Chapter-4

Materials and Methods
MATERIALS AND METHODS:

Sample Size:

A total number of 31 subjects with a confirmed diagnosis of either Gaucher disease, Neiman Pick Disease and MPS were evaluated over a period of 4 years, as the prevalence of these disorders is very less. The control group included 60 healthy, age and sex matched subjects who were not siblings of the index cases and hence could have been heterozygous or carriers. The sample size calculation was based on likely number to be referred to a center for reference of inborn errors of metabolism rather than a statistical calculation considering that all these are subgrouped as “orphan diseases”

Sample Collection:

Human serum samples was collected by clean venipuncture and immediately added to Becton Dickinson serum separator tubes, allowed to clot for 30 min at room temperature, and centrifuged for 15 min at 3000g at room temperature. Samples was aliquoted immediately and stored at -80 °C. Human plasma samples was collected in Becton Dickinson citrated plasma collection tubes and centrifuged for 15 min at 3000g at 4 °C. Three ml EDTA blood was collected for enzymatic assays from 60 control subjects and 31 patients during the study period and 3 ml of blood in a plain vacutainer as described was used for the respective ELISA. Informed consent has been taken from all subjects according to the Declaration of Helsinki.
Methodology

We had collected 3 ml EDTA blood and Centrifuged at 4°C at 3,000 RPM for 10 minutes and took 1 ml Buffy coat from the middle of the blood in 15 ml tube and kept in ice, then 2 ml of chilled lysis buffer was added and mixed gently and kept for 15 minutes. It was mixed twice in between and centrifuged at 4°C at 2500 RPM for 10 minutes. The supernatant was discarded and the pellets were kept in ice. Two ml lysis buffer was added in the tube and mixed gently, the tubes were left for 5 minutes in ice. Four ml 0.9% chilled NaCl was added in the tube, mixed gently and centrifuged at 4°C at 2500 RPM for 10 minutes. The supernatant was discarded and the pellets were kept in ice and 4 ml 0.9% chilled NaCl was added, mixed gently and left for 5 minutes in ice. Tubes were centrifuged at 4°C at 6000 RPM for 15 minutes. The supernatant was discarded and the WBC was stored at -20°C until use. The above blood samples were also collected twice more in follow up (one year after 1st contact, two year after 1st contact) using only ELISA sample for assay apart from evaluating their basic biochemical status i.e., complete Hemogram, Serum alkaline phosphatase etc.
PREPARATION OF LEUKOCYTES LYSATE

Initially a 0.01% Triton X solution was prepared. The Leukocytes were allowed to thaw and the distilled water was poured on the top of the leukocytes (without disturbing white cells) and these were then suspended in 400-500ul of 0.01% Triton X. During the assay, the leukocytes were disrupted by repeated freezing and thawing using 5-6 cycles. The Leukocytes were centrifuged at 6000 rpm at 4°C for 15 minutes and transferred the supernatant in small tube. These were stored at -20°C till use.

Protein Determination:

We diluted 0.05ml of cell lysate with 0.30ml deionized water to make it to a final dilution of 1:7 in duplicate tubes. 0.10ml of each diluted lysate was added. 0.10ml water was used for the blank control. Then we have added 0.20ml deionized water and 1.0ml of solution A and incubated for 10 minutes at room temperature. 0.10ml of solution B was added, vortexed, and incubated for 30 minutes at room temperature. We read the absorbance at 750 nm on a spectrophotometer.

Standard Curve for Lowery Assays

The standard curve was established and reassessed to ensure accuracy of results. It was linear over its entire range of 0.10-0.50 mg/ml as linearity cannot be assumed above these values.
We prepared bovine serum albumin (BSA) standard solution of 0.10, 0.20, 0.30, 0.40 and 0.50 mg/ml by weight in deionized water. These were stored at -20°C for several months. We followed the procedure for the Lowery assays, using 0.10 ml of the BSA standard solutions instead of the cell lysate. For the blank, 0.10 ml of water was used instead of the solution. The concentration of BSA standards vs the absorbency was plotted at 750 nm. The absorbance of 1.0 mg/ml was calculated.

**Determination of Protein in the Samples**

The following equation was used to estimate the protein concentration in the samples.

\[
\text{Protein concentration (mg/ml)} = \frac{\text{absorbance of sample X}}{7 \times \text{Absorbance of 1 mg/ml standard (BSA)}}
\]

**Measurement of Beta-Glucosidase Activity For Gaucher Disease**

For the above stated estimation, 0.1 ml Triton X-100 was diluted in 12.4 ml methanol. 125 mg sodium taurocholate was added. 0.05 ml of the detergent mixture was placed into 12 x 75 mm test tubes according to the number of patients and controls to be evaluated, and into two extra tubes for the blanks. All tubes were placed in a 60°C oven for several hours until completely dry. Each tube was containing approximately 0.5 mg sodium taurocholate and 0.4 mg Triton X-100.

The substrate was 5 mM 4-MU-β-D-glucopyranoside (1.7 mg/ml) in 0.2 M phosphate-citrate buffer at a pH of 5.4.
The assay required 0.030 mg protein for leukocytes. 0.10 ml of tissue lysate diluted to achieve the necessary protein concentration was placed into the 12 x 75 mm test tubes containing the detergent mixture as prepared above. Duplicate tubes were prepared for each sample.

0.10 ml of the substrate solution was then added at time zero and incubated at 37°C for 1 hour. The reaction was stopped by adding 1.30 ml of 0.17 M glycine-carbonate buffer, pH 9.8.

For the blank, substrate was incubated alone. The reaction was stopped by adding the glycine-carbonate and only then control lysate preparation diluted to the appropriate range of protein concentration was added. The fluorescence was measured at excitation wavelength 360 nm and emission wavelength 415 nm. The readings were taken in triplicate and if a variance of > 15% was found the samples were re-evaluated.

The activity was calculated as follows:

\[
\text{Leukocyte activity} = \frac{\text{fluorescence of (sample – black tubes)}}{\text{mg protein per assay} \times \text{fluorescence of 1 nmol 4-MU}} \times \text{mg protein per assay x fluorescence of 1 nmol 4-MU}
\]

Control range established from healthy controls in our lab was: 6.0 – 9.0 nmol/hr/mg
Measurement of Sphingomyelinase Activity for Niemann Pick Disease

ENZYME SOURCE: WBC Lysate was prepared as described above

ASSAY PROCEDURE: Initially the Test tubes were labeled as test (‘T’), Blank (‘B’) and Standard (S1-S5). 25 µl of acetate buffer was added to all the tubes. 50 µl of enzyme source (WBC lysate = 70+/- 10 µg protein) was added to tube labeled as test (‘T’). 50µl of water was added to reagent blank (‘B’). 50µl each of working standards (WS1-WS5) of 2-N-hexadecanoylamino 4- nitrophenol was transferred to respectively labeled tubes (S1-S5). All tubes were pre- incubated at 37°C for 10 minutes. The enzyme reaction was initiated by transferring 25µl of substrate to all tubes. All tubes were allowed to incubate for 60 minutes at 37°C. After 30 minutes of incubation, 0.15 ml glycine buffer was added and mixed. 1ml of ethanol was added in all tubes and vortexed. 50 µl of enzyme source was added to the blank tube.

All tubes were centrifuged at 5000rpm for 15 minutes and the supernatant was collected. The absorbance was taken at 410 nm against the reagent blank. The test reading was related to standards curve to deduce the amount of substrate formed (nmols) by enzyme solution. The activity was converted to specific activity by expressing the enzyme activity as nmols/mg protein/minute using the formula stated below.

Product (nmols) formed/ ml enzyme solution

i.e. Specific Activity* = -------------------------------
Protein content of enzyme source (mg/ml) X Time (Min.)

Specific Activity was expressed as nmols of product formed by mg protein/ minute.
Measurement of α-Iduronidase Activity (Mps I- Hurler Syndrome)

Deficient activity in: Hurler Syndrome for α-Iduronidase activity was assayed in Leukocytes. The substrate 5 mM 4-MU-α-L-iduronide (2.258 mg/ml) was dissolved in 0.5 M phosphate-citrate buffer, pH 4.3 and stored at -20°C for 3 month. The assay required 0.10-0.15 mg protein per assay. 100ul lysate diluted with deionized water was placed to achieve the necessary protein concentration into 12 X 75 mm test tubes. Duplicates tubes were prepared for each sample. 50ul of substrate solution was added at time zero and incubated at 37°C for 1 hour. The reaction was stopped by adding 1.35 ml of 0.17 M glycine carbonate buffer, pH 9.8. For the blank, substrate was incubated alone. The “reaction” was stopped by adding the glycine carbonate and then 100ul control lysate preparation diluted to appropriate range of protein concentration was added. Fluorescence was measured at excitation wavelength 360 nm and emission wavelength 415 nm. Average the duplicate readings. The activity of enzyme was calculated as follows:

\[
\text{Lysate activity} = \frac{\text{fluorescence of (sample – blank tubes)}}{(\text{nmol/hr/mg})} \times \frac{\text{mg protein per assay}}{1 \text{ nmol 4-MU}}
\]

Control range developed in the lab in Leukocytes ranged from 11-26 nmol/hr/mg
Measurement of Iduronate Sulfatase Activity (Mps II-Hunter Syndrome)

This utilized plasma for estimation as the enzyme had a sulfate residue. Completely thawed and mixed plasma or serum samples were used. Plasma samples were diluted 1:5 in distilled water. Two Eppendorf tubes per specimen and two additional tubes (as blanks) for the whole run were used and kept in ice-water. Eppendorf tubes were used to minimize evaporation due to the prolonged incubation times in this assay. The first step of incubation included 10μl plasma and 20μl buffered substrate were added to all tubes. All tubes were incubated for 4 hrs at 37°C in the water bath. In the second incubation, tubes were removed from the water bath and centrifuged for one minute at 5000rpm, in micro centrifuge to remove moisture from lids. 40 μl (dilute) McIlvain’s buffer pH 4.5 was added to all tubes. 10 μL LEBT was added to each tube and mixed. All tubes were incubated at 37°C for a further 24 hours. 920 μl stopping buffer was added in all tubes and mixed. Fluorescence was measured at 355/460 nm on the fluorometer. The calculation of specific gravity was done as indicated below.

\[
\text{Test} - \text{Blank} \times 2.5 \times \text{vol} (1) \times 1000 \times 5(\text{dil}) \times 1 \times \frac{1}{100} \times \frac{1.6}{\text{vol} \text{ plasma} \times 10} \times \text{time} (4) = \text{nmol/hr/ml}
\]

Reference ranges for normal population ranged from: 600 – 1610 nmol/hr/ml plasma
Assays for Total Glycosaminoglycans (GAG’s) Estimation

The specimen used was a spot sample though patients were advised to collect 24 hours urine if possible to decrease variation. Sample preparation: in the initial step the urine sample was centrifuged at 5000 rpm for 15 min. The supernatant was collected for analysis. The Assay procedure includes the following steps. Initial steps revolved quantitative precipitation of GAGs. Initially, 0.1 ml of urine was taken in an eppendorf tube labeled as ‘T’ (test). 0.1 ml of the series of reference standards (5-500 µg/ml) were taken to tubes labeled as ‘S’ (standard). 0.5 ml of GAGs precipitating reagent was added to all the tubes and mixed gently. It was observed if the bluish color still remained. More reagents were added, if necessary, to get the bluish tinge, to ascertain that DMB dye is in excess to effect the quantitative precipitation of the GAGs. The tubes were allowed to precipitate at room temperature for 20 minutes. The tubes were centrifuged at 5000 rpm for 15 min. The supernatant was discarded. 0.1 ml of SDS solution was added in all tubes and vortexed to dissolve the precipitant. 50 µl of 200 mg% solution of BSA was added and mixed gently. 1.0 ml of ethanol (95% w/v) was added, mixed gently and allowed to stand in an ice bucket for 20 minutes. The tubes were centrifuged at 5000 rpm for 15 minutes and decant/siphon off the supernatant. 100 µl of normal saline was added and vortex to dissolve the precipitate. 1 ml of protein based DMB dye reagent was added and mixed it allows to develop metachromasia for 10 minutes.
The absorbance was measured at 520 nm against reagent blank. The Test readings were computed against the standard curve prepared by using reference standard series to arrive at the GAGs content of urine. The values were expressed/reported the as the GAGs content /g creatinine or as 24 hrs excretion.

**CHITOTRIOSIDASE ELISA:**

Plasma Chitotriosidase enzyme levels were measured by a sandwich ELISA using a commercially available CircuLex (CycLex Co. Ltd, Nagano, Japan) is designed to measure the concentration of human chitotriosidase in human serum or plasma. The sample preparation was done as indicated. Serum or Plasma samples required a 50-fold dilution. The standard assay procedure: is depicted and included the following steps. The appropriate number of microtiter wells were removed from the foil pouch and placed into the well holder. Unused wells were returned to the foil pouch and stored at 4°C. Serum sample was diluted 1:50 ratio with Dilution Buffer (e.g. 6µL serum sample + 294 µL Dilution Buffer). 100 µL of Human Chitotriosisadase Standards (Std1-Std7, Blank) and diluted serum samples were added in duplicates, into the appropriate wells. The plate was incubated at room temperature (ca. 20°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker. The wells were washed 4-times by filling with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer. 100 µL of HRP conjugated Detection Antibody was added into each well. The plate was incubated at room temperature (20°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
The wells were washed 4-times by filling with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer. 100 µL of Substrate Reagent was added. (Exposing the microplate to direct sunlight was avoided. The plate was covered with aluminum foil or polyfilm sealer Substrate Reagent was returned to 2-8°C immediately after the necessary volume was removed. The plate was incubated at room temperature for 15-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker. 100 µL of Stop Solution was added to each well in the same order as the previously added substrate Reagent. The absorbance of each well was measured by using a spectrophotometric microplate reader at dual wavelengths of 450/550 nm.

CCL18/PARC ELISA

Plasma CCL18 levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available CytoSet (Biosource International, Camarillo, CA), consisting of a capture antibody, a biotinylated detection antibody, recombinant CCL18/PARC standard, and streptavidin-horseradish peroxidase (HRP) conjugate. Assay conditions were exactly as described by the manufacturer. The plate preparation was done as follows. Capture antibody was diluted to the working concentration in PBS buffer without carrier protein. The 96 well micro plate immediately coated with 100 µl per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. The wells were aspirated and washed with wash buffer; the washing process was repeated two times for a total of three washes. After the last wash, remaining wash buffer was removed by
aspirating or by inverting the plate and blotted it against clean paper towels. The plate was blocked by adding 300 µl of reagent diluents to each well and incubated at room temperature for a minimum of 1 hour. The aspiration/wash was repeated as in step 2.

Assays Procedure: The Assay procedure included the following steps. 100 µl of sample or plasma or standards were added in reagent diluents. Plate was covered with an adhesive strip and incubated 2 hours at room temperature. The aspiration/wash step was repeated as in step 2 of plate preparation. 100 µl of the detection antibody, diluted in reagent diluents were added to each well. The plate was covered and incubated 2 hours at room temperature. The plate was aspirated / washed as in step 2 of plate preparation. 100 µl of the working dilution of streptividin- HRP was added to each well. The plate was covered and incubated for 20 minutes at room temperature. The plate was aspirated / washed as in step 2 of plate preparation. 100 µl of the substrate solution was added to each well and incubated for 20 minutes at room temperature. 50 µl of stop solution was added to each well and gently taped to ensure thorough mixing. The optical density of each well was determined immediately by using a microplate reader set to 450 nm.
**Materials & Methods**

**Heparin Thrombin Cofactor II complex (THCII) ELISA**

**Assay Procedure:** The Assay procedure included the following steps. Preparation of Thrombin-THCII complex reference standard involved Purified THCII (330 µg/mL = 5 µM) in 20mM Tris-HCl, 0.15 M NaCl, pH 7.4, 1 mM EDTA and 0.05 U/ml heparin, was incubated with a limited amount of thrombin (37 µg/ml = 1µM) at 37°C for 30 minutes. Complete inhibition was confirmed by plasma clot time or chromogenic assay. A series of standards were made by diluting this stock T-THCII complex in THCII-deficient plasma. Standards containing 600, 200, 60 and 20 pM respectively were prepared. For coating of plates: The capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) was diluted and 100 µl was immediately added to every well in the plate. The plate was incubated 2 hours at room temperature or overnight at 2-8°C. For Blocking: The contents of plate were discarded and 150µl of blocking buffer was added to every well and incubated for 90 minutes at 22°C. The plate was washed 3 times with wash buffer. Standards and Test Samples were analysed as described: Test samples and reference standards prepared above were rediluted ¼ in sample diluents. 100 µl of standard and sample were applied per well in plate and incubated at 22°C for 120 minutes. The plate was washed 3 times with wash buffer. For Detecting Antibody: The detecting antibody was diluted 1/100 in sample diluents and 100µl applied to each well. The plate was incubated at 22°C for 60 minutes. The plate was washed 3 times with wash buffer. OPD Substrate: 100 µl of freshly prepared OPD substrate was applied to every well. Color was allowed to develop for 10-15 minutes then the color reaction was stopped with the addition of 50 µl/well of 2.5 M H₂SO₄. The plate wavelength was read at 490 nm.
Statistical Analysis:

The data was tabulated and analyzed. Statistical analysis was carried out by using software STATA 11.0 version (College Station, Texas, USA). Data were presented as median (range). Wilcoxon rank-sum (Mann-Whitney) test was used to compare the difference in median between the groups. The changes in Chitotriosidase, CCL18/PARC and Heparin cofactor II Thrombin over time points was compared using Wilcoxon signed-rank test. The p values < 0.05 was considered statistically significant.