Chapter 5

DISCUSSION
During the past three decades, major advances have been made in the elucidation and characterization of the molecular pathologies of inherited metabolic diseases. The specific enzymatic defects have been identified in more than 400 inborn errors of metabolism. For many of these disorders, demonstration of the enzymatic deficiency has led to the accurate diagnosis of the disease, carrier detection and prenatal diagnosis.

In India the screening, diagnosis, evaluation and comprehensive management of MPS is still in its infancy (Bharucha et al., 1998). The follow up of the patients and their family members are extremely difficult, since there are no defined follow up systems established for such genetic disorders. Also this is compounded by the non co-operation, low literacy and poverty levels of most of the patients and their family. Hence the entire task becomes individual perseverance.

There have been several scattered reports on MPS from India, which are mainly case reports and some extended to biochemical analysis. Mutational analysis of MPS is a virgin field in India and ours is the first attempt to study the molecular pathology of MPS in India, especially South India.

Our laboratory has been working on MPS for the past 10 years. In our experience, MPS III is found to be the most common type in India, followed by MPS IV, MPS I, MPS II and MPS VI respectively (Maya Sundari et al.,
1998). We have standardized the differential diagnosis of MPS based on the quantitative and qualitative analysis of urinary GAGs and definitive diagnosis by the assay of the pertinent lysosomal enzymes. Recently we have ventured to study the molecular pathology of MPS in India.

5.1 BIOCHEMICAL STUDIES

5.1.1 Urinary GAG analysis

Only the specific enzyme assay achieves the confirmatory diagnosis of MPS. There are 11 different enzyme defects in MPS (Neufeld and Meunzer 2001). It is not practical to assay all known enzyme defects in every suspected case considering the cost and time involved in the procedure. Hence urinary GAG analysis is a must to reduce the number of enzymes to be assayed in a suspected case.

5.1.2 Collection of urine sample

A 24 hour urine collection is considered to be the best choice for urinary GAG analysis. However most of the children referred are in their early childhood and are treated as out patient by the hospitals, making 24 hour collection problematic. This has been overcome by relating the urinary GAG excretion with urinary creatinine. Since the GAG / creatinine ratio obtained during the waking hours are considered to be similar to those obtained on complete 24 hour collection (Pennock et al., 1976). Creatinine excretion varies during 24 hour period and the GAG / creatinine ratio is increased at night (Di Ferrante and Lipscomb 1970). But the excretion of both substances
remain fairly constant during waking hours making analysis of random samples possible (Pennock et al., 1973; Pennock, 1976).

In our study, collection of infected urine samples and addition of preservatives to the urine were avoided since they are known to interfere with the isolation of urinary GAGs by lowering the efficiency of complex formation (DiFerrante, 1967). Hence fresh urine samples collected were stored frozen at -70°C until analysis which was usually within a week.

5.1.3 Quantitative analysis

The acid alcian blue complex formation method used for the quantitation of isolated urinary GAG is a simple, rapid and reproducible method for the estimation of sulfated GAGs, even in the presence of other polyanions or non sulfated hyaluronic acid (Gold 1981, Dembure and Roesel 1991). This method yields the same colour intensity with equal amount of different GAGs and permits direct measurement of the soluble alcian blue – GAG complex which has an altered absorption spectra compared to the pure dye. The earlier methods used (Whiteman 1973b) were cumbersome since they involved the isolation of the complex and its solubilization before spectrophotometric measurements. Also the present method is superior to the carbazole – sulfuric acid or borate modification method (Bitter and Muir 1962) which quantitate the GAG by measurement of their hexuronic acid content and hence misses KS which lacks hexuronic acid.
5.1.4 Qualitative analysis

Although quantitation of urinary GAG is effective in the detection of MPS patients, quantitation alone may lead to false positive and false negative results (Piraud et al., 1993). Also quantitation will not throw any light on the confirmatory diagnosis and typing of the patients. Hence it has to be coupled with qualitative analysis which mainly include CAME (Elango et al., 1998) and TLC (Dembure and Roesel 1991).

The present study utilized a discontinuous unidirectional electrophoretic method developed in our laboratory for the separation of standard and urinary GAGs (Elango et al., 1998). This method is a simplification of previously reported procedure (Hopwood and Harrison 1982; Dembure and Roesel 1991). In these methods, the separation was based on the differential solubilities of GAGs in ethanol and their mobilities in barium acetate at pH 5.0. In the earlier methods unless great care was exercised regarding constant cooling, it resulted in drying of the strips during electrophoresis due to salt deposition, hindering the buffer flow. On the other hand, our method has overcome this problem by reducing both the voltage (15 V/cm) and the buffer molarity (100 mM). The merits of our method rests in the simple and easy to handle apparatus, non-requirement of elaborate cooling system, sensitivity and high reproducibility of the results.

The GAG separation patterns obtained by us were slightly different from those reported earlier (Hopwood and Harrison 1982). Earlier workers observed an additional fast moving band (FDS 2) in the case of DS.
But we observed 3 bands (DS, FDS1 and FDS2) of varying intensities which may be due to variations in their concentration. In our hands KS always moved slower than CS in contrast to earlier reports (Cappelletti et al., 1979, Hopwood and Harrison 1982). Varying the concentration of ethanol or buffer molarity did not improve the mobility of KS. However this did not interfere with grouping of the patients for enzyme assays.

The data shown in the table 16 emphasizes the importance of a combined qualitative and quantitative analysis of urinary GAG in the diagnosis of MPS.

5.1.6 **Lysosomal enzyme assays**

5.1.6.1 **Lysosomal enzyme activity in Group I patients**

Considering the assay of arylsulfatase B for the diagnosis of MPS VI great care must be executed. The leukocyte lysates contain both arylsulfatases A and B, which act on the same substrate, hence care must be taken to differentially assay these enzymes. In arylsulfatase A assay, arylsulfatase B activity was inhibited and the kinetic anomaly was suppressed by pyrophosphate and high concentration of sodium chloride. In arylsulfatase B assay, the A activity was suppressed by high pH and an inhibitory substrate concentration. Under these conditions arylsulfatase A was largely inactivated by 30 min and reactivation of the enzyme by sulfate was prevented by barium ions. Arylsulfatase B activity was estimated from the reaction occurring in the 90 minus 30 min interval, with a correction for the
residual activity of the enzyme. In addition, arylsulfatase A was estimated in all MPS VI patients to rule out multiple sulfatase deficiency.

Also it was observed that arylsulfatase B activity showed a significant increase in MPS II patients (table 14a). This observation may be due to the visceral organs getting preferentially affected, resulting, for example, in hepatopathy and the diseased liver cells failing to clear acid hydrolyses from the circulation. Lysosomal ‘overloading’ also causes a dysfunction of the lysosomal apparatus. It is also reported that in MPS, the accumulated GAG may bind to the lysosomal hydrolases and increase their activity by protecting them against proteinase action (Kint et al., 1973).

5.1.6.2 Lysosomal enzyme assay in Group II patients

In the case of GALNS assay (MPS IV) great care was taken with respect to the blanks. Sulfates and phosphates are known powerful inhibitors for lysosomal sulfatases, hence the blanks (phosphate blanks) used in the present study contained, in addition to other components, 0.1M sodium phosphate, which completely inhibited GALNS activity. Also the leukocyte lysates used were extensively dialyzed to remove the “competitive inhibitor” which is known to substantially decrease the apparent Km value of the enzyme (Van Diggelen et al., 1990). These precautions helped a great deal in the proper assay of the enzyme and also helped in economizing the expensive substrate.

The 33 non MPS children excreted only CS in their urine, which is the pattern shown by normal children, but in spite of this they were not
normal. This may be due to a number of other pathological conditions like connective tissue disease, various bone disease, some type of oligosaccharidoses, Marfan syndrome (Endreffi and Dicso 1988; Piraud et al., 1993) are known where CS is the main GAG excreted.

5.2 CARRIER DETECTION

Carrier testing is the service most frequently requested by MPS families, second only to demands for effective therapy. Identification of carriers by the assay of pertinent lysosomal enzymes in the leukocytes are most reliable when the carrier levels are in the low range, mutually excluded from the normal range and least reliable when the activity overlaps with that of the normal range. The latter is the situation seen in the case of most of the MPS disorders. However, these biochemical detections will be soon superseded by molecular methods as and when the mutational data become available and also less expensive (Neufeld and Meunier, 2001).

At present there is no effective treatment for MPS making heterozygote identification a must, followed by genetic counseling in the families that already have an affected child. Studies have already demonstrated that the biochemical characterization of the enzyme from normal individual and heterozygotes could be highly useful for the detection of carriers (Dulaney et al., 1979; Momoi et al., 1977; Minami et al., 1980).
5.2.1 Carrier detection in MPS I families

In the present study, carrier levels of the enzyme $\alpha$-L-iduronidase was estimated in 10 obligate heterozygotes to prove / disprove the earlier observation from our laboratory using 20 obligate heterozygotes. The data obtained confirmed the earlier observation and the combined data showed the activity range of this enzyme in the 30 carriers (obligate heterozygotes) was mutually exclusive from the normal and patient ranges and also confined a narrow range. This makes the measurement of IDUA levels, a highly reliable method for carrier detection in our population, an observation also reported by earlier workers (Dulaney et al., 1979; Momoi et al., 1977; Minami et al., 1980).

5.2.2 Carrier detection in MPS VI families

Carrier levels of the enzyme arylsulfatase B were estimated in 1C obligate heterozygotes. It was observed that the activity range in the carriers completely overlapped with that of the controls. This suggests that the measurement of arylsulfatase B is highly unreliable method for carrier detection as has been reported earlier by others (Beratis, et al., 1975; Haskin: et al 1979).}

5. Consanguinity and MPS

A recessive gene disease requires the inheritance of a mutant allele at the same genetic locus from each parent. When the genes are rare th
possibility of unrelated parents being carriers for the same defect is small. If the parents have a common ancestor who carried a recessive gene, the possibility that the parents inherited the same allele is enhanced. Hence, the less frequent the recessive gene, the stronger the possibility that an affected individual is the product of a consanguineous mating. On the other hand when recessive genes are common in the population, the likelihood of two unrelated parents being carriers is great enough to minimize the role of consanguinity. For common traits, such as sickle cell anemia, phenylketonuria, cystic fibrosis and Tay-Sachs disease, consanguinity is uncommon in the parents (Neufeld and Meunzur 2001).

In India consanguinity is still widely prevalent (Appaji Rao et al., 1998). Consanguineous marriages are still strongly preferred in Karnataka, and high in parts of Tamil Nadu and Andhra Pradesh (Appaji Rao, 1998; Dronamraju and Khan 1963; Joshua 1974; Veerraju et al., 1978). It has a large number of endogamous castes, tribes and religious communities following different marriage patterns and are largely governed by three important regulations namely endogamy, exogamy and consanguineous marriages (Basu 1983). Also there exists a difference between North and South Indians regarding marriage regulations. By comparison Dravidian Hindus of South India, strongly favour marriages between biological kin. A study conducted in early 90’s has estimated that cumulative consanguinity – associated mortality upto early adulthood is, on an average 4.4% higher in
first cousin progeny than in non-consanguineous progeny (Bittles and Neel, 1994).

Contradictory reports have been published in recent years. Some workers indicate that consanguinity has very little or no role in congenital malformations, physical, mental and other heritable disorders. However it is a matter of common knowledge that when closely related individuals marry the chance of similar alleles combining increase many fold (Goswami, 1983) and the probability of abnormalities of embryonic deaths by recessive genes in the progeny of consanguineous marriages is much greater than in unrelated marriages (Basu, 1983).

Consanguinity was analysed by in a very limited study group (53 cases). Inspite of this the trends observed showed a strong link between MPS and consanguinity (MPS I, 67%; MPS VI, 80%). On the other hand the percent consanguinity was relatively low in the case of controls (30-35%) and an intermediate relationship (about 56%) was seen in the case of non MPS patients who may be suffering from other lysosomal disorders (oligosacchariduria, mannosidosis, mucolipidosis, sialidosis, etc.). These observations substantiate the view that the less frequent the recessive gene the stronger the possibility that an affected individual is the product of consanguineous mating. MPS being a rare genetic disorder, may be contributing to the above observation.
5.4 MUTATIONAL ANALYSIS

5.4.1 Methodology

The identification of disease causing mutations in a patient is the first step in examining genotype–phenotype correlation. The gene mutations can be divided into two categories, category one is gross rearrangements, large insertions, duplications or inversions. Such mutations can be detected by Southern blot or fluorescence in situ hybridization (FISH). Another category is single base substitutions or rearrangements, affecting only a few base pairs. For the analysis of this group of alterations, the polymerase chain reaction (PCR) is an important tool.

With increasing numbers of patients to be analyzed for mutations, simple and reliable screening methods are necessary. During the last few years screening methods like chemical cleavage mismatch (CCM), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) have been developed. CCM is time consuming and requires both radiolabelled and toxic chemicals. DGGE requires a special, relatively expensive apparatus and PCR primers, designed especially for this purpose. On the other hand, SSCP analysis is straightforward and does not require complicated laboratory equipment, it has become popular and represents the most frequently used screening method (Bunge et al., 1996). Based on the observation in our laboratory we use a simple and nonisotopic method of SSCP to screen the mutations.
Single stranded confirmational polymorphism (SSCP) is a convenient method of screening mutations before sequencing the gene. The principle of this method is that single stranded DNA (ssDNA) has a folded confirmation which is determined by intramolecular interactions of its bases. When electrophoresed on nondenaturing polyacrylamide gel, the ssDNA will have a specific mobility depending on the base sequence. Any difference in the base sequence of a ssDNA sample, due to a mutation or polymorphism will be detected as a mobility shift and will produce a different band pattern when compared to the normal (Orita *et al.*, 1989).

It is known that complementary single strands are better separated in gels with low cross linkage. The same rule holds true for SSCP gels in detecting mutation. It has been found empirically that the presence of low concentration (5-10%) of glycerol in a gel frequently improve separation of mutated sequences (Orita *et al.*, 1989). The reason for this is unknown, perhaps glycerol because of its weak denaturing action on nucleic acids partially opens the folded structure of single stranded nucleic acids so that more surface area of the molecule is exposed and thus there is more chance for acrylamide fibres to sense locally confirmed structural difference caused by mutation. It should be noted however that in rare cases mutated sequence show mobility shift only in gels without glycerol (Hayashi, 1991).

Occasionally one strand is separated into two or more bands in the SSCP gel even though the sequence is the same (Orita *et al.*, 1989). This suggests that strands having the same sequence can have different stable conformations. In rare cases one or both strands are detected as smears rather
than bands (Hyashi, 1991). This was observed in our study also (exon VII and exon XIII and XIV).

An advantage of PCR – SSCP analysis over other PCR based techniques for mutation detection is its simplicity. In PCR – SSCP, mutations are detected by the presence of shifted bands rather than by the absence of signals as is the case in some other detection methods (Hyashi, 1991).

5.4.2 Mutational Studies

5.4.2.1 Exon IX

About 30 polymorphisms and / or nonpathogenic sequence variants have been detected in IDUA gene. This is an extraordinarily large number of polymorphisms compared to many other disease genes of similar size (Hopwood et al., 1993). Of the 30 nonpathogenic sequence variations only one, which was detected in an Iranian patient, involves an insertion of a C in an string of nine Cs in intron 8, along with the severe mutation 1277 ins 9 in exon VIII (Bunge et al., 1995). In our study we did not find any mutation in exon VIII in the 16 MPS I patients analysed or in the control. Whereas the polymorphism / sequence variation, namely ins C in intron 8, was observed by us in 2 of our controls or normals and not in the patients. Hence this forms the first report of an ins C in intron 8 of normals.

The polymorphisms G 409 R, T 410 and V 454 I are reported in the exon IX of MPS I patients (Bach et al., 1993, Scott et al., 1993 and Bunge et al., 1994). The polymorphisms T 410 and V 454 I are also reported in normals with a frequency of 0.22 and 0.26 respectively (Scott et al., 1995). In
our study we found these two polymorphisms in controls (T410 and V454), but the same was not found in our patients.

5.4.2.2 Exon X

The polymorphism t → c at 2795 nucleotide position of intron 9 (Scott et al., 1993), C → T at 2915 nucleotide position of exon X (Bunge et al., 1994) and g → t at 3013 nucleotide position of intron 10 (Scott et al., 1993) are reported in MPS I patients. In our study we find these three polymorphisms / sequence variation in two of our MPS I patients and in a normal. It is interesting to add that these 3 polymorphism / sequence variations seen in our patients is also seen in our normal. This is the first report regarding the presence / identification of these polymorphisms in the normals (controls).

It is not known what effect any of the polymorphisms may have individually or in combination, on the stability and processing of the IDUA mRNA, alternative splicing, the rate of translation of the IDUA protein etc. However the polymorphisms are likely to be a contributing factor to variation in IDUA activity in healthy individuals and it is also likely that they could modify the severity of MPS I mutations (Scott et al., 1991; 1993).

A MPS I patient of Asian Indian origin was studied for mutation and result showed L 490 P, a T → C transition in codon 490 converting leucine (CTG) to proline (CCG). This mutation gave the intermediate phenotype, Hurler-Scheie. The parents of the patient were found to be non consanguineous. Therefore it is reported that the mutation L 490 P is
relatively common among Indian MPS I patients (Tieu et al., 1995). However the patient analysed was a Pakistani. from our study this mutation was not found in the 16 patients analysed.

It is accepted that differences in environmental and genetic backgrounds may in part explain differences in the clinical phenotypes of patients with the same disease genotype (Neufeld 1991). Indeed, most of the mutations for MPSI that have been observed more than once, are associated with more than one haplotype which is evidence of differences in genetic background, including differences within the IDUA gene (Scott et al., 1992, 1993). Based on the above discussion it appears that the disease causing mutations of our patients may be different from other populations reported.

DNA polymorphisms occur with even greater frequency outside of coding regions in parts of the genome that have little or no effect on gene expression. There is great interest in trying to use SNPs (Single Nucleotide Polymorphisms) to unravel the genetic contribution to complex traits (Collins et al., 1999). These polymorphisms might themselves contribute to disease susceptibility or be in linkage disequilibrium with disease-related variations.