Mucopolysaccharidoses are rare genetic disorders. To date 7 MPS types caused due to deficiency of 11 different lysosomal enzymes are known. Clinical diagnosis of the disease is highly problematic due to high degree of overlap between the clinical phenotypes among the MPS types and between MPS and other LSDs. Hence biochemical analysis becomes a must. In India, the screening, diagnosis, evaluation and comprehensive management of MPS is still in its infancy (Bharucha, 1998). The follow-up of the patients and their family members is extremely difficult, since there are no defined follow-up system 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 families. Hence the entire task depends on individual perseverance.

There have been several scattered reports on MPS from India (Ganguly et al., 1986, Balamurugan, 1990), which are mainly case reports and some include biochemical analysis. Mutational analysis is a virgin field in India and ours is the first attempt to study the molecular pathology of MPS in India, especially South India.

In our laboratory we have standardized the methods for differential diagnosis of MPS based on quantitative and qualitative analysis of urinary GAGs followed by definitive diagnosis by the assay of pertinent lysosomal enzymes. Mutational study on MPS I has been initiated in our laboratory and recently preliminary mutational analysis of MPS III has been initiated.
5.1 URINARY GAG ANALYSIS

The main objective for analyzing urinary GAG is to facilitate selection of probable defective enzyme to be examined for final diagnosis (Dembure and Roesel, 1991). Because it is not practical to assay all possible enzymes in every suspected case, in view of the cost and time involved in the procedures.

5.1.1 Collection of urine samples

A 24 hr urine collection is considered to be the best choice for urinary GAG analysis. However most of the children referred were in their early childhood and were treated only as out patients by the hospitals, making 24 hr collection difficult. This problem has been overcome by normalizing the GAG excretion to urinary creatinine. The GAG/creatinine ratio obtained during waking hours is considered to be similar to those obtained in complete 24 hr collection (Pennock, 1976) and this ratio increases at night (Di Ferrante and Lipscomb, 1970). But, the excretion of both substances remains fairly constant during waking hours making analysis of the ratio in random samples a possibility (Pennock et al., 1973, 1976).

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

The amount of GAG in urine is small because of which its prior isolation is required. In the alcian blue complex formation method (White and Young, 1977) used in the present study, urinary GAGs were isolated by complex formation with alcian blue 8GX. This dye, a copper containing cation, forms insoluble complexes with anionic GAGs under suitable conditions. Maximum complex formation takes place at pH 5.8 and in the presence of 50 mM magnesium chloride (Whiteman, 1973a,b; Whiteman and Young, 1977). This method is based on charge rather than composition of GAG backbone, precipitates GAGs below 3000 molecular weight, unlike CPC method and hence is more reliable. This consideration is very critical since, the urine of some MPS patients may contain 2 or more GAGs with low average molecular weight which could be missed by the CPC method (Whiteman, 1973a,b).

5.1.3 Quantitative analysis

The acid alcian blue complex formation method used for the quantitation of isolated urinary GAGs is a simple, rapid and reproducible method for the estimation of sulfated GAGs even in the presence of other polyanions and nonsulfated hyaluronic acid (Gold, 1981; Dembure and Roesel, 1981). This method yields the same color 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 (Hsu et al., 1971; Whiteman, 1973a,b).
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 (Dishe, 1947; Bitter and Muir, 1962) which estimate the GAGs by the measurement of their hexuronic acid content and hence fails to detect KS, which lacks hexuronic acid.

5.1.4 Qualitative analysis

Although estimation of urinary GAGs is effective in the detection of MPS patients, this alone may lead to false negative results, especially in MPS III and MPS IV patients, where the GAG excretion is very low (Sewell, 1988; Piraud, 1993). Also quantitative estimation will not throw any light on the confirmatory diagnosis and typing of the patients. Hence it has to be coupled with qualitative analysis. Even with the qualitative analysis no single separation method can identify all GAGs (Dembure and Roesel, 1991), hence electrophoretic and chromatographic methods are coupled in the present study.

The differential diagnosis is based in part upon the detection of abnormal distribution of sulfated GAGs in urine. Normal urine contains mostly chondroitin sulfate with small amounts of heparan sulfate and dermatan sulfate. Patterns obtained with MPS patients are clearly different from normal (Cohen et al., 1977). 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. In these methods, the separation is based on the 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 (15v/cm) and the buffer molarity (100 mM). The merits of our method rests in the simple and easy to handle apparatus, non-requirement of an elaborate cooling system, sensitivity and high reproducibility of the results. The GAG separation patterns obtained by us are slightly different from what was reported earlier (Hopwood and Harrison, 1982). Earlier workers observed an additional fast moving band (FDS2) 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 concentrations.

KS and HS gave very faint bands even for the standards. The limited capacity of cellulose acetate membrane strips to retain GAGs make detection of small amount of KS and HS difficult by this method. Also keratan sulfate excreted by MPS IV patients may be only 5 -16 % of urinary GAGs (Huang et al., 1985) and thus its detection on cellulose acetate membrane may require overloading.

The multisolvent thin layer chromatography is an excellent alternative method for detection of KS and HS. However by this method HS forms a
diffused band that is not well separated from chondroitin sulfate (Dembure and Roesel, 1991). Hence both the qualitative methods are used in the present study in the identification of MPS III and MPS IV patients.

Excretion of HS / HS and CS is unique to MPS III, thus they can be identified at this stage but enzyme estimations will help in the identification of their subtypes.

On the other hand KS/ KS and CS/ CS alone excreting patients were classified under same group and assigned MPS IV or non MPS as their probable MPS type. All these patients were estimated for MPS IV A and MPS IV B enzymes for 3 reasons.

1. KS and CS do not always separate because mobility of KS is very sensitive to small changes in the ethanol concentration.
2. KS excreting other lysosomal storage disorders are reported.
3. In adult Morquio cases, the KS excretion may even be normal (Pennock, 1976).

Hence enzyme assays can only differentiate MPS IV and non MPS patients.

5.2 ANALYSIS OF URINARY N-ACETYL GLUCOSAMINE SULFATES

The excretion of sulfated N-acetyl monosaccharides in the urine was first shown in Sanfilippo D patients (Hopwood and Elliot, 1983). Later this
excretory product was used to distinguish between MPS III A, III D, IV A, VI and multiple sulfatase deficiency (MSD) patients. However the procedure appeared more sensitive for MPS III D and MSD patients who show a N-acetyl glucosamine-6-sulfate sulfatase deficiency (Nowakowski et al., 1990).

In the present study urinary N-acetyl glucosamine sulfate analysis was undertaken to subtype the already identified MPS III patients (by TLC and CAME). The method does not identify MPS III B and III C patients who do not excrete GluNAc6S in their urine. MPS III A patients who give a mild positive reaction are identified and differentiated from MPS III D patients who give a strong positive reaction. The method was found to give very low false positive results (Nowakowski et al., 1990).

5.3 BLOOD ANALYSIS

Leukocytes and fibroblasts are reported to be the most reliable sources for lysosomal enzymes (Wenger and William, 1991). In the present study leukocytes were chosen for the assay of lysosomal enzymes. Leukocytes were isolated within 8 hours of blood collection. Though a variety of techniques are reported for the isolation of leukocytes from whole blood, dextran sedimentation method which yields 70% leukocytes and removes 99% of erythrocytes was chosen as the method of leukocyte isolation in the present study.
5.3.1 Lysosomal enzyme assays

The lysosomal enzymes were assayed by fluorimetric procedures. These procedures though expensive, are very sensitive and highly reliable. Using these procedures the normal activity ranges of the enzymes were determined in our population. Since the activities are known to vary with race, geographical location etc. In all our studies the normal ranges determined by us were used for comparison.

The activity range of heparan sulfamidase determined by us was comparable to the literature value. Normally this enzyme shows low activity in leukocytes due to its degradation by granulocyte proteases. Hence great care was taken to include pepabloc, a protease inhibitor and elevated assay temperature (Whiteman and Young, 1977; Hopwood and Elliot, 1981 a, 1982) to increase the activity of sulfamidase (Karpova et al., 1996). Dialysis of leukocyte lysate did not increase the activity of the enzyme. Hence S12 was used directly as the source of heparan sulfamidase in our assays. The residual activity in total leukocyte from MPS III A patients was 0-5.3 % of mean control value, whereas 0-10% has been reported in a study with 8 patients (Karpova et al., 1996).

In the case of GALNS assay (MPS IV A) 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, 0.1 M 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 in the use of the expensive substrate.

5.3.2 Carrier detection

At present there is no effective treatment for MPS making carrier identification a must, followed by genetic counseling of 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 (Dulany et al., 1976). In carrier detection studies, leukocytes are found to be better source for enzyme assay compared to fibroblasts. The results are most reliable when the enzyme activity is in the lower range and least reliable when the activity is in the control range.

5.3.2.1 Carrier detection in MPS III and MPS IV families

In the case of MPS III A families, the mean enzyme activity of obligate heterozygotes (2.37± 1.19) was below that of the control (5.65± 1.9). But when the individual values were compared there is an overlap between the ranges of obligate heterozygotes (1.19 – 5.17 nmol/ 17hr/ mg protein) and the control (2 – 9 nmol / 17 hr / mg protein). Out of 13 carriers analysed only 6 showed values not overlapping with the control value. Thus limiting its usefulness in carrier detection. There are also reports of carrier detection for
MPS III A (Schmidt et al., 1977; Hopwood and Elliot, 1982) where the mean activity for obligate heterozygotes was below the control values but showed a considerable overlap (Toone and Applegarth, 1988).

In the case of MPS III B, the range of α-N-acetylglucosaminidase activity in the 13 obligate heterozygotes (2.3 – 6.8 nmol / 17 hr / mg protein) was found to be mutually exclusive from the control range (8 – 20 nmol / 17 hr / mg protein). This makes the assay of α-N-acetylglucosaminidase, a highly reliable method for carrier detection.

In the case of GALNS, like that of heparan sulfamidase there is an overlap between the control and heterozygote ranges. In the present study out of 11 obligate heterozygotes analyzed, 72.5 % showed the values overlapping with control range. This suggests that the measurement of GALNS is a highly unreliable method for carrier detection as has been reported earlier from our laboratory and by others (Fukuda et al., 1992).

5.4 PREVALENCE OF MPS

MPS III is reported to be the most common MPS type in many populations (Spranger, 2001) and our laboratory data also is in accordance with this observation. Sixty seven out of 147 MPS cases analyzed (46%) by us are MPS III patients. MPS IV is the second most prevalent in our population and 36 out of 147 cases analyzed (24%) are MPS IV patients. Thus MPS III and MPS IV are found to be the most prevalent MPS types in our population.
Among the MPS III patients analyzed in the present study, MPS III A and III B patients are found to be more common than MPS III C and III D patients. MPS III B patients contributed 34% and MPS III A patients contributed 28%. The rest were MPS III C and III D patients. This observation is in agreement with the literature report that subtype B is the most common with a predominance in Greece and Italy (Weber et al., 1997) whereas in united kingdom (Whiteman & Young, 1977), Netherlands (Van De Kamp et al., 1981) and in Northern Europe, MPS III A is more prevalent.

5.5 CLINICAL FEATURES OF MPS III, IV & NON MPS PATIENTS

Analysis of clinical phenotypes in MPS III patients showed that combination of features like developmental delay, hyperactivity and progressive dementia in many cases. The skeletal pathology for MPS III is relatively mild and develops often after a diagnosis is established. The minimal skeletal involvement together with inability to detect heparan sulfaturia often leads to delayed diagnosis (Neufeld and Muenzer, 1983). As in other MPS conditions, considerable variation in onset and the severity of the clinical phenotype are observed.

In the case of Morquio patients, skeletal abnormality with normal intelligence was clearly seen but many of the nonMPS cases who were referred in suspicion of MPS also showed similar symptoms.
Clinical features observed in our MPS III and IV patients were similar to those observed in the literature and no feature specific to our population has been observed.

Diagnosis of MPS patients based on the clinical features is not really feasible because of the overlapping clinical features shown by them not only among MPS types but also with other lysosomal storage disorders. Also diagnosis depends on the clinician and the recorded feature may not be the actual phenotype of the patient. Therefore these records can not be used in analyzing patients (Weber et al., 1997).

5.6 CONSANGUINITY AND MPS

In India consanguinity is still widely prevalent (Appaji Rao, 1998). Consanguineous marriages are still strongly preferred in Karnataka and high in parts of Tamil Nadu and Andhra Pradesh (Appaji Rao, 1998; Joshua, 1974; Veeraraju, 1978). India has a large number of endogamous castes, tribes and religious communities following different marriage patterns which are largely governed by three important regulations namely endogamy, exogamy and consanguineous marriages (Basu, 1983). There is a difference between North and South Indians regarding marriage preferences. Dravidian Hindus of South India, strongly favour marriages between biological kins (Bittles and Neel, 1994).

Consanguinity was analyzed by us in a very limited study group (76 cases). In spite of this, the trends observed showed a strong link between MPS and consanguinity. In the present study biochemically confirmed MPS
patients showed 68% of consanguinity (MPS III + MPS IV). This reflects the fact that in any autosomal recessive disorder consanguineous marriages have a greater chance of inheriting identical copies of a mutant gene or genes from a common ancestor (Bittles et al., 1991, 1994).

The non-MPS families also showed high percentage of consanguinity (69.5%). This suggests that these families may also be carrying genes for other autosomal recessive disorders. These patients were clinically suspected to be suffering from MPS and many of the lysosomal storage disorders exhibit overlapping clinical features with MPS and also excrete GAGs in their urine. Hence it will be worthwhile analysing these patients for other lysosomal storage disorders.

5.7 MUTATIONAL ANALYSIS

5.7.1 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 number 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 stranded conformational 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 these observations in our laboratory we use a simple and nonisotopic method of SSCP to screen the mutations.

Single stranded conformational 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 conformation which is determined by intramolecular interactions of its bases. When electrophoresed on a 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 structural difference caused by mutation. It should be noted however that in rare cases mutated sequence show mobility shift only in gels with out 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 the 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).

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 in some other detection methods (Hyashi, 1991).

5.7.2 Mutational analysis of MPS III A Patients

The identification of mutations that are particularly frequent in certain populations will greatly assist molecular diagnosis and carrier detection (Weber et al. 1997). MPS III A results from heterogeneous mutations as well as prevalent mutations (Montfort et al., 1998; Di Natale et al., 1998; Blanch et al., 1997; Weber et al., 1997; Bunge et al., 1997). Among the prevalent mutations, R245H, R74C, 1091 del C and S66W are the most frequent mutations in Dutch (56.7%), Polish (56 %), Spanish (45.5%) and Italian
(33%) populations respectively (Beasley et al., 2000). A search of SGSH mutations in the human gene mutation data base (HGMD) indicates a total of 68 small alterations including 52 single nucleotide substitutions (missense or nonsense mutations), 1 splice site mutation, 8 small deletions and 7 small insertions.

In the present study 3 out of 8 exons of SGSH gene have been chosen for analysis. These exons (II, VI, and VIII) were chosen since they are reported to have the most common mutations (S66W, R74C, R245H and 1091 del C) and 63% of the reported mutations are present in these 3 exons.

These 3 exons were analyzed for SSCP by PAGE followed by silver staining. Silver staining is a safer method which avoids the hazardous radioactive labeling and at the same time it is a very sensitive method capable of detecting very small amounts of DNA. Any positive band shift obtained using the above method were considered to indicate mutation / polymorphism and were subjected to direct DNA sequencing.

Of the 3 exons analysed only exon VI showed polymorphism.

5.7.2.1 SSCP analysis of exon VI

We obtained 3 different band patterns when the exon VI was analyzed by the above method (ph.13). Of this only one band pattern (P9) showed sequence variation / polymorphism. The variations observed were a silent mutation at codon 227 and an insertional polymorphism in intron 5. Ins CT (676-37) polymorphism in the intron 5 has already been identified in one
patient (Di Natale et al., 1998). The silent mutation at codon 227 observed by us (P227P) has not been reported in literature but a pathogenic mutation P227R at the same nucleotide position resulting in severe form of MPS III A has been reported (Di Natale et al., 1998). R465H a common polymorphism (Blanch et al., 1997) reported by others was not observed in the present study. Reports are also available to show that the presence of many polymorphisms in intron 5 complicate the SSCP pattern of exon VI and their interpretation (Bunge et al., 1997).

Even though many common mutations are reported in these 3 exons and a high mutational rate, no pathogenic mutation was identified in all the 9 sulfamidase deficient patients studied. This may be due to the following reasons.

1. The methodology used by us may not be sensitive enough to pick up the mutations or the band shifts may be very small which was missed by us.

2. Silver staining may not be as sensitive as radiolabelling (Di Natale et al., 1998).

3. In our population, mutation in the enhancer/promoter region of the gene or intronic insertion/deletion which influence the transcription may be present (Weber et al., 1997).
4. The size of the PCR product could have hindered the identification. Except exon VI, the PCR products were >200 bp in our study and SSCP analysis is known to detect up to 90% of the mutation only if the PCR product analyzed is <200 bp (Hayashi and Yandell, 1993). The likelihood of detecting a base pair change is reduced significantly as the size of the analyzed fragment is increased (Weber et al., 1997).

5. Last but not the least, the mutations may be present in the other 5 exons which were not included in the present study.