Study of Clinical Profile and Molecular Basis of Niemann Pick Diseases in Cases from Local Population

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STUDY OF CLINICAL PROFILE AND MOLECULAR BASIS OF NIEMANN PICK DISEASE IN CASES FROM LOCAL POPULATION

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Molecular Biology

By

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In the name of Allah Almighty, The Most Gracious, The Most Beneficent, The Most Merciful

CERTIFICATE

This dissertation "Study of clinical profile and molecular basis of Niemann Pick disease in cases from local population" submitted by **Ms. Samra Aamir,** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Molecular Biology.

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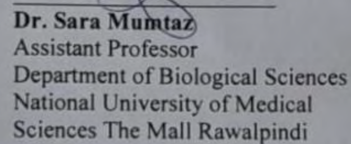

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DECLARATION

I hereby declare that the work presented in this thesis is the result of my own efforts and research work, carried out in Molecular Biology Lab, Department of Zoology, Quaid-I-Azam University Islamabad.

This thesis is my own composition and no part of it has been presented for any degree previously, nor does it contain, without proper acknowledgement or reference, any material from the published resources to the best of my knowledge.

Samra Aamir

DEDICATED

TO

MY BELOVED PARENTS WHO HAVE BEEN PILLARS OF SUPPORT, GUIDANCE AND LOVE IN MY LIFE

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LIST OF ABBREVIATIONS

Abbreviation	**Full Forms**
LY	Lysosome
LE	Late endosome
LAMP	Lysosomal Associated Membrane Protein
CLEAR	Coordinated Lysosomal Expression and Regulation
TEFB	Transcription factor EB
HLH	Helix Loop Helix
PtdIns3P	Phosphatidylinositol 3-phosphate
PtdIns (3,5)P_2	Phosphatidylinositol 3,5-bisphosphate
ER	Endoplasmic reticulum
TGN	Trans Golgi network
MPR	mannose 6-P receptor
LYNUS	lysosomal nutrient-sensing complex
Mtor	Mammalian Target of Rapamycin
LSDs	Lipid storage disorder
GM1	Gangliosidosis Type 1
GM2	Gangliosidosis Type 2
FD	Fabry disease
GLA	Galactosidase A
GD	Gaucher disease
GCase	Glucocerebrosidase
GlcCer	Glucosylceramide
KD	Krabbe disease
GALC	B-galactocerebrosidase gene
GalC	β-galactocerebrosidase
NPD	Niemann Pick Disease
GLD	Globoids Cell Leukodystrophy
SMPD1	Sphingomyelin Phosphodiesterase 1 gene
ASM	Acid Sphingomyeline
NPD-A	Niemann Pick Disease Type A
NPD-B	Niemann Pick Disease Type B
NPD-C	Niemann Pick Disease Type C
NPD-D	Niemann Pick Disease Type D
GGs	Gangliosides
GSLs	Glycosphingolipids
VSGP	Vertical Supranuclear Gaze Palsy
HGMD	Human Gene Mutation Database
ZNT5	Zn^{+2} transporter 5
NTD	N-terminal Domain
MLD	Middle Luminal Domain

CTD	C-terminal Domain
SSD	Sterol Sensing Domain
LAL	Lysosomal Acid Lipase
DBS	Dried Blood Spots
MS	Mass Spectrometry
LDL	Low Density Lipid
ACAT	Acyl CoA: Cholesterol Acyl Transferase
7-KC	7-ketocholesterol
C-triol	5β-cholestan-3β,5α,6β-triol
Lys-SM 509	Lysosphingomylin-509
CT	Chitotriosidase
CVS	Chronic Villus Sampling
SRT	Substrate Reduction therapy
FDA	Food and Drug Authority
CD	Cyclodextrin
ERT	Enzyme replacement therapy
AAV	Adeno Virus-Associated Virus
IRE	International Review Board
GIT	Gastrointestinal Track (GIT)
I.Q	Intellectual Quotient
CBC	Complete Blood Count
LFT	Liver Function Test
RFT	Renal Function Test
MRI	Magnetic Resonance Imaging
EDTA	Ethylenediaminetetraacetic Acid
PCR	Polymerase Chain Reaction
Rmp	Revolutions Per Minute
PK	Proteinase K
SDS	Sodium Dodecyl Sulphate
TE	Tris EDTA
TBE	Tris Borate EDTA

ABSTRACT

Lysosomes are heterogeneous organelles, containing different hydrolytic enzymes, operating at acidic pH, used to catalysed different biological molecules. Deficiency in these enzymes lead to storage of molecules results in lysosomal storage disorders. There are many lipids storage disorders, of which Niemann Pick Disease (NPD) is one. Initially it is reported by Albert Niemann in 1914 and transmitted in autosomal recessive manner which means two homozygous alleles must be present to cause disease phenotype. It has been divided into two groups, Group 1 having subtypes, Niemann pick type A and B, is due to deficiency of enzyme acid sphingomyelinase (ASM), caused by mutation in gene *sphingomyeline phosphodiesterase* 1 (*SMPD1)* whereas Group 2 having subtypes, C and D, caused due to mutation in gene *NPC Intracellular Cholesterol Transporter 1 (NPC1)* (95%) and *NPC Intracellular Cholesterol Transporter 2* (*NPC2)* (5%). *NPC1* gene encodes a trafficking protein, mutation in which lead to defective trafficking of acid sphingomyeline. Niemann Pick Disease type A (NPD-A) and Niemann Pick Disease type C (NPD-C) has neuropathic involvement whereas Niemann Pick disease type B (NPD-B) is non-neuropathic. NPD-A is severe form as it does not respond to any treatment whereas NPD-B can be treatable to some extent through enzyme replacement therapy. NPD-C is also difficult to diagnose and treat. The subtypes of NPD largely overlap in clinical manifestations.

In this study three NPD affected families were enrolled. Family history and clinical reports and blood sample were collected after informed consent. Clinical symptoms of patients include hepatosplenomegaly, developmental delay, respiratory infection, liver problems, short height, anaemic and jaundice. Clinical profile of patients, lab reports, ultrasound, X-rays and enzymes analysis reports were collected. Enzymes analysis shows normal levels of ASM in patients of NPD families. This suggested the presence of mutation in gene responsible for NPD type C or type D. As *NPC1* gene is responsible for Type C NPD in 95% of cases therefore it is selected. DNA was extracted. Primers were designed for mutational hot spot exon 21 of *NPC1* gene and polymerase chain reaction (PCR) and Sanger Sequencing were performed. Sequenced data was analysed and pathogenicity of the variant was checked through various *In-Silico* tools. Sequencing results of *NPC1* selected exon revealed a novel homozygous disease-causing missense variant c.3230G>C causing arginine into proline (p.Arg1077Pro) in one patient of NP005 family.

The *NPC1* mutational profile for Pakistani population is not well recognised. As NPD has autosomal recessive mode of inheritance. High frequency of consanguineous marriages (60%)

caused increased incidence of autosomal recessive disorder. Our result necessities the analysis of other exons of *NPC1* gene as well as *NPC2* gene for other two affected families to identify disease which will pave the way to targeted treatment options. Further comprehensive molecular genetic studies are required to be conducted to get data for genotype-phenotype correlation, new-born screening program and novel treatment strategies in future.

1. INTRODUCTION

1.1 Lysosomes (LY)

Lysosomes are single membrane bound heterogeneous organelles discovered in 1955 by Christian Du Duve contain hydrolytic enzymes that metabolize biological molecules such as lipid, proteins, polysaccharides and nucleic acid (Duve *et al.,* 1955 and Wang *et al.,* 2018). Hydrolytic enzymes of lysosomes operate at acidic pH of 5-6 and have ability to degrade the materials by endocytosis, autophagy and phagocytosis (Pu *et al.,* 2016). The product formed by the degradation of material in lysosome is transferred to Golgi apparatus for reuse or for release of material out of the cell through exocytosis which is important for immune processes. Furthermore, lysosomes plays role in the cellular processes such as metabolic processes and nutrients sensing (Bonam *et al.,* 2019).

1.2 Lysosomes Biogenesis

There are more than 100 genes that encode lysosomal membrane protein, lysosomal accessory proteins and hydrolases etc. Most of the lysosomal genes contain one or more CLEAR (Coordinated Lysosomal Expression and Regulation) GTCACGTGAC sequence in their promoter region to which transcription factor EB (TFEB) binds that is member of helix Loop Helix (HLH) leucine zipper family of transcription factor (Fisher *et al.,* 1991).TEFB comes to nucleus when there is need of expression of lysosomal genes depending upon cellular needs e.g. autophagy, starvation as shown in figure 1.1 (Fisher *et al.,* 1991; Sardiello & Ballabio, 2009).

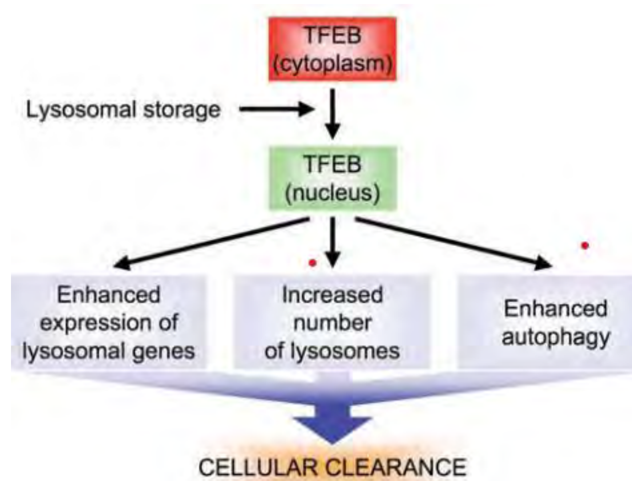


Figure 1.1 Nuclear translocation of TFEB into nucleus, enhances cellular clearance of storage undegraded material (Sardiello & Ballabio, 2009).

1.3 Lysosome membrane proteins

Lysosomes and late endosomes membrane proteins are mostly highly glycosylated trans membrane proteins (Hunziker & Geuze, 1996). These proteins are either transferred by direct pathway or indirect pathway. Indirect pathway involves the delivery of membranes proteins to plasma membrane before reaching to endosomes and direct pathway involves direct delivery to endosomes. The most abundant glycosylated membrane proteins of lysosomes are lysosomal associated membrane protein (LAMP), LAMP -1 and LAMP-2. Other proteins are LIMP-2/SCARB2, CD63, DIRC2, H^+ ATPase and chloride channel ClC-7 shown in figure 1.2. LAMP -1 and LAMP-2 are involved in autophagy pathways. When ligand and receptor dissociated, β-glucocerebrosidase is transported by LIMP-2/SCARB2. H^+ ATPase transported H^+ whereas chloride channel ClC-7 transported Cl^- into lysosomal lumen against the concentration gradient of proton. Fusion processes occurs due to presence of CD63 protein. Cathepsin mediated proteolysis occurs by DIRC2. For the delivery of lysosomal protein to later lysosomes, early endosomes are converted to late endosomes. For this purpose there is a mechanism that involves switching of Rab5 and phosphatidylinositol 3-phosphate (PtdIns3P) in early endosomes to Rab7 and phosphatidylinositol 3, 5-bisphosphate (PtdIns (3, 5) P_2 in late endosomes (Gillooly *et al.,* 2000; Helenius, 2011; Schink *et al.,* 2016 and Yang and Wang, 2021). Retromer dependant or independent pathway is used for the transfer of proteins to Golgi or plasma membrane that is not destined for lysosomes (McNally & Cullen, 2018). Rab switch consist of Rab5 and Rab7 in which inactivation of Rab5 and activation of Rab7 occurs by TBC-2 and Sand1-Ccz1 complex respectively for conversion of early endosomes to late endosomes (Pryor *et al*, 2000; Rink *et al.,* 2005; Li *et al.,* 2009, Chotard *et al.,* 2010; Nordmann *et al.,* 2010; Yang & Wang, 2021). Suppression and degradation of endosomal PtdIns3P occurs when Vps34 complex interact with WDR91/WDR81, allowing it to convert into PtdIns (3,5)P_2 (Liu *et al.,* 2016; Rapitaenu *et al*, 2016; Liu *et al*, 2017; Yang &Wang, 2021). Kiss-run mechanism is used for the fusion of late endosomes to lysosomes but sometimes fusion occurs when Rab7 accompanied with SNARE and homotypic fusion and protein sorting (HOPS) combined (Luzio *et al.,* 2010; Lurick *et al.,* 2018; Yang & Wang, 2021).

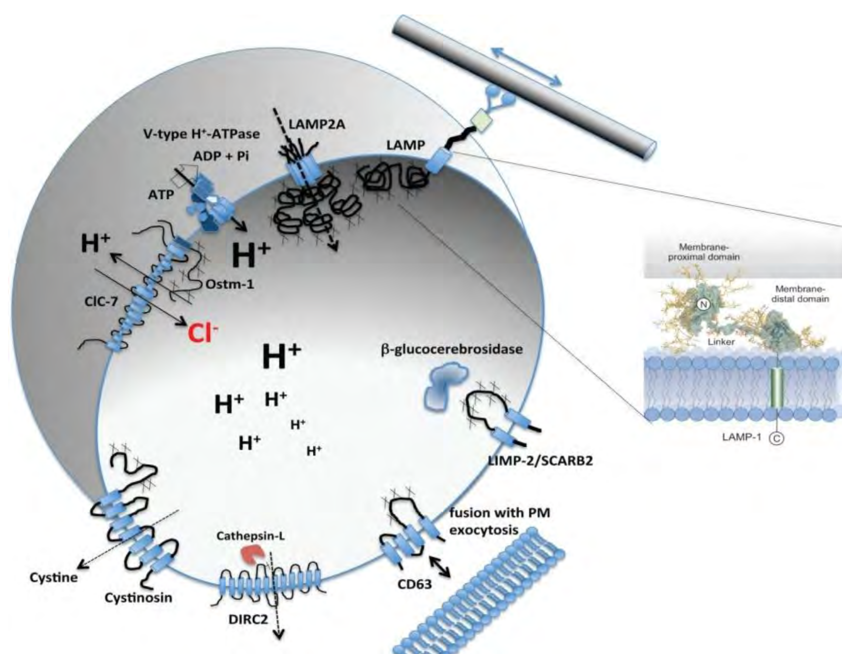


Figure 1.2 Lysosomal membrane proteins to regulate communication between lysosomal lumen and cytosol (Schwake, 2013).

Whereas lysosomal hydrolases are synthesized and glycosylated in endoplasmic reticulum (ER), and transferred to Golgi apparatus for the phosphorylation of mannose residue on glycosylated chain. Phosphorylated mannose residues are recognised by mannose 6-P receptor (MPR) in Trans Golgi network (TGN) and packed in clatherin coated vesicles. These are then transferred to early endosomes directly. Sometimes MPR independent pathway is also used for the delivery of hydrolases to endosomes (Braulke & Bonifacino, 2009; Luzio *et al.,* 2014 and Yang & Wang, 2021).

1.4 Function of lysosomes

Lysosomes play many roles like in cell death, plasma membrane repair, cell growth, degradation of worn out organelles, maintain cell homeostasis and many more (Settembre *et al.,* 2013). Phagocytosis/endocytosis and autophagy pathways are used for the degradation of material in lysosomes. Extracellular material destined to degrade, are first of all internalise by invagination of membrane, mature to become early endosomes then convert into late endosomes. In late endosomes extracellular materials are sorted in intra-laminal vesicle called multivesicle bodies (Huotari & Helenius, 2011).

Damaged proteins and worn out organelles are degraded in lysosomes by autophagy. Ingulfing of autophagic material form autophagosomes (double membrane) that fused with lysosomes to produce auto lysosomes. Cargo delivered to cytoplasm or to Golgi either through

autophagosomes or through endocytic pathway for further degradation. Catabolites formed, moves out of lysosomes through perimeter membrane exporter or through vesicular membrane trafficking for further processing. Here the material are further degraded or reused for the synthesis of complex molecules e.g. glycoshingolipid (Samie & Xu, 2014).

Nutrient sensation and cellular singling regulates both endocytic and autophagy pathways. Starvation triggered autophagy and stimulus is sensed by lysosomal nutrients sensing system (LYNUS). LYNUS consists of Rag GTPases, ATPase and the mammalian target of rapamycin (mTOR) complex (Settembre *et al.,* 2013).

1.5 Lipid storage disorder (LSDs)

Accumulation of undigested lipid and other bio molecules in lysosomes leads to metabolic disorders collectively called as lysosomal storage disorders (LSDs) (Lieberman *et al.,* 2012). LSDs are inborn error of metabolism with prevalence 1 in 1500 to 7000 live births (Staretz-Chacham *et al.,* 2009). Most common LSDs are lipidoses. Lipids may also be accumulated due to secondary mechanism like accumulation of protein/carbohydrates and membrane trafficking (Settembre *et al.,* 2013). LSDs consist of almost 60 different genetic diseases (Samie & Xu, 2014). Most LSDs are caused by mutation in hydrolases. Some may occurs by defect in catabolites exporter and membrane trafficking proteins that lead to cause of secondary lipid storage in lysosomes. Mutations in non lysosomal proteins also cause LSDs. Sometimes insoluble lipids accumulate, slow down membrane trafficking, precipitate in lysosomes and affect hydrolytic enzymes by reducing their activity (Samie & Xu, 2014). LSDs cause severe pathology due to accumulation of primary storage molecules that disrupt others cellular and biological functions (Lawrence *et al.,* 2010). Most of the LSDs has autosomal mode of inheritance. LSDs onsets in childhood in most of the cases but sometimes adult-onset form may occurs. LSDs affect different organs frequently and leads to progressive neuro degenerative disorders (Platt *et al.,* 2018).There are many lipid storage disorders some of them are mentioned below:

1.5.1 Gangliosidosis Type 1 and Type 2 (GM1 , GM2)

It is autosomal recessive disorder in which complex glycolipid accumulates in brain and other tissues. It has two types GM1 gangliosidosis and GM2 gangliosidosis. Deficient activity of β-galactosidase causes GMI gangliosidosis whereas β-hexosaminadase deficiency causes GM2 gangliosidosis. Hexosaminadase exist in 3 isoforms hexosaminadase A having α/β subunit and hexosaminadase B having two β subunit and hexosaminadase S have two α subunits.α subunit is

coded by *HEXA A* gene locted at 15q23-24 where as β subunit is codes by *HEXA B* gene located at 5q13.Tay Sach disease is the example of GM2 caused by the deficiency of hexosamindase A. Symptoms of GM1 are coarse facial feature ,edema, developmental delay, dysostosis (abnormalities in single or group of bones) and organomegaly of different organs (Patterson, 2013).

1.5.2 Fabry disease (FD)

FD has X-linked mode of inheritance in which there is complete or partial absence of α-galactosidase enzymes (GLA) that would lead to accumulation of glycosphingholipid in nerve, renal ,capillary endothelial and cardiac cell. Its prevalence is 1 in 100,000 live births (Germain, 2010). Alpha-galactosidase enzyme (GLA) is encoded by *α-galactosidasea A (GLA)* gene located at chromosome Xq22.1 that is 12kb long with seven exons (Schiffmann, 2009). GLA encodes a 429 amino acid protein that processed to form mature 370 amino acid protein (Garman & Garboczi, 2004). It is asymptomatic during first year of life. Symptoms appears bewteen the age of 3-10 years. Symptoms involve damage to various organs like cardiovascular problem, cerebrovascular complication, kidney and endothelial cell damage, cellular deathand dysmorphic fgacial abnormalities (coarse features, shallow mid face, prominent nasal bridges, bushy eyebrows, prominent lobules of ear etc) abnormalities in extremities, pain episodes in extremity , fever with unknown reason, pain in abdomen , and diarrhea with fatty food intake ,angiokeratoma, corneal opacities and oxidative stress (Schiffmann, 2009 and Germain, 2010).

1.5.3 Gaucher disease (GD)

GD is rare autosomal recessive disorder in which mutation in *GBA1* gene cause decreased in glucocerebrosidase (GCase, also called as glucosylceramidase) activity leads to accumulation of glucosylceramide (GlcCer) in gaucher cell. This gene is located on chromosome 1 at 1q21. Gaucher cell are scavenger cells called macrophages. Action of this enzymes converts glucosylceramide into ceramide and glucose. GD is the most common sphingolipidoses. In very rare cases, activator protein (saposin C) caused gaucher disease due to mutation in *PSAP* gene. Its prevalence is 1/40,000 or 1/60,000 in live births. In some cases due to defiency of GCase, an alternative pathway metabolize GlcCer into glycocylsphingosine. It is coverted to sphingosine and then sphingosine-1-phosphate by the action of second GCase encoded by *GBA2* gene **.**Sphingosine is toxic to bone (Stirnemann *et al.,* 2017). GD has 3 main type GD 1, GD 2 and GD 3. Symptom of GD are enlargement of spleen and liver, anemia, thromocytopenia, pulmonaryhypertension. Symptoms vary according to disease type e.g. GD 1 is non-neuropathic,

GD2 is acute neuropathic with survival rate limited to 2 to 3 years of life and visceral symptoms. GD 3 involves neural involvement but in later stage. Other symptoms involve are dementia, seizure, abnormal eye involvement and ataxia. Patient with GD 3 can survive upto three or four decades (Linari & Castaman, 2015; Gul *et al.,* 2021).

1.5.4 Krabbe disease (KD)

It is rare autosomal recessive neurodegenerative disorders caused due to mutation in the *β-galactocerebrosidase gene (GALC)* (located on chromosome 14) leads to accumulation of β-galactocerebrosidase (GalC) (Zlotogora *et al.,* 1990; Graziano & Cardile, 2015). This enzyme is important for the degradation of galactolipid including galactosylceramide and psychosine (Wenger & Luzi, 2020). KD is also named as globoids cell leukodystrophy (GLD) that affect white matter of central nervous system (CNS) and peripheral nervous system (PNS) (Graziano & Cardile, 2015). Symptoms of KD vary from early onset to late onset of disease. Early symptoms include increased intensity of dentate/cerebellar white matter and change in deep cerebral white matter. Late onset does not involve dentate/cerebellar white matter, posterior corpus callosum and parietal-occipital region (Abdelhalim *et al.,* 2014).

1.5.5 Niemann Pick Disease (NPD) and its types:

Niemann Pick Disease (NPD) is lipid storage autosomal recessive disorder characterised into two distinct groups; Group 1 includes acid sphingomyelinase-deficient diseases (ASM-deficient NPD) e.g. Niemann Pick Disease type A (NPD-A) and Niemann Pick Disease type B (NPD-B), resulted due to accumulation of acid sphingomyeline (ASM) due to mutation in gene *Sphingomyelin phosphodiesterase 1 (SMPD1)* whereas Group 2 includes Niemann Pick Disease Type C (NPD-C) and Niemann Pick Disease Type D (NPD-D), resulted due to mutation in *NPC1* and *NPC2* gene that encodes either NPC1 or NPC2 transport proteins. Both subtypes followed autosomal recessive pattern of inheritance (Vanier, 2013). Besides primary accumulation of lipid, secondary accumulation of other lipids may also occurred e.g. gangliosides (GGs) and glycosphingolipids (GSLs) accumulation is also prominent in NPD (Brieden & Sandhoff, 2020). In case of Type C NPD, 95% families shows mutation in *NPC1* gene and only 5% showed mutation in *NPC2* gene (Vanier, 2013; Vanier & Millat, 2003). *NPC1* gene encodes integral membrane protein involves in intracellular transport of cholesterol and homeostasis whereas *NPC2* gene encodes the 132 amino acid protein involves in binding of cholesterol (Yanjanin *et al.,* 2010). Both Niemann Pick Disease types are lysosomal lipid storage disorders that encountered with variety of visceral as well as neurovisceral manifestations

(Vanier, 2013). Crooker proposed four subtypes of NPD range from type A-D (CROCKER & FARBER,1958). Patient of type A have severe neuro visceral involvement where as Type B patients showed only visceral involvement. Both types shows deficiency of acid sphingomylinase (ASMD). The extent of ASM deficiency shows remarkable differences among type A and type B NPD. Patients with Type A shows little to no functional ASM (1%) whereas patient with type B shows retain some level of ASM activity (10%) (Schultz et al, 2016). Patient with type C and D shows subacute neurologic symptoms, mild level of lipid storage with no deficiency of ASM, less severe organomegaly than type A and B. Both Type C and D are shows abonormalities in intracelllular trafficking of endocytosed unesterified cholestrol in late endosomes and lysosomes (Vanier & Millat, 2003).

1.5.5.1 History of Niemann Pick Disease

NPD type A is initially reported by German pediatrician Albert Niemann in 1914 in an Ashkenazi Jewish infant who died at the age of 18 months, was reported with massive hepatosplenomegaly and neurodegenerative courses (Niemann, 1914; Schuchman & Desnick, 2017). Niemann observed massive lipid-storage in reticuloendothelial cells on autopsy (Niemann, 1914). Ernst Klenk named this storage compound “sphingomyeline” (Klenk, 1934).whereas Pflander and Dusendschon reported NPD type B in two Swiss brothers, with massive hepatosplenomegaly and no neurodegenerative abnormalities and were died at the age of 29 and 33 (Dusendschon, 1946; Pfandler, 1946; Schuchman & Desnick, 2017). Brady classified the group of patients that showed the overlapping clinical and biochemical features similar to that of type A and B into type C, and D Niemann Pick Disease (NPD). These patients showed symptoms of hepatomegaly, foamy macrophage infiltration into bone marrow and tissue, moderate accumulation of acid sphingomyeline (AS), partial deficiency of ASM and variable neurological abnormalities. In 1980, a mouse model, reported by Brady and Pentchev showed symptoms and acid sphingomyeline deficiency (ASMD deficiency) similar to these patients, Brady classified these deficiencies were due to primary to secondary defects in cholesterol esterification (Pentchev *et al.,* 1980; Pentchev *et al.,* 1984; Schuchman & Desnick, 2017. In 1985, they take some cells from patients infected with Type C NPD, cultured them in laboratory and reported cholesterol esterification defects distinguishing Type C from Type A and B NPD. Defect in two cholesterol binding proteins (NPC1 or NPC2) caused Type C NPD. Type D NPD patients were reported in only descendants of couple married in 1700 in south western Nova Scotia, Acadian (Winsor &Welch, 1978; Greer et al 1998; Vanier, 2010) Type D and E nosology were yet not described (Pentchev *et al.,* 1985Schuchman & Desnick, 2017).

1.5.5.2 Age onset of Niemann Pick Disease

Type A NPD subtype shows early age onset like 5-6 months and patients dies within 2-3 years (McGovern et al, 2006). Type B NPD onset and rate of disease progression is variable. It is more common among adults and patient with Type B NPD can survive upto six decades (Schuchman & Desnick, 2017). Type C onsets vary from foetal to adult depending upon variability of symptoms (Yanjanin *et al.,* 2010).

1.5.5.3 Clinical Manifestations of Niemann Pick disease (NPD)

Clinical feature varies depending on disease type and their age of onset:

- **Clinical features of Type A Niemann Pick Disease**:

Patients affected with Type A NPD subtype showed severe hepatosplenomegaly (in first year of life) with prominent abdominal distention and central nervous system involvement. Severe neurodegenerative diseases, cherry red spot in macula (50% infants), progressive psychomotor retardation, fail to attain milestone and profound hypotonia (Schuchman & Wasserstein, 2015). They never develop the ability to sit in dependently, unable to consume adequate calories due to hypotonia and stomach compression. Most die within 2-3 years due to respiratory failures (Schuchman & Desnick, 2017).

- **Clinical feature of type B Niemann Pick Disease:**

Patient affected with type B NPD showed hepatosplenomegaly with no central nervous system involvement (CNS) accompanied with liver failure in early stage of life, reddish brown Halo surrounding in macula of eyes, and pulmonary function compromised. Serum triglyceride level and LDL-cholesterol are mostly raised whereas HDL-cholesterol level are low in these patients (Schuchman & Wasserstein, 2015). Patient also showed the symptoms like delayed onset of puberty, restricted growth, fatigue, bone and joint pains (Wasserstein *et al.,* 2003), osteopenia, thrombocytopenia and leukopenia (worsen with time) (Iaselli *et al.,* 2011). Some variant showed neurological feature like ataxia, gross motor delay and difficulties in learning (Wasserstein *et al.,* 2006). They show life threatening problem e.g. haemorrhage, liver failure, oxygen dependency, coronary artery and valvular heart disease and splenic rupture (Cassiman *et al.,* 2016). Most of the patients with type B NPD subtype die due to failure of lungs and liver. Foam cell of lymph nodes, liver, spleen, lung air passage way, adrenal cortex, and bone marrows are mostly laden with lipid (sphingomyeline). Patients with type B NPD subtype showed the

variable symptoms depending upon age of onset and can survive upto adulthood (Schuchman & Desnick, 2017).

- **Clinical feature of Type C Niemann Pick Disease:**

Patients with type C NPD subtype showed neurovisceral symptoms. Clinical symptoms vary from neonatal fatal disorders to adult chronic neurodegenerative diseases. Disease severity depends on neurological involvement and proceeded by systemic diseases. Systemic diseases are almost absent in all or half of the adult onset patients and also not very severe except in prenatal onset of disease. Splenomegaly is observed and showed fluctuation (decrease) with time. In very few cases lung involvement are reported. Visceral organs involves in it are liver, spleen and sometimes lungs. Symptoms vary from prenatal forms to adults form. Neurological symptom varies with age of onset of disease e.g. developmental delay (in early infantile period), problem in learning and school problem, falls, clumsiness, cataplexy and gait problems (in late infantile and juvenile periods), ataxia and psychiatrics disturbances (in adult form). NP-C patients show most commonly vertical supranuclear gaze palsy (VSGP) (Solomon *et al.,* 2005). Psychiatric problems are more common in patient with late age. Other neurological disorders include ataxia, seizure, tremor, dysarthria, dystonia, dysphagia and dementia (Vanier, 2010).

1. Prenatal representation

NPD-C patient shows the symptoms of liver diseases, hepatosplenomegaly, fetal hydrops (Spiegel *et al.,* 2009) and cholestatic jaundice proceeding with the onset of neurological symptoms. Some of the patients die within 6 months due to worsen cholestatic jaundice. Patient having mutation in *NPC2 gene* showed severe respiratory insufficiency accompanied with liver disease and hepatosplenomegaly (Vanier, 2010).

2. Early Infantile Periods (2 months-2 years)

Patients showed the symptoms of hepatosplenomegaly along with delayed milestone sand hypotonia as first neurological sign. Patients are unable to acquire motor skill, unable to walk, tremors are frequent, seizures are uncommon and supranuclear gaze palsy is not observed. Leukodystrophy and cerebral atrophy are observed by MRI. Patients never survive more than 5 years (Vanier, 2010).

3. Late infantile period (2-6 years)

Patients showed the symptoms of hepatosplenomegaly that vary with time, language delay, gait problems, falls and cluminess due to ataxia. As ataxia worsen, dysphagia, dysarthria and

dementia becomes common. Others symptoms includes cataplexy, severe epileptic shocks, impairment in motor development, spasticity and swallowing problems deaths occur between the age range of 7-12 years (Vanier, 2010).

4. Juvenile periods (6-15 years)

Patient in this periods showed moderate splenomegaly sometimes hepatosplenomegaly, difficulties in learning and walking, clumsiness, falls, ataxia, dysarthria, and dysphagia. Cataplexy with/without narcolepsy (laughter induced) is common. Other symptoms includes are motor impairment, decline in intellect, dystonia, dysarthria worsen and patient lose the ability to talk. At later stage spasticity and swallowing problems arise require gastrostomy. Patients' survival is variable and can survive up to 30 years or more (Wraith *et al.,* 2009; Vanier, 2010).

5. Adolescence and adult (>15 years)

Patient in this period showed psychiatric sign with visual and audio hallucination, interpretative thoughts, behavioural problems (aggressiveness and social cut-off). Other features are cerebral ataxia, dysarthria, problems in movement, thalmoplegia, dysphagia and dystonia. Epilepsy is rarely observed (Vanier, 2010).

Table 1.1 Symptoms of Niemann Pick Diseases Type C according to age (Vanier, 2010)

Type NPC	Early infantile	Late infantile	Late infantile	Adult
Systemic involvement	Hepatosplenomegaly May regress with age Absent in ~15% of cases			
Neurological involvement	- Delay in motor milestones - Hypotonia - VSGP	- Gait problems - Clumsiness - Speech delay - Cataplexy - VSGP	- School problems - Ataxia - Seizures - Cataplexy - VSGP	Psychiatric problems - Ataxia - Dystonia - Dementia - VSGP

NPD: Niemann Pick disease,

1.5.5.4 Prevalence

Prevalence of Type A and B NPD in live birth is 1 in 250,000 individuals whereas for type C, it is 1 in 120,000 (Abghari *et al.,* 2019). Type A NPD is most prevalent among Ashkenazi Jewish individuals, Type B NPD is most common in descents of Turkish, Arabic and North African individuals whereas Type C NPD is most common among descent of French Acadian in Nova Scotia (Simonaro *et al.,* 2002; Bianconi *et al.,* 2019).

1.5.5.5 Molecular genetic of Niemann Pick Disease TYPE A and Type B

Type A and B is caused by *SMPD1* whose cytogenetic location is 11p15.1-11p15.4, length of 6kb with 6 exons and five intron, reading frame of 1890 bp, encoded the protein of 631 amino acid and several transcript (Abghari *et al.,* 2019). Acid Sphingomyelinase (ASM) is glycoprotein with six N-glycosylation sides and eight disulphides bridges and two zinc ion involves in proper folding and maintaining stability of ASM (Brieden & Sandhoff, 2020; Zhou *et al.,* 2016). According to HGMD (Human Gene Mutation Database) accessed on 17.01.2023, there are 250 reported mutation of SMPD1. Most are missense/non sense (183), some are small deletion (4), some are small deletions (45), small insertion (13), indels (4) and gross insertion /duplication (1).

1.5.5.6 Domains of SMPD1

ASM has 4 domains mentioned in figure 1.3. First one is N-terminal Saposin Domain consist of four alpha helix stabilized by three disulphides bonds. It has 83-165 residues. This domain is playing role in substrate activation for catalytic reaction. Second is Proline-rich linker domain with 166 to 198 residues that link saposin domain to last domain. Last domain is catalytic domain with 199-611 residues having 6 strands of β-sheets surrounded by α-helices. Fourth domain is C-terminal domain. Some of the lysosomal enzymes used saposin as cofactor for performing reactions. Some lysosomal hydrolases require saposin protein that provide lipid to the active site of enzymes for catalysis but saposin in saposin domain is quite enough for the hydrolysis of sphingomyline without external saposin protein (Kolter & Sandhoff, 2005; Schulze & Sandhoff, 2014; Xiong *et al.,* 2016; Zhou *et al.,* 2016).

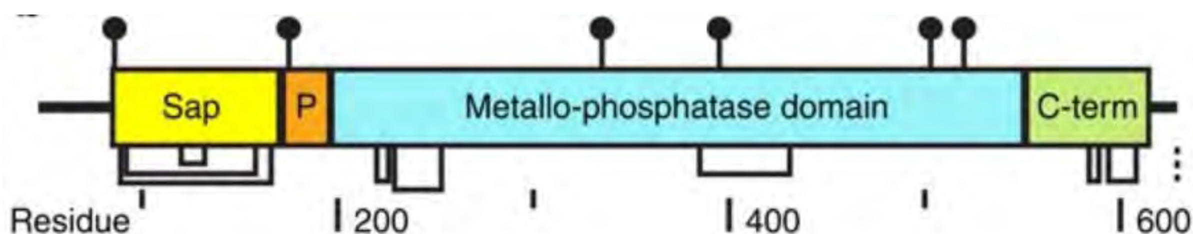


Figure 1.3 Domains of ASM with yellow saposin, orange showing proline-rich linker and green C-terminal domain (Zhou *et al.,* 2016)

Human ASM requires zinc ion, multiple membrane post-translational modification and acidic pH of 5 for the proper functioning (Jenkins *et al.,* 2009). ASM exist in two forms, intracellular lysosomal form and other one is extracellular secreted form. Both forms require zinc ion. Zinc bind to catalytic side of ASM. Zinc ion is preloaded in intracellular lysosomal form whereas exogenous zinc is required for secreted form. In the compartments of the early secretory pathway Zn^{+2} transporter 5 (ZNT5)- Zn^{+2} transporter 6 (ZNT6) heterodimers and Zn^{+2} transporter (ZNT7) homodimers are localized that play essential roles in SMPD1 activation, contribute to metabolism of cellular sphingolipid whereas cells with lack of the functions of these two complexes exhibited a reduced ceramide to sphingomyeline content ratio but how zinc and other factors effect enzymes activity is still not discovered/understood (Schissel *et al.,* 1996; Schissel *et al.,* 1998; Zhou *et al.,* 2016; Ueda *et al.,* 2022).

1.5.5.7 Mechanism of ASM

Acid sphingomyelinase is lipid hydrolase that catalyses sphingomyelin (SM) to phosphorylcholine and ceramide mentioned in figure 1.4. Acid sphingomyelinases (ASMases) produces the phophorylcholine head group that generates ceramide by cutting the phosphodiester bond of SM. Deficiency or inactivity of ASMase leads to accumulation of SM resulting in Niemann-Pick disease (Schuchman & Wasserstein, 2015; Cheema *et al.,* 2020). Within lysosomes, ASM interact with other hydrolases and maintains sphingolipid homeostasis (Falcone *et al.,* 2004).

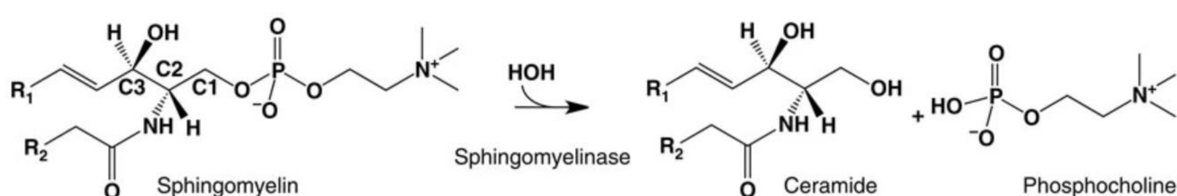


Figure 1.4 Catalysis of Sphingomyeline by Sphingomyelinase (Zhou *et al.,* 2016).

1.5.5.8 Molecular genetic of Niemann Pick Disease Type C

Type C is caused by *NPC1* and *NPC2* gene. *NPC1* gene has 25 exons and 24 introns, cytogenetic location is 18q11.2, encoded a protein of 1278 amino acid with molecular size of 142 kilo Dalton (kDa), located in limited lysosomal membrane whereas *NPC2* has 5 exons and 4 introns, with cytogenetic location 14q24.3, encoded a protein of 151 small soluble protein located in lysosome with molecular weight of 16 kilo Dalton (kDa) having high affinity for cholesterol binding with in endosomes and lysosomes (Ko *et al.,* 2003; McKay *et al.,* 2014; Evans & Hendriksz, 2017; Newton *et al.,* 2018). According to HGMD accessed on17.01.2023, there are 458 reported mutations of NPC1. Most are missense/nonsense (306), some are splicing (38), small deletion (63), small insertions (39), gross deletion (9), small indels (2) and gross insertion/duplication (1). There are 27 reported mutations of NPC2. Most are missense/nonsense (19), splicing (4), small deletion (3) and gross deletion (1).

1.5.5.9 Domains of NPC1

NPC1 is glycoprotein with 13 trans membrane helices forming 3 large luminal loop, 4 small luminal loops, 6 small cytoplasmic loop and a cytoplasmic tail shown in figure 1.5 (David & Ioannou, 2000). NPC1 has sterol sensing domain (SSD) that act as a cavity, accessible to both lipid bilayer and lysosomal lumen, required for the docking of individual cholesterol molecules and movement of cholesterol from endosomal /lysosomal lumen to within luminal leaflet of lipid bilayer (Kwon *et al.,* al., 2009; Li *et al.,* 2016). First large luminal domain called N-terminal domain (NTD) that consisted of 240 amino acids. Other 2 large luminal domains are middle luminal domain (MLD), C-terminal domain (CTD) (also called as cysteine rich domain) lies within 2-3 and 8-9 trans-membrane helices mentioned in figure 1.5 (Infante *et al.,* 2008a; Infante *et al.,* 2008b). These luminal loops undergoes high glycosylation to prevent proteolytic cleavage from lysosomal proteases. N terminal domain (NTD) has leucine zipper motif where cholesterol binds (Kwon *et al.,* 2009). SSD has the site for cholesterol binding. C-terminus has a dileucine motif that is important for the NPC1 localization in lysosome (LY) or late endosomes (LE). Loop 2 called MLD directly binds to cholesterol loaded-NPC2 to transfer cholesterol to NTD of NPC1, Loop 3 has ring finger motif (Yu *et al.,* 2014). The transformation required the interaction of NTD with omega (Ω) loop of CTD (Li *et al.,* 2017).

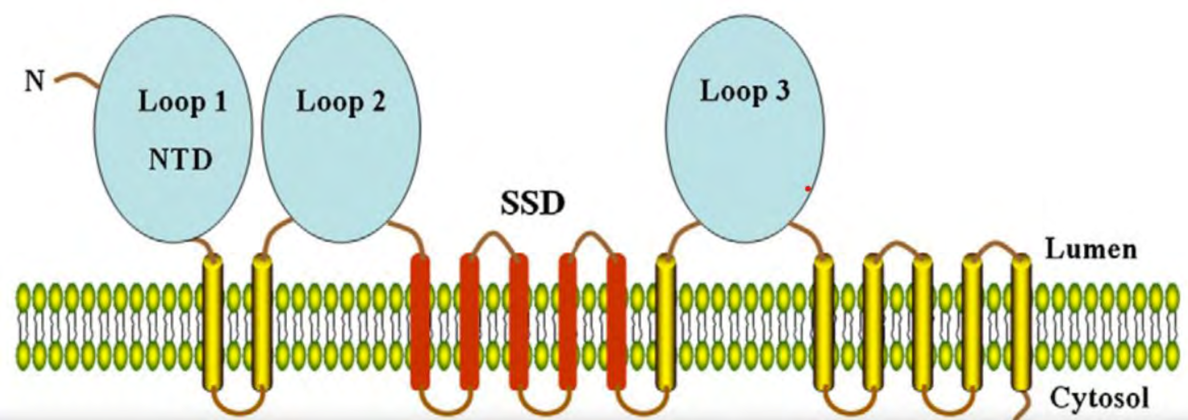


Figure 1.5 NPC1 protein structure having 13 transmembrane domains, with three large loops and SSD (Yu *et al.,* 2014).

NPC2/HE1 encodes small soluble proteins that bind cholesterol with micro affinity and has a signal peptide and prolein rich region, six cysteine residues and three Asn-linked glycosylation sites. It is ubiquitiously expressed in human tissues (Nakamura *et al.,* 2000). NPC2/HE1 has ML domain that is important in innate immunity as well as in lipid metabolism (Nakamura *et al.,* 2000).

1.5.5.10 Mechanism of transport by NPC2 and NPC1

In normal cell, free cholesterol is formed by the hydrolysis of endocytosed low density lipoprotein. This cholesterol is effluxes from lysosomes/ late endosomes through NPCI and NPC2 and transferred to other organelles e.g. plasma membrane endoplasmic reticulum (Nakamura *et al.,* 2000) and endocytic recycling compartment (Rosenbaum & Maxfield, 2011). NPC2 transferred cholesterol to NTD of NPC1 (Infant, Wang, *et al.,* 2008b). NPC2 structure showed that cholesterol bind to it with iso-octyl chain inward and sulphate facing outward to the solvent. This lead to a problem because in cholesterol extraction, cholesterol is positioned in such position that b-OH group facing hydrophilic solvent and iso-octyl side chain facing hydrophobic bilayer (Nakatani *et al.,* 1996). So NPC2 need to reverse the direction of cholesterol before binding. Esterified cholesterol bind to Lysosomal acid lipase (LAL) binding pocket of lysosomal acid lipase (LAL) in such an orientation that facilitated the hydrolysis of esterified cholesterol by LAL to free cholesterol and then transferred it to NPC2 and from NPC2 to NPC1, and then out of lysosomal compartment as mention in figure 1.6 (Inamaka *et al.,* 1984; Kwon *et al.,* 2009). Full NPC1 bind with nanomolar affinity with cholesterol and oxysterol 25-OH and 27-OH. NPC1 (NTD) does not have the ability to bind sterols that are modified at 3β- hydroxyl position. NPC1 (NTD) binds to cholesterol that is opposite in position to that NPC2 with 3βhydroxyl group of cholesterol faces the interior of NPC1 (NTD) and the iso-octyl side chain

faces outside (Infante *et s.,* 2008b). In NPC, unesterified cholesterol transport will be defective resulted in accumulation of unesterified cholesterol (Kwon *et al.,* 2009).

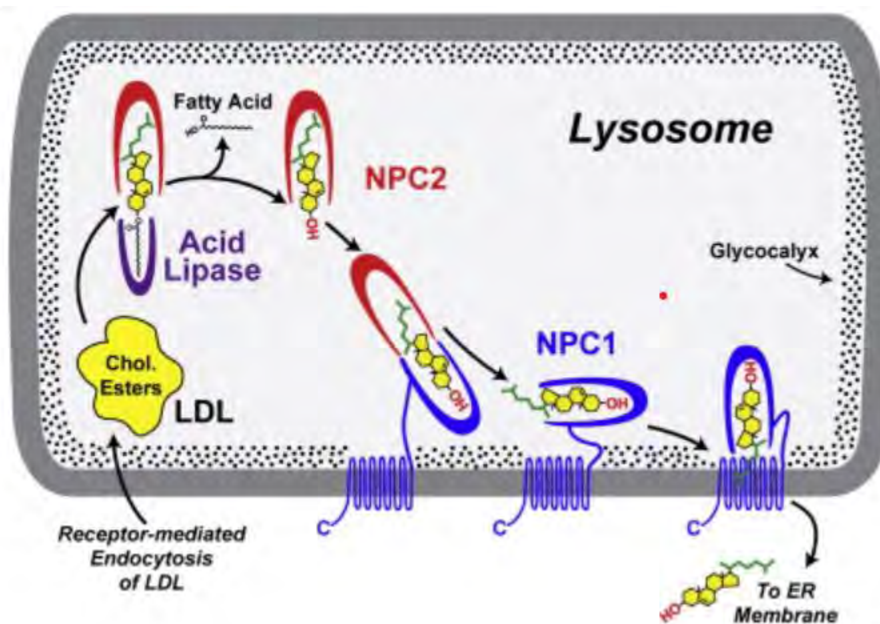


Figure 1.6 Pathway showing the transfer of cholesterol from LDL to NPC2, then to NPC1 to membranes (Kwon *et al.,* 2009).

1.6. Diagnosis of NPD

Diagnosis of NPD can be made by different methods mentioned below:

1.6.1 Acid sphingomyelinase enzyme assay

Tandem mass spectrometry (MS/MS) is used for analysis of ASM enzymes activity by using leukocytes and fibroblast cell through dried blood spots (DBS). This method is suitable for laboratory diagnosis of NPD including high throughput screening of at-risk populations and potentially for newborn screening (Legnini *et al.,* 2012).

1.6.2 Fillipin test

This test is considered as gold standard assay for the diagnosis of NPC (Vanier, 2010). This method involves actually the staining of accumulated cholesterol in culture cell of fibroblast and bone marrow by fillip and visualizes it under florescence microscope. It has some limitation as it is complicated, takes long time and required invasive skin biopsy. Sometimes it provides false negative result (Vanier *et al.,* 1991; Vanier & Latour, 2015). Accumulated cholesterol is shown in fibroblast cell in figure 1.7 through fillipin test (Walterfang *et al.,* 2006).

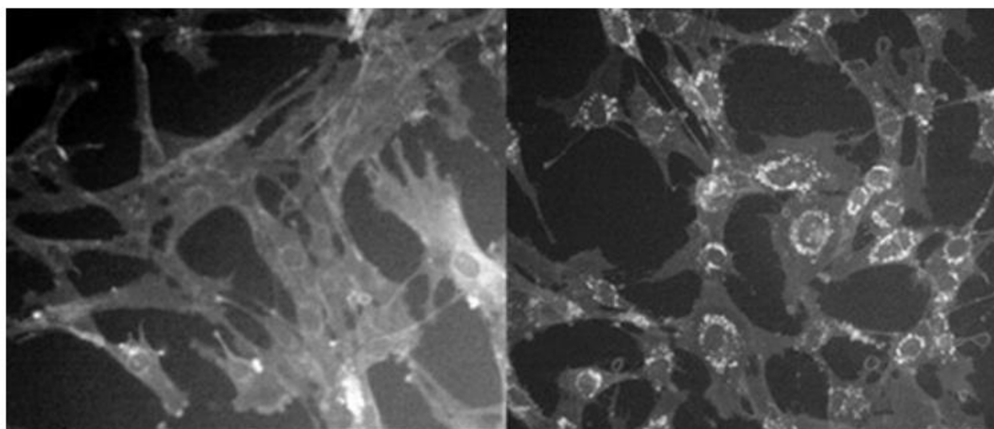


Figure 1.7 Fillipin staining of fibroblasts, normal (left) and patient (right) shown increase level of cholesterol accumulation in patient (Walterfang *et al.,* 2006).

1.6.3 Cholesteryl esterification test

This test is used to detect early formation of cholesteryl ester by loading of low density lipid (LDL) in cholesterol depleted cells .This test is useful in the detection of mutation in NPC1/NPC2. This test require living cell, is time taking and costly. In NPD-C patients, cholesterol get stored on late endosomes or lysosome that will reduced cholesteryl ester formation by acyl CoA: cholesterol acyl transferase (ACAT) so caused cholesterol storage and impaired cholesterol homeostasis in NPC cell (Pentchev *et al.,* 1987).

1.6.4 Biomarker

- **Oxy-sterol**

One the conventional diagnostic method for NPC is biomarker. In 2000s many biomarkers has been discovered. First biomarker is oxysterol, that is an oxidized cholesterol molecule produced in response to oxidative stress in NPC cell (due to accumulation of cholesterol in NPC cells) has positive predictive value of 97%.There are many oxidative markers like 7β-OHC, 7-ketocholesterol (7-KC) and 5β-cholestan-3β,5α,6β-triol (C-triol) produced through non-enzymatic pathway. Above mentioned bio markers are highly elevated in plasma of NPC as well as in NPD-A and NPD-B patients and can be determine by mass spectrometry. These biomarkers are also elevated in other diseases as well. For neurological pathophysiology, 24S-OHC is used as a biomarker (Zampieri *et al.,* 2009; Garg & Smith, 2017; Ory *et al.,* 2017). Lysosphingomylein (lyso SM), a metabolite of sphingomyelin, is also elevated in the plasma of patient with NP. A blood biomarker is (lysosphingomylin-509) has been used recently (Welford*et al.,* 2014; Giese *et al.,* 2015). Lysosphingomylin-509 levels are high in both ASMD

and NPC diseases so we cannot differentiate between them whereas use of lysosphingomyeline (lyso-SM) (also known as sphingosyl-phosphorylcholine), its level is high only in NPC diseases not in ASMD so combine assay of lyso-SM 509 and lyso-SM is used for the differentiation of NPC and ASMD and high ratio b/w them confirm NPC (Voorink-Moret *et al.,* 2018).

1.6.5 Radiological findings

Patient showing neuropsychiatric manifestation of NPD are evaluated through MRI and CT scan. MRI of patient affected with Niemann pick disease type A may be normal or sometimes showed cerebral atrophy on T_2-weighted imaging (McGovern *et al.,* 2006) pulmonary involvement in Type B NPD can be find out by high resolution CT scan (Simpson Jr *et al.,* 2010). CT and MRI of brain in patients affected by NPC showed atrophy, enlargement of 4 ventricles having cellular atrophy and hypoplasia of carpus collosum in figure 1.8 (Fink *et al.,* 1989).

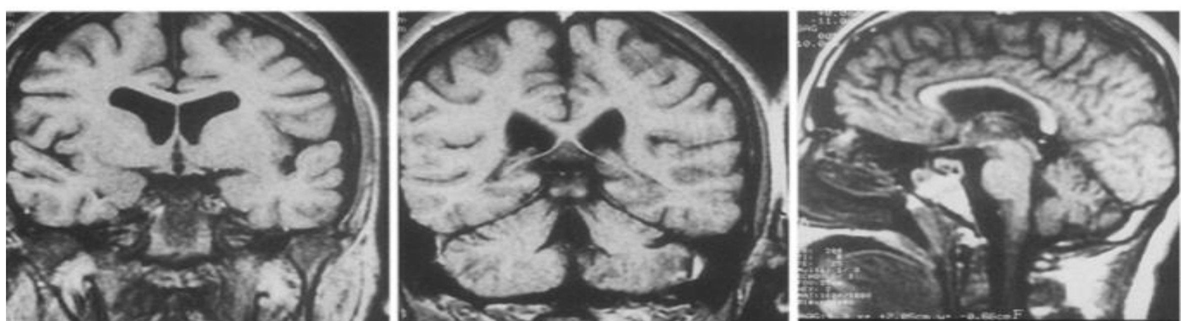


Figure 1.8 MRI scan of NPC patient brain showing cerebral atrophy, and hypoplasia of corpus callosum (Fink *et al.,* 1989).

1.6.6 Chitotriosidase (CT) assay

This assay performed for type NPD-C can be mildly positive, or normal. This test is unpredictable in 10% population due to pseudo deficiency mutation. Increased in CT activity is high in both gaucher diseases as well as in Niemann pick disease .Very high levels are present in GD whereas mild elevated level are present in Niemann Pick disease (Gou *et al.,* 1995; Ries *et al.,* 2006). Median chitotriosidase activity was 12,655 nmol/h per ml in Gaucher disease (GD), 780 nmol/h per ml in acid sphingomyelinase (ASM) and 925 nmol/h per ml in NP-C patients (Ries *et al.,* 2006). Chitotriosidase activity remained normal in NPC patient and observed only in late onset of disease. This assay lack specificity for NPC as chitotriosidase level remained high in other diseases like stroke, diabetes type 2 (Bustamante *et al.,* 2014; Elmonem *et al.,* 2016).

1.6.7 Bone marrow

In NPC patient, macrophages are laden with abnormal cholesterol called foam cell that can be easily detected in bone marrow. Foam cells showed high level of unesterified cholesterol. This test is not effective as features are not properly identified (Rodrigues *et al.,* 2006).

1.6.8 Prenatal diagnosis

Couple with already effected children should be recommended for pre-natal diagnosis that is performed using chronic villus sampling (CVS) at 10-12 week of pregnancy (Vanier, 2002)

1.6.9 Genetic testing

Genetic analysis is necessary and best confirmatory diagnostic method for the detection of NP disease (NPD) that show abnormal gene that caused Niemann Pick Types A,B and C (Patterson *et al.,* 2012).

1.7 Treatment of NPD

There is no proper cure of NPD but following treatments are recommended for that enhances up survival. Following are some treatment mentioned below:

1.7.1 Substrate Reduction therapy (SRT)

In SRT, metabolic homeostasis is maintained by decreases production of substrate that is needed to be catalysed by mutated enzymes. As substrate is present less, mutated enzymes can easily remove it through its limited catalytic activity during hydrolysis. Statin (drug that reduces cholesterol level), low cholesterol diet and ezetimibe (drug that reduces cholesterol in blood) are substrate reduction treatments. Another SRT involves the reduction of cholesterol production through hydrolysis of cholesteryl ester by LDL (Liu *et al.,* 2009; Rosenbaum *et al.,* 2009).

1.7.2 Miglustat

This is the only drug used for the treatment of patient affecting from Niemann Pick Disease type C. This drug inhibits the synthesis of glucosphingolipid by inhibiting the enzymes glucosylceramide that catalyse it. This drug has ability to cross blood barrier and proved effected in delaying of neurological symptoms in both adults and paediatric NPC patients. This treatment is effective for different age groups (Wraith & 2009b; Wraith *et al.,* 2010)

1.7.3 Arimoclomol

Arimoclomol is used as an effective drug approved by FDA for the treatment of NP-C patients. It enhances up the heat shock protein response but is bin clinical trials (Vite *et al.,* 2015; Schultz *et al.,* 2016).

1.7.4 Hydroxypropyl-beta-cyclodextrin (cyclodextrin) (CD)

Administration of cyclodextrin will reduce accumulated cholesterol from systematic organ but this has less ability to cross blood barrier so it hinders correction of central nervous system (CNS) for correction of neurological symptoms, direction administration of cyclodextrin is needed (Ramirez et a., 2010). This drug is in clinical trial. CDs replaces cholesterol transport protein by making complex with cholesterol and make it mobilize. It is also in but in clinical trials (Ingemann & Kirkegard, 2014; Szente & Szejtli, 2004).

1.7.5 Enzyme replacement therapy

No proper therapy for treating NPD is still discovered. However, enzyme replacement therapy (ERT) with recombinant acid sphingomyelinase (olipudase alfa) is used for the treatment of Niemann Pick Disease (NPD). After therapy, lipid profile in NP-B patients were checked that showed improvement with ERT. ERT is effective only in NPD-B as there is partial deficiency of ASM and no neurological symptoms are not present in it (Wasserstein *et al.,* 2015).

1.7.6 Gene Therapy

Gene therapy is recommended for the treatment of Niemann Pick Disease and is in clinical trials. Gene therapy with vector (adeno virus-associated virus (AAV)) was done on mouse model. Vector is either inserted systemically or intracerebroventricularly. Gene therapy lead to improved neurological symptoms, but life span is still limited (Chandler *et al.,* 2017).

1.7.7 Genetic counselling

Parents that are heterozygous should be counselled about the molecular inheritance of Niemann pick disease. As Niemann Pick Disease has autosomal recessive mode of inheritance so each live birth has 25% probability of affected and 50% probability to be carrier. Now a days, gene and molecular test are also used for the prenatal assessment of enzyme acid sphingomyelinase in Niemann pick type A, B and C (Patterson *et al.,* 2007).

1.8 Aims and Objectives of study

The main objectives of present study is

1.To study the clinical profile and molecular analysis of Niemann Pick Disease in cases from local population

The main objectives of present study are as following:

1. Molecular analysis of NPD patients for identification of prevalent disease causing mutations existing in Pakistani population.

2. Establishment of genotypic phenotypic correlation among disease causing variants.

3. Clinical characterization of Niemann Pick Diseases in patients enrolled in study.

2. MATERIALS AND METHODS

Genetic and molecular study of Niemann Pick Disease (NPD) consists of 4 main steps that are:

- Study approval
- Identification and enrollment of families
- Sample collection and Pedigree designing
- Molecular analysis

2.1 Study Approval

This study was approved by the Supervisory Committee at Department of Zoology, Faculty of Biological Science and Institutional Review Board (IRB), Quaid-i-Azam University, Islamabad, Pakistan. Patients diagnosed with Niemann Pick Disease (NPD) were collected from Children Hospital, of Pakistan Institute of Medical Science (PIMS), Islamabad, Pakistan and Holy family Hospital Rawalpindi, Punjab, Pakistan.

2.2 Identification and Enrolment of Families

During current study, three families (NP003, NP004, and NP005) each with at least single affected individual with Niemann Pick Disease (NPD) were enrolled after diagnosis by expert physicians from above mentioned tertiary care hospitals.

2.3 Consent Form

Before recruitment, consent form was designed according to Declaration of Helsinki, 2013 (Association, 2013) and signed by the accompanying parents guardian of the patients confirming the willingness to provide information related to disease, family history, photographs (where necessary) and, clinical laboratory reports. The purpose of current study was made clear to all the recruited families. All gathered informations were kept confidential.

2.4 Questionnaire

All the details related to each patients family history and clinical history were recorded on designed questionnaire by interviewing the accompanying family members. It took almost 20 minutes to complete the questionnaire. Questionnaire includes following details of patients:

It includes patient hospital ID, patient name, suspected disease type, age of patient, blood group, father name, parental consanguinity and any other diseases.

2.4.1 Recorded Medical Complaints Related to Patient

All the medical complaints that were briefed by the accompanying family members of the patients were noted. Complaints recorded were, delay in milestone achieving, abdominal distention, aggressive behaviour, speech delay, gibbus formation, short stature, respiratory/ear infection, eye clouding, cherry red spot in macula of eyes.

2.4.2 Examination

It includes the examination of height, weight, head circumference, facial features of patients as well as locomotion, gastrointestinal track (GIT) including the examination of spleen and liver. Neurological examination includes the examination of intellectual quotient (I.Q) and intellectual disability, dementia, dysphagia, dysarthria etc. Locomotory examination includes examination of gait (manner of walking), joint contractures, trunk and spine (scoliosis/kyphosis).

2.4.3 Laboratories

All the laboratory reports of the patients were collected that may consist of complete blood count (CBC), liver function test (LFT),renal function test (RFT), magnetic resonance imaging (MRI), enzymes analysis, radiological finding (X-ray) and ultrasound.

2.4.4 Pedigree Designing

Multiple family members were interviewed to get the history of the disease. After that pedigrees were drawn in order to determine inheritance pattern of the disease. Pedigrees were drawn using HaploPainter V.1.043.

2.5 Sample Collection

For the genetic and molecular analysis of the patient and accompanying family member, 5ml of venous blood was taken by the hospital staff members, using 5ml sterilized syringes with needle size of 23G x 1” and transferred it to the labelled tube having ethylenediaminetetraacetic acid (EDTA). EDTA prevents the blood from coagulation. Samples after collection were transferred to Cell and Molecular Biology Laboratory at Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, and kept at 20°C till DNA extraction.

2.6 Molecular Analysis

For the molecular analysis of the collected families, following steps were performed:-

- DNA Extraction from Blood Samples
- Agarose Gel Electrophoresis
- Primer Designing for Polymerase Chain Reaction (PCR)
- Primer Reconjugation
- PCR Amplification of Exons by SOLIS BIODYNE KIT
- Gel Electrophoresis of PCR Amplified Product
- Purification of PCR Product
- *NPC1* Sanger Sequencing
- Mutational Analysis of Sequenced Data

2.6.1 DNA Extraction from Blood Samples

For the extraction of DNA, **Phenol Chloroform Method** (Sambrook & Russell, 2006) was used and it took three days.

DAY 01

1. Blood samples were thawed at room temperature for the lysis of Red Blood Cells (RBCs).

2. Take 1.5ml Eppendorf (Ambion), labelled it with assigned (UAI) number of the patient.

3. Add 750µl of blood and poured 750µl of solution A (Table 2.1) in it.

4. Mixed thoroughly solution A and blood by inverting it several times. Leave it for 20 minutes at room temperature for approximate lysis of cells.

5. After that, centrifuge the samples in micro centrifuge (BECKMAN COULTER MICROFUGE 16) for 10 minutes at 13000 revolutions per minute (rpm).

6. After centrifugation, half of the supernatant were discarded properly in beaker containing bleach to avoid any contamination.

7. Nuclear pellet was dissolved in 400µl solution A and centrifuge it again for 10 minutes at 13000 revolutions per minute (rpm) in micro centrifuge.

8. Again discard the half supernatant, dissolve the nuclear pellet again in 400µl solution A and centrifuge it again for 10 minutes at 13000 rpm in microfuge.

9. After centrifugation discard the supernatant completely and again dissolve it in 400µl solution A again and centrifuge it again for 10 minutes at 13000 rpm in microfuge.

10. Repeat the same procedure for the fourth time, if the pellet contains any traces of haemoglobin.

11. Now discard the supernatant completely, add 400µl solution B (Table 2.1), 25µl of 20% SDS (Table 2.1) and 10µl Proteinase K (PK) (Table 2.1).

12. Incubate the samples overnight in incubator at 37°C for the digestion of protein in the pellet of white blood cells (WBCs).

DAY 2

1. On the next day, prepare fresh solution C (Table 2.1) and solution D (Table 2.1) by taking equal volume of both solutions (50:50).

2. Remove the samples from incubator and vortex them so the pellet will completely dissolve.

3. Add 500µl of freshly prepared solution C in each sample and centrifuge it at 13000 rpm for 10 minutes in microfuge.

4. After centrifugation two layers (upper and lower) were formed. Upper layer is transparent containing DNA and lower layer contain protein, lipid and cellular debris.

5. Remove the upper layer carefully and transferred it to newly labelled eppendrof tubes.

6. Add 500µl of solution D in each eppendrof tube. Centrifuge it again for 10 minutes at 13000 rpm in microfuge.

7. Again two layers were formed. Upper transparent layer was taken and transferred it to newly labelled eppendrof tube.

8. Add 60µl sodium acetate (Table 2.1) and 500µl chilled isopropanol (Table 2.1) in each labelled eppendrof tube. Tubes were inverted several times for the condensation of genomic DNA.

9. After DNA condensation, it appears as white thread in each eppendrof tube. Centrifuge it at 13000 rpm for 10 minutes for the formation of pellet.

10. After centrifugation, pellet stuck to the wall of eppendorf tube, supernatant was completely discarded.

11. Add 200µl of 70% ethanol (Table 2.1) in each eppendorf tube and centrifuge it for 10 minutes for 13000 rpm.

12. Discard the supernatant, mark the pellet on tube and allow it to dry at room temperature by placing the tube in vacuum concentrator.

13. DNA was air dried and 200µl of TE (Tris EDTA) buffer (Table 2.1) was added in each eppendorf tube and incubate the sample overnight in incubator at 37°C for DNA dissolution.

DAY 3

1. On the next day, take the sample out of incubator and vortex them for 2 minutes.

2. Heat shock the genomic DNA to deactivate the left over nucleases in water bath at 70°C for 1 hour after wrapping the lid of eppendorf tubes with parafilm.

3. Allow the samples to cool at room temperature and DNA samples were short spun at 1000 revolutions per minutes (rpm) for 2 minutes and transferred them to screw cap tubes (1.5ml, Axygen, USA) that are labelled with same UAI number of patients as eppendrof tubes.

5. Concentration of genomic DNA was estimated by nanodrop DNA quantification method (Titertek BERTHOLD, Germany) and also by agarose gel electrophoresis and then samples were stored in labelled cryobox at -20°C for further use.

Table 2.1: Composition of solutions used for Genomic DNA extraction through Phenol-Chloroform Method.

S.No	Solutions	Concentration and Chemical Composition
1	Solution A	Sucrose (0.32M), $MgCl_2$ (5Mm), Tris (10mM), Triton X (1%v/v)
2	Solution B	Tris (10mM), EDTA (2Mm), NaCl (400mM)
3	Solution C	Phenol (25), Chloroform(24), isoamyl-alcohol (1)
4	Solution D	Chloroform, Isoamyl-alcohol (24:1)
5	SDS	20%
6	Proteinase K (PK)	10mg/ml
7	TE Buffer	Tris (0.08g), EDTA (0.014G), Distilled water (50ml)
8	Sodium acetate salt ($NaCH_3COO$)	3M, pH 5-6
9	Isopropanol (chilled)	100%
10	Ethanol	70%

EDTA; Ethylenediamine tetra acetic acid, $MgCl_2$; Magnesium dichloride, ($NaCH_3COO$); Sodium acetate, SDS; Sodium dodecyl sulphate, NaCl; Sodium chloride, TE; Tris EDTA buffer, NaCl; Sodium chloride.

2.6.2 Agarose Gel Electrophoresis

1. Concentration and quantification of DNA was analysed by gel electrophoresis.

2. 1X TBE (Tris Borate EDTA) (Table 2.2 column A) was prepared by adding 100ml of 10X TBE buffer (Table 2.2 column A) and 900ml of distilled water in 1 litre reagent bottle and mixed thoroughly.

3. Mixing of 0.5 g of agarose in 50ml of TBE buffer (1X) in graduated cylinder to make 1% agarose gel. Cover it with aluminium foil and heated in microwave oven for 2 minutes.

4. Immediately after heating the mixtures, 3μl of 1% Ethidium Bromide (Table 2.2 column A) was added in the melted agarose gel and mixed by gently shaking the flask.

5. Allow the mixture to cool at room temperature and then poured the gel in casting tray to avoid bubble formation

6 Combs were placed in gel for well formation. Then the gel was allowed to set for 30 minutes to ensure polymerization at room temperature.

7 After polymerization, the combs were detached carefully from the gel and gel was positioned in gel tank (CS-300V, Cleaver Scientific Limited) containing 1X TBE buffer (Table 2.2 column A).

8. In first well 4μl of standard DNA sample (DNA ladder/marker) having concentration of 25ng/μl was loaded as reference.

9. Then 3μl of loading dye (Table 2.2 column A) and 4μl of genomic DNA sample were mixed thoroughly by micropipette and loaded in wells.

10. Samples were allowed to electrophorese for 30 minutes at 120V in gel tank containing 1-X buffer.

11. After 30 minutes, the gel with loaded DNA samples was removed from gel tank and DNA bands were analysed in gel documentation system (Biometra, Gottigen, Germany).

12. Record of gel was preserved in the form of picture.

Table 2.2: Composition of Agarose gel and Other Required Chemicals.

S. No	Solutions	Column A Compositions	Column B Compositions
1	Agarose gel	10X TBE (5ml)	10X TBE (5ml)
		Agarose (1%) (0.5 g)	Agarose (2%)(1.0 g)
		Ethidium Bromide (5µl)	Ethidium Bromide (5µl)
		Distilled Water (45µl)	Distilled Water (45µl)
2	Gel Preparation buffer (10X)	Boric acid (27.5µl)	Boric acid (27.5µl)
		EDTA (3.65 g)	EDTA (3.65 g)
		Tris (54 g)	Tris (54 g)
		Deionized water (500 ml)	Deionized water (500 ml)
3	Gel Running Buffer (1X TBE)	10X TBE (1 part)	10X TBE (1 part)
		Distilled water (9 part)	Distilled water (9 part)
4	Ethidium Bromide (50 ml)	Auto- claved filtered water (50 ml)	Auto- claved filtered water (50 ml)
		Ethidium Bromide (0.5 g)	Ethidium Bromide (0.5 g)
5	Loading Dye (25ml)	Auto- claved filtered water (25 ml)	Auto- claved filtered water (25 ml)
		Bromo-phenol blue (0.0875 g)	Bromo-phenol blue (0.0875 g)
		Sucrose (10 g)	Sucrose (10 g)

EDTA; Ethylenediamine tetra acetic acid, TBE; Tris borate EDTA, EDTA; Ethylenediamine tetra acid.

2.6.3 Primer Designing for PCR

Both forward and reverse primers were designed, for the amplification of exon 21 of *NPCI* gene. Ensemble genome browser (https://www.ensembl.org/index.html) was used to get full length transcript sequence of *NPC1* gene (NM_000271.5). Primers were designed by using primer 3 software (https://primer3.ut.ee/) from the flanking region of exon 21 of *NPC1* gene using exon intron junction. The primer size, melting temperature and GC content were checked by *In-Silico* PCR Tool (https://genome.ucsc.edu/cgi-bin/hgPcr). Single hit as well as specificity of exon 21 was checked by blast (https://www.ncbi.nlm.nih.gov/BLAST/) alignment tool (BLAT) and Primer Stat (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). Selected primers with their forward and reverse sequence, product size, primer length, melting temperature and GC content are mentioned in table 2.3.

Table 2.3 Details of primer pair used to amplify exon 21 of *NPC1* gene.

Primer	5′-3′ Sequence	Primer length (bp)	Melting temperature (°C)	GC Content (%)	Product Size (bp)
21 F	CAAGACCTGGACTCTCTTG AC	21	54	52.38	386
21 R	GATATACTGCCCTGTGCTC AG	21	54	52.38	

bp; base pair, F; Forward, GC content; Guanine-Cytosine Content, R; Reverse.

2.6.4 Primer Reconjugation

1. Primers are in dried freeze form so first of all placed them in room temperature for 40 to 50 minutes.

2. Then vortex the primers for 2-3 minutes from all sides.

3. After vortex, short spin the primers for 2-3 minutes at 3000 revolution per minutes (rpm) in microfuge so they settled down at the bottom.

4. Add 300µl ultrapure water in both primers tubes. Again vortex them and gave them short spin for 10-15 seconds at 3000rpm in microfuge.

5. Placed them at room temperature for at least 1 hour so the primer dissolved properly. It will be the stock solution of primer with concentration of 100pm/µl. After that store it in the freezer at -20µ°C.

6. Before use, make 20µl working solution of each primer from the stock solutions of by adding 2µl of stock solution and 18µl of ultrapure water in separate eppendrof tube and labelled them properly.

7. Stored it in freezer and used it during Polymerase Chain Reaction (PCR).

2.6.5 PCR Amplification of Exons (SOLIS BIODYNE KIT)

1. Polymerase chain reaction (PCR) was performed in 200µl PCR tubes (Axygen, USA).

2. Final volume of reaction mixture in PCR tubes was 25µl and consists of following components mentioned in table 2.4.

Table 2.4: Chemicals used in PCR mixture

Sr. No	Chemicals	Concentration	Volume(µl) for single PCR reaction
1	Sample DNA	>100 ng/µl	2µl
2	10X Taq Buffer $(NH_4)_2$ SO_4) and $MgCl_2$	10X , 25mM	2.5µl
3	DNTPs	2.5mM	2.5µl
4	Forward primer	10pm/µl	0.5µl
5	Reverse primer	10pm/µl	0.5µl
6	Taq polymerase	**N/A**	0.5µl
7	PCR water	**N/A**	16.5µl
	Total volume		**25 µl**

DNA; Deoxyribonucleic acid, dNTPs; Deoxyribonucleoside triphosphate; PCR; Polymerase chain reaction; $MgCl_2$; Magnesium dichloride.

Took PCR tubes and labelled them. After labelling them, added all above mentioned constituents in PCR tube to get final volume of 25µl then mixture was centrifuged (BECKMAN COULTER Microfuge) for 2-3 minutes. After that, PCR tubes were kept in the thermocycler (Bio-Rad T100) for amplification of target DNA segment that is exon 21 of *NPC1* gene for almost 3 hours. For the amplification of exon 21 of *NPC1* gene, Touch down PCR was performed that is operated between temperature ranges of 65°C-55°C. In touch down PCR, annealing temperature was kept 5-10°C higher than the optimal melting temperature(Tm) of primer and is gradually reduced in subsequent cycle that reduced the chance of non-specific binding. The conditions of thermo cycler for amplification are given in table 2.4 and figure 2.1.

Table 2.5: Thermo Cycler Profile for Touch Down PCR at 65 to 55°C.

Steps	Temperature	Time	Cycle
Initial Denaturation	96 °C	5 minutes	1X
Denaturation	95 °C	45 seconds	35X
Annealing	65 to 55°C	45 seconds	
Extension	72 °C	1 minute	
Final Extension	72 °C	8 minutes	1X
Hold	25 °C	∞	

°C; Centigrade, ∞; infinity, X; Times,

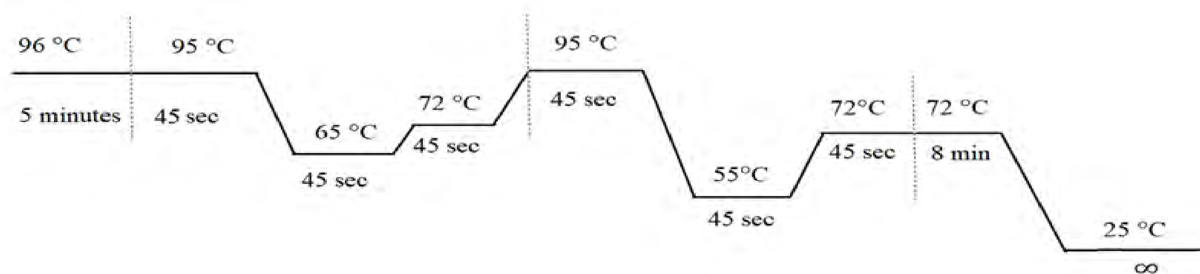


Figure 2.1: Touch Down PCR Thermal Cycling Conditions at 65-55°C.

2.6.6 Gel Electrophoresis for PCR Amplified Product

After PCR amplification, product of PCR was confirmed by gel electrophoresis. For this purpose amplified PCR products were electrophoresed on 2% agarose gel.

1. 2% agarose gel (Table 2.2 column B) was prepared by weighing 1g of agarose powder and put it in 50ml glass flask. After that add 50ml TBE buffer (1X) (Table 2.5). Wrap the flask with aluminium foil and heated in microwave oven for 2 minutes.

2. Immediately after heating the mixture, 3µl of 1% Ethidium Bromide (Table 2.2 column B) was added in the melted gel and mixed by gently shaking the flask.

3. Allowed the mixture to cool and then poured the gel in casting tray to avoid bubble formation. Combs were placed in gel for well formation. Then the gel was allowed to set for 30 minutes to ensure polymerization at room temperature.

4. After polymerization the combs were detached carefully from the gel and gel was positioned in gel tank (CS-300V, Cleaver Scientific Limited) containing 1X TBE (Table 2.2 column B) buffer as running buffer.

5. In the first well 2µl of DNA ladder (1kb) was loaded as size standard.

6. Then 3µl of dye (Table 2.2 column A) and 4µl of amplified DNA sample were mixed thoroughly by micropipette and loaded in wells.

7. Samples were allowed to electrophorese for 30 minutes at 120V in gel tank containing 1 X TBE buffer.

8. After electropherosis, the gel loaded with amplified DNA samples was removed from gel tank and DNA bands were analysed in gel documentation system (Biometra, Gottigen, Germany).

2.6.7 Purification of PCR Product

Purification of PCR product was done by WizPrep Purification Mini Kit. It involves following steps that are:

1.Take 20µl of each PCR product in 1.5µl eppendrof tube (Ambion) and added 100µl of GP buffer in it.

2. Placed the tubes at room temperature for 2 minutes.

3. After that, picked the mixture and shifted to labelled spin column and centrifuge it for 2 minutes at 13000 rpm.

4. Then added 350µl of washing buffer to each spin column.

5. Again centrifuged the mixture for 2 minutes for 13000 rpm and filter it. Discarded the filtrate.

6. Repeated the above 3 steps for complete purification of PCR product.

7. Centrifuged the mixture for 2 minutes to remove ethanol again and shifted them into newly labelled eppendrof tube.

8. Prepared 30µl elution buffer and applied it to column matrix centre, placed it at room temperature for 2 minutes and then centrifuged it at 3000 rpm for 3 minutes for the extraction of DNA product from the spin column to eppendrof tube

9. To estimate the purity and concentration, 5µl of PCR product were electrophorese on 2% agarose gel.

2.6.8 *NPC1* Sanger Sequencing

1. After purification of PCR product, 40µl of forward primer along with 16µl of purified PCR products were sent for commercial sequencing.

2. Each tube was sealed properly and labelled.

3. To accomplish the sequencing reaction Big Dye Terminator chemistry (an automated ABI PRISM ® 3730 Genetic analyzer) was applied.

4. Capillary electrophoresis method was used for separation of labelled fragments of DNA and was detected by spectrum analyzer.

5. Specific dyes were used for labelling of each nucleotide (thymine, guanine, cytosine and adenine) for documentation.

2.6.9 Mutational Analysis of Sequenced Data

Different software's were used for the analysis of mutations. Reference sequence was copied from ensemble genome browser (https://www.ensembl.org/index.html). Sequences were put in

Bio-edit (v.7.0.5) and aligned with the references sequence, taken from ensemble (https://www.ensembl.org/index.html). For chromatogram analysis, Chromas2.66 was used. After that applied different *In-Silico* tools such as mutation taster (https://www.mutationtaster.org/) to check whether the identified genetic variations are benign or have tendency to cause disease phenotype in Niemann Pick Diseases (NPD) Patients. Have Your Protein Explained (HOPE) was used to check the effect of substituted amino acid on protein, their configuration and biochemical nature. Uniport (https://www.uniprot.org/) was consulted to check variant sequence at protein level. Further other software like plyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Phdsnp (https://snps.biofold.org/phd-snp/phd-snp.html), SIFT (https://sift.bii.a-star.edu.sg/), Mu-pro (https://mupro.proteomics.ics.uci.edu) , panther (http://www.pantherdb.org/), and I-Mutant (https://folding.biofold.org/i-mutant/i-mutant2.0.html) were also used to check the pathogenicity of variant.

3. RESULTS

3.1 Clinical and Phenotypic Characterization of Niemann Pick Disease (NPD) Patients

Blood sample and Clinical data were collected from Niemen Pick Disease patients (NPD) and their guardians with the help of designed performa, from March 2022 to June 2022, by visiting paediatrics department of Pakistan Institute of Medical Science (PIMS), Islamabad, Pakistan and Holy Family Hospital, Rawalpindi, Punjab, Pakistan. Based on clinical profile, total three families were recruited for blood sampling. Each family is with single affected individual and all affected come from consanguineous mating. All the reports of patients were collected from the paediatrics department. Their personal information is kept confidential and patient were assigned IDs starting from NP-003 to NP-005.

3.2 Family Description

Clinical data obtained from three affected families showed different clinical manifestations like hepatosplenomegaly, delay in milestone achieving, short stature, aggressive behaviour, abdominal distention, eye shape difference, weak muscle tone and anaemia as mentioned in table 3.1. Brief history of each family along with pedigree is as following:

3.2.1 Family NP 003

First family (NP003) included in this study is a Pakhtoon family, belong from Tarnol, Islamabad. A male patient aged two years was admitted in children ward of Holy Family, Rawalpindi, Pakistan. On detailed physical examinations, biochemical tests and bone marrow analysis, Niemann Pick Disease was diagnosed in this patient. His sister, father and mother were phenotypically normal. He was born to a consanguineous couple. This family has four generations pedigree shown in figure 3.1A with single affected member (IV-1) in generation four. Parents of the proband were normal phenotypically and clinically which represent autosomal pattern of inheritance of the disease.

3.2.2 Family NP 004

Second family (NP004) included was also a Pakhtoon Family, belonged from District Kohat, Khyber Pakhtunkhawa, Pakistan. The family has one year old female patient admitted in PIMS, west paediatric ward due to severe hepatosplenomegly and anaemia. One of her sister died from same phenotypic and clinical conditions and other two siblings were phenotypically normal. On bone marrow analysis, Niemann Pick (NP) disease was confirmed. The parents were first degree cousin and family shows seven generations pedigree with two affected members (VII-1, VII-2) shown in figure 3.1C**.** Individual VII-1 was proband here and was selected for the molecular and genetic analysis of disease. Parents of affected individual were normal phenotypically and clinically, representing autosomal recessive inheritance pattern of disease transmission.

3.2.3 Family NP-005

This family (NP-005) is from Islamabad. A male patient of aged 2 years, with no family history of disease visited the PIMS hospital. Other siblings and parents were phenotypically normal. On bone marrow analysis, Niemann Pick Disease was confirmed. He was born to a consanguineous couple. This family has four generations pedigree with single affected member (IV-1) shown in Figure 3.1B. This member was proband and was selected for the molecular and genetic analysis of disease. Parents of the patient were normal which represents autosomal recessive inheritance pattern of disease transmission. All the clinical record of patient is mentioned in table 3.1.

Table 3.1 Clinical Data of Niemann-Pick Disease (NPD) Affected Patients Enrolled During Current Study.

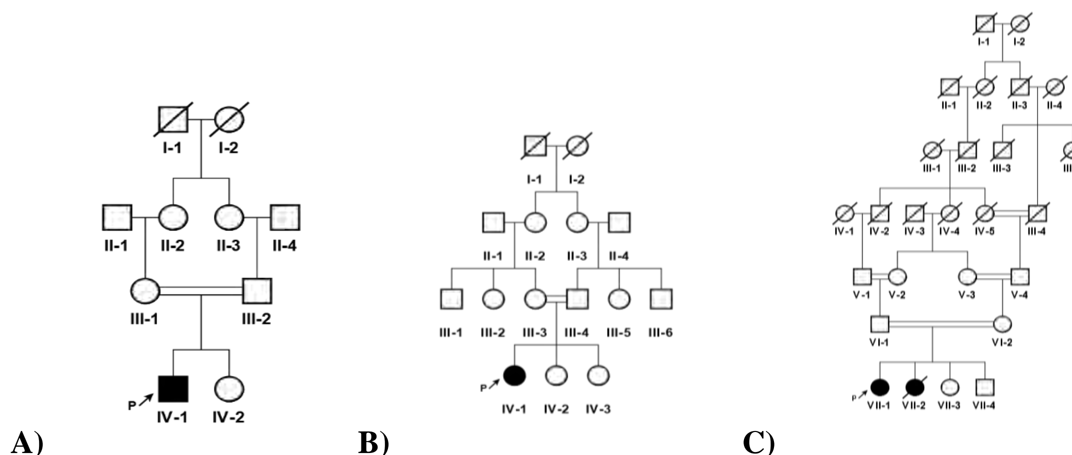
Family IDs	**NP 003**	**NP 004**	**NP 005**
Proband ID	IV-1	VII-1	1V-1
Gender	Male	Female	Male
Age at enrollment	2 years	1 year	2 year
Age at diagnosis	1.5 year	1 year	1.5 year
Consanguinity	Yes	Yes	Yes
Abdominal distention	Yes	Yes	Yes
Speech delay	Yes	Yes	Yes
Hepatosplenomagly	Yes(liver =8cm, spleen =5cm)	Yes	Yes
Developmental delay	Yes	Yes	Yes
Aggressive behaviour	Yes	No	No
Short stature	Yes	Yes	No
Corneal eye clouding	No	No	No
Height	Short	Short	Normal

Weight	6.8 kg	6 kg	9.8 kg
Dysphagia	No	Yes	No
Respiratory infection	Yes	Yes	Yes
Pallor Facial feature	Yes/ Coarse facial feature	Yes/ Coarse facial feature	No/ Coarse facial feature
Intellectual disability	N/A	N/A	N/A
Spine (scliosis/kyphosis)	Yes	Yes	Yes
Gait(manner of walking)	Yes	No	No
Joint contracture	No	No	No
Anaemia	Yes	Yes	Yes
Thrombocytopenia/ thrombocytosis	No/Yes	No/No	No/No
Deficiency of acid sphingomyelinase (ASM)	No	No	No
Cherry red spot in eye	No	No	No
Jaundice	Yes	No	No
Dementia	No	No	No
Ataxia	No	No	No
Tremors/seizures	No	No	Yes
LDL/Triglycerides level	N/A	N/A	N/A
Psychiatric problems	No	No	No
Hypotonia (decreased muscle tone)	Yes	Yes	No
Leukopenia/Leukocyt osis	No/Yes	No/No	No/No
Haemoglobin	less than normal	Less than normal	Less than normal

3.3 Pedigrees

Pedigrees were drawn through HaploPainter V.1.043. The square in the pedigree represents male members whereas circles represent female members. Affected members are shown with

filled circle/square. Normal individuals were shown by hollow squares and circles. Double line represents consanguinity. The deceased members in the family were represented by diagonal on circle or square. Roman numbers shows the generation number whereas Arabic numbers shows individual number in that generation mentioned in figure 3.2.



A) B) C)

Fig 3.1(A): Pedigree of Family NP003. (B) Pedigree of Family NP005. (C) Pedigree of Family NP004.

Normal male

Normal female

Affected male

Affected female

Person under study

Deceased

Marriage

Consanguineous marriage

Parents with offspring

Generation Roman numeral

Individual Arabic numeral

Figure 3.2 Key chart of symbols used in pedigrees of figure 3.1.

3.4 Genetic Characterization of Niemann Pick Disease (NPD) Patients

During this study, only exon 21 of *NPC1* gene is studied as it is considered as mutational hotspot for causing NPD type C. On Sanger Sequencing data analysis, a homozygous missense mutation was identified located at chr18, (NM_000271.5): c.3230G>C: p.(Arg1077Pro), in affected member VI-1 of family NP005. Mutation taster also predicted the pathogenicity of mutation. Parents were heterozygous for the variant and patient was homozygous mutant shown

in(Figure 3.3). As the variant is neither reported in ExAC/gnomAD nor in 1000G browser so it is likely to be the first report of this variant. Different *In-Silico* tools also predicted the variant as pathogenic. Difference of amino acid change is shown in figure 3.4. The remaining two families NP003 and NP 004 could not be identified with any pathogenic variant in the exon 21 of *NPC1* gene.

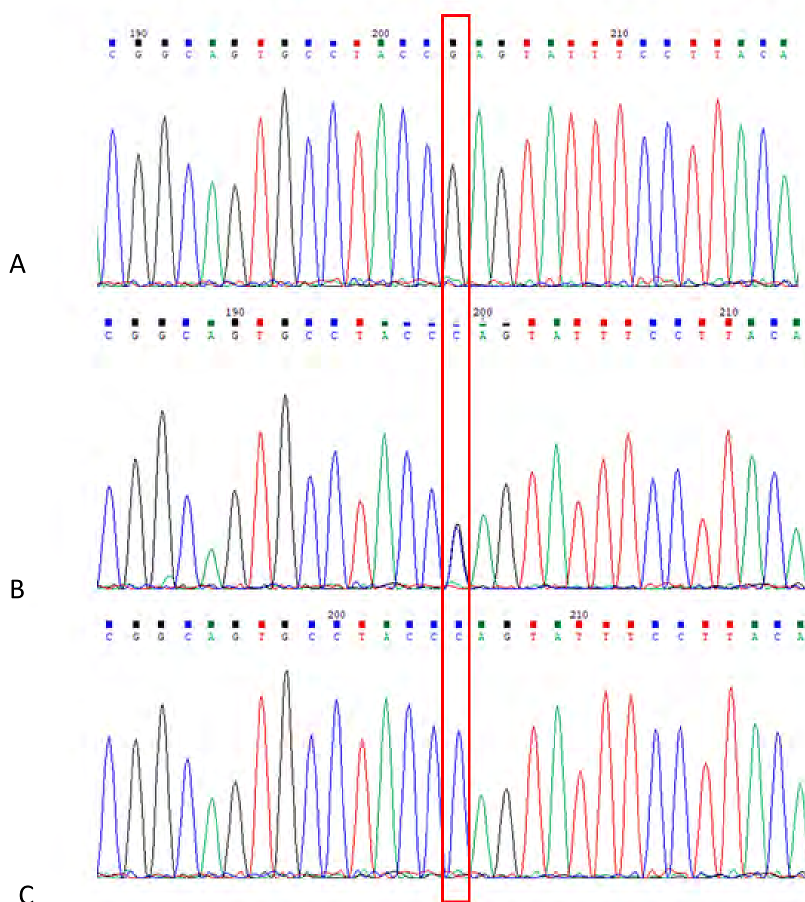


Figure 3.3: Sequence chromatogram showing novel variant (NM_000271.5): c.3230G>C causing p.Arg1077Pro (A) Chromatogram of homozygous wild type individual. (B) Chromatogram of heterozygous carrier parent. (C) Chromatogram of homozygous mutant patient.

3.5 *In Silico* analysis of Identified Disease Causing Variant

HOPE (Have Our Protein Explained) is software that is used for mutational analysis. Molecular origin of diseases related phenotype by mutation in protein is determined by HOPE. It shows protein 3D structure of normal and mutated protein and also predicts the structural and functional effects of mutation on the protein (Vanselaar *et al.,* 2010) HOPE analysis of our mutated amino acid is shown is figure 3.4.Disease causing missense pathogenic variant is further confirmed by using different *In-Silico* tools like i-mutant, Mu-pro, PolyPhen-2, Phdsnp and

SIFT. I-mutant predicts the decrease stability of protein that shows negative effect of that protein in this case and its expression is going to be decreased. Mupro is another tool is also used to check the stability of variant. In this case it gave the delta G value -1.1443002 that shows the decreased stability same as I-mutant. Pathogenicity of variant is checked through Polyphen-2, in this case variant is shown as probably damaging with the score 0.995 (sensitivity **0.68**; specificity: **0.97**) it means protein is damaging. According to Phd-snp, this variant is affecting the protein function and involved in causing disease. According to SIFT (Sorting Intolerant from Tolerant) variant is tolerant that is not affecting the protein stability.

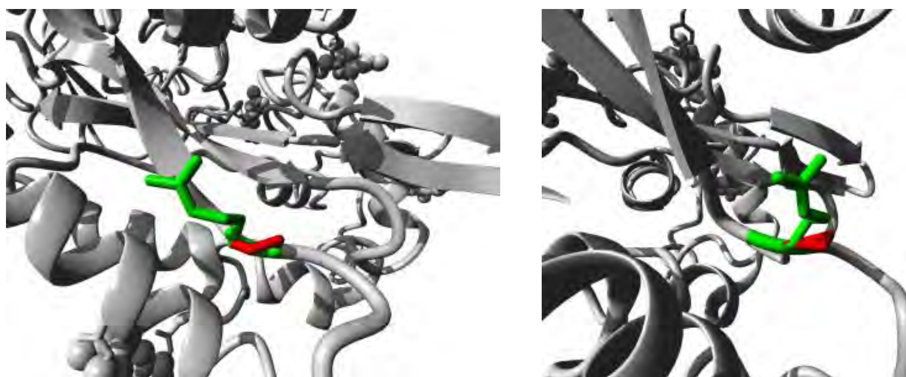


Figure 3.4:HOPE analysis shows amino acid substitution in protein structure where red color is representing the mutant amino acid (Proline) and green color is representing wild type amino acid (Arginine) .

4. DISCUSSION

Niemann Pick Diseases (NPD) is a heredity disease which has autosomal recessive mode of inheritance. The condition develops either due to total or partial deficiency of lysosomal enzymes, acid sphingomyelinase (ASM), which hydrolyse sphingomyeline(SM) into ceramide and phosphorylcholine (Schuchman & Desnick., 2017) or due to mutation in membrane trafficking protein that leads to accumulation of glycosphingolipid and cholesterol in endosomal lysosomal system (Alvarez *et al.,* 2008). Type A & B can be diagnosed through enzymes assay whereas type C can be diagnosed through filipin test (Legninii *et al.,* 2012; Patterson *et al.,* 2012).To prevent the prevalence of NPD early diagnosis and proper treatment is required. For the proper diagnosis of suspected patients and for screening of asymptomatic sibling, molecular analysis of disease-causing gene is crucial.

In case of NPD if all variants are in homozygous form then they will caused the disease. It means parents of patients must be heterozygous carrier of disease causing variant. Therefore population having consanguineous marriage would be at higher risk to develop the disease. In Pakistan 60% are the consanguineous marriages out of which 80% were first cousin marriages (Hussain & Bittles, 1998).Recessive disorders were more common in Pakistan due to consanguineous marriages that's why metabolic disorders are very common among Pakistan (Corry *et al.,* 2014; Gul *et al.,* 2019).

Only one variant was found in patients enrolled for this study in the CTD (also called Cystein rich domain) region of *NPC1* protein as a result of mutational analysis. It is missense variant, and this region is considered mutational hotspot as previously two known pathogenic variants has been identified by Fancelli *et al.,* 2009 and Zhan *et al.,* 2016 in same exon 21 of NPC1 gene and even same nucleotide position is same i.e. c.3230G>C but the replacement of amino acid is different (Fancelli *et al.,* 2009; Zhan *et al.,* 2016). Fancelli *et al.,* 2009 reported G (Guanine) nucleotide changes into A (Adenine) leading to change in codon CGA to CAA that is transition leading to p.R1077Q whereas Zhan reported CGA replaces by stop codon in chineses population that leads to premature termination of protein. (Fancelli *et al.,* 2009; Zhan *et al.,* 2016).Our variant p.Arg1077Pro is also on this position but replaced amino acids vary from already reported variants. Fancelli also reported missense variant c.3056A>G leading to p.Y1019C in Italian population in exon 21 of *NPC1* gene (Fancelli *et al.,* 2009). There are many other missense variants reported on exon 21 of *NPC1* gene worldwide like Sun *et al.,* 2001 reported c.3068T>G leading to p.V1023G (Sun *et al.,* 2001).

Millat *et al.,* 1999 reported c.3182T>C leading to p.I1061T in western European population and is most frequently found in this population. Same mutation is reported 20% in France and UK and 15% in US (Millat *et al.,* 1999; Millet *et al.,* 2005).In Europe p.P1007A is the second most frequent variant found in *NPC1* gene (Millet *et al.,* 2005).There are many missense variant i.e c.3182T>C leading to p.I1061T, c.3107C>T leading to p.T1036M, c.3185C>T (novel mutation) leading to p.Q421X were identified in exon 21 of *NPC1* gene (Millet *et al.,* 2005). Carstea *et al.,* 1997 reported c.3107C>T leading to p.T1036M in exon 21of *NPC1* gene (Carstea *et al.,* 1997). Novel SNP c.3185C>T leading to p.G1073G is also reported in exon 21 of *NPC1* gene (Millet *et al.,* 2005).In Japanese population missense variant c.3182T>C leading to p.I1061T and silent polymorphism C3159T is also reported on exon 21 of *NPC1* gene.(Yamamoto *et al.,* 2000). In Millet *et al.,* 2001, a missense variant c.3160G>A leading to p.A1054T is reported in exon 21 of NPC1 gene is reported that affects the cysteine rich loop in different ethnic Groups British, German, Turkish and Dutch (Millet *et al.,* 2001). In exon 21 of *NPC1* gene exonic SNP g.48727 C>T (c.3146) leading to A1048V is a novel variant reported in Caucasian population (Bauer *et al.,* 2002).

Mutational hotspots affect the function and structure of amino acid, folding and stability of protein, protein and protein interaction, protein expression and subcellular localization of protein and protein domains (Reva *et al.,* 2011).To find out the functional impact of amino acid change, different approaches are used. These approaches determine the physiochemical change of amino acid (e.g. change in size and polarity), structural changes due to change in Hydrophobicity) or evolutionary conservation of mutated residue (Adzhubei *et al.,* 2010; Reva *et al.,* 2011).Our variant p.Arg1077Pro varies in size, charge and hydrophobicity than wild type. Mutant aminoacid proline is small, neutral, and more hydrophobic than arginine. Wild type can form hydrogen bonding with glutamic acid at position 391.Change in hydrophobicity and smaller size of mutant type affects the hydrogen bonding as mutant residue not in corrected position to form hydrogen bond as wild type residue forms. Wild type residue can form salt bridges with glutamic acid at 391, 894 and 1089 position but mutant residue is unable to form these interactions due to disturbances in ionic bridges Mutated residue is in region that is important for the activity of protein and in contact with other protein domain that is important for the activity of protein. The interactions are disturbed due to this mutation that will affect the function of protein. Wild type residue is not conserved at this position as predicted by HOPE and *In-Silico* tools in figure 3.4.

According to previously published literature, there was no mutation reported in *NPC1* gene from Pakistani population but a few missense variants (c.1718G>C) (p.Trp573Ser), (c.1267C>T) (p.His423Tyr), (c.1493G>A) (p.Arg498His) and nonsense variant (c.1327C>T) (p.Arg443Term) in *SMPD1* gene were reported by Cheema *et al.,* 2020 (Cheema *et al.,* 2020).

4.1 Conclusion:

It is concluded that our Pakistani population is highly inbred and consanguineous marriages are most frequent here. There is high ratio of autosomal recessive disorders in Pakistan and Pakistani population is less explored for the genetic basis of these heredity diseases, there is no proper scanning including enzymatic analysis, genetic and molecular diagnosis for these disorders. In such scenario the best diagnosis is genetic testing for such complicated genetic disorders, which may reveal novel variants for diagnostics and treatment of these recessive disorders

4.2 Future perspective:

No sequence variant was found in exon 21 of proband of NP003 and NP004. It can be assumed that the pathogenic variant causing NPD-C can be found in other coding/non-coding or untranslated regions (UTR's) of *NPC1* or *SMPD1* gene. In future we will scan others exons of these unsolved families and hopefully they will be solved.

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