Identifying the Type of Niemann Pick Disease through Next Generation Sequencing Technology

Misbah Naeem Khan

Reg. No: 02272111015

A thesis submitted in partial fulfillment of the requirements of Quaid-i-Azam University for the degree of Master of Philosophy in Biotechnology

Department of Biotechnology
Faculty of Biological Sciences
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In the name of Allah, The Most Gracious, The Most Merciful.

CERTIFICATE

It is certified that the research work presented in this thesis titled "Identifying the Type of Niemann Pick Disease through Next Generation Sequencing Technology" was conducted by Misbah Naeem Khan. This thesis, submitted to the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the requirements for the thesis necessary for the partial fulfillment of the degree of Master of Philosophy in Biotechnology.

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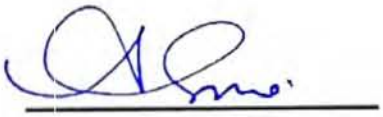

Dr. Asma Gul

Chairperson

Dr. Javaria Qazi

Dated

26th May, 2023

“Proclaim! (Or read!) in the name of thy Lord and Cherisher, Who created – Created man, out of a (mere) clot of congealed blood: Proclaim! And thy Lord is Most Bountiful, He Who taught (the use of) the pen, Taught man that which he knew not”

[Quran, 96:1-5].

Declaration

I hereby declare that the work presented in this thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously published or presented for any other degree or certificate.

Dedication

To my parents,

Mr. and Mrs. Naeem (late**)**

For always being close to my heart and a source of love, encouragement and inspiration, whose efforts and struggles have helped me to pursue all my academic goals, what I am doing in my career owes to them.

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Misbah Naeem Khan

Declaration of Originality

I hereby declare that the work accomplished in this thesis is my own research effort carried out in the Medical Genetics Research Laboratory, Department of Biotechnology, Quaid-i-Azam University Islamabad and is written and composed by me.

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Name: **Misbah Naeem Khan**

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LIST OF ABREVIATIONS

Abbreviations	Descriptions
%	Percentage
°C	Degree centigrade
Amp	Amperes
ACMG	American college of medical genetics and genomics
µg	Microgram
AR	Autosomal recessive
bp	Base pair
C	Cytosine
G	Guanine
Leu	Leucine
Val	Valine
HMD	Hereditary Metabolic Diseases
NPD	Niemann Pick Disease
EDTA	Ethylene diamine tetra acetic acid
mA	Milliampere
Mg	Milligram
mM	Millimolar
NaCl	Sodium chloride
ng	Nano gram

nM	Nano molar
OMIM	Online Mendelian inheritance in man
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
SIFT	Sorting Intolerant from Tolerant

ABSTRACT

Niemann Pick Type A Disease is an autosomal recessive disorder which is caused by mutations in SMPD1 gene encoding ASM protein. The gene is responsible for hydrolysis of Sphingomyelin into Ceramide within lysosomes. SMPD1 is located on chromosome 11 in a region between p15.1-p15.4. The type A NPD is a severe illness, characterized by neurovisceral symptoms, mainly hepatosplenomegaly, poor growth, cherry red spot and respiratory failure and a short life span. In this study, a Pakistani family was recruited from PIMS Hospital, Islamabad with symptoms supportive of NPD. The main objective of this study was to analyze the responsible variant through whole exome sequencing. Molecular diagnosis was utilized to filter the specific variant resulting in clinical manifestation of NPD. The specific variant for the disorder shows autosomal recessive inheritance pattern and proband shows missense mutation in exon 1 which is likely the cause of NPD in the family. The results will provide insight into further analysis and characterization of NPD and its related diseases. Protein expression studies are required to illustrate the effect of variant on protein stability, structure, function, and its role in development of phenotypic characteristics.

1. Introduction

1.1. Hereditary Metabolic Diseases

Defects in the catalytic activity of different enzymes are the root cause of hereditary metabolic diseases (HMD). One of the most common autosomal recessive illnesses in humans, HMDs has been the subject of extensive research. There are over 800 different varieties of HMDs (Scriver et al., 2001).

The prevalence of HMDs in various forms ranges from 1 in 10,000 newborns to $1:10^5$-10^6, each distinguished from one another by marked differences in their occurrence among different ethnicities. HMDs are subdivided into 22 groups based upon the type of particular compromised metabolic pathway (metabolic disorders of lipids, steroids, carbohydrates and other hormones, pyrimidines and purines, porphyrin and bilirubin, among others aminoacidopathy, organic aciduria,), or on the basis of a disorder's intracellular location (lysosomal, mitochondrial, and peroxisomal) (Zakharova, 2012).

1.2. Lysosomal storage diseases

LSDs is basically a group of 60 different nosologically recessive illnesses, that hold distinction among HMDs (Gorbunova, 1997; Scriver, 2001). They are all caused by inherited defects in lysosomes affecting their normal functions. Most biological macromolecules inside cells, like glycosaminoglycans, glycoproteins, or glycolipids are subjected to hydrolysis by lysosomes (Zakharova, 2012).

The Golgi apparatus controls formation of primary lysosome, which in turn combine with other membrane vesicles to form secondary lysosomes. During autophagy, secondary lysosomes are enclosed with material that have entered the cell either via endocytosis or directly absorbed to the cell surface. To bring about autophagy, endosomal-lysosomal system that works closely with chaperone-mediator system, is centralized to lysosomes. Lysosomal enzymes are basically acid hydrolases that split macromolecules into their constituents: amino acids, monosaccharides, fatty acids, and nucleic acids. Synthesis of hydrolases occur in endoplasmic reticulumn, which are subsequently further modified post-translationally, such as glycosylation with oligosaccharides in

order to obtain a terminal residue of the sugar, mannose-6-phosphate. At any point during the synthesis as well as maturation of these enzymes, genetic diseases cause the related specific substrates, such as glycoproteins, lipids, gangliosides, mucopolysaccharides and so on, to accumulate in lysosomes. The formation of the foam cells is morphological evidence, of an increase in the number of lysosomes as a result of this process. Non-split macromolecules buildup can result in significant accumulations, particularly in tissues and organs that regenerate at a faster rate. Activator proteins responsible for breakdown of insoluble substances like glycolipids, and proteins that control the mobility of vesicles enclosing lysosomal enzymes and substrates subjected for degradation, and proteins that govern the formation of lysosomes are all affected by genetic faults in some LSD patients. Certain lysosomal biogenesis-related proteins, activator proteins that break down insoluble substrates like glycolipids, and proteins that control the mobility of vesicles (containing hydrolysis or substrates lysosomal enzymes), all may have genetic defects that can lead to the development of LSDs. (Gorbunova ,2013; Scriver et al, 2001)

Lysosomes are abundant in mononuclear phagocytic system cells, which is why they are frequently engaged in the pathogenic process in LSDs. The natural locations of the associated macromolecule destruction are the target organs. As a result, the brain's white matter is affected when myelin catabolism is compromised, and the buildup of un-split macromolecules in CNS tissues typically promotes the onset of neurodegenerative diseases and mental retardation. Multiple dysostoses is caused by accumulation of any pathogenic matter in bony tissues, and mucopolysaccharides, which are found in most tissues, that lead to systemic organ and organ system damage. Thrombocytopenia, anaemia, and hepatosplenomegaly may be the result of accumulations in parenchymal tissues (Scriver et. al, 2001). Neuronal ceroid lipofuscinosis, mucopolysaccharidoses (MPS), oligosaccharidosis, lipidoses, and mucolipidoses are among the certain types of LSDs that have been reported (Gorbunova, 2013, 2015; Novikov, 2014).

Sphingolipids in lysosomes are hydrolyzed by Glycohydrolases sequentially isolating their terminal sugars from their core ceramide. Sphingolipidoses comprises a group of illnesses known as gangliosidosis, cerebrosidosis, and leukodystrophy respectively.

Cerebrosidosis includes conditions including Fabry disease (Novikov et al., 2015), glucosylceramide lipidosis or Gaucher disease (Lukin et al., 2014), lipogranulomatosis also called

Farber disease, and sphingomyelin lipidosis (also known as Niemann Pick disease) (Kutsev, 2015; Gorbunova et al., 2022).

1.3. Niemann-Pick disease (NPD)

The word Niemann-Pick disease (NPD) is a group of rare neurovisceral lipid storage disorders that are primarily categorized by the deposition of sphingomyelin, other lipid species, and cholesterol (and their respective derivatives) within the reticuloendothelial system, with or without central nervous system (CNS) involvement (Vanier 2010).

1.3.1. Types

There are three main types of NPD depending upon the type of genes involved and severity of symptoms;

- Type A which is severe type, appears in young children and is more prevalent in Jewish households. Other signs often include severe neurological impairment around six months of age. Rarely do children of this sort live past the age of 18 months.
- Type B, commonly referred to as juvenile onset, generally shows symptoms in adolescence, including ataxia and peripheral neuropathy (nerve damage and disrupted signaling). The brain is often unaffected. Sphingomyelin, a fatty molecule present in every cell of the body, accumulates toxically in types A and B due to insufficient enzyme activity. Children with type B may have comparatively longer lives, but because of lung damage, they may require breathing care.
- Type C might appear as an adult or as a kid, depending on when it develops. It is brought on by a lack of the NPC1 or NPC2 proteins. Neurological problems include comprehensive brain damage, which can cause problems with looking up and down, walking and eating difficulty, and a continuous loss of vision and hearing. Depending on their circumstances, some people die as children while others live to adulthood (https://www.ninds.nih.gov)

1.3.2 History

Albert Niemann (1914), a German pediatrician, studied a Jewish infant with cerebral and nervous system impairment and hepatosplenomegaly.

Ludwig Pick analyzed tissues from children who died with similar symptoms and found a new condition that differ from the previously described lipid storage disorders.

Crocker and Farber published a case study on patients among different forms of Niemann-Pick disease catagorized by the presence of 'foam cells' (lipid-laden macrophages) and high tissue sphingomyelin level in 1958. Crocker subsequently classified Niemann-Pick into four distinct groups based upon biochemical and clinical parameters since their patients' comprised children with typical neurological symptoms as well as those with no neurological symptoms.

Kanfer et al(1966) reported that the primary metabolic errors in NPD type A and type B was the result of a significant shortage in ASM deficiency (but not NPC).

The results, which included the deposition of complex glycosphingolipids in NP other than sphingomyelin, suggested that NPC should be treated as a different illness from NPA and NPB. NPD type C was distinguished from type A and B, by an expert consensus in Prague in 1982 (Schuchman & Desnick,2001)

1.3.3 Genes Involved in Niemann-Pick disease

1.3.3.1. SMPD1

Pathogenic mutations in *SMPD1* often lead to acid sphingomyelinase deficiency (ASMD), also known as Niemann-Pick disease (NPD) type A and B , which is one of the type of recessive LSDs .The acid sphingomyelinase 5-6 kb long gene , contains six exons, and is located on the p15.1-p15.4 region of chromosome 11. It is denoted by SMPD1 symbol in humans and Smpd1 in mouse (da Veiga Pereira et al. 1991). It encodes the human ASM protein (Uniprot ID: P17405), a phosphodiesterase 631

amino acids long having a saposin domain, which is basically a proline-rich linker, a C-terminal domain and a metallophosphatase catalytic domain (Zhou et al., 2016). According to HGMD, Total number of reported mutation up till now is 405. In Pakistani population there are five variants identified in *SMPD1* up till now (Cheema et al.,2020) as shown in table 1.

SMPD1 Mutation	*Amino Acid Change*	*Patient ID*	*Gender*	*Age at enrollment*	*Type of Mutation*	*Exons*	*gnomAD Frequency*	*ExAC*	*Consanguinity*	*Clinical Symptoms*	*Area*
c. 1718G>C	p. Trp573Ser	PKNP1	Male	12 months	Missense	6	0	0	Yes	Abdominal Distention Recurrent Chest infection PCV transfusion for once	Mianwali
		PKNP4	Male	13 months					Yes	Abdominal Distention Respiratory tract infection Mild to Moderate Developmental Delay	KPK
		PKNP6	Female	3 years					Yes	Abdominal Distention Malena 4 times PCV Transfusion Recurrent Respiratory tract infection Moderate Developmental Delay	KPK, Afghan Border
c. 1267C>T	p. His423Tyr				Missense	3	(0/1/143266) 6.98e-6	0			
		PKNP7	Female	21 months					Yes	Progressive Pallor Abdominal Distension PCV transfusion twice Recurrent Chest infection Underweight Stunted	Sindh
c. 1327C>T	p. Arg443Term	PKNP2	Female	19 months	Nonsense	3	(0/6/143204) 4.19e-5	8.25e-6	Yes	Progressive Pallor followed by abdominal distension Recurrent respiratory tract infection Mild to Moderate Developmental Delay Anicteric Abdomen distended	Hyderabad Sindh
c. 1493G>A	p.Arg498His	PKNP5	Female	13 months	Missense	4	0	0	Yes	Mild Development Delay Abdominal Distention Recurrent Respiratory tract infection Failure to thrive	Baluchistan
c.740G>A; 1493G>A	p. Gly247Asp; Arg498His	PKNP3	Female	17 months	Missense	2, 4	0,0	0,0	Yes	Progressive Abdominal Distention 3 times PCV Transfusion Recurrent Respiratory tract infection	Lahore

Figure 1- 1: Mutation in SMPD1 identified in Pakistani Patients with NPD

1.3.3.2. NPC1 and NPC2

According to Rosenbaum et al. (2011), mutations in NPC1 or NPC2 genes cause Niemann-Pick disease, type C (NPC), which accounts for roughly 95% of cases. The 56 kb long NPC1 gene, which has 25 exons and is located on chromosome 18q11–q12, is mapped. According to Millat et al. (1999), one mutant allele, p.I1061T, is particularly prevalent. (About 20% to 25% of alleles in patients with diagnoses from France or the UK). The NPC2 gene (formerly known as HE1) spans 13.5 Kb and has 5 exons. It is located on chromosome 14q24.3 [Naureckiene et al., 2000]. According to Verot et al. (2007) and Millat et al. (2001), one nonsense mutation (E20X) appears to be relatively common.

The large transmembrane protein NPC1 (encoded by NPC1) is 1278 amino acids long and is found in late endosomes and lysosomes. It has 13 transmembrane domains, three luminal domains, a lysosomal signal region, and more [Kwon et al., 2009]. High levels of glycosylation, a cholesterol-binding area, and a sterol-sensing domain (SSD) are all features of the luminal domains [Infante et al., 2008; Ioannou, 2000]. NPC2, a soluble cholesterol-binding protein, contains 151 amino acids and four highly conserved domains that control the binding and release of cholesterol [Ko et al., 2003; Friedland et al., 2003; Infante et al., 2008].

It is believed that within the lysosomal lumen, the soluble intra-lysosomal NPC2 protein binds the unesterified cholesterol that was originally transported into the lysosomes as a component of the endocytosed LDL particles (i.e., exogenously derived cholesterol). Finally, NPC2 transfers the free cholesterol to the membrane-bound tag-team member NPC1, who then transports the free cholesterol outside the lysosome (Subramanian and Balch 2008)

1.3.4 Incidence/ Epidemiology

Low ASM expression, often less than 10% of control values, and associated mutations in each copy of the gene are the main characteristics of ASM deficiencies (Niemann-Pick

disorders, types A and B) [McGovern et al., 2006]. The sole gene associated with ASM deficit is SMPD1 [Jones et al., 2008]. With incidence rates ranging from 0.5 to 1.0 per 100,000 persons, such autosomal recessive illnesses are frequently less prevalent [Schuchman, 2009]. Acid sphingomyelinase breaks down sphingomyelin (SM) into phosphorylcholine and ceramide. The hydrolase known as acid sphingomyelinases (ASMases) cleaves the phosphodiester bond in SM, releasing the phosphorylcholine head group and ceramide in the process. Niemann-Pick disease is brought on by Sphingomyelin deposition, which is caused by ASMase deficiency (OMIM; NPD-A: 257200, NPD-B: 607616). (Schuchman and colleagues, 2015&2017).

Type A ASMD has a generally uniform natural history, with rapid manifestation and a short life expectancy (McGovern et al.,2013 &2017). Those with ASMD type B, on the other hand, have a broad spectrum of disease symptoms, with a varying rate of disease progression, degree of severity, as well as life span. (Wasserstein et al.,2004; Lipiski et al. 2019; Hollak et al.,2012)

Niemann-Pick Type C is a neurovisceral condition caused by delayed transport of endosomal/lysosomal cholesterol and glycosphingolipids [Vanier,2010]. Mutations in one of two autosomal recessive genes, NPC1 (the majority of cases) or NPC2 (4% of cases), cause the disorder. Despite the fact that the phenotypes resulting from mutations in the two genes are nearly indistinguishable, the disease forms can sometimes be referred to as NPC1 and NPC2 to indicate the underlying mutation. The likelihood of developing NPC is one in 20,000. [Vanier,2010].

1.3.5 Symptoms

Type A Patient NPD display hepatosplenomegaly and underachievement within the first year of life. About 50% of these infants have a cherry-red patch in their macula. Rapid neurological progression, severe hypotonia, and failure to meet milestones are the disease's defining features. According to McGovern et al. (2006) and Schuchman et al. (2001), the majority of type A newborns do not live past their third year of life.

Except for mature red blood cells, all other cells contain lysosomes. As a result, ASM deficiency will have an effect on all cells. However, because monocyte/macrophage cells

are generally active in phagocytosis and rich in lysosomes, they are the mainly affected by ASMD. A large number of lipid-laden macrophages infiltrating the tissues are seen in histological imaging of reticuloendothelial organs, such as the liver and spleen (Pick.,1927). The same is true in case of lungs, where macrophages may be found both in the airways and the interstitial tissue, The central nervous system, lymphatic and haematopoietic systems, skeletal system, and, heart, all may be get affected in patients with ASMD (Elleder & Cihula,1983; Rodriguez &Vanier,1999; Thurberg et al.,2012).

Type B patients, on the other hand, show severe signs of hepatosplenomegaly accompanied by liver failure but with no apparent CNS involvement (Wasserstein et al.,2004; McGovern et al.,2008, Hollak et al.,2012]. It is also seen that there are elevated Triglycerides and LDL cholesterol, whereas low HDL cholesterol. Type B involves lungs, with compromised pulmonary function. These patients' eyes may also have a reddish-brown halo around the macula, however, in certain cases, a clear cherry red spot can also be observed. Also, NPD patients with intermediate symptoms between types A and B are also reported [Pavl et al, 2005].

According to Patterson et al. (2012) and Wraith et al. (2009), the clinical range and illness course are incredibly variable. In addition, the principal disease symptoms have been shown to depend on age, therefore NPC is now categorized into three main "forms" based on the age at which they first manifest: infantile, juvenile, and adult forms respectively (Vanier, 2010; Patterson, 1993). In the first year of life, visceromegaly—typically hepatosplenomegaly—and cholestatic jaundice are present in more than 40% of patients with all variants of NPC [Vanier & M.T., 2010, & Patterson et al., 2012]. Although most patients' jaundice and hepatomegaly resolve within 6 to 12 months, splenomegaly typically persists [Garver et al., 2007; Stampfer et al., 2013]. Furthermore, foetal onset NPC is another distinct phenotype, which manifests as foetal ascites or nonimmune hydrops fetalis [Vanier & M.T.,2010, Geberhiwot et al.,2018, Spiegel et al.,2009 & Maconochie et al., 1989 & Surmeli-Onay et al.,2013].

Followed by a phase of normal or rather a delayed growth, neurological symptoms may appear in majority of the patients. While at first there may be some nonspecific symptoms, such as clumsiness, developmental delay, hypotonia, and also regression may gradually

occur in patients, with loss of motor and cognitive abilities [Vanier &M.T.,2010, Patterson et al., 2012, Mengel et al.,2013]. According to several studies, NPC is more severe in patients with early-onset, than those with later-onset neurological illness (Vanier & M. T., 2010, Geberhiwot et al., 2018, Spiegel et al., 2009, Patterson et al., 2017, Rego et al., 2019).

1.3.6 Disease mechanism

Patients with NPD types A and B commonly tend to accumulate sphingomyelin (ceramidephosphocholine) lipids. (Schuchman & Desnick,2001). Cell membranes largely consist of sphingomyelin, an important phospholipid found in the myelin sheath (Quinn, 2014; Jana & Pahan,2010). Along with sphingomyelin, higher levels of bis(monoacylglycero)phosphate and lyso-sphingomyelin have also been reported (Kirkegaard et al., 2010; Chuang et al., 2014).

The enzyme acid sphingomyelinase (EC 3.1.4.12), which is absent in types A and B NPD, works at low pH and hydrolyze the sphingomyelin into phosphocholine and ceramide (N-fatty acylsphingosine) within Lysosomes (Henry et al.,2013; Schuchman,2010). Within the lysosomes, where it plays an essential function, ASM helps in membrane turnover and proper sphingolipid balance. However, ASM immediately migrates from the lysosomes to the outside of the plasma membrane under stress (such as heat shock or radiation), where it then causes the hydrolysis of sphingomyelin into ceramide. (Charruyer et al.,2005, Falcone et al.,2004). As a result, subsequent signaling pathways are stimulated, and "raft" structures, which are basically membrane microdomains, are reorganized. As a result, lipid abnormalities in the plasma membrane, in addition to lipid accumulation within lysosomes, may be responsible for clinical symptoms in types A and B NPD. All such membrane anomalies may then result in a variety of downstream changes in receptors functioning, cellular signaling pathways, and inefficient transport systems. It has been well known that the brains of ASM knock-out mice exhibit impaired synapse formation and functioning due to sphingomyelin deposition in the neuronal membranes (Buccinna et al.,2009; Scandroglio et al.,2008; Galvan et al.,2008).

The CNS is harmed and exhibits neuronal death in Niemann-Pick A, and as well as in Niemann-Pick C, and Gaucher diseases 2 and 3. Sphingomyelin accumulates in lysosomes as a result of Acid Sphingomyelinase deficiency, which results in Niemann Pick type A illness (NPAD). Mutations in the two genes, NPC1 and NPC2 genes, which encode the cholesterol transporter proteins in lysosomes and control the outflow of free cholesterol from this compartment, result in Niemann Pick type C disease (NPCD). As a result, cholesterol builds up in lysosomes in NPC cells. Mutations in the gene encoding the glucocerebroside 1 (GBA1) enzyme, that is in charge of breaking down the glucosylceramide (GlcCer), glycolipids and glucosylsphingosine, may result in Gaucher disease (GD) (GlcSph). The accumulations of Glycosphingolipids (GSLs) within the lysosomes is the result of deficiency of this particular enzyme. It's interesting to note that secondary lipids commonly build in these three disorders, such as cholesterol in NPA and GD, as indicated in figure 1 (Yaez et al., 2020).

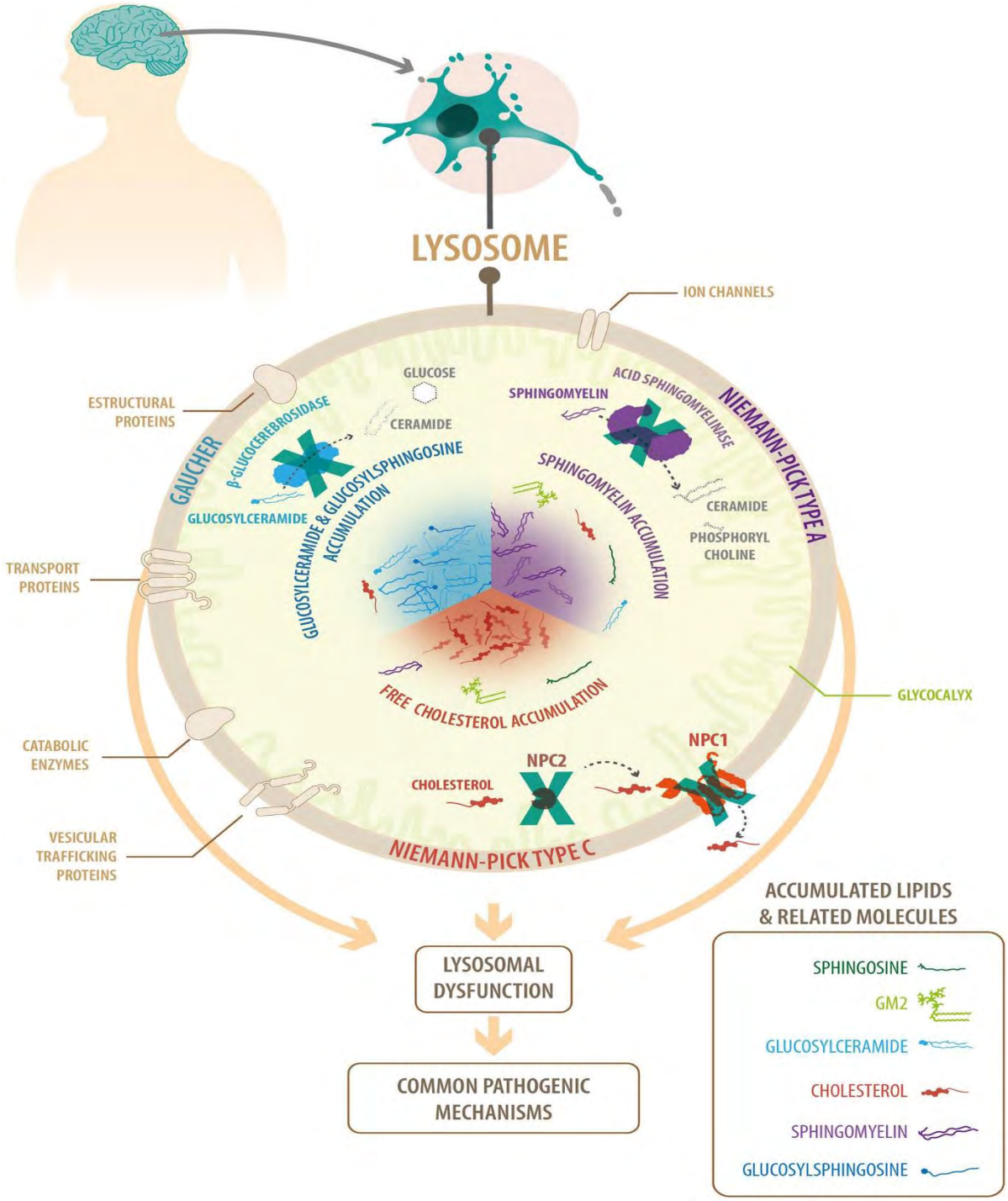

Figure 1- 2: Features of lysosomal neurodegenerative diseases: Niemann-Pick A, Niemann-Pick C

1.3.7 Diagnosis

ASM activity can be used to confirm suspected cases of ASM deficiency in cultured EBV-transformed lymphoblasts, white blood cells, or in primary skin fibroblasts. The most reliable and source for enzymatic testing of ASM-deficient NPD are skin fibroblasts, which typically have the high level of regular ASM activity. There are several techniques available to measure ASM activity within cellular extracts. The residual ASM activity in NPD patients with ASM deficiency is often less than 10%. However, there is no as such reliable relation between residual ASM activity and its clinical presentation, and the residual activities may be more pronounced in white blood cells. The enzymatic diagnosis should always be confirmed with SMPD1 mutation analysis, which may also have some added use for phenotypic prediction. Since most of the potential DNA alterations that have not been still expressed and may instead represent polymorphisms that result in normal or nearly normal levels of ASM activities, mutation analysis therefore alone should not be employed for diagnosis of ASM impaired NPD. For prenatal screening of ASM deficiency, mutation analysis and enzyme activity may also be done on amniotic cells or chorionic villi (Mellissa et al.,2022)

Feature	Type A	Type B
Age at onset/diagnosis	Early infancy	Childhood/ adolescence
Neurodegenerative course	+	–
Cherry-red macula	+	+/–
Dyslipidemia	+	+
Hepatosplenomegaly	+	+
Marrow NPD cells	+	+
Pulmonary disease	+	+
Liver disease	+	+/–
Age at death	2–3 years	Childhood/ adulthood
Autosomal recessive inheritance	+	+
Ashkenazic Jewish predilection	+	–
Acid sphingomyelinase activity	<5%	<10%

Figure 1- 3: Typical Clinical & Laboratory findings of ASM-deficient NPD.

There are no effective treatments for ASMD at the moment. Amniotic cell and Bone marrow transplant, thorough lung lavage, and are a few experimental approaches that are employed, but have no favorable benefit/risk ratio due to their unpredictable impact on long-term and short- term disease outcomes and complications [Schuchman EH, Wasserstein MP., 2015].

Splenectomy may also be necessary in certain types of severe splenic necrosis with functional loss [Lidove et al., 2015] or rupture [Chen et al., 2008, Simes and Maia, 2015], although it is typically not advised because it may aggravate pulmonary illness [McGovern et al., 2008]. So, supportive care and palliation are the only treatments available for patients with ASMD, which calls for a multidisciplinary approach [Pastores & Hughes, 2015].

In newborns with NPD A, physical and occupational therapy may be helpful, but it should be planned with reasonable objectives. Chronic oxygen therapy and alteration of everyday routines may be necessary for patients with progressive pulmonary illness. To reduce the risk of pneumonia, vaccination against certain strains of Streptococcus pneumoniae and influenza should be considered. Adult patients with lipid abnormalities linked with ASMD should be treated with conventional lipid-lowering medications. Although some patients have received growth hormone therapy to correct their small height and speed up their linear growth (Wasserstein et al.,2003)

For the treatment of ASMD, enzyme replacement therapy (ERT) using recombinant human ASM may have the ability to change the course of the disease. With other lysosomal storage disorders, ERT represents an established mechanism of action that has also been employed successfully [Parenti et al.,2015].

Results of a 26-week phase 1b study in five adult patients with type B NPD provided preliminary evidence of the efficacy as well as safety of recombinant human ASM in the patient population. These results included decrease in sphingomyelin storage as seen in spleen and liver volumes, liver biopsies, and serum chitotriosidase activity, as well as improved infiltrative lung disease, platelet counts, lipid profiles, and quality of life assessments [A phase 1/2 clinical trial is currently underway in paediatric patients, as is a phase 2/3 trial in adult patients with ASMD (https://www. clinicaltrialsregister.eu/ctr)

The primary diagnostic test for NP-C is the demonstration of defective intracellular transport of cholesterol and homeostasis. These studies necessitate the use of living cells, such as skin fibroblast culture. They should be carried out in specialized facilities with the necessary experience. The most sensitive and also most specific assay is the filipin test. Fibroblasts are grown in an LDL-enriched medium before being fixed and stained with filipin (a compound that forms specific complexes with unesterified cholesterol). Fluorescence microscopy of NP-C positive cells usually reveals a plethora of strongly fluorescent (cholesterol-filled) perinuclear vesicles. In approximately 80-85% of cases, this "classical" storage approach is observed. Even under optimal conditions, a lower (and variable) level of storage is observed in the remaining cases, which are described as having a "variant" biochemical phenotype (Vanier et al.,1995; Argoff et al.,1991). This "variant" biochemical phenotype is known to be caused by a number of recurrent NPC1 mutations, as was previously discussed. It should be noted that a similar, mildly aberrant filipin pattern has been seen in a number of heterozygotes (Vanier,1997; Vanier& Suzuki,1998), as well as occasionally in individuals with acid sphingomyelinase deficits. In cell lines with a "classical" biochemical phenotype, the LDL-induced rate of cholesteryl ester production was found to be very low, while only a modest or non-significant impairment was found in cell lines with a "variant" phenotype until recently (Vanier et al.,1995; Argoff et al.,1991).

All newly diagnosed patients are strongly advised to undertake gene testing, as molecular genetic research is currently the primary tool for prenatal diagnosis and the only accurate way to discover carriers in blood relations. Additionally, as was previously mentioned, gene testing may be necessary to support or disprove the diagnosis of NP-C. Since cell hybridization and other testing require more effort than gene sequencing, genetic complementation studies, which were once employed in a few laboratories to identify the afflicted gene, are no longer used. It is unfortunate that severe NPC1 mutations impact more than 95% of NP-C patients since sequencing of all exons and borders is more challenging for the NPC1 gene (25 exons) than the NPC2 gene (5 short exons). Rapid techniques to check for the two most common mutations have been reported (Millat et al.,1999 &2001).

1.3.8 Mutation Spectrum

The reported range of SMPD1 variants related with ASMD is incredibly diverse. In single families and in compound heterozygosity, the majority of mutations have been discovered. As a result, it is challenging to link the genotype to the phenotype. However, based on the functional examination of single mutants and for recurrent mutations discovered in homozygosity, certain inferences can be drawn (Zampieri et al.,2016). It is easier to establish a correlation between a pathogenetic missense variant and a particular clinical phenotype than it is for massive deletions, nonsense variants, or mutations that cause a reading frameshift in both alleles, which are all associated with the severe neurovisceral phenotype. However, the evidence at hand suggests that patients with at least one SMPD1 pathogenic genotype producing a partly active ASM protein also have the visceral non-neurological phenotype (Dardis et al.,2005) L304P and R498L, the two most common missense variations found in Ashkenazi Jews, have been linked to the severe neuronopathic phenotype in this population (Levran et al.,1991&1992).

1.4. Genetic counselling

Genetic counselling should be offered to the families at risk. Information about NPD must be provided to the family. Moreover, the inheritance must be discussed. This disease is inherited in autosomal recessive pattern so there is 50% chance that both the male and female offspring will be affected or normal.

It is recommended that pre-natal and pre-implantation testing should be followed in future. Genetic counselling and carrier testing should also be offered to the carriers, affected adults and the individuals who are at the risk of being a carrier.

1.5. Aims and objectives

Aims and objectives of this study were to find the types of variants in the suspected case of Niemann Pick Disease. The proband was diagnosed as the suspected case of NPD by analyzing the family history, clinical phenotypes, and biochemical testings. But when WES was done a reported variant was found in SMPD1 gene and the disease was. Based on these results pre-

symptomatic treatment and supportive therapy to prevent the proband from regression will be done. Moreover, genetic counselling of the family would be done, and prenatal testing would be recommended in case of pregnancy in future.

2. Material and Methods

2.1. Family Description

The present study includes a family from PIMS Hospital, Islamabad having suspected case of SMPD1. The blood samples of the patient(proband) were taken. The family was coded as Family I and with four children and two unaffected children with two affected children of physically healthy parents. Diagnostic reports, and family history and family were also taken at the time of sampling. Ethical consent of the family for conducting molecular analysis of the affected daughter was taken.

2.2. Pedigree Construction

All of the data required to create a pedigree was gathered from family members during the interview. Microsoft PowerPoint 365 was used to make the pedigree. In the pedigree, several shapes were employed to represent gender, illness state, and consanguinity. A circle is used to represent female and a square is used to represent male. The impacted family members are represented by shady circles and squares. A cross on a square or circle symbol represents a deceased individual. The proband is represented by an arrow. Consanguinity is shown by the two lines separating the partners. Arabic numbers are used to symbolise each family member within a generation, while Roman numerals are used to represent generations as a whole.

2.3. Sample Collection and Ethical consent

Due to the patient's children's incapacity to give informed consent, blood was obtained from the patient as well as every member of their immediate family. Blood was drawn using a BD syringes and two 3ml Ethylene Diamine Tetra Acetate (EDTA) tubes to prevent clotting. The blood samples were then delivered to the MGRL lab , department of Biotechnology in Quaid-i-Azam University, Islamabad where they were preserved in a freezer at 4°C.

2.4. DNA Extraction from the genome

The genomic DNA of the Family 1 is extracted from human blood using the organic (Phenol-Chloroform) approach.

2.4.1. Composition of solutions

The composition of solutions used in extraction of DNA through Phenol-Chloroform method is shown in the Figure 2.1

Table 2- 1: Preparation and Composition Solution

Solution	Composition	Quantity
Solution A	Sucrose (0.3M) Tri-HCL $MgCl_2$ (5mM) TritonX-100 Distilled water	57.4g, 5ml 2.5ml 5ml 400ml
Solution B	NaCl (400mM) Tris-HCl (10mM) EDTA (2mM) Distilled water	40ml 5ml 1ml Required to make total volume of 500ml
Solution C	Phenol Hydroxyquinone Tris-HCl B-merceptoethanol	100ml 10mg 10ml
Solution D	Chloroform Isoamyl alcohol	24ml 1ml

2.4.2 Phenol Chloroform Method

Blood samples from each member of the Family 1 are taken out of the refrigerator and left at room temperature (37oC) for an hour before to DNA extraction. The steps that follow are used to extract DNA.

1. A 1.5ml Eppendorf microcentrifuge tube was used to mix an equivalent volume of blood, 750l, and solution A. This was done by repeatedly inverting the tube.

2. After that, the tubes were kept at room temperature for 10 to 15 minutes. After that, the sample was centrifuged at 13000 rpm for one minute.

3. To dissolve the pellet, 400 l of solution A were added to a microcentrifuge tube after the supernatant was discarded. A 13000-rpm centrifugation session lasting one minute came next.

4. The pellet was dissolved using 400 l of solution B, 25 l of 10% SDS, and 5-8 l of Proteinase K after once more discarding the supernatant.

5. The pellet was broken up by tapping because it was hard and overnight it was stored at 37°C in an incubator.

6. The incubated microcentrifuge tube received equal volumes of Solution C and Solution D the following day (500 l). Centrifugation for 10 minutes at a speed of 13000 rpm came next.

7. The top aqueous layer was collected in a brand-new, sterile microcentrifuge tube.

8. Next, 500 l of solution D was added to a tube, and it was centrifuged at 13000 rpm for 10 minutes.

9. The top aqueous layer was once more transferred to a brand-new microcentrifuge tube that was sterile.

10. A microcentrifuge tube was filled with 500 l of -20 °C iso-propanol and 55 l of sodium acetate in order to precipitate the DNA. The tube was then repeatedly turned upside down.

11. The mixture was then centrifuged for 10 minutes at 13000 rpm.

12. Only the supernatant was discarded; the DNA pellet remained intact.

13. 200 l of 70% ethanol that had been frozen to -20 oC was added to the microcentrifuge tube to wash the DNA pellet. centrifugation for 7 minutes at a speed of 13000 rpm. After the ethanol was removed, the pellet was dried in the concentrator for approximately 10 minutes at 45 °C with the tube left open.

15. Finally. Rehydrating the DNA required adding 80–100 l of deionized water, which was then incubated at 37 oC overnight.

2.5. Molecular Analysis of Extracted DNA Agarose Gel Electrophoresis

DNA Extraction and Molecular Analysis The collected DNA was subjected to molecular analysis using agarose gel electrophoresis.

The process was completed in three steps: loading, gel setup, and gel preparation.

1. **Gel Preparation**

1. Making gel the genomic DNA is examined on a 1% agarose gel. The components required to manufacture 1% agarose gel are as follows:

2. 0.5g agarose and 45ml of distilled water

- 10X Tris-Borate EDTA in 5 ml (TBE)

3. The following are the steps for creating 1% agarose gel.

- 0.5g of agarose and 5ml of 10X TBE were added to 45ml of distilled water.
- To completely dissolve the agarose and create a clear solution, the mixture is then microwaved for two to three minutes.
- Let this mixture cool to room temperature for 5-7 minutes.
- 5 l of ethidium bromide was added and stirred into the solution once it cooled.

2. Gel setting

The gel solution was poured into a casting tray.

- Wells are created in the gel by using the appropriate combs. I inserted the combs to make wells in the gel as a result.
- After being poured into the casting tray, the solution was left to sit for 20 to 25 minutes at room temperature to solidify.
- After the gel had formed, the casting tray containing the solidified tray was put in the electrophoretic tank with 10X TBE as the electrolyte.

3. Sample loading

Bromophenol blue, a loading dye, is used to monitor DNA mobility inside the gel. The electrodes were attached, and an equal volume (3 to l) of bromophenol blue and extracted DNA was carefully fed into the wells without damaging the gel.

2.6. Gene Sequencing

The electrodes were connected to the electrophoretic tank, and a Bio-Rad power supply delivered the voltage.

• The duration was set at 25 to 30 minutes, and the voltage and current were set at about 120 volts and 400 amps, respectively.

• We will remove the electrodes and turn off the current once we can see that the bands have travelled a certain distance. After that, a UV Trans-illuminator was used to see the bands and their intensity after the gel had been properly removed. The extracted DNA was combined with 60 l of PCR water after being diluted to a final concentration of 40ng/l.

2.6.1 Sequencing of the Entire Exome

Whole exome sequencing was used to sequence the DNA of the proband. The SureSelect V5- post kit was used to create exome libraries (Agilent Technologies, Santa Clara, CA, USA). An Illumina HiSeq 400 was used for sequencing (Illumine San Diego, CA, USA). For 150 bp termination reads, the average depth of the target area was 142X. The resultant reads were mapped to the human reference genome using the BWA-men alignment program(http://biobwa.sourceforge.net/bwa.shtml) (UCSC, human genome assembly 19). For variant calling, the Genome Analysis Toolkit (GATK) was utilized. Any duplicate readings were eliminated using Picard tools. The Haplotype Caller pipeline and SnpEff (SnpEff v4.1) were used to annotate variant alleles in the VCF form5nset t. The subsequent stages of whole exome sequencing are used to separate the disease-related variation from the thousands of other variants.

• Several options were chosen, including frame shift, non-synonymous/synonymous, and other variations.

• The presence of an exon or intron variant that affects coding.

• Modifications to stop codon numbers

• Variants in the gnomAD-genome aggregate database with allele frequencies (accessed on 8 August 2022 at https://gnomad.broadinstitute.org).

• It is possible to determine if a variant will be harmful or pathogenic using in silico tools like Mutation Taster, SIFT, and Polyphen2.

• Following filtering, several variations were found in the proband.

A specific disease-causing variant will be confirmed using Sanger sequencing.

• The affected individuals must be homozygous for the candidate gene (SMPD1). It should be possible to see heterozygous peaks in carriers of this variant.

• The variant should be present in the coding regions of the exon.

• The altered text must be illegible or misspelt. Using in-silico techniques, the pathogenicity of the variants would be evaluated.

• According to the 1000 Genome Project, a variant's pathogenicity, or minor allele frequency, should be less than 0.01. (http://www.1000genomes.org)

2.6.2 Primer Designing

The SMPD1 gene's exon 1 was determined to have the mutation [c.103 C>G(p.Leu35Val)] using the ensembles genome browser (accessed on June 10, 2022). The precise parameters of the primers were analyzed using Oligo Calc and Sequence Manipulation Suite, PCR Primer Stats (SMS) (accessed on June 10, 2022) (https://biotools.nubic.northwestern.edu/OligoCalc.html and https://www.bioinformatics.org/sms2/pcr primer stats.html). BLAST (https://asia.ensembl.org/Homo sapiens/Tools/Blast/, retrieved on June 10, 2022) is used to examine the homology and hits of the primers. In-silico PCR was also used to measure product size to see if a virtual PCR utilizing the selected genomic sequence, forward and reverse primers, is possible or not. The chosen primer combination must be acquired from MACROGEN Korea.

Table 2- 2: List or SMPD1 Gene variant

Exon no.	**Type F/R**	**Primer sequence 5' to 3'**	**Primer length (bp)**	**T_m (c)**	**Gc content (%)**	**Product size (bp)**
01	F	AGAAGGGTAATCGGGTGTCC	20	58	55%	367
	R	CCACCCAAAGACATCTCGGA	20	59	55%	367

3. Results

3.1. Description of Family 1

Family 1 was recruited from Pakistan Institute of Medical Sciences, Islamabad, having suspected case of Nieman Pick Disease. Medical history and blood samples of the family were taken and pedigree was drawn in order to study the pattern of inheritance in the family.

3.2. Pedigree analysis of family 1

Pedigree revealed that the family 1 has a history of consanguineous marriage in third generation. As a result of consanguineous marriage, the couple was normal with no obvious signs of NPD, and had four children out of which one of their daughters (IV:1) and a son (IV:3) affected in 4th generation. The proband (IV:1) was 6 months old at the time of sampling, showing usual symptoms of Type A NPD at the time of sampling such as hepatosplenomegaly, feeding difficulty, enlargement of abdominal region and very thin extremities during routine pediatric visits. Through biochemical testing, low ASM levels in white blood cells of the proband were also suggestive of Type A NPD. The parents of the proband had normal phenotypic expression and the pattern of inheritance for the disease was assumed to be autosomal recessive.

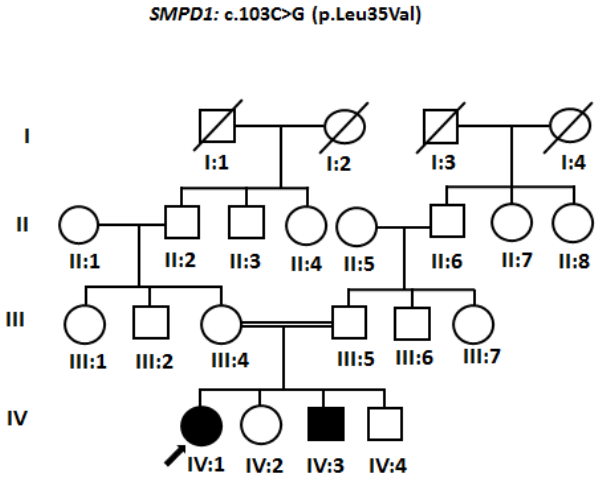

Figure 3- 1: Pedigree of Family 1

Figure 3.1. Pedigree of family 1, all females has been represented with circles and males with squares. The unaffected are represented by empty symbols, while the affected are represented by full symbols. In these four generations, there was one consanguinity found.

3.3. Gel Electrophoresis:

DNA from family 1 was isolated and then subjected to a gel electrophoresis analysis to determine its concentration. The bands visible on the gel were a sign of excellent quality and were appropriate for use in the exome sequencing procedure.

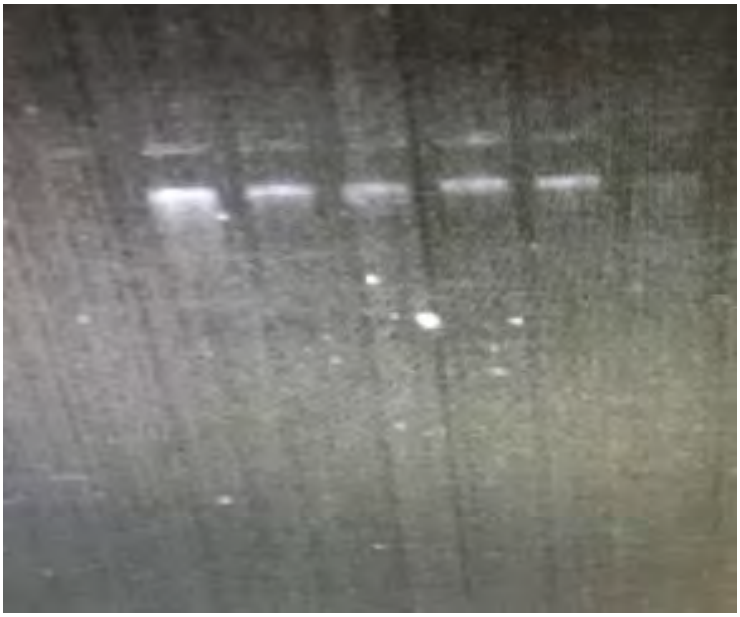

Figure 3- 2: Family 1's extracted DNA is distributed from right to left.

3.4. DNA sequencing

3.4.1 Analysis of whole exome sequencing data

The whole exome sequencing of SMPD1 gene with transcript id ENST00000342245.9, a variant with missense mutation c.103C>G and amino acid change is p.Leu35Val, has been reported in ClinVar in 2015. This amino acid change can alter the protein conformation of

ASM protein which cause inheritance of NPD condition. Different In silico tools were applied for the identification of CYP1B1 variant, some of these tools were Mutation Taster, PolyPhen 2.0, SIFT, PROVEAN, MuPRO and phd-SNP. Mutation Taster predicts variant and its amino acid sequence change and how it affects its protein structure and protein functions. SIFT and PolyPhen 2.0. indicates conflicted interpretations on the pathogenicity of the variant. MuPRO tool was used to predict alternative structure stability either its decrease or not due to amino acid change as in case of SMPD1 variant if amino acid change occurs in heme binding helix it can decrease the stability of protein molecule and affect ocular function of eye. PROVEAN and phd-SNP tool was used to predict deleterious effects of the variant. Some other In Silico tools were also used to further analyze to analyze protein structure and function. Conserved Protein Domain Family which generates the data for ASM protein and its related compounds. This tool show hierarchy of related conserve domain models in form of hierarchy tree. Another tool Clustal Omega was used to further check the alignment between divergent sequences. Conservative analysis of *SMPD1* variant was done COBALT (constrain based multiple alignment tool) provide data for multiple protein sequence alignment using conserved domain and local sequence similarity. It varies between highly conserved and non-conserved regions. Reported mutation region has been aligned and homology of this protein is checked with other organisms. The mutated sequence is highly conserved in all organisms.

Total 47,354 variants were identified in the proband exome sample, 15,674 variants that were potentially affecting protein were kept. 520 variants with allele frequency >0.01 in the 1000 genome , ExAC and Gnom AD were excluded. 25 Homozygous variants were picked. Using insilico tools, 13 missense variants were filtered out, 1 variant (c. 103C>G) with different pathogenic interpretations was found.

Discussion

Nieman Pick Disease is a class of lysosomal storage diseases inherited in an autosomal recessive manner. It is subdivided into three main types; Type A and B which arise from mutations in a SMPD1 gene and Type C which is caused by two genes C1 and C2. NPD is a rare neurovisceral condition or lipid storage disorder collectively characterized by the accumulation of different kinds of lipids, primarily sphingomyelin, cholesterol and their derivatives, inside the reticuloendothelial system with or without CNS involvement (Vanier 2010). These lysosomal storage diseases are also known as acid sphingomyelinase deficiencies with related mutations in each copy of the gene and expressing low ASM activity, usually less than 10% of control values [McGovern et al., 2006]. Within the first year of life, Type A Patient NPD exhibit hepatosplenomegaly and underachievement. A cherry-red patch in the macula is present in about 50% of these babies. The hallmarks of the condition include rapid neurological deterioration, severe hypotonia, and failure to achieve milestones (McGovern et al., 2006). Lungs with impaired pulmonary function are involved in type B. The macula of these individuals' eyes may also have a reddish-brown halo, although in some circumstances, a clear cherry red spot can also be seen. There are additional reports of NPD patients who exhibit symptoms that fall between categories A and B [Pavl et al, 2005].

SMPD1 gene encodes ASM protein which hydrolysis Sphingomyelin into its end products ceramide and phosphocholine in lysosomes respectively. SMPD1 is 5-6kb long protein coding gene, located on chromosome 11. Human ASM protein is a phosphodiesterase enzyme made up of 631 amino acids.

In this research study, a variant of SMPD1 gene was investigated in a Pakistani family. Proband was a 6 months old baby girl manifesting symptoms of NPD type A. One of her brother was also affected. The parents were normal and two other siblings were also not manifesting any disease symptoms.

Whole exome analysis of SMPD1 gene of the proband showed a missense mutation with conflicted interpretations. The mutation c.103C>G in exon 1 of SMPD1 of the proband is responsible for the amino acid change p.Leu35Val, leading to development of type A NPD. Proband was showing

symptoms like hepatosplenomegaly, enlarged abdomen with thin extremities, feeding difficulty, respiratory issues and poor growth.

Conclusion

According to research findings, Nieman Pick Disease is a rare autosomal recessive metabolic disorder with neurovisceral clinical symptoms and a hereditary impairment of sphingomyelin metabolism and cholesterol transport. Consanguinity is thought to increase the severity of the phenotypic and consequently the rate of occurrence. For an accurate diagnosis and genetic counselling, a genetic analysis of the patient and other family members, including the patient's parents, is necessary. Whole exome of the proband sequence reveals a missense mutation with varying pathogenicity interpretations. Sanger Sequencing of the remaining family members would be done for further confirmatory studies.

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List of Electronic Databases

Ensembl	https://asia.ensembl.org/index.html
OligoCalc	https://biotools.nubic.northwestern.edu/OligoCalc.html
SMS -Primer Stats	https://www.bioinformatics.org/sms2
NCBI-BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
UCSC Insilico PCR	https://genome.ucsc.edu
Mutation taster	https://www.mutationtaster.org
PROVEAN	http://genetics.bwh.harvard.edu/pph2
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