DISCUSSION

Objective-1

To standardize enzyme based study from leucocytes or plasma for postnatal diagnosis and cultured trophoblats (CT cells) or cultured amniotic fluid cells (AF cells) for prenatal diagnosis of Tay-Sachs disease (TSD) and Sandhoff disease (SD).

The Hexosaminidase-A (Hex-A) and total-Hexosaminidase (total-Hex) enzyme study are the most approachable as well as convenient method for identification of TSD and SD respectively (Wendeler and Sandhoff, 2009; Mahuran, 1999). The enzyme testing remains the accepted gold standard for TSD screening and diagnosis, with 98% sensitivity, and can be used in pan-ethnic populations and in the presence of rare and/or founder mutations (Monaghan *et al.,* 2008).

In our study, we have found all cases with infantile form of TSD and the enzyme activity was in accordance with the previous observations of infantile TSD cases (Kaback, 2000; Nalini and Christopher, 2004). However, MUG substrate is cleaved by all three isozymes (Hex-A, B and S) and therefore is used to measure total-Hex activity. MUGS substrate is cleaved primarily by the α-subunit containing isozymes (Hex-A and S). However, the β-active site can slowly hydrolyze this substrate with MUG/MUGS ratios of ~ 300:1 for Hex-B and 3.7:1 for Hex-A (Hou *et al.,* 1996; Sheth *et al.,* 2014b). Thus each of the %-residual Hex-A values reported in *table 4.15 (refer chapter 4; table 4.15)* was multiplied by 3.7 and varied from 0% to 3.1%, which is in accordance with the previously reported value.

In present study, TSD carriers had enzyme activity of <62% compared to non-carriers where enzyme activity was >64%. However, enzyme testing has technical limitations in overlapping reference intervals indicating that there is an inconclusive range of enzyme activity (62% to 64%) in which it is difficult to distinguish TSD carriers from non-carriers in our study. This study is also in accordance with previous study described by Monaghan *et al.,* (2008) where they have described <52% activity in carrier and >60% activity in non-carrier. Furthermore, Schneider *et al.,* (2009) also described technical limitations in overlapping

reference intervals mean that there is an inconclusive range of enzyme activity (52% to 60%) in which it is difficult to distinguish TSD carriers from non-carriers. Rarely, false negative and false positive results can occur in presence of B1 allele (Kytzia and Sandhoff, 1985; Fernandes *et al.,* 1992) and pseudodeficiency alleles (Triggs-Raine *et al.,* 1992). Medications including oral contraceptives may affect detected enzyme levels (D'Souza *et al.,* 2002) though we have not encountered this problem in present study.

Subjects of our study showed that loss of cognitive and physical milestones; cherry red spot in macula and abnormal muscle tones were the most common symptoms at early onset of disease (*Figure 4.7*). Seizures, hyperacusis, hearing impairment were frequently reported as well as mild hepatomegaly and mongolion spot were rarely reported symptoms at disease onset (*Figure 4.7*). In general, the age of onset and the clinical features of infantile TSD seen in our set of patients were consistent with the defined phenotype, except for the presence of prominent mongolian spots on the back even beyond infancy in two patients. However, mongolian spots are also reported in association with many of the patients with other LSDs (Sheth *et al.*, 2014a; Staretz-Chacham *et al.*, 2009) and in our cases it could be an accidental finding.

During fundus examinations, the cherry red spot of the fovea centralis of the macula of the retina, showed in 54/56 cases with TSD; 8/54 cases showed vision loss or poor vision, and 6/54 cases showed poor vision and cherry red spot, which consists with literature that reported cherry red spot are present in virtually all cases with TSD (Kaback and Desnick, 1999). The cherry red spot, common to many of the lysosomal storage diseases including GM1 and GM2 gangliosidoses, arise from the accumulation of gangliosides within the retinal ganglion cells, found in highest concentration within the macula. Since the fovea lacks ganglion cells, this allows for a contrast in coloration from the opacified ganglion cells, resulting in the cherry red spot (*Figure 5.1*). Long-term follow-up of such patients revealed a decrease in prominence of the cherry red spot as a result of ganglion cell death and resultant gliosis (Nakaya-Onishi *et al.*, 2000).

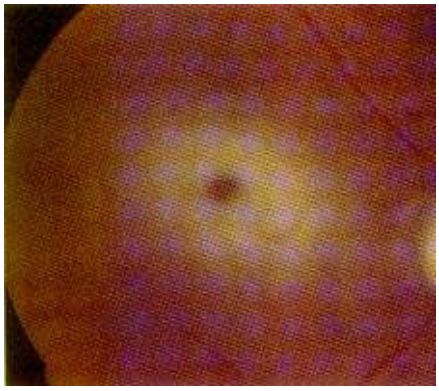
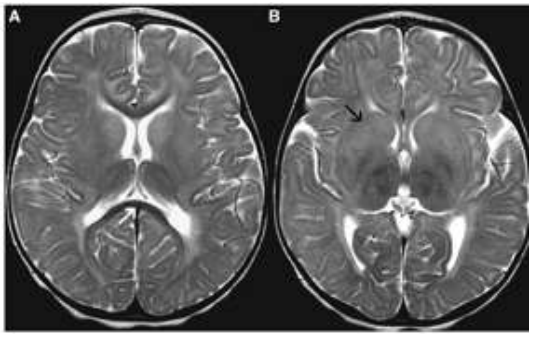


Figure 5.1: *Retinal photograph showing the cherry red spot at the center of the macula*

Brain imaging studies were available in 30/56 cases and showed characteristic finding of decreased in thalami and decreased attenuation of basal ganglia isodense with white matter, and few cases had dysmyelination (*Figure 5.2*), which also consist with reported finding on the same chronic subtypes of GM2 gangliosidosis (Karimzadeh *et al.,* 2014; Schiffmann and van der Knaap, 2009).

Figure 5.2: *Axial T_2-weighted images of patients with GM2 gangliosidosis (A) display hypointensity of the corpus callosum (white arrow) and (B) T_2 hyperintensity of the basal ganglia (black arrow)*



An electroencephalogram (EEG) study available in 17/56 cases, showed slow epileptic discharge and/or hyperarrhythmia in all TSD cases, which is also in consistency with recent publication that showed right posterior-temporal spike wave discharges that were significantly potentiated by sleep (Georgiou *et al.,* 2014).

TSD patients found in this study represented all regions (North, South, East, and West) of India. Majority of TSD patients (76%) were from western region while none of the patient were found from central region of India (*Figure 5.3*), could be due to either presence of founder mutation for TSD in Gujarat (Mistri *et al.,* 2012) or referral bias due to lack of investigation facility at other places like in this region. The place of origin of TSD patients of the present study could also be traced to majority states of India, which indicated that TSD was not confined to any particular geographical area of India. Furthermore, the prevalence of TSD

accounted for 8.4% in our study, while Verma *et al.* (2012) have shown the prevalence of TSD in nearly 6% of cases in his study from India. High prevalence of GM2 gangliosidosis (TSD and SD) has also been reported in children with neurological disorders from the southern region of India, where consanguinity is more common (Nalini and Christopher, 2004). However, TSD accounted in Indian literature was slightly lesser than the Portugal cohort especially for TSD (13.8%), while higher than the prevalence was reported from Australia and Czech Republic (4.1% and 2.5%, respectively) as shown in *figure 5.4* (Meikle *et al.*, 1999; Pinto *et al.*, 2004; Poupetova *et al.*, 2010).

Additionally, in these countries (Australia, Portugal, and Czech Republic) special working groups on LSDs increase awareness among the medical fraternity. Also patient support groups advocate the disease magnitude and support the affected families (Meikle *et al.*, 1999; Pinto *et al.*, 2004; Poupetova *et al.*, 2010). In India, very recently Indian Council of Medical Research (ICMR) has set up a special task force on LSDs which will focus on the magnitude of these disorders in different parts of the country (*Figure 5.5*), increase awareness among the clinicians by organizing regional training program, and establish common mutation spectrum for different LSDs.

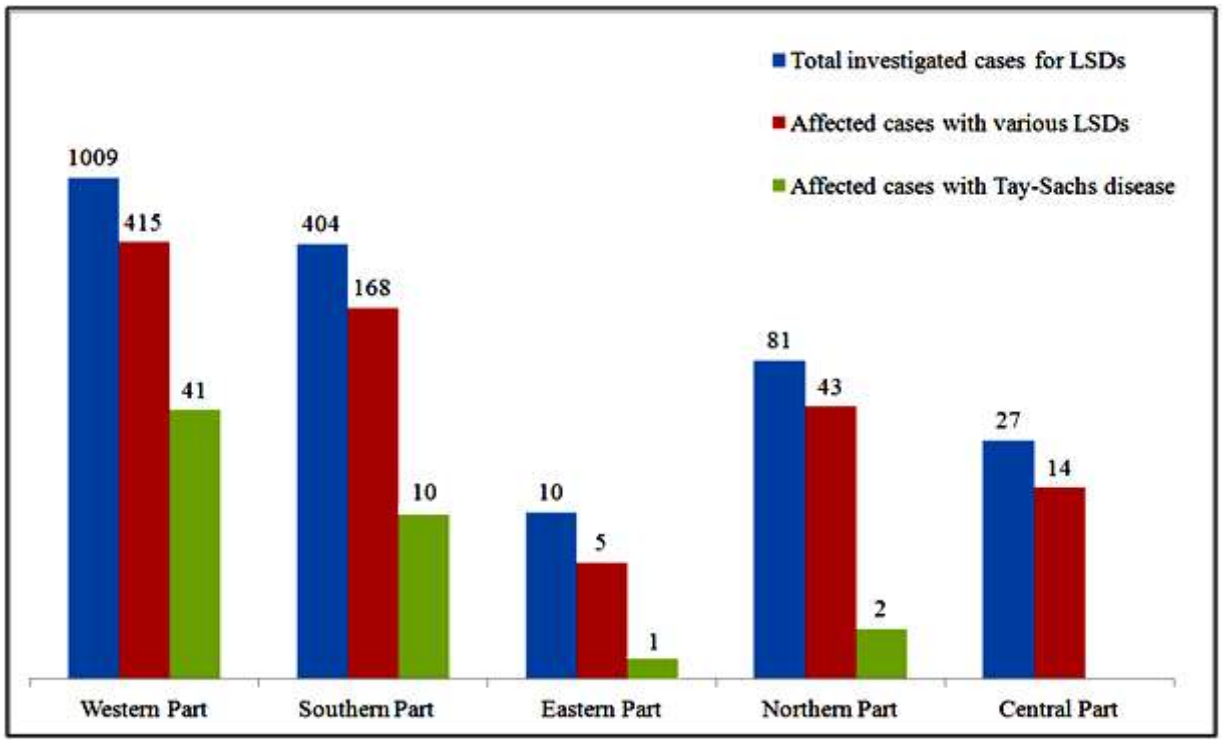


Figure 5.3: *Frequency of different LSDs followed by TSD in various part of country*

The ethnicity predilection is also known to be associated with a specific lysosomal disease like Gaucher, Tay-Sachs, Niemann-Pick type-A and Mucolipidosis-IV disease in Ashkenazi Jewish descendants (Marsden and Levy, 2010); Salla disease and Aspartylglucosaminuria in Finnish population; and Gaucher type III disease in people of Swedish descent (Marsden and Levy, 2010). In India, the prevalence of Gaucher disease was found to be higher in Maharashtra region, whereas TSD was more in Gujarat province as shown by our study (Sheth *et al.*, 2014a).

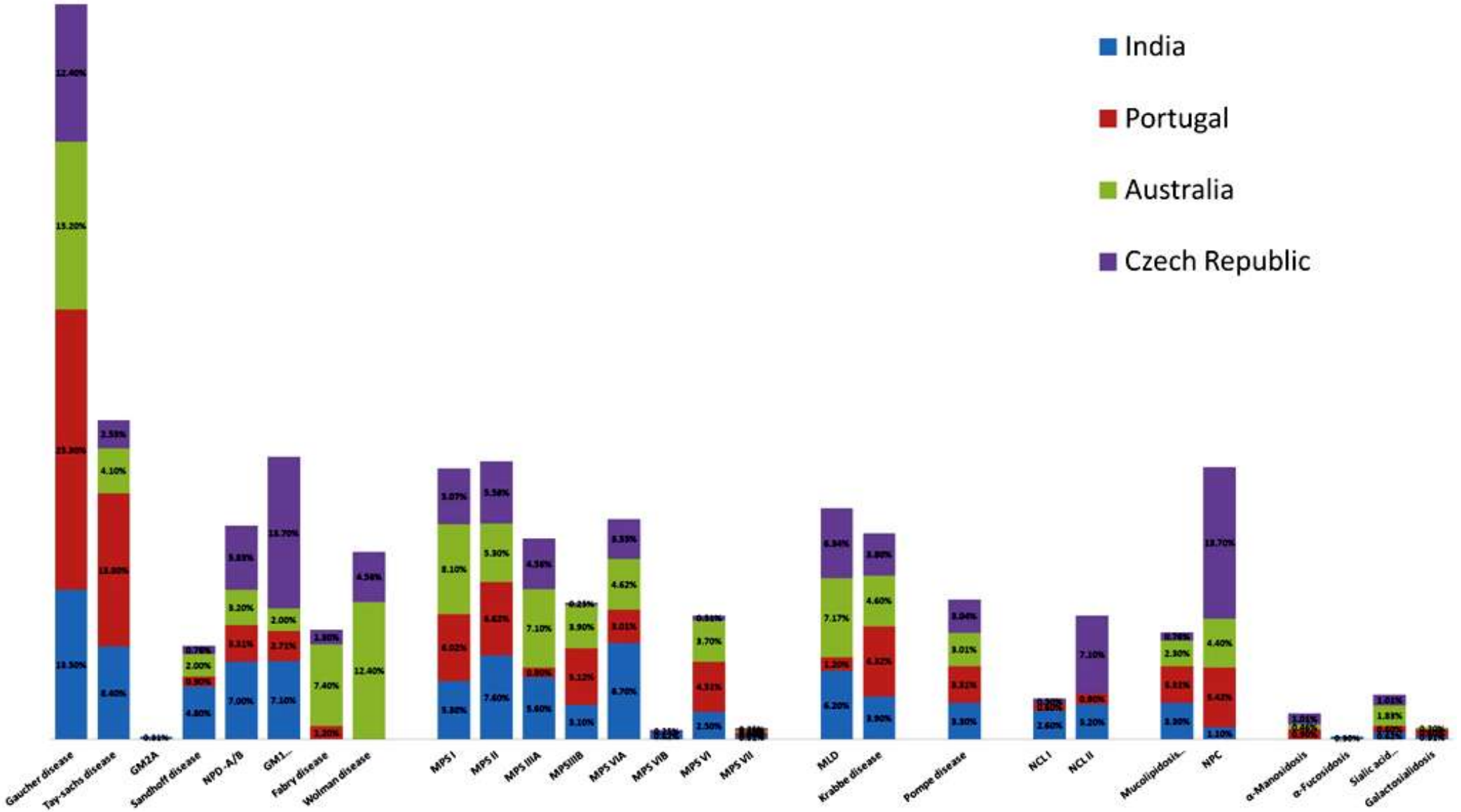


Figure 5.4: *Prevalence of TSD, SD, GM2Aand other LSDs in India*

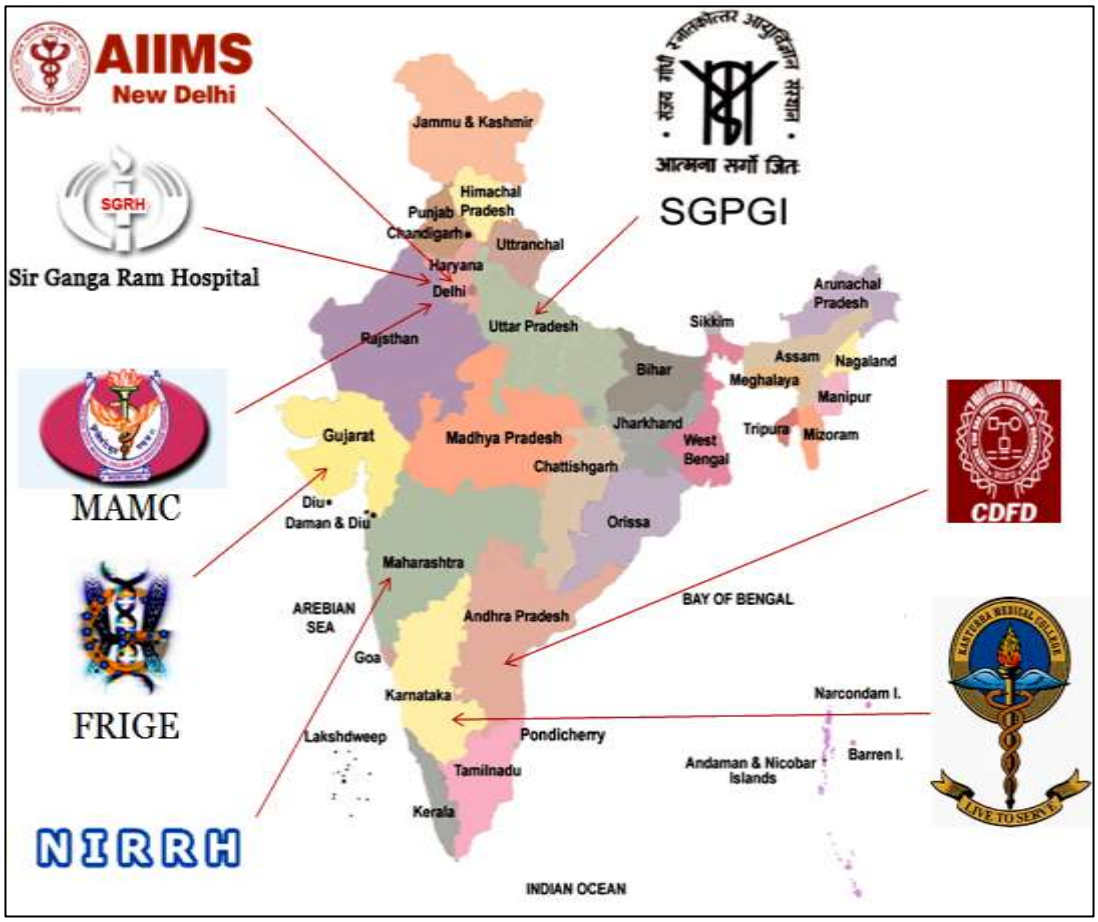


Figure 5.5: Indian Council of Medical Research (ICMR) has set up a special task force on LSDs at different parts of the country

Objective-2

To study the mutational spectrum of *HEXA* gene in Indian patients with Tay-Sachs disease and elucidate the pathogenic effect of novel mutations

Objective-5

To evolve a cost effective DNA based disagnosis for prevalent mutations in our population through identification of the carrier frequency by mass screening

No molecular studies had ever been reported from India, so there is no data regarding the sequence variation profile of TSD patients. In the present study, molecular analysis was carried out in 56 cases that includes 46 cases with classical TSD (deficient activity of Hex-A in

leucocytes), 2 cases with AB variant TSD/GM2A activator deficiency (normal activity of Hex-A and total-hex), with carrier detection (%Hex-A) in 8/56 parents where proband was not alive but was earlier diagnosed as a TSD by enzyme study.

In present study, overall 36 different variants were identified 34 in *HEXA* gene and 2 in *GM2A* gene that includes 23 (63.8%) novel and 13 (36.1%) previously known variants. They were further classified as 31 (86.1%) point sequence variants, 2 (5.5%) large deletions, 2 (5.5%) small deletions and 1(2.8%) small insertion in TSD patients. Majority of patients were carrying the missense variants in 18 (50%) patients followed by nonsense variants in 6 (16.6%), splice site variants in 7 (19.4%), frameshift variants in 3 (8.3%) and large deletions in 2 (5.5%) patients. Most of the point variants include transition of G>A (31%) and transition of C>T (24%) followed by transversion G>C (17%), transversion A>C (10.3%), transversion T>G (6.9%), transversion T>C (6.9%) and transition A>T (3.4%).

These data also indicate that the 4-bp insertion c.1278insTATC in exon 11, which accounts for the 75% of Ashkenazi Jews, 92% of Cajuns, 2% of Moroccan Jews and 20% of Non-Jews TSD alleles (Paw *et al.,* 1990; Myerowitz and Costigan, 1988; Harmon *et al.,* 1993; Kaufman *et al.,* 1997; Mc Dowell *et al.,* 1992) is responsible for 6/54 (11%) in the Indian population. The remaining common mutations [c.1421+1 G>C, 7.6 kb deletion, the pseudo-deficiency alleles (p.R247W and p.R249W)] were not detected in our study.

Over all 18 missense point variants (13 novel and 5 previously known) were identified in present study. Within novel variants, p.E114K was found as homozygous in Infantile TSD; here the acidic residue Glutamine is replaced by basic residue Lysine at codon 114 of exon-2 which is close to proximal region N-glycosylation site α-N115 (Lemieux *et al.,* 2006) and structural analysis also revealed conformational changes of the protein (*Figure 4.13(a)*).

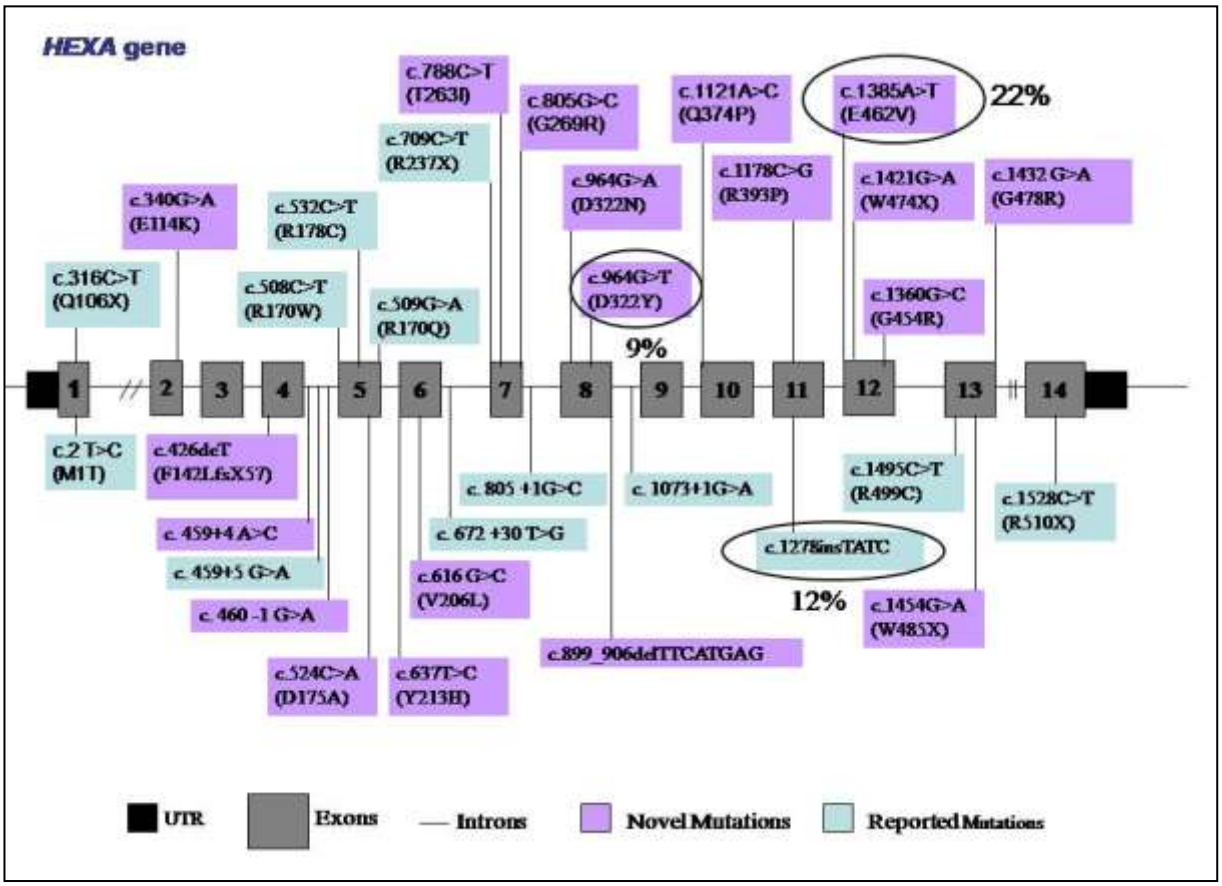


Figure 5.6: *Mutation spectrum of TSD in present study from India*

In this study, variants p.D175A and p.G269R were found as compound heterozygous in infantile TSD patients. The missense variant p.D175A disrupts the β-sheet due to an amino acid change from a positively charged Aspartic acid to a hydrophobic Alanine residue at codon 175 of exon 5 *(Figure 4.13(g)*. At the same time, p.G269R variant creates over-packing and backbone disruption due to the replacement of a small hydrophilic group residue Glycine with a positively charged residue Arginine at codon 269 of exon-7 *(Figure 4.13(h))*. Therefore, disruption of the backbone from αGly269Arg may result in the movement of αHis262, with the likelihood of disrupting the coordination of residues in the active site. Additionally, p.G269 is also the site of the mutation in late onset Tay-Sachs (p.G269S) with high frequency in the Ashkenazi Jews and resulted in an unstable α-subunit precursor. This failed to associate with the β-subunit, suggesting that the mutation reported here, found in a patient with the infantile acute disease, and has a more deleterious impact on the enzyme or its expression (Ohno *et al.*, 2008).

We had also identified two novel variants p.V206L and p.Y213H in exon-6, also found as a homozygous in infantile TSD patients. The missense variant p.V206L disrupts the β-sheet and

over packing of residues due to an amino acid change from, a relatively medium side chain group, uncharged amino acid residue Valine is replaced by non-polar, large side chain group amino acid Leucine at codon 206 in exon-6 *(Figure 4.13(i))*. Similarly, in variant p.Y213H, an aromatic, cyclic uncharged residues Tyrosine is replaced by a positively charged Histidine residue and disrupts the β-sheet *(Figure 4.13(j))* Furthermore, all the variants identified so far in residues proximal to Val206 and Tyr213 (V200M, W203G, H204R, D207E, S2101R, F211S) have been shown to cause acute TSD phenotypes (Lemieux *et al.*, 2006; Ohno *et al.*, 2008; Giraud *et al.*, 2010).

The missense variants p.T263I was also found as homozygous in Infantile TSD patient. The mutation occurs due to the polar residue Threonine that is replaced by non-polar residues Isoleucine. This variant occurred at next to active residue αHis262 (Lemieux *et al.*, 2006) therefore, disruption of αThr263Ile results in the movement of, with the likelihood of disrupting the coordination of residues in the active site *(Figure 4.13(k))*.

We had found three novel deleterious missense variants (p.D322N, p.D322Y, p.E462V) occurring at the functionally active site of the alpha subunit of Hex-A. We were especially interested in analyzing the p.E462V variant because it was identified in eleven unrelated families from one ethnic group but, to our knowledge, has never been identified in TSD patients of other ethnic origin. The sequence alignment of Hex-A from various species (*refer to figure 4.12*) reveal that the residue p.E462 is highly conserved. The RMSD of the modelled mutant is also significantly higher as compared to the wild-type protein (*refer to table 4.10 and figure 4.14(d)*). This mutation was present in homozygous state in nine patients while heterozygous state in two patients with different second allele exhibiting infantile TSD. This is in accordance with previous observations that missense mutations responsible for infantile TSD were generally located in a functionally importance region, such as the active site and the dimer interface (Ohno *et al.*, 2008).

The novel variant p.Q374P was found in infantile TSD, caused by the polar residue Glutamine is replaced by non-polar residues Proline at codon 374 of exon 10. The structural

analysis predicts that this variant might disturb the position of active sight residue. Another mutation at the same codon (p.Q374R) is reported in Italian ancestry (Montalvo *et al.*, 2005).

One more novel variant p.R393P in exon 11 was found as homozygous status in infantile TSD. The large positively charged amino acid residue Arginine is replaced by small uncharged amino acid Proline which causes the conformational changes in protein structure of *HEXA* gene.

The two novel nonsense variants p.W474X and p.W485X were detected in homozygous state might causing premature termination of the protein and mRNA reduction and should be deleterious. Other mutations at same codons (p.W474C) and (p.W485R) respectively, causing infantile TSD is also in reported in literature (Petroulakis *et al.,* 1998; Akalin *et al.,* 1992).

The deletions observed in (c. 426delT and c.899_906delTTCATGAG) two cases led to two frameshifts mutations (p.F142LfsX57 and p.F300HfsX21). The c.426delT was observed in exon 4 in an infantile TSD patient that changes the reading frame at codon 142 and introduces a premature stop at codon 199 (p.F142LfsX57), leading to a truncated protein or a nonsense-mediated mRNA decay (NMD). Nonetheless, in a non-Jewish French child, a 2 bp deletion in the same codon (codon-142) is reported by Akli *et al.,* in 1993. Similarly, deletion c.899_906delTTCATGAG was observed in exon 8 in an infantile TSD patients that change the reading frame at codon 300 and introduce a premature stop at codon 321 (p.F300HfsX21), leading to a truncated protein which would be rapidly degraded. Both the frameshift mutations were also evaluated by Mutation taster and was found to be disease causing.

The two novel splice site variants affecting mRNA splicing, c.459+4 A>C and c.460-1 G>A, were detected in two cases (*refer table 4.7*). In one case, the novel splice site variant c.459+4 A>C was found with previously reported mutation p.R178C in a compound heterozygous state. This mutation is in close proximity to the reported splice site mutation c.459+5 G>A in Argentinean and Spanish populations (Zampieri *et al.,* 2012; Gort *et al.,* 2012). One more novel splice site variant c.460-1 G>T was also found homozygous in one case. Another mutation at same position c.460-1 G>A, causing infantile TSD reported in American Black patient (Mules *et al.,* 1991). Both the splice site variants found in present study were evaluated

for pathogenicity using Mutation taster and was found to be disease causing; and was predicted to lead to a loss of aberrant donor splice site at this position (score 0.87 and 0.65 respectively). The patient was not available for further functional study of the mutation. As per our knowledge, these novel mutations have never been identified in TSD patients from other ethnic groups. However, several splicing mutations have been reported in *HEXA* gene which occurs at the exon-intron boundaries, either in homozygosity or as compound heterozygotes with a second null/ deleterious allele, resulted in very low or undetectable mRNA levels. Both the splicing variants found in this work were associated with the acute phenotypes of the disease, as it has been previously reported (Mahuran, 1999; Gravel *et al.,* 2001).

Among 34 variants demonstrated here in *HEXA* gene, 13 have been previously reported in different populations that includes 5 missense mutations c.2T>C (p.M1T) has been previously reported in non-Jewish English patients where T to C transition in the initiation codon, expected to produce no α-subunit and therefore a classical infantile phenotype (Harmon *et al.,* 1993). The mutation c.508C>T (p.R170W) has been previously reported in different ethnic groups [Japanese, French-Canadian (Estree region, Quebec) and Italian patients] (Tanaka *et al.,* 1999; Fernandes *et al.,* 1992; Akli *et al.,* 1993a). The side chain of R170 in domain-II forms a hydrogen bond with the E141 in domain-I. The substitution of R with W with a bulkier side chain has a large effect on the interface of domains-I and -II. This would destabilize the domain interface and cause degradation of the a-subunit (Tanaka *et al.,* 1999). The mutation c.509 C>A (p.R170Q) has been previously reported in Japanese (Nakano *et al.,* 1990) and Moroccan patients (Drucker *et al.,* 1992) and expression studies show inactive, unstable and shortened mature protein. An interesting effect of mutation occurring in c.509 C>A (p.R170Q) is activation of a cryptic acceptor splice site in exon 5 to produce aberrant transcript with 51bp deleted at 5' end of exon 5 (Myerowitz, 1997).

The mutation c.532C>T (p.R178C) has been previously reported from Czechoslovakia in B1-variant TSD (Tanaka *et al.,* 1990a). Experiments with site directed mutagenesis and expression study showed that either of these mutations (p.R178C) abolishes the catalytic activity.

Thus, Arg178 must be critical for the catalytic activity of the enzyme (Tanaka *et al.*, 1990a). However, in present study p.R178C was found as a compound heterozygous with novel variants c.459+4 A>C as second allele in Infantile form of TSD. The mutation c.1495C>T (p.R499C) has been previously reported in different ethnic group [Slavic, Irish, English and Polish] and the resulting phenotype is the severe from (Paw *et al.*, 1991). Appearance of Cysteine residue may create an illegitimate disulfide bond and destroy the proper tertiary structure of the enzyme wiping out all enzyme activity.

We had also found 3 previously known nonsense mutation p.Q106X mutation has been previously reported in non-Jewish population (Akerman *et al.*, 1997). The nonsense mutation p.Q237X was previously reported as benign in accordance with the NCBI SNP database. However, a subunit lacking its last 293 C-terminal residues is highly likely to make the protein non-functional. In the present study this mutation was observed in both parents with the history of an affected child with TSD. Though, mutation study in the affected child could not be carried out due to sudden death and unavailability of a sample. The National Center for Biotechnology Information's (NCBI) single nucleotide polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs150675340) reveals that the minor allele count and pathogenic potential of this polymorphism (rs150675340) are not available. The nonsense mutation c.1528 C>T (p.R510X) has been also reported in Saudi Arabia (Kaya *et al.*, 2011) and Iranian population (Jamali *et al.*, 2014). Generally, nonsense and frame-shift mutations result in the reduction of mRNA in the *HEXA* gene. Diminished amounts of mRNA have been reported in several mutations in the *HEXA* gene (Drucker *et al.*, 1992).

We have also found 4 previously known intronic variants in present study, splice mutation c.459+5 G>A has been previously reported from Argentinean (Zampieri *et al.*, 2012) and Spanish populations (Gort *et al.*, 2012). The intronic mutation c.672+30 T>G has been previously reported in an obligate carrier individual detected on biochemical screening. The mutation has been reported to be benign in this paper as it was found in combination with another disease

causing mutation in an obligate carrier (Triggs-Raine *et al.,* 1995). The National Center for Biotechnology Information (NCBI) single nucleotide polymorphism database (dbSNP) (http: //www.ncbi.nlm.nih.gov/SNP/index. html) revealed that the minor allele count of this polymorphism (rs117160567) is minimal (C=0.0103/13). However this allele has not been reported in homozygous state till date. Thus, the pathogenic potential of this mutation remains to be explored by functional analysis. The splice site mutation c.805+1 G>C has been reported previously from Portuguese patients (Ribeiro *et al.,* 1995). Another substitution at the same nucleotide c.805+1 G>A is common in French-Canadian patients from the Quebec region in Canada (Hechtman *et al.,* 1992). Both mutations are known to be associated with the infantile form of TSD. The abnormal splicing mutation c.1073+1 G>A cause aberrant transcript was homozygously found in one Infantile case of TSD. This mutation has been absent among the Jewish population, however it is found in about 15% of the non-Jewish carriers (Akerman *et al.*, 1992). This mutation has been also reported in Iranian patient (Montalvo *et al.,* 2005).

In one of our patients, no mutation could be identified while two patients were heterozygous for p.E462V mutant allele with unknown second allele. Hence, we set up a MLPA assay to ascertain accidental misgenotyping in our TSD cases and to analyze the alleles that remained uncharacterized after being studied by conventional techniques. This experimental approach was effective in identifying two large deletions in an Indian TSD patient for the first time that include homozygous deletion of exon 2 & 3 in one case and compound heterozygous deletion of exon 1 with second novel p.E462V mutant allele in two cases (*refer chapter 4; table 4.9*). Mutation was not identified in one case even after MLPA analysis, suggesting the presence of a deep intronic mutation that may affect the mRNA splicing process.

Furthermore, we present two cases having a novel mutation in *GM2A* gene. Clinically, proband was indistinguishable from classical infantile TSD. However, due to the normal activity of hex-A and total-hex enzymes in leucocytes, the c.DNA analysis of *GM2A* gene was carried out. The cases under study showed homoallelic nonsense mutation c.472G>T (p.E158X) and

c.242-2 A>T in *GM2A* gene. These are a novel pathogenic variants found to be responsible for a GM2AP deficiency in our patient with AB variant of TSD.

As per our knowledge, this mutations c.472G>T (p.E158X) and c.242-2 A>T are neither reported in any ethnic group, nor present in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). This variation has not been reported in the 1000 genome database and this region is conserved across species. *In-silico* analysis using the Mutation taster program suggests a probably damaging nature of the mutation. In nonsense mutation c.472G>T (p.E158X) a subunit lacking the last 36 C-terminal residues is likely to make the protein non-functional. This premature stop codon is likely to have produced a truncated protein as the stop codon occurred near the C-terminal, which leads to unstable mRNAs. It is also likely that the proteins synthesized in the endothelium reticulum (ER) undergo a quality control check by the resident ER system that recognizes abnormal proteins and degrades them (Henderson *et al.*, 1196). Nonsense point mutation in the present case seems to have produced a premature stop codon thus affecting the stability of the mutated protein or its Hex-A binding capabilities (Xie *et al.,* 1998) or perhaps leading to early degradation in the ER or Golgi bodies (Schepers *et al.*, 1996), thus portraying the clinical presentation.

All novel missense mutations were confirmed to be pathogenic using various bioinformatics tools like SIFT, Polyphen2 and Mutation taster algorithms (*refer chapter 4; table 4.10*) while splice site mutations were confirmed by Mutation taster and NNSplice 0.9 algorithms (*refer chapter 4; table 4.11*). Protein homology modeling study was carried out for p.E114K, p.D175A, p.V206M, p.Y213H, p.T263I, p.G269R, p.D322N, p.D322Y, p.Q374P, p.R393P, p.G454R, p.E462V and p.G478R to further establish the effect of novel mutation that occurred at highly evolutionarily conserved and functionally active domain residues in the protein leading to conformational changes or mRNA producing truncated protein. The RMSD of the modelled mutant is also significantly higher as compared to the wild-type protein except p.E114K mutations (*refer chapter 4; table 4.10*). The sequence alignment of Hex-A from various species reveal that the all residue highly conserved *(refer chapter 4; figure 4.12*). By protein modeling of

the mutations, we see a disruption in the hydrogen bonding network in the active site that would reduce G_{M2} binding energy and severely affect the activity of the mutant α-active site of Hex-A as shown in *table 4.12 and 4.13*.

In present study, nearly 80% of variants were occuring in C-terminal domain (Domain-II) comprising a core TIM barrel fold ($(\beta,\alpha)_8$-barrel) that cover active pocket while remaining 20% of variants were arising in N-terminal domain (Domain-I) (*refer chapter 4; table 4.7 & table 4.8*). We have also observed that nearly 37% (19/51) of cases had shown variants at αArg178, αAsp322, and αGlu462 which are functionally the active residues of *HEXA* gene (Lemieux *et al.*, 2006).

Furthermore, seven of the 29 point mutations, or ~25% (*refer chapter 4; table 4.7 & table 4.8*), occur at CpG dinucleotides which are generally known to be mutational "hotspots" (Cooper and Yousoufian, 1988).

A large number of gene mutations causing TSD have been previously reported in the *HEXA* mutation database (http://www.hexdb.mcgill.ca/). Among them, mutations resulting in gross alterations in the Hex α-subunit sequence are generally found in the severe infantile form of TSD. However, missense mutations causing amino acid substitutions have been found in both the infantile and late onset phenotypes (Zampieri *et al.*, 2012). Mahuran (1999) reported that the detrimental effect of most mutations is on overall folding and/or transport of the protein rather than on the functional sites (Gort *et al.*, 2012). Some of the mutations identified in our series affect crucial active residues or cause significant structural aberrations leading to the infantile form of the disease.

Objective-3

To understand effect of genotype on phenotypic development

The clinical presentation of the patients studied at the time of diagnosis was characterized by regression of milestones (motor skills and cognitive regression), cherry red spot, poor vision and/ or vision loss, abnormal muscle tone, seizures, hearing impairment and hyperaccusis similar to

what have been previously reported phenotypes in other studies (Nalini and Christopher, 2002; Frenandes Filho and Shapiro, 2004; Sheth *et al.,* 2014a; Verma *et al,* 2012).

However, our study demonstrated presence of mongolian spots in 3.7% (2/56) of the cases. Neuroimaging study of the brain of the proband was available in 24/56 patients and showed characteristic findings of putaminal hyperintensity and thalamic hypointensity, and few patients had dysmyelination. MRI of brain was also found to be normal in 7/54 patients, while in remaining patients neuroimaging study was not available. Nonetheless, the clinical and neuroimaging features of infantile TSD seen in our patients were consistent with the defined phenotype, except for the presence of prominent mongolian spots in few cases.

Moreover, in present study all TSD patients showed significant decreased level of residual activity of the Hex-A enzymes in early age correlating with infantile form of the disease. The results of enzyme activity measurements (Hex-A expressed as percentage of total-Hex activity) varied from 0%-1.29% (*refer chapter 4; table 4.15*). This is consistent with previous observations that infantile TSD patients had values ranging from 0-2% (Kaback and Desnick, 1999).

In present study, we have found novel deleterious mutations (p.D322N, p.D322Y and p.E462V) at the functionally active site of the α-subunit of β-Hexosaminidase-A which causes the infantile form of TSD with early and/or severe phenotype and markedly deficient enzyme activity as compared to mutations occurring at other sites (*refer figure 5.7*). Majority of the mutations identified in our series affect crucial active residues or cause significant structural aberrations leading to the infantile form of the disease. This is in accordance with the previous observations that the missense mutations responsible for the infantile form of TSD are generally located on the functionally important region such as active site and the dimer interface (Mahuran, 1999; Tropak and Mahuran, 2010). Furthermore, a large proportion of novel mutations [21(62%) of 34] were identified in the study. The higher number of novel mutations highlights the heterogeneity in the Indian population, and the new mutations correlate with the distinctness of gene pool (Dalal *et al.,* 2012).

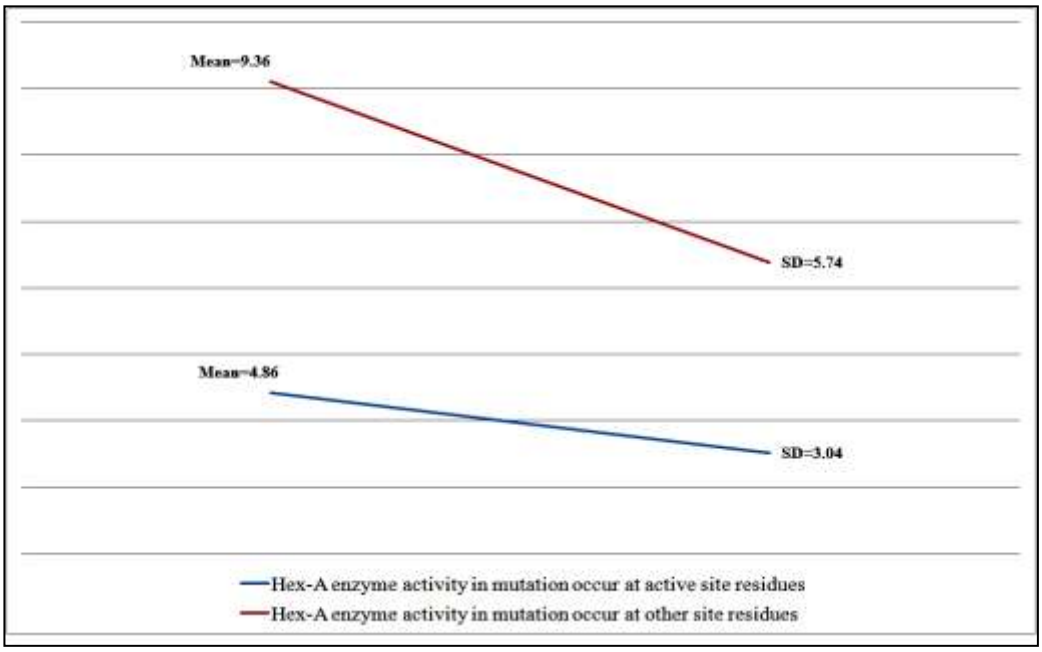


Figure 5.7: *Markedly deficient enzyme activity in mutations occur at functionally active site as compared to mutations occurring at other sites*

There are many studies of genotype-phenotype correlation in TSD patients but results vary in consistent so some studies reported several differences and other reported no differences (Montalvo *et al.*, 2005; Tropak and Mahuran, 2010; Mahuran, 1999). Correlating genotype with phenotype is possible with some of the more common *HEXA* mutations but becomes increasingly difficult when the mutations are rare. Many of the reported mutations are functionally pathogenic, preventing the formation of the Hex-A protein, and are therefore predicted to cause the classical form of TSD. Others are associated with sub-acute and chronic forms of the disease, which have a more variable phenotype and vary markedly in age of onset and severity.

In general our study clearly demonstrate that irrespective of the genotype, TSD patients in India are of infantile severe phenotype with missense mutation being the most common variant observed in *HEXA* gene.

Objective-4

To provide genetic counseling to the affected families and prevent the disease by prenatal diagnosis

In the present study prenatal study was carried out in 8 TSD cases by lysosomal enzymes and

mutation study. All had known mutations in an index case. All cases had shown concordance with enzymes and molecular study confirming that enzymes study in prenatal tissue is as reliable and specific as molecular investigation. This is very important in country like India where once an index case is diagnosed with confirmative enzymes study further work up for carrier detection and molecular confirmation depends on how the referring clinician and laboratory provide counseling to the families and also financial burden prohibits many families to undergo molecular analysis.

Nonetheless, enzyme based PD for some of the TSD often pose a problem of presence of pseudo-deficiencies (Thomas, 1994). In such cases, mutation analysis as well as enzyme activity is necessary in the parents and the index case before offering PD. The failure to study the presence of a pseudo-deficiency allele in a family could lead to an incorrect PD, which is a major limitation of the enzyme study.

Our experience confirms the usefulness of DNA testing for prenatal diagnosis of TSD. The identification of *HEXA* gene mutations enables PD by DNA analysis for most couples at risk for TSD. Moreover, the identification of the type of mutation, whether it is associated with classic infantile disease or with adult-onset variants, may provide useful information about the outlook for an affected child in other extended family members and community.