Metachromatic Leukodystrophy: A Bioinformatics Approach through Protein-Protein Interaction Network Analysis

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Submitted by

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DECLARATION

I hereby declare that this project titled "**METACHROMATIC LEUKODYSTROPHY: A BIOINFORMATICS APPROACH THROUGH PROTEIN-PROTEIN INTERACTION NETWORK ANALYSIS**", submitted to Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), Delhi is the original and independent work carried out by me under the guidance of Dr. Punit Kaur, Additional Professor, Head of the Department, Department of Biophysics, All India Institute of Medical Sciences, New Delhi, in partial fulfilment of the requirements for the award of Degree of Master of Technology in Bioinformatics and this dissertation or part of it has not been submitted by me for any other degree /diploma in any University/ Institute.

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Shri Ram

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LIST OF ABBREVIATIONS

MLD	Metachromatic Leukodystrophy
CNS	Central Nervous System
PNS	Peripheral Nervous System
ASA	Arylsulfatase A
GM2	Type of Gangliosidoses
GM3	Monosialodihexosylganglioside
MBP	Myelin Basic Protein
PLP	Proteolipid Protein
SCS	Schwann Cells
SAP-1	Sphingolipid Activator Protein-1
ADHD	Attention-Deficit Hyperactivity Disorder
MRI	Magnetic Resonance Image
ERT	Enzyme Replacement Therapy
SRT	Substrate Reduction Therapy
EET	Enzyme Enhancement Therapy
SQL	Structured Query Language
MiMI	Molecular Interactions from Michigan Molecular Interactions
BiNGO	Biological Networks Gene Ontology Tool
FWER	Family-wise Error Rate
FDR	False Discovery Rate

ClusterONE	Clustering with Overlapping Neighborhood Expansion
EPC	Edge Percolated Component
MNC	Maximum Neighborhood Component
DMNC	Density Of Maximum Neighborhood Component
MCC	Maximal Clique Centrality
BN	Bottleneck
GAD	Genetic Association Database
RDBMS	Relational Database Management System
DAVID	Databasefor Annotation, Visualization and Integrated Discovery
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
BIND	Biomolecular Interaction Network Database
DIP	Database of Interacting Proteins
MINT	Molecular Interaction Database
BioGRID	Biological General Repository for Interaction Datasets
HPRD	Human Protein Reference Database
OMIM	Online Mendelian Inheritance in Man
BH	Benjamini Hochberg
HECT	Homologous to the E6-AP Carboxyl Terminus
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy

Metachromatic Leukodystrophy: A Bioinformatics Approach through Protein-Protein Interaction Network Analysis

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1. ABSTRACT

System networks help in identifying new drug targets which in turn will generate more in-depth understanding of the underlying mechanism of diseases. Network based approach to study the pathogenesis of a disease is emerging as an important paradigm for analysis of biological systems. In this work the interaction network for Metachromatic Leukodystrophy (MLD) was built using Cytoscape and the analysis of network was carried out to find the drug targets. The most functional and highly interconnected sub-networks in the network were found which could help in understanding the mechanism of MLD.

Metachromatic Leukodystrophy (MLD) is one of a group of genetic disorders called the leukodystrophies. These diseases impair the growth or development of the myelin sheath, the fatty covering that acts as an insulator around nerve fibers. MLD is caused by a deficiency of the enzyme arylsulfatase A (ARSA). Lacking of complete knowledge of gene Co-expression network and pathogenesis for Metachromatic Leukodystrophy motivated us to develop an interaction network for this disorder. The *in-silico* prediction of potential interactions between nodes (genes) and target genes are of core importance for the identification of new drugs or novel targets for existing drugs. One of the aims of the study was to identify the functional and highly interconnected nodes in the network. Identifying the important sub-networks in the system could provide useful insights into the underlying molecular mechanism for Metachromatic Leukodystrophy. Another aim of the study was to identify candidate genes with high centrality score and perform their disease and pathway enrichment analysis to find out the disease classes in which these genes are involved and the pathways

they are affecting. An additional aspect of study was chromosome enrichment analysis of candidate genes to calculate the distribution of genes across different chromosomes.

Clique analysis was performed on the network and the highly interconnected clusters were found. SMAD9, PSAP, BMPR2, ARSA, UBE3A which may be the functional modules and can be identified as highly interconnected sub-graphs in the network. Most important potential drug targets found were TAF1, SMAD2, BRCA1, HNF4A, AR, SMAD9, CDC2, RB1, UBC, CDK2, UBB, PSAP, CDC23, MYC, MNAT1, CCNH, CDK7.

2. INTRODUCTION

Metachromatic Leukodystrophy (MLD) is an autosomal-recessive inherited lysosomal disorder, which belongs to the family of leukodystrophies as well as the sphingolipidoses as it affects the metabolism of sphingolipids. The MLD was discovered by Scholz Greenfield in 1925. MLD is rare, about 1 in 100,000 people has this disorder. MLD is also known as Scholz's disease or Arylsulfatase A deficiency **[**Barboura I, Ferchichi S, 2010]. Deficiency of the enzyme arylsulfatase-A (ARSA), or, more rarely, of its activator protein saposin-B leads to MLD [Holtschmidt H *et al.*, 1991, Aicardı` J (ed),1998, Von Figura K,2001]. In partial deficiency of ARSA, known as ARSA pseudodeficiency, which is sometimes also found in non-affected individuals, ARSA enzyme activity that is 5% to 20% of normal controls does not cause MLD [Arvan L Fluharty,2011].

The function of enzyme ARSA is to metabolise sulfatides by hydrolysing the 3-O ester bond of galactosyl and lactosyl sulfatides. The deficiency of enzyme ARSA leads to the accumulation of these substrates in metachromatic granules in oligodendrocytes, macrophages and some subtypes of neurons in the CNS, in Schwann cells and macrophages in the peripheral nervous system (PNS), and in visceral organs such as adrenal cortex, liver, pancreas, testes, kidney, sweat glands, , gallbladder and rectal tissue.

Four types of diseases are currently distinguished (according to the age at onset):

- Late infantile
- Early juvenile
- Late juvenile
- Adult forms

The late infantile form of the disease becomes evident in the second year of life, usually at 15–24 months. The juvenile form usually begins between 4 and 15 years of age and is further subdivided into early juvenile and late juvenile depending on whether the onset is before or after 6 years of age. Adult forms refer to the disease type which begins after 16 years of age. Adult-onset MLD progresses more slowly than the late infantile and juvenile forms, with a protracted course of a decade or more.

Late infantile and early juvenile cases are the most frequent, having a more severe phenotype and rapid progression. Characteristic symptoms of disease variants, because of the involvement of both CNS and PNS, include muscle wasting and weakness, muscle rigidity, developmental delays, convulsions, impaired swallowing, paralysis, dementia, dysphagia, blindness and deafness, seizures, diffuse atrophy and weakness with areflexia. Prognosis is severe, leading to death a few years after the onset of symptoms. In late juvenile cases, cognitive difficulties generally precede gait disturbances and the progression is slower. In the adult form, behavioral and intellectual changes are often the presenting symptoms, followed by spastic paraparesis and incontinence[Hyde TM,1992, Aicardı` J (ed),1998].

The infantile form, which presents a fairly constant clinical and pathological pattern, was first described by Greenfield (1933) who recorded two cases, and four further examples were reported by Russell Brain and Greenfield (1950). Jervis (1960) found 10 cases in the literature with pathological observations and described two more. To these should be added one recorded by Jacobi (1947) in which metachromasia was subsequently demonstrated by Diezel (1957), and another briefly reported by Peiffer and Hirsch (1955). Other cases have recently been described by Norman, Urich, and Tingey (1960) and by Hagberg, Sourander, Svennerholm, and Voss (1960) [J. W. Black and J. N. Cumings,1961].

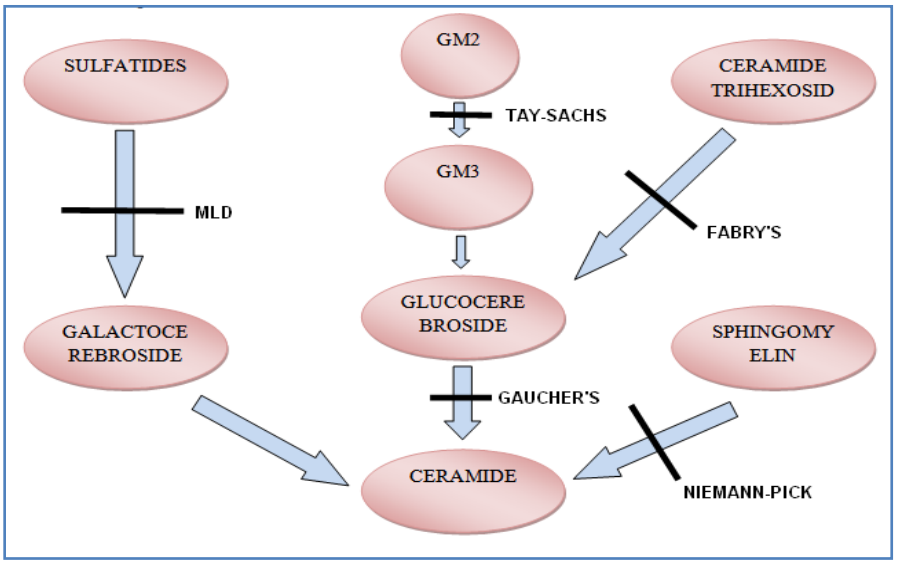


Figure 1: A diagram of Metachromatic Leukodystrophy and some other inborn errors of metabolism, showing the pathways and the names of the diseases when deficient.

At present, there is no successful treatment available to overcome the deterioration and loss of function caused by metachromatic leukodystrophy (MLD). In case of late infantile and early juvenile forms of the disease, bone marrow or cord blood transplantation may stabilize neurocognitive function [Krivit W,2004, Martin PL, Carter SL,2006] however, symptoms of motor function loss frequently progress. Mildly symptomatic and asymptomatic late juvenile and adult-onset forms are slower in progression, so are more likely to be stabilized with bone marrow transplantation.

In this study, we performed a system analysis of gene co-expression network in Metachromatic Leukodystrophy to investigate the topological characteristics of modulated genes in biological interaction network. In an effort to understand the molecular basis of genetic disease, it is important to discover their casual genes. Genes related to same disease are also known to have protein products that physically interact. A class of computational approaches has recently been proposed that exploit these two sources of information, physical interaction networks and linkage intervals, to predict associations between genes and diseases. The study of gene co-expression network is essential to define the molecular networks that contribute to maintain homeostasis of an organism's body functions. Disruptions in the gene networks results in disease in both human and animals. In effort to understand underlying mechanism of Metachromatic Leukodystrophy, Cytoscape, its plug-ins and various databases were used.

3. REVIEW OF LITERATURE

The word Leukodystrophy is coined from the Greek word "leuko" meaning white which refers to the white matter of the nervous system and "dystrophy" meaning inadequate development or growth. in medicinal terminology "dystrophy" imply to a condition which is progressive; that is, as the patient gets older the condition tends to get worse.

German neurologist Alzheimer published the first description of a patient with MLD in the year 1910. Later on, Metachromatic granules in liver, kidney and testis apart from brain were reported by Witte in 1921. Earlier MLD was known as a diffuse brain sclerosis. In 1938 Einarson and Neel renamed it as MLD [von Figura *et al*., 2001]. ASA deficiency in MLD patients was reported by Austin in 1963. Later on, Mehl and Jatzkewitz studied sulfatide metabolism and showed a block in the metabolism of sulfatide [Mehl and Jatzkewitz, 1965]. In 1975, very low levels of ASA were reported in some of the healthy individuals with a family history of MLD. Afterwards it was recognized that there is a moderately common allele of the ASA gene that leads to low expression of the enzyme [von Figura *et al*., 2001].

Metachromatic Leukodystrophy (MLD) is an uncommon type of diffuse cerebral sclerosis, characterized by the accretion of fats called sulfatides in cells, principally cells of the nervous system. [J.W.Black, J.N. Cumings,1961]. MLD is also known as Scholz's disease or Arylsulfatase A deficiency [Barboura I, Ferchichi S, 2010]. It is basically an autosomal-recessive neurometabolic disorder which belongs to the family of leukodystrophies as well as the sphingolipidoses. The accretion of fats results in progressive demolition of white matter of the brain, which consists of nerve fibers covered by myelin sheath. Myelin is a substance that insulates and protects nerves.

Metachromatic leukodystrophy is inherited in an autosomal recessive pattern and is caused by deficiency of enzyme arylsulfatase-A (ARSA), or, more rarely, of its activator protein saposin-B. Mutations in the ARSA and PSAP genes result in a decreased ability of enzyme arylsulfatase-A (ARSA), and protein saposin-B to break down sulfatides, resulting in the accretion of these substances in cells. Surplus sulfatides are toxic to the nervous system. This accretion progressively destroys myelin-producing cells, leads to the destruction of nervous system function [Holtschmidt H *et al.*, 1991, Aicardı` J (ed),1998, Von Figura K,2001].

The clinical features include progressive weakening of motor skills such as the ability to walk and intellectual functions. Loss of sensation in the extremities, incontinence, seizures, blindness, paralysis, inability to speak, and hearing loss is also observed. In due course patient lose consciousness of their surroundings and become unresponsive [R Macfaul *et al.*, 1982]. The signs of this disorder are most easily confused with similar symptoms of other degenerative nerve conditions such as Krabbe disease, childhood disintegrative disorder, and Gaucher's disease.

Some cases with partial deficiency of enzyme arylsulfatase A and no evident symptoms of MLD are also reported. This partial deficiency of enzyme ARSA is known as ARSA pseudo-deficiency. In case of Pseudo-deficiency, low enzyme activity (5% to 20% of normal controls) is reported but sulfatide is processed normally so MLD symptoms do not exist. [Arvan L Fluharty,2011].

3.1 GENETICS

Metachromatic leukodystrophy (MLD) is inherited in an autosomal recessive pattern. If both copies of the gene in each cell have mutations than probability to have this disorder is quit soaring. If individual carry one copy of the mutated gene than he/she typically do not show signs and symptoms of the MLD. The inheritance probabilities of Metachromatic leukodystrophy per birth are as follows:

- Case 1 : When One parent is affected and one is free of MLD
    - ✓ 0% children will have MLD because of normal gene is inherited from other parent.
    - ✓ 100% children will be carriers of MLD
- Case 2: When One parent is a carrier and the other is free of MLD
    - ✓ 50% children will be carriers of MLD
    - ✓ 50% children will be free from MLD

❖ Case 3: When Both parents are carriers:

- ✓ 25% children will be affected with MLD.
- ✓ 50% children will be carriers of MLD.
- ✓ 25% children will be free from MLD.

3.2 EPIDEMIOLOGY

Epidemiological surveys shows the incidence of metachromatic leukodystrophy per 100 000 live births varied from 0.6 to 2.5 [Ługowska A *et al.,* 2011]. The incidence of metachromatic leukodystrophy is estimated to occur in 1 in 40,000 to 1 in 160,000 individuals worldwide. The epidemiological studies revealed the non-random geographical distribution of metachromatic leukodystrophy prevalence. The increased frequency of MLD observed in Habbanite community (a small group of Jews who immigrated to Israel from southern Arabia), in which consanguineous marriages are relatively common (genetically isolated populations), has reported an incidence of metachromatic leukodystrophy of 1.3% and a 17% carrier frequency (Zlotogora *et al*.,1980).

The geographical variability of metachromatic leukodystrophy is shown in **Table 1**. This gives an impression that the prevalence of metachromatic leukodystrophy is highly variable. However, it should be noted that ost of the population-based studies have shown metachromatic leukodystrophy incidence being in range from 1:40,000 to 1:160,000.

Table1: Geographical variability of Metachromatic Leukodystrophy in some countries/communities.

Countries/communities	**Prevalence of MLD**	**Reference**
Northern Sweden	1:40000	Gustavson and Hagberg 1971
United States	1:100000	Kolodny and Fluharty 2001; Bonkowsky *et al* 2010
Habbanite community	1:75	Zlotogora *et al.,* 1980
Navajo Nation	1:2500	Genetics Home Reference. Reviewed September 2007
Arab groups in Israel	1:8000	Genetics Home Reference. Reviewed September 2007

3.3 CLINICAL MANIFESTATIONS:

Metachromatic Leukodystrophy (MLD) is a neurometabolic, autosomal recessive disease. It is a rare disorder and the diagnosis is often missed or delayed because it is not the first cause that comes to mind when a patient presents with symptoms.MLD is usually classified according to the age of onset:

- Late infantile
- Early juvenile
- Late juvenile
- Adult forms.

The late infantile and early juvenile forms are the most common in nature.

3.3.1 LATE INFANTILE:

This form becomes evident in the second year of life, usually at 15–24 months. Hagberg subdivided the clinical course into 4 stages (Hagberg *et al.*, 1962).

- **Stage I** , Sign and symptoms are stuble and easily overlooked during the first stage, which starts between 15-24 months of age. A child who has previously learned to walk becomes unsteady and requires support to stand. Hypotonia and flaccid weakness and occur in the legs or all 4 limbs. Muscle stretch reflexes may be diminished or absent. This stage lasts from a few months to a year or more.
- **Stage II**, In this stage child experienced problem to stand. However child can sit but no longer stand; cognitive deterioration begins; ataxia and nystagmus are noted; spasticity develops in the legs and possibly the arms and speech is also impaired due to a combination of dysarthria and aphasia. Intermittent pain in the arms and legs may also be reported. This stage may last only a few months
- **Stage III**, Quadriplegic and bedridden is characteristic features of this stage. Difficulty in maintaining normal breathing and dystonic posturing also observed .Speech is no longer distinct but the child may still be able to smile and respond.

- **Stage IV,** This stage lasts from a few months to several years. The child became deaf, blind, speechless and volitional movement.

3.3.2 EARLY JUVENILE

If age of onset is before 6 years of age it is characterized as early juvenile stage.
characteristic features of this form are :

- Impaired school performance
- Behavioral disturbances, such as confusion or bizarre behavior, followed by incontinence.
- Dysarthria , and extrapyramidal dysfunction.

The disease progresses to states that correspond to stages 3 and 4 of the late infantile form (Gordon 1978). Late infantile and early juvenile cases are the most frequent, having a more severe phenotype and rapid progression.

3.3.3 LATE JUVENILE

In this case age of onset is marked between 6 years of age to 12 years of age. The late juvenile form progresses more slowly than does the late infantile and early juvenile variants. Duration of the illness may be longer than 20 years (Mahmood *et al.,* 2010). In late juvenile cases, cognitive difficulties generally precede gait disturbances.

3.3.4 ADULT FORMS

The adult form age of onset can range from 12 to over 70 years. In the adult form, behavioral and intellectual changes are often the presenting symptoms, followed by spastic paraparesis and incontinence [Hyde TM,1992, Aicardı` J (ed),1998].
Other characteristic features of this form are as follows:

- Manifesting as a change in personality
- Poor school or work performance.
- Anxiety and emotional liability.
- Poor memory, and disorganized thinking
- Depression.
- Schizophrenia-like psychosis,

❖ Feelings of depersonalization etc

Table 2: Natural history of Metachromatic Leukodystrophy (MLD) subtypes

Source: GeneticsIN Medicine, Volume 13, Number 5, May 2011

	Late infantile type	**Juvenile type**	**Adult type**
General	40–60% of MLD cases, onset 6 months to 4 years, uniform disease course, and death within 5 years of onset.	20–35% of MLD cases, onset 4 to 16 years, more variable disease progression, and death within 10–20 years of onset.	15–25% of MLD cases, onset following puberty, variable velocity of progression, death within 5–20 years of onset
Initial symptoms	Peripheral neuropathy, muscle weakness, appendicular hyptonia, and hypo- or areflexia	Decline in school performance, emotional, behavioral disturbances, dysarthria/ataxia, and hyperreflexia	Decline in school/job performance, emotional lability, disorganized thinking, and hallucinations /delusions
Subsequent symptoms	Mental regression, visual and auditory impairment, loss of ambulation, evolution to hypertonia, dysarthria/ ataxia, bulbar paresis/dysphagia, and seizure disorder (25%)	Mental regression, optic atrophy, loss of ambulation, spastic quadriplegia, incontinence, bulbar paresis/dysphagia, and seizure disorder (50%)	Clumsiness, incontinence, spastic quadriplegia, choreiform movements, dystonia, bulbar dysfunction, and seizures rare
End stage	Vegetative state, complete loss of interaction, and death from aspiration pneumonia	Vegetative state and decerebrate posturing	Vegetative state

3.4 SULFATIDE BIOCHEMISTRY

Sulfatide accumulation is a major concern in case of Metachromatic Leukodystrophy. So in order to have a deep insight of the work, it is necessary to have an overview about sulfatide. The term sulfatide which is actually sulfur containing glycolipid is coined by Thudichum. 87.5% of the total lipids of myelin are sulfatide [Norton and Poduslo, 1982]. In 1933, Blix found that sulfatide is a mixture of equimolar amounts of cerebronic acid, sphingosine, galactose and sulfate [von Figura *et al*., 2001]. In sulfatide, an ester linkage is found between C-3 hydroxyl of galactose and sulfate [Stoffyn and Stoffyn, 1963]. C-18 sphingosine is the base of sulfatide [Stoffyn, 1966]. High proportion of long chain fatty acids and of fatty acids having 2-hydroxy groups are found in sulfatide and galactocerebroside. These two glycolipids constitute a major portion of 2- hydroxy fatty acids found in brain. After brain, kidney is second in relative abundance of sulfatides, but sulfatide concentration in kidney is around only 10% of the brain.

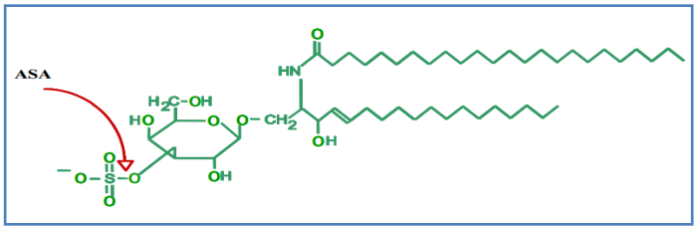


Figure 2: Chemical structure of Cerebroside 3-sulfate (Sulfatide). Sulfatide is a sulfate ester of cerebroside. Sulfatide has a sphingosine back bone with 3-O–beta–sulfo-D-galactose head group.

Synthesis of sulfatide involves sulfation of galactosylceramide (also designated as galactocerebroside) by a reaction with 3'-phosphoadenosine -5'-phosphosulfate (PAPS). Microsomal sulfotransferase catalyses the reaction [Farrell, 1974]. Biosynthesis of galactosylceramide which is the precursor of sulfatide is catalysed by UDP galactose : ceramide galactosyltransferase (CGT). Sulfates galactosylceramide to sulfatide is coverted with the help of 3'-phosphoadenosine-5'-phosphosulfate-cerebroside sulfotransferase (CST). During myelination its synthesis is maximum and in adults it proceeds more slowly. Microscopically, in affected cells from MLD patients, sulfatide is visualized as weird shaped massive inclusions.

Hydrophilic and hydrophobic interactions are found in sulfated glycolipids. Electrical neutrality of membranes is maintained through their anionic charge interactions with inorganic cations or organic amines. Sulfatide is bound to myelin basic protein (MBP) and proteolipid protein (PLP) by strong ionic interactions as it is located at the surface of the myelin membrane. Insulation feature of membranebilayer is due to galactosylceramides and sulfatides [Arvanitis *et al*., 1992; Norton and Poduslo, 1973; Vacher *et al*., 1989].Sulfatides works as a cofactor for Na/K ATPase in active sodium transport [Rintoul and Welti, 1989]. Sulfatides have very high affinity for various cellular adhesion molecules like laminin, thrombospondin, tenascin R etc. Sulfatides The anticoagulant activity in serum is also due to sulfatides.

3.5 MOLECULAR KNOWLEDGE ON MLD

Mutations in the ARSA gene may lead to the deficiency of ARSA causing MLD. The ARSA gene is located on human chromosome 22 at location 22q13. It is about 32 kb long with a coding sequence of 1521 bp. Eight exons encoding the 507 amino acid enzyme are present in the ARSA gene[Stein et al, 1989].

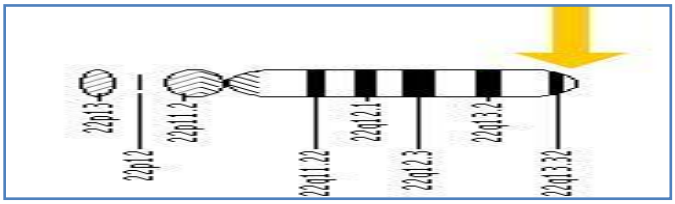


Figure 3: Showing chromosomal location of ARSA gene on the long (q) arm of chromosome 22 at position 13.33.

The ARSA gene is transcribed into three mRNA species, a major species of 2.1 kb, and two minor species of 3.7 and 4.8 kb. More than 90 missense mutations and polymorphisms have been identified in the ARSA gene [Kelly J Perkins *et al.,* 2005]. The most common mutation causing MLD, 459 +1 G--A, is a G--A transition destroying the splice donor site of exon 2. This mutation is associated with a severe phenotype of MLD and has never been found in the homozygous state in patients with juvenile or adult MLD [Joel Zlotogora *et al.*, 1994]. However, most common mutations in the general population: the IVS2 + 1G> A mutation which associated with the late infantile form, the P426L mutation is frequently

associated with the juvenile form, I179S mutation is associated with adult form and pseudodeficiency: N350 and 1524 +95 A → G (poly A-) [Clouter-Mackie & Gagnier, 2003].

Till date, total eight MLD-causing ARSA alleles have been characterized [Gieselmann *et al*. 1991] which can be divided into two groups. One of them encode for low residual enzyme activities and in other alleles is associated with the absence of enzyme activity. Genotype-phenotype correlation study has been done in reference to distribution of these alleles among patients. This study reveals that patients with two alleles associated with no residual activity suffer from the severe, late-infantile type of the disease and those with one or two alleles with low enzyme activity have the intermediate juvenile or mild adult type of the disease, respectively [Polten *et al*., 1991]. Biochemical data have been presented that support this genotype-phenotype correlation [Kappler *et al*., 1991; Leinekugel *et al*., 1992].

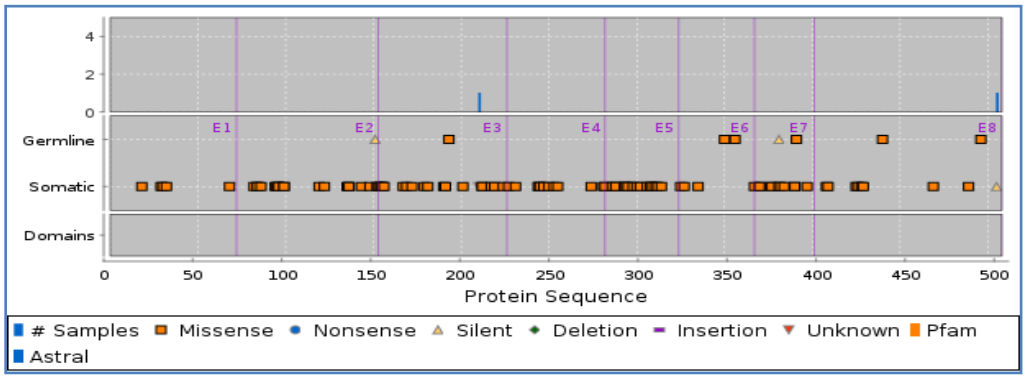


Figure 4: Distribution of mutations in ARSA gene.

Source: RCG Database http://rcgdb.bioinf.unisb.de/MutomeWeb/SimpleQuery?query=ARSA#space_5

MLD is found to be mainly caused by somatic mutations in ARSA gene. There are around 98 somatic mutations in ARSA reported and no germline mutations are found till date.

3.6 ARSA FUNCTION

The principle function of ARSA enzyme is the hydrolysis of sulfatides commonly called cerebroside-3-sulfate or 3-O sulfogalactosylceramide in galactocerebroside and sulfate. The successful working of ARSA necessarily depends on the presence of saposine B which forms a complex with the substrate [Ilhem Barboura *et al.*, 2010]. Myelin sheath is made up of membrane lipids like galactosyl-3-sulfate ceramide (cerebroside sulfate or sulfatide) and

ARSA catalyzes the first step in the degradation pathway of these membrane lipids. Insufficiency of this enzyme causes the storage of the substrate in lysosomes and an increase of sulfatide in the myelin membranes of affected individuals. Oligodendrocytes and Schwann cells of the nervous system are mainly effected due to accumulation of sulfatides but storage in many other organs is also reported[Joachim Kreysing *et al.*,1993] .

Housekeeping enzyme ASA'S expression can be seen in almost all tissues. Liver, placenta and urine has been reported as the major sources for the enzyme. The enzyme has a stumpy isoelectric point.

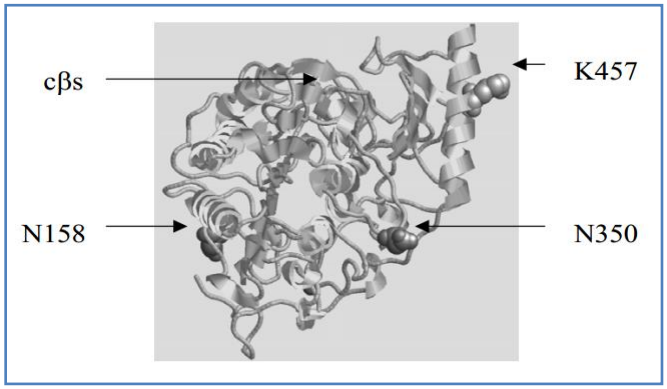


Figure 5: Three dimensional structure of housekeeping enzyme-ASA. Synthesized as a 62-kDa polypeptide, ASA consists of three N-linked oligosaccharideside chains,from which two (N-158 and N-350) are accessible by phosphotransferase and are needed for the activity of ASA. The N-terminal helix of the enzyme contains lysine which is represented here as K457 and Cβs represents the central β-pleated sheet [Yaghootfam *et al*., 2003].

Sulfatides are the major physiological substrates present in various sulfates broken up by ASA. Other than this, lactosyl ceramide 3-sulfate, seminolipid and psychosine sulfate are three major types of 3-O galactosyl sulfates cleaved by ASA.

ASA contains N-oligosaccharide side chains at each of the three potential N-glycosylation sites because of the hydrolyses of signal peptide in the lumen of the endoplasmic reticulum. ASA is recognized as lysosomal enzyme by phosphotransferase as it enters into the Golgi apparatus in fully folded form [von Figura et.al., 2001]. The presence of mannose-6-phosphate residues on the first and the third oligosaccharide side chains and the ineffective phosphorylation of only second N-glycosylation site has been reported after the in-depth examination of the three N-linked oligosaccharide side chains of ASA.

3.7 MYELIN AND SCHWANN CELLS

3.7.1 MYELIN

Oligodendrocytes and Schwann cells are the chief factories producing highly specialised plasma membrane i.e Myelin. Myelin acts as an insulator by wrapping itself around axon and thus is responsible for the fast axonal transmission of the action potential. One of the reasons behind proper functioning of the nervous system is the specific molecular organisation of myelin. Cholesterol and the glycosphingolipids, galactosylceramide and sulfatide forms the major portion of the lipid composition in multi lamellar structure of Myelin. Importance of these lipids in the formation and maintenance of myelin has been clinically proved [Bosio *et al*., 1996; Coetzee *et al*., 1996; Honke *et al*., 2002)].

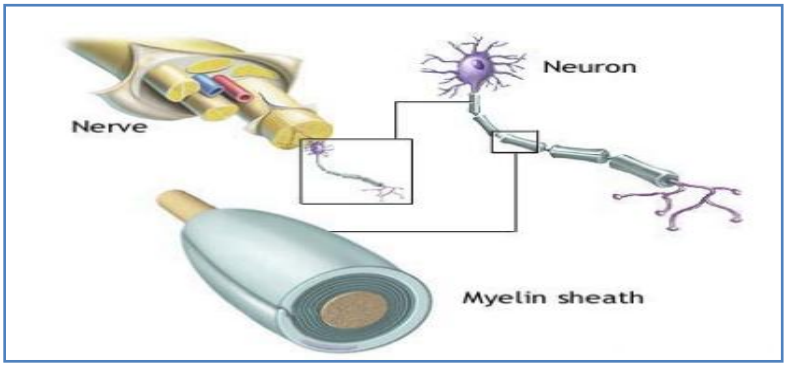


Figure 6: Neuron and Myelin. Diagrammatic representation of the nerve cell neuron and myelin insulating axon.

Source: http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/9682.jpg

Neurological disorders usually occur due to changes in myelin architechture which may be a result of a hereditary or acquired disease. Alterations in genes encoding myelin protein or myelin lipid metabolism may cause Genetic diseases[Anderson *et al*., 1998; Nave, 1994; Werner *et al*., 1998] . Disturbance in myelin lipid metabolism particularly causes two major lysosomal storage diseases - Krabbe disease and MLD [Gieselmann, 2003; Suzuki, 2003; von Figura *et al*., 2001; Wenger *et al*., 2001]. ßgalactocerebrosidase deficiency causes Krabbe disease, also known as Globoid cell Leukodystrophy and results in accumulation of galactosylceramide. One of the treatments of MLD may include the prevention of demyelination. This can help in the treatment of the disease to a great extent.

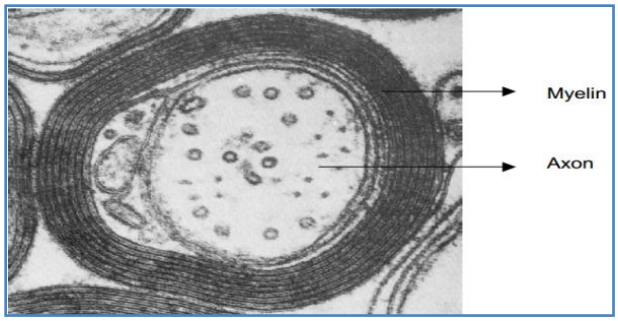


Figure 7: Ultrastructure of myelin. Electron micrograph of axon enwrapped by myelin.
Source: www.cytochemistry.net

3.7.2 SCHWANN CELLS (SCS)

Till date, an appropriate model for cell culture which can store sulfatides is unavailable and it is still unclear how at the molecular level the metabolism of a cell is affected by sulfatide storage [Gieselmann *et al*., 2003]. However only minute information is available for the demyelination machinery and the abnormal metabolic pathway of the causative molecule. Deficiency of particular lysosomal enzyme which causes metabolic defects in the cell can be cross corrected by an exogenously applied enzyme and this has also been reported by certain previous cell culture experiments [Neufeld, 1991]. Degradation of the substrate present in the lysosomes is caused by the internalization of the enzyme by mannose 6-phosphate receptors into the lysosomal compartment. In glial and neuronal cells, endocytosis of lysosomal enzymes is dependent on mannose 6-phosphate [Schluff *et al*., 1998; Stewart *et al*., 1997].

SCs, which sustain neurons, play an important role in the development of axons, its renaissance and myelination, are the principal accessory cells of the peripheral nervous system. Peripheral nervous system's SCs and central nervous system's oligodendrocytes form Myelin. It helps in the salutatory nerve conduction [Guenard *et al*., 1994]. Demyelinating and remyelinating lesions and characteristic lamellar inclusions in SCs and macrophages were reported in the low ASA activity in leukocytes and fibroblasts culture of a 38 year old man [Fressinaud *et al*., 1992]. Later, for MLD patients with peripheral neuropathy, similar reports were published [Coulter-Mackie *et al*., 2002; Hansen *et al*., 1994; Martinez *et al*., 1975].

In many laboratories, pure neonatal SCs are prepared using numerous methods. Till date, spontaneous transfection or immortalisation using oncogenes more than fifteen SC lines have been produced [Bolin *et al*., 1992; Boutry *et al*., 1992; Chen *et al*., 1987; Goda *et al*., 1991; Jirsova *et al*., 1997; Li *et al*., 1996; Porter *et al*., 1987; Ridley *et al*., 1989; Tennekoon *et al*., 1987; Thi *et al*., 1998; Toda *et al*., 1994; Watabe *et al*., 1995; Watabe *et al*., 1990). In each cell line, the extent of phenotype expression and differentiation differ from one other [Hai *et al*., 2002]. SCs from ASA KO and wild type (WT) mice need to be isolated for an in vitro cell culture system for MLD and this became possible with the technology which was established using above mentioned reports.

3.8 PROSAPOSIN

In humans PSAP gene encodes for Prosaposin (also known as PSAP) protein. Prosaposin is a highly preserved glycoprotein and it plays an important role in numerous biologically significant functions like the development of the nervous system and the reproductive system.

When Prosaposin is broken down, it produces four smaller proteins : Saposin A, B, C and D. Saposin is an contraction for Sphingolipid Activator **P**r**O**[**S**]te**IN**s [Morimoto S *et al.*, 1990]. Cysteine residues and glycosylation sites are at almost similar places in each domain of the prosaposin and each domain is found to be approximately 80 amino acid residues long.. PSAP not only carries out the neurotrophic activities but also serves as an integral membrane protein and secretory protein.

3.8.1 FAMILY MEMBERS

- **Saposin A**: Before isolation, Saposin A was considered as an N-terminal domain in the prosaposin cDNA. It has been reported that enzymatic degradation of 4-methlyumbelliferyl-β-glucoside, glucocerebroside, and galactocerebroside is motivated by Saposin A [Morimoto S *et al.*, 1989].

- **Saposin B**: It was the first small prosaposin protein to be discovered. Arylsulfatase A hydrolyses sulfatides but for the first time it was found that ARSA requires a heat stable factor Saposin B for this hydrolysis. It is recognized by a list of names like sulfatide

activator protein, sphingolipid activator protein-1 (SAP-1), dispersin, GM1 ganglioside activator, and nonspecific[O'Brien JS, Kishimoto Y, 1991]. It has been experimentally shown that many enzymes get activated when saposin interacts with the substrates instead of interacting with enymes themselves.

- **Saposin C**: Glycosylceramidase hydrolyses glycocerebroside and galactoslyceramidase degrades galactocerebroside with the help of Saposin C. This was the second small prosaposin protein to be discovered.
- **Saposin D**: Till date, not much is known about Saposin D, more exploration needs to be done. cDNA sequence of prosaposin lead to the prediction of this protein. Saposin-D is a specific sphingomyelin phosphodiesterase activator [Kishimoto Y, 1992].

3.8.2 STRUCTURE

Eighty amino acid residues long every saposin has six uniformly positioned cytesines, two prolines, and a glycosylation site (two in saposin A, one each in saposins B, C, and D) [Kishimoto Y, 1992]. Saposins which are enormously dense and tightly disulfide-linked molecules have intense heat-stability, plenty of disulfide linkages, and resistance to most proteases. α-helical structure of each saposin which is seen in all (especially with the first region), is maximal at a pH of 4.5 and thus is important for stimulation[Kishimoto Y, 1992]. The first 24 amino acids of N-terminal of saposin gives it a β-sheet configuration [O'Brien JS, Kishimoto Y , 1991].

The PSAP gene is located on human chromosome 10 at location 10q21.The molecular location of this gene on chromosome 10 is found at base pairs 73,576,054 to 73,611,081. In particular, on chromosome 10 PSAP gene is located from base pair 73,576,054 to base pair 73,611,081.

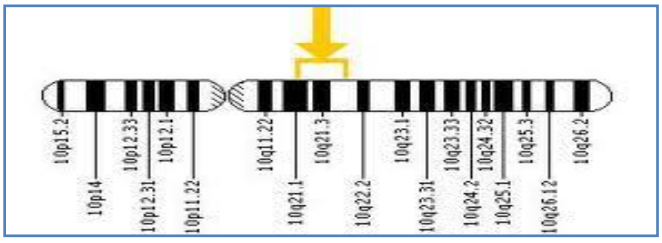


Figure 8: Showing chromosomal location of PSAP gene on the long (q) arm of chromosome 10 between positions 21 and 22

To understand the origination of the locus that codes the four highly conserved sphingolipids activator proteins or saposins, sequence analysis of chromosomal DNA which helped in characterizing the gene for prosaposin, was carried out. It comprises of 13 exons ranging from 57 to 1200 bp and introns range from 91 to 3812 bp in length. Among the four saposins, the regions encoding saposins A, B, and D each have three exons and region encoding saposin C have only two. The 3′ untranslated region, the four mature prosaposin proteins and carboxy terminus of the signal peptide were present in the sequence which was used. [Efrat Gavrieli Rorman *et al.,* 1992].

3.8.3 FUNCTION

The cell's recycling centers called lysosomes are the primary home for saposins. Sphingolipids (fatty substances) are broken down by saposins with the help of lysosomal enzymes.

- **Saposin A-D:** In lysosomal section, the catabolism of glycosphingolipids with tiny oligosaccharide groups is carried out primarily with the help of Saposins A-D
- **Saposin B:** Saposin B protein breaks down sphingolipids with the help of several enzymes. Saposin B protein when coupled with enzyme arylsulfatase A carries out tts most critical biological role. The nervous system's white matter which comprises of nerve fibers insulated with myelin, contains huge amount of sphingolipids and a subgroup of these sphingolipids called sulfatides further is cleaved by enzyme Saposin B. Transportation of lipids to the external surface of the cell may be carried out by Saposin B.
- **Saposin C:** Enzyme beta-glucocerebrosidase break down another sphingolipid glucocerebroside with the help of saposin C protein.
- **Saposins A and D:** These are also found to deal with sphingolipids.

3.9 SYMPTOMS

1. HYPERTONIA

Resting muscle tone: The muscles of the body are normally in a state of tension or resistance. Brain modulates this state by sending messages to the muscles which are contracting and those which are relaxing. Resting state of tension is required for a person to move and

respond. Abnormal tone occurs due to disrupted or incorrect messages from the brain. Damage to the brain or spinal cord results in this abnormal tone. Hypertonic muscles, also referred to as Spastic, feel hard or stiff. In this state the muscles and joints feel resistant to movement.

2. BEHAVIOR PROBLEMS

Behavioral problems often occurs in the adult form of Metachromatic Leukodystrophy and this is the first sign which appears during the teenage years or later. Symptoms may include alcoholism, drug abuse, or difficulties at school or work, anger & defiance, arguing & fighting, self-esteem, power, struggles, substance abuse & risky behavior, disrespect, aggression, outbursts & temper tantrums.

3. DECREASED COGNITIVE FUNCTION

Cognitive function describes a person's alertness and orientation, memory and attention span. In case of MLD, person's ability to interact with his physical environment goes down and leads to a state of confusion. Memory which is the most important cognitive ability is also diminished. In terms of attention span, patients' ability to make decisions, reasoning, his knowledge and use of language also gets declined.

4. INCONTINENCE

Incontinence is inability to control the excretion of urine or feces. Urination system relies on normal function in pelvic and abdominal muscles, diaphragm, and control nerves. In case of babies' nervous systems are too immature for urinary control. Incontinence in adults is assumed to be a disorder. In case of MLD, incontinence is seen due to malfunctioning of nervous system.

5. IRRITABILITY

Inconsolability/Irritability makes young ill children fussy, whiny, and fretful. This may be an early symptom of any serious problems. Irritability does not indicate any specific illness, it only shows that something is wrong with the patient. Children with MLD become increasingly agitated and irritable as it gets older.

6. SEIZURE

Disorders associated with central nervous system often results in changes in the physical behavior of the patient and this change is referred to as seizure s. In MLD, seizure causes the patient's muscles to contract and relax repeatedly. There are many different types of seizures and symptoms may vary from person to person.

7. DIFFICULTY WITH SWALLOWING

Mouth, throat area, and esophagus (the muscular tube that moves food to the stomach) are the majorly involved in the complex act of Swallowing and functioning of these body parts is controlled by different nerves and muscles. Swallowing is basically a voluntary control, but it also involves a lot of involuntary control. In MLD, patient finds difficulty with swallowing food as it gets stuck in the throat, or from the neck down to just above the abdomen behind the breastbone (sternum).

8. LOSS OF NORMAL MOVEMENT AND COORDINATION

This is the first symptom which is often seen in early and late infantile forms of MLD. In this, the most significant loss of movement skills (motor skills) usually starts between 12 to 18 months age. The first signs often include a decrease of body control and especially decrease in the ability to crawl or walk normally. At first, this loss of abilities occurs rapidly and then continues more gradually.

3.10 DIAGNOSIS

In case of Metachromatic leukodystrophy (MLD), diagnosis is often missed or delayed because it shares many common symptoms with other prone diseases and MLD itself is a rare disorder. Cerebral palsy or other causes of developmental delay often leads to a state of confusion and makes it difficult to diagnose MLD in young children. Batten disease, attention-deficit hyperactivity disorder (ADHD), or adolescent/puberty-related mood/behavioral changes also cause the same problem in case of older children. In adults, psychological conditions are often suspected. In case of MLD, a variety of tests must be performed to correctly diagnose the disease.

1. MRI

First indicative test that is performed is magnetic resonance image (MRI) of the brain. In MRI, demyelination is detected around the nerve cells which is basically the presence or absence of myelin sheaths around the nerve cells.

2. BLOOD AND URINE TEST

It is a formal diagnosis test for MLD because MRI test is not absolute in nature and abnormal MRI test results may be due to other reasons. It mainly consists of two tests :

- Measuring blood arylsulfatase A (ASA) enzyme levels
- Measuring urine sulfatide levels

Low levels of ASA are also found in a condition called ASA pseudodeficiency so it is not considered as a definitive test for MLD. So the diagnosis of MLD must be confirmed with other tests, including urinary sulfatide measurement. An increased amount of sulfatide in the urine indicates that the ASA enzyme is not breaking down sulfatides.

3. NERVE CONDUCTION VELOCITY TEST

The diagnosis of MLD may also include an evoked potential test and a nerve conduction velocity test. In this test, measurement of nerve stimulation due to brain's activity shows a delayed or impaired response because of the demyelination that occurs in MLD. In case of nerve conduction velocity test, the speed of signals which travels along the nerves is measured. It only measures electrical signals along the nerves in the arms and legs. A slower-than-normal signal may indicate loss of myelin, suggesting MLD.

4. DNA TEST

Diagnosis of MLD also includes molecular analysis, also called DNA testing. In this test, DNA profiling especially for ARSA gene is done and compared with normal one.With the help of this test, ARSA allele, responsible for MLD can be identified. This can be useful if the patient has a family history of MLD.

5. SCREENING TEST

Screening test is more popular to diagnose many genetic disorders nowadays. In this test disease can be diagnosed with the help of single drop of blood. It is more efficient test as compared to other one but it is not available for diagnosis of MLD. It is still in development phase.

3.11 TREATMENT

There is no effective treatment or cure available for MLD. Present care focus on treating symptoms and preserving the patient's quality of life. Currently treatment is only limited to pain and symptom management. However, there are two options available for Presymptomatic late infantile MLD patients, as well as those with juvenile or adult MLD, displaying mild to moderate symptoms.

- Bone marrow transplantation
- Stem cell transplantation

Even these two methods are in developmental phase and under investigation to see if it may slow down progression of the disease or stop its progression in the central nervous system.

- Gene therapy,
- Enzyme replacement therapy (ERT),
- Substrate reduction therapy (SRT), and
- Potentially enzyme enhancement therapy (EET)

These four methods may have future potential to cure or better management of the MLD. These methods are in process of investigation.

3.12 PROGNOSIS

Metachromatic Leukodystrophy is a progressive disorder and gets worsened over time. Eventually, patient loses all mental and muscle functions. Life span is variable for every individual and depends on at what age the condition has started. Course of the disease usually runs three to twenty years or more.

3.13 PREVENTION

If person have a family history of MLD, Reproductive planning in conjunction with genetic counseling is principle method for the prevention of metachromatic leukodystrophy. Carrier of MLD may be identified with the help of arylsulfatase A deficiency assay [Zlotogora *et al.* 1980], and affected fetus can be identified prenatally [Eto *et al.,* 1982]. Prenatal diagnosis and carrier identification is necessary to distinguish metachromatic leukodystrophy from the benign pseudodeficiency condition. Mutation analysis provides the most accurate method for prenatal diagnosis and carrier identification and helps in preimplantation genetic diagnosis [Braude *et al.,* 2002].

4. METHODOLOGY

In an effort to understand the interaction between various genes and gene pathways which are involved or getting affected in Metachromatic Leukodystrophy, the gene-gene interaction network map was built using Cytoscape and the network was analyzed using various plug-ins in Cytoscape. The genes responsible for MLD from the various databases were searched. Journals with published literature and reports on MLD were also referred. There are around 70 genes interacting with the candidate genes (ARSA and PSAP), found through literature and various database searches. In this study fallowing software and their corresponding applications was used to create and analyze the protein interaction network for MLD.

4.1 CYTOSCAPE

Cytoscape is an open source software platform for integrating and visualizing bimolecular interaction networks with high-throughput expression data, and integrating these networks with annotations and other state data into a unified conceptual framework. Cytoscape was designed at the Institute of Systems Biology in Seattle in 2002. Now, it is developed by an international consortium of open source developers [Paul Shannon *et al.*, 2003]. Cytoscape was initially made public in July, 2002 (v0.8).As of March 2013, there are three versions of Cytoscape: Cytoscape 2.x, Cytoscape 3.x, and cytoscape.js. Cytoscape 2.x is a stable, production version of Cytoscape. In current study we have used Cytoscape version 2.8.3.

Cytoscape's base is in Systems Biology, where it is used for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data. It is a project dedicated to building open-source network visualization and analysis software. A software "Core" provides basic functionality to layout and query the network and to visually integrate the network with state data. The Core is extensible through a plug-in architecture; align rapid development of additional computational analysis and features [Raman 2010].

A network (graph), which includes genes, proteins, and molecules which are represented as nodes and interactions represented as links, i.e. edges between nodes, is the central organizing metaphor of Cytoscape. Cytoscape is continuously progressing, both in core feature set and developer community. Molecular networks can be effectively visualized by

using various Cytoscape applications. In order to reduce the complexity of network, subsets of nodes and edges can be selected according to variety of criteria.

4.2 MiMI PLUG-IN

This plug-in is a java based interactive visualization tool for analyzing protein interactions and their biological effects. It is freely available in public domain. It integrates Cytoscape with MiMI database, a database that uses an intelligent deep-merging approach to integrate data from multiple protein interaction database. The MiMI database has data on 119,880 molecules, 330,153 interactions, and 579 complexes. The MiMI plug-in communicates with the MiMI database using a PHP server that acts as a database interface. The interface is implemented using stored procedures to modularize the code and avoid embedding of SQL queries in PHP scripts. In this way, the database query is isolated from the Cytoscape MiMI plug-in. This allows changes and upgrades to be made to the database without affecting the MiMI plug-in [Jing Gao *et al.,* 2009]. MiMI plug-in allows to access the integrated molecular data assembled in the database and retrieve graphics which contains protein/gene interactions.

4.3 BiNGO (The Biological Networks Gene Ontology Tool)

Among various Gene Ontology categories, in order to determine which are significantly statistically overrepresented in a cluster of genes or sub-graph of biological network, this java based plug-in is used. The functional themes of a gene cluster on the GO hierarchy can be mapped using BiNGO and by taking advantage of Cytoscape's versatile visualization environment BiNGO returns this mapping as a graph. Sub-graphs of biological networks or list of genes pasted as text can be used as input.

BiNGO features include:

- Assessing underrepresentation or overrepresentation of GO categories
- Hypergeometric or binomial test for overrepresentation
- Batch mode : analyze several clusters simultaneously using same settings
- Multiple testing correction using Bonferroni (FWER) or Benjamini&Hochberg (FDR) correction
- Wide range of organisms

4.4 NETWORK ANALYZER

Molecular interactions through statistical analysis of a network can be studied with the help of this java based visualization plug-in. This plug-in provides overall network topology via computing following parameters:

- Average number of neighbors
- Number of connected pair of nodes
- Node degrees
- Average clustering coefficient
- Topological coefficient
- Shortest path lengths
- Betweenness centrality
- Closeness centrality
- Stress centrality

It plots distribution curves based on Correlation coefficient and R-square value and these results can be saved as images or text files.

4.5 ClusterONE (Clustering with Overlapping Neighborhood Expansion)

ClusterONE, available as a standalone command-line application, or as a plug-in to Cytoscape**,** is a graph clustering algorithm. It is mainly useful for detecting protein complexes in protein-protein interaction networks with associated confidence values. It has the ability to handle weighted graphs and it can also generate overlapping clusters.

ClusterONE works by "growing" dense regions out of small seeds (typically one or two vertices), driven by a quality function called cohesiveness. The network quality can be assessed by the number of internal edges divided by the sum of the number of internal and boundary edges. This quality measure is based on the fact that a well-defined network should have many internal edges and only a few boundary edges. This quality measure is known as cohesiveness.

ClusterONE search for groups of high cohesiveness. Greedy algorithm is used to achieve this objective. Single seed vertex (a small set of vertices that are strongly bound together), is selected and step by step group is extended with new vertices addition so that the newly

added vertex always increases the cohesiveness of a group as much as possible. If removing a vertex from the group increases its cohesiveness, it is also permitted. When it is not possible to increase the cohesiveness of the group by adding another external boundary vertex or removing an internal boundary vertex, the process terminates.

4.6 CYTOHUBBA

Due to the availability of plenty of high throughput experimental data, one of the critical issues of post genome era is to reconstruct the proteomic and genomic interacting networks. Identification of important nodes or hubs in these interactomes is a key to explore significant characteristics inside biochemical pathways or complex networks. These essential nodes/hubs may serve as potential drug-targets for developing novel therapy of human diseases [Chung-Yen Lin *et al.*, 2008].

We can explore important nodes/hubs and fragile motifs in an interactome network by several topological algorithms such as:

- Degree
- Edge Percolated Component (EPC)
- Maximum Neighborhood Component (MNC)
- Density of Maximum Neighborhood Component (DMNC)
- Maximal Clique Centrality (MCC)
- Bottleneck (BN)
- EcCentricity
- Radiality
- Betweenness
- Stress

Since the analysis methods of hubba are based on topology, it can also be used on other kinds of networks to explore the essential nodes.

4.7 VENN AND EULER DIAGRAMS

This is a java based data visualization tool which provides a diagram view and a detailed view for comparing two or more Cytoscape groups at a time. It consists of a symmetrical layout that supports two to four Cytoscape groups. For two and three Cytoscape groups,

Venn diagram views use two and three circles respectively. For four or more than four Cytoscape groups, a symmetrical construction of four ellipses is used. The Euler diagram view uses an area-proportional layout that supports two or more Cytoscape groups. It is similar to a Venn diagram but does not display all possible intersections.

4.8 GENETIC ASSOCIATION DATABASE (GAD)

Human genetic association studies of complex diseases and disorders has compiled in Genetic Association Database. It is freely available in public domain. Summary data extracted from published papers in peer reviewed journals on candidate gene and GWAS studies are assembled in GAD. The objective of database is rapid identification of medically relevant polymorphism from the large volume of polymorphism and mutational data, in the context of standardized nomenclature.

Additional molecular reference numbers and links are also integrated with study data and recorded in the context of official human gene nomenclature. Characteristic feature of this database is that, It is gene centered, each record is a record of a gene or marker. It is freely available, anyone may access this database and anyone may also submit records.

There are three main components of GAD:

- Web interface,
- Perl modules and
- Database, which uses the Oracle RDBMS.

The database has three layers; gene and disease data are organized into a large fact table in a middle layer with dimensional views on the top layer. The bottom layer contains the tools for adding, editing, batch loading and downloading data to and from the database [Kevin G Becker *et al.*, 2004].

4.9 THE DATABASEFOR ANNOTATION, VISUALIZATION AND INTEGRATED DISCOVERY (DAVID)

High throughput genomics, proteomics and bioinformatics approaches have generated a huge amount of data. To extract valuable information from this data, functional annotation of differentially expressed genes is a necessary and critical step in his era. Biological knowledge is distributed in nature and frequently requires researchers to navigate through numerous web-accessible databases gathering information one gene at a time. DAVID has provided a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes.

Characteristic features of DAVID:

- Convert gene identifiers from one type to another.
- Identify enriched biological themes, particularly GO terms.
- Cluster redundant annotation terms.
- Discover enriched functional-related gene groups.
- Visualize genes on BioCarta & KEGG pathway maps.
- Display related many-genes-to-many-terms on 2-D view.
- Search for other functionally related genes not in the list.
- Link gene-disease associations.

Tools:

- Functional Annotation
- Gene Functional Classification
- Gene ID Conversion
- Gene Name Batch Viewer

DAVID Analyze results and the graphical displays remain dynamically linked to primary data and external data repositories, thereby furnishing in-depth as well as broad-based data coverage. The functionality provided by DAVID accelerates the analysis of genome-scale datasets by facilitating the transition from data collection to biological meaning [Dennis G Jr,*et.al*,2003].

In this study, we collected and manually curated over 40-50 review and other articles involved in Metachromatic Leukodystrophy and built a protein interaction network using Cytoscape 2.8.3. Various plug-ins were used to analyze manually curated network. All the associated biological pathways such as metabolic, lysosome-acid hydrolases, glycosphingolipid metabolism etc have been studied. This study was aimed at getting a better insight into Metachromatic Leukodystrophy pathogenesis and identifying the important drug targets for this disorder. Another aim of study was to identify the functional, highly interconnected nodes and bottleneck nodes in the network, identifying the important sub networks in the system could be helpful in understanding the underlying molecular mechanism of Metachromatic Leukodystrophy. The study was also focused on identification of candidate genes with high centrality score and perform their disease and pathway enrichment analysis to find out the disease classes in which these genes are involved and the pathways they are affecting. An additional aspect of study was chromosome enrichment analysis of candidate genes to calculate the distribution of genes across different chromosomes. The objective of this study was to build the network using Cytoscape and analyze the statistical significance of the network, and using its plug-ins, the important nodes (genes) which could be functional, were found.

4.10 CONSTRUCTION AND ANALYSIS OF THE MLD RISK GENES NETWORK

Metachromatic leukodystrophy (MLD) is inherited in an autosomal recessive pattern and is caused by deficiency of enzyme arylsulfatase-A (ARSA), or, more rarely, of its activator protein saposin-B (PSAP). Mutations in the ARSA and PSAP genes result in a decreased ability of enzyme arylsulfatase-A (ARSA), and protein saposin-B to break down sulfatides, resulting in the accretion of these substances in cells. High quality interactions with a confidence score of 0.7 including all the types of associations between the ARSA and PSAP with other genes and proteins were considered. Some of the interacting partners were not given in STRING and hence other databases and literature was thoroughly searched for the possible interactions. Genes extracted from various databases are given in **Table 3** and which are taken from literature are given in **Table 4** with their Pubmed IDs. Other databases such as KEGG, PubMed, BIND, DIP, MINT and BioGRID were also searched. To avoid false

positive results which may have obtained while using text mining procedures, PolySearch [Dean Cheng *et al.*, 2008] and Phenopred [Radivojac P *et al.*, 2008] tools were also used. Thereafter MiMI plug-in in Cytoscape was used to build gene-gene interaction network taking these 70 genes as input. The direct or close interactions of these query genes with the nearest neighbors were found. The interaction data was processed with the help of Oracle 10g database software and were saved in excel sheet which was then imported in Cytoscape 2.8.3 for further analysis. The top 20-30 genes obtained from the scoring were selected and analyzed individually for their association with MLD related scientific evidences in literature. The interaction search was restricted to *homo sapiens* and included all the kinds of experimental as well as predictive interactions. The curation of final database was performed manually to remove the duplicate interactions and uniform isoform notations with unique genes. The final network thereafter obtained consisted of 1050 nodes and 8019 edges. **Figure 9** showing the dialogue box for MiMI plugin.

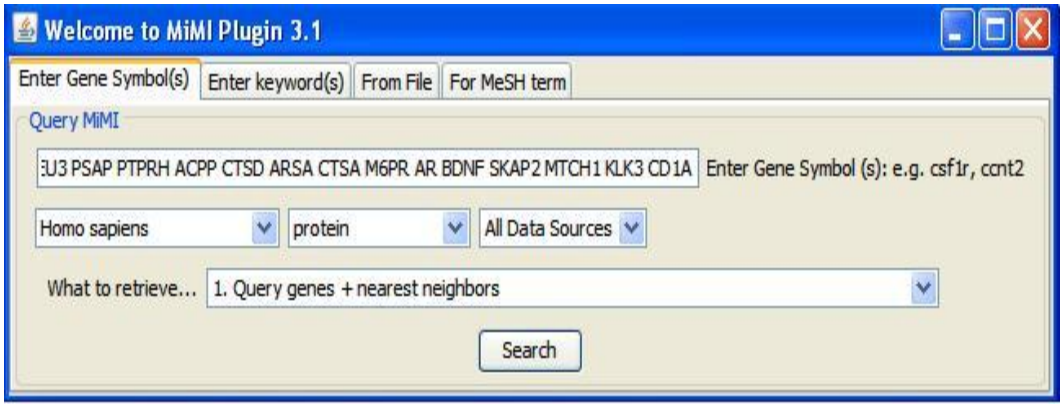


Figure 9: The dialogue box of MiMI plug-in with the query genes and type of information retrieval is shown.

Table 3: Genes known to interact with ARSA and PSAP taken from databases.

GENES	DATABASE
BMPR2, BRCA1, CD1A, CTSL1, SMAD2, SMAD9, SMAD9	HPRD
CETN2, CFTR, CLC4G, CLEC4G, COPS6, PPIC, SGCG	INTACT
GHR, UBC, UBE3A, VCP	PREDICTOME
ACPP, ALAS2, ARSD, ARSE, ARSF, ARSH, ARSI, ARSJ, ARSK, ASNA1, GAL3ST1, KLK3, MAFF, MTCH1, NEU1, NEU2, NEU3, NEU4, SGK223, SKAP2, SLURP1, UGT8, ZBED1	STRING

Table 4: Genes known to interact with ARSA and PSAP with their PMID's.

GENES	PMID
AR	PMID: 17044040
ARSA	PMID: 2320574
ARSB	PMID: 22216298,PMID: 4698279
ARSG	PMID: 18283100
ASAH1	PMID: 11104761
BDNF	PMID: 9748612
CD1B	PMID: 14716313
CD1D	PMID: 14716312
CTSA	PMID: 8206147 ;PMID: 18480170 ;PMID: 10484606
CTSD	PMID: 23108186
DEGS1	PMID: 19471889
GALC	PMID: 2717620, PMID: 21709,PMID: 19267410
GBA	PMID: 8206997
GBAP1	PMID: 17353235
GLA	PMID: 19267410
GLB1	PMID: 2320574, PMID:36575,PMID: 22216298
GM2A	PMID: 20510729
GUSB	PMID:6766092
M6PR	PMID: 17848585
PSAP	PMCID: PMC53725,PMID: 7866401 ,PMID: 2764035
PTPRH	PMID:8311994,PMID: 2320574,PMID: 1689485
SMPD1	PMID: 11856752

SORT1	PMID: 20571055, PMID: 19732768
STS	PMID:15561802,PMID: 4363968
SUMF1	PMID:17080200,PMID: 15962010
TFPI2	PMID: 21943334
TPX2	PMID: 15948238
TSG101	PMID: 22315426
VPS4A	PMID: 12915562

4.11 ANALYSIS OF THE INTERACTION NETWORK

Once the network was obtained for MLD it was analyzed for it topology and biological significance. To confirm the presence of molecular systems structures that may better explain missing linkage in the mechanism of MLD, systems biology approach was used to study the genetic risk gene networks as a whole rather4 than the risk genes individually. Till date only two candidate risk genes (ARSA and PSAP) are known in causing MLD. It was only imperative to study the system using the available bioinformatics resources and tools available. Cytoscape's base is in Systems Biology, where it is used for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data. The software core is extensible through a plug-in architecture; align rapid development of additional computational analysis and features [Raman 2010]. In the current study we have used cytoscape version 2.8.3.

Network Analyzer, a java based freely available Cytoscape application for the analysis of various kinds of networks. It can be used for both directed and un-directed kinds of networks. It is a statistical tool to compute various topological network parameters. Parameters computed by this plug-in can be characterized in two groups given below in **Table 5**:

Table 5: List of parameters computed by Network Analyzer

Simple Network Parameters	Complex Network Parameters
Number of connected components	Betweenness centrality
Parameters related to shortest paths	Closeness centrality
Parameters related to neighborhood	Degree distribution
Clustering coefficient	Neighborhood connectivity
	Shortest path
	Stress centrality
	Topological coefficient

These parameters obtained are further discussed in results and discussion section and provided us comprehensively about the topology of the network obtained. The understanding of network topology of a biological network can directly give an insight into various network characteristics. Further cluster analysis was carried out which provided a better topology description of the network including the location of highly connected sub-graphs and/or overlapping modules that usually correspond with relevant biological information. Cluster analysis was done using ClusterONE**.** Cluster ONE strives to discover densely connected and possibly overlapping regions within the Cytoscape network. These dense regions usually correspond to protein complexes or fractions of them. Cluster ONE works by "growing" dense regions out of small seeds (typically one or two vertices), driven by a quality function called cohesiveness. Highly inter connected nodes in the network were obtained suggesting their role in the pathogenesis of the disease. Density threshold was set 0.5 for unweighted networks. The network was un-weighted and the global clustering coefficient was set smaller than 0.1. The node penalty value was set to 2, that excludes the weaker connections in the network and thus were not added to the cluster.

Proteins with special graph (network) characters in an interactome may play critical roles in controlling or regulating cellular responses to a special physiological stimulus. For the present study cyto-Hubba (Hub Objects Analyzer) plug-in was used to explore important nodes/hubs in an interactome based on previous framework, Hub object Analyzer. To identify essential nodes/hubs the protein networks is a way to decipher the critical key controllers inside biochemical pathways or complex networks. These essential nodes/hubs may serve as candidates of drug-targets for developing novel therapy of human diseases.

Several centrality indexes were evaluated using CytoHubba. A normalized centrality score was calculated using ten centrality indexes Betweenness, Bottleneck, DMNC, MNC, MCC, EPC, Eccentricity, Radiality and Stress. To obtain normalized centrality score (Score *I)* following formula was used:

$$\text{Score}I = \sum_{i=1}^{Nc} \left(1 - \frac{\max(Ici) - Ici}{\max(Ici) - \min(Ici)}\right).100$$

Where Ic_i is the values of centrality indexes and i=1...Nc, and is the number of calculated centrality indexes (N= 10). Through this analysis all 1050 genes were ranked according to their score I value.

4.12 STATISTICAL SIGNIFICANCE TESTING OF MLD RISK GENE PROTEIN NETWORK

Biological Network Gene Ontology (BiNGO) [Maere S *et al.*, 2005], a plugin in Cytoscape was used to identify ontologies in the gene protein network. The overrepresented processes were assessed using hypergeometric statistical test. A FDR multiple hypothesis test adjustment was carried out using the Benjamini Hochberg (BH) procedure Benjamin and Hochberg false discovery rate (FDR) correction with a significance level of 0.05. BiNGO analysis identified three ontologies (cellular component, molecular function and biological processes).

4.13 GENE FUNCTIONAL CLASSIFICATION AND FUNCTIONAL ENRICHMENT ANALYSIS USING DAVID

To analyze a biological network, it is essential to concentrate on the larger biological network rather than at the level of an individual gene. DAVID gene functional classification tool was used to explore the functionally related genes together on the basis of their annotation term co-occurrence. The Uniprot IDs of 126 candidate genes were given as input in DAVID. The DAVID gene ID Conversion Tool was used to convert the gene IDs to relevant gene names with biological annotation in the DAVID database. 120 out of 126 genes were recognized and converted to DAVID id from their Uniprot IDs. To control the behavior of DAVID fuzzy clustering, the classification stringency was set to medium for balanced

results [Da Wei Huang *et al.,* 2008]. The Kappa Similarity Term Overlap was set to 4 and Kappa Similarity Threshold was set to 0.35. Six clusters were obtained out of which four were analyzed further with an enrichment score > 1.3. The highly interconnected clusters were further explored by Gene ontology (GO) analysis.

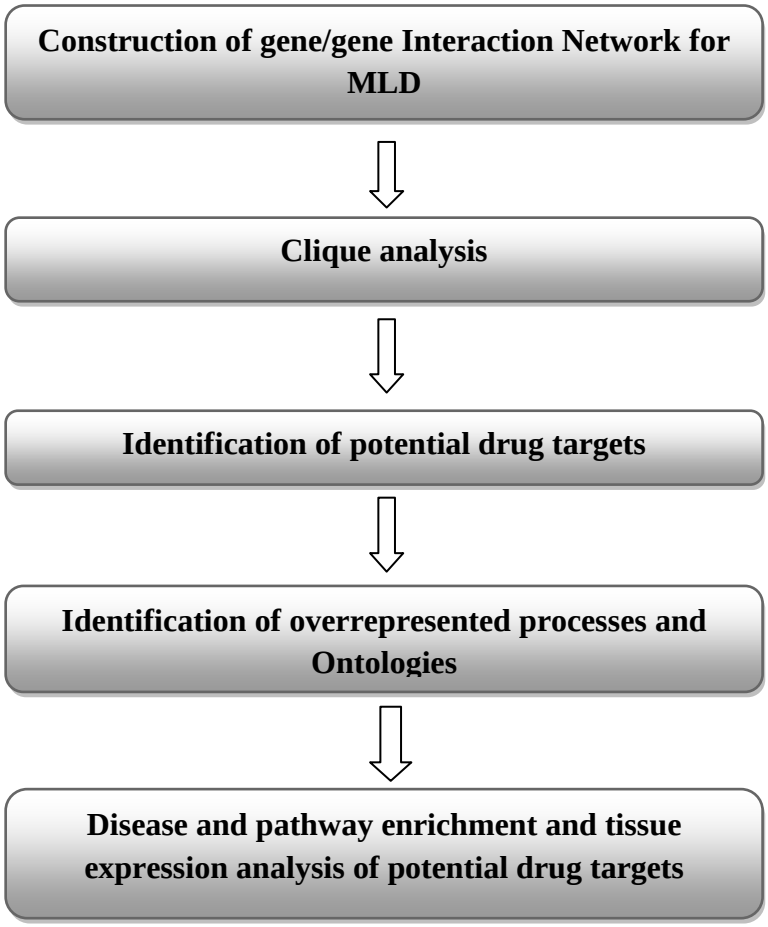
4.14 PATHWAY AND DISEASE ENRICHMENT ANALYSIS

To further analyze the results and explore the functional connections, 153 genes were mapped onto known gene sets. For this purpose DAVID was used to search for enriched KEGG pathways. To carry out the disease enrichment analysis DAVID extensively uses the genetic disease related databases such as Genetic Association Database (GAD) and OMIM. In addition a substantial statistical description in regards to the wide range of enrichment analysis has also been provided using DAVID. The analysis was carried out considering all the genes/proteins involved in the pathogenesis of MLD which were obtained from cluster and hub analysis using Cytoscape as well as proteins/genes which were found through literature.

4.15 TISSUE GENE EXPRESSION AND CHROMOSOME ENRICHMENT ANALYSIS

Gene activation patterns vary widely in complexity. While some genes are known to express in a straightforward and static manner, some are extraordinarily intricate and difficult to predict and model, with expression fluctuating wildly from minute to minute or from cell to cell [Shestopalov *et al.,* 2011]. Tissue gene expression profile and chromosome enrichment analysis have been widely used to study disease states. Tissue-specific gene expression profiles and chromosomal distribution of genes was studied through DAVID and GAD respectively. The analysis was carried out considering all the genes/proteins involved in the pathogenesis of MLD which were obtained from cluster and hub analysis using Cytoscape as well as proteins/genes which were found through literature.

The Overall work flow for gene interaction network and further analysis of network is shown below:



5. RESULTS

A ring like lattice network was obtained with 1050 nodes representing genes and 8019 edges representing the interactions between these genes. Edge Weighted Force Directed (BioLayout) network is shown in **Figure 10**. The Figure shows most of the nodes (genes/proteins) are present at the central core in the network while few nodes are lying at the periphery with lesser number of connections.

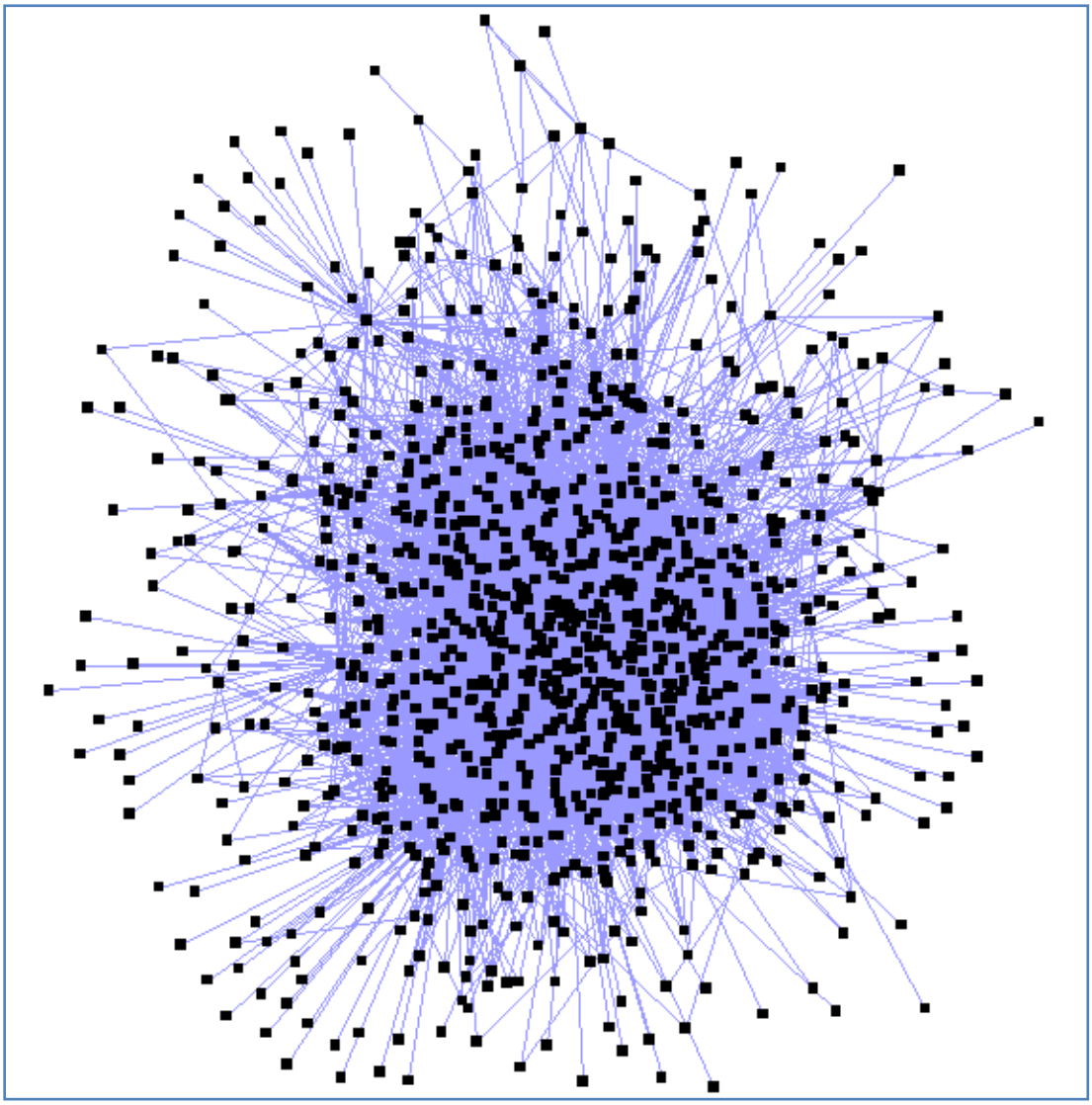


Figure 10: Protein interaction network having 1050 nodes and 8019 edges showing a ring-like lattice.

The candidate genes which are responsible for MLD were searched from published literature and their interactions were found from DIP, HPRD, BIND, STRING, MINT, IntAct, PUBMED, STRING, and Reactome databases. Journals with published literature and reports on MLD were also referred. There are around 70 genes responsible for this multiple system disease in human beings.

Literature survey and various databases search like HPRD, KEGG, etc was performed to get an insight about various disorders caused by mutations in genes interacting with ARSA and PSAP. The genes which play important role in causing disorders in different biological systems are shown in **Figure 11**. In MLD, Central Nervous System, Reproductive System, Respiratory System and Circulatory System with various other systems were found to be affected (**Figure 11**). MLD is known to be a neuro-degenerative disorder, the genes which are expressed in Nervous System were found to be highly affected. Genes associated with particular disorder are shown in corresponding color.

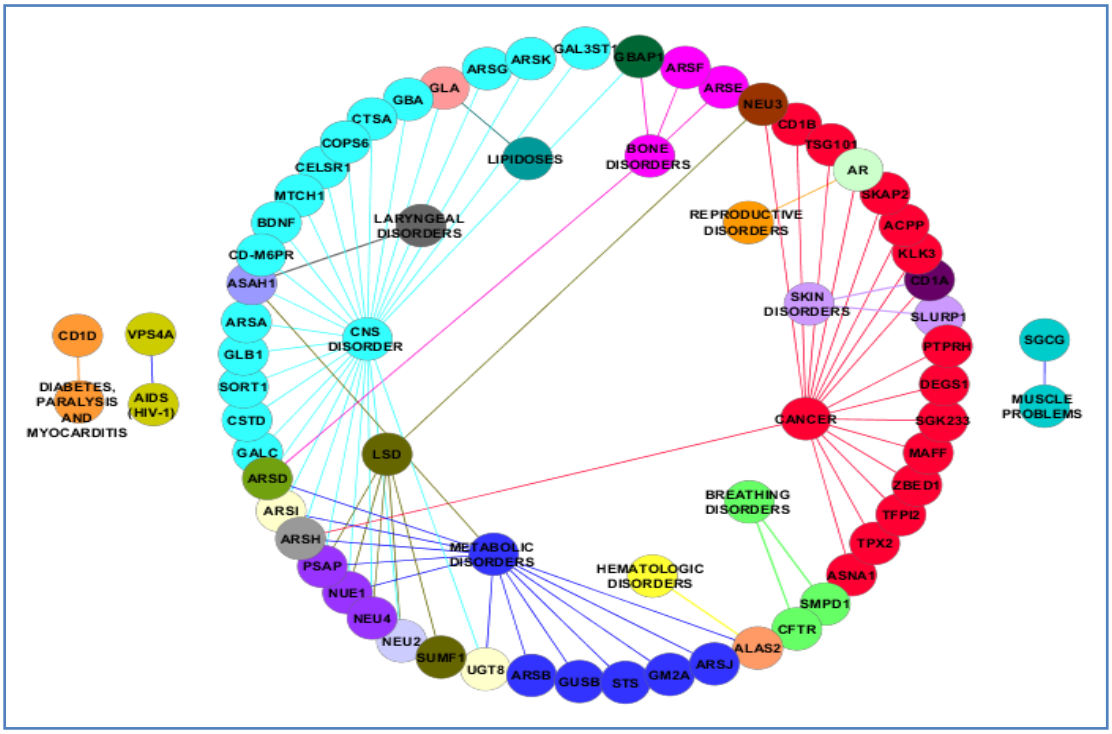


Figure 11: Disorders caused by genes interacting with ARSA and PSAP are shown. Out of 71 only 57 genes are found through literature and various databases search like HPRD, KEGG, etc.

The biological gene interaction network and its topological properties were studied and analyzed using Network analyzer. The analysis of network was done treating it as an undirected graph. For the network obtained, the overall calculated clustering coefficient is 0.288 indicating very limited number of connections in the neighborhood but nodes may be distantly connected. Centralization gives important information about how central the network's most central node is in relation to how central all the other nodes are. The value 0.253 of centralization indicates that the network topology have a few characteristic features of star topology. Network heterogeneity value 1.45 represents that network has tendency of having hubs (essential) nodes. The important hubs were identified in the network. In order to obtain these hubs the node degree value was taken into consideration. Nodes having degree more than 150 were considered and the genes which were found with larger node degree are TAF1(276), HNF4A(210), SMAD2(195), BRCA1(178), AR(163). Through literature study it was found that PSAP increases the activity of few of these genes [Koochekpour S *et al.*, 2007]. Any mutation in PSAP gene may cause the dysfunction of these genes. We were able to identify the significant genes which could play important roles in the underlying mechanism of MLD. The decreasing trend of neighborhood connectivity indicates that network is dominated by edges between low and highly connected nodes. When the power law curve function was fitted ($y=ax^b$) to closeness centrality and degree distribution, it is transformed into linear model ln y= ln b + a * ln x. A correlation value of 0.806 and R-square value 0.626 was obtained for closeness centrality. Similarly, for degree distribution the correlation value obtained was 0.779 and R-square was 0.852. The significant value of correlation and R-square shows that these two variables are negatively correlated and power law function is best fitted to this node degree distribution data which is the characteristic feature of Small world network model and can be seen in **Figure 12 and Figure 13**.These results signify the accuracy of the obtained network and imply that the network is highly significant and can be considered for further analysis.

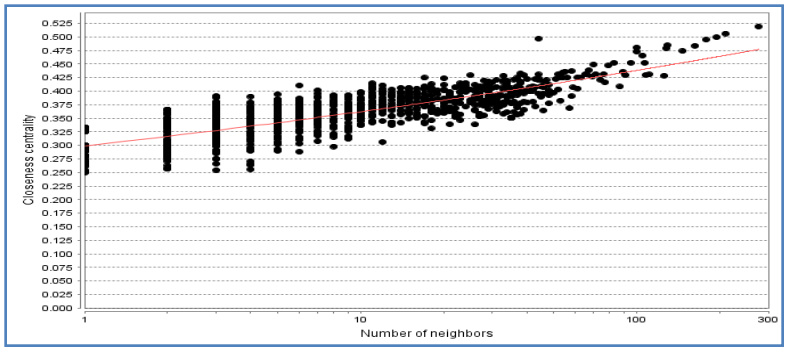


Figure 12: Linear model of power law function between closeness centrality and number of neighbors

The stress centrality parameter was visualized to observe the nodes which have greater stress implying larger number of edges passing through those nodes. The nodes having high stress were found: TAF1 (1772108.0), HNF4A (1202948.0), SMAD9 (107708), BRCA1 (989502.0), indicating that these nodes travel by high number of shortest path. It was found that PSAP node is connected through TAF1, SMAD9 and BRCA1 but ARSA is not connected to these stressed nodes.

We had calculated following parameters to determine the overall topology of network:

- Power law node degree distribution

$$p(k)=k^{\alpha}$$

- Characteristic path length

$$L=\log N$$

When the power law curve function was fitted ($y=ax^b$) to degree distribution, it is transformed into linear model ln y= ln b + a * ln x with a correlation value of 0.779 and R-square of 0.852 (**Figure 13**).

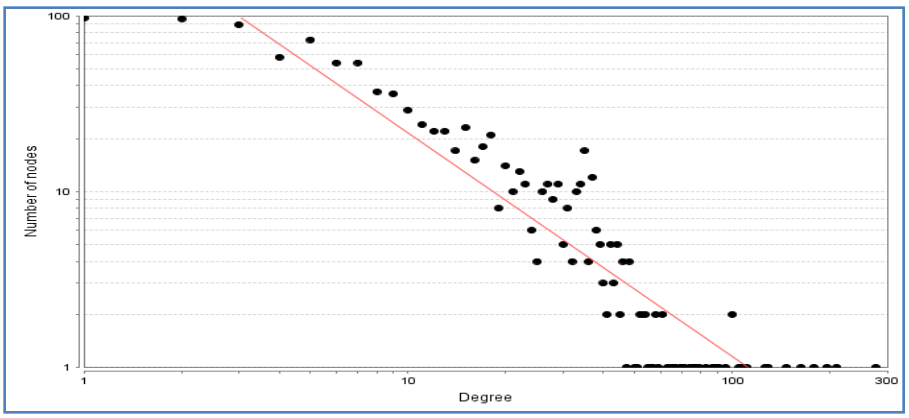


Figure 13: Linear model of power law function between degree and number of nodes**.**

The significant value of correlation and R-square shows that these two variables are negatively correlated and power law function is best fitted to this node degree distribution data which is the characteristic feature of Small world network model.

5.1 CHARACTERISTIC PATH LENGTH

We have calculated characteristic path length using the formula "Log N" which is a feature of small world network model [Danielle Smith Bassett and Ed Bullmore, 2006].

We have also compared this value with the value obtained from Network analyzer.

- Characteristic path length from network analyzer = 2.840
- Characteristic path length of Small World network model = log N
  = log (1050)
  = 3.02

The values obtained from network analyzer and the computed values are almost comparable.

Our network clearly resembles the Small world network model which was determined from power law degree distribution, characteristic path length, ring lattice like network. Additionally, it was also observed that the individual nodes were found to have few neighbors and most nodes can be reached from one another through few steps.

Cluster analysis for an interaction network is a crucial step in network analysis. Densely connected regions in graphs are most frequently identified by some unsupervised clustering method. However, standard clustering is not ideal for PPI networks: proteins may have multiple functions, and therefore the corresponding nodes may belong to more than one

cluster [Tamás Nepusz *et al.,* 2012]. Thus ClusterONE [Fan S *et al*., 2012] was used to identify the clusters with highly interconnected nodes. As a result, 145 clusters were obtained from which the cluster with density 34.436 and 126 nodes with 3.208E-9 p-value was selected for further analysis. Further study of the cluster showed that SMAD9, PSAP, BMPR2, ARSA, UBE3A genes were highly interconnected in the cluster and shown in **Figure 14**.

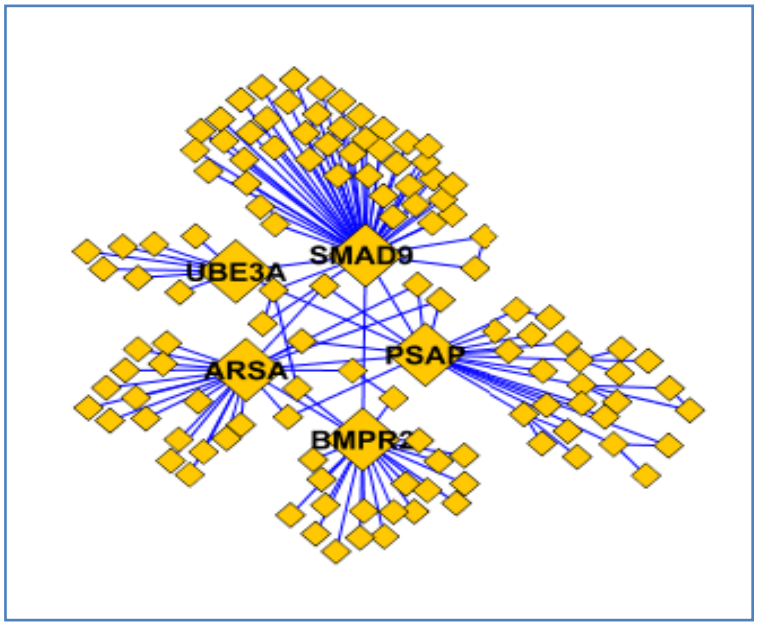


Figure 14: Cluster of 126 nodes representing highly interconnected genes

Disease-causing aberrations in the normal function of a gene define that gene as a disease gene. Proving a causal link between a gene and a disease experimentally is expensive and time-consuming. Comprehensive prioritization of candidate genes prior to experimental testing drastically reduces the associated costs. Computational gene prioritization is based on various pieces of correlative evidence that associate each gene with the given disease and suggest possible causal links. Candidate gene identification and prioritization of genes was done using Cytohubba plug-in in Cytoscape to find out essential nodes in the network which are also known as hub nodes or essential nodes. There are a number of methodologies available for hubs and essential genes identification, and all of them are available with the respective advantage and limitations [Chung-Yen L *et al.,* 2008, Kong W *et al.,* 2011, Mishra H *et al.,* 2011, Rio G *et al.,* 2009]. Some strategies are the use of genetic algorithm or machine learning procedures, however, the centrality approaches are by far the most applied

procedures even by simplicity and because several studies had being pointed out its applicability. Therefore, several centrality indexes were evaluated to obtain the potential genes: Betweenness, bottleneck, density of maximum neighborhood component (DMNC), eccentricity, maximal clique centrality (MCC),maximum neighborhood component (MNC), node degree, edge percolation component (EPC), radiality and stress [Chung-Yen L *et al.*, 2008]. On the basis of this normalized centrality index score, the top 50 genes with highest score I are considered as potential target genes **(Table 6).**

Normalized centrality index score is the combined percentage value of all ten centrality indexes. In this analysis we found MLD related genes like PSAP and ARSA in addition with other genes with high score I value like TAF1, SMAD2, BRCA1, HNF4A, AR, SMAD9 are also found which may be proved as potential drug targets for MLD (**Table 6**). In this analysis score I obtained for ARSA gene is found to be low as compared to other genes because it does not comprise any bigger hub center in protein network but it plays a critical role in passing information between hub centers.

Two hypothetical genes LOC729948 and LOC730314 were found to be among important genes. It is found that these hypothetical genes are similar to CDC26 subunit of anaphase promoting complex, so it is possible that they may further interact with CDC27, PTTG1, CCNB1, CDC16, ANAPC2, ANAPC16, ANAPC13, ANAPC5, CDC23, ANAPC7. Emphasize should be given to these hypothetical genes because these are found to be among top 50 candidate genes (**Table 6**). These genes should be further explored through Micro array data analysis to check their expression profile with respect to MLD.

Table 6: Top 50 genes obtained according to normalized centrality index.

Gene Symbol	Chromosomal location	Score I	Gene Symbol	Chromosomal location	Score I
TAF1	X	747.5212087	CDC26	9	367.7928177
SMAD2	18	646.9450308	FZR1	19	367.0962171
BRCA1	17	562.4871301	SMAD3	15	366.886843
HNF4A	20	553.005314	LOC729948	7	366.8359585
AR	X	484.9451925	ANAPC4	4	366.2309342
SMAD9	13	419.2151898	LOC730314	7	365.6949616
CDC2	10	416.0214789	CDKN1B	12	365.2963005
RB1	13	415.9210528	ANAPC2	9	364.6175973
UBC	12	415.0906131	ANAPC1	2	364.3057073
CDK2	12	413.5876164	ANAPC7	12	364.2794019
UBB	17	413.5730965	CCNB1	5	361.1748873
PSAP	10	397.6550792	ANAPC11	17	361.0961297
ANAPC10	4	392.6502057	ANAPC5	12	360.2940108
CDC23	5	391.3418829	CDC6	9	356.515173
MYC	8	387.2418442	CDC20	1	356.1679529
MNAT1	14	383.831744	UBE2E1	3	355.9524951
CCNH	5	382.3370698	BUB3	10	355.2756671
CDK7	5	381.3404889	ORC1L	1	355.1152718
CDC27	17	381.2638475	BUB1B	15	354.3007697
TP53	17	379.242666	MAD2L1	4	353.4248162
CDC16	13	377.5941342	PTTG1	5	353.3694199
CDKN1A	6	374.3586452	CDC25B	20	351.9598547
CDC25A	3	371.6090863	ORC5L	7	350.3714013
UBE2D1	10	368.7256054	ORC3L	6	349.6877287
UBE2C	20	368.1183305	PLK1	16	349.5862661

5.2 GENE ONTOLOGY ANALYSIS

GO describes gene and gene product attributes in term of their associated biological processes, cellular components and molecular functions. The size (area) of the nodes is proportional to the number of genes in the test set which are annotated to that node. The color of node represents the (corrected) p-values which are more than 5 order of magnitude smaller

than the chosen significance level. We performed molecular network analysis for the entire network using BiNGO application. From this analysis we found gene distribution across different molecular functional categories like protein binding, catalytic activity, etc (**Figure 15**). Binding node was found to be statistically significant with p-value 7.5649E-42 and 95.0% frequency (**Table 7)**. The binding node encompasses multiple types of molecular interactions, including protein binding, nucleic acid binding and nucleoside binding. The binding node is the most significant node because it encompasses 997 genes of 1050 genes. Out of 1050 genes in the MLD molecular function network, 892 genes are involved in protein binding. The binding node is further linked to nucleic acid binding node (304 genes), and nucleoside binding node (189 genes). Genes responsible for MLD i.e ARSA and PSAP were found to be involved in statistically significant node Binding. The secong ost important node was found to be the catalytic activity node. It was also found that ARSA and PSAP both are also involved in catalytic activity node encompassing 420 genes. However, when molecular function analysis was performed on the cluster with density 34.436 and 126 nodes, catalytic activity was found to be most statistically significant encompassing 63 genes out of 126 genes. Catalytic activity is further linked to statistically significant hydrolase activity node. Hydrolase activity node is further shown to be linked with arylsulfatase activity node. Though the size of the arylsulfatase node is comparatively small but it is statistically significant and is responsible for causing MLD. Further, the node for binding has size larger than catalytic activity but it is not found to be statistically significant but the binding node is further linked to HECT domain binding node which is found to be statistically significant **(Figure 16).**

Table 7: Molecular function analysis of MLD network

GO Description	Frequency	P-value
Binding	95.0%	7.5649E-42
Protein Binding	84.4%	0.0000E-100
Nucleic acid Binding	29.3%	2.0448E-9
Nucleoside Binding	17.9%	4.5978E-12
Catalytic activity	40.6%	1.7005E-6
Transcription regulatory activity	23.2%	1.09333E-36
Enzyme Regulatory activity	8.4%	6.2882E-4

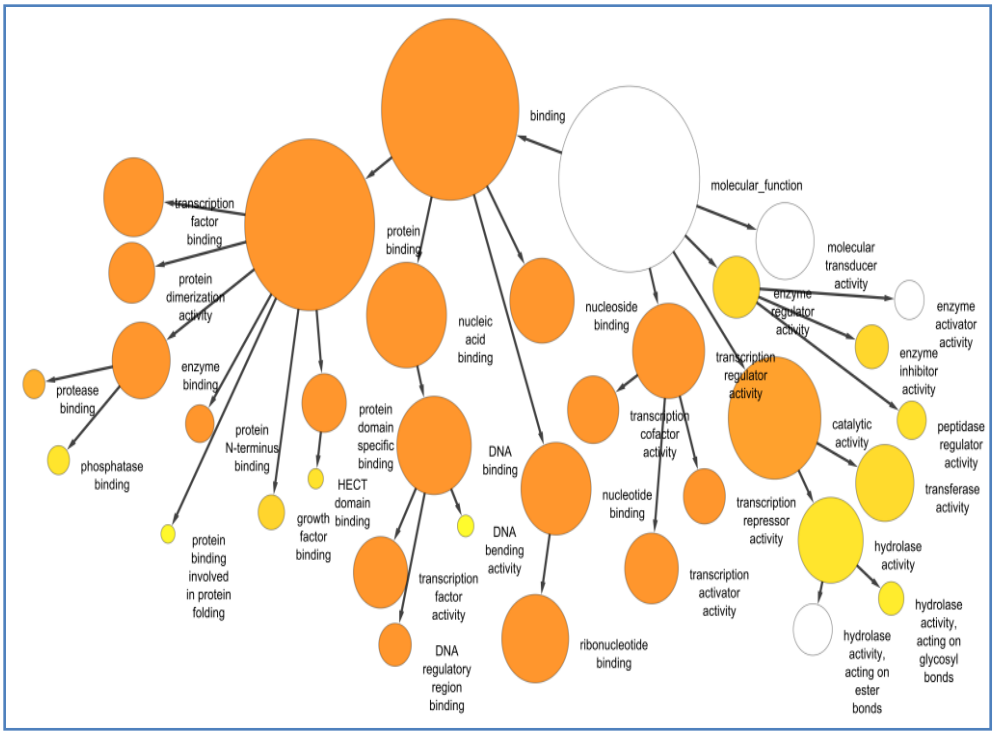


Figure 15: Showing major molecular function categories for MLD network**.**

Most important genes which are responsible for MLD like ARSA and PSAP belong to binding molecular function category. More specifically ARSA is responsible for Arylsulfatase activity, catalytic activity and hydrolase activity and PSAP is responsible for protein binding and enzyme regulatory activity.

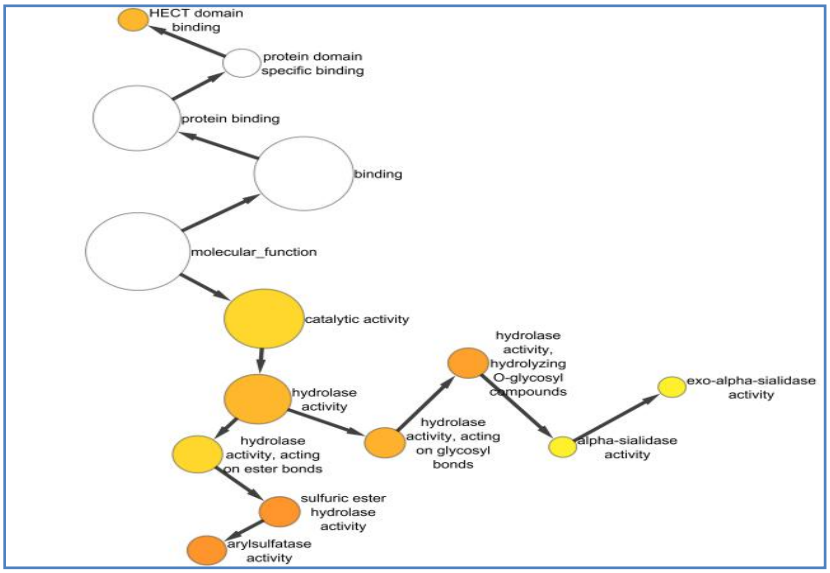


Figure 16: Showing major molecular function categories for ClusterONE result.

It is important to consider that several genes in the network do not present a known relation with specific disease. So in order to identify the disease classes to which these potential genes belong, disease enrichment analysis was performed. Several types of disease classes were found statistically significant in the enrichment analysis. In the GAD database, out of 126 genes, only 88 genes were found and were classified against their major disease classes. Various disease classes were obtained from DAVID, four were considered important for further analysis on the basis of number of genes in each disease class. As expected neurological and metabolic diseases classes were found to be significant in MLD. Surprisingly our results revealed the presence of significant number of genes which may be involved in Cancer pathways. Few of them are TAF1, BMPR2, BRCA1, PSAP, SMAD2. Immune disease class was also found to be significant. Genes such as HNF4A, SMAD2, BMPR2, BRCA1, CTSD were found under this class. A summarized result with the corresponding p-values for each disease subclass is shown in **Table 8.** The genes which are involved in major disease classes are shown in **Figure 17**.

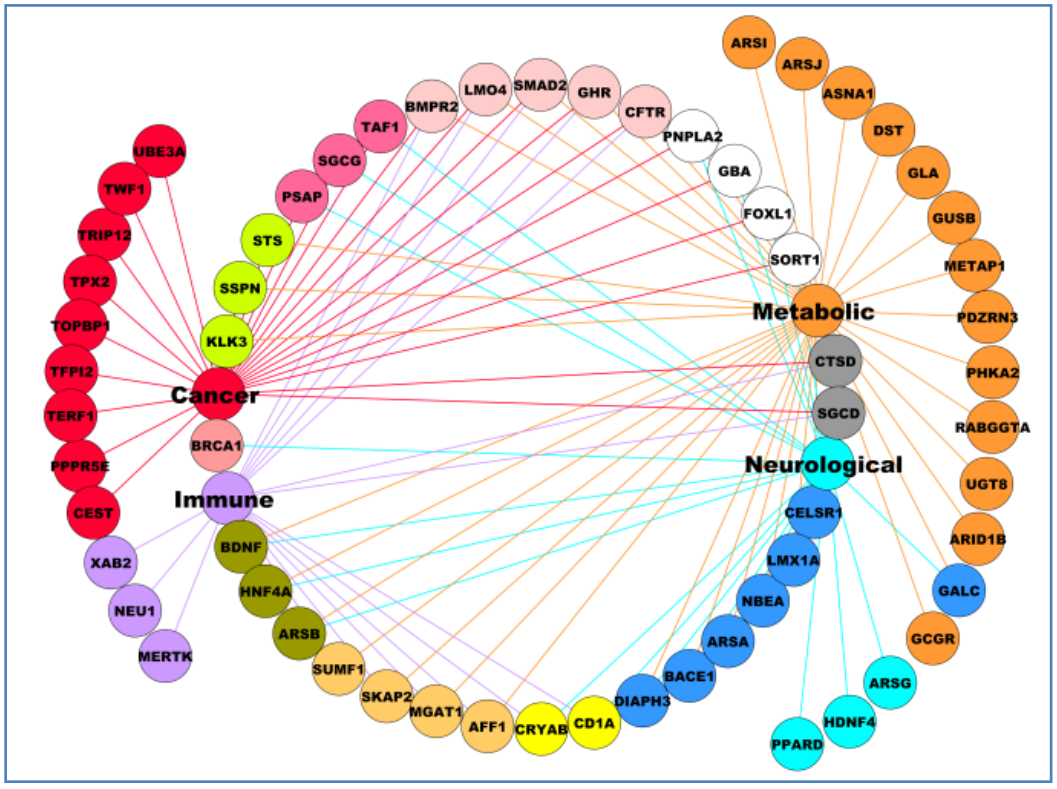


Figure 17: Showing Disease Enrichment Analysis for genes interacting with ARSA and PSAP genes.

Table 8: The disease enrichment analysis

Major Disease classes obtained from DAVID	P-Value	Disease
Metabolic	5.9e-4	Cholestrol, Hdl Body Weight Changes Diabetes Type 2 Lipoprotein,Ldl Triglycerides Bone Mineral Density
Neurological	2.9e-3	Alzheimer's Disease Stroke Parkinson's Disease
Cancer	8.2e-3	Breast Cancer Lung Cancer Prostate Cancer Ovarian Cancer
Immune	1.8e-2	Multiple Sclerosis Human Immuno Monocytes Diabetes Type 1

For better understanding and fine representation of disease enrichment analysis results, Venn and Euler diagram plug-in [Wilkinson L., 2012] in Cytoscape was used. The graphical interface shows the gene distribution among four major disease classes. From this analysis we found that out of 88 genes, 27 are involved in Cancer, 41 are involved in Metabolic, 23 are involved in Neurological and 20 are involved in Immune class (**Figure 18**). The gene distribution shared by various classes is also shown in tabular format (**Table 9**).

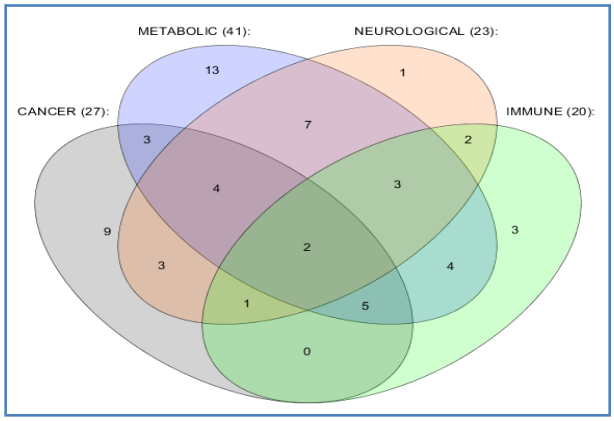


Figure 18: Venn Diagram representing number of common genes involved in various disease classes.

Table 9: Genes shared by various disease classes.

Disease Class	Number of Gene	Gene Name
Cancer + Metabolic	3	KLK3, STS, SSPN
Cancer + Neurological	3	PSAP, TAF1, SGCG
Metabolic + Neurological	7	DIAPH3, LMX1A, GALC, BACE1, CELSR1, ARSA, NBEA
Metabolic + Immune	4	SUMF1, SKAP2, MGAT1, AFF1
Neurological + Immune	2	CRYAB, CD1A
Cancer + Metabolic + Neurological	4	FOXL1, SORT1, GBA, PNPLA2
Cancer + Metabolic + Immune	5	GHR, CFTR, BMPR2, LMO4, SMAD2
Cancer + Neurological + Immune	1	BRCA1
Metabolic + Neurological + Immune	3	BDNF, HNF4A, ARSB
Cancer + Metabolic + Neurological + Immune	2	CTSD, SGCD

From **Table 9** it is evident that one of the major candidate gene, PSAP which is a well known gene in MLD is also involved in cancer disease class.

To calculate the distribution of potential genes across different chromosomes, we performed chromosomal enrichment analysis of top 50 potential genes obtained from normalized centrality score analysis (Score *I*) (**Table 6).** We observed that potential genes are distributed on different chromosomes with chromosome 17 having maximum number of candidate genes implying its importance in the pathogenesis of MLD. There are six genes: ANAPC11, BRCA1, UBB, CDC27, TP53, CDC6 found on chromosome 17 and all are related to cell cycle and ubiquitin mediated proteolysis pathways. 5 genes were found on chromosomes 12 and 5. CDKN1B, ANAPC7, ANAPC5, UBC, CDK2 genes are present on chromosome 12. PTTG1, CCNB1, CDC23, CCNH, CDK7 genes are found on chromosome 5. Interestingly, these genes on both the chromosomes are known to play role in cell cycle and cancer pathways. PSAP, BUB3, CDC2, UBE2D1 genes are found to be present on chromosome 10 and are involved in cell cycle, metabolic pathways and proteolysis (**Table 6)**.

To find out the pathways which are getting affected with the genes involved in MLD network, pathway enrichment analysis was performed through DAVID bioinformatics resource. The analysis was carried out on 126 genes present in the cluster obtained from ClusterONE. Out of 126 potential genes, 120 genes were found in DAVID, which shows DAVID database is the most representative of our gene space and query coverage was around 96%. It was found that Sphingolipid metabolic, lysosomal, proteolytic and ARVC pathways get mostly affected with the genes involved in MLD network (**Table 10**). Genes related to their corresponding pathways are shown in **Figure 2.**

Table 10: Pathway enrichment analysis of potential genes found through DAVID.

Gene Ontology term	Genes involved	Gene Names	%	p-value
Sphingolipid metabolism	14	ASAH1,UGT8,ARSA,DEGS1,GAL3ST1, GLA,GLB1,GALC,GBA,NEU1,NEU2, NEU3,NEU4,SMPD1	9.5	1.2E-15
Lysosome	19	GM2A,ASAH1,ARSA,ARSB,ARSG,CTS A,CTSD,CTSL1,GLA,GLB1,GALC,GBA, GUSB,M6PR,MAN2B1,PSAP,NEU1, SORT1,SMPD1	12.9	7.5E-15
Other glycan degradation	7	GLB1,GBA,MAN2B1,NEU1,NEU2,NEU3 ,NEU4	4.8	3.9E-8
Ubiquitin mediated Proteolysis	8	BRCA1,CUL7,TRIP12,UBE3A,UBE2Q1, UBE2L6,UBE2M,UBE2MP1,UBA6	5.4	2.6E-3
Glycosaminoglycan Degradation	3	ARSB,GLB1,GUSB	2.0	3.3E-2
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4	PKP2,SGCB,SGCD,SGCG0	2.7	8.6E-2

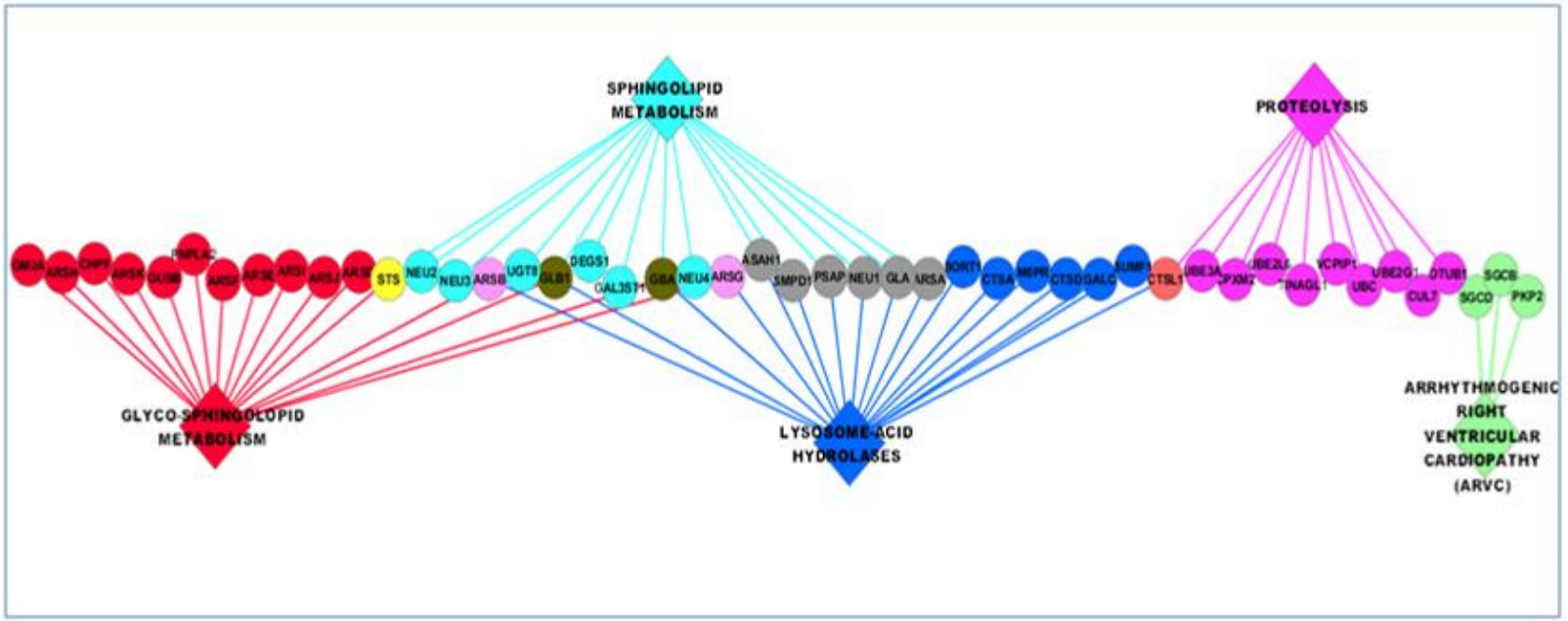


Fig 2 : Figure shows genes involved in major pathways obtained from Pathway Enrichment Analysis using Cytoscape

Tissue gene expression analysis was performed for better understanding of important genes which may express in various organs in MLD. Genes are distributed and are getting expressed mainly in Liver, Placenta, Fetal Brain, Small Intestine, Urine and Nervous System (**Table 11**). Surprisingly, in addition to Nervous system and excretory system, major number of genes were getting expressed in Placenta and Liver.

Table 11: Tissue Gene Expression Analysis Using DAVID.

Term	%	Gene	Gene Name	p-value
LIVER	22.4	33	CLEC4G,ASAH1,SIL1,TPX2,ARSA,ARSB,ARSD,ARSE,ALAS2,CES1,CES3,CTSA,CTSD,CTSL1,GAL,GLB1,GCGR,GBA,GUSB,QARS,GHR,HNF4A,M6PR,MAN2B1,PSAP,PHKA2,SGCB,NEU1,TERF1,SLC9A6,SKAP2,SUMF1,TINAGL1	4.2E-5
PLACENTA	30.6	45	AFF1,COPS6,GM2A,SIL1,SMAD2,ACPP,ALAS2,ARS,BRCA1,CES1,CTSA,CEP135,DEGS1,DST,EXPH5,GLB1,GALC,GCGR,GBA,GUSB,QARS,GHR,HERVV1,MAN2B1,METAP1,MTMR10,PPARD,PKP2,POMGNT1,PPP2R5E,SGCB,SGCD,SLURP1,NEU1,PLEC,UBASH3A,SUMF1,TFPI2,TINAGL1,TSG101,UBE2M,MAFF,MOS,VPS8,ZSCAN4	5.3E-5
FIBROBLAST	4.8	7	ASAH1,GLA,GUSB,NEU1,NEU4,SMPD1,TFPI2	4.2E-4
SMALL INTESTINE	5.4	8	ACTR8,ARHGAP9,ARSA,CHPF,KDM6A,MTMR11,NEU4,MAFF	1.2E-2
FETAL BRAIN	8.8	13	GPSM1,RABGGTA,SMAD9,TAF1,ARSF,CETN2,C10ORF2,DYNLT1,DST,SGK223,PPP2R5E,UBE3A,UBE2G1	1.4E-2
NERVOUS SYSTEM	2.0	3	BRCA1,DST,PSAP	6.7E-2
EXCRETORY SYSTEM	2.0	3	CRYAB,GAL3ST1,UBE3A	7.8E-2
LEUKOCYTE	2.7	4	BDNF,KLK3,M6PR,SKAP2	9.6E-2

6. DISCUSSION

The aim of the study was to built and analyzes the protein-protein interaction network for MLD. With the help of MiMI application in Cytoscape 2.8.3, protein-protein interaction network was built for MLD. The obtained network has Small World Network Model characteristics. Five genes were found to be highly inter-connected, namely SMAD9, PSAP, BMPR2, ARSA, UBE3A from ClusterONE analysis, which may play a major role in pathogenesis for this disease. The main objective behind this study was to identify probable drug targets for MLD. These were found using Cytohubba. The proteins encoded by the identified genes: TAF1, SMAD2, BRCA1, HNF4A, AR, SMAD9, CDC2, RB1, UBC, CDK2, UBB, PSAP, CDC23, MYC, MNAT1, CCNH, CDK7 can be potential drug targets for this disorder. Normalized centrality index score also supports potential gene targets obtained from Cytohubba algorithms.

Molecular function of these genes was studied using BiNGO. It was found that the genes causing MLD (ARSA and PSAP) and other important candidate genes are mostly involved in Binding and Catalytic activity. Major pathways which may be affected in MLD are Sphingolipid Metabolism, Lysosome, Proteolysis, Arrhythmogenic right Ventricular cardiomyopathy (ARVC). Organs like Liver, Small Intestine, Brain and Nervous System may get affected in MLD. Disease enrichment analysis shows that MLD has a possible link with metabolism, immune disorders and cancer.

7. CONCLUSION AND FUTURE PERSPECTIVE

The molecular interaction study of MLD was done using Cytoscape tool and its plug-ins. This study focuses on building and analyzing the protein-protein interaction network for MLD. MiMI was used to retrieve interactions of genes which were involved in causing MLD. Network was analyzed by Network Analyzer and we found that the query protein-protein interaction network have a Small World Network Model characteristics. From the list of clusters obtained through ClusterONE, the cluster with 34.436 density was selected. This highest ranking sub-network was found to have 126 genes, from which five genes were found to be highly inter-connected, namely SMAD9, PSAP, BMPR2, ARSA, UBE3A which may play a major role in pathogenesis for this disease. The main idea behind this study was to identify probable drug targets for MLD. These were found using Cytohubba. The proteins encoded by the identified genes: TAF1, SMAD2, BRCA1, HNF4A, AR, SMAD9, CDC2, RB1, UBC, CDK2, UBB, PSAP, CDC23, MYC, MNAT1, CCNH, CDK7 can be potential drug targets for this disorder. These genes have been poorly explored or unknown in the current state of the art of MLD physiopathology and should be further validated using experiments such as microarray, or genome wide association studies. The lack of gene expression data or microarray studies make it all the more important to study the MLD system in different tissues and cell lines. Structure of SMAD9 and MNAT1 proteins are still unknown, they need to be further explored in near future. Normalized centrality index score also supports potential gene targets obtained from Cytohubba algorithms. In this analysis score obtained for ARSA gene is found to be low as compared to other genes because it does not comprise any bigger hub center in protein network but it plays a critical role in passing information between hub centers which is also confirmed by Bottleneck algorithm. Molecular function of these genes was studied using BiNGO. It was found that the genes causing MLD (ARSA and PSAP) and other important candidate genes are mostly involved in Binding and Catalytic activity.

Pathway enrichment analysis was done to get deeper insight into the molecular mechanism of MLD. From this analysis we conclude that major pathways which may be affected in MLD are Sphingolipid Metabolism, Lysosome, Proteolysis, Arrhythmogenic right Ventricular cardiomyopathy (ARVC). Through tissue gene expression analysis we found that Organs like

Liver, Small Intestine, Brain and Nervous System may get affected in MLD. The comprehensive study was also done to find out other disease linked to MLD. Disease enrichment analysis was carried out to find to which disease class these potential genes belong. Through disease enrichment analysis we corroborated that MLD is a well known neurological disorder but we also found a possible link of MLD with metabolism, immune and cancer.

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9. APPENDIX

Appendix I: SQL queries for processing protein–protein interaction data obtained from MiMI plugin, in Oracle 10g Database Management System.

```
CREATE TABLE  "AIIMS1"
   (   "ID" NUMBER,
       "GENE" VARCHAR2(30),
        CONSTRAINT "AIIMS1_PK" PRIMARY KEY ("ID") ENABLE
   )
/
CREATE TABLE  "AIIMS2"
   (   "ID" NUMBER,
       "ID1" NUMBER,
       "ID2" NUMBER,
       "GENE_NAME1" VARCHAR2(4000),
       "GENE_NAME2" VARCHAR2(4000),
        CONSTRAINT "AIIMS2_PK" PRIMARY KEY ("ID") ENABLE
   )
/
CREATE OR REPLACE TRIGGER  "bi_AIIMS2"
  before insert on "AIIMS2"
  for each row
begin
  for c1 in (
    select "AIIMS2_SEQ".nextval next_val
    from dual
  ) loop
    :new."ID" :=  c1.next_val;
  end loop;
end;

/
ALTER TRIGGER  "bi_AIIMS2" ENABLE
/

SELECT GENE
FROM AIIMS1,AIIMS2
WHERE AIIMS1.ID = AIIMS2.ID1;

SELECT GENE
FROM AIIMS1,AIIMS2
WHERE AIIMS1.ID = AIIMS2.ID2;
```

Appendix II: **Normalized centrality index (SCORE *I*) for all genes involved in MLD protein- protein interaction network.**

GENE NAME	***SCORE I***
TAF1	747.5212087
SMAD2	646.9450308
BRCA1	562.4871301
HNF4A	553.005314
AR	484.9451925
SMAD9	419.2151898
CDC2	416.0214789
RB1	415.9210528
UBC	415.0906131
CDK2	413.5876164
UBB	413.5730965
PSAP	397.6550792
ANAPC10	392.6502057
CDC23	391.3418829
MYC	387.2418442
MNAT1	383.831744
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