added using DNAnexus. Filtering focused on rare non-synonymous or splice variants with <0.01 frequency in control databases.

The analysis identified a homozygous missense variant, c.205C>T (p.Arg69Trp), in exon 2 of the *RNASEH2C* gene. Allele-specific primers were designed with Primer3, and PCR with dideoxy sequencing confirmed that this variant co-segregated with the disease in the family.

3.2 Family B

A four-generation consanguineous family (Family B) from a remote area of Khyber Pakhtun Khuwa (KPK) province, Pakistan, was recruited for the study. The family was visited at their residence. The pedigree analysis revealed an autosomal recessive inheritance pattern for microcephaly. For genetic analysis, blood samples were collected from both affected individuals (IV-3 and IV-11) and healthy individuals (III-1, III-2, IV-1, IV-2, IV-4, IV-5, IV-7, IV-8, IV-9, and IV-10) (Figures 3.4 & 3.5).

3.2.1 Clinical Features

Affected members IV-3 and IV-11 were diagnosed with microcephaly at birth, following an autosomal recessive inheritance pattern within a consanguineous Pakistani family (Figure 3.4). Both individuals were delivered normally and initially showed no signs of neurological deficits. However, by the age of one, they exhibited a sloping forehead and severe intellectual disability. Both patients demonstrated developmental delays, speech impairments, and no notable facial dysmorphism or additional neurological deficits. Significant delays in walking and signs of hypertonia were also observed. Detailed interviews and observations confirmed that both individuals suffer from severe intellectual disability (Table 3.2).

3.2.2 Whole Exome Sequencing

Whole Exome Sequencing (WES) was performed on DNA from one affected individual (IV-3) using the Agilent 2100 Bioanalyzer/Illumina HiSeq2000. Exome enrichment was achieved with the SureSelect Human All Exon V4 kit, targeting a 30X mean read depth. Data were aligned with BWA-MEM, and processed with Picard and GATK for variant calling and quality recalibration. Custom annotations were done with DNAnexus.

Filtration focused on rare non-synonymous or splice variants with <0.01 frequency in gnomAD, ExAC, and the 1000 Genomes Project. The analysis identified a homozygous nonsense variant, c.3978G>A (p.Trp1326*), in exon 17 of the *ASPM* gene. Allele-specific primers designed with

Primer3 and PCR/dideoxy sequencing confirmed this variant co-segregates with the condition, being homozygous in affected individuals and heterozygous in asymptomatic ones.

3.3 Family C

A four-generation consanguineous family C was recruited from a remote area in the South Punjab province of Pakistan. The family was visited at their residence, where it was observed that the pedigree demonstrates an autosomal recessive inheritance pattern for intellectual disability. For genetic analysis, blood samples were collected from both affected individuals (IV-1 and IV-4) as well as healthy individuals (III-2, III-3, and IV-3) (Figures 3.7 & 3.8).

3.3.1 Clinical Features

Our study investigated a consanguineous family from Pakistan with two affected male siblings, IV-1 and IV-2. Individual IV-1 was assessed at 12 years of age. He was born full-term, with a birth weight of 2.6 kg. At the time of evaluation, IV-1 measured 139 cm in height and weighed 31 kg, with a head circumference of 43 cm. He exhibited severe intellectual disability and was non-verbal, reflecting significant speech and developmental delays. IV-1 was unable to bear weight or walk independently, highlighting his motor limitations. Additionally, he experienced seizures, hypotonia, and behavioral issues, which impacted his daily functioning and facial dysmorphism (micrognathia). Notably, he presented with severe cataracts from birth.

Individual IV-2 was assessed at 11 years of age and was also born full-term, weighing 2.7 kg at birth. During evaluation, IV-2 measured 126 cm in height and weighed 27 kg, with a head circumference of 42 cm. Similar to his brother, he displayed severe intellectual disability and was non-verbal, demonstrating pronounced developmental delays. IV-2 faced comparable motor challenges, being unable to bear weight or walk independently. He also experienced seizures, hypotonia, facial dysmorphism (micrognathia), and behavioral problems, which significantly affected his social interactions and quality of life. Unlike his brother, IV-2's severe cataracts developed progressively after the age of 2 (Table 3.3).

Despite the significant challenges they face, including severe intellectual disabilities and other clinical manifestations, the family has not pursued medical intervention due to difficult socioeconomic circumstances, which limit their access to necessary healthcare services.

3.3.2 Whole Exome Sequencing

Whole exome sequencing (WES) was conducted on DNA samples from both siblings using the Agilent 2100 Bioanalyser/Illumina HiSeq2000 platform, following the acquisition of informed consent.

Disease-Phenotype-driven data filtration was applied to identify rare non-synonymous exonic or splice site variants with a population frequency of <0.01 across control databases, including gnomAD, ExAC, and the 1000 Genomes Project. This left us with nine homozygous variants among which two candidates were the most compelling to explain the phenotype: missense homozygous mutations in *INTS1* (Chr7(GRCh37):g.1525021C>A; NM_001080453.3:c.3061G>T; p.Asp1021Tyr) and *COPB1* (Chr11(GRCh37): g.14480187C>A; NM_001144061.1:c.2693G>T; p.Arg898Leu).

While the *INTS1* variant did not co-segregate, Sanger sequencing confirmed the presence of a homozygous missense variant in *COPB1* (Chr11(GRCh37): g.14480187C>A; NM_001144061.1:c.2693G>T; p.Arg898Leu) in the affected siblings. Both parents and the unaffected sibling were found to be heterozygous for this variant, consistent with the hypothesis that homozygosity for p.Arg898Leu leads to the Baralle-Macken syndrome phenotype.

3.4 Family D

A four-generation consanguineous family (Family D) was recruited from a remote area in the Khyber Pakhtun Khuwa (KPK) province of Pakistan. The family was visited at their home, where the pedigree revealed an autosomal recessive inheritance pattern of intellectual disability. For genetic analysis, blood samples were collected from all four affected individuals (IV-1, IV-2, IV-4, and IV-5) as well as from healthy individuals (III-1, III-2, and IV-3) (Figures 3.10 & 3.11).

3.4.1 Clinical Features

Family D is a consanguineous Pakistani family with four members (IV-1, IV-2, IV-4, and IV-5) who exhibit severe intellectual disability, along with global developmental delays and significant behavioral issues such as aggression, hyperactivity, speech impairment, and an inability to recognize family members or acquire new skills. All affected siblings were born through normal delivery at full term. Despite achieving the ability to walk by age 5, they failed to meet other

cognitive milestones and are observed to be lethargic, physically weak, and disinterested in their environment. In contrast, one sibling, IV-3, is unaffected (Table 3.4).

3.4.2 Whole Exome Sequencing

Whole exome sequencing (WES) was performed on DNA from affected individual IV-1 using the Agilent 2100 Bioanalyzer and Illumina HiSeq2000, with exome enrichment by SureSelect Human All Exon V4 at 30X read depth. Data were aligned with BWA-MEM and processed with Picard and GATK for variant calling and quality control. Custom annotation was done with DNAnexus. Data filtering targeted rare non-synonymous or splice variants with <0.01 frequency in gnomAD, ExAC, and 1000 Genomes Project, but no homozygous variants of interest were found.

3.5 Family E

A five-generation, multi-nuclear consanguineous family (Family E) was recruited from a remote area of the KPK province of Pakistan. The family was visited at their residence. The pedigree analysis revealed an autosomal recessive pattern of inheritance for intellectual disability. For genetic analysis, blood samples were collected from all five affected individuals (IV-1, IV-4, IV-6, IV-11, and IV-14) as well as from healthy individuals (III-9, III-10, III-12, III-13, IV-2, IV-3, IV-5, IV-8, and IV-9) (Figure 3.13).

3.5.1 Clinical Features

Family E is a large consanguineous family from Pakistan, comprising five individuals (IV-1, IV-4, IV-6, IV-11, and IV-14) who exhibit a range of symptoms including moderate to severe intellectual disability, global developmental delay, hypotonia, and behavioral issues such as aggression, hyperactivity, speech impairment, and difficulty in recognizing family members and learning. In the first branch of the family (IV-1, IV-4, and IV-6), individuals have been observed with severe intellectual disability and febrile seizures from infancy. The remaining two members (IV-11 and IV-14) present with moderate intellectual disability since birth (Table 3.5).

3.5.2 Whole Exome Sequencing

Whole Exome Sequencing (WES) on DNA from affected individual IV-1 used the Agilent 2100 Bioanalyzer and Illumina HiSeq2000, with exome enrichment via the SureSelect Human All Exon

V4 kit at 30X read depth. Data were aligned with BWA-MEM, processed with Picard and GATK, and annotated using DNAnexus.

The analysis identified two candidate variants: c.620C>T (p.Ala207Val) in *GPSM1* and c.337A>T (p.Ile113Phe) in *IARS*. Allele-specific primers from Primer3 and PCR/dideoxy sequencing were used to check co-segregation, but both variants showed inconsistent co-segregation patterns among affected and asymptomatic family members.

3.6 Family F

A four-generation consanguineous family (Family F) was recruited from a remote area in the Khyber Pakhtun Khuwa (KPK) province of Pakistan. The family was visited in their residential area, and the pedigree analysis revealed an autosomal recessive inheritance pattern of Autism Spectrum Disorder (ASD). For genetic analysis, blood samples were collected from all affected individuals (IV-1, IV-2, IV-3, and IV-6) as well as from healthy individuals (III-2, III-3, IV-4, IV-5, and IV-7) (Figure 3.14 & 3.15).

3.6.1 Clinical Features

Family F consists of four affected individuals (IV-1, IV-2, IV-3, and IV-6) and three unaffected siblings (IV-4, IV-5, and IV-7), all born to a consanguineous couple (III-2 and III-3) within the same larger family (Figure 3.13). Each member of the family was born at full term without complications. However, the affected individuals have been clinically diagnosed with Autism Spectrum Disorder (ASD) and exhibit developmental delays without any noticeable muscular or facial dysmorphism. The affected individuals demonstrate delayed developmental milestones, including difficulty in supporting themselves while sitting and walking, compromised speech abilities, severe intellectual disability, microcephaly, early-onset seizures, and a lack of social engagement (Table 3.6).

3.6.2 Whole Exome Sequencing

Whole Exome Sequencing (WES) on DNA from affected individual IV-6 used the Agilent 2100 Bioanalyzer and Illumina HiSeq2000, with exome enrichment by SureSelect Human All Exon V4 at 30X read depth. Data were aligned with BWA-MEM, processed with Picard and GATK, and annotated with DNAnexus. Whole exome sequencing (WES) analysis pinpointed three candidate pathogenic variants showing neurological phenotypes consistent with those observed in the affected

individuals of this family. The variants include a missense mutation in *DMRTA2* (Chr1 [GRCh37]: g.50884846C>G; NM_032110.2; c.1120G>C; p.Gly374Arg), *MAST2* gene (Chr1[GRCh37]:g.46498278A>T; NM_001319245.1:c.3425A>T; p.Asp1142Val) and another in *ZSWIM5* (Chr1 [GRCh37]: g.45486401A>G; NM_020883.1; c.2509T>C; p.Ser837Pro).

However, only *ZSWIM5* (Chr1 [GRCh37]: g.45486401A>G; NM_020883.1; c.2509T>C; p.Ser837Pro). was absent from major databases including gnomAD, ClinVar, HGMDPro, and Decipher databases. Cosegregation analysis, confirmed via Sanger sequencing of all family members, showed that the segregation pattern of the *ZSWIM5* variant aligns with the phenotypes observed in the affected individuals.

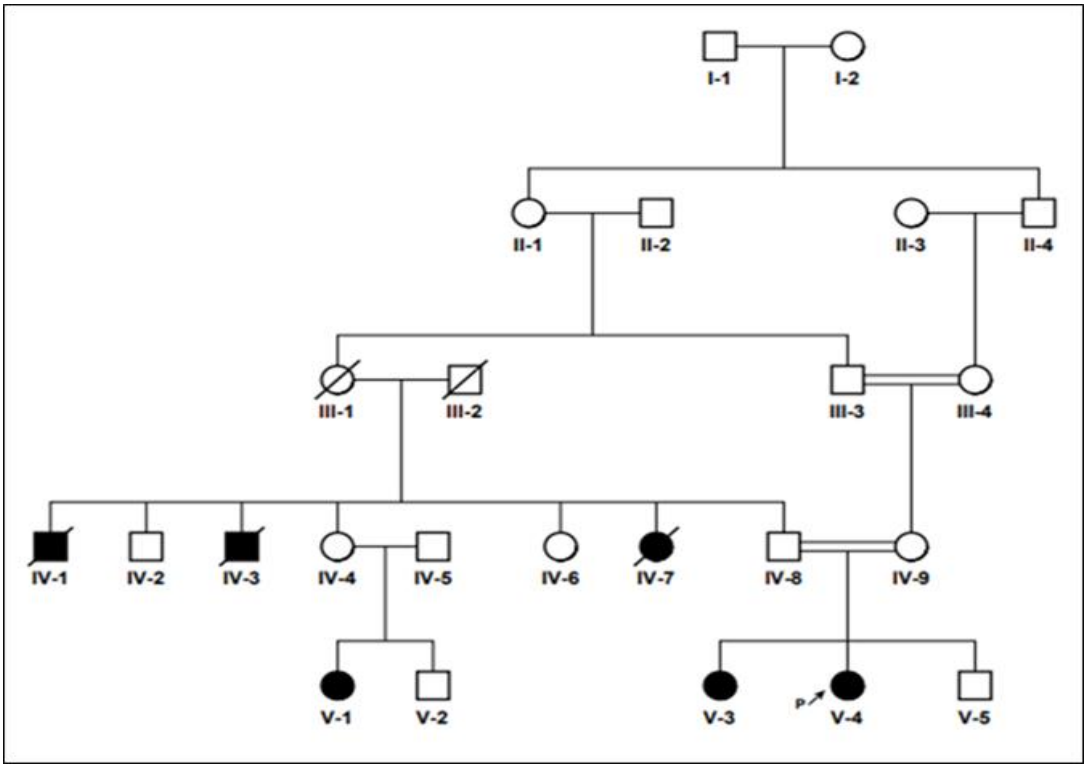

Figure 3.1 Pedigree of family A, with two affected siblings (V-3 & V-4) with Aicardi–Goutières Syndrome (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.1 Clinical features of Family A.

Family	**A**	
Individual	**V-3**	**V-4***
Disorder	Microcephaly	
Sex	F	F
Age of assessment	7	2
Gestation weeks	FT	FT
Birth weight kg	1.8	2.5
Height cm	92	81
Weight kg	7.5	8.2
Head circumference cm	41	42
Development features		
Intellectual Disability	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal
Development delay	+	+
Age walking	N/A	N/A
Neurological features		
Autistic features	-	-
Microcephaly	+	+
Seizures	+	+
Hypotonia	-	-
Behavioral problems	+	+
Other features	Visual impairment	Visual impairment

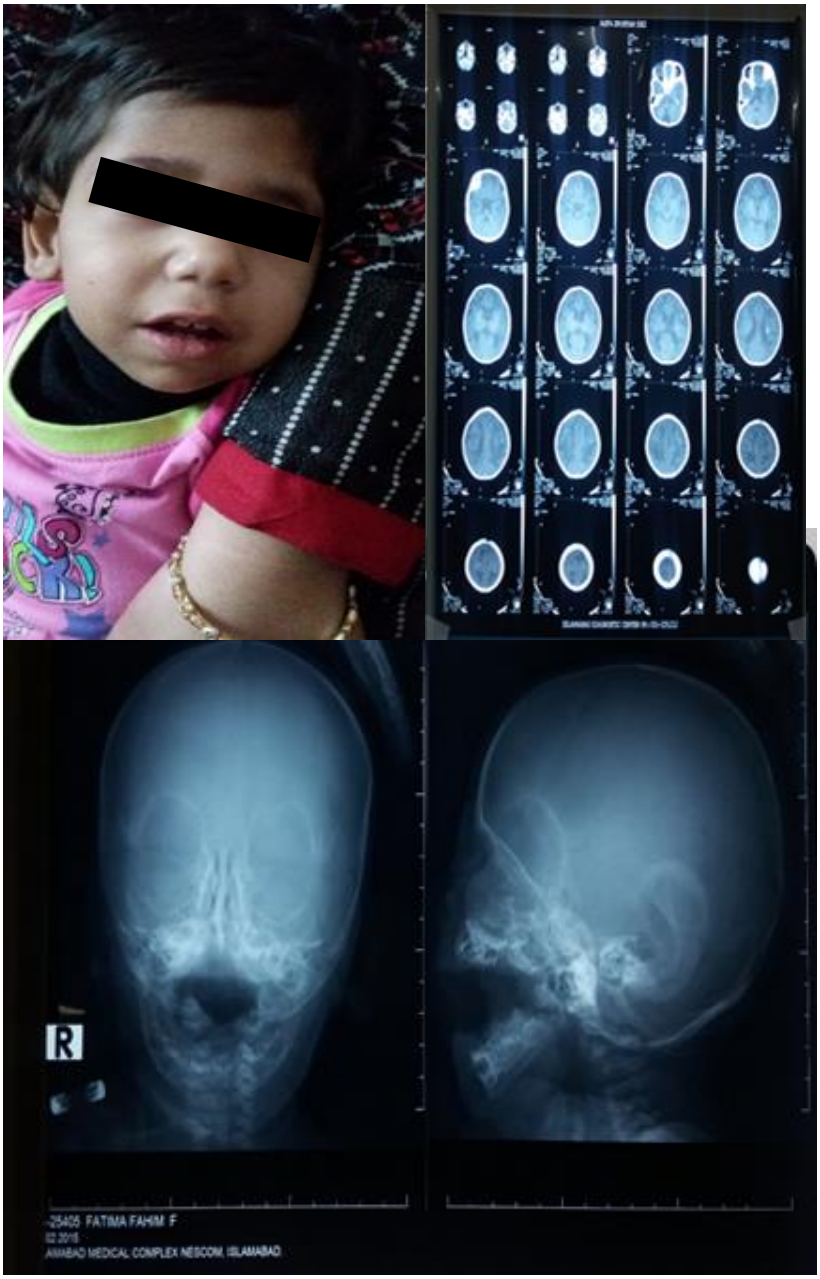

Figure 3.2 The photograph of affected individual and proband V-4 with facial dysmorphism, and developmental delay and ID; along with MRI scans.

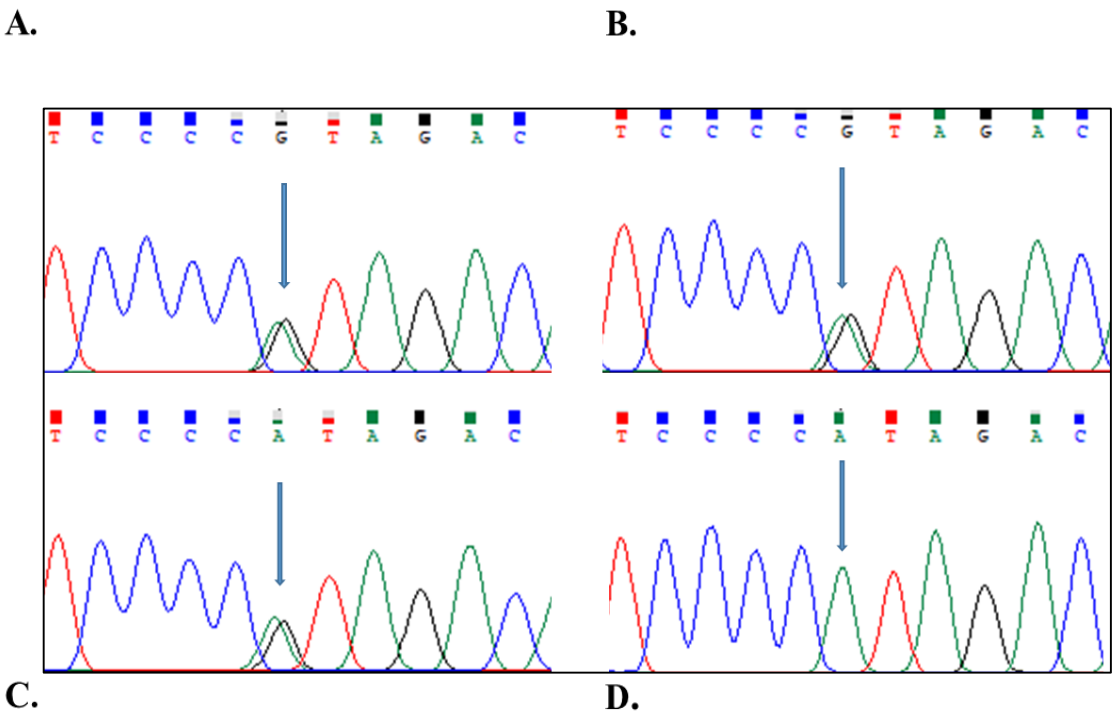

Figure 3.3 Chromatogram of Family A showing results for missense variant in *RNASEH2C*; c.205C>T (p.Arg69Trp) in A. heterozygous father; B. heterozygous mother; C. heterozygous sibling; D. homozygous proband.

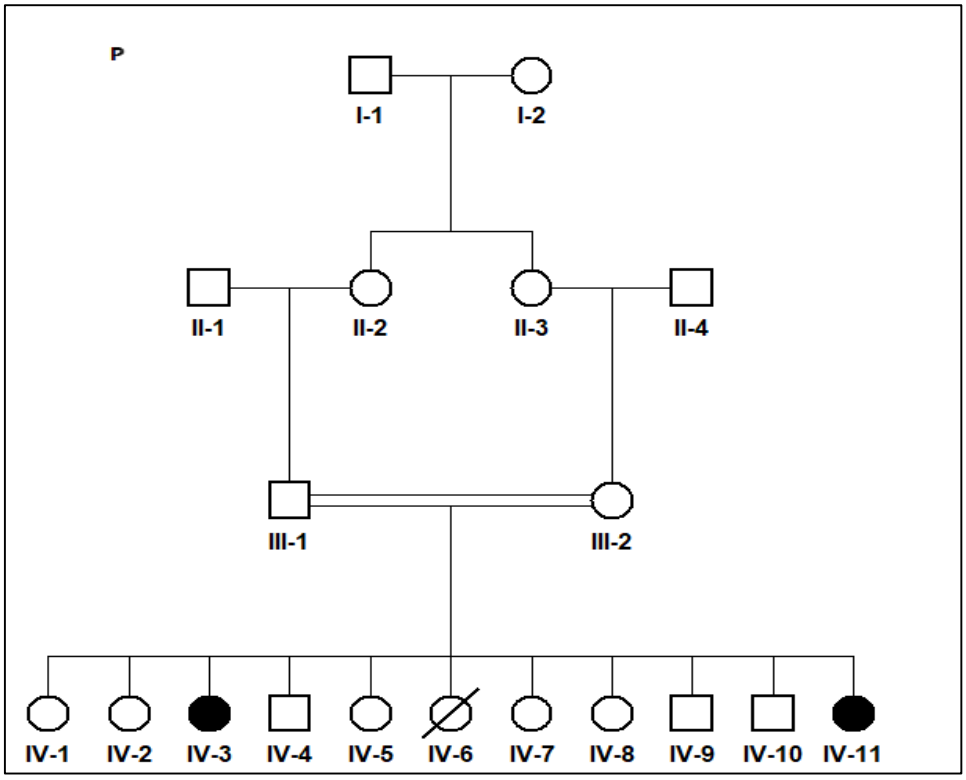

Figure 3.4 Pedigree of the family B, with two affected siblings (IV-3 & IV-11) with Microcephaly (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.2 Clinical features of Family B.

Family	**B**	
Individual	**IV-3***	**IV-11**
Disorder	Microcephaly	
Sex	F	F
Age of assessment	9	2
Gestation weeks	FT	FT
Birth weight kg	2.3	2.5
Height cm	99	79
Weight kg	8.5	7.7
Head circumference cm	39	34
Development features		
Intellectual Disability	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal
Development delay	+	+
Age walking	N/A	N/A
Neurological features		
Autistic features	+	+
Microcephaly	+	+
Seizures	+	+
Hypotonia	-	-
Behavioral problems	+	+
Other features	Visual impairment	Visual impairment

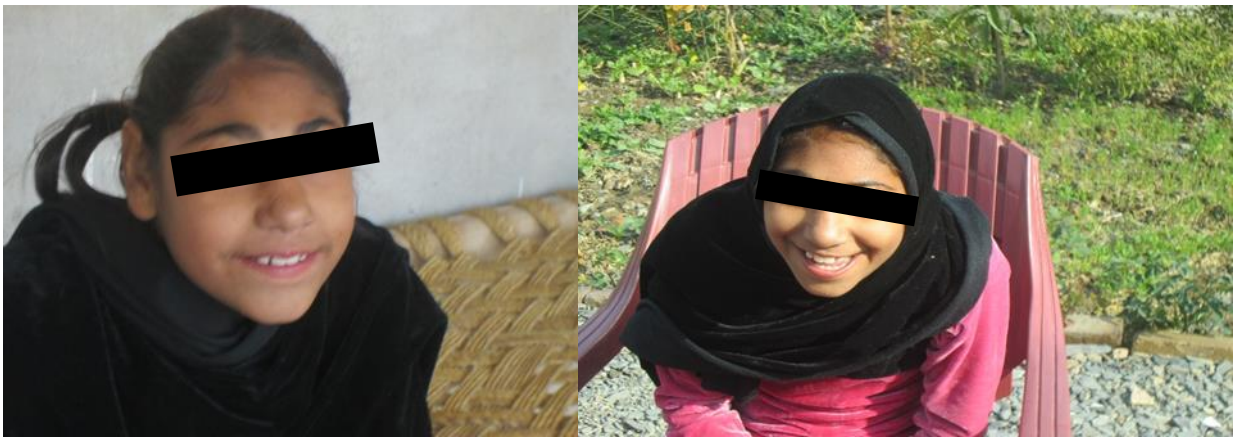

Figure 3.5 The photograph of affected individual and proband IV-3 with facial dysmorphism, and developmental delay and ID.

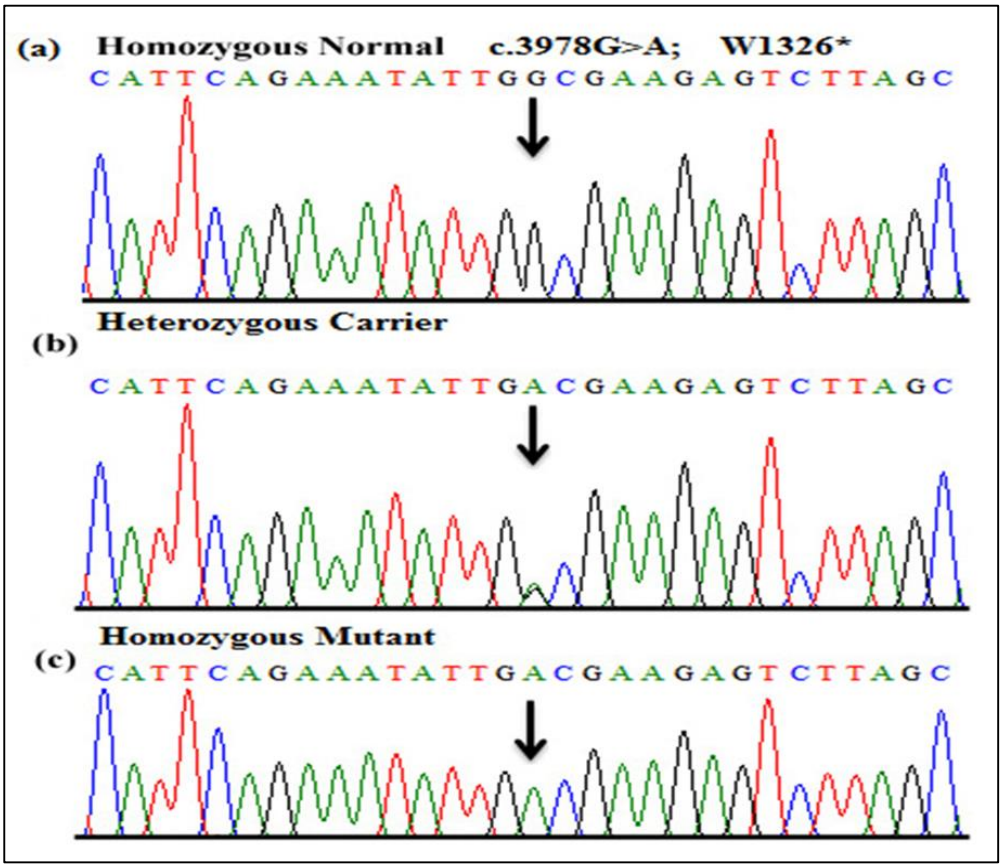

Figure 3.6 Chromatogram of Family B showing results for missense variant in *ASPM*; c.3978G>A (p. Trp1326*) in homozygous father; B. heterozygous mother; C. homozygous proband.

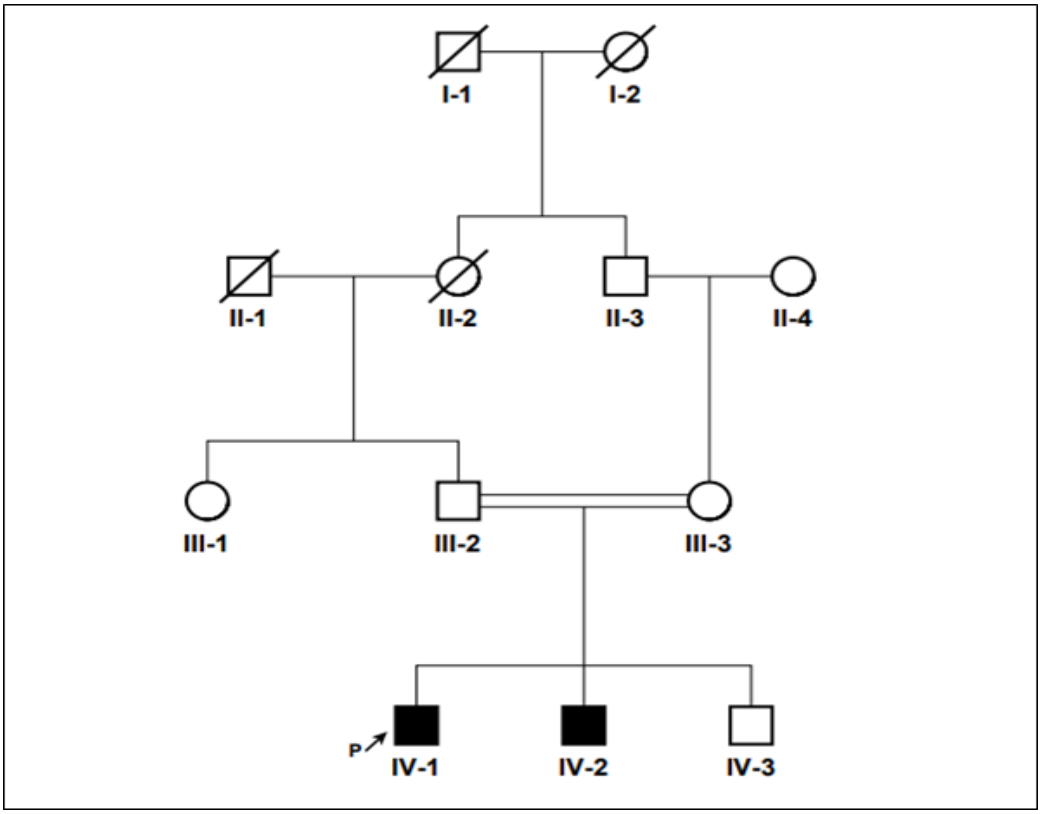

Figure 3.7 Pedigree of family C, with two affected siblings (IV-1 & IV-2) with intellectual disability (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.3 Clinical features of Family C.

Family	**C**	
Individual	IV-1*	IV-2
Disorder	Intellectual Disability	
Sex	M	M
Age of assessment	12	11
Gestation weeks	FT	FT
Birth weight kg	2.6	2.7
Height cm	139	126
Weight kg	31	27
Head circumference cm	43	42
Development features		
Intellectual Disability	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal
Development delay	+	+
Age walking	No weight bearing	No weight bearing
Neurological features		
Autistic features	-	-
Microcephaly	-	-
Seizures	+	+
Hypotonia	+	+
Behavioral problems	+	+
Other features	Severe cataracts	Severe cataracts

Figure 3.8 The photograph of affected individual and probandIV-1 with facial dysmorphism, and developmental delay, cataracts and ID.

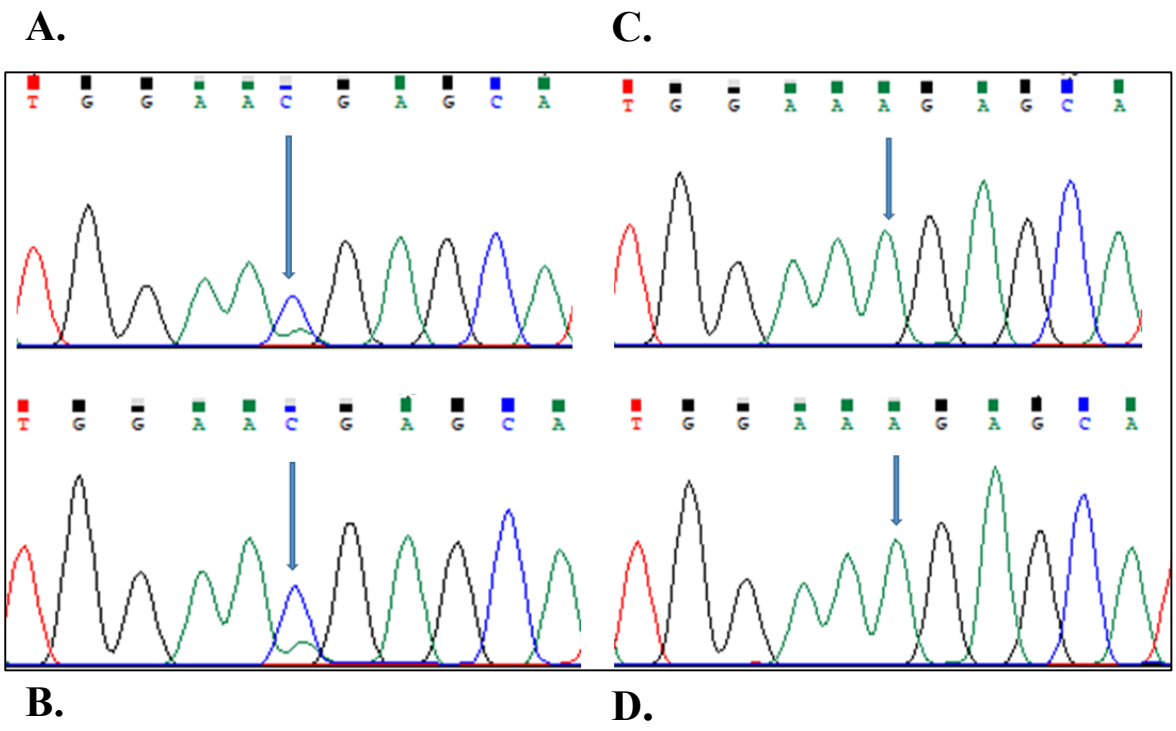

Figure 3.9 Chromatogram of Family C showing results for missense variant in *COPB1*; c.2693G>T (p.Arg898Leu) in A. heterozygous father; B. heterozygous sibling; C. homozygous proband; D. homozygous sibling.

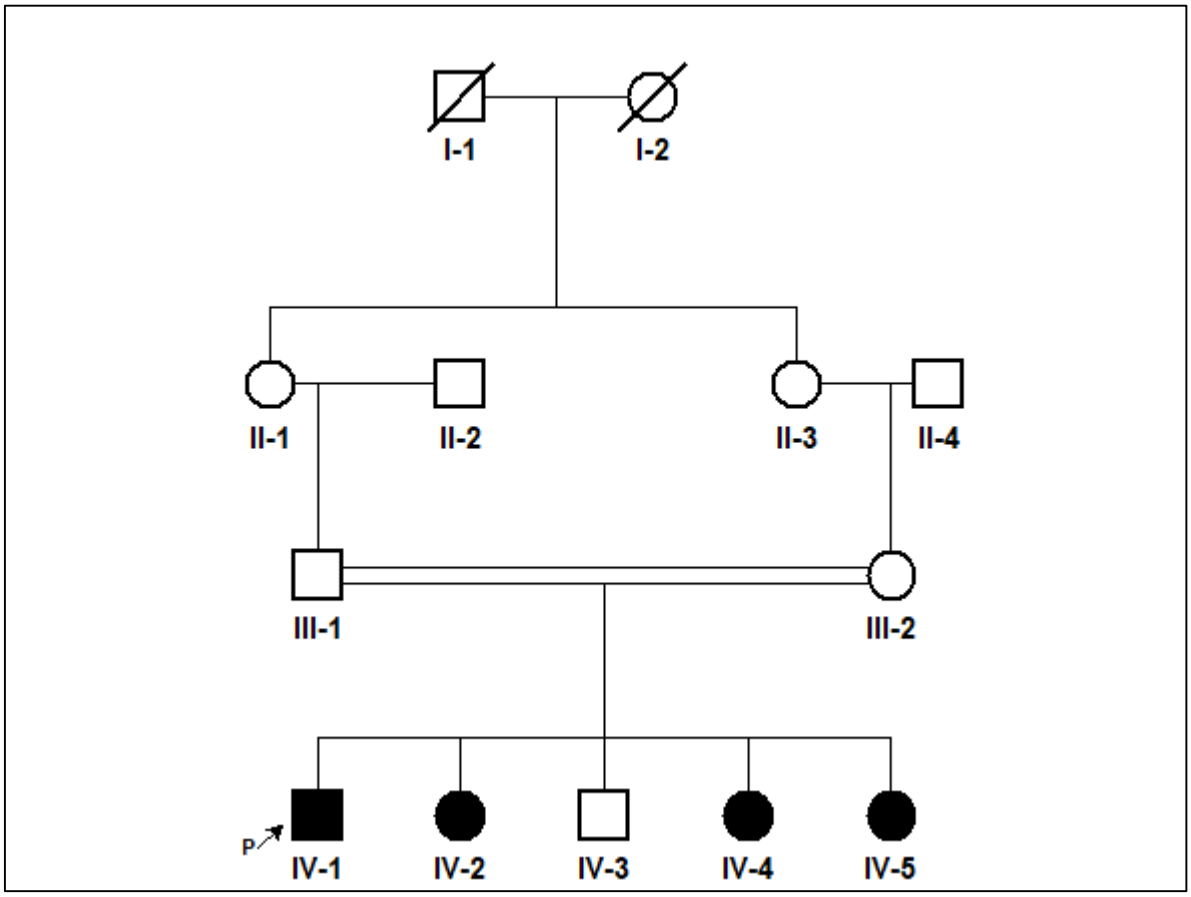

Figure 3.10 Pedigree of family D, with four affected siblings (IV-1, IV-2, IV-4 & IV-5) with Intellectual disability (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.4 Clinical features of Family D.

Family	**D**			
Individual	IV-1*	IV-2	IV-4	IV-5
Disorder	Intellectual Disability			
Sex	M	F	F	F
Age of assessment	12	11	9	8
Gestation weeks	FT	FT	FT	FT
Birth weight kg	2.0	2.1	2.5	2.4
Height cm	59	50	49	49
Weight kg	30	25	23	24
Head circumference cm	21	20.5	19	19
Development features				
Intellectual Disability	Severe	Severe	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal	Non-verbal	Non-verbal
Development delay	+	+	+	+
Age walking	No weight bearing	No weight bearing	No weight bearing	No weight bearing
Neurological features				
Autistic features	-	-	-	-
Microcephaly	-	-	-	-
Seizures	-	-	-	-
Hypotonia	-	-	-	-
Behavioral problems	+	+	+	+
Other features	Visual impairment	Visual impairment	Visual impairment	Visual impairment

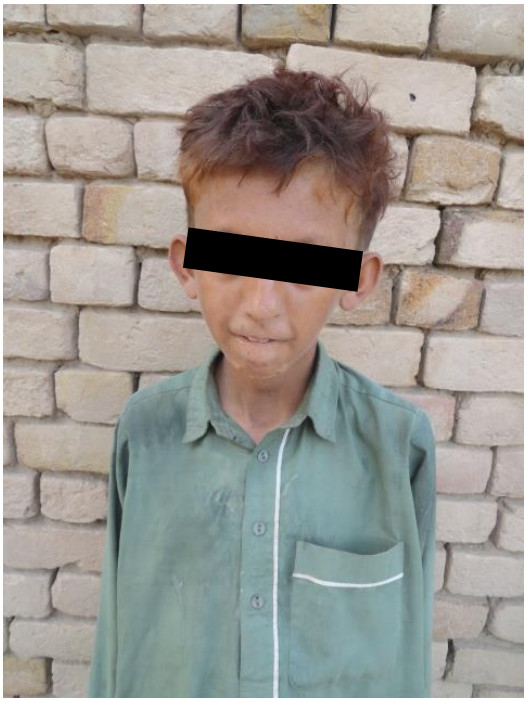

Figure 3.11 The photograph of affected individual and probandIV-1 with developmental delay, speech impairment and ID.

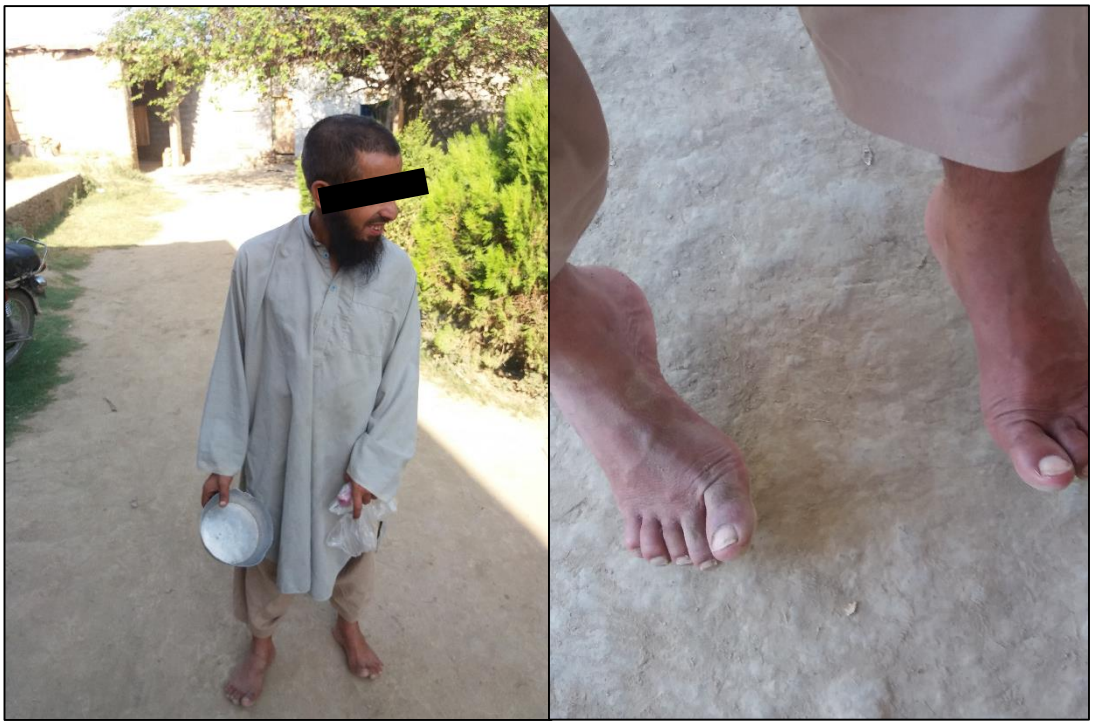

Figure 3.12 The photograph of affected individual and proband IV-1 with developmental delay, speech impairment and ID.

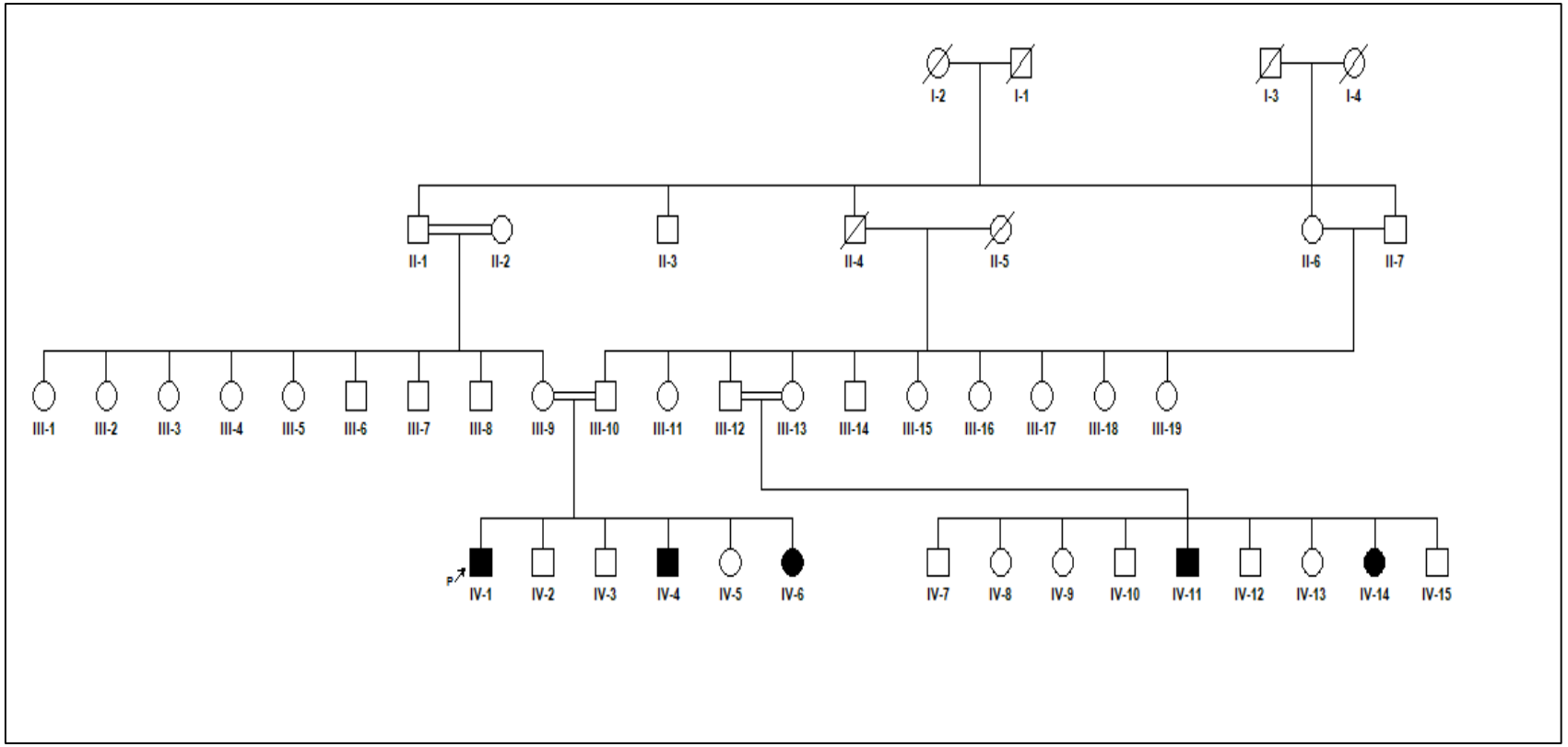

Figure 3.13 Pedigree of family E, with four affected individuals (IV-1, IV-4, IV-6, IV-11 and IV-14) with Intellectual disability (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.5 Clinical features of Family E.

Family	**E**				
Individual	IV-1*	IV-4	IV-6	IV-11	IV-14
Disorder	Intellectual Disability				
Sex	M	M	F	M	F
Age of assessment	25	12	09	14	10
Gestation weeks	FT	FT	FT	FT	FT
Birth weight kg	2.5	2.0	2.3	2.4	2.5
Height cm	139	126	122	124	126
Weight kg	2.5	2.7	2.5	2.4	2.5
Head circumference cm	43	42	43	42	41
Development features					
Intellectual Disability	Severe	Severe	Severe	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal	Non-verbal	Non-verbal	Non-verbal
Development delay	+	+	+	+	+
Age walking	No weight bearing	No weight bearing	No weight bearing	No weight bearing	No weight bearing
Neurological features					
Autistic features	-	-	-	-	-
Microcephaly	-	-	-	-	-
Seizures	+	+	+	+	+
Hypotonia	-	-	-	-	-
Behavioral problems	+	+	+	+	+
Other features	Visual impairment	Visual impairment	Visual impairment	Visual impairment	Visual impairment

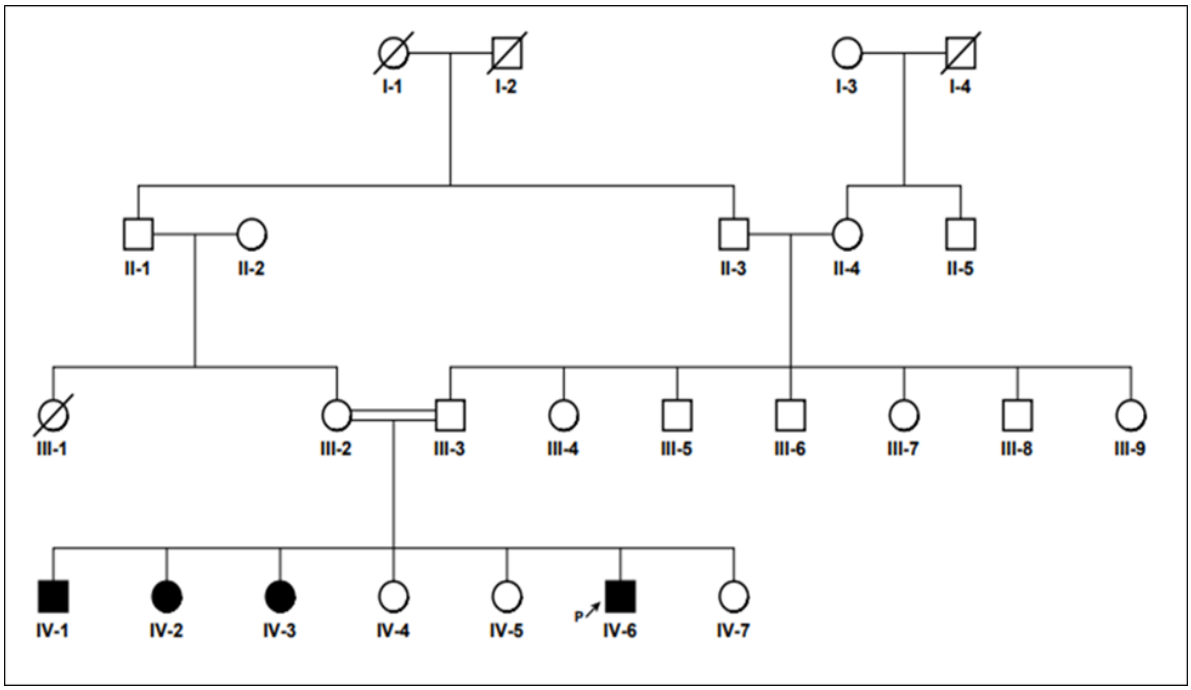

Figure 3.14 Pedigree of family F, with four affected siblings (IV-1, IV-2, IV-3, and IV-6) with Autism Spectrum Disorder (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.6 Clinical features of Family F.

Family	**ASD**			
Individual	IV-1	IV-2	IV-3	IV-6*
Disorder	Autism Spectrum Disorder			
Sex	M	F	F	M
Age of assessment	24	20	16	12
Gestation weeks	FT	FT	FT	FT
Birth weight kg	2.5	2.5	3	2.7
Height cm	134.6	80	115.6	135
Weight kg	36	11	19	35
Head circumference cm	48	46	44	42
Development features				
Intellectual Disability	Severe	Severe	Severe	Severe
Speech delay/impairment	Severe	Severe	Severe	Severe
Development delay	+	+	+	+
Age walking	4	4	3.5	4
Neurological features				
Autistic features	+	+	+	+
Microcephaly	+	+	+	+
Seizures	+	-	+	-
Hypotonia	+	+	+	+
Behavioral problems	+	+	+	+
Other features	Pointed nose	-	Pointed nose	Pointed nose

Figure 3.15 The photograph of affected individual and proband IV-6 with developmental delay, speech impairment, autism and ID.

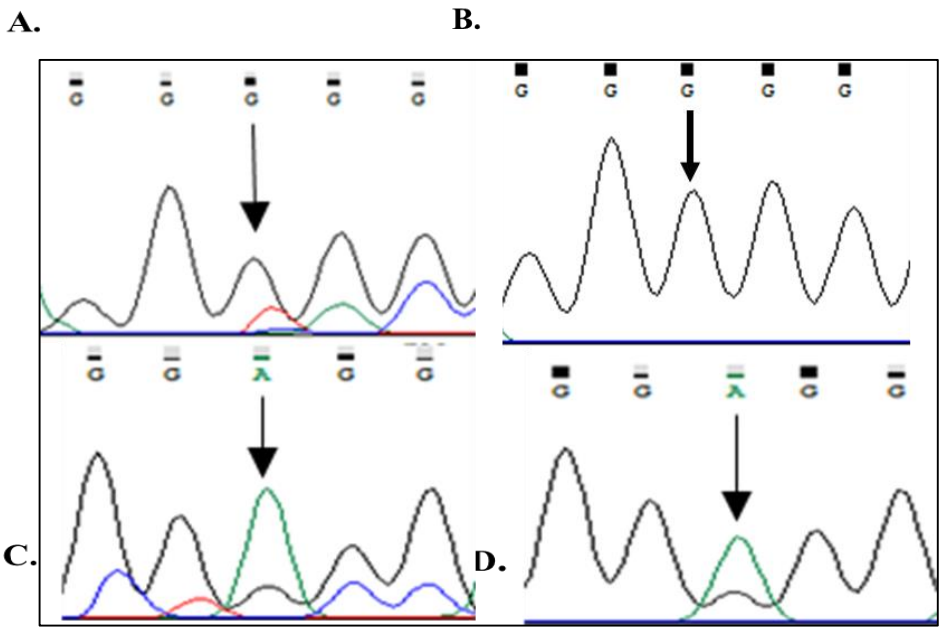

Figure 3.16 Chromatogram of Family F showing results of *ZSWIM5* missense variant c.2509T>C (p.Ser837Pro) in A and B while C and D show heterozygous sibling and mother for *ZSWIM5* respectively.

Families with Metabolic Disorder

(Family G and H)

3.7 Family G

A four-generation consanguineous family G was recruited from a remote area in South Punjab, Pakistan. The family, visited at their residence, displays an autosomal recessive inheritance pattern of Wilson's disease. For genetic analysis, blood samples were collected from the affected individual (IV-4) and healthy individuals (III-6, III-7, IV-2, and IV-3) (Figure 3.17).

3.7.1 Clinical Features

The family in question has a case of Wilson's disease involving one affected child (IV-4) and two unaffected siblings (IV-2 and IV-3). The parents (III-6 and III-7) are consanguineous and otherwise healthy. The affected child was born via normal delivery and did not exhibit symptoms of Wilson's disease until the age of two. Since then, the condition has progressed, and the child now displays a range of behavioral abnormalities, including anxiety, disinterest in surroundings, and difficulty with speech. Additionally, the child suffers from involuntary muscle movements, inability to walk, irregular menstrual cycles, and abdominal pain. The patient has been on a regimen of regular medications, including copper chelation and zinc sulfate supplements, since the diagnosis (Table 3.7).

3.7.2 Whole Exome Sequencing

Whole-exome sequencing (WES) on DNA from affected individual IV-4 used the Agilent 2100 Bioanalyzer and Illumina HiSeq2000, with exome enrichment by SureSelect Human All Exon V4 at 30X read depth. Data were aligned with BWA-MEM and processed with Picard and GATK for variant calling and quality control. Custom annotation was done with DNAnexus. Filtration focused on rare non-synonymous or splice variants with <0.01 frequency in gnomAD, ExAC, and 1000 Genomes Project. No homozygous variants of interest were found.

3.8 Family H

A four-generation consanguineous family H was recruited from a remote area of South Punjab province, Pakistan. The family was visited in their residential area, and the pedigree analysis revealed an autosomal recessive inheritance pattern of Gaucher disease. For genetic analysis, blood samples were collected from the affected individual (IV-3) and healthy individuals (III-3, III-4, IV-1, IV-2, IV-4, and IV-5) (Figure 3.18).

3.8.1 Clinical Features

The affected individual (IV-3) with Gaucher disease is a child from a consanguineous couple (III-3 and III-4) who also have two healthy children (IV-4 and IV-5). Although the child was asymptomatic at birth, symptoms began to appear around the age of one. By the time of sample collection, the disease had progressed significantly. The child presented with multiple medical issues, including recurrent diarrhea accompanied by abdominal pain and distension, frequent urinary tract infections, and bruising. Additionally, the child had ventricular septal defects, periodic fever, and febrile seizures. There were also developmental delays, a lack of interest in surroundings, feeding difficulties, involuntary eye movements, and sleep disturbances. The patient could not walk for more than a few minutes and is currently undergoing enzyme replacement therapy for Gaucher disease (Table 3.7).

3.8.2 Whole Exome Sequencing

Whole exome sequencing (WES) was performed on DNA from affected individual IV-3 using the Agilent 2100 Bioanalyzer and Illumina HiSeq2000, with enrichment by SureSelect Human All Exon V4 at 30X depth. Data were aligned with BWA-MEM and processed with Picard and GATK for variant calling and quality control. Custom annotation was done with DNAnexus. Filtration targeted rare non-synonymous or splice variants with <0.01 frequency in gnomAD, ExAC, and 1000 Genomes Project. No homozygous variants of interest were found.

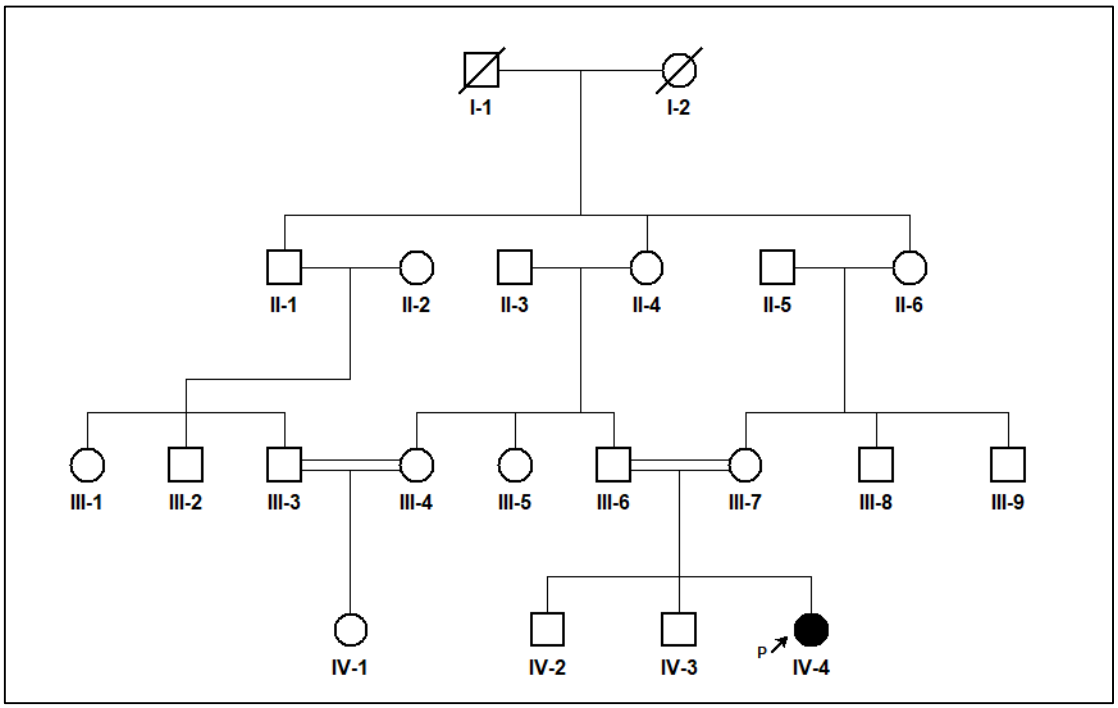

Figure 3.17 Pedigree of family G, with one affected individual (IV-4) with Wilson's disease (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

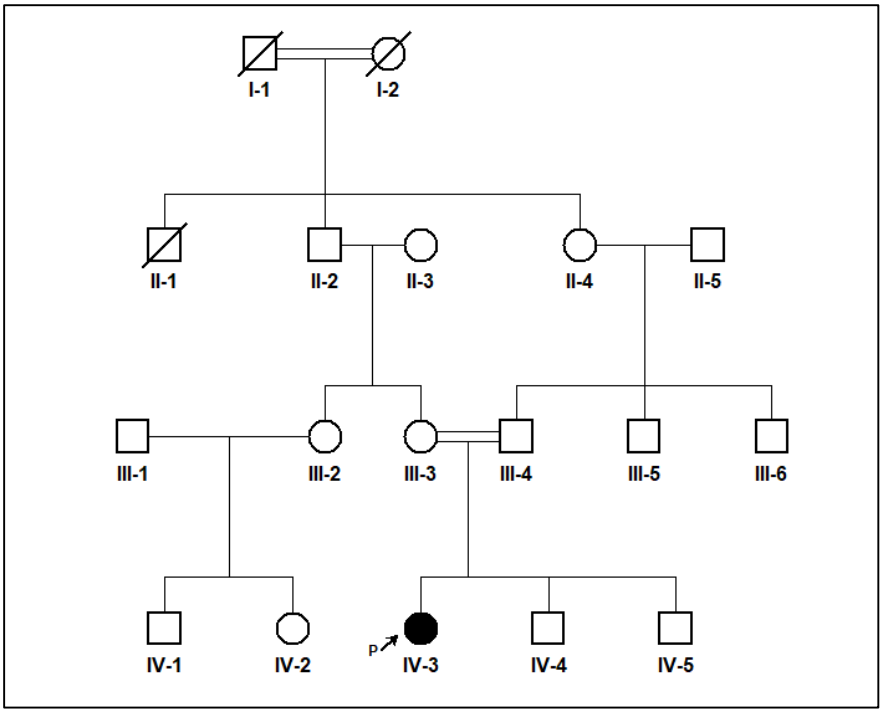

Figure 3.18 Pedigree of family H, with one affected individual (IV-3) with Wilson's disease (filled symbols) while unfilled symbols indicate unaffected individuals. **P** indicates the proband for this study.

Table 3.7 Clinical features of Family G & H.

Family	**G**	**H**
Individual	**IV-4***	**IV-3***
Disorder	Wilson Disease	Gaucher Disease
Sex	F	F
Age of assessment	7	2
Gestation weeks	FT	FT
Birth weight kg	1.8	2.5
Height cm	92	81
Weight kg	7.5	8.2
Head circumference cm	41	42
Development features		
Intellectual Disability	Severe	Severe
Speech delay/impairment	Non-verbal	Non-verbal
Development delay	+	+
Age walking	N/A	N/A
Neurological features		
Autistic features	-	-
Microcephaly	-	-
Seizures	-	-
Hypotonia	+	+
Behavioral problems	+	+
Other features	Visual impairment	Visual impairment

Figure 3.19 The photograph of affected individuals of family G and H with Wilson Disease and Gaucher Disease respectively.

Table 3.8 General details of all recruited families.

Family ID	Phenotype	Ethnic Group	Affected Individuals
A	Microcephaly	KPK	2
B	Microcephaly	KPK	2
C	Intellectual Disability	South Punjab	2
D	Intellectual Disability	KPK	4
E	Intellectual Disability	KPK	5
F	Autism Spectrum Disorder	KPK	4
G	Wilson Disease	South Punjab	1
H	Gaucher Disease	South Punjab	1

Table 3.9. Primers for candidate genes.

	Left Primer	***Right Primer***	***Location***
RNASEH2C	TGCTGAACACCATAACGAGC	GCTTTCGACATGCCTGGAAT	chr11:65487231+65488454
ASPM	TGCTGAACACCATAACGAGC	GCTTTCGACATGCCTGGAAT	chr1:197086753+197087285
COPB1	CCCCTTTGTCAGTCCAGAGA	CAGGCCCTTTCTGGTTACTG	chr11:14480031+14480236
INTS1	CTGCGGCCCTCTCATCTTTC	GCCCAGCTCATGAGTGTCTG	chr11:14480031+14480236
GPSM1	CTAGCCACCCTCTTTCCAGG	ATACCACCCTGCCCTTTCAG	chr11:14480031+14480236
IARS	GCACAAGGGACCATAAACCA	ACGTTGACTGTCAGACCTTCT	chr11:14480031+14480236
MAST2	ATCATCCTTCCAGCTTCCCC	ACTCCCCTAACTGCCATCAC	chr11:14480031+14480236
DMRTA2	TAAAGGCGTAGTCCATGGGT	AGGCTGACAAAGAAGAGGGT	chr11:14480031+14480236
ZSWIM5	CTTGTGTTCTGGGCCCAAAG	GGAAGGAGCCTCAAATGTGA	chr11:14480031+14480236

Chapter 4

Discussion

4.0 Discussion

We studied eight unrelated families with complex neurological and metabolic disorders to identify the causative pathogenic alterations on a genetic level to establish a molecular diagnosis using whole exome sequencing (WES). Clinical assessment revealed the diagnosis of Microcephaly in family A and B, Intellectual disability in family C, D and E and Autism Spectrum Disorder (ASD) in family F, Wilson's Disease in Family G and Gaucher Disease in family H.

In ***Family A***, we identified and confirmed, through cosegregation analysis, a single homozygous variant of *RNASEH2C* (c.205C>T; p. Arg69Trp). *RNASEH2C* gene is responsible for the expression of one of the three subunits (*RNASEH2A, RNASEH2B,* and *RNASEH2C)* of RNase H2 activity-based enzymatic protein complex which cleaves ribonucleotides from RNA: DNA duplexes to maintain genomic integrity (Deasy et al., 2019; Vogt et al., 2013; Reijns et al., 2011).

Mutations in the *RNASEH2A, RNASEH2B,* and *RNASEH2C* genes are linked to Aicardi–Goutières syndrome (AGS) (MIM #225750), a severe monogenic encephalopathy (Boulanger et al., 2021; Garau et al., 2021). This condition is an autosomal recessive neurological disorder characterized by autoinflammation. It results from defects in proteins that are crucial for nucleic acid metabolism and detection, leading to the accumulation of defective intracellular molecules (Uyur-Yalcin et al., 2015; Cristini et al., 2020; Giordano et al., 2022). With microcephaly as a primary phenotype, the characteristics of AGS, dysmorphic features, seizures, poor vision, and spasticity, are consistent with the clinical features recorded in our affected individuals (Supplementary Data 1). Due to its heterogeneous nature, nine known mutations have been reported in the literature: seven missense, one splice site variation, one indel, and one deletion respectively in *TREX1*, *SAMHD1*, *ADAR1*, *IFIH1*, and *RNU7-1* in addition to *RNASEH2* gene family (Giordano et al., 2022). All AGS-associated genes are crucial in the innate immune response which explains why AGS clinically imitates the phenotypes of *in utero*viral infection. While the exact pathophysiology of the syndrome is unclear, early AGS advancement characterized by high levels of interferon α in the spinal fluid suggests defective endogenous nucleic acid processing leading to the accumulation of defective DNA initiating an innate autoimmune response (Uyur-Yalcin et al., 2015; Cristini et al., 2020). The p.Arg69Trp mutation has been exclusively reported in South Asian populations and accounts for 72% of Aicardi–Goutières syndrome (AGS) type 3 cases, with an allele frequency of 0.0006822 in this demographic. Our study corroborates the previously established association of the *RNASEH2C* mutation c.205C>T as a founder mutation in South Asians for Aicardi–Goutières syndrome (Livingston et al., 2016).

In Family B, which exhibits a range of phenotypes for Primary Microcephaly (MCPH), including reduced head circumference and intellectual disability, Whole Exome Sequencing (WES) revealed a single homozygous nonsense variant, c.3978G>A (p.Trp1326*),located in exon 17 of the *ASPM* gene in the affected individuals.

MCPH is a condition caused by insufficient production of neurons in the developing neocortex due to issues with progenitor cell proliferation and/or apoptosis. There are approximately 25 genes associated with MCPH, and *ASPM* mutations are commonly linked to it, responsible for around 40% of MCPH cases (Razuvaeva et al., 2023).

The ASPM protein is composed of four distinct domains: a microtubule-binding domain (MTBD) at the N-terminal (NT), two calponin homology domains (CH), an isoleucine and glutamine domain (IQ motif), and a conserved C-terminal region. The MTBD is crucial for regulating microtubules by mediating *ASPM's* interaction with them during cell division and neurogenesis. The CH domains, which are often found in actin-binding proteins, are thought to facilitate contact between *ASPM* and either the actin cytoskeleton or microtubules (Jiang et al., 2017; van der Voet et al., 2009). Additionally, the IQ domain enables interactions with calmodulin and related proteins, playing a role in mitotic spindle function (Türkyılmaz & Sager, 2022; Hu et al., 2014).

ASPM is essential for normal mitotic progression and neurogenesis, interacting with several associates including *MCPH* proteins (such as CITK and MCPH2), katanin, calmodulin, cyclin E, FOXO, and UBE3A. Mutations or loss of *ASPM* disrupt normal mitotic events in model organisms like Drosophila, mice, ferrets, and in human cell cultures, likely due to defects in spindle assembly checkpoint function or mitotic slippage (Wu et al., 2023).

The nonsense mutation p. Trp1326X causes protein truncation precisely at the 1326th amino acid in the IQ domain. This leads to asymmetrical cell division during neurogenesis. *ASPM* is a centrosomal protein that is associated with neurogenesis and brain size (Chen et al., 2020). Functions in cell division, neurogenesis, genome stability, and disease development have been annotated for *ASPM* (Xu et al., 2021; Wu et al., 2022; Liu et al., 2018).

In ***Family C,*** with congenital cataracts and severe intellectual disability as significant clinical features (Supplementary data 1), we recognized a *COPB1* homozygous missense variation (c.2693G>T; p. Arg898Leu) (rs780448237) in a highly conserved region present on cytogenic band 11p15.2 in the affected individuals of a consanguineous couple. It has an allele frequency

0.00003587 according to Gnomad database and PolyPhen predicts it to be possibly damaging (0.65) and SIFT as deleterious. Three-dimensional (3D) structural analysis from the Protein Data Bank (AF_AFP53618F1) (https://www.rcsb.org/) shows that the p.Arg898Leu substitution occurs within a highly conserved beta-strand (Figure 4.1). Predictive modeling from the AlphaFold protein structure database (https://alphafold.ebi.ac.uk) further supports the pathogenic nature of this variant, assigning a high pathogenicity score of 0.777 to the R898L substitution (Figure 4.2 and 4.3).

COPB1 gene is a 22 exons based gene which encodes a 107 kDa protein forming one of the seven subunits (α, β, β', γ, δ, ε and ζ) of the COPI coatomer: the beta-subunit (β-COP). (Gao et al., 2023). It is engaged in facilitating the transportation of a variety of proteins and lipids between different cellular components including neurons for processing (Wang et al., 2020; Verrier et al., 2008; Todd et al., 2013). COPI efficiently regulates the formation of carrier vesicles and monitors transport routes from the Golgi apparatus to the Endoplasmic Reticulum (ER) as opposed to the ER to Golgi apparatus directional flow monitored by its counterpart, COPII. The recruitment of the coatomer at the plasma membrane is made possible by the GTPase activity of the plasma membrane-associated ADP-ribosylation factors (ARFs) at the trunk domains of β-COP and γ-COP subunit of the COPI. The complex can return to its free state as soon as the vesicle is formed and released. Other important functions of COPI include maintaining Golgi structural integrity as well as processing lipid droplet formation and lipolysis (Gao et al., 2023). Any defects in *COPB1* functioning interfere with efficient cargo transport, ERGIC trans-Golgi network compartmentalization, integrity of Golgi and recycling endosomes (Dell'Angelica et al., 2019; Kobayashi et al., 2015).

Localized protein synthesis requires distribution of subcellular RNA by trafficking mechanisms into different cellular components including the neuronal cells. These proteins participate in crucial functions in development, survival and maintaining cellular polarity and defects in RNA processing, transport or translation within the neuron causes human pathologies. Coatomer protein (COPI) are also involved in mobilization of cytoplasmic RNAs to the neurons and defects in the COPI complex components lead to neurodegeneration and irregular RNA displacement (Verrier et al., 2008; Todd et al., 2013). Consequently, COPI complex has been associated with disorders such as severe microcephaly syndrome (MIM # 617800) (DiStasio et al., 2017), Alzheimer disease (Bettayeb et al., 2016), a unique craniofacial syndrome (MIM # 617164) (Izumi et al., 2016), auto-inflammatory arthritis and lung disease (MIM # 616414) (Jensson et al., 2017) and periventricular heterotopia (MIM # 618185) (Ge et al., 2016). Recently, a mutation in the *COPB1* gene, c.1651T>G p. Phe551Val, has been linked to a novel recessive neurological disorder, Baralle-Macken syndrome

(MIM # 619255) (BARMACS), characterized by development delay, impaired intellectual disability, early-onset cataracts and, in some cases, variable microcephaly or dismorphic features (Macken et al., 2021).

The association of *COPB1* with intellectual disability is a relatively recent finding with just a single identified variant (c.1651T>G p. Phe551Val) in two unrelated families (Macken et al., 2021). Our analysis underscores the evolutionary significance of *COPB1* function. Rare missense mutations, such as the p.Arg898Leu variant, are known to disrupt various aspects of protein functionality, including structural stability, hydrogen bonding, and dynamic behavior, all of which are essential for optimal activity (Stefani, 2008). The precise folding of the polypeptide chain into a unique 3D structure, dictated by its primary amino acid sequence, is fundamental to protein function. Even minor disruptions can lead to severe, sometimes lethal, consequences, and in extreme cases, such mutations may abolish the protein's biological activity entirely (Hartl, 2017; Dobson, 2001; Ajmal, 2023).

Given the critical nature of the R898 position, the p.Arg898Leu mutation is likely to significantly impact intermolecular interactions within protein complexes, thereby impairing the functional behavior of *COPB1*. This particular amino acid substitution could reduce the protein's binding affinity to the COPI coatomer complex, a key player in mediating intracellular cargo transport between the Golgi apparatus and the endoplasmic reticulum (ER). Destabilization of this complex could disrupt the entire cargo transport system. Moreover, the p.Arg898Leu mutation may interfere with the recruitment of the coatomer complex to the plasma membrane, where interactions between plasma membrane-associated ARFs and the COPB1 trunk domain are essential for efficient vesicle formation and cargo sorting. Such disruptions could lead to broader cellular dysfunction, suggesting that this mutation has considerable pathogenic potential, likely compromising *COPB1*'s role in maintaining intracellular transport, a process vital for proper neuronal and cellular function, thereby contributing to the pathogenesis of the associated neurodevelopmental disorder.

The identification of the p.Arg898Leu variant in *COPB1* represents only the third known mutation linked to Baralle-Macken syndrome, with the previous two reported in female individuals from distinct populations. This study is the first to document the variant in a Pakistani population and the first to report affected males. This novel missense mutation expands our understanding of the genetic basis of Baralle-Macken syndrome, especially in consanguineous populations, emphasizing the urgent need for broader genomic research in underrepresented groups. Furthermore, this case highlights the importance of continued investigations into the functional consequences of *COPB1*

mutations. Future research should include functional assays to better elucidate their impact on protein transport and neuronal development.

In ***Family D***, no candidates of interest were established while in ***Family E***, WES data identified two candidates, a missense variant; c.620C>T (p. Ala207Val) present in *GPSM1* (Chr9(GRCh37): g.139231939C>T), and a missense variant; c.337A>T (p. Ile113Phe) in *IARS* gene (Chr9(GRCh37): g.95050132T>A). Co-segregation of these variants was not confirmed in all family members, with homozygosity identified in all affected individuals and asymptomatic individuals.

In ***Family F***, we report the involvement of a distinct cosegregating missense variant: a highly conserved *ZSWIM5* (c.2509T>C; p. Ser837Pro) present in all affected individuals in the family while unaffected parents and siblings were heterozygous however this single nucleotide variation is not present in gnomAD, however, Polyphen and SIFT predict it to be possibly damaging and damaging, respectively. Additionally, this variation has not been reported previously in coalition with ASD, making this a novel occurrence in the Pakistani ethnicity. ASD shows a high degree of locus heterogeneity (Paulsen et al., 2022), and among more than a hundred discovered genes, most of them functionally belong to either, synaptic structure/function, modification of chromatin, fetal brain expression, transcription factors, or as DNA/RNA binding proteins (Manoli & State, 2021).

Zinc-finger proteins (ZNFs) are distinguished by their unique and stable finger-like spatial conformation. This structure is stabilized by the presence of one or more zinc ions, which intricately link cysteine and histidine residues, resulting in the formation of a characteristic secondary structure known as the ββα fold (Cassandri et al., 2017). This structural motif is crucial for the function of ZNFs, as it facilitates their role in binding to DNA, RNA, or proteins (Eom et al., 2016).

ZNFs are categorized based on the specific configurations of their zinc-finger domains, which vary significantly in their structural details. To date, approximately 30 distinct types of zinc-finger domains have been identified, reflecting the diverse functional roles these proteins play in cellular processes (Hassan et al., 2023). Among these, the SWIM-type zinc-finger is particularly notable. The SWIM domain is characterized by a highly conserved Zn-chelating CxCnCxH motif, which, despite its conserved nature, remains functionally enigmatic (Xu et al., 2018).

In humans, the SWIM (SWI2/SNF2 and MuDR) family comprises nine genes, designated *ZSWIM1* through *ZSWIM9*, each of which plays a unique role in various biological processes. *ZSWIM1*, for instance, has been identified as a crucial biomarker for T helper cell differentiation, highlighting its significance in the immune response and potential implications in immunological disorders (Ko et

al., 2014). *ZSWIM2*, on the other hand, is involved in the regulation of MEX E3 ubiquitin ligase activity within the testis, pointing to its critical function in the regulation of protein turnover and stability during spermatogenesis (Nishito et al., 2006).

ZSWIM4 contributes to the ubiquitination process, a post-translational modification that tags proteins for degradation or regulates their function, underscoring its importance in maintaining cellular homeostasis (Forde et al., 2012). The role of *ZSWIM5* extends into the realm of oncology, where it has been found to inhibit the progression of non-small-cell lung cancer, suggesting a potential therapeutic target for cancer treatment (Xu et al., 2018).

A point mutation in *ZSWIM6* has been associated with acromelic frontonasal dysostosis, a rare congenital disorder marked by craniofacial and limb abnormalities, highlighting its essential role in developmental processes (Twigg et al., 2016). Meanwhile, *ZSWIM8* has garnered attention for its involvement in microRNA (miRNA) degradation. It facilitates the destabilization of Argonaute proteins, which are crucial components of the miRNA-induced silencing complex, thus affecting gene expression at the post-transcriptional level (Shi et al., 2020).

ZSWIM5 is a relatively newly characterized gene that consists of 14 exons, encoding a nuclear protein with a molecular weight of approximately 130 kDa and comprising 1188 amino acids (Xu et al., 2018). The structural intricacies of the *ZSWIM5* protein, specifically the wild-type human variant (Q9P217), have been detailed through three-dimensional (3D) structural analysis available from the Protein Data Bank (PDB ID: AF_AFQ9P217F1) (https://www.rcsb.org/). This resource provides a comprehensive view of the protein's spatial configuration.

Detailed structural analysis reveals that the amino acid substitution p. Ser837Pro is located within a highly conserved helix structure, which is preserved across different species (see Figures 4.4 and 4.5). This conservation underscores the functional importance of this region in maintaining the structural integrity of the protein.

Further predictive modeling from the AlphaFold protein structure database (https://alphafold.ebi.ac.uk) assigns a high pathogenicity score of 0.924 to the S837P substitution. This elevated score indicates that this specific amino acid change is likely to have a profound impact on the protein's function and stability (Figure 4.6). Such missense mutations, particularly those that are rare, are known to disrupt various aspects of protein function. They can affect protein stability, alter hydrogen bonding patterns, interfere with dynamic behavior, and impair overall protein activity. These disruptions can lead to a cascade of deleterious effects, contributing to disease development and progression (Stefl et al., 2013).

ZSWIM5 is predominantly expressed in the medial ganglionic eminence (MGE) of the developing forebrain, where it serves as a critical marker for progenitor cells that will develop into cortical interneurons (Mayer et al., 2018). Its expression is especially prominent during neurodevelopment, underscoring its role in primary cortical development. Studies on *ZSWIM5* mRNA in the developing mouse forebrain have highlighted its essential involvement in tangential migration pathways, which are crucial for the correct positioning and integration of neurons in the neocortex. *ZSWIM5* works in coordination with co-localized transcription factors, such as NKx2.1 and Lhx6, which are well-known regulators of forebrain neuron development. These factors together guide the migration and differentiation of progenitor cells from the MGE into the neocortex (Canitano & Pallagrosi, 2017). Once in the neocortex, these progenitor cells mature into GABAergic interneurons, which are crucial for maintaining the balance between excitatory and inhibitory synaptic activity essential for proper brain function (Nelson & Valakh, 2015). Disruptions in this excitatory/inhibitory (E/I) balance are well-documented in various neurodevelopmental disorders, such as autism spectrum disorders and schizophrenia (Canitano & Pallagrosi, 2017; Nelson & Valakh, 2015; Ramamoorthi & Lin, 2011; Sohal & Rubenstein, 2019). These conditions often present as abnormalities in neural circuit function and connectivity, highlighting the importance of precise regulation of neurodevelopmental processes involving genes like *ZSWIM5*.

ZSWIM5 expression is promptly upregulated as progenitor cells exit the cell cycle and transition to a postmitotic state. Given that the cell fate of cortical interneurons is determined shortly after they become postmitotic, the timing of *ZSWIM5* expression suggests that it may play a crucial role in regulating neurogenesis and the tangential migration of cortical interneurons (Chang et al., 2020). Additionally, *ZSWIM5* shows highly specific and dynamic expression patterns throughout embryogenesis, being expressed in several major regions of the brain, including the forebrain, midbrain, and hindbrain (Hassan et al., 2023).

ZSWIM6, much like its paralogue *ZSWIM5*, remains a gene with an unclear function. Both proteins feature the highly conserved zinc-finger-like SWIM domain, which is predicted to interact with DNA or proteins in various biological contexts (Makarova et al., 2002; Tischfield et al., 2017). *ZSWIM6* has a third paralogue, *ZSWIM8*, which has been studied in *C. elegans*. *ZSWIM8*, known as EBAX-1, serves as a substrate recognition subunit in the BC-box Cullin-RING E3 ligase (CRL) complex, targeting the misfolded SAX-3/Robo protein for proteasomal degradation (Wang et al., 2013). Given that *ZSWIM6* shares similar BC- and Cul2-box motifs, it may also function as an E3 ubiquitin ligase (Mahrour et al., 2008). Notably, *ZSWIM6* interacts with the E3 ubiquitin ligase

HECW2, whose mutations are associated with intellectual disability, epilepsy, and repetitive motor behaviors (Acharya et al., 2022; Hyder et al., 2021; Lu et al., 2013).

This study is the first to report the involvement of *ZSWIM5* in a neurological disorder, underscoring the critical importance of maintaining a delicate balance in synaptic signaling for proper cognitive function. Our analysis suggests that the identified *ZSWIM5* variant disrupts protein-protein interactions that are crucial for neurodevelopment, thereby supporting the gene's role in cognitive processes. Disruptions in similar proteins, such as *ZSWIM6*, have already been implicated in neurodevelopmental disorders like autism spectrum disorder and schizophrenia (Sohal & Rubenstein, 2019; Canitano & Pallagrosi, 2017). Given the high degree of homology between *ZSWIM5* and *ZSWIM6*, it is reasonable to hypothesize that *ZSWIM5* may function similarly, particularly in regulating the excitatory/inhibitory balance critical to neurodevelopment. This assumption is further supported by the shared structural domains and interaction networks between these paralogues, which may influence similar pathways in the brain. Consequently, our findings not only highlight the potential involvement of *ZSWIM5* in the pathophysiology of neurodevelopmental disorders but also underscore the need for further research to explore its precise mechanisms of action. Future studies focusing on *ZSWIM5* could provide deeper insights into its role in neurodevelopment and open new avenues for targeted therapeutic interventions in conditions associated with synaptic dysregulation.

In ***Family G*** and ***H***, no candidates of interest were established either.

The complexity of the nervous system renders it highly susceptible to various genetic variations. While elucidating the relationship between genes, proteins, and their functions is valuable, the core challenge of understanding disease pathology remains elusive. This complexity contributes to the slow progress in bridging gene discovery with therapeutic applications.

In conclusion, this study significantly extends previous genetic investigations by not only reaffirming known associations—such as the *RNASEH2C* founder mutation linked to AGS in South Asian populations and the *ASPM* truncation variant in primary microcephaly—but also unveiling novel pathogenic variants with compelling implications. Notably, we report the first Pakistani male cases of Baralle-Macken syndrome associated with a newly identified *COPB1* missense variant, thereby broadening the phenotypic and ethnic spectrum of the disease. Furthermore, the discovery of a previously unreported *ZSWIM5* variant in individuals with ASD introduces a novel candidate gene in the context of neurodevelopmental disorders, underscoring the genetic heterogeneity and complexity of ASD etiology. These findings represent a meaningful advancement from earlier

studies by offering both population-specific insights and expanding the mutational landscape of rare neurological and metabolic disorders. This highlights the indispensable role of WES in underrepresented populations and reinforces the value of functional validation and further research to clarify pathogenic mechanisms and inform targeted therapies.

In summary, this study provides a comprehensive analysis of genetic variants associated with the disease in question. Through a combination of genome-wide association studies (GWAS) and whole-exome sequencing (WES), we have identified several variants that appear to confer increased risk. Notably, some of these variants are located in previously unreported genomic regions, highlighting the importance of continued exploration in this area.

Our findings contribute valuable data to the existing body of genetic research and may inform future diagnostic and therapeutic strategies. However, the complexity of genetic architecture and the interplay of environmental factors necessitate cautious interpretation.

Future research should include functional studies of the novel variants identified in this study to elucidate their biological roles and mechanisms of action. Additionally, larger-scale population studies are warranted to validate these associations across diverse genetic backgrounds and to improve the generalizability of our findings.

Ultimately, these efforts will deepen our understanding of the genetic underpinnings of the disease and pave the way for more effective, personalized medical interventions.

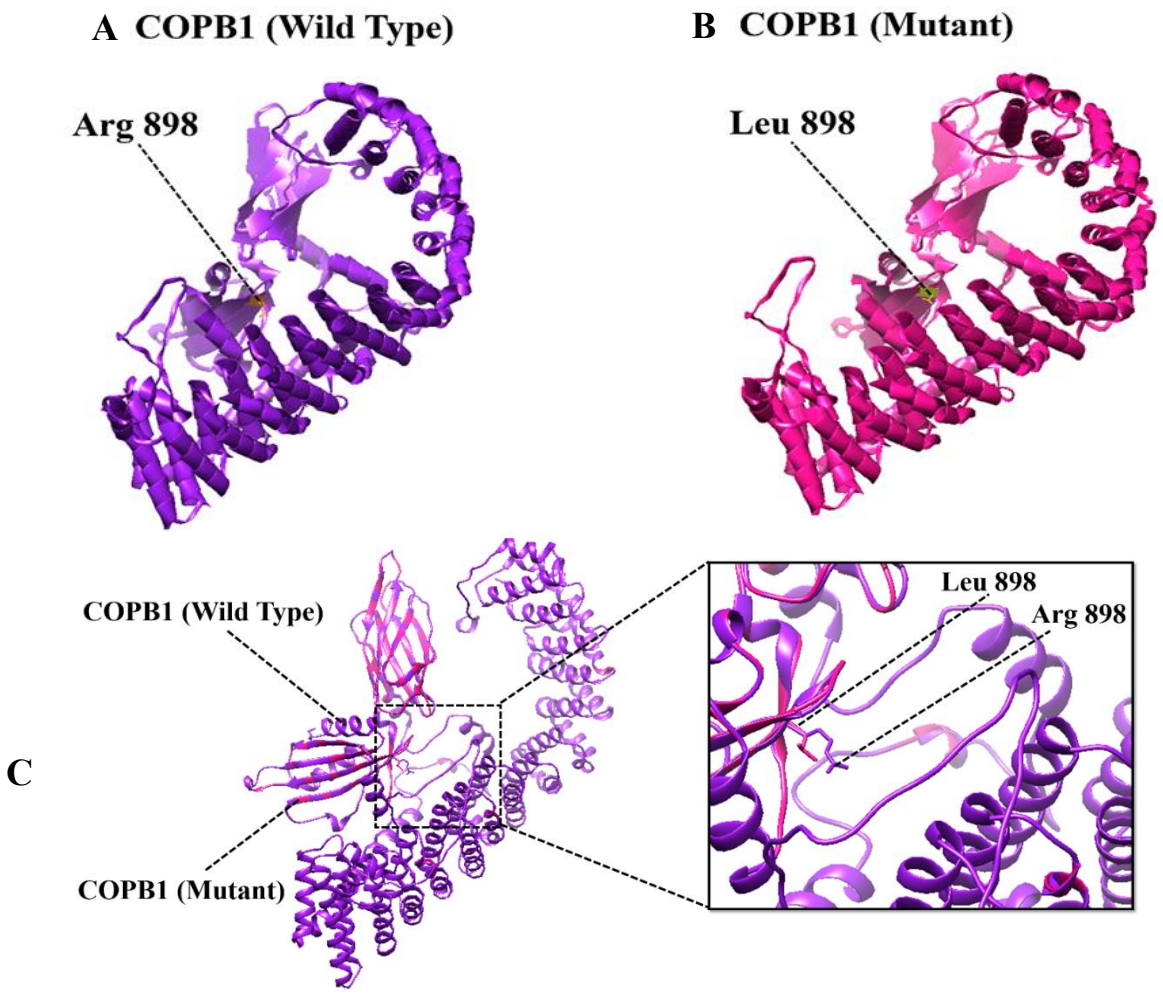

***Figure 4.1*: Three-dimensional structural representation of *COPB1* wild type and variant proteins. A: 3D structure of *COPB1* (wild type) in forest purple with Arg 898 in orange; B: 3D structure of *COPB1* (mutant) in hot pink with Leu 898 in yellow; C: Structural superposition of wild type and mutant proteins. *COPB1* (wild-type) is represented in purple whereas, *COPB1* (mutant) is shown in deep pink color.**

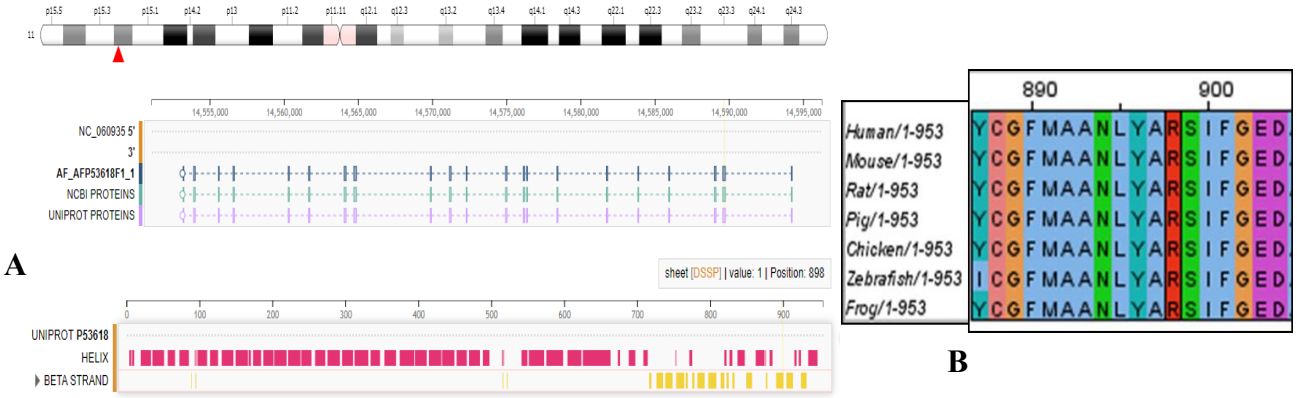

Figure 4.2. **A. Structural analysis of the homozygous missense variant in the *COPB1* gene (Chr11(GRCh37): g.14480187C>A; c.2693G>T; p.Arg898Leu) located on exon 21/22 within the highly conserved cytogenetic band 11p15.2. B. Highly conserved amino acid region forms the Beta strand of COPB1 protein.**

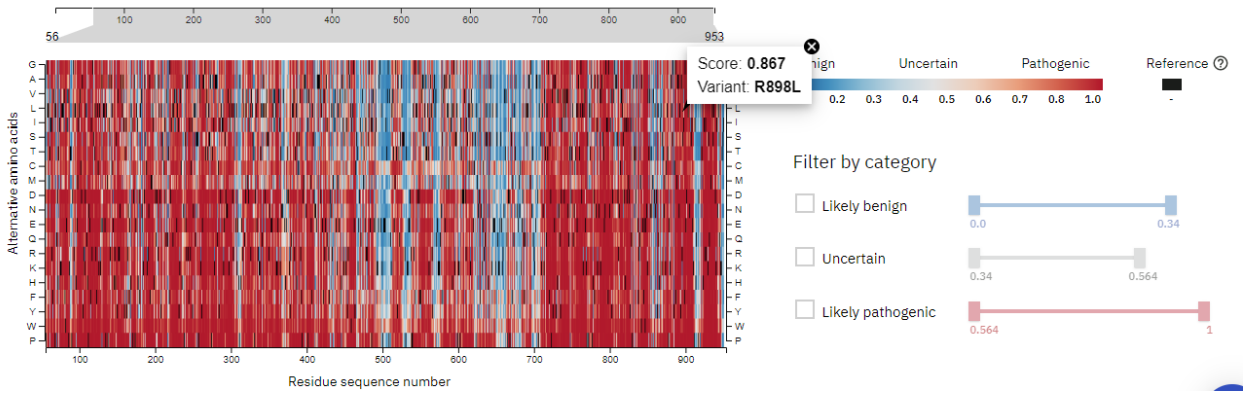

Figure 4.3. **AlphaFold protein structure database indicates the pathogenic nature of the *COPB1* variant (p.Arg898Leu) with a high pathogenicity score of 0.777.**

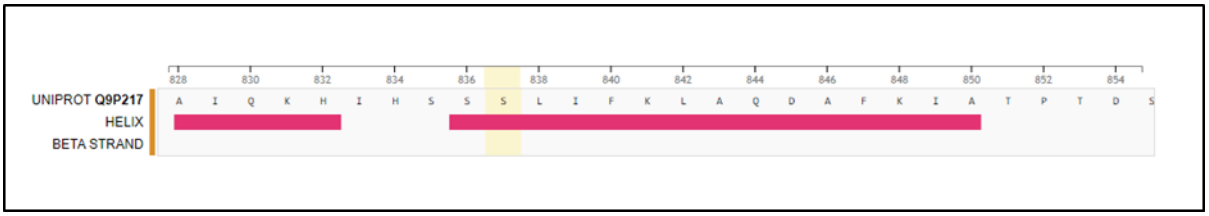

Figure 4.4 **Structural analysis of p. Ser837Pro shows it to be a part of a helix structure.**

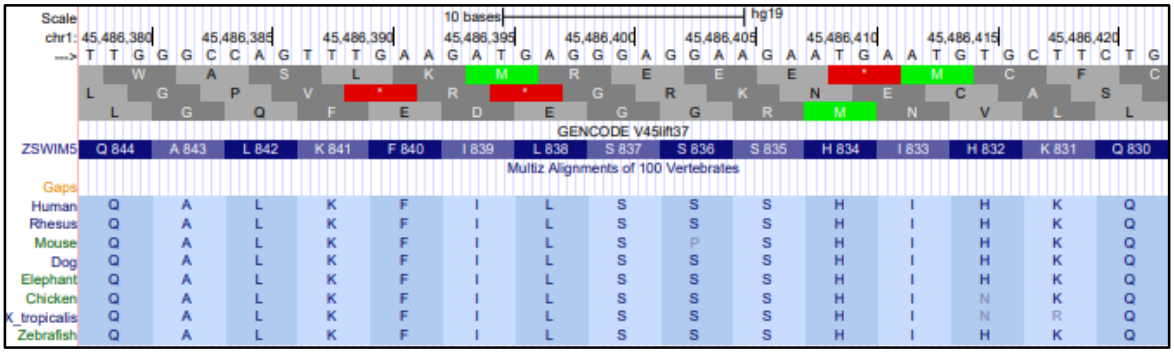

Figure 4.5 **Positional analysis of p. Ser837 shows it to be a highly conserved region.**

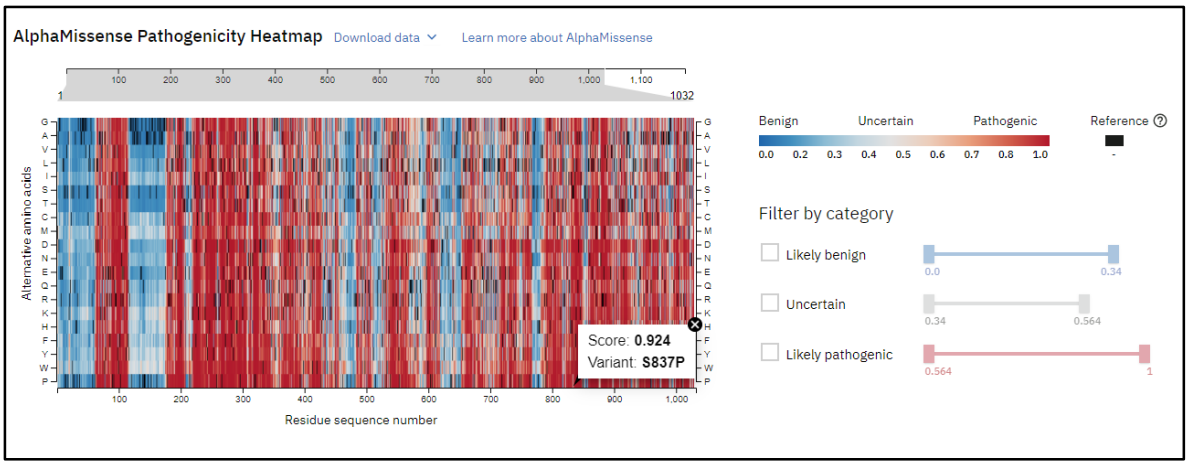

Figure 4.6 **The AlphaFold protein structure database predicts a high pathogenicity score of 0.924 for the S837P substitution.**

Chapter 5

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5.0 References

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