Chapter 6
Discussion
6.1. Morquio A syndrome

We have analyzed 93 patients with MPS IVA from 17 collaborating centers across India and have identified 51 mutations. We also have documented the non-pathogenic sequence variations observed in this study (Table 5.7). Though our center offers mutation analysis of both GALNS and GLB1 causing MPS IVB, we did not get any referrals for MPS IVB, thus suggesting rarity of MPS IVB.

We observed that 82% of mutations are missense and the others are indels (8%), splice site (6%), and nonsense (4%). Similar observations were seen in other studies where missense mutations were commonly found (Wang et al., 2010; Pajares et al., 2012; Dung et al., 2013; Morrone et al., 2014b). We observed homozygous mutations in all 37 patients with proven consanguineous marriages (Table 5.3). These findings highlight that remote consanguinity is likely in our population as many groups practice marriages within the same caste and religion. Similar observations were made in our work on another autosomal recessive condition (Dalal et al., 2012).

In this study, 80% of the patients have homozygous mutations and 20% have compound heterozygous mutations. This trend is different among 514 MPS IVA patients described in a review by Morrone et al., (Morrone et al., 2014a). We could not identify one allele in one patient with MPS IVA (patient No. 89), however the identification of single alleles have been described in 13% of patients in other studies (Morrone et al., 2014a). Mutations remain unidentified in two patients who display the clinical and radiological features of Morquio syndrome and low GALNS activity. This disparity is similar to observations made by others (Yamada et al., 1998; Terzioglu et al., 2002; Tomatsu et al., 2004a; Wang et al., 2010; Dung et al., 2013) and it has been reported that the mutation is not identified in up to 15% of patients with MPS IVA (Tomatsu et al., 2005b). In contrast to other MPS diseases, like MPS I, caused by the mutations in alpha-L-iduronidase gene (IDUA), one study reported that both mutations have been identified in 95% of patients with this disease (Beesley et al., 2001). The unidentified mutation could be present in deep introns or in the regulatory regions. Rarely, there can be large deletions or duplications that can escape detection by Sanger sequencing of coding regions.
Out of the 28 novel mutations in GALNS, 10 of them (p.Asn32Thr, p.Leu36Arg, p.Pro52Leu, p.Cys79Arg, p.Asn204Thr, p.Phe216Ser, p.Ala291Ser, p.Gly317Arg, p.Phe216Ser, p.Ala291Ser, p.Glu450Gly and c.1003-3C>G) were observed in more than one patient and the remaining novel mutations were only found in a single patient. Thirteen mutations, p.Asn32Thr, p.Leu36Arg, c.120+1G>C, p.Pro77Arg, p.Cys79Arg, p.His142Pro, p.Asn204Thr, p.Phe216Leu, p.Trp230Cys, p.Ala291Ser, p.Arg386Ser, p.Glu450Gly and p.Trp520Ser, were located at the same site that has been described in different populations earlier with a different amino acid change: p.Asn32Asp, p.Leu36Pro, c.120+1G>A, p.Pro77Arg, p.Cys79Tyr, p.His142Arg, p.Asn204Lys, p.Phe216Ser, p.Trp230Gly, p.Ala291Asp, p.Arg386His, p.Arg386Cys, p.Glu450Val and p.Trp520* (Ogawa et al., 1995; Tomatsu et al., 1995a; Bunge et al., 1997; Tomatsu et al., 2004c; Tomatsu et al., 2005b; Laradi et al., 2006; Wang et al., 2010; Ye et al., 2013). These previously described mutations at the same amino acid residues may strengthen the link between these mutations and the disease. The trend of having same site mutations was described in another study (Morrone et al., 2014b).

In our study, p.Ser287Leu was the most common mutation and has been shown to be less prevalent [20/1,091 alleles reported (1.9%)] among American Caucasian, Austrian, Greek, Macedonian, Middle Eastern, Polish, Spanish and Turkish patients (Bunge et al., 1997; Tomatsu et al., 2004c; Pajares et al., 2012; Dung et al., 2013; Morrone et al., 2014b). It has been previously described that the p.Ser287Leu mutation alters the hydrophobic core, packaging of the protein, and the Ser287 residue, thus leading to the reduction of GALNS activity (Sukegawa et al., 2000). The second, third and fourth most common mutations were p.Phe216Leu, c.1003-3C>G and p.Asn32Thr respectively, which were not reported in the literature.

Sixteen of the 42 missense mutations reside in the CpG dinucleotide site (Table 5.5). Mutations due to the transition of the CpG dinucleotide accounts for 38% of all missense mutations. This percentage was marginally higher in our study when compared to the other MPS IVA study (Tomatsu et al., 2004c). Interestingly, recurrent mutation p.Ser287Leu occurred from transitions at the CpG doublet, resulting in the deamination of cytosine and providing a possible explanation for the recurrence of this mutation.
Identification of 28 novel mutations and the absence of the most common mutations [c.1156C>T (p.Arg386Cys), c.337A>T (p.Ile113Phe), c.901G>T (p.Gly301Cys)] in our study, that was identified in other MPS IVA patients studied (Tomatsu et al., 2005b; Dung et al., 2013; Morrone et al., 2014b). These findings suggest that Indian patients with MPS IVA have a wide allelic heterogeneity and a distinct mutation spectrum when compared to other MPS IVA patients studied.

We identified a c.3G>A (p.Met1Ile) mutation in three patients, two in the homozygous state (patient 33 and 66) and the other in a compound heterozygous state with p.Pro151Leu (patient 55). The p.Met1Ile mutation may alter the start codon of GALNS and activate a downstream ATG which may be used for the initiation of translation. The p.Met1Ile mutation has been reported in a Filipino patient with severe bone deformity (Dung et al., 2013). Mutations p.Ser82Pro and p.Thr313Met were detected in the homozygous condition in patient 46 and patient 36 respectively, and the same mutations were present in Caucasian Canadian patients (Dung et al., 2013).

Majority of MPS IVA (64/72) had the severe (classical) phenotype, 6/72 had an attenuated phenotype and only 2 had mild phenotype. In the remaining 22 patients, we did not have information on growth parameters, and could not classify the severity. Homozygous mutations were present in 52 patients with severe phenotype and 10 severe patients had compound heterozygous mutations, another 2 patients with severe phenotype did not have the mutations. This demonstrated that severe phenotype has more severity due to homozygous mutations than compound heterozygous mutations (52 homozygous mutations in 64 severe phenotype). We observed p.Asn32Thr (1 allele in patient 42), p.Trp230Cys (1 allele in patient 25), p.Arg251Gln (1 allele each in patients 11, 42, 53), p.Ala291Ser (1 allele in patient 11), p.Arg386Ser (1 allele in patient 9), c.1003-3C>G (1 allele each in patients 9, 25, 53 and 2 alleles in patient 10) mutations in patients with an attenuated and mild phenotypes of MPS IVA. Hence, three of six patients with the attenuated phenotype and two patients with mild phenotype have compound heterozygous mutations. However, p.Asn32Thr, p.Ala291Ser, c.1003-3C>G mutations were also found in the homozygous state in those with a severe phenotype. The p.Arg251Gln mutation was found in one patient with a severe phenotype in compound heterozygosity with c.1003-3C>G (patient 17). His height was 110.5 cm. This
mutation was observed earlier with attenuated phenotype in a Korean patient (Park et al., 2013).

Exon 1 was the most frequent to harbor a mutation, followed by exon 8 and exon 7. These exons seem to be more susceptible to mutations in patients with MPS IVA in India and 39.8% of the mutations were clustered in these exons. This finding might facilitate devising a cost-effective diagnostic strategy in Indian patients with MPS IVA. Three novel missense mutations p.Cys79Arg, p.Arg83Trp, p.His142Pro were present in the active site of the GALNS enzyme (Rivera-Colón et al., 2012). p.Cys79Arg mutation may have an impact on loss of nucleophile.

The novel missense mutations discovered through this study were subjected to the prediction of their pathogenicity by a set of well-known tools available on the public domain. While a majority of the mutations were unanimously predicted to be pathogenic by all the tools, some were predicted inconsistently. Differences in predictions can be attributed to the inherent differences in the algorithms used by different tools, as well as the sequence-structure attributes used for characterization of wild type and mutant residues. It has also been suggested that alignment depth and quality used by the methods also impact their predictions (Tavtigian et al., 2008). Nonetheless, pathogenicity predictions provide a basis for further in-depth sequence and structural analyses of the mutations.

The sequence profile analysis of MSA of human GALNS with some of its orthologues revealed that the novel mutations are evolutionarily less preferred than their wild-type counter parts. A detailed structural analysis revealed that majority of the mutant sites are buried in the protein structure. The adverse structural/functional effects that could be associated to the novel mutations could be any of the following: (a) disruption of side chain mediated hydrogen-bonding/salt-bridge/disulfide bridge interactions, (b) disruption of the hydrophobic core by a non-polar to polar residue substitution, (c) introduction of unfavorable steric clashes due to the substitution of small residues to bulky residues and (d) substitution of functionally important residues. All mutations except p.Pro52Leu affect the structural and functional integrity of the individual subunits, and p.Pro52Leu specifically seems to hamper the biologically important homo dimer formation.
6.2. GM1 gangliosidosis

GM1 gangliosidosis is a heterogeneous rare disease that is characterized by neurodegeneration. It is difficult to draw a genotype-phenotype correlation for GM1 gangliosidosis due to the heterogeneity of the disease and mutations (Caciotti et al., 2011). The onset and severity may vary due to the variable accumulation of GM1 ganglioside and glycosaminoglycan in nervous and skeletal tissues (Hofer et al., 2009). Most of the patients in this study have infantile (71%) and juvenile (27%) forms which varies from the trends highlighted by Brunetti and Scaglia (Brunetti-Pierri and Scaglia, 2008). Central nervous system involvement was observed in all patients in this study. The clinical features we noted are classically observed in this condition and have been described in other studies (Brunetti-Pierri and Scaglia, 2008; Sperb et al., 2013). We did not, however, observe any good genotype-phenotype correlation. From our experience, GM1 gangliosidosis can be diagnosed by the presence of developmental delay, hypotonia and Mongolian spots. We have observed that the number of hyperpigmented macules increase with the progression of the disease, and that Mongolian spots have been noted in other storage disorders as well. The presence of cherry red spots is of great help to clinically narrow down the differential diagnoses. Thick gums and mild dysostoses need to be found, although it may be difficult to note in routine practice.

The mutation spectrum is known for several other populations (Nishimoto et al., 1991; Yoshida et al., 1992; Boustany et al., 1993; Silva et al., 1999; Georgiou et al., 2004; Sinigerska et al., 2006; Santamaria et al., 2007; Yang et al., 2010; Sperb et al., 2013). We report, for the first time, the mutations in GLB1 gene in GM1 gangliosidosis patients from India. Mutational analysis revealed 36 different mutations in 53 patients.

We observed that 66% of the mutations are missense/nonsense and the others are indels (17%) and splice site (17%). A similar observation was seen in the total number of mutations identified in GLB1 causing GM1 gangliosidosis (Figure 3.6). We observed homozygous mutations in 42 patients, of which 26 were born to consanguineous couples. Similar observations have been found in our previous studies on other autosomal recessive disorders (Dalal et al., 2012; Bidchol et al., 2014). This
pattern is likely for other recessive disorders in our country given the high degree of inbreeding.

The most common mutation in this study was c.75+2InsT which were present in nine infantile patients and one carrier (15% of the alleles). Six of these patients were homozygous and four were compound heterozygous with p.Trp527Leufs*5, p.Leu337Pro, p.Ile450Thrfs*11 and c.734-8A>G mutations. The c.75+2InsT mutation was reported to cause a 20 nucleotide insertion derived from the 5’ end of intron 1 of GLB1 which led to the abnormal splicing of pre-mRNA and gave rise to an abnormal protein product (Morrone et al., 1994). The second most frequent mutation was p.Leu337Pro (9.5% of the alleles) which was observed in six patients and has also been reported previously in the infantile form of GM1 gangliosidosis (Lei et al., 2012).

The p.Arg482His mutation found in three infantile patients in the homozygous state (patient 1, 2 and 27) has been reported earlier in the infantile form of GM1 gangliosidosis in Japanese and Maltese patients (Ishii et al., 1995; Caciotti et al., 2005b; Caciotti et al., 2007) and was found in the carrier status in the Cyprus population (Georgiou et al., 2005). This mutation interestingly, was reported in patients with MPS IVB when p.Arg482His was associated with p.Trp273Leu or p.Trp509Cys mutations (Oshima et al., 1991). The p.Arg482His was expressed in fibroblasts or COS-1 cells and produced a β-Gal protein with little or no residual activity (Ishii et al., 1995). The in vivo biochemical effect of p.Arg482His is difficult to determine, because this mutation occurs frequently in a compound heterozygous state. A different amino acid change is present in the same site at the 482 position (p.Arg482Cys) and also does not show β-Gal residual activity after its expression in fibroblasts of patients with GM1 gangliosidosis (Ishii et al., 1995).

Homozygous p.Arg49Cys and p.Gly123Arg mutations were detected in patients 26 and 25 respectively (Table 5.11). These mutations have also been reported in a Japanese patient with the infantile form of the disease, although one allele could not be identified (Nishimoto et al., 1991; Yoshida et al., 1991). The mutation p.Arg68Trp was detected in a carrier (family 5) and patient 50 (previously reported in Juvenile form of GM1 gangliosidosis) and has shown no β GAL activity in a mutant vector expressed in COS1 cells (Caciotti et al., 2003).
The p.Arg148Cys mutation was present in patient 47 in combination with a novel mutation: p.Gly134Arg. This mutation was previously identified in homozygous and heterozygous states and showed no residual β-GAL enzyme activity when expressed in COS-1 cells (Caciotti et al., 2007). The p.Asp332Asn mutation was detected in patient 39 and was found to have reduced catalytic activity of the β-GAL enzyme due to the mutation being present in the active site (Zhang et al., 2000). This mutation is more frequently detected in GM1 gangliosidosis carriers in Cypriot, Cyprus (Georgiou et al., 2005).

The p.Trp92* mutation was present in the infantile form in patients 37, 48 (homozygous) and 42 (heterozygous with p.Met1?). This mutation was previously detected in two patients with different phenotypes (Caciotti et al., 2011). The p.Arg457* mutation was identified in four infantile patients (patient 29, 40, 43 and 45) in the homozygous state and has been previously reported in a Japanese patient with the infantile form of GM1 gangliosidosis (Nishimoto et al., 1991). The p.Arg457* mutation leads to premature mRNA decay and was not detectable in COS1 cells (Nishimoto et al., 1991). The p.Trp527Leufs*5 mutation was observed in three patients with the infantile form (patient 12 and 15) and one carrier (family 22) of the same form of the disease. This p.Trp527Leufs*5 mutation was the most common mutation in the Brazilian patients with a frequency of 62.5% (Silva et al., 1999; Sperb et al., 2013). Another highly prevalent mutation in Brazilian, Iberian, and Roman patients was p.Arg59His (Santamaria et al., 2006; Sinigerska et al., 2006), but this mutation was absent in the present study.

A novel p.Arg201Pro was identified in the infantile form in patient 52 in this study. Other studies have reported a different amino acid change in the same site (p.Arg201His, p.Arg201Cys) in juvenile patients (Yoshida et al., 1991; Kaye et al., 1997).

Out of 22 novel mutations in GLB1, only one (Met1?) was observed in more than one patient and the remaining novel mutations were noted in single patients. These mutations were absent in databases, 537 Indians (covered in a next generation sequencing panel) and 50 normal Indian individuals. This absence suggests that the novel mutations reported here are likely to be pathogenic.
The functional domains (TBD, βD1 and βD2) carried 83% (30/36 of mutations) of mutations, suggesting an impact on protein function. A higher frequency of mutations (52% of the alleles) was observed in exons 1, 14 and 10, but in other studies frequency of mutations were clustered in exons 2, 6 and 15 (Brunetti-Pierri and Scaglia, 2008; Hofer et al., 2010). This information may be used for a cost-effective sequencing strategy to find the mutations in Indian patients with GM1 gangliosidosis.